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Assessment of the Role of the μ Opioid Receptor in Ethanol-Induced Hyperprolactinemia in Mice

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Abstract: The purpose of this study was to examine the role of the endogenous opiate system, specifically the opiate receptor subtypes, in response to ethanol induced prolactin secretion in male Swiss-Webster mice. In order to determine the role of μ opiate receptors in ethanol induced hyperprolactinemia, animals were treated with ethanol with and without pretreatment with the opiate receptor antagonist β-Funaltrexamine. Additional animals were also treated with β-Endorphin under similar conditions as a positive control of opiate receptor activity and prolactin secretion. Opiate receptor antagonists successfully ameliorated ethanol induced hyperprolactinemia. This research could provide insight into ethanol's role in prolactin secretion and indirectly monitor changes in reproductive and immunological functions.

Key words: Ethanol, prolactin, opiates, β-Funaltrexamine, β-Endorphin

INTRODUCTION

Prolactin (PRL) is a proteinaceous hormone secreted by specialized cells known as lactotrophs in the anterior pituitary gland. The hormone is found in all members of the vertebrate classes and has influences on reproduction, lactation, parental behavior, metabolism, osmoregulation and migration (Horseman, 2001). While the principal target of prolactin is the mammary gland, prolactin receptors have been found in nearly every tissue in the body. Prolactin secretion during the estrous cycle is generally predictable, with a slight rise around ovulation, echoing the peak in luteinizing hormone (Vekemans, *et al*., 1977). During and after childbirth PRL levels rise, leading to milk synthesis and lactation (Horseman, 2001).

The highly complex and tightly regulated pathway of prolactin secretion has been already characterized in humans and animals. Alterations in prolactin levels results in various reproductive problems and may play an important role in some cancers. Hyperprolactinemia, or hypersecretion of PRL, is one of the most common pituitary disorders. High levels of PRL in women as well as men inhibit reproductive function. Both sexes may suffer from loss of libido and men may experience impotence. In women, PRL indirectly inhibits ovarian folliculogenesis by reducing luteinizing hormone secretion. Ovulation is also suppressed since prolactin indirectly increases progesterone secretion (Horseman, 2001). Hyperprolactinemia also affects non-reproductive tissues. Decreased bone density has been demonstrated when PRL levels are elevated and alteration in PRL may have important effects on immune function.

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Prolactin is negatively regulated by the tuberoinfundibular dopaminergic (TIDA) neurons via the release of dopamine. Dopamine released from TIDA neurons in the hypothalamus acts as the major prolactin inhibiting factor on lactotrophs at the anterior pituitary. Lactotrophs, unlike other endocrine cells, have a high basal secretory activity and thus require basal inhibition. In the absence of positive stimulatory factors (e.g., estrogen), dopamine exerts a tonic inhibitory effect. Thus, decreased dopamine levels in the anterior pituitary result in increased prolactin levels.

It has been demonstrated that both chronic and acute alcohol consumption can result in hyperprolactinemia. Seiliovich *et al*. (1984) demonstrated hyperprolactinemia in pituitary cells *in vitro* as a result of ethanol administration. Laboratory rats (De *et al*., 2002) and humans (Soyka *et al*., 1991) also show increased prolactin levels after ethanol consumption. However, the mechanism of this ethanol stimulated prolactin release remains unclear.

Also of interest is the fact that the endogenous opioid neuronal system may act as an indirect regulator of prolactin secretion. The endogenous opioid peptides are divided into three families (the endorphins, the enkephalins and the dynorphins) and are mediated by three major types of receptors: mu (μ), kappa (κ) and delta (δ) receptors (Minami and Satoh, 1995). The endogenous opioid peptides provide major afferent input to TIDA neurons and regulate PRL secretion by inhibiting the release of dopamine (Ben-Jonathan and Hnasko, 2001). Several studies have attempted to determine which receptor subtype (μ, κ, or δ) on the TIDA neurons is primarily responsible for PRL regulation (Andrews and Grattan, 2003; Soaje and Deis, 1999). These studies suggest that the μ- and κ-receptors mediate prolactin secretion by their influence on TIDA neurons and when these receptors were activated by endogenous opioids or other agonists, dopamine release is inhibited. This inhibition results in increased levels of circulating prolactin (Moore and Lookingland, 2000).

Ethanol is known to stimulate the endogenous opioid system, resulting in the release of endogenous opiates (Di Chiara *et al*., 1996). Since the ingestion of ethanol increases β-endorphin release and endogenous opiates have been shown to induce PRL secretion, it follows that antagonizing the μ-receptor following ethanol administration could attenuate the hyperprolactinemia usually associated with ethanol ingestion. The drug β-Funaltrexamine (β-FNA) is an irreversible μ-receptor antagonist and as such was used in this experiment to establish a link between the signaling pathway related to ethanol induced PRL secretion and the role of the mu opiate receptors in mice.

MATERIALS AND METHODS

Animals Experiments

Thirty male Swiss-Webster mice and weighing 30-60 g were housed under controlled temperature and lighting and supplied with food and water *ad libitum*. Male mice were used in order to eliminate possible distortion of results caused by circulating endogenous estrogens or stress following ovariectomization. Age related differences were also minimized by randomly selecting animals from ages ranging from 3 to 30 months and subsequent age matching of the animals. We determined that Swiss-Webster mice were a superior animal model due to their rapid response times and sharp peak Blood Ethanol Concentrations (BEC) at 60 min after ethanol administration. We also found that ethanol administration intraperitoneally (IP) resulted in higher BECs than i.p. gavage and this method of administration was selected due to both its ease of and the rapid increased BEC.

The Swiss-Webster mice were divided into 5 groups containing six mice each. Mice (outbred stock) used for this project varied in age, from 3 months old to 29 months of age. Because of the concern that age may play a factor in prolactin levels, the mice were sorted into groups so that the average age and weights for each group were matched. Each group received different drug treatments

as detailed below. All drugs or saline were delivered IP with a tuberculin syringe. Each mouse in Group A, the control group, received 0.5 mL of saline administered IP. Because some hormone levels (including prolactin and the endogenous opiates) can increase due to stress and handling, it was important to minimize handling stress and to treat the controls in a manner similar to the treated groups to normalize for such stresses. Mice in Group B received a single IP injection of a 10% ethanol/saline solution at a dose of 2.0 g ethanol kg^{-1} body weight to achieve a blood alcohol level of approximately 0.10 g ethanol 100 mL^{-1} blood. Mice in Group C received a single IP injection of β-endorphin. The treatment was administered as a saline solution (25 μg β-endorphin mL^{-1}saline solution) at a dosage of 250 mg kg^{-1} of body weight. To examine μ-receptor involvement in ethanol-induced prolactin secretion, mice (groups D and E) were pretreated with the μ-receptor antagonist β-Funaltrexamine HCl (β-FNA). β-FNA antagonizes μ-receptor mediated prolactin secretion at an injected dose of 5.0 μg dissolved in 5 μL sterile saline. Since β-FNA is a long acting, irreversible antagonist, only one injection was necessary. Pretreatment drugs were administered 30 min before the stimulatory dose. Each group received one stimulatory dose of ethanol, β-endorphin, or saline. Thirty minutes after the pre-treatment, Groups D and E were administered ethanol and β-endorphin, respectively. Mice in Group D received an equivalent dosage of ethanol as Group B; mice in Group E received the same dosage of β-endorphin as Group C (Table 1)

Mice were sacrificed 60 min after stimulatory dose via CO_2 asphyxiation and subsequent cervical dislocation. Blood was drawn from cardiac puncture with sterile tuberculin syringe into pediatric blood collection tubes. On average, about 1 milliliter of blood from each mouse was collected and centrifuged at 7300 rpm (5000 g) at 4°C for 10 min. Serum was collected and used for subsequent analysis..

Prolactin Measurement

Mouse serum prolactin concentrations were measured by radioimmunoassay (RIA) in triplicate (NIDDK National Hormone and Peptide Program, Torrance, CA). The assay is based on the competition between unlabelled PRL and a fixed quantity of [^{125}I]-labeled PRL for a limited number of binding sites on a PRL specific antibody. With fixed amounts of antibody and radioactive ligand, the amount of radioactive ligand bound by the antibody will be inversely proportional to the concentration of added non-radioactive ligand. The antibody bound PRL is then reacted with a second antibody reagent which contains a second antibody that is bound to magnetizable polymer particles. Separation of the antibody is effected by magnetic separation and decantation of the supernatant. Measurement of the radioactivity in the pellet allows the amount of labeled PRL in the bound fraction to be calculated. Following the radioimmunoassay procedure, the radioactivity of each sample and standard was determined using a gamma scintillation counter. The concentration of unlabelled PRL in the sample is then determined by interpolation from a standard curve.

Table 1: Treatments groups detail

Animals n = 6 per group	Average age (months)	Average weight (g)	Drug dosage	Average PRL (ng mL^{-1})
A	12	49.9	Control saline (0.5 mL)	1.95
B	8.0	49.2	2.0 g ethanol kg^{-1} body weight	11.54
C	7.2	40.3	β-Endorphin 250 mg kg^{-1} of body weight	14.38
D	7.8	43.2	β-Funaltrexamine pretreatment (0.5 mL) + 2.0 g ethanol kg^{-1} body weight	2.04
E	7.2	49.8	β-Funaltrexamine pretreatment (0.5 mL) + β-Endorphin 250 mg kg^{-1} of body weight	3.31

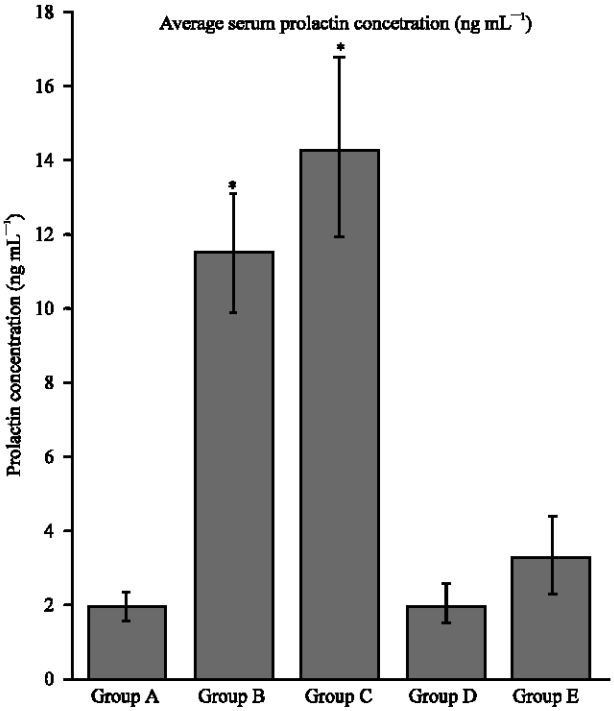

Fig. 1: Mean prolactin concentration (ng mL^{-1}) * = p<0.05

RESULTS

Prolactin Levels

All of the serum prolactin levels fell within the range of 0.7-28.7 ng mL^{-1}. Student's t-test was performed to assess significant differences between the means of the controls and treated animals. The prolactin levels in the ethanol only and β-endorphin only treated animals were significantly ($p<0.05$) different when compared to those of the controls (Fig. 1). Conversely, there was no statistical difference between the means of the control group and the animals pretreated with the opiate antagonist, β-FNA.

An analysis of variance (ANOVA) was also conducted in order to determine if the differences in the means between the groups were statistically significant. The single-factor ANOVA performed on all of the groups showed that there was a significant difference between two or more of the means: the F ratio of 16.25 was greater than the F critical value of 2.78. The p-value was 0.0000014, or a 0.000014% possibility that the F value was due to random chance.

DISCUSSION

This study examined the role of the μ-opioid receptor on prolactin secretion in male mice. It was found that control group mice, with no drugs administered, had an average circulating prolactin level of 1.95 ng mL^{-1}. Mice that were administered either ethanol or the endogenous opiate β-endorphin had a significantly higher level of circulating prolactin, at 11.54 and 14.38 ng mL^{-1}, respectively. The animals that received either β-endorphin or ethanol alone showed an average increase in PRL by approximately five times that of the controls. Mice that received the μ-receptor antagonist β-funaltrexamine prior to a stimulatory ethanol or β-endorphin treatment showed no statistically

significant increase in their prolactin levels and averaged 2.04 and 3.31 ng mL $^{-1}$, respectively when compared to the saline treated animals and almost completely reversed the stimulatory effects. These data support the hypothesis that the ingestion of ethanol indirectly results in hyperprolactinemia through the action of the μ-receptors on the TIDA neurons. There was no statistically significant difference between the control animals and the mice that received pre-treatment doses of β-FNA. This suggests that the opiate antagonist blocked the ethanol induced reduction in dopamine levels at the TIDA neurons. Several studies have suggested that the euphoric, rewarding and reinforcing effects associated with alcohol consumption may be due in part to endogenous opiate activity and dopamine. Furthermore, it has been shown that acute ethanol administration increases endorphin and enkephalin gene expression and increases the release of these peptides from the brain and pituitary of rodents (Oswald and Wand, 2004). Thus, it follows that ethanol ingestion would result in increased levels of the endogenous opioid β-endorphin. The increase in activation of μ-receptor would diminish the release of dopamine from the TIDA neurons and result in increased levels of circulating prolactin. This study has successfully illustrated that endogenous opiate pathways are, at least in part, related to ethanol induced increased prolactin secretion. Since the ingestion of ethanol increases β-endorphin release and endogenous opiates have been shown to induce PRL secretion, it follows that antagonizing the μ-receptor following ethanol administration would attenuate the hyperprolactinemia associated with ethanol ingestion. The irreversible μ-receptor antagonist drug β-Funaltrexamine (β-FNA) was able to attenuate this effect and thereby establish a link between the signaling pathway related to ethanol induced PRL secretion and the role of the mu opiate receptors in mice.

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