

**ZONULA OCCLUDENS-1 (ZO-1) AND ZO-1-ASSOCIATED
NUCLEIC ACID-BINDING PROTEIN (ZONAB) AT GLIAL
GAP JUNCTIONS IN MOUSE CNS**

By

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A Thesis Submitted to
The Faculty of Graduate Studies
In Partial Fulfillment of the Requirements
For the Degree of

MASTER OF SCIENCE

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TABLE OF CONTENTS

	Page
I. ACKNOWLEDGEMENTS	3
II. ABSTRACT	5
III. LIST OF ABBREVIATIONS	7
IV. GENERAL INTRODUCTION	9
IV.1. Short history of gap junctions	9
IV.2. Intercellular gap junctions in peripheral tissues	9
IV.3 The family of connexin proteins	11
IV.4. Trafficking and assembly of connexins	13
IV.5. Gap Junction Functions	14
IV.6. Gap Junctions in the CNS	16
IV.7. Astrocytic and oligodendrocytic gap junctions	17
IV.8. Neuronal gap junctions	18
IV.9. Connexins at CNS gap junctions	19
IV.10. Functions of glial gap junctions	22
IV.11. CNS connexins, gap junctions and disease	24
IV.12. Zonula occludens-1 protein at gap junctions	26
IV.13. MsY-box3 transcription factor ZONAB	28
IV.14. Aims of the present investigation	30

V. PROJECT: Expression of zonula occludens-1 (ZO-1) and the transcription factor ZO-1-associated nucleic acid-binding protein (ZONAB/MsY3) in glial cells and co-localization at oligodendrocyte and astrocyte gap junctions in mouse brain

Abstract	34
Introduction	35
Material and methods	36
Results	43
Discussion	49
Acknowledgements	55
Figures	59
 VI. GENERAL DISCUSSION	 69
 VII. REFERENCES	 73

I. ACKNOWLEDGEMENTS

If I could just step into a moment of divine inspiration and somehow manage to design the gap junction pill. Twice a day, till the symptoms fade away. That would be the prescription. Most probably it would sell big. Why is that? Because this pill will have more to it than the ability to cure organic diseases. Following the model of coupled cells, perfectly coordinated to assure simple but essential day-to-day functions, I will design this drug to establish the premises of communication between mind, body and soul. Once this state is achieved, once conscience is awakened and constantly alert, maybe some of the greed, frustration, aggression, some of the ill-intended, irrational behavior will simply cease to exist. Maybe somehow, the finger that pulls the trigger will refuse to move. My concern grows stronger thinking of the consequences. Probably the struggle for creating the antidote will be unleashed, because let's face it, the world of today is not ready for such a change yet, it's a jungle out there, and we are just predators enjoying every minute of it.

I would like to take this moment and thank my parents who have guided me so well through this "jungle", who provided moral and financial support when I was down.

A special thank you goes to my supervisor, Dr. James Nagy who showed me what research is really about. By offering me a position in his lab, Jim gave me the opportunity to experience science at its peak, in one of the top gap junction labs in the world.

I couldn't have done this, without the excellent technical assistance from Nora Nolette, Brett Mclean, Xinbo Li, Carl Olson, and without the constant advice and guidance from my committee members: Dr. Elissavet Kardami, Dr. Larry Jordan and Dr. Gunnar Valdimarsson.

Last but not least, I would like to thank Dr. Janice Dodd, the department head, a true leader to all of us, Gail McIndless and Judy Olfert.

I dedicate this thesis to Cristina.

II. ABSTRACT

With the increasing number of connexins shown to associate with the scaffolding phospho-protein zonula occudens-1 (ZO-1), including neuronal connexin 36 (Cx36) and oligodendrocytic Cx47, having suggested an extensive ZO-1 distribution in CNS, we have explored additional possible interactions between ZO-1 and astrocytic connexins Cx43 and Cx30.

The association of Cx30 and Cx43 with ZO-1 was confirmed by co-immunoprecipitation of these connexins with ZO-1 from mouse brain. In addition, Cx30 and Cx43 were co-localized with ZO-1 in mouse brain, providing evidence for ZO-1 expression in astrocytes in the CNS, and ZO-1 association with gap junctions between these cells. In separate studies, we deduced that Y-box transcription factor 3 (MsY3) is the mouse ortholog of the protein ZONAB. ZONAB was previously reported to interact with ZO-1 in canine MDCK cells and to regulate expression of the proto-oncogene ErbB2 in these cells. Confocal double immunofluorescence labelling with newly developed antibodies against ZONAB/MsY3 revealed association of this protein with Cx30, Cx43 and ZO-1 at astrocytic gap junctions, and with Cx32, Cx47 and ZO-1 at gap junctions formed by oligodendrocytes. These results provide new avenues for consideration of GJIC regulation via functions associated with ZO-1. In these glial cells, ErbB2 serves as a receptor of neuregulin, and neuroregulin/ErbB2 signalling is essential for astrocyte and oligodendrocyte survival and differentiation.

We speculate that functions of ErbB2 signalling in glial cells may, in part, be regulated by interactions between connexins, ZO-1 and the transcription factor ZONAB at glial gap

junctions during CNS development and in adult brain. In addition to ZO-1/ZONAB interactions at gap junctions, we have found in vivo association between the two at other locations, including peripheral tight junctions.

III. LIST OF ABBREVIATIONS

aa, amino acids

A/A, astrocyte-to-astrocyte

Ab, antibody

A/O, astrocyte-to-oligodendrocyte

CMT, Charcot-Marie-Tooth disease

CNPase, monoclonal anti-2,'3'-cyclic nucleotide 3' phosphodiesterase

CNS, central nervous system

Cx, connexin

E, extracellular loop of a multi-pass transmembrane protein

EM, electron microscopy

FITC, fluorescein isothiocyanate

FRIL, freeze-fracture replica immunogold labelling

GFAP, glial fibrillary acidic protein

GJIC, gap junction intercellular communication

IP, immunoprecipitation

kDa, kilodalton

KO, knock out

LM, light microscopy

M, molar

MAGUK, membrane associated guanylate kinase

MDCK, Madin-Darby Canine Kidney

ml, milliliter

mM, millimolar

mRNA, messenger ribonucleic acid

MsY3, mouse Y-box transcription factor 3

NGS, normal goat serum

NICHHD, National Institute of Child Health and Development

PB, phosphate buffer

PDZ, postsynaptic protein PSD-95/Drosophila junction protein Disc-large/tight junction protein ZO-1

pH, potential of hydrogen

SDS-PAGE, sodium dodecylsulphate polyacrylamide gel electrophoresis

TBS, 50 mM Tris-HCl, pH 7.4 with 1.5% sodium chloride

TBSTr, 50 mM Tris-HCl, pH 7.4, 1.5% NaCl, 0.3% Triton X-100

TBSTw, 20 mM Tris-HCl, pH 7.4, 150 mM NaCl with 0.2% Tween-20

μm , micron

ZO-1, zonula occludens-1

ZONAB, ZO-1 associated nucleic acid-binding protein

WT, wild type

IV. GENERAL INTRODUCTION

IV.1. Short history of gap junctions

“Communication is everything” may sound like one of those old-fashioned, redundant theories, but the exquisite coordination and functioning of the central nervous system (CNS) is totally dependant upon intercellular circuitry, achieved by means of chemical and electrical coupling. Gap junctions are clusters of intercellular channels that directly connect the cytoplasm of adjacent cells, allowing intercellular communication and providing the speed and synchrony needed for electrical and metabolic signalling in the CNS.

Gap junction history goes as far back as the 1960s, when these specialized membrane structure were discovered as an outcome of unrelated studies of low resistance ionic pathways (Loewenstein, 1967) and of fast excitatory transmission in the crayfish giant axon (Furshpan and Potter, 1968). The term “gap junction” arose from a 1967 study describing intercellular junctions in heart and liver (Revel and Karnovsky, 1967). Since then, more than three decades of research have been necessary to elucidate important aspects of their structure and function.

IV.2. Intercellular gap junctions in peripheral tissues

By now, gap junctions have been described in almost all species and identified in most vertebrate tissues, with the exception of erythrocytes, skeletal muscle cells, platelets and spermatocytes, which do not share this type of communication (Rozental et al., 2000; Evans and Martin, 2002).

Freeze fracture analyses present gap junctions as dense collection of particles in the plasma membrane of one cell, having corresponding pits in the closely opposing membrane of the neighbouring cell (White et al., 1995; Rash et al., 1998; Rash et al., 2000). Each particle and pit corresponds to an individual hemmichannel also termed a connexon. Connexons are approximately 60-65 Å in diameter and dock with corresponding connexons on adjacent cells, forming a perfectly sealed conduit of only 15 Å. These plaque forming gap junction channels have been studied by means of freeze fracture replica immunogold labelling (FRIL) (Rash et al., 1998; Rash et al., 2001), by fluorescently tagged proteins (Bukauskas et al., 2000) and by immunocytochemistry (Yeager and Nicholson, 1996; Severs et al., 2001; Nagy et al., 2001, 2003; Li et al., 2004a,b)

From a simplistic point of view, the hemichannel can be divided into three functional domains: the cytoplasmic domain modulating the channel properties, the membrane spanning domain responsible for forming the pore, and the extracellular module bridging across to link the two connexons. Cell adhesion proteins like catenins and cadherins have been described to influence the docking process, as cells need to establish mechanical coupling before assembling intercellular communicating channels (Giepmans, 2004; Herve et al., 2004)

In addition to transmission of electrical impulses, ions and metabolites with a molecular mass of less than 1000 Da can diffuse between coupled cells (Paul, 1995; Kumar and Gilula, 1996; Sohl and Willecke, 2004). Each connexon is a hexamer built of protein subunits called inxins in invertebrates and connexins in vertebrates (Sohl and Willecke, 2004). Connexons can be homomeric (oligomerization of one connexin type)

or heteromeric (containing more than one different connexin), whereas gap junctions are divided into homotypic (two connexons of the same kind in apposing cells) and heterotypic (opposing connexons each composed of a different connexin) (Kumar and Gilula, 1996; Rozental et al., 2000).

IV.3 The family of connexin proteins

Connexins are family of proteins characterized by an intricate tissue distribution. The same connexin can be present in histologically different tissues, while the same cell type can express multiple connexins, which can or can not form functional gap junction channels together. Furthermore connexin diversity in most organs is regulated throughout development (White et al., 1995; Rozental et al., 2000).

As far as nomenclature is concerned, connexins have been classified into α , β , and γ subgroups (based on sequence identity and length of the cytoplasmic loop), or have been named referring to the species and the expected molecular weight in kDa (for example mCx43 describes the 43 kDa mouse connexin43). So far, 21 connexin genes have been described in the human genome and 20 in the mouse genome (Willecke et al., 2002; Sohl and Willecke, 2004). Although most of them have orthologous pairs between the two species, there are connexin genes in humans that are unaccounted for in mouse and vice-versa.

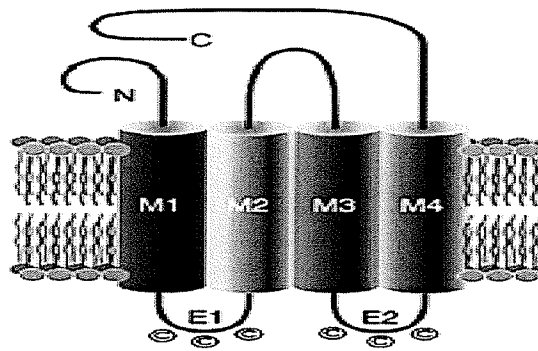


Fig. 1

A topological connexin model with the amino and carboxy-terminals facing the cytoplasm, with 4 transmembrane domains (M1 to M4), two extracellular loops (E1 and E2) and one cytoplasmic loop (Duffy et al., 2002; Herve et al., 2004), is schematically presented in figure 1 (reproduced after Sohl and Willecke, 2004). The amino acid sequences of all the connexins are highly conserved in the extracellular loops and in the transmembrane domains, while variation occurs in the C-terminal domain and the cytoplasmic loop, accounting for the variations in connexin molecular weight (Beyer et al., 1990; Bruzzone et al., 1996; Willecke et al., 2002). The M1-M4 domains are thought to be α helices and are approximately 20 amino-acids in length. Both E1 and E2 contain a significant amount of hydrophobic amino acids and three cysteine residues, capable of forming intra-monomer/molecular disulfide bridges (Goodenough et al, 1996; Evans and Martin, 2002). The extracellular loops are responsible for recognition and docking of the two opposing connexons, and for partially determining the voltage gating properties of the channel (Goodenough et al., 1996). Each connexin isoform can form channels that have unique size and charge dependant permeabilities, different unitary conductances determined mainly by the carboxy terminal domain sequence, and different

pH or voltage gating sensitivity, influenced by sequences in the amino terminal, cytoplasmic loop or extracellular domains (Bruzzone et al., 1996; Goodenough et al., 1996; Plum et al., 2000). The three different domains interact and influence one another; for example, amino acid charge substitutions at the M1-E1 border can reverse gating polarity (Bruzzone et al., 1996).

IV.4. Trafficking and assembly of connexins

The process of gap junction formation is a highly complex one, with many regulatory events involved. Connexins are synthesized in the endoplasmic reticulum (ER) and then follow the cellular secretory pathway (Bruzzone et al., 1996). It has been suggested that assembly of compatible connexin subunits into connexons takes place after the connexins have exited the ER, most probably in the trans Golgi network (Musil and Goodenough, 1993). However, this is a controversial issue. Some studies suggest that oligomerization takes place in ER membranes (Hurtley and Helenius, 1989), while others support the ER-Golgi-intermediate compartment (Diez et al., 1999). More recent evidence indicates that different connexin subtypes can oligomerize at different locations (Segretain and Falk, 2004). Once formed, a connexon remains closed to prevent changes between the luminal and cytosolic compartments. Physiological and biochemical studies have revealed that connexons are added to the plasma membrane right outside the junctional areas and that at this stage they become functional. These hemichannels can move laterally in the plasma membrane and the feeding of newly formed connexons to the junctional plaques takes place at the outer cluster margins (Lauf et al., 2002). Inter-connexon self recognition and binding are essential for the formation of the channel. Gap junctions are

dynamic structures as the connexin turnover is quite fast, with half-lives generally ranging from 1 to 5 hours (Musil et al., 2000; Saffitz et al., 2000; Segretain and Falk, 2004). As new channels are constantly added, older channels are removed from the plaque center and “digested” by intracellular degradation pathways (Segretain and Falk, 2004).

IV.5. Gap Junction Functions

Although gap junctions were initially regarded and described as passive conduits between neighboring cells, at this point there is hardly any mammalian tissue or organ left where extensive gap junction related regulatory events have not been described. Furthermore, if a certain cell or tissue is proven unable to develop functional communicating channels, the connexons may take over the responsibility and participate in cell regulatory events (Ebihara, 2003). Indeed, recent studies have shown that under certain circumstances the opening of single hemichannels in the plasma membrane is followed by release or uptake of metabolites (John et al., 1999; Ebihara, 2003).

The formation of communicating compartments, allowing direct exchange of ions and second messengers like cAMP, cGMP, Ca²⁺ and IP₃ (Kumar and Gilula, 1996) sets the premise for coordinated cellular activity and homeostasis. Different systems such as the “Xenopus Oocyte Assay” (2 cells paired after injection of specific RNA) and “Communication Deficient Cell Lines” (where connexin DNA can be transfected), together with mouse “knock-out” (KO) and “knock-in” technologies, have been employed to study connexins and their functions. KO mice are excellent models for investigating the patho-physiology of disease and can replicate human genetic disorders.

Knocking out a connexin gene may have different outcomes and is not always compatible with life. Therefore, conditional KO animals, where a certain connexin gene is “deleted” only in a particular tissue of interest, are sometimes used.

In peripheral tissues, intercellular junctions contribute to the regulation of diverse functions such as propagation of excitation in cardiac and smooth muscle, metabolic cooperation during embryonic development, regulation of pancreatic secretion and regulation of cell growth and carcinogenesis (Page and Shibata, 1981; Meda et al., 1984; Paul, 1995; Evans and Martin, 2002). In addition, connexins have been implicated in hematopoiesis, inflammatory reactions, atherosclerotic plaque formation (Kwak et al., 2003; Oviedo-Orta and Evans, 2004; Nakase and Naus, 2004) as well as in regulating alveolar lung function (Boitano et al., 2004).

A close analysis of several genetic abnormalities has revealed a close association between certain human inherited diseases and specific connexin mutations. X-linked Charcot-Marie-Tooth disease, a progressive degenerative neuropathy resulting from myelin disruption and axonal degeneration, was the first such disease shown to result from mutations in Cx32 (David Paul, 1995; Menichella et al., 2003; Wang et al., 2004). Interestingly, specific Cx32 mutations impair the peripheral nervous system (PNS), but do not cause other consistent pathologies, although the wide tissue distribution of Cx32 has been extensively described (Fairweather et al., 1994, Bruzzone et al., 1994). Perhaps in the context of multiple connexins expressed in the same cell types, other connexins can take over the defective function in a compensatory mechanism. However, besides shared functions, connexins can have unique tissue-dependant physiological properties, as shown by recent knock-in studies, where replacement of the Cx43 coding region with the

coding region of Cx40 restores normal cardiac architecture and function but “produces a fate worse than death” according to Thomas White (2003). This indicates that Cx40 could successfully substitute for Cx43 only in heart, leaving the animals sterile, as Cx43 functions in testis were not replaced; the animals were also underweight and had additional health problems.

Depending on the particular connexin domain where the alteration occurs, some connexin mutations may be linked to more than one disorder, for example faults in the synthesis of Cx26, Cx30 and Cx31 can be related to both deafness and skin disorders.

Missence mutations in Cx46 and Cx50 are associated with dominant congenital cataract in humans, Cx26 mutations may cause genetic deafness or dominant epidermal disease, Cx30 and Cx30.3 mutations can cause skin diseases, while mutations in Cx43 result in severe congenital cardiac malformation (Britz-Cunningham et al., 1995; Kelsell et al., 1997; White et al., 1998; Kelsell et al., 2001).

IV.6. Gap Junctions in the CNS

Various cell types in the CNS are extensively coupled by means of gap junctions, which are constantly regulated during development. The major cell types in the CNS are neurons and glial cells, the latter being represented by astrocytes, oligodendrocytes and microglia. Gap junctions in the CNS are present between electrically coupled cells, allowing transmission of neuronal signals at electrotonic synapses, but also between non-excitabile cells with roles in providing immediate ionic transfer and metabolic cooperation for the non-neuronal network.

Homologous gap junctions occurring between the same cell types have been described in neurons, astrocytes, ependymal cells and microglia (Rouach et al., 2002; Nagy and Rash, 2000) and supposedly in oligodendrocytes in culture (Kettenmann and Ransom, 1988). Heterologous gap junctions are present in the nervous system between astrocytes and oligodendrocytes, astrocytes and neurons, astrocytes and retinal Muller glial cells, and astrocytes and ependymal cells (Massa and Mugnaini, 1982; Nedergaard, 1994; Nadarajah et al., 1996, Nagy and Rush, 2000; Nagy et al., 2003). However the neuronal-glial coupling is still a controversial issue, since most of the supporting data has been weak (see review by Nagy et al., 2004). Possible functions of heterotypic/heterologous gap junctions include K^+ and water spatial buffering around neurons and the propagation of intercellular Ca^{2+} waves between astrocytes and other glial cells (Charles et al., 1996; Rouach et al., 2002; Stout and Charles, 2003)

IV.7. Astrocytic and oligodendrocytic gap junctions

As the most abundant type of glial cells, astrocytes, named after their star-like appearance, have numerous projections that form the astrocytic matrix and play both supportive and active roles in the brain functioning. Astrocytes in white matter are known as fibrous astrocytes while those in grey matter are referred to as protoplasmic astrocytes. By triggering maturation and development of adult neural stem cell, astrocytes may have a far more active role in neurogenesis than previously thought (Song et al., 2002). Electrophysiological and dye-transfer approaches have shown the presence of extensive, ubiquitous junctional coupling between astrocytes (Giaume and McCarthy, 1996; Rash et

al., 1997; Nagy and Rash, 2000), while more recent studies suggested their impact on normal CNS functioning (Nakase and Naus, 2004).

Oligodendrocytes are responsible for generation and maintaining myelin sheaths in nervous tissue, providing support and insulation to neighbouring axons. Normal myelination is dependant on connexin expression in oligodendrocytes and Schwann cells (Menichella et al., 2003; Odermatt et al., 2003). Oligodendrocytes form extensive gap junctions with astrocytes and become part of the astrocytic network, while autologous gap junctions between oligodendrocytes (coupling between processes of the same cell) or homologous oligodendrocytic gap junctions are infrequently described (Nagy and Rash, 2000).

IV.8. Neuronal gap junctions

Initially described decades ago in crayfish, inter-neuronal electrical synapses have become the target of intense research in mammals with the discovery of the Cx36 gene. Neuronal gap junctions are present during embryonic development, are abundant postpartum and still present in several adult brain regions including cortex, thalamus, striatum, inferior olive, olfactory bulb and retina (Rozental et al., 2000; Rouach et al., 2002; Nagy et al., 2004). The diverse functions fulfilled by these neuronal gap junctions at different stages in development and at different locations include neuronal differentiation (Rozental et al., 2000), electrical coupling and the contribution to generation and maintenance of synchronous neuronal activity (Nagy et al., 2004). Gap junctions are also plentiful in the developing and adult meninges, ependymal cells but seldom in microglial cells (Rouach et al., 2002; Sohl and Willecke, 2004).

IV.9. Connexins at CNS gap junctions

Eight different connexins (Cx26, Cx32, Cx36, Cx37, Cx40, Cx43, Cx45, Cx47) have been reported in neurons so far (Rouach et al., 2002). However, the characterization of neuronal connexins has been a controversial issue and available data is still unclear. For example, Cx45 mRNA has been detected in neural tissue and transgenic mice supported the idea of Cx45 expression in neonatal and adult neural tissue, although initial immunofluorescence studies failed to detect Cx45 immunoreactivity in neurons (Dermietzel et al., 1997; Zhang and Restrepo, 2002; Maxeiner et al., 2005). Of these eight connexins, to date only Cx36 has been categorized by EM procedures to be in neuronal gap junctions (Nagy et al., 2004). As stated by Rash and collaborators (Rash et al., 2000), the limitations of light microscopy resolution, the small size of glial processes surrounding neurons and the extreme proximity between the two, makes the process of localizing connexins as belonging to one cell type or the other almost impossible.

More recent studies provide evidence for the absence of Cx32, Cx43 and Cx26 in neuronal gap junctions (Rash et al., 2000; Nagy et al., 2004).

As far as CNS macroglia are concerned, FRIL studies together with immunohistochemical and molecular biology approaches have described six different types of glial connexins, with Cx26, Cx30 and Cx43 present in astrocytes and Cx29, Cx32 and Cx47 in oligodendrocytes (Yamamoto et al., 1990; Nagy et al., 1997; Dermietzel et al., 1997; Nagy et al., 2003).

Cx43 is 'presented' as the major/predominant astrocytic connexin by in vitro studies showing dependence of junctional communication on Cx43 in cultured astrocytes

(Giaume et al., 1991; Dermietzel et al., 1991), with massive reduction in coupling efficiency in cultures prepared from Cx43 KO animals. Nonetheless, it is noteworthy that the fairly heterogeneous Cx43 distribution in brain (Rouach et al., 2002), together with varying levels for Cx43 during development (Nagy and Rash, 2000), may influence Cx43 expression at certain stages and locations, and therefore have an effect on intercellular communication in cultured astrocytes. Cx43 was shown to be co-expressed with Cx30 in gap junctions of mature protoplasmic astrocytes in brain and spinal cord (Nagy et al., 1999; Nagy et al., 2003). Initially described in the CNS as a leptomeningeal connexin, Cx26 has since been documented in neurons and astrocytes of developing and adult rodent brain (Nadarajah et al., 1996; Solomon et al., 2001). Although its neuronal presence during early developmental stages needs further clarification, recent available data indicates Cx26 expression in astrocytes by showing its association with astrocytic Cx43 and Cx30 (Nagy et al., 2003) and with the specific astrocytic marker glial fibrillary acidic protein (GFAP). In addition, the absence of Cx26 from neuronal junctions in adult animals has been noticed (Nagy et al., 2001).

Variations of Cx43, Cx30 and Cx26 expression in grey and white matter regions indicate a heterogeneous distribution of astrocytic connexins throughout the CNS (Nagy et al., 2004). Astrocytes can mediate indirect coupling between oligodendrocytes at O/A/O (oligodendrocyte-to-astrocyte-to-oligodendrocyte) junctions (Rash et al., 2001). Oligodendrocytes express at least three connexins, Cx47, Cx32 and Cx29. Initially reported to be expressed by neurons (Teubner et al., 2001), Cx47 has been recently recognized as an oligodendrocytic connexin (Odermatt et al., 2003; Menichella et al., 2003; Li et al., 2004b). Quantitative studies show that the vast majority of

oligodendrocytes express all three connexins with differential subcellular distribution (Li et al., 2004b). By immunofluorescence, Cx47- and Cx32-positive puncta are plentiful and co-localize on oligodendrocyte somata and initial processes, while myelinated fibers contain an abundance of Cx29 and Cx32 (Li et al., 1997; Nagy et al., 2003). Cx32 and Cx47, present on the oligodendrocytic side of astrocyte-to-oligodendrocyte junctions (Li et al., 1997; Rash et al., 2001), are co-associated with Cx43, Cx30 and Cx29 present on the astrocytic side (Li et al., 1997; Rash et al., 2000, 2001; Nagy et al., 2004). The same astrocytic connexins contribute to the formation of both A/A and A/O junctions, thus raising the possibility of several coupling combinations between compatible binding partners at homologous or heterologous gap junctions. Cx29 has been reported as non-permissive with itself or with Cx32 (Altevogt et al., 2002) and presumably plays only a minor role in the formation of intercellular channels or of channels within myelin (Nagy et al., 2003; Altevogt et al., 2002).

Cx32 has been described as non-compatible with Cx43 (White et al., 1995; Nagy et al., 2003), and studies in transgenic animals revealed that indeed, only the expression of Cx30 (dramatically reduced levels) and Cx26 (only partially reduced levels) is influenced after knocking out the Cx32 gene (Nagy et al., 2003). Although not completely elucidated for heterologous junctions, probable binding partners for Cx32 are Cx30 and to a lower extent Cx26, while Cx47 seems to be permissive with Cx43 (Nagy et al., 2003).

From the control of connexin gene expression, to the trafficking and assembly into functional channels, gap junctions in CNS are highly regulated in accordance with changes in cellular activity. Factors including pH, connexin phosphorylation, Ca^{2+} , different hormones and the membrane potential modulate channel conductance by

opening or closing existent channels (Nagy and Dermietzel, 2000; Rouach et al., 2002; Segretain and Falk, 2004). Taken separately each of these factors can either reduce or increase coupling, and a good example is that of connexin phosphorylation whose effect is dependent on the type of phosphokinase involved.

Recent studies have confirmed that both charge and size are contributing factors to the permeability of gap junctional channels (Gong and Nicholson, 2001). By eliminating the effect of charge, Gong and Nicholson examined the physical exclusion limits of channels formed by Cx26 and Cx32 in *Xenopus* oocytes. In the case of Cx32 the size cut-off is between 11.2 Å and 9.6 Å, although the single channel conductance for Cx32 is rather small, approximately 55 pS. Cx26 has a 130 pS single channel conductance and a size exclusion limit of around 8 Å. Other studies reported a unitary conductance of 55 pS for gap junction channels composed of Cx47 and one of only 15 pS for those composed of Cx36 (Teubner et al., 2001).

The permeability properties of a gap junction channel are specific to the different connexins expressed in a particular cell or tissue and while electrical conductance and ionic selectivity may vary by one order of magnitude from one channel to the other, further studies are required to provide additional information as to how these different permeabilities and conductances might affect brain function.

IV.10. Functions of glial gap junctions

The astroglial syncytium seems to play critical roles in the physio-pathological functioning of the brain, and serves to maintain water-electrolyte balance, the blood-brain barrier, and mechanical and metabolic support to neurons (Rouach et al., 2002; Simard

and Nedergaard, 2004). Several of these proposed functions are achieved through means of direct intercellular signalling and metabolite exchange, bringing gap junctional channels and hemichannels into the spotlight. Astrocytes are important in spatial buffering of the K^+ and H^+ ions, as well as in the uptake of glutamate and its further conversion into glutamine (Fonseca et al., 2005; Leis et al., 2005; Wada et al., 2005). During periods of sustained neuronal activity, large amounts of K^+ are released by neurons and rapidly taken up by astrocytes. In consequence, neurotoxicity and alterations of neuronal conductance due to hyperexcitability are substantially reduced. The excessive K^+ is further redistributed by the astrocytic matrix to neighboring regions with a lesser K^+ concentration (Laming, 2000).

Another aspect worth mentioning is that of gap junctional calcium signal communication between different cell types of the brain. This involves the direct diffusion of either Ca^{2+} or inositol triphosphate (IP3), a calcium mobilizing messenger, through intercellular channels. Inter-astrocytic calcium waves are increases in astrocytic calcium levels that originate in one cell and sequentially propagate to neighboring astrocytes or neurons. Calcium waves are known to be present in cultures and in acute brain slices (Charles et al., 1991; Schipke et al., 2002) and spread through gap junctions or via the paracrine pathway (Sneyd et al., 1994; Hassinger et al., 1996). The exact roles of calcium signalling in astrocytes need further investigations, although involvement in buffering ions and neurotransmitters, and in astrocyte proliferation and differentiation has been proposed (Newman, 1986; Gallo and Ghiani, 2000).

Above and beyond the beneficial outcomes, calcium waves are also thought to be connected to pathological events like spreading depression (SD), a reversible cessation of

neuronal activity in response to noxious stimulation of the brain (Basarsky et al., 1998; Kunkler and Kraig, 1998). Interestingly, in cultures, heptanol as well as other gap junction inhibitors/blockers, can block Ca waves and prevent onset of SD (Rawanduzy et al., 1997; Kunkler and Kraig, 1998). Work with connexin knock-out animals, with transfected versus wild type glial cell lines and with gap junctional uncouplers will be required to further clarify aspects of neuronal-glial-vascular calcium signalling communication.

IV.11. CNS connexins, gap junctions and disease

Functional gap junctions, hemichannels and connexins are involved in a series of CNS disorders including brain ischemia, epilepsy, malignancy and Alzheimer's disease (Nagy et al., 1996; Rouach et al., 2002; Evans and Martin, 2002). One of the characteristics of brain inflammation is reactive gliosis, an excessive proliferation of astrocytes and microglia replacing neurons in the area of a degenerative lesion. It has been documented that these changes are frequently accompanied by changes in astrocytic gap junctional intercellular communication (Rouach et al., 2002). Inflammatory mediators and nitric oxide released during periods of brain trauma, together with the activation of different ligand receptor complexes, can down or up-regulate connexin expression as well as junctional properties. For example, chronic exposure to interleukin-1 (IL-1) down-regulates Cx43 mRNA and inhibits propagation of calcium waves in cultured astrocytes (John et al., 1999), while an increase in Cx43 expression was observed at sites of amyloid plaques in patients with Alzheimer's disease (Nagy et al., 1996; Nakase and Naus, 2004).

Presence of gap junctional coupling between healthy and unhealthy cells can in some instances increase the survival rate of the partially-injured cells, or in others increase the

death of the perfectly healthy cells, depending on the cell ratio and on the balance of neurotoxic to neuroprotective factors involved (Contreras et al., 2004; Farahani et al., 2005). This is due to the fact that, while in contact, injured cells exchange molecules with normal neighboring cells. Harmful signals and pro-inflammatory mediators spread towards normal cells 'in exchange' for health promoting factors (Rouach et al., 2002; Contreras et al., 2004). In their battle to save the injured, the healthy cells can find themselves drained of their basic energy resources and become subject to delayed apoptotic death. The final outcome can be both beneficial and dramatic. To better describe this, Farahani and colleagues proposed the "good samaritan" neuroprotective model and the "bystander" executioner model when referring to possible astrocytic roles in managing hypoxia-ischemia-induced oxidative stress (Farahani et al., 2005).

In epilepsy, electrical coupling through neuronal gap junctions, essential for the synchrony of bursting firing patterns, may be important in the propagation of the epileptic wave (Velazquez and Carlen, 2000). Evidence from in vitro models, where gap junctional blockers diminish and enhancers increase the seizure, further strengthens this concept (Carlen et al., 2000). The relation between gap junctions and brain tumors is still ambiguous. One concept is that in gliomas, transformed cells aggregate and further invade the astrocytic matrix via gap junctions (Lin et al., 2002). However, it should be noted that higher grade gliomas show a down-regulation in Cx43 expression (Huang et al., 1999) and that the effect of up-regulating gap junctions in cultures tend to reduce proliferation (Ozog et al., 2002). Recent data suggests an active role in tumor growth suppression for Cx43, independently of gap junction formation (Moorby and Patel, 2001; Zhang et al., 2003). Further research is needed to clarify whether this effect comes from

Cx43 interference in the cell cycle (Zhang et al., 2003) and to elucidate the importance of the C-terminal Cx43 domain in this suppressing outcome.

IV.12. Zonula occludens-1 protein at gap junctions

Most vertebrate connexins are known to interact with diverse proteins at gap junctions, forming multi-protein complexes (Duffy et al., 2002; Giepmans 2004). These proteins are believed to regulate connexin function and to participate in complex modulation of junctional communication. An increasing number of connexin interacting partners have been identified including kinases, phosphatases, cell signaling molecules and scaffolding proteins (Singh and Lampe, 2003); this is consistent with mass spectrometry studies for Cx43, for example, where nineteen proteins have been suggested to interact with Cx43.

One such scaffolding phospho-protein is the 220 kDa zonula occludens-1 (ZO-1). With three PDZ domains (PDZ1-PDZ3), one SH3 domain and one guanylate kinase (GK) domain (Fig. 1 of Project), ZO-1 is a member of the membrane-associated guanylate kinase (MAGUK) family of proteins (Gonzalez-Mariscal et al., 2000). The PDZ domains are structurally conserved modules (80 to 90 amino-acids) named after the first three proteins within which they were identified: PSD-95, Dlg-A and ZO-1 (Gonzalez-Mariscal et al., 2000). The SH3 (Src homology 3) domains usually contain a smaller number of amino-acids (50-70) and are important in the substrate-enzyme binding, or in binding to the GK modules of other proteins (Gonzalez-Mariscal et al., 2003). The GK module seems to be enzymatically inactive and has protein binding functions (Gonzalez-Mariscal et al., 2003). Besides the conserved domains, ZO-1 also contains a proline rich domain. Its nuclear localization signals (NLS) and nuclear exporting signals (NES) reveal

ZO-1 capacity of traveling back and forth between the plasma membrane and the nucleus (Gonzales-Mariscal et al., 2000). ZO-1 has initially been characterized at tight (Stevenson et al., 1986; Mitic and Anderson 1998) and adherens junctions (Itoh et al., 1991; Howarth et al., 1992) and has been recently associated with gap junctions (Li et al., 2004a,b). In the CNS, ZO-1 was originally detected as part of blood vessel tight junctions (Petrov et al., 1994; Wolburg and Lippoldt, 2002) and olfactory sensory neurons (Miragal et al., 1994) and its expression was considered restricted. Additional studies revealed ZO-1 expression in developing neuroepithelial cells (Saitou et al., 1997) and in mossy fiber terminals of hippocampus (Inagaki et al., 2003). It is well established now, with the characterization of neuronal and oligodendrocytic ZO-1 (Inagaki et al., 2003; Li et al., 2004a, b), that its distribution is far wider than originally considered, as suggested by Nagy and collaborators (Li et al., 2004a,b). ZO-1 has also been shown to associate with an increasing number of PDZ-binding-motif-containing connexins. Connexins such as Cx31.9, Cx43, Cx45, Cx46, Cx47 and Cx50 interact with the second PDZ domain of ZO-1, leaving Cx36 the only one to bind PDZ1 of ZO-1. After showing direct association between ZO-1 and neuronal Cx36, our group pursued a more thorough investigation of ZO-1 expression in brain. Immunohistochemistry and immunoprecipitation approaches together with pull-down assays further identified oligodendrocytic Cx47 and more recently astrocytic Cx30 as possible ZO-1 binding partners (Li et al., 2004b; Penes et al., 2005). Cultured astrocytes have been reported to express ZO-1 (Howarth et al., 1992; Song et al., 2005). By showing ZO-1 co-localization with markers of astrocyte-to-astrocyte gap junctions like Cx43 and Cx30, and ZO-1 co-immunoprecipitation with these

two astrocytic connexins, we provided evidence for ZO-1 expression in astrocytes in vivo.

The exact functionality of ZO-1 or of other connexin binding proteins at gap junctions is currently under investigation, but possible scaffolding or signalling roles can be suggested by analyzing known roles for these proteins at different locations (Mitic and Anderson 1998; Gonzalez-Mariscal et al., 2000, 2003; Giepmans, 2004).

The modular organization of ZO-1 allows it to associate with the cytoskeleton, with important transmembrane proteins present either at tight or at gap junctions and to bind signal transduction molecules. ZO-1 can shuttle between the plasma membrane and the nucleus where it is involved in regulation of gene expression (Gonzalez-Mariscal et al., 2000). The role of ZO-1 in tumorigenesis is still unknown, although beneficial effects have been described (Hoover and Liao, 1998).

IV.13. MsY-box3 transcription factor ZONAB

ZO-1 specifically interacts with diverse proteins through its SH3 domain. One such important protein is ZONAB (ZO-1-associated nucleic acid-binding protein). ZONAB is a Y-box transcription factor that binds to promoter sequences of cell-cycle regulators. ZONAB has been extensively described in MDCK cells by Balda and collaborators (Balda and Matter, 2000; Balda et al, 2003), but little is known about its expression patterns in vivo. In cultured cells, ZONAB was found both at the plasma membrane and in the nucleus, and its localization and cellular activity was closely regulated by ZO-1. ZONAB has two isoforms, long ZONAB B and an alternatively spliced shorter ZONAB A, with deletion of 68 amino acids; both of which are capable of binding ZO-1 (Balda

and Matter, 2000). Considering the sequence similarities between canine ZONAB and mouse MsY3, together with the presence of a highly conserved cold shock domain in both proteins, and in accordance with sequence alignment results, MsY3 has been considered the ZONAB mouse ortholog (Penes et al., 2005, PubMed - in process). With ZONAB binding the promoter of the proto-oncogene coding for ErbB2, ZO-1-ZONAB interaction has been shown to regulate ErbB2 expression in MDCK cells (Balda and Matter, 2000). ErbB2, also referred to as HER2 or Neu, is a tyrosine kinase receptor with important functions in the complex processes of cell growth and cell differentiation (Holbro & Hynes, 2004, Marmor et al., 2004). Specific ZONAB actions on cellular proliferation seem to be dependant upon the confluency of the cells involved, ranging from very little influence on growing cells to an ErbB2 repressor role in confluent cells (Balda et al, 2003). It was suggested that ZONAB acts as a derepressor of ErbB2 activity when sequestered at the plasma membrane via its binding to ZO-1 (Balda and Matter, 2000).

In oligodendrocytes, the ligand-receptor Neuregulin-ErBb2 signaling axis is important in oligodendrocytic survival, differentiation and myelination (Tokita et al., 2001; Park et al., 2001; Patten et al., 2003; Kim et al., 2003). Our recent description of ZO-1/ZONAB co-association with oligodendrocytic and astrocytic connexins at glial gap junctions raises questions as to the possible implications of these interacting complexes in ErbB2 signalling at these locations.

ZO-1-ZONAB interaction may also be involved in controlling paracellular permeability, as suggested by recent studies measuring the paracellular flux of radiolabelled substances in different systems. Although little is known about detailed

mechanisms, it was shown that cells over-expressing ZO-1 had an increased flux of paracellular manitol, while cells over-expressing both ZO-1 and ZONAB had almost normal permeability (Balda and Matter, 2003).

In MDCK cells, ZONAB has been reported to interact with other important proteins like CDK4 (cell division kinase 4) and RalA, a member of the Ras family of GTPases involved in key signalling pathways (Balda et al., 2003; Frankel et al., 2005). As suggested by studies showing diminished CDK4 nuclear pools as an outcome of reduced ZONAB accumulation in the nucleus, changes in the cellular levels of ZO-1 and/or ZONAB was suggested to control the pace of CDK4 nuclear import and therefore influence progression of the cell cycle (Balda et al., 2003).

IV.14. Aims of the present investigation

While previously studying ZO-1 expression in mouse brain, observations were made of widespread ZO-1 immunopositive puncta that could not be explained by the tight junctional localization of ZO-1 or by ZO-1 presence in neurons or oligodendrocytes, and indicated ZO-1 expression in as yet unidentified structures in the CNS (Li et al., 2004a,b). The discrepancies between these finding in our lab and earlier studies identifying a restricted ZO-1 distribution with ZO-1 mostly located along blood vessels could perhaps be explained by a lower ZO-1 abundance at these locations, but which were detected by our use of various anti-ZO-1 antibodies under different fixation conditions, with the consideration that ZO-1 immunostaining tended to be very sensitive to overfixation.

As earlier reports pointed out ZO-1 presence in cultured astrocytes, as well as Cx43 interaction with the PDZ2 domain of ZO-1, and considering that our previous unpublished data revealed labelling for ZO-1 resembling the distribution of astrocytic connexins, we investigated ZO-1 expression by astrocytes in vivo.

One important goal of our research is to understand more about connexin functions and the complex regulatory mechanisms controlling gap junctions. In attempting to do so, we first needed to identify and describe possible connexin and/or ZO-1 interacting proteins, which could provide new insights into deciphering the intricate signalling mechanisms present at intercellular junctions. One such functionally important protein is ZONAB, a transcription factor described to extensively associate with ZO-1 in culture. Some of the ZONAB related questions in our study can be stated as follows. At first, we wanted to determine whether ZONAB is expressed in the mouse brain in vivo. If so, in what cell types is it expressed? Further, we were interested in the subcellular localization of ZONAB with emphasis on its presence at intercellular gap junctions. Another aspect was that of possible ZONAB-ZO-1 interaction as suggested by in vitro studies. Last but not least, our aim was to determine ZONAB association with glial connexins and to characterize discrepancies between ZONAB presence in MDCK cells and ZONAB expression in mouse CNS in vivo.

Hypotheses:

1. ZO-1 is expressed by astrocytes in vivo and associates with astrocytic Cx30 and Cx43.

2. ZONAB is expressed in mouse CNS in vivo, and associates with ZO-1, Cx32, Cx47 and Cx43 at glial gap junctions.

Contribution for the current Project

My personal contribution to this project consists of the following:

- I have provided data presented in figures 1 to 4 and 8 to 10 respectively.
- I was responsible for writing the first draft of the manuscript in collaboration and under the supervision of Dr. J.I. Nagy.
- I was responsible for making sure that all suggested corrections are included in the final version of the submitted manuscript.

Xinbo Li has provided all the molecular data resulting in figures 5-7 and 8A.

V. PROJECT: Expression of zonula occludens-1 (ZO-1) and the transcription factor ZO-1-associated nucleic acid-binding protein (ZONAB/MsY3) in glial cells and co-localization at oligodendrocyte and astrocyte gap junctions in mouse brain

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ABSTRACT

The PDZ domain-containing protein zonula occludens-1 (ZO-1) interacts with several members of the connexin (Cx) family of gap junction forming proteins and has been localized to gap junctions, including those containing Cx47 in oligodendrocytes. We now provide evidence for ZO-1 expression in astrocytes and association with astrocytic connexins *in vivo* by confocal immunofluorescence demonstration of ZO-1 co-localization with astrocytic Cx30 and Cx43, and by ZO-1 co-immunoprecipitation with Cx30 and Cx43. Evidence for direct interaction of Cx30 with ZO-1 was obtained by pull-down assays that indicated binding of Cx30 to the second of the three PDZ domains in ZO-1. Further, we investigated mouse Y-box transcription factor MsY3, the canine ortholog of which has been termed ZO-1 associated nucleic acid-binding protein (ZONAB) and previously reported to interact with ZO-1. By immunofluorescence using specific anti-mouse ZONAB antibody, ZONAB was found to be associated with oligodendrocytes throughout mouse brain and spinal cord, and to be co-localized with oligodendrocytic Cx47 and Cx32, as well as with astrocytic Cx43. Our results extend the CNS cell types that express the multi-functional protein ZO-1, demonstrate an additional connexin (Cx30) that directly interacts with ZO-1, and show for the first time the association of a transcription factor (ZONAB) with ZO-1 localized to oligodendrocyte and astrocyte gap junctions. Given previous observations that ZONAB and ZO-1 in combination regulate expression of the tyrosine receptor kinase ErbB2, our results suggest possible roles of glial gap junction-mediated anchoring of signalling molecules (ZO-1 and ZONAB) that may regulate glial proliferation, differentiation and homeostatic processes.

INTRODUCTION

Gap junctions are clusters of intercellular channels that directly connect the cytoplasm of adjacent cells, allowing selective passage of ions and small molecules between coupled cells (Yeager & Nicholson, 1996, 2000). Each neighboring cell contributes hexameric hemichannels or connexons that are composed of various members of the family of gap junction forming connexins (Cx) (Bruzzone *et al.*, 1996; Goodenough *et al.*, 1996). Twenty mammalian connexins exhibiting differential tissue expression have been identified (Willecke *et al.*, 2002; Sohl & Willecke, 2004), and at least eight of these are differentially expressed in a variety of cell types in the CNS (Nakase & Naus, 2004). Among these, Cx26, Cx30 and Cx43 are expressed in astrocytes, and have been localized to homologous astrocyte-to-astrocyte gap junctions (A/A gap junctions), as well as to the astrocyte side of heterologous astrocyte-to-oligodendrocyte (A/O gap junctions) (Nagy *et al.*, 1997, 1999, 2001; Rash *et al.*, 2001), and Cx29, Cx32, Cx47 are expressed in oligodendrocytes, with the latter two localized in the oligodendrocyte side of A/O gap junctions (Altevogt *et al.*, 2002; Nagy *et al.*, 2003; Li *et al.*, 2004b; Odermatt *et al.*, 2003).

A wide range of functions have been attributed to gap junctional intercellular communication (GJIC) during development and in mature tissues (Evans & Martin, 2002; Nakase & Naus, 2004). Analyses of these functions have been advanced by identification of proteins that interact either with connexins or with other gap junction-associated proteins (Herve *et al.*, 2004; Giepmans, 2004), and that may contribute to processes governing GJIC, such as connexin cellular trafficking, gap junction assembly and/or regulation of channel conductance. Zonula occludens-1 (ZO-1), originally found to associate with tight and adherens junctions (Stevenson *et al.*, 1986; Itoh *et al.*, 1991), interacts with several connexins, including

Cx31.9, Cx36, Cx43, Cx45, Cx46 and Cx50 (Giepmans & Moolenaar, 1998; Toyofuku *et al.*, 1998; Kausalya *et al.*, 2001; Laing *et al.*, 2001; Nielsen *et al.*, 2002, 2003; Li *et al.*, 2004a). In the CNS, ZO-1 occurs at tight junctions along blood vessels (Wolburg & Lippoldt, 2002), and was found only recently to be expressed in neurons and oligodendrocytes (Inagaki *et al.*, 2003; Li *et al.*, 2004b). In oligodendrocytes, ZO-1 was found to be associated with A/O gap junctions and to interact with Cx47 via its second PDZ domains (Li *et al.*, 2004b). ZO-1 was reported to be expressed in astrocytes in vitro, but not in vivo (Howarth *et al.*, 1992; Song *et al.*, 2005).

In the present study, we re-examined ZO-1 localization in the CNS by immunofluorescence using a number of different anti-ZO-1 antibodies, and now provide evidence for ZO-1 expression in astrocytes in vivo. Double immunofluorescence labelling and immunoprecipitation (IP) approaches were used to demonstrate association of ZO-1 with astrocytic Cx30 and Cx43 in mouse brain, and pull-down assays were used to establish direct interaction of ZO-1 with Cx30. In addition, antibodies were developed against Y-box transcription factor 3 (MsY3), which was identified as the mouse ortholog of canine ZONAB (ZO-1-associated nucleic acid binding protein). We used double and triple immunofluorescence analyses to determine expression patterns of ZONAB in mouse CNS, and to demonstrate its co-localization with Cx32, Cx47 and ZO-1 at oligodendrocytic gap junctions, and with Cx43 at astrocytic gap junctions.

MATERIALS AND METHODS

Antibodies and animals

The antibodies used in this study, including specificity, dilutions employed, source and references to previous characterization are listed in Table 1. Most of the anti-connexin

antibodies used were obtained from Zymed Laboratories (South San Francisco, CA, USA), except polyclonal goat anti-Cx43 and polyclonal goat anti-Cx32, which were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal anti-2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase), used as a marker for immunolabelling oligodendrocytes, was obtained from Sternberger Monoclonals (Baltimore, MD, USA). We tested a series of ten different anti-ZO-1 antibodies for efficacy of ZO-1 detection in the immunohistochemical protocols described below. Four of these were obtained from Zymed Laboratories, one from Chemicon International (Temecula, CA, USA), three from Santa Cruz Biotechnology, one from Sanko Junyaku (Tokyo, Japan) and one from the National Institute of Child Health and Development (NICHD) (Iowa, IA, USA). These antibodies were generated against different sequences in ZO-1 and, where known, are indicated in Table 1, and diagrammatically in Fig. 1.

The rabbit polyclonal anti-ZONAB antibody was obtained from Zymed Laboratories (Cat No. 40-2800). This antibody was newly developed against what was deduced to be the mouse ortholog of canine ZONAB, and is listed in GenBank database as mouse Y-box transcription factor 3 (MsY3), or alternatively, cold shock domain protein. The antibody was generated against a region of ZONAB that is present in both the long isoform of this protein, as well as its shorter alternatively spliced isoform (ZONAB A, accession AAF 72335; ZONAB B, accession AAF 72336; MsY-box 3 short form, accession AAG14419; MsY-box long form, accession AAG14418; cold shock domain protein A short form, accession AAH62377; cold shock domain protein A long form, accession AAH48242).

Forty-two adult male CD1 mice (25-35 g) and six adult Sprague-Dawley rats (300-350 g) were obtained from the University of Manitoba Central Animal Service and were treated respecting the approved protocols of the Central Animal Care Committee. The experiments

were conducted in such a manner as to minimize the exposure of animals to stress and to other non-physiological stimuli.

Western blotting

Following decapitation of mice, brain samples were rapidly frozen on dry ice and stored at -80 °C. Brain tissues were homogenized in immunoprecipitation (IP) buffer containing 20 mM Tris-HCl, pH 8.0, 140 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM EGTA, 1.5 mM MgCl₂, 1 mM dithiothreitol, 1 mM phenylmethylsulphonyl fluoride, and 5 µg/mL each of the protease inhibitors leupeptin, pepstatin A and aprotinin. Tissue homogenates were sonicated and briefly centrifuged, followed by protein determination using a kit (Bio-Rad Laboratories, Hercules, CA, USA). Sample protein (20 µg) was separated electrophoretically in 7% and 12.5% polyacrylamide gels for detection of ZO-1 and ZONAB, respectively, and transblotted to polyvinylidene difluoride membranes (Bio-Rad) in standard Tris-glycine transfer buffer (pH 8.3) containing 0.5% sodium dodecylsulphate. Membranes were blocked for 2 h at room temperature in TBSTw buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl with 0.2% Tween-20) containing 5% non-fat milk powder, briefly washed with TBSTw, and then incubated for 16 h at 4 °C with anti-ZO-1 antibody (1 µg/mL) in TBSTw containing 1% non-fat milk powder. Membranes were then washed four times in TBSTw over a total of 40 min, incubated with horseradish peroxidase-conjugated donkey anti-rabbit or anti-mouse IgG diluted 1:5000 (Sigma-Aldrich Canada, Oakville, ON, Canada) in TBSTw containing 1% non-fat milk powder, washed with TBSTw four times over 40 min, and visualized by chemiluminescence (ECL, Amersham PB, Baie d'Urfe, Quebec, Canada).

Immunoprecipitation

Tissues from brain homogenized in IP buffer were used for immunoprecipitation procedures as previously described (Zhurinsky et al., 2000; Li et al., 2004a,b). Briefly, homogenates were sonicated, centrifuged at 20,000 g for 10 min at 4 °C, and 2 mg of supernatant protein was washed for 1 h at 4 °C with 20 µL of protein A-coated agarose beads (Santa Cruz Biotechnology), followed by centrifugation at 20,000 g for 10 min at 4 °C. Supernatants were incubated with 2 µg of monoclonal anti-ZO-1 antibody with shaking for 2 h at 4 °C, followed by 1 h incubation with 20 µL protein-A-coated agarose beads, and then centrifugation at 20,000 g for 10 min at 4 °C. The beads were washed vigorously five times with 1 mL washing buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl and 0.5% NP-40) and then boiled for 3 min in sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer containing 10% β-mercaptoethanol. Samples were processed by SDS-PAGE and immunoblot membranes were probed with anti-connexin antibodies.

Pull-down assay with GST-PDZ fusion proteins of ZO-1

Three plasmids (pGEX-3X) separately containing either the PDZ1, PDZ2 or PDZ3 domains of ZO-1 were kindly provided by Dr. Giepmans (University of California, San Diego). GST-PDZ domain fusion protein expression, coating to glutathione-agarose 4B beads and pull-down assays were conducted as previously described (Nielsen et al., 2002,2003; Li, et al., 2004). Briefly, pGEX-3X plasmids were transformed into *Escherichia coli* DH5α, and individual clones were selected and grown in LB media supplemented with 100 µg/mL ampicillin at 37 °C, and then further supplemented with isopropyl-β-D-thiogalactopyranoside (1 mM) for induction of GST-PDZ protein expression. Bacterial cells were harvested and washed with

50 mM Tris-HCl, pH 7.6, centrifuged at 10,000 g for 20 min, and pellets were stored at -80°C . Pellets were re-suspended in buffer (10 mM sodium phosphate, pH 8.0, containing 150 mM NaCl, 1% Triton X-100, 5% glycerol, 1 mM phenylmethylsulphonyl fluoride, 1 mM EDTA, 5 $\mu\text{g/mL}$ each of aprotinin, pepstatin A, leupeptin), centrifuged for 10 min at 20,000 g, and the supernatant was added to glutathione-agarose 4B beads with shaking at 4°C for 2 h. The mixture was centrifuged at 2,000 g for 5 min and the beads were washed five times with PBS buffer containing 1% Triton X-100. The beads with bound PDZ domain fusion proteins were incubated overnight with tissue from mouse thalamus homogenized in IP buffer. After washing extensively five times in sodium phosphate buffer, pH 7.4, containing 0.9% saline and 1% Triton X-100, protein bound to the PDZ domains linked to the agarose beads were eluted with SDS-PAGE sample buffer, separated by SDS-PAGE, and transferred to polyvinylidene difluoride membrane. Immunoblot membranes were probed with polyclonal anti-Cx30 antibody (Zymed Laboratories, 71-2200) diluted at 1:1,000, followed by chemiluminescence detection of immunoreactive bands. Membranes were then stripped and reprobed with anti-GST antibody diluted at 1:10,000 to confirm equal loading of GST-PDZ domain fusion protein.

Light microscopic immunofluorescence

Mice were deeply anesthetized with equithesin (3 ml/kg) and then perfused transcardially with 3 ml of pre-fixative solution consisting of cold (4°C) 50 mM sodium phosphate buffer (PB), pH 7.4, 0.9% NaCl, 0.1 sodium nitrate and heparin (1 unit/ml). This was followed by perfusion of animals with 40 ml of fixative solution containing cold 0.16 M sodium PB, pH 7.1, 0.2% picric acid and either 1%, 2% or 4% formaldehyde. The animals were then perfused with 10 ml

of PB containing 10% sucrose. Brains were removed and stored at 4°C for 48 to 72 h in cryoprotectant solution consisting of the final perfusate. Sections 10 µm thick were cut on a cryostat, collected on gelatinized glass slides and stored at -34°C until use for immunohistochemistry. Sections were washed for 20 min in 50 mM of Tris-HCl, pH 7.4, containing 1.5% sodium chloride (TBS) and 0.3% Triton X-100 (TBSTr). All primary antibodies were used at concentrations indicated in Table 1, and were diluted in TBSTr containing 5% normal goat or normal donkey serum. For single immunofluorescence labelling, sections were incubated for 24 h at 4°C with either monoclonal anti-ZO-1 antibody or rabbit polyclonal anti-ZONAB antibody, then washed for 1 h in TBSTr and incubated with secondary antibody for 1.5 h at room temperature. The secondary antibodies used were Cy3-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Groove, PA, USA) diluted 1:200, FITC-conjugated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA) diluted 1:100, and Alexa Fluor 488-conjugated donkey anti-rabbit IgG (Vector Laboratories) diluted 1:1000.

For double immunofluorescence labelling involving Cx30 and Cx43, sections were incubated simultaneously with anti-ZO-1 and either anti-Cx43 or anti-Cx30. For double immunofluorescence labelling in studies involving ZONAB, sections were incubated simultaneously with polyclonal anti-ZONAB antibody and either monoclonal anti-CNP-ase, anti-ZO-1, anti-Cx43, anti-Cx32 or anti-Cx47, followed by simultaneous incubation with combinations of two secondary antibodies as described below. For triple immunofluorescence labelling, sections were simultaneously incubated as above with rabbit anti-ZONAB, mouse anti-CNPase and goat anti-Cx32, or alternatively with anti-rabbit ZONAB, mouse anti-ZO-1 and goat anti-Cx32. After washing, sections were simultaneously incubated with Cy5-

conjugated donkey anti-mouse IgG, (Jackson ImmunoResearch Laboratories), diluted 1:200, Cy3-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories) diluted 1:200 and Alexa Fluor 488-conjugated donkey anti-goat IgG (Molecular Probes, Eugene, Oregon, USA) diluted 1:1000. After secondary antibody incubations, all sections were washed in TBSTr for 20 min, followed by two 15 min washes in 50 mM Tris-HCl buffer, pH 7.4, and coverslipped with antifade medium. In order to confirm the absence of inappropriate cross-reactions between primary and secondary antibodies used in double and triple labelling studies, single primary antibody omissions with inclusion of the complete set of secondary antibodies were performed for each of the combinations used.

The proportion of CNPase-immunopositive cells that were also immunopositive for ZONAB was determined by counts of double labelled cells in fields of cerebral cortex, striatum, hippocampus, hypothalamus and thalamus, with ZONAB-positive cells expressed as a percentage of CNPase-positive cells. The number of cells counted per area ranged from 55 to 75 with a mean of 300 CNPase-positive cells examined per animal in a total of five mice. Immunofluorescence was examined on a Zeiss Axioskop2 fluorescence microscope, using Axiovision 3.0 software (Carl Zeiss Canada, Toronto, Ontario) for image capture. For confocal immunofluorescence analysis, double- and triple-labelled sections were laser scanned using an Olympus Fluoview IX70 confocal microscope, and images were captured with Olympus Fluoview software. The final images were assembled using Photoshop 6.0 (Adobe Systems, San Jose, CA, USA), Corel Draw 8 and Northern Eclipse software (Empix Imaging, Missisagua, Ontario, Canada).

RESULTS

Immunofluorescence localization of ZO-1 in brain

In an attempt to gain confidence in cellular expression patterns of ZO-1 observed by immunofluorescence in the mouse CNS, various anti-ZO-1 antibodies directed against different as well as partially overlapping sequences in ZO-1 were employed. These antibodies and the amino acid sequences in ZO-1 used as immunogen, based on information provided by suppliers, are indicated in Fig. 1 and Table 1. Immunofluorescence labelling with each antibody was examined in liver and brain under various fixation conditions, with results summarized in Table 2. Similar immunofluorescence labelling patterns were obtained with most of the anti-ZO-1 antibodies, suggesting specificity of ZO-1 detection. Anti-ZO-1 antibodies obtained from Zymed Laboratories Inc, Sanko Junyaku and NICHD all produced strong labelling of ZO-1 associated with tight junctions in liver and along blood vessels in brain, and moderately to weak, dispersed punctate labelling in brain parenchyma, with little background. The Chemicon anti-ZO-1 antibody produced robust labelling of tight junctions in liver and brain, but gave only weak punctate labelling associated with oligodendrocytes in brain. Anti-ZO-1 antibodies from Santa Cruz Biotechnology gave either weak or an absence of tight junction labelling in liver and brain. Differences in labelling may be due to antibody quality or to differences in fixation protocols required for each antibody, and poor results with some of the antibodies may be due to our use of inappropriate tissue fixation procedures. As in previous studies (Li et al., 2004b), we found detection of ZO-1 to be highly sensitive to overfixation, such that detection of ZO-1-positive puncta in brain was best achieved with the weakest fixation conditions tested.

In liver, examples of ZO-1 labelling patterns at tight junctions are shown with antibody 61-7300 (Fig. 2A), Ab01033 (Fig. 2B) and 33-9100 (Fig. 2C). In CNS, an example of ZO-1 labelling along a blood vessel is shown with antibody 61-7300 (Fig. 2D). Dense, punctate labelling was obtained with this latter antibody throughout the brain, as shown in globus pallidus (Fig. 2E), subthalamic nucleus (Fig. 2F) and the lateral pons (Fig. 2G). Consistent with ZO-1 localization in mossy fiber terminals in the CA3 area of the hippocampus (Inagaki *et al.*, 2003) and in tight junctions of choroid plexus (Wolburg *et al.*, 2001), ZO-1 in these regions was detected with several antibodies (Table 2), as shown in hippocampus with antibody 33-9100 (Fig. 2H), and around choroidal ependymal cells with antibody 61-7300 (Fig. 2I). ZO-1 was also detected in white matter with some of these antibodies (Fig. 2I).

Co-localization of ZO-1 with astrocytic Cx43 and Cx30

Anti-ZO-1 antibodies that produced widely distributed punctate labelling in brain parenchyma were used in laser scanning confocal double immunofluorescence studies to examine the localization of ZO-1 in relation to that of the astrocytic connexins Cx43 and Cx30. In sections labelled for Cx43 with monoclonal 35-5000 and ZO-1 with polyclonal 61-7300, substantial co-localization of immunofluorescent puncta was observed in most brain regions, as illustrated in the cerebral cortex (Fig. 3A), hippocampus (Fig. 3B), globus pallidus (Fig. 3C) and thalamus (Fig. 3D). Sections double labelled with a second combination of antibodies, polyclonal anti-Cx43 71-0700 and monoclonal anti-ZO-1 33-9100, also displayed ZO-1/Cx43 punctate co-localization, as shown in cerebral cortex (Fig. 3E), striatum (Fig. 3F) and thalamus (Fig. 3G). Similar results were obtained in sections double labelled with a third combination of antibodies, polyclonal anti-Cx43 71-0700 and monoclonal anti-ZO-1 Ab01033, as shown in cerebral

cortex (Fig. 3H). However, not all Cx43-immunopositive puncta were ZO-1 positive and, conversely not all ZO-1-positive puncta were Cx43-positive. Moreover, individual Cx43-positive puncta were often only partially labelled for ZO-1. Oligodendrocyte somata and their initial processes were densely decorated with intense punctate immunolabelling for Cx43 and ZO-1, as previously described (Li et al., 2004), and were readily identified in some of the images shown in Figure 3, thus confirming ZO-1 association with oligodendrocytes using additional anti-ZO-1 antibodies.

Based on findings of extensive co-association of Cx43 with Cx30 in brain, it appeared likely that ZO-1 may also be co-localized with Cx30 (Rash *et al.*, 2001). This was confirmed by laser scanning confocal immunofluorescence analysis of sections double labelled with polyclonal anti-Cx30 71-2200 and monoclonal anti-ZO-1 33-9100, as shown in the globus pallidus (Fig. 4A) and hypothalamus (Fig. 4B). In order to establish that anti-ZO-1 antibodies directed against different sequences in ZO-1 produce labelling of identical punctate structures, brain sections were double labelled with polyclonal anti-ZO-1 61-7300 and monoclonal anti-ZO-1 Ab01033. As shown in hypothalamus (Fig. 4C) and subthalamic nucleus (Fig. 4D), a high degree of co-localization was observed with the two antibodies. In view of our report of ZO-1 localization at oligodendrocyte gap junctions (Li et al., 2004b), and association of astrocytic Cx30 and Cx43 with these junctions (Rash et al., 2001; Nagy et al., 2003), it was possible that ZO-1/Cx43 and ZO-1/Cx30 co-localization simply reflected oligodendrocytic ZO-1 association with astrocytic connexins at heterologous A/O gap junctions. This was tested by triple immunolabelling for Cx30, Cx32 and ZO-1, where Cx32 was used as a marker for oligodendrocyte gap junctions. As demonstrated in hippocampus (Fig. 4E), dense punctate labelling for Cx30 and ZO-1 were found to be co-associated with Cx32 on oligodendrocyte

somata, but labelling for Cx32 was much more restricted, and numerous puncta lacking Cx32 were seen to display Cx30/ZO-1 co-localization.

ZO-1 co-IP with Cx43 and Cx30, and interaction with Cx30

Homogenates of various mouse brain regions as well as whole brain were used to confirm ZO-1 detection by western blotting, and to test efficacy of antibody for IP of ZO-1. In cerebral cortex, thalamus, medulla and whole brain, anti-ZO-1 antibody Ab33-9100 detected a band between 210 and 220 kDa (Fig. 5A), which corresponds to the molecular weight of ZO-1. As shown in Figure 5B, immunoblots probed with anti-ZO-1 antibody 61-7300 also detected ZO-1 in homogenates of thalamus, as well as in IP material obtained with anti-ZO-1 Ab33-9100 from homogenate of thalamus.

To examine molecular association of Cx43 and Cx30 with ZO-1, homogenates of mouse brain regions were taken for IP of ZO-1 with anti-ZO-1 antibody 33-9100, and separate immunoblots of precipitates were probed with either anti-Cx43 antibody 18A or with anti-Cx30 antibody 71-2200. Immunoprecipitated protein consistently showed the presence of both Cx43 (Fig. 6A) and Cx30 (Fig. 6B).

In vitro pull-down assays involving incubation of thalamus tissue homogenates with plasmid-derived fusion proteins containing GST linked separately with each one of the three PDZ domains in ZO-1 were conducted to determine whether Cx30 directly interacts with the PDZ domains of ZO-1. Immunoblots loaded with material eluted from thalamus proteins that bound the PDZ2 domain of ZO-1 and probed with anti-Cx30 Ab71-2200 revealed the presence of Cx30 (Fig. 7A, lane 3), with blots of thalamus homogenates serving as positive controls for Cx30 detection (Fig. 7A, lane 1). Blots loaded with material bound to the PDZ1 and PDZ3

domains of ZO-1 showed an absence of Cx30 (Fig. 7A, lanes 2 and 4). Immunoblot membranes stripped and reprobed with anti-GST antibody confirmed the presence of GST-PDZ fusion protein with expected molecular weight (40-42 kDa) in material applied to the blots (Fig. 7B, lanes 2, 3 and 4), and its absence in homogenates of thalamus (Fig. 7B, lane 1).

Expression and immunolabelling of ZONAB in mouse brain

A newly generated antibody against ZONAB was used to examine ZONAB expression in brain by western blotting and immunofluorescence. As shown in Figure 8A, immunoblots loaded with homogenates of thalamus, cerebral cortex and cerebellum from mouse brain and probed with anti-ZONAB antibody showed detection of a 30-32 kDa band, which corresponds to the predicted molecular weight (30.8 kDa) of the shorter alternatively spliced form of MsY3.

Immunofluorescence labelling with anti-ZONAB antibody in mouse brain revealed association of intense labelling with small round and ovoid shaped cells, resembling oligodendrocytes, as well as with densely distributed puncta in both gray and white matter, as shown in the thalamus (Fig. 8B) and fimbria (Fig. 8C), respectively. Similar results were obtained in various areas of rat CNS (not shown). To establish ZONAB association with oligodendrocytes, sections from mouse were double-labelled with ZONAB and either with the oligodendrocyte marker CNPase, or with the oligodendrocyte gap junction proteins Cx47 and Cx32. As shown in low magnification images of various brain regions, virtually all CNPase-positive cells were immunopositive for ZONAB (Fig. 9A), nearly all Cx47-positive cells in gray matter (Fig. 9B) and white matter (Fig. 9C) were immunopositive for ZONAB, and nearly all Cx32-positive cells were immunopositive for ZONAB (Fig. 9D). Additional labelling of

ZONAB not associated with oligodendrocytes in these images consisted of immunopositive puncta that was poorly resolved at low magnification.

By confocal microscopy, immunolabelling of ZONAB associated with CNPase-positive cells appeared as puncta distributed around the cell periphery (Fig. 9E). Oligodendrocytes in all brain regions could be readily identified by their intense ZONAB immunoreactivity. However, in sections from brains fixed with 2% paraformaldehyde, these cells displayed both immunopositive puncta as well as intense, diffuse intracellular labelling that tended to obscure puncta at the cell periphery. In sections from brains fixed with 1% paraformaldehyde, oligodendrocytes displayed robust immunofluorescent punctate, reduced diffuse cytoplasmic labelling, and some intracellular punctate labelling. Dispersed punctate labelling for ZONAB not associated with oligodendrocytes appeared the same with both fixation protocols. In double labelled sections, counts of more than 1500 CNPase-positive oligodendrocytes in five different brain regions of five adult CD1 mice indicated that up to 98% of these cells were also immunolabelled for ZONAB (Table 3).

Co-localization of ZONAB with Cx47, Cx32, ZO-1 and Cx43

Double labelling confocal microscopy was used to examine ZONAB association with Cx47 and Cx32, which were previously shown to be localized at the oligodendrocyte side of A/O gap junctions (Li et al., 2004b). At all levels of CNS, punctate labelling of Cx47 associated with oligodendrocyte somata and their initial processes was invariably co-localized with ZONAB-immunopositive puncta, as shown in the cerebral cortex (Fig. 9F), thalamus (Fig. 9G) and amygdala (Fig. 9H). Similarly, triple immunofluorescence labelling revealed that Cx32-positive puncta on oligodendrocyte somata was co-localized with punctate labelling for

ZONAB around the periphery of CNPase-positive oligodendrocytes, as shown in the hippocampus (Fig. 10A) and thalamus (Fig. 10B). Double labelling in the same brain areas as well as others examined indicated near total co-localization of ZO-1-positive puncta with ZONAB-positive puncta on oligodendrocyte somata (Fig. 10C,D). Co-localization of Cx32 with ZONAB and with ZO-1 on oligodendrocytes, together with Cx47/ZONAB co-localization shown in Figure 9F-H, is consistent with previous demonstrations of Cx47 co-localization with Cx32 and with ZO-1 on oligodendrocyte somata (Li et al., 2004b).

Evidence for ZONAB expression in astrocytes and its localization at astrocytic gap junctions was sought in sections double labelled for Cx43 and ZONAB. As shown by low magnification in brain areas containing a high density of punctate labelling for both of these proteins, including the thalamus (Fig. 10E) and the subthalamic nucleus (Fig. 10F), Cx43 was substantially co-localized with ZONAB. However, there appeared to be a greater proportion of ZONAB-immunopositive puncta that lacked overlap with Cx43. As in the case of individual puncta containing only partial overlap of ZO-1 with either Cx43 or Cx30, a similar partial overlap at distinct puncta was observed with Cx43/ZONAB (Fig. 10E,F).

DISCUSSION

The present results confirm and extend our recent reports on ZO-1 expression in the mouse brain (Li et al., 2004a, b). In particular, evidence for ZO-1 expression in astrocytes *in vivo* was provided by its co-localization with Cx30 and Cx43, which are largely concentrated in gap junctions between astrocytes. Results demonstrating co-IP of ZO-1 with both Cx30 and Cx43 suggested molecular association of ZO-1 with each of these connexins, and *in vitro* pull-down assays demonstrated the capability of direct interaction of Cx30 with the second PDZ domain

of ZO-1. Our investigations of ZONAB revealed the presence of this Y-box transcription factor at macroglial gap junctions in vivo, as indicated by its co-localization with Cx32 and Cx47 in oligodendrocytes, and with Cx43 in astrocytes. These anatomical and biochemical data provide new avenues for consideration of GJIC regulation via functions associated with ZO-1, and suggest that possible functions of gap junctionally localized ZO-1 in glial cells is linked to reported cellular activities of ZONAB.

Oligodendrocytic and astrocytic expression of ZO-1

Although initially identified at tight junctions (Stevenson *et al.*, 1986; Mitic & Anderson, 1998; Gonzalez-Mariscal *et al.*, 2003), ZO-1 was subsequently found at adherens junctions and gap junctions in a variety of peripheral tissues (Itoh *et al.*, 1991; Thomas *et al.*, 2002; Giepmans, 2004). In the CNS, ZO-1 has been well documented in tight junctions of endothelial cells (Petrov *et al.*, 1994; Wolburg & Lippoldt, 2002), but also occurs in neuroepithelial cells (Saitou *et al.*, 1997), olfactory sensory neurons (Miragall *et al.*, 1994) and hippocampal mossy fiber terminals (Inagaki *et al.*, 2003), as well as at gap junctions formed by neurons and by oligodendrocytes (Li *et al.*, 2004a, b). The present findings now include astrocytes in the repertoire of CNS cell types that express ZO-1. Although earlier studies failed to detect ZO-1 expression in astrocytes in vivo, an abundance of this protein was found in cultured astrocytes (Howarth *et al.*, 1992; Song *et al.*, 2005), where it was shown to be directly associated with Cx43 (Duffy *et al.*, 2004).

Our immunofluorescence studies of ZO-1 using a variety of commercially available anti-ZO-1 antibodies represent an attempt to confirm the increasingly broader ZO-1 expression patterns identified in the CNS, and reconcile this with earlier literature indicating its restricted

distribution largely along blood vessels. Most but not all of the antibodies gave labelling of ZO-1 in tissues used as controls for ZO-1 detection, and most gave various intensities of punctate labelling that was localized to oligodendrocytes or that resembled the distribution of astrocytic connexins. However, labelling of ZO-1 associated with glial structures tended to be weaker and more sensitive to tissue fixation conditions, perhaps due to a lower abundance of ZO-1 or partial blockade of antibody epitopes by ZO-1 interacting proteins at these structures, which may explain earlier difficulties in ZO-1 detection in CNS macroglial cells.

Localization of ZO-1 at glial gap junctions

In correlative light microscope (LM) and electron microscope (EM) studies, we found that Cx30 and Cx43 in gray matter of brain parenchyma are co-localized in the vast majority of A/A gap junctions and are contained largely within these junctions, with very little detection of these proteins intracellularly (Yamamoto *et al.*, 1990a, b; Nagy *et al.*, 1999; Rash *et al.*, 2001). Consequently, these connexins may be regarded as markers for gap junctions between gray matter astrocytes, and it can thus be inferred that co-localization of Cx30 or Cx43 with dispersed punctate labelling of ZO-1 indicates the association of ZO-1 with A/A gap junctions. However, since a proportion of dense punctate labelling for ZO-1 occurred independently of these two connexins in some brain regions, we cannot exclude the possibility that ZO-1 is absent in some recently described subpopulations of A/A gap junctions (Altevogt & Paul, 2004), or that ZO-1 is also a component of other punctate-like cellular structures in the CNS, e.g., adherens junctions.

Cx30 and Cx43 in brain are also co-localized in the astrocyte side of A/O gap junctions, and freeze-fracture replica immunogold labelling studies have demonstrated the presence of

ZO-1 co-localized with Cx32 and Cx47 on the oligodendrocyte side of these junctions (Li *et al.*, 2004b). It is thus noteworthy that our finding of ZO-1 association with Cx30 and Cx43 does not simply reflect the exclusive presence of these connexins and ZO-1 in A/O gap junctions because Cx30/ZO-1 and Cx43/ZO-1 co-localization was far more widely distributed than oligodendrocytic Cx47/ZO-1 or Cx32/ZO-1 co-localization. Further, our triple immunofluorescence labelling demonstrated that Cx43/ZO-1 co-localization occurred independently of labelling for oligodendrocytic Cx32 at A/O gap junctions. In view of the presence of ZO-1 on the oligodendrocyte side of A/O gap junctions (Li *et al.*, 2004b), and given limits of LM resolution that preclude assignment of immunofluorescence labelled proteins to one or the other side of gap junctions, EM analyses will be required to determine whether ZO-1 also occurs on the astrocyte side of these A/O junctions.

Cx43 and Cx30 interaction with ZO-1

Based on reports that Cx43 directly interacts with the second PDZ domain of ZO-1 (Giepmans & Moolenaar, 1998; Toyofuku *et al.*, 1998), together with our findings of Cx30 and Cx43 co-localization and co-IP with ZO-1, and our demonstration that Cx30 also has the capability to interact with the second PDZ domain of ZO-1, it appears that both Cx30 and Cx43 at A/A gap junctions interact with ZO-1. Cx30/ZO-1 interaction is consistent with the presence of a PDZ consensus binding motif in the carboxy terminus sequence of Cx30, namely amino acids SFPS, which likely mediates interaction with ZO-1, but remains to be confirmed by pull-down assay after mutational deletion of the motif. Among the twenty mammalian connexin proteins identified, a total of seven have now been found to interact with PDZ domains of ZO-1.

Unlike the near total overlap of immunofluorescence labelling for Cx47 and ZO-1 previously observed at individual A/O gap junctions around oligodendrocyte somata (Li et al., 2004b), here we found immunofluorescence labelling of ZO-1 restricted to subportions of individual A/A gap junctions labelled for either Cx30 or Cx43. It is unlikely that this is due to segregation of Cx30 and Cx43 in these junctions, with a greater degree of ZO-1 association with one or the other of these connexins, because double labelling studies have indicated a uniform distribution of Cx30 and Cx43 in A/A gap junctions (Rash et al., 2001). Rather, expression of ZO-1 in astrocytes may be limited to levels insufficient for its complete binding to what may be a relatively greater abundance of connexins in these cells. Alternatively, ZO-1 may be selectively targeted to specific subdomains of A/A gap junctions, such as structurally stable regions or to areas actively involved in connexin turnover, which have been described in various cell systems (Lauf *et al.*, 2002; Segretain & Falk, 2004).

ZONAB expression in glia and localization at gap junctions

ZONAB is a Y-box transcription factor that has been well studied in relation to tight junctions in canine MDCK cells, and reported to interact with the SH3 domain of ZO-1 (Balda & Matter, 2000; Balda *et al.*, 2003). ZONAB has a long (ZONAB B) and an alternatively spliced isoform (ZONAB A) lacking sixty-eight amino acids. Canine ZONAB B and ZONAB A exhibit 86% homology with a long and short isoform of mouse MsY3 (GeneBank accession AAG14418, AAG14419.1), the sixty-eight amino acid sequence absent in canine ZONAB has a corresponding sequence absent in the mouse short isoform, and these proteins in both species contain a highly conserved cold shock domain. Thus, we inferred that MsY3 transcription factor is the mouse ortholog of canine ZONAB, and chose to refer to this ortholog using the

more descriptive term ZONAB. Our results showing co-localization of ZONAB with astrocytic and oligodendrocytic connexins, as well as with ZO-1, indicates expression of this protein in glial cells, its association with glial gap junctions, and its likely interaction with ZO-1 at these junctions. The presence of a known ZO-1 interacting protein at glial gap junctions provides additional indirect support for the presence of ZO-1 at these junctions. Although immunoblots showed detection of only ZONAB A in brain, more detailed biochemical and molecular studies of both ZONAB isoforms in neural tissues are required to determine precise isoform expression patterns.

Since the functional role of ZO-1 at gap junctions is largely unknown, cellular functions attributed to ZONAB in other systems may provide clues to regulatory and/or scaffolding functions of ZO-1 at gap junctions. In canine MDCK cells (Balda & Matter., 2000; Balda *et al.*, 2003), the transcription factor ZONAB acts to repress expression of the tyrosine kinase receptor ErbB2 (a.k.a. Neu or HER2), which has diverse roles in regulation of cell growth and differentiation (Casalini *et al.*, 2004; Holbro and Hynes, 2004; Marmor *et al.*, 2004). It was proposed that sequestration of ZONAB via association with ZO-1 at tight junctions of these cells derepresses ErbB2 expression and thereby promotes ErbB2-mediated cell differentiation (Balda & Matter, 2000). In oligodendrocytes and astrocytes, ErbB2 serves as a receptor of neuregulin, and neuregulin/ErbB2 signalling is essential for oligodendrocyte survival, differentiation and myelination (Tokita *et al.*, 2001; Park *et al.*, 2001; Schmid *et al.*, 2003; Patten *et al.*, 2003; Kim *et al.*, 2003). Based on these observations, together with the present findings, we speculate that these and other functions of ErbB2 signalling in glial cells may, in part, be regulated by interactions between connexins, ZO-1 and ZONAB at glial gap junctions.

Various junctional complexes at cell-cell contacts, including tight junctions, adherens junctions and desmosomes, have been described as key platforms for signalling, modulation of transcriptional activity and regulation of diverse and vital cellular processes such as cell proliferation and differentiation (Zahraoui *et al.*, 2000; Balda and Matter, 2003; Wei *et al.*, 2004). Recently, it has been considered that such regulatory platforms may include gap junctions specifically composed of Cx43 and attendant kinases, phosphatases, membrane receptors and signalling molecules (Singh & Lampe, 2003, Wei *et al.*, 2004). This idea is supported by the presence of ZO-1/ZONAB at glial gap junctions, and may be further extended to include gap junctions composed of other connexins and regulatory proteins.

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Table 1. Antibodies used for western blotting and immunohistochemistry

Antibody	Type	Species	Epitope*; Designation	Dilution	Source; Reference
Cx30	polyclonal	Rabbit	aa 241-261; 71-2200	2 µg/ml	Zymed; Nagy et al., 1999
Cx32	monoclonal	Mouse	aa 235-246; 35-8900	2 µg/ml	Zymed; Nagy et al., 2003
Cx32	polyclonal	Goat	c-terminus; sc-7258	2 µg/ml	Santa Cruz Biotech
Cx43	monoclonal	Mouse	c-terminus; 35-5000	2 µg/ml	Zymed; Nagy et al., 2003
Cx43	polyclonal	Goat	c-terminus; sc-6560	2 µg/ml	Santa Cruz Biotech
Cx43	polyclonal	Rabbit	c-terminus; 71-0700	0.25 µg/ml	Zymed; Li et al., 1998
Cx43	polyclonal	Rabbit	aa 346-363; 18A	1:1000	Yamamoto et al., 1990a
Cx47	monoclonal	Mouse	aa 407-420; 37-4500	2 µg/ml	Zymed
Cx47	polyclonal	Rabbit	c-terminus; 36-4700	2 µg/ml	Zymed; Li et al., 2004b
ZO-1	monoclonal	Mouse	aa 334-634; 33-9100	2.5 µg/ml	Zymed
ZO-1	polyclonal	Rabbit	aa 463-1109; 61-7300	3 µg/ml	Zymed
ZO-1	polyclonal	Rabbit	n-terminus; 40-2200	2 µg/ml	Zymed
ZO-1	polyclonal	Rabbit	mid-region; 40-2300	2 µg/ml	Zymed
ZO-1	monoclonal	Rat	protein; AB1520	1:100	Chemicon
ZO-1	monoclonal	Rat	protein; R26.4C	1:1	NICHD
ZO-1	monoclonal	mouse	aa 1-862; AB01033	1:500	Sanko Junyaku
ZO-1	polyclonal	Rabbit	aa 1437-1736; sc-10804	1:100	Santa Cruz Biotech
ZO-1	polyclonal	Goat	aa 2-20; sc-8147	1:100	Santa Cruz Biotech
ZO-1	polyclonal	Goat	aa 1728-1746; sc-8146	1:100	Santa Cruz Biotech
ZONAB	polyclonal	Rabbit	c-terminus; 40-2800	2 µg/ml	Zymed
CNPase	monoclonal	Mouse	whole protein	1:5000	Sternberger Monoclonals

Table 2. Immunofluorescence results for the anti-ZO-1 antibodies used

ZO-1 antibody	Tissues and structures examined					
	liver TJ	brain BV TJ	choroid plexus TJ	mossy fiber terminals	oligodendrocyte somata	Dispersed Puncta
Monoclonal 33-9100 Zymed	++++	++++	+++	+++	++++	+++
Polyclonal 61-7300 Zymed	+++	+++	++++	++	++++	+++
Polyclonal 40-2200 Zymed	++++	++++	++++	++++	++	++
Polyclonal 40-2300 Zymed	++++	++++	+++	+++	++	++
Monoclonal R26.4C NICHD	++++	++++	++++	+++	++	+
Monoclonal Ab01033 Sanko Junyaku	++++	++++	++++	+++	++	+
Monoclonal MAb1520 Chemicon	+++	+++	+++	0	+	0 *
Polyclonal sc-10804 Santa Cruz	+	++	+	0	0	0
Polyclonal sc-8147 Santa Cruz	0	0	0	0	0	0 **
Polyclonal sc-8146 Santa Cruz	0	0	0	0	0	0

Labelling intensity is indicated by: ++++ very intense; +++ intense; ++ moderate; + weak

* high background may have obscured weak labelling

** labelled all dendrites and all neuronal somata

Table 3. Percentage of CNPase-positive oligodendrocytes immunolabelled for ZONAB

Brain area	Oligodendrocytes immunolabelled for ZONAB
Cortex	98.3 $\pm$ 0.3 (412)
Striatum	98.7 $\pm$ 0.4 (309)
Hippocampus	98.9 $\pm$ 0.5 (290)
Hypothalamus	99.2 $\pm$ 0.1 (239)
Thalamus	98.4 $\pm$ 0.5 (318)

Values represent means $\pm$ S.E.M. of the percentage of CNPase-positive oligodendrocytes that were immunopositive for ZONAB. Numbers in parentheses show the total number of CNPase-positive oligodendrocytes examined in brain regions of five CD1 adult mice.

FIGURES

Fig. 1. Diagram illustrating anti-ZO-1 antibodies used in this study, and degree of overlap of sequences against which antibodies were generated. Exact sequences, where known, are given in Table 1. Also indicated are the relative location of these sequences in relation to the three PDZ domains (PDZ1, PDZ2, PDZ3) and one SH3 domain in ZO-1.

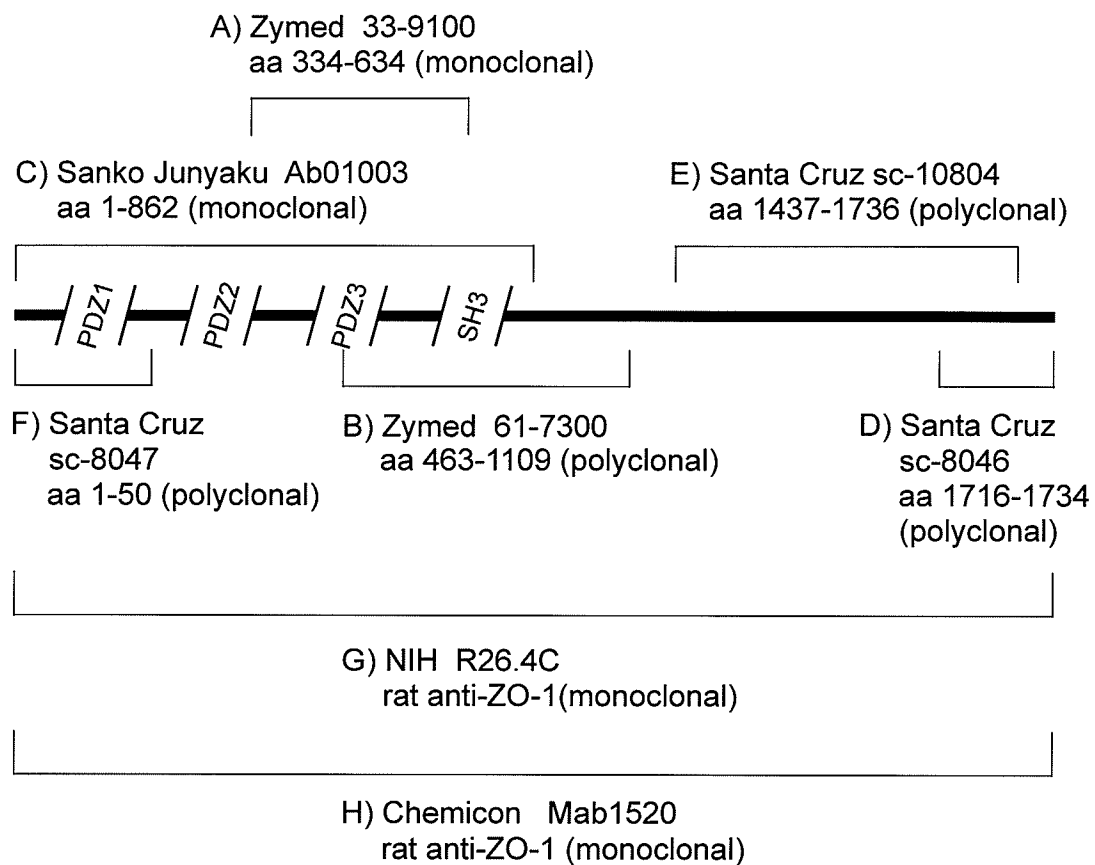


Fig.1

Fig. 2. Low magnification of immunofluorescence labelling patterns obtained with various anti-ZO-1 antibodies in liver and brain. (A-C) Similar labelling of ZO-1 at presumptive tight junctions in liver is shown with polyclonal 61-7300 (A), monoclonal Ab01033 (B) and monoclonal 33-9100 (C). (D) Blood vessel in thalamus showing continuous, linear strands of labelling with polyclonal anti-ZO-1 61-7300 at presumptive tight junctions. (E-G) Micrographs illustrating dense, punctate labelling for ZO-1 with polyclonal anti-ZO-1 61-7300 in globus pallidus (E), subthalamic nucleus (F) and lateral pontine nucleus (G). (H) Hippocampus CA3 region showing dense labelling with monoclonal anti-ZO-1 33-9100 in the mossy fiber terminal field (arrows). (I) Choroid plexus (arrow) and adjacent fimbria (asterisk) showing continuous labelling for ZO-1 around choroidal ependymal cells, and dispersed punctate labelling in fimbria white matter with polyclonal anti-ZO-1 61-7300. Scale bars: 50 μ m.

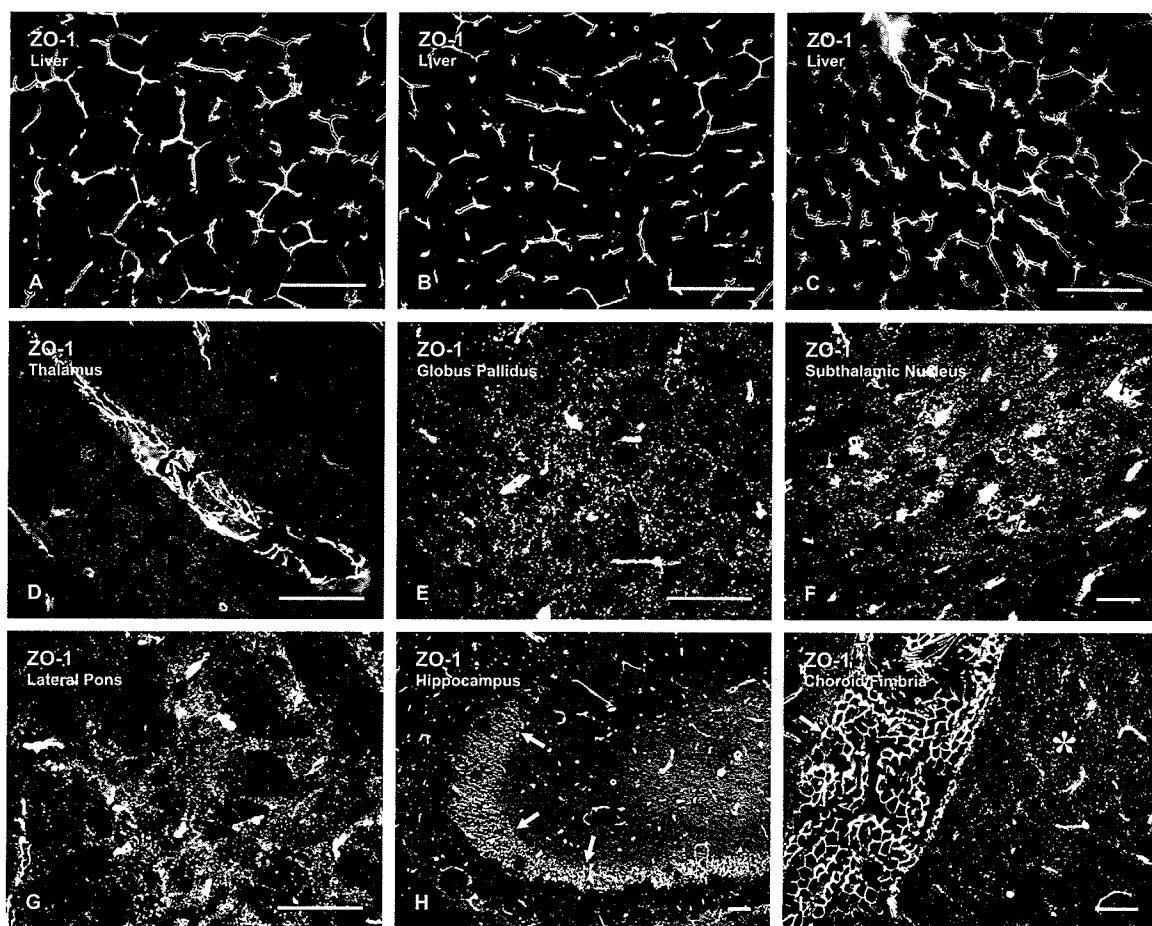


Fig. 2

Fig. 3. Laser scanning confocal double immunofluorescence labelling with various combinations of anti-Cx43 and anti-ZO-1 antibodies in regions of adult mouse brain. (A-D) Double immunolabelling with monoclonal anti-Cx43 35-5000 and polyclonal anti-ZO-1 61-7300 in cerebral cortex (A), hippocampus (B), globus pallidus (C) and thalamus (D), where densely distributed punctate labelling for Cx43 and ZO-1 at presumptive astrocytic gap junctions is substantially, though not totally, co-localized (A3-D3, yellow). Also seen is Cx43/ZO-1 co-localization around an oligodendrocyte (B, arrow). (E-G) Double immunolabelling with polyclonal anti-Cx43 71-0700 and monoclonal anti-ZO-1 33-9100 in cerebral cortex (E), striatum (F) and thalamus (G), with overlays showing co-localization of labelling (yellow in E3-G3) at widely dispersed puncta, as well as around oligodendrocyte somata (arrows) and their initial processes (arrowheads). (H) Double immunolabelling with polyclonal anti-Cx43 71-0700 and monoclonal anti-ZO-1 Ab01033 in cerebral cortex, with overlay (H3) showing Cx43/ZO-1 co-localization at dispersed puncta and on oligodendrocyte somata (arrow) and processes (arrowhead). Scale bars: 10 μ m.

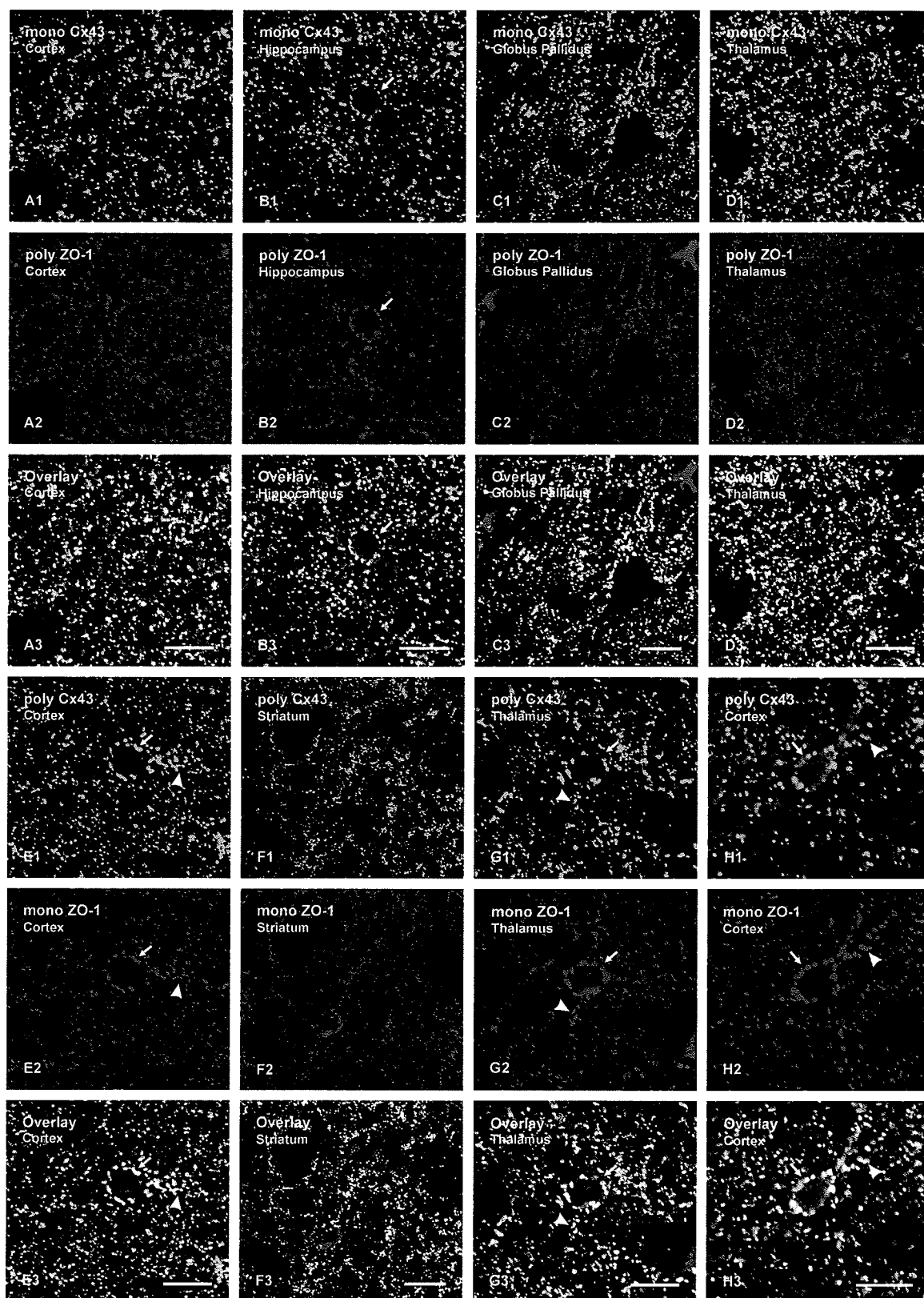


Fig. 3

Fig. 4. Laser scanning confocal double immunofluorescence of Cx30 and ZO-1 in adult mouse brain. (A,B) Double immunolabelling with polyclonal anti-Cx30 71-2200 and monoclonal anti-ZO-1 33-9100 in the globus pallidus (A) and hypothalamus (B) showing a high degree of Cx30-positive puncta co-localized with ZO-1-positive puncta (A3,B3, yellow). (C,D) Double immunolabelling with monoclonal anti-ZO-1 Ab01033 and polyclonal anti-ZO-1 61-7300 in hypothalamus (C) and subthalamic nucleus (D) showing co-localization of immunofluorescent puncta (C3,D3, yellow). Larger puncta in D are associated with oligodendrocytes. (E) Triple confocal immunofluorescence labelling in hippocampus showing co-localized punctate labelling for Cx30, ZO-1 and Cx32 (E, white in overlay) associated with an oligodendrocyte somata (arrows), and absence of oligodendrocytic Cx32 in surrounding puncta displaying co-localization of Cx30/ZO-1 at presumptive astrocytic gap junctions. Scale bars: 10 μ m.

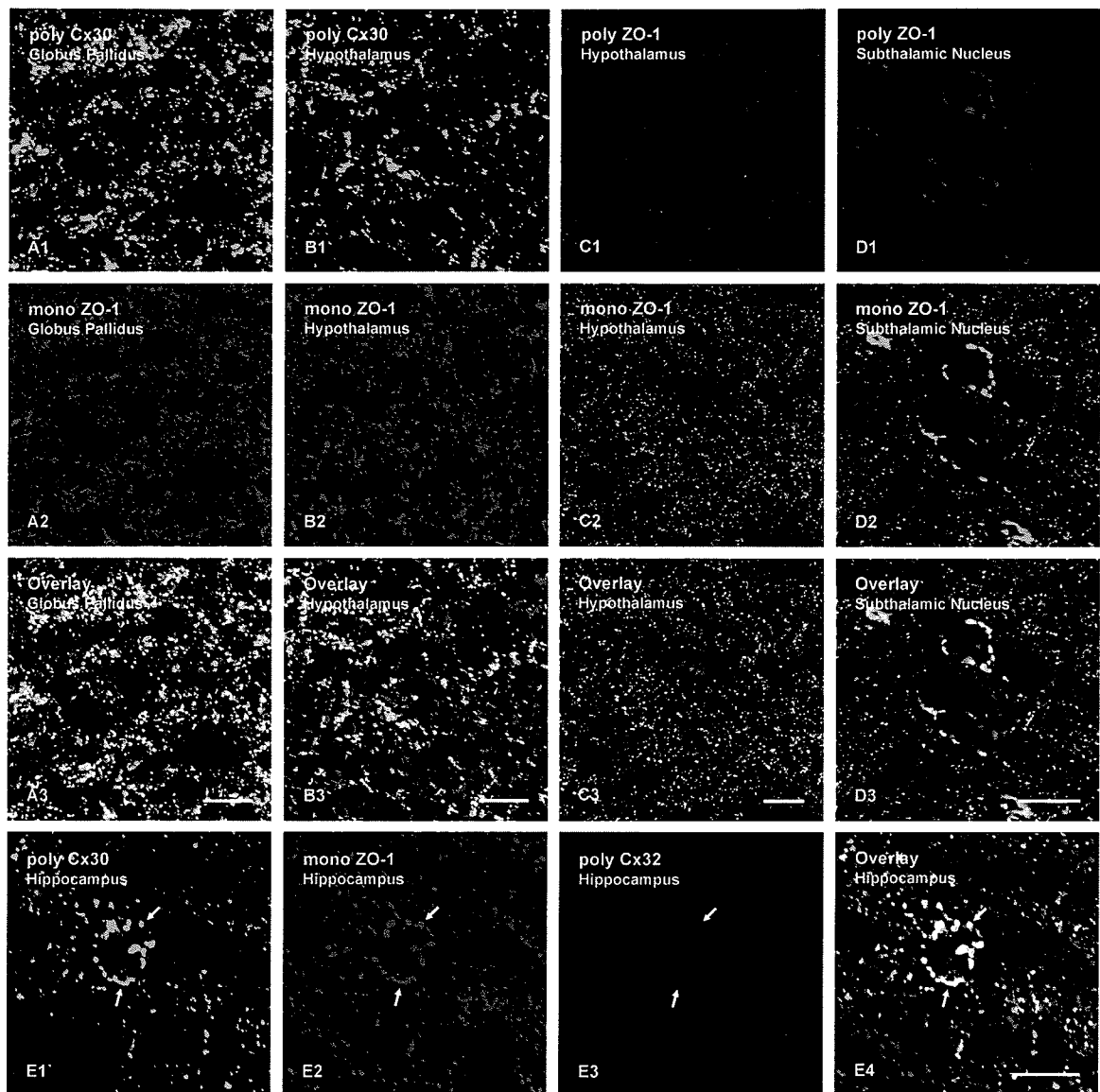


Fig. 4

Fig. 5. Western blots confirming ZO-1 antibody detection in adult mouse brain. (A) Immunoblot with monoclonal anti-ZO-1 33-9100 showing a major band at 210-220 kDa in cerebral cortex, thalamus, medulla and whole brain. (B) Immunoblot showing that the ZO-1 band detected with monoclonal anti-ZO-1 33-9100 in thalamus (lane 1) corresponds to the same band detected by this antibody after IP of ZO-1 from thalamus with polyclonal anti-ZO-1 61-7300 (lane 2).

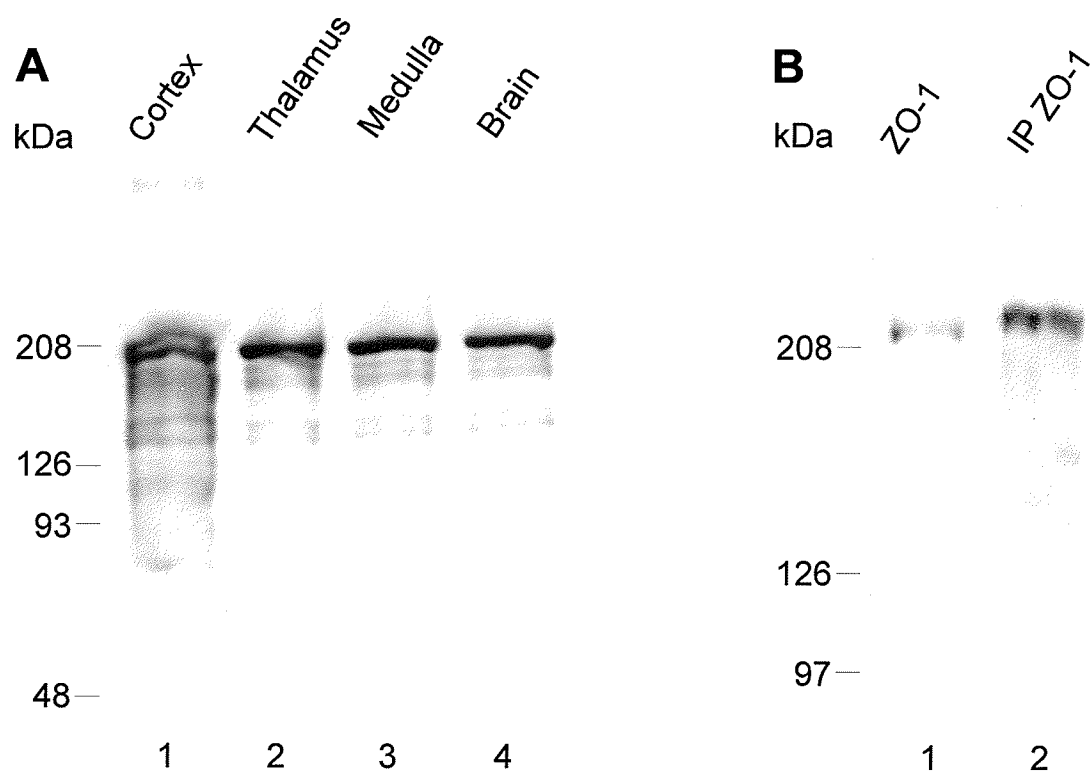
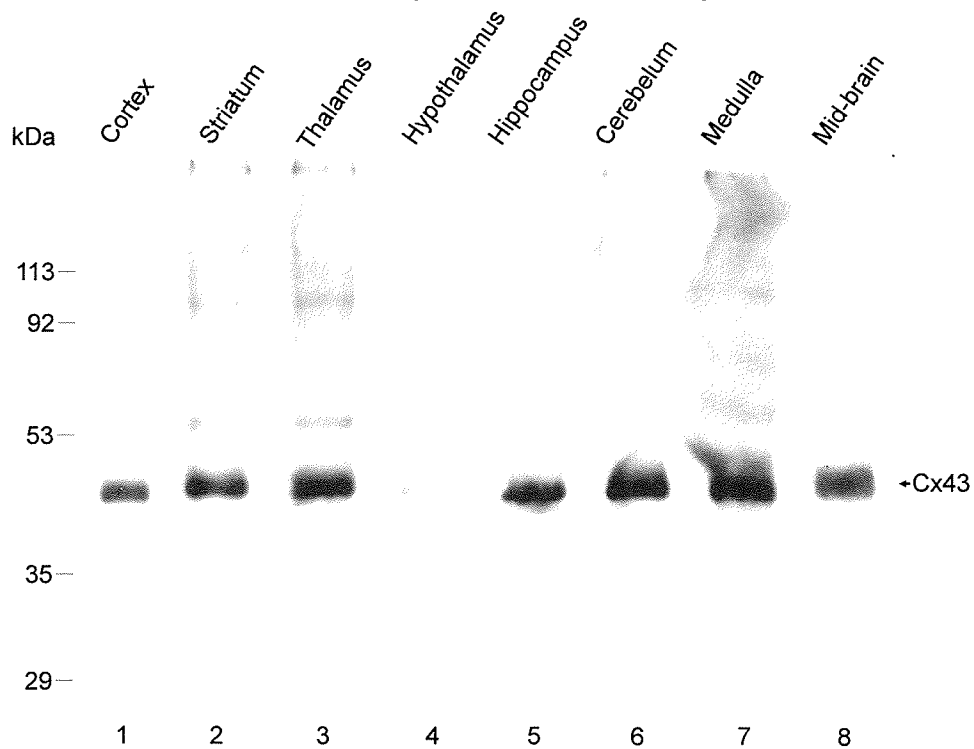


Fig. 5

Fig. 6. Co-immunoprecipitation of ZO-1 with astrocytic Cx43 and Cx30. (A) Immunoblot detection of Cx43 with polyclonal anti-Cx43 18A after IP of ZO-1 with monoclonal anti-ZO-1 33-9100 from the various brain regions indicated. (B) Immunoblot detection of Cx30 with polyclonal 71-2200 after IP of ZO-1 with monoclonal anti-ZO-1 33-9100 from the various brain regions indicated.

A IP mono anti-Z0-1, probed with anti-pCx43



B IP mono Z0-1, probed with anti-pCx30

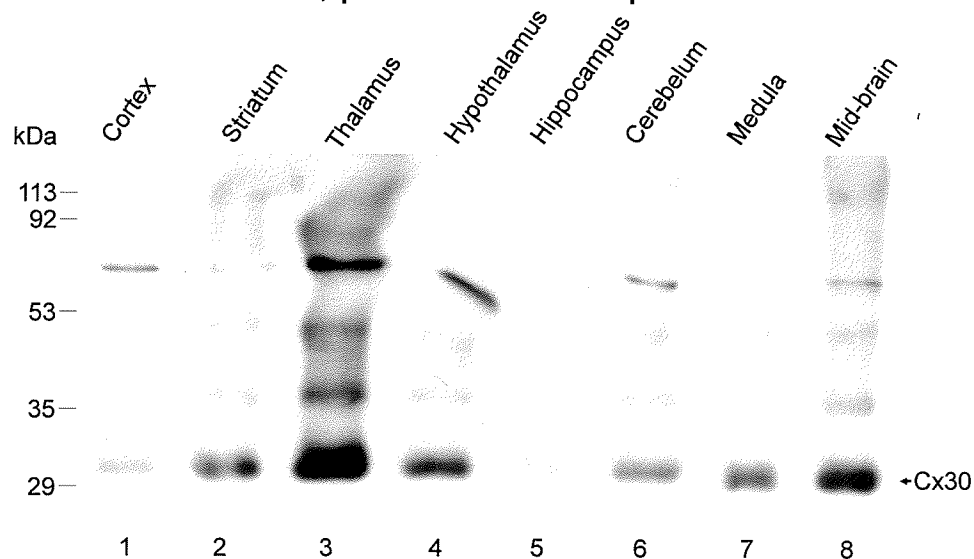


Fig.6

Fig. 7. Analysis of ZO-1 interaction with Cx30 by in vitro pull-down assay. (A) Immunoblot loaded with whole homogenate of thalamus and with thalamus homogenate incubated with GST fusion proteins containing the PDZ1, PDZ2 or PDZ3 domains of ZO-1, followed by probing of bound protein with polyclonal anti-Cx30 antibody. Blots show a dense band at 30 kDa in thalamus (lane 1), used as control for Cx30 detection, a corresponding band following pull-down with GST-PDZ2 (lane 3), and absence of pull-down with either GST-PDZ1 (lane 2) or GST-PDZ3 (lane 4). (B) The same immunoblot as in (A) after stripping and reprobing with anti-GST antibody shows equal loading of GST-PDZ fusion proteins (lanes 1, 2, 3). No GST is detected in thalamus (lane 1).

A Cx30/ZO-1 interaction in vitro

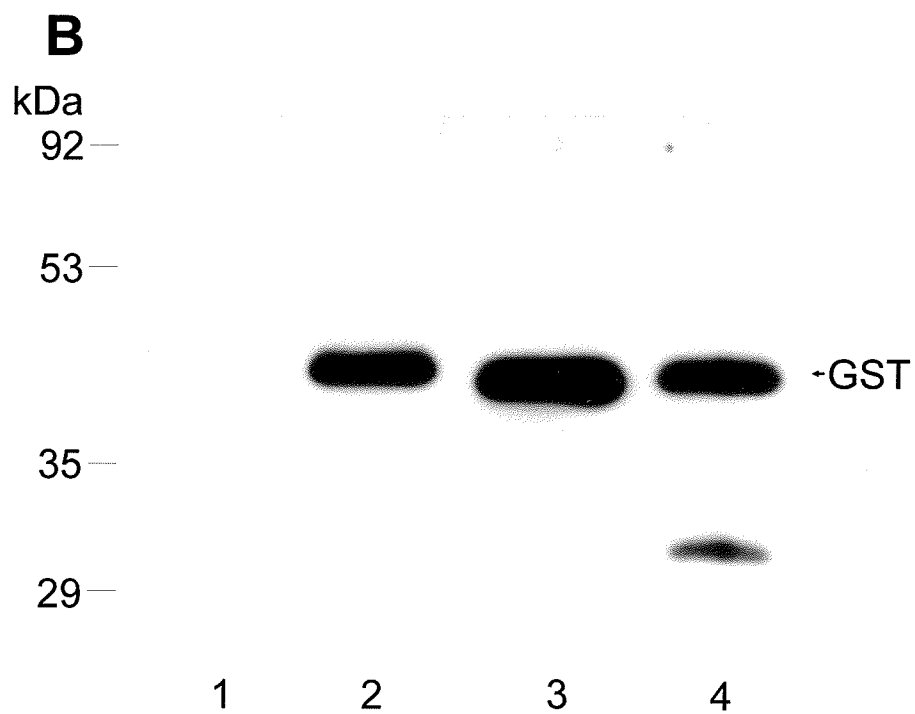
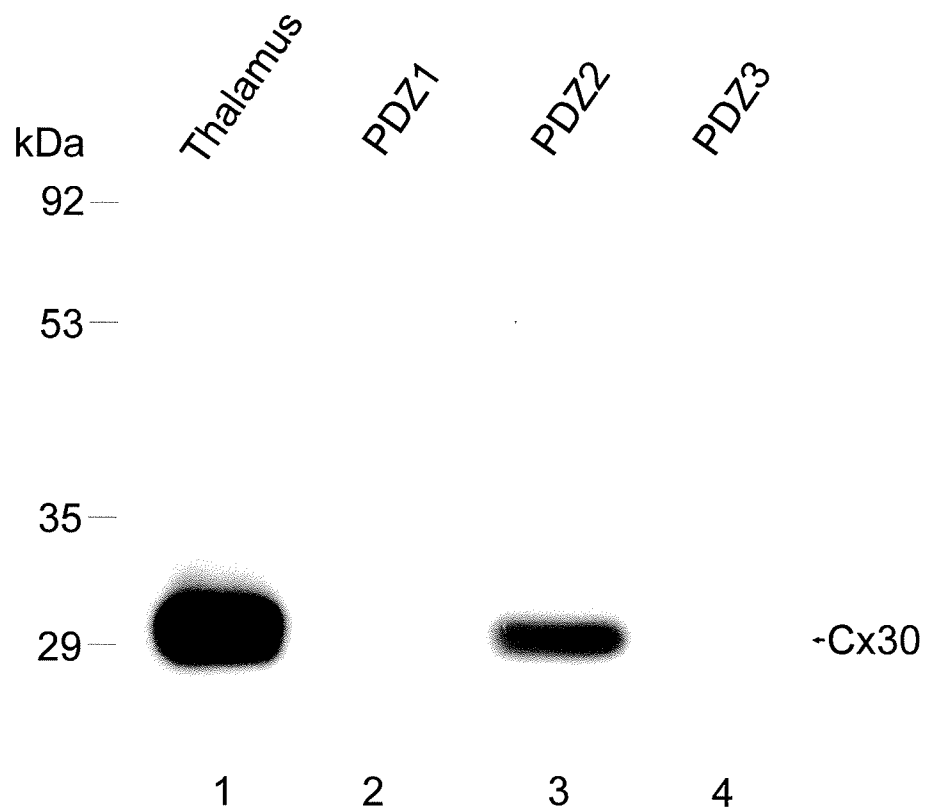


Fig. 7

Fig. 8. ZONAB expression in adult mouse brain. (A) Immunoblot loaded with homogenate of thalamus (lane 1), cerebral cortex (lane 2) and cerebellum (lane 3), showing detection of ZONAB as a band migrating at approximately 30 to 32 kDa. (B,C) Low magnification immunofluorescence micrographs showing the general distribution of labelling for ZONAB associated with small cells (arrows) in gray matter of the thalamus (B) and fimbria white matter (C). Diffuse punctate labeling in both areas is also evident. Scale bars: B,C, 100 μ m.

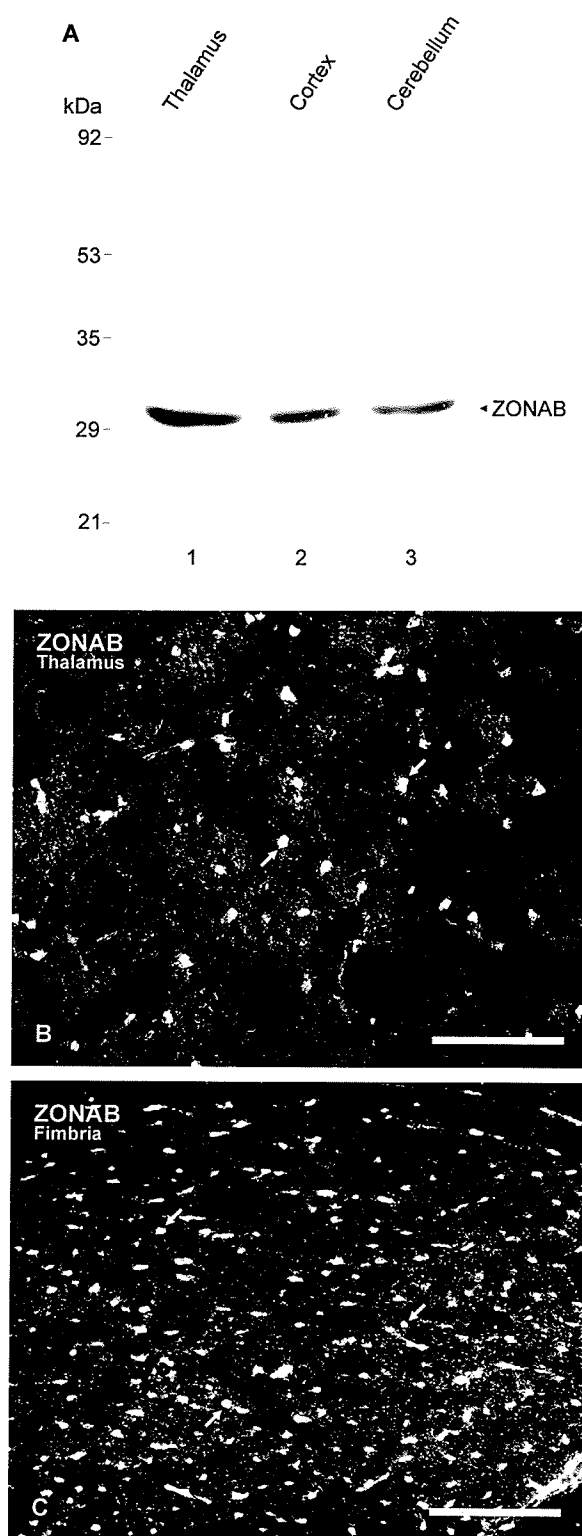


Fig. 8

Fig. 9. Double immunofluorescence co-localization of ZONAB with CNPase, Cx47 and Cx32 in oligodendrocytes. (A) Low magnification micrograph showing CNPase-positive oligodendrocytes (A1, arrows) immunolabelled for ZONAB (A2, arrows) in the cerebral cortex (A3, overlay). (B,C) Low magnification fields showing Cx47-positive cells (B1,C1, arrows) labelled for ZONAB (B2,C2, arrows) in the habenula (B) and internal capsule (C), with extensive co-localization, as seen in overlay (B3,C3, arrows). (D) Low magnification images showing Cx32-positive cells (D1, arrows) labelled for ZONAB (D2, arrows) in the thalamus (D3, overlay). (E) Higher magnification laser scanning confocal double immunofluorescence showing a CNPase-positive oligodendrocyte (E1, arrowhead) with punctate labelling for ZONAB around its periphery (E2, arrows), as seen in overlay (E3, arrows). (F-H) Confocal double immunofluorescence labelling for Cx47 and ZONAB in the cerebral cortex (F), thalamus (G) and amygdala (H), showing co-localization of punctate labelling associated with oligodendrocyte somata and their initial processes (arrows), as seen by yellow in overlay (F3,G3,H3, arrows). Scale bars: A,C, 50 μ m; B, 200 μ m; D, 100 μ m; E-H, 10 μ m.

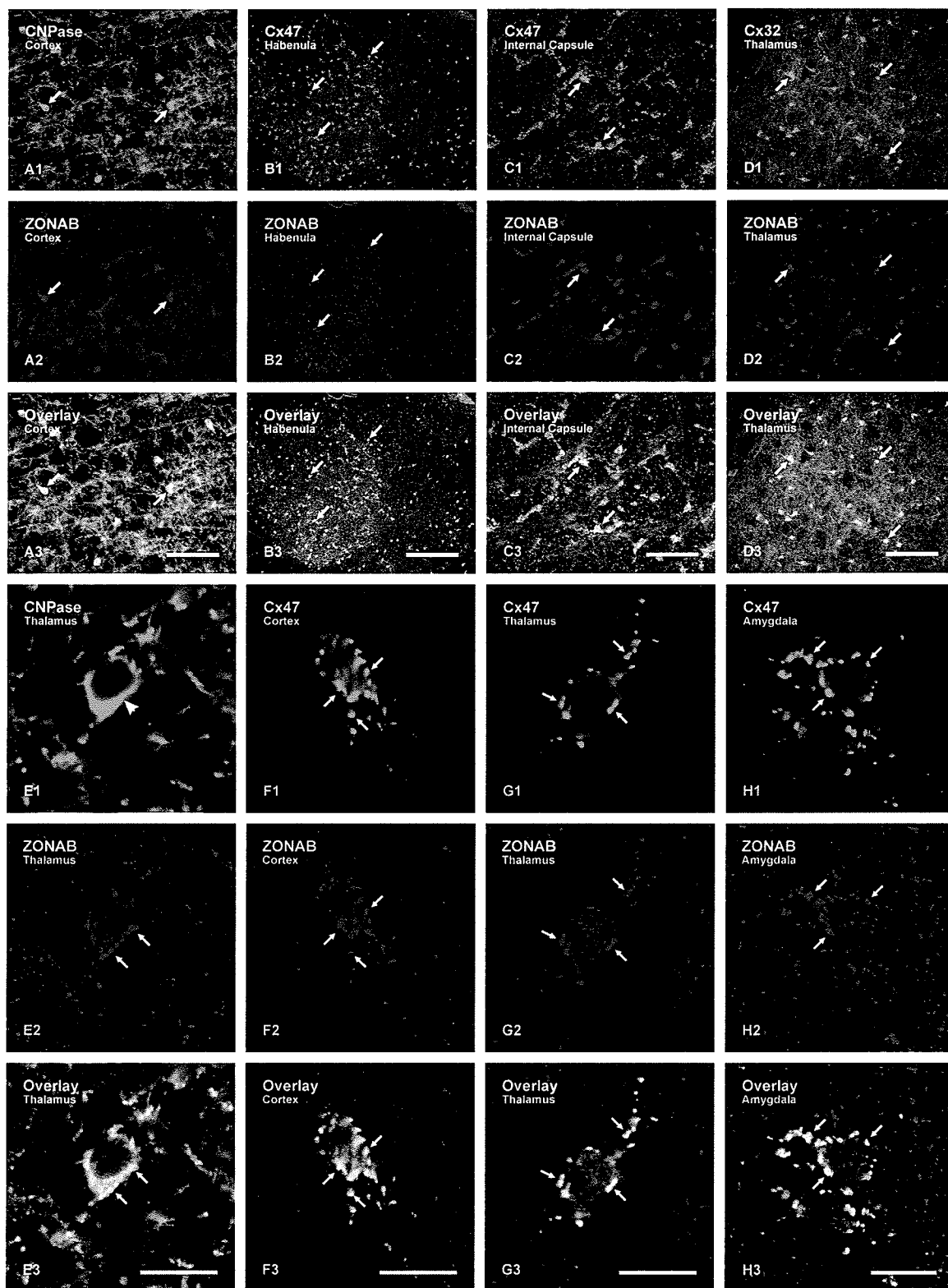


Fig.9

Fig. 10. Laser scanning confocal immunofluorescence co-localization of ZONAB with Cx32, ZO-1 and Cx43. (A,B) Triple immunofluorescence labelling in hippocampus (A) and thalamus (B) showing Cx32-positive puncta (A1,B1, arrows) and ZONAB-positive puncta (A2,B2, arrows) associated with CNPase-positive oligodendrocyte (A3,B3, arrowheads), as seen in overlay (A4,B4). Labelling for ZONAB is present both at the periphery of oligodendrocytes as well as intracellularly. (C,D) Double immunofluorescence in hippocampus (C) and thalamus (D) showing punctate co-localization of ZO-1 (C1,D1, arrows) and ZONAB (C2,D2, arrows) on oligodendrocytes, as seen in overlay (C3,D3, arrows). (E,F) Double labelling in the thalamus (E) and subthalamic nucleus (F) showing dense punctate labelling for Cx43 (E1,F1) and ZONAB (E2,F2), with substantial though not total co-localization of labelling, as seen by yellow in overlay (E3,F3, arrows) Scale bars: A-E, 5 μ m; F, 10 μ m.

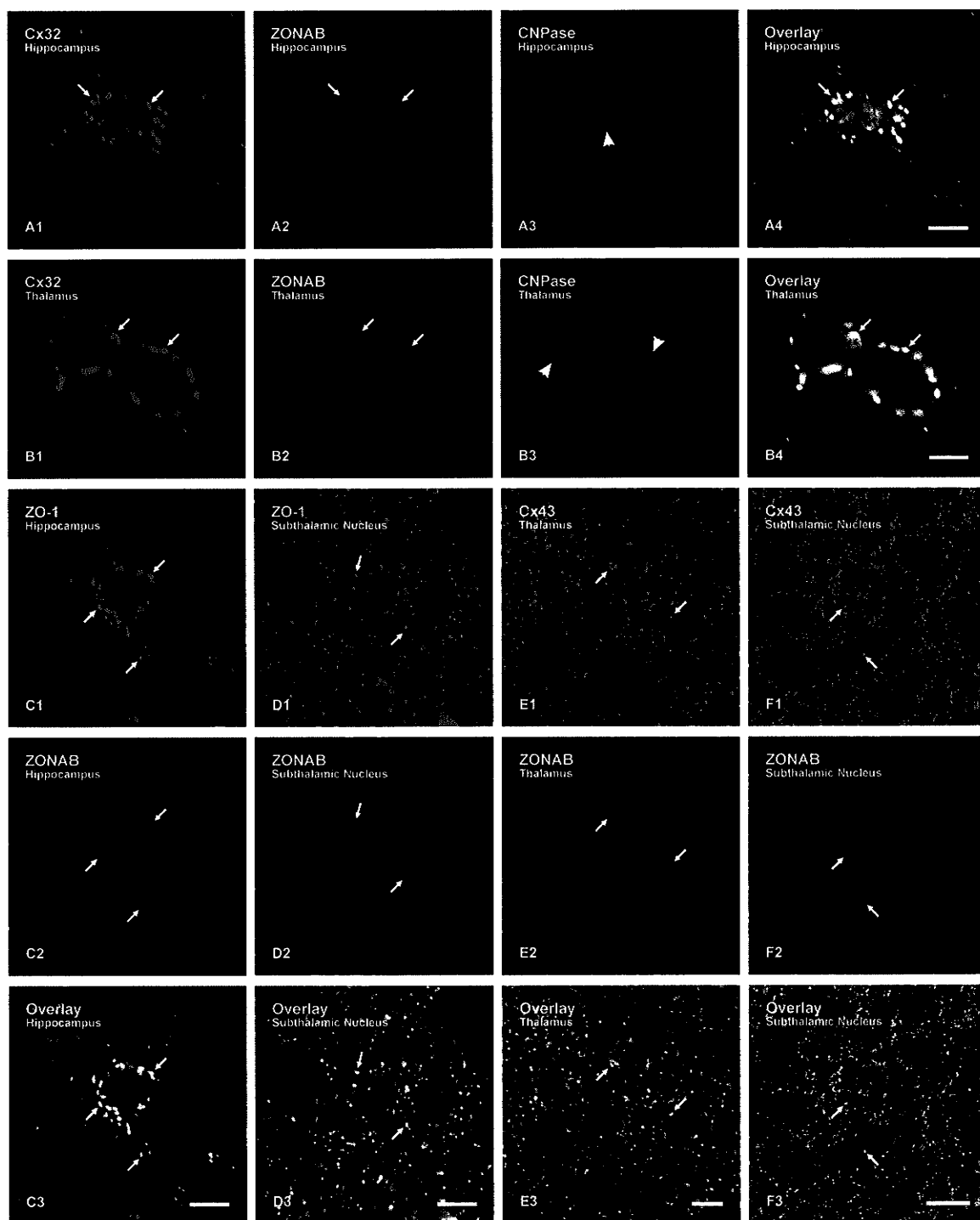


Fig. 10

VI. GENERAL DISCUSSION

With the increasing number of studies indicating the relevance of gap junction-mediated regulation of CNS and peripheral physiopathology, unraveling the close interactions between the diverse proteins present in complexes at intercellular junctional sites has become an important challenge. Evidence for such functional complexes, termed “platforms of regulation” have been provided by studies involving tight junctions, desmosomes, adherens junctions and more recently gap junctions (Zahraoui et al., 2000; Balda and Matter, 2003; Singh & Lampe, 2003; Wei et al., 2004). Our previous and current attempts towards identifying possible functional proteins associated with connexins at cell-to-cell junctions at various locations is in accordance with these findings and provide additional vital information.

Although the exact roles of ZO-1, ZONAB and ZO-1/ZONAB interaction at astrocytic and oligodendrocytic gap junctions are unclear, their presence, together with their important regulatory roles described at other locations, suggests their active involvement in managing signaling pathways at gap junctions. At tight junctions, ZONAB participates in the regulation of gene expression and control of cell growth and differentiation (Balda and Matter., 2000; Balda et al., 2003).

Therefore, we believe that ZONAB presence at glial gap junctions may be particularly important. That is because we consider that perhaps some of the above observations could be extrapolated to gap junctions, as tight and gap junctions appear to share not only structural and functional proteins, but also similar regulatory mechanisms (Giepmans et al., 2004).

Astrocyte Domains in the CNS

Our current understanding of astrocyte to astrocyte spatial relationship and interaction in the CNS is incomplete. Based primarily on studies using metal-impregnation techniques or the astrocytic marker glial fibrillary acidic protein (GFAP), astrocytic processes were initially thought to extensively interdigitate (Wolff, 1976; Rohlfmann & Wolff, 1996; Bushong et al., 2002). However, using these techniques alone has limitations in visualizing complex astrocytic interactions, since for example, GFAP delineates only 15-20% of the total astrocytic volume (Bushong et al., 2002). Recent more complex studies, using large groups of astrocytes filled with fluorescent dyes that provide a more accurate discrimination between neighbouring processes, describe individual astrocytes as “inhabiting” characteristic volumes of tissue (Bushong et al., 2002). In addition, astrocytic morphology appears to be influenced by their neighbors. Astrocytic processes may actually occupy separate anatomical domains of the neuropil, and contact seems to occur just at the tips of neighboring processes (Bushong *et al.*, 2002, Nagy & Rash, 2003). Gap junctional coupling is present at these restricted borders between neighbouring domains.

With evidence of TJ dependent cellular proliferation, differentiation and contact inhibition being mediated by ZONAB (Balda *et al.*, 2003), it may be interesting to elucidate whether ZONAB present at gap junctions connecting the tips of these distinct domains is involved in mediating contact dependant inhibition in astrocytes.

Further functional implications of ZO-1 and ZONAB at glial gap junctions

As previously mentioned, ZO-1-ZONAB interaction is involved in regulating ErbB2 levels at tight junctions in culture. ErbB2 is a tyrosine kinase receptor also referred to as

Neu or HER2 (Balda and Matter., 2000) that transduces neuregulin (growth and differentiation factor) signals in oligodendrocytes and Schwann cells with the critical roles described above (Park et al., 2001; Kim et al., 2003; Patten et al., 2003). Signaling pathways between axons of the peripheral nervous system and Schwann cells conveying encoded information about the axon caliber have been described as critical in the process of regulating myelin sheath thickness, with Neuregulin-1 and ErbB2/ErbB3 acting as ligand-receptor complexes (Michailov et al., 2004). These stated, we can only speculate on the possible ZONAB roles at oligodendrocytic junctions at this point, roles such as influencing glial differentiation or the myelination process through control of ErbB-2. Nonetheless, clarification of the exact ZO-1-ZONAB function and of the cascade of regulatory events remains to be determined.

Aspects not included in the current investigation

We showed ZONAB expression in glial cells in vivo and co-localization with astrocytic and oligodendrocytic connexins and with ZO-1, but further work is required to demonstrate ZONAB-ZO-1 and possibly ZONAB-connexin direct binding. We did not investigate whether in mouse CNS in vivo, ZONAB binds to the ErbB2 promoter or to other specific inverted CCAAT box-containing sequences found in other promoters. Further, previous investigations described either the existence of distinct subpopulations of astrocytes in the adult CNS, called complex and passive astrocytes (Walz and Lang, 1998; Walz, 2000) or the presence of astrocytic subclasses (Altevogt & Paul, 2004). We did not investigate whether ZO-1 or ZONAB expression is restricted to such subdivisions, since we consider that more data is required to clarify this issue of astrocytic classes.

Future directions

Currently, we aim to identify ZONAB at other locations in vivo including peripheral tight junctions, and we have preliminary data suggesting its association with ZO-1 and with peripheral connexins in liver, blood vessels, choroids plexus, uterus, lung, retina, heart and kidney. It would be important to determine changes in ZONAB expression patterns in KO animals and to investigate whether the up or down-regulation of ZO-1 and/or ZONAB in normal or transformed cells influences connexin expression and the formation of functional gap junctions. With the availability of cultured cell lines engineered in such a way as to express specific connexins (those thought to associate with ZONAB), it would be of interest to determine effects of ZONAB transfection as well as ZONAB up or down regulation in these cultures. Other aims of future research in the field of gap junctions, could be related to the specific functions of non-junctional hemichannels and their importance in cellular physiology.

Last but not least, I would like to go back to the association between connexin mutations and disease. It has already been proved that functional replacement of mutated/malfunctioning connexin genes could be manipulated towards life saving events (White, 2003). Future research investigating regulation of intercellular communication from a patho-pharmacological point of view could provide the knowledge required for the development of specifically targeted drugs that can modulate GJIC in connexin related diseases by closing or opening of channels and increasing the overall beneficial effects of adaptive tissue response.

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