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Induction of *Gnrh* mRNA expression by the ω-3 polyunsaturated fatty acid docosahexaenoic acid and the saturated fatty acid palmitate in a GnRH-synthesizing neuronal cell model, mHypoA-GnRH/GFP

Dean Q. Tran [a], Ernesto H. Ramos [a], Denise D. Belsham [a, b, c, *]

[a] Department of Physiology, Faculty of Medicine, University of Toronto, Toronto, ON M5S 1A8, Canada
[b] Department of Medicine, Faculty of Medicine, University of Toronto, Toronto, ON M5S 1A8, Canada
[c] Department of Obstetrics and Gynaecology, Faculty of Medicine, University of Toronto, Toronto, ON M5S 1A8, Canada

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ABSTRACT

Gonadotropin-releasing hormone (GnRH) neurons coordinate reproduction. However, whether GnRH neurons directly sense free fatty acids (FFAs) is unknown. We investigated the individual effects of the FFAs docosahexaenoic acid (DHA), palmitate, palmitoleate, and oleate (100 μM each) on *Gnrh* mRNA expression in the mHypoA-GnRH/GFP neuronal cell model. We report that 2 h exposure to palmitate or DHA increases *Gnrh* transcription. Using the inhibitors AH7614, K252c, U0126, wortmannin, and LY294002, we demonstrate that the effect of DHA is mediated through GPR120 to downstream PKC/MAPK and PI3K signaling. Our results indicate that the effect of palmitate may depend on palmitoyl-coA synthesis and PI3K signaling. Finally, we demonstrate that both DHA and palmitate increase *Gnrh* enhancer-derived RNA levels. Overall, these studies provide evidence that GnRH neurons directly sense FFAs. This will advance our understanding of the mechanisms underlying FFA sensing in the brain and provides insight into the links between nutrition and reproductive function.

1. Introduction

Reproduction and nutrition are closely coupled, but we do not fully understand the molecular mechanisms involved. The reproductive system is coordinated by the hypothalamic–pituitary–gonadal (HPG) axis, which comprises three regulatory cores: the hypothalamic gonadotropin-releasing hormone (GnRH) neurons, the anterior pituitary gonadotropes, and the gonads. These components process external and internal cues and communicate with each other to tightly regulate reproductive function. If and how the HPG axis detects nutrients, such as fatty acids, is a focus of ongoing research. Recent studies report that unsaturated free fatty acids (FFAs) directly regulate gonadotropin biosynthesis and secretion in LβT2 immortalized mouse gonadotropes and primary rat pituitary cell cultures (Sharma et al., 2013; Garrel et al., 2011). Moreover, it has also been reported that the FFAs palmitate, stearate, and oleate regulate cell proliferation and steroidogenesis in bovine gonadal granulosa cells (Vanholder et al., 2005). Currently, however, it is unknown if GnRH neurons also directly sense FFAs.

Long-chain fatty acids (LCFAs), FFAs with twelve or more carbon atoms, include saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), and polyunsaturated fatty acids (PUFAs). SFAs, MUFAs, and PUFAs seem to have distinct physiological functions. For instance, the SFA palmitate (C16:0) upregulates the oscillatory amplitude of *Bmal1* expression in mHypoE-37 hypothalamic neurons while the ω-3 polyunsaturated fatty acid (PUFA) docosahexaenoic acid (DHA; C22:6n-3) has no effect on *Bmal1* amplitude (Greco et al., 2014). Additionally, while palmitate stimulates pro-inflammatory signaling, the MUFAs oleate (C18:1n-9) and palmitoleate (C16:1n-7) both attenuate palmitate-induced pro-inflammatory signaling in neuronal cells and macrophages (Chan et al., 2015; Kwon et al., 2014).

The receptors GPR120 and GPR40 detect LCFAs (Hirasawa et al., 2005). DHA, for instance, binds and activates GPR120, which signals to activate phosphoinositide 3-kinase (PI3K) and protein kinase C (PKC) (Hirasawa et al., 2005; Oh et al., 2010). PKC subsequently activates the MAPK/ERK pathway (PKC/MAPK

* Corresponding author. Department of Physiology, University of Toronto, Medical Sciences Building 3344C, 1 King's College Circle, Toronto, ON M5S 1A8, Canada.
E-mail address: d.belsham@utoronto.ca (D.D. Belsham).

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pathway), and PI3K signals to induce Akt phosphorylation. In addition, SFAs such as palmitate can transactivate the toll-like receptor 4 (TLR4), which signals to activate the NF-κB pathway (Milanski et al., 2009). NF-κB, a transcription factor, regulates expression of target genes such as *Nfkbia* and *Nfkb1* (Sun et al., 1993; Ten et al., 1992). Recent reports have suggested that NF-κB may regulate *Gnrh* (Zhang et al., 2013). FFAs can also be metabolized to form other lipid signaling molecules; for example, palmitate is used in the biosynthesis of ceramides, which have been reported to regulate expression of genes such as *interleukin 6* (*Il6*) (Laulederkind et al., 1995). Fatty acid signaling in GnRH neurons and the direct effects of fatty acids on GnRH neuron function has not been studied, however.

The function of GnRH neurons is in part determined by GnRH biosynthesis (Clarke and Pompolo, 2005), which is transcriptionally regulated. *Gnrh* transcription is controlled by upstream regulatory elements: a proximal promoter (Nelson et al., 1998), a proximal enhancer (GnRH-E1) (Whyte et al., 1995), and two distal enhancers (GnRH-E2 and GnRH-E3) (Iyer et al., 2010). It has been reported that RNA polymerase II (RNA PII) occupies *Gnrh* enhancers and transcribes enhancer RNAs within GnRH-E1 (Iyer et al., 2011). eRNA expression positively correlates with enhancer activity and nearby gene expression (Kim et al., 2010). The function of eRNAs is currently being investigated (Natoli and Andrau, 2012), and it has been suggested that eRNAs may promote transcription by stabilizing chromatin-looping-mediated enhancer–promoter interaction (Li et al., 2013).

Studying the direct effects of fatty acids on GnRH neurons is difficult *in vivo*: the hypothalamus is a complex, heterogeneous tissue, and GnRH neurons are sparsely distributed across the hypothalamus (Gross, 1976). Moreover, the GnRH neuron population is heterogeneous (Constantin et al., 2013). Previous studies of GnRH neurons have used immortalized GnRH neuronal cell models, such as the GT1-7 and Gn11 cell lines. GT1-7 cells are GnRH-secreting neurons derived from a mouse neuronal tumor (Mellon et al., 1990), and Gn11 cells are immature, pre-migratory GnRH-secreting neurons (Radovick et al., 1991). These cell lines are clonal, however, and they may not represent adult GnRH neurons. Recently, our laboratory has established and described the adult mouse-derived, non-clonal GnRH-synthesizing and -secreting mHypoA-GnRH/GFP cell line (Gojska et al., 2014; McFadden et al., 2013). In brief, primary hypothalamic culture from a 2-month-old, female transgenic GnRH/GFP mouse was immortalized using the simian virus 40 T antigen (SV40 TAg); the cells were then fluorescence-activated cell sorted to generate an adult-derived, non-clonal population of GnRH/GFP-expressing neurons.

To show that GnRH neurons directly sense FFAs, we investigated the individual effects of DHA, palmitate, palmitoleate, and oleate on *Gnrh* mRNA expression in mHypoA-GnRH/GFP neurons. We report that DHA and palmitate upregulate *Gnrh* mRNA expression. Using PKC, MEK1/2, and PI3K inhibitors, we demonstrate that the effect of DHA is mediated by PKC/MAPK and PI3K signaling while the effect of palmitate is at least partially mediated by PI3K signaling. In addition, we used GPR120 and TLR4 antagonists to reveal that the effect of DHA is mediated by GPR120 and the effect of palmitate is not mediated by GPR120 or TLR4. We instead propose that palmitate-mediated increases in *Gnrh* mRNA expression depend on palmitate metabolism; however, by inhibiting *de novo* ceramide synthesis, we demonstrate that the effect of palmitate is not mediated by the palmitate metabolite ceramide. Finally, we provide evidence that DHA and palmitate increase *Gnrh* expression through transcriptional activation and that DHA and palmitate both increase expression of GnRH-E1-derived eRNA.

2. Materials and methods

2.1. Cell culture and reagents

mHypoA-GnRH/GFP cells were cultured in DMEM (Sigma–Aldrich; Oakville, ON, Canada) containing 5.5 mM glucose and supplemented with 5% FBS (Gibco, Burlington, ON, Canada) and 1% penicillin-streptomycin (Gibco), as previously described (Gojska et al., 2014; McFadden et al., 2013; Belsham et al., 2004). K252c, U0126, LY294002, Wortmannin, AH 7614, 5, 6-dichlorobenzimidazole riboside (DRB), TAK-242, fumonisin B_1, and PS1145 were purchased from Tocris Bioscience, Ellisville, MO, USA, and reconstituted in dimethyl sulfoxide (DMSO; Sigma–Aldrich). All inhibitors were used at concentrations previously reported by our laboratory, and utilized at levels that ensured specificity. Actinomycin D (ActD) and myriocin were purchased from Sigma–Aldrich and also reconstituted in DMSO. Inhibitor and agonist stock solutions were diluted in cell culture media for treatment. C16 ceramide (N-palmitoyl-D-erythro-sphingosine; Sigma–Aldrich) was prepared in 95% EtOH at 60 °C. Lipopolysaccharide (LPS; Sigma–Aldrich) was reconstituted in 1X phosphate-buffered saline (PBS), and was used at a final concentration of 100 ng/mL. Vehicle controls were used at 0. 1% DMSO and 0.01% EtOH as described. Basal GnRH mRNA levels were not significantly changed by vehicle controls at these concentrations over a 24 h timecourse.

2.2. Fatty acid preparation

Sodium palmitate, sodium oleate, and methyl palmitate (Sigma–Aldrich) stock solutions (100 mM) were prepared in molecular grade water (Thermo Scientific; Nepean, Ontario, Canada) by heating at 70 °C. In brief, sodium palmitate powder in solution was heated using a block heater buffered with water and set at 70 °C. Docosahexaenoic acid (DHA; Sigma–Aldrich) stock solution (100 mM) was prepared in DMSO. Palmitoleic acid (Sigma–Aldrich) stock solution (200 mM) was prepared in 50% EtOH at 60 °C. Fatty acid stock solutions were then diluted in heated cell culture media for treatment. Solubility was maintained in heated medium. Since the medium contained FBS, no further carrier molecule (ie. BSA) was required. It is important to note that previous studies from our laboratory and others has detected changes in gene expression with endotoxin-free BSA alone (Erridge and Samani, 2009), thus it was determined that LCFA without the BSA carrier was optimal for these experiments in cell culture, as reported by other studies (Huang et al., 2012). All concentrations of LCFAs were chosen after analysis of cell toxicity. Specifically, LCFAs at higher levels than 100 μM caused cell death in the mHypoA-GnRH/GFP neurons at 24 h.

2.3. Quantitative RT-PCR

Total RNA was isolated using the guanidium isothiocynate-phenol-chloroform extraction method with Turbo DNase (Ambion; Streetsville, Ontario, Canada) treatment, as previously described (Belsham et al., 2004), or the PureLink RNA Kit with on-column PureLink DNase (Ambion). cDNA was synthesized using the high-capacity cDNA reverse transcription kit (Applied Biosystems). 25 ng cDNA was amplified using a qRT-PCR master mix (Platinum SYBR Green qPCR SuperMix-UDG with ROX; Invitrogen), as previously described (Gojska et al., 2014). Samples were loaded in triplicate and run in an Applied Biosystems Prism 7000 Sequence Detection System machine. qRT-PCR data was analyzed using the standard curve method and normalized to histone 3a as a reference