Insulin signaling inhibits the 5-HT\textsubscript{2C} receptor in choroid plexus via MAP kinase

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Abstract

Background: G protein-coupled receptors (GPCRs) interact with heterotrimeric GTP-binding proteins (G proteins) to modulate acute changes in intracellular messenger levels and ion channel activity. In contrast, long-term changes in cellular growth, proliferation and differentiation are often mediated by tyrosine kinase receptors and certain GPCRs by activation of mitogen-activated protein (MAP) kinases. Complex interactions occur between these signaling pathways, but the specific mechanisms of such regulatory events are not well-understood. In particular it is not clear whether GPCRs are modulated by tyrosine kinase receptor-MAP kinase pathways.

Results: Here we describe tyrosine kinase receptor regulation of a GPCR via MAP kinase. Insulin reduced the activity of the 5-HT\textsubscript{2C} receptor in choroid plexus cells which was blocked by the MAP kinase kinase (MEK) inhibitor, PD 098059. We demonstrate that the inhibitory effect of insulin and insulin-like growth factor type 1 (IGF-1) on the 5-HT\textsubscript{2C} receptor is dependent on tyrosine kinase, RAS and MAP kinase. The effect may be receptor-specific: insulin had no effect on another GPCR that shares the same G protein signaling pathway as the 5-HT\textsubscript{2C} receptor. This effect is also direct: activated MAP kinase mimicked the effect of insulin, and removing a putative MAP kinase site from the 5-HT\textsubscript{2C} receptor abolished the effect of insulin.

Conclusion: These results show that insulin signaling can inhibit 5-HT\textsubscript{2C} receptor activity and suggest that MAP kinase may play a direct role in regulating the function of a specific GPCR.

Background

It was originally thought that GPCRs and tyrosine kinase receptors functioned independently to mediate different signaling events, but it has become clear in recent years that some functions and signaling pathways are shared (for reviews, see Marinissen and Gutkind, 2001 [1]; Luttrell, 2002 [2]; and van Biesen et al., 1996 [3]). For example, some classical neurotransmitters such as 5-HT have short-term effects on ion channels and other effectors such as adenylyl cyclase but also have growth factor-like
effects in developing brain [4] and mitogenic effects on fibroblasts (reviewed in Gerhardt and van Heerikhuizen, 1997 [5]). The peptide hormones insulin and IGF-1 have both short-term metabolic effects and long-term actions on cell growth and differentiation. Insulin and IGF-1 bind and stimulate tyrosine kinase receptors which interact with a large number of effectors [6,7]. Complex interactions occur between these two types of signaling pathways that are the subject of intense investigation.

The 5-HT2c receptor displays a heterogeneous distribution in the CNS [8] and is not found in peripheral tissues. It is abundant in choroid plexus where it modulates the production of cerebrospinal fluid (CSF), and in limbic regions and hypothalamus where it may play a role in motor behavior and appetite control. The 5-HT2c receptor has been implicated in anxiety, migraine, movement disorders, eating disorders and neuroendocrine regulation [9]. The importance of the 5-HT2c receptor in regulation of food intake is evident in a knockout mouse developed by Tecott et al. [10]. The obesity found in these mice is due to excessive food intake and phenotype along with increased plasma levels of insulin and leptin is analogous to Type 2 diabetes. The knockout mouse is also susceptible to epileptic-like seizures – suggesting that the 5-HT2c receptor has a role in tonic inhibition of neuronal excitability.

5-HT2c receptors and insulin/IGF-1 receptors share some functional roles; both have trophic effects in the brain and modulate appetite. 5-HT2c receptors [8] and insulin/IGF-1 receptors [11] co-localize in several areas in the brain including choroid plexus, olfactory bulb, cerebral cortex, hypothalamus, and hippocampus. Interactions between insulin and serotonergic pathways may have important consequences for their known roles in appetite modulation and trophic actions in the brain. We chose to look for these types of interactions in choroid plexus because both 5-HT2c receptors and insulin/IGF-1 receptors are abundant in this tissue and because the 5-HT2c receptor is the only 5-HT receptor present in these cells.

The 5-HT2c receptor is a member of the GPCR family. GPCRs stimulate heterotrimeric G proteins which release activated Gα and Gβγ subunits to interact with a variety of effectors. The function of GPCRs is tightly regulated by phosphorylation by second messenger activated kinases (protein kinase A and protein kinase C) and G protein-coupled receptor-specific kinases (GRKs). Arrestins bind phosphorylated receptors and further down-regulate receptor activity by inhibiting G protein interaction. It is well-known that GPCRs can regulate the activity of tyrosine kinase/MAP kinase pathways. However, there is little evidence for reciprocal regulation: MAP kinase effects on GPCR function. Here we report such evidence – insulin-mediated MAP kinase regulation of 5-HT2c receptor activity.

Results

Effect of insulin signaling on 5HT2c receptor function in choroid plexus cells

We examined the effects of insulin signaling on 5-HT2c receptor function in isolated rat choroid plexus cells. Activation of 5-HT2c receptors in choroid plexus stimulates phosphoinositide hydrolysis [12] and increases intracellular calcium [13]. We measured changes in intracellular calcium in response to serotonin (5-HT) with the calcium indicator, fura. A robust increase in intracellular calcium was seen in response to 5-HT (Fig. 1a and 1c) and this response could be completely blocked with the selective 5-HT2c receptor antagonist, mesulergine (data not shown). The response to 5-HT was significantly attenuated (40% inhibition) when choroid plexus cells were treated with insulin before superfusing serotonin (Fig. 1b and 1c). Pretreatment with PD 098059, a MEK inhibitor blocked the inhibitory effect of insulin (Fig. 1c) but had no effect on the serotonin response when used alone. These data indicate that insulin inhibits 5-HT2c receptor activity and MEK is an obligatory component of insulin’s action, implicating the involvement of the MAP kinase pathway.

Characterization of the mechanism of insulin’s effect on 5HT2c receptor function in Xenopus oocytes

To delineate the mechanism of insulin’s action on the 5-HT2c receptor, we used the Xenopus oocyte expression system. Oocytes contain endogenous insulin and IGF-1 receptors [14]. They also have many of the proteins in the RAS and MAP kinase pathways, which have been implicated in cell cycle control and re-entry into meiosis [15,16]. We expressed two GPCRs, a serotonin receptor (5-HT2c) and an M1 muscarinic acetylcholine (Ach) receptor. Both receptors utilize G proteins for signaling; they stimulate phosphoinositide hydrolysis and increased phospholipase C activity which leads to transient activation of a Ca2+-dependent Cl− current in the oocytes. Activation of either receptor can cross-desensitize the other, suggesting that they share the same G protein pathway.

To determine the effect of insulin receptor or IGF-1 receptor activation on GPCR’s, cells were treated with insulin or IGF-1. The Cl− currents elicited by subsequent 5-HT or Ach stimulation were compared with those without insulin/IGF-1 treatment. As shown in Fig. 2, treatment with insulin or IGF-1 resulted in a significant reduction of the peak Cl− current in response to 5-HT, but did not affect the response to Ach. Insulin alone did not induce an ionic current under these conditions (data not shown). Insulin treatment did not affect the general characteristics of the Cl− current induced by 5-HT or Ach, i.e., a depolarizing
Insulin and IGF-1 receptors activate several different regulatory proteins, including RAS, MAP kinase, PI3 kinase, S6 kinase and protein tyrosine phosphatase [6,7,17]. Initiation of this transduction cascade requires the intrinsic tyrosine kinase activity of the insulin/IGF-1 receptors. To determine whether or not tyrosine kinase activity was required for the action of insulin/IGF-1, the tyrosine kinase inhibitor genistein was used. As shown in Fig. 3a, in oocytes pretreated with genistein before IGF-1 administration, the reduction in 5-HT-induced Cl− current was abolished, whereas genistein treatment alone had no effect on the 5-HT response in oocytes not treated with IGF-1.

The insulin/IGF-1 pathway diverges into RAS-dependent and -independent paths. To identify which path is involved, an anti-RAS antibody that can neutralize RAS activity was injected into oocytes before IGF-1 treatment. This RAS antibody (Y13-259) has been shown in oocytes to block the re-entry into meiosis or germinal vesicle breakdown induced by IGF-1 [18]. Antibody injection blocked the effect of IGF-1 on the 5-HT response (Fig. 3b), demonstrating that the inhibitory effect of insulin/IGF-1 was RAS-dependent.

RAS activates a cascade of kinases, including RAF, MAP kinase kinase (MEK) and MAP kinase. The involvement of this pathway in regulation of 5-HT receptor function was examined. Pretreating cells with PD 098059, a specific MEK kinase inhibitor [19], blocked the effect of IGF-1 on the 5-HT response, whereas inhibition of protein kinase C did not (Fig. 4a). These results suggest that MEK is involved in mediating the inhibitory effect of the insulin/IGF-1 pathway.

MEK’s primary role is activation of MAP kinase through threonine and tyrosine phosphorylation of MAP kinase which in turn phosphorylates and regulates the function of several nuclear proteins, such as transcription factors, as well as cytosolic and membrane-associated proteins, such as the insulin and EGF receptors. To determine whether MAP kinase is involved in mediating the insulin/IGF-1 effect, activated MAP kinase [20] was injected into oocytes before testing the 5-HT response. Active MAP kinase reduced the 5-HT response by 56% compared to untreated controls (Fig. 4b), demonstrating that active MAP kinase is capable of mimicking the inhibitory effect of insulin/IGF-1 on the 5-HT_{2C} receptor.

The 5-HT_{2C} receptor contains a putative MAP kinase site [21] at serine 159 in the second intracellular loop between the 3rd and 4th transmembrane domains. To ascertain whether MAP kinase directly modulates the receptor function, this amino acid was changed to alanine by site-directed mutagenesis. As shown in Fig. 4c, insulin no
longer inhibited the peak Cl\textsuperscript{-} current activated by the mutant receptor S159A, indicating that serine 159 is likely the site of MAP kinase modulation.

**Discussion**

The 5-HT\textsubscript{2C} receptor is a member of the G protein-coupled receptor superfamily and one of over 30 cloned serotonin receptors. Except for the 5-HT\textsubscript{3} receptor which is an oligomeric ion channel, all serotonin receptors are GPCRs and are divided into seven subfamilies based on sequence homology, gene structure, and signaling pathways [22,23]. The 5-HT\textsubscript{2C} receptor, like other members of the 5-HT\textsubscript{2} family, stimulates phospholipase C, and increases IP\textsubscript{3} and DAG which leads to increased intracellular calcium and mediates many cellular processes. In general 5-HT\textsubscript{2} receptor types are growth-regulatory and stimulate cell

**Figure 2**

Effect of insulin and IGF-1 on the peak Cl\textsuperscript{-} current in response to serotonin or acetylcholine in Xenopus oocytes expressing 5-HT\textsubscript{2C} and M1 acetylcholine receptors. (A) Effects of insulin or IGF-1 on the peak Cl\textsuperscript{-} current in response to 5-HT. Top left, representative Cl\textsuperscript{-} current trace after stimulation with 5-HT. Top right, representative Cl\textsuperscript{-} current trace after stimulation with 5-HT in an oocyte pre-treated with insulin. Horizontal bars above the current traces indicate bath application of 1 µM 5-HT. Middle, effects of insulin on the peak Cl\textsuperscript{-} current induced by 5-HT. Bottom, effects of IGF-1 on the peak Cl\textsuperscript{-} current induced by 5-HT. (B) Effects of insulin or IGF-1 on the peak Cl\textsuperscript{-} current in response to Ach. Top left, representative current trace after stimulation with Ach. Top right, representative current trace after stimulation with Ach in an oocyte previously treated with insulin. Horizontal bars above the current traces indicate bath application of 10 µM Ach. Middle, effects of insulin on the peak Cl\textsuperscript{-} current induced by Ach. Bottom, effects of IGF-1 on the peak Cl\textsuperscript{-} current induced by Ach. The data are presented as mean ± S.E.M. of 12-16 oocytes (*, P < 0.05).
division. However, unlike the 5-HT\textsubscript{2A} [24] and 5-HT\textsubscript{2B} [25] receptors, the 5-HT\textsubscript{2C} receptor is not known to activate MAP kinase. The 5-HT\textsubscript{2C} receptor activates multiple signal transduction cascades \textit{in vivo} and in heterologous expression systems (see Raymond et al 2001 [22]; and Gerhardt and van Heerikhuizen, 1997 ([5], for reviews). In choroid plexus, the 5-HT\textsubscript{2C} receptor stimulates PI turnover, increases cGMP formation and activates Cl channels. In heterologous expression systems, the 5-HT\textsubscript{2C} receptor can couple to two different K\textsuperscript{+} channels [26] and in some cases either activation or inactivation of adenylyl cyclase has been demonstrated in addition to the well-known activation of phospholipase C and PI turnover.

The regulation of the 5-HT\textsubscript{2C} receptor has been the focus of several studies, but the results have been difficult to interpret for several reasons including the complex pharmacology of this receptor. The presence of spare 5-HT\textsubscript{2C} receptors [27] in choroid plexus has also complicated the study of desensitization \textit{in vivo}. However, it has been demonstrated that denervation of 5-HT neurons supersensitizes 5-HT\textsubscript{2C} receptors [28] in choroid plexus, suggesting that these receptors are under tonic control by 5-HT. In primary cultures of choroid plexus cells, repeated 5-HT application desensitizes 5-HT induced increases in intracellular calcium [13]. Down-regulation of receptor binding sites in choroid plexus cells is seen after agonist treatment [29]. However, inverse agonists can either down- [29] or up-regulate [30] 5-HT\textsubscript{2C} receptor binding sites dependent upon cellular context. The mechanisms

Figure 3
Effects of tyrosine kinase inhibitor and dominant-negative RAS antibody on IGF-1 effects on the serotonin response in \textit{Xenopus} oocytes. Shown are peak Cl\textsuperscript{-} currents in response to 5-HT after the indicated treatments. (A) Genistein, a tyrosine kinase inhibitor, blocks the inhibitory effect of IGF-1 on the 5-HT response. The data are presented as mean ± S.E.M. of 11-12 oocytes (*, P < 0.05). (B) RAS antibody, Y13-259, blocks the inhibitory effect of IGF-1 on the 5-HT response. The data are presented as mean ± S.E.M. of 6 oocytes (*, P < 0.05).

Figure 4
Role of MAP kinase in the inhibitory effect of IGF-1 on the 5-HT response in \textit{Xenopus} oocytes. Plotted are peak Cl\textsuperscript{-} currents in response to 5-HT after the indicated treatments. (A) The MEK kinase inhibitor, PD 098059, but not the protein kinase C inhibitor (PKC I), blocks the inhibitory effect of IGF-1 on the 5-HT response. The data are presented as the mean ± S.E.M. of 6 oocytes (*, P < 0.05). (B) Activated MAP kinase mimics the IGF-1 inhibitory effect on the 5-HT response. The data are presented as the mean ± S.E.M. of 8-12 oocytes (*, P < 0.05). (C) Mutation of the putative MAP kinase site on the 5-HT\textsubscript{2C} receptor abolishes the insulin effect. In oocytes expressing the wild-type 5-HT\textsubscript{2C} receptor (left two bars), insulin treatment resulted in a significant reduction of the peak Cl\textsuperscript{-} current in response to 5-HT. In cells expressing the MAP kinase site mutation S159A (right two bars), insulin had no effect on the 5-HT-activated Cl\textsuperscript{-} current. The data are presented as the mean ± S.E.M. of 6 oocytes (*, P < 0.05).
underlying desensitization or down-regulation of the 5-HT$_{2C}$ receptor are unknown but these reports suggest finely tuned control of 5-HT$_{2C}$ receptor function and density.

Several studies have utilized heterologous expression systems to identify mechanisms of 5-HT$_{2C}$ receptor regulation. Phosphorylation most likely has a prominent role in regulation of the 5-HT$_{2C}$ receptor. Westphal and colleagues [31] demonstrated that the 5-HT$_{2C}$ receptor exhibits basal phosphorylation, and that agonist treatment was associated with increased phosphorylation and receptor desensitization in transfected cells. The expressed 5-HT$_{2C}$ receptor exhibits homologous and heterologous desensitization in oocytes [32] mediated by protein kinase C [33–35]. A calmodulin-dependent kinase may also be involved in homologous desensitization [36]. In contrast, a recent study indicates that neither PKC nor calmodulin-dependent protein kinase II are involved, instead desensitization of the 5-HT$_{2C}$ Receptor is dependent on GRK activity [37]. Interestingly, tyrosine kinase involvement in the agonist-mediated phosphorylation of the rat 5-HT$_{3C}$ receptor has also been reported [38], although as yet no report has shown that the 5-HT$_{2C}$ receptor directly activates tyrosine kinases.

The 5-HT$_{2C}$ receptors are subject to another form of regulation by means of mRNA editing [39]. RNA editing [40] is a type of posttranscriptional modification that occurs when double-stranded RNA deaminase converts genomics encoded adenosines to inosines, thereby changing the coding sequence of specific RNA transcripts. RNA editing of the rat 5-HT$_{2C}$ receptor was first described by Burns et al. [39] and editing at four nucleotide sites alters three amino acids within the second intracellular loop. RNA editing of the 5-HT$_{2C}$ receptor is conserved among species [41] and at least 7 major isoforms with tissue-specific expression patterns have been described in rat brain [39] suggesting a functional importance. The mouse cDNA clone used in our study codes for the fully edited form (Val157-Ser159-Val161) of the receptor. The putative MAP kinase site (Ser159) we identified and mutated in this study is also a site of mRNA editing in this receptor; i.e. Ser159 is only present in edited forms. The fully edited 5-HT$_{2C}$ receptor exhibits homologous and heterologous desensitization in oocytes [32] mediated by protein kinase C [33–35]. A calmodulin-dependent kinase may also be involved in homologous desensitization [36]. In contrast, a recent study indicates that neither PKC nor calmodulin-dependent protein kinase II are involved, instead desensitization of the 5-HT$_{2C}$ Receptor is dependent on GRK activity [37]. Interestingly, tyrosine kinase involvement in the agonist-mediated phosphorylation of the rat 5-HT$_{3C}$ receptor has also been reported [38], although as yet no report has shown that the 5-HT$_{2C}$ receptor directly activates tyrosine kinases.

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The 5-HT$_{2C}$ receptor is very abundant in choroid plexus [8,42], and is the only 5-HT receptor present on these cells. Choroid plexus cells line the cerebral ventricles, form the blood-CSF barrier, and are responsible for the production of cerebrospinal fluid (CSF) [43]. Although the mechanisms have not been delineated, the 5-HT$_{2C}$ receptor is thought to regulate CSF production and the expression of transferrin. For example, upon intraventricular injection, 5-HT$_{2C}$ agonists decrease the production of CSF [44] and in primary cultures of choroid plexus, 5-HT agonists increase the expression of transferrin [45,46], an iron carrier protein which has trophic effects on the brain. In addition, choroid plexus expresses insulin, IGF-I, and IGF-II receptors [11,47], as well as large amounts of IGF-II that is secreted into the CSF [47]. CSF contains insulin/IGFs and 5-HT [48] in concentrations high enough to activate their respective receptors in choroid plexus, suggesting that this signaling mechanism may occur in vivo. It has been suggested that 5-HT$_{2C}$ receptors in choroid plexus may be tonically activated, due to CSF 5-HT concentrations near the EC$_{50}$ thus tonically inhibiting CSF production. Our data suggest that insulin may reduce 5-HT$_{2C}$ receptor activity, thereby increasing CSF production and decreasing transferrin production. As a consequence, insulin inhibition of 5-HT$_{2C}$ receptor activity may change the volume and composition of CSF and indirectly may alter the concentrations of many potentially important signaling molecules in CSF.

MAP kinase regulation is potentially an important mechanism of modulating GPCR function, however the prevalence of this type of modulation is unknown. Our results suggest that the 5-HT$_{2C}$ receptor is a MAP kinase substrate, whereas the M1 Ach receptor is not. The optimal consensus site for MAP kinase phosphorylation is Pro-X-(Ser/Thr)-Pro, however in some cases the upstream Pro is not required and a minimal sequence of Ser-Pro or Thr-Pro is phosphorylated (for review, see Davis, 1993[21]). The 5-HT$_{2C}$ receptor site is just such a minimal sequence of Ser-Pro and it is the only MAP kinase site we identified within the receptor's presumed intracellular domains. This site is not conserved within the M1 Ach receptor or other 5-HT$_{2}$ type receptors. Although all of these receptors contain Ser-Pro or Thr-Pro sequences within intracellular domains it is difficult to determine the importance of
these sites. For example, despite the fact that insulin did not inhibit Ach receptor signalng in our experiment, the rat M1 Ach receptor contains several MAP kinase sites, including one Thr-Pro sequence in the 2nd intracellular loop and 3 Ser-Pro and one Thr-Pro in the 3rd intracellular loop. The reason for this apparent discrepancy is unclear but may be related to a "spare receptor" phenomenon in the oocyte expression system. Alternatively, additional sequence or regulatory elements, which have not been identified, may be required for MAP kinase phosphorylation. Determining the prevalence and specificity of MAP kinase modulation of GPCR's will await future studies.

Conclusions
Taken together, our results demonstrate that insulin/IGF-1 signaling down-regulates 5-HT2C receptor function in choroid plexus and *Xenopus* oocytes via MAP kinase, and that the 5-HT2C receptor is a substrate for MAP kinase. To our knowledge, this is the first description of MAP kinase regulation of a GPCR and suggests that this form of regulation may be more wide-spread than has been previously recognized. Furthermore, since some GPCRs can modulate MAP kinase activity, MAP kinase regulation of GPCR function may then serve as a form of negative feedback control in much the same way that protein kinase A, protein kinase C and the G protein-coupled receptor kinases (GRKs) regulate GPCRs.

Methods
Chemicals and Reagents
Cell culture reagents and enzymes including MEM, HAM's F12, D-valine, dialyzed fetal calf serum, Pronase and DNase I are from Life Technologies (Grand Island, NY) except as noted. All other chemicals are from Sigma (St. Louis, MO) except as noted. PD 098059 was kindly provided by Dr. A. R. Saltiel (Parke-Davis Pharmaceutical Research, Ann Arbor, Michigan). Purified ERK2 was kindly provided by Dr. M. H. Cobb (Department of Pharmacology, UT Southwestern Medical Center, Dallas, TX).

Choroid plexus cell isolation and culture
All animal experiments in this work have been carried out in accordance with the Guide for the Care and Use of Laboratory Animals as required by the U.S. National Institutes of Health. Choroid plexus cells were isolated as described [46]. Briefly, choroid plexus from adult male Sprague-Dawley rats (175-200 g) were dissected into cold Locke's buffer, rinsed and enzymatically dissociated for 10 minutes at 37°C in Hank’s Balanced Salt Solution (HBSS) containing 330 ug/ml Pronase I and 260 ug/ml DNase I. The cells were pelleted and choroid plexus cells were released after an additional digest with 130 ug/ml DNase I in HBSS. The cells were resuspended in MEM containing D-valine and 15% dialyzed fetal calf serum and plated on concavalin A-coated round glass coverslips. Four days later the media was replaced with Ham’s F12 medium.

Intracellular calcium measurements
After 7 days in culture, cells were loaded with 1.5 µM fura-2 AM (Molecular Probes, Eugene, OR) in HBSS and 1 mg/ml bovine serum albumin (BSA) for 30 minutes at 22°C and washed in HBSS for 30 minutes at 22°C. Cells were continuosly superperfused with HBSS containing the indicated drugs. Insulin (10 or 20 µM) was diluted into HBSS containing 1 mg/ml BSA, and 5-HT (2 µM) was diluted into HBSS. Some cells were pretreated with 50 µM PD 098059 for 30 minutes before intracellular calcium measurements. Fura imaging was done using software kindly provided by Dr. Eric Gruenstein of University of Cincinnati and was carried out as previously described [49]. Peak intracellular calcium responses were measured from individual cells and pooled for statistical analysis.

Oocyte injection and drug treatment
Oocytes were injected with *in vitro* transcribed RNA for the mouse 5-HT2C [50] and the M1 acetylcholine (kindly provided by Dr. T. Bonner) receptors for the experiment in Fig. 2 or the 5-HT2C receptor alone for all other experiments. Three days after RNA injection, oocytes were stimulated with 5-HT or Ach, and the chloride current was measured using a two-electrode voltage-clamp (Axoclamp-2A, Axon Instruments, Foster City, CA). Oocytes were subjected to different treatments as follows. For insulin or IGF-1 treatment, oocytes were treated with 1 µM insulin or 100 nM IGF-1 in ND96 (96 mM NaCl, 2 mM KCl, 1 mM MgCl2, 5 mM HEPES, pH 7.6) and 1 mg/ml BSA for 5 minutes, then rinsed in ND96 for 2 minutes before stimulation with 1 µM 5-HT or 10 µM Ach. For the experiments in Fig. 3, some oocytes were pretreated with 1 µM genistein for 60 minutes or were injected with RAS antibody (0.65 µg/50 nl) 5 minutes before 5-HT stimulation or treatment with IGF-1 followed by 5-HT stimulation. For the experiments in Fig. 4, some oocytes were pretreated with either PD 098059 [19] (50 µM for 30 minutes) or protein kinase C inhibitor, myristoylated EGF-R (Calbiochem, La Jolla, CA), (5 µM for 30 minutes) before 5-HT stimulation or treatment with IGF-1 followed by stimulation with 5-HT. In Fig. 4b, some oocytes were injected with active MAP kinase (ERK2, 10 ng/cell) or MAP kinase buffer 5 minutes before 5-HT stimulation or treatment with IGF-1 followed by stimulation with 5-HT. For Fig. 4c, oocytes were injected with *in vitro* transcribed RNA for the 5-HT2C receptor or the 5-HT2C S149A mutant receptor. The oocytes were stimulated with 5-HT or treated with insulin followed by stimulation with 5-HT.

Site-directed mutagenesis
The putative MAP kinase site on the 5-HT2C receptor, serine 159, was mutated to alanine by PCR with primers con-
taining an alanine codon at the place for serine 159. The PCR product was subcloned into the same vector as the wild-type 5-HT2C receptor, and the mutation confirmed by DNA sequencing.

**Authors' contributions**

JH carried out fura imaging and oocyte experiments and drafted the manuscript. SZ participated in fura imaging and oocyte experiments and carried out the site-directed mutagenesis. LB participated in oocyte experiments. MM participated in the design of the study and generated the Ras antibody. AD purified the MAP kinase and participated in the design of the study. KG participated in the design of the study. AF participated in the fura experiments and in the design of the study. LY conceived of the study and participated in its design and coordination. All authors read and approved the final manuscript.

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