ACTIVITY OF MUSCARINIC, GALANIN AND CANNABINOID RECEPTORS IN THE PRODROMAL AND ADVANCED STAGES IN THE TRIPLE TRANSGENIC MICE MODEL OF ALZHEIMER'S DISEASE

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Abstract—Neurochemical alterations in Alzheimer's disease (AD) include cholinergic neuronal loss in the nucleus basalis of Meynert (nbM) and a decrease in densities of the M_2 muscarinic receptor subtype in areas related to learning and memory. Neuromodulators present in the cholinergic pathways, such as neuropeptides and neurolipids, control these cognitive processes and have become targets of research in order to understand and treat the pathophysiological and clinical stages of the disease. This is the case of the endocannabinoid and galaninergic systems, which have been found to be up-regulated in AD, and could therefore have a neuroprotective role. In the present study, the functional coupling of $G_{i/o}$ protein-coupled receptors to $GalR_1$, and the CB_1 receptor subtype for endocannabinoids were analyzed in the 3xTg-AD mice model of AD. In addition, the activity mediated by $G_{i/o}$ protein-coupled $M_{2/4}$ muscarinic receptor subtypes was also analyzed in brain areas involved in anxiety and cognition. Thus, male mice were studied at 4 and 15 months of age (prodromal and advanced stages, respectively) and compared to age-matched non-transgenic (NTg) mice (adult and old, respectively). In 4-month-old 3xTg-AD mice, the $[^{35}S]GTP\gamma S$ binding stimulated by galanin was significantly increased in the hypothalamus, but a decrease of functional $M_{2/4}$ receptors was observed in the posterior amygdala. The CB_1 cannabinoid receptor activity was up-regulated in the anterior thalamus at that age. In 15-month-old 3xTg-AD mice, muscarinic receptor activity was found to be increased in motor cortex, while CB_1 activity was decreased in nbM. No changes were found in $GalR_1$-mediated activity at this age. Our results provide further evidence of the relevance of limbic areas in the prodromal stage of AD, the profile of which is characterized by anxiety. The up-regulation of galaninergic and endocannabinoid systems support the hypothesis of their neuroprotective roles, and these are established prior to the onset of clear clinical cognitive symptoms of the disease. © 2016 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: cholinergic, neuropeptides, neurolipids, Alzheimer, G protein, autoradiography.

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Abbreviations: βA, β-amyloid; AD, Alzheimer's disease; EGTA, ethylene glycol tetraacetic acid; NTg, non-transgenic.

INTRODUCTION

Alterations in cholinergic neurotransmission seem to be one of the most characteristic hallmarks of Alzheimer's disease (AD), as was firstly described, due to the impairment of cholinergic neurons in the basal nucleus of Meynert (nbM) (Whitehouse et al., 1982). Moreover, a decrease in the density of M_2 muscarinic receptors has been described in areas related to memory and learning, such as the hippocampus and entorhinal cortex (Rodríguez-Puertas et al., 1997a). However, neurochemical alterations also include the impairment of noradrenergic (Marcyniuk et al., 1986) and serotonergic (Palmer et al., 1987) systems. Other neuromodulators, such as neuropeptides and neurolipids, present in cholinergic pathways, have become targets of research in order to understand, prevent or treat the disease. This is the case of the galaninergic and endocannabinoid systems, which have been found to be up-regulated in AD and could have a neuroprotective role (Manuel et al., 2014; Rodríguez-Puertas et al., 1997b). In fact, it has been described that cholinergic cells of nbM are hyperinnervated by galanin (Chan-Palay, 1988). Besides, an increase of ^{125}I-galanin binding was observed in AD brains in the hippocampus and entorhinal cortex, the same areas in which M_2 receptor density was reduced (Rodríguez-Puertas et al., 1997b). In nbM and amygdala an increase in galanin ^{125}I-binding has also been observed (Mufson et al., 2000; Pérez et al., 2002). In the case of cannabinoid neurotransmission, there is a reduction in CB_1 receptors in advanced Braak stages of the disease in different layers of the hippocampus (Westlake et al., 1994). Furthermore, there is an increase in both the activity and density of CB_1 receptors in early and moderate stages (Manuel et al., 2014). Nevertheless, cannabinoids are involved in neuroprotective functions against excitotoxic damage (Marsicano et al., 2003). In the same way, cannabinoids

are also involved in the suppression of neuroinflammatory processes in AD (Ehrhart et al., 2005).

Interestingly, pathophysiological processes underlying AD, seem to occur almost a decade prior to obvious clinical symptoms. The study of this long preclinical phase is now considered to be of crucial importance, together with classical research focused on the study of more advanced stages of the disease (Sperling et al., 2013). The identification of early pathophysiological alterations is necessary in order to lead to an effective therapy prior to the onset of the first clinical symptoms of AD. In the prodromal stage of the disease, not only biological markers specific for AD neuropathology and oxidative stress, but also non-specific markers of neuronal degeneration and inflammation are explored as targets for early drug therapies for AD (Bailey, 2007). As clearly stated by Welsh-Bohmer (2008), in the very early stages, the neuropsychological symptoms may not be apparent at all (latent phase), but as the pathology worsens, the first symptoms emerge (prodromal stage) followed by a full manifestation of the clinical disease (dementia stage). This growing concern for the initial stages of AD has lead to basic research on the validity of animal models to reproduce the wide spectrum of alterations found in AD patients, ranging from the pathological processes to the cognitive deficits and alterations in behavior (Giménez-Llort et al., 2007). Due to the genetic background of transgenic mice models of familial AD mutations, they may prove to be a useful tool for the study of the pathophysiological processes involved in AD, from the asymptomatic stages to the prodromal and the advanced stages of the disease. This is the case of the triple transgenic mice model of AD (3xTg-AD) harboring βAPP_{Swe}, $PS1_{M146V}$ and tau_{P301L} human transgenes, which mimics the development of the main histopathological features of the disease (Oddo et al., 2003). These mice progressively develop the neuropathological markers of AD in a temporal and regional-specific profile similar to that found in the brain of AD patients. At 4 months of age, 3xTg-AD mice develop intraneuronal β-amyloid (βA) deposits and this age could be compared to the prodromal stages of the disease. Indeed, we have consistently reported the presence of an anxious-like profile, together with some of the first cognitive deficits (Billings et al., 2005; Giménez-Llort et al., 2006, 2007; Cañete et al., 2015). The presence of intracellular βA is initially detected in the hippocampal CA_1 region, but at 6 months of age, it is also visible in the cortex (Billings et al., 2005) and in the basolateral amygdala (España et al., 2010), and is clearly related to cognitive deficits and behavioral alterations. At 12 months of age the appearance of extraneuronal βA is described and is followed, at 15 months of age, by concomitant hyperphosphorylated tau protein in the hippocampus (Oddo et al., 2003). At the neurochemical level, the basal forebrain cholinergic system starts to be affected in 3xTg-AD mice, early in the intracellular βA stage (Pérez et al., 2011). Histopathological alterations in the primary motor cortex also appear soon after 3 months of age (Mastrangelo and Bowers, 2008).

Within this context, the present work aims to describe the activity of muscarinic, galanin and cannabinoid receptors in 4- and 15-month-old 3xTg-AD mice. These ages were chosen as those which mimic the above-mentioned stages in the progress of the disease, that is, the prodromal stage and the advanced stages, respectively. Age-matched non-transgenic mice (NTg) were used as controls. Therefore, the objective of the study is to validate the neurochemical alterations in this animal model at different stages, but also to contribute to the understanding of the neuroanatomical substrates which are involved in the prodromal and advanced stages of the disease.

EXPERIMENTAL PROCEDURES

Animals

The group of Dr. Frank LaFerla (Dept. of Neurobiology and Behavior, University of California Irvine, USA) created 3xTg-AD transgenic mice as described by Oddo et al. (2003). Brain samples used in this study were from 4- and 15-month-old homozygous male 3xTg-AD mice and age-matched NTg mice ($n = 6$ in each group) of the colonies established at Universitat Autònoma de Barcelona, Spain. All animals were born and bred in the animal housing facilities in the Medical Psychology Unit and maintained under standard laboratory conditions in a 12-h light/dark cycle, $22 \pm 2\,°C$, 50–60% humidity, with food and water *ad libitum.*

[^{35}S]GTPγS binding assay

Brains were quickly removed and sectioned down the midline into the right and left hemispheres. Then tissues were frozen on dry ice, and kept at $-80\,°C$. The brains were cut in a Microm cryostat (HM 550, Thermo) to obtain 20 μm sections that were mounted onto gelatin-coated slides and these were stored at $-20\,°C$ until used.

The tissue sections were air-dried for 15 min and then immersed in a Tris–HCl buffer 50 mM (pH 7.4) with 100 mM NaCl, 3 mM $MgCl_2$ and 0.2 mM EGTA, and preincubated in the previous mentioned Tris–HCl buffer 50 mM supplemented with 2 mM GDP and 1 mM DL-dithiothreitol (DTT) and adenosine deaminase (3 mU/ml). Later, sections were incubated for 2 h at 30 °C in the same buffer containing 0.04 nM [^{35}S]GTPγS (1250 Ci/mmol; Perkin Elmer, Boston, MA, USA). The agonist-stimulated binding was measured under the same conditions in the presence of the specific GPCR agonist: carbachol (100 μM) for $M_{2/4}$ muscarinic receptors, galanin (1 μM) for $GalR_1$ receptors and WIN 55212,2 (100 μM) for CB_1 receptors The receptor subtype specificity was demonstrated by inhibiting the [^{35}S]GTPγS binding by co-incubation with atropine (10 μM), M15 (1 μM) and SR141716 (10 μM), as respective antagonists, together with carbachol, galanin and WIN 55212,2. The chosen concentrations of agonists and antagonists were similar to those used in previous studies, and the criteria was based on the potencies of the different compounds to produce a receptor-mediated effect. Therefore, the concentrations are more similar to physiological studies (EC50) than to receptor binding analysis (affinity, Kd). Note that