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ARTICLENeuronal connectivity between habenular
glutamate–kisspeptin1 co-expressing neurons
and the raphe 5-HT system

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Selangor, Malaysia***Abstract**

The habenula, located on the dorsal thalamic surface, is an emotional and reward processing center. As in the mammalian brain, the zebrafish habenula is divided into dorsal (dHb) and ventral (vHb) subdivisions that project to the interpeduncular nucleus and median raphe (MR) respectively. Previously, we have shown that kisspeptin 1 (Kiss1) expressing in the vHb, regulates the serotonin (5-HT) system in the MR. However, the connectivity between the Kiss1 neurons and the 5-HT system remains unknown. To resolve this issue, we generated a specific antibody against zebrafish Kiss1 receptor (Kiss-R1); using this primary antibody we found intense immunohistochemical labeling in the ventro-anterior corner of the MR

(vaMR) but not in 5-HT neurons, suggesting the potential involvement of interneurons in 5-HT modulation by Kiss1. Double-fluorescence labeling showed that the majority of habenular Kiss1 neurons are glutamatergic. In the MR region, Kiss1 fibers were mainly seen in close association with glutamatergic neurons and only scarcely within GABAergic and 5-HT neurons. Our findings indicate that the habenular Kiss1 neurons potentially modulate the 5-HT system primarily through glutamatergic neurotransmission via as yet uncharacterized interneurons.

Keywords: GPR54, kisspeptin 1, serotonin, ventro-anterior MR, zebrafish.

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The habenula constitutes the dorsal diencephalic conduction system, relaying information from forebrain regions to the boundary dividing the midbrain from hindbrain regions, further connecting to monoaminergic neurons including serotonergic and dopaminergic neurons (Sutherland 1982). The basic connectivity and structure of the habenula is well conserved throughout the mammalian lineage with subdivisions of the medial habenula (MHb) projecting to the interpeduncular nucleus (IPN) and the lateral habenula (LHb) to midbrain regions and the raphe nuclei, implicated in serotonin (5-hydroxytryptophan, 5-HT) release (Wang and Aghajanian 1977; Hikosaka 2010). Similarly, in non-mammalian vertebrates, the habenula is divided into the dorsal (dHb) and ventral (vHb) subnuclei (Braitenberg and Kemali 1970; Kemali and Guglielmotti 1977). The dHb is further subdivided into the medial (dHb_M) and lateral (dHb_L) subnuclei projecting to the ventral (vIPN) and dorsal IPN (dIPN) respectively, while the vHb projects to the median raphe (MR), accounting for the homologous nature of the dHb to the MHb and the vHb to the LHb (Amo *et al.* 2010). It is well known that significant

homologies exist between mammals and fish in terms of neuroanatomy, physiology and behaviours (Chowdhary *et al.* 1998; Lieschke and Currie 2007). In most teleost, the 5-HT-containing raphe region is divided into the superior (SR) and inferior (IR) raphe nuclei (Lillesaar 2011) and the SR is further subdivided into the MR (caudal of the IPN) and dorsal raphe (DR, superior to the dIPN) based on cytoarchitectural structuring (Lillesaar *et al.* 2007; Amo *et al.* 2010, 2014; Okamoto *et al.* 2012). In the zebrafish (*Danio rerio*), the dHb expresses transmitters such as tachykinin, glutamate (DeCarvalho *et al.* 2014) and acetylcholine (Hong *et al.* 2013) along with their receptors

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Abbreviations used: 5-HT, serotonin; GABA, γ -aminobutyric acid; GFP, green fluorescent protein; Kiss1, kisspeptin1; Kiss-R1, Kiss1 receptor; MR, median raphe; vHb, ventral habenula.

while the vHb neurons has been shown to project mainly to the vIPN potentially including the MR region (Servili *et al.* 2011; Ogawa *et al.* 2012), suggesting its potential role in 5-HT modulation (Ogawa *et al.* 2012).

Kisspeptin and its receptor (Kiss-R) homologues have been identified in several mammalian and non-mammalian vertebrates (Felip *et al.* 2009; Lee *et al.* 2009; Um *et al.* 2010; Gopurappilly *et al.* 2013). Zebrafish possess two genes encoding kisspeptin in the brain, *kiss1*, predominantly expressed in the habenula (Ogawa *et al.* 2012) and *kiss2*, in the hypothalamus (Kitahashi *et al.* 2009), with preferential affinity for their respective receptors, Kiss-R1 and -R2 (Lee *et al.* 2009). While Kiss2 in teleosts is a major regulator of reproduction (Kitahashi *et al.* 2009; Akazome *et al.* 2010; Tena-Sempere *et al.* 2012; Gopurappilly *et al.* 2013), our recent findings showed that kisspeptin 1 (Kiss1)/Kiss-R1 system modulates alarm substance-evoked fear potentially via the 5-HT system (Ogawa *et al.* 2014), involving the interaction of 5-HT_{1A} and 5-HT₂ receptors (Nathan *et al.* 2015). However, the neural pathway underlying this modulatory effect is poorly understood, as morphological analysis of Kiss1 in the zebrafish failed to identify dense connections between the vHb and 5-HT neurons in the SR regions, although a sparse plexus of Kiss1-positive fibers was seen (Rink and Wullimann 2001; Amo *et al.* 2010; Servili *et al.* 2011; Ogawa *et al.* 2012; Song *et al.* 2015). Based on recent evidences on the projection of the vHb to the MR (Amo *et al.* 2010, 2014; Ogawa *et al.* 2012; Broms *et al.* 2015), we hypothesize that Kiss1 neurons indirectly act on 5-HT neurons via non-serotonergic interneurons in the MR, similar to pathways of LHb neurons to the 5-HT neurons located within the DR via the MR in rats (Wang and Aghajanian 1977; Vertes *et al.* 1999).

In this study, to map out the potential neuronal pathway underlying the neuroanatomical association of Kiss1 terminals in the MR and the raphe 5-HT neuronal population in the zebrafish brain, we generated a specific antibody for zebrafish Kiss-R1 which we used to reveal the cellular location of its expression. The specificity of Kiss-R1 immunoreactivity was confirmed by comparison with expression patterns of *kissr1* gene. Furthermore, as the epitope of the Kiss-R1 antibody is also found in *kissr1b*-derived protein 2 (KRBDP2), an alternative splice variant of the *kissr1* gene (Onuma and Duan 2012), we performed *in situ* hybridization of *KRBDP2* mRNA transcripts. Using double-label immunofluorescence in transgenic (Tg) zebrafish lines, we determined the associations between Kiss1 neurons and the MR neuronal populations. To further identify the potential mechanism of Kiss1 signal transmission, we co-localized habenular Kiss1 and Kiss-R1 in neurons also expressing markers for glutamatergic, γ -aminobutyric acid (GABA)-ergic and acetylcholinergic neurons.

Materials and methods

Animals

Adult (6 month) wild-type male short-finned zebrafish were obtained from local suppliers. Transgenic zebrafish, [Tg (*bm3a-hsp70:GFP*)^{rw0110b}; expressing green fluorescent protein (GFP) in dHb_M-vIPN pathway] (Aizawa *et al.* 2005; Sato *et al.* 2007), [Tg (*-3.2pet1:eGFP*)^{ne0214}; expresses enhanced GFP in 5-HT neurons in the SR] (Lillesaar *et al.* 2009) and [Tg (*gad1b:GFP*); expressing GFP in GABAergic neurons] (Satou *et al.* 2013) were provided by Prof. Hitoshi Okamoto (RIKEN, Japan), Dr Christina Lillesaar (Institute of Developmental Genetics, Germany), and Dr Shin-ichi Higashijima (National Institute of Natural Science, Japan) respectively (Table 1). Fish were housed in 20 L tanks with a controlled temperature of 28°C ± 0.5°C and a 14/10 h light/dark phase. Fish were killed by immersion in 0.01% MS222 (tricaine methanesulfonate, Sigma, St Louis, MO, USA) solution before dissecting out the brain regions. All fish were maintained and research was carried out after obtaining ethical approval from Monash University Animal Ethics Committee (MAR/P/2012/093).

Generation of antibody for zebrafish Kiss-R1

Polyclonal antiserum for zebrafish Kiss-R1 (Antibody ID, #PA4217) was generated in rabbits against a synthetic peptide, QRSTEPLATYNREMFLSS (the carboxy-terminus of zebrafish Kiss-R1: GenBank accession number: EU047918). The antigen was conjugated with keyhole limpet haemocyanine which was used to immunize rabbits according to standard commercial procedures by Open Biosystems, USA. After an immune response had been verified, blood samples were centrifuged, and the antibody fraction was purified by the affinity purification column that was constructed by coupling the antigen to a gel. The purified antibody fraction was stored at -80°C. Specificity of the Kiss-R1 immunoreactivity was confirmed by comparison with expression patterns of *kissr1* and *KRBDP2* mRNA transcripts (see below).

Immunohistochemistry of zebrafish Kiss1 and Kiss-R1

Immunohistochemical localization of zebrafish Kiss1 and Kiss-R1 were performed as previously described (Soga *et al.* 2005). Anti-zebrafish Kiss1 (Antibody ID, #PAS 15133/15134) previously generated in our lab, specifically recognizes the Kiss1 cells in the vHb and axons in the MR (Ogawa *et al.*, 2014). For the controls for immunohistochemistry, alternate coronal brain sections, 15 μ m ($n = 1$) were incubated with either anti-zebrafish Kiss1 or anti-zebrafish Kiss-R1 antisera or those pre-absorbed with 10 μ g/mL of respective antigens at the working dilution for a 24 h period. For Kiss-R1 detection, signals were enhanced with tyramide signal amplification (TSA) Plus Biotin Kit (PerkinElmer/NEN Life Science Products, Wellesley, MA, USA). Sections were scanned and images captured using a Zeiss MIRAX Slide scanning system (Zeiss, GmbH, Göttingen, Germany) with the Mirax Viewer Image Software (3DTech, Budapest, Hungary) at a resolution of 230 nm with 20 \times and 40 \times objectives. Nomenclature of brain regions were adopted from Aizawa *et al.*, 2011; Amo *et al.* 2010, 2014; Yamamoto and Ito 2008; Wullimann *et al.*, 1996 and Wullimann and Rink 2002.

Table 1 List of transgenic zebrafish lines used in this study

Abbreviation of transgenic fish used in the manuscript	Name of transgenic line	Characteristic	References	Source
Tg <i>brn3a</i>	(<i>brn3a-hsp70:GFP</i>) ^{rw0110b}	Express GFP in the dHb _M -VIPN pathway under enhancer element control of <i>brn3a</i> , expressed in the habenula as a POU domain transcription-factor-encoding gene, upstream of the basal promoter at ambient temperature, <i>hsp70</i>	Aizawa <i>et al.</i> (2005), Sato <i>et al.</i> (2007)	Dr Hitoshi Okamoto (RIKEN, Japan)
Tg <i>pet1</i>	(<i>3.2pet1:eGFP</i>) ^{ne0214}	Express enhanced GFP (eGFP) at 3.2-kb fragment upstream of the <i>pet1</i> gene in 5-HT neurons in the SR	Lillesaar <i>et al.</i> (2007)	Dr Christina Lillesaar (Institute of Developmental Genetics, Germany)
Tg <i>gad1b</i>	<i>gad1b:GFP</i>	Express GFP in GABA-producing neurons	Satou <i>et al.</i> (2013)	Dr Shin-ichi Higashijima (National Institute of Natural Science, Japan)

DIG-*in situ* hybridization

Expression of *kissr1* and *KRBDP2* mRNA transcripts and marker genes for glutamatergic (*slc17a6b*), GABAergic (*gad1b* and *gad2*), and cholinergic (*chat*) neurons were examined by DIG-*in situ* hybridization. DIG-labeled riboprobes for *kissr1* were prepared as described previously (Ogawa *et al.* 2012). As for marker genes of neurotransmitters, RNA probes were synthesized via *in vitro* transcription from pGEM-T Easy vector (Promega, Madison, WI, USA) containing fragments from zebrafish cDNA of *slc17a6b*, *gad1b*, *gad2* and *chat*, with DIG or biotin RNA Labeling Mix (Roche Diagnostics, Mannheim, Germany). Gene abbreviations, the primer sequences used for probe synthesis, probe size and GenBank accession numbers are listed in Table S1. DIG-*in situ* hybridization was performed as previously described (Ogawa *et al.* 2012).

Immunofluorescence

Kiss1 and Kiss-R1 immunoreactivities were analyzed in the brain of Tg *brn3a* zebrafish that express GFP in the dHb_M-VIPN pathway (Aizawa *et al.* 2005; Sato *et al.* 2007), Tg *pet1* zebrafish that express GFP in 5-HT neurons in the SR (Lillesaar *et al.* 2009) Sagittal [Tg *brn3a*] and coronal [Tg *pet1*] brain sections (15 µm) were prepared as described above and immunoreacted with anti-zebrafish Kiss1 (1 : 500; #PAS 15133/15134) and anti-zebrafish Kiss-R1 antiserum (1 : 500). The zebrafish Kiss1 and Kiss-R1 immunoreactivity was visualized after development with Alexa Fluor 594-labeled anti-rabbit IgG (Cat# A11012; 1 : 500 dilution; Invitrogen, Eugene, OR, USA). To visualize 5-HT neurons in Tg *pet1* zebrafish, GFP signals were enhanced using a mouse anti-GFP monoclonal antibody (Cat# A11120; Invitrogen) at 1 : 250 dilution followed by development with Alexa-Fluor 488-labeled anti-mouse IgG (1 : 500 dilution; Invitrogen) and counterstained with 300 nM 4',6-diamino-2-phenylindole (Cat# D3571; DAPI; Invitrogen).

Co-localization of marker genes for glutamatergic and GABAergic neurons in habenular Kiss1/Kiss-R1 neurons and 5-HT neurons in the MR were examined by double-labeling combining fluorescence *in situ* hybridization and immunofluorescence. Tg *gad1b* zebrafish (Satou *et al.* 2013) was also used to examine association between Kiss1/Kiss-R1 with GABAergic neurons in the MR as

above. DIG- and biotin-labeled *slc17a6b* (glutamate) and *gad2* (GABA) riboprobes were applied to the brain sections of wild type fish (for Kiss1, *n* = 3) and Tg *pet1* zebrafish (for 5-HT, *n* = 3), respectively. *slc17a6b* and *gad2* riboprobes were detected with TSA Plus kit (PerkinElmer/NEN Life Science Products) according to the manufacturer's instructions. For double-labeling with Kiss1 and Kiss-R1, TSA-labeled sections were rinsed in 0.01 M phosphate-buffered saline and then incubated with either zebrafish Kiss1/Kiss-R1 antiserum (1 : 500) or Alexa Fluor 594-labeled anti-rabbit IgG (1 : 500; Invitrogen). For double-labeling with GFP-labeled 5-HT neurons, signals for the *slc17a6b* and *gad2* probes were detected using TSA Plus Cyanin3 (Cy3) Kit (PerkinElmer/NEN Life Science Products), while 5-HT neurons were detected as GFP signals.

Image and data analysis

The sections were coverslipped with Vectashield (Vector Laboratories Inc., Burlingame, CA, USA), viewed and images were captured under a fluorescent microscope (90i, Nikon, Tokyo, Japan) attached to a digital camera (DXM 1200c, Nikon) with commercial software (NIS Elements, D v4.0, Nikon). For this examination we used the appropriate excitation and emission wavelengths for GFP, Alexa Fluor 488, Alexa Fluor 594 and DAPI. Higher magnification (60× water immersion plus optical zooming 1.5×) images were obtained with confocal microscopy (C1si, Nikon) attached to an inverted microscope (ECLIPSE TE2000-E, Nikon). Neurons of interest were aligned in X, Y and Z planes and optical sections through the z-axis were obtained at 0.15 µm intervals. For high-resolution imaging, 60× water immersion objective (N.A. = s1.4) with an additional 1.5 × optical zoom to give a final magnification of 79.8 × was utilized at a single-plane with z-axis optical sections at 0.15 µm z-step size involving 7 steps each. Co-localization was determined as described by Corson and Erisir 2013. Z-stacks containing the neuron of interest were aligned in X, Y and Z planes. An apposition was identified by the presence of a two- or three-voxel wide overlap between two fluorophore-filled objects. If the labeled voxels seemed apposed but did not overlap, the presence of an actual space of at least 0.15 µm between the cell and the dendrite indicated this (Corson and Erisir

2013). This yielded a voxel size of $0.15 \mu\text{m}^3$. Percentage of co-localization of Kiss1/Kiss-R1 cells with neurotransmitter makers was determined by the formula, (co-localizing cells/total number of cells in area) $\times 100\%$ for a total number of 4 (habenula) and 3 (raphe) sections for each examination.

Color modification (adjusting gamma parameter and input intensity range) of multi-channel images was set with the look-up tables, and color rendering and color merging was performed using commercial software (NIS Elements, AR v4.1, Nikon). The brightness, contrast, color balance, and final size of the images were adjusted using Adobe Photoshop CS5.1 (Adobe Systems, San Jose, CA) and figures were prepared with Adobe Illustrator CS5.1 (Adobe Systems).

Results

Localization of zebrafish Kiss1- and Kiss-R1-immunoreactivity in the brain

Zebrafish Kiss1-immunoreactive (-ir) cells were observed in the vHb, giving rise to axonal projections bypassing the vIPN and terminating at the ventro-anterior corner of the MR (vaMR) through the fasciculus retroflexus (FR) (Fig. 1a–c). Double-immunofluorescence in the brain of Tg *brn3a* zebrafish confirmed the presence of axon terminals of Kiss1-ir fibers in the vaMR but not as a part of the vIPN (Fig. 1d–j). No co-localization was noted between Kiss1

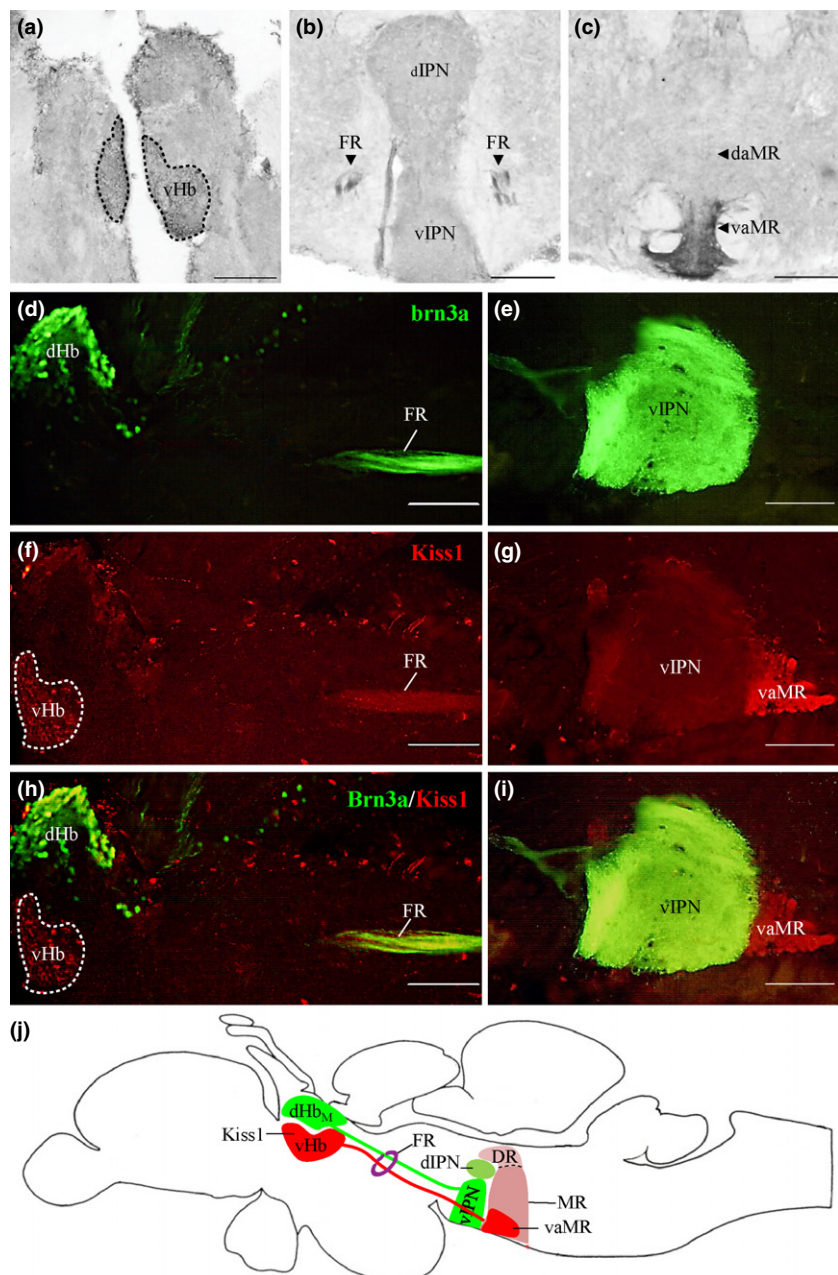


Fig. 1 Kisspeptin 1 (Kiss1) projection in the zebrafish brain. (a–c) Kiss1-immunoreactive cells observed in the vHb (a) project through the fasciculus retroflexus (FR) (b) down to the vaMR (c). (d and e), Photomicrograph of sagittal section of Tg *brn3a* zebrafish expressing green fluorescent protein (GFP) in dHb_M-vIPN pathway. (f–i) Kiss1-immunoreactive cells noted in vHb (red) and not in the dHb (green) with axonal projections coursing through the FR and terminating at the vaMR, a structure following the GFP-expressing vIPN. (j) Illustration depicts projections of dHb_M and vHb through the FR to the vIPN and vaMR (a subregion of the MR) respectively as a representation of the sagittal images. Scale bars, 100 μm .

immunoreactivities and GFP in the dHb or vIPN (Fig. 1h and i). Specificity of the antiserum against zebrafish Kiss-R1 was further confirmed via expression of DIG-labeled *kissr1* mRNA-expressing cells in the vHb of the anti-sense riboprobe (Fig. 2a) in comparison to the sense riboprobe (Fig. 2b). Similar to the expression patterns of Kiss1-ir, zebrafish Kiss-R1-ir cells were predominantly observed in the vHb with axons projecting through the FR and terminating in the vaMR (Fig. 2c–f). In addition, some Kiss1 or Kiss-R1-ir fibers and varicosities were also observed in the dorsal MR (dMR) region (Fig. 2f). Preabsorption of the primary antiserum with antigen for zebrafish Kiss-R1

peptide successfully abolished all immunoreactivity in the zebrafish brain (Fig. 2c inset).

Some Kiss-R1-ir cells were observed in the telencephalon, diencephalon and spinal cord regions with staining at varying intensities (Fig. 3a1–d1 and Figure S1a1–c1), as described below. The distribution patterns of Kiss-R1-ir cells are as illustrated in Fig. 4. Confirming the expression of Kiss-R1 neurons in these regions, we also saw the expression of *KRBPD2* mRNA at varying intensities in these neurons (Fig. 3a2–d2 and Figure S1a2–d2). The specificity of the riboprobe was confirmed by the absence of expression in the corresponding regions by the sense riboprobe (Fig. 3a3–d3

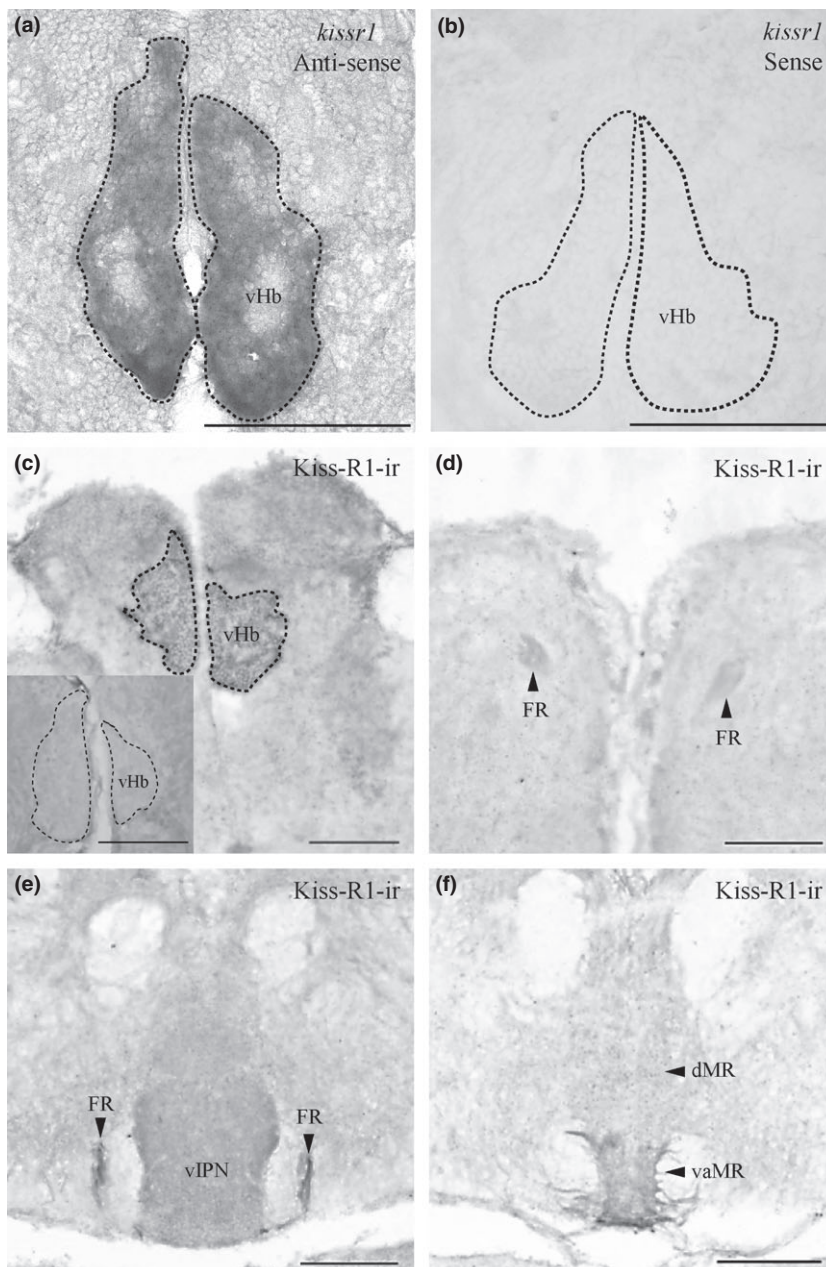


Fig. 2 Expression of *kissr1* mRNA and Kiss-R1 projection in the zebrafish brain. Coronal sections where *kissr1* mRNA is noted in the ventral subnuclei of the habenula (a) and no cells were observed in the sense strand (b). Kiss-R1-immunoreactive (-ir) cells observed in the vHb (c) send projections through the fasciculus retroflexus (FR) (d and e) down to the vaMR (f). Preabsorption with antigen showed no Kiss-R1-ir fibers or cells (C inset). Scale bars, 100 μm.

and S1a3–d3). In the telencephalic region, Kiss-R1-ir and *KRBDP2* mRNA-expressing cells were observed in the medial, central and posterior zones of the dorsal telencephalon as well as in the medial olfactory tract (Fig. 3a1, a2, b1, b2, Figure S1a1–c1 and 4). In the diencephalon, some Kiss-R1 immunoreactivity was observed in the central posterior thalamic nucleus (Fig. 3c1 and c2). Some cells were noted in regions surrounding the FR and in the posterior commissure (Fig. 3c1). A few Kiss-R1-ir cells were noted in the dorsal region of the trigeminal motor nucleus and the lateral longitudinal fascicle (Fig. 3d1 and d2, and 4). Both Kiss-R1-ir and *KRBDP2* cell somata were present in the inner arcuate fibers and the intermediate reticular formation, although the intensity was relatively low (Figure S1d1 and d2, and 4).

Kiss1/Kiss-R1-ir in relation to 5-HT neurons in the MR

Triple-label immunofluorescence in the brain of Tg *pet1* zebrafish showed neither direct associations with Kiss1-ir fibers (Fig. 5a–c) nor co-expression of Kiss-R1-ir in GFP-labeled 5-HT neurons (Fig. 5f–h) in the MR. However, at a higher magnification (79.8 \times), in the MR, we found a few 5-HT neurons in close association (no actual space observed more than 0.15 μ m between the fibers and cells as explained above) with Kiss1 and Kiss-R1-ir fibers (Fig. 5d and i). Confocal imaging (60 \times plus 1.5 \times optical zoom; N.A. = 1.4; z-step = 0.15 μ m) showed associations between both Kiss1 and Kiss-R1-ir fibers in DAPI-positive (non-serotonergic) cells in the vaMR region (Fig. 5e and j). In addition, confocal imaging showed close associations between 5-HT fibers with Kiss1-ir fibers within the vaMR and dMR regions (Fig. 5k and l). There were some 5-HT neurons in the ventral region of the dMR region, but not in the vaMR region (Fig. 5m).

Expression of markers for neurotransmitters in habenular Kiss1 neurons and raphe nucleus

In the habenular region, *slc17a6b* (glutamatergic neuronal marker) mRNA expression was observed in the dHb and vHb (Fig. S2a). As for GABAergic neurons, while *gad1b* signals were not detected by our probe (data not shown), *gad2* mRNA expression was seen only in the most ventral region of the vHb and in the lateral dorsal nucleus of the thalamus (Fig. S2b). A low expression of *chat* (acetylcholinergic) mRNA was observed in the dHb but not in the vHb (Fig. S2c). In the raphe region, *slc17a6b* mRNA-containing cells were observed in the MR, and sparsely in the DR (Fig. S2d). In the Tg *gad1b*, cells were observed only in the dMR region (Fig. 7e), while *gad2* was only observed in the dMR (Fig. S2e). There was weak expression of *chat* mRNA in the SR (MR and DR) (Fig. S2f).

Dual-fluorescence *in situ* hybridization coupled with immunofluorescence showed 80% co-expression (approximately 40/50 cells per section of area examined) of *slc17a6b* mRNA with Kiss1-ir cells in the vHb (Fig. 6a–c), while no

co-expression of *gad2* mRNA in Kiss1-ir cells (Fig. 6d–f) was observed. In the raphe nuclei, double-immunofluorescence showed Kiss1-ir fibers in close association with *slc17a6b* mRNA-containing neurons in the dMR (Fig. 7a–c). However, no close association was observed between Kiss-ir fibers with GFP-labeled *Gad1b* neurons (Fig. 7e–g), but there rarely are close associations with *gad2* mRNA (Fig. 7h–j) expressing GABAergic neurons in the dMR. Similar expression patterns were noted for Kiss-R1 in relation to these neurotransmitter markers in the raphe nucleus (Figure S3). In addition, double-labeling in the brain of Tg *pet1* zebrafish showed 25% (approximately 5/20 cells) of *slc17a6b* (Figure S4a–c) and 25.8% (approximately 6/20 cells) of *gad2* (Figure S4d–f) mRNA co-expressed in some GFP-labeled 5-HT neurons.

Discussion

We report the connectivity of neurons in the vHb that co-expresses Kiss1 and Kiss-R1 in the zebrafish and describe the localization of some of the important neurotransmitters involved in Kiss1 modulation of the 5-HT system in the MR alongside with the localization of Kiss-R1 immunoreactivity in the zebrafish.

In teleosts, the localization of multiple Kiss-R types in the brain have been demonstrated by RT-PCR (Biran *et al.* 2008; Filby *et al.* 2008; Shahjahan *et al.* 2010) and *in situ* hybridization (Ogawa and Parhar 2012; Ogawa *et al.* 2012; Escobar *et al.* 2013; Kanda *et al.* 2013) but there is limited information available on their neuroanatomical distribution. In addition, subcellular localization of G-protein coupled receptor (GPCR) cannot be defined by mRNA localization because GPCR protein products translocate within subcellular regions due to the post-translational GPCR trafficking (Ulloa-Aguirre *et al.* 2006). The present study is the first report demonstrating the detailed neuroanatomical as well as subcellular localization of Kiss-R1 in any species, either mammalian or non-mammalian. We also observed additional Kiss-R1-ir cells in the telencephalon, synencephalon and spinal cord regions where no *kissr1* mRNA was detected by *in situ* hybridization. However, in our previous report employing laser-capture microdissection coupled with real-time PCR, we have shown low amounts of *kissr1* mRNA expressions in the preoptic area, midbrain, hypothalamus, vIPN, cerebellum, optic tectum and spinal cord (Ogawa *et al.* 2012), much of which could potentially correspond to the very same Kiss-R1-ir cells detected by our antibody. Recently, zebrafish *kissr1* (also referred as *kiss1rb*) gene has been shown to produce 4 additional alternative splice variants encoding different protein lengths (*kiss1rb*-derived protein; KRBDP 1–4) (Onuma and Duan 2012). All the truncated forms can be translated, but none are functionally capable of mediating kisspeptin-derived cellular responses (Namba *et al.* 1993). KRBDP2 consists of 4 exons including

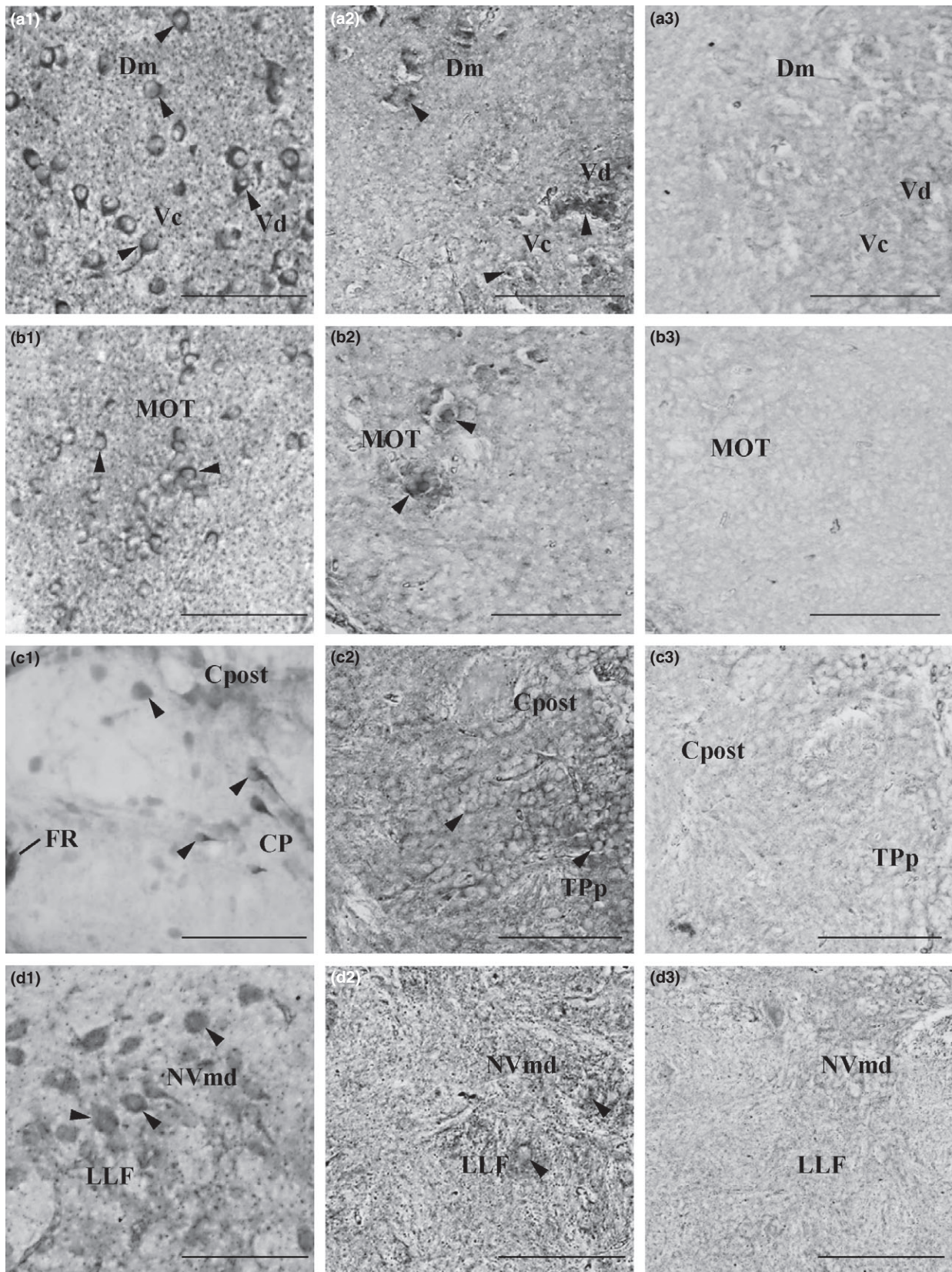


Fig. 3 Kiss-R1-ir localization and *kissr1b*-derived protein 2 (*KRBDP2*) expression in other regions of the zebrafish brain. a1–d1, Kiss-R1-immunoreactive (-ir) cell somata observed outside of the habenula such as the forebrain (a and b), midbrain (c) and hindbrain (d) regions.

a2–d2, *KRBDP2* mRNA expression was also observed in same regions as Kiss-R1-ir cells. a3–d3, The sense riboprobe showed no expression of *KRBDP2* mRNA. For abbreviations, see (Table 2). Scale bars, 100 μ m.

the C-terminal region of the Kiss-R1 protein, the epitope of our Kiss-R1 antibody. Hence, our antibody may recognize the full-length of Kiss-R1 and KRBDP2 in the zebrafish brain, explaining the presence of Kiss-R1 immunoreactive cells outside the habenula-MR region, which only express KRBDP2 mRNA, justified by our *in situ* hybridization findings.

Fluorescence labeling in Tg *brn3a* zebrafish confirmed the presence of Kiss1 in the vaMR. Our results indicate Kiss1 neurons in the vHb mainly project to the MR, particularly to the vaMR. These findings implicate the vHb-vaMR as the main pathway of habenular Kiss1 neurons; this corresponds to the projection of vHb neurons in the zebrafish (Amo *et al.* 2010), a finding that was recently confirmed to involve

specifically the vaMR (Amo *et al.* 2014) as well as Kiss1 projection seen in *kiss1:mCherry* transgenic zebrafish (Song *et al.* 2015). Projections from these neurons with soma in the habenula converged at the vaMR with some fibers observed projecting towards the dMR (Bianco *et al.* 2008). The co-localization of Kiss-R1 and Kiss1 in the vHb-vaMR corresponds with previous findings (Ogawa *et al.* 2010, 2012; Servili *et al.* 2011). Although these studies report the projection to the vIPN, we emphasize on the basis of the present results in the Tg *brn3a* that the MR is not part of the vIPN, but instead a division of the SR (Lillesaar *et al.* 2007; Amo *et al.* 2010), and though the projections bypass the vIPN, the fibers terminate at the MR (Amo *et al.* 2010), specifically the ventro-anterior corner, a subregion which has

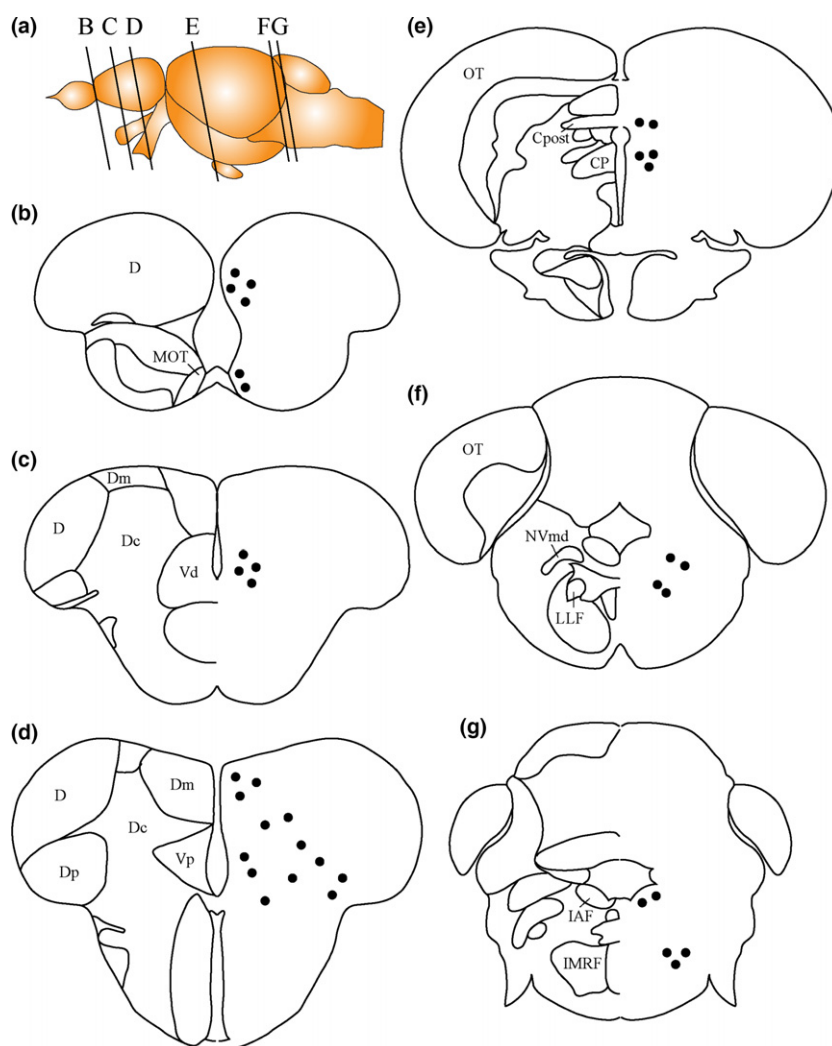


Fig. 4 Schematic representation of Kiss-R1-ir and *kissr1b*-derived protein 2 (*KRBDP2*) cells in the zebrafish brain. Black dots indicate Kiss-R1-immunoreactive cells and the same sites of expression of *KRBDP2*. For abbreviations, see (Table 2). Schematic diagram of brain and naming of regions adapted from Yamamoto and Ito (2008), Wullimann and Rink (2002) and Wullimann *et al.* (1996).

Table 2 List of abbreviations

5-HT	5-hydroxytryptamine; serotonin
CP	Central posterior thalamic nucleus
Cpost	Posterior commissure
D	Dorsal telencephalic area
daMR	Dorso-anterior corner of the MR
Dc	Central zone of dorsal telencephalic area
dHb	Dorsal habenula
Dm	Medial zone of dorsal telencephalic area
Dp	Posterior zone of dorsal telencephalic area
DR	Dorsal raphe
FR	Fasciculus retroflexus
Gad	Glutamic acid decarboxylase
IAF	Inner arcuate nucleus
IMRF	Intermediate reticular formation
LLF	Lateral longitudinal fascicle
MOT	medial olfactory tract
MR	Median raphe
NVmd	Dorsal region of trigeminal motor nucleus
OT	Optic tectum
Slc17a6b	Solute carrier family 17 sodium-dependent inorganic phosphate cotransporter 6 transcript variant 2
SR	Superior raphe
TPp	periventricular nucleus of posterior tuberculum
vaMR	Ventro-anterior corner of the MR
Vc	Caudal nucleus of ventral telencephalic area
Vd	Dorsal nucleus of ventral telencephalic area
vHb	Ventral habenula
Vp	Postcommissural nucleus of ventral telencephalic area

also been identified and recently re-affirmed (Amo *et al.* 2014). Our previous conclusion (Ogawa *et al.* 2012) was based on previous findings by Amo *et al.* (2010) and Servili *et al.* (2011), which however failed to clearly describe the terminating region due to lack of information on the MR but instead more on the IPN. The Tg *brn3a* zebrafish showed clear distinction between both these structures with the vHb-expressing Kiss-ir fibers terminating at the MR and not the vIPN. The current findings corroborates with our previous findings (Ogawa *et al.* 2012) where we mentioned the potential inclusion of the MR. The presence of Kiss1 and Kiss-R1 in the same cells and axon terminals at the MR denote their possible presynaptic role, although it remains unknown whether Kiss1 peptides are released from Kiss1 terminals and act presynaptically at Kiss-R1 (i.e. autoreceptor or retrograde transmission). Co-localization of a neu-

ropeptide and its cognate receptor in the same neurons has been also observed for kisspeptin (Arai 2009; Ogawa *et al.* 2012), oxytocin (Freund-Mercier *et al.* 1994) and vasopressin (Hurbin *et al.* 2002).

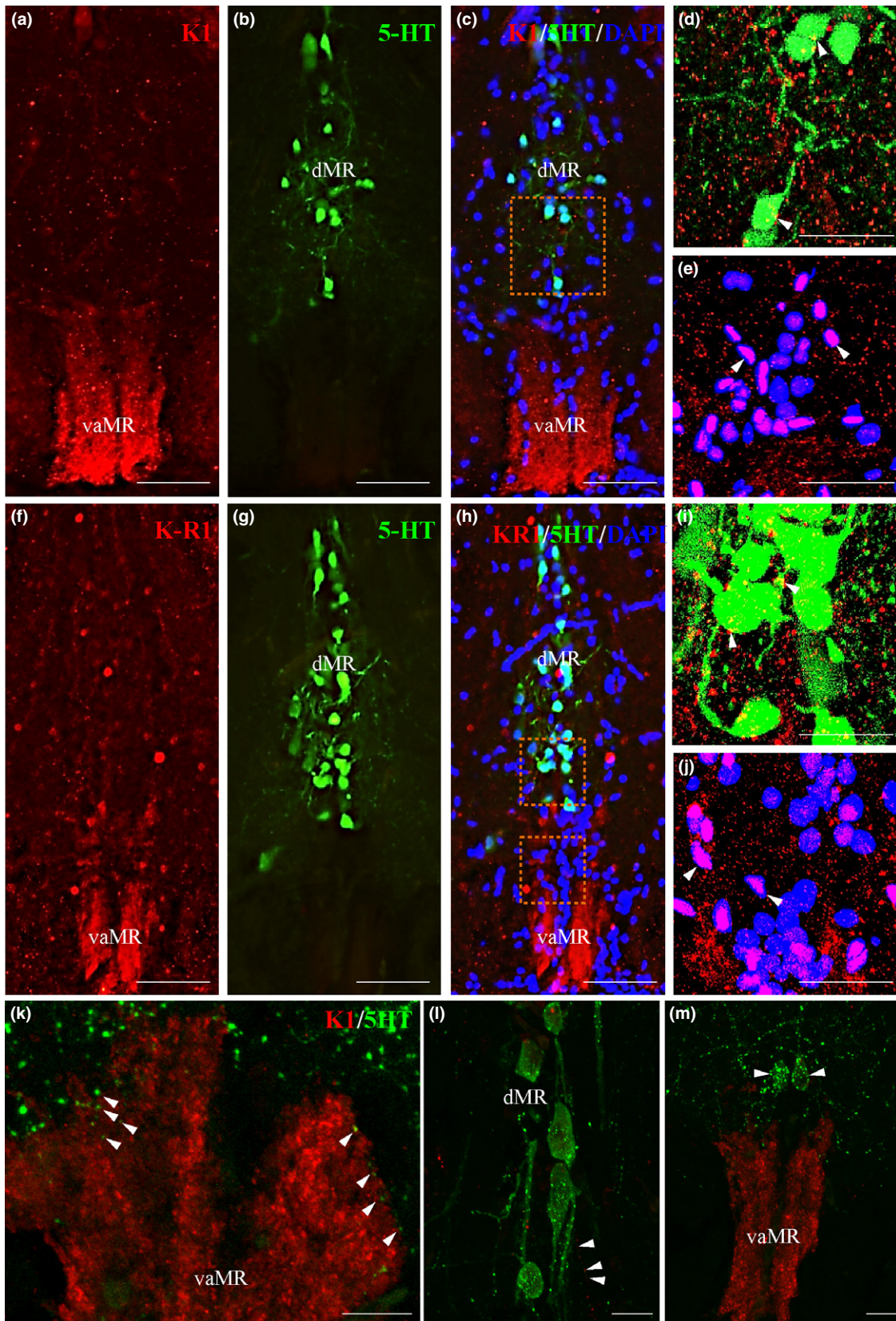
The SR 5-HT system comprises of the median (MR) and dorsal (DR) divisions (Lillesaar *et al.* 2007; Amo *et al.* 2010) where MR neurons occupy the midline region and DR neurons are located more dorsally, and lateral to the midline region (Lillesaar *et al.* 2007). Although 5-HT neurons are the main population occupying these regions, a recent study in the zebrafish shows that the vaMR region, where the vHb terminates, is absent of 5-HT neuronal cell bodies (Amo *et al.* 2014). Functional studies have implicated the teleost raphe serotonergic system in tolerance, resistance, adaptation or appropriate 'coping' strategies to acute or chronic stress (Deakin 1996). These findings indicate that during basal and stressful conditions the habenular Kiss1 system that modulates the raphe 5-HT system (Ogawa *et al.* 2014) may play a role in the hypothalamic-pituitary-adrenal axis, which is extensively and selectively modulated by serotonergic and non-serotonergic neuronal projections.

Our findings in the Tg *pet1* zebrafish show that very few Kiss1 fibers are in close association with 5-HT cell bodies in the MR, whereas there was no expression of Kiss-R1 in 5-HT neurons. This suggests that Kiss1 is likely to act indirectly on 5-HT neurons. However, a few individual fibers potentially originating from a subset of Kiss1 cells in the vHb were in direct contact with 5-HT neuronal cell bodies. We observed the presence of non-serotonergic cells surrounded by Kiss1 and Kiss-R1-ir axonal varicosities in the vaMR. However, based on the recent findings by Amo *et al.* (2014) that showed non-serotonergic cells in the MR do not respond to the vHb axonal stimulation in comparison to some of the 5-HT neurons that responded (Amo *et al.* 2014), these cells may not be involved in the regulation of the 5-HT system. Based on our immunohistochemical findings, Kiss1 occupies the entire vHb with some of the axons terminating on 5-HT neurons while a sparse population terminate on non-5HT neurons which could further act on the 5-HT neurons in the dMR. Additionally, we also observed close associations between Kiss1-fibers with 5-HT fibers in the vaMR, suggesting the possibility of their axon-axon interactions, which remains to be examined.

Our *in situ* hybridization study revealed that the majority of habenular Kiss1 cells are glutamatergic and not GABAergic.

Fig. 5 Neural association of kisspeptin 1 (Kiss1) and Kiss-R1 fibers (red) with serotonergic (green) and non-serotonergic (blue) raphe neurons in *pet1* Tg zebrafish. (a–c) and F–H: Kiss1 (a–c) and Kiss-R1 (f–h) fiber terminal was seen in the vaMR, while *pet1*-green fluorescent protein (GFP) labeled 5-HT neurons were noted in the MR. (d, e, i and j) Confocal image of double-labeling showed close association of Kiss1 (d and e) and Kiss-R1 (i and j) fibers with 5-HT neurons in the dMR (d

and i) and non-5-HT cells (DAPI) in the vaMR (e and j). There was no co-expression of Kiss-R1 in 5-HT cells. (60× plus 1.5× optical zoom; N.A. = 1.4; z-step = 0.15 μm). (k–m) 5-HT fibers were seen in close association with Kiss1 fibers in the vaMR (k) and in the dMR (l). There were few 5-HT cells seen near the vaMR region, but not inside the vMR (m). Scale bars, (a–c) and (f–g) 100 μm; (d, e, i and j) 20 μm; (k–m) 10 μm.



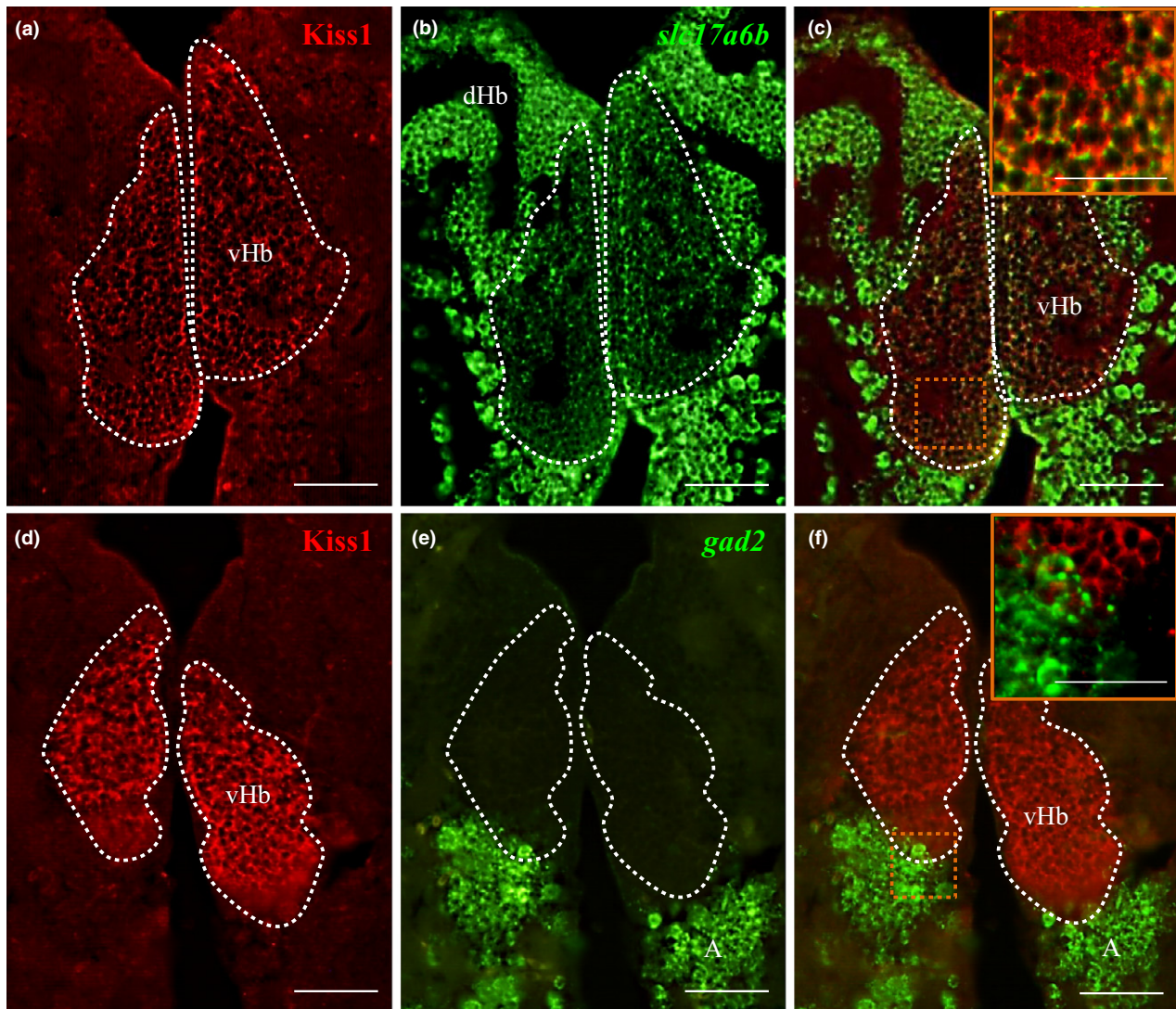


Fig. 6 Dual-fluorescence labeling of kisspeptin 1 (Kiss1) and Kiss-R1 (red) in glutamatergic and GABAergic neurons (green) in the habenula. (a–c) Photomicrographs showing some populations of Kiss1-immunoreactive (-ir) cells in the vHb that co-localized with cells expressing *slc17a6b* mRNA. Some individual Kiss1-ir fibers originating from the vHb co-localized with *slc17a6b* mRNA (as

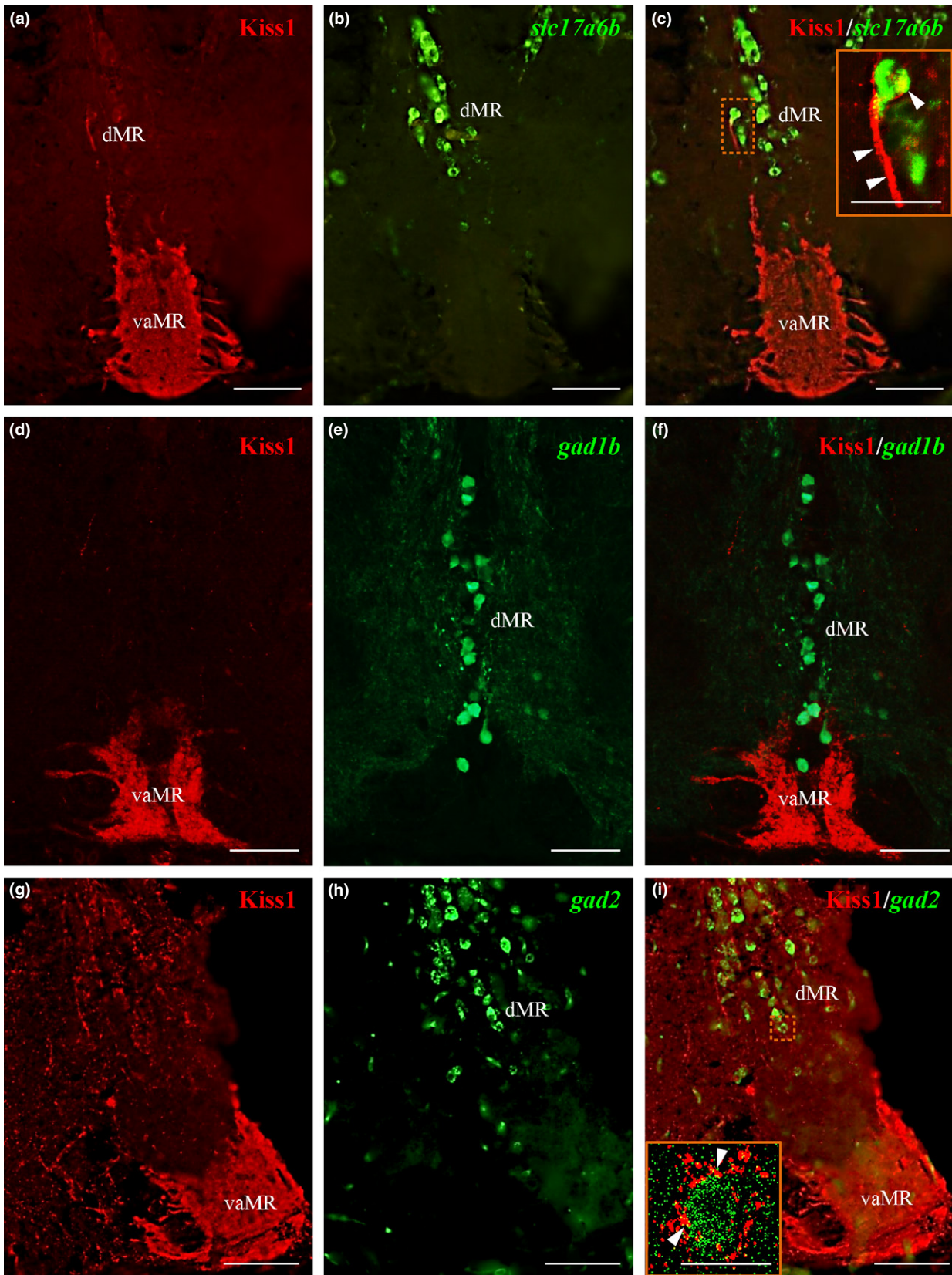
seen in the magnified confocal inset representing the region enclosed by the orange-dotted box; 60× plus 1.5× optical zoom; N.A. = 1.4; z-step = 0.15 μm). No co-localization was observed between Kiss1-ir cells and *gad2* mRNA-expressing cells. A: anterior thalamic nucleus. Scale bars, (a–f) 100 μm and inset c and f: 50 μm.

LHb projections utilize glutamate as a main excitatory neurotransmitter (Kalen *et al.* 1986; Behzadi *et al.* 1990; Ferraro *et al.* 1996) and there are cases in which the autocrine regulation of Kiss1 has been found to activate glutamate

release (Arai 2009), further supporting the idea that the majority of Kiss1 neurons in the zebrafish vHb are glutamatergic. Recent observations in zebrafish have shown that glutamatergic neuronal projections from the vHb exclusively

Fig. 7 Dual-fluorescence labeling of kisspeptin 1 (Kiss1) (red) in glutamatergic and GABAergic neurons (green) in raphe nuclei. (a–c) In the raphe nuclei, Kiss1-immunoreactive (-ir) fibers were seen in close association with *slc17a6b* mRNA expressing neurons in the dMR as denoted by the confocal image (inset C). (d–f) green fluorescent protein (GFP)-labeled Gad1b neurons were observed only in the dMR

with no close associations observed with Kiss1-ir fibers. G–I: Kiss1-ir fibers were seen in close association with *gad2* mRNA expressing neurons in the dMR, denoted by the confocal image (inset I; 60× plus 1.5× optical zoom; N.A. = 1.4; z-step = 0.15 μm). Presence of actual space of at least 0.15 μm noted between fibers and cells. Scale bars, (a–i): 100 μm; inset C and I: 50 μm.



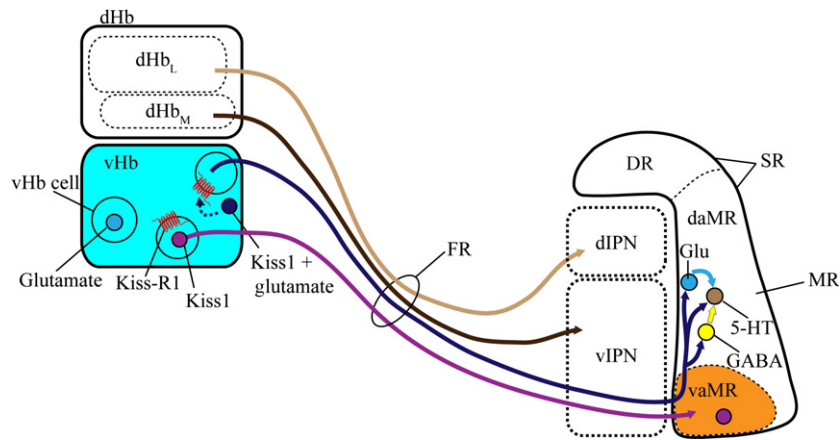


Fig. 8 Schematic drawing of hypothetical neural circuit of habenular kisspeptin 1 (Kiss1) neurons. The lateral subnucleus of the dHb projects to the dIPN while the medial subnucleus of the dHb projects to the vIPN. Some cells in the vHb express glutamate (light blue dot). Both Kiss1 (purple dot) and Kiss-R1 express in the vHb and project down (purple line) to the vaMR, a subregion of the MR [a division of the Superior raphe

(SR)], through the fasciculus retroflexus (FR). The presynaptic action of the Kiss1/K-R1 system causes the release of glutamate in co-expressing cells (dark blue dot) from the vHb (dark blue line) that potentially regulates the 5-HT system in the dMR directly or via glutamatergic (light blue line) and GABAergic neurotransmission (yellow line). Modified from Amo *et al.* (2010, 2014) and Lillesaar *et al.* (2007).

target the vaMR (Amo *et al.* 2014), where we observe the majority of Kiss1/Kiss-R1 axons. However, we also found some additional Kiss1/Kiss-R1 fibers and varicosities in the dorso-anterior corner of the MR, which are making contact with glutamatergic cells. This discrepancy may indicate that the majority of glutamatergic habenular Kiss1 neurons are terminating in the vaMR, but additional fibers derived from another subset of Kiss1 neurons further extend their projections to the dorso-anterior corner of the MR. Additionally, Amo *et al.* (2014) show activation of MR 5-HT neurons through excitatory glutamatergic projections from the vHb, where we also observed glutamatergic expression. Co-occurrence as well as co-release of neuropeptides and neurotransmitters in a single neural cell have been reported in various neurons (Reiner and Anderson 1990). The co-existence is necessary and seen in higher brain regions where a neuropeptide enhances or depresses the release of amino acid transmitters in order to produce a subset of responses to a similar modulatory outcome (van den Pol *et al.* 1998). Co-existing transmitters may work together to evoke physiological responses such that they may activate pre- and post-synaptic receptors, while the second transmitter may work in an inhibitory manner. Based on similar expression patterns observed for *gad1b* and *gad2* (Martin *et al.* 1998), double-labeling with *gad1b* and Kiss1/Kiss-R1 was only carried out in the MR to further clarify the extent of GABAergic neurotransmission involvement in 5-HT modulation. Due to the relatively weak staining of *chat* (marker for cholinergic neurons) observed in comparison to that of glutamate in the habenula, and based on recent findings in the zebrafish vHb (Amo *et al.* 2014), we did not progress with double-labeling of Kiss1/Kiss-R1 and *chat*. Hence, we speculate that Kiss1

could presynaptically activate Kiss-R1 that could alter glutamatergic levels in co-localizing cells in the vHb. This could further regulate 5-HT levels in the MR directly or indirectly, via GABAergic or glutamatergic MR neurons (Valentino *et al.* 2003; Ebner and Singewald 2006).

Our *in situ* hybridization showed some 5-HT neurons co-express with markers of glutamatergic and GABAergic neurons in the MR indicating that these neurons could transmit information to fear-regulating regions via the co-release of GABA and glutamate (El Mestikawy *et al.* 2011). Subsets of 5-HT neurons co-expressing the serotonin transporter project to various regions involved in depressive and frightful behaviours, such as the periaqueductal gray, amygdala and hypothalamus in rats (Shutoh *et al.* 2008; El Mestikawy *et al.* 2011). Co-expression of glutamic acid decarboxylase (GAD) and 5-HT has also been reported in various studies (Gaspar and Lillesaar 2012; Gocho *et al.* 2013), although a very small population is noted in the raphe nuclei, explaining the lesser extent of co-expression that we noted of *gad2* and 5-HT neurons in the MR.

In conclusion, Kiss-R1 expressing cells in the vHb project down through the FR where they terminate specifically at the vaMR. This projection pattern, along with that of Kiss1 and the lack of Kiss-R1 expressing cell soma in the MR suggests a presynaptic action of Kiss1 in the zebrafish. The autocrine regulation of Kiss1 may activate glutamate co-release that projects to the vaMR to interact with the 5-HT system in the MR (Fig. 8). Taken together, our findings indicate that the action of Kiss1/Kiss-R1 system on 5-HT neurons in the MR requires the interaction of glutamate as the main excitatory neurotransmitter and to a lesser extent, GABA.

Acknowledgments and conflict of interest disclosure

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All experiments were conducted in compliance with the ARRIVE guidelines.

Author contributions

S.O and I.S.P designed research; F.M.N performed research; S.O and F.M.N analyse data; F.M.N, S.O and I.S.P wrote the paper.

Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Figure S1. Kiss-R1-ir localization and *KRBDP2* expression in other regions of the zebrafish brain.

Figure S2. Expression of *slc17a6b*, *gad2* and *chat* mRNAs in the habenula and raphe regions.

Figure S3. Dual-fluorescence labeling of Kiss-R1 (red) in glutamatergic and GABAergic neurons (green) in raphe nuclei.

Figure S4. Co-expression of *slc17a6b* and *gad2* (red) with 5-HT (green).

Table S1. Gene abbreviations, the primer sequences, probe size and GenBank accession numbers for probes for DIG-*in situ* hybridization.

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