Brain Research Bulletin 137 (2018) 23–34

Contents lists available at ScienceDirect

Brain Research Bulletin

journal homepage: www.elsevier.com/locate/brainresbull

Sexually diergic hypothalamic-pituitary-adrenal axis responses to selective and non-selective muscarinic antagonists prior to cholinergic stimulation by physostigmine in rats

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ARTICLE INFO

Keywords:
Cholinergic
Methoctramine
Muscarinic
Pirenzepine
Scopolamine
Sexual diergism

ABSTRACT

Central cholinergic systems regulate the hypothalamic-pituitary-adrenal (HPA) axis differentially in males and females (sexual diergism). We previously investigated the role of muscarinic receptors in this regulation by administering physostigmine (PHYSO), an acetylcholinesterase inhibitor, to male and female rats pretreated with scopolamine (SCOP), a nonselective muscarinic antagonist. SCOP pretreatment enhanced adrenocorticotropic hormone (ACTH) and corticosterone (CORT) responses in both sexes; males had greater ACTH responses while females had greater CORT responses. In the present study, we further explored the role of muscarinic receptor subtypes in HPA axis regulation by administering PHYSO to male and female rats following SCOP or various doses of either the M1 or the M2 selective muscarinic receptor antagonists, pirenzepine (PIREN) or methoctramine (METHO), respectively. Blood sampling occurred before and at multiple times after PHYSO. ACTH and CORT were determined by highly specific immunoassays. PIREN + PHYSO resulted in sustained, dose-dependent increases in ACTH and CORT: ACTH responses were similar in both sexes, CORT responses were greater in females, and percent changes from baseline for both hormones were greater in males. METHO + PHYSO resulted in overall decreases in ACTH and CORT: ACTH and CORT responses were higher in females but lower than those caused by PIREN or SCOP in both sexes, and percent changes from baseline were lower in males. Area under the curve analyses further supported these sexually diergic effects. These results suggest that specific muscarinic receptor subtypes differentially influence the HPA axis in a sexually diergic manner.

1. Introduction

Muscarinic cholinergic receptors are located throughout the body and perform a wide variety of functions. Within the central nervous system (CNS), the five molecularly identified subtypes (M1-M5) of muscarinic receptors are widely expressed, with M1-M4 receptors expressed most predominantly (Abrams et al., 2006; Nathanson, 2008). M1, M3, and M5 receptors are coupled to $G_{q/11}$ subunits and are primarily postsynaptic; M2 and M4 receptors are coupled to $G_{i/o}$ subunits and are primarily presynaptic, functioning as autoreceptors (Rhodes et al., 2005; Abrams et al., 2006; Nathanson, 2008; Scarr, 2012; Jeon et al., 2015). In regions such as the hippocampus, prefrontal cortex, amygdala, and brainstem, muscarinic receptors are involved in cognition, memory, sleep, motor control, and various behaviors (Hughes and Dragunow, 1993; Aura et al., 1997; Bhatnagar et al., 1997; Brazhnik et al., 2004; Abrams et al., 2006; Cousens and Beckley, 2007; Li et al., 2007; Scarr, 2012; Terzioğlu et al., 2013; Ishibashi et al., 2014).

Central cholinergic systems also regulate the hypothalamic-pituitary-adrenal (HPA) axis, the neuroendocrine axis that controls an organism's stress response, and re-establishes homeostasis following stress (Tsagarakis and Grossman, 1990; Rhodes and Rubin, 1999; Smith and Vale, 2006; Steiner and Wotjak, 2008; Gentile et al., 2011; Gadek-Michalska et al., 2015). Muscarinic receptors have been implicated in a variety of mental disorders associated with HPA dysfunction, including depression, anxiety, Alzheimer's disease, and post-traumatic stress

Abbreviations: ACh, acetylcholine; ACTH, adrenocorticotropic hormone; ANOVA, analysis of variance; AUC, area under the curve; BBB, blood brain barrier; BNST, bed nucleus of the stria terminalis; CNS, central nervous system; CORT, corticosterone; CRH, corticotropin-releasing hormone; eCB, endocannabinoid; GABA, gamma-aminobutyric acid; HPA, hypothalamic-pituitary-adrenal; IP, intraperitoneal; KO, knockout; METHO, methoctramine; mPFC, medial prefrontal cortex; NE, norepinephrine; NMDA, *N*-methyl-D-aspartate; PHYSO, physostigmine; PIREN, pirenzepine; PVN, paraventricular nucleus; SAL, saline; SCOP, scopolamine; SEM, standard error of the mean

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https://doi.org/10.1016/j.brainresbull.2017.11.002
Received 23 July 2017; Received in revised form 21 October 2017; Accepted 2 November 2017
Available online 07 November 2017
0361-9230/

disorder (Marino et al., 1998; Harvey et al., 2004; Scarr, 2012; Terzioğlu et al., 2013; Witkin et al., 2014; Jeon et al., 2015), but their precise role in HPA axis activity has not been widely studied (Bhatnagar et al., 1997; Rhodes et al., 2001a,b, 2005, 2008; Hoeller et al., 2016), the majority of these studies being from our laboratory. Because depression, anxiety disorders, and post-traumatic stress disorder are over twice as prevalent in women as they are in men (Rhodes and Rubin, 1999; Reich et al., 2009; Guo et al., 2012; Scarr, 2012; Babb et al., 2013; Pisu et al., 2016; Wiersielis et al., 2016), sexually diergic mechanisms underlying muscarinic regulation of HPA axis activity are pertinent areas of study. As well, the potential role of muscarinic receptors in the pathophysiology of mood disorders has triggered an interest in muscarinic receptors as a possible target for antidepressant therapy (Scarr, 2012; Witkin et al., 2014; Jeon et al., 2015), further underscoring the importance of studies addressing sexual diergism and muscarinic regulation of stress pathways.

In our previous studies with jugular vein-cannulated rats, pretreatment with the nonselective muscarinic antagonist scopolamine (SCOP) increased HPA axis responses to the acetylcholinesterase inhibitor physostigmine (PHYSO) in both males and females (Rhodes et al., 2001a). The effects of SCOP were sexually diergic: adrenocorticotropic hormone (ACTH) responses were greater in males, and corticosterone (CORT) responses were greater in females (Rhodes et al., 2001a,b). We also have used M1 and M2 muscarinic receptor knockout (KO) mice to clarify the role of individual muscarinic receptor subtypes in HPA axis responses. The results of these studies suggested that M2 receptors play an important role in HPA axis regulation, consistent with the sexually diergic findings of our studies in rats (Rhodes et al., 2005, 2008).

The goal of the present study was to further characterize the sexually diergic role of M1 and M2 muscarinic receptors in HPA axis regulation by administering PHYSO to male and female rats following various doses of either nonselective, M1 selective, or M2 selective muscarinic receptor antagonists: SCOP, pirenzepine (PIREN), and methoctramine (METHO), respectively.

2. Materials and methods

2.1. Animals

One hundred and forty-one male and 137 female, eight-week old, jugular vein-cannulated, Sprague-Dawley rats weighing 220–225 g were obtained from Taconic Farms, Inc. (Germantown, NY, USA). Animals were housed singly in a well-ventilated, temperature- and humidity-controlled environment (22–25 °C, 50–75% humidity) under a standard 12-h light/dark cycle (lights on at 0700 h). Laboratory rat chow and water were available *ad libitum*. Estrous cycle was not controlled in the present study, but staging of the female rats for *post-hoc* analysis was done by light-microscopic examination of daily vaginal smears. Animals were allowed 4–5 days to acclimate to the housing conditions and the blood sampling paradigm via routine handling and flushing of their cannulae. Experiments were performed between 0900 h and 1300 h to minimize circadian variations in plasma hormone concentrations. All experiments were approved by the Allegheny-Singer Research Institute Animal Care and Use Committee and were conducted in accordance with National Institutes of Health guidelines for the care and use of laboratory animals. All efforts were made to minimize the number of animals used and their suffering.

2.2. Drug administration

All drugs were administered intraperitoneally (IP). PHYSO (physostigmine salicyclate; Forest Pharmaceuticals, St. Louis, MO, USA), SCOP (scopolamine HCl; Sigma, St. Louis, MO, USA), PIREN (pirenzepine dihydrochloride; Sigma, St. Louis, MO, USA), and METHO (methoctramine tetrachloride; Sigma, St. Louis, MO, USA) were freshly prepared in saline (SAL) before injection. Dosing times and concentrations for PHYSO and SCOP (Somani and Khalique, 1986, 1987; Jung et al., 1988a,b; O'Neill et al., 1994; Rhodes et al., 2001b), PIREN (Hughes and Dragunow, 1993; Tobin, 1998; Tobin et al., 2002; Witkin et al., 2014), and METHO (Wess et al., 1988; Watson et al., 1992; Hirose et al., 2002; Tobin et al., 2002; Furuta et al., 2016) were based on their onset of action, half-life, and elimination in rats. SCOP (0.3 mg/kg) was used because that dose produced significant, sexually diergic HPA axis responses in our earlier studies (Rhodes et al., 2001a,b).

In all experiments, PHYSO (0.1 mg/kg – represented as PHYSO in figures, tables, and text) was administered at 0 min. In the antagonist + PHYSO studies, antagonists were administered at −25 min and included SCOP (0 or 0.3 mg/kg – represented as SAL, SCOP 0.3), PIREN (0, 10, 30, or 70 mg/kg – represented as SAL, PIREN 10, PIREN 30, PIREN 70), and METHO (0, 0.3, 1, 3 mg/kg – represented as SAL, METHO 0.3, METHO 1, METHO 3). SAL was substituted for PHYSO or for the antagonists in the control groups (e.g., SAL + SAL, SAL + PHYSO, SCOP + SAL, PIREN + SAL, METHO + SAL), so that all animals always received two injections.

2.3. Blood sampling

A standard, two-person procedure for blood sampling from cannulated animals that was established in our laboratory was used. One person gently held the animal in a stationary position, while the other person collected the blood sample. Animals remained calm subsequent to daily handling by these same individuals. Each blood sample was collected in less than 1 min. To maintain cannula patency, twice each week the stainless-steel cannula plug was removed, the heparin-polyvinylpyrrolidone (PVP; 100 IU/ml) lock solution (Sigma, St. Louis, MO, USA) was aspirated, and 0.1 ml buffered normal SAL was injected, followed by replacement of 0.02 ml lock solution. A similar procedure was followed for blood sampling: The lock solution was aspirated, and 300–325 µl blood was withdrawn into a 1-ml tuberculin syringe, immediately transferred into microcollection tubes, and stored on ice. Following blood sample collection, replacement solution of warm (37 °C) buffered normal SAL, equal to the amount of blood withdrawn, was immediately infused through the cannula, the cannula was injected with 0.02 ml lock solution, and the stainless-steel plug was reinserted.

The plasma was separated by centrifugation, immediately frozen at −80 °C, and stored until hormone analyses. Baseline blood samples were collected at −25 min (immediately prior to the administration of the first drug) and at −15 min. The two baseline hormone values were averaged and are presented in the figures as one average baseline value at −20 min. Four additional blood samples were collected at 10 min, 20 min, 40 min, and 60 min following administration of the second drug (PHYSO or SAL) at 0 min.

2.4. Hormone assays

Plasma samples were analyzed in singlet for ACTH and in duplicate for CORT. ACTH was determined by a highly specific immunoradiometric assay (Nichols Institute, San Juan Capistrans, CA, USA). Inter- and intra-assay coefficients of variation were 6% and 4% respectively. The minimum detectable ACTH concentration was 1.5 pg/ml. CORT was analyzed by radioimmunoassay kits (ICN Pharmaceuticals, Costa Mesa, CA, USA). The CORT antibody cross-reacted less than 0.5% with other steroids. Inter- and intra-assay coefficients of variation were both 4%. The minimum detectable CORT concentration was 1.1 ng/ml.

2.5. Statistical analysis

Group Ns varied due to insufficient sample for the analysis of both hormones from some animals and/or loss of cannula patency. Data are presented as mean ± standard error of the mean (SEM). Areas under the curve (AUC) were used as additional measures of hormone