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Changes in the female arcuate nucleus morphology and neurochemistry after chronic ethanol consumption and long-term withdrawal

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ABSTRACT

Ethanol is a macronutrient whose intake is a form of ingestive behavior, sharing physiological mechanisms with food intake. Chronic ethanol consumption is detrimental to the brain, inducing gender-dependent neuronal damage. The hypothalamic arcuate nucleus (ARN) is a modulator of food intake that expresses feeding-regulatory neuropeptides, such as alpha melanocyte-stimulating hormone (α-MSH) and neuropeptide Y (NPY). Despite its involvement in pathways associated with eating disorders and ethanol abuse, the impact of ethanol consumption and withdrawal in the ARN structure and neurochemistry in females is unknown. We used female rat models of 20% ethanol consumption for six months and of subsequent ethanol withdrawal for two months. Food intake and body weights were measured. ARN morphology was stereologically analyzed to estimate its volume, total number of neurons and total number of neurons expressing NPY, α-MSH, tyrosine hydroxylase (TH) and estrogen receptor alpha (ERα). Ethanol decreased energy intake and body weights. However, it did not change the ARN morphology or the expression of NPY, α-MSH and TH, while increasing ERα expression. Withdrawal induced a significant volume and neuron loss that was accompanied by an increase in NPY expression without affecting α-MSH and TH expression. These findings indicate that the female ARN is more vulnerable to withdrawal than to excess alcohol. The data also support the hypothesis that the same pathways that regulate the expression of NPY and α-MSH in long-term ethanol intake may regulate food intake. The present model of long-term ethanol intake and withdrawal induces new physiological conditions with adaptive responses.

1. Introduction

Alcohol consumption is a worldwide health problem with serious socio-economic consequences. Research carried out in our laboratory and by others has considerably contributed for the now widely accepted view that chronic and excessive ethanol consumption disrupts the structure and the function of the central nervous system (CNS) both in humans as in experimental animals (Harper, 2009; Mukherjee, 2013; Madeira and Paula-Barbosa, 1999; Silva et al., 2002a). Marked degenerative alterations, including cell and synapse loss, and changes in a variety of neurotransmitter systems have been noticed in numerous regions of the brain, including the hypothalamus (Harper, 2009; Madeira et al., 1993; Silva et al., 2002a; Tavares and Paula-Barbosa, 1984). Ethanol-induced malady may be either permanent (Silva et al., 2002a, 2009; Silva and Madeira, 2012) or reversible upon abstinence (Pereira et al., 2014; Silva et al., 2002b, 2009). Despite the evidence that ethanol-induced brain injury is sexually dimorphic, with females evidencing higher susceptibility for brain

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damage (Silva et al., 2009; Silva and Madeira, 2012; Sohrabji, 2002; Sullivan et al., 2002), few studies have addressed the effects of ethanol consumption on neurotransmitter systems in the female brain.

Accumulating evidence indicates that the regulation of ethanol consumption is controlled by the same hypothalamic peptides and brain circuits that regulate food intake (Egli, 2003; Thiele et al., 2003). The orexigenic neuropeptide Y (NPY) and the anorexigenic α-melanocyte-stimulating hormone (α-MSH) are widely known for their critical role in the regulation of feeding behavior (Morton et al., 2006; Meister, 2007; Vianna and Coppari, 2011). These neuropeptides were also shown to be involved in ethanol consumption and in the neurobiological responses to ethanol intake and withdrawal. In effect, NPY is involved with the neuronal mechanisms of alcohol preference (Caberlotto et al., 2001; Carvajal et al., 2006; Thiele et al., 2003) and in the modulation of alcohol withdrawal symptoms (Carvajal et al., 2006; Roy and Pandey, 2002). α-MSH and melanocortin receptors have been implicated in the regulation of responses to ethanol consumption and withdrawal and in the modulation of the dopaminergic reward system (Olney et al., 2014; Shelkar et al., 2015; Thiele et al., 2003). Ethanol was also shown to disturb the dopaminergic system, increasing the craving effect of ethanol (Kim et al., 1997; Oomizu et al., 2003). The largest collection of hypothalamic neurons expressing NPY and α-MSH are located in the arcuate nucleus (ARN) (Meister, 2007; Vianna and Coppari, 2011). This nucleus is one of the main central modulators of food intake (Bouret et al., 2004; Morton et al., 2006) and shares connections with other nuclei involved in the control of feeding behavior and ethanol consumption (Thiele et al., 2003; Valassi et al., 2008; Volkow et al., 2011). Moreover, ARN neurons release dopamine (Moore et al., 1987) and express dopamine receptors (Kim et al., 2010; Romero-Fernandez et al., 2014), whose activation decreases food intake and body weight (Guo et al., 2014; van de Giessen et al., 2014; Volkow et al., 2011).

Estradiol is the most relevant hormone affecting feeding behavior and body weight both in women (Racine et al., 2012) and female rats (Butera, 2010). This ovarian hormone exerts its actions through the activation of estrogen receptors (ER) (McEwen et al., 2012), which are abundantly expressed in the ARN (Chakraborty et al., 2003; Shughrue et al., 1996; Simerly et al., 1990) and co-localize with NPY, POMC and dopamine (de Souza et al., 2011; Leite et al., 2008; Sar et al., 1990). It has been shown that estradiol administration upon ovariectomy induces a decrease in NPY levels (Baskin et al., 1995; Shimizu et al., 1996; Silva et al., 2010) and an increase in POMC and α-MSH expression in the ARN (de Souza et al., 2011; Hirosawa et al., 2008; Medina et al., 1998). Estradiol also affects the dopaminergic system (Ben-Jonathan and Hnasko, 2001; DeMaria et al., 2000; Oomizu et al., 2003) and reduces the activity of tyrosine hidroxilase (TH) (Arbogast and Hyde, 2000; Jones and Naftolin, 1990).

Because central modulation of ethanol and food intake shares the same neuronal pathways, we sought to analyze the impact of heavy chronic ethanol consumption and long-term withdrawal in the structure and neurochemistry of the ARN. Knowing that estradiol, by acting through the ERα, can change the synthesis and expression of ARN neuropeptides, and that females are particularly susceptible the deleterious effects of ethanol, we have also assessed the effect of ethanol and withdrawal in the expression of ERα. For this purpose, we have used stereological methods to estimate the volume of, and the total number of neurons in the ARN as well as the total number of ARN neurons expressing NPY, α-MSH, TH and ERα in randomly cycling female rats upon six months of ethanol consumption or two months of withdrawal.

2. Material and methods

2.1. Animals and treatments

Female Wistar rats were kept in a controlled room with 22 °C of temperature, 55% of humidity and under a 12 h light/dark cycle (lights on at 7 a.m.). Solid diet (4RF21/C Mucedola, Milan, Italy) and water were freely available. Only females exhibiting consecutive 4- to 5-day estrous cycles were used. At 2 months of age, rats at all stages of the estrous cycle were randomly assigned to the ethanol-treated (Et) group, which had an alcoholic solution as the only liquid available, or the control group. The ethanol concentration was progressively increased in the course of 2 weeks, starting with a 5% (v/v) solution and rising by 1% per day to a final 20% (v/v). After 6 months of treatment, half of the Et rats were euthanized (n = 10) and the remaining (n = 10) were switched to tap water for a further 2 months – withdrawal (Wd) group. The shift from ethanol treatment to water intake was performed gradually over a 2-week period by progressively reducing the ethanol concentration in the drinking solution by 1% per day. The control group (isocaloric control, Ic, n = 10) was isocaloric to the Et group. These rats had access to the same amount of food consumed by Et rats plus an additional quantity of pellets that was calculated taking into account the ethanol caloric value (1 gm = 7 Kcal) and the mean volume of ethanol solution consumed daily by Et rats (Madeira et al., 1997). All animals received beverage ad libitum and Et and Wd rats also received food ad libitum. The amount of solid and fluid intake was calculated daily and rats were weighed weekly. During the last 15 days of the experiments, the estrous cycle was monitored by daily collection of vaginal smears and histological examination. The experiments were performed according to the European Communities Council Directives of 22 September 2010 (2010/63/EU) and Portuguese Act n° 113/13. All efforts were made to minimize the number of animals used and their suffering.

2.2. Tissue preparation

2.2.1. Histological procedures

Animals (n = 5 per group) were anesthetized (3 ml/kg b.w. of a sodium pentobarbital solution, i.p.) and perfused transcardially, between 1 p.m. and 2 p.m., with a fixative solution containing 1% paraformaldehyde and 1% glutaraldehyde in 0.12 M phosphate buffer (pH 7.2). The brains were removed from the skulls, weighed and post-fixed for 15 days in fresh fixative. After removal of the frontal and occipital poles, the blocks containing both the right and left hypothalami were dehydrated through a graded series of ethanol solutions, embedded in glycolmethacrylate (hydroxyethylmethacrylate, Technovit 7100, Kulzer and Co., Wehrheim, Germany) and sectioned in the coronal plane at 40 μm. The sections were mounted serially and stained with a Giemsa solution (Fig. 1A) modified for use in glycolmethacrylate-embedded material (West et al., 1991). Finally, the sections were coverslipped with histomount mounting medium (National Diagnostics, Atlanta, GA, USA). These sections were used for the estimation of total number of neurons and the volume of the ARN.

2.2.2. Immunohistochemistry

Rats (n = 5 per group) were anesthetized with promethazine (10 mg/kg bw, s.c.), xylazine (2.6 mg/kg bw, i.m.) and ketamine (50 mg/kg bw, i.m.) and stereotaxically injected, into the lateral ventricle, with colchicine (10 μl of a 0.25% solution in physiological saline; C9654, Sigma-Aldrich Ltd., Madrid, Spain). Because previous studies have shown that without the use of colchicine only NPY and α-MSH fibers are stained (Boyer et al., 1994; Maolood and Meister, 2008; Lucas et al., 2014), colchicine was administered in order to inhibit axonal transport and to increase NPY and α-MSH