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GENETIC BLOCKADE OF THE DOPAMINE D_3 RECEPTOR ENHANCES HIPPOCAMPAL EXPRESSION OF PACAP AND RECEPTORS AND ALTERS THEIR CORTICAL DISTRIBUTION

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Abstract—Dopamine D_3 receptors (D_3Rs) are implicated in several aspects of cognition, but their role in aversive conditioning has only been marginally uncovered. Investigations have reported that blockade of D_3Rs enhances the acquisition of fear memories, a phenomenon tightly linked to the neuropeptide pituitary adenylate cyclase-activating peptide (PACAP). However, the impact of D_3R ablation on the PACAPergic system in regions critical for the formation of new memories remains unexplored. To address this issue, levels of PACAP and its receptors were compared in the hippocampus and cerebral cortex (CX) of mice devoid of functional D_3Rs ($D_3R^{-/-}$) and wild-types (WTs) using a series of comparative immunohistochemical and biochemical analyses. Morphometric and stereological data revealed increased hippocampal area and volume in $D_3R^{-/-}$ mice, and augmented neuronal density in CA1 and CA2/3 subfields. PACAP levels were increased in the hippocampus of $D_3R^{-/-}$ mice. Expression of PACAP receptors was also heightened in mutant mice. In the CX, PACAP immunoreactivity (IR), was restricted to cortical layer V in WTs, but was distributed throughout layers IV–VI in $D_3R^{-/-}$ mice, along with increased mRNAs, protein concentration and staining scores. Consistently, PAC1, VPAC1 and VPAC2 IRs were variably redistributed in CX, with a general upregulation in cortical layers II–IV in knockout animals. Our interpretation of these findings is that disturbed dopamine neurotransmission due to genetic D_3R blockade may enhance the PACAP/PAC1–VPAC axis, a key endogenous system for the processing of fear memories. This could explain, at least in part, the facilitated acquisition and consolidation of aversive memories in $D_3R^{-/-}$ mice. **© 2015 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: dopamine D_3 receptor, PACAP, PAC1/VPAC receptors, aversive conditioning, hippocampus, cerebral cortex.

INTRODUCTION

Dopamine (DA) is a neurotransmitter whose activities in the central nervous system (CNS) govern a myriad of behavioral responses, depending on the DA receptor engaged and the brain circuitry involved. Such DA-guided behaviors include drug craving (Pilla et al., 1999; Payer et al., 2014; Morales et al., 2015), tolerance to anxiolytic drugs (Leggio et al., 2011, 2013, 2015), nicotine sensitization (Smith et al., 2015) and alcohol preference (Leggio et al., 2014), among others. A number of reports have also supported a key contribution of the DAergic system in regulating certain aspects of memory function, like appetitive (Phillips et al., 2002) and especially aversive conditioning (Inoue et al., 2005; Micale et al., 2010; Castorina et al., 2011, 2013; D'Amico et al., 2013a,b). These activities of DA are mediated by two subtypes of G-protein-coupled receptors, classified according to their structural similarities and pharmacological properties: D_1-like (comprising D_1R and D_5R) and D_2-like (D_2R, D_3R, and D_4R) (Missale et al., 1998; Karasinska et al., 2005). Among these receptors, the dopamine D_3 receptor (D_3R) has particularly captured scientific interest in recent years. In fact, the development of more selective ligands has contributed significantly in unraveling new and interesting functions associated with the D_3R receptor. The D_3R, cloned by Sokoloff two and a half decades ago (Sokoloff et al., 1990), exhibits peculiar biochemical features that make it an attractive pharmacological target to ameliorate specific behavioral disturbances: the receptor

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Abbreviations: ANOVA, analysis of variance; BSA, bovine serum albumin; CX, cerebral cortex; $D_3R^{-/-}$, dopamine D_3 receptor knockout; D_3Rs, dopamine D_3 receptors; DA, dopamine; DG, dentate gyrus; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; ELISA, enzyme-linked immunosorbent assay; H_2O_2, hydrogen peroxide; IHC, immunohistochemistry; IR, immunoreactivity; PA, passive avoidance conditioning; PACAP, pituitary adenylate cyclase-activating peptide; PBS, phosphate-buffered saline; ROI, region of interest; tPA, tissue plasminogen activator; VIP, vasoactive intestinal peptide; WTs, wild-types.

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couples to $G\alpha_{i/0}$ proteins to inhibit adenylyl cyclase activity; it has the highest affinity for DA compared to other DA receptors, and; it is detectable at various levels in the striatum (Ikeda et al., 2013; Leggio et al., 2015). Furthermore, the receptor is also localized in extra-striatal brain structures known to be involved in the processing and generation of new memories, specifically the hippocampus and cerebral cortex (CX) (Sokoloff et al., 1992, 2006; Levant, 1998; Cho et al., 2010; Castorina et al., 2011, 2013; D'Amico et al., 2013a, 2013b) and, although with a restricted pattern, it can also be detected in lobules 9 and 10 of the cerebellum (Barik and de Beaurepaire, 2005).

Available data on mouse D_3R knockout models ($D_3R^{-/-}$) have helped to partially delineate a behavioral profile for these mice. $D_3R^{-/-}$ mice retain normal spatial memory function (Xing et al., 2010) and emotional behavior (Chourbaji et al., 2008), but do demonstrate enhanced acquisition and consolidation of aversive-related memories, as shown by the enhanced retention of foot shock-induced conditioning in avoidance tasks (Micale et al., 2010; D'Amico et al., 2013a,b). However, to date the underlying neurochemical events that may explain the facilitated acquisition of fear-associated conditioning by these knockout mice have not been elucidated. Currently, the most compelling hypothesis remains that genetic ablation of D_3Rs (which normally act as negative feedback regulators of DA release, by pre-synaptic modulation of DA outflow) probably generates a hyperDAergic environment that provokes changes in key molecular determinants of synaptic plasticity and neuronal remodeling in regions critical for the formation of the conditioned fear response, such as the previously identified tissue plasminogen activator (tPA) (Teesalu et al., 2002; Castorina et al., 2013). Unfortunately, while changes in hippocampal tPA are not sufficient to specifically address the neurochemical basis underlying the enhanced acquisition of this type of fear conditioning, as tPA activities affect multiple downstream targets, and result in a generalized enhancement of memory performance in a variety of behavioral tasks beside passive avoidance conditioning (PA) (Calabresi et al., 2000). Therefore, the gap still needs to be filled.

The neuropeptide pituitary adenylate cyclase-activating peptide (PACAP) is widely distributed in almost every brain region (Watanabe et al., 2007; Marzagalli et al., 2015). It belongs to the secretin/glucagon/peptide histidine-isoleucine superfamily, which also includes a structurally related peptide with 68% homology to PACAP, named vasoactive intestinal peptide (VIP) (reviewed by Vaudry et al., 2009; Harmar et al., 2012). The actions of PACAP are mediated by two classes of receptors, PAC1 and VPAC subtypes (including VPAC1 and VPAC2), the former has the highest affinity for PACAP, and the latter shows equal and high affinity for both PACAP and VIP.

PACAP in particular is a pleiotropic molecule that emerges as the critical target implicated in the development of fear-associated memories (Ressler et al., 2011; Stevens et al., 2014). Furthermore, there are now sufficient data to claim that PACAP administration, even at low doses, can specifically enhance memory retention in PA (Sacchetti et al., 2001) – an effect that, importantly, appears to also engage the DAergic system (Telegdy and Kokavszky, 2000). With this idea in mind, we hypothesized that the constitutively disturbed DA environment caused by functional inactivation of the D_3R (Accili et al., 1996) could alter the basal hippocampal and cortical PACAPergic systems in mutant mice. The present study therefore characterized and quantified differences in the PACAP/PAC1–VPAC1-2 axis of wild-type (WT) vs $D_3R^{-/-}$ mice using a battery of comparative morphological, stereological, immunohistochemical and biomolecular experiments.

EXPERIMENTAL PROCEDURES

Animals

Experiments were carried out on $D_3R^{-/-}$ mice and their WT littermates (total of 23 male mice 8–12 weeks old per genotype). Mice were housed four per cage and fed with standard laboratory chow and allowed free access to water *ad libitum*, in an air-conditioned environment with a 12-h light–dark cycle, as described elsewhere (Castorina et al., 2011). All the experimental procedures were performed during the light cycle (between 10 a.m. and 2 p.m.). $D_3R^{-/-}$ mice used in these experiments were 5th–8th generation of congenic C57BL/6J mice, generated by a backcrossing strategy. The genotypes of the D_3R mutant and WT mice were identified by a PCR method by using two pairs of primers flanking either exon 3 of the WT D_3R or the PGK (phosphoglycerate kinase 1 gene promoter) cassette of the mutated gene (Accili et al., 1996). All animals were used only once in the experiments, which were carried out according to the European Community Council Directive 86/609/EEC. Efforts were made to minimize animal suffering and to reduce the number of animals used. The design, methods and rationale of these experiments were, approved by the Ethics Committee for Animal Research of the University of Catania.

Stereological measurements

General premises. To estimate the number of cells in the hippocampus, we combined the fractionator and the optical disector method as suggested by others (West et al., 1991; West, 1999). The entire counting process was done with the help of a commercially available stereology system (Stereo Investigator™; Micro-BrightField Europe, Magdeburg, Germany). In brief, serial coronal sections were collected from the entire hippocampus and every fifth section of the right hippocampus was analyzed. The choice to use the right hippocampus was done empirically since no difference on preliminary measurements was appreciable between left and right hippocampi. The first section for analysis was selected using a random number table. On each section, the dentate gyrus (DG), area CA2/3, area CA1, our region of interest (ROI) were defined and a grid was placed randomly within the ROI. At regularly predetermined positions of the grid, cells were counted within

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