A regulatory loop between miR-132 and miR-125b involved in gonadotrope cells desensitization to GnRH

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The GnRH neurohormone is the main activator of the pituitary gonadotropins, LH and FSH. Here we investigated the contribution of microRNAs in mediating GnRH activation. We first established that miR-125b targets several actors of Gαq/11 signalling pathway, without altering Gαs pathway. We then showed that a Gαs-mediated, PKA-dependent phosphorylation of NSun2 methyltransferase leads to miR-125b methylation and thereby induces its down-regulation. We demonstrated that NSun2 mRNA is a target of miR-132 and that NSun2 may be inactivated by the PP1α phosphatase. Time-course analysis of GnRH treatment revealed an initial NSun2-dependent down-regulation of miR-125b with consecutive up-regulation of LH and FSH expression. Increase of miR-132 and of the catalytic subunit of PP1α then contributed to NSun2 inactivation and to the return of miR-125b to its steady-state level. The Gαq/11-dependent pathway was thus again silenced, provoking the down-regulation of LH, FSH and miR-132. Overall, this study reveals that a regulatory loop that tends to maintain or restore high and low levels of miR-125b and miR-132, respectively, is responsible for gonadotrope cells desensitization to sustained GnRH. A dysregulation of this loop might be responsible for the inverted dynamics of these two miRNAs reported in several neuronal and non-neuronal pathologies.

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female infertility\(^{16,17}\). GnRH treatment of murine immortalized LβT2 gonadotrope cells was shown to modulate expression of several miRNAs\(^{18,19}\). We recently demonstrated that the rise in two of the most induced-ones, miR-132 and miR-212 was necessary for the stimulation of FSH expression\(^{20}\). Conversely, miR-125b was found to be among the most repressed miRNAs\(^{18,19}\). This was particularly interesting as miR-125b and miR-132 have been shown to exhibit opposing effects on dendritic spine morphology and synaptic physiology in hippocampal neurons\(^{21}\). If such opposing effects were to occur in gonadotrope cells, the inverse behaviour following GnRH exposure, i.e. increased miR-132 and decreased miR-125b, should contribute to the activation of gonadotropins expression. Encouragingly, miR-125b was demonstrated on different cell models to target the mRNA of several cellular components like MAP2K7\(^{22-23}\), p38\(^{23}\) and JUN\(^{24}\), all three known to be involved in Gαq/11-mediated GnRH signalling\(^{4}\). The present study was aimed to address the role of miR-125b in the GnRH signalling with a particular attention on a possible contribution to the desensitization mechanism.

**Results**

**miR-125b inhibits gonadotropins expression.** Overexpression of miR-125b in control or GnRHa-treated (1 nM for 4 h) rat pituitary cells significantly decreased basal LH secretion and prevented GnRH-induced secretion of both LH and FSH (Fig. 1a). Lhb and Fshb steady-state mRNA levels were strongly reduced when miR-125b was overexpressed in control as well as in GnRH-treated rat pituitary cells (Fig. 1b).

Gonadotrope cells represent less than 15% of pituitary cells. To decipher the mechanism of miR-125b action, we thus used the murine LβT2 gonadotrope cell model. Overexpression of miR-125b in LβT2 cells (Fig. S1a) had no effect on basal gonadotropin α subunits mRNA level (Fig. 1c), probably because of an already low (hundred times lower) expression level. However, as in rat pituitary cells, it abolished the GnRH-induced (10 nM for 4 h) increase observed in control cells (Fig. 1c). Conversely, blocking miR-125b action prevented the increase in Lhb and Fshb mRNA level (Fig. 1d). These effects occurred at a transcriptional level as either overexpression or blocking miR-125b significantly altered both Fshb and Lhb promoter activities in LβT2 cells (Fig. S1b).

**miR-125b prevents GnRH activation of Gαq/11, but not Gαs-dependent pathway.** A number of potential targets for miR-125b that were predicted from *in silico* analyses are known to be involved in GnRH signalling\(^{25}\). Some of these potential targets (Map2k7, Jun and Mapk14 (encoding p38), all depending on PLC3 activation) were confirmed in different cell types\(^{22-24}\). In addition to these targets, we investigated the potential effect of miR-125b on Gαq/11, to which GnRH receptor couples, CACNA1C that controls calcium entry, ELK1, IP3-R (encoded by Itpr1) and CAMK2a that are responsible for and activated by calcium release, respectively (see Fig. S1c for a schematic presentation of GnRH signalling pathways).

We first looked for the presence of miR-125b and its potential target mRNAs in RISC. Each complex contains a given targeted mRNA together with its targeting miRNA\(^{26}\). The complexes were immunoprecipitated by using a pan-AGO antibody. In addition to miR-125b, mRNAs for Gna11 (which encodes Gαq/11), Map2k7, Elk1, Jun and Camk2a were shown to be present in the immunoprecipitated complexes (Fig. 2a) in control cells. All co-immunoprecipitated miRNAs exhibited reduced levels when cells were treated with GnRHa, together with the reduction of miR-125b. In order to confirm a role of miR-125b in the GnRH-induced captures into RISC, we looked for the effects of both overexpression and blocking of miR-125b on signalling effectors content. We confirmed that miR-125b overexpression decreased the protein level of MAP2K7, ELK1 and Gαq/11 (Fig. 2b; see Fig. S2a for representative blots). JUN level was too low in un-stimulated cells but overexpression of miR-125b prevented its GnRH-induced rise (Fig. 2b). Blocking miR-125b increased protein levels of Gαq/11, p38, ELK1, and GnRH-induced JUN (Fig. 2c and Fig. S2b). Unlike most miRNAs, miR-125b usually provokes a down-regulation of its target messengers, by inducing their rapid deadenylation\(^{22}\). We showed that overexpression of miR-125b induced a decrease in the basal level of Map2k7, Elk1, Gna11 and Mapk14 mRNAs and a marked decline in GnRH-induced Jun and Camk2a mRNAs (Fig. S1d), in agreement with the variations observed in their cellular encoded protein levels. In addition, Itpr1 and Cacna1c mRNAs were also reduced (Fig. S1d) suggesting that their encoded proteins would also be down-regulated. Conversely, blocking miR-125b enhanced the cellular content in Map2k7, Mapk14, Itpr1, Cacna1c, Gna11 and Jun mRNAs (Fig. S1e). If Elk1 (and Camk2a) mRNA level did not appear significantly increased when miR-125b action was prevented, the level of the encoded ELK1 protein was actually increased (Fig. 2c). Conversely, MAP2K7 level was not increased (rather the reverse) when miR-125b action was prevented despite the rise of its encoding mRNA, suggesting that other factors affecting MAP2K7 turnover may be altered by the treatment. A discrepancy between mRNA (Mapk14) and protein levels of p38 was also observed when miR-125b was overexpressed.

*In silico* analysis indicates that CREB is not a potential target of miR-125b. It has been shown that GnRH treatment does not alter the level of CREB but stimulates its PKA-mediated phosphorylation\(^{25,27}\). Neither overexpression (Fig. 2d and Fig. S2c) nor blocking (Fig. 2e and Fig. S2d) miR-125b had any effect on the GnRH-induced level and phosphorylation state of CREB after 30 min of treatments showing that the coupling to Gαs is still able to convey a GnRH stimulatory signal through the cAMP pathway.

Altogether, these results show that miR-125b targets into RISC mRNAs of a number of GnRH signalling effectors associated with coupling to the Gαq/11-calcium-activated pathway, likely exerting a silencing effect on most GnRH signalling mediators. The absence of effect on the Gαq/cAMP activated pathway keeps an entry door open for GnRH. GnRH treatment leads to a decrease in miR-125b level, allowing full activation of its Gαq/11-dependent signalling and allowing enhanced gonadotropin subunits expression.

**miR-125b is repressed by an NSun2-mediated methylation in gonadotrope cells.** NOP2/Sun RNA methyltransferase (NSun2) or MISU for myc-induced SUN-domain-containing protein\(^{28}\) was first characterized as responsible for transferring a methyl group from S-adenosyl-L-methionine to cytosine residues (m5C) of transfer RNAs\(^{29}\). However, NSun2 has recently been shown to be involved in the repression of miR-125b...
actions in Hela cells by methylating adenosine into N6-methyladenosine (m6A) on specific positions of primary and pre-miR-125b transcripts, thereby inhibiting the processing30. NSun2 was also shown to be responsible for the methylation of the mature form of miR-125b, which attenuates the recruitment of miR-125b into RISC30.

Figure 1. miR-125b is involved in the GnRH induction of LH and FSH expression. (a,b) Rat primary pituitary cells were electroporated with a miR-125b expressing vector or an empty vector and then treated with 1 nM GnRHa for 4 hours. (a) The concentration of accumulated LH and FSH into the medium was measured by ELISA. Overexpressing miR-125b significantly reduced basal secretion of LH and prevented the GnRHa-induced secretion of LH and FSH (n = 4). (b) Lhb and Fshb mRNA levels were measured by qRT-PCR and normalized to Gapdh mRNA. Overexpressing miR-125b nearly abolished basal expression of both Lhb and Fshb mRNA and prevented their GnRH-induced expression (n = 3). (c) LβT2 cells were electroporated with a miR-125b expressing vector or an empty vector and then treated with 10 nM GnRHa for 4 h. Overexpression of miR125 prevented the GnRHa-increased Lhb and Fshb mRNA expression (n = 7). (d) LβT2 cells were electroporated with anti-miR-125b or scrambled LNA. Blocking miR-125b increased both Lhb and Fshb mRNA levels (n ≥ 10). Different letters illustrate significant differences. *P < 0.05; **P < 0.01; ***P < 0.001.
Overexpression of *Nsun2* in LβT2 cells, which reached 2.5-fold its level in control cells (Figure S3a), led to a decrease in miR-125b level down to 60% of its steady-state level (Fig. 3a) and induced an increase in *Fshb* expression (Fig. 3a) and in all identified miR-125b mRNA targets (Fig. S3c). Conversely, the silencing of *Nsun2* down to 30% of its cellular content using an anti-*Nsun2* LNA (Fig. S3b) induced a doubling in miR-125b level (Fig. 3b), the repression of *Fshb* subunit expression (Fig. 3b) and a decline in most GnRH signalling miR-125b target effectors (Figure S3d). Unlike when altering directly miR-125b levels, either overexpression or blocking *Nsun2* had no significant effect on *Lhb* endogenous mRNA (Fig. 3a,b). Overexpression of *Nsun2* had a lower effect on miR-125b level as compared to when blocked using a LNA (down to 60% compared to 10%). *Lhb* was suggested to be more sensitive to the GnRH-mediated stimulation than *Fshb* (see refs 3–5 for review) so that moderate alteration of

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**Figure 2.** miR-125b prevents GnRH activation of Goq/11-, but not Gαs-dependent pathways. (a) Effect of a GnRHa treatment on the recruitment into RISC of Goq/11-mediated pathway factors. Levels of miR-125b and selected potential target mRNAs were determined by qRT-PCR on pan-AGO-immunoprecipitated samples and normalized to input level. GnRHa treatment (10 nM for 4 h) of LβT2 cells reduced the recruitment of miR-125b and of all tested target mRNAs (n = 5). (b,c) Effect of alteration of miR-125b on the protein level of Goq/11-related pathway factors in LβT2 cells. b; Cells were electroporated with a miR-125b expressing vector or an empty vector and protein levels were quantified on harvested cells by Western blot analysis. Overexpression of miR-125b led to a significant reduction of most basal or GnRH-induced selected Goq/11 expressing vector or an empty vector and protein levels were quantified on harvested cells by Western blot analysis. Overexpression of miR-125b had a lower effect on miR-125b level as compared to when blocked using a LNA (down to 60% compared to 10%).
Gαq/11 alone might not be sufficient to significantly affect Lhb mRNA level. In addition, overexpression of Nsun2 would not only lead to a decrease in miR-125b but may also have a large panel of effects as this methyltransferase is notably known to also methylate messenger as well as transfer RNAs. These additional effects might interfere with those due to the decrease of miR-125b.

To assess a possible role for the methylation of miR-125b in the regulation of gonadotropin subunits expression by GnRH, RNA isolated from LβT2 cells was immunoprecipitated using a highly specific anti-m6A antibody. The methylated fraction of miR-125b increased in the immunoprecipitated fraction in response to GnRHa treatment as well as in cells in which Nsun2 was overexpressed (Fig. 3c).

GnRH activates Nsun2 by a PKA-dependent phosphorylation. Both Nsun2 protein (Fig. 4a and Fig. S2e) and mRNA levels (Fig. 4b) were reduced 4 h after a GnRHa treatment indicating that, in our gonadotrope cell model, the modification of the Nsun2 level could hardly be accounted for its activation in response to GnRH. The Aurora-B kinase has been shown to phosphorylate Nsun2 on Ser139. Using a phosphoprotein enrichment method, we observed that the level of Nsun2 increased in the phosphoprotein fraction after a GnRHa treatment of LβT2 cells (Fig. 4c,d and Fig. S2f), demonstrating that GnRH induces phosphorylation of Nsun2.

**Figure 3.** miR-125b is repressed by an Nsun2-mediated methylation in gonadotrope cells. (a,b) Effect of overexpressing or blocking Nsun2. LβT2 cells were electroporated with Nsun2 or control expressing vector and miR-125b, Fshb and Lhb mRNA levels were quantified by qRT-PCR and normalized to snU6 (miR-125b) or to Gapdh. (a) Overexpression of Nsun2 reduced mi-125b and increased Fshb mRNA level. Lhb mRNA level was not affected (n = 9). (b) Blocking Nsun2 using an anti-Nsun2 LNA led to inverse effects compared to cells electroporated with a scramble LNA (n = 9). (c) Effect of a GnRHa treatment and of overexpressing Nsun2 on the methylation status of miR-125b. LβT2 cells were either treated by GnRH (10 nM for 4 h) or electroporated with an Nsun2 expressing vector and harvested. Total RNA extract was immunoprecipitated using an anti-m6A antibody and miR-125b was quantified in the immunoprecipitated fraction and normalized to input miR-125b level. Overexpression of Nsun2 had a similar stimulatory effect as the GnRHa treatment on the methylation status of miR-125b (n ≥ 4). *P < 0.05; **P < 0.01; ***P < 0.001.
As CREB phosphorylation was not affected by modulating miR-125b in LβT2 cells, we investigated the potential role of the cAMP-dependent pathway in the GnRH-induced miR-125b methylation. We observed that increasing intracellular cAMP level using 8Br-cAMP significantly amplified the phosphorylated fraction of NSun2 in LβT2 cells treated with GnRH (10 nM for 4 h). The same treatments had corresponding inverse effects on miR-125b cellular content. The catalytic subunit of PKA was associated with NSun2 10 min after the GnRH-treatment. Representative western blot images are given on Fig. S2. *P < 0.05; **P < 0.01; ***P < 0.001.
of NSun2 (Fig. 4d and Fig. S2f) as well as the methylated form of miR-125b (Fig. 4e and Fig. S2g) and reduced miR-125b cellular content (Fig. 4f). Conversely, inhibiting PKA pathway by the inhibitor (H89) did not significantly affect the level of phosphorylation of NSun2 in control cells (Fig. 4g), showing that the basal phosphorylation state of NSun2 is not mediated by PKA. However, the presence of inhibitor prevented the GnRH-induced rise in the phosphorylation state of NSun2 (Fig. 4g). In agreement, pre-treatment with the PKA inhibitor Rp-cAMP suppressed the GnRH-induced miR-125b increase in the methylated fraction (Fig. 4e) and its cellular decrease (Fig. 4f). PKA was shown to be activated within 5–10 min of a GnRH treatment of LiiT2 cells12. Co-immunoprecipitation experiments using an anti-NSun2 antibody showed that the catalytic subunit of PKA (PRKACA) was associated with Nsun2, 10 min after a GnRH treatment (Fig. 4h), further confirming that PKA was directly responsible for the GnRH-induced phosphorylation of NSun2.

miR-125b/miR-132 opposite regulation in gonadotrope cells. In a previous study, we demonstrated that a rise of miR-132 in GnRH-treated gonadotrope cells was necessary for the stimulation of Fshb expression20. It was therefore interesting to determine whether the elevation of miR-132 in GnRH-treated gonadotrope cells was induced, or allowed, by the down regulation of miR-125b. Increasing AMP effect alone was inefficient in enhancing mature miR-125 level (Fig. 5a). However, the GnRH-induced rise in miR-132 was prevented when cells were pre-treated with the PKA inhibitor Rp-cAMP (Fig. 5a), attesting an indispensable role of this pathway in the activation of miR-132 by GnRH. The methylation status of miR-125b is altered in response to PKA activation, potentially allowing an involvement of miR-125b in the regulation of miR-132. Lowering miR-125b level by either using anti-miR-125b LNA or overexpressing NSun2 increased miR-132 level (Fig. 5b,c). Conversely, overexpression of miR-125b prevented the GnRH-induced rise of miR-132 level (Fig. 5d), indicating that miR-125b represses miR-132 expression. In cortical neurons, miR-132 expression is stimulated by the activation of ERK signalling25. We show here that overexpression of miR-125b prevented the GnRH-induced phosphorylation of ERK1/2 (Fig. 5e and Fig. S2h). In addition, blocking ERK1/2 phosphorylation by treatment with U0126, a MEK1/2 inhibitor prevented the GnRH-induced rise in the expression of AK005051 transcript encoding pri-miR-125/212 (Fig. 5f). This result indicates that the GnRH-induced down-regulation of miR-125b would allow the Gaoq/11-mediated MEK1/2-induced activation of ERK1/2 and its stimulatory action on miR-132/212 gene transcription.

Conversely, blocking miR-132 induced a decrease in miR-125b level (Fig. 5g) whereas miR-132 overexpression increased miR-125b cellular content (Fig. 5h). Taken together these results demonstrate the existence of a regulatory loop between miR-125b and miR-132, two miRNAs that have opposing effects on gonadotropins expression. In this loop, the GnRH-induced decrease in miR-125b would lead to an up-regulation of miR-132 expression which in turn has a stimulatory effect on miR-125b expression. Such a loop should contribute to a return to steady-state levels of these miRNAs latterly after GnRH stimulation.

Dynamics of the miR-125b/miR-132 regulatory loop. To get a better insight into the temporal sequence of the GnRH response, we performed kinetics experiments. RISC content of miR-125b appeared to rapidly decrease in response to GnRHa stimulation (Fig. 6a). As expected, miR-132 recruitment quickly followed, likely resulting from enhanced gene expression (Fig. 6a). After eight hours of treatment, when the RISC content of miR-132 was at its highest level, miR-125b started to be re-captured and the level of NSun2 mRNA into RISC started to rise (Fig. 6a) while the cellular content of NSun2 protein decreased (Fig. 6b and Fig. 2i). Interestingly, in silico analysis indicated that NSun2 is a potential target of miR-132. In agreement, when miR-132 was overexpressed, NSun2 mRNA level increased in AGO-immunoprecipitated fraction (Fig. S4a) and the cellular content of NSun2 protein was lowered (Fig. S4b) while its messenger RNA was unaffected (Fig. S4c). These results confirm that the rise of miR-132 contributes to the return of miR-125b to its steady-state level through an inhibition of NSun2 expression.

As NSun2 protein level does not necessarily reflect its potential activity, we looked at the phosphorylation status of NSun2 throughout the GnRhA treatment. The NSun2 phosphorylation level was already increased 2 h after GnRH exposure (Fig. 6c and Fig. S2i) in agreement with the observed negative effect on the recruitment of miR-125b into RISC and on its cellular content. After 6 h of treatment, the phosphorylated fraction of NSun2 was lowered and miR-125b started to rise and be recruited again into RISC.

NSun2 is inactivated by a PP1α-dependent dephosphorylation. The decrease in the phosphorylation status of NSun2 suggests that it is submitted to the action of a phosphatase. We quantified the mRNA level of a number of phosphatases by qRT-PCR among which the catalytic subunit of PP2, PP2CA (Figure S4d) but only the messenger for the catalytic subunit of PP1α, PPP1CA, exhibited a profile in agreement with the level of phosphorylation of NSun2. Pppla1α increased significantly after 6 h of GnRH treatment (Fig. 6d) i.e. just before the decrease of the phosphorylated form of NSun2. Blocking MEK1/2 by using U0126 decreases basal level of Ppp1ca and, as for miR-132 primary transcript expression, prevented its GnRH-induced rise (Fig. 6e). This shows that the rise of both Pppla1α and miR-132 relies on the activation of ERK1/2 which is allowed by the lifting of the miR-125b-mediated inhibition of the Gaoq/11 pathway. Overexpression of PPP1CA countered the GnRH-induced phosphorylation of NSun2 after 4 h of treatment without affecting the basal level of its phosphorylation (Fig. 6f and Fig. S2k). Co-immunoprecipitation experiment using an anti-NSun2 antibody showed that PPP1CA was associated with NSun2 after 8 h of GnRH treatment (Fig. 6g). These results strongly suggest that the activation of Pppla1α is responsible for the inactivation of NSun2, the return of miR-125b to its steady-state level and the silencing of the Gaoq/11 signalling pathway. This silencing effect is attested by a decrease in Lhb and Fshb mRNA levels (Fig. 6h).
Discussion

Although mammalian GnRHR is resistant to homologous desensitization, gonadotropin secretion is significantly reduced or abolished by sustained treatment with GnRH agonists. In the rhesus monkey with radio-frequency induced lesions of the medial basal hypothalamus which ablates most GnRH-secreting neurons, gonadotropin secretion are restored when GnRH is administered intermittently but not when infused continuously. Similarly, in vivo treatment with long-lasting GnRH analogues, reduces levels of Lhb and Fshb transcripts in rat pituitary gland and the secretion of gonadotropin hormones. More interestingly, in ewes in which GnRH levels are monitored in the pituitary portal blood during the course of the follicular phase, LH secretion surge appears to end several hours before the amplitude and frequency of the GnRH pulses decline, indicating that desensitization also occurs in physiological conditions.

Figure 5. miR-125b/miR-132 opposite regulation in gonadotrope cells. (a) LβT2 cells were either treated by GnRHa (10 nM for 4 h) or the cAMP analogue 8Br-cAMP or were pre-treated with the PKA inhibitor Rp-cAMP before the same GnRHa treatment. Inhibition of PKA prevented the GnRH induction of miR-132 (n = 8). (b–d) Effect of alteration of miR-125b on miR-132 level. (b) LβT2 cells were electroporated with anti-miR-125b or scrambled LNA. Blocking miR-125b increased miR-132 level (n = 8). (c) Cells were electroporated with NSun2 or empty expressing vector. Overexpression of NSun2 led to a significant increase expression (n = 9). (d) Cells were electroporated with miR-125b or empty expression vector before a GnRHa treatment (10 nM for 4 h). Overexpression of miR-125b led to a significant reduction of the GnRHa-induced miR-132 expression (n = 4). (e) Effect of alteration of miR-125b on ERK1/2 phosphorylation status. LβT2 cells were electroporated with miR-125b or empty expression vector and then treated with 10 nM GnRHa for 2 h. Extracted proteins were analysed by western blotting using anti-ERK1 and anti-pERK1/2, successively. A representative blot is given on Fig. S2h). The antiserum recognizes both ERK1 (upper band) and ERK2 (lower band). The two signals were summed for quantification. Overexpression of miR-125b prevented the GnRH-induced phosphorylation of ERK1/2 (n = 3). (f) LβT2 cells were either treated by GnRHa (10 nM for 8 h) or were pre-treated with the MAPK kinase (MEK)1/2 (U0126) inhibitors before the same GnRHa treatment. Inhibition of ERK1/2 phosphorylation prevented the GnRH-induced expression of AK006051 mRNA encoding miR-132 and miR-212 (n = 3). (g,h) Effect of alteration of miR-132 on the miR-125b level in LβT2 cells. (g) Cells were electroporated with anti-miR-132 or scrambled LNA. Blocking miR-132 led to a significant decrease in miR-125b level (n = 6). (h) Cells were electroporated with a miR-132 expressing vector or an empty vector. Overexpression of miR-132 led to a significant increase of miR-125b expression (n = 5). *P < 0.05; **P < 0.01; ***P < 0.001.
Figure 6. miR-125b/miR-132 regulatory loop involvement in gonadotrope cell desensitization to GnRH. (a–d) Dynamics of the GnRH-induced miR-125b/miR-132 regulatory loop. LβT2 cells were treated with 10 nM GnRHa. (a) Effect on the recruitment into RISC. miR-125b, miR-132 and NSun2 mRNA levels were determined on pan-AGO-immunoprecipitated samples and normalized to input level. Recruitment of miR-125b was quickly reduced and kept low until 8 h of treatment. Recruitment of miR-132 was delayed until 2 h and maintained for up to 8 h. NSun2 mRNA was recruited only after 8 h (n ≥ 5). (b) GnRHa treatment induces a decrease in NSun2 protein level (n ≥ 4). (c) Protein extracts were eluted from a phosphoprotein enrichment column and analysed by western blotting using an anti-NSun2 antibody. The GnRHa-treatment rapidly induced a strong increase of NSun2 in the phosphoprotein fraction, peaking at 4 h (n ≥ 4). (d–f) NSun2 is inactivated by a PP1α-dependent dephosphorylation. d; LβT2 cells were treated with 10 nM GnRHa. Ppp1ca mRNA was significantly induced after 6 h of treatment (n ≥ 5). (e) LβT2 cells were either pre-treated with the MEK1/2 inhibitors (U0126) or not before treatment with GnRHa (10 nM for 8 h). Inhibition of ERK1/2 phosphorylation prevented the GnRH-induced expression of Ppp1ca mRNA (n = 3). (f) Cells were electroporated with PP1CA or empty expression vector followed by a GnRHa treatment (10 nM for 4 h). PP1CA overexpression alone had no effect on basal phosphorylation level but prevented the GnRH-induced phosphorylation of NSun2 (n ≥ 3). (g) Cells were treated by GnRHa (10 nM for 4 or 8 h). Protein extracts were immunoprecipitated using
Desensitization of gonadotrope cells to sustained GnRH stimulation has first been attributed to a lack of calcium response possibly through decreased expression of the IP3 receptor. Expression of several signalling factors was shown to be down-regulated upon prolonged GnRHR stimulation. This is notably the case for $G_{q/11}$, PLC$\beta_1$ and several isoforms of PKC$\alpha$. Overexpression of a constitutively active mutant of $G_{q/11}$ induces resistance to GnRH further indicating that desensitization to GnRH involves a $G_{q/11}$-mediated pathway.

It was shown that treatment with GnRH of murine L$\beta$T2 gonadotrope-derived cells or porcine pituitary cells induces considerable changes in the microtranscriptome. In this study, we show that miR-125b content in RISC dropped within 1 h and was maintained low for 6 h of sustained GnRH exposure and then progressively re-increased to reach its steady-state level after 24 h. This indicated that the repressive effects of miR-125b on targets are transiently lifted for a limited time after the beginning of the GnRH treatment. miR-125b was demonstrated to target several components of the $G_{q/11}$-mediated pathway. If MAP2K7, p38$\alpha$ and JUN were already described as effective targets of miR-125b, others, like, $G_{q/11}$, ITTP1, CamK2a or ELK1 were here revealed for the first time to our knowledge. Blocking miR-125b induced an increase in these targets and a stimulation of LH and FSH expression, indicating that high levels of miR-125b are high enough in unstimulated cells to convey its silencing effect on the $G_{q/11}$-mediated pathway. In contrast, the $G_{q/11}$-mediated pathway remained fully open to stimulation even when miR-125b was overexpressed, in agreement with previous reports showing that only the $G_{q/11}$-mediated pathway is submitted to desensitization. By targeting $G_{q/11}$ and several downstream components of the $G_{q/11}$ activated pathway, the effects of miR-125b are likely to account for most if not all the previously described alterations provoked by sustained exposure to GnRH. Importantly, owing to its silencing effects on $G_{q/11}$-mediated signalisation, miR-125b may be involved in the regulation of a number of G protein-coupled receptors that activate this pathway.

The desensitization effect is due to a return of miR-125b to its steady-state, silencing level, a few hours after the beginning of a continuous exposure to GnRH. The decrease of miR-125b was generated by its methyltransferase activity on adenosine to NSun2. Nevertheless, one cannot exclude that the phosphorylation of NSun2 may induce a conformational change that allows interaction with another enzyme that would bear the methyltransferase activity attributed to NSun2.

If miR-132 up-regulation and miR-125b down-regulation are necessary for GnRH to enhance gonadotrope expression, these regulations are expected to occur also when gonadotrope cells are submitted to a pulsatile mode of stimulation. It is likely that new steady-state levels are reached with miR-125b low enough for the $G_{q/11}$-mediated pathway to exert its transcriptional stimulation, allowing miR-132 to be up-regulated. miR-125b down-regulation is dependent on NSun2 activation by a PKA-mediated phosphorylation. Tsutsumi and coll demonstrated that multiple pulses of GnRH caused multiple pulses of cAMP and PKA activation without desensitization. Such intermittent stimulation of PKA may activate NSun2 intermittedly, allowing a fine-tuning in the level of miR-125b. This hypothesis will have to be investigated using perfused gonadotrope cells submitted to a GnRH pulsatile challenge.

The desensitization effect was shown in this study to rely on a regulatory loop between miR-125b and miR-132 that tends to restore steady-state levels of miR-125b and miR-132. This regulatory loop depends on a PKA-mediated activation of NSun2 and a subsequent deactivation induced by PP1$\alpha$. Since PKA, NSun2 and PP1$\alpha$ are considered ubiquitous, such a regulatory loop should be activated in a number of tissues. miR-125b and miR-132 have opposite effects on dendritic spine morphology and synaptic physiology in hippocampal neurons. It would be interesting to investigate whether the regulatory loop is operating in hippocampal neurons where miR-132 is shown to be down-regulated in Alzheimer's disease patients. More generally, owing to their opposite effects on synaptic physiology, such a loop might be important during brain development. It is also tempting to speculate that a deregulation of this loop might be responsible for the inverted dynamics between miR-132 and miR-125b (one is up-regulated whereas the other is down-regulated) described in adrenal tissue submitted to an estradiol treatment or in pituitary adenomas when compared to normal pituitary tissues. Consistent with this hypothesis, the same inverted regulation was observed in human dermal fibroblasts from patients carrying a homozygous loss-of-function mutation in the NSun2 gene when compared to fibroblasts heterozygous for the mutation (see Supplemental Table 6 from Hussain et al.).

In conclusion, in this paper, we identified a number of new targets for miR-125b showing that miR-125b is able to silence the $G_{q/11}$-mediated signalling of the GnRH response. We also showed that NSun2 is a target of NSun2 antibody. Co-immunoprecipitated proteins were analysed by western blotting using anti-PP1CA. Representative blot of immunoprecipitated NSun2 proteins from GnRH-treated compared to control cells.
miR-132. We revealed that the NSun2-mediated methylation on m6A is activated by a PKA-mediated phosphorylation and inactivated by a PP1-catalyzed dephosphorylation. These three enzymes participate in a regulatory loop between miR-125b and miR-132 that tends to restore steady-state levels of miR-125b and miR-132. The activation of this regulatory loop is shown to be responsible for the desensitization of gonadotrope cells to GnRH.

**Materials and Methods**

**Products, plasmid constructs and antibodies.** GnRH agonist (GnRHa), [D-Trp6]-LHRH or triptorelin was purchased from Sigma-Aldrich. PKA activator 8-Br-cAMP and inhibitors Rp-cAMP and H89 were purchased from VWR International. MAPK kinase (MEK)1/2 (U0126) inhibitor was purchased from Sigma. Cell culture reagents were from Life Technologies. Plasmid expressing miR-125b-1 (pmCherry) is from Addgene (#58990). Plasmid expressing miR-132 (pDsRed2-C1) was previously described26. The NSUN2 expression vector (human Nsun2 coding sequence inserted into pCDNA4-Myc His A) was a gift of Dr. M. Frye (Cancer Research, Cambridge, UK). The PP1CA expressing vector (Myc-His tagged PP1CA in pcDNA3-1) was a gift of Dr. L. Neckers (Center for Cancer Research, NCI, Bethesda, MD). The (−2000/+698) rat Fshb-Luc reporter vector was a gift from Pr. UB Kaiser (Harvard Medical School, Boston, MA). The (−2016/ +76) rat Lhb promoter sequence was cloned from rat genomic DNA using primers listed in Table S1. The amplified sequence was inserted 5' to the coding sequence of luciferase into the multiple cloning site of pGL3 vector (Clontech).

**Animals, cell culture and treatment.** Experiments were conducted according to a protocol that was approved by the institutional animal care and use committee of Paris Diderot University (CEEA40).

Wistar rats anterior pituitary glands were dissected from adult (300–450 g) male rats (Janvier, CERJ) and cells were dispersed as previously described26. Cells were electroporated (1.2 × 105 cells/tip) with pmCherry-miR-125b or empty vector as described below and seeded in Ham F-10 medium supplemented with 10% fetal bovine serum (FBS). After overnight incubation, the medium was replaced by the same medium complemented with 0.5% penicillin/streptomycin (P/S - Sigma-Aldrich). After 48 h, cells were starved overnight in serum-free -medium then treated for 4 h with GnRHa in the starvation medium.

Mouse pituitary gonadotrope L/T2 cells, provided by Pr. P. Mellon (Department of Reproductive Medicine, University of California, San Diego, CA)26,29, were maintained in monolayer cultures with DMEM (Invitrogen) supplemented with 10 FBS, 0.5% P/S. Cells at passages 13–19 were plated at a density of 0.5 × 106 cells/cm² in triplicates in multi-well plates or 6-cm dishes coated with 30 μg/mL poly-L-lysine (Sigma-Aldrich). For GnRHa stimulation, cells were starved overnight in DMEM containing 0.5% P/S and then incubated for 2 to 24 h in fresh starving medium supplemented with 10 mM GnRHa. Cells were also treated 4 h with 1 mM 8Br-cAMP or pre-treated with 1 mM Rp-cAMP, 10μM H89 or 10μM U0126 for 30 min before stimulation with GnRHa.

**Electroporation.** pcDNA4-Myc-His-hNSUN, pmCherry-miR-125b and pDsRed-miR-132 miRNA expression or control (empty) vectors as well as anti-NSUN2 LNA (CATGTCATGTGTCACATCTCTT) and scrambled-S (TCATACTA), respectively (underlined LNA (TCACAAGTTAGGGTCTCAGGGA), anti-miR-132/212 LNA (AGACTGTT) and corresponding controls, were dispersed as previously described48. Cells were electroporated into L/T2 or rat primary pituitary gonadotrope cells using the Neon® Transfection System (Invitrogen) as previously described20.

**Immunoprecipitation of AGO-associated mRNA and RISC Complex.** Experimental conditions for RISC immunoprecipitation (RIP) were previously described26. Briefly, cells were cross-linked in 1% formaldehyde solution. Ten μg of anti-pan AGO antibody, 2 μg of cellular protein extract and 50 μl of Dynabeads® Protein G (Invitrogen) were incubated at 4°C overnight. The immunoprecipitated (IP) beads were then washed 3 times and subjected to DNase I (Promega) treatment at 37°C for 20 min. The reaction was stopped and the cross-linking reversed by heating at 70°C for 60 min. Co-immunoprecipitated RNAs as well as “input” RNAs were extracted using TRIzol reagent (Invitrogen) following manufacturer’s instructions and subjected to DNase I digestion as described above.

**Measurement of in vivo methylation.** One μg of anti-m6A antibody, 20μg of cellular RNA extract and 20 μl of Dynabeads® Protein G (Invitrogen) were incubated in 200 μl of IPP buffer (150 mM NaCl, 0.1% NP-40, 10 mM Tris-HCl [pH 7.4]) containing 1 U/μl of RNasin at 4°C for 2 h. The immunoprecipitated (IP) beads were then washed 5 times in IPP buffer. miRNAs isolated from the IP beads were subject to poly-A tailing and reverse transcription followed by real-time quantitative PCR analysis as described below.

**Gonadotropin assays.** LH and FSH concentrations were measured in cell media using an ELISA method. Micro-titration plates (High binding, Greiner Bio-one) were coated overnight at 4°C with 10 ng of purified rat LH (NIH I-10) or 3 days with 15 ng of purified rat FSH (NIH I-6) diluted in carbonate buffer. Rat LH (NIH RP3) or rat-FSH (NIH RP2) used as standards or culture media were incubated overnight with anti-rat LH (NIH S11 at 1:4000) or anti-rat FSH (NIH S11 at 1:8000) at 4°C. Plates were rinsed with PBS containing 1% BSA and 0.1% Tween 20 for 1 h at RT and washed with PBS-0.1% Tween 20 before addition of standards or samples for competition binding (2 h at 4°C). After removal of unbound material, phosphatase alkaline-labelled secondary antibody (dilution 1:2000, Thermo Scientific) was added and phosphatase alkaline activity was revealed after 1 h
at 4 °C with SigmaFast pNPP reagent. The minimum detectable LH and FSH concentrations were 0.2 and 1 ng/ml, respectively. The inter-assay coefficient of variation were <10%.

**Transcript quantification.** Total RNA was collected using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Real-time PCR was carried out in duplicates in the LightCycler 480 Instrument (Roche Diagnostics) as previously described using primers given in Table S1. Expression levels were normalized to Gapdh mRNA level.

**Micro RNA quantification.** One µg of total RNA was poly-adenylated by incubating with 5 U of poly (A) polymerase (New England Biolabs) at 37 °C for 30 min in a final volume of 10 µl. First-strand cDNA was synthesized from poly(A)-tailed RNA using a poly(T)-tailed universal primer (3′UP, Table S1) and SuperScript II (Invitrogen) according to the manufacturer's protocol. Amplification was carried out in 5 µl of LightCycler 480 SYBR Green I Master Mix (Roche Diagnostics) using miRNA-specific primers and a primer mix (LUP:SUP/1:5) so as to increase specificity, as indicated in Table S1. MicroRNA expression levels were calculated as for mRNA quantification (see above) and normalized to snU6 RNA level.

**Protein extraction and immunoblotting.** LβT2 cells were washed after GnRHa treatment or electroporation with ice-cold 50 mM HEPES pH 7.0 and homogenized in RIPA lysis buffer (20 mM Tris-HCl; pH 7.5, 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4 and 1 μg/ml leupeptin) supplemented with protease and phosphatase inhibitors (Roche) and 5 mM nicotinamide (Sigma-Aldrich). Homogenates were cleared at 20,000 x g for 30 min at 4 °C. Total proteins from supernatant were quantified using BCA assay (Pierce). Equal amounts of protein (15–20 μg) were separated on a 10% SDS-PAGE. After transfer onto nitrocellulose membrane, anti-NSun2 (1/1000), anti-MAP2K7 (1/1000), anti-p38 MAPK (1/1000), anti-PP1CA (1/1000), anti-PRKACA (1/1000), anti-P104 (1/2000) and anti-GAPDH HRP (1/1000) antibodies were successively used in TBS containing 0.1% Tween 20 (TBS-T) supplemented with 5% BSA. Membranes were washed three times in TBS-T and then incubated with horseradish peroxidase-conjugated secondary antibody in TBS-T/5% BSA for 60 min at room temperature and then washed three times in TBS-T. Proteins were detected by enhanced chemiluminescence detection system (GE Healthcare). Blots were analysed with a Fuji LAS-4000 imager and quantified using ImageJ software. Total CRED and GAPDH were used as internal loading control for pCREB and other proteins expression, respectively. Membranes were stripped in stripping buffer (2% SDS, 62.5 mM Tris pH 6.8, 114 mM 3-mercaptoethanol) for 30 min at 50 °C between each detection.

**NSun2 co-immunoprecipitation of PKA catalytic subunit.** Co-immunoprecipitation of endogenously expressed proteins was performed using LβT2 cells incubated with or without 10 nM GnRHa for 10 min. Cells were harvested in RIPA lysis buffer (see above). Extracts were incubated overnight with 5 μg of anti-Nsun2 antibody in the presence of Protein G magnetic Dynabeads® (Invitrogen) and resulting complexes were washed, denatured and eluted according to the manufacturer's instruction.

**NSun2 co-immunoprecipitation of PP1α catalytic subunit.** Co-immunoprecipitation of endogenously expressed proteins was performed using LβT2 cells incubated with 10 nM GnRHa for 4 h or 8 h. Cells were cross-linked in 1% formaldehyde solution for 10 min. Crosslinking was stopped using glycerine pH 7 for 5 min. Cells were then harvested in RIPA lysis buffer (see above). Extracts were incubated overnight with 5 μg of anti-Nsun2 antibody in the presence of Protein G magnetic Dynabeads® (Invitrogen) and resulting complexes were washed, uncross-linked and denatured at 95 °C during 30 min and then eluted in 2x sample buffer (4% SDS, 20% Glycerol, 0.12 M Tris pH 6.8, and 10% 3-mercaptoethanol).

**Phosphoprotein enrichment.** Following cell lysis, proteins were enriched in a phosphoprotein enrichment column (Thermo Scientific Phosphoprotein Enrichment Kit, Thermo Fisher Scientific), according to the manufacturer's instructions. Briefly, 3.5 mg of protein from each LβT2 cell lysate was applied to a phosphoprotein column containing a proprietary enrichment gel. The samples were incubated in the column for 30 min at 4 °C and washed. Retained proteins were eluted with five column washes with elution buffer. Phosphoprotein content, typically yielding 15–25% of the total protein loaded, were determined using the BCA assay.

**Luciferase assay.** Cells were electroporated using the Neon® Transfection System (Invitrogen) as previously described and plated into 96-well plates (1.5 x 105 cells/well) in DMEM with 10% FBS. Cells were co-transfected with 0.2 μg of Fshb-Luc or Lhb-Luc reporter or control vectors, 0.05 μg of Renilla luciferase-reporter (Promega), a reporter plasmid driven by a Herpes virus thymidine kinase (TK) promoter used as a control for transfection efficiency and 0.2 μg of miRNA expressing or control vector as well as 100 nM of LNA-anti-miR or scrambled anti-miR. After overnight incubation, cells were harvested and luciferase activities were measured using the Dual-Luciferase® Reporter Assay System (Promega) as instructed by the manufacturer.

**Statistical analysis.** All values are given as mean ± S.E.M. of at least three (the actual number is given by the value “n”) independent experiments. Statistical differences were first determined using one way ANOVA (GraphPad software) followed by Dunnett's t test for multiple comparisons and Student's t test for pair-wise comparisons. Statistical significance was defined as P < 0.05.
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Author Contributions
J.L. and B.Q. conceived and designed the experiments. J.L., D.L., A.F.-V., G.G. and J.-N.L. performed the experiments. J.L., J.C.-T. and B.Q. analysed the data and wrote the paper. All authors discussed the results and reviewed the manuscript.

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Corrigendum: A regulatory loop between miR-132 and miR-125b involved in gonadotrope cells desensitization to GnRH

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The original version of this Article contained a typographical error in the spelling of the author Joëlle Cohen-Tannoudji, which was incorrectly given as Joelle-Cohen-Tannoudji.

The Author Contributions statement,

“J.L. and B.Q. conceived and designed the experiments. J.L., D.L., A.F.-V., G.G. and J.-N.L. performed the experiments. J.L., J.-C.-T. and B.Q. analysed the data and wrote the paper. All authors discussed the results and reviewed the manuscript”.

now reads:

“J.L. and B.Q. conceived and designed the experiments. J.L., D.L., A.F.-V., G.G. and J.-N.L. performed the experiments. J.L., J.C.-T. and B.Q. analysed the data and wrote the paper. All authors discussed the results and reviewed the manuscript”.

These errors have now been corrected in the PDF and HTML versions of the Article.

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