Amygdala Kisspeptin Neurons: Putative Mediators of Olfactory Control of the Gonadotropic Axis

Rafael Pineda a, Fabrice Plaisier a, Robert P. Millar a–d, Mike Ludwig a

a Centre for Integrative Physiology, University of Edinburgh, Edinburgh, UK; b Mammal Research Institute, Department of Zoology and Entomology, and c Centre for Neuroendocrinology, University of Pretoria, Pretoria, and d MRC/UCT Receptor Biology Unit, Institute for Infectious Disease and Molecular Medicine, University of Cape Town, Cape Town, South Africa

Key Words
Kisspeptin · Amygdala · Olfactory bulb · Gonadotropic axis

Abstract
Kisspeptins and their receptors are potent regulators of the gonadotropic axis. Kisspeptin neurons are found mainly in the hypothalamic arcuate nucleus and the anteroventral periventricular nucleus. However, there is also a third population of kisspeptin neurons, located in the amygdala. We used fluorescence immunohistochemistry to quantify and localize the amygdala kisspeptin neurons and to reveal close apposition and putative innervations by vasopressinergic and tyrosine hydroxylase-positive dopaminergic neurons. Using microinjections of retro- and anterograde tracers, and viral transfection systems in rats and transgenic mice, we showed reciprocal connectivity between the accessory olfactory bulb and the amygdala kisspeptin neurons. In vitro recordings indicate an inhibitory action of kisspeptin on mitral cells in the accessory olfactory bulb. Using viral specific-cell gene expression in transgenic mice in combination with double immunofluorescence histochemistry, we found that the amygdala kisspeptin neurons also project to gonadotropin-releasing hormone (GnRH) neurons in the preoptic area. Our neuroanatomical and electrophysiological data suggest that amygdala kisspeptin neurons integrate social behaviour and olfactory information into GnRH neurons in the preoptic area to coordinate the gonadotropic axis and the appropriate output behaviour to olfactory cues.

Introduction
Kisspeptins and their receptors are critical components in the control of the gonadotropic axis [1–3]. Kisspeptins act via the G protein-coupled receptor Kiss1r (also known as GPR54) [4, 5], which is expressed on the majority (>90%) of GnRH neurons [6]. Kisspeptin administration strongly stimulates gonadotropin secretion [4, 5], suggesting that kisspeptin is a major regulator of gonadotropin-releasing hormone (GnRH)/gonadotropin secretion and a key determinant of sex steroid production and secretion by the gonads. In rodents, kisspeptin-expressing neurons are found in two areas of the hypothalamus: the arcuate nucleus and the anteroventral periventricular nucleus [7–9]. These mediate negative and positive feedback (in females), respectively, from the sex steroids onto GnRH neurons [10, 11].

For reproduction to fully function, the gonadotropic/endocrine axis must be accompanied by appropriate behaviours. Interestingly, kisspeptin may also be involved
in the regulation of behaviours through a third population of kisspeptin neurons found in the medial amygdala \[8, 12\]. The medial amygdala is a key part of the limbic system responsible for complex social behaviours \[13–15\]; it integrates signals relayed by several neuropeptides important for social behaviours, notably vasopressin, corticotrophin-releasing factor, and oxytocin \[16\]. Vasopressin plays a fundamental role in social behaviours, including affiliation, social cognition, aggression, and anxiety/stress responses \[17–19\]. In addition, changes in dopamine levels increase motivation and reward in these behaviours \[20–22\].

Most social behaviours in mammals require recognition of individuals which is largely mediated visually and by odours. The olfactory bulb is the main part of the brain involved in receiving and sending odour information to brain areas involved in behaviours and memory, including the amygdala \[23, 24\]. Anatomically and functionally, the olfactory bulb is organized in two systems: the main olfactory system and the accessory olfactory system \[23, 25\]. The accessory olfactory system consists of the vomeronasal organ, localized in the nasal cavity, and the accessory olfactory bulb (AOB) in the posterior-dorsal area of the olfactory bulb. Vomeronasal cells project to the AOB, where they make contact with mitral cells \[26\]. Mitral cells in the AOB project to the amygdala \[27\], and cells in the amygdala send projections back to the AOB \[28, 29\], suggesting a feedback control of olfaction by the amygdala.

Our work was aimed at characterizing the localization of kisspeptin neurons in the amygdala and their innervation by the dopamine and vasopressin systems, and to determine whether the amygdala kisspeptin neurons project to the olfactory system and affect olfactory neuron activity. In addition, using viral specific-cell gene expression in combination with double immunofluorescence histochemistry, we aimed to characterize whether amygdala kisspeptin neurons project to GnRH neurons in the preoptic area (POA).

**Materials and Methods**

**Animals**

Adult male Sprague-Dawley rats (290–300 g) and adult male transgenic Kiss1-CreGFP mice (Kiss1tm1.1(creEGFP)Stei, Jackson Laboratory, stock No. 017701; 28–30 g) were housed on a 12:12-hour light:dark cycle (lights off at 19:00 h) with free access to food and water. All experiments were conducted in accordance with a UK Home Office project licence that was reviewed by the University of Edinburgh Ethics Committee.

**Amygdala Kisspeptin Neurons: Localization and Inputs**

To localize the kisspeptin neurons in the amygdala and the inputs from dopamine and vasopressin neurons, adult male rats (290–300 g) were injected intraperitoneally with a lethal dose of pentobarbital sodium and perfused transcardially with heparinized (129 mg/l) 0.9% saline, followed by 4% paraformaldehyde (PFA) in PBS (pH 7.2–7.4). The brains were removed and post-fixed overnight at 4°C in a 2% PFA + 15% sucrose solution and then cryoprotected in 30% sucrose in PBS with 0.01% sodium azide. Coronal sections (40 μm) were cut on a freezing microtome. Each brain was divided into three sets of sections; the first set of each animal was used for double kisspeptin and tyrosine hydroxylase (TH, the rate-limiting enzyme of catecholamine biosynthesis converting tyrosine into the precursor of dopamine) immunofluorescence histochemistry (see below), the second set for kisspeptin and vasopressin, and the last set for kisspeptin staining only.

**Retrograde Tracing Study**

The injection method we used is described in detail elsewhere \[30, 31\]. For retrograde tracing experiments, we used red RetroBeads (Lumafluor Inc.). Sprague-Dawley rats (290–300 g) were first anesthetized with 4% isoflurane and then maintained on 1.5–2% isoflurane throughout surgery. The rats were placed in a stereotaxic frame, and a glass capillary (Drummond Scientific Company; cat. No. 2-000-005) was implanted in the posterodorsal area of the medial amygdala using the following coordinates with reference to the bregma \[32\]: anteroposterior (AP) –3.4 mm, mediolateral (ML) 3.8 mm, and dorsoventral (DV) 8.5 mm. Red RetroBeads were pressure injected (200 nl, 100 nl/min). After injection the wound was sutured, and the rats were given buprenorphine (0.03 mg/kg) subcutaneously for pain relief during the recovery period. One week after injection, the rats were perfused transcardially as above, and the olfactory bulbs were removed and processed as described later.

**Anterograde Tracing Studies**

For anterograde tracing experiments, we used fluoro-Ruby (10% in PBS, D-1817; Life Technologies). Under stereotoxic conditions, a glass capillary was implanted in the rat AOB using the following coordinates: AP 6.0 mm, ML 1.5 mm, and DV (from brain surface) 1.0 mm. In 4 rats, 50–100 nl of fluoro-Ruby was injected into the AOB by iontophoresis.

To show the bidirectional connectivity between the olfactory bulb and the amygdala, we used 300 nl of a combination of red RetroBeads and an adeno-associated virus (AAV) expressing a green fluorescent protein (GFP) sequence under the ubiquitous cytomegalovirus immediate-early enhancer/chicken β-actin hybrid (CAG) promoter (serotype I/2) (AAV1/2-CAG-GFP). AAVs have been commonly used to deliver genes of interest into adult neurons in the central nervous system in vivo \[33\]. The mix was injected using the same coordinates as for the beads in the retrograde study, but in this case the rats were left for 4 weeks for viral expression of GFP in the medial amygdala.

**Kiss1-CreGFP mice** were transfected with AAV to evaluate the potential projections from amygdala kisspeptin neurons to the olfactory system and to GnRH neurons in the POA. We used a cre-dependent AAV to deliver a fluorescence reporter specifically to amygdala kisspeptin neurons \[34, 35\]. In this system, an inverted yellow fluorescent protein (YFP) reporter sequence was floxed by two LoxP sequences oppositely oriented (fig. 5a). Thus after the cre
recombinant step, YFP was orientated to the correct sense and transduced specifically into kisspeptin cells under the constitutive elongation factor 1-alpha promoter (Ef1a) [36, 37]. This type of AAV is commonly known as double-floxed inverse open reading frame (DIO) type and was purchased from the Gene Therapy Center Vector Core (University of North Carolina; serotype 5, AAV5-DIO-YFP).

Adult male heterozygotic Kiss1-CreGFP mice (28–30 g) were injected unilaterally with 400 nl (100 nl/min) of AAV5-DIO-YFP into the medioamygdala following the coordinates of the reference atlas [38]: AP –1.9 mm, ML 2.0 mm, and DV 4.9 mm. Three weeks after injection, the mice were perfused transcardially with heparinized saline followed by PFA as above, and the brains were removed and processed for double immunofluorescence histochemistry as described below. Six sections from each mouse (n = 3) – corresponding to bregma 0.38, 0.50, 0.62, 0.74, 0.86, and 0.98 – were evaluated, and the percentage of GnRH neurons receiving YFP (amygdala kisspeptin projection) appositions was determined.

**Immunofluorescence Protocols**

The sections were treated as described before [31]. Briefly, they were mounted in order, caudal to rostral, on SuperFrost® Plus slides (VWR; cat. No. 631-0108) and dried for 1 h at 37°C. They were then washed in PBS-T (0.1% Tween 20) for 10 min and subjected to heat-induced epitope retrieval using 10 mM sodium citrate (pH 6) for 10 min at 90°C. The heat-induced epitope retrieval step greatly increases amygdala kisspeptin staining, but in the tracing experiments the step was omitted since it compromised detection of the RetroBeads, fluoro-Ruby, and fluorescent reporters (GFP and YFP). After cooling to room temperature in a water bath and a 5-min wash in PBS, the sections were preincubated in blocking buffer [3% of the appropriate serum (goat or donkey) + 0.4% Triton X-100 in PBS] for 45 min. Next, the sections were incubated with primary antibodies (table 1) in blocking buffer for 2 days at 4°C. Afterwards, the sections were washed for 3 × 10 min in PBS and then incubated with secondary antibodies (table 1), diluted in blocking buffer for 1 h at 37°C, and then washed 3 × 10 min in PBS. Nuclear DNA was stained with Hoechst 33342 (10 μg/ml in PBS for 5 min and washed 3 times for 5 min in PBS). The slides were briefly immersed in double-distilled water and covered-slipped using Fluormount Aqueous Mounting Medium (F4680; Sigma). All steps were performed at room temperature unless otherwise stated. No signal was detected in control sections after applying secondary antibodies in the absence of primary antibodies. Also, no kisspeptin signal was detected after preincubation of tissue with 1 μM kisspeptin-10 in blocking buffer (Tocris; cat. No. 4243).

Immunoreactivity was visualized with the Nikon A1R FLIM confocal system. Z stacks were condensed to maximum intensity projections using NIS-Elements software. Images were exported to the ImageJ software. To aid colour-blind readers [39], in a specific case (revealing kisspeptin fibres in the olfactory bulb) the red channel was recoloured to magenta; an overlay of green and magenta channels will result in white colour.

**Analysis of Fibre Appositions**

Dopaminergic and vasopressinergic inputs to the amygdala kisspeptin neurons, and amygdala kisspeptin projection to GnRH neurons in the POA, were quantified as described elsewhere [7]. Briefly, sections were examined under epifluorescence microscopy using a ×40 objective. Kisspeptin and GnRH neurons were then evaluated for close appositions of TH or vasopressin and YFP fibres, respectively. We considered fibres to be in close apposition only when directly adjacent to GnRH neurons and in the same focal plane. For qualitative evaluation of fibres’ appositions, selected kisspeptin or GnRH neurons were imaged using confocal microscopy, and 3D reconstructions were generated using Imaris software (Bitplane).

**Kiss1r mRNA Detection in the Olfactory System by Final RT-PCR**

To determine Kiss1r expression in the olfactory system, total RNA was isolated from parts of microdissected AOBs and the hy-

---

**Table 1. Primary and secondary antibodies used in the immunofluorescence assays**

| Antibodies (Primary) | Code | Supplier | Dilution | Raised in |
|----------------------|------|----------|----------|-----------|
| Kisspeptin           | #564 | Dr. A. Caraty | 1/10,000 | rabbit |
| Vasopressin-neurophysin | PS41 | Dr. H. Gainer | 1/1,500 | mouse |
| TH                   | MAB318 | Merck Millipore | 1/1,500 | mouse |
| MAP2                 | ab5392 | Abcam | 1/1,500 | chicken |
| GFP/YFP              | ab13970 | Abcam | 1/10,000 | chicken |
| GnRH                 | MAB5456 | Merck Millipore | 1/1,000 | mouse |

**Secondary antibodies**

| Antibodies (Secondary) | Code | Supplier | Dilution | Raised in |
|------------------------|------|----------|----------|-----------|
| Streptavidin-Alexa Fluor® 555 conjugate | S-32355 | Life Technologies | 1/500 | – |
| Streptavidin-Alexa Fluor® 488 conjugate | S-32354 | Life Technologies | 1/500 | – |
| Alexa Fluor® 488 anti-mouse | A-21202 | Life Technologies | 1/500 | donkey |
| Alexa Fluor® 555 anti-rabbit | A-31572 | Life Technologies | 1/500 | donkey |
| Alexa Fluor® 488 anti-chicken | A-11039 | Life Technologies | 1/500 | goat |
| Alexa Fluor® 555 anti-mouse | A-21422 | Life Technologies | 1/500 | goat |

Amygdala Kisspeptin Neurons
pithalum, kidney, liver, and lung using TRIzol® Reagent (Thermo Fisher Scientific; cat. No. 15596-026) as instructed by the manufacturer. Further, DNase treatment and RNA purification was done using the High Pure RNA Tissue Kit (Roche; cat. No. 12033674001). Then, 0.5 μg of total RNA was used for cDNA synthesis using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche; cat. No. 05081955001). cDNA was amplified using GoTaq® G2 Green Master Mix (Promega; cat. No. M7822). Rat Kiss1r mRNA (NM_023992.1) was detected by final RT-PCR, using the following PCR primer pair: Kiss1r – forward (5′-CTT CAC CGC GCT CCT CTA TC-3′) and Kiss1r – reverse (5′-CGG GAA CAC AGT CAC GTA GC-3′); amplicon size 151 bp. Cycling PCR conditions consisted of a first denaturing cycle at 98°C for 30 s, followed by 35 (Kiss1r) or 30 (rat ribosomal protein S11) cycles of amplification, defined by denaturation at 98°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. A final extension cycle of 72°C for 5 min was included. For an internal RT reaction control, amplification of a 240-bp fragment of rat ribosomal protein S11 mRNA (NM_031110.1) was performed in parallel in each sample using the following primer pair: S11 – forward (5′-CAT TCA GAC GGA GCG TGC TTA C-3′) and S11 – reverse (5′-TGC ATC TTC ATC TTC GTC AC-3′). The specificity of the PCR products was confirmed by sequencing (Source BioScience sequencing service).

**Slice Preparation for in vitro Electrophysiology**

Recordings were performed on brain slices from adult Sprague-Dawley rats (2–3 months old). Rats were anesthetized with isoflurane and decapitated. The olfactory bulbs were removed, and 300-μm sagittal sections were cut in ice-cold sucrose artificial cerebrospinal fluid (aCSF; composition in mM: 86 NaCl, 1.2 NaH2PO4, 2.5 KCl, 25 NaHCO3, 25 glucose, 50 sucrose, 0.5 CaCl2, and 7 MgCl2; saturated with 95% O2/5% CO2, pH 7.2–7.4, 300 mOsm). The slices were transferred to ACSF to equilibrate for 30–40 min at 35 ± 1°C (composition in mM: 124 NaCl, 1.2 NaH2PO4, 2.5 KCl, 25 NaHCO3, 20 glucose, 2 CaCl2, and 1 MgCl2; saturated with 95% O2/5% CO2, pH 7.2–7.4, 300 mOsm).

Mitral cells were recorded in loose-patch-clamp configuration (seal resistance 20–30 MΩ) with aCSF-filled patch pipettes (3–6 MΩ) pulled from borosilicate glass capillaries (model GC150F-10; Harvard Apparatus) with a horizontal puller (P-97; Sutter Instruments). Spontaneous action potentials (spikes) were recorded using an Axopatch 200B amplifier (Axon Instruments) in track mode.

All mitral cell recordings were conducted at 25 ± 2°C during constant aCSF perfusion (2 ml/min). Slices were visualized with an upright microscope (Zeiss Axioskop) equipped with ×10 and ×40 immersion objectives and infrared differential interference contrast (IR-DIC). Images were acquired using a Hamamatsu Orca-ER camera controlled by Simple PCI software (Digipixel). AOB mitral cells were identified by the cells dorsal to the lateral olfactory tract. Only mitral cells that showed a spontaneous firing rate were recorded. It has previously been shown in mice that 94% of AOB mitral cells were spontaneously active [40].

Rat kisspeptin-10 (Tocris; cat. No. 4243) was prepared at 100-μM stock solution in double-distilled water, stored at −20°C, and diluted 1,000 times to a 100-nM working solution in aCSF just before being applied to the bath by perfusion for 2 min [6, 41].

**Biocytin Filling**

In some recordings, the electrode solution (aCSF) contained (0.2% w/v) biocytin (B-1592; Life Technologies) to confirm the localization of the recorded cell. After the loose-patch recordings, the cells were filled for 3–4 min with biocytin in the whole-cell configuration and the slices were incubated in 4% PFA in PBS overnight at 4°C. The following day, the slices were washed 3 times for 10 min with PBS, then washed with PBS-T for 10 min and incubated for 1 h in a streptavidin incubation buffer (0.4% Triton X-100 in PBS). They were then incubated 4 h at room temperature with 1/500 Streptavidin-Alexa Fluor® 488 conjugate (see table 1 for reference) in incubation buffer, and later washed 3 times for 10 min in PBS and mounted using Fluoromount.

**Data Collection and Recording Analysis**

Recorded signals were low-pass filtered at 2 kHz and digitized at 5 kHz with an A/D converter (Digidata 1322A; Axon Instruments). pClamp software (Molecular Devices) was used to record and analyse the data. The absolute mean firing rate and the relative percentage of change during 3 min before and after kisspeptin infusion were analysed. Statistical differences were calculated using a two-tailed paired t test analysis in GraphPad Prism software. Significance was set at * p ≤ 0.05 and ** p ≤ 0.01.

**Results**

**Amygdala Kisspeptin Neurons: Localization and Inputs**

The total number of kisspeptin neurons in the amygdala, plus their localization within the amygdala, was evaluated in 3 animals (fig. 1). Kisspeptin neurons were restricted to the caudal portions of the posterodorsal area of the medial amygdala (MePD), with an average of 149 ± 8 kisspeptin neurons per amygdala (range 120–172 kisspeptin neurons per amygdala). The maximum number of kisspeptin neurons was found at bregma −3.48. No kisspeptin neurons were found rostral to bregma point −3.12, even though the MePD extends up to bregma −2.40 [32], indicating that amygdala kisspeptin neurons are localized only in the caudal portions of the MePD.

To evaluate inputs to the amygdala kisspeptin neurons, we used immunohistochemistry to co-stain sections for TH and vasopressin. Twenty-five percent (77 of 305) of the identified amygdala kisspeptin neurons showed appositions with TH fibres, and 11% (31 of 294) with vasopressin fibres (fig. 2).

**Retrograde Tracing**

To determine whether amygdala kisspeptin neurons receive inputs from the olfactory system, we injected RetroBeads into the MePD area where kisspeptin neurons were found. The RetroBeads labelled mitral cells in

**DOI:** 10.1159/000445895

**Neuroendocrinology 2017;104:223–238**
Fig. 1. Rat amygdala kisspeptin population: localization and numbers. a Expression of kisspeptin (Kp) neurons in a coronal section of the rat amygdala at bregma level –3.48. b Distribution and average number of kisspeptin neurons (red dots) throughout the rat medial amygdala (atlas templates from Paxinos and Watson [32]). c Total number of kisspeptin (Kp) neurons at different levels. d Total number of kisspeptin neurons per amygdala (n = 6). Values are expressed as means ± SEM. opt = Optic track.
(For legend see next page.)
the AOB (fig. 3a) but not in the main olfactory bulb. We observed a different pattern of RetroBead distribution in the mitral cell layer of the AOB, with the anterior part showing a more intense optical density (amount of beads) than the posterior part (fig. 3b). Quantification of the pixel intensity using ImageJ (fig. 3b) revealed a significant difference ($p \leq 0.01$, two-tailed unpaired t test) in optical density between the two parts of the AOB (anterior part $100 \pm 14.35$ vs. posterior part $46.20 \pm 6.36$, expressed as mean ± SEM; 3 animals, 2 sections per animal; fig. 3c).

### Anterograde Tracing Assay

To confirm the existence of a pathway from the AOB to the amygdala, we used the anterograde tracer fluoro-Ruby (fig. 4a, b). Figure 4c shows strong labelling of fibres.
Amygdala Kisspeptin Projection to the Olfactory Bulb

To test whether the amygdala kisspeptin neurons project to the olfactory system, we injected an AAV system into the amygdala to express YFP specifically in kisspeptin neurons (fig. 5a). The YFP reporter was not expressed in response to virus injection in wild-type mice (not shown). Immunohistochemistry for YFP indicated a clear projec-

Fig. 4. Connectivity between mitral cells of the AOB and amygdala kisspeptin (Kp) neurons. a Injection of the anterograde tracer fluoro-Ruby into the AOB. b Enlarged view of the yellow square in a. c Labelled fibres from the AOB project towards the amygdala along layer 1 of the posterolateral cortical amygdaloid nucleus (white arrowhead) and adjacent to the MePD. d Some AOB fibres penetrate into the MePD and can be seen adjacent to kisspeptin neurons (green, indicated with white arrowheads). e Enlarged view of the kisspeptin neuron (green) indicated with a blue arrowhead in d. Some non-kisspeptin cells also seemed to be contacted by multiple boutons (d, white arrowheads). MTCL = Mitral cell layer; GCL = granule cell layer; opt = optic track.
Fig. 5. Amygdala kisspeptin projections to the AOB. a Scheme of the experimental procedure. a1 AAV expressing an inverted YFP reporter sequence floxed by two LoxP sequences oppositely oriented (AAV5-DIO-YFP). a2 AAV expressing the GFP sequence under the ubiquitous CAG promoter AAV1/2-CAG-GFP. b AAV5-DIO-YFP injection into mouse medial amygdala shows kisspeptin projection (yellow) in the AOB. Hoechst DNA nuclear marker in blue. c, d Combination of red RetroBeads and AAV1/2-CAG-GFP injection into rat MePD revealed a bidirectional connection between the AOB and the MePD. d Enlarged view of the white square in c. e–g Immunohistochemistry confirming kisspeptin-expressing fibres (magenta) in the AOB. MAP2, a specific marker for neuronal dendrites, in green. ITR = Inverted terminal repeat sequence; WPRE = woodchuck hepatitis post-transcriptional regulatory element (expression enhancer); pA = polyadenylation signal sequence.
tion from the amygdala kisspeptin neurons to the AOB (fig. 5b) but no projections to the main olfactory bulb (data not shown). Combination of red RetroBeads and AAV1/2-CAG-GFP injection into the MePD revealed a bidirectional connection between mitral cells of the AOB and the MePD (fig. 5c, d). Interestingly, the kisspeptin fibre innervations of the AOB appear to be different in mice and rats. In mice, kisspeptin projections innervate the mitral and granule cell layer, whereas in rats only granule cells are innervated. Whether this reflects differences between species or just differences in degree of expression or technique (e.g. AAV serotype) needs to be determined.

We confirmed the presence of a kisspeptin projection in the AOB using immunohistochemistry for kisspeptin (fig. 5e–g). To exclude the possibility that these fibres were dendrites from the amygdala kisspeptin neurons, we used a specific marker for neuronal dendrites, microtubule-associated protein 2 (MAP2) [43]. The lack of staining with MAP2 confirms that the fibres were axonal projections.

**Kisspeptin Receptor Expression in the AOB**

To determine kisspeptin receptor expression in the AOB, we performed an expression analysis of the Kiss1r transcript in microdissected samples from the AOB and other tissues using RT-PCR (fig. 6). The RT-PCR results show Kiss1r mRNA in the AOB and hypothalamus but not in other tissues such as the kidney, liver, and lung.

**In vitro Recordings**

Seventeen AOB mitral cells from 8 male rats were recorded using patch-clamp electrophysiology. Following kisspeptin administration, the mean firing rate and the percentage of change were determined. Nine of the 17 cells (53%) had reduced their firing rate by more than 10%, whereas 8 cells were unchanged (fig. 7).

**Amygdala Kisspeptin Projection to GnRH Neurons in the POA**

The animals in which the AAV5-DIO-YFP system was injected into the amygdala were also used to determine whether amygdala kisspeptin neurons project to the GnRH neurons in the POA. Double immunofluorescence histochemistry for YFP and GnRH was performed, and the percentage of GnRH neurons receiving YFP appositions was quantified in appropriate sections at coordinates of the brain atlas (fig. 8g). We found that the amygdala kisspeptin neurons project via the stria terminalis (fig. 8a–c) to reach the GnRH neurons in the POA (fig. 8d, e). About 15% (11 of 71) of the identified GnRH neurons in the POA appeared to receive inputs from amygdala kisspeptin neurons (fig. 8f), with the highest number of connections at bregma level 0.50 (33 ± 7%; n = 3 mice, 6 sections per mouse; fig. 8g).

**Discussion**

Although mRNA for kisspeptin in the amygdala has been reported previously [8, 12], the specific localization was not shown. We demonstrate here that many kisspeptin neurons are located in the caudal areas of the MePD. The function of kisspeptin neurons in the amygdala is unknown, but the amygdala has been implicated in numerous physiological and behavioural processes, including those related to reward, social behaviours, and reproduction [13–15, 44].

We found TH-immunoreactive fibres (TH is commonly used as a marker for dopaminergic neurons) adjacent to amygdala kisspeptin neurons. Our current retrograde studies (not shown), using FluoroGold, indicate...
Fig. 7. Changes in electrical activity in mitral cells of the AOB in response to kisspeptin infusion. a Biocytin-filled mitral cell in the AOB after patch-clamp recording. Right panel: enlarged view of the red square. b Examples of mitral cell recording showing a lack of response (b1) or reduction in firing rate of greater than 10% after kisspeptin infusion (b2). c Absolute and percentage change in firing rate from all cells recorded (* p ≤ 0.05). GL = Glomerular layer; MTCL = mitral cell layer; LOT = lateral olfactory tract; Kp10 = kisspeptin-10.
Fig. 8. Amygdala kisspeptin projections to GnRH neurons in the POA. a AAV5-DIO-YFP injection into the amygdala shows that the amygdala kisspeptin neurons project via the stria terminalis (red arrowhead) to reach the GnRH neurons in the POA (b, c). The white arrowhead in a indicates the layer 1 of the posterolateral cortical amygdaloid nucleus pathway to the olfactory system. d, e Two examples of confocal images and 3D reconstructions of GnRH neurons (red) showing appositions of fibres from the amygdala kisspeptin neurons (yellow). Hoechst DNA nuclear marker in blue. f Number of GnRH neurons identified receiving YFP (amygdala kisspeptin) appositions. g Percentages of GnRH neurons with YFP appositions for different coordinates from the brain atlas [38]. Values are expressed as means ± SEM.

(For fig. 8f–g see next page.)
that at least some of the dopaminergic fibres originate in the midbrain dopaminergic population of the ventral tegmental area (VTA). Connections between the amygdala and the VTA have been described previously [45–50]. The VTA is a key brain area of the reward system [20] and has been linked with social behaviours, including affiliate behaviour and attachment [51]. We also found vasopressin fibres in close apposition with amygdala kisspeptin neurons. It is believed that these fibres come from parvocellular vasopressin neurons in the paraventricular nucleus [52]. Vasopressin plays an important role in the regulation of social bonding [53–55] and aggressive behaviours [56]. In conjunction, social bonding and aggression are critical behaviours for species perpetuation. Aggression allows better access to resources (food mainly), while affiliative interactions are necessary for reproductive behaviours, and vasopressin is important in the control of both [19]. The secretion of testosterone is involved in both aggressive and reproductive behaviours [57], and kisspeptin neurons control its production and secretion. Thus, kisspeptin neurons in the amygdala may link appropriate behaviours modulated by vasopressin and dopamine with the reproductive state of the gonadotropic axis.

In rodents, most behaviours, including social behaviours, are initiated by olfactory cues. To evaluate connectivity between the olfactory system and the amygdala kisspeptin neurons, we used microinjections of retro- and anterograde tracers, and viral transfection in rats and transgenic mice. The experiments with the retrograde tracer showed that mitral cells in the anterior part of the AOB have more prominent projections to the MePD than the mitral cells in the posterior region. The AOB is involved in sensing pheromonal cues from the vomeronasal organ [58]. Anatomically and functionally, the AOB is divided into an anterior and a posterior part [25, 59]. The apical layer of the vomeronasal organ, which expresses receptors of the V1R family, projects to the anterior AOB, while neurons located in the basal layer express receptors of the V2R family and project to the posterior AOB [23, 60]. The two parts of the AOB display different patterns of neuronal activation, as indicated by expression of the immediate early gene c-fos [61], suggesting that the two parts play different roles in an individual’s odour recognition.

Half of the mitral cells recorded electrophysiologically were inhibited by kisspeptin administration. We found expression of the kisspeptin receptor transcript (mRNA) in microdissected samples of the AOB. However, the precise anatomical localization of the kisspeptin receptor has yet to be described in the olfactory system, and it is possible that the inhibitory response is mediated indirectly, via granule cells of the AOB. Currently, it is believed that Kiss1r is a G_{q/11} -coupled receptor, and therefore we would have expected that any direct effects of kisspeptin on mitral cells would be excitatory. However, we cannot exclude the possibility that kisspeptin couples to G proteins in a cell-specific manner [62], and if Kiss1r on mitral cells is G_i coupled, it may explain the observed inhibitory effect on electrical activity.

Alternatively, granule cells are the most common inhibitory (GABA) cell type in the olfactory bulb, and they
control the activity of mitral cells through dendrodendritic inhibition [63, 64]. Thus, it is possible that Kiss1r is expressed in granule cells of the AOB and the inhibitory effect seen is indirect. Amygdala kisspeptin expression changes during the oestrous cycle, with significantly higher levels of mRNA expression during the phase of pro-oestrus [12]. Mating in rodents is restricted to the late pro-oestrus/early oestrus phase of the cycle. The increase in secretion of kisspeptin during proestrus may stimulate granule cell activity and hence block/decrease the activity of mitral cells filtering out odour cues in the AOB, as has been shown for other neuropeptides in the main olfactory bulb [65].

The MePD is enriched with androgen and oestrogen receptors [66, 67] and is connected to brain areas implicated in reproductive behaviours [28], including the POA, where most GnRH neurons are located. It was shown that the kisspeptin neurons from the arcuate nucleus and anteroventral periventricular nucleus project to the POA [68]. Using a cell-specific viral targeting approach, we showed here that the amygdala kisspeptin neurons also project to GnRH neurons in the POA. Most GnRH neurons express the kisspeptin receptor, and kisspeptin is believed to be one of the most potent regulators of GnRH neuron activity [6]. In this study, we found that the amygdala kisspeptin neurons project via the striatal terminalis (fig. 5), the major pathway from the amygdala region [29] to reach GnRH neurons in the POA. Taken together, the three kisspeptin populations projecting to the POA [68] form a complex network to control GnRH neurons.

The described neurocircuitries between the AOB, amygdala kisspeptin neurons, and the GnRH neurons in the POA may help to explain several behaviours related to pheromonal cue-induced re-organization of the gonadotropic axis. For example, the Whitten effect, where groups of females start cycling in synchrony when they are exposed to a male or his odour [69], and the Bruce effect, an abrupt abortion of pregnancy in response to the smell of a male who is not the father [70], suggest involvement of the pheromone/odour pathway in the control of the gonadotropic axis, and the amygdala kisspeptin neurons may link the two functions, i.e. olfaction and reproduction. The kisspeptin neurons in the amygdala may also link appropriate behaviours modulated by vasopressin and dopamine with the reproductive state of the gonadotropic axis.

In addition to the neuroanatomical data presented here, recent functional studies have shown that kisspeptin signalling in the amygdala regulates gonadotropin secretion [44]. Together, these data provide evidence of a role for the amygdala kisspeptin neurons in the control of the gonadotropic axis, thus integrating limbic circuits and reproductive hormone secretion. However, further work is required to show that these kisspeptin cells in particular do indeed modulate GnRH secretion.

Acknowledgements

This work was supported by an MRC grant (to M.L.), by the Newton International Fellowship programme (R.P.; ref. No. NF130516), co-funded by the Royal Society and the British Academy, and by the British Society for Neuroendocrinology (Project Support Grant). We also would like to thank to A. Kabasik-Thayil and U. Wiegand (IMPACT imaging facility, University of Edinburgh) for their help with confocal microscopy, Dr. A. Caraty and Dr. H. Gainer for kindly providing us with some primary antibodies, Dr. C. McClure for supply of the AAV1/2-CAG-GFP virus, and Prof. G. Leng for critical reading of the manuscript.

References

1 Seminara SB, Messager S, Chatzidaki EE, Thresher RR, Acierno JS Jr, Shagoury JK, Bo-Abbas Y, Kuohung W, Schwinof KM, Hendrick AG, Zahn D, Dixon J, Kaiser UB, Slaug- genhaut SA, Gusella JF, O’Rahilly S, Carlton MB, Crowley WF Jr, Aparicio SA, Collode WH: The GPR54 gene as a regulator of puberty. N Engl J Med 2003;349:1614–1627.
2 de Roux N, Genin E, Carel JC, Matsuda F, Abbas Y, Kuohung W, Schwinof KM, Hendrick AG, Zahn D, Dixon J, Kaiser UB, Slau- genhaut SA, Gusella JF, O’Rahilly S, Carlton MB, Crowley WF Jr, Aparicio SA, Collode WH: The GPR54 gene as a regulator of puberty. N Engl J Med 2003;349:1614–1627.
3 Funes S, Hedrick JA, Vassileva G, Markowitz L, Abbondanzo S, Golovko A, Yang S, Mons-ma FJ, Gustafson EL: The KISS-1 receptor GPR54 is essential for the development of the murine reproductive system. Biochem Biophys Res Commun 2003;312:1357–1363.
4 Pineda R, Aguilar E, Pinilla L, Tena-Sempere M: Physiological roles of the kisspeptin/ GPR54 system in the neuroendocrine control of reproduction. Prog Brain Res 2010;181:55–77.
5 Pinilla L, Aguilar E, Dieguez C, Millar RP, Tena-Sempere M: Kisspeptins and reproduc tion: physiological roles and regulatory mecha nisms. Physiol Rev 2012;92:1235–1316.
6 Han S-K, Gottsch ML, Lee KJ, Popa SM, Smith JT, Jakawich SK, Clifton DK, Steiner RA, Herbison AE: Activation of gonadotro pin-releasing hormone neurons by kisspeptin as a neuroendocrine switch for the onset of puberty. J Neurosci 2005;25:11349–11356.
7 Clarkson J, d’Anglemont de Tassigny X, Collode WH, Caraty A, Herbison AE: Distribu tion of kisspeptin neurones in the adult female mouse brain. J Neuroendocrinol 2009; 21:673–682.
8 Gottsch M, Cunningham M, Smith J, Popa S, Acohiba D, Crowley W, Seminara S, Clif- ton D, Steiner R: A role for kisspeptins in the regulation of gonadotropin secretion in the mouse. Endocrinology 2004;145:4073– 4077.
Amygdala Kisspeptin Neurons

Neuroendocrinology 2017;104:223–238
DOI: 10.1159/000445895
Brennan PA, Zufall F: Pheromonal communication in vertebrates. Nature 2006;444:308–315.

Yokosuka M: Histological properties of the glomerular layer in the mouse accessory olfactory bulb. Exp Anim 2012;61:13–24.

Mombaerts P: Genes and ligands for odorant, vomeronasal and taste receptors. Nat Rev Neurosci 2004;5:263–278.

Norlin EM, Gussing F, Berghard A: Vomeronasal phenotype and behavioral alterations in Gαi2 mutant mice. Curr Biol 2003;13:1214–1219.

Millar RP, Babwah AV: Kiss1r: hallmarks of an effective regulator of the neuroendocrine axis. Neuroendocrinology 2015;101:193–210.

Chen WR, Xiong W, Shepherd GM: Analysis of relations between NMDA receptors and GABA release at olfactory bulb reciprocal synapses. Neuron 2000;25:625–633.

Egger V, Svoboda K, Mainen ZF: Mechanisms of lateral inhibition in the olfactory bulb: efficiency and modulation of spike-evoked calcium influx into granule cells. J Neurosci 2003;23:7551–7558.

Tobin VA, Hashimoto H, Wacker DW, Takayanagi Y, Langnaese K, Caquineau C, Noack J, Landgraf R, Onaka T, Leng G, Meddle SL, Engelmann M, Ludwig M: An intrinsic vasopressin system in the olfactory bulb is involved in social recognition. Nature 2010;464:413–417.

Simerly RB, Chang C, Muramatsu M, Swanson LW: Distribution of androgen and estrogen receptor mRNA-containing cells in the rat brain: an in situ hybridization study. J Comp Neurol 1990;294:76–95.

Cooke BM: Steroid-dependent plasticity in the medial amygdala. Neuroscience 2006;138:997–1005.

Yeo S-H, Herbison AE: Projections of arcuate nucleus and rostral periventricular kisspeptin neurons in the adult female mouse brain. Endocrinology 2011;152:2387–2399.

Whitten WK: Modification of the oestrous cycle of the mouse by external stimuli associated with the male. J Endocrinol 1956;13:399–404.

Gangrade BK, Dominic CJ: Studies of the male-originating pheromones involved in the Whitten effect and Bruce effect in mice. Biol Reprod 1984;31:89–96.