Occludin is overexpressed in Alzheimer’s disease and vascular dementia

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Abstract

The tight junctions (TJs) are key players in the control of blood-brain barrier (BBB) properties, the most complex TJs in the vascular system being found in the endothelial cells of brain capillaries. One of the main TJs proteins is occludin, which anchors plasma membranes of neighbour cells and is present in large amounts in the brain endothelia. Previous studies demonstrated that disruption of BBB in various pathological situations associates with changes in occludin expression, and this change could be responsible for malfunction of BBB. Therefore in this study, applying an immunohistochemical approach, we decided to explore the occludin expression in frontal cortex (FC) and basal ganglia in ageing control, Alzheimer’s disease (AD), and vascular dementia (VD) brains, as far as all these pathologies associate microangiopathy and disruption of BBB. Strikingly, we found selected neurons, astrocytes and oligodendrocytes expressing occludin, in all cases studied. To estimate the number of occludin-expressing neurons, we applied a stereological approach with random systematic sampling and the unbiased optical fractionator method. We report here a significant increase in ratio of occludin-expressing neurons in FC and basal ganglia regions in both AD and VD as compared to ageing controls. Within the cerebral cortex, occludin was selectively expressed by pyramidal neurons, which are the ones responsible for cognitive processes and affected by AD pathology. Our findings could be important in unravelling new pathogenic pathways in dementia disorders and new functions of occludin and TJs.

Keywords: occludin • blood brain barrier • intercellular tight junctions • Alzheimer • vascular dementia • cerebral angiopathy • CADASIL • quantification/stereology

Introduction

The blood brain barrier (BBB) is a physical and metabolic barrier, a specialized system of capillary endothelial cells essential for the maintenance and regulation of the neuronal microenvironment, anatomically formed by components of the ‘neurovascular unit’ [1, 2, 3]. The permeability of the BBB is determined by the interendothelial junctions. In mammalian cells, the intercellular junctions are categorized in: tight junctions (TJs), adherent junctions and gap junctions [4].

One of the most important characteristics of the microvasculature of the central nervous system (CNS) is the cell-to-cell contact between two adjacent
endothelial cells of the cerebral capillaries through the TJs [5]. In the brain of vertebrates there are two types of cells that bear TJs or TJs-like structures: vascular endothelial cells and oligodendrocytes [6]. The TJs of endothelial cells consists of narrow belt-like structures in the apical region of the lateral plasma membrane circumferentially wrapping each cell and connecting the neighbours [7]. The function of TJs is not only to separate the apical from the basolateral plasma membrane ('fence function'), but also to restrict the flow through the paracellular pathway ('gate/barrier function') [8].

At the BBB level, the endothelial cells of brain capillaries possess the most complex TJs in the vascular system [9], junctions that are thought to be one of the main factors responsible for the tightness of the BBB [10]. The molecular components of TJs are important factors controlling the intramembrane diffusion and maintaining the structural and functional polarity of the endothelial cells [11, 12]. Stabilization of TJs involves a complex network of transmembrane and peripheral proteins, such as occludin, claudin family members, junctional adhesion molecules (JAMs) 1–3, cingulin, spectrin and zonula occludens (ZO)-family members, in which these proteins bind to each other and to the actin cytoskeleton [13].

Occludin is a 65 kD transmembrane protein localized to TJs which anchors ZO-1 and ZO-2 proteins and the plasma membranes of adjacent cells [4]. Occludin is present in large amounts in brain endothelial cells, being undetectable in non-neuronal endothelia [14]. Since it was suggested that the occludin molecules tightly obliterate the interendothelial space, occludin is used as the marker of choice in most pathological studies of BBB [15, 16]. It has also been demonstrated that disruption of BBB in various pathological situations associates with changes in occludin expression [17–20]. A detectable occludin expression was also identified in primary and secondary cultures of neurons and astrocytes from adult mouse [21], being suggested that astrocytes and neurons are capable of retaining or regaining their neuroepithelial characteristics in vitro.

Aging in human beings is associated with significant structural and functional alterations in the BBB and dysregulation of specific TJs proteins was suggested [22, 23]. Age has a strong impact on dementia incidence, an exponential rise to the age of 90 being reported in epidemiological studies [24]. Disruption of BBB is also thought to play an important role in the pathogenesis of cerebral microangiopathy [25] and patients with microangiopathy present white matter changes that ultimately lead to impairment of neuronal flow and cognitive decline in both vascular dementia (VD) and Alzheimer’s disease (AD) [26]. Moreover, in addition to cerebral amyloid angiopathy AD patients show profound changes in cerebral microvessels often independent of amyloid deposition [27]. Conversely, it was suggested that amyloid beta (Aβ) effects on TJs protein complexes might be responsible for alteration of BBB integrity and neuropathological findings in AD [28].

Hypertension is one of the main risk factors for VD and a disturbed fence function of the TJs was demonstrated in stroke hypertensive rats at the level of blood–brain barrier endothelial cells [29]. Furthermore oxidative stress, a common pathogenic feature of both dementia types [30], induces a down regulation of the occludin expression that leads to BBB breakdown [31].

The role of TJs proteins is poorly understood in dementia disorders. It is also uncertain whether changes in BBB are a cause or an effect of disease progression [32]. The aim of this study was to comparatively analyse the expression of occludin, one of the main TJs proteins, in human brains of control cases and of several dementia conditions: AD, VD, cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) and congophilic amyloid angiopathy (CAA).

Materials and methods

The brain material was obtained from the Huddinge Brain Bank, Stockholm, Sweden, in accordance with Swedish law and with the permission of the Karolinska University Hospital Ethical Committee. Two brain regions of interest were analysed, with specific anatomical and haemodynamic conditions: cerebral cortex (frontal, Brodmann’s area 46/9) and the head of the nucleus caudatus.

The study was based on evaluation of five aging control, 4 AD, 6 VD, 2 CADASIL and 2 CAA post-mortem human brains. The control group included the brains of traffic-accident patients or of patients who had no history of long-term illness or neuropsychiatric disorders. The AD group included brains from patients with clinically and pathologically confirmed AD. Clinical diagnosis was based on combined Diagnostic and Statistical Manual of Mental
Disorders (DSM)-III-R [33] and National Institute of Neurological and Communicative Diseases and Stroke/Alzheimer’s Disease and Related Disorders Association (NINCDS-ADRDA) criteria [34]. The definite neuropathological diagnosis of AD was determined by using Consortium to Establish a Registry for Alzheimer’s Disease (CERAD) and National Institute on Aging (NIA)-Reagan Institute Criteria [35, 36]. The VD group included brains from patients with clinically and pathologically diagnosed VD. The VD group consisted of two cases of single strategic stroke, three cases with dementia multi-infarct and one case with diffuse white matter changes. We also analysed qualitatively two cases of CADASIL and two cases of CAA. Age and sex of all cases are presented in Table 1.

Table 1: Age and sex of cases analyzed in each study group

| Group   | Age | Sex |
|---------|-----|-----|
| Control |     |     |
|         | 90  | F   |
|         | 88  | F   |
|         | 86  | F   |
|         | 56  | M   |
|         | 70  | M   |
| AD      |     |     |
|         | 87  | F   |
|         | 86  | M   |
|         | 91  | F   |
|         | 72  | F   |
| VD      |     |     |
|         | 87  | F   |
|         | 86  | F   |
|         | 65  | M   |
|         | 72  | M   |
|         | 89  | F   |
|         | 77  | F   |
| CADASIL |     |     |
|         | 33  | M   |
|         | 63  | F   |
| ICA     |     |     |
|         | 79  | M   |
|         | 78  | M   |

Sectioning and histology

Immunostaining with antibodies raised against occludin was performed on buffered formaldehyde-fixed embedded sections (long-fixation time sections were used). A series of thin sections (7 μm) were cut exhaustively in a randomly chosen sagittal plane on the microtome. After the deparafinization and rehydration procedures the sections were rinsed in distilled water for 5 min. and then heated at 80°C for 20 min in 0.1 M citrate buffer of pH 9. After cooling, the sections were incubated with the primary antibody solution overnight (approximately 16 hrs, dilution 1:100) at 4°C. The primary antibody was a rabbit polyclonal antiserum raised against the C-terminal 150 amino acids of human occludin (Zymed Laboratories, Inc., South San Francisco, CA, USA). Thereafter, the sections were treated with a biotinylated secondary antibody (1:300, Vector Laboratories, Burlingame, CA, USA) and with the avidin–biotin-peroxidase complex kit (Vector, Burlingame, CA, USA) with 3,3’-diaminobenzidine-4HCl/H2O2 (DAB, Sigma, St.Louis, MO, USA) as a substrate. Next, the sections were counterstained with haematoxylin–eosin stain for background. Two types of negative controls slides were run for all the sections (for both frontal and striatal regions). The negative controls were run in an identical manner except the incubation with the primary antibody. For the first set of negative controls we omitted the primary antibody, whereas for the second set we added instead of the primary antibody an unspecific immunoglobulin serum at the same concentration. The sections were then mounted and examined under a Nikon microscope.

Quantification of neurons

To estimate the number of occludin-expressing neurons, we applied a stereological approach on 2-dimension quantification with random systematic sampling and the unbiased optical fractionator method [37], similarly to a previous study [38]. It is known that with an optical dissector probe it is possible to sample isolated particles, in our case neurons, with a uniform probability in the bi-dimensional space regardless the size, shape or orientation of the tissue [37]. In brief, the area of interest was delineated in low magnification (x 2.5) using the cursor. Due to the clear anatomical borders, we were able to distinguish the grey matter from the white matter. A meander sampling function of the GRID v2.0 program (Olympus, Denmark) was used for stepping through the delineated region with a chosen counting frame. Then, a 100-x oil-immersion objective with a numerical aperture of 1.40 was moved into place and the appropriate counting frame superimposed on the screen. The desired horizontal and vertical step lengths, assisted by a highly precise servo-controlled motorized microscopy stage, were dimensioned for the appropriate distance [231.22 μm (x-step) x 231.22 μm (y-step)] in between the counting frames (1603.9 μm²). The cells in the space were counted by an optical dissector probe (z-axis). The optical dissector uses the simple rule that a neuron is counted if its body cell is in the counter-frame. This procedure ensured the selection of a systematic random sample of sections. The number of neurons was express as a number per squared millimetre.

The neurons could be easily distinguished from the other types of cells due to the presence of the round nucleus with visible cytoplasm and a single large nucleolus, no heterochromatin [39].
Statistical analysis

Statistical analyses of the results were performed using JMP 4 software (SAS Institute, Cary, NC, USA). The neuronal ratio was defined as (number of occludin-expressing neurons / total number of neurons) x 100. To analyse the global variation of the neuronal ratio one-way ANOVA was used. To compare the neuronal ratio between controls, AD and VD (two by two) the Tukey–Kramer honestly significant difference (HSD) test has been used. The CADASIL and CAA cases were not used for statistical analysis, due to the small sample size, but we used them instead for a qualitative description. To compare the neuronal ratio between the frontal and striatal regions, we used the paired Student’s t-test. Precision of estimates was evaluated by calculating the coefficient of error (CE = SEM/mean, where SEM represents the standard error of mean).

Results

Occludin expression in neuronal cells

Our first observation was that expression of occludin was increased in neurons from AD and VD brains as compared to control brains, in both frontal and striatum (STR) regions (Table 2 and Fig. 1). The occludin-expressing neurons were in all cases predominantly pyramidal. The neuronal fibres were also stained more intensely in AD and VD as compared to control (Fig. 1). In CADASIL and CAA, we did not notice any difference in neuronal occludin expression as compared to control cases (Fig. 1).

Subsequently, we used a quantitative approach in order to estimate the number of occludin-expressing neurons in control, AD and VD groups, in FC and STR regions. Table 2 presents comparatively the quantitative data of neurons / mm² and occludin-positive neurons / mm² in FC and STR of control, AD and VD groups. The number of neurons did not significantly differ between the groups analysed. Conversely, the statistical analysis showed that there was a significant difference (P < 0.01) for the occludin-positive neurons concerning the global variation both in frontal and striatal region. The area chosen for counting was similar in all cases (with around 100 counting frames / area) and there was no significant difference in the density (number of neurons / area) between the cases analysed. Qualitatively there were no analysed areas where heterogeneity in distribution of occludin was observed.

In the control group, we found a significantly higher ratio of occludin-positive neurons in the FC region (23.97 ± 4.52%) as compared to the striatal region (3.32 ± 0.21%), as shown in Fig. 3 (P < 0.01). In contrast, in AD and VD groups we found no significant difference in ratio of occludin-positive neurons between the frontal and striatal regions (Fig. 3).

The ratio of occludin-expressing neurons was significantly higher in the FC region and in the striatal region in the AD group (54.25 ± 5.49, P < 0.05 and 54.49 ± 5.22, P < 0.05, respectively) and in the VD group (51.71 ± 6.13, P < 0.05 and 34.86 ± 27.66, P < 0.05, respectively) as compared to the control group (23.97 ± 4.52 and 3.32 ± 0.21, respectively).

![Table 2](image)

Table 2. Quantification of total number of neurons and occludin-expressing neurons (positive neurons) in frontal cortex (FC) and striatum (STR) regions in control (5 cases), Alzheimer’s disease (AD, 4 cases) and vascular dementia (VD, 6 cases) groups. All values were truncated to two decimals.

| Group | Region | Total number of neurons | Number of positive neurons |
|-------|--------|-------------------------|---------------------------|
|       |        | mean        | SD  | SEM  | CE  | mean | SD  | SEM  | CE  |
| CONTROL | FC    | 23.17       | 3.14 | 1.57 | 6.79 | 5.58 | 1.52 | 0.76 | 13.65 |
|        | STR   | 31.58       | 4.32 | 2.16 | 6.84 | 1.04 | 0.08 | 0.04 | 4   |
| AD     | FC    | 20.67       | 4.55 | 2.27 | 11   | 11.25 | 3.13 | 1.57 | 13.92 |
|        | STR   | 34.33       | 4.22 | 2.11 | 6.14 | 18.88 | 2.76 | 1.38 | 7.31 |
| VD     | FC    | 22          | 5.56 | 2.78 | 12.65 | 11.25 | 2.71 | 1.36 | 12.06 |
|        | STR   | 24.8        | 10.57 | 4.32 | 17.78 | 9.28 | 8.57 | 3.5  | 37.69 |

SD = standard deviation, SEM = standard error of the mean, CE = coefficient of error (multiplied by 100).
as shown in Fig. 3. We did not find any significant differences in neuronal ratios between AD and VD groups in FC and in striatal regions.

We also noticed that within VD group, the highest ratio of occludin-expressing neurons in the STR was obtained for the dementia multi-infarct cases (multiple basal ganglia lesions), as compared with the single strategic infarct and diffuse white matter cases, but due to the limited number of cases per each subgroup the statistical analysis was not performed separately.

**Occludin expression in glial cells**

Occludin expression in glial cells was evaluated qualitatively only. In the grey matter of the FC, selective occludin staining of the oligodendroglial cells was found in all diseased and control brains. In the basal ganglia, more astrocytes were positive for occludin than oligodendrocytes. In this region, a positive occludin expression was obvious preferentially in the nucleus (Fig. 1). In the frontal white matter, we were able to identify more occludin-positive oligodendrocytes and astrocytes in AD and VD as compared to the control group (Fig. 2).

**Occludin expression in vessels**

At the brain microvasculature level, in the FC of AD and control brains, we observed that occludin expression localized selectively in some of the endothelial cells or in the media layer (Fig. 1, C insert). Conversely, in VD and CADASIL brains, occludin expression localized in most of the vessel wall (Fig. 1, E insert and GG). No occludin staining was noted within the striatal arteries wall.

**Occludin in CADASIL and CAA**

Observation of the CADASIL and CAA cases showed that the expression of occludin in neurons, oligodendrocytes and astrocytes was similar to control in both regions analysed. However, as compared to control, a more intense occludin staining was obvious throughout the entire arterial wall in CADASIL FC, but not in CADASIL basal ganglia (Fig. 1, GG and H). In CAA, we found a patchy-like occludin staining in the endothelial region of cortical arteries, but a similar to control staining pattern in the basal ganglia. (Fig. 1, I and J).

**Discussion**

Several lines of evidence suggest that occludin is a protein located selectively in the cerebral endothelium at the level of TJs [29, 40], where it seems to regulate BBB functions [41, 42]. However, occludin expression was also identified in primary and secondary cultures of neurons and astrocytes from adult mouse [21] and it has been demonstrated that neurons and astrocytes regulate synthesis and distribution of occludin in brain capillary endothelial cells in co-cultures [43, 44]. However, other functions were suggested for occludin as well, like involvement in glucose-transport [45], recovery of the blood–nerve barrier [46] and developmental remodelling of the neuroepithelium [47].

To our knowledge, in this study we show for the first time that occludin is expressed by neurons in human brain. In AD brains, we found an increased occludin expression in neurons in both FC and basal ganglia regions in an area- and cell-specific manner. It is particularly remarkable that in the aged control brains almost 1/4th of neuronal cells (mainly pyramidal neurons) in the FC express occludin. In contrast, only 3% of the neurons in basal ganglia are occludin-positive. In dementia conditions, such as AD and VD, expression of occludin in neurons doubled or increased for at least 15 times in cortex and basal ganglia, respectively. This many-fold differences could actually prove a region-specific difference in neural plasticity potential.

The distinct ratio of occludin-expressing neurons in FC and basal ganglia regions in controls could be explained by distinct microvasculature properties and a dissimilar regulation of the neurovascular unit. It is well known, for instance, that corpus STR is more sensitive to ischaemic injury than the cerebral cortex [48] and that striatal arteries are of end-type, with a poor collateral network [49]. Therefore, it is perfectly possible that occludin expression in neurons is regulated also by factors depending on neurovascular coupling, which is different in the striatal and cortical areas. Furthermore, our study shows a rise in the ratio of occludin-expressing neurons in the basal ganglia in AD and VD as compared
It is now largely accepted that vascular lesions in the territory of lenticulostriate arteries are a common pathological finding not only in VD, but also in AD [50]. It is also worthy to note that occludin expression in basal ganglia differs among the subtypes of VD. Despite the clinical diagnose of VD, it is to believe that the patho-morphological base of each VD subtype is distinct and therefore it could associate a different occludin expression pattern.

Many studies showed that all mitogen-activated protein kinase (MAPK) pathways are activated in vulnerable neurons in AD brains [51] and a recent report identified p38 MAPK and extracellular signal-regulated protein kinase (ERK)1/ERK2 as being responsible for regulation of occludin expression [52]. Under these circumstances, it is probable that MAPK-pathways upregulation is responsible for the two-fold-increase in neuronal occludin expression in AD. Moreover, the level of basic fibroblast growth factor (FGF-2) is markedly elevated in AD brains [53] and, on the other hand, in FGF-2/-/-/FGF-5/-/- double mutant mice, a decrease of occludin expression and a defect in BBB functioning were reported [54]. Therefore, it is tempting to speculate that in AD, the high FGF levels trigger an increase in occludin expression, with a possible protective role.
The similarities of high occludin expression and distribution found in both AD and VD could be explained by signalling factors that are equally up regulated in these diseases. One example is the vascular endothelial growth factor (VEGF), which is itself a hypoxia-inducible angiogenic factor that was found elevated in cerebrospinal fluid (CSF) of AD and VD patients as compared to controls [55]. Moreover, it was suggested that polymorphisms within the promoter region of the VEGF gene confer greater risk for AD [56] and neuronal availability of VEGF seems to be reduced by deposition within the senile plaques [57]. A recent report demonstrated that VEGF increases the endothelial permeability by protein kinase C (PKC)-dependent phosphorylation of occludin [58]. Therefore, it is possible that a high VEGF production dysregulates the occludin to a higher and non-functional phosphorylation state in both AD and VD. In turn, an increased synthesis could occur to compensate the TJs function.

Moreover, we found a high expression of occludin not only in neurons, but also in astrocytes and oligodendrocytes, which means that in different types of CNS cells the mechanisms regulating occludin expression are similar, as far as they respond to the same pathogenic or compensatory factors. In contrast, regulation of occludin in the cerebral microvasculature did not parallel its regulation in neurons, as far as we found no marked difference in the vessel-staining pattern between control and AD brains. Instead, we showed qualitatively an increase of vascular occludin expression in VD, CADASIL and CAA. These distinct pathways of regulation of the same protein in different brain structures is not new to CNS biology, being seen for instance with the endothelial and neuronal isoforms of nitric oxide synthase [59]. Furthermore, it was recently shown that treatment with the fibrillogenic form of β-amyloid peptide decreases endothelial expression of occludin [28]. We may speculate that this mechanism could be involved in the regional and cellular differences seen in our study between occludin expression in microvasculature in AD and VD. However, it was recently reported that in cultured cerebral endothelial cells, experimentally-induced oxidative stress down-regulates occludin expression [31], result that is in apparent conflict to the increased microvascular occludin expression found in VD, CADASIL and CAA brains in our study. Even though oxidative stress could occur in endothelial cells in virtually all vascular brain pathologies, the temporal evolution and intensity of exposure to oxidative stress is completely different in these diseases as compared to an experimental paradigm observed for hours. For instance, it could be the case that oxidative stress down-regulates occludin by an early effect on transcription which results as well in BBB permeability alteration, followed by an adaptive increase in occludin expression at a later stage.

We have also to consider that besides the canonical, 504 amino acids occludin form, other alternative transcripts have been reported, as at least a shorter variant, lacking the fourth transmembrane domain (TM4) [60] and a longer variant, with a N-terminal sequence of 56 amino acids (1B) [61]. Due to the fact that the antiserum used in our study recognizes the C-terminal 150 amino acids of occludin, it is very probable not to distinguish between the canonical form of occludin and the variant transcripts. Therefore, based on the results of this study, we cannot...
conclude on the type of occludin transcript which is found in human brain cells or which is responsible for the increase of expression in AD and VD.

In the end, we would like to comment on two other aspects linked to occludin localization showed in our study. First, the intranuclear presence of occludin in astrocytes from basal ganglia suggests that occludin can be transported from cytoplasm to nucleus, a phenomenon seen with factors regulating transcription. Second, the identification of occludin expression selectively in pyramidal neurons, the ones bearing neurofibrillary tangles and being responsible for high cognitive processing, might suggest a role for occludin in cellular responses to neurodegeneration.

In conclusion we show, to our knowledge for the first time, occludin-expressing neurons, astrocytes and oligodendrocytes in human brains. In AD and VD, neurons in FC and basal ganglia overexpress occludin as compared to controls. At the level of the cerebral microvasculature, there is an increase of occludin expression in the predominantly 'vascular-based' types of neurodegeneration: VD, CADASIL and CAA, and least in AD. Our findings could be important to elucidate new pathogenic pathways in dementia disorders and open a new horizon for occludin functions in the CNS.

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