Interplay between the dopaminergic system and the extracellular matrix in synaptic plasticity

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Summary

The most challenging task of our brain is to keep the balance between tenacity and plasticity. On the one hand, it is necessary that the brain networks possess a high degree of plasticity to be able to process new information. On the other hand, the brain needs the ability to stabilize structures for long-term information storage and memory formation. During development synapses and neuronal networks get stabilized over time which is paralleled by the maturation of the extracellular matrix (ECM). The brain's ECM is formed by glia cells as well as neurons, enwraps and stabilizes synapses and is well-known as a key player in diverse plasticity processes, including learning and memory formation, as shown e.g. in mouse mutants lacking key ECM molecules. In turn, the activity status of a given neuronal network seems to induce remodeling of ECM structures to allow plastic restructuring, but the molecular mechanisms underlying this remodeling are largely unknown. The neuromodulator dopamine (DA) is a potent modulator of motivated learning processes, acting through five distinct but closely related G protein-coupled receptors (D1-D5). It was shown to enhance learning performance, e.g. in an auditory task in Mongolian gerbils when D1-like receptors were activated. Enzymatic degradation of the ECM has also been shown to enhance learning performance of Mongolian gerbils in a frequency-modulated (FM) discrimination task and to restore juvenile-like structural plasticity. Moreover, stimulation of D1-like DA receptors was found to increase extracellular activity of the serine protease tPA (tissue-type plasminogen activator) being able to restructure the ECM. Therefore, I hypothesized that pharmacological stimulation of D1-like DA receptors will lead to an activation of ECM-modifying proteases, especially ADAMTS 4 and ADAMTS 5, and a restructuring of the ECM, thus contributing to synaptic plasticity. I investigated the most abundant chondroitin sulfate proteoglycans (CSPGs) of the lectican family in the mature ECM- brevican (BC) and aggrecan (Acan). Indeed, I could show that systemic activation of D1-like DA receptors with the D1 receptor agonist SKF38393 results in enhanced BC and Acan cleavage in synaptosomal fractions of rat prefrontal cortex.

To shed light on the underlying molecular mechanism, I performed *in vitro* experiments using rat dissociated cortical cultures at DIV21 when the ECM is considered to be mature. I was able to confirm the obtained *in vivo* results. Furthermore, I could demonstrate that BC and Acan cleavage appears only at excitatory synapses. CSPGs of the

lectican family, especially BC and Acan, are well known targets for enzymes of the ADAMTS family of proteases. Here, I could demonstrate in a knockdown approach that ADAMTS 4 as well as ADAMTS 5 are essential for DA-dependent cleavage of BC. Increased perisynaptic BC cleavage upon D1-like DA receptor activation is based on network activity and activity of postsynaptic sites, since sodium channels and NR2B-containing NMDARs are involved in the signalling. Furthermore, using optogenetic and pharmacological tools I could show that D1-like DA receptor-induced perisynaptic BC cleavage requires a co-signalling through PKA and CaMKII.

Taken together, the results of this thesis contribute to a further understanding of potential molecular mechanisms of synaptic ECM restructuring in DA-dependent processes. Furthermore, they provide a better understanding of DA-dependent remodeling of mature ECM under physiological conditions due to activation of ADAMTS 4 and ADAMTS 5. Interestingly, molecules of the ECM as well as their cell surface receptors, cell adhesion molecules (CAMs) and ECM-modifying proteases are entangled in processes related to major brain diseases such as Alzheimer's and Parkinson's disease, or schizophrenia and epilepsy. Therefore, the here identified ECM-modifying proteases as well as proteolysis-derived ECM fragments and their appropriate cell surface receptors could serve as potential therapeutic targets to alleviate symptoms of e.g. neurodegenerative diseases.

Zusammenfassung

Eine der wohl anspruchsvollsten Aufgaben unseres Gehirns ist es, eine Balance zwischen Stabilität und Plastizität zu wahren. Auf der einen Seite ist es wichtig, dass das Gehirn einen hohen Grad an Plastizität aufweist, um die Möglichkeit zu haben neue Informationen zu prozessieren. Aufgrund von Informationsspeicherung und Gedächtnisbildung benötigt das Gehirn andererseits die Möglichkeit Strukturen und synaptische Verbindungen zu stabilisieren. Während der Entwicklung werden Synapsen und ganze neuronale Netzwerke über die Zeit stabilisiert. Dieser Prozess geht einher mit der Heranreifung der Extrazellulären Matrix (EZM). Die EZM des Gehirns wird sowohl durch Gliazellen als auch Neuronen geformt, umgibt und stabilisiert Synapsen und ist als einer der Hauptakteure in Plastizitätsprozessen und damit für Lernen und Gedächtnis bekannt. Dies wurde zum Beispiel in Mausmutanten, denen zentrale EZM-Moleküle fehlten, gezeigt. Der Aktivitätsstatus eines bestimmten neuronalen Netzwerkes wiederum scheint den Umbau von EZM-Strukturen zu begünstigen und damit plastische Umstrukturierungen zu erlauben. Allerdings sind die zugrundeliegenden molekularen Mechanismen weitesgehend unbekannt. Der Neuromodulator Dopamin (DA), welcher mittels fünf unterschiedlicher, aber sehr nah verwandter, G protein-gekoppelter Rezeptoren (D1-D5) im Gehirn agiert, ist wichtig für Motivations-basierte Lernprozesse. So wurde zum Beispiel gezeigt, dass in einer auditorischen Aufgabe mongolische Wüstenrennmäuse durch Aktivierung D1-ähnlicher DA Rezeptoren ein besseres Lernverhalten zeigen als die Kontrollgruppe. Basierend auf dieser Studie wurde in dieser Arbeit das mögliche Zusammenspiel zwischen dopaminergem System und der EZM des Gehirns, sowie ihr Einfluss auf synaptische Plastizität näher untersucht. Der enzymatische Abbau der EZM trägt zu einer verbesserten Lernleistung in einer FM Diskriminierungsaufgabe in Mongolischen Wüstenrennmäusen bei und besitzt die Fähigkeit eine juvenil-ähnliche strukturelle Plastizität wiederherzustellen. Darüber hinaus wurde gezeigt, dass die Aktivierung D1-ähnlicher DA Rezeptoren die extrazelluläre Aktivität der Serinprotease tPA (Gewebespezifischer Plasminogenaktivator), welche möglicherweise fähig ist die EZM umzubauen, erhöht. Daraus folgend vermute ich, dass eine chemische Stimulierung D1-ähnlicher Rezeptoren zu einer Aktivierung EZM-modifizierender Proteasen, wie zum Beispiel ADAMTS 4 und ADAMTS 5, und zu einem daraus folgenden Umbau der EZM, sowie zu synaptischer Plastizität führt. Dafür habe ich die am häufigsten exprimierten Chondroitinsulfatproteoglykane (CSPGs) der Lectican-Familie im adulten Ge-

hirn Brevican (BC) und Aggrecan (Acan) untersucht. Tatsächlich konnte ich im Präfrontalen Cortex von Ratten sowohl eine erhöhte BC- als auch Acan-Spaltung nach systemischer Aktivierung D1-ähnlicher DA Rezeptoren mittels des D1 Rezeptor Agonisten SKF38393 in der Synaptosomenfraktion feststellen. Um Aufschluss über den zugrundeliegenden molekularen Mechanismus zu erlangen, habe ich *in vitro* Experimente in dissoziierten kortikalen Rattenkulturen an Tag 21 *in vitro* (DIV21) durchgeführt. Zu diesem Zeitpunkt ist die EZM vollständig entwickelt. Zunächst konnte ich die im *in vivo* Experiment erhaltenen Resultate bestätigen. Des Weiteren gelang es mir aufzuzeigen, dass die Spaltung der Lecticane BC und Acan ausschließlich an exzitatorischen Synapsen auftritt. CSPGs der Lectican-Familie, insbesondere BC und Acan, sind bekannte Zielmoleküle der Enzyme der ADAMTS-Familie. In dieser Arbeit konnte ich durch die Methode des Knockdowns zeigen, dass ADAMTS 4 und ADAMTS 5 essentiell für die DA-abhängige BC-Spaltung sind. Diese perisynaptische BC-Spaltung ist nicht nur abhängig von der Aktivität des gesamten Netzwerkes, sondern auch von postsynaptischer Aktivität, da sowohl Natriumkanäle als auch NR2B-containing NMDARs am Signalweg beteiligt sind. Zudem konnte ich unter zu Hilfenahme optogentischer und pharamkologischer Werkzeuge zeigen, dass die perisynaptische BC-Spaltung, welche durch Aktivierung D1-ähnlicher DA Rezeptoren induzierbar ist, einen Co-Signalweg mittels PKA und CaMKII benötigt.

Die in dieser Arbeit erhaltenen Resultate tragen zu einem besseren Verständnis potentieller molekularer Mechanismen hinsichtlich eines Umbaus der synaptischen EZM in DA-abhängigen Prozessen bei. Weiterhin bekommt man einen Eindruck der Umstrukturierung der gereiften EZM aufgrund der Aktivierung der EZM-modifizierenden Proteasen ADAMTS 4 und ADAMTS 5 basierend auf der Stimulierung D1-ähnlicher DA Rezeptoren. Interessanterweise scheinen Moleküle der EZM, ebenso wie ihr Zelloberflächenrezeptoren, Zelladhäsionsmoleküle und EZM-modifizeirende Proteasen in Prozesse involviert zu sein, welche in Verbindung mit den Haupterkrankungen des Gehirns stehen, wie zum Beispiel Alzheimer und Parkinson oder Schizophrenie und Epilepsie. Somit könnten die hier identifizierten Proteasen, sowie ihre generierten Fragmente und deren mögliche Zelloberflächenrezeptoren potentielle therapeutische Ziele darstellen, um Symptome neurodegenerativer Erkrankungen zu mildern.

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Abbreviations

AC	adenylate cyclase
Acan	aggrecan
ADAM	A disintegrin and metalloproteinase
ADAMTS	A disintegrin and metalloproteinase with thrombospondin motifs
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
AP-5	(2R)-amino-5-phosphonovaleric acid
BC	brevican
bPAC	bacterial photo-activatable adenylyl cyclase
Bral1	brain-specific link protein 1
CaMKII	Ca^{2+}/Calmodulin-dependent protein kinase II
cAMP	cyclic adenosine monophosphate
CEMIP	cell migration-inducing protein
ChABC	Chondroitinase ABC
CNS	central nervous system
CREB	cAMP response element-binding protein
CSPG	chondroitin sulfate proteoglycan
Ctl	control
DA	dopamine
D1R	D1 dopamine receptor
D2R	D2 dopamine receptor
DIV	days in vitro
ECM	extracellular matrix
E-LTP	early phase long-term potentiation
FM	frequency modulation
GABA	γ-aminobutyric acid
GAD65	glutamate decarboxylase 65
GPCR	G-protein-coupled receptor
HAPLN	hyaluronan and proteoglycan link protein
HA	hyaluronic acid
Ifen	Ifenprodil
KO	knock out
LED	light-emitting diode
LGI1	leucine-rich, glioma inactivated 1
LRP-1	low-density lipoprotein-related protein 1
LTD	long-term depression
LTP	long-term potentiation
L-LTP	late phase long-term potentiation
MAP2	microtubule-associated protein 2
MAPK	mitogen-activated protein kinase
MEK	mitogen-activated protein kinase kinase
mGluR	metabotropic glutamate receptor

MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
NAc	nucleus accumbens
Ncan	neurocan
NMDAR	N-methyl-D-aspartic acid receptor
NR2B	N-methyl-D-aspartic acid receptor subtype 2B
PACE4	furin/paired basic amino acid-cleaving enzyme 4
PC 5/6	prohormone convertase 5/6
PKA	protein kinase A
PKC	protein kinase C
PNN	perineuronal net
PPC	proprotein convertase
PSD95	postsynaptic density protein 95
PV	parvalbumin
rAAV	recombinant adeno-associated virus
RT	room temperature
SCI	spinal cord injury
shRNA	small hairpin ribonucleic acid
SN	substantia nigra
TARP	transmembrane AMPA receptor regulatory protein
TGFβ	transforming growth factor β
TIMP3	tissue inhibitor of metalloproteinase 3
TMEM2	transmembrane protein 2
TNC	tenascin C
TNR	tenascin R
tPA	tissue-type plasminogen activator
TSR	thrombospondin type 1 sequence repeat
TTX	Tetrodotoxin
unpubl.	unpublished
Vcan	versican
VGCC	voltage-gated calcium channel
VMAT2	vesicular monoamine transporter 2
VTA	ventral tegmental area
WT	wild type

1 Introduction

1.1 Synaptic Plasticity

It is well known that synapses, the small connections between neurons, are able to respond to activity changes with strengthening or weakening over time. This adaptational process is called synaptic plasticity (reviewed in (Hughes, 1958)). Based on the *Hebbian Theory*, synaptic plasticity is a fundamental cellular mechanism underlying learning and memory. To achieve synaptic plasticity which depends on postsynaptic calcium release several molecular mechanisms need to cooperate, such as the amount of presynaptically released neurotransmitter and the ability of the postsynaptic cell to respond to it (Gaiarsa et al., 2002; Gerrow and Triller, 2010). Synapses perform those plastic changes often by altering the number of receptors located in a synapse (Gerrow and Triller, 2010). On a short timescale of tens of milliseconds up to a few minutes short-term synaptic plasticity acts to either strengthen or weaken synapses (reviewed in (Zucker and Regehr, 2002)). However, on a longer timescale two major forms of long-term synaptic plasticity have been identified: long-term potentiation (LTP) and long-term depression (LTD) which can last for minutes to hours or even days (Gerrow and Triller, 2010).

In general, LTD reduces the efficacy of synapses for hours or longer in an activity-dependent way. This process is necessary to weaken specific synapses to benefit from synaptic strengthening which is caused by LTP (reviewed in (Massey and Bashir, 2007)). Typically, homosynaptic LTD being restricted to single synapses and activated by a low frequency stimulus needs not only the activation of NMDA receptors (NMDARs), but also postsynaptic Ca^{2+} influx and a phosphatase cascade that needs to be activated (Mulkey and Malenka, 1992; Mulkey et al., 1993, 1994; Escobar and Derrick, 2007). The required Ca^{2+} could enter via voltage-gated calcium channels (VGCC) or be released by intracellular stores via activation of metabotropic glutamate receptors (mGluRs), thus activation of NMDARs is not essential for LTD (reviewed in (Anwyl, 2006)) (Figure 1). In addition to glutamate, other neurotransmitters such as acetylcholine and dopamine are implicated in LTD (reviewed in (Massey and Bashir, 2007)).

LTP, on the other hand, was first described by Terje Lomø in 1966 (reviewed in (Lømo, 2003); (Bliss and Lomo, 1973)). It is divided into two phases, an early phase (E-LTP) independent of protein synthesis and a late phase (L-LTP) including activation of tran-

scription factors and protein synthesis. Here, the structural changes are obvious. E-LTP lasts for a few hours, while L-LTP can last up to 24h (Frey et al., 1993; Abel et al., 1997). Important players in LTP formation are NMDARs and AMPA receptors (AMPARs). Since the pore of NMDARs is blocked by a Mg^{2+} ion, it is essential that the postsynaptic membrane is depolarized to release Mg^{2+}, open the pore and allow Na^{+}, K^{+} and especially Ca^{2+} to pass. This depolarization is achieved either by simultaneous stimulation or via a strong stimulus alone (reviewed in (Baltaci et al., 2019)).

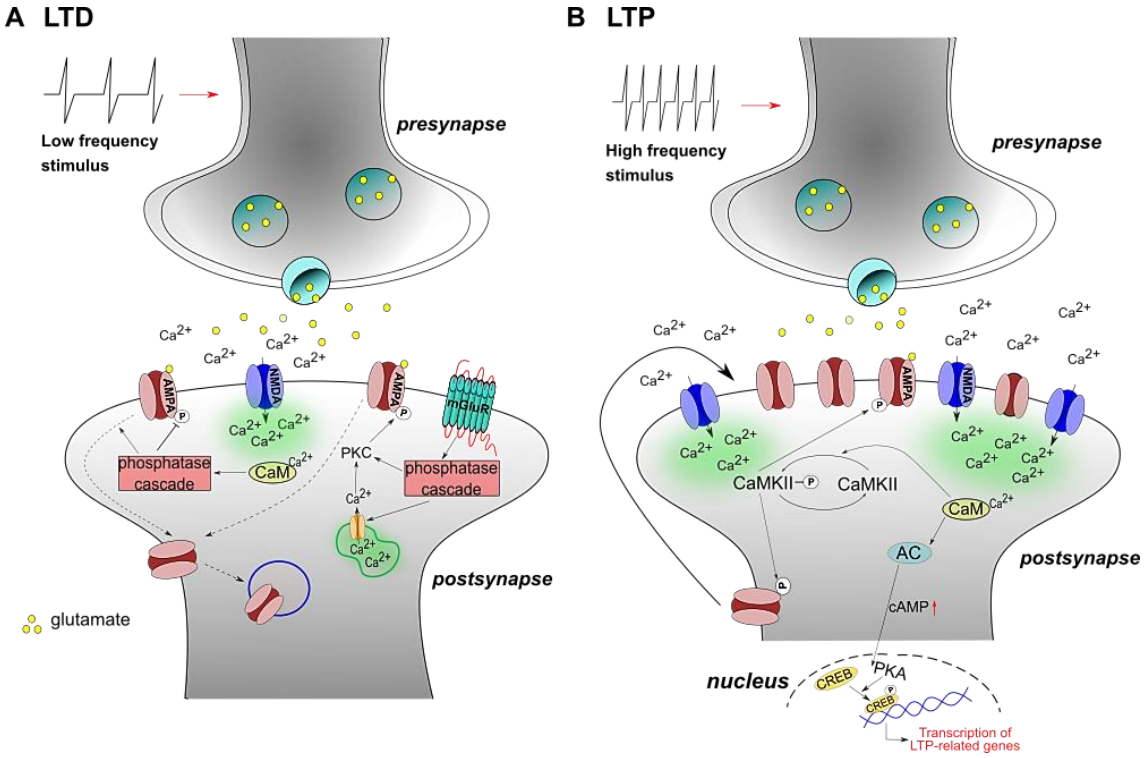

Figure 1: LTD and LTP are two major forms of long-term synaptic plasticity.

(A) LTD is induced by a low frequency stimulation resulting in modest activation of postsynaptic NMDARs through which only few Ca^{2+} ions can enter. In the postsynaptic terminal moderately enhanced Ca^{2+} levels activate calmodulin (CaM) and a cascade of phosphatases that can lead to the internalization of AMPARs. Beside the NMDAR-CaM pathway, a mechanism via mGluRs and protein kinase C (PKC) could result in receptor internalization as well (Man et al., 2000; Wang and Linden, 2000).
(B) A high frequency stimulus leads to activation of NMDARs, thus to an enhanced Ca^{2+} influx into postsynaptic terminals. Increased intracellular Ca^{2+} levels lead to the activation of Ca^{2+}/calmodulin-dependent kinase II (CaMKII) via Ca^{2+}/CaM. Phosphorylated and thus active CaMKII is able to phosphorylate the GluR1 subunit of AMPARs resulting in increased membrane trafficking and enhanced conductance of AMPARs. In addition, the Ca^{2+}/CaM complex activates adenylate cyclase (AC) leading to enhanced levels of cyclic adenosine monophosphate (cAMP) and the activation and translocation of protein kinase A (PKA) into the nucleus. Here, PKA is able to phosphorylate and activate transcription factors (e.g. CREB), thus transcription of LTP-related genes (Kristensen et al., 2011; Incontro et al., 2018).

The activation of NMDARs and the enhanced calcium influx result in activation of CaMKII via Ca^{2+}/CaM. Once activated CaMKII translocates to the synapse, binds to the NR2B subunit of NMDARs and phosphorylates target proteins in the postsynaptic den-

sity (PSD) (Figure 1) (Leonard et al., 2002; Incontro et al., 2018). In addition, CaMKII displays an interaction site in the C-tail of the GluA1 subunit of AMPARs. Phosphorylation of the serine residue S831 within this C-tail during LTP is increasing which than results in increased conductance of AMPARs (Lee et al., 2000; Kristensen et al., 2011). Furthermore, phosphorylation of the GluA1 C-tail by CaMKII is also believed to have an influence on AMPAR trafficking into membranes (Figure 1) (reviewed in (Opazo and Choquet, 2011)). To stabilize AMPARs in the membrane for further potentiation, it is necessary that these receptors are bound to PSD proteins. AMPARs are associated with TARPs (transmembrane AMPA receptor regulatory proteins) carrying a PDZ binding domain within their C-terminal tail which in turn can bind to the PDZ domain of PSD95 getting receptors trapped in the PSD (Sumioka et al., 2011). This signalling cascade appears in E-LTP which is independent of protein synthesis. In turn, L-LTP occurs after repeated stimuli and can last up to 24h *in vitro*. Here, changes in the gene expression, *de novo* protein and mRNA synthesis are key aspects (Abraham, 2003; Bosch and Hayashi, 2012). Interestingly, in the hippocampus of P15 rats dendrites display more spines and synapses, whereas adults show fewer spines but larger synapses after LTP induction (Bailey et al., 2015). Especially in the hippocampus L-LTP depends on PKA, MAPK (mitogen-activated protein kinase) and CREB (cAMP response element-binding protein) (Frey et al., 1993). The most important pathway for L-LTP induction in the hippocampus follows the cyclic AMP-PKA-CREB way. Therefore, AC is activated either via Ca^{2+}/CaM or by ligands binding to G protein-coupled receptors positively coupled to AC, like e.g. dopamine (Eliot et al., 1989; Tang and Gilman, 1991). PKA activated by cAMP relocates to the nucleus to phosphorylate the transcription factor CREB (Figure 1). Thus, genes related to CRE and being part of the L-LTP are transcribed (reviewed in (Nguyen and Woo, 2003)). Another crucial pathway involves MAPK. PKA initiates the assembly of the small GTPase Rap-1 and the kinase B-Raf phosphorylating MEK which in turn phosphorylates MAPK (Vossler et al., 1997; Morozov et al., 2003). Phosphorylated MAPK moves into the nucleus to activate transcription factors which in turn bind to DNA sequences encoding for immediate early genes playing important roles in synaptic plasticity (Bozon et al., 2003). To study molecular mechanisms of LTP *in vitro*, it is also possible to chemically induce LTP in neuronal cultures or acute slices by using 50 µM picrotoxin, 50 µM forskolin and 100 nM rolipram (Singh, 2017).

Beside short-term and long-term synaptic plasticity homeostatic plasticity is a third well-known plasticity form in the brain. In brief, homeostatic plasticity refers to the ability of neurons to preserve a certain level of activity considering prolonged alterations in synaptic stimulation. It is suggested that homeostatic regulation of the receptor number shares a signalling pathway together with the receptor insertion into membranes after LTP induction. There are several protocols known to induce homeostatic plasticity pharmacologically, for instance, in neuronal cultures using AMPAR blockers or TTX. However, it is essential that homeostatic mechanisms take place to prevent neuronal network activity from being driven towards runaway activity or silence (Turrigiano and Nelson, 2004).

Altogether, in the brain several forms of plasticity have been identified and key modulators and molecules have been studied. In this thesis I will focus on the neuromodulator dopamine acting via G protein-coupled receptors and the cAMP-PKA pathway and that constitute an important player in LTP-type synaptic plasticity.

1.2 Dopamine and its receptors

1.2.1 The neuromodulator dopamine

Dopamine (DA) is one of the most important known monoamine neurotransmitters in the brain. It is synthesized in a limited set of neurons located in the ventral tegmental area (VTA) and the substantia nigra (SN) as well as in the periphery (Seeman, 2010). The primary metabolic pathway is the synthesis of DA out of the essential amino acid L-phenylalanine which is converted into the amino acid L-tyrosine in the kidney. After, tyrosine is taken up by the brain via an active transport mechanism (Musacchio, 1975). Within monoaminergic neurons L-DOPA as precursor of DA is synthesized out of tyrosine via tyrosine hydroxylase. This enzymatic reaction step is rate limiting and inhibited by high levels of catecholamines (end-product inhibition) (Musacchio, 1975). DA by itself is also found in food; however it is incapable to cross the blood brain barrier. Therefore, it is essential that DA is synthesized in the brain to perform its neuronal activity. After its synthesis in the cytosol, DA is transported into vesicles via VMAT2 (vesicular monoamine transporter 2) and stored there until its release into the synaptic cleft upon an action potential (Eiden et al., 2004). Once released, DA binds to its specific receptors, mostly located at postsynaptic sites, and activates them. Receptor activation results in a series of downstream effects, for example excitation, synaptic plasticity,

learning and memory and motor control (reviewed in (Beaulieu and Gainetdinov, 2011)). When the signalling cascade is activated, DA molecules get quickly unbound from their receptors and need to be restored in presynaptic boutons. Therefore, they are absorbed back in the presynaptic terminal via a reuptake mediated either by DA transporters or plasma membrane monoamine transporters (reviewed in (Torres et al., 2003)). Back in the cytosol DA is degraded via monoamine oxidase or repacked into vesicles by VMAT2 to be available for subsequent releases (Eiden et al., 2004).

1.2.2 Family of Dopamine Receptors

Upon an action potential DA gets released in the synaptic cleft and acts via seven transmembrane G protein-coupled receptors which are divided into two main groups. The group of D1-like DA receptors consists of D1 and D5 DA receptors, while D2, D3 and D4 DA receptors are members of the D2-like DA receptor group (Andersen et al., 1990; Niznik and Van Tol, 1992; Vallone et al., 2000). Both groups are closely related to each other so that for example both receptor classes contain phosphorylation and palmitoylation sites at their C-terminus being involved in agonist-dependent desensitization of the receptors (Journot et al., 1987; Bates et al., 1991; Ng et al., 1994).

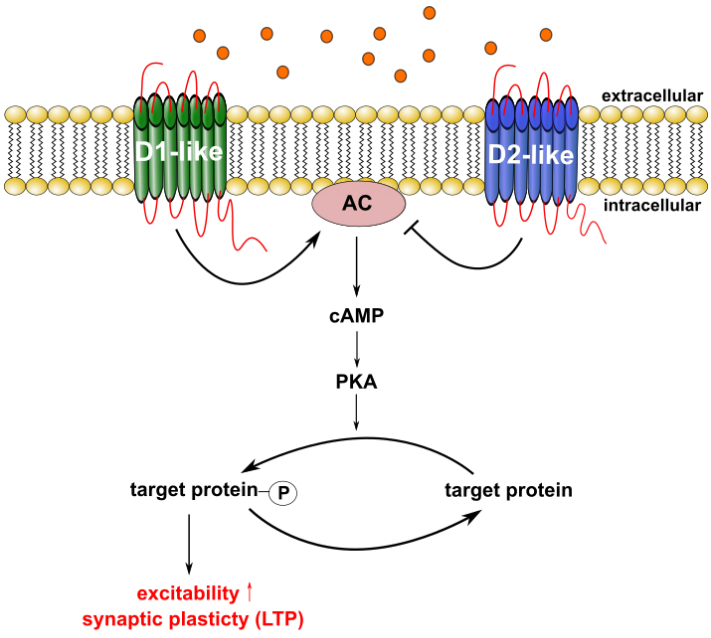

Figure 2: Schematic illustration of DA signalling through DA receptors

The neuromodulator DA signals via two families of DA receptors, either D1-like DA receptors or D2-like DA receptors. Members of the D1-like DA receptor family are positively coupled to AC and enhance intracellular cAMP levels. Thus, PKA activity increases and target proteins get phosphorylated. This cascade is resulting in enhanced excitability and synaptic plasticity. D2-like DA receptors inhibit AC and do not enhance excitability.

D1-like DA receptors are positively coupled to AC resulting in enhanced levels of intracellular cAMP levels. Elevated cAMP levels are leading to increased activity of PKA that in turn can phosphorylate and activate its downstream target molecules (Figure 2). Different from D1-like DA receptors, D2-like DA receptors inhibit AC and the down-

stream PKA-dependent signalling pathway (reviewed in (Beaulieu and Gainetdinov, 2011)) (Figure 2). All DA receptors are expressed at postsynaptic sites but D2 and D3 DA receptors can be found presynaptically as well (Hersch et al., 1995; Yung et al., 1995; Sokoloff et al., 2006; Yao et al., 2008).

D1 and D2 DA receptors are able to build heterodimers with each other following a phospholipase C (PLC) pathway. Activation of PLC was shown to result in the production of inositol triphosphate (IP3) as well as diacylglycerol (DAG) leading to enhanced intracellular calcium levels and activation of protein kinase C (PKC). Beside their interaction with one another DA receptors are known to interact with other receptors and ion channels located at the synapse.

1.2.3 Interaction with other receptors and ion channels

Especially D1 DA receptors are found to functionally and physically interact with different types of glutamate receptors such as NMDARs and AMPARs as well as GABA receptors (reviewed in (Beaulieu and Gainetdinov, 2011)). Thus, the carboxyl group of D1 DA receptors was found to interact with the NR1 and NR2A subunit of NMDA receptors in cultured hippocampal neurons and transfected COS-7 cells (Lee et al., 2002; Fiorentini et al., 2003). Disruption of the D1R/NR1 interaction results in activation of mechanisms for cell survival. The interaction between D1Rs and NR2As has been shown to inhibit NMDAR currents via a reduction of NMDAR cell surface expression (Lee et al., 2002). Activation of D1Rs affects the trafficking as well as the surface expression of NMDARs, thus synaptic plasticity. But vice versa NMDAR activation modulates D1R surface expression and signalling as well (Gao and Wolf, 2008; Hu et al., 2010; Li et al., 2010).

D2 DA receptors can interact with the NR2B subunit of NMDARs upon cocaine abuse. This D2R/NR2B interaction disrupts the association of NMDARs and CaMKII inhibiting the phosphorylation of the receptor as well as NMDAR-mediated currents (Liu et al. 2006). Interestingly, D5 DA receptors have been shown to interact with the second intracellular loop of GABA-A receptors. This interaction seems to be specific for D5Rs, since it does not occur with D1Rs (Liu et al., 2000). Beside NMDARs, AMPARs have been shown to interact with DA receptors at least to some extent in specific brain regions. Thus, it has been shown that D1Rs and D2Rs differentially regulate phosphorylation of GluA1 AMPARs in the striatum (Xue et al., 2017). Furthermore, functional

communication between AMPARs and D2Rs is essential to allow for learning of novel spatial information in the ventral striatum (Coccurello et al., 2012).

DA receptors can not only interact or influence receptors but also ion channels, especially calcium channels. D1Rs have been found to affect the activity of calcium channels. D1 agonists were able to increase calcium currents via L-type calcium channels in rat striatal neurons. This effect could be mimicked by cAMP analogs and inhibited by PKA blockers suggesting that there might be a PKA-mediated phosphorylation of calcium channels (Liu et al., 1992; Surmeier et al., 1995).
All described receptor-receptor and receptor-channel interactions are quite complex and not yet fully understood but suggested to be key players in several types of synaptic plasticity.

1.2.4 Dopamine in synaptic plasticity

DA by itself plays a key role in classical forms of synaptic plasticity, but was recently found to be also important in newly identified ones such as neo-Hebbian and Spike Timing-dependent plasticity (Tritsch and Sabatini, 2012; Lisman, 2017). To shape synaptic plasticity, the neuromodulator DA modulates presynaptic neurotransmitter release, for instance. Thus, DA typically acts through a decrease in neurotransmitter release at synapses that respond to dopaminergic modulation (reviewed in (Tritsch and Sabatini, 2012)). However, in the cortex DA influences GABAergic transmission from fast-spiking interneurons and non-fast spiking interneurons differentially. Thus, it is potentiating inhibitory postsynaptic potentials that are initiated by non-fast spiking interneurons, while hindering GABA release from fast spiking ones (Gao et al., 2003). Furthermore, synaptic plasticity is shaped by the modulation of postsynaptic receptors through DA. Fast synaptic transmission is mediated via glutamate receptors as well as GABA receptors. Interestingly, both receptor types display either a PKA or a PKC phosphorylation site being fundamental for the function. Since DA is acting via these kinases it is likely that those receptors are targets for the neuromodulator.

Activation of D1 DA receptors was shown to potentiate extrasynaptically as well as synaptically located NMDAR functions due to receptor phosphorylation and lateral diffusion (Braithwaite et al., 2006; Gao and Wolf, 2008). Beside NMDARS, AMPARs are essential for synaptic plasticity. It has been shown that D1 receptors acting via PKA promote the PKA-dependent phosphorylation of AMPARs resulting in enhanced sur-

face expression and electrophysiological properties of the receptor (Sun et al., 2005) and reviewed in (Shepherd and Huganir, 2007)). Finally, DA is shaping excitability of different neuronal subtypes in the PFC (reviewed in (Tritsch and Sabatini, 2012). However, the complete cellular and molecular mechanism of DA-dependent plasticity is still elusive.

1.2.5 Pharmacology of D1-like DA receptors

To shed light on the underlying pathways and to investigate, for example, molecular mechanisms of DA receptor signalling *in vitro*, numerous pharmacological agents are known and used. Here, I will focus on the pharmacological activation and inhibition of D1-like DA receptors. Most D1-like DA receptor agonists and antagonists belong to the family of benzazepines. Thus, SKF38393, for instance, is well-known as a partial activator of D1-like DA receptors and triggers D1-mediated cAMP accumulation in cells (Conroy et al., 2015). SKF81297, another often used agonist, has been shown to be as efficient as dopamine itself in terms of D1-mediated intracellular cAMP accumulation (Conroy et al., 2015). Beside stimulation of AC and cAMP accumulation, there are pharmacological compounds that have been shown to activate the PLC pathway. Especially SKF83959 was identified as a high-affinity activator for D1 DA receptors coupled to PLC. Interestingly, this compound showed little or no activation of AC-coupled receptors and could be used in fact to antagonize DA-dependent cAMP accumulation (Rashid et al., 2007). SCH23390, the most common used D1-selective antagonist, has been found to completely block cAMP accumulation *in vitro* (Conroy et al., 2015). In *in vivo* studies SCH23390 was also found to abolish generalized seizures evoked by, for example, pilocarpine (reviewed in (Bourne, 2001)). However, the here described pharmacological compounds are helpful tools for *in vivo* and *in vitro* investigations of the dopaminergic system.

1.3 The brain's ECM

The human brain consists of around 100 billion neurons being connected via synapses and surrounded by glial cells (reviewed in (Herculano-Houzel, 2009)). Approximately 20 % of the total brain volume is extracellular space (Nicholson and Syková, 1998). This space is filled with a highly organized molecular structure called the extracellular matrix (ECM). Neurons as well as astrocytes express and secrete components of the ECM wrapping somata of neurons and pre- and postsynapses as well (Jaworski et al.,

1999; Seidenbecher et al., 2002; Zhang et al., 2014). Beside perineuronal nets (PNNs) which occur as a highly condensed, aggrecan-based structure and were first described by Camillo Golgi (reviewed in (Celio et al., 1998), brevican-based axonal coats were described recently as a different form of ECM structures which were found to be small, round- or oval-shaped and are reminiscent of pearl laces (Lendvai et al., 2012; Morawski et al., 2012b). In this thesis, I will focus on a third form the perisynaptic, hyaluronan-based ECM.

1.3.1 Molecular composition of the Hyaluronan-based ECM

The more diffuse perisynaptic, hyaluronan-based ECM contains hyaluronic acid (HA), a high molecular weight, negatively charged, non-sulfated glycosaminoglycan, forming the backbone of the ECM (reviewed in (Galtrey and Fawcett, 2007; Dityatev et al., 2010; Kwok et al., 2011)) (Figure 3). Hyaluronic acid synthases (HAS) located in the plasma membrane synthesize HA which is transported directly into the extracellular space (Schulz et al., 2007) (Figure 3). Interestingly, HA has been shown to have an influence on the lateral diffusion of AMPARs and the activity of L-type Ca^{2+} channels (Frischknecht et al., 2009a; Kochlamazashvili et al., 2010). Heparan and chondroitin sulfate proteoglycans (HSPGs and CSPGS, respectively) are prominent components of the ECM. The proper function of secreted proteins of the Wnt or TGF-β families has been shown to be dependent on HSPGs. Furthermore, HSPGs are involved in developmental processes such as axon guidance (reviewed in ((de Wit and Verhaagen, 2007)). CSPGs, on the other hand, represent the most prominent binding partners of HA in the central nervous system (CNS). CSPGs are bound to HA via their N-terminal tail (Fox and Caterson, 2002). By itself, CSPGs are composed of a glycoprotein representing the core protein and covalently bound sulfated glycosaminoglycan (GAG) side chains in a varying number (reviewed in (Zimmermann and Dours-Zimmermann, 2008)). Well known are members of the lectican family of CSPGs such as aggrecan (Acan), brevican (BC), neurocan (Ncan) or versican (Vcan).

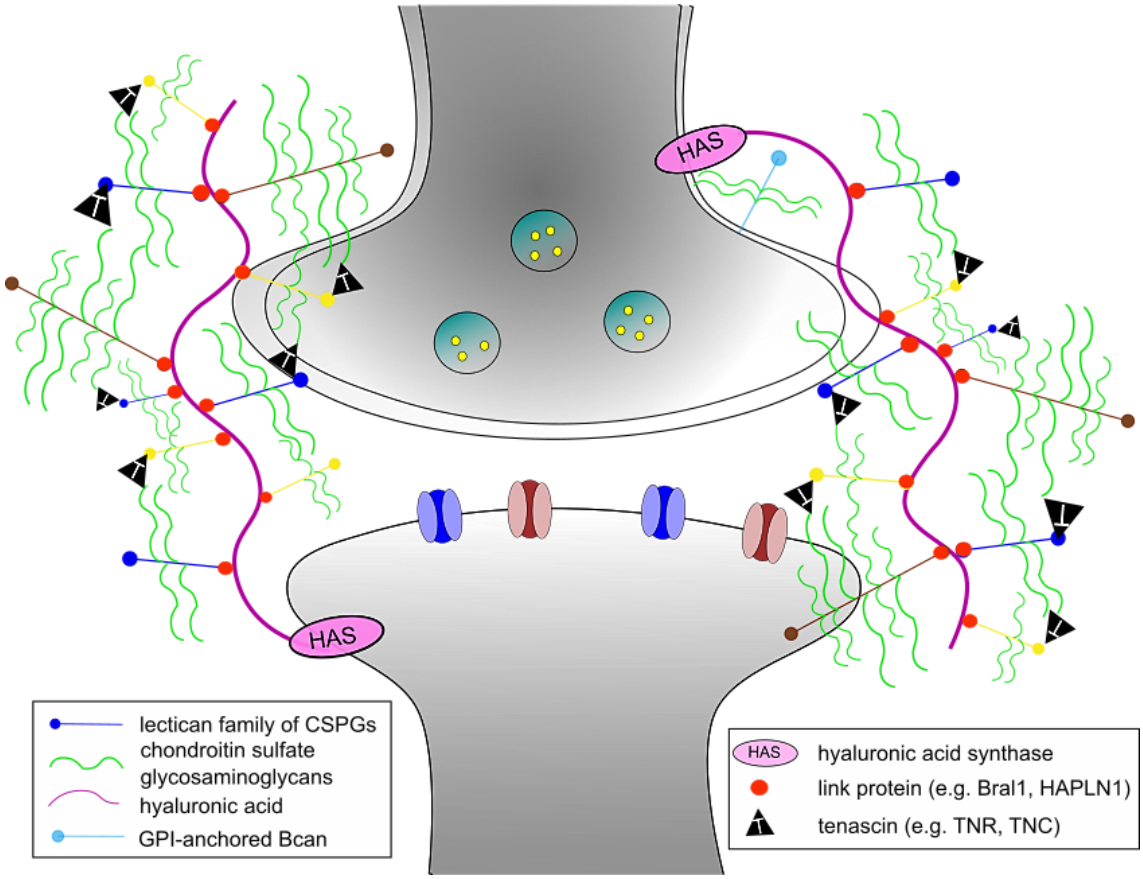

Figure 3: Schematic illustration of the molecular composition of the perisynaptic ECM

The brain's ECM consists of HA that is synthesized and released by HAS. CSPGs of the lectican family, such as BC, Acan, Ncan or Vcan are bound to HA via their N-terminus. To strengthen this bond, HA and CSPGs are bound together via link proteins. BC can be also expressed in a GPI-anchored form. Tenascins link the C-terminus of CSPGs with GAG side chains of CSPGs to build up the net-like structure surrounding synapses.

All CSPGs of the lectican family are connected with tenascins, such as tenascin R (TNR) or tenascin C (TNC), via their C-terminal G3 domain (reviewed in (Miyata and Kitagawa, 2017)) (Figure 3). Another glycoprotein namely reelin can be also found in the ECM structure. Reelin has been found to be secreted at synapses and to control NMDAR surface mobility. Furthermore, it is involved in the regulation of neuronal progenitor migration (reviewed in (Dityatev et al., 2010)). To strengthen the bond between HA and CSPGs link proteins like Bral1 (brain link protein 1) or HAPLNs (hyaluronan and proteoglycan binding link protein) connect both HA and CSPGs (Hirakawa et al., 2000; Spicer et al., 2003) (Figure 3).

1.3.2 The lecticans BC and Acan

The members of the lectican family of CSPGs are quite large molecules which display highly homologous G1 and G3 domains (Mörgelin et al., 1989). The most prominent

CSPGs in the rodent brain are BC and Acan. BC is the shortest member of the so called Acan-Vcan family of CSPGs. It contains like all other family members a N-terminal located G1 domain binding HA and a C-terminal G3 globular domain but no G2 domain such as Acan (Yamada et al., 1994). BC is specifically expressed in the brain and its expression is developmentally regulated. Thus, only small amounts of expressed BC can be found in rats during the first postnatal days. The expression of BC is increased gradually and reaches a plateau in the adult (Seidenbecher et al., 1998). After its expression by neurons or glia cells, BC is either incorporated into the neuronal ECM or bound at the cell surface (Carulli et al., 2006; Hedstrom et al., 2007; Frischknecht et al., 2009a). BC was found to contain a number of various cleavage sites for different ECM-modifying proteases. All cleavage sites are located between the G1 and G3 domains (Nakamura et al., 2000). Matrix metalloproteases (MMPs) were shown to cleave BC into fragments with different sizes, while ADAMTS 4 was found to generate two fragments of a similar size range. However, upon cleavage N-terminal HA-attached fragments of a size around 50 kDa and C-terminal fragments of approximately 80 kDa are generated. Since both fragments can be found in brain lysates, it is suggested that BC cleavage occurs *in vivo* to a large extent (Yamada et al., 1994; Seidenbecher et al., 1995; Matthews et al., 2000). It is suggested that cleavage via those proteases is responsible for the physiological turnover of BC as well as its pathological degradation (Nakamura et al., 2000). Especially, the ADAMTS 4-derived 50 kDa fragment has been found dramatically upregulated under conditions of kainite-induced lesions in rats (Yuan et al., 2002).

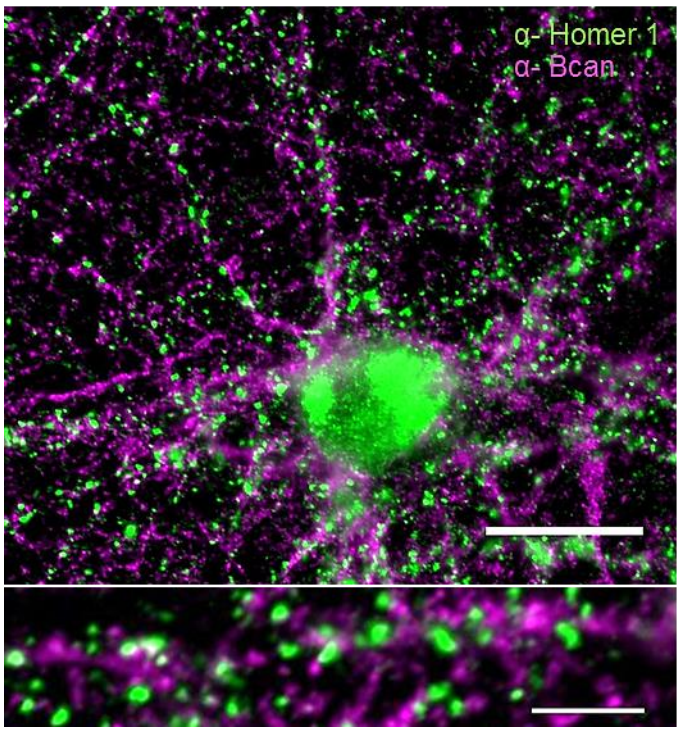

Figure 4: Expression pattern of extracellularly located BC in dissociated cortical rat cultures

Dissociated cortical neurons (DIV21) are stained for the excitatory synaptic marker Homer 1 (green) and extracellular BC (magenta). BC nicely enwraps the soma as well as Homer 1-positive synapses (scale bar: 20 µm, close-up: 5 µm).

The largest member of the lectican family in the brain Acan contains beside the globular domains G1 and G3 an additional globular domain G2 (Wiedemann et al., 1984; Paulsson et al., 1987). It has been shown that G1 and G2 display so-called link protein domains, known for their ability to bind HA (Watanabe et al., 1995). Interestingly, Acan is almost exclusively found in PNNs. Therefore, it is thought to be important for the formation and function of these structures (Morawski et al., 2012b). Just as BC, Acan expression is strictly regulated during development. In early developmental stages Acan protein expression is low and peaks in early adolescence in the CNS (Morawski et al., 2012). However, only a small subset of neurons does express Acan protein. For example in the cerebral cortex, especially parvalbumin-positive fast-spiking interneurons express this lectican (McRae et al., 2007; Morawski et al., 2012). Similar to BC, Acan displays similar cleavage sites for MMPs and ADAMTS 4 in the interglobular domains (Fosang et al., 1992). Especially, in ostheoarthritis ADAMTS-derived fragments have been found to be enriched (reviewed in (Fosang and Little, 2008)). Furthermore, in rat brains under conditions of status epilepticus (SE) Acan-based PNNs were shown to be dramatically degraded due to an increased MMP activity up to two months after SE (Rankin-Gee et al., 2015). Thus, degradation of Acan through proteases is not only necessary for its physiological turnover but seems to play a role in different pathologies as well.

Altogether, the brain's ECM as a quite complex structure seems to fulfill a number of different functions in the healthy and the diseased brain.

1.3.3 Functions of the brain's ECM

The ECM can form tight, net-like structures surrounding dendrites and synapses, which fulfill a number of important functions in the CNS. It is well-known to function as a diffusion barrier. Thus, the volume transmission of soluble molecules is massively influenced by the molecular composition of the ECM (reviewed in (Dityatev et al., 2010)). The afore-mentioned link protein Bral1, for example, was shown to hinder the diffusion of water-soluble molecules, especially in the white matter of the brain (Bekku et al., 2010). Enzymatic removal of chondroitin sulfate side chains using Chondroitinase ABC (ChABC) has been found to influence the diffusion of extracellular Ca^{2+} ions as well as their local concentration (Hrabětová et al., 2009). Because of their charged nature, PNNs are thought to function as a kind of neuroprotector against oxidative stress to neurons (Morawski et al., 2004). The ECM functions not only as a diffusion barrier for extracellular ions or neurotransmitters, but seems to be a physical barrier for the lateral

diffusion of receptors. Frischknecht et al. could demonstrate that enzymatic degradation of HA by hyaluronidase in hippocampal neurons results in increased lateral mobility of AMPARs in the postsynaptic membrane. In addition, AMPAR exchange from synaptic and extrasynaptic sites was more rapid (Frischknecht et al., 2009a). Beside AMPARs the ECM also has an influence on NMDARs. Reelin, a secreted glycoprotein, is involved in the control of the surface mobility of NR2B-containing NMDARs (Groc et al., 2007). Those receptors get stuck in the postsynaptic site for a long period of time when reelin activity is inhibited (Groc et al., 2004). However, beside its physiological functions the neuronal ECM occupies also a structural one by stabilizing synaptic formations. It is not only the net-like structure which stabilizes synapses but there are also a number of ECM molecules, such as LGI1, ADAM22 or 23, connecting pre- and postsynapses as trans-synaptic "cross-linkers". Those ECM molecules are highly important, since a loss can result, for example in specific forms of epilepsy (reviewed in (Dityatev et al., 2010)).

The neuronal ECM does not only play a role in health but has been found to undergo dramatic changes in several brain diseases such as Alzheimer's disease (AD) or epilepsy as well as in brain injuries. In AD, for instance, Acan-based PNNs have been shown to remain unchanged in their distribution, structure and molecular properties. But perisynaptic BC-based axonal coats were exclusively lost in the core of amyloid plaques in parallel to the loss of synapses (Morawski et al., 2012). In models of epilepsy Acan mRNA was found to be reduced up to two month after status epilepticus induced in rats. Interestingly, Ncan expression which is less in the adult has been shown to be increased after seizures (reviewed in (McRae and Porter, 2012)). However, enhanced expression of CSPGs in CNS scar tissue composed of glial cells has been found to be related to acute brain injuries such as stroke or spinal cord injuries (SCI) and inhibits regeneration (reviewed in (Wiese et al., 2012)). ChABC treatment in turn has been shown to remove the inhibitory effect of CSPGs and allow for axon regeneration (Zuo et al., 1998).

In the last years, the ECM and its possible functions in synaptic plasticity went more and more in the focus of investigations.

1.3.4 The ECM of the brain in plasticity

Important players in LTP formation and synaptic plasticity like AMPARs and NMDARs and an increased Ca^{2+} influx into postsynaptic sites are influenced by the

ECM. Experimental degradation of the ECM has been shown to influence the synaptic mobility of AMPARs, and thus short-term plasticity (Heine et al., 2008; Frischknecht et al., 2009a). Furthermore, enzymatic ECM degradation has an influence on VGCC and tunes synaptic short-term plasticity as well (Kochlamazashvili et al., 2010). There is more evidence that the ECM is a key player in synaptic plasticity, since some studies performed with animals lacking certain components of the ECM showed deficits in some forms of plasticity. Mice that are lacking TNR display impairment in LTP but not in LTD. Furthermore, when TNR is missing a disinhibition in the CA1 region of the hippocampus is observed as well as an increase in the threshold for LTP induction (Bukalo et al., 2007). It was also shown that mice deficient in BC or Ncan display impairments in the maintenance of LTP (Zhou et al., 2001; Brakebusch et al., 2002). Interestingly, mice deficient in BC, Ncan, TNR and TNC showed altered profiles of synaptic potentiation and depression in the dentate gyrus (DG). Especially, E-LTP (<75 min after tetanization) was absent in those mice. Nevertheless, these quadruple KO mice seem to have an intact synaptic plasticity, but this is fundamentally different from normal WTs (Jansen et al., 2017).

In further *in vivo* approaches the experimental weakening of the ECM has been shown to influence plasticity for example in the visual cortex or enhance cognitive flexibility. Thus, Pizzorusso and colleagues could demonstrate that in young animals a shift in ocular dominance plasticity occurs after monocular deprivation that is absent after the critical period in adults. However, adult rats displayed this shift after ChABC treatment. Thus, the authors suggested that the mature ECM prevents experience-dependent plasticity, but degradation of CSPGs again allows for plasticity (Pizzorusso et al., 2002). Another study performed with Mongolian gerbils revealed that injection of ChABC in the auditory cortex resulted in an enhanced performance in an auditory learning task. In this task animals learned to discriminate linear frequency-modulated (FM) tones due to active avoidance in a Go/NoGo paradigm performed in a two-way shuttle-box (Happel et al., 2014). Furthermore, the same group observed changes in the ECM after mice performed the same FM discrimination task, thus suggest that endogenous mechanisms through specific proteases need to be present allowing for restructuring of the neuronal ECM (Niekisch et al., 2019).

In search for mechanisms of plasticity-induced endogenous modulation of the ECM a study from our lab could show that under conditions of homeostatic plasticity the

cleaved fragment of the lectican BC is increased in different brain regions and around synapses in hippocampal cultures. This suggests that local proteolysis of perisynaptic ECM might be able to alter synaptic functions and influence several forms of synaptic plasticity (Valenzuela et al., 2014). Those observed endogenous changes in the composition of the ECM occur via ECM-modifying proteases expressed by brain cells.

1.4 ECM-modifying proteases

Several ECM-modifying proteases, e.g. glycosidases like hyaluronidase or ChABC are able to change the ECM enzymatically. Hyaluronidases can be found endogenously in the brain (reviewed in (Lepperdinger et al., 2001)). Beside enzymes cleaving the ECM's backbone or GAG side chains, several proteases were found to cleave CSPGs of the lectican family within the core protein region. There are different types of protease families, most prominent the family of matrix metalloproteinases (MMPs), the family of A Disintegrin and Metalloproteinases (ADAMs) and the A Disintegrin and Metalloproteinase with Thrombospondin motifs (ADAMTS) family. All of them belong to the metzincin protease superfamily which is named according to the conserved methionine residue close to the metalloprotease active site being zinc ion-dependent (reviewed in (Kelwick et al., 2015a)).

1.4.1 The ADAMTS family of proteases

In my thesis, I focused on the ADAMTS family of proteases, which were found in multicellular, eukaryotic organisms (for review see (Kelwick et al., 2015)) and well-known to be potent candidate proteases for cleaving CSPGs in the brain. All family members are secreted, extracellular enzymes. The basic organization of a signal peptide followed by a pro-region of varying length, a metalloproteinase domain, a disintegrin-like domain, a central thrombospondin type 1 sequence repeat (TSR) motif and a cysteine-rich domain which is followed by a spacer region is indicated by ADAMTS 4 (Figure 5). This basic structure is extended at the C-terminus by additional TSRs in other family members (reviewed in (Kelwick et al., 2015)).
The first ADAMTS enzyme ADAMTS 1 was identified in 1997 (Kuno et al., 1997). In total there are 19 human ADAMTS proteins being assembled in eight "clades" based on their domain organization and known functions. ADAMTS 1, 4, 5, 8 and 15 belong to the so called aggrecanase and proteoglycanase clade that can cleave hyaluronan-binding CSPGs (Sandy et al., 1991; Nakamura et al., 2000). ADAMTS enzymes are expressed

as zymogens carrying an N-terminal pro-domain which contains at least one cleavage site for furin or furin-like proprotein convertases (PPCs).

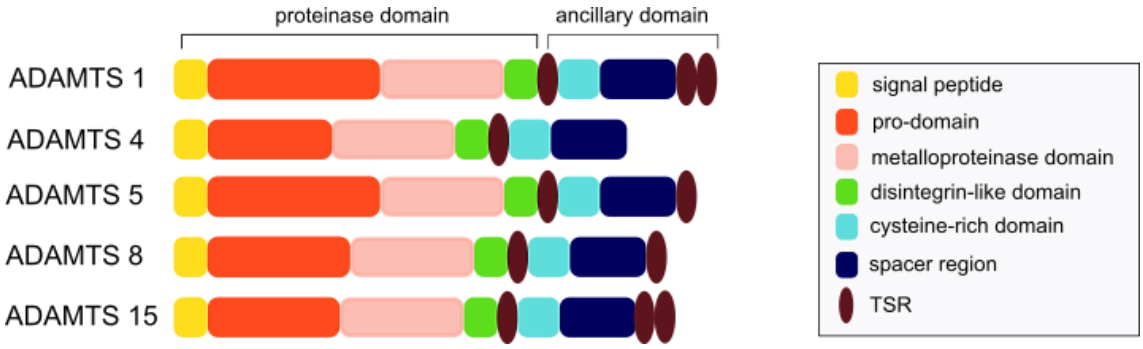

Figure 5: Domain organization of the aggrecanase and proteoglycanase clade

The ECM-remodeling proteases ADAMTS 1, 4, 5, 8 and 15 are members of the so-called aggrecanase and proteoglycanase clade. ADAMTS 4 displays the prototypic structure of all family members. The proteinase domain consists of a signal peptide (yellow) followed by the pro-domain (orange), a metalloproteinase domain (light-pink) and a disintegrin-like domain (green). At the C-terminus the ancillary domain contains a TSR motif (brown), a cysteine-rich domain (cyan) and a spacer region (dark blue). This basic structure is just extended by several TSR motifs at the C-terminus of the appropriate protease.

Activation of ADAMTS pro-enzymes takes place in the *trans*-Golgi network, close to the membrane or extracellularly. Thus, for instance ADAMTS 1 and ADAMTS 4 have been shown to be activated by PPCs in the *trans*-Golgi network followed by the secretion of the active enzyme (Rodriguez-Manzaneque et al., 2000; Wang et al., 2004; Tortorella et al., 2005). By contrast the precursor of ADAMTS 5 is extracellularly activated by furin (Longpré et al., 2009). One important motif of all ADAMTS enzymes is the ancillary domain. This carboxy-terminal domain is crucial for their ECM association, activity regulation and it is important for their substrate-binding preferences (reviewed in (Kelwick et al., 2015a)). ADAMTS enzymes are crucial in development and important key players in plasticity-dependent changes of the ECM.

1.4.2 The ECM-modifying enzymes ADAMTS 4 and ADAMTS 5

Especially the enzymes ADAMTS 4 and ADAMTS 5 were found to be prominent ECM-modifying proteases in the brain (reviewed in (Kelwick et al., 2015)). ADAMTS 4 and ADAMTS 5, two prominent representatives of the ADAMTS enzyme family, were first described as aggrecanase-1 and aggrecanase-2 in cartilage, thus named after their potential to cleave the proteoglycan Acan in the core protein region (Abbaszade et al., 1999). Later, those enzymes were also identified in the brain playing a crucial role

in the restructuring of the ECM (Valenzuela et al., 2014). However, due to the high expression of ADAMTS 4 and 5 in cartilage, these enzymes were thought to be the major players in arthritis (reviewed in (Fosang and Little, 2008; Troeberg and Nagase, 2012). Indeed, both enzymes seem to be crucial for the pathogenesis of the arthritis, with a more critical role for ADAMTS 5. ADAMTS 5 $^{-/-}$ mice show an enhanced reduction in joint destruction when compared with WT or ADAMTS 4 $^{-/-}$ (Glasson et al., 2005; Stanton et al., 2005). Moreover, both enzymes were found in several types of cancer. While, for instance, ADAMTS 5 is downregulated in breast cancer, ADAMTS 4 expression is enhanced (Porter et al., 2004). In glioblastoma, in turn ADAMTS 4 and also ADAMTS 5 were found to be upregulated and are suggested to be responsible for the invasive nature of this type of brain tumor (Held-Feindt et al., 2006). Members of the ADAMTS enzyme family do not only play a role in diseases but also in development. Thus, ADAMTS 5, for instance, was shown to be essential for heart development. It is required for the correct clearance of the early Vcan-rich ECM. Mice deficient for ADAMTS 5 show developmental defects in the cleavage of Vcan. Thus, ADAMTS 5 null mice display magnified heart valves in late fetal stages due to reduced Vcan cleavage (Dupuis et al., 2011).

Importantly, ADAMTS enzymatic activity is strictly controlled. On one side, the activity of ADAMTS enzymes is controlled by their inhibition via tissue inhibitors of metalloproteinases (TIMPs). The most effective one seems to be TIMP3 which can interact with the ancillary domains of ADAMTS 4 and 5 to promote enzyme inhibition (Hashimoto et al., 2001). On the other side enzyme activity is also controlled by internalization and degradation. It has been shown that ADAMTS 4 as well as 5 are able to interact with the low-density lipoprotein-related protein 1 (LRP-1) resulting in different half-lives of the extracellular enzymes. All in all, ADAMTS 4 and ADAMTS 5 display several important functions throughout the body and both enzymes are strictly regulated in their activity state. However, since most studies focus on pathological functions of these enzymes, not much is known about their function in, for instance, learning processes in a healthy brain.

1.5 Aims of the thesis

Synaptic plasticity, fundamental for learning and memory, is based on the regulation of neurotransmitter release and the response of receptors at the postsynaptic site, but also

on structural reorganization of synaptic connections. The neuromodulator DA is well-known to be a key player in plasticity, for instance in reward learning. It has been shown that Mongolian gerbils injected with a D1 agonist showed enhanced performance in a FM discrimination task compared to control animals (Schicknick et al., 2008). Synapses are enwrapped by a meshwork of proteins called the ECM. However, during postnatal development the loose premature ECM turns into a tight, net-like structure. To allow for plasticity and learning and memory in the adult, it is essential that the ECM has the ability to be restructured. It has been shown that in a FM discrimination task Mongolian gerbils performed better after enzymatic ECM removal in the auditory cortex than control animals (Happel et al., 2014). Furthermore, in an *in vitro* approach upon conditions of homeostatic plasticity the amount of cleaved BC is enhanced around synapses (Valenzuela et al., 2014). Based on these studies, I searched for a functional connection between the dopaminergic system and the ECM integrity that in fact might be essential for synaptic plasticity. Therefore, I addressed the following questions:

1. Does systemic activation of DA D1 receptors affect ECM integrity *in vivo*?
2. If changes in the ECM composition occur, which ECM-modifying proteases are active and/or released upon DA receptor activation?
3. What is the underlying molecular mechanism?

2 Material and Methods

All chemicals with analytical grade quality were purchased from Roth or the companies indicated.

2.1. Neuronal cell cultures

Rat cortical cultures were prepared by LIN Dept. Neurochemistry technicians Isabel Herbert, Anita Heine, and Kathrin Hartung. All media and solutions were pre-warmed at 37°C.

Table 1: Media and reagents for primary neuronal cell culture

Media and reagents	Ingredients/Provider
DMEM complete	10 % FCS (Gibco); 1 % Penicillin/Streptomycin 100x (Gibco); 2 mM L-Glutamine 100x (Gibco) in DMEM (Gibco)
NB+ (Neurobasal)	2 % B27 (Gibco); 2 mM L-Glutamine 100x (Gibco); 1 % Penicillin/Streptomycin 100x (Gibco) in Neurobasal (Gibco)
Distilled water	Gibco/Millipore
HBSS+ (with Mg2+ and Ca2+)	Gibco
HBSS-	Gibco
10x Trypsin	Gibco
Poly-D-Lysine	100 mg/l poly-D-lysine in 100 mM boric acid, pH 8.5, sterile filtered

2.1.1. Preparation of glass coverslips

Glass coverslips used for dissociated cortical cultures were sterilized without washing beforehand, coated with poly-D-lysine, washed with ultra-pure water and stored in HBSS- until day of preparation.

2.1.2. Preparation of dissociated cortical cultures

Embryos of Wistar rats (E18-19) were decapitated. Both hemispheres were isolated and freed from the meninges in ice-cold HBSS+. After three washing steps with HBSS-, 10x trypsin was added to both cortices and incubated for 20 min at 37 °C. The tissue was washed again with HBSS- and dissociated with two syringes of different diameter (first 0.9 mm, followed by 0.45 mm). Cells were further dissociated using a cell mesh followed by dilution in DMEM complete to different plating concentrations. Dissociated cortical cultures were plated in 24- and 6-well plates with a density of 50,000 or

500,000 cells per well, respectively. One day after plating DMEM complete was replaced by NB+. Once per week, fresh NB+ medium was added.

2.2. Antibodies

Table 2: List of primary antibodies, origin and working concentration

Primary antibody	Species	Company	Cat.-No.	ICC dilution	WB dilution
α- ADAMTS 4	rb	Abcam	ab185722	1:200	1:500
α- ADAMTS 5	rb	OriGene Technologies	TA321798	1:200	1:500
α- brevican	ms	BD Biosciences	610894		1:1000
α- brevican	gp	LIN/ Seidenbecher		1:1000	
α- Rb399	rb	LIN/Frischknecht		1:500	1:1000
α- Homer 1	ms	Synaptic Systems	160 011	1:1000	
α- GAD65	ms	Abcam	ab26113	1:2000	
α- MAP2	ms	Synaptic Systems	188 011	1:2000	
α- Bassoon	rb	Synaptic Systems	141 002	1:2000	
α- PSD95 K28/43	ms	NeuroMab	P78352	1:1000	
α- Synaptotagmin-I 550 Oyster	ms	Synaptic Systems	105 311C3	1:750	
α- D1 receptor	rt	Sigma-Aldrich	D2944	1:240	
α- GAPDH	rb	Synaptic Systems	247 002		1:1000
α- GFAP	ms	Synaptic Systems	173 011	1:500	
α- D2 receptor	rb	Abcam	ab21218	1:1000	
α- aggrecan	rb	Merck Millipore	AB1013	1:1000	1:1000
α- aggrecan neo	rb	Novus Biologicals	74350	1:500	

Species: rabbit (rb), mouse (ms), guinea pig (gp), rat (rt).

Fluorescently labeled secondary antibodies used for ICC were purchased from Invitrogen (Alexa Fluor 488, 568, 647; dilution 1:1000) or from Dianova (Cy3, Cy5; dilution 1:1000). Secondary antibodies labeled with peroxidase (POD) used for quantitative immunoblotting were purchased from Jackson Immuno Research.

2.3. Drugs

Table 3: List of used drugs, origin and working concentration

Compound	Company	Cat.-No.	Working conc.
cAMPS-Rp, triethylammonium salt	Tocris Bioscience	1337	15 µM
D-(-)-2-Amino-5-phosphonopentanoic acid (D-AP5)	Tocris Bioscience	0106	50 µM
Diltiazem hydrochloride	Tocris Bioscience	0685	20 µM
Forskolin	Tocris Bioscience	1099	50 µM
Hexa-D-Arginine	Tocris Bioscience	4711	0.58 µM
Ifenprodil	Tocris	0545	3 µM
KN93 phosphate	Tocris Bioscience	5215	2 µM
(-)- Quinpirole hydrochloride	Tocris Bioscience	1061	1 µM
Rolipram	Tocris Bioscience	0905	0.1 µM
SCH23390 hydrochloride	Abcam	ab120597	10 µM
SKF 38393 hydrobromide	Tocris Bioscience	0922	5 mg/kg body weight
SKF 81297 hydrobromide	Tocris Bioscience	1447	1 µM
Tetrodotoxin (TTX)	Sigma-Aldrich	T8024	1 µM
TIMP-3	R&D Systems		7.5 nM

2.4. Immunocytochemistry (ICC)

Table 4: Buffers and solutions for immunocytochemistry

Buffer	Composition
4 % PFA (w/v)	4 % PFA in PBS, pH 7.4
Blocking Solution	10 % FCS in PBS, 0.1 % Glycin, 0.1 % Triton-X 100
Mowiol (96 ml)	9.6 g Mowiol, 24 g Glycerol, 24 ml H_20 → stirring 24-48 h, 48 ml 0.2 M Tris (pH 8.5) → 10 min 50 °C, 2.5 g DABCO → 24 h stirring
1x PBS	2.7 mM KCl, 1.5 mM KH_2PO_4, 137 mM NaCl, 8 mM Na_2HPO_4, pH 7.4

2.4.1. Immunocytochemical staining of dissociated cortical cultures

Rat cortical cultures were grown on poly-D-lysine-coated coverslips (Ø 24 mm) in NB+ medium. For staining of ECM proteins and cell surface proteins antibodies were diluted in NB+ medium. A wet chamber was covered with Parafilm® and small drops of antibody solution were pipetted on it. Coverslips were turned - cells facing down - on top of the drop and incubated at 37 °C for 20 min. After fixation with 4 % PFA for 5 min at room temperature (RT) cells were incubated in Blocking Solution to stop PFA reactivity and block unspecific binding sites as well as to permeabilize the cells. Primary antibodies to stain for intracellular proteins were diluted in Blocking Solution, dropped on Parafilm and coverslips with cells facing down were incubated with primary antibodies overnight at 4°C. Hereafter cells were washed three times with 1x PBS. Fluorescently labeled secondary antibodies were also diluted in Blocking Solution and cells incubated for 45 min at room temperature in darkness. Following three washing steps with 1x PBS, coverslips with cells were mounted using Mowiol and stored at 4°C until analysis. When intracellular proteins/epitopes were stained, the protocol started with PFA fixation of the cells.

2.4.2. Synaptotagmin antibody uptake

In order to investigate synaptic activity an antibody against the luminal domain of synaptotagmin-1 coupled with a fluorophore was used similarly as antibodies for surface-expressed receptors. Dissociated cortical cultures (DIV21) were treated with the D1-like DA receptor agonist SKF81297 (Table 3) for 15 min followed by a 20 min-incubation with the synaptotagmin antibody listed in Table 2. Non-treated neurons served as control group. The synaptic marker Bassoon was used to identify presynapses. Thus, the fluorescence intensity of this antibody reflects the activity state of single presynapses.

2.4.3. Generation of ADAMTS 4 and 5 shRNAs

Table 5: shRNA sequences of scramble, ADAMTS 4 and ADAMTS 5

rat ADAMTS 4	shRNA 1: 5'- ATCGTGACCACATCGCTGT -3' shRNA 2: 5'- TATAGCGCAAGCTGACTGC -3'
rat ADAMTS 5	**shRNA 1: 5'- TAGCGCGCATGCTTGACTG -3'** **shRNA 2: 5'- ATCCCCGTAAACTCGTTCG -3'**
control siRNA (scramble)	5'- CGGCTGAAACAAGAGTTGG -3'

Constructs of small hairpin RNA to knock down specific proteases were designed by Dr. Rahul Kaushik in the lab of A. Dityatev at the DZNE. Virus production, purification and virus titration were performed together with Dr. Rahul Kaushik and Jennifer Schneeberg at the DZNE. In brief, to knock down rat ADAMTS 4 (GeneID: 66015) and ADAMTS 5 (GeneID: 304135) shRNA plasmids were cloned by the insertion of the siRNA sequences (Dharmacon, Horizon Discovery) targeting the open reading frame (ORF) of rat ADAMTS 4 and ADAMTS 5 into AAV U6 GFP (Cell Biolabs Inc.) using BamH1 (New England Biolabs) and EcoR1 (New England Biolabs) restriction sites. Positive clones were sequenced and used for the production of recombinant adeno-associated particles as described previously in (McClure et al., 2011). In brief, HEK 293T cells were transfected using calcium phosphate method with an equimolar mixture of the shRNA-encoding AAV U6 GFP, pHelper (Cell Biolabs Inc.) and RapCap DJ plasmids (Cell Biolabs Inc.). 48 h after transfection cells were lysed using freeze-thaw cycles and treated with benzonase (50 U/ml) for 1 h at 37 °C. Lysates were centrifuged at 8,000 x g at 4 °C. Supernatants were collected and filtered using a 0.2 micron filter. Filtrates were passed through pre-equilibrated HiTrap Heparin HP affinity columns (GE HealthCare Life science), followed by washing with Wash Buffer 1 (20 mM Tris, 100 mM NaCl, pH 8.0; sterile filtered). Columns were additionally washed with Wash Buffer 2 (20 mM Tris, 250 mM NaCl, pH 8.0; sterile filtered). Viral particles were eluted using Elution Buffer (20 mM Tris, 500 mM NaCl, pH 8.0; sterile filtered). To exchange Elution Buffer with sterile PBS Amicon Ultra-4 centrifugal filters with 100,000 Da molecular weight cutoff (Merck Millipore) were used. Finally, viral particles were filtered through 0.22 µm Nalgene® syringe filter units (sterile, PSE, Sigma Aldrich), aliquoted and stored at -80 °C until use.

2.4.4. Knockdown of ADAMTS 4 and 5 in neuronal cultures

For immunocytochemical and western blot analysis rat dissociated cortical cultures DIV14 were infected with different AAV- shRNAs and incubated for 1 week at 37°C. Every 2-3 days 50% of the medium was replaced by a fresh aliquot. For immunostaining of proteins neurons were processed according to 2.4.1. In addition, cells were used for western blot to determine the knock-down efficiency at the protein level. Therefore, cells were washed three times with ice-cold 1x PBS, scraped off and centrifuged at 700 x g for 10 min at 4°C. After cells were incubated with an appropriate amount of lysis buffer on ice for 30 min. Samples were cleared by centrifugation at 10,000 x g for

20 min at 4°C. Lysates were incubated with Chondroitinase ABC (0.1 U/µl) for 30 min at 37°C in a thermo shaker, mixed with 5x SDS sample buffer and processed according to 2.6.1.

2.4.5. Optogenetic modulation of cAMP in dissociated cortical neurons

Dissociated rat cortical neurons (DIV 14) were infected with AAV2/7.Syn-bPAC-2A-tdimer (a gift from Christine Gee, ZMNH Hamburg). At DIV 21 infected cultures were treated as described in (Stierl et al., 2011). In brief, cells were treated with a 500-ms flash of a 455 nm LED (0.9 milliwatt*mm^{-2}). Afterwards cells were kept at 37°C followed by staining for the synaptic marker Homer 1 and Rb399 at different time points (5 min, 10 min, 15 min, 30 min, 60 min, 120 min and 180 min). Brevican cleavage was analyzed at Homer 1-positive synapses as described in 2.4.6.

2.4.6. Microscopy and Image analysis

Images were taken using a Zeiss Axioplan 2 epifluorescence microscope equipped with a camera (CoolSNAP™ MYO CCD camera, Visitron Systems) and VisiView® software (Visitron Systems). Images were processed and analyzed using ImageJ (MacBiophotonics, ImageJ Version 1.51w) and OpenView software (Version 1.5 from Noam Ziv). In brief, to analyze the synaptic staining selectively, microscope images were processed to 512px x 512px images using ImageJ. The tool "Box_puncta_Ex" of the OpenView software was used to define synapses. This mask was used to measure intensities of immunoreactivities of interest around defined synapses. To analyze puncta in close vicinity of other puncta the tool "Match_Set1" was used in addition. Here, images rotated by 90° were used as quality control.

2.5. *In vivo* Pharmacology

Adult male Wistar rats were injected with SKF38393 (5 mg/kg body weight, i.p.) or vehicle as described in (Schicknick et al., 2008). 1h after injection rats were anesthetized with isoflurane followed by decapitation with a guillotine (This part of the experiment was done by H. Niekisch in the lab of M. Happel at LIN). For further use the prefrontal cortex (PFC), hippocampus and rest of the brain were dissected and stored at -80 °C.

All experimental procedures were carried out in accordance with the EU Council Directive 86/609/EEC and were approved and authorized by the local Committee for Eth-

ics and Animal Research (Landesverwaltungsamt Halle, Germany) in accordance with the international NIH Guidelines for Animals in Research.

2.6. Biochemistry

Table 6: Buffers for biochemistry

Buffer	Composition
Buffer A	0.32 M sucrose, 5 mM HEPES, pH 7.4
Buffer B	0.32 M sucrose, 5 mM Tris, pH 8.1
5x SDS sample buffer	250 mM Tris/HCl pH 6.8, 7.5 % (w/v) SDS, 30 % (w/v) glycerol, 0.25 % (w/v) bromophenol blue, 0.5 M DTT
1x Electrophoresis buffer	192 mM glycine, 0.1 % (w/v) SDS, 25 mM Tris pH 8.3
Separation gel (20%)	8.25 ml 1.5 M Tris/HCl pH 8.8, 7.5 ml 87 % glycerol, 16.5 ml 40 % acrylamide, 330 µl 10 % SDS, 330 µl 0.2 M EDTA, 22 µl TEMED, 120 µl 0.5 % bromophenol blue, 75 µl 10 % APS, 1.2 % (v/v) 2,2,2-trichlorethanol (TCE)
Separation gel (5%)	8.25 ml 1.5 M Tris/HCl pH 8.8, 17.94 ml dH_2O, 1.89 ml 87% glycerol, 4.12 ml 40 % acrylamide, 330 µl 0.2 M EDTA, 330 µl 10 % SDS, 22 µl TEMED, 118 µl 10 % APS, 1.2 % (v/v) TCE
Stacking gel (5%)	6 ml 0.5 M Tris/HCl pH 6.8, 7.84 ml dH_2O, 5.52 ml 87 glycerol, 3.90 ml 30 % acrylamide, 240 µl 0.2 M EDTA, 240 µl 10 % SDS, 17.2 µl TEMED, 140 µl phenol red, 137 µl 10% APS
1x Blotting Buffer	192 mM glycine, 0.2 % (v/v) SDS, 10 % (v/v) methanol, 25 mM Tris pH 8.3
1x TBS-T	50 mM Tris, 150 mM NaCl, 0.1% Tween-20, pH 7.6

2.6.1. Brain fractionation

For brain fractionation the PFC samples were weighed and homogenized with a Potter S (900 rpm, 12 strokes) in 10 ml/g_{tissue} Buffer A containing a protease inhibitor cocktail (Complete ULTRA Tablets, EDTA-free, EASYpack, Roche Diagnostics). The homogenate was centrifuged at 1,000 x g for 10 min, afterwards the obtained pellet was diluted again in Buffer A (same volume as in the first step) followed by centrifugation at 1,000 x g for 10 min. The obtained supernatant (S1) was centrifuged at 12,000 x g for 20 min. Again the pellet (P2) was re-diluted in Buffer A and centrifuged at 12,000 x g for 20

min. P2 was diluted in Buffer B, added to sucrose gradient and centrifuged at 85,000 x g for 2h. The obtained synaptosomal fraction was incubated with Chondroitinase ABC (Sigma Aldrich) at 37 °C for 30 min. Afterwards samples were prepared for SDS-PAGE by adding 5x SDS loading buffer and heating at 95°C for 10 minutes.

2.6.2. SDS-PAGE with the Laemmli system

Proteins were separated by use of one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under fully denaturing conditions. Gradient gels were used with the separating gel having an acrylamide concentration of 5% on top and 20% at the bottom. The separating gel was covered by the stacking gel to focus the proteins. Gels were placed in an electrophoresis chamber (Mighty Small II MINI Vertical Electrophoresis Unit, Hoefer) and covered with electrophoresis buffer. Protein samples were prepared while diluted with 5x SDS sample buffer and boiled at 95 °C for 5 min, shortly centrifuged at full speed to load the supernatants on the gel. The gel run was performed with 8 mA constantly within the stacking gel and 12 mA during the separation phase. Afterwards gels were activated for 5 min using UV light and used for Western Blotting.

2.6.3. Western Blotting and loading normalization

After SDS-PAGE proteins were electrotransferred from polyacrylamide gels to a Roti®-polyvinylidene fluoride membrane (PVDF membrane, 0.45 µm). Before transfer the membrane was activated in methanol for a few seconds. The protein transfer was performed in a Western Blot chamber (Mighty Small Transfer Tank, Hoefer) filled with 1x Blotting Buffer for 1:45 h at 200 mA. During the transfer the system was cooled at 4 °C. Afterwards the membrane was rinsed with methanol, imaged with ultraviolet (UV) light and processed for immunoblot detection.

TCE incorporation into the SDS-PAGE gels provides detection of proteins after UV light activation. Protein detection on PVDF membranes is possible as well after a short UV exposure. The obtained image was used to normalize sample loading using ImageJ software (MacBiophotonics, ImageJ Version 1.51w) according to (Singh, 2017).

2.6.4. Immunoblot detection

The PVDF membrane was blocked using 5 % non-fat milk powder in 1x TBS-T for 30 min at RT. After the membrane was shortly rinsed with 1x TBS-T and incubated

with primary antibodies diluted in 5% non-fat milk powder in 1x TBS-T overnight at 4 °C. Following three washing steps with 1x TBS-T the membrane was probed with peroxidase-labeled secondary antibodies (Table 2) diluted in 5% non-fat milk powder in 1x TBS-T for 1h at RT. Before detection the membrane was washed three times with 1x TBS-T. Immunoblot detection was then performed with an ECL Chemocam Imager (INTAS Science Imaging Instruments GmbH, Goettingen, Germany).

2.7. Statistical Analysis and graphical representation

For statistical analysis and graphical representation Graphpad Prism 5 for Windows (version 5.02, December 17, 2008) was used. The respective statistical test used is indicated in the figure captions in the Results part. For immunocytochemical analysis four independent experiments were performed, two coverslips per condition prepared and five cells per coverslip imaged and analyzed. Here, the indicated n-number in each caption represents the number of analyzed coverslips. In the case of western blot analysis four independent experiments were analyzed, respectively. Here, the indicated n-number in each caption represents the number of analyzed independent experiments.

3 Results

3.1. BC cleavage is increased at synapses after D1-like DA receptor activation

It has been shown that Mongolian gerbils performed better in a FM discrimination task when the D1/D5 DA receptor agonist SKF38393 was injected systemically shortly before, shortly after or 1 day before conditioning (Schicknick et al., 2008). Furthermore, it has been demonstrated that mice display changes in their ECM composition after the performance of a FM discrimination task (Niekisch et al., 2019). To study if activation of D1-like DA receptors induces changes in the ECM composition in adult rats, D1-like DA receptors were activated pharmacologically using a single dose of SKF38393 (5 mg/kg body weight, i.p.; collaboration with Dr. M. Happel and Dr. H. Niekisch). Afterwards, I investigated if the ECM is altered after receptor activation. I chose the PFC since D1Rs are highly expressed in this brain region (reviewed in (Beaulieu and Gainetdinov, 2011)). To investigate changes in the ECM composition, I analyzed expression levels of the most abundant lecticans BC as well as Acan in the homogenate and synaptosomal fractions of the PFC by western blot analysis. I found that the amount of full-length and cleaved BC is unaltered in the homogenate fraction of control and SKF38393-treated animals (Figure 6D). In the synaptosomal fraction the expression level of full-length BC was decreased by ~ 41 %, while the cleaved fragment was upregulated by nearly 50 % in SKF38393-treated animals (Figure 6D, E). Using the neoepitope-specific antibody Rb399 which detects the N-terminal ADAMTS-derived fragment of BC, I also could detect an increase in the amount of cleaved BC by around 50 % in the synaptosomal fraction, while levels in the homogenate remain unchanged (Figure 6F). Similarly, levels of full-length and cleaved Acan were unaltered in the homogenate (Figure 6G, H). However, in synaptosomal fractions of SKF38393-treated animals the amount of cleaved Acan was significantly increased (Figure 6G). Levels of full-length Acan tend to be reduced in the synaptosomal fraction (Figure 6H). Indeed, activation of D1-like DA receptors affects molecules of the ECM at least in synaptosomal fractions.

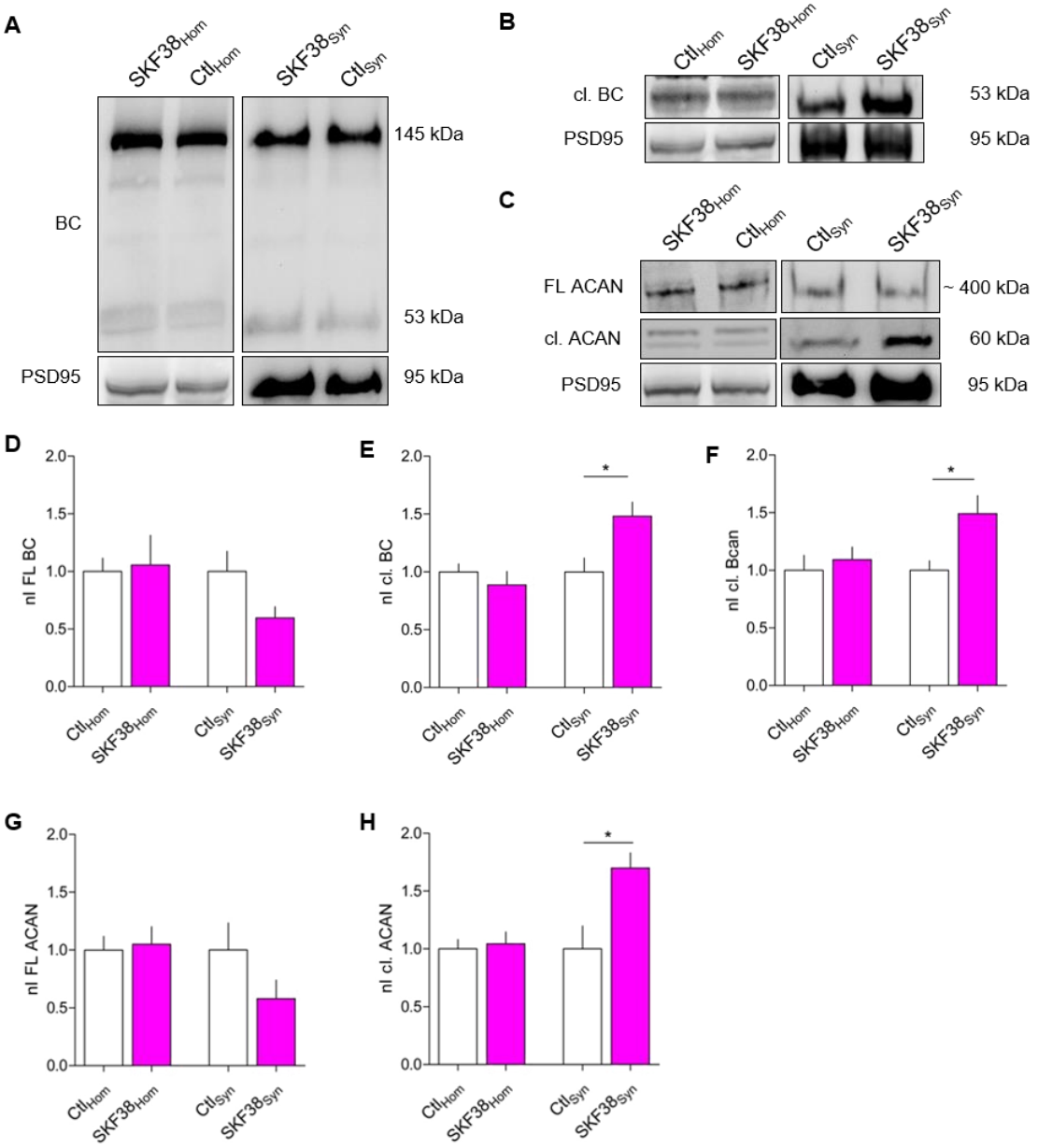

Figure 6: The ECM is altered in synaptosomal fractions of rat PFC after systemic activation of D1-like DA receptors in adult Wistar rats

(A) Representative western blots showing the expression of BC in homogenate (left) and synaptosomal fraction (right). **(A-C)** PSD95 illustrates the efficient enrichment of synaptic structures in the synaptosomal fractions. **(B)** Representative western blots for the expression of aggrecan and its 60 kDa cleaved fragment in the homogenate and synaptosomal fractions. **(C)** Represantitive western blots for the expression of cleaved BC using the neo-epitope-specific antibody Rb399 in the homogenate and the synaptosomal fractions. **(D-E)** Quantification of normalized band intensities. **(D)** The levels of full-length BC are unalterd in the homogenate of control (Ctl) and SKF38393-injected rats (SKF38). In the synaptosomal fraction full-length BC is reduced after SKF38393 injection (homogenate: Ctl, 1 ± 0.1157, n = 4; SKF38, 1.056 ± 0.2574, n = 4; average ± SEM; Unpaired t test; P = 0.8491; synaptosomes: Ctl, 1 ± 0.1745, n = 4; SKF38, 0.5980 ± 0.0961, n = 4; average ± SEM; Unpaired t test; P = 0.0901). **(E)** The expression of cleaved BC detected by ms α-brevican (Table 2) is unalterd in the homogenate after D1-like DA receptor activation, but significantly increased in the synaptosomal fraction (homogenate: Ctl, 1 ± 0.0696, n = 4; SKF38, 0.8875 ± 0.116, n = 4; average ± SEM; Unpaired t test; P = 0.4373; synaptosomes: Ctl, 1 ± 0.12, n = 4; SKF38, 1.481 ± 0.1221, n = 4; average ± SEM; Unpaired t test; * P = 0.0308). **(F)** The rise in cleaved BC in the synaptosomal fraction

can also be detected by the neo-epitope-specific antibody Rb399 (homogenate: Ctl, 1 ± 0.1301, n = 4; SKF38, 1.092 ± 0.1098, n = 4; average ± SEM; Unpaired t test; P = 0.8014; synaptosomes: Ctl, 1 ± 0.0826, n = 4; SKF38, 1.491 ± 0.1563, n = 4; average ± SEM; Unpaired t test; * P = 0.032). **(G)** Full-length Acan is unaltered in the homogenate after SKF38393 treatment. The slight decrease in the synaptosomal fraction is not significant (homogenate: Ctl, 1 ± 0.1176, n = 4; SKF38, 1.05 ± 0.1511, n = 4; average ± SEM; Unpaired t test; P = 0.8014; synaptosomes: Ctl, 1 ± 0.2342, n = 4; SKF38, 0.5798 ± 0.1598, n = 4; average ± SEM; Unpaired t test; P = 0.1889). **(H)** The expression of cleaved Acan is unalterd in the homogenate after D1-like DA receptor activation, but significantly increased in the synaptosomal fraction (homogenate: Ctl, 1 ± 0.0815, n = 4; SKF38, 1.044 ± 0.1017, n = 4; average ± SEM; Unpaired t test; P = 0.7458; synaptosomes: Ctl, 1 ± 0.1985, n = 4; SKF38, 1.7 ± 0.1317, n = 4; average ± SEM; Unpaired t test; * P = 0.0261). (nI FL BC/ FL Acan = normalized intesity of full-length BC/ full-length Acan; nI cl. BC/Acan = normalized intensity of cleaved BC/cleaved Acan)

3.2. Dissociated cortical cultures express DA receptors of the D1 and D2 type

Since I could show that *in vivo* the ECM molecules BC and Acan are altered after the activation of DA D1-like receptors, I was interested in investigating the underlying molecular signalling mechanism. As a model system I used primary rat cortical neurons and verified first the expression of D1Rs but also D2Rs in this system. Therefore, cells (DIV21) were stained for either D1Rs or D2Rs in combination with Homer 1 as a synaptic and MAP2 as a somato-dendritic marker. I observed that, indeed, rat dissociated cortical neurons (DIV21) do express D1Rs appearing as tiny, bright puncta along dendrites (Figure 7A). In addition, D1Rs were also found in close vicinity of Homer 1-positive synaptic puncta (Homer 1+) (Figure 7B). Around 44 % of D1R-positive puncta (D1R+) appear in close vicinity of Homer 1+ puncta, while only 22 % of Homer 1+ puncta are located in close vicinity of D1R+ puncta. As a quality control I also validated images rotated by 90° according to (Dunn et al., 2011). These values were significantly lower than for the original images indicating a specific co-distribution of D1R+ and Homer 1+ puncta close to each other (Figure 7C, D). In addition to D1Rs also D2Rs are well expressed in dissociated cortical cultures, as shown previously in (Sun et al., 2005). I could confirm the expression of D2Rs as small bright puncta along dendrites and in close vicinity of Homer 1+ excitatory synapses (Figure 8A, B). Again, I analyzed the amount of D2Rs being located in close vicinity of Homer 1+ synapses and used images rotated by 90° as quality control. Here, around 47 % of D2Rs appear in close vicinity of Homer 1+ excitatory synapses (Figure 8C). Nearly 25 % of Homer 1+ synapses are found in close vicinity of D2Rs (Figure 8D). Taken together, I could demonstrate that D1Rs as well as D2Rs are expressed in dissociated cortical neurons. From previous studies it is known that this cul-

ture system also expresses a fully-fledged ECM and can thus be used to study the molecular signalling mechanisms underlying DA-induced matrix trimming.

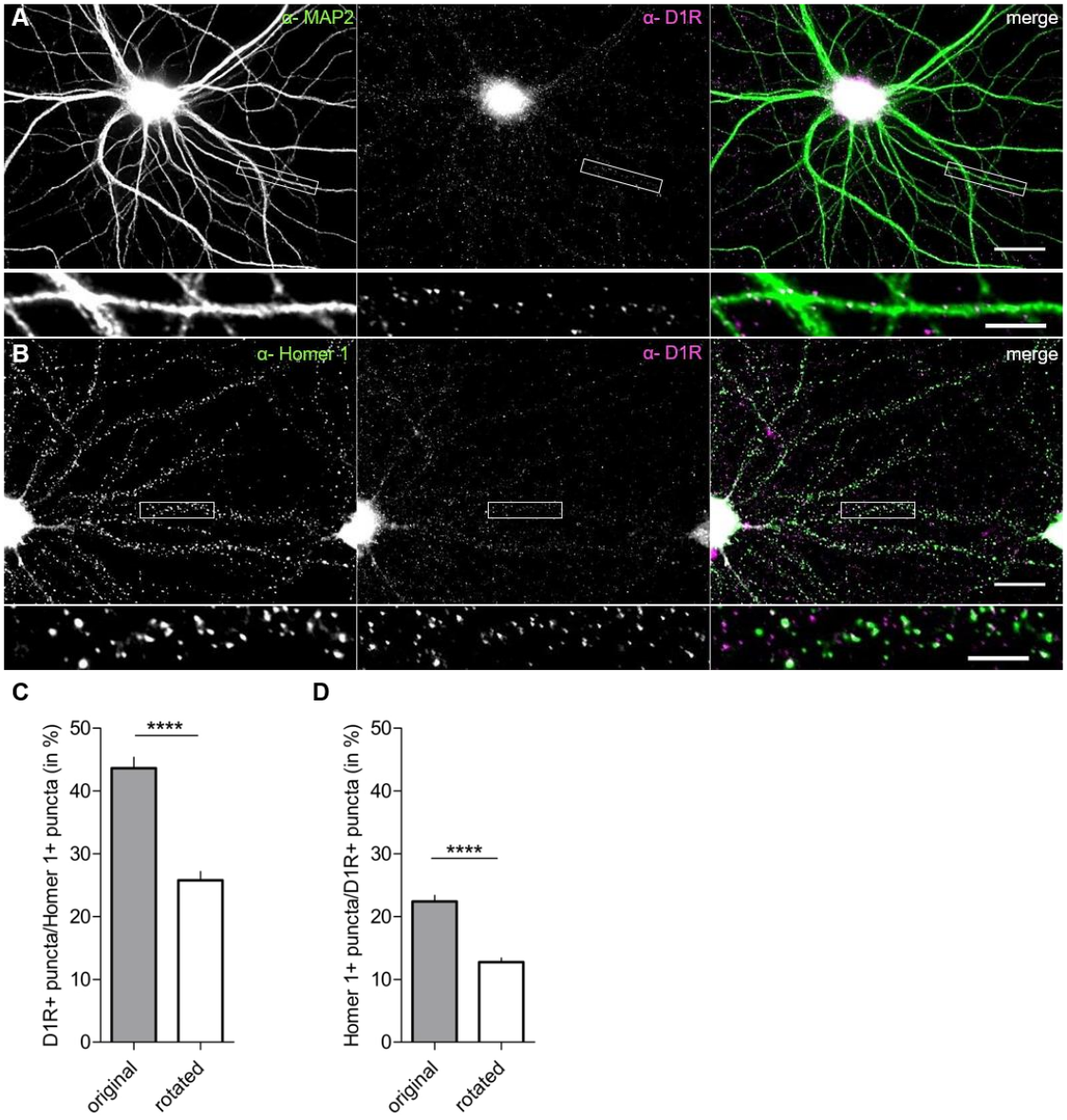

Figure 7: D1Rs are expressed in rat dissociated cortical cultures

(A-B) Rat dissociated cortical neurons (DIV 21) were stained for the somato-dendritic marker MAP2 (A, green) or the excitatory synaptic marker Homer 1 (B, green) and DA D1 receptors (D1Rs, magenta). D1Rs appear as bright puncta along dendrites (scale bar: 20 µm, close-up: 5 µm) and can be found in close vicinity of Homer 1-positive puncta. Some D1R- and Homer 1-positive puncta show an overlap (scale bar: 20 µm, close-up: 5 µm). **(C-D)** Quantification of D1R-positive puncta. **(C)** In rat dissociated cortical cultures (DIV21) around 44 % D1 DA receptor-positive puncta are in close vicinity of Homer 1-positive synaptic puncta (original, 43.65 ± 1.727, n = 30; rotated, 25.79 ± 1.389, n = 30; average % ± SEM %; Paired t test; *** P < 0.0001) (n = number of analyzed neurons). **(D)** Around 22 % of Homer 1-positive synaptic puncta are in close vicinity of D1R-positive puncta (original, 22.41 ± 0.9848, n = 30; rotated, 12.75 ± 0.6574, n = 30; average % ± SEM %; Unpaired t test; *** P < 0.0001) (n = number of analyzed neurons).

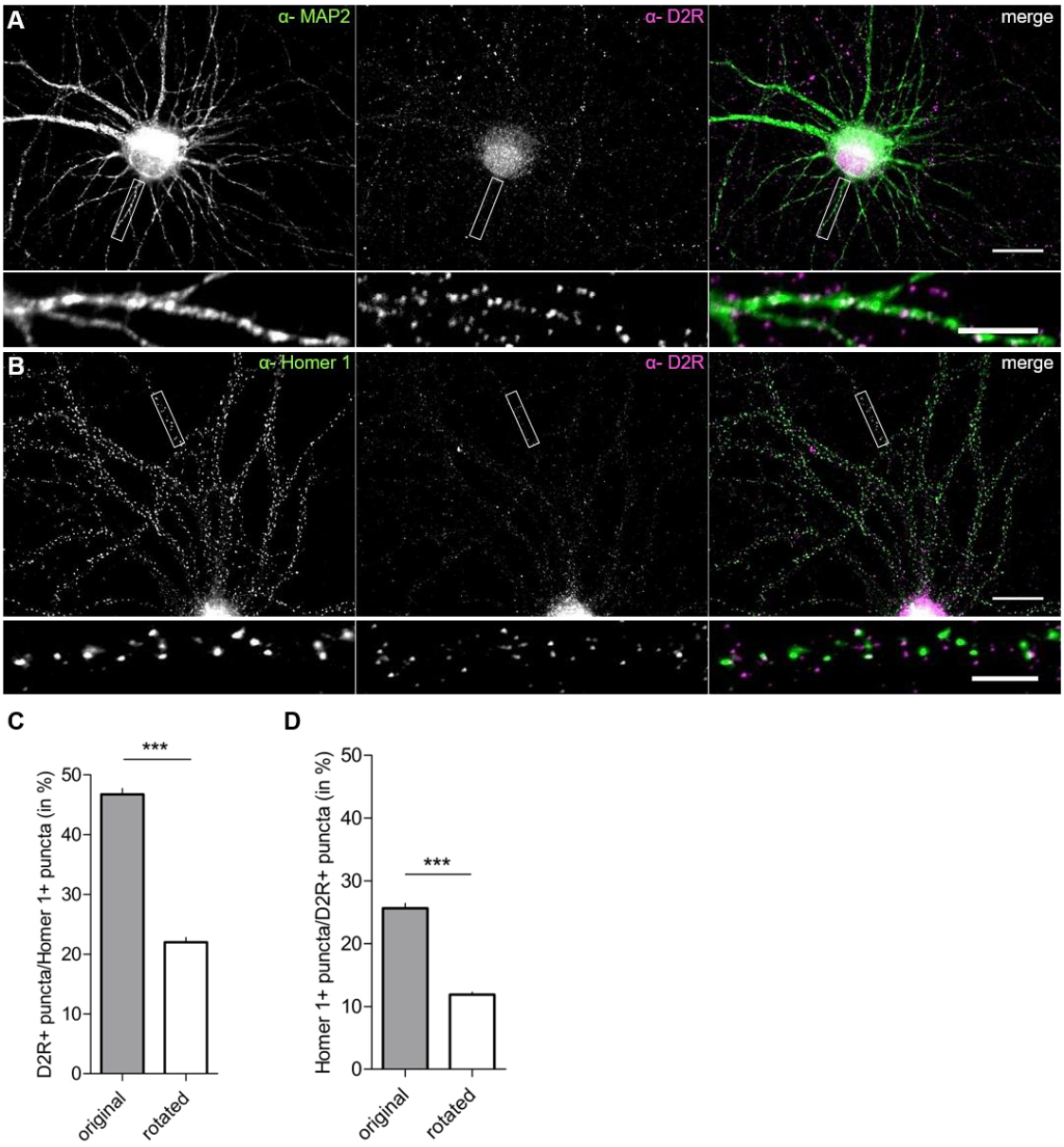

Figure 8: D2-type DA receptors are expressed in rat dissociated cortical cultures

(A-B) Rat dissociated cortical neurons (DIV21) were stained for the somato-dendritic marker MAP2 (A, green) or the excitatory synaptic marker Homer 1 (B, green) and D2 DA receptors (D2Rs, magenta). D2Rs appear as bright puncta along dendrites (scale bar: 20 µm; close-up: 5 µm) and in close vicinity of Homer 1-positive puncta (scale bar: 20 µm; close-up: 5 µm)**.** **(C-D)** Quantification of D2R-positive puncta. **(C)** Around 47 % of D2R-positive puncta are in close vicinity of Homer 1-positive synaptic puncta (original, 46.75 ± 0.9586, n = 40; rotated, 21.99 ± 0.8152, n = 40; average % ± SEM %; Paired t test; *** P < 0.0001) (n = number of analyzed neurons). **(D)** Around 25 % of Homer 1-positive synaptic puncta are in close vicinity of D2R-positive puncta (original, 25.66 ± 0.7576, n = 40; rotated, 11.87 ± 0.3838, n = 40; average % ± SEM %; Paired t test; *** P < 0.0001) (n = number of analyzed neurons).

3.3. Activation of D1-like but not D2- like DA receptors augments perisynaptic cleavage of BC

To investigate the molecular signalling pathway underlying the DA-dependent ECM remodeling, I first verified if the observed *in vivo* effect can be confirmed in rat dissociated cortical cultures. According to pharmacological approaches described in the literature, I used a different agonist for the *in vitro* approach. SKF38393 is often used in *in vivo* studies (Schicknick et al., 2008; Farahmandfar et al., 2016; Deliano et al., 2018; Pekcec et al., 2018; Ruan et al., 2019), while the now used SKF81297 is used mostly for *in vitro* investigations (Chen et al., 2007; Dai et al., 2008; Jürgensen et al., 2011; Li et al., 2016). For the performed experiments I used dissociated cortical cultures at DIV21 when the ECM is considered to be mature (Miyata et al., 2005; John et al., 2006; Dityatev et al., 2007; Frischknecht et al., 2009). I treated dissociated rat cortical cultures (DIV 21) with the agonist for D1-like DA receptors SKF81297 (SKF) and to test for D2-like DA receptors contributions I used Quinpirole (Quin). Afterwards, I analyzed the perisynaptic amount of total and cleaved BC at excitatory and inhibitory synapses and along dendrites. BC cleavage was significantly increased by around 86 % around excitatory synapses only after stimulation of D1-like DA receptors (Figure 9B, right graph). Here, the amount of total BC was unaltered in comparison to control conditions upon activation of either D1-like or D2-like DA receptors (Figure 9B, left graph). Surprisingly, at GAD65-positive inhibitory synapses I could not detect any changes neither in the amount of total nor in cleaved BC after stimulation of the afore-mentioned receptors (Figure 9D). Furthermore, I could not identify any changes in the amount of total as well as cleaved BC along dendrites in neither condition, D1-like and D2-like DA receptor activation (Figure 9E). Since in the *in vivo* experiment I did not only observe an increase in BC cleavage but also in the cleavage of Acan after D1-like DA receptor activation, I tested for Acan cleavage *in vitro* as well. Indeed, Acan cleavage also increased only after D1-like DA receptor stimulation. SKF-dependent Acan cleavage appeared to be twice as high then under control conditions (Figure 9H, right graph). However, activation of D2-like DA receptors resulted in a slight, but not significant increase (Figure 9H, left graph). Thus, I could verify that DA-dependent BC and Acan cleavage appears only at excitatory synapses after D1-like DA receptor activation. Activation of D2-like DA receptors did not affect ECM remodelling in my experiments at all. Therefore, in

the following experiments I focussed on excitatory synapses and on D1-like DA receptor stimulation and used BC as model molecule.

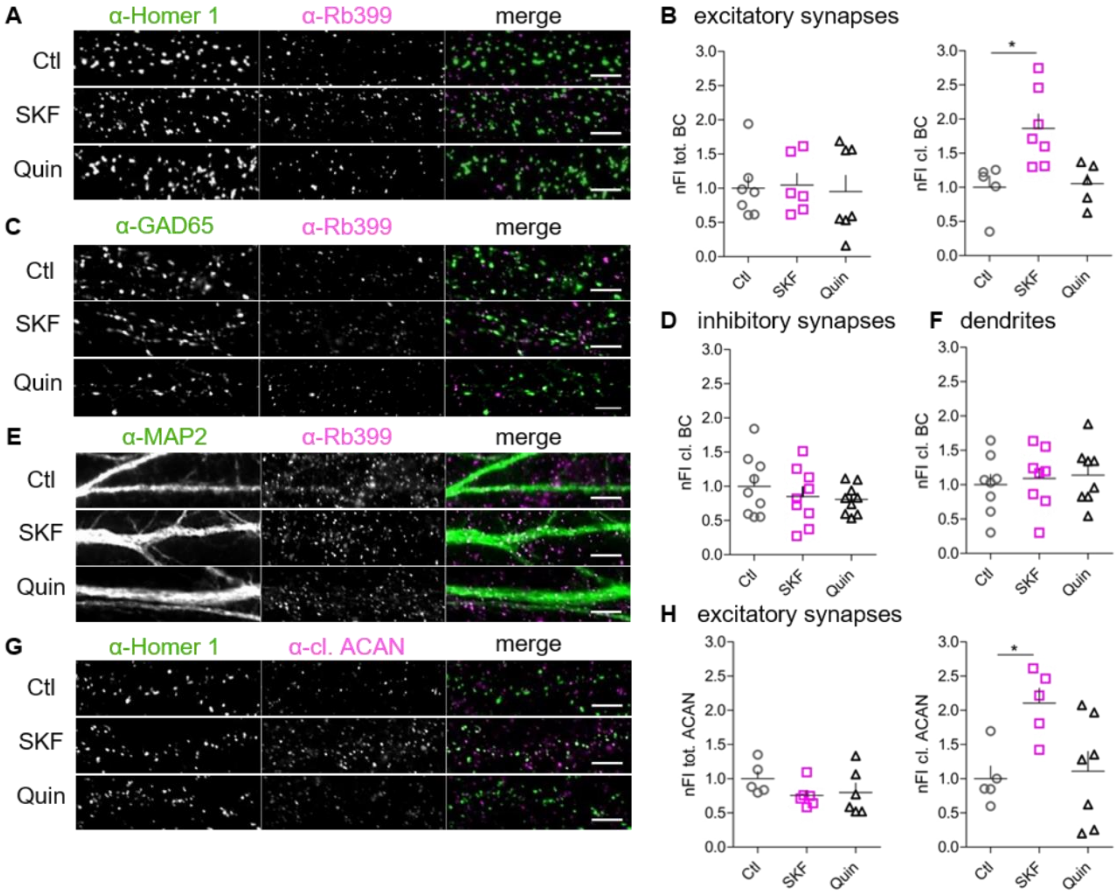

Figure 9: Modification of perisynaptic ECM at excitatory synapses is DA D1-like receptor-dependent

(A,C,F,G) Rat dissociated cortical neurons (DIV21) were either non-treated (Ctl) or treated with the D1-like DA receptor agonist SKF or the D2-like DA receptor agonist Quin and stained for the excitatory synaptic marker Homer 1 (A+G, green), the inhibitory synaptic marker GAD65 (C, green) or the somato-dendritic marker MAP2 (F, green) and extracellular cleaved BC detected by the neo-epitope-specific antibody Rb399 (A,C,F, magenta) or extracellular cleaved Acan (G, magenta) (scale bar: 5 µm). **(B)** At Homer 1-positive excitatory synapses the amount of cleaved BC is significantly increased after D1-like DA receptor activation but not after D2-like DA receptor activation (tot. BC: Ctl, 1 ± 0.1742, n = 7; SKF, 1.045 ± 0.1745, n = 6; Quin, 0.9502 ± 0.2380, n = 7; average ± SEM; One-way ANOVA; P = 0.9471; cl. BC: Ctl, 1 ± 0.1673, n = 5; SKF, 1.864 ± 0.21, n = 7; Quin, 1.054 ± 0.1403, n = 5; average ± SEM; One-way ANOVA; P = 0.007; Dunnett's Multiple Comparison Test; ** P<0.05). **(D, E)** The amount of cleaved BC remained unaltered at inhibitory synapses (D) and on dendrites (E) after DA receptor activation ((D): Ctl, 1 ± 0.149, n = 9; SKF, 0.8521 ± 0.1359, n = 9; Quin, 0.8077 ± 0.0715, n = 9; average ± SEM; One-way ANOVA; P = 0.5239; (E): Ctl, 1 ± 0.1511, n = 8; SKF, 1.09 ± 0.1542, n = 8; Quin, 1.139 ± 0.1514, n = 8; average ± SEM; One-way ANOVA; P = 0.809). **(H)** Acan cleavage was also increased at Homer 1-positive excitatory synapses after D1-like, but not D2-like DA receptor activation. The amount of total Acan was unaltered (tot. Acan: Ctl, 1 ± 0.1055, n = 5; SKF, 0.7565 ± 0.0728, n = 6; Quin, 0.7995 ± 0.0728, n = 6; average ± SEM; One-way ANOVA; P = 0.2938; cl. Acan: Ctl, 1 ± 0.1859, n = 5; SKF, 2.105 ± 0.2179, n = 5; Quin, 1.108 ± 0.291, n = 7; average ± SEM; One-way ANOVA; P = 0.0204; Dunnett's Multiple Comparison Test; * P<0.05). (nFI tot. BC/Acan = normalized fluorescent intensity of total BC/total Acan; nFI cl. BC/cl. Acan = normalized fluorescent intensity of cleaved BC/cleaved Acan)

3.4. Perisynaptic BC cleavage is indeed DA-dependent

To now investigate if the observed effect is truly D1-like DA receptor-dependent, I treated neuronal cultures (DIV 21) with SCH23390, an antagonist for D1-like DA receptors, 30 minutes before stimulation and analyzed again the amount of cleaved BC in the perisynaptic area of excitatory and inhibitory synapses and along dendrites. As expected, inhibitory synapses and dendrites revealed no changes in BC cleavage neither following DA receptor stimulation nor inhibition (Figure 10B, C). Inhibition of DA D1-like receptors alone and inhibition followed by stimulation of the receptors revealed no significant changes in the perisynaptic amount of cleaved BC compared to control conditions at excitatory synapses (Figure 10A). Thus, these results suggest that the observed effect of increased BC cleavage is truly D1-like DA receptor-dependent.

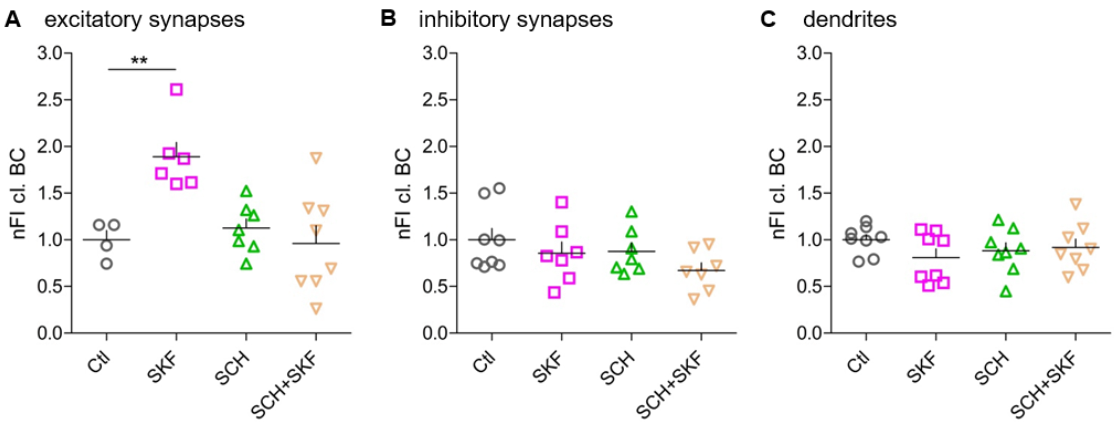

Figure 10: Inhibition of D1-like DA receptors abolishes SKF-induced BC cleavage

(A) Treatment with the D1-like DA receptor antagonist SCH23390 abolishes the SKF-induced increase in perisynaptic BC cleavage (Ctl, 1 ± 0.0999, n = 4; SKF, 1.888 ± 0.1542, n = 6; SCH, 1.125 ± 0.0998, n = 7; SKF+SCH, 0.9609 ± 0.1892, n = 8; average ± SEM; One-way ANOVA; P = 0.0014; Dunnett's Multiple Comparison Test; ** P<0.001). **(B, C)** The amount of cleaved BC was unaltered at inhibitory synapses (B) and on dendrites (C) ((B): Ctl, 1 ± 0.1217, n = 8; SKF, 0.8552 ± 0.1203, n = 7; SCH, 0.8754 ± 0.0924, n = 7; SCH+SKF, 0.6704 ± 0.0823, n = 7; average ± SEM; One-way ANOVA; P = 0.2052; (C): Ctl, 1 ± 0.054, n = 8; SKF, 0.8099 ± 0.0934, n = 8; SCH, 0.883 ± 0.0848, n = 8; SCH+SKF, 0.919 ± 0.0893, n = 8; average ± SEM; One-way ANOVA; P = 0.4384) (nFI cl. BC = normalized fluorescent intensity of cleaved BC).

3.5. D1Rs are less expressed at GAD65-positive inhibitory synapses

Stimulation of D1-like DA receptors resulted in enhanced perisynaptic BC and Acan cleavage only at excitatory synapses. I hypothesized that differential expression levels of D1Rs at inhibitory versus excitatory synapses could explain this finding. Therefore, I stained dissociated cortical neurons (DIV21) for GAD65 and D1Rs and investigated the expression of D1Rs in close vicinity of GAD65-positive (GAD65+) synaptic puncta.

Indeed, I could find less D1Rs expressed in close vicinity of GAD65+ inhibitory synapses compared to Homer 1+ excitatory synapses.

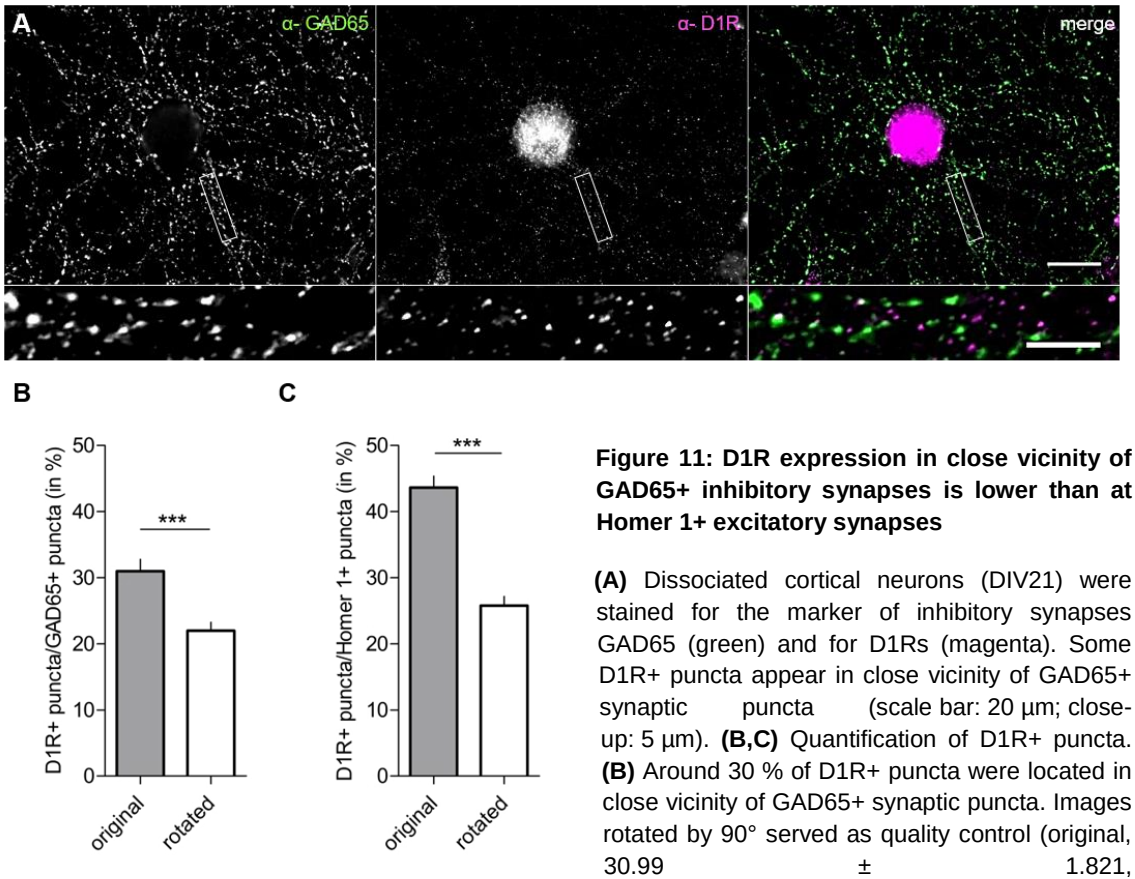

Figure 11: D1R expression in close vicinity of GAD65+ inhibitory synapses is lower than at Homer 1+ excitatory synapses

(A) Dissociated cortical neurons (DIV21) were stained for the marker of inhibitory synapses GAD65 (green) and for D1Rs (magenta). Some D1R+ puncta appear in close vicinity of GAD65+ synaptic puncta (scale bar: 20 µm; close-up: 5 µm). **(B,C)** Quantification of D1R+ puncta. **(B)** Around 30 % of D1R+ puncta were located in close vicinity of GAD65+ synaptic puncta. Images rotated by 90° served as quality control (original, 30.99 ± 1.821, n = 34; rotated, 21.99 ± 1.29, n = 34; average % ± SEM %; Paired t test; *** P = 0.0002) (n = number of analyzed neurons). **(C)** In rat dissociated cortical cultures (DIV21) around 44 % D1R+ puncta are in close vicinity of Homer 1+ synaptic puncta (original, 43.65 ± 1.727, n = 30; rotated, 25.79 ± 1.389, n = 30; average % ± SEM %; Paired t test; *** P < 0.0001) (n = number of analyzed neurons).

I could detect around 15 % more D1R+ puncta in close vicinity of Homer 1+ excitatory synapses compared to GAD65+ inhibitory synapses. This differential expression may contribute to the SKF-induced increasing effect of BC and Acan cleavage at excitatory synapses.

3.6. ECM-modifying proteases are involved in SKF-dependent BC cleavage

Increased amounts of cleaved BC after D1-like DA receptor stimulation are pointing to ECM-modifying proteases being activated and/or released to degrade CSPGs of the perisynaptic ECM upon DA signalling. Based on previous findings the most promising candidate proteases for cleaving BC are ADAMTS 4 and ADAMTS 5 (Matthews et al., 2000; Yuan et al., 2002; Hamel et al., 2005a; Viapiano et al., 2008; Valenzuela et al.,

2014). To test, whether the ECM-modifying proteases ADAMTS 4 and 5 are involved in SKF-dependent BC cleavage, I treated rat dissociated cortical cultures (DIV 21) with TIMP3 (tissue inhibitor of metalloproteinases 3), an inhibitor of these proteases, 15 minutes before the stimulation. Neurons treated with TIMP3 alone showed a slight decrease in BC cleavage by nearly 30 % indicating that there might be a pool of already active proteases in the extracellular space (Figure 12A). Stimulation of D1-like DA receptors, while ADAMTS 4 and 5 are still blocked, resulted in a 40 % decrease in BC cleavage around excitatory synapses (Figure 12A) as compared to unblocked cells. These results lead to the assumption that TIMP3-sensitive proteases like ADAMTS 4 and ADAMTS 5 are essential for perisynaptic BC cleavage after stimulation of D1-like DA receptors.

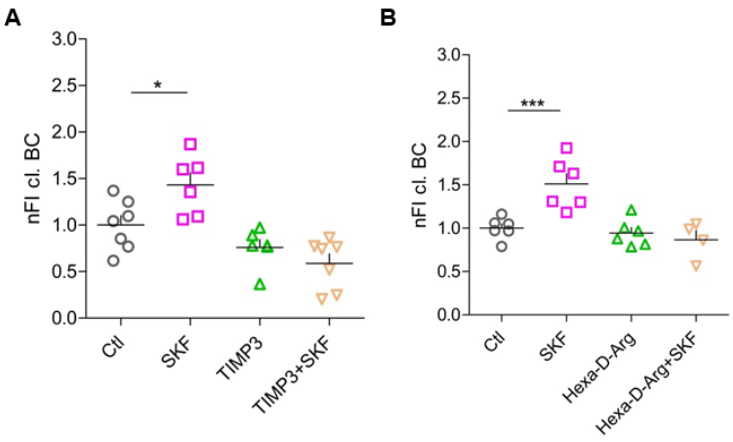

Figure 12: ADAMTS 4 and 5 are involved in SKF-induced perisynaptic BC cleavage

(A) Inhibition of ADAMTS 4 and 5 with TIMP3 results in a decrease in BC cleavage around excitatory synapses (Ctl, 1 ± 0.1013, n = 7; SKF, 1.623 ± 0.2566, n = 6; TIMP3, 0.7602 ± 0.0851, n = 6; SKF+TIMP3, 0.5882 ± 0.1017, n = 7; average ± SEM; One-way ANOVA; P = 0.0489; Dunnett's Multiple Comparison Test; * P<0.05).

(B) Inhibition of the pro-protein convertase PACE 4 with Hexa-D-Arginine (Hexa-D-Arg) diminished SKF-induced BC cleavage at excitatory synapses (Ctl, 1 ± 0.051, n = 6; SKF, 1.509 ± 0.1183, n = 6; Hexa-D-Arg, 0.9441 ± 0.0636, n = 6; Hexa-D-Arg+SKF, 0.8651 ± 0.1077, n = 4; average ± SEM; One-way ANOVA; P = 0.0002; Dunnett's Multiple Comparison Test; ** P<0.05) (nFI cl. BC = normalized fluorescent intensity of cleaved BC).

However, all members of the ADAMTS enzyme family are expressed in their inactive form carrying an N-terminal pro-domain which needs to be cleaved off before activation (Tortorella et al., 2005; (reviewed in (Kelwick et al., 2015)). Proprotein convertases (PPCs), such as furin and furin-like PPCs (e.g PACE 4 and PC5/6) are able to remove the N-terminal pro-domain efficiently. The mentioned furin-like PPCs cleave at residues Arg^{212}/Phe^{213} (Tortorella et al., 2005), thus I concluded to pre-treat my cultures for 10 min with Hexa-D-Arginine (Hexa-D-Arg) which consists of six consecutive arginine residues and inhibits furin-like PPCs. Neurons either treated with the inhibitor alone or with inhibitor and SKF revealed an unaltered amount of cleaved BC around excitatory synapses (Figure 12B). In sum, ADAMTS 4 and ADAMTS 5 might be activated by furin or furin-like PPCs in a SKF-dependent manner.

3.7. Validation of shRNAs of ADAMTS 4 and ADAMTS 5

Since TIMP3 inhibits both ADAMTS 4 and 5 and also several ADAMs (e.g. ADAM12, ADAM17) (Fröhlich et al., 2013; Uchikawa et al., 2015) or MMPs such as MMP2 or MMP9 (Zhai et al., 2018), I performed knockdown experiments using small hairpin RNAs (shRNAs) against ADAMTS 4 and 5 to nail down individual contributions of both proteases for the SKF-induced BC cleavage. To increase the efficiency of the knockdown, we produced recombinant adeno-associated viruses (rAAVs) of the aforementioned shRNAs in cooperation with Dr. Rahul Kaushik from DZNE Magdeburg. Altogether, I tested two different shRNA constructs for each protease. Therefore, I infected rat dissociated cortical cultures either with shADAMTS 4 or shADAMTS 5 at DIV 14. A scramble construct was used as negative control and was also infected at DIV 14. At DIV 21 neurons were stained for the synaptic marker Homer 1 and the ECM-modifying proteases ADAMTS 4 or ADAMTS 5 according to 2.4.1 to analyse knockdown efficiency at synapses (Figure 13E, F). In addition, total ADAMTS 4 and 5 protein levels were determined according to 2.6.1 and 2.6.3 (Figure 13A, B). In both cases, construct no.1 did not show an efficient knockdown neither at total protein levels nor at synapses (Figure 13C, D, G and H). Constructs no. 2 for both proteases displayed a reasonable knockdown efficiency. The construct shADAMTS 4.2 led to reduction of overall expression by around 75 %, while the synaptic amount of ADAMTS 4 showed a reduction of nearly 70 % (Figure 13C, G). Cultures infected with the construct shADAMTS 5.2 revealed a reduction in the total expression of ADAMTS 5 by nearly 65 %, while the synaptic expression was reduced by nearly 75 %. All following experiments were performed with the constructs no. 2 (Figure 13D, H).

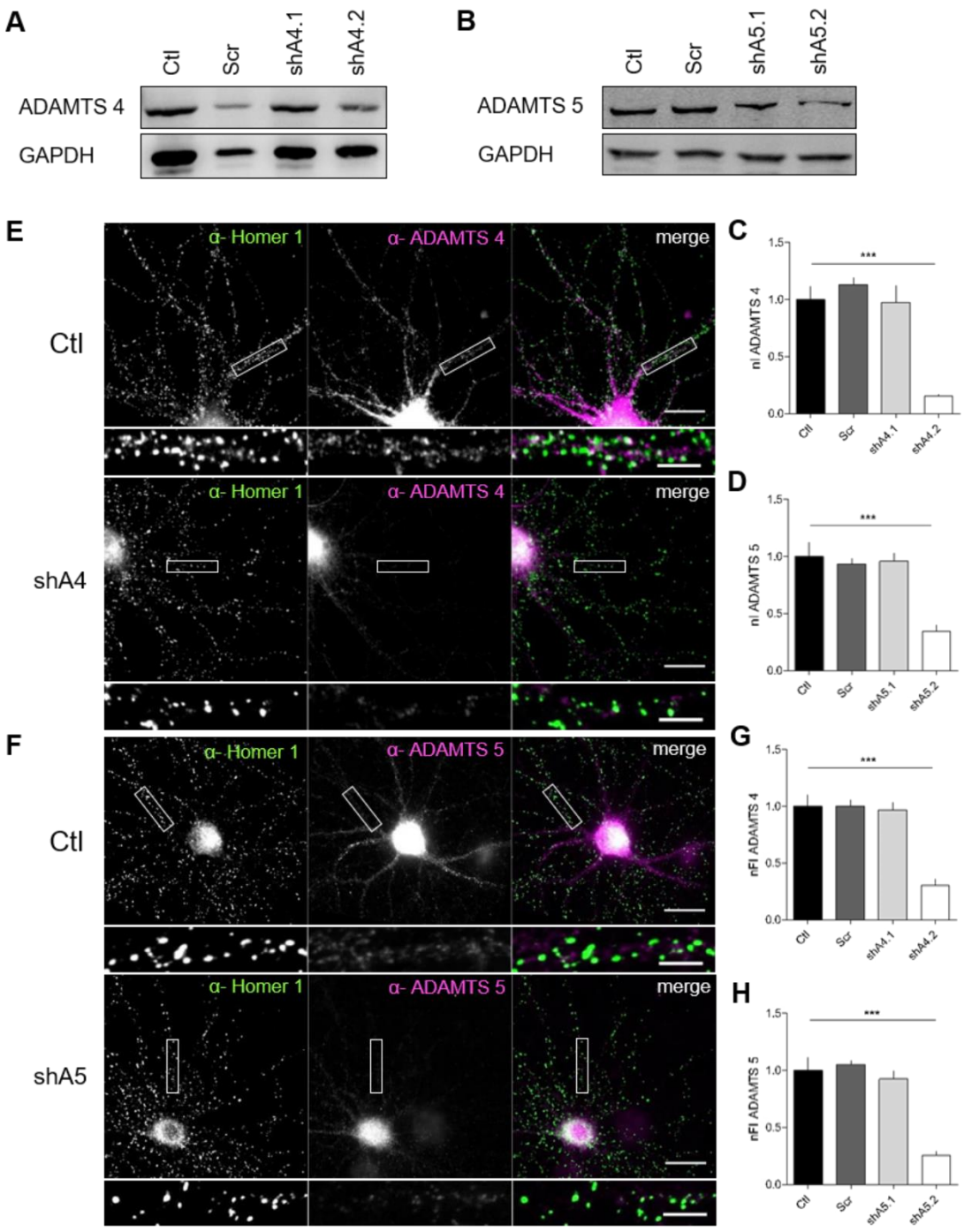

Figure 13: For each protease one shRNA construct out of two tested showed a significant knockdown

(A,B) Representative western blots to illustrate the knockdown efficiency of shRNA construct for ADAMTS 4 (A) and ADAMTS 5 (B). GAPDH was used as loading control (lower panel). **(C,D,G,H)** Quantitative analysis of total ADAMTS 4 and 5 protein levels. **(C)** Construct shADAMTS 4.2 (shA4.2) showed a high knockdown efficiency on total protein level, while construct shADAMTS 4.1 (shA4.1) showed no knockdown at all (Ctl, 1 ± 0.113, n = 5; Scr, 0.9759 ± 0.0246, n = 5; shA4.1, 0.887 ± 0.0535, n = 3; shA4.2, 0.2461 ± 0.0188, n = 3; average ± SEM; One-way ANOVA; P = 0.0001; Dunnett's Multiple Comparison Test; *** P<0.05). **(D)** On total ADAMTS protein level construct shADAMTS 5.2 (shA5.2) is the only construct that shows an efficient knockdown (Ctl, 1 ± 0.1234, n = 6; Scr, 0.9407 ± 0.0379, n = 6; shA5.1, 0.958 ± 0.0696, n = 4; shA5.2, 0.3465 ± 0.0487, n = 4; average ± SEM; One-way ANOVA; P = 0.0003; Dunnett's Multiple Comparison Test; *** P<0.05).

(E) Rat dissociated cortical cultures (DIV14) were either non-infected (Ctl) or infected with shADAMTS 4.2 (E, shA4) or shADAMTS 5.2 (F, shA5). At DIV21 cultures were stained for the excitatory synaptic marker Homer 1 (E,F, green) and ADAMTS 4 (E, magenta) or ADAMTS 5 (F, magenta) (scale bar: 20 µm; close-up: 5 µm). **(G,H)** Quantification of ADAMTS 4 and 5 synaptic expression levels. **(G)** Validation of knock-down efficiency at Homer 1-positive synapses revealed that only construct shADAMTS 4.2 (shA4.2) shows an efficient knockdown (Ctl, 1 ± 0.0995, n = 5; Scr, 1.002 ± 0.0546, n = 5; shA4.1, 0.9697 ± 0.062, n = 5; shA4.2, 0.3034 ± 0.0543, n = 6; average ± SEM; One-way ANOVA; P = 0.0001; Dunnett's Multiple Comparison Test; *** P<0.05) (nFI = normalized fluorescent intensity). **(H)** Also, at Homer 1-positive synapses only construct shADAMTS 5.2 displays an efficient knockdown for ADAMTS 5 (Ctl, 1 ± 0.1133, n = 5; Scr, 1.052 ± 0.0352, n = 4; shA5.1, 0.9238 ± 0.0717, n = 4; shA5.2, 0.2588 ± 0.0326, n = 4; average ± SEM; One-way ANOVA; P < 0.0001; Dunnett's Multiple Comparison Test; *** P<0.05). (nI = normalized intensity); (nFI = normalized fluorescent intensity)

3.8. Exclusively neurons were infected by the used rAAVs

To knockdown ADAMTS 4 and ADAMTS 5, we cloned shRNAs into AAV U6 GFP (2.4.3). Since the expression of the cloned shRNAs is under the control of a U6 promotor, both neurons and astrocytes were expected to be infected. Surprisingly, under the conditions used neurons were perfectly infected as indicated by a strong GFP signal, but I was not able to get astrocytes infected with the used AAV (Figure 14).

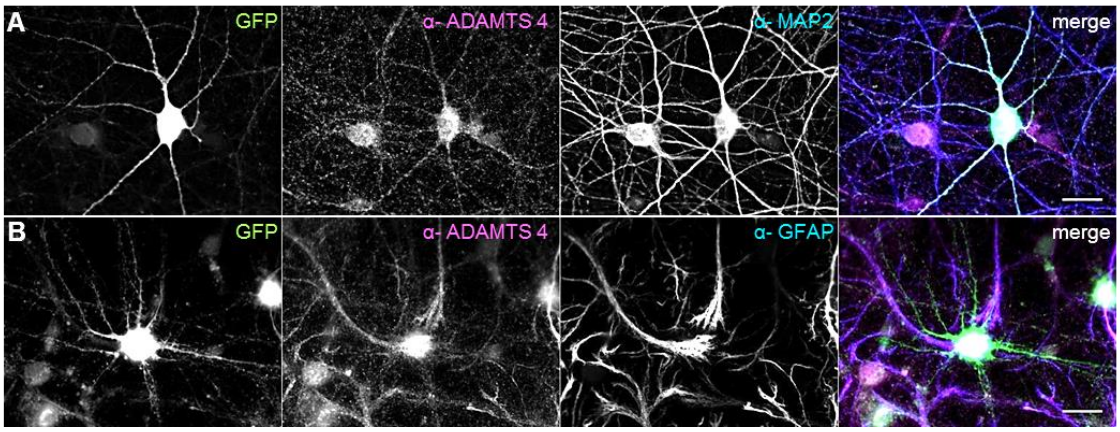

Figure 14: Exclusively neurons were infected by the used rAAVs

(A,B) As an example, dissociated cortical cultures were infected with shADAMTS4.1-AAV GFP at DIV14. At DIV 21 neurons were stained for the somato-dendritic marker MAP2 (A, blue) or the astrocytic marker GFAP (B, blue) and the ECM-modifying protease ADAMTS 4 (magenta). The strong GFP signal (green) indicates the successful infection with the used AAV. Astrocytes display no GFP signal suggesting no infection with the used rAAV (scale bar: 20 µm).

Both neurons and astrocytes do express the ECM-modifying protease ADAMTS 4 (Figure 14). In a recent paper, it was shown that also ADAMTS 5 is expressed by both cell types (Zhang et al., 2014). Since I was only able to detect infected neurons but no astrocytes (Figure 14), I could just make a point about the effects of neuronal expressed ADAMTS 4 and ADAMTS 5 in the following experiment.

3.9. Neuronal ADAMTS 4 and ADAMTS 5 are essential for SKF-dependent perisynaptic BC cleavage

Since TIMP3 is an inhibitor for both ADAMTS 4 and 5 but is not able to distinguish between these enzymes. I infected neuronal cultures (DIV 14) using the under 3.7 validated shRNA constructs as described in 2.4.4. At DIV 21 cultures were stained for Homer 1 and cleaved BC as indicated in 2.4.1. Non-infected cultures (Ctl) and cultures infected with scramble-AAV as a negative control showed the expectable significant increase in BC cleavage upon D1-like DA receptor activation showing that the virus infection does not interfere with the cellular mechanism (Figure 15).

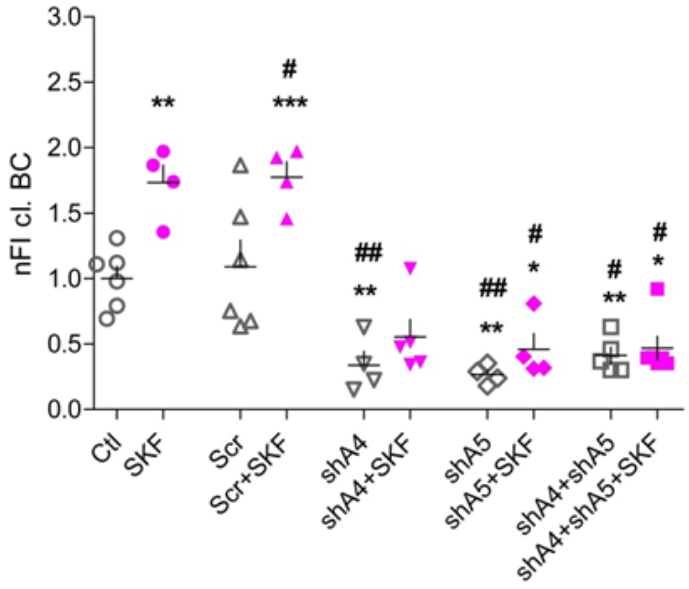

Figure 15: Both ADAMTS 4 and ADAMTS 5 are essential for SKF-dependent BC cleavage

Knockdown of ADAMTS 4, ADAMTS 5 or both proteases together leads to a significant decrease in D1-like DA receptor-induced BC cleavage (Ctl, 1 ± 0.0929, n = 6; SKF, 1.732 ± 0.134, n = 4; Scr, 1.091 ± 0.2033, n = 6; Scr+SKF, 1.773 ± 0.1169, n = 4; shA4, 0.3385 ± 0.1052, n=4; shA4+SKF, 0.5532 ± 0.1343, n= 5; shA5, 0.2658 ± 0.0362, n=4; shA5+SKF, 0.4606 ± 0.1183, n=4; shA4+shA5, 0.4122 ± 0.0619, n=5; shA4+shA5+SKF, 0.4680 ± 0.0911, n=6; average ± SEM; One-way ANOVA; P < 0.0001; Dunnett's Multiple Comparison Test; *** P<0.001). (* = significance compared to Ctl; # = significance compared to Scr; nFI cl. BC = normalized fluorescent intensity of cleaved BC).

Both constructs shADAMTS 4.2 and shADAMTS 5.2 showed a significant decrease in perisynaptic BC cleavage by around 70 % compared to control and scramble conditions. Infected cultures displayed a slight but not significant increase in BC cleavage upon D1-like DA receptor activation (Figure 15). Since there is still cleaved BC detectable, it might be possible that either astrocytic proteases take over or other members of the ADAMTS family are activated and cleave BC. The slight increase in BC cleavage upon D1-like DA receptor activation could indicate that either the knockdown efficiency is too low or there is another source of ADAMTS 4 and also ADAMTS 5 activation, for instance astrocytes within the culture. Despite the open questions, these results indicate that both proteases ADAMTS 4 and ADAMTS 5 are involved in SKF-induced perisynaptic BC cleavage. Surprisingly, cultures infected with both shRNA constructs simulta-

neously revealed nearly comparable results for BC cleavage as obtained for each single infection. Following activation of D1-like DA receptors the amount of cleaved BC was similar to the results for ADAMTS 4 or ADAMTS 5 alone (Figure 15). Based on these results, ADAMTS 4 and ADAMTS 5 seem to be two essential ECM-modifying proteases involved in SKF-induced perisynaptic BC cleavage.

3.10. Network activity and postsynaptic activity are essential for BC cleavage upon D1-like DA receptor activation

To further disentangle the molecular mechanism of this DA-dependent ECM modulation I studied activity prerequisites for it to occur.
In a first approach, I used the synaptotagmin-1 antibody uptake assay (2.4.2) to determine the activity state of single synapses within the culture system (Figure 16A). Bassoon was used as synaptic marker. Cultures treated with the D1-like DA receptor agonist showed a significantly higher fluorescence intensity for synaptotagmin-1 than untreated controls, thus suggesting that the presynaptic activity is significantly increased after D1-like DA receptor stimulation (Figure 16B).

Based on this finding, in a next step I focused on the network activity. Therefore, I silenced the cultures using TTX 10 minutes before activating D1-like DA receptors. The amount of cleaved BC was unaltered after network silencing alone. Network silencing before D1-like DA receptor abolished the rise in BC cleavage (Figure 16C). Thus, I suggest that network activity is essential for SKF-dependent BC cleavage. To shed more light on the underlying molecular mechanism, I first investigated potential other receptors on the postsynaptic site. Since D1-like DA receptors are able to directly and functionally interact with postsynaptically located NMDARs (Haddad, 2005; Missale et al., 2006), I was interested if this type of glutamate receptors is involved in SKF-dependent BC cleavage. First, all types of NMDARs were under investigation and blocked by using AP-5. Here, I could observe that by inhibiting all NMDARs the SKF-dependent BC cleavage was abolished (Figure 16D). In addition, it is well known that NR2B-containing NMDARs are localized in close vicinity of D1 DA receptors in the PFC of rats (Hu et al., 2010). Therefore, I tested if especially NR2B-containing NMDARs might play a role in DA-dependent BC cleavage. Treatment with Ifenprodil revealed that the SKF effect is abolished (Figure 16E), thus this specific subtype of NMDARs could be a potential interaction partner in the underlying signalling pathway.

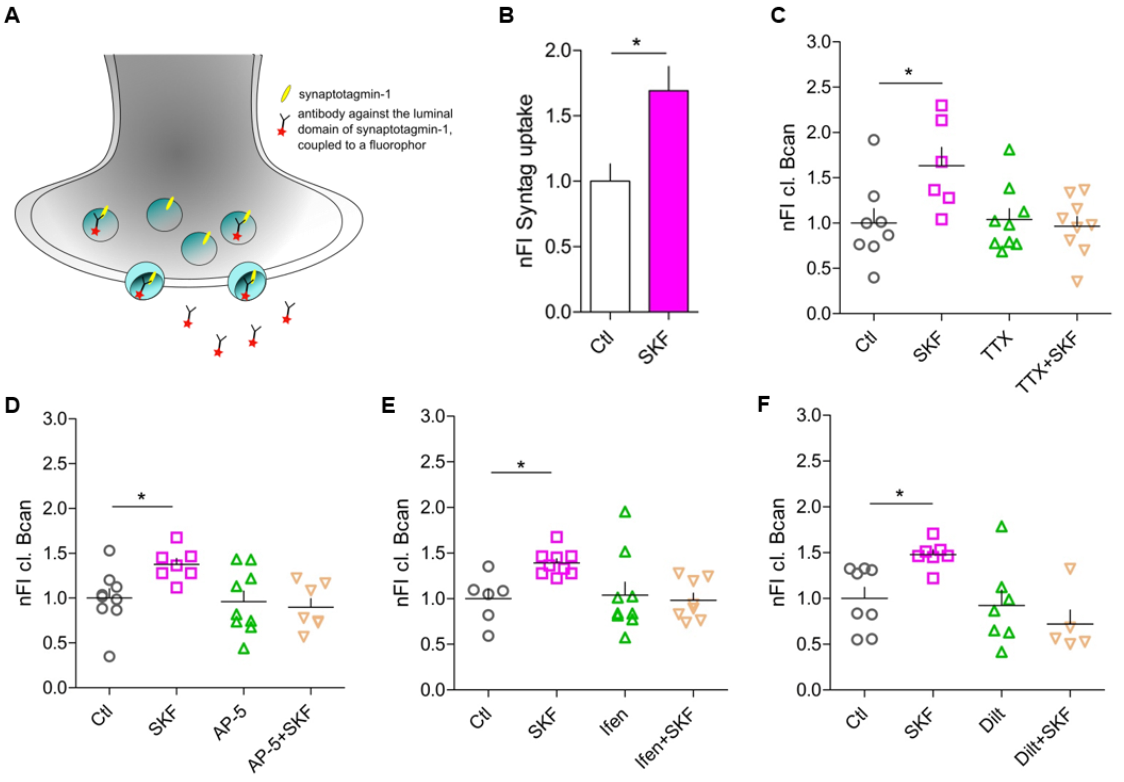

Figure 16: Network activity as well as activity of postsynapses is essential for SKF-induced BC cleavage

(A) Scheme to illustrate the principle of the synaptotagmin-1 antibody uptake assay. Fluorescently labeled antibodies against the luminal domain of synaptotagmin-1 were added to the medium of dissociated cortical neurons (DIV21). When a neurotransmitter vesicle is fused with the presynaptic membrane the antibody is taken up and recycled. Thus, presynaptic activity is visualized by the fluorescent intensity of the antibodies taken up (nFl Syntag uptake = normalized fluorescent intensity of synaptotagmin-1 antibody uptake). **(B)** Presynaptic activity is enhanced after stimulation of D1-like DA receptors by SKF (Ctl, 1± 0.1326, n = 6; SKF, 1,692 ± 0.1849, n = 6; average ± SEM; Unpaired t test; * P = 0.0124) (nFl Syntag uptake = normalized fluorescent intensity of synaptotagmin uptake). **(C)** Silencing neuronal networks with TTX results in an unaltered BC cleavage (Ctl, 1 ± 0.1599, n = 8; SKF, 1.632 ± 0.2036, n = 6; TTX, 1.039 ± 0.1211, n = 9; SKF+TTX, 0.9663 ± 0.1061, n = 9; average ± SEM; One-way ANOVA; P=0.0168; Dunnetti's Multiple Comparison Test; * P<0.05). **(D)** Inhibition of all types of NMDARs with AP-5 abolishes SKF-induced perisynaptic BC cleavage (Ctl, 1 ± 0.105, n = 9; SKF, 1.376 ± 0.0671, n = 7; AP-5, 0.9599 ± 0.1183, n = 9; SKF+AP-5, 0.8976 ± 0.0964, n = 7; average ± SEM; One-way ANOVA; P = 0.0187; Dunnett's Multiple Comparison Test; * P<0.05). **(E)** Especially, inhibition of NR2B-containing NMDARs results in unaltered BC cleavage (Ctl, 1 ± 0.107, n = 6; SKF, 1.392 ± 0.046, n = 9; Ifen, 1.039 ± 0.1436, n = 9; SKF+Ifen, 0.984 ± 0.0782, n = 8; average ± SEM; One-way ANOVA; P = 0.0199; Dunnett's Multiple Comparison Test; * P<0.05). **(F)** SKF-induced perisynaptic BC cleavage is subjected to L-type VGCC calcium signalling (Ctl, 1 ± 0.1223, n = 8; SKF, 1.478 ± 0.0542, n = 7; Dilt, 0.9228 ± 0.1698, n = 7; Dilt + SKF, 0.7188 ± 0.1541, n = 5; average ± SEM; One-way ANOVA; P = 0.0046; Dunnett's Multiple Comparison Test; * P<0.05) (nFl cl. BC = normalized fluorescent intensity of cleaved BC).

Upon activity calcium ions enter the cell not only via NMDA receptors but also through VGCCs (reviewed in (Hansen et al., 2018; Heine et al., 2019)). Described physiological interactions of D1-like DA receptors and L-type VGCC suggested a possible influence of these channels in the investigated DA-dependent perisynaptic BC cleavage (Surmeier et al., 1995; Young and Yang, 2004; Chen et al., 2007). Thus, I blocked postsynaptic L-

type VGCC using Diltiazem hydrochloride 10 min prior D1-like DA receptor stimulation. Calcium channel inhibition followed by the stimulation of D1-like DA receptors abolished SKF-induced perisynaptic BC cleavage (Figure 16F). Those results suggest that the activity of the entire neuronal network, the activity of postsynaptic sites as well as the influx of calcium through L-type VGCC are necessary to lead to enhanced perisynaptic BC cleavage after D1-like DA receptor activation.

3.11. DA-dependent BC cleavage requires elevated intracellular cAMP levels

Stimulation of D1-like DA receptors results in an activation of AC leading to an intracellular increase in levels of the second messenger cAMP. These enhanced levels of cAMP trigger activation of PKA causing phosphorylation of downstream proteins (reviewed in (Beaulieu and Gainetdinov, 2011)). Thus, I investigated if SKF-dependent BC cleavage might also follow a PKA-dependent mechanism. To test this, I first blocked PKA using cAMPS-Rp, a cell-permeable cAMP analog which acts as a competitive antagonist of cAMP-induced PKA activation. This inhibitor interacts with the cAMP binding site of the regulatory site but is resistant to hydrolysis by phosphodiesterases. Inhibition of PKA prior to the stimulation of D1-like DA receptors revealed no significant changes in levels of cleaved BC around excitatory synapses (

Figure 17A). Further, I was interested, if elevated intracellular cAMP levels are sufficient for enhanced perisynaptic BC cleavage. Therefore, I stimulated AC intracellularly by forskolin (Fo) while at the same time blocking phosphodiesterase-4 using rolipram (Ro) to abolish the degradation of newly formed cAMP. Pharmacological activation of AC showed a significant increase in perisynaptic BC cleavage comparable to SKF-induced BC cleavage. AC activation and additional stimulation of D1-like DA receptors revealed no further enhancement in levels of cleaved BC suggesting that the culture system might be saturated. Again, silencing network activity abolished BC cleavage (

Figure 17B). However, activation of AC resulting in elevated levels of intracellular cAMP seems to be sufficient for increased perisynaptic BC cleavage.

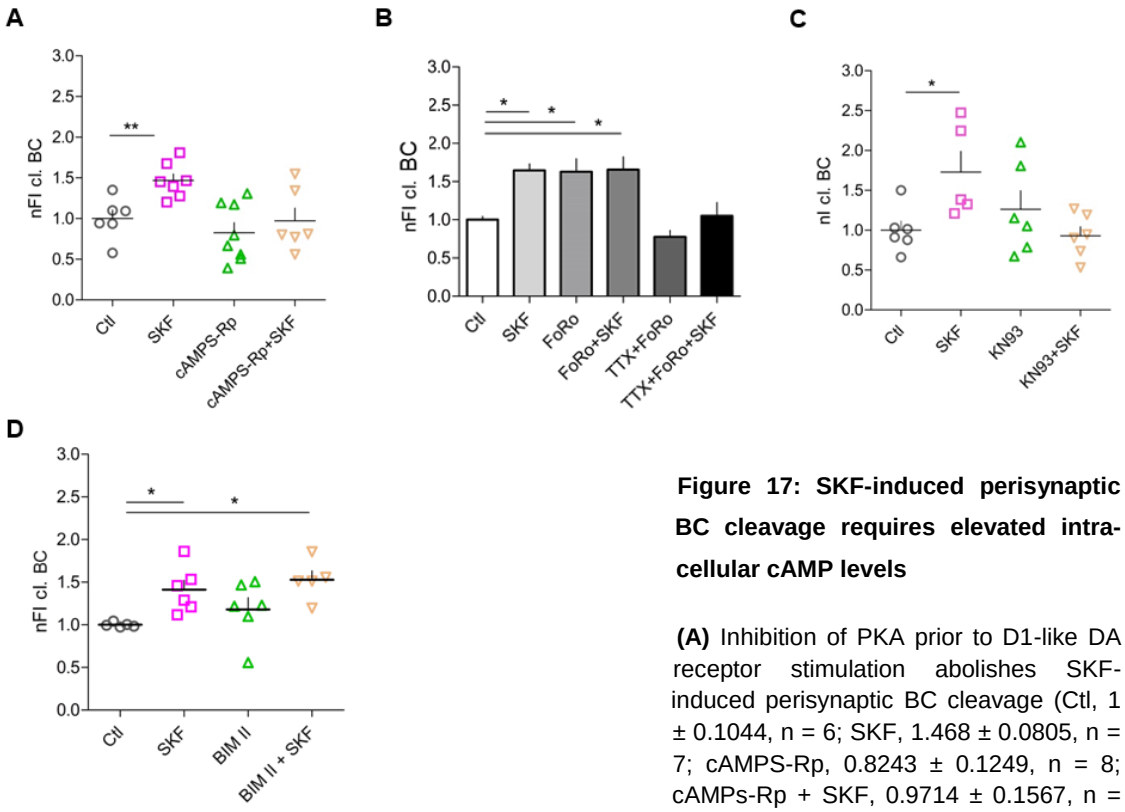

Figure 17: SKF-induced perisynaptic BC cleavage requires elevated intracellular cAMP levels

(A) Inhibition of PKA prior to D1-like DA receptor stimulation abolishes SKF-induced perisynaptic BC cleavage (Ctl, 1 ± 0.1044, n = 6; SKF, 1.468 ± 0.0805, n = 7; cAMPS-Rp, 0.8243 ± 0.1249, n = 8; cAMPs-Rp + SKF, 0.9714 ± 0.1567, n = 6; average ± SEM; One-way ANOVA; P = 0.0041; Dunnett's Multiple Comparison Test; ** P<0.05).
(B) Increased AC activity is sufficient for increased BC cleavage around excitatory synapses (Ctl, 1 ± 0.0437, n = 4; SKF, 1.646 ± 0.0858, n = 4; FoRo, 1.63 ± 0.1683, n = 5; FoRo + SKF, 1.656 ± 0.1679, n = 4; TTX + FoRo, 0.7763 ± 0.0858, n = 4; TTX + FoRo + SKF, 1.051 ± 0.1741, n = 3; average ± SEM; One-way ANOVA; P = 0.0002; Dunnett's Multiple Comparison Test; *** P<0.05).
(C) Different from PKA, Proteinkinase C (PKC) is not involved in SKF-induced BC cleavage (Ctl, 1 ± 0.012, n = 5; SKF, 1.412 ± 0.1096, n = 6; BIM II, 1.179 ± 0.1401, n = 6; BIM II + SKF, 1.527 ± 0.1049, n = 5; average ± SEM; One-way ANOVA; P = 0.0171; Dunnett's Multiple Comparison Test; * P<0.05).
(D) SKF-induced perisynaptic BC cleavage is subjected to intracellular calcium signalling via CaMKII (Ctl, 1 ± 0.1139, n = 6; SKF, 1.648 ± 0.2978, n = 5; KN93, 0.9165 ± 0.1118, n = 4; SKF+KN93, 0.9303 ± 0.1126, n = 6; average ± SEM; One-way ANOVA; P = 0.0269; Dunnett's Multiple Comparison Test; * P<0.05) (nFI cl. BC = normalized fluorescent intensity of cleaved brevican).

To confirm this, I used an optogenetic tool provided by Dr. C. Gee from ZMNH. Thus, I infected rat dissociated cortical cultures (DIV 14) with AAV2/7.Syn-bPAC-2A-tdimer. With this virus containing a bacterial photo-activatable adenylyl cyclase (bPAC) I was able to modulate cAMP levels optogenetically in the culture system as previously described in (Stierl et al., 2011). In brief, bPAC is activated by a light flash of approximately 450 nm. Afterwards, it produces cAMP out of ATP, thus intracellular cAMP levels are elevated (Figure 18A).

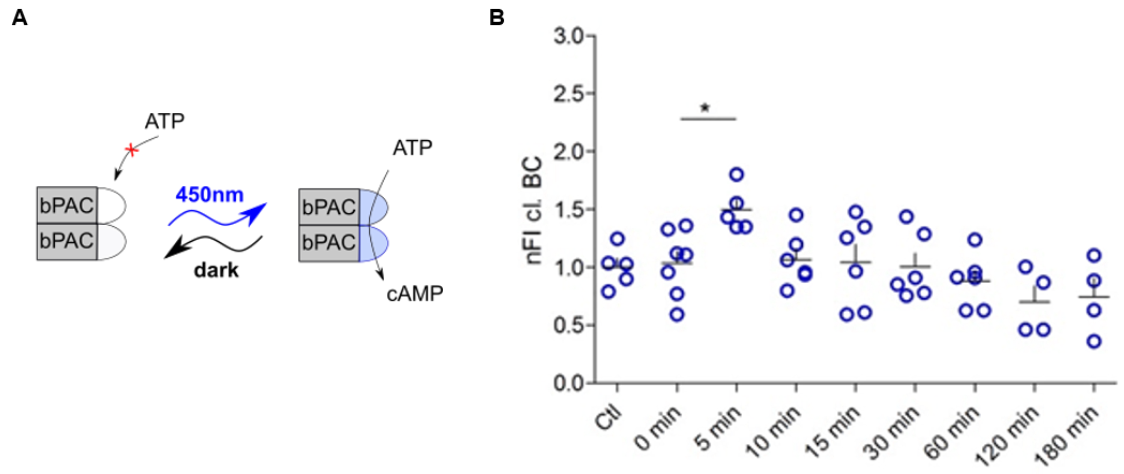

Figure 18: Optogenetic modulation of intracellular cAMP levels resulted in increased BC cleavage

(A) Schematic illustration of the functional principle of bPAC. When light with a wavelength of 450 nm shines on bPAC, it gets activated and produces cAMP out of ATP. Thus, intracellular cAMP levels get elevated. **(B)** Elevated intracellular cAMP levels are crucial for an increase in perisynaptic BC cleavage (Ctl, 1 ± 0.0765, n = 5; 0 min, 1.033 ± 0.1064, n = 7; 5 0.1172, n = 6; 60 min, 0.8787 ± 0.0938, n = 6; 120 min, 0.6993 ± 0.1398, n = 4; 180 min, 0.7450 ± 0.1605, n = 4; average ± SEM; One-way ANOVA; P = 0.0049; Dunnett's Multiple Comparison Test; ** P<0.05). (nFl cl. BC = normalized fluorescent intensity of cleaved brevican)

At DIV 21 cells were activated with a 500-ms flash of a 455 nm LED (0.9 mW mm^{-2}) and the amount of perisynaptically cleaved BC analyzed at different time points. Infected but not activated cells served as a control and indicated time point 0. Already 5 min after stimulation, I could observe a significant rise in levels of cleaved BC around excitatory synapses (Figure 18B). The amount was comparable to SKF-induced BC cleavage. Interestingly, 10 min after blue light stimulation levels of cleaved BC returned back to baseline and did not change significantly over a longer period of time (up to 180 min) (Figure 18B). In conclusion, elevated intracellular cAMP levels are sufficient to induce BC cleavage around excitatory synapses.

3.12. Perisynaptic BC cleavage depends on intracellular calcium signalling via CaMKII

Calcium influx into the postsynaptic terminal is important for long-term potentiation. Calcium ions can enter the cell either through activated NMDARs or via VGCC. Within this thesis I could show that both NMDARs and L-type VGCC are involved in the signalling mechanism. Therefore, I tested if intracellular calcium signalling may be necessary for the observed SKF effect. Calcium is known to activate downstream proteins. A major downstream effector is CaMKII. This enzyme is a multifunctional serine/ threonine kinase that is well known as a key regulator in synaptic plasticity (Silva et al.,

1992; Mayford et al., 1996). To inhibit CaMKII, cultures were treated with KN 93 phosphate, an inhibitor for the aforementioned kinase, 10 min before D1-like DA receptors were stimulated. Inhibition of CaMKII alone and prior to D1-like DA receptor activation revealed an unaltered amount of cleaved BC around excitatory synapses (

Figure 17C). Beside CaMKII, calcium also acts on PKC. I was wondering if this calcium-dependent kinase might also be involved in the signalling. Therefore, I blocked PKC by using Bisindolylmaleimide II (BIM II) 15 min before D1-like DA receptor stimulation. In my experiments PKC turned out not to play a role in SKF-induced BC cleavage, since inhibition of the kinase followed by stimulation of D1-like DA receptors revealed a significant increase in perisynaptic BC cleavage as seen for SKF alone (

Figure 17D). Thus, SKF-induced BC cleavage depends on intracellular calcium signalling via CaMKII, but the calcium-dependent kinase PKC is not involved in the signalling.

4 Discussion

The aim of this thesis was to investigate the interplay between the dopaminergic system and ECM integrity in synaptic plasticity. I was able to show that activation of D1-like DA receptors results in a restructuring of the HA-based perisynaptic ECM *in vitro* and *in vivo*. I focused on the ECM components BC and Acan, two of the most abundant lecticans in the adult brain, expressed and secreted by neurons and astrocytes (Seidenbecher et al., 1998; Jaworski et al., 1999; Zhang et al., 2014). The underlying signalling pathway requires a co-signalling via PKA and CaMKII. In the following I will answer and discuss the questions outlined in 1.5.

4.1. Systemic activation of D1-like DA receptors *in vivo* affects ECM integrity at synaptosomes

Here, I could show that *in vivo* systemic activation of D1-like DA receptors results in enhanced DA-dependent cleavage of BC and Acan, the most abundant lecticans in the rodent brain in synaptosomes. This finding is interesting with respect to several studies that investigated either the effect of dopaminergic modulation on learning performance or the experimental removal of the ECM in the context of structural plasticity and learning.

In an FM discrimination task, Mongolian gerbils which have been injected with the D1-like DA receptor agonist SKF38393 either shortly before, shortly after or one day before the training revealed a better performance than control animals (Schicknick et al., 2008). The experimental breakdown of the ECM by hyaluronidase has been shown to modulate synaptic short-term plasticity through enhanced diffusion of postsynaptic glutamate receptors such as NMDARS or AMPARs (Heine et al., 2008; Frischknecht et al., 2009). Furthermore, VGCC were identified to be affected after enzymatic ECM removal which is thought to modulate short-term synaptic plasticity as well (Kochlamazashvili et al., 2010). Another animal study demonstrated that enzymatic degradation of the ECM in the auditory cortex of Mongolian gerbils resulted in enhanced cognitive flexibility in reversal learning in a FM discrimination task (Happel et al., 2014). Since ECM cleavage with exogenous glycosidases is an artificial model resulting in conditions which do probably not occur in the brain it is interesting to compare results to those obtained in knock out models of different ECM. Animals lacking components of the ECM show impaired synaptic plasticity, for example in the adult mouse brain (for re-

view see (Dityatev and Fellin, 2008). Mice deficient in TNR, for instance, exhibit impaired LTP but normal LTD. In addition, the lack of TNR in these mice leads to disinhibition of the CA1 region of the hippocampus and to a shift in the threshold for induction of LTP (Bukalo et al., 2007). It also has been reported that mice lacking the CSPGs BC and Ncan show impairments in LTP 30 min and 2h after induction, respectively (Zhou et al., 2001; Brakebusch et al., 2002). Unfortunately, there is almost no literature about reward learning and changes in the ECM structure. However, one study has shown that activation of MMP-9 is required for reward learning in the central amygdala. MMP-9 KO mice displayed appetitively motivated conditioning (Knapska et al., 2013).

Since most studies investigated either the dopaminergic system or the ECM in terms of contribution to plasticity and learning, this thesis providing first evidence that the dopaminergic system affects ECM integrity can help to link both lines of research. This interplay might be a key component of synaptic plasticity and motivated learning.

4.2. Neuronal ADAMTS 4 and ADAMTS 5 are essential for DA-dependent perisynaptic ECM re-modeling

I was also able to show that neuronal proteases ADAMTS 4 and ADAMTS 5 are essential for the DA-dependent increase in BC cleavage.
It is well known that BC can be found perisynaptically associated with synaptic proteins (Hagihara et al., 1999; Seidenbecher et al., 2002), while Acan is almost exclusively located in PNNs (Matthews et al., 2002). However, the observed increase in perisynaptic BC and Acan cleavage occurs already 15 min after receptor stimulation in dissociated cortical cultures. This led to the assumption that the effect must result either from proteolytic cleavage or from a displacement of cleaved BC right to excitatory synapses rather than from *de novo* protein synthesis and subsequent cleavage. Several studies have demonstrated that the ECM is built and remodeled in an activity-dependent way (reviewed in (Dityatev and Fellin, 2008)). In a recent study BC was found being expressed by a large fraction of parvalbumin-positive (PV+) cells. Here, BC levels have been shown to vary in response to network activity which is required for synaptic plasticity (Favuzzi et al., 2017). This observation goes in line with my findings where I could show that short network silencing abolished the SKF-induced BC cleavage. However, a few years ago our group was able to show that after induction of homeostatic plasticity in neuronal cell cultures through extended network silencing BC processing is enhanced

at synapses (Valenzuela et al., 2014). These findings suggest a strict regulation of BC-processing proteases in a time-dependent manner. There are several ECM-modifying proteases being endogenously expressed in the brain and well known to process ECM molecules. Beside MMPs, enzymes of the ADAMTS family are excellent candidate proteases for endogenous cleavage of CSPGs. All family members have been shown to be secreted and extracellularly active. But, only a few of them, such as ADAMTS 4 and ADAMTS 5, are well known to cleave Acan and BC (Kelwick et al. 2015; Stanton et al. 2011). As mentioned before, we have shown that BC processing is enhanced under conditions of homeostatic plasticity. Here, the N-terminal proteolytic fragment of BC generated by ADAMTS 4 has been shown to be located perisynaptically. Furthermore, we could identify ADAMTS 4 as the potential main BC-processing protease located in synapses (Valenzuela et al., 2014). Within my thesis, I could show the involvement of ADAMTS 4 in perisynaptic BC processing at excitatory synapses even after acute stimulation of D1-like DA receptors. In addition, I could identify ADAMTS 5 as a second candidate protease for BC processing in the brain.
Several studies could identify especially ADAMTS 4 as the main protease for processing BC (reviewed in (Kelwick et al., 2015)). Thus, ADAMTS 4 was found to be expressed in neuronal as well as in astrocytic cultures. Its activity in astrocytic cultures seems to be regulated, for instance, by the transforming growth factor β (TGF β) that is often released in response to a number of injuries. Interestingly, astrocytic cultures challenged with a number of different cytokines and treated with TGFβ displayed a reduction in ADAMTS-derived BC fragments as well as a reduced ADAMTS activity (Hamel et al., 2005).

Enzymes of the ADAMTS family are synthesized as zymogens carrying a pro-domain at their N-terminus. These pro-ADAMTS proteinases can either be cleaved at their C- or N-terminal fragments by furin or pro-protein convertases such as PACE 4 or PC5/6. Cleaving of the pro-domain results in mature and potentially active enzymes (Flannery et al., 2002; Lemarchant et al., 2013). ADAMTS 5 was shown to be exclusively activated extracellularly (Longpré et al., 2009). However, there are contrary points of view concerning the compartment where ADAMTS 4 is activated. One study claimed the activation of ADAMTS 4 in the *trans*-Golgi network via furin (Wang et al., 2004). One year later Tortorella and colleagues demonstrated that cleaving off the pro-domain is strongly depending on the pH optimum of the activating protease. Thus, at neutral pH

furin is less efficient than PACE 4 in activating pro-ADAMTS 4. Interestingly, at acidic pH PACE 4 is the only PPC efficiently activating ADAMTS 4 (Tortorella et al., 2005). In the knockdown experiment in this thesis I could show that only knocking down ADAMTS 4 or ADAMTS 5 results in a dramatic decrease in BC cleavage compared to control conditions. These findings suggest that there might be a pool of inactive proteases present in the extracellular space. This hypothesis is underlined by a recent finding in our group. We could demonstrate that in acute hippocampal slices proteases were activated by removing their pro-domain. This extracellular activation alone was sufficient to increase the amount of cleaved BC suggesting that indeed there need to be inactive proteases stored extracellularly, ready for fast activation (Singh, 2017). However, not only furin and furin-like PPCs have been found to activate pro-ADAMTS enzymes. A recent study could identify tPA (tissue-type plasminogen activator) as another potential activator for ADAMTS 4. In spinal cord injury (SCI) it was shown that tPA can activate pro-ADAMTS 4 leading to degradation of inhibitory CSPGs (Lemarchant et al., 2014). Interestingly, tPA has been shown to display enhanced extracellular activity depending on the activation of postsynaptic D1Rs in the nucleus accumbens (NAc) (Ito et al., 2007). Thus, it might be that pro-ADAMTS 4 is activated by tPA extracellularly upon D1-like DA receptor activation. However, the specific PPC and the exact place for activation still need to be investigated.

4.3. DA-dependent, perisynaptic BC cleavage requires co-signalling through PKA and CaMKII

DA receptors reveal a broad expression pattern in the brain as well as in the periphery. D1Rs were shown to be the most prominent DA receptor subtypes in prefrontal cortex, while D2Rs are mostly expressed at high levels in the striatum or in the NAc. Interestingly, both receptor subtypes are rarely found to be co-expressed in same cells (reviewed in (Missale et al., 1998)). D1Rs were shown to be positively coupled to AC and increased PKA activity, while D2Rs inhibit AC activity and reduce cAMP levels and PKA activity (reviewed in (Beaulieu and Gainetdinov, 2011)). However, Ito and his colleagues could identify the D1-cAMP-PKA pathway as the underlying mechanism for enhanced extracellular tPA activity (Ito et al., 2007). Based on my observations that enhanced intracellular cAMP levels and PKA are essential for perisynaptic BC cleavage, it is quite striking that DA-dependent remodeling of the perisynaptic ECM might follow this pathway as well.

It is well known that plasticity of excitatory synapses needs both activation of postsynaptic NMDARs and an increase in intracellular calcium. A strong depolarization of the postsynaptic site removes the magnesium block of NMDARs resulting in an activation of those receptors. The high calcium permeability of NMDARs leads to an increased calcium influx into the postsynaptic compartment resulting in changes in synaptic strength. PKA was shown to regulate this calcium permeability of NMDARs in dissociated hippocampal neurons and in hippocampal slices to promote lasting changes in synaptic strength (Skeberdis et al., 2006). The elevation of NMDAR-mediated postsynaptic Ca^{2+} levels activates well described pathways of kinases and phosphatases involving for example CaMKII (Gardoni et al., 1998; Strack and Colbran, 1998; Leonard et al., 2002; Barria and Malinow, 2005). A possible mechanism could be that PKA phosphorylates the serine residue 897 (Ser897) of the NR1 subunit of NMDARs or receptor-associated proteins, thus changing the geometry of the NMDA channel pore (Dudman et al., 2003; Skeberdis et al., 2006). Furthermore, calcium influx via L-type VGCC was also shown to be responsible for LTP induction in hippocampus and was proposed to be influenced by PKA (Hell et al., 1995; Meunier et al., 2017). Just as neuronal ones, cardiac L-type VGCC have been found to be activated by PKA-dependent phosphorylation resulting in an increased influx of calcium ions (McDonald et al., 1994). Beside neurons astrocytes were moved more and more in the focus of investigations. Astrocytes are able to express both DA receptors and DA transporters. Some culture studies could show with strong evidence that D1 and D5 receptors are expressed in rat astrocytes (Hösli and Hösli, 1986; Zanassi et al., 1999; Brito et al., 2004; Miyazaki et al., 2004). Interestingly, the used D1-like DA receptor agonist SKF81297 has been found to increase intracellular cAMP levels in astrocytes (Vermeulen et al., 1994; Zanassi et al., 1999; Requardt et al., 2010). Thus, it could be that astrocytes are players in the mechanism as well. In addition, ADAMTS 4 and ADAMTS 5 have been shown to be expressed not only by neurons but also in astrocytes at least at RNA levels (Zhang et al., 2014). Here, I could show that ADAMTS 4 protein is expressed in astrocytes and neurons in dissociated cortical cultures (Figure 14B). So, one could speculate that both neuronal and astrocytic D1-like DA receptors are stimulated resulting in an increase in extracellular active proteases, thus in a remodeling of the perisynaptic ECM. Unfortunately, there is no literature directly examining astrocyte-mediated effects of DA on neurons in healthy brain function such as synaptic plasticity (reviewed in (Jennings and Rusakov, 2016)). All studies suggesting a direct dopaminergic effect on neurons via

astrocytes considered astrocyte-mediated neurotoxicity (Shao et al., 2013; Ding et al., 2016). So far, the full effects of DA on astrocytic physiology and astrocyte-mediated effects of DA on neurons are still uncertain.

4.4. ECM fragments as matricryptins

Within this thesis I could show that the amount of cleaved BC and Acan is enhanced after SKF-induction. But what will be the fate of these ADAMTS-derived fragments at perisynaptic sites? There is more and more evidence that fragments of ECM molecules are not only internalized and degraded but may act as biologically active molecules (Davis et al., 2000). Davis and co-workers defined a new concept of matricryptic sites. This term is specifically used for biologically active ECM fragments containing a cryptic site which is normally not exposed in the intact molecule (Davis et al., 2000). Well studied are the effects of HA fragments in different tissues. The biological functions of those fragments have been shown to be strictly size-dependent and organ-specific. Furthermore, it is hypothesized that fragments of different sizes influence their affinity to specific receptors, the clustering of receptors in the membrane or the HA uptake, thus intracellular signalling cascades (reviewed in (Cyphert et al., 2015)).

As mentioned before, beside ECM-modifying proteases like ADAMTS family members, also hyaluronidases are endogenously expressed in the brain which can degrade HA. Since all known enzymes for HA degradation seem to act intracellularly, CEMIP (Cell migration-inducing protein) and TMEM2 (Transmembrane protein 2), two newly described proteins with HA binding and degrading capacity acting extracellularly or at the cell surface, became the focus of attention. CEMIP was found to be involved in HA depolymerisation. It has been shown to bind and degrade high-MW HA into intermediate size HA fragments (Yoshida et al., 2014). CEMIP seems to act independently of endogenous hyaluronidases, since its pH optimum is in the range of 6-7 and knockdown of CEMIP did not influence the expression of common hyaluronidases (Yoshino et al., 2017). Furthermore, mice deficient in CEMIP showed enhanced levels in the overall amount of HA in the brain as well as in the average molecular weight of HA fragments. In addition, those mice performed worse in the Morris water maze task suggesting CEMIP to be involved in learning and memory processes (Yoshino et al., 2017). Unlike CEMIP, less is known about TMEM2 and its function in the brain. However, it is a transmembrane protein and acts as a cell surface hyaluronidase that degrades high-MW HA into fragments of 5 kDa size. TMEM2 seems to be highly specific for HA frag-

ments and acts in a calcium-dependent manner. Apparently, it does not need living cells to be enzymatically active. But, contact to HA has been shown to be absolutely essential for its performance (Yamamoto et al., 2017). Nothing is known about its functions in learning and memory processes but it was observed that in mice deficient for TMEM2 CEMIP might be able to take over functions of TMEM2 (Yoshino et al., 2017). Several cell surface receptors were identified mediating signalling of ECM fragments into the cell such as integrins, growth factor receptors or membrane proteoglycans (reviewed in (Ricard-Blum and Vallet, 2019)). Integrins are the most frequently identified and characterized receptors for ECM fragments. They were shown to be the major adhesion receptors for the ECM involved in ECM assembly, cell-matrix interactions and cell migration (Missan and DiPersio, 2012; Xiong et al., 2013). However, several ECM fragments can bind to different receptors and different fragments might share the same receptor(s) (reviewed in (Ricard-Blum and Vallet, 2019)). Since I could observe a decline in cleaved BC already 10 min after stimulation of intracellular cAMP levels (Figure 17B), it might be possible that some fragments are further degraded and others act as bioactive molecules via, for instance, integrins inducing downstream signalling cascades, thus plastic changes of synapses. Nevertheless, the underlying molecular and signalling mechanisms are elusive and need further investigation.

4.5. The brain's ECM, ECM-modifying proteases and ECM fragments in the context of pathological diseases

However, BC and its ADAMTS-derived fragments are not only important for proper brain functions but also have been found to be involved in the building and cell invasion of tumors such as malignant glioma, the most common and deadly primary brain tumor. Up-regulation of BC and its predominantly ADAMTS-derived fragments has been shown to promote glioma cell invasion. In this study, they suggest that BC and its cleaved fragments might act independently from each other (Viapiano et al., 2008). Glioma cell lines with invasive properties have been found to express ADAMTS 4 and 5 mRNA (Matthews et al., 2000; Held-Feindt et al., 2006). Thus, compounds inhibiting

ADAMTS-dependent BC cleavage could help to reduce glioma migration and allow for surgical removal.

Beside tumor invasion, ADAMTS enzymes have been found to be involved in recovery from SCI. SCI results in a glial scar which mainly consists of CSPGs of the lectican family diminishing functional recovery. Mice following SCI showed increased mRNA expression of ADAMTS 1, 5 and 9 associated with enhanced BC, Acan and Vcan cleavage. Surprisingly, ADAMTS 4 mRNA was not upregulated (Demircan et al., 2014). However, another study performed in adult rat demonstrated upregulation of ADAMTS 4 mRNA and Acan cleavage after SCI. Here, ADAMTS 4 infusion into the spinal cord after SCI promoted functional recovery similar to treatment with ChABC (Tauchi et al., 2012). Recently, it has been found that tPA has the potential to cleave pro-ADAMTS 4 into its active form. Interestingly, either infusion of tPA or active ADAMTS 4 at the lesion site after SCI resulted in axonal sprouting as well as functional recovery. Altogether, especially ADAMTS 4 could serve as a potential therapeutic target to promote functional recovery after SCI when delivered soon after the injury.

There is also increasing evidence that ADAMTS and ADAMTS-derived fragments play a crucial role in epilepsy. In the dentate gyrus of rats injected with kainic acid to induce seizures, for instance, a rise in ADAMTS-derived BC fragments in combination with a dramatic loss of synaptic density was detectable. BC cleavage by ADAMTS 4 and enhanced levels of BC fragment occurred already 8h post-seizure (Yuan et al., 2002). On the other hand, our group was able to show that in an epileptic mouse model lacking the presynaptic scaffold protein bassoon the expression of full-length BC is reduced in the forebrain. This reduction in BC expression is correlated with the number of seizures, thus the less BC is expressed the more seizures appear. Surprisingly, changes in BC cleavage as well as in ADAMTS 4 expression were not detectable (Blondiaux et al., unpubl.).

ADAMTS enzymes and changes in the ECM composition were linked to neurological diseases such as AD. A recent study could demonstrate that two different AD mouse models overexpressing amyloid precursor protein showed a defect in ocular dominance plasticity at one month of age. This defect was associated with enhanced ECM deposition (William et al., 2012). Furthermore, elevated levels of BC protein has been found at early stages of AD in a mouse model. These mice displayed a loss of contextual fear memory and a defect in LTP that could be restored using ChABC (Végh et al., 2014). A

recent study could show that Acan-based perineuronal nets are unaltered in their distribution, structure and molecular properties in AD. Interestingly, those intact perineuronal nets were found in close vicinity of amyloid plaques. However, BC-based perisynaptic ECM was lost in the core of these plaques associated with a loss of synapses. Thus, it might be that Acan-based perineuronal nets serve as a protection for further neuronal degradation (Morawski et al., 2012b). This protective effect is underpinned by another study demonstrating that neurons surrounded by perineuronal nets are less frequently affected by Tau-internalization than neurons without perineuronal nets (Suttkus et al., 2016).

In sum, all these findings highlight that regulated processing of components of the mature ECM has important functional implications in health and disease. Furthermore, fragments of ECM components as well as ECM-modifying proteases that are implicated in numerous pathological diseases might serve as potential therapeutic targets in the future.

4.6. Conclusion and Outlook

In conclusion, I was able to show that activation of D1-like DA receptors influences perisynaptic ECM integrity *in vivo* and *in vitro* by enhanced BC and Acan cleavage. This alteration in the ECM composition via ADAMTS 4 and 5 required a co-signalling through PKA and CaMKII. Furthermore, DA-dependent remodeling of the perisynaptic ECM needs network activity as well as activity of postsynaptic sites. Although the whole molecular mechanism is still elusive, identified pathways are depicted in

Figure 19.

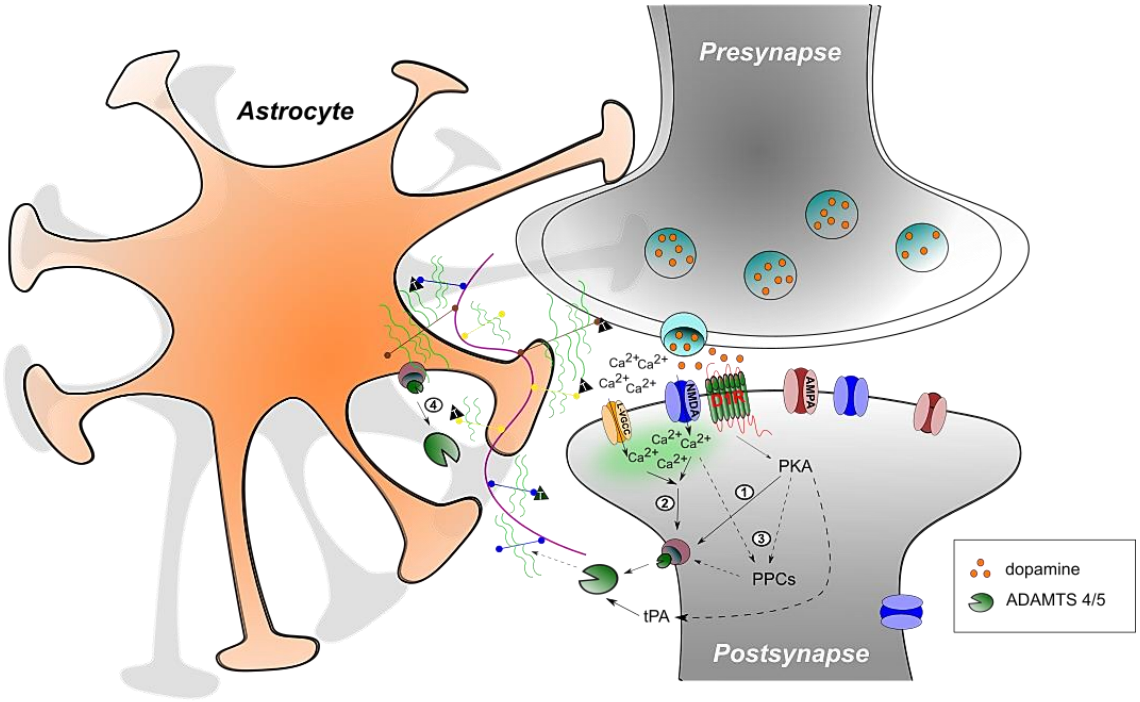

Figure 19: Schematic illustration of possible molecular mechanisms underlying DA-dependent BC cleavage

Activation of D1-like DA receptors results in increased intracellular cAMP levels leading to downstream activation of PKA. Further, active PKA might be able to release already active proteases, here ADAMTS 4 and ADAMTS 5, in the extracellular space to remodel the perisynaptic ECM (①). Besides its downstream signalling, stimulation of D1-like DA receptors has an influence on Ca^{2+} influx through NMDARs and L-type VGCC. Enhanced Ca^{2+} influx activates downstream CaMKII that might lead to the release of ADAMTS 4 and 5 into the extracellular space (②). However, D1-like DA receptor stimulation might result in a co-signalling of pathways ① and ②. Furthermore, all ADAMTS enzymes carry a pro-domain at the N-terminus to keep them in an inactive state. Thus, after D1-like DA receptor stimulation PKA activation and/or enhanced Ca^{2+} levels could result in the activation of PPCs. Active PPCs themselves could cleave off the pro-domain of ADAMTS 4 and 5 resulting in active enzymes being released (③). However, ADAMTS 4 and ADAMTS 5 are expressed by astrocytes as well. Therefore, activation of astrocytic D1-like DA receptors might also cause a release of these enzymes by astrocytes (④).

My hypothesis is underpinned by a study in 2016 where it has been shown that activation of D1-like DA receptors results in increased MMP activity in extracts of striatal slices. Furthermore, they could show that a 1h pretreatment of slices with SKF81297 potentiated NMDA-stimulated calcium influx (Li et al., 2016). Thus, the illustrated pathways are likely to occur. Regarding the signalling pathway, one could further test, if activation of D1-like DA receptors leads to enhanced surface expression of glutamate receptors which has been shown to be enhanced after experimental breakdown of the ECM (Heine et al., 2008; Frischknecht et al., 2009; Schweitzer, 2015). Furthermore, it would be of high interest to investigate if ADAMTS-derived fragments are biologically active molecules and if so what receptors might be involved in the signalling pathways.

I only investigated ADAMTS 4 and ADAMTS 5 in this thesis but other brain-expressed ADAMTS enzymes, like for instance ADAMTS 8 or ADAMTS 15, belonging to the aggrecanase clade have been shown to cleave at least Acan. It might be worth to test these enzymes to get an even better understanding of BC cleavage. However, the possible influence of astrocytic-expressed ADAMTS 4 and ADAMTS 5 in DA-dependent BC cleavage needs also further investigation. In addition, it might be of high interest to clarify where pro-ADAMTS enzymes are possibly activated.

Here, the molecular mechanism was investigated in a culture system, thus the obtained results need to be confirmed in an *in vivo* approach. For that it would be of high interest to investigate, for instance, ADAMTS and lectican levels in synaptosomal fractions after a DA-dependent learning task. Furthermore, the here used shRNAs for ADAMTS 4 and ADAMTS 5 could be injected into mice or rats and learning performances in a DA-dependent task could be investigated.

However, the results of my thesis may contribute to a further understanding of underlying molecular mechanisms in motivated learning processes. Furthermore, one will get a better understanding of activity-dependent remodeling of mature ECM due to activation of ADAMTS 4 and ADAMTS 5. The here identified ECM-modifying proteases as well as produced ECM fragments or their possible cell surface receptors could serve as potential therapeutic targets to moderate neurodegenerative diseases such as epilepsy or AD.

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Declaration of Honor

Ich versichere hiermit, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; verwendete fremde und eigene Quellen sind als solche kenntlich gemacht.

Ich habe insbesondere nicht wissentlich:

- Ergebnisse erfunden oder widersprüchliche Ergebnisse verschwiegen,
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Ich erkläre mich damit einverstanden, dass die Arbeit ggf. mit Mitteln der elektronischen Datenverarbeitung auf Plagiate überprüft werden kann.

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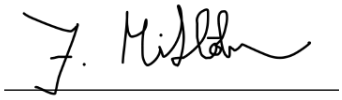

Wellen, den 29.01.2020 (Jessica Mitlöhner)