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Analysis of calretinin early expression in the rat hippocampus after beta amyloid (1–42) peptide injection

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ABSTRACT

It has already been reported that cannabinoids are neuroprotective agents against excitotoxicity in vitro and increase after acute brain damage in vivo. This background prompted us to study the localization and expression of the calcium -binding protein calretinin in a condition similar to Alzheimer disease and its possible relationship with cannabinoids and their supposed protective role.

We carried out quantitative analysis of the transient changes in calretinin expression shown by hybridochemistry within neuronal cell populations in the hippocampus of a beta amyloid-treated rat model of Alzheimer's disease and their correlation with endocannabinoid increase.

Calretinin expression increases throughout the first week after cortical amyloid-beta peptide injection, and then decreases towards normal levels in the rat hippocampus during the following weeks, indicating that decreased calretinin gene expression may be associated with either increase of endocannabinoids or VDM11-induced accumulation of endocannabinoids. In contrast, SR1, an antagonist, which limits the cannabinoid effect by selective binding to the cannabinoid receptor CB1, up-regulates calretinin expression with respect to non-treated rats.

This could mean that the SR1 endocannabinoid-blocking action through CB1 receptors, that are normally stimulated by endocannabinoids to inhibit calcium increase,

Abbreviations: (AD), Alzheimer's disease; (SP), senile plaques; (NFT), neurofibrillary tangles; (CBPs), calcium-binding proteins; (CR), calretinin; (EC), endogenous cannabinoids; (ECS), endocannabinoid system; (CB), cannabinoid; (CA1), cornus ammnonis 1; (VDM11), N-arachidonoyl-(2-methyl-4-hydroxyphenyl) amine; (SR1), SR141716A [N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide]; (FFPE), formalin fixed paraffin embedded

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might cause a higher calretinin expression. This would allow us to speculate on a possible reverse relationship between endocannabinoid and calretinin levels in the hippocampal calcium-homeostasis balance.

1. Introduction

The histo-pathological features of Alzheimer's disease (AD) are characterized by the presence of two types of abnormal deposits: "senile plaques" (SP) and "neurofibrillary tangles" (NFT). SPs are extra-cellular lesions which have been shown to contain amyloid fibrils by electron microscopic studies (Selkoe, 1991, 2001). Accumulation of beta amyloid peptide (βA) is considered as a key event in the pathogenesis of AD (Hardy and Allsop, 1991; Lee et al., 2004; Esteban, 2004; Parihar and Hemnani, 2004) and induces cell death in different cerebral regions, including the hippocampus, a region that is heavily affected in AD and critically involved in learning and memory (Braak and Braak, 1998; Thal et al., 2002; Burgess et al., 2002). These abnormal deposits of βA cause alterations of different molecular and cellular mechanisms, one of these being the alteration of calcium homeostasis that may be central to the pathogenesis of AD (LaFerla, 2002; Mattson and Chan, 2003; Smith et al., 2005; Stutzmann, 2005; Wenk, 2006). The toxicity of βA seems to cause an increase of Ca^{2+} in response to environmental stimuli such as excitatory amino acids and membrane depolarization that elevate intracellular calcium levels (Mattson et al., 1992, 1993). Though Ca^{2+} is important, prolonged high concentrations of Ca^{2+} can be toxic to neurons; in particular, they can trigger apoptotic cell death (Blaustein, 1988). Thus, βA peptide may also render neurons vulnerable to different kinds of intra-cellular insults by disrupting normal Ca^{2+} homeostasis. It has been suggested that impaired regulation of Ca^{2+} by calcium-binding proteins (CBPs) contributes to neurodegenerative processes (Heizmann and Braun, 1992; Schäfer and Heizmann, 1996). Changes in intracellular Ca^{2+} are modulated by CBPs, such as calretinin (CR), calbindin and parvalbumin that act like Ca^{2+} buffers (Baimbridge et al., 1992). These proteins are likely to have a neuroprotective role. CR belongs to a family of low molecular weight CBPs. Their suggested functions include a role in neuroprotection against excitotoxicity or a calcium-buffering function in the cells expressing this protein in the brain. Studies performed in vitro have demonstrated that calretinin-immunoreactive neurons are resistant to βA toxicity (Pike and Cotman, 1995). CR-positive neurons appeared normal in the hippocampus but had a reduced dendritic tree in the entorhinal cortex. Dystrophic CR-immunoreactive fibers were often observed in the outer molecular layer of the gyrus dentatus and in the CA4 sector in Alzheimer's disease. Most neurons containing NFT were not CR- immunoreactive and most senile plaques were not associated with CR-positive fibers. These results show that entorhinal CR-positive neurons are affected in Alzheimer's disease in spite of an absence of systematic association with neurofibrillary tangles and senile plaques (Brion and Résibois, 1994). In the prefrontal cortex as well as in the inferior temporal cortex, no difference was observed in the density of calretinin-immunoreactive neurons in Alzheimer's disease brains as compared to control cases. Moreover, the cellular morphology of these neurons was well preserved in the Alzheimer's disease cases. These data suggest that CR-immunoreactive neurons, like other calcium-binding protein-containing interneurons, are resistant to degeneration in Alzheimer's disease(Hof et al., 1993).

A great deal of evidence demonstrates that the brain possesses various endogenous protection factors against neurodegenerative and neurotoxic insults. A link between endogenous cannabinoids (EC) and neurodegenerative diseases has been repeatedly suggested (Mechoulam et al., 2002; Mechoulam, 2002). Indeed, the endocannabinoid system (ECS), including cannabinoid (CB) receptors and their endogenous ligands, has been reported to exert neuroprotective effects in several models of neuronal injury (Iuvone et al., 2007; Marsicano et al., 2002; Milton, 2002; van der Stelt and Di Marzo, 2005). The identification of two different types of CB receptors, CB1 and CB2, clarified, at least in part, the molecular mechanisms that mediate many of the well known effects of natural CB, including delta-9-tetrahydrocannabinol. The type 1 cannabinoid receptor, CB1, is one of the most abundant G-coupled receptors in the brain. It is especially abundant in the basal ganglia, hippocampus, cerebellum, and cortex; whereas the CB2 receptor is restricted to cells of the immune system. This pattern of distribution matches well with the known effects of CB on motor and cognitive functions (Pertwee, 1997). The evidence showing that exogenous anandamide affords neuroprotection in models of excitotoxicity (van der Stelt et al., 2001), as well as the observation that mice with defective CB1 receptor genes are more vulnerable to neuronal damage (Parmentier-Batteur et al., 2002), have further reinforced the hypothesis that the ECS may represent a preservation system for the brain during neurotoxicity (Irvin and Greenberg, 2000). Experimental data suggest that ECs are involved in the neurotoxicity induced by βA in models of AD. Some of us have shown in fact that EC levels increase after βA injection (van der Stelt et al., 2006).

It has been suggested that destabilization of calcium is central to the pathogenesis of AD. 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 AD. There are several other demonstrations of the involvement of CBPs in AD, too many to be referred to here. For example, the hippocampal loss of calcium-binding protein-positive neurons in AD has been reported (Baglietto-Vargas et al., 2010; Stepanichev et al., 2006; Takahashi et al., 2010). However, the relationship of CBPs and cannabinoids has not

been examined. Therefore, in the present study we investigated whether the destabilization of the early expression of CR in the rat hippocampus via a βA-triggered increase in cannabinoid levels could help to identify possible mechanisms underlying the disturbances of hippocampal function observed in this disease.

2. Results

We chose the hippocampus as the CNS area in which to examine the effects of βA, because this area is affected in AD, is a very homogeneous brain area that allows quantification of βA-induced cellular changes, and has well defined neuronal subpopulations that have already been quantified by immunocytochemistry. SP were not identified in our model of AD as revealed by the study of Congo red examination of coronal, serial sections of the hippocampus.

2.1. *ISH. normal and sham-operated rats*

First, we examined the localization of calretinin mRNA within the normal rat hippocampus, which apparently has not been examined before by ISH, although brain calretinin has been fully characterized by the immunocytochemical methods. CR is a calcium-binding protein found in aspiny interneurons throughout the hippocampus, particularly in the stratum pyramidale and radiatum of the cornus ammnonis 1 (CA1) area as well as in spiny interneurons of the stratum lucidum of area CA3. (Jacobowitz and Winsky, 1991; Blaustein, 1988).

In Fig. 1, calretinin ISH of a normal rat hippocampus section is shown: normal rats express low detectable levels of CR in the CA1–CA3 and DG regions of the hippocampus (Fig. 1A). Although the resolution of the radiogram images was not excellent for morphological evaluation, the labeling could be seen to be restricted to the regions containing neuronal cell bodies and could not apparently be found in the neuropil between the multiple subfields'.

Sham-operated rats also expressed CR (Fig. 1B) as seen in rat brain in baseline conditions (Fig. 1A).

The sense probe gave no signal nor any general background.

2.2. *ISH. βA injected rats*

CR expression was clearly seen throughout the hippocampus of the rat brain after injection of 3 μL βA 1–42 fragment, although the amyloid was injected into the deep frontal cortex. Shortly afterwards, positive neurons were detectable in all the subfields of the CA1–CA3 and DG regions reaching the highest level after 5 days from injection (Fig. 1C). The optical density of CR mRNA-labeled hippocampus neurons declined during the second week after treatment and continued to decrease gradually in the third week until it reached the normal mRNA expression levels (Figs. 2 and 3). Keeping in mind that injections were performed deep into the frontal cortex close to hippocampus, no significant difference in the optical density of CR expression was found between the left and right halves of the examined hippocampi, with the exception of CA2, CA3 and DG at 5 days-treatment, where

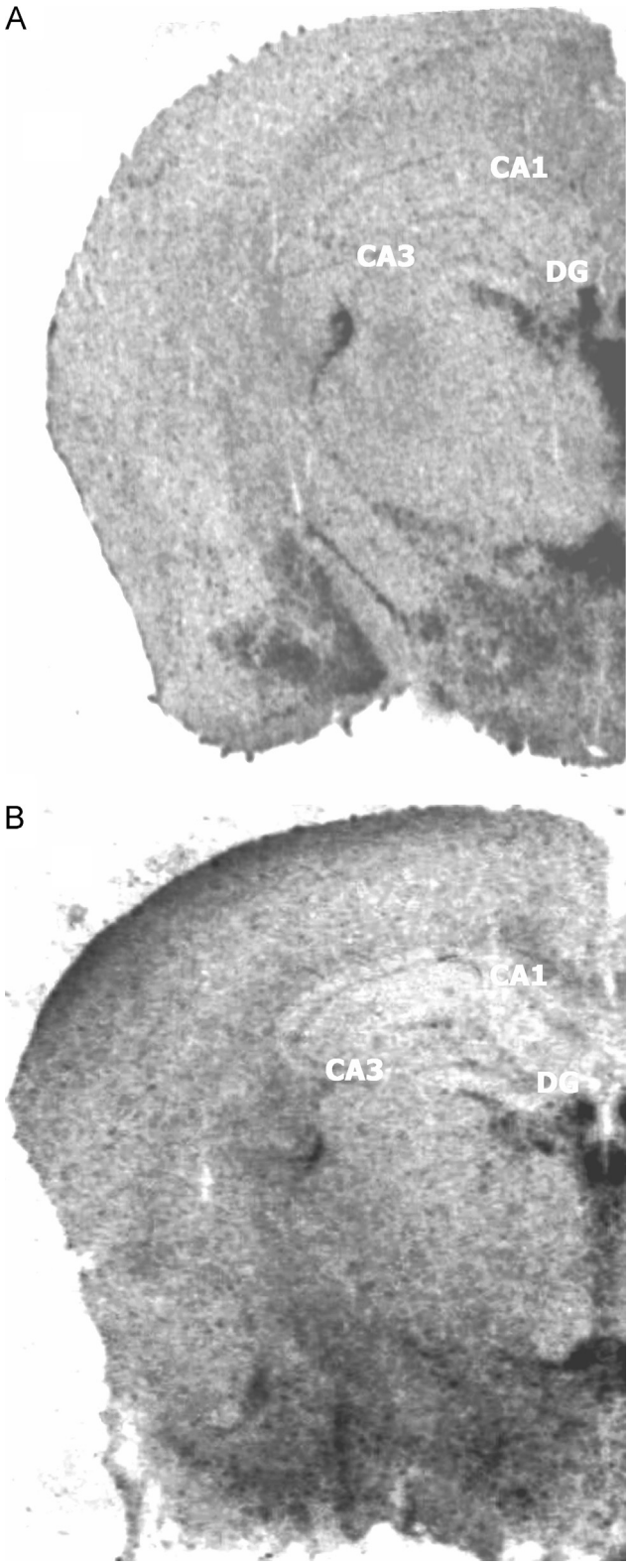

Fig. 1 – X-ray films showing labeling of CR-expressing neurons in the left hippocampus. Normal (A) and sham operated (B) rat brains show a very moderate labeling of CR neurons.

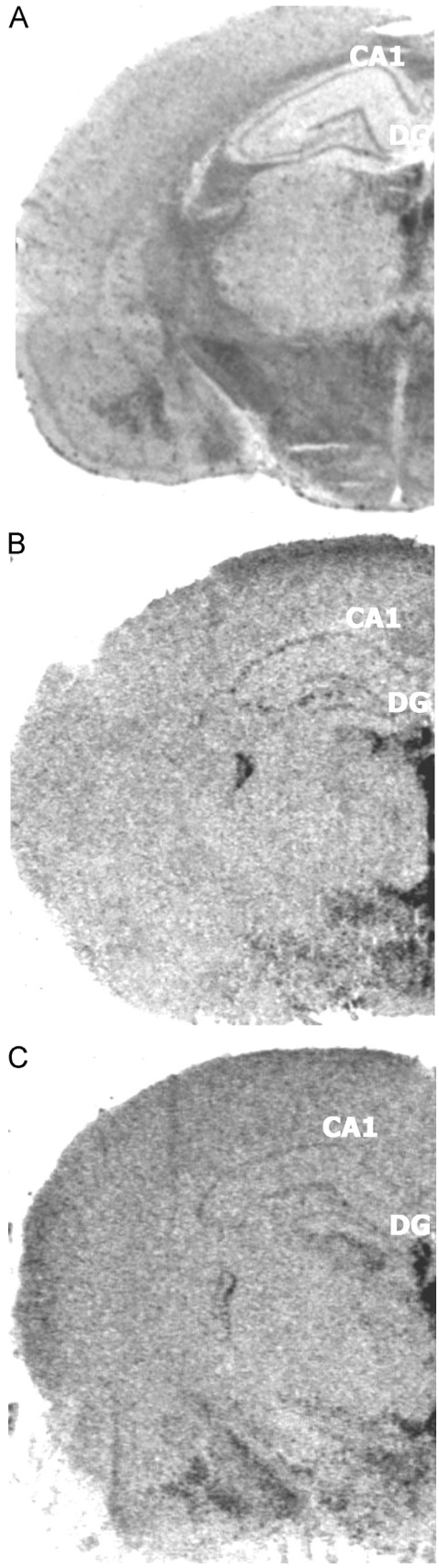

Fig. 2 – After βA treatment, CR expression peaked at 5 days (A) and gradually decreased at 12 (B) and 20 days (C).

differences from the average values were balanced by their higher standard error (Fig. 4). Therefore, the optical density measurements were performed on one half only of the hippocampus.

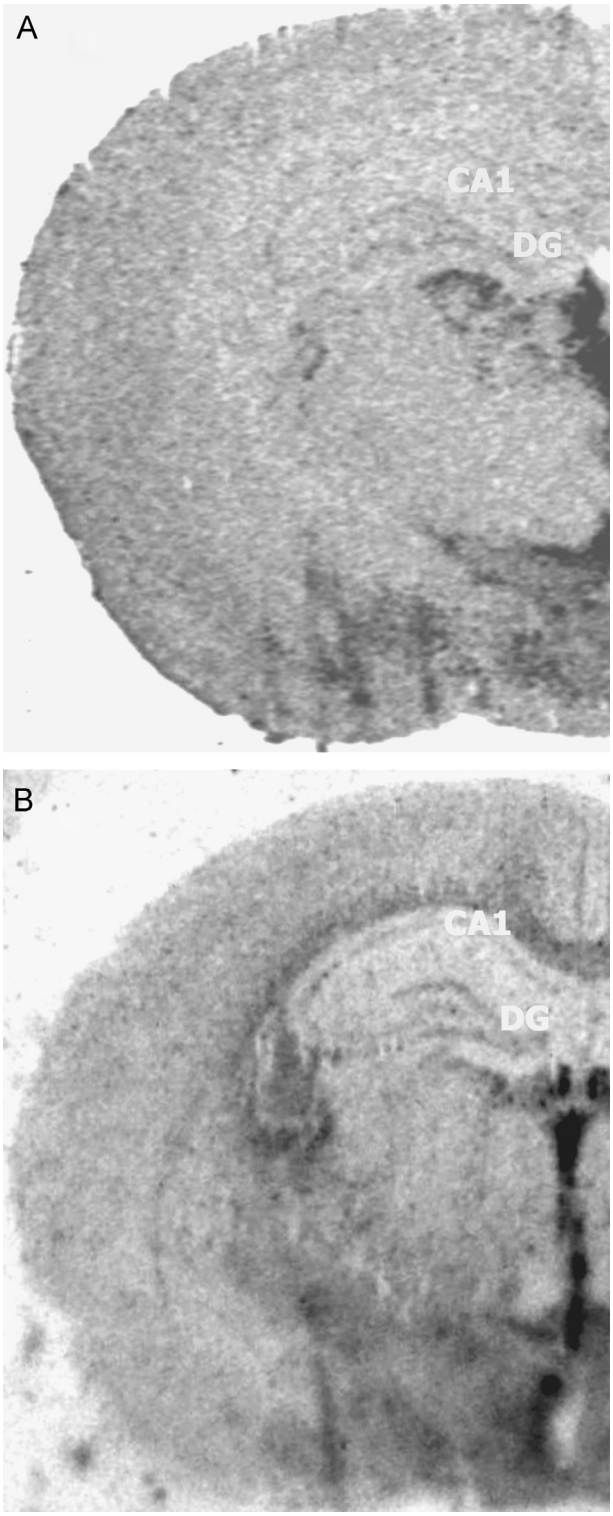

Fig. 3 – βA+VDM11 treated animals (A) showed labeling very similar to brain in normal conditions, while βA+SR1 up-regulated CR expression (B).

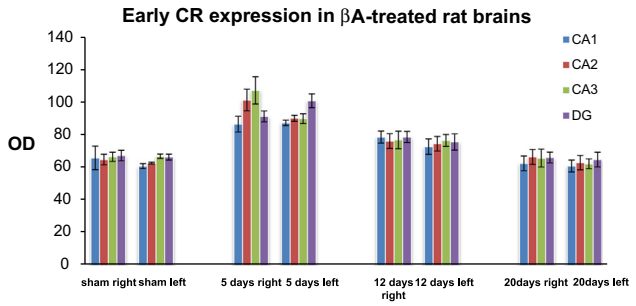

Fig. 4 – O.D. measurement values corresponding to hippocampal CR expression of sham-operated and βA-treated rats. Comparison between left and right halves. It is shown that values of right and left O.D.s are only slightly different in either sham-operated and βA-treated brains, except in CA1 and CA2 subfield at 5 and 12 days βA-treatment: this prompted us to measure the O.D. of one hippocampal half only in the following experiments. $p<0.05$ in 5 days vs sham, and 20 days vs 5 days.

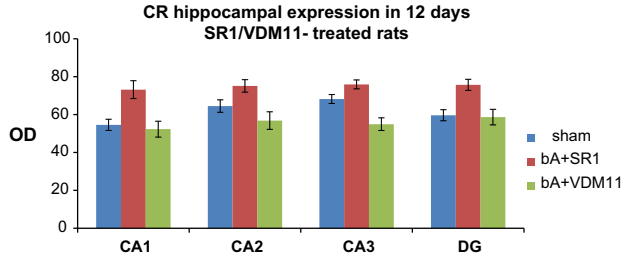

Fig. 5 – CR hippocampal expression of animals injected peritoneally with βA+SR1 and βA+VDM11. This graph shows evidence that βA+VDM11 treatment is associated with a down-regulated CR hippocampal expression, while βA+SR1 up-regulates it. $p<0.05$ in βA+SR1 vs sham and vs βA+VDM11.

Congo Red preparations did not show evidence of SP or NFT.

2.3. *ISH. βA/VDM11/SR1 treatment*

Following the treatment with 3 μL of 1–42 β-Amyloid, the endocannabinoid re-uptake inhibitor, VDM1 (N-arachidonoyl-(2-methyl-4-hydroxyphenyl) amine), was injected intraperitoneally (5 mg/kg) every day, until the 7th day, and the animals were sacrificed at the 12th day. No morphological alterations were observed after treatment with either VDM11 or SR1 (SR141716A [N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide]). CR hippocampal expression was significantly increased in SR1-treated rats (p Values were <0.05) (Figs. 3B and 5), with a very similar optical density value throughout the hippocampus except in the CA1 where it was slightly lower. In contrast, after intraperitoneal injection with VDM11, the optical density in the hippocampus was on average lower than the middle value in sham-operated animals (p Values were <0.05) (Figs. 3A and 5) with slight differences between the different areas.

3. Discussion

In order to perform this study, we tried to get a reliable AD-like condition. Several investigators have administered βA directly by intracranial injection. We needed at the beginning to establish whether the intracranial injection per se could have caused a difference between the left and right halves of the hippocampus. According to the Tukey post-hoc statistical results, there was no significant difference between left and right hippocampal CR expression during the two weeks following βA treatment, with the exception of the right CA2 and CA3 hippocampus, and left DG that apparently expressed a higher amount of CR compared with the contralateral subfield at the fifth day of treatment. However, statistical analysis showed that the higher standard error of the right CA2 and CA3, and left DG with respect to the average standard error made this difference meaningless and we therefore decided to measure the optical density of only one half of the hippocampus in subsequent experiments.

The model we used mimics many features of AD, including robust neuroinflammation, synaptic damage and neuronal loss in the hippocampus as has also been shown in our institution. The loss of CR neurons that has been recently reported (Baglietto-Vargas et al., 2010; Stepanichev et al., 2006; Takahashi et al., 2010) refers to a different experimental condition, where PS1/AbetaPP transgenic mice were used and the observations were carried out on 2–12 month old mice. However, even if the differences could be species-specific, we agree with these authors that CR-containing hippocampal neurons are early targets of βA pathology, induced in our study by central administration.

In addition, a recent discovery (Stepanichev et al., 2006) is the possible link between amnesia and neurodegenerative changes in the rat hippocampus induced by single intracerebroventricular injections of βA 25–35 at a dose of 15 nmoles. In those experiments, training was followed by histological assessment of the state of the hippocampus on brain sections stained with haematoxylin and eosin. Parallel to βA (25–35)-induced impairments in long-term and working memory, there was a moderate reduction in the number of neurons in hippocampal field CA1; there was no change in the number of cells in the field CA3. Thus, this was the first demonstration that impairments of learning and memory induced by single doses of βA are specifically associated with neurodegenerative changes in rat hippocampal field CA1. In our experience tissue alterations such as NFT and SP were not shown even with specific staining, while sparse, occasional swelling neurons were observed in haematoxylin eosin preparations (not shown). The effectiveness of βA frontal cortex injection was demonstrated by the neurotoxicity-induced hippocampal CR upregulation itself, although we do not know, at present, whether amyloid causes frontal cortex neuronal dysregulation that will then have downstream effects on either cannabinoid release in the hippocampus and then on the CR system, or viceversa. Furthermore, the up-regulated CR expression in CA1 with more in CA2, CA3 and DG, reached its highest peak during the first week after the βA injection. The constant difference of CR expression between CA1 and the other subfields is notable. In fact, CR is

highly expressed in CA1 with respect to normal brain of βA- and SR1-treated rats, but less than in CA2, CA3 and DG. Therefore, assuming that CR-containing neurons are less vulnerable to calcium destabilization following βA (Hof et al., 1993), it seems that both CA1 and CA3 are better preserved because of their higher CR expression with respect to normal conditions, even though other authors have clearly shown that impairments of learning and memory are associated with specific neurodegenerative changes.

The other question we tried to answer was whether there were links between endocannabinoids and a calcium-binding protein. Some of us (van der Stelt et al., 2006) have previously investigated the involvement of endocannabinoids in the control of neuronal damage and memory loss in rodents treated with the βA peptide (1–42). Briefly, twelve days after stereotaxic injection of βA peptide into the rat cortex, and concomitant with the appearance in the hippocampus of neuronal damage markers, the level of 2-arachidonoyl glycerol, but not anandamide, was enhanced in the hippocampus. VDM-11 (5 mg/kg, i.p.), an inhibitor of endocannabinoid cellular re-uptake, significantly enhanced rat hippocampal and mouse brain endocannabinoid levels when administered sub-chronically from 3 or 7 days after βA peptide injection up to the 12–14th day. VDM-11 concomitantly reversed hippocampal damage in rats and loss of memory retention in the passive avoidance test in mice, but only when administered from the 3rd day after βA peptide injection. Thus, it is suggested that early, as opposed to late, CR enhancement, in parallel with the increase of endocannabinoid levels, might protect against βA neurotoxicity and its consequences.

In the current study, in order to show any possible relationship between endocannabinoids and CR, we needed to increase endocannabinoid levels, but did not need to check the 2-AG level enhancement as this had previously been well assessed by van der Stelt et al. (2006). 0ur data show that endocannabinoids in vivo may affect CR levels, but no other papers have studied this yet. Therefore in vitro studies could help assessing whether CR expression can be increased by limiting reuptake of endocannabinoids.

The question of whether calcium-binding proteins can be responsible for molecular alterations, that are reported in some way to reflect AD-related cognitive impairments, was also answered. One of the components involved in the regulation of calcium homeostasis is given by calcium binding proteins that exert their function by buffering the intracellular calcium concentration (Blaustein, 1988). Reduction of the expression of these proteins could lead to neuronal death due to a 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 probably have a neuroprotective role (Hof et al., 1991, 1993). There are only a few studies that have examined changes in calcium- binding proteins in the dentate gyrus of AD patients and there is also a relative lack of data relating to the distribution and expression of calcium binding proteins in the DG of βA-induced AD-like conditions. Our data show that the CR level in the whole hippocampus increases very soon after βA administration, and that its distribution within the hippocampal layers and sub regions expands in the first week and then gradually declines during the following two weeks. Now, as far as we know, alterations of the ECS in obesity can alter CR hippocampal levels (Massa et al., 2010); furthermore, an age-dependent hippocampal CR decrease corresponds to a change in the endocannabinoid system (Lee et al., 2010). Therefore, our hypothesis, consistent with the data of the cited literature, is that CR and the ECS may be related.

In conclusion, the aim of the present study was to investigate the gene expression of a calcium -binding protein, calretinin, throughout the normal hippocampus, and the early calretinin expression in βA-treated hippocampus, keeping well in mind that not all hippocampal neurons contain CR (Miettinen et al., 1992). It has been reported that other calcium-binding protein, such as parvalbumin, S-100 and calbindin may have a role in AD, and our data reflect that of the literature. The early in vivo effect of βA-induced neurotoxic and inflammatory activity on the etiopathogenesis and development of AD has been evaluated and, in such a context, experiments were carried out to evaluate the role of the cannabinoid system by inhibiting its receptors and to analyze possible relations between ECs and calretinin.

4. Experimental procedure

Adult male Wistar rats (300–350 g), aged from 15 to 18 weeks, were used after at least 1 week of habituation in the facilities. All experiments were carried out according to the European Community Council Directive 86/609/EEC and efforts were made to minimize animal suffering and to reduce the number of animals used. Three to five animals were used for each data point and for each type of experiment (i.e. histochemical analysis, routine staining) (Table 1).

4.1. *Drug administration to rats*

A βA 1–42 fragment was prepared as a stock solution in sterile 0.1 M phosphate-buffered saline (PBS; pH 7.4), aliquoted, frozen in liquid nitrogen and stored at −90 °C until used. 3 μl of newly thawed βA 1–42 fragment solution (10 ng/μl) were used for each injection. Sterile PBS was injected into control animals. Rats were anesthetized with sodium pentobarbital (60 mg/kg) which was supplemented throughout the surgery as required. They were placed in a stereotaxic frame and βA 1–42 fragment or PBS was injected into the right, deep frontal cortex or hippocampus according to literature (Kowall et al., 1991; Stéphan et al., 2001). Injections were made over 1 min using a 10-μl Hamilton syringe fitted with a 30-gauge blunt-tipped needle, and the needle was left in place for an additional 2 min. The animals were then sacrificed at the 5th, 12th and 20th day from injection in accordance with results from preliminary experiments. Similarly treated animals were used in the experiments with the endocannabinoid reuptake inhibitor, VDM11 (De Petrocellis et al., 2000). The compound was dissolved in DMSO/H_2O (95:100 volume ratio, 5 mg/0.1 ml) and injected intraperitoneally (5 mg/kg) every day, starting from the 3rd or the 7th day from the operation until the 12th day when animals were sacrificed. It turned out that CR, in parallel with endocannabinoids, raises soon after the βA injection. Further studies could verify whether CR can

Table 1 – Synoptic representation of treatments, time points and histological procedures.

Time point	Administered drug			Fixation procedure for ISH	Routine staining
	Intracranial injection	Intraperitoneal injection			
Day 5th	β-amyloid	–	–	freezing	
Day 12th	β-amyloid	β-amyloid+SR1	β-amyloid+BDM11	freezing	FFPE
Day 20th	β-amyloid	–	–	freezing	
Normal	–	–	–	freezing	FFPE
Sham				freezing	FFPE

Provided that three to five brains were used for each time point/treatment, the different drugs administered and the histological procedures are synoptically illustrated.
FFPE (formalin fixed paraffin embedded).

be considered a sentinel molecule of a neurodegenerative disease. When experiments with the CB1 receptor antagonist SR1 were carried out, the DMSO-dissolved compound was also injected intraperitoneally as for VDM11. We knew from previous experiments that there was no point in continuing evaluations after the 12th day (unpublished observations).

4.2. *Histological study*

Rats ($n=5$ for each experimental group) were randomly assigned to a treatment group with βA peptide fragment (1–42). On the 5th and 12th day rats were sacrificed by decapitation and the whole brain was rapidly removed, quickly frozen on powdered dry ice, and stored at −80 °C.

Serial coronal sections of 12 μm were cut on a cryostat at −18 °C at the level of the hippocampus, using the Rat Brain Atlas of Paxinos and Watson (2007) as an anatomical reference. Unfixed frozen, serial, coronal sections were cut from the region of interest and stored at −80 °C until subsequent analysis. Three serial sections/slide for each animal were processed for hybridocytochemistry.

A number of whole brains, for each different treatment and time-point, were also fixed by perfusion with 10% buffered formalin–paraffin embedding for routine staining (cresyl violet, haematoxylin eosin and Congo red).

4.3. *In situ hybridization (ISH)*

For ISH, the calretinin probe was a 45-base oligodeoxyribonucleotide complementary to bases 4835–4879 of the mouse mRNA (GenBank Accession no. AB037964). The probe was labeled at 3′ using [α35S] dATP, terminal deoxynucleotidyl transferase (15 units/ml), and tailing buffer. The unincorporated nucleotides were separated from radiolabeled DNA using Sephadex G-50 chromatographic columns. A labeled sense probe was used as a negative control.

The procedure for in situ hybridization was taken from several standard published protocols (Arai et al., 1994; Cimini et al., 1997; Cicale et al., 2002; Isaacs et al., 1995; Winsky et al., 1989). Briefly, 10–12 μm-thick sections were fixed in 1.5% formaldehyde in 0.12 M sodium phosphate buffer (pH 7.2–7.4), quickly rinsed three times with 1X PBS, and placed in 0.25% acetic anhydride in 0.1 M triethanolamine/0.9% NaCl, pH 8.0, for 10 min. Next, the sections were dehydrated in 70%, 80%, 90% and 100% ethanol, delipidated in chloroform for 5 min, then rinsed again in 100% and 95% ethanol and air-dried. Sections were hybridized with 0.4–0.6 × 106 cpm of radiolabeled oligonucleotide in buffer containing 50% formamide, 600 mM NaCl, 80 mM Tris–HCl (pH 7.5), 4 mM EDTA, 0.1% pyrophosphate, 0.2 mg/ml heparin sulfate, and 10% dextran sulfate. Incubations were carried out at 37 °C in a humid chamber for 20 h. After hybridization the sections were processed through four 15 min washes in 2X SSC/50% formamide at 40 °C, followed by two 30 min washes with 1X SSC at 40 °C. The slides were rapidly rinsed in distilled water and then in 70% ethanol. The sections were dried and exposed to Biomax MR X-ray film (Kodak Scientific Imaging Film, USA). The time of exposure was chosen to maximize the signal to noise ratio and also to avoid optical density approaching the limits of saturation. The evaluation of in situ hybridization experiments was done on radiograms obtained by inverting the scanned x-ray film so that the white areas corresponded to labeled structures.

4.4. *Statistical analysis*

Data were expressed as the mean ± the standard error of the mean (S.E.M.). One-way ANOVA was used to analyze treatment effects. In all analyses, a p Value of <0.05 was considered statistically significant. Tukey's post-hoc test was used to determine the locus of effects for significant ANOVA results.

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