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Abnormal hypothalamic oxytocin system in fibroblast growth factor 8-deficient mice

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Abstract

Oxytocin (OT) is a nonapeptide essential for maternal care. The development of the OT neuroendocrine system is a multi-step process dependent on the action of many transcription factors, but upstream signaling molecules regulating this process are still poorly understood. In this study, we examined if fibroblast growth factor 8 (FGF8), a signaling molecule critical for forebrain development, is essential for the proper formation of the OT system. Using immunohistochemistry, we showed a significant reduction in the number of neurons immunoreactive for the mature OT peptide in the paraventricular nucleus (PVN) and supraoptic nucleus (SON) in the hypothalamus of homozygous (HOMO) *FGF8* hypomorphic mice compared to wild-type mice. The number of neurons positive for oxyphysin prohormone in the SON but not the PVN was also significantly reduced in *FGF8* HOMO hypomorphs. However, steady-state mRNA levels of the *oxyphysin* prohormone were not significantly different between *FGF8* hypomorphs and WT mice. These data suggest that a global reduction in FGF8 signaling leads to an overall reduction of mature OT and oxyphysin prohormone levels that may have resulted from defects in multiple stages of the hormone-synthesis pathway. Since proper hormone synthesis is a hallmark of mature OT neurons, this study suggests that FGF8 signaling may contribute to the phenotypic maturation of a neuroendocrine system that originates within the diencephalon.

Keywords

Fibroblast growth factors (FGF); FGF8; Oxytocin; Oxyphysin; Neurophysin; Development

Introduction

One of the most well-characterized endocrine functions of the oxytocin (OT) system is to stimulate mammary myoepithelial contraction, which leads to milk letdown. OT-knockout mice produce milk but cannot nurse their pups, demonstrating a critical role of this neurohormone in maternal care [1]. OT acting within the brain has also been implicated in the onset of maternal behaviors such as nesting, sniffing and exploration of pups, pup retrieval, licking, and grooming [2]. Defects in the formation or maturation of this system could lead to disruption of behaviors critical for offspring survival.

OT neuron development involves a number of steps. Magnocellular neuroendocrine OT neurons are derived from the neuroepithelium lining the third ventricle by embryonic day 10.5 (E10.5). OT neurons then migrate either laterally to form the paraventricular nucleus (PVN) or ventro-laterally to form the supraoptic nucleus (SON) in the hypothalamus

(HYPO) from E10.5 to E14.5 [3–5]. After migration, OT neurons target their axons to the pars nervosa of the neurohypophysis, where the mature nonapeptide is secreted into the circulation. The proper formation and maturation of OT neurons are guided by precise spatial and temporal signaling. Many studies have shown that transcription factors such as *Otp*, the POU transcription factor *Brn2*, and the PAS domain transcription factors *Sim1* and *Arnt2* [6–8] are critical for the development of the PVN and SON. However, to our knowledge, no studies to date have examined the role of upstream signaling molecules in the formation of OT system in mice.

We hypothesize that members of the fibroblast growth factor (FGF) family may serve as signaling molecules for the maturation of OT neurons. FGFs are widely active in the developing central nervous system. This family of 22 ligands and 4 transmembrane receptors regulates neuronal growth, survival, migration, and patterning [9]. One of the FGFs, FGF8, has early developmental effects on central tissues such as the midbrain/hindbrain junction [10, 11] and the telencephalon [12]. Further, FGF8 is indispensable for the development of a peripheral structure, the olfactory placode, which gives rise to important neuroendocrine neurons [13, 14].

Less clear is the role of FGF8 in the development of neuroendocrine systems, such as the OT system, that originate in the HYPO. However, indirect evidence suggests such a role of FGF8 is possible. First, *FGF8* is expressed in the ventral diencephalon and infundibulum during development [15]. Second, FGF receptors are present on magnocellular SON and PVN neurons [16, 17]. Lastly, *FGF8* expression in the infundibulum modulates the induction of Rathke's pouch and differentiation of dorsal cell types of the adenohypophysis [18–20], thereby implicating *FGF8* in the development of the pituitary. It is therefore possible that FGF8 participates in the development of OT neurons that originate in the presumptive hypothalamic region adjacent to the *FGF8*-expressing infundibulum.

In this study, we investigated the role of FGF8 signaling in the development of OT neurons. The evidence that FGF8 is important for the development of the numerous structures, including the pituitary, led to our hypothesis that a reduction in FGF8 signaling may have deleterious effects on the development of OT neurons. Using the techniques of immunohistochemistry (IHC) and quantitative reverse transcriptase polymerase chain (qRT-PCR) reaction, we found significant defects in the OT system of mice deficient in FGF8 signaling, suggesting that FGF8 is involved in the phenotypic maturation of OT neurons.

Results

OT-immunoreactive neurons

In order to determine whether FGF8 signaling is critical for the normal presence of the OT system, IHC was performed to detect mature OT peptide-containing neurons in the PVN and SON of postnatal day (PN) 0 wild-type (WT), heterozygous (HET), and homozygous (HOMO) *FGF8* hypomorphic mice. Representative photomicrographs of the PVN and SON of each genotype are shown in Figs. 1a–c and 2a–c, respectively. OT-immunoreactive (IR) neurons were quantified in all PVN- and SON-containing sections from half of the section series (Fig. 3a–c). The mean PVN OT neuron counts \pm SEM for each genotype were 180 ± 9 in WT, 167 ± 17 in HET, and 71 ± 17 in HOMO. OT-IR neurons in the PVN of HOMO mice were significantly different between HOMO and WT, as well as between HOMO and HET (Fig. 3a). The mean SON OT neuron counts \pm SEM for each genotype were 56 ± 5 in WT, 28 ± 8 in HET, and 11 ± 3 in HOMO. OT-IR neurons in the SON of WT mice were significantly different between WT and HET, as well as between WT and HOMO (Fig. 3b). There was also a significant reduction in total OT-IR neurons in the HYPO (the sum of neurons in SON and PVN) in HOMO mice compared to WT and HET (Fig. 3c). During the

course of neuron counting, we did not notice any unusual ectopic accumulation of OT-IR neurons in the preoptic/hypothalamic regions of either HET or HOMO compared to WT (data not shown).

NP-1-IR neurons

The peptides NP-1 and OT are cleaved from the same prohormone. Whereas the anti-OT antiserum detects specifically the mature OT nonapeptide, the NP-1 antiserum can detect the unprocessed prohormone as well as the cleaved NP-1. This makes NP-1 an ideal marker for detecting oxytocinergic neurons regardless of whether the prohormone has been processed into mature OT. IHC using a NP-1 antibody was performed to examine if a reduction in OT-IR neurons in HOMO mice is accompanied by a parallel decrease in the NP-1-IR neurons. Representative photomicrographs of the PVN (Fig. 1d–f) and SON (Fig. 2d–f) and the quantification of NP-1-IR neurons in the PVN and SON of each genotype are shown in Fig. 3d–f. Sections used for NP-1 IHC represent half of the section series derived from the same animals used for OT IHC above. The mean PVN NP-1 neuron counts \pm SEM were 376 ± 23 in WT, 369 ± 20 in HET, and 307 ± 53 in HOMO. No significant differences in NP-1-IR neurons were found among the genotypes in the PVN (Fig. 3d). The mean SON neuron counts \pm SEM were 227 ± 15 in WT, 227 ± 18 in HET, and 96 ± 27 in HOMO. There was a significant reduction of NP-1-IR neurons in the SON of HOMO between HOMO and WT and HOMO and HET (Fig. 3e). There was also a significant reduction in total NP-1-IR neurons in the HYPO of HOMO mice when compared to WT (Fig. 3f). During the course of neuron counting, we did not notice any unusual ectopic accumulation of NP-1-IR neurons in the preoptic/hypothalamic regions of either HET or HOMO compared to WT (data not shown).

In order to verify that the lower number of OT-IR neurons compared to NP-1-IR neurons in pups of all genotypes was due to a developmental delay in peptide processing instead of an IHC artifact, we performed OT and NP-1 IHC in an adult animal. As expected, the number of OT-IR neurons in the PVN was comparable to that of NP-1-IR neurons in the adult brain (data not shown).

Oxyphysin prohormone mRNA

In order to examine whether the reduction in OT- and NP-1 neurons in *FGF8* hypomorphic mice was caused by reduced *oxyphysin* prohormone mRNA levels, qRT-PCR was performed on cDNA from the hypothalamus of PN0 mice. One-way ANOVA did not reveal significant differences ($P = 0.4360$) in hypothalamic *oxyphysin* prohormone mRNA levels among genotypes. The mean copy numbers ($\times 10^6$) of *oxyphysin* prohormone mRNA per microgram total RNA were 3.9 ± 0.31 in WT, 3.4 ± 0.19 in HET, and 3.5 ± 0.19 in HOMO (Fig. 4).

Discussion

The maturation of OT neurons involves a number of steps ranging from progenitor proliferation, migration, and axon path finding to mRNA transcription, prohormone synthesis and processing, and vesicular transport. These steps eventually culminate in a vesicular pool of releasable mature OT nonapeptide [21]. Dysregulation of any these steps could alter the functionality of the OT system. By demonstrating that *FGF8* hypomorphs had reduced OT-IR and NP-1-IR neurons in hypothalamic regions, this study suggests that *FGF8* signaling deficiency impairs the formation of the OT system. Both the PVN and SON are affected by *FGF8* hypomorphy, but the impact appears more severe in the SON. The levels of hypothalamic *oxyphysin* prohormone mRNA do not differ among genotypes, suggesting *FGF8* deficiency does not significantly alter the specification of OT neuronal

fate. These data are the first to demonstrate that *FGF8* is required for a normal population of OT neurons.

As detection of mature OT at PN0 is limited [22, 23], we used NP-1 IHC to examine whether *FGF8* hypomorphy led to reduced number of neurons able to synthesize the oxyphysin prohormone. Further, we performed IHC to examine whether the oxyphysin-synthesizing neurons acquired the ability to synthesize the mature OT, a process that primarily takes place after birth [21, 24]. We found that *FGF8* deficiency caused an overall reduction of NP-1-IR neurons in the HYPO of HOMO *FGF8* hypomorphs. As expected, this reduction was accompanied by a parallel reduction in OT neurons in the HYPO. However, the reduction of OT neurons in the SON of HET (Fig. 3a) and PVN of HOMO (Fig. 3b), neither of which exhibited reduced NP-1-IR neurons, raised the possibility that several post-transcriptional steps leading to the formation of mature OT may be affected by *FGF8* signaling deficiency.

At present, the most parsimonious explanation for our data is that [1] the translation of *oxyphysin* transcript into the oxyphysin prohormone and [2] the processing of oxyphysin prohormone into the mature OT peptide are both impaired in *FGF8* hypomorphs. We observed normal *oxyphysin* transcript levels in all genotypes, but the total number of neurons positive for NP-1 (also indicative of neurons positive for oxyphysin prohormone) was reduced in HYPO of *FGF8* hypomorphs, suggesting decreased efficacy of translation. After translation, OT is cleaved from NP-1 by a prohormone convertase. The resulting products are a three amino acid C-terminally extended OT (OT-Gly-Lys-Arg) and NP-1. The terminally extended OT is then cleaved sequentially by an endopeptidase and a carboxypeptidase to remove the remaining three amino acids. The resultant peptide is considered mature after the C-terminal amidation [25, 26]. In our study, normal numbers of NP-1-IR neurons in PVN of HOMO and SON of HET were accompanied by reduced OT-IR neurons, suggesting that the efficacy of this post-translational processing is also reduced. The finding that FGF signaling might influence post-translational processing events is not unprecedented. Like OT, gonadotropin-releasing hormone (GnRH) peptide is also cleaved from a larger prohormone. FGF2 treatment was reported to alter GnRH prohormone processing by increasing the proportion of C-terminally extended intermediate products [27].

In rats, the detection of mature OT lags behind that of oxyphysin prohormone during development. In fact, the C-terminally extended OT peptide is the prominent form of OT in the fetal brain with the final amidated peptide not becoming evident until birth [22, 24]. These results suggest that the capacity to process the oxyphysin prohormone into OT is associated with the phenotypic maturation of the neurons [22, 23] and can be used as a marker to gauge the maturity of these neurons. Our current data that NP-1-IR cells in all genotypes are consistently more numerous and intensely stained at PN0 than OT-IR neurons suggest murine OT neurons undergo similar phenotypic maturation around the time of birth. The lower efficacy of posttranslational processing in *FGF8* hypomorphs could therefore be a consequence of impaired or delayed phenotypic maturation of OT neurons. It would be ideal to examine whether this is a permanent defect or only a transient delay. However, our HOMO mice do not survive past PN0, thus other models of *FGF8* deficiency will need to be explored. At present, a prohormone processing defect is the most parsimonious explanation for the mismatch between NP-1-IR and OT-IR neuron numbers, but other possibilities such as disruption in the stability, folding, or transport of the mature OT peptide in *FGF8* hypomorphs cannot be ruled out.

An alternative hypothesis for the cause of reduced OT neuronal population in *FGF8* hypomorphs is that *FGF8* could promote the migration of OT neurons out of their birthplace,

the subventricular zone. Due to the greater distance of SON from the birthplace, the arrival of these neurons in SON may be impacted more than PVN when FGF8 is deficient. Supporting this, *FGF8* is important for the organization of the fibers that guide GnRH neuronal migration from the olfactory placode to the hypothalamus [28]. Further, *FGF8* is expressed near the presumptive SON in optic stalks, optic nerves, and chiasmatic area until E14.5 [15]. *FGF8* expression in these zones coincides temporally with the arrival of OT neurons in the SON, suggesting an involvement of FGF8 in the proper positioning of these neurons. However, if cells destined for the SON do not receive the proper signal to migrate, one might expect them to become arrested in or near the PVN. The lack of ectopic accumulation of OT neurons as well as the normal number of NP-1-IR neurons in the PVN did not support this hypothesis.

In this study, we demonstrated that FGF8 deficiency decreased the number of OT-IR and NP-1-IR neurons. The pattern of neuronal loss was similar between PVN and SON in that the significant decrease in OT-IR neurons was accompanied by the seemingly less affected NP-1-IR neurons in both nuclei. Although the precise mechanism underlying this neuronal loss is unclear, reduced efficacy in translational and post-translational events that ultimately decreases hormone production is the most plausible explanation. Further, the disruption in this OT synthesis pathway could be a consequence of impaired or delayed OT neuronal maturation resulting from FGF8 deficiency.

Materials and methods

Animals

FGF8 hypomorphic mice [29] (129p2/OlaHsd* CD-1; obtained from Mouse Regional Resource Centers, Davis, CA, USA) kept on a 12-h light and 12-h dark cycle and fed water and rodent chow ad libitum were bred in our animal care facility. These animals contain a neomycin-resistance element between Exons 1 and 2 of the *FGF8* gene. This element contains a false splice site which leads to a ~55% reduction in the level of functional *FGF8* transcript under HOMO condition [29]. Possibly due to the absence of olfactory bulbs [13] and inability to suckle, HOMO hypomorphs do not survive past the first day of birth. Therefore, pups were killed and genotyped on PN0. WT, HET, and HOMO animals are represented in this study. The brains were processed for IHC or qRT-PCR for the quantification of oxyphysin prohormone message. All animal procedures complied with the protocols approved by the Institutional Animal Care and Use Committee at the University of Colorado.

Immunohistochemistry (IHC)

Tissue preparation—For IHC, the brains were dissected and immersion-fixed in 5% acrolein for 5 h at room temperature and then cryoprotected in 30% sucrose until sectioning.

IHC—Two peptides, OT and neurophysin-1 (NP-1), are derived from the post-translational cleavage of the oxyphysin prohormone. In order to visualize neurons IR for these two peptides, 40- μ m coronal frozen sections through the hypothalamus were cut and used for IHC. One half of these sections were incubated with a mouse monoclonal anti-OT antibody (1:10,000, 4°C, 3 nights) (Millipore, Billerica, MA, USA) and the other half with a goat polyclonal anti-NP-1 antibody (1:500, 4°C, 3 nights) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The anti-OT antibody detects only the mature OT, whereas the anti-NP-1 antibody detects both NP-1 and oxyphysin prohormone. All sections were then incubated sequentially with a biotinylated secondary antibody (1:500), avidin–biotin complex (Vector Labs, Burlingame, CA, USA), and reacted with diaminobenzidine for color detection. Sections were then mounted on slides, counter-stained with methyl green, cleared with

Histoclear (National Diagnostics, Atlanta, GA, USA), and coverslipped. The negative controls included sections incubated with OT primary antibody pre-adsorbed with 10 μ M OT and without primary antibodies. The best antibody concentrations were chosen based on pilot studies conducted with three serial dilutions (1:5000, 1:10000, and 1:20000) of the primary antibodies.

Quantification of neurons and data analysis—An investigator blind to the identities of the slides quantified OT-IR and NP-1-IR neurons in PVN and SON. The total number of OT neurons in the PVN and SON were combined and called HYPO. In mice, it is also not possible to differentiate between the magnocellular and parvocellular OT neurons [30], thus all OT-positive neurons were scored. All data were analyzed by one-way ANOVA followed by Tukey's multiple comparison test. Differences were considered significant when $P < 0.05$.

Reverse transcriptase quantitative polymerase chain reaction

Tissue preparation—Pups were killed by decapitation and the brains were dissected and blocked to obtain the hypothalamus, which encompasses the brain region between the optic chiasm and caudal mammillary body. Hypothalamic blocks were immediately placed on dry ice and kept frozen at -70°C until processed.

RNA isolation and cDNA synthesis—Tissues were homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and RNA was isolated according to manufacturer's instructions. The concentration and quality of RNA were evaluated by spectrophotometric reading at wavelengths of 260 and 280 nm. 1 μ g of total RNA was used for the synthesis of cDNA using Superscript III First-Strand cDNA Synthesis kit (Invitrogen) following manufacturer's instructions.

qRT-PCR of oxyphysin prohormone transcript—For each PCR reaction, FastStart SYBR Green Master mix (Roche, Indianapolis, IN, USA) was combined with primers and sample cDNA or standard to achieve a final volume of 22 μ l. The standard used was a 433-bp oxyphysin prohormone cDNA inserted into pT7T3D-Pac vector (I.M.A.G.E. cDNA Clones, Invitrogen). Reactions were run in a 96-well plate in duplicates. The final reaction mixture consists of 4 μ l cDNA template, 18 μ l SYBR Green Master mix, and 0.25 μ M of forward and reverse primers each. The primer sequences were 5'-CAGGGCGAAGGCAGGTAGTT-3' for the forward primer, and 5'-GTCTCGCTTGCTGCCTGCTT-3' for the reverse primer (Integrated DNA Technologies, San Diego, CA, USA). The reaction was performed by a Mastercycler® ep *realplex** using a three-step PCR amplification program. The following program was used: hold at 95°C for 10 min prior to amplification, the denaturing (95°C), annealing (65°C), and elongation (72°C) phases were held at their respective temperatures for 30 s each and were repeated for 40 cycles. After amplification, a melting curve was included to verify amplification of the correct product.

Quantification and data analysis—The threshold of the Mastercycler was set to ten times the standard deviation above the noise of the automatic baseline. The data points of the exponential phase of the amplification curves were then used to calculate sample C_T values. The software used linear regression of the standard curve to determine the mean copies/well of the samples. An investigator blind to the sample identity excluded those that did not have any product in the melting curve, or whose melting curve profile did not match the standard amplicon. The mean copies/well for each sample was then normalized by the amount of starting RNA used for cDNA synthesis. Data were analyzed by one-way ANOVA

followed by Tukey's multiple comparison test. Differences were considered significant when $P < 0.05$.

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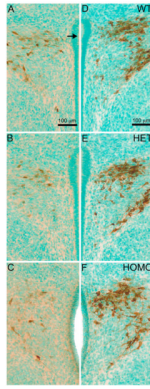


Fig. 1. Representative photomicrographs of OT-IR (**a–c**) and NP-1-IR (**d–f**) neurons in the PVN of PNO WT (**a, d**), HET (**b, e**), and HOMO (**c, f**) *FGF8* hypomorphs. *Brown color* represents OT immunostaining. *Green color* represents methyl green nuclear counterstain. Note the visual reduction of OT-IR neurons in (**b**) and (**c**) compared to **a**. *Arrow points* toward the third ventricle. *Scale bar* = 100 μ m. (Color figure online)

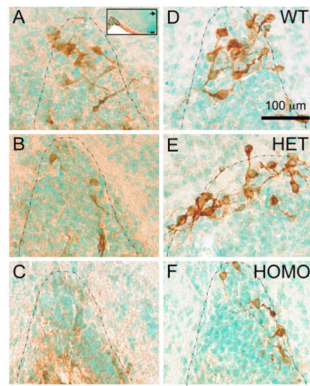


Fig. 2.

Representative photomicrographs of OT-IR (**a–c**) and NP-1-IR (**d–f**) neurons in the SON of PNO WT (**a, d**), HET (**b, e**), and HOMO (**c, f**) *FGF8* hypomorphs. The boundary of SON is delineated by the *dotted line*. *Brown color* represents OT immunostaining. *Green color* represents methyl green nuclear counterstain. Inset in (**a**) is a low-magnification photomicrograph of the same section, with the SON delineated by *dotted line*. Note the visual reduction of OT-IR neurons in (**b**) and (**c**) compared to (**a**), and the reduction of NP-1-IR neurons in (**f**) compared to (**d**) and (**e**). *Arrow points* toward the third ventricle. *Scale bars* = 100 μm . (Color figure online)

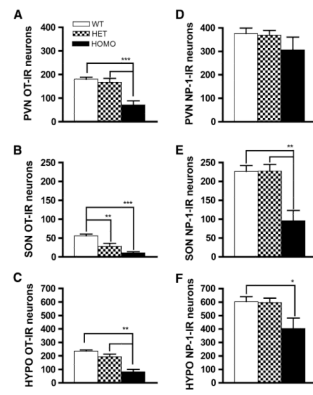


Fig. 3. Quantification of OT-IR (a–c) and NP-1-IR (d–f) neurons in WT, HET, and HOMO *FGF8* hypomorphic mice. Data are represented as mean \pm SEM. $N = 6-7$ per genotype. Significant difference between groups is indicated by brackets above bars. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

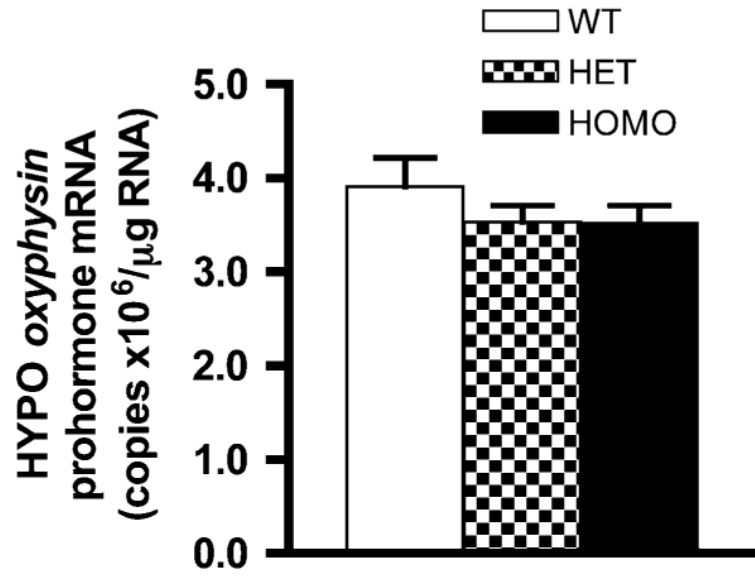


Fig. 4. Hypothalamic *oxyphysin* prohormone mRNA levels are not significantly different between genotypes. Data are represented as mean \pm SEM. $N = 6$ per genotype