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The role of gap junctional intercellular communication and connexins in the differentiation of human neural progenitor cells

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ABBREVIATIONS

- 1-oct – 1-octanol
- ADP/ ADT – adenosine di/triphosphate
- bFGF – basic fibroblast growth factor
- CFL – 5(6)-carboxyfluorescein diacetate
- Cx – connexin
- DIC - Differential interference contrast
- DMEM - Dulbecco's Modified Eagle's Medium
- DNA – deoxyribonucleic acid
- EDTA - ethylenediaminetetraacetic acid
- EdU - 5-ethynyl-2'-deoxyuridine
- EGF/R – epidermal growth factor/ receptor
- EGTA - ethylene glycol tetraacetic acid
- ER – endoplasmic reticulum
- ES cells – embryonic stem cells
- FITC - fluorescein isothiocyanate
- FRAP –fluorescence recovery after photobleaching
- GFAP – glial fibrillary acidic protein
- GFP – green fluorescent protein
- GJ – gap junction
- GJIC - gap junctional intercellular communication
- HuC/D – human neuronal protein
- MTT - 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
- RFP – red fluorescent protein
- SDS-PAGE – sodium dodecyl sulphate polyacrylamide gel electrophoresis
- SEM – standard error of mean
- siRNA – small interfering RNA
- T/TBS – Tween/ Tris-buffered solution
- Tris - tris(hydroxymethyl)-aminomethan
- TRITC - tetramethylrhodamine isothiocyanate
- YFP – yellow fluorescent protein
- ZO-1 – Zonula occludens-1 protein

ABSTRACT

During the last decade, intense research in the field of neural stem cell development has been provided relevant information about both proliferation and differentiation of progenitor cells. The mechanisms that trigger the elaboration of specific cell phenotypes are of great interest but largely unknown. It was suggested that gap junctional intercellular communication (GJIC) and connexin (Cx) proteins play a crucial role in the development of neural progenitors. However, the mechanisms of cell-cell coupling in regulating cell fate during embryogenesis are poorly understood.

To elucidate the role of GJIC in proliferation and differentiation, we used a human neural progenitor cell line derived from the ventral mesencephalon that enabled us to study alterations in GJIC and Cx expression in a time-dependent manner throughout the whole process of differentiation.

Using fluorescence recovery after photobleaching (FRAP) we found that neural progenitors are coupled via gap junctions (GJs). Moreover, dye coupling was extensive in proliferating cells but diminished after the induction of differentiation as indicated by a 2.5-fold increase of the half-time of fluorescence recovery. Notably, recovery half-time strongly decreased in the later stage of differentiation by 5-fold.

Western blot analysis revealed the presence of at least three different Cx isotypes, namely Cx43, Cx31 and Cx59. Although all Cxs were found to show different expression patterns, only Cx43 demonstrated a time-dependent expression profile that was similar to the alteration of GJIC. Accordingly, confocal microscopy revealed Cx43 as the main GJ-forming protein in ReNcell VM197. Interestingly, large amounts of cytoplasmic Cx43 were retained mainly in the Golgi network during proliferation but decreased when differentiation was induced.

Pharmacological blockage of GJIC demonstrated a strong impact of cell-cell coupling on proliferation and neuronal cell fate. While cell growth was inhibited by 15%, the elaboration of neuronal phenotypes was reduced by 25%. A specific role for Cx43 on progenitor development was investigated by down-regulation of Cx43, leading to a marked decrease of functional cell-cell coupling by 60%. Upon Cx43 knockdown, both the number of S-phase cells and neuronal differentiation efficiency were reduced by 50%.

Furthermore, the epidermal growth factor (EGF) was found to maintain the high level of Cx43 in proliferating progenitors. Likewise, GJIC was stimulated by EGF due to an increase of GJ plaques formation. Conversely, knockdown of Cx43 using small interfering RNA (siRNA) abolished the mitogenic activity of EGF, resulting in a 50% decrease of the proliferation rate.

Taken together, our findings provide a dual function of Cx43 and GJIC in the neural development of ReNcell VM197 human progenitor cells. 1) GJIC accompanied by high Cx43 expression is necessary to maintain cells in a proliferative state by mediating the mitogenic activity of growth factors. 2) Additionally, gap junctional coupling is required to complete neuronal differentiation, including the establishment of a neural network. However, uncoupling of cells is crucial in the early stage of differentiation during the exit from cell cycle and cell fate commitment[12].

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1 INTRODUCTION

1.1 STEM CELLS

Stem cells in the mammalian body represent a remarkable cell population that is crucial for tissue organization and development. Although the concept of stem cells was already theoretically postulated in the 19th century, the isolation of mouse embryonic stem cells (ES cells) was firstly described in 1981 (Evans & Kaufmann, 1981; Martin, 1981). There is no doubt that research on human cells profited substantially from the experiences gained by the work with mouse cells. However, despite many similarities, results obtained with mouse ES cells are not fully applicable to human ES cells. Thus, the first isolation of ES stem cells, derived from human tissue was an important step in stem cell research (Thomson, 1998).

The key feature of stem cells is their capability to choose between self-renewal state and multilineage differentiation (Odorico *et al.*, 2001). Embryonic stem cells, originated from the inner cell mass of the blastocyst during early embryogenesis, demonstrated pluripotency i.e. giving rise to all distinct cell types. Human ES cells express telomerase activity and several specific markers, including octamer-binding protein, CD9 and SOX2 (Mimeault & Batra, 2006). ES cells can be triggered to differentiate into certain progenitors or mature cells of ectodermal, endodermal and mesodermal germ layers (Odorico *et al.*, 2001).

Further development rapidly declines the frequency of ES cells due to the formation of restricted progenitors or terminally differentiated cells. However, a few cells retain their stem cell behaviour in postnatal individuals and become a reserve precursor cell. These tissue-specific adult stem cells possess a multipotent character and thus their differentiation ability is restricted to certain cell lineages (Mimeault *et al.*, 2007). In contrast to ES cells, adult stem/progenitor cells are required to maintain tissue homeostasis and contribute to regeneration and repair of damaged tissue by replacement of specialized mature cells within the tissue they reside (Young & Black, 2004).

1.2 NEURAL PROGENITOR CELLS

For many years it was believed that the adult brain represents a static entity without self-renewal capacity and that the generation of neurons was limited to the prenatal stage of neural development. Nowadays, it is well accepted that the formation of neurons and glial cells and their integration into the complex brain circuitry occurs during all stages of development, giving evidence for a pool of neural precursor cells in the central nervous system. In the embryo, neural progenitors have been found to be located in different parts of the developing brain, including hippocampus, cerebellum or cerebral cortex (Temple, 2001). In the adult brain both the subventricular zone of the lateral ventricle and the

subgranular zone of the hippocampal dentate gyrus have been shown to contain progenitors capable to induce neuron and glial cell formation (Martino & Pluchino, 2006). The process of differentiation and maturation, emanating from neural progenitors, depends on several parameters that regulate the fate of progeny. Cell-cell interactions were shown to influence the maintenance, activation and differentiation of stem cells (Doetsch, 2003). A prominent role was assumed for astrocytes that were found to stimulate neurogenesis of precursor cells. Due to their meandering cell protrusions and branches, astrocytes can translate signals between the vasculature system and other cells. Additionally, they are highly coupled via gap junctions (GJs), which promotes the propagation of signalling within a stem cell cluster (Doetsch, 2003).

To gain deeper insight into the behaviour of neural progenitors and to reveal the mechanisms of stem cell differentiation, several neural stem cell lines have been generated by viral introduction of immortalized genes (*c-myc*, *v-myc*) in concert with growth factor stimulation (Donato *et al.*, 2007; De Filippis *et al.*, 2008; Villa *et al.*, 2009). Growth factors such as epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) are commonly used as mitogens to maintain neural stem cell lines in a proliferative state. Upon removal of growth factors these cell lines undergo differentiation along distinct cell lineages (Zhao *et al.*, 2004).

As in vitro models, immortalized cell lines possess different features, making them superior to primary cell cultures. They are characterized by cellular and genetic homogeneity and can be produced in large quantities. Further, deoxyribonucleic acid (DNA) modifications are much more feasible compared to primary precursors and gene expression can be maintained over passages (Vescovi & Snyder, 1999). Based on these advantages, immortalized cell lines are a good tool to study the development of neural precursors. Using HiB6 cells, a cell line originally isolated from the E16 rat brain, it was demonstrated that the protein kinase A pathway is critical for the hippocampal neurogenesis (Kim *et al.*, 2002). Furthermore, to elucidate the detailed mechanism responsible for neural migration, researchers derived benefits from the migratory properties of the ST14A clone, obtained from the E14 striatum of rat (Cacci *et al.*, 2003). However, neural stem cell lines are not only used to study neuronal differentiation, but also help to reveal the cellular defects underlying neurodegenerative diseases (Ehrlich *et al.*, 2001). Moreover, immortalized neural stem cells have been discovered to be suitable for testing cell toxicity of biomaterials. Considering transplantation of neural progenitors, biomaterials were suggested to support the adherence, differentiation and migration of engrafted cells. Thus, biocompatibility of these materials, like polymers or nanofibres, is an important issue that can be addressed by the use of neural progenitor cell lines (He *et al.*, 2010; Bhang *et al.*, 2012). In summary, these findings give a general idea

about the possibilities that are provided by immortalized neural stem cells and highlight their importance in regenerative medicine.

1.3 THERAPEUTIC VALUE OF NEURAL PROGENITOR CELLS

Neurodegenerative diseases such as Parkinson´s disease, Alzheimer´s diseases or stroke are characterized by a loss of region-specific neurons due to progressive cell death. Regenerative medicine has been faced with the development of strategies to prevent neuronal deficiency and to promote functional recovery. The use of neural stem cells may provide a promising tool for cell replacement therapies and tissue engineering approaches, but it seems to be quite difficult for transplanted cells to differentiate under the "mature" conditions of the adult brain. However, many studies have been successfully demonstrated that stem cell based therapy could be applied to treat several human diseases (Lindvall *et al.*, 2004). Transplantation of fetal, mesencephalic tissue into the brain of patients suffering from Parkinson´s disease showed that neuronal replacement can work in in the human central nervous system (Kordower *et al.*, 1995). Work on animal models support the notion about the great potential of neural stem cells in tissue protection and replacement of damaged cells when engrafted into degenerated regions of the brain. For example, the immortalized cell line RN33B was shown to survive up to 24 weeks after transplantation into the cerebral cortex. Subsequent differentiation resulted in the formation neuron, capable of firing action potentials and receiving input from other synapses (Shihabuddin *et al.*, 1995; Englund *et al.*, 2002). Surprisingly, the functional recovery resulting from transplantation of neural stem cells does not correlate with the number of transplantation-derived newly generated neurons, suggesting additional positive bystander effects of engrafted neural progenitors (Richardson *et al.*, 2005; Ryu *et al.*, 2005).

As mentioned above, neurodegenerative diseases like Parkinson´s diseases are caused by a loss of dopaminergic neurons. Thus, researchers have been encouraged to reinforce the capability of neural progenitors to differentiate into neuronal phenotypes to increase the therapeutic value for Parkinson`s diseases animal models. Following genetic modification, inducing overexpression of certain enzymes and transcription factors relevant for neuronal development, neural stem cells were found to generate more dopaminergic neurons (Ryu *et al.*, 2005; Li *et al.*, 2007). Moreover, transplantation into the striatum of parkinsonian rats markedly improved their behaviour compared to the control individuals.

A role of neural progenitors was also proposed for the treatment of central nervous system traumata. Apart from the direct damage of neural tissue, brain traumata are accompanied by secondary effects, which arisen from the toxic effects of several modulators and enhance the initial brain damage. Glutamate, calcium and nitric were identified to impair the ability of

neural tissue for regeneration und increase neuronal cell death (Lynch & Dawson, 1994). Immortalized neural progenitors were shown to survive and differentiate after injection in damaged brain tissue, despite the critical conditions such as high glutamate and cytokine concentration (Riess *et al.*, 2002). To improve these benefits, neural progenitors were induced to express neurotrophic factors, which support the neuroprotective effects and functional recovery of animals suffering from traumatic injury (Bakshi *et al.*, 2006). The HiB5 progenitor clone that has been retrovirally transduced to produce nerve growth factor was demonstrated to increase the cognitive and neuromotor function and decreased apoptosis of host hippocampal neurons (Philips *et al.*, 2001).

Advances have also been made in stem cell-based therapy of spinal cord injuries to repair tissue damage. Transplantation of immortalized neural stem cells into injured rat spinal cord can stimulate the extensive growth of host axons by secretion of trophic factors that positively influence their environment (Lu *et al.*, 2003). In addition, neural stem cell clones were reported to share similar properties as radial glial. They migrate into the injured spinal cord and protect host cells from accumulation of macrophages and loss of axons and myelin (Hasegawa *et al.*, 2005).

Beside their application in the treatment of neurological disorder, neural stem cell lines are able to suppress tumour growth. Upon co-transplantation with tumours into animals, neural stem cells demonstrated enhanced migration towards the cancer cells that even occurred as neural progenitors were injected into peripheral vasculatures (Brown *et al.*, 2003; Staflin *et al.*, 2004). This homing capacity is an important feature for potential anti-cancer therapies to directly deliver therapeutic agents into the tumour.

Researchers have made an effort to isolate and characterize neural stem cells in order to blaze the trail for their future career in regenerative medicine. The findings above give evidence that neural progenitor cell lines hold enormous potential for the treatment of neurodegenerative diseases. However, their clinical application requires precise knowledge about the mechanisms that govern the elaboration of neuronal and non-neuronal phenotypes. This in turn may help to identify a practical and effective strategy for specifically stimulating neuronal differentiation of progenitors, which is crucial for the treatment of Parkinson´s or Alzheimer´s disease.

1.4 THE DIVERSITY OF CELL JUNCTIONS

Proper cell adhesion and intercellular communication are essential for the development of multicellular tissues and organisms as cell fate determination of stem cells is regulated by them (Tsai & McKay, 2000; Fuchs *et al.*, 2004). Cell-cell contacts are provided by three

different types of protein complexes, called cell junctions: 1) tight junctions, 2) adherens junctions and desmosoms and 3) GJs.

Tight junctions are close contacts between adjacent cells and thus create a primary barrier to diffusion of solutes via the intercellular pathway (Powell, 2012). Additionally, they maintain cell polarity by preventing lateral diffusion of cellular membrane proteins. Tight junctions are mainly composed of occludins and claudins and are found in epithelial and endothelial cells where they are located in the apical part of the cell, along the intercellular cleft respectively (Dejana, 2004).

Adherens junctions and desmosomes are located below tight junctions and belong to the anchoring junctions, which transmit mechanical stability. While adherens junctions are mainly linked to the actin cytoskeleton, desmosomes were shown to interact with intermediate filaments (Nagafuchi, 2001).

While tight junctions and anchoring junctions provide mechanical resistance, GJs are channel forming cell-cell contacts that allow direct communication between neighbouring cells. The direct connection of cytoplasm of adjacent cells enables the exchange of ions and molecules. GJs were found in most vertebrate tissue, except for some terminally differentiated cells like skeletal muscle cells, erythrocytes, circulating lymphocytes, and mature sperm cells (Meşe *et al.*, 2007; Rackauskas *et al.*, 2010).

1.5   STRUCTURE OF GJS

1.5.1   CONNEXINS IN VERTEBRATES

Connexins (Cxs) are a family of structurally related transmembrane proteins that are able to form a channel inside the plasma membrane to connect the cytoplasm of adjacent cells. To date, 20 members of the Cx gene family have been identified in the mouse and 21 in the human genome (Söhl & Willecke, 2004). The expression of Cxs is very variable and can change during the development of different tissues. Among all Cx isotypes, Cx43 has been found to be the most abundant Cx in vertebrates, indicating an important function for cellular development. All Cxs share a similar structural topology that is characterized by 4 alpha helical transmembrane domains (M1-4, Figure 1A) connected by 2 extracellular loops, 1 intracellular loop linking M2 and M3 and a cytoplasmic N-and C-terminal region (Figure 1). Although the third transmembrane domain was proposed to be the major channel-lining segment, there was evidence for a contribution by amino acids sequences of other domains of the Cx protein (Zhou *et al.*, 1997; Fleishman *et al.*, 2004). The extracellular loops are involved in cell-cell interactions between GJ hemichannels of two neighbouring cells. Three highly conserved cysteine residues enable the formation of disulphide bonds, which are crucial for proper gap junctional communication (Maeda & Tsukihara, 2011). The C-terminal

domain and the intracellular loop show great diversity in terms of sequence and length within the connexin family. However, the C-terminus contains different important phosphorylation sites that are important for the regulation of connexin degradation, transport or GJ assembly (Krutovskikh & Yamasaki, 2000).

Cx isotypes can be classified by their degree of homology and length of the cytoplasmic loop (Willecke *et al.*, 2002) or by their molecular mass (ranging from 23 to 62kD), which is the more common nomenclature and also used in this thesis.
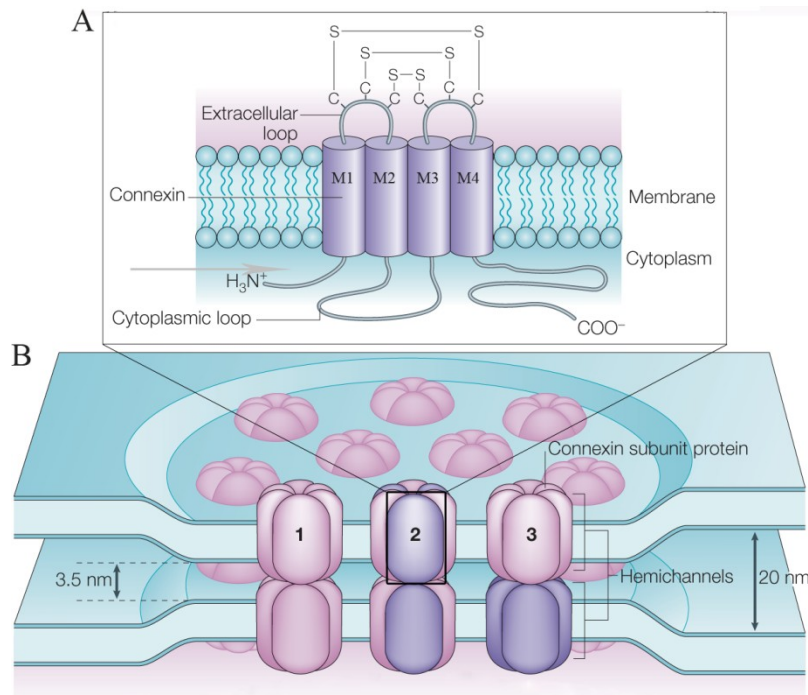
Figure 1 Molecular organization and topology of a GJ plaque. (**A**) Cx proteins consist of 4 membrane spanning domains (M1-M4), connected by 1 cytoplasmic and 2 extracellular loops that are crucial for docking. S-S represents conserved cysteine disulphide bonds. Cx proteins vary mainly in their C- and N-terminal region. (**B**) Hemichannels, formed by 6 Cx subunits, can dock to each other and establish a GJ channel. Three different types of GJs are known, depending on their molecular composition. 1) homomeric/homotypic, 2) heteromeric and 3) heterotypic. Homotypic and heterotypic GJs are composed of two identical or two different hemichannels. Homomeric or heteromeric hemichannels comprise one or more Cx isotypes. (adapted from Söhl *et al.*, 2005)

1.5.2 GJ – THE INTERCELLULAR CHANNEL

GJs are specialized protein structures in the plasma membrane that contain clusters of channels, mediating direct communication between adjacent cells. They allow diffusional exchange of small molecules up to a molecular mass of 1kD, including metabolites, ions, second messengers or siRNA (Evans & Martin, 2002). GJ channels consist of 2 connexons (hemichannels), each provided by one of the neighbouring cells (Figure 1B). A connexon is formed by the oligomerization of 6 Cx proteins, transported to the cell surface through microtubules and finally fused to the plasma membrane. Hemichannels can either exist as a non-junctional channel or diffuse into the areas of cell-cell contacts where it forms a

complete GJ channel with a connexon of the adjacent cell. The expression of different connexin isotypes results in a great variance of GJ channel composition. Connexons can consist of either one single Cx isoform leading to homomeric connexons or they contain different connexin types and form heteromeric structures (Meşe *et al.*, 2007). The diversity of GJ channels is enhanced by the possibility that hemichannels can connect to homomeric or heteromeric hemichannels resulting in homotypic channels, or connexons can form heterotypic channels by docking to different homomeric or heteromeric connexons. However, several studies have shown that certain isoforms do not interact with each other, determining connexin oligomerization and GJ formation as a connexin isotype specific process (Falk *et al.*, 1997; Segretain & Falk, 2004). Once a single GJ channel has formed, clustering leads to the establishment of large GJ plaques that can contain up to many thousands of GJs with several micrometres in size (Segretain & Falk, 2004).

1.6 THE CONNEXIN LIFE CYCLE – GJ FORMATION, REMOVAL AND DEGRADATION

Remarkably, Cxs proteins have a short half-life of only a few hours (Beardslee *et al.*, 1998; Hervé *et al.*, 2007). It is proposed that this short lifespan enables rapid response to physiological changes by in- or decreasing gap junctional intercellular communication (GJIC). Thus, alterations in Cx expression, assembly and degradation allow regulation beside the channel opening and closure mechanisms that are associated with channel gating (Bukauskas & Verselis, 2004). Figure 2 schematically illustrates the different steps of the Cx life cycle, including synthesis, GJ formation and degradation. Consistent with classical integral membrane proteins, Cx are synthesized by involvement of the endoplasmic reticulum (ER). During integration into the ER membrane Cxs obtain their final functional transmembrane topology (Falk, 2000). The location of subsequent connexin oligomerization into connexons is controversially discussed and seems to depend on specific Cx isotypes. Cx assembly has been demonstrated to occur in the ER (Falk *et al.*, 1997). However, Cx43 and Cx46 were also reported to be present as monomers in the ER and Golgi network and assembly only started during their residence in the trans-Golgi Network (Musil & Goodenough, 1993; Koval *et al.*, 1997). Further studies are required to solve the question which compartment is involved in the final oligomerization of Cxs. After exiting the ER, connexons pass the ER-Golgi-intermediate compartment before entering the Golgi stacks and the trans-Golgi network (Laird, 1996). However, other studies suggest a Golgi-independent pathway for Cx26 transport (George *et al.*, 1999; Martin *et al.*, 2001). Trafficking of assembled Cxs to the cell surface is mediated by microtubules and actin filaments (Olk *et al.*, 2009). Once fused with the membrane, connexons freely diffuse until

docking with a connexon from the adjacent cell to form a GJ channel. The clustering into GJ plaques is a very rapid and dynamic process, whereas renewal occurs from the outer margin of the GJ plaques as revealed by studies using green fluorescent protein-tagged Cx (GFP-Cx) (Lauf *et al.*, 2002). As GJ coupling is not directly correlated to the clear microscopic observations of GJ plaques, small GJ clusters or even individual GJ channels will also contribute to functional coupling. Alternatively, connexons may also act as hemichannels, allowing molecule exchange between the extra – and intracellular space. Degradation starts with the internalization of the entire GJ or a fragment of it. As GJs cannot be separated again into connexons under physiological conditions, they are removed from the centre of the plaque by internalization into vesicular double-membrane structures, so-called annular junctions (Laird, 1996). Once internalized, further degradation involves lysosomal and proteasomal degradation pathways (Segretain & Falk, 2004). However, other pathways of GJ internalization may coexist that did not require the formation of annular junctions (Laird, 1996).
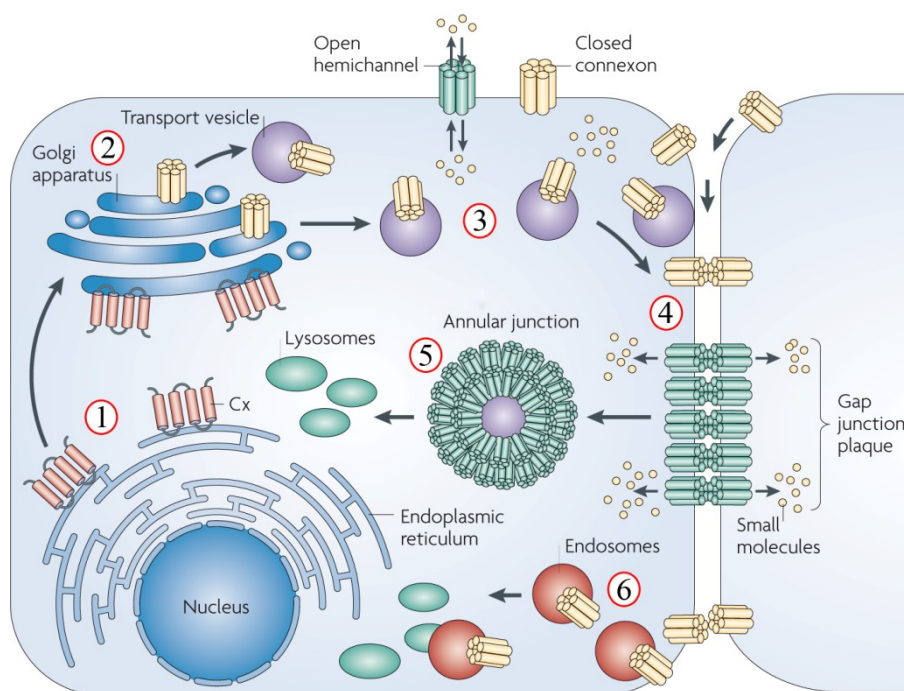
Figure 2 Overview about the Cx life cycle. (**1**) Cxs insert into the ER where they commonly reach their final folded state. (**2**) On trafficking to the Golgi network Cxs proteins oligomerize into hemichannels. (**3**) After microtubule-dependent transport to the cell surface, (**4**) connexons of adjacent cells can either dock to each other to form a GJ channel or exist as hemichannel, connecting intra- and extracellular space. (**5**) After a few hours, GJs or fragments of GJs are internalized into one of two neighboring cells as annular junctions and further degraded in lysosomes. (**6**) However, other mechanisms of GJ degradation may coexist where connexons enter the cell by the classical endocytic pathway. (adapted from Naus & Laird, 2010)

1.7 FUNCTIONS OF CONNEXINS

1.7.1 FUNCTIONS WITHOUT JUNCTIONS – GJ-INDEPENDENT ACTIVITY OF CONNEXINS

Many studies have been done elucidating the function of connexins as GJ forming proteins that mediate the exchange of molecules between connected cells. However, Cxs may also act in a channel independent manner by direct or indirect interaction with a variety of different proteins.

Cxs were found to play a role in the regulation of the cytoskeleton. Elias *et al.* (2007) reported that interaction of Cxs with the actin cytoskeleton is necessary for the migration of neuronal precursor cells along radial fibres during brain development. Accordingly, administration of 1-octanol (1-oct) and 18-α-glycyrrhetinic acid, two well-known GJ blockers, induce disorganization of actin stress-fibres in astrocytes (Yamane *et al.*, 1999). Furthermore, knockout of Cx43 in cardiac neural crest cells impaired the actin organization and reduced cell migration and directionality (Xu, 2001). Although the precise mechanisms of actin regulation are quite unknown, it was suggested that drebrin and zonula occludens-1 (ZO-1) protein may act as a linker between the C-terminal tail of Cxs and actin filaments (Giepmans, 2004). However, several studies have also indicated a direct interaction of Cxs and the cytoskeleton as observed for tubulin. Gap junctional Cx43 directly interacts with α- and β-tubulin in the cell periphery, regulating the dynamic of microtubules (Giepmans *et al.*, 2001).

Beside ZO-1, Cxs may also associate with other cell-cell contact proteins. E-cadherin and α-catenin colocalized with newly formed GJs, indicating a close relationship between adherens junction and GJ formation (Fujimoto *et al.*, 1997). Moreover, Cx was suggested to regulate cell development by interaction with β-catenin, a key protein of the Wnt signalling pathway (Ai *et al.*, 2000). The role of Cxs in cell signalling is further supported by studies showing strong proximity with Caveolin in Lipid Rafts that are important in clustering signalling molecules to ensure proper signal transduction (Schubert *et al.*, 2002). Proteins of the CCN (CeflO/Cyr61 and NOV) group were also found to interact with Cxs (Fu *et al.*, 2004). Members of the CCN family are required for diverse biological processes, including differentiation, adhesion and migration.

The examples mentioned demonstrate that Cxs can regulate cellular processes in a way that is not exclusively linked to their GJ-forming ability. Although many different binding partners have been identified the C-terminal domain of Cx seems to be mainly involved in mediating these protein interactions.

1.7.2 CHANNEL-DEPENDENT FUNCTIONS - REGULATION OF GJ ACTIVITY

As noted before, GJs connect the cytoplasm of adjacent cells and mediate the exchange of ions, second messengers and metabolites including inositol trisphosphate, ATP (adenosine triphosphate) or glutamate (Rackauskas *et al.*, 2010). Due to this electrical and chemical communication GJs are important regulators for tissue and organ coordination (Evans & Martin, 2002). Cx expression is cell- and tissue-dependent and Cx diversity as well as their combinatorial complexity in hemichannels and GJs leads to a great multiplicity of channels with unique properties concerning conductance, gating and permeability (Meşe *et al.*, 2007). Selective permeability can be visualized using fluorescent tracers that might only diffuse across GJ channels and therefore provide a suitable tool for studying GJIC (Elfgang *et al.*, 1995). Selective permeability has been reported for inositol polyphosphates (Niessen *et al.*, 2000) or ADP/ATP and is dictated by several parameters, including molecular weight, size, shape and net charge of the crossing molecule (Goldberg *et al.*, 2004). As GJs have a slightly negative charge, a preference could be observed for cations (L. Harris, 2001). Additionally, the interaction between Cxs and other molecules can affect channel selectivity. It has been shown, that cyclic adenosine monophosphate and cyclic guanosine monophosphate close specific channels while other nucleotides do not induce a change of permeability (Bevans & Harris, 1999). The extent of gap junctional communication is also regulated by voltage, which is particularly prominent in excitable cells. Depending on Cx composition, GJ closure can either be induced by hyperpolarization or upon depolarization (González *et al.*, 2007). Further, calcium, an important ion that mediates different cellular processes, also regulates GJ activity. The closure of GJs by calcium during certain pathological conditions prevents depolarization and leakage of metabolites by uncoupling of damaged cells (Rackauskas *et al.*, 2010). Although it is not clear whether calcium acts directly or indirectly on GJs, there has been evidence for an involvement of calmodulin in calcium-mediated channel gating (Peracchia *et al.*, 2000). Chemical gating of GJs is also affected by, H^+ ions, whereas sensitivity of GJs is dependent on Cx isoform and cell type (Francis *et al.*, 1999). Thus, variation of the intracellular pH results in altered cell-cell coupling most probably due to direct protonation of histidine residues located in the carboxyl tail of the Cx protein (Rackauskas *et al.*, 2010).

Many cellular events were shown to require phosphorylation, leading to a change of protein conformation and activity. A large number of Cxs are known to be phosphoproteins that are commonly phosphorylated by several protein kinases on serine, threonine and tyrosine residues of the cytoplasmic C-tail or the cytoplasmic loop (Lampe & Lau, 2000; Urschel *et al.*, 2006). Phosphorylation of Cxs induces changes of the molecular protein structure, which affects channel behaviour, including the unitary conductance and mean open time (Moreno

et al., 1992; Cottrell *et al.*, 2002). In addition, phosphorylation has also been discussed to be linked to GJ assembly, internalization and degradation (Solan & Lampe, 2005; Laird, 2006). The channel-dependent function of Cxs is not exclusively restricted to its role as a GJ-forming protein. As hemichannels in the plasma membrane, Cxs provide a pathway for the release or uptake of molecules and ions between the intracellular and extracellular environment, indicating its physiological importance in signalling functions and cellular homoeostasis. Their permeability characteristics are similar to that of GJs (Stout *et al.*, 2004). However, in contrast to GJs, which remain mostly in an open state, hemichannels were found to have a low open probability under resting conditions that can be increased by certain stimuli (Goodenough & Paul, 2003). Previous studies have shown that Cx hemichannels can be opened by low extracellular calcium concentration, membrane depolarization, mechanical stress, activation of purinergic receptors or intracellular activity of kinases (Trexler *et al.*, 1996; Stout *et al.*, 2004; Cotrina *et al.*, 2008). Yet, the mechanistic principles of the gating processes still have to be elucidated. Several experiments have been performed to investigate the kinds of molecules capable to pass hemichannels, and an important finding was the paracrine release of ATP, resulting from a reduction of the extracellular calcium level (Braet *et al.*, 2003; Coco *et al.*, 2003). Consistently, several different messenger molecules were also suggested to pass hemichannels such as glutamate, nicotinamide adenine dinucleotide or prostaglandins (Bruzzone *et al.*, 2001; Parpura *et al.*, 2004; Cherian *et al.*, 2005). In astrocytes, 15% of the entirely expressed Cx43 was found to be located in hemichannels in non-junctional membrane areas (Retamal *et al.*, 2006). These observations and the fact that hemichannels allow regulated release of signaling molecules indicate a strong biological importance in mediating glial-glial or neuron-glial interactions within neural networks.

1.7.3 ROLE OF CONNEXINS IN CELL PROLIFERATION

A pronounced role of Cxs in cell growth has been assumed soon after the discovery of gap junctional coupling (Loewenstein, 1968). Later on, several studies reported that cell growth is inhibited by intercellular communication and that GJ blockage increased proliferation rate, suggesting that cell growth correlates inversely with the extent of cellular communication (Mehta *et al.*, 1986; Loewenstein & Rose, 1992; Tabernero *et al.*, 2001). Consistently, cells exposed to mitogens responded with inhibition of cell communication (Maldonado *et al.*, 1988; Moorby *et al.*, 1995), leading to the assumption that adjacent cells have to decrease gap junctional contacts before entering mitosis. Likewise, Cx was found to regulate proliferation in tumour cells. While cancer cells demonstrated reduced GJIC (Yamasaki *et al.*,

1995), forced Cx expression inhibited proliferation, supposing a role for Cxs in tumour suppression (Zhu *et al.*, 1991; Mehta *et al.*, 1999).

However, the regulation of proliferating events by Cxs is controversially discussed and a large number of studies have observed a stimulating function of Cxs and GJIC on cell growth. Cx43 null astrocytes showed a reduced growth rate compared to wild-type cells that were characterized by extensive GJIC, mediated by Cx43-formed GJs (Naus *et al.*, 1997). Moreover, in neural progenitors Cx43 and GJIC were demonstrated to be crucial for maintaining cells in a proliferative state when cultured in the presence of growth factors. Conversely, inhibition of GJIC abolished the mitogenic activity of growth factors resulting in an increased cell death (Cheng *et al.*, 2004). Growth factors like bFGF, in turn, can promote Cx43 expression and increase the level of GJIC (Nadarajah *et al.*, 1998). Thus, proper cell-cell coupling is necessary for the signalling of mitogens, suggesting a function of GJIC in providing cell synchrony and regulation of proliferation and cell contiguity. These different findings support the hypothesis that Cxs and GJs exhibit their effect on proliferation in a cell-type-dependent manner. Although the underlying mechanism that controls Cx functionality has not been revealed, the C-terminus seems to play a major role in the selective activity of Cx proteins (Dang *et al.*, 2003).

1.7.4 ROLE OF CONNEXINS IN NEURAL DIFFERENTIATION AND DEVELOPMENT

Many previous reports have demonstrated a link between GJIC and differentiation, indicating that Cxs and GJ-mediated intercellular communication is required for both cellular development and tissue differentiation. GJIC plays a critical role during embryogenesis, as cell-cell coupling in the developing embryo enables the formation of communication compartments and developmentally important domains (Kalimi & Lo, 1988; Bagnall *et al.*, 1992; Dahl *et al.*, 1996). These different compartments allow the establishment of morphogen gradients and the electrical and metabolic synchronization, which is important for embryo patterning and proper differentiation (Levin, 2002). The specific and coordinated functions of Cx proteins and gap junctional coupling during development are emphasized by the fact that Cxs are differentially regulated during differentiation. Keratinocytes demonstrated an altered Cx expression pattern while their differentiation and migration through the epidermal layers (Butterweck *et al.*, 1994). Similar data were obtained for neural tissue. The detection of Cxs in the developing and mature rat brain revealed that Cx composition changes during the differentiation process. While Cx26 largely disappeared from the immature brain after 3-6 weeks, the level of Cx32 increased (Dermietzel *et al.*, 1989). This might suggest a correlation between expressed Cxs and specific steps in neural development. Cxs expressed at the early stage might indicate their involvement in

coordinated migration of dopaminergic and other cells from the ventricular zone to other areas in the developing brain. High prenatal levels could give evidence for a pronounced role in gap junctional coupling of neurons (Leung *et al.*, 2002).

Neural development starts from a certain cell population within the embryo that is capable to differentiate into neurons and glial cells. These neural progenitors were found to require proper GJIC and Cx expression for their development into neural phenotypes. Neural stem cells express several Cx isotypes and are capable to communicate via GJs (Wong *et al.*, 2008). Moreover, Cx levels and the extent of GJIC vary during their differentiation. In the developing neocortex, both neuroblasts and proliferating cells are highly coupled with each other, whereas differentiated cells demonstrate low GJIC (Lo Turco & Kriegstein, 1991; Bittman *et al.*, 1997). In a human teratocarcinoma cell line that is similar to neural precursors and able to differentiate into post-mitotic neurons, Cx43 protein level and dye coupling progressively decreased after induction of differentiation and were absent in differentiated neurons (Bani-Yaghoub *et al.*, 1997). Furthermore, GJ blockage led to a decreased number of differentiated neurons (Bani-Yaghoub, Underhill, *et al.*, 1999). Accordingly, Cx36 can regulate the cell fate of neural progenitors towards the neuronal lineage (Hartfield *et al.*, 2011). However, Cxs also influence the differentiation of non-neuronal cell types. The lack of Cx43 expression was found to induce a deficiency in oligodendrocyte differentiation and an increase in astrocytes (Wong *et al.*, 2008) .

As the reader can appreciate, GJs and Cxs are crucial regulators of development and indispensable for neural differentiation including cell fate commitment. The challenge is now to unravel the mechanisms by which Cxs contribute to neural development of human neural progenitors.

1.8 AIM OF THE STUDY

During the last few years, neural stem cells have become of great interest for regenerative medicine by providing the possibility of different therapeutically useful cell types. One major aspect in stem cell research deals with the question which cellular events trigger cell fate commitment during the differentiation process of neural progenitors. Precise knowledge about the mechanisms that control the elaboration of neuronal phenotypes may provide a powerful approach for therapies of neurodegenerative diseases. Several findings led to the assumption that Cxs and gap junctional communication might be involved in neural progenitor development. In this study, we used an immortalized neural human progenitor cell line to gain deeper insight into the role of Cx-mediated intercellular communication during proliferation and differentiation. In particular, we wanted to address the following main questions:

➢ Are ReNcell VM197 human neural progenitors coupled via GJs? And if so, is there a difference in the extent of intercellular communication between proliferating progenitors and differentiated cells?

➢ What Cx isotypes mediate gap junctional communication in neural progenitors? Further we asked whether Cx expression may change after induction of differentiation.

➢ Does Cx–mediated cell-cell coupling affect the proliferative ability of neural progenitors and can it regulate neuronal cell fate?

2 MATERIAL AND METHODS

2.1 CULTIVATION AND DIFFERENTIATION OF ReNcell VM197

All experiments were performed using the human neural progenitor cell line ReNcell VM197 (Millipore, Schwalbach, Germany, SCC008 ReNeuron, Ltd, Guilford, UK). This cell line was derived from the ventral midbrain of a 10-week-old human fetus and immortalized by retroviral transduction with *v-myc*. In this study ReNcell VM197 grows as monolayer in the presence of the growth factors EGF and bFGF. Cells have a doubling time of 25-30h and are able to rapidly differentiate into neural phenotypes after growth factor removal.

Cells were cultivated as described previously (Hoffrogge *et al.*, 2006). Briefly, cells were grown on laminin coated culture vessels in media containing DMEM/F12 (Dulbecco's Modified Eagle's Medium) supplemented with B27 media supplement, 2mM glutamine, 10 units/ml heparin and 50µg/ml gentamycin (all Invitrogen, Karlsruhe, Germany) and maintained at 37°C in 5% CO_2 humid atmosphere. For proliferation, 20ng/ml EGF and 10ng/ml bFGF were added (Invitrogen). Cells were passaged at confluence of 70-80% every 3-4 d. To avoid changes in differentiation characteristics, cells were only used up to passage 30. Cells were differentiated according to standard differentiation protocol, where differentiation was induced by withdrawal of growth factors (Donato *et al.*, 2007) at a confluence of approximately 70%.

Sub-confluent cell layers were passaged by using trypsin EDTA (in HBSS without Ca^{2+} and Mg^{2+}; Invitrogen; benzonase, 25U/ml, Merck, Darmstadt, Germany) for 3min at 37°C, followed by addition of trypsin inhibitor (0.55mg/ml trypsin inhibitor, Sigma-Aldrich, Steinheim, Germany; 1% human serum albumin, Grifols, Langen, Germany; benzonase 25U/ml; in DMEM/F12) to stop the reaction. After centrifugation at 126g for 5min, cells were resuspended in 1ml proliferation medium, counted and seeded at desired cell density. For most experiments, cells were grown for 2-4d before differentiation was initialized.

For microscopy studies, cells were cultured on glass coverslips that were flame-treated for sterilization, followed by incubation with poly-D-lysine (Sigma) overnight. After removal of poly-D-lysine, coverslips were washed with dH_2O, sterilized by irradiation with UV light for 20min and air-dried. All cell culture flasks, well plates and coverslips were coated with laminin (dilution 1:100 in DMEM/F12, Trevigen, Eching, Germany) for at least 4h to facilitate cell attachment.

2.2 siRNA TRANSFECTION

Down regulation of Cx43 gene expression was carried out using Cx43 siRNA (Sigma). Different transfection parameters were tested to estimate best transfection efficiency, including low cytotoxicity. Finally, proliferating cells were transfected with 100nM Cx43 siRNA and mock siRNA by electroporation with the Neon Transfection System (Invitrogen, 1300V, width 10ms, 3 pulses) according to the manufacturer's instructions. Cells were cultured for another 2d in the presence of growth factors before used for further experiments.

2.3 MTT-ASSAY

The MTT assay is a calorimetric test to estimate the viability and proliferation of cells. This assay is based on the reduction of the yellow tetrazole MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to purple formazan in living cells by an enzymatic reaction (Mosmann, 1983). The absorbance correlates with the cell viability and can be quantified by measuring the OD at 550nm.

For analysing cytotoxicity of 5(6)-carboxyfluorescein diacetate (CFL) cells were cultured in 96-well plates. Different concentrations of CFL were applied either to proliferating cells or on 2d differentiated cells. CFL was diluted in 200µl growth- or differentiation medium per well (8 wells per concentration) and incubated at 37°C for 5h. Two hours before CFL-exposure ended, 0.5 mg/ml MTT solution (Roth, Karlsruhe, Germany) was added to each well. After incubation, medium was removed and formazan crystals were solubilized by adding 100µl DMSO (Sigma) containing 10% SDS and 0.6% acetic acid (Roth). Finally, culture plates were mixed on a plate shaker and extinction was measured at 550nm versus blank (only DMSO-lysis buffer) using an EL 808 Ultra Microplate Reader (Bio-Tek Instruments Inc., Highland Park, USA, 158840). Estimation of cytotoxicity was calculated and statistically analysed by MS Excel 2010 (Microsoft).

2.4 MICROSCOPIC ANALYSIS OF RENCELL VM197

2.4.1 CELL FIXATION AND IMMUNOLABELLING FOR CONFOCAL FLUORESCENCE MICROSCOPY

The whole process of fixation and antibody labelling was performed at room temperature. For microscopic studies cells were grown in 24-well plates on glass coverslips and fixed with phosphate buffered saline (PBS; 10x: 80 g/l NaCl, 1.5 g/l Na_2HPO_4 x 2 H2O, all AppliChem, 2 g/l KCl, 2 g/l KH_2PO_4, all Sigma; all in dH_2O; pH 7.4) containing 4% paraformaldehyde (Sigma)

for 20min. Afterwards, paraformaldehyde was quenched using PBS containing 50mM NH_4Cl (Roth) for 10min.

In case of Golgi staining, proliferating cells were transfected with Golgi-GFP (20 particles per cell) for 24h and, if required, differentiated for further experiments. Fixation was performed as described above. Expression of the Golgi protein *N*-acetylgalactosaminyltransferase-2 was carried out as outlined by the manufacturer (Invitrogen). If necessary, fixed cells were permeabilised by incubation with PBS containing 0.2% Triton X-100 (Sigma) for 5min. Generally, Cxs were labelled without previous permeabilization. To reduce unspecific binding, cells were treated with 1% gelatin (Sigma, in PBS) for 1 hour, followed by labelling with a primary antibody (see Table 1) diluted in 1% gelatin (in PBS) for 1 hour. Subsequently, cells were rinsed twice in 0.2% gelatin (in PBS) for 5min and incubated with appropriate secondary antibodies (see Table 1) diluted in 1% gelatin for 45min.

Cell nuclei or actin cytoskeleton were stained with Hoechst and 1µg/ml phalloidin tetramethylrhodamine isothiocyanate (TRITC), respectively. After rinsing in 0.2% gelatin, PBS and dH_2O, cells were mounted on microscope slides using ProLong® Gold antifade reagent (Invitrogen).

For Triton X-100 *in situ* extraction of Cxs (Musil & Goodenough, 1991; Xu, 2001), cells were incubated with PBS containing 0.5% paraformaldehyde and 0.3% Triton X-100 for 15min on an orbital shaker, followed by postfixation with 4% paraformaldehyde in PBS for additional 15min. Subsequently, cells were immunolabelled as described above.

antibody	host	dilution	supplier and Cat.nr.
primary			
anti-α-tubulin	mouse	1:1000	Sigma, T5168
anti-β(II) tubulin-FITC	mouse	1:80	Abcam, ab25770
anti-nestin	mouse	1:400	BD, Heidelberg, Germany, 611658
anti-connexin 31	mouse	1:300	Sigma, WH0002707M1
anti-connexin 43	rabbit	1:400	Abcam, ab11370
anti-connexin 59	rabbit	1:100	Sigma, SAB1300563
anti-Human Neuronal Protein (HuC/D)	mouse	1:300	Invitrogen, A21271
anti-EGF receptor	mouse	1:200	Santa Cruz, sc-73511
anti-ZO-1- Alexa Fluor®594	mouse	1:200	Invitrogen, 339194

glial fibrillary acid protein(GFAP)-Cy3	mouse	1:500	Sigma, C9205
secondary			
anti-mouse Alexa Fluor®488 and 594	goat	1:300	Invitrogen, A11001 and A11032
anti-rabbit Alexa Fluor®488 and 594	rabbit	1:300	Invitrogen, A11034 and A11012

fluorescent dyes		**dilution**	**supplier and Cat.nr.**
Hoechst 2495 (1mg/ml)		1:1000	Sigma, 862096
Phalloidin TRITC		1:500	Sigma, P1951
fluorescent protein based reagent			
Golgi-GFP		20 particles per cell	Invitrogen, C10592

Table 1 Antibodies and fluorescent dyes used for immunolabelling.

2.4.2 QUANTITATIVE ANALYSIS OF PROLIFERATION

Proliferation analysis was performed using EdU (5-ethynyl-2'-deoxyuridine, Invitrogen), a nucleoside analogue of thymidine that is incorporated into the DNA during S-phase, allowing quantitative estimation of proliferating cells. Detection of EdU is based on a copper-catalysed click reaction between an alkyne group of EdU and an azide group conjugated to a fluorescent dye (Alexa Fluor®594, Invitrogen, Karlsruhe, Germany).

For proliferation analysis cells were cultured on glass coverslips and exposed to 5µM EdU for 2h. After fixation and permeabilization with 0.2% Trion X-100, cells were incubated for 30min in EdU reaction buffer containing 1µM Alexa Fluor®594 carboxamido-6-Azidohexanyl (Invitrogen), 100mM TrisHCl (Sigma, pH 7.9), 2mM copper sulphate and 100mM ascorbic acid (all Roth). Finally, cells were mounted in ProLong® Gold antifade reagent. For microscopic quantification EdU positive cells were counted out of at least 1000 cells in three independent experiments and the percentage was calculated using MS Excel.

2.4.3 QUANTITATIVE ANALYSIS OF NEURONAL DIFFERENTIATION AND UNCOUPLING EXPERIMENTS

To study neuronal development in ReNcell VM197 cells were labelled for the neuron specific markers β(III) tubulin and HuC/D. Neurons were quantified by counting of HuC/D and β(III) tubulin positive cells out of at least 2000 cells in three independent experiments.

Pharmacological inhibition of GJIC was achieved with 50μM and 100μM carbenoxolone (CBX) diluted in distilled water and 500μM 1-oct. For analysing the effect of GJ blockage on proliferation cells were exposed to the uncoupling agents for 24h and subjected to EdU-staining as described above. To assess the impact of GJIC on neuronal differentiation cells were grown to 70% confluence before differentiation was induced and incubated with CBX or 1-oct, followed by fixation after 24, 48, 72 and 96h and staining for β(III) tubulin and HuC/D. To ensure constant concentration conditions differentiation media containing CBX or 1-oct was replaced every day. Neurons were quantified

2.4.4 MICROSCOPIC IMAGE ACQUISITION OF FIXED CELLS

Fluorescent images of fixed cells were obtained using a Nikon A1 confocal imaging system with a 40x or 60x/NA 1.4 oil objective (Nikon, Japan). Images were acquired from z-stacks of 30 images with 0.25μm thickness of each section. Finally, maximum projections were created with Nikon NIS Elements software. For protein expression analysis of Triton X-100 extracted Cx43, parameter settings (pinhole size, gain, offset, laser intensity and section thickness) were kept constant for image acquisition of all time points.

For surface rendering of confocal z-stacks, Imaris software (V6.0-6.3, Bitplane, Zurich, Switzerland) was used. Surfaces were created on the basis of fluorescence intensity signals.

2.5 LIVE CELL IMAGING STUDIES

2.5.1 LONG-TERM ANALYSIS OF NEURAL DIFFERENTIATION

Morphological alterations during the differentiation process of ReNcell VM197 were observed by differential interference contrast (DIC)-confocal microscopy. Cells were seeded on 4-well LabTek chambers and differentiation was induced as cell confluence reached 70%. Images were taken every 10min for 4d by use of the Nikon A1 imaging system with a 40x or 60x/NA 1.4 oil objective (Nikon).

2.5.2 FLUORESCENCE RECOVERY AFTER PHOTOBLEACHING

To study GJIC cells were loaded with 20μM CFL for 25min (Sigma), followed by rinsing with Hanks´ balanced salt solution (Invitrogen). Fluorescence recovery after photobleaching

(FRAP) measurements were performed by using a Nikon A1 confocal imaging system equipped with a CO_2 cage incubator (Nikon, Japan) and images were obtained with a 60x/NA 1.4 oil objective. A single cell within a cluster was bleached for 50 sec using a 488nm laser beam and recovery of fluorescence was recorded at 30 sec intervals for a total of 10min. Two unbleached cells in the same visual field were selected as reference to subtract the loss of fluorescence due to photobleaching during the acquisition process. Subsequent analysis of recorded frame sequences was done by Nikon NIS Elements Software (Nikon), including reference correction and background subtraction. The fluorescence intensity of every timepoint was normalized to the average prebleach and postbleach intensity (Abbaci *et al.*, 2007). Halftime of recovery (t_{half}) was calculated by fitting the fluorescence recovery curve to the following exponential equation: $F(t) = A(1 - e^{\wedge}(-\tau * t))$, where t is the time [s] after photobleaching, $F(t)$ is the normalized fluorescence intensity [%], A is the asymptotic value which the final fluorescence intensity reaches. τ is the fitted parameter that is used to calculate the corresponding t_{half} by the equation $\ln(0.5)/\tau$. Graph fitting and t_{half} calculations were performed by use of GraphPadPrism5 software (GraphPad Prism, Inc., San Diego, CA, USA). All FRAP measurements were performed on 30-50 cells per experiment and repeated at least three times.

2.5.3 PLASMID PREPARATION AND TRANSFECTION

The red fluorescent protein-Cx43 (RFP-Cx43) plasmid was originally obtained as a gift from PD Dr. Irina Majoul, Institute of Biology Centre for Structural & Cell Biology in Medicine, University of Lübeck, Germany. Plasmid amplification of RFP-Cx43 and yellow fluorescent protein-α-tubulin (YFP-α-tubulin; Clontech, CA, USA) was carried out using chemically competent *E.coli* One Shot®TOP10 (Invitrogen) according to the manufactures protocol.

For plasmid isolation transformed *E.coli* were centrifuged at 2800g for 15min, resuspended in 600µl dH_2O and lysed by incubation with 600µl 2x lysis buffer (1% SDS (Invitrogen), 0.14M NaOH and 25mM EDTA (all Sigma)) for 5min. Subsequently, lysed bacteria were diluted in 300µl 2M $MgCl_2$ (Applichem) and centrifuged at 27000g for 5min at 4°C. After addition of 300µl 5M sodium acetate (Sigma), lysates were placed on ice for 10min and subjected to centrifugation at 42000g for 10min at 4°C. The supernatant was collected, diluted with 5% polyethylene glycol (stock solution 30%, Roth) and incubated on ice for 2h. Following centrifugation at 20000g for 20min at 4°C, plasmids were resuspended in 0.4ml TE buffer and 1ml 99% ethanol and incubated overnight at -20°C. Afterwards, eluted DNA was pelleted by centrifugation at 20000g for 20min at 4°C, resuspended in 70% ethanol and centrifuged again. Pellet was dried and suspended in 20µl TE-buffer, including 1µg/ml RNAse. DNA

concentration was estimated according to its optical density at 260nm using a UV-spectrophotometer (Thermo scientific, Rochester, NY, USA).

Plasmid transfection into ReNcell VM197 was performed using the electroporation device GenePulser II (BioRad). Cells were washed with pre-warmed HBSS (Invitrogen), trypsinized as described above and centrifuged at 126g for 5min at room temperature. For each transfection $2x10^6$ cells were transferred in separate tubes and centrifuged again, followed by resuspension in 100µl electroporation buffer containing 140mM Na_2PO_4, 5mM KCl, 10mM $MgCl_2$; pH 7.4. Finally, 4µg plasmid were added and incubated for 3min at room temperature. Electroporation was performed in a 0.2cm electroporation cell (Lonza, Basel, Switzerland) with two subsequent electric shocks in a GenePulser II: (1) 1000V for 0.1-0.2msec; GenePulser parameters: U = 0.95kV, capacity = 10µF; (2) 100V for 15-30msec; GenePulser parameters: U = 0.1kV, capacity = 0.5µF x 1000 at "high cap" setting. After electroporation 500µl growth medium were added and cells were seeded on 4 well LabTek chambers for live cell imaging analysis

2.5.4 ANALYSIS OF MICROTUBULE-DEPENDENT CX43 MOVEMENT

For live cell imaging studies of Cx43 movement, cells were double transfected with RFP-tagged Cx43 and YFP-tagged α-tubulin as described in 2.5.3. Cells were seeded on LabTek chambers and cultured for a least 1d to allow plasmid transcription and analysed with a Nikon A1 imaging system as mentioned in 2.5.1. For visualization of RFP-Cx43 and YFP-α-tubulin constructs, fluorophores were excited with a 488nm (YFP) and 594nm (RFP) laser beam. To maintain cell viability cells were kept at 37°C in 5% CO_2 atmosphere. Calculation of particle velocity was done by computational tracking of Cx43 vesicles using the Nikon NIS elements software.

2.6 BIOCHEMISTRY

2.6.1 PURIFICATION OF MEMBRANE-BOUND CX43

Membrane fractions were isolated according to an adapted protocol reported by Hand *et al.*, (2002). All steps of preparations were performed on ice and with pre-chilled buffer solutions. Cells, grown in 2x T75 culture flasks, were rinsed with Tris-EGTA buffer containing 5mM Tris, 1mM EGTA, 1mM PMSF (all Sigma); pH 8.5. The solution was removed and cells were scraped in the presence of Tris-EGTA buffer containing 0.25mM sucrose (Sigma). Cells were collected by centrifugation at 126g for 10min at 4°C and pellet was suspended in 1ml Tris-EGTA buffer supplemented with 8.7% sucrose, 1mM EDTA, 10mM triethanolamine (all Sigma)), 10mM acetic acid (Roth); pH 7.4. Cell lysis was supported by pipetting up and down

using a syringe with a 25G needle and subsequently approved by microscopic evaluation. Lysates of two flasks were pooled together and centrifuged at 126g for 10min at 4°C to separate membrane fraction (supernatant). A three-step gradient was prepared containing 2.6ml of 50% sucrose solution at the bottom step and 4.2ml of 27% sucrose solution at the middle step. Finally, 2ml of the sample were layered on top. The gradient was ultracentrifuged at 192000g for 2h at 4°C and the 27%/50% interface was collected into a new tube and diluted 1:1 with Tris-EGTA buffer. The crude membrane fraction was spun down at 86000g for 30min at 4°C. The pellet was resuspended in 200µl Tris-EGTA buffer and centrifuged again to remove 8.7% sucrose, followed by suspension in 100µl Tris-EGTA. Samples were lyophilized for 4h using a vacuum centrifuge and pellet was suspended in 50µl dH$_2$O. Protein concentration was estimated using Pierce BCA Protein Assay Kit (Thermo Scientific). Finally, samples were diluted 1:4 in 4x SDS buffer containing 0.25M Tris (Sigma), 40% glycerin (AppliChem, Darmstadt, Germany), 20% β-mercaptoethanol (Bio-Rad, Munich, Germany), 10% sodium dodecyl sulphate (SDS) (Invitrogen), 0.01% bromphenol blue sodium salt (Serva, Heidelberg, German); pH 6.8) and boiled for 5min at 98°C.

2.6.2 PREPARATION OF TOTAL CELL LYSATES AND DETERGENT EXTRACTION OF CX43

For preparation of total lysates, cells cultured in 6-well plates were washed twice with PBS and lysed in 200µl 2xSDS buffer (see 2.6.1), followed by sonication and boiling for 5min at 98°C.

For Triton X-100 extraction of Cx43, cells were cultured in T75 culture flasks. Preparation of lysates was carried out on ice and with pre-chilled buffer solutions. Cells were rinsed with PBS, followed by scraping and subsequent centrifugation at 126g for 10min at 4°C. Cell disruption was induced by suspension of the cell pellet with 500µl lysis buffer containing 150mM NaCl (AppliChem); 1mM Na$_3$O$_4$V (Acros/ThermoFisher Scientific, Rochester, USA); 1mM EDTA, 1mM EGTA, 1mM NaF, 1% Triton X-100, 1mM PMSF, 1µg/ml Pepstatin, 1µg/ml Aprotinin, 1µg/ml Leupeptin (all Sigma). Prior subsequent centrifugation, 166µl sample was retained as total cell lysate, whereas the remaining lysate was subjected to ultracentrifugation at 100000g for 30min at 4°C. Triton X-100 soluble fraction (supernatant) was collected and Triton X-100 insoluble pellet was suspended in 100µl lysis buffer. Samples were lyophilized and suspended in 50µl dH$_2$O and Protein concentration was estimated using Pierce BCA Protein Assay Kit (Thermo Scientific). For sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), samples were diluted 1:4 in 4x SDS sample buffer.

2.6.3 SDS-PAGE AND IMMUNOASSAY

Proteins were separated by SDS-gel electrophoresis using 10% polyacrylamide gel (separation gel: 13.33ml 30% acrylamide; 10ml 4x separation gel buffer; 16.47 ml dH$_2$O; 140µl TEMED (Sigma); 64µl 12.5% ammonium peroxide sulphate in dH$_2$O, Acros, Nidderau, Germany) and 4% stacking gel (0.575ml 30% acrylamide; 1.25ml 4x loading gel buffer; 3.109ml dH$_2$O; 46µl TEMED; 20µl 12.5% ammonium peroxide sulphate). A gel electrophoresis chamber (Mighty Small 200 Hoefer electrophoresis device; GE Healthcare, Solingen, Germany) was filled with running buffer (10x: 0.25mM Tris; 1.92M glycine; 1% SDS (Sigma); in dH$_2$O.; pH 8.2), gels were loaded with specific samples and constant 20mA for each gel was applied for 60-70min.

After separation of lysates by SDS-PAGE, proteins were transferred onto nitrocellulose membrane by tank blotting and detected using protein specific antibodies. Acrylamide gel, NC membrane, filter papers and fibre pads were equilibrated in blot buffer containing 200mM glycine; 25mM Tris and 20% methanol (AppliChem; in dH$_2$O; pH 8.6) for 20min and assembled according to the manufacturer´s instructions with the Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). Proteins were transferred onto NC-membrane at constant 30V overnight. Successful transfer was controlled by staining with Ponceau S solution (3% trichloroacetic acid, Merck; 0.1% Ponceau S, Sigma) for 3min , followed by washing with dH$_2$O to remove excessive Ponceau S. Membranes were treated for 1 hour with TTBS (10x: 100mM Tris; 1.49mM NaCl; 1% Tween® 20, Sigma; in dH$_2$O; pH 7.4) containing 5% non-fat dry milk (AMPI, New Ulm, USA) in order to block unspecific binding sites, followed by washing with TTBS 3 times for 5min. Subsequently, membranes were incubated with primary antibodies (see Table 2) diluted in TTBS including 5% non-fat dry milk for 2h at room temperature. After rinsing with TTBS 3 times, membranes were incubated with horseradish-conjugated secondary antibody (see Table 2) diluted in TTBS containing 2.5% non-fat dry milk and washed 3 times with TTBS and TBS. Bound antibodies were detected with ECL western blot detection reagent (GE Healthcare, Freiburg, Germany). Finally, membranes were exposed to light sensitive films (GE Healthcare), which were developed (Kodak Professional D-19 Developer, Sigma-Aldrich, Hamburg, Germany and fixative, Filmfabrik Wolfen, Germany) and scanned for quantification. The quantification of proteins signals was performed by ImageJ software (NIH/USA). Whole lanes were selected and grey levels were measured automatically. All samples were normalized to the loading control α-tubulin or actin. To illustrate the presence of Cx43, Cx31 and Cx59 in ReNcell VM197 (Figure 8B), the membrane was simultaneously incubated with three different Cx antibodies. However, antibody specificity was tested before by separate incubation of the membrane to exclude cross reactivity.

antibody	host	dilution	supplier and Cat.nr.
primary			
anti-α-tubulin	mouse	1:400000	Sigma, T5168
anti-β-actin	mouse	1:250000	Sigma, A5441
anti-Cx31	rabbit	1:400	Sigma, SAB1100240
anti-Cx43	rabbit	1:8000	Abcam, Cambridge, UK, ab11370
anti-Cx59	rabbit	1:100	Abcam, ab86414
anti-Cx36	goat	1:200	Santa Cruz, Heidelberg, Germany, sc-14904
anti-Cx45	goat	1:200	Santa Cruz, sc-7679
secondary			
anti-mouse IgG, horseradish peroxidase (HRP)	sheep	1:10000	GE Healthcare, Munich, Germany, Cat. NA931V
anti-rabbit IgG, HRP	goat	1:80000	Sigma, A9169
anti-goat IgG, HRP	donkey	1:50000	Santa Cruz, sc-2020

Table 2 Antibodies used for immunoblotting.

2.7 STATISTICAL ANALYSIS

All results are shown as mean ± standard error of mean (SEM) of at least three independent experiments. Statistical significance between two parameters was estimated with student´s *t*-test. Time-dependent protein expression patterns and FRAP data, including timeline analysis and calculated halftimes, were analysed by one-way ANOVA. Statistical significance between fluorescence recovery curves was calculated by two-way ANOVA. *Post-hoc* test was performed using Bonferroni´s test for multiple comparisons between proliferating and differentiated cells or untreated and treated cells. For analysing statistical significance of neuronal differentiation, *post-hoc* test was carried out for multiple comparisons between 1d differentiated cells and 2-5d differentiated cells. The probability levels considered as statistically significant were $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$. Calculations were made with GraphPadPrism5 software (GraphPad Prism, Inc.) or MS Excel (Microsoft, Redmond, WA, USA).

3 RESULTS

3.1 CHARACTERIZATION OF ReNcell VM197

In the present study we determine a role for GJIC in proliferation and differentiation of human neural progenitors using the immortalized human progenitor cell line ReNcell VM197. This cell line is well characterized and has been used in several studies where it was shown to be a simple and accepted model to investigate different aspects of neural differentiation *in vitro* (Lange *et al.*, 2011; Mazemondet *et al.*, 2011). ReNcell VM197 is able to readily differentiate into neurons and glial cells within 3d after growth factor removal, a fact that allows long term analysis of different stages of differentiation.
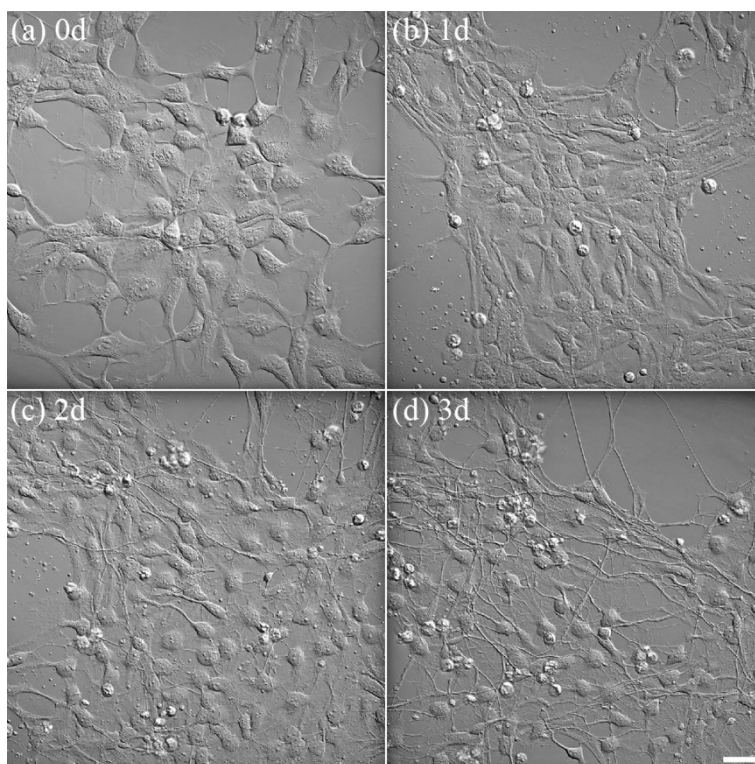

Figure 3 Neural differentiation of ReNcell VM197. Long-term DIC-microscopy of differentiating ReNcell VM197 demonstrated strong morphological changes after induction of differentiation. While proliferating progenitors showed a round cellular shape (a), growth factor removal induced the formation of cell protrusions within 1d (b). Progressive cell branching was observed after 2d (c), which resulted in the formation of a dense neural meshwork after 3d (d), Bar 50μm.

The differentiation process is accompanied by strong morphological changes of the cellular architecture that finally results in the establishment of a dense neural network. Long-term DIC-confocal microscopy of ReNcell VM197 undergoing differentiation demonstrated the alterations in cell morphology (Figure 3). While proliferating progenitors were characterized by a plump and round cellular shape, induction of differentiation led to the development of long cell protrusions and cell branches within 1d (Figure 3a-b). Proceeding differentiation finally resulted in the formation of a stable meshwork after 3-4d.
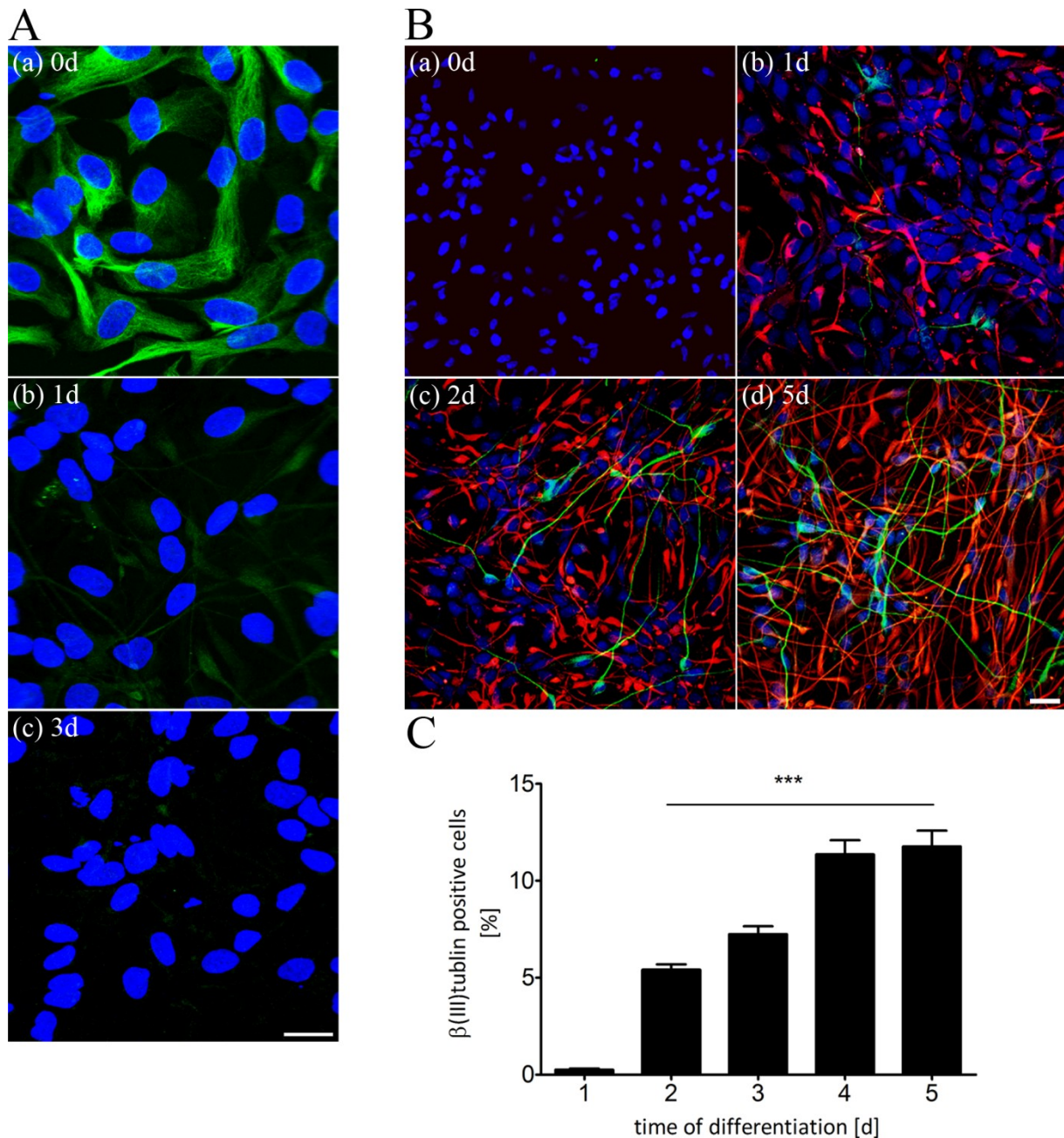
Figure 4 Altered expression of progenitor and neural differentiation markers during ReNcell VM197 development. (**A**) Proliferating ReNcell VM197 progenitors demonstrated strong expression of the neural precursor marker nestin (0d). However, as cells were induced to differentiate, expression of nestin decreased (1d-3d). Cells were labelled with anti-nestin antibody. Nuclei were stained with Hoechst, Bar 20μm (**B**) Rapid Neuronal and glial cell differentiation in ReNcell VM197. Proliferating progenitors showed no differentiated phenotypes (0d). Induction of differentiation by growth factor removal led to the formation of neurons and glial cells beginning after 1d (b). An increasing number of neurons and glial cells was observed after 2d and a dense neural network was established after 5d of differentiation (c, d). (**C**) Microscopic quantification revealed an increase of the neuron number over time, reaching a maximum value of 10-12% at the end of differentiation after 4-5d. Cells were stained for β(III) tubulin (green) and GFAP (red). Nuclei were stained with Hoechst, Bar 50μm. Graph represents data of 3 independent experiments, shown as mean±SEM. Time course of neuronal development was analysed by one way ANOVA $P<0.0001$, followed by Bonferroni *post-hoc* test. Bar 20μm

The expression of nestin, a class VI filament protein, is a common feature of neural progenitors, indicating their undifferentiated and self-renewal state. Proliferating ReNcell VM197 demonstrated strong nestin expression, which was progressively decreased as differentiation proceeded (Figure 4A). After differentiation, nestin expression was replaced by the expression of glial and neuronal-specific proteins. Therefore, cells were immunolabeled with anti-glial fibrillary acidic protein (GFAP), a glial specific marker and anti-β(III) tubulin to reveal neuronal phenotypes. As shown in Figure 4B, proliferating progenitors were characterized by the absence of neural markers. After the removal of growth factors, the majority of progenitors differentiated into glial cells, with branches forming a network soon after 1d of differentiation (Figure 4B, b). Moreover, neuronal cells were found that expand above the glial carpet and whose number increased as differentiation proceeded (Figure 4B, b-d). Microscopic quantification revealed a rising number of neurons during differentiation, reaching a maximum value of 10-12% after 4d, which indicates the completion of neuronal development (Figure 4C).

3.2 GJIC IN ReNcell VM197

3.2.1 GJIC MEASUREMENT BY FRAP MICROSCOPY

To investigate the extent of GJIC in neural progenitors, we performed FRAP microscopy using CFL, a tracer that is often used to analyse functionality of GJs. Once inside the cell, the non-fluorescent form of CFL is hydrolysed by intercellular esterases to a membrane-impermeable fluorescent anion that is retained by the cell and can only diffuse via GJs. A prerequisite of
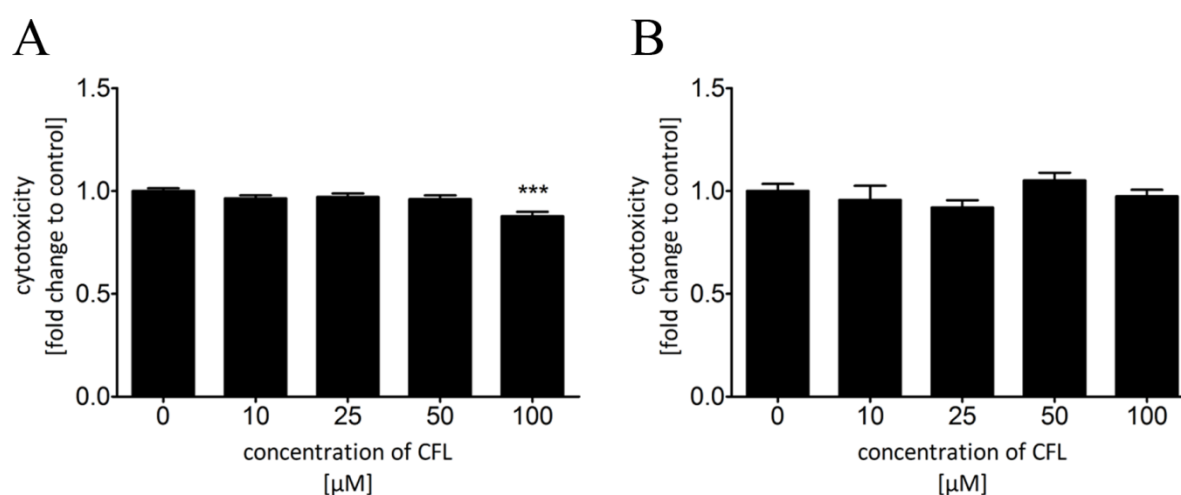
Figure 5 Cytotoxicity of CFL in proliferating and differentiated ReNcell VM197. Proliferating or 3d differentiated cells were loaded with different concentrations of CFL for 5h and subjected to MTT assay. (**A**) In proliferating progenitors CFL exerts its cytotoxic effect at a concentration of 100μM. (**B**) No cytotoxic effects of CFL on cell viability were detected in differentiated cells. Graphs represent data of 3 independent experiments, shown as mean±SEM. Statistical differences were calculated by Student´s t-test, ***$P<0.001$.

fluorescent dyes in live cell imaging is the absence of cytotoxic effects that cause cell damage and alter cellular physiology. To avoid undesired side effects of the fluorescent probe and to ensure accurate FRAP measurements, we initially tested cell toxicity of CFL by a calorimetric MTT assay. As cell sensitivity can change during differentiation, both proliferating and 3d differentiated cells were loaded with different concentrations of the fluorescent probe for 5h. CFL was found to exhibit its cytotoxic effect on proliferating cells at a concentration of 100µM (Figure 5A). In contrast, no effect on cell viability was detected in differentiated cells (Figure 5B). Based on these data, all FRAP experiments were performed with CFL at a concentration of 20µM to exclude cytotoxic effects.

As demonstrated in Figure 6A, a selected cell within a cluster of cells was photobleached by an argon laser and diffusion-dependent fluorescence recovery was recorded for 10min.
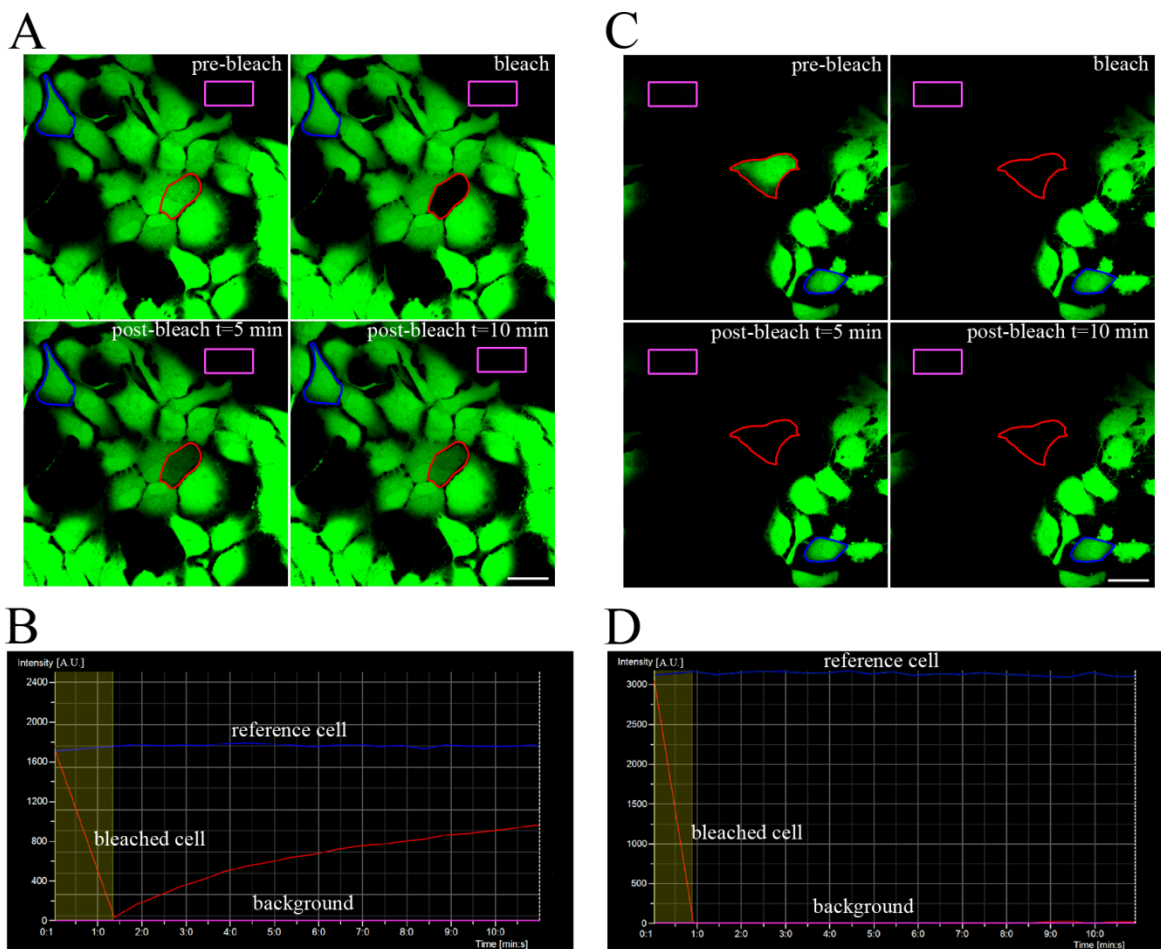
Figure 6 FRAP microscopy as a tool to analyse GJIC. (**A**) Cells were loaded with CFL, followed by photobleaching of a selected cell (red frame) within a cluster of cells. Fluorescence recovery was recorded for 10min. (**B**) Typical course of fluorescence recovery of a bleached cell under control conditions due to GJ-mediated CFL diffusion from adjacent cells. Background intensity (purple frame) and intensity within a reference cell (blue frame) were recorded to encompass bleaching effects caused by the acquisition process. (**C, D**) No fluorescence recovery was observed in bleached single cells, indicating that recovery was dependent on dye diffusion from neighboring cells, Bar 20µm.

Background fluorescence (purple frame) and fluorescence intensity of a reference cell (blue frame) were measured to include the loss of fluorescence induced by the acquisition process (Figure 6A). Since the hydrolysed form of CFL might only diffuse across GJs, the time course of fluorescence recovery, shown in Figure 6B, represents a distinct feature for the extent of GJIC of a cell population. Bleaching of a single cell without any adjacent cells did not result in fluorescence recovery (Figure 6C, D). These data clearly demonstrate that fluorescence recovery measured by FRAP was exclusively arisen from dye diffusion of neighbouring cells into the bleached area. Thus, this microscopic technique is an appropriate tool to study the intercellular communication via GJs in neural progenitors.

3.2.2 GJIC ALTERS DURING DIFFERENTIATION OF ReNcell VM197

Next, we carried out a detailed long-term temporal analysis of GJIC during the differentiation process. FRAP microscopy analysis revealed strong dye coupling when cells were kept in a proliferative state in the presence of EGF and bFGF (Figure 7A). However, induction of differentiation by withdrawal of growth factors led to a profound decrease of GJIC within the first 24h (Figure 7A). This early stage of differentiation is characterized by the exit from cell cycle and crucial for initiating cell fate commitment (Galderisi *et al.*, 2003). Proceeding differentiation characterized by glial cell formation and neuronal maturation was accompanied by an increase of GJIC. Moreover, dye coupling was even 1.5-fold stronger than observed in proliferating progenitors (Figure 7A), indicating a higher degree of intercellular communication. The comparison of calculated halftimes by analysis of fluorescence recovery curves confirmed the different extent of cell coupling in ReNcell VM197 (Figure 7B, C). Compared to proliferating cells, the speed of fluorescence recovery declined by more than 50% after differentiation was initialized. However, proceeding differentiation resulted in a stronger cell-cell coupling that increased the flow through of CFL into the bleached cell. This different, time-dependent modulation of GJIC implies its importance for proper differentiation in neural progenitor cells. The observed modulation of GJIC directly reflects the change of GJ channel activity. This would encompass either a switch of selective channel permeability or the rearrangement of GJ plaques by regulating Cx43 gene expression or degradation processes (Cottrell *et al.*, 2002; Rackauskas *et al.*, 2010).
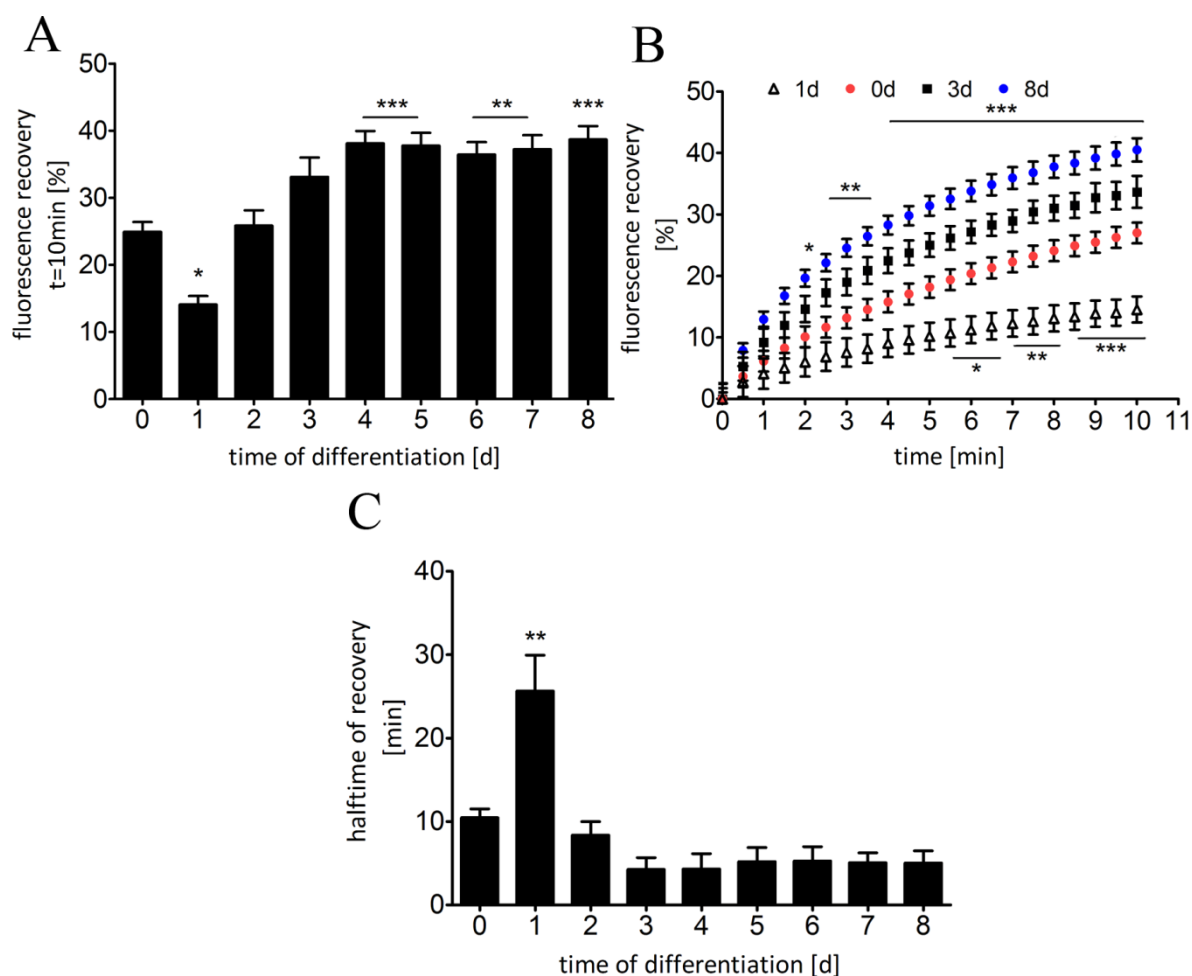
Figure 7 Alterations in cell-cell coupling during proliferation and differentiation. (**A**) Dye coupling was measured by FRAP. GJIC was strong in proliferating neural progenitors, but decreased after differentiation was induced, followed by an increase in the later stage of differentiation. Graph represents fluorescence recovery values after 10min. (**B**) Average fluorescence recovery curves of proliferating cells (0d) and differentiated progenitors (1d, 3d, 8d) confirm the different extent of GJIC. (**C**) Halftimes of recovery were calculated by fitting datasets to an exponential equation, indicating the speed of recovery during differentiation. Graphs represent data of ≥3 independent experiments, shown as mean±SEM. Time course of GJIC and halftimes of recovery were analysed by one-way ANOVA $P<0.0001$. Differences between fluorescence recovery curves were estimated using two-way ANOVA $P<0.0001$. Multiple comparisons were performed using Bonferroni *post-hoc* test. Halftimes of recovery were calculated by data fitting as described in material and methods.

3.3 EXPRESSION OF CXS DURING DIFFERENTIATION OF ReNcell VM197

3.3.1 SCREENING FOR DIFFERENT CX ISOTYPES

As gap junctional coupling requires the presence of Cxs, we wanted to confirm the kinetic profile of GJIC at the molecular level by analysing Cx expression throughout the whole process of differentiation. Due to several reports, showing the presence of different Cx isoforms in embryonic stem cells and human neural tissue (Nadarajah *et al.*, 1997; Söhl & Willecke, 2004; Huettner *et al.*, 2006; Wörsdörfer *et al.*, 2008), we initially screened for the
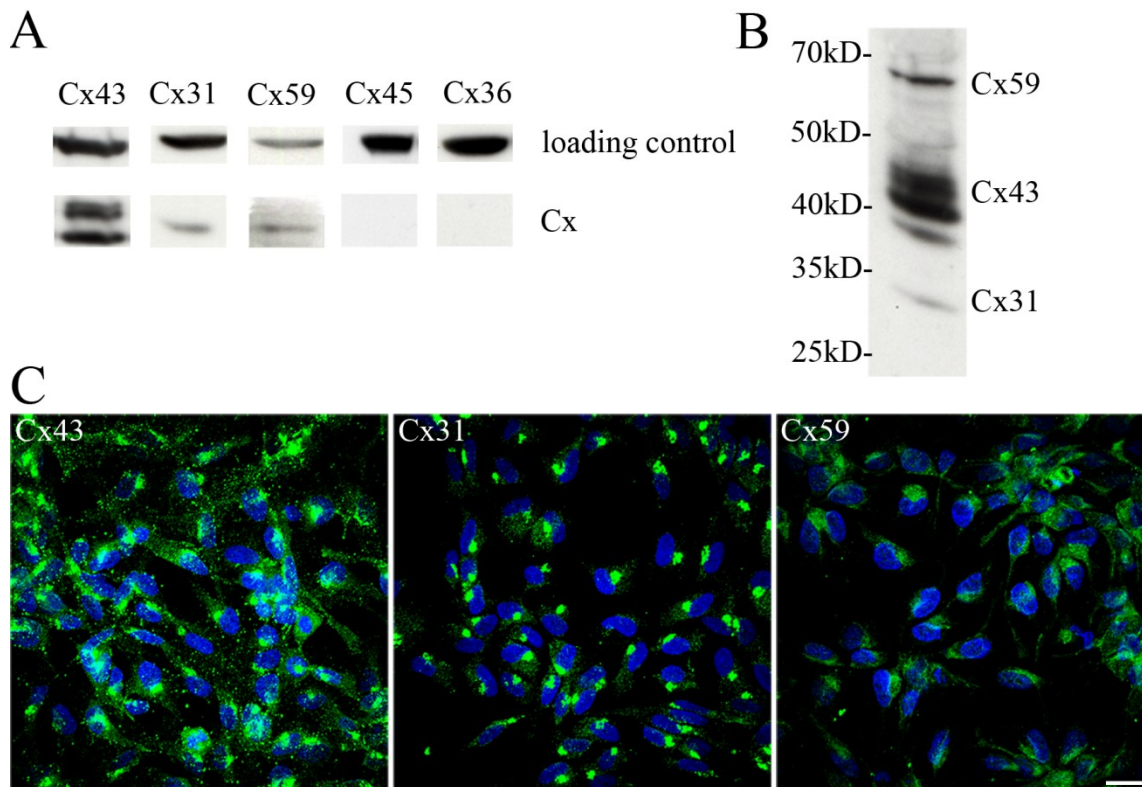
Figure 8 Expression of Cxs in proliferating progenitors. (**A**) Screening for several Cx isotypes revealed the expression of Cx43, Cx31 and Cx59. (**B**) Western blot image, showing the presence of Cxs in the same lysate. (**C**) Confocal microscopy of Cx-labeled cells demonstrated high levels of Cx43 in proliferating progenitors. Cells were immunolabeled with Cx antibodies and nuclei were stained with Hoechst, Bar 20µm.

expression of Cx31, Cx36, Cx43, Cx45 and Cx59 in proliferating ReNcell VM197 progenitors. Western blot data give evidence for the presence of only three different Cx isotypes, namely Cx43, Cx31 and Cx59 (Figure 8A, B). Note, three phosphorylation states were found for Cx43, as indicated by three different bands. As shown in Figure 8A, most of Cx43 is unphosphorylated, while a smaller amount is present in the P1- and P2-form. Cell immunolabelling with different antibodies confirmed the presence of these three Cxs and showed high levels of Cx43 expression, compared to Cx31 and Cx59 (Figure 8C). Based on these data, detailed time-dependent gene expression analysis of whole cell lysates was performed, showing specific expression pattern for each Cx isotype during the differentiation process.

3.3.2 CXS ARE DIFFERENTIALLY EXPRESSED DURING NEURAL DIFFERENTIATION

Cx43. Expression of Cx43 was monitored for 10d and we observed a dynamic change of Cx43 expression during proliferation and differentiation. Proliferating cells showed a high expression level of Cx43, but it was down-regulated by 80% after the induction of

differentiation. However, expression of Cx43 showed a 2.5-fold re-increase after 5d when neural differentiation was completed (Figure 9A). The dynamic expression pattern of Cx43 was visualized by immunofluorescence microscopy (Figure 9B). Proliferating cells that were labeled with Cx43 antibody demonstrated strong fluorescence intensity (Figure 9B, a) that progressively declined within 4d of differentiation (Figure 9B, b-c), followed by a marked increase (Figure 9B, e-f).
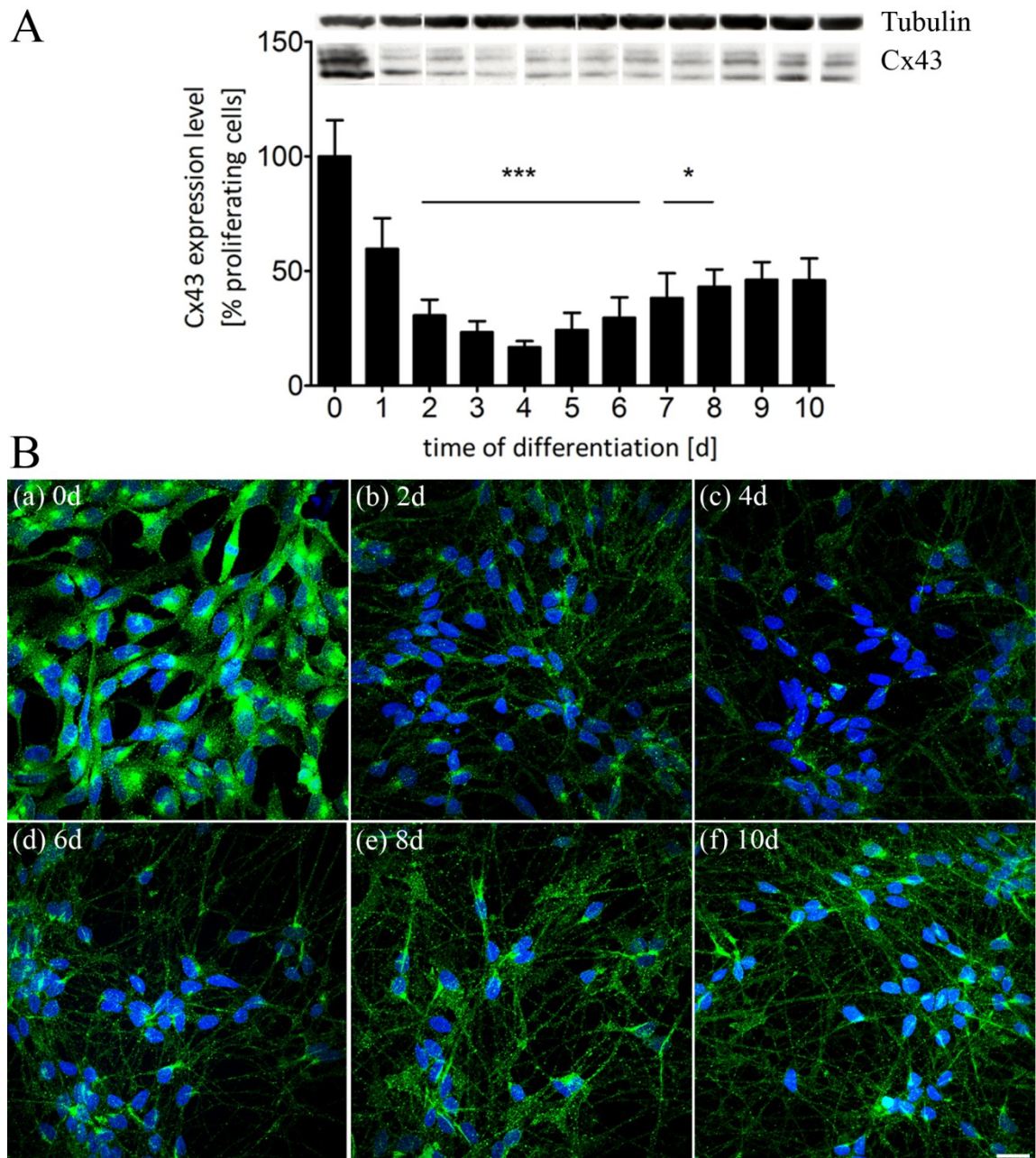
Figure 9 Expression of Cx43 during proliferation and differentiation. (**A**) Detailed time-dependent analysis showed progressive down-regulation of Cx43 protein during the first days of differentiation. However, gene expression of Cx43 increased with proceeding differentiation after 4-5d. (**B**) Confocal microscopy of proliferating progenitors and differentiated cells confirmed western blot results and indicated cytoplasmic and membranous localization of Cx43 in ReNcell VM197, Bar 20μm. Graph represents data of 4 independent experiments, shown as mean±SEM, one-way ANOVA, $P<0.0001$, followed by Bonferroni *post-hoc* test.

Cx31. A similar dynamic course of gene expression was detected for Cx31. The level of Cx31 decreased by 40% within the first 2d after growth factor removal. Indeed, this reduction of gene expression was less pronounced compared to Cx43 (Figure 10A). Proceeding differentiation of neural progenitors induced a consistent up-regulation of Cx31, which resulted in a 3.5-fold increase of the protein level in the late stage of differentiation. These alterations in the expression profile were supported by confocal microscopy of Cx31-labeled cells. As demonstrated in Figure 10B the fluorescence signal of Cx31 was reduced when differentiation was initialized (Figure 10, a-c). However, fluorescence intensity progressively increased after 6d.

Cx59. Compared to western blot data obtained for Cx43 and Cx31, Cx59 protein expression showed no pronounced alterations during the differentiation process (Figure 11A). Although these data give evidence for a wave-like expression pattern, we did not detect significant changes between tested timepoints. In contrast to these results, microscopic analysis demonstrated a slight decrease of Cx59 after growth factor removal that persisted up to 6d of differentiation (Figure 11B, a-d). As shown for Cx31 and Cx43, fluorescence intensity of Cx59 increased in the late stage of differentiation.

Comparison of the time-dependent Cx expression patterns and the dynamic behaviour of gap junctional coupling during differentiation revealed that protein levels of Cx43 and Cx31 changed in way that is similar to the GJIC profile obtained by FRAP microscopy (Figure 6Figure 9Figure 10). However, the gene expression kinetic did not exactly reflect the changes in cell-cell coupling that occur during the differentiation process. The observed decrease of Cx43 level was more dramatic as the reduction of GJIC, whereas up-regulation of Cx43 in the late stage of differentiation was less pronounced than the increase of gap junctional communication. Furthermore, the alteration of the Cx43 level showed a 3d delay compared to the GJIC profile. A 1d delay was detected for Cx31, which was down-regulated by 40% within the first 2d of differentiation (Figure 10). The subsequent, progressive increase of Cx31 was stronger, but slower compared to the GJIC profile. Indeed, this 1d delay of the Cx31 expression pattern was shorter than observed for Cx43. While Cx31 and Cx43 were found to share similar expression profiles, no significant changes of the protein level were detected for Cx59. However, the temporal correlation of both the expression of Cx43 and Cx31 and GJIC might suggest a modulating function of these Cxs for GJ coupling during neural differentiation.
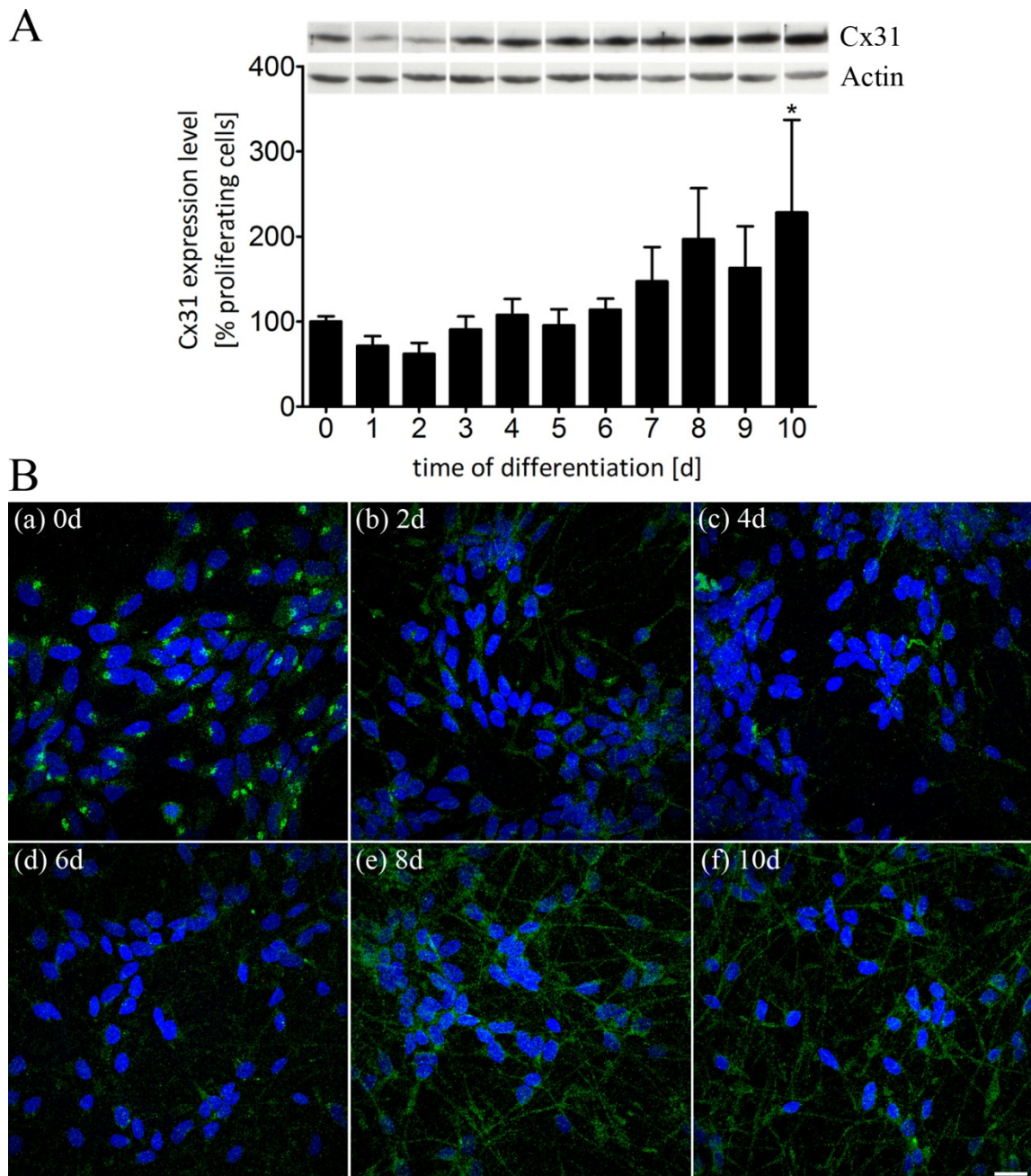
Figure 10 Expression of Cx31 during proliferation and differentiation. (**A**) Time-dependent western blot analysis demonstrated a down-regulation of Cx31 during the first 2d of differentiation. As differentiation proceeded, Cx31 protein level increased, reaching a 4-fold up-regulation after 10d. (**B**) Confocal microscopy of proliferating progenitors and differentiated cells showed similar alteration in protein expression as revealed by western blotting, Bar 20μm. Graph represents data of 3 independent experiments, shown as mean±SEM, one-way ANOVA, *P*<0.01, followed by Bonferroni *post-hoc* test.
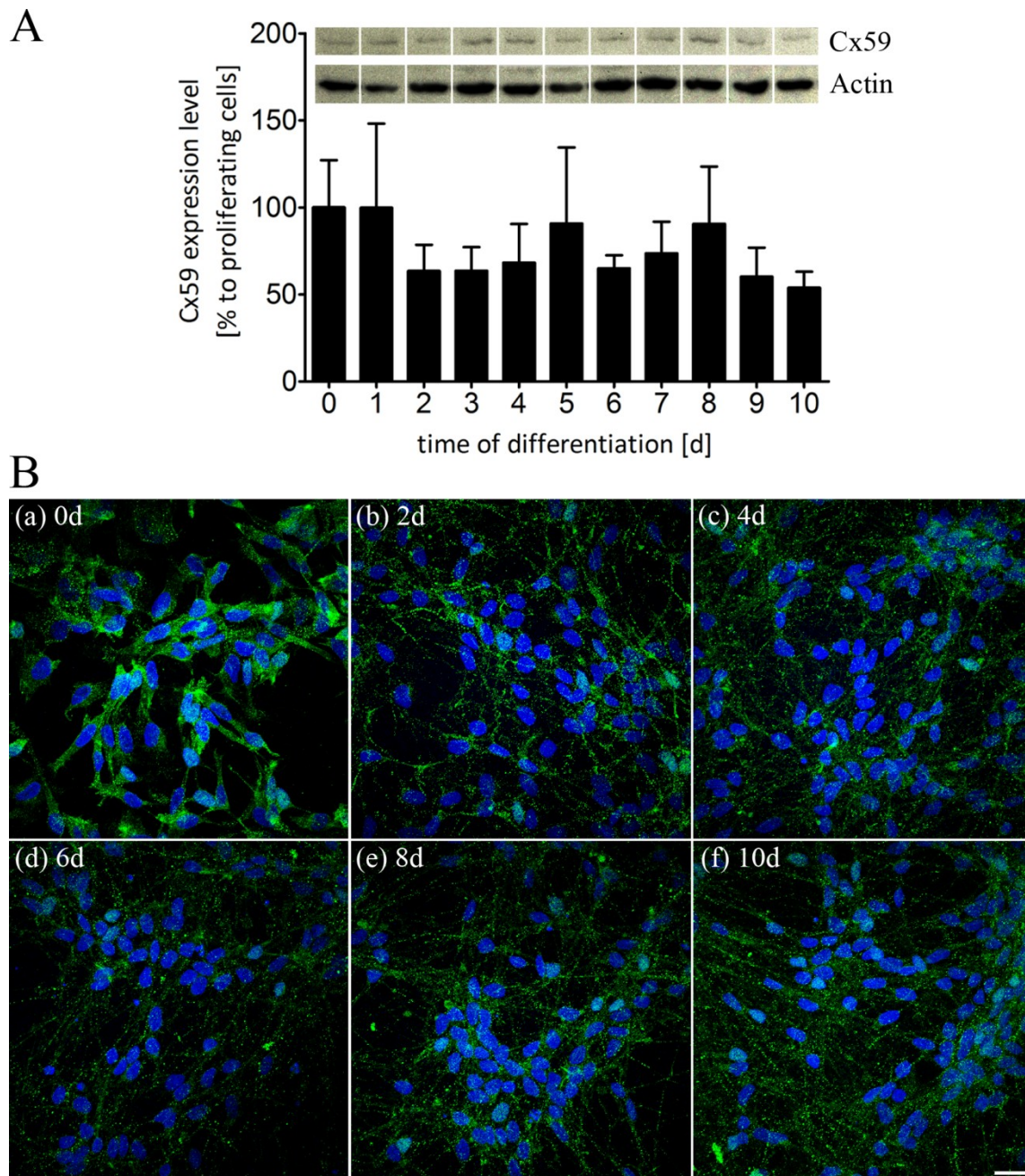
Figure 11 Expression of Cx59 during proliferation and differentiation. (**A**) Time-dependent analysis of full cell lysates by western blotting showed no significant changes in Cx59 expression during the differentiation of neural progenitors. (**B**) However, microscopic observations of proliferating and differentiated cells suggested a down-regulation of Cx59 after induction of differentiation and subsequent increase of Cx59 protein level in the late stage of differentiation, Bar 20μm. Graph represents data of 3 independent experiments, shown as mean±SEM, one-way ANOVA, $P>0.05$.

3.3.3   CX EXPRESSION IN NEURONS AND GLIAL CELLS

Specific Cx isoforms are differentially expressed during the development in different cell types. As ReNcell VM197 can differentiate into neurons and glial cells, we investigated the presence of Cx proteins in non-neuronal and neuronal cells. Therefore, cells were differentiated for 4d and stained for GFAP and β(III) tubulin to distinguish neurons and glial phenotypes. Confocal images, presented in Figure 12A, show that fluorescence signals of all Cxs were obtained in glial cells throughout the whole cell body, including cell protrusions. Neurons were also found to express all three Cx isotypes (Figure 12B), indicating that Cx expression was not restricted to certain cell types during differentiation.

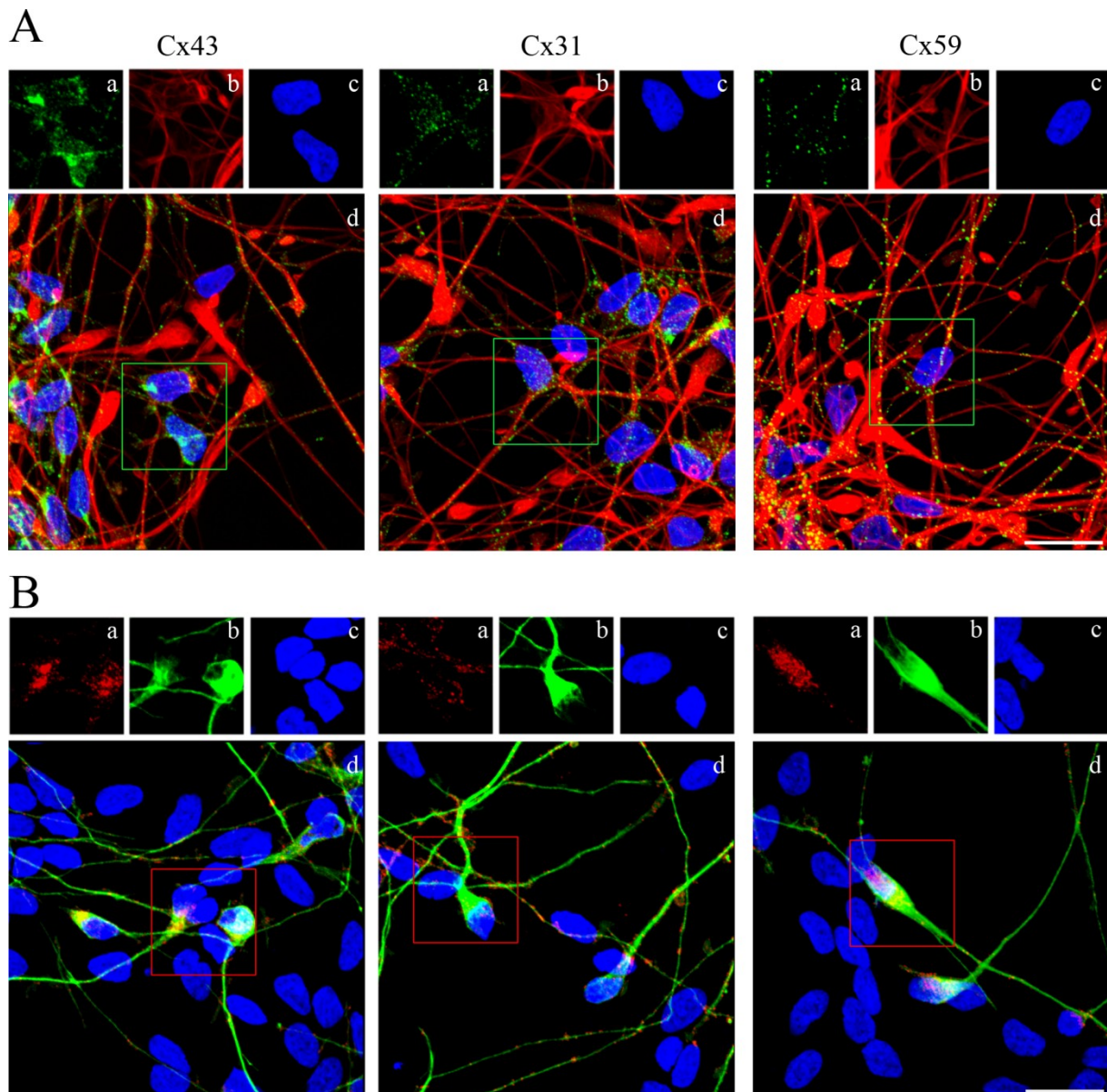**Figure 12 Expression of Cxs in glial cells and neurons.** (**A**) Double labelling of cells with glial-specific anti-GFAP (red) and anti-Cx (green) demonstrated the expression of Cx43, Cx31 and Cx59 in non-neuronal cells. (**B**) Cxs were also found in neurons as shown by Cx (red) and neuronal β(III) tubulin labelling (green). (a) Cx, (b) GFAP or β(III) tubulin, (c) DNA, (d) merged, Bar 20µm.

3.3.4   INTRACELLULAR AND SUBCELLULAR DISTRIBUTION OF CXS

3.3.4.1   CYTOPLASMIC DISTRIBUTION OF CXS

As Cx proteins are not exclusively involved in GJ formation and therefore not only located in the cell membrane, the analysis of whole cell lysates (Figure 9-11) might not exactly represent the amount of Cxs within GJ plaques. Thus, we focused on the intra- and subcellular distribution of expressed Cxs in ReNcell VM197.

Cx43. Cx43 was distributed throughout the whole cell in both proliferating and differentiated ReNcell VM197 progenitors (Figure 9). Notably, we observed a strong accumulation in a region near the nucleus that was particularly prominent in proliferating cells. Based on several studies reporting an involvement of the Golgi-network in Cx life cycle, we assumed the Golgi apparatus as a subcellular store for Cx43 (Das Sarma *et al.*, 2001; Kennedy *et al.*, 2003). This was confirmed by incubation with Golgi-GFP that targets the human Golgi-resident enzyme N-acetylgalactosaminyltransferase 2. Confocal images presented in Figure 13A show that both Cx43 and the Golgi network colocalized to the same perinuclear region of the cell (Figure 13A, proliferating cells). As differentiation was induced the number of cells containing strong intracellular accumulations of Cx43 drastically declined (Figure 13A, 3d). However, a recharge of these intracellular Golgi-stores was observed in the later stage of differentiation when Cx43 is up-regulated (Figure 13A, 8d). This indicates that an intracellular pool of Cx43 is retained in the Golgi-network, especially in proliferating progenitors, which is depleted when differentiation is induced.

Cx31. Microscopic observations of Cx31-immunolablled cells showed a similar intracellular distribution pattern as observed for Cx43 (Figure 13B). However, while most of Cx31 is accumulated in the Golgi-apparatus, only a small portion of intracellular Cx31 is distributed throughout the cytoplasm (Figure 13B, proliferating cells). Induction of differentiation by removal of growth factors resulted in a depletion of the Golgi-stores that were refilled as differentiation proceeded (Figure 13B, 3d, 8d).

Cx59. In contrast to Cx43 and Cx31, which were mainly located in the Golgi network, Cx59 demonstrated a uniform, ubiquitous distribution pattern (Figure 13C). Neither in proliferating progenitors nor in differentiated cells Cx59 proteins were found to accumulate and colocalize with the Golgi-apparatus.
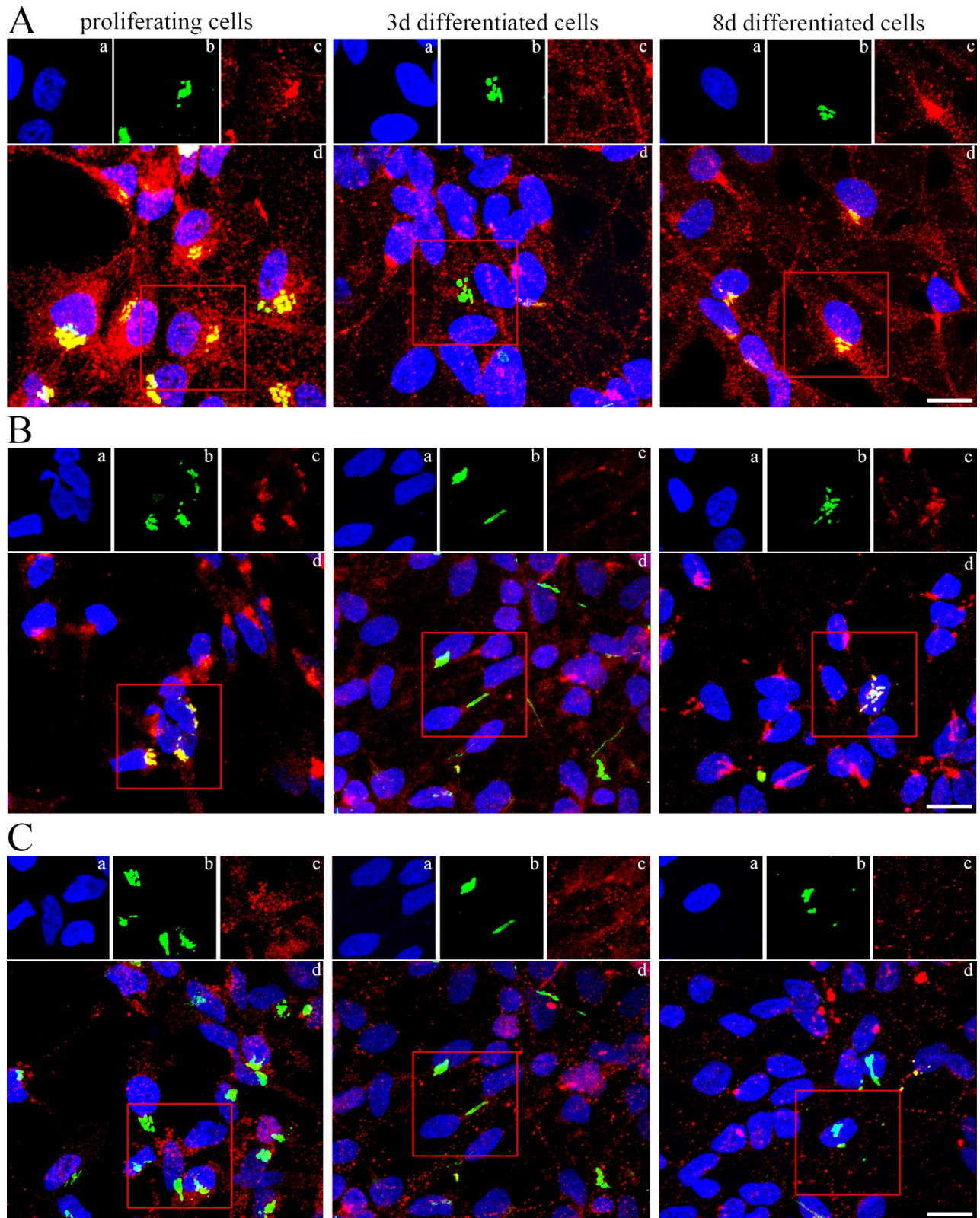
Figure 13 Intracellular localization of Cxs in proliferating and differentiated cells. (**A**) In proliferating progenitor cells Cx43 was found to be located in the Golgi-apparatus, shown by colocalization of Cx43 and Golgi-GFP. A weak signal of Cx43 in the Golgi area was detected in early differentiated cells (3d), which re-increased as differentiation proceeded (8d). (**B**) Cx31 also accumulates in the Golgi network in proliferating cells. Accumulation disappeared in the early stage of differentiation (3d) and re-established with continuing differentiation (8d). (**C**) Cx59 showed ubiquitous distribution pattern with no accumulations in proliferating and differentiated cells. Cells were incubated with Golgi-GFP (b, green), fixed and co-stained with Cx antibodies (c, red) and Hoechst (a), Bar 20μm.

To further define the subcellular localization of Cxs, proliferating progenitors were treated with Brefeldin A (BFA) for 30min prior immunolabelling to stimulate disruption of the Golgi-network (Figure 14). The bright, perinuclear fluorescence of Cx43 and Cx31 (arrows) disappeared, resulting in a diffuse staining pattern (asterisks) after BFA incubation due to dispersion of Cx molecules throughout the cytoplasm. As Cx59 did not accumulate in a perinuclear compartment, administration of BFA only induced a decrease of fluorescence intensity. The effect of BFA on the Golgi-Network was visualized by Golgi-GFP transfection, showing disassembly of GFP-tagged Golgi stacks after BFA incubation (inserted images).

These results support our finding that most of the intracellular Cx43 and Cx31 were located in the Golgi apparatus, which suggest that both Cxs might be trafficking through the secretory pathway and probably assemble in the trans-Golgi-network.
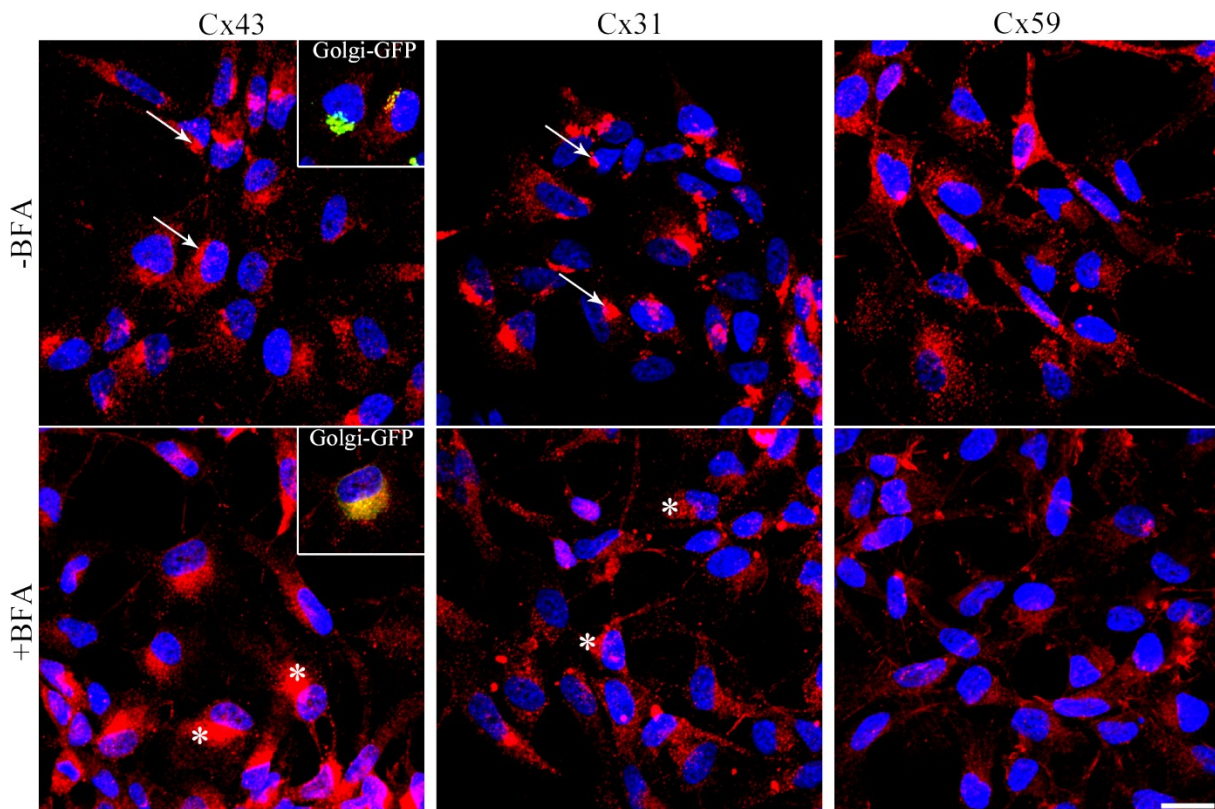
Figure 14 Effect of Golgi disruption on Cx distribution. While control cells showed strong accumulations of Cx43 and Cx31 in the Golgi apparatus (arrows), treatment with the Golgi-disrupting agent BFA caused strong dispersion of Cx43 and Cx31 throughout the cytoplasm (asterisks). Minor changes in cellular distribution were observed for Cx59. BFA only induced a decrease in fluorescence intensity. Inserted images show Golgi-GFP labelled cell when untreated or treated with BFA. Cells were incubated with Golgi-GFP (green), fixed and co-stained with Cx43 antibody (red) and Hoechst (a), Bar 20µm.

3.3.4.2 INVOLVEMENT OF CXS IN GJ AND HEMICHANNEL FORMATION

As shown by FRAP microscopy, neural progenitor cells were able to communicate by functional GJs and demonstrated expression of three Cx isotypes (Figure 7, 8). Therefore, we clarified the role of Cxs in GJ formation. Musil and Goodenough showed that most of the Cx already assembled in GJs is phosphorylated and resistant to Triton-X solubilization (Musil and Goodenough, 1991). We performed a Triton X-100 in situ extraction assay using 0.3% Triton X-100 in the fixative to remove unphosphorylated, cytoplasmic Cx and to clearly delineate membrane-bound Cx. As determined by confocal microscopy, bright spots of Cx43 were predominantly located in regions of cell-cell contacts, indicating the formation of GJ plaques in the plasma membrane of proliferating progenitors (Figure 15).This was confirmed by double immunolabelling with ZO-1, a cell junction protein that was previously reported to interact with gap junctional Cx43 (Giepmans, 2006). The slice view and DIC-overlay revealed that Cx43 plaques were clearly located between adjacent cells (arrows). In contrast, only few, small dots of Cx31 had been identified between neighbouring cells, colocalized with ZO-1 (Figure 15). Further, number, size and intensity of Cx31-formed GJs were distinctly reduced when compared with the Cx43 staining pattern. The extraction by Triton X-100 induced complete removal of Cx59, indicating that Cx59 is not involved in GJ formation (Figure 15).

In addition to GJ formation, Cxs can also be incorporated into the plasma membrane to form hemichannels and thus regulating the shuttling between the cytoplasm and the extracellular space. To distinguish between intracellular Cx and Cx-hemichannels located in the plasma membrane, we performed confocal microscopy, followed by 3D reconstruction. Compared to GJ plaques, hemichannels are smaller in size and difficult to detect by microscopic imaging (Figure 16A). Therefore, we applied surface rendering based on the fluorescence signal obtained by co-staining of the actin cytoskeleton (Figure 16, white surface) and Cx (red dots). As the actin network spans the whole cell and is linked to the plasma membrane, it represents an appropriate marker for determining the cell surface area. We found that large amounts of Cx43 were closely attached to the cell membrane, suggesting the presence of Cx43 hemichannels in ReNcell VM197. In contrast, Cx31 and Cx59 were not located at the cell surface area (Figure 16).

These microscopic observations identified Cx43 as the main channel forming protein in ReNcell VM197, including both GJ and hemichannel formation. However, only a small portion of the entire expressed Cx43 and was transported to the membrane and contributed to the establishment of GJ and hemichannels, whereas most Cx remained in the cytosol and the Golgi apparatus.
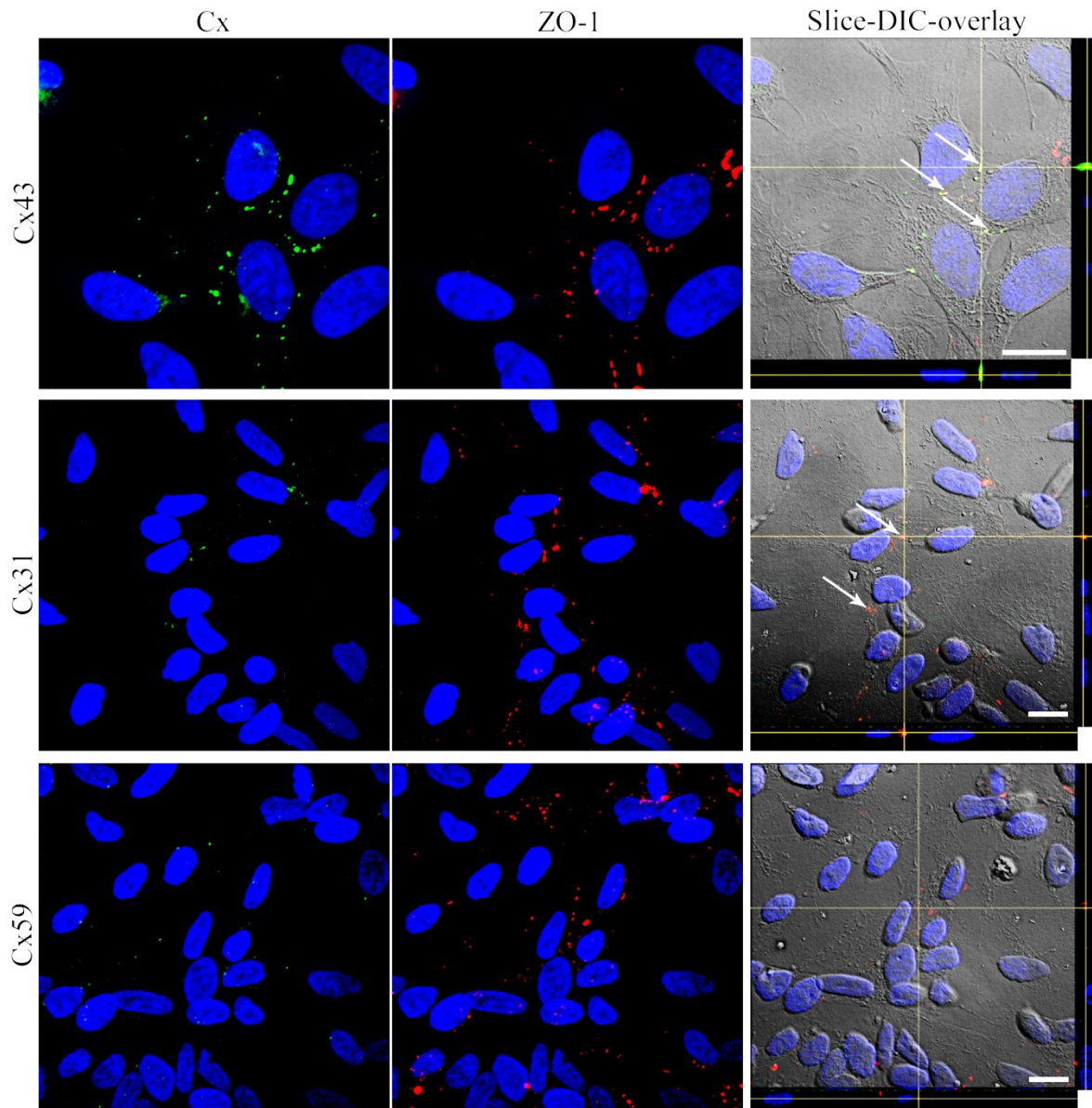
Figure 15 Localization of Cxs in GJ plaques. The removal of cytoplasmic Cx by Triton X-100 allowed clear delineation of GJ plaques between adjacent cells. Double immunolabelling of Cx and ZO-1, another GJ-associated protein, and subsequent confocal analysis of recorded z-stacks including DIC-overlay confirmed the presence of GJs at cell borders (arrows). Note the colocalization of bright Cx43 spots and ZO-1 at cell borders. Less GJ-forming activity was observed for Cx31. Cx59 exhibited no resistance to Triton X-100, indicating no involvement in GJ coupling. Cells were subjected to Triton X-100 extraction as described in material and methods, followed by immunolabelling with Cx antibody (green) and cy3-conjugated ZO-1 antibody (red), nuclei were stained with Hoechst, Bar 20µm.
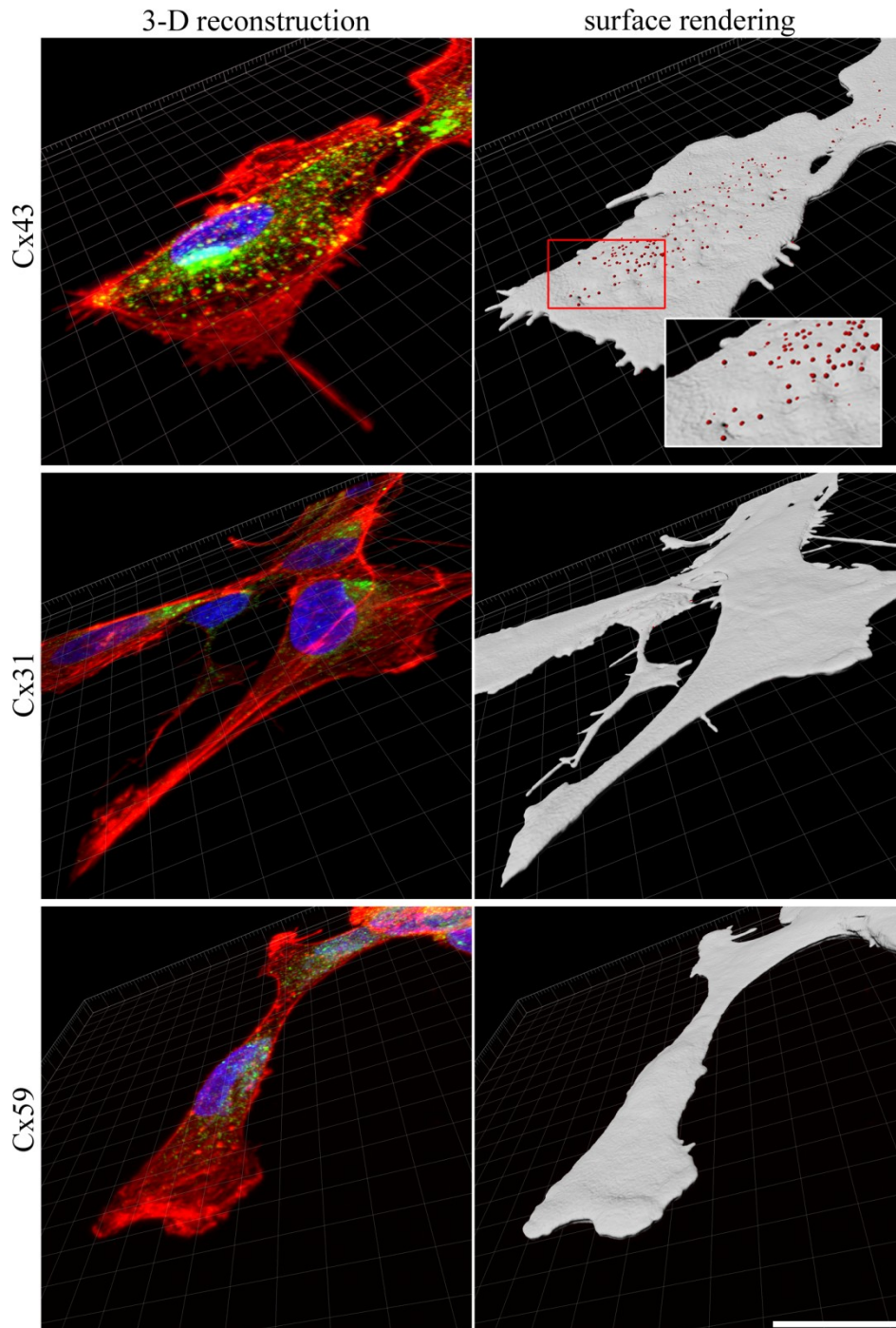
Figure 16 Hemichannel formation in proliferating progenitors. Cells were immunolabelled for Cxs and actin to reveal the cell surface area, followed by confocal 3-D reconstruction. Subsequent surface rendering allowed the visualization of Cx proteins (red dots) closely attached to the cell surface (white surface). In contrast to Cx31 and Cx59, Cx43 was found to localize close to the membrane, which indicates the presence of hemichannels. Note that the size of surface-bound Cx43 dots was increased for better visualization of hemichannels. Cells were labelled with Cx43 antibody (green), Rhodamine/Phalloidin (red) and Hoechst, Bar 20 µm

3.4 CX43 – A KEY PLAYER IN GJIC OF ReNcell VM197

3.4.1 NEURAL DIFFERENTIATION INDUCED ALTERATIONS OF GAP JUNCTIONAL CX43

We have shown that the extent of gap junctional coupling changed during the differentiation process and that Cx43 expression was characterized by a similar time-dependent pattern (Figure 7, 9). Due to the fact that Cx43 is the main GJ-forming protein and therefore might be a key protein in regulating GJIC, we carried out time-dependent expression analysis of triton-insoluble, gap junctional Cx43 to compare both the entire expressed connexin and connexin located in GJs. First, we performed biochemical analysis of cytosolic and membrane fractions to confirm our confocal data, revealing the localization of Cx43 proteins in the plasma membrane (Figure 15). As presented in Figure 17A, Cx43 was detected in both membrane and cytosolic fraction. Accuracy of membrane isolation was verified by detection of E-cadherin, a transmembrane protein that was only found in the membrane fraction. Moreover, we observed a minor signal of cytoskeletal α-tubulin, giving also evidence for successful membrane fractionation.

The solubility of non-phosphorylated and phosphorylated forms of Cx43 in Triton X-100 was proven by western blot analysis of total cell lysates that were subjected to treatment with Triton X-100. Subsequent ultracentrifugation allowed separation into soluble and insoluble fractions. Compared to total cell lysates, Triton X-100-soluble fractions showed less amounts of phosphorylated Cx43 in both proliferating and differentiated cells (Figure 17B). In contrast, the phosphorylated form of Cx43, which is mainly located within GJ plaques, was resistant to detergent extraction. These data confirmed that unphosphorylated Cx is rather sensitive to detergent extraction than the phosphorylated forms of Cxs.

In order to investigate changes in expression and distribution of gap junctional Cx43, Triton X-100 extracted cells were stained with anti-Cx43 and subjected to 3D image analysis to quantify fluorescence intensity. As confocal images demonstrated, most Triton-X resistant Cx43 was found in proliferating cells (Figure 17C, 0d). However, the amount of membranous Cx43 was reduced after growth factor removal as cells started to undergo differentiation (Figure 17C, 2d-6d). After 4d differentiated neural progenitors showed an increasing signal of detergent-resistant Cx43 (Figure 17C, 4d-10d), but Cx43 spots were smaller in size and less pronounced compared to those observed in proliferating cells. Quantitative analysis, summarized in Figure 17D, illustrated in detail the time-dependent alterations of fluorescence intensity. Neural differentiation induced a decrease of gap junctional Cx43 by 70% that was maintained up to 4d of differentiation, followed by a progressive 2-fold re-increase of protein expression as differentiation proceeded. These time-dependent alterations of Triton X-100 resistant Cx43 are similar to the expression pattern of the entire

expressed Cx43 (Figure 9, 17), indicating that the regulation of Cx43 expression directly affects the incorporation of Cx proteins into GJs, which in turn results in a change of gap junctional communication.
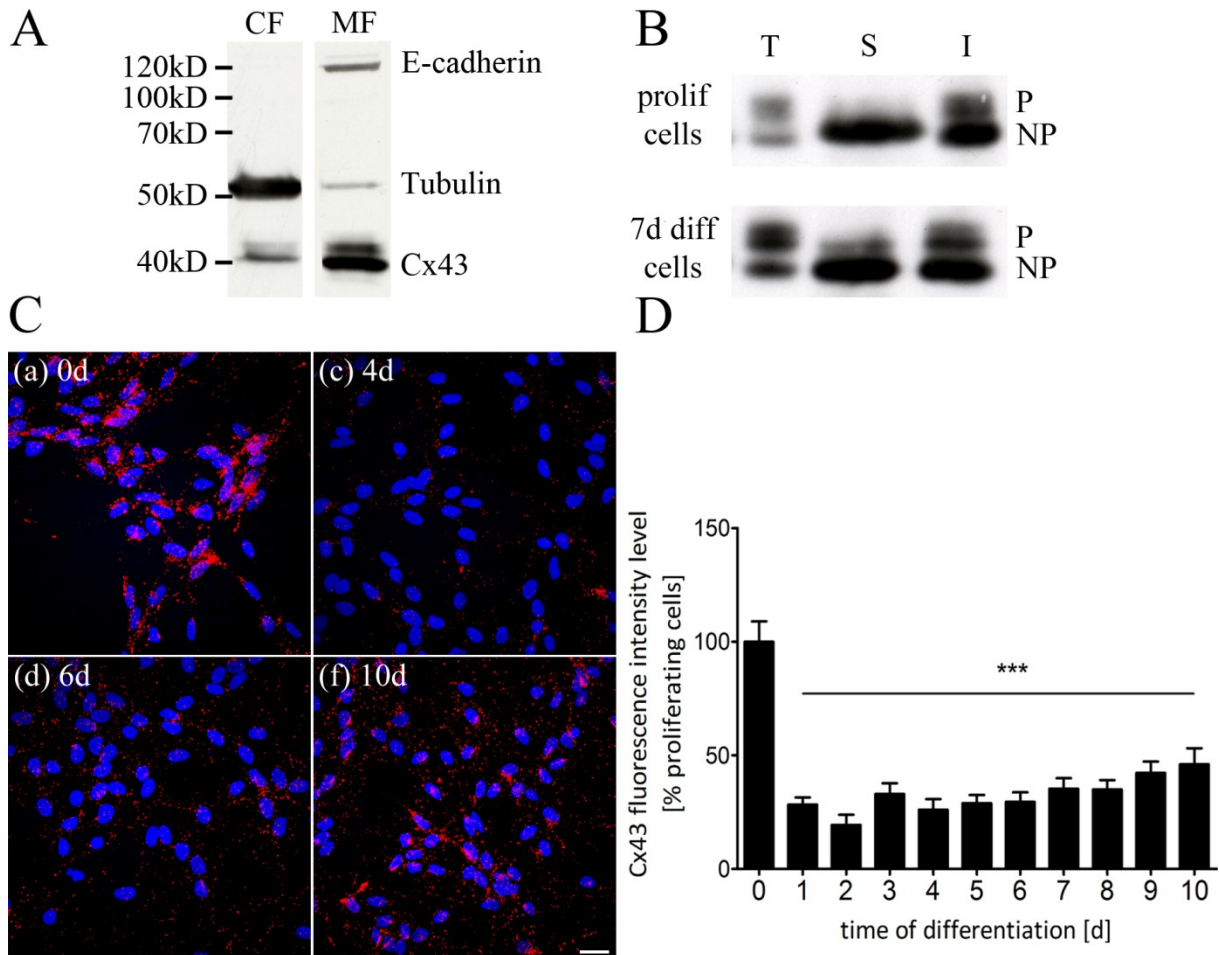
Figure 17 Expression of gap junctional Cx43 during proliferation and differentiation. (**A**) Western blot analysis of cytoplasmic (CF) and membrane fractions (MF) demonstrated the presence of membrane-bound Cx43 in proliferating progenitors. E-cadherin and α-tubulin were used as control to ensure proper fractionation (**B**) Western blotting confirmed the effect of Triton X-100 extraction on Cx43 in proliferating and differentiated cells. While total cell lysates (T) and Triton-insoluble fractions (I) contained large amounts of phosphorylated (P) and non-phosphorylated (NP) Cx43, Triton X-100-soluble fractions (S) showed a reduced signal for the phosphorylated form due to its detergent insolubility. (**C**) Confocal microscopy of Cx43 labelled cells revealed that most Triton X-100 insoluble Cx43 was found in (0d) proliferating cells. Induction of differentiation led to a decrease of gap junctional Cx43 (2d-4d), followed by an increase in the later stage of differentiation (6d-10d). (**D**) Microscopic quantification of confocal images indicated strong reduction of GJ bound Cx43 as differentiation was initialized and a subsequent progressive increase up to 10d of differentiation. Cellular fractionation and western blotting were performed as described in material and methods. Cells were labelled with Cx43 antibody (red) and Hoechst, Bar 20µm. Graph represents data of three independent experiments, one-way ANOVA, *P*<0.0001, followed by Bonferroni *post hoc* test.

3.4.2 MICROTUBULE-DEPENDENT TRANSPORT OF CX43

As the cytoskeleton is an important player in mediating the transport of proteins that traffic through the secretory pathway, we analysed the involvement of microtubules in Cx43 movement. Thus, cells were treated with the microtubule-depolymerizing agent nocodazole (10μM), fixed and labeled with anti-Cx43 antibody. Under control conditions Cx43 was distributed throughout the whole cell body and strongly accumulated in the Golgi network (Figure 18).

Administration of nocodazole induced pronounced changes in Cx43 distribution, leading to a loss of intracellular accumulations and a diffuse staining pattern (Figure 18). Inserted images demonstrated the effect of nocodazole on microtubule behaviour. While microtubules formed a dense network in control cells, nocodazole treatment resulted in depolymerisation and complete disruption of microtubule filaments. The susceptibility of Cx43 distribution to nocodazole indicates that Cx43 behaviour and transport are directly influenced by the microtubule network.

The movement of Cx43 vesicles along microtubules was demonstrated by live cell imaging of proliferating and differentiated cells expressing Cx43-tagged RFP and α-tubulin-tagged YFP. Figure 18B presents a recorded frame sequence revealing the microtubule-dependent transport of Cx43. The Images illustrate the track of movement of two selected particles over time. To visualize the covered distance, Cx43 vesicles were marked by arrows and their starting points are shown as bars. Computational tracking analysis demonstrated that particles moved along microtubules at an average velocity of 0.62 μm/s. The speed of Cx43 movement remained constant during differentiation as both proliferating and differentiated cells showed no significant changes in velocity (Figure 18C). Direct involvement of the cytoskeleton was emphasized by the fact that inhibition of microtubule dynamics by colchicine did markedly reduce the average particle velocity by 70% (Figure 18D). Taken together, these data suggest that Cx43 interacts with microtubules for transporting Cx vesicles from the Golgi-apparatus throughout the cytoplasm and to the plasma membrane.
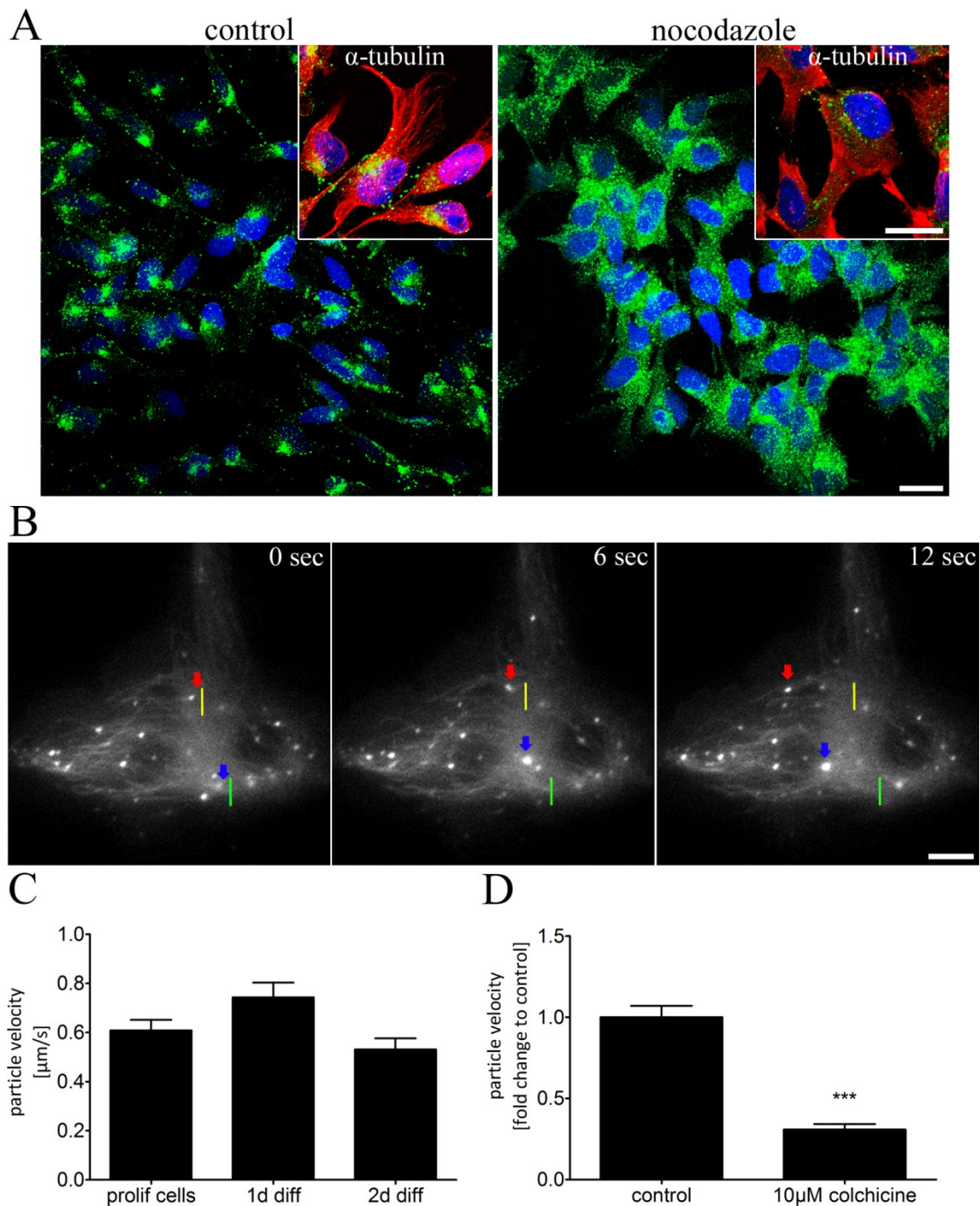
Figure 18 Involvement of microtubules in Cx43 distribution and transport. (**A**) Compared to control cells, treatment with the microtubule depolymerizing agent nocodazole for 1 hour induced a loss of Cx43 accumulations leading to a diffuse staining pattern. Inserted images approve the effect of nocodazole on microtubules. As untreated cells showed a dense microtubule network, drug administration resulted in complete disruption. Cells were labelled with Cx43 (green) and α-tubulin antibody (red). Nuclei were stained with Hoechst, Bar 20μm. (**B**) Movement of Cx43-tagged RFP vesicles in proliferating progenitors along YFP-tagged microtubules. Two selected vesicles were labeled by red and blue arrows. Begin of movement was marked with yellow and green bars, Bar 20μm. (**C**) Quantification of computational vesicle tracking revealed an average velocity of 0.62 μm/s, which was constant in proliferating and differentiated cells. (**D**) Incubation with colchicine, a microtubule-depolymerizing agent, distinctly reduced the average speed of Cx43 vesicle movement. Graphs represent data of 3 independent experiments, shown as mean±SEM. Statistical differences were calculated by Student´s t-test (***$P<0.001$)

3.5 IMPACT OF GJIC AND CX43 ON PROLIFERATION AND NEURONAL DIFFERENTIATION OF ReNcell VM197

3.5.1 PHARMACOLOGICAL INHIBITION OF GJIC

As shown in section 3.2.2, GJIC is differentially modulated in human neural progenitors during differentiation and the dynamic Cx expression is involved in mediating this effect. This implies a pronounced role of proper gap junctional coupling in proliferation and in particular in the neuronal differentiation process.

The administration of uncoupling agents enabled us to study the effect of impaired communication on cell growth and neuronal development. Here we used two well-established GJ blockers, including CBX and 1-oct to inhibit GJIC. We initially evaluated the efficiency of used blockers in affecting intercellular communication. CBX was found to inhibit GJIC by 20% when applied at a concentration of 50µM (Figure 19). The increase of CBX concentration to 100µM enhanced the effect of GJ blockage, resulting in a reduction of GJ coupling by 60%. Administration of 1-oct, an uncoupling agent belonging to the chemical group of alcohols, also impaired GJIC. FRAP data showed that cell-cell coupling decreased by 25% when cells were treated with 500µM 1-oct.
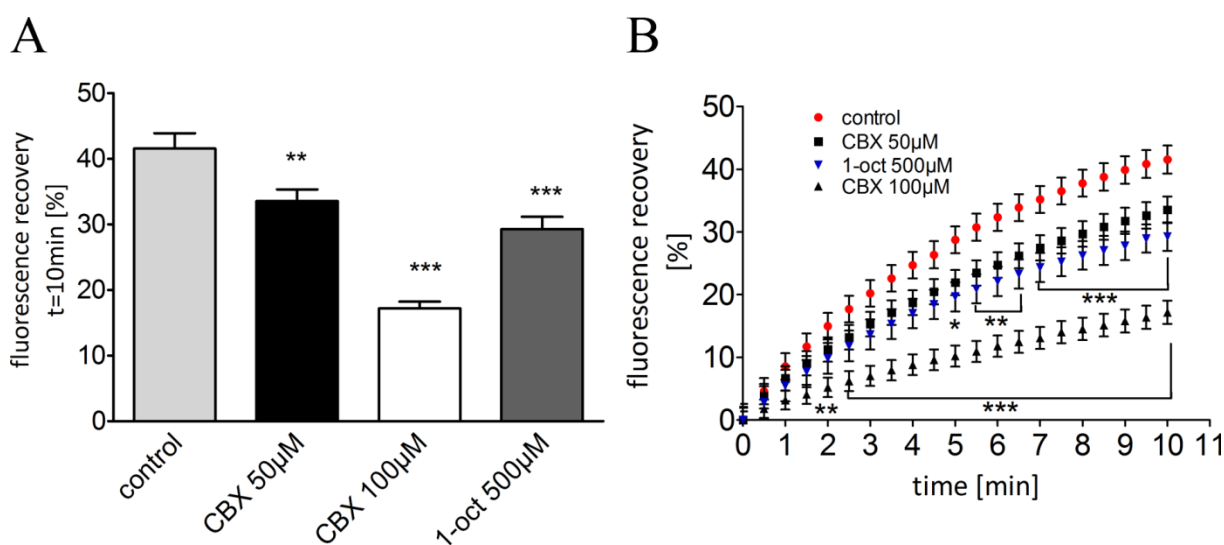
Figure 19 Pharmacological Inhibition of GJIC in proliferating progenitors. (**A**) Treatment with CBX and 1-oct significantly reduced the extent of gap junctional coupling in proliferating cells. (**B**) Average fluorescence recovery curves indicate the reduced speed of dye flux as GJ-blockers were applied. Graphs represent data of 3 independent experiments, shown as mean±SEM. Statistical differences were calculated by (A) Student´s *t*-test (**$P<0.01$, ***$P<0.001$) or (B) two-way ANOVA $P<0.001$ followed by Bonferroni *post hoc* test.

Based on this data, we wanted to ascertain if reduced GJIC affects the proliferation of ReNcell VM197 neural progenitors. Thus, cells were incubated with CBX and 1-oct for 24h and treated with EdU, a nucleoside analogue that is incorporated into the DNA during S-phase, which indicates the extent of proliferation (Figure 20A). Results, presented in

Figure 20B indicated a significant decrease of EdU-positive cells after CBX exposure by 11-16%. By contrast, 1-oct did not exhibit an inhibiting effect on cell growth.

Next, we investigated the impact of GJIC on neuronal differentiation. Cells were differentiated for 4d in the presence of CBX and 1-oct, followed by microscopic quantification of neuronal phenotypes that were recognized by labelling of β(III) tubulin and HuC/D (Figure 20C). In order to maintain GJ blockage during the entire course of the experiment, differentiation medium containing CBX and 1-oct was replaced every day. As seen in Figure 20D, we detected approximately 25% fewer neurons after 4d in cells incubated with the CBX. While CBX influenced neural cell fate at concentrations of 50µM and 100 µM, 1-oct was not able to induce reduction of the neuronal number. Although both CBX and 1-oct were shown to inhibit GJIC, only CBX was found to decrease the proliferation rate and impaired the elaboration of neuronal phenotypes. However, these results suggest the requirement of proper GJIC for the regulation of proliferation and neuronal development in neural progenitors.
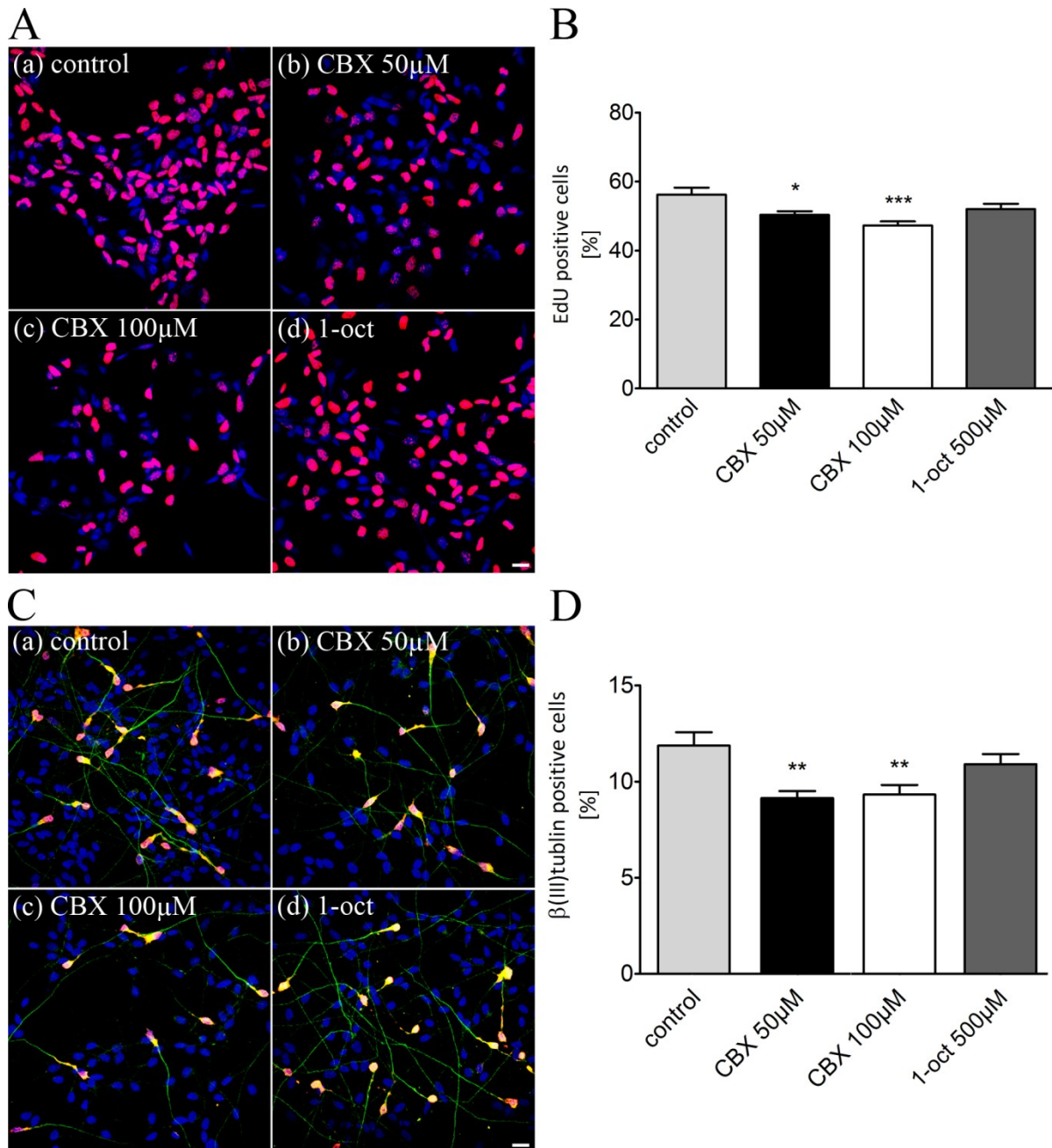
Figure 20 Pharmacological inhibition of GJIC affects proliferation and neuronal differentiation. (**A**) Cells were either left untreated (a) or incubated with 50μM CBX (b), 100μM CBX (c) and 500μM 1-oct (d) for 24h, followed by incubation with EdU (red) to analyse proliferation, Bar 20μm. (**B**) Microscopic quantification revealed a significantly reduced incorporation of EdU in cells treated with the GJ blocker CBX. However, no proliferation-inhibiting effect was observed for 1-oct. (**C**) For analysing the effect on neuronal development, cells were differentiated for 4d in the presence of CBX (b, c) and 1-oct (d). Subsequent visualization of neurons by immunolabelling demonstrated impaired elaboration of neuronal phenotypes in CBX treated cells. (**D**) Incubation with 50μM and 100μM CBX reduced the neuron number by 25%. In contrast,1-oct did not affect neuronal development. Cells were labelled with neuron specific anti-HuC/D and anti-β(III) tubulin. Nuclei were stained with Hoechst, Bar 20μm. Graphs represent data of 3 independent experiments, shown as mean±SEM. Statistical differences were calculated by Student´s t-test (*$P<0.05$;**$P<0.01$;***$P<0.001$).

3.5.2 IMPACT OF CX43 ON PROLIFERATION AND DIFFERENTIATION

As some functions of Cx43 are not attributed to its ability to form GJs and to mediate the intercellular exchange of molecules, we performed knockdown experiments using Cx43 siRNA to study the specific role of Cx43 in proliferation and differentiation of neural progenitors. This allows us to encompass both the channel-dependent and channel-independent function of this protein.

3.5.2.1 VALIDATION OF KNOCKDOWN EFFICIENCY

Initially, different transfection parameters were evaluated to prevent an electroporation-induced increase of cell death. Proliferating progenitor cells were transfected by electroporation and subjected to differentiation conditions after 2d. To validate knockdown efficiency, we carried out western blot analysis, revealing down regulation of Cx43 by 80% in proliferating progenitors transfected with Cx43 siRNA (Figure 21A). Moreover, the knockdown was stable throughout the tested time course up to 4d after differentiation was induced. Immunolabelling with anti-Cx43 antibody showed a distinct reduction of fluorescence intensity in proliferating and differentiated Cx43 knockdown cells, which corresponds to our western blot data (Figure 21B). For functional validation, FRAP microscopy was performed to demonstrate the impact on gap junctional coupling. As mock transfected cells showed unaltered dye recovery in photobleached cells, knockdown of Cx43 distinctly decreased fluorescence recovery (Figure 21C). Analysis of pooled data demonstrated that dye recovery was markedly inhibited by 50% when Cx43 was down-regulated (Figure 21D). Average fluorescence recovery curves confirmed the different speed of GJ-dependent dye diffusion, showing a much slower influx of CFL into Cx43-siRNA transfected cells (Figure 21D). These results confirmed our microscopic observations, suggesting Cx43 as the major GJ-forming protein and highlight the involvement of Cx43 in ensuring the dynamic changes of GJIC in the differentiation process of human neural progenitors.

3.5.3 EFFECT OF CX43 KNOCKDOWN ON GROWTH FACTOR-DEPENDENT PROLIFERATION

After verifying the impact of Cx43 knockdown on GJIC, we investigated the importance of Cx43 for cell growth of neural progenitors. Cell differentiation is always accompanied by changes in the cell cycle, leading to a stop of cell-division and $G_{1/0}$-arrest. Initially, we analysed the alteration of proliferation rate that occurred as cells undergo differentiation under control conditions. Proliferating cells were induced to differentiate by the removal of
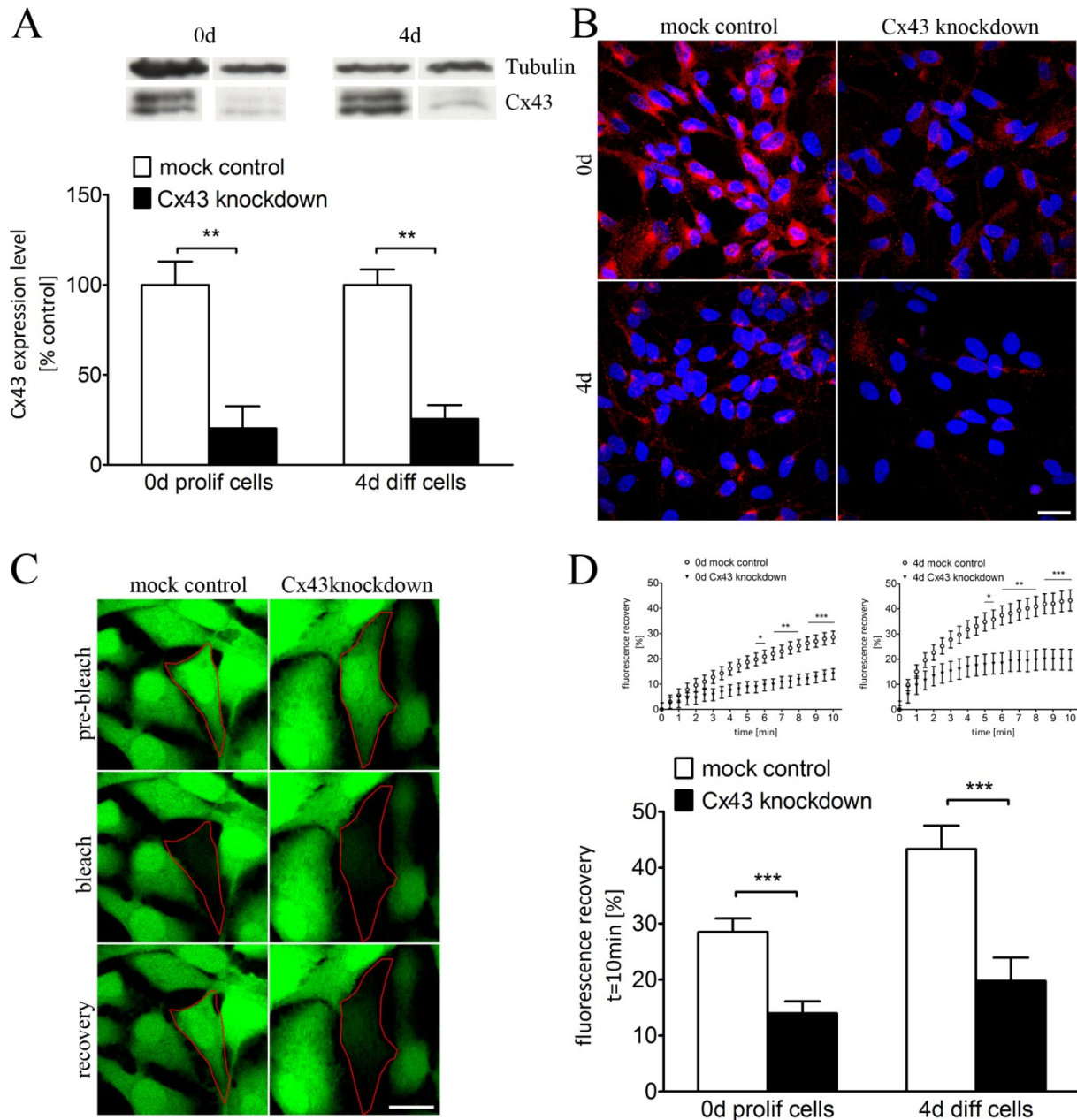
Figure 21 Effect of Cx43 knockdown on GJIC in proliferating and differentiated progenitor cells.
(**A, B**) Proliferating cells were transfected with Cx43 siRNA. As determined by western blotting and confocal microscopy of Cx43 labeled cells, knockdown efficiency was about 75% in both proliferating and 4d differentiated cells, Bar 50μm. (**C**) As revealed by FRAP analysis GJIC was distinctly reduced by 45% in knockdown cells, Bar 20μm. (**D**) While fluorescence recovery was strong in control cells loaded with CFL, it was impaired in Cx43 siRNA transfected cells. Inserted graphs illustrate the fluorescence recovery during measurement, showing the reduction of GJIC in Cx43 knockdown cells. Graphs represent data of 3 independent experiments, shown as mean±SEM. Statistical differences in Cx43 expression and GJIC between mock and knockdown cells were calculated by Student´s *t*-test **$P<0.01$, ***$P<0.001$. Fluorescence recovery curves were analysed by two-way ANOVA, $P<0.0001$, followed by Bonferroni *post-hoc* test.

growth factors and subjected to an EdU-assay after 24h (Figure 22A). As cells were maintained in the presence of bFGF and EGF, 40-50% of cells were found to incorporate EdU into the DNA. However, differentiation led to a strong decrease of cell division. The number of S-phase cells was markedly reduced by 50% after 12h and proliferation almost stopped after 1d, indicated by less than 1% EdU-positive cells (Figure 22B).

As ReNcell VM197 is kept in a proliferative state by both EGF and bFGF, we assumed that Cx43 might affect cell proliferation by mediating the mitogenic activity of these growth factors. Cx43 knockdown cells were either cultured in complete proliferation medium or in EGF or bFGF alone, followed by EdU treatment and subsequent microscopic quantification of S-phase cells. Compared to mock control, down-regulation of Cx43 caused a 40-50% decrease of cell growth under all culture conditions, suggesting Cx43 as an important positive regulator for cell growth by mediating the proliferative effects of bFGF and EGF (Figure 22C).

The finding that Cx43 stimulates proliferation encouraged us to investigate whether knockdown of Cx43 influences the stop of cell division after initializing differentiation by growth factor removal. Interestingly, siRNA-mediated down-regulation caused contradictory effects in differentiated cells. While down-regulation of Cx43 in proliferating progenitors reduced the number of EdU positive cells, a 9-fold increase of EdU positive cells was detected after 1d of differentiation (Figure 22D).

Taken together, this points to a major role of Cx43 in regulating proliferation of neural progenitors. Our data lead to the assumption that Cx43 might have opposite effects on cell growth under different conditions. In proliferating progenitors Cx43 is needed to promote growth factor-driven proliferation. However, under differentiation conditions, Cx43 was found to support the exit from the cell cycle, preventing cells from further proliferation. As pharmacological inhibition of GJIC only induced a slight decrease of the proliferation rate, Cx43 seems to control cell growth by a mechanism that is not exclusively attributed to their GJ-forming activity.
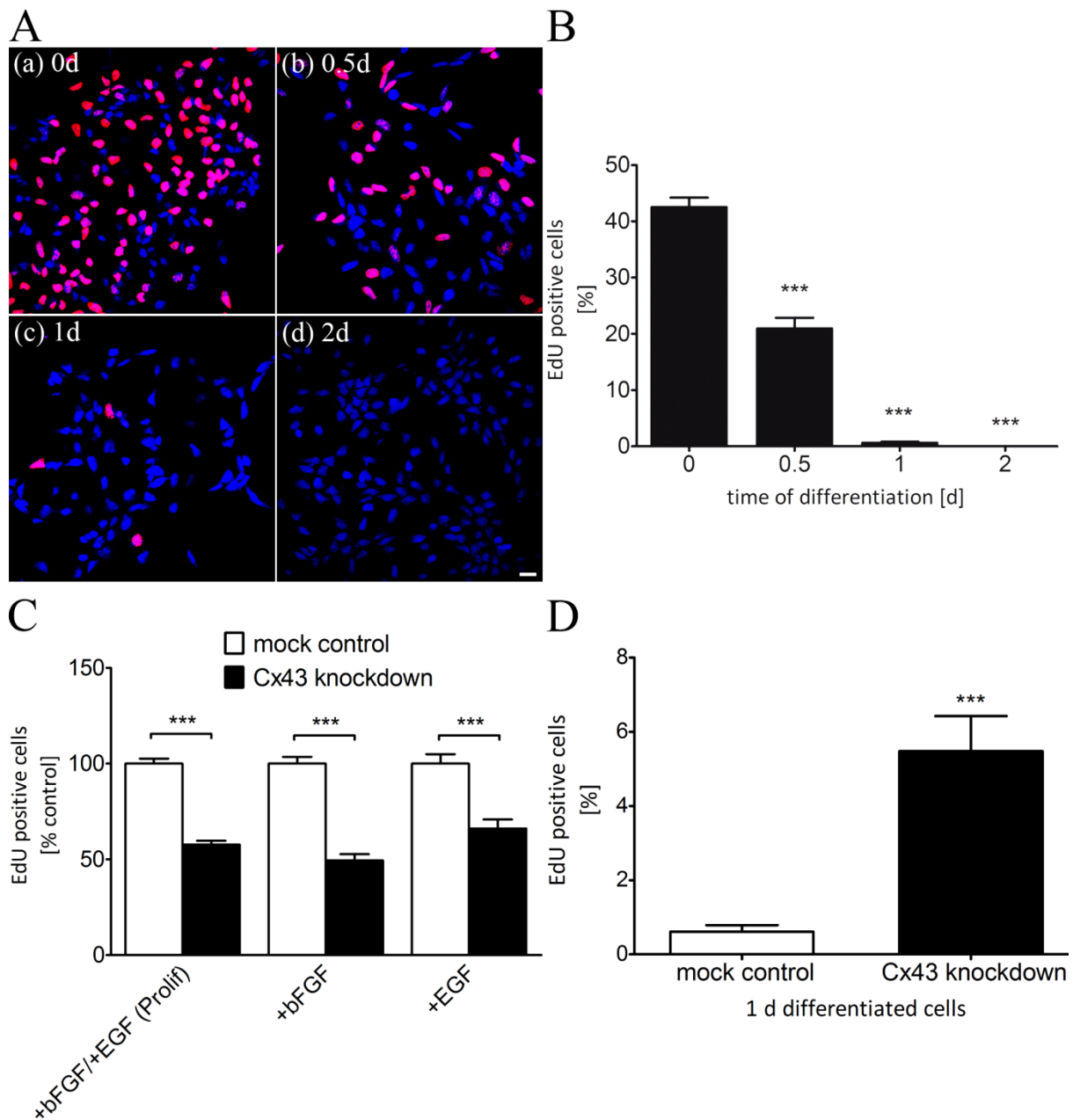
Figure 22 Cx43 regulates proliferation. Proliferation rate was measured using an EdU-assay. (**A**) Under control conditions the ability of cells to incorporate EdU (red) decreased as differentiation was induced. (**B**) Microscopic quantification revealed a progressive decline of mitotic cells, resulting in a complete stop of proliferation after 2d. (**C**) Proliferating progenitors were transfected with Cx43 siRNA and cultured in both EGF and bFGF or in EGF or bFGF alone. EdU-assay showed that siRNA-mediated knockdown of Cx43 resulted in an inhibition of cell growth by 50%-60% under all tested proliferation conditions. (**D**) However, down-regulation of Cx43 impaired the stop of proliferation as cells were induced to differentiate. In 1d differentiated cells Cx43 knockdown produced a 9-fold increase of mitotic cells compared to mock control. Cells were incubated with EDU and labelled with Alexa594®, Bar 20μm. Graph represents data of 3 independent experiments, shown as mean±SEM. Statistical differences were analysed by (B) one-way ANOVA, $P<0.0001$, followed by Bonferroni´s *post hoc* test or (C, D) Student´s *t*-test, ***$P<0.001$.

3.5.3.1 EFFECT OF CX43 KNOCKDOWN ON NEURONAL CELL FATE

Beside its regulating function on proliferation, Cx43 was also found to influence neuronal cell fate. Proliferating progenitors were transfected with Cx43-siRNA and subjected to differentiation conditions. We monitored the amount of neurons during 4d of differentiation by double labelling of β(III) tubulin and HuC/D positive cells. Fluorescence images acquired by confocal microscopy showed the impaired differentiation in ReNcell VM197 (Figure 23A). While cells were able to establish a dense neuronal network under control conditions, Cx43 knockdown prevented the formation of such a network. In control cells, the percentage of neurons progressively increased with continuing differentiation, reaching a maximum value of 10-12% (Figure 23B). Cells lacking Cx43 due to siRNA transfection demonstrated a slower increase of β(III) tubulin positive cells over time. Moreover, the number of neurons was markedly decreased by 50-65% compared to mock control (Figure 23B). Similar to our proliferation analysis, knockdown of Cx43 induced more powerful effects than inhibition of GJIC by pharmacological blockage. Thus, Cx43 might also exert its effect on neuronal cell fate by a channel-independent activity.

The decrease in neuronal efficiency led us to study whether cells that failed to differentiate into neurons might have an alternative cell fate. The labelling of cells using anti-GFAP antibodies revealed that both Cx43 knockdown and mock cells establish the same dense glial-network after induction of differentiation (Figure 24A). Additionally, we monitored the expression of the neural progenitor marker nestin and observed a progressive decrease during differentiation, showing that both control and knockdown cells lost their progenitor cell character and left the proliferative state (Figure 24B). These data indicate that Cx43 knockdown neither disturbs gliogenesis nor prevents neural progenitors from differentiation.
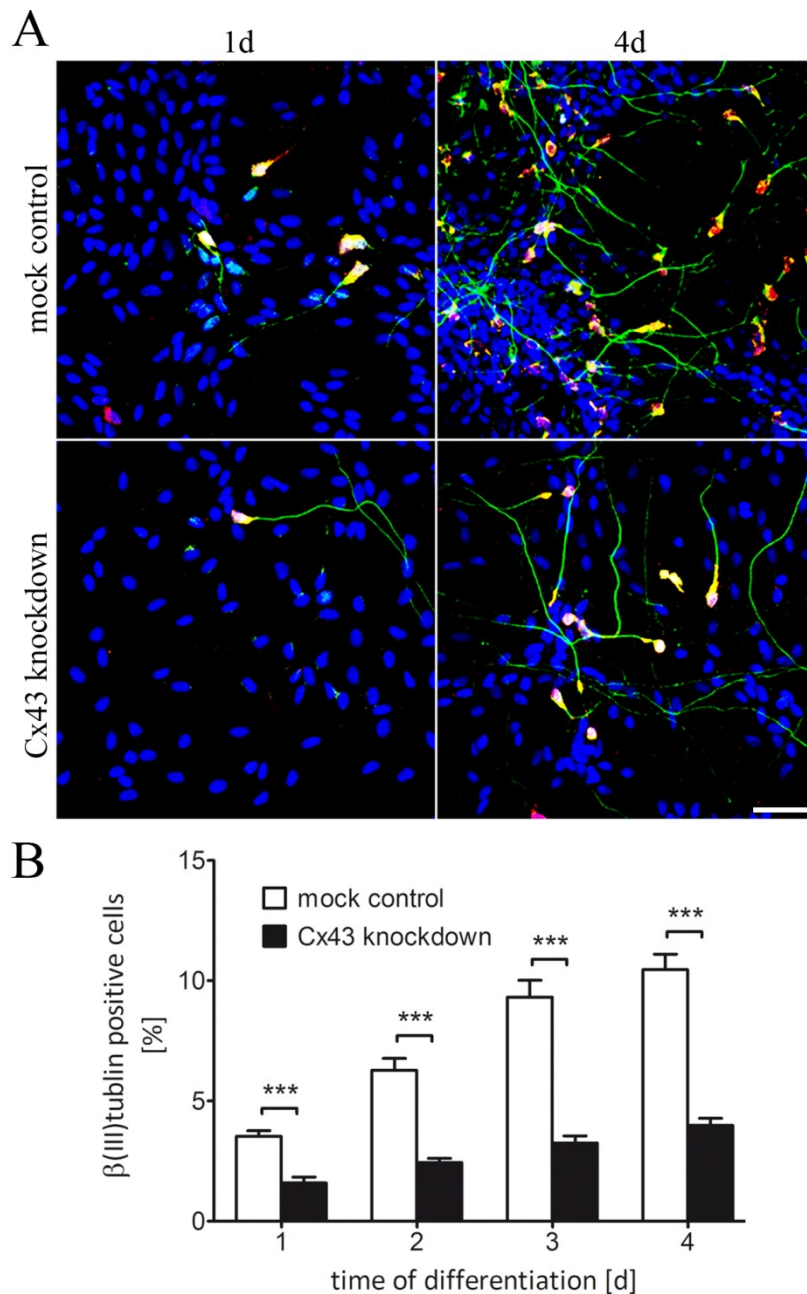
Figure 23 Cx43 regulates neuronal cell fate. (**A**) Proliferating progenitors were transfected with Cx43 siRNA and subjected to differentiation conditions. Cx43 knockdown markedly reduced the number of neurons and prevented the formation of a neuronal network as revealed by double labeling of β(III) tubulin (green) and HuC/D positive cells (red), Bar 50μm (**B**) Subsequent microscopic quantification of β(III) tubulin positive cells showed an decrease of neurons by more than 50% from the beginning of differentiation. Graph represents data of 3 independent. Statistical differences were calculated by Student´s *t*-test, ***$P<0.001$.
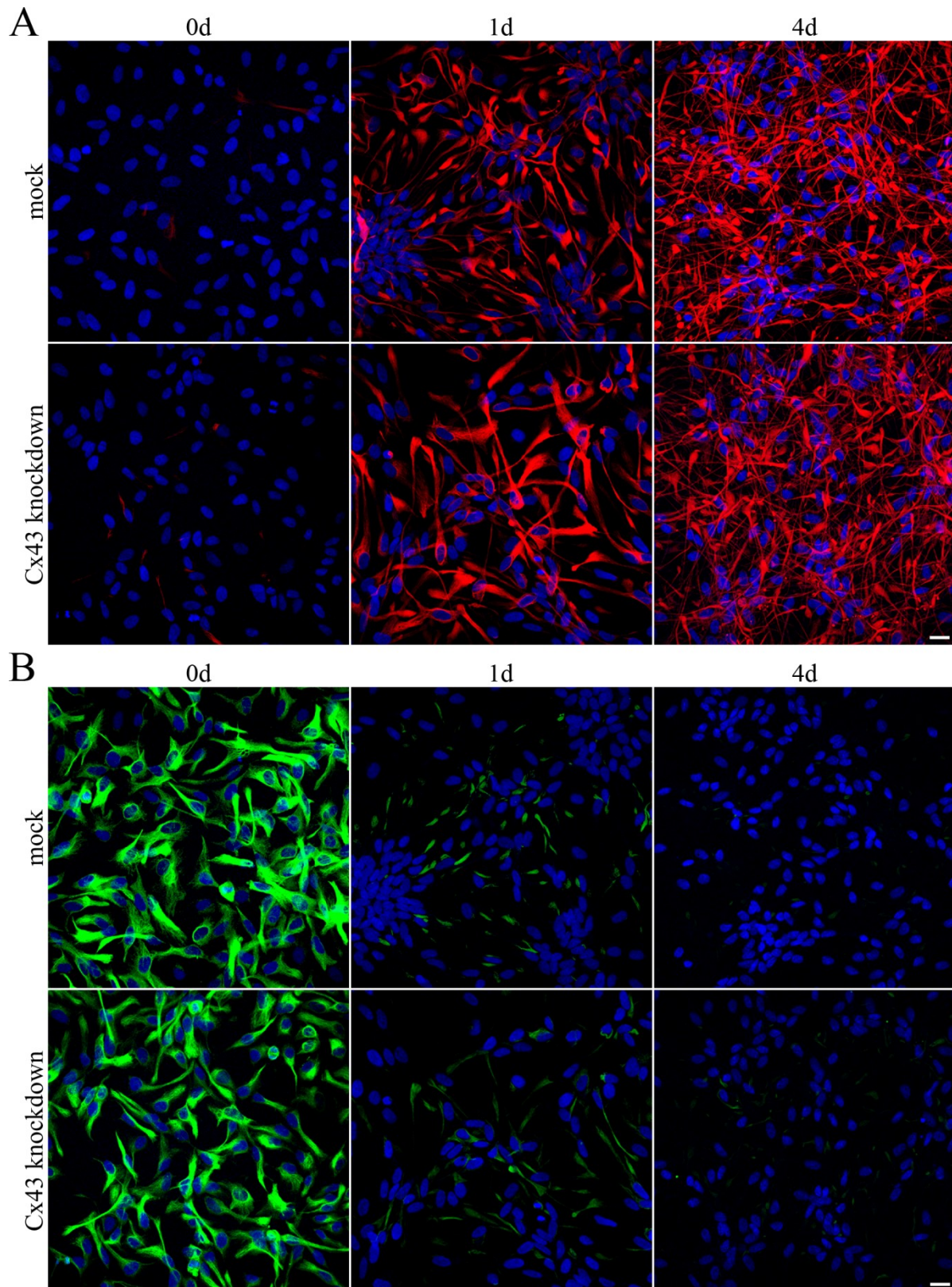
Figure 24 Effect of Cx43 knockdown on glial cell differentiation and nestin expression. (**A**) Cx43 knockdown did not affect gliogenesis. A dense glial multilayer network was established in control and knockdown cells after the induction of differentiation. (**B**) The expression pattern of nestin was similar in both mock and knockdown cells. As nestin was strongly expressed in proliferating cells, differentiation led to progressive down-regulation, Bar 20µm.

3.6 ROLE OF EGF IN THE REGULATION OF CX43 BEHAVIOR

3.6.1 EGF AS A MODULATOR OF CX43 EXPRESSION

The strong dynamic alterations in Cx43 expression and distribution during differentiation of ReNcell VM197 led us to investigate possible mechanisms that are involved in mediating these dynamic changes. According to our western blot data (Figure 9) the protein level of Cx43 declined once differentiation was induced by removal of bFGF and EGF. This suggests a growth factor-dependent regulation of Cx43. To address this issue, cells were subjected to different growth conditions and Cx43 levels were estimated by western blotting. As expected, complete removal of growth factors for 1d decreased the amount of Cx43 by 75% compared to cells cultured in the presence of both EGF and bFGF (Figure 25A). We also observed a similar reduction of protein expression when cells were treated with bFGF alone. However, incubation with medium containing EGF maintained the amount of Cx43 and prevented down-regulation. Immunolabelling with Cx43 antibody and subsequent confocal microscopy supported the finding that EGF promoted Cx43 expression under proliferation conditions (Figure 25B). While fluorescence intensity was markedly reduced in differentiated cells (Figure 25B, b) and in cells treated with bFGF (Figure 25B, c), administration of EGF alone (Figure 25B, d) maintained a similar high Cx43 expression as observed in cells, grown in complete proliferation medium (Figure 25B, a).

To verify the stimulating effect of EGF on Cx43 expression, cells were cultured in the absence of growth factors, in complete proliferation medium or in bFGF alone for 1d, followed by additional treatment with 20ng/ml EGF for 5h. As shown in Figure 26, incubation with EGF for 5h promoted Cx43 expression in differentiated cells and in cells cultured in bFGF. The latter ones demonstrated a 3.5-fold increase of the Cx43 level compared to control cells where no EGF was applied (Figure 26A, B). Interestingly, we found that EGF induced a 9-fold up-regulation of Cx43 in 1d differentiated cells, suggesting that EGF also transduced its mitogenic signal to cells that had left the proliferative state. Taken together, these data provide evidence for a stimulating function of EGF on Cx43 expression under proliferation conditions. This is in agreement with our finding showing a strong decrease of Cx43 after induction of neural differentiation by removal of EGF and bFGF. However, the mechanism for the observed up-regulation in the late stage of differentiation remains elusive.
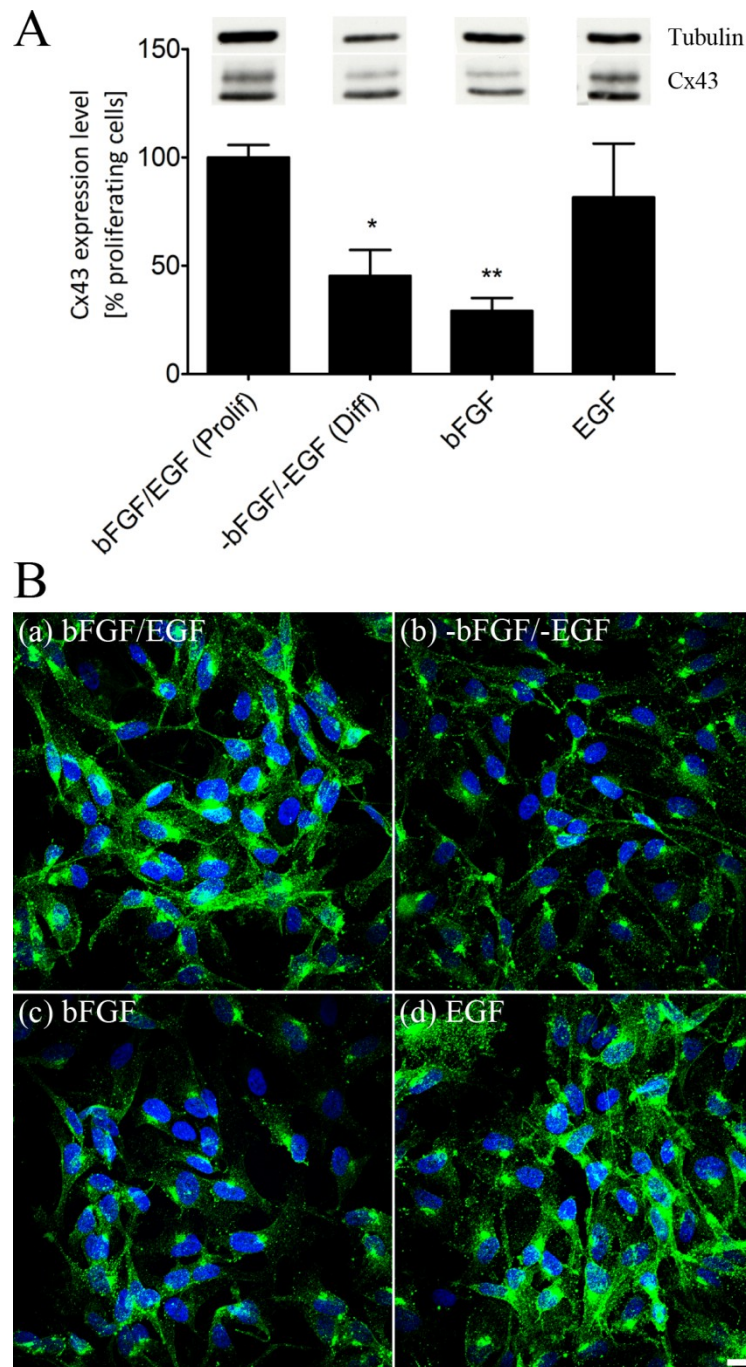
Figure 25 Growth factor dependent regulation of Cx43 expression in neural progenitors.
(**A**) Compared to progenitors grown in complete proliferation medium, culturing cells in bFGF alone or in
the absence of growth factors reduced the expression of Cx43 to 50%. However, treatment with EGF alone
was sufficient to maintain strong Cx43 expression. (**B**) Confocal images confirmed these observations,
showing similar levels of Cx43 in proliferating cells (a) and EGF treated cells (d). Incubation without
growth factors (b) or with bFGF alone (c) resulted in a decreased Cx43 level. Cells were cultured in
different growth conditions for 1d, followed by immunolabelling with Cx43 antibody. Nuclei were stained
with Hoechst, Bar 20μm. Graph represents data of 3 independent experiments, shown as mean±SEM.
Statistical differences were calculated by Student´s *t*-test *$P< 0.5$, **$P<0.01$.
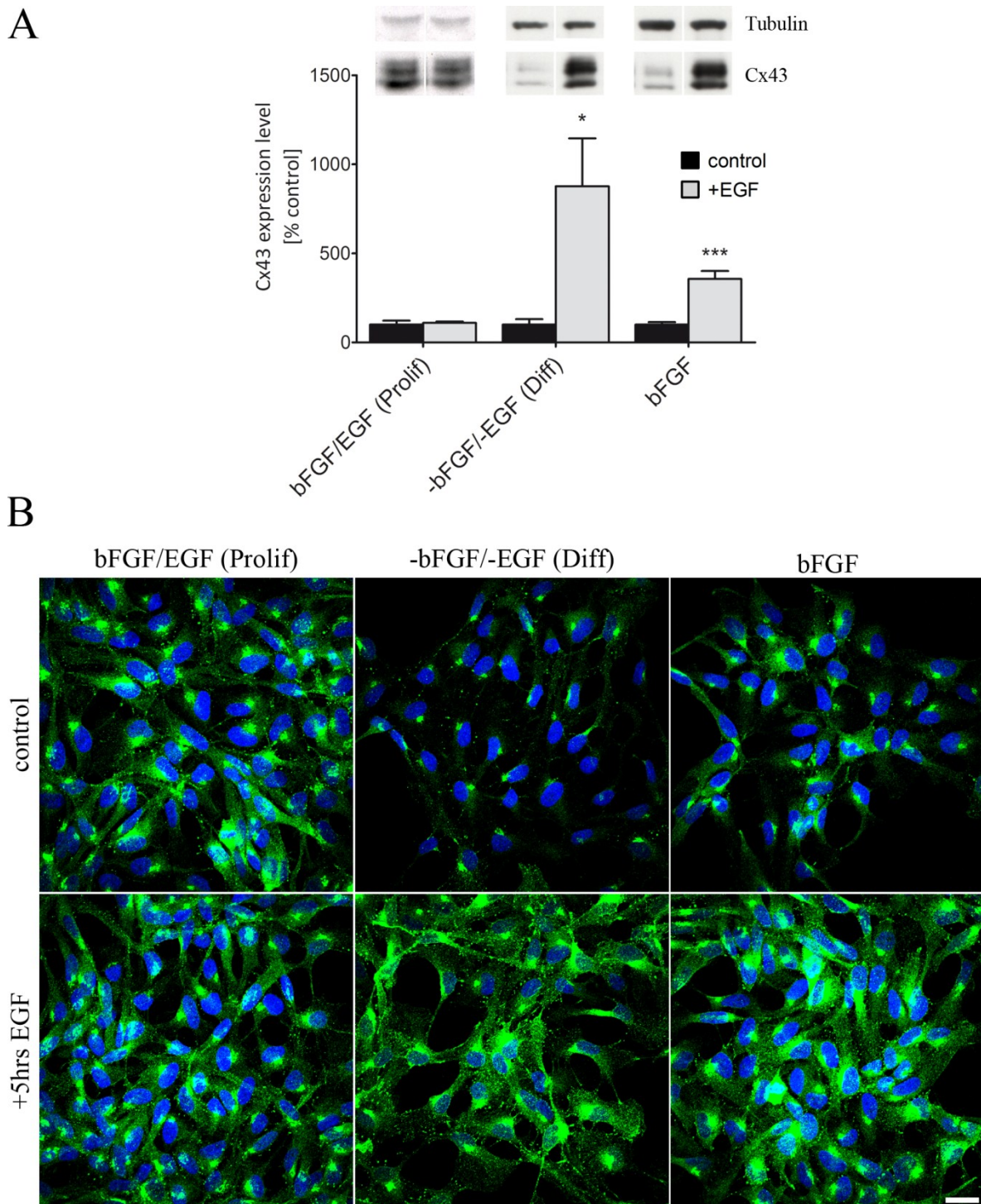
Figure 26 EGF stimulates the expression of Cx43. Cells were cultured in complete proliferation medium, in differentiation medium without growth factors and in bFGF alone (control) for 1d, followed by additional treatment with EGF for 5h (+EGF). (**A**) EGF stimulated the expression of Cx43 in differentiated cells and in bFGF cultured cells. Note, EGF induced a 8-fold up-regulation of Cx43 in differentiated cells. (**B**) Western blot results were confirmed by confocal microscopy of cells labelled with Cx43 antibody, showing a pronounced increase of fluorescence intensity after EGF-stimulation of differentiated and bFGF cultured cells, Bar 20µm. Graph represents data of 3 independent experiments, shown as mean±SEM. Statistical differences were calculated by Student´s *t*-test *P<0.05, **P<0.01.

3.6.2 EGF STIMULATES CX43 EXPRESSION VIA AN EGFR-DEPENDENT PATHWAY

Next, we asked why differentiated cells showed a stronger response to EGF concerning Cx43 expression than proliferating progenitors (Figure 26). The signalling cascade induced by growth factors usually starts with the receptor-ligand interaction. It is well known that EGF transduces its signal via a receptor specific pathway (Lo *et al.*, 2006). Confocal microscopy of fluorescent-labeled proliferating cells revealed that the EGF receptor (EGFR) exhibits a spot-like distribution pattern throughout the whole cell (Figure 27). Using additional fluorescence labelling of the actin cytoskeleton and 3D-image reconstruction, EGFR-spots were found to be located at the cell surface (Figure 27, merged). Surprisingly, detailed time-dependent protein expression analysis gave evidence for an increasing expression of EGFR after induction of differentiation (Figure 28). EGFR was massively up-regulated during the differentiation process, reaching a maximum value after 8d, which was 60-fold higher compared to proliferating cells (Figure 28A). The alterations in gene expression were confirmed by confocal microscopy showing a progressive increase of the EGFR level after initializing differentiation (Figure 28B).
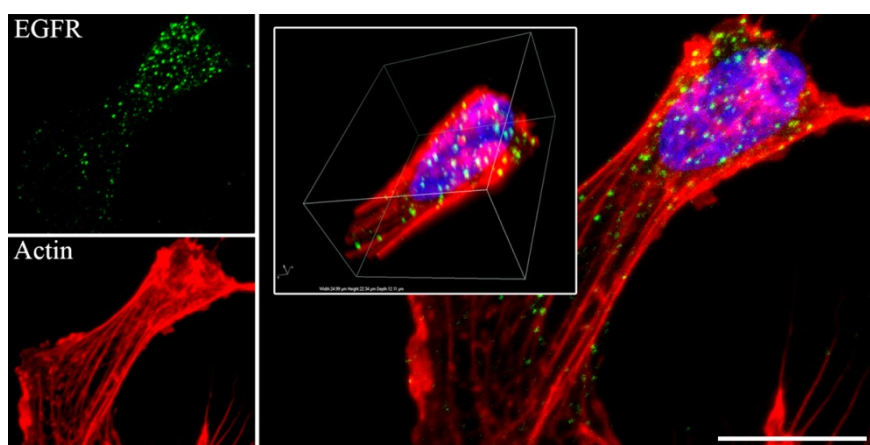
Figure 27 Localization of EGFR in proliferating progenitors. EGFR demonstrated a spot-like distribution pattern throughout the whole cell. Inserted 3D-image revealed that EGFR is located at the cell surface. Proliferating progenitors were labeled with EGFR-antibody (green). Actin was stained with Rhodamine/Phalloidin (red) to visualize cell borders. DNA was stained with Hoechst, Bar 20μm.

By culturing cells under different conditions, EGF removal was identified as the trigger for the up-regulation of EGFR (Figure 28C). No significant differences in EGFR expression were found in cells cultured in complete proliferation media or in EGF alone. As EGF was removed and cells were induced to proliferate only in the presence of bFGF, EGFR expression demonstrated a 13-fold increase that was much stronger compared to differentiated cells where EGFR was 5-fold up-regulated (Figure 28C). This indicates that only EGF and not bFGF regulates the level of EGFR when cells are kept in a proliferative state. While the administration of EGF supressed the expression of its own receptor, the removal triggered the up-regulation of EGFR during neural differentiation.
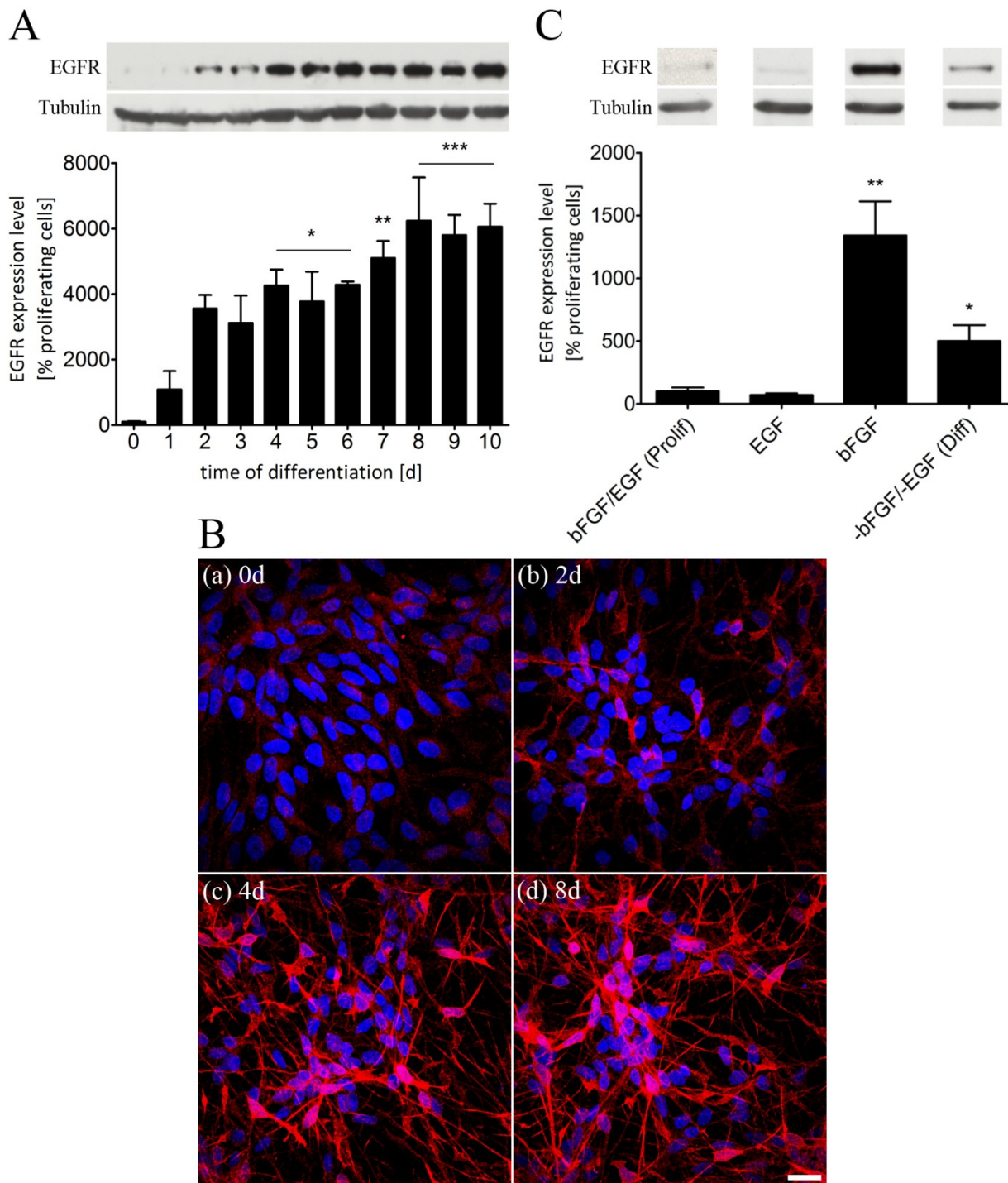
Figure 28 EGF regulates the expression of EGFR. (**A**) Western blot analysis showed a massive increase of EGFR after induction of differentiation, resulting in a 60-fold up-regulation after 8d. (**B**) Confocal microscopy of proliferating cells (a), 2d, (b) 4d (c) and 8d (d) differentiated cells confirmed the increasing level of EGFR. (**C**) The increase of EGFR expression was triggered by EGF removal. Culturing cells in complete proliferation medium and in EGF alone did not alter EGFR expression. However, treatment with bFGF alone resulted in an up-regulation that was even higher than observed in differentiated cells, suggesting that EGF negatively regulates the expression of its own receptor. Cells were immunolabelled with EGFR-antibody and Hoechst, Bar 20μm. Graphs represent data of 3 independent experiments, shown as mean±SEM. Time-dependent EGFR expression was analysed by one-way ANOVA, $P<0.0001$, followed by Bonferroni *post hoc* test. Data presented in (C) were analysed by Student′s *t*-test *$P<0.05$, **$P<0.005$.

The use of AG1478, a potent EGFR-kinase inhibitor, enabled us to demonstrate that EGF acts on Cx43 expression via EGFR. Therefore, cells were cultured in bFGF for 1d to attenuate EGF-dependent growth control, followed by pre-treatment with AG1478 for 2h and subsequent adminstration of EGF for 5h (Figure 29). Confocal imaging of cells labelled with anti-Cx43 antibody and western blot data showed that EGF enhanced the expression of Cx43 when applied to bFGF cultured cells (Figure 29A, B). However, pre-treatment with the EGFR-inhibitor completley counteracted the stimulationg effect of EGF and retained the level of Cx43 similar to that observed in cells cultured in bFGF alone (Figure 29).
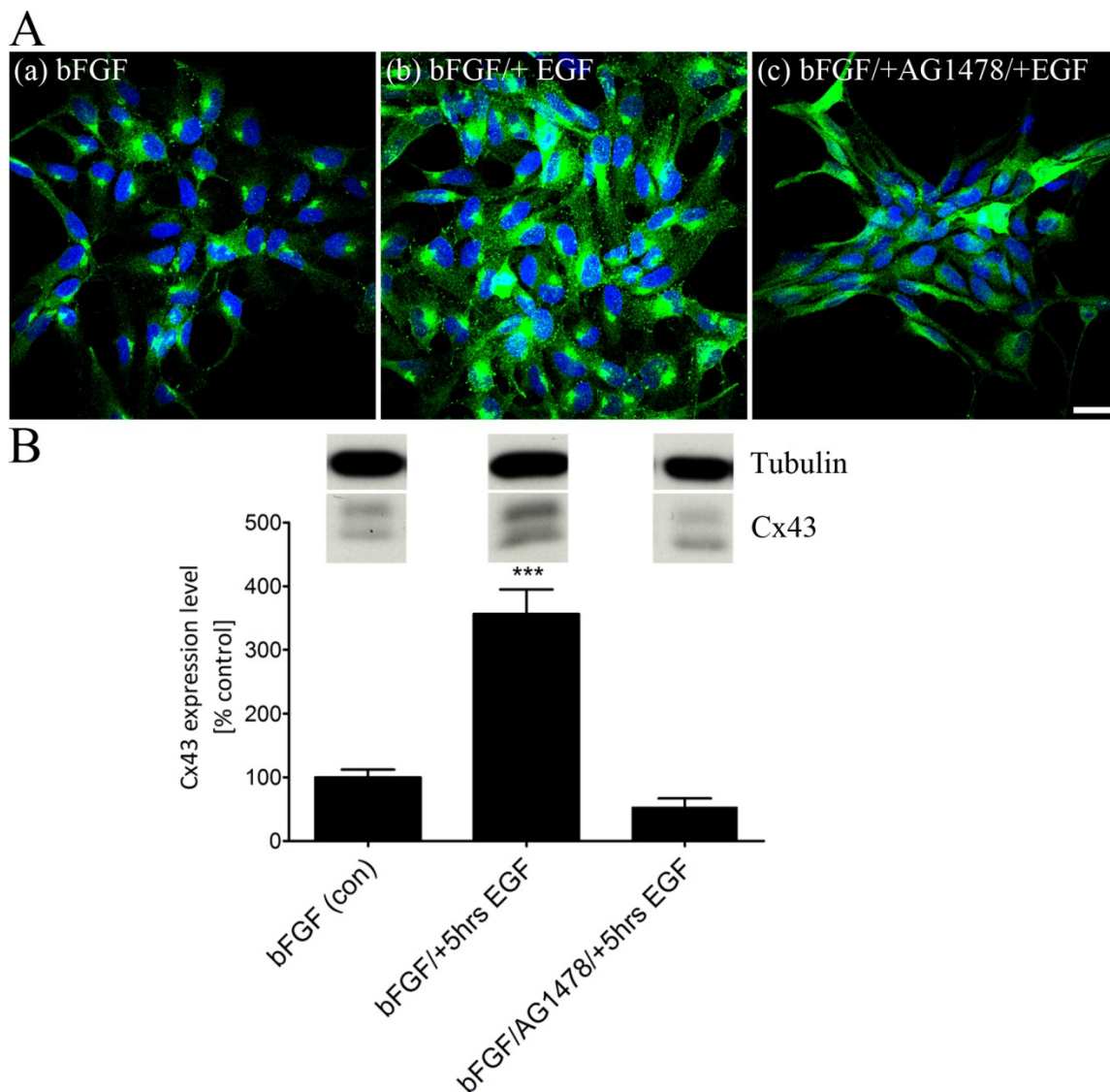
Figure 29 Effect of EGFR inhibition on Cx43 expression. (**A**) Compared to cells treated with bFGF alone alone (a) additional administration of EGF (b) increased the level of Cx43. Pre-treatment with the EGFR-inhibitor AG1478 (c) counteracted the stimulating effect of EGF, indicating the involvement of an EGFR-dependent pathway in the regulation of Cx43 expression. Cells were stained with anti-Cx43 antibody and Hoechst, Bar 20μm. (**B**) Quantitative estimation of Cx43 expression by western blotting demonstrated that AG1478 abolished the effect of EGF, resulting in a similar level of Cx43 as shown for bFGF cultured cells (con). Graph represents data of 3 independent experiments, shown as mean±SEM. Statistical differences were calculated by Student´s t-test ***$P<0.001$.

These data indicate that Cx43 expression in proliferating progenitors is mainly regulated by EGF via an EGFR-dependent pathway. Moreover, the high level of EGFR after EGF-removal might explain the strong impact of EGF on Cx43 expression in differentiated cells (Figure 26).

3.6.3 FUNCTIONAL REGULATION OF CX43 BY EGF

As EGF treatment was shown to enhance Cx43 expression, we evaluated a possible impact of EGF on GJIC by FRAP-microscopy. We assumed that EGF-induced up-regulation of Cx43 might promote gap junctional coupling due to increased GJ plaques formation. Compared to proliferating progenitors grown in EGF and bFGF, GJIC was significantly decreased by 35% when cells were cultured in bFGF alone (Figure 30A). However, addition of EGF for 3h recovered GJIC to the same extent as observed in proliferating control cells. The fact that EGF promoted intercellular communication was verified by administration of AG1487. The EGFR-inhibitor completely counteracted the effect of EGF, resulting in a similar low level of GJIC as shown for bFGF cultured cells. The average fluorescence recovery curves, presented in Figure 30B, demonstrated significant inhibition of gap junctional coupling by EGF withdrawal or EGFR blockage. Accordingly, the comparison of the speed of recovery showed a 2-3-fold increase of recovery halftime after removal of EGF or treatment with AG1478 (Figure 30C).

We already demonstrated that Cx43 is up-regulated by EGF (Figure 25, 26). Using confocal microscopy of Trion X-100 extracted cells, we found that EGF also increased the amount of gap Junctional Cx43 (Figure 31). High amounts of large GJ plaques were observed in cells grown in the presence of EGF and bFGF (Figure 31a) or in EGF alone (Figure 31c). In contrast, removal of EGF strongly reduced the amount of Triton X-100-insoluble Cx43 (Figure 31b). Similar images were acquired when AG1478 was applied to prevent transduction of the EGF signal (Figure 31d).

Taken together, EGF was found to exhibits its stimulating impact on GJIC by increasing the number of functional GJs formed by Cx43.
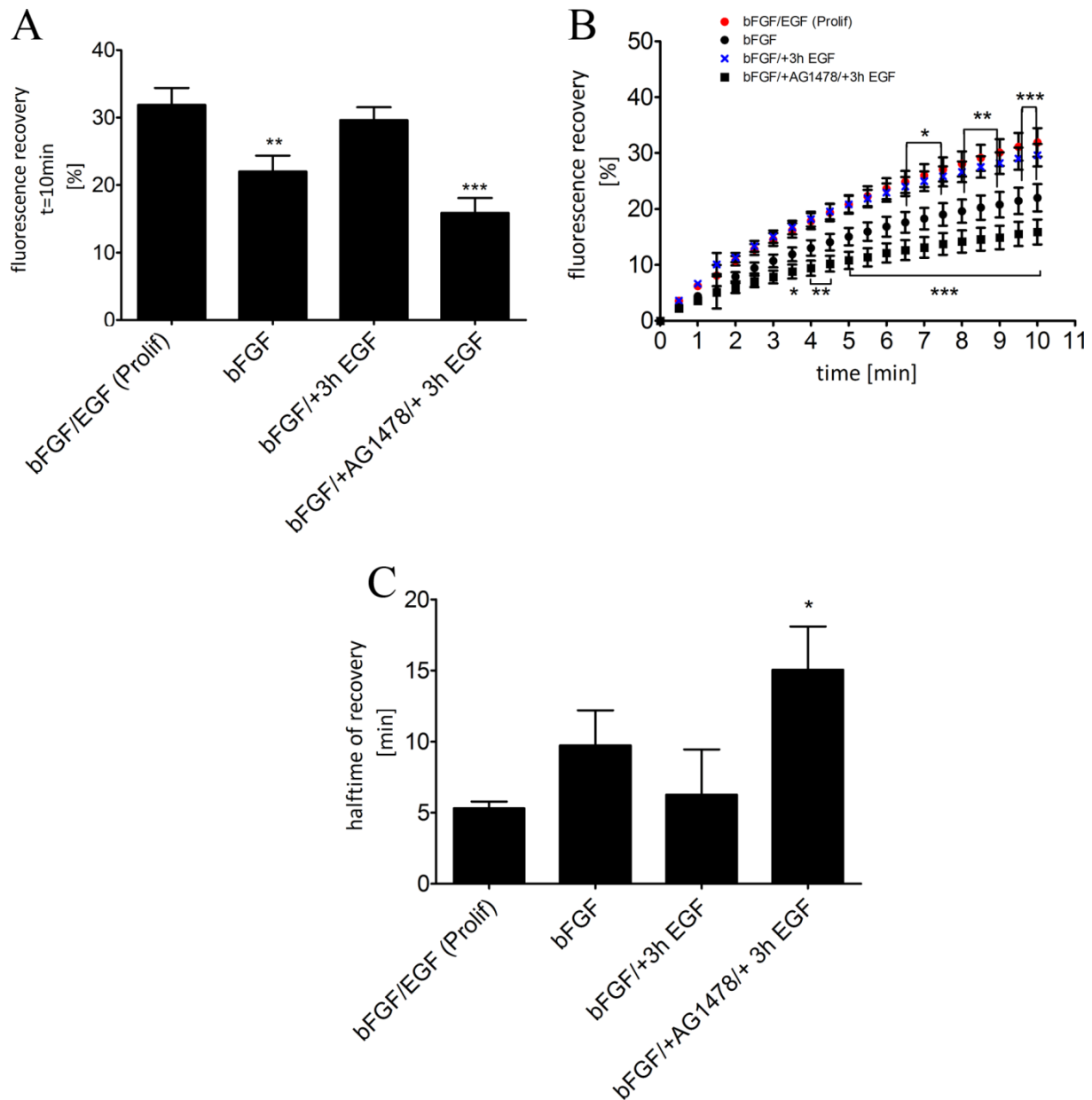
Figure 30 EGF affects GJIC in ReNcell VM197. (**A**) FRAP analysis revealed that treatment with bFGF alone reduced GJIC compared to proliferating cells cultured in both bFGF and EGF. While incubation with EGF for 3h stimulated GJIC, pre-treatment with the EGFR inhibitor AG1478 abolished this effect. (**B**) Average fluorescence recovery curves of cells treated different growth media. (**C**) Halftimes of recovery were calculated by fitting data sets to an exponential equation, indicating a decrease of dye diffusion in cells cultured in bFGF alone or pre-treated with AG1487. Graphs represent data of 3 independent experiments, shown as mean±SEM. Statistical differences were calculated by (A,C) Student´s *t*-test (*$P<0.05$; **$P<0.01$, ***$P<0.001$) or (B) two-way ANOVA $P<0.001$ followed by Bonferroni *post hoc* test.
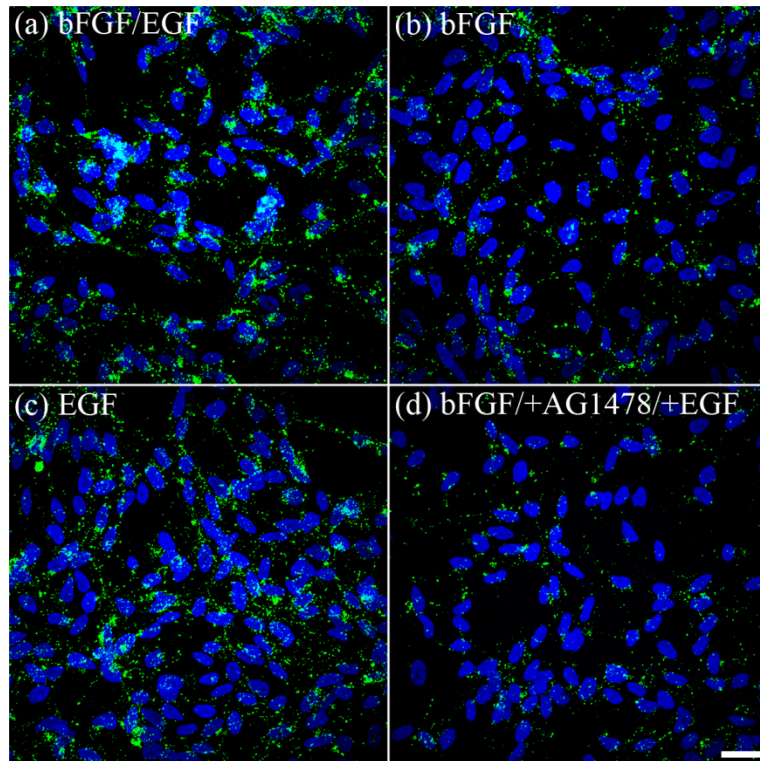
Figure 31 Effect of EGF on gap junctional Cx43. Compared to cells cultured in the presence of both growth factors (a) and EGF alone (c), the amount of Triton X-100-resistant Cx43 drastically declined when cells were grown in bFGF (b). Treatment with AG1487 (d) diminished EGF-induced effects, resulting in a reduced number and size of Cx43-formed GJs. Cells were cultured in different growth condition for 1d and subjected to Triton X-100 extraction. Cells were labelled with Cx43 antibody and Hoechst, Bar 20µm.

3.7 SUMMARY

This work provides evidence for a prominent role of Cx43 and Cx-mediated intercellular communication in regulating the proliferation and differentiation of human neural progenitor cells. We showed that neural differentiation was accompanied by dynamic alterations in gap junctional coupling. As cells were kept in a proliferative state, the extent of GJIC was maintained at a high level. However, induction of differentiation caused strong inhibition of intercellular communication by 50%, followed by a re-increase in the late stage of differentiation, resulting in a more extensive dye coupling as observed in proliferating cells.

Although Cx31 and Cx59 were also expressed by ReNcell VM197, Cx43 was found to be the major GJ-forming protein, which modulates the changes in GJIC during neural development. According to the GJIC profile, proliferating cells demonstrated high expression of Cx43 that was distributed throughout the whole cell with strong accumulation in the Golgi network. Neural differentiation resulted in a decline of Cx43 expression by 80% that re-increased in the late stage of differentiation.

Pharmacological blockage and knockdown experiments using Cx43 siRNA revealed an important function of Cx43, acting on proliferation and differentiation in a channel-independent and channel-dependent manner. While GJ blockage reduces proliferation rate by 16%, knockdown of Cx43 decreased the number of S-phase cells by 50%, suggesting that Cx43 mediated the mitogenic activity of bFGF and EGF. Additionally, we found a strong influence of GJIC and Cx43 on neuronal efficiency. Similar to proliferation analysis, pharmacological blockage reduced neuronal number to a lower extent than Cx43 knockdown. Upon transfection with Cx43 siRNA, we detected a profound decrease of neuronal cells by 50-60%, indicating a strong impact of Cx43 on neuronal cell fate.

We identified EGF as a positive regulator of GJIC that up-regulates Cx43 and thus increases the number of GJ plaques in the plasma membrane. Culturing cells in the absence of EGF or EGFR-blockage retained Cx43 expression at a low level and reduced the extent of GJIC.

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4   DISCUSSION

4.1   ReNcell VM197 – AN *IN VITRO* MODEL SYSTEM TO STUDY THE ROLE OF GJIC DURING NEURAL DEVELOPMENT

The effect of Cx-mediated GJIC on neural differentiation was analysed using the human embryonic neural progenitor cell line ReNcell VM197. Embryonic progenitor cells derived from the early midbrain have similar properties in cell coupling that occur during development. A number of studies had been shown ReNcell VM197 as a simple and accepted model for investigating different aspects of the neural differentiation process *in vitro* (Morgan *et al.*, 2009; Hübner *et al.*, 2010; Lange *et al.*, 2011; Mazemondet *et al.*, 2011). This cell line is easy to manipulate, including high transfection efficiency. ReNcell VM197 allows rapid and controlled differentiation (within days) at a high degree of reproducibility that facilitates long-term study of all different stages of neuronal differentiation.

Moreover, the cell line demonstrates a broad similarity to primary neural progenitors. Cells are maintained in a self-renewal and proliferative state in the presence of EGF and bFGF. This is in agreement with several reports, showing that mitogen treatment of primary progenitors stimulates proliferation and repress differentiation (Johe *et al.*, 1996; Tropepe *et al.*, 1999; Seaberg & van der Kooy, 2002). As cells were kept under proliferation conditions, we detected a strong expression of nestin (Figure 4), a common marker for identifying neural progenitor cells *in vivo* and *in vitro* (Lendahl *et al.*, 1990; Mignone *et al.*, 2004; Crespel *et al.*, 2005).

Moreover, cells can specifically be induced to undergo terminal differentiation at any time by removal of growth factors, resulting in the elaboration of neuronal and glial cell phenotypes, similar to primary precursors (Johe *et al.*, 1996; Dhara & Stice, 2008). While differentiation of primary cells lasts several weeks (Reubinoff *et al.*, 2001), formation of neuronal and glial phenotypes in ReNcell VM197 is already observed after 1d (Figure 4). The fact that immortalized neural progenitors represent a genetically homogenous cell population with an expandable developmental window is also an important benefit of this cell line.

Pharmacological inhibition of GJIC impaired neuronal differentiation of ReNcell VM197, resulting in a lower number of neuronal phenotypes (Figure 20). These data are consistent with several previous reports, indicating an important role of GJIC in neural differentiation (Lo Turco & Kriegstein, 1991; Bittman *et al.*, 1997; Bechberger, *et al.*, 1999; Fields & Stevens-Graham, 2002).Thus, ReNcell VM197 is an appropriate and convenient to use cell model for investigating the impact of gap junctional coupling on neural development.

4.2 ALTERATIONS IN GJ COUPLING ARE MODULATED BY DYNAMIC CHANGES OF CX EXPRESSION

4.2.1 GJIC AND CX43 ARE DOWN-REGULATED AFTER INDUCTION OF DIFFERENTIATION

Neural differentiation in the developing brain is a complex process that is known to be mediated by several intrinsic pathways, by extracellular signals and by cell-cell contacts that can all be regulated by Cx proteins (Gage, 2000; Reubinoff *et al.*, 2001; Belliveau *et al.*, 2006). This regulation can be either achieved by the channel-dependent function of Cx as a GJ component or by direct signalling activity of Cx proteins, which in turn affects the differentiation process (Moorby & Patel, 2001; Wong *et al.*, 2008). A variety of different Cx isotypes was found in stem cells and neural tissue (Nadarajah *et al.*, 1997; de Rivero Vaccari *et al.*, 2007). Cx43 was shown to be the most abundant Cx protein that is widely distributed throughout the central nervous system, i.e. in human stem cells, neurons, astrocytes and oligodendrocytes (Rouach *et al.*, 2002; Huettner *et al.*, 2006). Due to their ability to mediate transport of nucleotides, second messengers, ions or metabolites, GJs allow metabolic and electrical homeostasis and facilitate the flow of information between adjacent cells. This enables the coordination and synchronization of several processes such as cell growth, death, survival and differentiation (Krutovskikh & Yamasaki, 2000; Tabernero *et al.*, 2001).

In the present study we provide evidence for a pronounced role of Cxs and gap junctional communication in regulating both the proliferation and neural differentiation of human neural progenitor cells. We analysed in detail the time-dependent changes in GJIC and Cx expression that occur as cells undergo differentiation. Using FRAP microscopy we demonstrated a dynamic change in GJ coupling of ReNcell VM197 under proliferation and differentiation conditions (Figure 7). While proliferating progenitors showed extensive dye coupling, GJIC was strongly reduced upon initiation of differentiation. This is consistent with studies using a human pluripotential teratocarcinoma cell line NT2/D1, where retinoic acid treatment caused neuronal differentiation that was accompanied by a decrease of dye coupling (Bani-Yaghoub *et al.*, 1997; Bani-Yaghoub, Bechberger, *et al.*, 1999). However, the requirement of decreasing GJIC for differentiation is not only limited to neural cells. Brissette *et al.* (1994) reported that the intercellular transfer of GJ-permeable dyes is significantly reduced during the differentiation of keratinocytes. Our observation that undifferentiated ReNcell VM197 demonstrated extensive GJIC points to an important role of gap junctional coupling in growth factor dependent proliferation of human neural progenitors (discussed in 4.3).

The extent of GJIC can be controlled by several cellular mechanisms. Changes in Cx expression affect the amount and composition of functional GJs, which in turn modulates intercellular communication (Kanaporis *et al.*, 2008). We showed that Cx43, Cx31 and Cx59 are expressed by ReNcell VM197, whereas Cx43 mainly contributed to GJ formation (Figure 8, 15). During the last decades Cx43 was identified as the most widely expressed Cx isomer. It was found to be expressed in at least 34 different tissues, including neuronal and non-neuronal cell types (Rackauskas *et al.*, 2010). Several studies demonstrated the presence of Cx43 in brain tissue, including embryonic mouse neural progenitors, astrocytes and neurons (Nadarajah *et al.*, 1997; Simbürger *et al.*, 1997; Rash *et al.*, 2001; Duval *et al.*, 2002).

In contrast, Cx31 is mainly located in skin and the inner ear and little is known about its function in neural development (Evans & Martin, 2002). Jungbluth *et al.* (2002) described the expression of Cx31 in certain subpopulations within the mouse embryonic hindbrain. A role for Cx31 in neurite outgrowth, a characteristic feature of neuronal differentiation, was shown by Unsworth and colleagues using the human SH-SY5Y neuroblastoma cell line (Unsworth *et al.*, 2007). However, this effect on neuronal differentiation was not associated with the channel-dependent function of Cx31.

In addition to Cx43 and Cx31, Cx59 was also revealed to be expressed in ReNcell VM197 (Figure 8. As one of two Cxs, Cx59 is restricted to human tissue, since no orthologous connexin was found in the rodent genome. Although little is known about this recently discovered Cx, there has been evidence for the localization of Cx59 in the human retina, which is considered by researchers as the "natures brain slice" (Söhl *et al.*, 2010). Compared to Cx43 and Cx31, the expression pattern of Cx59 exhibited no significant changes during the differentiation process (Figure 11). This might suggest that Cx59 does not affect neural differentiation in ReNcell VM197 in a serious manner.

The observed changes in GJIC after induction of differentiation by growth factor removal strongly correlated with a decrease of Cx43 and Cx31 expression (Figure 7, 9, 10) Alhough the expression pattern of Cx31 is similar to the GJIC profile, we detected only few amounts of membrane-bound gap junctional Cx31 (Figure 15). Thus, we excluded a pronounced role of Cx31 in gap junctional coupling of ReNcell VM197. In contrast, Cx43 is the most prominent Cx and we could demonstrate that down-regulation resulted in decreased dye coupling most likely due to impaired GJ plaques formation (Figure 21). This suggests Cx43 as a key target protein in the regulation of intercellular trafficking via GJs.

Changes in Cx expression seem to correlate with distinct steps of neural differentiation. Previous reports demonstrated a dynamic Cx expression rather than constant levels of Cx during neural development (Gulisano *et al.*, 2000; Xia *et al.*, 2000; Leung *et al.*, 2002). In the

developing brain, Cxs were shown to be down-regulated when cells start to differentiate (Rozental *et al.*, 1998; Leung *et al.*, 2002). These considerations strongly suggest a reciprocal relationship between the extent of GJ coupling and Cx43 expression, respectively, and the development of neural phenotypes. As ReNcell VM197 neural progenitors can rapidly differentiate into neurons and glial cells, cell fate commitment already occurs in the first 24h, when gap junctional coupling was reduced (Figure 4, 7). The early stage of differentiation is characterized by exiting from the cell cycle, switch to G1/0 phase and triggering the elaboration of specialized neural phenotypes (Galderisi *et al.*, 2003). This indicates a regulatory function of Cxs and GJs on the cell cycle. A possibility that was confirmed by Bittman *et al.* (1997), reporting that reduced cell coupling decreased the probability of cells to enter S-phase during neurogenesis. Consequently, the G1 phase is prolonged, which promotes neural differentiation. This is in agreement with the observation that the level of ReNcell VM197 in G1/0 reached 96 % within the first 24h of differentiation (Mazemondet *et al.*, 2011). Studies on human lung fibroblasts also described the importance of proper GJIC for cell cycling, showing that GJ closure by cisplatin was associated with a G1 arrest and initiation of premature senescence (Zhao *et al.*, 2004). Since GJs provide a pathway for direct signalling of cell cycle regulating molecules, the decrease of GJIC in differentiating ReNcell VM197 cells could restrain signal transduction, which promotes cell cycle exit. A possible candidate that can be transferred via GJs and regulate cellular processes includes calcium. It was demonstrated by our group that calcium levels change as differentiation is initialized, indicating its importance for neural differentiation (unpublished data). Additionally, it was found that increased intercellular trafficking of calcium facilitates the G1 cell cycle block in human hepatoma cells (Liu *et al.*, 2009). Moreover, other second messengers such as inositol trisphosphate and cyclic adenosine monophosphate are known to be determinants for cell cycling. However, the mechanisms beyond Cx43-mediated cell cycle regulation are also related to the channel –independent function of Cxs, resulting from direct and indirect interactions with different cell cycle-involved proteins (Omori & Yamasaki, 1999; Qin *et al.*, 2002; Johnstone *et al.*, 2010).

The switch in Cx43 expression that occurred as ReNcell VM197 was subjected to differentiation conditions might also suggest an interaction between Cx43 and several key homeostasis proteins. Previous studies have reported that proteins of the Wnt family are crucial for the maintenance of proliferation and for triggering differentiation of neural progenitors (Muroyama *et al.*, 2002; Hirabayashi *et al.*, 2004; Davidson *et al.*, 2007). The importance of Wnt-signalling in regulating cellular behaviour was also shown in ReNcell VM197 (Hübner *et al.*, 2010; Lange *et al.*, 2011). Wnt molecules transduce the signal via β-catenin, which is a key player of the Wnt cascade, acting as a transcription regulating factor

(Moon *et al.*, 2002). Once the Wnt pathway has activated, β-catenin is accumulated in the cytosol and transported to the nucleus, where it can regulate a number of genes that code for cell cycle regulators like cyclin D1 and *c-myc* (Davidson *et al.*, 2009; MacDonald *et al.*, 2009). Using ReNcell VM197, Mazemondet *et al.* (2011) gave evidence for an increase of nuclear β-catenin after induction of differentiation that enhanced by exogenous stimulation with Wnt molecules. As Cx43 was shown to interact with β-catenin at cell-cell contact areas, we assume that the observed high amount of Cx43 in proliferating cells (Figure 9) reduced the pool of free β-catenin available for Wnt signalling (Ai *et al.*, 2000). Consequently, sequestration of β-catenin from the Wnt pathway by Cx43 might prevent the Wnt driven neural differentiation. Interestingly, the Cx43 gene itself is controlled by Wnt, as indicated by association of β-catenin with the promoter of Cx43 (Xia *et al.*, 2010). These data suggest a bidirectional regulation between Wnt/β-catenin signalling and Cx43 expression, which in turn influences the development of neural progenitors.

4.2.2 COMPLETION OF NEURAL DIFFERENTIATION REQUIRES UP-REGULATION OF GJIC AND CX43

Strikingly, we found a distinct re-increase of dye coupling with proceeding differentiation (Figure 7). Likewise, Cx43 expression increased in the late stage of differentiation, supporting the notion that Cx43 modulates gap junctional coupling in ReNcell VM197. Real time PCR analysis of Cx43 mRNA expression during rat-midbrain development revealed similar alterations, showing a decrease of Cx43 mRNA, followed by up-regulation in the late stage of differentiation and mature brain (Leung *et al.*, 2002). However, this dynamic expression pattern of Cx43 has not been reported before in human neural progenitors. Compared to undifferentiated progenitors where GJIC is needed to keep them in a proliferative state, we hypothesize that gap junctional coupling is now required for establishing a functional neural network. Beside metabolic coupling, GJs provide electrotonic interconnections between neurons and glial cells (Bennett & Zukin, 2004). Proper GJIC was found to synchronize the Na$^+$ level of hippocampal astrocytes, which serves to coordinate the physiological responses that depends on Na$^+$ ions and membrane potentials (Rose & Ransom, 1997). Moreover, the frequency of oscillations of the membrane potential in glial networks was shown to modulate the activity of neuronal cells (Alvarez-Maubecin *et al.*, 2000a). In neurons, GJs form electrical synapses that may contribute to generation, maintenance and synchronization of neuronal firing patterns (Nagy *et al.*, 2004). Note that only 10-12% of cells differentiate into neurons (Figure 4B, C). Thus, the extent of coupling measured by FRAP mainly relies on glial cell communication. Studies performed in cultures and brain slices have demonstrated that astrocytes form a functional syncytium through gap junctional coupling

(Dermietzel & Spray, 1998; Giaume & Venance, 1998). A leading part for astrocytic GJs has been attributed to calcium signalling (Giaume & Venance, 1998; Verkhratsky *et al.*, 1998; Weissman *et al.*, 2004). The propagation of calcium waves allows transduction of information within the glial network and it was shown to be involved in neuronal activity (Verkhratsky *et al.*, 1998). This raises the possibility that the re-increase of GJIC after 2d (Figure 7) is important to ensure an accurate exchange of calcium ions between mature glia cells, which promotes the establishment of proper glial-neuron interactions and participates in signalling functions of the neuronal network. Although the majority of ReNcell VM197 progenitors acquire a glial cell fate, we do not exclude the involvement of neurons in GJIC of differentiated neural progenitors, since neurons were also demonstrated to interact via GJs (Nagy *et al.*, 2004; Söhl *et al.*, 2005).

However, the expression profile of Cx43 does not exactly correspond to the kinetic profile of GJIC. The re-increase of the Cx43 level was distinctly smaller than the re-increase of GJIC during the late phase of differentiation (Figure 7, 9). Moreover, the increase of gap junctional coupling was accompanied by a delayed up-regulation of Cx43. As mentioned above, several studies have shown that specific Cx subtypes can be differentially expressed during differentiation which results in altered cell-cell coupling (Dermietzel *et al.*, 1989; Nadarajah *et al.*, 1997; Cina *et al.*, 2007). Based on western blot data and subsequent analysis by immunofluorescence microscopy we now provide evidence for the presence of 2 more Cxs in ReNcell VM197 neural progenitors, namely Cx59 and Cx31 (Figure 8). Thus, the difference between the dynamic profiles of GJIC and the Cx43 level might result from an altered expression of Cx59 and Cx31 that modulates GJIC, which in turn may contribute to proper neural differentiation. Expression analysis showed a marked up-regulation of Cx31 after 3d of differentiation (Figure 10). However, very small amounts of Cx31 and the absence of Cx59 in GJs, suggested that these Cxs are not involved in channel-dependent functions (Figure 15). The large variety of Cx isotypes in neural progenitor populations and the alteration of Cx expression during neural development indicate the presence of further, unknown Cxs that might control GJIC in ReNcell VM197. Beside transcriptional regulation, GJ gating can be influenced more rapidly by changing the phosphorylation status of Cxs (Kanemitsu & Lau, 1993; Lampe & Lau, 2000). Likewise, Cx composition, pH or voltage can affect junctional conductance (Francis *et al.*, 1999; Kanaporis *et al.*, 2008). Alterations of these parameters during differentiation may lead to selective activation of GJs, so that they become functional under certain physiological conditions. This might also explain why up-regulation of Cx43 protein at the end of neural differentiation (5-8d) is not accompanied by an additional increase of gap junctional communication in ReNcell VM197.

4.3 ROLE OF GJIC AND CX43 IN PROLIFERATION

4.3.1 EGF REGULATES GJIC AND CX43 EXPRESSION

Several different soluble factors are present in the developing brain, regulating proliferation and differentiation of neural progenitors. These comprise neurotrophins, neurotransmitters, extracellular matrix proteins and growth factors (Cattaneo & McKay, 1990; Davis & Temple, 1994; LoTurco *et al.*, 1995; Kuhn *et al.*, 1997). Here, we used bFGF and EGF to maintain neural progenitors in a proliferative state. Beside its role in driving proliferation, growth factor signalling can also modulatory affect functional activity and expression of Cx proteins and GJIC (Maldonado *et al.*, 1988; Rozental *et al.*, 1998). EGF is a powerful mitogen that is capable of stimulating cell growth, cell survival and differentiation (Carpenter & Cohen, 1979; Yamada *et al.*, 1997). It was found to be synthesized in several regions of the brain, e.g. hippocampus, cortex and cerebellum, where it mediates cell growth (Yamada *et al.*, 1997). Moreover, it plays a pivotal role in the proliferation of neural stem cells, derived from embryonic or adult brain (Reynolds *et al.*, 1992; Gritti *et al.*, 1995). Using radioimmunoassay, EGF was detected at concentrations ranging from 0.33 to 0.99ng/ml wet weight of adult brain tissue (Schaudiessb *et al.*, 1989). Although a non-physiological dose of EGF was used for culturing ReNcell VM197 progenitors, previous stem cell studies for proliferation or differentiation revealed that cells grown in EGF at concentrations up to 50ng/ml maintain their physiological self-renewal and neural differentiation potential (Kitchens *et al.*, 1994; Yu *et al.*, 2006; Hartfield *et al.*, 2011). In our study, treatment with 20ng/ml EGF maintained the expression of Cx43 at high level. Moreover, additional incubation with EGF was shown to up-regulate Cx43, which in turn increased GJIC (Figure 25, 26). The impact of EGF on GJ coupling is controversially discussed for a number of different cell types, including neural and non-neural cells. Ueki *et al.* (2001) found that EGF caused down-regulation of Cx43 in cortical rodent astrocytes. This is supported by studies with HEK cells and liver epithelial cells, where EGF was demonstrated to inhibit Cx43-mediated gap junctional communication (Cameron *et al.*, 2003; Leithe & Rivedal, 2004). Similar observations were made by Park and colleagues, showing a negative effect of EGF on GJIC in embryonic mouse stem cells (Park *et al.*, 2008). Conversely, EGF is known to induce an enhancement of gap junctional communication and Cx43 expression in kidney epithelial cells and granulosa cells (Vikhamar *et al.*, 1998; Kennedy *et al.*, 2003). Although less studies have implicated EGF in stimulating GJIC of stem cells, it was reported that additional treatment with mitogens in neural progenitors can promote Cx43 expression and GJIC (Nadarajah *et al.*, 1998). In agreement with these findings, we identified EGF as a positive regulator for Cx43 expression in human neural progenitor cells that increase gap junctional coupling under proliferating conditions (Figure 30).

The findings above suggest that EGF exerts its effects on GJIC in a cell type-dependent manner. Although the mechanism of action is not well understood, two major principles were found to control cell-cell coupling: 1) through synthesis, degradation and incorporation of Cx proteins in the plasma membrane or 2) by local regulation of the gating of existing GJs. Our results demonstrated that EGF-induced up-regulation of Cx43 expression and led to an increased insertion of newly synthesized Cx43 proteins in the plasma membrane (Figure 26Figure 31). However, there are additional regulatory mechanisms that can change GJ permeability more rapidly than alterations at the protein level. Phosphorylating events were demonstrated to be important in regulating Cx processing and function. A number of Cxs, including Cx43, 31, 50 and 56 are phosphorylated in the C-terminal region that is located in the cytoplasm (Lampe & Lau, 2000). We found that EGF transduced its signal via an EGFR-dependent pathway (Figure 29) that is known to recruit and phosphorylate/activate a number of important signalling elements such as phospholipase C or MAP kinases (Lo *et al.*, 2006). *In vitro* studies gave evidence that Cx43 is a substrate of these kinases and phosphorylation can influence GJIC in a positive or negative manner (Sáez *et al.*, 1998; Lampe *et al.*, 2000). The phosphorylation of specific serine sites in the Cx43 protein can attenuate GJIC very quickly (Kanemitsu & Lau, 1993). In contrast, phosphorylation is important to maintain cell-cell coupling as the majority of gap junctional Cx43 was found to be phosphorylated. Nevertheless, EGF-stimulated phosphorylation might occur over the basal level of serine phosphorylation and thus led to changes in GJ permeability. However, we assume that EGF achieves positive changes in GJ conductance by up-regulation of Cx43 rather than by phosphorylation.

4.3.2   GJIC MAINTAINS NEURAL PROGENITORS IN A PROLIFERATIVE STATE

Previous studies have shown that proliferating neural progenitors communicate via GJs and pharmacological blockage, using halothane or 1-oct, decreased the number of cells entering S-phase (Bittman *et al.*, 1997; Cai *et al.*, 1997). This relationship between mitogenic activity of neural progenitors and gap junctional coupling is also supported by our data. Proliferation of ReNcell VM197 progenitors was maintained by administration of both bFGF and EGF at concentrations that were generally used for culturing of neural progenitors (Kitchens *et al.*, 1994; Yu *et al.*, 2006; Hartfield *et al.*, 2011). The fact that proliferating cells demonstrated strong Cx43 expression, accompanied by extensive GJIC, highlights its importance for proper GJ coupling during cell growth (Figure 7, 9). Moreover, we demonstrated that the presence of EGF in the culture media was associated with a high expression level of Cx43 (Figure 26). Treatment with the well-known GJ blocker CBX significantly reduced the mitogenic effects of

EGF and bFGF on proliferating neural progenitors most probably due to reduced GJ coupling (Figure 20).

Although many studies reported about a growth factor-dependent regulation of Cxs and GJIC, less is known about an involvement of Cxs in mediating the proliferative effects of EGF and bFGF, which allows self-renewal of neural progenitors. However, it is possible that mitogens or survival factors induced by bFGF/EGF can be transferred via GJs to repress neural differentiation and promote proliferation. The requirement of cell-cell interactions for precursor cell growth was confirmed by the fact that singly dissociated cells from the mouse embryo demonstrated no proliferative activity in the absence of growth factors, whereas those in clusters proliferate for several days before undergoing differentiation (Ghosh & Greenberg, 1995). Consistently, the effect of bFGF was found to be only evident when cells are in contact with each other. A relationship between growth factors and Cx43 in ReNcell VM197 was proven by the fact that EGF stimulated Cx43 expression, leading to increased GJ plaques formation (Figure 26, 30, 31). Moreover, the proliferative activity of both EGF and bFGF was reduced as cells were transfected with Cx43 siRNA (Figure 22). This is in agreement with observations made by Cheng and colleagues showing that the mitogenic activity of bFGF increased as proliferating progenitors were cultured at high density and GJ blockage abolished this effect (Cheng *et al.*, 2004). Hence, we assume that Cx43 promotes the growth factor driven proliferation of neural progenitors due to a signalling mechanism that requires the presence of gap junctional cell-cell contacts.

The exchange of molecules between adjacent cells is predominately dependent on the diameter of the GJ channel and thus the intercellular transfer is limited to molecules up to 1kD in size. The identity of compounds that are transported via GJs and promote the survival of neural progenitors is largely unknown. However, some small molecules were suggested to be involved in mediating growth factor signalling via GJs, including calcium and ATP. The former one is a central key player in the signal transduction system of neural stem cells that can easily pass GJs (Pearson *et al.*, 2004) .EGF and bFGF were shown to elevate calcium levels in several different cell types, e.g. neural crest neurons and ganglion neurons (Distasi *et al.*, 1995, 1998; Munaron *et al.*, 1995; Ma & Sansom, 2001). Accordingly, triggering of calcium responses by growth factors was found to be critical in maintaining the self-renewal state of rodent neural precursors (Maric *et al.*, 2003). Calcium-imaging studies in the developing retina revealed that GJs orchestrate proliferation of progenitors by propagation of calcium, indicating that functional GJs are required for calcium signalling (Pearson *et al.*, 2004).

Like calcium, ATP is also capable to cross GJ channels and trigger cell growth-specific events in connected cells. Nucleotides represent an important type of extracellular ligands that are

indispensable for neural development, including regulation of migration, proliferation and differentiation (Neary & Zimmermann, 2009). In addition, nucleotides were reported to interact with growth factor receptor-dependent pathways. Neural stem cells, cultured in the presence of bFGF and EGF, demonstrated an increase of proliferation when ATP was co-applied (Mishra *et al.*, 2006). Moreover, this mitogenic effect of ATP were also observed in astrocytes and radial glia (Fields & Stevens-Graham, 2002; Weissman *et al.*, 2004). Most studies have revealed that ATP exerts its stimulating effect on proliferation mainly by an extracellular mechanism. GJ hemichannels that connect intra- and extracellular spaces represent an important source for ATP release in neural progenitors (Pearson *et al.*, 2005). In astrocytes, ATP is released via hemichannels that comprise Cx43, similar to the hemichannels found in our cell line (Figure 16) (Stout *et al.*, 2002). In summary, these findings raise the possibility for a Cx43-dependent transport of ATP between adjacent cells or into the extracellular environment, which promotes the growth factor-dependent proliferation of neural progenitor cells.

The impact of GJ coupling on cell growth was analysed using pharmacological GJ blockage and Cx43 knockdown. Both treatment with CBX and Cx43 knockdown reduced GJIC to a similar extent (50-60%, Figure 19, 21). Interestingly, siRNA-mediated gene silencing resulted in much stronger decrease of cell growth than observed after treatment with CBX. These data led us to assume that growth factor signalling in neural progenitors is also affected by a mechanism that is unrelated to the channel function of Cx43. This functional activity in the control of proliferation seems to be mediated by several interactions between Cx43 and other important proteins that possess signal transduction abilities. We found that ZO-1 colocalized with Cx43 in GJ plaques (Figure 15). ZO-1 has been linked to regulation of cell cycle progression and proliferation by activating gene transcription (Balda *et al.*, 2003; Sourisseau *et al.*, 2006). Although ZO-1 was originally reported as a tight junction protein, the interaction with a number of Cxs is now well characterized (Giepmans, 2004). It was proposed that ZO-1 serves as a scaffold protein, recruiting other yet unidentified signalling molecules, which arise from growth factor stimulation (Giepmans & Moolenaar, 1998). Thus, reduction of Cx43 by gene knockdown might prevent proper proliferation due to impaired ZO-1 mediated growth factor signalling.

Cx43 was also reported to directly bind α- and β-tubulin and microtubules at the cell periphery were shown to terminate at Cx43-formed GJs (Giepmans *et al.*, 2001). Consistently, we gave evidence for a relationship between Cx43 and the microtubule cytoskeleton as revealed by administration of nocodazole that caused both disruption of microtubules and severe changes in Cx43 distribution (Figure 18). As microtubules form a

dense network and span from the plasma membrane to the nucleus, they provide a suitable system for the directional flow of information. EGF and bFGF transduce their signal by different extracellular regulated kinases that interact with microtubules *in vivo* and *in vitro* (Mandelkow *et al.*, 1992; Reszka *et al.*, 1997). Hence, alterations in microtubule properties will change the efficiency of growth factor signal transmission. It is undisputed that Cx vesicles are transported along microtubules throughout the whole cell. However, there is increasing evidence for an influence of Cx binding on tubulin dynamics. Microtubules were found to grow more frequently to Cx43 membrane plaques, where they persist 3.5 times longer (Shaw *et al.*, 2007). Cx43 mutants, lacking the tubulin binding domain demonstrated altered cytoskeletal architecture, which emphasizes a modulating function of Cx43 in microtubule behaviour that in turn might affect protein kinases activated by EGF/bFGF (Francis *et al.*, 2011).

In the context of the findings above, our data led to the assumption that Cx43 positively influences bFGF and EGF-mediated proliferation in neural progenitors by a mechanism that is quite different from its channel-forming properties. Most of the channel-independent functions of Cx43 were associated with the cytoplasmic C-terminal tail, suggesting it as a regulatory region for Cx43-mediated cell growth. Truncation of the C-terminus of Cx43 maintained GJIC, indicating that it is not essential for GJ formation, including assembly, membrane insertion and gating (Omori & Yamasaki, 1999). However, several studies demonstrated an impact of extra- and intracellular signals on GJ gating that was mediated by the C-terminus (Liu *et al.*, 1993; Homma *et al.*, 1998). Interestingly, the C-terminus of Cx43 has also been shown to be localized in the nucleus. Exogenous expression of the C-terminal portion of Cx43 in transformed HeLa cells and cardiomyocytes revealed the ability of the C-terminus to enter the nucleus and affect proliferation (Dang *et al.*, 2003). This indicates a possible role for Cx43 in regulating the expression of cell growth-regulatory genes by interacting directly or indirectly with chromatin or transcription-factor complexes. Although, there is no data about the influence of the carboxyl-tail on gene transcription in neural progenitors, EGF or bFGF might induce cleavage of Cx43, resulting in the release of the C-terminus fragment that is translocated into the nucleus to activate or inhibit specific target genes. Accordingly, using microarray, several growth-regulatory genes were identified that are synergistically expressed with Cx43 (Kardami *et al.*, 2007).

Additionally, Cxs were found to control gene expression by sequestration of transcription factors in the cytoplasm. Thus, changes in Cx expression alter the balance between factors bound to Cx and those free to activate genes in the nucleus. This was reported for the Wnt pathway that is known to regulate proliferation and differentiation in neural stem cells (Viti *et al.*, 2003; Zechner *et al.*, 2003). The transcription factor β-catenin is the key protein in the

Wnt-cascade and it directly binds to Cx43 (Giepmans, 2004). Wnt and growth factor signalling cross regulate each other by several mechanisms, which alter the intracellular distribution of β-catenin (Ciruna & Rossant, 2001; Huber & Weis, 2001). Israsena *et al.* (2004) reported that treatment with bFGF can control the expression of β-catenin which promotes the self-renewal state of neural precursors. Hence, binding of unbound β-catenin by Cx43 might represent a mechanism that controls growth factor-induced signal transduction. This is in agreement with the finding that the level of β-catenin in the nucleus of ReNcell VM197 neural progenitors augmented after removal of EGF and bFGF (Mazemondet *et al.*, 2011), accompanied by a strong decrease of Cx43 expression (Figure 9). The lower level of Cx43 in the cell might result in a higher amount of unbound β-catenin, which facilitates the nuclear shuttling and gene regulation.

Likewise, the transcription factor ZONAB colocalizes with ZO-1 and Cx43 in neural cell types (Kardami *et al.*, 2007). ZONAB interacts with gap junctional Cx via the linker ZO-1 (Vinken *et al.*, 2011), and colocalization of the later ones was demonstrated in proliferating ReNcell VM197 (Figure 15). ZONAB was found to accumulate in the nucleus and bind to promoter regions of genes encoding a number of proteins responsible for proliferation, including cyclin D1 or cdk4 (Dbpa *et al.*, 2006). Although these indications might point to an involvement of Cx43 in regulating the transcriptional efficiency of ZONAB by sequestration, there are no reports showing alteration in ZONAB activity upon treatment with EGF or bFGF. Additional studies are required to unravel whether Cx binding of ZONAB is involved in growth factor signalling.

In summary, the high expression level of Cx43 in proliferating neural progenitors is suggested to promote the undifferentiated and proliferative state. On the one hand, GJs provide a pathway that mediates the mitogenic activity of EGF and bFGF. The increased gap junctional coupling allowed intercellular exchange of survival factors that are either related or unrelated to growth factor signalling. On the other hand, Cx43 facilitates the signal transduction process induced by EGF and bFGF by a channel-independent mechanism that encompasses the interaction with several important signalling proteins.

4.4 CX43 INFLUENCES NEURONAL DIFFERENTIATION IN NEURAL PROGENITORS

One hallmark in the formation of a functional neural network during development is the generation of correct cell types in the appropriate region of the central nervous system. This process of neural cell fate determinations is controlled by different extra and intracellular signals (Cepko *et al.*, 1996; Edlund & Jessell, 1999). Emerging evidence suggests that Cxs, in particular their GJ-forming ability, are involved in cell fate decisions of neural stem cells.

Approximately half of the 21 Cxs were found to be expressed in the central nervous system and they are differentially regulated during neural development with specific expression patterns dependent on stage of development and cell type (Huettner *et al.*, 2006). Neural progenitors maintained in culture are capable to differentiate into neurons and astrocytes, similar to the neural development in the brain. In this study, we showed that neuronal differentiation of ReNcell VM197 is accompanied by dynamic alterations of GJIC (Figure 4, 7), which suggests that the extent of GJ coupling is an important factor for the elaboration of neuronal phenotypes. This was confirmed by pharmacological inhibition of GJIC, leading to a decrease in neuronal efficiency by 25% (Figure 19). Our observations are supported by the group of Bani-Yaghoub *et al.* (1999), using the human pluripotential teratocarcinoma cell line NT2/D1, which possesses many features of neuronal progenitor cells. They demonstrated that administration of CBX represses the ability of cells to acquire a neuronal cell fate. Consistently, GJ blockage also interferes with astroglial and neuronal differentiation of P19 embryo carcinoma cells (Bani-Yaghoub, Underhill, *et al.*, 1999). In proliferating mouse neural progenitors, CBX was reported to induce alterations in cell density and cellular morphology (Duval *et al.*, 2002). In contrast, these negative effects of GJ blockage were not detected when cells were allowed to differentiate. The finding indicates that proper GJIC might be crucial for the early stage of neural development when cells leave their proliferative state and undergo differentiation.

How GJ coupling can modulate the differentiation process and trigger the elaboration of certain cell types is controversially discussed in literature. Since GJs provide a pathway that allows flux of information, specific molecules can cross GJ channels and activate signal cascades in the target cell, leading to cell fate determination. The small amino acid taurine might be an attractive candidate for a cell fate regulator that can be transferred via GJs between adjacent cells (Spoerri *et al.*, 1990; Wu *et al.*, 2005). It has been proposed that taurine acts as trophic factor in the retina and in the central nervous system, promoting the survival of neuronal phenotypes (Chen *et al.*, 1998). Additionally, taurine induced an increase of neurons, formed during the differentiation process of mouse neural progenitors, which is similar to the development of human progenitors. This suggests a possible involvement of taurine in ReNcell VM197 differentiation.

Further, there is emerging evidence that GJs are permeable for small RNAs, which can regulate important aspects of development and physiology by controlling gene expression (Pasquinelli & Ruvkun, 2002; Bartel *et al.*, 2004; Valiunas *et al.*, 2005). MicroRNAs were demonstrated to be expressed in embryonic stem cells where they were attributed to the control of self-renewal and pluripotency (Suh *et al.*, 2004; Wolvetang *et al.*, 2007).

Exogenously-induced alterations of the small RNA processing complex impaired cell cycle progression, leading to a decrease of proliferation rate compared to the wildtype cells (Murchison *et al.*, 2005). Moreover, microRNAs were found to be required for the effective gene silencing of stem cell markers as cells undergo differentiation (Wang *et al.*, 2007). The transfer of RNA oligonucleotides occurs in a Cx-specific manner. While GJs formed by Cx32 and Cx26 are not permeable to siRNA, channels composed of Cx43 allow passage of these oligonucleotides (Valiunas *et al.*, 2005). We have found that Cx43 acts as a major GJ-forming protein in ReNcell VM197 (Figure 15, 21), raising the possibility for a gap junctional shuttling of small RNAs in human neural progenitors that influences transcription of genes coding for proteins critical in progenitor proliferation and neural development.

Although we did not observe a strong increase of apoptotic cells upon Cx43 knockdown, we cannot exclude the possibility that down-regulation resulted in an increase of neuronal cell death rather than in altered cell fate decisions. Hence, this might imply a neuroprotective function of GJIC in neural progenitors. Under control conditions ReNcell VM197 was shown to differentiate into two distinct cell types 1) neurons and 2) glial cells, identified by β(III) tubulin and GFAP. Approximately 10-12% of cells differentiated into neurons, whereas 88-90% acquired a glial cell fate (Figure 4C). In differentiated progenitors, these large amounts of glial cells formed an extensive syncytium in which neurons were embedded (Figure 4B). Functional assays have demonstrated that cytoplasms of astrocytes and neurons are coupled via GJs (Alvarez-Maubecin *et al.*, 2000b). Glial-neuron interactions have been implicated in neuroprotection, suggesting that the observed decrease of neurons upon Cx43 knockdown (Figure 23) might also result from a loss of intercellular coupling between early neuronal and non-neuronal cells (Kirchhoff *et al.*, 2001). Even though we did not show specific dye coupling between neurons and glial cells, we gave evidence for the expression of the GJ-forming protein Cx43 in both GFAP and β(III) tubulin positive cells (Figure 12), which is consistent with several previous reports (Bittman & LoTurco, 1999; Rouach *et al.*, 2002; Nagy *et al.*, 2004). The neuroprotective impact of glial GJIC was investigated by comparison of neuronal sensitivity to oxidative stress. The blockage of gap junctional coupling in glial cells increased neuronal cell death after exposure to oxidative agents (Blanc *et al.*, 1998). In accordance, using a mixed neural culture of neurons and Cx43-deficient astrocytes, Naus *et al.* (2001) were able to clarify a correlation between astrocytic GJs and neuronal viability. It was suggested that GJIC impede cell death by shuttling of survival factors between healthy cells and their dying neighbours or by dilution of toxic substances via GJs (Nakase *et al.*, 2003; Contreras *et al.*, 2004).

Taken together, our data enabled us to propose a model that implies a dual function of GJIC during the neural differentiation process of human embryonic progenitors (Figure 32). In

proliferating progenitors, strong GJIC is necessary to maintain the self-renewal state by mediating the proliferative effects of growth factors. As growth factors like EGF and bFGF induce an increase of intracellular mitogenic molecules, GJs enable the rapid exchange of these molecules within coupled progenitor populations that in turn enhances their proliferative activity. In contrast, GJ coupling is reduced in the early stage of differentiation, which facilitates the exit from cell cycle and cell fate commitment to specific neural phenotypes. However, a re-increase of GJIC is necessary to complete neural differentiation and allow the establishment of a functional neural network. GJs may provide a pathway for the exchange of ions, metabolites and other molecules capable to modulate certain properties of the neural network, including electrotonic coupling or cell composition.
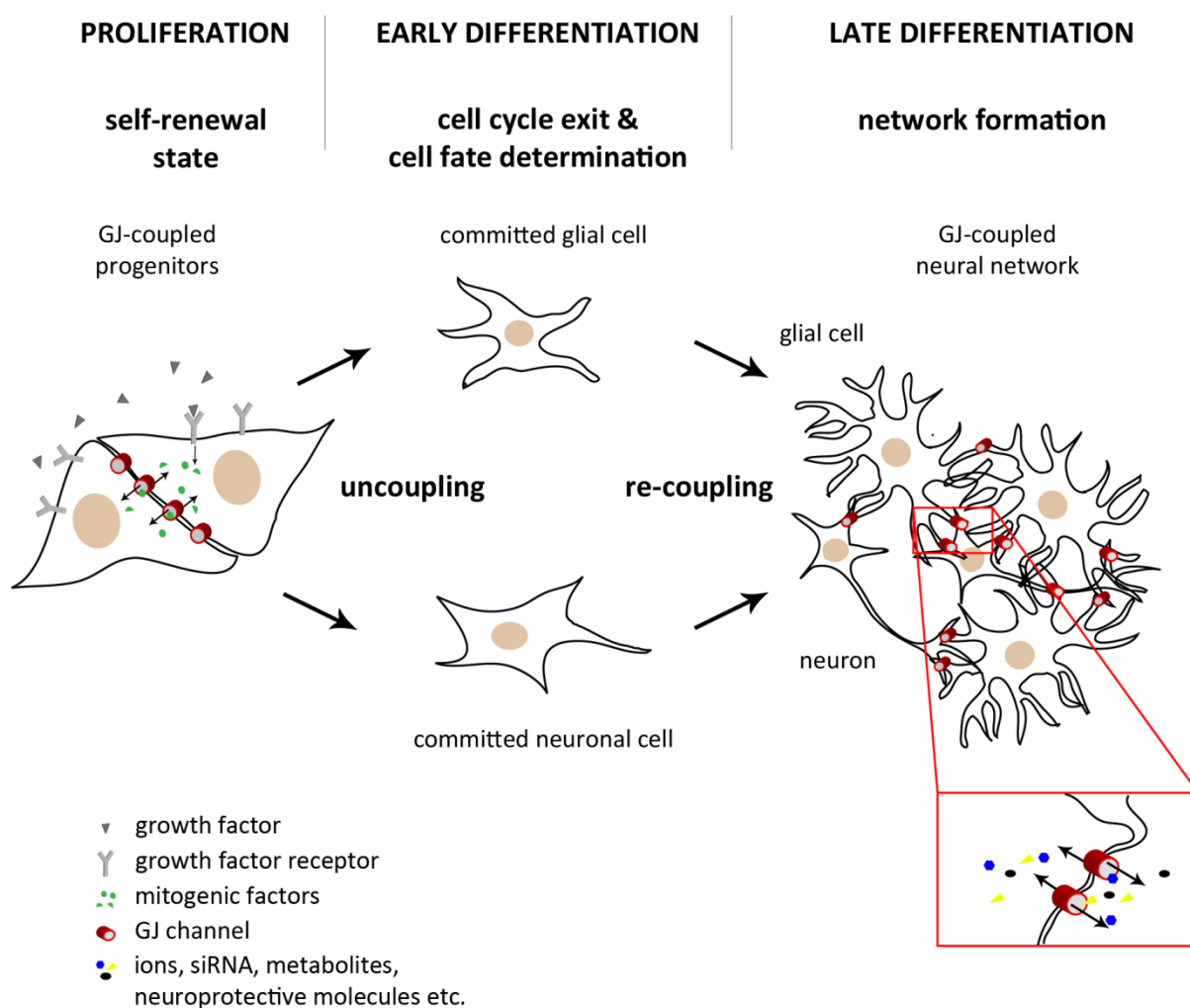
Figure 32 Proposed model for a dual function of GJIC in the development of human neural progenitors. In proliferating progenitors, GJIC is required to maintain cells in a self-renewal state by mediating the proliferative effects of growth factors. Growth factor-induced signaling produces an increase of mitogenic molecules that are exchanged via GJs and enhance proliferation in coupled neural progenitors. In the early stage of differentiation GJIC is reduced, which facilitates both the exit from cell cycle and cell commitment to either a glial or neuronal lineage. However, a late re-increase of GJIC is necessary to complete differentiation and allow the establishment of a functional neural network, by providing a pathway for the exchange of ions, metabolites and other important molecules that modulates the neural network.

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This model only encompasses the channel-forming ability of Cx proteins, but several reports also implicated a modulating function of Cxs on neuronal differentiation that is not directly correlated with GJ coupling and molecule exchange between adjacent cells. We demonstrated that down-regulation of Cx43 by siRNA induced more severe effects on neuronal development than pharmacological inhibition of GJIC. Although both CBX treatment and knockdown of Cx43 decreased GJIC in a similar extent, impaired Cx43 expression reduced the neuronal number by 50%, which was 2 times stronger as observed after pharmacological blockage (Figure 19, 20, 21, 23). Our findings are in agreement with data showing an involvement of Cx43 in neuronal differentiation of embryonic cells that was not associated to its channel-forming activity (Santiago *et al.,*2010). Accordingly, Cx36 was also reported to influence neuronal cell fate. While shRNA-mediated knockdown of Cx36 decreased the number of neurons, overexpression increased neurogenesis (Hartfield *et al.,* 2011). It was suggested that the presence of Cxs might enhance the amount of cell-cell contacts, which in turn stimulates neuronal differentiation. Using multipotent cortical stem cells, Tsai and colleague revealed a strong impact of cell density on cell-type composition and Cx43 was identified as an important molecule that is involved in cell-cell contact mediated signalling (Tsai & McKay, 2000; Parekkadan *et al.*, 2008).

The ability of Cx43 to directly or indirectly interact with a number of proteins supports the notion of Cx43 as a mediator in cell signalling events (Giepmans, 2006). As reviewed by Edlund & Jessell (1999), cell fate decisions in progenitor cells are driven by growth factors, neurotrophic factors, cytokines and other soluble or insoluble cues and Cxs themselves can be regulated by them. Thus, alterations in Cx43 expression may change the way in which neural progenitor cells respond to extracellular signals resulting in altered cell fate decisions as we observed by a reduced neuron number in Cx43-deficient cells (Figure 23). The nerve growth factor was reported to promote the survival and differentiation of neurons in the central and peripheral nervous system via a mechanism that includes the phosphorylation of Cx43 and a subsequent increase of GJIC (Cushing *et al.*, 2005). Although, there is no data about endogenous regulation of nerve growth factor and its receptor in ReNcell VM197 development, these findings support the hypothesis that Cx43 might mediate neurotrophin signalling in human progenitors. As discussed above, neuronal differentiation of ReNcell VM197 progenitors is largely driven by Wnt signalling (Mazemondet *et al.*, 2011). Due the interaction with β-catenin, Cx43 is suggested to be part of the signal transduction process that lead to cell fate determination (Giepmans, 2006).

In summary, our data suggest an important role for Cx43 in regulating the development of human neural progenitor cells. Although GJs provide an important pathway for small diffusible cell fate regulators, neuroprotective molecules and metabolites, Cx43 also

mediates neural differentiation in a channel-independent manner by direct protein-protein interactions.

4.5 PERSPECTIVES

Investigations on human neural embryonic cells are still at an early stage and intense research is required to elucidate the mechanisms that trigger differentiation and ensure proper communication within a cell population. We have demonstrated ReNcell VM187 as suitable *in vitro* model to study the impact of Cxs and gap junctional communication on neural development. Cx43 and GJs were found to play a major role in cell growth and differentiation of ReNcell VM197. However, additional data is needed to clarify the involvement of other Cx isotypes in the development of neural progenitors of the ventral midbrain. Screening for Cxs revealed the presence of at least three different Cxs. Although we provide extensive data for an involvement of Cx43 in neural differentiation, the precise functions of Cx31 and Cx59 remain elusive. The expression pattern of Cx31 showed an increase during differentiation, indicating a role of Cx31 in neural development. Since confocal microscopy revealed no/less amounts in GJ plaques, these Cxs might exert its effects by a channel-independent mechanism. Co-localization analysis will clarify potential interaction candidates, which provide basis for further investigations.

Interestingly, the expression of these two Cxs in human tissue derived from the central nervous system has not been reported before. Cx31 is known to be expressed especially in the skin and in the inner ear, but it is also present in human embryonic stem cells (Evans & Martin, 2002; Huettner *et al.*, 2006). Mutations in Cx31 have been attributed to peripheral neuropathy, which might imply possible functions in neural development (López-Bigas *et al.*, 2001). However, expression of Cx31 is unlikely to be essential for neuronal differentiation as Cx31 knockout mice show no altered neuronal development or behaviour. It was assumed that Cx31 deficiency can be compensated by other Cxs (Plum *et al.*, 2001). Nevertheless, Cx31 was found to increase during differentiation of neuroblastoma cells and it affected neurite outgrowth (Unsworth *et al.*, 2007). Knockdown experiments or overexpression of Cx31 have to be performed to create evidence for an involvement of Cx31 in neurite outgrowth or cell migration in human neural progenitors.

We showed that GJIC is crucial to mediate the mitogenic activity of EGF in proliferating progenitors. Moreover, the strong extent of GJIC in differentiated cells indicates an increase of molecule shuttling in neuronal networks. However, we did not address the key question what kinds of molecules are transferred via GJs to maintain the cells in a proliferative state, promoting cell survival or regulate cell fate. This is a difficult question to answer as the number of molecules capable of passing GJs and altering proliferative activity or cell fate is

enormous. Further, whether these cues act as a death or survival factor might depend on the cell type and environmental conditions of the cell that receives the signal. For example, Ca^{2+} is an important modulating second messenger that was shown to trigger development and differentiation, but also stimulates apoptosis (Berridge *et al.*, 1998). The notion that cell fate regulators are transferred via GJs is supported by the finding that communicating cells die in clusters (Krysko *et al.*, 2005). Consistently, GJs were found to enhance neuronal vulnerability to traumatic brain injury, probably by shuttling of pro-apoptotic cues. In contrast, pharmacological blockage of GJIC decreased the extent of post-traumatic cell death and prevented the spread of injury (Frantseva *et al.*, 2002). As discussed above taurine and siRNA have also been assumed to act as cell fate modulators that cross GJs to reach adjacent cells and affect neural differentiation (Chen *et al.*, 1998; Valiunas *et al.*, 2005). The precise identification of these signalling molecules is a prerequisite for a specific exogenous-induced increase of differentiation towards the neuronal lineage or a reduction of cell death when cell replacement therapy is the strategy of choice in the treatment of brain injuries or neurological disorders (Lindvall *et al.*, 2004). Future work will now involve the development of therapeutic strategies to reduce the secondary effects caused by traumatic brain injuries and to maximize functional recovery (Rozental *et al.*, 2001).

Initially, Cxs were thought to be the only proteins capable to form channels between adjacent cells. However, a novel group of proteins, called pannexins (Panxs), was recently identified to share similar functions with Cxs (D'hondt *et al.*, 2009). Panxs are abundantly expressed in the vertebrate nervous system, but they were shown to be involved in hemichannel formation rather than in GJ formation (Scemes *et al.*, 2009). There is emerging evidence that ATP-release, previously reported to occur via Cx-hemichannels, is predominately mediated by Panxs hemichannels (D'hondt *et al.*, 2009). However, little is known about the biology of Panxs in neural stem cells. In addition to Cxs, Panxs were shown to be up-regulated during the development of hippocampal neural progenitors. Moreover, they were assumed to be necessary for proper neuronal differentiation and proliferation (Swayne *et al.*, 2010; Wicki-Stordeur *et al.*, 2012). Unfortunately, it is difficult to experimentally distinguish between Cx- and Panxs channels as specific blockers are not available and cells can express several isoforms of Panxs and Cxs. This raises the possibility that channel functions, actually attributed to Cxs, are mediated by Panx proteins. Thus, further studies are now needed to increase the knowledge about the properties of Panx channels in neural stem cells. Moreover, future work should address the question how they are regulated during neural development or how Panxs themselves are capable to alter the differentiation process.

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Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit selbständig und nur unter Zuhilfenahme der angegebenen Quellen angefertigt habe. Die aus den genutzten Werken wörtlich und inhaltlich entnommenen Stellen (Text und Abbildungen) wurden als Entlehnung kenntlich gemacht.

Rostock, d. 03.04.2013

Heiko Lemcke

Publikationsliste

<u>Lemcke, H.</u> & Kuznetsov, S.A. (2013) Involvement of connexin43 in the EGF/EGFR signalling during self-renewal and differentiation of neural progenitor cells. Cell. Signal., **25**, 2676–2684.

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Kiselyov, A.S., Semenova, M.N., Chernyshova, N.B., Leitao, A., Samet, A. V, Kislyi, K. a, Raihstat, M.M., Oprea, T., **<u>Lemcke, H.</u>**, Lantow, M., Weiss, D.G., Ikizalp, N.N., Kuznetsov, S. a, & Semenov, V. V (2010) Novel derivatives of 1,3,4-oxadiazoles are potent mitostatic agents featuring strong microtubule depolymerizing activity in the sea urchin embryo and cell culture assays. European journal of medicinal chemistry, **45**, 1683–1697.

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