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mRNA expression of ion channels in GnRH neurons: Subtype-specific regulation by 17β-estradiol

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ABSTRACT

Burst firing of neurons optimizes neurotransmitter release. GnRH neurons exhibit burst firing activity and T-type calcium channels, which are vital for burst firing activity, are regulated by 17β-estradiol (E2) in GnRH neurons. To further elucidate ion channel expression and E2 regulation during positive and negative feedback on GnRH neurosecretion, we used single cell RT-PCR and real-time qPCR to quantify channel mRNA expression in GnRH neurons. GFP-GnRH neurons expressed numerous ion channels important for burst firing activity. E2-treatment sufficient to induce an LH surge increased mRNA expression of HCN1 channels, which underlie the pacemaker current, the calcium-permeable $Ca_V1.3$, $Ca_V2.2$, $Ca_V2.3$ channels, and TRPC4 channels, which mediate the kisspeptin excitatory response. E2 also decreased mRNA expression of SK3 channels underlying the medium AHP current. Therefore, E2 exerts fundamental changes in ion channel expression in GnRH neurons, to prime them to respond to incoming stimuli with increased excitability at the time of the surge.

1. Introduction

As demonstrated in a number of species including the rat, sheep and rhesus monkey, the preovulatory luteinizing hormone (LH) surge is accompanied by a surge in gonadotropin-releasing hormone (GnRH) (Caraty et al., 1989; Chappell and Levine, 2000; Levine and Ramirez, 1982; Pau et al., 1993), suggesting increased activity of GnRH neurons at the time of the GnRH surge. This increased activity is primarily due to increased estradiol levels, because treatment with 17β-estradiol (E2) in ovariectomized (OVX) females can mimic the positive feedback regulation of GnRH and LH secretion (Caraty et al., 1989; Chappell and Levine, 2000). Cell-attached single-unit extracellular recording to evaluate GnRH neuronal firing activity during negative as compared to positive feedback has revealed that the GnRH neuronal firing rate is low during the morning negative feedback period and significantly higher during the evening positive feedback period (Christian et al., 2005). Collectively, these findings would indicate that there are fundamental changes in GnRH neuronal firing activity during the different stages of the ovulatory cycle and during E2-induced negative and positive feedback. The timing of the GnRH (LH) surge and thus the increased activity of GnRH neurons at the time of the surge is E2-dependent, but it is also entrained to a circadian input from the suprachiasmatic nucleus (SCN) at least in some rodent species (Chappell et al., 2009; Christian et al., 2005; Christian and Moenter, 2008; Legan et al., 1975). The mechanism by which the SCN affects GnRH neuronal activity is not known, but circadian cues may involve vasopressin input to kisspeptin neurons and vasoactive intestinal peptide input to GnRH neurons (Christian and Moenter, 2008; Vida et al., 2010; Ward et al., 2009).

In addition to GnRH neurons, kisspeptin neurons are essential for reproductive development and reproductive competence (Oakley et al., 2009). These hypothalamic neurons express ERα, are affected by E2 feedback, and are strongly excitatory to GnRH neurons (Oakley et al., 2009). Kisspeptin excites GnRH neurons by actions on the G protein-coupled receptor 54 (GPR54), also called kisspeptin receptor (Zhang et al., 2008). Evidence from GT1–7 GnRH neuronal cells suggests that GPR54 exhibits an E2-dependent diurnal variation in mRNA expression (Tonsfeldt et al., 2011). These findings, however, have not been confirmed in native GnRH neurons.

Abbreviations: AHP, afterhyperpolarization; E2, 17β-estradiol; GnRH, gonadotropin-releasing hormone; GPR54, G protein-coupled receptor 54; HCN, hyperpolarization-activated cyclic nucleotide-gated; HVA, high voltage activated; LH, luteinizing hormone; POA, preoptic area; qPCR, quantitative real-time PCR; RT, reverse transcription; sc-PCR, single cell PCR; SK, small-conductance calcium-activated potassium channels; TRPC, canonical transient receptor potential; ZT, zeitgeber time.

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Based on a model similar to that described and validated for thalamocortical relay neurons and hypothalamic neurosecretory neurons (Chemin et al., 2002; Erickson et al., 1993b; Kelly and Rønnekleiv, 1994; Kim et al., 2001), we had predicted that T-type calcium channels together with the hyperpolarization-activated, cyclic nucleotide-gated channels (HCN) are essential for induction of burst firing in GnRH neurons, and that the calcium-dependent, small-conductance calcium-activated potassium channels (SK) - type channels, which underlie afterhyperpolarization (AHP) are crucial for allowing repetitive cycles of burst firing (Kelly and Rønnekleiv, 1994; Kelly and Wagner, 2002). All of these channels are active in GnRH neurons and contribute significantly to their signaling pattern (Bosch et al., 2002; Chu et al., 2009, 2010; Kato et al., 2006; Lee et al., 2010; Liu and Herbison, 2008; Spergel, 2007; Zhang et al., 2007, 2009). Additional channels important for GnRH neuronal firing include canonical transient receptor potential (TRPC) channels, which are activated by kisspeptin, and high voltage activated (HVA) calcium channels, which are important for calcium homeostasis and peptide release (Sun et al., 2010; Zhang et al., 2008).

While previous studies have demonstrated that E2 regulates the expression and/or function of a number of channels in GnRH neurons including T-type and L-type calcium channels (Sun et al., 2010; Zhang et al., 2009), little is known about the channel subtype expression and the E2 and diurnal regulation of the majority of ion channels in GnRH neurons. To begin to understand the E2-induced changes in GnRH neurons, we have explored the mRNA expression of HCN, TRPC, SK, and HVA calcium channels in the morning (negative feedback) and the expression in the evening (positive feedback) in oil- and E2-treated females. Indeed, we have found an E2-induced increased mRNA expression of HCN1, TRPC4, $Ca_V1.3$ (L), $Ca_V2.2$ (N) and $Ca_V2.3$ (R)-type calcium channels in GnRH neurons. In contrast, SK3 mRNA was decreased in GnRH neurons, whereas GPR54 mRNA was not altered at any time-points. These findings indicate that the rising E2-levels exert specific fundamental changes in ion channels expression in GnRH neurons, to prime these neurons for altered responsiveness to incoming stimuli leading to changes in excitability in an E2-dependent manner.

2. Materials and methods

2.1. Animals

Adult female CBB6 mice (GnRH-GFP) (Suter et al., 2000) were maintained under constant temperature and lights. Two different lighting cycles were used, where lights were on between 0600 h (zeitgeber time (ZT) 0) and 1800 h (ZT 12) or where lights were on between 0200 h (ZT 0) and 1400 h (ZT 12) local time. The breeders and most of the research animals were kept permanently under reversed lighting schedule, and these animals were used for the majority of evening experiments with some exceptions as noted below. At time of weaning additional research animals were moved to an adjacent room with a regular lighting schedule (0600–1800 h) and were kept there until adulthood (at least 60 days of age). These animals were used for all of the morning experiments and selected evening experiments. We found no evidence for differences between the two lighting schedules. Food and water were provided *ad libitum*. The females were exposed to male bedding to establish normal estrous cycle prior to bilateral ovariectomy (OVX) and increased response to E2 afterwards (Bronson and Whitten, 1968; Dalal et al., 2001). The animals were OVXed under isofluorane inhalant anesthesia 5–7 days before experimentation, and were given a dose of 4 mg/kg carprofen (Rimadyl, Pfizer Animal Health, New York) immediately following surgery for analgesia. All animal procedures were according to NIH standards and were approved by the Institutional (Oregon Health and Science University) Animal Care and Use Committee.

2.2. Experimental design

2.2.1. Induction of the LH surge

For induction of positive feedback regulation of LH by ovarian steroids in mice, different models have been developed. One model, based on studies by Bronson and colleagues, used E2 implants at the time of OVX combined with a surge-inducing E2 injection 6 days later (Bronson and Vom Saal, 1979). Another similar model (Gee et al., 1984) used an E2 priming implant and an E2 surge implant of differing doses. Both of these models are critically dependent on the "appropriate" concentrations of E2, such that too little or too high levels of E2 will reduce or prevent the LH surge (Bronson and Vom Saal, 1979). We found that, with such sensitivity, we had too many inconsistencies inducing an LH surge in mice. Therefore, we have developed a two-step E2-injection procedure utilizing a priming E2 dose followed by a surge-inducing E2 dose. On day 5 following OVX, the animals were given a subcutaneous injection of a priming dose of 17β-estradiol benzoate (0.25 μg in 50 μl oil) or oil vehicle (50 μl) at ZT 4–5. On day 6, the animals were given a surge dose of 17β-estradiol benzoate (1.0–1.5 μg in 50 μl oil) or oil-vehicle (50 μl) at ZT 4–5. The animals were used for experimentation the following day. The LH surge was induced under both lighting conditions with similar results. When intact animals were used, estrous stage was confirmed by vaginal smears.

2.2.2. mRNA quantification in GnRH neuronal pools

In initial experiments we tested the linearity of mRNA expression in single cells compared to pools of GnRH neurons. Since the mRNA expressions of GnRH and GPR54 are quite high in GnRH neurons and these RNAs can be quantified even in single cells, we compared the expression in single cells versus pools of 2, 4 and 8 cells in two intact animals killed during the second day of diestrus. The expression of β-actin was used as control as described below. In addition, we also tested the linearity of one of the high-expressing ion channels, SK3. Since this transcript could only be quantified in pools consisting of at least 5-cells, we compared the expression in pools of 5 and 10 GnRH neurons. For this experiment we used OVX animals, since the SK current (medium I_{AHP}) is reduced acutely with E2 application in GnRH neurons (Chu et al., 2009) and E2-treatment *in vivo* leads to inhibition of the mI_{AHP} in POA GABAergic neurons (Wagner et al., 2001).

2.2.3. Distribution of mRNAs in single GnRH neurons and quantitative mRNA measurements in GnRH neuronal pools

These studies were aimed at investigating the mRNA expression in GnRH neurons of a number of ion channels of which there exist several subunits. In order to determine which subtype is expressed in GnRH neurons, individual GnRH neurons were acutely dispersed, harvested and subjected to single cell RT-PCR using primers selective for each channel subtype (see Table 1). In addition, pools of 5 or 10 neurons were harvested and subjected to real-time qPCR in order to determine the quantitative expression of channel subtypes in GnRH neurons.

2.2.4. Effects of oil- and E2-treatment on mRNA expression in GnRH neurons

In initial experiments, we tested whether channel mRNA expression in GnRH neurons was different between morning and evening in ovariectomized (OVX) oil-treated animals. The analysis of 4 neuronal pools (5 cells each) from 3 animals revealed that there were no differences in mRNA expression between the two time-points (Table 2). Since oil-treatment did not change the mRNA expression irrespective of time of day, E2 treatment was