Brief Communications

The Metabolic Regulator PGC-1α Directly Controls the Expression of the Hypothalamic Neuropeptide Oxytocin

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The transcriptional coactivator PGC-1α is a key regulator of cellular energy expenditure in peripheral tissues. Recent studies report that PGC-1α-null mice develop late-onset obesity and that the neuronal inactivation of PGC-1α causes increased food intake. However, the exact role of PGC-1α in the CNS remains unclear. Here we show that PGC-1α directly regulates the expression of the hypothalamic neuropeptide oxytocin, a known central regulator of appetite. We developed a unique genetic approach in the zebrafish, allowing us to monitor and manipulate PGC-1α activity in oxytocinergic neurons. We found that PGC-1α is coexpressed with oxytocin in the zebrafish hypothalamus. Targeted knockdown of the zebrafish PGC-1α gene activity caused a marked decrease in oxytocin mRNA levels and inhibited the expression of a transgenic GFP reporter driven by the oxytocin promoter. The effect of PGC-1α loss of function on oxytocin gene activity was rescued by tissue-specific re-expression of either PGC-1α or oxytocin precursor in zebrafish oxytocinergic neurons. PGC-1α activated the oxytocin promoter in a heterologous cell culture system, and overexpression of PGC-1α induced ectopic expression of oxytocin in muscles and neurons. Finally, PGC-1α forms an *in vivo* complex with the oxytocin promoter in fed but not fasted animals. These findings demonstrate that PGC-1α is both necessary and sufficient for the production of oxytocin, implicating hypothalamic PGC-1α in the direct activation of a hypothalamic hormone known to control energy intake.

Introduction

An animal's state of energy homeostasis is achieved when the energy it expends is balanced by the energy derived from the food it ingests. Hypothalamic neurons regulate energy intake by secreting hypothalamic neuropeptides that affect the feelings of hunger and satiety (Horvath, 2005; Lam et al., 2005). Prominent among these is the nonapeptide oxytocin, which has been found to regulate energy intake by reducing appetite (Leng et al., 2008; Maejima et al., 2009; Olszewski et al., 2010; Zhang et al., 2011). Oxytocinergic neurons residing in the paraventricular nucleus (PVN) of the hypothalamus transmit hypothalamic adiposity signals to the nucleus of the solitary tract (NTS), a brain area known to integrate satiety signals from the gut and hypothalamus (Verbalis et al., 1986; Kirchgessner et al., 1988; Rinaman, 1998; Blevins et al., 2004). Moreover, Prader-Willi syndrome (PWS), a condition characterized by deficits in oxytocinergic neurons, is known to cause hyperphagia and severe obesity (Swaab et al., 1995; Höybye et al., 2003).

At the cellular level, the energetic state of an individual cell is sensed and balanced by means of intracellular signaling pathways that regulate the transcriptional programs controlling energy expenditure (Cantó and Auwerx, 2009). The peroxisome proliferator-activated receptor gamma coactivator-1-α (PGC-1α, also known as PPARγC1α) is a transcriptional coactivator of many factors induced under conditions that cause energy expenditure, such as cold, fasting, and exercise (Lin et al., 2005).

Genetic perturbations of either oxytocin and its receptor or PGC-1α lead to similar abnormalities in the regulation of body weight and metabolism: Both PGC-1α$^{-/-}$ and oxytocin receptor–deficient mice exhibit impaired thermoregulation and develop late-onset obesity (Wu et al., 1999; Lin et al., 2004; Leone et al., 2005; Takayanagi et al., 2008). Defects in oxytocin secretion were found to affect food intake and cause anorexia (Maejima et al., 2009; Zhang et al., 2011). Accordingly, knock-out of the oxytocin peptide causes increased intake of animals' selected diets (Amico et al., 2005; Kasahara et al., 2007; Miedlar et al., 2007). Similarly, neuronal inactivation of PGC-1α leads to increased food intake (Ma et al., 2010).

Despite the above-mentioned evidence that PGC-1α and oxytocin regulate common physiological activities, a direct link between these factors has not been shown. We undertook a unique genetic approach in the zebrafish that allowed us to monitor and manipulate PGC-1α activity at single-cell resolution. We present here the first genetic and biochemical evidence that the gene encoding oxytocin is a direct target of PGC-1α.

Materials and Methods

Antibodies and transgenesis plasmid constructs. Rabbit polyclonal antibody directed to a conserved C-terminal peptide of PGC-1α was purchased from Abcam (Abcam, Cat. #ab54481). Anti-enhanced green fluorescent protein (EGFP) antibody was from Invitrogen. The Tol2kit transposon-based vector system (Kwan et al., 2007) was used for site-specific recombination-based cloning of cDNAs encoding the *oxtl* precursor and

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pgc-1α, downstream of the Gal4-responsive 10×UAS element and basal promoter and for constructing the oxytocinergic EGFP reporter and Gal4 driver. A multicistronic gene expression cassette, in which the viral 2A peptide sequence was placed between the gene of interest and mCherry fluorescent protein, was used to report the expression of the transgene (Provost et al., 2007). The *oxtl* promoter/enhancer fragment contained a 644 bp upstream region, including the first exon of the *oxtl* gene (Zv9 chr5:75,201,279–75,201,922).

RNA preparation, gene expression analyses, and transcriptional activation assay. *In situ* hybridization and immunostaining were performed as previously described (Blechman et al., 2007; Machluf and Levkowitz, 2011). Total RNA was prepared from fish larvae using the TRI-REAGENT (Molecular Research Center) according to the manufacturer's instructions. Quantitative PCR (qPCR) was performed using a 7300 real-time system (Applied Biosystems). Total cDNA was amplified using a SYBR Green qPCR kit (Finnzymes). The relative quantity (RQ) was normalized to a control sample and to the *β-actin* standard. Oligonucleotide primers for qPCR were as follows: *oxtl* (ccctccagaatccaccaatga; actaccagcatctagtcttac), *cs* (acgctgtgctgaggaagacaga; attgggaaggtgcttgaggg), *ucp1* (agagggacccacagctttctaca; cacgaacatcaccacgttcca), *prkaa1* (tgtgaggacgcagcaaaagg; gaggtaagagaagaggccag), and *β-actin* (gaggctctcttccagccttc; cggatgtccacgtcgcacttc). Transcriptional activation assay was performed in HEK-293T cells, which were transfected using calcium phosphate in the presence or absence of a PGC-1α expression vector together with pGL3-basic luciferase reporter under control of the *oxtl* promoter, and a renilla expression vector for normalization (Promega).

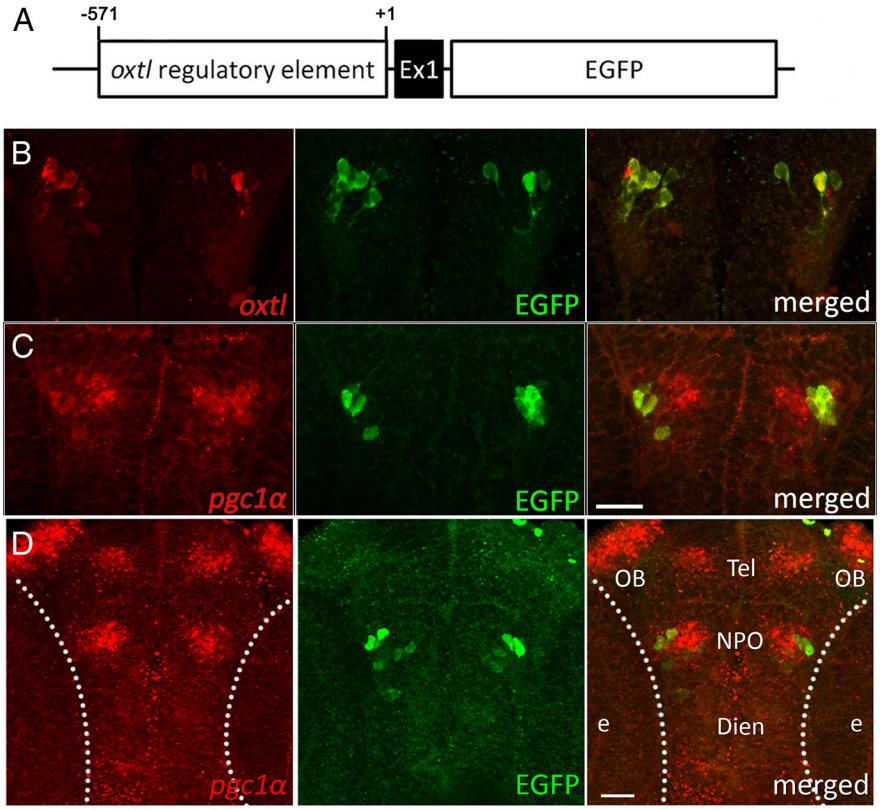

Figure 1. PGC-1α is expressed in oxytocinergic neurons. ***A***, Scheme depicting the transgenic DNA construct *oxtl*:EGFP. The transgenic vector contained a regulatory region including the first exon (Ex1) of the *oxtl* gene upstream to EGFP reporter. ***B***, ***C***, Single-plane confocal images of the NPO in zebrafish embryos harboring the *oxtl*:EGFP transgene. Embryos were subjected to fluorescent *in situ* hybridization with either *oxtl* (***B***) or *pgc*-1α (***C***) probes followed by anti-EGFP antibody to detect the EGFP oxytocinergic reporter. ***D***, Confocal *z*-stack of the zebrafish brain showing spatial expression of *pgc*-1α mRNA in the zebrafish Tg(*oxtl*:EGFP) reporter transgene. Dien, Diencephalon; e, eye; NPO, neurosecretory preoptic; OB, olfactory bulb; Tel, telencephalon. Scale bar, 25 μm.

Chromatin immunoprecipitation. Pools of 50 larvae (of either sex) per treatment or adult male brains were cross-linked with 2.2% formaldehyde for 20 min at room temperature (RT), washed twice with 0.125 M glycine and once with cold PBS, followed by homogenization in 0.6 ml of cell lysis buffer (10 mM Tris, pH 8.1, 10 mM NaCl, 0.5% NP-40), and incubation for 20 min on ice. After 5 min of centrifugation (3500 rpm), the pellet was transferred to a new tube, resuspended in 0.2 ml of lysis buffer (50 mM Tris, pH 8.1, 10 mM EDTA, 1% SDS), and incubated on ice for 20 min. The extracted volume was completed to 1 ml with IP buffer (16.7 mM Tris pH 8.1, 167 mM NaCl, 1.2 mM EDTA, 1.1% Triton, 0.01% SDS) and sonicated. A 10 μl sample of the soluble extract was analyzed on agarose gel to confirm an average size of 500 kb for the DNA. The extract was clarified by centrifugation for 15 min at 4°C and diluted threefold with buffer solution (20 mM Tris, pH 8.1, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100) to yield the solubilized chromatin. Next, salmon sperm DNA (50 μg/ml), tRNA1 (100 μg/ml), and bovine serum albumin (1 mg/ml) were added, and the extract was precleared by addition of 50 μl of protein A/G Sepharose suspension beads per milliliter and incubation for 30 min on a rotator at 4°C. After a 15 min centrifugation at 4°C, the extract was transferred to a new tube. For immunoprecipitation, 4 μl of anti-PGC-1α antibody or control IgG was added to 1 ml of the soluble chromatin and incubated overnight at 4°C. After centrifugation, the extracts were transferred to a new tube containing 30 μl of 50% protein A/G Sepharose suspension and incubated at 4°C for 1 h in a rotating wheel. The beads were then washed sequentially with TSE-150 mM NaCl (TSE is 20 mM Tris, pH 8.1, 2 mM EDTA, 1% Triton X-100, 0.1% SDS, containing 500 mM NaCl), TSE-500 mM NaCl, buffer III (10 mM Tris, pH 8.1, 0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA), and three times with TE (10 mM Tris, pH 8, 1 mM EDTA). Complexes were eluted by incubating the beads three times with 100 μl of elution buffer (1% SDS, 0.1 M $NaHCO_3$, 20 μg of glycogen per ml) for 10 min each. The combined eluates were heated overnight at 65°C to reverse the formaldehyde cross-links, extracted with phenol-chloroform followed by ethanol precipitation, and finally resuspended in 20 μl of TE. Immunoprecipitated DNA and 1.2% of the input DNA were subjected to qPCR analysis using primers specific to the *oxtl* promoter (gattagttcctgaaagctgt; cagtgaaccggtcactacac) or *oxtl* exon 4 (ccctccagaatccaccaatga; actaccagcatctagtcttac). Results were normalized to both input and control antibody.

Adult dietary regime. One-year-old male zebrafish (*Tupfel long fin*) were separated to individual 2 L tanks in a recirculating aquatic system under a 13 h light:11 h dark cycle. Fish were either fed twice a day with Artemia for 84 h or left unfed throughout the same period. Thereafter, the brains were dissected and subjected to either chromatin immunoprecipitation (ChIP) or mRNA qPCR analyses.

Bioinformatics and statistical analyses. Promoter regions of zebrafish (Zv9/danRer7 chr5:75,201,279–75,201,922), mouse (mm9 chr2:130,401,250–130,401,949), and human (hg19 chr20:3,051,606–3,052,305) oxytocin genes were extracted from the UCSC genome browser. The carp sequence was taken from GenBank (AF322651.2, 1157–1856). Multiple alignment was performed with the Muscle algorithm (version 3.8.31), and transcription factor binding site analysis was performed with MatInspector in the Genomatix Genome Analyzer package (Matrix Library version 8.3).

Statistical significances were determined by one-way ANOVA test followed by Tukey method for comparison of means using JMP software (SAS Institute). The nontreated controls of the various treatments were pooled together after we determined (by Tukey analysis) that there was no significant difference between the various control samples.

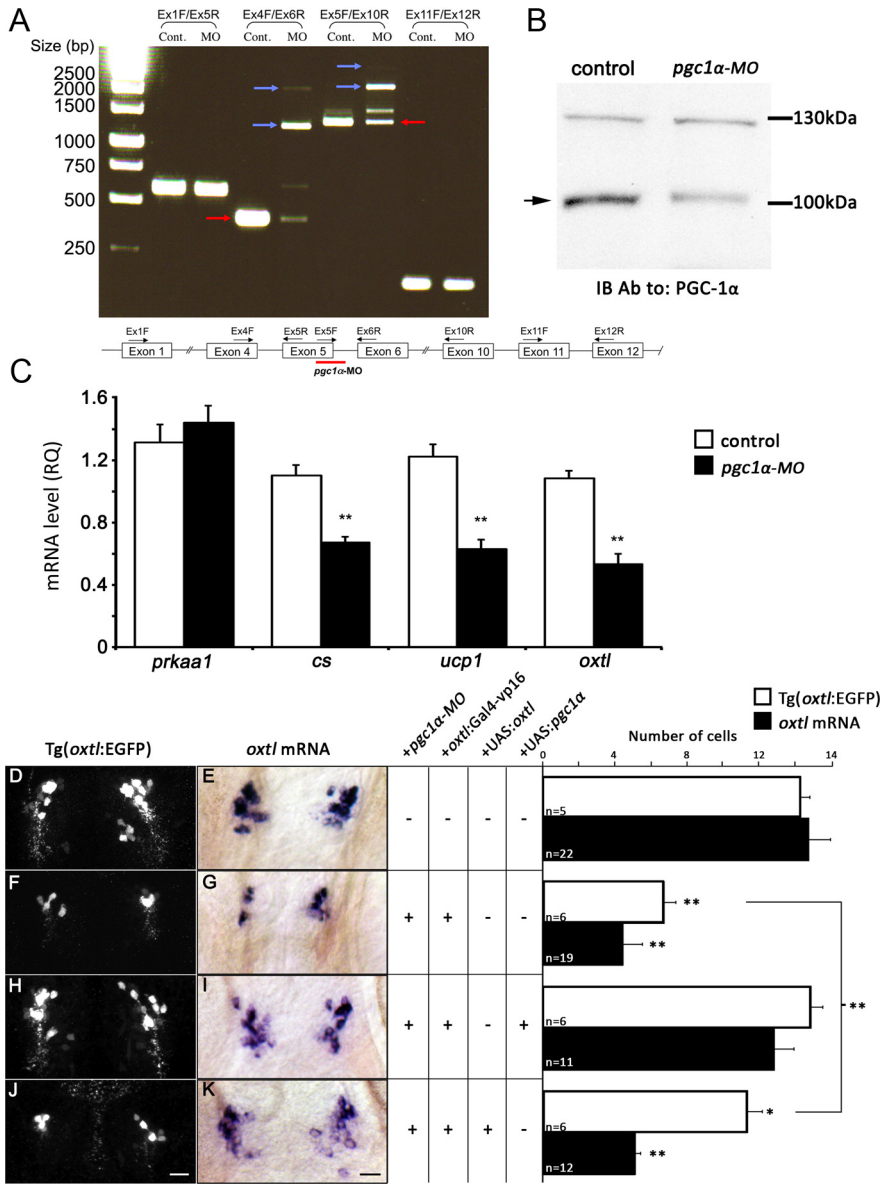

Figure 2. PGC-1α knockdown causes deficiency in oxytocin-positive neurons. ***A***, Gel electrophoresis image showing amplified mRNA splicing products in embryos injected with a synthetic splice-blocking antisense MO oligonucleotide (5′-CTGGCTGCCTGGCTCTCACCTCGCT-3′; Gene Tools) directed to *pgc-1α* (*pgc-1α*-MO; at 1.5 ng per 1.7 nl). Primer pairs used to amplify the respective exons (Ex) are indicated at the top. The red arrow points to the correctly spliced mRNA in the control (Cont.) treatment, whereas blue arrows indicate altered splicing products following injection of the *pgc-1α*-MO. Amplification of exons 1–5 (Ex1F/Ex5R) and exons 11 and 12 (Ex11F/Ex12R) was used as control to demonstrate untargeted constitutive exons. A scheme depicting *pgc-1α* gene structure, indicating the *pgc-1α*-MO binding site as well as the PCR primers used to amplify the various mRNA products, is shown below. ***B***, Reduction of PGC-1α protein expression following its knockdown. Proteins were extracted from a pool of 25 larvae per treatment. Equal amounts of the resulting protein extracts were subjected to 10% SDS-PAGE, followed by immunoblotting with an antibody directed to PGC-1α. The position of the ~105 kDa PGC-1α protein band is indicated by an arrow. ***C***, qPCR analysis of mRNAs encoding to AMP kinase α 1 catalytic subunit (*prkaa1*), citrate synthase (*cs*), uncoupling protein 1 (*ucp1*), and oxytocin (*oxtl*) in embryos (pools of 5 embryos per treatment) injected with antisense MO oligonucleotide directed against *pgc-1α* (*pgc-1α-MO*). $^{**}p < 0.05$, $n = 6$. ***D–K***, Representative images and histograms showing the expression of either EGFP protein or *oxtl* mRNA in mock-injected embryos (***D***, ***E***) and following microinjection of antisense MO oligonucleotide directed against *pgc-1α* (*pgc-1α*-MO; ***F–K***). Oxytocinergic-specific expression of PGC-1α and the oxytocin precursor were mediated by coinjections of transposon-based plasmid vectors containing 10 UAS elements upstream of either *pgc-1α* (***H***, ***I***) or *oxtl* (***J***, ***K***) cDNAs together with *oxtl*:Gal4 driver. $^{**}p < 0.05$, $^{*}p < 0.01$, scale bar, 20 μm.

Results

The physiological activities of fish oxytocin-like peptide (also known as isotocin) are conserved in all vertebrates [for review, see Peter and Fryer (1983) and Leng et al. (2008)]. To identify the molecular events underlying the synthesis of oxytocin, we screened the zebrafish model organism database (ZFIN) (Bradford et al., 2011) for transcriptional regulators expressed in the neurosecretory preoptic area (NPO), which includes neurons expressing the zebrafish homolog of oxytocin, termed oxytocin-like (Oxtl) (Blechman et al., 2007). One such candidate was the gene encoding the zebrafish ortholog of the transcriptional coactivator PGC-1α (LeMoine et al., 2010). We next generated a transgenic zebrafish line, Tg[*oxtl*:EGFP], that expresses EGFP in oxytocinergic neurons (Fig. 1*A*,*B*). This transgenic line facilitated high-resolution coexpression analysis of PGC-1α and Oxtl.

In situ hybridization of *oxtl* mRNA followed by immunostaining of EGFP verified the precise expression of our reporter transgene in *oxtl*+ neurons (Fig. 1*B*). Using this reporter line, we found that the expression of the gene encoding PGC-1α is highly enriched in the NPO, which includes the EGFP+ oxytocinergic neurons (Fig. 1*C*,*D*). Our analysis showed that nearly all oxytocinergic neurons contained the *pgc-1α* transcript (Fig. 1*C*).

As mentioned above, previous studies have implicated both PGC-1α and oxytocin in the regulation of energy balance. Given the known biochemical role of PGC-1α in regulating gene transcription, we hypothesized that PGC-1α may directly regulate the synthesis of oxytocin. To address this theory, we set out to analyze the expression of oxytocin mRNA following targeted gene knockdown of *pgc-1α*. We designed an antisense morpholino (MO) oligonucleotide, which causes an aberrant pgc-1α splice product and ~70% decrease in PGC-1α protein levels (Fig. 2*A*,*B*).

Knockdown of *pgc-1α* led to significant decrease in the expression of the mitochondrial genes citrate synthase (*cs*) and uncoupling protein 1 (*ucp1*), two known downstream effectors of PGC-1α (Lin et al., 2005) (Fig. 2*C*). As expected, *pgc-1α* knockdown did not affect the expression of the AMP kinase α1 catalytic subunit (*prkaa1*), which is an upstream regulator of PGC-1α (Cantó and Auwerx, 2009) (Fig. 2*C*). We further found that knockdown of *pgc-1α* led to a marked decrease in the expression of both *oxtl* mRNA and the *oxtl*:EGFP reporter transgene (Fig. 2*C–G*). These deficits could have been explained by either a decrease in *oxtl* promoter activity or a loss of oxytocinergic cells. To resolve these possibilities, we constructed a tissue-specific Gal4 driver, denoted *oxtl*:Gal4, that enables conditional gene expression in oxytocinergic neurons. Injection of a transposon-based transgenic vector *oxtl*:Gal4 together with a construct harboring the *pgc-1α* cDNA under the control of multiple Gal4 upstream activation sequences (10×UAS) rescued the deficits caused by *pgc-1α* knockdown; both

EGFP+ and *oxtl* mRNA+ neurons were rescued (Fig. 2*H*,*I*). In agreement with our notion that PGC-1α regulates *oxtl* transcription, re-expression of *oxtl* mRNA in oxytocinergic neurons of *pgc-1α* knocked-down animals (*oxtl*:Gal4;UAS:*oxtl*) rescued the *oxtl* mRNA deficit but did not recover the loss of EGFP expression (Fig. 2*J*,*K*). These results suggest that oxytocinergic neurons survive without *pgc-1α* gene activity but express compromised levels of the *oxtl* mRNA.

PGC-1α regulates cellular metabolism by means of its recruitment to transcriptional complexes that bind to the promoter regions of genes involved in energy expenditure (Lin et al., 2005; Cantó and Auwerx, 2009). We examined whether PGC-1α directly binds to and affects *oxtl* promoter activity. First, we performed a ChIP assay using an anti-PGC-1α antibody, followed by promoter-specific qPCR to detect *in vivo* binding of PGC-1α to the oxytocin promoter. This analysis showed that PGC-1α exists in complex with the *oxtl* promoter *in vivo* (Fig. 3*A*). In line with the aforementioned finding that PGC-1α knockdown leads to impaired *oxtl* mRNA expression, specific overexpression of PGC-1α in oxytocinergic neurons using the *oxtl*:Gal4 driving the UAS:*pgc-1α* cassette led to a threefold increase in the levels of *oxtl* mRNA (Fig. 3*B*). Moreover, experiments in a heterologous cell culture (HEK-293T) system, in which *pgc-1α* was cotransfected with a reporter construct harboring a luciferase gene under the control of the *oxtl* promoter, revealed a dose-dependent activation of the *oxtl* promoter by PGC-1α (Fig. 3*C*).

To examine whether overexpression of PGC-1α *in vivo* affects oxytocin promoter activity, we ectopically induced the expression of the UAS:*pgc-1α* cassette with the constitutive *ef1α*:Gal4 driver. This misexpression of PGC-1α in the trunk of zebrafish embryos induced ectopic expression of the *oxtl*:EGFP reporter transgene, which was detected in both muscles and neurons (Fig. 3*D*). Notably, overexpression of PGC-1α caused abnormal expression of the endogenous *oxtl* mRNA in ectopic locations in ~10–12% of the injected samples (Fig. 3*E*).

Expression of oxytocin mRNA is known to be regulated by feeding/fasting (Kublaoui et al., 2008; Tung et al., 2008). We examined whether the association of PGC-1α with the *oxtl* promoter is correlated with nutrient regulation of oxytocin mRNA in adult zebrafish. To this end, we performed a quantitative ChIP assay in fed versus fasted states. This analysis demonstrated that the association of PGC-1α protein with the proximal *oxtl* regulatory regions we identified is correlated with higher oxytocin mRNA levels in fed versus fasted states (Fig. 4*A*). The specificity of this association was further demonstrated by the lack of PGC-1α binding to a remote downstream region (exon 4) within the *oxtl* gene (Fig. 4*A*,*B*). Together our results demonstrate that PGC-1α regulates oxytocin expression by interacting with its promoter and activating *oxtl* transcription.

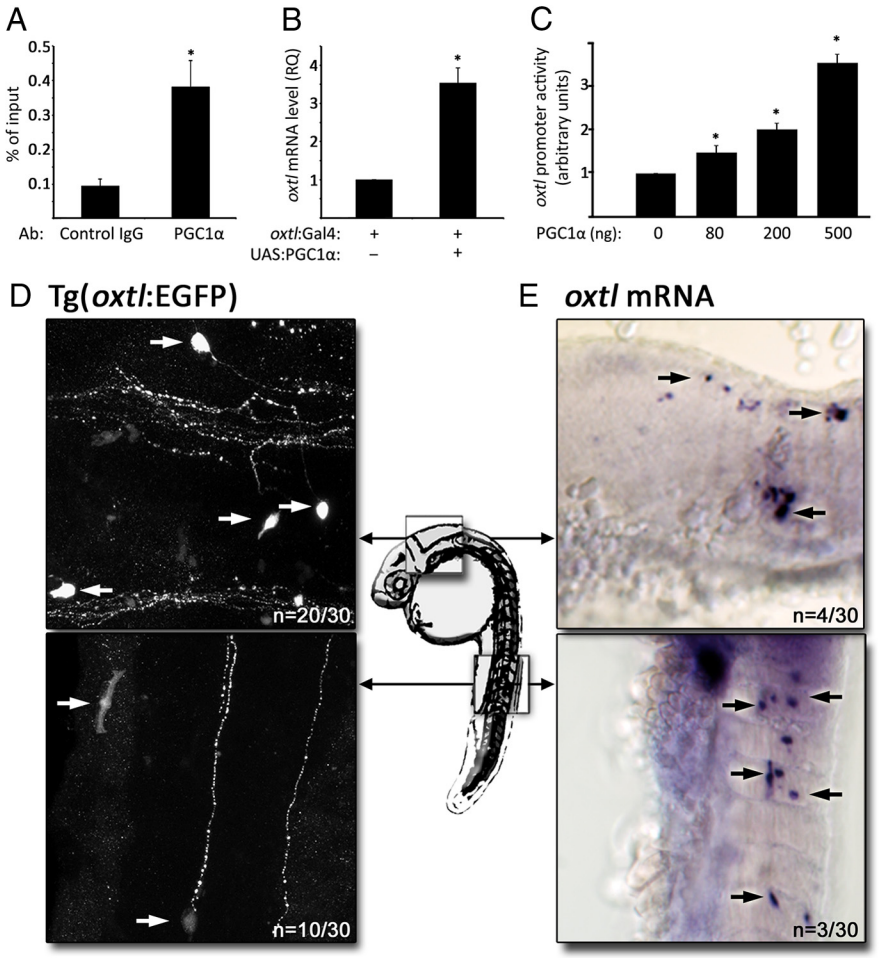

Figure 3. PGC-1α regulates oxytocin transcription. ***A***, Quantitative ChIP analysis of the recruitment of PGC-1α to the *oxtl* promoter. Chromatin was extracted from pools of 50 larvae per antibody followed by ChIP of either control IgG or anti-PGC-1α. $^*p < 0.05$, $n = 4$. ***B***, qPCR analysis of *oxtl* mRNA in embryos (pools of 10 larvae per treatment) injected with either transposon-based *oxtl*:Gal4 driver construct alone or together with a construct containing the Gal4-responsive 10 UAS driving the expression of PGC-1α. $^*p < 0.05$, $n = 4$. ***C***, Transcriptional activation measurements of the *oxtl* promoter showing relative luciferase reporter activity following transient transfection to HEK-293T cells of either *oxtl*:luciferase construct alone or together with increasing concentrations of a plasmid containing PGC-1α under the control of a viral CMV promoter. $^*p < 0.05$, $n = 8$. ***D***, ***E***, Gain of function of PGC-1α in ectopic locations. Transgenic *oxtl*:EGFP embryos were injected with a construct containing Gal4 under the control of the constitutive *ef1α* promoter together with a UAS:*pgc-1α* construct. Ectopic expression of the *oxtl*:EGFP transgene (***D***) or *oxtl* mRNA (***E***) in the hindbrain and trunk (arrows) was analyzed 24 h after injection. Scale bars: 25 μm in ***D***, 50 μm in ***E***.

Discussion

Neuropeptide production is a key step of terminal differentiation of hypothalamic neurons and is critical for physiological adaptation to homeostatic challenges. In the zebrafish, each brain hemisphere contains a single cell cluster of merely 10–20 oxytocinergic neurons, as compared to larger clusters of cells containing tens of thousands of neurons in mammals (Machluf et al., 2011). We took advantage of this feature to study the regulation of oxytocin synthesis at a single-cell resolution. Our study identifies a novel biochemical mechanism for the transcriptional regulation of the hypothalamic peptide oxytocin by PGC-1α, which is a major intracellular regulator of cellular metabolism.

The activity of PGC-1α in the brain is primarily associated with its role in supplying the high energy demands of neurons (Lin et al., 2004; Leone et al., 2005). It has been recently shown that genetic ablation of PGC-1α leads to impaired induction of orexigenic peptides, such as agouti-related protein (AgRP) and neuropeptide Y (NPY), under fasting conditions (Ma et al., 2010). These results are consistent with the idea that PGC-1α coordinates the expression of

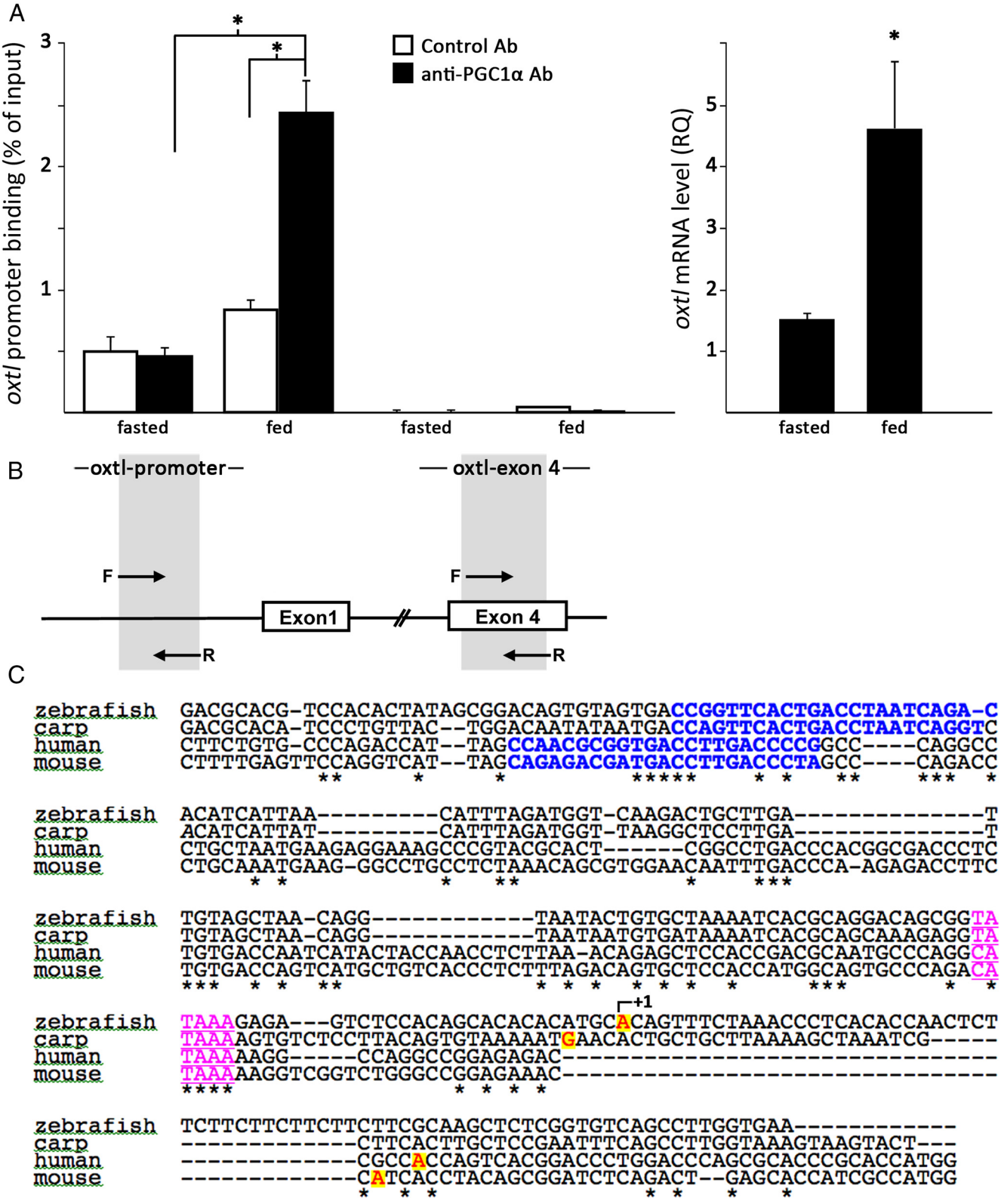

Figure 4. Nutrient-dependent association of PGC-1α with the *oxtl* promoter. ***A***, Histograms showing quantitative ChIP analyses of the recruitment of PGC-1α to the *oxtl* promoter (left) and *oxtl* mRNA level (right) in brain extracts derived from 1-year-old fasted and fed fish. Ab, Antibody. *$p < 0.05$, $n = 8$. ***B***, A scheme depicting *oxtl* gene structure indicating the respective positions of the forward (F) and reverse (R) primers used for quantitative ChIP analysis. ***C***, Comparative analysis of oxytocin promoter regions. A multiple alignment of human, mouse, zebrafish, and carp is shown. The conserved RORα site in bold type blue letters is taken from MatInspector predictions. Transcription start sites are highlighted in red with yellow background and the TATA box is in underlined magenta.

hypothalamic neuropeptides to cope with changes in energy levels in the body (Coppari et al., 2009). However, a direct interaction between PGC-1α and the promoter regions of these hypothalamic peptides has never been demonstrated. Our study now provides strong genetic and biochemical evidence showing that PGC-1α is both necessary and sufficient for the production of the anorexigenic neuropeptide oxytocin in the zebrafish hypothalamus.

An important question is whether the regulation described in this manuscript is conserved in mammals. Comparative oxytocin promoter analysis of two mammals (human and mouse) and two fish (zebrafish and carp) revealed a conserved putative transcription factor binding site for retinoid-related orphan receptor α (RORα) (Fig. 4*C*). This binding site is homologous to the previously identified composite hormone response element, which mediates the transactivation of the rat oxytocin gene (Adan et al., 1993). RORα is known to be coactivated by PGC-1α and mediates the effects of PGC-1α on metabolic and clock gene expression; the former is implicated in the direct activation of the mouse oxytocin promoter (Chu and Zingg, 1999; Lau et al., 2004; Liu et al., 2007). It remains to be determined whether neuronal PGC-1α and RORα also regulate the expression of oxytocin and other anorexigenic neuropeptides during energy surplus conditions.

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