Altered expression of the CB1 cannabinoid receptor in the triple transgenic mouse model of Alzheimer's disease

Gaurav Bedse[a*], Adele Romano[a*], Silvia Cianci[a], Angelo M. Lavecchia[a], Pace Lorenzo[f], Maurice R. Elphick[b], Frank M. LaFerla[c], Gianluigi Vendemiale[d], Caterina Grillo[e], Fabio Altieri[e], Tommaso Cassano[f§] and Silvana Gaetani[a]

[a]Department of Physiology and Pharmacology, Sapienza University of Rome, Rome, 00185, Italy

[b]School of Biological and Chemical Sciences, Queen Mary University of London, Mile End Road, London E1 4NS, UK

[c]Department of Neurobiology and Behaviour, University of California, Irvine, CA 92697-4545, USA

[d]Department of Medical and Surgical Sciences, University of Foggia, Foggia, 71100, Italy

[e]Istituto Pasteur-Fondazione Cenci Bolognetti - Department of Biochemical Sciences, Sapienza University of Rome, Rome, 00185, Italy

[f]Department of Clinical and Experimental Medicine, University of Foggia, Foggia, 71100, Italy

[*]These are co-first authors

Running title: Altered CB1 receptor expression in 3×Tg-AD

[§]to whom correspondence should be addressed: Department of Clinical and Experimental Medicine, University of Foggia, Viale Luigi Pinto 1, Foggia - 71100, ITALY

Tel.: +39 0881 588042; Fax: +39 0881 188 0432

e-mail: tommaso.cassano@unifg.it

Abstract

The endocannabinoid system (ECS) has gained much attention as a new potential pharmacotherapeutic target in various neurodegenerative diseases, including Alzheimer's disease (AD). However, the association between CB1 alterations and the development of AD neuropathology is unclear and often contradictory. In this study, brain CB1 mRNA and CB1 protein levels were analysed in 3×Tg-AD mice and compared to wild-type littermates at 2, 6 and 12 months of age, using in-situ hybridization and immunohistochemistry, respectively. Semiquantitative analysis of CB1 expression focused on the prefrontal cortex (PFC), prelimbic cortex (PrL), dorsal hippocampus (DH), basolateral amygdala complex (BLA) and ventral hippocampus (VH), all areas with high CB1 densities that are strongly affected by neuropathology in 3×Tg-AD mice. At 2 months of age, there was no change in CB1 mRNA and protein levels in 3×Tg-AD mice compared to Non-Tg mice in all brain areas analyzed. However, at 6 and 12 months of age, CB1 mRNA levels were significantly higher in PFC, DH, BLA and lower in VH in 3×Tg-AD mice compared to wild-type littermates. CB1 immunohistochemistry revealed that CB1 protein expression was unchanged in 3×Tg-AD at 2 and 6 months of age, while a significant decrease in CB1 receptor immunoreactivity was detected in the BLA and DH of 12-month-old 3×Tg-AD mice, with no sign of alteration in other brain areas. The altered CB1 levels appear, rather, to be age-and/or pathology-dependent, indicating an involvement of the ECS in AD pathology and supporting the ECS as a potential novel therapeutic target for treatment of AD.

Keywords:

3×Tg-AD mice; Alzheimer’s disease; CB1 mRNA; CB1 receptor; Basolateral amygdala complex; Hippocampus; Prefrontal cortex; Endocannabinoid system

Introduction

Alzheimer's disease (AD) is progressive, degenerative and irreversible neurological disorder that causes deterioration of memory, judgment and reasoning in the elderly. AD is characterized by accumulation of extracellular insoluble plaques, intracellular neurofibrillary tangles (NFTs) in the brain and selective synaptic and neuronal loss. Extracellular plaques consist of amyloid-β (Aβ) protein and NFTs are composed of hyperphosphorylated tau protein [1]. Although Aβ plaques and NFTs pathology are prominent, other pathological alterations in neurotransmitter systems and concomitant changes in synthetic enzymes and associated receptors are also an important feature of AD. For example, cholinergic and glutamatergic neurotransmitter systems are known to be affected by AD [2].

The endocannabinoid system (ECS) has gained much attention as a new potential pharmacotherapeutic target in various neurodegenerative diseases including AD. The CB1-type cannabinoid receptor (CB1) is the most abundant G protein–coupled receptor expressed in the central nervous system (CNS) and through the activation of CB1 receptors in the CNS, the ECS exerts important functions such as retrograde inhibition of neurotransmitter release, control of neuronal excitability, and regulation of various forms of synaptic plasticity [3]. Aberrant patterns of brain CB1 receptor expression and densities have been observed postmortem in patients suffering from AD and in animal models of AD. However, these observations are sparse and often contradictory [4-8], so the relationship between alterations in CB1 expression and the development of AD neuropathology is still unclear.

Oddo and his colleagues developed a triple transgenic mouse model of AD (3×Tg-AD) harbouring three mutant human genes $PS1_{M146V}$, APP_{Swe}, and Tau_{P301L} [9]. This model mimics critical aspects of AD neuropathology observed in the human AD patients [10, 11]: it progressively develops both plaques and tangles in AD relevant brain regions (mainly cortex, hippocampus and amygdala); it exhibits early deficits in synaptic plasticity, including long-

term potentiation; it shows selective loss of α7 neuronal nicotinic acetylcholine receptors [9, 12], severe deficits in glutamatergic neurotransmission and altered mitochondrial functions in hippocampus and cortex [13].

The aim of the present study was to evaluate whether brain CB1 expression is altered in 3×Tg-AD mice in comparison with wild type littermates (Non-Tg). Moreover, to investigate whether the temporal and regional patterns of such possible alterations might overlap with those of Aβ and tau pathology in this AD model, brain CB1 expression was analysed at different ages [9]. As a consequence, by studying the temporal expression of CB1 in the wild type littermates, our study has also allowed us to analyse the impact of aging on CB1 levels. Our analyses were conducted on both CB1 mRNA and CB1 protein levels in 3×Tg-AD and wild-type mice at 2, 6 and 12 months of age, by *in situ* hybridization and immunohistochemistry/immunofluorescence, respectively, followed by the semi-quantitative analysis of the respective signals obtained in prefrontal cortex (PFC), prelimbic cortex (PrL), dorsal hippocampus (DH), basolateral amygdala complex (BLA) and ventral hippocampus (VH), all areas strongly affected by the neuropathology and characterized by high CB1 densities.

Materials and Methods

Animals

Male 3×Tg-AD and Non-Tg mice aged 2-, 6-, and 12-months old were used in this study. The 3×Tg-AD mice harboring $PS1_{M146V}$, APP_{Swe}, and Tau_{P301L} transgenes were genetically engineered by LaFerla and colleagues at the Department of Neurobiology and Behavior, University of California, Irvine [9]. Colonies of 3×Tg-AD mice and Non-Tg littermates were established at the vivarium of the Puglia and Basilicata Experimental Zooprophylactic Institute (Foggia, Italy). The 3×Tg-AD mice background strain is C57BL6/129SvJ hybrid and genotypes were confirmed from tail biopsy, according to the procedures described previously [9, 14]. The housing conditions were controlled (temperature 22°C, light from 07:00 –19:00, humidity 50%–60%), and fresh food and water were freely available.

In situ hybridization

In situ hybridization was performed on coronal sections of brains using a ^{35}S-labeled RNA probe complementary to rat CB1 mRNA. Riboprobes in antisense and sense orientation were generated from linearized vector constructs (520 bp, a kind gift of Dr. Jin Fu, Xiamen University) by *in vitro* transcription using the appropriate RNA polymerases [15].

Mice (n = 5 per group) were euthanized by decapitation; their brains were rapidly removed, snap frozen in 2-methylbutane (-50°C) and stored at -80°C. Brain sections (20 µm) were cut on a cryostat (-20°C) and thaw-mounted on RNAse-free positively charged slides to be hybridized at 60°C for 16 h in a buffer containing [^{35}S]cRNA (45,000 dpm ml^{-1}), 10% dextran sulfate, 50% formamide, 1× Denhardt's solution, 100 µgml^{-1} denatured salmon sperm DNA, 0.15 mg ml^{-1} tRNA and 40 mM dithiothreitol. After hybridization, the sections were exposed to Kodak Biomax film (Sigma-Aldrich) for 3 days. Autoradiography films were first scanned (Epson perfection 3200 PHOTO) at high resolution (900 dpi). Optical densities were

converted to radioactivity measurements (μCi) by densitometric analysis of ^{14}C-microscale standards that were used to create a calibration curve.

Immunohistochemistry and immunofluorescence

Mice (n = 3 per group) were intra-cardioventricularly perfused with saline followed by fixation solution (4% paraformaldehyde in 0.1 M phosphate buffer, PB, pH 7.4) at a flow rate of 36 ml min^{-1}. Then brains were fixed for 48 hours in 4% paraformaldehyde. Free-floating coronal sections of 50 μm thickness were obtained using a vibratome slicing system (microM, Walldorf, Germany) and stored at 4°C in 0.02% sodium azide in phosphate buffered (PB). The endogenous peroxidase activity was quenched for 30 minutes in 0.3% H_2O_2. The brain sections were blocked with 10% normal goat serum/PBS with 0.3% Triton X-100 and then incubated with CB1 2825.3 antiserum (C-terminus residues 461–473, generously provided by Dr. Maurice Elphick, Queen Mary College) (1:1500 dilution) for overnight at 4°C [16]. Evidence of the selectivity of this antiserum in revealing CB1 expression in the rat nervous system has been previously obtained by pre-absorption tests with the CB1 C-terminal peptide antigen and by Western blotting, which reveals a band in rat brain homogenates (~53 kDa) consistent with the expected molecular mass for CB1 [16, 17]. Furthermore, the selectivity of the antiserum for CB1 has been previously confirmed by analysis of brain tissue and dorsal root ganglia from CB1-knockout mice [17, 18]. After removing the primary antiserum in excess, sections were incubated with secondary antibody (Biotin-SP-conjugated fragment donkey anti rabbit IgG) for 1 h at room temperature. After washing excess of antibody, sections were treated with avidin–biotin–peroxidase complex (ABC, 1:200 dilution, Vector Laboratories) and then developed with diaminobenzidine (DAB) substrate using the avidin-biotin horseradish peroxidase system (Vector Laboratories).

For immunofluorescence staining, free floating coronal brain sections of 30 µm thickness were obtained using a cryostat (Microm HM550, Thermo scientific) and stored at 4°C in 0.02% sodium azide in PB. The brain sections were treated with 90% of formic acid for 7 min followed by PB washes. Then the brain sections were blocked in a solution containing 5% normal goat serum and 0.3% Triton X-100 in PB and then incubated with both CB1 2825.3 antiserum (1:1500 dilution) and Aβ monoclonal antibody (6E10, Covance, 1:1500 dilution) for 16 h at 4°C. After removing primary antibodies, sections were incubated with both secondary antibodies Alexa Fluor 555 donkey anti-rabbit IgG (1:250 dilution) and Alexa Fluor 488 goat anti-mouse IgG (1:250 dilution) for 1 h and 30 min at room temperature. All washes after this step were carried out in dark. After washing excess of antibodies, sections were treated with Hoechst, Sigma (1:5000 dilution). After washing excess Hoechst with PB, brain slices were mounted on slides. Furthermore, to confirm the background staining level, an immunofluorescent staining for CB1 was also carried out without the primary antibody.

All immunohistochemically-stained sections were viewed using a Nikon 80i Eclipse microscope equipped with a DS-U1 digital camera, and NIS-elements BR software (Nikon, Tokyo, Japan). Immunofluorescent slices were observed under the confocal microscope Olimpus FV-1000.

Semiquantitative analyses of the autoradiographic signal of hybridized CB1 mRNA and of CB1 DAB-immunostaining or immunofluorescence were performed using freeware software from the National Institutes of Health (Scion Image software) and were expressed as optical densities.

Statistical analysis

The optical densities obtained by the semiquantitative analyses were analyzed by two way analysis of variance (ANOVA), with genotype and age as variables. Tukey's honestly

significant difference test was used for multiple *post hoc* comparisons. The correlation analysis between Aβ and CB1 protein levels was performed on the respective optical densities measured on double immunofluorescent slices and expressed as percentage of those measured in Non-Tg mice, by using the Pearson correlation test. Statistical significance threshold was set at $p<0.05$.

Results

CB1 mRNA expression

Representative images of CB1 mRNA distribution in the mouse brain is shown in Fig. 1A and quantitative analysis of CB1 mRNA expression in PFC, PrL, DH, VH and BLA is shown in Fig 1B-F. The results from ANOVA revealed an overall effect of genotype [$F_{(genotype)1,122}$ = 31.992, $p < 0.001$], age [$F_{(age)2,122} = 16.177$, $p < 0.001$] and genotype × age interaction [$F_{(age\ x\ genotype)2,122} = 4.288$, $p < 0.05$] on CB1 mRNA expression in PFC (Fig. 1B). Post hoc comparisons revealed that CB1 mRNA expression was significantly higher in 3×Tg-AD mice compared to Non-Tg mice at 6 months (+56%, $p<0.05$) and 12 months (+15%, $p<0.05$) of age. Different results were obtained for PrL, where a significant overall effect of age was observed [$F_{(age)2,113} = 18.212$, $p < 0.001$], with no significant overall effect of genotype [$F_{(genotype)1,113} = 1.161$, n.s.] and genotype by age interaction [$F_{(age\ x\ genotype)2,113} = 0.871$, n.s.] (Fig. 1C). ANOVA analysis of CB1 mRNA expression in DH and VH demonstrated a significant overall effect of age, genotype and age by genotype interaction [DH: $F_{(age)2,151}$ = 81.052, $p < 0.001$; $F_{(age\ x\ genotype)2,151} = 3.166$, $p < 0.05$; $F_{(genotype)1,151} = 19.079$, $p < 0.001$; VH: $F_{(age)2,182} = 10.431$, $p < 0.001$; $F_{(age\ x\ genotype)2,182} = 6.987$, $p < 0.001$; $F_{(genotype)1,182} = 10.116$, $p < 0.01$]. Interestingly, post hoc comparisons revealed a clear dissociation between the dorsal and ventral hippocampus (Fig. 1D and E, respectively). In particular, the former showed a significantly higher expression of CB1 mRNA in the 3×Tg-AD mice compared to Non-Tg mice both at 6 months (+29%, $p<0.05$) and 12 months (+33%, $p<0.05$) of age, while in the latter there was a significant decrease in CB1 mRNA expression in the transgenic mice compared to the control group (-40% and -35%, respectively at 6 and 12 months of age; $p<0.05$). Statistical analysis of CB1 mRNA expression in the BLA revealed a significant overall effect of age [$F_{(age)2,160} = 14.888$, $p < 0.001$], genotype [$F_{(genotype)1,160} = 31.774$, $p < 0.001$] and age by genotype interaction [$F_{(age\ x\ genotype)2,160} = 8.916$, $p < 0.001$] (Fig. 1F). Post

hoc comparisons revealed that CB1 mRNA expression was significantly higher in 3×Tg-AD mice compared to Non-Tg mice at 6 months (+78%, $p<0.05$) and 12 months (+49%, $p<0.05$) of age.

CB1 protein expression

Representative microphotographs of CB1 immunostaining are shown in Fig. 2A. Fig. 2B-F shows the semiquantitative analysis of CB1 protein expression in the PFC, PrL, DH, VH and BLA. The results from ANOVA revealed an overall effect of genotype [$F_{(genotype)1,314} = 12.687$, $p < 0.001$] and age [$F_{(age)2,314} = 59.579$, $p < 0.001$] in DH, with no significant overall effect of genotype × age interaction [$F_{(age\ x\ genotype)2,314} = 0.345$, n.s.] on CB1 protein expression (Fig. 2D). Post hoc comparisons revealed that CB1 protein levels were significantly lower in 3×Tg-AD mice compared to Non-Tg mice at 12 months of age (-20%, $p<0.05$) and that, in within-genotype comparisons, both groups of mice at 12 months of age showed significantly lower CB1 protein levels compared to 2- and 6-month old mice.

For the BLA, ANOVA showed an overall effect of age [$F_{(age)2,67} = 6.735$, $p < 0.01$], with no significant overall effect of genotype [$F_{(genotype)1,67} = 2.736$, n.s.] and significant genotype × age interaction [$F_{(age\ x\ genotype)2,67} = 3.279$, $p < 0.05$] on CB1 protein expression (Fig. 2F). Interestingly, at 12 months of age 3×Tg-AD mice showed (i) lower CB1 protein expression compared to age-matched Non-Tg mice (-42%), and (ii) significantly lower CB1 protein levels compared to 2-month-old (-48%, $p<0.05$) and 6-month-old (-47%, $p<0.05$) transgenic mice.Finally, no significant difference was found between genotypes at 2, 6 and 12 months of age in PFC, PrL and VH (Fig. 2B, C and E).

Lowered CB1 protein expression in DH and BLA were further confirmed by immunofluorescent staining (Fig.3 A, B lower panel). At 12 months of age, 3×Tg-AD mice showed lower CB1 protein levels in DH (-22%, $p<0.05$) and BLA (-48 %, $p<0.05$) compared

to Non-Tg mice (Fig.3 C, D). Moreover, by performing a double immunofluorescence for CB1 and Aβ (Fig.3 E, F), we could semiquantitatively measure both protein levels and find an inverse correlation between the decline of CB1 receptor expression and the build up of Aβ pathology in both the DH (Fig.3 G, DH: $\rho = -0.7599$, $p<0.0001$) and the BLA (Fig.3 H, BLA: $\rho = -0.5052$, $p<0.001$, Pearson Correlation test).

Discussion

In this study the general pattern of CB1 mRNA expression and of CB1 protein distribution throughout the mouse brain revealed similarity with previous reports [16, 19, 20]. Furthermore, this study has revealed for the first time that CB1 mRNA and CB1 protein expression in 3×Tg-AD mice is altered in brain areas particularly involved in learning and memory processes and where the impact of AD neuropathology is more prominent. More specifically, a significant increase of CB1 mRNA levels in PFC, DH, BLA and a reduction in VH were found in 3×Tg-AD mice compared to Non-Tg mice at 6 and 12 months of age. Such differences were found to be opposite for CB1 protein levels in the DH and BLA, where CB1 protein levels were lower in 12-month-old 3×Tg-AD mice compared to their age-matched Non-Tg mice. No differences between genotypes were found in the brains of 2-month-old mice.

Furthermore, the comparisons within mice from the same genotype at different ages revealed significant effects of aging on both CB1 mRNA and CB1 protein levels in several brain regions. In particular, we observed an age-dependent increase of CB1 mRNA levels in most areas for both genotypes (except BLA for Non-Tg mice and VH for 3×Tg-AD mice), while a decrease of CB1 protein expression was detected in two brain areas of aged mice (the DH for both genotypes and the BLA for 3×Tg-AD mice) as compared to 2- and 6-months-old mice of the respective genotype.

In this study, the correlation between CB1 mRNA and protein levels observed was not direct. This observation is not surprising, as it was previously demonstrated that in general mRNA levels do not necessarily predict the respective protein levels [21]. Moreover, the discrepant results obtained here are complex to interpret considering also that CB1 receptors are expressed mostly on synaptic terminals whilst CB1 mRNA is synthesized mostly in the cell body. For example, CB1 receptors are abundantly expressed on GABAergic interneurons of

several brain areas that receive also the nerve terminals expressing CB1 protein from other structures. In this case the CB1 protein levels will result more abundant that the respective CB1 mRNA level. Conversely, other sites contain only CB1 expressing terminals with no cell body expressing CB1 mRNA. Therefore, in these areas not necessarily CB1 protein levels correspond to CB1 mRNA levels. However, the most obvious hypothesis arising from our results is that this discrepancy might be due to modifications at translational and/or post-translational levels, occurring at the three different ages considered.

Two months of age in our murine AD-model corresponds to a pre-pathologic phase characterized by the absence of any Aβ and tau pathological expression [9]. The lack of differences in CB1 expression between genotypes at this age suggests that 3×Tg-AD mice do not have inborn altered CB1 expression in the brain regions analysed. Therefore, we speculate that the altered pattern of CB1 expression found at older ages in their brains can be interpreted as age- and/or pathology-dependent. In accordance with this hypothesis, an extensive set of age-related and pathology-related alterations are described in our murine model (see table 1).

At 6 months of age, extracellular Aβ deposits first become apparent in the frontal cortex of 3×Tg-AD mice, while intracellular Aβ immunoreactivity starts to build-up in hippocampus, cortex and amygdala [9, 22]. At 12 months of age extracellular Aβ deposits are readily evident in frontal cortex, amygdala, DH and VH; the immunoreactivity for hyperphosphorylated tau starts to be evident in CA1 neurons of hippocampus, particularly at the somatodendritic level of pyramidal neurons (progressing later to involve cortical structures) [9, 14].

From our results, alterations of CB1 mRNA but not protein levels appear at 6 months of age, when the AD neuropathology seems to impact on CB1 expression first at transcriptional levels. Alterations in CB1 expression become more evident at 12 months of age, when they involve also the protein levels in the BLA and DH, remaining unaltered in the other areas.

Interestingly, the temporal pattern of the changes of CB1 protein expression observed in our study seemed to correlate with the temporal pattern of the development of Aβ pathology, at least in the two brain areas analysed, namely the DH and the BLA. Previous studies corroborate our finding that CB1 receptors in cortex are unchanged [4, 8] and lowered in DH [5, 7] in AD. However, some reports showed that CB1 levels are altered in cortex [23, 24] and unaltered in DH of AD patients [4, 6, 8]. These discrepancies might be due to different disease models used in each study. Until now, much emphasis has been given to the role of the ECS in cortex and hippocampus in AD pathology, while leaving the BLA poorly investigated, in spite of its well-known role in learning, its involvement in AD neuropathology and its quite high expression of CB1 receptors.

Age-related changes of CB1 mRNA expression in the rodent brain have been already reported in the literature, although data are still sparse and in some cases discrepant from our results. In particular, CB1 mRNA was observed to increase steadily throughout neuronal development of rats and mice until animals reach 2 months of age [25, 26]. Conversely, a decrease of CB1 mRNA has been described in hippocampus and BLA, with no change in cortex, when rats are 24-months-old [27]. These discrepancies with our results might be due to different species used in these studies.

CB1 receptors play important roles in neuroprotection and the enhancement of endocannabinoid tone is now considered an attractive therapeutic approach to treat AD. It has been demonstrated, indeed, that the enhancement of brain endocannabinoid tone is able to reverse memory impairment and neurotoxic effects triggered by soluble Aβ in murine models of AD [28]. The neuroprotective function of cannabinoid system is thought to occur through variety of mechanisms. For example, through CB1 receptor activation anandamide was recently shown to positively regulate Notch-1 pathway, which plays a key role in neurogenesis, long term memory and neuronal development, and thus restore AD

304 neurodegeneration and memory impairments [29]. Moreover, ECS was also demonstrated to
305 be involved in clearing Aβ from the blood brain barrier, as demonstrated in vitro by
306 Bachmeier et through the incubation with cannabinoid receptor agonist or inhibitors of
307 endocannabinoid-degrading enzymes [30]. Based on our results, we speculate that increasing
308 the endocannabinoid tone or hyperactivating CB1 receptors might produce such ameliorating
309 effects by counterbalancing the loss of CB1 receptors in selected brain areas, such as the BLA
310 and the DH.
311 In this latter area, we recently observed a dramatic deficit of glutamate neurotransmission in
312 aged 3×Tg-AD mice. These lower levels of glutamate did not appear to be due to synaptic
313 loss, as synaptophysin, a presynaptic vesicle marker of synaptic density, was not altered [13,
314 31-34]. Within the hippocampus, CB1 receptors are highly expressed by GABAergic
315 interneurons [35], where they negatively control GABA release on excitatory glutamatergic
316 neurons. Therefore, it can be hypothesized that the reduced glutamatergic neurotransmission
317 in this area might result from the reduced CB1 expression on GABA terminals and the
318 consequent excessive GABA-mediated inhibition of glutamatergic neurons.
319 Recently, CB1 was found to be expressed in mitochondria, and a novel role for CB1 receptors
320 in the regulation of energy metabolism in the brain was proposed [36]. Aged 3×Tg-AD mice
321 show severe mitochondrial impairment, as was previously shown by our group and by others
322 [13, 37], and the hippocampus is the most severely affected area. This previous observation is
323 in line with the current findings of reduced CB1 levels in DH of aged mutant mice.
324 Apart from genetic factors, stress has also been suggested as a risk factor in developing AD
325 and severe cognitive decline in AD patients. HPA axis dysregulation and elevated cortisol
326 levels have been described in a substantial proportion of patients with AD [38-40]. Moreover,
327 animal studies, including some performed on 3×Tg-AD mice, suggest some sort of interaction
328 between corticosterone, dysregulation of the HPA axis and Aβ/tau pathology in AD [41, 42],

although the mechanisms underlying this interaction remain unknown. In particular, when corticosterone levels in 3×Tg-AD mice were evaluated, Green and colleagues found that basal corticosterone levels were unchanged until 9 months of age compared to aged matched non transgenic mice. After 9 months of age, corticosterone levels were significantly elevated in 3×Tg-AD mice compared to age-matched non transgenic mice [42]. Although corticosterone levels were normal at early age, these mice showed activated HPA axis in 3-4-month-old 3×Tg-AD. At this age increased mRNA levels of mineralocorticoid receptor and glucocorticoids receptor were also observed in the hippocampus and PVN with no change in the amygdala, while the mRNA of corticotropin releasing hormone decreased in the PVN and increased in both the central nucleus of the amygdala and the bed nucleus of the stria terminalis [43].

There is evidence that the ECS regulates the HPA axis by negatively modulating its activation induced by the exposure to stress [44-46]. Among other areas, CB1 receptors expressed in DH and BLA seem to be involved in negative feedback of glucocorticoids in these brain regions [47]. As a consequence, CB1 receptor blockade with the antagonist, SR141716, results in activation of the HPA axis as measured by an increase in plasma corticosterone levels in rodents [44]. Apart from dysregulated HPA axis, increased emotionality and depressive like behavior are reported in these mice [48]. We have observed depressive like behavior in these mice when subjected to a forced swimming and tail suspension test (unpublished data). Moreover, these mice are reported to show symptoms of anxiety and fear associated with spatial memory deficits. Authors proposed a deleterious role of intraneuronal Aβ on amygdala-dependent emotional responses [49]. A similar behavioral phenotype was observed in CB1-knockout mice ($CB1^{-/-}$), which show also increased circulating levels of adrenocorticotropic hormone [46], corticosterone [50, 51], anxiety like and fear responses [52-54] as well as depressive like behavior [55, 56]. Our result might suggest that the

354 decreased CB1 like immunoreactivity found in DH and BLA in 3×Tg-AD mice could play a
355 role in the hippocampus-related memory deficits and amygdala-related behavioural
356 alterations.
357 Interestingly, a recent study by Stumm et al, showed that the lack of CB1 receptors in $CB1^{-/-}$
358 mice over-expressing APP23 can result in reduction of amyloid plaque load, reduced *in situ*
359 inflammation and impaired learning and memory in aged mice [34]. We propose that lowered
360 CB1 receptor expression might contribute to the cognitive impairments and dysregulated
361 HPA axis found in 3×Tg-AD mice.
362 Overall our results show that 3×Tg-AD do not have inborn altered CB1 mRNA and protein
363 expression, as they did not show any alteration at 2 months of age when their phenotype is
364 still normal. The altered CB1 mRNA/protein levels appear, rather, to be age-and/or
365 pathology-dependent, thus supporting the idea of a critical role of the ECS in AD and its
366 possible impact as novel pharmacological target. How AD pathology exactly affects CB1
367 receptors and whether CB1 receptors and AD pathology are directly or indirectly linked needs
368 to be further explored.
369

Acknowledgements

This study was supported by PRIN (2009) (to GV). The authors thank Dr. Antonio Petrella from the Puglia and Basilicata Experimental Zooprophylactic Institute (Foggia, Italy) for his invaluable veterinary assistance. The authors disclose no conflicts. All experiments were performed in strict compliance with the Italian National Laws (DL 116/92), the European Communities Council Directives (86/609/EEC). All efforts were made to minimize the number of animals used in the study and their suffering.

References

[1] Selkoe DJ (2001) Alzheimer's disease: genes, proteins, and therapy. *Physiol Rev* **81**, 741-766.

[2] Whitehouse PJ, Price DL, Struble RG, Clark AW, Coyle JT, Delon MR (1982) Alzheimer's disease and senile dementia: loss of neurons in the basal forebrain. *Science* **215**, 1237-1239.

[3] Freund TF, Katona I, Piomelli D (2003) Role of endogenous cannabinoids in synaptic signaling. *Physiol Rev* **83**, 1017-1066.

[4] Lee JH, Agacinski G, Williams JH, Wilcock GK, Esiri MM, Francis PT, Wong PT, Chen CP, Lai MK (2010) Intact cannabinoid CB1 receptors in the Alzheimer's disease cortex. *Neurochem Int* **57**, 985-989.

[5] Westlake TM, Howlett AC, Bonner TI, Matsuda LA, Herkenham M (1994) Cannabinoid receptor binding and messenger RNA expression in human brain: an in vitro receptor autoradiography and in situ hybridization histochemistry study of normal aged and Alzheimer's brains. *Neuroscience* **63**, 637-652.

[6] Mulder J, Zilberter M, Pasquare SJ, Alpar A, Schulte G, Ferreira SG, Kofalvi A, Martin-Moreno AM, Keimpema E, Tanila H, Watanabe M, Mackie K, Hortobagyi T, de Ceballos ML, Harkany T (2011) Molecular reorganization of endocannabinoid signalling in Alzheimer's disease. *Brain* **134**, 1041-1060.

[7] Kalifa S, Polston EK, Allard JS, Manaye KF (2011) Distribution patterns of cannabinoid CB1 receptors in the hippocampus of APPswe/PS1DeltaE9 double transgenic mice. *Brain Res* **1376**, 94-100.

[8] Benito C, Nunez E, Tolon RM, Carrier EJ, Rabano A, Hillard CJ, Romero J (2003) Cannabinoid CB2 receptors and fatty acid amide hydrolase are selectively overexpressed in neuritic plaque-associated glia in Alzheimer's disease brains. *J Neurosci* **23**, 11136-11141.

[9] Oddo S, Caccamo A, Shepherd JD, Murphy MP, Golde TE, Kayed R, Metherate R, Mattson MP, Akbari Y, LaFerla FM (2003) Triple-transgenic model of Alzheimer's disease with plaques and tangles: intracellular Abeta and synaptic dysfunction. *Neuron* **39**, 409-421.

[10] Mesulam MM (1999) Neuroplasticity failure in Alzheimer's disease: bridging the gap between plaques and tangles. *Neuron* **24**, 521-529.

[11] Mesulam MM (2000) A plasticity-based theory of the pathogenesis of Alzheimer's disease. *Ann N Y Acad Sci* **924**, 42-52.

[12] Oddo S, Caccamo A, Green KN, Liang K, Tran L, Chen Y, Leslie FM, LaFerla FM (2005) Chronic nicotine administration exacerbates tau pathology in a transgenic model of Alzheimer's disease. *Proc Natl Acad Sci U S A* **102**, 3046-3051.

[13] Cassano T, Serviddio G, Gaetani S, Romano A, Dipasquale P, Cianci S, Bellanti F, Laconca L, Romano AD, Padalino I, LaFerla FM, Nicoletti F, Cuomo V, Vendemiale G (2012) Glutamatergic alterations and mitochondrial impairment in a murine model of Alzheimer disease. *Neurobiol Aging* **33**, 1121.e1121-1112.

[14] Oddo S, Caccamo A, Kitazawa M, Tseng BP, LaFerla FM (2003) Amyloid deposition precedes tangle formation in a triple transgenic model of Alzheimer's disease. *Neurobiol Aging* **24**, 1063-1070.

[15] van Rijn CM, Gaetani S, Santolini I, Badura A, Gabova A, Fu J, Watanabe M, Cuomo V, van Luijtelaar G, Nicoletti F, Ngomba RT (2010) WAG/Rij rats show a reduced expression of CB(1) receptors in thalamic nuclei and respond to the CB(1) receptor agonist, R(+)WIN55,212-2, with a reduced incidence of spike-wave discharges. *Epilepsia* **51**, 1511-1521.

[16] Egertova M, Elphick MR (2000) Localisation of cannabinoid receptors in the rat brain using antibodies to the intracellular C-terminal tail of CB. *J Comp Neurol* **422**, 159-171.

[17] Bridges D, Rice AS, Egertova M, Elphick MR, Winter J, Michael GJ (2003) Localisation of cannabinoid receptor 1 in rat dorsal root ganglion using in situ hybridisation and immunohistochemistry. *Neuroscience* **119**, 803-812.

[18] Monory K, Massa F, Egertova M, Eder M, Blaudzun H, Westenbroek R, Kelsch W, Jacob W, Marsch R, Ekker M, Long J, Rubenstein JL, Goebbels S, Nave KA, During M, Klugmann M, Wolfel B, Dodt HU, Zieglgansberger W, Wotjak CT, Mackie K, Elphick MR, Marsicano G,

Lutz B (2006) The endocannabinoid system controls key epileptogenic circuits in the hippocampus. *Neuron* **51**, 455-466.

[19] Marsicano G, Lutz B (1999) Expression of the cannabinoid receptor CB1 in distinct neuronal subpopulations in the adult mouse forebrain. *Eur J Neurosci* **11**, 4213-4225.

[20] Egertova M, Cravatt BF, Elphick MR (2003) Comparative analysis of fatty acid amide hydrolase and cb(1) cannabinoid receptor expression in the mouse brain: evidence of a widespread role for fatty acid amide hydrolase in regulation of endocannabinoid signaling. *Neuroscience* **119**, 481-496.

[21] Pascal LE, True LD, Campbell DS, Deutsch EW, Risk M, Coleman IM, Eichner LJ, Nelson PS, Liu AY (2008) Correlation of mRNA and protein levels: cell type-specific gene expression of cluster designation antigens in the prostate. *BMC Genomics* **9**, 246.

[22] Kitazawa M, Oddo S, Yamasaki TR, Green KN, LaFerla FM (2005) Lipopolysaccharide-induced inflammation exacerbates tau pathology by a cyclin-dependent kinase 5-mediated pathway in a transgenic model of Alzheimer's disease. *J Neurosci* **25**, 8843-8853.

[23] Ramirez BG, Blazquez C, Gomez del Pulgar T, Guzman M, de Ceballos ML (2005) Prevention of Alzheimer's disease pathology by cannabinoids: neuroprotection mediated by blockade of microglial activation. *J Neurosci* **25**, 1904-1913.

[24] Solas M, Francis PT, Franco R, Ramirez MJ (2013) CB2 receptor and amyloid pathology in frontal cortex of Alzheimer's disease patients. *Neurobiol Aging* **34**, 805-808.

[25] Psychoyos D, Vinod KY, Cao J, Xie S, Hyson RL, Wlodarczyk B, He W, Cooper TB, Hungund BL, Finnell RH (2012) Cannabinoid receptor 1 signaling in embryo neurodevelopment. *Birth Defects Res B Dev Reprod Toxicol* **95**, 137-150.

[26] Romero J, Garcia-Palomero E, Berrendero F, Garcia-Gil L, Hernandez ML, Ramos JA, Fernandez-Ruiz JJ (1997) Atypical location of cannabinoid receptors in white matter areas during rat brain development. *Synapse* **26**, 317-323.

[27] Berrendero F, Romero J, Garcia-Gil L, Suarez I, De la Cruz P, Ramos JA, Fernandez-Ruiz JJ (1998) Changes in cannabinoid receptor binding and mRNA levels in several brain regions of aged rats. *Biochim Biophys Acta* **1407**, 205-214.

[28] van der Stelt M, Mazzola C, Esposito G, Matias I, Petrosino S, De Filippis D, Micale V, Steardo L, Drago F, Iuvone T, Di Marzo V (2006) Endocannabinoids and beta-amyloid-induced neurotoxicity in vivo: effect of pharmacological elevation of endocannabinoid levels. *Cell Mol Life Sci* **63**, 1410-1424.

[29] Tanveer R, Gowran A, Noonan J, Keating SE, Bowie AG, Campbell VA (2012) The endocannabinoid, anandamide, augments Notch-1 signaling in cultured cortical neurons exposed to amyloid-beta and in the cortex of aged rats. *J Biol Chem* **287**, 34709-34721.

[30] Bachmeier C, Beaulieu-Abdelahad D, Mullan M, Paris D (2013) Role of the cannabinoid system in the transit of beta-amyloid across the blood-brain barrier. *Mol Cell Neurosci* **56**, 255-262.

[31] Arsenault D, Dal-Pan A, Tremblay C, Bennett DA, Guitton MJ, De Koninck Y, Tonegawa S, Calon F (2013) PAK inactivation impairs social recognition in 3xTg-AD Mice without increasing brain deposition of tau and Abeta. *J Neurosci* **33**, 10729-10740.

[32] Noristani HN, Meadows RS, Olabarria M, Verkhratsky A, Rodriguez JJ (2011) Increased hippocampal CA1 density of serotonergic terminals in a triple transgenic mouse model of Alzheimer's disease: an ultrastructural study. *Cell Death Dis* **2**, e210.

[33] Rodriguez-Ortiz CJ, Hoshino H, Cheng D, Liu-Yescevitz L, Blurton-Jones M, Wolozin B, LaFerla FM, Kitazawa M (2013) Neuronal-specific overexpression of a mutant valosin-containing protein associated with IBMPFD promotes aberrant ubiquitin and TDP-43 accumulation and cognitive dysfunction in transgenic mice. *Am J Pathol* **183**, 504-515.

[34] Stumm C, Hiebel C, Hanstein R, Purrio M, Nagel H, Conrad A, Lutz B, Behl C, Clement AB (2013) Cannabinoid receptor 1 deficiency in a mouse model of Alzheimer's disease leads to enhanced cognitive impairment despite of a reduction in amyloid deposition. *Neurobiol Aging* **34**, 2574-2584.

[35] Pertwee RG (2008) The diverse CB1 and CB2 receptor pharmacology of three plant cannabinoids: delta9-tetrahydrocannabinol, cannabidiol and delta9-tetrahydrocannabivarin. *Br J Pharmacol* **153**, 199-215.

[36] Benard G, Massa F, Puente N, Lourenco J, Bellocchio L, Soria-Gomez E, Matias I, Delamarre A, Metna-Laurent M, Cannich A, Hebert-Chatelain E, Mulle C, Ortega-Gutierrez S, Martin-Fontecha M, Klugmann M, Guggenhuber S, Lutz B, Gertsch J, Chaouloff F, Lopez-Rodriguez ML, Grandes P, Rossignol R, Marsicano G (2012) Mitochondrial CB(1) receptors regulate neuronal energy metabolism. *Nat Neurosci* **15**, 558-564.

[37] Yao J, Irwin RW, Zhao L, Nilsen J, Hamilton RT, Brinton RD (2009) Mitochondrial bioenergetic deficit precedes Alzheimer's pathology in female mouse model of Alzheimer's disease. *Proc Natl Acad Sci U S A* **106**, 14670-14675.

[38] Davis KL, Davis BM, Greenwald BS, Mohs RC, Mathe AA, Johns CA, Horvath TB (1986) Cortisol and Alzheimer's disease, I: Basal studies. *Am J Psychiatry* **143**, 300-305.

[39] Masugi F, Ogihara T, Sakaguchi K, Otsuka A, Tsuchiya Y, Morimoto S, Kumahara Y, Saeki S, Nishide M (1989) High plasma levels of cortisol in patients with senile dementia of the Alzheimer's type. *Methods Find Exp Clin Pharmacol* **11**, 707-710.

[40] Swanwick GR, Kirby M, Bruce I, Buggy F, Coen RF, Coakley D, Lawlor BA (1998) Hypothalamic-pituitary-adrenal axis dysfunction in Alzheimer's disease: lack of association between longitudinal and cross-sectional findings. *Am J Psychiatry* **155**, 286-289.

[41] Elliott EM, Mattson MP, Vanderklish P, Lynch G, Chang I, Sapolsky RM (1993) Corticosterone exacerbates kainate-induced alterations in hippocampal tau immunoreactivity and spectrin proteolysis in vivo. *J Neurochem* **61**, 57-67.

[42] Green KN, Billings LM, Roozendaal B, McGaugh JL, LaFerla FM (2006) Glucocorticoids increase amyloid-beta and tau pathology in a mouse model of Alzheimer's disease. *J Neurosci* **26**, 9047-9056.

[43] Hebda-Bauer EK, Simmons TA, Sugg A, Ural E, Stewart JA, Beals JL, Wei Q, Watson SJ, Akil H (2013) 3xTg-AD mice exhibit an activated central stress axis during early-stage pathology. *J Alzheimers Dis* **33**, 407-422.

[44] Patel S, Roelke CT, Rademacher DJ, Cullinan WE, Hillard CJ (2004) Endocannabinoid signaling negatively modulates stress-induced activation of the hypothalamic-pituitary-adrenal axis. *Endocrinology* **145**, 5431-5438.

[45] Hill MN, McLaughlin RJ, Bingham B, Shrestha L, Lee TT, Gray JM, Hillard CJ, Gorzalka BB, Viau V (2010) Endogenous cannabinoid signaling is essential for stress adaptation. *Proc Natl Acad Sci U S A* **107**, 9406-9411.

[46] Barna I, Zelena D, Arszovszki AC, Ledent C (2004) The role of endogenous cannabinoids in the hypothalamo-pituitary-adrenal axis regulation: in vivo and in vitro studies in CB1 receptor knockout mice. *Life Sci* **75**, 2959-2970.

[47] De Kloet ER, Vreugdenhil E, Oitzl MS, Joels M (1998) Brain corticosteroid receptor balance in health and disease. *Endocr Rev* **19**, 269-301.

[48] Gimenez-Llort L, Blazquez G, Canete T, Johansson B, Oddo S, Tobena A, LaFerla FM, Fernandez-Teruel A (2007) Modeling behavioral and neuronal symptoms of Alzheimer's disease in mice: a role for intraneuronal amyloid. *Neurosci Biobehav Rev* **31**, 125-147.

[49] Espana J, Gimenez-Llort L, Valero J, Minano A, Rabano A, Rodriguez-Alvarez J, LaFerla FM, Saura CA (2010) Intraneuronal beta-amyloid accumulation in the amygdala enhances fear and anxiety in Alzheimer's disease transgenic mice. *Biol Psychiatry* **67**, 513-521.

[50] Cota D, Steiner MA, Marsicano G, Cervino C, Herman JP, Grubler Y, Stalla J, Pasquali R, Lutz B, Stalla GK, Pagotto U (2007) Requirement of cannabinoid receptor type 1 for the basal modulation of hypothalamic-pituitary-adrenal axis function. *Endocrinology* **148**, 1574-1581.

[51] Steiner MA, Marsicano G, Wotjak CT, Lutz B (2008) Conditional cannabinoid receptor type 1 mutants reveal neuron subpopulation-specific effects on behavioral and neuroendocrine stress responses. *Psychoneuroendocrinology* **33**, 1165-1170.

[52] Haller J, Bakos N, Szirmay M, Ledent C, Freund TF (2002) The effects of genetic and pharmacological blockade of the CB1 cannabinoid receptor on anxiety. *Eur J Neurosci* **16**, 1395-1398.

[53] Maccarrone M, Valverde O, Barbaccia ML, Castane A, Maldonado R, Ledent C, Parmentier M, Finazzi-Agro A (2002) Age-related changes of anandamide metabolism in CB1 cannabinoid receptor knockout mice: correlation with behaviour. *Eur J Neurosci* **15**, 1178-1186.

[54] Martin M, Ledent C, Parmentier M, Maldonado R, Valverde O (2002) Involvement of CB1 cannabinoid receptors in emotional behaviour. *Psychopharmacology (Berl)* **159**, 379-387.
[55] Aso E, Ozaita A, Serra MA, Maldonado R (2011) Genes differentially expressed in CB1 knockout mice: involvement in the depressive-like phenotype. *Eur Neuropsychopharmacol* **21**, 11-22.
[56] Valverde O, Torrens M (2012) CB1 receptor-deficient mice as a model for depression. *Neuroscience* **204**, 193-206.
[57] Billings LM, Oddo S, Green KN, McGaugh JL, LaFerla FM (2005) Intraneuronal Abeta causes the onset of early Alzheimer's disease-related cognitive deficits in transgenic mice. *Neuron* **45**, 675-688.

553 **Table 1**. Summary of age related molecular and behavioral changes in 3×Tg-AD mice

Age of 3×Tg-AD mice	CB1 receptor	Molecular and behavioral observation	Ref
2 months	mRNA and protein unchanged	-no Aβ and tau pathology -cognitively unimpaired	[9]
		-intraneuronal Aβ in hippocampus and amygdala -cognitively impaired	[9, 42, 43, 57]
4 months	-	-activated central HPA axis -normal corticosterone levels -altered mRNA levels of corticoid receptors and CRH	
6 months	Increased mRNA in PFC, DH, BLA Decreased in mRNA in VH Protein unchanged	-extracellular Aβ in neocortex -intraneuronal buildup in hippocampus, amygdala and cortex -impaired LTP -synaptic dysfunction	[9, 14]
9 months	-	-increased corticosterone levels	[42]
12 months	Increased mRNA in PFC, PrL, DH, BLA Decreased mRNA in VH Decreased protein in DH and BLA Protein unchanged in PFC, PrL and VH	extracellular Aβ deposits is evident in frontal cortex, amygdala, DH and VH -Tau pathology evident in hippocampus	[9, 14]
18 months	-	- deficits in glutamate neurotransmission and mitochondrial functions in prefrontal cortex and hippocampus - emotionality and depressive like behavior	[13, 48]

554 LTP, long-term potentiation; HPA, hypothalamic-pituitary-adrenal; CRH, corticotropin-
555 releasing hormone

Figure legends

Fig.1. CB1 mRNA distribution pattern in Non-Tg and 3xTg-AD mice. (A) Representative micrographs of coronal sections from mouse brain showing distribution of CB1 mRNA scanned from autoradiographic film exposed for 3 days. The dashed lines indicate the brain regions where the optical density was measured. (B-F) CB1 mRNA expression levels in Non-Tg (open bars) and 3xTg-AD mice (black bars) at 2, 6 and 12 months (2M, 6M, 12M, respectively) of age in PFC (B), PrL (C), DH (D), VH (E) and BLA (F). The data are expressed as means ± SEM * $p < 0.05$ vs Non-Tg and °$p < 0.05$ ($n = 5$ per group).

Fig.2. CB1 protein distribution pattern in Non-Tg and 3xTg-AD mice. (A) Representative microphotographs of brain coronal sections showing CB1 immunostaining in the selected brain areas. The dashed lines indicate the brain regions where the optical density was measured. (B-F) CB1 protein expression levels in Non-Tg (open bars) and 3xTg-AD mice (black bars) at 2, 6 and 12 months (2M, 6M, 12M, respectively) in PFC (B), PrL (C), DH (D), VH (E) and BLA (F). The data are expressed as means ± SEM * $p< 0.05$ vs Non-Tg and °$p<0.05$ ($n = 3$ per group).

Fig.3. CB1 protein expression by immunofluorescence staining in 12 months old 3xTg-AD mice. (A, B) Representative microphotographs of brain coronal sections showing nuclear staining with Hoechst (blue) and CB1 immunofluorescence staining (red) in the DH (A) and BLA (B) without or with CB1 antiserum incubation step. (C, D) CB1 protein expression levels measured in the DH (C) and in the BLA (D) of 12 month-old Non-Tg (open bars) and 3xTg-AD mice (black bars). (E, F) Representative photographs for CB1 protein (red), Aβ protein (green) and nuclear staining with Hoechst (blue) in DH (E) and BLA (F). (G, H) Scatterplot of Aβ protein levels vs CB1 protein levels showing an inverse correlationship

581 (Pearson test) in both the DH (G, ρ= -0.7599, p<0.0001) and the BLA (H, ρ= -0.5052,

582 p<0.001) The data are expressed as means ± SEM * p < 0.05 vs Non-Tg (n = 3 per group).

Figure 1

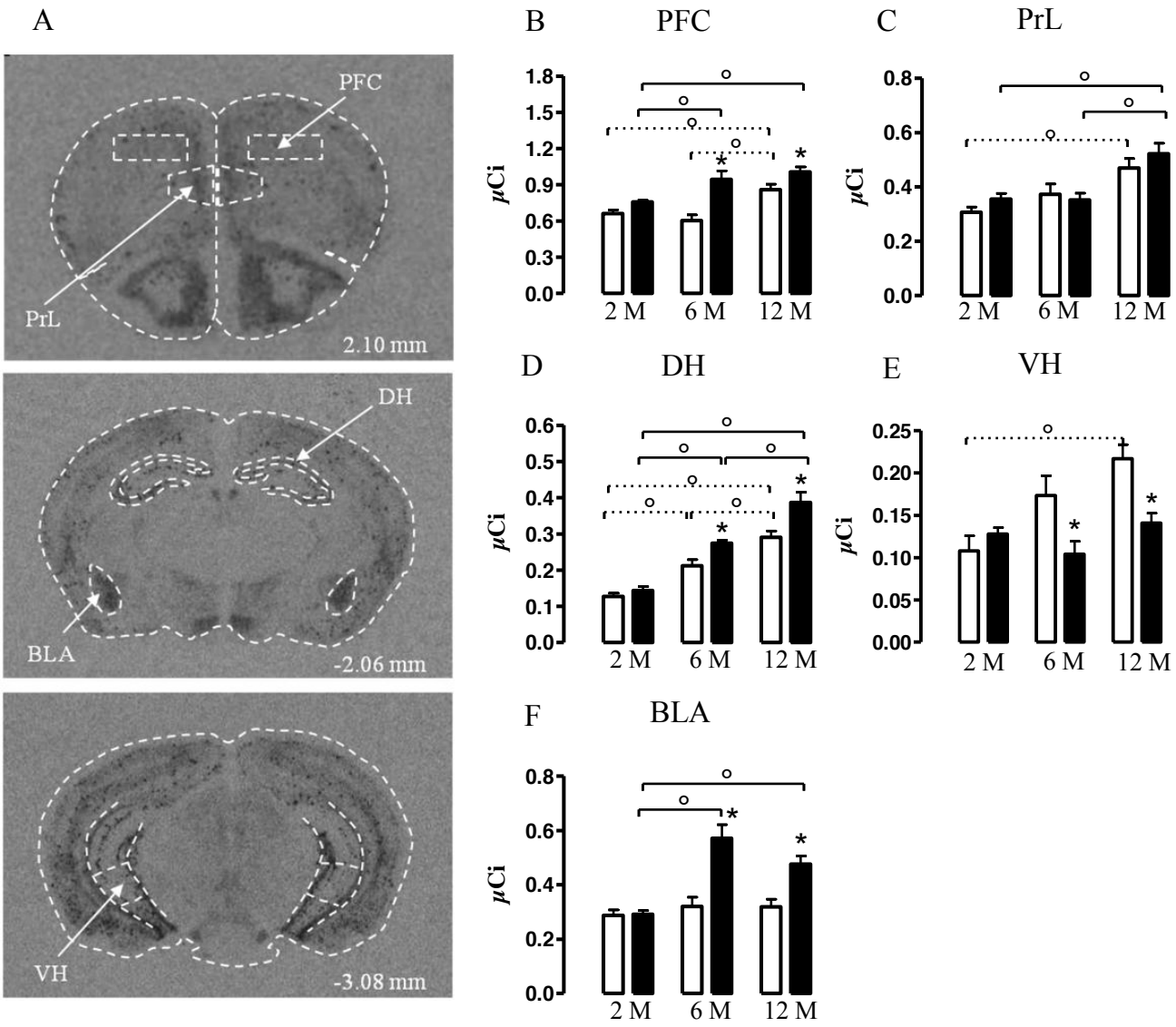

A
PFC
PrL
2.10 mm
DH
BLA
-2.06 mm
VH
-3.08 mm
B
PFC
C
PrL
D
DH
E
VH
F
BLA
µCi
2 M
6 M
12 M

Figure 2

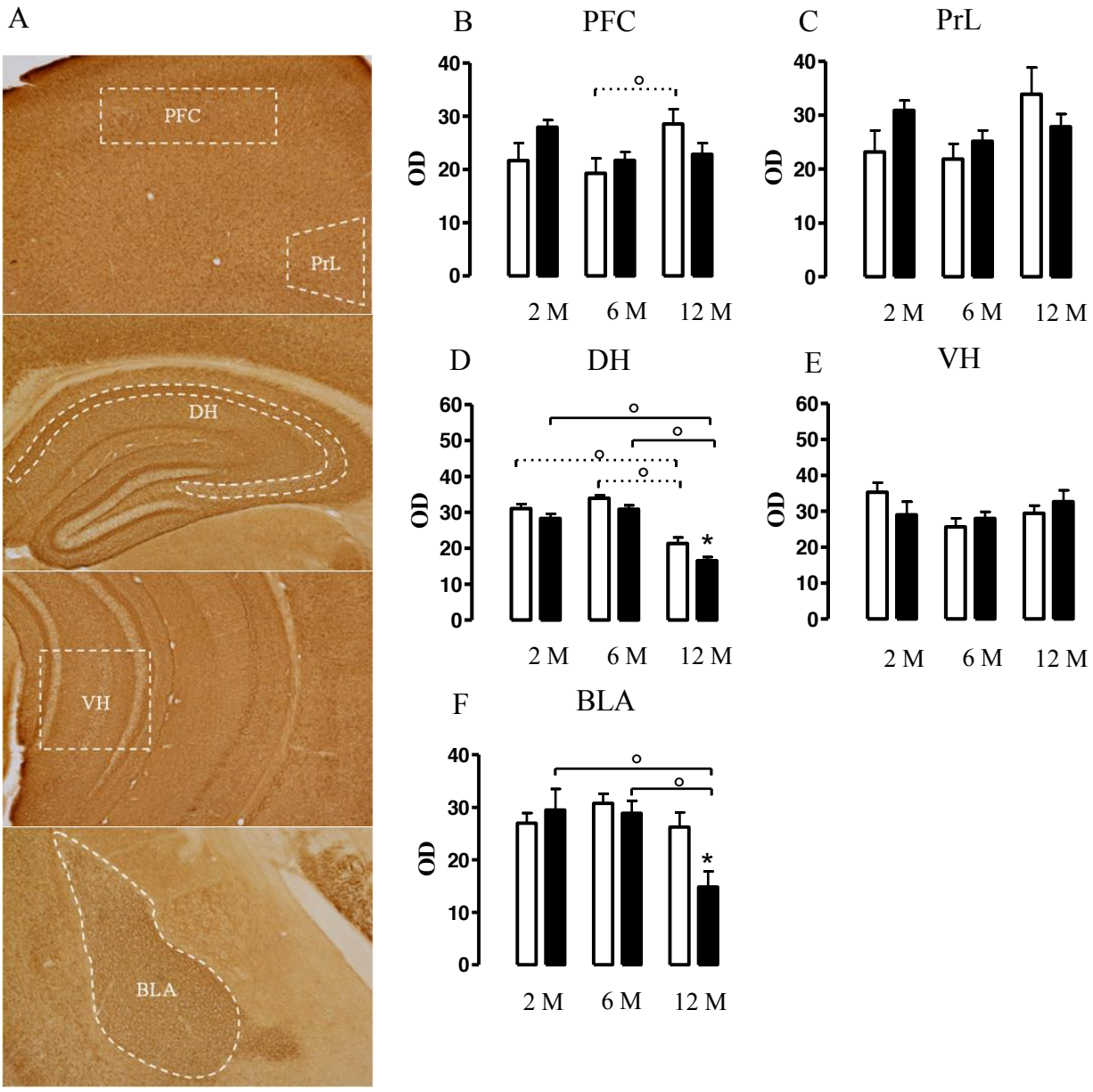
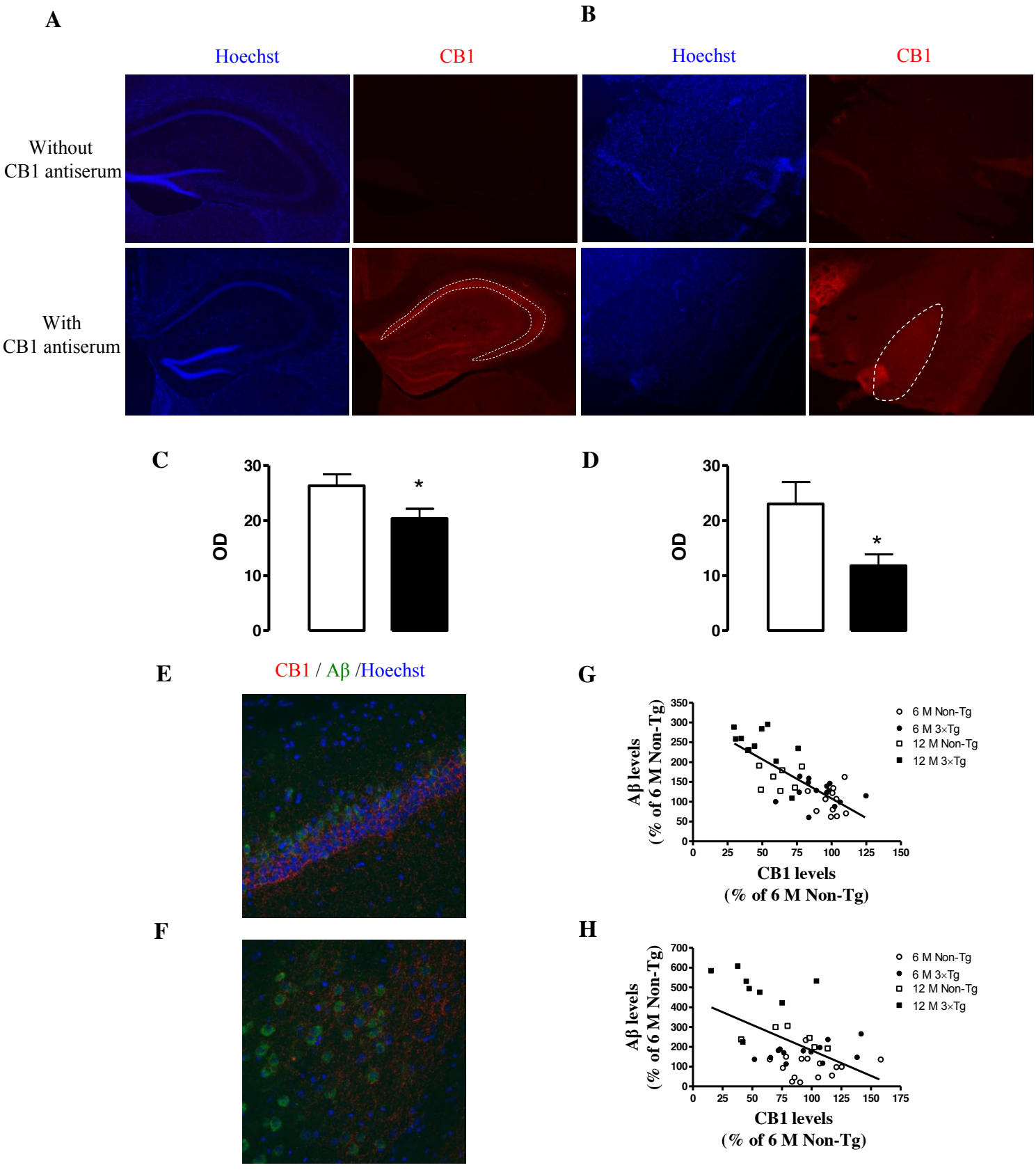


Figure 3


A
B
Hoechst
CB1
Hoechst
CB1
Without CB1 antiserum
With CB1 antiserum
C
OD
30
20
10
0
*
D
OD
30
20
10
0
*
E
CB1 / Aβ /Hoechst
F
G
Aβ levels (% of 6 M Non-Tg)
CB1 levels (% of 6 M Non-Tg)
6 M Non-Tg
6 M 3×Tg
12 M Non-Tg
12 M 3×Tg
H
Aβ levels (% of 6 M Non-Tg)
CB1 levels (% of 6 M Non-Tg)
6 M Non-Tg
6 M 3×Tg
12 M Non-Tg
12 M 3×Tg