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Amygdala kisspeptin neurons: putative mediators of olfactory control of the gonadotropic axis

Rafael Pineda[1], Fabrice Plaisier[1], Robert P. Millar[1,2,3] and Mike Ludwig[1]

[1]Centre for Integrative Physiology, University of Edinburgh, Hugh Robson Building, George Square, Edinburgh, EH8 9XD, UK
[2]Mammal Research Institute, Department of Zoology and Entomology, and Centre for Neuroendocrinology, University of Pretoria, Pretoria 0001, South Africa
[3]MRC/UCT Receptor Biology Unit, Institute for Infectious Disease and Molecular Medicine, University of Cape Town, Cape Town, South Africa

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 odour information to GnRH neurons in the preoptic area to coordinate the gonadotropic axis and the appropriate output behaviour to odour cues.

Introduction

Kisspeptins (Kp) 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 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 (ARC) and the anteroventral periventricular nucleus (AVPV) [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 accessory olfactory system [23, 25]. The accessory olfactory system consists of the vomeronasal organ, localised 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 (290-300g) and adult male transgenic *Kiss1*-CreGFP mice (*Kiss1*$^{tm1.1(cre/EGFP)Stei}$, Jackson laboratory, stock No: 017701) (28-30g) were housed on a 12:12 h 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 – 300g) were injected intraperitoneally with a lethal dose of pentobarbital sodium and perfused transcardially with heparinised (129mg/l) 0.9% saline, followed by 4% paraformaldehyde (PFA) in PBS (pH 7.2-7.4). Brains were removed and post-fixed overnight at 4ºC 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 in 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 to the precursor of dopamine) immunofluorescence histochemistry (see below), the second set for kisspeptin and vasopressin and the last set was used 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 retro-beads (Lumafluor, Inc). Sprague Dawley rats (290 – 300g) were first anesthetized with 4% isoflurane and then maintained on 1.5 – 2 % isoflurane throughout surgery. 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 bregma [32]: anterioposterior (AP) -3.4mm, mediolateral (ML) 3.8mm, and dorsoventral (DV) 8.5mm. Red retro-beads were pressure injected (200nl, 100nl/min). After injection the wound was sutured and the rats were given buprenorphine (0.03mg/kg) subcutaneously for pain relief during the recovery period. One week after injection, 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 stereotaxic conditions, a glass capillary was implanted in the rat AOB using the following coordinates: AP 6.0mm, ML 1.5mm, and DV (from brain surface) 1.0mm. In four rats, 50-100nl of fluoro-Ruby was injected into the AOB by iontophoresis.

To show the bidirectional connectivity between the olfactory bulb and the amygdala, we used 300nl of a combination of red retro-beads and an adeno-associated virus (AAV) expressing green fluorescent protein (GFP) sequence under the ubiquitous cytomegalovirus immediate-early enhancer/chicken beta-actin hybrid (CAG) promoter (serotype 1/2), 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 the beads in the retrograde study, but in this case rats were left for four 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 opposite oriented (Fig. 5A). Thus after the cre recombinant step, YFP was orientated to the correct sense and transduced specifically in kisspeptin cells under the constitutively elongation factor 1-alpha promoter (Ef1a) [36, 37]. The type of AAV is commonly known as double-floxed inverse open reading frame (DIO) type and was purchased from Gene Therapy Center Vector Core (University of North Carolina), serotype 5, AAV5-DIO-YFP.

Adult heterozygotes *Kiss1*-CreGFP male mice (28–30g) were injected unilaterally with 400nl (100nl/min) of the AAV5-DIO-YFP into the medial amygdala following the coordinates of the reference atlas [38]: AP -1.9mm, ML 2.0mm, DV 4.9mm. Three weeks after injection, the mice were perfused transcardially with heparinised saline followed by PFA as above, the brains 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

Sections were treated as described before [31]. Briefly, sections were mounted in order, caudal to rostral, on SuperFrost® Plus slides (VWR, Cat. No. 631-0108) and dried for 1h at 37°C. They were then washed in PBS-T (0.1% Tween-20) for 10min, and subjected to heat induced epitope retrieval (HIER) using 10mM sodium citrate pH 6 for 10min at 90°C. The HIER step greatly increases amygdala kisspeptin staining, but in the tracing experiments the step was omitted since it compromised detection of the retro-beads, fluoro-Ruby and the fluorescent reporters (GFP and YFP). After cooling to room temperature in a water bath and a 5min wash in PBS, the sections were then preincubated in blocking buffer (3% of the appropriate serum (goat or donkey) + 0.4% Triton X-100 in PBS) for 45min. 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 3x 10min in PBS and then incubated with secondary antibodies (Table 1), diluted in blocking buffer for 1h at 37°C and then washed 3x 10min in PBS. Nuclear DNA was stained with Hoechst 33342 (10μg/ml in PBS for 5min and washed 3x for 5min in PBS). Slides were briefly immersed in double-distilled water and coverslipped using Fluoromount Aqueous Mounting Medium (Sigma, F4680). 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 1μM 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 facilitate colour-blind readers [39], in a specific case (revealing kisspeptin fibres in the olfactory bulb) the red channel was recoloured to magenta; 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 40x 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 micro dissected accessory olfactory bulbs and the hypothalamus, 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 High Pure RNA Tissue Kit (Roche, Cat. No.12033674001). 0.5µg of total RNA was used for cDNA synthesis using Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Cat. No. 05081955001). cDNA was amplified using GoTaq® G2 Green Master Mix (Progema, 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´); Kiss1r-reverse (5´-CGG GAA CAC AGT CAC GTA CC-3´); amplicon size, 151bp. Cycling PCR conditions consisted in a first denaturing cycle at 98°C for 30s, followed by 35 (Kiss1r) or 30 (S11) cycles of amplification, defined by denaturation at 98°C for 30s, annealing at 60°C for 30s, and extension at 72°C for 30s. A final extension cycle of 72°C for 5min was included. For internal Reverse Transcription (RT) reaction control, amplification of a 240bp fragment of the 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′); S11-reverse (5′- TGC ATC TTC ATC TTC GTC AC -3′) {Pinilla:2011by}. Specificity of PCR products was confirmed by sequencing (Source BioScience sequencing service).

Slices preparation for in vitro electrophysiology

Recordings were performed in brain slices from adult Sprague Dawley rats (2 - 3 months old). Rats were anesthetised with isoflurane and decapitated. The olfactory bulbs were removed and 300μm sagittal sections were cut in ice-cold sucrose artificial cerebrospinal fluid (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). Slices were transferred to artificial cerebrospinal fluid (aCSF) to equilibrate for 30-40 min at 35±2°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 and 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 Axo-patch 200B amplifier (Axon Instruments, USA) in track mode.

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

Rat kisspeptin-10 (Tocris, Cat. No. 4243) was prepared at 100µM stock solution in double distilled water, stored at -20ºC and diluted x1000 times to 100nM working solution in aCSF just before being applied to the bath by perfusion for 2min [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 incubated in 4% PAF in PBS overnight at 4°C. The following day the slice was washed 3x for 10min with PBS, then washed with PBS-T for 10min and incubated for 1h in a streptavidin incubation buffer (0.4% Triton X-100 in PBS). Slices were then incubated 4h at room temperature with 1/500 Streptavidin-Alexa Fluor® 488 conjugate (see antibodies table for reference) in incubation buffer and later washed 3x for 10min in PBS and mounted using Fluoromount.

Data collection and recording analysis

Recorded signals were low pass filtered at 2kHz, digitized at 5kHz 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 3min before and after kisspeptin infusion was analysed. Statistical differences were calculated using a two tailed paired *t*-test analysis in GraphPad Prism software. Significance was set at * $P \leq 0.05$, ** $P \leq 0.01$.

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 three 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 to 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 show 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 retro-beads into the MePD area where kisspeptin neurons were found. The retro-beads labelled mitral cells in the AOB (Fig. 3A), but not in the main olfactory bulb. We observed a different pattern of retro-beads distribution in the mitral cell layer of the AOB, with the anterior part showing a more intense optical density (amount of beads) compared to 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 100±14.35 vs. posterior part 46.20±6.36, expressed as mean ± SEM; 3 animals, 2 sections/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 projecting towards the amygdala along the layer 1 of the posterolateral cortical amygdaloid nucleus (PLCo1) [42]. Several fibres penetrate into the MePD and labelled boutons be seen adjacent to kisspeptin neurons (Fig. 4D,E). Interestingly, some non-kisspeptin cells in the MePD also seemed to be contacted by multiple boutons.

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 projection from the amygdala kisspeptin neurons to the AOB (Fig. 5B), but no projections to the main olfactory bulb (data not shown). Combination of red retro-beads and the AAV1/2-CAG-GFP injection in the MePD revealed a bidirectional connection between the mitral cells of the AOB and the MePD (Fig. 5C,D). Interestingly, the kisspeptin fibre innervations of the AOB appear to be different between 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 species differences or just degree of expression or technique differences (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 are dendrites from the amygdala kisspeptin neurons, we used the specific marker for neuronal dendrites, microtubule-associated protein 2 (MAP2) [43]. The lack of staining with MAP2 confirms that the fibres are axonal projections.

Kisspeptin receptor expression in the AOB

To determine kisspeptin receptor expression in the AOB, we performed expression analysis of the Kiss1r transcript in micro dissected 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 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 was determined. Nine of the seventeen cells (53%) reduced their firing rate by more than 10%, whereas eight 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 (Figs. 8A-C) to reach the GnRH neurons in the POA (Figs. 8D,E). About

15% (11 of 71) of identified GnRH neurons in the POA appear to receive inputs from amygdala kisspeptin neurons (Fig. 8F), with the highest number of connections at the bregma level 0.50 (33% ± 7; n=3 mice, 6 sections/mouse, Fig. 8G).

Discussion

Although mRNA for kisspeptin in the amygdala has been reported previously [8, 12], the specific localisation was not shown. We show here that many kisspeptin neurons are located in the caudal areas of the posterodorsal medial amygdala (MePD). The function of kisspeptin neurons in the amygdala is unknown, but the amygdala has been implicated in numerous physiological and behavioral processes, including those relating to reward, social behaviors and reproduction [13-15, 44].

We found TH-immunoreactive fibres (TH is commonly used as marker for dopaminergic neurons) adjacent to amygdala kisspeptin neurons. Our current retrograde studies (not shown), using fluorogold, indicate 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 behaviors for the perpetuation of the species. 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 the 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. 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 sensing pheromonal cues from the vomeronasal organ (VNO) [58]. Anatomically and functionally the AOB is divided into the anterior and the posterior parts [25, 59]. The VNO apical layer, 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 the 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 micro-dissected 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 the Kiss1r receptor is a Gq/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 Gi-coupled then it may explain the observed inhibitory effect on electrical activity.

Alternatively, granule cells are the most common inhibitory (GABA) cells type in the olfactory bulb, and they control the activity of mitral cells through dendrodendritic inhibition [63, 64]. Thus, is possible that the 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 proestrus [12]. Mating in rodents is restricted to the late proestrus/early oestrus phase of the cycle. The increase in the 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 MOB [65].

The posterodorsal area of the medial amygdala is enriched with androgen and oestrogen receptors [66, 67], and is connected with brain areas implicated in reproductive behaviours [28], including the POA where most GnRH neurons are located. It has been shown that the kisspeptin neurons from ARC and AVPV project to the POA [68]. Using a cell-specific viral targeting approach we show 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 stria terminals (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 its 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, olfaction and reproduction. The kisspeptin neurons in the amygdala may also link appropriate behaviors modulated by vasopressin and dopamine with the reproductive state of the gonadotropic axis.

In addition to our neuroanatomical data presented here, recent functional studies have shown that kisspeptin signaling 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 hormones secretion. However, further work is required to show that these kisspeptin cells in particular do indeed modulate GnRH secretion.

Figure 1: Rat amygdala kisspeptin population: localization and numbers. (**A**) Expression of kisspeptin 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 [32]). (**C**) Total number of kisspeptin neurons at different levels and (**D**) total number of kisspeptin neurons per amygdala (n=6). Values are expressed as mean ± SEM. opt, optic track.

Figure 2: Rat amygdala kisspeptin population: dopaminergic and vasopressinergic inputs. Confocal images and 3D reconstructions of two examples of amygdala kisspeptin neurons (red) receiving (**A**) TH and (**B**) vasopressin appositions (green). Hoechst DNA nuclear marker in blue.

Figure 3: Pattern of connectivity between mitral cell layer of the AOB and the MePD. (**A**) Sagittal section of a rat AOB show retro-beads (red) distribution only in the mitral cell layer after injection in the MePD (Hoechst DNA nuclear marker in blue). (**B**) Show surface plot image of retro-beads in the MTCL from an image used for fluorescent intensity quantification. (**C**) Optical density quantification of the anterior (aAOB) and posterior AOB (pAOB) revealed significant differences between the two parts (**$P \leq 0.01$). Values are expressed as mean ± SEM; VNL - vomeronasal nerve layer, GL - glomerular layer, EPL - external plexiform layer, MTCL - mitral cell layer, LOT - lateral olfactory tract, GCL - granule cell layer.

Figure 4: Connectivity between mitral cells of the AOB and amygdala kisspeptin 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 the layer 1 of the posterolateral cortical amygdaloid nucleus (white arrow) 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 arrows). (**E**) Enlarged view of the kisspeptin neuron (green) indicated by a blue arrow in **(D)**. Some non-kisspeptin cells also seemed to be contacted by multiple boutons (white arrows). MTCL - mitral cell layer, GCL - granule cell layer, opt - optic track.

Figure 5: Amygdala kisspeptin projection to the AOB. (**A**) Scheme of experimental procedure. (Ai) AAV expressing an inverted yellow fluorescent protein (YFP) reporter sequence floxed by two LoxP sequences opposite oriented – AAV5-DIO-YFP. (Aii) AAV expressing green fluorescent protein (GFP) sequence under the ubiquitous CAG promoter – AAV1/2-CAG-GFP. **(B**) AAV5-DIO-YFP injection into the mouse medial amygdala shows kisspeptin projection (yellow) in the AOB. Hoechst DNA nuclear marker in blue. (**C, D)** Combination of red retro-beads and the AAV1/2-CAG-GFP injection in the 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. Specific marker for neuronal dendrites, microtubule-associated protein 2 (MAP2) in green.

Ef1a - elongation factor 1-alpha promoter, CAG - cytomegalovirus immediate-early enhancer/chicken beta-actin hybrid promoter, ITR - inverted terminal repeat sequence, WPRE - woodchuck hepatitis post-transcriptional regulatory element (expression enhancer), pA - polyadenylation signal sequence.

Figure 6: Kisspeptin receptor expression in the AOB. Images of RT-PCR showing (**A**) expression of the kisspeptin receptor (Kiss1r) and (**B**) ribosomal protein S11 mRNA in microdissected samples from the AOB and other tissues.

Figure 7: Changes in electrical activity in mitral cells of 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 (**Bi**) a lack of response or (**Bii**) reduction in firing rate of greater than 10% after kisspeptin infusion. (**C**) Absolute and percentage change of firing rate from all cells recorded (*P ≤0.05).

Figure 8: Amygdala kisspeptin projection to GnRH neurons in the POA. (A) AAV5-DIO-YFP injection into the amygdala shows that the amygdala kisspeptin neurons project via the stria terminals (red arrow) to reach the GnRH neurons in the POA **(B, C)**. White arrow 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 mean + SEM.

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

Primary Abs	**Code**	**Supplier**	**Dilution**	**Raised in**
Kp	#564	Dr A. Caraty	1/10,000	rabbit
VP-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 Abs	**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

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