DIPLOMARBEIT

Titel der Diplomarbeit

Analysis of growth cone collapse and axon retraction in primary mouse neurons by time-lapse and fluorescence microscopy.

verfasst von

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angestrebter akademischer Grad

Magister der Naturwissenschaften (Mag.rer.nat.)

Wien, 2013

Studienkennzahl lt. Studienblatt: A 490
Studienrichtung lt. Studienblatt: Diplomstudium Molekulare Biologie
Betreut von: ao. Univ.-Prof. Dr. Friedrich Propst
What’ve they got in there, King Kong?
– Dr. Ian Malcolm, Jurassic Park (1993)
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1 Introduction

1.1 The nervous system

The nervous system is an integral part of almost all multicellular organisms. The central nervous system (CNS), comprised of the brain and spinal cord, is distinguished from the peripheral nervous system (PNS) which describes all components not included in the CNS, namely sensory and motor nerves that spread out into the body and the ganglia. It is composed of two main families of cells, neurons and glia, that in unison provide a network of pathways where information such as sensory impressions and movement commands can be transmitted from the periphery towards the center and vice versa. Neurons are the main transmitters of information whereas glia, even though more numerous, are mainly believed to have assistive functions. However, recent knowledge about the participation of glial cells in neurotransmission and synaptic function indicates an influence (Auld and Robitaille, 2003).

A typical neuron has a cell body that contains the nucleus and most of the organelles. From that central part several cell protrusions, summarized as neurites, extend (Bear et al., 2007; Levitan and Kaczmarek, 2002). Neurites are divided into the (shorter) ones responsible for receiving signals called dendrites and a single (long) one deemed to transmit signals, called the axon. In some cases, for example the sciatic nerve, originating in the lumbar vertebrae innervating the lower extremities, these axons can reach lengths of up to 1 m.

Glial cells offer structural and paracrine support to the neurons which they surround and protect (Bear et al., 2007; Levitan and Kaczmarek, 2002). Astrocytes are the main component of brain tissue and embed the various types of neurons. Myelination, the process of forming myelin sheaths around axons, is established by Oligodendrocytes in the CNS and Schwann cells in the PNS. The insulation provided by myelin greatly increases nerve conduction velocity by enabling saltatory conduction. Microglia are the equivalents of macrophages in the CNS and clean up dead cells and debris.
1.2 Dorsal root ganglion neurons

Dorsal root ganglia (DRGs) contain the cell bodies of peripheral sensory neurons and are located dorsally inside the vertebral column. Two are symmetrically placed in each vertebral segment and correspond to a characteristic part of the body that is innervated by the associated nerves. DRG neurons are pseudo-unipolar which means that one axon exits the soma and bifurcates shortly thereafter. The peripheral branch receives sensory signals either directly or from other nerves and conveys them towards the proximal branch which forwards it onwards, at the same time bypassing the soma.

DRG neurons have been used in research for years and are easily obtained from various sources such as mice, rats and chicks. They can be cultured as explants with their surrounding native tissue or after enzymatically removing said tissue for dissociated culture. Several methods for preparation and culture of DRGs are found in literature, commonly referring to laminin-coated surfaces as a necessary prerequisite for neuron survival.

![Figure 1.1: Cross Section of Spinal Cord.](http://cnx.org/content/m44751/latest/)
1.3 Cortical neurons

Cortical neurons (CN) are a mixture of mainly pyramidal neurons as well as stellate cells and other neurons in smaller parts. CN culture always contains glial cells, which can be reduced in number by carefully choosing the time of preparation. It is advisable to use brains no older than 1 day (P0) but embryonic brains (E14–17) are generally preferred. CN originate from glial progenitor cells and polarize in vivo during migration to their target layer in the neocortex (E11–E17). They are completely polarized after birth, which is an important difference between embryonic and neonatal brains (Barnes and Polleux, 2009). Figure 1.2 shows polarization in vitro which is different but comparable to this pattern. Cells start by forming multiple short lamellipodia that transform into less but more defined short neurites. Symmetry break occurs after 2–4 d in culture, indicated by one neurite growing ahead of the others and forming the axon. Continued culture enables the study of dendrite branching and synapse formation. Several culture methods and protocols are described in literature (Beaudoin et al., 2012; Eide and McMurray, 2005; Kapfhammer et al., 2007; Potter and DeMarse, 2001).

Figure 1.2: Polarization of cortical neurons in vitro. Stages 1–5 of polarization as seen in cultured cortical neurons obtained from embryonic brains 14 days after fertilization (E14) after culture for x days in vitro (DIV). Adapted from Barnes and Polleux (2009).

1.4 The neuronal cytoskeleton

The cytoskeleton is of utmost importance for a multitude of cellular processes in every cell. The cytoskeleton is not rigid but able to adjust to new requirements and environments. The special shape and size of neurons is specifically attributable to the coordinated interaction of the cytoskeletal partners. Neuronal migration, for example, plays an important role during differentiation and polarization of neurons. Also, the exceptionally long cell
protrusions, axons and dendrites, that separate the location of gene transcription, the
nucleus, from potential destinations for gene products, synapses and growth cones, bring
about major challenges for intracellular transport processes that have to be overcome.
The main components of the neuronal cytoskeleton are microtubules, actin filaments and
neurofilaments as well as associated proteins.

1.4.1 Actin

Actin is a globular ATP-binding protein with intrinsic ATPase activity. Three different
classes of globular (G)-actin (α, β and γ) are known in mammals (Alberts, 2008). As
described there, they are all capable of forming filaments and occur in different types of
cells. The ATP-bound monomeric form of actin can be incorporated into the filament at
the barbed (plus) end. Shortly after incorporation ATP is hydrolyzed to ADP. In a process
called treadmilling, subunits are constantly added at the plus end and removed at the
minus end without changing the net-length of the actin filament. Filamentous (F)-actin
is often found in cell protrusions such as filopodia and lamellipodia where they form
bundles and two-dimensional networks that are the main driving force behind membrane
extension at these sites. Thus, actin-based movement is the main cause of growth cone
motility. It seems that lack of F-actin does not prevent axon growth but rather growth
cone turning and its ability to react to guidance cues (Dent et al., 2011). The motor
protein myosin-II is thought to be a major factor in the generation of force that pushes
the membranes forward. A permanent retrograde flow of F-actin is achieved by myosin
movement.

1.4.2 Microtubules

Microtubules are structures reminiscent of a pipe in which the outer wall is formed by 13
protofilaments assembled in parallel around a hollow center (Alberts, 2008). Further, the
protofilaments are assembled by heterodimers of α- and β-tubulin subunits that are
positioned in such a way that in the final filament α- and β-subunits alternate. This causes
the whole tubule to be polar, since the two ends exclusively exhibit either α- or β-tubulin.
The two ends are known as plus and minus ends and associated with several characteristics.
The minus end is usually positioned towards the cell center where, next to the nucleus,
the microtubule organizing center (MTOC) is located. The MTOC is the site of nucleation
for new microtubules, ensures their correct directionality as well as positioning of the nucleus. The plus ends are the sites of polymerization and usually point outwards where they are often found close to the cell membrane. The mechanism by which new subunits are incorporated into the growing filaments is based on the GTPase-activity of β-tubulin. Hydrolysis of $\text{GTP} \rightarrow \text{GDP} + \text{P}_i$ changes the conformation of the heterodimer slightly by introducing a light bend that carries over into the respective protofilament. Because hydrolysis happens with a slight delay, a so-called $\text{GTP cap}$ of GTP-bound heterodimers remains at the tip of the growing microtubule. As long as the addition of new subunits is faster than hydrolysis the microtubule grows at the plus end. However, if hydrolysis catches up, the protofilaments exercise a force due to their inherently curved structure. This leads to depolymerization and is called catastrophic shrinkage. The process can be reversed by repeated formation of a protective GTP-cap, rescuing the microtubule. Plus ends can be protected from depolymerization by a protein cap, anchoring it at a specific location in the cell and thereby introducing a stable link between the centrosome and this specific region. The process of alternating catastrophe and rescue events is known as dynamic instability and characteristic for microtubules. Microtubules are especially important in nascent neurons where they orchestrate migration. Process polarity in developed neurons is then established and maintained by microtubule orientation. The centrosome orients itself between the nucleus and axon where microtubules are mostly oriented with their plus ends towards the distal end. This orientation is usually reversed in dentrites (Sakakibara et al., 2013).

**Microtubule-associated proteins**

Prominent examples of microtubule-associated proteins (MAPs) are the ones with structural implications such as the members of the MAP1 family (MAP1A, -B and -S), MAP2, and tau as well as molecular motors such as kinesin and dynein (Dent et al., 2011; Halpain and Dehmelt, 2006). They have binding sites for tubulin as well as other cellular components and can thereby act as linkers. MAPs are also important for the regulation of stability and dynamic properties of microtubules (Neukirchen and Bradke, 2011). These functions are regulated by posttranslational modifications such as phosphorylation and S-nitrosylation. MAP1B is a target for S-nitrosylation, which was shown to convey axon retraction-related signals (Stroissnigg et al., 2007). Phosphorylation of MAP1B alters its affinity to microtubules depending on site of the modification as well as the modifying
enzyme. MAP2 and tau react to phosphorylation by decreasing their affinity to microtubules. Distribution of MAPs in axons and neurites is tightly regulated and an influence on polarization is suspected. MAP1-family proteins are located in axons, dendrites and the soma. tau is preferentially found in axons where it is implicated in protection (Qiang et al., 2006) and MAP2 is a marker for dendrites (Stiess and Bradke, 2011).

1.4.3 Neurofilaments

Intermediate filaments found in neurons are called neurofilaments. They are thought to provide structural support to the neuron, especially the axon, in a similar manner as keratins do in epithelial cells (Alberts, 2008).

1.5 Axon guidance

During growth and development of the organism growing neurons have to cover immense distances compared to their size which is achieved by cell movement and the extending axon. During this growth phase the axon is guided along its path by extracellular signals called guidance cues. These signals can be surface-bound or soluble factors that act in an either attractive or repulsive way. A combination of different factors in varying concentrations or gradients elicit a finely tuned influence on axons in their reach. Different neurons can respond differently to the same signaling environment. Laminin and fibronectin are part of the extracellular matrix (ECM) and examples of substrate-bound attractants whereas slits, ephrins or chondroitin sulphate proteoglycans repel. Together they form a figurative corridor on which a growing axon can move forward. The direction is influenced in detail by diffusible guidance cues such as semaphorins and ephrins as well as growth factors and morphogens along the way (Lowery and Van Vactor, 2009).

1.5.1 The growth cone

On the tip of every growing axon exists a characteristic structure called the growth cone which was first described by Santiago Ramón y Cajal in 1890. In cultured neurons it appears as a flat and highly motile structure that continually probes the environment by forming and retracting filopodia and lamellipodia. It reacts to guidance cues accordingly by directional turning towards attractive signals or away from repellent ones. Motion of
the structure is primarily accomplished through actin rearrangements with microtubules mainly following the given direction. Evidence suggests that microtubules may be guided along bundles of F-actin and proteins that can bind to both actin and tubulin form connections between the two to further strengthen the structure (Dent et al., 2011; Lowery and Van Vactor, 2009). Growth cone turning can be influenced by directly manipulating microtubule dynamics, also proving interaction and influence between the two cytoskeletal components in this regard. As shown in Figure 1.3 the central (C) domain is rich in microtubules that extend from the axon shaft and lie parallel to each other. The peripheral (P) domain includes the highly motile cell protrusions. Filopodia house F-actin bundles that may be accompanied by microtubules that explore the newly formed structures. The lamellipodia-like veils are situated between the filopodial spikes and are filled with a meshwork of actin filaments. Between these two lies the transitional (T) zone, formed by a ring of contractile actomyosin structures.

![Figure 1.3: Schematic representation of a growth cone.](image)

Repulsive guidance cues or unfavorable conditions induce turning of the growth cone away from the source. This is achieved by retraction of the part facing the repellent source through depolymerization or increase of net retrograde flow of F-actin as well as increased catastrophe events of microtubules (Dent et al., 2011). Movement towards an attractant resembles an amoeboid movement pattern, as described by Lowery and Van Vactor (2009). Recognition of an attractive surface-bound structure (e.g. the ECM protein laminin) by a membrane-bound receptor (e.g. integrin) induces intracellular signaling (e.g. through Rho GTPases) that induces a change in the cytoskeletal composition at that
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point. The actin network is anchored to this receptor and the leading edge of the growth cone protrudes into the direction of the point of anchoring. As the attachment point is overgrown by the moving growth cone, intracellular rearrangements lead to advancement of microtubules into the newly occupied region. A new piece of axon, almost devoid of actin and equipped with parallel microtubules, is formed at the lagging side of the growth cone by the involvement of myosin II-contraction.

1.5.2 Signaling

The plasma membrane of the growth cone is decorated with various receptors for guidance signals. The four classic and best studied ones are netrins, semaphorins, ephrins and slits. They convey their signals through interaction with their respective ligands (netrins: DCC family proteins, semaphorins: plexins, ephrins: Eph-receptors, slits: Robo) and may need further processing such as endocytosis or proteolytic cleavage for full effect or modulation of the strength of impact (Bashaw and Klein, 2010; Dickson, 2002; Huber et al., 2003). Cell-adhesion receptors such as cadherins, integrins or cell-adhesion molecules provide connection points to extracellular matrix (ECM) proteins for the cytoskeleton and are also involved in signaling. Receptor signaling can be direct or passed on via second messengers such as calcium or cyclic nucleotides. These two systems are heavily interlinked and influence each other. Increase of intracellular Ca\textsuperscript{2+}-levels induces the production of cyclic nucleotides and activates NOS, while cyclic nucleotides influence calcium-channels in the plasma membrane and intracellular calcium storages.

Ultimately, many signals converge on the actin cytoskeleton by altering the activity of Rho-family GTPases such as RhoA, RAC1 and CDC42. Src family kinases and focal adhesion kinase are also known to convey guidance signals from ephrin and netrin respectively, but due to their involvement in many different signaling cascades unrelated to growth cone steering, their importance herein remains unclear (Myers et al., 2011). Second messengers affect the composition of surface receptors which implicates a feedback regulatory mechanism. A regulation of surface receptors for the reciprocal action and influence with ECM proteins is a proposed method of influence on growth cone steering by diffusible guidance cues (Bashaw and Klein, 2010).
Semaphorin3A

Semaphorins are a family of guidance molecules that are freely diffusible or associated to the plasma membrane and categorized into 8 groups on the basis of their structural features (Dickson, 2002; Pasterkamp and Giger, 2009). They are expressed in various tissues and many have been found to influence neuron growth negatively (Giger et al., 2010) making them important targets for research. The secreted guidance cue Semaphorin3A is of crucial importance during development with severe phenotypes in case of its absence. It has a repellent effect on axons (Dickson, 2002) and attracts dendrites of pyramidal neurons (Polleux et al., 2000). Its repulsive effect on growth cones can be converted into an attractive one by cGMP (Song et al., 1998). Semaphorin3A–/– mice have multiple developmental defects such as skeletal and heart malformation (Behar et al., 1996) or in neuronal polarity (Lerman et al., 2007) and they die soon after birth (Behar et al., 1996).

1.6 Axon retraction

Retraction is an active process through which neurons are able to reduce the net length of neurites. This happens during development as well as under pathological circumstances and is accomplished by concerted energy-consuming processes that include backwards directed force generation, membrane uptake and dismantling of cytoskeletal structures. The mechanism is thought to be important for correct wiring of the brain by directed removal of redundant connections and for learning and memory as well as in pathological situations of nerve atrophy (Baas and Ahmad, 2001; Luo and O’Leary, 2005).

It was proposed by Baas and Ahmad (2001) that opposing forces generated by dynein/-microtubule and myosin/actin interaction are the main cause for axon progression or retraction. The actin and microtubule-cytoskeleton are linked, among others, by motor proteins that move microtubules towards the distal tips of neurites. Both actin and tubulin-based movement are needed for the full effect. The authors describe bead-like swellings and sinusoidal arrangement of the retracted axon that are reminiscent of the retraction phenotype observed in the lab (Descovich, 2009; Ebner, 2012; Krupa, 2009; Stroissnigg et al., 2007; Tranciková, 2007) and elsewhere (He et al., 2002; Orlova et al., 2007).
1.7 Neuronal nitric oxide synthase

Neuronal nitric oxide synthase (nNOS) is a 160 kDa protein and the neuronal isoform of nitric oxide (NO) synthase enzymes. Other members are expressed in different cells and tissues and are called endothelial (eNOS) and inducible (iNOS) NOS. All three generate NO from L-arginine and O₂. nNOS is mainly expressed in the brain and spinal cord but has been detected in kidney, pancreas and vascular smooth muscle cells as well. nNOS plays an integral part in NO-signaling in the nervous system. After stimulation by Ca²⁺ it catalyses the synthesis of NO. It is implicated in synaptic plasticity, learning and memory rather than immediate neurotransmission and involved in various physiological and pathophysiological processes (Förstermann and Sessa, 2012; Steinert et al., 2010).

1.7.1 Nitric Oxide

NO is a short-lived signaling molecule that cannot be stored in vesicles or be recycled in any way, thus it has to be produced in close proximity to the site of effect and the synthesis must be strictly regulated (Snyder, 1995). Its prime signaling target in physiological conditions is soluble guanylyl cyclase, thus increase of cGMP and consequential effects are its principal although indirect means of action. NO can also act by direct S-nitrosylation of proteins and DNA or in neighboring cells through diffusion (Förstermann and Sessa, 2012). S-nitrosylation is the reversible covalent binding of NO to a thiol group and is highly specific (Gao, 2010). Recognition motifs may be necessary in the selection of S-nitrosylation targets. S-nitrosothiols are light sensitive and release NO upon exposure to visible light (Sexton et al., 1994).

1.8 Soluble guanylyl cyclase

Soluble guanylyl cyclase (sGC) is a 72 kDa heterodimer of the subunits α₁ or α₂ with β₁. Polypeptide β₂ was not yet found to participate in the formation of active enzymes and is potentially a pseudogene. Only αβ-heterodimers are functional in vivo (Friebe and Koesling, 2009) although other dimers can form in vitro (Poulos, 2006). Thus, deletion of the β₁ gene suffices to create complete sGC knockout mice (Friebe and Koesling, 2009). A functional heterodimeric enzyme has a N-terminal regulatory domain that contains a heme group which reacts to nitric oxide. Upon NO-binding a conformational change
activates the C-terminal catalytic domain which then induces an increase in production of cyclic guanosine monophosphate (cGMP) (Poulos, 2006). sGC deficient mice have severe phenotypes in several tissues including impaired smooth muscle relaxation, elevated arterial blood pressure, effects on vasodilation, gastrointestinal motility and platelet aggregation as well as neuronal implications. Most newborns die within 48 h (Friebe and Koesling, 2009). sGC is implicated in axon guidance (Descovich, 2009; Dickson, 2002; Stroissnigg et al., 2007) and growth cone turning (Tojima et al., 2009). Expression of soluble guanylyl cyclase varies in axons and dendrites, giving rise to differences in local cGMP-levels which can lead to opposing effects of exposure to guidance molecules in axons and dendrites (Polleux et al., 2000).

### 1.8.1 Cyclic guanosine monophosphate

Cyclic guanosine monophosphate (cGMP) is synthesized from GTP by soluble or membrane-bound guanylyl cyclases after activation. sGC is activated by NO whereas membrane-bound GCs are receptors for various ligands (Derbyshire and Marletta, 2012). cGMP acts as a secondary messenger and is implicated in various cellular processes and tissues.

Cyclic guanosine monophosphate (cGMP) plays a role in neurite growth and growth cone mobility (Murray et al., 2009) as well as axon branching (Schmidt and Rathjen, 2010; Zhao et al., 2009). cGMP and cyclic adenosine monophosphate (cAMP) often act in opposing ways, indicating a fine-tuning mechanism by control of cGMP/cAMP ratio (Shelly et al., 2010). Attraction and repulsion can be reversed by activating the respective cAMP and cGMP-signaling pathways (Song et al., 1998).

### 1.9 Cyclin-dependent kinase 5

Ncdk, the holoenzyme comprised of Cdk5 and its neuronal activator p35, is responsible for phosphorylation of several cytoskeletal targets and is important during neuronal development, migration and axon formation (Paglini and Cáceres, 2001). One such target is MAP1B which, upon phosphorylation, exhibits changed binding to microtubules and thereby influences microtubule dynamics.

Cdk5 is implicated in a multitude of events during maturation of the nervous system, including migration, maturation and differentiation of neurons (Jessberger et al., 2008).
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Nikolic et al., 1996) . It is expressed in both the embryonic and adult brain (Jessberger et al., 2009; Su and Tsai, 2011) where it remains important for synapse formation and plasticity and neuron survival. It may have a role in various neurodegenerative diseases (Su and Tsai, 2011).

1.10 Previous results and aim of this work

It was previously shown that Ca\(^{2+}\)-induced activation of nNOS leads to axon retraction via S-nitrosylation of MAP1B-LC1. This effect was reproduced by replacing this endogenous NO-source with the direct NO donor SNAP (Stroissnigg et al., 2007). As mentioned, NO mainly acts by inducing cGMP synthesis by sGC which leads to phosphorylation of MAP1B and induces axon retraction as well. Evidence for a previously suggested (Dinerman et al., 1994) negative feedback loop from cyclic-GMP dependent protein kinase (PKG) to nNOS was found by former members of the lab. Significantly increased axon retraction was observed in wild-type neurons after treatment with LY83583 and ODQ alone (Descovich, 2009). Simultaneous inhibition of nNOS returned the number of retracting cells back to untreated levels. Krupa (2009) and Stroissnigg et al. (2007) showed that inhibition of nNOS with NPA reduced Calcimycin-induced axon retraction in wild-type DRG neurons, demonstrating that activation of nNOS is involved in Ca\(^{2+}\)-induced axon retraction.

Previous data by Krupa (2009) showed that treating DRG neurons with Roscovitine leads to MAP1B-dependent axon retraction that does not involve nNOS activation. Moreover, a Poly-l-lysine (PLL)/laminin-coated surface is required for the effect which does not work on PLL alone.

Several kinases that are involved in the other mentioned signaling cascades such as PKA and PKG as well as GSK3\(\beta\) and Cdk5 are implicated in Semaphorin3A signaling as well. These findings made it an obvious candidate for investigation concerning its involvement in nitric oxide signaling. After treatment with Semaphorin3A, a MAP1B dependent reduction in neurite outgrowth was previously observed by Descovich (2009). Semaphorin3A treatment with simultaneous inhibition of nNOS returned the neurite outgrowth back to untreated levels.

These previous findings were on the one hand mostly obtained by analysis of fixed cells and on the other hand not completely coherent. Therefore, we wanted to support the
results with live-imaging data to deepen our knowledge of the underlying signaling mechanisms.

A schematic overview, showing the important participants in the involved signaling cascades including the involved enzymes and their respective inhibitors, is shown in Figure 1.4.

**Figure 1.4: Pathways leading to axon retraction with relevance to this work.** The most important known participants of signaling cascades involved in NO/sGC/cGMP-signaling are represented here schematically. → indicates direct causative impact, ← indicates inhibition, ↑ x indicates increase of x.
2 Methods

2.1 DRG techniques

2.1.1 Isolation and culture

One mouse at a time was anesthetized for approximately 1 min with 150 µl Isoflurane (dispensed in a closed glass beaker lined with green towels) and immediately killed by decapitation with household scissors. All tools were sterilized with 70% EtOH before use. The mouse was fixed for dissection on a styrofoam plate covered in green towels using needles. To sterilize the fur it was thoroughly soaked with 70% EtOH before the first incision at the dorsal midline. The skin was removed using pincers and a scalpel while ensuring that the newly exposed subcutaneous tissue was not contaminated. The dorsal part of the vertebrae was cut with angled scissors, starting from the neck.

After removal of the bone the mouse was transferred onto a dissection microscope. There, the ganglia were removed from the spinal duct and immediately put into ice cold F-12 medium. DRGs were dissociated by digestion with collagenase (2 mg/ml in F-12 medium supplemented with 2% Horse serum for the duration of 1.5 h at 37°C, 5% CO₂) and subsequent simultaneous trypsinization (0.025%) and DNaseI treatment (50 µg/ml, in PBS). The reaction was stopped by addition of prewarmed F-12 supplemented with 20% horse serum. Two Pasteur pipettes were narrowed to different diameters by gentle exposure to a gas flame and used to homogenize the DRGs by repeatedly resuspending the solution. The cells were washed twice with preincubated F-12 medium after low-speed centrifugation and finally resuspended in growth medium.

2.1.2 Time-lapse microscopy

6-well plate preparation

Square 13 mm-coverslips were first washed with ddH₂O and EtOH-abs. then baked dry at 180°C over night to sterilize, before being stored in an air tight container at 4°C until
use. 6-well plates were prepared by drilling a hole of 10 mm in diameter at the center of each well. Immediately before use they were warmed on a heating block and turned bottom up. Paraffin was melted while glass coverslips as well as a blue pipetting tip were preheated. One glass platelet per well was positioned over the hole from the outside to allow drops of paraffin, pipetted to the edge of the coverslip, to be sucked in by adhesion. After solidification, the 6-well plate was placed under UV light for at least 30 min before washing with ddH$_2$O twice. Afterwards, the coverslips were incubated with PLL (10 µg/µl) for 1 h, washed twice with PBS and finally incubated with Laminin (10 µg/µl) for 3 h, each step done in the incubator (100 % humidity, 5 % CO$_2$). PLL incubation could be extended for hours, up to over night, depending on experimental schedules.

**Image acquisition**

Videos were recorded using a 32× phase contrast objective in a “Zeiss Axio Observer” microscope equipped with an incubation chamber, capable of maintaining constant temperature and atmospheric conditions (37 °C, 100 % humidity, 5 % CO$_2$). The whole setup (sans cells) was kept running for hours before use to heat up the stage and optics to working temperature and reduce fluctuations in experimental conditions.

The 6-well plate containing cells was put in the chamber and fixed with the provided screw as well as additional pieces of foam material. The old growth medium was completely removed to remove debris and substituted with 2.7 ml of preheated new medium. Multiple positions were marked in each well and a series of images was taken over 20 min. Meanwhile, inhibitors and vehicle controls were prepared in 300 µl growth medium in ten-fold excess, to obtain the experimental concentrations after subsequent addition to the wells. The order was decided randomly and noted down for tracking by a colleague. Video acquisition was continued for another 40–100 min in 10–20 min-intervals, producing a series of 5 (untreated) and 3–6 (treated) images.

**Video analysis**

Time-lapse videos of samples before treatment were scanned for growing neurites, defined by outward moving neurite tips in the last frame of the video. Those neurites were marked and kept track of in the image series taken after treatment. They were then assessed and categorized into retracting (backwards motion towards the cell body, possibly but not
necessarily along the previous path), extending/pausing (forward motion or standstill, but no backwards motion) or collapse (loss of membrane integrity and complete dissipation of the neurite or cell). Percentages were calculated and evaluated.

2.1.3 Immunocytochemistry

24-well plate preparation and plating

Round 13 mm-coverslips were first washed with ddH₂O and EtOH-abs., then baked dry at 180°C over night to sterilize before being kept in an air tight container at 4°C until use. One platelet was put in each well and washed twice with ddH₂O. Afterwards, the coverslips were incubated with PLL (10 µg/µl) for 1 h, washed twice with PBS and finally incubated with Laminin (10 µg/µl) for 3 h, each time at 100% humidity and 5% CO₂. PLL incubation could be extended for hours, up to over night, depending on experimental schedules.

Prepared coverslips were washed twice with F-12, the medium was removed and the cell suspension of desired density was added to each well. Cells were incubated (100% humidity, 5% CO₂) for 12–16 h until treatment.

Fixing and staining

Cells were fixed with 4% PFA/11% Sucrose by incubating for 20 min, using a preheated solution of 8% PFA/22% Sucrose in PBS that was administered to the wells in equal amounts compared to the growth medium within. Work was continued at room temperature from this point onwards. The cells were rinsed twice with PBS, permeabilized with 0.1% Triton-X 100 for 10 min and washed once with PBST. Blocking was achieved by incubation with 2% BSA in PBST for 30–60 min. Primary antibodies were diluted in 1% BSA and applied for 1,5–2 h while providing 100% humidity in a wet chamber. After three washing steps with PBST, secondary antibodies as well as other staining agents when applicable, were prepared by dilution as before. Incubation proceeded in the same wet chamber in complete darkness. The coverslips were then rinsed with ddH₂O, the residual water dabbed off gently followed by mounting on glass microscopy slides using 7µl Mowiol. The slides were left to dry over night in the dark.
2 Methods

Fixed cell analysis

Fixed cells were assessed by immunofluorescence microscopy. Labels on the microscopy slides were covered with opaque tape and randomly re-labeled with numbers by a colleague. The coverslip was scanned in a meandering fashion, starting from either top or bottom and moving to the opposing side in a straight line, shifting to either left or right by more than one field of sight and repeating the process until the total number of assessed neurons reached roughly 100. Neurons were categorized as either retracting, unchanged or collapsed when the majority of all cell protrusions exhibited similar behavior. Retracting neurons were defined as showing the characteristic retraction bulbs and sinusoidal bundles of microtubules. To be categorized as unchanged the neurons had to have spread growth cones, intact microtubule filaments and show no sign of the aforementioned retraction. Collapsed neurons were identified by their loss of membrane integrity and complete absence of cellular shape. Their occurrence was used as a rough measure for experimental success, since collapse tends to increase dramatically in case of suboptimal culture conditions or handling errors.

2.2 Cortical Neuron techniques

2.2.1 Isolation and culture

Newborn mouse pups (P0) were killed one at a time by decapitation, the skull was removed with pincers and the whole brain immediately transferred to a 6 cm-dish containing ice cold dissection buffer. All tools were sterilized with 70% EtOH before use, further preparation was done under a dissection microscope. Meninges were removed using microforceps, followed by careful removal of unwanted brain regions, such as the olfactory bulb, hippocampus and cerebellum. The tissues were treated with Trypsin solution for 20 min. The supernatant was removed and plating medium was added to inhibit trypsin, followed by homogenization of the tissue by resuspending repeatedly with Pasteur pipettes of different diameters. Cell pellets were washed twice with fresh medium, passed through a cell strainer (40 µm mesh size) to remove undigested tissue and finally resuspended in plating medium.
2 Methods

2.2.2 Immunocytochemistry

24-well plate preparation and plating

Coverslips coated with PLL and Laminin (see above) were washed twice with plating medium. Cells were plated at a density of 50000 cells per well and incubated at 37 °C and 5% CO₂. After 2 h, cells were checked for sufficient attachment using a phase contrast microscope and if so, the medium was changed to feeding medium. Cells were further incubated (100% humidity, 5% CO₂) for a total of 60–65 h until treatment.

Fixing and staining of cells

Fixing and staining was conducted analogous to DRG neurons, see above.

Growth cone collapse assay

Fixed cell analysis was conducted in accordance to Kapfhammer et al. (2007), with slight modifications. At least 50 protrusions longer than 20 µm of cells without any contact to surrounding cells were assessed. Growth cones were categorized as collapsed when no lamellipodia and at most two filopodia were present while at the same time the neurite tips stained positive for tubulin. Data for axons was collected if one neurite was at least twice as long compared to the second longest neurite and therefore clearly distinguishable from the rest.

2.3 Statistical analysis

2.3.1 Chi Square ($\chi^2$) test

Percentages of neurons in each condition were calculated and tested for independence using Pearson’s Chi Square test [http://math.hws.edu/javamath/ryan/ChiSquare.html](http://math.hws.edu/javamath/ryan/ChiSquare.html).

2.3.2 Independent Student’s t-test

Independent Student’s t-tests were performed using Microsoft Excel for Mac (Version 14.3.7). Data shown as mean values ± standard error of the mean.
3 Materials

3.1 Isolation & Cell Culture of Neurons

3.1.1 Reagents

All reagents were stored cool and dark in the fridge with sterility maintained where necessary.

Table 3.1: List of Reagents

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Company</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-27 supplement (50×)</td>
<td></td>
<td>Gibco</td>
<td>17504</td>
</tr>
<tr>
<td>Collagenase</td>
<td>10 µg/ml</td>
<td>Sigma</td>
<td>A2058</td>
</tr>
<tr>
<td>DMSO</td>
<td></td>
<td>Sigma</td>
<td>41639</td>
</tr>
<tr>
<td>Fetal Calf Serum</td>
<td></td>
<td>Sigma</td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ham’s F-12 Nutrient Mix, GlutaMAX™</td>
<td></td>
<td>Gibco</td>
<td>31765</td>
</tr>
<tr>
<td>HBSS</td>
<td></td>
<td>Gibco</td>
<td>14170</td>
</tr>
<tr>
<td>Horse Serum</td>
<td></td>
<td>Gibco</td>
<td></td>
</tr>
<tr>
<td>Isoflurane (IsoFlo®)</td>
<td></td>
<td>Abbott</td>
<td>B506</td>
</tr>
<tr>
<td>Laminin</td>
<td>10 µg/ml</td>
<td>Sigma</td>
<td>L2020</td>
</tr>
<tr>
<td>Neurobasal medium</td>
<td></td>
<td>Gibco</td>
<td>10888</td>
</tr>
<tr>
<td>Poly-L-Lysine</td>
<td>10 µg/ml</td>
<td>Sigma</td>
<td>P1524</td>
</tr>
<tr>
<td>Trypsin-EDTA</td>
<td></td>
<td>Sigma</td>
<td>T4049</td>
</tr>
</tbody>
</table>

3.1.2 N3 supplement

N3 supplement for neuron culture was made following the protocol for a 100 × stock solution, originally based on Romijn et al., [1981]. Briefly, chemicals in Table 3.2 were prepared and/or dissolved immediately before use, mixed in the appropriate amounts, filtered sterile and stored in aliquots at −20°C until needed. For each batch of cultured
neurons the necessary amount was added to the respective growth medium to yield a 1 ×
final concentration.

Table 3.2: N3 supplement composition

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Company</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBSS</td>
<td>—</td>
<td>Gibco</td>
<td>14170</td>
</tr>
<tr>
<td>Apotransferrin</td>
<td>100 µg/ml</td>
<td>Sigma</td>
<td>T1147</td>
</tr>
<tr>
<td>BSA</td>
<td>10 µg/ml</td>
<td>Sigma</td>
<td>A2058</td>
</tr>
<tr>
<td>Putrescine</td>
<td>0.2 mM</td>
<td>Sigma</td>
<td>P5780</td>
</tr>
<tr>
<td>Insuline</td>
<td>1.74 µM</td>
<td>Sigma</td>
<td>I1882</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>0.12 µM</td>
<td>Sigma</td>
<td>C2505</td>
</tr>
<tr>
<td>Tri-iodothyronine</td>
<td>29.7 nM</td>
<td>Sigma</td>
<td>T6397</td>
</tr>
<tr>
<td>Progesterone</td>
<td>39.8 nM</td>
<td>Sigma</td>
<td>P6149</td>
</tr>
<tr>
<td>Na-Selenite</td>
<td>57.8 nM</td>
<td>Sigma</td>
<td>S9133</td>
</tr>
</tbody>
</table>

3.1.3 Media for cortical neuron culture

Media were prepared according to Anilkumar et al.,[2013] with minor adjustments. In
short, supplements were added to the 500 ml media bottles and thoroughly mixed. The
contents was filtered sterile and stored in aliquots of 50 ml at 4 °C until use.

Dissection Buffer

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>HBSS</td>
<td>500 ml</td>
<td></td>
</tr>
<tr>
<td>50 U/ml PenStrep</td>
<td>5 ml (100 ×)</td>
<td></td>
</tr>
<tr>
<td>7 mM Hepes</td>
<td>3.5 ml (1 M, pH 7.3)</td>
<td></td>
</tr>
<tr>
<td>2 mM L-Glutamine</td>
<td>5 ml</td>
<td></td>
</tr>
</tbody>
</table>

Feeding Medium

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurobasal Medium</td>
<td>500 ml</td>
<td></td>
</tr>
<tr>
<td>50 U/ml PenStrep</td>
<td>5 ml (100 ×)</td>
<td></td>
</tr>
<tr>
<td>2 mM L-Glutamine</td>
<td>5 ml</td>
<td></td>
</tr>
<tr>
<td>2 % B27 supplement</td>
<td>10 ml</td>
<td></td>
</tr>
</tbody>
</table>
**3 Materials**

**Plating Medium**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM + GlutaMax</td>
<td>500 ml</td>
</tr>
<tr>
<td>50 U/ml PenStrep</td>
<td>5 ml (100 ×)</td>
</tr>
<tr>
<td>5 % FCS</td>
<td>25 ml</td>
</tr>
<tr>
<td>5 % HS</td>
<td>25 ml</td>
</tr>
<tr>
<td>2 % B27 supplement</td>
<td>10 ml</td>
</tr>
</tbody>
</table>

**3.2 Immunocytochemistry**

**3.2.1 Antibodies and staining reagents**

Antibodies and staining reagents were diluted with 1 % BSA in PBS prior to application.

**Table 3.3: List of Antibodies and staining reagents**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company</th>
<th>Number</th>
<th>Dilution factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse anti-α-tubulin B512</td>
<td>Sigma</td>
<td>T5168</td>
<td>1:200</td>
</tr>
<tr>
<td>Goat anti-mouse IgG Alexa</td>
<td>Molecular Probes</td>
<td>A-11029</td>
<td>1:1000</td>
</tr>
<tr>
<td>Mouse anti-βIII-tubulin</td>
<td>Covance</td>
<td>MMS-435P</td>
<td>1:1000</td>
</tr>
<tr>
<td>Phalloidin Texas Red</td>
<td>Molecular Probes</td>
<td>T-7471</td>
<td>1:200</td>
</tr>
<tr>
<td>Hoechst</td>
<td></td>
<td></td>
<td>1:2000</td>
</tr>
</tbody>
</table>

**3.2.2 Mowiol mounting medium**

**Table 3.4: Mowiol mounting medium composition**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mowiol 4-88</td>
<td>2.4 g</td>
</tr>
<tr>
<td>Glycerol</td>
<td>6 g</td>
</tr>
<tr>
<td>ddH_{2}O</td>
<td>6 ml</td>
</tr>
<tr>
<td>0.2 M Tris-HCl pH 8.5</td>
<td>12 ml</td>
</tr>
</tbody>
</table>

Mowiol powder was added to glycerol in a conical 50 ml centrifuge tube. After thorough stirring water was added and left to rest for 2 h at room temperature. After addition of Tris the mixture was heated to 53 °C and incubated with occasional stirring until Mowiol...
was almost completely dissolved. After centrifugation at 4000–5000 rpm for 20 min the supernatant was aliquoted into 1.5 ml-Eppendorf tubes and stored at \(-20^\circ\text{C}\) until use.

### 3.3 Compounds used for cell treatments

The substances were prepared following manufacturers instructions, aliquoted and stored at \(-20^\circ\text{C}\) until use. Most were dissolved in DMSO, except NPA which was dissolved in ddH\(_2\)O and Semaphorin3A which was dissolved in PBS supplemented with 0.1 % BSA.

#### Table 3.5: List of substances for treatments and respective vehicle controls

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Vehicle</th>
<th>Company</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>—</td>
<td>Sigma</td>
<td>41639</td>
</tr>
<tr>
<td>Calcimycin</td>
<td>DMSO</td>
<td>Invitrogen</td>
<td>A1493</td>
</tr>
<tr>
<td>LY83583</td>
<td>DMSO</td>
<td>Calbiochem</td>
<td>440205</td>
</tr>
<tr>
<td>NPA</td>
<td>H(_2)O</td>
<td>Tocris</td>
<td>1200</td>
</tr>
<tr>
<td>ODQ</td>
<td>DMSO</td>
<td>Sigma</td>
<td>O3636</td>
</tr>
<tr>
<td>Roscovitine</td>
<td>DMSO</td>
<td>Sigma</td>
<td>R7772</td>
</tr>
<tr>
<td>Semaphorin3A</td>
<td>PBS/BSA</td>
<td>R&amp;D Systems</td>
<td>5926-S3</td>
</tr>
</tbody>
</table>

### 3.4 Mice

#### 3.4.1 Ethics Statement

Mouse tissues were prepared in adherence to Austrian laws relevant for laboratory animals: Tierversuchsgesetz, BGBl. Nr. 501/1989 and BGBl. I Nr. 162/2005.

#### 3.4.2 Genetic background

Mouse line 5B22F1 was created by deletion of the MAP1B gene and backcrossing to C57BL/6OlaHsd and 129P2/Ola mice (Meixner et al., 2000), respectively. Heterozygous mice (females of subline 5B22BL6 and males of subline 5B22Ola) are mated to efficiently produce MAP1B knockouts. Mating their respective wild-type equivalents produces wild-type controls that are genetically almost identical (less than 0.1 % difference) to each
other and to wild-type littermates of the F1 knockouts mentioned before. These wild-type littermates were used for my experiments.

### 3.4.3 Individuals

Mouse numbers taken from database. Genotype was confirmed by PCR.

<table>
<thead>
<tr>
<th>mouse</th>
<th>line</th>
<th>sex</th>
<th>genotype</th>
<th>age at culling (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1274</td>
<td>5B22F1</td>
<td>male</td>
<td>wt</td>
<td>101</td>
</tr>
<tr>
<td>1275</td>
<td>5B22F1</td>
<td>male</td>
<td>wt</td>
<td>101</td>
</tr>
<tr>
<td>1378</td>
<td>5B22F1</td>
<td>female</td>
<td>wt</td>
<td>100</td>
</tr>
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<td>1379</td>
<td>5B22F1</td>
<td>female</td>
<td>wt</td>
<td>107</td>
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<td>wt</td>
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<td>male</td>
<td>wt</td>
<td>120</td>
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<tr>
<td>1382</td>
<td>5B22F1</td>
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<td>5B22F1</td>
<td>male</td>
<td>wt</td>
<td>132</td>
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<td>5B22F1</td>
<td>male</td>
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<td>220</td>
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<td>5B22F1</td>
<td>male</td>
<td>wt</td>
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</tr>
<tr>
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<td>5B22F1</td>
<td>male</td>
<td>wt</td>
<td>201</td>
</tr>
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</table>
4 Results

4.1 Roscovitine induced Cdk5 inhibition in DRG neurons leads to neurite retraction

Roscovitine is a purine analog of the olomoucine family of cyclin-dependent kinase (Cdk) inhibitors. It is a strong inhibitor of Cdk1, Cdk2, Cdk5 and Cdk7 but not of others and acts by competing with ATP in the active site (Bain et al., 2007; Meijer et al., 1997). Previous data from the lab showed that treating adult dorsal root ganglion (DRG) neurons with Roscovitine leads to MAP1B-dependent axon retraction that involves GSK3β, Rho-associated kinase (ROCK) and myosin but not nNOS activation. Moreover, a Poly-L-lysine (PLL)/laminin-coated surface is required for the effect which does not work on PLL alone (Krupa, 2009). These results were based on PFA-fixed cells and immunofluorescence, although it was shown that similar phenotypes of cell retraction could also be observed in time-lapse microscopy (Krupa, 2009). These observations were, however, not quantified.

Therefore, we wanted to elaborate upon these results and further support them with live-imaging results to deepen our knowledge of the underlying signaling mechanisms. As a consequence the first step was to repeat the experiments dealing with Roscovitine induced axon retraction in adult DRG neurons.

4.1.1 Roscovitine induced neurite retraction can be observed in time-lapse microscopy

Adult DRG neurons were prepared as described [section 2.1] and cultured for 24 h. The microscope stage and live-cell imaging incubation chamber were started 4 h before use to ensure stable conditions and reduce focus shifts due to heat changes in the stage, culture dishes and optics. The cells were left undisturbed for 3.5 h to recover after the 6-well culture dish was transferred onto the microscope. Imaging was started 20 min before treatment in 5 min intervals to monitor cell growth. After treatment with 50 µM Roscovitine or 0.2 % DMSO (as vehicle control) further images were taken in 20 min intervals over the
next 100 min. Neuron behavior after treatment was categorized as growth, retraction or collapse. Figure 4.2 shows examples of these three types of reaction. Time points –20’–0’ in all three columns show growing neurites before treatment (asterisks), which was an essential prerequisite to be counted.

After treatment (time-points 0’–100’) the reactions differ dramatically: Column A shows neurites that continue to grow steadily throughout the duration of the experiment and appear to be completely undisturbed. Neurites in Column B start to retract upon exposure to Roscovitine. The net length of neurites decreases immediately, combined with the formation of retraction bulbs (arrowheads), sinusoidal bundles (thin arrow) and trailing remnants (thick arrow). At time-point 100’ some neurites have completely disappeared while other, as yet extensively branched neurites were reduced to filopodia-like cell protrusions with only a fraction of their former length. Column C shows a neuron that was counted as “collapsed”. Complete loss of membrane integrity in all of its cell protrusions after treatment produced a beads-on-a-string-like appearance of the remaining cellular components that disintegrated further over time (time-points 60’–100’).

Figure 4.1: Cdk5 inhibition leads to increase in neurite retraction in DRG neurons. DRG neurons grown in PLL/Laminin coated plastic 6 well-plates for 24 h were set up in a live imaging chamber providing a humidified atmosphere with 37°C and 5% CO₂ during the experiment. They were monitored by phase contrast microscopy for 20 min, treated with either DMSO (0.2 %) or Roscovitine (50 µM) or left untreated. After further imaging for 100 min in 20 min intervals, solely neurites growing before drug application were quantified after treatment and sorted into categories growth, retraction or collapse, the respective percentages are shown.

About 18 % of neurites of untreated cells showed signs of retraction (Figure 4.1). Addition of the solvent control (DMSO) increased the rate of retraction only slightly to 24 % whereas treatment with Roscovitine more than doubled that number to 56 %. The behavior of neurons was similar to those described by Krupa (2009), but no quantification of live cell imaging was conducted back then. My result can therefore only be compared to the previ-
Figure 4.2: Neurite reaction to Cdk5 inhibition with Roscovitine in living cells. DRG neurons grown in PLL/Laminin coated plastic 6 well-plates for 24 h were set up in a live imaging chamber providing a humidified atmosphere with 37°C and 5% CO₂ during the experiment. They were monitored by phase contrast microscopy for 20 min (time points −20′–0′), treated with either DMSO (0.2%) or Roscovitine (50 µm). After further imaging for 100 min in 20 min intervals (time points 0′–100′), solely neurites growing before drug application were quantified after treatment and sorted into categories growth (column A), retraction (B) or collapse (C).
ous data obtained from fixed cells, saying that 48% of wild-type DRG neurons retracted when exposed to 50 µm Roscovitine. Both results are in the same order of magnitude for total retraction (48 and 56%) while increase from vehicle control to Roscovitine treatment is smaller in my case (+32%) compared to previous results (+47%). In both cases retraction did not increase significantly between untreated samples and vehicle control and only a small percentage of neurons collapsed.

4.1.2 Roscovitine induced neurite retraction can be observed in fixed cells

The experimental data previously gathered in the lab was mainly based on analysis of fixed cells, which was why I also tried to obtain data using the same method. I first wanted to ensure that my results were comparable to those acquired previously in the lab, therefore I repeated the experiment involving Roscovitine induced Cdk5-inhibition.

Cells were acquired as described [section 2.1], cultured on PLL/laminin coated coverslips for 24 h and treated with 50 µm Roscovitine. After fixing with 4% PFA and staining for tubulin, analysis was conducted via fluorescence microscopy. Treating DRG neurons with 50 µm Roscovitine induced distinct changes in growth cone and neurite appearance. The hallmarks of axon retraction (retraction bulbs, sinusoidal microtubule bundles and trailing remnants) could all be seen, however not all of them appeared together at the same time. For the most part retraction bulbs and sinusoidal bundles appeared together whereas trailing remnants were absent in most cases. This could arguably be due to the fact that trailing remnants do not always withstand the manipulations during fixing.

Figure 4.3 (A–C) shows intact neurons that were counted as unchanged. Most cells have long, undisturbed neurites ending in intact, spread growth cones. Compared to these, the retracted cells look clearly different: Neurites have a wavy appearance (F, H, I) while neurite tips lack flat growth cones but seem to end in a filopodium (D, H) or in bulbs (D, F, I). Collapsed neurons (G) are the most drastic reaction to changed environmental conditions or drug treatments. They have completely lost their membrane integrity and look like intertwined beads-on-a-string with many light dots disrupted by regions with no fluorescence signal.
Figure 4.3: Neurite reaction to Cdk5 inhibition with Roscovitine in fixed cells. DRG neurons grown on PLL/Laminin coated glass coverslips for 24 h were treated with either DMSO (0.2%) or Roscovitine (50 µM), fixed with 4% PFA, immuno-stained for tubulin, quantified by fluorescence microscopy and sorted into categories unchanged (column A–C), retracted (D–F, H–I) or collapsed (G). Unchanged neurites were defined as having spread growth cones, intact microtubule filaments and show no sign of retracting neurites, which featured the characteristic hallmarks described as retraction bulbs (D, F), trailing remnants (D, E) and sinusoidal microtubule bundles (F). Cells were counted as either unchanged, retracted or collapsed if the majority of neurites displayed the respective features.

Figure 4.4: Cdk5 inhibition leads to increase in neurite retraction in DRG neurons. DRG neurons grown on PLL/Laminin coated glass coverslips for 24 h were treated with either DMSO (0.2%) or Roscovitine (50 µM), fixed with 4% PFA, immuno-stained for tubulin, quantified by fluorescence microscopy and sorted into categories unchanged, retracted or collapsed, the respective percentages are shown and were tested for independence using Pearson’s Chi Square test.
4 Results

Neurons were counted as retracted when the majority of protrusions exhibited characteristic phenotypes (retraction bulb, sinusoidal bundling, trailing remnant). In case of neurons treated with only DMSO, about 14% showed signs of retraction (Figure 4.4) while exposure to 50 µM Roscovitine in the medium resulted in 39% of neurons retracting. Total retraction as well as relative increase (24.6%) from vehicle control to Roscovitine treatment were lower compared to both the experimental data from Krupa (2009) and my own live-imaging result.

In both experiments an increase of retraction was observed after Roscovitine treatment.

4.2 Custom microscope modification for light sensitive experiments

Since the signaling pathways I was interested in involved on the one hand the production of nitric oxide (NO) by neuronal nitric oxide synthase (nNOS) and S-nitrosylation of proteins such as MAP1B-LC1 (Stroissnigg et al., 2007), and on the other hand soluble guanylyl cyclase (sGC), a target of NO-signaling that is also implicated in axon guidance events (Dickson, 2002), the fact that S-nitrosothiols are light sensitive and release NO upon exposure to visible light (Sexton et al., 1994) was taken into consideration. Previous experiments involving NO-donors such as S-nitroso-N-acetylpenicillamine (SNAP) were conducted in the dark (Stroissnigg et al., 2007), thus to also provide constant experimental conditions and rule out as many uncontrollable factors as possible, we decided to perform further experiments in complete darkness.

Therefore, I constructed a custom built cover from cardboard, black paper and a lanyard (Figure 4.5) that was compatible with the Zeiss AxioObserver Microscope used. It completely blocked any direct light from the computer screen and hallway lights while also absorbing reflective light produced during experiments with its blackened side facing the cells. It did not interfere with the microscope stage or the specimen in any way.

In addition to this setup, the experiments were conducted in complete darkness, except for a darkroom lamp which provided just enough red light to be able to perform necessary manipulations during treatments. Furthermore, I ensured that exposure to light was minimized in general, and set the necessary exposure times for each taken picture to the
Figure 4.5: Microscope setup for light sensitive experiments. The microscope was adapted for light sensitive reactions to ensure complete blocking of ambient light during experiments (A) while allowing access to the 6well-plate in the incubation chamber for necessary manipulations (B). A cardboard shield of appropriate size was cut out and lined with blackened paper around the edges as well as on the inside facing the cells (C) to ensure complete absorption of scattered light originating from each exposure. The plate was attached to the condenser from the bottom and fixed with a lanyard from the top (D).

lowest possible but still usable setting and increased the intervals for taking pictures after treatment to 20 min from 5 min before treatment.
4.3 Investigating a possible cGMP dependent feedback loop involving nNOS

Neuronal nitric oxide synthase (nNOS) plays an integral part in NO-signaling in the nervous system. After stimulation by Ca\(^{2+}\) it catalyses the synthesis of nitric oxide (NO) from L-arginine and oxygen. It is involved in various physiological and pathophysiological processes (Förstermann and Sessa, 2012). It was previously shown that Ca\(^{2+}\)-induced activation of nNOS leads to axon retraction via S-nitrosylation of MAP1B-LC1. This effect was reproduced by replacing this endogenous NO-source with the direct NO donor SNAP (Stroissnigg et al., 2007). However, S-nitrosylation of proteins is not the only way in which nitric oxide can influence axon movement. NO targets sGC, which is also implicated in axon guidance (Descovich, 2009; Dickson, 2002; Stroissnigg et al., 2007) and growth cone turning (Tojima et al., 2009). Upon activation by NO, sGC increases production of cyclic guanosine monophosphate (cGMP) manyfold (Poulos, 2006), which by itself plays a role in neurite growth and growth cone mobility (Murray et al., 2009) as well as axon branching (Zhao et al., 2009). Evidence for a previously suggested (Dinerman et al., 1994) negative feedback loop from cyclic-GMP dependent protein kinase (PKG) to nNOS was found by former members of the lab (Descovich, 2009; Krupa, 2009; Stroissnigg et al., 2007). Again, these results were based on PFA-fixed cells and immunofluorescence. Additional data from live-imaging to supplement these end-point analyses was missing.

4.3.1 Lack of significant effects of sGC inhibition in time-lapse microscopy

Direct inhibition of sGC with LY83583 and ODQ was compared to Ca\(^{2+}\)-induced axon retraction by Calcimycin. Adult DRG neurons were prepared as described (section 2.1) and cultured for 24 h. The microscope stage and live-cell imaging incubation chamber were started 4 h before use to ensure stable conditions and reduce focus shifts due to heat changes in the stage, culture dishes and optics. The cells were left undisturbed for 3 h to recover after the 6-well culture dish was transferred onto the microscope. Imaging was started 20 min before treatment in 5 min intervals to monitor cell growth. After treatment with either 0.1% DMSO (as vehicle control), 1 or 3 µM Calcimycin, 10 µM ODQ, 2 µM LY83583 or medium alone, further images were taken in 20 min intervals over the next 40–100 min. Neuron behavior after treatment was categorized as growth, retraction or collapse.
The results in Figure 4.6 show that about 25% of cells showed signs of retraction in both untreated (medium without any drugs added) and vehicle control samples. Treatment with 1 µm Calcimycin led to 29.3% of neurites retracting. Increasing the concentration of Calcimycin to 3 µm caused a reduction of this number to 18.2% but induced widespread collapsing activities: 34.3% of all cell protrusions that were growing (and therefore fell into the category of assessed cells) before treatment, disintegrated afterwards. Both, the exposure to ODQ and LY83583 were devoid of any significant effect compared to vehicle treatment. Retraction was measured as 25.1 and 28.3%, respectively. In case of ODQ, however, 9.3% of cells collapsed.

![Graph](image.png)

**Figure 4.6: Lack of significant effects of sGC inhibition in time-lapse microscopy.** DRG neurons grown in PLL/Laminin coated plastic 6 well-plates for 18–21 h were set up in a live imaging chamber providing a humidified atmosphere with 37°C and 5% CO₂ during the experiment. They were monitored by phase contrast microscopy for 20 min, treated with either DMSO (0.1%), Calcimycin (1 or 3 µm), ODQ (10 µm), LY83583 (2 µm) or left untreated. After further imaging for 40–100 min in 20 min intervals, solely neurites growing before drug application were quantified after treatment and sorted into categories growth, retraction or collapse. Data from three (untreated, LY83583) or four (DMSO, Calcimycin, ODQ) individual experiments was combined, the respective percentages are shown as mean values ± SEM.

### 4.3.2 Ca²⁺-induced axon retraction is not influenced by sGC inhibition

After the ambiguous results of time-lapse microscopy I set up similar experiments using immunocytochemistry. Cells were acquired as described (section 2.1), cultured on PLL/laminin coated coverslips for 20–23 h and treated with either 0.1% DMSO, 10 µm Calcimycin, 2 µm LY83583 or both. After fixing with 4% PFA and staining for tubulin, analysis was conducted via fluorescence microscopy. Neurons were counted as retracted when the majority of protrusions exhibited characteristic phenotypes (retraction bulb, sinusoidal bundling, trailing remnant).
In case of neurons treated with only DMSO, 10% showed signs of retraction [Figure 4.7] while exposure to 10 µm Calcimycin in the medium resulted in a significant increase to 26%. Both, a combination of Calcimycin and LY83583 as well as treatment with LY83583 alone reduced retraction to about 17% and 13%, respectively. These changes were not significant but showed a tendency in the opposite direction of the results acquired by Descovich (2009). Less than 3% collapsed neurons were observed in all the samples, which is closer to what was previously observed in the lab but in stark contrast to the behavior observed by me by live-cell imaging.

![Figure 4.7: Ca\(^{2+}\)-induced axon retraction is not influenced by sGC inhibition.](image)

**Figure 4.7:** Ca\(^{2+}\)-induced axon retraction is not influenced by sGC inhibition. DRG neurons grown on PLL/Laminin coated glass coverslips for 20–23 h were treated with either DMSO (0.1%), Calcimycin (10 µM), LY83583 (2 µM) or both, fixed with 4% PFA, immuno-stained for tubulin, quantified by fluorescence microscopy and sorted into categories unchanged, retracted or collapsed. Data of two separate treatments in two individual experiments were combined, the respective percentages are shown as mean values ± SEM.

### 4.3.3 Absence of significant effects of nNOS inhibition in time-lapse microscopy

Inhibition of nNOS with N-ω-propyl-l-arginine (NPA) in the presence or absence of Calcimycin was assayed to investigate effects on neurite growth. Adult DRG neurons were prepared as described [section 2.1] and cultured for 23–27 h. The microscope stage and live-cell imaging incubation chamber were started 4 h before use to ensure stable conditions and reduce focus shifts due to heat changes in the stage, culture dishes and optics. The cells were left undisturbed for 3 h to recover after the 6-well culture dish was transferred onto the microscope. Imaging was started 20 min before treatment in 5 min
intervals to monitor cell growth. After treatment with either 0.1 % DMSO (as vehicle control), 3 or 5 µM Calcimycin, 300 µM NPA or both, further images were taken in 20 min intervals over the next 40 min. Neuron behavior after treatment was categorized as growth, retraction or collapse.

Retraction of neurites subjected to vehicle control DMSO was with 26.3 % (Figure 4.8) again in the range of previous experiments. Treatment with 3 or 5 µM Calcimycin induced cell collapse of up to 32% in one experiment but left neurite retraction unchanged in comparison to DMSO. NPA treatment did lead to a decrease in retraction to 12 %, compared to vehicle treatment, however this reduction was not significant. Combined treatment with both substances manifested in a return of the number of retracting neurons to the range of DMSO-levels (28.1 %). Interestingly, no collapsed neurons were observed in this case, although the cells were exposed to NPA in addition to Calcimycin.

![Figure 4.8: Absence of significant effects of nNOS inhibition with NPA in time-lapse microscopy. DRG neurons grown in PLL/Laminin coated plastic 6 well-plates for 23–27 h were set up in a live imaging chamber providing a humidified atmosphere with 37°C and 5 % CO₂ during the experiment. They were monitored by phase contrast microscopy for 20 min, treated with either DMSO (0.1 %), Calcimycin (3 or 5 µM), NPA (300 µM) or both. After further imaging for 40 min in 20 min intervals, solely neurites growing before drug application were quantified after treatment and sorted into categories growth, retraction or collapse. Data from two individual experiments was combined, the respective percentages are shown as mean values ± SEM.]

4.3.4 Ca²⁺-induced axon retraction is unaffected by nNOS inhibition

The previous experiment involving the inhibition of nNOS with NPA in the presence or absence of Calcimycin was repeated using immunocytochemistry. Cells were acquired as described (section 2.1), cultured on PLL/laminin coated coverslips for 23 h and treated
with either 0.1 % DMSO, 10 µm Calcimycin, 300 µm NPA or both. After fixing with 4 % PFA and staining for tubulin, analysis was conducted via fluorescence microscopy.

Neurons were counted as retracted when the majority of protrusions exhibited characteristic phenotypes (retraction bulb, sinusoidal bundling, trailing remnant). In case of neurons treated with only DMSO, 9.6 % showed signs of retraction while exposure to 10 µm Calcimycin in the medium resulted in 20.6 % of neurons retracting (Figure 4.9). Exposing cells to 300 µm NPA did not increase retraction, compared to vehicle control while the combined treatment with Calcimycin and NPA produced a significant increase in axon retraction to 28.1 %. This was slightly more than Calcimycin alone (+7.5 %) but significantly more than DMSO (+18.5 %) and NPA alone (+17.7 %).

In both experiments involving nNOS inhibition it appeared that treatment with NPA alone shows a tendency to a decrease in axon retraction compared to Calcimycin treatment, but it does not in the presence of Calcimycin. This contradicts previous results (Krupa, 2009; Stroissnigg et al., 2007).

Figure 4.9: Inhibition of nNOS with NPA does not prevent Ca\(^{2+}\)-induced axon retraction. DRG neurons grown on PLL/Laminin coated glass coverslips for 23 h were treated with either DMSO (0.1 %), Calcimycin (10 µM), NPA (300 µM) or both, fixed with 4 % PFA, immuno-stained for tubulin, quantified by fluorescence microscopy and sorted into categories unchanged, retracted or collapsed, the respective percentages are shown and were tested for independence using Pearson’s Chi Square test.
4.4 Effects of Semaphorin 3A on primary cortical neurons

The secreted guidance cue Semaphorin3A is of crucial importance during development with severe phenotypes in case of its absence (Behar et al., 1996). Semaphorin3A is a repellent guidance cue for axons (Dickson, 2002) and attracts dendrites of pyramidal neurons (Polleux et al., 2000). Its repulsive effect on growth cones can be converted into an attractive one by cGMP (Song et al., 1998). Expression of soluble guanylyl cyclase varies in axons and dendrites, giving rise to differences in local cGMP-levels which presumably lead to the opposing effect of exposure to Semaphorin3A in axons and dendrites (Polleux et al., 2000). Several kinases that were already mentioned previously such as PKA andPKG (Dontchev and Letourneau, 2002) as well as GSK3β and Cdk5 (Roth et al., 2009) are implicated in Semaphorin3A signaling as well. These findings made it an obvious candidate for investigation concerning its involvement in nitric oxide signaling.

After treatment with Semaphorin3A for 2 to 3 days, a MAP1B dependent reduction in neurite outgrowth was previously observed in the lab in hippocampal and DRG explants, but not dissociated cells of either hippocampal or DRG origin (Descovich, 2009). Semaphorin3A treatment with simultaneous inhibition of nNOS returned the neurite outgrowth back to untreated levels in explants, whereas in case of dissociated cells no effects were observed. Most of those experiments were performed with treatments over the course of several days except those involving dissociated cells in which case they were fixed and assessed after 30 min of treatment. The lack of an observable effect in dissociated neurons as opposed to the significant effects in explants led to the conclusion that dissociated neurons do not respond to treatment with Semaphorin3A in this way.

Since many groups showed that Semaphorin3A induces immediate reactions in mouse neurons (Brown and Bridgman, 2009; Li et al., 2009; Schlatter et al., 2008), we reasoned that a putative immediate effect induced by Semaphorin3A in dissociated neurons may have been missed in the previous experimental design. Thus I set up experiments with short treatment periods (15–60 min) using growth cone collapse as a readout. The intention was to find a method for assessing short-term effects on growth cones that could ultimately also be transferred to a live-imaging setup, such as in Kapfhammer et al. (2007). They describe protocols for assessing growth cone collapse in both time-lapse and immuno-fluorescence microscopy using the same neuron source. I also wanted to check in my experiments if a difference in growth cone collapse was observable if axons were compared to the total numbers of neurites.
4 Results

4.4.1 Primary cortical neurons were successfully cultured and stained

The preparation and immuno-staining of mouse cortical neurons was first learned from colleagues and further optimized. Seeding density proved to be of crucial importance. Neurons grew poorly if the seeding density was lower than 40000 cells per 13 mm coverslip, arguably due to lacking paracrine support from glia cells. When the cells grew too densely, however, it was impossible to assess growth cones for their appearance. Also, the experimental prerequisite that assessed cells may not touch others was hardly ever met and therefore too few cells per coverslips could be counted. A density of 50000 cells per 13 mm coverslip proved to be a good balance between cell health and assessability and was repeatedly used in the following experiments.

Cells were acquired as described (section 2.2) and cultured on PLL/laminin coated coverslips for 60 h. After fixing with 4 % PFA and staining for $\beta_{III}$-tubulin, actin and DNA (Hoechst) analysis was conducted via fluorescence microscopy.

![Figure 4.10](image)

Figure 4.10 shows fixed cells immuno-stained for actin (red), $\beta_{III}$-tubulin (green) and DNA (blue). Neurons (A–D) express $\beta_{III}$-tubulin and are clearly discernible from glial cells (C, D, arrowheads) that do not, which is indicated by the absence of tubulin-staining (green). Neurons showed signs of differentiation, as described in literature. Barnes and Polleux (2009) characterize Stage 1 of cortical neuron polarization as having short protrusions mainly originating from actin activity (lamellipodia, filopodia) which fits the cell shown in Figure 4.10 (A).

![Figure 4.10](image)

Figure 4.10 (B) shows a cell corresponding to Stage 2, with neurites that are approximately the same length but were already formed with the involvement of the microtubule machinery, apparent by the intense green staining along the length of the protrusion. Growth cones were categorized as either intact (B–D, thick arrows) or collapsed (B, C, thin arrows). Collapsed growth cones were defined as having no lamellipodia and at most two filopodia while at the same time the neurite tips stained positive for tubulin.

Neurons in (C, D) exhibit features characteristic for symmetry break (Stage 3), where one neurite grows ahead of the others and develops into the axon. According to Barnes and Polleux (2009) this happens after 2–4 d in culture in case of cortical neurons, which is in line with the time these neurons had to grow (60 h = 2.5 d).
Figure 4.10: Cultured primary cortical neurons show stages of polarization and are distinguishable from glial cells. Cortical neurons grown on PLL/Laminin coated glass coverslips for 60 h fixed with 4% PFA, immuno-stained for βIII-tubulin, actin and DNA (Hoechst) and analyzed by fluorescence microscopy. Growth cones were categorized as intact (B–D, thick arrows) or collapsed (B–C, thin arrows). Glia cells were distinguishable from neurons due to their lack of tubulin-staining (C–D, arrowheads). Axons (D) were defined as being at least twice as long as the second longest neurite of the same cell.

4.4.2 Treatment with 100 ng/ml Semaphorin3A does not alter growth cone appearance

The concentration of 100 ng/ml Semaphorin3A that I first used was also applied previously by Descovich (2009) but her experimental setup was different. Previously, the cells were cultured in media supplemented with Semaphorin3A for several days whereas I used short periods (0–30 min) for treatments to observe potential immediate effects on growth cones.

Cells were acquired, analyzed and counted as described before.
Figure 4.11: Treatment with 100 ng/ml Semaphorin3A does not significantly alter growth cone appearance. Cortical neurons grown on PLL/Laminin coated glass coverslips for 60 h were treated with Semaphorin3A (100 ng/ml), fixed with 4% PFA, immuno-stained for βIII-tubulin, actin and Hoechst and analyzed by fluorescence microscopy. Growth cones were assessed individually and defined as collapsed when no lamellipodia and at most two filopodia were present while at the same time the neurite tips stained positive for tubulin. Axons (b) were counted when clearly identifiable, defined by being at least twice as long as the second longest neurite of the same cell. Data of two separate treatments in two individual experiments were combined, the respective percentages are shown as mean values ± SEM.

Figure 4.11(a) shows the results of treatment with 100 ng/ml Semaphorin3A compared to untreated samples. Growth cones collapsed in 55% of cells if left untreated and 52% or 53% after treatment for 15 or 30 min, respectively. Significant differences were not observed, neither when all neurites nor when axons only were considered. Slightly less axons exhibited collapsed growth cones when left untreated (48%, Figure 4.11(b)) whereas after treatment for 15 or 30 min these numbers changed to 37 and 54%, respectively.
4.4.3 Bath application of 500 ng/ml Semaphorin3A induces growth cone collapse

Despite the mechanistic differences to previous experiments I was convinced that a Semaphorin3A-induced effect on growth cones should be observable with this approach. Examples of the application of higher concentrations in literature prompted me to repeat the experiments with an increased concentration of 500 ng/ml and also try a later time point for fixation (60 min). Manns et al. (2012) used up to 700 ng/ml for 30 min on chick DRG neurons whereas Brown and Bridgman (2009) treated embryonic mouse DRG neurons with 500 ng/ml for 30 min to assess growth cone collapse. Nangle and Keast (2011) saw Semaphorin3A-induced growth cone collapse after as much as 60 min in rat DRG neurons. These changes would still allow to adapt the setup for live-imaging in future experiments to acquire additional data.

Figure 4.12(a) shows the results of treatment with 500 ng/ml Semaphorin3A compared to untreated samples. Again, about half of the observed growth cones collapsed 55% in the untreated sample (53%) which did not increase significantly after 15 min (57%). After 30 min, however, growth cone collapse increased significantly to 70% and remained at a high 61% 30 min later. When only axons were counted (Figure 4.12(b)) this picture changed slightly, as the untreated collapse decreased to 40%. Growth cone collapse was also very high after 30 min, however, this increase was not calculated as significant.
Figure 4.12: Bath application of 500 ng/ml Semaphorin3A induces growth cone collapse after 30 min. Cortical neurons grown on PLL/Laminin coated glass coverslips for 60 h were treated with Semaphorin3A (500 ng/µl), fixed with 4 % PFA, immuno-stained for βIII-tubulin, actin and Hoechst and analyzed by fluorescence microscopy. Growth cones were assessed individually and defined as collapsed when no lamellipodia and at most two filopodia were present while at the same time the neurite tips stained positive for tubulin. Axons were counted when clearly identifiable, defined by being at least twice as long as the second longest neurite of the same cell. Data of two separate treatments in two individual experiments were combined, the respective percentages are shown and were tested for independence using Pearson’s Chi Square test.

A direct comparison of growth cone collapse in axons and total neurites does not reveal any significant differences between axons and total neurites of cultured dissociated cortical neurons after short-term treatment with 100 (a) or 500 ng/ml (b) Semaphorin3A.
Figure 4.13: Growth cone collapse after treatment with 100 (a) or 500 ng/ml (b) Semaphorin3A does not differ between axons and total neurites. Cortical neurons grown on PLL/Laminin coated glass coverslips for 60 h were treated with Semaphorin3A, fixed with 4% PFA, immuno-stained for βIII-tubulin, actin and with Hoechst and analyzed by fluorescence microscopy. Growth cones were assessed individually and defined as collapsed when no lamellipodia and at most two filopodia were present while at the same time the neurite tips stained positive for tubulin. Axons were counted when clearly identifiable, defined by being at least twice as long as the second longest neurite of the same cell.
5 Discussion

This diploma work was an attempt to gain new insights into several different aspects of neurite growth and growth cone behavior involving nitric oxide and cyclic nucleotide signaling. For that reason, several ways to influence the signaling pathways of nitric oxide synthase and soluble guanylyl cyclase were assessed in different model systems. Among the goals was the necessity to reproduce data of previous lab members using their methods as a starting point for further experiments. Additional data acquired using different methods should then be used to strengthen previous results and answer further questions in the same areas previously approached. Putative delicate and/or short-lived phenomena involving growth cone appearance, mobility or even collapse were hard to observe with the hitherto primarily used experimental readout for neurite growth, namely fluorescence microscopy after PFA fixation and immuno-staining. Therefore, a robust and reproducible live-imaging protocol that promised to help gain insight into immediate and short-term effects of growing neurites was needed.

5.1 Establishing the method

Axon retraction induced by inhibition of Cyclin-dependent kinase 5 was the topic of extensive research in the lab (Krupa, 2009). Ncdk, the holoenzyme comprised of Cdk5 and it’s neuronal activator p35, is responsible for phosphorylation of several cytoskeletal targets and is important during neuronal development, migration and axon formation (Paglini and Cáceres, 2001). One such target is MAP1B which, upon phosphorylation, exhibits changed binding to microtubules and thereby influencing microtubule dynamics. The previous data showed that inhibition of Cdk5 with Roscovitine leads to MAP1B-dependent axon retraction that involves GSK3β, ROCK and myosin but not nNOS activation. Moreover, a PLL/laminin-coated surface is required for the effect which does not work on PLL alone (Krupa, 2009).

I confirmed that wild-type DRG neurons retracted after treatment with 50 µm Roscovitine and my data, acquired from time-lapse and fluorescence microscopy, is in accordance to
previous findings in both cases. An increase of retraction to 56% was observed in live cell-imaging while 39% of neurons were counted as retracting after fixation and immunostaining. The canonical hallmarks of retraction described elsewhere (Ebner, 2012; He et al., 2002; Orlova et al., 2007; Stroissnigg et al., 2007) were identified in both living and fixed cells. Neurites readily reacted to treatments by formation of retraction bulbs, sinusoidal microtubule bundles and trailing remnants. The experiments were insofar successful as the primary goal, to reproducibly induce axon retraction in cultured neurons and be able to detect and count retracted neurons, was met. However, retraction in cells left untreated or treated only with the solvent control DMSO were higher in my experiments, compared to almost zero in previous findings (Krupa, 2009; Stroissnigg et al., 2007). A reason for that was thought to be the lack of experience with handling of primary neurons as opposed to a pivotal difference in experimental execution. It is also known that neuron cultures suffer dramatically from hyperosmolarity of the culture medium (Potter and DeMarse, 2001) which may easily be caused by water evaporation. This is mainly an issue in long-term cultures but may also be an important aspect for shorter culture-periods in incubators that can not adequately keep constant humidity levels or when opened too frequently. A further possible explanation for the differences may lie in the scoring procedure. Although everything was done to ensure that the previously applied classification of neurites and whole neurons was adopted, absolute consensus could only have been guaranteed by working side-by-side on the same samples while carefully attuning the assessment routine. It may therefore be argued that counting of the phenotypes was not done in a completely congruent fashion.

Nevertheless, results were deemed acceptable because neurite retraction was not significantly different in untreated and vehicle treated cells, even if they were unusually elevated in the first place. As a consequence, the relative increase of retraction from vehicle to Roscovitine treatment was lower in fixed (+24.6%) and live cells (+32%) when compared to previous results by Krupa (2009) (+47%). Completely collapsed cells, indicated by total loss of membrane integrity and dissipation of the neurites into bead-like swellings, was observed previously (Descovich, 2009; Ebner, 2012; Krupa, 2009; Stroissnigg et al., 2007) and also in my experiments. The frequency of occurrence in untreated cells was used as a measure for cell health for it noticeably increased in case of bad culture conditions.

Taken together, it was concluded that the results were a suitable proof-of-concept for the method and sufficiently reproducible for further application.
5.2 Lack of supporting evidence for putative feedback loop from PKG to nNOS

A previously suggested negative feedback loop from cyclic-GMP dependent protein kinase (PKG) to neuronal nitric oxide synthase (nNOS) suggesting a backwards inhibitory effect of PKG, a downstream target of soluble guanylyl cyclase (sGC), on nNOS (Dinerman et al., 1994) was substantiated by results of former members of the lab (Descovich, 2009; Tranciková, 2007). I tried to gain further insight into the finely tuned interplay between the participating proteins by observing the growth of cultured neurons after inhibition or stimulation of several of the participants in the proposed signaling cascade.

Calcium ionophore Calcimycin was used in the lab before to increase intracellular levels of Ca\(^{2+}\) and I closely followed those protocols. Unlike then, I was unable to apply Calcimycin in time-lapse microscopy at a concentration of 10 \(\mu\)M due to widespread cell death. Lower concentrations did not show a significant increase in axon retraction compared to vehicle control. The variation between experiments was much higher for cell collapse than for neurite retraction. A slight increase in concentration could sometimes induce a disproportionate increase in collapsing cells, suggesting a highly unfavorable impact of Calcimycin on cultured DRG neurons. This was in stark contrast to previous data from the lab (Krupa, 2009), which was based on analysis of fixed cells. There, treatment with 10 \(\mu\)M Calcimycin usually led to about 40% of neurons showing signs of retraction. Interestingly, when I also assessed fixed cells my results were closer to those previous observations. The fraction of collapsed cells was never as high in immuno-stained samples as in live-cell imaging and the results were more consistent between experiments compared to time-lapse imaging. Also, application of 10 \(\mu\)M Calcimycin induced a significant increase in neurite retraction.

The striking difference in cell collapse is interesting for various reasons. It can be argued that the cultured cells should grow and react in a comparable way due to the similar culture conditions and treatments, but there must still be some difference that leads to the different results. The PFA-fixation protocol corresponds to an end-point observation of the processes that are observed with time-lapse microscopy while they are happening, thus a possibly drastic initial reaction is only seen in the latter. Furthermore, collapsed cells may be washed away with the multiple washing steps during the PFA-fixation protocol and therefore not seen in the immuno-stained samples. Dead cells are mostly missed out on in
the fixation protocol and only those cells that survive are assessed for their configuration. As a consequence, the two protocols emphasize different phenotypes although the cells are otherwise cultured and treated similarly putting a bias on either outcome depending on the method used.

This may be important, because the increase of intracellular Ca\textsuperscript{2+}-levels may not be the only consequence of Calcimycin treatment due to a potential inherent cytotoxic effect described in the following examples. Bath application of Calcimycin leads to significant cell death in rat hippocampal slice cultures (Bickler and Fahlman, 2004) and retinal ganglion cells (RGC-5) (McKernan et al., 2007). A calcium-independent increase in cell death after Calcimycin treatment was observed in MSN neuroblastoma cells (Chan et al., 1998). These cytotoxic or pro-apoptotic effects of Calcimycin may further reduce viability in case of subpar culture conditions.

To investigate the influence of sGC activity on axon retraction I used the inhibitors LY83583 and ODQ in my experiments. Previously, a MAP1B-dependent increase in axon retraction after sGC-inhibition was observed (Descovich, 2009) and attributed to the lacking feedback inhibition of PKG towards nNOS that was proposed by Dinerman et al. (1994). Evidence suggested that further production of nitric oxide enabled continuing S-nitrosylation of MAP1B, culminating in axon retraction. Previously, significantly increased axon retraction was observed in wild-type neurons after treatment with LY83583 and ODQ alone (Descovich, 2009). Simultaneous inhibition of nNOS returned the number of retracting cells back to untreated levels which further substantiated the feedback model. These data were obtained using fixed cells, so in order to support these findings with live-imaging data I started by repeating the experiments for sGC-inhibition using time-lapse microscopy. However, my results did not show hints of the feedback loop. No significant changes in axon retraction could be observed after treatment with either LY83583 or ODQ. Based on the similar results of time-lapse and fluorescence microscopy in case of Cdk5-inhibition, inhibition of sGC with LY83583 was compared to Ca\textsuperscript{2+}-induced axon retraction using Calcimycin following the fixed-cell protocol. But again, treatment with LY83583 had no effect, neither in the presence nor absence of Calcimycin. Similar to before, counted numbers were generally lower when fixed cells were analyzed as opposed to the evaluation of time-lapse videos. Nevertheless, the insignificant relative change in axon retraction induced by LY83583 treatment was virtually identical (+3 % increase compared to vehicle) in both datasets. These results contradicted previous findings by Descovich
Discussion

(2009), where axon retraction was significantly increased after treatment with LY83583 by about 30%.

NPA was used before to directly inhibit nNOS, a target of the cGMP-activated kinase PKG. To be able to further delve into it’s involvement in axon retraction I first had to set up the basis for further experiments by repeating previous approaches (Krupa, 2009; Stroissnigg et al., 2007). They showed that inhibition of nNOS with NPA reduced Calcimycin-induced axon retraction in wild-type DRG neurons, demonstrating that activation of nNOS is involved in Ca²⁺-induced axon retraction. This was contrary to my observations in time-lapse microscopy, for I could not see any significant effects on axon retraction that was attributable to NPA treatment. One reason for that may have been the reappearing difficulties with Calcimycin treatment that led to highly fluctuating numbers of collapsing cells, thereby potentially masking other phenotypes. NPA had no significant effect when applied alone and did not inhibit a suggested but insignificant increase in axon retraction by Calcimycin when applied together. Similar results were obtained by repeating these treatments in an assay using PFA-fixation and immuno-staining. The faint suggestion that NPA was not able to inhibit Ca²⁺-induced axon retraction was thereby solidified. The percentage of cells exhibiting signs of retraction was indistinguishable when comparing NPA to vehicle treatment but showed a significant increase in the concurrent presence of Calcimycin.

As to why I was not able to find supporting evidence for the supposed feedback loop, I have no definite answers. The complexity of signaling cascades is difficult to grasp as a whole and mostly reduced to the necessary essentials when one tries to investigate them. This may also be the case in the present example potentially missing other influences. Several key enzymes have a multitude of interaction partners and modes of action which are the topic of ongoing research (Förstermann and Sessa, 2012; Nossaman et al., 2012; Steinert et al., 2010). One such finely regulated key enzyme, nNOS, is targeted by several kinases leading to changes in activity after phosphorylation (Garthwaite, 2008). These changes are, as reported there, not completely understood and the findings are sometimes even conflicting. For example, Bredt et al. (1992) observe reduced nNOS activity after PKC-induced phosphorylation whereas an increase is reported by Nakane et al. (1991). The importance of nNOS for DRG neurons presumably decreases with age, as indicated by reduced expression in adults (Tojima et al., 2009). This could indicate significantly different levels of nNOS in cultured DRG neurons, leading to variations in responsiveness.
to Ca\(^{2+}\)-signals and NO-production. Even when only considering my experiments, the age at culling of the used mice ranged from 101 to 227 days. This range should increase further when consolidated with previous data.

In light of the fact that NO acts as the most important activator of sGC (Derbyshire and Marletta, 2012; Jurado et al., 2004) an unexpected initial reaction of nNOS to increased Ca\(^{2+}\)-levels would further translate and amplify through cGMP-signaling. sGC is a target for S-nitrosylation which leads to desensitization to NO stimuli as soon as after the first exposure (Derbyshire and Marletta, 2012) potentially posing consequences for long-term experiments involving continued stimulation of nNOS or direct NO donors.

Another thought that arose during my thesis-work was, that some unknown but fundamental experimental parameter was different to the years before. In cell culture, it is generally advisable to maintain stable conditions throughout a series of linked experiments to guarantee reproducible results, for example by using sera from the same batch. I took great care to apply methods and protocols as closely matched to before as possible but the drastically diverging results persisted. Many previous findings could not be repeated or were even contradicted by my results.

One potential source of interference was thought to be the extracellular matrix protein laminin, which was used for coating all culture dishes and glass coverslips. Laminin has particular important for neuronal outgrowth and axon guidance (Luckenbill-Edds, 1997). The propagation of Ca\(^{2+}\) signals is modulated by intracellular levels of NO and cGMP which in turn depend on laminin (Tojima et al., 2009). This was substantiated by Descovich (2009) who describes that the presence of Laminin is necessary for Calcimycin-induced axon retraction. The presence of Laminin-1 can reverse attraction to repulsion towards Netrin-1 (Höpker et al., 1999) which was shown to be dependent on nNOS (Descovich, 2009). Krupa (2009) showed that CaMK-II is not involved in Calcimycin induced axon retraction. Nevertheless, an influence of CaMK-II on axon stability and turning after activation by laminin was described by Easley et al. (2006). Soluble laminin in the culture medium increased branching while reducing organelle transport and growth of primary neurites of cultured mouse DRG neurons (Kohno et al., 2005). Concentration changes in surface-bound laminin were shown to alter directional growth in rat hippocampal neurons (Xing et al., 2010). This specifically demonstrates that small changes in experimental conditions have the power to dramatically change the behavior of neurons and thereby influence experimental outcome. It is conceivable that by manually coating culture dishes
such differences are unknowingly incorporated, exposing the growing neurites to different concentrations than in previous experiments, thereby greatly altering the result. It is well known among users of cell culture that different batches of cell culture supplies, such as serum, can result in unexpected problems such as in cell growth or behavior to treatments. It is therefore conceivable that also differences in production between batches of laminin that are unknown to the user may influence results.

5.3 Influence of Semaphorin3A on growth cone conformation

The final part of this thesis work dealt with a possible influence of Semaphorin3A treatment on growth cone behavior in neonatal mouse cortical neurons. The choice of cortical neurons as a model system was due to a different ongoing project in the lab and for potential comparability of the results. I also wanted to learn how to prepare and culture neonatal cortical neurons which I successfully accomplished. It was possible for me to assess growth cone appearance by immuno-staining for βIII-tubulin and actin.

Semaphorin3A is a repellent guidance cue for axons (Dickson, 2002) but can exert attractive properties as well (Polleux et al., 2000). It was deemed to have implications in nitric oxide-signaling before and thus topic of previous investigation. A MAP1B-dependent reduction in neurite outgrowth was observed in hippocampal and DRG explants but no effect was observed in dissociated cells of either origin after treatment with Semaphorin3A (Descovich, 2009).

I repeated the treatments on cortical neurons and assessed growth cone appearance in comparison to untreated cells. The combined data of treatments with 100 ng/ml Semaphorin3A lacks any observable effect. Growth cone collapse was around 50 % whether cells were treated or not. Increasing the concentration to 500 ng/ml Semaphorin3A significantly elevated growth cone collapse at time points 30 and 60 min to about 70 %. This suggests that higher concentrations of Semaphorin3A may be needed to induce effects in these dissociated cultures. However, this experiment was not repeated, therefore no certain point can be made. Other groups have used as much as 500 ng/ml (Manns et al., 2012; Nangle and Keast, 2011) or 700 ng/ml (Brown and Bridgman, 2009) successfully. Curiously, the amount of Semaphorin3A-induced growth cone collapse reported in literature (Manns et al., 2012; Nangle and Keast, 2011) was similar to my own findings, as opposed to the results for untreated samples which were always higher in my case. The relative increase
from this basal level was thus lower than compared to those other results. Descovich (2009) saw significant effects in explant cultures only, which may fit an observation of Manns et al. (2012), who say that involvement of GSK3β in Semaphorin3A-dependent growth cone collapse is subjected to a concentration-based choice of different signaling-pathways. Their model suggests a difference for high or low concentrations of Semaphorin3A which may arguably exist between explant culture, where cells remain embedded in their surrounding tissue, and dissociated culture, where single cells are exposed to the medium directly.

Taken together my results are not yet conclusive. A proposed model of direct signaling via sGC and cyclic GMP with implications to growth cone collapse (Nangle and Keast, 2011) points to a possible connecting to previous data from the lab concerning nitric oxide signaling and MAP1B dependent axon retraction. In light of this suggested mechanism one could look at Semaphorin3A-signaling with respect to the sGC signaling cascade and may clarify similarities or differences between growth cone collapse and axon retraction in cortical neurons and dorsal root ganglions.
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>°C</td>
<td>degree Celsius</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CaMK-II</td>
<td>Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II</td>
</tr>
<tr>
<td>Cdk</td>
<td>cyclin-dependent kinase</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>CN</td>
<td>cortical neuron</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>ddH\textsubscript{2}O</td>
<td>double deionized water</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DRG</td>
<td>dorsal root ganglion</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>FCS</td>
<td>foetal calf serum</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-[2-hydroxyethyl]piperazine-N’-[2-emanesulphonic acid]</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s balanced salt solution</td>
</tr>
<tr>
<td>HS</td>
<td>horse serum</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Dalton</td>
</tr>
<tr>
<td>LPA</td>
<td>lysophosphatidic acid</td>
</tr>
<tr>
<td>LY83583</td>
<td>6-(phenylamino)-5,8-quinolinedione</td>
</tr>
<tr>
<td>MAP</td>
<td>microtubule-associated protein</td>
</tr>
<tr>
<td>m</td>
<td>molar</td>
</tr>
<tr>
<td>MT</td>
<td>microtubule</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NPA</td>
<td>N-ω-propyl-L-arginine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>ODQ</td>
<td>1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>pH</td>
<td>potentium hydrogenii</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
</tbody>
</table>
| PLL          | Poly-
-lysine Hydrobromide |
| PNS          | peripheral nervous system |
| RNA          | ribonucleic acid |
| RT           | room temperature |
| sGC          | soluble guanylyl cyclase |
| SNAP         | S-nitroso-N-acetylpenicillamine |
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and is involved in the development of the central and peripheral nervous system.” In: The Journal of cell biology 151.6, pp. 1169–1178.


Abstract

Nitric oxide (NO)-signaling is implicated in various physiological processes including neuronal growth and maturation. NO is synthesized by neuronal nitric oxide synthase (nNOS) after activation by Ca\(^{2+}\) and can act directly through S-nitrosylation of proteins. It was demonstrated that this impacts axon retraction but a different NO-dependent mechanism also plays a role. The signaling cascade involving NO, soluble guanylyl cyclase (sGC) and cyclic guanosine monophosphate (cGMP) has been the topic of extensive research in the lab. Previous results obtained by analysis of fixed cells substantiated a proposed feedback inhibitory regulation from cGMP-dependent protein kinase (PKG) towards nNOS. I wanted to further support these results with live-imaging data. Therefore, the previously established method of analysis had to be adapted for time-lapse microscopy. Using adult mouse dorsal root ganglion neurons I examined the rate of axon retraction as indicated by the classical hallmarks (retraction bulb, sinusoidal bundling of the axon shaft and trailing remnant) after treatment with different inhibitory substances for key enzymes in the pathway.

I confirmed that wild-type DRG neurons retracted after treatment with Roscovitine using both time-lapse and fluorescence microscopy. Calcium ionophore Calcimycin was used to increase intracellular levels of Ca\(^{2+}\) but unlike previous lab members, I was unable to apply Calcimycin in time-lapse microscopy at a concentration of 10 µM due to widespread cell death. Lower concentrations did not show a significant effect compared to vehicle control. To investigate the influence of sGC activity on axon retraction I used the inhibitors LY83583 and ODQ in my experiments but no significant changes in axon retraction could be observed after treatment in either case. Also when using the fixed-cell protocol, treatment with LY83583 had no effect, neither in the presence nor absence of Calcimycin. NPA was used to directly inhibit nNOS. I could not see any significant effects on axon retraction that was attributable to NPA treatment in live-imaging and PFA-fixed cells. Taken together, my data did not support the putative inhibitory feedback loop from PKG to nNOS.
The second part of this thesis dealt with a possible influence of Semaphorin3A treatment on growth cone behavior in neonatal mouse cortical neurons. Semaphorin3A is a repellent guidance cue for axons but can exert attractive properties on neurites as well. A MAP1B-dependent reduction in neurite outgrowth was observed previously in the lab in hippocampal and DRG explants but no effect was observed in dissociated cells of either origin after treatment. I repeated the treatments of cortical neurons and assessed growth cone appearance in comparison to untreated cells. The combined results of treatments with 100 ng/ml Semaphorin3A lack any observable effect. Increasing the concentration to 500 ng/ml Semaphorin3A significantly elevated growth cone collapse at several time points. Taken together my results are not yet conclusive but suggest that higher concentrations of Semaphorin3A may be needed to induce effects in these dissociated cultures.
Zusammenfassung


Ich bestätigte in Zeitraffer- und Immunfluoreszenzmikroskopie, dass wildtyp-DRG Neuronen sich nach der Behandlung mit Roscovitin zurückzogen. Die Calcium-Ionophore Calcimycin wurde zur Erhöhung der intrazellulären Konzentration von Ca²⁺ verwendet, aber im Gegensatz zu früheren Laborkollegen war es mir nicht möglich die Konzentration von 10 µM in der Zeitraffermikroskopie anzuwenden, da das den Großteil der Zellen tötete. Niedrigere Konzentrationen hatten im Vergleich zur Kontrolle mit Lösungsmittel keinen signifikanten Effekt. Um den Einfluss der sGC auf die Axon-Retraktion zu untersuchen verwendete ich die Inhibitoren LY83583 und ODQ in meinen Experimenten, konnte aber in keinem Fall signifikante Effekte beobachten. Auch unter Anwendung des Protokolls für fixierte Zellen hatte die Behandlung mit LY83583 keinen Effekt, weder in An- noch Abwesenheit von Calcimycin. NPA wurde zur direkten Inhibition von nNOS verwendet. Ich konnte keine signifikanten Auswirkungen auf die Axon-Reaktion, die mit NPA in...
Zusammenfassung

Verbindung gebracht werden konnte, feststellen. Zusammengefasst haben meine Daten die mutmaßlich hemmende Rückkoppelungsschleife von PKG zu nNOS nicht bekräftigt.

Acknowledgments

I want to thank several people who, in one way or another, helped me along the way.

First and foremost I have to extend my gratitude to Univ. Prof. Friedrich Propst, who gave me the opportunity to work on this project. Without his continued help during my time at the bench and critically reading and discussing this manuscript, this Thesis would not exist.

A big Thank you! to my colleagues Petronela, Raji, Waltraud, Zsuzsi, Anton and Michael as well as all the people in the Department of Cell Biology who were always helpful and supportive.

Many thanks go out to my friends and especially those who offered advice and support during the final steps of the way: Birgit, Karin, Mirjam and Klemens.

I am deeply grateful to my parents, Karin and Christian, who supported me throughout the years of changing interests and various degrees of diligence as well as my brothers, Andreas and Maximilian, who are in the unfortunate position that I am the oldest of us three.

Finally, I want to thank my boyfriend Markus for his patience and continued encouragement throughout the years.

Stay classy, everybody.
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