Stress-induced inhibition of LH pulses in female rats: role of GABA in arcuate nucleus

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Abstract

Stress exerts profound inhibitory effects on reproductive function by suppression of the pulsatile release of GnRH and therefore LH. Besides the corticotrophin-releasing factor (CRF), this effect also might be mediated via GABAergic signaling within the arcuate nucleus (ARC) since its inhibitory effects on LH pulses and increased activity during stress. In the present study, we investigated the role of endogenous GABAergic signaling within the ARC in stress-induced suppression of LH pulses. Ovariectomised oestradiol-replaced rats were implanted with bilateral and unilateral cannulae targeting toward the ARC and lateral cerebral ventricle respectively. Blood samples (25 µl) were taken via chronically implanted cardiac catheters every 5 min for 6 h for measurement of LH pulses. Intra-ARC infusion of GABA_A receptor antagonist, bicuculline (0.2 pmol in 200 nl artificial cerebrospinal fluid (aCSF) each side, three times at 20-min intervals) markedly attenuated the inhibitory effect of lipopolysaccharide (LPS; 25 µg/kg i.v.) but not restraint (1 h) stress on pulsatile LH secretion. In contrast, restraint but not LPS stress-induced suppression of LH pulse frequency was reversed by intra-ARC administration of GABA_B receptor antagonist, CGP-35348 (1.5 nmol in 200 nl aCSF each side, three times at 20-min intervals). Moreover, intra-ARC application of either bicuculline or CGP-35348 attenuated the inhibitory effect of CRF (1 nmol in 4 µl aCSF, i.c.v.) on the LH pulses. These data indicate a pivotal and differential role of endogenous GABA_A and GABA_B signaling mechanisms in the ARC with respect to mediating immunological and psychological stress-induced suppression of the GnRH pulse generator respectively.

Key Words

- GABA
- arcuate nucleus
- corticotrophin-releasing factor
- luteinizing hormone
- stress

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Introduction

Gamma-aminobutyric acid (GABA), the principal inhibitory neurotransmitter in the CNS, has been implicated in the modulation of the reproductive system. Its physiological function is majorly mediated via two major receptor subtypes: ionotropic GABA_A receptor (GABA_AR) and metabotropic GABA_B receptor (GABA_BR). Although investigations on the role of GABA in the

gonadotrophin-releasing hormone (GnRH) pulse generator have focused on the effect of manipulating GABA levels in the medial preoptic area (mPOA) (Li *et al.* 2011, Lin *et al.* 2012), which is the major GnRH neurons site in the rat, a caveat to consider is the overwhelming evidence that the neural construct comprising the GnRH pulse generator resides in the arcuate nucleus (ARC) of the mediobasal

hypothalamus (MBH) in rat and other species such as primates (Plant *et al.* 1978, Ohkura *et al.* 1991). Indeed, GABA has been demonstrated to alter the release of GnRH from the ARC-median eminence fragments incubated *in vitro* (Masotto *et al.* 1989), and administration of GABA directly into the ARC significantly inhibit pulsatile luteinizing hormone (LH) secretion in rats (Nishihara & Kimura 1987). Furthermore, both GABA receptor subtypes in the ARC appear to be involved in the modulation of pulsatile LH secretion. The suppression of LH pulse frequency in response to intra-ARC of GABA_AR agonist or antagonist in castrated rams has led to the suggestion that normal operation of the GnRH pulse generator may require tonic GABAergic input and deviation from this results in dysfunction of the hypothalamic oscillator (Ferreira *et al.* 1996, 1998). However, controversially, Kimura *et al.* (1993) reported that continuous systemic injection of GABA_AR antagonist, readily elicits seizures that are associated with MUA volley frequency, but in the absence of behavioral seizures, GnRH pulse generator frequency is not affected in the rat. As regards to the GABA_BR, although infusion of GABA_BR agonist or antagonist into the ARC-median eminence region is shown to have no effect on LH pulse frequency in castrated sheep or female monkeys (Ferreira *et al.* 1996, Mitsushima *et al.* 2003), GABA_BR activation in the ARC/MBH is demonstrated to increase LH pulse amplitude (Ferreira *et al.* 1996) and reverse the negative feedback effect of oestradiol (E₂) or testosterone on LH release (Jackson & Kuehl 2002) in sheep.

Stress exerts profound inhibitory effects on reproductive function by suppressing the pulsatile release of GnRH and therefore LH, with different stressors acting through distinct neural pathways and involving various neurotransmitter systems (Li *et al.* 2010). Recently, we demonstrated that endogenous GABA signaling within the mPOA is involved in the stress-induced suppression of LH pulses (Lin *et al.* 2012). Nevertheless, neurons in the ARC receive dense direct GABAergic inputs (Decavel & van den Pol 1990), contain both GABA receptor subtypes (Anderson & Mitchell 1986) and display altered excitability in response to GABA receptor activation (Wagner *et al.* 1999, Muroya *et al.* 2005). Moreover, levels of GABA and/or its synthesizing enzyme glutamic acid decarboxylase₆₇ (GAD₆₇) are increased in the ARC in response to various stressors, including immunological challenge such as lipopolysaccharide (LPS) and interleukin-1 (Akema *et al.* 2005, Sirivelu *et al.* 2009), as well as psychogenic restraint stress (Bowers *et al.* 1998). More importantly, we demonstrated that activation of GABAergic neurons in ARC may

contribute to restraint stress-induced suppression of pulsatile LH secretion (Y S Lin, X F Li and K T O'Byrne, unpublished observation). In addition, bacterial endotoxin-induced suppression of GnRH release from the incubated hypothalamic POA/MBH fragments *in vitro* is mediated by both GABA receptor subtypes (Feleder *et al.* 1996). Therefore, there is no reason to exclude the possibility that GABAergic signaling within the ARC might contribute to the modulation of pulsatile LH secretion under stressful conditions.

Corticotrophin-releasing factor (CRF) has been demonstrated to play a pivotal role in stress-induced suppression of pulsatile LH secretion (Rivier & Vale 1985, Li *et al.* 2010). However, the failure to find any back-filled CRF cells from major CRF neuronal populations to GnRH perikarya using tract-tracing in rats (Hahn *et al.* 2003), suggests the CRF-mediated suppression of GnRH pulse generator activity may primarily involve indirect regulatory mechanisms. CRF has been shown to stimulate the release of GABA in a dose-related manner in some stress-related sites (Kash *et al.* 1999, Roberto *et al.* 2010). Moreover, the suppression of pulsatile LH secretion in response to i.c.v. administration of CRF is associated with activation of GABAergic neurons in the ARC. Nevertheless, it remains to be established whether GABA signaling within the ARC participates in the suppression of CRF on LH pulses.

The present study was designed to investigate the role of ARC GABAergic signaling in stress- and CRF-induced suppression of GnRH pulse generator by determining whether intra-ARC administration of selective GABA_AR or GABA_BR antagonist blocks the suppression pulsatile LH secretion in rats. We further investigated whether GABA_AR or GABA_BR antagonists have a differential role in suppression of pulsatile LH secretion in response to different types of stressful challenge, such as restraint and LPS.

Material and methods

Animals and surgical procedures

Adult female Sprague-Dawley rats (weighting 220–250 g) obtained from Charles River (Manston, UK) were housed under controlled conditions (12 h light:12 h darkness cycle; lights on at 0700 h; temperature at 22±2 °C) and provided with food and water *ad libitum*. All animal procedures were performed in accordance with the United Kingdom Home Office Regulations. All surgical procedures were carried out under ketamine anaesthesia (100 mg/kg

i.p.; Pharmacia and Upjohn Ltd, Crawley, UK) and xylazine (10 mg/kg i.p.; Bayer). Rats were bilaterally ovariectomised and implanted with a Silastic capsule (Sanitech, Havant, UK), filled to a length of 25 mm with E₂ (Sigma–Aldrich Ltd) dissolved at a concentration of 20 µg/ml arachis oil (Sigma–Aldrich). The E₂-containing capsules produced circulating concentrations of E₂ within the range observed during the diestrous phase of the estrous cycle ($\sim 38.8 \pm 1.2$ pg/ml) as previously described (Cagampang *et al.* 1991).

To investigate the effect of intra-ARC application of GABA receptor antagonists on stress- and CRF-induced suppression of pulsatile LH secretion, rats were implanted with bilateral guide cannula (22 gauge; Plastics One, Roanoke, VA, USA) directed towards the ARC, following the coordinates: 0.4 mm lateral, 3.3 mm posterior to bregma and 10.2 mm below the surface of the dura (Paxinos & Watson 1986). For experiments involving CRF administration, separate groups of rats were fitted with unilateral i.c.v. guide cannula (22 gauge; Plastics One) positioned towards the left lateral cerebral ventricle, following the coordinates: 4.4 mm lateral, 3.7 mm posterior to bregma and 5.9 mm below the surface of the dura (Paxinos & Watson 1986), in addition to the intra-ARC cannulae. The guide cannulae were secured using dental cement (Dental Filling Ltd, Swindon, UK) and fitted with dummy cannulae (Plastics One) to maintain patency. All brain cannulae were implanted at the same time of ovariectomy. After a 10-day recovery period, the rats were fitted with two indwelling cardiac catheters via the jugular veins, as described previously (Lin *et al.* 2011). Experimentation commenced 3 days later. Cannula placement was verified by histological inspection at the end of experiment, and animals with cannulae tips located outside the ARC were excluded from analysis.

Intra-ARC infusion of bicuculline or CGP-35348 on stress- or CRF-induced suppression of the GnRH pulse generator

To test whether the stress-induced suppression of LH pulses was mediated by endogenous GABAergic signaling in the ARC, we examined the effect of intra-ARC administration of selective GABA_AR (bicuculline; Sigma–Aldrich) or GABA_BR (CGP-35348; Sigma–Aldrich) antagonist on the restraint and LPS stress-induced suppression of pulsatile LH secretion. With respect to the evidence from other studies (Kimura *et al.* 1993, Hiruma *et al.* 1994), we established a regimen of bicuculline in preliminary studies (three injections of 0.2 pmol bicuculline/ 200 nl artificial cerebrospinal fluid (aCSF) per injection bilaterally into the

ARC) that did not cause seizures. Similarly, the selection of the dosage for CGP-35348 was also based on the evidence from other studies and our preliminary studies (Scott & Clarke 1993a, Sirivelu *et al.* 2009). On the morning of experimentation, a bilateral internal injection cannula (Plastics One) with extension tubing, preloaded with bicuculline, CGP-35348 or aCSF as vehicle control was inserted into the guide cannula, extending 1.0 mm beyond its tip to reach the ARC. The distal ends of the tubing were extended outside of the animal cage and connected to 5-µl Hamilton syringes (Waters Ltd, Elstree, UK), thereby allowing remote infusion without disturbing the animals during the experiment. Rats were attached via one of the two cardiac catheters to a computer-controlled automated blood sampling system, which allows for the intermittent withdrawal of blood samples (25 µl) without disturbing the animals. Once connected, animals were left undisturbed for 1 h, after which blood sampling for LH measurement commenced at ~ 1000 h and samples were collected every 5 min for 6 h. After removal of each 25 µl blood sample, an equal volume of heparinized saline (5 U/ml normal saline; CP Pharmaceuticals Ltd, Wrexham, UK) was automatically infused into the animal to maintain blood volume. For restraint stress, after 100 min of controlled blood sampling, bicuculline, (0.2 pmol in 200 nl aCSF for each side; $n=9$), CGP-35348 (1.5 nmol in 200 nl aCSF for each side; $n=10$) or aCSF (200 nl for each side; $n=9$) was administered over 5 min via the intra-ARC cannulae. Additional same doses of bicuculline, CGP-35348 or aCSF were given at 20 and 40 min after the initial injection. Twenty minutes after the initial intra-ARC injection, the rats were placed in custom-built restraint devices for 1 h. This was necessary since commercially available restraint devices would not be suitable for mobile animals with the head tether attachment used for the automated blood sampling in the experiment. Automated blood sampling continued uninterrupted during restraint and the 3-h post-restraint period. Additional controls were left to roam freely around their enclosure after the intra-ARC infusions of aCSF ($n=8$), bicuculline ($n=9$) or CGP-35348 ($n=9$). For the immunological stress, LPS (25 µg/kg in 0.3 ml saline, Sigma–Aldrich) was injected via the second i.v. catheter 20 min after the first intra-ARC injection of bicuculline ($n=10$), CGP-35348 ($n=10$) or aCSF ($n=9$). Two additional injections of the drug were given at 20 and 40 min after the initial injection. Control rats were given bilateral intra-ARC injections of aCSF ($n=8$), bicuculline ($n=9$) or CGP-35348 ($n=8$) followed by injection of 0.3 ml of saline instead of LPS.

For CRF experiment, rats were prepared following the procedure as described for the stress experiments. At the same time, an i.c.v. internal cannula (Plastics One) with extension tubing, preloaded with CRF or aCSF, was inserted into the unilateral guide cannula. The distant end of the tubing connected to the i.c.v. cannula was extended outside of the animal cage and attached to a 25- μ l Hamilton syringe (Waters Ltd). Blood sampling continued for 6 h. Bicuculline (0.2 pmol; $n=11$), CGP-35348 (1.5 nmol; $n=11$) or aCSF (0.2 μ l; $n=10$) was bilaterally administered into the ARC as described for the stress experiments. Twenty minutes after the first injection into the ARC, CRF (1 nmol in 4 μ l aCSF) was infused i.c.v. over a period of 5 min. Additional control rats were given bilateral intra-ARC injections of aCSF ($n=8$), bicuculline ($n=10$) or CGP-35348 ($n=9$), with the same doses and procedures, followed by i.c.v. injection of 4 μ l aCSF.

ARC cannula placement

Correct cannula placement in the ARC was confirmed by injection of 200 nl India ink through the internal guide cannula, followed by cresyl violet staining and microscopic inspection of 30- μ m frozen brain sections. Only data from animals with correct cannula placement was analyzed.

RIA for LH measurement

A double-antibody RIA supplied by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK, Bethesda, MD, USA) was used to determine the LH concentration in the 25- μ l whole blood sample. Reference preparation was rLH-RP-3. The sensitivity of the assay was 0.093 ng/ml. The intra-assay variation was 6.8%, and the inter-assay variation was 8.0%.

Statistical analysis

The algorithm ULTRA was used to establish the detection of LH pulses (Van Cauter 1988). Two intra-assay coefficients of variation of the assay were used as the reference threshold for the pulse detection. Briefly, ULTRA functions by comparing each data point to the two points before and after, and determining if a significant rise and fall has occurred in the profile; this is consistent with a pulse. The inhibitory effect of LPS stress or i.c.v. infusion of CRF on pulsatile LH secretion was calculated by comparing the mean LH pulse interval before treatment with that during the first 2 h post-LPS or CRF

administration. Additionally, the mean LH pulse interval during the 3rd plus 4th h post-LPS or CRF treatment period was compared with the 2-h baseline control period. The inhibitory effect of restraint stress on LH pulses was calculated by comparing the mean LH pulse interval before stress with the first prolonged interval, which corresponded to the first LH pulse interval, after stress onset. For the 2-h pre-restraint baseline and post-restraint recovery periods, the LH interpulse interval was calculated by dividing the appropriate period duration by the number of pulses. In the absence of stressors or CRF infusion, the LH interpulse intervals within the corresponding time periods were compared. Statistical significance was tested using one-way repeated measures ANOVA followed by Dunnett's test. $P<0.05$ was considered statistically significant.

Results

Correct ARC cannula placement

Figure 1 shows the location of individual bilateral cannula that was targeted in the ARC, as well as misplaced injection sites. Data from animals with incorrect cannula placement were analyzed separately.

Effects of intra-ARC infusion of bicuculline or CGP-35348 on restraint stress-induced suppression of LH pulses

In the restraint stress experiments, there was no significant difference between the LH pulse interval within the 2-h baseline period of control ($n=6$) and restrained ($n=7$) rats receiving aCSF, with group means $\pm$ S.E.M. of 21.11 ± 0.51 and 24.31 ± 0.87 min respectively (Fig. 2A, B and G). Restraint stress resulted in an immediate interruption of pulsatile LH secretion in aCSF-treated animals (Fig. 2B and G; $P<0.05$). Bilateral intra-ARC administration of GABA_BR antagonist, CGP-35348 (Fig. 2F and G; $n=8$; $P<0.05$) but not GABA_AR antagonist, bicuculline (Fig. 2D and G; $n=7$; $P=0.84$) 20 min before the onset of restraint stress and at 20 and 40 min after the initial injection significantly reduced restraint stress-induced suppression of LH pulses. Intra-ARC administration of bicuculline ($n=7$) or CGP-35348 ($n=7$) *per se* had no effect on LH pulse frequency (Fig. 2, C, E and G).

The data from rats with incorrect cannula placement were analyzed separately. The restraint stress-induced suppression of LH pulses was not attenuated by the administration of CGP-35348 ($n=2$) or bicuculline ($n=2$), with group means $\pm$ S.E.M. of 52.50 ± 7.50 and

Figure 1

Schematic illustration and photomicrograph of the infusion site targeted to the arcuate nucleus (ARC). (A and B) Schematic illustration and photomicrograph showing the target site for bilateral cannulation of the ARC. Bars, 500 μ m. (C, D and E) Schematic drawings of the ARC illustrating the individual sites of injection for the restraint, LPS stress or CRF infusion

experiments respectively. Numbers in each drawing indicate the distance (mm) to bregma. Open circles show the injection sites, open triangles show the misplaced injection sites. mt, mammillothalamic tract; 3V, third ventricle; ME, median eminence.

52.50 $\pm$ 2.50 min for the first prolonged LH pulse interval post restraint stress, respectively.

Effects of bicuculline or CGP-35348 on LPS stress-induced suppression of LH pulses

For the systemic stress experiments, in the 2-h basal control period, no differences in LH pulse interval were detected between the aCSF- ($n=7$), bicuculline- ($n=8$) or CGP-35348- ($n=6$) treated animals with saline co-injection (Fig. 3A, C, E and G). Administration of LPS (25 μ g/kg in 0.3 ml, i.v.) into aCSF-treated rats evoked a profound inhibitory effect on pulsatile LH release, which was observed after a delay of $\sim$ 30 min with the first prolonged LH pulse interval lasting more than 1 h, followed by a general recovery to normal LH pulse frequency (Fig. 3B and G; $n=7$; $P<0.05$). The animals treated with bicuculline demonstrated an attenuated LH pulse suppression in response to LPS stress (Fig. 3D and G; $n=9$; $P<0.05$), with a group mean $\pm$ s.e.m. of 31.14 $\pm$ 2.02 min for the LH pulse interval in the first 2 h following injection of LPS; this was not observed in the animals treated with CGP-35348

(Fig. 3F and G; $n=8$; $P=0.11$), with a group mean $\pm$ s.e.m. of 50.63 $\pm$ 5.02 min for the LH pulse interval in the same period. The administration of saline (0.3 ml, i.v.) had no effect on the LH pulse interval in animals treated with aCSF (Fig. 3A and G), bicuculline (Fig. 3C and G) or CGP-35348 (Fig. 3E and G). LPS induced similar suppression of LH pulses in one bicuculline-treated rats and two CGP-35348-treated rats that had cannulae located outside the ARC.

Effects of bicuculline or CGP-35348 on CRF-induced suppression of LH pulses

In the 2-h basal control period, no differences in LH pulse interval were observed in rats infused with aCSF vehicle ($n=8$), bicuculline ($n=9$) or CGP-35348 ($n=9$) into the ARC with centrally administrated CRF, with group means $\pm$ s.e.m. of 22.50 $\pm$ 0.84, 23.33 $\pm$ 1.13 and 23.61 $\pm$ 1.35 min, respectively (Fig. 4B, D, F and G). Administration of CRF (1 nmol, i.c.v.) increased the LH release interval (Fig. 4B and G; $P<0.05$). Bilateral intra-ARC administration of either bicuculline (Fig. 4D and G;

Figure 2

Representative examples illustrating the effect of intra-ARC administration of bicuculline (BIC) or CGP-35348 (CGP), on restraint stress-induced suppression of LH pulses in ovx, E₂-treated rats. Representative examples showing effects of bilateral intra-ARC infusion (down arrow) of aCSF (A and B), BIC (C and D) or CGP (E and F) on LH pulse frequency in the

absence and presence (horizontal line; 1 h) of restraint stress, respectively. (G) Summary showing the effect of intra-ARC infusion of selective GABA receptor subtype antagonists on restraint stress-induced suppression of pulsatile LH secretion. **P*<0.05 vs pre-restraint control period within the same group. **P*<0.05 vs restrained controls at the same time point.

P<0.05) or CGP-35348 (Fig. 4F and G; *P*<0.05) significantly reduced the inhibitory effect of CRF on pulsatile LH release. No significant differences were found between the effect of bicuculline or CGP-35348 on the attenuation of the suppression of LH pulses in response to CRF (Fig. 4D, F and G; *P*=0.53). The i.c.v. administration of aCSF had no effect on the LH pulse interval in animals treated with intra-ARC injection of aCSF (Fig. 4A and G; *n*=6), bicuculline (Fig. 4C and G; *n*=8) or CGP-35348 (Fig. 4E and G; *n*=7).

CRF induced a similar suppression of LH pulses in two bicuculline and two CGP-35348-treated rats in which the cannulae were located outside the ARC, with groups mean $\pm$ S.E.M. of 46.50 ± 3.50 and 47.50 ± 6.50 min for LH pulse interval in the first 2 h post injection of CRF respectively.

Discussion

The present study not only shows that endogenous GABA signaling within the ARC mediates stress-induced suppression of GnRH pulse generator, but also provides evidence for a stressor-specific manner of GABA receptor subtypes in

the regulation of the GnRH pulse generator. We found that administration of the selective GABA_AR antagonist, bicuculline, into the ARC markedly attenuated the inhibitory effect of LPS, but not restraint stress on pulsatile LH secretion. In contrast, restraint but not LPS stress-induced suppression of LH pulse frequency was reversed by intra-ARC infusion of the specific GABA_BR antagonist, CGP-35348. This is consistent with the evidence showing that various stressors, including restraint and LPS, increase GABAergic activity in the ARC (Bowers *et al.* 1998, Akema *et al.* 2005, Sirivelu *et al.* 2009). Consistently, a similar differential role has also been reported for mPOA GABA_AR and GABA_BR in various stressors-induced suppression of the pulse generator (Lin *et al.* 2012). In the present study, the radius of spread of dye from the centre of the injection site in the ARC was ~ 400 μ m (data not shown), making sure that the GABA antagonist is limited with the ARC region but not reach other critical stress-related sites such as mPOA (Herbison *et al.* 1995, Lohman *et al.* 2005, Lin *et al.* 2012). It is further supported by the evidence showing that perfusion of GABA antagonists via misplaced ARC cannulae was ineffective in blocking the inhibitory responses.

Figure 3

Representative examples illustrating the effect of intra-ARC administration of GABA_A receptor antagonist, bicuculline (BIC) or GABA_B receptor antagonist, CGP-35348 (CGP), on LPS stress-induced suppression of pulsatile LH secretion in ovx, E₂-treated rats. Representative examples showing effects of bilateral intra-ARC infusion (down arrow) of aCSF (A and B), BIC (C and D) or CGP (E and F) on LH pulse frequency in animals injected saline

(0.3 ml; down-headed open triangle) or LPS (25 µg/kg, in 0.3 ml saline; down-headed filled triangle) respectively. (G) Summary showing the effect of intra-ARC infusion of specific GABA receptor subtype antagonists on LPS stress-induced suppression of pulsatile LH secretion. [#]*P* < 0.05 vs pre-LPS control period within the same group. ^{*}*P* < 0.05 vs LPS-treated controls at the same time point.

A stressor-specific property of GABA receptor subtypes in the regulation of the GnRH pulse generator has been demonstrated in the present study. Consistently, previous studies have shown that GABA_AR antagonist, bicuculline blocks IL-1β (Sirivelu *et al.* 2009) but not restraint (Roozendaal *et al.* 1997) stress-induced suppression of preovulatory LH surge. On the other hand, the GABA_BR antagonist, CGP-35348, is without effect on systemic stress response (Sirivelu *et al.* 2009). Moreover, GABA_BR has been reported to be involved in psychogenic disorders like anxiety and depression (Nakagawa *et al.* 1996, Nowak *et al.* 2006). Nevertheless, the physiological significance of the differential effects of the GABA receptor subtypes observed in the present and previous study remains to be unknown. The identification of the ARC as a common site for GABA receptor modulation of restraint and LPS might suggest a convergence of pathways that are activated by different types of stressors, including psychological and immunological, which is similar to our previous observation with the region of mPOA (Lin *et al.* 2012). It is worth noting that the suppressive effect of LPS and

restraint on pulsatile LH secretion was not completely blocked by intra-ARC administration of the selective GABA receptor antagonists, raising the possibility that the GABAergic signalling within the ARC might just play a partial role in stress-induced suppression of GnRH pulse frequency.

Our previous study demonstrated that endogenous GABA signaling within the mPOA is involved in the stress-induced suppression of LH pulses, indicating the direct actions of GABA on GnRH neurons (Lin *et al.* 2012), since the GnRH soma are located in the mPOA. Although no GnRH neurons are observed in the ARC region in the rat (Merchenthaler *et al.* 1984), recent neuroanatomical studies demonstrated that GABAergic inputs heavily contact the entire length of GnRH dendron which include dendrite and axon in the arcuate-median eminence region (Campbell *et al.* 2005, Herde *et al.* 2013, Moore *et al.* 2015), indicating that the GABA may also act directly on GnRH dendrons in the mediation of LH pulses during stress. Additionally, as the critical excitatory player in the control of GnRH pulse generator activity (Goodman *et al.* 2007,

Figure 4

Representative examples illustrating the effect of intra-ARC administration of GABA_A receptor antagonist, bicuculline (BIC) or GABA_B receptor antagonist, CGP-35348 (CGP) on CRF-induced suppression of pulsatile LH secretion in ovx, E₂-treated rats. Representative examples showing effects of bilateral intra-mPOA infusion (down arrow) of aCSF (A and B), BIC (C and D) or CGP (E and F) on LH pulse frequency in animals injected with

aCSF (4 µl; down-headed open triangle) or CRF (1 nmol in 4 µl aCSF; down-headed filled triangle) respectively. (G) Summary showing the effect of intra-ARC infusion of selective GABA receptor subtype antagonists on CRF-induced suppression of pulsatile LH secretion. **P* < 0.05 vs pre-CRF control period within the same group. **P* < 0.05 vs CRF-treated controls at the same time point.

Li *et al.* 2009), kisspeptin neuronal system in the ARC is down-regulated by central administration of CRF and by various stressors, including restraint, LPS and hypoglycaemic stress (Kinsey-Jones *et al.* 2009, Castellano *et al.* 2010), suggesting a reduced kisspeptin system may be a contributing factor in stress-induced suppression of GnRH pulse generator frequency. However, the mechanisms causing the stress-induced decrease of kisspeptin remain to be explored. One recent study demonstrated a functional relationship between the kisspeptin and GABAergic neuronal network by showing that blockage of endogenous GABAergic signalling with bicuculline dramatically increases kisspeptin release (Terasawa *et al.* 2010). Therefore, GABAergic signalling might, in part, suppress the kisspeptin neuronal network in the ARC, and thereby GnRH pulse generator activity, during stress.

It is well established that CRF plays a pivotal role in stress-induced suppression of pulsatile LH secretion in various species (Li *et al.* 2010). Although the synaptic connections between CRF and GnRH neurons are observed in the mPOA of rats (MacLusky *et al.* 1988), providing the anatomical substrate for direct functional interaction between CRF and GnRH neurons; the failure to

find any back-filled CRF cells from major CRF populations to GnRH perikarya using tract-tracing in rats (Hahn *et al.* 2003) suggests that CRF-mediated suppression of GnRH pulse generator frequency may primarily involve indirect regulatory mechanisms. The present data, showing that infusion of GABA receptor antagonist into the ARC blocked the inhibitory effect of i.c.v. administered CRF on pulsatile LH secretion, indicates that GABAergic signaling within the ARC regions may be lower in the functional hierarchy to CRF in regulating GnRH pulse generator activity. Although whether GABAergic neurons in the mPOA receive direct CRF inputs has not been examined, CRF is demonstrated to augment the post-synaptic response to GABA via increasing GABA release in other brain regions, including the central nucleus of amygdala and bed nucleus of stria terminalis (Kash & Winder 2006, Roberto *et al.* 2010). Nevertheless, the possibility of direct actions of CRF at the level of the GnRH or GABA cell bodies could not be excluded, since infusion of CRF directly into ARC has been observed to suppress pulsatile LH secretion in ovx, E₂-primed rats (Li *et al.* 2010). This observation extends the studies demonstrating that the opioidergic signaling acts as a

downstream player to CRF in stress-induced suppression of GnRH pulse generator activity.

Although the main focus of the present study was to determine the role of the ARC GABAergic receptor signaling in the stress-induced suppression of pulsatile LH secretion, an inevitable adjunct was the effects of manipulating GABA signaling on GnRH pulse generator frequency *per se*. We have shown in the present study that intra-ARC administration of GABA_AR or GABA_BR antagonists at doses that block stress-induced suppression of LH pulses do not affect pulsatile LH secretion *per se*, suggesting that GABAergic signaling in the ARC might not be critical for regulation of GnRH pulse generator frequency under normal non-stress conditions. However, this suggestion has to be tempered because there is considerable evidence that the gonadal steroid milieu modulates GABAergic control of GnRH/LH secretion (Scott & Clarke 1993b). Indeed, dioestrous-like levels of E₂ replacement (Cagampang *et al.* 1991) were used in the present study to provide an enhanced preoptic GABA tone compared to an ovariectomised alone model (Mansky *et al.* 1982), so the results might have been different with the use of an alternative gonadal steroid replacement regimen or an intact rat model. Although some studies reported the inhibitory effect of GABA_AR antagonism with bicuculline on GnRH pulse generator frequency, it is possibly a consequence of CNS disturbances, at least in the rat (Kimura *et al.* 1993). It is further supported by evidence showing that a 70–90% reduction in the levels of GABA_AR activity at the GnRH neuron after knockdown of the γ 2-subunit in these neurons did not affect basal levels of LH or fertility in the mouse (Lee *et al.* 2010). With regard to GABA_BR antagonism in the ARC, we similarly failed to demonstrate an effect on pulsatile LH secretion, confirming observations in the sheep (Scott & Clarke 1993b). Thus, based on the preceding results, it appears that GABAergic signaling *per se* are not involved in the normal regulation of LH pulses under nonstressed conditions, but nonetheless their activation under stressful conditions consequently inhibits GnRH pulse generator activity.

The present study and our previous study demonstrate the role of GABA signaling in the ARC and mPOA in stress-induced suppression of LH pulses respectively. As the essential sites involving reproductive function, there is no reason to exclude the possibility that either the mPOA or the ARC might contribute to the modulation of pulsatile LH secretion under stressful conditions. Indeed, electrochemical stimulation of both the mPOA and ARC has been shown to affect LH release in rats (Kawakami *et al.* 1982). Moreover, CRF can act on both the mPOA and ARC to

inhibit pulsatile LH secretion in rats (Li *et al.* 2010). The consistent results regarding a role for the endogenous GABAergic system in the mPOA and ARC in the mediation of the LH pulse frequency during stress, presented in the pharmacological studies, emphasize that both regions may act as major sites involving stress-induced suppression of GnRH pulse generator frequency. In addition to the communication at the level of the median eminence that fibers originating in the ARC are shown to innervate GnRH fibers arising within the mPOA (Ramaswamy *et al.* 2008), the ARC also provides a direct or indirect route to GnRH perikarya within the mPOA (Pompolo *et al.* 2005, Wintermantel *et al.* 2006). Therefore, it is reasonable to speculate that the mPOA and ARC might not act independently but interact with each other in the modulation of GnRH pulse generator activity during stress.

Taken together, the results of this study indicate a pivotal and differential role of endogenous GABA_AR and GABA_BR signaling mechanisms in the ARC with respect to mediating immunological and psychological stress-induced suppression of the GnRH pulse generator respectively. Moreover, this observation demonstrates that the GABAergic signaling acts as a downstream player to CRF in stress-induced suppression of GnRH pulse generator activity. Nevertheless, more studies need to be conducted to characterize the mechanisms by which stress inhibits the GnRH pulse generator.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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