Biological Psychiatry: Celebrating 50 Years

Archival Report

De Novo Protein Synthesis Mediated by the Eukaryotic Elongation Factor 2 Is Required for the Anxiolytic Effect of Oxytocin

Stefanie Martinetz, Carl-Philipp Meinung, Benjamin Jurek, David von Schack, Erwin H. van den Burg, David A. Slattery, and Inga D. Neumann

ABSTRACT

BACKGROUND: The neuropeptide oxytocin (OXT) mediates its actions, including anxiolysis, via its G protein–coupled OXT receptor. Within the paraventricular nucleus of the hypothalamus (PVN), OXT-induced anxiolysis is mediated, at least in part, via activation of the mitogen-activated protein kinase pathway following calcium influx through transient receptor potential cation channel subfamily V member 2 channels. In the periphery, OXT activates eukaryotic elongation factor 2 (eEF2), an essential mediator of protein synthesis.

METHODS: In order to study whether OXT activates eEF2 also in neurons to exert its anxiolytic properties in the PVN, we performed in vivo and cell culture experiments.

RESULTS: We demonstrate that OXT, in a protein kinase C–dependent manner, activates eEF2 both in a hypothalamic cell line and in vivo within the PVN. Next, we reveal that OXT stimulates de novo protein synthesis, while inhibition of protein synthesis within the PVN prevents the anxiolytic effect of OXT in male rats. Moreover, activation of eEF2 within the PVN conveyed an anxiolytic effect supporting a role of OXT-induced eEF2 activation and protein synthesis for its anxiolysis. Finally, we show that one of the proteins that is upregulated by OXT is the neuropeptide Y receptor 5. Infusion of a specific neuropeptide Y receptor 5 agonist into the PVN consequently led to decreased anxiety-related behavior, while pretreatment with a neuropeptide Y receptor 5 antagonist prevented the anxiolytic effect of OXT.

CONCLUSIONS: Taken together, these results show that OXT recruits several intracellular signaling cascades to induce protein synthesis, which mediates the anxiolytic effects of OXT within the PVN and suggests that eEF2 represents a novel target for anxiety-related disorders.

Keywords: Anxiety, De novo protein synthesis, eEF2, NPY5R, Oxytocin, PVN

https://doi.org/10.1016/j.biopsych.2019.01.010

The neuropeptide oxytocin (OXT) is currently discussed as a treatment option for a series of psychiatric disorders owing to its prosocial, stress-attenuating, and anxiolytic properties (1–3). OXT, when infused into the paraventricular nucleus of the hypothalamus (PVN), reduces anxiety- and fear-related behaviors in both rats and mice (4–8). The neuronal connections underlying its anxiolytic and fear-attenuating actions within various brain regions have progressively been revealed (4,8–10). In parallel, knowledge of intraneuronal signaling pathways coupled to the OXT receptor (OXTR) that underlie anxiolysis is growing, but gaps remain in our understanding concerning the molecular mechanisms that causally link OXTR recruitment and anxiolysis (7). Therefore, in order to fully establish OXT as a potential treatment option, the complete pathways, their target genes, and their contribution to the acute or longer lasting anxiolytic effects of OXT need to be revealed.

The principle effect of OXT upon binding to its G protein–coupled receptor (GPCR) is generation of inositol-3-phosphate, which triggers calcium ion (Ca^{2+}) release from intracellular stores (7,11). However, GPCRs can engage additional intracellular pathways that would be beyond their reach based on Gα and Gβ/γ subunit recruitment per se. A potential explanation is the formation of receptor heterodimers or transactivation of other receptors that are able to recruit additional pathways depending on the cell type–specific repertoire of the expressed GPCRs (12–16). Such flexibility would enable OXT to induce appropriate biochemical and behavioral responses to meet the demands imposed by the internal or external milieu.

To address the effectors underlying the anxiolytic properties of OXT, we have already partially elucidated the intracellular signaling coupled to activation of the OXTR in the PVN. For instance, OXTR activation leads to increased intracellular Ca^{2+} levels through influx of extracellular Ca^{2+} via transient receptor potential cation channel subfamily V member 2 (TRPV2) channels (17). This is a prerequisite for the activation of mitogen-activated protein kinase kinase 1 and 2 (MEK1/2) and the subsequent anxiolytic effect of OXT (5,18). OXT can also

 ISSN: 0006-3223

act, although not in the context of anxiety, via neuronal TRPV1 (19).

In peripheral cells, OXT has been shown to engage additional intracellular signaling pathways. For example, in myometrial cells, the recruitment of eukaryotic elongation factor 2 (eEF2) in a protein kinase C (PKC)–dependent manner is necessary for uterine contractility and successful parturition (20). Dephosphorylation of eEF2, or inhibition of phosphorylation at threonine 56, is equivalent to eEF2 activation, whereas eEF2 is generally inactive in its phosphorylated form (p-eEF2). In neurons, eEF2 is a key factor controlling dendritic protein synthesis from local messenger RNA (mRNA) pools, which might be important for synaptic plasticity (21,22). However, it is presently not known whether OXTR activation promotes the activity of eEF2 and subsequent protein synthesis in neurons. As eEF2 activation depends on OXTR-coupled PKC in myometrial cells (7), this hypothesis seems likely. In addition to PKC, mitogen-activated protein kinase (MAPK) can activate eEF2, as shown in cardiomyocytes (23,24). This finding is of particular interest, since we have shown that OXT exerts its anxiolytic activity through transactivation of the epidermal growth factor receptor (EGFR) (5) and subsequent recruitment of MAPK (5,17,18). While the precise mechanisms underlying EGFR transactivation have not been fully elucidated, an involvement of Ca^{2+} and PKC has been repeatedly implicated (25). Moreover, PKC directly activates Raf-1 proto-oncogene, serine/threonine kinase, both in vitro and in vivo (26), and regulates Raf kinase inhibitory protein, leading to MAPK activation (27). Thus, it is possible that both TRPV2 channel opening and PKC activation are upstream of MAPK recruitment by OXT, and hence both play a role in the anxiolytic activity of OXT.

Here we reveal that OXT, via two distinct pathways, activates eEF2 and subsequent protein synthesis. In detail, 1) activation of the OXTR leads—via the $G\alpha_q$ subunit of the G protein—to the activation of PKC. Additionally, 2) via $G\beta/\gamma$ recruitment, OXT prompts the MAPK pathway to induce full eEF2 translational activity. Having shown that OXT stimulates protein synthesis, we performed a microarray to identify candidate targets for the OXT-mediated protein synthesis. While numerous genes within the rat PVN were regulated by intracerebroventricular (icv) OXT, we identified that neuropeptide Y (NPY) receptor 5 (NPY5R) expression was upregulated following OXT administration in vitro and in vivo, and that NPY5R activity is sufficient for anxiolysis in general and necessary for the anxiolytic effect of OXT in particular.

METHODS AND MATERIALS

Animals and Surgical Procedures

Adult male Wistar rats (250–300 g) (Charles River Laboratories, Sulzfeld, Germany) or virgin female Wistar rats (for microarray analysis only) (Charles River Laboratories) were housed under standard laboratory conditions (12-hour light/dark cycle, 22°C–24°C, lights on at 6 AM, food and water ad libitum). All behavioral tests were performed in the light phase (8 AM to noon). The studies were conducted in accordance with the European Communities Council Directive (86/609/EEC) and approved by the government of Oberpfalz, Germany (AZ 54–2531.2–16/08). Surgical procedures were performed under isoflurane anesthesia, local anesthesia, and sterile conditions, supplemented by analgesic (buprenorphine, 0.05 mg/kg; Bayer, Leverkusen, Germany) and antibiotic (enrofloxacin; Bayer) treatments. After surgery, animals were single housed and handled daily to habituate them to the respective infusion procedure.

For analysis of neuronal responses to icv OXT or local intervention within the PVN, rats were implanted with a guide cannula (21 gauge, 12-mm length) 2 mm above the right lateral ventricle or above the target brain region (PVN) as described previously (5,18,28).

Animal Experimental Protocols

For signaling studies, animals received icv infusions of either vehicle (Veh) or 1 nmol/5 μL OXT and were killed 10, 20, 30, 60, or 90 minutes later. Punches were taken from a brain slice of 2 mm (anterior/posterior bregma −1 to −2) with a tissue puncher (2-mm diameter). Proteins were extracted from PVN tissue and quantified by Western blot (Supplement 1).

To determine whether protein synthesis in general, and NPY5R synthesis in particular, is necessary for OXT to exert its anxiolytic effect, separate groups of rats received bilateral intra-PVN infusions of the protein synthesis inhibitor anisomycin (29), the NPY5R antagonist L152,804 (0.82 nmol/0.5 μL in 0.1% dimethyl sulfoxide) (30), or the respective Veh 20 minutes prior to OXT or Veh infusion (0.01 nmol in Ringer's solution) (5,17). Thirty minutes after the last infusion, anxiety-related behavior was tested in the light/dark box (LDB) as previously described (17,18).

To reveal the specific involvement of eEF2 activation in the regulation of anxiety-related behavior, separate groups of rats received bilateral intra-PVN infusions of the eEF2 kinase inhibitor A484954 (0.005 nmol in 0.1% dimethyl sulfoxide; MilliporeSigma, Burlington, MA) (31) or Veh before testing in the LDB 10 minutes later.

To reveal the effects of OXT on gene expression levels within the PVN (RNA microarray), separate groups of rats received an icv infusion of 1 nmol/5 μL OXT or Veh, and they were killed 30 minutes later (see below and Supplement 1).

To assess whether NPY5R activation within the PVN modulates anxiety-related behavior, separate groups of rats received bilateral intra-PVN infusions of the NPY5R agonist [cPP]hPP (0.02 nmol in Ringer's solution; Bachem, Bubendorf, Switzerland) (32) or Veh and were tested in the LDB 10 minutes later.

Cell Culture and Experimental Protocols

Cell culture experiments were performed with either the rat hypothalamic cell line H32 (33) or primary hypothalamic neurons (34,35) (see Methods in Supplement 1). Isolation of de novo synthesized proteins was performed in primary hypothalamic neurons and H32 cells based on tagging and/or isolation of a synthetic Methionine-analogue according to Dieterich *et al.* (36) (see Methods in Supplement 1).

For analyses of signaling pathways in vitro, H32 cells were stimulated with Ringer's solution (Veh) or 200 nmol/L OXT (5,17,18) in Ringer's solution (MilliporeSigma) for 10, 20, 30, 60, and 90 minutes. In addition, cells were preincubated for 10 minutes with either 1 μmol/L of the PKC inhibitor Gö6983, 10 μmol/L of the MEK1/2 blocker U0126, or Veh (all in 0.1%

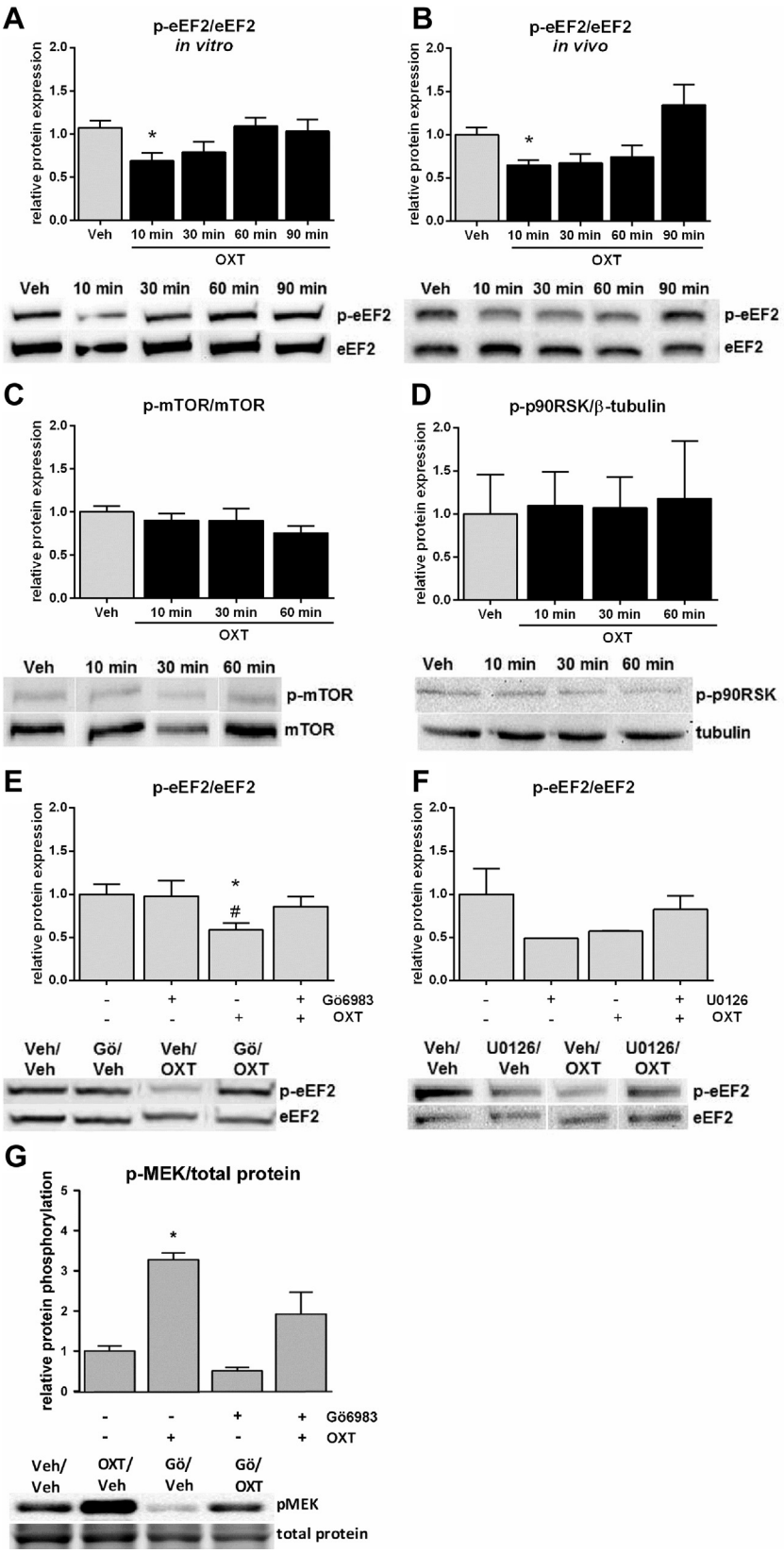

Figure 1. Eukaryotic elongation factor 2 (eEF2) is activated, i.e., dephosphorylated, by oxytocin (OXT) via the protein kinase C pathway. Time-dependent decrease of inactive eEF2 phosphorylated at threonine 56 (p-eEF2) **(A)** in hypothalamic cells (vehicle [Veh], $n = 10$; 10 minutes, $n = 5$; 30 minutes, $n = 4$; 60 minutes, $n = 4$; 90 minutes, $n = 4$) and **(B)** within the hypothalamic paraventricular nucleus of male rats (Veh, $n = 26$; 10 minutes, $n = 10$; 30 minutes, $n = 4$; 60 minutes, $n = 6$; 90 minutes, $n = 5$) after OXT stimulation (in vitro: 250 nmol/L; intracerebroventricular: 1 nmol/5 μL) compared with Veh treatment. **(C)** Levels of phosphorylated mammalian target of rapamycin (p-mTOR) or **(D)** phosphorylated p90 ribosomal s6 kinase (p-p90RSK) were not influenced by OXT. **(E)** Blockade of OXT-induced downregulation of p-eEF2 by preincubation of hypothalamic cells with Gö6983 (Gö), a protein kinase C inhibitor ($n = 3$ or 4). **(F)** The mitogen-activated protein kinase 1 and 2 (MEK1/2) inhibitor U0126 reduced p-eEF2 levels and prevented OXT-induced reduction of p-eEF2 levels in H32 cells ($n = 3$). **(G)** Blockade of OXT-induced MEK phosphorylation by preincubation of H32 cells with Gö6983 ($n = 3$). Bars represent mean + SEM, $^*p < .05$ vs. Veh, $\#p < .05$ vs. Gö6983/OXT.

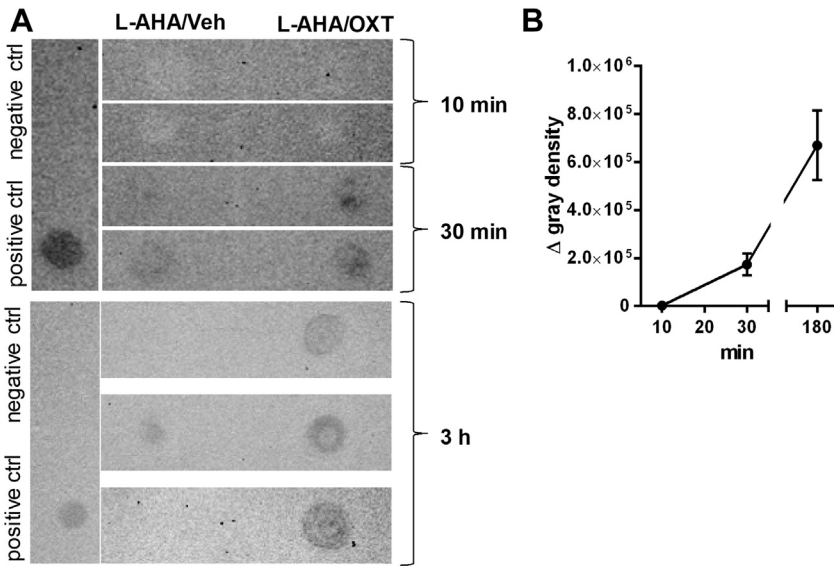

Figure 2. Oxytocin (OXT) increases de novo protein synthesis in hypothalamic cells. The synthetic amino acid L-azidohomoalanine (L-AHA) gets incorporated into newly synthesized proteins and is detected via a click azide–alkyne reaction with biotin alkyne. **(A)** Dot blot analysis of separate labeling experiments in primary hypothalamic cells 10, 30, and 180 minutes after OXT stimulation (250 nmol/L; $n = 2$ or 3). **(B)** Time-dependent increase of detectable newly synthesized proteins compared with vehicle (Veh)–treated cells. Line represents mean ± SEM ($n = 2$ or 3). ctrl, control.

dimethyl sulfoxide in Ringer's solution), before 200 nmol/L OXT or Veh was applied. To assess the effects of eEF2 activation on NPY5R protein levels, H32 cells were stimulated with the eEF2 kinase inhibitor A484954 (MilliporeSigma) 10 μmol/L or 50 μmol/L for 3 hours with Veh as control. In all cases, proteins were isolated 10 minutes after the last stimulation and subjected to Western blot (see Methods and Table S1 in Supplement 1).

Microarray and Complementary DNA Analysis

To identify genome-wide transcript expression changes within the PVN following OXT administration, rats received an icv infusion of either OXT or Veh, and PVN tissue was collected 30 minutes later for an Affymetrix microarray (Thermo Fisher Scientific, Waltham, MA) study (see Methods in Supplement 1). Subsequently, upregulation of *Npy5r* mRNA was confirmed by quantitative polymerase chain reaction quantification relative to the expression of glyceraldehyde-3-phosphate dehydrogenase (37); see Table S2 in Supplement 1 for primer sequences.

Statistics

Behavioral and biochemical data were analyzed using either a Student's *t* test, one-way analysis of variance followed by least significant difference post hoc test, or two-way analysis of variance (pretreatment; treatment) followed by least significant difference post hoc test using the software package SPSS (version 19.0; IBM Corp., Armonk, NY). Data represent mean and SEM; significance was accepted at $p < .05$.

RESULTS

OXT Activates eEF2 Through PKC and MEK1/2

To determine whether OXT recruits eEF2, we assessed the level of eEF2 phosphorylated at threonine 56, both in cell culture and in the PVN. The p-eEF2 level was reduced in H32 cells 10 minutes following incubation with 200 nmol/L OXT ($F_{4,27} = 3.104$, $p = .036$), indicating activation, and the level returned to baseline after 30 to 60 minutes (Figure 1A). Similarly, icv infusion of OXT transiently reduced p-eEF2 levels in the PVN within 10 minutes ($F_{4,51} = 4.336$, $p = .005$), which reversed to baseline from 30 minutes onward (Figure 1B). Thus, OXT activates eEF2 both in a hypothalamic cell line and within the PVN.

eEF2 phosphorylation and dephosphorylation are controlled by multiple intracellular messengers. In order to determine which messengers are employed by OXT, we analyzed the recruitment of the most regularly reported ones, including PKC, since it has already been shown to activate eEF2 upon OXT stimulation in myometrial cells (20). We found that the mammalian target of rapamycin and p90 ribosomal s6 kinase were not activated by OXT in H32 cells, as evidenced by a lack of alteration of their phosphorylation level, arguing against a role in the control of p-eEF2 levels (Figure 1C, D). In contrast, blocking PKC activity with Gö6983 abolished the effect of OXT on MEK1/2 ($F_{3,13} = 17.597$, $p < .001$) (Figure 1G) and eEF2 ($F_{3,13} = 5.697$, $p = .018$) phosphorylation in H32 cells (Figure 1E, F). This finding suggests a PKC-dependent signaling cascade that leads to the OXT-induced activation of MEK1/2 and subsequent dephosphorylation (or reduced phosphorylation) of eEF2. However, while the MEK1/2 blocker U0126 applied alone reduced p-eEF2 levels, it prevented OXT-induced eEF2 dephosphorylation ($F_{3,13} = 7.937$, $p = .037$) (Figure 1F). Together these findings suggest that eEF2 is under inhibitory control of MEK1/2 under basal conditions and that MEK1/2 activation by OXT removes this brake. This is consistent with earlier observations that OXT-induced MEK1/2 activation exerts actions that are quite different from those exerted by MEK1/2 that is active in the absence of OXT. The most striking example is the lack of phosphorylation of extracellular signal-regulated kinases 1 and 2 under OXT (18), despite this representing the most consistently characterized downstream consequence of MEK1/2 activation (38). Together, these results suggest that eEF2 is dephosphorylated

(or less phosphorylated) through direct coupling of OXTR activation to PKC and subsequent transactivation of the EGFR and MEK1/2 recruitment.

OXT Promotes Protein Synthesis in Hypothalamic Cells

Given that eEF2 is an important regulator of protein synthesis, we next determined whether OXT administration leads to increased protein synthesis in vitro. By measuring the incorporation of the synthetic amino acid L-azidohomoalanine into newly synthesized proteins, we show that OXT increased protein synthesis within 30 minutes until at least 3 hours (p = .01 for primary cells; p = .003 for H32 cells) (Figure 2). Basal protein synthesis in Veh-treated H32 cells showed a slow increase of newly synthesized proteins over time, becoming detectable at the 3-hour time point, which reflects the phenomenon of permanent cell activity in immortalized cell lines (39). Thus, OXT does indeed stimulate protein synthesis in hypothalamic cells.

OXT-Induced Protein Synthesis Is Involved in Anxiolysis

To assess whether de novo protein synthesis is involved in OXT-induced anxiolysis after 30 minutes, male rats were pretreated with the protein synthesis inhibitor anisomycin, or Veh, and then tested in the LDB. We could confirm the established anxiolytic effect of intra-PVN OXT (5,18), as reflected by an increased time spent in the light chamber ($F_{3,43}$ = 4.824, p = .006; Veh/OXT vs. Veh/Veh, p = .042) (Figure 3A). Anisomycin alone did not affect behavior, but it did block the anxiolytic effect of OXT (Veh/OXT vs. anisomycin/OXT, p = .002) (Figure 3A, B). Thus, the anxiolytic effect observed 30 minutes after intra-PVN OXT infusion requires active protein synthesis.

Direct eEF2 Activation Within the PVN Is Anxiolytic

Next, we hypothesized that increased protein synthesis via eEF2 activation is anxiolytic, and we tested whether inhibition of eEF2 kinase, resulting in activation of eEF2, is sufficient to decrease anxiety. Bilateral infusion of the eEF2 kinase inhibitor A484954 into the PVN increased the number of entries into the light chamber and reduced the latency to re-enter the light chamber of the LDB ($p < .05$). Furthermore, we found a tendency toward an increase in the time spent in the light chamber (p = .065) (Figure 3B). Thus, stimulation of protein synthesis within the PVN, via activation of eEF2, is sufficient to reduce anxiety-like behavior.

Determination of Gene Expression and Proteins Affected by Intra-PVN OXT Administration

In icv OXT-treated rats, we identified 157 upregulated and 204 downregulated known genes within the PVN (see Figure 4, Table 1, and Table S3 in Supplement 2 and the Gene Expression Omnibus DataSet). Among the upregulated genes, we focused on NPY5R, since the NPY system, especially those mechanisms involving NPY1R and NPY5R, has been implicated in the regulation of anxiety-like behavior (32,40). We confirmed, via quantitative polymerase chain reaction and Western blot analysis, that OXT increases both NPY5R gene expression and protein synthesis 30 minutes and 180 minutes

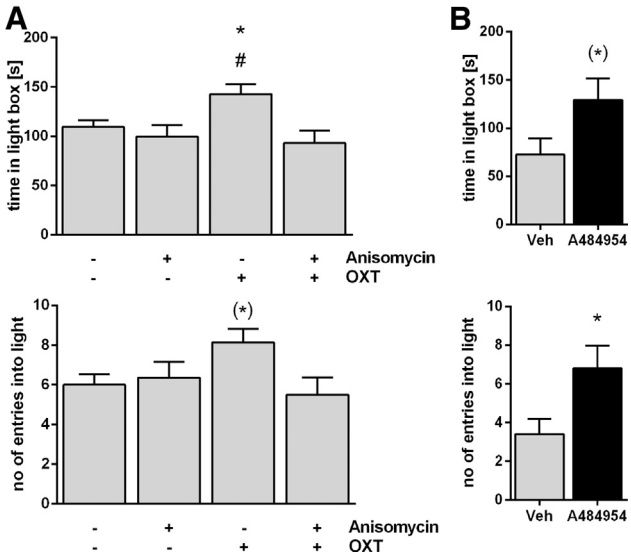

Figure 3. Local inhibition of protein synthesis by anisomycin abolishes the anxiolytic effect of oxytocin (OXT) within the paraventricular nucleus, while activation of eukaryotic elongation factor 2 (and protein synthesis) decreases anxiety-related behavior in the light/dark box. **(A)** Intra–paraventricular nucleus infusion of the protein synthesis inhibitor anisomycin (23.5 μmol/0.5 μL) prior to OXT infusion (0.01 nmol/0.5 μL) abolishes the anxiolytic effect of OXT (−/−, n = 8; +/−, n = 11; −/+, n = 14; +/+, n = 10) indicated by a significant increase in time spent in the light box but no difference to basal levels in the presence of anisomycin. **(B)** Intra–paraventricular nucleus infusion of the eukaryotic elongation factor 2 kinase inhibitor A484954 (0.005 nmol/0.5 μL) decreases anxiety-related behavior as reflected by increased time spent in the light and more entries performed into the light box (n = 7–9). Bars represent mean + SEM, $^{*}p < .05$ vs. vehicle (Veh), $\#p < .05$ vs. anisomycin/OXT, $^{(*)}p \leq .065$. no, number.

after intra-PVN OXT infusion in male rats ($p < .05$) (Figure 5A). To assess whether direct eEF2 activation would mimic the OXT-induced increase in NPY5R protein levels, H32 cells were treated with the eEF2 kinase inhibitor A484954 for 3 hours. A484954 dose-dependently increased NPY5R protein levels, thereby showing a similar effect on NPY5R expression as OXT ($F_{2,15}$ = 13.706, $p < .001$; Veh vs. 10 μmol/L, p = .007; Veh vs. 50 μmol/L, $p < .001$) (Figure 6A).

NPY5R Activity Within the PVN Is Anxiolytic and Required for the Anxiolytic Effect of OXT

To assess whether NPY5R plays a role in anxiety-related behavior within the PVN, we bilaterally infused the specific NPY5R agonist [cPP]hPP into the PVN 10 minutes before testing rats in the LDB. Local [cPP]hPP increased the time spent in the light chamber of the LDB (p = .007) (Figure 5B) without changing locomotor activity. Next, we asked whether NPY5R is involved in OXT-induced anxiolysis. Male rats were pretreated with the selective NPY5R antagonist L152,804 or Veh prior to local OXT infusion, and then they were tested in the LDB. We replicated the anxiolytic effect of OXT, as reflected by a strong trend toward increased time spent in the light chamber of the LDB; while L152,804 alone did not affect any behavioral parameter, it blocked the anxiolytic effect of OXT ($F_{3,20}$ = 4.107, p = .02; Veh/Veh vs. Veh/OXT, p = .053;

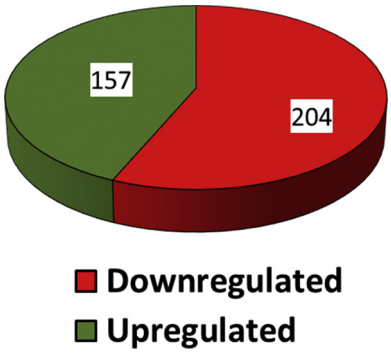

Figure 4. Oxytocin infusion leads to the upregulation of 157 known genes and the downregulation of 204 known genes as assessed using Affymetrix HGU133 Plus 2.0 arrays.

Veh/OXT vs. L152/OXT, p = .035) (Figure 6B). These data reveal that OXT increases the synthesis of proteins, such as NPY5R, whose activity is sufficient for anxiolysis in general and necessary for the anxiolytic effect of OXT in particular.

DISCUSSION

One of the well-established actions of centrally released OXT is anxiolysis; however, the underlying intracellular mechanisms remain poorly elucidated. In this study, we show that the anxiolytic effect of intra-PVN OXT, as witnessed 30 minutes after infusion, requires active protein synthesis. Furthermore, we have shown that OXT, through activation of PKC and MEK1/2, engages eEF2 to induce protein synthesis. A microarray assessment revealed a number of genes that are potentially regulated by OXT. Specifically, we validated the OXT-induced upregulation of NPY5R and revealed its anxiolytic properties within the PVN. These results support a novel role for eEF2, namely anxiety regulation within the hypothalamus.

Central to this study is the cascade of events coupled to OXTR activation and reduced eEF2 phosphorylation. Indeed, using specific pharmacological tools, we could identify several factors that are up- or downstream of eEF2 recruitment, resulting in protein synthesis and anxiolysis. Although the use of anisomycin to block protein synthesis may affect protein phosphorylation and thus seems rather unspecific, it did not affect anxiety-related behavior. Additionally, we have assessed the role of eEF2 and NPY5R in behavior with highly specific pharmacological modulators. Therefore, it appears that OXT-induced eEF2 activation with subsequent protein synthesis is required for the anxiolytic effect of OXT in the PVN.

In contrast to nonneural cell types (22,41–43), eEF2 is under the control of PKC, rather than mammalian target of rapamycin and p90 ribosomal s6 kinase, when recruited by OXTR. In support, this OXT-eEF2 pathway has been reported in myometrial cells (20). Since the PKC family of protein kinases comprises multiple subtypes, future studies are required to investigate the exact molecular identity of involved PKC isoforms.

The identified OXTR-eEF2 signaling pathway adds to the complexity of OXTR-mediated intracellular effects. However, eEF2 may be a downstream effector of the TRPV2-MEK1/2 cascade previously described to be necessary for OXT-induced anxiolysis (17). Our data further suggest that PKC and MEK1/2 are part of the same cascade, considering that both prevent the OXT-induced reduction of p-eEF2 levels to a similar extent. This finding is consistent with the generally accepted notion that PKC is a mediator of EGFR transactivation (25,44). Therefore, we propose a two-tiered intracellular signaling cascade that is coupled to the OXTR and brings about anxiolysis. The first tier depends on activation of the $G\alpha_q$ protein subunit and involves PKC, while the second tier requires the activity of the $G\beta/\gamma$ protein subunit, which promotes extracellular Ca^{2+} influx through TRPV2 channels that is necessary for EGFR transactivation. Thus, the combined actions of the different G protein subunits seem necessary for anxiolysis through transactivation of the EGFR and subsequent recruitment of downstream intracellular effectors (Figure 7). This conceptual model is in line with the demonstration of eEF2 activation by other GPCRs in myocytes. For example, stimulation of adrenergic (24) and angiotensin (23) receptors also leads to MEK1/2 phosphorylation and subsequent dephosphorylation of eEF2 kinase, the principal inhibitor of eEF2 (45). On the other hand, application of the MEK1/2 inhibitor U0126 in the absence of OXT promoted eEF2 dephosphorylation. This finding indicates an inhibitory action of MEK1/2 on eEF2 dephosphorylation. Alternatively, this finding could indicate a stimulatory action of eEF2

Table 1. Excerpt of Genes Significantly Upregulated or Downregulated Within the Rat Paraventricular Nucleus of the Hypothalamus 30 Minutes After Intracerebroventricular Infusion of Oxytocin

Biological Process	Upregulated Genes	Downregulated Genes
Synaptic Activity/ Plasticity	*Grm4* *Gabra5* *Dpys14* *Rxr*	*Gria3, Gria4* *Gabrg3* *Glrb* *Smcx* *Mecp2*
Neuronal Excitability	*Kcnc3* *Prkcd* *Kcnk4* *Kcnj11*	*Kcnc1/Kcncq1* *Kcnab1* *Kcnip4* *Dlg2* *Snx27*
Neuropeptides	*Hcrt* *Npy, Npy5r* *Adcyap1* *Ghrh* Rfamide related peptide	*Crf* *Oxt* *Avp*
Cell Adhesion	*Necl-1* *Pard3* *Amigo2* *Adamts1* *Efemp2*	*Ncam* *Nrxn3* *Pcdh9 and Pcdha13* *Cdh6 and Cdh11* *Negr1* *Obcam* *Cntn3* *Caspr* *Adgra3* *Itgb6* *Flrt3* *Dcc*

Assessment of the regulated genes identified a number of potential target genes involved in biological processes known to be regulated by oxytocin (for the full list of altered genes see Table S3 in Supplement 2).

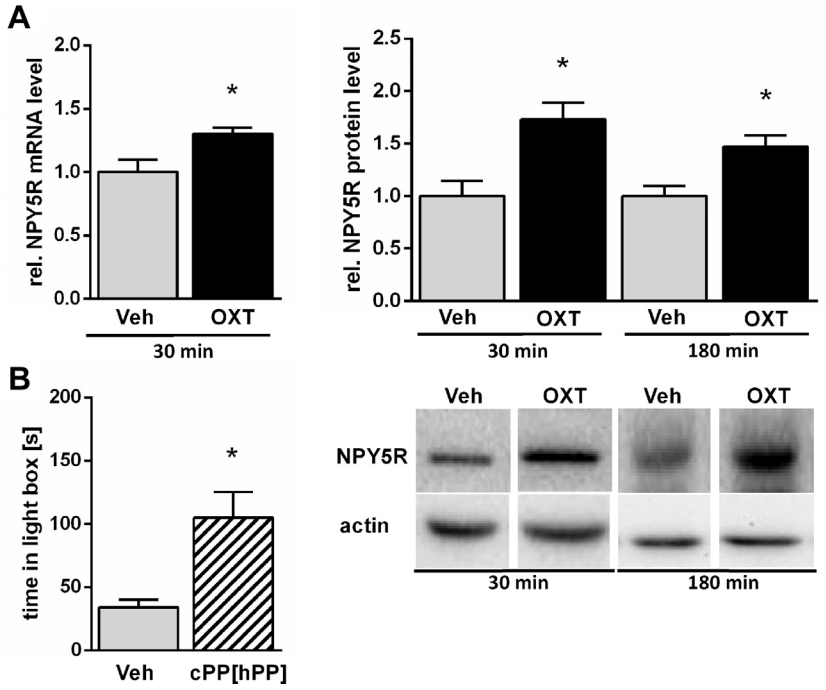

Figure 5. **(A)** Intracerebroventricular oxytocin (OXT) increases relative (rel.) messenger RNA (mRNA) and protein expression of neuropeptide Y receptor 5 (NPY5R) in the paraventricular nucleus of male rats 30 minutes and 180 minutes after infusion; *$p < .05$, $n = 3–7$. **(B)** Activation of NPY5R within the paraventricular nucleus via local infusion of the NPY5R agonist [cPP]hPP decreases anxiety-related behavior in the light/dark box 10 minutes after infusion. Bars represent mean + SEM. *$p < .05$, $n = 6$. Veh, vehicle.

phosphorylation under basal conditions. This effect of MEK1/2 signaling has not received much attention, as MEK1/2 is generally considered to be a mitogenic agent, mainly involved in proliferation and cell survival (38) but also in the onset and progression of many types of cancer (46). However, the antiproliferative effects of some drugs (sulforaphane, pioglitazone) have been associated with upregulated MEK1/2 phosphorylation (47,48), suggesting that MEK1/2 negatively affects mitogenic intracellular signaling pathways that would require protein synthesis for cell proliferation. The precise mechanisms underlying these effects of MEK1/2 have not been elucidated to date, but they warrant further investigation.

The observed onset of eEF2 dephosphorylation within 10 minutes is in accordance with the time frame for the activation of both PKC and MEK1/2 (5,49). In dendrites, protein synthesis can occur within 3 minutes following stimulation of

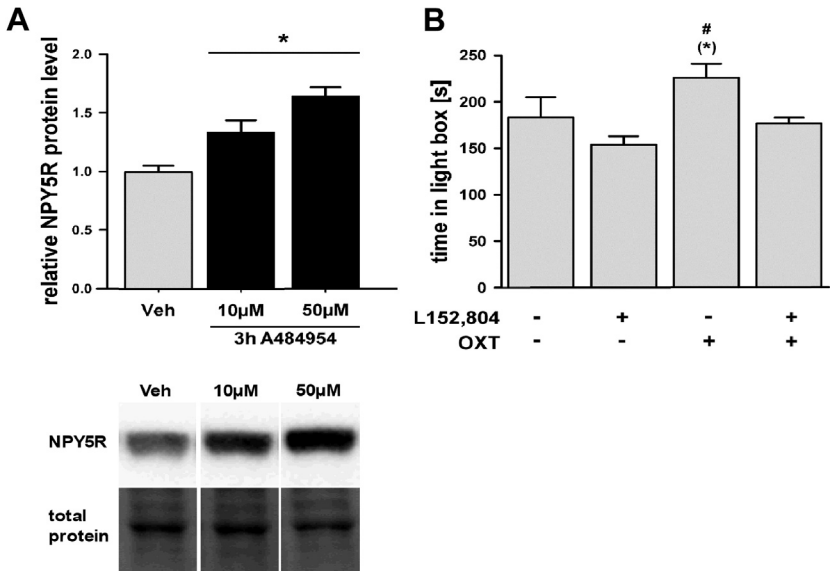

Figure 6. Activation of eukaryotic elongation factor 2 increases neuropeptide Y receptor 5 (NPY5R) levels in vitro, while preadministration of an NPY5R antagonist abolishes the anxiolytic effect of oxytocin (OXT) in vivo within the paraventricular nucleus. **(A)** The eukaryotic elongation factor 2 kinase inhibitor A484954 (10 μmol/L [μM], 30 μmol/L) increased NPY5R protein levels in the hypothalamic cell line H32 in a dose-dependent manner (vehicle [Veh], $n = 7$; 10 μm, $n = 7$; 50 μm, $n = 4$). **(B)** Intra–paraventricular nucleus infusion of the NPY5R antagonist L152,804 (0.82 nmol/0.5 μL) prior to OXT (0.01 nmol/0.5 μL) abolished the anxiolytic effect of OXT (−/−, $n = 8$; +/−, $n = 6$; −/+, $n = 5$; +/+, $n = 6$), indicated by an increased time spent in the light box. Bars represent mean + SEM. *$p < .05$ vs. Veh, $^{(*)}p = .053$ vs. Veh, $^{\#}p < .05$ vs. L152,804/OXT and vs. L152,804/Veh.

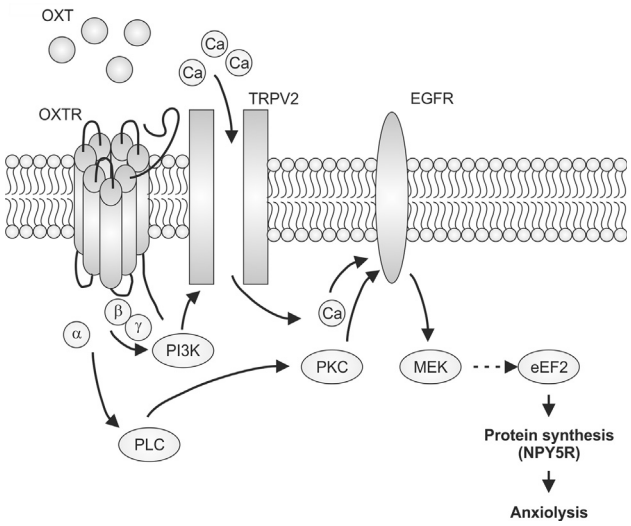

Figure 7. Schematic representation of the proposed two-tiered intracellular signaling cascade leading to oxytocin (OXT)–induced anxiolysis. Upon binding to its receptor, OXT leads to the activation of protein kinase C (PKC) via the $G\alpha_q$ protein subunit, which subsequently transactivates the epidermal growth factor receptor (EGFR) (5). Secondly, via the $G\beta/\gamma$ protein subunit, OXT promotes extracellular calcium ion (Ca) influx through transient receptor potential cation channel subfamily V member 2 (TRPV2) channels (17), which is necessary for EGFR transactivation and mitogen-activated protein kinase pathway activation (35). The combined actions of the different G protein subunits seem thus necessary for anxiolysis via convergence on eukaryotic elongation factor 2 (eEF2) and the resultant stimulation of protein synthesis. The dashed line from mitogen-activated protein kinase kinase (MEK) to eEF2 represents uncertainty concerning the mechanism by which MEK1/2 inhibits eEF2 phosphorylation. NPY5R, neuropeptide Y receptor 5; OXTR, OXT receptor; PI3K, phosphatidylinositide-3 kinase; PLC, phospholipase C.

N-methyl-D-aspartate receptors (50,51), which is important for synaptic plasticity and learning throughout the brain (52). This also applies to fear learning in the lateral amygdala, where both long-term potentiation and memory consolidation depend on protein synthesis (53). Importantly, eEF2 has been shown to induce rapid effects on translational regulation (54).

Similar to the effects of the de novo synthesized proteins on stabilizing potentiated synapses, the proteins formed following OXTR binding and eEF2 activation might consolidate the anxiolytic phenotype of OXT activity. In support, a longer-term (4-hour) anxiolytic effect of a single endogenous OXT surge in the PVN following successful mating has been observed (55). As a proof of principle to determine whether OXT induces the production of proteins with anxiolytic activity, we studied NPY5R expression, since NPY5R agonists have anxiolytic properties (56). *Npy5r* mRNA was markedly upregulated by OXT as measured by microarray and quantitative polymerase chain reaction. In confirmation, NPY5R protein levels were more abundant 30 minutes following OXT treatment. Thus, OXT may promote the translation of the already existing *Npy5r* mRNA pool and may increase this pool even further during prolonged exposure. Moreover, the specific eEF2 kinase inhibitor A484954 also increased NPY5R protein levels, supporting its role in the observed effects of OXT.

Our observation that intra-PVN NPY5R activation, by means of a specific agonist, induces anxiolysis demonstrates that OXT is able to promote the synthesis of proteins that exert anxiolytic activity for at least 4 hours. Furthermore, the anxiolytic effect of OXT was prevented by pretreatment with a specific NPY5R antagonist. This finding suggests that neuropeptides within the PVN act in concert to prevent anxiety-like behavior. Indeed, we recently found that neuropeptide S released from locus coeruleus axon terminals in the PVN induces local somatodendritic OXT release, demonstrating an additional interaction between OXT and other neuropeptides (57). Local OXT may expose many, if not all, PVN neurons to OXT. However, it is at present not clear which of these neurons, such as corticotropin-releasing factor, vasopressin, or OXT cells, express the OXTR or NPY5R. Thus, the cell type–specific responses of PVN neurons and their contribution to the regulation of anxiety-related behavior need to be addressed in future studies.

The anxiolytic activity of NPY5R in the PVN has not been described before, but such activity has been described in the amygdala (32,58). Thus, the present results further support the roles of NPY5R and the PVN in anxiety regulation. Further, binding of NPY to NPY1R or NPY5R on OXTergic neurons in the PVN inhibits the secretion of anorexigenic neuropeptides (59). This finding, taken together with our findings, indicates complex interactions between the OXT and NPY system in the context of anxiety and feeding behaviors at the hypothalamic level.

In conclusion, we have shown that OXT exerts its anxiolytic activity in the PVN through protein synthesis, which is mediated through eEF2 activation. Importantly, two distinct GPCR-coupled signaling pathways converge to activate eEF2—i.e., 1) the G protein α_q subunit and PKC activation (necessary for EGFR transactivation) and 2) the β/γ subunit—to promote extracellular Ca^{2+} influx and MEK1/2 activation. A role of eEF2 in anxiolysis was recently demonstrated in mice with a dysfunctional gamma-aminobutyric acid A receptor on somatostatin-positive neurons. These mice displayed decreased eEF2 phosphorylation in the hippocampus and medial prefrontal cortex and showed reduced levels of anxiety (60). Thus, it appears that eEF2 activation and subsequent protein synthesis are generally implicated in the control of anxiety-like behavior, including OXT-induced anxiolysis. Specifically, we can show that OXT stimulates NPY5R synthesis, with NPY5R activation exerting a clear anxiolytic effect and being necessary for the anxiolytic effect induced by OXT. Taken together, our study has identified novel effectors of OXT-induced anxiolysis in the PVN that lend themselves to further study of anxiety disorders and development of pharmacological therapies in the future.

ACKNOWLEDGMENTS AND DISCLOSURES

This work was supported by the German Research Foundation (Grant No. GRK 2147 [to IDN, BJ, C-PM], Grant No. JU3039/1–1 [to BJ], Grant Nos. NE 465/27-1 and NE 465/31–1 [to IDN], Grant No. NE 465/19–1 [to IDN, EvdB], and Grant No. SL 141/4–1 [to DAS]), as well as the Bundesministeriums für Bildung und Forschung (Grant OptiMD to [IDN, DAS]) and European Union (Grant No. 60240 FemNAT-CD [to IDN]). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the article.

We thank Anna-Kristina Schmidtner and Rodrigue Maloumby (University of Regensburg) for excellent technical support.

The authors report no biomedical financial interests or potential conflicts of interest.

ARTICLE INFORMATION

From the Department of Behavioral and Molecular Neurobiology (SM, C-PM, BJ, DAS, IDN), Regensburg Center of Neuroscience, University of Regensburg, Regensburg; Department of Psychiatry, Psychosomatic Medicine and Psychotherapy (DAS), University Hospital, Goethe University, Frankfurt am Main, Germany; Biotherapeutics Clinical Research and Development (DvS), Precision Medicine, New York, New York; and Centre de Neurosciences Psychiatriques (EHvdB), CHUV, Lausanne, Switzerland.

SM and C-PM contributed equally to this work.

DAS and IDN contributed equally to this work.

Address correspondence to Inga D. Neumann, Ph.D., Department of Behavioral and Molecular Neurobiology, University of Regensburg, 93053 Regensburg, Germany; E-mail: inga.neumann@biologie.uni-regensburg.de.

Received Jul 19, 2018; revised Dec 17, 2018; accepted Jan 2, 2019.

Supplementary material cited in this article is available online at https://doi.org/10.1016/j.biopsych.2019.01.010.

REFERENCES

1. Meyer-Lindenberg A, Domes G, Kirsch P, Heinrichs M (2011): Oxytocin and vasopressin in the human brain: Social neuropeptides for translational medicine. Nat Rev Neurosci 12:524–538.
2. Neumann ID, Slattery DA (2016): Oxytocin in general anxiety and social fear: A translational approach. Biol Psychiatry 79:213–221.
3. Martinetz S, Neumann ID (2016): The potential of oxytocin as a therapeutic target for psychiatric disorders. Expert Opin Ther Targets 20:515–518.
4. Knobloch HS, Charlet A, Hoffmann LC, Eliava M, Khrulev S, Cetin AH, *et al.* (2012): Evoked axonal oxytocin release in the central amygdala attenuates fear response. Neuron 73:553–566.
5. Blume A, Bosch OJ, Miklos S, Torner L, Wales L, Waldherr M, *et al.* (2008): Oxytocin reduces anxiety via ERK1/2 activation: Local effect within the rat hypothalamic paraventricular nucleus. Eur J Neurosci 27:1947–1956.
6. Zoicas I, Slattery DA, Neumann ID (2014): Brain oxytocin in social fear conditioning and its extinction: Involvement of the lateral septum. Neuropsychopharmacology 39:3027–3035.
7. Jurek B, Neumann ID (2018): The oxytocin receptor: From intracellular signaling to behavior. Physiol Rev 98:1805–1908.
8. Menon R, Grund T, Zoicas I, Althammer F, Fiedler D, Biermeier V, *et al.* (2018): Oxytocin signaling in the lateral septum prevents social fear during lactation. Curr Biol 28:1066–1078, e1066.
9. Guzman YF, Tronson NC, Jovasevic V, Sato K, Guedea AL, Mizukami H, *et al.* (2013): Fear-enhancing effects of septal oxytocin receptors. Nat Neurosci 16:1185–1187.
10. Huber D, Veinante P, Stoop R (2005): Vasopressin and oxytocin excite distinct neuronal populations in the central amygdala. Science 308:245–248.
11. Sanborn BM, Dodge K, Monga M, Qian A, Wang W, Yue C (1998): Molecular mechanisms regulating the effects of oxytocin on myometrial intracellular calcium. Adv Exp Med Biol 449:277–286.
12. Liebmann C (2011): EGF receptor activation by GPCRs: An universal pathway reveals different versions. Mol Cell Endocrinol 331:222–231.
13. Romero-Fernandez W, Borroto-Escuela DO, Agnati LF, Fuxe K (2013): Evidence for the existence of dopamine d2-oxytocin receptor heteromers in the ventral and dorsal striatum with facilitatory receptor-receptor interactions. Mol Psychiatry 18:849–850.
14. Terrillon S, Bouvier M (2004): Roles of G-protein-coupled receptor dimerization. EMBO Rep 5:30–34.
15. Busnelli M, Kleinau G, Muttenthaler M, Stoev S, Manning M, Bibic L, *et al.* (2016): Design and characterization of superpotent bivalent ligands targeting oxytocin receptor dimers via a channel-like structure. J Med Chem 59:7152–7166.
16. Busnelli M, Chini B (2018): Molecular basis of oxytocin receptor signalling in the brain: What we know and what we need to know. Curr Top Behav Neurosci 35:3–29.
17. van den Burg EH, Stindl J, Grund T, Neumann ID, Strauss O (2015): Oxytocin stimulates extracellular Ca^{2+} influx through TRPV2 channels in hypothalamic neurons to exert its anxiolytic effects. Neuropsychopharmacology 40:2938–2947.
18. Jurek B, Slattery DA, Maloumby R, Hillerer K, Koszinowski S, Neumann ID, *et al.* (2012): Differential contribution of hypothalamic MAPK activity to anxiety-like behaviour in virgin and lactating rats. PLoS One 7:e37060.
19. Nersesyan Y, Demirkhanyan L, Cabezas-Bratesco D, Oakes V, Kusuda R, Dawson T, *et al.* (2017): Oxytocin modulates nociception as an agonist of pain-sensing TRPV1. Cell Rep 21:1681–1691.
20. Devost D, Carrier ME, Zingg HH (2008): Oxytocin-induced activation of eukaryotic elongation factor 2 in myometrial cells is mediated by protein kinase C. Endocrinology 149:131–138.
21. Heise C, Gardoni F, Culotta L, di Luca M, Verpelli C, Sala C (2014): Elongation factor-2 phosphorylation in dendrites and the regulation of dendritic mRNA translation in neurons. Front Cell Neurosci 8:35.
22. Taha E, Gildish I, Gal-Ben-Ari S, Rosenblum K (2013): The role of eEF2 pathway in learning and synaptic plasticity. Neurobiol Learn Mem 105:100–106.
23. Everett AD, Stoops TD, Nairn AC, Brautigan D (2001): Angiotensin II regulates phosphorylation of translation elongation factor-2 in cardiac myocytes. Am J Physiol Heart Circ Physiol 281:H161–H167.
24. Wang L, Proud CG (2002): Regulation of the phosphorylation of elongation factor 2 by MEK-dependent signalling in adult rat cardiomyocytes. FEBS Lett 531:285–289.
25. George AJ, Hannan RD, Thomas WG (2013): Unravelling the molecular complexity of GPCR-mediated EGFR transactivation using functional genomics approaches. FEBS J 280:5258–5268.
26. Kolch W, Heidecker G, Kochs G, Hummel R, Vahidi H, Mischak H, *et al.* (1993): Protein kinase C alpha activates RAF-1 by direct phosphorylation. Nature 364:249–252.
27. Yamamoto Y, Lee D, Kim Y, Lee B, Seo C, Kawasaki H, *et al.* (2012): Raf kinase inhibitory protein is required for cerebellar long-term synaptic depression by mediating PKC-dependent MAPK activation. J Neurosci 32:14254–14264.
28. Paxinos G, Watson CR (1998): The Rat Brain in Stereotaxic Coordinates, 4th ed. San Diego, CA: Academic Press.
29. Lopez J, Gamache K, Schneider R, Nader K (2015): Memory retrieval requires ongoing protein synthesis and NMDA receptor activity-mediated AMPA receptor trafficking. J Neurosci 35:2465–2475.
30. Nakazato M, Murakami N, Date Y, Kojima M, Matsuo H, Kangawa K, Matsukura S (2001): A role for ghrelin in the central regulation of feeding. Nature 409:194–198.
31. Chen Z, Gopalakrishnan SM, Bui MH, Soni NB, Warrior U, Johnson EF, *et al.* (2011): 1-Benzyl-3-cetyl-2-methylimidazolium iodide (NH125) induces phosphorylation of eukaryotic elongation factor-2 (eEF2): A cautionary note on the anticancer mechanism of an eEF2 kinase inhibitor. J Biol Chem 286:43951–43958.
32. Sorensen G, Lindberg C, Wortwein G, Bolwig TG, Woldbye DP (2004): Differential roles for neuropeptide Y Y1 and Y5 receptors in anxiety and sedation. J Neurosci Res 77:723–729.
33. Mugele K, Kugler H, Spiess J (1993): Immortalization of a fetal rat brain cell line that expresses corticotropin-releasing factor mRNA. DNA Cell Biol 12:119–126.
34. Jurek B, Slattery DA, Hiraoka Y, Liu Y, Nishimori K, Aguilera G, *et al.* (2015): Oxytocin regulates stress-induced *Crf* gene transcription through CREB-regulated transcription coactivator 3. J Neurosci 35:12248–12260.
35. Liu Y, Kamitakahara A, Kim AJ, Aguilera G (2008): Cyclic adenosine 3', 5'-monophosphate responsive element binding protein phosphorylation is required but not sufficient for activation of corticotropin-releasing hormone transcription. Endocrinology 149:3512–3520.

36. Dieterich DC, Lee JJ, Link AJ, Graumann J, Tirrell DA, Schuman EM (2007): Labeling, detection and identification of newly synthesized proteomes with bioorthogonal non-canonical amino-acid tagging. Nat Protoc 2:532–540.
37. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, *et al.* (2009): The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55:611–622.
38. Kyriakis JM, Avruch J (2012): Mammalian MAPK signal transduction pathways activated by stress and inflammation: A 10-year update. Physiol Rev 92:689–737.
39. Maqsood MI, Matin MM, Bahrami AR, Ghasroldasht MM (2013): Immortality of cell lines: Challenges and advantages of establishment. Cell Biol Int 37:1038–1045.
40. Heilig M (2004): The NPY system in stress, anxiety and depression. Neuropeptides 38:213–224.
41. Browne GJ, Proud CG (2004): A novel mTOR-regulated phosphorylation site in elongation factor 2 kinase modulates the activity of the kinase and its binding to calmodulin. Mol Cell Biol 24:2986–2997.
42. Leprivier G, Remke M, Rotblat B, Dubuc A, Mateo AR, Kool M, *et al.* (2013): The eEF2 kinase confers resistance to nutrient deprivation by blocking translation elongation. Cell 153:1064–1079.
43. Proud CG (2007): Signalling to translation: How signal transduction pathways control the protein synthetic machinery. Biochem J 403:217–234.
44. Neumann ID, van den Burg EH (2013): Oxytocin and vasopressin release and their receptor-mediated intracellular pathways that determine their behavioral effects. In: Choleris E, Pfaff DW, Kavaliers M, editors. Oxytocin, Vasopressin and Related Peptides in the Regulation of Behavior. Cambridge, UK: Cambridge University Press, 27–43.
45. Wang X, Regufe da Mota S, Liu R, Moore CE, Xie J, Lanucara F, *et al.* (2014): Eukaryotic elongation factor 2 kinase activity is controlled by multiple inputs from oncogenic signaling. Mol Cell Biol 34:4088–4103.
46. Mandal R, Becker S, Strebhardt K (2016): Stamping out RAF and MEK1/2 to inhibit the ERK1/2 pathway: An emerging threat to anticancer therapy. Oncogene 35:2547–2561.
47. Hsu YC, Chang SJ, Wang MY, Chen YL, Huang TY (2013): Growth inhibition and apoptosis of neuroblastoma cells through ROS-independent MEK/ERK activation by sulforaphane. Cell Biochem Biophys 66:765–774.
48. Kole L, Sarkar M, Deb A, Giri B (2016): Pioglitazone, an anti-diabetic drug requires sustained MAPK activation for its anti-tumor activity in MCF7 breast cancer cells, independent of PPAR-gamma pathway. Pharmacol Rep 68:144–154.
49. Egan JJ, Saltis J, Wek SA, Simpson IA, Londos C (1990): Insulin, oxytocin, and vasopressin stimulate protein kinase C activity in adipocyte plasma membranes. Proc Natl Acad Sci U S A 87:1052–1056.
50. Brown A, Baird MR, Yip MC, Murray J, Shao S (2018): Structures of translationally inactive mammalian ribosomes. Elife 7:e40486.
51. Scheetz AJ, Nairn AC, Constantine-Paton M (2000): NMDA receptor-mediated control of protein synthesis at developing synapses. Nat Neurosci 3:211–216.
52. Buffington SA, Huang W, Costa-Mattioli M (2014): Translational control in synaptic plasticity and cognitive dysfunction. Annu Rev Neurosci 37:17–38.
53. Schafe GE, LeDoux JE (2000): Memory consolidation of auditory pavlovian fear conditioning requires protein synthesis and protein kinase A in the amygdala. J Neurosci 20:RC96.
54. Tsokas P, Grace EA, Chan P, Ma T, Sealfon SC, Iyengar R, *et al.* (2005): Local protein synthesis mediates a rapid increase in dendritic elongation factor 1A after induction of late long-term potentiation. J Neurosci 25:5833–5843.
55. Waldherr M, Neumann ID (2007): Centrally released oxytocin mediates mating-induced anxiolysis in male rats. Proc Natl Acad Sci U S A 104:16681–16684.
56. Morales-Medina JC, Dominguez-Lopez S, Gobbi G, Beck-Sickinger AG, Quirion R (2012): The selective neuropeptide Y Y5 agonist [cPP(1–7),NPY(19–23),Ala31,Aib32,Gln34]hPP differently modulates emotional processes and body weight in the rat. Behav Brain Res 233:298–304.
57. Grund T, Goyon S, Li Y, Eliava M, Liu H, Charlet A, *et al.* (2017): Neuropeptide S activates paraventricular oxytocin neurons to induce anxiolysis. J Neurosci 37:12214–12225.
58. Sajdyk TJ, Schober DA, Gehlert DR (2002): Neuropeptide Y receptor subtypes in the basolateral nucleus of the amygdala modulate anxiogenic responses in rats. Neuropharmacology 43:1165–1172.
59. Valassi E, Scacchi M, Cavagnini F (2008): Neuroendocrine control of food intake. Nutr Metab Cardiovasc Dis 18:158–168.
60. Fuchs T, Jefferson SJ, Hooper A, Yee PH, Maguire J, Luscher B (2016): Disinhibition of somatostatin-positive GABAergic interneurons results in an anxiolytic and antidepressant-like brain state. Mol Psychiatry 22:920–930.