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MORFOLOGÍA Y BIOLOGÍA CELULAR

CANALES IONICOS TRP Y DEG/ENa^{+}C EN LOS ORGANOS SENSITIVOS DEL PEZ CEBRA

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RESUMEN DEL CONTENIDO DE TESIS DOCTORAL

1.- Título de la Tesis Doctoral	
Español/Otro Idioma: Canales Iónicos TRP y DEG/ENAC en órganos sensitivos del pez cebra	Inglés: TRP Ion Channels and DEG / ENaC in sensory organs in zebrafish

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RESUMEN (en español)

La capacidad de los vertebrados para detectar y discriminar entre los estímulos del entorno, y del medio interno, se consigue gracias a órganos sensoriales y receptores sensitivos dispersos por todo el cuerpo que dependen de la activación de canales iónicos para transducir los estímulos específicos en señales eléctricas.

El presente trabajo de tesis se inscribe en una línea de investigación desarrollada en el Grupo SINPOS del Departamento de Morfología y Biología Celular de la Universidad de Oviedo, en colaboración con el Departamento de Scienze Veterinarie de la Universidad de Messina (Italia), y se ha diseñado para estudiar la distribución tisular del canal iónico de receptor de potencial transitorio vanilloide de tipo 4 (TRPV4) y de los canales iónicos sensibles a la concentración de protones (ASIC) en los órganos sensoriales (roseta olfatoria, retina, oído interno, papilas gustativas, y neuromastos de la línea lateral) de *Danio rerio* adulto. Este modelo animal es uno de los más utilizados en neurobiología. Sus sistemas sensoriales han sido caracterizados a nivel morfológico, estructural y de expresión génica para algunas moléculas implicadas en los procesos sensoriales, pero hasta la fecha existe escasa información sobre los canales iónicos que intervienen en la detección de estímulos específicos. Los resultados obtenidos han sido publicados en cuatro artículos referenciados en el Journal Citation Index [1].

Se ha detectado el canal TRPV4 en el pez cebra adulto a nivel de mRNA y proteína. Su distribución en los órganos de los sentidos se circunscribe a: una subpoblación de células ciliadas en los neuromastos y el oído, las neuronas crípticas y ciliadas del epitelio olfatorio, células sensoriales de las papilas gustativas y células amacrinas en la retina.

Las células sensoriales de las papilas gustativas del pez cebra adulto expresan ASIC4, mientras que los nervios que las suplen expresan ASIC2. Mediante Western-blot también se demostró la presencia de ASIC1, pero no se detectó en las papilas gustativas.

La roseta olfatoria de *Danio rerio* expresa ASIC2 mRNA y la proteína, localizados en los cilios del epitelio no sensorial. Proponemos que el epitelio olfatorio de los peces se clasifique en olfativo y no olfativo en lugar de sensorial y no sensorial, atendiendo a la

distribución positiva de los canalesTRPV4 y ASIC2 asociadas con diferentes funciones sensoriales.

En la retina del pez cebra existen mRNAs para zASIC2 y zASIC4.2 y sus proteínas, que se distribuyen por la totalidad de las capas de la retina, siendo ASIC4.2 exclusivo de los fotorreceptores, mientras que ASIC2 predomina en las capas plexiformes; Las células ganglionares expresan ambos canales iónicos.
[1]Cell Tissue Res, 2015; Histochem Cell Biol 143: 59-68; Neurosci Lett 536:35-40, 2013; Microsc Res Tech 75:89-96

RESUMEN (en Inglés)

In vertebrates, the ability to detect and discriminate between stimuli of the environment, as well as the internal environment, is achieved through sense organs and sensory receptors dispersed throughout the body which depend on the activation of ion channels to transduce the specific stimuli into electrical signals.

The present Thesis is a part of the results of a line of research developed in the SINPOS research group at the Department of Morphology and Cell Biology of the University of Oviedo, in collaboration with the Department of Veterinary Sciences of the University of Messina (Italy). The studies were designed to analyze the occurrence and the tissue distribution of the ion channel transient receptor potential vanilloid 4 (TRPV4), and the acid-sensing ion channels (ASICs) in the sensory organs (olfactory rosette, retina, inner ear, taste buds, and neuromasts of the lateral line system) of adult *Danio rerio*. This animal model is widely used in neurobiology. Their sensory systems of *Danio rerio* have been characterized at the morphological, structural and molecular levels for some molecules involved in sensory processes. But to date there is little information on the ion channels involved in the detection of specific stimuli. The results have been published in four publications included in the Journal Citation Index.
TRPV4 was detected in adult zebrafish at mRNA and protein levels. Its distribution in the sensory organs was confined to: a subpopulation of hairy cells in the neuromasts and the ear, the cryptic and ciliated neurons of the olfactory epithelium, the sensory cells of taste buds, and the retinal amacrine cells.

The sensory cells of the taste buds of the adult zebrafish express ASIC4, while nerves supplying them express ASIC2. By Westernblot ASIC1 was also detected, but it was not detected by immunohistochemistry in the taste buds.

The epithelium of the olfactory rosette expresses ASIC2 mRNA and protein, located in the cilia of the non-sensory epithelium. We propose that fish olfactory epithelium should be divided into olfactory and not olfactory epithelium instead of sensory and not sensory epithelium, attending to the expression of TRPV4 and ASIC2.

In the retina of *Danio rerio* there are mRNAs for zASIC2 and zASIC4.2 and their

proteins, which are distributed in all the layers of the retina, being ASIC4.2 exclusive of the photoreceptors, while ASIC2 predominates in the plexiform layers; the ganglion cells express both ion channels.

[1]Cell Tissue Res, 2015; Histochem Cell Biol 143: 59-68; Neurosci Lett 536:35-40, 2013; Microsc Res Tech 75:89-96

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A la mujer de mi vida, Oli, incomprendida y nunca valorada como mujer y como profesional.
A mi hijo Samuel, mi esperanza.
A mis directores de Tesis Doctoral, siempre generosos y benévolos.
A mis amigos y compañerosd de trabajo, por su paciencia y porconvertirse en revisores denodados.

ABREVIATURAS

BCIP/NBT:	Nitroazul-tetrasodio / 5-Bromo-4-cloro-3-indocil-fosfato
dNTP:	Desoxirribonucleótidos trifosfato
dpf	Días post-fertilización
EDTA:	Acido etildiaminotetraacético
GenBank	National Center for Biotechnology Information, Rockville Pike, Bethesda (Estados Unidos)
GRD:	Ganglio raquídeo dorsal
h:	Horas
hpf	Horas post-fertilización
IgG	Inmunoglobilina G
IHC:	Inmunohistoquímica, siglas del inglés "Immunohistochemistry"
ISH:	Hibridación in situ, siglas del inglés "In situ hybridization"
LB/O:	luz blanca-oscuridad
LBC:	luz blanca continua
min:	minutos
n/a:	No aplicable- no comprobado
ORN	Neuronas receptoras olfatorias, siglas del inglés "olfactory receptor neuron"
PAP:	Peroxidasa/Anti-Peroxidasa
PBS:	Fosfato salino, de las siglas en inglés "phosphate buffered saline"

RT-PCR:	Reacción en cadena de la polimerasa con transcriptasa inversa, siglas del inglés “Reverse transcription polymerase chain reaction”
SINPOS:	Sistema Nervioso Periférico y órganos de los Sentidos
SNC:	Sistema nervioso central
SNP:	Sistema nervioso periférico
Taq:	*Thermus aquaticus*
TBS	Solución salina tamponada con tris(hidroximetil)aminometan, siglas del inglés “Tris-buffered saline”
Tris	Tris[hidroximetil]aminometano
Tween-20	polioxietilen(20)sorbitanmonolaurato
WB:	Western blot, inmunoblot o electrotransferencia

INDICE

INTRODUCCION

1. INTRODUCCION

"Nada hay en mi intelecto que no haya pasado por mis sentidos", esta frase de Aristóteles pone en evidencia que ya desde la antigüedad se conocía que los sistemas sensoriales clásicos (auditivo, olfatorio, somatosensorial, gustativo y visual) son los que proporcionan a los organismos vivos información sobre el medio que los rodea. Cada uno de ellos es capaz de detectar un tipo específico de estímulo, codificar la información y transmitirla al cerebro; allí se procesa y se integra mediante el proceso de percepción sensorial.

En los vertebrados la capacidad para detectar y discriminar entre la gran variedad de estímulos del entorno, y del medio interno, se consigue gracias a estructuras especializadas que actúan como receptores sensoriales: son los órganos sensoriales y los receptores sensitivos dispersos que existen por todo el cuerpo.

Durante las dos últimas décadas se han producido notables progresos en el conocimiento de los mecanismos celulares y moleculares de los diferentes tipos de sensibilidad y en todos ellos intervienen de manera determinante diferentes familias de canales iónicos (ver para una revisión Belmonte y Viana, 2008; Damann y Cols., 2008). La evidencia científica acumulada sugiere que los sistemas sensores dependen de la activación de canales iónicos para transducir los estímulos específicos en señales eléctricas (Montell, 2005; Bandell y Cols., 2007). Muchos de estos canales iónicos se expresan en los órganos sensitivos; además, los resultados de los trabajos efectuados con animales manipulados en los genes de dichos canales, muestran variaciones en diferentes tipos de sensibilidad (Price y Cols., 2000; Liedtke y Cols., 2003, Mizuno y Cols., 2003; Suzuki y Cols., 2003; Lee y Cols., 2005).

Desde hace más de 30 años el grupo de investigación en el que he realizado la tesis doctoral se ocupa del estudio de los órganos sensoriales de diferentes especies animales, y en la última década ha analizado algunos aspectos de la morfología y biología de estos órganos en el pez cebra (*Danio rerio*), debido a la importancia de este modelo animal en el campo de la neurobiología (Yoshihara, 2009). Los sistemas sensoriales del *Danio rerio* han sido caracterizados a nivel estructural, morfológico y también de expresión génica para algunas moléculas implicadas en los procesos sensoriales, pero hasta la fecha existe escasa información sobre los canales iónicos que intervienen en la detección de estímulos específicos.

Inicialmente se pensó que cada tipo de sensibilidad estaba asociada a un tipo concreto y específico de canal iónico. Sin embargo, la situación actual sugiere que cada canal iónico puede relacionarse con más de un tipo de sensibilidad y que las células sensoriales transductoras de un tipo concreto de sensibilidad expresan más de un tipo de canal iónico. Por tanto, la función de cada tipo celular sensorial dependería de la combinación y la presencia de varios canales iónicos en la misma célula (ver para una revisión Belmonte y Viana, 2008).

En base a lo anteriormente expuesto, el presente trabajo de tesis se ha diseñado para estudiar la distribución tisular de algunos canales iónicos de las familias de receptores de potencial transitorio (TRP) y de los canales iónicos sensibles a la concentración de protones (ASIC) en los órganos sensoriales (roseta olfatoria, retina, oído interno, papilas gustativas, y neuromastos de la línea lateral) de *Danio rerio*. Se pondrá especial interés en la identificación exacta del tipo de célula sensorial que expresa cada uno de los canales analizados y la posible co-localización de más de uno de ellos en el mismo tipo celular.

Los resultados que se obtengan constituirán el punto de partida de futuras investigaciones sobre patologías sensoriales que implican a estos canales, ya que *Danio rerio* es un excelente modelo animal para realizar pre-screaning de moléculas (antagonista, agonistas de los canales), estudios de toxicidad o de manipulación genética.

ESTADO ACTUAL DEL PROBLEMA

2. ESTADO ACTUAL DEL PROBLEMA

2.1. La sensibilidad general de los vertebrados

2.1.1. Introducción general

La supervivencia y el correcto funcionamiento de los órganos en los vertebrados depende en gran medida de la obtención de una información adecuada del medio externo (donde encuentra el alimento y residen los posibles peligros) y del estado de las funciones internas. La capacidad para detectar y examinar estímulos procedentes tanto del ambiente como del medio interno depende de una parte especializada del sistema nervioso que reconoce, integra, interpreta y responde de forma adecuada a dichos cambios. Se trata del sistema sensorial o aferente formado por cadenas de neuronas y sinapsis, excitatorias e inhibitorias, que van desde la periferia hasta los niveles más elevados del sistema nervioso central. La entrada de la información al sistema nervioso requiere la presencia de sistemas receptores que, en conjunto, reciben el nombre de sensores o receptores sensoriales; estos deben de tener capacidad para detectar y discriminar distintos estímulos o formas de energía (mecánica, química, visual, etc.), responder de forma diferencial según el grado de intensidad del estímulo y elaborar una respuesta adecuada y rápida

Figura 1. *La vía de la información sensorial*

El proceso sensorial consta de cuatro pasos: a) estimulación: un estímulo incide sobre un receptor conectado al extremo terminal del axón de una neurona sensitiva; b) transducción: transformación del estímulo en un potencial de acción electroquímico; c) conducción a través de la cadena de neuronas de la vía; y d) sensación y percepción por áreas específicas del cerebro.

El efecto particular que resulta de la activación funcional de un tipo de receptor sensorial se denomina modalidad sensorial y viene determinada por el tipo de energía transmitida por el estímulo y los receptores especializados para detectarla, así como sus vías centrales y áreas cerebrales. Ya en 1826, Johannes Müller avanzó sus "leyes de energías sensoriales específicas." Postuló que la cualidad de la sensación no depende tanto del tipo de estímulo que afecta a los sentidos como del tipo de fibra nerviosa que interviene en la percepción. Es decir, que si se estimula el sistema visual se producen sensaciones visuales, y si se estimulan los nervios especializados en provocar las sensaciones de calor se produce, independientemente de cual sea el estímulo.

De los cinco sistemas sensoriales clásicos (auditivo, olfatorio, somatosensorial, gustativo y visual) solo el sistema somatosensorial es multimodal; es decir, es capaz de detectar diferentes tipos de estímulos específicos, como la posición de las articulaciones (propiocepción), estímulos nocivos, temperatura y tacto; este último, a su vez, incluye una gran variedad de componentes entre los que se encuentran la detección de la curvatura, dureza, forma, textura y presión.

No todas las especies animales poseen las mismas capacidades sensoriales sino que existe una gran diversidad debido a la presencia en las células receptoras de un número limitado de mecanismos de transducción, basados en la forma de energía a la que son predominantemente sensibles. Estos mecanismos son la

mecanotransducción, la fototransducción, la quimiotransducción, la termotransducción, la electrotransducción y la magnetotransducción. Algunas sensaciones más complejas, como la hidrosensibilidad o sensibilidad a la humedad, poseen mecanismos de transducción completamente desconocidos.

2.1.2. Receptores específicos de la sensibilidad, órganos de los sentidos

Los receptores sensoriales son estructuras que contienen células especializadas en detectar determinados tipos de estímulos del medio ambiente. Los estudios electrofisiológicos de la segunda mitad del siglo XX hicieron pensar que cada tipo de receptor sensorial poseía un mecanismo transductor para la detección de una sola forma de energía y atendiendo a esta idea se catalogaron según la naturaleza física del estímulo en:

a) Mecanorreceptores: que son estimulados cuando se produce la deformación mecánica del receptor o de las células adyacentes a éste. En el sistema somatosensor median el sentido del tacto, las sensaciones propioceptivas (contracción y relajación muscular) y la posición corporal. Los mecanorreceptores del oído interno se ocupan del oído y del sentido del equilibrio.

b) Termorreceptores cutáneos: que detectan los cambios en la temperatura, tanto corporal, como de los objetos que se tocan.

c) Nociceptores: se activan por el daño producido en los tejidos, ya sea por mecanismos físicos o químicos.

d) Fotorreceptores: son sensibles a la incidencia de luz sobre la retina del ojo.

e) Quimiorreceptores: están implicados en la detección de las sensaciones de dolor, picor, gusto y olfato.

Atendiendo a su localización, los receptores sensoriales se dividen en exteroceptores (receptores externos), interoceptores (receptores viscerales) y propioceptores (receptores musculares y articulares).

Estímulo	Receptor	Localización	Estructura
INTEROCEPCION			
Temperatura	Receptores calor-frío	Piel, hipotálamo	Terminaciones nerviosas libres
Tacto	Corpúsculos de Meissner Células de Merckel	Piel glabra	Terminación nerviosa encapsulada
Vibración	Corpúsculos de Pacini	Dermis profunda	Terminación nerviosa encapsulada
Dolor	Nociceptores	A lo largo del cuerpo	Terminaciones nerviosas libres
Estiramiento muscular	Receptores de estiramiento	Músculos	Terminaciones nerviosas alrededor del huso neuromuscular
Presión sanguínea	Barorreceptores	Ramas arteriales	Terminaciones nerviosas pared arterial (delgada)
EXTEROCEPCION			
Gravedad	Estatocistos	Cámaras exteriores del oído interno	Otolitos y células ciliadas
Movimiento	Cúpula	Canal semicircular del oído interno	Colección células ciliadas
	Órgano de la línea lateral	Ranuras profundas en el cuerpo del pez	Colección células ciliadas
Sabor	Células de las papilas gustativas	Boca y piel de los peces	Quimiorreceptores de células epiteliales con microvilli
Olor	Neuronas olfatorias	Canal nasal	Quimiorreceptores de neuronas ciliadas
Audición	Órgano de Corti	Cóclea del oído interno	Células ciliadas entre membrana basilar y tectoria
Visión	Conos y bastones	Retina del ojo	Pigmentos fotosensibles
Calor	"Pit órgan"	Cara de la serpiente y peces	Receptor temperatura en 2 cámaras
Electricidad	Ampollas de Lorenzini	Dentro de piel de pez	Vesículas cerradas con canal iónico de distribución asimétrica
Magnetismo	Desconocido	Desconocido	Desconocido

Tabla 1.*Transducción sensorial en los vertebrados (Modificado de Raven PH, Johnson G B, editores. Biología 6ª ed. Boston: McGraw-Hill; 2002).*

Los cinco sentidos clásicos pertenecen al primer grupo, pero existen además receptores internos que informan del estado de contracción de los músculos, de la distensión de las vísceras, de la composición química de la sangre, etc.

Finalmente, los receptores sensoriales también se pueden clasificar atendiendo a su complejidad estructural. De acuerdo con la clasificacióngeneral de los sensores propuesta por Malinovsky (1996) todas las estructuras que sirven para la percepción deben ser llamadas sensores, y se diferencian sensores con zonas dendríticas formadas de un axón de células pseudomonopolares y sensores con células neuroectodérmicas, que son capaces de transformar la energía de un estímulo en impulsos nerviosos.

El primer grupo incluye 15 tipos de formaciones sensoriales que representan diferentes modalidades y cualidades: mecanosensores (baro -, preso -, osmo), nocisensores, sensores térmicos, sensores químicos y electrosensores. El segundo grupo incluye sensores o células sensoriales primarias (células neurepiteliales bipolares: olfatosensores, oftalmosensores) y células sensoriales secundarias (células neuroectodérmicas especializadas conectadas con una neurona: otosensores, vestibulosensores, gustosensores). También pertenece a este grupo la línea lateral, un sistema mecano- y oto-sensor en cyclostomos, peces y algunos anfibios.

Los receptores sensoriales se pueden disponer repartidos por el cuerpo, como sucede con los receptores sensoriales de la temperatura, o bien estar agrupados en órganos de alta complejidad, los denominados órganos de los sentidos, como los que se encuentran en el ojo o el oído. Cada órgano de los sentidos es una estructura especializada que consta de uno o varios tipos de células receptoras y estructuras de soporte, pero siempre en conexión con neuronas cuyos axones alcanzan, directa o

indirectamente, el sistema nervioso central. Así, el sentido del gusto asienta en unas estructuras llamadas papilas gustativas en la lengua donde se localizan los receptores denomimados botones gustativos que se ponen en contacto con los alimentos. El del olfato se sitúa en el epitelio olfatorio donde se encuentran los receptores o neuronas olfatorias. La visión depende de la retina, situada en la parte posterior del globo ocular, que contiene una serie de células sensibles a la luz. Y por último la audición y el equilibrio, son responsabilidad del oído. Las células ciliadas del órgano de Corti del caracol del odio interno son responsables de la audición, mientras que las células sensitivas de las crestas ampulares y las máculas estáticas del órgano vestibular son las encargadas del equilibrio.

Sistema sensor	Modalidad	Energía estímulo	Tipo de receptor	Células receptoras
Visual	Visión	Luz	Fotorreceptor	Conos y bastones
Auditivo	Oído	Sonido	Mecanorreceptor	Célula ciliada (cóclea)
Vestibular	Equilibrio	Gravedad	Mecanorreceptor	Célula ciliada (laberinto vestibular)
Somatosensor	Tacto	Presión	Mecanorreceptor	Receptores cutáneos
	Propiocepción	"Displacement"	Mecanorreceptor	Receptor músculo / articular
	Temperatura	Térmico	Termorreceptor	Receptor de frío / calor
	Dolor	Mecánico	Mecanorreceptor	Nociceptores
		Químico	Quimiorreceptor	
		Térmico	Termorreceptor	
	Picor	Químico	Quimiorreceptor	Nociceptor químico
Gustativo	Gusto	Químico	Quimiorreceptor	Papilas gustativas
Olfativo	Olfato	Químico	Quimiorreceptor	Neuronas olfatorias

Tabla 2. *Sistemas sensoriales y modalidades. (Modificado de Gardner EP, Martin JH. Codificación sensorial. En: Kandel ER, Schwartz JH, Jessell TM, Siegelbaum SA, Hudspeth AJ. Principios de neurociencia. 5ª edición: McGraw-Hill; 2000. P449-473).*

2.2. El pez cebra (*Danio rerio*) como modelo en estudios de biomedicina

2.2.1. Introducción

El *Danio rerio*, es un pez tropical de agua fría perteneciente a la familia de los Cyprinidae división Teleostei (Nelson, 2006), que se caracteriza por la presencia de 5 bandas horizontales de color oscuro a lo largo del cuerpo, razón por la que recibe el nombre de pez cebra. Fue reconocido como modelo de investigación en vertebrados a partir de los trabajos del grupo de George Streisinger que lo utilizó en sus estudios sobre las funciones de los genes en los procesos biológicos (Streisinger y Cols., 1981; Chakrabarti y Cols., 1983; Walker y Streisinger, 1983; Grunwald y Streisinger, 1992).

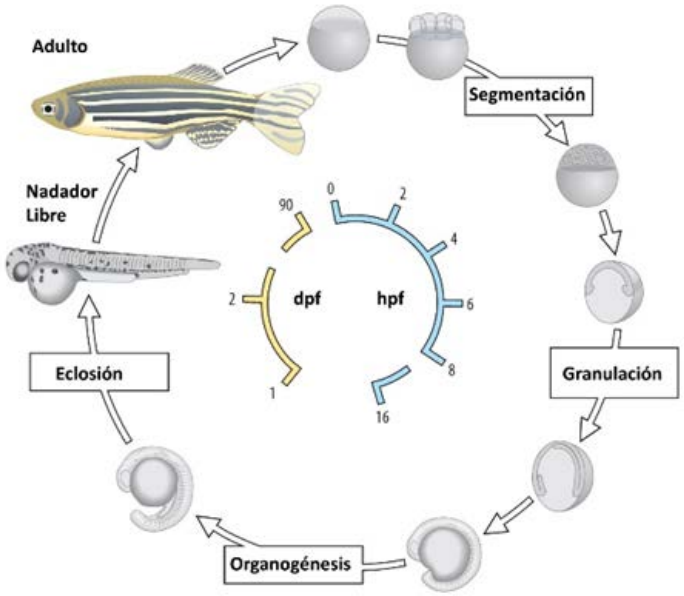

Figura 2. *Etapas del desarrollo del pez cebra (recuperado y modificado el 14 de Febrero de 2015 de http:/www.Mun.ca/biology/desmid/Brian/BIOL3530/DEVO_03/ch03f09.jpg).*

Desde entonces su importancia ha ido aumentando debido a sus características biológicas, entre las que cabe destacar: a) pequeño tamaño, que permite criar y mantener un gran número de individuos en un espacio reducido, con costes inferiores a cualquier mamífero; b) se reproduce todo el año, suele alcanzar la madurez sexual en 3-4 meses, y una pareja reproductora puede producir 4.200 huevos fecundados por apareamiento; c) la fertilización externa y que el óvulo y el embrión sean transparentes facilita la identificación visual de los movimientos morfogenéticos y de la organogénesis con microscopio de disección; d) el desarrollo es rápido y 24 horas después de la fertilización (hpf) los principales órganos y sistemas están formados, a las 48 hpf se pueden observar las primeras respuestas de comportamiento, y a los 3 días se alimenta de forma autónoma y activa (Kimmel y Cols., 1995).

Más del 80% de sus genes comparten un alto grado de homología con vertebrados y entre un 50-80% de identidad con humanos; además, han sido clonados muchos ortólogos del pez cebra de genes de mamíferos (Kabashi y Cols., 2010), encontrándose funciones similares (revisión de Yoshihara, 2009). A partir de ese punto se han creado peces cebra manipulados genéticamente, habiéndose obtenido en algunos casos, mutantes con fenotipos similares a los de enfermedades humanas.

Por todo lo anteriormente expuesto, el pez cebra es en la actualidad uno de los modelos más útiles en el estudio de biología del desarrollo, toxicidad de drogas, respuesta a xenobióticos y generación de estrés oxidativo (Ali y Cols., 2011). Además está aceptado por la comunidad científica para el estudio de enfermedades humanas en numerosas áreas de investigación.

Áreas de investigación		Referencia bibliográfica
Cáncer	Angiogénesis tumoral	Tobia y Cols.,2011
	Sarcoma de tejidos blandos	Dodd, 2010
	Leucemia	Teittinem y Cols., 2012
Circulatorio	Desarrollo y enfermedad cardiaca	Bakkers, 2011
	Cardiopatías congénitas	Wang, 2011
Digestivo	Enfermedad inflamatoria intestinal	Love y Cols., 2007
	Enfermedad hepática	Wilkins y Pack, 2013
Endocrino	Mucolipidosis tipo II	Flanagan-Steet y Cols., 2009
	Complicaciones diabéticas	Jörgens y Cols., 2012
Envejecimiento	Orígenes y desarrollo del envejecimiento	Kishi, 2014
Genética	Organogénesis vertebrados y trastornos humanos	Ackermann y Paw, 2003
	Genética del comportamiento	Norton y Cols., 2010
Hematología	Modificación genética de plaquetas	Thijs y Cols., 2012
	Hemostasia y trombosis	Jagadeeswaran y Cols., 2005
Locomotor	Distrofia muscular	Berger y Currie, 2012
Neurología	Alzheimer	Newman, 2014
	Epilepsia	Stewart y Cols., 2012
Psiquiatría	Esquizofrenia	Morris, 2009
Toxicología	Toxicidad farmacológica	Raldúa y Piña, 2014

Tabla 3. *Áreas de investigación en las que se ha usado el pez cebra.*

2.2.2. El pez cebra como modelo en patologías de la sensibilidad y órganos de los Sentidos

El pez cebra presenta una anatomía del sistema nervio central bastante compleja (cerebro anterior, medio y posterior, incluyendo diencéfalo, telencéfalo y cerebelo), todos los componentes sensitivos y motores del sistema nervioso periférico y órganos sensitivos especializados (ojo, sistema olfatorio y oído; Lieschke y Cols., 2007). En opinión de muchos investigadores es el modelo ideal de vertebrado para el estudio de los sistemas sensoriales debido a su tamaño relativamente pequeño para el examen microscópico, su potencial para la manipulación genética y la

similitud morfológica, fisiológica y de expresión génica de sus órganos sensitivos con los de los humanos (Lieschke y Cols., 2007).

Áreas de investigación	Referencias bibliográficas
Enfermedad ocular humana	Gestri y Cols., 2012
Glaucoma	Veth y Cols., 2011
Retinopatía vascular	Van Rooijen y Cols., 2010
Ototoxicidad	Eimon, 2009
Audición y sordera	Whitfield, 2002
Anosmia	Garaffo y Cols., 2013
Ciliopatías	McIntyre y Cols., 2012
Detección de tóxicos ambientales	Lee y Cols., 2014
Regeneración de órganos mecanosensores	Dufourcq y Cols., 2006

Tabla 4. *Patologías de los órganos de los sentidos estudiadas en pez cebra.*

2.2.3. Los órganos de los sentidos del pez cebra

A principios del siglo XX, el biólogo alemán Jakob von Uexküll, propuso la idea de que *"cada especie animal tiene su propio universo sensorial".* Sin duda, el universo sensorial de los peces es diferente al nuestro, *"Los peces tienen excelentes sentidos de la vista y olfato y un exclusivo sistema de la línea lateral, que con exquisita sensibilidad detecta corrientes de agua y vibraciones proporcionando un tacto a distancia en el agua"* (Hickman, 2009).

Los peces han realizado adaptaciones evolutivas para sobrevivir en el medio acuático, y en ese sentido han desarrollado órganos de los sentidos con características peculiares**.** Sus sistemas sensoriales sufren cambios durante la ontogenia, ya que larvas, juveniles y adultos deben

enfrentarse, de acuerdo con los requerimientos de cada etapa de la vida, a diferentes universos sensoriales. El *Danio rerio* posee todas las modalidades sensoriales clásicas, mecanorrecepción en la línea lateral y oído, fotorrecepción en la retina y quimiorrecepción en las papilas gustativas y roseta olfatoria.

En las páginas que siguen se hará una breve descripción de la estructura de los órganos sensoriales del *Danio rerio* y de dos familias de los canales iónicos ASIC y TRPV (TRP subfamilia vanilloide) objetivo de la presente tesis doctoral, haciendo especial énfasis en los datos disponibles sobre la presencia de este tipo de canales en los órganos de los sentidos en los vertebrados con el fin de poder interpretar y discutir de forma adecuada los resultados que se obtengan.

2.2.3.1. Papilas gustativas

El gusto es una modalidad sensitiva química que permite a los organismos identificar los nutrientes adecuados, y evitar las toxinas y sustancias indigeribles. En los peces, el gusto comienza cuando los componentes disueltos en el agua se ponen en contacto con la parte apical de las células sensoras (células presinápticas) que se encuentran alojadas en los órganos especializadas del gusto, las papilas gustativas (Chaudhari y Roper, 2010).

Las papilas gustativas de los teleósteos adultos son órganos sensoriales intraepiteliales con forma de pera o cebolla, formadas por unas 30-40 células de dos tipos: sensitivas y basales. Se encuentran distribuidas en la cavidad orofaríngea, en el epitelio de los labios, en los 2 pares de agallas, epitelio de la cabeza y algunas veces por toda la superficie del cuerpo (Kotrschal, 2000; Hansen y Cols., 2002). El epitelio sensorial de las papilas gustativas está formado por células epiteliales modificadas, alargadas y orientadas verticalmente.

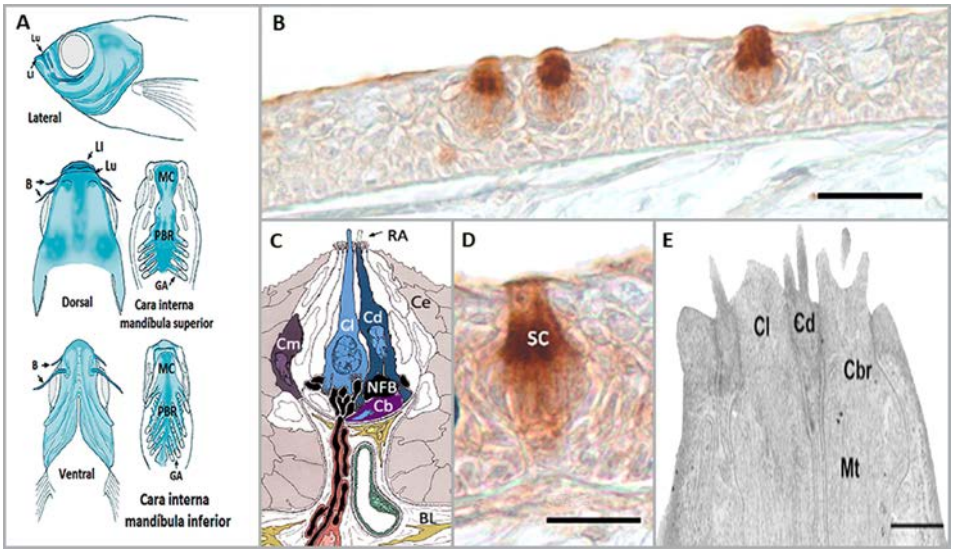

***Figura 3**. Distribución y estructura de las papilas gustativas en pez cebra (juvenil y adulto), **A**: Distribución espacial; cabeza, barbillas (B), labio superior (Lu) e inferior (Ll), cavidad bucal (MC), región faríngeo (branquial) (PBR), arcos branquiales (GA). **C:** Esquema de una papila gustativa de teleósteo: células oscuras (Cd), células claras (Cl), células basales de Merkel (Cb), células marginales (Cm), células epiteliales (Ce), nervio (TB), plexo nervioso (NFB), lamina basal (BL), área receptora (RA). **B** y **D:** Sección de papilas gustativas de pez cebra adulto usando marcaje inmunohistoquímico (tomado de Amato y Cols., 2012). **D:** Microfotografía electrónica de transmisión del polo apical de una papila gustativa de larva de pez cebra de 10 días. (A, C y E Tomados de Hansen y Cols., 2002).*

Atendiendo a la densidad en el microscopio electrónico de transmisión (TEM) y a sus terminaciones apicales, se diferencian como células oscuras (con microvilli pequeños) y células claras (con un único microvilli largo; Reutter, 1978, 1982; Jakubowski y Whitear, 1990). En las larvas se describe un tercer tipo celular, de densidad electrónica más ligera que las células oscuras y de microvillis más largos que forman una terminación en cepillo (Hansen y Cols., 2002). Entre las células claras y oscuras y la lámina basal, se extienden horizontalmente las células basales. Éstas se asemejan a las células de Merkel, tienen sinapsis y pueden servir como mecanorreceptores, o como células neuroendocrinas o paracrinas (Reutter y Witt, 1993; Roper, 1993; Hansen y Zielinski, 2002; Germana` y Cols., 2004).

La información química detectada por las células gustativas, es transmitida al sistema nervioso central a través de un plexo nervioso presente en la parte basal de la papila, alrededor de las células basales Merkel-like (Zachar y Jonz, 2012).

2.2.3.2. Roseta olfatoria

En los peces, cuya vida se desarrolla en un medio relativamente homogéneo y uniforme como el agua, el olfato regula gran parte de sus procesos vitales: reproducción, alimentación, comunicación social, huida, migración, etc… (Hamdani y Doving, 2007).

El órgano del olfato de los peces teleósteos asienta en un epitelio que se organiza en una estructura diferenciada, llamada roseta olfativa. Las células sensitivas del mismo responden a una amplia variedad de moléculas disueltas en el agua entre las que se incluyen aminoácidos, ácidos biliares, péptidos o ácidos nucleicos (Hara, 1992; Lindsay y Vogt, 2004). Además, son sensibles a las feromonas sexuales (Van der Hurk y Cols., 1987), de alarma (Hall y Suboski, 1995) y a las moléculas de la denominada “memoria olfatoria” (Whitlock, 2006).

El epitelio olfativo se encuentra situado en una cámara ósea en el extremo anterior de la cavidad craneal, denominada cámara olfatoria, que presenta orificios de entrada y salida (narinas anterior y posterior) lo que permite el flujo del agua en contacto con el epitelio olfativo. El movimiento del agua en la cámara olfatoria lo crea el propio movimiento del pez, pero también los cilios presentes en su epitelio (Whitlock, 2006). En el interior de esta cavidad ósea se encuentra la roseta olfatoria, compuesta por una serie de láminas o lamelas olfatorias que se originan en el suelo de la cavidad; la morfología de la roseta y el número de láminas que la forman varía de unas especies a otras (Hansen y Zeiske, 1993, 1998; Hansen y Cols., 2005).

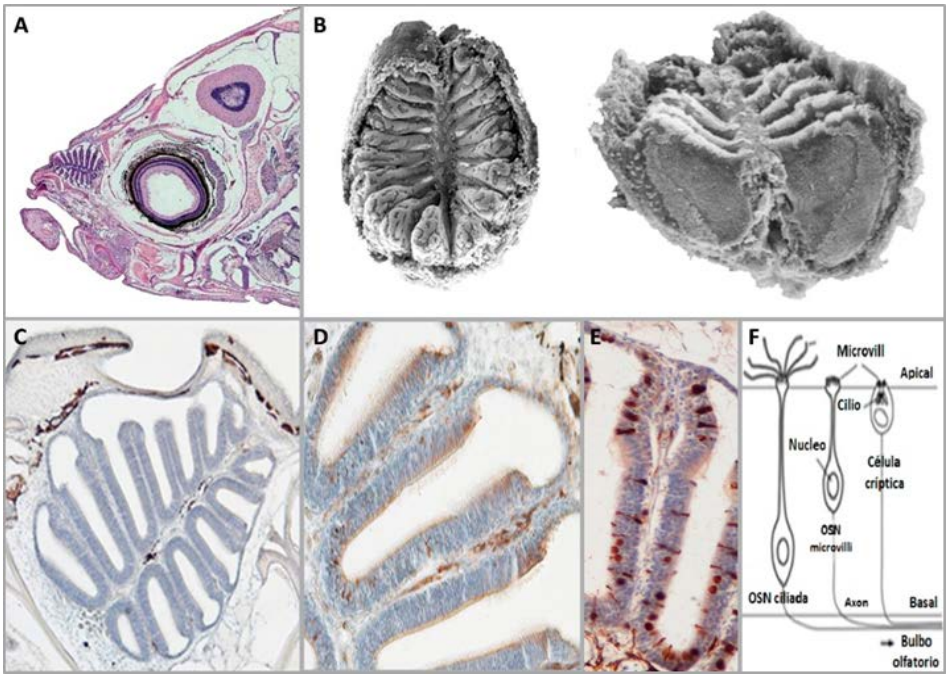

Figura 4. *Organización del epitelio olfatorio del pez cebra adulto.* ***A:*** *Sección histológica de cabeza teñida con hematoxilina-eosina* ***B****: Micrigrafía de microscopía electrónica de barrido de roseta olfatoria (Tomada de Weth y Cols., 1996).* ***C, D y E****: Secciónes histológicas sagitales de roseta olfatoria: epitelio sensorial (se), epitelio no sensorial (nse). (C y D cortesía del Profesor José Antonio Vega, B tomada de Amato y Cols., 2013).* ***E:*** *Representación esquemática de los cuatro tipos de neuronas del epitelio olfatorio (Dibujo tomado de Yoshihara 2009).*

Cada lamela está formada por un eje conectivo tapizado de epitelio sensorial y no sensorial. Dentro del primero se localizan las neuronas olfativas que proyectan diferenciaciones de membrana (cilios, microvilli) a la superficie del epitelio y que por su otro extremo sinaptan con las dendritas de las neuronas olfatorias mitrales del bulbo olfatorio, formando los glomérulos en los que se produce la integración de las señales olfatorias. En el caso concreto de *Danio rerio*, hay tres tipos morfológicos de neuronas receptoras olfativas repartidas, y aparentemente superpuestas dentro del epitelio sensorial, con características morfológicas y bioquímicas propias: neuronas ciliadas (que presentan largas dendritas y pocos cilios), neuronas con microvilli (con dendritas cortas y microvilli) y neuronas crípticas, (de forma

redondeada y con pocos cilios y microvilli) (Thommesen, 1983; Hansen y Finger, 2000). Recientemente, Ahuja y Cols. (2014) han descrito una nueva población de neuronas sensoriales olfativas en el pez cebra, las neuronas kappa, llamadas así por su forma característica. Hay unos pocos cientos de ellas en el epitelio olfativo, su morfología es significativamente diferente, y sus axones se proyectan a un solo glomérulo del bulbo olfatorio. No expresan los marcadores propios de las neuronas ciliadas o de las crípticas, pero parecen tener microvilli.

Las diferentes longitudes de las dendritas de las neuronas olfativas hacen que sus somas se encuentren a distintas profundidades dentro del epitelio por lo que éste adopta un patrón pseudoestratificado. En general, los somas de las células crípticas son de localización superficial, los de las neuronas con microvilli asientan en zonas centrales, y los de las ciliadas en las capas más profundas. Respecto a su distribución dentro de las láminas de la roseta, las ciliadas son más abundantes en la regiones periféricas mientras que las de microvilli se encuentran principalmente en la parte central (Hansen y Cols., 2004).
La proyección central de las neuronas sensoriales olfativas es muy ordenada: todas las neuronas que expresan un determinado receptor convergen en la misma región en el bulbo olfatorio ipsilateral y terminan en un solo glomérulo (Vassar y Cols., 1994; Hansen y Cols., 2003). En la organización funcional del sistema olfatorio de los peces se han descrito tres vías paralelas para la conducción de los estímulos olfativos desde el bulbo olfatorio, vía fascículo olfatorio, hasta el telencéfalo. Una de las vías está en relación con las señales sociales, otra con las feromonas y actividad sexual y la tercera con los olores de los alimentos y la conducta alimentaria (ver Hamdani y Doving, 2007).

Los cilios y microvilli de las neuronas olfativas son el asiento de los receptores-sensores que responden a las moléculas odoríferas que contiene el agua (Sato y Suzuki, 2001; Hansen y Zielinski, 2005). En el órgano del olfato de los mamíferos se han encontrado cuatro familias de

genes que codifican para receptores olfativos, todos ellos acoplados a proteínas G: receptor del olor (OR), receptores del órgano vomeronasal (V1R y V2R) y receptores de trazadores asociados de aminas (TAARs). En los peces, incluidos los teleósteos, se han detectado ortólogos de estas cuatro familias (Korsching, 2009).

2.2.3.3. Ojo

En los vertebrados en general, y en los peces en particular, el sistema visual cumple dos funciones bien diferenciadas: visual, para buscar comida y evitar depredadores, y no visual, que agrupa a un conjunto de actividades diversas que van desde la regulación de ciclos circadianos hasta el control de conductas reproductivas.

El sistema visual del pez cebra es similar al del resto de los vertebrados (Roberts y Ellis, 2001; Koppang y Bjerkas, 2006). La luz atraviesa la córnea, el cristalino y los medios líquidos del ojo, y llega a la retina, la capa más interna del globo ocular, encargada de la recepción y el procesamiento inicial de la información visual. La retina tiene una disposición laminar muy organizada. Los somas neuronales se localizan en las denominadas capas nucleares, mientras que sus prolongaciones y las conexiones entre ellos constituyen las capas plexiformes.

En la retina se han descrito los siguientes tipos celulares (Gestri G y Cols., 2012): a) epiteliales pigmentadas, que constituyen la porción externa de la retina, y forman la capa basal de la misma; b) tres tipos básicos de células gliales, astrocitos, microglía, y un tipo especial de glía radial denominada células de Müller, que sirven para mantener el balance iónico dentro de la retina (Newman y Zahs, 1997); en algunas especies también se han encontrado oligodendrocitos; c) los fotorreceptores y neuronas (bipolares y ganglionares); d) varios tipos de interneuronas (bipolares, horizontales, interplexiformes y amacrinas).

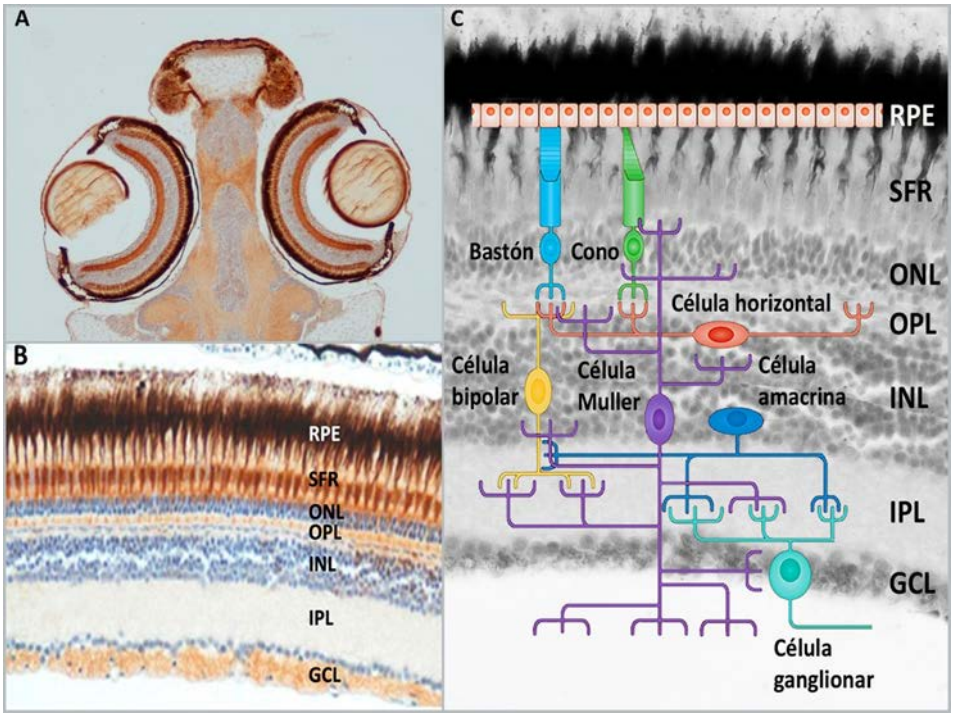

Figura 5. *Estructura anatómica de la retina del pez cebra. A: Sección histológica de una larva de pez cebra, (Cortesía del profesor José Antonio Vega). B: Sección histológica de retina de pez cebra mostrando las capas de la retina: Epitelio pigmentario (RPE), Segmento fotorreceptor (SFR), capa nuclear externa (ONL), capa plexiforme externa (OPL), capa nuclear interna (INL), capa plexiforme interna (IPL) y capa de células ganglionares (GCL) (Tomado de Amato y Cols., 2013)* ***C:*** *Esquema de los tipos celulares y su organización en la retina, (tomado de Goldman 2014).*

Por lo que respecta a la organización en capas, en la nuclear externa (ONL) están los somas de los fotorreceptores (conos y bastones), en la nuclear interna (INL) se localizan las celulas horizontales, bipolares, amacrinas y los cuerpos celulares de las células gliales de Muller, y en la de las células ganglionares (GCL) están los somas celulares de las células ganglionares cuyos axones forman el fascículo óptico. Estas tres capas están separadas entre sí por las capas plexiformes interna (IPL) y externa (OPL). El pez cebra posee un tipo de bastones y cuatro tipos de conos atendiendo a su espectro de absorción y morfología (Endeman y Cols., 2013). Como en otros órganos sensoriales y al contrario que los mamíferos, el pez cebra adulto regenera todas las neuronas de las retina después de sufrir un

daño mediante la activación, desdiferenciación y proliferación de la células de Muller (Bernardos y Cols., 2007; Fimbel y Cols., 2007).

2.2.3.4. Sistema de la línea lateral (SLL)

"*Los peces tienen un exclusivo sistema de la línea lateral, que con exquisita sensibilidad detecta corrientes de agua y vibraciones proporcionando un tacto a distancia en el agua*" (Hickman, 2009).

La línea lateral consiste en una serie de órganos mecanosensores llamados neuromastos que detectan turbulencias en el agua, ayudando a los peces a percibir corrientes, capturar presas, evitar obstáculos y a los predadores, y a algunos tipos de peces les permite mantener la posición "en school", es decir, grupos altamente coordinados que se desplazan al unísono (Wubbells y Schellart, 1998). Este sistema sensoral toma su nombre de la disposición céfalo-caudal de los neuromastos a lo largo de las caras laterales del cuerpo, desde las cercanías del opérculo hasta la base de la cola. Además en algunas especies, incluido el pez cebra, existe un sistema de la línea lateral cefálico.

Se diferecian en el SLL dos partes: los neuromastos superficiales, dispersos en la piel, "pit organs", y los neuromastos profundos o canaliculares, situados en canales subdérmicos a lo largo del tronco (canal de la línea lateral). La parte cefálica del SLL está situada en canales óseos de los huesos (canales cefálicos). Ambos tipos de canales se abren en el agua circundante por medio de pequeños poros (Satou y Cols., 1994).

Cada neuromasto está compuesto por un núcleo de 15 o 20 células ciliadas rodeadas por 2 tipos celulares accesorios, células de soporte y células envolventes o del manto, todas cubiertas por una cúpula gelatinosa segregada por éstas últimas (Rouse y Pickeles 1991). El ramillete ciliar apical de las células ciliadas está compuesto por un largo kinocilio (9+2 de configuración de microtubulos) y un grupo de pequeños esterocilios graduados en altura decreciente desde el kinocilio (Cernuda-

Cernuda y García-Fernandez, 1992). La cúpula se desplaza con los movimientos del agua y a su vez mueve los cilios de las células ciliadas produciendo señales que son enviadas al cerebro.

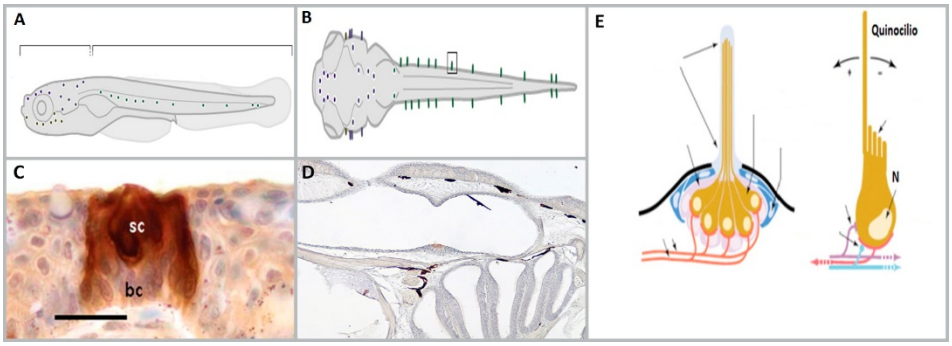

Figura 6. *Morfología del sistema dela línea lateral del pez cebra.* ***A y B****: Vista dorsal y lateral de la distribución de los neuromastos a lo largo del cuerpo de una larva de pez cebra. (tomado de Harris y Cols.,2003).* ***C y D****: Secciónes histológicas de neuromasto superficial y profundo respectivamente: células sensoriales (SC), células basales (bc). (Tomadas de Amato y Cols., 2012, y cortesía del Profesor José Antonio Vega).* ***E****: Esquema de un neuromasto que ilustra los diferentes tipos de células que están involucradas y su organización. (Tomado de Ghysen y Dambly-Chaudière, 2004).*

2.2.3.5. Oído interno

El oído de un pez cebra adulto consiste en tres canales semicirculares unidos ortogonalmente (anterior, posterior y horizontal/lateral), y tres órganos otolíticos (utrículo, sáculo y lagena) que contienen cada uno un parche sensorial o mácula. Estas cámaras están revestidas por un epitelio sensorial y rellenas por endolinfa. Los canales semicirculares y el utrículo, junto con el conducto endolinfático, forman la *pars superior* relacionada con el equilibrio, la aceleración y la detección de la gravedad. El sáculo y la lagena, con sus receptores sensoriales ciliados, son la *pars inferior*, responsable de la audición (Popper y Combs., 1980; Popper y Cols., 2003).

Cada canal semicircular termina en una ampolla, un ensanchamiento del canal que contiene un cresta sensorial perpendicular a su eje; Los extremos no ampulares de los canales anteriores y posteriores se fusionan para formar medialmente la cruz común, en cuya base se

localiza la mácula neglecta, un par de pequeños parches sensoriales sin otolitos, dentro de la pars superior.
Aunque los peces no tienen oído externo ni medio, y carecen de un aparato de audición especializado como la cóclea en los mamíferos, los órganos del equilibrio y de detección de la gravedad son funcionalmente equivalentes a los de todos los grupos de vertebrados (Fay y Popper 1999a).

Por su lado, los órganos otolíticos están cubiertos por un otolito, una masa proteica calcificada suspendida sobre la mácula sensorial subyacente, amortiguado por la matriz gelatinosa de la membrana otolítica. Las máculas se espesan en parches de epitelio que contienen células de soporte intercaladas con células ciliadas sensoriales, que son células epiteliales especializadas con un cilio apical formado por un kinocilio y numerosos estereocilios. La estructura, desarrollo y función de la célula ciliada está bien conservada entre los peces y los mamíferos (Nayak et al. 2007).

El oído interno del pez cebra adulto presenta varias máculas en: utrículo, sáculo y la lagena, la pequeña mácula neglecta, y las tres crestas ampulares (anterior, lateral y posterior) situadas en la base de su conducto semicircular. En este animal, la diferentes maculas están involucrados en la detección de la aceleración lineal así como en la audición (Nicolson, 2005, Popper y Fay, 1993, Popper y Platt, 1993; Bang y Cols., 2001; Whitfield y Cols., 2002). Las crestas ampulares de los canales semicirculares son responsables del control de la postura y la aceleración angular (Ernest y Cols., 2000, Nicolson, 2005, Popper y Fay, 1993, Popper y Platt, 1993 y Whitfield y Cols., 2002),

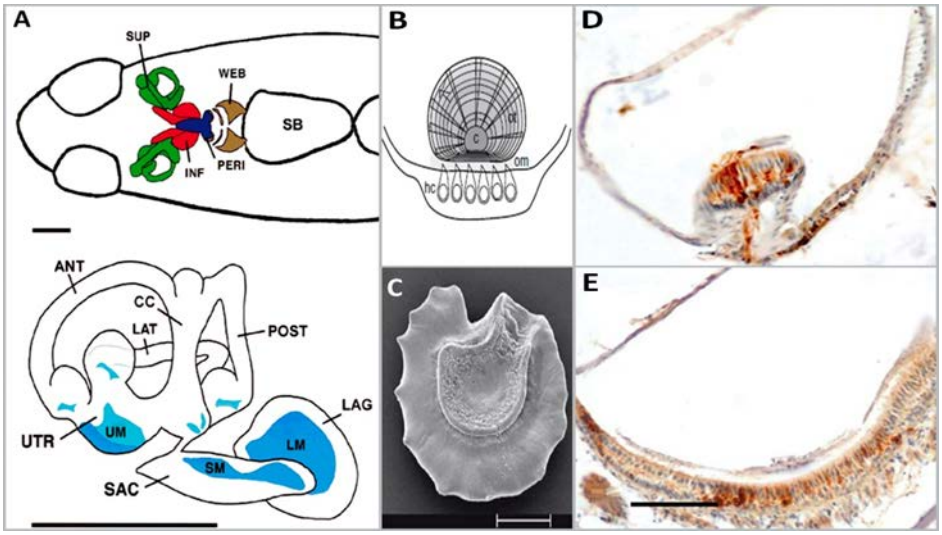

Figura7. *El oído del pez cebra adulto.* ***A:*** *ilustración esquemática de la ubicación, tamaño y orientación del oído y estructuras accesorias auditivas. Canal semicircular anterior (ANT); cruz común (CC); lagena (GAL); mácula lagenar (LM); canal semicircular lateral (LAT); espacio perilinfático (PERI-azul); canal semicircular posterior (POST);mácula sacular (SM); sáculo (SAC); pars inferior (INF-rojo); pars superior(SUP-verdes); vejiga natatoria (SB); mácula utricular (UM); Utrículo (UTR); huesecillos weberianos (WEB-marrón). (Tomado de Bang y Cols., 2001).* ***B****: Diagrama esquemático de una sección transversal de un otolito en la segunda semana del desarrollo. El otolito (ot) se sienta sobre la mácula sensorial de las células ciliadas (HC), embebido en una membrana otolítica (OM).* ***C****: micrografía electrónica de barrido del otolito lagenar de pez cebra adulto. (B y D Tomados de Abbas y Cols., 2010).* ***D y E****: Secciónes histológicas del oído del pez cebra adulto, a nivel de las crestas ampulares de los canales semicirculares (D) y de La mácula del utriculo, sáculo y lagena (E), y. (Tomado de Amato y Cols., 2012).*

En consecuencia, el epitelio macular vibra con respecto al otolito y los haces cilio-sensoriales se desvían; es el estímulo “directo” del oído, análogo a la conducción ósea de otros vertebrados, y representa el único mecanismo de audición disponible en muchas especies de peces (Popper y Liu 2000).

Los peces cuentan además con una estructura anatómica que contribuye a la estimulación "indirecta" del oído por una onda de sonido que viaje a través del agua, la vejiga natatoria, cuya oscilación es funcionalmente análoga a la vibración de la membrana timpánica en el medio tetrápodo. La vejiga natatoria está conectada al sáculo por los osículos de Weber,

que son funcionalmente equivalentes a los huesos del oído medio (Bird y Mabee 2003; Higgs Cols.. 2003; Grande y Young 2004).

2.3. Canales iónicos en la base de la sensibilidad

2.3.1. Conceptos generales

A lo largo de la evolución, los organismos superiores han desarrollado sistemas sensores cuya funcionalidad, desde hace pocos años, se ha asociado a la activación de canales iónicos para transducir estímulos específicos en señales eléctricas. La activación puede producirse directamente como en el caso del sentido del tacto (Goodman y Schwarz, 2003; Sukharev y Corey, 2004) o indirectamente por componentes químicos de una cascada de trasducción como sucede para la luz o los estímulos olorosos (Hardie 2003, Montell, 2003; Nakamura, 2000). Los estímulos sensitivos dan lugar a la apertura y/o cierre de canales iónicos, provocando cambios en las concentraciones de iones en el interior y el exterior de la célula; estos gradientes hacen posible un sistema de señalización eléctrica que produce un cambio en el potencial de membrana de la célula receptora.

Los canales iónicos son estructuras de membrana formados por agregados de proteínas que contienen un poro central acuoso que permite el intercambio iónico entre la célula y el medio externo.
Todos comparten tres caracterísiticas: conducen iones, reconocen y seleccionan iones específicos y se abren o cierran en respuesta a señales eléctricas, mecánicas o químicas (Kandel y Cols., 2000). Se han identificado diferentes tipos de canales íonicos en la membrana celular que pueden ser clasificados según su mecanismo de activación: los que no se pueden abrir o cerrar y los que sí lo hacen y son dependientes de ligando (ionotrópicos), dependientes de protones, controlados por segundos mensajeros (metabotrópicos) y dependientes de voltaje.

De todas las familias de canales iónicos existentes los que parecen estar más involucrados en la transducción de la señal de los diferentes órganos de los sentidos, desde los nemátodos a los mamiferos (Montell, 2005, Bandell y Cols., 2007), son: la superfamilia de canales de receptor de potencial transitorio (TRP; ver Eid y Cortrigh, 2009; Julius, 2013; Tóth, 2014; Voets, 2014; Zufall, 2014) y la de degenerinas-canales epiteliales de Na^+ (epitelial amiloride-sensitive Na^+ channel and degenerin; DEG/ENaC), y dentro de ésta la familia de los canales iónicos sensibles a la concentración de protones (acid-sensing ion channels, ASICs; Sluka y Cols., 2009; Holzer, 2009, 2011, 2015; Benarroch, 2014; Kristal, 2015).

Identidad	Familia	Modos de activación	Rango
TRPV1	TRPV	Térmica, osmótica	>42ºC
TRPV4	TRPV	Térmica, osmótica	>27-34ºC
TRPM8	TRPM	Térmica	<28ºC
TRPC6	TRPC		
ASIC1a	DEG/ENa^+C	Mécanica (Tacto)	pH 6.2-6.8
ASIC1b	DEG/ENa^+C	Mécanica (Tacto)	pH 5.1-6.2
ASIC2a	DEG/ENa^+C	Mecánica (Tacto)	pH 4.1-5.0
ASIC2b	DEG/ENa^+C	Mécanica (Tacto)	n/a
ASIC3	DEG/ENa^+C	Mecánica (Tacto/nocicepción)	pH 6.2-6.7
ASIC4	DEG/ENa^+C	Térmica, osmótica	n/a

***Tabla 5**. Propiedades de los canales TRP y ASIC (modificado de Lingueglia, 2007).*

2.3.2. Canales de la superfamilia TRP

Los canales iónicos TRP constituyen una extensa familia formada por 28 miembros agrupados en 8 subfamilias, atendiendo a su homología de secuencia y sus similitudes funcionales: TRPC1-7 (familia canónica o clásica), TRPM1-8 (melastatina), TRPV1-6 (vanilloide), TRPA1 (anquirina), TRPP1-3 (policistina) y TRPML1-3 (mucolipina); TRPN presente en invertebrados (Clapham y Cols., 2005) hasta el momento, el único miembro de esta familia identificado en los vertebrados es de pez cebra (Sidi y Cols., 2003). Finalmente, existe una familia adicional (TRPY)

evolutivamente más distanciada de las anteriores y que se expresa en levaduras (Venkatachalan y Montell, 2007).

Todos los receptores TRP son canales catiónicos que permiten el flujo de Ca^{2+} y Na^{+}, el cual se modifica según la isoforma, la permeabilidad y la selectividad para cationes mono o divalentes (Pedersen y Cols., 2005). Su patrón de distribución tisular es muy amplio, habiéndose detectado en prácticamente todos los tejidos, especialmente en el sistema nervioso central y periférico, donde juegan un papel crucial en la transducción sensorial (Venkatachalan y Montell, 2007). Además su permeabilidad para el catión Ca^{+2} implica la activación de señales de transducción celular que también contribuyen a la transmisión sensorial. Estudios de asociación genética han relacionado mutaciones en estos receptores con enfermedades humanas.

CANAL	ENFERMEDAD	SINTOMAS
TRPC3	Degeneración retiniana	Muerte de fotorreceptores
TRPC6	Glomeruloesclerosis focal y segmental	Pérdida renal
TRPM2	Extrés oxidativo y neurodegeneración	Neurodegeneración
TRPM6	Hipomagnesemia con hipocalcemia	Pérdida renal secundaria
TRPM7	Extrés oxidativo y neurodegeneración	Neurodegeneración
TRPP2 **TRPP1**	Policistitis renal autosómica dominante	Fallo renal, quistes renales
TRPML1	Mucolipidosis tipo IV	Retraso mental, neurodegeneración, degeneración retiniana

Tabla 6.- *Enfermedades humanas asociadas a canales TRPs. Información tomada de Venkatachalan y Montell 2007, Nilius y Cols., 2007.*

Estructuralmente, los canales TRP son homo o hetero-oligómeros (formados por diferentes subunidades de distintos canales TRP) constituidos por la asociación de cuatro subunidades alrededor de un eje de simetría central que coincide con el poro iónico (Dietrich y Cols., 2006).

Cada subunidad está compuesta por 6 segmentos transmembrana (S1-S6) flanqueados por extremos intracelulares en los extremos aminoterminal y carboxiterminal (N y C). Los dominios de transmembrana son las zonas más conservadas a lo largo de toda la familia, presentando la mayor variabilidad en los extremos N- y C-terminal, que es donde existen los dominios característicos de cada subfamilia (Montel, 2005).

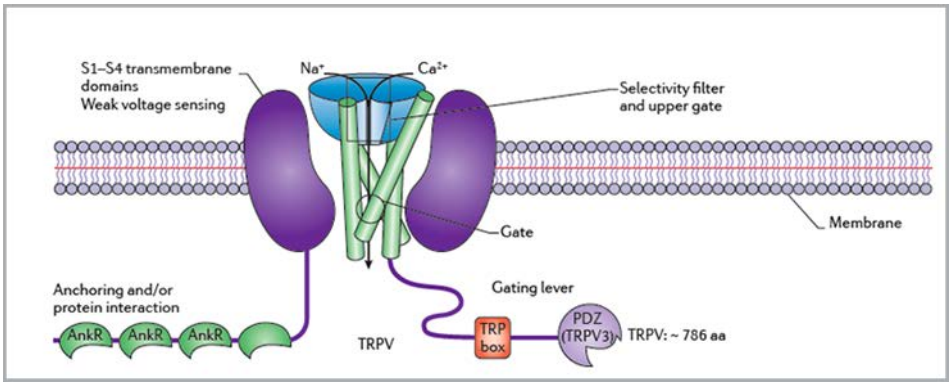

***Figura 8**. Esquema de la estructura de los canales iónicos de la familia TRPV. (Tomado de Moran y Cols., 2011)*

Los TRP juegan un papel fundamental en la transducción de las distintas modalidades sensoriales. Se les ha implicado en una gran variedad de funciones tales como la osmotransducción, la percepción del calor, de la presión, del dolor, del tacto, del gusto, de la audición, la homeostasis del Ca^{2+} y del Mg^{2+}, la angiogénesis, el mantenimiento del tono vascular, el crecimiento, la proliferación y la muerte celular, y la detección del flujo laminar (Montel,2005;Huang , 2004;Ramsey y Cols., 2006;Minke y Cook, 2002; Yao y Garland, 2005). Seguramente aún quedan muchas otras funciones por descubrir debido a la reciente identificación de la mayoría de los miembros de esta familia.

Por lo que respecta a la subfamilia de TRPV, en los mamíferos consta de 6 miembros divididos en 2 grupos según el grado de homología, a saber, TRPV1-4 (reconocen estímulos térmicos) y TRPV5-6. La activación polimodal alcanza su máxima expresión en los miembros del grupo TRPV1-V4 de esta subfamilia (O'Neil y Brown, 2003; Nilius y Cols., 2004). De entre los diferentes tipos de estímulos, la activación por estímulos mecánicos y térmicos parece ser una característica presente en este grupo de canales (Mutai y Heller, 2003; O'Neil y Heller, 2005; Tominaga y Caterian, 2004; O'Neil y Brown, 2003). En el caso del pez cebra solamente se han identificado los ortólogos del TRPV1 y TRPV4 (Shigeru y Ryuzo, 2006).

El presente trabajo de tesis doctoral se ha centrado en el estudio del TRPV4, prototipo de canal iónico de activación polimodal (O'Neil y Brown, 2003) ya que puede activarse frente a un amplio rango de estímulos físicos y químicos, considerándose un canal integrador de estímulos capaz de participar en una gran variedad de respuestas fisiológicas y celulares (O'Neil y Brown, 2003; Nilius y Cols., 2004). Hasta la fecha, se ha demostrado que este canal responde a cambios de temperatura (Chung y Cols., 2003; Watanabe y Cols., 2002), a citrato (Suzuki y Cols., 2003), a variaciones del pH (Suzuki y Cols., 2003) y a estrés mecánico (Gao y Cols., 2003), entre otras.

2.3.2. Canales de la superfamilia ASIC

Los ASIC fueron denominados inicialmente canales de sodio cerebrales (brain Na^+ channels, BNaC) ya que fueron descubiertos en las neuronas (Benos y Stanton, 1999); Pertenecen a la familia DEG/ENa^+C (Kellenberger y Schild, 2002; Krishtal, 2003; Holzer, 2009, 2011), son insensibles al voltaje y se activan por protones extracelulares.
Las proteínas ASIC son codificadas por 4 genes diferentes, que codifican 6 proteínas (Wemmie y Cols., 2006; Lingueglia y Cols., 2006) ya que existe *splicing* alternativo de *asic1* y *asic2* (Bassler y Cols., 2001). De la seis

proteínas ASIC, solamente 4 son activadas por pH ácido: ASIC1a, ASIC1b, ASIC2a y ASIC3. Estos son los más implicados en el control las de las fluctuaciones de pH, pero también se cree que pueden responder a acidificaciones prolongadas y bajas (Lingueglia, 2007).

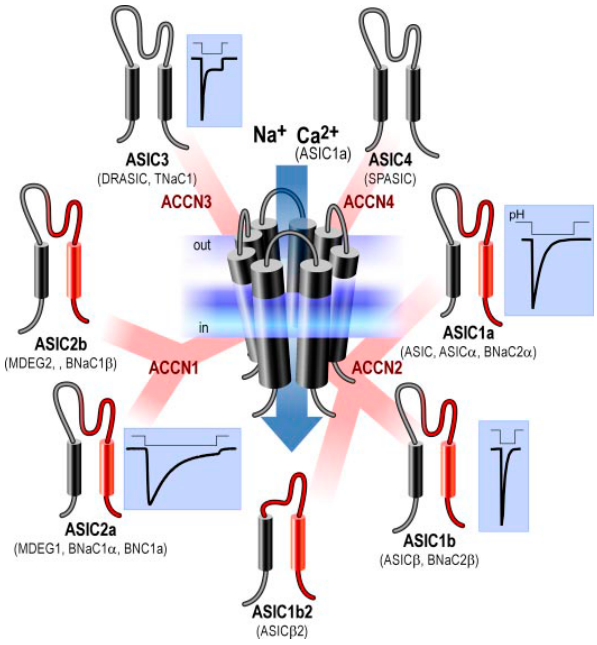

***Figura 9**. La familia de canales iónicos ASIC. Tomado de E. Lingueglia (2007)*

Los ASICs son proteínas de bajo peso molecular, de unos 500 aminoácidos, normalmente glicosiladas y caracterizadas por la presencia de dos secuencias de membrana helicoidal (dominios transmembrana 1 y 2), un "*loop*" extracelular rico en cisteína altamente conservado y un corto segmento N-terminal y C-terminal (Waldmann y Lazundski, 1998; Kellenberger y Schild, 2002; Welsh y Cols., 2002; Lingueglia, 2007). Las diferentes subunidades forman complejos homomultímeros y heteromultímeros con diferentes cinéticas, sensibilidad al pH extracelular, distribución tisular y propiedades farmacológicas

(Waldmann y Cols., 1999; Alvarez de la Rosa y Cols., 2002; Benson y Cols., 2002; Welsh y Cols., 2002; Wemmie y Cols., 2006).

Isoforma	Distribución	pH	Fisiología		Patología
ASIC1a	Cerebro Médula espinal SNP Retina Células del gusto Hueso	6.2 l 6.8	Mecanorrecepción visceral Transducción visual Plasticidad sináptica Nocicepción:SNC / SNP Condicionamiento del miedo	↑ ↓ →	Inflamación: GRD/médula espinal Daño post- isquemia cerebral Dolor neuropático Epilepsia (rata) Gliomas
ASIC1b	SNP Célula del gusto Célula aérea cóclea	5.1 l 6.2	Nocicepción?	↑	Inflamación: GRD
Asic2a	SNP mecanorreceptores especializados Cerebro Médula espinal Ganglio espiral coclear Célula gusto (ratas) Hueso	4.1 l 5	Modula ASIC1 enSNC Transducción visual Mecanorrecepción cutánea/visual Audición supraumbral Gusto	↑	Inflamación: médula espinal Protección degeneración retiniana
ASIC2b	Cerebro Médula espinal SNP Retina Células del gusto	n/a	Modulación ASIC2a/ASIC3	↑ ↓	Inflamación:GRD Epilepsia(rata)
ASIC3	SNP Mecanorreceptores especializados Células del gusto Retina Testículo Célula epitelial pulmonar Oído interno Hueso		Nocicepción Mecanorrecepción cutánea y visceral Oído	↑	Inflamación:GRD Hiperalgesia mecánicadelmúsculo Sensaciónacida
ASIC4	SNP (bajo), cerebro, médula espinal, glándula pituitaria, oído interno	n/a	?		?

***Tabla 7**. Propiedades de los canales ASICs-Tomada de Lingueglia (2007).*

A los canales iónicos ASIC se les ha implicado en la percepción del dolor (Reeh y Cols., 2001 ; Basbaum y Cols., 2009; Jeong y Cols., 2013; Deval y Cols., 2008), del tacto (Price y Cols., 2001; Chen y cols 2002), del gusto (Lin y Cols., 2002; Ugawa y Cols., 2003), de la audición (Couloigner y Cols., 2001; Zhong y Liu, 2004) y especialmente en la mecanotransducción (Hoger y French, 2002; Hildebrand y Cols., 2004; Roza y Cols., 2004; Delmas y Cols., 2011; Chen y Wong, 2013).

En el pez cebra existen proteínas ASIC semejantes a las de los mamíferos que se denominan zASIC: zASIC1.1, zASIC1.2, zASIC1.3, zASIC2, zASIC4.1, y zASIC4.2 (Paukert y Cols., 2004). El análisis filogenético ha demostrado que *zASIC1.1, zASIC2* and *zASIC4.1* son ortólogos de los de los mamiferos, mientras *zASIC1.2* y *zASIC1.3* son paralógos; hasta la fecha no se ha identificado en esta especie ningún ortólogo de ASIC3. Los zASICs activados por protones extracelulares lo hacen de una forma similar a la de los mamíferos, mientras que el zASIC2 y el zASIC4.2 no se activan por los descensos de pH (Paukert y Cols., 2004). Así, la insensibilidad del zASIC4.2 a los protones es una reminiscencia del patrón restringido del ASIC4 en mamíferos y la activación del ASIC4.1 por los protones podría ayudar a identificar estructuras que dependen de los mismos para su activación (Paukert y Cols., 2004).

2.4. Canales iónicos en los órganos de los sentidos de los vertebrados

2.4.1. Humanos y otros mamíferos

Los canales iónicos TRPV4 y ASICs juegan un papel fundamental en la transducción de las distintas modalidades somatosensoriales, incluyendo el tacto, gusto, olfato, osmolaridad y sensación térmica (Montell, 2005, Bandell y Cols., 2007). Estas funciones se basan en gran medida por la presencia de dichos canales en los dístintos órganos de los sentidos (ver tablas 7 y 8).

Por otro lado, los estudios realizados con ratones manipulados genéticamente apoyan la participación de estos canales en la transducción de la señal sensorial en diferentes órganos de los sentidos. En los ratones carentes de los genes que codifican para los canales iónicos que son objeto del presente estudio se han demostrado cambios en la funcionalidad de algún órgano sensitivo.

Como se muestra en la tabla 7 ASIC2 y ASIC3 parecen estar implicados en la mecanotransducción (ver Peng y Cols., 2004; Montaño y Cols., 2009; Calavia y Cols, 2010) ya que han sido detectados en diferentes morfotipos de mecanorreceptores. Además, los ratones knockout para los canales ASIC2a y ASIC3 presentan una disminución en la sensación táctil (Price y Cols., 2000; 2001). ASIC3 interviene también en la percepción del dolor (Sutherland y Cols., 2001).

También se ha sugerido que ASIC2 participa en la respuesta gustativa debido a su presencia en los quimiorreceptores linguales (Huque y Cols., 2003; Ugawa y Cols., 2003; Shimada y Cols., 2006). Sin embargo los ratones carentes de la subunidad ASIC2a parecen no tener alteraciones en la sensibilidad gustativa (Ritcher y Cols., 2004) por lo que el déficit de ASIC2a podría ser compensada por otro canal. No obstante, Huque y Cols. (2009) sustentan la importancia de ASIC2 en el sentido del gusto porque mutaciones en este gen producen ageusia en humanos.

Diversos grupos de investigación han estudiado la presencia de los canales ASIC en el oído interno y los resultados obtenidos son muy controvertidos. El modelo murino knockout para ASIC2 sugiere que este canal podría participar en la modulacion de estímulos auditivos supraumbrales (Peng y Cols., 2004) o que no participa en el proceso de audición (Roza y Cols., 2004). Respecto al ASIC3, los animales carentes de este gen desarrollan prematuramente perdida de oído (Hildebrand y Cols., 2004).

Por último, los trabajos realizados sobre ratones deficientes en ASIC2 demuestran que son más sensibles a la degeneración retiniana inducida por la luz (Ettaiche y Cols., 2004), mientras que los resultados obtenidos en los que carecen de ASIC3 sugieren que éste está implicado en el mantenimiento de la integridad retiniana (Lilley y Cols., 2004; Ettaiche y Cols., 2006).

ÓRGANO	TIPO CELULAR	TECNICA	ESPECIE	REFERENCIA
Oído	Células ciliadas: internas/externas del órgano de Corti, de canales semicirculares, mácula utricular Estría vascular de la cóclea	ISH	Ratón rata pollo humano	Liedtke y Cols.,2000
	Células ciliadas del oído, ganglio espiral	RT-PCR IHQ	Ratón	Shen y Cols., 2006
	células pilosas/ soporte órgano de Corti, marginales de estría vascular, vestibulares del ganglio espiral y vestibular, epiteliales de saco endolinfático	IHQ	Cobaya	Takumida y Cols., 2005
Piel	Queratinocitos basales y suprabasales	IHQ,	HUMANO	Radtke y Cols.,2011 Fusi y Cols.,2014
	Corpúsculos de Meissner.	IHQ	Ratón	Suzuki y Cols., 2003
	Células de Merkel-vibrisas	ISH	Ratón. rata	Liedtke y Cols..2000
	Corpúsculo de Herbst	IHQ	Ave	Cabo y Cols., 2013
Articulación Pluma	Corpúsculos de Herbs-Axón y núcleo interno	IHQ	Ave	Cabo y Cols., 2013
Ojo	Retina: células ganglionares	RT-PCR IHQ	Ratón Ratón	Gillian Y Wensel, 2011 Ryskamp y Cols., 2011
	Cornea: células epiteliales	IHQ RT-PCR WB	Humano	Pan y Cols., 2008,
Olfatorio	ORN, cilios, células basales, de soporte y lámina propia	IHQ	Ratón	Ahamed y Cols., 2009

***Tabla 8**. Canal TRPV4 en los órganos de los sentidos en vertebrados*

El canal TRPV4 tambien participa en la fisiología sensorial y su alteración funcional mediante mutaciones corrobora esta afirmación. Así, los ratones *knockout* para TRPV4 presentan déficits del equilibrio osmótico: ingieren menos agua, tienen una presión osmótica más elevada, y los

niveles de vasopresina están alterados (Liedtke y Friedman 2003); también muestran disregulaciones en otras funciones sensoriales: la percepción de la presión (Suzuki y Cols., 2003), la percepción del sonido con una mayor susceptibilidad a sufrir daños acústicos (Tabuchi y Cols., 2005) y la percepción del dolor asociado a un cambio osmótico mediado a través de neuronas nociceptivas aferentes y potenciado durante la inflamación (Liedtke y Friedman, 2003; Suzuki y Cols., 2003).

2.4.2. Pez cebra y otros teleósteos

Los datos disponibles sobre la presencia de los canales iónicos ASICs y TRPV4 en los órganos sensoriales de los peces son muy escasos, y la mayoría de las investigaciones en este campo se han llevado a cabo en estadios larvarios.
El canal TRPV4 se ha detectado en larvas de pez cebra en estructuras sensoras situadas en la línea lateral posterior (Dambly-Chaudiere y Cols., 2003) y en las capas nuclear interna y de las células ganglionares de la retina (Sanchez-Ramos y Cols., 2012); en adultos, se ha encontrado en las células ciliadas de los neuromastos (Lee y Cols., 2013) y de los "*pit organs*" (Mangos y Cols., 2007) y en las células ganglionares de la retina (Sanchez-Ramos y Cols., 2012).

Respecto a los ASICs, los datos en este modelo animal son similares a los obtenidos en los mamíferos. La mayoría de los zASIC se expresan con una amplia distribución en el sistema nervioso central mientras que su presencia en los órganos periféricos es limitada (Paukert y Cols., 2004). Los datos disponibles se restringen al periodo larvario; todos los zASIC se expresan en el sistema nervioso entre las 24 hpf y las 48 hpf lo que sugiere un papel en el desarrollo del sistema nervioso; respecto a los órganos de los sentidos, zASIC1.1 se presenta en las neuronas óticas, mientras que zASIC2, zASIC4.1 y zASIC4.2 se expresan en las células ganglionares de la retina las cuales reciben el input de los fotorreceptores en las larvas de pez cebra a partir de 48 hpf (Paukert y Cols., 2004).

En nuestra línea de investigación con *Danio rerio* adulto hemos detectado la presencia de ASIC2 y ASIC4 en las papilas gustativas y en los nervios que las suplen (Viña y Cols., 2013), en la retina y en el epitelio olfatorio (Viña y Cols., 2015a, 2015b). Respecto a TRPV4 lo hemos hallado en retina, papilas gustativas, neuromastos y roseta olfatoria (Amato y Cols., 2012). Estos hallazgos constituyen el cuerpo de esta tesis doctoral y se exponen en detalle en capítulo de resultados.

OBJETIVOS

3. OBJETIVOS

Los datos científicos disponibles establecen que los canales iónicos ASIC y TRPV desempeñan papeles esenciales en la transducción nerviosa que ocurre en la mayoría de las especies animales, y que los sistemas sensoriales dependen de la activación de canales iónicos para transducir los estímulos específicos en señales eléctricas.

Actualmente se considera que la capacidad de los diferentes tipos de neuronas sensitivas y células sensitivas para detectar y codificar estímulos específicos resulta de la combinación de la actividad de diferentes canales iónicos.

Por otro lado, los canales iónicos TRPV y ASIC están presentes en las diferentes estructuras sensoriales de los vertebrados, incluido *Danio rerio*. Sin embargo, hasta el momento, no se ha realizado un estudio sistemático sobre la presencia de estos canales en los órganos sensoriales de este modelo animal, uno de los más utilizados en investigación biomédica.

Los objetivos planeados son los siguientes:

1.- Examinar la expresión y distribución del canal iónico TRPV4, mediante inmunohistoquímica, en los órganos de los sentidos del pez cebra adulto (línea lateral, oído, papilas gustativas, epitelio olfatorio y retina).

2.- Establecer la expresión y distribución de los canales iónicos de la familia ASIC (ASIC1, ASIC2, y ASIC4) en las papilas gustativas externas e internas del *Dario rerio* adulto.

3.- Establecer la morfología y estructura del epitelio olfatorio del pez zebra adulto. Determinar la expresión de la canal iónico ASIC2 en la roseta olfatoria a nivel de mRNA y proteína. Caracterizar la localización celular de este canal en el epitelio olfatorio mediante inmunohistoquímica.

4.- Comprobar la presencia y distribución en la retina del *Dario rerio* adulto de los mRNA para los canales zASIC, zACIC4.1 y zASIC4.2. Determinar la presencia de estas proteínas, así como su localización celular y finalmente comprobar su concordancia con los resultados previos de la hibridación in situ realizada con sondas específicas para *Danio rerio*.

MATERIAL Y METODOS

4. MATERIAL Y METODOS

4.1. Material

4.1.1. Muestras de pez cebra *(Danio rerio)*

Para la realización del presente estudio se utilizaron 95 *Danio rerio* adultos de ambos sexos obtenidos del CSIS (Centro Sperimentale Ittiopatologia de Sicilia, Universidad de Messina, Italia) criados a una temperatura constante de 28,5°C, y alimentados dos veces al día (Westerfield, 1995). Los animales fueron anestesiados por inmersión en una solución de MS222 (metanosulfato de tricaina; 0,4 g / l) y sacrificados por decapitación. Todos los experimentos se llevaron a cabo en conformidad con las directrices europeas vigentes en materia de experimentación animal.

Nº animales	Órgano sensorial	Proteina	Nª animales	Técnica
15	Papila gustativa	ASIC2	5	Western-blot
			10	IHQ
20	Neuromasto	TRPV4	10	IHQ
	Papila gustativa			
	Oído interno		5	Western-blot
	Roseta olfatoria		5	RT-PCR
	Retina			
20	Retina	ASIC2/ASIC4	10	IHQ / ISH
			10	RT-PCR / Western-blot
25	Roseta olfatoria	ASIC2	10	IHQ / ISH Microscopía electrónica
			5	RT-PCR / Western-blot
Experimentos con ciclos de luz blanca-oscuridad / Blanca continua				
15	Retina	ASIC2 / ASIC4	5 LB/O	RT-PCR / Western-blot
			5 LB/O	RT-PCR / Western-blot
			5 LBC	RT-PCR / Western-blot

***Tabla 10**.- Material utilizado para la realización del trabajo.*

Para el trabajo sobre retina se realizaron tres grupos de animales: grupo control en el que los animales se mantuvieron con ciclos de luz blanca/oscuridad (12/ 12 horas, n = 5), y grupos experimentales que se mantuvieron con luz blanca continua (n = 5) o en oscuridad continua (n = 5).

Los animales expuestos a luz continua se colocaron en una caja plástica transparente con la fuente de luz colocada 3 cm por encima del nivel del agua, y se mantuvieron en esta situación durante 10 días. La fuente de luz blanca (Tª = 4.000ºK) fue de Philips MASTER TL- D 18W/840 Reflex (Philips ConsumerLifestyle, España). La irradiación de 28,57 W/m^2 se midió utilizando un photoradiometricTektronix J1800 (TekLumaColor Inc, USA) y la composición espectral de la fuente de luz se determinó utilizando un espectrofotómetro de fibra óptica Spectrawiz EPP2000 (SterllarNet Inc., USA). El grupo de animales de oscuridad continua permaneció el mismo tiempo en una caja plástica pintada de negro (ver para detalles Sánchez-Ramos y Cols., 2012).

4.2. Técnicas

4.2.1. Inmunohistoquímica

4.2.1.1. Tratamiento de las muestras para inclusión en parafina

Los tejidos fueron fijados por inmersión en Bouin durante 24 horas, deshidratados y procesados para su inclusión rutinaria en parafina. Los bloques se cortaron con un micrótomo a 10 µm de espesor y las secciones obtenidas se montaron en portaobjetos gelatinizados. Este material fue utilizado para los estudios de inmunohistoquímica y de hibridación in situ.

4.2.1.2. Inmunohistoquímica simple mediante método indirecto de PAP

Las secciones histológicas se diafanizaron con xilol y se rehidrataron mediante alcoholes de gradación decreciente hasta agua. A continuación, se trataron con H_2O_2 al 3% durante 10 minutos para bloquear la actividad peroxidasa endógena. Posteriormente se lavaron con tampón PBS 1M, pH 7.6, que contenía Tween-20 al 0.5% (PBS-T) para permeabilizar las membranas celulares, y seguidamente se incubaron con albúmina de suero bovino (BSA) al 3 % durante 30 minutos para eliminar las uniones inespecíficas.

La incubación con los anticuerpos primarios se llevó a cabo durante toda la noche, a 4°C en una cámara húmeda. Para la obtención de resultados óptimos con el anticuerpo anti-TRPV4 fue necesario realizar recaptación antigénica o desenmascaramiento del epítopo con el fin de aumentar la permeabilización de las membranas celulares y de exponer el antígeno al anticuerpo primario. Para ello, los cortes hidratados y antes del bloqueo de la actividad peroxidasa endógena, se incubaron en tampón de extracción (DakoEnVision Flex Target Retrieval solution high pH), en baño a 90°C, durante 20 min, seguido de 20 min a temperatura ambiente.

Tras la incubación con los anticuerpos primarios, las secciones se lavaron con el tampón PBS-T durante 20 min y después se incubaron durante 90 min a temperatura ambiente con el anticuerpo secundario (Dako EnVision labelled polymer-HR anti-conejo IgG o de IgG anti-ratón). Finalmente, tras un lavado en PBS-T se reveló la inmunorreacción con una solución de 3-3’ diaminobencidina (DAB, kit de revelado de Dako). Las secciones se lavaron en agua, se deshidrataron en una batería de etanol a concentración creciente, se diafanizaron en xilol y se montaron con Entellan®. Las preparaciones se fotografiaron en un microscopio óptico Nikon Eclipse 80i acoplado a una cámara Nokia DS-5M.

Los controles de la especificidad de la inmunorreacción se realizaron por exclusión del anticuerpo primario o por incubación con suero no inmune de conejo o de ratón en lugar del anticuerpo primario. En ambos casos el inmunomarcaje fue negativo.

Anticuerpo	Origen	Dilución	Proveedor
zfTRPV4 (transient receptor potential cation channel, subfamily V, member 4)	Conejo	1:200	Abcam (Cambridge, Reino Unido)
TRPV4 (transient receptor potential cation channel, subfamily V, member 4)	Conejo	1:200	Abcam (Cambridge, Reino Unido)
ASIC2 (Acid Sensing Ion Channel 2)	Conejo	1:100	LifeSpan Bioscience (Seattle, USA)
ASIC4 (Acid Sensing Ion Channel 4)	Conejo	1:100	LifeSpan Bioscience (Seattle, USA)
Asic1 (Acid Sensing Ion Channel 1)	Conejo	1:200	Abcam (Cambridge, ReinoUnido)
S-100	Conejo	1:1000	Dako, Glostrup, Denmark; code Nº. Z0311
β-tubulin	Mouse	1:300	clone TUB 2.1, Sigma, St Louis, MS, USA
calretinin	Mouse	1:100	clone 6B3, Swant, Marly, Switzerland
zASIC4	Conejo	1:50	Cortesia del Dr. Greg Goss, Universidad de Alberta, Canada

Tabla 11. *Anticuerpos primarios utilizados en estos estudios*

4.2.1.3 Doble inmunohistoquímica fluorescente con microscopía confocal

Las secciones se desparafinaron y rehidrataron como se describió en el apartado anterior. Seguidamente, se lavaron 20 min en PBS-T y se incubaron con una mezcla 1:1 de dos anticuerpos primarios seleccionados para la detección simultánea de dos antígenos (inmunohistoquímica doble), durante toda la noche a 4°C en cámara húmeda. Tras lavar las preparaciones con PBS-T durante 30min, los cortes se incubaron con los anticuerpos secundarios durante 90 min: primero con Alexa Fluor 488 (Invitrogen, Paisley, Reino Unido), IgG de cabra anti-conejo a una dilución 1:200 en PBS; y a continuación con Cy3, IgG de asno anti-ratón (Jackson Immunoresearch Laboratories, Pensilvania, USA), diluido 1:50 en PBS. La incubación con los anticuerpos secundarios se llevó a cabo en cámara húmeda oscura a temperatura ambiente. Entre

las dos incubaciones y *a posteriori* se realizó un lavado en PBS-T. Las preparaciones fueron montadas con Fluoromount-G (Southern-Biotech, Alabama, USA).

La inmunofluorescencia fue detectada mediante un microscopio automático de fluorescencia Leica DMR-XA acoplado a un software de Captación de Fluorescencia LeikaQfluoro (Unidad de Microscopía Fotónica y Proceso de Imágenes, Universidad de Oviedo). Los controles de la especificidad de la reacción consistieron en omitir el anticuerpo primario, el secundario o ambos. Además, se llevaron a cabo controles adicionales con el fin de confirmar la ausencia de procesos de autofluorescencia del tejido o producidos por el proceso de fijación omitiendo ambos anticuerpos.

4.2.2. Hibridación in situ cromogénica

4.2.2.1. Diseño y marcaje de sondas para hibridación in situ

En el pez cebra zASIC2 tiene una única isoforma, mientras que el ASIC4 presenta dos isoformas, zASIC4.1 y zASIC4.2 (Chen y Cols., 2007). En el presente trabajo se han diseñado sondas específicas de DNA para cada uno de ellos en base a la secuencia de mRNA del GenBank

Gene		Probe
zASIC2 (NM_214788.1)	Sense	5`GAAGAGGGGAAGACTTTAAGGACTACAATGAAAG3`
	Antisense	5`GTGATATATTTCTCGGATCGGTTGAACTTCTTCTC3`
zASIC4a (NM_214787.2)	Sense	5`GCCAACACCATCCTCCCGAATCACCATCACCACCAC3`
	Antisense	5`GTGGTGGTGATGGTGATTCGGGAGGATGGTGTTGGC3`
zASIC4b (NM_214786.1	Sense	5`CCAGAGAACCAAGAACCTGCGGGAGCAGAACCTG3`
	Antisense	5`CAGGTTCTGTTCCCGCAGGTTCTTGGTTCTCTGG3`

***Tabla 12**. Secuencias de las sondas utilizadas para la hibridación in situ a partir de la secuencia de mRNA del GenBank (números de acceso*)

En todos los casos, las sondas tenían entre 34 y 36 nucleótidos, y la temperatura de "*melting*" fué de 72ºC para ASIC2 y de 84°C para ambas sondas de ASIC4. Las sondas se marcaron con biotina mediante el kit 3`End DNA Labeling Kit (Thermo Fisher Scientific Inc., USA) según el protocolo específicado por la casa comercial.

4.2.2.2. Hibridación in situ

Las secciones fueron desparafinizadas y rehidratadas, y se realizó recaptación antigénica en tampón de extracción (Dako EnVision Flex Target Retrieval solution high pH) en baño a 95ºC durante 10 min seguido de otros 10 min a temperatura ambiente. A continuación los tejidos se lavaron con PBS, se incubaron con HCl 0,2N durante 15 min, y se lavaron con PBS estéril durante 5 min y PBS-T (Triton-X100 0,03%) durante 15 min. Posteriormente los cortes se trataron con proteinasa K 10µg/ml durante 7 min a 37°C para facilitar la exposición de los ácidos ribonucleicos. Después las secciones se lavaron con PBS estéril durante 10 min para bloquear la actividad enzimática. Finalmente, los tejidos se incubaron con la sonda a una dilución 1:3 en agua MillQ, en un horno de hibridación Hybridizer Instrument for in situ hybridization (DAKO). El programa de hibridación consistía en: 5 min a 95ºC para separar la cadena de ADN, seguido de 16 h a 55ºC para las sondas de asic4 y 17 h a 47ºC para ASIC2.

Tras la incubación, las muestras se lavaron con TBS durante 10 min para eliminar los restos de sondas. Se realizaron lavados astringentes con la solución SSC (clorihidrato citrato de sodio) a 60º-65ºC según la sonda usada, durante 15 min. Los siguientes pasos de lavado y bloqueo se realizaron con Dig Wash and Block Buffer Set de acuerdo a las especificaciones del fabricante (Roche Applied Science, Penzberg-Alemania). Los tejidos se incubaron con un conjugado de streptavidina-AP (Sistema de detección de hibridación in situ para sondas biotiniladas, Dako) a temperatura ambiente durante una hora. La reacción se reveló

con una solución BCIP/NBT (Sigma), tras lo cual se lavaron en TBS y se montaron con "inmuno in situ mount" (Santa Cruz Biotechnology, USA).

4.2.3. Western-blot

4.2.3.1. Extracción de proteínas total de tejido

Las muestras de cerebro, roseta y ojos se homogenizaron (1:2, w/v) en una solución Tris-HCl solución salina tamponada (TBS, 0,1M, pH 7,5) que contenia leupeptina 1 mM, pepstatina 10 mM y 2 mM de fluoruro de fenilmetilsulfonilo. Los homogeneizados se centrifugaron a 25.000 rpm durante 15 min a 4ºC y el pellet resultante se disolvió en Tris-HCl 10 mM pH 6,8, SDS al 2%, ditiotreitol 100 mM y 10 % de glicerol a 4ºC.

4.2.3.2. Western Blot

La electroforesis de las proteína se realizó según el método de Laemmli (Laemmli, 1970). La electroforesis y posterior transferencia se llevaron a cabo en un equipo Miniprotean3 (BioRad, Hércules-California-USA) a amperaje constante. Tras la electroforesis se transfirieron las proteínas a una membrana de nitrocelulosa a una intensidad de 125 mA por gel a 4ºC durante toda la noche.
Posteriormente, se bloqueó la membrana por inmersión durante 3 h en PBS que contenía leche en polvo al 5 %, y 0,1 % de Tween-20. Las membranas se incubaron entonces a 4ºC durante 2 h con un anticuerpo contra los anticuerpos ASIC2 y TRPV4 (ver la Tabla 11). Después de la incubación, las membranas se lavaron con TBS pH 7,6 que contiene 20 % de Tween-20, y se incubaron de nuevo durante 1 h con el IgG de cabra anti-conejo diluido 1:100 (Santa Cruz Biotechnology, Santa Cruz-USA) a temperatura ambiente. Las membranas se lavaron de nuevo y se incubaron con el complejo PAP diluido 1:100 durante 1 h a temperatura ambiente. Finalmente, la reacción se visualizó usando un reactivo quimioluminiscente (ECL, Amersham Pharmacia Biotech, Buckinghamshire, Reino Unido). Las proteínas marcadoras se visualizaron por tinción con azul brillante.

4.2.4. Transcripción reversa de ARN acoplada a PCR (RT-PCR)

4.2.4.1. Extracción del ARN

El RNA se extrajo a partir de ojos, roseta y cerebro (control positivo) de peces adultos usando un kit comercial (Trizol Reagant, invitrogen, Carlsbad, CA, USA) de acuerdo con las especificaciones de la casa comercial. El RNA se precipitó y lavó con etanol frio y después se secó y se disolvió en un volumen adecuado de tampón Tris-EDTA (10mM Tris-HCl pH8.0 y 1mM EDTANa^2). Cada una de las muestras fue tratada con 1 U de DNase durante 1 h a 37^{o}C para digerir el DNA genómico. Los RNAs se precipitaron, lavaron y disolvieron de nuevo en el mismo tampón. La concentración de RNA se midió a 260 nm (Biomate 3, Thermo Electron, Corporation, Waltham, MA, USA) y su pureza fue valorada por un lector de ratio 269/280 nm. Se uso el kit High capacity cDNA archive (Applied Biosystems, Carlsbad, CA, USA) para obtener cDNA a partir de 10µg de RNA total a 42^{o}C durante 90 min siguiendo las especificaciones del productor.

4.2.4.2. RT-PCR

Los cDNAs se usaron para realizar la PCR cuantitativa. Los oligonucleótidos específicos para la amplificación de cada ADN particular se diseñaron de acuerdo a las secuencias publicadas para *Danio rerio* zASIC2, zASIC4a, y zASIC4b en el genBank (números de acceso de GeneBank NM_214788.1, NM_214787.2 y NM_214786.1, respectivamente) y *Danio rerio* β-actina (número de acceso GenBank NM 131031).

Las condiciones de amplificación fueron las siguientes: 2 U de Taq ADN polimerasa (Promega, Madison, WI, USA), 1 µM cebadores, 10 ng de cerebro de pez cebra o retina ADNc, 0,2 mM de cada dNTP en 15 µL de tampón Taq ADN polimerasa.

Gene	Forward	Reverse
zASIC2 (NM_214788.1)	5´- TTGTTGGCCAGCTTAGTCCT-3´	5´- GGGTCGGTAGTTAGTGAAGT- 3´
zASIC4a (NM_214787.2)	5´- ACGATGTTGCAGGATTATTAGG-3´	5´- TTTGTCATCTCTTTGTGGCCA--3´
zASIC4b (NM_214786.1)	5´- GCAAGTACCAGAAGTCAGAGGA-3´	5´- CTGTCCACCAATGTCTCCTAAC-3´
B-actin (NM 131031)	5´- CACAGATCATGTTCGAGACC- 3´	5´ - GGTCAGGATCTTCATCAGGT-3´

***Tabla 13**. Primers diseñados según las secuencias consenso del Danio rerio del GenBank (números de acceso).*

La reacción se realizó en un termociclador (Hyband Th. Ciclador) con el siguiente programa:

	10 ciclos			20 ciclos			
1 min 94ºC	1 min 94ºC	30 seg 65ºC	45 seg 72ºC	1 min 94ºC	30 seg 61ºC	45 seg 72ºC	5 min 72ºC

Los productos de la PCR se visualizaron por tinción con bromuro de etidio bajo luz UV después de la electroforesis en un 2 % gel de agarosa con marcador Ready Load 1KB plus DNA ladder de ADN (Invitrogen, Carlsband, USA).

4.2.4.3. RT-PCR cuantitativa (qRT-PCR)

La PCR cuantitativa se llevó a cabo usando 1 µg de cDNA. Los primers utilizados aparecen en la tabla 15. Las reacciones de PCR se realizaron con TaqMan Universal PCR Master Mix (Applied Biosystems) usando 5 pmol de cada primer y 9 pmol de las sondas de ASIC2, ASIC4.2 y actina. Los ensayos se realizaron por triplicado utilizando una 7500 PCR real-time system (Applied Biosystem, Foster City, California, USA) y la cuantificación se calculó usando el algoritmo $2^{-\Delta\Delta Ct}$. La media del valor obtenido por los animales sometidos a 12horas de luz/ 12horas de oscuridad fue considerada como control, las diferencias estadísticas entre el grupo

control comparado con los experimentales (oscuridad continua y luz continua) fue determinada usando el análisis de varianza (ANOVA) ($p \leq 0.05$)

4.2.5. Microscopía electrónica de transmisión

Las rosetas olfatorias aisladas (n = 5) se embebieron en resina Durcupan ACM (Fluka) atendiendo al siguiente protocolo: Fijación en glutaraldehido al 2,5% en 0.2M PBS (pH7.4) durante 2 h a 4^{o}C; lavado repetido y post-fijación en tetróxido de osmio al 1% en PBS durante 1 hora a 4^{o}C. A continuación los tejidos fueron deshidratados a través de pasos por concentraciones crecientes de acetonas y se embebieron en Durcupan ACM. Las piezas se cortaron con un ultramicrotomo Reichardt Jung ULtracut E y las secciones semifinas, de 1 µm, se tiñeron con azul de toluidina y se examinaron al microscopio óptico. Las secciones ultrafinas (700Å) se seleccionaron de áreas concretas de los cortes semifinos, se tiñeron con acetato de uranilo y se examinan con un microscopio electrónico de trasmisión Jeol Jem 100SX (Departamento de Ciencias Veterinarias, Sección de Anatomía, Universidad de Messina, Italia).

4.2.6. Microscopía electrónica de barrido

Las rosetas olfatorias aisladas (n = 5) fueron fijadas en 2,5% de glutaraldehido en tampon fosfato 0,1M de Sörensen. Despues de lavarse varias veces en el mismo tampon, fueron deshidratadas con el uso de una barería de alcoholes crecientes, se secarón en Balzers CPD 030, se recubrieron con 3 nm gold en Balzers BAL-TEC SCD 050 y se estudiaron en un Cambridge Stereoscan 240 electron microscope (Zeiss ex Cambridge Instruments, Cambridge, UK), que opera con una aceleración de voltage de 20 kV.

RESULTADOS

5. RESULTADOS. APORTACIONES

5.1. Amato V, **Viña E**, Calavia MG, Guerrera MC, Laurà R, Navarro M, De Carlos F, Cobo J, Germanà A, Vega JA. **TRPV4 in the sensory organs of adult zebrafish.** Microsc Res Tech. 2012; 75(1):89-96.

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5.4. Viña E, Parisi V, Sánchez-Ramos C, Cabo R, Guerrera MC, Quirós LM, Germanà A, Vega JA, García-Suárez O. **Acid-sensing ion channels (ASICs) 2 and 4.2 are expressed in the retina of the adult zebrafish.** Cell Tissue Res. 2015 Jan 15. [Epub ahead of print]. DOI 10.1007/s00441-014-2084-5

TRPV4 in the Sensory Organs of Adult Zebrafish

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KEY WORDS *TRPV4*; zebrafish; neuromasts; inner ear; taste buds; olfactory epithelium; retina

ABSTRACT *TRPV4* is a nonselective cation channel that belongs to the vanilloid (V) subfamily of transient receptor potential (TRP) ion channels. While TRP channels have been found to be involved in sensing temperature, light, pressure, and chemical stimuli, TPRV4 is believed to be primarily a mechanosensor although it can also respond to warm temperatures, acidic pH, and several chemical compounds. In zebrafish, the expression of *trpv4* has been studied during embryonic development, whereas its pattern of TPRV4 expression during the adult life has not been thoroughly analyzed. In this study, the occurrence of *TRPV4* was addressed in the zebrafish sensory organs at the mRNA (RT-PCR) and protein (Westernblot) levels. Once the occurrence of *TRPV4* was demonstrated, the *TRPV4* positive cells were identified by using immunohistochemistry. TPRV4 was detected in mantle and sensory cells of neuromasts, in a subpopulation of hair sensory cells in the macula and in the cristae ampullaris of the inner ear, in sensory cells in the taste buds, in crypt neurons and ciliated sensory neurons of the olfactory epithelium, and in cells of the retina. These results demonstrate the presence of *TRPV4* in all sensory organs of adult zebrafish and are consistent with the multiple physiological functions suspected for *TRPV4* in mammals (mechanosensation, hearing, and temperature sensing), but furthermore suggest potential roles in olfaction and vision in zebrafish. *Microsc. Res. Tech. 00:000–000, 2011.*

INTRODUCTION

The sensory system of teleosts, including zebrafish, consists of specialized sensory organs able to detect mechanical and chemical environmental stimuli, as well as light (Ostrander, 2000). The mechanosensory cells are grouped into superficial and deep neuromasts that form the lateral line system (LLS; Coombs et al., 2002), and in the sensory epithelia of the inner ear (Bang et al., 2001). The chemosensory cells are grouped into taste buds (Hansen et al., 2002), and sparse in the lamellae of the olfactory epithelium (Hansen and Zielinski, 2005). Moreover scattered solitary chemosensory cells are present in the skin (Kotrschal et al., 1997). Finally, the light-sensitive cells form a part of the retina, which is structured as in other vertebrates (Marc and Cameron, 2001; Yazulla and Studholme, 2001). All these sensory cells contain ion channels that are at the basis of the organ-specific transduction that converts different types of stimuli into electric energy.

The superfamily of transient receptor potential (TRP) ion channels is formed by seven subfamilies of nonselective cation channels, which have been identified in almost all living organisms (see for a review Eid and Cortright, 2009). Functionally, TRP channels are involved in sensing temperature, light, pressure, as well as in detecting several chemical stimuli (see Damann et al., 2008). *TRPV4* is a Ca^{2+} entry channel belonging to the vanilloid subfamily, that works as a mechanosensor including detection of osmotic stimuli, but also responds to warm temperatures, acidic pH, and several chemical compounds (see Claphan, 2003; Liedtke, 2007a,b,c; Plant and Strottmann, 2007). Importantly, mechanical and chemical sensitivity of *TRPV4* is modulated by temperature (Gao et al., 2003). All these data suggest that *TRPV4* is probably expressed in sensory organs, although limited information regarding expression of *TRPV4* is available. In mammals, the expression of *TRPV4* at the mRNA levels is diffuse (see for references-Facer et al., 2007; Plant and Strottmann, 2007), and consistently with its putative role in sensory transduction it has been detected in sensory ganglia (Alessandri-Haber et al., 2003; Delany et al., 2001; Liedtke et al., 2000), and in some types of cutaneous (Suzuki et al., 2003) and inner ear mechanoreceptors (Liedtke et al., 2000; Takumida et al., 2005). The phenotype observed in *TRPV4* deficient animals confirmed some but not all the functions attributed to *TRPV4* (see Plant and Strottmann, 2007; Ramsey et al., 2006; Suzuki et al., 2003).

In zebrafish five TRP channel genes have been described, including *trpv4*, which is 72% identical and 82% similar to the human *TRPV4* (Mangos et al.,

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2007). Whole mount in situ hybridization in zebrafish embryos revealed expression of *trpv4* in sensory organs such as the neuromasts of LLS and the olfactory pit (Mangos et al., 2007). Whether the expression of *TRPV4* in zebrafish remains unchanged or it is different in adult life has not been investigated. This is a potentially important area of research because mutations in different TRP channels have been linked to a variety of diseases (for a review see Nilius et al., 2007) and zebrafish is a common model used to investigate several human diseases involving sensory organ physiology like vision (Goldsmith and Harris, 2003), hearing and balance (Ghysen and Dambly-Chaudière, 2007; Nicolson, 2005; Whitfield, 2002), or olfaction (Orlando, 2001). In this manuscript, we addressed this issue by investigating the expression of *TRPV4* and its localization in sensory organs of adult zebrafish.

MATERIALS AND METHODS

Animals and Treatment of the Tissues

Twenty adult zebrafish (*Danio rerio*) 5 months old were used in this study. Animals were bred under standard conditions, and obtained from CISS (Center of Experimental Ichthyopathology of Sicily), University of Messina, Italy. They were anesthetized with MS222 (Tricaine methanesulfonate; 0.4 g/L) and sacrificed by decapitation. The heads of 10 animals were placed in Bouin's fixative for 24 h, then processed for paraffin embedding, and used for immunohistochemical analysis. The heads from other five animals were used for PCR, and the remaining five heads were used for Westernblot.

RNA Isolation and Reverse Transcriptase PCR

Total RNAs were extracted from fresh heads using Trizol reagent (Sigma; St. Louis, MO). The integrity of RNA was checked using agarose gel electrophoresis. RNA extracted was reverse-transcribed in a final volume of 20 l L using 20 U of Superscript RNA-ase H2 Reverse Transcriptase (Gibco BRL, Gaithersburg, MD) in the manufacturer's buffer containing 2 l g RNA, 5 l M oligo (dT), 12–18 mM dNTPS, 40 U RNA-ase inhibitor (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK), 0.1 l g/lL BSA and 10 mM DTT. The reaction took place at 42°C for 90 min. The sequences of the oligonucleotide primers were based upon the published sequences for *Danio rerio TRPV4* (GenBank accession number NM_001042730) and *Danio rerio* β-actin (GenBank accession number NM 131031), and were for *TRPV4* forward: 5′-CAGAGATG AGGGTGGATACT-3′, reverse: 5′-CGGGTAGAGTTTG GCACATT-3′; and for β-actin forward: 5′-CACAGATC ATGTTCGAGACC-3′, reverse: 5′-GGTCAGGATCTTCA TCAGGT-3′.

The conditions of amplification were as follows: 2 U Taq DNA Polymerase (Promega, Madison, WI), 1 l M primers, 10 ng zebrafish brain cDNA, 0.2 mM each dNTP in 15 l LTaq DNA Polymerase buffer. The reaction was performed in a thermal cycler (Hyband Th. Cycler) with the following program: 1 min 94°C initial denaturation, then 10 cycles of 94°C 1 min, 65°C 30 s, and 72°C 45 s, followed by 20 cycles of 94°C 1 min, 61°C 30 s, 72°C 45 s and a 5-min final extension at 72°C. The PCR products were visualized by ethidium bromide staining under UV light following electrophoresis on a 2% agarose gel with a marker Ready Load 1 kb plus DNA ladder (Invitrogen, Carlsband, CA).

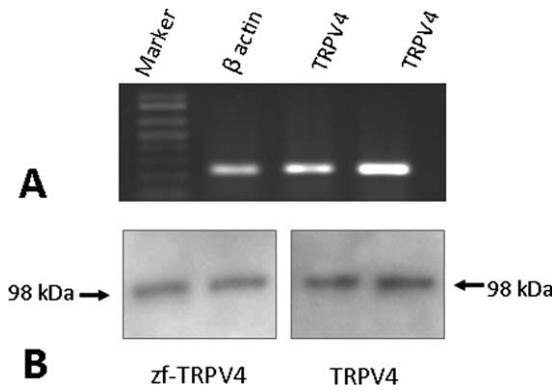

Fig. 1. **A**: RT-PCR analysis of *TRPV4* and β-actin mRNA expression in the head of adult zebrafish. A 250 b.p. fragment of *TRPV4* mRNA was detected. The integrity of the samples was demonstrated by the presence of a 220 b.p. fragment of β-actin mRNA. **B**: Westernblot detection of *TRPV4* in head homogenates from adult zebrafish. The anti-*TRPV4* antibodies used recognize a protein band of about 98 kDa consistent with the complete *TRPV4*.

Westernblot

To characterize the antibodies used in the study, we used Western blots of lysates prepared from head homogenates following procedures previously described (Germanà et al., 2010). Briefly, the samples were pooled and homogenized (1:2, wt/vol) in Tris-HCl buffered saline (TBS, 0.1 M, pH 7.5) containing 1 μM leupeptin, 10 μM pepstatin, and 2 mM phenylmethylsulfonyl fluoride. The homogenates were centrifuged at 25,000 rpm for 15 min at 4°C, and the resulting pellet dissolved in Tris-HCl 10 mM pH 6.8, 2% SDS, 100 mM dithiothreitol, and 10% glycerol at 4°C. The lysates were analyzed by electrophoresis in 15% discontinuous polyacrylamide SDS gels. After electrophoresis, proteins were transferred to a nitrocellulose membrane and antibody nonspecific binding was blocked by immersion for 3 h in PBS containing 5% dry milk, and 0.1% Tween-20. The membranes were then incubated at 4°C for 2 h with two different antibodies against *TRPV4*. One was a rabbit-anti zebrafish polyclonal antibody (Abcam, Cambridge, UK, catalogue number ab69094; used diluted 1:500) against a synthetic peptide as a part of zebrafish *TRPV4* conjugated to an immunogenic carrier protein (it is referred throughout the manuscript as zf*TRPV4*). The other antibody used was a rabbit polyclonal antibody (Abcam, catalogue number ab63003, used diluted 1:500) against a synthetic peptide derived from the cytoplasmic n-terminus conserved in mouse, human, and rat *TRPV4* conjugated to immunogenic carrier protein (it is referred throughout the manuscript as *TRPV4*). After incubation, the membranes were washed with TBS pH 7.6 containing 20% Tween-20, and incubated again for 1 h with the goat antirabbit IgG (diluted 1:100) at room temperature. Membranes were washed again and incubated with the PAP complex diluted 1:100 for 1 h at room temperature. Finally, the reaction was developed using a chemiluminescent reagent (ECL, Amersham

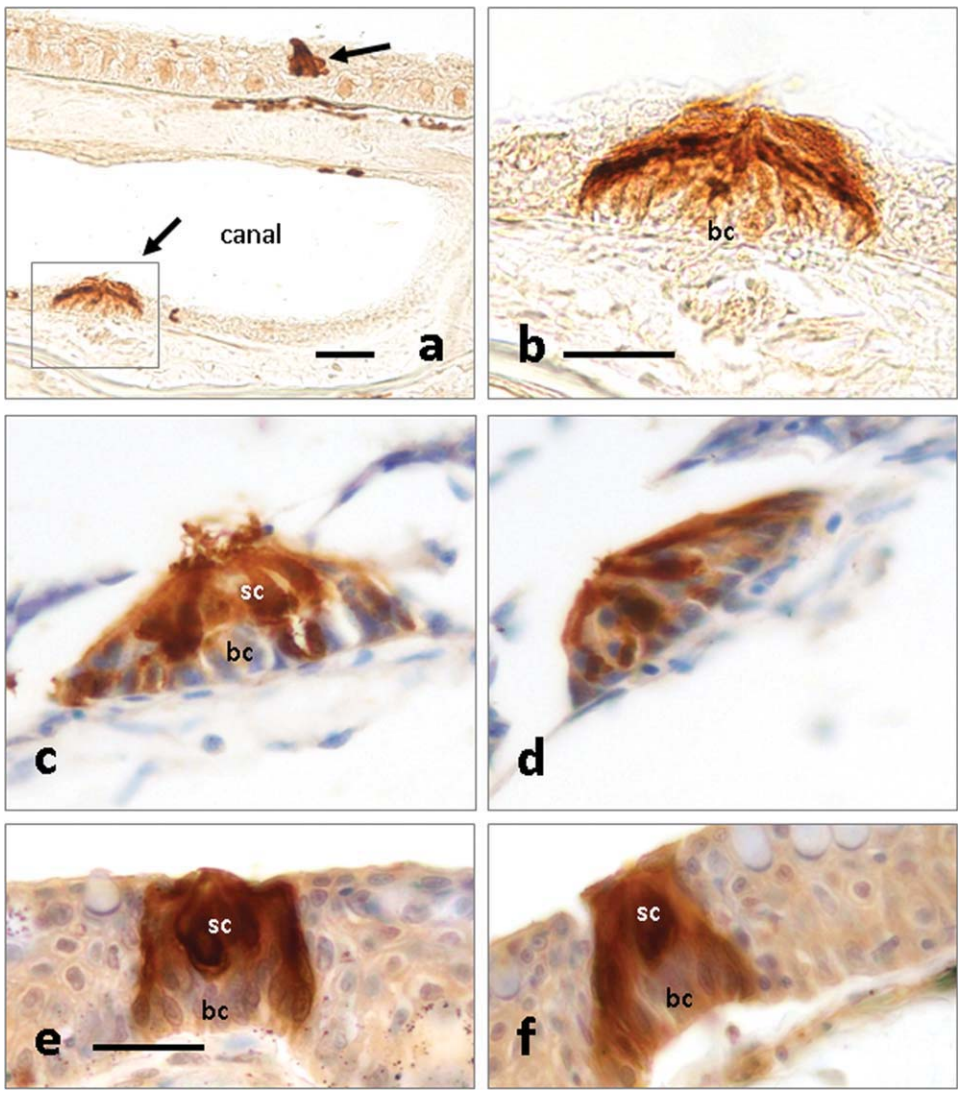

Fig. 2. Localization of *TRPV4* in neuromasts of the lateral line system of zebrafish using immunohistochemistry. TPRV4 was found in the canal (**a**–**d**) and superficial (**e**,**f**) neuromasts. In some neuromasts *TRPV4* expression was found in mantle cells (a), in addition to hair sensory cells expressed. Arrows in (a) indicate a canal neuromasts and a superficial taste bud. bc, basal cells; sc, sensory cells. Scale bar: 50 μm for a; 20 μm for b–d; 15 μm for e and f. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Pharmacia Biotech, Buckinghamshire, UK) and exposed to Hyperfilm. Marker proteins were visualized by staining with Brilliant Blue.

Immunohistochemistry

To investigate the distribution of *TRPV4* in the sensory organs of adult zebrafish 10 μm thick serial transversal, horizontal, and sagittal sections were obtained and collected on gelatin coated microscope slides. The sections were then processed for indirect peroxidase immunohistochemistry using the EnVision kit (Dako, Copenhagen, Denmark). Briefly, deparaffinized and rehydrated sections were heated in Envision FLEX target retrieval solution high pH (Dako) at 65°C for 20 min followed by incubation for 20 min at room temperature in the same solution. The sections were then rinsed in Tris-HCl buffer (0.05 M, pH 7.5) containing 0.1% bovine serum albumin and 0.2% Triton-X 100. The endogenous peroxidase activity and nonspecific binding were blocked (3% H_2O_2 and 25% fetal calf serum, respectively) and sections were incubated overnight with the primary antibodies anti-*TRPV4* and anti-zf*TRPV4* described above, both used diluted 1:200. The slides were then rinsed and incubated with the peroxidase-labeled, polymer-conjugated goat anti-rabbit for 30 min, and after two 5-min washes, the final reaction product was visualized with the DAB solution provided in the kit. Negative controls were carried out by exclusion of the primary antibody or by incubation with nonimmune rabbit serum. In both cases, immunolabeling was completely abolished.

RESULTS

Expression of *TRPV4* mRNA

Total RNA was retro-transcribed and, in order to assess its integrity the β-actin gene was amplified by

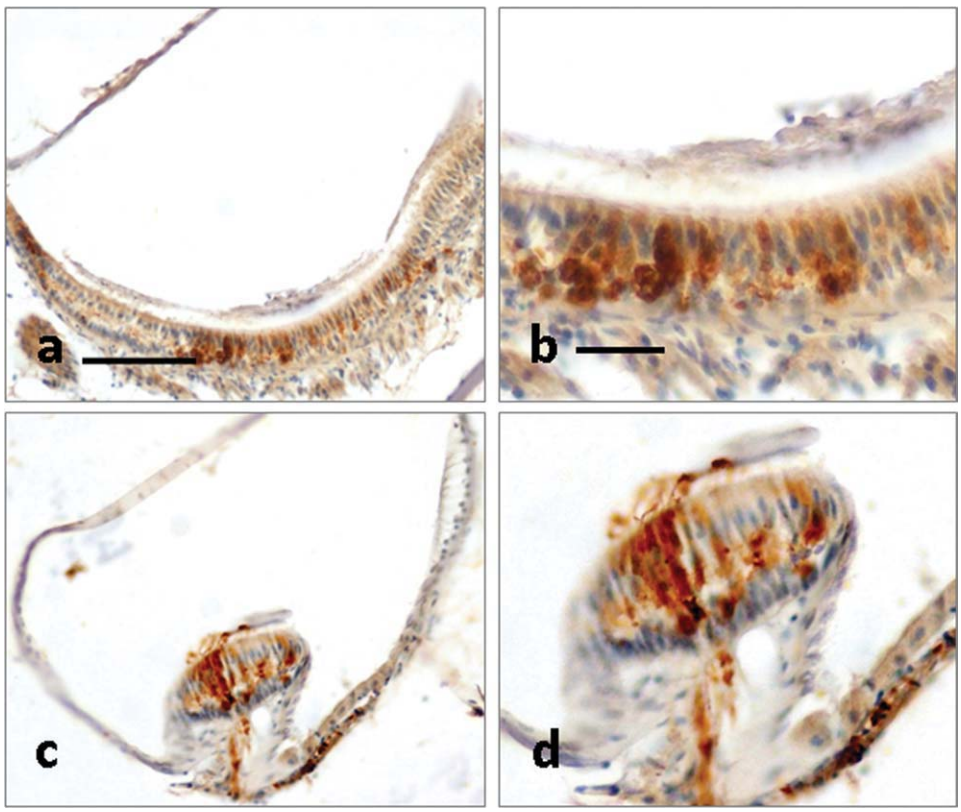

Fig. 3. Localization of *TRPV4* in the inner ear of zebrafish using immunohistochemistry. In the macula of the utriculus, sacculus, and lagena some hairy cells expressed *TRPV4* (**a** and **b**), and a subpopulation of hair sensory cells in the cristae ampullaris of the three semicircular canals displayed *TRPV4* (**c** and **d**). Scale bar: 20 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

PCR in the resulting cDNA. All experiments were performed in triplicate. The Figure 1A shows the analysis of zebrafish *TRPV4* mRNA and of zebrafish β-actin mRNA isolated from heads of adult animals. The size of the amplified fragments was of 250 b.p. and 220 b.p., respectively.

Western Blot

Western blot analysis was performed in homogenates of head of adult zebrafish. The two antibodies anti-*TRPV4* used recognized a single band of about 98 kDa (Fig. 1B). The molecular weight of the protein bands revealed by the anti-*TRPV4* antibodies was determined on the basis of their electrophoretic migration in relation to the molecular weight standards including myosin (212 cDNA), α-macroglobulin (170 kDa), β-galactosidase (116 kDa), and transferrin (76 kDa).

Distribution of *TRPV4* Immunoreactivity

The presence of *TRPV4* immunoreactivity was systematically studied in the cephalic sensory organs of adult zebrafish. The pattern of immunolabeling was identical with both the used antibodies, and the images illustrating the results were all them obtained with the anti zf*TRPV4* antibody.

Lateral Line System: Canal and Superficial Neuromasts

The neuromasts of the LLS located within the cephalic canals were found to have variable morphology and cell density. They are composed of sensory hair cells and nonsensory cells, the later including supporting cells, mantle cells, and basal cells. Neuromasts showed two patterns of distribution of TPRV4 immunoreactivity (Fig. 2): in some cases it was found primarily in mantle cells (Figs. 2a and 2b), whereas in others a subset of hair sensory cells displayed TPRV4 in addition to the mantle cells (Figs. 2c and 2d). The cutaneous pit organs also displayed *TRPV4* immunostaining in both mantle and hair cells (Fig. 2e and 2f). Since the adult neuromasts in zebrafish contain different types of hair cells, it is possible that the heterogeneity in the expression of TPRV4 protein could be related to restricted expression in a particular hair cell subtype.

Inner Ear

The sensory epithelium of the membranous sacs (utriculus, sacculus, and lagena) and of the semicircular canals of the inner ear, known as maculae and cristae ampullaris, respectively, share the same basic structure but there are differences in the microstructure of the hair sensory cells (Higgs et al., 2003). In the maculae some hair cells expressed *TRPV4* immunoreactivity (Figs. 3a and 3b), and a subpopulation of hair cells in the cristae ampullaris of the three semicircular canals also displayed *TRPV4* immunoreactivity (Figs. 3c and 3d). Given that these neuroepithelia contain more than one type of hair cells it seems that *TRPV4* immunoreactivity is only present in a subtype of those cells.

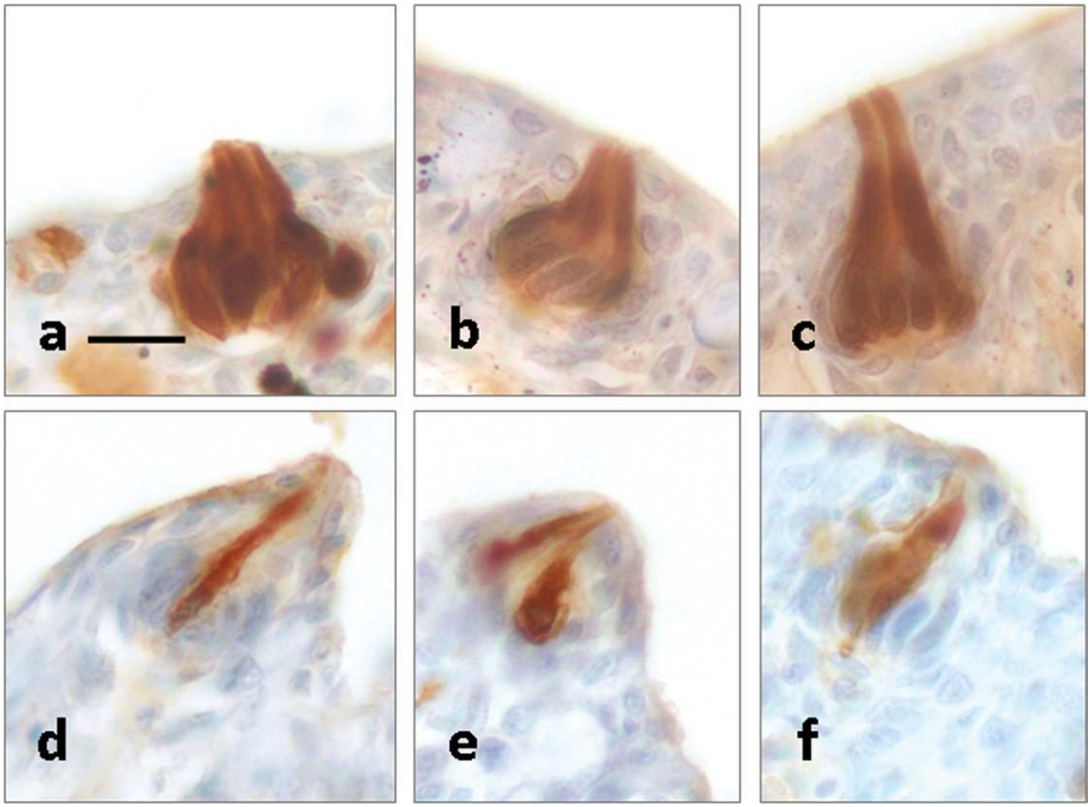

Fig. 4. Localization of *TRPV4* in cutaneous and oral taste buds of zebrafish using immunohistochemistry. *TRPV4* in skin taste buds (**a**–**c**) was expressed in elongated and vertically oriented cells identified as sensory cells. This pattern of expression was slightly different from the pattern observed in taste buds from the initial segments of the digestive tract (**d**–**f**), in which only a subpopulation of sensory cells expressed *TRPV4*. Scale bar: 15 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Taste Buds

The taste buds in zebrafish are intraepithelial sensory organs distributed in the lips, oral cavity, gills, pharyngeal tract, and through the skin surface. They consist of dark and light sensory cells, and basal cells. In the cutaneous taste buds (Figs. 4a–4c) TPRV4 was detected in all sensory cells. This pattern of expression was slightly different in the taste buds from oral or pharyngeal localization (Figs. 4d–4f), in which only a subpopulation of sensory cells displayed TPRV4 immunoreactivity.

Scattered in the skin, and also in the initial segments of the alimentary tract, isolated cells with globular morphology were found to show high intensity of *TRPV4* immunostaining (data not shown). Based on the anatomical distribution and morphology, they were identified as solitary chemosensory cells (Kotrschal et al., 1997).

Olfactory Epithelium

Positive TPRV4 cells were observed scattered in the lamellae of sensory epithelium in the olfactory rosette (Figs. 5a–5c). The cells showing TPRV4 immunoreactivity were identified as crypt neurons and ciliated sensory neurons. The crypt cells are spherical or pear-shaped, placed in the basal segments of the lamellae closely to the epithelial surface (Fig. 5e3). The ciliated sensory neurons have the soma in the basal layer of the sensory epithelium and have long dendrites and a few cilia (Fig. 5e2).

In addition to these sensory cells the nonsensory olfactory epithelium contained abundant *TRPV4* positive cells with different shape and size, which were not identified (Figs. 5d and 5e1).

Retina

The only structure in the eye that contain *TRPV4* positive cells was the retina. Specific immunoreactivity was detected in the soma and processes of cells localized within the internal nuclear layer (INL) and the ganglion cell layer (GCL; Figs. 6a and 6b). *TRPV4* was expressed mainly in the soma of these cells, although higher magnification revealed that some of the cellular processes in the inner plexiform layer (IPL) also expressed *TRPV4* (Fig. 6b). Based on their localization and morphology the retinal *TRPV4* positive cells were identified as a subtype of amacrine cells, but a neuronal nature cannot be ruled out.

In addition to the sensory organs, *TRPV4* immunostaining was detected in segments of craniofacial bones and in brain nuclei (data not shown).

DISCUSSION

TRPV4 channel is a member of the TRP vanilloid subfamily involved in mechanic and chemical sensation that also responds to warm temperatures and

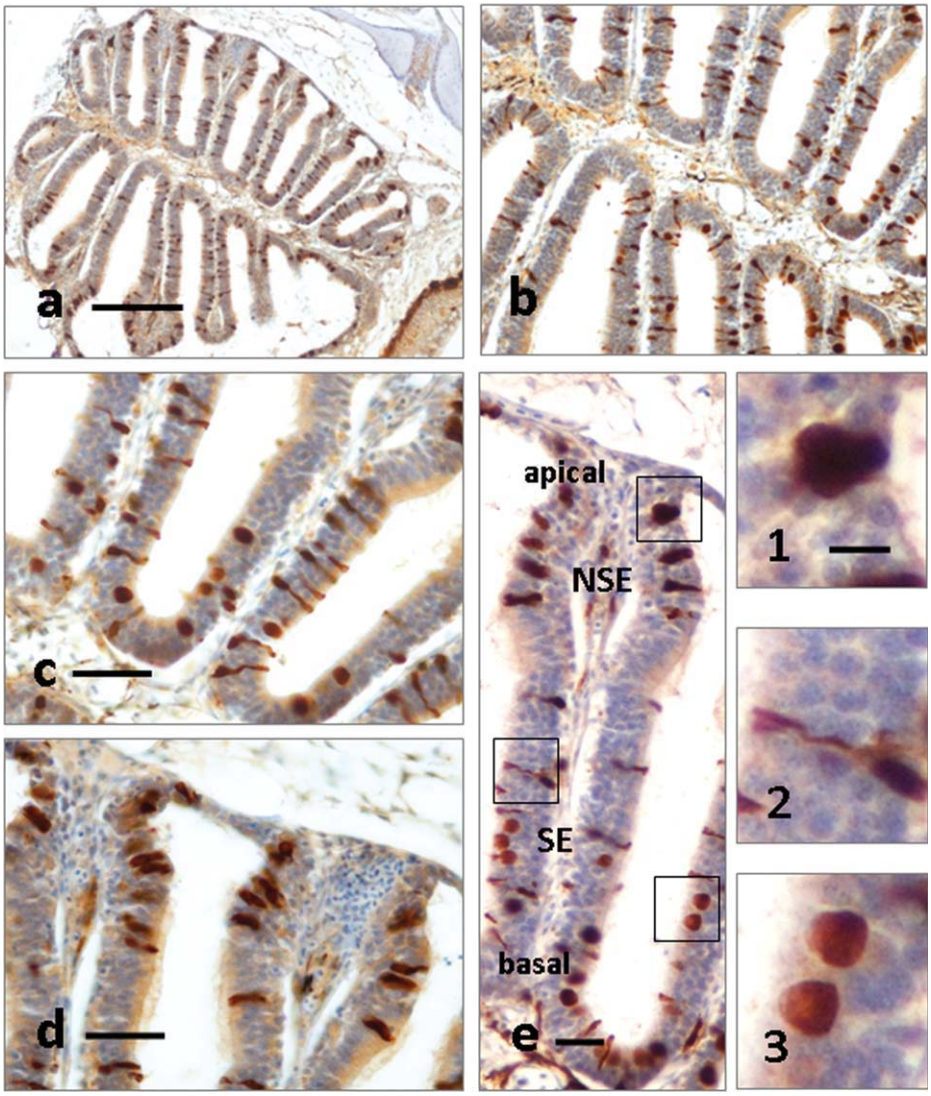

Fig. 5. Localization of *TRPV4* in olfactory epithelium of zebrafish using immunohistochemistry. In the olfactory rosette TPRV4 immunoreactivity was observed in cells of both the sensory (**a**–**c**) and non-sensory epithelium. At higher magnification, the cells that expressed TPRV4 were identified as crypt neurons and ciliated sensory neurons (**e**), but *TRPV4* positive cells unidentified were observed. e1–e3 are enlargement of *TRPV4* positive cells in e. standard error (SE), sensory epithelium; NSE, non sensory epithelium. Scale bar: 80 μm for a; 50 μm for b; 20 μm for c–e; 10 μm for e1–e3. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

acidic pH (see Plant and Strottmann, 2007). In this study, we investigated expression of *TRPV4* in sensory organs of the adult zebrafish, and we demonstrate that all then contain cells that express this TRP vanilloid ion channel.

Using PCR we have demonstrated that the adult zebrafish express specific *TRPV4* mRNA, as previously observed in zebrafish embryos (Mangos et al., 2007) thus suggesting that this ion channel is expressed during the entire lifespan of the animal. Moreover, we have observed by Westernblot that the antibodies used recognize a single protein with an estimated molecular weight of 94 kDa, which is in the range of that expected for *TRPV4* in vertebrates (Mangos et al., 2007; Plant and Strotmann, 2004).

Regarding the distribution of *TRPV4* in head sections of adult zebrafish we detected positive immunostaining in cells within all sensory organs, which nature was determined on the basis of previous structural studies in zebrafish: neuromasts (see Ledent, 2002; Webb and Shirey, 2003), the inner ear sensory epithelia (Bang et al., 2001; Higgs et al., 2003), the taste buds (Hansen et al., 2002), the olfactory epithelium (Hansen and Zeiske, 1998; Hansen and Zielinski, 2005), the retina (Bilotta and Saszik, 2001), and the solitary chemosensory cells (Kotrschal et al., 1997).

A subpopulation of hair cells of the neuromasts as well as of the sensory epithelia of the inner ear displayed *TRPV4* immunoreactivity. The expression of *TRPV4* in mantle cells of neuromasts in zebrafish embryos was previously reported by Mangos et al. (2007). We now confirm these results in adults and add new data demonstrating the presence of *TRPV4* in some hair sensory cells. However, the reason why the

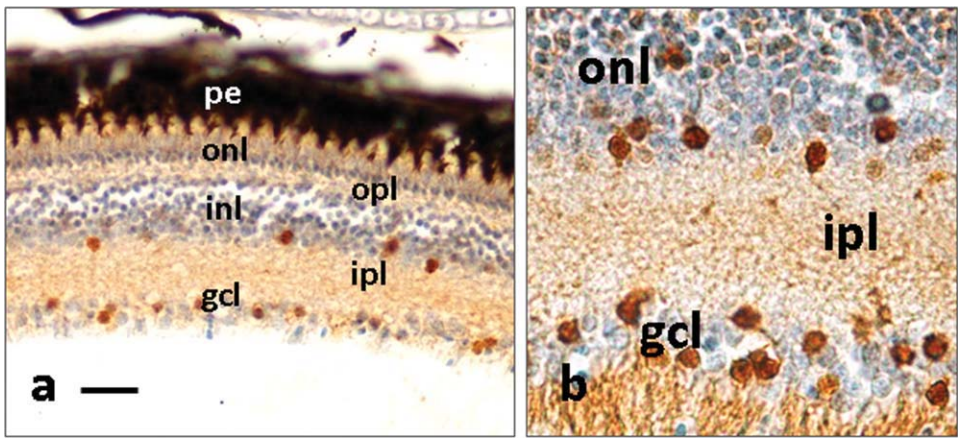

Fig. 6. Localization of *TRPV4* in the retina of zebrafish using immunohistochemistry. Specific immunoreactivity for *TRPV4* was detected in the soma and processes of amacrine cells within the internal nuclear layer and the ganglion cell layer (**a**). *TRPV4* was expressed mainly in the soma of these cells types, although high magnification revealed that some of the cellular processes in the inner plexiform layer (**b**). pe, pigmented epithelium; onl, outer nuclear layer; opl, outer plexiform layer; inl, inner nuclear layer; IPL, inner plexiform layer; gcl, ganglion cell layer. Scale bar: 25 μm for a; 10 μm for b. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

expression of *TRPV4* differs among superficial and canal neuromasts, and it is so heterogeneous in hair cells of the inner ear is unknown, but this phenomenon has been observed in these cells for other proteins like S100 protein (Germanà et al., 2007). The presence of *TRPV4* in sensory cells of the neuromasts and of the inner ear claims for an involvement of *TRPV4* in mechanotransduction as suggested before (Damann et al., 2008; Orr et al., 2006; Plant and Strotmann, 2007; Tsunozaki and Bautista, 2009). The occurrence of *TRPV4* in these cells is of particular importance since recent experiments suggest that TRP channels potentially play significant roles in the physiology of the inner ear and in human hearing (Cuajungco et al., 2007). However, although *TRPV4* mRNA expression has been detected in the cochlear and vestibular ganglia of the rat (Kitahara et al., 2005), we failed to detect *TRPV4* in these structures in zebrafish.

The taste buds and the olfactory system detect chemical changes in the environment. Although there are considerable variations in the organization of the peripheral olfactory organs among different species of fishes, the structure the olfactory sensory epithelium is extremely consistent (Hansen and Zielinski, 2002). However, the taste buds are also similar from a structural point of view among teleostean (see for a review Hansen et al., 2002; Hara, 1994; Kotrschal, 2000). The occurrence of *TRPV4* in the olfactory epithelium of zebrafish was observed during the embryonic period (Mangos et al., 2007) but the cells expressing *TRPV4* were not identified. In our study, *TRPV4* immunoreactivity was observed in the crypt and ciliated olfactory neurons, as well as in unknown cells placed in the non-sensory olfactory epithelium. All together, these results strongly suggest that *TRPV4* can be involved in olfactory sensation. Recently, Ahmed et al. (2009) and Nakashimo et al. (2010) detected TRPV1, TRPV2, TRPV3, and *TRPV4* immunoreactivity in the olfactory epithelium of mice, and they suggest that TRPV channels may contribute to olfactory chemosensation. Furthermore, we have observed for the first time in the present study that *TRPV4* is present in all sensory cells forming the cutaneous taste buds and in a subset of sensory cells in the oropharyngeal ones. Despite the similarities, taste buds contain different types of sensory cells (Hansen et al., 2002), depending on the location. This may explain why the pattern of expression of TPRV4 is variable in different locations, as it occurs for S100 protein or calretinin (Germanà et al., 2007). Therefore, the occurrence of *TRPV4* in the olfactory epithelium as well as in taste buds argues for a chemosensor role of *TRPV4*.

We also detected expression of *TRPV4* in retinal cells morphologically identified as amacrine cells, which somata were placed in the INL and GCL layers. Present results added new data to the occurrence of TRPV channels in the vertebrate retina (Leonelli et al., 2009). It has been suggested that the retinal TRPV channels have specific roles in visual processing, although it remain to be established. Thus, in addition to the mechanosensor and chemosensor functions suspected for *TRPV4*, a role in the visual system, at least in the adult zebrafish, can be hypothesized.

However, from the pioneer researches *TRPV4* was related to signaling transduction in response to osmotic stimuli (see for review Liedtke, 2007a,b,c). We have not investigated this functional aspect of *TRPV4*, and there are no direct evidences that *TRPV4* controls any osmotic function in sensory cells. Nevertheless, an association between *TRPV4* and the water channel aquaporin 5 has been suggested (Liu et al., 2006), and we have recently found expression of aquaporin 4 (Zichichi et al., 2011) in the same sensory cells of adult zebrafish that express *TRPV4*. The possible relationship between these molecules, if any, remains to be clarified.

In summary, we have detected expression of *TRPV4* in specialized sensory cells of all sensory organs of adult zebrafish what is consistent with the multiple physiological functions that are suspected from different studies and that include the regulation of body osmolality, mechanosensation, temperature sensing, vascular regulation, or hearing. Nevertheless, although this results suggest specific sensory roles of *TRPV4*

because its localization in sensory cells, this not necessarily indicates whether it is functionally relevant in a given signaling cascade, and if even which position it assumes. More studies are necessary to elucidate these topics.

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Acid-sensing ion channels (ASICs) in the taste buds of adult zebrafish

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HIGHLIGHTS

- Acid-sensing ion channels (ASICs) are involved in acid sensing, and probably also in mechanotransduction, in mammals.
- ASICs were examined by Western blot and immunohistochemistry in the adult zebrafish taste buds.
- ASIC2 and ASIC4 immunoreactivities were detected in taste sensory cells, and ASIC2 in nerves supplying them.
- ASIC1 and ASIC3 were absent from taste buds.
- ASIC2 and ASIC4 could play a role in taste in adult zebrafish.

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ABSTRACT

In detecting chemical properties of food, different molecules and ion channels are involved including members of the acid-sensing ion channels (ASICs) family. Consistently ASICs are present in sensory cells of taste buds of mammals. In the present study the presence of ASICs (ASIC1, ASIC2, ASIC3 and ASIC4) was investigated in the taste buds of adult zebrafish (zASICs) using Western blot and immunohistochemistry. zASIC1 and zASIC3 were regularly absent from taste buds, whereas faint zASIC2 and robust zASIC4 immunoreactivities were detected in sensory cells. Moreover, zASIC2 also immunolabelled nerves supplying taste buds. The present results demonstrate for the first time the presence of zASICs in taste buds of teleosts, with different patterns to that occurring in mammals, probably due to the function of taste buds in aquatic environment and feeding. Nevertheless, the role of zASICs in taste remains to be demonstrated.

1. Introduction

The taste in fishes is a major chemosensory system not only devoted to the evaluation of food composition but also to the detection of variations in the environmental chemical composition [5,31]. The morpho-functional sites for taste are specialized organs called taste buds, which are distributed not only in the external skin surface of the head, lips, and barbells but also in the intra-oral cavity, including anterior branchial apparatus [4,5].

In detecting chemical properties a great number of ion channels participate in the mammalian taste cells. They include 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB)-sensitive chloride channels, hyperpolarization-activated channels HCN1 and HCN4, potassium (K2P) channels, leakage-type K^+ channels, the transient receptor potential family members PKD1L3 and PKD2L1, and acid-sensing ionic channels (ASICs) [8,9,14,18,22,23,25–27; see also [17]].

ASICs are H^+-gated, voltage-insensitive cation channels belonging to the amiloride-sensitive degenerin/epithelial Na^+ channel (DEG/ENaC) superfamily. Structurally, ASICs consist of two transmembrane domains and a large extracellular loop [6,7,24,30]. Functionally, ASICs not only monitor deviations from the physiological values of extracellular pH, but also participate in mechanoception and nociception [6,7,24].

In mammals, six ASICs (ASIC1a, ASIC1b, ASIC2a, ASIC2b, ASIC3, and ASIC4) are encoded by four different genes (*asic1*, *asic2*, *asi3*, and *asic4*) and are widely expressed in both the central and peripheral nervous systems (for Refs. [6,15]). Similarly as in mammals, six ASICs have been identified in zebrafish (*z*ASICs; ZASIC1.1, zASIC1.2, zASIC1.3, zASIC2, zASIC4.1, and zASIC4.2), but codified by six different genes. The proteins codified by *zasics* have similar predicted molecular masses (around 60 kDa) and share 60–75% of amino acid identity with rat and human ASICs [21].

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Because of the importance of acid sensing in taste it can be expected that taste cells express ASICs. In taste buds it is thought that extracellular H^+ activate ASICs resulting in an influx of extracellular Ca^{2+} which in turn is presumed to lead to neurotransmitter release onto the synapses of gustatory sensory afferent fibres (see for a review [25]). In agreement with this view, the presence of ASIC1, ASIC2 and ASIC3, but not ASIC4, in the taste buds of mammals has been documented [23,28,29,25]. Conversely, no data exist about the occurrence of ASICs in taste buds of fishes in spite of the essential role of taste in the fish biology. Therefore, we decide to investigate the distribution of ASICs in taste buds of adult zebrafish using Western blot and immunohistochemistry. The main goal of the research is to contribute to the molecular mechanisms of taste in fishes.

2. Materials and methods

Adult zebrafish (*Danio rerio*; $n = 15$), 6 months old, were obtained from CISS (Centro Ittiopatologia Sperimentale Sicilia), University of Messina, Italy. The specimens were anaesthetized with MS222 (ethyl-m-amino benzoate; 0.4 g L^{-1}) and sacrificed by decapitation. The heads of 5 animals were isolated, cleaned in cold saline solution, and processed for Western blot. The heads of the remaining 10 animals were fixed in Bouin's fixative for 24 h and then routinely processed for paraffin embedding. The pieces were cut 10 μm thick in serial sections, and collected on gelatine-coated microscope slides.

For Western blot lysates prepared from whole head homogenates were processed following procedures previously described [3]. Briefly, the samples were pooled and homogenized (1:2, w/v) in Tris–HCl buffered saline (TBS, 0.1 M, pH 7.5) containing 1 μM leupeptin, 10 μM pepstatin and 2 mM phenylmethylsulfonyl fluoride. The homogenates were centrifuged at 25,000 rpm for 15 min at 4 °C and the resulting pellet dissolved in Tris–HCl 10 mM pH 6.8, 2% SDS, 100 mM dithiothreitol, and 10% glycerol at 4 °C. The lysates were analyzed by electrophoresis in 12% discontinuous polyacrylamide SDS gels. After electrophoresis, proteins were transferred to a nitrocellulose membrane and antibody non-specific binding was blocked by immersion for 3 h in PBS containing 5% dry milk, and 0.1% Tween-20. The membranes were then incubated at 4 °C for 2 h with a rabbit polyclonal antibody against ASICs (see Table 1). After incubation, the membranes were washed with TBS pH 7.6 containing 20% Tween-20, and incubated again for 1 h with the goat anti-rabbit IgG (diluted 1:100) at room temperature. Membranes were washed again and incubated with the PAP complex diluted 1:100 for 1 h at room temperature. Finally, the reaction was developed using a chemiluminescent reagent (ECL, Amersham Pharmacia Biotech, Buckinghamshire, UK) and exposed to Hyperfilm. Marker proteins were visualized by staining with Brilliant Blue.

To investigate the distribution of ASICs deparaffinised and rehydrated sections were processed for indirect peroxidase immunohistochemistry as follows: deparaffinised and rehydrated sections were rinsed in Tris–HCl buffer (0.05 M, pH 7.5) containing 0.1% bovine serum albumin and 0.2% Triton-X 100. The endogenous peroxidase activity and nonspecific binding were blocked (3% H_2O_2 and 25% foetal calf serum, respectively) and sections were incubated overnight at 4 °C with the primary antibodies included in Table 1. Then, sections were rinsed in the same buffer, and incubated with goat anti-rabbit IgG HRP-conjugated (Amersham, UK) diluted 1:100 for 1 h at room temperature. Finally, sections were washed, the immunoreaction was visualized using 3-3′DAB as a chromogen, and the sections were slightly counterstained with Harris haematoxylin to ascertain structural details. The specificity of the immunoreactivity developed was tested substituting the primary antibody by a non-immune serum, omitting the primary antibody. Some of these antibodies have been proved in zebrafish for both Western blot and immunohistochemistry in paraffin-embedded tissues [12].

Table 1
Primary antibodies used in the study.

Antigen	Origin	Dilution	Supplier
ASIC1	Rabbit	1:200	Abcam pcl[1]
ASIC2	Rabbit	1:200	Lifespan Biosciences[2]
ASIC3	Rabbit	1:200	Abcam pcl[1]
ASIC4	Rabbit	1:200	Lifespan Biosciences[2]
Calretinin	Mouse	1:100	Swant[3]
β-Tubulin	Mouse	1:100	Sigma[4]

ASIC: acid-sensing ion channels 1, 2, 3 and 4.
[1]Cambridge, UK.
[2]Seattle, WA, USA.
[3]Switzerland.
[4]SMissouri, USA.
Anti-ASIC1: raised against a synthetic peptide to an extracellular domain epitope of human ASIC1, conjugated to immunogenic carrier protein; recognizes both subtype1 and subtype2. ab65698.
Anti-ASIC2: antibody raised against a synthetic peptide from the extracellular domain of mouse ASIC2 conjugated to an immunogenic carrier protein. LS-B156/12883.
Anti-ASIC3: antibody raised against a synthetic peptide to an internal sequence of human ASIC3 conjugated to immunogenic carrier protein. Ab65697.
Anti-ASIC4: antibody raised against a synthetic peptide from the cytoplasmic domain of human ASIC4 conjugated to immunogenic carrier protein used as an immunogen. LS-B920.
Anti-calretinin: antibody against and epitope within the first 4 EF-hands domains common to both calretinin and calretinin-22 K. 6B3.
Anti-β-tubulin: antibody recognizes an epitope in the carboxy-terminal part of all five isoforms of beta-tubulin (between amino acids 281–446). Clone tub 2.1.

To ascertain the true nature of structures displaying ASIC immunoreactivity in taste buds, sections were processed for simultaneous detection of ASIC2 and β-tubulin (used to immunolabel nerves [1,11], or ASIC4 proteins and calretinin (used to immunolabel sensory cells in taste buds [2]) [19]. Double immunostaining was performed on 10 μm thick deparaffinized and rehydrated sections. Non-specific binding was reduced by incubation for 30 min with a solution of 1% bovine serum albumin in tris buffer solution (TBS). The sections were then incubated overnight (10 μm thick sections), at 4 °C in a humid chamber with a 1:1 mixture of anti-ASIC2 antibody (diluted 1:100) and anti-β-tubulin antibody (clone TUB 2.1, Sigma, St Louis, MS, USA, diluted 1:300 in the blocking solution), or anti-ASIC4 antibody (diluted 1:100) and anti-calretinin antibody (clone 6B3, Swant, Marly, Switzerland, diluted 1:100 in the blocking solution). After rinsing with TBS, the sections were incubated for 1 h with Alexa fluor 488-conjugated goat anti-rabbit IgG (Serotec, Oxford, UK), diluted 1:1000 in TBS containing 5% mouse serum (Serotec), then rinsed again and incubated for another hour with Cy™3-conjugated donkey anti-mouse antibody (Jackson-ImmunoResearch, Baltimore, MD, USA) diluted 1:50 in TBS. Both steps were performed at room temperature in a dark humid chamber. Sections were then washed, dehydrated and mounted with Entellan®. Double staining was detected using a Leica DMR-XA automatic fluorescence microscope (Servicio de Análisis de Imágenes, Universidad de Oviedo) coupled with a Leica Confocal Software, version 2.5 (Leica Microsystems, Heidelberg GmbH, Germany) and the images captured were processed using the software Image J version 1.43 g Master Biophotonics Facility, Mac Master University Ontario (www.macbiophotonics.ca; see also the legend of supplementary material).

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.neulet.2013.01.006.

For control purposes representative sections were processed in the same way as described above using non-immune rabbit sera instead of the primary antibodies, or omitting the primaries antibodies in the incubation. Moreover, additional experiments

using pre-absorbed antibodies (10 μg of the blocking peptide in 1 ml of the antibody working solution) were carried out for ASIC2 and ASIC4; the blocking peptides were provided by LifeSpan BioSciences (Cambridge, UK). Under these conditions no positive immunostaining was observed (data not shown).

3. Results

In head lysates the only antibodies offering positive results in Western blot experiments were those against ASIC1, ASIC2 and ASIC4, whereas the antibody against ASIC1 and ASIC3 did not detect any protein. The anti-ASIC1 antibody detected a protein band with an estimated molecular weight of about 60 kDa, the anti-ASIC2 antibody detected a single and robust protein band with an estimated molecular weight of about 64 kDa, whereas the antibody against ASIC4 identified a faint and single band with an estimated molecular weight of 64 kDa (Fig. 1a). The anti-ASIC3 antibody did not detect any protein by Western blot (data not shown). The molecular weight of the protein bands was determined on the basis of their electrophoretic migration in relation to the molecular weight

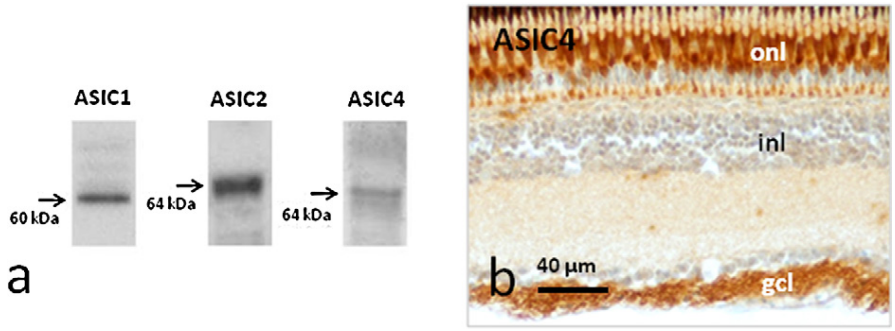

Fig. 1. (a) Western blot detection of ASIC1, ASIC2 and ASIC4 (right) in head homogenates of adult *Danio rerio*. The anti-ASIC1, anti-ASIC2 and anti-ASIC4 antibodies used recognize protein bands of ~60 kDa, ~64 kDa, and ~64 kDa, respectively. Immunohistochemical analyses with the same antibodies showed positive ASIC1 immunoreactivity in the lateral line ganglia (b), widespread immunoreactivity for ASIC2 in the brain (c), and ASIC4 immunoreactivity in the photoreceptors and ganglion cells layer of the retina (d).

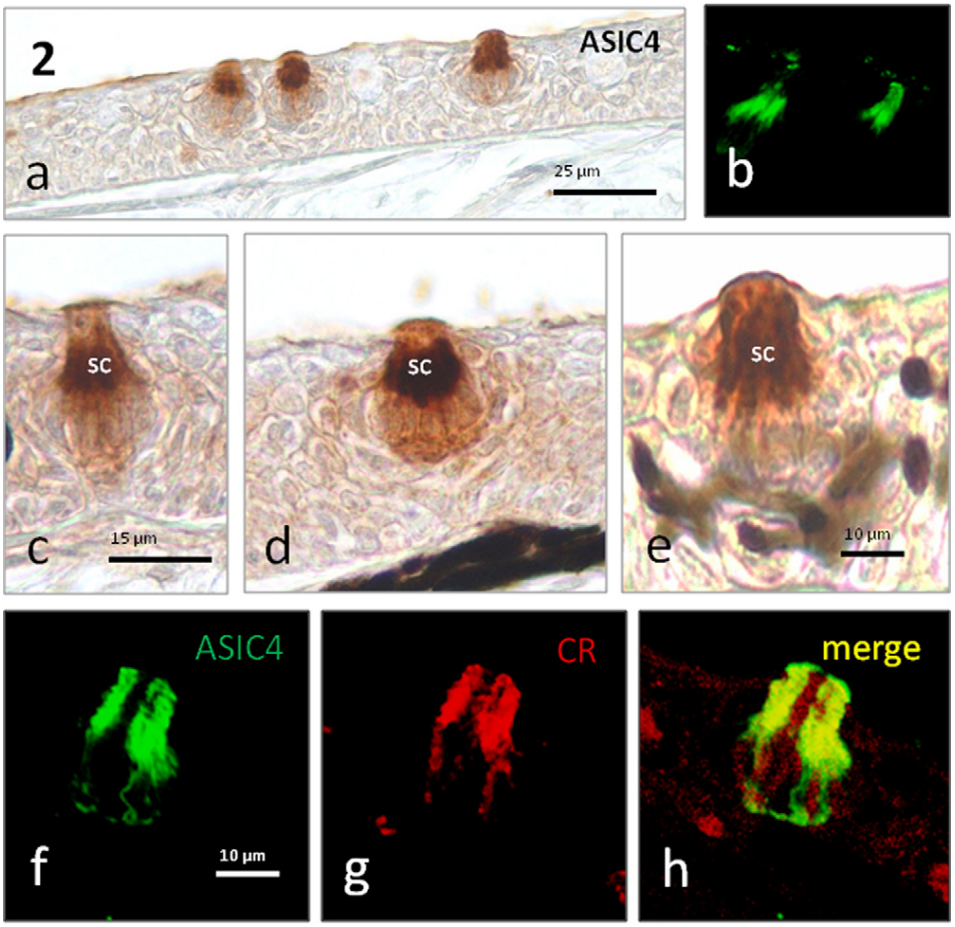

Fig. 2. Immunohistochemical localization of ASIC4 in cutaneous (a, c, d, e) and oral (b, f, g, h) taste buds of adult zebrafish. In taste buds immunolabelled with anti-ASIC4 antibody/Alexa fluor 488-conjugated goat anti-rabbit IgG (green fluorescence), and with anti-calretinin antibody/Cy™3-conjugated donkey anti-mouse antibody (red fluorescence) a subpopulation of sensory cells are labelled. When the images were overlapped there was a partial co-localization of ASIC4 and calretinin in the same cells. SC: sensory cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

standards including myosin (212 kDa), α-macroglobulin (170 kDa), β-galactosidase (116 kDa) and transferrin (76 kDa), and the molecular masses of the proteins detected were consistent with the predicted molecular masses of ASIC proteins in zebrafish [21].

The positive histological controls we have analyzed in the study (the brain, the lateral line ganglia, and the retina) displayed positive immunostaining for ASIC1 in the lateral line ganglia (data not shown), for ASIC2 in the brain and the ganglion cell layer of the retina (data not shown), and for ASIC4 in the photoreceptive cells and ganglion cell layer of the retina (Fig. 1b). These positive controls were selected on the basis of the pattern of gene expression reported by Paukert et al. [21].

In the study of adult zebrafish taste buds, first of all we investigated their structure in both the oral cavity and skin surface. Structurally the taste buds of adult zebrafish, independently of their anatomical localization, consist of onion-shaped clusters of sensory cells, supporting cells and basal cells (Fig. 1 supplementary material). Sensory cells are elongated and two types can be distinguished on the basis of their electron density characteristics: clear cells and dark cells, whose apical poles reach the free surface, whereas the basal poles are profusely innervated (Fig. 1 supplementary material).

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.neulet.2013.01.006.

In total agreement with the results obtained by Western blot, ASIC3 positive immunostaining was never detected in head sections of adult zebrafish heads including taste buds or sensory structures related to them (data not shown). Moreover, specific ASIC1 immunoreactivity was always absent in taste buds (data not shown). Conversely ASIC2 and ASIC4 were always detected in taste buds or in the nerves supplying them. Strong ASIC4 immunoreactivity was observed in all taste buds, independently of their localization in the oral cavity or the body surface (Fig. 2). The pattern of immunostaining was cytoplasmic and showed polarization, the immunoreactivity being localized in the superficial-apical segment of the cells (Fig. 2a–e). In most taste buds ASIC4 immunoreactivity

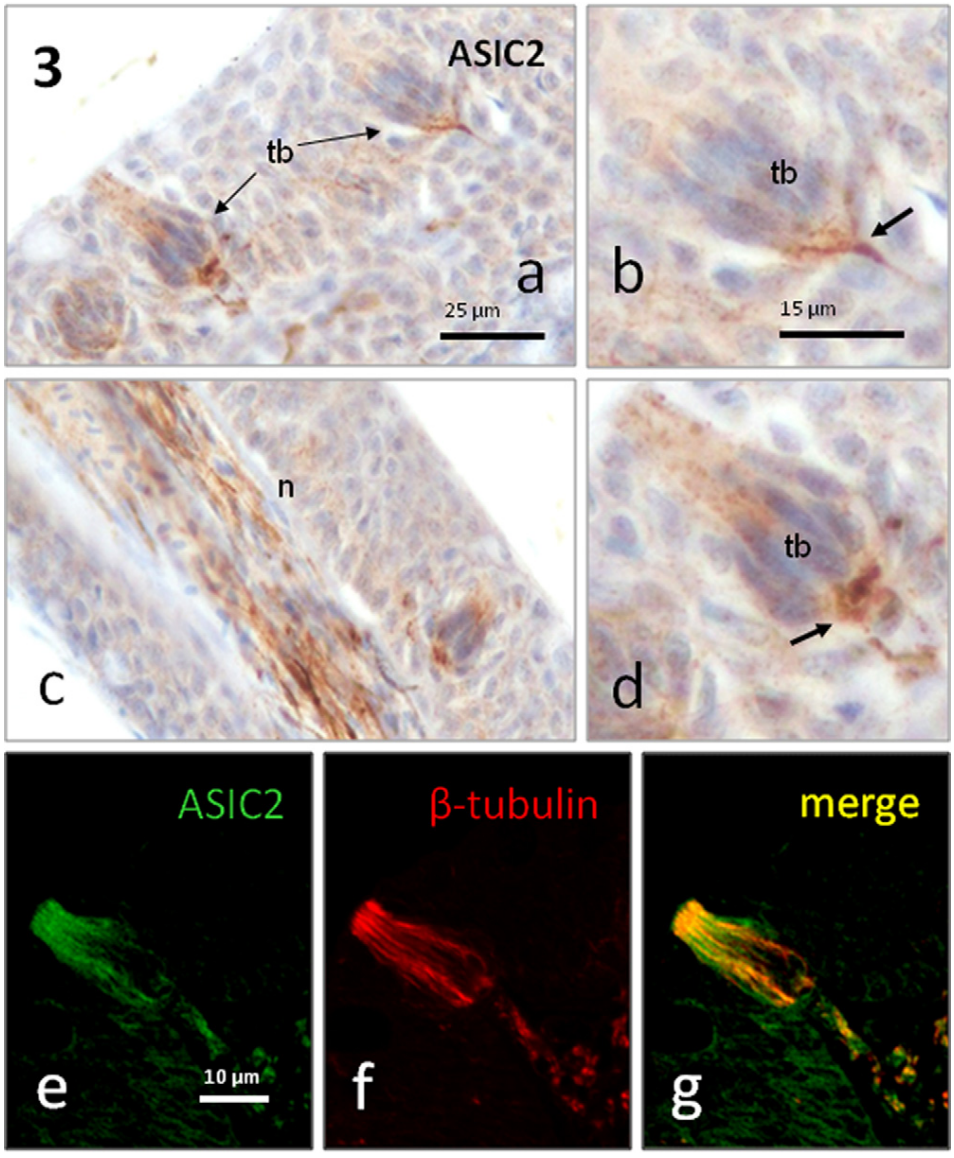

Fig. 3. Immunohistochemical localization of ASIC2 in cutaneous (a–d) and oral (e–g) taste buds (tb) of adult zebrafish. Faint immunoreactivity for ASIC2 was detected distributed not only in the cytoplasm of sensory cells, but also in nerve bundle profiles below the epidermis. The nerves supplying taste buds were also ASIC2 immunoreactive (arrows in b and d). In taste buds immnolabelled with anti-ASIC2 antibody/Alexa fluor 488-conjugated goat anti-rabbit IgG (green fluorescence), and with anti-β tubulin antibody/Cy™3-conjugated donkey anti-mouse antibody (red fluorescence) a subpopulation of sensory cells are labelled. When the images were overlapped there was a partial co-localization of ASIC2 and β tubulin in the same cells. n: nerves, tb: taste buds.

immunolabelled all cells, but in some cases ASIC4 was restricted to a subtype of taste cells which also expressed calretinin (Fig. 2f–h). On the basis of their morphology alone the correspondence of ASIC4+/calretinina+ taste cells with clear or dark sensory cells, cannot be established.

Regarding ASIC2, it was detected in both nerves and taste cells. Behind the epidermis nerve trunk profiles displaying ASIC2 immunoreactivity were regularly observed. They emitted thick ASIC2 positive branches which contacted the basal pole of the taste buds (Fig. 3a–d). Moreover a faint but specific granular ASIC2 immunoreactivity was observed in the external segments of the sensory cells (Fig. 3b–d). The nerve profiles were also immunolabelled with the antibody against β-tubulin, and both ASIC2 and β-tubulin were co-localized in nerves. Moreover, β-tubulin filled the cytoplasm of a subpopulation of sensory cells which occasionally also displayed ASIC2 immunoreactivity (Fig. 3e–g).

4. Discussion

The molecular mechanisms of taste in fishes are still poorly known, although in the last years they began to be understood [9,20]. Taste buds are the chemosensory organs where chemical stimuli are transduced into electrical signals carried to the central nervous system. The structure and distribution of taste buds is now well known in zebrafish. In agreement with Hansen et al. [4] we identified two main types of sensory taste cells, denominated clear and dark, in the taste buds of adult zebrafish but we were unable to identify the third type of sensory cells described by those authors. Research is in progress in our laboratory to identify structurally and immunohistochemically the sensory cell subtypes present in the taste buds of adult zebrafish.

The main goal of the present research was to investigate the occurrence of ASICs in taste buds of adult zebrafish. The occurrence of ASICs in the taste buds of mammals is now well documented, but the occurrence of these ion channels in fishes, in particular in the teleost zebrafish, is reported here for the first time. As of now six zASICs have been cloned in zebrafish, although the ortholog of the mammalian ASIC3 has not been found; zASIC have predicted molecular masses similar to those of mammals, and 60–75% of amino acid identity, and are well preserved during evolution [21]. Our results in Western blot are consistent with the predicted molecular masses for ASIC1, ASIC2 and ASIC4. Importantly, the isoforms of the proteins identified by Western blot remains elusive although it is possible that the antibodies detect all of them because the localization of the epitope is immunolabelled. As expected, in agreement with the absence of zASIC3 in zebrafish, the antibody against ASIC3 did not identify any protein neither by Western blot nor by immunohistochemistry. Thus, our results lend further support to the absence of ASIC3 in zebrafish.

In our hands, only ASIC2 and ASIC4 were detected in zebrafish taste buds, and the antibody against ASIC1 showed negative results although the protein was detected by Western blot and labelled cells in the brain and peripheral nervous system. In comparing these results with that previously reported in mammals notable differences exists. In fact, whereas ASIC4 was not detectable in the taste buds of rat and mouse [25] we have regularly detected it in zebrafish. In contrast, ASIC1 and ASIC3 were always absent in zebrafish and both have been found at the mRNA level in both rat and mouse [25]. Regarding ASIC2 the present results basically agree with those in rat taste buds but not with those in mouse taste buds [22,23,25,29]. Although more studies are required the environment and the feeding may account for these differences between mammals and fishes.

The presence of ASICs not only in taste buds but also some functional studies strongly argues for a role of these ion channels in taste. Nevertheless, their roles in taste are still not clear. So, although ASIC2 has been proposed as a mammalian sour (acid) taste receptor [13,14,16,27–29] behavioural responses to acid taste stimuli remained unaltered in mice lacking the ASIC2 gene [10,23] demonstrating that ASIC2 is not an acid taste receptor in mice, although acid transduction in rats is consistent with the properties of ASIC2 (for review [28]).

It is believed that protons act on ASICs and depolarize taste receptor cells resulting in an influx of extracellular Ca^{2+}, which, in turn, is presumed to lead to neurotransmitter release onto the synapses of gustatory sensory afferent fibres [18,22,26,27,29]. However functional experiments have demonstrated that zASIC1.1, zASIC1.2, zASIC1.3 or zASIC14.1 are acid sensible, but zASIC2 and zASIC4.2 cannot be activated by acid test solutions [21]. Therefore, the possible role of ASICs in taste buds of zebrafish is elusive. In any case since ASICs are present in taste cells and they depend on distinct calcium signals to generate appropriate cellular responses through different mechanisms [1] it can be hypothesized a role of these ion channels in taste cells in taste or in other unknown functions.

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ACID-SENSING ION CHANNELS (ASICs) IN TASTE BUDS OF ADULT ZEBRAFISH

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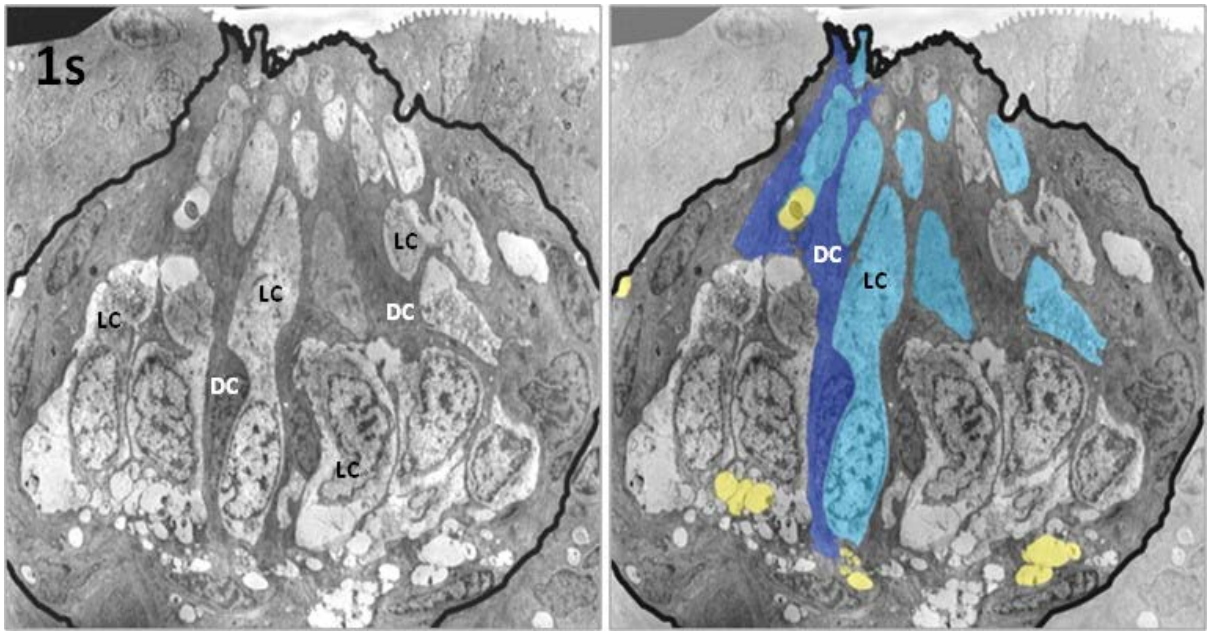
SUPPLEMENTARY MATERIAL

Figure 1s.- Transmission electron microphotographs of one cutaneous taste bud in adult zebrafish. **A**. Taste buds are onion-shaper chemosensory organs composed of elongate taste receptor cells classically divided into light (LC) and dark (DC), the former representing the primary gustatory cells. The nerves enter taste buds to form a nerve plexus. **B**. The two main types of sensory cells in taste buds are coloured in navy blue (dark cells) and clear (light cells), whereas some nerve profiles are coloured in yellow. Original magnification x2500.



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ORIGINAL PAPER

Acid-sensing ion channel 2 (ASIC2) is selectively localized in the cilia of the non-sensory olfactory epithelium of adult zebrafish

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Abstract Ionic channels play key roles in the sensory cells, such as transducing specific stimuli into electrical signals. The acid-sensing ion channel (ASIC) family is voltage-insensitive, amiloride-sensitive, proton-gated cation channels involved in several sensory functions. ASIC2, in particular, has a dual function as mechano- and chemo-sensor. In this study, we explored the possible role of zebrafish ASIC2 in olfaction. RT-PCR, Western blot, chromogenic in situ hybridization and immunohistochemistry, as well as ultrastructural analysis, were performed on the olfactory rosette of adult zebrafish. ASIC2 mRNA and protein were detected in homogenates of olfactory rosettes. Specific ASIC2 hybridization was observed in the luminal pole of the non-sensory epithelium, especially in the cilia basal bodies, and immunoreactivity for ASIC2 was restricted to the cilia of the non-sensory cells where it was co-localized with the cilia marker tubulin. ASIC2 expression was always absent in the olfactory cells. These findings demonstrate for the first time the expression of ASIC2 in the olfactory epithelium of adult zebrafish and suggest that it is not involved in olfaction. Since the cilium sense and transduce mechanical and chemical stimuli, ASIC2 expression in this location might be related to detection of aquatic environment pH variations or to detection of water movement through the nasal cavity.

Keywords Acid-sensing ion channel 2 · Olfactory epithelium · Cilium · Zebrafish

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Introduction

The peripheral olfactory system of vertebrates consists of bipolar olfactory receptor neurons (ORNs), localized in the olfactory epithelium, and conducts information to the brain via the first cranial nerve. In teleosts, the olfactory epithelium is arranged in several lamellae that converge into a midline raphe, forming an oval-shaped rosette; sensory and non-sensory regions are separated on each lamella. The sensory, i.e., olfactory, epithelium contains three types of ORNs named ciliated, microvillous and crypt neurons, which are distributed differently in different species (Hansen and Zielinski 2005). Very recently, a fourth type of ORN, kappe neurons, has been identified in the olfactory epithelium of zebrafish (Ahuja et al. 2014). ORNs can be distinguished on the basis of their morphology

and ultrastructural features (Hansen and Zielinski 2005), the expression of different families of odorant receptor molecules and G-proteins (Hansen et al. 2003; Oka et al. 2012; Ahuja et al. 2014), the presence of receptors for neurotrophic factors (Catania et al. 2003), the occurrence of several cytosolic Ca^{2+}-binding proteins (Germanà et al. 2004, 2007) or ion channels (Amato et al. 2012; Parisi et al. 2014), and the central projections to the central nervous system (Gayoso et al. 2012; Ahuja et al. 2013, 2014).

Different studies in non-vertebrates and vertebrates have identified several ion channels that are responsible for detecting a range of thermal, chemical or mechanical stimuli (see for a review Damann et al. 2008). The capability of the sensory cells to detect and to encode the specific stimuli is due to the combination of different ion channels (French et al. 2010; Liedtke 2007; Ezak et al. 2010). In particular, the acid-sensing ion channels (ASICs) are H^+-gated voltage-insensitive, amiloride-sensitive ion channels that belong to the degenerin/epithelial Na^+ channel (DEG/ENaC) superfamily of cation channels. They monitor moderate deviations from the physiological values of extracellular pH, but also participate in mechanoreception and nociception (Wemmie et al. 2006; Holzer 2009, 2011; Sherwood et al. 2012; Zha 2013).

The zebrafish is an attractive model for studying the neurological, cellular and molecular basis of behaviors, including those in which the olfaction plays a key role such as feeding and reproduction (Laberge and Hara 2001; Valentincic et al. 2005; Whitlock 2006; Braubach et al. 2009; Yoshihara 2009). Six ASICs have been identified in zebrafish (denominated *z*ASICs; zASIC1.1, zASIC1.2, zASIC1.3, zASIC2, zASIC4.1 and zASIC4.2; Paukert et al. 2004), encoded by six different genes. The zASICs have similar predicted molecular masses (~60 kDa) and share 60–75 % of amino acid identity with rat and human ASICs (Paukert et al. 2004). ASICs are widely distributed in both nervous and no-nervous tissues of mammals (see Sherwood et al. 2012). In zebrafish, the expression of ASICs at the mRNA or protein levels has been reported in the central and peripheral nervous system, the eye and taste buds (Paukert et al. 2004; Chen et al. 2007; Levanti et al. 2011; Viña et al. 2013). In the present study, we investigate the possible involvement of ASIC2 in olfaction in fishes. The study was aimed to contribute to the knowledge of the cellular and molecular basis of olfaction in fish using RT-PCR, Western blot, in situ hybridization and immunohistochemistry on the olfactory epithelium of adult zebrafish.

Materials and methods

Adult zebrafish (*Danio rerio*; $n = 25$), 6 months old, were obtained from CISS (Centro Ittiopatologia Sperimentale Sicilia), University of Messina, Italy. The specimens were anaesthetized with MS222 (ethyl-m-amino benzoate; 0.4 g L^{-1}) and killed by decapitation. The heads of 10 animals were fixed in Bouin's fixative for 24 h and then routinely processed for paraffin embedding; thereafter the pieces were cut 10 μm thick in serial sections and collected on gelatine-coated microscope slides. This material was used for in situ hybridization and immunohistochemistry. The olfactory rosettes were isolated from the heads of other 5 animals and processed for transmission electron microscopy or scanning electron microscopy. Finally, the isolated olfactory rosettes of the remaining 10 animals were used for Western blot and RT-PCR. The isolated brains from 4 animals were used as positive controls for Western blot (Viña et al. 2013).

RT-PCR

Total RNA was extracted from the olfactory rosettes of adult zebrafish using a commercial kit (Trizol Reagent, Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. After precipitation and cold ethanol washing, RNA was dried and dissolved in an appropriate volume of Tris–EDTA buffer (10 mM Tris–HCl pH 8.0 and 1 mM EDTA-Na_2). Each sample was digested with 1 U of DNase I for 1 h at 37 °C to digest genomic DNA. The RNA was precipitated, washed, and dissolved again in the same buffer. RNA solution was quantified at 260 nm (Biomate 3, Thermo Electron Corporation, Waltham, MA, USA), and its purity was assessed by the ratio at 260/280 nm. We used the High Capacity cDNA archive kit (Applied Biosystems, Carlsbad, CA, USA), random hexamers and 10 μg of total RNA to make cDNA following the manufacturer's instructions. The reaction was performed at 42 °C for 90 min.

cDNA obtained from zebrafish rosettes was used to perform a PCR. Specific oligonucleotide primers were designed based on published sequences for *Danio rerio* ASIC2 (GenBank accession number NM_214788.1) and *Danio rerio* β-actin (GenBank accession number NM_131031). The sequences were as follows: for zASIC2 forward: 5′-TTGTTGGCCAGCTTAGTCCT-3′, reverse: 5′-GGGTCGGTAGTTAGTGAAGT-3′; for β-actin forward: 5′-CACAGATCATGTTCGAGACC-3′, reverse: 5′-GGTCAGGATCTTCATCAGGT-3′.

The conditions of amplification were as follows: 2 U Taq DNA polymerase (Promega, Madison, WI), 1 μM primers, 10 ng zebrafish brain or retina cDNA, 0.2 mM each dNTP in 15 μL Taq DNA polymerase buffer. The reaction was performed in a thermal cycler (Hyband Th. Cycler) with the following program: 1 min 94 °C initial denaturation, then 10 cycles of 94 °C 1 min, 65 °C 30 s and 72 °C 45 s, followed by 20 cycles of 94 °C 1 min, 61 °C 30 s, 72 °C 45 s and a 5 min final extension at 72 °C. The PCR products were visualized

by ethidium bromide staining under UV light following electrophoresis on a 2 % agarose gel with a marker Ready-Load 1 kb plus DNA ladder (Invitrogen, Carlsbad, USA).

Western blot

For Western blot, lysates prepared from olfactory rosettes and brain homogenates were processed following procedures previously described (Germanà et al. 2010). Briefly, the samples were pooled and homogenized (1:2, w/v) in Tris–HCl-buffered saline (TBS, 0.1 M, pH 7.5) containing 1 μM leupeptin, 10 μM pepstatin and 2 mM phenylmethylsulfonyl fluoride. The homogenates were centrifuged at 25,000 rpm for 15 min at 4 °C, and the resulting pellet dissolved in Tris HCl 10 mM pH 6.8, 2 % SDS, 100 mM dithiothreitol and 10 % glycerol at 4 °C. The lysates were analyzed by electrophoresis in 12 % discontinuous polyacrylamide SDS gels. After electrophoresis, proteins were transferred to a nitrocellulose membrane, and antibody nonspecific binding was blocked by immersion for 3 h in PBS containing 5 % dry milk and 0.1 % Tween-20. The membranes were then incubated at 4 °C for 2 h with an antibody against ASIC2. This antibody was a rabbit polyclonal antibody raised against a synthetic peptide from the C-terminus of human ASIC2 (LS-B156/12883; Lifespan Biosciences, Seattle, WA, USA) and used diluted 1:200. After incubation, the membranes were washed with TBS pH 7.6 containing 20 % Tween-20 and incubated again for 1 h with the goat anti-rabbit IgG (diluted 1:100) at room temperature. Membranes were washed again and incubated with the PAP complex diluted 1:100 for 1 h at room temperature. Finally, the reaction was developed using a chemiluminescent reagent (ECL, Amersham Pharmacia Biotech, Buckinghamshire, UK) and exposed to Hyperfilm. Marker proteins were visualized by staining with Brilliant Blue.

DNA probe preparation

Specific sense and antisense DNA probes for zASIC2 were designed on the basis of its mRNA sequence (GenBank accession number NM_214788.1). The probe sequences were sense 5′-GAAGAGGGGAAGACTTTAA GGACTACAATGAAAG-3′ and antisense 5′-GTGATA TATTTCTCGGATCGGTTGAACTTCTTCTC-3′, having a melting temperature above 72 °C and a length of 35 bp and 34 bp, respectively. Biotin 3′End DNA Labeling Kit was used for labeling probes following the manufacturer's specifications (Thermo Fisher Scientific Inc., MA, USA).

Chromogenic in situ hybridization (CISH)

To perform the hybridizations, paraffin-embedded tissue sections were treated with xylene and then rehydrated by incubation through decreasing alcohol concentrations. The samples were pre-treated by incubation with antigen retrieval buffer (DAKO, Germany) for 10 min at 95 °C and for 10 min at room temperature. After rinsing with PBS, they were immersed in a HCl 0.2 N solution for 15 min and washed in PBS for 5 min and in PBS-T (Triton-X100 0.3 %) for 15 min. Then, tissues were incubated with proteinase K 10 μg/ml for 7 min at 37 °C to facilitate the exposure of cellular ribonucleic acid. The enzyme activity was blocked by washing the samples twice with PBS for 5 min. Finally, preparations were incubated with labeled probes at a dilution of 1:3 in MillQ Water in a DAKO hybridization oven. The hybridization program consisted of a denaturation step performed at 95 °C for 5 min followed by the hybridization step carried out at 47 °C for 17 h. Then, the samples were washed twice in TBS for 10 min. Stringent washings were performed using a series of sodium citrated chloride (SSC) solutions for 15 min at 60 °C. Washing and blocking steps were performed using Dig Wash and Block Buffer Set according to the manufacturer's specifications (Roche Applied Science, Germany). The tissues were incubated with Streptavidin–AP conjugate for 1 h at room temperature and then washed twice for 15 min. BICP-NBT was used as a chromogen. Sections were mounted with Immuno In Situ Mount (Santa Cruz Biotechnology, Texas, USA).

Immunohistochemistry

Deparaffinized and rehydrated sections were rinsed in Tris–HCl buffer (0.05 M, pH 7.5) containing 0.1 % bovine serum albumin and 0.2 % Triton-X 100. The endogenous peroxidase activity and non-specific binding were blocked (3 % H_2O_2 and 25 % fetal calf serum, respectively), and the sections were incubated overnight at 4 °C with the primary antibody against ASIC2 described above. This antibody has been tested in zebrafish for both Western blot and immunohistochemistry in paraffin-embedded tissues (Viña et al. 2013). Afterward, sections were rinsed in the same buffer and incubated with goat anti-rabbit IgG HRP-conjugated (Amersham, UK) diluted 1:100 for 1 h at room temperature. Finally, sections were washed, the immunoreaction visualized using 3-3′ DAB as a chromogen, and the sections slightly counterstained with Harris hematoxylin to ascertain structural details. The specificity of the immunoreactivity developed was tested incubating representative sections with specifically pre-absorbed antibody (5 μm/ml of working solution; LifeSpan BioSciences, reference LS-PB156). Under these conditions, no positive immunostaining was observed.

To accurately identify the structures displaying ASIC2 immunoreactivity in the olfactory epithelium, sections were processed for simultaneous detection of ASIC2 and β-tubulin. Double immunostaining was performed on

deparaffinized and rehydrated sections. Non-specific binding was reduced by incubation for 30 min with a solution of 1 % bovine serum albumin in Tris buffer solution (TBS). The sections were then incubated overnight, at 4 °C in a humid chamber with a 1:1 mixture of anti-ASIC2 antibody (diluted 1:100) and anti-β-tubulin antibody (clone TUB 2.1, Sigma, St Louis, MS, USA, diluted 1:300 in the blocking solution). After rinsing with TBS, the sections were incubated for 1 h with Alexa fluor 488-conjugated goat anti-rabbit IgG (Serotec, Oxford, UK), diluted 1:1000 in TBS containing 5 % mouse serum (Serotec), then rinsed again and incubated for another hour with Cy™3-conjugated donkey anti-mouse antibody (Jackson-ImmunoResearch, Baltimore, MD, USA) diluted 1:50 in TBS. Both steps were performed at room temperature in a dark humid chamber. Sections were then washed, dehydrated and mounted with Entellan®. Double staining was detected using a Leica DMR-XA automatic fluorescence microscope (Servicio de Análisis de Imágenes, Universidad de Oviedo) coupled with a Leica Confocal Software, version 2.5 (Leica Microsystems, Heidelberg GmbH, Germany), and the images captured were processed using the software Image J version 1.43 g Master Biophotonics Facility, Mac Master University Ontario (www.macbiophotonics.ca).

For control purposes, representative sections were processed in the same way as described for immunohistochemistry. Under these conditions, no positive immunostaining was observed (data not shown).

Transmission electron microscopy

Isolated olfactory rosettes ($n = 5$) were processed for Durcupan ACM (Fluka) resin embedding as follows: pieces fixed in 2.5 % glutaraldehyde in 0.2 M phosphate-buffered saline (pH 7.4), for 2 h at 4 °C, were washed repeatedly in the same buffer and postfixed in 1 % osmium tetroxide in 0.2 M phosphate-buffered saline for 1 h at 4 °C. Then, the tissues were dehydrated with increasing alcohol concentrations. The dehydrated pieces were embedded in Durcupan ACM. Finally, the sections were obtained with a Reichert Jung Ultracut E. Semi-thin sections. (1 μm) were stained with toluidine blue and examined with a light microscope. Ultrathin sections (700 Å) were obtained from selected areas of the semi-thin sections, stained with uranyl acetate and lead citrate, and examined and photographed with a Jeol-Jem 100 SX transmission electron microscope.

Scanning electron microscopy

Isolated olfactory rosettes ($n = 5$) were fixed in 2.5 % glutaraldehyde in Sörensen phosphate buffer 0.1 M. After several rinsing in the same phosphate buffer, they were dehydrated in a graded alcohols series, critical-point dried in a Balzers CPD 030, sputter coated with 3 nm gold in a Balzers BAL-TEC SCD 050 and examined under a Cambridge Stereoscan 240 electron microscope (Zeiss ex Cambridge Instruments, Cambridge, UK) operating with an accelerating voltage of 20 kV.

Results

Morphology and structure of the olfactory epithelium in adult zebrafish

The olfactory rosette of adult zebrafish observed by scanning electron microscopy shows that it is formed by a series of lamellae, all inserted in a midline raphe (Fig. 1a–c). Two different zones can be distinguished in each lamella: The peripheral part is filled with long and numerous cilia, which characterize the non-sensory epithelium (Fig. 1d and e), and the central part which shows numerous cilia and microvilli typical of sensory epithelium (Fig. 1f). The midline raphe contains numerous microvilli and the interlamellar epithelium, which shows scarce microvilli and protrusions of the goblet cells (data not shown).

The ciliated non-sensory cells in the olfactory rosette were also studied by transmission electron microscopy. High columnar cells extend from the basal lamina to the epithelial surface (Fig. 2a). They have typical characteristics of ciliated epithelial cells and possess a large number of long cilia (Fig. 2b) rising from the broad, flat apex of the cell. The cilia are peculiar kinocilia with characteristic 9 + 2 axonemes, and basal body at the basis (Fig. 2b–e). In addition to these cells, the non-sensory epithelium contains scarce cells with poor microvillous protrusions as well as goblet cells (data not shown).

zASIC2 mRNA is expressed in the zebrafish olfactory rosette

Polymerase chain reaction assay was used to detect specific expression of zASIC2 using total cDNA obtained from isolated zebrafish rosettes. The amplification of zebrafish β-actin was used as positive control. After electrophoresis, amplified fragments were visualized by UV light. Bands of expected lengths corresponding to zASIC2 and β-actin cDNAs, 300 bp and 220 bp, respectively, were observed (Fig. 3a). Hence, the peripheral olfactory organ of adult zebrafish expresses zASIC2.

To localize the expression of zASIC2 mRNA within the olfactory epithelium, in situ hybridization was performed. High level of expression was observed in the luminal border of the non-sensory epithelium in all the lamellae of the olfactory rosette (Fig. 4a–c). Controls performed with sense oligos were always negative (Fig. 4d and e).

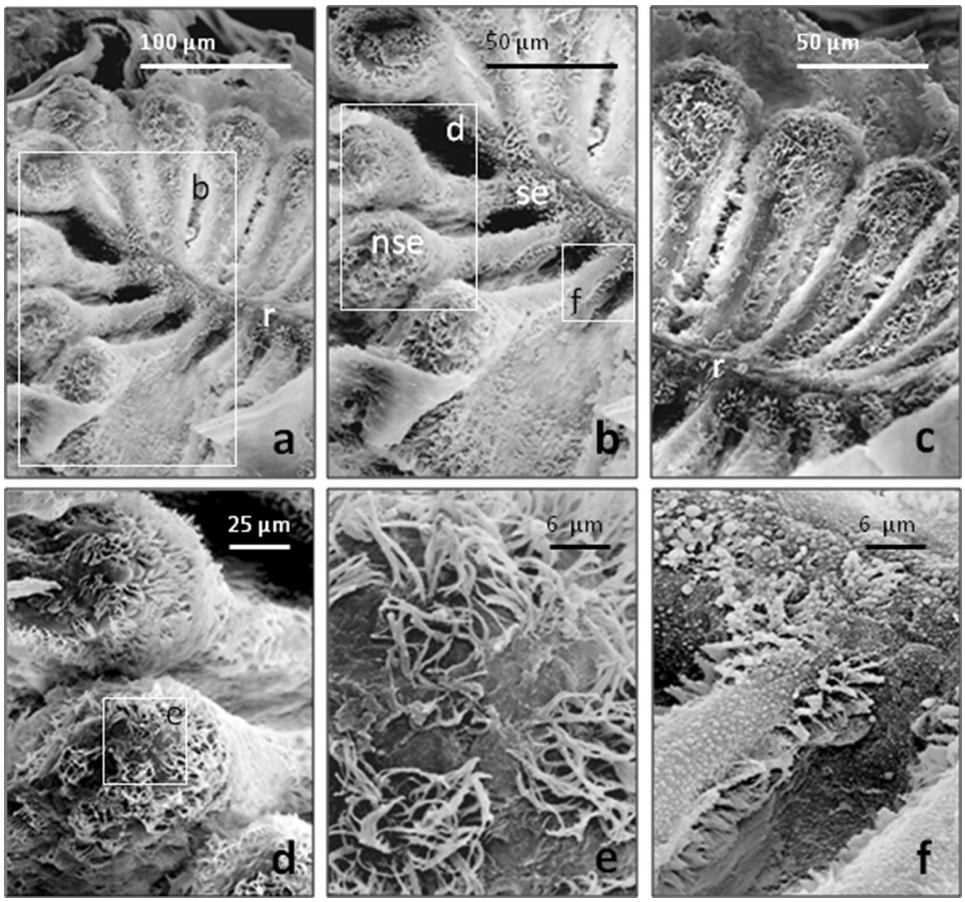

Fig. 1 Scanning electron micrographs showing the morphology of the olfactory rosette (**a**, **b**, **c**). Each lamella is divided into two parts: sensory and non-sensory. The sensory epithelium contains numerous cilia (**f**), whereas the non-sensory epithelium is filled with long kinocilia (**d, e**). The non-sensory epithelium covering the midline raphe contains numerous microvilli (**f**). **b** is a detail of **a**, and **d** is a detail of **b**. *nse* non-sensory epithelium, *r* median raphe, *se* sensory epithelium

ASIC2 is present in the zebrafish olfactory rosette

We performed Western blot analysis to confirm the RT-PCR results and to test the anti-ASIC2 antibody using lysates obtained from zebrafish rosettes and brains. The results demonstrated that ASIC2 is expressed in adult zebrafish rosette and brain through the identification of one single and robust protein band with an estimated molecular weight of about 62 kDa (Fig. 3b), which is in the range of the molecular weight expected for ASIC2. The molecular weight of this protein band was determined on the basis of its electrophoretic migration in relation to an appropriate molecular weight standard.

The distribution of ASIC2 protein in the olfactory epithelium of adult zebrafish was analyzed by immunohistochemistry (Fig. 5a–f). A strong and specific ASIC2 immunoreaction was detected and found to be restricted to the cilia of the peripheral parts of the olfactory lamellae that correspond to the non-sensory epithelium (Fig. 5a and b, d–f). Sections incubated with specifically pre-absorbed anti-ASIC2 did not displayed positive immunostaining (Fig. 5c).

To ascertain that the ASIC2-positive structures are cilia, some sections were processed for simultaneous detection of ASIC2 and β-tubulin, a typical for the microtubules of the cilia. In these sections, we found a specific immunoreaction for ASIC2 (Fig. 6a, b, d) and β-tubulin (Fig. 6c). The merge and masque images obtained using laser confocal microscopy showed co-localization of β-tubulin and ASIC2 (Fig. 6e and f) confirming the expression of ASIC2 in the cilia of the non-sensory olfactory epithelium.

Discussion

The expression and distribution of the ion channel ASIC2 in the olfactory epithelium of adult zebrafish were analyzed in order to determine whether or not they participate

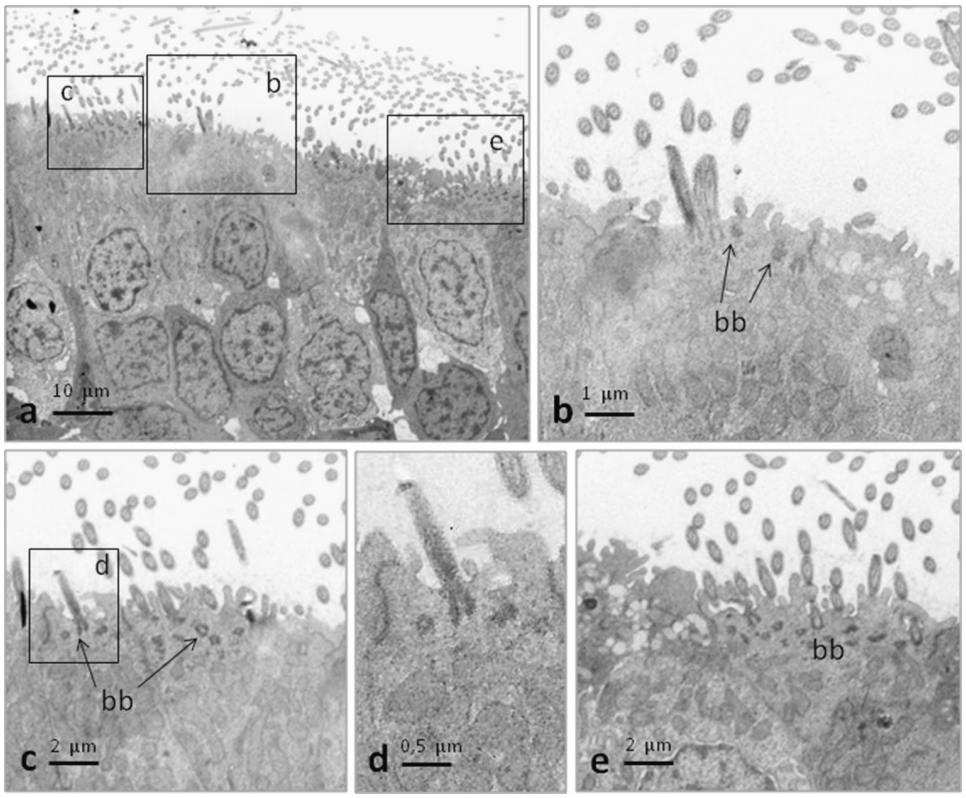

Fig. 2 Transmission electron micrographs of the non-sensory epithelium of the olfactory organ (**a**). Figures **b**, **c** and **e** are details of **a**; **d** is an enlargement of **c**. The ciliated non-sensory cells have numerous kinocilia and few microvillous protrusions. Long kinocilia show the central doublets of axoneme oriented in the same direction, and basal bodies (*bb*) were present at the base of kinocilia

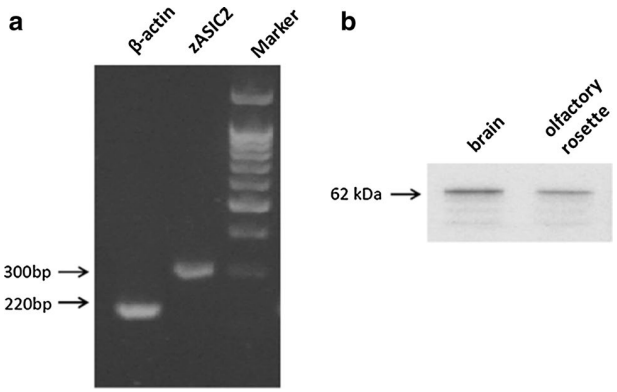

Fig. 3 **a** RT-PCR analysis of zASIC2 and β-actin mRNA expression in isolated olfactory rosette of adult zebrafish. A 300-bp fragment of ASIC2 mRNA was detected. The integrity of the samples was demonstrated by the presence of a 220-bp fragment of β-actin mRNA. **b** Western blot detection of ASIC2 in isolated brain and olfactory rosette of adult zebrafish. The anti-ASIC2 antibody used recognizes a single protein band of about 62 kDa consistent with the complete ASIC2

in olfaction. We assumed that the presence of ASIC2 in one or more types of ORNs is an indirect evidence of its involvement in the physiology of these cells, and therefore in olfaction. ASIC2 is widely expressed in the central and peripheral nervous system (see Holzer 2009; Sherwood et al. 2012) as well as in some non-nervous tissues (Babinsky et al. 2000; Jahr et al. 2005; Cuesta et al. 2014). In zebrafish, ASIC2, at the mRNA or protein level, has been detected in developing and adult brain (Paukert et al. 2004; Levanti et al. 2011), in the enteric nervous system and peripheral nerves (Levanti et al. 2011; Viña et al. 2013) and in the nerve layers of the retina (Viña el al, unpublished data). As far as we know, ASIC2 has not been detected outside the nervous system in zebrafish.

We have used specific and sensible methods to perform our research, and all of them yielded consistent results: The expression and localization of the zASIC2 mRNA, as well as the occurrence and localization of ASIC2 protein, were restricted to the non-sensory epithelium, in particular in the cilium of the ciliated epithelial cells (see Pedersen et al. 2012; Ishikawa and Marshall 2013). Thus, based on the present results, ASIC2 in the olfactory epithelium does not participate in olfaction, but, presumably, in some other unknown functions. The occurrence of ASIC2 in epithelial cells has been reported previously in the ciliated and stereociliated cells of different organs (trachea, oviduct,

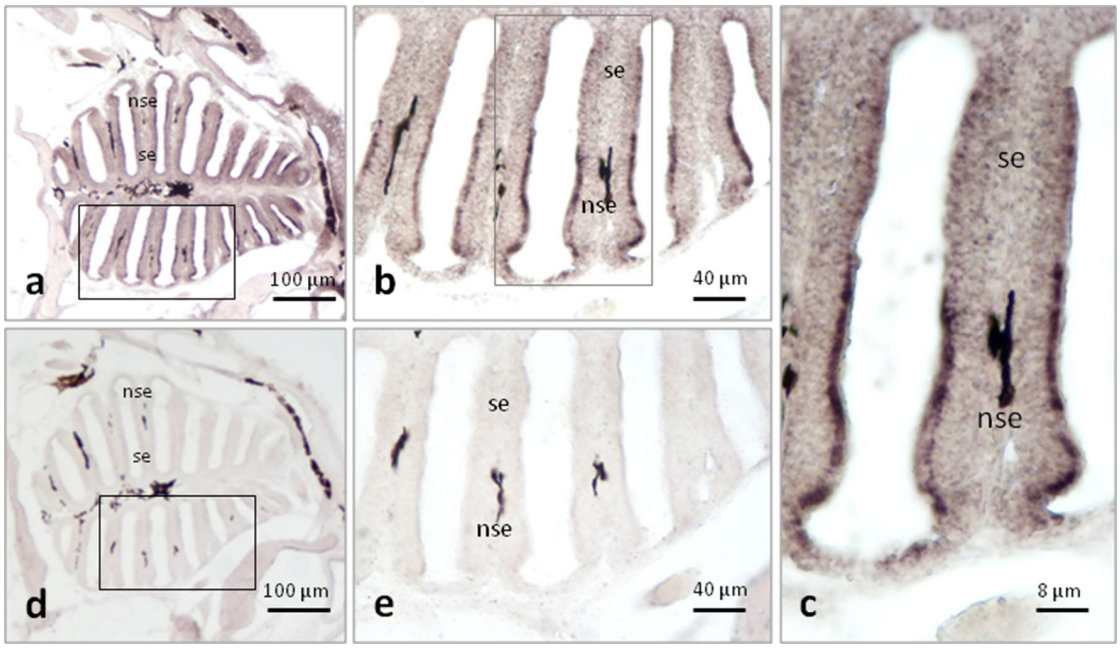

Fig. 4 Chromogenic in situ hybridization for zASIC2 in adult zebrafish olfactory rosette. zASIC2 antisense strand shows a specific staining in the apical zone of the epithelial cells in the non-sensory epithelium (**a–c**), whereas sense strand does not labeled the olfactory rosette (**d, e**). *nse* non-sensory epithelium, *se* sensory epithelium

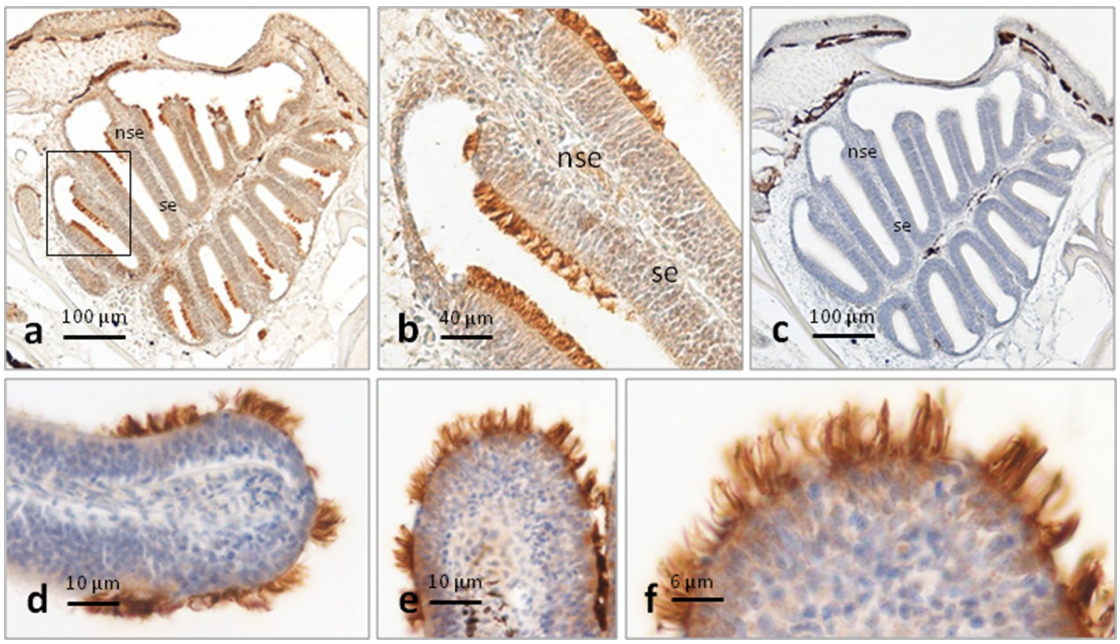

Fig. 5 Immunohistochemical localization of ASIC2 in the olfactory epithelium of adult zebrafish. Specific ASIC2 immunoreactivity (**a, b, d–f**) was detected restricted to the kinocilia of the non-sensory epithelium. No immunostaining was detected in control sections incubated with preabsorbed anti-ASIC2 antibody (**c**). *nse* non-sensory epithelium, *se* sensory epithelium

epididymis, Corti's organ and ampullary crest) in developing and adult rat, including the olfactory epithelial septum (Kikuchi et al. 2008, 2010). These authors observed that the ciliated cells have ASIC2 channels on the cilia membrane whereas stereociliated cells exhibited ASIC2 in the cytoplasm. ASIC1 was also found in the insertion portion

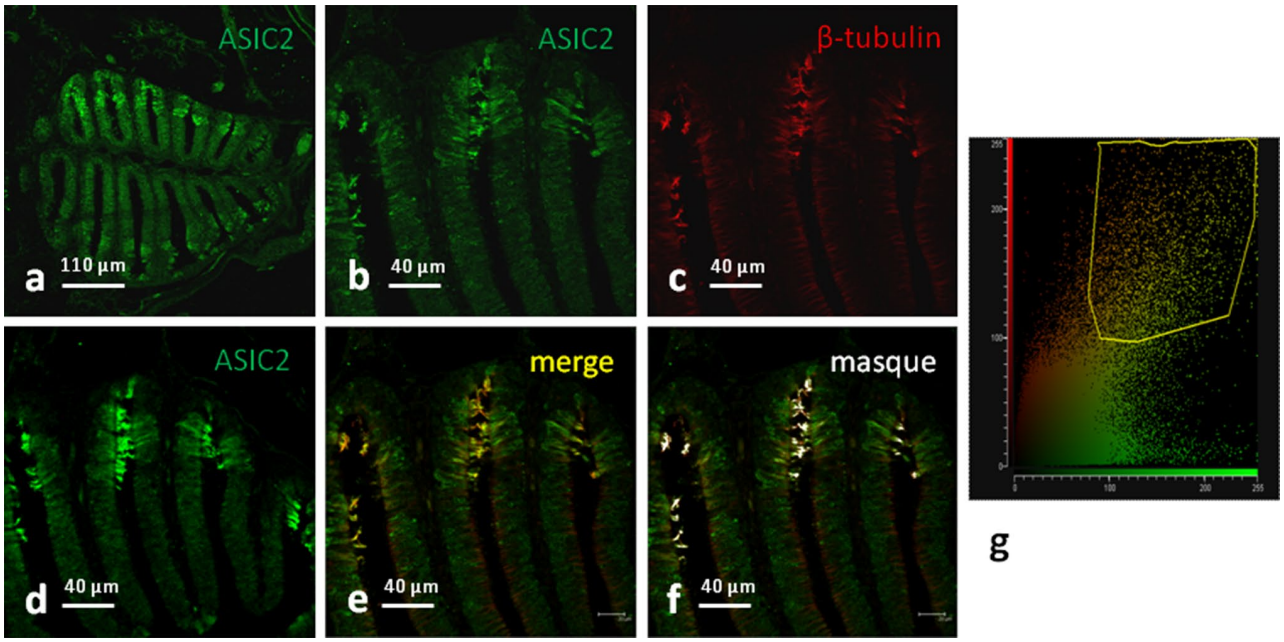

Fig. 6 Confocal laser-scanning images of ASIC2 (*green*, **a, b, d**) and β-tubulin (*red*, **c**) in adult zebrafish olfactory epithelium demonstrating that ASIC and β-tubulin co-localize in the kinocilia of the non-sensory epithelium (**e**). Figure **f** is a merge image of ASIC, and β-tubulin covered by mask (*white dots*). Mask represents the regions of interest drawn on the cytofluorogram around the high *red signal dots*. Figure **g** shows a 2D cytofluorogram from the two detection channels from the original image. Every *dot* of cytofluorogram represents an intensity value pair from the two detection channels. *Red signal dots* are not close to the *Y*-axis because the *green signal* has been acquired with higher gain amplification than the *red signal*. A region of interest on the cytofluorogram around the high *red signal dots* had been drawn, and it is represented as a mask (*white dots*) on the merge image (*center bottom*)

of the stereocilia of mouse cochlear outer hair cells (Ugawa et al. 2006). Our results demonstrate that ASIC2 is never present in the cytoplasm of the ciliate cells but only in the membrane and cilia.

The non-sensory epithelium of the olfactory rosette in teleosts, including zebrafish, is primarily filled with ciliated cells, and their cilia show structural organization of cytoskeletal microtubules as 9 + 2. Our results regarding the structure of these cells are in total agreement with those reported earlier by Hansen and Zeiske (1998).

The primary cilium is actually regarded as a system by which cells sense and transduce mechanical and chemical stimuli, sensing fluids in a large number of tissues and organs (see Prasad et al. 2014). It converts extracellular stimulations to intracellular signals (Muhammad et al. 2012). It is also noteworthy that the membrane of the cilium contains multiple sensory and channel proteins, including a variety of Ca^{2+}-ion permeable that play a role in Ca^{2+}-mediated fluid-flow mechanosensation (Anishkin and Kung 2013). Among these ion channels, there is very probably ASIC2 (Kikuchi et al. 2008, 2010). But, what is the role of ASIC2 in the cilium? Also, are the functions attributed to the cilium associated with the presence of ASIC2 in this structure? The mechano- and chemo-sensory functions of the cilium (Prasad et al. 2014) are the same proposed for ASIC2 (see Sherwood et al. 2012). Thus, it can be speculated that ASIC2 plays important roles in the molecular basis of the cilium functions. Nevertheless, the mechanosensory role of ASIC2 has not been definitively clarified (see Lingueglia 2007). In fact, data obtained from ASIC2 null mice (Price et al. 2000) have shown that ASIC2 is a central component of a mechanosensory complex, but ASIC2 is not a part of mechanically gated ion channels for mechanosensation (Roza et al. 2004). In fish, the detection of water movement is attributed to the hairy cells of the neuromasts in the lateral line system (Bleckmann and Zelick 2009; Montgomery et al. 2009), but present results suggest that the movement of water entering the nasal fossa in fish may be also detected by the cilia of the olfactory rosette.

On the other hand, ASIC2 channel is gated by pH variations. In mammals, two isoforms of ASIC2 exist, ASIC2a and ASIC2b, which have different maximal activation pH values (Lingueglia et al. 1996; Holzer 2009). Nevertheless, in zebrafish, only one ASIC2 was cloned (Paukert et al. 2004) presumably assuming the same role as in mammals. Given the very high levels of expression of ASIC2 in the cilia of the zebrafish olfactory epithelium, it appears unlikely that their role is limited to the detection of pH changes, since the aquatic environmental pH for fish must

be unvaried. Therefore, the function of ASIC2 in the olfactory epithelium of adult zebrafish remains to be elucidated. In mammals, Kikuchi et al. (2010) suggested that ASIC2 may function for the survival and retention of ciliated cells of the rat nasal septum against dynamic changes in the pH environment. This role is difficult to apply in fish because the pH of the aquatic environment must be unchanged.

Classically, the olfactory epithelium in fish has been divided into sensory and non-sensory epithelium. However, if the occurrence of ASIC2 in the olfactory epithelium of zebrafish is an evidence of mechano- and/or chemo-detection by the non-sensory epithelium, all the sectors of the rosette lamellae are sensory. Therefore, we propose to divide the epithelia of the lamellae into olfactory and non-olfactory instead of sensory and non-sensory. This assumption is also supported by the presence of TRPV4 positive cells in the non-sensory epithelium (Viña et al. 2013; Parisi et al. 2014).

In conclusion, the present study demonstrated the expression of ASIC2 in ciliated cells of the olfactory epithelium of zebrafish, which can be related to detection of water movement or acidic environment pH. Further studies are necessary to definitively clarify the role of ASIC2 in the olfactory system.

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REGULAR ARTICLE

Acid-sensing ion channels (ASICs) 2 and 4.2 are expressed in the retina of the adult zebrafish

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Abstract Acid-sensing ion channels (ASICs) are H^+-gated, voltage-insensitive cation channels involved in synaptic transmission, mechanosensation and nociception. Different ASICs have been detected in the retina of mammals but it is not known whether they are expressed in adult zebrafish, a commonly used animal model to study the retina in both normal and pathological conditions. We study the expression and distribution of ASIC2 and ASIC4 in the retina of adult zebrafish and its regulation by light using PCR, in situ hybridization, western blot and immunohistochemistry. We detected mRNA encoding zASIC2 and zASIC4.2 but not zASIC4.1. ASIC2, at the mRNA or protein level, was detected in the outer nuclear layer, the outer plexiform layer, the inner plexiform layer, the retinal ganglion cell layer and the optic nerve. ASIC4 was expressed in the photoreceptors layer and to a lesser extent in the retinal ganglion cell layer. Furthermore, the expression of both ASIC2 and ASIC4.2 was down-regulated by light and darkness. These results are the first demonstration that ASIC2 and ASIC4 are expressed in the adult zebrafish retina and suggest that zebrafish could be used as a model organism for studying retinal pathologies involving ASICs.

Keywords Retina · Acid-sensing ion channels · Light · Zebrafish

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Introduction

Acid-sensing ion channels (ASICs) are H^+-gated, voltage-insensitive cation channels that belong to the amiloride-sensitive degenerin/epithelial Na^+ channel (DEG/ENaC) superfamily. The physiological role of ASICs is to monitor variations in extracellular pH and to participate in synaptic transmission, mechanosensation and nociception (see Holzer 2009, 2011; Sherwod et al. 2012; Zha 2013). Six different ASICs have been identified in zebrafish (referred to as *z*ASICs): zASIC1.1, zASIC1.2, zASIC1.3, zASIC2, zASIC4.1 and zASIC4.2. These six proteins have similar predicted molecular masses (~60 kDa) and share 60–75 % amino acid sequence with rat and human ASICs. The zASIC genes are expressed throughout the central nervous system but their expression in peripheral organs is limited (Paukert et al. 2004). Recently, using immunohistochemistry we detected in the adult zebrafish ASIC2 and ASIC4 in taste buds and in the nerves supplying them, respectively (Viña et al. 2013) and ASIC2 in the neurons of the enteric

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nervous system (Levanti et al. 2011). Different members of the DEG/ENaC superfamily, including ASICs, are expressed in most layers of the mammalian retina (Table 1) and in the retina of zebrafish larvae zASIC2 and zASIC4 are expressed in the retinal ganglion cells (RGC; Paukert et al. 2004).

The function of ASICs in the retina in both normal and pathological conditions remains unknown. Fluctuations in pH play an important role in the retina and for that reason ASICs are thought to be involved in the fine tuning of visual perception, as well as in the adaptation of the retinal responses to different light intensities (see Ettaiche et al. 2004). As pH variations are also associated with pathological conditions, ASICs are likely to be involved in the pathogenesis of retinal diseases. For instance, ASIC1a seems to plays a role in RGC death induced by hypoxia (Tan et al. 2011) and protection of the retinal pigmentary epithelium (RPE) against oxidative stress (Tan et al. 2013). ASIC2 is a negative modulator of rod phototransduction and is beneficial for the maintenance of retinal integrity. The ASIC2 knockout mice are more sensitive to light-induced retinal degeneration (Ettaiche et al. 2004). Moreover, the blockage of ASICs may have a potential neuroprotective effect in ocular ischemic diseases (Miyake et al. 2013).

Table 1 Localization of acid-sensing ion channels (ASIC) and degenerin-epithelial Na^+ channel (DEG-ENaC) subunits in the retina of different species

	DEG/ENaC	ASIC1	ASIC2	ASIC3	ASIC4
RPE	Rat[a]				
	Human[b]	Cell line[g]			
	Bovine[c*]				
ONL	Rat[a,b]		Mouse[f]		*Zebrafish*
			Zebrafish		**Zebrafish**
OPL	Rat[b]		**Zebrafish**		
	Human[b]				
INL	Rat[a,b]	Rabbit[e]	Rabbit[e]	Rabbit[e]	Rabbit[e]
	Rabbit[e]		Mouse[f]		
			Zebrafish		
			Zebrafish		
IPL	Rat		**Zebrafish**		
	Human[b]				
RGC	Rat[a,b]		Rabbit[e]		Rabbit[e]
	Human[b]	Rabbit[e]	Mouse[f]	Rabbit[e]	*Zebrafish*
			Zebrafish		
			Zebrafish I[i**]		*Zebrafish I*[i**]
MG	Rat[d*]	Rabbit[e]	Rabbit[e]	Rabbit[e]	Rabbit[e]
	Rabbit[e]	Rabbit[h]			

Results of the present study are included in the table: *zebrafish* indicate results at the mRNA level and **Zebrafish** at the protein level

RPE retinal pigmentary epithelium; *ONL* outer nuclear layer; *OPL* outer plexiform layer; *INL* inner nuclear layer; *IPL* inner plexiform layer; *RGC* retinal ganglion cells layer; *MG* Müller glia

*in culture

**zebrafish larvae

[a] Matsuo 1998; [b] Mirshahi et al. (1999); [c] Mirshahi et al. (2000); [d] Golestaneh et al. (2001); [e] Brockway et al. (2002); [f] Ettaiche et al. (2004); [g] Tan et al. (2013); [h] Tan et al. (2011); [i] Paukert et al. (2004)

In the last decade, zebrafish has emerged as a model to study the biology and pathophysiology of visual disorders, including some in which ASICs could be involved (Goldsmith and Harris 2003; Collery et al. 2006; Gross and Perkins 2008). Nevertheless, neither the expression of ASICs in the retina of adult zebrafish nor the regulation of its expression by light has been analyzed. Here, we used PCR, in situ hybridization, western blot and immunohistochemistry to examine the patterns of expression of ASIC2 and ASIC4 in the retina of adult zebrafish. We chose to focus on these channels because they are expressed in the retina of zebrafish larvae (Paukert et al. 2004). Moreover, the effects of continuous light or dark exposition in the expression of these ion channels were evaluated using quantitative-PCR. This study intends to contribute to the understanding of the molecular mechanisms of vision and may serve as a base line for future studies in retinal biology and pathology using zebrafish as a model.

Material and methods

Animals

Adult zebrafish (*Danio rerio*; $n=35$), 8 months old, were obtained from CISS (Centro Ittiopatologia Sperimentale Sicilia), University of Messina, Italy. The specimens were anaesthetized with MS222 (ethyl-m-amino benzoate; 0.4 g/L–) and sacrificed by decapitation. The eyes of 10 animals were enucleated, washed in cold saline solution and used for RT-PCR or western blot. The heads of another 10 animals were fixed in Bouin's fixative for 24 h and processed for paraffin embedding. These tissues were cut into 10-μm-thick serial sections and mounted on gelatin-coated microscope slides and used for in situ hybridization and immunohistochemistry.

An additional group of animals ($n=15$) was kept in a white light-darkness cycle (12/12 h; $n=5$), continuous white light ($n=5$), or continuous darkness ($n=5$). Briefly, the animals were placed in a transparent plastic cage with the light source placed 3 cm above for 10 days. The white light ($T^a=4000°K$) source was Philips MASTER TL-D Reflex 18 W/840 (Philips, Consumer Lifestyle, Spain). The irradiance was estimated to be 28.57 W/m^2 using a photoradiometric Tektronix J1800 (TekLumaColor, USA) and the spectral composition of the light source was determined using an optic fiber spectrophotometer Spectrawiz EPP2000 (SterllarNet Inc., USA). A group of animals was exposed to darkness in a special black-painted cage (for details, see Sánchez-Ramos et al. 2013). At the end of the experiment, the eyes were quickly enucleated and used for qRT-PCR.

RT-PCR

Total RNA was extracted from the eyes of adult zebrafish using a commercial kit (Trizol Reagent; Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. After precipitation and washing with cold ethanol, RNA was dried and dissolved in an appropriate volume of Tris–EDTA buffer (10 mM Tris–HCl pH 8.0 and 1 mM EDTANa2). Each sample was digested with 1 U of DNase I for 1 h at 37 °C to digest genomic DNA. The RNA was precipitated, washed and dissolved again in the same buffer. RNA concentration was measured at 260 nm (Biomate 3; Thermo Electron, Waltham, MA, USA) and its purity was assessed by the ratio 260/280 nm reading. We used the High Capacity cDNA archive kit (Applied Biosystems, Carlsbad, CA, USA) and random hexamers to make cDNA from 10 μg of total RNA at 42 °C for 90 min following the manufacturer's instructions.

Complementary DNA prepared from eyes and brain (considered as the positive control) was used to perform qPCR. Specific oligonucleotide primers were designed based on the consensus sequences of *Danio rerio* zASIC2, zASIC4.1 and zASIC4.2 (GeneBank accession numbers NM_214788.1, NM_214787.2 and NM_214786.1, respectively) and *Danio rerio* β-actin (GenBank accession number NM 131031). The primers used were: zASIC2 forward: 5′-TTGTTGGCCAGCTTAGTCCT-3′, zASIC2 reverse: 5′-GGGTCGGTAGTTAGTGAAGT-3′; for zASIC4.1 forward: 5′-ACGATGTTGCAGGATTATTAGG-3′, zASIC4.1 reverse: 5′-TTTGTCATCTCTTTGTGGCCA-3′; for zASIC4.2 forward: 5′-GCAAGTACCAGAAGTCAGAGGA-3′, zASIC4.2 reverse: 5′-CTGTCCACCAATGTCTCCTAAC-3′; and for β-actin forward: 5′-CACAGATCATGTTCGAGA CC-3′, β-actin reverse: 5′-GGTCAGGATCTTCATCAGGT-3′.

The PCR reaction consisted of 2 U Taq DNA Polymerase (Promega, Madison, WI, USA), 1 μM primers, 10 ng zebrafish brain or retina cDNA, 0.2 mM each dNTP in 15 μL Taq DNA Polymerase buffer. The reaction was performed in a thermal cycler (Hyband Th. Cycler) with the following program: 1 min 94 °C initial denaturation, then 10 cycles of 94 °C 1 min, 65 °C 30 s and 72 °C 45 s, followed by 20 cycles of 94 °C 1 min, 61 °C 30 s, 72 °C 45 s and a 5-min final extension at 72 °C. The PCR products were visualized by ethidium bromide staining under UV light following electrophoresis on a 2 % agarose gel with a marker Ready Load 1 kb plus DNA ladder (Invitrogen, Carlsbad, USA).

qRT-PCR

qPCR was performed using 1 μg of the cDNA to detect zASIC2 and zASIC4.2 expression. The primers used were based upon the published mRNA sequences for *Danio rerio* ASIC2 and ASIC4.2 (GenBank accession numbers NM_214788.1 and NM_214786.1) and *Danio rerio* β-actin (GenBank accession number NM_131031) and were for ASIC2 forward : 5′-TTGTTGGCCAGCTTAGTCCT-3′, reverse: 5′-GGGTCGGTAGTTAGTGAAGT-3′, for ASIC4.2 forward: 5′-GCAAGTACCAGAAGTCAGAGGA-3′, reverse: 5′-CTGTCCACCAATGTCTCCTAAC-3′ and for β-actin forward: 5′-CACAGATCATGTTCGAGACC-3′, reverse: 5′-GGTCAGGATCTTCATCAGGT-3′.

The homemade TaqMan probes were labeled at the 50 with 60FAM fluorochromes for zASIC2 and zASIC4.2 and VIC fluorochrome for β-actin, while the 3′ ends were labeled with the Minor Groove Binder (MGB) quencher. The PCR reactions were performed using the TaqMan Universal PCR Master Mix (Applied Biosystems) using 5 pmol of each primer and 9 pmol of both target and β-actin probe. The assays were performed in triplicate in independent experiments using a 7500 PCR real-time System (Applied Biosystems) and quantification was calculated using the $2^{-\Delta\Delta Ct}$ algorithm. The average value in animals exposed to a 12-h white light/12-h darkness was considered as the control and the final results were expressed as fold difference compared with the control (relative expression). Statistical differences between experimental groups compared with the standard were determined using analysis of variance (ANOVA) ($p \leq 0.05$).

In situ hybridization

In zebrafish, zASIC2 is expressed as a unique isoform, while zASIC4 has two isoforms zASIC4.1 and zASIC4.2 (Chen et al. 2007). Specific sense and antisense DNA probes for zASIC2 (sense 5′-GAAGAGGGGAAGACTTTAAGGACTACAATG AAAG-3′, antisense 5′-GTGATATATTTCTCGGATCGGTTG AACTTCTTCTC-3′), zASIC4.1 (5′-GCCAACACCATCCT CCCGAATCACCATCACCACCAC-3′, antisense 5′-GTGG TGGTGATGGTGATTCGGGAGGATGGTGTTGGC-3′) and zASIC4.2 (5′-CCAGAGAACCAAGAACCTGCGGGAGC AGAACCTG-3′, antisense 5′-CAGGTTCTGCTCCCGCAG GTTCTTGGTTCTCTGG-3′) were synthesized.

In all cases, the length of the probes was kept between 34 and 36 nucleotides with melting temperatures above 84 °C. The probes were labeled with the 3′ End DNA Labeling Kit according to the manufacturer's specifications.

To perform chromogenic in situ hybridizations, paraffin-embedded tissue sections were treated with xylene, decreasing concentrations of ethanol and water. Then, the samples were incubated with 0.2 N HCl for 15 min, washed with sterile PBS for 5 min and PBS-T (Triton ×-100, 0.3 %) for 15 min and incubated with proteinase K 10 μg/ml for 5 min at 37 °C to facilitate the exposure of cellular ribonucleic acid. Then, preparations were washed with PBS for 10 min. Finally, the preparations were incubated with labeled probes diluted 1:3 in sterile water in a DAKO hybridization oven for 5 min at 95 °C and at 16 h at 55 °C. The samples were washed with TBS twice and with sodium citrate-sodium chloride (SSC) solutions for 15 min at 65 °C. Washing steps and blocking

reactions were performed with the Dig Wash & Block Buffer Set according to the manufacturer's specifications (Roche Applied Science, Mannheim, Germany). The tissues were incubated with streptavidin-AP-conjugate antibody for 30 min at room temperature and washed for 15 min followed by incubation and developing. BICP-NBT was used as a chromogen. Sections were mounted with Immuno in situ mount (Santa Cruz Biotechnology).

Western blot

Lysates prepared from whole eye homogenates were processed following procedures previously described (Germanà et al. 2010). Briefly, the samples were pooled and homogenized (1:2, w/v) in Tris–HCl buffered saline (TBS, 0.1 M, pH 7.5) containing 1 μM leupeptin, 10 μM pepstatin and 2 mM phenylmethylsulfonyl fluoride. The homogenates were centrifuged at 25,000 rpm for 15 min at 4 °C and the resulting pellet dissolved in Tris HCl 10 mM pH 6.8, 2 % SDS, 100 mM dithiothreitol and 10 % glycerol at 4 °C. The proteins were separated by electrophoresis in 12 % polyacrylamide SDS gels and transferred to a nitrocellulose membrane. Antibody non-specific binding was blocked by incubation for 3 h in PBS containing 5 % dry milk and 0.1 % Tween-20. The membranes were then incubated at 4 °C for 2 h with rabbit anti-ASIC2 and anti-ASIC4 polyclonal antibodies. The anti-ASIC2 antibody was a rabbit polyclonal antibody from the extracellular region of mouse ASIC2 conjugated to an immunogen carrier protein (LS-B156/12883; Lifespan Biosciences, Seatle, WA, USA). The antibody against ASIC4 was a rabbit polyclonal antibody from the cytoplasmatic domain of human ASIC4 conjugated to immunogenic carrier protein used as an immunogen (LS-C94536-100; Lifespan Biosciences). Both antibodies were used diluted 1:200. After incubation, the membranes were washed with TBS pH 7.6 containing 20 % Tween-20 and incubated for 1 h with goat anti-rabbit IgG (diluted 1:100) at room temperature. Membranes were washed again and incubated with the PAP complex diluted 1:100 for 1 h at room temperature. The reaction was developed using a chemiluminescent reagent (ECL; Amersham Pharmacia Biotech, Buckinghamshire, UK) and exposed to Hyperfilm. Marker proteins were visualized by staining with Brilliant Blue.

Immunohistochemistry

To investigate the distribution of ASIC2 and ASIC4 in the zebrafish retina, deparaffinized and rehydrated sections from whole heads were processed for indirect peroxidase immunohistochemistry as follows: sections were rinsed in Tris–HCl buffer (0.05 M, pH 7.5) containing 0.1 % bovine serum albumin and 0.2 % Triton-× 100. The endogenous peroxidase activity and nonspecific binding were blocked (3 % H_2O_2 and 25 % fetal calf serum, respectively) and sections were incubated overnight at 4 °C with the same primary antibodies described above diluted 1:100. Sections were then rinsed and incubated with goat anti-rabbit IgG (Amersham) diluted 1:100 for 1 h at room temperature. Finally, sections were washed, the immunoreaction visualized using 3–3′DAB as a chromogen and counterstained with Harris hematoxylin to ascertain structural details. The specificity of the immunoreactivity was tested by replacing the primary antibody with a pre-absorbed antiserum (5 μg of the blocking peptide in 1 ml of the working solution). The control peptides were obtained from Lifespan Biosciences (LS-PB156 for ASIC2 and LS-PC 94536 for ASIC4). Brain sections were also probed in an identical way and were used as positive controls.

An additional immunohistochemical analysis was also carried out as described above using one anti-zebrafish zASIC4.2 polyclonal antibody, raised in rabbit directed against one region near the NH2 terminus (aminoacids 146–160; PKSRKGHRPSELQYP) (Dymowska et al. 2014).

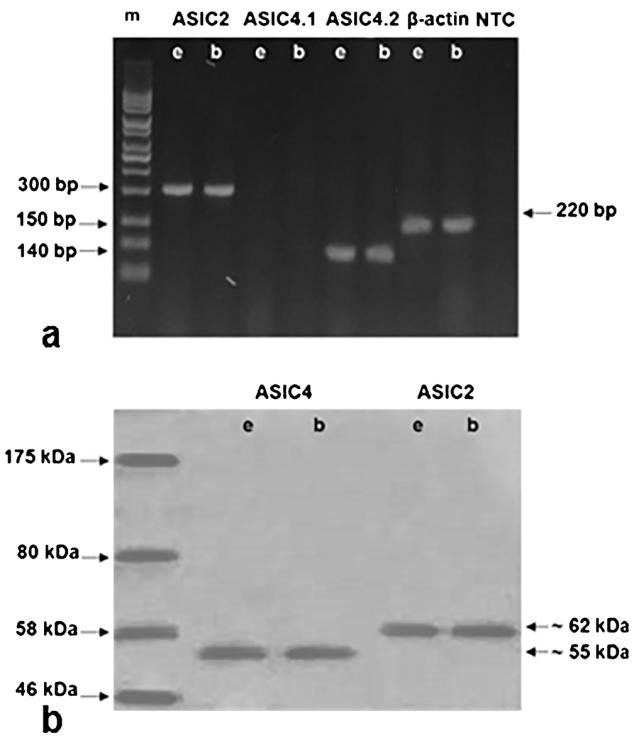

Fig. 1 **a** RT-PCR analysis of ASIC2, ASIC4.1, ASIC4.2 and β-actin mRNA expression in the brain (control) and eye of adult zebrafish. A 300- and 146-pb fragment of ASIC2 and ASIC4.2 mRNA, respectively, was detected. ASIC4.1 mRNA was absent, no band was detected. The integrity of the samples was demonstrated by the presence of a 220-pb fragment of β-actin mRNA. **b** Western blot detection of ASIC2 and ASIC4 in brain and eye homogenates from adult zebrafish. The anti-ASIC2 and anti-ASIC4 antibodies used recognize a single protein band of about 62 kDa and 55 kDa, respectively, consistent with the complete ASIC2 and 4. Since ASIC4.1 mRNA was not found, it can be assumed that the ASIC4 protein band corresponds to the ASIC4.2 isoform

Results

zASIC2 and zASIC4.2 mRNAs are expressed in the eye

We used cDNA prepared from RNA obtained from zebrafish eyes and brains (used as a control) to detect expression of *zASIC2*, *zASIC4.1* and *zASIC4.2* using PCR (Fig. 1a). The results demonstrated that *zASIC2* (300 bp) and *zASIC4.2* (146 bp) are expressed in the eye of adult zebrafish, whereas *zASIC4.1* was absent. Identical results were obtained in the brain (Fig. 1a).

ASIC2 and ASIC4 proteins are expressed in the eye

To elucidate whether ASIC2 and ASIC4 proteins are expressed in the zebrafish retina, western blot was done in eye and brain homogenates using anti-ASIC2 and anti-ASIC4 antibodies (Fig. 1b). We detected two proteins with the estimated molecular weights of about 62 kDa (ASIC2) and 55 kDa (ASIC4) in both organs. The results were consistent with the findings using PCR and demonstrated that both ASIC2 and ASIC4 are expressed in the eye of adult zebrafish.

Distribution of zASIC2 and zASIC4.2 mRNAs in the retina

The retinal localization of the zASIC2 and zASIC4 mRNA in adult zebrafish was analyzed by in situ hybridization. We observed high and specific expression of each zASIC when compared with the control. The results showed a different spatial localization in specific retinal layers. zASIC2 expression was observed in the outer nuclear layer (ONL), the inner nuclear layer (INL) and in the RGC layer (Fig. 2d–f; Fig. S1 in Supplementary Material). zASIC4 has two isoforms, zASIC4.1 *and* zASIC4.2 although only zASIC4.2 was detected by RT-PCR. In order to confirm those results in situ hybridization with probes specific for each mRNA was performed. Expression of zASIC4.2 was detected mainly in the photoreceptors layer (PhL) but also in the RGC layer (Fig. 3d–f). zASIC2 and zASIC4.2 were expressed exclusively in the nuclear layer and never in the plexiform layers. Controls performed with of sense oligos were negative for both zASIC2 (Fig. 2-c) and zASIC4.2 (Fig. 3a–c; Fig. S2 in Supplementary Material).

Immunohistochemical detection of ASIC2 and ASIC4 in the retina

The distribution of ASIC2 and ASIC4 proteins in the retina of adult zebrafish was analyzed using immunohistochemistry.

In animals exposed to a 12 h/12 h rhythm of white light-darkness ASIC2 immunoreactivity was detected in the ONL, the outer plexiform layer (OPL), the inner plexiform layer (IPL) and the RGL (Fig. 4a-c). Immunolabeling was restricted to the cell body of photoreceptors in the ONL. In the IPL the immunoreactivity was observed in fibers of three strata: two in the ON inner sublamina and one in the OFF outer sublamina (Fig. 4b–d; for a detailed description see Jusuf and Harris 2009). Finally, the bodies and dendrites of the RGC also

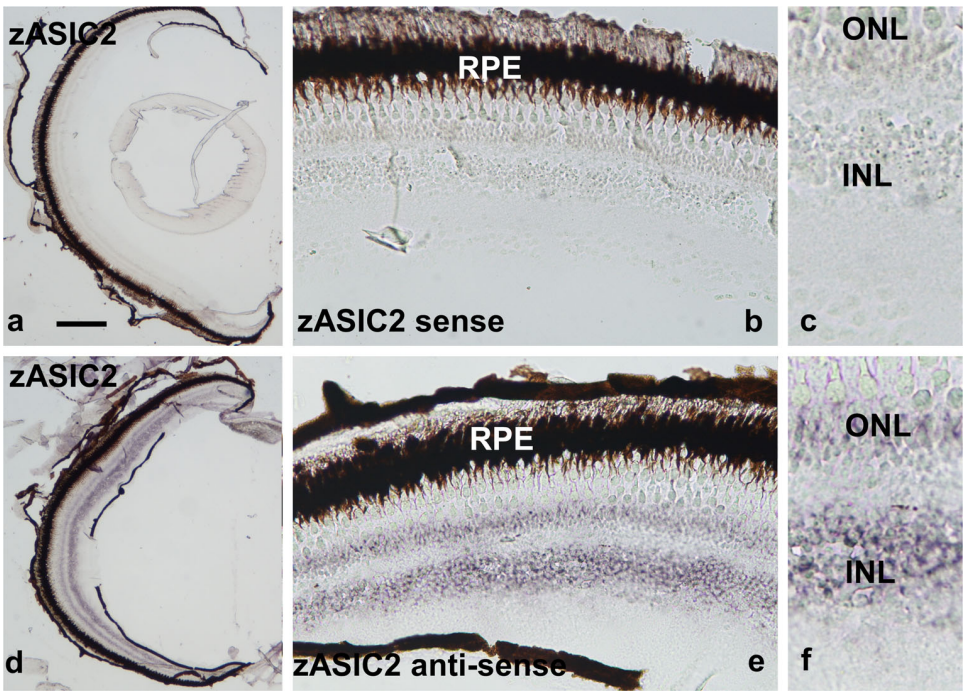

Fig. 2 Chromogenic in situ hybridization for zASIC2 in adult zebrafish retina. zASIC2 sense strand control (**a**–**c**) does not labeled the zebrafish retina whereas the zASIC2 anti-sense showed a specific staining in ONL and INL layers (**d**–**f**). Low magnification showed that probes recognized whole both layers (**d**). *Scale bars* (**a**, **d**) 100 μm, (**b**, **e**) 40 μm, (**c**, **f**) 10 μm

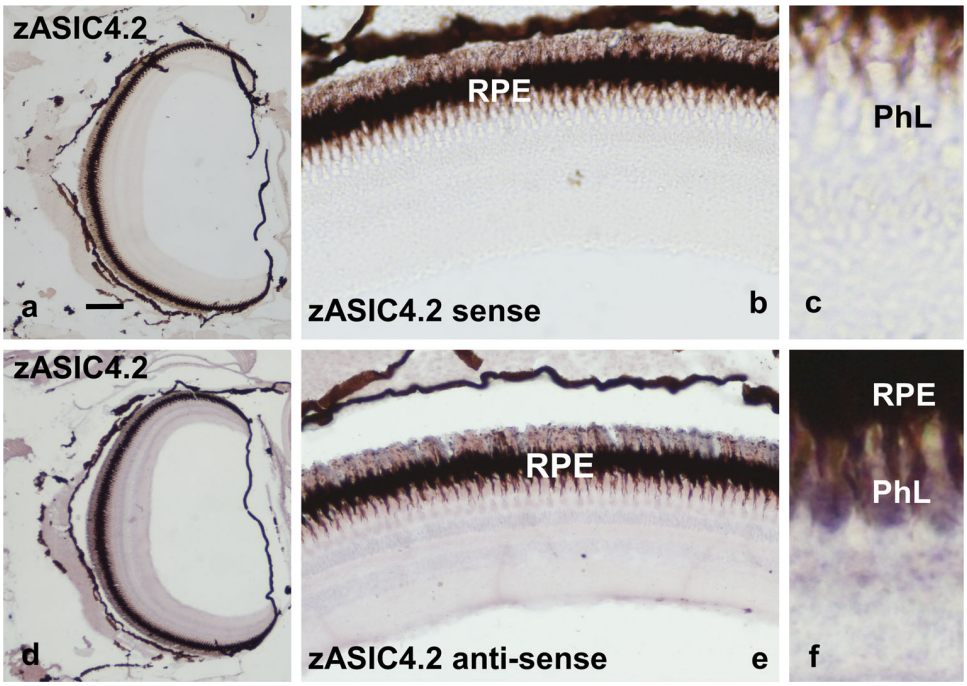

Fig. 3 Chromogenic in situ hybridization showing zASIC4.2 expression in adult zebrafish retina. Labeling with zASIC4.2 sense was not detected (**a-c**) while zASIC4.2 antisense signal was restricted to photoreceptors (PhL) and with a low intensity in retinal ganglion cells (**d-f**). *Scale bars* (**a**, **d**) 100 μm, (**b**, **e**) 40 μm, (**c**, **f**) 10 μm

expressed ASIC2, which sometimes projected to the IPL or the optic nerve layer (Fig. 4e, f). The nerve fibers of the optic nerve also displayed a strong ASIC2 immunoreactivity.

Expression of ASIC4 immunoreactivity was restricted to the external segment of the PhL without difference between cones and rods. We also detected weak but specific ASIC4 immunoreactivity in RGC and the optic nerve (tangential fibers) layer (Fig. 5a-c). Similarly, the localization of zASIC4.2 immunoreactivity was in the photoreceptors and GGC layers (Fig. 5d and e) but the homogeneity in labelling photoreceptors and the intensity of immunostaining was lower than when we used unspecific polyclonal antibody. Finally, strong ASIC2 and weak ASIC4 immunoreactivity was also observed in the brain as well as in other non-neuronal tissues used as positive controls (Figs. S3 and S4 in the Supplementary Material).

The pattern of immunoreactivity described in the retina of adult zebrafish for both ASIC2 and ASIC4 was almost identical to that observed in zebrafish larvae at the different post fecundations days (Figs. S3 and S4 in the Supplementary Material).

(see Figs. S3 and S4 in Supplementary Material).

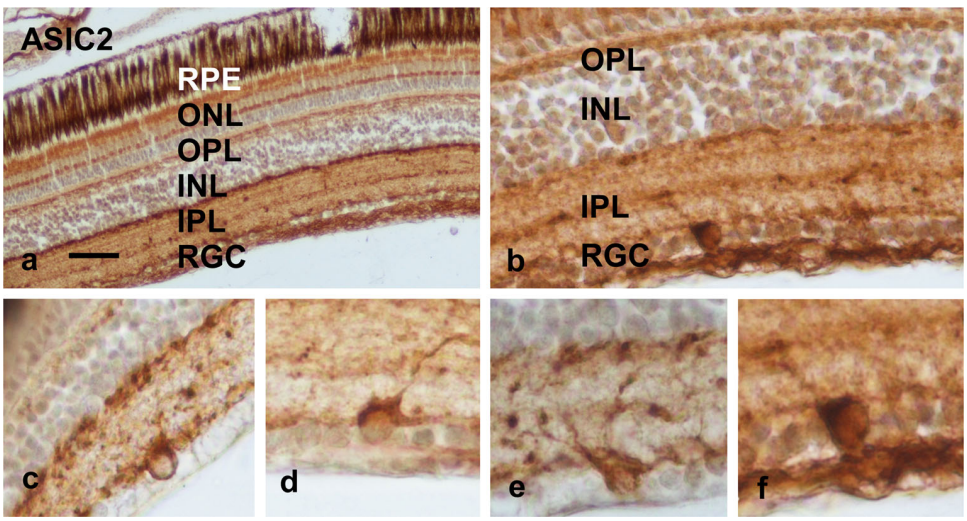

Fig. 4 Immunohistochemical localization of ASIC2 and ASIC4 in the retina of adult zebrafish. ASIC2 immunoreactivity was detected in the outer nuclear layer (*ONL*), outer plexiform layer (*OPL*), inner nuclear layer (*INL*), inner plexiform layer (*IPL*) and retinal ganglion cell (*RGC*) (**a**,**b**). Specific immunoreactivity was detected in the soma and processes of cells localized within RGC layer (**c**–**f**). Scale bars (**a**) 40 μm, (**b**) 25 μm, (**c**–**f**) 10 μm

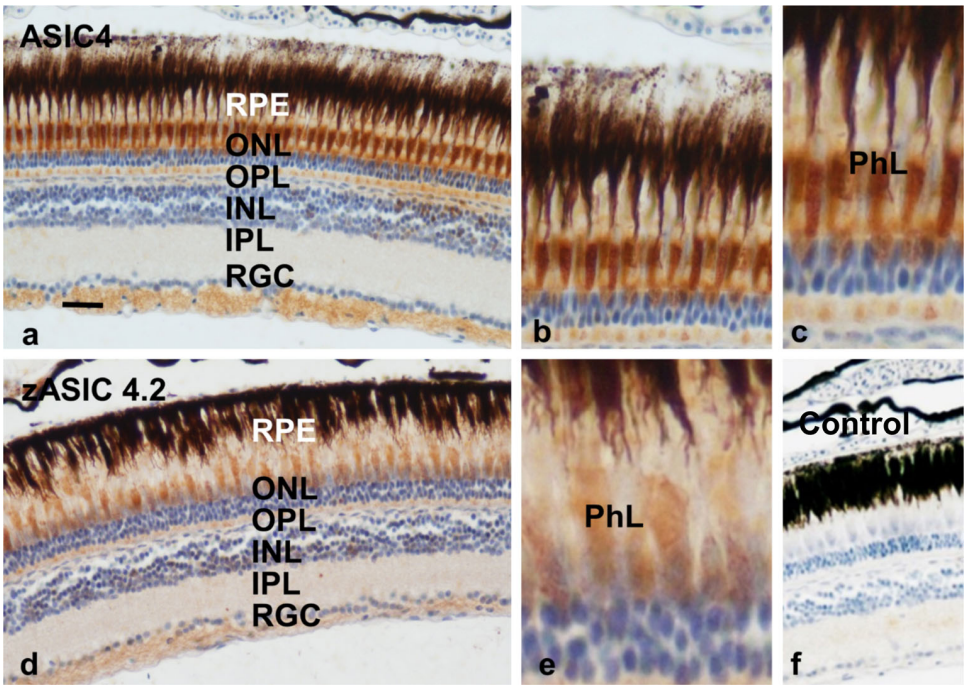

Fig. 5 Immunohistochemical localization of ASIC4 (**a-c**) and zASIC4.2 (**d**,**e**) in the retina of adult zebrafish. In both cases the immunoreactivity was detected in the photoreceptors (PhL) and retinal ganglion cell (RGC) layers. Pre-adsorbed anti-ASIC4 antibody with the specific peptide resulted in total absence of immunostaining (**f**). Scale bars (**a**, **d**, **f**) 40 μm, (**b**) 25 μm, (**c**, **e**) 10 μm

Effects of continuous light and darkness exposures in the expression of zASIC2 and zASIC4.2

The analysis of zASIC2 and zASIC4.2 mRNA expression was performed by qPCR in animals exposed to different light conditions: 12/12 h light/dark, continuous light or continuous dark. zASIC2 expression was decreased in the groups maintained under continuous light or continuous darkness in comparison with the control group (12 h light /12 h dark), without significant differences between them (Fig. 6a). zASIC4.2 mRNA expression was decreased in both experimental groups although no significant differences were observed (Fig. 6b).

Discussion

ASICs are a large family of ion channels with key roles in cell physiology since they regulate cell Na^+ and Ca^{2+} entry into the cell in response to extracellular pH variations and are involved in different physiological processes including synaptic transmission. In the present study, we demonstrated the expression and cell localization of ASIC2 and ASIC4.2 channels in the retina of adult zebrafish, based on the following evidence. First, zASIC2 and zASIC4.2 mRNAs were detected by RT-PCR in the eye and using in situ hybridization in the retina. Second, ASIC2 and ASIC4 proteins were found in the eyes by western blot. Third, the immunohistochemistry demonstrated expression of both proteins in different layers of the retina. In addition, we observed that exposure to continuous white light or darkness slightly down-regulates the expression pattern of these ion channels.

Other groups have reported that ASICs are widely expressed in both the central and peripheral nervous system as well as in some non-neuronal tissues. Of particular interest is the expression of ASICs in different sensory cells, suggesting that they are involved in several modalities of sensitivity, including light sensitivity (Hildebrand et al. 2004; Huang et al. 2006; Ettaiche et al. 2009; Del Valle et al. 2012; Viña et al. 2013). ASICs have been found in all layers of the mammalian retina (see Table 1) but, as far as we know, this is the first study reporting the distribution of ASIC2 and ASIC4 in the retina of adult zebrafish (for a review, see Bibliowicz et al. 2011; Gestri et al. 2012).

A previous study in zebrafish larvae reported expression of zASIC2 and zASIC4 mRNA restricted to the RGC (Paukert et al. 2004). The present results in adults show remarkable differences. In fact, while in larvae zASIC2 mRNA was detected only in RGC, in adult animals it was detected in ONL, INL and GRC and at the protein level was observed in ONL, RGC, both plexiform layers and optic nerve. Interestingly, the distribution on ASIC2 in larvae was almost identical to that observed in adults, thus suggesting that ASIC2 reaches a mature pattern of distribution in the zebrafish retina early in development, presumably when synaptic contact is established. The occurrence of ASIC2 in all these localizations has been demonstrated in the mammalian retina (Table 1).

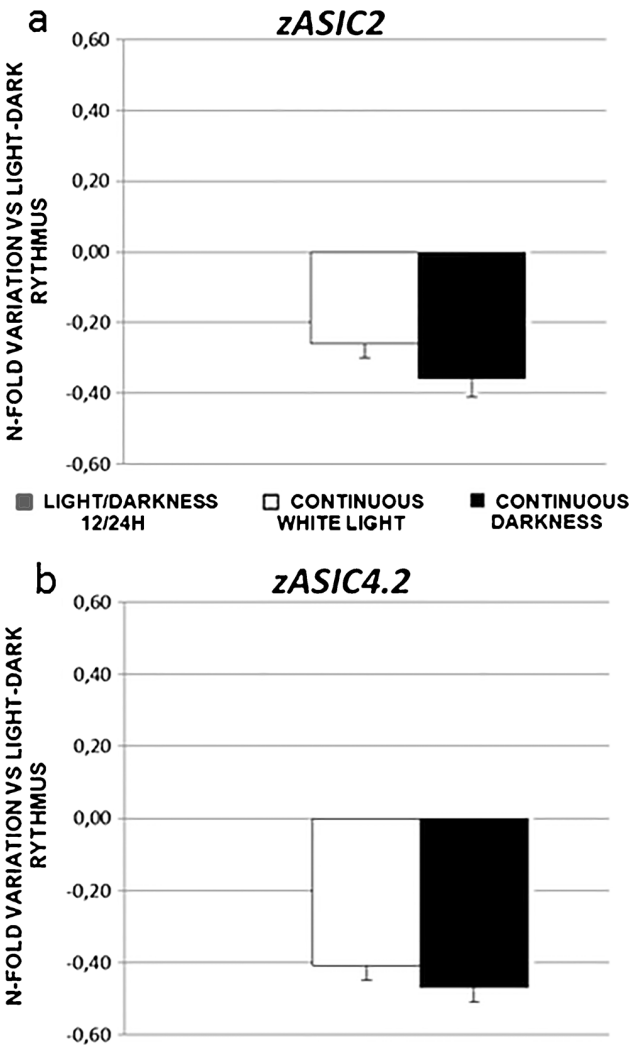

Fig. 6 Expression levels of zASIC2 (**a**) and zASIC4.2 (**b**) mRNAs in the eye of adult zebrafish after exposition for 10 days to continuous white light illumination and darkness. Values in white light–darkness (12 h/12 h) rhythm were considered as base levels. zASIC2 and zASIC4.2 mRNA levels were always decreased in all experimental groups. Continuous white light exposure: *white box*; continuous dark exposure: *black box*. Data presented as the mean ± standard error of the mean

Regarding ASIC4, there was a noticeable difference in the isoforms expressed between larvae and adults. In larvae, both isoforms of zASIC4 were detected, whereas in adults we only found zASIC4.2. It can be speculated that this is a consequence of retinal maturation and that only ASIC4.2 is necessary during adult life. Other differences between larvae and adults regard the distribution of ASIC4 mRNA. Whereas zASIC4 mRNA is expressed only in RGC of larvae, in adults both photoreceptors and RGC express ASIC4 at both mRNA and protein levels. Immunoreactivity for ASIC4 was also detected in the photoreceptors of zebrafish larvae but not in RGC. These findings suggest that the expression of ASIC4 in the zebrafish retina undergoes maturational changes but detailed age-related studies are necessary to elucidate this aspect. Expression of ASIC4 in RGC has also been reported in rabbits (see Table 1).

The biological role of ASICs in the retina of vertebrates and zebrafish is still poorly understood. ASICs respond to extracellular acidification by regulating transmembrane Na^+, K^+, or Ca^{2+} flux (Kress and Waldmann 2006). Since ASICs are sensors of dynamic transient fluctuations in local pH, it is likely that small pH variations around photoreceptors after light exposure (Chesler and Kaila 1992; DeVries 2001) will activate ASICs present in retinal cells. It is known that inactivation of the ASIC2 gene increases the light response of the retina and makes the retina more sensitive to light-induced degeneration (Ettaiche et al. 2004), therefore activation of ASIC2 in the retina after light exposure could modulate synaptic activity and protect cells against light-induced damage. In our experiments, there was a tendency for down-regulation of both ASIC2 and ASIC4.2 mRNAs after exposition to continuous light or continuous dark compared with those exposed to a 12-h cycle of light/dark. Nevertheless, more experiments are necessary to establish whether or not light has any role in regulating the expression of ASICs in the retina of adult zebrafish.

During the course of several retinal diseases, there is decreased extracellular pH in the retina, thus suggesting that ASICs may be a target worth pursuing (Fadool and Dowling 2008). The data described in this manuscript suggest that zebrafish could be used as a model organism for drug testing targeted to ASICs.

Acknowledgment The antibody against ASIC4.2 zebrafish was kindly provided by Dr. Greg Goss, University of Alberta, Canada.

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ACID-SENSING ION CHANNELS (ASICs) 2 and 4.2 ARE EXPRESSED IN THE RETINA OF THE ADULT ZEBRAFISH

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SUPPLEMENTARY MATERIAL

MATERIAL AND METHODS

Animals and treatment of the tissues.- Forty zebrafish (*Danio rerio*) at larval stage 7, 10, 20, and 30 days post-fertilization (dpf) were used in this study. The age was determined according to Kimmel et al. (1995). Five animals per age group were used for immunohistochemistry. The fish were obtained from CISS, crossed, maintained and sacrificed as described in the main text.

Immunohistochemistry.- The animals were fixed *in toto* in Bouin's fixative for 24 h at 4°C, and then the heads removed and routinely processed for paraffin embedding. The pieces were cut in serial horizontal sections 10 µm thick, and collected on gelatin-coated microscope slides as previously described (Germanà et al., 2010). The sections were then processed for indirect peroxidase immunohistochemistry as described in the main text, using the same antibodies and incubation conditions.

RESULTS

Immunohistochemical localization of ASIC2 and ASIC4 in the retina of zebrafish larvae.- The localization of ASIC2 in zebrafish larvae from 7 to 30 dpf demonstrate the presence of this protein in ONL, OPL, IPL and RGC, as well as in the nerve fibers of the optic nerve (Fig. S3). Intense ASIC2 immunoreactivity was also detected in the brain and the nerve fibres of the cranial nerve ganglia; in these places some sensory neurons also displayed ASIC2 immunoreactivity.

The distribution of ASIC4 in the retina of zebrafish larvae was restricted to the photoreceptors at all ages sampled (7 to 30 pfd; Fig. S4). In adult animals positive immunostaining was observed on some mechanosensory cells of the cristae ampullaris (Fig. S4c) and superficial taste buds (Figs. S4d,e). The presence of ASIC4 in these localization has been previously demonstrated (Viña et al., 2013).

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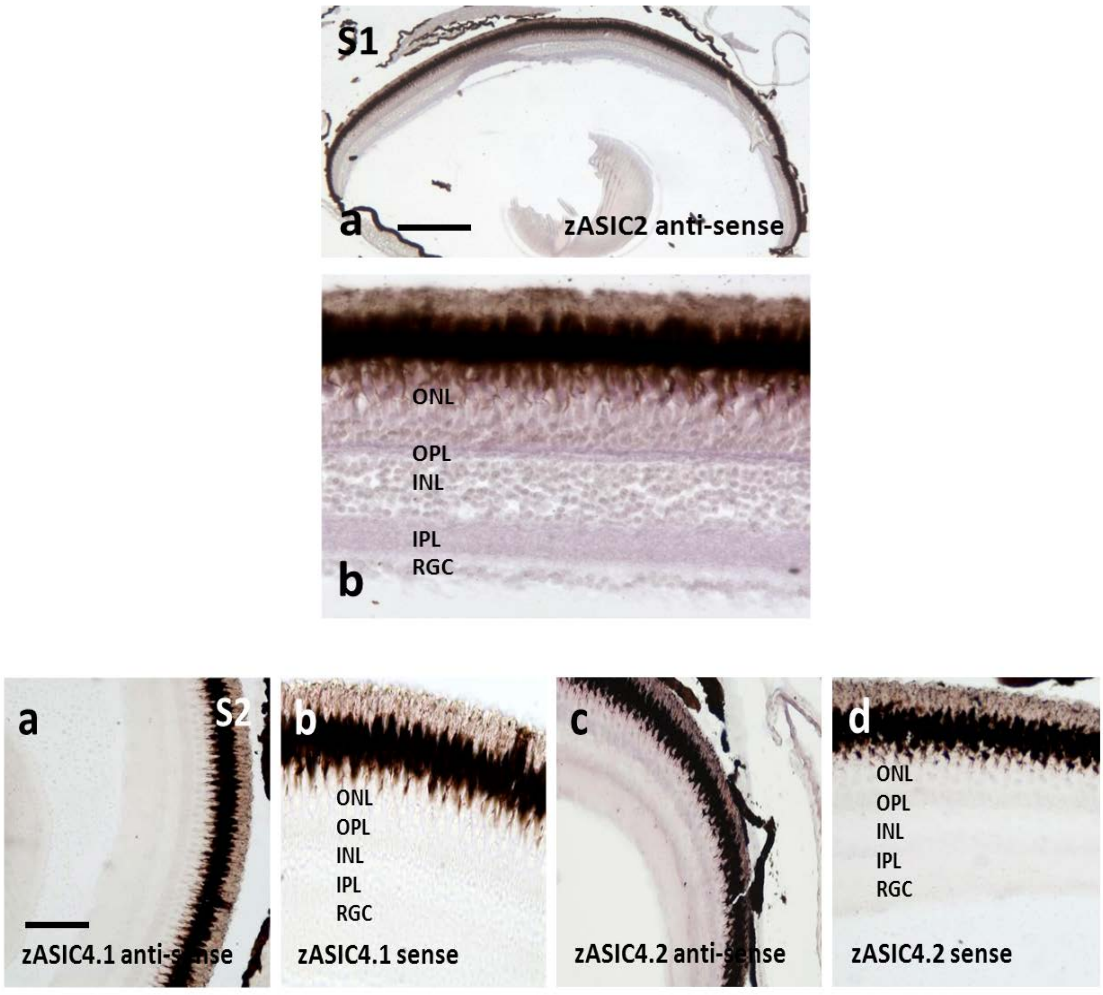
Figure S1. Chromogenic *in situ* hybridization for *zASIC2* in adult zebrafish retina. *zASIC2* anti-sense showed a specific staining in ONL, INL and RGC layers. Low magnification showed that probes recognized whole both layers. ONL: outer nuclear layer, OPL: outer plexiform layer, INL: inner nuclear layer, IPL: inner plexiform layer, RGC: retinal ganglion cells. Scale bar: 100 µm for **a**, and 10 µm for **b**.

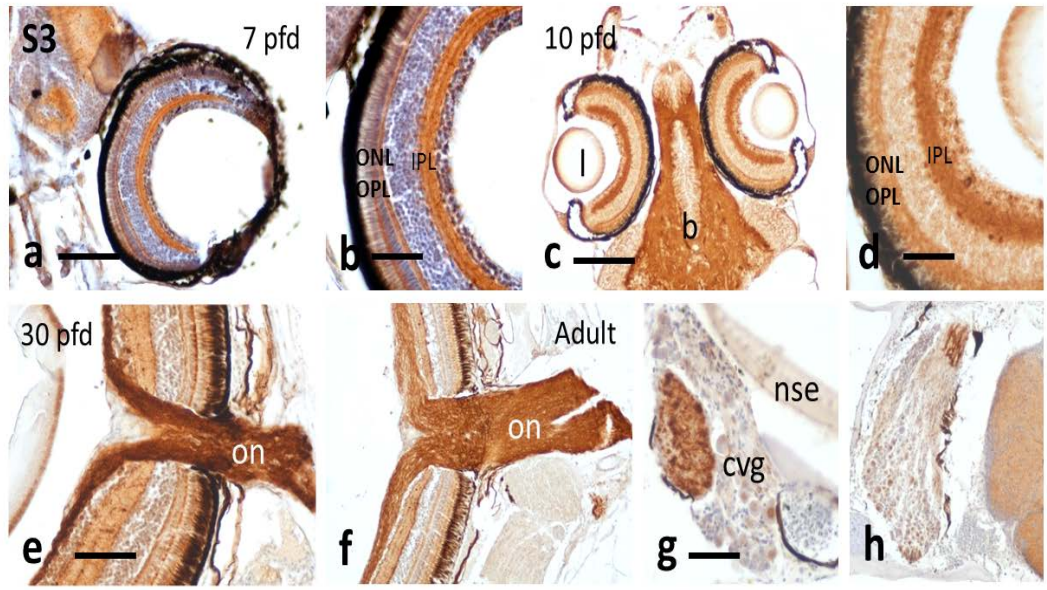
Figure S2. Chromogenic *in situ* hybridization for *zASIC4.2* in adult zebrafish retina. Labeling with zASIC4.1 sense (**b**) or zASIC.1 anti-sense (**a**) was not detected while zASIC4.2 anti-sense signal was observed in photoreceptors (PhL) and with a low intensity in retinal ganglion cells (**d**), but not with zASIC4.2 sense. Scale bar: 40 µm for **a**, **c** and **d**; 25 µm for **b**.

Figure S3. Immunohistochemical localization of ASIC2 in the retina, the brain, and the sensory ganglia of zebrafish larvae and adults. In 7 pfd (**a**,**b**) ASIC2 immunoreactivty was present in ONL, both plexiform layers, and in isolated cells of the RGC layer. By 10 pfd (**c**,**d**) the distribution of ASIC2 was identical. By 30 days (**e**) pfd and later, until adult life (**f**) the patter of ASIC2 immunostaining in the retina was unchanged. Intense immunostaining was observed in the nerve fibers of the optic nerve. Outside the retina

ASIC2 was strongly expressed in the brain (**c**) and the nerve fibers of the cranial nerve sensory ganglia (**g**, **h**). Faint ASIC2 was also found in some sensory neurons of those ganglia. b: brain; cvg: cochleovestibular ganglion; on: optic nerve; nse: neurosensoy epithelium of the utricle. Scale bar: 70 µm for **a**; 100 µm for **c**; 50 µm for **b** and **d**; 40 µm for **e** and **f**; 100 µm for **g** and **h**.

Figure S4. Immunohistochemical localization of ASIC4 in the retina, the brain, and sensory organs of zebrafish larvae and adults. In 7 pfd (**a,b**) and 10 pfd (**c,d**) larvae retinal ASIC4 immunoreactivity was restricted to the photoreceptors. In some sensory cells of the ampullary crest of the inner ear (**e**), and of the taste buds (**f,g**) of adult zebrafish high expression of ASIC4 was observed. ac: ampullary crest; tb: taste buds. Scale bar: 100 µm for a and b; 40 µm for **e** and **f;** 10 µm for **g**.

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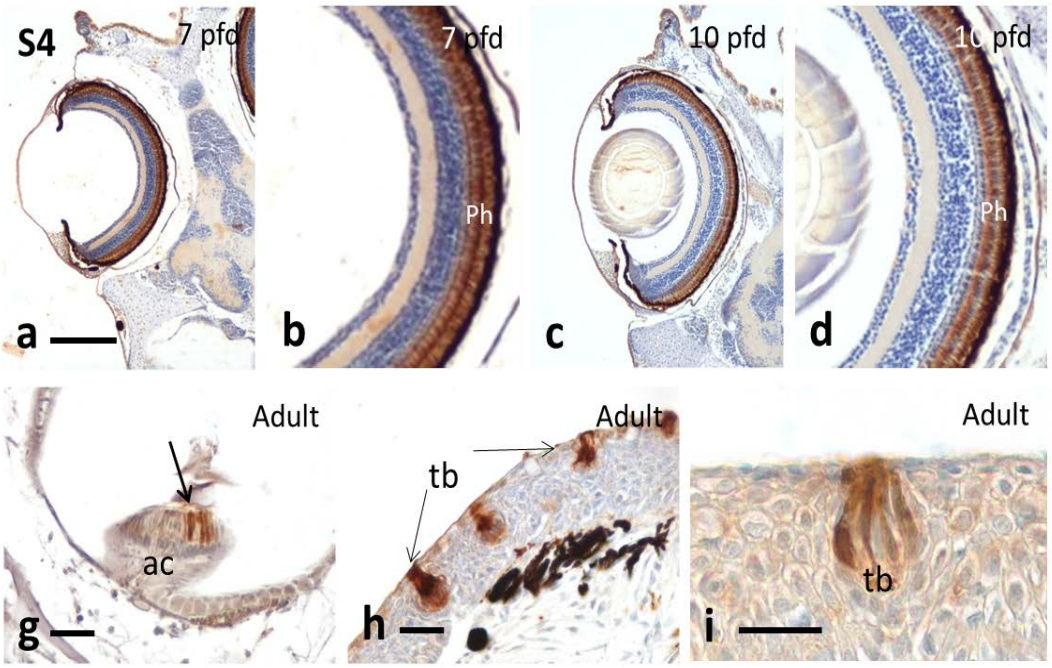
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5.5. INFORME FACTOR DE IMPACTO DE LAS PUBLICACIONES

Tesis Doctoral presentada por Don Eliseo Viña Fernández como compendio de publicaciones.

Número de artículos publicados: 4

Factor de impacto de las publicaciones según el Journal Citation Report:

Revista	Año	Factor de Impact	Categoría	Puesto en la categoría	Revistas en la categorí
Microscopy Research andTechnique	2012	1.593	Anatomy & Morpholgy	10	21
Neuroscience Letters	2013	2.055	Neuroscienc es	212	252
Histochemistry and Cell Biology.	2015 *	2927	Microscopy	2	11
Cell and Tissue Research	2015 *	3.333	Cell Biology	60	185

***iSi web of Knowledge 2013

Oviedo, a 12 de Marzo de 2015

Directores de la Tesis Tutor de la Tesis

Olivia García Suárez Roberto Cabo Pérez José Antonio Vega Alvarez

DISCUSION

6. DISCUSION

El presente estudio se inscribe en la línea de investigación que durante los últimos 10 años se desarrolla en el Grupo SINPOS del Departamento de Morfología y Biología Celular de la Universidad de Oviedo, en colaboración con el Departamento de Scienze Veterinarie de la Universidad de Messina (Italia) y que está dedicada al estudio de los órganos sensoriales de *Danio rerio*. Las contribuciones de esta línea de estudio se recogen en sucesivas publicaciones internacionales a partir del año 2002. En este trabajo de tesis doctoral se aportan nuevos datos sobre la presencia, localización celular y posible función de dos tipos canales iónicos (ASICs y TRPV4) en este modelo animal.

La supervivencia de un organismo depende en gran medida de su capacidad de respuesta y adaptación al medio ambiente que lo rodea y una de las formas de interactuar es a través de las distintas modalidades sensoriales. Para ello, todos las especies animales han desarrollado estructuras sensitivas especializadas, los órganos sensoriales, los cuales presentan receptores específicos que les permiten detectar diferentes estímulos (Lieschtke y Cols., 2007; Hickman, 2009).

Actualmente se conoce que los canales iónicos TRP y ASIC juegan un papel fundamental en la transducción de distintas modalidades somatosensoriales, incluidas el tacto, gusto, olfato, osmolaridad y sensación térmica, en un gran rango de especies animales, desde mamíferos a peces y nemátodos (Montell, 2005, Bandell y Cols., 2007). En este sentido, se considera que la capacidad de los diferentes tipos de

neuronas sensitivas para detectar y traducir estímulos específicos resulta de la activación de diferentes canales iónicos simultaneamente (ver Belmonte y Viana, 2008).

Por tanto, el trabajo de esta tesis se planteó teniendo en cuenta que la función transductora de los canales viene determinada por su distribución anatómica.

En el primer artículo incluido en esta investigación se analizó la presencia del canal TRPV4 en los órganos sensoriales del pez cebra adulto. El TRPV4 es un miembro de la sub-familia TRP vanilloide implicado en la mecano- y quimio-sensación, que también responde a las temperaturas cálidas (umbral de 25-35^{o}C) y a los pH ácidos (Plant y Strottmann, 2007).

Usando la técnica de la PCR se demostró la existencia mRNA para TRPV4 en la cabeza de *Danio rerio* adultos. Este hallazgo concuerda con los datos aportados en larvas de este teleósteo por Mangos y Cols. (2007), lo que sugiere queTRPV4 se expresa a lo largo de toda la vida del individuo. Mediante Western-blot se detectó una banda de 94 kDa que se corresponde con el peso molecular estimado para TRPV4 en mamíferos, hecho que está en consonancia con la elevada preservación de los canales TRP en la evolución filogenética (Plant y Strotmann, 2007).

Los sistemas sensores de los teleósteos, incluido el pez cebra, consisten en estructuras especializadas capaces de detectar estímulos mecánicos, químicos o la luz (Ostrander, 2000). Las células mecanosensoras están

agrupadas en neuromastos profundos y superficiales que forman el sistema de la línea lateral y en el epitelio del oído interno; las células quimiosensoras están agrupadas en las papilas gustativas y en el epitelio olfatorio; y las células sensibles a la luz están en la retina. En este primer artículo se estudió, por tanto, la distribución del canal TRPV4 en los órganos sensoriales.

Respecto a los mecanorreceptores, se observó que el TRPV4 se localiza en una subpoblación de células ciliadas, tanto en los neuromastos como en el epitelio sensitivo del oido interno. La expressión de TRPV4 en los neuromastos de pez cebra había sido previamente demostrado por Mangos y Cols. (2007) en las células del manto de larvas. Nuestro trabajo en adultos, añade datos nuevos ya que también lo hemos detectado en algunas células ciliadas. Por otro lado, ponen de manifiesto que la expresión de este canal se mantiene durante toda la vida del animal. Un dato sorprendente y que merece ser destacado es que la presencia de TRPV4 difiere entre los dos tipos topográficos de neuromastos: los superficiales y los que se encuentran dentro del canal. La razón de este hecho es desconocida, aunque ya se había observado esta misma variabilidad para la expresión de otras proteínas como la S100 (Germanà y Cols., 2007). La presencia de TRPV4 en las células sensitivas de los neuromastos y del oido interno hace pensar que este canal iónico está implicado en la mecanotrasducción como se había sugerido anteriormente por diferentes autores (Orr y cols., 2006; Plant y Strottman, 2007; Damann y cols., 2008; Tsunozaki y Bautista, 2009). Esta hipótesis resulta más atractiva teniendo en cuenta que algunos

experimentos sugieren que los canales TRP juegan un papel en la fisiología del oído interno y en la audición en humanos (Cuajungco y Cols., 2007). Sin embargo, en nuestro estudio no se ha encontrado TRPV4 ni en el epitelio sensorial ni en el ganglio cocleo-vestibular, como sucede en la rata (Kitahara y Cols., 2005).

Hasta el momento, en el pez cebra se han clonados seis zASICs, y no se ha identificado el ortólogo del ASIC3 de mamífero. Los zASIC tienen masas moleculares similares a las de los mamíferos, un 60 a 75% de homología entre aminoácidos y presentan un alto grado de conservación en la evolución (Paukert y Cols., 2004). En el presente trabajo de tesis doctoral se abordó en primer lugar el estudio de la presencia de los ASIC a nivel proteico en el pez cebra mediante estudios de Western-blot. Nuestros resultados son coincidentes con las masas moleculares predeterminadas para ASIC1, ASIC2 y ASIC 4, como cabía esperar, con el anticuerpo contra ASIC3 no se identificó ninguna proteína.

En cuanto a la distribución celular de los ASICs en las papilas gustativas existen diferencias entre el pez cebra y los mamíferos. En nuestro trabajo se detectaron ASIC2 y ASIC4, pero no ASIC1, aunque la proteína se encontró por Western-blot y se observó inmunorreacción para ASIC1 en células situadas en el cerebro y en el sistema nervioso periférico. Nuestros resultados no concuerdan con los aportados por Huque y Cols. (2009) en humanos que sí observaron expresión de ASIC1 mRNA en papilas gustativas. Respecto al ASIC4, todos los tipos celulares de las papilas, independientemente de su localización, fueron positivas aunque

en algunos casos ASIC4 solo se detectó en las células sensoriales. Estos datos son semejantes a los observados en humanos (Huque y Cols., 2009). En cuanto al ASIC2, los resultados en pez cebra demuestran que solo está presente en los nervios que suplen las papilas, lo que coincide básicamente con los datos existentes para la rata, pero no el ratón (Richter y Cols., 2004a; Ritcher y Cols., 2004b; Shimada y Cols., 2006; Ugawa y Cols., 2003).

En general, las diferencias interespecíficas en la expresión de los ASICs en las papilas gustativas se podrían atribuir a los diferentes medios en que se desenvuelve la vida de cada especie (acuático VS. terrestre) así como a los distintos hábitos alimentarios, aunque es necesario realizar más estudios con el fin de arrojar luz sobre esta cuestión.

La presencia de los ASIC en las papilas gustativas, confirma la idea que sostienen algunos estudios funcionales sobre un papel de estos canales iónicos en el gusto. Así, aunque ASIC2 se ha relacionado con la detección del sabor ácido en mamíferos (Lin y Cols., 2002; Lin y Cols., 2004; Liu y Simon 2001; Ugawa y Cols., 1998; Ugawa, 2003; Ugawa y Cols., 2003), las respuestas de comportamiento a los estímulos con sabor ácido no se alteran en ratones que carecen del gen ASIC2 (Kinnamon y Cols., 2000; Ugawa y Cols., 2003) lo que demuestra que ASIC2 no es un receptor del sabor ácido en ratones, aunque la transducción de ácido en ratas coincide con las propiedades de los ASIC2 (para una revisión ver Ugawa , 2003). Se cree que los protones actúan sobre los canales ASIC y despolarizan células receptoras gustativas dando lugar a una afluencia de Ca^{2+} extracelular lo

que, a su vez, llevá a liberar los neurotransmisores en las sinapsis de las fibras aferentes sensoriales gustativas (Miyamoto y Cols., 2000; Ritchter y Cols., 2004a; Stevens y Cols., 2001; Ugawa y Cols., 1988, 2003). Sin embargo, en pez cebra los experimentos funcionales han demostrado que zASIC1.1, zASIC1.2, zASIC1.3 o zASIC14.1 son sensibles al ácido, mientras que zASIC2 y zASIC4.2 no (Paukert y Cols., 2004). Por lo tanto, el posible papel de ASIC2 y 4 en las papilas gustativas de pez cebra es difícil de hipotetizar. En cualquier caso, ya que los ASIC están presentes en las células gustativas y dependen de distintas señales de Ca^{2+} para generar respuestas celulares adecuadas (Dvorscak y Marfurt, 2008) se puede hipótetizar que estos canales iónicos en las células del gusto están relacionados con la detección del sabor o con otras funciones desconocidas.

Otros de los aspectos abordados en la presente tesis doctoral fue el de la expresión y distribución del ASIC2 en el epitelio olfatorio de pez cebra adulto. Se partió de la premisa de que la presencia de ASIC2 en uno o más tipos de neuronas olfatorias es una evidencia indirecta de la implicación de este canal iónico en la olfación. ASIC2 tiene una amplia distribución en el sistema nervioso central y periférico (Ver Holzer, 2009; y Sherwood y Cols., 2012) además de en tejidos no neuronales (Babinsky y Cols., 2000; Jahr y Cols., 2005; Cuesta y Cols., 2014). En el pez cebra, se ha detectado en el cerebro, tanto de individuos adultos como en desarrollo (Paukert y cols., 2004; Levanti y cols., 2011), en el sistema nervioso entérico y en los nervios periféricos (Levanti y Cols., 2011; Viña y Cols., 2013). Sin embargo,

ASIC 2 no se ha localizado fuera del sistema nervioso en este modelo animal.

Nuestro trabajo ha demostrado por primera vez que ASIC2 se encuentra en la roseta olfatoria del pez cebra tanto a nivel de mRNA como de proteína, quedando restringida su expresión al epitelio no sensorial de la misma, concretamente los cilios de las células epiteliales ciliadas. Este resultado se verifico mediante doble inmunohistoquímica con microscopía confocal, al observarse que ASIC2 se co-localiza con β-tubulina, marcador del citoesqueleto de los cilios. A la luz de estos resultados puede deducirse que ASIC2 no participa en la olfación pero sí probablemente en otras funciones desconocidas hasta el momento. Existen datos previos sobre la presencia del ASIC2 en las células ciliadas y esteriociliadas de diferentes órganos de rata (traquea, oviducto, epididimo, órgano de Corti y cresta ampular), incluido el septo epitelial olfatorio (Kikuchi y Cols., 2008, 2010). Estos estudios demostraron que las células ciliadas tenían canales ASIC2 en la membrana ciliar mientras que las células con estereocilios presentaban ASIC2 en el citoplama. Nuestros resultados muestran que el ASIC2 no se encuentra nunca en el citoplasma de las células ciliadas sino en la membrana y en los cilios.

Hansen y Zeiske, (1998) observaron que el epitelio no sensorial de la roseta olfatoria de los teleósteos, incluido el pez cebra está tapizado con células ciliadas típicas, y sus cilios presentan una organización estructural con un patrón de microtubulos 9+2. Nuestros resultados confirman estas observaciones.

Actualmente, el cilio primario se considera como un sistema mediante el cual las células sienten y transducen los estímulos mecánicos y químicos (Prasad y Cols., 2014) y se tranforman los estímulos extracelulares en señales intracelulares (Muhammad y Cols., 2012). Hay que hacer notar que la membrana de los cilios contiene múltiples proteinas sensoras y canales iónicos, incluidos canales permeables al Ca^{2+} que juegan un papel en la mecanosensación mediada por Ca^{2+} (Anishkin y Kung, 2013). Entre estos canales iónicos muy probablemente se encuentra el ASIC2 (Kikuchi y cols., 2008, 2010). Pero, su posible papel en el cilio está por aclarar. Las funciones mecano y quimio sensoras del cilio (Prasad y cols., 2014) son las mismas que las atribuidas al ASIC2 (Sherwood y cols., 2012) lo que da pie a especular que el ASIC2 es la base molecular de las funciones del cilio.

Respecto a la función quimiosensora del ASIC2, se reconoce que responde a variaciones de pH. En mamíferos existen dos isoformas de ASIC2, ASIC2a y ASIC2b, las cuales tienen diferentes valores de activación por pH (Lingueglia y cols., 1996; Holzer y cols., 2009). Sin embargo, en el pez cebra solo existe una isoforma de ASIC2 (Pauker y cols., 2004) que presumiblemente asume todos los papeles de las dos isoformas de los mamíferos. Nosotros hemos observado en este trabajo que ASIC2 está localizado en los cilios del epitelio olfatorio del pez cebra. Kikuchi y cols. (2010) proponen que el ASIC2 puede estar en relación con la supervivencia y capacidad de las células ciliadas del septo nasal de la rata para detectar los cambios dinámicos del pH del ambiente. Sin embargo, este papel es difícil de extrapolar a los peces ya que el pH en los ambientes acuáticos es bastante homogéneo.

El posible papel mecanosensor del ASIC2 no ha sido definitivamente clarificado (Lingueglia, 2007). De hecho, los datos obtenidos para los ratones null de ASIC2 (Price y cols., 2000) indicavan que el ASIC2 es un componente central del complejo mecanosensor, pero no es una parte del canal abierto mecánicamente para la mecanosensación (Roza y cols., 2004).En los peces la detección de los movimientos del agua esta habitualmente atribuida a las células ciliadas de los neuromastos del sistema de la línea lateral (Bleckmann y Zelick, 2009; Montgomery y cols., 2009) pero los hallazgos aportados por nuestro estudio sugieren que los movimientos del agua que entra en la fosa nasal en el pez pueden ser también detectado por los cilios de la roseta olfatoria. Por tanto, a pesar de los intensos esfuerzos por detectar esta molécula en la roseta olfatoria, queda por delante una línea de investigación para poder dilucidar su función en el epitelio olfatorio.

Clásicamente el epitelio olfatorio en peces se dividía en epitelio sensorial y no sensorial. Sin embargo si la presencia de ASIC2 en el epitelio olfatorio del pez cebra es una evidencia de detección mecánica o química por el epitelio no sensorial, toda la lamela de la roseta olfativa sería sensorial. En base a ello proponemos la división del epitelio en olfativo y no olfativo, en vez de sensorial y no sensorial. Esta asunción viene avalada, además, por la primera aportación de esta tesis en la que se encontraron células TRPV4 positivas en el epitelio no sensorial de la roseta (Viña y cols., 2013; Parisi y Cols., 2014).

La parte final de nuestro trabajo se centró en el estudio de los canales iónicos ASIC en la retina. Estos canales se han detectado en todas las capas y tipos celulares de la retina de los mamíferos. Sin embargo, los datos en *Danio rerio* son muy escasos y la distribución de ASIC2 y ASIC4 en la retina del pez cebra adulto no se ha estudiado previamente, a pesar de que esta especie animal está consolidada como modelo en la investigación de patologías asociadas a la retina en humanos (ver para revision Bibliowicz y cols., 2011; Gestri y cols., 2012).

Como se ha mencionado previamente en el estado actual del tema, en el pez cebra existe una isoforma de ASIC2 (zASIC2) y dos de ASIC4 (zASIC4.1 y zASIC4.2). A nivel de mRNA, mediante las técnicas de RT-PCR e hibridación in situ, hemos demostrado que en el pez cebra adulto existe solamente zASIC2 y zASIC4.2. Estos resultados no coinciden con los de Pauker y cols. (2004) que describen todas las isoformas para estos genes en las larvas del *Danio rerio*. Ello lleva a hipotetizar que la desaparación de ASIC4.1 en el adulto está en relación con la maduración retiniana y que solamente ASIC4.2 es necesario en la vida adulta. Nuestra aportación respecto a la localización celular de los mRNA de ASIC2 y ASIC4.2 evidencia que la expresión en adultos es más amplia que en las larvas. El ASIC4.2 solo se observaba en las RCG de la retina en estado embrionario (Paukert y cols., 2004), mientras que en los animales adultos se encontró también en los fotorreceptores. Así pues, se puede asumir que la maduración de los fotorreceptores en el pez cebra esta acoplado a la expresión de ASIC4, aunque el papel que juega en estas células todavía es desconocido.

Respecto a la expresión de ASIC2 mRNA estudiada mediante hibridación in situ, en el pez cebra adulto se detectó en ONL, INL, y GCL, mientras que en larvas Paukert y Cols.(2004) solo lo observaron en GCL, lo que nos sugiere que la maduración de la retina causa una reorganización en las células que expresan zASIC2.

Los hallazgos del presente estudio son los primeros que identifican y localizan ASIC2 y ASIC4.2 a nivel proteico en la retina de *Danio rerio* adulto. En homogenizados de ojo se encontró ASIC2 y ASIC4 mediante Western-blot y por inmunohistoquímica se comprobó la distribución celular. ASIC2 se localizó en GCL y ambas capas plexiformes, mientras que ASIC4 estaba localizado en los fotorreceptores y la GCL. Existe una concordancia total para ASIC4 entre la distribución del mRNA y la proteína. Sin embargo aparecen unas ligeras disferencias respecto al ASIC2 que son fáciles de explicar: la presencia de la proteína en las capas plexiformes puede ser debida a su exportación a la zona sináptica de las neuronas bipolares o a los fotorreceptores.

Hasta la fecha, existe poca información respecto a la función de los ASICs en la retina. Se sabe que en ratones la inactivación de ASIC2 incrementa la respuesta a la luz y la sensibiliza hacia una degeneración inducida por la misma (Ettaiche y Cols., 2004).

En la retina tienen lugar cambios en el pH extracelular que afectan directamente a su actividad y a la fototransducción. En este sentido las fluctuaciones de pH se cree que están implicadas en el ajuste de la percepción visual y en la adaptación de las respuestas de la retina a las diferentes intensidades de luz (Ettaiche y Cos., 2004). Por su parte, los

ASIC son canales iónicos sensibles al ácido que responden a H^+ y son sensibles a fluctuaciones transitorias de pH locales. Así pues, las variaciones en las concentraciones de pH asociadas con la actividad de los fotorreceptores son probablemente la principal causa de activación de los ASIC presentes en las células fotorreceptoras retinianas. La luz causa alcalinización sináptica (Chesler y Cols., 1992) mientras que la activación de los canales de Na^+ dependientes de ligando produce acidificación. Mientras la acidificación se previene bloqueando la bomba de protones, la re-alcalinización puede anularse bloqueando los canales iónicos permeables a protones. Estos cambios ocurren en la OPL, que es donde se encuentra el ASIC2 y por tanto este canal iónico podría estar implicado en estos procesos (Wang y Cols., 2014). Además, en las capas plexiformes es donde tienen lugar los principales contactos sinápticos de la retina, y la actividad neuronal genera cambios de pH (Kristhal, 2003). Finalmente, ASIC2 y ASI4.2 también aparecen en las células ganglionares, las cuales presentan una alta actividad metabólica que produce una acidificación intersticial transitoria entorno del nervio óptico durante la neurotransmisión en la hendidura sináptica (Palmer y cols., 2003). Por otro lado, se han encontrado evidencias de que el pH de la retina sigue un ritmo circadiano (Dmitriev y Mangel 2001) y que la estimulación de la retina por luz induce cambios significativos en el pH extracelular modulando el flujo de información visual dentro de la retina (Barnes y Cols., 1993;). Al analizar el efecto de la exposición continua a luz y oscuridad sobre la expresión de zASIC2 y zASIC4.2 se observó un significativo descenso en los mRNAs para ambas moléculas en las dos condiciones experimentales sugiriendo que una variación de pH produce

cambios significativos en ambos canales en la retina el pez cebra adulto. Algunas patologías retinianas se correlacionan con una disminución del pH extracelular y por tanto los canales iónicos sensibles al ácido representan un objetivo a valorar (Fadool y Douling, 2008). En particular ASIC1a parece jugar un papel en la muerte de GCL inducida por hypoxia (Tan y Cols., 2011) y protege al epitelio retinal pigmentario del estres oxidativo (Tan y Cols., 2013). ASIC2 es un modulador negativo de la fototrasducción en los bastones, y participa en el mantenimiento de la integridad retiniana, ya que los ratones *ASIC2* knock-out son más sensibles a la degeneración retiniana inducida por la luz (Ettaiche y Cols., 2004). Además, el bloqueo de los ASICs podría tener un potencial efecto neuroprotector en las enfermedades debidas a la isquemia ocular (Miyake y Cols., 2013).

Con este trabajo de tesis doctoral creemos haber contribuido de manera notable al conocimiento de los canales iónicos ASICs y TRPV4 en los órganos sensoriales del *Danio rerio*. Las evidencias científicas indican que cada vez está más claro que estos canales iónicos son fundamentales en la fisiología sensorial. Los resultados aportados por nuestro estudio demuestran que tanto el TRPV4 como los ASIC2 y ASIC4 se encuentran en todos los órganos sensitivos del pez cebra a estudio, asociados principalmente a células sensoriales, si bien en el epitelio olfatorio también se detectan en el denominado epitelio no sensorial. Dado que su función trasductora viene defendida en gran medida por su distribución anatómica, nuestros datos refuerzan la importancia de estas proteínas en

la fisiología de las distintas modalidades sensoriales, así como en la transmisión sináptica.

La demostración de la presencia y distribución de los canales iónicos TRPV4, ASIC2 y ASIC4.2 en un modelo animal de tan fácil manejo como el pez cebra es de gran interés porque pueden ayudar a definir el papel funcional de estos canales en el ámbito de la fisiología animal y encontrar nuevas alternativas terapeúticas basadas en el bloque de dichos canales para el el tratamiento del dolor, glaucoma, sordera…etc.

De todo lo anteriormente expuesto se deduce que el estudio de estos canales resulta tan interesante como necesario ya que son posibles dianas terapeúticas farmacológicas. El desarrollo de compuestos que reviertan o controlen su actividad tanto en condiciones de normalidad como patológicas es un campo que ofrece gran potencialidad de investigación, y este estudio ha validado al *Danio rerio* como modelo animal para llevarlos a cabo.

CONCLUSIONES

7. Conclusiones

En base a los resultados obtenidos y a la oportuna discusión de los mismos, hemos llegado a las siguientes conclusiones:

1. El canal TRPV4 se detecta en el pez cebra adulto a nivel de mRNA y proteína. Su distribución en los órganos de los sentidos se circunscribe a: una subpoblación de células ciliadas en los neuromastos y el oído, las neuronas crípticas y ciliadas del epitelio olfatorio, células sensoriales de las papilas gustativas y células amacrinas en la retina.

2. Las células sensoriales de las papilas gustativas del pez cebra adulto expresan ASIC4, mientras que los nervios que las suplen expresan ASIC2. Mediante Western-blot también se demostró la presencia de ASIC1, pero no se detectó en las papilas gustativas.

3. La roseta olfatoria de *Danio rerio* expresa ASIC2 mRNA y la proteína, localizados en los cilios del epitelio no sensorial. Proponemos que el epitelio olfatorio de los peces se clasifique en olfativo y no olfativo en lugar de sensorial y no sensorial, atendiendo a la distribución positiva de los canalesTRPV4 y ASIC2 asociadas con diferentes funciones sensoriales.

4. En la retina del pez cebra existen mRNAs para zASIC2 y zASIC4.2 y sus proteínas, que se distribuyen por la totalidad de las capas de la retina, siendo ASIC4.2 exclusivo de los fotorreceptores, mientras que ASIC2 predomina en las capas plexiformes; Las células ganglionares expresan ambos canales iónicos.

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