Implicación de las proteínas accesorias de los receptores de melanocortinas (MRAPs) en la respuesta al estrés en peces

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Informa que la Tesis Doctoral titulada IMPLICACIÓN DE LAS PROTEÍNAS ACCESORIAS DE LOS RECEPTORES DE MELANOCORTINAS (MRAPS) EN LA RESPUESTA AL ESTRÉS EN PECES ha sido realizada por Don Raúl Antonio Cortés Barrera en el Instituto de Acuicultura de Torre de la Sal (IATS) bajo su dirección y que, una vez revisado y comprobado el trabajo, considera que reúne los requisitos necesarios para la obtención del grado de Doctor, por lo que autoriza su presentación.

Para que así conste

José Miguel Cerdá Reverter.

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Índice

Abreviaturas frecuentes

-/-	Referencia a un organismo Knock Out para algún gen
Ac	Núcleo Arcuato
ACTH	Hormona adenocorticotropa
AMPc	Adenosín monofosfato cíclico
ANOVA	Análisis de la varianza
AGRP	Proteína relacionada con agouti
agrp	Proteína relacionada con agouti (gen)
ARC	Núcleo arqueado del hipotálamo
ASIP	Proteína de señalización agouti
asip	Proteína de señalización agouti (gen)
atrn	Atractina (gen)
β-actin	β-actina (gen)
bp	Pares de bases (inglés)
CART	Transcrito regulado por cocaína y anfetaminas
cart	Transcrito regulado por cocaína y anfetaminas (gen)
CCK	Colecistoquinina
cDNA	ADN complementario
CHO	Células de ovario chino
COS-7	Células de riñón de mono verde africano
CREB	Elementos de respuesta a AMPc
CRF	Factor liberador de corticotropina
CSIC	Consejo Superior de Investigaciones Científicas
CTRL	Control
DIG	Digoxigenina
DMSO	Dimetilsulfóxido
DNA	Ácido desoxirribonucleico
ECL	Extracelular
EDTA	Ácido etilendiaminotetraacético

EF-1α (efα)	Factor de elongación 1α (gen)
ER	Retículo endoplásmico
FGD	Deficiencia familiar de glucorticoides
FLU	Fluoresceína
G	Proteína de guanidinio
gapdh	Gliceraldehído-3-fosfato deshidrogenasa (gen)
GFP	Proteína fluorescente verde
goat	Ghrelina-O-acil transferasa (gen)
GR	Receptor de glucocorticoide
gr	Receptor de glucocorticoide (gen)
grelin	Grelina (gen)
Hd	Hipotálamo dorsal
HEK-293	Línea celular de células embrionarias de riñón humano
HPA	Hipotálamo-pituitario-adrenal
IATS	Instituto de Acuicultura de Torre la Sal
ICK	Nudo de cisteína
ICL	Intracelular
ICV	Intracerebroventricular
ISH	Hibridación *in situ*
LPH	Lipotropina
MCR	Receptor de melanocortinas
mgrn1	Mahogunina o Mahogunin RING-finger (gen)
mr	Receptor de mineralocorticoides (gen)
MRAP	Proteína accesoria del receptor de melanocortinas
mrap	Proteína accesoria del receptor de melanocortinas (gen)
mRNA	RNA mensajero
MS-222	Metasulfonato de tricaína
MSH	Hormona estimuladora de los melanocitos
mtif	Factor de transcripción microftalmia (gen)
MTII	Melanotan II

NEFA	Ácidos grasos no esterificados
NPO	Núcleo preóptico
NPY	Neuropéptido Y
npy	Neuropéptido Y (gen)
NTS	Núcleo *tractus solitarius*
ORF	Marco de lectura abierta
PAF	Paraformaldehido
PAM	Monooxigenasa amidante de peptidil-glicina
PB	Tampón fosfato
PBS	Tampón fosfato salino
PC1	Prohormona convertasa 1
pc1/3	Prohormona convertasa 1 (gen)
PC2	Prohormona convertasa 2
pc2	Prohormona convertasa 2 (gen)
PCR	Reacción en cadena de la polimerasa
pepck	Fosfoenolpiruvato carboxiquinasa (gen)
PFA	Paraformaldehido
PI	*pars intermedia*
PKA	Fosfoquinasa A
POC	Variante del POMC identificada en las lampreas
POM	Variante del POMC identificada en las lampreas
POMC	Propopiomelanocortina
pomc	Propopiomelanocortina (gen)
PPARα	Receptores activados por proliferadores peroxisómicos
Ppa	Área preóptica anterior
PVN	Núcleo paraventricular
qPCR	Reacción en cadena de la polimerasa cuantitativa en tiempo real
RACE	"Rapid Amplification cDNA Ends" (técnica genómica)
RNA	Ácido Ribonucleico
rPD	*pars distalis rostral*

RP-HPLC	Cromatografía liquida de alta eficiencia
rpl13	Proteína ribosómica 60S L13 (gen)
RT	Temperatura ambiente
RT-PCR	Reaccion de Retrotranscripción seguida de una PCR
SEM	Error estándar de la media
SNC	Sistema nervioso central
SNS	Sistema nervioso simpático
SNP	Polimorfismo de único nucleótido
T3	Triyodotironina
t3	Triyodotironina (gen)
T4	Tiroxina
tat	Tirosina aminotransferasa (gen)
TM	Transmembrana
TESPA	3-aminopropiltrietoxisilano
TU	"Tübingen", cepa de peces cebra
tub	Alfa tubulina (gen)
tyr	Tirosinasa (gen)
WT	"Wild-Type", peces cebra con fenotipo salvaje
1R	Primer evento de duplicación génica en los cordados
2R	Segundo evento de duplicación génica en los cordados
3R	Tercer evento de duplicación génica en Teleósteos
18S	Sub unidad ribosomal 18 (gen)

Preámbulo

El sistema de melanocortinas es, sin duda, uno de los sistemas hormonales más complejos que existen en la naturaleza. Sus diversas implicaciones en procesos tales como la pigmentación, la respuesta inmunológica, el estrés, el control de la ingesta de alimentos, con la concomitante importancia de la incidencia de la obesidad en la salud de las poblaciones occidentales, gravitan aún más en su importancia y la necesidad de profundización en su conocimiento.

La expansión en la investigación de este sistema hormonal despegó con la caracterización de los receptores de melanocortinas al inicio de los años 90, en el laboratorio del Dr. Roger D. Cone (Vollum Institute for Advanced Biomedical Research, Oregon Health Sciences University, Portland). Su asociación con el desarrollo de la obesidad todavía propulsó más la investigación dada la repercusión de esta pandemia del siglo XXI en los sistemas de salud pública. Hoy en día las mutaciones en el receptor 4 de melanocortinas (MC4R), son la principal causa de obesidad monogénica en la población humana. Sin embargo, el sistema de melanocortinas también está implicado en los mecanismos de respuesta al estrés. De hecho, el eje hipotálamo-hipófisis-adrenal es crítico en la elaboración de la respuesta al estrés regulando la síntesis y secreción de glucocorticoides adrenales (cortisol o corticosterona dependiendo de las especies).

Así el sistema de melanocortinas ofrece un puente de unión entre los mecanismos de respuesta al estrés y los mecanismos que controlan el balance energético, es decir un puente para la integración de información ambiental/corporal sobre los mecanismos que gobiernan la disponibilidad de energía metabólica.

La investigación en el sistema de melanocortinas sufrió un segundo auge con el descubrimiento de las proteínas accesorias que interaccionan con los receptores para dotarlos de actividad funcional, como en el caso del MC2R, o bien para modificar su actividad. Su descubrimiento dio respuesta a una grave enfermedad conocida por su resistencia a la hormona adrenocorticotropa (ACTH) y denominada como “deficiencia familiar de glucocorticoides” (FGD). En ella, los pacientes presentan altos niveles plasmáticos de ACTH, pero inexistentes de glucocorticoides. La interacción de estas proteínas con los receptores, agregan otro punto más de complejidad al sistema hormonal pero también otro punto más de regulación de la actividad del sistema.

El uso del pez cebra, presenta muchas ventajas en investigación debido a las facilidades genómicas disponibles hoy en día, su trasparencia durante los primeros días de desarrollo, la facilidad de mantenimiento y reproducción en instalaciones adecuadas. Además, la distancia genética que separa al pez cebra de nuestra especie es mucho menor que aquella que la separa de otros organismos modelo como la mosca del vinagre, “*Drosophila meanogaster*”, o el nemátodo “*Caenorhabditis elegans*”. Mi trabajo también se ha beneficiado del uso del pez cebra en investigación, el cual ha facilitado la entrega de respuestas a algunas incógnitas que presentaba el sistema de melanocortinas.

La presente tesis doctoral ha dado origen a seis publicaciones de arbitraje internacional, las cuales conforman los seis capítulos de esta tesis. Además, ha dado lugar a un capítulo del libro “Saberes para Chile” (ISBN: 9788416611348 pp221-246, 2015) y diez presentaciones en congresos nacionales e internacionales, en los cuales se ha dado a conocer el rol de las MRAPs en el sistema de melanocortinas.

Resumen

Resumen

El sistema de melanocortinas es un sistema hormonal, autocrino, paracrino y endocrino compuesto por agonistas (hormonas), receptores, y paradójicamente, por antagonistas del mismo sistema. A estos componentes del sistema se le ha adicionado recientemente sus proteínas accesorias, las cuales conforman un nuevo punto de regulación conocido. La actividad de este sistema hormonal está relacionada con diversas funciones fisiológicas que incluyen principalmente: el control de la pigmentación, la regulación del balance energético y la regulación de la respuesta al estrés.

Los experimentos realizados en el laboratorio donde he desarrollado mi tesis doctoral, fueron pioneros en la descripción de las proteínas accesorias de los receptores de melanocortinas (MRAPs) en peces. Durante la experimentación se demostró la existencia de dos proteínas accesorias en el genoma del pez cebra (MRAP1 y MRAP2), la segunda de las cuales está duplicada en esta especie (MRAP2a y MRAP2b), modelo que ha sido utilizado en la experimentación presentada. La MRAP1 interacciona con el receptor 2 de melanocortinas (MC2R) para permitir su expresión funcional, pero la función de las MRAP2s era desconocida y acotar su función ha sido el principal objetivo de la tesis doctoral. Los resultados presentados demuestran que el MC2R de la lubina también requiere de la interacción con MRAP1 para poder responder a la hormona adrenocorticotropa (ACTH), adquiriendo así su expresión funcional. Además, demostramos que los glucocorticoides son capaces de inhibir la expresión del MC2R en las glándulas interrenales, lo que supone un mecanismo de retroalimentación con posible relevancia en la adaptación al estrés.

Por su parte las MRAP2 no tienen efectos sobre la actividad del MC2R, pero como demostramos en la tesis doctoral, la MRAP2a, pero no la MRPA2b, es capaz de convertir al MC4R, un receptor canónico de la hormona estimuladora de los melanocitos (MSH) expresado en el cerebro, en un receptor de ACTH. La ACTH se libera desde la hipófisis en respuesta a estímulos hipotalámicos que integran información relativa a condiciones estresantes. Mientras que el MC4R está fundamentalmente implicado en el control central de la regulación de la ingesta. Por tanto, la modificación del perfil farmacológico del MC4R tras interacción con MRAP2a otorga un mecanismo molecular para la integración de la información del estrés sobre los circuitos centrales que controlan la ingesta. Es decir, otorga una base molecular directa para las relaciones estrés-ingesta. Este cambio del perfil farmacológico no se observa tras la interacción con el MC1R ni con el MC5R, si bien la interacción MC1R-MRAP2a provoca una disminución de la sensibilidad del receptor a la MSH.

Finalmente, hemos caracterizado la respuesta central y periférica del pez cebra al estrés agudo evaluando los niveles de expresión de diversos componentes del sistema de melanocortinas y demostrando la sensibilidad de este sistema a los elementos estresantes.

Resum

El sistema de melanocortines és un sistema hormonal, autocrí, paracrí i endocrí compost per agonistes (hormones), receptors i paradóxicament, per antagonistes del mateix sistema. A aquests components del sistema se'ls ha addicionat recentement les seues proteïnes accesòries, les quals conformen un noa punt de regulació conegut. L'activitat d'aquest sistema hormonal està relacionada amb diverses funcions fisiològiques que inclouen principalment: el control de la pigmentació, la regulació del balanç energètic i la regulació de la resposta a l'estrès.

Els experiments realitzats al laboratori on he desenvolupat la meua tesi doctoral, van ser pioners en la descripció de les proteïnes accesòries dels receptors de melanocortines (MRAPs) en peixos. Durant l'experimentació se va demostrar l'existència de dues proteïnes accesòries en el genoma del peix zebra (MRAP1 y MRAP2), la segona de les quals està duplicada en aquesta espècie (MRAP2a y MRAP2b), model que ha sigut utilitzat en l'experimentació presentada. La MRAP1 interacciona amb el receptor 2 de melanocortines (MC2R) per a permetre la seua expressió funcional, però la funció de les MRAP2s era desconeguda i acotar la seua funció ha sigut el principal objectiu de la tesi doctoral. Els resultats presentats demostren que el MC2R del llobarro també requeireix de la interacció amb MRAP1 per a poder respondre a la hormona adrenocorticotropa (ACTH), adquirint així la seua expressió funcional. A més a més, demostrem que els glucocorticoides son capaços d'inhibir l'expressió del MC2R a les glàndules interrenals, la qual cosa suposa un mecanisme de retroalimentació amb possible relevància en l'adaptació a l'estrès.

Per la seua part les MRAP2 no tenen efectes sobre l'activitat del MC2R, però com demostrem a la tesi doctoral, la MRAP2a, però no la MRPA2b, és capaç de convertir al MC4R, un receptor canònic de la hormona estimuladora dels melanocitos (MSH) expressat en el cervell, en un receptor de ACTH. La ACTH es allibera des de la hipòfisi com a resposta a estímuls hipotalàmics que integren informació relativa a condicions estressants. Mentres que el MC4R està fonamentalment implicat en el control central de la regulació de la ingesta. Per tant, la modificació del perfil farmacològic del MC4R tras interacció amb MRAP2a atorga un mecanisme molecular per la integració de la informació de l'estrès sobre els circuits centrals que controlen la ingesta. És a dir, atorga una base molecular directa per a les relacions estrès-ingesta. Aquest canvi del perfil farmacològic no s'observa tras la interacció amb el MC1R ni amb el MC5R, encara que la interacció MC1R-MRAP2a provoca una disminució de la sensibilitat del receptor a la MSH.

Finalment, hem caracteritzat la resposta central i perifèrica del peix zebra a l'estrès agut avaluant els nivels d'expressió de diversos components del sistema de melanocortines i demostrant la sensibilitat d'aquest sistema als elements estressants.

Abstract

Abstract

The melanocortin system is an endocrine, paracrine and autocrine hormonal system composed by agonist (hormones), receptors, and atypically endogenous antagonists. Recently, the so-called melanocortin receptor-accessory proteins (MRAPs) have been integrated as a part of the system. These proteins conform a new regulatory point of the system. The activity of the hormonal system is related to several physiological functions including control of pigment patters as well as the regulation of energy balance and the stress response.

Experiments done in the lab in which I have run my PhD studies were pioneer in the description of fish MRAPs. It was demonstrated that the genome of zebrafish includes two accessory proteins (MRAP1 and MRAP2) and the second one is duplicated into MRAP2a and MRAP2b forms. MRAP1 interacts with the melanocortin 2 receptor (MC2R) allowing its functional expression but the function of MRAP2 was unknown and delimiting its function has been the main objective of this doctoral thesis. Results demonstrated that sea bass MC2R also requires the interaction with MRAP1 in order to be activated by the adrenocorticotropic hormone (ACTH), reaching therefore its functional expression. We also demonstrated that glucocorticoids are able to inhibit MC2R expression in the interrenal tissue suggesting the existence of a negative feedback with potential relevance in the stress adaptation.

The MPRA2 has no effects on MC2R activity, but as we demonstrate, the MRAP2a, but not MRAP2b, in able to transform the MC4R, a canonical melanocyte- stimulating hormone (MSH) receptor expressed in the brain, into an ACTH receptor. ACTH is released form the pituitary in response to stressful stimuli integrated in the hypothalamus. On the other hand, MC4R is mainly involved in the central control of food intake and energy balance. Therefore, the MC4R pharmacological profile after interaction with MRAP2a provides a molecular mechanism for the integration of stress information into the central circuits controlling energy balance. That means, molecular basis for the stress-food intake interaction. This pharmacological modification seems to be specific for MC4R since the interaction of MRAP2a with MC5R or MC1R does not provide ACTH sensitivity to the receptor. However, MC1R-MRAP2a interaction induces a decrease in the sensibility of the receptor to MSH. Finally, we have characterized the central and peripheral response of zebrafish to acute stress by evaluating the expression levels of several components of the melanocortin system thus demonstrating the sensibility of this system to stressful agents.

Introducción General

1. Proopiomelanocortina (POMC)

Los trabajos que integran la presente tesis doctoral se centran en el estudio del sistema de melanocortinas de los peces. Este conjunto de pequeñas hormonas peptídicas se caracteriza por mostrar actividad melanotropa y/o corticotropa. Por actividad melanotropa se entiende la estimulación de la síntesis de melanina, mientras que por actividad corticotropa entendemos la estimulación de la actividad del córtex adrenal induciendo la síntesis y liberación de glucocorticoides (Bernier *et al.* 2009). Todas ellas derivan de un precursor común llamado proopiomelanocortina (POMC) que se sintetiza fundamentalmente en la hipófisis, tanto en las células melanotropas de la *pars intermedia* como en las células corticotropas de la *pars distalis* (Bernier and Peter 2001; Cerdá-Reverter and Canosa 2009).

El POMC puede organizarse desde el punto de vista estructural en tres dominios principales y cada uno de ellos integra una hormona estimuladora de los melanocitos (MSH), que se caracterizan por la presencia del tetrapéptido "HFRW" (Castro and Morrison 1997). El dominio amino (N-) terminal integra la γ-MSH. El dominio central está ocupado por la hormona adrenocorticotropa (ACTH) cuyos primeros 13 aminoácidos corresponden a la α-MSH. El domino carboxilo (C-terminal) contiene a la β-lipotrofina, que a su vez integra a la β-MSH y, en su extremo terminal, un péptido del sistema opioide denominado β-endorfina.

La liberación de unos u otros péptidos hormonales, depende del tipo de procesado al que será sometido el precursor en el tejido de expresión. En las células corticotropas, la acción de la proconvertasa 1 (PC1/3) induce la liberación de ACTH y β-lipotrofina, mientras que en las células melanotropas la acción combinada de PC1/3 y PC2 provoca la fractura de la prohormona en α-MSH y β-endorfina (Coll and Tung 2009; Millington 2007; Morash *et al.* 2009; Seidah and Chretien 1999). Por tanto, mediante este procesado diferencial dependiente de la batería de convertasas celulares se pueden obtener diferentes péptidos específicos desde un precursor común.

Las MSHs sufren además un procesado postranscripcional que genera diferentes isoformas en base a su grado de acetilación. La primera modificación que sufren conlleva un proceso de amidación, en virtud de la acción de la monooxigenasa amidante de peptidil-glicina (PAM), característica de la mayor parte de péptidos bioactivos (Kumar *et al.* 2016). Seguidamente, las MSHs son acetiladas en su región N-terminal, un proceso que aumenta la estabilidad de las hormonas protegiéndolas contra la acción de las aminopeptidasas, incrementando su vida media. Por el contrario, la acetilación de la β-endorfina abole completamente su actividad opioide (Cawley *et al.* 2016). El grado de acetilación es

diferente pudiendo encontrar formas no acetiladas, monoacetiladas o diacetiladas que exhiben una farmacología sensiblemente diferente.

El *pomc* se expresa también en el sistema nervioso central (SNC), en dos poblaciones neuronales concretas; el núcleo arqueado del hipotálamo (ARC) y el núcleo del tracto solitario (NTS) del cerebro posterior (Bagnol *et al.* 1999). En el SNC el POMC se procesa fundamentalmente hacia la síntesis de α-MSH y β-endorfina por acción de ambas convertasas (Castro and Morrison 1997).

La presencia de ACTH en el SNC es bastante controvertida. Diversos estudios han caracterizado la presencia de inmunoreactividad a la ACTH en fracciones obtenidas mediante cromatografía liquida de alta eficiencia (RP-HPLC) de extractos hipotalámicos (Smith and Funder 1988). Los experimentos que han utilizado anticuerpos específicos contra la ACTH también sugieren la presencia de la hormona en las neuronas hipotalámicas del ratón. De hecho, las células de ACTH marcadas inmunoreactivamente son las mismas que las marcadas con anticuerpos específicos contra la α-MSH, ya que ambas hormonas comparten los mismos epítopos, pero la inmunoreactividad a ACTH se localiza principalmente en los cuerpos neuronales mientras que la inmunoreactividad contra la α-MSH se localiza en las terminales nerviosas (Hentges *et al.* 2009). Las proyecciones α-MSH de las neuronas POMC alcanzan las principales áreas del SNC, incluyendo importantes áreas telencefálicas implicadas en la regulación del comportamiento, así como áreas hipotalámicas implicadas en el control neuroendocrino (Bagnol *et al.* 1999).

2. Los receptores de melanocortinas

Aunque la ACTH (Dixon and Li 1956; Li *et al.* 1955) y la α-MSH (Dhariwal *et al.* 1966) fueron purificadas hace aproximadamente 60 años y el POMC caracterizado mediante clonación molecular hace aproximadamente 40 años (Nakanishi *et al.* 1976), sus receptores no fueron descubiertos hasta inicios de los años 90 (Mountjoy *et al.* 1992). Actualmente, se han caracterizado cinco receptores de melanocortinas (MC1R-MC5R) en tetrápodos, aunque su número varia en vertebrados no tetrápodos.

Los receptores de melanocortinas pertenecen a la subfamilia A13 de los receptores similares a la rodopsina (Isberg *et al.* 2014), pertenecientes a la familia de receptores de 7 dominios transmembrana (TM1-TM7) acoplados a la proteína de guanidinio (G). Todos ellos se diferencian por su perfil farmacológico y dominios de expresión. El MC2R, también conocido como receptor de ACTH, es el único que no une MSHs. El resto de los receptores unen las diversas MSHs con diferente afinidad, pero son prácticamente insensibles a la ACTH, excepto el MC1R (ver más adelante).

Tabla I. Familias de proteínas Gα pertenecientes a las proteínas G heterotriméricas.

Familia	Tipo de proteína G	Efectos
I		
	G_s	Activación de adenilato ciclasa y canales de Ca^{2+}
	G_{olf}	Activación de adenilato ciclasa en neuronas olfativas
II		
	G_I	Inhibición de adenilato ciclasa y activación de canales de K^+
	G_o	Activación de canales de K^+, inactiva canales de Ca^{2+} y activa fosfolipasa C-β
	G_t	Activa fosfodiesterasas de GMP-cíclico en fotorreceptores de bastón en vertebrados
III		
	G_q	Activación de fosfolipasa C-β

(modificado de Alberts *et al.* 2010; Taleisnik 2006).

Las proteínas G de los GPCR son heterotriméricas, compuestas por tres sub unidades (α, β y γ), y su activación provoca una disociación de la subunidad α para posteriormente activar a la adenil ciclasa, encargada de producir el adenosín monofosfato cíclico (AMPc) o activar la fosfolipasa C-β, aumentando los niveles de Ca^{2+} intracelular (Karp *et al.* 2005; Lodish *et al.* 2005). Las proteínas G se agrupan en tres familias diferentes (Tabla I), de las cuales dos interactúan con los MCRs (Chen *et al.* 1995): las proteínas Gs (con todos los MCRs) y las proteínas Gq (MC1R, Van Raamsdonk *et al.* (2009); MC3R, Konda *et al.* (1994) y MC4R, Li *et al.* (2016)).

2.1.MC1R

El MC1R se expresa fundamentalmente en el folículo piloso y ocupa el locus genético denominado "*extensión*" (*E*) (Robbins *et al.* 1993). Su acción es crítica en el control de la melanogénesis, y por extensión, en el control del patrón de pigmentación de los vertebrados (revisado por García-Borrón *et al.* 2014; García-Borrón *et al.* 2005). Es el que mayor afinidad presenta por la α-MSH, por ese motivo en ocasiones se le denomina como receptor de α-MSH, pero en humanos, y en menor extensión en ratones, se acopla a la vía del AMPc de forma independiente a la activación por agonistas, es decir se activa de forma constitutiva (revisado por Herraiz *et al.* 2018).

La activación del receptor provoca la activación de fosfoquinasa A (PKA) y la fosforilación dependiente de PKA de la proteína CREB (proteína de unión a los elementos de repuesta al AMPc), que finalmente deriva en la activación de un gen "master" de la melanogénesis denominado factor de transcripción asociado con microftalmia (*mitf*). *mitf* regula al alza la expresión de genes clave y limitantes de la melanogénesis como la tirosinasa (*tyr*) y las proteínas relacionadas con la tirosinasa 1 y 2 (dopacromo tautomerasa) que dirigen la síntesis de eumelanina, un pigmento de color negro o marrón oscuro (Bagnara and Matsumoto 2007; Garcia-Borron *et al.* 2014; Lin and Fisher 2007).

El MC1R se une también con cierta afinidad la ACTH, pero nunca con igual afinidad al MC2R. De hecho, uno de los diagnósticos diferenciales de la enfermedad de Cushing, provocada por elevados niveles de ACTH derivados de un adenoma hipofisiario, es la hiperpigmentación debida a la activación del MC1R por la ACTH (Chan *et al.* 2011).

2.2.MC2R

El MC2R es probablemente el receptor más peculiar de la familia, se expresa casi exclusivamente en la zona reticulada de la corteza o "córtex" adrenal donde se sintetizan prioritariamente los glucocorticoides (Charmandari *et al.* 2005; Faught *et al.* 2016). Forma, por tanto, una parte importante del eje hipotálamo-pituitario-adrenal (HPA) donde se regula una porción importante de la respuesta al estrés en vertebrados (Barton 2002; Mommsen *et al.* 1999; Pankhurst 2011; Wendelaar Bonga 1997). Ante un agente/estímulo estresante se induce la síntesis y liberación/secreción desde el hipotálamo del factor liberador de corticotropina (CRF), que estimula la síntesis de POMC y liberación de ACTH en las células corticotropas de la pituitaria (revisado por Flik *et al.* 2006). La ACTH viaja por el torrente sanguíneo hasta alcanzar el córtex adrenal donde induce la síntesis y secreción de glucocorticoides específicos de la especie (cortisol en el caso de humanos y peces y cortisona en el caso de roedores).

Una de las preguntas que más intriga ha generado en el campo farmacológico de esta familia de receptores es, como el MC2R puede discriminar entre ACTH y α-MSH estando la secuencia α-MSH contenida en la estructura de la ACTH. Las hipótesis actuales consideran que el receptor es

activado, como el resto de receptores de la familia, por la secuencia "core" HFRW de MSH pero que la región de contacto/unión entre el tetrapéptido y el receptor, conocida como "binding pocket", está estructuralmente cerrada y requiere de la interacción entre secuencias específicas presentes en la región C-terminal de la ACTH (KKRRP) y un segundo "binding pocket" en el MC2R (Dores and Liang 2014). La interacción entre KKRRP y este segundo "binding pocket" abrirá el primer "binding pocket" que debe interactuar con el tetrapéptido para producirse la activación del receptor.

El MC2R requiere además de la interacción con una proteína accesoria del MC2R (MRAP) para adquirir la completa expresión funcional (Metherell *et al.* 2005). En su ausencia, el receptor no puede alcanzar la membrana plasmática y, por tanto, no puede unir la ACTH (ver después). Está característica ha retrasado mucho el análisis de su perfil farmacológico puesto que el receptor no es funcional en las líneas celulares que se utilizan habitualmente en farmacología (HEK-293, CHO, COS-7).

2.3.MC3R

El MC3R es, quizás, el receptor más enigmático de la familia. También se le conoce como receptor de γ-MSH ya que es el agonista por el que muestra mayor sensibilidad (Roselli-Rehfuss *et al.* 1993). El perfil de unión en MC3R de humano es el siguiente: γ-MSH>α-MSH>β-MSH>ACTH mientras que en rata es γ-MSH>α-MSH>ACTH>β-MSH (revisado por Schioth *et al.* 2005). Su dominio de expresión es fundamentalmente el SNC y principalmente las neuronas hipotalámicas, pero también se ha detectado su producción periférica en la placenta y el intestino (Gantz *et al.* 1993), además de una interesante expresión en macrófagos diferenciados del peritoneo (Getting *et al.* 2003).

El MC3R está implicado en la regulación del peso corporal y probablemente del particionado de energía en los diferentes depósitos corporales, tejido adiposo/muscular. La deleción del MC3R no produce hiperfagia o condición hipometabólica en situación normal, pero conlleva un aumento de la masa adiposa y una disminución de la masa muscular corporal (Chen *et al.* 2000). De hecho, la liberación de γ-MSH en el hipotálamo de rata reduce la cantidad de tejido adiposo (Eerola *et al.* 2018).

Investigaciones recientes sugieren que el MC3R regula el balance energético de forma reostática, mediante el control de la señalización de las neuronas hipotalámicas que expresan MC4R (Ghamari-Langroudi *et al.* 2018). Además, diversos estudios han mostrado que el entrenamiento de los ritmos de alimentación y la actividad alimenticia anticipatoria requieren una expresión funcional del MC3R (Sutton *et al.* 2010; Sutton *et al.* 2008), desvelando así su implicación en los ritmos circadianos de alimentación.

2.4.MC4R

El MC4R es probablemente uno de los receptores del sistema mejor estudiado, en virtud de su impacto en la regulación del balance energético. Los trabajos sobre el MC4R lideraron, en cierta

medida, el estudio de las melanocortinas tras la primera caracterización de los receptores (Mountjoy *et al.* 1992).

La manifiesta obesidad mórbida del ratón carente de expresión funcional del MC4R (Huszar *et al.* 1997), focalizó el interés sobre las funciones centrales del sistema de melanocortinas y su implicación en la regulación del balance energético. Los efectos del sistema de melanocortinas sobre la ingesta son principalmente mediados a través del MC4R, ya que se ha visto que la inhibición de la ingesta producida por las inyecciones de MTII (un agonista promiscuo de los diferentes MCRs), no tienen efecto en los ratones $MC4R^{-/-}$ (Marsh *et al.* 1999). Los ratones $MC4R^{-/-}$ no solamente exhiben un incremento de los acúmulos de grasa abdominal debido a la hiperfagia, sino que, además, presentan una disminución de la tasa metabólica basal, hiperinsulinemia y un incremento en el crecimiento lineal cercano al 15%. Actualmente, se sabe que las mutaciones en este receptor son la principal causa de obesidad monogénica en humanos (Farooqi 2008; Farooqi *et al.* 2003).

Diversos experimentos han demostrado que este receptor señaliza de forma constitutiva, es decir, no requiere de la unión a ligando para su activación (Nijenhuis *et al.* 2001; Srinivasan *et al.* 2004). Esta actividad constitutiva parece mantenerse gracias a la unión del segmento/extremo N-terminal del propio receptor (Ersoy *et al.* 2012; Srinivasan *et al.* 2004; Vaisse *et al.* 2000). Todo apunta a que el sistema de melanocortinas, vía la activación constitutiva del MC4R, supone un freno a la ingesta y mantiene la tasa metabólica dentro de un rango coherente con la adquisición de energía. Las mutaciones que eliminan este freno provocan, por tanto, hiperfagia y obesidad. Las situaciones que requieren un balance energético positivo, aumento de la ingesta y reducción el balance energético, deben sobrepasar la actividad constitutiva del receptor mediante la acción de agonistas inversos (ver más adelante).

La β-MSH es el agonista endógeno más potente del MC4R, aunque también se activa mediante la unión de la α-MSH y en menor medida por la γ-MSH (Coll and Tung 2009). Curiosamente, este receptor también se activa mediante la unión de péptidos que no pertenecen a la familia de las melanocortinas como la β-defensina y la lipocalina. Este último péptido se expresa en el tejido óseo y parece ser el responsable de su metabolismo (Rucci *et al.* 2015)

El MC4R también se expresa tímidamente en tejidos periféricos, siendo destacable su presencia en el intestino (Engelstoft *et al.* 2013). Su expresión inhibe la motilidad, aumentando el tiempo de tránsito intestinal y por tanto redundando en la inhibición de la ingesta (Panaro *et al.* 2014).

2.5.MC5R

El MC5R es el receptor con mayor presencia periférica, aunque siempre con niveles de expresión muy bajos. Su función siempre fue un poco esquiva a las investigaciones, incluso tras generar el ratón deficiente en su expresión $MC5R^{-/-}$. Finalmente Chen *et al.* (1997) demostraron que el MC5R

está implicado en el control de la secreción de las glándulas exocrinas. Así, los ratones MC5R$^{-/-}$ tenían un defecto en la impermeabilización al agua y la termorregulación debido a una disminución en la producción de lípidos de las glándulas sebáceas. Su perfil de expresión en los órganos involucrados en síntesis y/o liberación de feromonas hizo suponer su participación en estos procesos (Morgan and Cone 2006; Morgan *et al.* 2004a; Morgan *et al.* 2004b). Además, se le ha señalado como mediador de efectos provocados por hormonas melanocortinas sobre el sistema inmunológico, regulando la expresión de citoquinas (Jun *et al.* 2010) y en los efectos de α-MSH sobre la actividad de células presentadoras de antígeno (Lee and Taylor 2011).

3. Antagonistas Endógenos de los Receptores de Melanocortinas

El sistema de melanocortinas es el único sistema hormonal conocido que presenta antagonistas endógenos de sus receptores, los cuales compiten con los agonistas derivados del POMC por la unión y activación de los receptores. Esta característica revela la exquisita regulación que requiere el sistema de melanocortinas y por extensión, la importancia de sus funciones fisiológicas.

Hasta la fecha se han descrito dos miembros del sistema que actúan como antagonistas endógenos, la proteína de señalización agutí (ASIP) y la proteína relacionada con agutí (AGRP). Tienen perfiles de expresión y funciones fisiológicas distintas, pero están estructural y evolutivamente relacionados.

Por un lado, ASIP se expresa principalmente en la piel, participando en la regulación de la pigmentación, compitiendo con α-MSH por la unión al MC1R. Esto tiene como resultado que en los melanocitos se produzca feomelanina, un pigmento de color amarillo-rojizo. En cambio, AGRP se expresa en el hipotálamo donde regula la homeostasis energética por medio de su competición con α y β-MSH por unión al MC4R (Cone 1999; Cone 2006).

3.1.ASIP

La proteína de señalización agutí, denominada agutí exclusivamente en el caso del ratón, fue el primer antagonista conocido. Estructuralmente se caracteriza por poseer una región amino (N)-terminal compuesta principalmente por residuos básicos con una alta proporción de arginina (R) y lisina (K) que precede una zona rica en residuos de prolina (P) y a su región funcional carboxilo (C)-terminal rica en residuos de cisteínas (C) (Cerda-Reverter *et al.* 2005; Guillot *et al.* 2012).

Figura 1. Ratón con pigmentación agutí obtenido de Cone (2006).

La proteína debe su nombre al pelaje agutí característico de una cepa de caballos españoles, así como de un grupo de roedores sudamericanos pertenecientes al género *Dasyprocta* y llamados comúnmente como agutíes. El color de este pelaje es negro, pero se caracteriza por la presencia de una banda subapical amarilla que genera un aspecto pardo presente en muchos mamíferos (figura 1). El color negro viene determinado por la presencia de eumelanina, un pigmento de color marrón oscuro-negro, mientras que el color amarillo se debe a la presencia de otra forma química de melanina denominada feomelanina.

La activación del MC1R por medio de la α-MSH provoca la síntesis de melanina mientras que un pulso de síntesis de ASIP en el folículo piloso y la antagonización del MC1R provoca el intercambio hacia la síntesis de feomelanina, responsable de la banda subapical (Cone *et al.* 1996; Slominski *et al.* 2005). El gen agutí es, además, responsable de la polaridad de pigmentación dorsoventral característica de muchas especies de mamíferos que presentan una tonalidad dorsal más oscura que el pelaje ventral (Vrieling *et al.* 1994). El gen está bajo el control de diversos promotores y su activación regula su expresión dorso-ventral y su expresión pulsátil durante el ciclo del crecimiento piloso.

La cepa de ratones agutí amarillos presentan un pelaje completamente amarillo, sin polaridad dorso-ventral, además de ser obesos y presentar diferentes trastornos metabólicos asociados a su obesidad mórbida. Dicho fenotipo se debe a una activación constitutiva provocada por una mutación delectiva que deja al gen agutí bajo el control del promotor de otro gen llamado *rely*. Este último gen codifica una proteína de unión al RNA que se expresa constitutivamente de forma ubicua provocando que ASIP haga lo propio, induciendo así la síntesis continua y ubicua de feomelanina. Su obesidad se debe a la antagonización de ASIP sobre el MC4R central. *asip* se expresa en la piel y normalmente no tiene acceso al MC4R central, pero en el caso del ratón agutí amarillo donde se expresa de forma ubicua, se produce dicha antagonización que lleva a la ingesta aumentada y la consecuente obesidad.

La intensidad en la pigmentación agutí, se relaciona directamente con la región básica (Miltenberger *et al.* 1999), así como en la regulación de células pigmentarias en *Xenopus* (Ollmann and Barsh 1999). Aunque también existen antecedentes respecto a la disminución en afinidad al

receptor está controlado por la Atractina, una proteína transmembrana simple, ubicuamente expresada y que suprime la pigmentación agutí (Gunn *et al.* 1999; He *et al.* 2001).

ASIP es también responsable del patrón de pigmentación dorsoventral, en el cual el color del pelaje de la zona dorsal es oscuro por la mayor producción de melanina (pigmentación negra/café), además de la baja expresión de *asip*. Por el contrario, en la zona ventral la pigmentación dominante es de color claro, debido a la alta producción de feomelanina provocada por la mayor expresión de *asip* (Sakai *et al.* 1997; Vrieling *et al.* 1994).

Figura 2. Efecto de la sobreexpresión de *agrp* humana, bajo el control del promotor de la *β-actina*. A la izquierda un ratón sin mutación y a la derecha un ratón obeso que sobre-expresa *agrp*. Nótese que la pigmentación de ambos ratones es similar (Wilson *et al.* 1999).

3.2.AGRP

La obesidad del ratón agutí amarillo debida al antagonismo del MC1R hizo suponer la existencia de un sistema equivalente en el cerebro que regulase la ingesta. Mediante el estudio de una librería de "expressed sequence tags" (ESTs) de ratón y de las características estructurales de las proteínas codificadas se descubrió un gen que codificaba una proteína de estructura similar a ASIP, con el mismo patrón de cisteínas en su región C-terminal. Su expresión ubicua mediante sistemas de transgénesis resulta en obesidad, pero un pelaje normal indicando su independencia de los sistemas de pigmentación y de la actividad del MC1R (Ollmann *et al.* 1997) (figura 2).

agrp se expresa exclusivamente en las neuronas hipotalámicas (Bagnol *et al.* 1999) GABAérgicas del núcleo arqueado en las que colocaliza, en un 98% de ocasiones, con otro péptido orexigénico, el neuropéptido Y (NPY) (Hahn *et al.* 1998). La ablación dirigida de estas neuronas NPY/AGRP inducen un rápido decremento de la ingesta que conduce al ayuno (Luquet *et al.* 2005). Las proyecciones GABAérgicas de las neuronas NPY/AGRP regulan, a su vez, la expresión de las neuronas POMC del propio arqueado (Horvath *et al.* 1997) y el comportamiento alimenticio en el núcleo parabraquial (Wu *et al.* 2009). El ayuno produce un fuerte incremento en la expresión

hipotalámica de *agrp* (Ollmann *et al.* 1997) mientras que la inyección ICV de AGRP induce la ingesta en rata (Rossi *et al.* 1998).

AGRP actúa como un antagonista competitivo de α-MSH compitiendo por la unión al MC3R y MC4R pero además también actúa, independientemente de α-MSH, disminuyendo la actividad constitutiva del receptor como hace un agonista inverso (Nijenhuis *et al.* 2001; Srinivasan *et al.* 2004; Sánchez *et al.*, 2009). La explicación fisiológica de la actividad constitutiva del MC4R está relacionada con una inhibición constante de la ingesta siendo necesario el bloqueo de esta actividad vía la sobreexpresión de *agrp* durante el ayuno y su agonismo inverso sobre MC4R para sobrepasar la inhibición estimulando la ingesta del animal (revisado por Cone 2006).

4. Proteínas accesorias del receptor de melanocortinas tipo 2 (MRAP)

La caracterización farmacología del MC2R fue problemática durante años debido a los requisitos celulares del receptor para su expresión funcional. A diferencia del resto de receptores, el *mc2r* no se expresa de forma funcional en líneas celulares convencionales tipo HEK-293 (células embriónicas de riñón humano), COS-7 (células de riñón de mono verde africano) o CHO (células ovario de hámster chino) y solamente lo hace en líneas celulares derivadas del córtex adrenal (Y6 o OS3) (Schimmer *et al.* 1995). El receptor es incapaz de alcanzar la membrana plasmática cuando se expresa en estas líneas convencionales quedando atrapado en el complejo reticular-Golgi (ver Figura 3), confirmando la necesidad de un factor presente en las células adrenocorticales que es capaz de facilitar su tráfico a la membrana plasmática.

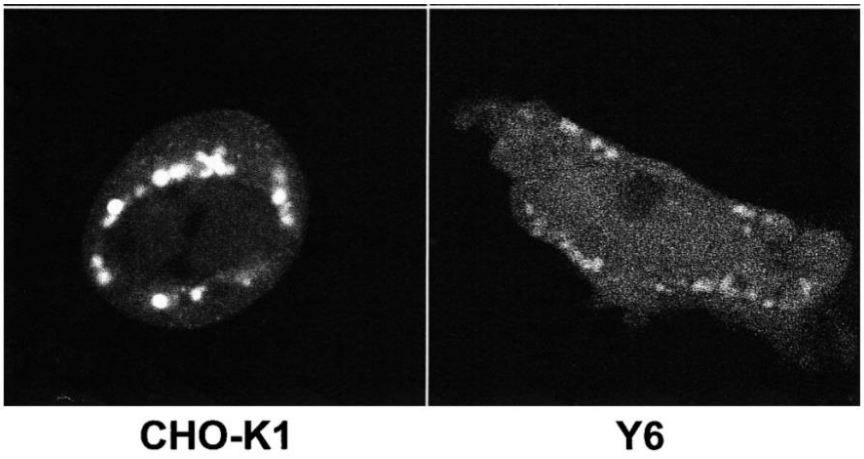

Figura 3. Imágenes de MC2R marcado con GFP transfectado transitoriamente en células CHO-K1 (línea celular no adrenal) y en células Y6 (línea celular adrenal), obtenidas mediante microscopia confocal (modificado de Noon *et al.* 2002). En la imagen podemos observar que en la célula CHO-K1, el MC2R se encuentra en el citoplasma o en la zona perinuclear. Por el contrario, en la célula Y6, el receptor marcado se encuentra en la membrana plasmática.

Dicho factor no fue descubierto hasta el año 2005 gracias al estudio de una enfermedad rara denominada deficiencia familiar de glucocorticoides (FGD) (Metherell *et al.* 2005). Una enfermedad de origen autosómico recesiva, en la cual los pacientes poseen una elevada cantidad de ACTH que contrasta con bajos niveles de glucocorticoides en sangre. Asociado a esta patología, los pacientes exhiben hiperpigmentación debida a la interacción del exceso de ACTH sistémica con los MC1Rs presentes en los melanocitos (Jackson *et al.*, 2015). La primera causa reconocida de FGD fueron las mutaciones que provocaban la inactivación del MC2R y, por lo tanto, la inefectividad de la ACTH a la hora de inducir las síntesis de glucocorticoides. Sin embargo, un porcentaje de pacientes sigue exhibiendo FGD en ausencia de mutaciones del *mc2r*. A este segundo tipo se le denominó FGD tipo 2, en contraposición a la FGD tipo 1 dependiente de mutaciones en el MC2R (revisado por Jackson *et al.* 2015; Meimaridou *et al.* 2013; Novoselova *et al.* 2013). El mapeo de polimorfismos de único nucleótido o "single nucleotide polymorphism (SNP)" en pacientes que exhibían FGD tipo 2, llevó a la identificación de un gen cuya proteína presentaba altos niveles de expresión en el cortex adrenal. Esta proteína, realmente, ya había sido caracterizada anteriormente en fibroblastos 3T3-L1 y fue denominada como proteína de bajo peso molecular específica del tejido adiposo (Xu *et al.* 2002), aunque posteriormente se renombró como proteína accesoria del MC2R (MRAP). Su caracterización estructural reveló una proteína de membrana con un único dominio transmembranal que forma dímeros antiparalelos (figura 4), e interactúa físicamente con el receptor permitiendo su tráfico a la membrana plasmática donde intercala sus siete dominios transmembrana y se acopla a la proteína G tras interacción con el agonista (ACTH) para disparar la cascada de señalización intracelular.

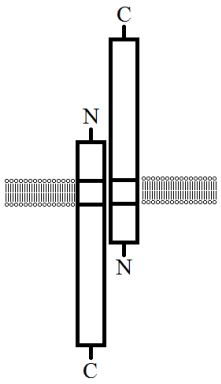

Figura 4. Representación esquemática de la orientación de las MRAPs y formación de dímeros anti paralelos descrito por Sebag and Hinkle (2009b).

Cooray *et al.* (2011) demostraron que esta interacción MC2R-MRAP es más compleja de lo esperado y deriva en la formación de un complejo integrado por dos moléculas de MC2R y dos dímeros de MRAP para formar un complejo hexámerico indispensable para asegurar la migración a la membrana plasmática. La MRAP también interfiere negativamente en la dimerización de otros receptores de melanocortinas comprometiendo la correcta expresión de MC5R en la membrana plasmática (Sebag and Hinkle, 2009a) y reduce la respuesta de MC1R, MC3R, MC4R y MC5R al análogo NDP-α-MSH (Chan *et al.* 2009).

En el estudio de caracterización de la MRAP, el grupo de Adrian Clark ya apuntó la existencia de un fragmento abierto de lectura ("orf") con cierta homología de secuencia a la MRAP cuya proteína codificada presentaba una relación estructural con la MRAP. Durante los primeros años todo el esfuerzo de investigación fue invertido en la MRAP, sin embargo, unos años más tarde se puso foco en la función de esta segunda proteína denominada MRAP2 (Chan *et al.* 2009). Los experimentos realizados por el grupo de Clark demostraron que la MRAP2 era capaz de interactuar con todos los receptores de melanocortinas modificando su afinidad por los diferentes agonistas, pero su función fisiológica distaba de ser reconocida (Chan *et al.* 2009).

El campo de las proteínas accesorias no pasó inadvertido para la endocrinología comparada y nuestro grupo de investigación realizó una prospección "*in silico*" de los posibles ortólogos de la MRAP en los genomas de peces disponibles (figura 5). Las búsquedas comparativas dieron como fruto la caracterización de dos MRAPs en el pez cebra, que se denominaron MRAP1 y MRAP2 con alta relación de identidad con la MRAP y MRAP2 de mamíferos, respectivamente. La MRAP2 además presentaba un parálogo en el genoma del pez cebra, lo que llevo a diferenciar ambos genes como *mrap2a* y *mrap2b* (Agulleiro *et al.* 2010). A partir de este momento se propuso una nueva nomenclatura para las proteínas accesorias pasando a denominarse MRAP1 y MRAP2 en lugar de MRAP y MRAP2, aunque en parte de la bibliografía sigue manteniéndose la nomenclatura inicial más clásica (Cerdá-Reverter *et al.* 2011).

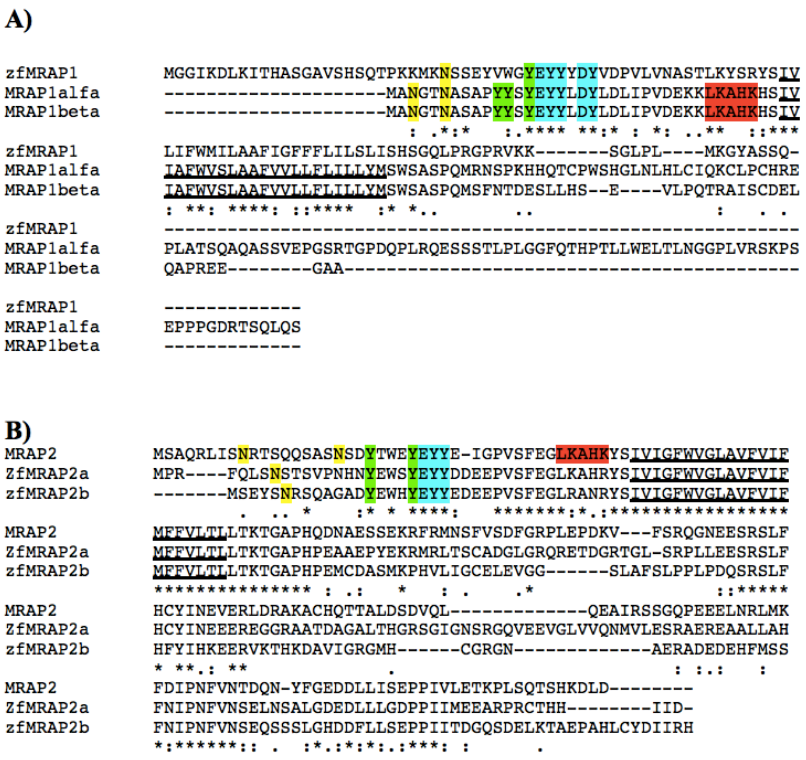

Figura 5. Secuencia de aminoácidos de MRAPs de humano y pez cebra (Zf). A) secuencias de MRAP1 y B) secuencias de MRAP2. En amarillo sitios de consenso para N-glicosilación, en verde zonas ricas en tirosina, en calipso dominios conservados en todos las MRAPs de los vertebrados (Agulleiro *et al.* 2010). En rojo dominio involucrado en la formación de topología dual y subrayado probable zona transmembrana (Sebag and Hinkle 2007).

Al igual que en mamíferos, la MRAP1 es necesaria para la expresión funcional del MC2R. Los experimentos de inmunoprecipitación demostraron el contacto entre las dos proteínas, o a través de un intermediario. De forma similar, los experimentos inmunocitoquímicos revelaron la presencia en membrana del receptor tras la interacción con MRAP1 y los experimentos farmacológicos demostraron la activación del receptor por ACTH en presencia de MRAP1, pero no en ausencia de la proteína accesoria. Por el contrario, la MRPA2a y MRPA2b no modifican la actividad farmacológica del receptor y al igual que en mamíferos su función era una incógnita.

5. Respuesta al estrés y melanocortinas

El estrés se define como “la respuesta no específica del organismo a cualquier demanda que se le impone” (Selye, 1955). También podríamos definirlo como la situación en la cual el ambiente ejerce presión sobre la fisiología de un organismo, al exponerlo a condiciones ambientales distintas de sus parámetros óptimos. Tal es el caso de los peces, cuyo metabolismo está directamente relacionado

con parámetros de su entorno como son la temperatura, salinidad o el oxígeno disponible (Carneiro & Urbinati, 2001; Madeira *et al.*, 2013; Valenzuela *et al.*, 2005). Determinado por el tiempo de duración, podemos dividir el estrés en agudo y crónico. El estrés agudo, más común en la naturaleza, es de duración breve y con una intensidad relativamente alta (dependiendo del factor). Está asociado a factores ambientales como cambios inesperados en el clima, la disponibilidad de alimento en períodos de tiempo breves y a la presencia de depredadores. Por el contrario, el estrés crónico es menos común en la naturaleza, su intensidad puede ser menor pero su larga duración provoca un aumento sostenido en la demanda energética afectando de manera más severa y negativa en comparación con el estrés agudo (Tort, 2011). Los vertebrados median la respuesta al estrés por medio de dos ejes o vías, el eje controlado por el sistema nervioso simpático (SNS), denominada vía/eje simpático-cromafín (SC) (Eiden, 2013; Kvetnansky *et al.*, 2013) y desde el sistema nervioso central (SNC) mediante el control del eje hipotálamo-pituitario-adrenal (interrenal en peces teleósteos) (Barton, 2002; Mommsen *et al.*, 1999; Pankhurst, 2011; Wendelaar Bonga, 1997).

La ruta controlada por el SNC comienza en el núcleo preóptico (NPO). El NPO es el equivalente del núcleo paraventricular (PVN) hipotalámico en mamíferos, una zona de integración de información interna (corporal) y externa (ambiental) donde se encuentran las neuronas productoras del factor liberador de corticotropina (CRF). Las terminales de estas neuronas inervan directamente las células corticotropas de la *pars distalis rostral* (rPD) donde estimulan la síntesis de POMC y procesado hacia la liberación sistémica de ACTH (revisado por Huising *et al.*, 2004; Flik *et al.*, 2006). La ACTH sistémica alcanza la interrenal donde induce la señalización, vía MC2R-MRAP1, que derivará en la estimulación de la esteroideogénesis adrenal/interrenal, que conlleva a su vez, la síntesis y liberación de glucocorticoides a partir del colesterol (Alsop & Vijayan, 2008; Cerdá-Reverter *et al.*, 2011).

El sistema de melanocortinas es, por tanto, un punto clave en la respuesta de los organismos a las condiciones de estrés. MRAP1 modula la expresión funcional de MC2R, mientras que MRAP2 pueden modificar el perfil farmacológico de los receptores de melanocortinas, por tanto, es lógico pensar que las proteínas accesorias constituyan un nuevo punto de regulación de la respuesta al estrés.

Objetivos

El objetivo general de esta tesis fue profundizar en el estudio de las MRAPs, investigando el rol fisiológico de la MRAP2 en vertebrados, así como la implicación del sistema de proteínas accesorias en la respuesta al estrés de los peces desarrollando los siguientes puntos:

1) El primer objetivo fue realizar sendas revisiones bibliográficas sobre la evolución del sistema de melanocortinas y lo que se conocía, en el momento, sobre las proteínas accesorias en vertebrados.

2) La única especie vertebrados no mamíferos donde se había demostrado la dependencia de la MRAP1 del MC2R hasta el momento había sido el pez cebra, por tanto, estudiamos la dependencia del MC2R de otras especies de peces, con el fin de comprobar la conservación evolutiva de la interacción.

3) El siguiente objetivo fue estudiar la función fisiología de las MRAP2s en el pez cebra analizando la interacción con otros receptores de melanocortinas.

4) El último objetivo fue estudiar la respuesta al estrés agudo del sistema de melanocortinas en el pez cebra.

CAPÍTULO I: Evolution of the melanocortin system

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Resumen

El sistema de melanocortinas es uno de los más complejos sistemas hormonales. Involucra diferentes agonistas codificados en el precursor proopiomelanocortina (POMC) o en diferentes genes como *β-defensinas*, antagonistas endógenos, como la proteína de señalización agouti (ASIP) o proteína relacionada con agouti (AGRP), y cinco receptores de melanocortina (MCR). Eventos de duplicación genómica completa han precedido a la diversificación funcional y molecular de la familia, además se han propuesto algunos procesos de duplicación co-evolutivos y en tándem. Los patrones evolutivos de los diferentes componentes son controvertidos y diferentes hipótesis han surgido de estudios de secuenciación genómica. En esta revisión, resumimos las diferentes hipótesis evolutivas propuestas para los diferentes componentes del sistema de melanocortina.

Abstract

The melancortin system is one of the most complex of the hormonal systems. It involves different agonists encoded in the mutiplex precursor proopiomelanocortin (POMC) or in different genes as *β-defensins*, endogenous antagonist, like agouti-signalling protein (ASIP) or agouti-related protein (AGRP), and five different melanocortin receptors (MCRs). Rounds of whole genome duplication events have preceded the functional and molecular diversification of the family in addition some co-evolutionary and tandem duplication processes have been proposed. The evolutionary patterns of the different partners are controversial and different hypotheses have emerged from a study of the sequenced genomes. In this review, we summarize the different evolutionary hypotheses proposed for the different melanocortin partners.

Introduction

The melanocortin system is one of the most complex hormonal systems in vertebrates. The system integrates diverse agonists encoded in the multiplex precursor proopiomelanocortin (POMC; Nakanishi *et al.* 1979). Melanocortins bind to a family of G-protein-coupled receptors that positively couple to the intracellular cAMP production. The family comprises five receptors (MC1R-MC5R) that preferentially bind the different melancortin agonists (Schioth *et al.* 2005). The signaling and pharmacological profile of the MCRs is further modulated by a group of transmembrane proteins called melanocortin receptor accessory proteins (MRAPs; Cerdá-Reverter *et al.* 2013). MRAP1 physically interacts with MC2R to allow its trafficking to the plasma membrane for the binding of adrenocorticotropic hormone (ACTH), whereas MRAP2 binds MC4R to provide ACTH sensitivity as well as to decrease its constitutive activity (Agulleiro *et al.* 2013a; Sebag *et al.* 2013). Atypically, the melanocortin system is regulated also by endogenous antagonists that compete with the agonists to bind to the receptor, thus decreasing agonist-induced cAMP production, or work as inverse agonists, thus decreasing the activity of the constitutively activated MCRs. Agouti-signaling protein (ASIP) binds MC1R and MC4R, whereas agouti-related protein (AGRP) antagonizes the melanocortin agonist effect at MC3R and MC4R (Table I).

Table I: Agonist and antagonist of melanocortin receptors. For review and references, see Cerdá-Reverter *et al.* (2013); Cerdá-Reverter *et al.* (2011); Schioth *et al.* (2005).

Receptor	Agonists/Antagonist	Accessory Protein	Main Tissue Expression	Main Function
MC1R	MSHs, β-defensin 103/ASIP	MRAP2	Skin	Melanogenesis
MC2R	ACTH	MRAP1	Adrenal gland	Stress response
MC3R	MSHs/AGRP	MRAP2	Brain	Energy Balance
MC4R	MSHs/AGRP	MRAP2	Brain	Energy Balance
MC5R	MSHs	MRAP2	Non-specific	Exocrine secretion

In addition, some non-POMC-derived melanocortin, responsible for different pigment phenotypes in mammalian species have been described. For example, β-defensin 103 (CBD103), which belongs to a protein family previously implicated in innate immunity, has been shown to display high affinity for the MC1R (Candille *et al.* 2007). Additionally, mutations in mahogany (attractin, *atrn*) and mahoganoid (mahogunin ring finger 1, *mgrn1*) loci also rescue pigmentation anomalies and the obesity phenotype of agouti mice, working downstream of agouti gene and upstream or at the level of MC1R/MC4R. Both *atrn* and *mgrn1* regulate MC4R degradation in the lysosome and mutations of these genes promote the trafficking of the receptor back to the membrane (Overton and Leibel 2011).

Obviously, this molecular complexity has resulted in numerous pathways being proposed for the regulation of the system and, by extension, for the regulation of the multiple physiological processes in which melanocortins are involved. The study of the melanocortin system has yielded a conceptual revolution for endocrinology and the system nowadays is one of the key areas for the understanding of human obesity (Cone 2006). In this paper, we will provide a description of the molecular structure of the different members of the melanocortin family within an evolutionary framework to suggest potential co-evolutionary processes within this complex hormonal system.

Proopiomelanocortin (POMC)

Proopiomelanocortin (POMC) gene encodes a protein precursor whose posttranscriptional processing yields melanocortins among a number of biologically active peptides. This precursor is comprised of three main domains in tetrapod species: The N-terminal pro-γ-melanocyte stimulating hormone (MSH), the central adrenocorticotropic hormone (ACTH) and C-terminal β-lipotropin. Each domain contains one MSH peptide delineated by a core sequence HFRW: i.e. γ-MSH in pro-γ-MSH, α-MSH as N-terminal sequence of ACTH and β-MSH in the β-lipotropin domain. The latter domain further includes the C-terminal β-endorphin, an endogenous opioid peptide (Nakanishi *et al.* 1979). POMC is mainly produced in the pituitary and its postranslational processing occurs in a tissue-specific manner. The proteolytic cleavage of POMC by prohormone convertase 1 (PC1) generates ACTH and β-lipotropin in the corticotrophs of the anterior pituitary, whereas cleavage by PC1 and PC2 produces α-MSH and β-endorphin in the melanotrophs of the pars intermedia (Castro and Morrison 1997).

Because tetrapod POMC sequences contain three MSH-core sequences (the above mentioned α-MSH, β-MSH and γ-MSH), the POMC gene has been thought to evolve through intragenic duplication of an ancestral MSH gene (Nakanishi *et al.* 1979). In sarcopterygian fish (lobe-finned fish), the POMC precursor exhibits the same three MSH domains (Amemiya *et al.* 1999; Dores *et al.* 1999). The γ-MSH domain occurs only as a remnant in non-teleost actinopterygians, including sturgeons (Alrubaian *et al.* 1999; Amemiya *et al.* 1997), and is absent in the most derived group, the teleosts (Arends *et al.* 1998; Cerdá-Reverter *et al.* 2003b; Salbert *et al.* 1992). In contrast, elasmobranch fish have an additional fourth MSH domain, termed δ-MSH, which probably arose as a duplication from the β-MSH-β-endorphin segment. This duplication process seems to be unique in chondricthyans, where it may have occurred after the divergence from the osteichthyan lineage (Amemiya *et al.* 1999). In a very recent paper, a novel melanocortin peptide, termed ε-MSH, has been characterized in cichlid and pomacentrid species and seems to be the result of a tandem duplication of the segment encoding α-MSH-ACTH (Harris *et al.* 2014).

POMC belongs to the opioid/orphanin gene family, in which all genes encode at least one chemical signal that has either the opioid core sequence, YGGF, or the orphanin/nociceptin core sequence, FGGF (Dores and Lecaude 2005). The gene family includes *proenkephalin* (PENK), *prodinorphyn* (PDYN) and *proorphanin* (PNOC) as well as POMC (Danielson and Dores 1999). To date neither opioid/orphanin-related genes nor melanocortin-receptor related genes have been described in the genome of cephalochordates (amphioxus and tunicates), while two different POMC orthologues have been cloned in agnatha (lamprey); proopiomelanotropin (POM) and proopiocortin (POC). POM only exhibits an MSH-core and opioid sequences, whereas POC encodes ACTH and β-endorphin (Takahashi *et al.* 2005). In this framework, it is reasonable to speculate that the ancestral gene of the family emerged in the protochordate lineage. Following the general agreement that two sequential rounds of genome duplication (R) occurred before ancestral gnathostome, it has been proposed that proenkephalin and prodynorphin are the result of the 2R, whereas proorphanin seems to be the result of a local duplication of POMC that could take place after 2R. Timing of the origin of POMC is uncertain, an while it may be claimed that POMC arose before the lamprey/gnathostome division, it is not possible to say that POMC arose before the 2R since it is still unclear whether the lampreys underwent one or two tetraploidizations (Sundström *et al.*, 2010). In fact, Dores (2013) has proposed a new model that assumes that lampreys are 2R organisms, which provides more convincing support for the presence of two POMC orthologues in lamprey. This model predicts the presence of still unidentified proenkephalin and prodynorphyn genes in lampreys. Supporting this idea, enkephalin-like peptides have been described in the lamprey brain (Dores 2013), but definitive confirmation depends on completion of the lamprey genome sequencing project. Sequencing of the lamprey genome has provided strong evidence that lampreys have undergone 2R (Smith *et al.* 2013) but probably also an independent 3R event (Mehta *et al.* 2013). It is then possible that POC and POM may have arisen in this specific lamprey duplication event.

It is also well accepted that teleost fish underwent a third genome duplication process (3R) and duplicated genes have frequently endured (Meyer and Van de Peer 2005). These new gene copies can undergo neofunctionalization (by the new copy), subfunctionalization (both copies share the function of the original copy) or pseudofunctionalization (the sequence of the new copy degenerates and loses its function). This is also the case for the opioid/orphanin gene family as POMC duplicates have been reported in all teleost species in which the genome has been sequenced (see Sundstrom *et al.* 2010 for references) and subfunctionalization has been demostrated in Tetraodon (de Souza *et al.* 2005). In some species like barfin flounder (Verasper moseri) (Takahashi *et al.* 2005), Burton's mouthbrooder (Haplochromis burtoni) or medaka (Oryzias latipes) (Harris *et al.* 2014) three different genes have been found but they seem to be the result of lineage-specific gene duplication events other than genome duplication (Sundstrom *et al.* 2010).

Melanocortin Antagonist

Agouti-signaling (ASIP) and agouti-related (AGRP) protein are endogenous antagonists of melanocortin receptors (MCRs), which compete with melanocyte-stimulating hormones (MSH) and adrenocorticotropic hormone (ACTH). In mice, ASIP is expressed by dermal papillae cells and acts within the hair follicle microenvironment to control the switch between the production of eumelanin (black-brown pigment) and phaeomelanin (yellow-red pigment) by antagonizing α-MSH effects on MC1R in the follicle melanocytes (Cone 2006). In contrast, AGRP is expressed mainly in the central nervous system (CNS), where it antagonizes α-MSH at central MC3R and MC4R to regulate food intake, energy expenditure and linear growth (Ollmann *et al.* 1997). AGRP works also as an inverse agonist in constitutively activated MC4R (Ersoy *et al.* 2012; Nijenhuis *et al.* 2001; Sánchez *et al.* 2009). ASIP and AGRP molecules share a cysteine-rich C-terminal domain, consisting of 10 spatially conserved cysteine residues that form 5 disulfide bridges, which are essential for the conformational stability and biological functions of the molecule. This cysteine pattern resembles that of the conotoxins and agatoxins, suggesting that agouti-like proteins adopt an inhibitory cysteine knot (ICK) fold. The N-terminal region of ASIP is filled by a basic domain with a high proportion of arginine and lysine residues. This region leads a proline-rich area that immediately precedes the cysteine rich C-terminal domain (figure 1). The integrity of this basic domain is also essential for the full activity of the ASIP protein (Miltenberger *et al.* 1999; Miltenberger *et al.* 2002).

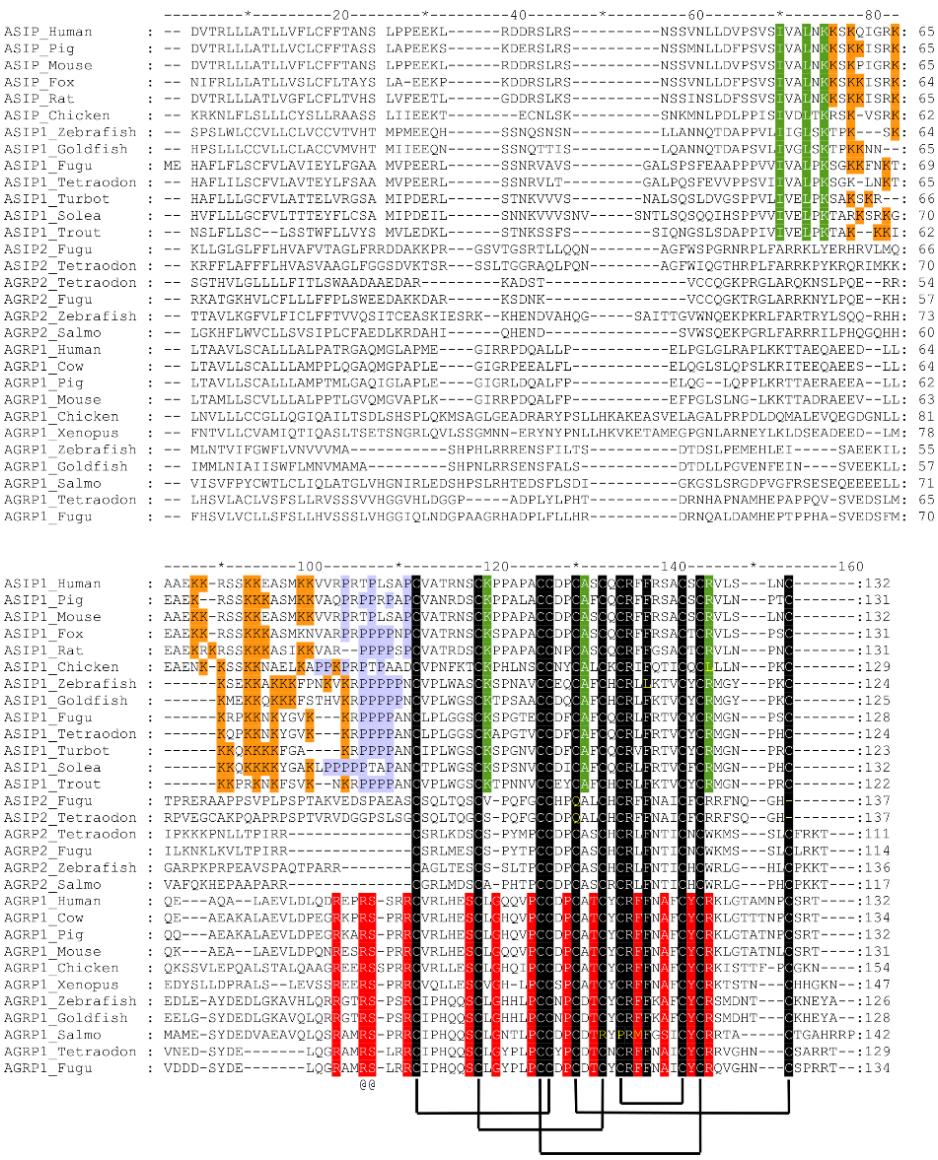

Figure 1. Alignment of agouti-signaling protein (ASIP) and agouti-related protein (AGRP) amino acid sequences. Dashes were introduced to improve alignment. Black boxes show amino acid residues conserved in all sequences. Green boxes show residues only conserved in ASIP1 sequences. Red boxes indicate residues only conserved in AGRP1 sequences. Orange boxes indicate basic residues before proline domain in ASIP1 sequences. Purple boxes indicate proline residues before cysteine domain in ASIP1 sequences. Lines joining cysteine residues indicate putative disulfide bonds forming the cysteine domain. Alignment positions 106-107 marked with @ are conserved for AGRP1 post-transcriptional processing. Yellow letters indicate exceptions in the AGRP1 sequence in salmonids (positions 133, 135 and 137) and ASIP2 sequences in Fugu and Tetraodon (position 130).

The N-terminal region has been shown to down-regulate melanocortin receptor signaling in *Xenopus* melanophore (Ollmann and Barsh 1999) and is thought to mediate low affinity interactions with the product of the mahogany locus, i.e atracttin (He *et al.* 2001). On the contrary, the N-terminal region of the AGRP sequences displays low level of identity and exhibits and consensus processing site prior to the cysteine domain where the propeptide is cleaved (Creemers *et al.* 2006).

Tetrapod species have a single copy of *asip* and *agrp* genes but the genome of teleost fish have four endogenous antagonists: *asip1*, *asip2*, *agrp1* and *agrp2*. Both AGRP1 and ASIP1 share a cysteine

knot with the structure C-x(6)-C-x(6)-C, whereas AGRP2 and ASIP2 exhibit a shortened version of the cystine knot C-x(6)-C-x(5)-C and were originally named as A2 peptides (Klovins and Schioth 2005). The evolutionary relationship among peptides remains to be unraveled (Braasch and Postlethwait 2011; Guillot *et al.* 2012; Schioth *et al.* 2011; Vastermark *et al.* 2012) and alternative evolutionary hypotheses suggest different nomenclatures for the peptides. Studies have suggested that ASIP2 and AGRP2 are ohnolog genes (duplicated genes resulting form genome doubling) of ASIP1 and AGRP1, respectively, which are generated during teleost-specific genome duplication (3R, figure 2A) (Kurokawa *et al.* 2006). Recent studies support the view that the AGRP2 chromosomal region does not share a synteny relationship with fish AGRP1 or with tetrapod AGRP. The AGRP2 and ASIP2 regions show conserved synteny with a region of human chromosome 8, which, in turn, shares paralogies with the ASIP region on chromosome 20.

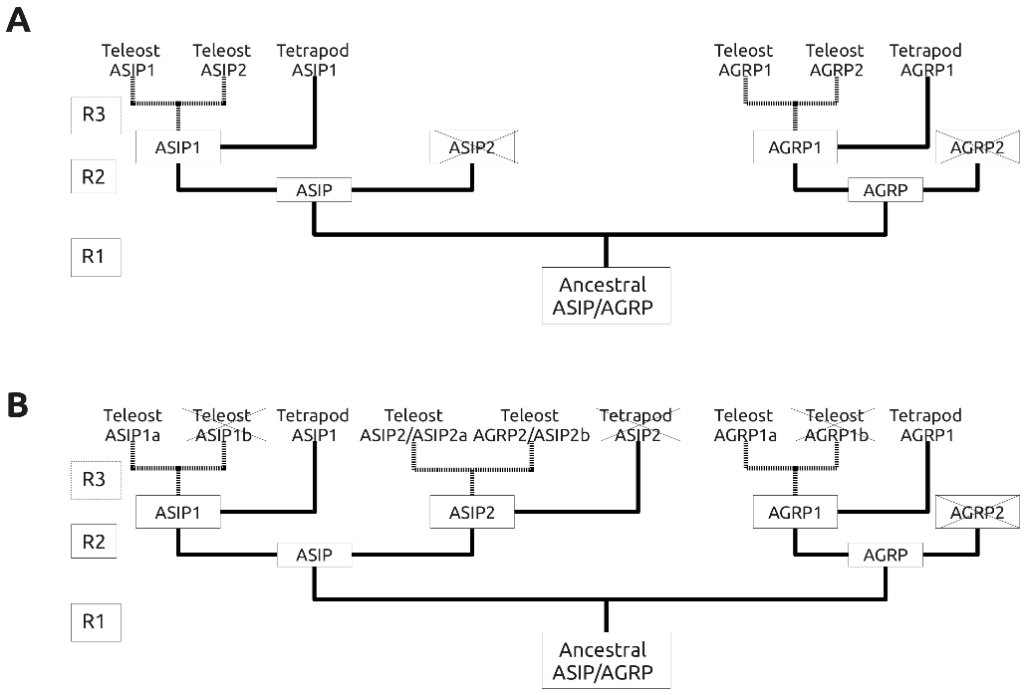

Figure 2. Evolutionary schemes showing alternative hypotheses for the evolution of melanocortin endogenous antagonists. A) Evolutionary hypothesis and nomenclature supported by Kurokawa *et al.* (2006). B) Evolutionary hypothesis and nomenclature proposed by Braasch and Postlethwait (2011). See text for details. Dashed lines indicate the third round of genome duplication in teleost fish.

The model proposes that the AGRP/ASIP precursor was duplicated twice during the two rounds of vertebrate genome duplication (R1, R2). Both AGRP2 and ASIP2 were lost in the tetrapod genome but ASIP2 was retained in the teleost genome. After the teleost-specific genome duplication, the additional copy of *agrp* gene was likewise lost from the teleost genome but both copies of the *asip2* gene were retained (figure 2B). These copies are named *asip2* and *agrp2* (Kurokawa *et al.* 2006) but the new model proposes naming them *asip2a* and *asip2b*, respectively (Braasch and Postlethwait 2011). Schiöth and collaborators rebuilt the phylogeny by introducing a sequence from elephant shark (Schioth *et al.* 2011). If AGRP is used to root the tree, the results support Braasch and Postlehwait's

hypothesis but if the tree is rooted by the shark sequence, the AGRP2 and ASIP2 clusters group with the AGRP cluster, supporting the previous nomenclature (Kurokawa *et al.* 2006).

Melanocortin Receptor Accessory Proteins (MRAPs)

MRAPs were discovered when the MC2R was seen to be unable to reach the plasma membrane when expressed in conventional cell lines. This indicated the presence of an MC2R-specific transport system in the adrenocortical cells. Homozygosity mapping using single nucleotide polymorphism (SNP) microarrays and RT-PCR identified a gene which was exclusively expressed in the adrenal cortex. When coexpressed with MC2R in CHO cells, this protein allowed functional expression of the receptor (Metherell *et al.* 2005). In fact, this gene had been characterized previously as an upregulated gene during the differentiation of mouse 3T3-L1 cells from fibroblasts into adipocytes, and named fat tissue-specific low molecular weight protein (Xu *et al.* 2002) before being renamed melanocortin 2 receptor accessory protein (MRAP). Human (h) MRAP is a small protein exhibiting a hydrophobic transmembrane domain (residues 38-60). The *hmrap* gene is alternatively spliced into two different proteins, *hmrap*α and *hmrap*β, which contain 172 and 102 amino acids, respectively. Both proteins are identical in the first 69 amino acids that contain the N-terminal region, the transmembrane domain and the first section of the C-terminal region. hMRAP has no putative signal peptide, exhibits potential N-linked glycosylation sites and has a tyrosine-rich region within the N-terminal domain. Blasting human genome with the MRAP sequence pointed to another gene (C6orf117) encoding a small single transmembrane–domain protein that is more than 27% identical to hMRAP in the N-terminal and transmembrane domains but differs at the C-terminus (Metherell *et al.* 2005). This gene encodes a 205 amino acid protein now known as MRAP2. This introduced a degree of confusion into the nomenclature since most authors continue to call the first characterized protein MRAP rather than MRAP1. Subsequent studies identified *mrap* orthologues in different species. We identified three different MRAPs in zebrafish, one MRAP grouped with tetrapod MRAP1s and the other two named as MRAP2a and MRAP2b. We also found *mrap1* and *mrap2* orthologues in most fish genomes screened but only zebrafish exhibited *mrap2* paralogues (reviewed by Cerdá-Reverter *et al.* 2013).

An MRAP2-like sequence has also been identified in elephant shark (Callorhinchus milii) and sea lamprey (Petromyzon marinus) but not in the amphioxius (Brachiostoma floridae) (Vastermark and Schioth 2011). Following this phylogenetic distribution, it would be reasonable to propose that the MRAP system emerged in some now extinct protochordate lineage, similar to that proposed for opioid/orphaning-related genes and melanocortin receptors (see above). However, both MRAP1 and MRAP2 were detected only after radiation of the osteichthyes, suggesting that the ancestral *mrap2* was duplicated at some point after the divergence of elephant shark from other jawed vertebrates (Valsalan *et al.* 2013). *mrap1* sequences seem to be absent in some genomes including medaka, Atlantic cod, tilapia, coelacanth, frog and reptiles, suggesting a gene loss process, although it cannot ruled out that it is an artifact of incomplete databases (Valsalan *et al.* 2013). Taken together, all this suggests that

MRAP2 has retained the original function, whereas the function of MRAP1 has diverged. Accordingly, the functional expression of elephant shark MC2R is independent of the action of MRAP1 or MRAP2 (Reinick *et al.* 2012b). Dores (2013) suggested that the ancestral MC2R, present in the early gnathostome did not require MRAP1 for signaling. At some point after the diversification of cartilaginous and bony fish, the MC2R lost its function, particularly the capacity to traffic to the plasma membrane, but this function was subsequently rescued by MRAP1 in a nice example of gene co-evolution.

Melanocortin Receptors

Melanocortin exerts its physiological role by binding to a family of specific G protein-coupled receptors (GPCR) that positively couple to adenylyl cyclase. MCRs belong to the A-13 family of the rodopshin class of GPCRs. This family of receptors seems to be a chordate gene family since orthologues have been annotated in the genomes of hagfish, lamprey, elasmobranch, teleost and tetrapod species but they are absent in the genome of echinoderms, cephalocordates or urochordates as well as in protostomes (Vastermark and Schioth 2011). Tetrapod species have five melanocortin receptors (MC1R-MC5R). In mammalian systems, melanocortin MC2R receptor is specific for ACTH. The four other MCRs bind to MSHs, with MC1R and MC3R exhibiting the highest affinity for α-MSH and γ-MSH, respectively (reviewed by Schioth *et al.* 2005). However, in elasmobranchs, all five MCR orthologues have not been characterized in a single species. MC1R, MC2R and MC3R has been characterized in elephant shark (Callorhynchus milii, Vastermark and Schioth 2011) whereas MC3, MC4R and MC5R have been cloned in spiny dogfish (Squalus acanthias, Klovins *et al.* 2004b; Ringholm *et al.* 2003). The number of receptors diverges in teleost fish. Zebrafish has six MCRs, with two copies of the melanocortin MC5R, while pufferfish has only four, with no melanocortin MC3R and only one copy of melanocortin MC5R (Logan *et al.* 2003). The evolution of this family of receptors has been reviewed previously (Dores 2013; Kumar *et al.* 2011; Liang *et al.* 2013; Logan *et al.* 2003; Schioth *et al.* 2005; Takahashi and Kawauchi 2006; Vastermark and Schioth 2011) but discrepancies have arisen when explaining the evolutionary relationship and the origin of MC5R. If we follow the 2R script, the ancestral gnathostome should have at least four paralogues and it is plausible to think that one of these receptors arose from a local duplication event (figure 3).

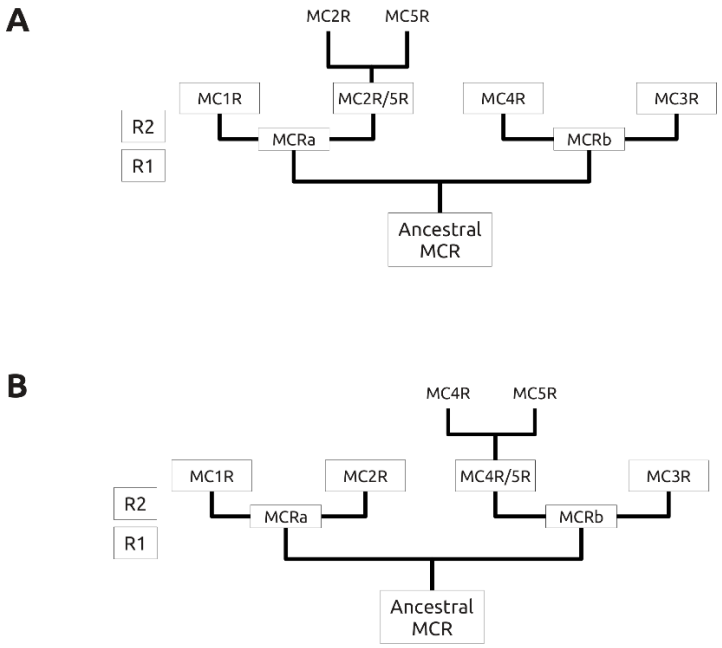

Figure 3. Evolutionary schemes showing alternative hypotheses for the evolution of melanocortin receptors. A) Evolutionary hypothesis and nomenclature proposed by Baron *et al.* (2009). B) Evolutionary hypothesis and nomenclature proposed by Vastermark and Schioth (2011). See text for details.

The analysis of several vertebrate genomes revealed that *mc1r*, *mc2r*, *mc3r*, and *mc4r* are all located in different chromosomes, suggesting that these receptors were the result of genome duplications (R). In contrast, *mc5r* is found always on the same chromosome as MC2R but in the opposite direction (Logan *et al.* 2003; Schioth *et al.* 2005). This tail to tail localization suggests that MC2R and MC5R could be descendants of a common ancestor (MC2R/MC5R) by a local duplication event. This event might have occurred in the ancestral gnathostome since elasmobranchs exhibit both MC2R and MC5R. Supporting this idea, it has been shown that melanocortin receptors lack introns with the exception of a spliced variant of MC1R. However, fugu MC2R and MC5R exhibit one and three introns, respectively. The position of the MC2R intron in the DRY motif is conserved with the position of one of the introns in MC5R, suggesting the close phylogenetic relationship between both receptors. Logan *et al.* (2003) suggested that this retained intron in Fugu was common to the whole MCR family but was lost from all members of the gene family. However, the genetic code in the DRY motif can form a protosplice site, which would seem to be a target for the insertion of spliceosomal introns through a process called reverse splicing (Vastermark and Schioth 2011). Introns in this same sequence DRY can be found in many GPCRs, suggesting that these introns may have been inserted later in the evolution of Fugu as a result of recent insertions (Schioth *et al.* 2005). Based on chromosome localization and the gene structure of MCRs, Baron *et al.* (2009) proposed the evolutionary sequence shown in figure 3A, in which the most ancient known versions of MCRs are found in lampreys, i.e. MCRa and MCRb (Haitina *et al.*, 2007). These receptors represent two branches of the ancestral MCR duplication that probably took place during 1R. MCRa was then duplicated into MC1R and the ancestral MC2R/MC5R, whereas MCRb split into MC3R and MC4R (figure 3A). The ancestral form of MC2R/MC5R was then duplicated into a local event to render MC2R and MC5R.

Recently, Vastermark and Schioth (2011) have challenged this hypothesis using the latest phylogenetic methods and shown that MC4R and MC5R are the most closely related members and MC2R and MC5R are the most divergent. Therefore, MC4R and MC5R cluster together with MC3R and lamprey MCRb. On the other hand, MC1R and MC2R cluster together with lamprey MCRa as basal sequence. These phylogenetic relationships have led authors to reconsider the evolutionary hypothesis of the MCR family, as shown in figure 3B. In this scheme, the sequences of the cyclostome/gnathostome ancestor generated the two main evolutionary branches of MCRs after 1R. MCRa was then duplicated into MC1R and MC2R, whereas MCRb split into MC3R and the ancestral version of MC4R/MC5R, which later split into MC4R and MC5R. Therefore, this hypothesis lends weight to the idea that MC5R originated as a result of a local duplication of MC4R, probably in the ancestral gnathostome, and then the MC5R locus was moved to the chromosome carrying the MC2R locus. Alignment of melanocortin receptor amino acid sequences and phylogenetic reconstructions are provided in supplementary figures 1 and 2, respectively. Additional reconstructions were reported in Logan *et al.* (2003), Schioth *et al.* (2005), Kumar *et al.* (2011) and Vastermark and Schioth (2011). Gene neighbor analysis have been reported in Logan *et al.* (2003), Schioth *et al.* (2005) and Kumar *et al.* (2011).

The other intriguing aspect of the MCR family evolution is the absence of MC3R in most teleost fishes but this receptor is present in the genome of cartilaginous, amphibian, avian and mammalian species (Schioth *et al.* 2005; Vastermark and Schioth 2011). Some fishes also exhibit MC3R including spotted gar and coelacanth (Vastermark and Schioth 2011) but the only known teleost fish exhibiting MC3R is the zebrafish (Logan *et al.* 2003) and goldfish. Kumar *et al.* (2011) reported that zebrafish MC3R does not share synteny relationship with the tetrapod MC3R. After analysis of the presumed zebrafish MC3R locus and posterior comparison with the genome of other ray-finned fishes, the locus is filled by the thyrotropin-releasing hormone receptor 3 (TRHR3). In addition, Bayesian phylogeny predicts the branching out of zebrafish MC3R earlier that elephant shark more suggesting that MC3R has a different origin. MC3R has been reported as the mammalian γ-MSH receptor (Roselli-Rehfuss *et al.* 1993). Interestingly, teleost fish lacks melanocortin MC3R receptor and γ-MSH domain in the POMC gene, suggesting a coevolutionary process of the peptide/receptor system.

Concluding remarks

The melanocortin system is a complex hormonal system of the vertebrates that probably arose prior to the emergence of jawless vertebrates, over 500 MYA ago. The system was diversified during the two genome duplication rounds. Several evolutionary processes are represented within this family including internal tandem duplications within the POMC gene to give rise to up to four different MSH sequence cores or local duplication to generate two different receptors MC2R/MC5R or MC4R/MC5R according to Dores's or Schiöth's hypothesis, respectively. New intron acquisition by reverse splicing and, of course, gene loss processes after genome duplication. The evolution of the family is linked also to putative co-evolutionary processes nicely represented by the lack of MC3R and the main

receptor ligand γ-MSH in teleost fish and the possible rescue of MC2R function by the functional divergence of the MRAP2 copy, now named MRAP1. Recently, we demonstrated that MC4R/MRAP2 interaction provides ACTH sensitivity to the receptor (Agulleiro *et al.* 2013a). We also think that these interactive processes have evolutionary importance since the receptor/accessory protein interaction could provide new mechanisms to the GPCRs for modifying the binding spectrum and also result in sensitivity of new target tissues to the peptide hormones.

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CAPÍTULO II: Involvement of melanocortin receptor accessory proteins (MRAPs) in the function of melanocortin receptors

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Resumen

El sistema de melanocortina integra diferentes agonistas, agonistas competitivos o inversos y receptores. Investigaciones recientes también han descubierto un sistema específico de proteínas accesorias de los receptores de melanocortina (MRAP), las cuales están involucradas en la regulación de la expresión funcional de estos receptores. Las mutaciones de MRAP1 son responsables de la deficiencia familiar de glucocorticoides tipo 2 (FGD2), un raro trastorno autosómico que se caracteriza por altos niveles plasmáticos de hormona adrenocorticotropina (ACTH), pero una severa deficiencia de cortisol. La ACTH se une al receptor de melanocortina 2 (MC2R), un receptor acoplado a proteína G, en la glándula suprarrenal para promover la síntesis de corticosteroides. En ausencia de MRAP1, MC2R no puede translocarse desde el retículo endoplásmico a la membrana plasmática y la señalización inducida por ACTH se extingue. Una segunda proteína MRAP, llamada MRAP2, también modula la actividad de MC2R. Los MRAP también interactúan con los otros receptores de melanocortina, ajustando sus propiedades farmacológicas. En este artículo, revisamos brevemente el sistema MRAP y su interacción con los receptores de melanocortina.

Abstract

The melanocortin system integrates different agonists, competitive or inverse agonists, and receptors. Recent investigations have also discovered a specific system of melanocortin receptor accessory proteins (MRAPs) that are involved in the regulation of the functional expression of these receptors. MRAP1 mutations are responsible for type 2 familial glucocorticoid deficiency (FGD2), a rare autosomal disorder characterized by high plasma adrenocorticotropin hormone (ACTH) levels but severe cortisol deficiency. ACTH binds melanocortin 2 receptor (MC2R), a G protein-coupled receptor, in the adrenal gland to promote corticosteroid synthesis. In the absence of MRAP1, MC2R cannot translocate from the endoplasmic reticulum to the plasma membrane and ACTH-induced signaling is extinguished. A second MRAP protein, called MRAP2, also modulates MC2R activity. MRAPs also interact with the other melanocortin receptors, adjusting their pharmacological properties. In this paper, we briefly review the MRAP system and its interaction with melanocortin receptors.

Melanocortin system

Melanocortins are posttranscriptional products of a complex precursor named proopiomelanocortin (POMC). They are mainly comprised of an adrenocorticotropic hormone (ACTH) and melanocyte-stimulating hormones (α-, β- γ- and δ-MSH). Tetrapod POMC precursor comprises three main domains: The N-terminal pro-γ-MSH, the central ACTH and the C-terminal β-lipotropin. Each domain contains one MSH peptide delineated by a core sequence HFRW: γ-MSH in pro-γ-MSH, β-MSH as N-terminal sequence of ACTH and β-MSH in β-lipotropin domain (Nakanishi *et al.* 1979).

Tetrapod POMC is mainly produced in the vertebrate pituitary and its posttranslational processing occurs in a tissue-specific manner. The proteolytic cleavage of POMC by prohormone convertase 1 (PC1) generates ACTH and β-lipotropin (β-LPH) in the corticotrophs of the anterior pituitary, whereas cleavage by PC1 and PC2 produces α-MSH and β-endorphin in the melanotrophs of the *pars intermedia*. POMC is also centrally produced and mainly processed to α-MSH and β-endorphin (Castro and Morrison 1997). In rodents, two discrete groups of neurons in the hypothalamus (arcuate nucleus) and the medulla (nucleus of the *tractus solitarius*) also produce POMC (Bagnol *et al.* 1999). POMC mRNA expression within the mediobasal hypothalamus has been conserved throughout vertebrate evolution, as POMC transcripts have been detected in hypothalamic neuronal systems of amphibians (Tuinhof *et al.* 1998), birds (Gerets *et al.* 2000) and fish (Cerdá-Reverter and Peter 2003). However, no POMC expression within the vagal lobe, the teleostean nucleus of the *tractus solitarius*, has been reported in any teleost species.

Melanocortin exerts its physiological role by binding to a family of specific G protein-coupled receptors that positively couple to adenylyl cyclase. Tetrapod species have five melanocortin receptors (MC1R-MC5R). The extracellular and cytoplasmic tails of all of the melanocortin receptors are unusually short. In fact, the MC2 receptor is the smallest of the hundreds of identified GPCRs. In mammalian systems, MC2R is specific for ACTH. By contrast, the MSHs bind to the other four MCRs, with MC1R and MC3R exhibiting the highest affinity for α-MSH and γ-MSH, respectively (Schioth *et al.* 2005). Atypically, melanocortin signalling is not exclusively regulated by the binding of endogenous agonists, as naturally occurring antagonists, agouti-signalling protein (ASP) and agouti-related protein (AGRP) compete with melanocortin peptides by binding to MCRs. ASP is a potent melanocortin antagonist at melanocortin MC1R and MC4R receptors and relatively weak at MC3R. In contrast, AGRP is mainly produced within the hypothalamic arcuate nucleus and the adrenal gland, where it strongly inhibits melanocortin signalling at MC3R and MC4R, but it is not active at MC1R (Cone 2006). Several studies have demonstrated that mammalian ASIP and AGRP work as inverse agonists at MC4R, which is constitutively activated (Chai *et al.* 2003; Nijenhuis *et al.* 2001). Therefore, MC4R signals in the absence of agonist binding. AGRP or ASIP reduces constitutive MC4R signalling in a dose-dependent manner. The presence of melanocortin endogenous antagonist including AGRP and ASIP in fish was first demonstrated in goldfish (Cerda-Reverter *et al.* 2005; Cerdá-Reverter and Peter 2003) and later in several teleost species (Kurokawa *et al.* 2006; Murashita *et al.* 2009; Song *et al.* 2003).

Discovery of melanocortin receptor accessory protein (MRAP)

Of the five melanocortin receptors, only the activation MC2R fails when expressed in the conventional heterologous cell lines typically used to screen the activity of G protein-coupled receptors (GPCRs). Suitable expression data were obtained only when MC2R was expressed in adrenocortical-derived cell lines (Y6 or OS3), in which endogenous MCR expression is absent (Schimmer *et al.* 1995). The experiments in a range of non-adrenal cells suggested that the receptor cannot reach the plasma

membrane but is retained in the endoplasmic reticulum (ER). It seems that transmembrane domains (TM) 3 and 4 of the MC2R comprise an ER retention signal that can be transferred to other MCRs (Fridmanis *et al.* 2010). Therefore, the presence of an accessory factor that works as an MC2R-specific transport system in the adrenocortical cells was assumed (Noon *et al.* 2002). The response to this hypothesis was provided by Clark's group, who studied familial glucocorticoid deficiency (FGD) (Metherell *et al.* 2005). FGD is a rare autosomal disorder characterized by high plasma ACTH levels but severe cortisol deficiency with normal mineralocorticoid levels. Untreated patients may die because of hypoglycemia or recurrent infections. Mutations of MC2R only explain around 25% of FGD cases, and FGD caused by receptor mutations has been termed as type 1. Linkage studies provided evidence of a second FGD locus (Chan *et al.* 2011; Cooray and Clark 2011). Homozygosity mapping using single nucleotide polymorphism (SNP) microarrays identified a third locus on chromosome 21 in some families where FGD exhibited no MC2R mutations. Data analysis provided a single candidate region at 21q22.1. About thirty known/predicted genes were localized within the region but none was a reasonable candidate for FGD given its function. However, after RT-PCR expression screening only one of these genes was exclusively expressed in the adrenal cortex. Actually, this gene had been characterized previously as an upregulated gene during the differentiation of mouse 3T3-L1 cells from fibroblasts into adipocytes, and named as fat tissue-specific low molecular weight protein (Xu *et al.* 2002) before being renamed melanocortin 2 receptor accessory protein (Metherell *et al.* 2005). MRAP seems to be responsible for about 20% of FGD, and FGD due to mutations in MRAP is now known as FGD type 2. Metherell *et al.* (2005) showed that human (h) MRAP is similar to another gene (C6orf117) encoding a small single transmembrane–domain protein that is more than 27% identical to hMRAP in the N-terminal and transmembrane domains but differs at the C-terminus. This gene encodes a 205 amino acid protein now known as MRAP2 (Chan *et al.* 2009; Sebag and Hinkle 2009b). This introduces a degree of confusion into the nomenclature since most authors continue to call the first characterized protein MRAP rather than MRAP1. Subsequent studies identified MRAP orthologues in different species. We identified three different MRAPs in zebrafish, one MRAP grouped with tetrapod MRAP1s, and the two other MRAP sequences were classified as MRAP2a and MRAP2b. We also found MRAP1 and MRAP2 orthologues in most fish genomes screened but only zebrafish exhibited MRAP2 paralogues (Agulleiro *et al.* 2010; Cerdá-Reverter *et al.* 2011).

Structure of MRAPs and interaction with MC2R

Human MRAP1 is a small protein exhibiting a hydrophobic transmembrane domain (residues 38-60). MRAP1 is mainly expressed in the adrenal cortex but also in the testis, liver, brown and white fat, brain thyroid, breast and lymph nodes (Metherell *et al.* 2005; Xu *et al.* 2002). The hMRAP1 gene is alternatively spliced into two different proteins, i.e. hMRAPα and hMRAPβ, which contain 172 and 102 amino acids, respectively. Both proteins are identical in the first 69 amino acids that contain the N-terminal region (37 amino acids), the transmembrane domain (23 amino acids) and the first section of the C-terminal region (9 residues). The rest of the C-terminal region is fully divergent and seems to be involved in the fine tuning of MRAP intracellular localization and ACTH-induced cAMP

production (Roy *et al.* 2007; Roy *et al.* 2012). It has also been suggested that the C-terminal region can play a role in the amount of the receptor that reaches the cell surface because deletion of the C-terminus increases MC2R cell surface expression (Webb *et al.* 2009). hMRAP1 has no putative signal peptide, exhibits potential N-linked glycosylation sites (Asn-X-Ser/Thr) at position 3 and 6 and has a tyrosine-rich region within the N-terminal domain (residues 9-24).

The topology of the molecule suggests that MRAP1 is a type II integral membrane protein with an exoplasmic C-terminus. This means that the C-terminus faces the internal region of the endoplasmic reticulum (ER) and Golgi apparatus but turns towards the cell exterior when the protein reaches the plasma membrane. Metherell *et al.* (2005) corroborated this orientation by tagging the C-terminal domain of the hMRAP1 in CHO cells. Subsequent studies demonstrated that MRAP1 variants exhibit dual topology, meaning that both the N- and C-terminal regions can be found facing outward from the CHO (Sebag and Hinkle 2007) or HEK293 cells (Roy *et al.* 2007). Dual topology was demonstrated at different expression levels and the two orientations, i.e. N_{exo}-C_{cyt} and N_{cyt}-C_{exo}, are present in roughly a 1:1 ratio (Sebag and Hinkle 2007). On SDS-PAGE, hMRAP1 migrates with lower mobility than suggested by its molecular weight. Co-immunoprecipitation and mass spectroscopy studies in CHO and adrenal mouse Y1 cell lines suggest that MRAP exists as a homodimer (Cooray *et al.* 2008; Metherell *et al.* 2005). Sebag and Hinkle (2007) elegantly demonstrated that MRAP1 forms antiparallel homodimers and that both types of antiparallel complexes are able to interact with the MC2R in a similar way. Subsequently, Cooray *et al.* (2011) corroborated the formation of antiparallel homodimers by bioluminescence resonance energy transfer (BRET) but also demonstrated that MC2R forms homodimers in the ER. These MC2R homodimers form a complex with at least two antiparallel MRAP1 homodimers, making a hexameric complex that is probably stimulated by two ACTH molecules. Ligand binding induces a rapid conformational change and subsequent relaxation of the complex (Cooray *et al.* 2011). Sebag and Hinkle (2009b) also studied the structural requirements of MRAP1 to acquire the dual topology and to allow receptor trafficking and signaling. Deletion of the first 30 amino acids of the N-terminus or 64 of the 67 residues in the C-terminus did not affect the dual topology. However, residues localized just prior to the transmembrane domain (31-37, LKANKHS) are critical for the dual topology to be adopted. The deletion of these residues forced MRAP into a N_{exo}-C_{cyt} topology but also prevented dimer formation, suggesting that the dual topology is required for dimerization. These residues, together with the transmembrane domain, are also involved in the functional expression of the receptor. When the MC2R is co-expressed with a mutant MRAP1 lacking the transmembrane domain, the receptor is trapped in the ER. Therefore, a transmembrane domain is sufficient for interaction with the MC2R but is insufficient to promote cell surface expression (Webb *et al.* 2009). The same occurred when the receptor was coexpressed with a single topology and monomeric MRAP1 mutant lacking residues 31-37. Interestingly, when MC2R is coexpressed with the mutant MRAP1 lacking residues 1 to 30, the receptor is unable to produce an ACTH-induced cAMP increase although easily reaches the plasma membrane (Sebag and Hinkle 2009b). Smaller deletions demonstrated that the motif between amino acids 18 and 21 (LDYI) is a key

region for ACTH-induced signaling. Therefore, when these amino acids were mutated to alanine the receptor reached the membrane but was unable to signal via cAMP after stimulation with ACTH. It seems that the inability to produce cAMP is a consequence of deficient ACTH binding, i.e. the binding of ACTH to MC2R co-expressing mutated MRAP1 (18-21A) is negligible. These results contrast with those reported by Webb *et al.* (2009), who showed that deletions of the fragment (9-24), that includes the tyrosine-rich region, precludes MC2R trafficking. This region is not needed for MRAP trafficking to the membrane but it could play a role in the stabilization of the MRAP1/MC2R complex or, alternatively, could interact with other proteins that regulate the surface expression of the complex (Webb *et al.* 2009). Within the latter domain, the motif EYY is fully conserved in all MRAP1 sequences characterized to date and could play a key role in the control of receptor trafficking (Agulleiro *et al.* 2010).

Using a mutagenesis approach involving the systematic construction of chimeric MC2R/MC4R, Fridmanis *et al.* (2010) hypothesized a model for the interaction of MRAP and MC2R. Data suggest that MRAP1 interacts with the TM4 and TM5 of the MC2R, which are important for the binding of the four basic amino acids in ACTH. However, data reported by Hinkle *et al.* (2011) did not support the model since the substitution of TM4 and TM5 of the MC2R with the MC4R did not impair MRAP dependence.

The structure of MRAP2 and interaction with the MCRs has not been studied in depth. The human MRAP2 gene consists of 4 exons that encode a 205 amino acid protein with a predicted molecular mass of 23.5 kD (Chan *et al.* 2009). Molecular modeling also predicts the presence of a transmembrane domain which is fully conserved among MRAP2 sequences (Agulleiro *et al.* 2010). Similar to MRAP1, the C-terminal domain is not conserved but it is noticeable that five amino acids within this region (IPNFV) are fully conserved in all MRAP2 sequences. MRAP2 is also a glycosylated protein and exhibits a tyrosine-rich domain in the N-terminal region. The residues localized immediately before the transmembrane domain of hMRAP1 (31-37, LKANKHS), which are critical for the dual topology to be adopted, only show one substitution compared with hMRAP1. However, the region containing the motif LDYI, responsible for ACTH binding and induced signaling, is not conserved (Agulleiro *et al.* 2010; Chan *et al.* 2009). In fact, orientation analysis in CHO cells demonstrates that both ends of the MRAP2 are detectable on the extracellular side of the membrane. MRAP2 facilitates MC2R trafficking to the membrane (Agulleiro *et al.* 2010; Chan *et al.* 2009; Sebag and Hinkle 2009b) but data on functional activation by ACTH are controversial. Co-immunoprecipitation studies demonstrated that MRAP2 interacts with the MC2R and fully rescues the functional expression of the receptor. In addition, non-glycosylated MRAP2 assists MC2R to reach the plasma membrane but the receptor cannot respond to the ACTH stimulation. These data suggest that MRAP2 has two independent functions, (i) assisting the traffic of MC2R to the plasma membrane and (ii) enabling the receptor to respond to ACTH (Chan *et al.* 2009). However, Sebag and Hinkle (2009b) reported that MC2R reaches the cell membrane in the presence of MRAP2 but that the increase in ACTH-induced cAMP was much lower (8-fold) than that exhibited in cells expressing

both MC2R and MRAP1. When the LDYI motif was introduced into the MRAP2 sequence, the co-expressed MC2R was able to respond to ACTH to a similar extent to that observed in cells co-expressing MC2R/MRAP1. Subsequently, it was demonstrated that MRAP2 is an endogenous inhibitor of MC2R activation and competes with MRAP1 to bind to the receptor, thus decreasing the ability of ACTH to stimulate cAMP production. Also, ACTH binds to MC2R with high affinity when co-expressed with MRAP1, but not with MRAP2 (Sebag and Hinkle 2010).

In our hands, zfMRAP2a was able to partially promote the presence of zfMC2R in the membrane but not ACTH-induced cAMP synthesis, while the co-expression of zfMRAP2a with zfMRAP1 enhanced the response of MC2R to ACTH stimulation (Agulleiro *et al.* 2010). Zebrafish genome also exhibits a paralogue gene of MRAP2 named MRAP2b. The protein is well conserved within the N-terminal domain but, again, it shows high divergence within the C-terminal region, also when compared with zfMRAP2a. Co-expression of MRAP2b with zfMC2R does not promote the functional expression of the receptor. Tagging experiments demonstrated that MRAP2b co-localized with the zfMC2R in a perinuclear region and in the cytoplasm and was therefore unable to promote zfMC2R traffic to the membrane and an increase in ACTH-induced cAMP. When Doufexis *et al.* (2007) investigated the interaction of the C-terminal tail of the MC2R with nucleoporin 50, they found that the stimulation of HEK cells expressing MC2R with ACTH promoted a gradual translocation of the MC2R-Nup50 complex from the membrane to the nucleus. It is thus possible that MRAP2b could traffic zfMC2R to the nuclear membrane or play a role in the nuclear translocation process (Agulleiro *et al.* 2010). Both zfMRAP2a and zfMRAP2b cooperate with MRAP1 to intensify the ACTH-induced cAMP increase mediated by zfMC2R (Agulleiro *et al.* 2010).

Recent research in cartilaginous fish have characterized the first MC2R that can be activated independently of MRAP in CHO cells. Therefore, trafficking to the membrane and functional activation do not require co-expression with exogenous MRAPs (Reinick *et al.* 2012b).

MRAP effects on the function of other MCRs

If the MRAP system assists MC2R functional expression, an obvious question arises as to whether this system is also involved in the function of the remaining melanocortin receptors. Immunoprecipitation studies demonstrated that both MRAP1 and MRAP2 interact physically with all five melanocortin receptors (Chan *et al.* 2009). Both MRAP1 and MRAP2 reduce the expression level of MC4R and MC5R but not of MC1R and MC3R in the plasma membrane. Accordingly, both MRAP1 and MRAP2 decrease NDP-MSH-stimulated cAMP production in cells expressing MC3R, MC4R and MC5R, but only MRAP2 was able to induce a similar effect on MC1R (Chan *et al.* 2009). In contrast to this, Sebag and Hinkle (2010) reported no effect of MRAP1 or MRAP1+MRAP2 on NDP-MSH-stimulated cAMP production mediated by MC4R expression in CHO cells. Studies on MC5R demonstrated that the receptor rapidly traffics to the membrane, but it is trapped intracellularly when coexpressed with MRAP1. The MC5R homodimerizes in the absence of MRAP1, as does MC2R, but the coexpression of MRAP1 inhibits MC5R homodimerization, unlike MC2R (Sebag and

Hinkle 2009a). Therefore, MRAP1 has diametrically opposite effects on two different MCRs. The MRAP1 promotes MC2R traffic to the plasma membrane but prevents the functional expression of MC5R. Experiments in cartilaginous fish (Scualus acanthias) have shown that MRAP1 can also increase the sensitivity of the MC5R to ACTH. In this species, the MC5R is activated with higher potency by ACTH (1-25) than other MSH peptides (Reinick *et al.* 2012a).

Concluding remarks and future directions

The melanocortin system is probably one of the most complex endocrine systems as it exhibits a complex precursor encoding different agonists (MSHs and ACTH) and a non-agonist (β-endorphin), five different receptors, the constitutive activity of some receptors (MC4R), endogenous inverse agonists (AGRP and ASIP) and receptor accessory proteins (MRAPs) that promote the presence of the receptor in the plasma membrane. The discovery of these accessory proteins has allowed proper characterization of the MC2R and also of the other MCRs. The new challenge will be to study how these MRAPs can modulate the function of the melanocortin receptors by regulating their mRNA expression, functional expression, agonist binding and signaling. In fact, MRAPs are excellent candidates for regulating MC2R sensitivity by modulating the functional expression. This regulatory function might be physiologically relevant in the modulation of stress responses, especially stress accommodation processes. The MRAP system could limit the presence of MC2R in the membrane and therefore decrease the response to ACTH. The same might also be true for the functions regulated by the remaining MCRs.

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CAPÍTULO III: Molecular Characterization and Functional Regulation of Melanocortin 2 Receptor (MC2R) in the Sea Bass. A Putative Role in the Adaptation to Stress

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Resumen

La activación del receptor de melanocortina 2 (MC2R) por ACTH, controla la cascada de señalización que conduce a la síntesis de esteroides en el tejido interrenal (análogo a la corteza suprarrenal en mamíferos) de los peces. Sin embargo, se sabe poco sobre la regulación funcional de este receptor en peces. En este trabajo, se describe la clonación del MC2R de lubina europea a partir de ADNc de hígado. SbMC2R requiere de la proteína accesoria del receptor de melanocortina 2 (MRAP) para su expresión funcional. El cortisol adicionado en el alimento, pero no los protocolos de estrés a largo plazo, disminuyeron la expresión de sbMC2R interrenal. Los datos sugieren la existencia de una retroalimentación negativa sobre la expresión de sbMC2R interrenal, impuesta por glucocorticoides locales o sistémicos. Esta retroalimentación podría estar involucrada en la adaptación al estrés a largo plazo, al regular la sensibilidad interrenal a la ACTH. La activación de MC2R inducida por ACTH estimula la lipólisis hepática, lo que sugiere que la ACTH puede mediar los efectos inducidos por el estrés "aguas arriba" de la liberación de cortisol.

Abstract

The activation of melanocortin 2 receptor (MC2R) by ACTH mediates the signaling cascade leading to steroid synthesis in the interrenal tissue (analogous to the adrenal cortex in mammals) of fish. However, little is known about the functional regulation of this receptor in fish. In this work described, we cloned sea bass MC2R from a liver cDNA. SbMC2R requires the melanocortin 2 receptor accessory protein (MRAP) for its functional expression. Dietary cortisol but not long-term stress protocols downregulated interrenal sbMC2R expression. Data suggest the existence of a negative feedback on interrenal sbMC2R expression imposed by local or systemic glucocorticoids. This feedback could be involved in long-term stress adaptation by regulating interrenal sensitivity to ACTH. ACTH-induced MC2R activation stimulates hepatic lipolysis, suggesting that ACTH may mediate stress-induced effects upstream of cortisol release.

Introduction

Melanocortin peptides are processed from the complex peptide precursor named proopiomelanocortin (POMC). They are mainly comprised of adrenocorticotropic hormone (ACTH) and melanocyte-stimulating hormones (α-, β-, γ- and δ-MSH). POMC is mainly produced in the pituitary and its posttranslational processing occurs in a tissue-specific manner (Castro and Morrison 1997). The proteolytic cleavage of POMC by prohormone convertase 1 (PC1) generates ACTH and β-lipotropin in the corticotrophs of the anterior pituitary, whereas cleavage by PC1 and PC2 produces α-MSH and β-endorphin in the melanotrophs of the pars intermedia (Seidah and Chretien 1999). The melanocortins exert its physiological role by binding to a family of specific G protein-coupled receptors (GPCR) that positively couple to adenylyl cyclase. Tetrapod species have five melanocortin receptors (MC1R-MC5R), although the number of receptors diverges in teleost fish. For example,

pufferfish have only 4 receptors with no melanocortin MC3R, while zebrafish (Danio rerio) has six MCRs, with MC3R and two copies of the melanocortin MC5R (Cerdá-Reverter *et al.* 2011).

The MC2R is specifically activated by ACTH, while the other MCRs can be activated by the MSHs as well as ACTH (Schioth *et al.* 2005). Cell surface expression of a functional MC2R strictly requires the presence of a single-transmembrane domain accessory protein called the MC2R accessory protein (MRAP). This protein interacts with the receptor to facilitate correct folding, subsequent glycosylation and cell surface expression (Metherell *et al.* 2005) but it is also essential for ACTH binding and ACTH-induced cAMP production (Agulleiro *et al.* 2010; Roy *et al.* 2007; Sebag and Hinkle 2009b). A recent study indicates that MC2R is present as a homodimer in the plasma membrane. This homodimer oligodimerizes with two MRAP homodimers to form a hexameric complex (Cooray *et al.* 2011). In the absence of MRAP, the MC2R is retained at the endoplasmic reticulum where cells cannot be stimulated by ACTH. In mammals, the alternative splicing of the last two exons of MRAP gene gives rise to two isoforms that differ in the C-terminal region (MRAPα and MRAPβ) (Metherell *et al.* 2005) but MRAP isoforms were not found in zebrafish (Agulleiro *et al.* 2010). In the latter species, MRAP and MC2R are mainly expressed within the interrenal (analogous to the adrenal cortex in tetrapods) and hepatic tissue, where they play a key role in interrenal stereoideogenesis and probably the hepatic lipid metabolism (Agulleiro *et al.* 2010). An additional paralogue of MRAP known as MRAP2 is also expressed in vertebrate species, including fish (Cerdá-Reverter *et al.* 2011). Because of the finding of MRAP2, the original protein responsible for the traffic of the MC2R to the plasma membrane should be called MRAP1 instead of MRAP.

As in other vertebrates, MC2R activation also plays an essential role in the stress response in fish. Following stressor exposure, the hypothalamic neurons release corticotrophin-releasing factor (CRF) to the anterior pituitary, which stimulates ACTH production and release from corticotrophs. With the permission of MRAP1, plasma ACTH activates interrenal MC2R, which results in the increased synthesis of cortisol, the main glucocorticoid in fish. Cortisol is released to the blood to regulate a wide array of systems in both stressed and non-stressed animals (Alsop and Vijayan 2009; Bernier 2006; Wendelaar Bonga 1997). Although *in vitro* and *in vivo* ACTH-induced interrenal steroideogenesis (Wendelaar Bonga 1997) is well established, few studies has focused on the MC2R function and its characterization in fish (Agulleiro *et al.* 2010; Aluru and Vijayan 2008; Klovins *et al.* 2004a; Metz *et al.* 2005). Our previous work demonstrated that sea bass (Dicentrarchus labrax) is very sensitive to stressful conditions (Rubio *et al.* 2010), which induce a severe decline in food intake levels and growth performance (Leal *et al.* 2011). To better understand this sea bass response to stressful conditions, we cloned MC2R (sbMC2R) and studied the liver and interrenal response to metabolic and chronic physical stress, respectively, as well as the cortisol-induced effects on MC2R interrenal expression.

Results

Molecular cloning sbMC2R

By means of RT-PCR and using degenerate primers designed against conserved regions of fish melanocortin receptor sequences, we cloned a 214 bp fragment showing high identity to the MC2Rs reported in other vertebrate species. The full cDNA sequence was obtained by screening a liver cDNA library with the fragment obtained by RT-PCR. The excised fragment contained an open reading frame of 909 bp that encodes a protein of 303 amino acid residues length protein with seven potential transmembrane domains. Similar to other melanocortin receptors, the sbMC2R orthologue exhibits short extracellular (ECL) and intracellular (ICL) loops, and shares cysteine residues at positions 234, 248 and 254 (sea bass numbering), which are fully conserved in all melanocortin receptors (Fig. 1 and http://www.gpcr.org). The deduced amino acid sequence displays a potential N-glycosylation site within the N-terminal domain at position 7 (Fig. 1). Sea bass MC2R shares the PMY motif in the first ICLD, which is conserved in most melanocortin receptors. The motif DRY in the ICL2, a consensus motif of the class A of rodopshin-like G-protein coupled receptor, is also present. Consensus recognition sites for protein kinase C (PKC) were found at positions 133 (ICL2), 217 (ICL3) and 289 in the extended intracellular tail (ICL4). Similarly, one cAMP- and cGMP-dependent protein kinases and two casein kinase II were found at positions 300 (extended intracellular tail), 28 (TM1) and 272 (TM7), respectively. The presence of consensus sites for diverse kinases suggests regulation of the receptor by phosphorylation. The identity of the deduced amino acid sequence ranged from 55-79% compared with other fish MC2R sequences, but was less than 44% compared with the tetrapod MC2R sequences. The identity was also unequally distributed. The average identity for the different segments of the receptor was calculated following the alignment shown in figure 1. The average identity for the different TMs was 54.1 % (TM1), 62.2 % (TM2), 68.6 % (TM3), 48.4 % (TM4), 48.9 % (TM5), 58.5 % (TM6) and 65.6 % (TM7). The highest identity values were found within ICL1 (73%), ICL2 (82.2%) and ECL4 (77.4%).

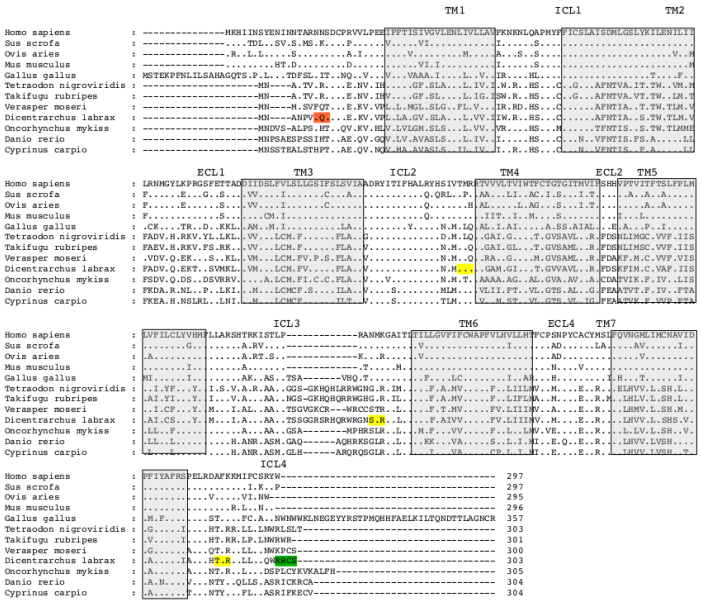

Figure 1. Alignment of MC2R amino acid sequences. Sea bass MC2R sequence is highlighted in bold letters. Dots indicate amino acids identical to the top sequence. Dashes were introduced to improve alignment. Grey boxes show putative transmembrane domains. Putative N-linked glycosylation sites are coloured orange. Blue indicates potential casein kinase II phosphorylation sites. Putative protein kinase C phosphorylation sites are coloured yellow. Green color frames show cAMP- and cGMP-dependent protein kinase phosphorylation sites. Sea bass MC2R sequence accession number FR870225.

Peripheral and central distribution of sbMC2R mRNA

Higher sbMC2R mRNA levels were detected by real time PCR in the liver, testis and head-kidney but expression level was also detected in the pituitary and spleen. Low levels were distinguished in the fat, muscle and skin (figure. 2).

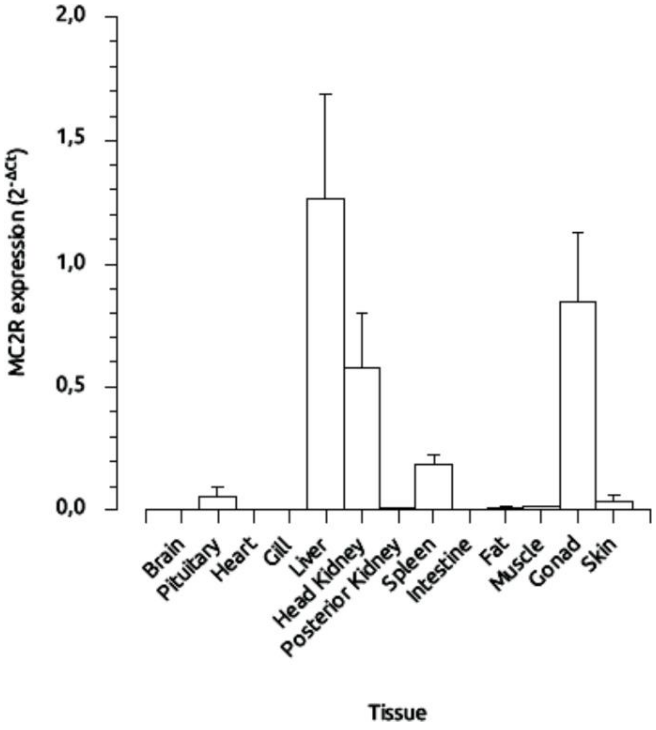

Figure 2. Distribution of sea bass MCR2 mRNA expression in different tissues, as revealed by q-PCR. β-actin was used as reference gene. See material and methods for details.

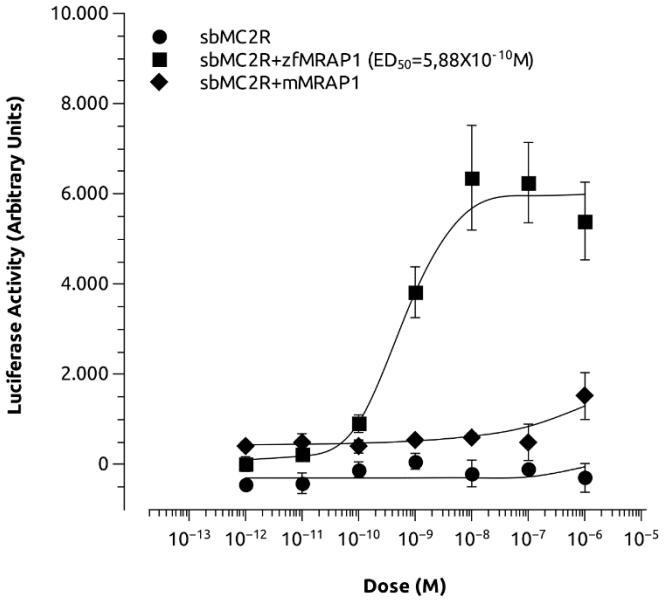

Figure 3. Effects of human ACTH (1-24) on luciferase activity in CHO cells transiently expressing sbMC2R, and zebrafish (zf)MRAP1 or mouse (m)MRAP1. Cells were transiently transfected also with a cAMP-responsive luciferase reporter gene.

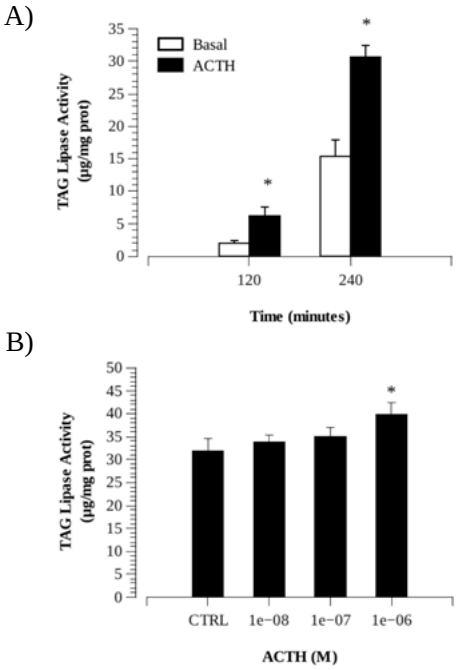

Figure 4. Effects of melanocortin agonist ACTH on hepatic lipolysis measured as releasing of non-esterified fatty acids (NEFA) after hormonal treatment to the culture media. A) Time course experiments using human ACTH at 10^{-6} M after 2 and 4 hours incubation. B) Dose-response experiments after 4h incubation.

SbMC2R activation by ACTH

For functional characterization of the sbMC2R, the coding region was directionally ligated into pcDNA3 and transiently expressed in CHO cells also producing luciferase under the control of cAMP responsive elements. The functional expression of MC2R requires the presence MRAP1 which was co-transfected with the sea bass receptor. Since sea bass MRAPs have not been characterized, we used mammalian (mouse) and zebrafish MRAP1. The stimulation of sbMC2R with hACTH(1-24) in the absence of MRAP1s did not induce any change in the luciferase activity. However, when zfMRAP1 was co-transfected the sbMC2R-induced luciferase activity increased in a dose response manner (ED_{50}=5,88 X 10^{-10}) (figure 3). When co-expressed with mMRAP1, the sbMC2R was able to respond only to the highest hACTH(1-24) doses (10^{-6} M).

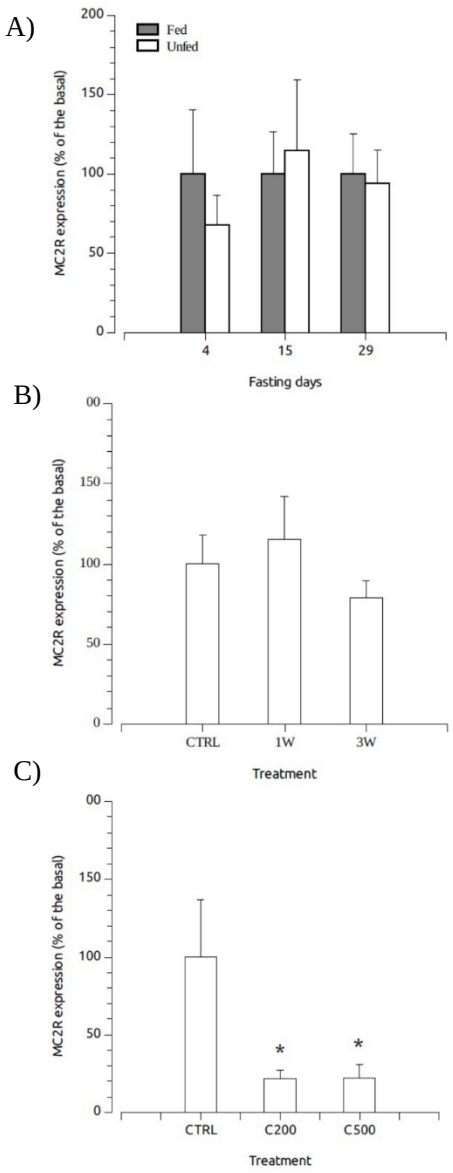

Figure 5. Physiological and hormonal regulation of MC2R expression. A) Effects of progressive fasting on liver MC2R expression. Data were expressed as percentage of the control level. B) MC2R expression levels in interrenal tissue of the sea bass after repetitive physical disturbance imposed by routine cleaning protocols with animals inside the tanks once (1W) or three times a week (3W) during 33 days. Control fish (CTRL) were never disturbed C) Interrenal MC2R expression levels in sea bass fed with cortisol-containing diets at doses of 0 (CTRL), 200 (C200) and 500 (C500) µg/g food. Animals were fed during 32 days using self-feeding systems. Asterisk indicates significant differences between control and treated fish after ANOVA followed by Tukey´s multiple range test ($P<0.05$). Gene expression levels were expressed as ratio specific mRNA/β-actin. Similar results were obtained when the expression levels were expressed as ratio specific mRNA/18S RNA. Data are mean ± SEM (n= 8).

ACTH effects on hepatic lipid metabolism

The incubation of liver slides with hACTH 10^{-6} M in HBS resulted in a significant increase in total NEFA concentration in the culture medium after 2 and 4 h (figure 4A). Only the highest hACTH concentration significantly stimulated NEFA production *in vitro* (figure 4B).

Effects of fasting and stress on sbMC2R expression

Quantitative PCR yielded no significant differences in hepatic MC2R-mRNA expression levels when fed and fasted animals were compared after 4, 15 and 29 days of fasting (figure 5A). Similarly, repetitive physical stressors induced no significant differences in the interrenal expression of the receptor (figure 5B). In contrast, long-term administration of cortisol-containing diets induced a significant decrease in interrenal sbMC2R expression (figure 5C).

Discussion

The present study demonstrates that sbMC2R requires the presence of MRAP1 to reach its functional expression. In the interrenal tissue, dietary cortisol decreased MC2R expression but chronic physical stressors had no effect. In addition, the receptor was profusely expressed in the sea bass liver where it stimulated lipolysis. Together, these data suggest that MC2R/MRAP1 activation by ACTH is essential in the regulation of fish stress response. Such a response could be partially orchestrated upstream of the concomitant ACTH-induced cortisol release. However, the regulation of MC2R by dietary cortisol suggests the existence of a short-loop feedback that could play an important role in the adaptation to stress.

Sequence comparisons show that the cloned receptor in the sea bass displays high identity to other vertebrate MC2R. Sequence analysis reveals that MC2Rs have a very short N-terminal region. In the sea bass, this region expands only the first 19 amino acids and exhibits one potential glycosylation site which is conserved in all MC2R molecules. The N-terminal region is critical in receptor trafficking since it partially determines the presence of the receptor in the plasma membrane (Fridmanis *et al.* 2010; Roy *et al.* 2010). The MC2R is intracellular arrested when expressed in non-adrenal cells and MRAP1 expression allows its cell surface functional expression (Metherell *et al.* 2005). The replacement of the MC2R N-terminal region by that from MC4R permits the surface expression, albeit inefficiently, demonstrating that the N-terminal region plays a key role in the intracellular retention of the receptor (Fridmanis *et al.* 2010). N-linked glycans are also required for plasma membrane targeting of MC2R. The absence of N-glycosylation in the human MC2R blocks the surface expression of the receptor (Roy *et al.* 2010). It has been proposed that the low N-glycosylation level of MC2R could constrain the ability of the receptor to reach the cell membrane. Oligomerization with two antiparallel MRAP1 molecules (Cooray *et al.* 2011; Sebag and Hinkle 2007) could increase the N-glycosylation level of the MC2R-MRAP1 complex, since MRAP1 exhibits two sites for N-linked glycosylation, thus facilitating surface expression (Chen *et al.* 2007; Fridmanis *et al.* 2010; Hinkle *et al.* 2011).

Pharmacological experiments demonstrated that sbMC2R requires the presence of MRAP1 for its functional expression in CHO cells. In the absence of MRAP1, sbMC2R did not respond to hACTH(1-24) stimulation, whereas co-expression of mammalian MRAP1 in the cell system allowed

only a very discrete receptor response. Therefore, the MRAP1 dependence of MC2R could be an ancestral requirement of this receptor but recent experiments with elephant shark (Callorhinchus milii) have reported that elasmobranch MC2R responds to ACTH independently of MRAP1 (Reinick *et al.* 2012b). We have previously demonstrated that zfMC2R requires zfMRAP1 coexpression to traffic to the plasma membrane and to generate a signal in response to ACTH (Agulleiro *et al.* 2010). Here, we further shown that mammalian MRAP1 cannot interact efficiently with sbMC2R, to allow an intracellular signal in response to hACTH(1-24). Similar results were obtained also in rainbow trout (Onchorynchus mykiss) (Liang *et al.* 2011). In some species, such as tilapia (Oreochromis mossambicus) or barfin flounder (Verasper moseri), MC2R was shown to be activated by different isoforms of MSH by using interrenal primary cell cultures but interrenal MC5R expression was also reported (Kobayashi *et al.* 2011; Lamers *et al.* 1992). We did not test the effect of MSHs on sbMC2R activity but our previous studies in zebrafish demonstrated that α-MSH was not able to stimulate the receptor (Agulleiro *et al.* 2010). Similar results were also obtained in trout and carp by using primary cultures and measuring the cortisol released to the culture medium (Aluru and Vijayan 2008; Metz *et al.* 2005).

In mammals, MC2R is predominantly expressed in the adrenal cortex, where it regulates adrenal steroid synthesis and secretion. In teleosts, the steroidogenic cells, together with closely intermingled chromaffin cells, are embedded in the head kidney forming the interrenal organ, homologue of the mammalian adrenal gland (To *et al.* 2007). We show that sbMC2R is densely expressed in the head kidney but also in liver, spleen, and testis. In addition, some level of expression was detected in the pituitary. A similar distribution was reported in trout, carp, zebrafish and flatfish (Agulleiro *et al.* 2010; Aluru and Vijayan 2008; Kobayashi *et al.* 2011; Metz *et al.* 2005). Both head-kidney and spleen contain a high number of macrophages (Verburg-van Kemenade *et al.* 2011), suggesting that MC2R activation could be involved in the modulation of the sea bass immune system, as previously proposed (Mola *et al.* 2005). Microarray studies have demonstrated the effects of handling stress on the expression of immune system-related genes in fish (Krasnov *et al.* 2005). In addition, ACTH has been shown to modulate cytokine expression in the gilthead seabream (Sparus aurata) (Castillo *et al.* 2009). Therefore, ACTH-MC2R-MRAP1 interaction in the immune tissue could represent an endocrine pathway that regulates the effect of stress on the immune response upstream of cortisol release by interrenal tissue.

In our experimental conditions, the chronic physical stressors could not regulate interrenal sbMC2R expression but when cortisol was added to the diet daily, a significant reduction in the interrenal expression levels was consistently detected. Experiments in dogs have demonstrated that the abundance of the mRNA encoding MC2R is significantly downregulated in cortisol-secreting adrenocortical carcinomas (Galac *et al.* 2010). This suggests that cortisol regulates interrenal expression of the receptor and by extension the sensitivity to ACTH in a negative short-loop feedback.

In vitro experiments in rainbow trout have demonstrated that ACTH can upregulate interrenal receptor expression (Aluru and Vijayan 2008). Therefore, ACTH could increase its own interrenal responsiveness but cortisol, synthesized and secreted in response to the systemic ACTH, would modulate negatively the system to reach the original receptor levels prior to stimulation. This regulatory system could take part in the stress adaptation of the HPI axis. The existence of a negative intra-adrenal feedback loop has previously been suggested in mammals (Gummow *et al.* 2006). Studies in carp have shown that restraint stress for 1 day did not induce changes in the interrenal MC2R expression but a significant reduction was seen after 7 days of treatment (Metz *et al.* 2005). Results obtained in trout also demonstrated an acute-stressor-induced elevation of plasma ACTH and interrenal MC2R expression (Aluru and Vijayan 2008) 4 hours post-stressor. In our experiment, sea bass were physically disturbed once or three times a week for 33 days. At the end of the experiment, the plasma cortisol levels were monitored and no differences were found. However, previous pilot experiments demonstrated that this stress protocol induced a significant increase in plasma cortisol after 2 hours (Leal *et al.* 2011). It is therefore plausible that during chronic stress, sea bass cannot maintain high corticosteroid levels, because of metabolic implications. These high cortisol levels could counteract ACTH-induced MC2R autoregulation, leading to a normalization of interrenal receptor levels, ACTH responsiveness and, by extension, to the normalization of cortisol levels. Previous studies in the sea bass showed that short-term confinement (4 h) did not induce a loss of interrenal sensitivity to ACTH (Rotllant *et al.* 2003). However, long-term crowding (23 days) in gilthead seabream reduced the sensitivity of interrenal tissue to ACTH stimulation (Rotllant *et al.* 2000). This hypothesis would support the absence of differences in cortisol plasma levels and MC2R mRNA during long-term stress protocols in the sea bass (Leal *et al.* 2011).

There are no studies reporting the functional involvement of MC2R in the brain of vertebrates. In mammals and avian species, MC2R does not seem to be expressed in the brain (Jacobs *et al.* 2002; Takeuchi *et al.* 1998; Xia and Wikberg 1996) but several studies in fish, including rainbow trout (Oncorhynchus mykiss) (Aluru and Vijayan 2008), Fugu (Takifugu rubripes) (Klovins *et al.* 2004a) and barfin flounder, (Verasper moseri) (Kobayashi *et al.* 2011), have demonstrated that this receptor is profusely expressed in the central nervous system. However, studies in carp (Cyprinus carpio) (Metz *et al.* 2005) and zebrafish (Danio rerio) (Chen *et al.* 2007) failed to show central MC2R expression. The expression of MC2R in the fish brain is intriguing and it could be relevant in the regulation of the central response to stressors upstream of cortisol release. However, more studies involving *in situ* hybridization and or ACTH binding are required.

In the gonadal tissue, sbMC2R was expressed in the testis and residual levels were found in the ovary (not shown). Experiments in rat have demonstrated that ACTH stimulates testosterone production in neonates, while adult Leydig cells were insensitive to melanocortin peptides (O'Shaughnessy *et al.* 2003). Interestingly, gonadotropin-independent precocious puberty has been

reported in boys with a congenital mutation in *DAX 1* that results in adrenocrotical hypoplasia, increased testosterone and ACTH levels (Domenice *et al.* 2001). Although stress effects of fish reproduction are documented (Schreck 2010), to the best of our knowledge, no studies have focused on the effects of ACTH on the fish male reproductive axis. However, studies in zebrafish have reported the involvement of ACTH in gonadotropin-stimulated estradiol release form ovarian follicles (Alsop *et al.* 2009). Altogether, MC2R expression in the sea bass testis suggests that ACTH could be involved in the regulation of the testicular function and, particularly, in the regulation of the steroidogenic pathways.

In a previous work, we demonstrated the effect of MTII (melanotan II) on the hepatic lipid metabolism of the sea bass (Sanchez *et al.* 2009), an effect probably mediated by the expression of MC5R in the liver. Following the same protocol, we now demonstrate that ACTH can also induce hepatic lipolysis, measured as free fatty acid release into the culture medium after melanocortin agonist exposure of liver fragments. The lipolytic effects were only detected when the highest doses of hACTH (10^{-6} M) were used. It contrasts with the high sensitivity of the receptor by ACTH measured by *in vitro* expression of the MC2R in CHO cells. This discrepancy can be attributable to the different culture systems (monolayer cell culture *vs* tissue fragments), MC2R expression levels and/or evaluated response (luciferase activity *vs* lypolysis). It is well known that stress activation of the hypothalamus-pituitary-interrenal (HPI) axis contributes in a key way to restoring energy homeostasis by mobilizing fuel stores to make energy available for increased metabolic demand (Mommsen *et al.* 1999). Chronic stress induces a severe decline of the hepatosomatic and mesenteric fatty index in the sea bass as well as in the food intake levels and growth. However, no differences in plasma cortisol levels were detected (Leal *et al.* 2011). It is thus conceivable that ACTH promotes hepatic lipolysis independently of cortisol release. We thought that fasting might regulate hepatic sensitivity to ACTH by modulating receptor expression levels, but long-term fasting had no effect on hepatic sbMC2R expression.

In summary, the sbMC2R orthologue was cloned form a liver cDNA library. Comparative analysis of MC2R sequences suggests that ICL2 and ICL3 are key areas of receptor activation, whereas ECL4 could be involved in agonist binding. Functional expression in non-adrenal cells requires the presence of MRAP1. Interrenal expression was modulated by dietary cortisol, suggesting the existence of a negative feedback on MC2R expression imposed by local or systemic glucocorticoids. This feedback could be involved in long-term stress adaptation by regulating interrenal sensitivity to ACTH via the modulation of MC2R expression, resulting in normalized plasma glucocorticoid levels. The fact that ACTH-induced MC2R activation stimulates hepatic lipolysis suggests that ACTH mediates stress-induced effects upstream of cortisol release.

Material and methods

Animals and reagents

One year old immature sea bass were maintained in 2000L tanks supplied with continuously aerated running sea water and equipped with an automatic feeder activated by a string sensor placed 3 cm below the water surface. The feeders were connected to a computer system that recorded the date, the time and the tank from which each food demand originated. The number of demands was integrated every 5 minutes. Animals were maintained under natural conditions for at least six months and self-fed with a commercial diet (Mistral 21, Proaqua Nutrición, S.A.; 43% protein, 23% fat, 20% carbohydrates, 6% ash, gross energy 22.5 kJ/g, in 3 mm standard pellets). Before the experiments, fish were placed in experimental 500 L tanks, continuously supplied with running seawater and provided with identical self-feeding systems and acclimated for at least one week. Prior to netting, animals were pre-anaesthetized in 2-phenoxy-ethanol (0.005%) for 3-5 minutes in their home tanks. Subsequently, the animals were removed from their home tanks and anesthetized for 2 min in the same anesthetic (0.05%) in the sampling tank. The day before samplings, sensors were removed from the water at 10.00 am, the time at which sampling always started. When required, the experimental animals were sacrificed by rapid decapitation. All experiments were carried out in accordance with the principles published in the European animal directive (86/609/EEC) for the protection of experimental animals and approved by the Consejo Superior de Investigaciones Científicas (CSIC) ethics committee (project number AGL2007-65744-C03-02). Unless otherwise indicated, all reagents were purchased from Sigma (St Louis MO, USA).

Cloning procedure

Genomic DNA isolated from blood was used as template for touchdown PCR reactions with Taq DNA polymerase (Invitrogen) and degenerate primers designed against conserved regions of the known MC2R sequences. The 5´ primer (MCR2Fw2) was a 18-mer with the sequence: 5´ CCATGCCMRSARGATTGC 3´. The 3' primer (MCR2Rv2) had the sequence 5´ TGYAGCTSRAASAKAGATCGGT 3´. PCR products of about 214 base pair (bp) were isolated from low melting point (LMP) Nusieve GTG agarose gel (FMC) ligated into pGEM-T easy vector (Promega) and subsequently transformed into XLI-Blue E. coli. One clone that contained an insert of the expected size was sequenced. Subsequently, filters from a sea bass liver ZAP cDNA library (kindly supported by Dr. Giovanni Bernardini from the Department of Biotechnology and Molecular Sciences of University of Insubria, Italy) containing approximately $5x10^5$ clones were screened with the previous sbMC2R fragment obtained by PCR (see above). Membranes were prehybridized for at least 3 hours in hybridization solution (50 % formamide, 6X SSPE, 0.5 % SDS, 5 X Denhardt's solution and 10 mg/ml yeast tRNA type III (Sigma, St Louis, MO), 1 X SSPE containing 150 mM NaCl, 1 mM EDTA, 9 mM NaH2PO4, pH = 7.4). The probe was labeled with dCTP [α-32P] (Amersham Biosciences), using the random primer labeling kit (Invitrogen). Hybridization was carried out

overnight in fresh hybridization solution containing 10^6 cpm/ ml dCTP [α-32P] at 42 °C. Final washes were performed in 0.1 X SSPE at 60 °C. After three purification rounds, six positive phages were selected for *in vivo* excision. The DNA insert in excised pBluescript II SK vector was sequenced on both strands. The nucleotide sequence of sbMC2R has been deposited with EMBL Nucleotide Sequence Database under accession number FR870225.

Real time quantitative PCRs

For tissue distribution of sbMC2R expression, total RNA was purified from fresh tissues (testis, intestine, fat, liver, muscle, spleen, head kidney, posterior kidney, gill, skin, heart, pituitary and brain) and treated with RQ1-DNAse (Promega). Five micrograms were retrotranscribed using superscript II reverse transcriptase (Invitrogen) and random hexaprimers (Invitrogen). To evaluate gene mRNA levels from individual liver or head kidney total RNA was treated with RQ1-DNAse. Subsequently, three microgram aliquots were used as template for cDNA synthesis, which was primed as before.

One microlitre of cDNA (sbMC2R) or diluted cDNA (18S RNA and β-Actin) and primers (70 nM) were added to 7.5 µl of Sybr green PCR master mix (ABgene, Thermo Scientific, Spain) and the volume was adjusted to 15 µl with water. Primer sequence were qPCR_MC2R_F1 (5' TTGCAGTGGACCGTTACATC3') and qPCR_MC2R_R1 (5' GGCAACGAAGCAGATCATGA 3'). PCRs were carried out on an iCycler (Bio-Rad, Madrid, Spain). Data were analyzed with the ΔΔCt (cycle threshold) method. As internal controls fragments of the sea bass β-actin mRNA and 18S RNA were amplified, using primers qPCR_β-actin_Forw/qPCR_β-actin_Rev and qPCR_18S_Forw/qPCR_18S_Rev primers, respectively. Sequences were as follows: qPCR_β-actin_Forw 5' GCCGCGACCTTACAGACTAC 3' qPCR_β-actin_Rev 5' AGCACAGTGTTGGCGTACAG 3', 18S_Forw 5’ GCATGCCGGAGTCTCGTT 3’ and 18S_Rev 5´ TGCATG GCCGTTCTTAGTTG 3´. All samples were processed in duplicate.

Constructs and pharmacological experiments

The full coding region of the sbMC2R was amplified by PCR using the vector isolated from the library as template and the primers Hind-MC2R-Forwad primer (5' TATAAGCTTATGAATGCTAACCCAGTG 3') and XhoI-MC2R-Reverse (5' TTACTCGAGGTAAAGCACATATAAAGTGT 3'). The receptor was then directionally subcloned into pcDNA3 (Invitrogen) and sequenced on both strands. The synthesis of zfMRAP1, mMRAP1 and hMC2R constructs was described previously (Liang *et al.* 2011).

Experiments were done in transiently transfected CHO cells. CHO cells were grown in DMEM/F12 with 5% fetal calf serum at 37ºC in a humidified 5% CO_2 incubator. Cells were plated in 96-well dishes and transfected with 40 ng/well total DNAusing FugeneHD according to the specifications of the manufacturer. Typically, transfections were done with 13,3 ng/well of a cDNA

dependent luciferase reporter containing multiple copies of cAMP responsive element (CRE) from rat insulin promoter, 21,3 ng/well sbMC2R DNA and 5,3 ng/well MRAP1 or GFP DNA. After 24 hours the medium was replaced and 40 µl fresh media containing 20 µM forskolin or hACTH (1-24) (Phoenix Biochemicals). After 5 hours medium was removed and One step luciferase Step Reargent (Nanolight Technologies) was added. Luminiscence was read on a BioTek platereader. Values shown represent the mean SEM from triplicate wells in a representative experiment (see Liang *et al.* 2011; Reinick *et al.* 2012b for more details).

Effects of ACTH on hepatic lipolysis

To evaluate the effects of melanocortin agonist on hepatic lypolysis, the animals were sacrificed, and their livers carefully removed. Small liver slices (50-80 mg) were dissected and incubated in 1 ml HB medium (136.9 mM NaCl, 5.4 mM KCl, 0.81 nM $MgS0_4$, 0.44 mM KH_2PO_4, 0.33 mM Na_2HPO_4, 5 mM $NaHCO_3$, pH= 7.6), containing 5mM glucose, 1.5 mM $CaCl_2$ and 2.5% fat-free BSA for 60 min at 25ºC. Subsequently, the medium was removed and slices were incubated with 1 ml of HBS containing human ACTH 10^{-6} M (Bachem). After 2 and 4 h at 25ºC the medium was removed for the determination of non-esterified fatty acids (NEFA) using commercial kits (WAKO Diagnostics) and following the supplier's recommendations. Liver slides were mechanically homogenized for protein determination using the BCA protein assay kit (Pierce). Subsequently, dose-response studies were made by incubating liver slides with hACTH in HB ranging from 10^{-6} to 10^{-9} M for 4 h. Experiments were always performed in quadruplicate wells and repeated at least three times independently.

Effects of progressive fasting on liver sbMC2R expression

To evaluate the effects of fasting on liver sbMC2R expression, ten groups of 10 fish each [body weight (BW) = 117±1.54 g] were adapted for one-week to individual 500-litre aquaria and fed *ad libitum* at 9.00 a.m. After this acclimation period, five groups were hand fed at the same ratio, and the other five were fasted. Fed and fasted groups were sampled at 12:00 (3 hours post-feeding in the case of the fed groups) at 4 and 15 and 29 days. Fish were decapitated and liver samples were dissected for immediate total RNA extraction. RNA samples were kept at -80ºC in 75% ethanol until cDNA synthesis for quantitative PCRs (see above).

Effects of cortisol and chronic physical stress on interrenal sbMC2R expression

A first trial was designed to evaluate the effects of chronic physical stress on interrenal sbMC2R expression. One hundred animals (BW= 221.82 ± 1.32 g L= 25.95 ± 0.047 cm) were distributed into 10 experimental tanks (n= 10) provided with automatic self-feeders. Three tanks were cleaned once a week (1W) and three tanks were cleaned three times a week (3W), whereas the four control tanks were never cleaned (CTRL). The cleaning protocol was always performed at 10:00 am and involved draining and brushing the tanks with the animals inside. The tanks were emptied until

the dorsal fins of the fish were exposed and then brushed for 2 minutes and immediately refilled. After 33 days, nine animals/treatment were sampled to obtain interrenal tissue samples.

A second trial was designed to evaluate the effects of cortisol administration on sbMC2R expression. Ninety animals (BW= 136 ± 0.96 g L= 22.63 ± 0.05 cm) were distributed into 9 experimental tanks (n= 10) provided with automatic self-feeders. The fish of three tanks were fed the control diet (CTRL), three tanks the cortisol-contaning food at 200 µg/g food (C200) and the remaining three tanks with cortisol-enriched diet at 500 µg/g food (C500) for 32 days. The animals were sampled as above, and tissue samples were kept at -80ºC until used. For detailed experimental design see Leal *et al.* (2011).

Data analysis and statistics

Sequence comparisons and alignments were performed using ClustalX. A phylogenetic tree was derived using public domain CulstalX, which uses the Neighbor-Joining method on a matrix of distances. The membrane protein secondary structure was predicted using the Split 4.0 Server (http://split.pmfst.hr/split/4/). In gene expression studies, specific mRNA levels were normalized as a ratio to 18S RNA. Statistical analysis was conducted by one-way analysis of the variance followed by Tukey´s multiple range test ($p<0.05$). Putative transmembrane domains were inferred by TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/).

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CAPÍTULO IV:

Melanocortin 4 receptor becomes an ACTH receptor by coexpression of melanocortin accessory protein 2

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Resumen

El receptor 2 de melanocortina (MC2R), es el único receptor canónico de ACTH. Su expresión funcional requiere la presencia de una proteína accesoria, conocida como proteína accesoria del receptor 2 de melanocortina 1 (MRAP1). El genoma de los vertebrados exhibe un gen parálogo llamado MRAP2, que se duplica en el pez cebra (MRAP2a y MRAP2b), aunque su función sigue siendo desconocida. En este artículo, demostramos que MRAP2a permite que MC4R, un receptor canónico de MSH, sea activado por ACTH con una sensibilidad similar a la exhibida por MC2R. Ambas proteínas interactúan físicamente y se coexpresan en las neuronas del área preóptica, una región clave en el control del equilibrio energético y la secreción hipofisaria en los peces. Inyecciones de ACTH inhiben la ingesta en peces cebra de tipo salvaje, pero no en peces cebra que carecen de MC4R funcional. Tanto MRAP1 como MRAP2a están regulados hormonalmente, lo que sugiere que estas proteínas son sustratos para las vías reguladoras de retroalimentación en la señalización de melanocortinas. El ayuno no tiene ningún efecto sobre la expresión central de MRAP2a pero estimula la expresión de MRAP2b. Esta proteína interactúa y se localiza junto con MC4R en las neuronas hipotalámicas tuberales, pero no tiene ningún efecto sobre el perfil farmacológico de MC4R. Sin embargo, MRPA2b es capaz de disminuir la actividad informadora basal en líneas celulares que expresan MC4R. Es plausible que MRAP2b disminuya la actividad constitutiva del MC4R durante los períodos de ayuno, conduciendo al animal hacia un balance energético positivo. Nuestros datos indican que las MRAP2 controlan la actividad de MC4R, abriendo nuevas vías para la regulación de la señalización de melanocortina y, por extensión, para la regulación del equilibrio energético y la obesidad.

Abstract

Melanocortin 2 receptor (MC2R) is the only canonical ACTH receptor. Its functional expression requires the presence of an accessory protein, known as melanocortin receptor 2 accessory protein 1 (MRAP1). The vertebrate genome exhibits a paralogue gene called MRAP2, which is duplicated in zebrafish (MRAP2a and MRAP2b), although its function remains unknown. In this paper, we demonstrate that MRAP2a enables MC4R, a canonical MSH receptor, to be activated by ACTH with a similar sensitivity to that exhibited by MC2R. Both proteins physically interact and are co-expressed in the neurons of the preoptic area, a key region in the control of the energy balance and hypophyseal secretion in fish. ACTH injections inhibit food intake in wild-type zebrafish but not in fish lacking functional MC4R. Both MRAP1 and MRAP2a are hormonally regulated, suggesting that these proteins are substrates for feedback regulatory pathways of melanocortin signaling. Fasting has no effect on the central expression of MRAP2a but stimulates MRAP2b expression. This protein interacts and is co-localized with MC4R in the tuberal hypothalamic neurons but has no effect on the pharmacological profile of MC4R. However, MRPA2b is able to decrease basal reporter activity in cell lines expressing MC4R. It is plausible that MRAP2b decreases the constitutive activity of the MC4R during fasting periods, driving the animal toward a positive energy balance. Our data indicate that MRAP2s control the activity of MC4R, opening up new pathways for the regulation of melanocortin signaling and, by extension, for the regulation of the energy balance and obesity.

Introduction

Melanocortins, which are the posttranscriptional products of a complex precursor named proopiomelanocortin (POMC), are mainly composed of an adrenocorticotropic hormone (ACTH) and melanocyte-stimulating hormones (α-, β-, γ- and δ-MSH) (Nakanishi *et al.* 1979). POMC is mainly produced in the pituitary and its posttranslational processing occurs in a tissue-specific manner. The proteolytic cleavage of POMC generates ACTH in the corticotrophs of the anterior pituitary, whereas POMC cleavage produces α-MSH and β-endorphin in the melanotrophs of the *pars intermedia*. POMC is also centrally produced in the arcuate nucleus and the nucleus of the *tractus solitarius*, where it is mainly processed to α-MSH and β-endorphin (Castro and Morrison 1997).

Melanocortin exerts its physiological role by binding to a family of specific G protein-coupled receptors that positively couple to adenylyl cyclase. Tetrapod species have five melanocortin receptors (MC1R-MC5R). MC2R is specific for ACTH, whereas the MSHs bind to the other four MCRs, with MC1R and MC3R exhibiting the highest affinity for α-MSH and γ-MSH, respectively (Schioth *et al.* 2005). Atypically, melanocortin signaling is not exclusively regulated by the binding of endogenous agonists, since naturally occurring antagonists, agouti-signalling protein (ASIP) and agouti-related protein (AGRP) compete with melanocortin peptides by binding to MCRs.

Melanocortin signaling participates in the regulation of multiple physiological functions (Schioth *et al.* 2005) but its involvement in the control of corticosteroid synthesis, via MC2R (Chan *et al.* 2011), and in the control of energy balance via MC3R and MC4R (Cone 2006), are the most studied facets of such signaling. Central activation of MC3R and MC4R is thought to mediate the effects of melanocortin on the energy balance (Cone 2006) since both MC3R knockout rat (Chen *et al.* 2000) and MC4R knockout mice (Huszar *et al.* 1997) display severe alterations in energy homeostasis. Interruption of α-MSH central signaling by the ubiquitous constitutive expression of agouti gene in obese yellow mice (*Ay*) results in hyperphagia, hyperinsulinemia, increased linear growth, maturity-onset obesity and yellow fur (Lu *et al.* 1994). A similar metabolic syndrome is also observed in transgenic mice ubiquitously overexpressing AGRP (Ollmann *et al.* 1997), and in MC4R knockout mice (Huszar *et al.* 1997). The central administration of the C-terminal fragment of AGRP (Rossi *et al.* 1998) or chemical antagonists for MC3R and MC4R increase food intake in rodents (Fan *et al.* 1997), and intracerebroventricular (ICV) injections of the melanocortin receptor agonist, MTII, produces a dose-dependent reduction in food intake in mice (Fan *et al.* 1997). However, MC4R-deficient mice do not respond to the anorectic effects of MTII suggesting that α-MSH inhibits feeding primarily by activating MC4R (Marsh *et al.* 1999).

Of the five melanocortin receptors, only the activation MC2R fails when expressed in the conventional heterologous cell lines. In non-adrenal cells, the receptor is retarded in the endoplasmic reticulum, and its functional expression requires the presence of an accessory protein, known as melanocortin receptor 2 accessory protein (MRAP), which works as an MC2R-specific transport system to the plasma membrane (Metherell *et al.* 2005). MRAP is a small protein exhibiting a hydrophobic transmembrane domain mainly expressed in the adrenal cortex. The knockdown of endogenous MRAP in Y1 adrenocortical

cells leads to insensitivity to ACTH, demonstrating that MRAP is essential for producing an ACTH responsive MC2R (Cooray *et al.* 2008). MRAP interacts with the MC2R to facilitate correct folding, and subsequent glycosylation and receptor cell surface expression (Metherell *et al.* 2005) but it is also essential for ACTH binding and ACTH-induced cAMP production (Roy *et al.* 2007; Sebag and Hinkle 2009b).

Vertebrate genome has an MRAP paralogue that also encodes a small single transmembrane-domain protein (Valsalan *et al.* 2013), now named MRAP2 (Chan *et al.* 2009; Metherell *et al.* 2005; Sebag and Hinkle 2009b). Most authors continue to call the first characterized protein MRAP rather than MRAP1, but hereinafter we shall use the numerical nomenclature. The function of MRAP2 is controversial (Cerdá-Reverter *et al.* 2013). Co-immunoprecipitation studies demonstrate that MRAP2 interacts with the MC2R and fully rescues the functional expression of the receptor (Chan *et al.* 2009). However, Sebag and Hinkle (2009b) have reported that MC2R reaches the cell membrane in the presence of MRAP2 but that the increase in ACTH-induced cAMP was much lower (8-fold) than that exhibited in cells expressing both MC2R and MRAP1. Subsequent studies have demonstrated that MRAP2 is an endogenous inhibitor of MC2R activation and competes with MRAP1 to bind to the receptor, thus decreasing the ability of ACTH to stimulate cAMP production (Sebag and Hinkle 2010).

Very recent studies suggest that MRAPs can also modulate the function of other melanocortin receptors. Immunoprecipitation studies have demonstrated that both MRAP1 and MRAP2 interact physically with all five melanocortin receptors (Chan *et al.* 2009; Sebag and Hinkle 2009b). Both MRAP1 and MRAP2 reduce the functional expression of MC4R and MC5R but not of MC1R and MC3R in the plasma membrane. Accordingly, both MRAP1 and MRAP2 decrease [Nle4,D-Phe7]-α-MSH-stimulated cAMP production in cells expressing MC3R, MC4R and MC5R, but only MRAP2 was able to induce a similar effect on MC1R (Chan *et al.* 2009). However, the physiological involvement of these interactions is unknown.

The aim of this study was to investigate the interaction of MRAPs with the MCR system as well as the physiological involvement of these interactions using zebrafish (zf) as model. We studied the hormonal and physiological regulation of MRAP expression as a potential pathway for the regulation of melanocortin signaling. Zebrafish genome has six MCRs since MC5R is duplicated (MC5Ra and MC5Rb) and three different MRAPs. One MRAP group has tetrapod MRAP1s, and the two other MRAP sequences are classified as MRAP2 paralogues (MRAP2a and MRAP2b) (Agulleiro *et al.* 2010; Cerdá-Reverter *et al.* 2011). We demonstrated that MC4R can be activated by ACTH when the receptor is co-expressed with MRAP2a, exhibiting similar sensitivity to that shown by MC2R. This pharmacological finding has a clear physiological significance since both proteins, i.e. MC4R and MRAP2a, physically interacts and are coexpressed in the same neurons of the preoptic area. ACTH injection inhibits short-term food intake in wild-type zebrafish, as MSH does in other closely related species (Cerdá-Reverter *et al.* 2003a), but not in zebrafish lacking functional MC4R (*Sa122*). It demonstrates that MC4R is required for the anorexic effects of ACTH. The expression of MRAP2b also co-localizes with MC4R in the tuberal hypothalamus but it has no effect on agonist binding. Central MRAP2b expression increases during fasting, suggesting that this

protein can depress constitutive MC4R signaling during starvation. Finally, we also demonstrate that MRAPs are hormonally regulated, suggesting a new pathway for the fine tuning of melanocortin signaling.

Material and methods

Animals, reagents and primers

Wild-type TU strain zebrafish were raised at 24-28ºC, with 14h light/12h dark cycle. MC4R$^{-/-}$ mutant strain *sa122* were obtained from the Sanger Institute Zebrafish Mutation Project and genotyped as previously described (Zhang *et al.* 2012). Prior any manipulation, animals were netted and anaesthetized for 1 minute in 2-phenoxy-ethanol (0.05%) in the sampling tank. When required animals were sacrificed by rapid decapitation after anesthesia. All experiments were carried out in accordance with the principles published in the European animal directive (86/609/EEC) for the protection of experimental animals and approved by the Consejo Superior de Investigaciones Científicas (CSIC) ethics committee (project numbers AGL2010-22247-C03-01 and CSD 2007-00002 to JM C-R). Unless otherwise indicated, all reagents were purchased from Sigma (St Louis MO, USA). Primers used in the experiments are summarized in supplementary table 1.

Supplementary Table 1. Sequence of primers and probes used for cloning MC4R and MC5Ra and for evaluation of gene expression by qPCR.

Primer	Sequence (5´-3´)
Hind_zfMC4RFw	TATAAGCTTATGAACACCTCACATCATCATG
Hind_zf_Myc_MC4RFw	TATAAGCTTATGGAACAAAAACTCATCTCAGAAGAGGATCTGATGAACACCTCACATCATCA
Xho_zfMC4RRv	TTACTCGAGTTATACACACAGAGATGCAAGTC
Hind_zfMC5aRFw	TATAAGCTTATGAACACTTCTGAGACCAC
Hind_zf_Myc_MC5aRFw	TATAAGCTTATGGAACAAAAACTCATCTCAGAAGAGGATCTGATGAACACTTCTGAGACCAC
Xho_zfMC5aRRv	TTACTCGAGTTACCTAGACATTCCAAAGACG
Zf_1_Taqman_Fw	CTTCTTCTTGATTTTGTCACTTATTTCAC
Zf_1_Taqman_Rv	TCTTTACTGAGATGATGCATAACCTTTC
Zf_1_Taqman_Probe	[6FAM]CCCTCGAGTCAAAAAATCCGGTTTGC[TAM]
Zf_2a_Taqman_Fw	AGAGCCGCCACTGATGCT
Zf_2a_Taqman_Rv	CCACTTGGCCTCTGGAGTTG
Zf_2a_Taqman_Probe	[6FAM]CTCTCACCCATGGACGATCAGGCA[TAM]
Zf_2b_Taqman_Fw	TTGGCTGTGAGCTGGAAGTG
Zf_2b_Taqman_Rv	TGAAAGAGGGAACGTGATTGG
Zf_2b_Taqman_Probe	[6FAM]CATTTTCTCTGCCACCGCTGCCTG[TAM]
Zf_mc2r_Taqman_Fw	CCTGTTAGCACGCCATCATG
Zf_mc2r_Taqman_Rv	AGGCCGCTTTTCCTGTGTT
Zf_mc2r_Taqman_Probe	[6FAM]AAACCGAATCGCGTCTATGCCTGGT[TAM]
Zf_mc4r_Taqman_Fw	GCCTCGCTCTACGTCCACAT
Zf_mc4r_Taqman_Rv	CGGCGATCCGTTTCATG
Zf_mc4r_Taqman_Probe	[6FAM]TTCCTTCTAGCCCGGCTG[TAM]
zebrafish_bAct_Fw	CTCTTCCAGCCTTCCTTCCT
zebrafish_bAct_Rv	CTTCTGCATACGGTCAGCAA

Cloning procedure

The full coding regions of the zebrafish MCRs genes were obtained from public databases (http://www.ensembl.org/index.html), subcloned in pGEM-T easy vector (Promega) and subsequently subcloned directionally into Hind III/Xho I restricted pcDNA5/FRT (Invitrogen). Primers sequences are shown in supplementary Table 1. MRAP constructs were obtained as previously described (Agulleiro *et al.* 2010). Briefly different N- or C-terminal epitope tagged proteins (MRAPs and MCR) were made by PCR using Taq DNA Polymerase (Invitrogen) and pcDNA5/zfMRAP1, pcDNA3/zfMRAP2a, pcDNA3/zfMRAP2b, pcDNA5/zfMCRs constructs as templates. Proteins were N- or C-terminally tagged with Flag (DYKDDDDKC) or C-Myc (EQKLISEEDL) epitopes. The expected size products were cloned directionally into Hind III and Xho I restricted pcDNA5/FRT vector and sequenced.

Tissue expression experiments

Total RNA was purified from fresh tissues (testis, ovary, intestine, liver, muscle, spleen, head

kidney and post kidney, gills, skin, eyes, heart, brain and whole fish) with Tri-Reagent (Sigma) and 1µg was used for cDNA synthesis with Superscript III reverse transcriptase (Invitrogen) primed with random hexamers and oligo(dT)12-18 (Invitrogen). The cDNA of five tissues from different animals (n=5/tissue) was subsequently used as template for quantitative real-time PCR (qPCR). For MRAP expression quantifications, 1 µl of cDNA was added to 10 µl of 2X Taqman PCR master mix (ABgene, Thermo Scientific, Spain), primers and probes concentrations were 300 nM and 200 nM, respectively. As internal controls, a fragment of β-actin, elongation factor 1 (EF-1α) and 18S were amplified. One microlitre cDNA (1/100) and 250 nM primers were added to 7.5 µl of 2X Sybrgreen PCR master mix (ABgene, Thermo Scientific). Reactions were carried out in duplicate in a Realplex Mastercycler (Eppendorf). Primer sequences are shown in supplementary Table 1.

Double *in situ* hybridization

Animals were anaesthetized, sacrificed and tissues carefully dissected. Brains were fixed with paraformaldehyde (PAF, 4%) in phospate buffer (PB, 0.1 M pH 7.4) overnight, dehydrated, and embedded in Paraplast (Sherwood, St Louis, MO). Serial 6 µm cross sections were cut using a rotary microtome. Sections were mounted on 3-aminopropyltriethoxylane (TESPA)-treated slides and then air-dried at room temperature (RT) overnight. Sections were stored at 4ºC under dry conditions and used for hybridization within one month. The double *in situ* hybridization procedure was according to (Escobar *et al.* 2013).

Before hybridization, sections were deparaffinized, rehydrated and post-fixed in 4% PAF for 20 minutes. Slides were then rinsed twice in PB for 7 minutes and treated with a Proteinase-K solution (20 µg/ml in 50 mM Tris-HCl, 5mM EDTA, pH= 8) for 5 minutes at RT. Slides were then washed in PB and post-fixed again in PAF for 5 minutes, subsequently rinsed in sterile water and acetylated in a triethanolamine (0.1 M, pH=8)/acetic anhydride solution. Sections were then dehydrated and dried at RT. Nonisotopic riboprobes for full-length zfMC4R and zfMRAP2a were synthesized using a digoxigenin (DIG) and fluorescein (FLU)-RNA labeling mix (Roche Diagnostics), respectively, according to the manufacturer's instructions. After 7 minutes incubation at 75ºC, riboprobes were diluted simultaneously in hybridization buffer containing 50% formamide, 300 mM NaCl, 20 mM Tris-HCl (pH 8), 5 mM EDTA (pH 8), 10% Dextran sulphate, 1x Denhardt´s solution and 0.5 µg/µl yeast RNA type III. Subsequently, 100 µl of hybridization solution was added to each pre-treated slide (see above), which were cover-slipped and incubated in a humidified chamber at 55ºC overnight. The following day coverslips were removed by incubating slides in a solution containing 5x standard saline citrate buffer (SSC, 1x SSC containing 150 mM NaCl, 15 mM sodium citrate, pH 7), for 30 minutes at 55ºC. The slides were then rinsed in 2xSSC, 50% formamide for 30 minutes at 65ºC and three times immersed in NTE buffer (500 mM NaCl, 10 mM Tris-HCl, 5 mM EDTA, pH 7.5) for 10 minutes at 37ºC. After RNAse treatment 2 µg/ml RNAse in NTE) for 30 minutes at 37ºC, slides were rinsed once in NTE buffer for 10 minutes at 37ºC, once in 2x SSC, 50% formamide, for 30 minutes at 65ºC, once in 2xSSC for 10 minutes at RT and twice in 0.1x SSC for 15 minutes at RT. Then slides were washed twice for 10 min at room temperature in buffer A (100 mMTris-HCl, pH 7.5; 150 mM NaCl) and incubated in blocking solution (2% blocking reagent, Roche Diagnostics

in buffer A) for 30 min at room temperature. Subsequently, the slides were incubated with anti-DIG alkaline phosphatase-conjugated sheep Fab fragments and anti-FLU horse-radish peroxidase (POD) sheep Fab fragments (Roche Diagnostics) diluted in blocking solution. On the next day, sections were washed twice for 10 min in buffer B (100mM Tris pH=9.5, 100mM NaCl), incubated for fluorescent detection of POD activity with tyramide-biotine amplification diluent (Perkin Elmer) for 20 minutes and subsequently washed in buffer B with 0.1% Triton for 15 minutes and exposed overnight at room temperature to Streptavidin Alexa 488 diluted 1:300 in blocking buffer. Next day, slides were incubated with HNPP (2-hydroxy-3-naphtoic acid-2'-phenylanilide phosphate) in HNPP/FastRED solution (Roche Diagnostic) during 3 hours for fluorescence detection of alkaline phosphatase activity. Finally, slides were cover slipped with Vectashield Hard Set mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen). Anatomical locations were confirmed by reference to a brain atlas of zebrafish (Wullimann *et al.* 1996).

Cell culture and transfection

HEK cells were maintained in DMEM media (Gibco) supplemented with 10% FBS (Gibco) and 1% penicillin/streptomycin mixture (Gibco) in a humidified atmosphere of 5% CO2 at 37ºC. Transient transfections were carried out using Lipofectamine LTX (Invitrogen) according to the manufacturer's instructions with 100ng of each construct, and total amounts of DNA were kept constant in 2µg with pBSSK plasmid.

Pharmacological experiments

A HEK-293 cell clone (clone Q), stably expressing β-galactosidase under the control of a vasoactive intestinal peptide promoter placed downstream of tandem repetitions of cAMP responsive elements (CRE) was used to evaluate receptor activation (CRE-GAL) (Sánchez *et al.* 2009).

ZfMRAP constructs alone or in combination were transiently transfected together with zfMC4R and zfMC5aR constructs in the clon Q. A construct carrying luciferase gene under the control of a constitutive promoter was also transfected to standardize transfection levels. The following day, cells were split up into 96-well plates and stimulated with human α-MSH (Bachem) and ACTH 1-24 (Bachem) ranging from 10^{-7} to 10^{-10} M or forskolin 10^{-6} in assay medium at 48h post-transfection. After 6h, the medium was removed, cells were lysed and galactosidase activity measured as previously described (Sánchez *et al.* 2009). The effect of zebrafish AGRP (zfAGRP, kindly donated by Dr. Millhauser from Department of Chemistry, University of California, USA), 10^{-7} M on ACTH-stimulated MC4R/MRAP2a activity was studied also. Measurements were normalized for the protein content, the luciferase activity and forskolin-induced galactosidase activity. Protein content was determined using the BCA protein assay kit (Pierce). Luciferase activity was determined using the luciferase assay kit (Promega) following provider instructions.

In order to corroborate the effect of MRAP2b on MC4R basal activity a cell clone Flp recombinase-mediated homologous recombination system (Flp-lnTM) was used to produce cells lines stably expressing MC4R in HEK-293/FRT cells, a cell line with single genome-integrated FRT (Agulleiro *et al.* 2010). The development of isogenic cell lines was carried out according to the manufacturer recommendations.

Subsequently, cells were transiently transfected with 500 ng CRE-GAL alone or together with 20 ng of MRAP2b construct. Basal galactosidase levels in unstimulated cells transfected with MC4R or MC4R+MRAP2b were determined as above. Transfection levels were standardized as before.

Western blotting and co-immunoprecipitation

Whole-cell lysates were prepared 24 h after transfection. Cells were washed once with cold PBS and lysates generated using lysis buffer, briefly: 50mM Tris-HCl, 500mM NaCl, 0,5% TritonX-100 and 1mM EDTA with protease and phosphatase inhibitors and incubated for 30 min on ice. Samples were then spun for 20 min at 16,000 g at 4°C. The supernatant was mixed with Laemmli Sample buffer 2X before use for Western blotting or incubated overnight at 4°C with anti-FLAG magnetic beads (Sigma), or anti-MYC agarose beads (Sigma) for Co-IP. After incubation, agarose was washed 4 times in lysis buffer, supernatant removed and SDS loading buffer added. Magnetic beads were treated as manufacturer instructions and also resuspended in SDS loading buffer. After boiling for 3 min, samples were run in SDS-polyacrylamide gel. Western blotting was performed with anti-FLAG (Sigma), or anti-MYC (Abcam) antibodies used at dilutions of 1/1000 and 1/5000, respectively and detected by HRP chemiluminiscence reaction of secondary antibody (SuperSignal West Femto, Pierce).

Immunofluorescence microscopy

HEK cells grown onto poly-L-lysine-coated coverslips were transiently transfected with 0.2 µg/well of Myc-MC4R and 0.2 µg/well Flag-MRAP2a, or Flag-MRAP2b constructs. Twenty four hours later, cells were fixed and permeabilized by incubation in methanol for 5 min and subsequently in acetone for 1 min. Then, cells were rehydrated, washed in PBS, blocked and incubated with mouse anti-C-Myc and rabbit anti-Flag antibodies. Primary antibodies were detected with goat anti-mouse or anti-rabbit secondary antibodies coupled to Alexa-Fluor 488 or Alexa-Fluo 594 (Invitrogen) as required. DAPI (2 µM) was used to stain nuclei. Coverslips were mounted in Prolong mounting medium for fluorescence (Invitrogen). Cells were also examined with a laser-scanning confocal microscope (Olympus FV1000).

Cell surface ELISA

To measure cell surface receptor expression, 293/FRT/Myc-zfMC4R cells were seeded in poly-L-lysine coated 24-well plate (1×10^5 per well) and transfected independently with pcDNA3/zfMRAP2a or pcDNA5/zfMRAP2b. Twenty four hours after transfection, cells were washed with phosphate saline buffer (PBS), fixed on ice for 15 min with 1.85% formaldehyde to evaluate the presence of the receptor in the plasma membrane, or for 5 min with methanol for total receptor measurements. Cells were then processed for ELISA as previously described (Agulleiro *et al.* 2010). Non-specific OD_{492} values were determined by transfecting the untagged versions of each construct when possible or with EGFP. Experiments were repeated three independent times in triplicate.

***In silico* analysis of the MRAP1 5'-flanking region**

As a first approach to understand the hormonal regulation of the MRAPs, the first 5 kb of the 5' flanking region of the zfMRAP1 were obtained from Ensembl database

(http://www.ensembl.org/index.html) and analyzed for the presence of putative cis-acting elements using MathInspector (Genomatix, http://www.genomatix.de/) and Transcription Element Search System (Tess, http://www.cbil.upenn.edu/cgi-bin/tess/tess) software.

Hormonal and physiological regulation of MRAP expression

Twenty fish per treatment were reared in individual aquariums and fed twice a day during one week at 4% of body weight with control food or the same diet containing 500µg/g of T_3 (3,3',5-triiodo-L-thyronine, Sigma), cortisol (hydrocortisone, Sigma) or bezafibrate (Sigma), an agonist of the peroxisome proliferator-activated receptor alpha (PPARα). After 7 days, fish were sacrificed and whole body was quickly frozen in dry ice. Total RNA of the whole fish was purified with Maxwell 16LEV Simply RNA Tissue Kit (Promega) as described by the manufacturer. Quantitative PCRs and data analysis were as before but 3 µg of RNA were used as template for cDNA synthesis. For fasting experiments, thirty animals were reared in two tanks (n=15/tank) and fed for 14 days at 4% of the body weight. Thirty additional animals, split up into two individual aquaria, were fasted and sampled at 7 and 14 days. For stress experiments thirty animals were maintained during 7 and 14 days in 1/20 of water volume when compared with the control group. After the experimental period, brains were removed carefully and total RNA was extracted using Tri-Reagent. cDNA synthesis, PCR quantification of MRAP expression and data analysis were done as before.

Food intake experiments

Adult female and male zebrafish were placed individually in 2-litre tanks for 4 consecutive days and food intake level was daily recorded. An excess of quantified food pellets (Supervit granulat, Tropical) were added to the tank at 10 am and the number of pellets was quantified after 2 and 4 hours. These measurements provided a base line of food intake levels for each individual fish. The fifth day animals were injected intraperitoneally with saline, 0.1, 2 or 10 µg of hACTH (1-24). A minimal of 10 fish were injected for each treatment. After 15 minutes, food intake levels were recorded in the same manner. Food intake levels of each treated fish were expressed as the percentage of the base line (average of the food intake levels during the previous four days). The same protocol was used with the zebrafish strain *Sa122*, obtained from the Zebrafish Mutation Project (Welcome Trust Sanger Institute, UK), which lacks a functional MC4R. This zebrafish mutant has a different genetic background so we injected both wildtype and mutant fish with saline or 10µg hACTH (1-24)/fish (the effective doses obtained in the previous experiments). Total food intake was recorded after 4 hours.

Data analysis and statistics

Receptor activation data were fitted to logistic curves using QtiPlot free-software for LINUX (http://soft.proindependent.com/qtiplot.html). For graphical representation, the response average for each dose from three independent experiments was calculated and data were fitted to logistic and ED_{50} values were resumed in Table 1 and 2. For statistical comparisons, data form each independent experiment (n= 3) were fitted to dose response curves and ED_{50} average values compared and significant differences were indicated by asterisk in Table 1 and 2. qPCR data were analyzed with the ΔΔCt (cycle threshold) method.

Statistical analysis was conducted by one-way analysis of the variance followed by Tukey´s multiple range test ($p<0.05$).

Results

MC4R and MRAP expression

All four mRNAs, i.e. MC4R, MRAP1, MRAP2a mad MRAP2b, were expressed in the head kidney but only transcripts of MC4R and MRAP2s were found abundantly in the brain. All three MRAPs, but no MC4R, were expressed in the zebrafish testis. In addition, MRAP1 was expressed also in the muscle and spleen whereas MRAP2b was expressed in the eye. Residual levels were also found in some peripheral tissues (figure 1).

In order to corroborate the expression of MC4R and MRAP2a in the brain but also to demonstrate the coexpression of these genes in the same neurons, double *in situ* hybridization with non-isotopic probes was done (figure 2). MC4R and MRAP2a colocalized in the preoptic area, at the level of anterior part of the parvicellular preoptic nucleus (Ppa) also in the dorsal hypothalamus (Hd), particularly in the lateral extension of the third ventricle (data not shown) and the periventricular gray zone of the optic tectum (PGZ, data not shown). On the contrary, MC4R and MRAP2b colocalized mainly in cells that coat the third ventricle within the medial area of the tuberal hypothalamus (figure 2).

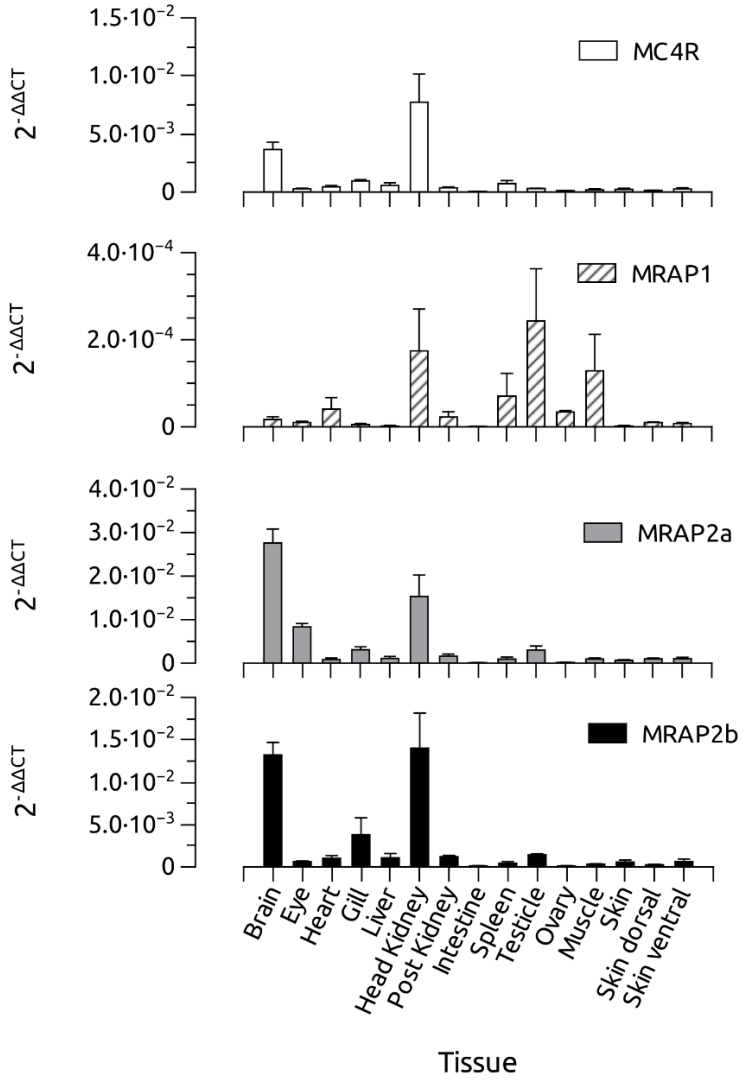


Figure 1. Distribution of MC4R and MRAPs mRNA expression in different zebrafish tissues, as revealed by qPCR. Amplifications of β-actin, 18S and EF1α mRNAs were used as internal control of the reverse transcription.

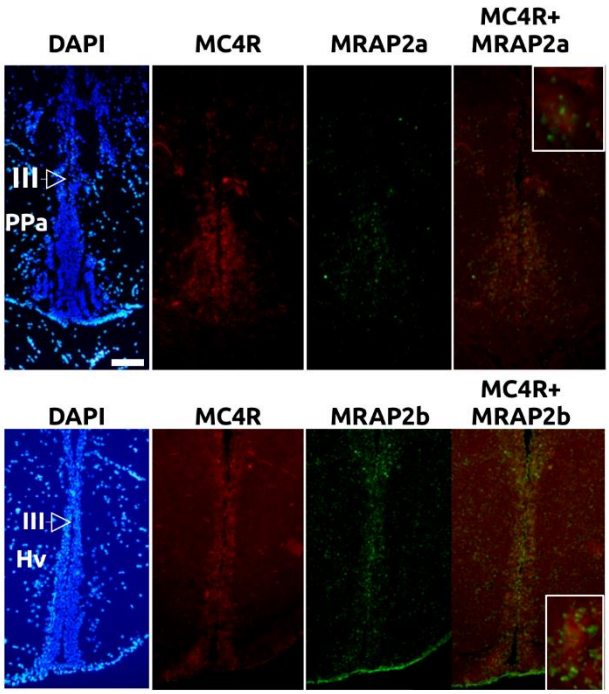


Figure 2. Double *in situ* hybridization of MC4R (red) and MRAP2a (green) at the level of preoptic area (upper panels) or MC4R (red) and MRAP2b (green) at the level of tuberal hypothalamus (lower panels). Samples were aslo stained with 4`6-diamidino-2-phenylindole (DAPI) for morphologic studies. III, third ventricle; HV, ventral hypothalamus; Ppa, anterior preoptic area. Insets in the right panels show magnification of MC4R and MRAP colocalization. Scale bar, 200 μm.

MC4R and MRAP2s physically interact as demonstrated by immunoprecipitation an immunofluorescence studies

To determine if MC4R and accessory proteins directly or closely interact, we tested whether receptor co-immunoprecipitated with each MRAP. Both zfMC4 and MC5aR co-immunoprecipitated with MRAP2a and MRAP2b but receptors did not interact with MRAP1 (figure 3 and supplementary figure 1). When coexpressed *in vitro*, all three MC4R, MRAP2a and MRAP2b proteins were detected by immunocytochemical techniques. High levels of MC4R MRAP2a (figure 4 upper panels) and MRAP2b (not shown) were detected in the surrounding area of the cellular nucleus matching the position of the endoplasmic reticulum/golgi complex. All three proteins were detected also in the nuclear membrane (figure 4, not shown for MRAP2b). MC4R was targeted partially to the plasma membrane where was found to colocalize partially with MRAP2a and or in close apposition/colocalization with MRAP2b (figure 4).

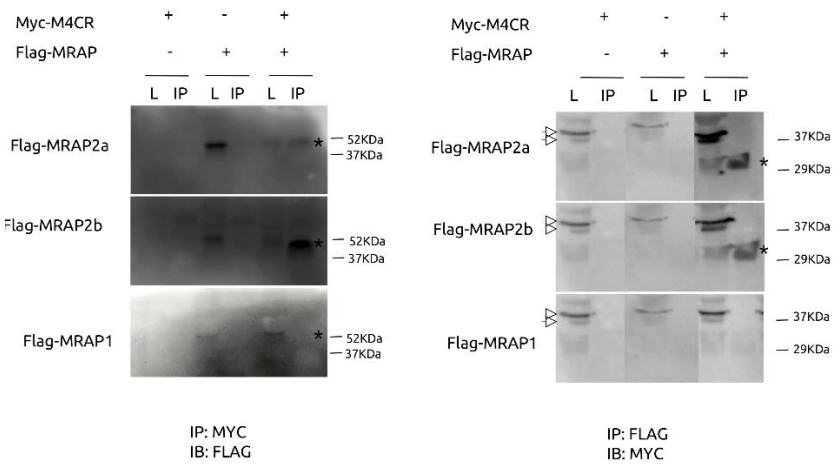
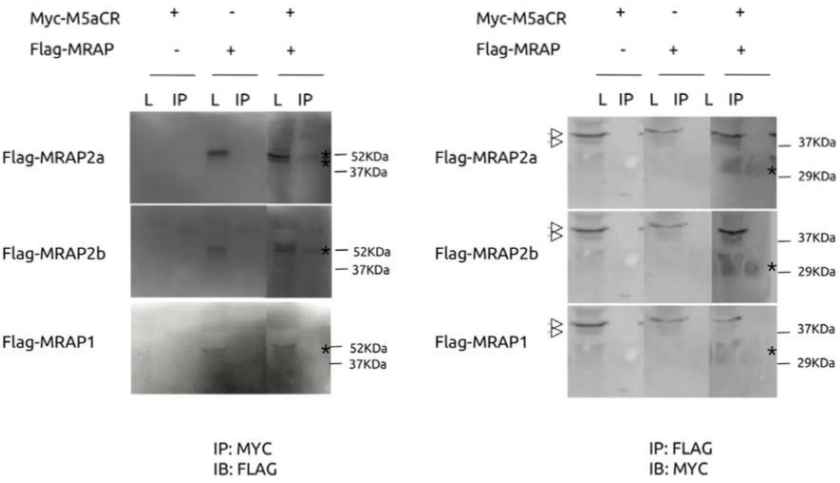


Figure 3. MRAP2s and MC4R interactions. HEK-cells were transfected with Flag-tagged MRAPs and/or Myc-tagged MC4R. Whole-cell lysates were prepared 24 h after transfection and used for Western blot or incubated with anti-FLAG magnetic beads or anti-MYC agarose beads for coimmunoprecipitation. Both Flag-MRAP2a and Flag-MRAP2b but not Flag-MRAP1 interact with Myc-MC4R as seen by immunoblotting with anti-Flag and anti-Myc, respectively after immunoprecipitation with anti-Myc. Asterisks indicate positive bands in crude lysates and immunoprecipitated samples whereas white arrowheads show unspecific bands.



Supplemental Figure 1. MRAP2s and MC5R interactions. Both Flag-MRAP2a and Flag-MRAP2b but not Flag-MRAP1 interact with Myc-MC5R as seems by immunoblotting with anti-Flag and anti-Myc, respectively after immunoprecipitation with anti-Myc.

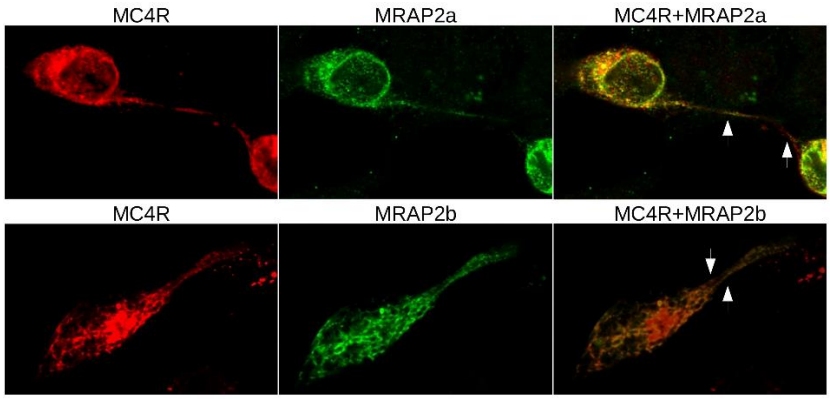


Figure 4. Immunofluorescence assays in live cells showing the expression of MRAPs (green) and/or zfMC4R (red). N-terminally Flag-tagged MRAPs and N-terminally Myc-tagged MC4R were transiently expressed in HEK-293 cells. Photomicrographs are taken with a 60 X objective and are from a single optical section obtained within an acquisition of z stacks (0.10 µm/slice). Arrows indicate regions of the plasma membrane where MRAPs and MC4R are potentially colocalizated.

Table I. MC4R or MC5R were expressed alone or in combination with one of the different MRAPs in HEK-293 cells expressing a reporter gene under the control of cAMP-responsive elements (CRE).

	MC4R	MC4R+MRAP1	MC4R+MRAP2a	MC4R+MRAP2b	MC5R	MC5R+MRAP1	MC5R+MRAP2a	MC5R+MRAP2b
α-MSH	1.30 × 10^{-8} [±2.49 × 10−8]	8.49 × 10^{-9} [±3.3 × 10−9]	2.14 × 10^{-8} [±3.92 × 10−8]	1.70 × 10^{-8} [±2.43 × 10−8]	1.16 × 10^{-9} [±3.19 × 10−9]	1.09 × 10^{-9} [±1.07 × 1010]	1.37 × 10^{-9} [±1.78 × 10−9]	8.78 × 10^{-10} [±9.6 × 10−10]
ACTH(1–24)	1.77 × 10^{-7} [±6.26 × 10−7]	6.15 × 10^{-7} [±3.84 × 10−7]	8.49 × 10^{-9a} [±3.37× 10−9]	4.9 × 10^{-8} [±5.1 × 10−8]	9.73 × 10^{-10} [±3.02 × 10−10]	9.01 × 10^{-10} [±1.47 × 10−10]	1.41 × 10^{-9} [±6.68 × 10−9]	6.80 × 10^{-10} [±2.08 × 10−10]
ACTH (1–24) + AGRP			3.57 × 10^{-7b} [±6.77 × 10−7]					

The mean of the reporter activation, expressed as percentage of the basal level, for each concentration of melanocortin agonist (hACTH1–24 or α-MSH), was calculated from 3 independent experiments and the resultant data were fitted to logistic curves using Qtiplot free software. For statistical comparisons, data from each independent experiment (n= 3) were fitted to dose-response curves and ED50 average values (M) compared by one-way ANOVA and significant differences were indicated. Numbers in brackets are with the 95% confidence intervals of nonlinear fittings. ED50 values from gene reporter activation for melanocortin analogues on zebrafish MC4R expressed in HEK 293 cells.

[a] Significant differences ($P < 0.05$) from MC4R transfected cells. [b] Significant differences between cells transfected with MC4R and MRAP2a treated with ACTH or ACTH+AGRP after t test ($P< 0.05$).

Pharmacological implication of MC4R/MRAPs interaction

To determinate if the interaction zfMC4R-MRAP has some pharmacological implication, we co-expressed both the receptor and the accessory proteins in HEK cells and stimulated the cells with increasing α-MSH or ACTH concentrations. Co-expression of MC4R and MRAP1 or MRAP2b, but no MRAP2a, slightly increased the sensitivity of the receptor by α-MSH. However, coexpression of MC4R and MRAP2a significantly increased the sensitivity of the receptor to hACTH (1-24). This effect was no evident when the receptor was expressed together with MRAP1 or MRAP2b (Fig. 5, Table1). When MC4R was expressed with a combination of MRAPs (MRAP2a+MRAP1, MRAP2a+MRAP2b, MRAP1+MRAP2b or MRAP2a+MRAP1+MRAP2b) the receptor only show sensitivity to ACTH when MRAP2a was present in the combination (figure 6, Table 2). AGRP worked as a competitive antagonist of ACTH at MC4R when

coexpressed with MRAP2a since its presence in the media decreased the ACTH-induced galactosidase activity (figure 7). MRAPs had no effect on the α-MSH or ACTH-induced activation of MC5Ra thus supporting previous results on MC4R. When MRAP2b was transfected into HEK-293 FRT cells stably expressing MC4R, basal activity levels decreased about 20% showing that MRAP2b is able to reduce light but significantly the constitutive activity of the receptor. MRAP2a had no effect on the basal activity of the receptor (figure 8A).

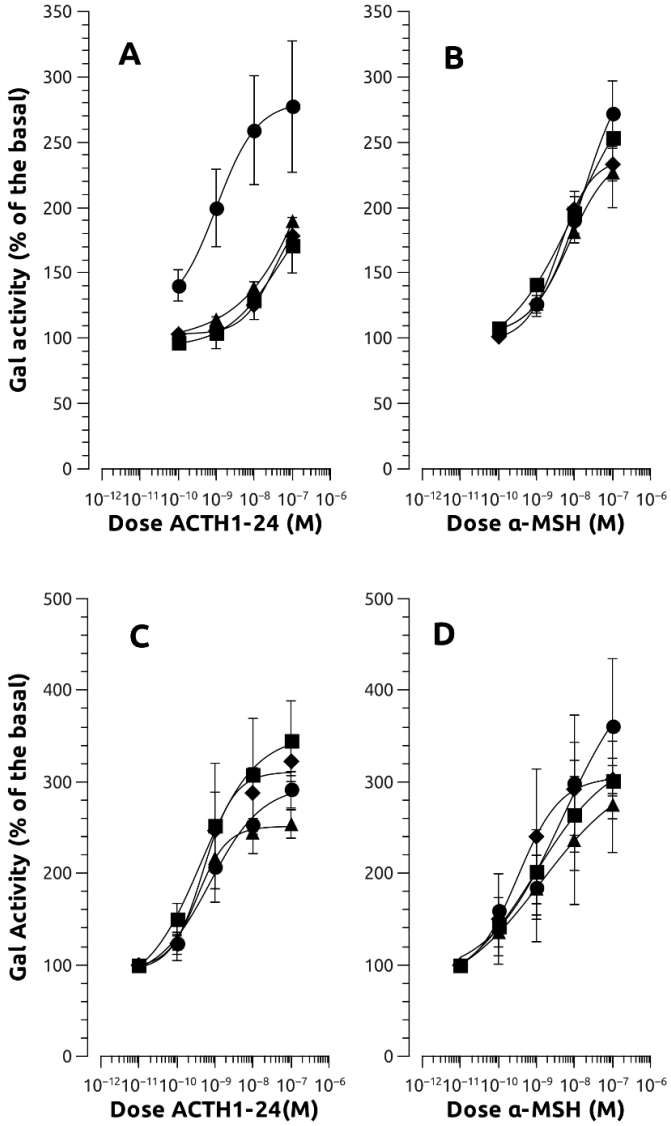


Figure 5. Pharmacologic properties of melanocortin agonist, α-MSH and hACTH (1–24) at HEK-293 transiently expressing both MC4R (upper panels) or MC5Ra (lower panels) and different MRAPs (■, MC4R; ♦, MC4R+MRAP1; ●, MC4R+MRAP2a; ▲, MC4R+MRAP2b) but stably expressing a cAMP-responsive β-galactosidase reporter gene. Data were normalized to protein levels and expressed as percentage of the basal levels. A construct carrying luciferase gene under the control of a constitutive promoter was also transfected to standardize the transfection levels. Experiments were performed using quadruplicate data points and repeated at least 3 times independently. Data are mean ± SEM of the 3 independent experiments.

Table II. MC4R Was Expressed Alone or in Combination with one or several MRAPs in HEK-293 cells expressing a eeporter eene ender the control of CREs.

	MC4R	MC4R+ MRAP2a	MC4R+ MRAP2a+ MRAP1	MC4R+ MRAP2a+ MRAP2b	MC4R+ MRAP2a+ MRAP2b+ MRAP1	MC4R+ MRAP2b+ MRAP1	MC2R	MC2R+ MRAP1	MC2R+ MRAP2a+ MRAP1	MC2R+ MRAP2a
ACTH (1–24)	8.6×10^{-7}	9.59×10^{-9} [a]	9.87×10^{-9} [a]	4.97×10^{-9} [a]	9.64×10^{-8} [a]	—	—	1.37×10^{-9}	1.05×10^{-9}	—
	[$\pm 0.36 \times 10^{-6}$]	[$\pm 6.56 \times 10^{-9}$]	[$\pm 3.39 \times 10^{-9}$]	[$\pm 0.97 \times 10^{-9}$]	[$\pm 1.88 \times 10^{-9}$]			[$\pm 0.21 \times 10^{-9}$]	[$\pm 1.13 \times 10^{-9}$]	

Data were treated as in Table 1. ED_{50} values from gene reporter activation for ACTH(1–24) on zebrafish MC4R or MC2R and MRAPs expressed in HEK 293 cells. Dashes indicates non-significant fitting.

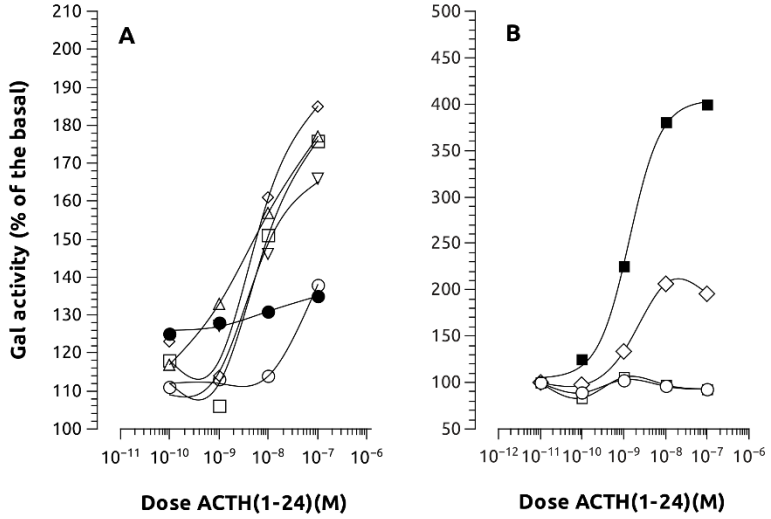


Figure 6. Effects of different MRAP combination on MC4R-induced galactosidase activity. A, Pharmacologic properties of hACTH(1–24) at HEK-293 transiently expressing both MC4Rs with different combinations of MRAPs (○, MC4R; □, MC4R+MRAP2a; ◊ , MC4R+MRAP2a+MRAP1; △, MC4R+MRAP2a+MRAP2b; ▿, MC4R+MRAP2a+MRAP2b +MRAP1; ●, MC4R+MRAP2b+MRAP1, and stably expressing a cAMP-responsive β-galactosidase reporter gene. Only when MRAP2a was present in the combination, the MC4R was able to respond to ACTH stimulation. B, Pharmacologic properties of hACTH(1–24) at HEK-293 transiently expressing both MC2R with different MRAPs (○, MC2R; ■, MC2R+MRAP1; □, MC2R+MRAP2a; ◊, MC2R+MRAP1+MRAP2a). Data were normalized to protein levels and expressed as percentage of the basal levels. Error bars were omitted to facilitate the graph vision. A construct carrying luciferase gene under the control of a constitutive promoter was also transfected to standardize transfection levels. Experiments were performed using quadruplicate data points and repeated 2 times independently.

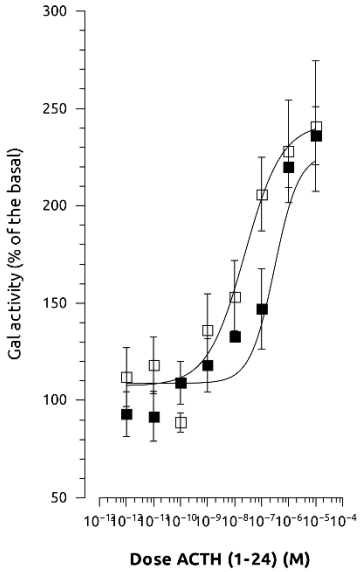


Figure 7. Effects of AGRP on hACTH(1–24)-stimulated galactosidase activity in HEK-293 cells transiently expressing both MC4R and MRAP2a but stably expressing a cAMP-responsive β-galactosidase reporter gene (□, MC4R+MRAP2a; ■, MC4R+MRAP2a+AGRP). Data were normalized to protein levels and expressed as percentage of the basal levels. A construct carrying luciferase gene under the control of a constitutive promoter was also transfected to standardize transfection levels. Experiments were performed using quadruplicate data points and repeated at least 2

independent times.

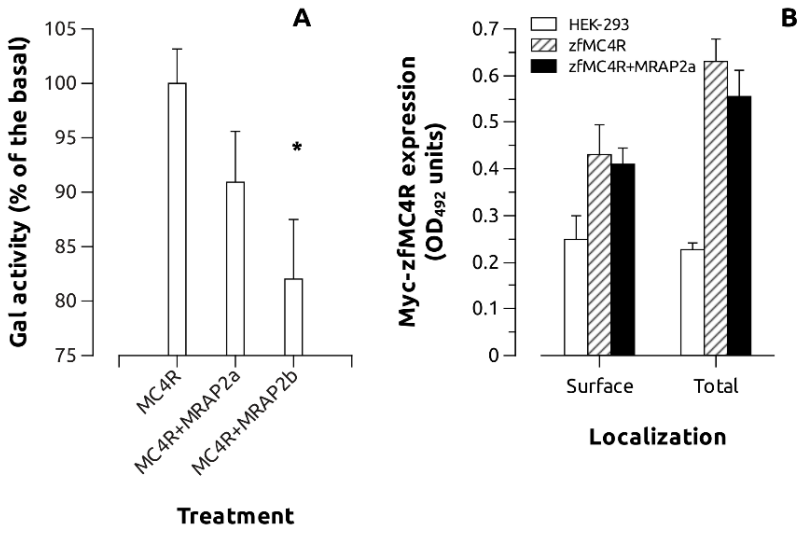


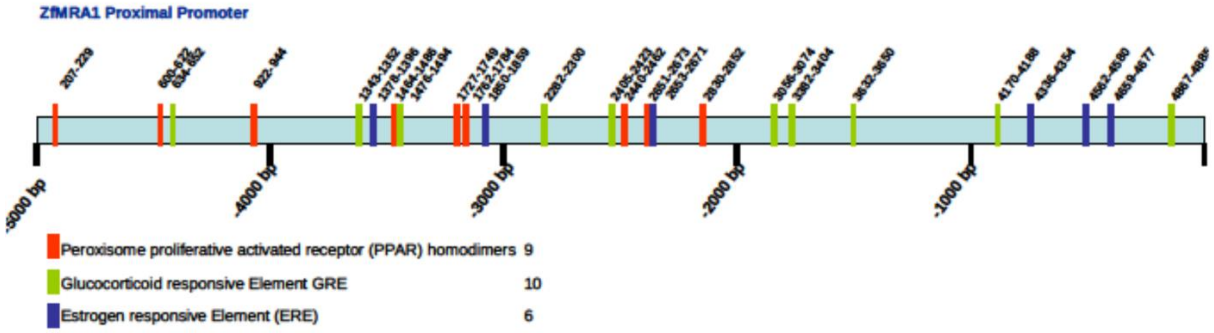
Figure 8. A, Effects of MRAP2a and MRAP2b on MC4R-induced galactosidase basal activity in HEK-293 cells stably expressing MC4R but transiently expressing galactosidase gene under the control of a constitutive promoter carrying several CRE sites (see Material and Methods for more details). A construct carrying luciferase gene under the control of a constitutive promoter was also transfected to standardize transfection levels. Experiments were performed using quadruplicate data points and repeated 4 independent times. Asterisks show significant differences after Student's t test ($P< 0.05$). B, Total and cell surface detection of Myc-zfMC4R using anti-Myc antibodies. Control corresponds to nontransfected HEK-293 cells. Cells were transiently transfected with MC4R or MC4R+MRAP2a and assayed for total and extracellular C-Myc detection by whole-cell ELISA. The results represent the mean ± SEM of 3 independent experiments, each performed in triplicate.

ZfMC4R surface expression

zfMRAP2a expression had no effect on zfMC4R surface or total expression (figure 8B).

MRAP1 5'-flanking region

In order to obtain information on putative hormonal systems regulating MRAP system, we analyzed the 5'-flanking region of the MRAP1. The proposed promoter sequence contained a number of sites that corresponded to homologs of the consensus sequences of various hormone responsive elements, in particular, 10 putative glucocorticoid response elements (GRE), 6 potential estrogen response element (ERE). We also highlighted the presence of 9 putative peroxisome proliferative activated receptor (PPAR) homodimers (see supplementary figure 2).



Supplementary Figure 2. Sequencing analysis of the zebrafish MRAP1 promoter. The 5' flanking region of the zfMRAP1 was obtained from Ensembl database (http://www.ensembl.org/index.html). The promoter was analyzed by using MathInspector (Genomatix, http://www.genomatix.de/) and Transcription Element Search System (Tess, http://www.cbil.upenn.edu/cgi-bin/tess/tess) software. Only potential glucocorticoid response elements (GRE), estrogen response elements (ERE) and peroxisome proliferative activated receptor (PPAR) homodimers are shown.

Hormonal and physiological regulation of zfMRAPs

In order to evaluate if the MRAP system can be a regulatory node of the melanocortin system activity, we studied the expression response of the all three MRAPs to different hormonal systems. We took advantage of the previous promoter analysis of MRAP1 (see above). Both orally administrated cortisol and bezafibrate for a week significantly inhibited the expression of MRAP1 and MRAP2a. A similar effect was also observed on MRAP2b expression, but levels did not reach statistical significance. T3 had no effect on MRAP system expression but its oral administration tends to decrease the expression levels of MRAP1 and MRAP2a (figure 9A).

We also explore the effect of fasting and stress on central expression of the MRAP2s and MC4R. Long-term fasting significantly increased MC4R expression at 1 week but such increase was abolished at 2 weeks. On the contrary, MRAP2b expression was increased significantly during the whole experimental period. Stress by density had no effects on central MRAP2 expression after 1 or 2 weeks (figure 9B).

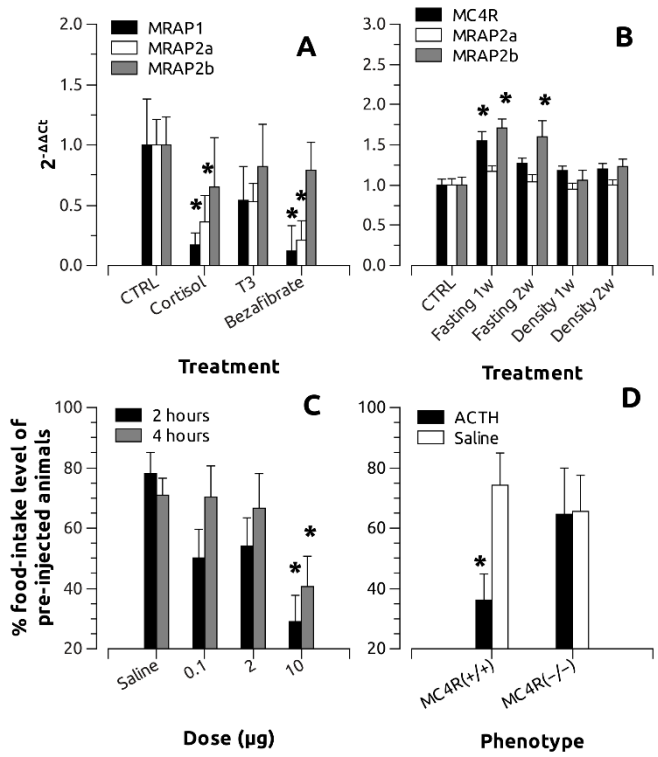


Figure 9. A, Effects of cortisol, T_3, or bezafibrate, a PPAR agonist, on MRAP expression. Animals were fed with food pellets containing 500 µg/g of the different hormones and humanely destroyed after 7 days. Total RNA of the whole fish was purified and used for cDNA synthesis. MRAP1, MRAP2a, and MRAP2b expression was analyzed with the ΔΔCt (cycle threshold) method. B, Fasting and rearing density effects on brain expression of MRAP2a and MRAP2b. Animals were fed for 14 days at 4% of the body weight and subsequently fasted for 7 and 14 days. For density experiments animals were maintained during 7 and 14 days in 1/20 of water volume when compared with the control (CTRL) group. MRAP2s expression data were treated as before. C, Effects of hACTH(1–24) on zebrafish

(wild-type TU strain) food intake levels. Food intake levels were recorded during 4 consecutive days to establish a base line for each intact fish. The fifth day animals were injected ip with hACTH(1–24). After 15 minutes, food intake levels of each treated fish were recorded after 2 and 4 hours and expressed as the percentage of the base line (average of the food intake levels during the previous 4 days). D, Effects of hACTH (1–24) on zebrafish sa122 food intake levels. Food intake levels were recorded and calculated as before (see Figure 12). Asterisks show significant differences after one-way ANOVA and Tukey's method ($P < 0.05$).

ACTH effects on zebrafish food intake

In order to evaluate the effects of ACTH on food intake levels, we injected intraperitoneally adult zebrafish (mean body weight= 0,421 ± 0,0013 g) with saline or increasing doses of hACTH (1-24). Animals injected with saline ate about 80% of the previous food intake after 2 or 4 hours. However, animals injected with 10 µg of hACTH (1-24) eat only about 30 and 40% after 2 or 4 hours, respectively. No significant differences were observed when fish were injected with lower doses (0,1 or 2 µg) (figure 9C). When zebrafish lacking a functional MC4R were injected with the effective doses of hACTH (1-24) no effects on food intake were recorded at 4 hours post-injection. However, the same doses of hACTH(1-24) injection induced a significant reduction of food intake levels in wild-type animals of the same genetic background (figure 9D).

Discussion

Genome vertebrate contains from 4 to 6 melanocortin receptors but only MC2R is considered the ACTH receptor (Cerdá-Reverter *et al.* 2011). The functional expression of MC2R requires the coexpression of MRAP1 which promotes receptor traffic to the membrane (Metherell *et al.* 2005). MRAP1 has an paralogue called MRAP2 which has been duplicated again in zebrafish (MRAP2a and MRPA2b) (Agulleiro *et al.* 2010). Although MRAP2 has been shown to interact with other melanocortin receptors its function remains unclear (Chan *et al.* 2009; Sebag and Hinkle 2009b). In this paper, we demonstrate that MRAP2a confers to MC4R, a canonical MSH receptor, the capability to be activated by ACTH with a similar sensitivity to that exhibited by MC2R (Agulleiro *et al.* 2010). However, the coexpression of MRAP2a has no effect on α-MSH-induced MC4R activation. It means that MRAP2a is able to transform a MSH receptor into an ACTH receptor. This capability is specific for the tandem MRAP2a/MC4R since coexpression of the receptor with MRAP1 or MRAP2b had no effect on ACTH- or α-MSH-induced cAMP production. When several MRAPs were expressed in combination, MC4R was able to respond to ACTH only when MRAP2a was present in the combination, further corroborating this specificity. From the evolutionary point of view, the results here reported provides a new aspects of the functional evolution of the G-protein coupled receptors. Therefore, the coexpression of an accessory protein can supply a new function to the receptor by widening the binding spectrum. These new binding properties allow the receptor to signal new physiological conditions. The expression of an accessory protein could expand also the tissue response to new hormonal systems or new molecules.

This ability of MRAP2a is restricted to the MC4R since the coexpression of the different MRAPs with the MC5Ra had no effect on the pharmacological profile of the receptor. We have tested also the interaction of the different MRAPs with the other MCRs and no effects on ACTH sensitivity were recorded (R Cortes, MJ Agulleiro and JM Cerdá-Reverter, unpublished results). In a recent paper, Sebag and Hinkle (2010) reported that hMRAP2 compete with hMRAP1 for binding to hMC2R decreasing the potency of ACTH. Similar to our results, hMRAP1 and hMRAP2 had no effects on NDP-α-MSH-induced activation of hMC4R but ACTH-induced receptor activation was not tested. Reinick *et al.* (2012a) reported that dogfish MC5R (Scualus acanthias) responds with higher affinity to ACTH (1-25) than α-MSH and sensitivity of the receptor to ACTH(1-25) increased by coexpression with zebrafish or mouse MRAP1. However, the coexpression of cartilaginous MRAP2 had no impact on MC5R function. The present experiments cannot discriminate the mechanism by which MRAP2a promotes the ACTH-induced MC4R activation, but immunoprecipitation and immunofluorescence experiments demonstrate that both proteins interact directly or closely by means of an intermediary protein. As expected, this interaction did not modify the surface functional expression of the receptor since α-MSH-induced activation remains unaltered after MRAP2a/MC4R co-expression. This result suggests that MRAP2a modifies the MC4R tertiary structure to increase ACTH-binding affinity.

In contrast to the activation data, MRAP2a interaction is not specific to MC4R since it also immunoprecipitates with MC5Ra. Similarly, MRAP2b binds both receptors, i.e. MC4R and MC5Ra but MRAP1 does not. Accordingly, hMRAP2 has been shown to interact with all five hMCRs (Chan *et al.* 2009). Our results suggest that MRAP2b could have some effect on MC4R function regardless of agonist binding. The MC4R is a constitutive receptor that signals in absence of ligand. AGRP works as an inverse agonist, decreasing basal activity of the receptor, but also as competitive antagonist, inhibiting MSH-induced activation (Ersoy *et al.* 2012; Nijenhuis *et al.* 2001; Sánchez *et al.* 2009). Crosstalk between α-MSH and AGRP regulates the activity of the MC4R thus controlling output effects on energy balance and growth (Cone 2006). Decreased MC4R signaling in MC4R knockout mice (Huszar *et al.* 1997), AGRP overexpressing mice (Ollmann *et al.* 1997), agouti mice (Lu *et al.* 1994; Nijenhuis *et al.* 2001) or morpholino zebrafish (Zhang *et al.* 2012) results in hyperphagia, obesity and increased linear growth. Our results demonstrate that fasting severely increases central MRAP2b expression. Therefore, it is plausible that MRAP2b could decrease constitutive activity of the MC4R during fasting periods driving the animal toward positive energy balance. Further supporting this idea, we demonstrate that transient transfection of low quantities of MRPA2b is able to decrease basal galactosidase activity in cells lines stably expressing MC4R. In addition, double ISH experiments show that both MC4R and MRAP2b expression colocalize in the neurons of the ventral hypothalamus, a brain area involved in the regulation of pituitary secretion but also in the control of energy balance. In fact, the ventral hypothalamus seems to be the homologue of the mammalian arcuate nucleus (Ersoy *et al.* 2012). Colocalization studies provide an anatomical support to the

immunoprecipitation experiments but also a physiological role to the protein interaction. We anticipate that overexpression of MRAP2b in zebrafish could result in increased linear growth as observed in AGRP transgenic zebrafish (Klebig *et al.* 1995) or MC4R knockout zebrafish (Zhang *et al.* 2012).

Both MC4R and MRAP2a mRNAs co-localize also in the preoptic area, tuberal hypothalamus and optic tectum. The interaction between both proteins in the brain suggests that ACTH could be involved in the regulation of food intake and growth in zebrafish. In support of this, we demonstrate that peripheral administration of ACTH inhibits short-term food intake in wild animals but no in the zebrafish strain *sa122* that lacks of a functional MC4R. It demonstrates that MC4R, but not MC2R, mediates anorexic effects of ACTH. In mammals, ACTH is synthesized mainly in the pituitary but central POMC is mainly processed to α-MSH and β-endorphin (Castro and Morrison 1997). Therefore, it suggests that peripheral ACTH could reach central structures controlling food intake, particularly brain areas expressing both MC4R and MRAP2a, to inhibit food intake. However, immunohistochemical experiments in carp, a very close related species to zebrafish, have reported the presence of ACTH in the preoptic area but expression studies demonstrated that POMC is exclusively expressed in the tuberal hypothalamus of goldfish (Cerdá-Reverter and Canosa 2009). It suggests that hypothalamic POMC can be processed into ACTH and projected to preoptic area, where MC4R/MRAP2a are expressed, to modulate melanocortin signaling. Alternatively, ACTH could stimulate cortisol secretion, via interrenal MC2R, and inhibits food intake as observed in other fish species (Song and Cone 2007). However, it is unlikely because *sa122* animals exhibit a functional MC2R but food intake levels remain unaltered. In addition, cortisol treatment exhibits long lasting effects on feeding response (Klebig *et al.* 1995). However, our results show that ACTH-treated animals reduce food intake levels after two hours making thus improbable that cortisol mediates ACTH effects on food intake. Central effects of ACTH on MC4R are not exempt of AGRP competitive antagonist since the presence of this protein can decrease the ACTH-induced MC4R activation. This result lends weight to the central action of ACTH since AGRP is basically expressed in the zebrafish brain (Cerdá-Reverter *et al.* 2003b).

The MRAP system provides a mechanism for the fine tuning of the melanocortin signaling throughout the regulation of the receptor activity and/or response. Supporting this idea, we demonstrate that MRAP1 and MRAP2a expression, both proteins involved in the regulation of ACTH responsiveness via MC2R and MC4R, respectively, are downregulated by cortisol, T_3 and bezafibrate. All three compounds could modulate the response to ACTH, regulating the presence of MC2R in the membrane, by decreasing MRAP1 expression, or regulating ACTH binding to MC4R, by down regulating MRAP2a expression. This fact acquires special relevance in regulatory feedback systems. The MC4R, together with MC2R (Zhang *et al.* 2012), is also highly expressed in the head-kidney where interrenal tissue, the equivalent of mammalian adrenal tissue, is intermingled. Increased cortisol

levels could decrease interrenal MRAP1 and MRAP2a expression providing a mechanism for a local negative feedback by decreasing the sensitivity to systemic ACTH.

In summary, we demonstrate that MC4R, a canonical MSH receptor involved in the control of energy balance, becomes an ACTH receptor-like when co-expressed with MRAP2a. Both proteins, MRAP2a and MC4R physically interact and are co-expressed at the central nervous system, in key areas involved in the regulation of energy balance. In addition, ACTH administration inhibits food intake in wild type animals but not in MC4R deficient zebrafish suggesting that MC4R mediates the anorexigenic effects of ACTH. MRAP2a is regulated by several hormonal systems including corticosteroid and thyroid hormones thus providing an excellent substrate for the fine tuning of melanocortin activity. MRAP2b is also able to interact with MC4R and both proteins are co-expressed in the brain, in similar areas where MC4R and MRAP2a are coexpressed. MRAP2b cannot modify the receptor response to the agonist but is upregulated during chronic fasting suggesting that the protein can decrease the constitutive activity of the receptor during fasting. This result support a role for MRAP2b in the control of energy balance and suggest that protein dysfunction would result in increased growth and/or obesity.

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CAPÍTULO V:

Melanocortin receptor accessory protein 2 (MRAP2) interplays with the zebrafish melanocortin 1 receptor (MC1R) but has no effect on its pharmacological profile

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Resumen

El sistema de melanocortina es probablemente uno de los sistemas hormonales más complejos ya que integra agonistas codificados en el precursor proopiomelanocortina, antagonistas endógenos, proteína de señalización de agouti y proteína relacionada con agouti, cinco diferentes receptores acoplados a proteína G y dos proteínas accesorias. Estas proteínas accesorias interactúan con los receptores de melanocortina para permitir el tráfico hacia la membrana plasmática o para regular el perfil farmacológico. El MC1R ocupa el locus extensión, que es el principal responsable de la regulación de la pigmentación. En el pez cebra, tanto el sistema MC1R como el MRAP2 se expresan en la piel. Demostramos que el MC1R del pez cebra interactúa física o estrechamente con el sistema MRAP2, aunque esta interacción no resulta en la modificación del perfil farmacológico estudiado. Sin embargo, el ayuno progresivo indujo el oscurecimiento de la piel, pero también una regulación positiva de la expresión de MRAP2 en la piel, lo que sugiere un papel desconocido para MRAP2a que podría involucrar procesos de desensibilización del receptor. También demostramos que el estrés por hacinamiento induce el oscurecimiento de la piel y una regulación negativa de la expresión de MC1R en la piel.

Abstract

The melanocortin system is probably one of the most complex hormonal systems since it integrates agonist, encoded in the proopiomelanocortin precursor, endogenous antagonist, agouti signaling protein and agouti-related protein, five different G-protein coupled receptors and two accessory proteins. These accessory proteins interact with melanocortin receptors to allow traffic to the plasma membrane or to regulate the pharmacological profile. The MC1R fill the extension locus, which is primarily responsible for the regulation of pigmentation. In zebrafish, both MC1R and MRAP2 system are expressed in the skin. We demonstrate that zebrafish MC1R physically, or closely, interacts with the MRAP2 system, although this interaction did not result in modification of the studied pharmacological profile. However, progressive fasting induced skin darkening but also an upregulation of the MRAP2 expression in the skin, suggesting an unknown role for MRAP2a that could involve receptor desensitization processes. We also demonstrate that crowding stress induces skin darkening and a downregulation of MC1R expression in the skin.

Introduction

The melanocortin system is formed by proopiomelanocortin POMC-derived peptides, mainly melanocyte-stimulating hormones (MSHs) and adrenocorticotropic hormone (ACTH) (Cerdá-Reverter *et al.* 2013; Sebag and Hinkle 2009a). The physiological functions of these small peptides are mediated by a family of G-protein-coupled receptors (Cone 2006). Tetrapod species exhibit five melanocortin receptors (MCR1-5) but the number diverges in fish. Zebrafish exhibit an additional copy of the MC5R but pufferfish lack MC3R and only have one copy of the MC5R (Schioth *et al.*

2005). The MC2R specifically binds ACTH while the remaining receptors bind ACTH and MSHs with different affinities. Melanocortin signaling can be also be modulated by an endogenous melanocortin antagonist named agouti-signaling protein (ASIP) and agouti-related protein (AGRP) (reviewed by Cerdá-Reverter *et al.* 2013; Cerdá-Reverter *et al.* 2011). In addition, recent research has demonstrated that the functional expression of these receptors can be regulated by a group of proteins, known as melanocortin receptor accessory proteins (MRAPs), that interact with the MCRs to modulate receptor traffic to the membrane, agonist binding and cAMP production (Cerdá-Reverter *et al.* 2013). Tetrapod species have two MRAP paralogues (MRAP1 and MRAP2) but, once again, the genome of fish can have additional paralogues, e.g. MRPA2 is duplicated in zebrafish as MRAP2a and MRAP2b (Agulleiro *et al.* 2010).

MRAPs were discovered because of the inability of MC2R to reach the plasma membrane and, consequently, to respond to ACTH when expressed in non-adrenal cells (Rached *et al.* 2005). Homozygosity mapping using single nucleotide polymorphism microarrays identified one gene, now named MRAP1, which is exclusively expressed in the adrenal cortex responsible for the MC2R traffic to the plasma membrane (Metherell *et al.* 2005). This small protein exhibits a hydrophobic transmembrane domain and displays a dual topology, meaning that both the N- and C-terminal regions can be found facing outward from the cell (Roy *et al.* 2007; Sebag and Hinkle 2007). MRAP1 forms antiparallel homodimers (Sebag and Hinkle 2007) as MC2R homodimerizes in the endoplasmatic reticulum (ER). These MC2R homodimers form a complex with at least two antiparallel MRAP1 homodimers, making a hexameric complex that is probably stimulated by two ACTH molecules (Cooray and Clark 2011). MRAP1 is also essential for correct folding, subsequent glycosylation, ACTH binding and ACTH-induced cAMP production (Roy *et al.* 2007; Sebag and Hinkle 2009b). The MRAP system is also involved in the function of the remaining melanocortin receptors. Immunoprecipitation studies in mammalian species have demonstrated that both MRAP1 and MRAP2 physically, or closely, interact with all melanocortin receptors (Chan *et al.* 2009). Both MRAPs reduce the functional expression of MC4R and MC5R but not of MC1R and MC3R in mammals (Chan *et al.* 2009). Accordingly, MRAP1 and MRAP2 reduce melanocortin-stimulated cAMP production in cells expressing MC3R, MC4R and MC5R, but only MRAP2 is able to induce a similar effect in MC1R (Chan *et al*. 2009). Our recent studies in zebrafish have demonstrated that MRAP2a transforms MC4R, a canonical MSH receptor, into an ACTH receptor when co-expressed in HEK cells (Agulleiro *et al.* 2013a) and MRAP2b is able to reduce MC4R constitutive activity (Agulleiro *et al.* 2013a; Sebag *et al.* 2013).

In mammals, MC1R is mainly expressed in the follicle melanocytes where the activation by α-MSH stimulates the synthesis of eumelanin (black-brown pigment), leading to skin darkening. ASIP antagonizes α-MSH effects on MC1R, switching the production of eumelanin to pheomelanin (yellow-red pigment) (Cone 2006). Suggestively, MC1R has very high affinity for α-MSH and ACTH

(Schioth *et al.* 2005). Similarly, the skin of some fish species is darkened under stress conditions (Kittilsen *et al.* 2009). It is thus plausible that MRAP system modify MC1R sensitivity to ACTH and/or MSH, as it does for MC4R in zebrafish (Agulleiro *et al.* 2013a).

In this paper, we explore the interaction between the MRAP system and MC1R using zebrafish as model. In addition, we also study the physiological regulation of skin MC1R and MRAP system expression under stressful conditions. Immunoprecipitation and immunolocalization studies showed that MRAP system physically interacts with MC1R in vitro. However, α-MSH- and ACTH-induced cAMP production in cells overexpressing MC1R was not modified by coexpression of the MRAP system. Finally, skin MRAP2b and MC1R expression were modulated under stressful conditions.

Material and methods

Animals

The wild-type zebrafish (TU) used in this experiment were reared at the facilities of the Instituto de Acuicultura de Torre la Sal (Castellón-Spain) under standard conditions. Fish were randomly captured, sacrificed in ice-cold water and quickly decapitated before any other manipulation. Experiments were carried out in accordance with the principles published in the European Animal Directive (86/609/CEE) concerning the protection of experimental animals and were approved by the internal committee.

Table I. Primers and probes used for tissue expression analysis, qPCR and cloning.

Primer	Sequence (5'-3')
Hind_zf_MC1R_Fw	TATAAGCTTATGAACGACTCTTCGCGCCA
Xho_zf_MC1R_Rv	TTACTCGAGTTACACTGCAAAGCACCACGAA
Hind_zf_Myc_MC1R_Fw	TATAAGCTTATGGAACAAAAACTCATCTCAGAAGAGGATCTGATGAACGACTCTTCGCG
zf_MC1R_Fw	TCCCACAAACCCTTACTGCAAG
zf_MC1R_Rv	TACACTGCAAAGCACCACGAAC
zf_MRAP2a_Fw	AGAGCCGCCACTGATGCT
zf_MRAP2a_Rv	CCACTTGGCCTCTGGAGTTG
zf_MRAP2b_Fw	TTGGCTGTGAGCTGGAAGTG
zf_MRAP2b_Rv	TGAAAGAGGGAACGTGATTGG
zf_β-actine_Fw	CTCTTCCAGCCTTCCTTCCT
zf_β-actine_Rv	CTTCTGCATACGGTCAGCAA
zf_18S_Fw	TGCATGGCCGTTCTTAGTTG
zf_18S_Rv	AGTCTCGTTCGTTATCGGAATGA
zf_Rpl13α_Fw	TCTGGAGGACTGTAAGAGGTATGC
zf_Rpl13α_Rv	AGACGCACAATCTTGAGAGCAG
zf_EF1α_Fw	CTGGAGGCCAGCTCAAACAT
zf_EF1α_Rv	ATCAAGAAGAGTAGTACCGCTAGCATTAC
zf_MRAP2a_Taqman_Probe	[6FAM]CTCTCACCCATGGACGATCAGGCA[TAM]
zf_MRAP2b_Taqman_Probe	[6FAM]CATTTTCTCTGCCACCGCTGCCTG[TAM]

MC1R, melanocortin receptor 1; MRAP2a, melanocortin receptor accessory protein 2 subtype a; MRAP2b, melanocortin receptor accessory protein 2 subtype b; 18S, 18S ribosomal RNA; Rpl13, 60S ribosomal protein L13; EF1α, elongation factor 1α.

Tissue expression experiments

To determine the tissue distribution of zebrafish MC1R (zfMC1R), total RNA was purified from fresh tissues (brain, eyes, heart, gills, liver, head kidney, post kidney, intestine, spleen, testis, ovary, muscle, total skin, dorsal skin and ventral skin) of five fish using TRI-reargent (Sigma) and treated with RQ1-DNAse (Promega). For testis and ovaries, only three fish from each sex were used. RNA (0.5 μg) was retro-transcribed to cDNA using Superscript IIITM reverse transcriptase (Invitrogen) primed with oligo-dT (12-18) (Promega). cDNA was used then as a template for quantitative PCR (qPCR). Elongation factor 1α (EF1α), β-actin and 18S levels were used to normalize zfMC1R expression. Amplifications were carried out in final reaction volume of 20 μl containing 10 μl 2X Sybrgreen® PCR master mix (ABgene, Thermo Scientific). Specific primer concentrations (see Table 1 for sequences) were as follows: zfMC1R, 250 nM; EF1α, 125nM; β-actin, 300nM and 18S, 150 nM). Sequence primers of house-keeping genes were obtained from Tang *et al.* (2007) and Koven and Schulte (2012). The volume of cDNA was 0.5 μl for zfMC1R, 1μl for EF1α, 1 μl (1/10) for β-actin and 1 μl (1/10000) for 18S. Reactions were carried out in a Realplex2 Mastercycler (Eppendorf) in triplicate. The tissue distribution of MRAPs in zebrafish obtained by qPCR have been published previously (Agulleiro *et al.* 2013a).

Co-immunoprecipitation studies

In order to analyze whether MRAPs physically or closely interact with the MC1R, co-immunoprecipitation (Co-IP) assays were performed. Myc-zfMC1R alone or in combination with Flag-zfMRAP2a or Flag-zfMRAP2b (Agulleiro *et al.* 2010) was transfected with lipofectamine in HEK-293 cells. The whole-cell lysates were prepared 24-h post-transfection, as described by Agulleiro *et al.* (2013a). Myc-human(h)MC2R and hFlag-MRAP1 were used as positive controls. Subsequently, samples were centrifuged for 20 min at 16000 g, 4°C. 20 µl of the supernatant were mixed with Laemmli Sample Buffer (LBS) 2X before being used for Western blotting and the rest of the sample were incubated overnight at 4°C with anti-MYC magnetic beads (MBL) for Co-IP. After incubation, the beads were washed 3 times in lysis buffer, the supernatant was removed and Laemmli 2X loading buffer was added. After boiling for 3 min, samples were run in SDS-polyacrylamide gel. Immunodetection was performed with anti-MYC (Abcam) antibody (1:5000) and detected by HRP chemiluminescence reaction coupled to the secondary antibody (Pierce).

Immunofluorescence microscopy

HEK cells grown on poly-L-lysine-coated coverslips were transiently transfected with 0.2 µg/well of Myc-MC1R and 0.2 µg/well Flag-MRAP2a, or Flag-MRAP2b constructs. Twenty-four hours later, cells were fixed and permeabilized by incubation in methanol for 5 min and subsequently in acetone for 1 min. The cells were then rehydrated, washed in PBS, blocked and incubated with mouse anti-C-Myc and rabbit anti-Flag antibodies. Primary antibodies were detected with goat anti-mouse or anti-rabbit secondary antibodies coupled to Alexa-Fluor 488 or Alexa-Fluo 594 (Invitrogen) as required. DAPI (2 µM) was used to stain nuclei. The coverslips were mounted in Prolong mounting medium for fluorescence (Invitrogen). Cells were examined with an Olympus BX-41microscope.

Effects of MRAPs on zfMC1R pharmacological profile

The full coding regions of the zebrafish MC1R (zfMC1R) genes were obtained from public databases (http://www.ensembl.org/index.html). cDNA from the whole fish was amplified with Taq DNA polymerase (Invitrogen) using specific primers targeting the extremes of the coding region (see Table 1). The expected size products were purified and cloned directionally in pcDNA5/FRT. The same procedures were performed to obtain plasmid MC1R tagged with an MYC (EQKLISEEDL) sequence at the N-terminal but using a modified FW primer (Table 1). MRAP constructs were taken from Agulleiro *et al.* (2010). The effect of the different zebrafish MRAPs (MRAP1, MRAP2a and MRAP2b) on the MSH- and ACTH-induced zfMC1R activity was measured by transient co-transfection of zfMC1R with the individual MRAPs. 100 ng of each specific construct was transfected using Lipofectamine TM LTX with PLUS TM Reagent (Invitrogen), according to the manufacturer's instructions, in HEK-293 cells stably expressing β-galactosidase under the control of vasoactive intestinal peptide promoter placed downstream of tandem repetitions of cAMP-responsive elements

(Clon Q) (Sanchez *et al.* 2009). In addition, 100 ng of luciferase construct were transfected for standardization of the transfection efficiency (Agulleiro *et al.* 2013a). The total transfection amount of DNA was kept constant at 2 μg with pBSSK plasmid (Stratagene). Cells were maintained in DMEM medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and 1% penicillin/streptomycin (Invitrogen) in a humidified atmosphere of 5% CO2 at 37 °C. The antibiotics were removed 24-hours prior to transfection and FBS was replaced by bovine serum albumin (BSA) for the stimulation protocols. 24 hours post transfection, cells were divided into 96 well plates and 48 hours post-transfection they were stimulated with human α-MSH (Bachem), ACTH(1-24) ranging from 10^{-5} to 10^{-10} M or forskolin 10^{-6}. Six hours post-stimulation, the medium was removed and cells were lysed, measuring the galactosidase activity measured as previously described (Sanchez *et al.* 2009). Receptor activation assays were performed in quadruplicate wells and repeated in at least 3 independent experiments. Luciferase levels were measured using commercial assays (Promega) and following the manufacturer's recommendations.

Effects of stressful conditions on the skin melanocortin system

The effects of fasting and high stocking density on the relative expression levels of zfMC1R and zfMRAPs were evaluated. For this, 90 zebrafish were acclimatized to laboratory conditions for 15 days and subsequently divided into three 20-liter aquaria (n= 30 fish/treatment). Body weight (BW) and length were recorded for biometrical purposes. The control group, at a density of 10 fish/liter, was fed twice a day for two weeks with a commercial feed (4% BW). Animals of the second group were reared in similar density conditions but fasted throughout the experimental period. Finally, the third group were fed twice a day for two weeks at 4% BW but kept at a higher density (60 fish/l). To reach this density fish were placed in a 0.5-liter holey plastic container inside a 20-liter aquarium. In this way, fish were kept at a higher density, but under similar conditions of water quality in all the experimental groups. Seven and fourteen days after the experiment, 10 fish per group were weighed, measured and dissected to remove skin samples. Total RNA was individually extracted as mentioned previously and 0.5 μg was to synthesize cDNA. For MC1R, β-actin, and 18S qPCR amplifications were carried out in a 20 μl final reaction volume as before. For RPL13α, the final volume of the reaction was 15 μl, containing 7.5 μl of 2X Sybrgreen® PCR master mix (ABgene, Thermo Scientific), 1 μl of cDNA in at dilution of 1:100 and primer concentration of 200 nM. The RPL13α primers used were obtained from Tang *et al.* (2007), while MRAP2a and MRAP2b determination followed Agulleiro *et al.* (2010): briefly, 10 μl of 2X Taqman® PCR master mix (ABgene, Thermo Scientific, Spain), and 0.5 μl of cDNA was used. Primer and probe concentrations were 300 nM and 250 nM, respectively. Reactions were carried out in tripicate. The primer sequences are shown in Table 1.

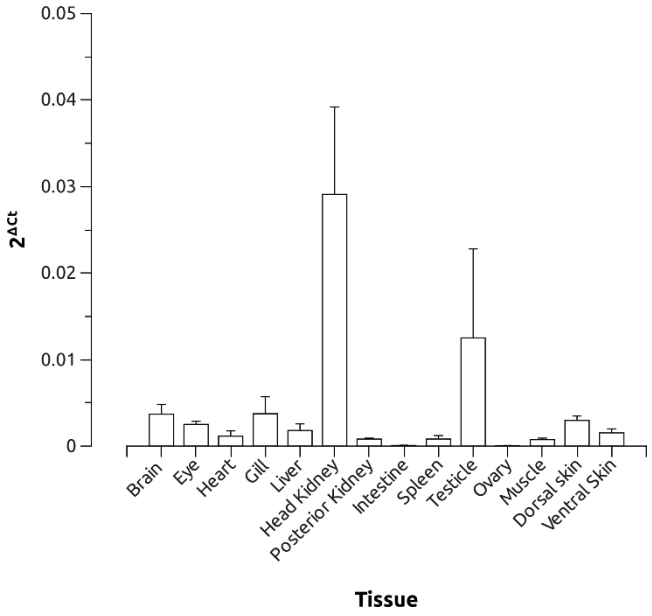

Figure 1. Expression of MC1R in different tissues analyzed by real-time PCR. The cDNA of five tissues from different animals (n=5/tissue; n=3/tissue for ovary) was used as template for quantitative real-time PCR. Data were analyzed using ΔCt method and geometric average of EL1α, β-actin, and 18S for normalization (Vandesompele *et al.* 2002).

Data analysis and statistics

Tissue expression data were analyzed using the ΔCt method and the geometric average of EL1α, β-actin, and 18S for normalization (Vandesompele *et al.* 2002). Skin expression data were analyzed using the ΔΔCt method and the geometric average of β-actin, RPL13-α and 18S for normalization. Statistical differences were tested using one way ANOVA, followed by the HSD post hoc test ($p<0.05$) using SPSS. Receptor activation data were fitted to logistic curves using free QtiPlot software for LINUX (http://soft.proindependent.com/qtiplot.html). For the graphical representation, the response average for each dose from three independent experiments was calculated and data were fitted to logistic curves. The qPCR data were analyzed with the ΔΔCt (cycle threshold) method. Statistical analysis was conducted by one-way analysis of the variance followed by Tukey´s multiple range test ($p< 0.05$).

Results

Tissue expression of MC1R

By means of RT-PCR using specific primers, we cloned a 981 bp fragment showing 99% identity to zebrafish MC1R (accession numbers BC162836.1, BC162848.1, NM_180970.1 and AY161847.1). The highest MC1R expression levels were detected in the head kidney and testis. In addition, a certain level of expression was found in the brain, eye, gill, heart, liver, posterior kidney and muscle. As expected, MC1R was also found in both dorsal and ventral skin but no expression was found in the intestine and ovary (figure 1).

MC1R and MRAP system interplay, as demonstrated by immunoprecipitation and immuncolocalization, in HEK-cells

The interaction of zfMC1R and accessory proteins was studied primarily by co-immunoprecipitation (Co-IP). Zebrafish MC1R co-immunoprecipitaed with MRAP2a and MRAP2b but not with MRAP1 (figure 2). When coexpressed in vitro, all three proteins (MC4R, MRAP2a and MRAP2b) were detected by immunocytochemical techniques. High levels of MRAP2a and MRAP2b (figure 3) were detected in the surrounding area of the cell nucleus matching the position of the endoplasmic reticulum/Golgi complex. MC1R was easily detected in the cellular membrane where it was found closely colocalized with MRAP2a (figure 3). In contrast, MC1R and MRAP2b did not seem to colocalize in the plasma membrane but only in the cellular cytoplasm (figure 3).

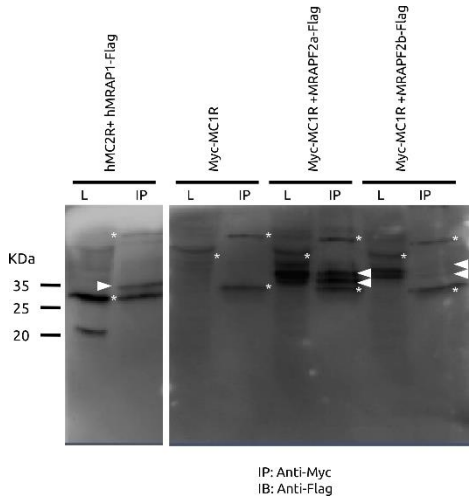

Figure 2. MRAP2s and MC1R interactions. HEK-cells were transfected with Flag-tagged MRAPs and/or Myc-tagged MC4R. Whole-cell lysates were prepared 24 h after transfection and used for Western blot or incubated with anti-MYC agarose beads for coimmunoprecipitation. Both Flag-MRAP2a and Flag-MRAP2b but not Flag-MRAP1 interact with Myc-MC1R as seen by immunoblotting with anti-Flag after immunoprecipitation with anti-Myc. Asterisks indicate unspecific bands whereas arrowheads indicate positive bands in crude lysates and immunoprecipitated samples.

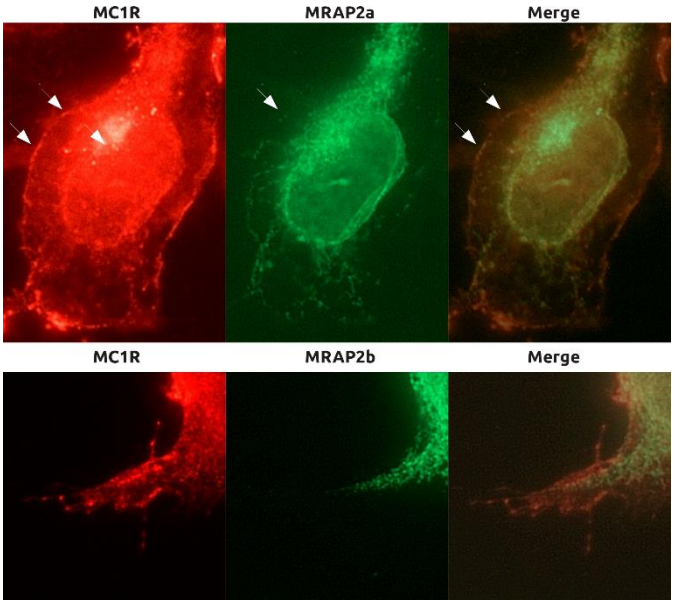

Figure 3. Immunofluorescence assays in live cells showing the expression of MRAP2s (green) and/or zfMC1R (red). N-terminally Flag-tagged MRAPs and N-terminally Myc-tagged MC4R were transiently expressed in HEK-293 cells. Photomicrographs are taken with a 100X objective using a conventional Olympus BX41. Arrows indicate regions of the plasma membrane where MRAP2a and zfMC1R are potentially colocalizated.

Effects of MRAPs on zfMC1R pharmacological profile

Zebrafish MC1R was not able to respond to ACTH (1-24) when expressed in HEK-293 cells. A significant increase in ACTH (1-24)-induced cAMP production was detected in cells stimulated at 10-5 M (Fig. 4). On the contrary, MC1R-expressing cells were stimulated with α-MSH but no differences were found when MC1R was expressed alone or in combination with the different MRAPs (Fig. 4). ED50 values were as follows: ED50 MC1R=5.1 X 10-7 ± 3.46 X 10-7; MC1R+MRAP1=1.89 X 10 -7 ± 2.33 X 10-7; MC1R+MRAP2a=1.78 X 10-7 ± 1.15 X 10-7; MC1R+MRAP2b=2.48 X 10-7 ± 1.66 X 10-7.

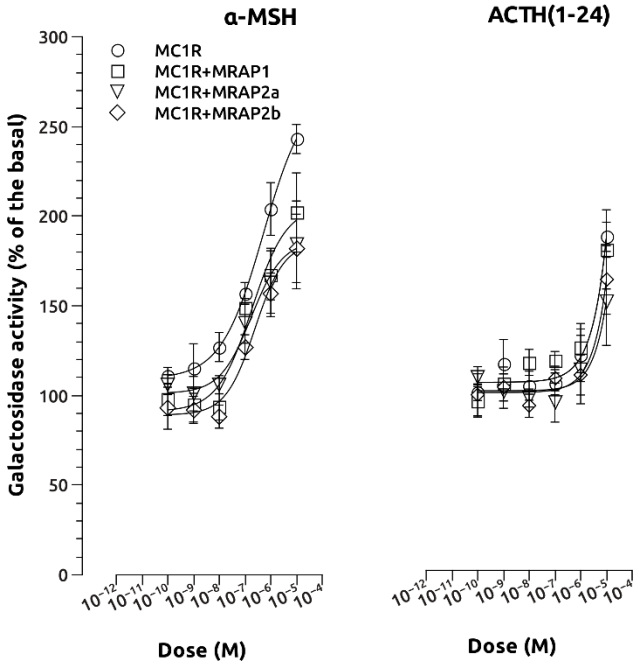

Figure 4. Pharmacological properties of melanocortin agonist, α-MSH and hACTH (1-24) at HEK-293 transiently expressing both MC1R and different MRAPs and stably expressing a cAMP-responsive β-galactosidase reporter gene. Data were normalized to protein levels and expressed as percentage of the basal levels. A construct carrying luciferase gene under the control of a constitutive promoter was also transfected to standardize the transfection levels. Experiments were performed using quadruplicate data points and repeated at least three times independently. Data are mean ± SEM of the tree independent experiments.

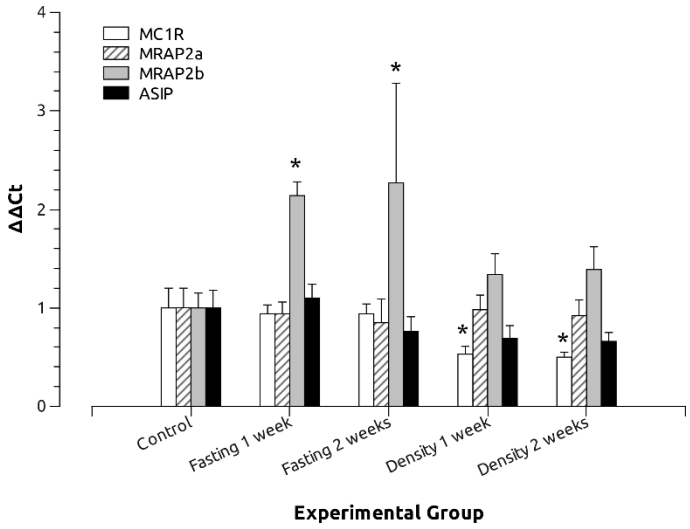

Figure 5. The effects of progressive fasting and high stocking density on the relative expression levels of zfMC1R and zfMRAPs were evaluated. Total skin RNA was purified and used for cDNA synthesis. Data were analyzed with the ΔΔCt (cycle threshold) method.

Physiological regulation of zfMC1R and MRAP expression in the skin

The expression of MC1R, MRAP2 a, MRAP2b and ASIP1 in skin was evaluated by qPCR after progressive fasting but also in fish subjected to two different densities of culture. MRAP2b

expression in the skin increased significantly after one or two weeks of fasting, and MC1R expression levels were depressed at the higher rearing density after one and two weeks (figure 5). Interestingly, the fish kept in the higher density conditions and those subjected to progressive fasting exhibited a darkening of the skin (data not shown).

Discussion

Our previous studies have demonstrated that MRAP system interacts with the melanocortin receptors to allow receptor trafficking to the plasma membrane (MC2R) (Agulleiro *et al.* 2010), modify the pharmacological profile or attenuate the constitutive activity of the receptor (MC4R) (Agulleiro *et al.* 2013a; Sebag *et al.* 2013). Studies in mammalian systems have shown that the MRAP system also interacts with other melanocortin receptors, including MC1R, MC3R and MC5R (Chan *et al.* 2009). The interaction between MC1R and MRAP2 is responsible for a decrease in NDP-MSH-stimulated cAMP production compared with cells that only express the receptor (Chan *et al.* 2009). In this study, we demonstrate by co-immunoprecipitation and immunological studies that both zfMRAP2a and zfMRAP2b interact with the zfMC1R although the interactions have no effect on the pharmacological profile of the receptor. However, MRAP2b expression in the skin increases significantly after fasting-induced stress, suggesting as-yet unknown functions for the MRAP2/MCR interaction.

The darkening of zebrafish skin as a result of long-term crowding or fasting suggests that stimulation of the hypothalamic-pituitary-interrenal (HPI) axis could be responsible for the MC1R activation that leads to melanin synthesis and/or pigment reorganization. It is known that MC1R signaling is critical for fish pigmentation since nonsense mutations results in albinism (Gross *et al.* 2009). In addition, MC1R mediates MSH-induced pigment dispersion in goldfish chromatophores (Kobayashi *et al.* 2011), while HPI activation after exposure to stressful conditions induces glucocorticoid synthesis and secretion via increased ACTH plasma secretion and binding to MC2R in the interrenal cells (Bernier 2006). It is therefore plausible that increased ACTH plasma levels after exposure to stressful conditions could bind MC1R to stimulate melanin synthesis and/or reorganization. Suggestively, it has been demonstrated that human and fugu MC1R bind ACTH with high affinity (Schioth *et al.* 2005). Furthermore, several endocrine pathologies involving increased pituitary ACTH production and secretion, like Cushing's and Addison's diseases and familial autosomal glucocorticoid deficiency (FGD), are characterized by skin hyperpigmentation (Novoselova *et al.* 2013). With this in mind, we explored the sensitivity of zebrafish MC1R to ACTH(1-24) by studying the ACTH-induced cAMP production in HEK-cells overexpressing MC1R. Our results show that zfMC1R responded to ACTH very weakly and only pharmacological doses close to μM were able to induce some degree of hormone-induced cAMP production. Similarly, seabass MC1R was barely activated by ACTH (1-39) (Sanchez *et al.* 2010) but fugu MC1R was activated by ACTH(1-24) with a high degree of sensitivity (Schioth *et al.* 2005). A previous work of ours

demonstrated that MRAP2a enables MC4R, a canonical MSH receptor, to be activated by ACTH with similar sensitivity to that exhibited by MC2R (Agulleiro *et al.* 2013a). Similarly, MRAP2b is able to reduce the constitutive activity of MC4R (Agulleiro *et al.* 2013a; Sánchez *et al.* 2009; Sebag *et al.* 2013). Since MRAP2/MC4R interaction confers ACTH sensitivity to the receptor, we hypothesized that the interaction between MRAP2 and MC1R systems could sensitize zebrafish MC1R to ACTH. However, coexpression of MRAP2a or MRAP2b did not change MSH- or ACTH-induced cAMP production, suggesting that the MRAP2 systems have no effect on MC1R pharmacology. We cannot exclude an effect of the binding profile since MC1R has been shown to bind other melanocortin peptides, including β-MSH, and to display differential sensitivity depending on the degree of acetylation of the α-MSH (Sanchez *et al.* 2010). One possibility is that the MRAP2 system could regulate the intracellular MC1R trafficking pathways in the pigment cells. Preliminary results in our lab show that MRAP2/MC1R coexpression does not modify receptor density in the plasma membrane, as also reported in mammalian systems (Chan *et al.* 2009). However, the increase of MRAP2b expression in the skin during progressive fasting suggests an elusive physiological role for the MRAP2 system. Accessory proteins are also known to be associated with their receptors throughout their life cycle and are part of the desentization and internalization processes (Chan *et al.* 2009). Mammalian MC5R traffics to the membrane in the absence of MRAPs but is trapped intracellularly when coexpressed with MRAP1. In addition, the coexpression of the MC5R with MRAP1 blocks receptor dimerization (Sebag and Hinkle 2009a). Therefore, it is possible that the interaction between the MRAP2/MC1R systems affects the desentization processes to modify receptor responses to persistent circulating MSHs during stressful conditions, although more experiments are required to explore this hypothesis.

Several G-protein-coupled receptors (GPCRs) have been seen to require accessory proteins for complete functional expression, including D1 dopamine receptor, calcitonin-like receptor, odorant receptors and calcium sensing receptor (Cooray *et al.* 2009). Therefore, the dopamine receptor interacting protein 78 (DRiP78) is involved in the trafficking of D1 dopamine receptor (Bermak and Zhou 2001). DRiP78 binds to a transport motif within the C-terminus of the D1 receptor that has been found to be conserved in many GPCRs. Consequently, it has been suggested that this accessory protein may also regulate the export of other GPCRs. Studies have demonstrated a role for DRiP78 in the trafficking of AT1 angiotensin II receptors and M2 muscarinic acetylcholine receptors to the plasma membrane (Leclerc *et al.* 2002). It was recently suggested that DRiP78 also plays a role as a molecular chaperone in the assembly of the G protein subunits (Dupre *et al.* 2007). It is therefore plausible that MRAP2 system has functions independent of the melanocortin system, but which are still unknown.

Finally, we also explored the skin expression of MC1R following long-term stressful conditions. MC1R expression was not affected by progressive fasting but was significantly down-regulated after 1 and 2 weeks of crowding stress. Plasma α-MSH levels rise in chronically crowding-

stressed fish (Flik *et al.* 2006). It is therefore arguable that high levels of α-MSH are responsible for the skin darkening in stressed fish but also for a down-regulation of the MC1R expression. This further supports the idea that fish darkening might be used as a stress maker in zebrafish.

In summary, we have demonstrated that the zebrafish MC1R and MRAP2 systems physically, or closely, interact but that this interaction has no effect of the pharmacological profile or sensitivity to the main receptor agonist (α-MSH). However, we also show that MRAP2b is upregulated in the skin during progressive fasting, suggesting an unknown physiological role for this protein. Finally, we demonstrate that crowding stress induces skin darkening and a down-regulation of the MC1R expression in the skin.

Acknowledgments

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CAPÍTULO VI: Effects of acute handling stress on short-term central expression of orexigenic/anorexigenic genes in zebrafish

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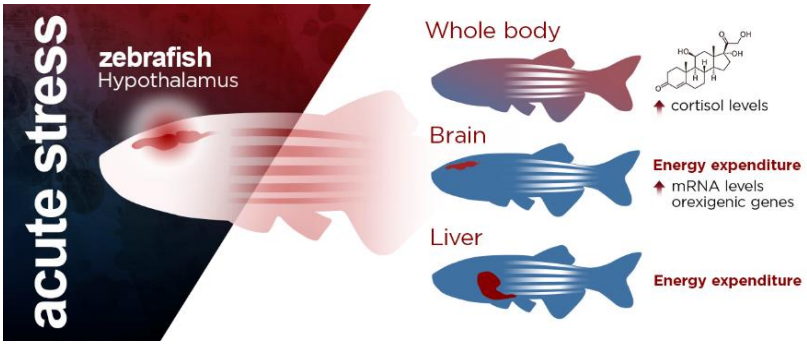

Keywords: Brain; Cart; Feeding behavior; Food intake; Melanocortin; Npy

Fish Physiol Biochem. 2018 Feb;44(1):257-272.

Resumen

Los mecanismos fisiológicos que impulsan la respuesta al estrés en los vertebrados están conservados evolutivamente. Estos mecanismos implican la activación de los ejes hipotálamo-simpático-cromafín (HSC) como hipotálamo-hipofisario-adrenal (HPA). En los peces, la reducción de los niveles de ingesta es una característica común en la respuesta conductual al estrés, pero los mecanismos centrales que coordinan la respuesta energética aún no son bien conocidos. En este trabajo, exploramos los efectos del estrés agudo en los sistemas centrales clave que regulan la ingesta en los peces, así como en los niveles de cortisol y glucosa. Mostramos que el estrés agudo induce un rápido aumento en el cortisol sin cambios en los niveles de glucosa, al mismo tiempo que promueve una respuesta central rápida activando las vías neuronales. Los tres péptidos orexigénicos investigados, el neuropéptido Y (*npy*), la proteína relacionada con agouti (*agrp*) y la *grelina*, aumentaron su nivel de expresión central, lo que sugiere que estos sistemas neuronales no están involucrados en los efectos inhibitorios de la alimentación a corto plazo provocados por el estrés agudo. Por el contrario, los precursores anorexigénicos investigados, *cart* y *pomc*, mostraron una mayor expresión después del estrés agudo, lo que sugiere su participación en los efectos anorexigénicos.

Abstract

Physiological mechanisms driving stress response in vertebrates are evolutionary conserved. These mechanisms involve the activation of both the hypothalamic-sympathetic-chromaffin cell (HSC) and the hypothalamic-pituitary-adrenal (HPA) axes. In fish, the reduction of food intake levels is a common feature of the behavioral response to stress but the central mechanisms coordinating the energetic response are not well understood yet. In this work, we explore the effects of acute stress on key central systems regulating food intake in fish as well as on total body cortisol and glucose levels. We show that acute stress induced a rapid increase in total body cortisol with no changes in body glucose, at the same time promoting a prompt central response by activating neuronal pathways. All three orexigenic peptides examined, i.e. neuropeptide y (*npy*), agouti-realted protein (*agrp*) and *ghrelin*, increased their central expression level suggesting that these neuronal systems are not involved in the short-term feeding inhibitory effects of acute stress. By contrast, the anorexigenic precursors tested, i.e *cart* peptides and *pomc*, exhibited increased expression after acute stress, suggesting their involvement in the anorexigenic effects.

Introduction

Stress response is highly conserved among vertebrates and can be classified from the temporal point of view into acute and chronic stress (for a review see Schreck and Tort 2016). Acute stress, which is more common in nature, is short-lived, relatively intense, and associated with environmental factors, such as temperature, oxygen levels, unpredictable changes, food limitation during short-time

periods or the presence of predators (McEwen and Wingfield 2003; Tort 2011). The acute stress response involves the activation of both the hypothalamic-sympathetic-chromaffin cell (HSC) and the hypothalamic-pituitary-adrenal (HPA) axes. HSC activation is fast but difficult to quantify due to rapid changes in catecholamine levels (Eiden 2013; Kvetnansky *et al.* 2013). The response mediated by the HPA axis is slower than the HSC response and characterized by the release of glucocorticoids, one of the main hormonal stress indicators in vertebrates (Tort 2011; Tsigos and Chrousos 2002).

In fish, the hypothalamic-pituitary-interrenal response (HPI, the equivalent of the tetrapod HPA axis) begins with the production of corticotropin-releasing factor (CRF) in neurons of the preoptic area (POA) and culminates in the production of cortisol, the main fish glucocorticoid, in the interrenal cells (for a review see Gorissen and Flik 2016; Mommsen *et al.* 1999; Wendelaar Bonga 1997). Preoptic CRF neurons innervate corticotropic cells of the anterior pituitary to stimulate proopiomelanocortin (POMC) production (Huising *et al.* 2004). POMC is processed by subtilisin-like enzymes, known as prohormone convertase 1/3 (PC1/3) (Seidah and Chretien 1999), resulting in adrenocorticotropic hormone (ACTH) production. Subsequently, ACTH is released into the bloodstream and reaches interrenal cells, where it interacts with the melanocortin 2 receptor (MC2R), in the presence of the melanocortin receptor accessory protein 1 (MRAP1) (Metherell *et al.* 2005). MRAP1 acts as an accessory factor for the correct folding and transport of MC2R to the plasma membrane (Agulleiro *et al.* 2010; Metherell *et al.* 2005). In turn, MC2R activation promotes the production of glucocorticoids such as cortisol in all vertebrates (Alsop and Vijayan 2008; Cerdá-Reverter *et al.* 2013). An increase in cortisol plasma levels is one of the most evolutionary conserved stress responses in vertebrates and is commonly used as an indicator of the degree of stress experienced (Leal *et al.* 2011). In addition to MRAP1, most vertebrate genomes exhibit a paralogue gene called MRAP2 (Chan *et al.* 2009), whose functions remain poorly understood (Cerdá-Reverter *et al.* 2013). MRAP2 is structurally related to MRAP1 but has no a main effect on MC2R functionality. In the zebrafish, MRAP2 is duplicated into MRAP2a and MRAP2b (Agulleiro *et al.* 2010). MRAP2a is able to change MC4R pharmacological profile by increasing receptor affinity to ACTH to similar levels than those reported for MC2R (Agullerio *et al.*, 2013b). On the contrary, MRAP2b seems to decrease the binding of melanocortin to MC4R (Sebag *et al.* 2013) as well as the constitutive activity of this receptor in zebrafish (Agulleiro *et al.* 2013a).

Once in the bloodstream, cortisol triggers a plethora of mechanisms that participate to partly restore homeostasis by making energy available for increased metabolic requirements. These mechanisms include the modulation of food intake, a common feature of the behavioral response to stress in fish (Bernier 2006; Bernier and Peter 2001). Experiments have demonstrated that low intraperitoneal doses increase food intake levels in goldfish (Bernier *et al.* 2004), whereas higher doses profoundly inhibit feeding behavior in goldfish (Bernier *et al.* 2004) and other species including rainbow trout (Gregory and Wood 1999) and sea bass (Leal *et al.* 2011). Exogenous doses of cortisol

inhibits CRF but stimulates neuropeptide Y (*npy*) expression within the preoptic area of the goldfish (Bernier *et al.* 2004). This scenario could explain the orexigenic effects of low cortisol doses since NPY stimulates and CRF inhibits food intake in goldfish, respectively (Volkoff *et al.* 2005, see below). Experiments in rainbow trout have demonstrated that implants of cortisol inhibit preoptic expression of both CRF and NPY but do not prevent the stimulation of neuropeptide expression under stress, suggesting that the responsiveness to acute stressors is maintained under chronic stress (Doyon *et al.* 2006). Overall, the data suggest that the fish neural system involved in the control of food intake is responsive to stressful situations and increased plasma cortisol levels but that pathways other than NPY and CRF mediate the anorexic effects of cortisol in a more significant manner. Although the interaction between stress response and the regulation of food intake in fish has been highly reported (Bernier *et al.* 2004; Guillot *et al.* 2016; Leal *et al.* 2011; Rubio *et al.* 2010), the central mechanisms coordinating the energetic and neural response to stress are still not well understood (Bernier 2006).

Our recent experiments demonstrated that under stressful conditions, zebrafish eat less but that feeding levels recover after three days (Guillot *et al.* 2016). Similar to tetrapod species, the hypothalamus is a key area of the fish brain in the regulation of the energy balance. Hypothalamic neurons integrate peripheral (body) and environmental information to elaborate a coordinated metabolic and behavioral response. These neurons express diverse hormone receptors and produce neuropeptides and neurotransmitters that increase hunger/satiety and, accordingly, promote feeding-related behavior (Volkoff *et al.* 2009). Both POMC (Cerdá-Reverter *et al.* 2003b) and cocaine and amphetamine-related transcript (CART) (Volkoff and Peter 2000) are potent anorexigenic (inhibiting food intake) factors of the fish brain. The *pomc* is expressed in a discrete region of the *lateral tuberal nucleus*, the teleostean homologue of the *arcuate nucleus* (Cerdá-Reverter and Canosa 2009), where it is processed to produce melanocyte-stimulating hormone (α-MSH) and β-endorphin. The central administration of chemical agonists or antagonists of the melanocortin receptors inhibit or stimulate food intake in fasted or fed animals, respectively (Cerdá-Reverter *et al.* 2003b). On the contrary, NPY (López-Patiño *et al.* 1999) and agouti-related protein (AGRP) (Cerdá-Reverter and Peter 2003) are a potent orexigenic factors (stimulating food intake) in the fish hypothalamus. AGRP is an inverse agonist of the constitutively activated MC4R (Sánchez *et al.* 2009). Ghrelin is the only peripheral peptide hormone stimulating food intake and adiposity in mammals but its central synthesis is controversial (Jönsson 2013). Experiments in fish also suggest that central and peripheral ghrelin is involved in the control of the energy balance (Jönsson 2013). Ghrelin administration in cyprinids stimulates food intake and progressive fasting increases central and gastrointestinal *ghrelin* expression in the goldfish and zebrafish (Amole and Unniappan 2009; Unniappan *et al.* 2002). Similarly, the administration of ghrelin to the brain increases food intake levels in rainbow trout (Velasco *et al.* 2016), although a previous work reported the opposite effect (reviewed by Jönsson 2013).

In this study, we evaluate the effects of acute stress on the transcription of key genes involved in the central and hepatic control of energy homeostasis and food intake. Acute stress-dependent transcriptional effects were combined into an integrated biological response index (IBRv2). This method is commonly used as a multi-biomarker approach to assess ecosystem health and to identify the impact of environmental stress on organisms (Sanchez *et al.* 2013). Our data show that acute stress increases whole-body cortisol levels but also promotes a central response by rapidly activating neuronal pathways. Concomitantly, the expression of orexigenic genes, including *npy*, *agrp* and *ghrelin*, increased, suggesting that these neuronal systems are not involved in the short-term feeding inhibitory effects of acute stress. By contrast, the tested anorexigenic precursors, i.e *cart* peptides and *pomc*, exhibited increased expression after acute stress, suggesting that they are involved in the anorexigenic effects.

Materials and methods

Fish

Adult zebrafish (0.25 ±0.04 g), wild-type TU strain, were obtained from the zebrafish facility in the Instituto de Acuicultura de Torre de la Sal (IATS-CSIC), (Castellón-Spain). Fish were kept in 20 L glass aquaria, at 27 ±1°C and fed twice a day with a commercial diet (TetraMin) at 4% of body weight for 2 weeks. In all the aquaria, the rearing density was one fish per liter and the photoperiod was set at 14 h light/10 h dark. All experiments were performed at 10:00 h to avoid changes related with circadian rhythms and fish were fasted 24 h before the beginning of the assay. Water conditions were monitored daily and the measured values were considered acceptable considering the requirements of this species (Lawrence 2007). The experiment was carried out in accordance with the principles published in the European Animal Directive (86/609/CEE) concerning the protection of experimental animals and were approved by the internal committee of Consejo Superior de Investigaciones Científicas (CSIC).

Acute stress experiment

The acute stress was induced by slightly netting the fish, following the protocol described in Ramsay *et al.* (2009) with some modifications. Sixty fish were maintained under controlled conditions (non-stressed fish) and the remaining sixty fish were captured and maintained in an air-exposed net for 3 minutes (min) and then released back into the home tank for three minutes. This protocol was repeated five consecutive times. The fish were maintained in resting conditions in their home tanks and sampled 15, 60 and 180 min post-stress. At the end of the post-stress period, twenty fish per sampling time were sacrificed by anesthetic overdose with 2-phenoxy-ethanol (0.05%). Ten fish per group were immediately frozen at -80 ºC and used for whole body cortisol and glucose measurements. The liver and brain of the remaining fish was dissected and immediately frozen at -80°C until total RNA extraction.

Whole-body cortisol extraction

Individual frozen zebrafish were thawed, weighed and placed in a test tube, where each animal was homogenized in 1 mL of phosphate saline buffer (PBS, 50 mM, pH 7.6) using a Polytron PT1200E Manual Disperser (Kinematica). The homogenized samples were centrifuged for 10 min at 16000 g and the supernatant was recovered. An aliquot of 10 µL was reserved for the glucose analysis. For the whole-body cortisol analysis, 200 µL of the supernatant were transferred to a borosilicate 5 mL test tube. Each sample was extracted twice with 1.8 mL of diethyl ether (Sigma-Aldrich, Spain). For each extraction, tubes were stirred by vortex for 60 s. The aqueous layer was frozen in dry ice for 30 s and the ether layer was transferred to a new borosilicate test tube. The organic layer of both extractions was pooled in the same test tube and dried by applying a nitrogen stream while the tubes were heated at 40°C. The dry extract was reconstituted in 250 µL of PBS-gelatin (1%).

Whole-body cortisol and glucose measurement

Whole-body cortisol was assayed by radioimmunoassay as described in described by Rotllant *et al.* (2006), quantifying the radioactivity using a liquid scintillation counter (Scintillation Counter Wallac 1409, PerkinElmer). The antibody (ref. 07-121016, MP Biomedicals, Solon, OH, USA) was used at a final dilution of 1:4500 (MO Biomedicals LLC, OH, USA). Antibody cross-reactivity with cortisol is 100% (manufacturer's specification) and the lower detection limit of the cortisol assay was 0.16 ng/mL. The possible cross-reactivity with other steroid hormones varies from 1.6% for corticosterone and is lower than 0.7% for 17α-hydroxyprogesterone, cortisone, desoxycorticosterone, 17α-hydroxypregnenolone, dexamethasone, progesterone, aldosterone, cholesterol, estradiol and testosterone. Whole-body glucose was measured by spectrophotometry by the glucose-oxidase method with a commercial kit and following the manufacturer's indications (Glucose RTU, Biomerieux, Spain).

RNA isolation and reverse transcription

Total RNA was isolated from brain and liver using TRI Reagent (Sigma) and treated with RQ1-DNAse (Promega) following the manufacturer's instructions. The RNA concentrations were measured using a NanoDropND-1000 Spectrophotometer (Thermo Fisher Scientific, USA). Reverse transcription (RT), to generate complementary DNA (cDNA), was performed using 1 µg of total RNA, denatured 70°C, 10 min, Oligo dT15primer (Promega) and SuperScript™ III Reverse Transcriptase enzyme (Invitrogen), in the presence of the recombinant ribonuclease inhibitor RNaseOUT™ (Invitrogen), in a final volume of 20 µL. The resultant cDNA was stored at -20°C until use. All procedures were performed following the manufacturer's protocols.

Transcriptional analysis and normalization strategy

The GenBank identification numbers, primer sequences and efficiencies are shown in Table 1. Efficiency of the amplification was determined for each primer pair using serial 5-fold dilutions of pooled cDNA. The efficiency was calculated as E=10(-1/s), where *s* is the slope generated from the serial dilutions when Log dilution is plotted against ΔCT (threshold cycle number) (Pfaffl 2001). RT-qPCR was performed using iTaqTM Universal SYBR® Green Supermix (Bio-Rad). Reactions were assembled according to manufacturer's instructions with individual 20 µL reactions consisting of 10 µL SYBR® Green PCR master mix (2x), 200 nM primers and 2 µL of cDNA template. All reactions were run in the Bio-Rad CFX384 Real-Time PCR Detection System (Bio-Rad Laboratories, USA), under the following protocol: 95ºC for 5 min, 40 cycles of 95ºC for 30 s, 60ºC for 30 s. All samples were run in triplicate. The expression data obtained from three independent biological replicates were used to calculate the threshold cycle (Ct) value. After checking for primer efficiency, RT-qPCR analysis of all the individual samples was determined following the same protocol as described above. The expression levels of the following genes were measured in the brain: agouti-related protein 1 and 2 (*agrp1* and *2*), cocaine and amphetamine-related transcript 1, 2, 3 and 4 (*cart 1, 2, 3* and *4*), FBJ murine osteosarcoma viral oncogene homolog (*fosab*), glucocorticoid receptor (*gr*), *ghrelin* and ghrelin-O-acyl transferase (*goat*), melanocortin receptor type 4 (*mc4r*), neuropeptide y (*npy*), melanocortin receptor accessory protein type 2a and 2b (*mrap 2a, 2b*), mineralocorticoid receptor (*mr*), proconvertase type 1 and 2 (*pc1 and 2*) and proopiomelanocortin a and b (*pomca* and *b*). In the liver, the expression levels of glucocorticoid receptor (*gr*), melanocortin receptor accessory protein type 1 (*mrap1*), mineralocorticoid receptor (*mr*), phosphoenolpyruvate carboxykinase 2 (*pepck*) and tyrosine aminotransferase (*tat*) were measured. In addition, eukaryotic translation elongation factor 1 alpha 1 (*ef*α), β-actin (*β-actin*), alpha-tubulin (*tub*), and glyceraldehyde 3-phosphate dehydrogenase (*gapdh*) genes were tested for gene expression stability using the NormFinder application (Andersen *et al.* 2004), which is based on an algorithm for identifying the most appropriate normalization gene among a set of candidates (Andersen *et al.* 2004). According to NormFinder results, the most stable gene was *gapdh*, which was then selected as housekeeping gene in the normalization procedure. Relative gene expression was calculated according to Pfaffl (2001).

Statistical analysis

Results were expressed as mean ± SE (standard error). Statistical data analysis was carried out using SPSS software 20 (SPSS Inc, IBM, IL, USA). For the gene expression analysis, one-way analysis of variance (one-way ANOVA) was performed in order to assess differences followed by a post-hoc Dunnett's test to signal significant differences between groups (Zar 1999). For the physiological parameters (cortisol and glucose), statistical differences between post-stressed groups and the respective controls were assessed using a Student t-test. To analyze statistically significant

differences between the post-stress groups, one-way analysis of variance (one-way ANOVA) followed by a post-hoc Tukey were performed. Statistical significance of the results was accepted for $p<0.05$.

In order to integrate the alterations induced by the acute stress at the transcriptional level of the selected genes, the integrated biological response (IBRv2) index was calculated as described by Sanchez *et al.* (2013) using all gene expression data (brain and liver), as well as expression data from individual tissues. The results obtained for the IBR calculation are shown as star plot charts, representing the deviation of all post stressed fish compared with the control (0).

Results

No mortality or morbidity was observed in fish subjected to the acute stress experiments.

Whole-body cortisol and glucose levels

Cortisol and glucose levels measured in the whole-body of *Danio rerio* are shown in figure 1. An analysis of the whole-body cortisol levels revealed that cortisol in the stressed groups significantly decreased with time (15 min> 60 min>180 min), whereas whole-body glucose levels remained unaltered throughout the experiment. Acute stress induced a statistically significant increase in whole-body cortisol, which reached maximum levels 15 min post-stress (40.57±3.98 ng cortisol/g fish). Cortisol levels in the whole-body 60 min post-stress were lower than at 15 min, but still significantly higher than in the respective control groups (11.80±3.87 ng cortisol/g fish). After 180 min, cortisol levels had fallen to basal levels, suggesting that animals recovered from stress (2.07±0.20 ng cortisol/g fish). There was no significant difference in cortisol levels between the control groups.

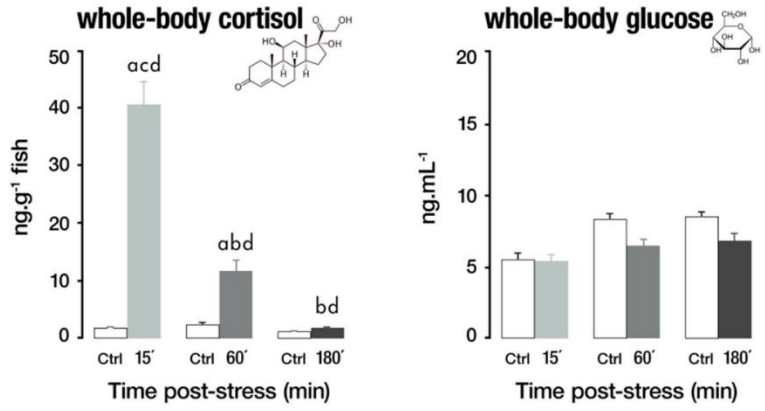

Figure 1. Whole body cortisol (left panel) and glucose (right panel) in zebrafish after acute handling stress. Ten fish per group were frozen at -80ºC after anaesthetic overdose and used for whole body cortisol and glucose measurement. White and coloured bars indicate control (Ctrl) and stressed fish (15, 60, 180 minutes), respectively. a, b, c and d indicate differences vs Ctrl, 15, 60 and 180 minutes, respectively ($p<0.05$).

Transcriptional regulation by acute stress

The overall profile obtained for gene expression is displayed as a heatmap (figure 2). Brain *pomc a* expression levels exhibited a significant increase 15, 60 and 180 min post-stress, whereas *pomc b* levels only increased significantly 15 and 180 min post-stress (figure 3A). On the other hand, brain mRNA levels of the genes coding for enzymes that perform the proteolytic cleavage of POMC, i.e. *pc1/3* and *pc2*, were significantly higher15 min post-stress, but had fallen to basal levels by the following sampling times (Fig. 3A). By contrast, *cart 1* and *cart2a* expression in the brain remained unaltered during the entire experimental time but both *cart3* and *cart4* exhibited significant upregulation 15 and 60 min post-stress. Central *mc4r* as well as *mrap2a* expression levels remained unchanged after the stress protocol, whereas *mrap2b* expression was significantly higher than basal levels at 15 min post-stress (figure 3A).

In the liver (figure 3D), the expression of the melanocortin receptor accessory protein 1 (*mrap1*) had increased significantly by 180 min post-stress, while the levels were similar to those of control animals at the previous sampling times. Furthermore, *gr* and *mr* expression levels remained unaltered throughout the experiment (figure 3D). Phosphoenolpyruvate carboxykinase 2 (*pepck*) showed significant overexpression only at 180 min after acute stress but tyrosine aminotransferase (*tat*) mRNA levels were upregulated at 60 min post-stress (figure 3E). The IBR index, calculated using liver expression data, increased with the post-stress period, reaching maximal values at 180 min. The first post-stress sampling time (15 min) led to alterations corresponding to an IBR value of 3.8, reaching 7.2 at 60 min and a maximum of 11.6 at 180 min (figure 4B). Based on all the gene expression data, the IBR value pointed to a similar impact on gene transcription at 15 and 180 min (figure 5).

With regards to orexigenic genes (figure 3B), *agrp1* mRNA levels had significantly increased in the zebrafish brain 15 min post-stress, returning to basal levels afterwards. *agrp2* expression levels in stressed animals were always similar to those of control animals. Brain *npy* and *ghrelin* expression levels increased significantly at all tested times, whereas *goat* mRNA levels were only higher 60 and 180 min post-stress. The mRNA levels of the *fosab* gene, previously known as *c-fos* (FBJ murine osteosarcoma viral oncogene homolog) were upregulated at 15 and 60 min post-stress but returned to basal levels after 180 min. Glucocorticoid and mineralocorticoid receptors (*gr* and *mr*, respectively) showed a similar response pattern, exhibiting significantly depressed levels only 180 min after acute stress (figure 3C). The integrated biological response index (IBR), calculated using brain expression data (figure 4A), revealed a higher impact on the selected brain genes at 15 min post-stress (30.2), which slightly decreased with sampling times (24.8 and 22.5 for 60 and 180, respectively).

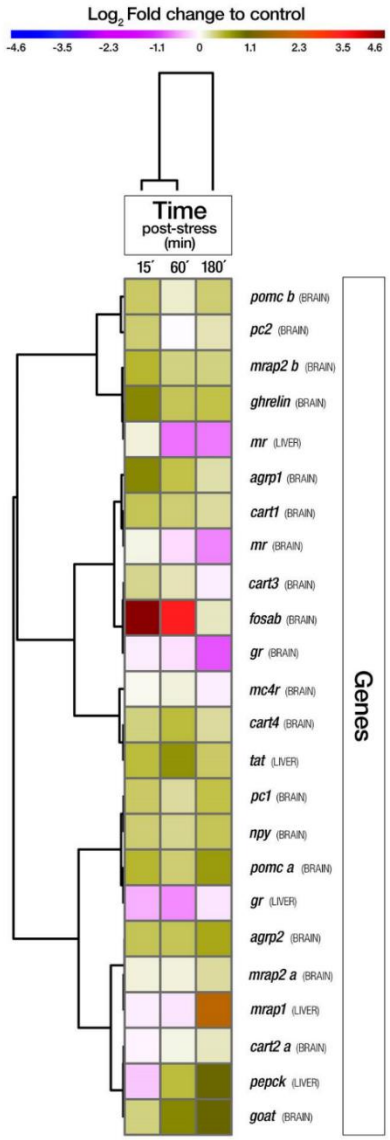

Figure 2. Heat map generated using gene expression levels of several genes related with feeding behavior or metabolism regulation in the brain and liver of zebrafish. *agrp1 and 2* (agouti-related protein subtype 1 and 2), *cart 1,2,3 and 4* (cocaine and amphetamine-related transcript subtype 1,2,3 and 4), *fosab* (FBJ murine osteosarcoma viral oncogene homolog), *goat* (ghrelin-O-acyl transferase), *gr* (glucocorticoid receptor), *mc4r* (melanocortin receptor type 4), *mr* (mineralocorticoid receptor), *mrap1, 2a and 2b* (melanocortin receptor accessory protein subtype 1, 2a and 2b), *npy* (neuropeptide Y), *pepck* (phosphoenolpyruvate carboxykinase 2), *pomc a and b* (proopiomelanocortin subtype a and b), *pc 1 and 2* (proconvertase subtype 1 and 2), *tat* (tyrosine aminotransferase).

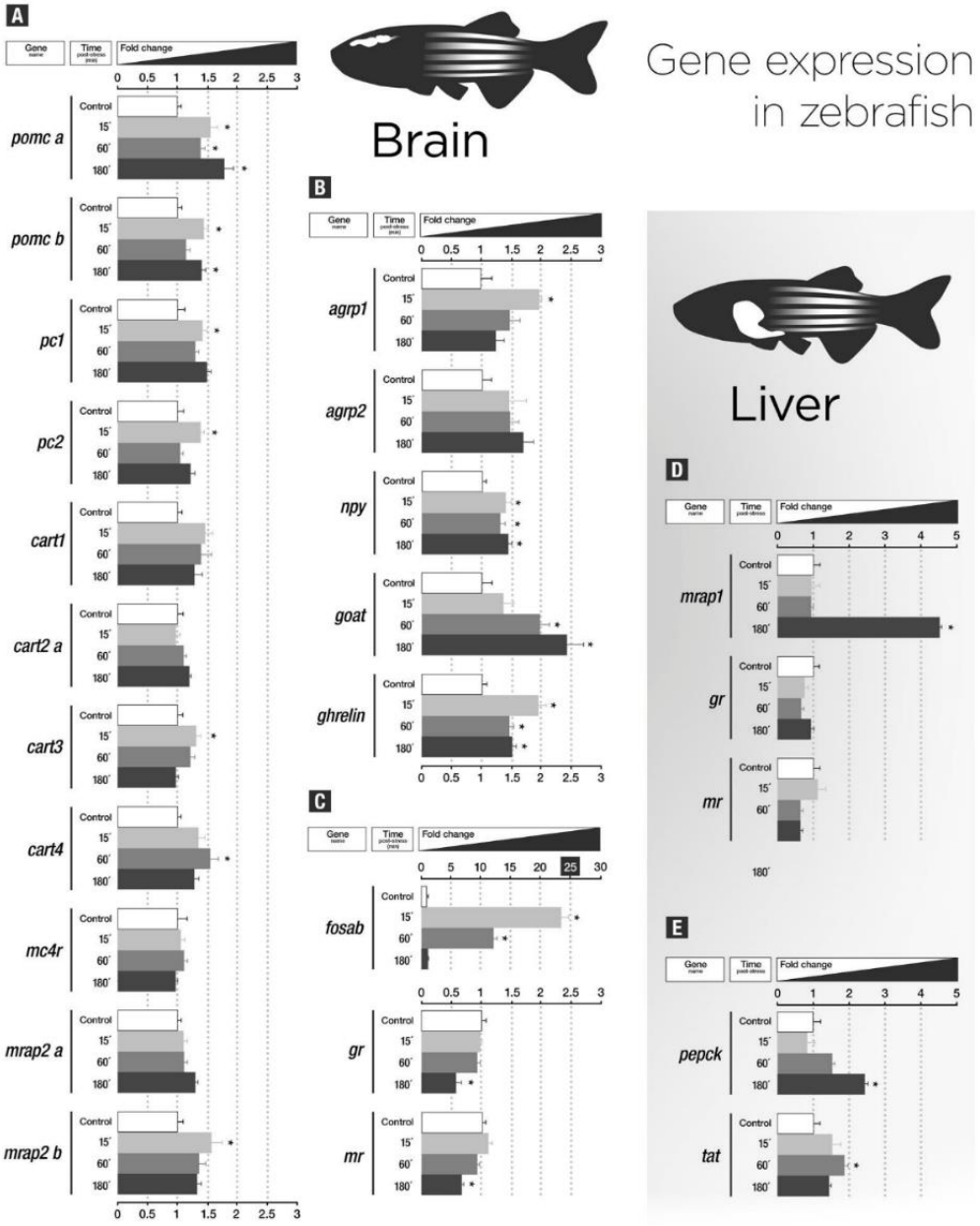

Figure 3. Expression level of different genes involved in the regulation of energy balance in the brain (A, B and C) and liver (D and E) of zebrafish as revealed by quantitative real-time PCR (qPCR). Amplifications of *gapdh* were used as internal control of the reverse transcription. See figure 2 for abbreviations.

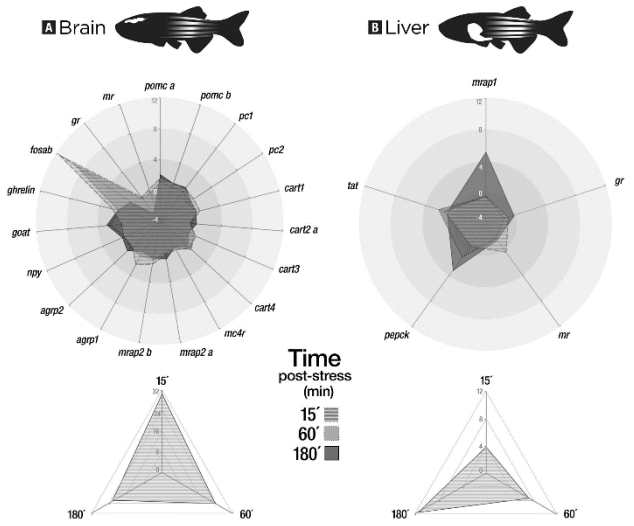

Figure 4 Assessed endpoints star plots and integrated biomarker response (IBR) in A) brain and B) liver, 15, 60 and 180 min after acute stress.

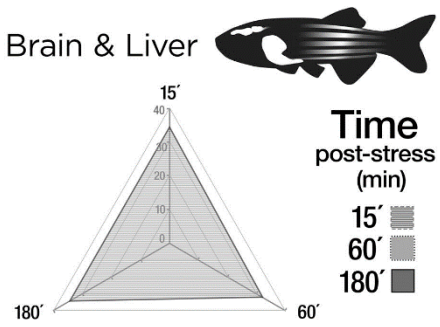

Figure 5. Integrated biomarker response (IBR) calculated with brain and liver data combined.

Discussion

Our previous results demonstrated that stressful conditions drastically reduced food intake levels in zebrafish but, once the stressor had ceased, animals recovered and gradually returned to pre-stressor feeding levels (Guillot *et al.* 2016). The present results add to these findings by showing short-term transcriptional changes in some central systems involved in the control of food intake as well as the expression of metabolic genes in the liver. As previously reported, the acute stress induced by netting and air exposure induced a significant increase in whole body cortisol levels, which peaked 15 min post-stress. Fish started to recover 60 min post stress, as suggested by the decrease in cortisol levels that reached basal levels at 180 min post-stress. This stress response is in agreement with previous studies in zebrafish although quantitative differences in cortisol levels may be related with slight differences due to the experimental protocols followed. Changes in carbohydrate metabolism and glucose levels have been used as general stress indicators in fish (reviewed by Mommsen *et al.* 1999). Under the conditions of acute stress described in the present study, the increase in cortisol levels was accompanied by unaltered whole-body glucose levels throughout the experiment, a pattern of

response already observed in previous studies with stressed fish (reviewed by Mommsen *et al.* 1999). These results support the idea that there is considerable variability in fish responses under stress conditions, mostly related to variations in stressor exposure and individual differences (Rotllant and Tort 1997; Teles *et al.* 2006).

The results further suggest that neuronal systems are rapidly activated under acute stress conditions as indicated by the increase in *fosab* (also known as *c-fos*) gene expression, an immediate early gene marker commonly used as an indicator of neuronal activity (Calfun *et al.* 2016). The rapid increase in *fosab* expression coincided with increased whole-body cortisol levels, suggesting that cortisol is partially responsible for the rapid activation of neuronal systems after acute stress. Comparable results have been obtained in zebrafish experiments using similar acute stress protocols (Pavlidis *et al.* 2015). Previous studies in mammals reported that *fosab* expression increased in the *arcuate nucleus* after stress events, suggesting that neuronal circuits controlling the energy balance are directly affected by stressful conditions (Keshavarzy *et al.* 2015; Kwon *et al.* 2006). The regulation of food intake and energy homeostasis is highly conserved throughout the evolutionary history of vertebrates (Cerdá-Reverter and Canosa 2009; Cerdá-Reverter *et al.* 2003b; Forlano and Cone 2007). In the present study, we show that acute stress induces a significant increase in whole-body cortisol levels and modulates the short-term central expression of genes involved in the regulation of food intake. Such effects on both systems persist after 180 min, when cortisol levels have returned to basal values.

Concerning *pomca* and *pomcb* mRNA levels, they remained high throughout the 180 min post-stress period. An increase in brain *pomc* mRNA levels after acute stress protocols was reported previously in zebrafish (Pavlidis *et al.* 2015). POMC is a complex precursor that encodes several neuroactive peptides, whose release depends on the set of prohormone convertases that cleave the peptide precursor (Castro and Morrison 1997). Our results demonstrate that *pc1* and *pc2* were upregulated at 15 min post-stress. The combined postranscriptional action of both convertases on POMC precursor results in the release of both α-MSH and β-endorphin (Seidah and Chretien 1999), which suggests that increased levels of central *pomc* after acute stress leads to the production of both α-MSH and β-endorpohin. In agreement with this, our previous experiments demonstrated that the central administration of an agonist inhibits food intake in fasted goldfish (Cerdá-Reverter *et al.* 2003a; Cerdá-Reverter *et al.* 2003b), whereas a chemical antagonist increases food intake levels in fed animals. The melanocortin α-MSH has also been demonstrated to increase energy expenditure in zebrafish larvae (Renquist *et al.* 2013), suggesting that the melanocortin system plays a role during energy mobilization, leading to the restoration of homeostasis after stressful situations (Mommsen *et al.* 1999). By contrast, β-endorphin has been shown to increase food intake levels after central administration (de Pedro *et al.* 1995). The complexity of the POMC precursor as well as the functional diversity of the encoded peptides makes it difficult to reach firm conclusions by exclusively studying

gene expression. We hypothesized that central level of *pomc*-encoded melanocortin peptides could help to unravel melanocortin involvement in the stress-induced feeding response. Accordingly, we studied also *mc4r, mrap2a* and *2b* and *agrp* expression in the brain after acute stress. MC4R has a key role in regulating the energy balance and growth in vertebrates including fish (Cerdá-Reverter *et al.* 2003a). The central administration of a chemical antagonist of the receptor increased food intake in satiated goldfish (Cerdá-Reverter *et al.* 2003b) and endogenous antagonist over expression in transgenic systems increased food intake levels and growth in zebrafish (Guillot *et al.* 2016). The present results show that only *mrap2b* expression was upregulated at 15 min post-stress while receptor expression remained unchanged. Similar results were found during progressive fasting in zebrafish (Agulleiro *et al.* 2013a). Our previous studies and others have demonstrated that MC4R is constitutively activated (Sánchez *et al.* 2009) and that the interaction with MRAP2b decreases the activity level of the receptor (Agulleiro *et al.* 2013a; Sebag *et al.* 2013). Both receptor and accessory protein expression co-localize in the *lateral tuberal nucleus* (Agulleiro *et al.* 2013a), suggesting that the constitutive activity of MC4R could be indirectly regulated by *mrap2b* expression. However, the observed response in MRAP2b could decrease the constitutive activity of MC4R, inducing the animal to increase feeding levels, an opposite reaction to that deduced from the increase in *pomc* expression. We also studied the effects of acute stress on the central expression of melanocortin endogenous antagonists and only *agrp1* mRNA levels were seen to be stimulated 15 min after stress. Within the brain, *agrp1* is exclusively expressed in the ventral region of the *lateral tuberal nucleus* (Agulleiro *et al.* 2014). AGRP works as an inverse agonist for MC4R, thus reducing the constitutive activity of the receptor (Sánchez *et al.* 2009). Although the data seem to indicate that the central melanocortin system is not involved in the short-term anorectic effects of acute stress, more experiments are required to elucidate the effects of increased *pomc* expression as well as the potential reduction of the MC4R constitutive activity imposed by both *mrap2b* and *agrp* increased expression.

A similar response to that obtained for brain *agrp* was observed in other central orexigenic pathways. Both *npy* and *ghrelin* central mRNA levels increased at all sampling times, whereas *goat* levels increased 60 and 180 min post stress. *npy* is expressed in the ventral region of the *lateral tuberal* nucleus (Cerdá-Reverter *et al.* 2000) in the same region where AGRP-neurons are localized, but co-localization studies are absent in fish. The expression of *ghrelin* in the brain itself is somewhat controversial. Quantitative PCR experiments have demonstrated *ghrelin* expression in the fish brain but hybridization techniques failed to detect *ghrelin* mRNA transcripts (Kaiya *et al.* 2005; Unniappan and Peter 2005), suggesting that ghrelin is present in the brain only in low quantities. By contrast, GOAT, the enzyme acylating ghrelin, is widely synthesized in the brain and its production is related to energy *status* (Blanco *et al.* 2017; Hatef *et al.* 2015). The role of GOAT in the postranscriptional processing of central ghrelin is uncertain and other roles including lipid acyl transferase cannot be ruled out. Our results showing a concomitant increase of the central *ghrelin* and *goat* expression suggest a role of the central GOAT in acylation of the centrally produced or peripheral incorporated

ghrelin in the fish brain. Both NPY and ghrelin have been shown to increase food intake in fish after central administration (López-Patiño *et al.* 1999; Unniappan *et al.* 2002). Accordingly, central *npy* (Narnaware *et al.* 2000) and *ghrelin* expression, but also gastrointestinal ghrelin production, increase with fasting (Unniappan and Peter 2005), suggesting a role in the compensatory feeding after food deprivation periods. Similarly no effect of acute stress was observed on *npy* transcription levels in tilapia (Oreochromis mossambicus) (Upton and Riley 2013). Again, our results are contrary to what was expected since the expression of all three genes is upregulated after stress, when feeding levels dramatically decrease in zebrafish (Guillot *et al.* 2016). This suggests that the central Ghrelinergic and NPYergic pathways rapidly respond to acute stress but are not involved in short-term anorectic effects. Alternatively, the increased *npy*, *ghrelin* and *agrp* mRNA levels in brain after acute stress might suggest that the zebrafish brain immediately triggers a coordinated response to counteract the inhibitory effects of stress on food intake. Interestingly, increased expression of *orexin*/*hypocretin* mRNA levels, an orexigenic neuronal system of the fish brain, has been previously reported in zebrafish (Pavlidis *et al.* 2015).

Similar to POMC-derived peptides, CART peptides are also involved in the negative regulation of food intake levels in fish (Volkoff and Peter 2000). Zebrafish brain produces four different cart peptide (CART1-CART4) (Akash *et al.* 2014), and the expression of all four is downregulated after fasting in pre-metamorphic stages (Nishio *et al.* 2012). However, studies in adult zebrafish have shown that only *cart2* and *4* are downregulated after 5 days of starvation (Akash *et al.* 2014). Our experiments show that only *cart 3* and *4* are upregulated in zebrafish brain after acute stress, suggesting that the different *cart* copies conceal the functional diversity of the system, as expected after the subfunctionalization of duplicated genes (Meyer and Van de Peer 2005; Murashita and Kurokawa 2011). The expression of *cart* 3 is restricted to the *entopeduncular nucleus*, whereas *cart* 4 is widely expressed in the telencephalic region and *caudal tuberal* hypothalamus. Our results showing a downregulation of *cart* expression in the brain after acute stress suggest the involvement of cart system in the stress-induced feeding response.

The effects of cortisol are mediated through corticosteroid receptors, including glucocorticoid and mineralocorticoid receptors located in all tissues (Takeo *et al.* 1996; Teles *et al.* 2013). In several fish species, two copies of the *gr* have been reported, but zebrafish genome only exhibited single copies for both *gr* and *mr* (reviewed by Schaaf *et al.* 2009). After binding to cortisol, these nuclear receptors translocate to the nucleus to regulate gene expression in response to stressful conditions. In our experiment, the expression of *gr* and *mr* was downregulated at 180 min post-stress, when cortisol levels were similar to control levels. In the conditions of the experiment, acute stress induced a rapid activation of the HPI axis, as demonstrated by increased cortisol levels that, in turn, induced a depression of *gr* and *mr* central expression. Previous results in zebrafish reported a slight but significant increase of *gr* brain expression 15 min after acute stress and no effect on *mr* central

expression (Pavlidis *et al.* 2015). In tilapia, cortisol administration by intraperitoneal injection, mimicking acute stress conditions, resulted in decreased *gr* mRNA levels in the intestine (Takahashi *et al.* 2006). The downregulation of the corticosteroid receptors may represent a mechanism to decrease tissue responsiveness to high cortisol levels, after acute stress. The present finding is in good agreement with previous results in mammals, in which reduced *gr* transcriptional levels were reported after acute cortisol treatment (Yudt and Cidlowski 2002).

The liver is the main energy store in cyprinid species (Huising *et al.* 2006) and activation of the HPI axis has been demonstrated to promote energy availability or an increased metabolic demand (Ashley 2007; Mommsen *et al.* 1999; Wendelaar Bonga 1997). Experiments in sea bass have reported that long-term stressful conditions have no effect on hepatic *mc2r* expression, while oral cortisol administration sharply decrease receptor mRNA levels (Agulleiro *et al.* 2013b). Our data demonstrate that *mrap1* liver expression was dramatically increased 180 min post-stress. Our previous studies in sea bass showed that ACTH induces hepatic lipase activity, suggesting that MC2R has a key role in the hepatic energy mobilization to restore body homeostasis after stressful conditions. The functional activity of MC2R is linked to the interaction with MRAP1 that allows the trafficking of the receptor to the plasma membrane (Agulleiro *et al.* 2010). Increased *mrap1* expression in the liver after acute stress could increase the presence of MC2R in the plasma membrane of hepatocytes, thus increasing liver sensitivity to systemic ACTH released after acute stress. Alternatively, *mrap1* expression could also be modulated by increased cortisol levels, since *in silico* analysis of the *mrap1* proximal promoter revealed the presence of several putative glucocorticoid responsive elements (Agulleiro *et al.* 2013a). However, *mrap1* expression in the whole-body zebrafish was shown to decrease after cortisol treatment in zebrafish (Agulleiro *et al.* 2013a). No effects were recorded in hepatic expression of *gr* and *mr*. *pepck* encodes for a gene that catalyzes the conversion of oxaloacetate to phosphoenolpyruvate and is the rate-limiting step in the metabolic pathway that produces glucose from lactate and other precursors derived from the citric acid cycle (Matte *et al.* 1997; Panserat *et al.* 2001). *tat* encodes a mitochondrial protein tyrosine aminotransferase that catalyzes the conversion of L-tyrosine into p-hydroxyphenylpyruvate (Rettenmeier *et al.* 1990). In the present study, the acute stress induced an upregulation of both *pepck* and *tat,* supporting the activation of hepatic gluconeogenesis in the absence of changes in glucose levels. These results agree with previous data showing increased activities of gluconeogenic enzymes in cortisol-treated fish, concomitantly with no change in plasma glucose levels (reviewed by Mommsen *et al.* 1999).

Integrated expression studies using IBR index provide information concerning the deviation of overall responses compared to control. The data suggest a different time-frame in the tissue response to acute stress. Cluster analysis of the three sampling times indicates that brain response occurs mainly during the first 15-60 min, whereas the liver response is delayed until 60-180 min.

In summary, we show that acute stress induced a rapid increase in total body cortisol with no changes in body glucose, at the same time promoting a prompt central response by activating neuronal pathways, as demonstrated by increased *fosab* expression 15 min post-stress. All three orexigenic peptides, i.e. *npy*, *agrp* and *ghrelin*, increased their central expression level suggesting that these neuronal systems are not involved in the short-term feeding inhibitory effects of acute stress. By contrast, the anorexigenic precursors tested, i.e *cart* peptides and *pomc*, exhibited increased expression after acute stress, suggesting their involvement in the anorexigenic effects. In the liver, the increased expression of *peck* and *tat* genes suggest the activation of gluconeogenic pathways but also an increased sensitivity to HPI activation induced by the raised *mrap1* mRNA levels.

Acknowledgements

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Discusión General

1. Respuesta del MC2R a ACTH en presencia de una MRAP1 heteróloga

Los diversos resultados obtenidos hasta la fecha confirman que, en mamíferos, la MRAP1 está involucrada en el tráfico y la señalización del MC2R (Chan *et al.* 2009; Sebag and Hinkle 2007; Sebag and Hinkle 2009b; Webb *et al.* 2009). Estas funciones de señalización y transporte están conservadas en actinopterigios, como lo han confirmado resultados previos obtenidos en nuestro laboratorio (Agulleiro *et al.* 2010). Sin embargo, esta dependencia entre receptor y proteína accesoria no existe en los peces cartilaginosos ya que el MC2R del tiburón elefante (*Callorhinchus mili*) es capaz de responder a ACTH en ausencia de cualquier tipo de MRAP (Reinick *et al.* 2012b).

Parte del trabajo desarrollado en el laboratorio se ha enfocado la caracterización farmacológica de los receptores de melanocortinas en diferentes especies de peces, incluyendo la lubina y mi tesis doctoral no ha sido una excepción. De hecho, fue la ausencia de respuesta *in vitro* del MC2R de lubina la que nos llevó a caracterizar las proteínas accesorias por primera vez en peces. Sin embargo, utilizamos el pez cebra por sus facilidades, fundamentalmente la secuenciación del genoma (Agulleiro *et al.* 2010). Dado que las secuencias de MRAP de la lubina no estaban caracterizadas, utilice MRAPs heterólogas para la caracterización demostrando que el MC2R de lubina, al igual que la mayor parte de vertebrados, requiere de la interacción con la MRAP1 para su expresión funcional. Los resultados apoyan la hipótesis de Dores (2013) que respalda la existencia de una coevolución de ambas proteínas (MC2R y MRAP1). Dores defiende que a lo largo de la evolución se produjo una mutación en el antecesor de los vertebrados óseos que invalidó la función del MC2R y fue la MRAP1 la que rescato la funcionalidad de este receptor.

Los resultados también demuestran especificidad del receptor con referencia a su interacción con la MRAP, ya que el MC2R de lubina responde a la ACTH en presencia de la MRAP de pez cebra, pero lo hace de forma muy modesta en presencia de la MRPA1 de mamíferos, algo que también fue observado en el MC2R de trucha arco iris (Liang *et al.* 2011).

2. Interacción entre el MC4R y la MRAP2

La publicación original que demostró la existencia de la MRAP1 ya citaba la presencia de un fragmento abierto de lectura cuya proteína potencial mostraba cierta similitud a la MRAP1. Esta proteína se le conoció como MRAP2 y su publicación hizo replantearse la nomenclatura de la familia. La mayor parte de autores siguieron denominando a las dos proteínas como MRAP y MRAP2, respectivamente, aunque nuestro grupo de investigación planteó su denominación como MRAP1 y MRAP2. Tras su publicación empezó una carrera por averiguar la función de esta nueva proteína y su relación potencial con el sistema de melanocortinas. Chan *et al.* (2009) demostraron que la MRAP2 era capaz de interaccionar con todos los receptores de melanocortinas inhibiendo la producción de AMPc estimulada por NDP-MSH. Los trabajos realizados en nuestro laboratorio demostraron, además, que las dos versiones de MRAP2 del pez cebra eran capaces de interaccionar con el MCR2 si bien no podían mediar la señalización dependiente de AMPc.

Mi planteamiento inicial fue conocer si las MRAP2s del pez cebra eran capaces de modificar el perfil farmacológico de los receptores de melanocortinas. Con este fin, realicé una batería de experimentos farmacológicos analizando la respuesta de los diferentes receptores a los agonistas de melanocortinas (α-MSH y ACTH) en presencia o ausencia de MRAP2a o MRAP2b. Los resultados demostraron claramente que, aunque la co-expresión de receptores no tenía ningún efecto sobre la producción de AMPc estimulada por α-MSH, la MRAP2a era capaz de trasformar al MC4R en un receptor de ACTH. Este resultado fue corroborado posteriormente en nuestro laboratorio analizando la conservación evolutiva de la interacción MC4R-MRAP2 (Soletto *et al.* 2019). Sebag *et al.* (2013), en un estudio paralelo en el pez cebra, mostró que la MRAP2a reducía la capacidad del MC4R para unir α-MSH, sin cambiar la afinidad del receptor por la hormona. Por el contrario, los estudios de Asai *et al.* (2013) mostraron que la coexpresion de MRAP2 con el MC4R de ratón produce un aumento de la síntesis de AMPc tras estimulación con α-MSH, resultados opuestos a los obtenidos tras estimulación con otro agonista químico de las melancortinas, el NDP-MSH (Chan *et al.* 2009). Ninguno de estos autores estudió el efecto de la interacción sobre la afinidad del MC4R por ACTH. Sin embargo, los resultados obtenidos en esta tesis, tras estimulación con ACTH, fueron posteriormente corroborados usando el MC4R de pollo (Zhang *et al.* 2017) y de humanos (Soletto *et al.* 2019).

Como he destacado en la introducción general, el MC2R es el único receptor que responde únicamente a la ACTH y nunca a las diferentes formas de MSH, con la salvedad de los

elasmobranquios (Reinick *et al.* 2012b). El resto de los receptores presenta baja afinidad por la ACTH (Schioth *et al.* 2005), sin embargo, nuestros datos demuestran que el MC4R del pez cebra se convierte en un receptor altamente sensible a la ACTH tras interacción con la MRAP2a. Esto no ocurría con otros receptores, particularmente la forma a del MC5R o el MC1R, apoyando así la especificidad de la interacción MC4R-MRAP2a y, junto con la conservación evolutiva, sugiriendo la existencia de un rol fisiológico. Por el contrario, la MRAP2b no modificaba el perfil farmacológico del MC4R, pero si es capaz de disminuir sensiblemente la actividad constitutiva del receptor. Este hecho fue también corroborado en los estudios de Sebag *et al.* (2013) en pez cebra y por los de Asai *et al.* (2013) en ratón, aunque los niveles de disminución de la actividad constitutiva fueron mucho mayores en ambas especies que los encontrados en nuestros experimentos. Mediante técnicas de inmunoprecipitación demostramos, además, que ambas MRAPs eran capaces de interactuar físicamente con el MC4R apoyando más la interacción física con el receptor bien directa o a través de otra proteína. La interacción física demostrada mediante inmunoprecipitacion también se produce con el MC5Ra y el MC1R, sin embargo, no produce ningún efecto farmacológico sobre los receptores anteriores.

Para buscar la significación biológica de dicha interacción estudié los perfiles de expresión de ambas proteínas accesorias junto con el perfil del MC4R. Los tres genes se expresan profusamente en el cerebro y el tejido interrenal. Es cierto que la escasez de tejido interrenal podría provocar algún artefacto sobre los niveles de expresión, pero este no es el caso del cerebro, donde la cantidad de tejido es apreciable. La coexpresion en el cerebro nos llevó a estudiar mediante técnicas inmunohistoquímicas los lugares de localización cerebral. La técnica de doble hibridación *in situ* reveló que MRPA2a colocaliza fundamentalmente con MC4R en el núcleo preóptico, una región equiparable al núcleo paraventricular hipotalámico de mamíferos (Herget and Ryu 2015; Herget *et al.* 2014). Por el contrario MRPA2b colocaliza principalmente con el MC4R el núcleo lateral tuberal, una región equivalente a el núcleo arqueado (Soengas *et al.* 2018). Ambas regiones están implicadas en la regulación del balance energético tanto en mamíferos como en peces (Delgado *et al.* 2017), lo que nos hizo suponer que la interacción central MC4R-MRAP2a podría estar relacionada con el control de la ingesta en peces. Para comprobar esta hipótesis afrontamos dos aproximaciones, por un lado, estudiamos la expresión hipotalámica de MRAP2a y MRAP2b en condiciones de ayuno progresivo y observamos que el ayuno inducia la expresión del MRAP2b en el cerebro, pero no tenía efectos sobre la expresión de MRAP2a. Por el contrario, la expresión de MRAP2a es muy sensible a diferentes tratamientos hormonales incluido el cortisol. Dado que MRAP2b disminuye la actividad constitutiva del MC4R es factible pensar que la sobreexpresión durante el ayuno pueda disminuir la actividad constitutiva del receptor durante los periodos de balance energético negativo. Esto provocaría una reducción en la inhibición constitutiva del hambre mediada por el MC4R, dirigiendo al animal hacia la ingesta o comportamientos que promuevan la ingesta. Como segunda aproximación estudiamos el efecto de la administración periférica de ACTH sobre los niveles de ingesta. Como muestran los resultados, la administración intraperitoneal de ACTH disminuye los niveles de ingesta de forma dosis

dependiente en peces cebra salvajes, sin embargo, el efecto desaparece en peces carentes del MC4R (cepa *Sa122*, programa de mutación del pez cebra, ZMP). Por tanto, nuestros datos sugieren que el rol de la interacción MC4R-MRAP2 está relacionado con el control de la ingesta en peces y podría servir como sustrato de integración de los efectos del estrés sobre la ingesta (ver después).

Este punto genera una nueva cuestión ¿Hay ACTH en el cerebro de vertebrados? La mayor parte de los estudios convergen hacia la ausencia de ACTH central, ya que el procesado de POMC en el núcleo arqueado y el núcleo del tracto solitario genera principalmente α-MSH y β-endorfina (Castro and Morrison 1997). Sin embargo, algunos estudios han destacado la presencia de inmunoreactividad a la ACTH tras cromatografía líquida de alta eficiencia (HPLC) sugiriendo su presencia en el cerebro de rata (Smith and Funder 1988). De forma alternativa, la ACTH sistémica podría alcanzar los núcleos neuronales centrales transportando información periférica hacia las estructuras centrales. La administración intraperitoneal de ACTH, de nombre comercial "vigabatrin", es el único fármaco permitido por la "US Food and Drug Association" para el tratamiento de los espasmos infantiles, un síndrome neurológico epiléptico infantil (Iacobas *et al.* 2013). Los efectos no se median a través de los receptores de glucocorticoides ya que, tras su bloqueo químico, los efectos de la ACTH persisten (Brunson *et al.* 2002; Brunson *et al.* 2001; Iacobas *et al.* 2013). La administración periférica de ACTH y los efectos centrales sobre los espasmos infantiles sugieren una acción central de la ACTH periférica que podría explicar también los efectos sobre la ingesta de la administración periférica de ACTH, que demuestran nuestros experimentos. Los resultados ofertan, por tanto, un mecanismo molecular para la acción central de la ACTH que puede ayudar al entendimiento de esta patología neuronal y al desarrollo de nuevos fármacos que minimicen los efectos secundarios. Experimentos recientes del grupo de investigación han demostrado que la interacción entre el MC4R y la proteína accesoria de humanos también dotan de sensibilidad a la ACTH, demostrando la conservación evolutiva del mecanismo y apoyando sólidamente una función fisiológica de la cual deriva su conservación (Soletto *et al.* 2019).

3. Melanocortinas un "link" entre la ingesta y el estrés

La respuesta al estrés se media, fundamentalmente, a través de dos ejes, el hipotálamo-hipofisis-interrenal (HPI) y el hipotalámico-simpatico-cromafín (HSC). El sistema de melanocortinas es parte integral y crítica del eje HPI, regulando la producción y liberación de glucocorticoides, cortisol en el caso de los peces, vía MC2R/MRAP1 tras estimulación por la ACTH hipofisaria. Nuestros resultados demuestran que ni el ayuno ni el estrés físico inducido por la limpieza de tanques regulan la expresión interrenal del receptor, pero, por el contrario, el tratamiento de los animales con diferentes

dosis de cortisol inhibe la expresión de MC2R de forma severa. En función de los resultados, nosotros sugerimos la presencia de un bucle de regulación corto que podría tener implicaciones en la acomodación al estrés de los organismos. En virtud de este bucle los altos niveles de cortisol, tras el estrés, inducirían una inhibición de la expresión del receptor que conllevaría una desensibilización de la respuesta a ACTH. La persistencia de las condiciones de estrés y elevados niveles de ACTH no se traducirían, por tanto, en altos niveles de cortisol a largo plazo, preservando al organismo de los efectos secundarios de la exposición prolongada a glucocorticoides. Este proceso se conoce como acomodación al estrés.

Si el MC2R requiere de la proteína MRAP1 para su presencia en membrana y por lo tanto para su respuesta a la ACTH, es posible que la propia MRAP1 sea uno de los puntos de regulación del eje HPI y por extensión de la respuesta al estrés. Acorde a esta hipótesis, los resultados de la tesis demuestran que la inclusión de cortisol en los piensos disminuye la expresión de MRAP1 en el pez cebra. En este caso no pudimos acotar la expresión al tejido interrenal, dada la complejidad para su disección en esta especie, pero sí pudimos demostrar que la expresión corporal se reducía. Por tanto, la reducción en la expresión de MRAP1 tras el tratamiento con cortisol sugiere que además de una reducción en la expresión del receptor, la ausencia de MRAP1 podría condicionar la presencia de este en membrana y por tanto la respuesta a la ACTH, jugando, así, un rol potencial en la acomodación al estrés.

Como he expuesto anteriormente, la interacción MC4R/MRAP2a trasforma al MC4R en un receptor de ACTH. El MC4R se expresa fundamentalmente en el SNC y regula el balance energético incluyendo los niveles de ingesta. El POMC, precursor de la ACTH, se expresa fundamentalmente en hipófisis y se secreta desde los corticotropos en relación con los niveles de estrés del organismo induciendo la síntesis de cortisol. Por tanto, los resultados de esta tesis proveen un nuevo mecanismo molecular para explicar las interacciones entre el estrés y la ingesta. De hecho, la administración periférica de ACTH reduce significativamente la ingesta en el pez cebra, pero se requiere la presencia de un MC4R funcional, ya que los animales carentes no pueden responder a la ACTH periférica. Este resultado demuestra que los efectos de la ACTH sobre la ingesta son mediados por el MC4R, un receptor que solamente puede responder fisiológicamente a esta hormona en presencia de MRAP2a. Los resultados no solamente proveen un mecanismo molecular adicional que puede participar en la respuesta energética al estrés, sino que provee un mecanismo molecular por el cual MC4R puede mediar los efectos del estrés en ausencia de síntesis de esteroides y ofrece nuevos dominios tisulares diana para la ACTH. Es decir, aquellos tejidos que coexpresen MC4R y MRAP2 serán susceptibles de responder a la ACTH, una hormona que se secreta tras situaciones de estrés. Utilizando la disponibilidad de datos de expresión génica en humanos, se ha comprobado que ambas proteínas se expresan significativamente en el bazo sugiriendo un rol de la ACTH en la función inmune y/o hematopoyética (Soletto *et al.* 2019).

Conclusiones

1) Las relaciones filogenéticas de los receptores son inciertas. Las dos hipótesis actuales defienden la paralogía entre MC2R/MC5R o bien entre MC4R/MC5R. La situación en el mismo cromosoma de MC2R y MC5R sugiere que ambos receptores surgieron mediante duplicación de una forma ancestral.

2) El MC2R de lubina posee una elevada identidad con otros MC2R de vertebrados y, al igual que ellos, requiere la interacción con MRAP1 para señalización de la respuesta a la ACTH. Dicha interacción no está exenta de especificidad ya que la MRAP1 de mamíferos no induce la expresión funcional del receptor de lubina.

3) La inhibición de la expresión del MC2R por el aumento de glucocorticoides plasmáticos sugiere la existencia de un sistema de retroalimentación negativo que podría jugar un papel importante en a la adaptación al estrés, protegiendo, así, al organismo frente a la elevación crónica de glucocorticoides.

4) La interacción de MRAP2a con el MC4R convierte a este receptor de MSH en un receptor de ACTH mostrando una sensibilidad similar a la exhibida por el MC2R. Por el contrario, la MRAP2b no modifica el perfil farmacológico del receptor, pero si es capaz de reducir su actividad constitutiva. La respuesta del MC4R a la ACTH establece un mecanismo molecular para la mediación de los efectos del estrés sobre la ingesta.

5) La ACTH administrada periférica es capaz de inhibir la ingesta del pez cebra, pero requiere de un MC4R funcional, ya que los peces carentes de una forma activa del receptor no responden a la ACTH con una inhibición de los niveles de alimento ingerido

6) La modificación cualitativa del perfil farmacológico del MC4R es específica ya que, aunque las MRAPs son capaces de interaccionar físicamente con otros receptores de melanocortinas, MC1R y MC5Ra, ninguno de ellos adquiere competencias frente a la ACTH, si bien la sensibilidad de MC1R a la α-MSH se reduce significativamente.

7) El estrés agudo provoca un incremento de los niveles corporales de cortisol en el pez cebra induciendo una rápida respuesta en el sistema nervioso central, medida mediante la expresión de *fosab* (antes conocido como *c-fos*), que se traduce en un incremento de la expresión de diversos péptidos orexigéenicos (NPY, AgRP y grelina) y anorexigénicos (POMC, CART). Los resultados sugieren que los péptidos orexigenicos citados no están relacionados con la

inhibición de la ingesta a corto plazo inducida por el estrés agudo, como sí que lo están los péptidos anorexigénicos.

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