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Optogenetic stimulation of kisspeptin neurones within the posterodorsal medial amygdala increases LH pulse frequency in female mice

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Short title: Optogenetic stimulation of MePD Kiss1 increases LH pulse frequency

Abstract

Kisspeptin within the arcuate nucleus of the hypothalamus is a critical neuropeptide in the regulation of reproduction. Together with neurokinin B and dynorphin A, arcuate kisspeptin provides the oscillatory activity that drives the pulsatile secretion of GnRH, and therefore LH pulses, and is believed to be a central component of the GnRH pulse generator. It is well established that the amygdala also exerts an influence over gonadotrophic hormone secretion and reproductive physiology. The discovery of kisspeptin and its receptor within the posterodorsal medial amygdala (MePD), and our recent finding showing that intra-MePD administration of kisspeptin or a kisspeptin receptor antagonist results in increased LH secretion and decreased LH pulse frequency, respectively, suggests an important role for amygdala kisspeptin signalling in the regulation of the GnRH pulse generator. To further investigate the function of amygdala kisspeptin, the present study used an optogenetic approach to selectively stimulate MePD kisspeptin neurones and examine the effect on pulsatile LH secretion. MePD kisspeptin neurones in conscious Kiss1-Cre mice were virally infected to express the channelrhodopsin 2 protein and selectively stimulated by light via a chronically implanted fibre optic cannula. Continuous stimulation using 5 Hz resulted in an increased LH pulse frequency, which was not observed at the lower stimulation frequencies of 0.5 and 2 Hz. In wild-type animals, continuous stimulation at 5 Hz did not affect LH pulse frequency. These results demonstrate that selective activation of MePD *Kiss1* neurons can modulate hypothalamic GnRH pulse generator frequency.

Introduction

It is well established that hypothalamic kisspeptin (Kp) is a critical neuropeptide for reproduction. Inactivating mutations of the genes encoding *KISS1* or its receptor, *KISS1R (a.k.a. GPR54)*, result in hypogonadotrophic hypogonadism and a failure to progress through puberty in humans and rodent models (1, 2). Of the major hypothalamic populations of *Kiss1* neurones, in the anterior preoptic area (POA) and the arcuate nucleus (ARC), attention has focused on the ARC as a key component of the GnRH pulse generator (3, 4) These neurones, known as KNDy, because they co-express neurokinin B (NKB) and dynorphin A (DYN), innervate the distal processes of the GnRH neurones at the level of the median eminence (5) where kisspeptin can act directly to stimulate GnRH release (6). NKB acting through its receptor (TACR3) is thought to function as an excitatory signal to depolarise KNDy cells postsynaptically in the neural network, resulting in kisspeptin output to the GnRH neurones to initiate each GnRH pulse. The co-released DYN functions as an inhibitory signal within the KNDy neural network, acting presynaptically on kappa opioid receptors (KOR) to inhibit the release of NKB, thus terminating kisspeptin release and terminating the signal for GnRH secretion (7).

Despite the autonomous nature of the GnRH pulse generator, it is modulated by various signalling systems, including metabolic, circadian and stress, to regulate reproductive function. The amygdala, a key limbic brain structure commonly known for its role in higher-order emotional processing, is implicated in reproduction, including psychological stress-induced suppression of pulsatile LH secretion (8) and therefore it is reasonable to suggest that the amygdala is a component of upstream regulation of the hypothalamic GnRH pulse generator. The finding of extra-hypothalamic *Kiss1* expression and its receptor in the medial amygdala, and more specifically its posterodorsal subnucleus (MePD) (9), has opened up new possibilities concerning its role in reproductive function. Indeed, we have shown that the MePD *Kiss1* neurones are a key upstream regulator of pubertal timing (10), sexual motivation and social behaviour (11). Moreover, we have discovered through neuropharmacological studies that Kp signalling in the MePD *per se* robustly regulates hypothalamic GnRH pulse generator frequency (12), however, the underlying neural mechanisms are unknown.

In this study, we have used an optogenetic approach to selectively stimulate the MePD *Kiss1* neurones in fully-conscious mice to characterise the parameters that alter LH pulse frequency. Additionally, since *Kiss1* neurones in the ARC can be directly modulated by GABA (13, 14) and there are a large number of GABAergic neurones in the MePD (15, 16), this neurotransmitter might potentially mediate changes in LH pulse frequency observe during

optogenetic stimulation of *Kiss1* neurones in the MePD. Therefore, we will also determine whether tdTomato labelled *Kiss1* neurones in the MePD co-expressed GABA.

Materials and methods

Animals

Adult female mice (strain 129S6Sv/Ev) weighing between 19-23 g and aged between 6-8 weeks were used. They were bred in house at King's College London and genotyped with a multiplex PCR protocol to detect heterozygosity for the Kiss-Cre or wild-type allele (17). The primers to detect the wild-type allele were mKiss hetF3 (CCG TCA TCC AGC CTA AGT TTC TCA C) and mKiss hetR3 (ATA GGT GGC GAC ACA GAG GAG AAG C), and the primers to detect the mutant allele were mKiss a526 (GCT TTT ATT GCA CAA GTC TAG AAG CTC) and Asc403 (CAG CCG AAC TGT TCG CCA GGC TCA AGG). The Kiss-CRE;tdTomato mice carry a CRE-activated tdTomato transgene that is specifically expressed in *Kiss1* neurons (17). Mice were kept singularly housed under controlled conditions (12:12 h hour dark/light cycle, on at 07:00 h, 25 °C) and provided with food and water *ad libitum*. All animal procedures performed were approved by the Animal Welfare and Ethical Review Body (AWERB) Committee at King's College London, and in accordance with the UK Home Office Regulations.

Unilateral stereotaxic injection of channelrhodopsin viral construct and implantation of fibre optic cannula

All surgical procedures were performed under aseptic conditions. General anaesthesia was achieved using ketamine (Vetalar, 100 mg/kg, i.p.; Pfizer, Sandwich, UK) and xylazine (Rompun, 10 mg/kg, i.p.; Bayer, Leverkusen, Germany). Animals were firstly bilaterally ovariectomised immediately before being secured in a motorised Kopf stereotaxic frame. All cranial and brain surgical procedures were performed using a robot stereotaxic system (Neurostar, Tubingen, Germany). A small hole was drilled in the skull at a location above the MePD. The stereotaxic injection coordinates used to target the MePD were obtained from the mouse brain atlas of Paxinos and Franklin (18) (2.1 mm lateral, 1.70 mm posterior to bregma and at a depth of 5.1 mm). Using a 2-µL Hamilton micro-syringe (Esslab, Essex, UK) attached to the robot stereotaxic frame, 1 µl of the AAV-construct (AAV9.EF1.dflox.hChR2(H134R)-mCherry.SPRE.hGH (4.35 x 10^{13} GC/ml; Penn Vector Core, University of Pennsylvania, USA) was injected unilaterally into the MePD at a rate of 100 nl/min. The needle was left in position for a further 5 min and then removed slowly over 1 min. A fibre optic cannula (200 µm, 0.39NA,

1.25 mm ceramic ferrule; Thorlabs LTD, Ely, UK) was then inserted at the same co-ordinates as the injection site, but to a depth of 4.85 mm, so that the fibre optic cannula was situated immediately above the latter. A glue composite (RS Pro 20 g Super Glue, RS Components, Corby, UK) was then used to fix the cannula in place, and the skin incision closed with suture. All mice, both test (Cre+) and wild-type (Cre-), received the AAV injection and implantation of a fibre optic cannula. Following a 1 week recovery period from surgery, the mice were handled daily to acclimatise them to the tail-tip blood sampling procedure (19).

In vivo optogenetic stimulation of MePD kisspeptin neurones and blood samplings for LH measurement

All experiments were conducted in the absence of gonadal hormone replacement, in order to mitigate the modulatory effects of circulating oestrogen on the neuroendocrine control of the GnRH pulse generator. Experiments were carried out at least 4 weeks following surgery, to ensure sufficient opsin expression as well as to allow for sufficient habituation to the bleeding protocol. Prior to optogenetic stimulation, the very tip of the mouse's tail was excised using a sterile scalpel for subsequent blood sample collection (20). The chronically implanted fibre optic cannula was then attached via a ceramic mating sleeve to a multimode fibre optic rotary joint patch cables (Thorlabs), allowing freedom of movement of the animal, for delivery of blue light (473 nm wavelength) using a Grass SD9B stimulator controlled DPSS laser (Laserglow Technologies, Toronto, Canada). Laser intensity at the tip of the fibre optic patch cable was 5 mW. After 1 h acclimatisation, blood samples (4 µl) were collected every 5 min for 2.5 h. After 1 h controlled blood sampling, continuous optic stimulation (5-ms pulse width) was initiated at 0.5, 2 or 5 Hz for 90 min. To control for the effects of viral infection and surgical procedure, AAV-infected and fibre optic-implanted Kiss-Cre+ animals were bled for LH pulse detection in the absence of optic stimulation. Furthermore, to control for the effects of optogenetic stimulation, Cre- animals, which would therefore not express the ChR2, were bled in the presence of 5 Hz optic stimulation. The Kiss-Cre+ mice received all the stimulation protocols in random order, with at least two days, but typically one week, between experiments. Cre- (n = 5) animals received 5 Hz optic stimulation only.

The blood samples were processed by ELISA as reported previously (19). Mouse LH standard and antibody were purchased from Harbour-UCLA (California, USA) and secondary antibody (NA934) was from VWR International (Leicestershire, UK). The intra-assay and inter-assay variations were 4.6% and 10.2%, respectively.

Validation of AAV injection site and fibre optic cannula placement

After completion of experiments, mice were anaesthetised with a lethal dose of ketamine and transcardially perfused with heparinised saline for 5 min, followed by 10 min of ice-cold 4% paraformaldehyde (PFA) in phosphate buffer (pH 7.4) for 15 min using a pump (Minipuls, Gilson, Villiers Le Bel, France). Brains were rapidly collected and postfixed sequentially at 4 °C in 15% sucrose in 4% PFA and in 30% sucrose in phosphate-buffered saline until they sank. Afterwards, brains were snap-frozen on dry ice and stored at -80 °C until processing. Brains were coronally sectioned (30-µm) using a cryostat (Bright Instrument Co., Luton, UK) and every third section was collected between -1.34 mm to -2.70 mm from the bregma. Sections were mounted on microscope slides, air-dried and cover slipped with ProLong Antifade mounting medium (Molecular Probes, Inc. OR, USA). Precise injection sites were verified and evaluated and only animals expressing mCherry fluorescent protein in the MePD were included in the analysis. Positive neurones expressing mCherry fluorescent protein throughout the MePD were quantified using an Axioskop 2 Plus microscope (Zeiss, Gottingen, Germany). The neuroanatomical landmarks bordering the MePD were determined using a reference guide from the mouse brain atlas (17). The number of mCherry positive neurones was counted in the MePD of each animal using 5-6 section and the total number was used to calculate the group mean (mean ± SEM). Images were taken using Axioskop 2 Plus microscope (Zeiss) equipped with Axiovision version 4.7 (Zeiss). Additionally, correct fibre optic cannula placement in the MePD was confirmed by microscopic inspection of the same 30-µm brain sections. Only data from animals with correct AAV-injection and cannula placement were analysed

LH Pulses and Statistical Analysis

Detection of LH pulses was established by use of the Dynpeak algorithm (21). The effect of optogenetic stimulation on parameters of LH secretion was calculated by comparing the mean LH inter-pulse interval within the 90 min stimulation period with the 60 min pre-stimulation control period. For the non-stimulated control animals, the same timepoints were compared. The mean interval between LH pulses, within the 90 min stimulation period, or equivalent, was also compared between experimental groups. Statistical significance was tested using a two-tailed paired t-test. The LH pulse amplitude was calculated as the difference between the peak of an LH pulse and the baseline LH level, and the means were compared between the 60 min pre-stimulation period and 90 min stimulation period. The LH pulse amplitude was also compared between experimental groups within the 90 min stimulation period. $P < 0.05$ was considered statistically significant. Data are presented as the mean ± SEM.

Immunohistochemistry

Ovary intact 6-8 weeks old *Kiss/CRE - tdTomato* mice (strain 129S6Sv/Ev;C57B6) in dioestrus (determined via vaginal cytology) were perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS) pH 7.6. (Sigma-Aldrich, UK). The brains were removed and post-fixed in the same fixative at room temperature for 1 hour and then transferred into 30% sucrose solution in PBS until they sunk. The brains were cut on a microtome with a freezing stage into 30µm coronal sections and stored in cryoprotectant media at -20°C. Free-floating sections were washed in 1X Tris-buffered saline (TBS, pH 7.5) three times to remove the cryoprotectant media, then subjected to antigen retrieval in citrate buffer (pH 6.0) at 80°C for 30 min. The sections were washed in Tris-buffered saline containing 0.1% Triton X-100 (TBST) three times then blocked with 5% BSA (Sigma-Aldrich, UK) and 0.5% Triton X (Sigma-Aldrich, UK) for 30 min at room temperature on an orbital shaker. Sections were incubated with rabbit anti-GABA (A2052,1:600 dilution, Sigma-Aldrich, UK) in incubation media (0.25% Triton-X-100, 0.3% bovine serum albumin, 2% normal serum (NGS; Sigma-Aldrich, UK) in 1X TBS, pH 7.5) for 48 h at 4 °C on an orbital shaker. After the primary antibody incubation, sections were washed three times in TBST and then incubated with fluorophore 488-conjugated goat anti-rabbit (1:400 dilution; Thermo Fisher Scientific, UK) for 2 h at room temperature in the dark on an orbital shaker. Sections were then washed three times in 1X TBS and mounted on poly-lysine-coated slides (VWR, UK) in mounting medium (Fluoromount-G, ThermoFisher), cover slipped and stored at 4°C in the dark until being imaged using epi-fluorescence and confocal microscopy (Leica SP2 Laser Scanning Confocal Microscope, Cambridge Advanced Imaging Centre, UK). The brain sections were analysed for co-localization between the tdTomato fluorescence (red) and the GABA immunostaining signal (green) by photomicroscopy of each channel and combining the images for overlap of the signals indicated by a yellow colour. A signal was judged to be positive of the shape of the cell was co-incident between the two channels. To eliminate signals were not erroneously generated by overlapping cells, thin 30um sections were used and the focal plane of the signal was restricted by con-focal microscopy. For each section, the analysis was performed bilaterally.

Results

Validation of AAV injection site and fibre optic cannula placement

The AAV-ChR2 virus used to infect the cells in Kiss-Cre+ mice was tagged with fluorescent mCherry, allowing it to be visualised under a microscope. Analysis of images acquired from coronal sectioning of the mouse brains showed that 6 out of the 8 animals had successful stereotaxic injection of AAV-ChR2 virus into the MePD. The mean number of mCherry-

positive cells in unilaterally-injected brain sections was 22.00 ± 4.81 per animal. A representative example of a coronal brain section is presented in figure 1. Animals with misplaced injections did not have altered LH pulse frequencies following optogenetic stimulation (data not shown). mCherry cell bodies were not seen within the AVPV or ARC, indicating targeted ChR2 expression to *Kiss1* neurones in the MePD (Fig. 1D-G). Additionally, there was an absence of mCherry expression within the MePD of the AAV-ChR2 injected WT animals (data not shown). All fibre optic cannulae were correctly placed in the MePD.

Effects of continuous optogenetic stimulation of MePD kisspeptin neurones on LH pulse parameters

Following 1 h blood sampling with no light stimulation, MePD kisspeptin neurones were stimulated with blue light at 0.5, 2, or 5 Hz for 90 min. In Kiss1-Cre+ mice, only continuous stimulation at 5 Hz resulted in a significant increase in LH pulse frequency from a mean inter-pulse interval (IPI) of 26.07 ± 3.81 min to 16.64 ± 0.83 min (Fig. 2). No significant change in IPI was observed at 0.5 or 2 Hz optic stimulation, or in the non-stimulated controls (Fig. 2). Additionally, in Kiss1-Cre- mice, there was no change in LH pulse frequency at 5 Hz optic stimulation (Fig. 2). In terms of LH pulse amplitude, there was no statistically significant difference between the 60 min control period and the 90 min stimulation, or equivalent non-stimulation period for each group (Table 1).

***Kiss1* neurones in the medial amygdala do not co-express GABA.**

Kiss1 neurones in the ARC can be directly modulated by GABA (13, 14) and as there are a large number of GABAergic neurones in the MePD, we hypothesised that this neurotransmitter might mediate the changes in LH pulse frequency we observe after optogenetic activation of *Kiss1* neurones in the MePD. GABA expression was analysed by immunohistochemistry (IHC) in tdTomato positive *Kiss1* neurons in the MePD AVPV, and ARC (Fig. 3). The anti-GABA antibody has been used widely by other groups and its specificity is supported by IHC studies using tissues from GAD65 knock-out mice, which have reduced GABA production (22, 23) and in mice lacking both GAD65 and GAD67 where no GABA immunoreactivity is found (24). In the AVPV region, we found that 71% of tdTomato positive *Kiss1* neurones co-expressed GABA (n = 5 mice, 105 out of 147 neurones) but in the MePD, only 10% co-expression was found (n = 6 mice, 9 out of 93 neurones). In the ARC, 14% of tdTomato positive neurones expressed GABA (n = 3 mice, 139 out of 984 neurones).

Discussion

The present study demonstrates that optogenetic stimulation of *Kiss1* neurones within the MePD increases LH pulse frequency and provides clear evidence that MePD *Kiss1* neuronal activity can modulate the frequency of the hypothalamic GnRH pulse generator. These data extend our earlier neuropharmacological findings where intra-MePD administration of Kp dose-dependently increased LH levels (12) and more recent studies showing chemogenetic activation of MePD *Kiss1* neurones increased mean circulating levels of LH (25, 26). The relationship between the amygdala and its regulation of gonadotrophic hormone secretion has received considerable attention, with evidence to support the notion that the medial amygdala in fact has a negative output on the reproductive system. Stimulating the medial amygdala results in delayed pubertal onset (27), whilst lesions advance the onset of puberty (28) and increase the secretion of LH (29). Indeed, the findings that up to 70% of the neuronal outputs arising from the MePD are GABAergic (16), including a significant percentage of those reaching reproductive centres (15), support the idea of an MePD inhibitory modulation of reproduction. We have recently shown MePD neurones projecting directly onto ARC *Kiss1* neurones, although their neurochemical phenotype remains unknown (30). The identification of the latter connection to the KNDy network might help to establish the underlying mechanisms by which the MePD regulates the GnRH pulse generator. Interestingly, MePD *Kiss1* neurones have also been shown to project to GnRH neurones in the medial preoptic area (POA) (31). However, our previous data showing *Kiss1* receptor antagonism in the MePD reduced LH pulse frequency (12) would suggest that MePD *Kiss1* signalling *per se* rather than *Kiss1* output to the POA GnRH neurones underlies the upstream regulation of the hypothalamic GnRH pulse generator. Moreover, chemogenetic activation of MePD *Kiss1* fails to activate GnRH neurones (25). These data and our present finding that continuous lower-frequency optic stimulation increased the frequency of LH pulses supports the concept that MePD control of LH secretion is via activation of the KNDy neurones in the ARC, which are thought to be a major component of the neural construct underlying GnRH pulse generation (3, 4).

How can we reconcile the findings that classical stimulation and lesion studies suggest an inhibitory influence of the amygdala, whilst selective optical stimulation of MePD *Kiss1* enhances LH secretion? Clearly, these experimental manipulations are different, with lesion and stimulation studies affected a wider range of neurons than specific activation of *Kiss1* neurones. In addition, although GABAergic neurones are present in the MePD, we did not find extensive co-localisation between GABA and tdTomato labelled *Kiss1* neurones in this region. In contrast, we found that GABA was co-localized with around 70% of tdTomato positive *Kiss1*

neurons in the AVPV which is similar to that found by others (32). In the ARC, GABA staining was found in only 14% of *Kiss1* neurones, which is lower than the 25-50% reported elsewhere (32, 33). This discrepancy may reflect different methodological approaches as we have measured GABA directly while the other groups used less direct approaches such as *in situ* hybridization for *Gad67* expression (32) or reporter activity driven from the *Vgat* promoter (33). Alternatively, it is possible that the anti-GABA antibody may not detect all GABAergic neurones in the ARC although we consider this to be unlikely given the data for robust staining in the AVPV. Since very few *Kiss1* neurones in the MePD are GABAergic, we would not expect these to be inhibitory on LH secretion.

An alternative, but less simple explanation might involve a disinhibitory system whereby *Kiss1* in the MePD activates inhibitory GABAergic interneurons, which in turn inhibit GABAergic efferents to the ARC. Whilst this remains hypothetical, it is important to realise that the MePD is of pallidal origin (34), therefore suggesting that a neural circuit within this structure is, at least in part, of a classical GABAergic disinhibitory nature. Although the endogenous firing pattern of MePD *Kiss1* neurones is unknown, we have shown that continuous optogenetic activation of this population at the relatively low frequency of 5 Hz increased LH pulse frequency, thus demonstrating the minimal activational requirements to modulate the hypothalamic GnRH pulse generator. Further work is required to establish the functional link between MePD *Kiss1* neurone activation and GABAergic signalling to affect pulsatile LH secretion.

Evidence that *Kiss1* neurones within the MePD have a stimulatory role modulating the GnRH pulse generator is an important development in reproductive neuroendocrinology, as it provides further mechanistic insight into why KISS1R receptor antagonism in the MePD was shown to delay puberty, disrupt oestrous cyclicity and reduce the occurrence of the preovulatory LH surge in the rat (10). The MeA is highly-active in response to a range of external stressors such as restraint (35), footshock (36), and predatory odour (37), and is involved in the activation of the hypothalamic-pituitary-adrenal (HPA) axis in response to external anxiogenic stimuli; increased levels of ACTH and glucocorticoids are accompanied with stimulation of the MeA (38). Additionally, as part of the limbic brain, the MePD is an upstream regulator that likely acts as a neural hub integrating several external signals, including olfactory chemosignals and anxiogenic stimuli, with the function of the GnRH pulse generator. Kp and the amygdala have been shown to be critically involved in the integration of olfactory cues with the promotion of sexual behaviour. Lesioning of the MePD results in a diminution of sexual partner preference in rodents (39), and in the “ram effect”, whereby the introduction of a sexually active ram can overcome reproductive inactivity in anoestrous ewes,

the resurgence of functional LH pulsatility coincides with an increase in *Kiss1* neurone activity (FOS) in the ARC, POA, and MeA (40, 41). Our previous study using pharmacological activation of MePD *Kiss1* neurones with the DREADD (Designer Receptors Exclusively Activated by Designer Drugs) methodology built upon previous understanding of the facilitatory role of the MePD in sexual behaviour. We showed that stimulating MePD *Kiss1* neurones in this way produces a dual result of augmenting sexual partner preference whilst attenuating anxiety behaviour in male mice (11). Furthermore, in studies of men viewing sexual images, intravenous administration of kisspeptin results in increased neuronal activity in the amygdala, detected with fMRI neuroimaging, corresponding to a reduction in negative mood and reduced aversion to sex (42). Taken together, these are important developments in reproductive neuroendocrinology. Investigating kisspeptin in the limbic brain has been able to further our understanding of the elusive neural mechanisms governing reproductive physiology, including pubertal development, as well as the mechanisms by which stress has its inhibitory effects on reproductive capability.

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Brown RC, Thomas SA, Bloom SR, Dhillo WS. Kisspeptin modulates sexual and emotional brain processing in humans. *J Clin Invest*. 2017; **127**(2): 709-19.

Data Availability Statement: All data contained within the manuscript have been deposited in the King's Research Data Management System and are freely available to public access (www.kcl.ac.uk/library/researchsupport/research-data-management/preserve/deposit-your-data-with-kings3).

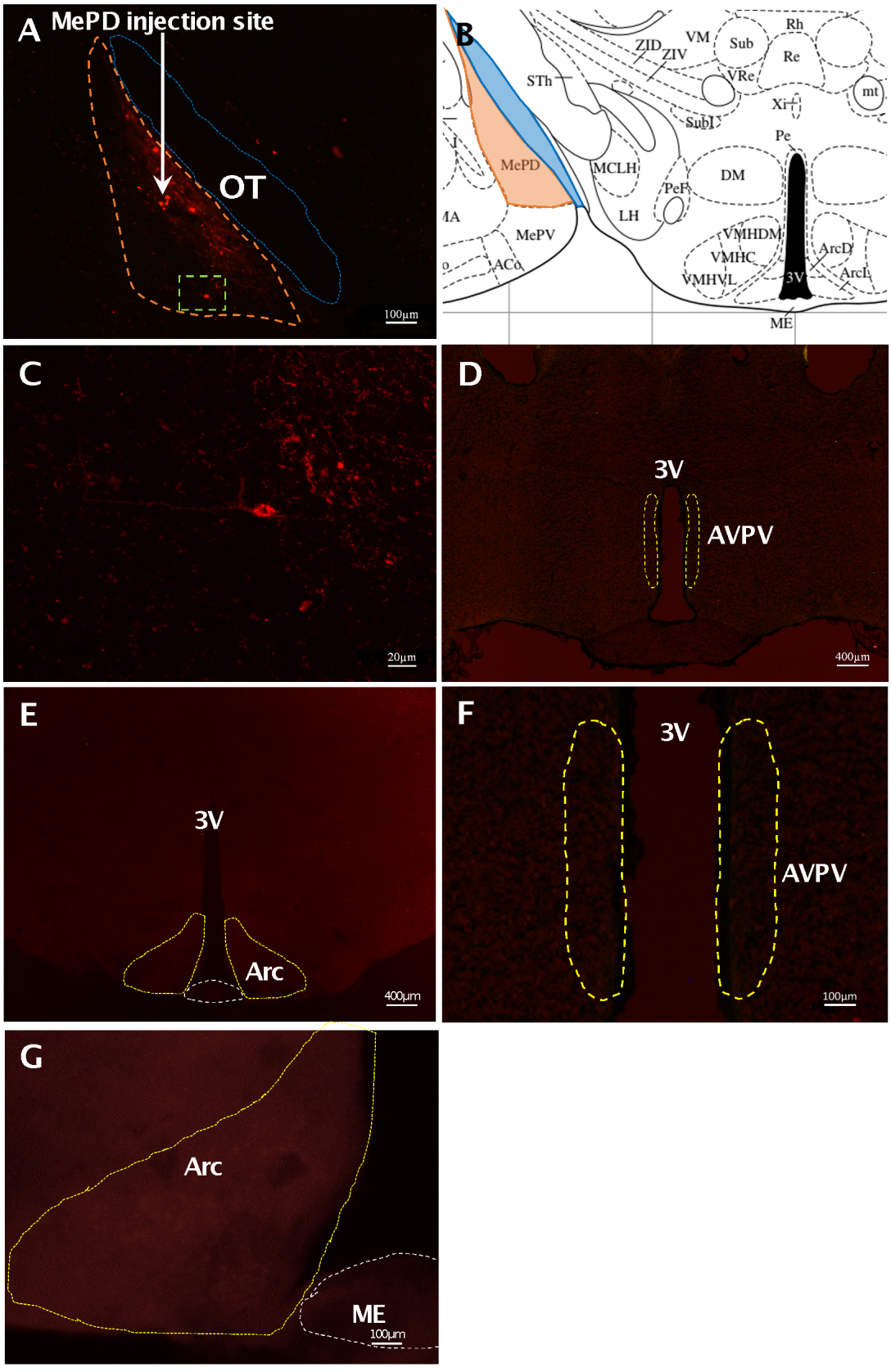
Figure 1: Expression of ChR2-mCherry in MePD kisspeptin neurones in Kiss-CRE mice. Coronal section showing red mCherry fluorescence positive neurones (orange line) in the MePD (A) and the white arrow indicates the injection site of AAV9.EF1.dflox.hChR2(H134R)-mCherry.WPRE.hGH into the MePD of Kiss-Cre mice in the same section. Higher-power view shows the MePD kisspeptin neurones tagged with mCherry (red fluorescence), which indicates ChR2 receptor expressing kisspeptin neurones (C); this image is a magnification of the area of Fig. 1A encased within the green dotted line. The absence of mCherry fluorescence in the AVPV (D) and ARC or median eminence (ME) (E) shows the specific infection of MePD kisspeptin cells with the channelrhodopsin. Higher-power view of the AVPV (F) and ARC (G) further confirms the absence of mCherry-infected cells in these regions. Schematic representation of MePD and its spatial relationship with the optic tract (B). ME, median eminence; OT, optic tract; 3V, third ventricle.

Figure 2: Effect of continuous optogenetic stimulation of MePD *Kiss1* neurones on LH pulse frequency. Representative examples showing the effects of no stimulation (A), 0.5 Hz (B), 2 Hz (C), 5 Hz stimulation (D) on pulsatile LH secretion in ovariectomised mice. A representative example is also shown for the effect of 5 Hz stimulation on ovariectomised wild-type mice (E). (F), The mean LH inter-pulse interval for increasing frequencies before and after the onset of stimulation. Continuous stimulation at 5 Hz resulted in a significant reduction in the LH inter-pulse interval. LH pulses detected by the DynePeak algorithm are indicated with an asterisk. $^{\#}P < 0.05$ vs control, (n = 6 per treatment group). Results represent mean ± SEM.

Figure 3: GABA expression in Kiss1-tdTomato labelled neurones in different regions of the hypothalamus. Brain sections from gonadally intact Kiss-CRE;tdTomato female mice were examined for co-localization between tdTomato and GABA. tdTomato was detected by intrinsic fluorescence in the brain slices and GABA by immunohistochemistry. In the MePD, the white filled arrowhead indicates a tdTomato neurone (red) that co-expresses GABA (green). Unfilled arrowhead indicates a tdTomato neurone (red) that does not co-express GABA. The fifth panel is a magnified area of the region shown by the orange and red box in the merged panel respectively. n = 6 mice, 7-11 sections per mouse. In the AVPV region, arrowheads indicate tdTomato neurones (red) that co-express GABA (green). The fifth panel is a magnified area of the region shown by the orange box in the merged panel. 3V, third ventricle. n = 5 mice, 4-5 sections per mouse. In the ARC region, very few GABA positive neurones were found and only 14% of tdTomato positive neurones expressed GABA. White filled arrowhead indicates Kiss1-tdTomato neurones (red) that co-express GABA (green).

Unfilled arrowheads indicate Kiss1-tdTomato neurones (red) that do not co-express GABA. The fifth panel is a magnified area of the area encased by the orange box in the merged panel. Scale bar =100μm. n = 3 mice, 4-5 sections per mouse. For all sections nuclei were counterstained with DAPI (blue).

Figure 1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MePD injection site
OT
100μm
B
STh
ZID
ZIV
VM
Sub
Rh
Re
VRe
Xi
mt
SubI
Pe
MePD
MCLH
DM
PeF
LH
MePV
VMHDM
VMHC
VMHVL
ArcD
ArcL
3V
ACo
ME
C
20μm
D
3V
AVPV
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E
3V
Arc
400μm
F
3V
AVPV
100μm
G
Arc
ME
100μm

Figure 2

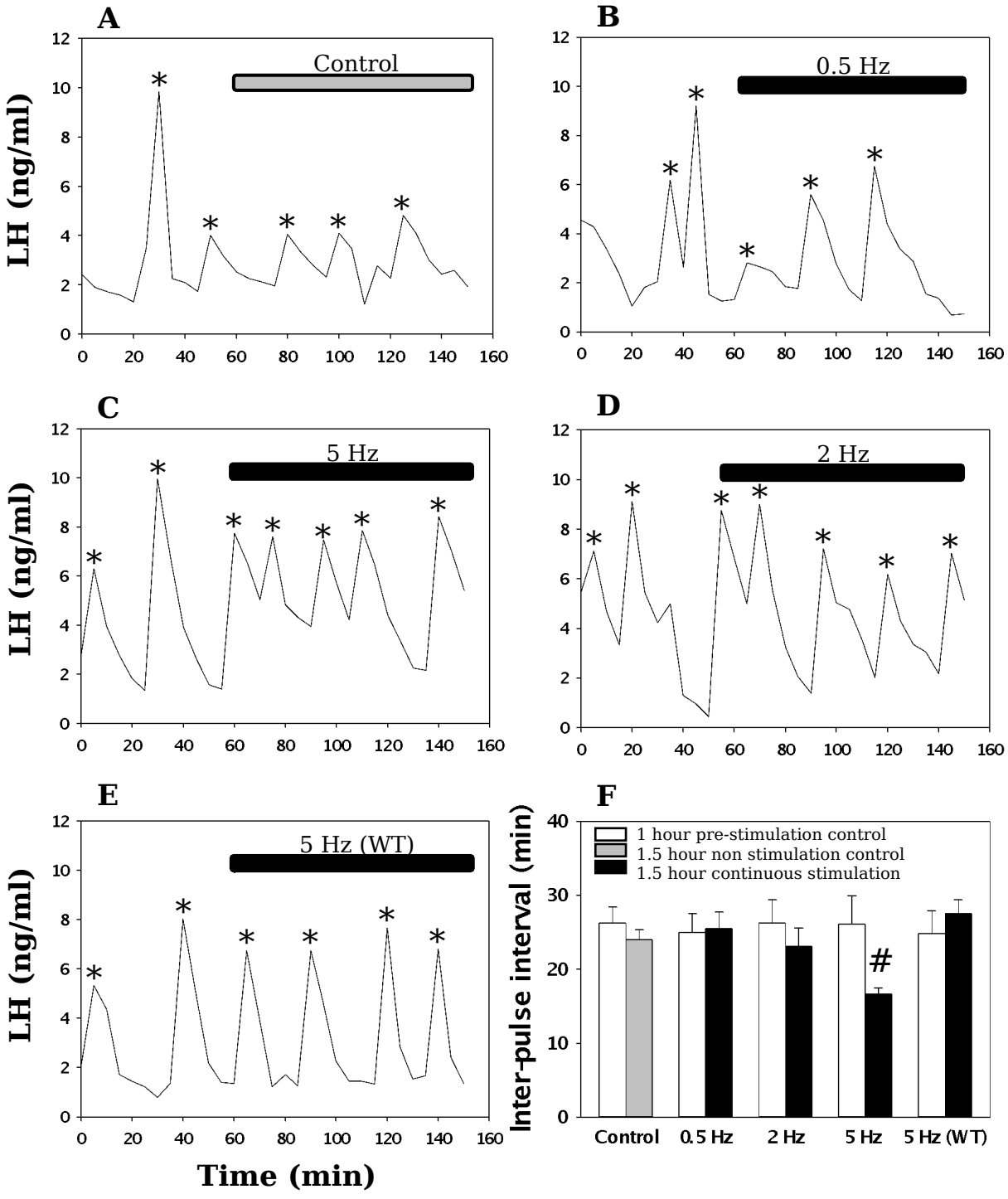
A
Control
B
0.5 Hz
C
5 Hz
D
2 Hz
E
5 Hz (WT)
LH (ng/ml)
Time (min)
F
1 hour pre-stimulation control
1.5 hour non stimulation control
1.5 hour continuous stimulation
Inter-pulse interval (min)
#
Control
0.5 Hz
2 Hz
5 Hz
5 Hz (WT)

Figure 3

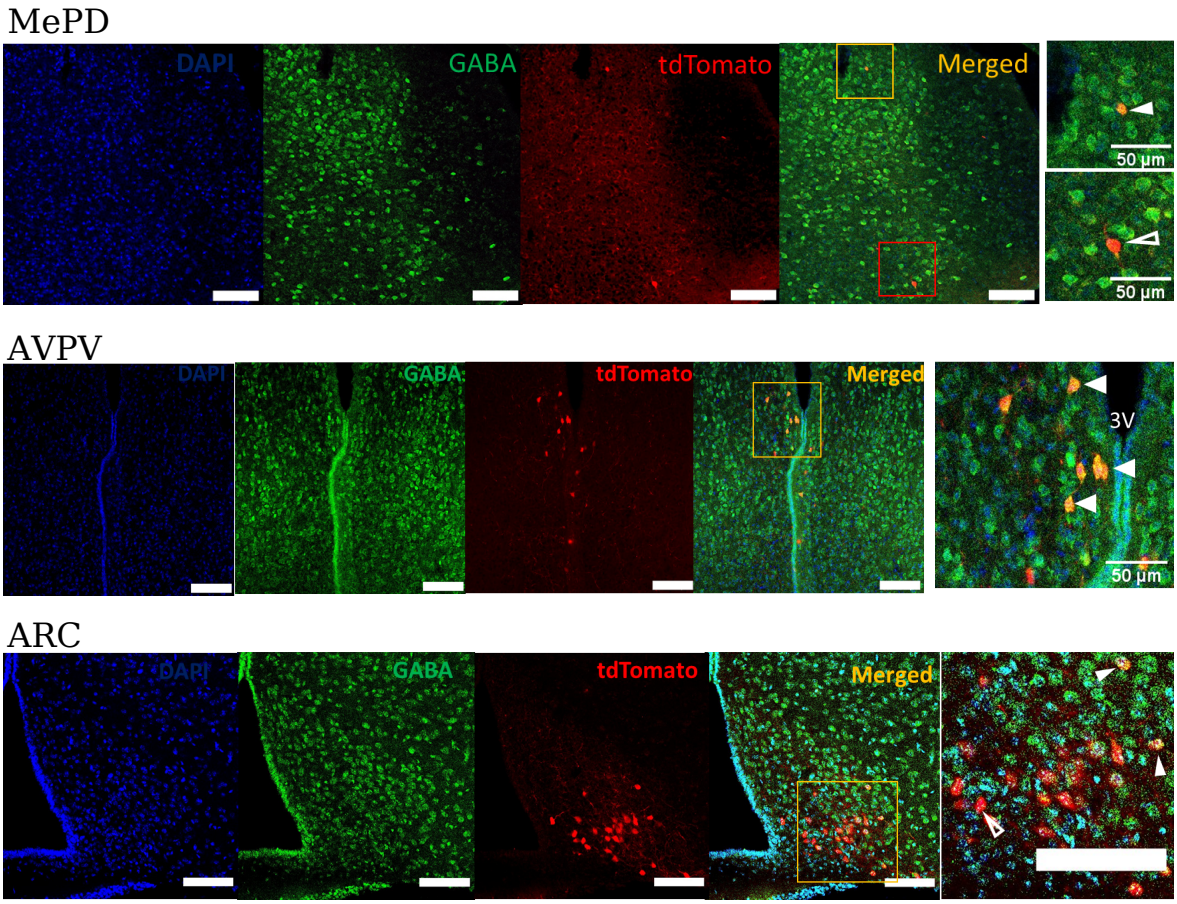
MePD
DAPI
GABA
tdTomato
Merged
50 µm
50 µm
AVPV
DAPI
GABA
tdTomato
Merged
3V
50 µm
ARC
DAPI
GABA
tdTomato
Merged

Table 1. LH pulse amplitude before and during optogenetic stimulation of the MePD *Kiss1* neurones. The mean (± SEM) values for LH pulse amplitude before and during optic stimulation showed no statistically significant difference in each experimental group between the mean LH pulse amplitude before and during 5 Hz optic stimulation n = 6 per group.

Frequency of optic stimulation	Mean LH pulse amplitude (ng/ml)	
	Control period	Stimulation period
Control (no optic stimulation)	3.50 ± 2.05	4.26 ± 1.91
0.5 Hz	3.31 ± 1.71	3.46 ± 0.76
1 Hz	3.19 ± 1.59	3.22 ± 1.20
2 Hz	3.91 ± 2.70	4.45 ± 2.88
5 Hz	4.38 ± 3.45	3.67 ± 2.34

Table 2. Number of *Kiss1*-tdTomato labelled neurones co-localised with GABA in different regions of the hypothalamus and amygdala. The mean (± SEM) values of co-localisation of GABA in *Kiss1*-tdTomato labelled neurones in each region. n = 3 - 6 animals per group.

Region	Number of Kiss1-tdTomato labelled cells	Number of colocalization with GABA
MePD	18.6 ± 3.5	1.8 ± 0.7
AVPV	36.75 ± 14.9	26.25 ± 10.9
ARC	328 ± 3.1	46.33 ± 5.7