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SUBFIELD AND LAYER-SPECIFIC DEPLETION IN CALBINDIN-D28K, CALRETININ AND PARVALBUMIN IMMUNOREACTIVITY IN THE DENTATE GYRUS OF APP/PS1 TRANSGENIC MICE

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Abstract

The depletion of neuronal calcium binding proteins deprives neurons of the capacity to buffer high levels of intracellular Ca^{2+} and this leaves them vulnerable to pathological processes, such as those present in Alzheimer's disease (AD). The aim of the present study was to investigate the expression of the calcium binding proteins, calbindin-D28K, calretinin and parvalbumin in the dentate gyrus (DG) of APP/PS1 transgenic mice and their non-Tg littermates, as well as the relation with the deposition of human A β . We measured the expression of these three proteins at seven different rostro-caudal levels, and in the molecular, granular and polymorphic layers of the DG. We found that, except in the most caudal part of the DG, there is a substantial loss of calbindin-D28K immunoreactivity in all three layers of the DG in APP/PS1 mice compared to the non-Tg mice. Significant loss of calretinin immunoreactivity is present in most of the polymorphic layer of the DG of APP/PS1 mice compared to the non-Tg mice, as well as in the rostral and intermediate part of the inner molecular layer. Compared to the non-Tg mice parvalbumin immunoreactivity is significantly reduced throughout the whole polymorphic layer as well as in the rostral and intermediate part of the granular layer of DG in APP/PS1 mice. The relatively preservation of calbindin immunoreactivity in the caudal part of molecular and granular layers as well as calretinin immunoreactivity in the caudal part of polymorphic layer of the DG is likely related to the lower A β expression in those parts of DG. The present data suggest an involvement of calcium-dependent pathways in the pathogenesis of AD and indicate that there exists a subfield and layer-specific decrease in immunoreactivity which is related to the type of calcium-binding protein in APP/PS1 mice. Moreover, it seems that APP expression affects more the calbindin expression than parvalbumin and calretinin expression in the DG of APP/PS1 transgenic mice.

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Keywords

Alzheimer's disease; calcium binding protein; hippocampus; human A β

Alzheimer's disease (AD) is a progressive and irreversible neurodegenerative disorder that leads to cognitive and behavioural impairments. The pathogenesis of AD is complex, and involves many molecular, cellular and physiological abnormalities. However, destabilization of calcium homeostasis has been suggested to be central to the pathogenesis of AD, implicating the most important molecular hallmarks of AD: β -amyloid and tau (for review, see Mattson et al., 2001, LaFerla, 2002; Mattson and Chan, 2003; Xie, 2004; Smith et al., 2005; Stutzmann, 2005; Wenk, 2006). One of the components involved in the regulation of calcium homeostasis are the calcium binding proteins, proteins that exert their function by buffering the intracellular calcium concentration (Blaustein, 1988). Reduction of their expression level could lead to neuronal death due to the failure to buffer the pathologically high calcium concentration that can occur during the development of AD (Stutzmann, 2005). Therefore, it has been suggested that these proteins likely have a neuroprotective role (Hof et al., 1991; 1993; Iacopino et al., 1994).

Calbindin-D28K (hereafter calbindin), calretinin and parvalbumin, high-affinity cytosolic calcium binding proteins, have been used extensively not only as markers in anatomical and developmental studies but also as markers for brain region specific selective neuronal resistance to degeneration in AD (Hof et al., 1991, 1993, 1999). However, in the literature, the presence or loss of calcium binding protein positive neurons in AD has not been confirmed unambiguously. The discrepancies between studies highly depends on 1) brain regions sampled, 2) variability between the quantitative methods used, and 3) variability in the severity of the disease between cases (Solodkin et al, 1996; Leuba et al., 1998; Palop et al., 2003; Ohm, 2007).

There are only a few studies that have examined changes in calcium binding protein in the dentate gyrus (DG) of AD patients. Thus, it has been found that in DG of AD patients there was a significant decrease in the number of parvalbumin-positive neurons, accompanied by a marked overall loss of immunoreactive processes throughout the neuropil. This decrease did not exhibit any difference between the specific layers of the DG, nor did the reduction appear to target a specific morphological type of parvalbumin-immunopositive neurons (Brady and Mufson, 1997). A significant decline of calretinin immunoreactivity in the DG of AD patients was shown to be layer specific, and was only found in the inner (Kaufmann et al., 1998) and outer molecular layer (Brion and Resibois, 1994). Palop et al. (2003) reported a marked reduction of calbindin immunoreactivity in human AD dentate gyrus with the main changes present in the granular layer. The same authors found that neuronal calbindin levels were significantly reduced in transgenic mice overexpressing human amyloid precursor proteins (hAPP). The changes were primarily in the granular layer of the DG as well as in the molecular layer. A significant reduction of calbindin expression, but not of parvalbumin and calretinin expression, in the granular layer of the DG was also found in another AD transgenic mice model: in the brain of the mice that express hAPP containing the Swedish and V717F Indiana mutations (Lee et al., 2004). The DG is resistant to the formation of plaques, tangles and neuronal death until late stages of AD. Furthermore, disturbances in associative memory that depend on the DG are observed already in early stages of AD. Whether the loss of calcium binding proteins expression in the DG of AD patients is related to memory deficits and development of the above mentioned AD pathological hallmarks is questioned (Ohm, 2007).

While there are few AD patient studies, there is a relative lack of data correlating subfield and layer distribution of calcium binding proteins in the DG of transgenic AD model mice,

especially in relation to amyloid β plaque density. Therefore, the aim of the present study was to investigate the expression of three calcium binding proteins, i.e., calbindin, calretinin and parvalbumin, throughout the rostrocaudal extent of the molecular, granular and polymorphic layers of the DG of APP/PS1 transgenic mice as well as their correlation with the deposition of human A β .

MATERIAL AND METHODS

Animals

Male mice carrying both human APP695swe mutation (K595N and M596L) and PS1 A246E mutation (APP/PS1 mice) (Borchelt et al., 1997) or their nontransgenic littermates (NT) were used. The mice were of hybrid origin (C57BL/6J $\times$ C3H) but were back-crossed to C57BL/6J for 10 generations. A total of 12 mice (NT = 6, APP/PS1 = 6) of 14 months of age were used. The animals were housed individually in a constant, controlled environment (temperature $21 \pm 1^\circ\text{C}$, humidity 50–60%, light period 07:00–19:00 h). Food and fresh water were available *ad libitum*. The experiments were conducted according to the Council of Europe (Directive 86/609) and Finnish guidelines, and approved by the State Provincial Office of Eastern Finland.

Histopathology

The first part of experiment, related to tissue preparation and immunostaining, was done at the Department of Neuroscience and Neurology, University of Kuopio, Finland. The laboratory environmental conditions were maintained equally throughout all experiments, and all material (i.e., for each Ca-binding antibody) was stained at the same time in the same tray.

Fixation and tissue preparation

At the age of 14 months, the mice were deeply anesthetized with sodium pentobarbital (70 mg/kg, i.p.) and perfused transcardially with phosphate buffered saline (PBS) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were removed from the skull and postfixed for 4 hr in the same fixative, at 4°C . Then, the brains were transferred to a 30% sucrose solution in PBS overnight at 4°C , they were cut using a freezing, sliding microtome in 30 μm thick coronal sections, which were collected in PBS (100 mM, pH 7.4). All animals were sacrificed on the same day, brains were stored in antifreeze (150 g Sucrose, 300 ml Ethylene glycol with 500 ml 0.05 M Na-phosphate buffer, pH 7.4 added) (Crawford et al., 1984) and all brains were cut in the same week.

Calbindin, calretinin, parvalbumin and human A β single immunocytochemistry

The sections were immunocytochemically stained for calbindin, calretinin, parvalbumin and for human A β , and alternate sections were stained with cresyl violet (Nissl stain) for cytoarchitectonic control purposes. Free-floating sections were washed 2×5 min in 0.1 M Tris-buffered saline (TBS, pH 7.6), with the addition of 0.3% Triton-X-100 (TBS-TX), preincubated at room temperature (RT) (20°C). Three series of 1 in 6 sections (destinated for the calcium binding protein immunostaining) were then incubated (free-floating) in either the rabbit-anticallbindin antibody solution (1:100,000, Swant, Bellinzona, Switzerland), or the rabbit-anticalretinin antibody solution (1:4000, Swant, Bellinzona, Switzerland) or the rabbit-antiparvalbumin antibody solution (1:100,000, Swant, Bellinzona, Switzerland) for 18 h at RT. Following incubation, the sections were washed 3×5 min in TBS-TX, and incubated at RT for 2 h in the secondary antibody (goat-antirabbit Ig*biotin; 1:500, Chemicon International, Inc., CA, USA). A fourth series was stained for A β (Mouse anti-human A β 4–9; W02, Genetics Company, Switzerland; Van Groen et al., 2003). In short, the sections were first pretreated for 30 min with hot (85°C) citrate buffer and then transferred to a solution containing the primary antibody (mouse anti-human A β at 1:20,000). Following incubation in this solution for 18 h

on a shaker table at room temperature (20°C) in the dark, the sections were rinsed three times in TBS-T and transferred to the solution containing the secondary antibody (goat anti-mouse-biotin, Sigma). After 3×5 min washes in TBS-TX, the sections of the fourth series were then incubated with mouse ExtrAvidin (1:1000, Sigma) for 2h at RT, then washed 10 min in PBS and 2×10 min in Tris/HCl. The reaction product was visualized with the diaminobenzidine (DAB)-H₂O₂-peroxidase reaction, completed during 3 min. The DAB reaction was stopped with three washes in Tris/HCl. The sections were then mounted on gelatine-covered slides, air dried, dehydrated in ethanol solutions and xylene, and coverslipped with DPX.

All sections with Ca-binding protein staining were counterstained with Congo-red to be able to analyse the relation between Ca-binding staining and amyloid β plaque deposition. In short, the sections were put overnight in 4 % paraformaldehyde, the next day they were rinsed for 5 min in dH₂O, treated for 20 min in an alcoholic NaCl-hydroxide solution, then stained in the Congo Red solution for 30 minutes. Following dehydration for 20 sec in 95 % alcohol, and 1 min in 100% alcohol, the slides were cleared in xylene, and coverslipped.

To minimize variations in staining intensity, all the staining procedures were performed in batches containing equal proportions of control and experimental sections. All sections in a 'batch' were processed in the same staining tray, under identical experimental conditions.

Image analysis and quantification protocol

Image analysis was performed at Servicio de Análisis de Imagen, Universidad de Murcia, Spain.

Digitized images were obtained with a Sony DXC151AP digital camera on a Leica DMLB100T microscope (Leica, Barcelona) using a low-power objective (2.5x). Images were acquired and digitalized with an identical microscope illumination and camera settings. The quantification of calcium binding proteins immunoreactivity was determined with the Q-500-MC Qwin software (Leica, Barcelona). Two types of calibration were done by an operator who was blinded to the experimental groups: arithmetic and densitometric. Arithmetic calibration was done to establish the correlation between distance in pixels and μm . In relation to densitometric calibration, the digital image consists of a 512×512 matrix of pixels, where each pixel referred to a number between 0 (black) and 255 (white), representing the intensity of transmitted light or grey level at a point. The data were expressed as integrated optical density (IOD) per $1 \mu\text{m}^2$ of area in the inverted values such the highest values corresponding to highest immunolabelling. The analysis covered the entire molecular, granular and polymorphic layers of the DG, through its rostrocaudal extent between -0.94 and -4.16 posterior to bregma (Paxinos and Franklin, 2001) (Figure 1). The DG was divided in seven rostro-caudal levels (Table 1); we analyzed 1–3 sections per level per animal. The data from each animal were grouped according to the level and group. Once IOD analysis was completed, the number of plaques consisting of human amyloid β with a Congo-red positive core in the molecular, granular and polymorphic layers of DG was assessed. The data obtained were grouped according to the above mentioned rostro-caudal levels.

The photomicrographs of Nissl staining, representing levels 2, 4 and 6 of the DG, (Figure 2) correspond to the same levels in photomicrographs of calcium binding proteins and W0-2 immunostaining presented in Figure 3, 5, 7 and 9.

Statistical analysis

The data are expressed as the mean of IOD/area $\pm$ S.D. The statistical analysis was made using the SPSS 13.0 statistical package. The data were analyzed with the General Linear Model

repeated measures analysis, followed by the two-tailed Student's t-test for independent samples. Differences were accepted as statistically significant when $p < 0.05$.

RESULTS

Cresyl violet staining

The DG has been divided into the molecular, granular and polymorphic layers (Figure 2), the changes in anatomy at the three main rostro-caudal parts (rostral, intermediate and caudal; Figure 1) are illustrated in Figure 2. The molecular layer of the DG has been subdivided into three layers, the outer, medial and inner layer, each consisting of approximately 33% of the total thickness of the molecular layer.

Calbindin immunocytochemistry

In the NT mouse, and in the APP/PS1 mouse, all layers of the DG were clearly immunopositive for calbindin (Figure 3). The molecular layer was stained uniformly dark, the granule cells were slightly darker stained, and below the granule cell body layer, the mossy fibers stained very dark. Repeated measures analysis showed that in both NT and APP/PS1 transgenic mice, there was a significant linear increase of the immunoreactivity from rostral to caudal in the molecular ($F_{(1,10)}=15,815$, $p < 0.003$) and granular ($F_{(1,10)}=29,949$, $p < 0.0001$) layers of the DG (Figure 4A). However, there were no significant changes in immunoreactivity from rostral to caudal in the polymorphic layer of NT and APP/PS1 mice ($F_{(1,10)}=1,375$, $p < 0.268$). Repeated measures analysis did not show significant group and level interaction for molecular layer ($F_{(1,10)}=2,594$, $p < 0.138$), granular ($F_{(1,10)}=0.118$, $p < 0.738$) and polymorphic layer ($F_{(1,10)}=2,450$, $p < 0.149$) suggesting that the calbindin immunoreactivity changed in the same manner in both groups along the rostro-caudal extension of DG. Repeated measures analysis also showed that there were significant group differences between NT and APP/PS1 transgenic mice in the molecular ($F_{(1,10)}=8,316$, $p < 0.016$), granular ($F_{(1,10)}=40,375$, $p < 0.0001$) and polymorphic layer ($F_{(1,10)}=5,649$, $p < 0.039$) of the DG. The post hoc analysis demonstrated that with the exception of the most caudal part of the DG (i.e., level 7), the APP/PS1 transgenic mice had a significantly lower calbindin expression compared to the NT mice (Figure 3 and 4A).

Calretinin

The inner molecular layer and the polymorphic layer of the DG showed strong calretinin immunoreactivity (Figure 5). In both, NT and APP/PS1 transgenic mice, the calretinin immunoreactivity in the polymorphic layer increased linearly from the rostral to caudal part of the DG ($F_{(1,10)}=45,157$, $p < 0.0001$; Figure 4B and 5). In contrast, in inner molecular layer, there was no significant linear increment of immunoreactivity from the rostral to caudal part of the DG ($F_{(1,10)}=3,683$; $p < 0.084$). Repeated measures analysis did not show significant group and level interaction for inner molecular layer ($F_{(1,10)}=0.031$, $p < 0.864$) and polymorphic layer ($F_{(1,10)}=0,920$, $p < 0.360$) suggesting that the calretinin immunoreactivity changed in the same manner in both groups along the rostro-caudal extension of DG. Repeated measures analysis showed that there were significant group differences between NT and APP/PS1 transgenic mice in the inner molecular ($F_{(1,10)}=6,060$, $p < 0.034$) and polymorphic layer ($F_{(1,10)}=31,794$, $p < 0.0001$) of the DG. The post hoc analysis demonstrated that the APP/PS1 transgenic mice had a significantly lower calretinin immunoreactivity at the rostral and intermediate levels of the inner molecular layer (levels 2, 3 and 4) as well as in almost the whole polymorphic layer (except level 6) of the DG compared to the NT mice (Figures 4B and 5).

Parvalbumin

The granular and polymorphic layers of the DG were parvalbumin immunoreactive (Figure 6). In contrast to the calbindin and calretinin staining, in the granular and polymorphic layers of the DG many neurons were labeled, with only light staining of the neuropil. In both, NT and APP/PS1 transgenic mice, parvalbumin immunoreactivity linearly increment from rostral to caudal levels of the granular ($F_{(1,10)}=5,070$, $p < 0.048$) and polymorphic layer ($F_{(1,10)}=40,538$, $p < 0.0001$) (Figure 4C and 6). Repeated measures analysis did not show significant group and level interaction for granular ($F_{(1,10)}=1,447$, $p < 0.257$) and polymorphic layer ($F_{(1,10)}=0,097$, $p < 0.762$) suggesting that the parvalbumin immunoreactivity changed in the same manner in both groups along the rostro-caudal extension of DG. Repeated measures analysis showed that there were significant group differences in the granular ($F_{(1,10)}=18,266$, $p < 0.002$) and polymorphic layer ($F_{(1,10)}=100.139$, $p < 0.0001$) of the DG. The post hoc analysis demonstrated a significant reduction of parvalbumin immunoreactivity in APP/PS1 transgenic mice throughout the polymorphic layer of the DG. Some areas of the intermediate (levels 3 and 5) and the entire caudal part (levels 6 and 7) of the granular layer of the DG of the APP/PS1 did not show loss of parvalbumin immunoreactivity (Figures 4C and 6), compared to controls.

Human A β immunoreactivity

Human A β immunoreactivity (stained with the W0-2 antibody) in APP/PS1 transgenic mice was detected in molecular, granular and polymorphic layers of the DG, with the highest levels present at the intermediate level (Figure 7). There was no significant linear correlation between the W0-2 immunoreactivity and the rostro-caudal level of the granular ($F_{(1,5)}=5,019$, $p < 0.075$), molecular ($F_{(1,5)}=0.033$, $p < 0.863$) and polymorphic layers ($F_{(1,5)}=0.016$, $p < 0.906$) (Figure 8). Repeated measures analysis showed significant effect of A β immunoreactivity and calbindin immunoreactivity and level for molecular layer ($F_{(1,10)}=6,091$, $p < 0.033$) and granular layer ($F_{(1,10)}=6,310$, $p < 0.031$) but not for polymorphic layer ($F_{(1,10)}=1,702$, $p < 0.221$) suggesting that the increase of calbindin immunoreactivity from level 5 to 7 of molecular and granular layers correlates with decreased A β immunoreactivity. Moreover, we found a significant effect of A β immunoreactivity and calretinin immunoreactivity for the polymorphic layer ($F_{(1,10)}=11,687$, $p < 0.007$) where a significant decrease of A β immunoreactivity from level 5 to 7 correlated with an increase of calretinin immunoreactivity. Repeated measures analysis did not show a significant effect between A β immunoreactivity and parvalbumin immunoreactivity for the granular layer ($F_{(1,10)}=0,548$, $p < 0.476$) and polymorphic layer ($F_{(1,10)}=4,276$, $p < 0.066$).

Congo-red and Ca-binding proteins

Plaques consisting of human amyloid β with a Congo-red positive core were present in the molecular, granular and polymorphic layers of the DG in APP/PS1 transgenic mice (Figure 9). However, the highest plaque density was in the molecular layer distributed almost equally from level 2 to 6. Congo-red positive cores were detected uniformly in polymorphic layer from level 4 to 7. The lowest number of plaques was found in granular layer, mostly from level 4 to 6. Generally, there was no significant correlation between the number of plaques and calcium binding protein expression (data not shown). The only exception was calretinin expression, where lower calretinin immunoreactivity in the intermediate part of inner molecular layer of DG correlated with higher deposition of Congo-red positive plaque cores ($F_{(1,10)}=13,546$, $p < 0.004$).

DISCUSSION

Several studies (Liu et al., 1996; Blasco-Ibañez and Freund, 1997; Fujise et al., 1998; Fujise and Kosaka, 1999; Domínguez et al., 2003; Mátyás et al., 2004; Tang et al., 2006) have shown

that the distribution of calcium binding proteins in the mouse DG is layer specific. The results of the present study confirm and extend these previous findings, we have shown that: 1) calbindin immunoreactivity is present in all layers of DG, 2) calretinin immunoreactivity was mainly expressed in the inner molecular layer and the polymorphic layer of the DG, and 3) parvalbumin immunoreactivity was present in the granular and polymorphic layers of the DG.

Semi-quantitative morphological studies (Liu et al., 1996; Blasco-Ibañez and Freund, 1997; Fujise et al., 1998) have suggested that calretinin immunoreactivity was higher in the ventral than in the dorsal part of the polymorphic layer of the DG. Similarly, quantitative morphological studies performed by Jinno and Kosaka (2002) suggested that the dorsoventral difference in calcium binding proteins expression in the mouse DG is layer specific. They found that the numerical density of parvalbumin immunoreactive neurons was higher in the ventral part of the molecular, granular and polymorphic layers of DG than in their respective dorsal parts. However, a higher numerical density of calretinin immunoreactive cells and calbindin immunoreactive cells was found only in ventral parts of the granular and polymorphic layers, respectively. Although, in the present study caudal parts of the DG contain both intermediate and ventral parts of DG, calbindin expression in the molecular and granular layers of the DG as well as calretinin and parvalbumin expression in the polymorphic layer of the DG were higher at the levels that incorporate both intermediate and ventral parts of DG than the levels that are represent only the dorsal parts of DG.

In this study, we demonstrate quantitatively that a significant correlation between calbindin, calretinin and parvalbumin immunoreactivity and the rostro-caudal extension of the DG exists, and that it is layer specific. In the present study we have shown that in NT mice, calbindin immunoreactivity in the molecular and granular layers, calretinin immunoreactivity in the polymorphic layer as well as parvalbumin immunoreactivity in the granular and polymorphic layers, significantly linearly increases from the rostral to the caudal part of DG. Similar significant linear increases in calcium binding protein immunoreactivity from the rostral to the caudal part of DG has also been demonstrated in APP/PS1 mice.

Recent studies have shown a significant reduction of calbindin immunoreactivity in the granular and molecular layers of dorsal part of the DG in transgenic APP mice (Palop et al., 2003; Lee et al., 2004). In the present study, besides the granular and molecular layer, a significant reduction of calbindin immunoreactivity was also found in the polymorphic layer. Furthermore, we show that a significant reduction of calbindin immunoreactivity is not only characteristic for the rostral part of the DG but affected almost the whole DG (with exception of the most caudal part). In the present study, relatively preservation of calbindin immunoreactivity in the most caudal part of molecular and granular layers of the DG in APP/PS1 coincides with a significant decrease of A β deposition in the caudal part of these layers. In contrast, there are no significant relation between increase in calbindin expression in the caudal part of the polymorphic layer of APP/PS1 mice on the one hand, and decrease in A β immunoreactivity on other hand.

In the inner molecular layer, the calretinin immunoreactivity is significantly level specific for NT mice and the highest density of calretinin immunoreactivity is located in the intermediate part of the DG. Therefore, our data are similar to those reported in the study of Liu et al. (1996). Although these authors did not perform a quantitative analysis of calretinin immunoreactivity, Figure 5 of the manuscript clearly indicates that the immunoreactivity increased from the rostral to the caudal extension of the polymorphic layer of the DG, and that the highest intensity of staining in the inner molecular layer is present in the intermediate part of the DG.

According to Lee et al. (2004), there are no significant differences in parvalbumin and calretinin expression between control mice and APP transgenic mice (mice that express hAPP containing the Swedish and V717F Indiana mutations) in the dorsal part of the DG. However, we found a significant reduction of calretinin immunoreactivity in rostral and intermedial levels of the inner molecular layer, as well as in almost the whole polymorphic layer of the DG of APP/PS1 mice, in comparison to NT mice. A significant reduction of parvalbumin immunoreactivity in APP/PS1 transgenic mice, in comparison to the control NT mice, is demonstrated throughout the polymorphic layer of the DG, while only the caudal part of the granular layer of these mice does not show a loss of parvalbumin immunoreactivity. The likely explanation for this differences are the transgenic mice that have been used in the studies, Lee et al., (2004) used transgenic mice that express only hAPP, while in our study the transgenic animals express both hAPP and PS1.

Moreover, in the present study we found that there was a significant correlation between increased A β expression on the one hand and increased calbindin (as well as parvalbumin expression) on other hand, in the polymorphic layer in APP/PS1 mice. Similar correlations between A β and parvalbumin expression from the rostral to the caudal level of the granular layer of APP/PS1 mice has also been found. In contrast, a significant increase of calretinin immunoreactivity from level 5 to 7 in the polymorphic layer and calbindin increase in the same levels in the molecular and granular layers coincides with a significant decrease of in A β immunoreactivity. These findings suggest that A β expression differently affects distinct calcium binding proteins depending on the layer of DG studied. Moreover, our data suggest that calbindin immunoreactivity is more affected than parvalbumin and calretinin immunoreactivity. It is possible that calretinin and parvalbumin cells are more resistant to beta amyloid toxicity than calbindin positive cells. This hypothesis is supported by in vitro studies that demonstrated that parvalbumin and calretinin positive cells are resistant to beta amyloid toxicity (Harkany et al., 1995; Pike et al., 1995) and that only neurons that had induced calbindin overexpression can counteract this neurotoxic effect (Prehn et al., 1996). Together, this suggests that the relatively high A β load in the DG in these mice overwhelms the capacity of the neurons containing Ca-binding proteins to compensate for the stress caused by the amyloid load.

Finally, it has been found that PS1 can potentiate the increase of intracellular calcium that is induced by beta amyloid, and that the overexpression of calbindin in the neurons stabilizes the intracellular calcium level, and prevents the proapoptotic actions of PS1 (Guo et al., 1998).

In conclusion, the vulnerability of calcium-binding proteins expression in the DG in APP/PS1 transgenic mice is subfield and layer-specific. The most profound changes were found in the rostro-intermediate part of the molecular, granular and polymorphic layers of the DG. Moreover, it seems that calbindin positive neurons are more affected by the concomitant APP and PS1 expression in transgenic mice than parvalbumin and calretinin positive neurons. Together, these data strongly suggest that the performance of a detailed, layer specific analysis of the rostro-caudal staining patterns of these three calcium binding proteins in the whole hippocampal formation in AD pathology would be desirable in future studies. Thus, in order to gain a better understanding of AD pathogenesis, we believe that the study of the time-course of 1) subfield and layer-specific changes in calcium binding proteins in the hippocampus, 2) cognitive decline dependent on the hippocampus and 3) the development of pathological hallmarks in the hippocampus (i.e., amyloid deposition, neuronal loss, etc) is needed. Further, the question can be raised whether the DG has an anatomical and functional subdivision along its dorso-ventral axis, since the expression of calcium binding proteins changes with the level of coronal sections.

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List of abbreviations

Aβ	amyloid β
AD	Alzheimer's disease
ANOVA	one way analysis of variance
APP	amyloid precursor protein
Ca²⁺	calcium
DAB	diaminobenzidine
DG	dentate gyrus
hAPP	human amyloid precursor protein
IOD	integrated optical density
NT	nontransgenic
PBS	phosphate buffered saline
PS1	presenilin 1
RT	room temperature
S.D	standard deviation
TBS	Tris buffered saline
Tg	transgenic
Tx	Triton, X-100

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Figure 1. Schematic drawing indicating the dentate gyrus, and representing levels 2 (A), 4 (B) and 6 (C) of the dentate gyrus as shown in Figure 2. (i.e., Nissl-stained) as well as in photomicrographs of calcium binding proteins and human A β immunostaining presented in Figure 3, 5, 7 and 9.

Figure 2.

Photomicrographs of the coronal Nissl stained sections from the rostral to the caudal part of the dentate gyrus of NT mice. Big dots represent the analyzed molecular layer, small dots represent analyzed polymorphic layer, the area between big and small dots represent the analyzed granular layer. Scale bar in panel A equals to 600 μm and applies to the panels A to C, scale bar in panel D equals to 150 μm and applies to the panels D to F.

Figure 3. Photomicrographs of coronal sections from the rostral to the caudal part of the dentate gyrus of NT (panels from A to F) and APP/PS1 transgenic mice (panels from G to L) immunolabeled for calbindin. ML-molecular layer, GL-granular layer and PL-polymorphic layer. Scale bar in panel C equals to 600 μ m and applies to the panels A to C and G to I, scale bar in panel L equals to 150 μ m and applies to the panels D to F and J to L.

Figure 4.

Layer-specific immunoreactivity of calbindin (A), calretinin (B) and parvalbumin (C) in the dentate gyrus of NT and APP/PS1 transgenic mice. Data (integrated optical density- IOD/area) are presented as mean $\pm$ S.D. Post hoc analysis at each level. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure 5.

Photomicrographs of coronal sections from the rostral to the caudal part of the dentate gyrus of NT (panels from A to F) and APP/PS1 transgenic mice (panels from G to L) immunolabeled for calretinin. iML-inner molecular layer, oML-out molecular layer, GL- granular layer and PL-polymorphic layer. Scale bar in panel C equals to 600 μ m and applies to the panels A to C and G to I, scale bar in panel F equals to 150 μ m and applies to the panels D to F and J to L.

Figure 6.

Photomicrographs of coronal sections from the rostral to the caudal part of the dentate gyrus of NT (panels from A to F) and APP/PS1 transgenic mice (panels from G to L) immunolabeled for parvalbumin. GL granular layer and PL-polymorphic layer. Scale bar in panel C equals to 600 μ m and applies to the panels A to C and G to I, scale bar in panel L equals to 150 μ m and applies to the panels D to F and J to L.

Figure 7.

Photomicrographs of coronal sections from the rostral to the caudal part of the dentate gyrus of APP/PS1 mice immunolabeled for WO-2 antibody against human A β . ML-molecular layer, GL- granular layer and PL-polymorphic layer. Scale bar in panel C equals to 600 μ m and applies to the panels A to C and G to I, scale bar in panel F equals to 150 μ m and applies to the panels D to F and J to L.

Figure 8.

Correlation between human Aβ immunoreactivity and calcium binding proteins immunoreactivity (calbindin, calretinin and parvalbumin) in dentate gyrus. Significant correlation between Aβ immunoreactivity and calretinin immunoreactivity was found in the polymorphic layer of dentate gyrus ($r=0.393$, $p < 0.007$) as well as between Aβ immunoreactivity and parvalbumin and calbindin immunoreactivities in the granular layer of dentate gyrus ($r=0.286$, $p < 0.048$; $r=0.330$, $p < 0.009$, respectively).

Figure 9.

Photomicrographs of coronal sections of intermedial part of the dentate gyrus of APP/PS1 mice that represent plaque deposition consisting of human amyloid β with a Congo-red positive core. A. calbindin, B. calretinin, C. parvalbumin. iML-inner molecular layer, oML-out molecular layer, ML-molecular layer, GL- granular layer and PL-polymorphic layer Scale bar in panel A equals to 150 μ m and applies to all.

Table 1

Bregma corresponding to the levels refer in Figures 1–8 throughout the rostro-caudal extension of the dentate gyrus

LEVELS	BREGMA	
	From	To
1	–0.94	–1.22
2	–1.34	–1.70
3	–1.82	–2.18
4	–2.30	–2.70
5	–2.80	–3.16
6	–3.28	–3.64
7	–3.80	–4.16