Evidence for an interaction between proinsulin C-peptide and GPR146

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Abstract

Microvascular diseases, such as retinopathies, neuropathies, and nephropathies, are a devastating consequence of type 1 and type 2 diabetes. The etiology of diabetes-associated microvascular dysfunction is poorly understood, and, likewise, treatment modalities for these disorders are limited. Interestingly, proinsulin C-peptide has been shown to play a protective role against diabetes-associated complications in experimental animals and in diabetic humans and is thus an attractive therapeutic target. However, an important step in the development of C-peptide-based therapeutics is identification of the C-peptide receptor, which is likely a G protein-coupled receptor (GPCR). Using a unique Deductive Ligand-Receptor Matching Strategy, we sought to determine whether one of the known orphan GPCRs is essential for C-peptide signaling. Knockdown of GPR146, but not GPR107 or GPR160, blocked C-peptide-induced cFos expression in KATOIII cells. Furthermore, stimulation with C-peptide caused internalization of GPR146, and examples of punctate colocalization were observed between C-peptide and GPR146 on KATOIII cell membranes. These data indicate that GPR146 is likely a part of the C-peptide signaling complex and provide a platform for the elucidation of the C-peptide signalosome.

Key Words

- ▶ C-peptide
- ▶ GPR146
- ▶ orphan GPCR
- ▶ insulin receptor

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Introduction

Diabetes is associated with the development of microvascular complications, such as nephropathy, neuropathy, and retinopathy, which can severely and negatively impact quality of life (Wahren *et al.* 2012). The etiology of diabetes-associated microvascular dysfunction is complex and poorly understood, and thus, therapeutic interventions to prevent or treat these disorders are limited. However, proinsulin C-peptide has emerged as a promising target for the treatment of diabetes-associated microvascular complications (Luppi *et al.* 2011, Wahren *et al.* 2012). Indeed, treatment with exogenous C-peptide was shown to prevent or reverse retinopathies (Ido *et al.* 1997), neuropathies (Wahren *et al.* 2012), and nephropathy (Wahren

et al. 2012) in experimental animals and in diabetic patients. Importantly, plasma levels of C-peptide strongly and negatively correlated with the incidence of microvascular complications in diabetic patients (Bo *et al.* 2012), indicating that endogenous C-peptide may play a protective role in the vasculature. However, an essential step in the development of C-peptide-based therapeutics is identification of the C-peptide receptor (CpepR).

Although C-peptide has been shown to bind specifically to human cell membranes (Rigler *et al.* 1999, Luppi *et al.* 2011), the receptor of C-peptide remains unknown. C-peptide has been shown to initiate intracellular signaling cascades that are usually associated with the activation

of a G protein-coupled receptor (GPCR), such as the activation of protein kinase C and the mobilization of calcium (Wahren *et al.* 2012). Furthermore, the effects of C-peptide were shown to be sensitive to pertussis toxin (Luppi *et al.* 2011, Wahren *et al.* 2012), strongly suggesting that the CpepR is a GPCR, perhaps one of the 136 orphan GPCRs cataloged by IUPHAR (Sharman *et al.* 2013). We have developed a Deductive Ligand-Receptor Matching Strategy that we recently used to identify the cognate receptor of the peptide hormone, neuronostatin (Samson *et al.* 2008), to be GPR107 (Yosten *et al.* 2012). In these initial studies, we sought to employ the same methodology to determine whether any of the known orphan GPCRs were essential for transmitting the C-peptide signal *in vitro* and thus provide a platform for elucidation of the C-peptide signalosome.

Materials and methods

Cell culture

KATOIII cells (ATCC, Manassas, VA, USA) were maintained in Iscove's Modified Dulbecco's Medium (IMDM; ATCC) with 20% fetal bovine serum (FBS) and 1% penicillin/streptomycin. HEK293 cells (a kind gift from M Rauchman, Saint Louis University) were cultured in DMEM (Sigma-Aldrich) with 20% FBS and 1% penicillin/streptomycin. TF-1 cells (ATCC) were maintained in RPMI-1640 (Sigma-Aldrich) with 10% FBS, 1% penicillin/streptomycin, and 2 ng/ml GM-CSF (Sigma-Aldrich). FBS was purchased from ATCC, and penicillin/streptomycin was purchased from Sigma-Aldrich.

PCR

Total RNA was collected from KATOIII, HEK293, and TF-1 cells using a Qiagen RNeasy RNA Isolation Kit. RNA was used as a template to produce cDNA with oligo(dT) and MMLV reverse transcriptase (Promega). Primers targeting orphan GPCRs were designed using PrimerQuest Software (Integrated DNA Technologies, Coralville, IA, USA) and specificity was confirmed using NCBI PrimerBLAST (www.ncbi.nlm.nih.gov/tools/primer-blast) and USC Genome Browser (genome.ucsc.edu). Primers (Supplementary Table 1, see section on supplementary data given at the end of this article) were purchased from Integrated DNA Technologies. PCRs were performed in an Applied Biosystems GeneAmp 2400 using the following protocol: 94 °C for 3 min, 94 °C for 30 s, 56 °C for 45 s, 72 °C for 45 s, GOTO Step 2×25 cycles, and 72 °C for 10 min.

Knockdown of orphan GPCRs

KATOIII cells (~80% confluent) were plated on 12-well, tissue culture-treated plates in IMDM with 20% FBS and no antibiotics and incubated at 37 °C under 5% CO₂ for 48 h. Cells were transfected with vehicle (media with Lipofectamine 2000 alone), or vehicle containing 1, 10, or 100 nM siRNA directed against GPR107 (Yosten *et al.* 2012), GPR146 (Yosten *et al.* 2012), GPR160, or eGFP as a control (Yosten *et al.* 2012), using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Constructs of siRNA were designed and validated by Integrated DNA Technologies (Supplementary Table 1, see section on supplementary data given at the end of this article). Five hours later, the medium was changed to serum-free IMDM in preparation for cell response assays, which were performed 24 h following transfection. Concentrations of siRNA used in these studies were determined in preliminary experiments using standard concentration-response protocols to identify the most effective concentrations (i.e. the lowest concentration of siRNA that consistently led to at least 50–60% knockdown; Yosten *et al.* 2012).

Cell response assays

KATOIII cells were serum-starved overnight (~18 h) and then exposed to vehicle (serum-free IMDM), 1 nM C-peptide in vehicle, or media containing 20% FBS (positive control) for 1 h at 37 °C under 5% CO₂. Cells were lysed and RNA was collected using the Qiagen RNeasy RNA Isolation Kit. Changes in cFos mRNA expression were evaluated by real-time PCR (RT-PCR). Experiments were performed at least three times in triplicate. Human C-peptide was purchased from Phoenix Pharmaceuticals (Burlingame, CA, USA).

Real-time PCR

Cells treated with C-peptide (see above section, Cell response assays) were lysed and RNA was collected using the Qiagen RNeasy RNA Isolation Kit. cDNA was produced using oligo(dT) and MMLV Reverse Transcriptase (Promega). RT-PCRs were performed using Bio-Rad iQ SybrGreen MasterMix, according to the manufacturer's directions, and primers specific for human cFos and GAPDH (Samson *et al.* 2008, Yosten *et al.* 2012) diluted 1:10 in nuclease-free water. RT-PCR was conducted using a Bio-Rad CFX96 Real Time thermocycler according to the following protocol: 95 °C for 5 min, 94 °C for 15 s, 60 °C for 1 min, GOTO 2×30, 72 °C for 10 min, melt curve

65–96 °C; and increment 1 °C for 1 s (Yosten *et al.* 2012). Data were analyzed using the $\Delta\Delta C_t$ method, as described previously (Yosten *et al.* 2012).

Immunofluorescence

KATOIII cells were cultured on chambered coverslips (Fisher Scientific, Pittsburgh, PA, USA) and treated with 1 or 5 nM C-peptide for 1, 5, 10, 15, 20, or 30 min or left unstimulated. Cells were washed in 1×PBS and fixed with 4% paraformaldehyde for 10 min followed by blocking with 10% donkey and human sera (each 10%, Sigma–Aldrich) in 1×PBS with 0.5% BSA for 30 min. Cells were stained with either antibodies to GPR146 (rabbit polyclonal, Antibodies Online, cat #ABIN213365) at 10 µg/ml, insulin receptor β (Abcam, Cambridge, MA, USA, cat #ab983) at 10 µg/ml, and/or C-peptide (mouse monoclonal, Abcam, cat #ab8297) at 2 µg/ml for 1 h followed by donkey anti-mouse conjugated with Dylight 549 or donkey anti-mouse conjugated with Dylight 488 secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA) also for 1 h. In some cases, wheat germ agglutinin (Molecular Probes, Invitrogen) was used to visualize the plasma membrane. All antibodies were diluted in blocking buffer.

Imaging

Immunostained KATOIII cells were visualized on a LSM510 confocal microscope platform (Zeiss, Germany) in the Molecular Microbiology Imaging Facility at Washington University in St Louis. Individual cells or groups of cells were visualized both in single plane images as well as Z stacks captured at 0.2 µm intervals. Differential interference contrast was used in some images to visualize

the structure of the cells. Images were post processed either in ImageJ (NIH, Bethesda, MD, USA) using global brightness/contrast adjustments or in the Huygens Essentials package (SVI, Hilversum, The Netherlands) using deconvolution algorithms. Where appropriate, Z stacks were reconstructed and a ray-traced surface projection of the three-dimensional relationship of the stacked data was produced (Huygens Essentials software package, SVI). Object colocalization was determined by the number of objects with greater than or equal to two coincident voxels also using the Huygens Essentials software package (SVI).

Statistical analysis

Data were analyzed using a Student's *t*-test, ANOVA, or a nonparametric test (Mann–Whitney *U* test), as appropriate (Zar 1984). The tests used for each experiment are reported in the figure legends. Statistical analyses were performed using SPSS Software (IBM).

Results

In order to identify CpepR candidates, we utilized our Deductive Ligand-Receptor Matching Strategy (Yosten *et al.* 2012). First, we identified three human cell lines (KATOIII, HEK293, and TF-1) that responded to treatment with C-peptide with an increase in cFos expression (Fig. 1A). These cell lines were screened for the expression of orphan GPCRs (Sharman *et al.* 2013). Of the 136 orphan GPCRs, 24 were expressed by all the three cell lines (Fig. 1B). In order to generate a 'short list' of candidate receptors from the 24 orphan GPCRs identified in our initial screen, we evaluated those 24 receptors based on homology and predicted function using bioinformatic databases, including NCBI (www.ncbi.nlm.nih.gov/), UniProt (www.uniprot.org/), and the PDSP GPCR database

Figure 1

Identification of GPR146 as a necessary component for C-peptide signaling. (A) The human gastric tumor cell line, KATOIII, the human embryonic kidney cell line, HEK293, and the human erythroleukemia cell line, TF-1, were treated with 1 nM C-peptide for 30 min (HEK293) or 60 min (KATOIII and TF-1), and changes in cFos expression were evaluated using RT-PCR. All the three cell lines responded to C-peptide with a significant increase in cFos expression. Data were analyzed using a Student's *t*-test, **P* < 0.05 and ***P* < 0.01 vs vehicle-treated control cells. (B) KATOIII, HEK293, and TF-1 cells were screened for the expression of orphan GPCRs using PCR. Of the 136 known orphan GPCRs, 24 were expressed by all the three cell lines. Boxes indicate orphan GPCRs expressed by all the three cell lines. Dotted lines indicate edges of separate gels. (C) Transfection of vehicle (Lipofectamine alone, no siRNA),

control siRNA (directed against green fluorescent protein, eGFP), or siRNA directed against GPR107 or GPR160 did not alter C-peptide-induced cFos expression in KATOIII cells. However, knockdown of GPR146 completely abolished the ability of KATOIII cells to respond to exposure with C-peptide. Effective concentrations of siRNA were determined in previous experiments (Yosten *et al.* 2012), and data were excluded if <50% knockdown (as determined by RT-PCR) of the intended target was achieved for each well. (D) KATOIII cells transfected as in (C) were exposed to 20% FBS and analyzed for changes in cFos mRNA expression. Knockdown of GPR146 abrogated FBS-induced cFos expression of those cells. Data for (C) and (D) were analyzed using a Student's *t*-test, **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 vs vehicle-transfected, vehicle-treated cells (C), or vs vehicle-transfected, C-peptide-treated cells (D).

(pdsp.med.unc.edu/). Through this process, we eliminated four receptors that had been deorphanized (GPR31 (NM_005299.2; Guo *et al.* 2011); LGR4 (NM018490; Glinka *et al.* 2011); LPHN1 (NM_014921; Silva *et al.* 2011); and Mas1 (NM_002377; Santos *et al.* 2013)). Eleven receptors were eliminated because they exhibited high homology to structural proteins (GPR125 (NM_145290; Bjarnadottir *et al.* 2004)), amino acid receptors (MRGPRD (NM_198923) and TAAR6 (NM_175067)), chemokine receptors (FY/DARC (NM_002036) and CMKLR1 (NM_004072)), proton sensors (GPR65 (NM_003608)), sphingosine receptors (GPR63 (NM_030784.2)), and sensory or nociceptive proteins (OPN3 (NM_014322); MRGPRX1 (NM_147199; Dong *et al.* 2001); MRGPRX4 (NM_054032; Dong *et al.* 2001); and TAAR8 (NM_053278); Sharman *et al.* 2013). We next compared the known expression profiles of the remaining orphan GPCRs (Regard *et al.* 2008) to the expected sites of expression, given C-peptide's known actions (i.e. kidney, fat, skeletal muscle, and pancreas), and generated a short list that included GPR160 and GPR146 as the top two candidates. These receptors, as well as the neuronostatin receptor, GPR107 (Yosten *et al.* 2012; expressed by KATOIII, HEK293, and TF-1 cells), were knocked down individually in KATOIII cells using siRNA (Yosten *et al.* 2012). Vehicle-treated (Lipofectamine 2000, no siRNA) or control siRNA (eGFP)-treated cells responded to treatment with C-peptide with a significant increase in cFos expression (Fig. 1C). Knockdown of either GPR107 or GPR160 did not affect C-peptide-induced cFos expression; however, knockdown of GPR146 completely blocked the ability of C-peptide to stimulate cFos expression in those cells (Fig. 1C). Knockdown of GPR146 also abrogated FBS-induced cFos expression in KATOIII cells (Fig. 1D).

KATOIII cells were stained using an antibody directed against the intercellular domain of GPR146. GPR146 could be detected throughout the cell membranes of KATOIII cells (Fig. 2A). Exposure to 1 nM C-peptide appeared to induce internalization of GPR146 over the course of 30 min (Fig. 2B). Furthermore, cells that were exposed to

C-peptide exhibited punctate regions of colocalization between C-peptide and GPR146 (Fig. 2C) that increased in number as a function of incubation time (Fig. 2D). Studies from several groups have indicated that C-peptide- and insulin-initiated signaling cascades functionally interact (Wahren *et al.* 2012). However, KATOIII cells exposed to C-peptide alone exhibited only infrequent, punctate colocalization between GPR146 and the insulin receptor (Fig. 2E).

Discussion

GPR146 is expressed in three C-peptide-responsive cells lines and appears to be essential for C-peptide-induced cFos expression in KATOIII cells. Furthermore, knock-down of GPR146 reduced FBS-induced cFos expression, indicating that GPR146 is essential for transmitting the signal of a factor present in FBS, such as C-peptide. Stimulation with C-peptide induced internalization of GPR146, and punctate colocalization was observed between C-peptide and GPR146 on KATOIII cell membranes. These initial observations indicate that GPR146 may be part of the C-peptide signalosome and is a potential candidate receptor for proinsulin C-peptide. It should be noted that while a functional interaction between C-peptide and GPR146 exists, we have not demonstrated a direct physical interaction between the two proteins, except by co-immunofluorescence. For this reason, confirmatory experiments must be performed in order to ascertain the specific role of GPR146 in transmitting the C-peptide signal. These necessary confirmatory experiments must include radioligand binding studies, co-immunoprecipitation, and *in vivo* functional assays. Although our initial observations do not conclusively demonstrate that GPR146 is a CpepR, the identification of GPR146 as essential for C-peptide-dependent signaling provides a platform for elucidation of the C-peptide signaling complex.

GPR146 may contribute to C-peptide signaling in several ways. GPR146 may act as a co-receptor of the

Figure 2

Staining of GPR146 on KATOIII cell membranes. (A) Three-dimensional reconstruction of a KATOIII cell stained with a polyclonal antibody directed against GPR146 (red) and with wheat germ agglutinin (WGA, blue). (B) KATOIII cells were exposed to C-peptide for 1, 5, 10, 15, 20, or 30 min and stained for GPR146 (red). Exposure to C-peptide appeared to induce internalization of GPR146 from the cell membrane (WGA, blue). (C) Cells were plated on glass chamber slides and exposed to 5 nM C-peptide for 30 min. After fixation, cells were stained for

GPR146 (green) and C-peptide (red), and punctate co-localizations were observed. The number of co-localized objects increased as a function of time (D). (E) KATOIII cells were exposed to C-peptide for 30 min, and cells were stained for GPR146 (green) and insulin receptor β (red). Only infrequent, punctate co-localized objects were observed. Black arrowheads indicate co-localized objects in (C) and (E). Data in (D) were analyzed by ANOVA with Scheffe's multiple comparison, $**P < 0.01$.

CpepR(s), as part of a C-peptide signaling complex or signalosome, or could act as a CpepR itself. Alternatively, GPR146 could be a promiscuous GPCR (activated by multiple ligands) or may interact with modulating proteins, such as RAMPs (Kadmiel *et al.* 2012), thus modifying receptor specificity. However, knockdown of GPR146 did not alter the responsiveness of KATOIII cells to neuronostatin (Yosten *et al.* 2012), thus suggesting specificity of GPR146 for C-peptide.

Little is known about GPR146. GPR146 was originally identified as a 150-amino acid, partial sequence, termed PGR8 (Gloriam *et al.* 2004). In humans, the gene for GPR146, now known to be a 333-amino acid protein, is encoded by a single exon located at 7p22.3 (Gloriam *et al.* 2004). Orthologs of GPR146 have been identified in monkey, mouse, rat, chicken, zebrafish, and fugu (Gloriam *et al.* 2004). The rodent orthologs of GPR146 share 74% homology with the human sequence (Gloriam *et al.* 2004). Interestingly, human and rodent C-peptide sequences only exhibit ~70% homology. Although over 150 single-nucleotide polymorphisms have been identified in the human *GPR146* gene (Sherry *et al.* 2001), no clinical associations have been reported thus far.

Several lines of evidence suggest that C-peptide- and insulin-initiated signaling cascades functionally interact (Luppi *et al.* 2011, Wahren *et al.* 2012). Some studies have reported that the insulin receptor interacts with a GPCR coupled to $G_{\alpha q}$ (Kisfalvi *et al.* 2007), and, interestingly, the actions of C-peptide are likely mediated by $G_{\alpha q}$ (Wahren *et al.* 2012). These data suggest that the insulin receptor and the CpepR might physically interact. However, we observed only infrequent colocalization of the insulin receptor and GPR146 following stimulation with C-peptide alone. Co-stimulation with C-peptide and insulin may be necessary to induce oligomerization of insulin receptor and GPR146, or GPR146 may interact with another membrane protein to transmit the C-peptide signal. Regardless, elucidation of the C-peptide signalosome is crucial for the development of C-peptide-based therapeutics for the treatment of diabetes-associated microvascular dysfunction. Although the studies presented here do not unequivocally demonstrate that GPR146 is a CpepR, they are an important first step in the elucidation of the C-peptide signaling complex.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JOE-13-0203>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

G R K planned and performed the experiments, contributed to the discussion of the results, and aided in the preparation of the manuscript. L J R planned and performed the experiments. W K S planned the experiments, contributed to the discussion of the results, and edited the manuscript. G L C Y planned and performed the experiments, contributed to the discussion of the results, and prepared the manuscript.

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References

- Bjarnadottir TK, Fredriksson R, Hoglund PJ, Gloriam DE, Lagerstrom MC & Schioth HB 2004 The human and mouse repertoire of the adhesion family of G-protein-coupled receptors. *Genomics* **84** 23–33. (doi:10.1016/j.ygeno.2003.12.004)
- Bo S, Gentile L, Castiglione A, Prandi V, Canil S, Ghigo E & Ciccone G 2012 C-peptide and the risk for incident complications and mortality in type 2 diabetic patients: a retrospective cohort study after a 14-year follow-up. *European Journal of Endocrinology* **167** 173–180. (doi:10.1530/EJE-12-0085)
- Dong X, Han S, Zyllka MJ, Simon MI & Anderson DJ 2001 A diverse family of GPCRs expressed in specific subsets of nociceptive sensory neurons. *Cell* **106** 619–632. (doi:10.1016/S0092-8674(01)00483-4)
- Glinka A, Dolde C, Kirsch N, Huang YL, Kazanskaya O, Ingelfinger D, Boutros M, Cruciat CM & Niehrs C 2011 LGR4 and LGR5 are R-spondin receptors mediating Wnt/ β -catenin and Wnt/PCP signaling. *EMBO Reports* **12** 1055–1061. (doi:10.1038/embor.2011.175)
- Gloriam DE, Schioth HB & Fredriksson R 2004 Nine new human Rhodopsin family G-protein coupled receptors: identification, sequence characterisation and evolutionary relationship. *Biochimica et Biophysica Acta* **1722** 235–246. (doi:10.1016/j.bbagen.2004.12.001)
- Guo Y, Zhang W, Giroux C, Cai Y, Ekambaram P, Dilly AK, Hsu A, Zhou S, Maddipati KR, Liu J *et al.* 2011 Identification of the orphan G protein-coupled receptor GPR31 as a receptor for 12-(S)-hydroxyeicosatetraenoic acid. *Journal of Biological Chemistry* **286** 33832–33840. (doi:10.1074/jbc.M110.216564)
- Ido Y, Vindigni A, Chang K, Stramm L, Chance R, Heath WF, DiMarchi RD, Di Cera E & Williamson JR 1997 Prevention of vascular and neural dysfunction in diabetic rats by C-peptide. *Science* **277** 563–566. (doi:10.1126/science.277.5325.563)
- Kadmiel M, Fritz-Six KL & Caron KM 2012 Understanding RAMPs through genetically engineered mouse models. *Advances in Experimental Medicine and Biology* **744** 49–60. (doi:10.1007/978-1-464-2364-5_5)
- Kisfalvi K, Rey O, Young SH, Sinnett-Smith J & Rozengurt E 2007 Insulin potentiates Ca^{2+} signaling and phosphatidylinositol 4,5-bisphosphate hydrolysis induced by Gq protein-coupled receptor agonists through

- an mTOR-dependent pathway. *Endocrinology* **148** 3246–3257. (doi:10.1210/en.2006-1711)
- Luppi P, Cifarelli V & Wahren J 2011 C-peptide and long-term complications of diabetes. *Pediatric Diabetes* **12** 276–292. (doi:10.1111/j.1399-5448.2010.00729.x)
- Regard JB, Sato IT & Coughlin SR 2008 Anatomical profiling of G protein-coupled receptor expression. *Cell* **135** 561–571. (doi:10.1016/j.cell.2008.08.040)
- Rigler R, Pramanik A, Jonasson P, Kratz G, Jansson OT, Nygren P, Stahl S, Ekberg K, Johansson B, Uhlen S *et al.* 1999 Specific binding of proinsulin C-peptide to human cell membranes. *PNAS* **96** 13318–13323. (doi:10.1073/pnas.96.23.13318)
- Santos RA, Ferreira AJ, Verano-Braga T & Bader M 2013 Angiotensin-converting enzyme 2, angiotensin-(1–7) and Mas: new players of the renin–angiotensin system. *Journal of Endocrinology* **216** R1–R17. (doi:10.1530/JOE-12-0341)
- Samson WK, Zhang JV, Avsian-Kretchmer O, Cui K, Yosten GLC, Klein C, Lyu RM, Wang YX, Chen XQ, Yang J *et al.* 2008 Neuronostatin encoded by the somatostatin gene regulates neuronal, cardiovascular, and metabolic functions. *Journal of Biological Chemistry* **283** 31949–31959. (doi:10.1074/jbc.M804784200)
- Sharman JL, Benson HE, Pawson AJ, Lukito V, Mpamhanga CP, Bombail V, Davenport AP, Peters JA, Spedding M, Harmar AJ *et al.* 2013 IUPHAR-DB: updated database content and new features. *Nucleic Acids Research* **41** D1082–D1088. (doi:10.1093/nar/gks960)
- Sherry ST, Ward MH, Kholodov M, Baker J, Phan L, Smigielski EM & Sirotkin K 2001 dbSNP: the NCBI database of genetic variation. *Nucleic Acids Research* **29** 308–311. (doi:10.1093/nar/29.1.308)
- Silva JP, Lelianaova VG, Ermolyuk YS, Vysokov N, Hitchen PG, Bernin-ghausen O, Rahman MA, Zangrandi A, Fidalgo S, Tonevisky AG *et al.* 2011 Latrophilin 1 and its endogenous ligand Lasso/teneurin-2 form a high-affinity transsynaptic receptor pair with signaling capabilities. *PNAS* **108** 12113–12118. (doi:10.1073/pnas.1019434108)
- Wahren J, Kallas A & Sima AA 2012 The clinical potential of C-peptide replacement in type 1 diabetes. *Diabetes* **61** 761–772. (doi:10.2337/db11-1423)
- Yosten GLC, Redlinger LJ & Samson WK 2012 Evidence for an interaction of neuronostatin with the orphan G protein-coupled receptor, GPR107. *American Journal of Physiology* **303** R941–R949. (doi:10.1152/ajpregu.00336.2012)
- Zar JH 1984 *In Biostatistical Analysis*, 2nd edn. Englewood Cliffs, NJ: Prentice Hall.

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