Muscle Lim Protein in naïve and injured neurons

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Evgeny Levin
aus Tscherepowetz

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aus dem Bereich Experimentelle Neurologie des Universitätsklinikums
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Referent: Prof. Dr. Dietmar Fischer

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Summary

Mature neurons of the central nervous tissue (CNS) do not normally regenerate their lesioned axons. Although neurons of the peripheral nervous system (PNS) are on the contrary able to regrow axons in the injured nerves, this regeneration often does not result in a complete functional restoration. The development of effective therapeutic strategies for the improvement of axonal regeneration requires the exploration of corresponding molecular mechanisms.

Muscle Lim Protein (MLP) has so far been mainly regarded as a muscle-specific protein with a particularly high abundance in heart myocytes and diverse cellular functions. Two studies also reported upregulation of Mlp-mRNA in axonally injured PNS neurons (Newton et al., 2000; Stam et al., 2007). However, neither the levels of MLP-protein, nor its spatiotemporal expression pattern or function in the intact or lesioned nervous tissue have been investigated until now.

Within the scope of this thesis, MLP expression was investigated in the dorsal root ganglia (DRG) of rats upon sciatic nerve crush (SNC). Thereby, we were able to confirm previous reports on upregulated Mlp-mRNA levels in this nerve injury model and also detected for the first time the presence of translated protein in about 10% of DRG neurons. This distinct subset of cells could not be assigned to any of the canonical neuronal subpopulations of DRG seven days after SNC. Nevertheless, further detailed investigations revealed that the majority of MLP expressing cells arose from the well-characterized subpopulation of Isolectin B4-positive nociceptors, which gradually downregulated their ability to bind the lectin upon axotomy.

Another objective of this thesis was to explore whether MLP is also induced during the embryonic and postnatal development of nervous tissue. To this end, embryonic and postnatal retinae of rats were analyzed using quantitative PCR, Western blot and immunohistochemical methods. We found upregulation of MLP in cholinergic amacrine cells between embryonic day 20 (E20) and postnatal day 14 (P14). Strikingly, all cholinergic amacrine interneurons were MLP-positive between P7 and P14. Thereafter, the expression of the protein decreased and was no longer detectable in the adult retinae.

Precise investigations on the possible role and the molecular mechanism of MLP in axonal regeneration would most likely involve extensive *in vitro* experiments with genetically modified adult neurons. However, mature neurons are notoriously insusceptible to genetic manipulations by the common *in vitro* gene delivery techniques. Therefore, the last part of this thesis focused on the establishment of an efficient baculovirus-based *in vitro* transduction method for primary postnatal and adult CNS and PNS neuron. VSV-G pseudotyped baculoviruses (BacMam) were used to deliver reporter genes, expressed under the regulation of CMV-promoter, to cultures of RGC and DRG neurons. Transduction rates of up to 80% were reproducibly achieved by this approach. Moreover, the induction of transgene expression was detected within only 24 hours following the application of viruses. If two different viruses were added simultaneously, almost all transduced cells co-expressed both reporter proteins, making the BacMam-system suitable for co-transduction experiments. Finally, using Cre- and hIL6-baculoviruses we confirmed fully functional transgene expression, enabling execution of neuronal assays *in vitro*.

Zusammenfassung

Neurone des adulten zentralen Nervensystems (ZNS) sind auf dem natürlichen Weg nicht in der Lage ihre durchtrennten Axone zu regenerieren. Obwohl die Neurone des peripheren Nervensystems (PNS) hingegen relativ effizient neue Axone bilden können, führt diese Regeneration jedoch oft nicht zu einer vollständigen Wiederherstellung der Funktion im betroffenem Gewebe. Die Entwicklung neuer Therapieoptionen, um die axonale Regeneration zu fördern, setzt ein detailliertes Verständnis der zugrundeliegenden molekularen Mechanismen voraus.

Muscle Lim Protein (MLP) gilt bislang als ein muskelspezifisches Protein. Es ist besonders stark im Herzgewebe exprimiert. Dort übernimmt MLP viele wichtige Funktionen. Zwei Studien haben bereits festgestellt, dass die mRNA von MLP auch in axotomierten Neuronen des PNS hochreguliert wird (Newton et al., 2000; Stam et al., 2007). Dennoch wurde noch nie untersucht, ob MLP dort tatsächlich als Protein vorliegt bzw. wie schnell, wie lange und in welchen Zellen genau es exprimiert wird. Auch über die Funktion von MLP in Neuronen ist nichts bekannt. Darüber hinaus wurde seine Expression im intakten Nervengewebe noch nie untersucht.

Im Rahmen dieser Doktorarbeit wurde die Expression von MLP in Spinalganglien der Ratten nach einer Verletzung des Ischiasnervs untersucht. Dabei konnte die bereits zuvor beschriebene Induktion der Mlp-mRNA bestätigt werden und darüber hinaus wurde in ca. 10% der axotomierten Neuronen zum ersten Mal auch MLP als Protein nachgewiesen. Die meisten von ihnen konnten sieben Tage nach einer Ischiasnervverletzung nicht einer der typischen Subpopulationen der Spinalganglienneurone zugeordnet werden. Weitere Untersuchungen ergaben jedoch, dass der Großteil der MLP-produzierenden Zellen aus der Subpopulation der Isolektin B4-positiven Nozizeptoren hervorgingen, welche in Folge der Axotomie ihre Fähigkeit das Lektin zu binden verloren haben.

In dem zweiten Teil dieser Doktorarbeit sollte überprüft werden, ob MLP auch während der embryonalen oder der postnatalen Entwicklung der Neurone exprimiert wird. Hierfür wurden embryonalen und postnatalen Retinae von Ratten mittels quantitativer PCR, Western Blot-Analyse und Immunhistochemie untersucht. Tatsächlich wurde

eine Hochregulation von MLP in den Retinae zwischen dem embryonalen Tag 20 (E20) und dem postnatalen Tag 14 (P14) festgestellt. Das Protein wurde ausschließlich in cholinergen amakrinen Zellen gebildet. Dabei waren alle diese Zellen positiv für MLP zwischen P7 und P14, regulierten die Expression des Proteins jedoch daraufhin komplett wieder herunter.

Für die zukünftige Aufklärung der Rolle und des Wirkmechanismus von MLP in der axonalen Regeneration wird unter anderem die Durchführung von Zellkultur-Assays mit genetisch veränderten Neuronen notwendig sein. Bekanntermaßen existiert bislang jedoch keine Methode, die ein effizientes Einschleusen vom genetischen Material in primäre adulte Neurone *in vitro* ermöglicht. Aus diesem Grund wurde dafür im Rahmen dieser Doktorarbeit ein neuer Ansatz basierend auf der Verwendung von Baculoviren etabliert. Die eingesetzten Viren (BacMam) waren mit dem Glykoprotein VSV-G pseudotypisiert und ermöglichten die Expression von Transgenen gesteuert durch CMV-Promotoren. BacMam-Vektoren erreichten reproduzierbare Trasduktionsraten von bis zu 80% in Zellkulturen der RGZ und der Spinalganglienneuronen. Dabei wurden die eingeschleusten Gene bereits innerhalb von 24 Stunden nach der Zugabe von Viren exprimiert. Darüber hinaus haben in Fällen, in denen Zellen mit zwei verschiedenen Viren gleichzeitig behandelt wurden, alle transduzierten Neurone beide Reportergene exprimiert, was die Verwendbarkeit des BacMam-Systems für Co-Transduktionsexperimente ermöglicht. Mit Cre- und hIL6-Bakuloviren haben wir zudem die Funktionalität der baculoviral exprimierten Proteine bestätigen können, was die Einsetzbarkeit der Methode für neuronale Assays untermauert.

Abbreviations

AAV	Adeno-associated virus
AC	Amacrine cell
Akt	Protein kinase B
Atf3	Activating transcription factor 3
BDNF	Brain-derived neurotrophic factor
cDNA	Complementary deoxyribonucleic acid
CGRP	Calcitonin gene-related peptide
ChAT	Choline acetyltransferase
CMV	Cytomegalovirus
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CRP	Cysteine-rich protein
CSPGs	Chondroitin sulfate proteoglycans
CSRP	Cysteine and glycine-rich protein
DRG	Dorsal root ganglion
dsDNA	Double-stranded deoxyribonucleic acid
eGFP	Enhanced green fluorescent protein
fGFP	Farnesylated green fluorescent protein
Gap-43	Growth-associated protein 43
GCL	Ganglion cell layer
GDNF	Glial-derived neurotrophic factor
Gp130	Glycoprotein 130
hIL6	Hyper-interleukin 6
IB4	Isolectin B4
IL6	Interleukin 6
INL	Inner nuclear layer
IPL	Inner plexiform layer
IS	Inflammatory stimulation
JAK	Janus kinase

kb	Kilobase
kDa	Kilo Dalton
LIF	Leukemia inhibitory factor
LV	Lentivirus
MLP	Muscle Lim Protein
mRNA	Messenger ribonucleic acid
NF	Neurofilament
NGF	Nerve growth factor
NT-4	Neurotrophin-4
ONC	Optic nerve crush
ONL	Outer nuclear layer
OPL	Outer plexiform layer
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PNS	Peripheral nervous system
PTEN	Phosphatase and tensin homolog
qPCR	Quantitative polymerase chain reaction
RGC	Retinal ganglion cell
shRNA	Short hairpin ribonucleic acid
SOCS3	Suppressor of cytokine signaling 3
SP	Substance P
Sprr1a	Small proline-reach repeat protein 1a
STAT3	Signal transducer and activator of transcription 3
TH	Tyrosine hydroxylase
VSV-G	Vesicular stomatitis virus envelope G-protein
WHO	World Health Organization

1. Introduction

1.1 Nerve injury and axonal regeneration

Regeneration failure of injured nerves in the central nervous system (CNS) is still one of the biggest challenges in modern medicine. Irreparable axon damage and neuronal cell death are common consequences of traumatic CNS injuries, neurodegenerative diseases as well as other disorders harmfully affecting nervous tissue. For example, based on epidemiological data provided by the World Health Organization (WHO), up to 500,000 people worldwide suffer from spinal cord injuries every year. This condition is not only associated with lifelong severe health and social implications for the patients but also markedly increases the risk of premature mortality (WHO, 2013). Moreover, nerve injuries also account for tremendous economic costs. In the United States, they are estimated to reach several billions of US Dollars (Shoichet et al., 2008; Grinsell and Keating, 2014). Despite some substantial progress in the understanding of molecular and physiological principles relevant in the context of neuronal regeneration, this knowledge has not yet resulted in effective and clinically validated therapeutic strategies neither for the protection of injured neurons from death and loss of function nor for the renewal of axonal connections. Hence, there is still a need for further research and identification of molecular mechanisms, which might be important for successful treatment of severe nerve injuries.

The poor regenerative capacity of CNS tissue is found not only in humans but is common for all mammals. The ability to extend neurites is high in embryonic and postnatal neurons but declines substantially in mature CNS (Argiro et al., 1984; Cai et al., 2001; Goldberg et al., 2002). Moreover, as already described in the pioneering work by Ramon y Cajal (Ramón y Cajal and May, 1928), neurons that die from injuries generally cannot be replaced by newly generated analogous cells, even though in the meantime neurogenesis-competent stem cells have been discovered in some regions of the adult brain (Ma et al., 2009). The lack of an effective regenerative process in the central nervous tissue is believed to be due to the complexity of precisely formed and purposively adjusted mature neuronal structures. The necessity to maintain and protect this unique architecture probably would not tolerate uncontrolled modifications, which

may be caused by regenerated neurons and their neurites. This argument is partially supported by the fact that the mammalian peripheral nervous system (PNS), a far less complex neuronal entity than the CNS, possesses an extensive ability to regrow injured axons.

Axonal injuries are associated with severe consequences for affected CNS neurons themselves and the surrounding tissue. First of all, some neurons, being detached from their axonal targets, undergo rapid apoptosis (Villegas-Perez et al., 1993; Berkelaar et al., 1994; Rabacchi et al., 1994; Fischer et al., 2000; Fischer et al., 2004). However, even neurons that survive axotomy are often functionally eliminated from neuronal networks as presynaptic terminals of respective input neurons are removed from their cell bodies (Blinzinger and Kreutzberg, 1968; Graeber et al., 1993; Linda et al., 2000). This process, referred to as synaptic stripping, is believed to be facilitated by active microglia (Blinzinger and Kreutzberg, 1968). Microglial cells typically become activated in response to neuronal injuries and are supposedly involved in the clearance of cellular debris in damaged tissue (Kreutzberg, 1996; Nakajima and Kohsaka, 2001; Hilla et al., 2017). In particular, in the CNS, microglia is responsible for the phagocytosis of fragments accruing from the severed (distal) axon stumps in the course of their degeneration process known as Wallerian degeneration (Neumann et al., 2009).

Factors limiting efficient axonal regeneration of the CNS neurons are usually grouped in two categories: insufficient intrinsic capability and unfavorable extra-neuronal/extra-axonal environment. The first category implicates missing or insufficient activation of relevant signaling pathways, insufficient anabolic activity as well as the poor supply of proper intracellular resources, which are indispensable for the formation of new neurites. Besides these intrinsic obstructions, extracellular space in the region of axonal injury provides additional obstacles for successful regeneration. On the one hand, slow and deficient clearance of myelin debris, which are produced as result of Wallerian degeneration, causes an extensive accumulation of inhibitory molecules capable of impeding neurite extension (George and Griffin, 1994; McKerracher et al., 1994; Mukhopadhyay et al., 1994; Chen et al., 2000; GrandPre et al., 2000; Wang et al., 2002; Gaudet et al., 2011). In addition, the site of axonal injury is rapidly being filled with reactive astrocytes. These invading cells form a so-called glial scar (Silver and Miller, 2004), which not only establishes a physical barrier for neurite extension, but also

provides a source for additional growth-inhibiting effector molecules, in particular, chondroitin sulfate proteoglycans (CSPGs) (McKeon et al., 1991; Niederost et al., 1999; Silver and Miller, 2004). The strongest evidence of how crucial the composition of extra-neuronal environment affects axonal regeneration was provided by the studies of Aguayo and co-workers: Where a peripheral nerve graft was transplanted into a CNS lesion, an extensive innervation of the graft by axons arising from injured neurons had been observed, indicating that in contrast to the CNS, the perineural tissue of the PNS is rather growth-permissive and favors axonal regeneration (Aguayo et al., 1981; So and Aguayo, 1985). This is partially attributed to a rapid clearance of myelin debris by Schwann cells, the major glial cell population of the PNS, and by macrophages (Gaudet et al., 2011). Astrocytes are not present in the PNS and therefore no glial scar is formed upon nerve injuries. However, axonal regeneration in PNS is not only supported by the absence of growth-inhibiting conditions but is also additionally boosted by growth-promoting mechanisms in the environment of outgrowing axons, which are missing in the CNS. In particular, Schwann cells start to proliferate in the damaged region of a peripheral nerve forming structures referred to as bands of Büngner, which support and guide axonal growth (Chen et al., 2007; Fex Svennigsen and Dahlin, 2013). At the same time, these Schwann cells upregulate the production of adhesion molecules and essential extracellular matrix proteins (e.g. laminin) resulting in a significantly accelerated axon extension (Nieke and Schachner, 1985; David et al., 1995; Chen and Strickland, 2003). Moreover, this process is supposedly also promoted by neurotrophins (e.g., nerve growth factor [NGF], brain-derived neurotrophic factor [BDNF] or neurotrophin-4 [NT-4]) that are delivered to regenerating fibers (Terenghi, 1999). All these extrinsic factors in combination with a profoundly activated intrinsic regeneration machinery account for robust and substantial axon regrowth by injured PNS neurons (Chen et al., 2007). Nevertheless, in the current medical practice, the repair of peripheral nerve lesions, e.g. those resulting from traumatic injuries, is often incomplete (Grinsell and Keating, 2014). This is particularly attributed to the limited speed and distance range of the axon growth, which become critical factors especially for regeneration over long distances. If the peripheral axons do not find and reinnervate their targets within a certain period of time, the functional loss in the target tissue often remains incurable (Grinsell and Keating, 2014; Faroni et al., 2015). Furthermore,

misguidance of axons and innervation of wrong targets contribute to incomplete functional regeneration (Fex Svennigsen and Dahlin, 2013). Hence, the need to find therapeutic options to improve axonal regeneration is still of vital importance also for PNS injuries. The development of effective therapeutic strategies, however, requires a deep molecular understanding of injury- and regeneration-associated processes. This knowledge is currently obtained from successive detailed examinations of relevant signaling pathways and identification of genes that are differentially regulated upon axonal injuries or in the course of axon regeneration.

1.2 Dorsal root ganglia

Dorsal root ganglia (DRG) contain cell bodies of peripheral sensory neurons (Burgess and Perl, 1967; Bessou and Perl, 1969; Christensen and Perl, 1970). These neurons are highly heterogeneous and can be assigned to different subpopulations according to their morphologic or phenotypic characteristics as well as the type and the conduction velocity of the information they perceive (Yoshida and Matsuda, 1979; Harper and Lawson, 1985; Lawson and Waddell, 1991; Stucky and Lewin, 1999). Nociceptive neurons are responsible for the perception of harmful stimuli. They are predominantly of small or medium size and possess thin unmyelinated C-fiber axons for slow transmission of electrical signals (Snider and McMahon, 1998). These neurons are further subdivided in a peptidergic and a nonpeptidergic subgroup. Peptidergic neurons express different peptide neurotransmitters such as calcitonin gene-related peptide (CGRP) and substance P (SP) (Nagy and Hunt, 1982; Ju et al., 1987), whereas nonpeptidergic nociceptors are on the contrary identified by labeling with Isolectin B4 (IB4) from *Griffonia simplicifolia* (Silverman and Kruger, 1990). The expression of the subgroup markers, however, partially overlaps: In rats, about 30% of IB4-positive DRG neurons are reportedly immunoreactive for CGRP, while about 45% of CGRP-positive neurons also bind IB4 (Price and Flores, 2007). Mechanoreceptors and proprioceptors, the third main subpopulation, are DRG neurons with large somata as well as with thick, heavily myelinated and rapidly conducting A-fiber axons. Characteristically, they express high levels of the neurofilament heavy chain (200 kDa NF) (Lawson and Waddell, 1991; Perry et al., 1991; Marmigere and Ernfors, 2007). Besides these three

dominating and well-characterized subpopulations, some rarely found, however functionally and phenotypically distinct neurons, can also express other specific markers, e.g. tyrosine hydroxylase (TH) (Price and Mudge, 1983; Brumovsky et al., 2006).

1.3 Sciatic nerve: Model system for axonal injury and regeneration in the PNS

Sciatic nerve crush resulting in axonotmesis is one of the most common models of peripheral nerve injuries in rodents. This is attributed to the relatively large diameter of the nerve and its simple, swift and minimally invasive accessibility for surgical manipulations (Oliveira et al., 2014). The sciatic nerve is one of the major nerves in lower limbs. Consequently, in rats, this nerve innervates both hind paws giving rise to four main distinct branches: tibial, sural, peroneal and cutaneous (Schmalbruch, 1986). Sensory fibers of the rat sciatic nerve originate from lumbar L4, L5 and L6 DRG (Swett et al., 1991). However, about 99% of them are provided solely by neurons from L4 and L5 (Devor et al., 1985; Swett et al., 1991). Therefore, L6 is often excluded from evaluation in studies with sciatic nerve neurons. The particular contribution of L4 and L5 to the overall number of sensory axons in the nerve varies between different rat strains: In Wistar rats, both DRG supply nearly equal numbers of afferents, whereas in Sprague-Dawley rats, two-thirds of contributing fibers are provided by the L5 DRG (Rigaud et al., 2008). The sciatic nerve of adult rats contains in total projections from about 10,000 DRG neurons, most of which (~76 %) innervate cutaneous tissue (Swett et al., 1991).

Crush injury of the sciatic nerve in rats causes extensive sensory and motoric impairment of the hind limbs. Remarkably, severed axons quickly start to regenerate, achieving growth rates of about 2-3 mm per day (Gold et al., 1995), and almost complete functional recovery is usually attained after three to four weeks (Vogelaar et al., 2004). During this time, however, DRG neurons affected by the injury undergo massive biochemical changes. In particular, axotomy of the sciatic nerve was shown to alter the expression of more than 100 genes (Xiao et al., 2002). Among others, these changes impinge the availability of the typical subpopulation markers, resulting, for instance, in a marked reduction in the percentages of IB4- or CGRP-positive neurons

(Bennett et al., 1998). More importantly, however, injured DRG neurons extensively upregulate a subset of genes that promote axon regrowth and therefore account for their profound intrinsic regenerative capability. Some of the most prominent and well-characterized regeneration-associated genes in DRG are *c-Jun* (Jenkins and Hunt, 1991), *Atf3* (Tsujino et al., 2000), *Gap-43* (Skene and Willard, 1981; Woolf et al., 1990) and *Sprr1a* (Bonilla et al., 2002). Upregulation of mRNA for the allegedly muscle-specific Muscle Lim Protein (*Mlp*) was found in axotomized DRG neurons of rats by two independent studies (Newton et al., 2000; Stam et al., 2007). However, these works neither explored whether detected transcripts are translated into a protein nor specified and characterized the population of neurons, which express it. As such information is indispensable before detailed examinations on the possible role of MLP in axonal injury and regeneration can be conducted, some preliminary investigations are needed to clarify these points.

1.4 Muscle Lim Protein

MLP, alternatively also referred to as cysteine and glycine-rich protein 3 (CSRP3) or cysteine-rich protein 3 (CRP3), is a member of the CRP-family of LIM domain proteins, which comprises two further closely related members: CRP1 and CRP2. Characteristically, all three CRP proteins contain two LIM domains connected by a long inter-domain region (Arber et al., 1994; Schallus et al., 2009).

LIM motifs that were initially found in Lin-11, Isl-1, and Mec-3 proteins utilize the consensus sequence $CX_2CX_{17-19}HX_2CX_2CX_2CX_{16-20}CX_2$(C/H/D) to establish two tandem zinc finger structures (Michelsen et al., 1993), which efficiently mediate protein-protein interactions (Schmeichel and Beckerle, 1994; Weiskirchen et al., 1995; Kong et al., 1997).

MLP was discovered in muscle tissue by Arber and co-workers in 1994 (Arber et al., 1994) and is considered a strictly muscle-specific protein since.

MLP is significantly upregulated during myogenesis in striated muscle tissue (Arber et al., 1994). In the absence of MLP, myocytes fail to differentiate properly (Arber et al., 1994). The crucial role of MLP in this process is linked to its functional interaction with

basic helix-loop-helix transcription factors, supporting the expression of muscle-specific genes (Kong et al., 1997). In adults, MLP remains highly expressed in cardiac myocytes and is involved there in the maintenance of cytoarchitecture and the function of the contractile apparatus (Arber et al., 1994; Arber et al., 1997; Hoffmann et al., 2014). An MLP knockout in mice causes extensive deterioration in cellular morphology of the heart muscle, resulting in severe cardiomyopathy and heart failure (Arber et al., 1997). Certain mutations of the MLP gene or its decreased expression were also linked to similar clinical conditions in humans (Zolk et al., 2000; Geier et al., 2008; Knoll et al., 2010). Furthermore, upregulation of MLP was reported in injured muscle tissue of other types, in particular, in denervated skeletal muscles as well as in vascular myocytes after mechanical injuries, where it is supposedly involved in vascular remodeling (Arber et al., 1994; Wang et al., 2006).

The molecular mechanisms responsible for the MLP affecting the development, structural properties or functionality of myocytes are highly complex and still incompletely understood. On the one hand, this is attributed to the dual subcellular localization of the protein in the nuclei and the cytosol of the cells. MLP accumulates notably in nuclei during the process of myogenic differentiation, but later predominantly resides in the cytoplasm of mature cardiomyocytes (Arber et al., 1994; Arber et al., 1997). However, upon stretch or mechanical overload of the heart muscle, MLP, an essential sensor and regulator of such stress conditions, again extensively translocates into nuclei (Knoll et al., 2002; Boateng et al., 2007; Buyandelger et al., 2011). On the other hand, interactions of MLP with multiple cellular players, efficiently provided through its two LIM domains (Arber et al., 1997), result in a plenty of diverse mechanistic tasks (Buyandelger et al., 2011). In the cytoplasm, MLP efficiently binds to f-actin and promotes cross-linking and stabilization of f-actin bundles (Arber et al., 1997; Hoffmann et al., 2014). Notably, the underlying mechanism involves dimerization of MLP molecules (Hoffmann et al., 2014). Moreover, MLP was shown to interact with some other ubiquitous actin-associated proteins like α-actinin, cofilin, β-spectrin or zyxin, possibly affecting their functions (Louis et al., 1997; Flick and Konieczny, 2000; Papalouka et al., 2009). Another important interaction of MLP with the muscle-specific protein T-cap occurs at the boundaries of myocytic contractile units (sarcomeric z-discs) and plays a crucial role in cardiac stretch sensing (Knoll et al., 2002). Some

studies also linked MLP to intracellular calcium signaling and metabolic processes like autophagy (Gupta et al., 2008; Kemecsei et al., 2010; Rashid et al., 2015). As a consequence, despite its relatively small size (merely 194 amino acids) and a simple structure (only two functional domains), MLP is now considered to be one of the most important and most versatile myocytic proteins.

1.5 Retina and visual system

The retina is the multilayered nervous tissue in the eye that is responsible for low-level visual processing and transmission of visual information to the brain. Different types of neurons which build up the retina are organized in three distinct layers: outer nuclear layer (ONL: photoreceptor neurons), inner nuclear layer (INL: horizontal, bipolar and amacrine cells) and ganglion cell layer (GCL: retinal ganglion cells [RGCs] and some displaced amacrine cells). ONL and INL are separated by a fiber layer, referred to as the outer plexiform layer (OPL), which contains dendrites and synapses of photoreceptors and bipolar cells. The inner plexiform layer (IPL) is located between the INL and the GCL and contains the dendrites and synapse arising from the corresponding cell populations (Kandel et al., 2013). In addition, further complexity of the retina is due to the subdivision of these five basic neuron types in further distinct subpopulations, which vary in their molecular phenotype, morphology, and function. For instance, there are at least 20 known RGC subtypes (Masland, 2012; Sanes and Masland, 2015) and at least 26 subtypes of amacrine cells (MacNeil and Masland, 1998; MacNeil et al., 1999). Retinogenesis in rodents occurs between embryonic days (E) 8/9 and postnatal day (P) 12 (Rapaport et al., 2004). RGCs, cone photoreceptors, as well as horizontal and amacrine cells are the first neuronal populations to be born (starting around E8/E9), whereas bipolar cells and rod photoreceptors emerge in later embryonic stages (E18- E21) (Rapaport et al., 2004; Voinescu et al., 2009)

The primary function of horizontal bipolar and amacrine cells is that of interneurons assimilating and modulating the visual information received by photoreceptors, before it eventually reaches the RGCs. RGCs, in turn, generate from this input action potentials that are conveyed to the areas of visual processing in the brain. Although

RGCs are believed to innervate at least 30 (Kandel et al., 2013) different brain regions, the main retinorecipient targets are the lateral geniculate nucleus in the diencephalon, the pretectum and the superior colliculus in the mesencephalon as well as the suprachiasmatic nucleus in the hypothalamus (Purves and Williams, 2001). The lateral geniculate nucleus, involved in the transmission of visual information to the cortex, is the main retinofugal target area in humans (Bear et al., 2009; Kandel et al., 2013). In contrast, the majority of RGCs in rodents and some other vertebrates project their axons to the superior colliculus, which is responsible for the movements of eyes, head and neck (Hofbauer and Drager, 1985; Bear et al., 2009).

1.6 RGCs: Model system for axonal injury and regeneration in the CNS

RGCs and optic nerve are an important general model system to study axonal injuries and axon regeneration in CNS tissue. In particular, the intraorbital optic nerve crush (ONC) surgery, which results in a complete transection of the axons without affecting the surrounding meningeal sheath, is commonly applied in rodents for investigations of RGC survival and axon regeneration.

Experimental intraorbital ONC in animal models causes an extensive apoptotic death of RGCs. Hence, just within 14 days after axotomy, the number of RGCs is reduced by more than 90% in rats (Berkelaar et al., 1994; Muller et al., 2007) and more than 80% in mice (Leibinger et al., 2009; Leibinger et al., 2013a). Of the surviving cells, only very few are able to regrow axons, which do not get far beyond the lesion site (Kiernan, 1985; Lucius et al., 1998; Berry et al., 1999; Chierzi et al., 1999; Leon et al., 2000). However, the survival and regenerative capacity of RGCs can be substantially improved through activation of Janus kinase/Signal Transducer and Activator of Transcription 3 (JAK/STAT3) and Phosphatidylinositol-4,5-bisphosphate 3-kinase/AKT (PI3K/AKT) pathways (Müller et al., 2007; Park et al., 2008; Smith et al., 2009; Leibinger et al., 2013a; Leibinger et al., 2013b; Zukor et al., 2013). Besides the deletion of the respective intrinsic breaks (suppressor of cytokine signaling 3 [SOCS3] for JAK/STAT3 and phosphatase and tensin homolog [PTEN] for PI3K/AKT signaling) (Park et al., 2008; Smith et al., 2009), this effect, especially on the JAK/STAT3 pathway, is achieved upon stimulation of the cells with IL6-type cytokines. A potent intravitreal supply with

such cytokines can be provided with a so-called inflammatory stimulation (IS). This term describes the secretion of inflammatory molecules from activated glial cells or macrophages. An intentional eye lens injury (puncture), performed simultaneously to axotomy, induces strong IS via the release of β/γ-crystallins in the vitreous chamber (Fischer et al., 2000; Leon et al., 2000; Fischer et al., 2008). These crystallins activate retinal glial cells (astrocytes and Müller cells) which start to produce IL6-like cytokines, in particular, ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF) and IL6 itself (Muller et al., 2007; Leibinger et al., 2009; Leibinger et al., 2013b). Subsequent activation of corresponding α-receptors and gp130 in the cell membrane of RGCs results in up to an eightfold increase in survival rate and dramatically enhanced axon growth (Fischer et al., 2000; Leon et al., 2000). A similar IS-related effect can be achieved by intravitreal application of toll-like receptor 2-agonists like zymosan and Pam3Cys (Yin et al., 2003; Hauk et al., 2010). However, the application of the designer cytokine hyper-IL6 (hIL6), a fusion protein of IL6 and IL6-receptor, which does not depend on the presence of respective α-receptors on the cell surface, exhibits effects even superior to those produced by a lens injury (Leibinger et al., 2016).

Insights on mechanisms involved in the axonal regeneration of RGCs in rodents are often obtained from *in vivo*-initiated studies utilizing the optic nerve crush model and intravitreal injections of adeno-associated viruses (AAVs) to transduce and thereby genetically modify the cells. However, these approaches require complex surgical interventions on living animals, take a long time until a readout is possible and are limited by some disadvantages of AAVs. A far less complicated, time-saving and often more suitable alternative for basic mechanistic investigation are experiments performed fully *in vitro* with dissociated RGC cultures. However, like other mature neurons, adult RGCs are generally unamenable to the common *in vitro* gene delivery methods. Therefore, a reliable, quick, and efficient way to transduce cultured RGCs is required to surmount this impediment.

1.7 Problems associated with gene delivery to mature neurons *in vitro*

Two most efficient options to explore the role of particular genes in axonal regeneration are knockout or overexpression of their coding DNA-sequences. As mentioned above, studies on exact function and mode of action of such genes are often carried out in cell culture. However, post-mitotic mature neurons (especially those obtained from the CNS) not only poorly endure *in vitro* cultivation, but are also insusceptible to most common gene delivery techniques. The most non-viral gene transfer methods like nucleofection, calcium phosphate precipitation or lipofection are suitable for embryonic or early postnatal neurons but work very inefficiently in adult neuronal cells, producing transduction rates mostly not exceeding 10% (Zeitelhofer et al., 2007; Karra and Dahm, 2010). The main reason for the restriction of these methods to embryonic or early postnatal neurons is that they require disintegrated nuclear membrane for efficient DNA delivery, which is the case in proliferating or just exited the mitosis cells, but not in mature non-dividing neurons (Karra and Dahm, 2010). Although nucleofection has arguably been developed to overcome this obstacle (Zeitelhofer et al., 2007), its efficiency is nevertheless too low in adult neuronal cell cultures. Hence, the research on axonal regeneration is often performed in neurons obtained from embryonic or postnatal tissue. However, these neurons differ significantly from their terminally differentiated counterparts, in particular, in their ability to extend neurites. Therefore, results gained from experiments with such immature cells are often not applicable to the adult nervous system.

Viral vectors, such as recombinant lentiviruses (LVs) or AAVs, are more suitable for genetic manipulations of mature cells because they actively permeate the nuclear envelope to deliver recombinant DNA. Nevertheless, especially high transduction rates for these vectors *in vitro* are also mainly reported in postnatal or embryonic neurons (Fleming et al., 2001; Gascon et al., 2008; Royo et al., 2008; Hutson et al., 2012; Ding and Kilpatrick, 2013), whereas their efficiency in adult neuronal cell cultures is often considerably lower. This could partially be attributed to some side effects of such viruses like toxicity in case of the use of too high titers as well as potential insertional mutations in the genome of host cells (Karra and Dahm, 2010). Moreover, recombinant genes delivered with these vectors are expressed with some delay: up to five days

post-transduction with LVs and up to 14 days post-transduction with AAVs (Karra and Dahm, 2010). Cells obtained from a mature neuronal tissue may not survive for such a long time period, e.g. RGCs normally start to die at day 5 *in vitro* (Grozdanov et al., 2010). A further important disadvantage of these viruses is the limited size of cDNA-insert they can bear: 2.5–5 kb for LVs and less than 2.5 kb for AAVs (Karra and Dahm, 2010). This factor substantially hampers the application of both vector systems for the delivery of long or multiple genes. The usability of LVs is further complicated by the requirement of biosafety level 2 laboratories.

1.8 Baculovirus-mediated gene delivery to mammalian cells

Baculoviruses are dsDNA-viruses that naturally infect only insect cells (Clem and Passarelli, 2013). Their outstanding properties, like good safety profile, easy producibility and large insert size capacity, established them in the last decades as one of the most frequently used biotechnological systems for recombinant protein expression in insect cell lines (Airenne et al., 2013; van Oers et al., 2015). The same advantages also made baculoviruses a suitable instrument for gene delivery to mammalian cells (Kost and Condreay, 2002; Kost et al., 2005). To this end, the gene of interest is supplemented by a mammalian promoter as well as by post-transcriptional regulatory elements and inserted in the circular genome of the virions (Hofmann et al., 1995; Boyce and Bucher, 1996). Baculoviral particles enter potential host cells via endocytosis fusing their envelopes with cell membranes (Airenne et al., 2013). Gp64 is the major envelope glycoprotein of native baculoviruses responsible for the fusion process (Okano et al., 2006). Pseudotyping of the envelope by other glycoproteins can, however, improve the ability of baculoviruses to enter cells from various species (Hu, 2005). Vesicular stomatitis virus envelope G-protein (VSV-G) is often used for this purpose, as it exhibits high tropism for a broad spectrum of mammalian and non-mammalian cell types (Barsoum et al., 1997; Kolangath et al., 2014). Baculoviruses that are genetically modified to efficiently transduce mammalian cells are referred to as BacMam. BacMam particles deliver transgene-cDNA into the nuclei but do not integrate the DNA into the genome of the host, hence avoiding a disruption of genomic sequences (Airenne et al., 2013). Importantly, mammalian cells cannot activate insect-

specific promoters. Therefore, BacMam-virions are not capable of replicating in these recipient cells, making them suitable for biosafety level 1 laboratories (Kost and Condreay, 2002; Kost et al., 2005). In addition, BacMam-system is easy to use *in vitro,* as the pure virus solution is added directly to the normal cell culture medium without any additional supplements (Kost et al., 2005). Therefore, the cultures are not subjected to unnecessary stress or irregular biochemical conditions. All these features, combined with potential insert capacities of more than 100 kb (Ghosh et al., 2002), simple scalable production process and low cytotoxicity, are the reason for the importance of BacMam-viruses as an efficient and versatile tool for gene delivery (Kost and Condreay, 2002; Hu, 2005; Kost et al., 2005; Mansouri et al., 2016). However, despite the obvious advantages and some brief reports on successful transduction of neurons *in vivo* (Haeseleer et al., 2001; Wang et al., 2005; Turunen et al., 2014), the usability of the BacMam-system has never been systematically evaluated and established for the transduction of mature CNS and PNS neurons in culture. Consequently, the potential application of this method for *in vitro* research on axonal regeneration has remained unexplored until now.

2. Thesis objectives

The significant upregulation of Mlp-mRNA reported by two studies in axotomized DRG neurons raised the question, whether this alleged muscle-specific protein is functionally involved in the regeneration process of injured PNS, and possibly also CNS, neurons. However, as already mentioned in the introduction, the actual presence of detectable MLP-protein levels has never been verified until now in any of the nerve injury paradigms. Moreover, nothing is known about MLP expression in naïve (uninjured) nervous tissue. Because of the complex multimodal function of MLP in myocytes, possible investigations on the exact mechanism of this protein in axonal regeneration may require extensive cell culture-based experiments with primary adult neurons. In particular, knockdown or exogenous MLP expression, as well as expression of its mutated forms or potential interaction partners, might prove helpful to explore relevant molecular interrelations. Therefore, there is a need for an efficient, versatile, high-throughput gene delivery approach, suitable for both, post-mitotic central and post-mitotic peripheral neurons, which can be applied to perform such genetic manipulations. With regard to these considerations, this thesis comprises following objectives:

1) To explore whether MLP is upregulated on protein level in axotomized DRG and to characterize the corresponding neuronal population
2) To explore whether MLP is expressed in naïve (uninjured) nervous tissue
3) To develop a robust, efficient *in vitro* transduction method for primary adult neurons based on the BacMam technology

3. Results and discussion

3.1 Induction of MLP expression in axotomized DRG neurons

This part of the result and discussion section is based on the publication in Scientific Reports with the title “Nociceptive DRG neurons express muscle lim protein upon axonal injury” (Levin et al., 2017). In this study, I was involved in the design of experiments. I performed some of the sciatic nerve injuries and carried out the Western Blot, the qPCR, and immunohistochemical examinations. Furthermore, I also performed all cell culture experiments with DRG neurons. In addition, my contribution also included the analysis and interpretation of data.

Two studies reported an upregulation of Mlp-mRNA in DRG neurons of rats after sciatic nerve injuries (Newton et al., 2000; Stam et al., 2007). To verify these reports, we performed a qPCR on L4/L5 DRG obtained from rats seven days after unilateral axonotmetic sciatic nerve crush (SNC). Indeed, we found an ~ 8-fold increase in Mlp expression in these DRG compared to uninjured controls. However, neither of the two previous studies had examined whether Mlp-mRNA is translated into a protein. To address these questions, we first performed some preliminary experiments to prove the usability of a commercially available polyclonal antibody for a specific detection of MLP protein. To this end, the antibody was tested in HEK293 cells co-expressing MLP and either a specific shRNA directed against MLP transcripts or a non-targeting scrambled control shRNA. The antibody strongly stained MLP in control cells, but not in cells co-transfected with MLP and the specific shRNA. In a subsequent Western blot analysis, the antibody detected a strong distinct band of about 21 kDa in a rat heart lysate (tissue with particular high MLP-content) as well as in lysates of HEK293 cells co-expressing MLP and the scrambled control, while the intensity of the band was expectedly reduced in HEK293 cells upon knockdown with the specific antisense construct. Strong MLP-bands were also visible in L4/L5 DRG-lysates four days after SNC, but not in uninjured controls, indicating that Mlp-mRNA had indeed been translated into the corresponding protein.

The validated polyclonal and a previously characterized monoclonal (Geier et al., 2008; Levin et al., 2014) antibodies were then used to perform immunohistochemical analysis of MLP expression on DRG sections. We observed no MLP signal on sections prepared from uninjured animals. However, MLP positive cells were clearly detectable in L4/L5 DRG obtained from animals two, four, seven and 14 days after SNC. Co-staining for neuronal marker βIII-tubulin confirmed that neurons were the exclusive source of MLP expression in the DRG. In addition, retrograde tracing with fluorogold dye from the proximal end of cut sciatic nerves as well as co-staining with the common injury marker ATF3 corroborated that MLP expression was restricted to injured neurons. While only about 3% of all DRG neurons were MLP-positive two days after SNC (initial phase of regeneration), their proportion reached 9% four days after SNC, but then remained nearly unchanged up to 14 days post-crush (in the advanced phase of the regeneration process). Hence, obviously, only a small percentage of DRG neurons is able to upregulate MLP. Therein, no significant differences were found between L4 and L5 DRG. Notably, Newton and co-workers (Newton et al., 2000) reported MLP expression in about 30% of L5 DRG neurons using *in situ* hybridization approach. Moreover, our measurements of cross-sectional cell area at the nuclear level revealed that the vast majority of MLP-positive cells were of small or medium size with an average cell area of 526 μm^2. In contrast, Newton et al. found MLP transcripts in neurons of all sizes. However, Newton et al. used another rat strain, another injury model (partial sciatic nerve ligation) and analyzed only mRNA production. Therefore, different experimental conditions, as well as posttranslational regulation of MLP expression in some neurons, could explain the discrepancies between our study and the work of Newton et al.

MLP protein was localized in the somata and the axons of DRG neurons. A detailed examination of MLP staining using confocal microscopy revealed that the protein had not been transported into the nuclei of the cell. This finding excludes the possibility that MLP could be involved in gene regulation of axotomized DRG neurons via the mechanisms described in myocytes (Kong et al., 1997).

In the next step, we investigated whether MLP expression is restricted to a certain subpopulation of neurons. To this end, we co-stained DRG sections for MLP and the most common subpopulation markers: CGRP/SP (peptidergic nociceptors), IB4 (nonpeptidergic nociceptors), NF200 (large mechanoreceptors/proprioceptors).

Evaluating DRG sections seven days after SNC, we surprisingly found that almost all MLP-expressing neurons (81%) were negative for the canonical subpopulation markers. In contrast, ~48% of these cells could be assigned to the IB4-subpopulation when analyzed two days post-crush. Therefore, we assumed that MLP-positive neurons gradually lose the ability to bind IB4. Indeed, the proportion of IB4-positive cells was markedly reduced seven days after SNC compared to day 2 or uninjured controls, whereas CGRP/SP- and NF200-subpopulations remained unchanged. This finding is consistent with other reports describing rapid downregulation of IB4-binding in axonally injured nonpeptidergic nociceptors (Bennett et al., 1998; Averill et al., 2002). Therefore, the majority of MLP-expressing neurons probably originated from the IB4-subgroup of nociceptors, which become unamenable for the labeling with the lectin upon axotomy. Notably, the soma size distribution of MLP-positive cells also fits in the size range previously reported for nociceptive neurons (Molliver et al., 1995). Although small DRG neurons, particularly in L5, can alternatively also belong to the subpopulation of TH-expressing cells, we were not able to find a co-localization of MLP with the TH staining (Price and Mudge, 1983; Brumovsky et al., 2006).

To verify whether our *in vivo* results can also be reproduced *in vitro*, we prepared neuronal cell cultures from cervical, thoracic, lumbar and sacral DRG of unlesioned rats. MLP was gradually induced and eventually became detectable in about 18% of these DRG neurons after three to five days in culture. Thus, the percentage of MLP-positive neurons was higher compared to those quantified *in vivo*. This finding could be explained by the influence of specific culture conditions or the use of further DRG in addition to L4 and L5. Nevertheless, the majority of MLP-expressing neurons (62%) was likewise not detectable by the canonical subpopulation markers five days after preparation of cells. Consistently, we found a reduction in the proportion of IB4-positive neurons between two hours and five days in culture, while the proportions of CGRP/SP- and NF200-subpopulations remained unchanged. Hence, MLP expression *in vitro* was apparently also upregulated predominantly in formerly IB4-positive nociceptors. A characteristic phenotypic attribute of these neurons is the expression of a functional receptor for glial-derived neurotrophic factor (GDNF) (Molliver et al., 1997; Bennett et al., 1998; Zwick et al., 2002). Moreover, the treatment of axotomized DRG with GDNF prevents axotomy-induced downregulation of IB4-binding (Bennett et al., 1998). To test

whether MLP-positive neurons express GDNF-receptor and whether GDNF could rescue their IB4-labeling, we added this cytokine to DRG cultures. Indeed, GDNF largely prevented the decline in the proportion of IB4-positive neuron after five days in culture and maintained IB4-binding in 51% of MLP-expressing cells (compared to 11% without GDNF). Hence, this finding further substantiated our hypothesis that the most MLP-positive cells originate from a distinct subset of nonpeptidergic nociceptive neurons, which lose their ability to bind IB4 upon axotomy. Therefore, MLP could serve as a novel marker for these injured neurons.

The role of MLP in axotomized DRG, however, remains a matter of future studies. It is possible that MLP is particularly important for the regrowth of lesioned axons. An attempt to address this question was made in the work of Stam et al. (Stam et al., 2007) using an RNAi-mediated knockdown approach in cultured DRG neurons. These experiments did not reveal any impact of MLP on axonal regeneration. However, this study did not take into consideration that MLP is expressed only in a small population of neurons. Hence, a possible effect could have been masked by the overwhelming majority of MLP-negative cells. Besides the probable implication in axonal growth, MLP-expression in corresponding nociceptors could also influence the sensation of noxious stimuli, which is often affected by the axotomy (Woolf and Salter, 2000).

To sum up, the necessary, extensive characterization of MLP-expression in axotomized DRG, presented in this study, finally paves the way for precise and efficient investigations on the function of this protein in the injured PNS tissue.

3.2 Expression of MLP in developing retinal neurons

This part of the result and discussion section is based on the publication in PloS One with the title “Neuronal Expression of Muscle LIM Protein in postnatal retinae of rodents” (Levin et al., 2014). I contributed to the study design and performed the experiments for this publication. I was also involved in the analysis and interpretation of data.

The upregulation of MLP discovered in axotomized DRG raised the question whether MLP could be also a part of the normal embryonic and postnatal development program

in neurons. In particular, the expression of MLP in developing CNS cells like RGCs could be a hint for a possible involvement of this protein in the morphologic or functional maturation of the central nervous tissue. To address this question, the expression of MLP was analyzed in embryonic (E18 - E20), postnatal (P1 - P14) and adult (P21 - P42) retinae of naïve rats. Based on qPCR and Western blot data, MLP levels increased between E20 and P14. The highest expression was measured at P14 with a ~7-fold upregulation of Mlp-mRNA compared to E18. However, MLP expression subsequently decreased in adult animals. In particular, mRNA levels returned to the embryonic state already at P21 and, correspondingly, no protein could be detected by the Western blot in rats older than this age. Subsequent immunohistochemical evaluation of retinal cross-sections using an MLP-specific antibody confirmed the temporal expression pattern previously unveiled by the molecular biology analysis and surprisingly revealed that MLP positive cells were equally spread between GCL and INL. Rather than for RGCs, such distribution is characteristic for cholinergic amacrine cells (ACs), which exist as a "normally" placed population in the INL and a displaced population in the GCL (Hayden et al., 1980; Vaney et al., 1981; Voigt, 1986). The displaced fraction is stimulated by enhancing illumination and therefore referred to as ON-cells. They project their dendrites to the ON-sublamina of the IPL. In contrast, cholinergic ACs of the INL are excited by decreasing light stimuli (OFF subpopulation). The dendrites of these cells correspondingly terminate in the OFF-sublamina of the IPL (Famiglietti, 1983). Cholinergic ACs synthesize high amounts of the neurotransmitter acetylcholine (Voigt, 1986) and therefore can be specifically stained with antibodies against the enzyme choline acetyltransferase (ChAT) (Eckenstein and Thoenen, 1982). Indeed, co-staining for MLP and ChAT revealed that all cholinergic ACs expressed MLP between P7 and P14, whereas in adult animals MLP signal almost completely disappeared in virtually all of these ChAT-positive neurons. MLP was detected in cytoplasms as well as in dendrites, and its staining exactly colocalized with the two ChAT-immunoreactive dendritic bands formed by the both subgroups of cholinergic ACs in the IPL. Interestingly, the nuclei of the cells did not contain MLP. Increasing expression of the protein was also detected in the cultured postnatal ChAT-positive ACs obtained from P1-rats with the same intracellular localization pattern restricted to their cytoplasms and sprouting dendrites. Therefore, upregulation of MLP is most likely

attributed to intrinsic maturation program rather than to extrinsic biochemical or electric stimulation of cholinergic ACs in the retinal parenchyma. In fact, the temporal pattern of MLP expression was well correlated with the morphological and functional maturation process of cholinergic ACs. These cells emerge in the retina of rodents around E8/E9. Their genesis peaks at E16/E17, but continues until at least P5/P7 (Rapaport et al., 2004; Voinescu et al., 2009). At E17, around the time point of detected MLP upregulation, they start to express ChAT and to extend dendrites which eventually establish two distinct cholinergic layers in the IPL (Kim et al., 2000). The synapses between cholinergic ACs and bipolar cells as well as their main target cell population, the direction-selective RGCs (Famiglietti, 1987; Taylor and Smith, 2012), are established between P3 and P15 (Fisher, 1979; Wei et al., 2011). This process partially coincides with the appearance of acetylcholine-driven waves of spontaneous excitatory activity (between P0 and P11), which supposedly affect the formation of synapses and neuronal circuits in the retina (Feller et al., 1996; Bansal et al., 2000; Ford and Feller, 2012). Maturation of cholinergic ACs is accomplished at the time point of the eye opening (around P13-P15 in rodents) (Kim et al., 2000; Zhang et al., 2005). Remarkably, this was exactly the age of the animals when retinal MLP levels began to decrease.

In conclusion, MLP is obviously expressed during a crucial phase of embryonic and postnatal development of cholinergic ACs and is possibly involved in this process. In this context, an influence of the protein on gene expression seems quite unlikely due to its absence in the nuclei. Instead, MLP could interact with various cytoplasmic proteins to regulate different cellular functions, which are important for morphologic and functional maturation of cholinergic ACs. For instance, it could promote the growth and stratification of dendritic arbors. Although further investigations are needed to elucidate the exact role of MLP in cholinergic ACs, this study for the first time described its expression in naïve nervous tissue. The particular upregulation in developing neurons potentially indicates the biological importance of MLP for the establishment of neuronal structures and might, therefore, explain its induction also in regenerating neurons.

3.3 Baculovirus-mediated transduction of primary adult PNS and CNS neurons

This part of the result and discussion section is based on the publication in Scientific Reports with the title “Highly efficient transduction of primary adult CNS and PNS neurons” (Levin et al., 2016). I was involved in the study design and prepared all baculoviruses except for the DsRed-virus. I also performed and evaluated the transduction and neurite outgrowth experiments. My contribution also includes the analysis and interpretation of data.

Mature primary neurons are not susceptible to transfections/transductions *in vitro* with virtually all so far exciting methods. Baculoviruses are generally known as an efficient gene delivery system but have never been thoroughly evaluated in this context for cultures of adult primary neuronal cells. To test the transduction efficiency of BacMam-vectors in DRG and RGC cultures, we prepared VSV-G-pseudotyped baculoviruses for the expression of farnesylated GFP (fGFP) or DsRed under the regulation of CMV promoter. Since gene delivery systems are normally more effective in developing neurons, we first tested the applicability of the BacMam-viruses in postnatal (P7) DRG neurons of rats. To this end, the fGFP-viruses were added directly to the cell cultures four hours after the preparation of the cells and two-thirds of the culture medium were replaced 16 hours later. The cells were monitored for up to 72 hours post-transduction. About 80% of postnatal DRG neurons were reproducibly transduced by the baculovirus. Remarkably, this high transduction rate was achieved only 24 hours after the application of the viral solution, indicating a rapid induction of transgene expression. Subsequent, similarly conducted experiments with adult rat and mouse DRG neurons resulted in a comparably efficient and swift transduction. Therefore, we did not detect any age- or species-specific differences in BacMam-mediated gene delivery to PNS neurons. Moreover, the survival of these cells in culture was not affected by the virus application. However, we noted some degradation of axons in cultures 72 hours post-transduction. This adverse effect was most likely attributed to the strong expression of the particular membrane-bound (farnesylated) GFP variant, which could impede neurite formation. Some previous reports also described apoptosis of neurons upon eGFP expression (Detrait et al., 2002). In support of this assumption, we did not

observe any detrimental impact on axon growth in a further series of experiments, where we applied a baculovirus for the expression of Cre recombinase in ROSA-tdTomato mouse DRG neurons. Cre/LoxP system is commonly used for conditional gene knockout and relies on rapid and strong expression of Cre. Notably, a previous study failed to detect successful genetic recombination in postnatal mouse neurons upon baculovirus-mediated transduction with Cre (Erbs et al., 2013). In contrast, we observed a pronounced induction of tdTomato, the reporter of successful Cre-specific gene editing in the ROSA-tdTomato model, in about 50% of neurons 24 hours post-transduction. This proportion further increased to 60% after 72 hours. Hence, baculoviruses have indeed been proven to be a suitable and efficient tool for experiments with Cre/LoxP-dependent gene knockout in neuronal cultures. Moreover, we also analyzed subpopulation-specificity of BacMam viruses in ROSA-tdTomato DRG, as this aspect could be an important issue for transduction experiments with peripheral neurons. However, we did not observe any differences in transduction rates of IB4 and NF200 subpopulations by the Cre-baculovirus, indicating no specific tropism for certain neuronal subgroups.

The transduction efficiency in primary adult CNS neurons was tested in RGC cultures obtained from rats, mice and zebrafish, using the same protocol as in DRG experiments. In opposite to our findings in DRG, we however, detected differences in transduction rates of RGCs between species. Whereas maximal transduction rates in rats were reliably above 80%, the corresponding proportion did not exceed ~50% in mice and ~20% in zebrafish. Moreover, the maximal transduction rates were reached slower than in DRG neurons (after 48 hours in rat and mice RGCs and after six days in zebrafish RGCs). Some optimization of experimental conditions, e.g. a higher multiplicity of infection, might be needed to further improve this outcome. Nevertheless, such reliable and high efficiency of gene delivery to adult CNS neurons *in vitro* is outstanding and has not been achieved so far using other techniques. Moreover, we did not observe any neurocytopathic or neurotoxic effects. We also tested whether neurons can still be transduced after several days in culture. Indeed, BacMam-particles transduced zebrafish RGCs, which were pre-incubated for four days, with an efficiency comparable to the immediate virus application. Although zebrafish RGCs were in general considerably less prone to BacMam-mediated transduction than the RGCs

isolated from rats and mice, the detected transduction rate would still be sufficient to perform evaluable overexpression or knockout experiments. Moreover, it should be noted that at the time of the conduct of our study, we did not find any other reports on successful gene delivery to zebrafish neurons *in vitro*, which are, remarkably, an important and unique model system to study axonal regeneration (Diekmann et al., 2015b, a). Limited transduction rate might even be advantageous for some experimental settings because in this case the transduced and the non-transduced cells can be evaluated and compared in the same well.

Some experiments might also require the concurrent delivery and expression of several genes. Therefore, we tested the transduction of RGCs with two different BacMam-viruses for expression of two different fluorescent reporter proteins (fGFP and DsRed). Consistent with previous reports using AAVs (Wu et al., 2015), almost all transduced cells expressed both reporters simultaneously. Hence, this particular feature opens unique opportunities for co-expression studies in cultured neurons.

An important question for the research on axonal regeneration was whether BacMam-viruses can be applied to perform *in vitro* neurite outgrowth assays, a useful experimental model to study regeneration-associated molecular mechanisms (Grozdanov et al., 2010). Using DsRed-baculovirus in a preliminary experiment, we found out that transduction of RGCs with BacMam-particles did not alter the neurite extension *per se* compared to non-transduced controls. We then used a baculovirus for the expression of the designer cytokine hIL6 to perform an exemplary neurite outgrowth assay. In a previous study, we already reported that hIL6 efficiently promotes neurite outgrowth of mouse RGCs *in vivo* and *in vitro* (Leibinger et al., 2016). As expected, RGCs transduced with the hIL6-baculovirus were also able to extend longer neurites than controls. Hence, we concluded that the BacMam-system is suitable for studies on gene functions in the context of axonal regeneration. Moreover, some further advantages of baculoviruses generally favor their use in laboratory research. They include easy producibility, biosafety level 1 handling, uncomplicated long-term storage (Wasilko et al., 2009) and simple non-toxic transduction process. In addition, high insert capacity (up to 38 kb for the particular system used in our study) makes BacMam-particles suitable for delivery of large genes or several different genes at once (Hu, 2005; Kost et al., 2005; Mansouri et al., 2016). Arguably, transduction with mammalian

baculoviruses is in general only transient because of the missing integration of delivered cDNA into the genome of host cells. However, this aspect is probably not of importance for non-dividing cells, like neurons. Notably, many neuronal assays are anyway accomplished within several days and even if a prolonged expression of a transgene is required, the cells can be readily re-transduced by the same baculovirus (Makkonen et al., 2015). Some modifications may be required to improve the tropism of baculoviruses, as we also detected some transduced non-neuronal cells in our cultures. Although the proportion of these cells was negligibly small, probably due to special neuron-optimized culture conditions, they could potentially hamper the evaluation of some experiments. However, the use of neuron-specific promoters instead of CMV might easily overcome this problem as well (Li et al., 2004; Wang, 2008). In summary, baculoviral transduction could become an efficient, indispensable tool to explore the role of proteins like MLP in injured RGC and DRG neurons.

4. Literature

Aguayo AJ, David S, Bray GM (1981) Influences of the glial environment on the elongation of axons after injury: transplantation studies in adult rodents. J Exp Biol 95:231-240.

Airenne KJ, Hu YC, Kost TA, Smith RH, Kotin RM, Ono C, Matsuura Y, Wang S, Yla-Herttuala S (2013) Baculovirus: an insect-derived vector for diverse gene transfer applications. Mol Ther 21:739-749.

Arber S, Halder G, Caroni P (1994) Muscle LIM protein, a novel essential regulator of myogenesis, promotes myogenic differentiation. Cell 79:221-231.

Arber S, Hunter JJ, Ross J, Jr., Hongo M, Sansig G, Borg J, Perriard JC, Chien KR, Caroni P (1997) MLP-deficient mice exhibit a disruption of cardiac cytoarchitectural organization, dilated cardiomyopathy, and heart failure. Cell 88:393-403.

Argiro V, Bunge MB, Johnson MI (1984) Correlation between growth form and movement and their dependence on neuronal age. The Journal of neuroscience : the official journal of the Society for Neuroscience 4:3051-3062.

Averill S, Davis DR, Shortland PJ, Priestley JV, Hunt SP (2002) Dynamic pattern of reg-2 expression in rat sensory neurons after peripheral nerve injury. The Journal of neuroscience : the official journal of the Society for Neuroscience 22:7493-7501.

Bansal A, Singer JH, Hwang BJ, Xu W, Beaudet A, Feller MB (2000) Mice lacking specific nicotinic acetylcholine receptor subunits exhibit dramatically altered spontaneous activity patterns and reveal a limited role for retinal waves in forming ON and OFF circuits in the inner retina. The Journal of neuroscience : the official journal of the Society for Neuroscience 20:7672-7681.

Barsoum J, Brown R, McKee M, Boyce FM (1997) Efficient transduction of mammalian cells by a recombinant baculovirus having the vesicular stomatitis virus G glycoprotein. Human gene therapy 8:2011-2018.

Bear MF, Connors BW, Paradiso MA (2009) Neurowissenschaften ein grundlegendes Lehrbuch für Biologie, Medizin und Psychologie, 3. Aufl. [der amerikan. Orig.-Ausg.] Edition. Heidelberg: Spektrum Akad. Verl.

Bennett DL, Michael GJ, Ramachandran N, Munson JB, Averill S, Yan Q, McMahon SB, Priestley JV (1998) A distinct subgroup of small DRG cells express GDNF receptor components and GDNF is protective for these neurons after nerve injury. The Journal of neuroscience : the official journal of the Society for Neuroscience 18:3059-3072.

Berkelaar M, Clarke DB, Wang YC, Bray GM, Aguayo AJ (1994) Axotomy results in delayed death and apoptosis of retinal ganglion cells in adult rats. The Journal of neuroscience : the official journal of the Society for Neuroscience 14:4368-4374.

Berry M, Carlile J, Hunter A, Tsang W, Rosenstiel P, Sievers J (1999) Optic nerve regeneration after intravitreal peripheral nerve implants: trajectories of axons regrowing through the optic chiasm into the optic tracts. Journal of neurocytology 28:721-741.

Bessou P, Perl ER (1969) Response of cutaneous sensory units with unmyelinated fibers to noxious stimuli. J Neurophysiol 32:1025-1043.

Blinzinger K, Kreutzberg G (1968) Displacement of synaptic terminals from regenerating motoneurons by microglial cells. Z Zellforsch Mikrosk Anat 85:145-157.

Boateng SY, Belin RJ, Geenen DL, Margulies KB, Martin JL, Hoshijima M, de Tombe PP, Russell B (2007) Cardiac dysfunction and heart failure are associated with abnormalities in the subcellular distribution and amounts of oligomeric muscle LIM protein. American journal of physiology Heart and circulatory physiology 292:H259-269.

Bonilla IE, Tanabe K, Strittmatter SM (2002) Small proline-rich repeat protein 1A is expressed by axotomized neurons and promotes axonal outgrowth. The Journal of neuroscience : the official journal of the Society for Neuroscience 22:1303-1315.

Boyce FM, Bucher NL (1996) Baculovirus-mediated gene transfer into mammalian cells. Proc Natl Acad Sci U S A 93:2348-2352.

Brumovsky P, Villar MJ, Hokfelt T (2006) Tyrosine hydroxylase is expressed in a subpopulation of small dorsal root ganglion neurons in the adult mouse. Experimental neurology 200:153-165.

Burgess PR, Perl ER (1967) Myelinated afferent fibres responding specifically to noxious stimulation of the skin. The Journal of physiology 190:541-562.

Buyandelger B, Ng KE, Miocic S, Piotrowska I, Gunkel S, Ku CH, Knoll R (2011) MLP (muscle LIM protein) as a stress sensor in the heart. Pflugers Archiv : European journal of physiology 462:135-142.

Cai D, Qiu J, Cao Z, McAtee M, Bregman BS, Filbin MT (2001) Neuronal cyclic AMP controls the developmental loss in ability of axons to regenerate. The Journal of neuroscience : the official journal of the Society for Neuroscience 21:4731-4739.

Chen MS, Huber AB, van der Haar ME, Frank M, Schnell L, Spillmann AA, Christ F, Schwab ME (2000) Nogo-A is a myelin-associated neurite outgrowth inhibitor and an antigen for monoclonal antibody IN-1. Nature 403:434-439.

Chen ZL, Strickland S (2003) Laminin gamma1 is critical for Schwann cell differentiation, axon myelination, and regeneration in the peripheral nerve. The Journal of cell biology 163:889-899.

Chen ZL, Yu WM, Strickland S (2007) Peripheral regeneration. Annu Rev Neurosci 30:209-233.

Chierzi S, Strettoi E, Cenni MC, Maffei L (1999) Optic nerve crush: axonal responses in wild-type and bcl-2 transgenic mice. The Journal of neuroscience : the official journal of the Society for Neuroscience 19:8367-8376.

Christensen BN, Perl ER (1970) Spinal neurons specifically excited by noxious or thermal stimuli: marginal zone of the dorsal horn. J Neurophysiol 33:293-307.

Clem RJ, Passarelli AL (2013) Baculoviruses: sophisticated pathogens of insects. PLoS Pathog 9:e1003729.

David S, Braun PE, Jackson DL, Kottis V, McKerracher L (1995) Laminin overrides the inhibitory effects of peripheral nervous system and central nervous system myelin-derived inhibitors of neurite growth. J Neurosci Res 42:594-602.

Detrait ER, Bowers WJ, Halterman MW, Giuliano RE, Bennice L, Federoff HJ, Richfield EK (2002) Reporter gene transfer induces apoptosis in primary cortical neurons. Mol Ther 5:723-730.
Devor M, Govrin-Lippmann R, Frank I, Raber P (1985) Proliferation of primary sensory neurons in adult rat dorsal root ganglion and the kinetics of retrograde cell loss after sciatic nerve section. Somatosens Res 3:139-167.
Diekmann H, Kalbhen P, Fischer D (2015a) Active mechanistic target of rapamycin plays an ancillary rather than essential role in zebrafish CNS axon regeneration. Front Cell Neurosci 9:251.
Diekmann H, Kalbhen P, Fischer D (2015b) Characterization of optic nerve regeneration using transgenic zebrafish. Front Cell Neurosci 9:118.
Ding B, Kilpatrick DL (2013) Lentiviral vector production, titration, and transduction of primary neurons. Methods in molecular biology 1018:119-131.
Eckenstein F, Thoenen H (1982) Production of specific antisera and monoclonal antibodies to choline acetyltransferase: characterization and use for identification of cholinergic neurons. EMBO J 1:363-368.
Erbs E, Pradhan AA, Matifas A, Kieffer BL, Massotte D (2013) Evaluation of cre recombinase delivery in mammalian cells using baculovirus infection. Journal of biotechnology 166:182-186.
Famiglietti EV (1987) Starburst amacrine cells in cat retina are associated with bistratified, presumed directionally selective, ganglion cells. Brain Res 413:404-408.
Famiglietti EV, Jr. (1983) 'Starburst' amacrine cells and cholinergic neurons: mirror-symmetric on and off amacrine cells of rabbit retina. Brain Res 261:138-144.
Faroni A, Mobasseri SA, Kingham PJ, Reid AJ (2015) Peripheral nerve regeneration: experimental strategies and future perspectives. Adv Drug Deliv Rev 82-83:160-167.
Feller MB, Wellis DP, Stellwagen D, Werblin FS, Shatz CJ (1996) Requirement for cholinergic synaptic transmission in the propagation of spontaneous retinal waves. Science 272:1182-1187.
Fex Svennigsen A, Dahlin LB (2013) Repair of the Peripheral Nerve-Remyelination that Works. Brain Sci 3:1182-1197.
Fischer D, Pavlidis M, Thanos S (2000) Cataractogenic lens injury prevents traumatic ganglion cell death and promotes axonal regeneration both in vivo and in culture. Invest Ophthalmol Vis Sci 41:3943-3954.
Fischer D, Petkova V, Thanos S, Benowitz LI (2004) Switching mature retinal ganglion cells to a robust growth state in vivo: gene expression and synergy with RhoA inactivation. The Journal of neuroscience : the official journal of the Society for Neuroscience 24:8726-8740.
Fischer D, Hauk TG, Muller A, Thanos S (2008) Crystallins of the beta/gamma-superfamily mimic the effects of lens injury and promote axon regeneration. Molecular and cellular neurosciences 37:471-479.
Fisher LJ (1979) Development of synaptic arrays in the inner plexiform layer of neonatal mouse retina. J Comp Neurol 187:359-372.
Fleming J, Ginn SL, Weinberger RP, Trahair TN, Smythe JA, Alexander IE (2001) Adeno-associated virus and lentivirus vectors mediate efficient and sustained

transduction of cultured mouse and human dorsal root ganglia sensory neurons. Human gene therapy 12:77-86.
Flick MJ, Konieczny SF (2000) The muscle regulatory and structural protein MLP is a cytoskeletal binding partner of beta1-spectrin. Journal of cell science 113 (Pt 9):1553-1564.
Ford KJ, Feller MB (2012) Assembly and disassembly of a retinal cholinergic network. Vis Neurosci 29:61-71.
Gascon S, Paez-Gomez JA, Diaz-Guerra M, Scheiffele P, Scholl FG (2008) Dual-promoter lentiviral vectors for constitutive and regulated gene expression in neurons. Journal of neuroscience methods 168:104-112.
Gaudet AD, Popovich PG, Ramer MS (2011) Wallerian degeneration: gaining perspective on inflammatory events after peripheral nerve injury. J Neuroinflammation 8:110.
Geier C et al. (2008) Beyond the sarcomere: CSRP3 mutations cause hypertrophic cardiomyopathy. Human molecular genetics 17:2753-2765.
George R, Griffin JW (1994) Delayed macrophage responses and myelin clearance during Wallerian degeneration in the central nervous system: the dorsal radiculotomy model. Experimental neurology 129:225-236.
Ghosh S, Parvez MK, Banerjee K, Sarin SK, Hasnain SE (2002) Baculovirus as mammalian cell expression vector for gene therapy: an emerging strategy. Mol Ther 6:5-11.
Gold BG, Katoh K, Storm-Dickerson T (1995) The immunosuppressant FK506 increases the rate of axonal regeneration in rat sciatic nerve. The Journal of neuroscience : the official journal of the Society for Neuroscience 15:7509-7516.
Goldberg JL, Klassen MP, Hua Y, Barres BA (2002) Amacrine-signaled loss of intrinsic axon growth ability by retinal ganglion cells. Science 296:1860-1864.
Graeber MB, Bise K, Mehraein P (1993) Synaptic stripping in the human facial nucleus. Acta Neuropathol 86:179-181.
GrandPre T, Nakamura F, Vartanian T, Strittmatter SM (2000) Identification of the Nogo inhibitor of axon regeneration as a Reticulon protein. Nature 403:439-444.
Grinsell D, Keating CP (2014) Peripheral nerve reconstruction after injury: a review of clinical and experimental therapies. Biomed Res Int 2014:698256.
Grozdanov V, Muller A, Sengottuvel V, Leibinger M, Fischer D (2010) A method for preparing primary retinal cell cultures for evaluating the neuroprotective and neuritogenic effect of factors on axotomized mature CNS neurons. Current protocols in neuroscience / editorial board, Jacqueline N Crawley [et al] Chapter 3:Unit3 22.
Gupta MP, Samant SA, Smith SH, Shroff SG (2008) HDAC4 and PCAF bind to cardiac sarcomeres and play a role in regulating myofilament contractile activity. The Journal of biological chemistry 283:10135-10146.
Haeseleer F, Imanishi Y, Saperstein DA, Palczewski K (2001) Gene transfer mediated by recombinant baculovirus into mouse eye. Invest Ophthalmol Vis Sci 42:3294-3300.
Harper AA, Lawson SN (1985) Conduction velocity is related to morphological cell type in rat dorsal root ganglion neurones. The Journal of physiology 359:31-46.

Hauk TG, Leibinger M, Muller A, Andreadaki A, Knippschild U, Fischer D (2010) Stimulation of axon regeneration in the mature optic nerve by intravitreal application of the toll-like receptor 2 agonist Pam3Cys. Invest Ophthalmol Vis Sci 51:459-464.

Hayden SA, Mills JW, Masland RM (1980) Acetylcholine synthesis by displaced amacrine cells. Science 210:435-437.

Hilla AM, Diekmann H, Fischer D (2017) Microglia are irrelevant for neuronal degeneration and axon regeneration after acute injury. The Journal of neuroscience : the official journal of the Society for Neuroscience.

Hofbauer A, Drager UC (1985) Depth segregation of retinal ganglion cells projecting to mouse superior colliculus. J Comp Neurol 234:465-474.

Hoffmann C, Moreau F, Moes M, Luthold C, Dieterle M, Goretti E, Neumann K, Steinmetz A, Thomas C (2014) Human muscle LIM protein dimerizes along the actin cytoskeleton and cross-links actin filaments. Molecular and cellular biology 34:3053-3065.

Hofmann C, Sandig V, Jennings G, Rudolph M, Schlag P, Strauss M (1995) Efficient gene transfer into human hepatocytes by baculovirus vectors. Proc Natl Acad Sci U S A 92:10099-10103.

Hu YC (2005) Baculovirus as a highly efficient expression vector in insect and mammalian cells. Acta pharmacologica Sinica 26:405-416.

Hutson TH, Foster E, Dawes JM, Hindges R, Yanez-Munoz RJ, Moon LD (2012) Lentiviral vectors encoding short hairpin RNAs efficiently transduce and knockdown LINGO-1 but induce an interferon response and cytotoxicity in central nervous system neurones. The journal of gene medicine 14:299-315.

Jenkins R, Hunt SP (1991) Long-term increase in the levels of c-jun mRNA and jun protein-like immunoreactivity in motor and sensory neurons following axon damage. Neuroscience letters 129:107-110.

Ju G, Hokfelt T, Brodin E, Fahrenkrug J, Fischer JA, Frey P, Elde RP, Brown JC (1987) Primary sensory neurons of the rat showing calcitonin gene-related peptide immunoreactivity and their relation to substance P-, somatostatin-, galanin-, vasoactive intestinal polypeptide- and cholecystokinin-immunoreactive ganglion cells. Cell and tissue research 247:417-431.

Kandel ER, Schwartz JH, Jessell TM, Siegelbaum S, Hudspeth AJ (2013) Principles of neural science, 5th Edition. New York, NY ; London: McGraw-Hill.

Karra D, Dahm R (2010) Transfection techniques for neuronal cells. The Journal of neuroscience : the official journal of the Society for Neuroscience 30:6171-6177.

Kemecsei P, Miklos Z, Biro T, Marincsak R, Toth BI, Komlodi-Pasztor E, Barnucz E, Mirk E, Van der Vusse GJ, Ligeti L, Ivanics T (2010) Hearts of surviving MLP-KO mice show transient changes of intracellular calcium handling. Mol Cell Biochem 342:251-260.

Kiernan JA (1985) Axonal and vascular changes following injury to the rat's optic nerve. J Anat 141:139-154.

Kim IB, Lee EJ, Kim MK, Park DK, Chun MH (2000) Choline acetyltransferase-immunoreactive neurons in the developing rat retina. J Comp Neurol 427:604-616.

Knoll R et al. (2002) The cardiac mechanical stretch sensor machinery involves a Z disc complex that is defective in a subset of human dilated cardiomyopathy. Cell 111:943-955.
Knoll R et al. (2010) A common MLP (muscle LIM protein) variant is associated with cardiomyopathy. Circ Res 106:695-704.
Kolangath SM, Basagoudanavar SH, Hosamani M, Saravanan P, Tamil Selvan RP (2014) Baculovirus mediated transduction: analysis of vesicular stomatitis virus glycoprotein pseudotyping. Virusdisease 25:441-446.
Kong Y, Flick MJ, Kudla AJ, Konieczny SF (1997) Muscle LIM protein promotes myogenesis by enhancing the activity of MyoD. Molecular and cellular biology 17:4750-4760.
Kost TA, Condreay JP (2002) Recombinant baculoviruses as mammalian cell gene-delivery vectors. Trends in biotechnology 20:173-180.
Kost TA, Condreay JP, Jarvis DL (2005) Baculovirus as versatile vectors for protein expression in insect and mammalian cells. Nat Biotechnol 23:567-575.
Kreutzberg GW (1996) Microglia: a sensor for pathological events in the CNS. Trends Neurosci 19:312-318.
Lawson SN, Waddell PJ (1991) Soma neurofilament immunoreactivity is related to cell size and fibre conduction velocity in rat primary sensory neurons. J Physiol 435:41-63.
Leibinger M, Andreadaki A, Diekmann H, Fischer D (2013a) Neuronal STAT3 activation is essential for CNTF- and inflammatory stimulation-induced CNS axon regeneration. Cell death & disease 4:e805.
Leibinger M, Muller A, Andreadaki A, Hauk TG, Kirsch M, Fischer D (2009) Neuroprotective and axon growth-promoting effects following inflammatory stimulation on mature retinal ganglion cells in mice depend on ciliary neurotrophic factor and leukemia inhibitory factor. The Journal of neuroscience : the official journal of the Society for Neuroscience 29:14334-14341.
Leibinger M, Muller A, Gobrecht P, Diekmann H, Andreadaki A, Fischer D (2013b) Interleukin-6 contributes to CNS axon regeneration upon inflammatory stimulation. Cell death & disease 4:e609.
Leibinger M, Andreadaki A, Gobrecht P, Levin E, Diekmann H, Fischer D (2016) Boosting Central Nervous System Axon Regeneration by Circumventing Limitations of Natural Cytokine Signaling. Mol Ther.
Leon S, Yin Y, Nguyen J, Irwin N, Benowitz LI (2000) Lens injury stimulates axon regeneration in the mature rat optic nerve. The Journal of neuroscience : the official journal of the Society for Neuroscience 20:4615-4626.
Levin E, Diekmann H, Fischer D (2016) Highly efficient transduction of primary adult CNS and PNS neurons. Sci Rep 6:38928.
Levin E, Leibinger M, Andreadaki A, Fischer D (2014) Neuronal expression of muscle LIM protein in postnatal retinae of rodents. PloS one 9:e100756.
Levin E, Andreadaki A, Gobrecht P, Bosse F, Fischer D (2017) Nociceptive DRG neurons express muscle lim protein upon axonal injury. Sci Rep 7:643.
Li Y, Wang X, Guo H, Wang S (2004) Axonal transport of recombinant baculovirus vectors. Mol Ther 10:1121-1129.
Linda H, Shupliakov O, Ornung G, Ottersen OP, Storm-Mathisen J, Risling M, Cullheim S (2000) Ultrastructural evidence for a preferential elimination of

glutamate-immunoreactive synaptic terminals from spinal motoneurons after intramedullary axotomy. The Journal of comparative neurology 425:10-23.
Louis HA, Pino JD, Schmeichel KL, Pomies P, Beckerle MC (1997) Comparison of three members of the cysteine-rich protein family reveals functional conservation and divergent patterns of gene expression. The Journal of biological chemistry 272:27484-27491.
Lucius R, Gallinat S, Rosenstiel P, Herdegen T, Sievers J, Unger T (1998) The angiotensin II type 2 (AT2) receptor promotes axonal regeneration in the optic nerve of adult rats. J Exp Med 188:661-670.
Ma DK, Bonaguidi MA, Ming GL, Song H (2009) Adult neural stem cells in the mammalian central nervous system. Cell Res 19:672-682.
MacNeil MA, Masland RH (1998) Extreme diversity among amacrine cells: implications for function. Neuron 20:971-982.
MacNeil MA, Heussy JK, Dacheux RF, Raviola E, Masland RH (1999) The shapes and numbers of amacrine cells: matching of photofilled with Golgi-stained cells in the rabbit retina and comparison with other mammalian species. J Comp Neurol 413:305-326.
Makkonen KE, Airenne K, Yla-Herttulala S (2015) Baculovirus-mediated gene delivery and RNAi applications. Viruses 7:2099-2125.
Mansouri M, Bellon-Echeverria I, Rizk A, Ehsaei Z, Cianciolo Cosentino C, Silva CS, Xie Y, Boyce FM, Davis MW, Neuhauss SC, Taylor V, Ballmer-Hofer K, Berger I, Berger P (2016) Highly efficient baculovirus-mediated multigene delivery in primary cells. Nature communications 7:11529.
Marmigere F, Ernfors P (2007) Specification and connectivity of neuronal subtypes in the sensory lineage. Nature reviews Neuroscience 8:114-127.
Masland RH (2012) The tasks of amacrine cells. Vis Neurosci 29:3-9.
McKeon RJ, Schreiber RC, Rudge JS, Silver J (1991) Reduction of neurite outgrowth in a model of glial scarring following CNS injury is correlated with the expression of inhibitory molecules on reactive astrocytes. The Journal of neuroscience : the official journal of the Society for Neuroscience 11:3398-3411.
McKerracher L, David S, Jackson DL, Kottis V, Dunn RJ, Braun PE (1994) Identification of myelin-associated glycoprotein as a major myelin-derived inhibitor of neurite growth. Neuron 13:805-811.
Michelsen JW, Schmeichel KL, Beckerle MC, Winge DR (1993) The LIM motif defines a specific zinc-binding protein domain. Proceedings of the National Academy of Sciences of the United States of America 90:4404-4408.
Molliver DC, Radeke MJ, Feinstein SC, Snider WD (1995) Presence or absence of TrkA protein distinguishes subsets of small sensory neurons with unique cytochemical characteristics and dorsal horn projections. J Comp Neurol 361:404-416.
Molliver DC, Wright DE, Leitner ML, Parsadanian AS, Doster K, Wen D, Yan Q, Snider WD (1997) IB4-binding DRG neurons switch from NGF to GDNF dependence in early postnatal life. Neuron 19:849-861.
Mukhopadhyay G, Doherty P, Walsh FS, Crocker PR, Filbin MT (1994) A novel role for myelin-associated glycoprotein as an inhibitor of axonal regeneration. Neuron 13:757-767.

Muller A, Hauk TG, Fischer D (2007) Astrocyte-derived CNTF switches mature RGCs to a regenerative state following inflammatory stimulation. Brain 130:3308-3320.

Müller A, Hauk TG, Fischer D (2007) Astrocyte-derived CNTF switches mature RGCs to a regenerative state following inflammatory stimulation. Brain 130:3308-3320.

Nagy JI, Hunt SP (1982) Fluoride-resistant acid phosphatase-containing neurones in dorsal root ganglia are separate from those containing substance P or somatostatin. Neuroscience 7:89-97.

Nakajima K, Kohsaka S (2001) Microglia: activation and their significance in the central nervous system. J Biochem 130:169-175.

Neumann H, Kotter MR, Franklin RJ (2009) Debris clearance by microglia: an essential link between degeneration and regeneration. Brain 132:288-295.

Newton RA, Bingham S, Davey PD, Medhurst AD, Piercy V, Raval P, Parsons AA, Sanger GJ, Case CP, Lawson SN (2000) Identification of differentially expressed genes in dorsal root ganglia following partial sciatic nerve injury. Neuroscience 95:1111-1120.

Niederost BP, Zimmermann DR, Schwab ME, Bandtlow CE (1999) Bovine CNS myelin contains neurite growth-inhibitory activity associated with chondroitin sulfate proteoglycans. The Journal of neuroscience : the official journal of the Society for Neuroscience 19:8979-8989.

Nieke J, Schachner M (1985) Expression of the neural cell adhesion molecules L1 and N-CAM and their common carbohydrate epitope L2/HNK-1 during development and after transection of the mouse sciatic nerve. Differentiation 30:141-151.

Okano K, Vanarsdall AL, Mikhailov VS, Rohrmann GF (2006) Conserved molecular systems of the Baculoviridae. Virology 344:77-87.

Oliveira J, Lopes F, de Almeida F, Martinez A (2014) Gene Therapy in Rodents Models of Traumatic Peripheral Nerve Injury. J Cell Sci Ther 5.

Papalouka V, Arvanitis DA, Vafiadaki E, Mavroidis M, Papadodima SA, Spiliopoulou CA, Kremastinos DT, Kranias EG, Sanoudou D (2009) Muscle LIM protein interacts with cofilin 2 and regulates F-actin dynamics in cardiac and skeletal muscle. Molecular and cellular biology 29:6046-6058.

Park KK, Liu K, Hu Y, Smith PD, Wang C, Cai B, Xu B, Connolly L, Kramvis I, Sahin M, He Z (2008) Promoting axon regeneration in the adult CNS by modulation of the PTEN/mTOR pathway. Science 322:963-966.

Perry MJ, Lawson SN, Robertson J (1991) Neurofilament immunoreactivity in populations of rat primary afferent neurons: a quantitative study of phosphorylated and non-phosphorylated subunits. Journal of neurocytology 20:746-758.

Price J, Mudge AW (1983) A subpopulation of rat dorsal root ganglion neurones is catecholaminergic. Nature 301:241-243.

Price TJ, Flores CM (2007) Critical evaluation of the colocalization between calcitonin gene-related peptide, substance P, transient receptor potential vanilloid subfamily type 1 immunoreactivities, and isolectin B4 binding in primary afferent neurons of the rat and mouse. The journal of pain : official journal of the American Pain Society 8:263-272.

Purves D, Williams SM (2001) Neuroscience, 2nd Edition. Sunderland, Mass.: Sinauer Associates.
Rabacchi SA, Bonfanti L, Liu XH, Maffei L (1994) Apoptotic cell death induced by optic nerve lesion in the neonatal rat. The Journal of neuroscience : the official journal of the Society for Neuroscience 14:5292-5301.
Ramón y Cajal S, May RM (1928) Degeneration and regeneration of the nervous system: Oxford University Press ; Humphrey Milford.
Rapaport DH, Wong LL, Wood ED, Yasumura D, LaVail MM (2004) Timing and topography of cell genesis in the rat retina. J Comp Neurol 474:304-324.
Rashid MM, Runci A, Polletta L, Carnevale I, Morgante E, Foglio E, Arcangeli T, Sansone L, Russo MA, Tafani M (2015) Muscle LIM protein/CSRP3: a mechanosensor with a role in autophagy. Cell death discovery 1:15014.
Rigaud M, Gemes G, Barabas ME, Chernoff DI, Abram SE, Stucky CL, Hogan QH (2008) Species and strain differences in rodent sciatic nerve anatomy: implications for studies of neuropathic pain. Pain 136:188-201.
Royo NC, Vandenberghe LH, Ma JY, Hauspurg A, Yu L, Maronski M, Johnston J, Dichter MA, Wilson JM, Watson DJ (2008) Specific AAV serotypes stably transduce primary hippocampal and cortical cultures with high efficiency and low toxicity. Brain Res 1190:15-22.
Sanes JR, Masland RH (2015) The types of retinal ganglion cells: current status and implications for neuronal classification. Annu Rev Neurosci 38:221-246.
Schallus T, Feher K, Ulrich AS, Stier G, Muhle-Goll C (2009) Structure and dynamics of the human muscle LIM protein. FEBS letters 583:1017-1022.
Schmalbruch H (1986) Fiber composition of the rat sciatic nerve. Anat Rec 215:71-81.
Schmeichel KL, Beckerle MC (1994) The LIM domain is a modular protein-binding interface. Cell 79:211-219.
Shoichet MS, Tate CC, Baumann MD, LaPlaca MC (2008) Strategies for Regeneration and Repair in the Injured Central Nervous System. In: Indwelling Neural Implants: Strategies for Contending with the In Vivo Environment (Reichert WM, ed). Boca Raton (FL).
Silver J, Miller JH (2004) Regeneration beyond the glial scar. Nature reviews 5:146-156.
Silverman JD, Kruger L (1990) Selective neuronal glycoconjugate expression in sensory and autonomic ganglia: relation of lectin reactivity to peptide and enzyme markers. Journal of neurocytology 19:789-801.
Skene JH, Willard M (1981) Axonally transported proteins associated with axon growth in rabbit central and peripheral nervous systems. The Journal of cell biology 89:96-103.
Smith PD, Sun F, Park KK, Cai B, Wang C, Kuwako K, Martinez-Carrasco I, Connolly L, He Z (2009) SOCS3 deletion promotes optic nerve regeneration in vivo. Neuron 64:617-623.
Snider WD, McMahon SB (1998) Tackling pain at the source: new ideas about nociceptors. Neuron 20:629-632.
So KF, Aguayo AJ (1985) Lengthy regrowth of cut axons from ganglion cells after peripheral nerve transplantation into the retina of adult rats. Brain Res 328:349-354.

Stam FJ, MacGillavry HD, Armstrong NJ, de Gunst MC, Zhang Y, van Kesteren RE, Smit AB, Verhaagen J (2007) Identification of candidate transcriptional modulators involved in successful regeneration after nerve injury. The European journal of neuroscience 25:3629-3637.
Stucky CL, Lewin GR (1999) Isolectin B(4)-positive and -negative nociceptors are functionally distinct. The Journal of neuroscience : the official journal of the Society for Neuroscience 19:6497-6505.
Swett JE, Torigoe Y, Elie VR, Bourassa CM, Miller PG (1991) Sensory neurons of the rat sciatic nerve. Experimental neurology 114:82-103.
Taylor WR, Smith RG (2012) The role of starburst amacrine cells in visual signal processing. Vis Neurosci 29:73-81.
Terenghi G (1999) Peripheral nerve regeneration and neurotrophic factors. J Anat 194 (Pt 1):1-14.
Tsujino H, Kondo E, Fukuoka T, Dai Y, Tokunaga A, Miki K, Yonenobu K, Ochi T, Noguchi K (2000) Activating transcription factor 3 (ATF3) induction by axotomy in sensory and motoneurons: A novel neuronal marker of nerve injury. Molecular and cellular neurosciences 15:170-182.
Turunen TA, Laakkonen JP, Alasaarela L, Airenne KJ, Yla-Herttuala S (2014) Sleeping Beauty-baculovirus hybrid vectors for long-term gene expression in the eye. The journal of gene medicine 16:40-53.
van Oers MM, Pijlman GP, Vlak JM (2015) Thirty years of baculovirus-insect cell protein expression: from dark horse to mainstream technology. J Gen Virol 96:6-23.
Vaney DI, Peichi L, Boycott BB (1981) Matching populations of amacrine cells in the inner nuclear and ganglion cell layers of the rabbit retina. J Comp Neurol 199:373-391.
Villegas-Perez MP, Vidal-Sanz M, Rasminsky M, Bray GM, Aguayo AJ (1993) Rapid and protracted phases of retinal ganglion cell loss follow axotomy in the optic nerve of adult rats. J Neurobiol 24:23-36.
Vogelaar CF, Vrinten DH, Hoekman MF, Brakkee JH, Burbach JP, Hamers FP (2004) Sciatic nerve regeneration in mice and rats: recovery of sensory innervation is followed by a slowly retreating neuropathic pain-like syndrome. Brain Res 1027:67-72.
Voigt T (1986) Cholinergic amacrine cells in the rat retina. J Comp Neurol 248:19-35.
Voinescu PE, Kay JN, Sanes JR (2009) Birthdays of retinal amacrine cell subtypes are systematically related to their molecular identity and soma position. J Comp Neurol 517:737-750.
Wang C (2008) Hybrid baculovirus-adeno-associated virus vectors for prolonged transgene expression in human neural cells. Journal of neurovirology 14:563-568.
Wang KC, Koprivica V, Kim JA, Sivasankaran R, Guo Y, Neve RL, He Z (2002) Oligodendrocyte-myelin glycoprotein is a Nogo receptor ligand that inhibits neurite outgrowth. Nature 417:941-944.
Wang X, Li Q, Adhikari N, Hall JL (2006) A role for muscle LIM protein (MLP) in vascular remodeling. Journal of molecular and cellular cardiology 40:503-509.

Wang X, Wang C, Zeng J, Xu X, Hwang PY, Yee WC, Ng YK, Wang S (2005) Gene transfer to dorsal root ganglia by intrathecal injection: effects on regeneration of peripheral nerves. Mol Ther 12:314-320.

Wasilko DJ, Lee SE, Stutzman-Engwall KJ, Reitz BA, Emmons TL, Mathis KJ, Bienkowski MJ, Tomasselli AG, Fischer HD (2009) The titerless infected-cells preservation and scale-up (TIPS) method for large-scale production of NO-sensitive human soluble guanylate cyclase (sGC) from insect cells infected with recombinant baculovirus. Protein expression and purification 65:122-132.

Wei W, Hamby AM, Zhou K, Feller MB (2011) Development of asymmetric inhibition underlying direction selectivity in the retina. Nature 469:402-406.

Weiskirchen R, Pino JD, Macalma T, Bister K, Beckerle MC (1995) The cysteine-rich protein family of highly related LIM domain proteins. The Journal of biological chemistry 270:28946-28954.

WHO (2013) Fact sheet N°384: Spinal cord injury. In: http://www.who.int/mediacentre/factsheets/fs384/en/.

Woolf CJ, Salter MW (2000) Neuronal plasticity: increasing the gain in pain. Science 288:1765-1769.

Woolf CJ, Reynolds ML, Molander C, O'Brien C, Lindsay RM, Benowitz LI (1990) The growth-associated protein GAP-43 appears in dorsal root ganglion cells and in the dorsal horn of the rat spinal cord following peripheral nerve injury. Neuroscience 34:465-478.

Wu D, Klaw MC, Connors T, Kholodilov N, Burke RE, Tom VJ (2015) Expressing Constitutively Active Rheb in Adult Neurons after a Complete Spinal Cord Injury Enhances Axonal Regeneration beyond a Chondroitinase-Treated Glial Scar. The Journal of neuroscience : the official journal of the Society for Neuroscience 35:11068-11080.

Xiao HS, Huang QH, Zhang FX, Bao L, Lu YJ, Guo C, Yang L, Huang WJ, Fu G, Xu SH, Cheng XP, Yan Q, Zhu ZD, Zhang X, Chen Z, Han ZG, Zhang X (2002) Identification of gene expression profile of dorsal root ganglion in the rat peripheral axotomy model of neuropathic pain. Proc Natl Acad Sci U S A 99:8360-8365.

Yin Y, Cui Q, Li Y, Irwin N, Fischer D, Harvey AR, Benowitz LI (2003) Macrophage-derived factors stimulate optic nerve regeneration. The Journal of neuroscience : the official journal of the Society for Neuroscience 23:2284-2293.

Yoshida S, Matsuda Y (1979) Studies on sensory neurons of the mouse with intracellular-recording and horseradish peroxidase-injection techniques. J Neurophysiol 42:1134-1145.

Zeitelhofer M, Vessey JP, Xie Y, Tubing F, Thomas S, Kiebler M, Dahm R (2007) High-efficiency transfection of mammalian neurons via nucleofection. Nature protocols 2:1692-1704.

Zhang J, Yang Z, Wu SM (2005) Development of cholinergic amacrine cells is visual activity-dependent in the postnatal mouse retina. J Comp Neurol 484:331-343.

Zolk O, Caroni P, Bohm M (2000) Decreased expression of the cardiac LIM domain protein MLP in chronic human heart failure. Circulation 101:2674-2677.

Zukor K, Belin S, Wang C, Keelan N, Wang X, He Z (2013) Short hairpin RNA against PTEN enhances regenerative growth of corticospinal tract axons after spinal

cord injury. The Journal of neuroscience : the official journal of the Society for Neuroscience 33:15350-15361.

Zwick M, Davis BM, Woodbury CJ, Burkett JN, Koerber HR, Simpson JF, Albers KM (2002) Glial cell line-derived neurotrophic factor is a survival factor for isolectin B4-positive, but not vanilloid receptor 1-positive, neurons in the mouse. The Journal of neuroscience : the official journal of the Society for Neuroscience 22:4057-4065.

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Eidesstaatliche Erklärung

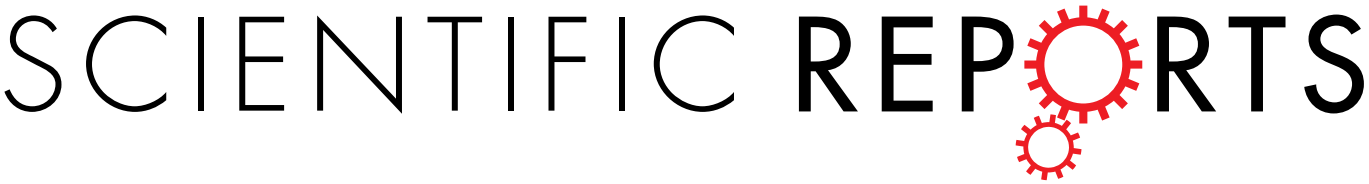
Ich versichere an Eides Statt, dass die Dissertation von mir selbständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf“ erstellt worden ist.

Berlin, den 21. Juni 2017

Appendix: original publications

Original publication 1

Nociceptive DRG neurons express muscle lim protein upon axonal injury



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Nociceptive DRG neurons express muscle lim protein upon axonal injury

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Evgeny Levin[1], Anastasia Andreadaki[1], Philipp Gobrecht[1], Frank Bosse[2] & Dietmar Fischer[1]

Muscle lim protein (MLP) has long been regarded as a cytosolic and nuclear muscular protein. Here, we show that MLP is also expressed in a subpopulation of adult rat dorsal root ganglia (DRG) neurons in response to axonal injury, while the protein was not detectable in naïve cells. Detailed immunohistochemical analysis of L4/L5 DRG revealed ~3% of MLP-positive neurons 2 days after complete sciatic nerve crush and maximum ~10% after 4–14 days. Similarly, in mixed cultures from cervical, thoracic, lumbar and sacral DRG ~6% of neurons were MLP-positive after 2 days and maximal 17% after 3 days. In both, histological sections and cell cultures, the protein was detected in the cytosol and axons of small diameter cells, while the nucleus remained devoid. Moreover, the vast majority could not be assigned to any of the well characterized canonical DRG subpopulations at 7 days after nerve injury. However, further analysis in cell culture revealed that the largest population of MLP expressing cells originated from non-peptidergic IB4-positive nociceptive neurons, which lose their ability to bind the lectin upon axotomy. Thus, MLP is mostly expressed in a subset of axotomized nociceptive neurons and can be used as a novel marker for this population of cells.

Muscle lim protein (MLP), also known as cysteine rich protein 3 (CRP3, CSRP3), is a member of the family of cysteine rich proteins (CRP). Since its discovery, MLP was supposedly expressed exclusively in muscle tissue. Although rat dorsal root ganglion (DRG) neurons reportedly express Mlp-RNA, the protein was never detected in these cells so that its translation remained questionable[1,2]. However, we recently found MLP transiently expressed in embryonic and postnatal, but not in mature retinal amacrine cells[3], suggesting that this protein may be involved in neuronal processes.

Previously, MLP protein is located in the cytosol and/or nucleus of cardiac and skeletal myocytes. There, it is essential for diverse biological processes, such as myocyte differentiation[4,5], response to mechanical stress[6,7], modulation of the actin-cytoskeleton[8], maintenance of the cytoarchitecture[9] and metabolism[10]. This multifunctional role was attributed to MLP's ability to associate with numerous intracellular interaction partners via its two LIM domains[11–13]. Nuclear MLP accumulation was primarily associated with differentiation or mechanical stress sensing in muscle cells[4,13,14].

DRG are clusters of highly heterogeneous populations of primary sensory neurons[15–17]. These neurons differ substantially in size, gene expression, electrophysiological properties[18–21] and the type of sensory information they convey. Small diameter neurons with unmyelinated C-fibers are predominantly nociceptors involved in the transduction of pain stimuli[22] that can be further subdivided into peptidergic and non-peptidergic neurons. While peptidergic nociceptors express neuropeptides such as calcitonin gene-related peptide (CGRP) or substance P (SP)[23,24], non-peptidergic neurons can instead be labelled by Isolectin B4 (IB4) from *Griffonia simplicifolia*[25]. In comparison, large diameter neurons, which are involved in proprioception and mechanosensation, bear heavily myelinated A-fibers and express the heavy chain of neurofilament (200 kDa NF)[20,26,27]. Although CGRP/SP, IB4 and 200 kDa NF are commonly used to distinguish DRG subpopulations, the presence of these markers might also overlap in some neuronal populations. For instance, ~30% of IB4-positive DRG neurons in rats are reportedly immunoreactive for CGRP while ~45% of CGRP-positive neurons also bind IB4[28].

Despite the detection of Mlp-mRNA[2], expression of the protein has so far been elusive in adult neurons. The current study provides first evidence that MLP protein is actually induced in some adult neurons upon axotomy.

[1]Division of Experimental Neurology, Department of Neurology, Heinrich-Heine-University of Düsseldorf, Merowingerplatz 1a, 40225, Düsseldorf, Germany. [2]Molecular Neurobiology Laboratory, Department of Neurology, Heinrich-Heine-University Moorenstrasse 5, 40225, Düsseldorf, Germany. Correspondence and requests for materials should be addressed to D.F. (email: dietmar.fischer@uni-duesseldorf.de)

More specifically, MLP was mainly detected in non-peptidergic, nociceptive DRG neurons that concurrently and inversely lost their ability to bind the IB4 upon axonal injury. Thus, MLP can be used as a novel marker for this subpopulation of neurons.

Materials and Methods

Animals. All experimental procedures were approved by the local animal care committee in Recklinghausen (LANUV, Germany) and conducted in compliance with federal and state guidelines for animal experiments in Germany (approval number: 84-02.04.2015.A290). Rats were maintained on a 12-hour light/dark cycle with *ad libitum* access to food and water. In total 24 rats were used. Animals were killed either by inhalation of CO_2 for preparations of cell cultures, Western blot lysates and mRNA isolations or by intraperitoneal application of ketamine (60–80 mg/kg; Pfizer) and xylazine (10–15 mg/kg; Bayer) and perfusion through the heart with cold PBS (Gibco) followed by paraformaldehyde (Sigma) (4% PFA in PBS) for immunohistochemical analyses.

Sciatic nerve crush. In total 18 adult Wistar male rats (200–230 g) were anesthetized by intraperitoneal injections of ketamine (60–80 mg/kg) and xylazine (10–15 mg/kg). A skin incision of about 10 mm was made over the left gluteal region. The ischiocrural musculature was carefully spread with minimal tissue damage to expose the sciatic nerve from the sciatic notch to the point of trifurcation. Severe axonotmetic crush injury was performed proximal to the tibial and peroneal divisions for 30 s using Dumont #5 forceps (Hermle). No crush injury was performed in sham-operated animals. For fluorogold tracing of neurons, the sciatic nerve was re-exposed 5 days after the initial surgery and cut proximal to the crush site. A piece of gel foam soaked in aqueous fluorogold solution (Thermo Fischer) was placed at the proximal stump prior to suturing the skin. Animals were sacrificed 2 days thereafter.

RNA isolation and quantitative real-time PCR. Total RNA was isolated from L4 and L5 DRG using the RNeasy kit (Qiagen) according to the manufacturer's protocol and 40 ng RNA were reverse transcribed using the Superscript II kit (Invitrogen). Quantification of Mlp expression was performed relative to the endogenous housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (Gapdh) using SYBR Green PCR Master Mix (Applied Biosystems) and QuantiTect primers Rn_Csrp3_1_SG (order-id: QT00183708) and Rn_Gapdh_1_SG (order-id: QT00199633; QuantiTect Primer Assay; Qiagen) and the Applied Biosystems 7500 real-time PCR System. DRG-derived cDNA was amplified during 45 cycles with amplification efficiency of about 100% according to the manufacturer's protocol. All reactions were performed in duplicate and at least three independent samples (3 animals) were analyzed per experimental group. Same numbers of sham-operated animals were used as uninjured controls. Relative Mlp expression was calculated using the comparative threshold cycle method ($\Delta\Delta C_t$)[29]. Specificity of PCR products was determined and verified for each run using the dissociation curve analysis feature of the Applied Biosystems 7500 software. Data are presented as means ± SEM. Data were tested for normal distribution and significances of intergroup differences calculated with GraphPad Prism software using Student's t-test.

MLP knockdown in HEK293 cells. Rat MLP cDNA was cloned into pcDNA3.1/V5-His Topo vector (Thermo Fischer). AVV-U6-EGFP plasmid containing scrambled shRNA was obtained from Vector Biolabs. Mlp-specific shRNA (5′-GATCCGGTTTACCATGCAGAAGAAATCTCGAG-ATTTCTTCTGCATGGTAAACCTTTT-AGATCTA-3′) was also cloned into AVV-U6-EGFP. HEK293 cells were seeded at ~3–5 × 10^4 cells per well in 96-well plates and co-transfected with MLP cDNA and shRNA-constructs using Lipofectamin 2000 (Thermo Fischer) the next day. Cells were stained for MLP protein using a goat anti-MLP antibody (1:500; C9001-23; US Biological; RRID: AB_2087783) after further 24 hours of incubation.

Immunohistochemistry and image analysis. In total, 8 rats were anesthetized and perfused through the heart with cold PBS followed by paraformaldehyde (4% PFA in PBS). L4/L5 DRG and sciatic nerves were isolated, post-fixed for 3 hours in 4% PFA, transferred to 30% sucrose overnight (4 °C) and embedded in KP cryo-compound (Klinipath). Serial sagittal sections (thickness of 14 µm) through the whole DRG including parts of the peripheral and central roots were prepared using a cryostat (Leica), thaw-mounted onto Superfrost plus glass slides (VWR) and stored at −20 °C until further use. For immunostaining, sections were exposed to 100% methanol for 10 min and then blocked with 2% bovine serum albumin (BSA, Sigma) and 5% donkey serum (Sigma) in PBS containing 0.05% Tween 20 (Sigma). Sections were incubated with primary antibodies dissolved in blocking solution for 16 hours. Primary antibodies included goat anti-MLP (1:400; C9001-23; US Biological; RRID: AB_2087783), mouse anti-MLP[30] [a kind gift from Dr. Geier, Max Delbrück Center for Molecular Medicine, Berlin, Germany], mouse anti-βIII-tubulin (1:1000; clone TUJ1; MMS-435P; Biolegend; RRID: AB_2313773), rabbit anti-βIII-tubulin (1:1000; clone TUJ1; MRB-435P; Biolegend; RRID: AB_10175616), rabbit anti-CGRP (1:100; BML-CA1134-0025; Enzo; RRID: AB_2050884), rabbit anti-Substance P (1:500; ABIN617879; ImmunoStar; RRID: AB_572266), mouse anti-200 kDa neurofilament (1:1000; ab78158, Abcam; RRID: AB_1566479), mouse anti-ATF3 (1:200; ab58668; Abcam; RRID: AB_879578) and rabbit anti-tyrosine hydroxylase (1:500; NB300-109; Novus Biologicals; RRID: AB_10077691). Sections were washed three times with PBS for 10 min, incubated with secondary antibodies dissolved in blocking solution as described above for 1 h and then washed three times with PBS. Secondary antibodies included donkey anti-mouse, donkey anti-rabbit and donkey anti-goat antibodies conjugated to either Alexa Fluor 488, Alexa Fluor 594 or Alexa Fluor 405 (1:1000; Thermo Fisher). For IB4 staining, some sections were subsequently incubated with IB4 lectin (FITC-conjugated, 25 µg/ml in PBS, L2895, Sigma) for 2 h. Stained sections were embedded in Mowiol mounting medium and the whole DRG section containing 300–600 neurons was photographed using a fluorescent microscope (Observer. D1, Zeiss) at 200x magnification (6–10 images per DRG section). For size determination of MLP-positive neurons, the cross-sectional cell area was measured at the level of the nucleus using ImageJ Software. Values were grouped into 50 µm^2 bins and respective proportions of MLP-positive neurons in each bin depicted as histogram.

For the quantification of neuronal subpopulations, CTCF (corrected total cell fluorescence) values were calculated for strongly subtype marker-positive neurons with visible nuclei co-stained with βIII-tubulin using the ImageJ software and the following formula: CTCF = integrated density of stained cell − (mean background fluorescence × cell area). Only labeled cells with CTCF of ≥100000 were counted. Four randomly selected sections per DRG were analyzed for each neuronal subpopulation. MLP fluorescence intensities in nuclei and cytoplasm were quantified using confocal images obtained with the Leica TCS SP8 confocal laser scanning microscope at 200x magnification. Integrated densities of same sized areas were measured in nuclei and cytoplasm and corrected for background fluorescence. Data were tested for normal distribution and significances of intergroup differences calculated with GraphPad Prism software using either one-way analysis of variance (ANOVA) with Holm-Sidak *post hoc* test or Student's t-test and are presented as means ± SEM.

DRG neuron cultures. DRG neurons were isolated from 6 adult male rats as previously described[31–33]. In brief, DRG were harvested, incubated in Dulbecco's modified Eagle medium (DMEM) containing 0.25% trypsin/EDTA (Thermo Fisher) and 0.3% collagenase type IA (Sigma) and mechanically dissociated. Cells were re-suspended in DMEM supplemented with 10% fetal bovine serum (FBS, GE Healthcare) and 500 U/ml penicillin/streptomycin (BioChrom) and plated into 96-well plates coated with poly-D-lysine (0.1 mg/ml, molecular weight <300,000 Da, Sigma) and 20 µg/ml laminin (Sigma). Some cultures were treated with 50 ng/ml human recombinant glial-derived neurotrophic factor (GDNF, Peprotech) immediately before plating and on day 3 in culture. Cells were cultured at 37 °C in 5% CO_2 for up to 5 days, fixed in 4% PFA for 25 min and then permeabilized in 100% methanol for 10 min. Cultures were then blocked with 2% bovine serum albumin (BSA, Sigma) and 5% donkey serum (Sigma) in PBS containing 0.05% Tween 20 (Sigma) and incubated with primary antibodies dissolved in blocking solution for 16 hours. Primary antibodies included goat anti-MLP (1:400; C9001-23; US Biological; RRID: AB_2087783), mouse anti-βIII-tubulin (1:1000; clone TUJ1; MMS-435P; Biolegend; RRID: AB_2313773), rabbit anti-βIII-tubulin (1:1000; clone TUJ1; MRB-435P; Biolegend; RRID: AB_10175616), rabbit anti-CGRP (1:100; BML-CA1134-0025; Enzo; RRID: AB_2050884), mouse anti-200 kDa neurofilament (1:1000; ab78158, Abcam; RRID: AB_1566479). Plates were washed three times with PBS for 10 min, incubated with secondary antibodies dissolved in the blocking solution as described above for 1 h and then washed three times with PBS. Secondary antibodies included donkey anti-mouse, donkey anti-rabbit and donkey anti-goat antibodies conjugated to either Alexa Fluor 488, Alexa Fluor 594 or Alexa Fluor 405 (1:1000; Thermo Fisher). For IB4 staining some sections were subsequently incubated with IB4 lectin (FITC-conjugated, 25 µg/ml in PBS, L2895, Sigma) for 2 h. Percentages of DRG neurons stained by the respective marker(s) were quantified using a fluorescent microscope (Observer.D1, Zeiss) in two independent experiments with at least three replicate wells for each subpopulation. Data were tested for normal distribution and significances of intergroup differences calculated with GraphPad Prism software using either one-way analysis of variance (ANOVA) with Holm-Sidak *post hoc* test or Student's t-test and are presented as means ± SEM.

Western blot assay. For protein lysate preparation, 2 rats with sciatic nerve crush (SNC) and 2 rats with sham-surgery were killed and DRG isolated. DRG were homogenized in lysis buffer (20 mM Tris/HCl pH 7.5, 10 mM KCl, 250 mM sucrose, 10 mM NaF, 1 mM DTT, 0.1 mM Na3VO4, 1% Triton X-100, 0.1% SDS) with protease inhibitors (Calbiochem, Darmstadt, Germany) and phosphatase inhibitors (Roche, Basel, Switzerland) using 5 sonication pulses at 40% power (Bandelin Sonoplus). Lysates were cleared by centrifugation in an Eppendorf tabletop centrifuge at 5000 rpm for 10 min at 4 °C. Lysates of heart were used as a positive control. Moreover, cell-lysates from HEK293 cells transfected with MLP expression plasmid and either scrambled control shRNA or Mlp-shRNA were prepared to verify the specificity of the antibody. Proteins were separated by sodium-dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), using Mini TGX gels (10%, BioRad, Hercules, USA) according to standard protocols and transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad). Blots were blocked in 5% dried milk with 1% bovine serum albumin (BSA) in phosphate- buffered saline with 0.05% Tween 20 (PBS-T) (Sigma) and incubated with antibodies against mouse anti-β-actin (1:4000; A5441; Sigma; RRID: AB_476744), or goat anti-MLP (1:500; C9001-23; US Biological; RRID: AB_2087783) at 4 °C overnight. All antibodies were diluted in 5% dried milk in PBS-T. Bound anti-MLP-antibody was visualized with anti-goat immunoglobulin G (IgG) secondary antibody conjugated to horseradish peroxidase (Sigma, 1:30 000) and the antigen-antibody complexes were detected by enhanced chemiluminescence (Biozyme) on a FluorChem E detection system (ProteinSimple). Bound anti-actin-antibody was visualized with anti-mouse infrared dye secondary antibody (IRDey 800 CW; LI-COR; 1:20 000) using Odyssey Infrared Imaging System (LI-COR).

Results

Induction of MLP expression in a subset of DRG neurons upon axotomy. Although Mlp mRNA was reportedly detected in axotomized dorsal root ganglia (DRG) of adult rats[1,2], it remained unknown whether these neuronal Mlp transcripts are actually translated into protein. In order to confirm this previous finding, we first performed quantitative Mlp RT-PCR on mRNA isolated from adult rat L4 and L5 DRG as these project axons into the sciatic nerve. Indeed, Mlp expression was strongly induced (~8-fold increase) at 7 days after sciatic nerve crush (SNC) in comparison to uninjured nerves (Fig. 1A). We then tested the applicability and specificity of a commercially available polyclonal MLP antibody (C9001-23 from US Biological) for immunohistochemistry and Western-Blot analysis. To this end, HEK293 cells were co-transfected with a MLP expression plasmid and either Mlp-shRNA or scrambled control shRNA. Immunocytochemistry of these cultures revealed strong and clear MLP staining in control-shRNA transfected cells, but no signal was detectable upon Mlp-shRNA expression (Fig. 1B). Similarly, the antibody detected a strong band at the size of 21 kD in heart-lysate and MLP-expressing HEK-lysates, which was markedly reduced by shRNA (Suppl. Fig. 1A). Therefore, this polyclonal serum and a

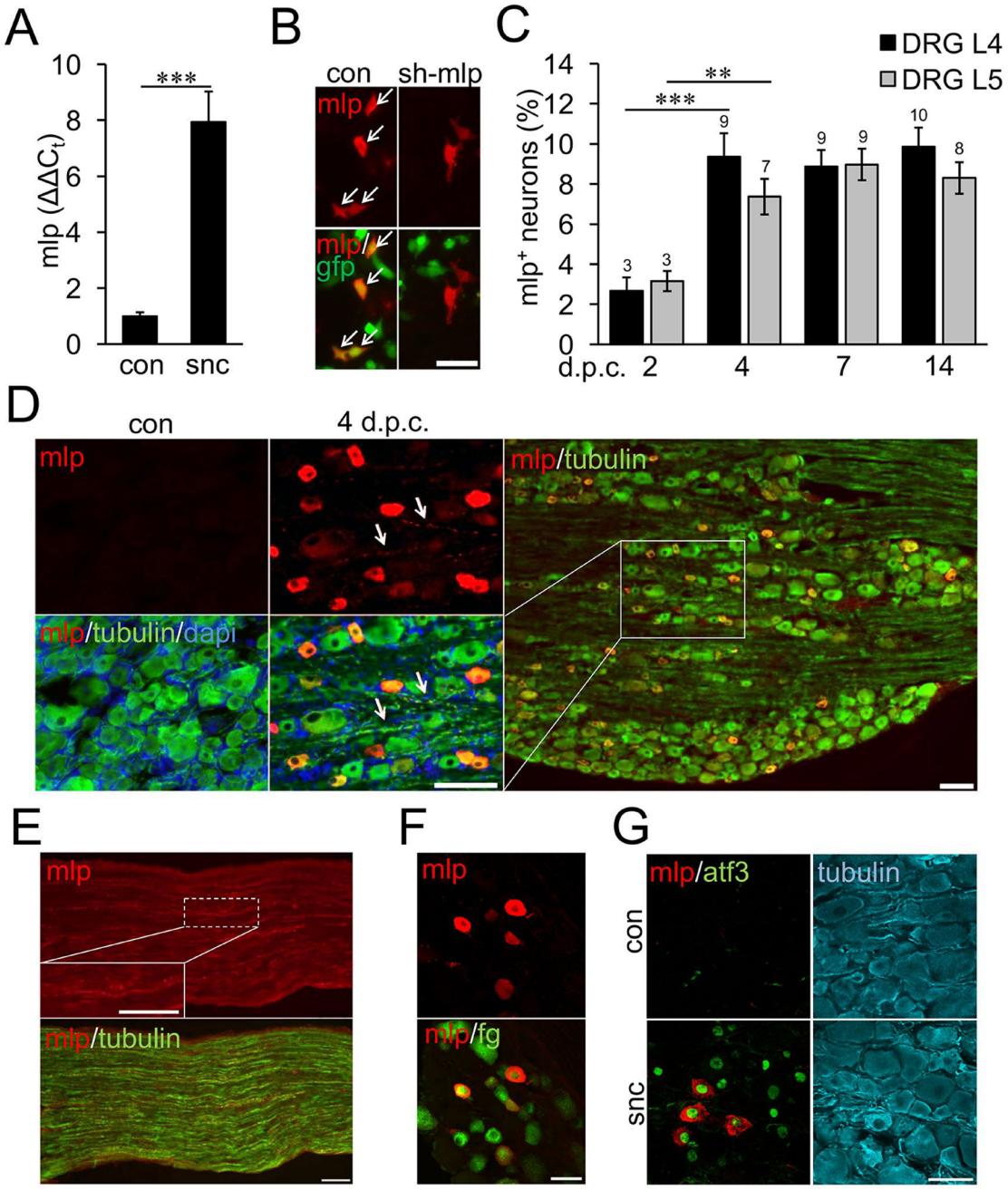

Figure 1. MLP is expressed in a subpopulation of DRG neurons. (**A**) Quantitative RT-PCR reveals strong induction of Mlp expression in adult male rat L4/L5-DRG 7 days after sciatic nerve crush (snc) in comparison to sham-operated controls (con). Samples of 3 animals per group (snc and con) were used. The Ct values for Mlp were in the range of 24 to 30 cycles and the Ct values for Gapdh of 18–19 cycles. Treatment effect: ***$p < 0.001$. (**B**) HEK293 cells were co-transfected with MLP expression plasmid and either scrambled control shRNA (con) or Mlp-shRNA (sh-Mlp). GFP expression indicates shRNA-transfected cells and MLP was detected 24 hours after transfection. Mlp-shRNA knocked down MLP expression. Scale bar: 50 µm. (**C**) Quantification of MLP-positive (mlp^+) L4 and L5 DRG neurons at 2, 4, 7 and 14 days post sciatic nerve crush (d.p.c.). Data represent mean percentages ± SEM of all βIII-tubulin-stained neurons on DRG sections as in (**D**). Two male rats and 4 sections per DRG were used for each group. Treatment effects: **$p < 0.01$, ***$p < 0.001$. (**D**) Immunohistochemical analysis of MLP expression on DRG sections of sham-operated controls (con) and 4 d.p.c. The sections were stained for MLP (red), βIII-tubulin (green) and DAPI (blue). MLP was detected in a subpopulation of βIII-tubulin-positive neurons and their axons (arrows). Scale bars: 50 µm. (**E**) MLP expression (red) was also detected in βIII-tubulin-positive axons (green) on proximal sciatic nerve sections at 4 d.p.c. The insert shows a higher magnification of a MLP-positive axon. Scale bars: 100 µm. (**F**) MLP immunostaining on DRG sections 7 days after sciatic nerve cut and retrograde fluorogold (fg) labeling. MLP expression was only detected in fg-positive injured neurons. Scale bar: 50 µm. (**G**) DRG sections of sham-operated controls (con) and 4 d.p.c were stained for MLP (red), ATF3 (green) and βIII-tubulin (cyan). ATF3 expression indicated axotomized DRG neurons. MLP was only detected in ATF3-positive neurons. Scale bar: 50 µm.

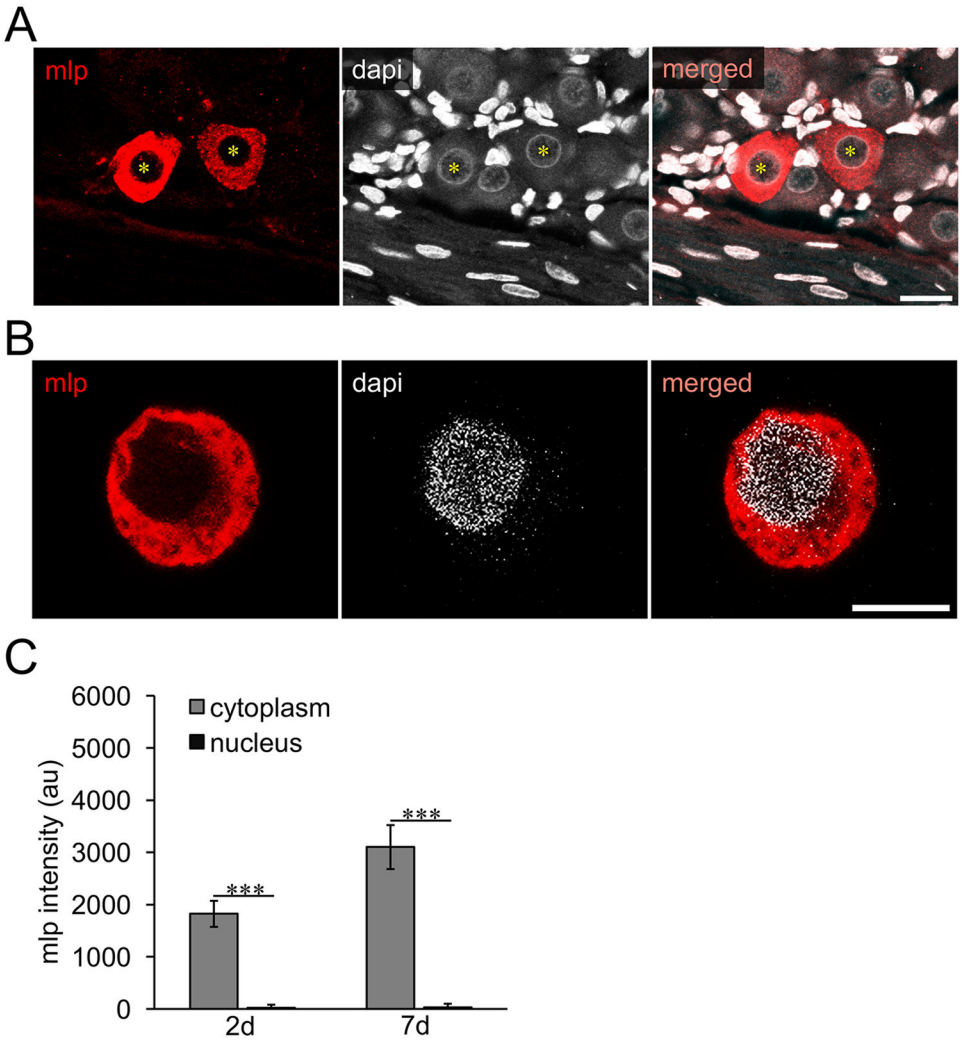

Figure 2. MLP is located in the cytosol, but not the nucleus. (**A**) Confocal images (4 merged z-stacks of 1.04 µm each) of MLP (red) and DAPI (white) stained DRG section 7 days after sciatic nerve crush. MLP was detected in the cytoplasm, but not in DAPI-positive nuclei of DRG neurons (stars). Scale bar: 20 µM. (**B**) Confocal image (3 merged z-stacks of 0.956 µm) of a MLP (red) and DAPI (white) stained DRG neuron after 3 days in culture. MLP was not detected in nuclei of cultured DRG neurons. Scale bar: 10 µM. (**C**) Intensities of MLP signals were quantified on confocal images 2 and 7 days after sciatic nerve crush stained as described in (**A**) in nuclei and cytoplasm of MLP-positive neurons. Data are indicated in arbitrary units (au) for background-corrected fluorescence intensities. Effects: ***$p < 0.001$.

previously characterized proprietary monoclonal antibody[30] were deemed suitable for immunohistochemical studies as well as Western-Blot analysis and subsequently used to specifically stain MLP on DRG sections. No staining was observed in control L4/L5 DRG without prior SNC, but a pronounced signal was detected in some of the βIII-tubulin-positive neurons 4 days post crush (d.p.c.) (Fig. 1C,D). These results were verified by Western-Blot analysis (Suppl. Fig. 1A). MLP staining was found in cell somata as well as in axons and some axonal profiles were accordingly stained in longitudinal sciatic nerve sections (Fig. 1E). The time course of MLP induction was established by the quantification of MLP-positive neurons in L4 and L5 DRG at different time points after sciatic nerve crush. Only 3% of all βIII-tubulin-positive neurons were also stained for MLP at 2 d.p.c. (initial stage of regeneration) and the level of 9% reached at 4 d.p.c. did not further increase up to 14 d.p.c. (later stage) (Fig. 1C). Moreover, retrograde fluorogold labeling from the cut sciatic nerve (Fig. 1F) and co-staining of MLP-positive neurons with neuronal injury marker ATF3 (Fig. 1G) confirmed that MLP was only expressed in axotomized DRG neurons (in ~15% of ATF-3 positive cells). Thus, MLP expression was induced in a relatively small number of axotomized DRG neurons. In addition, detailed confocal microscopy revealed that neuronal MLP expression was restricted to the cytoplasm and, in contrast to myocytes, not found in nuclei. Quantification of MLP-expression on histological DRG sections (Fig. 2A,C) as well as staining of isolated DRG neurons in culture (Fig. 2B) featured nuclei devoid of MLP protein. Therefore, induction of MLP is unlikely to contribute to gene expression changes upon nerve injury, but the protein might rather be involved in structural modification of the cytoskeleton.

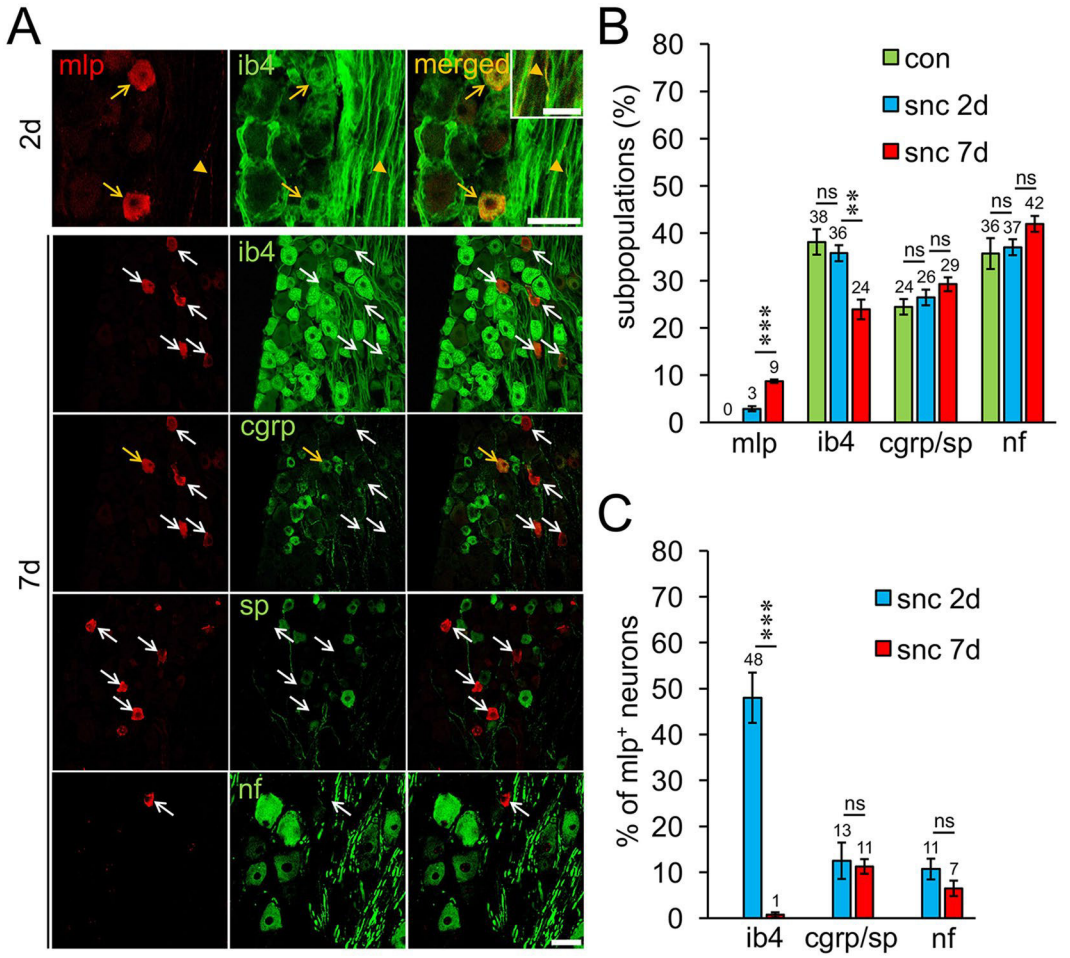

Figure 3. MLP is mainly expressed in nociceptive DRG neurons. (**A**) L4/L5 DRG sections prepared 2 and 7 days after sciatic nerve crush (snc) were co-stained for MLP (red) and one of four markers of neuronal DRG subpopulations (green): isolectin B4 (ib4), calcitonin gene related peptide (cgrp), substance P (sp) or 200 kDa neurofilament (nf). Many MLP-positive DRG neurons (yellow arrows) and their axons (yellow arrowhead) were positively stained for IB4 at 2 days after snc. In contrast, only very few double-positive cells (yellow arrow) were found at 7 days after snc, while most MLP-positive neurons did not co-express any of the four canonical markers (white arrows). Co-staining of MLP for IB4 and CGRP 7 days after snc was performed as triple staining on the same DRG section. Original blue color of CGRP signal was changed to green for a better visualization of positive cells. Scale bars: 50 µm, 25 µm for insert showing higher magnification of a MLP/IB4 double-positive axon. (**B**) Quantification of mlp-, ib4-, cgrp/sp- and nf-subpopulations in DRG from either untreated adult male rats (con) or at 2 and 7 d.p.c. in relation to all βIII-tubulin-positive neurons. Data represent mean percentages ± SEM from 2 rats per experimental group and 4 sections per DRG. Only the subpopulations of MLP- and IB4-positive neurons changed after sciatic nerve crush. (**C**) Percentage of neurons double-positive for MLP and either ib4, cgrp/sp or nf on sections as described in (**A**) at 2 and 7 days after snc. Treatment effects: $^{**}p < 0.01$, $^{***}p < 0.001$, ns: non-significant.

Predominant MLP expression in nociceptive DRG neurons. We next investigated whether MLP might be exclusively expressed in one of the three major subpopulations of DRG neurons. To this end, DRG sections prepared 2 or 7 days after SNC were co-stained for MLP and either IB4 (marker for non-peptidergic nociceptors), CGRP+SP (markers for peptidergic nociceptors) or 200 kDa neurofilament (marker for mostly large-sized neurons with myelinated fibers). These canonical markers labeled most neurons in L4/L5 DRG from uninjured rats: 38% of all βIII-tubulin-positive neurons were co-stained for IB4, 24% for CGRP or SP and 36% for neurofilament (Fig. 3A,B). With respect to MLP-positive neurons, only few (13% and 11%) were co-stained for CGRP/SP and neurofilament, respectively, at 2 days after SNC and this proportions were not significantly changed at 7 d.p.c. (Fig. 3C). Surprisingly, while 48% of MLP-positive neurons and their axons were co-stained for IB4 at 2 d.p.c. (Fig. 3A), this percentage strongly decreased to 1% at 7 d.p.c. (Fig. 3C). Thus, the vast majority (81%) of MLP-positive neurons did not co-localize with any of the applied markers 7 days after SNC (Fig. 3A,C). As the number of MLP-expressing neurons is still increasing from 2 to 7 d.p.c. (Fig. 1C), we speculated that DRG neurons might lose their canonical marker label. Indeed, the percentage of IB4-positive neurons significantly decreased 7 days after SNC

(24%) compared to 2 d.p.c. (36%) and uninjured controls (38%), while the proportion of CGRP/SP- and neurofilament-positive DRG neurons was unchanged (Fig. 3B). In conclusion, MLP expression seems to be predominantly induced in non-peptidergic nociceptors that lose their ability to bind IB4 upon axotomy[34]. Consistently, cell size analysis revealed that most of MLP-positive neurons were of small-medium-sizes (Suppl. Fig. 1B), being in the range previously described for nociceptive neurons[35]. Additionally, we analyzed MLP expression in tyrosine hydroxylase (th)-positive DRG neurons. This is another subpopulation of neurons that are also negative for the classical canonical markers, but only found in L5 DRGs[36,37]. However, these th-positive neurons did not express MLP, either (Suppl. Fig. 1C).

To substantiate these findings, we also investigated the induction of MLP expression in mixed cultures of cervical, thoracic, lumbar and sacral DRG of unlesioned controls. Consistent with the *in vivo* data, no MLP expression was detected 2 hours after culture preparation (Fig. 4A,B). First positive cells (~1%) were detected at 1 day in culture and their number increased to ~18% at 3–5 days in culture. Of these MLP-positive neurons, 25% were co-stained for CGRP, 6% for neurofilament and 7% for IB4, leaving 62% unassigned after 5 days in culture (Fig. 4C,D). Again, the percentage of IB4-positive neurons markedly decreased with prolonged culturing time (30% at 5 days in culture compared to 58% at 2 h after plating), while the proportion of CGRP- and neurofilament-positive cells remained unchanged (Fig. 4E). Thus, MLP expression was also induced mainly in formerly IB4-positive neurons. In order to substantiate this hypothesis, we exposed dissociated DRG cultures to glial-derived neurotrophic factor (GDNF) as this treatment reportedly delays axotomy-induced downregulation of IB4 detection[34]. Consistently, GDNF treatment significantly increased the percentage of IB4-positive neurons at 5 days in culture compared to untreated controls (Fig. 4F). At the same time the percentage of MLP-positive neurons co-labeled for IB4 increased from 11% to 51% (Fig. 4G), indicating that the vast majority of MLP-expressing neurons gradually lost their ability to bind IB4. The total number of DRG neurons was not affected by GDNF-treatment (Suppl. Fig. 1D). Altogether, these data suggest that MLP was predominantly induced in non-peptidergic nociceptors upon axotomy and might serve as a new marker for this subpopulation upon decline of IB4-staining.

Discussion

The current study shows for the first time that MLP expression in mature neurons upon axotomy. However, only a small percentage of DRG neurons expressed this protein, most of which represented non-peptidergic nociceptors. As this neuronal subpopulation lost its IB4 staining over time after injury, MLP could serve as a new marker to track the fate of these axotomized neurons.

Previous studies already reported upregulation of Mlp-RNA in adult rat DRG neurons upon partial sciatic nerve ligation[1] or after sciatic nerve crush[2] based on *in situ*-hybridization analysis. MLP induction was estimated in roughly 30% of L5 DRG neurons[1], but no data on protein levels or specific expression in neuronal subtypes were provided. To address the issue whether MLP transcripts are at all translated in neurons, the current study investigated the temporal and spatial expression pattern of the protein in DRG neurons after complete sciatic nerve crush using specific MLP antibodies[3]. Immunohistochemical analysis confirmed gradual induction of MLP protein in maximal 10% of L4/L5 DRG neurons within the first 4 days after injury. Thus, its expression is obviously restricted to a relatively small percentage of neurons *in vivo*, only a relatively small. In cell culture, slightly more (17%) neurons were positive for MLP, which might be due to the cultivation of additional (cervical, thoracic and sacral) DRG neurons compared to the analysis of L4/L5 DRG in *vivo* or the specific culture conditions. However, quantifications always remained significantly lower than 30% and was mainly restricted to small-diameter, nociceptive neurons. In contrast, Newton *et al.* reported MLP expression in DRG neurons of all sizes. This discrepancy might be based on different experimental conditions of the two studies. While Newton *et al*. used a partial ligation model, a different rat strain and *in situ*-hybridization, we performed complete sciatic nerve crush, used Wistar rats and analyzed the protein rather than mRNA expression. Nevertheless, we can neither exclude the possibility that under different experimental conditions MLP might be expressed in other neuronal cell types nor that MLP expression might be posttranslationally regulated. Albeit these remaining open questions, the data presented in the current study demonstrate that significant levels of MLP protein are expressed only in a small and distinct subpopulation of axonally injured DRG neurons.

We initially found that the vast majority of MLP-positive DRG neurons could not be assigned to any of the well characterized canonical DRG subpopulations at 7 days after SNC. However, sections that were prepared 2 days after SNC, when MLP expression had not yet reached maximal levels, approximately 50% of MLP-positive neurons co-labeled by IB4. It is well known that non-peptidergic nociceptive neurons gradually lose their ability to bind this lectin upon axotomy, making their identification difficult. Hence, most MLP-positive neurons detected 7 days after sciatic nerve injury likely originated from these nociceptive neurons. Consistently, the vast majority of MLP-positive neurons in culture could also not be detected by any of the canonical subpopulation markers 5 days after plating. Correspondingly, the proportion of IB4-positive neurons was dramatically reduced at this time point compared to 2 hours, while CGRP and 200 kDa neurofilament expressing subpopulations remained unchanged. To substantiate the hypothesis that most MLP-positive neurons originated from IB4-positive cells we treated the cultures with GDNF. Consistent with Bennett *et al.*, GDNF treatment strongly delayed the decline of IB4-staining in MLP-expressing neurons, thereby confirming that the MLP-positive cells express a functional GDNF receptor, which is another typical phenotypic feature of non-peptidergic DRG neurons[34,38,39]. Thus, the vast majority of MLP-positive neurons are non-peptidergic, nociceptive neurons.

An important question that needs to be addressed in the future is the functional role of MLP in these neurons. In myocytes, MLP has been shown to interact with at least 16 different partners thereby modulating various cellular functions[7,10,40]. In particular, the nuclear accumulation of MLP has been shown as a response to

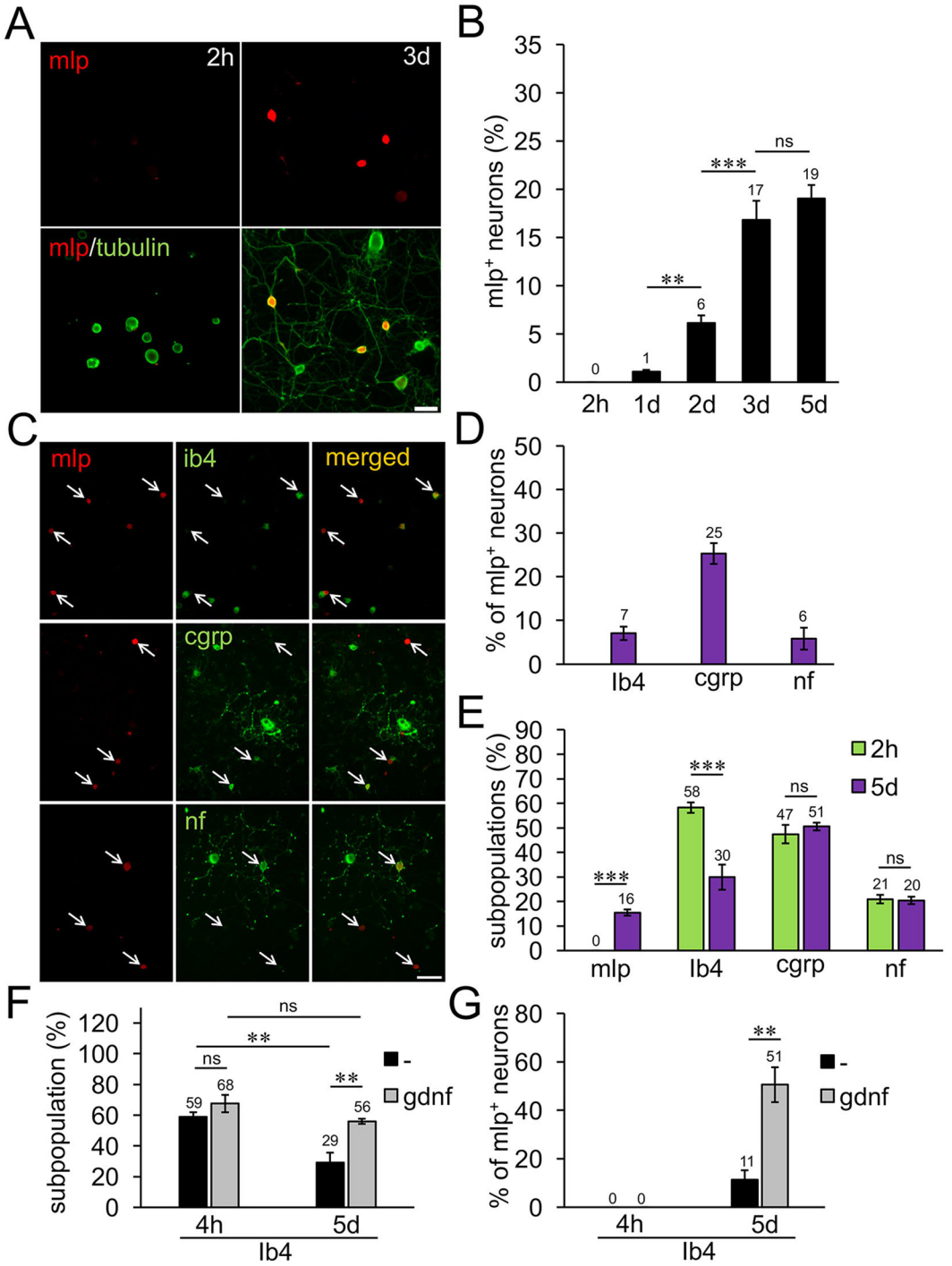

Figure 4. Induction of MLP expression in nociceptive DRG neurons in culture. (**A**) Cultures of dissociated adult rat DRG neurons were stained for MLP (red) and βIII-tubulin (green) 2 hours (2 h) and 3 days (3 d) after cell preparation. Scale bar: 50 µm. (**B**) Quantification of MLP-positive (mlp$^+$) DRG neurons in cultures as in (**A**) at 2 h and 1–5 days after plating. Data represent mean percentages ± SEM of 2 independent experiments each with 3–4 repeat wells. (**C**) Representative pictures of cultures co-stained for MLP (red, arrows) and either calcitonin gene-related peptide (cgrp), isolectin B4 (ib4) and 200 kDa neurofilament (nf) (green) after several days in culture. Scale bar: 100 µm. (**D**) Percentage of neurons double-positive for MLP and either ib4, cgrp or nf in DRG cultures as described in (**C**) at 5 d after plating. (**E**) Quantification of mlp-, ib4-, cgrp- and nf-subpopulations in relation to all βIII-tubulin-positive neurons as described in (**C**) at 2 h and 5 d. (**F**) Quantification of IB4-positive neurons cultured either with or without glial-derived neurotrophic factor (gdnf) for 4 h and 5 d after plating. (**G**) Quantification of MLP-positive neurons co-stained with IB4 in cultures as described in (**F**). Treatment effects: $^{**}p < 0.01$, $^{***}p < 0.001$, ns: non-significant.

mechanic stress[6, 14] and described being involved in processes regulating gene expression[5]. However, a similar role appears unlikely in neurons based on our finding that MLP protein was not detected in neuronal nuclei. Nevertheless, MLP could affect other injury-associated processes in DRG neurons, such as axon regeneration. In fact this possibility was already previously addressed using a knock-down approach with Mlp-RNA in cultured DRG neurons with no effect on axon growth[2]. However, based on our findings that MLP is only induced in so few neurons, we consider the detection of a potential effect in these studies impossible as a significant effect would be masked by the majority of negative neurons. A more detailed analysis considering this aspect may therefore lead to a different outcome. As MLP is predominantly expressed in nociceptors, an involvement in the transmission of noxious stimuli, which is altered in injured DRG neurons, is also conceivable[41]. These questions still await further analysis in the future. Nevertheless, our findings identify MLP as a novel marker for a subset of axotomized nociceptive rat DRG neurons that are not clearly recognizable by other established subpopulation markers.

References

1. Newton, R. A. *et al*. Identification of differentially expressed genes in dorsal root ganglia following partial sciatic nerve injury. *Neuroscience* **95**, 1111–1120 (2000).
2. Stam, F. J. *et al*. Identification of candidate transcriptional modulators involved in successful regeneration after nerve injury. *The European journal of neuroscience* **25**, 3629–3637, doi:10.1111/j.1460-9568.2007.05597.x (2007).
3. Levin, E., Leibinger, M., Andreadaki, A. & Fischer, D. Neuronal expression of muscle LIM protein in postnatal retinae of rodents. *PLoS One* **9**, e100756, doi:10.1371/journal.pone.0100756 (2014).
4. Arber, S., Halder, G. & Caroni, P. Muscle LIM protein, a novel essential regulator of myogenesis, promotes myogenic differentiation. *Cell* **79**, 221–231 (1994).
5. Kong, Y., Flick, M. J., Kudla, A. J. & Konieczny, S. F. Muscle LIM protein promotes myogenesis by enhancing the activity of MyoD. *Molecular and cellular biology* **17**, 4750–4760 (1997).
6. Knoll, R. *et al*. The cardiac mechanical stretch sensor machinery involves a Z disc complex that is defective in a subset of human dilated cardiomyopathy. *Cell* **111**, 943–955 (2002).
7. Buyandelger, B. *et al*. MLP (muscle LIM protein) as a stress sensor in the heart. *Pflugers Archiv: European journal of physiology* **462**, 135–142, doi:10.1007/s00424-011-0961-2 (2011).
8. Hoffmann, C. *et al*. Human muscle LIM protein dimerizes along the actin cytoskeleton and cross-links actin filaments. *Molecular and cellular biology* **34**, 3053–3065, doi:10.1128/MCB.00651-14 (2014).
9. Arber, S. *et al*. MLP-deficient mice exhibit a disruption of cardiac cytoarchitectural organization, dilated cardiomyopathy, and heart failure. *Cell* **88**, 393–403 (1997).
10. Rashid, M. M. *et al*. Muscle LIM protein/CSRP3: a mechanosensor with a role in autophagy. *Cell death discovery* **1**, 15014, doi:10.1038/cddiscovery.2015.14 (2015).
11. Weiskirchen, R., Pino, J. D., Macalma, T., Bister, K. & Beckerle, M. C. The cysteine-rich protein family of highly related LIM domain proteins. *The Journal of biological chemistry* **270**, 28946–28954 (1995).
12. Schallus, T., Feher, K., Ulrich, A. S., Stier, G. & Muhle-Goll, C. Structure and dynamics of the human muscle LIM protein. *FEBS Lett* **583**, 1017–1022, doi:10.1016/j.febslet.2009.02.021 (2009).
13. Arber, S. & Caroni, P. Specificity of single LIM motifs in targeting and LIM/LIM interactions *in situ*. *Genes Dev* **10**, 289–300 (1996).
14. Boateng, S. Y. *et al*. Cardiac dysfunction and heart failure are associated with abnormalities in the subcellular distribution and amounts of oligomeric muscle LIM protein. *American journal of physiology. Heart and circulatory physiology* **292**, H259–H269, doi:10.1152/ajpheart.00766.2006 (2007).
15. Burgess, P. R. & Perl, E. R. Myelinated afferent fibres responding specifically to noxious stimulation of the skin. *The Journal of physiology* **190**, 541–562 (1967).
16. Bessou, P. & Perl, E. R. Response of cutaneous sensory units with unmyelinated fibers to noxious stimuli. *J Neurophysiol* **32**, 1025–1043 (1969).
17. Christensen, B. N. & Perl, E. R. Spinal neurons specifically excited by noxious or thermal stimuli: marginal zone of the dorsal horn. *J Neurophysiol* **33**, 293–307 (1970).
18. Yoshida, S. & Matsuda, Y. Studies on sensory neurons of the mouse with intracellular-recording and horseradish peroxidase-injection techniques. *J Neurophysiol* **42**, 1134–1145 (1979).
19. Harper, A. A. & Lawson, S. N. Conduction velocity is related to morphological cell type in rat dorsal root ganglion neurones. *The Journal of physiology* **359**, 31–46 (1985).
20. Lawson, S. N. & Waddell, P. J. Soma neurofilament immunoreactivity is related to cell size and fibre conduction velocity in rat primary sensory neurons. *The Journal of physiology* **435**, 41–63 (1991).
21. Stucky, C. L. & Lewin, G. R. Isolectin B(4)-positive and -negative nociceptors are functionally distinct. *The Journal of neuroscience: the official journal of the Society for Neuroscience* **19**, 6497–6505 (1999).
22. Snider, W. D. & McMahon, S. B. Tackling pain at the source: new ideas about nociceptors. *Neuron* **20**, 629–632 (1998).
23. Ju, G. *et al*. Primary sensory neurons of the rat showing calcitonin gene-related peptide immunoreactivity and their relation to substance P-, somatostatin-, galanin-, vasoactive intestinal polypeptide- and cholecystokinin-immunoreactive ganglion cells. *Cell and tissue research* **247**, 417–431 (1987).
24. Nagy, J. I. & Hunt, S. P. Fluoride-resistant acid phosphatase-containing neurones in dorsal root ganglia are separate from those containing substance P or somatostatin. *Neuroscience* **7**, 89–97 (1982).
25. Silverman, J. D. & Kruger, L. Selective neuronal glycoconjugate expression in sensory and autonomic ganglia: relation of lectin reactivity to peptide and enzyme markers. *Journal of neurocytology* **19**, 789–801 (1990).
26. Perry, M. J., Lawson, S. N. & Robertson, J. Neurofilament immunoreactivity in populations of rat primary afferent neurons: a quantitative study of phosphorylated and non-phosphorylated subunits. *Journal of neurocytology* **20**, 746–758 (1991).
27. Marmigere, F. & Ernfors, P. Specification and connectivity of neuronal subtypes in the sensory lineage. *Nat Rev Neurosci* **8**, 114–127, doi:10.1038/nrn2057 (2007).
28. Price, T. J. & Flores, C. M. Critical evaluation of the colocalization between calcitonin gene-related peptide, substance P, transient receptor potential vanilloid subfamily type 1 immunoreactivities, and isolectin B4 binding in primary afferent neurons of the rat and mouse. *The journal of pain: official journal of the American Pain Society* **8**, 263–272, doi:10.1016/j.jpain.2006.09.005 (2007).
29. Leibinger, M. *et al*. Interleukin-6 contributes to CNS axon regeneration upon inflammatory stimulation. *Cell death & disease* **4**, e609, doi:10.1038/cddis.2013.126 (2013).
30. Geier, C. *et al*. Beyond the sarcomere: CSRP3 mutations cause hypertrophic cardiomyopathy. *Hum Mol Genet* **17**, 2753–2765, doi:10.1093/hmg/ddn160 (2008).
31. Gobrecht, P., Leibinger, M., Andreadaki, A. & Fischer, D. Sustained GSK3 activity markedly facilitates nerve regeneration. *Nature communications* **5**, 4561, doi:10.1038/ncomms5561 (2014).

32. Leibinger, M. *et al*. Boosting Central Nervous System Axon Regeneration by Circumventing Limitations of Natural Cytokine Signaling. *Mol Ther*, doi:10.1038/mt.2016.102 (2016).
33. Gobrecht, P. *et al*. Promotion of Functional Nerve Regeneration by Inhibition of Microtubule Detyrosination. *J Neurosci* **36**, 3890–3902, doi:10.1523/JNEUROSCI.4486-15.2016 (2016).
34. Bennett, D. L. *et al*. A distinct subgroup of small DRG cells express GDNF receptor components and GDNF is protective for these neurons after nerve injury. *The Journal of neuroscience: the official journal of the Society for Neuroscience* **18**, 3059–3072 (1998).
35. Molliver, D. C., Radeke, M. J., Feinstein, S. C. & Snider, W. D. Presence or absence of TrkA protein distinguishes subsets of small sensory neurons with unique cytochemical characteristics and dorsal horn projections. *The Journal of comparative neurology* **361**, 404–416, doi:10.1002/cne.903610305 (1995).
36. Price, J. & Mudge, A. W. A subpopulation of rat dorsal root ganglion neurones is catecholaminergic. *Nature* **301**, 241–243 (1983).
37. Brumovsky, P., Villar, M. J. & Hokfelt, T. Tyrosine hydroxylase is expressed in a subpopulation of small dorsal root ganglion neurons in the adult mouse. *Experimental neurology* **200**, 153–165, doi:10.1016/j.expneurol.2006.01.023 (2006).
38. Molliver, D. C. *et al*. IB4-binding DRG neurons switch from NGF to GDNF dependence in early postnatal life. *Neuron* **19**, 849–861 (1997).
39. Zwick, M. *et al*. Glial cell line-derived neurotrophic factor is a survival factor for isolectin B4-positive, but not vanilloid receptor 1-positive, neurons in the mouse. *The Journal of neuroscience*: *the official journal of the Society for Neuroscience* **22**, 4057–4065, doi:20026394 (2002).
40. Lange, S. *et al*. MLP and CARP are linked to chronic PKCalpha signalling in dilated cardiomyopathy. *Nature communications* **7**, 12120, doi:10.1038/ncomms12120 (2016).
41. Woolf, C. J. & Salter, M. W. Neuronal plasticity: increasing the gain in pain. *Science* **288**, 1765–1769 (2000).

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Author Contributions

D.F. and E.L. designed the project. E.L., P.G., A.A. and F.B. performed experiments. D.F. and E.L. analyzed the data. D.F. supervised the research; D.F. and E.L. wrote the paper.

Additional Information

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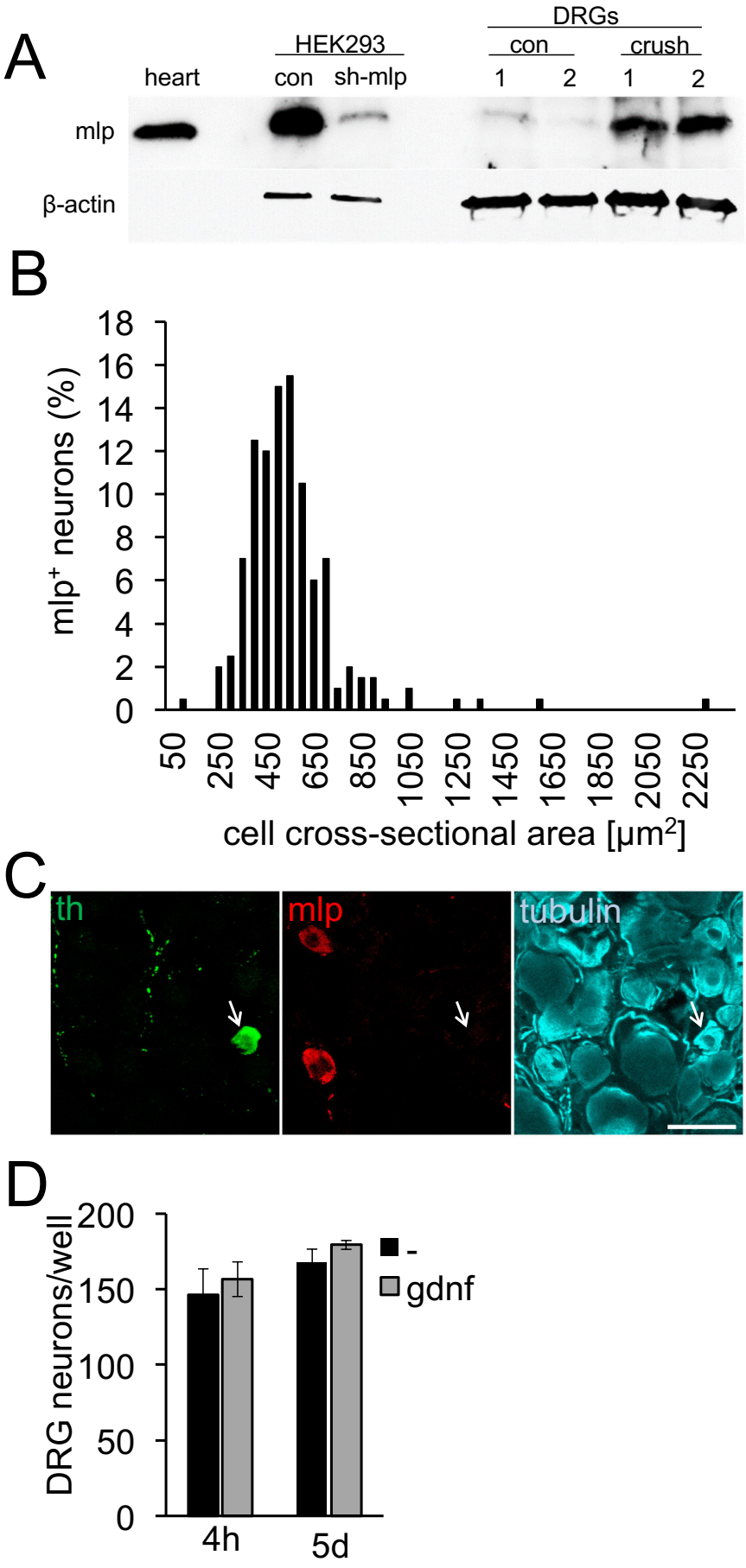
Nociceptive DRG neurons express muscle lim protein upon axonal injury

Evgeny Levin, Anastasia Andreadaki, Philipp Gobrecht, Frank Bosse and Dietmar Fischer

Supplementary Figure 1



Original publication 2

Neuronal expression of Muscle LIM Protein in postnatal retinae of rodents

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Neuronal Expression of Muscle LIM Protein in Postnatal Retinae of Rodents

Evgeny Levin, Marco Leibinger, Anastasia Andreadaki, Dietmar Fischer*

Division of Experimental Neurology, Department of Neurology, Heinrich-Heine-University Düsseldorf, Düsseldorf, Germany

Abstract

Muscle LIM protein (MLP) is a member of the cysteine rich protein family and has so far been regarded as a muscle-specific protein that is mainly involved in myogenesis and the organization of cytoskeletal structure in myocytes, respectively. The current study demonstrates for the first time that MLP expression is not restricted to muscle tissue, but is also found in the rat naive central nervous system. Using quantitative PCR, Western blot and immunohistochemical analyses we detected MLP in the postnatal rat retina, specifically in the somas and dendritic arbors of cholinergic amacrine cells (AC) of the inner nuclear layer and the ganglion cell layer (displaced AC). Induction of MLP expression started at embryonic day 20 and peaked between postnatal days 7 and 14. It subsequently decreased again to non-detectable protein levels after postnatal day 28. MLP was identified in the cytoplasm and dendrites but not in the nucleus of AC. Thus, retinal MLP expression correlates with the morphologic and functional development of cholinergic AC, suggesting a potential role of this protein in postnatal maturation and making MLP a suitable marker for these neurons.

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* E-mail: dietmar.fischer@uni-duesseldorf.de

Introduction

The family of cysteine rich proteins (CRP) comprises three closely related members, CRP1, CRP2, and CRP3. CRP3 is also known as muscle LIM protein (MLP) and has been postulated as a muscle-specific protein (mainly expressed in the heart). It is involved in the myogenesis and cytoskeletal organization of myocytes [1–5]. Accordingly, MLP deficiency leads to myocardial hypertrophy followed by cardiomyopathy and heart failure [6,7]. In myocytes, MLP is localized in the cytoplasm, where it is involved in cytoskeleton modulation [4]. In addition, MLP has been reported to translocate to the nucleus to modulate gene expression during myogenesis and as part of a biomechanical stress response [8,9]. A functional role of MLP in other tissues such as the central nervous system, such as the retina has not yet been described.

Amacrine cells (AC) are a heterogeneous group of retinal interneurons and synaptically interact with bipolar cells, retinal ganglion cells (RGCs) and other AC. Mammalian AC have been classified into more than 26 morphologic subtypes [10,11] that are distinguishable by specific molecular markers, morphology, size and their neurotransmitters [12]. Cholinergic AC produce the neurotransmitter acetylcholine [13] allowing their specific identification by antibodies against choline acetyltransferase (ChAT) [14]. In rodents, ChAT-immunoreactive AC are detected from embryonic day 17 and produce acetylcholine lifelong [15]. AC are either conventionally located in the inner nuclear layer (INL) at the border with the inner plexiform layer (IPL) or displaced to the ganglion cell layer (GCL) [13,16,17]. The cells of the displaced subpopulation are stimulated by light and therefore termed ON-cells. Their dendrites are restricted to the ON sublamina of the IPL, whereas the AC located in the INL are excited in the absence of light (OFF cells). These AC accumulate their dendrites in the OFF-sublamina. As a consequence of this strict stratification pattern, two distinct ChAT-immunoreactive dendritic layers of cholinergic AC are visible in the IPL [18]. Moreover, cholinergic AC induce spontaneous waves of action potentials in the developing retina, which reportedly facilitates the formation of visual circuits between retinal neurons. This process is apparently driven by the release of acetylcholine [19,20].

In mammals, the morphology of cholinergic AC proceeds development until the animals open their eyes between postnatal days 13–15 [15,21]. Concurrently, AC form synapses with direction-selective RGCs during the first two postnatal weeks to establish functional circuits [22]. Islet1, NeuroD or Math3 are reportedly among the few so far identified factors that promote genesis and differentiation of AC [23,24], while cell adhesion and guidance molecules such as semaphorins participate in the specific laminar stratification of AC [25,26].

The current study reports that MLP is expressed in the cytoplasm of cholinergic AC during the late embryonic and the postnatal maturation stage, thereby demonstrating that MLP is also markedly expressed in other tissue than muscle and that MLP is a specific marker for postnatal cholinergic AC with a potential role in AC maturation.

Materials and Methods

All experimental animal procedures were approved by the local animal care committee in Recklinghausen and conducted in compliance with federal and state guidelines for animal experiments in Germany (Permit Number: 84-02.04.2012.A300). Rats

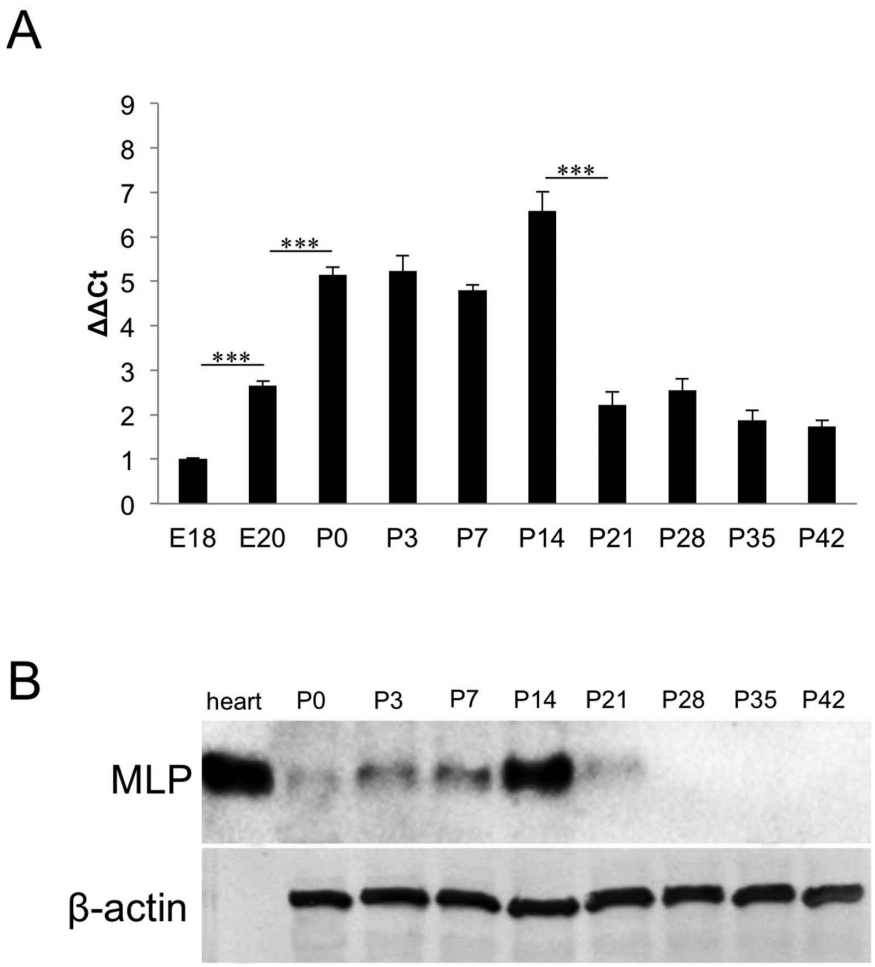

Figure 1. Transient MLP-expression in postnatal retinae. A: Quantitative real-time PCR of embryonic and postnatal rat retinae. Relative MLP expression is shown as fold change of the respective value at E18. MLP expression was increased at E20 compared to E18 and further increased significantly after birth. Highest MLP-expression was detected at P14 (a fold change of 6.6). MLP expression levels sharply decreased between P14 and P21 and reached a plateau afterwards. Comparison of relative expression: ***P≤0.05. **B**: Western Blot analysis of MLP protein in postnatal retinae (P0–P42). MLP levels increased slightly between P0 and P7 and peaked at P14. MLP levels were markedly decreased at P21 and were below detection in retinal lysates of P28 and older animals. MLP in heart muscle lysate served as positive control and β-actin as loading control.
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were maintained on a 12 hour light/dark cycle with ad libitum access to food and water. Rats were killed either by inhalation of CO_2 or intraperitoneal application of ketamine (60–80 mg/kg; Pfizer) and xylazine (10–15 mg/kg; Bayer) and perfused through the heart with cold PBS (Gibco) followed by paraformaldehyde (Sigma) (4% PFA in PBS).

RNA Isolation and Quantitative Real-time PCR

Total RNA was isolated from retinae of rats using the RNeasy kit (Qiagen) according to the manufacturer's protocol. Retina-derived RNA (10 ng) of 4 different animals per age group was combined and reversely transcribed using the superscript II kit (Invitrogen). The cDNA quantification of MLP and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression was performed with the SYBR Gree PCR Master Mix (Applied Biosystems) and QuantiTect primers (Rn_Csrp3_1_SG, Rn_Gapdh_1_SG QuantiTect Primer Assay (200); Qiagen) using the Real-Time PCR System (Applied Biosystems 7500). Retina-derived cDNA was amplified during 45 cycles according to the manufacturer's protocol. All reactions were performed in duplicate and as two independent runs. Quantitative analysis was performed using Applied Biosystems 7500 software, calculating the expression of MLP relative to the endogenous housekeeping gene GAPDH. Relative quantification was calculated using comparative threshold cycle method (ΔΔCt). The specificity of the PCR products from each run was determined and verified with the dissociation curve analysis feature of the Applied Biosystems 7500 software. The significance in pairwise comparisons between age groups was evaluated using Mann-Whitney Rank Sum Test.

Western Blot

For retinal lysate preparation, rat retinae were dissected and collected in lysis buffer (20 mM Tris/HCl (Sigma), pH 7.5, 10 mM KCl (AppliChem), 250 mM sucrose (Sigma), 10 mM NaF (Sigma), 1 mM DTT (Sigma), 0.1 mM Na_3VO_4 (Sigma), 1% Triton X-100 (Sigma), 0.1% SDS (Sigma)) with protease inhibitors (Calbiochem). Retinae were homogenized by sonification and centrifuged at 5000 rpm for 10 min. The supernatants obtained from 4 different animals per group were combined and used for western blot analysis. Separation of proteins was performed by 10% SDS polyacrylamide gel electrophoresis, according to standard protocols (Bio-Rad). Afterwards, proteins were trans-

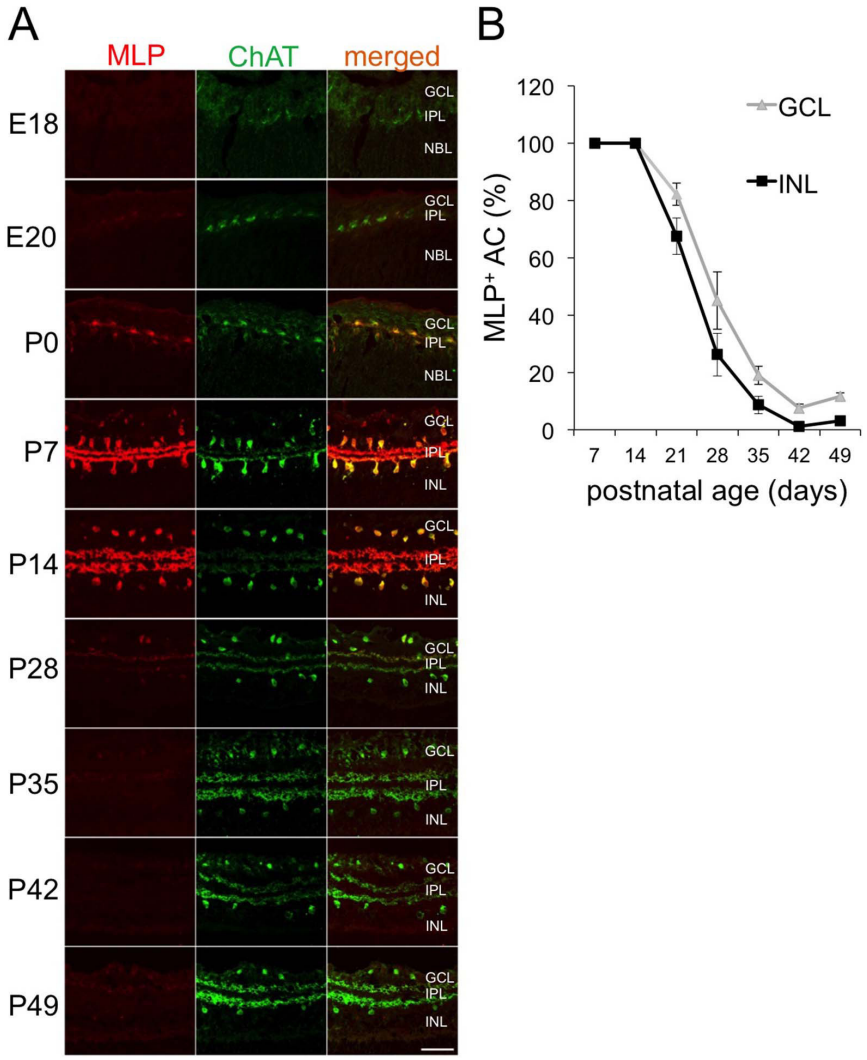

Figure 2. Expression of MLP in postnatal ChAT-positive AC. A: Confocal images were taken of E18, E20, P0, P7, P14, P28, P35, P42 and P49 retinae after co-staining with antibodies against ChAT and MLP. MLP expression becomes detectable at E20 in ChAT-positive AC located in the inner nuclear layer as well as ganglion cell layer. Levels reached a peak between P7 and P14. MLP expression dramatically decreases afterwards. GCL = ganglion cell layer, IPL = inner plexiform layer, INL = inner nuclear layer Scale bar: 50μm. **B**: Quantification of MLP-positive cholinergic AC in the inner nuclear layer (INL) and ganglion cell layer (GCL, displaced) of retinae of 7–49 days old rats. All cholinergic AC were positive for MLP at P7 and P14. Numbers of MLP-positive AC continuously decreased between P21 and P42. This attenuation proceeded slightly faster in the INL than in the GCL.
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ferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad). The blots were blocked in 5% dried milk with 1% bovine serum albumin (BSA) in Tris-buffered saline-Tween-20 and processed for immunostaining with either a polyclonal antibody against MLP (C9001-23; United States Biological; 1:500) or a monoclonal antibody against β-actin (AC-15; Sigma; 1:5000). Bound anti-MLP-antibody was visualized with anti-goat immunoglobulin G (IgG) secondary antibody conjugated to horseradish peroxidase (Sigma, 1:30 000) and the antigen–antibody complexes were detected by enhanced chemiluminescence (Bio-Rad). Bound anti-actin-antibody was visualized with anti-mouse infrared dye secondary antibody (IRDey 800 CW; LI-COR; 1:20 000) using Odyssey Infrared Imaging System (LI-COR).

Immunohistochemistry and Quantification of MLP-positive AC

Rats were anesthetized and perfused through the heart with cold PBS followed by paraformaldehyde (4% PFA in PBS). Eyes were enucleated, post-fixed for several hours in 4% PFA, transferred to 30% sucrose overnight (4°C), and embedded in Tissue-Tek (Sakura). Frozen sections were cut longitudinally on a cryostat, thaw-mounted onto coated glass slides (Superfrost plus, Fisher, Pittsburgh, PA, USA) and stored at −20°C until further use. A polyclonal antibody against ChAT (AB144P; Merck Millipore; 1:100) and a monoclonal antibody against MLP (a kind gift from Dr. Geier, Max Delbrück Center for Molecular Medicine, Berlin, Germany; 1:400) were used. Anti-goat IgG and anti-mouse IgG antibodies conjugated to Alexa Fluor 488 and Alexa Fluor 594, respectively, were used as secondary antibodies (Invitrogen; 1:1000). Stained sections were embedded with Mowiol (Calbiochem), covered using glass coverslips (VWR) and analyzed

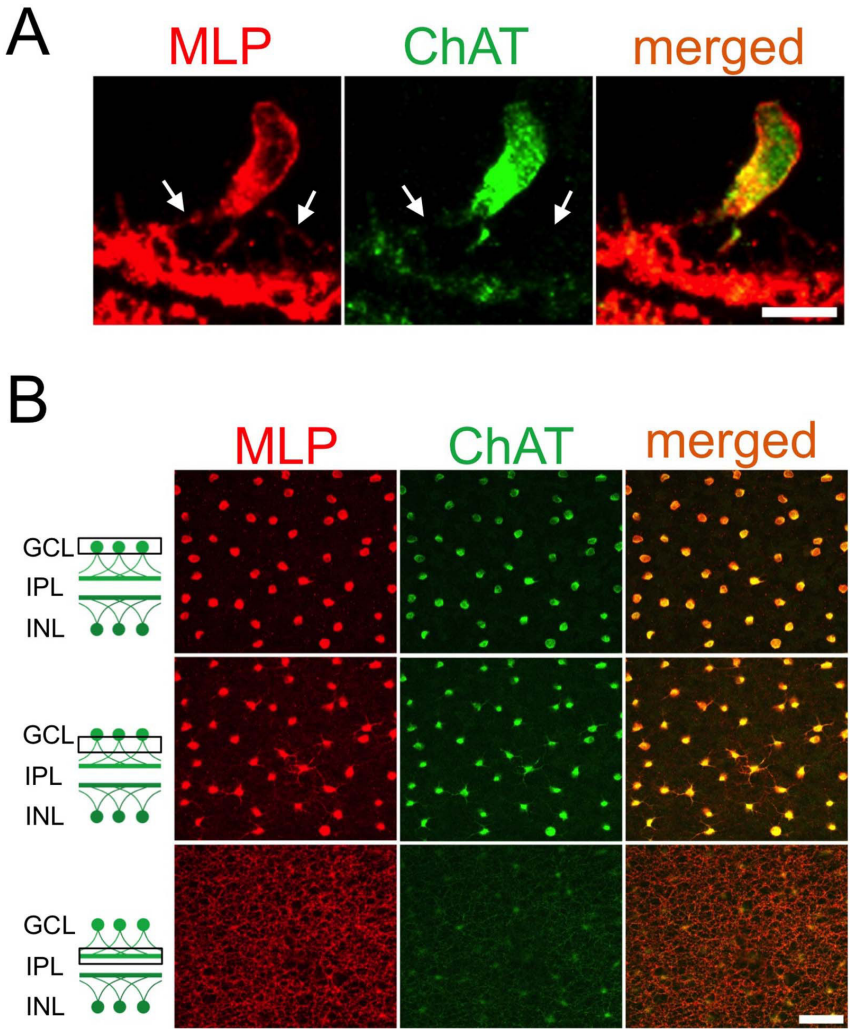

Figure 3. Subcellular localization of MLP in cholinergic AC. A: Confocal image of a MLP-positive cholinergic AC at P7, demonstrating MLP protein in the soma and the dendrites (arrows), but not in the nucleus. Scale bar: 10 µm. **B**: Confocal image of cholinergic AC in a retinal flatmount at P9, co-stained with antibodies against MLP and ChAT. The somas of all cholinergic amacrine cells as well as their proximal and distal dendrite segments are positive for MLP. Scale bar: 50 µm.
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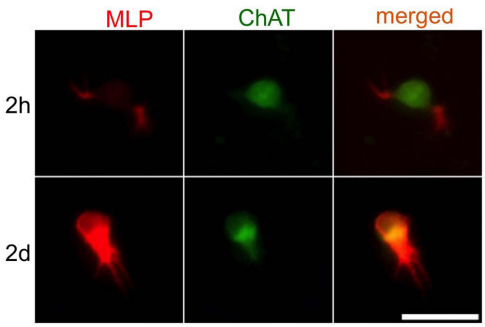

Figure 4. MLP-expression in cultured postnatal AC. Cultured cholinergic AC were obtained from the retina of a P1-rat. The cells were fixed and stained with antibodies against MLP and ChAT after 2 hours and 2 days in culture. After 2 hours, the cholinergic AC showed a week MLP expression in the soma. In sprouting dendrites, however, MLP-expression was more pronounced. After 2 days, the MLP levels dramatically increased in the soma as well as in the dendrites of the cholinergic AC. Scale bar: 25 µm.
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under a fluorescent microscope (Axiovision, Zeiss). For quantification of MLP-positive cholinergic AC, an intensity threshold was set to identify the positively stained cells. The percentage of MLP-expressing cholinergic AC was calculated as the ratio of MLP-positive to ChAT-positive cell numbers. Three animals per postnatal age and four sections per animal were quantified. Confocal images of fluorescent sections were obtained using confocal laser scanning microscope LSM 510 (Zeiss).

For retinal whole mount staining, rats were killed by decapitation and the retinae were rapidly dissected from the eyecups. Retinae were attached to nitrocellulose filter, fixed in 4% PFA for 30 min and treated with 2% TritonX (Sigma) for 1 h. The whole mounts were stained with antibodies against ChAT (1:100) and MLP (1:400), embedded with Mowiol and covered using glass coverslips. Confocal images were obtained using confocal laser scanning microscope LSM 510 (Zeiss).

Dissociated AC Culture

Tissue culture plates (four-well-plates; Nunc) were coated with poly-D-lysine (0.1 mg/ml, molecular weight 300 000 Da; Sigma),

rinsed with distilled water and air-dried. To prepare AC cultures, postnatal rats were killed by decapitation, retinae were rapidly dissected from the eyecups and incubated at 37°C for 30 min in a digestion solution containing papain (10 U/ml; Worthington) and L-cysteine (0.3 mg/ml; Sigma) in Neurobasal (NB) medium (Gibco). They were then rinsed with NB medium and triturated in 1 ml NB medium. Dissociated cells were passed through a cell strainer (40 mm) and 300 µl cell suspension in NB medium containing B27 supplement (1:50; Gibco), penicillin/streptomycin (0.2 mg/ml; Biochrom) and L-Glutamine (0.5 mM; Sigma) were added to each well. Cells remained in culture for 2 hours or 2 days and were fixed in 4% paraformaldehyde (PFA) solution in PBS for 25 min and then in 100% methanol (Sigma) for 10 min. AC were stained with antibodies against ChAT (1:100) and MLP (1:400).

Results

MLP Expression in Embryonic and Postnatal Rat Retina

Initial experiments revealed MLP expression in postnatal rat retinae. To assess the complete temporal pattern of MLP expression during retinal development, we examined embryonic, postnatal and adult rat retinae (E18-P42) using quantitative real-time PCR and western blot analysis. Real-time PCR revealed low expression levels of MLP in E18 and adult retinae (Fig. 1. A). Expression increased slightly in E20 retinae and was significantly elevated at P0 to P14, with peak expression at P14 (~7 fold). Thereafter, MLP expression rapidly decreased, falling to levels comparable to embryonic stages. (Fig. 1 A).

To verify the mRNA expression data at the protein level, we also performed western blot analysis on retinal lysates from P0–P42 rats. Sparse MLP protein was detectable at P0 (Fig. 1. B), but MLP levels increased between P3 and P14. In accordance with the results of the real-time PCR analysis, MLP expression peaked at P14 and markedly decreased at P21. No MLP protein was detected in retinal lysates at P28, P35 or P42.

MLP is Expressed in Cholinergic Amacrine Cells

We next investigated the cellular localization of MLP in embryonic and postnatal retinae. Immunohistochemical staining of retinal cross-sections identified MLP-positive cells in the GCL and the INL of P14 retinae (Fig. 2 A). Co-immunostaining with an antibody against ChAT, a specific marker for cholinergic AC, revealed rigorous co-localization of ChAT and MLP staining, demonstrating that MLP expression is restricted to cholinergic AC only (Fig. 2 A, Fig. 3 B). MLP staining was detected on postnatal AC somas and dendrites whereas the nucleus remained spared (Fig. 3 A, Fig. 3 B). In addition, both sublaminae of the IPL containing dendrites of cholinergic AC from the GCL and the INL, respectively, were prominently immunoreactive for MLP.

We next analyzed the time course of MLP expression and quantified MLP-positive cholinergic AC in late embryonic and postnatal retinae. Slight MLP expression co-localizing with ChAT-positive cells was first detected at E20 (Fig. 2 A). The intensity of MLP immunoreactivity and number of MLP-positive cells substantially increased at P0 close to a still barely developed IPL. At P7 and P14, all cholinergic AC expressed MLP protein (Fig. 2 A, Fig. 2 B) and two subpopulations of MLP-positive cells located either in the GCL or the INL were clearly distinguishable. MLP staining markedly decreased in both subpopulations of cholinergic AC at P28 (Fig. 2A, Fig. 2 B). This reduction was slightly more pronounced in the INL compared to the GCL. On average, 45% of ChAT-immunoreactive cells of the GCL and 26% in the INL were still faintly MLP-positive at P28. Consistent with the Western-blot results, MLP remained weakly expressed in only very few cholinergic AC at P42 and P49 (Fig. 2A, Fig. 2 B).

MLP is Expressed in Cultured Cholinergic AC

We tested whether MLP-Expression can also be observed in cultured postnatal cholinergic AC. To this end cultures of cholinergic AC were prepared from retinae of rats at P1. MLP-expression was observed in small sprouting dendrites of cholinergic ACs after 2 hours in culture (Fig. 4). After 2 days MLP levels markedly increased in the somas and in the dendrites (Fig. 4). The dendrites, however, were not immunoreactive for ChAT. These data indicate that MLP is a suitable marker for outgrowing dendrites of cultured postnatal cholinergic AC.

Discussion

MLP is a well-characterized protein, mainly expressed in heart tissue. It is involved in myogenesis and the organization of cytoskeletal structures in myocytes and has so far been regarded as muscle-specific [1,4,8]. The current study demonstrates to our knowledge for the first time that this protein is also markedly expressed in postnatal CNS tissue and therefore not restricted to muscle tissue. Western-blot analysis and quantitative PCR revealed a transient retinal MLP expression starting at E20, reaching a peak between P7 and P14 and absence in the adult retina. Immunohistochemical analysis confined MLP expression in cholinergic (ChAT-positive) AC. These neurons are one of at least 26 distinguishable AC subtypes with the ability to produce the neurotransmitter acetylcholine and are involved in the maturation of postnatal retina [19,20] as well as motion sensation by the visual apparatus [27,28].

In rodents, first AC are born at E8/E9, along with retinal ganglion cells (RGCs), horizontal cells and cone photoreceptors. AC genesis peaks at E16/E17, but proceeds at least until P5/P7 [29,30]. Consistent with the data presented in the current study, cholinergic AC start to express choline acetyltransferase (ChAT) at around E18 and therefore almost simultaneously to the observed induction of retinal MLP-expression [15]. However, in contrast to the lifelong expression of ChAT, MLP is only expressed throughout the first 3 weeks after birth and absent in adult AC. During this postnatal time period AC form dendrites (E18), establish two well-separated cholinergic dendritic layers in the IPL (P3) [15] and make synapses on RGCs and bipolar cells (P3–P15) [31]. This process also timely overlaps with the emergence of spontaneous acetylcholine dependent waves of excitatory activity between P0 and P11 [32], which are important for the formation of synapses and the establishment of neural circuits between retinal neurons. The formation of specific synapses between cholinergic AC and direction-selective RGCs, known to be crucial for motion sensing, occurs in the second postnatal week and is therefore timely correlated with morphologic maturation of AC [22]. Maturation of cholinergic AC is eventually accomplished at postnatal day 15, the time of eye opening [15,21] and of detected decrease of MLP expression, suggesting a potential functional involvement of this protein in this context. Future studies still have to investigate the potential role of MLP during AC development. To this end the morphology of AC or other retinal cells as well as the visual function of MLP deficient and wild-type mice could be compared with each other to identify potential differences.

In muscle tissue MLP is crucially involved in myogenesis and myocyte differentiation, requiring the localization of MLP in the nucleus, where it serves as transcriptional cofactor and modulates the expression of myocyte specific genes [8]. In addition, MLP is also located in the cytoplasm where it might promote the assembly

of cytoskeletal proteins along actin-based filaments [4]. As MLP was only detected in the cytoplasm of AC it appears more likely that it is rather involved in the organization of cytoskeleton than regulation of gene expression in these neurons. In this context MLP may support the growth and stratification of dendrites or synapse formation. MLP has been shown to interact with a broad variety of proteins belonging to different functional classes in muscle tissue [33,34]. In particular, interactions of MLP with α-actin [35], actin-binding proteins like cofilin 2 [2], spectrins (βI-spectrin) [5] or even metabolic enzymes like D-lactate dehydrogenase [36] were described. Therefore, the role of MLP in cholinergic AC might depend on the interaction with several partners. Further research is required to address these possibilities. Nevertheless, MLP expression can now be used as a novel marker for postnatal cholinergic AC.

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Author Contributions

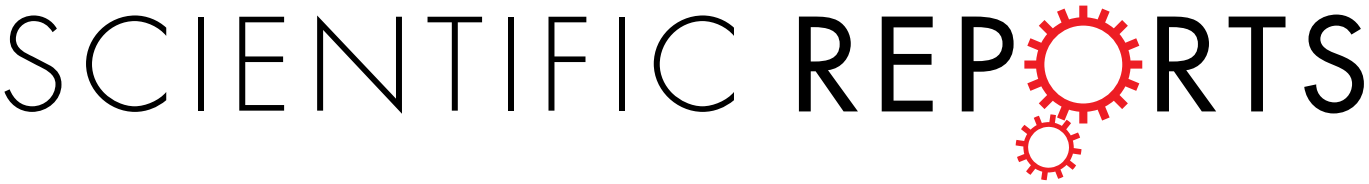
Conceived and designed the experiments: EL ML DF. Performed the experiments: EL ML AA. Analyzed the data: EL DF. Wrote the paper: EL DF.

References

1. Arber S, Halder G, Caroni P (1994) Muscle LIM protein, a novel essential regulator of myogenesis, promotes myogenic differentiation. Cell 79: 221–231.
2. Papalouka V, Arvanitis DA, Vafiadaki E, Mavroidis M, Papadodima SA, et al. (2009) Muscle LIM protein interacts with cofilin 2 and regulates F-actin dynamics in cardiac and skeletal muscle. Mol Cell Biol 29: 6046–6058.
3. Wang X, Li Q, Adhikari N, Hall JL (2006) A role for muscle LIM protein (MLP) in vascular remodeling. J Mol Cell Cardiol 40: 503–509.
4. Arber S, Caroni P (1996) Specificity of single LIM motifs in targeting and LIM/LIM interactions in situ. Genes Dev 10: 289–300.
5. Flick MJ, Konieczny SF (2000) The muscle regulatory and structural protein MLP is a cytoskeletal binding partner of betaI-spectrin. J Cell Sci 113 (Pt 9): 1553–1564.
6. Arber S, Hunter JJ, Ross J Jr, Hongo M, Sansig G, et al. (1997) MLP-deficient mice exhibit a disruption of cardiac cytoarchitectural organization, dilated cardiomyopathy, and heart failure. Cell 88: 393–403.
7. Geier C, Gehmlich K, Ehler E, Hassfeld S, Perrot A, et al. (2008) Beyond the sarcomere: CSRP3 mutations cause hypertrophic cardiomyopathy. Hum Mol Genet 17: 2753–2765.
8. Kong Y, Flick MJ, Kudla AJ, Konieczny SF (1997) Muscle LIM protein promotes myogenesis by enhancing the activity of MyoD. Mol Cell Biol 17: 4750–4760.
9. Boateng SY, Belin RJ, Geenen DL, Margulies KB, Martin JL, et al. (2007) Cardiac dysfunction and heart failure are associated with abnormalities in the subcellular distribution and amounts of oligomeric muscle LIM protein. Am J Physiol Heart Circ Physiol 292: H259–269.
10. MacNeil MA, Heussy JK, Dacheux RF, Raviola E, Masland RH (1999) The shapes and numbers of amacrine cells: matching of photofilled with Golgi-stained cells in the rabbit retina and comparison with other mammalian species. J Comp Neurol 413: 305–326.
11. MacNeil MA, Masland RH (1998) Extreme diversity among amacrine cells: implications for function. Neuron 20: 971–982.
12. Haverkamp S, Wassle H (2000) Immunocytochemical analysis of the mouse retina. J Comp Neurol 424: 1–23.
13. Voigt T (1986) Cholinergic amacrine cells in the rat retina. J Comp Neurol 248: 19–35.
14. Eckenstein F, Thoenen H (1982) Production of specific antisera and monoclonal antibodies to choline acetyltransferase: characterization and use for identification of cholinergic neurons. Embo J 1: 363–368.
15. Kim IB, Lee EJ, Kim MK, Park DK, Chun MH (2000) Choline acetyltransferase-immunoreactive neurons in the developing rat retina. J Comp Neurol 427: 604–616.
16. Hayden SA, Mills JW, Masland RM (1980) Acetylcholine synthesis by displaced amacrine cells. Science 210: 435–437.
17. Vaney DI, Peichi L, Boycott BB (1981) Matching populations of amacrine cells in the inner nuclear and ganglion cell layers of the rabbit retina. J Comp Neurol 199: 373–391.
18. Famiglietti EV Jr (1983) 'Starburst' amacrine cells and cholinergic neurons: mirror-symmetric on and off amacrine cells of rabbit retina. Brain Res 261: 138–144.
19. Feller MB, Wellis DP, Stellwagen D, Werblin FS, Shatz CJ (1996) Requirement for cholinergic synaptic transmission in the propagation of spontaneous retinal waves. Science 272: 1182–1187.
20. Ford KJ, Feller MB (2012) Assembly and disassembly of a retinal cholinergic network. Vis Neurosci 29: 61–71.
21. Zhang J, Yang Z, Wu SM (2005) Development of cholinergic amacrine cells is visual activity-dependent in the postnatal mouse retina. J Comp Neurol 484: 331–343.
22. Wei W, Hamby AM, Zhou K, Feller MB (2011) Development of asymmetric inhibition underlying direction selectivity in the retina. Nature 469: 402–406.
23. Elshatory Y, Everhart D, Deng M, Xie X, Barlow RB, et al. (2007) Islet-1 controls the differentiation of retinal bipolar and cholinergic amacrine cells. J Neurosci 27: 12707–12720.
24. Inoue T, Hojo M, Bessho Y, Tano Y, Lee JE, et al. (2002) Math3 and NeuroD regulate amacrine cell fate specification in the retina. Development 129: 831–842.
25. Yamagata M, Weiner JA, Sanes JR (2002) Sidekicks: synaptic adhesion molecules that promote lamina-specific connectivity in the retina. Cell 110: 649–660.
26. Matsuoka RL, Nguyen-Ba-Charvet KT, Parray A, Badea TC, Chedotal A, et al. (2011) Transmembrane semaphorin signalling controls laminar stratification in the mammalian retina. Nature 470: 259–263.
27. Famiglietti EV (1987) Starburst amacrine cells in cat retina are associated with bistratified, presumed directionally selective, ganglion cells. Brain Res 413: 404–408.
28. Taylor WR, Smith RG (2012) The role of starburst amacrine cells in visual signal processing. Vis Neurosci 29: 73–81. doi:10.1017/S0952523811000393.
29. Rapaport DH, Wong LL, Wood ED, Yasumura D, LaVail MM (2004) Timing and topography of cell genesis in the rat retina. J Comp Neurol 474: 304–324.
30. Voinescu PE, Kay JN, Sanes JR (2009) Birthdays of retinal amacrine cell subtypes are systematically related to their molecular identity and soma position. J Comp Neurol 517: 737–750.
31. Fisher LJ (1979) Development of synaptic arrays in the inner plexiform layer of neonatal mouse retina. J Comp Neurol 187: 359–372.
32. Bansal A, Singer JH, Hwang BJ, Xu W, Beaudet A, et al. (2000) Mice lacking specific nicotinic acetylcholine receptor subunits exhibit dramatically altered spontaneous activity patterns and reveal a limited role for retinal waves in forming ON and OFF circuits in the inner retina. J Neurosci 20: 7672–7681.
33. Gehmlich K, Geier C, Milting H, Furst D, Ehler E (2008) Back to square one: what do we know about the functions of muscle LIM protein in the heart? J Muscle Res Cell Motil 29: 155–158.
34. Buyandelger B, Ng KE, Miocic S, Piotrowska I, Gunkel S, et al. (2011) MLP (muscle LIM protein) as a stress sensor in the heart. Pflugers Arch 462: 135–142.
35. Louis HA, Pino JD, Schmeichel KL, Pomies P, Beckerle MC (1997) Comparison of three members of the cysteine-rich protein family reveals functional conservation and divergent patterns of gene expression. J Biol Chem 272: 27484–27491.
36. Flick MJ, Konieczny SF (2002) Identification of putative mammalian D-lactate dehydrogenase enzymes. Biochem Biophys Res Commun 295: 910–916.

Original publication 3

Highly efficient transduction of primary adult CNS and PNS neurons



OPEN

Highly efficient transduction of primary adult CNS and PNS neurons

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Evgeny Levin, Heike Diekmann & Dietmar Fischer

Delivery and expression of recombinant genes, a key methodology for many applications in biological research, remains a challenge especially for mature neurons. Here, we report easy, highly efficient and well tolerated transduction of adult peripheral and central neuronal populations of diverse species in culture using VSV-G pseudo-typed, recombinant baculovirus (BacMam). Transduction rates of up to 80% were reliably achieved at high multiplicity of infection without apparent neuro-cytopathic effects. Neurons could be transduced either shortly after plating or after several days in culture. Co-incubation with two different baculoviruses attained near complete co-localization of fluorescent protein expression, indicating multigene delivery. Finally, evidence for functional protein expression is provided by means of cre-mediated genetic recombination and neurite outgrowth assays. Recombinant protein was already detected within hours after transduction, thereby enabling functional readouts even in relatively short-lived neuronal cultures. Altogether, these results substantiate the usefulness of baculovirus-mediated transduction of mature neurons for future research in neuroscience.

Protein overexpression and gene knockout are key technologies for the study of molecular mechanisms in life sciences. However, post-mitotic and in particular adult neurons are generally difficult to culture and particularly resistant to the delivery and expression of recombinant genes, thereby often limiting experimental approaches. Therefore, an easy and reliable method to genetically manipulate cultured neurons would be highly desirable in order to facilitate research on the molecular basis of neuronal function under normal and pathological conditions.

Despite ongoing advances in physical, chemical and electrical methods of gene delivery, primary neurons still tend to be refractory to plasmid transfection in cell culture. Although nucleofection can achieve 60–80% efficiency after optimization, this technique is mainly restricted to freshly isolated, embryonic and postnatal neurons and requires relatively expensive equipment and reagents[1,2]. Calcium phosphate precipitation and lipofection methods achieve at best 5–10% transfection rates and are, moreover, associated with general toxicity and transient expression[2]. Viral gene delivery systems, such as recombinant lentivirus (LV) or adeno-associated virus (AAV), may overcome the problem of low efficient gene transfer particularly into non-dividing neurons. Some studies report significant transduction rates for embryonic or postnatal cerebellar and hippocampal neurons[3–7], but gene transfer into adult neurons can be considerably less efficient (5–10% for LV-transduction of adult dorsal root ganglion (DRG) neurons in our hands). Furthermore, the above-mentioned viral expression technologies have significant drawbacks, limiting their applicability for neuronal cell cultures. These shortcomings comprise cytotoxicity at high titers, risk of insertional mutations, late onset of transgene expression (notably for AAV at 5–14 days after transduction), limited insert size (<2.5 kb for AAV, 2.5–5 kb for LV) and requirement for biosafety level 2 for LV[2].

Baculoviruses offer several advantages compared to other viral gene delivery vectors in terms of safety, high insert size capacity and ease of production and have therefore been widely used for heterologous protein expression[8,9]. Natural baculoviruses infect insects and cannot replicate in mammalian cells. Increased tropism can be achieved via modification of the baculoviral envelope glycoprotein gp64[10]. For example, vesicular stomatitis virus envelope G-protein (VSV-G) -pseudo-typed virions feature more efficient cell entry and transduction of a variety of mammalian cells[11]. BacMam is a genetically engineered baculovirus with VSV-G-modified capsid protein that contains a DNA cassette for transgene expression in mammalian cells, which ignore insect-specific promoters and express only mammalian promoter-driven transgenes. BacMam virus transduction is generally well tolerated without apparent cytotoxicity, even at high multiplicity of infection (MOI)[10,12,13]. In addition,

Division of Experimental Neurology, Medical Faculty, Heinrich-Heine-University, Merowingerplatz 1a, 40225 Düsseldorf, Germany. Correspondence and requests for materials should be addressed to D.F. (email: dietmar.fischer@uni-duesseldorf.de)

baculoviruses are easy to use as they are simply incubated with cells in normal culture medium and can be handled in a biosafety level 1 facility. Therefore, the BacMam technology seemed a good candidate for gene transfer and expression in neurons[14–16]. To our surprise, we were unable to identify any published description of successful baculovirus-mediated transduction of adult neurons *in vitro*.

Here, we provide first evidence that primary neurons of the adult central nervous system (CNS) as well as peripheral nervous system (PNS) can be very efficiently transduced in culture using BacMam virus. Observed transduction rates markedly exceeded previously reported attempts with other gene transfer methods without obvious neuro-cytopathic effects. BacMam-induced expression of heterologous proteins was promptly initiated and proofed functional, as determined by successful cre-mediated genetic recombination and neurite outgrowth assays. Thus, this methodology provides novel experimental opportunities with cultured primary neurons, which could greatly advance future research in neuroscience.

Materials and Methods

Animals. All experimental animal procedures were approved by the local animal care committee (Umweltministerium NRW, Landesumweltamt, Recklinghausen) and conducted in compliance with federal and state guidelines for animal experiments in Germany. Adult (6–8 weeks old) male and female Wistar rats and mice of strains C57BL/6 and ROSA-tdTomato [C57BL/6;Cg-*Gt(ROSA)26Sor*$^{tm14(CAG-tdTomato)Hz}$]*w*ere maintained on a 12 h light/dark cycle with ad libitum access to food and water. Adult, 4–8 months old, homozygous Tg(fGAP43:GFP) zebrafish[17] were kept in the zebrafish facility of the University of Düsseldorf on a 14 h light/10 h dark cycle under standard conditions[18]. Rats were sacrificed by inhalation of CO_2, mice by cervical dislocation and zebrafish by immersion in MS222 (0.4 mg/l) and decapitation.

Vectors. Farnesylated EGFP (fGFP), which is targeted to the plasma membrane (kindly provided by Prof. Joost Verhaagen, Amsterdam), DsRed-Monomer (Clontech) and Cre-HA (kindly provided by Dr. Zhigang He, Boston) were directionally cloned into the Gateway pENTR 2B Dual Selection Vector (Thermo Fisher) using respective restriction enzymes. For the generation of a hyper-interleukin 6 (hIL6) and EGFP co-expression vector, an MCS-IRES-EGFP sequence was amplified using primers 5′-AATGAATTCCTCGAGCTAACGTTACTGGCCGAA-3′ and 5′-TCATTACTTGTACAGCTCGT-3′ and inserted into the pENTR 2B Gateway vector. HIL6[19] was then directionally cloned into this modified vector. All expression constructs were transferred into the BacMam pCMV-Dest vector using Gateway LR Clonase II Enzym-Mix (Thermo Fisher) and transformed into MaxEfficiency BH10Bac cells for transposition into a bacmid according to manufacturer's protocols. The BacMam pCMV-DEST vector contains the cytomegalovirus (CMV) promoter for high-level expression in mammalian cells, Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element (WPRE) for increased duration of gene expression, SV40 polyadenylation signal for efficient transcription termination and polyadenylation of mRNA and VSG protein for viral delivery into mammalian cells (Thermo Fischer).

Generation of recombinant baculovirus. Recombinant baculoviruses were produced using the ViraPower BacMam Expression System (Thermo Fisher) according to manufacturer's protocols. In brief, recombinant bacmid DNA was purified and transfected into adherent Sf9 cells (Thermo Fisher) using Cellfectin reagent to generate P1 recombinant baculovirus stock. Baculoviruses were amplified by inoculation of 50 ml Sf9 suspension cultures (10^6 cells/ml) in Sf-900 III SFM Medium supplemented with 12.5 U/ml penicillin/streptomycin (Biochrom) in 125 ml polycarbonate Erlenmeyer flasks with vent cap (Corning) with 1 ml virus stock solution and incubation at 27 °C and 130 rpm for 2–3 days. Amplified viruses were purified and concentrated by ultracentrifugation of 27 ml virus-containing supernatant underlayed with 2.7 ml sucrose solution (25% sucrose with 5 mM NaCl and 10 mM EDTA in H_2O) in OptiSeal polypropylene tubes (Beckmann Coulter) at 80,000 g and 4 °C for 80 min. Viral pellets were re-suspended in 0.5 ml PBS and passed through 0.22 μm low protein binding sterile syringe filters (Merck Millipore). Baculovirus preparations were pre-tested on HEK293 cells (seeded at ~3–5 × 10^4 cells per well in 96-well plates) by adding 1 μl virus per well overnight. Transduction efficiencies of $\geq$ 90% were regarded appropriate for further use. Otherwise, virus stock was subjected to further amplification cycles.

Baculovirus titration. Baculoviral titers were determined using the FastPlax Titer Kit (Merck Millipore). Sf9 cells (3 × 10^4 cells/well) were seeded into 96-well plates (Thermo Fisher) and inoculated with 20 μl serial virus dilutions (10^{-3} – 10^{-7} in duplicate) for 1 h at room temperature with gentle rocking. Thereafter, 100 μl culture medium was added to each well and cells were incubated at 27 °C for 30 h. After fixation in 4% paraformaldehyde (PFA, Sigma), transduced cells were stained with an antibody against the gp64 envelope protein and XGal staining according to the manufacturer's protocol and quantified using an inverted microscope (Observer.D1, Zeiss). All baculoviruses used in this study had calculated titers of ~10^8 pfu/ml.

DRG cultures. DRG neurons were isolated from rats and mice as described previously[19,20]. In brief, DRG were harvested, incubated in Dulbecco's modified Eagle medium (DMEM) containing 0.25% trypsin/EDTA (Thermo Fisher) and 0.3% collagenase type IA (Sigma) and mechanically dissociated. Cells were re-suspended in DMEM supplemented with 10% fetal bovine serum (FBS, GE Healthcare) and 500 U/ml penicillin/streptomycin (BioChrom) and 50 μl of this mixed population (containing ~100–500 DRG neurons) plated into 96-well plates coated with poly-D-lysine (0.1 mg/ml, molecular weight <300,000 Da, Sigma) and 20 μg/ml laminin (Sigma). The respective baculovirus (10 μl in PBS, ~10^6 pfu) was directly added 4 h after seeding and cells incubated at 37 °C and 5% CO_2. Two-thirds of the DRG culture medium was replaced with fresh medium 16 h after transduction. After 1, 2 or 3 days in culture, cells were fixed in 4% PFA for 25 min and permeabilized in 100% methanol for 10 min. DRG neurons were identified with TUJ-1 antibody against βIII-tubulin (1:2,000, BioLegend) and in some cases co-stained with either isolectin B4 (FITC-conjugated, 1:40, L2895, Sigma) or an antibody against 200 kD neurofilament (1:1000, ab7795, Abcam). All experiments were performed in duplicate with three replicate wells

for each experimental group. Percentages of fGFP- or tdTomato-positive, transduced DRG neurons in relation to TUJ-1, isolectin B4 and neurofilament counts, respectively, were quantified using a fluorescent microscope (Observer.D1, Zeiss) and presented as means ± SEM. Significances of intergroup differences were evaluated using one-way analysis of variance (ANOVA) with Holm-Sidak post hoc test.

Retinal cultures. Retinal cultures from rats, mice and zebrafish were prepared as described previously[21,22]. In brief, retinae were dissected from eyecups and digested in DMEM containing papain (16.4 U/ml for rat and zebrafish, 10 U/ml for mouse retinae, respectively; Worthington) and L-cysteine (0.3 mg/ml for rat and zebrafish, 0.2 mg/ml for mouse retinae, respectively; Sigma) at 37 °C for 30 minutes (rat and mice retinae) or at room temperature for 40 minutes (zebrafish retinae). Rat and mice retinae were triturated and washed by centrifugation in 50 ml DMEM (7 min at 900 g for rat and 7 min at 500 g for mouse retinae). Retinal pellets were re-suspended in DMEM (6.5 ml/rat retina, 1.5 ml/mouse retina) containing B27-supplement (1:50, Thermo Fisher) and 200 U/ml penicillin/streptomycin to contain ~1–2 × 10^3 retinal ganglion cells (RGC)/ml. Zebrafish retinae were rinsed with L15/salt solution (12.5% salt solution: 10 mM D-glucose, 1.26 mM CaCl2, 32 mM Hepes, pH 7.5/87.5% L15; Thermo Fisher) prior to trituration in 2 ml fish medium (2% FBS, 0.2 mg/ml penicillin/streptomycin in L15/salt solution). Cells were seeded into 96-well plates (50 µl, for the determination of transduction rates) or 4-well plates (300 µl, for outgrowth assays) coated with poly-D-lysine (0.1 mg/ml, molecular weight <300,000 Da, Sigma) and 20 µg/ml laminin (Sigma). The respective baculovirus was directly added 4 h after seeding and cells incubated at 37 °C and 5% CO_2 (rat and mice RGCs) or at 27 °C (zebrafish RGCs). Generally, 10^6 pfu (10 µl in PBS) were used per well for the determination of transduction rates, 5 × 10^5 pfu each for co-transduction and 10^5 pfu (1 µl in PBS) for neurite outgrowth assays. Two-thirds of the RGC culture medium was replaced with fresh medium 16 h after transduction. After 1–6 days in culture, cells were fixed in 4% PFA for 25 min and in case of subsequent immunocytochemistry permeabilized in 100% methanol for 10 min. Rat and mice RGCs were identified with TUJ-1 antibody (1:2,000; BioLegend), while zebrafish RGCs express EGFP[22]. All experiments were performed in duplicate with at least three replicate wells per experimental group. Percentages of GFP- or DsRed-positive, transduced RGCs in relation to TUJ-1 and EGFP, respectively, were quantified at 1, 2 and 3 days for rat and mice RGCs and 4 and 6 days for zebrafish RGCs using a fluorescent microscope (Observer.D1, Zeiss) and presented as means ± SEM. In addition, the number of surviving RGCs was quantified per well. Significances of intergroup differences were evaluated using one-way analysis of variance (ANOVA) with Holm-Sidak post hoc test or Student's t-test. For neurite outgrowth assays, RGCs at 4 days in culture with neurites longer than twice the soma diameter were photographed using a fluorescent microscope (200×, Observer.D1, Zeiss) and neurite length was determined using ImageJ software. In addition, βIII-tubulin-, DsRed- or GFP-positive RGCs were quantified per well. Average neurite length for transduced and non-transduced RGCs was determined by dividing the sum of neurite length by the respective RGC count and normalized to non-transduced cells. HIL6-induced neurite length is presented as fold change compared to DsRed-transduced RGCs. Significance of intergroup difference was evaluated using Student's t-test. Expression of hIL6 and phosphorylation of STAT3 in hIL6-baculovirus transduced RGCs was evaluated upon immunohistochemistry with antibodies against GFP (1:1000, NB100-1770, Novus Biologicals), IL-6 (1:500, ab6672, Abcam) and pSTAT3 (1:200, 9145 S, Cell Signaling Technology).

Results

Transduction of DRG neurons. Gene transfer generally tends to be more effective with younger primary neurons. Accordingly, baculovirus (bv)-mediated transduction of embryonic and postnatal neurons was reported with 20–30% efficiency[23–26]. Therefore, we first tested our BacMam preparations on dorsal root ganglion (DRG) neurons isolated from P7 rat pups. Virus particles were directly added to dissociated DRG neurons 4 h after seeding, ensuring sufficient time for cell attachment. The proportion of transduced (GFP-expressing) and βIII-tubulin-positive neurons was determined at 1, 2 and 3 days after transduction (d.a.t.). This approach reliably achieved transduction rates of ~80% for postnatal DRG neurons (Fig. 1a,b). Of note, no differences were observed for the different time points analyzed, indicating efficient transduction and fast induction of recombinant protein expression within 24 h. Despite these high transduction rates, counts of DRG neurons did not differ between vehicle- and bv-treated cultures (Fig. 1c), indicating low neurotoxicity of our BacMam preparation.

Based on this promising result with postnatal neurons, we also applied baculovirus particles to mature neuronal cultures. Strikingly, similar transduction efficiencies (~80%) were observed for DRG neurons isolated from adult rats (Fig. 1d,e) as well as adult mice (Fig. 1g,h), without any apparent signs of neurotoxicity (Fig. 1f,i). GFP expression was strong already at 1 d.a.t., allowing easy visualization of axonal processes. Therefore, BacMam-mediated gene delivery is an easy-to-use and reliable method to efficiently express heterologous proteins in cultured postnatal as well as adult peripheral sensory neurons of different mammalian species.

Transduction of mature retinal ganglion cells. Primary mature CNS neurons are usually very difficult to culture, but retinal ganglion cells (RGCs) isolated from adult mammals can be kept in culture for up to 5 days prior to the onset of neuronal degeneration[21]. These neurons are therefore frequently used for the study of age dependent processes, such as neuronal survival and axon regeneration[22,27,28]. However, efficient and fast *in vitro* gene transfer is, to our knowledge, still virtually impossible and hampers experimental approaches. Therefore, we next investigated whether adult RGCs would be equally amenable to BacMam transduction as DRG neurons. To this end, retinal cells isolated either from adult rats or mice were incubated with GFP-encoding BacMam virus (Fig. 2). GFP fluorescence became clearly visible within 24 h in βIII-tubulin positive RGCs, indicating efficient transduction and protein synthesis. The transduction rate for rat RGCs increased from ~50% at 1 d.a.t. to almost 90% at 2 and 3 d.a.t. (Fig. 2a,b). Transduction of mouse RGCs was less extensive, but still substantial with ~20% at 1 d.a.t. and ~50% at 2 and 3 d.a.t. (Fig. 2d,e). The number of RGCs was unchanged upon viral transduction compared to vehicle-treated controls (Fig. 2c,f), again demonstrating low cytotoxicity. To our knowledge, this is

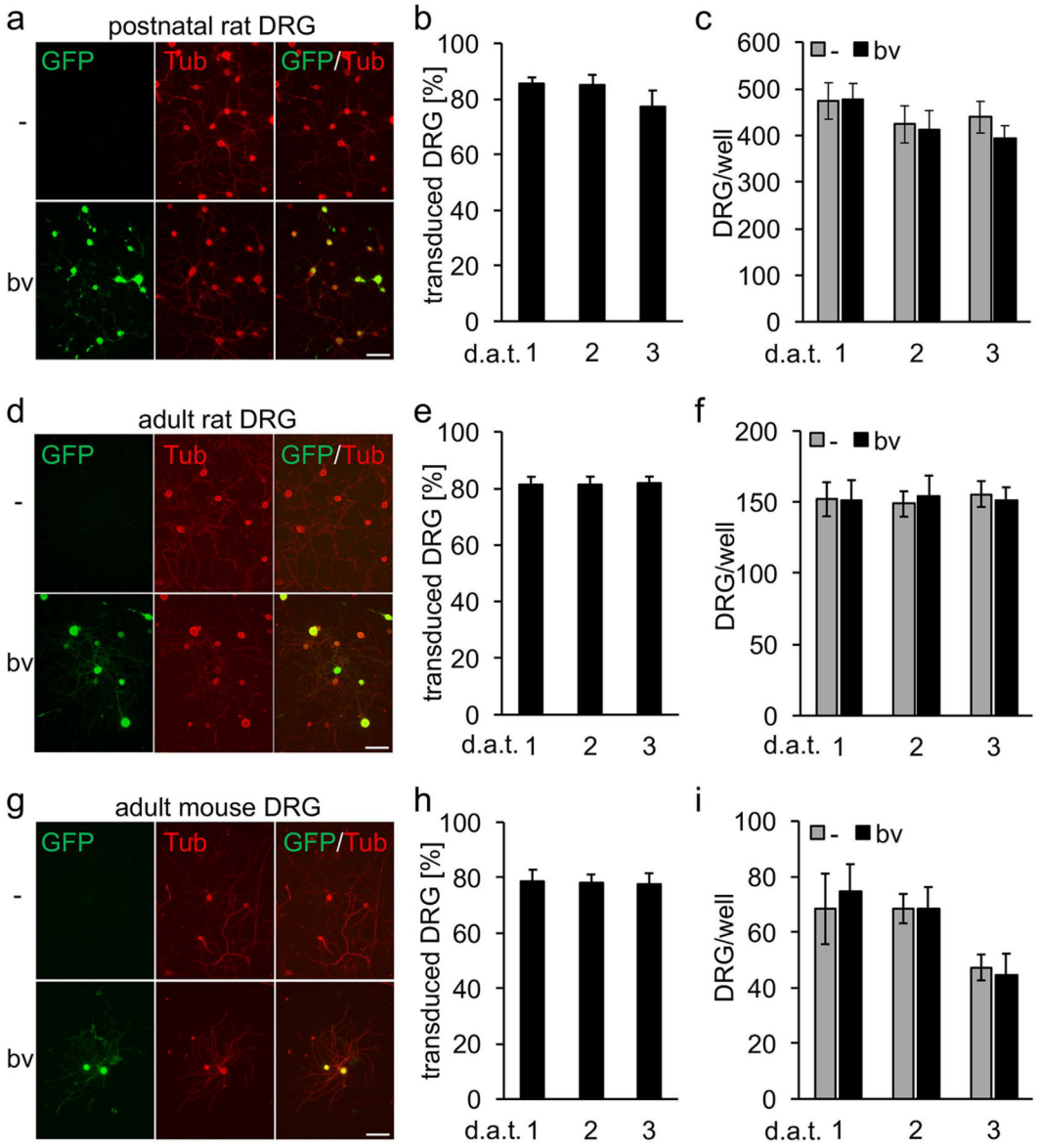

Figure 1. Transduction of dorsal root ganglion neurons. Dissociated dorsal root ganglion neurons (DRG) isolated from postnatal (**a**–**c**) or adult (**d**–**f**) rats and adult mice (**g**–**i**) were transduced with fGFP-baculovirus (bv). Representative pictures of vehicle (-) and bv -treated cultures (**a**,**d**,**g**) show transduced, GFP-expressing neurons (green) that were co-stained with the neuronal marker βIII-tubulin (Tub, red) at 2 days after transduction (d.a.t.). The percentage of transduced, GFP-expressing DRG neurons was determined at 1, 2 and 3 d.a.t. (**b**,**e**,**h**), revealing similar transduction efficiencies of 80–90% in postnatal and adult DRG neurons. Cell survival in postnatal (**c**) and adult (**f**, **i**) DRG cultures was not affected by bv-application compared to vehicle-treated controls (-). Scale bars: 100 μm.

the first report of efficient gene transfer into cultured adult mammalian RGCs. The fact that maximal transduction rates were only achieved delayed compared to DRG neurons suggest general slower induction of exogenous protein expression in RGCs.

Zebrafish RGCs have recently been established for the analyses of regeneration competent CNS neurons[22], but methods for their genetic manipulation are even more sparse than for mammalian neurons. As baculoviruses can reportedly transduce embryonic zebrafish cells *in vivo*[29], we also analyzed BacMam-mediated transduction of dissociated retinal cells isolated from adult zebrafish. Induction of DsRed transgene expression in zebrafish RGCs was considerably slower compared to mammalian RGCs and was detected in ~10% RGCs at 4 d.a.t. and ~20% at 6 d.a.t (Fig. 2g,h), without neurotoxic effects on cell counts. These varied proportions of transduced RGCs in rat, mice and zebrafish indicate species-specific differences in BacMam transduction rates. Some experimental approaches might favor gene transfer after extended culturing time, for example when neurons have already extended neurite processes. In order to test this possibility, BacMam virus was added to zebrafish RGCs after 4 days in culture (Fig. 2j). Remarkable, the transduction rate was with ~18% comparable to immediate virus

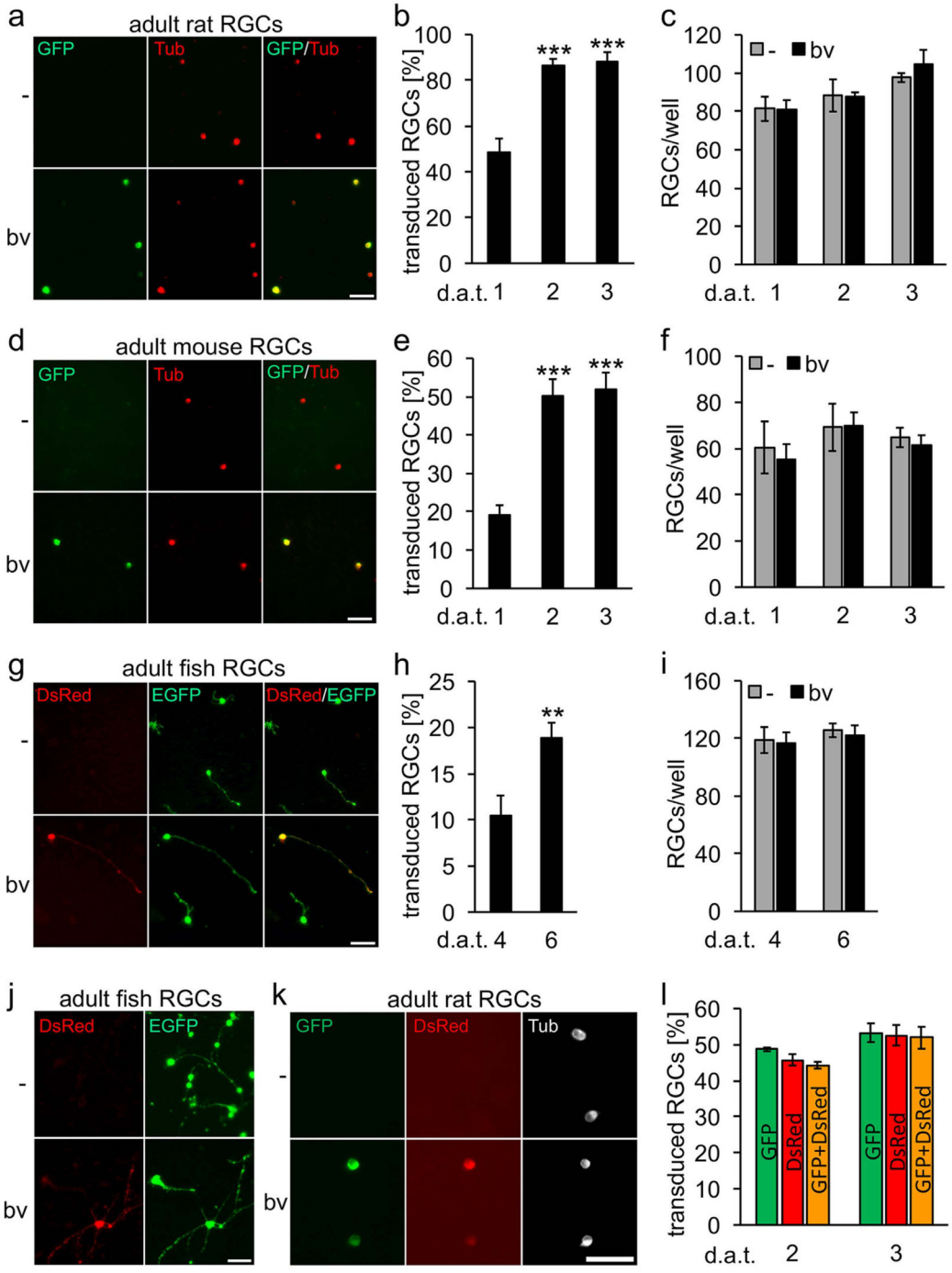

Figure 2. Transduction of mature retinal ganglion cells. Dissociated retinal ganglion cells (RGCs) isolated from adult rats (**a**–**c**), mice (**d**–**f**) and zebrafish (**g**–**i**) were transduced with baculovirus (bv) encoding fGFP (rat and mice) or DsRed (zebrafish). Representative pictures of vehicle- (-) and bv -treated cultures (**a**,**d**,**g**) visualize transduced RGCs (GFP and DsRed, respectively) that were either co-stained with the neuronal marker βIII-tubulin (Tub, red) at 2 days after transduction (d.a.t) for rat and mice RGCs (**a**,**d**) or identified by EGFP expression for zebrafish RGCs at 6 d.a.t. (**g**). The percentage of transduced RGCs was determined at 1, 2 and 3 days after transduction (d.a.t.) for rat and mice (**b**,**e**) and at 4 and 6 d.a.t. for zebrafish (**h**). Treatment effects compared to vehicle-treated controls: ***$p < 0.001$, **$p < 0.01$. Scale bars: 50 μm. RGC survival (**c**,**f**,**i**) was not affected by baculovirus application (bv) compared to vehicle-treated controls (-). (**j**) Delayed transduction of adult zebrafish RGCs with DsRed-bv after 4 days in culture. Scale bar: 50 μm. (**k**,**l**) Co-transduction of adult rat RGCs with fGFP-bv and DsRed-bv. The two viruses were added to retinal cultures simultaneously at half the concentration of single transductions. Representative pictures show co-transduced, fGFP- and DsRed-expressing RGCs (green and red, respectively) that were co-stained against the neuronal marker βIII-tubulin (white) at 2 d.a.t. Scale bar: 50 μm. (**k**). The percentage of transduced RGCs was determined at 2 and 3 d.a.t. (**l**). Non-significant difference in co-tranduction efficiency compared to single transductions.

application. Thus, neurons cannot only be efficiently transduced immediately after plating, but also several days later, leaving some leeway for the initiation of recombinant protein expression within a given experiment.

It is often desirable to induce expression of more than one heterologous protein in a neuron (e.g. the gene of interest and a fluorescent marker protein). Therefore, we also analyzed co-transductions with two BacMam viruses encoding different fluorescent marker proteins (GFP and DsRed) (Fig. 2k,l). Halving each virus concentration (~5×10^5 pfu each) compared to previous experiments, respective single transduction rates were expectedly reduced. Now, ~50% rat RGCs expressed either GFP or DsRed at 2 and 3 d.a.t. (Fig. 2l). As visible DsRed expression took longer to appear than for GFP, transduction rates were not analyzed at 1 d.a.t. in this experiment. Strikingly, almost every transduced RGC was double-positive for both marker proteins, indicating efficient and reliable co-transduction (Fig. 2k,l). All in all, adult RGCs across different species were readily transduced by BacMam viruses and the options of delayed gene transfer and virtual co-transduction might be advantageous for functional *in vitro* assays, facilitating novel experimental options.

Induction of cre-mediated recombination. In further experiments, we wanted to ensure the expression of functional heterologous proteins upon BacMam virus transduction using cre-mediated recombination and neurite outgrowth assays. The cre/loxP- system is widely used for targeted, conditional knockout experiments, which for one depends on efficient and timely cre recombinase expression, particularly in cell cultures. However, bv-mediated expression of cre recombinase in postnatal mouse striatal neurons was reportedly insufficient to induce genetic recombination, although the same construct was active in neuronal cell lines[30]. We therefore tested a BacMam virus encoding cre recombinase on adult DRG neurons isolated from ROSA-dtTomato mice. Expression of dtTomato indicates successful recombination upon induced enzymatic cre activity (Fig. 3a) and was detected in roughly 50% DRG neurons at 1 d.a.t., increasing to ~60% at day 3 (Fig. 3b). Therefore, cre-mediated recombination is quickly and efficiently initiated upon BacMam-transduction, which should enable the analysis of target gene-specific knockouts in primary neuronal cultures.

In separate experiments, we additionally quantified the transduced proportions of DRG subpopulations, which can be histologically classified by different marker expression (Fig. 3c,d). For instance, non-peptidergic small-diameter neurons bind isolectin B4 (IB4), while large-diameter neurons are immunoreactive for neurofilament heavy chain (NF). Overall, a transduction rate of ~70% was determined for all, βIII-tubulin-positive DRG neurons at 3 d.a.t. (Fig. 3d), which is comparable to transduction with GFP-encoding BacMam virus (Fig. 1). Similar (60–70%), not significantly different percentages were detected for NF- and IB4- positive neurons, indicating that different DRG subpopulations are equally efficiently transduced (Fig. 3d). Therefore, BacMam-mediated cre expression is functional in adult cultured neurons and does not show any preference for specific cell types.

Induction of hyper-IL6- mediated neurite growth. Application of the designer cytokine hyper-IL-6 (hIL6) activates the janus kinase/signal transducer and activator of transcription 3 (JAK/STAT3) pathway in cultured RGCs and markedly promotes their neurite growth[19,31]. We adapted this approach to further evaluate the functionality of heterologous proteins expressed upon BacMam-mediated gene delivery and to demonstrate the feasibility of assaying the role of a specific protein in established *in vitro* neurite outgrowth assays via viral-induced expression. To this end, DsRed-encoding control or hIL6-encoding BacMam virus was applied to mouse retinal cultures at 4 h after plating (Fig. 4). Quantification of mean RGC neurite length at 4 d.a.t revealed no significant difference for DsRed-bv compared to vehicle-treated cultures (Fig. 4a,b). This result, together with unchanged RGC numbers (Fig. 2f), confirmed neuronal tolerance for BacMam transduction. Expression of the designer cytokine hIL6 was expectedly detected in transduced, GFP-positive RGCs, but not in control cultures (Fig. 4c). Functional activity of hIL6 was established through increased phosphorylation of STAT3 in transduced RGCs (Fig. 4d). In addition, RGC neurite growth was ~6-fold longer upon hIL6 expression compared to DsRed-bv-transduced control RGCs (Fig. 4e,f). Again, no neurotoxicity was observed, as RGC survival was similar for both BacMam viruses (Fig. 4g). Therefore, active, recombinant proteins can be swiftly and efficiently expressed in adult neurons upon BacMam transduction, enabling versatile overexpression and knockdown studies in cultured neurons in the future.

Discussion

The current study provides a highly efficient, reliable and fast method to transfer recombinant DNA into adult primary neurons directly in culture. Although transduction/transfection of embryonic and postnatal neurons by various methods has been described previously, respective reports for primary adult neurons are still missing. Consistently, our own experience has shown that most approaches are not straightforwardly transferred to adult neurons. However, research aspects relating to the adult CNS or PNS are best examined using mature neurons. One example is regenerative axonal growth, which crucially depends on neuronal age[32,33]. Using the recombinant BacMam system, we could now demonstrate for the first time that mature neurons from the PNS (DRG neurons) as well as from the CNS (RGCs) of various species can be readily transduced with high efficiency in culture. Highly reproducible transduction rates of ~80% were detected for rat and mice adult DRG neurons and different neuronal subpopulations were transduced to similar extent without obvious preferences. In comparison, some species specificity was observed for RGCs. Whereas transduction rates of $\geq$ 80% were also observed for rat RGCs, ~50% mouse RGCs were transduced with the same viral stock under comparable culture conditions. Although it might be possible to further increase this percentage by applying higher MOIs, the achieved efficiency was more than adequate for our *in vitro* experiments. In fact, assay interpretation might be even more reliable if transduced and untransduced cells can be evaluated in the same well. Lowest efficiency of ~20% was established for zebrafish RGCs, which would still be sufficient to quantify the impact of heterologous protein expression[22,28]. In fact, this is to our knowledge the first report of successful gene transfer into primary zebrafish neurons in culture. All in all,

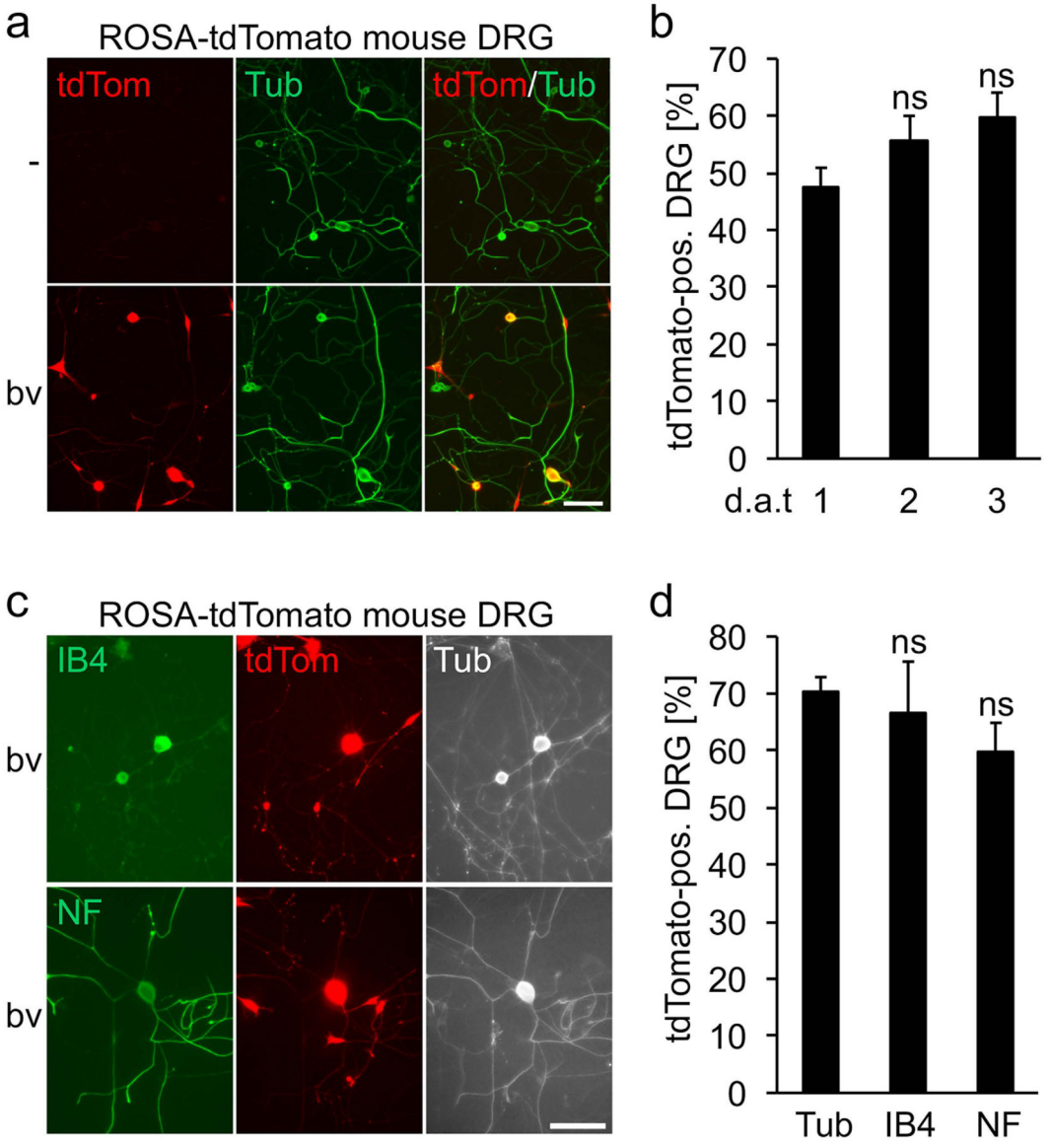

Figure 3. Induction of cre-mediated recombination. Adult dorsal root ganglion neurons (DRG) isolated from ROSA-tdTomato mice were transduced with baculovirus encoding cre recombinase (cre-bv) (**a**–**d**). Representative pictures of cre-bv-treated cultures (**a**,**c**) show transduced, tdTomato-expressing DRG (red) that were co-stained against the neuronal markers (green) βIII-tubulin (Tub) (**a**), neurofilament- (NF) and isolectine B4- (IB4) (**c**) at 3 days after transduction (d.a.t). The percentage of transduced cells in relation to βIII-tubulin was determined at 1, 2 and 3 d.a.t. with non-significant (ns) differences compared to 1 d.a.t. (**b**). In addition, the proportion of transduced NF- and IB4- positive DRG subpopulations was determined (**d**), revealing non-significant (ns) differences in transduction efficiencies compared to βIII-tubulin-positive neurons Scale bars: 100 μm.

unexpectedly high gene transfer rates were observed for all tested neurons, although variability between neuronal types and between species was noted, which may require individual pre-testing in new experimental settings.

Transduction of primary adult neurons was straightforward and simple, as viral inoculum was just added directly to neuronal cultures. Surprisingly, high transduction rates were observed without any application of histone deacetylase inhibitors such as butyrate, which is often used to increase baculoviral gene transfer and recombinant protein expression[10,12]. As this treatment was deemed unnecessary for our purposes, inhibitor-induced toxicity was avoided. Generally, baculoviruses are known for their low cytotoxicity and superior biosafety profile. Accordingly, numbers of surviving neurons in our cultures did not change over time compared to untreated controls. However, a partial change of culture medium at 10–20 h after virus application was essential to avoid a partial loss of cells. In addition, progressive loss of neurite processes was noted upon strong fGFP expression, as DRG axons became fragmented after 3 days in culture. Potentially, extensive intercalation of fGFP into the plasma membrane was detrimental to cultured neurons. This is consistent with previously reported EGFP-induced apoptosis[34], but was not observed upon monomeric DsRed expression. Nevertheless, MOI rates were reduced for all outgrowth assays in our study to elude any potential negative side effects.

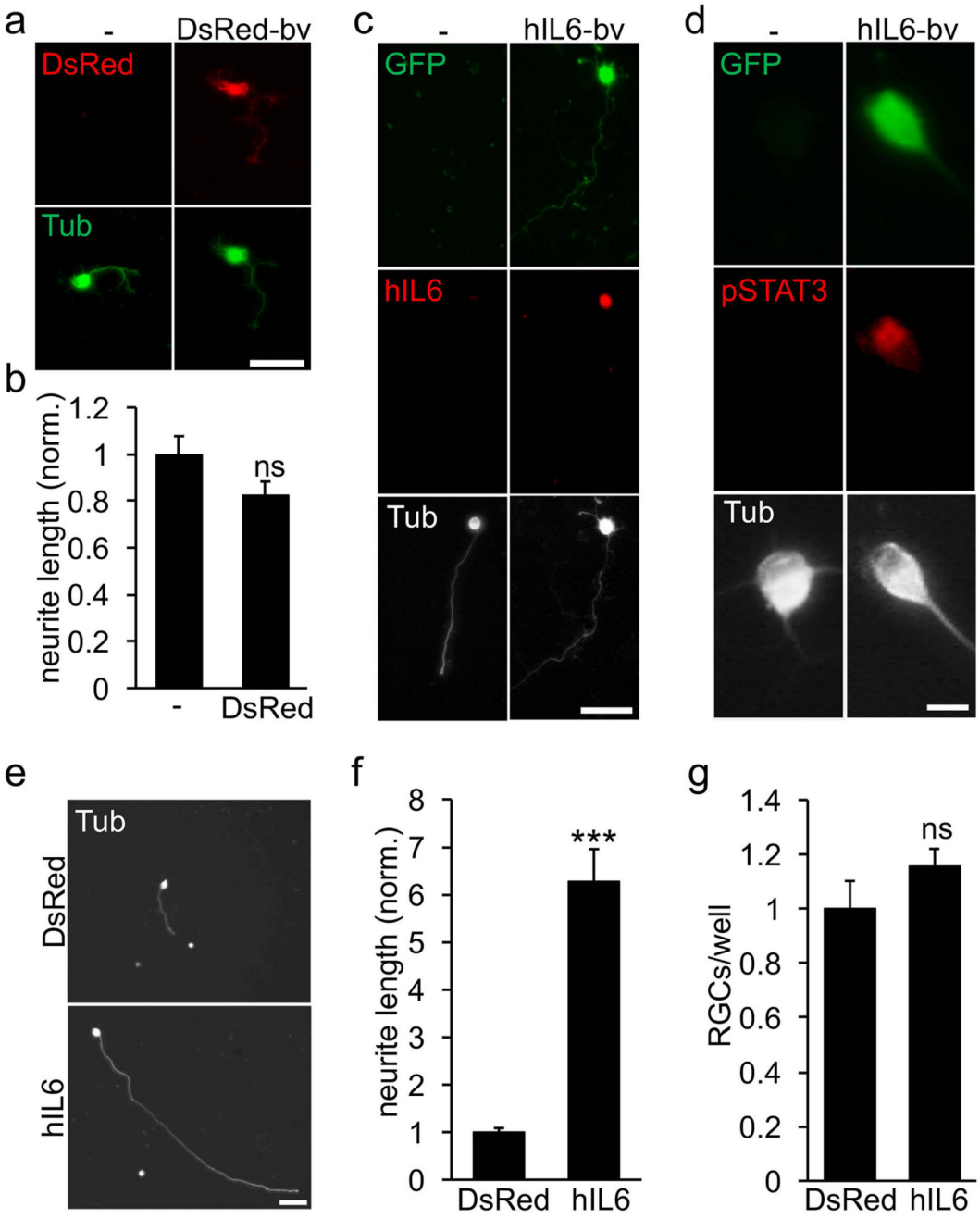

Figure 4. Induction of hIL6- mediated neurite growth. Adult mouse retinal cells were transduced either with control DsRed-bv (**a**,**b**,**e**–**g**) or hIL6-bv (baculovirus encoding hIL6 and EGFP) (**c**–**g**). (**a**) Representative pictures show vehicle- (-) and DsRed-bv-transduced retinal ganglion cells (RGCs) stained with the neuronal marker βIII-tubulin (Tub, green) at 4 days after transduction (d.a.t.). (**b**) Quantification of mean neurite length normalized to vehicle-treated controls (-) with an average neurite length of 20.3 μm/RGC reveal no generalized effect of control baculovirus on neurite outgrowth. Representative pictures of vehicle- (-) and hIL6-bv-treated RGCs at 4 d.a.t. (**c**,**d**) show expression of hIL6 (**c**) and induction of STAT3 phosphorylation (**d**) in transduced, GFP-expressing (green) RGCs that were co-stained against βIII-tubulin (white). Representative pictures of RGC neurite growth upon DsRed-bv or hIL6-bv transduction (**e**) and quantification of mean neurite length normalized to DsRed-bv-transduced RGCs with an average value of 23.1 μm/RGC (**f**) at 4 d.a.t. illustrate ~6-fold longer, βIII-tubulin-positive neurites upon hIL6 expression. Treatment effect: ***$p < 0.001$ (**g**) RGC survival was similar for both baculoviruses. ns = non-significant. Scale bars: 50 μm in a, c and e; 10 μm in d.

In accordance with the apparent indiscrimination of baculovirus hosts, transgene expression was also detected in some non-neuronal cells in our mixed cultures. However, their proportion was rather low, potentially due to the fact that our culture conditions favored the survival of neurons. Their presence did not interfere with assay evaluation, as they were clearly distinguishable upon neuronal marker staining. Alternatively, neuron-specific

regulatory elements could be inserted into the viral vectors to drive gene expression exclusively in neurons[25,35]. Fluorescent marker expression became already evident in rat and mice neurons within a few hours after transduction, although DsRed took slightly longer and maximum transduction levels required 2 days in RGCs. Nevertheless, this time course is considerably faster compared to LV-transduction, which takes 2–5 days in rat DRG neurons[36] and is even slower for AAVs[2]. Due to the limited survival time of most mature neurons in culture, slow induction of transgene expression might, however, not leave enough time to evaluate certain parameters, such as effects on neurite growth. For this reason, AAVs are generally not applied *in vitro*, but are injected *in vivo* several weeks prior to culture preparation[27]. Depending on the experimental setting, it might, however, be advantageous to analyze the immediate effects of heterologous protein expression without prolonged pre-treatment, as this could already impact neural physiology. This approach is now possible using recombinant BacMam viruses. Exemplary, we confirmed neurite growth-promotion upon BacMam-mediated hIL6 expression in cultured RGCs. Overall, this protocol is considerably more time- and cost-efficient compared to *in vivo* AAV transduction and achieved at least similar, if not better growth promotion (compare to ref. 19). The early onset of gene expression also enabled cre-mediated genome editing *in vitro*. In contrast to a previously reported unsuccessful attempt[30], we observed fast and efficient recombination in adult neurons. Thus, conditional gene knockout studies are possible with this approach, considerably extending experimental capabilities in culture.

Construction of recombinant BacMam virus is relatively easy, mostly requiring only a biosafety level 1 facility. Either purified virus or infected cells can be stored almost indefinitely[37] and high titers can be recurrently and reproducibly produced simply by re-infection of insect cells, hence providing ongoing supply at low effort. Another advantage is the large cloning capacity of baculovirus (up to 38 kbp) compared to LV- and AAV-vectors, facilitating expression of larger proteins and even multicomponent protein complexes, for example for crispr/cas-mediated genome engineering[10,12,13]. Alternatively, co-expression of several cDNAs can be achieved upon baculovirus co-transduction. Conveniently, virtually the same neurons were transduced upon application of two BacMam viruses, similar to previously described AAV-mediated transduction *in vivo*[38]. The prevalently transient nature of BacMam-mediated expression should not pose a drawback for neurons and avoids the risk of insertional mutations. Most assays using primary neurons are anyway completed within a few days. In addition, BacMam-mediated expression might even persist longer in differentiated neurons compared to mitotic cells, as cell-division-induced dilution does not occur. Accordingly, no decline in marker expression was detected up to 8 days in zebrafish RGC cultures (data not shown). Alternatively, transgene expression might be extended by means of BacMam re-application at later stages[39], as neurite-bearing neurons were also readily transduced. All in all, we consider the efficient BacMam-mediated gene transfer into cultured neurons a very versatile approach for a wide variety of applications in neurobiology research.

References

1. Zeitelhofer, M. *et al.* High-efficiency transfection of mammalian neurons via nucleofection. *Nat Protoc* **2,** 1692–1704, doi: 10.1038/nprot.2007.226 (2007).
2. Karra, D. & Dahm, R. Transfection techniques for neuronal cells. *J Neurosci* **30,** 6171–6177, doi: 10.1523/jneurosci.0183-10.2010 (2010).
3. Ding, B. & Kilpatrick, D. L. Lentiviral vector production, titration, and transduction of primary neurons. *Methods in molecular biology* **1018,** 119–131, doi: 10.1007/978-1-62703-444-9_12 (2013).
4. Gascon, S., Paez-Gomez, J. A., Diaz-Guerra, M., Scheiffele, P. & Scholl, F. G. Dual-promoter lentiviral vectors for constitutive and regulated gene expression in neurons. *Journal of neuroscience methods* **168,** 104–112, doi: 10.1016/j.jneumeth.2007.09.023 (2008).
5. Hutson, T. H. *et al.* Lentiviral vectors encoding short hairpin RNAs efficiently transduce and knockdown LINGO-1 but induce an interferon response and cytotoxicity in central nervous system neurones. *The journal of gene medicine* **14,** 299–315, doi: 10.1002/jgm.2626 (2012).
6. Fleming, J. *et al.* Adeno-associated virus and lentivirus vectors mediate efficient and sustained transduction of cultured mouse and human dorsal root ganglia sensory neurons. *Hum Gene Ther* **12,** 77–86, doi: 10.1089/104303401450997 (2001).
7. Royo, N. C. *et al.* Specific AAV serotypes stably transduce primary hippocampal and cortical cultures with high efficiency and low toxicity. *Brain Res* **1190,** 15–22, doi: 10.1016/j.brainres.2007.11.015 (2008).
8. Kost, T. A. & Condreay, J. P. Recombinant baculoviruses as mammalian cell gene-delivery vectors. *Trends in biotechnology* **20,** 173–180 (2002).
9. Airenne, K. J. *et al.* Baculovirus: an insect-derived vector for diverse gene transfer applications. *Mol Ther* **21,** 739–749, doi: 10.1038/mt.2012.286 (2013).
10. Hu, Y. C. Baculovirus as a highly efficient expression vector in insect and mammalian cells. *Acta pharmacologica Sinica* **26,** 405–416, doi: 10.1111/j.1745-7254.2005.00078.x (2005).
11. Barsoum, J., Brown, R., McKee, M. & Boyce, F. M. Efficient transduction of mammalian cells by a recombinant baculovirus having the vesicular stomatitis virus G glycoprotein. *Hum Gene Ther* **8,** 2011–2018, doi: 10.1089/hum.1997.8.17-2011 (1997).
12. Kost, T. A., Condreay, J. P. & Jarvis, D. L. Baculovirus as versatile vectors for protein expression in insect and mammalian cells. *Nat Biotechnol* **23,** 567–575, doi: 10.1038/nbt1095 (2005).
13. Mansouri, M. *et al.* Highly efficient baculovirus-mediated multigene delivery in primary cells. *Nature communications* **7,** 11529, doi: 10.1038/ncomms11529 (2016).
14. Haeseleer, F., Imanishi, Y., Saperstein, D. A. & Palczewski, K. Gene transfer mediated by recombinant baculovirus into mouse eye. *Invest Ophthalmol Vis Sci* **42,** 3294–3300 (2001).
15. Wang, X. *et al.* Gene transfer to dorsal root ganglia by intrathecal injection: effects on regeneration of peripheral nerves. *Mol Ther* **12,** 314–320, doi: 10.1016/j.ymthe.2005.03.032 (2005).
16. Turunen, T. A., Laakkonen, J. P., Alasaarela, L., Airenne, K. J. & Yla-Herttuala, S. Sleeping Beauty-baculovirus hybrid vectors for long-term gene expression in the eye. *The journal of gene medicine* **16,** 40–53, doi: 10.1002/jgm.2756 (2014).
17. Udvadia, A. J. 3.6 kb genomic sequence from Takifugu capable of promoting axon growth-associated gene expression in developing and regenerating zebrafish neurons. *Gene expression patterns : GEP* **8,** 382–388, doi: 10.1016/j.gep.2008.05.002 (2008).
18. Westerfield, M. *The zebrafish book: a guide for the laboratory use of zebrafish (Brachydanio rerio)*. Eugene: University of Oregon. (1989).
19. Leibinger, M. *et al.* Boosting Central Nervous System Axon Regeneration by Circumventing Limitations of Natural Cytokine Signaling. *Mol Ther*, doi: 10.1038/mt.2016.102 (2016).

20. Fischer, D. Hyper-IL-6: a potent and efficacious stimulator of RGC regeneration. *Eye (Lond).* 2016 Nov 25. doi: 10.1038/eye.2016.234. [Epub ahead of print].
21. Gobrecht, P., Leibinger, M., Andreadaki, A. & Fischer, D. Sustained GSK3 activity markedly facilitates nerve regeneration. *Nature communications* **5,** 4561, doi: 10.1038/ncomms5561 (2014).
22. Grozdanov, V., Muller, A., Sengottuvel, V., Leibinger, M. & Fischer, D. A method for preparing primary retinal cell cultures for evaluating the neuroprotective and neuritogenic effect of factors on axotomized mature CNS neurons. *Current protocols in neuroscience/editorial board, Jacqueline N. Crawley … [et al.]* Chapter 3, Unit3 22, doi: 10.1002/0471142301.ns0322s53 (2010).
23. Diekmann, H., Kalbhen, P. & Fischer, D. Characterization of optic nerve regeneration using transgenic zebrafish. *Frontiers in cellular neuroscience* **9,** 118, doi: 10.3389/fncel.2015.00118 (2015).
24. Sarkis, C. *et al.* Efficient transduction of neural cells *in vitro* and *in vivo* by a baculovirus-derived vector. *Proc Natl Acad Sci USA* **97,** 14638–14643, doi: 10.1073/pnas.260472897 (2000).
25. Tani, H. *et al. In vitro* and *in vivo* gene delivery by recombinant baculoviruses. *Journal of virology* **77,** 9799–9808 (2003).
26. Li, Y., Wang, X., Guo, H. & Wang, S. Axonal transport of recombinant baculovirus vectors. *Mol Ther* **10,** 1121–1129, doi: 10.1016/j.ymthe.2004.08.018 (2004).
27. Wang, J. *et al.* Efficient transduction of spiral ganglion neurons *in vitro* by baculovirus vectors. *Neuroreport* **18,** 1329–1333, doi: 10.1097/WNR.0b013e3282010b16 (2007).
28. Leibinger, M., Andreadaki, A., Diekmann, H. & Fischer, D. Neuronal STAT3 activation is essential for CNTF- and inflammatory stimulation-induced CNS axon regeneration. *Cell Death Dis* **4,** e805, doi: 10.1038/cddis.2013.310 (2013).
29. Diekmann, H., Kalbhen, P. & Fischer, D. Active mechanistic target of rapamycin plays an ancillary rather than essential role in zebrafish CNS axon regeneration. *Frontiers in cellular neuroscience* **9,** 251, doi: 10.3389/fncel.2015.00251 (2015).
30. Wagle, M. & Jesuthasan, S. Baculovirus-mediated gene expression in zebrafish. *Marine biotechnology* **5,** 58–63, doi: 10.1007/s10126-002-0050-9 (2003).
31. Erbs, E., Pradhan, A. A., Matifas, A., Kieffer, B. L. & Massotte, D. Evaluation of cre recombinase delivery in mammalian cells using baculovirus infection. *Journal of biotechnology* **166,** 182–186, doi: 10.1016/j.jbiotec.2013.05.011 (2013).
32. Schwab, M. E. & Bartholdi, D. Degeneration and regeneration of axons in the lesioned spinal cord. *Physiological reviews* **76,** 319–370 (1996).
33. Goldberg, J. L., Klassen, M. P., Hua, Y. & Barres, B. A. Amacrine-signaled loss of intrinsic axon growth ability by retinal ganglion cells. *Science* **296,** 1860–1864, doi: 10.1126/science.1068428 (2002).
34. Detrait, E. R. *et al.* Reporter gene transfer induces apoptosis in primary cortical neurons. *Mol Ther* **5,** 723–730, doi: 10.1006/mthe.2002.0609 (2002).
35. Wang, C. Hybrid baculovirus-adeno-associated virus vectors for prolonged transgene expression in human neural cells. *Journal of neurovirology* **14,** 563–568, doi: 10.1080/13550280802290606 (2008).
36. Yu, H. *et al.* Lentiviral gene transfer into the dorsal root ganglion of adult rats. *Molecular pain* **7,** 63, doi: 10.1186/1744-8069-7-63 (2011).
37. Wasilko, D. J. *et al.* The titerless infected-cells preservation and scale-up (TIPS) method for large-scale production of NO-sensitive human soluble guanylate cyclase (sGC) from insect cells infected with recombinant baculovirus. *Protein expression and purification* **65,** 122–132, doi: 10.1016/j.pep.2009.01.002 (2009).
38. Wu, D. *et al.* Expressing Constitutively Active Rheb in Adult Neurons after a Complete Spinal Cord Injury Enhances Axonal Regeneration beyond a Chondroitinase-Treated Glial Scar. *J Neurosci* **35,** 11068–11080, doi: 10.1523/JNEUROSCI.0719-15.2015 (2015).
39. Makkonen, K. E., Airenne, K. & Yla-Herttulala, S. Baculovirus-mediated gene delivery and RNAi applications. *Viruses* **7,** 2099–2125, doi: 10.3390/v7042099 (2015).

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Author Contributions

D.F., H.D. and E.L. designed the project. E.L. and H.D. performed the experiments. D.F., E.L. and H.D. analyzed the data. D.F. supervised the research; D.F., E.L. and H.D. wrote the paper.

Additional Information

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