„Expression and function of transglutaminase 1 in the brain"

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1. Introduction

The brain is of a stunning elaborateness. It harbours the largest diversities of cell types throughout the body. A total of about 80% of genes in the genome is expressed in the nervous system (Lein, Hawrylycz et al. 2007). But even this high degree of diversity is easily outshined by the complexity of connections between neuronal cells. In the human cerebral cortex about 1 billion synapses per cubic millimetre can be found (Alonso-Nanclares, Gonzalez-Soriano et al. 2008).

A major attribute of the brain is its neuronal plasticity, the ability to modulate and remodel neuronal circuits depending on external or internal inputs. These adaptive changes are not restricted to developmental processes, they also occur in adulthood, building the foundation of learning and memory (Martin, Grimwood et al. 2000). One of the most fascinating questions concerning the brain is how this concert of assembly and reduction, as well as the stabilisation of desired structures, is controlled.

In 1949 Hebb proposed a model, in which correlated activity dependent alteration of synaptic strength and formation of new synapses were underlying this plasticity (Hebb 1949). Experimental proof for Hebb's postulate was given in 1973. In the hippocampal formation a tetanic stimulation dependent form of synaptic plasticity, the so called long-term potentiation (LTP), was discovered (Bliss and Lomo 1973). LTP is the enhancement of synaptic transmission between a presynaptic and a postsynaptic neuron, triggered by brief tetanic pulses. LTP comprises biochemical changes, like recruitment of new neurotransmitter receptors (Collingridge, Isaac et al. 2004), and morphological alterations, such as enlargement of synapses or the formation of new ones (Yuste and Bonhoeffer 2001; Matsuzaki, Honkura et al. 2004; Park, Salgado et al. 2006).

The morphological changes are mainly investigated at dendritic spines, which form the postsynaptic contacts for most excitatory synapses in the central nervous system. Key factors for the synaptic remodelling are the cytoskeleton, mainly actin (Matus 1999), and adhesion proteins, especially cadherins (Okamura, Tanaka et al. 2004). Local remodelling of the actin network respectively stabilisation of actin fibres affect the morphological plasticity of dendritic spines, thereby attenuating the transmission efficiency. To put it simply, larger spines contain stronger synapses than smaller ones (Harris and Stevens 1989). LTP induction varies in different cell types and brain regions, it is even age dependent (Bliss and Lomo 1973; Matsuoka, Kaba et al. 1997; Rogan, Staubli et al. 1997;

Bence and Levelt 2005). One major signalling molecule in LTP is calcium (Lynch, Larson et al. 1983). Not surprisingly intracellular calcium levels have a great impact on actin fibre stability and the association of different proteins to the actin cytoskeleton (Matus 2000). Therefore in order to further enlighten LTP, the investigation of calcium dependent actin-modifying enzymes is of great interest. Possible players could be transglutaminases.

Transglutaminases present a family of calcium dependent cross-linking enzymes, catalysing a transamidation reaction, which links the carboxamide moiety of a protein-bound glutamine residue to a primary amine (Sarkar, Clarke et al. 1957; Lorand and Graham 2003). If the primary amine is a lysine a ε-(γ-glutamyl)lysine isopeptide bridge is formed (Pisano, Finlayson et al. 1968). Therefore, transglutaminases were mainly seen as "biological glues" for a long time, based on their ability to cross-link proteins in this way (Griffin, Casadio et al. 2002). But in the last years the number of known processes involving transglutaminase activity has been broadened significantly (Mehta, Fok et al. 2006).

Various transglutaminases have been found in tissues besides their initially known locations and by now transglutaminase activity is found to be associated with diverse processes like immune response (Novogrodsky, Quittner et al. 1978; Cordella-Miele, Miele et al. 1993; Mehta, Fok et al. 2006), apoptosis (Fesus and Szondy 2005) or cancer (Mehta 1994; Jiang, Ablin et al. 2009). Besides their *in vivo* roles, transglutaminases become increasingly important in industrial and medical applications like food processing (Lantto, Puolanne et al. 2005) or wound healing (Jurgensen, Aeschlimann et al. 1997).

One recent focus of transglutaminase research is the central nervous system. Different transglutaminases have been found in the human brain (Kim, Grant et al. 1999; Hadjivassiliou, Aeschlimann et al. 2008). They are involved in developmental processes (Tucholski, Kuret et al. 1999; Mahoney, Wilkinson et al. 2000; Tucholski and Johnson 2003) and synaptic plasticity (Friedrich, Fesus et al. 1991; Festoff, Suo et al. 2001). But probably most interestingly is the correlation of heightened transglutaminase activity in the brain and neurodegenerative disorders (Jeitner, Pinto et al. 2009). Nevertheless, our understanding of transglutaminase activity in the brain is still limited, especially as the investigation of transglutaminases in the nervous system has been concentrated on only one type, the tissue transglutaminases also called transglutaminase 2.

1.1 The transglutaminase family

Transglutaminases can be found throughout the tree of life, in microorganisms (Kanaji, Ozaki et al. 1993), plants (Serafini-Fracassini and Del Duca 2008), invertebrates (Singh and Mehta 1994) and vertebrates (Sarkar, Clarke et al. 1957; Puszkin and Raghuraman 1985; Yasueda, Nakanishi et al. 1995; Zhang and Masui 1997). In humans there are nine known members of the transglutaminase family, the transglutaminases 1 to 7, the factor XIIIa and the erythrocyte band 4.2 (Fig. 1). All members go back to a common ancestor (Grenard, Bates et al. 2001), possessing a high degree of structural homology.

Transglutaminase 1 is also named keratinocyte transglutaminase based on its first discovered location. It is expressed in terminal differentiating keratinocytes in the granular layer of the skin (Thacher 1989) and in cultured keratinocytes under differentiating conditions, like heightened calcium levels (Liew and Yamanishi 1992; Yada, Polakowska et al. 1993). The role of this enzyme in the skin is well studied. Transglutaminase 1 participates in the formation of the cornified cell envelope (CE), a ~15 nm thick structure around the cell membrane of dead corneocytes (terminal differentiated keratinocytes) in the cornified layer of the skin (Nemes and Steinert 1999). The CE consists of a 10 nm thick protein envelope (PE), made of cross-linked structural proteins (Rice and Green 1977), and a 5 nm thick lipid envelope (LE) linking the protein envelope to intercellular lipids. The CE provides mechanical stability and water impermeability to the outer layer of the skin. Transglutaminase 1 plays a critical role in assembling the PE (Candi, Melino et al. 1995; Candi, Tarcsa et al. 1999) and in the cross-linking of ceramides of the LE to proteins of the PE (Nemes, Marekov et al. 1999). Mutations in the transglutaminase 1 gene are linked with the severe disease of lamellar ichthyosis, leading to scaling of the skin and diminished skin barrier function (Russell, DiGiovanna et al. 1994). A transglutaminase 1 knock-out mouse model dies shortly after birth due to fatal water loss, based on a dysfunction in CE assembly (Matsuki, Yamashita et al. 1998).

Beside its localisation in the skin, transglutaminase 1 can be found in other epithelial tissues (Hiiragi, Sasaki et al. 1999; Martinet, Bonnard et al. 2003) and some parts of the vascular endothelium (Baumgartner, Golenhofen et al. 2004), where it is associated with intercellular junctions, most likely stabilising them by cross-linking activity (Baumgartner and Weth 2007).

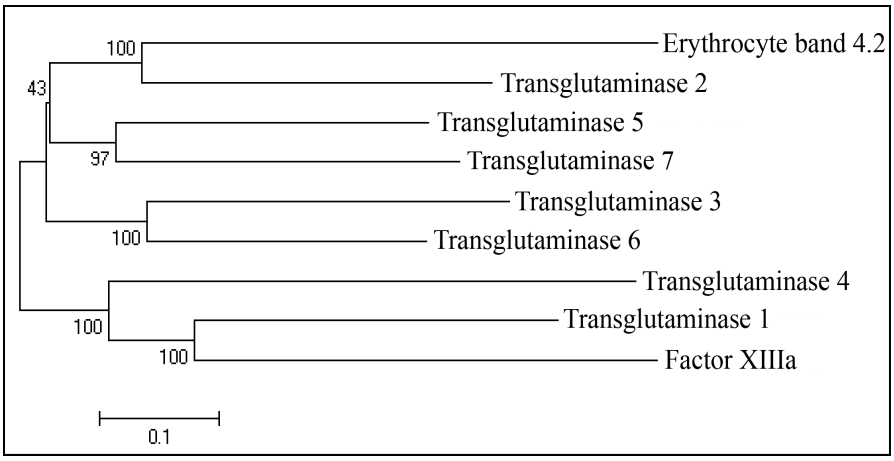


Fig. 1.1: Evolutionary distances between the members of the transglutaminase family. The evolutionary distances between the cDNA sequences of all nine human transglutaminases were calculated using MEGA 4.1 (www.megasoftware.net). The numbers at the joints are bootstrap values.

Transglutaminase 2, also named tissue transglutaminase, was the first enzyme discovered with transglutaminase activity (Sarkar, Clarke et al. 1957). It is the best investigated family member and expressed throughout the body. Besides its transamidating activity, it can also bind and hydrolyse guanosine triphosphate (GTP) (Nakaoka, Perez et al. 1994). Transglutaminase 2 is mainly an intracellular protein, localised in the cytosol. A small portion can also be found in the nucleus (Lesort, Attanavanich et al. 1998). Here it can modify histones (Ballestar, Abad et al. 1996) and transcription factors (Tatsukawa, Fukaya et al. 2009). Additionally transglutaminase 2 can partly be found extracellularly (Martinez, Chalupowicz et al. 1994). Given that transglutaminase 2 lacks a leader sequence, the enzyme is not secreted over the normal endoplasmatic reticulum/golgi pathway. Currently the export mechanism is still unknown. Extracellular transglutaminase 2 is mostly associated to membrane-proteins, primarily integrins (Akimov, Krylov et al. 2000).

The diverse localisations of transglutaminase 2 give an idea of the manifold functions this enzyme has. Transglutaminase 2 shows pro- as well as antiapoptotic properties (Fesus and Szondy 2005), depending on its localisation and activity. It plays a role in cell-matrix interactions (Zemskov, Janiak et al. 2006), is involved in signalling processes (Nakaoka, Perez et al. 1994; Kojima, Inui et al. 1997), inflammation (Lee, Kim et al. 2004) and can cross-link various cytoskeletal proteins, including actin (Nemes, Adany et al. 1997). Nevertheless, far more target proteins than functions for transglutaminase 2 are known. Additionally reports suggest that transglutaminase 2 can also act as a protein disulphide isomerase (Hasegawa, Suwa et al. 2003) and has an intrinsic kinase activity (Mishra and Murphy 2004).

Tranglutaminase 3 is expressed throughout the epidermis (Chung and Folk 1972; Ogawa and Goldsmith 1976; Tarcsa, Marekov et al. 1997). It is involved in the CE formation (Candi, Tarcsa et al. 1999) and it hardens the inner root sheath in hair follicles (Chung and Folk 1972; Tarcsa, Marekov et al. 1997).

In humans transglutaminase 4 is mainly expressed in the prostate (Dubbink, Verkaik et al. 1996). Its function is relatively unclear, but prostate cancer cells overexpressing transglutaminase 4 are more readily adhering to endothelial cells, resulting in a higher invasiveness (Jiang, Ablin et al. 2009). In rodents transglutaminase 4 is involved in the formation of the copulatory plug (Williams-Ashman 1984).

Transglutaminase 5, like transglutaminase 1 and 3, can be found in the skin (Candi, Oddi et al. 2001), where it seems to be important for the adhesion between the granular and cornified layer (Cassidy, van Steensel et al. 2005).

Transglutaminase 6 and 7 are largely uncharacterised (Grenard, Bates et al. 2001).

The enzymatic active A subunit of the blood clotting factor XIII, possesses transglutaminase activity and can cross-link fibrin (Lorand, Urayama et al. 1966), leading to blood clotting (Lorand 2001).

The erythrocyte band 4.2 is an enzymatic inactive member of the family and functions as a structural protein in membranes. In red blood cells it links CD47 to the cytoskeleton (Mouro-Chanteloup, Delaunay et al. 2003).

1.2 Structure and catalytic activity of transglutaminases

Typically transglutaminases consist of four domains, an N-terminal β-sandwich domain, a catalytic core and two C-terminal β-barrel domains (Yee, Pedersen et al. 1994; Liu, Cerione et al. 2002; Ahvazi and Steinert 2003). The core domain includes the reaction centre, comprising a catalytic triad of a cysteine, histidine and aspartate (Pedersen, Yee et al. 1994). The cysteine residue can attack the target glutamate, thereby forming a thiol-acyl enzyme intermediate, which is again attacked by a nucleophilic substrate (Folk 1969). Possible nucleophiles are a protein-bound lysine, a small primary amine, water or, at least in transglutaminase 1, a ω-hydroxy ceramide (see Fig. 2 for reaction mechanisms). Additionally a polyamine can be linked to the glutamine and in a second step another amino group of the polyamine is linked to a second glutamine, forming a cross-link

between two glutamines in a two step reaction (Martinet, Beninati et al. 1990). The transglutaminase reaction is relatively slow (Kim, Kim et al. 1994), with about one reaction cycle every three to four seconds. The substrate specificity is based mainly upon the glutamate residue (Coussons, Price et al. 1992). The specificity of different transglutaminases shows overlapping substrate spectra, but even at the same protein, it occurs that different transglutaminases target different glutamines (Hitomi, Horio et al. 2001).

Transglutaminases need high calcium levels, in the double digit µM range, to become fully active (Candi, Paradisi et al. 2004). The binding of three calcium ions leads to a conformational change in the protein, opening a pore to the catalytic core (Ahvazi and Steinert 2003), so that the enzyme becomes active. In the cytosol the transamidating activity is presumably blocked most of the time, whereas extracellular transglutaminases should always be active.

Besides calcium, at least transglutaminase 2 and 5 are able to bind and hydrolyse GTP (Achyuthan and Greenberg 1987; Candi, Paradisi et al. 2004). GTP blocks the calcium binding and, therefore, inhibits the transamidation activity of these transglutaminases (Hitomi, Ikura et al. 2000). From transglutaminase 2 it is also known, that it can act as a G-protein (Nakaoka, Perez et al. 1994) in signalling processes.

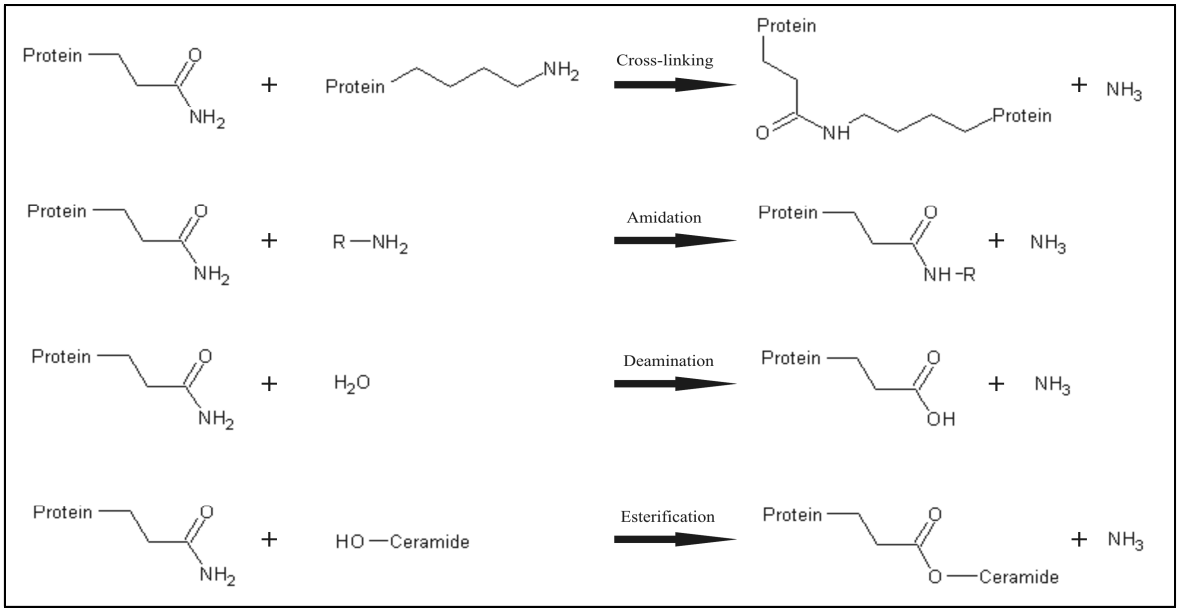


Fig. 1.2: Reaction mechanisms of transglutaminases. Transglutaminases target specific glutamine residues in proteins, which they bind covalently to a protein-bound lysine (cross-linking) or to small primary (poly)amines (amidation). With water the glutamine can be deaminated to a glutamate (deamination). At least transglutaminase 1 can create an ester-bond to ω-hydroxy ceramides (esterification).

But transglutaminase activity is not only modulated by calcium and GTP concentrations. Some transglutaminases (e.g. transglutaminase 1, 3 and factor XIII) must be proteolytically processed to become fully active (Lorand, Urayama et al. 1966; Kim, Gorman et al. 1993; Kim, Kim et al. 1994). Additionally various factors have been found to influence transglutaminase activity and substrate specificity (Lai, Bielawska et al. 1997; Nemes, Demeny et al. 2000; Sturniolo, Chandraratna et al. 2005; Antonyak, Li et al. 2009). This leads to the assumption, that transglutaminase activation *in vivo* can be much more complicated than expected.

Transglutaminase 1 is larger in size than other members of the transglutaminase family. Most additional sequences lie at the N- respectively C-terminus of the protein (Kim, Idler et al. 1991). As a unique feature, it can be bound to membranes, by acylation with myrisitc or palmitic acid (Chakravarty and Rice 1989). Therefore, transglutaminase 1 seems to be constitutively N-myristilated at a cluster of cysteine residues at the N-terminus of the protein. Additionally it can also be S-myristilated, or the myristilation can be exchanged with an S-palmytilation. At least in the skin, these changes seem to be dependent on the activity state of the enzyme (Steinert, Kim et al. 1996).

To become fully active, transglutaminase 1 has to be cleaved at two distinct sites by a protease, resulting in three fragments: a 10 kDa fragment containing the membrane anchor, a 67 kDa fragment with the catalytic core and a 33 kDa fragment spanning both β-barrel domains (Kim, Chung et al. 1995; Boeshans, Mueser et al. 2007). The three fragments can stay associated, limiting transglutaminase 1 activity to the membrane, or the 67 and 33 kDa fragments can detach together from the membrane anchor, resulting in cytosolic transglutaminase 1 activity. The activity of the membrane bound 67/33/10 kDa complex is about hundred times and that of the soluble 67/33 kDa complex is still about ten times higher than the full-length protein (Steinert, Chung et al. 1996).

1.3 Transglutaminases in the central nervous system

Besides the previous discussed locations of expression, transglutaminase 1, 2, 3 and 6 can also be found in the mammalian brain (Kim, Grant et al. 1999; Hadjivassiliou, Aeschlimann et al. 2008). The most abundant family member here is transglutaminase 2, at least two third of total transglutaminase activity in the mouse forebrain is based on transglutaminase 2 (Bailey, Graham et al. 2004). It was found in neuronal (Mahoney, Wilkinson et al. 2000) and glial (Monsonego, Shani et al. 1997) cell types.

One field of action for transglutaminase 2 seems to be the development of the brain. During the brain development the expression and activity of transglutaminase 2 changes significantly in various regions (Mahoney, Wilkinson et al. 2000; Citron, Zoloty et al. 2005). Transglutaminase 2 was found to stabilise tau by transamidation (Tucholski, Kuret et al. 1999) and could thereby affect axonal outgrowth. Furthermore, transglutaminase 2 can stabilise new developing neurites (Mahoney, Wilkinson et al. 2000), indicating its potential role in wiring of the brain. Transglutaminase 2 can also regulate cAMP response element-binding protein (CREB), giving it a role in neuronal cell differentiation (Tucholski and Johnson 2003).

Much less is known about the other members of the transglutaminase family in the brain. Transglutaminase 1 was found mainly in the cerebellum and the corpus callosum, transglutaminase 3 in the amygdala (Kim, Grant et al. 1999). Their functions in the central nervous system are unknown.

The best investigated field regarding transglutaminases in the brain are neurodegenerative disorders. Elevated transglutaminase expression and/or activity have been found in Alzheimer’s disease (AD), Huntington’s disease (HD), Parkinson’s disease (PD) and amyotrophic lateral sclerosis (ALS) (Fujita, Honda et al. 1998; Kim, Grant et al. 1999; Lesort, Chun et al. 1999; Junn, Ronchetti et al. 2003). Originally it was proposed that transglutaminase activity could facilitate protein aggregation (Kahlem, Terre et al. 1996; Junn, Ronchetti et al. 2003), a common characteristic of these disorders. In AD, amyloid beta and tau are targets for transglutaminase activity (Dudek and Johnson 1993; Dudek and Johnson 1994) and transglutaminase 2 expression and activity colocalises with senile plaques and neurofibrillary tangles in AD brains (Wilhelmus, Grunberg et al. 2009). But surprisingly in HD mouse models the knock-out of transglutaminase 2 produces even more protein aggregates (Lai, Tucker et al. 2004) and it was shown that transglutaminase activity

can inhibit the formation of amyloid-type protein aggregations in AD (Konno, Morii et al. 2005). Meanwhile soluble protein oligomers are seen as the toxic species in these disorders (Michalik and Van Broeckhoven 2003) and there are evidences that transglutaminase activity could stabilise these soluble oligomers (Konno, Morii et al. 2005).

An additional connection between neurodegenerative disorders and transglutaminases is indicated by a frequently dysregulation in the calcium homeostasis in this diseases (Palotas, Penke et al. 2004), probably leading to a pathologic overactivation of transglutaminases.

Nevertheless, the role of transglutaminases in the brain in general is poorly understood and their exact function in neurodegenerative diseases has still to be elucidated.

1.4 Aim of the thesis

The aim of this work is to further elucidate the expression pattern of transglutaminases in the central nervous system and their activity in neurons, with a special regard to transglutaminase 1. Investigations of neural transglutaminase 1 expression were so far restricted to the human brain in context of neurodegenerative disorders. In this work the expression of transglutaminase 1 in the murine brain is studied for the first time, using immunohistochemical stainings of cryostatic brain slices. Additionally the transamidating activity of transglutaminases in primary neuronal cell cultures of mice and chicken are to be investigated. Finally a substrate for neuronal transglutaminase 1 is identified and characterised.

2. Materials and Methods

2.1 Materials

2.1.1 Chemicals and enzymes

Chemical/Enzyme	Company
5-(biotinamido)pentylamine EZ-link	Fisher Scientific (Schwerte, Germany)
A23187 calcium ionophore	Sigma-Aldrich (St. Louis, MO, USA)
Acetone >99.5%	Applichem (Darmstadt, Germany)
Adenosine tri-phosphate magnesium salt >95%	Sigma-Aldrich (St. Louis, MO, USA)
Adenosine tri-phosphate sodium salt >98%	Applichem (Darmstadt, Germany)
Albumin fraction V (BSA) 98%	Carl Roth (Karlsruhe, Germany)
Albumin from bovine serum 96%	Sigma-Aldrich (St. Louis, MO, USA)
all trans-Retinal	Sigma-Aldrich (St. Louis, MO, USA)
Ammonium peroxide sulphate (APS)	Carl Roth (Karlsruhe, Germany)
Ampicillin sodium salt	Sigma-Aldrich (St. Louis, MO, USA)
Aprotinin	Applichem (Darmstadt, Germany)
Arabinosyl cytosine hydrochloride (araC)	Sigma-Aldrich (St. Louis, MO, USA)
Azure II	Fluka (St. Louis, MO, USA)
*Bam*HI	Fermentas (St. Leon-Rot, Germany)
Bovine brain acetone powder	Sigma-Aldrich (St. Louis, MO, USA)
Bovine calf serum	Hyclone (Logan, Utah, USA)
Bromophenol blue soidum salt	Carl Roth (Karlsruhe, Germany)
Calcium chloride dihydrate	Merck (Darmstadt, Germany)
Casein	Sigma-Aldrich (St. Louis, MO, USA)
Cellfectin	Invitrogen (Karslruhe, Germany)
Coelenterazine fcp	Sigma-Aldrich (St. Louis, MO, USA)
Developer LX24	Kodak (Stuttgart, Germany)
D-Glucose	Carl Roth (Karlsruhe, Germany)
Dimethyl caseine	Sigma-Aldrich (St. Louis, MO, USA)
Dimethyl sulfoxide	Applichem (Darmstadt, Germany)
Di-sodium hydrogen phosphate dihydrate	Carl Roth (Karlsruhe, Germany)
Di-sodium tetra borate 10-hydrat	Merck (Darmstadt, Germany)
Dithiothreitol	Carl Roth (Karlsruhe, Germany)
DMEM: Ham's F12	Invitrogen (Karslruhe, Germany)
Dnase I from bovine pancreas	Sigma-Aldrich (St. Louis, MO, USA)
Donor horse serum	Biochrom (Berlin, Germany)
Dulbeccos modified eagles medium (DMEM)	Lonza (Basel, Suisse)
*Eco*RI	Fermentas (St. Leon-Rot, Germany)
Ethanol >99.8%	Carl Roth (Karlsruhe, Germany)
Ethidium bromide solution 10mg/ml	Sigma-Aldrich (St. Louis, MO, USA)
Ethylene diamine tetracetic acid (EDTA)	Carl Roth (Karlsruhe, Germany)
Ethylene glycol tetracetic acid (EGTA)	Carl Roth (Karlsruhe, Germany)
Fetal calf serum (FCS)	Biochrom (Berlin, Germany)
Fixer AL4	Kodak (Stuttgart, Germany)

Gentamycin	Sigma-Aldrich (St. Louis, MO, USA)
Glacial acetic acid	Riedel-de Haën (Seelze, Germany)
Glycerol 86%	Carl Roth (Karlsruhe, Germany)
Glycine	Applichem (Darmstadt, Germany)
Grace's insect medium unsupplemented	Invitrogen (Karslruhe, Germany)
Hanks balance salts	Applichem (Darmstadt, Germany)
HEPES	Applichem (Darmstadt, Germany)
Herculase II polymerase	Stratagene (La Jolla, CA, USA)
Hexamine cobalt (III) chloride	Applichem (Darmstadt, Germany)
*Hind*III	Fermentas (St. Leon-Rot, Germany)
Hydrochloric acid 25%	Carl Roth (Karlsruhe, Germany)
Hydrogen peroxide 30%	Sigma-Aldrich (St. Louis, MO, USA)
Imidazole	Carl Roth (Karlsruhe, Germany)
IPTG	Carl Roth (Karlsruhe, Germany)
Isopentan	Applichem (Darmstadt, Germany)
Isopropanol	Carl Roth (Karlsruhe, Germany)
Kanamycin	Sigma-Aldrich (St. Louis, MO, USA)
Leupeptin	Sigma-Aldrich (St. Louis, MO, USA)
L-Glutamine	Applichem (Darmstadt, Germany)
Lipofectamine 2000	Invitrogen (Karslruhe, Germany)
Low melting agarose	Invitrogen (Karslruhe, Germany)
Luminol 98%	Fluka (St. Louis, MO, USA)
Lysozyme molecular grade	Applichem (Darmstadt, Germany)
Magnesium chloride hexahydrate >99%	Carl Roth (Karlsruhe, Germany)
Magnesium sulphate	Merck (Darmstadt, Germany)
Maleic acid	Sigma-Aldrich (St. Louis, MO, USA)
Manganese (II) chloride	Merck (Darmstadt, Germany)
Methanol 99%	Carl Roth (Karlsruhe, Germany)
Methylenblue hydrate	Riedel-de Haën (Seelze, Germany)
Neomycin	Carl Roth (Karlsruhe, Germany)
Nerve growth factor 7S from murine submaxil. gland	Sigma-Aldrich (St. Louis, MO, USA)
Neutral red	Carl Roth (Karlsruhe, Germany)
*Nhe*I	Fermentas (St. Leon-Rot, Germany)
Nickel-NTA agarose	Qiagen (Hilden, Germany)
Normal goat serum	Sigma-Aldrich (St. Louis, MO, USA)
*Not*I	Fermentas (St. Leon-Rot, Germany)
N-propyl gallat	Fluka (St. Louis, MO, USA)
Paraformaldehyde	Merck (Darmstadt, Germany)
p-Coumaric acid	Sigma-Aldrich (St. Louis, MO, USA)
Penicilline G potassium salt	Serva (Heidelberg, Germany)
Pepstatin A 99%	Applichem (Darmstadt, Germany)
Poly-L-lysine hydrobromide MW:30.000-70.000	Sigma-Aldrich (St. Louis, MO, USA)
Poly-L-lysine solution 0.1%	Sigma-Aldrich (St. Louis, MO, USA)
Ponceau S	Carl Roth (Karlsruhe, Germany)
Potassium acetate	Applichem (Darmstadt, Germany)
Potassium chloride	Carl Roth (Karlsruhe, Germany)
Potassium dihydrogen phosphate	Carl Roth (Karlsruhe, Germany)
RedTaq DNA Polymerase Premix	Sigma-Aldrich (St. Louis, MO, USA)
Rnase A Dnase free	Sigma-Aldrich (St. Louis, MO, USA)
Rotiphorese gel 30	Carl Roth (Karlsruhe, Germany)
Serva blue	Serva (Heidelberg, Germany)
Sf900 II SFM (1.3x)	Invitrogen (Karslruhe, Germany)
Sf-900 II SFM insect medium	Invitrogen (Karslruhe, Germany)

Skimmed milk	Applichem (Darmstadt, Germany)
Sodium acetate	Merck (Darmstadt, Germany)
Sodium azide	Carl Roth (Karlsruhe, Germany)
Sodium chloride	Carl Roth (Karlsruhe, Germany)
Sodium dicarbonate water free	Merck (Darmstadt, Germany)
Sodium dihydrogen phosphate dihydrate	Carl Roth (Karlsruhe, Germany)
Sodiumdodecylsulphate (SDS) ultra pure	Carl Roth (Karlsruhe, Germany)
Sodiumhydroxid	Carl Roth (Karlsruhe, Germany)
Streptomycine sulfate	Serva (Heidelberg, Germany)
Supplement B27	Invitrogen (Karslruhe, Germany)
T4 DNA ligase	Applichem (Darmstadt, Germany)
Tetracycline	Sigma-Aldrich (St. Louis, MO, USA)
Tetramethylethylenediamine (TEMED)	Carl Roth (Karlsruhe, Germany)
Trichlor acetic acid	Applichem (Darmstadt, Germany)
Tris ultra qualtiy	Carl Roth (Karlsruhe, Germany)
Tri-sodium citrate	Carl Roth (Karlsruhe, Germany)
Triton X-100	Carl Roth (Karlsruhe, Germany)
Trypan blue	Applichem (Darmstadt, Germany)
Trypsin 1:250	Applichem (Darmstadt, Germany)
Trypsin from bovine pancreas >9000 BAEE units/mg	Sigma-Aldrich (St. Louis, MO, USA)
Trypsin inhibitor from soybean, cell culture tested	Sigma-Aldrich (St. Louis, MO, USA)
Tween 20	Carl Roth (Karlsruhe, Germany)
X-Gal	Carl Roth (Karlsruhe, Germany)
*Xho*I	Fermentas (St. Leon-Rot, Germany)
Xylene cyanol FF for molecular biology	Sigma-Aldrich (St. Louis, MO, USA)
Yeast extract	Applichem (Darmstadt, Germany)

2.1.2 Devices

Device	**Company**
Thermocycler Primus 25	MWG (Ebersberg, Germany)
Power source PPC 300/200.4	Northumbria Biologicals Limited (Cramlington, UK)
Semidry blot apparatus Trans-Blot SD Cell	Bio-Rad (München, Germany)
PAGE aparatus Mini Protean II	Bio-Rad (München, Germany)
Horicontal shaker 3017	GFC (Burgwedel, Germany)
UV table TFP-M/WL	LTF Labortechnik (Wasserburg, Germany)
Heating block	Stuart Scientific (Stone, UK)
Exposing cassette Special/Rapid 200	Dr. Goos Suprema (Heidelberg, Germany)
Fluorescence microscope 102 M	Motic (Wetzlar, Germany)
Table top centrifuge 5415C	Eppendorf (Hamburg, Germany)
Environmental shaker ES-20	MS Laborgeräte (Wiesloch, Germany)
Incubator Hera Cell	Heraeus (Hanau, Germany)
Laminar flow cabinet BSB6	Gelaire (Syndney, AUS)
Centrifuge 3-15	Sigma (Osterode am Harz, Germany)
Ultracentrifuge TL-100	Beckmann (Fullerton, CA, USA)
Amplifier SEC-05LX	npi electronic GmbH (Tamm, Germany)
Oscilloscope HM 1507	Hameg Instruments (Mainhausen, Germany)
Cryostat CM 3050	Leica Microsystems (Wetzlar, Germany)

2.1.3 Kits

Kit	Company
Genomic DNA isolation: Geno/mini DNA Isolation Spin Kit	Applichen (Darmstadt, Germany)
Plasmid Isolation (mini): Zyppy Plasmid Mini Prep Kit	Zymo Research (Orange, CA, USA)
Plasmid Isolation (midi): Plasmid Midi Kit	Qiagen (Hilden, Germany)
DNA purification: High Pure PCR Product Purification Kit	Roche Applied Science (Penzberg, Germany)
Gel DNA extraction: Agarose Gel DNA Extraction Kit	Roche Applied Science (Penzberg, Germany)
Southern Blot: DIG High Prime DNA Labelling and Detection Starter Kit II	Roche Applied Science (Penzberg, Germany)

2.1.4 Antibodies

Antibody	Species	dilution	Clone/Cat. no.	Company
anti-Trans-glutaminase 1	rat	ICC/IHC/WB: undiluted	TG1F-1	Hybridoma supernatant [Hiiragi 1999]
anti-Mtap 2ab	mouse	ICC/IHC: 1:200	MT-01	Exbio (Vestec, Czech Republic)
anti-GFAP	rabbit	ICC/IHC: 1:5000	ab7260	Abcam (Cambridge, MA, USA)
anti-Synaptophysin	mouse	ICC/IHC: 1:1000	SVP-38	Sigma-Aldrich (St. Louis, MO, USA)
anti-Synapsin 1	rabbit	ICC/IHC: 1:500	S193	Sigma-Aldrich (St. Louis, MO, USA)
anti-β-actin	mouse	WB: 1:5000	AC-15	Sigma-Aldrich (St. Louis, MO, USA)
anti-GFP	rabbit	WB: 1:2000	598	MBL (Woburn, MA, USA)
anti-Rat-Cy2	goat	ICC/IHC: 1:200		Jackson ImmunoResearch (West Grove, PA, USA)
anti-Rat-Cy3	goat	ICC/IHC: 1:300		Jackson ImmunoResearch (West Grove, PA, USA)
anti-Rat-Pox	goat	WB: 1:30.000		Jackson ImmunoResearch (West Grove, PA, USA)
anti-Mouse-Cy2	goat	ICC/IHC: 1:200		Jackson ImmunoResearch (West Grove, PA, USA)
anti-Mouse-Pox	goat	WB: 1:30.000		Jackson ImmunoResearch (West Grove, PA, USA)
anti-rabbit-Cy2	goat	ICC/IHC: 1:200		Jackson ImmunoResearch (West Grove, PA, USA)
anti-Rabbit-Pox	goat	WB: 1:40.000		Jackson ImmunoResearch (West Grove, PA, USA)
Streptavidin-Cy2	/	ICC/IHC: 1:500		Jackson ImmunoResearch (West Grove, PA, USA)
Streptavidin-Cy3	/	ICC/IHC: 1:2.000		Jackson ImmunoResearch (West Grove, PA, USA)
Streptavidin-Pox	/	WB: 1:70.000		Jackson ImmunoResearch (West Grove, PA, USA)

Cy2: green fluorescent marker; Cy3: red fluorescent marker; Pox: horseradish peroxidase

2.1.5 Primer

Primer	Sequence	Annealing temp.
TG1 KO L fw	CTC GAG ACC GAT ATA TAC AGG GTT	47℃
TG1 KO L rev	CTC GAG ACT ATG AAT CCG GCA CCA	47℃
TG1 KO M fw	GTC GAC ATA GTG CTC CCC TAG TGC	47℃
TG1 KO M rev	GTC GAC GGT GGG TAC ATC TCT GTA A	47℃
TG1 KO R fw	GGA TCC CAC ATGV CCA CCA CTG GTC TT	51℃
TG1 KO R rev	GCG GCC GCA AAG CCA TAG TAC TTG GAT A	51℃
TG1 SB S1 fw	CGG ACT CTG TGA CCA TGC CT	50℃
TG1 SB S1 rev	CCG ACA TTG AGG ACC TTG GG	50℃
TG1 SB S2 fw	TAG CAA GGT GGA GAG GAG GTT TT	50℃
TG1 SB S2 rev	TTT ACA CCA CTG CCC CGA GA	50℃

fw: forward primer; rev: reverse primer

All primers were purchased at Eurofins MWG Operon (Ebersberg, Germany).

2.1.6 Animals

Mice (*Mus musculus*):

For cryostatic slices, the brains of adult male mice from the strain C57BL/6 were used. For cerebellar granule cell cultures brains of baby mice postnatal day five (P5) were used. The cultures used for the immocytochemical staining against transglutaminase 1 (Fig. 9) and for the transglutaminase activity staining (Fig. 13) were from BALB/c mice, those used for the immunocytochemical doublestainings (Fig. 10) were from C57BL/6 mice.

Chicken (*Gallus gallus*):

For the isolation of telencephalic cell cultures fertilised eggs from white leghorns were purchased from a local poultry and incubated in a forced draft incubator for 7 to 9 days before preparation.

2.2 Cell culture

2.2.1 General cell culture solutions

Phosphate-buffered salt solution (PBS):

80 g/L	Sodium chloride
0.2 g/L	Potassium chloride
1.7 g/L	Disodium hydrogen phosphate
0.2 g/L	Potassium dihydrogen phosphate

The pH was checked (should be between 7.2-7.4) and the solution was autoclaved.

Hanks balanced salt solution (HBSS):

9.82 g/L	Hanks Balanced Salts
0.35 g/L	Sodium bicarbonate

The solution was sterile filtrated.

Trypsin/EDTA:

0.5 g/L	Trypsin 1:250
0.2 g/L	Ethylenediaminetetraacetic acid (EDTA) dihydrate

Dissolved in PBS. The pH was adjusted to 7.2 with sodium hydroxide and the solution was sterile filtrated.

Pen/Strep (100x):

10 mg/ml	Penicillin G, sodium salt
10,000 U/ml	Streptomycin

Dissolved in PBS. The solution was sterile filtrated.

Trypan blue solution:

0.4% (w/v)	Trypan blue

Dissolved in PBS. The solution was filtrated through a 0.4 µm syringe filter.

2.2.2 PC12 cell culture

2.2.2.1 Maintenance of PC12 cell culture

Solutions

PC12 medium:

6% (v/v)	Donor horse serum
6% (v/v)	Bovine calf serum
1% (v/v)	Pen/Strep 100x (see 2.2.1)

The solutions were diluted under a clean bench in sterile Dulbecco's Modified Eagle Medium (DMEM) with high glucose and glutamine.

Procedure

To split a PC12 culture the medium was aspirated and the cells were washed one time with PBS. 1 ml (25 cm^2 flask) or 3 ml (75 cm^2 flask) Trypsin/EDTA was spread over the cell layer. Quickly the excessive liquid was aspirated and the cells were incubated for 1-2 min until they began to detach from the surface. The cells were washed of with 5 ml (25 cm^2 flask) or 15 ml (75 cm^2 flask) of 37°C warm PC12 medium and divided in a ratio of 1:4 to 1:10 into new flasks.

The cells were cultivated into 5 ml (25 cm^2 flask) or 15 ml (75c m^2 flask) PC12 medium at 37°C and 5% CO_2 in a humidified incubator. Every 2-3 days the medium was exchanged. Shortly before reaching confluency the cells were passaged again.

2.2.2.2 Transferring PC12 cells to glass cover slips

Solutions

Poly-l-lysine solution:

10 mg/ml	Poly-l-lysine hydrobromide

Dissolved under the clean bench in sterile double distilled water.

Procedure

The cover slips were cleaned and chemically sterilised in an ultrasonic cleaner in 80% ethanol (v/v). Four cover slips at a time were transferred in the 30 mm dishes and washed with sterile double distilled water. The glass slips were covered with poly-l-lysine solution and incubated at 37°C for 1-2 h. Afterwards the lysine solution was aspirated and the cover slips were washed three times for 10 min with sterile water. The cover slips were air dried over night under sterile conditions.

A PC12 culture (see 2.2.2.1) was splitted and transferred to a dish with lysine coated cover slips. After 30min the cells attached to the surface and they were cultivated in 2 ml PC12 medium as described in 2.2.2.1

2.2.2.3 Differentiating PC12 cells

Solutions

NGF solution (1000x):

50 µg/ml	Nerve growth factor 7S
1%	Bovine serum albumin

Dissolved in DMEM and sterile filtrated.

Procedure

To differentiate the PC12 cells into their neuronal phenotype the cell layer was washed one time with PBS. New PC12 medium laced with 50 ng/ml NGF was added to the cells. The cells were cultivated as described above (see 2.2.2.1) but medium with NGF was used. After 2 days the first neurites could be observed. About 10 days after the first addition of NGF the cells stopped dividing.

2.2.2.4 Transfection of PC12 cells

Procedure

$2x10^5$ PC12 cells per well were seeded in a 24-well plate and cultivated for one day. The culture medium was exchanged with 0.5 ml fresh PC12 medium. 2 µg vector DNA was diluted in DMEM, additionally 4 µl Lipofectamine 2000 was diluted in another 50 µl of DMEM. The solutions were incubated for 5 min at room temperature. Afterwards they were mixed together and incubated for another 20 min. The mixture was added to the cells and cultivated for 3 h. Afterwards the cells were washed one time with PC12 medium. Fresh medium was added and the cells were cultivated for 48-72 h.

Prior to analysis transfected cells containing the channelrhodopsin construct were incubated with all-trans retinal and cells containing the aequorin/GFP fusionprotein were incubated with coelenterazine fcp for 1h in the dark, to activate the proteins.

2.2.2.5 Whole cell current clamp recordings of PC12 cells

Solutions

Extracellular solution:

140 mM	Sodium chloride
2.8 mM	Potassium chloride
2 mM	Calcium chloride
2 mM	Magnesium chloride
10 mM	HEPES
10 mM	D-Glucose

The pH was adjusted to 7.2 with sodium hydroxide.

Intracellular solution:

140 mM	Potassium chloride
2 mM	Magnesium chloride
1 mM	Calcium chloride
11 mM	Ethylene glycol tetraacedic acid (EGTA)
10 mM	HEPES
2 mM	Disodium adenosine triphosphate
3mM	Magnesium adenosine triphosphate

The pH was adjusted to 7.2 with potassium hydroxide.

Procedure

PC12 cells were cultured on cover slips as described in 2.2.2.2. The cells were transferred to a chamber containing warm extracellular solution. Glass electrodes with an input resistance of 3-8 MΩ were made in a puller and filled with intracellular solution. The electrodes were droved up to the cells using a micro manipulator. A light negative pressure was applied at the electrode to achieve a seal between cell membrane and electrode. The membrane was severed locally by the application of a strong negative pressure. The successful access to the cell was controlled via its resting potential, which was typically in the range of -30 to -80 mV. Afterwards the reactions of the cell to depolarising or hyperpolarising pulses were recorded.

To record Channelrhodopsin-2 expressing cells, the cells were transfected with pBK-CMV Chop2-YFP like described in 2.2.2.4. 30 min before the recording was performed 1 µM all-trans retinal was added to the cells, and they were incubated at 37°C in the dark. The cells were transferred to the recording chamber and recorded like described above. To stimulate the cells they were illuminated with a mercury arc lamp.

2.2.3 Sf9 cell culture

Procedure

To passage Sf9 cells, the medium was aspired and the cells were washed of with 3 ml (25 cm^2 flask) or 10 ml (75 cm^2 flask) Sf-900 II SFM. The cells were split in a ratio of 1:2 to 1:4 into new flasks and cultivated in 5 ml (25 cm^2 flask) or 15 ml (75 cm^2 flask) of Sf-900 II SFM at 27°C in a humidified incubator. If necessary the medium was exchanged ever 4-5 days. At 80-90% confluency the cells were passaged again.

2.2.4 Isolation of chicken telencephalic cells

Solutions

Stop Buffer:

10% (v/v)	Fetal calf serum (FCS)

Dissolved in Dulbeccos Modified Eagles Medium/Hams F12 (1:1 mixture) under the clean bench.

Telencephalic Media:

2% (v/v)	50x B27-Supplement
50 U/ml	Penicillin
50 µg/ml	Streptomycin

Dissolved in Dulbeccos Modified Eagles Medium/Hams F12 (1:1 mixture) under the clean bench.

Procedure

5 ml and 15 ml of HBSS (see 2.2.1) were aliquoted in centrifugation tubes and cooled on ice. Two microscopy dishes were filled with cold HBSS. The egg was opened at the flat end and the embryo was fetched, decapitated and the head was transferred to one of the dishes. The skull was opened. The telencephalon was isolated and carried over to the second dish. The meninges were removed and the telencephalon was transferred to the tube containing 5ml HBSS. The tube was returned to the ice afterwards.

Under the clean bench the tissue was washed two times with 1ml cold HBSS. Afterwards the solution was replaced with 1 ml Trypsin/EDTA (see 2.2.1) and incubated eight minutes on ice and another eight minutes at 37°C in the incubator.

The Trypsin/EDTA was aspired and the digestion stopped with 1 ml Stop buffer. To separate the cells, the solution was passed 20-30 times through a 1000 µl Eppendorf pipette. The suspension was centrifuged 5 min at 300 g. The supernatant was discarded and the cells were resuspended in 1 ml HBSS. The solution was again centrifuged for 5 min at 300 g. The HBSS was aspired and the cells resuspended in10 ml warm telencephalic medium.

The cell suspension was counted and seeded in a concentration of 2.5-5x10^5 cells per cm^2 in poly-l-lysine coated dishes or flasks. The cells were cultivated at 37°C and 5% CO_2 in a water saturated incubator.

2.2.5 Isolation of murine cerebellar granule cells

Solutions

10x Krebs Ringer:

1.2 M	Sodium chloride
50 mM	Potassium chloride
12 mM	Potassium dihydrogen phosphate
0.25 M	Sodium bicarbonate
0.14 M	D-Glucose

A small amount (tip of a spatula) of phenolred was added.

$MgSO_4$-Solution:

3.82% (w/v)	Magnesium sulphate, 7 H_2O

$CaCl_2$-Solution:

1.6% (w/v)	Calcium chloride, 2 H_2O

KCl-Solution:

1.34 M	Potassium chloride

Dissolved in Basal Eagles Medium and sterile filtrated.

100x Gentamycin-Solution:

10 mg/ml	Gentamycin sulphate

Dissolved in double distilled water and sterile filtrated.

100x AraC-Solution:

1 mM	Cytosine-arabinofuranoside/HCl
16 µl/ml	KCl-Solution

Dissolved in Basal Eagles Medium and sterile filtrated.

Solution 1:

30 ml	10x Krebs-Ringer
0.9 g	Albumin from bovine serum, ≥96%, cell culture tested
2.4 ml	$MgSO_4$.-Solution

Dissolved in 300 ml double distilled water and sterile filtrated.

Solution T:

0.025% (w/v)	Trypsin from bovine pancreas, ≥9000 BAEE units/mg protein, cell culture tested.

Dissolved in Solution 1 and sterile filtrated.

Inhibitor-Solution:

0.008% (w/v)	Deoxyribonuclease I from bovine pancreas, ≥2000 Kunitz units/mg protein
0.052% (w/v)	Trypsin inhibitor from *Glycine max*, cell culture tested
1% (v/v)	$MgSO_4$-Solution

Dissolved in Solution 1 and sterile filtrated.

Thinned-Inhibitor-Solution:

3.2% (v/v)	Inhibitor-Solution

Dissolved in Solution 1 and sterile filtrated.

Solution Ca:

0.8% (v/v)	$MgSO_4$-Solution
0.12% (v/v)	$CaCl_2$-Solution

Dissolved in Solution 1 and sterile filtrated.

Granule Cell Medium:

10% (v/v)	Fetal Calf Serum
2 mM	L-Glutamine, cell culture tested
1% (v/v)	100x Gentamycin-Solution
1.65% (v/v)	KCl-Solution

Diluted in Basal Eagles Medium under the clean bench.

Procedure

The mouse was decapitated. The scull was opened and the cerebellum transferred to a dish with cold Solution 1. The meninges were removed and the tissue was transferred to a 15ml centrifugation tube containing 10ml cold Solution 1. The tube was kept on ice until further treatments. Up to five brains were pooled for further processing.

The solution with the tissue was transferred to a clean bench and poured in a 6 cm Petri dish. The brain was chopped with a scalpel and poured back into the tube. The solution was centrifuged for 3 min at 150 g and the supernatant was aspirated. The tissue was resuspended in 7 ml Solution T and transferred to a new 6 cm Petri dish. The tissue was incubated for 13 min at 37°C under occasional agitation.

To stop the trypsin reaction 7 ml of the Thinned Inhibitor Solution was added and the sample was transferred to a 15 ml centrifuge tube. It was centrifuged for 3 min at 150 g. The supernatant was aspirated and replaced with 2 ml of Inhibitor Solution. The cells were triturated by passing them 25 times through a flame polished Pasteur pipette. After 10 min the supernatant was transferred to a new centrifuge tube and 3 ml Solution Ca were added. To the pellet another 2 ml of Inhibitor-Solution was added and it was again passed 25 times through a flame polished pipette. Both cell suspensions were united. After another 10 min the supernatant was transferred to a new tube and centrifuged 10 min at 150 g. The supernatant was aspirated and the cells resuspended in warm Granule Cell Medium.

The cell suspension was counted and seeded in a concentration of $2.5x10^5$ cells per cm^2 in poly-l-lysine coated dishes or flasks. The cells were cultivated at 37°C and 5% CO_2 in a water saturated incubator. On the nest day 1% 100x AraC-Solution was added.

2.3 General methods

2.3.1 Discontinuous sodium dodecyl sulphate polyacrylamide gel electrophoresis

Solutions

Loading buffer (3x):

187 mM	Tris
208 mM	SDS
37.8% (w/v)	Glycerol

The chemicals were dissolved in double distilled water and the pH was adjusted to 6.9 with hydrochloric acid. 0.4 mg/ml bromophenol blue and for reductive conditions 1.5 mg/ml dithiotreithol were added.

Electrophoresis buffer (5x):

0.5%	SDS
0.96 M	Glycine
124 mM	Tris

Stacking gel:

3.6% (v/v)	Acrylamide/bisacrylamide (37.5:1)
125 mM	Tris/HCl pH 6.8
0.1% (w/v)	SDS
1.5 µl/ml	Tetramethylethylendiamine (TEMED)
3 µl/ml	10% ammonium persulphate (APS)

After the addition of TEMED and APS the gel was casted immediately.

Resolving gel (10%):

10% (v/v)	Acrylamide/bisacrylamide (37.5:1)
375 mM	Tris/HCl pH 8.8
0.1% (w/v)	SDS
1.5 µl/ml	TEMED
3 µl/ml	10% APS

After the addition of TEMED and APS the gel was casted immediately.

Procedure

The resolving gel was mixed and immediately poured between glass plates. The gel was overlaid with water. After 30 min the water was poured off, the stacking gel was mixed, poured immediately on top of the resolving gel and the comb was inserted. After another 30 min the comb was removed and the gel was transferred to a horizontal electrophoresis tank. The tank was filled with 1x electrophoresis buffer.

The samples were mixed with loading buffer and denatured at 95°C for 5 min. The denatured samples were filled into the wells of the gel. Additionally 4 µl of the protein ladder was injected into one well.

A voltage of 80 V was applied until the samples had left the stacking gel. Afterwards the voltage was raised to 100-160 V. The electrophoresis was stopped when the blue band of the loading buffer left the gel. After the electrophoresis the gel was stained with Coomassie blue (see 2.3.2) or the proteins were blotted on a nitrocellulose membrane (see 2.3.3)

2.3.2 Coomassie brilliant blue staining of polyacrylamide gels

Solutions

Staining solution:

2.5 g/L	Serva blue
45.4% (v/v)	Isopropanol
9.2% (v/v)	Glacial acetic acid

Destaining solution:

5% (v/v)	Glacial acetic acid
7.5% (v/v)	Ethanol

Procedure

The polyacrylamide gel was transferred to a dish with staining solution and stained for 10min on a horizontal shaker. The staining solution was transfused and replaced at first with tap water and afterwards with destaining solution. The gel was destained on a horizontal shaker and the destaining solution was regularly changed until the protein bands were clearly visible.

2.2.3 Western Blot

Solutions

Transfer buffer:

25 mM	Tris
192 mM	Glycine
20% (v/v)	Methanol
0.1% (w/v)	SDS

Ponceau S:

0.5% (w/v)	Ponceau S
3% (w/v)	Trichloracetic acid

PBST:

0.05% (w/v)	Tween 20

Dissolve in PBS (see 2.2.1)

Blocking solution:

5% (w/v)	skimmed milk

Dissolved in PBST

ECL I:

2.5 mM	Luminol
0.396 mM	p-Coumaric acid
0.1 M	Tris pH 8.5

ECL II:

0.0192% (v/v)	Hydrogen peroxide
0.1 M	Tris pH 8.5

Procedure

Six Whatman papers and a nitrocellulose membrane were soaked into transfer buffer and stacked with a polyacrylamide gel in the following order: three whatman papers, the nitrocellulose membrane, the gel and another three whatman papers. The stack was transferred to a semidry blotting chamber and blotted at 47 mA for 90 min.

After the blotting the membrane was transferred to a dish with Ponceau S solution and stained for about 1 min. The staining solution was poured back and the membrane was washed several times with double distilled water on a shaker, until the protein bands were clearly visible. If necessary the lanes were separated with a scalpel and marked with a pencil. To unstain the membrane completely, it was washed in PBS.

The PBS was exchanged with blocking solution and the membrane was blocked for 30 min on a shaker. The primary antibody was diluted in blocking solution. The membrane was transferred to a sealing bag and the antibody was added. The primary antibody was incubated for 3 h at room temperature or over night at 4°C on a shaker. The membrane was transferred to a dish with PBST and washed three times for 10 min under shaking. The peroxidase labelled secondary antibody was diluted in PBST. Afterwards the membrane was transferred again to a sealing bag containing the secondary antibody and incubated for 90 min at room temperature. The blot was finally washed three times for 10 min in a dish with PBST and stored in PBS until chemiluminescence detection.

For detection both ECL solutions were mixed in a ratio of 1:1 and the blot was incubated for 1 min in this solution. Afterwards the membrane was covered with saran wrap and an x-ray film. The bands of the protein weight marker were transferred with a pen to the film. The membrane was exposed for 10 sec to 15 min and the film was developed afterwards.

2.3.4 Polymerase chain reaction

Procedure

The template DNA was mixed with 0.25 µM of each Primer (200 pmol/µl) respectively, 10 µl of the Herculase reaction buffer and 0.5 µl of the dNTP Mix (25µM each dNTP) were added. The reaction volume was brought to 49 µl with double distilled water. Afterwards 1 µl of the polymerase was added. The tube was transferred to a thermocycler and following program was run.

Step	Temperature	Time	Cycles
Denaturing	95 °C	2 min	/
Denaturing	95°C	20 sec	25-35
Annealing	Primer dependent	20 sec	
Elongation	72°C	30 sec/kb DNA	

Tab. 2.1: Thermocycler program for polymerase chain reaction

The annealing temperature depended on the melting point of the used primer pair. The elongation time depended on the length of the amplified fragment.

2.3.5 Agarose gel electrophoresis

Solutions

50x TAE buffer:

2 M	Tris
1 M	Acetic acid
0.1 M	EDTA

The pH was adjusted to 8.3 with acetic acid.

6x Loading buffer:

10 mM	Tris pH 7.6
60 mM	EDTA pH 7.6
0.03% (w/v)	Bromophenolblue
0.03% (w/v)	Xylene cyanol FF
60% (w/v)	Glycerol

Ethidiumbromide staining solution:

0.5 µg/ml Ethidiumbromide

Dissolved in tap water. Ethidiumbromide is carcinogenic.

Procedure

Agarose was added to 1x TAE and dissolved by heating in a microwave oven. The concentration of the agarose in the solution varied from 0.8% (w/v) for large DNA fragments to 2% (w/v) for very small fragments. The solution was poured in the electrophoresis chamber and a comb was inserted. After the gel had solidified the comb was removed and the gel was overlaid with 1x TAE buffer.

The DNA was mixed with loading buffer and putted into a well. To be able to estimate the length of the DNA a DNA weight marker was added into an additional well. The DNA was separated at about 5V/cm electrode distance for 45 min to 90 min.

After the electrophoresis, the gel was transferred to a dish containing ethidiumbromide staining solution and stained for 15min in the dark. Afterwards it was moved to another dish with tap water, destained for another 15min in the dark and visualised on an UV table.

For preparatory purpose the desired DNA band was cut out of the gel with a clean scalpel and the DNA was isolated with a commercial available DNA extraction kit (e.g. Roche agarose gel DNA extraction kit).

2.4 Immunofluorescence stainings

2.4.1 Immunocytochemical stainings of cultured cells

Solutions

Formaldehyde fixative:

2% (w/v)	Paraformaldehyde

Mixed in PBS (see 2.2.1). The solution was heated up to 60°C. 1 N NaOH was slowly added until the Paraformaldehyde dissolved. Afterwards the solution was filtrated.

Permeabilistation buffer:

0.1% (w/v)	Triton X-100

Dissolved in PBS.

Blocking solution:

1% (w/v)	bovine albumin fraction V (BSA)
1% (v/v)	normal goat serum (NGS)

Dissolved in PBS.

Mounting solution:

1.5% (v/v)	N-propyl gallat
60% (w/v)	Glycerine

Dissolved in PBS.

Procedure

The cells were cultivated in 30 mm tissue culture dishes on polylysine coated glass cover slips. The cultures were washed two times with 37°C warm PBS. Afterwards they were fixed for 10 min at room temperature in 2% formaldehyde. Alternatively they were fixed in 100% Methanol at -20°C for 5 min. The fixed cells were washed three times with PBS at room temperature. Formaldehyde fixed cells were permeabilised with 0.1% Triton X-100 for 5 min at room temperature and were washed again three times with PBS, before they

were transferred to slides in the humid chamber and blocked with 25 µl blocking solution for 30 min at room temperature. Methanol fixed cells were blocked directly.

The blocking solution was replaced with 20 µl of the primary antibody diluted in an antibody dependent ratio in PBS. The antibody was incubated for 3 h at room temperature or over night at 4°C. Afterwards the cells were washed one time for 10 min and two times for 5 min with PBS at room temperature. For double-stainings the cells were incubated with the second primary antibody as described above for the first one.

The cells were incubated with 20 µl of the secondary antibody, diluted in PBS, for 90 min at room temperature in the dark. Afterwards they were washed three times for 5 min in the dark. The cover slips were shortly dipped into double distilled water and mounted on a drop of mounting solution on a slide with the cell covered surface facing downwards. The stainings were stored at 4°C in the dark and were observed at a fluorescence microscope.

2.4.2 Cryostatic brain slices and immunohistochemistry

Solutions

Methylenblue solution:

Solution 1:

1% (w/v)	Methyleneblue
1% (w/v)	Disodium tetraborate

Solution 2:

1% (w/v)	Azure II
1% (w/v)	Disodium tetraborate

Solution 1 and 2 are mixed in a ratio of 1:1 and filtered.

TG1-P incubation buffer:

0.1 M	Tris pH 8.0
5 mM	Calcium chloride
1 mM	Dithiothreitol

Stable for one week.

TG1-P control buffer:

0.1 M	Tris pH 8.0
1 mM	Dithiothreitol
1 mM	EDTA pH 8.0

Stable for one week.

Procedure

Isopentan was cooled down in liquid nitrogen to about -100°C. The animal was anesthetised and sacrificed. The brain was isolated and frozen in cold isopentan. Afterwards the brain was transferred directly into liquid nitrogen for at laest 10 min. Finally, the tissue was stored at -80°C in a deep temperature freezer.

The brain was cut into 7 µm thick slices using a cryostat. The slices were transferred to poly-l-lysine coated slides.

For methylenblue staining the slides were incubated at 30°C for 10 min and afterwards stained for about 2 min in methylenblue solution. The slides were washed with tap water and dried.

For staining with the transglutaminase 1 specific peptide TG1-P the slices were incubated at 30°C for 10 min and afterwards blocked for 30 min with blocking solution (see 2.4.1). 10 µM TG1-P was diluted in TG1-P incubation buffer (for the specific staining) or TG1-P control buffer (for the negative control). The blocking buffer was replaced with the TG1-P solutions and the slices were incubated for 90 min at 37°C in the dark. Afterwards the slides were washed 3 times for 10 min with PBS (see 2.2.1) in the dark.

For immunohistochemical stainings the slices were fixated at -20°C in acetone and washed three times for 5 min with PBS. Afterwards they were blocked for 30 min with blocking solution and incubated with primary antibody in a humid chamber for 3 h at room temperature or over night at 4°C. The slides were washed three times for 5 min with PBS and afterwards incubated for 90 min with the secondary antibody. The slides were washed three times for 5 min with PBS.

The stainings were overlaid with a drop of mounting solution (see 2.4.1) and a glass cover slip and were documented at a fluorescence microscope.

2.5 Analysis of transglutaminase activity

2.5.1 Biotinylation of transglutaminase target proteins in cell culture

Procedure

Mouse cerebellar granule or chicken telencephalic cells were cultured as described before (see 2.2.4 and 2.2.5). To analyse the transglutaminase activity the culture medium was renewed and supplemented with 1.3 mg/ml 5-(biotinamido)pentylamine (5-BPA) and 0.1 µl/ml R281 (a transglutaminase inhibitor). As negative control, only R281 was added to the medium. The cells were incubated over night and afterwards analysed by SDS-PAGE and Western Blot (see 2.3.1 and 2.3.2) with a streptavidin coupled to a peroxidase, or by imunocytochemistry (see 2.4.1) with a fluorophor coupled streptavidin.

2.5.2 Purification of biotinylated proteins

Solutions

Phosphate buffer:

0.1 M	Sodium dihydrogen phosphate
0.15 M	Sodium chloride

The pH was adjusted to 7.2.

Procedure

Cell cultures were labelled with 5-BPA as described in section 2.5.1. The cells were collected with the help of a rubber policeman in phosphate buffer containing protease inhibitors and homogenised by sonification. The cell lysate was pelleted at 10,000g for 10 min. The supernatant was transferred to a new tube. Streptavidin agarose was equilibrated for 30 min with phosphate buffer. The agarose was added to the cell lysate solution. The mixture was incubated over night at 4°C and additionally 3h at room temperature under constant agitation. The streptavidin agarose was pelleted at 4,000g for 5 min and washed two times with phosphate buffer. Afterwards the wash solution was exchanged with SDS loading buffer (see 2.3.1) containing dithiothreitol and the sample was incubated for 8 min

at 95°C. The agarose was pelleted at 14,000g for 1 min and the supernatant was transferred into a new tube. The supernatant was analysed by SDS-PAGE and Western blot with an antibody against β-actin or a streptavidin.

2.6 Generation and analysation of recombinant human transglutaminase 1

2.6.1 Cloning of TG1 cDNA into pFastBac1

Procedure

The cDNA sequence of human transglutaminase 1 containing an N-terminal His6-tag was excised out of the vector pGEM-T fl hTGk His via *Not*I and *Eco*RI. The DNA fragment containing the cDNA was purified over an agarose gel (see 2.3.5). The vector pFastBac 1 was also cut with *Not*I and *Eco*RI and column purified with a commercial available kit.
Afterwards the transglutaminase 1 cDNA was ligated into pFastBacI (see 2.7.4). The emerging clones were screened via analytical plasmid preparation (see 2.7.3) and restriction analyses and positive clones were sequenced, to verify the correct insert.

2.6.2 Generation of the recombinant bacmid

Solutions

LB Agar Plates:
See 2.7.2, after cooling to 60°C following solutions were added:

50 µg/ml	Kanamycin
7 µg/ml	Gentamicin
10 µg/ml	Tetracycline
40 µg/ml	IPTG
X µg/ml	X-Gal

The plates were poured immediately.

Procedure

The *E. coli* DH10Bac were thawed on ice. 100 µl DH10Bac suspension per transformation was transferred to a cooled 12 ml round bottom transformation tube. 1 ng of the pFastBacI construct or 5 µl sterile double distilled water for the negative control were added to the cells and mixed. The cell suspension was incubated for 30min on ice and afterwards heat shocked for exactly 45sec at 42°C without shaking. The cells were transferred back on ice and cooled down for 2 min. 900µl room temperature SOC medium was added and the tubes were shaked at 37°C at about 200rpm for 4 h.

A tenfold serial dilution (10^{-1} to 10^{-3}) of the cells in SOC medium was prepared and 100 µl of each dilution was plated on LB plates. The plates were incubated for 48h at 37°C and for about 1h at 4°C.

Ten white colonies were chosen from the plates and restreaked on fresh LB agar plates. The plates were incubated over night at 37°C. From a single colony confirmed to have a white phenotype the bacmid was isolated (see 2.6.3)

2.6.3 Isolation of recombinant bacmid

Solutions

LB media:

See 2.7.2, before inoculation following solutions were added:

50 µg/ml	Kanamycin
7 µg/ml	Gentamicin
10 µg/ml	Tetracycline

Solution I:

15 mM	Tris/HCl pH 8.0
10 mM	EDTA pH 8.0
100 µg/ml	RNase A, DNase free

The solution was filter sterilised.

Solution II:

0.2 M	NaOH
1% (w/v)	SDS

The solution was filter sterilised.

Potassium Acetate:

3 M	Potassium acetate

The pH was adjusted to 5.5 and the solution was autoclaved.

Procedure

2 ml LB media was inoculated with successfully transformed DH10Bac. The culture was grown over night at 37°C and about 200 rpm. 1.5 ml of the culture was transferred to a microcentrifuge tube and centrifuged for 1 min at 14,000 g. The supernatant was removed and the pellet resuspended in 300 µl of Solution I. To lyse the cells 300µl of Solution II was added and the sample was incubated for 5min at room temperature. Afterwards 300 µl 3M potassium acetate was slowly added and gently mixed in the meantime. The solution was incubated on ice for 5 to 10 min and afterwards centrifuged for 10 min at 14,000 g.

800 µl isopropanol was placed in a microcentrifuge tube and the supernatant was added. The solution was mixed by inverting it a few times. Afterwards it was incubated on ice for 5 to 10 min. The mixture was centrifuged for 15 min at 14,000 g. The supernatant was discarded and the pellet was washed with 500 µl 70% ethanol. After another centrifugation step of 5 min at 14,000 g, the supernatant was carefully removed completely and the pellet was air dried for about 10 min. The pellet, containing the desired DNA, was dissolved in 40 µl TE buffer and stored at 4°C.

The bacmid was analysed by a PCR (2.3.4) with specific primers and a subsequent gelelectrophoresis (2.3.5), to verify the correct insertion of the transglutaminase 1 sequence.

2.6.4 Transfecting Sf9 cells and isolating P1 viral stock

Procedure

$9x10^5$ cells per well in a six-well plate were seeded in Sf-900 II SFM. After 1h the cells had attach. 2 µg of the recombinant bacmid were diluted in 100 µl unsupplemented Grace's medium. Additionally 6 µl of cellfectin reagent were diluted in 100 µl unsupplemented Grace's medium. The DNA and the cellfectin solution were combined, gently mixed and incubated for about 30 min at room temperature. In the meantime the cells were washed once with unsupplemented Grace's medium.

800 µl of unsupplemented Grace's medium were added to the sample. The wash solution was removed from the cells and the DNA/cellfectin mixture was added. The cells were incubated for 5 h at 27°C. Afterwards the transfection media was replaced with 2 ml Sf-900 II SFM.

After 96 h the medium, containing the recombinant baculovirus, was collected and transferred to a 15 ml tube. To remove cells and debris, the medium was clarified for 5 min at 500 g. The supernatant was transferred to a new 15 ml tube. 2% (v/v) fetal bovine serum was added and this P1 viral stock was stored at 4°C in the dark.

2.6.5 Amplifying the baculoviral stock

Procedure

$1.4x10^7$ Sf9 cells were seeded in a 75 cm^2 flask. After 1 h $2.8x10^6$ virus particles (pfu) were added to the cells. The virus concentration of the P1 viral stock was assumed with $1x10^6$ pfu/ml. The cells were incubated for 96 h at 27°C. Afterwards the medium was removed and clarified at 500 g for 5 min. The supernatant was transferred to a new 15 ml tube and stored at 4°C in the dark. The virus titer of this P2 viral stock should be about ten times higher compared to the original stock. The virus concentration was determined by a viral plaque assay.

2.6.6 Viral plaque assay

Solutions

4% Agarose Gel:

4% (w/v)	Low melting agarose

The solution was autoclaved.

Neutral Red:

1 mg/ml	Neutral Red

Procedure

$1.2x10^6$ Sf9 cells per well are seeded in two 6-well plates. A tenfold serial dilution from 10^{-3} to 10^{-8} was prepared from the baculoviral stock in Sf-900 II SFM. After the cells had attached the medium in the 6-well plates was exchanged with 2 ml of the diluted viral solutions. Two wells per dilution were used and the cells were incubated for 1 h.

In the meanwhile the agarose gel was dissolved in a microwave oven and the 1.3x Sf-900 medium was heated to 37°C. Three parts of the 1.3x Sf-900 medium were combined with one part of the liquefied agarose. The solution was mixed and placed in a 37°C water bath.

Sequentially starting from the highest dilution, the viral solution was exchanged with 2 ml of Sf-900 agarose medium. The overlay was allowed to harden for about 15 min, afterwards the plates were moved to an incubator and incubated for 10 days at 27°C.

After 10 days 500 µl of neutral red solution was added to each well and incubated for 2 h. The excess stain was removed. Viral plaques appeared as bright spots in a red background. The viral titer of the baculoviral stock was calculated.

Viral titer: $\frac{\text{pfu}}{\text{ml}} = \frac{\text{number of plaques} * \text{dilution factor}}{\text{ml of inoculum}}$

Baculoviral stocks with a lower titer than $1x10^7$ pfu/ml were amplified again (see 2.6.5).

2.6.7 Expression of recombinant transglutaminase 1

Procedure

$2.25x10^7$ cells were seeded in a 75 cm^2 flask. After 1 h the cells were rinsed once with medium. Afterwards 15 ml fresh medium was added. The baculoviral stock solution was added to gain a viral titer of $4.5x10^7$ pfu per flask. The cells were incubated for 96 h. The medium was aspirated and the cells were harvested in 500 µl 3x SDS PAGE buffer. The expression was analysed by Western Blot using an anti-transglutaminase 1 antibody (see 2.3.3)

2.6.8 Purification of recombinant transglutaminase 1

Solutions

Lysis buffer:

50 mM	Sodium dihydrogen phosphate
300mM	Sodium chloride
10mM	Imidazole

The pH was adjusted to 8.0 with sodium hydroxide. After pH adjustment 1% (w/v) Nonidet P40 was added.

Wash buffer:

50 mM	Sodium dihydrogen phosphate
300mM	Sodium chloride
20mM	Imidazole

The pH was adjusted to 8.0 with sodium hydroxide.

Elution buffer:

50 mM	Sodium dihydrogen phosphate
300mM	Sodium chloride
250mM	Imidazole

The pH was adjusted to 8.0 with sodium hydroxide.

Procedure

The recombinant protein was expressed as described in section 2.6.7, but, instead, of 3x SDS PAGE buffer 8 ml lysis buffer was used to harvest the cells. The cell suspension was incubated for 10 min on ice and afterwards centrifuged for 10 min at 10,000 g and 4°C. The supernatant was transferred to a new tube.

400 µl of Ni-NTA agarose was equilibrated with 10 ml PBS. The equilibrated Ni-NTA agarose was transferred to the tube containing the cleared lysate and incubated under shaking for 2h at 4°C.

The mixture was loaded onto a 3 ml column and the flow-through was collected for analysis. The column was washed three times with 4 ml wash buffer, each time collecting the wash fraction for analysis. Afterwards the protein was eluted four times with 200 µl elution buffer. The eluates were collected separately. The purification was analysed by SDS-PAGE with Coomassie staining (see 2.3.2) and Western Blot (see 2.3.3).

Eluate fractions containing transglutaminase 1 were pooled and protease inhibitors were added to the solution.

2.6.9 Cross-linking of actin with recombinant transglutaminase 1

Solutions

Extraction buffer:

2 mM	Tris pH 8.0
0.2 mM	Adenosine triphosphate sodium salt
0.5 mM	Dithiothreitol
0.2 mM	Calcium chloride
0.01% (w/v)	Sodium azide

Procedure

20 ml extraction buffer was prechilled on ice water. 1 g acetone powder was added to the buffer and stirred on ice for 30 min. The solution was clarified for 10 min at 10.000 g. The supernatant was filtered through glass wool into a graduated cylinder and the volume was determined. Under stirring potassium chloride was added to a final concentration of 50 mM. Afterwards magnesium chloride was added to a concentration of 2 mM and adenosine triphosphate to a concentration of 1 mM.

The solution was incubated at room temperature for 30 min and for 90 min at 4°C without stirring. Afterwards the solution was stirred slowly at 4°C and potassium chloride was added to a final concentration of 0.6 M. The solution was incubated another 90 min at 4°C, this time with stirring. For experiments with F-actin this solution was used. Otherwise the solution was centrifuged for 3 h at 84.000 g and the supernatant was discarded. The pellet was washed one time with chilled extraction buffer and afterwards soaked in 1 ml buffer for 1 h. Subsequently the pellet was resuspended using a Dounce homogenizer. The suspension was transferred to a dialysing membrane and dialysed in extraction buffer at 4°C for 3 days. The buffer was changed every day. To clarify the solution it was centrifuged at 84.000 g for 3 h. The supernatant was transferred to a new tube and stored at -20°C.

To cross-link the purified actin, 1/10 volume of the actin solution and 100 ng/ml recombinant transglutaminase 1 was added to TG1-P incubation buffer and the solution was incubated for 2h at 37°C. To tag the target glutamate of actin with a biotin group additionally 1 mg/ml 5-(biotinamido)pentylamine was added.

2.7 Cloning of DNA fragments

2.7.1 Generation of competent cells

Solutions

FSB buffer:

100 mM	Potassium chloride
45 mM	Manganese dichloride
10 mM	Calcium dichloride
3 mM	Hexamine cobalt trichloride
10 mM	Potassium acetate
10% (w/v)	Glycerine

The pH was adjusted to 6.5 with acetate and the solution was sterile filtrated.

DMSO:

Aliquots of 500 µl of dimethyl sulfoxide (DMSO) were aerated with nitrogen and stored at -20°C.

SOB medium:

20 g/L	Casein
5 g/L	Yeast extract
10 mM	Sodium chloride
2.5 mM	Potassium chloride

The medium was autoclaved and after cooling following sterile solutions were added.

0.5% (v/v)	1 M magnesium chloride
0.5% (v/v)	1 M magnesium sulphate

SOB agar:

16 g/L agar were mixed with SOB basal medium and autoclaved. Afterwards magnesium chloride and sulphate were added and the agar plates were poured.

Procedure

A fresh SOB agar plate was inoculated with *Escherichia coli* DH5α and incubated at 37°C over night. 50 ml SOB medium was inoculated with five colonies from the agar plate and incubated at 37°C on a shaker. The OD_{600nm} of the culture was continuously checked and the incubation was stopped at an OD of 0.44-0.5 (about 4-6 h). The suspension was transferred to a Falcon tube and cooled on ice. Afterwards it was pelleted at about 1000 g for 12 min at 4°C. The supernatant was discarded and the pellet dissolved in 14 ml cold FSB buffer. The suspension was incubated for 15 min on ice and afterwards centrifuged again at 1000 g and 4°C for 10 min. The supernatant was discarded and the pellet resuspended in 3.36 ml FSB buffer. The solution was incubated on ice for 5 min. 117.6 µl DMSO were added, mixed well and incubated for another 5 min on ice. This step was repeated once. The suspension was portioned in 210 µl aliquots and immediately frozen in liquid nitrogen. Afterwards the aliquots were transferred to a -80°C freezer.

2.7.2 Transformation of competent cells

Solutions

LB medium:

10 g/L	Casein
5 g/L	Yeast extract
10 g/L	Sodium chloride

The pH was adjusted to 7.4 with sodium hydroxide and the solution was autoclaved.

Ampicillin solution:

50 mg/ml	Ampicillin

The solution was sterile filtrated.

Kanamycin solution:

70 mg/ml	Kanamycin

The solution was sterile filtrated.

LB agar plates:

15 g/L agar were added to LB medium and the solution was autoclaved. After the solution cooled down to about 60°C 1 µl/ml antibiotic solution was added (ampicillin or kanamycin) and the plates were poured.

SOC medium:

10 µl/ml sterile 2M glucose solution was added to SOB medium (see 3.2.1)

Procedure

Two agar plates per transformation and an additional plate for the negative control were poured. The competent cells were thawed on ice. 10 µl of a ligation reaction (see 2.7.4) were filled into a transformation tube and cooled on ice, for negative control additionally 10 µl sterile double distilled water were used. 100 µl competent cells were added to the tubes and incubated for 40 min on ice. The cells were heat shocked for exactly 90 sec in a 42°C water bath and afterwards cooled immediately on ice for 3 min. 900 µl SOC medium were added and incubated on a shaker at 37°C for 90 min. 100 µl of the solution was plated

on a agar plate, the remaining 900 µl were pelleted, resuspended in 100 µl SOC medium and plated on a second agar plate. The plates were incubated over night at 37°C. Clones were picked for analysis (see 2.7.3) and the plates were stored at 4°C.

2.7.3 Analytical plasmid preparation

Solutions

STET buffer:

0.1 M	Sodium chloride
10 mM	Tris/HCl pH 8.0
1 mM	Ethylene diamine tetraacetic acid pH 8.0 (EDTA)
5% (w/v)	Triton X-100

Lysozyme solution:

10 mg/ml	Lysozyme

Dissolved in STET buffer.

TE buffer:

10 mM	Trsi/HCl pH 8.0
1 mM	EDTA pH 8.0

RNase A solution:

20 µg/ml	Ribonuclease A DNase free

Dissolved in TE buffer.

Sodium acetate solution:

3 M	Sodium acetate

The pH was adjusted to 5.2 with acetate.

Procedure

5 ml of antibiotic containing LB media was inoculated with the desired bacteria and incubated over night at 37°C on a shaker. 1.5 ml of the culture was pelleted at 12000g for 1 min. The supernatant was discarded and resuspended in 350 µl STET buffer. 25 µl lysozyme solution was added. The tube was placed for 90 sec in a boiling water bath and afterwards centrifuged at 12000g for 10 min. The pellet was removed with a sterile toothpick and 40 µl sodium acetate and 420 µl isopropanol were added. After incubating 5 min at room temperature, the tube was centrifuged again at 12000g for 15 min. The supernatant was removed and the pellet washed with 1 ml 70% ethanol. After another centrifugation step at 12000g for 5 min the supernatant was completely removed and the pellet dried for 5-10 min and resuspended in 40 µl RNase A solution. The solution was incubated for 10 min at 37°C and stored at -20°C.

The isolated vector was investigated by restriction analysis with a commercial available restriction enzyme and subsequent agarose gel electrophoresis (see 2.3.5).

2.7.4 Ligation of DNA fragments into a vector

Procedure

The vector and insert DNA were digested with the same restriction enzymes and column or gel purified. 100ng of the vector were mixed with the 3-4 molar excess of the insert. The reaction volume was brought to 17.5 µl with double distilled water. The sample was incubated at 45°C for 5 min. 2 µl of 10x Ligase buffer and 0.5 µl of T4 DNA Ligase (0.5 units) was added. The ligation was performed at 16°C for 4 h or over night at 4°C. Afterwards the sample was heated to 65°C for 10 min. 10 µl of this ligation reaction was used for transformation (see 2.7.2).

2.8 Conditional transglutaminase 1 knock-out mouse

2.8.1 Assembly of the transglutaminase 1 knock-out vector

Procedure

Genomic DNA of C57BL/6 mice was isolated with a genomic DNA isolation kit. Via a PCR reaction (see 2.2.4) three DNA fragments (L, M and R) were amplified and cloned into a vector (see 2.7). The used primer pairs were TG1 KO L, TG1 KO M and TG1 KO R (see 2.1.5). With the help of the PCR primers specific restriction sites were inserted at the ends of the fragments (see 2.1.5, underlined sequences). With these restriction enzymes the fragments were consecutively subcloned into the destination vector pTarget.

The emerging vector pTarget TG1-KO was isolated from the bacteria with a commercial available midi prep kit and linearised with *Not*I. Afterwards the vector was electroporated into mouse stem cells and the cells were cultivated in medium containing the selective antibiotic G418. The evolving clones were singularised and their DNA was isolated and analysed.

2.8.2 Analysis of stem cell clones via Southern blot

Solutions

Depurination solution:

0.125 M	Hydrochloric acid

Denaturation solution:

87.66 g/L	Sodium chloride
20 g/L	Sodium hydroxide

Neutralisation buffer:

87.66 g/L	Sodium chloride
60.5 g/L	Tris

The pH was adjusted to 7.5 with hydrochloric acid.

20x SSC:

3 M	Sodium chloride
0.3 M	tri-Sodium citrate dihydrate

The pH was adjusted to 7.0 with hydrochloric acid and the solution was autoclaved.

Low stringency wash buffer:

0.1% (w/v)	Sodium dodecylsulphate
2x	SSC

High stringency wash buffer:

0.1 % (w/v)	Sodium dodecylsulphate
0.5x	SSC

Maleic acid buffer:

0.1 M	Maleic acid
0.15 M	Sodium chloride

The pH was adjusted to 7.5 with sodium hydroxide and the solution was autoclaved.

Antibody wash buffer:

0.3% (w/v)	Tween 20

Diluted in maleic acid buffer.

Blocking buffer:

10% (v/v)	10x blocking buffer (Roche)

Diluted in maleic acid buffer.

Detection buffer:

0.1 M	Tris
0.1 M	Sodium chloride

The pH was adjusted to 9.5 with hydrochloric acid.

Procedure

Genomic DNA of C57BL/6 mice was isolated with a commercial available kit and two DNA fragments (S1 and S2) were amplified over an PCR reaction (see 2.3.4) and cloned (see 2.7). The used primer pairs were TG1 SB S1 and TG1 SB S2. The constructs containing these fragments were isolated and the fragments were excised with *Bam*HI and *Not*I and gel purified. A Digoxygenin labelling reaction with the DIG high prime DNA labelling and detection kit was performed, following the random prime method. The labelled probes were purified with a PCR Purification kit.

The genomic DNA of the stem cell clones (see 2.8.1) was digested with *Bam*HI (for S1) or *Hind*III (for S2) and separated over an agarose gel (see 2.3.5). The gel was incubated for 10 min in depurination buffer, for 30 min in denaturation buffer, for 30 min in neutralisation buffer and finally for 15 min in 20xSSC. Afterwards the separated DNA was blotted in a capillary blot to a nylon membrane. Therefore, in a dish filled with 20xSSC a platform was created. On this platform two crossed bridges of whatman papers, their ends hanging into the 20xSSC solution, were build up. At the crossing of the bridges a pile of three soaked whatman papers, the gel, the membrane, another three soaked whatman papers and a 5 cm stack of paper towels was build up. On top of the pile a weight of about 500g was placed. The gel was blotted over night.

The next day the pile was dismantled and the nylon membrane was fixated at 80°C for 2 h. Afterwards the membrane was transferred to a hybridization bag and prehybridised with DIG Easy Hyb hybridization solution at 38°C for about 4 h under shaking. About 25 ng DIG labelled DNA probe per ml hybridization solution was transferred to a new microcentrifuge tube and denaturated in a boiling water bath for 5 min. Afterwards the probe was cooled on ice water and diluted in DIG Easy Hyb hybridisation solution. The prehybridisation solution was exchanged with the hybridisation solution and the blot was hybridised over night at 38°C under constant agitation.

The next day the blot was washed two times for 10 min in low stringency buffer at room temperature and two times for 30 min in high stringency buffer at 68°C on a shaker. Afterwards the blot was washed in antibody wash solution for 3 min at 38°C and blocked for 30 min in blocking solution. The blocking solution was exchanged with the antibody solution consisting of anti-Digoxygenin antibody diluted 1:10000 in blocking solution. The blot was incubated for another 30 min with the antibody at 38°C. Afterwards it was washed two times for 15 min at 38°C in antibody washing buffer and additionally incubated for 3 min with detection buffer.

The detection buffer was poured of and the hybridization bag was cut open. The membrane was covered with CSPD chemiluminescence buffer and immediately covered again with the bag. After 5 min of incubation the bag was sealed again and the blot was incubated for another 10 min. Afterwards the blot was exposed to X-ray film for 20-45 min.

3. Results

3.1 Transglutaminase 1 expression in the murine brain

So far only limited data are available for the expression pattern of tranglutaminase 1 in the brain. Transglutaminase 1 has been detected in the cerebral cortex, the corpus callosum and the cerebellum (Kim, Grant et al. 1999). It has been found in neuronal and glial cells. However, its definite expression pattern remains unknown.

To further elucidate the occurrence of transglutaminase 1 in the brain, 7 µm thick coronal sections of the brain of an adult male C57BL/6 mouse were prepared and immunohistochemically stained against transglutaminase 1 and microtubule-associated protein 2 (Mtap2) respectively glial fibrilary acidic protein (GFAP). Mtap2 is a neuronal marker. It appears mainly in dendritic microtubules (Friedrich and Aszodi 1991). GFAP on the other hand is a glial marker, primarily for astrocytes, occurring in intermediate filaments (Eng, Ghirnikar et al. 2000). To back up the findings with the anti-transglutaminase 1 antibody a synthetic peptide (TG1-P; sequence: YEQHKLPSSWPF), known to be a preferred substrate of transglutaminase 1 (Sugimura, Hosono et al. 2008), was used to visualise transglutaminase 1 via its activity. The glutamine in TG1-P is a target for transglutaminase 1 activity, but not for the transglutaminases 2 and 3, as well as factor XIIIa. Transglutaminase 1 cross-links the peptide to nearby proteins. This activity is visualised via a fluorescein marker at the C-terminus of the peptide.

Throughout the brain the anti-translgutaminase 1 antibody stained the endothelial lining of blood vessels (see Fig. 3.1). The signal was located at the membranes of endothelial cells. Additionally transglutaminase 1 stainings were also found in parts of the ventricular system in ependymal cells, like in the lateral ventricle in figure 3.1. Similar to the endothelium the signal was located at the membranes, but most common at the basal side. So it seems that a membrane-bound form of transglutaminase 1 is expressed in endothelial and ependymal cells of the brain.

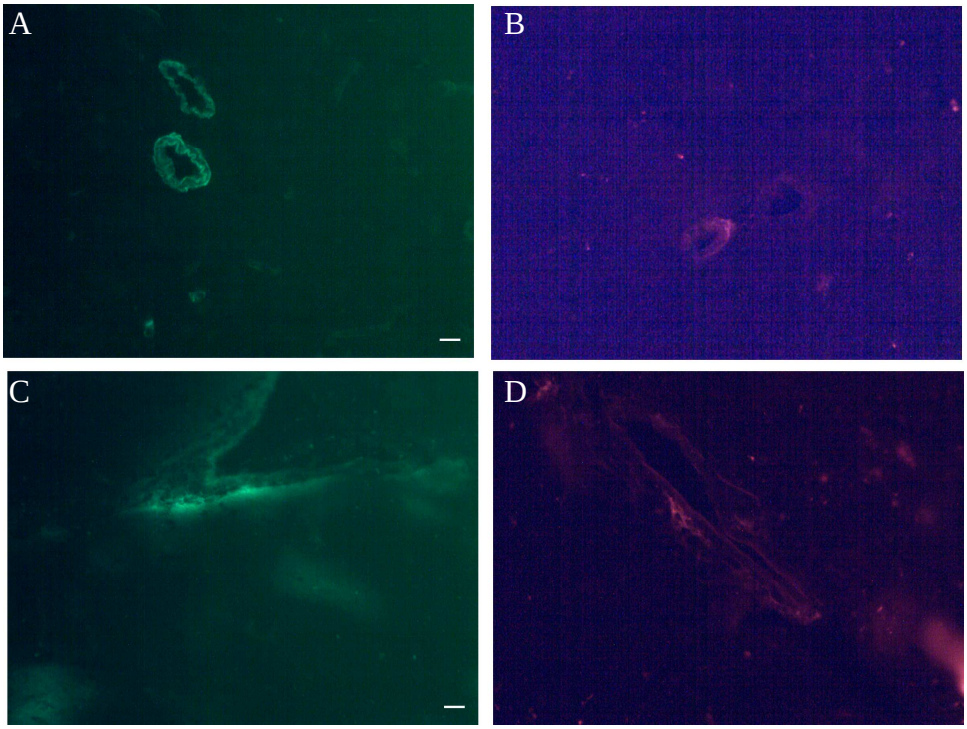


Fig. 3.1: Transglutaminase 1 expression in blood vessels and ventricles. Detection of transglutaminase 1 activity with TG1-P (A+C) and immunohistochemical stainings against transglutaminase 1 (B+D) in cryostatic slices of the mouse brain.
Scale bar: 10 µm

Other prominent signals of the anti-transglutaminase 1 antibody were found in different fibre tracts, like the corpus callosum (see Fig. 3.2), the amygdala capsule and the external capsule (see Fig. 3.3). A streak like staining was found. The intensity of these streaks decreased towards the borders and could not be linked to specific structures or cell borders, although they colocalised at least partly with GFAP stainings.

In addition transglutaminase 1 staining was found in parts of the caudoputamen (Fig. 3.3). Like the structures before the expression was mainly restricted to nerve fibres. But again the signal was only found sparsely in a streaked or punctuated pattern and not throughout all of the fibres.

Even though these stainings look rather unspecific, the immunohistochemcial findings were supported by transglutaminase 1 activity stainings using TG1-P. Additionally both negative controls, an immunohistochemical staining without primary antibody and a TG1-P staining with EDTA (to inhibit the calcium dependent transglutaminase activity), were negative in this regions. It seems that transglutaminase 1 is expressed in parts of nerve fibres. It is partially localised in astrocytes, but for the most parts in GFAP and Mtap2 negative regions, presumably axons.

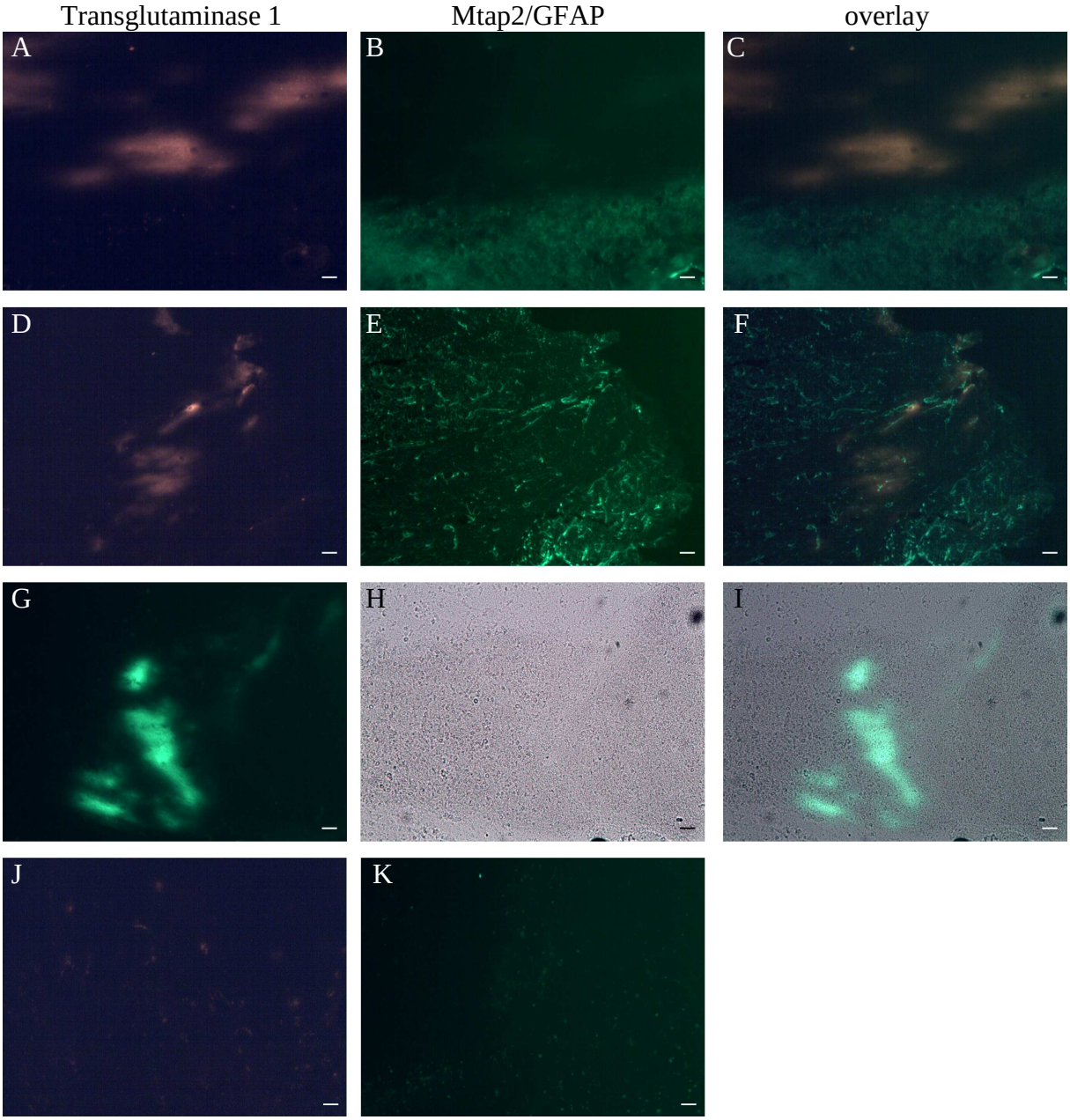
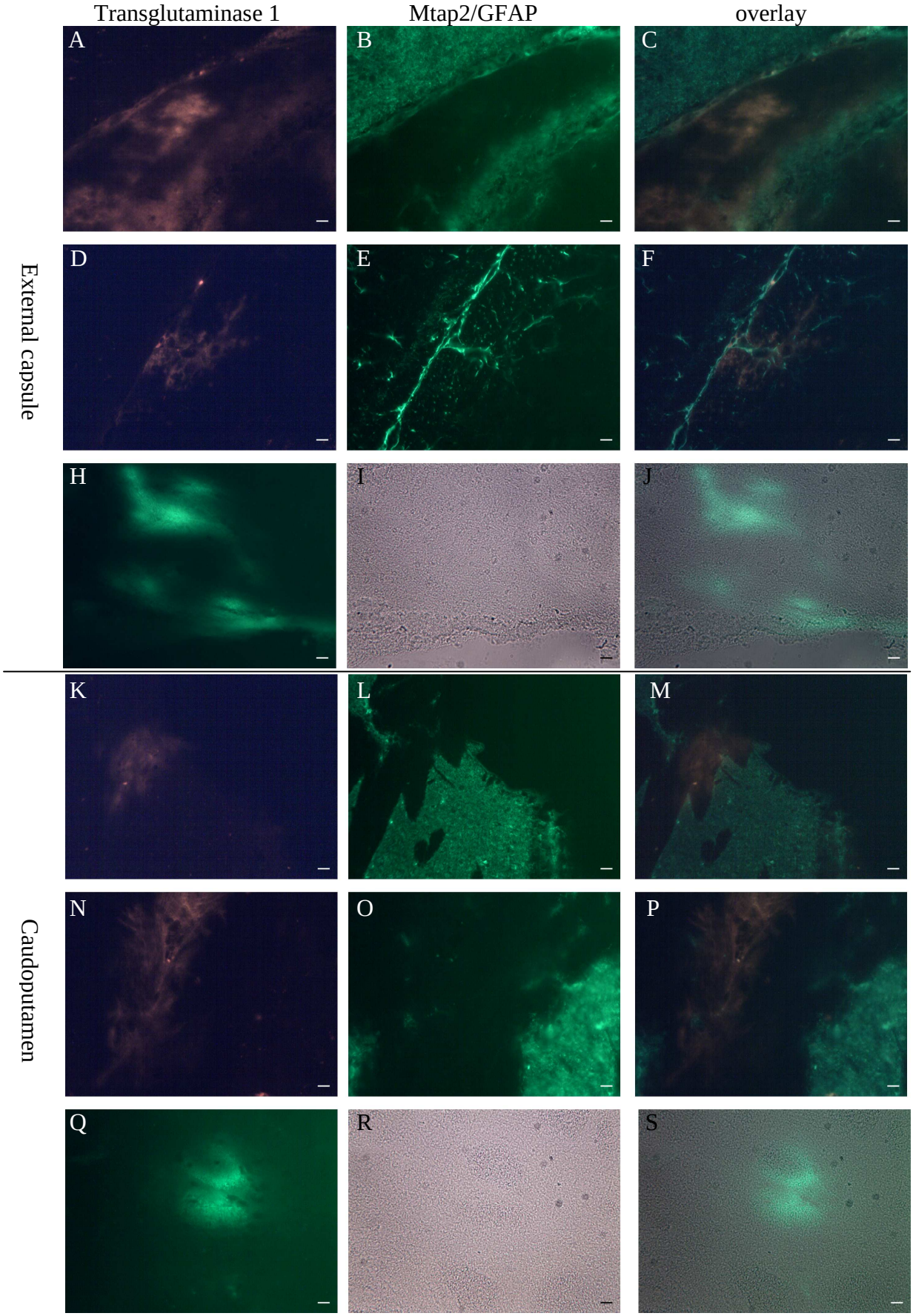


Fig. 3.2: Double staining in the region of the corpus callosum. Cryostatic slices of a mouse brain were immunohistochemically stained against A: transglutaminase 1, B: Mtap 2, C: overlay of A+B, D: transglutaminase 1, E: GFAP, F: overlay of D+E. G: Staining of transglutaminase activity with TG1-P, H: phase contrast image, I: overlay of G+H, J: negative control for anti-transglutaminase 1 staining (corpus callosum in the upper half), K: negative control for TG1-P staining (corpus callosum on the left side)
Scale bar: 10µm (A-C, J+K) or 20µm (D-I).


Transglutaminase 1
Mtap2/GFAP
overlay
External capsule
Caudoputamen
A
B
C
D
E
F
H
I
J
K
L
M
N
O
P
Q
R
S

⬆Fig. 3.3: Double staining in the region of the external capsule and the caudoputamen. Cryostatic slices of a mouse brain were immunohistochemically stained against transglutaminase 1 (A, D, K and N), Mtap 2 (B and L) and GFAP (E and O). Additionally transglutaminase activity was visualised with TG1-P (H and Q), I and R are phase contrast images of the TG1-P stainings. The pictures in the last row are overlays of the two previous pictures. The stainings A-J were in the region of the external capsule, the stainings K-S were in the caudoputamen.
Scale bar: 10µm.

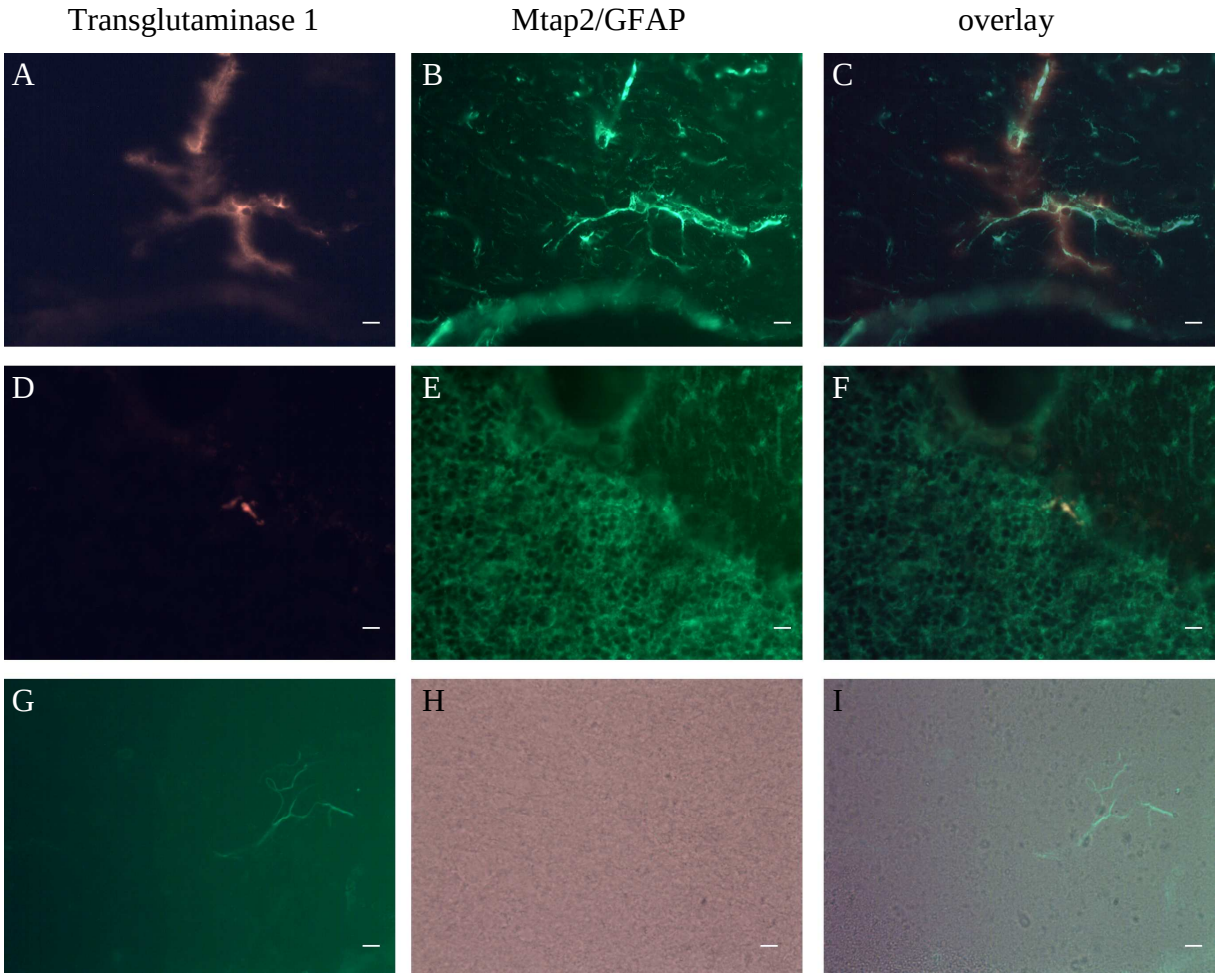


Fig. 3.4: Double staining of the cerebral cortex and the cerebellum. Cryostatic slices of a mouse brain were immunohistochemically stained against A: transglutaminase 1, B: Mtap 2, C: overlay of A+B, D: transglutaminase 1, E: GFAP, F: overlay of D+E. G: Staining of transglutaminase activity with TG1-P, H: phase contrast image, I: overlay of G+H.
Scale bar: 10µm (A-C and G-I) or 20µm (D-F)

Besides these prominent stainings far-scattered transglutaminase 1 positive astrocytes were found in the cerebral cortex and in the cerebellum (see Fig. 3.4). Few positive neurons were also found in the granular layer of the cerebellum (see Fig. 3.4). In contrast to the endothelial and ependymal cells, no clear membrane localisation of the signal was visible.

Summing up in the murine brain membrane-bound transglutaminase 1 was found in endothelial cells of the brain vascular system and partly in ependymal cells of the ventricular system. In neural cell types some astrocytes of the cerebral cortex and the cerebellum, as well as few neurons of the cerebellar granule layer were transglutaminase 1 positive. But most striking was the transglutaminase 1 staining found in different fibre tracts, most likely in projecting axons.

3.2 Transglutaminase 1 expression in cerebellar granule cell culture

To further investigate the role of transglutaminase 1 in the brain, considering its expression in some cells of the cerebellum, cell cultures of primary cerebellar granule cells were chosen. The cells were isolated form five days old baby mice and cultivated for up to four weeks. Figure 3.5 shows anti-transglutaminase 1 stainings at various time points in this culture.

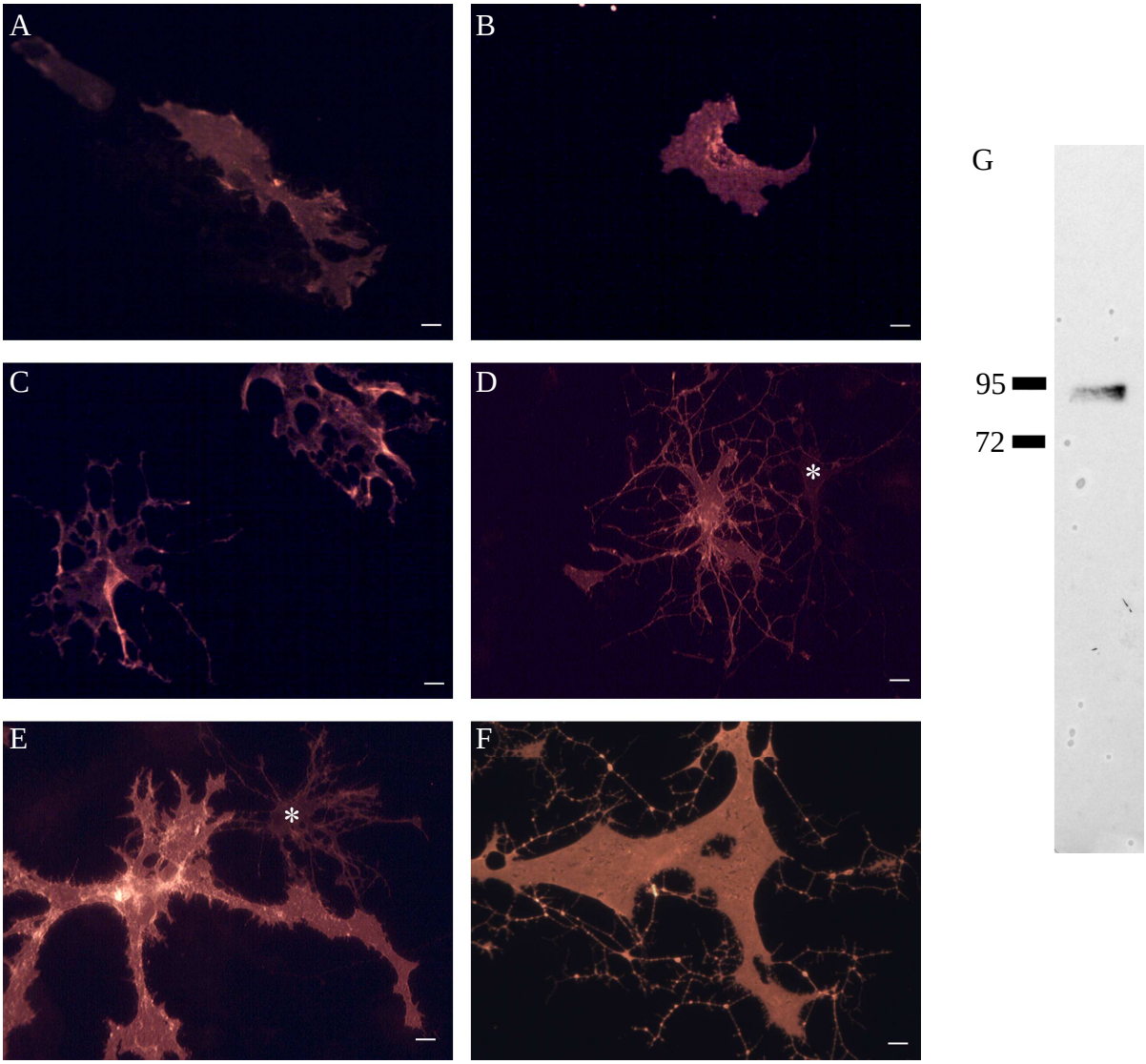


Fig. 3.5: Time dependent expression of transglutaminase 1 in murine cerebellar granule cells. Cell cultures of cerebellar granule cells where fixed and stained against transglutaminase 1 at various time points. A: DiV 1; B: DiV 3; C: DiV 5; D: DiV 9; E: DiV 14; F: DiV 28. The * marks cells with a weak transglutaminase 1 expression. G: Western Blot of 14 days old granule cell cultures against transglutaminase 1. 72 and 95 kDa are marked in the blot.
Scale bar: 10 µm

During the first five days only a faint staining of a handful of cells was visible, becoming stronger and more frequent over cultivation time. Interestingly the staining mostly showed no clear membrane localisation of transglutaminase 1, therein reflecting the immunohistochemical stainings, indicating that a high percentage of the expressed transglutaminase 1 exists in its soluble form. At later time point (>1 week) groups of transglutaminase 1 positive cells were observed frequently. The expression intensity could then be divided roughly in three groups, transglutaminase 1 negative cells, cells with a low expression of transglutaminase 1 (Fig. 3.5 D+E cells marked with an asterisk) and cells with a high expression of transglutaminase 1 (Fig 3.5 D+F). Western Blot analysis of later cultures with an anti-transglutaminase 1 antibody revealed a single band at about 92 kDa (Fig 3.5 G), which matched the calculated mass of the soluble transglutaminase 1 protein.

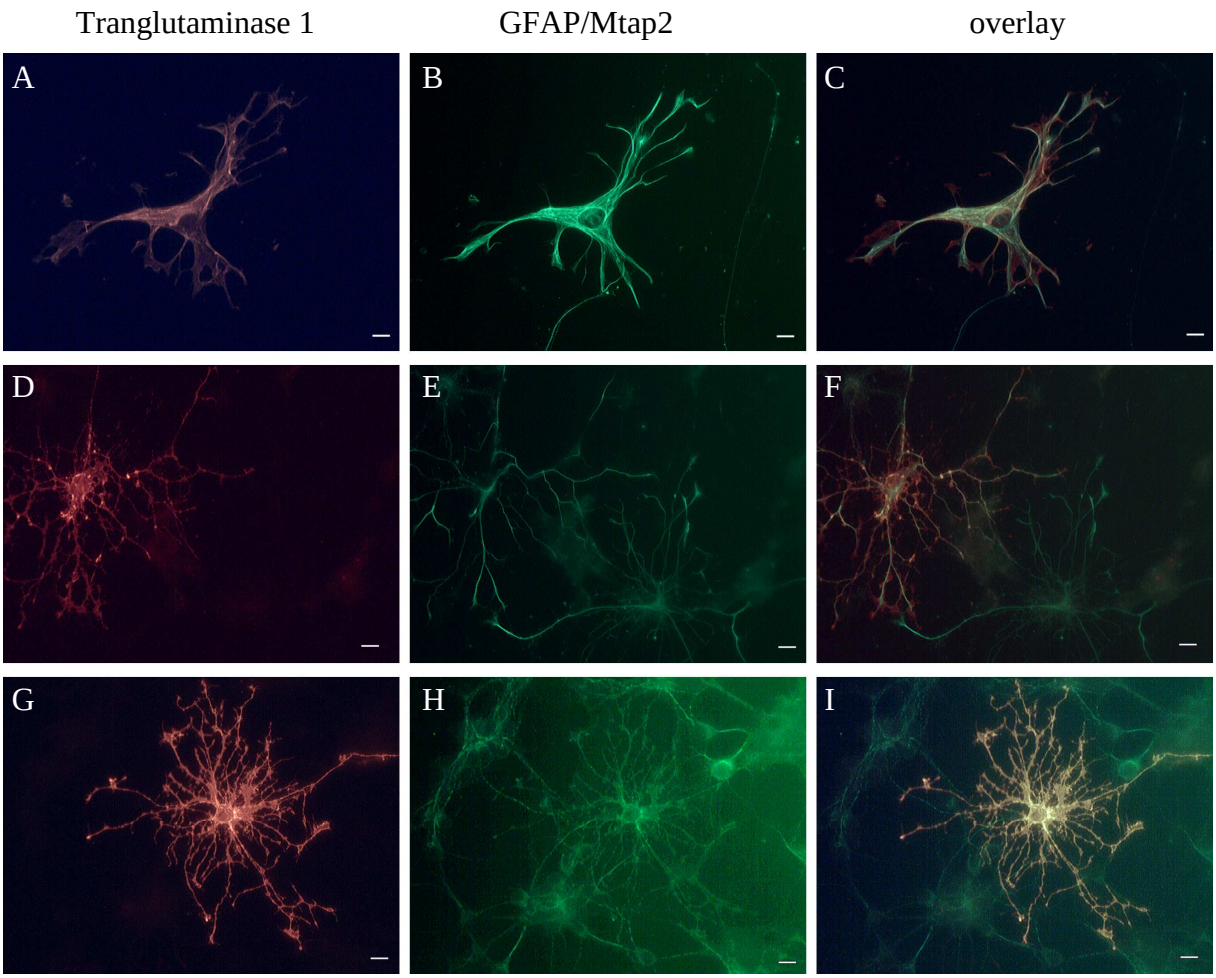


Fig. 3.6: Double stainings of murine cerebellar granule cells. 5 days old cultures (A-C) and 9 days old cultures (D-I) of cerebellar granule cells were stained against A: transglutaminase 1, B: GFAP, C: overlay of A+B, D: transglutaminase 1, E: GFAP, F: overlay D+E, G: transglutaminase 1, H: Mtap2, I: overlay of G+H.
Scale bar: 10µm.

Double stainings against transglutaminase 1 and Mtap2 respectively GFAP gave information about the cell types of transglutaminase 1 positive cells. In the first eight days of the culture only glial cells were found to be transglutaminase 1 positive (Fig. 3.6 A-C). At day nine in culture the first transglutaminase 1 positive neurons were found (Fig. 3.6 G-I). But even though the total number of neurons exceeded the number of glial cells in the culture, the number of transglutaminase 1 positive neurons remained relatively low during the cultivation time, compared to transglutaminase 1 positive astrocytes. Additionally the average signal strength in glial cells lay clearly above the expression in neuronal cell types.

3.3 Transglutaminase activity in neuronal cell cultures

Transglutaminase 1 is normally dormant intracellulary and has to be activated by heightened calcium levels. Therefore, the activity of transglutaminase 1 in the cell culture was analysed. A synthetic substrate for transglutaminases named 5-(biotinamido)pentylamine (5-BPA) was used for these experiments. The molecule consists of a small primary amine which gets linked to a target glutamine of transglutaminases by their activity. For detection purpose this amine is coupled to a biotin, which can be detected with streptavidin. The 5-BPA is membrane permeable and can be added directly to the cell culture. Given that 5-BPA is a substrate of all members of the transglutaminase family, prior to testing the activity, the expression of other transglutaminases known to be found in the cerebellum, namely transglutaminase 2, has to be checked. Therefore, granule cell cultures were stained against transglutaminase 2 (Fig. 3.7).

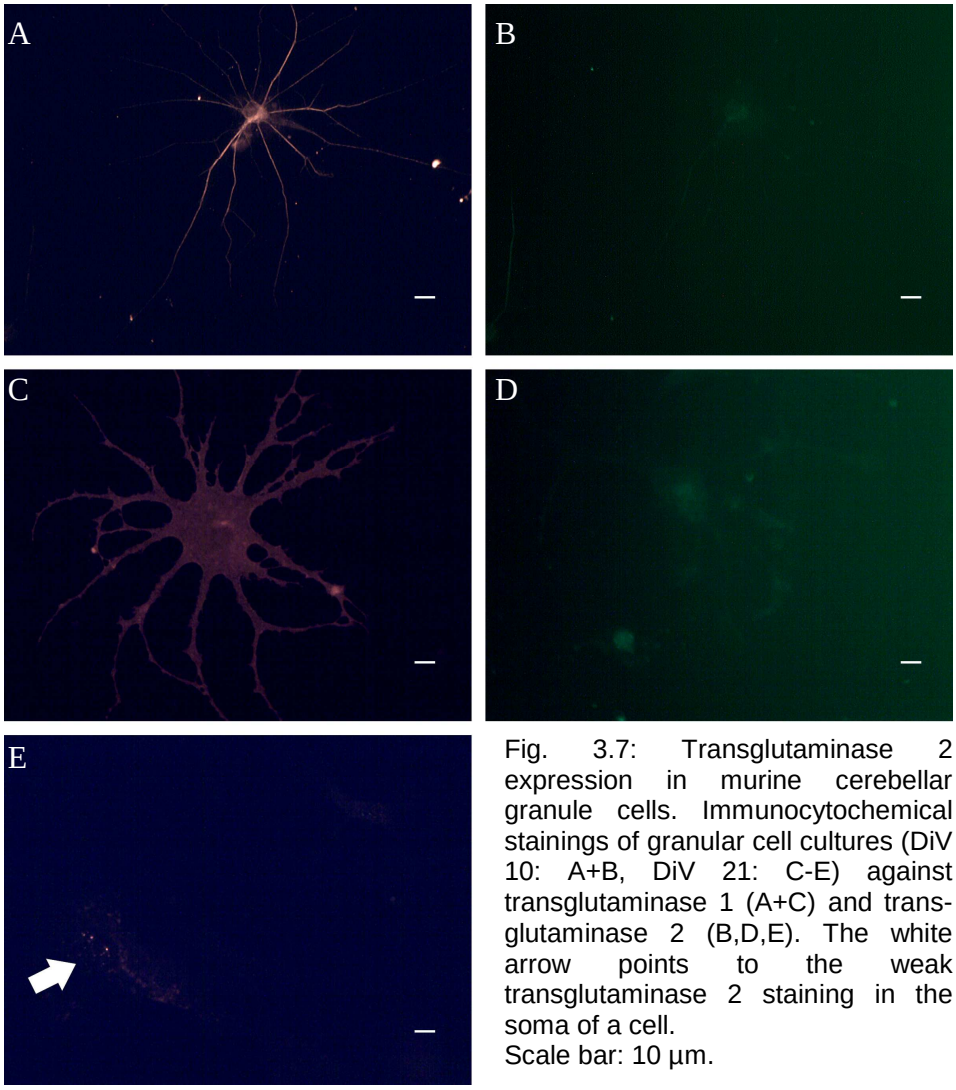


Fig. 3.7: Transglutaminase 2 expression in murine cerebellar granule cells. Immunocytochemical stainings of granular cell cultures (DiV 10: A+B, DiV 21: C-E) against transglutaminase 1 (A+C) and transglutaminase 2 (B,D,E). The white arrow points to the weak transglutaminase 2 staining in the soma of a cell.
Scale bar: 10 µm.

In young cultures there was no transglutaminase 2 signal detectable (Fig. 3.7 B). Only in cultures older than two weeks a spotted staining in the soma of some cells was visible (Fig. 3.7 E). Transglutaminase 2 is partly expressed extracellulary, so it is most likely that in these cells the transglutaminase 2 is located in some type of secretory vesicles. To block this presumed extracellular activity of transglutaminase 2 a membrane impermeable inhibitor of transglutaminases called R281 was added to the cultures in all activity experiments.

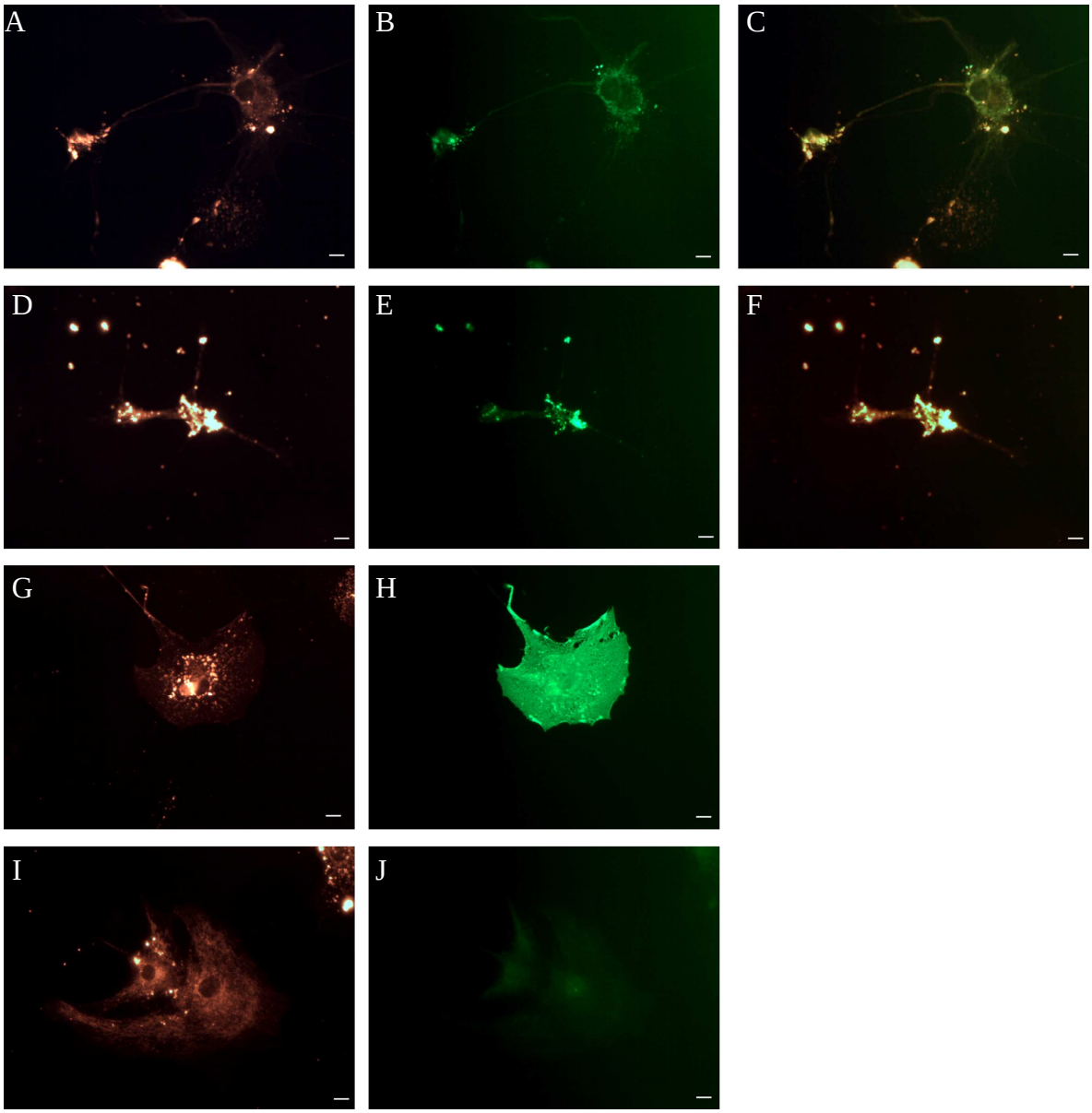


Fig. 3.8: Transglutaminase activity in murine cerebellar granule cells. Granule cell cultures (DiV 21) preincubated with 5-biotinamidopentylamine (A-C and G-J) or without preincubation (D-F), were stained against biotin (A,D,G,I) and synaptophysin (B+E) or transglutaminase 1 (H+J). C and F are overlays of A+B, respectively D+E.
Scale bar: 10 µm

For these experiments cultures were incubated with 0.1 mM 5-BPA for 16 hours, before the cells were fixed and stained with streptavidin, detecting the biotin group of the 5-BPA and thereby visualising the transglutaminase activity (Fig. 3.8). The streptavidin stainings showed a punctuated pattern colocalised with synaptic markers (Fig. 3.8 A-C) and a streaked pattern, suggesting staining of cytoskeletal structures. These staining patterns were also found in culture which were not preincubated with 5-BPA (Fig. 3.8 D-F). They most likely display naturally biotinylated proteins. The direct comparison of streptavidin stainings of cells expressing transglutaminase 1 (Fig. 3.8 G+H) with cells lacking transglutaminase 1 (Fig. 3.8 I+J), led to the conclusion that the transglutaminase 1 in this cell culture was inactive under normal cultivation conditions. So the cerebellar granule cell culture seems to be an improper model system to investigate the activity of transglutaminase 1 in neural cells.

Alternatively a telencephalic cell culture from the chicken was tested. These cells were isolated from chicken embryos at embryonic day eight to nine and cultured under serum free conditions. There is no specific antibody available against chicken transglutaminase 1 so far. Several different transglutaminase antibodies were tested in this cell culture, but no specific signal was detectable with one of them. To still be able to visualise the potential transglutaminase 1 expression in these cells the transglutaminase 1 specific peptide TG1-P was used. Staining with TG1-P revealed a solemnly membrane associated signal (see Fig. 3.9). This signal could be prevented by addition of EDTA or R281, so it seems to be based on transglutaminase activity. However, TG1-P was not tested with chicken transglutaminases, so these stainings are no clear evidence for transglutaminase 1 expression. Nevertheless, a membrane-bound transglutaminase was expressed in this cell culture, so it was tested for transglutaminase activity with 5-BPA (Fig. 3.10).

Streptavidin stainings of cultures incubated with 5-BPA showed a specific staining of some nuclei (Fig. 3.10 B), most likely due to transglutaminase 2 activity, which can also act as a transcription regulator in the nucleus. In addition a punctuated staining of neurites was found (Fig. 3.10 E). This staining was colocalised with the synaptic marker synapsin I. Thus transglutaminase activity is localised in synaptic endings of chicken telencephalic cells.

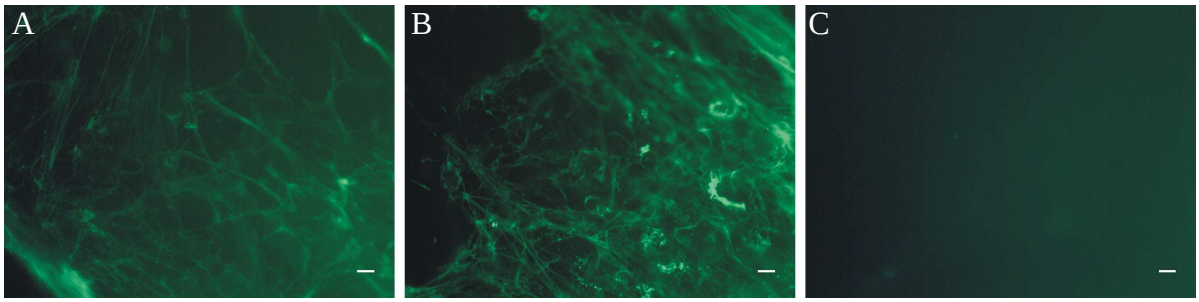
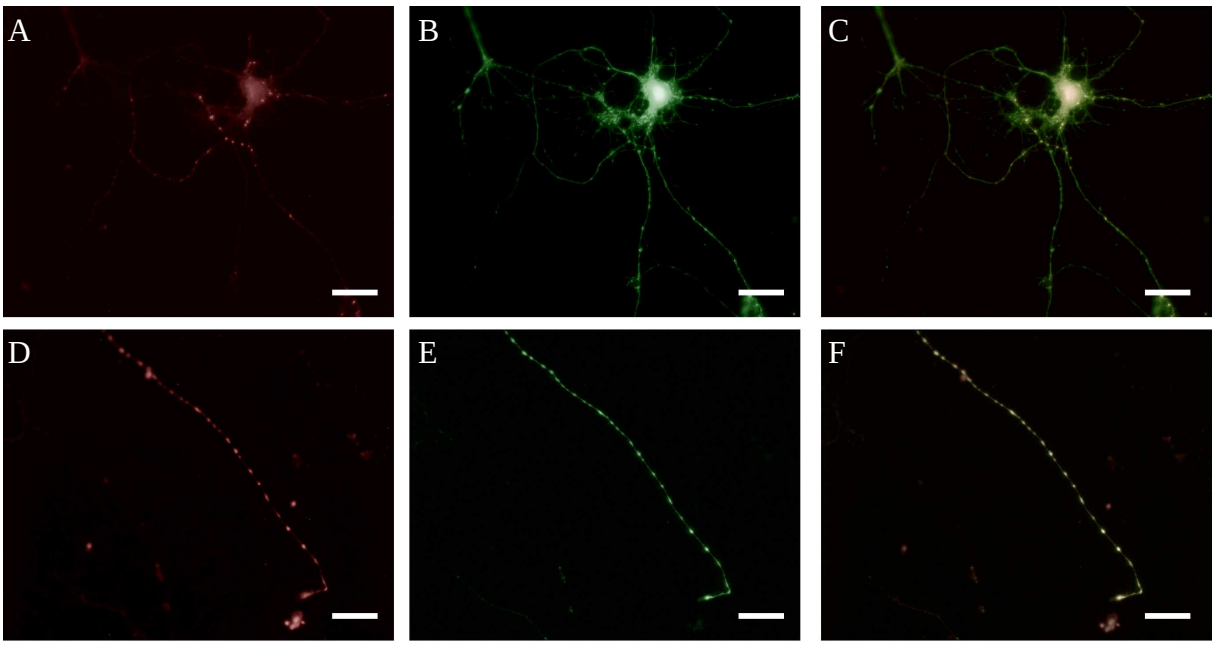


Fig. 3.9: TG1-P staining of chicken telencephalic cultures. Telencephalic cultures (DiV 13) were stained with TG1-P. The fluorescent labelled peptide is cross-linked to the membranes of the cells due to transglutaminase activity (A+B). By addition of R281 the transglutaminase activity is blocked and the staining disappears (C).
Scale bar: 10 µm



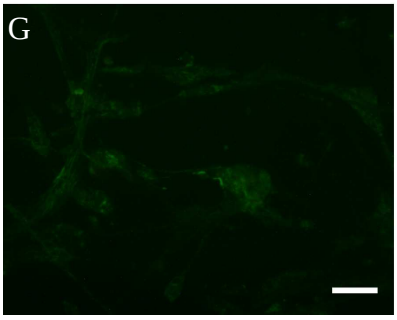
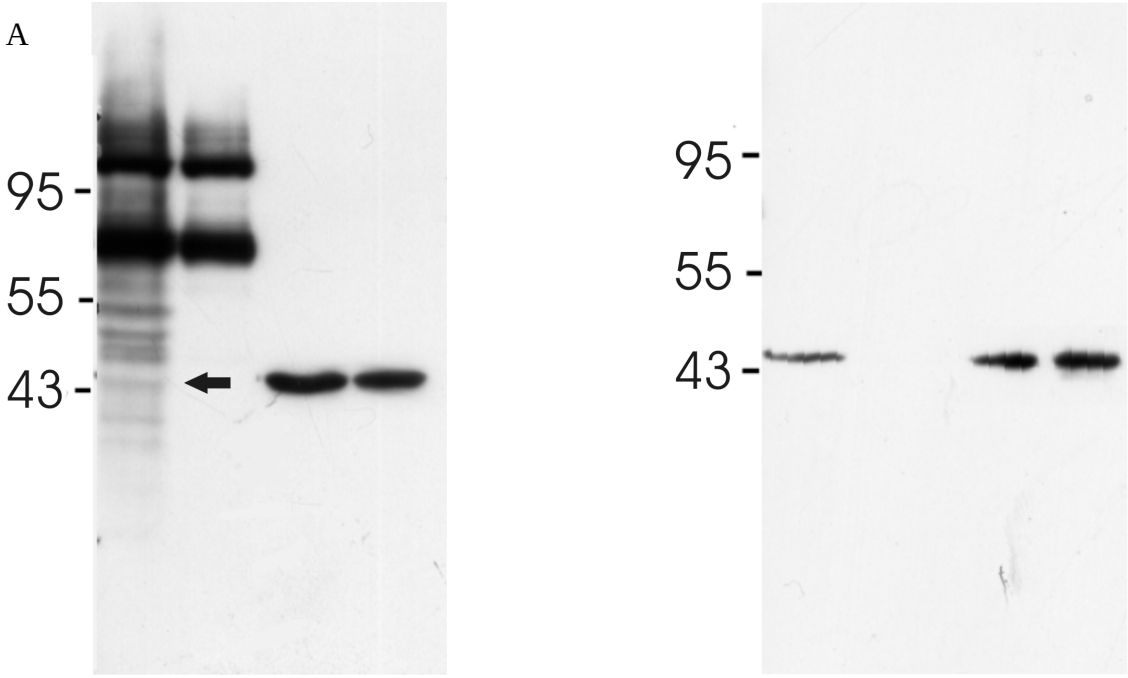


Fig. 3.10: Transglutaminase activity in chicken telencephalic cultures. Telencephalic cell cultures (DiV 18) preincubated with 5-biotinamidopentylamine (A-F) or without preincubation (G) and stained against synapsin I (A,D) or biotin (B,E,G). C and F are overlays of A+B respectively D+E.
Scale bar: 12.5 µm (A-C and G) or 5 µm (D-F).

A



+	-	+	-	Preincubation	+	-	+	-
S	S	A	A	Streptavidin/Actin	A	A	A	A
-	-	-	-	Column purification	+	+	-	-

B

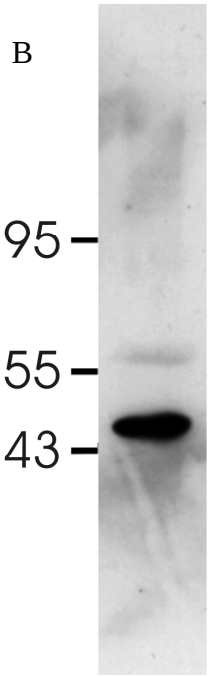


Fig. 3.11: Target proteins for transglutaminases in chicken telencephalic cultures. A: Cell lysates of telencephalic cell cultures (DiV 14) with (+) or without (-) 5-biotinamidopentylamine preincubation (see first row) were blotted against biotin (S) or β-actin (A) (see second row). In a second step the lysates were purified over a streptavidin column (see third row) and blotted afterwards. The black arrow points at the band of the same height as β-actin in the streptavidin blot. B: Cell lysates of organotypic cultures of chicken telencephalon cultivated for 20 days, incubated for one day with 5-BPA and purified over a streptavidin column. The eluate was blotted against β-actin. The numbers indicate the positions of a molecular weight marker in kDa.

To identify the proteins that are transamidated by transglutaminase activity, cells preincubated with 5-BPA were lysed. The proteins where separated by a polyacrylamide gel, blotted and stained against biotin via streptavidin (Fig. 3.11 A). The comparison between the streptavidin staining of cell lysates incubated with 5-BPA and the negative control showed several bands which only occur after addition of 5-BPA, indicating that these proteins were biotinylated over a transglutaminase dependent cross-linking of 5-BPA. One of the biotinylated bands in the streptavidin blot was found to be at the same height as β-actin (Fig. 3.11, black arrow). To clarify the nature of this band the cell lysate was purified over a streptavidin agarose column, thereby isolating all biotinylated proteins and the eluates of this column were blotted against β-actin (Fig. 3.11 A, second blot). In contrast to the negative control, in the eluate of cells preincubated with 5-BPA β-actin was detectable, although the addition of 5-BPA had no effect on the expression level of β-actin (Fig. 3.11 A, second blot lane 3 and 4). 5-BPA seemed to be cross-linked to β-actin via transglutaminase activity. Combining these facts it is most likely that β-actin is one target protein for transglutaminases at least in telencephalic cell cultures of the chicken. Regarding its membrane localisation transglutaminase 1 could be the enzyme cross-linking synaptic β-actin. Purification of 5-BPA incubated chicken organotypic slice cultures of the telencephalon over a streptavidin column showed the same results concerning the cross-linking of 5-BPA to β-actin (Fig. 3.11 B).

To unravel the exact cross linking mechanism of β-actin column purified lysates of telencephalic cultures incubated with 5-BPA were separated over a polyacrylamide gel and the β-actin band was cut out. The protein was extracted from the gel, digested with trypsin and analysed with a mass spectrometer (Tab. 3.1). The target glutamine becomes linked to the 5-BPA through transglutaminase activity and thereby the mass of the protein fragment containing the target glutamine should depart distinguishable from the normal value. Unfortunately only some fragments of the actin were found by the mass spectrometer, not including any altered one. But at least five glutamine residues can be excluded as targets for transglutaminase activity in the telencephalic cells, sparing seven potential target glutamines.

```
                10                  20                  30                  40                  50
MDDDI AALVV DNGSG MCKAG FAGDD APRAV FPSIV GRPRH QGVMV GMGQK
                60                  70                  80                  90                  100
DSYVG DEAQS KRGIL TLKYP IEHGI VTNWD DMEKI WHHTF YNELR VAPEE
                110                 120                 130                 140                 150
HPVLL TEAPL NPKAN REKMT QIMFE TFNTP AMYVA IQAVL SLYAS GRTTG
                160                 170                 180                 190                 200
IVMDS GDGVT HTVPI YEGYA LPHAI LRLDL AGRDL TDYLM KILTE RGYSF
                210                 220                 230                 240                 250
TTTAE REIVR DIKEK LCYVA LDFEQ EMATA ASSSS LEKSY ELPDG QVITI
                260                 270                 280                 290                 300
GNERF RCPEA LFQPS FLGME SCGIH ETTFN SIMKC DVDIR KDLYA NTVLS
                310                 320                 330                 340                 350
GGTTM YPGIA DRMQK EITAL APSTM KIKII APPER KYSVW IGGSI LASLS
                360                 370
TFQQM WISKQ EYDES GPSIV HRKCF
```

Tab. 3.1: Mass spectrometer analysis of β-actin from chicken. Marked in red are the protein fragments found in the mass spectrometer. Glutamines detected in the MS are marked green, undetected glutamines are marked blue.

To clarify if β-actin is really a target for transglutaminase 1, recombinant human transglutaminase 1 was expressed in Sf9 cells, with the help of the baculovirus method. The recombinant transglutaminase contains a His6-tag, so it could be purified over a Ni-NTA column. The transfected Sf9 cells showed a transglutaminase 1 positive band in the Western Blot at the predicted height (Fig. 3.12 A, lane 1). After column purification no other band was detectable via Coomassie staining. Surprisingly after prolonged storage the transglutaminase band at about 92 kDa diminishes and a new band appears with a much higher molecular weight, hardly entering the stacking gel (Fig 3.12 A, lane 4). This band is also transglutaminase 1 positive. The most likely explanation is that tranglutaminase 1 cross-links itself forming high molecular complexes, even though the storage buffer does not contain any calcium, so the activity level should be rather low.

To test the activity of the recombinant protein, dimethylcaseine, a known target for transglutaminases used in many activity assays, was incubated with 5-BPA and the expressed transglutaminase (Fig 3.13 A). Dimethylcaseine consists of several different proteins. The recombinant transglutaminase cross-linked the 5-BPA mainly to a protein of about 30 kDa, such cross-link was not found in samples without transglutaminase 1. Thus the recombinant transglutaminase was functional.

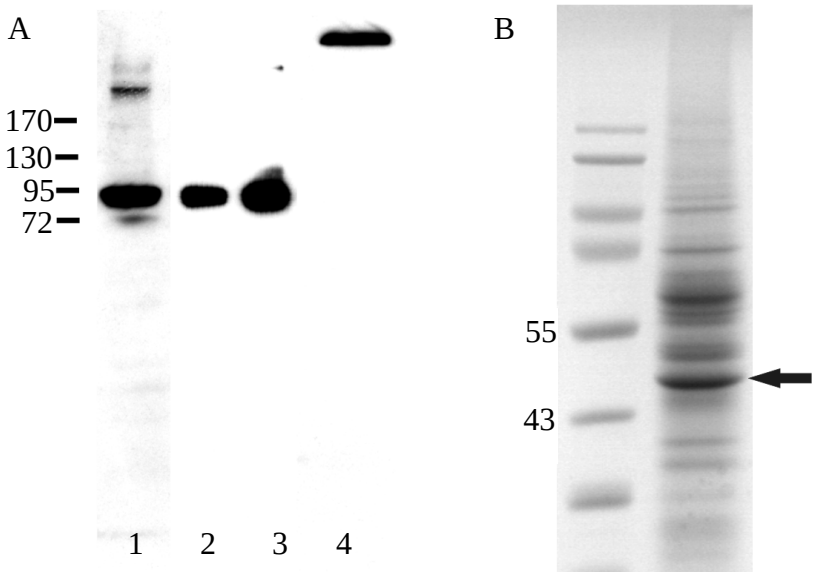


Fig. 3.12: Expression of recombinant transglutaminase 1 and extraction of actin. A: Western Blot of cell lysates of transglutaminase 1 expressing Sf9 cells (lane 1), eluate fractions 3 and 4 after purification of the lysates over a Ni-NTA column (lane 2+3) and eluate after prolonged storage (lane 4) the blot was stained against transglutaminase 1. B: Coomassie stained gel of an actin extraction from brain acetone powder. The arrow marks the actin band. The numbers indicate the mass of a molecular weight marker in kDa.

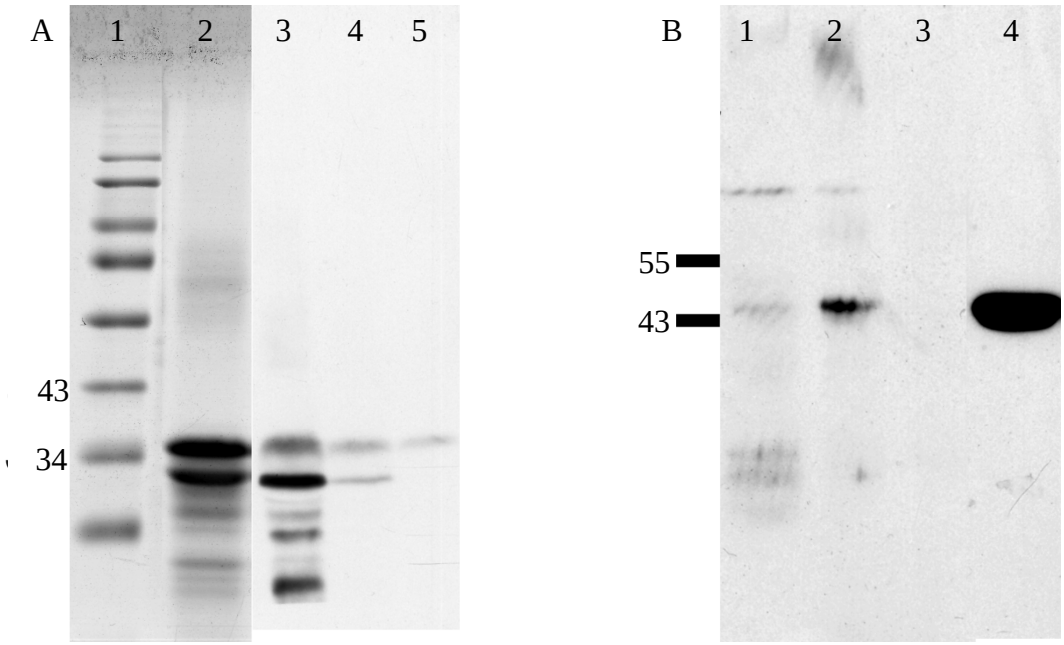


Fig. 3.13: Activity of recombinant transglutaminase 1. A: Molecular weight marker (lane 1), Coomassie staining of DMC (lane 2), DMC incubated with 100 ng transglutaminase 1 and 5-BPA (lane 3), DMC incubated with 10 ng transglutaminase 1 and 5-BPA (lane 4) and DMC incubated without transglutaminase 1 and with 5-BPA (lane 5). B: Western Blot stained with Streptavidin of G-actin with transglutaminase 1 and 5-BPA (lane 1), F-actin with transglutaminase 1 and 5-BPA (lane 2), F-actin without transglutaminase 1 but with 5-BPA (lane 3) and F-actin incubated with transglutaminase 1 and without 5-BPA (lane 4) stained against β-actin.

To check if the expressed transglutaminase also cross-links β-actin, actin was purified from brain acetone powder by successive polymerisation and depolymerisation (Fig. 3.12 B) and incubated with 5-BPA and the recombinant transglutaminase. Globular actin (G-actin) was only weakly cross-linked with 5-BPA (Fig 3.13 B, lane 1), in contrast F-actin was by far stronger labelled (Fig. 3.13 B, lane 2). So β-actin is a target for recombinant transglutaminase 1 *in vitro* and F-actin is a better substrate than G-actin. To clarify whether inter- or intramolecular bonds are catalysed by transglutaminase 1, incubations of F-actin and transglutaminase 1 without 5-BPA were performed. Subsequent Western blot analysis revealed only a single band of about 45 kDa (Fig 3.13 B, lane 4), representing an actin monomer, showing that actin was not cross-linked to any other protein in the sample, but rather intermolecular.

3.4 Synaptic activity dependent activation of transglutaminases

Combining the calcium dependency of transglutaminases with the synaptic localisation at least in chicken telencephalic cultures an elegant regulatory mechanism seems possible. In glutamatergic neurons synaptic activity leads to an influx of calcium in pre- and postsynaptic endings, heightening the local intracellular calcium level to an extent where transglutaminases could be activated.

To check this hypothesis about neuronal activity dependent transglutaminase activation, neurons in culture had to be stimulated over a prolonged time. To reach this goal a light gated cation channel named channelrhodopsin 2 (Chop2) was used (Nagel, Szellas et al. 2003). Chop2 is sensitive for blue light and leads after activation to a depolarisation of cells by influx of cations. This depolarisation should activate voltage gated channels in neurons, leading to an action potential (Boyden, Zhang et al. 2005). To easily detect this activation another construct was used, a fusion protein of aequorin and a modified green fluorescent protein (eGFP). Both originate from the jellyfish *Aequorea victoria* (Shimomura, Johnson et al. 1962; Chalfie 1995). Aequorin is a calcium dependent chemiluminescent protein (Jones, Hibbert et al. 1999), able to detect the calcium influx attending the activation of a neuron. The aequorin signal, however, is very weak and hard to detect. Therefore, an eGFP was fused to the aequorin. The energy of the excited aequorin is transferred radiationless via fluorescence resonance energy transfer (FRET) to the eGFP. The emerging green fluorescence is about 50 times stronger than the aequorin signal alone (Baubet, Le Mouellic et al. 2000).

Non dividing primary cells are very hard to transfect with a vector, therefore, a new model system was chosen for these experiments, a cell line called PC12. PC12s are rat pheochromocytoma cells. They can be reversibly differentiated into a neuronal phenotype (Greene and Tischler 1976), by addition of nerve growth factor (NGF) to the medium. It is known that PC12 cells express transglutaminase 1 and 2 (Byrd and Lichti 1987). To transfect these cells with Chop2 respectively aequorin/eGFP two constructs were used (Fig. 3.14), containing the proteins under the control of the cytomegalovirus immediately early promoter (CMV) (Boshart, Weber et al. 1985) for expression in eukaryotic cells. The vector pBK-CMV/Chop2-YFP contains additionally a yellow fluorescent protein (YFP) as a marker gene, to check the transfection and expression efficiency.

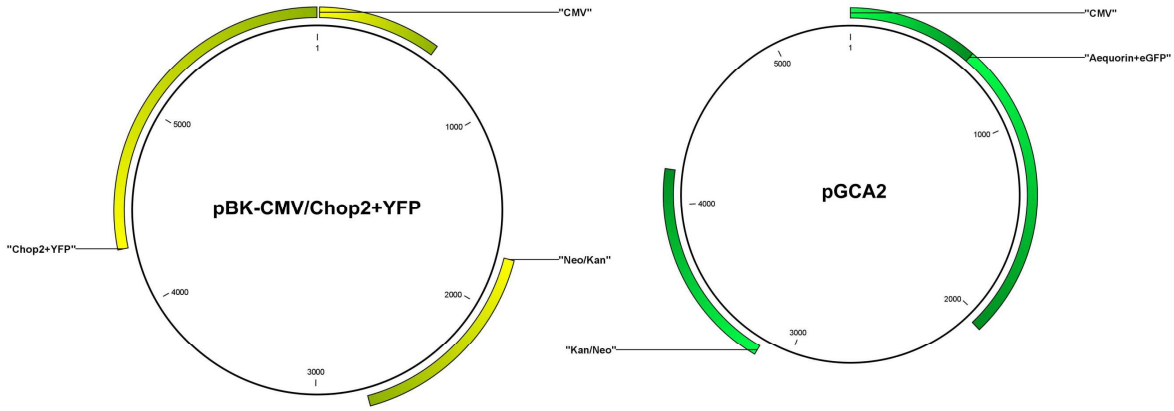


Fig. 3.14: Vector maps of pBK-CMV/Chop2+YFP and pGCA2. Maps of the vectors for expressing the Channelrhodopsin 2 YFP and the Aequorin eGFP fusion proteins in PC12 cells under the control of a CMV promoter.

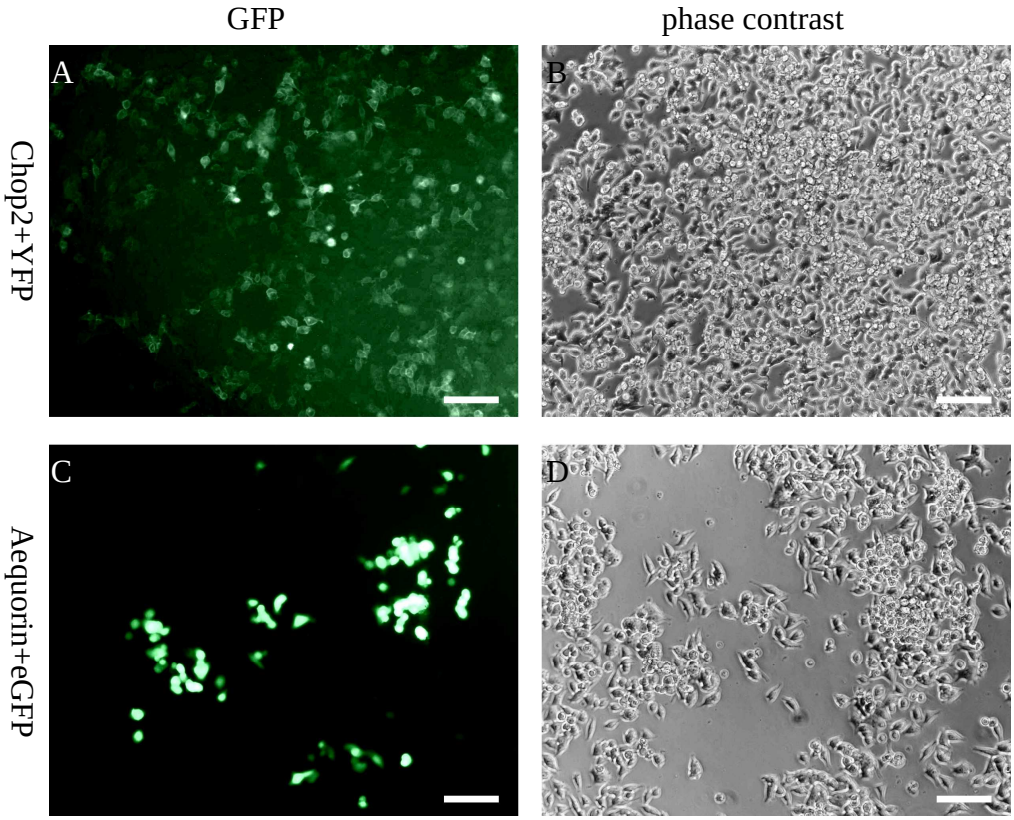


Fig. 3.15: Transfections of PC12 cells with Channelrhodopsin-2/YFP and Aequorin/eGFP. Undifferentiated PC12 cells were transfected with the vector pBK-CMV/Chop2+YFP (A+B) respectively pGCA2 (C+D). Scale bar: 50 µm.

PC12 cells were tranfected separately with both constructs (Fig. 3.15). The membrane bound localisation of the channelrhodopsin/YFP was clearly distinguishable from the cytosolic expression of the aequorin/eGFP. The expression strength varied within the culture, but the transfection efficiency seemed sufficient to induce network activity and monitor it respectively.

To check the Chop2 activity, differentiated PC12 cells were transfected with pBK-CMV/Chop2+YFP and recorded via patch clamp 72 hours after transfection, at the peak of Chop2 expression. Prior to recordings the cells were incubated with all-trans retinal for half an hour to activate the Chop2. Retinal is the co-factor of Chop2. Only cells showing a strong YFP signal were chosen for recording. These cells were stimulated with white light from a mercury-arc lamp, normally used for fluorescence excitation. Figure 3.16 shows recordings of two stimulated cells. Illumination resulted in an initial strong depolarisation to the extent of maximally 10 mV (black arrows), which declined to a solid state after about 100 ms. This behaviour fits well to the known desensitisation of the channel. There was no active response from the cell to the depolarisation visible. The first recording shows the behaviour in response to two consecutive illuminations, spaced by one second. The reaction to the second illumination had a lower initial depolarisation, demonstrating that the channel needed more time to recover completely. However, with recovery times above ten seconds the cells could be activated several times in a row showing a comparable response pattern. Nevertheless, no active response of the stimulated cells was recordable.

To further characterise the cells electrophysiologically, fully differentiated untransfected PC12 cells were recorded with the whole cell patch clamp technique (Fig. 3.18). The resting potentials of the recorded cells lay between -30 and -80 mV, with the median at about -40 mV. Not all recorded cells showed any response to depolarising stimuli. But in all positive cases, a depolarisation to -20 mV or above was necessary to evoke an active response. This shows that the depolarisation of maximal 10 mV, achieved by activation of Chop2, is not strong enough to stimulate the cells, in particular because most cells showed a reaction comparable to the second record of figure 3.16, with a depolarisation below 5 mV. Furthermore, no recorded PC12 cell showed a mature action potential. Only relative broad and low voltage peaks could be evoked, casting some doubt that the cells possess real synaptic activity. The reactivity of the aequorin/eGFP fusion protein was tested using the calcium ionophore A23187. This ionophore assembles itself into the membrane of cells

and allows divalent cations (mostly Mn^{2+}, Ca^{2+} and Mg^{2+}) to enter the cell (Reed and Lardy 1972).

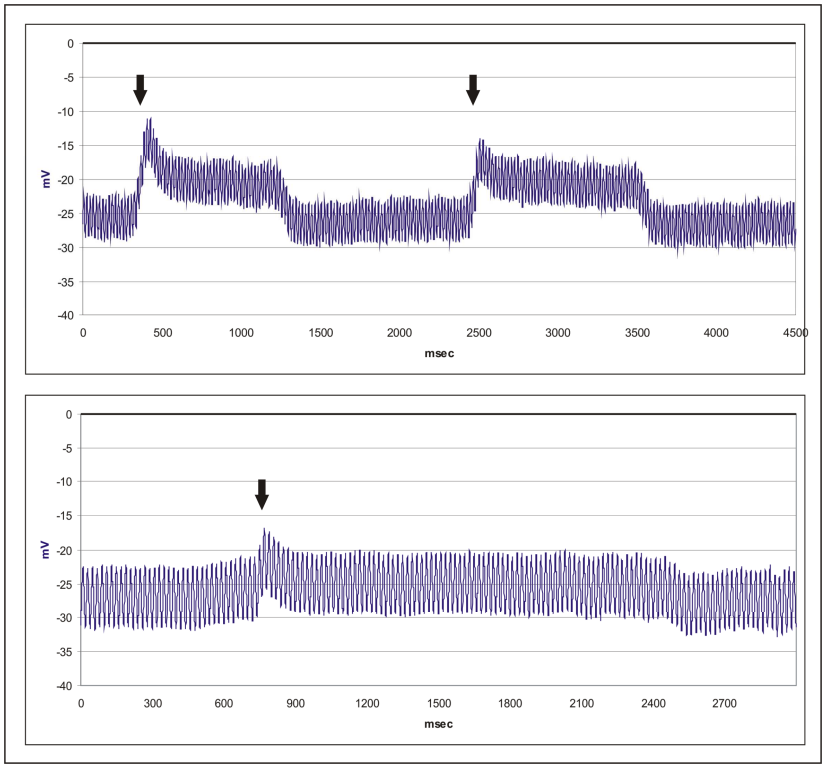


Fig. 3.16: Whole cell patch clamp recordings of Channelrhodopsin 2 transfected PC12 cells. Seven days differentiated PC12 cells where transfected with pBK-CMV/Chop2+YFP. 72h after transfection retinal was added. Under a fluorescence microscope transfected cells were selected and stimulated with white light. Electrophysiological recordings were performed intracellulary with a sharp electrode. The black arrows indicate the initial depolarization at stimulation.

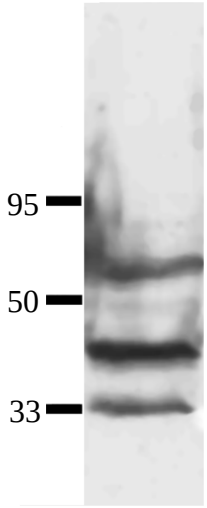


Fig. 3.17: Western Blot of PC12 cells transfected with Aequorin/eGFP. Undifferentiated PC12 cells were transfected with pGCA2 and 72h after transfection the cells were lysed and the lysates blotted against an anti-GFP antibody. The numbers indicate the mass of a molecular weight marker in kDa.

The cells were investigated under a microscope in the dark and the ionophore was added to the medium. The inflow of calcium should activate the aequorin and leads to a green fluorescence. However, no reaction was visible. Even with long exposed images of a camera, no signal was detectable. To investigate the functional expression of aequorin/eGFP, PC12 cells were transfected with pGCA2 and cell lysates were blotted against an anti-GFP antibody (Fig. 3.17). Instead, of a single band at about 50 kDa, representing the fusion protein, three different bands ranging from over 50 to 33 kDa were visible. The highest band presumably represents the complete protein. The smaller bands were most likely products of an incomplete transcription or degradation products of protease activity. So only about one third of the protein seems to be complete and thereby functional, probably this reduces the aequorin/eGFP signal under the detection limit.

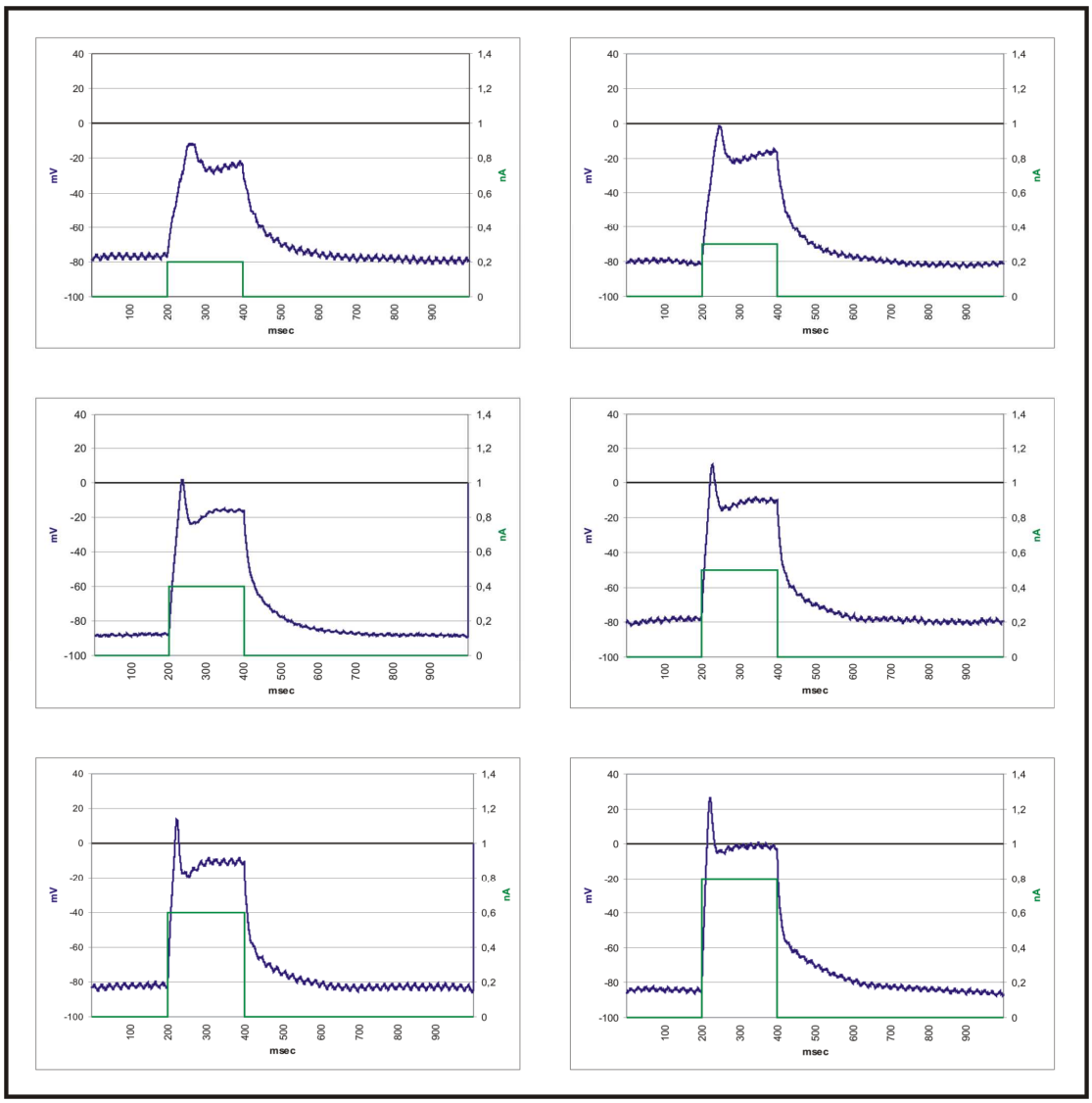


Fig. 3.18: Whole cell patch clamp recordings of PC12 cells. Electrophysiological recordings from ten days differentiated PC12 cells. The stimulus ranged from 0.2 nA to 0.8 nA and lasted for 200 ms.

3.5 Transglutaminase 1 knock-out mouse

Probably the best method to investigate the *in vivo* function of a protein is the generation of a knock-out mutant. The developing phenotype can reveal a lot about the role of a protein in an organism. The classical example for such a mutant is a knock-out mouse. Via recombination the original gene is replaced with a non-functional construct, leading to a system wide knock-out of this gene. In the case of transglutaminase 1 such a constitutively knock-out already exists (Matsuki, Yamashita et al. 1998). Unfortunately the developing mice are not viable postnatal due to skin defects leading to fatal water loss. To overcome this problem we plan to use a conditional knock-out mutant of transglutaminase 1 (Lobe and Nagy 1998), utilising the Cre/lox-system.

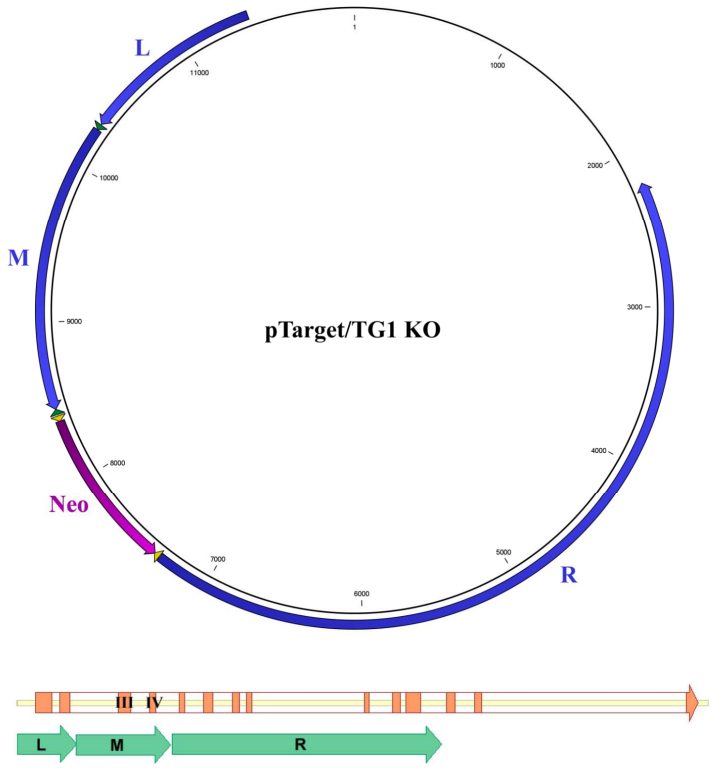


Fig. 3.19: Vector map of the transglutaminase 1 knock-out construct. The pTarget/TG1 KO vector containing both homology regions (L+R) the knockout region (M) flanked by loxP sites (in green) and the Neomycin resistance cassette flanked by FRT sites (in yellow). Under it the organisation of the tranglutaminase 1 gene with it's exons (orange boxes) and the regions spanned by the KO construct with the KO region (M) containing the exons III and IV.

To interrupt the gene about 1800 base pairs including the exons III and IV should be deleted (Fig 3.19). This leads to a frame-shift mutation in the coding sequence, introducing a premature stop codon. The remaining fragment lacks the catalytic core and should posses no enzymatic activity. To achieve this knock-out a vector was constructed (Fig. 3.19) containing a ~1 kb long homologous fragment upstream (fragment L) and a ~5 kb long homologous fragment downstream (fragment R) of the targeted knock-out fragment (fragment M). Fragment M is bordered by two loxP sites, enabling to excise this part by Cre recombinase activity. Additionally a neomycin resistance cassette was inserted, to screen the stem cells with G418 after transfection. This cassette is flanked by FRT sites to delete it after successful transfection via FLIP recombinase activity.

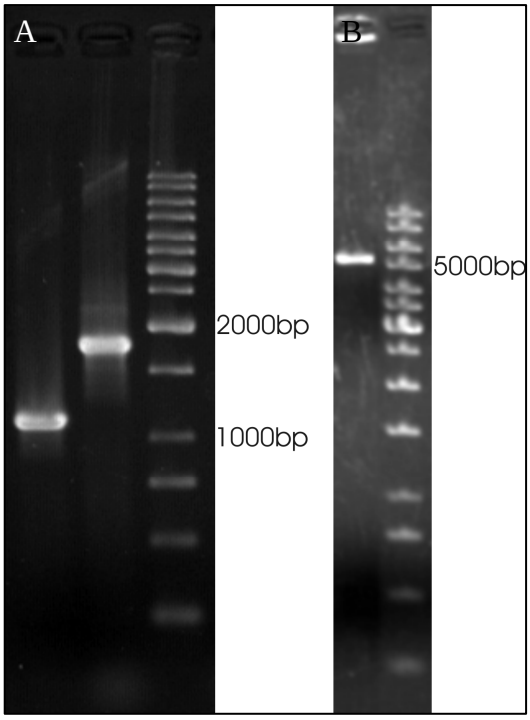


Fig. 3.20: Amplification of DNA fragments for the knock-out construct. A: Agarose electrophoresis of the PCR for the L (lane 1) and M (lane 2) fragment of pTarget/TG1 KO. B: Agarose gel electrophoresis of the PCR for the R (lane 1) fragment of pTarget/TG1 KO. The numbers are masses in base pairs of a molecular weight marker.

The fragments L, M and R were amplified from C57BL/6 genomic DNA (Fig. 3.20) and cloned individually into the vector pPCR-Script Amp. To validate the DNA sequences the cloned fragments were completely sequenced (see Appendix for sequence information). Afterwards the three fragments were excised with restriction enzymes and subcloned into the knock-out vector pTarget, which already contained loxP sites and the resistance cassette. The correct insertion of the fragments was again validated by sequencing. The complete construct was electroporated into mouse stem cells and the cells were cultured with the selective antibiotic G418. Evolving clones were separated and the genomic DNA of these clones was isolated.

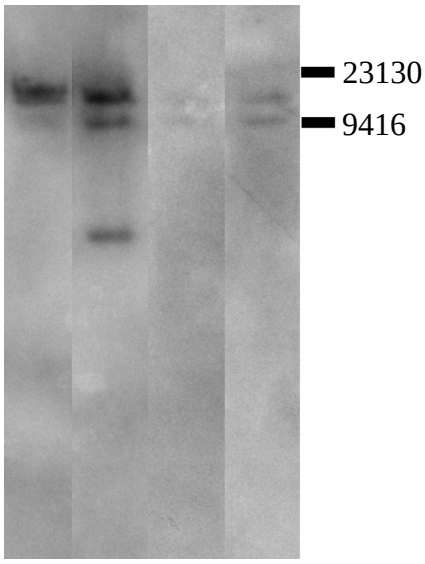


Fig 3.21: Southern Blot analysis of transglutaminase 1 knock-out clones. Genomic DNA of stem cell clones transfected with the knock-out construct were analysed via Southern Blot with probe 1. Four clones (14, 23, 30 and 132 from left to right) were positive for the recombination. The lower band is the recombinant one. The numbers are masses in basepairs of a molecular weight marker.

To analyse the clones for a correct recombination of the construct into the transglutaminase 1 gene, the genomic DNA was digested with *Bam*HI (for southern blot probe 1) or *Hind*III (for southern blot probe 2) and two southern blots were performed, detecting additionally binding sites for these restriction enzymes in positive clones. With probe 1 four positive clones (14, 23, 30 and 132) were detected (Fig. 3.21). This blot verifies that the part upstream of the knock-out fragment was successfully inserted into the gene. The analysis of a correct recombination of the part downstream of the knock-out fragment could not be completed in time.

4. Discussion

4.1 Expression of transglutaminase 1 in the murine brain and cerebellar granule cells

Until now studies regarding the expression of tranglutaminase 1 in the CNS were mainly restricted to the human brain in the context of neurodegenerative diseases (Kim, Grant et al. 1999; Zemaitaitis, Kim et al. 2003; Wilhelmus, Grunberg et al. 2009; Wilhelmus, Verhaar et al. 2009). To further elucidate the distribution of transglutaminase 1 in the murine CNS cryostatic slices of mouse brains were prepared and double-stained against transglutaminase 1 and Mtap2, respectively GFAP. Additionally the occurrence of transglutaminase 1 was investigated by its cross-linking activity with a fluoresceine labelled peptide (TG1-P). TG1-P is not efficiently cross-linked by the transglutaminases 2 and 3 as well as factor XIIIa (Sugimura, Hosono et al. 2008), therefore, at least in the brain it should be specific for transglutaminase 1.

A prominent location of transglutaminase 1 expression is the endothelium. Throughout the brain vascular system transglutaminase 1 can be found at the membranes of endothelial cells. Transglutaminase 1 expression has been previously described in the endothelium of the mouse myocard (Baumgartner, Golenhofen et al. 2004). It is shown to be localised at adherens junctions and one target of its cross-linking activity was β-actin (Baumgartner and Weth 2007). At adherens junctions clusters of cadherins are associated intracellulary over catenins with actin fibres (F-actin) (Hirano, Nose et al. 1987; Rimm, Koslov et al. 1995). The cadherin-dependent adhesion at these junctions depends on the association with the cytoskeleton (Baumgartner, Schutz et al. 2003; Waschke, Curry et al. 2005). In the endothelium inflammatory signals lead to the assembly of contractile actin stress fibres and disassembly of the junctional F-actin network. The weakened junctions can then be disrupted by the contractile apparatus, resulting in a heightened endothelial permeability. Elevated calcium levels are a key signal for this response (Curry 1992; Sandoval, Malik et al. 2001). *In vitro* it was shown that endothelial cells expressing transglutaminase 1 are more resistant to barrier breaking effects than endothelial cells with silenced transglutaminase 1 expression (Baumgartner, Golenhofen et al. 2004). It is hypothesised that the increased calcium concentration activates transglutaminase 1, which then can

cross-link and stabilise F-actin, resulting in an enhancement of the VE-cadherin adhesion at the adherens junctions.

In the brain endothelial cells form the blood-brain barrier, rigorously controlling the diffusion of molecules between blood stream and brain tissue. This barrier can also be disrupted by inflammatory signals (Abbott 2000). The transglutaminase 1 expression in the brain endothelium was not found to be concentrated at adherens junctions. Nevertheless, the most likely role of transglutaminase 1 here resembles its role in the myocard, stabilising adherens junctions by its cross-linking activity. Transglutaminase 1 could thereby be an important factor in sustaining the blood-brain barrier.

Besides the endothelium, transglutaminase 1 can be found in parts of the ependyma, the lining of the ventricular system. The expression is found at the membranes of ependymal cells and is mainly concentrated at the basal site. The ependyma resembles epithelial membranes in other tissues. It is assumed that it builds up a barrier between the cerebrospinal fluid and the brain, resembling the blood-brain barrier (Del Bigio 1995; Bruni 1998). So transglutaminase 1 could play a similar role here like in the endothelium, stabilising intercellular junctions. Regarding its location at the basal site it could also enhance the adhesion of the cells to the basal lamina. However, it remains unclear why transglutaminase 1 is restricted to certain parts of the ependyma.

Even more ambiguous is the localisation of transglutaminase 1 in parts of fibre tracts. It was reported before, that transglutaminase 1 is present in the corpus callosum (Kim, Grant et al. 1999). Nevertheless, the found expression pattern in the mouse brain did not resemble the pattern in human brain. Only partially the expression was limited to cellular structures, namely astrocytes. Most parts of the staining were streak-like and could not be ascribed to a distinct structure. The transglutaminase seems to be located in axons, yet only in parts of them, but not in defined tracts. Transglutaminase 1 positive neurons, projecting these axons were not found anywhere on the slices. The anti-transglutaminase 1 staining would be implausible, if the activity dependent staining would not display the same picture. It is not to be excluded that both stainings were unspecific, even though the negativity of the control staining with TG1-P and without calcium is a good argument for the specificity of the staining. Another possibility is that the antibody and the TG1-P could not penetrate the tissue completely, showing only parts of the existent transglutaminase 1. But this seems very unlikely, regarding the thickness of only 7 µm of the slices. However, it is hard to draw any conclusions further than, transglutaminase 1 is expressed at least in

parts of different fibre tracts, including the corpus callosum, the external capsule and parts of the caudoputamen.

The expression of transglutaminase 1 in glial cells seemed to be randomly scattered. Furthermore, it is unclear why only a handful of neurons in the cerebellum were expressing it. Therein these results mirror the literature, in which also a clear constriction of transglutaminase 1 expression to definite cell types of the brain is lacking. It seems certain that transglutaminase 1 is not permanently expressed in specific cell types. Rather unknown events have to initiate the expression. The transglutaminase 1 promoter contains a AP1-like site and two Sp1-like sites (Medvedev, Saunders et al. 1999; Phillips, Jessen et al. 2004). Transcription factors able to bind at these sites are involved in several different processes, leaving plenty of room for speculations. AP-1 and Sp1 activity is linked among other things to cell differentiation, proliferation, survival, migration, apoptosis and immune response (Opitz and Rustgi 2000; Herdegen and Waetzig 2001; Shaulian and Karin 2002; Kaczynski, Cook et al. 2003; Wagner and Eferl 2005). In the brain AP-1 activation corresponds to neuronal activity (Alberini 2009) and is found in neuronal development, as well as neurodegeneration and apoptosis (Herdegen and Leah 1998). Primary cell cultures are a promising tool to investigate the initiation of transglutaminase 1 expression.

In recent literature only transglutaminase 2 expression has been investigated in cerebellar granule cells cultivated on a specific substrate (Perry, Mahoney et al. 1995). In these cells transglutaminase 2 is found to be involved in neurite-outgrowth (Mahoney, Wilkinson et al. 2000) and excitotoxicity (Ientile, Caccamo et al. 2002). Transglutaminase 1 expression has been described generally in the cerebellum, but not in cerebellar granule cell cultures. In the cerebellum of baby mice (P5) as well as in young granule cell cultures transglutaminase 1 was only found in some astrocytes. Beginning from day nine *in vitro* (DiV9) the first neurons expressing transglutaminase 1 were found. The onset of transglutaminase 1 expression seems to depend on the differentiation process of the neurons in culture. Interestingly the expression was mainly cytosolic. Western blots of granule cell cultures showed only a single band, representing the full-length protein. Hence transglutaminase 1 was not proteolytically activated. Although it is known that even unprocessed transglutaminase 1 can cycle between a membrane-bound and a soluble state (Steinert, Chung et al. 1996), it is surprising that only small amounts of transglutaminase 1 were membrane bound in these cells. However, the constitutively myristilation of transglutaminase 1 is only shown in keratinocytes (Steinert, Kim et al. 1996), so probably

most parts of transglutaminase 1 in these cultures were not acylated by fatty acids, leading to the mainly cytosolic localisation.

The number of transglutaminase 1 positive cells, as well as the expression strength, rises with cultivation time. In later cultures clusters of transglutaminase 1 positive cells were found surprisingly frequently. One possible explanation would be a lateral induction of the expression. However, the mechanisms underlying the regulation of transglutaminase 1 remained unclear. Just as the differences between transglutaminase 1 positive and negative cells. To further characterise the neuronal population of the culture, stainings against vesicular glutamate transporter 1 (VGLUT-1), a presynaptic protein of glutamatergic synapses, were performed. VGLUT-1 is expressed by cerebellar granule cells *in vivo* (Hioki, Fujiyama et al. 2003). However, in contrast to VGLUT-1 expression found in cryostatic slices, no VGLUT-1 was detectable in the cell culture. The most likely explanation would be that at least no functional glutamatergic postsynaptic sites are formed. It remained unclear if the transglutaminase 1 positive neurons belong to a specific subtype. Also there were no phenotypic differences detectable between glial cells expressing transglutaminase 1 and those that do not.

Especially the *in vivo* expression of transglutaminase 1 remained enigmatic. Not only has the diffuse expression in projecting axons of fibre tracts raised questions, but also the absence of transglutaminase 1 in the somas of neurons outside the cerebellum was surprising. The later especially because in the literature there are evidences of neuronal transglutaminase 1 expression in the cerebral cortex (Kim, Grant et al. 1999; Wilhelmus, Verhaar et al. 2009). To further characterise the expression pattern of transglutaminase 1 in the brain, western blots and quantitative real time PCRs of interesting brain regions would be the methods of choice.

Granule cell cultures mirrored the transglutaminase 1 expression of the cerebellum quite well, even though the density of transglutaminase 1 positive cells in later cultures is significant higher than *in vivo*. The main question remaining is about the difference between transglutaminase 1 positive and negative cells. What processes or signalling molecules trigger the onset of the expression? Further characterisation of transglutaminase 1 expressing cells could reveal certain subtypes of neuronal or glial cells being positive for transglutaminase 1. More likely the transglutaminase 1 expression is locally induced. Identifying those induction mechanisms would be a hard task. But the investigation of transcription factors binding to AP1 or Sp1 elements could reveal the regulatory mechanism controlling transglutaminase 1 expression in these cells and in the brain.

4.2 Transglutaminase 1 knock-out mouse

The constitutive transglutaminase 1 knock-out mouse from Matsuki *et al*. (Matsuki, Yamashita et al. 1998) clarified the importance of transglutaminase 1 in the formation of the cornified cell envelope. But besides the defective stratum corneum of the skin no abnormalities in other transglutaminase 1 expressing tissues were found. It is known that transglutaminases can partially be replaced in their functions by other family members. For example a transglutaminase 2 knock-out mouse shows a nearly unaltered phenotype (De Laurenzi and Melino 2001), despite the various functions of transglutaminase 2. This could also be the case in the transglutaminase 1 knock-out. However, another possibility is that abnormalities would primary develop at later time points. The neonatal death of the knock-out mouse would hide those alterations. To avoid this problem we are developing a conditional knock-out of transglutaminase 1. Unfortunately the completion of the knock-out could not be accomplished in time. But at least a knock-out construct was cloned and successfully inserted into mouse stem cells. In the coming months the knock-out mouse has to be finished and analysed.

Hopefully, the knock-out gives insight into the role of transglutaminase 1 in the brain. It could clarify the expression and function of transglutaminase 1 in fibre tracts and it could probably reveal the events underlying the activation of transglutaminase 1 expression in glial cells and neurons.

4.3 Transglutaminase activity in neuronal cell cultures

Usually transglutaminase activity is monitored with labelled transglutaminase substrates, small primary amines in general. 5-(biotinamido)pentylamine (5-BPA) is such a molecule. It consists of a cadaverine linked to a biotin. 5-BPA is a known substrate of transglutaminases (Slaughter, Achyuthan et al. 1992). Due to its membrane permeability, it is a good tool to monitor intracellular transglutaminase activity in cell cultures. 5-BPA shows no specificity towards a certain transglutaminase subtype, so it can give no information about the transglutaminase it is cross-linked by.

In cerebellar granule cell cultures the most likely source for transglutaminase activity besides transglutaminase 1 is transglutaminase 2. *In vivo* transglutaminase 2 was found in the first two weeks postnatal in the cerebellum (Perry, Mahoney et al. 1995). *In vitro* it is found only under specific cultivation conditions, like the addition of retinoic acid or the cultivation on a special biomatrix (Perry, Mahoney et al. 1995; Mahoney, Wilkinson et al. 2000). Transglutaminase 2 stainings of granule cell cultures revealed only a faint expression in later cultures, located in vesicle like structures. Most likely transglutaminase 2 is secreted into the extracellular space. Its activity could thereby easily be blocked by the membrane impermeable transglutaminase inhibitor R281. Because transglutaminase 3 and 6 are not expressed in the cerebellum, transglutaminase 1 should be the only source of intracellular transglutaminase activity.

To display transglutaminase activity cerebellar granule cell cultures were incubated with 5-BPA and stained afterwards with a fluorophor coupled streptavidin. There was no visible differences in the staining of cells preincubated with 5-BPA compared to control cells, independent from the transglutaminase expression of these cells. Apparently the transglutaminase 1 in these cells was inactive, which is not much of a surprise considering the relatively low cytosolic calcium concentrations. For an activation of transglutaminase 1 at least a local elevation of calcium levels would be necessary.

In neurons such a heightened calcium level can be achieved by synaptic activity. The influx of calcium through glutamate receptors and voltage-gated calcium channels could activate the transglutaminase. Although the cerebellar granule cells in culture were positive for the synaptic markers synapsin 1 and synaptophysin, no VGLUT-1 expression was detectable, additionally they were also negative for postsynaptic density-95 (PSD95), a protein of the postsynaptic site. Hence it is questionable if these cells develop functional

synapses in culture. Additionally granule cells were cultivated at high potassium concentrations (Gallo, Kingsbury et al. 1987). *In vivo* mature granule cells need the association to purkinje cells, otherwise they die (Chen and Hillman 1989). Granule cells cultured under low potassium conditions also die. Therefore, the cells are kept in a premature state, by cultivating them under depolarising conditions, comparable to not fully differentiated granule cells (Okazawa, Abe et al. 2009). On the one hand such a treatment keeps them alive, on the other hand, these cells are not totally differentiated (Mellor, Merlo et al. 1998). In organotypic cultures of granule cells chronic depolarisation is shown to impair synaptogenesis (Okazawa, Abe et al. 2009), suggesting the conclusion that these cells fail to form fully functional synapses in the culture. Attempts to activate the transglutaminase *in vitro* by elevating the potassium levels to further depolarise the cells and evoke synaptic activity failed, just as attempts to further differentiate the granule cells by lowering the potassium concentrations after ten days in culture.

A further investigation of transglutaminase 1 activity in neurons was achieved with another cell culture model, using chicken telencephalic neurons. Cortical neurons of chicken are known to develop functional synapses in culture (Tokioka, Matsuo et al. 1993). A major drawback of this model system is that until now no transglutaminase 1 homologue was found in the chicken. Transglutaminases of the type 1 are known from mammals and some fish, but were not found in any birds or reptiles so far. The only evidence for transglutaminase 1 expression in the chicken is provided by a work on cornification proteins in the avian epidermis (Alibardi and Toni 2004). Immunohistochemical stainings of the chicken skin revealed a transglutaminase 1 like pattern of expression. Nevertheless, it remains unclear, if this staining was really based on transglutaminase 1 expression. Attempts using various transglutaminase 1 antibodies, as well as reverse transcription polymerase chain reactions with primers homologue to conserved regions of transglutaminase 1 remained unsuccessful.

A characterisation of a potential transglutaminase 1 expression in the telencephalic cultures nonetheless, was implemented by using TG1-P. This method showed a membrane-bound transglutaminase activity in these cells, although it remained unclear if this activity was really based on a transglutaminase 1 homologue protein. There are no reports about transglutaminases in the avian brain. However, transglutaminase 2 would be another candidate, besides transglutaminase 1, for this activity in telencephalic neurons.

5-BPA incubations showed transglutaminase activity in the nucleus and in synapses of chicken telencephalic cell cultures. Because transglutaminase 2 is the only family member

known to be transported into the nucleus, the nuclear activity originates, without much doubt, from it. The synaptic transglutaminase activity could be caused by the membrane-bound assumed transglutaminase 1. Although distributed over the whole membrane, the transglutaminase could be locally activated by synaptic activity.

4.4 β-Actin is a target for transglutaminase activity in chicken telencephalic cell cultures

Purification of telencephalic cell lysates over a streptavidin-column revealed that β-actin was only biotinylated after the addition of 5-BPA to the medium. This implied that 5-BPA was cross-linked to β-actin by synaptic transglutaminase activity in the culture. Additionally recombinant human transglutaminase 1 was able to cross-link 5-BPA to F-actin, purified from brain acetone powder. Actin is a known target for transglutaminase 1, 2 and factor XIIIa (Cohen, Blankenberg et al. 1980; Gorman and Folk 1980; Baumgartner and Weth 2007), but here it is shown for the first time that synaptic β-actin functioned as a substrate for transglutaminases. The cross-linking of 5-BPA to β-actin was also found in organotypic cultures of chicken forebrain (see Fig. 17 B), suggesting that synaptic transglutaminase activity also occurs *in vivo*.

The attempt to characterise the target glutamine within β-actin was unsuccessful, but at least some glutamate residues could be excluded. *In vitro* it was shown that transglutaminase 2 cross-links small primary amines to glutamine 41 of actin, resulting in a higher polymerisation rate and lower requirements on the actin concentration for the polymerisation initiation (Takashi 1988). Bacterial transglutaminases can form an intramolecular cross-link in globular actin between the glutamine residue 41 and the lysine residue 50 (Eli-Berchoer, Hegyi et al. 2000). This intermolecular bridge has no effect on the polymerisation of actin, but it leads to higher thermo-stability and an enhanced resistance against proteolysis. In endothelial cells it is shown, that transglutaminase 1 is able to stabilise the actin network (Baumgartner and Weth 2007). Additionally actin-associated proteins can be cross-linked to actin. Myosin is shown to be cross-linked to glutamine 41 of actin through transglutaminase activity (Eligula, Chuang et al. 1998).

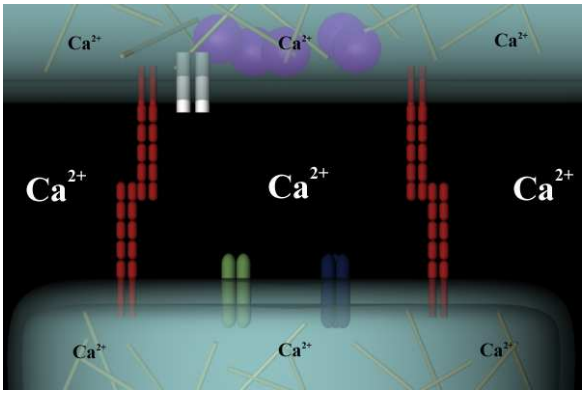
These cross-linking reactions all take place under artificial conditions and not intracellulary. Also transglutaminase 1 and 2 at least have partial differing substrate specificities (Sugimura, Hosono et al. 2006; Sugimura, Hosono et al. 2008). However, it is likely that glutamine 41 is a common target for transglutaminase activity and our mass spectroscopy data did not exclude this residue as the target glutamine in telencephalic cells. Although the identification of the cross-linked glutamine residue of β-actin would be very interesting, 5-BPA is an artificial cross-linking partner of actin, so the function of the *in vivo* cross-link can only be assumed. In Western blot experiments cell lysates of chicken telencephalic cultures showed only a single band of β-actin, which is contrary to the

possibility that actin is linked to an associated protein. Additionally incubation of β-actin with recombinant transglutaminase 1 showed no cross-linking of actin monomers among each other or to other proteins. So the most likely transamidating reaction is the formation of an intramolecular bond between glutamine 41 and lysine 50. Assuming that this cross-link is formed at synapses and that it stabilises F-actin against network disrupting mechanisms, still leaves a plenty of different possibilities for the effect of transglutaminase activity at synapses.

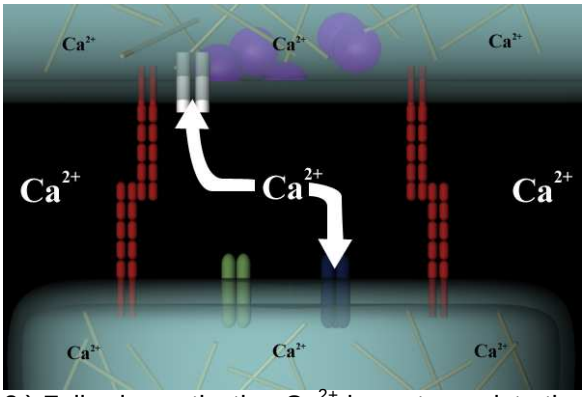
Actin is a key molecule for many modulating processes at the synapse. At the presynaptic site it was shown that the actin conformation influences the neurotransmitter release (Bernstein, DeWit et al. 1998). F-Actin disassembles at the active zone during synaptic activity and repolymerise again at prolonged depolarisation of the cell, leading to a decline in neurotransmitter release. Experiments with F-actin disrupting and stabilising agents show that F-actin can act as a physical barrier between the readily-releasable neurotransmitter vesicle pool at the active site and storage pools. Only after partial depolymerisation of the actin network at the active site, the readily-releasable pool can be replenished (Bernstein, DeWit et al. 1998).

At the postsynaptic site actin is associated with many proteins of the postsynaptic density. NMDA and AMPA receptors, the neurotransmitter receptors of glutamatergic synapses, are coupled to it. The activity of these receptors leads to an uncoupling of them from the cytoskeleton, resulting in a displacement of the receptors out of the active zone and thereby in an activity-dependent negative feedback loop (Rosenmund and Westbrook 1993; Furukawa, Fu et al. 1997). Besides these direct modulations of neurotransmitter signalling, actin reorganisation also underlies the morphological plasticity of dendritic spines (Fischer, Kaech et al. 1998). Therefore, actin is important for the formation of new spines, as well as the activity-dependent enlargement of existing ones (Nikonenko, Jourdain et al. 2002).

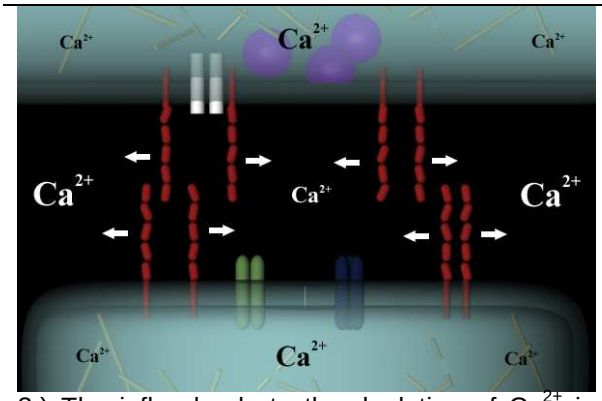
Interestingly most of the actin reorganisation processes underlying these modulatory functions are regulated by locally increased calcium concentrations, thus linking these mechanisms to synaptic, as well as transglutaminase activity. Therefore, transglutaminases could play a counterpart against calcium dependent disruption of the actin cytoskeleton by stabilising F-actin via cross-linking activity.



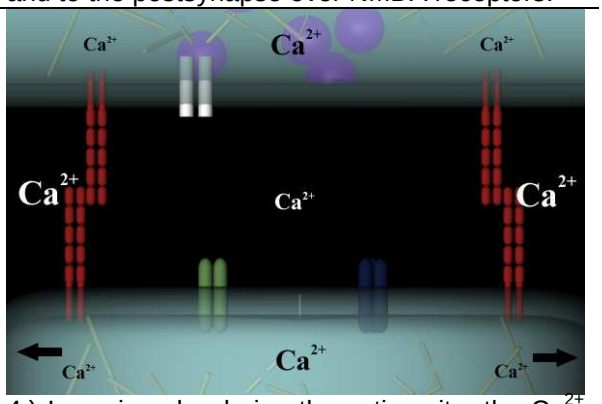
1.) In the dormant synapse cadherin dimers are flanking the active site.



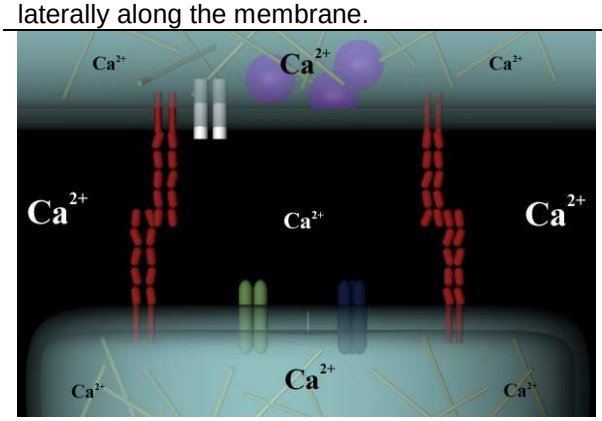
2.) Following activation Ca^{2+}-ions stream into the presynapse over voltage-gated Ca^{2+}-channels and to the postsynapse over NMDA receptors.



3.) The influx leads to the depletion of Ca^{2+} in the synaptic cleft, thus disrupting the cadherin adhesion. Intracellulary the heightened Ca^{2+} level activates actin severing proteins like gelsolin and F-actin depolymerises. Thereby the connection of cadherins to the actin cytoskeleton is disrupted. The cadherin monomers can diffuse laterally along the membrane.



4.) In regions bordering the active site, the Ca^{2+} concentrations intra- and extracellulary are at normal levels. Cadherin monomers diffusing in these regions will form dimers and they can attach to the intact cytoskeleton again.
Repetitive activations could consequently lead to an enlargement of the synapse.



3b) In neurons expressing transglutaminase 1, the transglutaminase is activated by the heightened calcium level. Actin is cross-linked intramoleculary and thereby stabilised. The depolymerisation of F-actin is prevented. The cadherin molecules stay attached to the cytoskeleton and the morphology of the synapse is preserved.

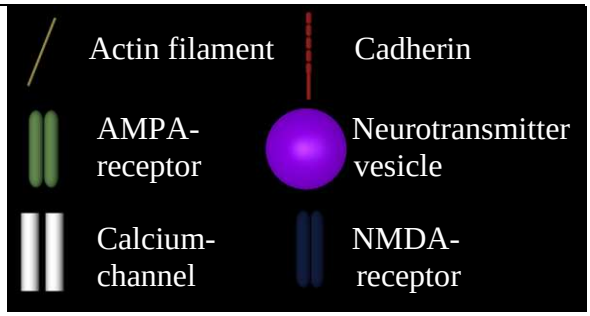


Fig. 4.1: Hypothesis for the role of transglutaminase 1 in synaptic endings.

But perhaps the most interesting potential function of actin stabilisation by transglutaminase activity relates to the adhesion proteins of synapses. Synaptic connections resemble adherens junctions as they can be found in epithelial cells (Tepass, Truong et al. 2000). Cadherins, mostly flank the active sites and are the main adhesion proteins of synapses (Uchida, Honjo et al. 1996). They are associated intracellulary to the actin cytoskeleton via catenins and actin binding proteins. Some published work, therefore, propose that the synapse is a specific adaptation of epithelial adherens junctions (Fannon and Colman 1996). The role of synaptic cadherins is not limited to the development and maintenance of synaptic endings. In the last years cell adhesion-dependant and –independent roles of cadherins in synaptic plasticity are revealed (Arikkath and Reichardt 2008; Tai, Kim et al. 2008).

NMDA receptor activity can trigger the expansion of spine heads, thereby enhancing the synaptic efficacy. This process is dependent on actin polymerisation and cadherin adhesion (Okamura, Tanaka et al. 2004). A possible mechanism underlying the spine enlargement is a calcium-dependent disruption and reconstitution of cadherin adhesion. Adhesion of cadherins is calcium-dependent. Upon calcium binding cis- and trans-dimerisation of cadherin molecules is stabilised and the adhesion is enhanced. During synaptic activity the extracellular calcium concentration in the synaptic cleft drops from 1.5 – 2 mM to 0.3 – 0.8 mM (Nicholson, ten Bruggencate et al. 1978; Rusakov and Fine 2003), due to the influx of calcium ions into the synapse. Such a drop in calcium levels leads to a reduction of the adhesion strength of 40% to 85% for N-Cadherin, the major cadherin of synapses (Heupel, Baumgartner et al. 2008). Simultaneously the intracellular heightened calcium concentrations could lead to a depolymerisation of synaptic actin, for example via gelsolin (Furukawa, Fu et al. 1997). Cadherin molecules would detach from their binding partners extracellulary and from the cytoskeleton intracellulary. The free cadherins could diffuse laterally in the synaptic cleft. In the periphery of the active zone the intra- and extracellular calcium concentrations would be normal. Free cadherins reaching this zone would dimerise again and attach to actin. Subsequent activations could thereby enlarge the area of cadherin adhesion around the active site. N-Cadherin binding is able to recruit AMPA receptors to the membrane (Saglietti, Dequidt et al. 2007), so the enlargement of the adhesive area could lead to an enlargement of the active zone of the synapse.

What would be the role of transglutaminase activity in such a model? Most likely the influx of calcium ions after the activation of the synapse would activate the previous dormant transglutaminase. Intercellular cross-linking of actin could stabilise F-actin and

thereby prevent the dissociation of the cadherin/catenin complex from the cytoskeleton. The cadherin-dependent adhesion would be strengthened and the lateral diffusion of cadherins should be prevented (see Fig 4.1 for a model). Therefore, synaptic transglutaminase activity could be a regulatory mechanism limiting the activity-driven cadherin remodelling at synapses. Interestingly, cross-links introduced by transglutaminases into a protein can not be reversed by any known proteinase. This means that the modification and so the stabilisation of actin would persist throughout the life of the protein, leading to a long lasting stabilisation of the synaptic morphology. The stabilising effect of transglutaminase 1 activity on cadherin adhesion is already hypothesised in endo- and epithelial adherens junctions (Hiiragi, Sasaki et al. 1999; Baumgartner, Golenhofen et al. 2004). Therefore, this role of transglutaminase activity at synapses would not be a totally new one.

The next step in investigating synaptic transglutaminase activity could be a further characterisation of actin cross-linking. It is still unknown if the assumed intramolecular cross-link is really formed. Furthermore, western blots of telencephalic cell lysates preincubated with 5-BPA showed various biotinylated proteins beside β-actin, which were not identified so far. It would be interesting to see if actin associated proteins like catenins or α-actinin, which links NMDA receptors to the cytoskeleton (Allison, Gelfand et al. 1998), are under these cross-linked proteins. Finally a characterisation of the *in vivo* cross-linking activity at synapses could be achieved by immunoprecipitation of synaptosomal proteins with an antibody against the ε-(γ-glutamyl)lysine isopeptide bridge, formed by transglutaminases. Unfortunately such an N-ε-(γ-glutamyl)lysine antibody tested in our lab, was found to be too unspecific for this task.

4.5 Synaptic activity dependent activation of transglutaminases

The induction of transglutaminase cross-linking activity by neuronal activation is an obvious assumption. Nevertheless, this hypothesis has to be verified. With the light-dependent cation-channel channelrhodopsin 2 (Chop2) a direct activation of neuronal cells is possible (Nagel, Szellas et al. 2003; Boyden, Zhang et al. 2005). To avoid the difficult transfection of primary neurons, the cell line PC12 was used. PC12 cells can be differentiated in a neuronal phenotype (Greene and Tischler 1976; Greene and Rein 1977). They express voltage-gated calcium and potassium channels (Streit and Lux 1987; Hoshi and Aldrich 1988), as well as transglutaminase 1 and 2 (Byrd and Lichti 1987). Hence light-induced depolarisation of Chop2-expressing PC12 cells should lead to an influx of calcium ions, thereby activating both transglutaminases.

Unfortunately the depolarisation achieved by Chop2-activity is not sufficient to cause an activation of these cells. The utilisation of a stronger light source could lead to a depolarisation strong enough to cross the threshold for activation. Alternatively and probably more promising, the replacement of PC12 cells with primary neurons could solve the problem. Even though this means that the tranfection method has to be changed. An adeno- or lentiviral system seems to be most suitable for such transfection. An exchange of the model system could also prevent the degradation of the aequorin/eGFP fusionprotein.

4.6 Conclusions

The results from murine and chicken neural cells showed a diverse picture. In the murine brain the situation remained inconclusive. The expression and even more the function of transglutaminase 1 in nerve fibre tracts are ambiguous, as well as the expression in neuronal and glial cells of the cerebellum. It seems certain that transglutaminase 1 is not permanently expressed in specific cell types. Rather unknown events have to initiate the expression. Otherwise in endothelial and ependymal cells the role of transglutaminase 1 is by far clearer. The predicted function as a stabiliser of intercellular junctions suits well to the membrane localisation and is consistent with the known role of transglutaminase 1 in other endothelial and epithelial tissues. The conditional transglutaminase 1 knock-out mouse is a promising tool to investigate the open questions.

In chicken forebrain cultures transglutaminase expression and activity could be described comparatively well, even though the final proof that the activity bases on transglutaminase 1 is still pending. However, transglutaminase activity was clearly localised at synaptic endings and β-actin is one of the substrates for the transglutaminase. β-Actin is at least a target for recombinant human transglutaminase 1. It seems likely that transglutaminase 1 also cross-links actin *in vivo*. The exact cross-linking mechanism as well as the possible connection with synaptic activity remained unclear. Even so an intracellular linkage between glutamine 41 and lysine 50 is not only the most likely possibility it is also in line with the experimental findings. The activity dependent stabilisation of the actin cytoskeleton is a consequential hypothesis and the stabilisation of synaptic intercellular junctions is an elegant model to link the potential role of transglutaminase 1 in endothelial and epithelial cells with its role in neurons.

5. Summary

The transglutaminases family includes calcium-dependent cross-linking enzymes catalysing a transamidation reaction between a protein-bound glutamine residue and a small primary amine or a protein-bound lysine residue. Transglutaminase 1, a member of this family, is expressed in different epithelial and endothelial tissues. Recently transglutaminase 1 was also identified in the brain. Here its activity was found to be up regulated in correlation with neurodegenerative diseases. However, little is known about the distribution and the function of transglutaminase 1 in the nervous system. The aim of this study was the characterisation of the expression of transglutaminase 1 in the brain and the analysis of transglutaminase activity in neural cell cultures.

To investigate the distribution of transglutaminase 1 in the central nervous system, cryostatic slices of mouse brains were immunohistochemically stained against transglutaminase 1 and neuronal, as well as glial markers. Transglutaminase 1 expression was found in scattered astrocytes throughout the cerebral cortex and the cerebellum, in few neurons inside the granular layer of the cerebellum, the caudoputamen and in parts of different fibre tracts, like the corpus callosum and the external capsule. The function of transglutaminase 1 in these cell types remained enigmatic.

Transglutaminase 1 was also found in endothelial cells of the brain vascular system and in parts of the ependymal lining of the ventricular system. Transglutaminase 1 is associated with adherens junctions in endothelial and epithelial cells of other tissues. Therefore we assume that the transglutaminase 1 found in the vascular and ventricular system of the brain is also involved in the stabilisation of intercellular junctions.

To clarify the role of transglutaminase 1 in the murine brain, a construct for a conditional knock-out mutant of transglutaminase 1 was cloned and successfully transfected into mouse stem cells. Unfortunately the knock-out mouse was not finished in time.

To investigate the activity of transglutaminase 1 two primary cell cultures, murine cerebellar granule cells and chicken telencephalic cells were established. Neurons and astrocytes of the granule cell culture were shown to partly express transglutaminase 1, but the enzyme was inactive in this culture. In the telencephalic cultures a membrane bound transglutaminase 1 staining was detected and a transglutaminase activity located in synaptic endings was found. In addition β-actin was found to be a substrate for this synaptic transglutaminase activity. This finding was supported with the expression of a

recombinant transglutaminase 1, which was able to cross-link a small primary amine to β-actin.

A model was proposed for the activation of transglutaminase 1 via calcium influx following synaptic activity and for the stabilisation of F-actin through transglutaminase 1 catalysed intramolecular cross-links between glutamine 41 and lysine 50 of β-actin. In this way transglutaminase 1 could stabilise the morphology of synaptic endings in a neuronal activity dependent fashion.

Transglutaminasen sind calciumabhängige Quervernetzungsproteine, die eine Transamidierungsreaktion zwischen spezifischen proteingebundenen Glutaminresten und kleinen primären Aminen, bzw. einem proteingebundenen Lysin, katalysieren können. Transglutaminase 1 wird in unterschiedlichen Epithelien und Endothelien expremiert. Des Weiteren wurde es auch im Gehirn entdeckt. Hier scheint seine Expression und Aktivität im Zusammenhang mit verschiedenen neurodegenrativen Erkrankungen, wie z.B. *Morbus Alzheimer* oder *Morbus Parkinson*, erhöht zu sein. Bisher liegen jedoch wenige Daten über die normale Expression und Funktion von Transglutaminase 1 im Gehirn vor. Ziel dieser Arbeit war daher die Untersuchung des Expressionsmusters von Transglutaminase 1 im Gehirn und seiner Aktivität in neuronalen Zellkulturen.

Zur Aufklärung der Verteilung von Transglutaminase 1 im Gehirn *in vivo* wurden Kryostatschnitte von Mäusehirnen angefertigt und immunhistochemisch gegen Transglutaminase 1 und verschiedene Marker für Neuronen und Gliazellen angefärbt. Vereinzelte Transglutaminase 1 positive Astrocyten wurden dabei im cerebralen Cortex und im Kleinhirn gefunden. Des Weiteren wurde Transglutaminase 1 in einzelnen Neuronen im Kleinhirn, sowie in verschieden Fasertrakten des Gehirns, wie dem Corpus Callosum, der externen Kapsel oder dem Caudoputamen festgestellt. Die Funktion der Transglutaminase in diesen Zellen blieb jedoch schleierhaft.

Zusätzlich wurde Transglutaminase 1 Expression auch in Endothelzellen der Blutgefäße und in Ependymzellen des Ventrikulärsystems entdeckt. Transglutaminase 1 ist in Endothelien und Epithelien anderer Gewebe mit Adherens Junctions assoziiert, daher nehmen wir an das auch im Gehirn interzelluläre Kontakte im Endothel und Ependym stabilisiert werden.

Für die nähere Untersuchung der Funktion von Transglutaminase 1 im murinen Gehirn wurde ein Vektorkonstrukt für eine konditionelle Transglutaminase 1 knockout Maus kloniert und erfolgreich in Mäusestammzellen rekombiniert. Leider konnte die knockout Maus nicht im Rahmen der Doktorarbeit fertig gestellt werden.

Die neuronale Aktivität von Transglutaminase 1 wurde mit Hilfe zweier primärer Zellkulturen, zum einen Körnerzellen aus dem Kleinhirn der Maus, zum anderen Telencephalonkulturen aus dem Hühnchen, untersucht. In der Körnerzellkultur wurden vereinzelte Transglutaminase 1 positive Neuronen und Astrocyten entdeckt, diese Transglutaminase 1 zeigte aber *in vitro* keinerlei Transamidierungsaktivität. In den Telencephalonkulturen wurde eine membranlokalisierte Transglutaminase 1 Färbung und eine synaptische Transglutaminaseaktivität nachgewiesen. Es wurde festgestellt, dass β-

Aktin ein Zielprotein für diese Transglutaminaseaktivität und das F-Aktin ein Substrat für rekombinant expremierte Transglutaminase 1 ist.

Eine Hypothese wurde vorgeschlagen, wonach neuronale Transglutaminase 1 durch aktivtätsabhängigen Einstrom von Calcium in Synapsen aktiviert wird und dort intramolekular Aktin zwischen Glutamin 41 und Lysin 50 quervernetzt. Die dadurch zustande kommende Stabiliserung von F-Aktin könnte einen Mechanismus darstellen, wodurch die Morphologie von Synapsen aktivitätsabhängig gefestigt wird.

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Appendix

Sequences of used constructs and vectors

pGEM-T TGase1fl-His6, in bold the insert

GGGCGAATTGGGCCCGACGTCGCATGCTCCCGGCCGCCATGGCCGCGGGAT**ACATAAGTCACTTACCAGGTCT**
GTCCCTGCGGCATCCAGTCTGTGGGTCCTGTCCCATCCATCCTGACCTGTTCCATCTCAGCCCCAGGACTCAG
TACTGCGGTTGCCAACACTGCTGCCAGGCATGATGGATGGGCCACGTTCCGATGTGGGCCGTTGGGGTGGCAA
CCCCTTGCAGCCCCCTACCACGCCATCTCCAGAGCCAGAGCCAGAGCCAGACGGACGCTCTCGCAGAGGAGGA
GGCCGTTCCTTCTGGGCTCGCTGCTGTGGCTGCTGTTCATGCCGAAATGCGGCAGATGACGACTGGGGACCTG
AACCCTCTGACTCCAGGGGTCGAGGGTCCAGCTCTGGCACTCGAAGACCTGGCTCCCGGGGCTCAGACTCCCG
CCGGCCTGTATCCCGGGGCAGCGGTGTCAATGCAGCTGGAGATGGCACCATCCGAGAGGGCATGCTAGTAGTG
AACGGTGTGGACTTGCTGAGCTCGCGCTCGGACCAGAACCGCCGAGAGCACCACACAGACGAGTATGAGTACG
ACGAGCTGATAGTGCGCCGCGGGCAGCCTTTCCATATGCTCCTCCTCCTGTCCCGGACCTATGAATCCTCTGA
TCGCATCACCCTTGAGTTACTCATCGGAAACAACCCCGAGGTGGGCAAGGGCACGCACGTGATCATCCCAGTG
GGCAAGGGGGGCAGTGGAGGCTGGAAAGCCCAGGTGGTCAAGGCCAGTGGGCAGAATCTGAACCTGCGGGTCC
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GTTGCCCTTTGACCCCCGCAATGAGATCTACATCCTCTTCAACCCCTGGTGCCCAGAGGACATTGTGTACGTG
GACCATGAGGATTGGCGGCAGGAGTATGTTCTTAATGAGTCTGGGAGAATTTACTACGGGACCGAAGCACAGA
TTGGTGAGCGGACCTGGAACTACGGCCAGTTTGACCACGGGGTGCTGGATGCCTGCTTATACATCCTGGACCG
GCGGGGGATGCCATATGGAGGCCGTGGAGACCCAGTCAATGTCTCCCGGGTCATCTCTGCCATGGTGAACTCC
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TGGGCAGCGTGGAGATCCTGCTTAGCTACCTACGCACGGGATATTCCGTCCCCTATGGCCAGTGCTGGGTCTT
TGCTGGCGTGACCACCACAGTGCTGCGCTGCCTGGGTCTGGCCACCCGTACTGTCACCAACTTCAACTCCGCC
CACGACACAGACACATCCCTTACCATGGACATCTACTTCGACGAGAACATGAAGCCCCTGGAGCACCTGAACC
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Insert of pPCR Script TG1 KO L, in bold the restriction sites for subcloning

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Insert of pPCR Script TG1 KO M, in bold the restriction sites for subcloning

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GTGGACCAGACTGGCCTTGAACTTACAGAGATCTACCCACC**GTCGAC**

Insert of pPCR Script TG1 KO R, in bold the restriction sites for subcloning

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CGGCCGC

Insert of pPCR Script TG1 SB S1

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AATGTCGG

Insert of pPCR Script TG1 SB S2

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AA

pTarget TG1 KO

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