Universitat Autònoma de Barcelona

Departament de Medicina

"EL MASTOCITO: CÉLULA CLAVE EN LA MICROINFLAMACIÓN INTESTINAL Y EN LA REGULACIÓN DE LA FUNCIÓN BARRERA MEDIADA POR EL ESTRÉS EN EL SÍNDROME DEL INTESTINO IRRITABLE CON PREDOMINIO DE DIARREA"

Tesis Doctoral

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Tesis Doctoral dirigida por Francisco Javier Santos Vicente,
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HACEN CONSTAR

Que la memoria titulada **"El mastocito: célula clave en la microinflamación intestinal y en la regulación de la función barrera mediada por el estrés en el Síndrome del Intestino Irritable con predominio de diarrea"** presentada por Mar Guilarte Clavero para optar al grado de Doctor, se ha realizado bajo su dirección, y al considerarla concluida, autorizan su presentación para ser juzgada por el tribunal correspondiente.

Y para que conste a los efectos firman la presente.

Barcelona, Junio de 2014

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INTRODUCCIÓN

1. MASTOCITOS

1.1. Características de los mastocitos

Los mastocitos son leucocitos derivados de células hematopoyéticas pluripotenciales $CD34^+$ de la médula ósea que, a diferencia de otras células mieloides, completan su diferenciación y maduración en los tejidos periféricos. Su crecimiento y diferenciación se producen bajo la influencia de las citocinas, IL-3, IL-4, IL-9, IL-10 y el factor de células madre (*stem cell factor*, SCF), factores de crecimiento, factor de crecimiento de nervios (*nerve growth factor,* NGF), prostaglandina E_2 (PGE_2) y por la interacción con determinadas moléculas de adhesión (Bischoff *et al.*, 2002; Metcalfe *et al.*, 1997). La presencia de los mastocitos en los tejidos depende de la acción de su receptor tirosin-cinasa, c-*kit*, y de su ligando, el SCF. En humanos, el SCF es la principal citocina implicada en la maduración, la activación y la quimiotaxis de los mastocitos (**figura 1**). Su maduración final está condicionada por la interacción con el microambiente del tejido en el que residan, siendo especialmente activa en el caso de existir inflamación local (Gurish MF *et al.*, 2001).

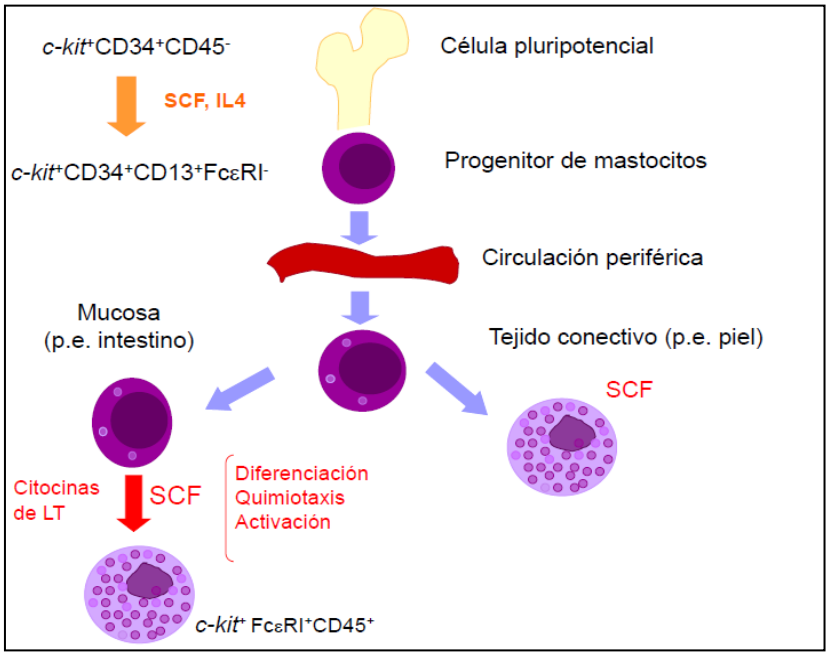

Figura 1: Desarrollo y diferenciación de los mastocitos.

Los mastocitos presentan una distribución ubicua en el organismo. Se encuentran localizados adyacentes a los vasos sanguíneos y linfáticos y a terminaciones nerviosas, pero son particularmente frecuentes cerca de las superficies epiteliales de la piel y en las mucosas de los tractos gastrointestinal, respiratorio y genitourinario, localización tisular estratégica que favorece la interacción óptima del mastocito con el medio externo. El mastocito es la principal célula efectora en la respuesta alérgica, pero también ejerce funciones determinantes en la reparación tisular y en el control de la homeostasis (Galli et Wershil 1996; Metcalfe *et al.*, 1997; Henz *et al.*, 2001). Se estima que la densidad de los mastocitos varía entre 500-4.000 células/mm^3 en los pulmones, de 7.000-12.000 células/mm^3 en la piel y, aproximadamente, 20.000 células/mm^3 en el tracto gastrointestinal (Wasserman *et al.*, 1989).

1.1.1. Heterogeneidad de los mastocitos

Tanto en humanos como en roedores, los mastocitos son una población celular muy heterogénea en lo que se refiere a características morfológicas, funcionales e histoquímicas. En roedores, los mastocitos maduros se dividen en dos subtipos, según su localización: los mastocitos del tejido conectivo (*Connective Tissue Mast Cells,* CTMC), que se encuentran principalmente en la piel, sobre todo alrededor de los vasos sanguíneos y en la cavidad peritoneal, contienen altas cantidades de histamina y están implicados en la respuesta innata frente a bacterias; los mastocitos de la mucosa (*Mucosal Mast Cells,* MMC), que son dependientes de citocinas derivadas de linfocitos T, se localizan fundamentalmente en la mucosa intestinal y pulmonar y su contenido en histamina es menor al de los CTMC. Ambos subtipos son ricos en proteasas granulares de secreción, que difieren según el tipo de mastocito: los mastocitos de la mucosa intestinal (IMMC) de rata expresan de forma selectiva RMCP-II (*Rat Mast Cell Protease*), que equivale a la quimasa de los humanos, y condroitin

sulfato, mientras los CTMC, que están más granulados expresan RMCP-I y V (equivale a quimasa) y RMCP-VI y VII (equivale a triptasa) y heparina (Stenton *et al.*, 2002).

En humanos (**Tabla I**), los mastocitos se diferencian en dos subtipos según las proteasas que contienen en sus gránulos y son histoquímicamente análogos a los subtipos de los roedores, aunque, a diferencia de estos, coexisten en distintos tejidos (Metcalfe *et al.*, 1997, Krishnaswamy *et al.*, 2005):

1) MC_{TC}: contienen triptasa, quimasa y carboxipeptidasa y predominan en piel y submucosa intestinal.

2) MC_T: la única proteasa neutra que contienen es la triptasa y predominan en la mucosa intestinal y en los alvéolos.

Los mastocitos humanos también presentan heterogeneidad en la expresión de citocinas, lo que sugiere distintas funciones biológicas de los MC_{TC} y los MC_T (Bradding *et al.*, 1995). El espectro de mediadores y citocinas producidas y liberadas por cada subpoblación de mastocitos depende del tipo de estimulación externa (Lorentz *et al.*, 2000, Yu *et al.*, 2001a). Mientras un alérgeno induce principalmente la degranulación y la liberación de mediadores pro-inflamatorios, como la histamina o eicosanoides, los productos bacterianos inducen la secreción de citocinas necesarias las respuestas inmunitarias innatas y adaptativas (Okumura *et al.*, 2003; Bischoff *et al.*, 2007b). Así mismo, la respuesta mastocitaria a secretagogos es distinta según su localización. Los mastocitos de la piel, MC_{TC}, son muy sensibles a la estimulación por sustancia P (SP), compuesto 48/80 (liberador de histamina) y morfina (Yamaoka *et al.*, 2007). En cambio en el pulmón, los mastocitos MC_T sólo responden al compuesto 48/80 y no a SP mientras que los mastocitos MC_{TC} responden a ambos secretagogos (Oskeritzian *et al.*, 2005). Por otra parte, los mastocitos intestinales, predominantemente la población MC_T, no reaccionan al compuesto 48/80, a sustancia P ni a morfina *in vitro* (Rees *et al.*, 1988). Las diferencias entre las subpoblaciones de mastocitos en los

distintos microambientes sugieren que tanto las células como los factores de estos microambientes son determinantes para la función efectora del mastocito.

Tabla I: Características de las subpoblaciones de mastocitos humanos (Adaptada de Metcalfe 1997, Krishnawamy 2005, Stenton *et al.*, 2002).

	MC_{TC}	**MC_T**
Distribución (%)		
Piel	>99	<1
Intestino		
Mucosa	20	80
Submucosa	77	23
Alveolos	7	93
Mucosa nasal	34	66
Dependencia de células T	no	si
Mediadores sintetizados		
Histamina	+++	+++
Quimasa	++	-
Triptasa	++	+++
Carboxipeptidasa A	++	-
LTC_4	++	++
PGD_2	++	+++
TNF-α	++	+++
IL-4, IL-5, IL-6, IL-13	++	+++
Inhibición por cromoglicato disódico	no	si

LTC_4: Leucotrieno C_4; PGD_2: prostaglandina D_2; TNF: *Tumor necrosis factor*; IL: interleucina

1.1.2. Mediadores de los mastocitos

Los mastocitos liberan y generan un grupo heterogéneo de mediadores que difieren en potencia y actividad biológica. La activación del mastocito da lugar a dos tipos de respuesta: la secreción del contenido pre-formado de los gránulos por exocitosis y la síntesis de nuevos mediadores. Los mediadores del mastocito se dividen en tres grupos (Metcalfe *et al.*, 1997):

A- Mediadores preformados, contenidos en los gránulos:

- Aminas biógenas: histamina, serotonina (5-HT).
- Proteasas neutras: triptasa, quimasa, carboxipeptidasa A, fosfolipasa.
- Proteoglicanos: heparina, sulfato de condroitina, ácido hialurónico.
- Péptidos: hormona liberadora de corticotropina (*Corticotropin Releasing Hormone,* CRH), bradicinina, sustancia P (SP), factor de crecimiento del endotelio vascular (*Vascular Endothelial Growth Factor*, VEGF), urocortina, péptido vasoactivo intestinal (*Vasoactive Intestinal Peptide*, VIP).
- Citocinas: factor de necrosis tumoral-α (*Tumor Necrosis Factor*, TNF-α) e interleucina-6 (IL-6).

B- Mediadores derivados de lípidos

- Derivados del ácido araquidónico: prostaglandina-D2 (PGD_2), leucotrienos (LT) LTB_4, LTC_4, LTD_4, LTE_4.
- Factor activador de plaquetas (*Platelet Activating Factor,* PAF).

C- Citocinas, quimiocinas y factores de crecimiento

- Citocinas: IL-1, IL-2, IL-3, IL-4, IL-5, IL-9, IL-10, IL-13, IL-16, interferon-γ (INF-γ).
- Quimiocinas: IL-8, quimiocina de regulación por activación expresada y secretada por los linfocitos T (*Regulated on Activation, Normal T cell Expressed and Secreted*, RANTES), proteína inflamatoria del macrófago (Macrophage inflammatory Protein, MIP); MIP-1α, MIP-1β, proteína quimiotáctica del monocito (*Monocyte Quemoattractant Protein*, MCP); MCP-1, MCP-3, MCP-4.

- Factores de crecimiento: factor estimulador de colonias de granulocitos y macrófagos (*Granulocyte Macrophage-Colony Stimulating Factor*, GM-CSF), factor de crecimiento de nervios (*Nerve Growth Factor*, NGF).

1.1.3. Activación de los mastocitos

La a*ctivación de los mas*tocitos se produce principalmente por la interacción de un antígeno con su anticuerpo IgE específico unido en la membrana celular a través de la activación del receptor de alta afinidad para la IgE (FcεRI). Además, los mastocitos también pueden ser activados de forma independiente de IgE, por sustancias del microambiente, como las citocinas, los neuropéptidos, diversos fármacos, componentes del complemento y productos bacterianos.

1.1.3.1. Activación del mastocito dependiente de IgE

La exposición a un antígeno, denominado alérgeno, activa a los linfocitos Th2 específicos para dicho alérgeno que, a su vez, interactúan con los linfocitos B específicos. Estos, se diferencian a células plasmáticas que producirán IgE específica para el alérgeno. La IgE secretada se unirá a la membrana celular de los mastocitos a través del receptor FcεRI. Está unión de la IgE específica con su receptor implica la sensibilización de los mastocitos. Una nueva exposición al alérgeno, que se une a las moléculas de IgE específica ancladas en la superficie de los mastocitos, da lugar a la agregación de FcεRI y a la activación del mastocito. Esta activación resulta en la degranulación mastocitaria, la liberación de sus mediadores preformados y la generación de mediadores lipídicos, citocinas y quimiocinas proinflamatorias. Mediante la liberación y la producción de moléculas proinflamatorias, los mastocitos activan la respuesta alérgica inmediata y tardía. Las consecuencias de la liberación de mediadores preformados suele ocurrir en pocos minutos, de ahí el término de

hipersensibilidad inmediata. Estos mediadores interactúan con diversos componentes del tejido circundante y producen la inflamación alérgica.

Además, el mastocito se puede activar por un mecanismo dependiente de IgE en ausencia de una reacción alérgica, cuya significación biológica es todavía desconocida. Por otra parte, la IgE regula la supervivencia del mastocito a través de su unión a FcεRI en ausencia de antígenos, suprimiendo la apoptosis inducida por la deprivación de factores de crecimiento (Asai *et al.*, 2001).

1.1.3.2. Activación del mastocito independiente de IgE

El mastocito puede activarse por mecanismos independientes de la IgE, clasificados en inmunitarios y no-inmunitarios:

- Estímulos no inmunitarios:

- Neuropéptidos y hormonas: ACTH (*Adenocorticotropin Hormone*), CRF (*Corticotropin Releasing Factor*), urocortina, SP, CGRP (*Calcitonin-Gene Related Peptide*), HRP (Histamine *Releasing* Peptide), VIP, somatostatina, neurotensina, neurocinina-1 (NK-1), GnRH (*Gonadotropin Releasing Hormone)*.
- Factores de crecimiento y moléculas angiogénicas: NGF, SCF, VEGF, factor de crecimiento del fibroblasto (*Fybroblast Growth Factor-2*, FGF-2), bradicinina.
- Neurotransmisores: dopamina, acetilcolina, norepinefrina.
- Estímulos físicos: calor, frío, vibraciones, presión, hipoxia, pH.
- Estímulos biológicos: toxinas bacterianas, parásitos o virus.
- Compuestos básicos: compuesto 48/80, polimixina B, polímeros de aminoácidos.
- Fármacos: morfina, relajantes musculares, medios de contraste yodados, quinolonas, cannabinoides.
- Otros: lectinas, dextranos, ionóforos de calcio, péptidos del veneno de himenópteros, lectinas de las plantas, galectinas.

- Estímulos inmunitarios:

- Citocinas: IL-1, IL-3, IL-8, SCF, TNF-α, IFN-γ, GM-CSF
- Quimiocinas: MIP-1α, RANTES, MCP-1-4
- Anafilotoxinas: C3a, C4a, C5a
- Cadenas ligeras de inmunoglobulinas
- Receptores de baja afinidad de la IgG

1.1.4. Degranulación del mastocito

Se han descrito dos tipos principales de degranulación mastocitaria: anafiláctica y selectiva o *piecemeal* (**Figura 2**).

La degranulación anafiláctica se produce de forma característica tras la activación del mastocito por IgE, mediante la secreción rápida y explosiva del contenido granular, que implica la fusión de la membrana intergranular y la membrana plasmática, formando conductos o canales de secreción para la liberación masiva del contenido al espacio pericelular. También se produce por otros mediadores inmunológicos como mediante las cadenas ligeras de Ig, superalérgenos, fracciones del complemento, diferentes citocinas y moléculas de adhesión.

En la degranulación *piecemeal,* la liberación de los mediadores del mastocito se produce de forma gradual. Consiste en un vaciamiento lento y selectivo de los gránulos, constatado por la pérdida de la densidad granular, con ausencia de la fusión intergranular. El contenido de los gránulos se libera al espacio pericelular por transporte microvesicular. Este proceso es típico de la activación por neuropéptidos y otros estímulos no inmunológicos como neurotransmisores, factores de crecimiento, ATP y otros agentes físicos, químicos y biológicos. Además, se ha descrito el fenómeno de *transgranulación,* por el cual los mastocitos interaccionan con otras células adyacentes, mediante la emisión de pseudópodos que contienen gránulos y remanentes de gránulos extruidos (que contienen heparina, quimasa,

carboxipeptidasa) (Greenberg *et al.*, 1983; Dvorak *et al.*, 2005; Wilhelm *et al.*, 2005) Este proceso de liberación de mediadores es selectivo y depende del estímulo de activación (Galli *et al.*, 2005).

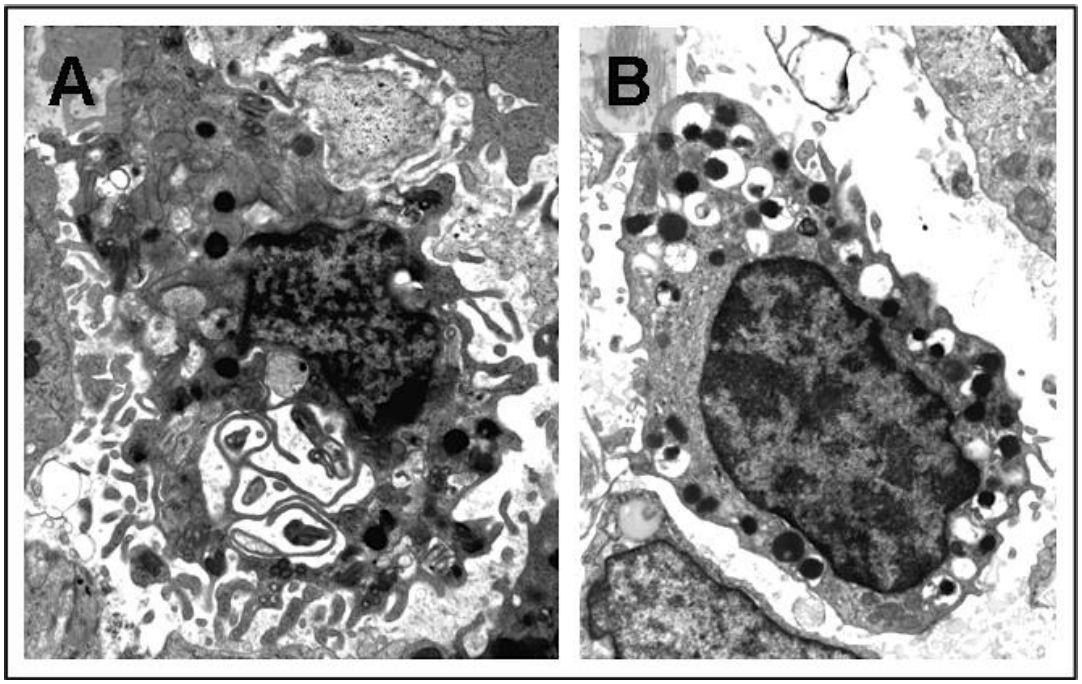

Figura 2: Imágenes de microscopía electrónica de transmisión de la ultraestructura del mastocito de la mucosa intestinal humana (15.000 aumentos) A. Degranulación anafiláctica. B. Degranulación selectiva o *piecemea*l.

1.2. Funciones de los mastocitos

El mastocito ejerce su función efectora mediante la liberación de sus mensajeros bioquímicos multifuncionales contenidos en sus gránulos. Los mastocitos son necesarios para el desarrollo de las reacciones alérgicas, a través del FcεRI, implicando la degranulación y la liberación de mediadores vasoactivos, proinflamatorios y nociceptivos, así como en respuestas fisiopatológicas no inmunitarias, como la homeostasis tisular, el remodelado tisular, la fibrosis y la angiogénesis (**Figura 3**).

1.2.1.Mastocitos e inmunidad innata

Los mecanismos de defensa innata incluyen mecanismos de barrera, secreciones y activación de determinadas células en el tejido. Los mastocitos se localizan preferentemente en la interfase entre el huésped y en ambiente externo, donde ejercen la función de "centinelas" de la inmunidad innata, estableciendo contacto con patógenos y activando la respuesta defensiva (Galli *et al.*, 1999). Gracias a la gran variedad de receptores de superficie que poseen pueden interactuar directamente con los microorganismos, como los receptores tipo "*toll" (Toll-Like Receptors, TLR*-1,3, 4, 6 y 9) y receptores manosilados, e indirectamente mediante los receptores Fc (FcγRI, FcγRII, FcγRIII) y los receptores del complemento (CR3 o CD11b-CD18, CR4 o CD11c-CD18, CR5 o CD88), que reconocen factores del complemento de la pared bacteriana conocidos como opsoninas (Marshall, 2004).

1.2.2. Mastocitos e inmunidad adquirida

El sistema inmunitario adaptativo está dirigido de forma específica contra el agente infeccioso. Los mastocitos cumplen todos los requisitos para ejercer una función en la inmunidad adquirida, ya que son capaces de fagocitar, procesar y captar antígenos, modular el crecimiento de linfocitos, su reclutamiento y la producción de Ig (Henz *et al.*, 2001), así como presentar antígenos por mecanismos dependientes de MCH de clase I y clase II y modular la migración, maduración y activación de las células dendríticas.

Los mastocitos expresan los receptores CD43, CD80, CD86, CD40L que permiten la interacción con linfocitos B y T. Además, secretan citocinas y quimiocinas como TNF-α, IL-1β, IL-4, IL-5, IL-8 y lL-13 que activan a linfocitos y macrófagos (Galli *et al.*, 2005). Además, los mastocitos no solo actúan como células efectoras proinflamatorias en las respuestas inmunitarias, sino que también contribuyen a su inicio y su regulación (Galli *et al.*, 2005).

1.2.3. Mastocitos y reparación tisular

El proceso de reparación de los tejidos es una respuesta fisiológica normal que conduce hacia la restauración de su estructura y función normales tras el daño tisular. En algunas condiciones, el proceso de reparación acarrea una restitución alterada que puede dar lugar a la fibrosis tisular. Los mastocitos están implicados en la patogenia de algunos procesos fibróticos. En el asma alérgico, el depósito de colágeno y otros componentes de la matriz bajo la membrana basal del epitelio respiratorio se ha relacionado con la estimulación repetida del mastocito (Oh, 2005). Los mastocitos también participan en la reparación fisiológica de heridas (Artuc *et al.*, 1999), contribuyendo a la migración y la proliferación de fibroblastos. Este proceso está mediado por la histamina, que interacciona con receptores H2 en los fibroblastos, la IL-4, TNF-α, NGF y FGF (Levi-Schaffer F *et al.*, 1990; Kupietzky *et al.*, 1996; Bonini *et al.*, 1999). Por su parte, las quimasas participan en la síntesis de colágeno y en la formación del tejido de granulación (Iba *et al.*, 2004). En este proceso de fibrosis es importante destacar que los fibroblastos aumentan la supervivencia de los mastocitos, probablemente por la expresión de SCF por parte de los fibroblastos y su interacción con el c-*kit* en los mastocitos (Krishnaswamy *et al.*, 2005).

1.2.4. Mastocitos y angiogénesis

La angiogénesis es un proceso que implica el crecimiento de nuevos vasos, así como la migración, la proliferación, la formación y la supervivencia de células endoteliales. Los factores angiogénicos de crecimiento son producidos por distintas células, tales como los fibroblastos, los linfocitos T, las células plasmáticas, los neutrófilos y los eosinófilos. Los mastocitos están implicados en la neovascularización tanto fisiológica como patológica, dada su localización próxima a capilares y a canales linfáticos en el tejido conectivo. Su incremento está, asimismo, relacionado con la angiogénesis

asociada a hemangiomas, neoplasias, reparación de heridas, ovulación y disfunción miocárdica (Metcalfe *et al.*, 1997). Los mediadores mastocitarios asociados a este proceso son la triptasa, la heparina y la histamina, así como el TNF-α y el VEGF (Metcalfe *et al.*, 1997).

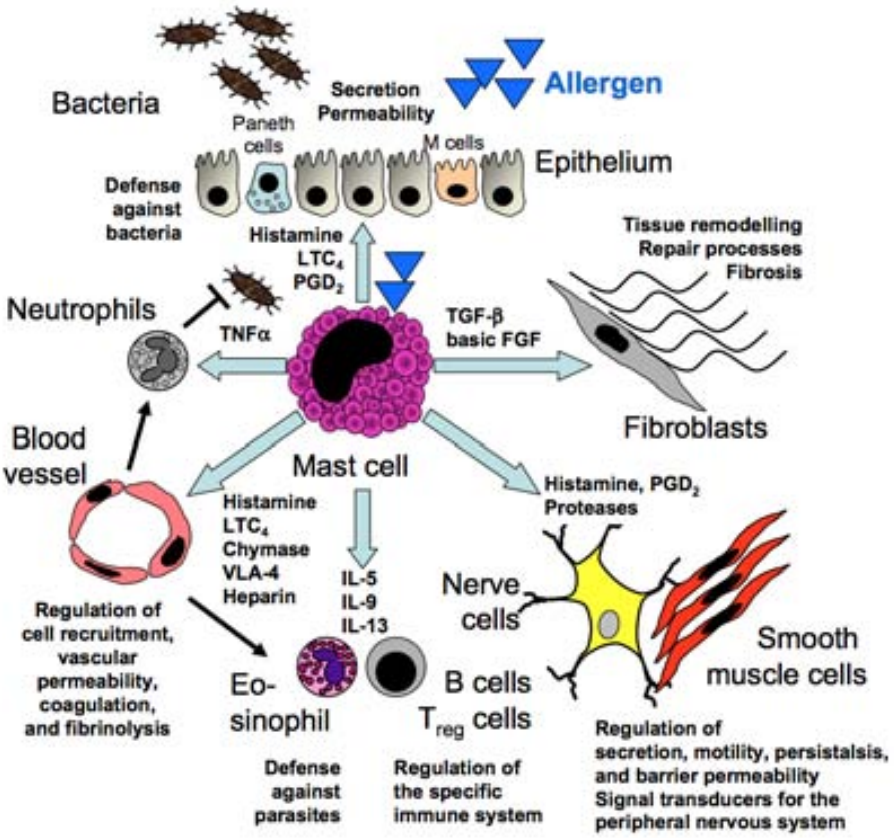

Figura 3: Funciones del mastocito (Fuente: Bischoff, 2009)

1.3. Mastocitos en el tracto gastrointestinal

Los mastocitos se distribuyen en todas las capas del tracto gastrointestinal, pero de forma mayoritaria en la lámina *propria* de la mucosa y en la submucosa, siendo predominantes los MC_T en la mucosa (70-80% *vs.* 10-20% MC_{TC}), mientras que en la submucosa, el fenotipo dominante es el MT_{CT} (60-80% *vs.* 15-20% MC_T). Globalmente, el 90-95% de los mastocitos de la mucosa intestinal produce triptasa y el 60-75%

produce quimasa (Stenton *et al.,* 2002). Los mastocitos gastrointestinales son únicos por su capacidad para modular su fenotipo (aun siendo células maduras), proceso denominado *transdiferenciación*, por el que liberan un nuevo perfil de mediadores en determinadas condiciones microambientales (Friend *et al.*, 1996; Frossi *et al.*, 2004).

Su localización, cerca de los vasos sanguíneos y de los nervios entéricos, tiene implicaciones tanto funcionales como morfológicas. En la mucosa intestinal, más del 50% de los mastocitos se sitúa cerca de terminaciones nerviosas. Cuando existe inflamación tisular esta relación neuro-anatómica se vuelve mucho más íntima, casi sináptica (distancia entre 20-200nm) (Barbara *et al.*, 2004), aumentando también el número total de mastocitos, como ocurre en patologías como la alergia alimentaria (Crowe et al., 1992), las parasitosis, la enfermedad inflamatoria intestinal (EII; Dvorak *et al.*, 1980; Bischoff *et al.*, 1996) y el síndrome del intestino irritable (SII; Barbara *et al.,* 2004). La situación estratégica y la proximidad neural habilitan la comunicación multidireccional entre el mastocito, los sistemas nerviosos entérico y central, el sistema inmunitario y la flora intestinal (Wood, 2004).

Los mastocitos intestinales regulan múltiples funciones tisulares de vital importancia para la función normal del tracto gastrointestinal. Así, los mastocitos se encuentran en situación de equilibrio entre las funciones fisiológicas y los efectos patológicos. Las funciones fisiológicas del mastocito en el intestino incluyen:

- Regulación de funciones epiteliales: secreción de agua y electrolitos e integridad de la barrera epitelial.
- Regulación de funciones endoteliales: flujo sanguíneo, vasoconstricción, coagulación/fibrinólisis, permeabilidad endotelial.
- Regulación del flujo de células en el tejido y función celular de neutrófilos, eosinófilos y linfocitos.
- Regulación de funciones neurológicas: interacciones neuro-inmunitarias, motilidad intestinal y percepción del dolor.

- Regulación de la transformación tisular: reparación heridas, fibrosis.
- Defensa frente al huésped: infecciones bacterianas, víricas, parasitarias

2. ESTRUCTURA Y FUNCIÓN DE LA BARRERA INTESTINAL

El epitelio intestinal constituye la mayor superficie (unos 250m^2) (Artis 2008) en contacto con el medio externo. Para asegurar la homeostasis, una sola capa de células epiteliales tiene la difícil tarea de permitir el paso de fluidos, electrolitos y nutrientes a la vez que prevenir el acceso de antígenos. Así, y debido a su particular estructura formada por multitud de vellosidades y criptas, debe desempeñar dos funciones básicas y fundamentales. Por un lado, la función digestiva: la digestión y absorción de los nutrientes, el transporte de agua y electrolitos y la secreción de proteínas a la luz intestinal. Por otro, debe actuar como barrera defensiva, impidiendo el paso de sustancias potencialmente tóxicas o nocivas, como microorganismos patógenos, antígenos alimentarios o factores proinflamatorios, desde la luz intestinal hacia el medio interno. La función de barrera intestinal es un componente esencial para la homeostasis intestinal y representa la primera línea de defensa contra distintos *"noxas"* del medio externo.

2.1. Anatomía de la pared del intestino delgado

La pared del intestino presenta una arquitectura específica, constituida por cuatro capas concéntricas (**Figura 4**): la mucosa, la submucosa, la muscular y la serosa (Pascual *et al.*, 2001).

a) Capa mucosa: es la más externa y en contacto directo con la luz intestinal. Se divide a su vez en tres subcapas concéntricas:

- Epitelio: reviste toda la superficie del intestino. Se compone de células digestivas absortivas (enterocitos), secretoras (células caliciformes, células de Paneth, células enteroendocrinas) e inmunológicas, principalmente linfocitos intraepiteliales (LIE).
- Lámina *propria*: formada por tejido conectivo que contiene numerosos vasos sanguíneos y linfáticos, así como fibras nerviosas. Está compuesta por una gran

variedad celular con funciones defensivas y de comunicación. Esta capa incluye el tejido linfoide difuso, formado por los linfocitos de la lamina propia (LLP) y los folículos linfoides prominentes. Ambos contienen multitud de células inmunocompetentes y es en esta región donde predominan los mastocitos del tracto gastrointestinal.

- *Muscularis mucosae*: es una fina capa de músculo que separa la mucosa de la submucosa y constituye el soporte físico de ésta. Incluye una capa circular interna y una capa longitudinal externa de músculo liso.

b) Capa submucosa: constituida por una densa capa de tejido conectivo que se encuentra por debajo de la mucosa. En ella existen vasos sanguíneos y linfáticos y el plexo submucoso o de Meissner, que forma parte del sistema nervioso entérico (SNE) y cuya función principal consiste en regular la secreción de las glándulas digestivas.

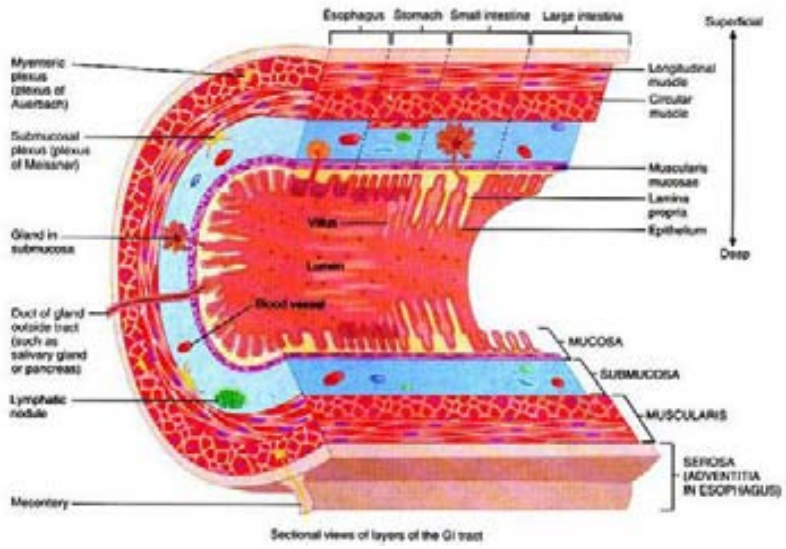

Figura 4: Histología del intestino delgado
(Fuente:http://apbrwww5.apsu.edu/thompsonj/Anatomy&Physiology)

c) Capa muscular: formada por células musculares lisas distribuidas estructuralmente en dos capas, la circular interna y la longitudinal externa. Entre estas dos capas se encuentra otro componente del SNE, el plexo mientérico o de Auerbach, que regula

los movimientos peristálticos que desplazan el contenido de la luz a lo largo del tubo digestivo.

d) Capa serosa: es la capa más externa y la que envuelve al intestino. Está formada básicamente por células epiteliales planas y tejido conectivo muy laxo.

2.2. Mecanismos de barrera intestinal

Existen distintos niveles de defensa en la barrera intestinal:

a- Luz intestinal: donde los ácidos gástricos y pancreáticos y las secreciones biliares degradan las bacterias y las proteínas. Aquí se encuentra la flora bacteriana, que inhibe la colonización por patógenos a través de la modificación del pH y mediante la competencia por nutrientes necesarios para el crecimiento de patógenos y por el nicho ecológico.

b- Microclima: incluye la secreción de agua e iones, encargados de arrastrar las sustancias nocivas de la luz intestinal, el glicocálix y la capa de *mucus* que contiene IgA que neutraliza patógenos y previene su adhesión al epitelio.

c- Epitelio: es una barrera física eficaz que presenta una permeabilidad selectiva, de la cual va a depender en gran medida la integridad de la barrera intestinal.

d- Lámina *propria*: que contiene células inmunitarias capaces de generar respuestas defensivas específicas e inespecíficas.

e- Peristaltismo: cuya finalidad es propulsar el contenido luminal.

2.2.1. El epitelio intestinal.

El epitelio intestinal forma una barrera selectiva que favorece el flujo de nutrientes, regula la secreción de agua e iones y limita el contacto con los antígenos dietarios y microbianos que recibe diariamente. Esta barrera no es totalmente impermeable a macromoléculas, por lo que pequeñas cantidades de antígenos alimentarios y microrganismos participan en la inducción de una respuesta inmunológica

homeostática que está dominada por la tolerancia inmunológica a estos antígenos (Ménard *et al.*, 2010).

El epitelio intestinal es una monocapa de células columnares que recubre toda la superficie del intestino. Está compuesto principalmente por células absortivas denominadas enterocitos, aunque también existen células secretoras, como las células caliciformes (células Globet), las células de Paneth y las células enteroendocrinas. Aparte de estas células secretoras existe también un tipo celular especializado, las células M, situadas en el epitelio que recubre los folículos linfoides, que transportan antígenos luminales hacia los folículos para su presentación antigénica. En el epitelio intestinal, también existen células inmunitarias, como los IEL, cuya principal función es mantener la homeostasis de la flora saprófita (Ismail *et al.*, 2011).

La parte apical del enterocito, que está en contacto con la luz intestinal, contiene las microvellosidades intestinales, que aumentan enormemente la superficie de absorción del intestino, pero también la superficie de contacto con antígenos. Los enterocitos están conectados unos con otros por un complejo sistema de uniones intercelulares que diferencia la membrana apical de la basolateral. Los bordes laterales están unidos a las células adyacentes por una serie de estructuras muy complejas: las uniones estrechas apicales (*tight junctions*, TJ) o "*zonula occludens*" y las uniones adherentes o "*zonula adherens*". Estas uniones son las responsables de la adhesión y la polaridad de las células, siendo las uniones estrechas apicales las que desempeñan un importante papel regulador de la permeabilidad intestinal (Turner, 2009).

2.3. Permeabilidad intestinal

La permeabilidad intestinal se refiere al movimiento o flujo de moléculas a través de la pared intestinal. El epitelio intestinal permite el paso de moléculas ya sea por

difusión pasiva, como iones o moléculas inertes de bajo peso molecular, como por un proceso activo desde la luz intestinal a la lámina *propria*. Este transporte se realiza por dos mecanismos distintos: paracelular, a través de las uniones estrechas, y transcelular, por mecanismos de endocitosis/exocitosis (transcitosis) mediados o no por receptores de membrana (**Figura 5**).

2.3.1. Vía de transporte transcelular

Parte de los antígenos alimentarios son transportados a través de los enterocitos por un mecanismo de transcitosis. Este mecanismo fue descrito inicialmente en el intestino delgado de ratas (Warshaw *et al.*, 1971) y posteriormente también se ha identificado en el yeyuno humano (Heyman *et al.,* 1988). Solo una pequeña cantidad de proteínas intactas son transcitosadas (aproximadamente un 0,1% del contenido luminal), lo que refleja la eficiencia de los enterocitos en la función barrera intestinal (Pascual *et al.,* 2001).

Las moléculas con peso molecular >600 Da, principalmente de naturaleza proteica, son captadas por las células epiteliales por endocitosis en la membrana apical y transferidas hacia la lámina *propria* mediante transcitosis. Durante la transcitosis, los péptidos no digeridos o las proteínas son parcialmente degradados en compartimentos endosomales o lisosomales y liberados totalmente (como animoácidos) o parcialmente degradados. La cuantificación por técnicas de radiocromatografía indica que las proteínas de gran tamaño captadas por los enterocitos son liberadas a la cara basal, como péptidos inmunogénicos (40%) o totalmente degradas a aminoácidos (50%) (Ménard *et al.*, 2010). Los péptidos de mayor tamaño o las proteínas liberadas intactas a la lámina *propria* serán captadas por células presentadoras de antígenos locales para inducir una respuesta inmunitaria específica.

El transporte transcelular de partículas microbianas de gran tamaño se realiza principalmente por las células epiteliales especializadas, las células M, situadas en la superficie de folículos linfoides. Las células dendríticas también pueden captar bacterias directamente de la luz intestinal mediante la extensión de sus dendritas entre las células epiteliales, facilitando la presentación antigénica y la activación inmunitaria en la mucosa intestinal (Turner, 2009).

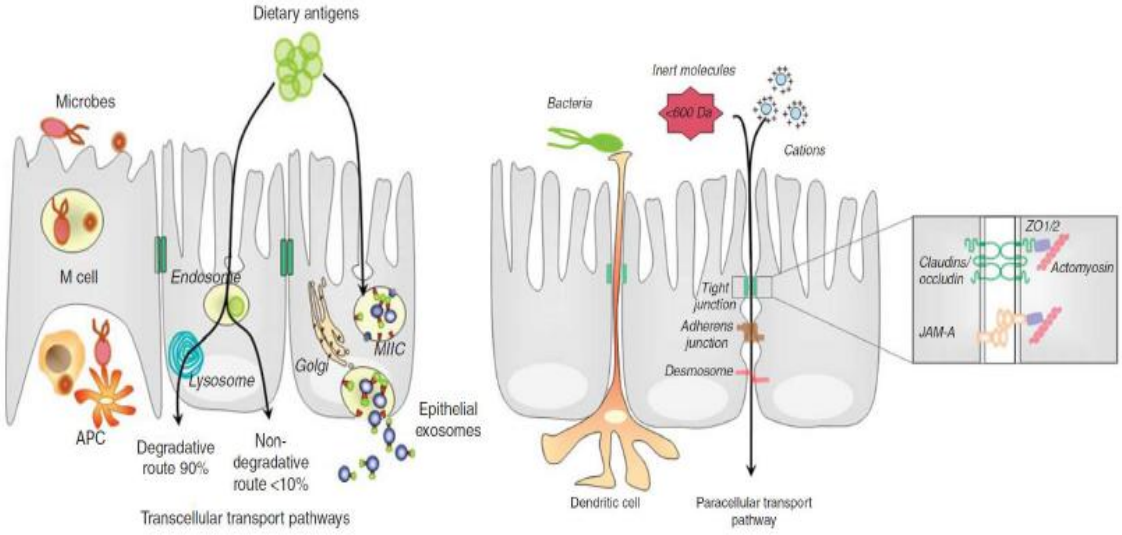

Figura 5: Vías de transporte epitelial: transcelular y paracelular (Reproducido de Ménard *et al.*, 2010)

2.3.2. Vía de transporte paracelular

Consiste en el transporte pasivo a través del espacio entre dos enterocitos adyacentes y está regulado por complejos proteicos intercelulares localizados en las uniones latero-apicales de las membranas celulares de los enterocitos. La vía de transporte paracelular limita el paso de moléculas a aquellas con un peso molecular <600Da. Esta limitación viene determinada por el tamaño de los poros de las uniones estrechas intercelulares, que tienen un diámetro de que oscila entre 0,4 a 8-9 nm (Watson *et al.*, 2001). La difusión de moléculas de pequeño tamaño se produce por movimientos de agua debidos a los gradientes osmóticos o electroquímicos, que

inducen el arrastre de solventes. La alteración de alguno de estos mecanismos permite el paso de macromoléculas al medio interno, que en condiciones fisiológicas se encontraría restringido.

2.3.3. Métodos de estudio de la permeabilidad intestinal

La permeabilidad intestinal se puede valorar mediante técnicas *in vivo* e *in vitro*. Para determinar la permeabilidad intestinal *in vivo* se usan marcadores bioquímicos o isotópicos. Entre los primeros, los más usados son los mono o disacáridos, como la ramnosa, el manitol, la lactulosa o la sucralosa y los polímeros del etilenglicol (PEG). Dentro de los marcadores isotópicos, los más extensamente usados son los quelatos no degradables marcados isotópicamente, como el ^{51}Cr-EDTA y el ^{99}Tc-DTPA. Tienen el inconveniente de ser radioactivos y se usan sobre todo en animales de experimentación, aunque también se han utilizado en estudios de investigación en humanos (Dunlop *et al.*, 2006; Wallon *et al.*, 2008). Otro método indirecto para estudiar la permeabilidad intestinal *in vivo* es la determinación de la liberación de albúmina a la luz intestinal en un segmento aislado del intestino, mediante la perfusión continua de la región intestinal (Alonso *et al.*, 2008; Alonso *et al.*, 2009). En animales de experimentación se estudia la permeabilidad intestinal *in vivo* mediante la determinación de macromoléculas como dextranos o azul de Evans en la sangre tras su administración por sonda enteral.

Para establecer la permeabilidad intestinal *in vitro* se utilizan macromoléculas fluorescentes o radioactivas (Ghandehari *et al.*, 1997; Keita *et al.*, 2006), las más utilizadas son las proteínas del rábano, como la peroxidasa (*horseradish peroxidase*, HRP; Santos *et al.*, 2001) o las bacterias marcadas (O´Brien *et al.*, 2002) en cultivos de monocapas de células epiteliales o en baño de órganos, como las cámaras de *Ussing* (Keita *et al.*, 2010).

2.3.3.1.Cámaras de Ussing

Las cámaras de Ussing fueron inventadas en 1.950 por Hans H. Ussing, para estudiar el transporte iónico a través de la piel de la rana. Cada cámara consta de dos piezas separadas que permiten colocar entre ambas un epitelio aislado y crear dos compartimentos, uno mucosal y otro serosal (**Figura 6**). La cámara está conectada a un reservorio en el que se introduce una solución nutritiva, que está continuamente oxigenada y a una temperatura de 37ºC, lo que permite la perfusión continua del tejido en condiciones fisiológicas.

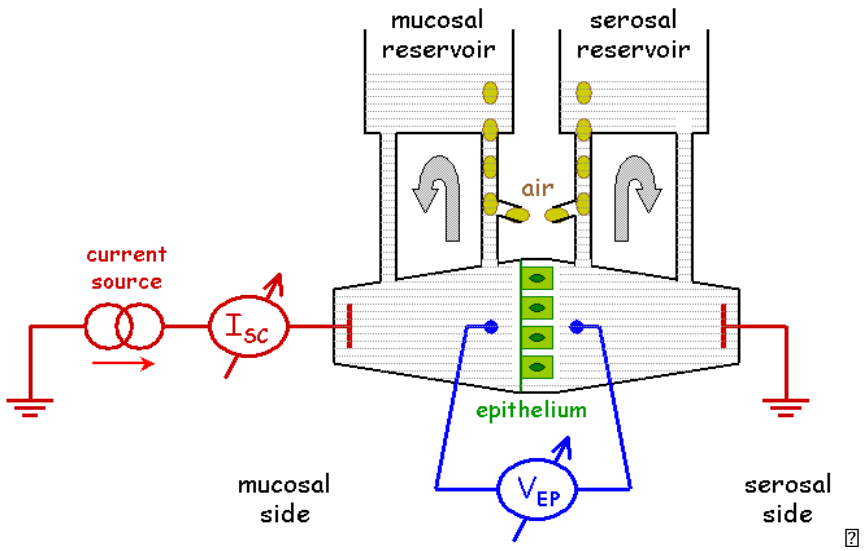

Figura 6: Representación esquemática del funcionamiento de las cámaras de Ussing (Fuente: http://www.fh-jena.de/~gitter/ussikamr.pdf)

Mediante dos electrodos, situados en cada compartimento, se determinan las propiedades electrofisiológicas del epitelio. Los parámetros que se evalúan son la corriente de cortocircuito (I_{sc}), que refleja el transporte activo de iones a través del epitelio y la conductancia, la inversa de la resistencia transepitelial, que indica el paso neto de iones y moléculas de bajo peso molecular, por lo que este parámetro se considera un indicador de la permeabilidad paracelular. Además, con la adición de una molécula a la solución nutritiva, se mide su flujo a través del tejido, siendo útil para estudiar la permeabilidad del intestino.

3. ESTRÉS Y FUNCIÓN INTESTINAL

3.1. Concepto de estrés

La reacción fisiológica coordinada del organismo frente a estímulos que amenazan la homeostasis se denomina estrés. El estrés psicológico puede estar desencadenado por varios estímulos como el miedo, la agresión, los cambios ambientales inesperados, el aislamiento social u otras condiciones patológicas. En respuesta al estrés, se generan respuestas tanto autonómicas, endocrinas como inmunológicas coordinadas para mantener la estabilidad del organismo. El exceso de estrés en individuos susceptibles altera esta respuesta adaptativa y puede predisponer a éstos al desarrollo de nuevas enfermedades o a exacerbar enfermedades ya existentes (Gunnar et Quevedo, 2007)

La percepción de esta amenaza por el individuo activa la respuesta organizada e inmediata de los principales sistemas efectores de defensa: el sistema simpático-adrenomedular (SAM) y el eje hipotálamo-hipofisario-adrenal (HPA). El SAM es la división simpática del sistema nervioso autónomo (SNA), constituido por las células cromafines de la médula adrenal que, como consecuencia de su activación, liberan adrenalina y noradrenalina. Estas catecolaminas activan los receptores adrenérgicos, situados en los órganos diana y preparan al organismo para reaccionar ante el estímulo, garantizando un flujo de sangre (aumento del gasto cardíaco, vasodilatación a nivel muscular y vasoconstricción a nivel cutáneo), para aumentar el aporte de oxígeno y energía a los tejidos donde sea necesario, mediante el incremento de la glucogenólisis, que favorece la utilización de la glucosa. Al mismo tiempo, la activación del eje HPA estimula las neuronas parvocelulares del núcleo paraventricular del hipotálamo para secretar el factor liberador de corticotropina (*corticotropin-releasing factor,* CRF) y arginina vasopresina (AVP) que son secretados a la circulación portal hipofisaria desde terminales axonales de la zona externa de la eminencia media. Estos péptidos viajan a la hipófisis anterior y promueven la síntesis de corticotropina (*adrenocorticotropin hormone*, ACTH). La ACTH es liberada a la

circulación sistémica y activa a la corteza suprarrenal la cual induce un aumento temporal en el torrente sanguíneo de glucocorticoides (cortisol y corticosterona) que circulan hacia los distintos tejidos, facilitando la coordinación entre el cerebro y sus efectos a nivel periférico (de Kloet *et al.*, 2003). El eje HPA está regulado por mecanismos de retroalimentación mediados por el cortisol en la hipófisis, el hipotálamo y el hipocampo. La activación crónica de este eje produce niveles elevados de cortisol en sangre (Belmaker *et al.,* 2008) que se ha implicado en distintas patologías como la depresión (Thomson *et al.*, 2008), la ansiedad (Quirin *et al.*, 2008) y el SII (Dinan *et al.*, 2006; Fukudo, 2007).

3.2. El CRF: principal mediador de la respuesta al estrés

El CRF es un péptido de 41 aminoácidos y es la molécula clave que integra los distintos niveles de respuesta al estrés (Hill *et al.*, 2013). A nivel neuroendocrino, es el responsable de la activación del eje HPA durante el estrés. Pero, además, la expresión de CRF en distintas zonas del cerebro de mamíferos, sugiere su participación en la respuesta visceral, inmunológica y de comportamiento al estrés (Koob, 1999). El CRF está implicado en los efectos cardiovasculares secundarios al estrés , como el aumento de la frecuencia cardíaca y respiratoria y el aumento de la presión arterial. A nivel inmunológico, modula el proceso inflamatorio actuando en los receptores localizados en los linfocitos T y en los macrófagos (Caso *et al.,* 2008). El CRF está también implicado en el desarrollo de las emociones relacionadas con el estrés, como la excitación y la ansiedad (Bale *et al.*, 2004).

El CRF comparte no solo homología de secuencia sino también propiedades biológicas con otros péptidos relacionados, como la sauvagina y las urocortinas 1-3. Estas moléculas tienen una función importante en procesos fisiológicos, tales como el balance energético y el metabolismo, el mantenimiento de la función cardiaca y el

tono vascular, funciones motoras a nivel gastrointestinal, la reproducción, el embarazo y el parto, la angiogénesis y la vascularización.

El CRF y sus péptidos relacionados se expresan en células inmunitarias, en las neuronas aferentes y en las células enterocromafines, por lo que se produce en órganos linfoides, en la placenta, en la piel así como en la mucosa intestinal, lo que sugiere la implicación del CRF también en la respuesta al estrés a nivel periférico.

3.3. Receptores del CRF

La acción del CRF y de las urocortinas está mediada por los receptores CRF-R1 y CRF-R2. La expresión de ambos receptores es específica del tejido, está regulada por condiciones fisiológicas y se ve afectada por estímulos ambientales. Las urocortinas en general presentan gran afinidad para ambos receptores, pero el CRF muestra una mayor afinidad para CRF-R1 y las urocortinas 2 y 3 para el receptor CRF-R2 (Taché *et al.*, 2009).

Los receptores CRF-R1 están ampliamente distribuidos por el SNC (córtex cerebral, cerebelo, amígdala, hipófisis anterior, bulbo olfatorio) y en tejidos periféricos, principalmente en la piel, pero también en testículos, ovarios, miometrio, bazo, corazón y en el intestino (Hillhouse *et al.*, 2006; Larauche *et al.*, 2009). Por el contrario, los receptores CRF-R2, se expresan en menor cantidad en el cerebro, principalmente en elementos no neurales del SNC, y se encuentran mayoritariamente distribuidos en órganos periféricos como el pulmón, el corazón, los músculos y el intestino (Kimura *et al.*, 2002; Bale *et al.*, 2004; Larauche *et al.*, 2009). Este patrón de distribución, sugiere distintas funciones fisiológicas para cada receptor, demostradas en animales de experimentación. Así, los ratones deficientes en CRF-R1 presentan menor comportamiento tipo ansiedad (*anxiety-like behaviour*) y una respuesta al estrés reducida (Timpl *et al.*, 1998; Trimble *et al.*, 2007), mientras que los deficientes en CRF-R2 muestran mayor ansiedad, mayor hipersensibilidad al

estrés y una función cardiovascular alterada respecto a los no deficientes en estos receptores (Bale *et al.*, 2000). Sin embargo, paradójicamente, la administración de agonistas y antagonistas de CRF-R2 a nivel cerebral genera actitudes ansiolíticas y ansiogénicas (Bale *et al.*, 2004).

Por otra parte, la expresión periférica de CRF-R1 y CRF-R2 varía en función de la especie estudiada. Diversos estudios sugieren que el CRF-R2 es el principal receptor expresado en los órganos periféricos en roedores (Bale et al., 2004; Hillhouse et al., 2006), sin embargo, en humanos, los órganos periféricos expresan tanto CRF-R1 como CRF-R2, indicando un alto grado de complejidad y sugiriendo funciones más sutiles para el CRF y las urocortinas en la fisiología y la fisiopatología humana. Por lo tanto, la expresión de CRF-R1 y CRF-R2 en los tejidos periféricos permite al CRF y a las urocortinas dar lugar a diversos y, a veces, opuestos efectos. (Hillhouse *et al.*, 2006).

3.4. Efecto del CRF sobre el tracto gastrointestinal

Los efectos del CRF en la regulación funcional del tracto gastrointestinal han sido demostrados tanto a nivel del SNC como periférico. En el tracto gastrointestinal se han descrito CRF-R1 y CRF-R2 en el colon de humanos y roedores y en el íleon de roedores (Larauche *et al.*, 2009). De hecho, la presencia de receptores para el CRF en el colon de los humanos sugiere un papel de las vías periféricas en la regulación de la función gastrointestinal durante el estrés (Larauche *et al.*, 2009). Además, la administración de CRF o de sus péptidos relacionados, las urocortinas, da lugar a acciones periféricas que mimetizan los efectos del estrés en el tracto digestivo (van den Elzen *et al.*, 2007; Gourcerol *et al.*, 2011). Sin embargo, la regulación de éstas moléculas en condiciones basales o en situaciones de estrés y en los trastornos funcionales gastrointestinales es todavía desconocida (Wallon *et al.,* 2008; Yuan *et al.*, 2012). En el colon de la rata, el CRF-R1 se expresa mayoritariamente en la cripta

de las células epiteliales y en las neuronas de la submucosa y el plexo mientérico y la expresión predominante de los CRF-R2 se produce en la superficie de las células epiteliales y en los vasos sanguíneos de la submucosa intestinal (Chatzaki *et al.*, 2004a, Chatzaki *et al.*, 2004b). En cambio, en el duodeno e íleon tanto CRF-R1 como CRF-R2 se expresan además en las células de la lámina propia (Porcher *et al.*, 2005; Larrauche *et al.*, 2009). En el intestino humano, solo se ha descrito la presencia de ambos receptores en el colon, con una expresión mayoritaria de CRF-R1 y se expresan en mayoritariamnemte en células de la lámina propia como mastocitos y macrófagos como en el plexo mientérico y submucoso (Muramatsu *et al.*, 2000; Yuan *et al.*, 2007; Wallon *et al.*, 2008; Yuan *et al.*, 2012).

La importancia del CRF en el tracto gastrointestinal queda reflejada en la capacidad del estrés tanto en el inicio como en la exacerbación de enfermedades digestivas tales como el SII o la EII (Suárez *et al.*, 2010).

3.4.1. Efecto del CRF en la motilidad y la sensibilidad visceral intestinal

El CRF tiene un papel determinante en la regulación de la motilidad gastrointestinal en respuesta al estrés. Varios estudios en animales de experimentación han demostrado la co-localización e interacción de los receptores CRF-R1 y CRF-R2 en el colon de la rata y en las neuronas del plexo mientérico (Gourcerol *et al.*, 2011), modulando la motilidad y la percepción del dolor de forma diferenciada. Por un lado, tanto la activación periférica como intracerebroventricular de CRF-R1 mediante la administración de CRF, produce un aumento de la motilidad del colon y de las contracciones gástricas, de la defecación y de la hipersensibilidad visceral (Martínez *et al.*, 2004; Larauche *et al.*, 2009; Gozu *et al.*, 2013). Los ratones deficientes en CRF-R1, no solo presentan menor comportamiento tipo ansiedad, como se ha comentado anteriormente, sino que también, a nivel gastrointestinal muestran una menor sensibilidad visceral (Trimble *et al.*, 2007) y una disminución de la defecación

(Martínez *et al.*, 2006). Estos cambios motores son bloqueados con antagonistas no selectivos del CRF, el CRF $_{9\text{-}41}$-α-helical o la astresina, con escasa penetrancia a nivel cerebral (Stengel et al., 2009; Maillot *et al.*, 2000; Martínez *et al.*, 2001; Santos *et al.*, 2008) y por antagonistas del CRF-R1, como la antalarmina (Greenwood-Van Meerveld *et al.*, 2005).

Por otro lado, y de forma contrapuesta, la activación de los CRF-R2 inhibe el vaciamiento gástrico (Nozu *et al.*, 2013), suprime la estimulación de la función motora del colon y protege del dolor visceral producido por la distensión colónica (Martínez *et al.*, 2002; Million *et al.*, 2006). El bloqueo del CRF-R2 por la astresina2b, un antagonista selectivo, no produce ningún efecto en el aumento de la respuesta defecatoria secundaria a la administración de CRF (Martínez *et al.*, 2004). Recientemente se ha demostrado en el colon de ratas, que la activación de CRF-R2 inhibe las señales de CRF-R1 en las neuronas del plexo mientérico y la respuesta motora colónica secundaria al estrés. (Gourcerol *et al.*, 2011).

En humanos, la administración periférica de CRF en voluntarios sanos produce hipersensibilidad visceral, observada mediante el uso de balones de distensión rectal (Lembo *et al.*, 1996). En pacientes con SII, el CRF administrado por vía periférica en pacientes con SII induce un aumento de la motilidad colónica, induce síntomas abdominales y estimula la secreción de ACTH (Fukudo *et al.*, 1998). Sin embargo, los efectos de los antagonistas del CRF son dispares. Por un lado en pacientes con SII con predominio de diarrea (SII-D), la administración del $CRF_{9\text{-}41}$ α-helical mejora la hipermotilidad gastrointestinal y el aumento de la percepción visceral producida por la estimulación eléctrica, sin afectar el eje HPA (Sagami *et al.*, 2004), pero el pexacerfont, un antagonista específico para el CRF-R1, no tiene ningún efecto en el tránsito intestinal ni en el colónico (Sweetser *et al.*, 2009).

3.4.2 Alteración de la función barrera intestinal por el CRF

Tanto el estrés agudo como el estrés crónico alteran la secreción y la permeabilidad epitelial en animales de experimentación y en humanos.

Diversos modelos animales de estrés han observado un aumento de la secreción iónica y de agua en el yeyuno y en el colon. Así, la administración directa de CRF en el colon de la rata, produce la secreción activa de cloro e implica los nervios adrenérgicos y colinérgicos y a los mastocitos (Santos *et al.,* 2000). Los cambios secretores producidos por el estrés agudo o la administración periférica de CRF son inhibidos con el tratamiento con CRF $_{9\text{-}41}$ α-helical (Santos *et al.,* 2008). El CRF-R1 es el receptor responsable del aumento de la actividad secretora y de la subsecuente aparición de diarrea producida por el estrés. Esto ha sido demostrado por la capacidad de revertir la diarrea con la administración de antagonistas del CRF-R1 (Saunders *et al.,* 2002) y por el aumento de la actividad secretora como respuesta a la administración de un agonista del CRF-R1, la estresina1 (Teitelbaum *et al.,* 2008).

En humanos, también se ha demostrado la secreción de agua aumentada en el yeyuno en relación con el estrés agudo, mediante técnicas de perfusión segmentaria, como por ejemplo tras un estrés psicológico producido por estímulos auditivos (Barclay *et al.,* 1987), o tras el estrés agudo producido por dolor al frío en voluntarios sanos (Santos *et al.*, 1998; Alonso *et al.*, 2008) y en pacientes con alergia a alimentos (Santos *et al.,* 1998).

Se ha observado también un incremento en la permeabilidad intestinal en relación con situaciones de estrés físico como la cirugía, los traumatismos o las infecciones gastrointestinales (Dunlop *et al.,* 2006). El estrés agudo por frío también aumenta la permeabilidad intestinal en mujeres sanas y, al contrario de lo que ocurre con la secreción intestinal, este incremento es superior de forma proporcional al nivel de estrés basal (Alonso *et al.,* 2008). El CRF ha sido implicado

como el principal mediador del aumento de permeabilidad intestinal producida por el estrés. La administración intraperitoneal o *ex vivo* en la mucosa del colon de CRF produce un aumento de la permeabilidad intestinal a macromoléculas (Santos *et al.,* 1999; Santos *et al.,* 2008; Wallon *et al.,* 2008), que es revertida con CRF $_{9\text{-}41}$ α-helical y dependiente de CRF-R1 (Moeser *et al.,* 2007). En el colon de ratas sometidas a un estrés crónico psicosocial, se produce un aumento de la expresión de receptores CRF-R1, que es dependiente del nivel de estrés. Estos cambios se acompañan de una activación del eje HPA, de la inflamación de la mucosa intestinal y de alteraciones en la permeabilidad epitelial (Vicario *et al*., 2012). El receptor CRF-R2 también modifica el paso de sustancias a través del epitelio y las respuestas inflamatorias en el colon (Teitelbaum *et al*., 2008). El aumento de la permeabilidad en respuesta al estrés puede implicar un aumento en la captación luminal de antígenos luminales, con el consiguiente inicio de la respuesta inflamatoria en la mucosa.

3.5. El eje estrés-mastocito y la regulación de la función barrera intestinal

Los mastocitos son células efectoras del eje cerebro-intestino, que traducen las señales de estrés en la liberación de un amplio rango de neurotransmisores, proteasas y citocinas proinflamatorias, que alteran la fisiología intestinal. Ello es debido, por un lado, a su localización adyacente a las terminaciones de los nervios y a que los mastocitos expresan receptores para el CRF (subtipos CRF-R1 y CRF-R2), la SP y las urocortinas, lo que indica que los mastocitos actúan como enlace entre el cerebro y los tejidos periféricos (Kempuraj *et al.,* 2004; Cao *et al*., 2005; van der Kleij *et al.,* 2003; Larauche *et al*., 2009).

La actividad de los mastocitos en la mucosa intestinal merece una especial atención dada su capital importancia en la alteración de la barrera epitelial bajo la influencia del estrés. Recientemente, en un modelo porcino se ha demostrado que el aumento de la permeabilidad paracelular secundario a la administración de CRF es

dependiente del TNF-α liberado por los mastocitos (Overman *et al.*, 2012). El aumento de la permeabilidad intestinal paracelular también depende de la liberación de NGF por parte del mastocito, mediada por el receptor CRF-R1 (Barreau *et al.*, 2007). La triptasa puede estimular los receptores PAR-2 (*protease-activated receptor* 2) que se expresan en las terminaciones nerviosas y en la membrana apical y basolateral de los enterocitos. La activación de estos receptores en el intestino produce una reacción inflamatoria local, un aumento de la permeabilidad paracelular, mediante la modulación de proteínas de unión transmembrana en el epitelio intestinal, un aumento de la secreción iónica (Jacob *et al.*, 2005; Bueno *et al.*, 2008), así como la alteración en la motilidad intestinal, la hipersensibilidad visceral y la hiperexcitabilidad de las neuronas entéricas (Vergnolle, 2005), alteraciones características del SII.

La participación específica del mastocito en estos fenómenos se ha demostrado a nivel experimental. El aumento de la secreción iónica y del transporte transepitelial de macromoléculas en el yeyuno y en el colon de la rata, tras el estímulo de estrés agudo o crónico, (Saunders *et al.*, 1994; Santos *et al.*, 1999), se inhibe con la administración de doxantrazol y no se observa en ratas deficientes en mastocitos (Santos *et al.*, 2000, Santos *et al.*, 2001). De la misma manera, en humanos, el estrés agudo produce un aumento de la secreción de agua y de la permeabilidad epitelial a nivel yeyunal, que se acompaña de la liberación a la luz del intestino delgado de mediadores mastocitarios como la histamina, la triptasa y la PGD2 (Santos *et al.*, 1998; Santos *et al.*, 1999).

El CRF induce un aumento de la secreción iónica y, a la vez, aumenta el flujo de HRP *ex vivo* y la liberación de RMCPII a la luz intestinal en ratas. Estas respuestas epiteliales son inhibidas por la astresina y el doxantrazol y se encuentran reducidas en el colon de ratas deficientes en mastocitos. (Santos *et al*. 2008). Además, estudios *in vitro* demuestran que el CRF, a través de la activación de los receptores CRF-R1 y

CRF-R2 presentes en los mastocitos subepiteliales, aumenta la captación de macromoléculas por vía transcelular en la mucosa de colon humano (Wallon *et al.*, 2008). Por último, el cromoglicato disódico, un estabilizador de la membrana de los mastocitos bloquea el aumento de la permeabilidad del intestino delgado producido tanto por el estrés psicológico como por la administración de CRF en voluntarios sanos (Vanuystel *et al.*, 2013).

Por todo ello, los mastocitos se consideran efectores clave en la respuesta intestinal al estrés.

4. SÍNDROME DEL INTESTINO IRRITABLE

El SII es un trastorno digestivo crónico que se caracteriza por la presencia de dolor o molestia abdominal (que mejora con la defecación), asociado al cambio en el número y/o consistencia de las deposiciones. Actualmente, es uno de los mayores problemas de salud en el mundo, en base a su elevada prevalencia, que varía según el país entre el 1% al 45% (Khan et Chang, 2010). Según una revisión sistemática reciente que incluye 81 publicaciones que representan un total de 260.960 pacientes, la prevalencia de SII es del 11.2% (Lovell *et* Ford, 2012a). Existe un ligero predominio en el género femenino, de 1,67 veces respecto al género masculino (Lovell *et* Ford, 2012b). Debido a su recurrencia y cronicidad, el SII tiene un gran impacto económico directo e indirecto, estimado en el 0.5-1% del gasto sanitario total en los países desarrollados (Fortea *et al.*, 2013). Además, existen pocas opciones terapéuticas claramente eficaces, por lo cual muchos pacientes padecen con frecuencia una mala calidad de vida (Camilleri *et al.*, 2010).

Existen diferentes subtipos clínicos en función del hábito deposicional: predominio de diarrea (SII-D), predominio de estreñimiento (SII-E), mixto (SII-M) y SII-inclasificable, con anormalidad de las heces, pero insuficiente para cumplir criterios de diarrea, estreñimiento o mixto (Longstreth *et al.*, 2006). A pesar de las diferencias en su presentación clínica, su etiología y fisiopatología no se han establecido. Las mujeres tienen mayor predominio del subtipo SII-E y menor de SII-D respecto a los varones con SII (Lovell *et* Ford 2012b). Por otro lado, en un 6-17% de pacientes, los síntomas de SII se desarrollan después de una gastroenteritis (Matricon *et al.*, 2012), por lo que se denomina SII post-infeccioso (SII-PI). Se ha observado que después de una epidemia de gastroenteritis por *Salmonella enteriditis* el riesgo de padecer SII está aumentado en 8 veces aproximadamente (Mearin *et al.*, 2005). El SII-PI también se ha observado después de infecciones por *Campylobacter, E coli* y *Shigella* (Spiller *et al.*, 2000; Wang *et al.*, 2004; Marshall *et al.*, 2006).

El SII se considera un trastorno intestinal funcional prototípico ya que no existen marcadores biológicos útiles para establecer su diagnóstico. Tampoco existen cambios macroscópicos en la endoscopia ni en la histología convencional. Por ello, su diagnóstico se basa en criterios clínicos. En la actualidad se utilizan los criterios de Roma (Longstreth *et al.*, 2006) y en la exclusión de enfermedades orgánicas.

4.1. Factores etiológicos del SII

Aunque el origen del SII se desconoce, se han identificado algunos determinantes asociados a su aparición y desarrollo. De hecho, el modelo bio-psico-social de Drossman (Tanaka *et al.*, 2011) asume que no hay una única causa para el SII y que éste se debe a complejas interacciones entre el huésped y el ambiente. Los factores inherentes al proprio huésped incluyen el género, la edad, factores genéticos y socioculturales, mientras que en los factores ambientales se encuentran el estrés psicosocial, las infecciones gastrointestinales, los antibióticos o la dieta (**Figura 7**).

- Factores genéticos: En gemelos, la probabilidad de padecer trastornos funcionales es debida en un 57% de los casos a factores genéticos (Morris-Yates *et al.*, 1998). Se ha observado, además, una agregación familiar del 33% en pacientes con SII (Locke *et al.*, 2000). Se han descrito polimorfismos en genes relacionados con la neurotransmisión y con la función neuro-inmunomoduladora (Gonsalkorale *et al.*, 2003; Zucchelli *et al.*, 2011). La asociación más estudiada es el gen *5-HTTLPR* (región promotora del gen que codifica para serotonina, 5-HT), así como el gen de TNF-α (Camilleri *et al.*, 2012).
- Factores ambientales: Los más importantes son las infecciones gastrointestinales previas, como se ha comentado anteriormente. Asimismo, se cree que los factores que modifican el microbioma intestinal también podrían contribuir al desarrollo del SII (Madden *et al.*, 2002). La detección, casi universal (hasta en el 84%), de sobre-crecimiento bacteriano en los test de aliento y la mejoría

sintomática tras la erradicación (Pimentel *et al.*, 2011) apoyan la relevancia de las bacterias en el desarrollo del SII.

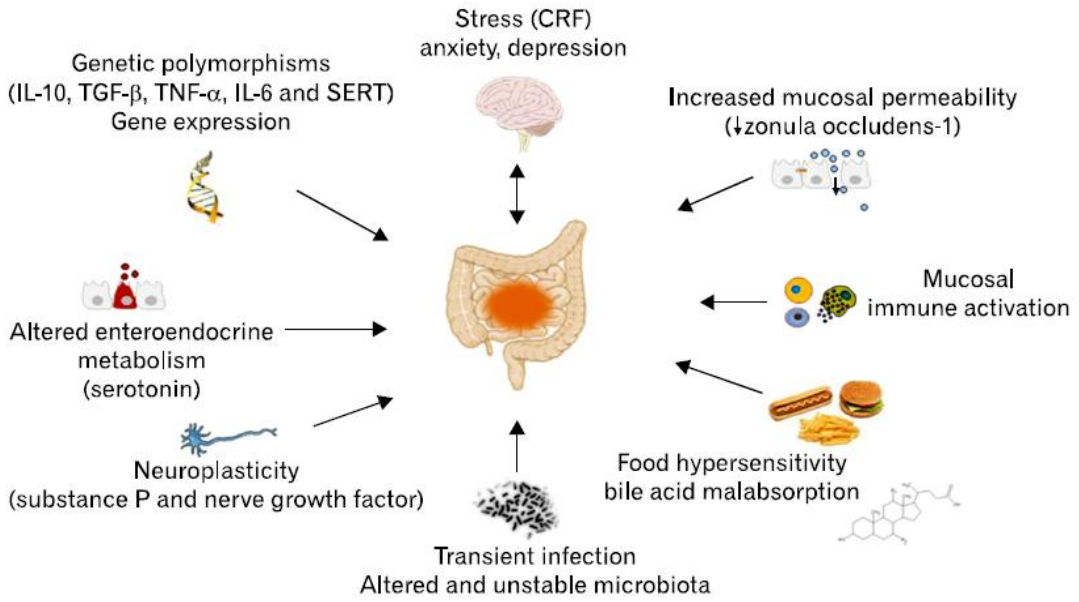

Figura 7: Posibles factores biológicos implicados en la patogenia del SII (Fuente: Barbara *et al.*, 2011)

- Factores psicosociales: En pacientes con SII hay mayor prevalencia de trastornos psiquiátricos, principalmente de ansiedad y depresión, respecto a los individuos sanos o a pacientes con enfermedades gastrointestinales orgánicas (Sudea-Blaga *et al.*, 2012). La existencia de antecedentes de abuso sexual u otros acontecimientos traumáticos, se ha relacionado en estudios epidemiológicos con la aparición posterior de SII (Irwin *et al.*, 1996, Delvaux *et al.*, 1997, Naliboff *et al.*, 2012, Sudea-Blaga *et al.*, 2012). Además, se ha observado una correlación entre la gravedad de los síntomas y las comorbilidades psiquiátricas (Fitzgerald *et al.*, 2008; Spiller *et al.*, 2004).
- Factores dietéticos: aunque algunos pacientes relacionan la aparición de los síntomas con la ingesta de determinados alimentos, la implicación de la dieta en

el origen del SII no se ha demostrado. La alergia alimentaria parece tener una relación, dados los estudios que demuestran una mayor frecuencia de historia familiar y personal de alergia a alimentos en pacientes con SII (Park *et al.*, 2006), así como mayor gravedad de los síntomas en el caso del SII-D (Vivinus-Nébot *et al.*, 2010).

4.2. Fisiopatología del SII

La base fisiopatológica responsable del SII no se conoce en la actualidad, no obstante las alteraciones de la motilidad intestinal, la alteración de la percepción visceral y la presencia de microinflamación en la mucosa se han postulado como los principales mecanismos de la disfunción intestinal existente en esta entidad.

4.2.1. Motilidad intestinal

La alteración del hábito deposicional es una característica clínica común de pacientes afectos de SII, lo que sugiere una alteración en la función motora. Durante muchos años se ha considerado a las alteraciones de la motilidad intestinal como un factor fisiopatológico de gran relevancia en el SII. Sin embargo, no existe un patrón motor específico del SII, sino más bien alteraciones de carácter cuantitativo respecto a personas sanas. El tiempo de tránsito colónico está disminuido en el 26% de los pacientes con SII-E y aumentado en el 33% de los pacientes con SII-D (Manabe *et al.*, 2010). Se ha observado el aumento de la respuesta motora frente al estrés respecto a los sujetos sanos (Fukudo *et al.*, 1998, Murray *et al.*, 2004) y la alteración del tráfico y de la tolerancia intestinal a la sobrecarga con gas (Serra *et al.*, 2002). Esta disfunción motora sugiere la alteración de las vías de neurotransmisión implicadas en su control. En pacientes con SII-D se ha observado un aumento de la concentración de 5-HT, mediador clave de los reflejos secretores y peristálticos gastrointestinales, y de sus metabolitos en el plasma (Atkinson *et al.*, 2006).

Sin embargo, las alteraciones motoras observadas en los pacientes con SII no son específicas ni consistentes y se correlacionan parcialmente con los síntomas, por lo tanto no pueden considerarse marcadores útiles para su diagnóstico (Quigley, 2005).

4.2.2. Hipersensibilidad visceral

La hipersensibilidad visceral también está implicada en la patogenia del SII. El aumento de la sensibilidad visceral se ha observado tanto en respuesta a un estímulo físico como fisiológico, como los movimientos intestinales. Los posibles mecanismos implicados son la alteración de las vías aferentes del eje cerebro-intestino, el procesamiento del dolor en el SNC e incluso la hiper-respuesta a estímulos ambientales como el estrés (Camilleri *et al.,* 2012). Cada vez surgen más evidencias que muestran alteraciones en los mecanismos de control y de procesamiento del dolor a nivel del SNC y la relación entre estas anomalías y las manifestaciones clínicas del SII (Seminowicz *et al.*, 2010; Ringel *et al.*, 2008; Elsenbruch, 2011). En estos pacientes existe un aumento de la expresión del receptor de potencial transitorio vaniloide 1 (*transient receptor potential vanilloid 1,* TRPV1) en las fibras nerviosas intestinales, que se correlaciona con el dolor abdominal (Akbar *et al.,* 2008). La activación de estos receptores en las neuronas sensitivas primarias estimula la liberación de SP y de CGRP en el tejido. Ambos neuropéptidos participan en la generación de la inflamación neurogénica y modulan la activación del mastocito, que libera proteasas que estimulan de nuevo los receptores nociceptores, lo que podría explicar la disminución del umbral de activación de estos nociceptores y, por lo tanto, generar la hiperalgesia.

4.2.3. Microinflamación intestinal

En los últimos años, el interés en el SII se ha centrado sobre todo en la demostración de la existencia de la inflamación mucosa de bajo grado, o microinflamación, y en su participación en la fisiopatología de esta entidad. Este hallazgo ha cambiado

totalmente el concepto de la enfermedad, considerada como prototipo de enfermedad intestinal funcional. La inflamación de la mucosa no se observa ni durante el procedimiento endoscópico ni en la histología convencional de biopsias mucosas. Sin embargo, mediante técnicas específicas, se ha descrito su presencia a lo largo del intestino, desde el yeyuno hasta el recto, sobre todo en el subtipo SII-D y en el SII-PI. Esta inflamación de bajo grado abarca la mucosa, la *muscularis* mucosa y los nervios entéricos e incluye la proliferación de distintas células inmunitarias de la lámina *propria*, como los linfocitos y los mastocitos, y de células enterocromafines (Ortiz-Lucas *et al.*, 2010a; Matricon *et al.*, 2012).

No obstante, no se conoce con exactitud qué grado de inflamación es necesario para que se altere la función gastrointestinal y ésta se traduzca en síntomas clínicos. Las células inflamatorias predominantes en el intestino de pacientes con SII difieren según el subtipo de SII; en los SII-D predomina la infiltración de mastocitos y linfocitos en la lámina *propia* y en los SII-PI los linfocitos T en la lámina *propia y* las células enterocromafines productoras de 5-HT en el epitelio *(*Dunlop *et al., 2003)*. En el SII-E y en el SII-M también se ha observado un aumento de linfocitos T y de mastocitos en la mucosa intestinal, aunque es necesario realizar más estudios en estos subtipos que confirmen estas observaciones (Matricon *et al*., 2012, Camilleri *et al*., 2012).

Tanto los linfocitos B como los T residentes en la mucosa intestinal tienen una función esencial en las respuestas inmunológicas adaptativas del intestino. Además, los LIE, por su localización característica, actúan como primera línea de defensa del huésped frente a patógenos. En una revisión sistemática reciente, se muestra la discrepancia entre los distintos estudios en cuanto al aumento de la densidad de linfocitos T en la lámina *propria* y de los LIE en pacientes con SII. Así, hay estudios que muestran una mayor densidad de linfocitos T en las distintas porciones del intestino, pero otros no observan diferencias respecto a los individuos sanos. Estas

diferencias son independientes del segmento del intestino evaluado (Matricon *et al.*, 2012), y podrían ser debidas al uso de distintos métodos de cuantificación y a distintos criterios en cuanto a la selección de pacientes. Por el contrario, no se ha observado el aumento de linfocitos B en la mucosa del colon de pacientes con SII (Piche *et al.,* 2008; Park *et al.,* 2006; O'Sullivan *et al.,* 2000) aunque si en el yeyuno de pacientes con SII-D (Vicario *et al.,* 2011). El aumento en la densidad de linfocitos T en la mucosa intestinal se ha observado más frecuentemente en el SII-PI (Gwee *et al.,* 1999; Spiller *et al.,* 2000; Kim *et al.,* 2010). En este subtipo también se ha observado el aumento de la permeabilidad intestinal (Spiller *et al.*, 2000; Marshall *et al.*, 2004), lo que sugiere que el defecto de la barrera intestinal favorece el desarrollo de la inflamación en la mucosa y la activación del sistema inmunitario local.

También se ha descrito, sobre todo en el colon y en el recto de pacientes con SII-PI tras enterocolitis aguda por *Campylobacter* y *Shigella,* un aumento de células enterocromafines (Spiller *et al.,* 2000; Kim *et al.*, 2010), que son las principales productoras de 5-HT del organismo. Pero, al igual que ocurre con otros subtipos celulares, este hallazgo no es consistente en todos los pacientes (Matricon *et al.*, 2010). Por otro lado, no se ha observado ningún incremento de esta estirpe celular en los segmentos proximales del intestino, como en el duodeno, el yeyuno o el íleon (Wang *et al.,* 2007; El-Salhy *et al.,* 2010).

El infiltrado inflamatorio observado es paralelo al aumento de moléculas pro-inflamatorias, como la IL-1β, el TNF-α, la IL-6, la IL-8 y la triptasa, en la mucosa, en el contenido luminal y en la circulación periférica (Liebregts *et al.,* 2007; Gwee *et al.,* 2003; Martínez *et al.*, 2012). También es paralelo a la disminución de citocinas antiinflamatorias (IL-10 TGF-β) en la mucosa del colon y del recto de pacientes con SII (O'Mahony *et al.*, 2005; Dinan *et al.*, 2006), lo que sugiere un aumento en las respuestas de la inmunidad innata. Pero, como ocurre con los linfocitos y las células

enterocromafines, los hallazgos de los distintos estudios no son consistentes. No existen cambios en la concentración de citocinas circulantes en los pacientes con SII (Ortiz-Lucas *et al.,* 2010b; Matricon *et al.,* 2012). Sin embargo, se ha observado la secreción de TNF-α, IL-1β e IL-6 en células mononucleadas aisladas de pacientes con SII cuando son expuestas a lipopolisacaridos de *E coli,* lo que indica una activación inmunitaria frente a infecciones aumentada en esta enfermedad (Liebregts T *et al.*, 2007).

Además, se ha descrito un aumento en la expresión de moléculas de adhesión en el endotelio del colon y de integrinas en los linfocitos de sangre periférica, sugiriendo un mayor reclutamiento de esta población al intestino de los pacientes de SII *(*Ohman *et al.,* 2005*)*.

4.3. Permeabilidad intestinal y SII

La pérdida de la integridad de la barrera intestinal epitelial permite la penetración de antígenos luminales, lo que conduce a la activación de respuestas inmunológicas en la mucosa del intestino. La disfunción de la barrera epitelial, junto con la inflamación de bajo grado y la activación inmunitaria, desempeñan un papel fundamental en la patogenia del SII. En este sentido, el aumento de la permeabilidad intestinal que se produce en el SII contribuye al proceso inflamatorio, ya que expone a la mucosa a antígenos de la dieta o bacterianos, los cuales promueven y mantienen la activación inmunitaria (Barbara, 2006). Por otro lado, es importante tener en cuenta que en otras enfermedades crónicas intestinales, como la enfermedad celíaca o la EII, en las que la activación del sistema inmunológico intestinal tiene un papel primordial en su patogenia, también se ha observado un aumento de la permeabilidad intestinal junto con la alteración de las proteínas estructurales esenciales de las uniones intercelulares (Arrieta *et al.*, 2006). Se ha observado que la permeabilidad intestinal es distinta según el subtipo de SII. Así, pacientes con SII con

SII-D y con SII-PI presentan alteración de la permeabilidad más prominente que en los pacientes con el subtipo SII-E (**tabla II**). Además, se han descrito anomalías tanto a nivel molecular como estructural en las uniones estrechas de pacientes con SII, que correlacionan con la sintomatología del SII (Piche et al., 2009; Martínez *et al.*, 2012; Martínez *et al.*, 2013).

Los mediadores presentes en el tejido de estos pacientes, como las serin-proteasas, dan lugar al cambios en la permeabilidad intestinal y promueven la hipersensibilidad visceral, tal como se demuestra en sobrenadantes de biospias de colon y en las heces de paciente con SII-D (Gecse *et al.*, 2008). Esto no implica que la alteración de la función barrera produzca *per se* dolor, sino que, como consecuencia de ésta, el fácil acceso del contenido luminal a la mucosa puede originar respuestas inflamatorias locales y la modulación de la función sensitivo-motora. Existe además, una correlación directa entre el aumento de la permeabilidad intestinal y el dolor abdominal, el síntoma principal de los pacientes con SII, que es más evidente en los pacientes con SII-D (Zhou *et al.*, 2009; Vivinus-Nebot *et al.*, 2012).

Tabla II: Resumen de los resultados de los estudios de la permeabilidad intestinal en pacientes con SII (Adaptado y modificado de Martínez *et al*, 2012).

	Método	*SII* vs. *V. Sanos*	*Resultado*	*Referencia*
In vivo				
Intestino Delgado	^{51}Cr-EDTA	30 *vs.* 15	↑PI en SII-D	Dunlop *et al.*, 2006
	^{51}Cr-EDTA	30 *vs.* 10	↓PI en SII-E (proximal). No diferencias a nivel distal	Gecse *et al.*, 2012
	L/M	31 *vs.* 12	↑PI en SII-PI	Spiller *et al.*, 2000
Colon	L/M	19 *vs.* 10	↑PI en SII-D(42%)	Zhou *et al.*, 2010
	L/M	54 *vs.* 22	↑PI en SII-D (39%)	Zhou *et al.*, 2009
	L/M	132 *vs.* 86	↑PI en SII-PI(36%) vs.19%	Marshall *et al.*, 2004
	^{51}Cr-EDTA	30 *vs.* 15	No diferencias entre SII-PI y SII-E	Dunlop *et al.*, 2006
	^{51}Cr-EDTA	30 *vs.* 10	↑PI en SII-D	Gecse *et al.*, 2012
In vitro				
Colon	Cámaras de Ussing	12 *vs.* 5	↑PI en biopsias de SII	Piche *et al.*, 2009
	Cámaras de Ussing	34 vs. 15	↑PI en biopsias de SII	Vivinus-Nébot, 2012
	Cámaras de Ussing	52 *vs.* 25	↑ en sobrenadantes de SII-D y SII-PI. No en SII-E	Gecse *et al.*, 2008

SII: Síndrome del Intestino Irritable; V.Sanos: voluntarios sanos; PI: permeabilidad intestinal; L/M: ratio lactulosa/manitol; SII-D: SII con predominio de diarrea; SII-PI: SII postinfeccioso; SII-E: SII con predominio de estreñimiento.

4.4. Mastocitos y SII

La mucosa intestinal de pacientes con SII presenta un aumento del número de mastocitos y/o de sus mediadores, principalmente triptasa e histamina, a lo largo de todo el tracto gastrointestinal, desde el duodeno hasta el recto (**Tabla III**). Este aumento de mastocitos se ha observado en todos los subtipos de SII, pero de manera predominante en pacientes con SII-D y con SII-PI (Weston *et al.*, 1993; O'Sullivan *et al.*, 2000; Park *et al.*, 2003;Wang *et al.*, 2004; Park *et al.*, 2006; Lee *et al.*, 2008: Goral *et al.*, 2010; Kim *et al.*, 2010). La mayoría de los estudios han sido realizados en el ciego y en el colon, probablemente debido a la mayor facilidad de acceso a muestras de tejido en estas porciones del intestino, donde la densidad de mastocitos es de un 50 a un 100% mayor que en los controles sanos (Matricon *et al.*, 2012). Sin embargo, y a diferencia de las estirpes celulares comentadas en el apartado anterior, muy pocos estudios no han demostrado este incremento del número de mastocitos. (Cenac *et al.*, 2007; Klooker *et al.*, 2010; Lee *et al.*, 2013), aunque en estos, sí se ha observado un aumento en la liberación de histamina o en la expresión de triptasa en la mucosa (Cenac *et al.*, 2007; Klooker *et al.*, 2010). Esta activación mastocitaria se correlaciona con el aumento del número y de la consistencia de las deposiciones en el SII-D (Martínez et al., 2012).

Varios estudios han mostrado una estrecha relación anatómica entre los mastocitos y las terminaciones nerviosas de la mucosa intestinal del colon de pacientes con SII, que se correlaciona con el nivel de degranulación de los mastocitos (Park *et al.*, 2003, Barbara *et al.*, 2004). Esta proximidad habilita la comunicación bidireccional entre el mastocito, el SNC y el SNE, que junto con el aumento del número de mastocitos y sus mediadores en el SII, puede contribuir a la generación de las manifestaciones clínicas de esta entidad (Barbara et al., 2004; Barbara et al., 2007). Asimismo, los mediadores liberados por los mastocitos intestinales pueden causar hiperexcitabilidad en las terminaciones neuronales del sistema nervioso

entérico y participar en el inicio de respuestas sensoriales y motoras en la mucosa intestinal (Barbara *et al.*, 2007).

La implicación del mastocito en la fisiopatología del SII queda reflejada en la similitud clínica del SII con los síntomas abdominales de la mastocitosis sistémica y los síndromes de activación mastocitaria (Frieri et al., 2013; Picard et al., 2013), así como con algunos pacientes con alergia a alimentos (Brandtzaeg et al., 2010). Los pacientes con mastocitosis sistémica presentan habitualmente dolor abdominal (Frieri *et al.*, 2013) e hipersensibilidad colónica a la distensión (Libel *et al.*, 1993), lo que refuerza la participación del mastocito en la hipersensibilidad visceral, condición característica del SII.

Por último, la demostración de la eficacia en el tratamiento del SII de fármacos estabilizadores del mastocito, como el cromoglicato disódico (Lobo, Tesis doctoral UAB, 2013) y el ketotifeno (Klooker *et al.*, 2010) sustenta el papel fundamental de esta célula en la patogenia del SII.

Tabla III: Densidad de mastocitos en la mucosa intestinal en el SII y en controles sanos (Adaptado de Matricon et al, 2012, Camilleri et al., 2012, Philpott et al., 2010)

Tipo de SII	SII vs. V. sanos	Lugar de biopsia	Capa del intestino	Número de mastocitos	Activación del mastocito	Referencia
SII-D	41 vs. 48	Duodeno	Mucosa	↑	NR	Walker *et al.*, 2009
SII-D	20 vs. 29	Duodeno	Mucosa	↑	Liberación de triptasa en sobrenadante	Foley *et al.*, 2011
SII-D+SII-E	20 vs. 15	Duodeno Yeyuno distal Ileon Terminal	Mucosa Mucosa Mucosa	= = ↑	NR NR NR	Wang et al., 2007
SII-D	20 vs. 15	Ileon	Mucosa	↑	NR	Weston *et al.*, 1993
SII-PI	56 vs. 12	Ileon terminal	Mucosa	↑	NR	Wang et al., 2004
SII-D	18 vs. 15	Ileon Colon Ascendente Recto	Mucosa Mucosa Mucosa	↑ ↑ ↑	% de MC degranulados (M.E)	Park *et al.* , 2006
SII	14 vs. 7	Ciego Colon Recto	Mucosa Mucosa Mucosa	↑ = =	NR NR NR	O'Sullivan *et al.*, 2000
SII-D	14 vs. 14	Ciego Recto	Lámina propia Lámina propia	↑ ↑	% de MC degranulados (M.E)	Park *et al.*, 2003
SII-D+SII-C	50 vs. 21	Ciego	Lámina propia	↑	NR	Piche et al., 2008
SII-D+SII-E +SSI-M	34 vs. 15	Ciego	Mucosa	↑	Liberación de triptasa espontánea en biopsias de SII-D y SII-M	Vivinus-Nebot *et al.*, 2012
SII-D+SII-C	72 VS. 50	Ciego Recto	Mucosa Mucosa	↑ en SII-D (= en SII-C)	NR	Goral *et al.*, 2010

Tabla III: (continuación)

Tipo de SII	SII vs. V. sanos	Lugar de biopsia	Capa del intestino	Número de mastocitos	Activación del mastocito	Referencia
SII-D+SII-E+SII-M	77 vs. 28	Colon ascendente, transverso y descendente	Mucosa	↑	NR	Chadwick *et al.*, 2002
		Recto	Mucosa	↑		
SII	44 vs. 22	Colon descendente	Mucosa	↑	% de MC degranulados.Liberación de triptasa e histamina	Barbara *et al.*, 2004
SII	15 vs. 7	Colon ascendente	Lámina propria	=	Aumento de la expresión de triptasa	Cenac *et al.*, 2007
		Recto	Lámina propria	=		
SII	23 vs. 22	Colon sigmoide	Mucosa	↑	NR	Akbar *et al.*, 2008
SII-D+SII-E	48 vs. 24	Colon	Mucosa	↑	NR	Cremon *et al.*, 2009
SII-D+SII-E	11 vs. 4	Colon	Mucosa	↑	Aumento de triptasa e histamina en sobrenadantes	Buhner *et al.*, 2009
SII-D+SII-E	25 vs. 18	Colon descendente	Mucosa	↑ en SII-D	Aumento de actividad *trypsin-like*	Coeffier et al., 2010
SII-PI+SII-D	11 vs. 10	Colon	Mucosa	↑ en SII-PI	NR	Kim et al., 2010
SII-D+SII-E	25 vs. 12	Colon	Mucosa	↑	Liberación espontánea de triptasa e histamina	Cremon *et al.*, 2011
SII-D+SII-E+ SII-M+SII-PI	28 vs. 34	Recto	Mucosa	↑ (excepto SII-PI)	NR	Dunlop *et al.*, 2003
SII-D	16 vs. 7	Recto	Mucosa	=	Liberación de triptasa en sobrenadante	Lee *et al.*, 2013

SII: Síndrome del Intestino Irritable; V. Sanos: voluntarios sanos; SII-D: SII con predominio de diarrea; SII-E: SII con predominio de estreñimiento; SII-M: SII mixto; SII-PI: SII post-infeccioso; NR: no realizado; M.E: microscopía electrónica; MC: mastocito

4.5. Mastocito: regulación de la permeabilidad intestinal y de la respuesta al estrés en el SII

Una posible causa que explique el aumento de permeabilidad intestinal regulada por el estrés en el SII son los mastocitos (**Figura 8**). Los mediadores del mastocito, como la histamina, la triptasa, la SP o la 5-HT son capaces de alterar la integridad de las uniones estrechas intercelulares y provocar la alteración de la permeabilidad transcelular, permitiendo el paso de macromoléculas que en condiciones fisiológicas verían restringido su paso. En pacientes con mastocitosis sistémica, la activación de los mastocitos intestinales causa diarrea (Frieri *et al.*, 2013), síntoma cardinal de los pacientes que padecen SII-D, lo que demuestra los efectos reguladores de los mediadores de los mastocitos en la función epitelial intestinal. En otras enfermedades en las que el mastocito también tiene una función primordial, como la alergia a alimentos, las citocinas y las quimiocinas secretadas por los mastocitos activados intervienen en el reclutamiento de células inflamatorias, contribuyendo al mantenimiento del aumento de la permeabilidad intestinal (Yu *et al.*, 2001a), independientemente de si el proceso ha sido iniciado por un alérgeno o por una bacteria.

En ratones deficientes en LMLCK (*long miosin light chain kinase*), una proteína del citoesqueleto, se ha observado que para que se produzca diarrea, es necesario la alteración de las uniones estrechas, que es dependiente de la activación de los linfocitos T (Clayburgh *et al.*, 2005). La alteración de la barrera intestinal en pacientes con SII-D está en relación con la menor expresión y la redistribución de proteínas relacionadas con las uniones estrechas, como la zonula occludens-1 y la ocludina (Piche *et al.*, 2009; Martínez *et al.*, 2012). Además, también se han observado el aumento en la expresión de la claudina-2 y la disminución de la fosforilación de la ocludina, así como alteraciones ultraestructurales en el complejo de unión apical, como la condensación del citoesqueleto periunional y el aumento de

la distancia intercelular (Martínez *et al.,* 2013) Todos estos cambios se correlacionan con el número y la activación de los mastocitos de la lámina propia y con los síntomas del SII, como el aumento del tránsito intestinal y la disminución en la consistencia de las heces (Martinez *et al.,* 2013). Recientemente se ha descrito, además, que el aumento de la permeabilidad paracelular a macromoléculas (en concreto a HRP) en la mucosa rectal de pacientes con SII-D se correlaciona positivamente con el número de mastocitos en la mucosa (Lee et al, 2013). El tratamiento oral prolongado con cromoglicato disódico mejora la diarrea y la consistencia de las deposiciones, entre los síntomas principales en pacientes con SII-D (Lobo, Tesis doctoral, 2013), sugiriendo la mejora de la función barrera intestinal en estos pacientes.

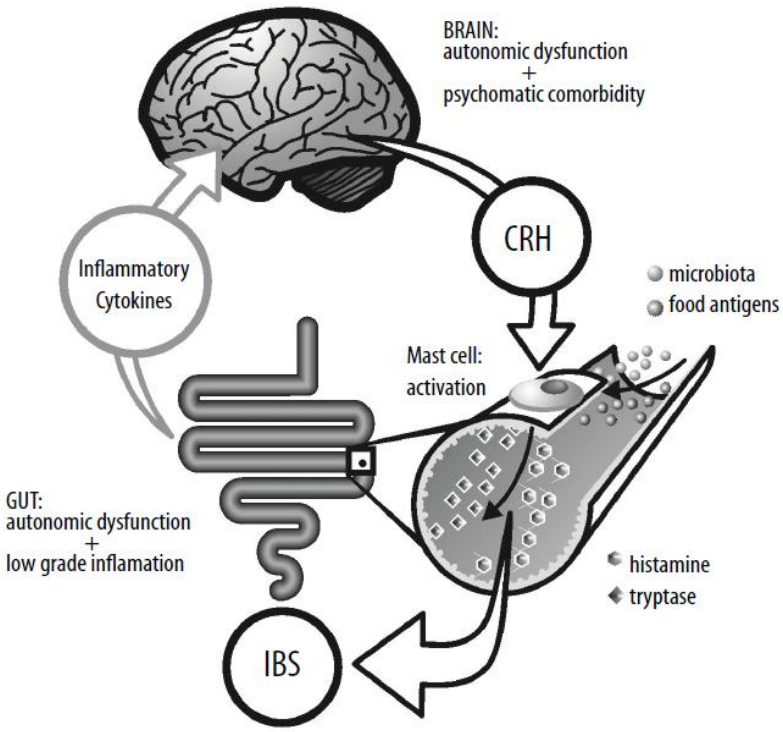

Figura 8: El eje cerebro-intestino-mastocito en el SII (Fuente: Philpott *et al.*, 2011)

Por otro lado, la alteración de la barrera intestinal producida por el estrés es crucial para el desarrollo de microinflamación intestinal. El estrés crónico es un determinante clave para la existencia de una barrera intestinal alterada en respuesta a un estrés agudo, lo que puede predisponer al desarrollo de una disfunción mucosa prolongada y a la posterior aparición de la inflamación de la mucosa y al desarrollo de los síntomas característicos del SII (Alonso et al, 2008). La sensibilidad al estrés es un factor crucial en la gravedad y en la persistencia de los síntomas del SII. Estos cambios en la función intestinal asociados al estrés están mediados por cambios tanto en la expresión como en la activación de receptores de CRF, ya que la administración de un antagonista no selectivo del CRF revierte los síntomas intestinales inducidos por el estrés (Sagami *et al*., 2004).

4.6. Tratamiento farmacológico del SII

En la actualidad no existe un tratamiento claramente eficaz para el SII. El manejo del SII implica dos tipos de abordajes terapéuticos: el uso de fármacos destinados estrictamente al control de los síntomas, como los espasmolíticos para el dolor abdominal o los opiodes para la diarrea y el uso de nuevas dianas terapeúticas basadas en los mecanismos fisiopatológicos del SII. Dentro de estos últimos están los fármacos antagonistas de los receptoes del CRF y de la serotonina y las terapias antiinflamatorias, como los fámacos estabilizadores del mastocito.

4.6.1. Antagonistas de los receptores de CRF

Se ha realizado un ensayo clínico con pexacerfort (antagonista del CRF-R1) en paciente con con SII-D y no se han observado beneficios frente al placebo ni en el tránsito colónico, ni en el dolor abdominal ni en la frecuencia y consistencia de las heces (Sweetser *et al*., 2009).

4. 6. 2. Antagonistas de los receptores de la serotonina

Se han realizado ensayos clínicos con antagonistas de los receptores de la serotonina,

como el alosetrón, antagonista del receptor 5-HT3, para evaluar sus efectos en relación a la motilidad intestinal y la sensibilidad visceral. Se ha observado que .prolonga el tiempo de tránsito colónico, con mínimos efectos en la percepción visceral en sujetos sanos y con acción sobre el SNC en relación a la ansiedad (Berman *et al.*, 2002) y la mejoría en la consistencia y el dolor abdominal (Mayer *et al.*, 2003). El estreñimiento es el efecto adverso más frecuente con este tratamiento, y la aparición de colitis isquémica restringe su uso a casos de SII con diarrea severa que no respondan al tratamiento convencional.

4.6.3. Farmacos estabilizadores del mastocito

En los últimos años se han desarrollado e implementado estrategia terapéuticas dirigidas al bloqueo de la activación mastocitaria.

a) Ketotifeno: El ketotifeno bloquea la liberación de mediadores por parte del mastocito al ser un antagonista no competitivo del receptor de la histamina H1. En un estudio controlado con placebo, realizado en pacientes de SII durante 8 semanas, aumentó el umbral de sensibilidad visceral, evaluada mediante distensión colorectal, sin modificar la liberación de triptasa basal determinada en los sobrenadantes procedentes de las biopsias rectales (Klooker et al., 2010).

b) Cromoglicato disódico: Su mecanismo de acción no se conoce en detalle, pero interfiere con el transportador de calcio en la membrana del mastocito, bloqueando así la liberación de sus mediadores. Tras el tratamiento durante 6 meses, el cromoglicato disódico mejora el dolor abdominal y la diarrea en pacientes con SII-D, comparado con placebo (Lobo, Tesis Doctoral, UAB, 2013) y esta mejoría se asocia a un restablecimiento de la homeostasis intestinal. En estudios realizados en pacientes con SII durante 4 y 8 semanas se observó un control más efectivo que el placebo en el control de síntomas, principalmente en pacientes sensibilizados a alergenos alimentarios, aunque sin diferencias con respecto a una dieta de exclusión (Lunardi

et al., 1991, Stefanini et al., 1995).

4.6.4. Otros fármacos antiinflamatorios

La mesalazina reduce el infiltrado leucocitario, incluidos los mastocitos, así como los niveles de triptasa, histamina e IL-1β en la mucosa intestinal en los pacientes con SII-D. Sin embargo, el único estudio que existe frente a placebo no mostró diferencias, debido a que el número de pacientes estudiados fue reducido, lo cual limita la interpretación de los resultados (Tuteja *et al.*, 2012).

4.7. Modelos animales de disfunción intestinal inducida por el estrés

El desarrollo de modelos animales para el estudio de las enfermedades digestivas asociadas al estrés ha evolucionado considerablemente en las últimas décadas, lo que ha contribuido a mejorar el conocimiento mecanicista de estas patologías (Kiank *et al.*, 2010). Un prototipo de este grupo de enfermedades es, sin duda, el SII, por su asociación con el estrés crónico, tanto en el inicio (Söderholm *et al.*, 2002) como en la perpetuación de sus manifestaciones clínicas (Collins *et al.* 1996).

Existen diferentes modelos animales de estrés crónico que reproducen algunas de las características clínicas más frecuentes del SII y que han aportado información muy valiosa acerca de los mecanismos implicados en la fisiopatología del SII. Estos modelos incluyen la deprivación materna (O'Mahony *et al.* 2009), donde las crías son separadas de su madre durante un determinado periodo de tiempo, y es útil para estudiar los efectos del estrés a edades tempranas en el tubo digestivo y sus consecuencias en la vida adulta. Otro modelo de estrés crónico es la aplicación repetida de estímulos homotípicos estresantes, como el estrés por evitación de agua (*water avoidance stress*, WAS; Bradesi et al., 2005) o el estrés por inmovilización (Liebregts et al. 2007). La aplicación repetida de un estímulo estresante refleja el patrón de estrés diario que experimentamos los humanos (Caso *et al.*, 2008).

A partir de estos modelos, se puede interpretar que la exposición aguda o repetitiva a un único estímulo estresante conduce a la alteración de la fisiología intestinal caracterizada por el aumento en la secreción de iones, la permeabilidad macromolecular, la inflamación microscópica, la hipersensibilidad visceral, la alteración de la motilidad, e incluso la penetración bacteriana (Collins *et* Bercik, 2009; Santos *et al.,* 2008b;. Söderholm *et al.*, 2002). Además, estas respuestas implican la desregulación del eje HPA y están mediadas por el CRF y los mastocitos (Santos *et al.*, 2008;. Keita *et al.*, 2010), un perfil que ha sido descrito también en pacientes con SII. Sin embargo, en las sociedades organizadas, como son la humana y la mayoría de mamíferos, existe una tendencia natural hacia la formación de grupos interactivos y jerarquías, donde los estímulos estresantes predominantes son de naturaleza mixta, por lo que, desde el punto de vista experimental, no parece que los modelos existentes representen claramente este contexto social (Tamashiro *et al.*, 2005). El estrés por aglomeración es un modelo de laboratorio ampliamente utilizado para evaluar la actividad del eje HPA en roedores (Bugajski *et al.,* 2003). Éste representa un modelo de estrés crónico psicológico en el que el establecimiento y el mantenimiento de la jerarquía social en ratas hacinadas implica la competencia por los recursos (espacio, comida y agua), que se traduce en experiencias psicológicas y físicas fuertes para todos los animales, incluso los dominantes y subordinados (Tamashiro *et al.*, 2005), lo que que podría reproducir mejor la naturaleza y la intensidad del estrés al que están expuestos a diario los animales de experimentación en su entorno natural (Sgoifo *et al.*, 1999), y podría también, representar el estrés vital al que están expuestos los seres humanos y, en concreto, los pacientes que desarrollan el SII.

HIPÓTESIS

HIPÓTESIS

El Síndrome del Intestino Irritable, clásicamente considerado una enfermedad del colon, es un trastorno altamente prevalente, con gran impacto en la calidad de vida y pocas opciones terapéuticas eficaces y satisfactorias. Aunque no se conoce en profundidad el origen de este síndrome, el estrés se ha asociado tanto al desarrollo como a la perpetuación de las manifestaciones clínicas cardinales. Aunque los mecanismos fisiopatológicos subyacentes todavía se están dilucidando, destacan, como hallazgos comunes relacionados con la sintomatología, por un lado, la alteración de la función de barrera intestinal y, por el otro, la presencia de inflamación de bajo grado en la mucosa intestinal. Debido a la carencia de biomarcadores diagnósticos útiles, el diagnóstico se basa en criterios clínicos y en la exclusión de otras patologías gastrointestinales que cursan con síntomas similares. En consecuencia, es de gran interés e importancia dirigir la investigación en este campo hacia la identificación de marcadores biológicos que faciliten el diagnóstico positivo y el diseño de nuevas estrategias terapéuticas. El mastocito cumple los requisitos como célula candidata a modular la fisiopatología del síndrome del intestino irritable, ya que a nivel intestinal interviene en la regulación de la motilidad, la sensibilidad visceral y la función de barrera epitelial. De hecho, la presentación clínica gastrointestinal de enfermedades relacionadas con el mastocito, como la mastocitosis sistémica o los síndromes de activación mastocitaria, es solapable en muchos aspectos con las manifestaciones clínicas del síndrome del intestino irritable.

En esta tesis se postula la siguiente hipótesis: “La activación del mastocito intestinal en respuesta al estrés es responsable del aumento de la permeabilidad epitelial en pacientes con Síndrome del Intestino Irritable con predominio de diarrea”

OBJETIVOS

OBJETIVOS

El objetivo principal de esta tesis es la identificación de mecanismos fisiopatológicos principales subyacentes al origen del síndrome del intestino irritable. Para ello, se plantean estudios en pacientes de esta patología y se desarrolla un modelo experimental de estrés crónico en ratas.

Objetivos específicos:

1. Estudiar si en el yeyuno de pacientes con síndrome del intestino irritable con predominio de diarrea existe un aumento en el número y la activación de mastocitos y establecer la utilidad del mastocito y sus productos biológicos como biomarcadores diagnósticos.

2. Determinar si el estrés crónico por aglomeración en ratas reproduce las deficiencias estructurales y funcionales del síndrome del intestino irritable y estudiar la función del mastocito en el desarrollo de estas anomalías.

3. Estudiar el papel regulador del mastocito en el yeyuno de pacientes con síndrome del intestino irritable con predominio de diarrea sobre el aumento de la secrecion iónica y la permeabilidad intestinal en respuesta al estrés agudo.

CAPÍTULO 1

“DIARRHEA-PREDOMINANT IBS PATIENTS SHOW MAST CELL ACTIVATION AND HYPERPLASIA IN THE JEJUNUM”

INTRODUCTION

Irritable bowel syndrome (IBS) is a highly prevalent disorder that comprises a heterogeneous group of patients suffering from chronic and recurrent abdominal pain usually associated with visceral hypersensitivity and altered bowel habit [1]. IBS has been considered a prototypic gut functional disorder since no reliable biological markers are readily available. Different clinical subtypes of IBS are apparent, although differences in their pathophysiology and aetiology have not been clearly established. Recently, interest has focused on the presence of mucosal inflammation and the putative role of immune cells and environmental factors, such as chronic stress, in the generation and perpetuation of this inflammatory process, factors that may be particularly valid for diarrhea-predominant IBS [2,3].

The role of stress and stressful events is well recognized in patients with functional gastrointestinal disorders, and IBS patients appear to be at risk to suffer from psychosocial stress [4]. Clinical and epidemiological studies also indicate that in certain diarrhea-prone IBS symptom intensity and durability depends, to a great extent, on the presence of chronic stress as a co-morbid factor [3,5]. Indeed, stress alters intestinal motility [6,7], enhances visceral perception [8,9], reactivates gut mucosal inflammation [10,11] and disturbs epithelial function [12], in both animal models and humans, and these changes have been also observed in diarrhea-predominant IBS patients. However, the pathway linking stress with IBS pathophysiological abnormalities and symptoms has not been precisely characterised.

The stress-mast cell axis is a putative pathway that may help understand the relationship of stress with the generation of certain pathophysiological characteristics of IBS. Mast cells participate in the regulation of intestinal motility, visceral sensitivity and mucosal and epithelial gut barrier function [13], and both acute [14] and chronic stress [15] induce intestinal mast cell activation. Anatomical

contacts between mast cells and enteric nerve fibers have been demonstrated in the human gastrointestinal mucosa and increase, when inflammation is present [9,16,17]. Moreover, increased mast cells numbers and mast cells products have been described in the terminal ileum and proximal and distal colon of IBS patients [17-19]. The mast cell-enteric nerve interaction provides a physical substrate for bidirectional communication between the central nervous system and the gut, by which stress might influence gastrointestinal physiology

Finally, epidemiological studies have shown that IBS patients often complain of dyspeptic symptoms [20,21]. Although preliminary findings may suggest enhanced activation of jejunal mast cells after administration of stress-like hormones in IBS-diarrhea [22] evaluation of upper gut mucosal inflammation and mast cell status in these patients is lacking. Thus, our aim was to investigate whether diarrhea-predominant IBS patients showed mast cell infiltration and activation in the jejunum.

MATERIAL and METHODS

Participants

Newly diagnosed diarrhea-predominant IBS (IBS-D) patients, fulfilling the Rome II criteria [23], and healthy volunteers were prospectively recruited from the outpatient gastroenterology clinic and by public advertising, respectively. A complete medical history and physical examination and compliance with functional dyspepsia Rome II criteria [24] were performed in all participants. All IBS-D experienced daily watery or mushy stools that varied in number (3 to 12/day) associated with abdominal pain or discomfort that was relieved with defecation. Candidates were also evaluated by allergists to rule out food and respiratory allergy. A battery of prick skin tests (Laboratorios Leti SA, Barcelona, Spain) for 32 common foodstuffs and 24 inhalants was performed prior to the biopsy using histamine and saline as positive and negative controls, respectively. Reasonable exclusion of other gastrointestinal diseases was achieved in IBS patients by means of a broad biochemical and serological profile including anti-transglutaminase and anti-endomisium antibodies, upper and lower endoscopy, abdominal sonography and barium studies, when considered necessary by the responsible physician (table I). Previous history of acute gastroenteritis and its relationship to the initiation of IBS symptoms was carefully recorded.

None of the participants was allowed to take antihistamines, ketotifen, nedocromil, cromolyn, acetylsalicylates, NSAIDs, anticholinergics, theophylline, β_2-agonists, codeine or opioid derivatives for at least 2 weeks prior to the intestinal biopsy to prevent any effect on mast cell numbers and activation. Patients having taken steroid or immunosupressive drugs anytime in the last 6 months were not included. Written informed consent was obtained from each participant. The study was approved by the Ethical Committee of the Hospital and conducted according the revised Declaration of Helsinki.

Table I. Investigations performed in study participants.

	Healthy volunteers	IBS-D
Upper endoscopy*	0	14/20
Colonoscopy & biopsy	0	4/20
Jejunal biopsy & aspirate	14/14	20/20
Blood biochemical profile	14/14	20/20
Celiac serology & thyroid function	0	20/20
Stool culture & microscopy	0	20/20
Skin prick tests	14/14	20/20
Abdominal imaging[§]	no	12/20

*Upper endoscopy was performed only in patients suffering from dyspepsia. In 4 of the IBS-D a colonoscopy and biopsy was performed showing <20 CD3+cells/100 surface epithelial cells, excluding lymphocytic colitis.[§] Abdominal imaging included barium studies, sonography and tomography

Baseline Stress and Depression Levels

Stress levels were measured using the Spanish version of the Modified Social Readjustment Scale of Holmes-Rahe [25]. This validated questionnaire reflects the occurrence of significant life events in the last year of life and allows stratification of participants as suffering from low (0-150), moderate (151-300) or severe (>300) stress.

Depression was evaluated using the Spanish version of the Beck's Depression Inventory [26] and participants classified as suffering from low (10-18), moderate (19-29) or severe (>30) depression. Both questionnaires were filled in by participants the same day of the jejunal biopsy.

Jejunal biopsy, fluid content aspirate and blood sample

Jejunal biopsy was performed within 3 weeks after inclusion. The day of the biopsy all patients were clinically symptomatic and severity of IBS was assessed using a modified visual analogical scale, from 0 (no affectation) to 10 (maximum), to evaluate the compromise of quality of life according to the self perception of abdominal pain intensity and diarrhea [27].Mucosal biopsies and aspirates were obtained using a Watson capsule with an attached 3 mm diameter aspiration tube. After an overnight fast, the instrument was orally inserted under fluoroscopic control, between 8-10 am, to the proximal jejunum, 5 to 10 cm distal to the Treitz's angle. Jejunal fluid (5 mL) was obtained by gentle aspiration with a 10 mL syringe, snap frozen and stored at -80º C until analyzed. Then, a tissue sample was obtained by suction with a 50 mL syringe, immediately embedded in 4% buffered formalin and processed for histology and immunohistochemistry. In addition, a 5 mL blood sample was taken at the end of the study and the serum recovered and frozen until analyzed.

Histology and Immunohistochemistry

An experienced gastrointestinal pathologist, who was blinded to the clinical diagnosis, examined the biopsy specimens. Jejunal sample biopsies were stained with hematoxylin & eosin for general histological examination and epithelial morphometry as performed in routine clinical practice. In particular, the presence of eosinophilic infiltration, epithelial abnormalities including villous atrophy and microorganisms were evaluated at x400 magnification. In order to further exclude celiac disease and lymphocytic enteritis, microscopic inflammation was also assessed by counting intraepithelial lymphocytes (IELs).

For immunohistochemistry, paraffinated samples were cut in 5 μm sections, paraffin removed with xylene and rehydrated. Endogenous peroxidase activity was

blocked with 0.2% hydrogen peroxidase solution and non-specific labelling was blocked in serum blocking solution. Sections were incubated in complete medium for 1 hour at room temperature with *c-kit* anti-human rabbit polyclonal antibody (CD117, Dako, Carpinteria, CA, USA) at a dilution of 1:50). As a negative control the primary antibody was omitted and replaced with phosphate-buffered saline. The reaction was revealed by the avidin-biotin complex peroxidase method (ABC Elite kit, Vector Burlingame, CA, USA) followed by staining with the peroxidase substrate 3.3 diaminobenzidine tetrachloride (DAB; Sigma GmbH, Deisenhofen, Germany). The slides were counterstained with 50% hematoxylin. Human gastrointestinal tumour tissue was used as a positive control for *c-kit* expression. Samples were also processed for CD3 immunohistochemistry (Dako, Carpinteria, CA, USA) at a dilution of 1:50, using the same technique. Quantitation of mast cells and IELs (CD3+cells) was performed on immunostained sections with a Leitz microscope (Laborlux S microscope, E. Leitz, Wetzlar, Germany) at x400 magnification. The number of cells stained for *c-kit* and CD3 were counted in 8 contiguous non-overlapping fields and expressed as mast cells per high power field (MC/hpf) and IELs/100 epithelial cells.

Inter-observer reproducibility of mast cells and IELs findings was tested by comparing counts against a second experienced pathologist. Similarly, intra-observer reproducibility was assessed comparing counts in two different days performed by the principal pathologist, using the Bland and Altman method [28]. Reproducibility in all cases was excellent (**Figure 1**) and hence results reported from now on are those of the principal pathologist.

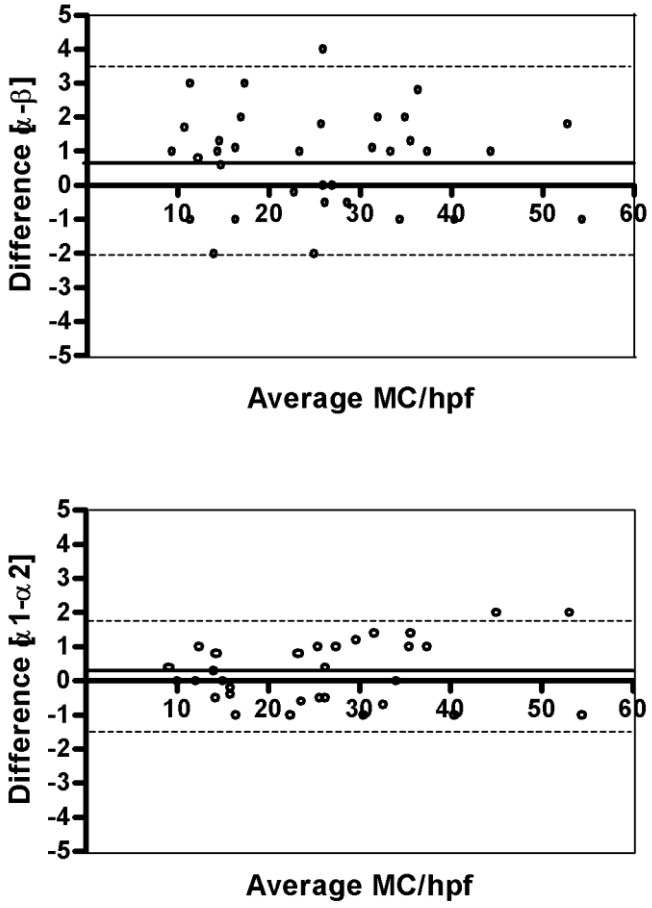

Figure 1: Reproducibility of mast cell counts
Reproducibility of mast cell counts was assessed comparing numbers of mast cells reported by two independent observers (α and β; upper panel) or by the same observer in two separate days (α1 and α2; lower panel). Dotted lines represent 95% limits of agreement (Upper panel: (-2;3.6), Lower panel: (-1.5;1.98)). Black solid lines represent the mean differences (Upper: 0.77 ± 1.45; Lower: 0.24 ± 0.88) and dots represent differences for each single measurement.

Blood and Jejunal Tryptase Concentration

Serum and jejunal concentration of the mast cell protease, tryptase, were assayed by means of a specific fluoroenzyme-immunoassay (FEIA-UniCAP, Pharmacia Diagnostics, Uppsala, Sweden). Serum samples were processed following manufacturer's instructions and concentration expressed as μg/L.

To determine tryptase in intestinal fluid, jejunal fluid samples were first lyophilized to increase concentration by a factor of 12 and reconstituted in phosphate buffer saline + 1% bovine serum albumin + multiprotease inhibitor cocktail (dilution 1:100), containing AEBSF 104 mM, aprotinin 0.08 mM, leupeptin 2 mM,

bastatin 4 mM, pepstatin 1.5 mM, E-64 1.4 mM (Sigma-Aldrich, St. Louis, MO). Samples were then centrifuged at 1000 x*g* for 10 minutes a t 4˚C, the supernatants were collected and tryptase concentration was assayed and expressed as µg/L after correction according to the concentration factor. Tryptase curves were not influenced by phosphate buffer saline, bovine serum albumin or protease inhibitors.

Data expression and Statistical Analysis

Data are expressed as mean ± SD [IC 95%], unless otherwise stated. Non-parametric tests were used as appropriate to increase statistical assurance (Mann-Whitney *U* test, Fisher's exact test and Spearman correlation test). *P* values of < 0.05 were considered significant.

RESULTS

Participants

Twenty-three newly diagnosed IBS-D patients and 14 healthy volunteers were selected. Three IBS-D patients were excluded due to inadequate jejunal tissue sampling (2 superficial biopsies and 1 gastric biopsy). There were no differences in age and gender proportion between healthy and IBS-D populations. Interestingly, most IBS-D patients also suffered from functional dyspepsia (70%), considered their bowel symptoms as quite severe and 30% definitely related onset of their IBS symptoms to a previous episode of acute gastroenteritis. Other demographic and clinical characteristics of the included participants are shown in table II.

Table II. Demographic and clinical characteristics of participants.

	Healthy volunteers	**IBS-D**
Number of subjects	14	20
Gender F:M	6:8	14:6
Age, years (range)	27.9 ± 7.8 (22-53)	32.8 ± 7.7 (21-56)
Symptom duration, months (range)	NA	17.9 ± 8.2 (8-36)
Severity (0-10)	NA	6.8 ± 1.5
Functional dyspepsia	0/14	14/20
Previous gastroenteritis	no	6/20
Other diseases	no	3/20*

Other diseases that presented IBS-D patients were: Hypothyroidism, Von Willenbrand Disease and Pernicious anemia. Data are expressed as mean ± SD. F, female; M, male; IBS-D, diarrhea-predominant irritable bowel syndrome; NA, non applicable.

Baseline stress and depression levels

IBS-D patients showed higher stress levels at baseline than healthy (IBS-D: 203 ± 114 [149-256] *vs.* H 112 ± 99 [55-169], p=0.019). IBS-D patients most frequently (13/20) scored as moderate (39%) to severe (28%) in the Holmes-Rahe Scale whereas only a few of the healthy (4/14) scored as moderate (18.2%) to severe (9.1%), (**Figure2**).

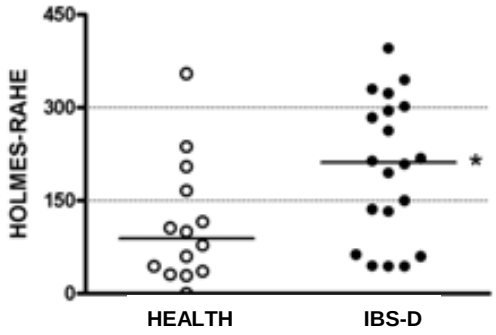

Figure 2: Baseline psychological stress levels (Modified Social Readjustment Scale of Holmes-Rahe). Dotted lines represent levels of stress: low (0-150), moderate (151-300) and severe (>301). Black lines represent median values*, p=0.019, Mann Whitney test.

The majority of our participants (75%) were not depressed and the incidence of depression in the IBS-D group (33.3%) was not statistically different to that of healthy (14.3%). However, when considering only depressed patients in both groups, IBS-D showed higher scores than healthy (**Figure 3**).

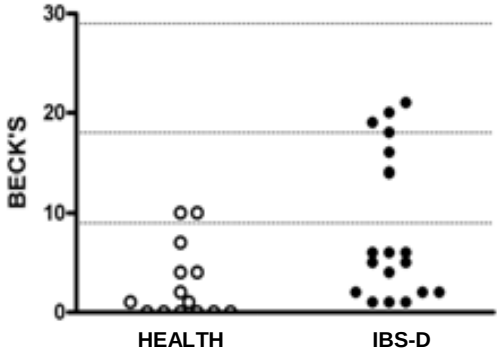

Figure 3. **Baseline Depression Levels** (Beck's Depression Inventory). Dotted lines represent depression levels: normal (0-9), low (10-18) and moderate (19-29).

Histology and mucosal inflammation

Routine histology disclosed normal epithelial architecture, no increase in the number of eosinophils and no parasites, microbial or viral inclusions and a normal or discrete lymphoplasmacitary infiltrate in the lamina propria and/or in the intraepithelial compartment with no apparent differences observed between IBS-D patients and the control group (**Figure 4A-4B**).

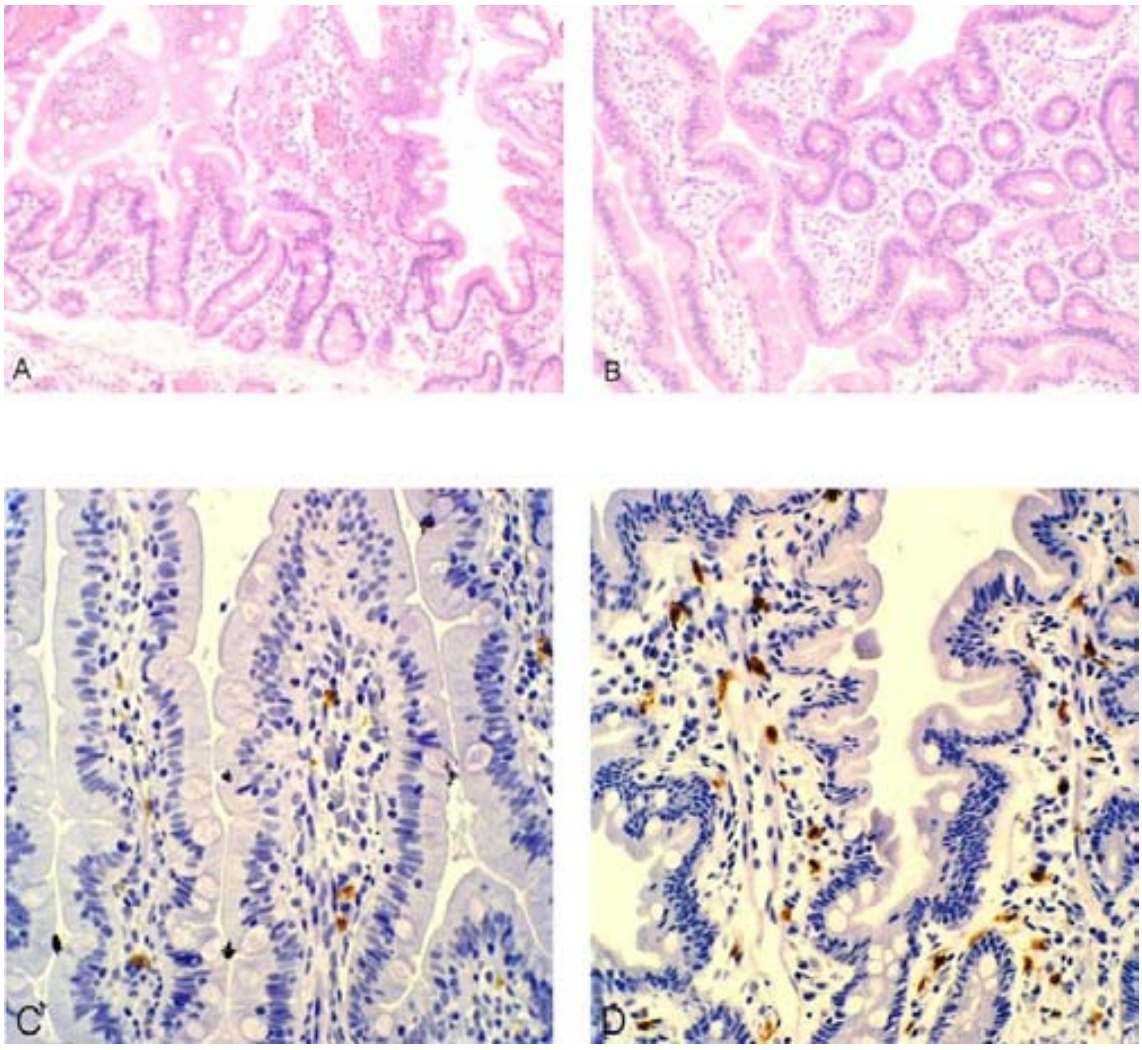

Figure 4. Jejunal histology and mast cell counts. Routine processing of jejunal samples for hematoxylin & eosin staining disclosed no apparent differences in epithelial architecture or the presence of inflammatory cells in the lamina propria, between healthy (A) and IBS-D patients (B). However, after immunohistochemistry for *c-kit* (CD117, brown cells) IBS-D patients (D) showed higher mast cell numbers compared to healthy (C). Slides shown (x400 magnification) are representative of common findings.

Immunohistochemistry for CD3+cells revealed mild increased in IELs numbers in IBS-D when compared to healthy volunteers (IBS-D: 15.3 ± 5.5 [12.7-17.9] *vs*. H: 10.3 ± 3.9 [8.0-12.5]; p=0.006) (**Figure5**).

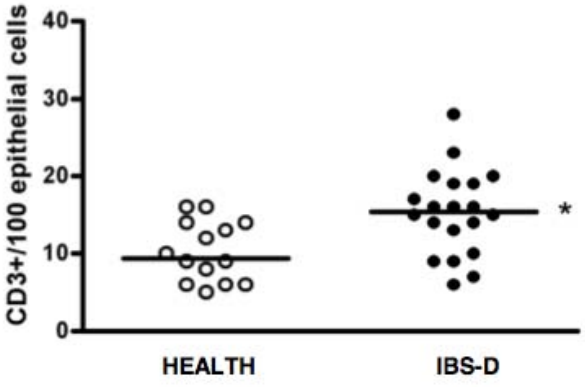

Figure 5. Jejunal intraepithelial lymphocytes (IELs) counts. IELs (CD3+ cells) were counted in 8 contiguous, non-overlapping, histological fields, at x400 and expressed as IELs/100 epithelial cells after immunohistochemistry for *CD3*. IBS-D patients (n=20) showed an increase of IELs compared to healthy (n=14). Black lines represent median values. *, p = 0.006, Mann-Whitney test.

Mast cells counts

Mast cells (CD117+) were markedly increased in the jejunal mucosa of IBS-D patients compared to healthy volunteers (IBS-D: 34.0 ± 9.3 [29.5-38.5] *vs*. H: 15.3 ± 4.4 [12.6-17.9] MC/hpf; p < 0.0001) (**Figure 6**) and the majority of these CD117+ cells were localized within the lamina propria (**Figure 4C-4D**). Notably, only one healthy but all the IBS-D showed more than 20 MC/hpf. IBS-D dyspeptic patients showed similar mast cell numbers than non-dyspeptic IBS-D patients (32.2 ± 9.0 [27.5-39.0] *vs*. 31.8 ± 7.7 [24.2-39.1] MC/hpf; p = NS). Moreover, no differences in mast cell numbers were detected between IBS-D patients with a previous history of acute gastroenteritis and patients without such antecedent (32.3 ± 5.9 [26.0 -38.5] *vs*. 34.7 ± 10.2 [28.8-40.6] MC/hpf; p = NS).

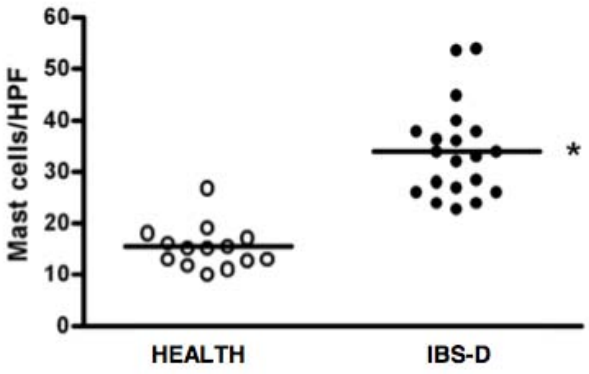

Figure 6: Jejunal mast cells count. Mast cells were counted in 8 contiguous, non-overlapping, histological fields, at 400*x*, and expressed as mast cells per high power field (HPF) after immunohistochemistry for *c-kit*. IBS-D patients (n=20) showed a significant increase of mast cell numbers compared to healthy (n=14). Black lines represent median values. *, $p < 0.0001$, Mann-Whitney test.

Notably, only one healthy but all the IBS-D showed more than 20 MC/hpf. IBS-D dyspeptic patients showed similar mast cell numbers than non-dyspeptic IBS-D patients (32.2 ± 9.0 [27.5-39.0] *vs.* 31.8 ± 7.7 [24.2-39.1] MC/hpf; p = NS). Moreover, no differences in mast cell numbers were detected between IBS-D patients with a previous history of acute gastroenteritis and patients without such antecedent (32.3 ± 5.9 [26.0 -38.5] *vs.* 34.7 ± 10.2 [28.8-40.6] MC/hpf; p = NS). Differences in mast cell numbers were not attributable to age (r = 0.328 [-0.033-0.61]; p = 0.066), stress levels (r = 0.35 [-0.02-0.638]; p = 0.056) or gender, as shown in **Table III**.

Table III. Gender effect on mast cell counts in the jejunal mucosa.

	Male	Female
IBS-D	36.8 ± 9.3 (230.3 ± 57.9)	32.7 ± 9.4(204.4 ± 58.8)
Healthy volunteers	15.9 ± 5.4(99.3 ± 33.9)	15.9 ± 5.4(99.3 ± 33.9)

IBS-D: male, n = 6; female, n = 14; Healthy: male, n = 8; female, n = 6. Mast cell numbers are expressed as cells/high power field. Mast cell counts are also expressed, for comparison, as cells/mm^2 (in parentheses). Conversion factor: 1 mm^2 = 6.249 high power fields. Values are expressed as mean ± SD.

Mast cell tryptase

Tryptase concentration in serum was within the normal range in all participants and similar in both groups (IBS-D: 5.52 ± 2.01 [4.52-6.53]; H: 5.40 ± 2.15 [3.96-6.85] □g/L). Notably, jejunal luminal tryptase was significantly higher in IBS compared to the control group (IBS-D: 0.45 ± 0.38 [0.20-0.69] *vs.* 0.09 ± 0.10□□g/L [0.02-0.177], p=0.005) indicating local activation of mast cells (Figure 7). No correlation was found between number of mast cells and levels of luminal tryptase. In addition, dyspeptic symptoms and IBS severity were not correlated to gender, mast cell numbers, jejunal tryptase, psychological stress or depression

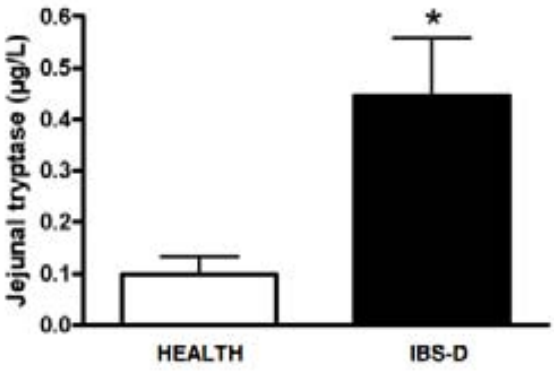

Figure 7: Jejunal luminal tryptase. Values are expressed as mean ± SEM. *, p = 0.005; Mann-Whitney test.

DISCUSSION

The present study provides evidence of increased mast cell numbers along with mast cell activation in the jejunum of diarrhea-prone IBS patients. These novel findings extend those of previous studies showing mast cell hyperplasia in terminal ileum and colon of IBS patients, indicating that mucosal inflammation in IBS-D patients is not limited to the lower gut. Moreover, activation of jejunal mast cells suggests that local mast cell-mediated inflammatory events contribute to functional disturbances and clinical manifestations of IBS-D.

Despite the increasing prevalence and importance of IBS there is no single distinctive biological marker and the diagnosis is based on clinical criteria and exclusion of other gastrointestinal disorders [24]. In recent years, the conventional view of IBS as a non-organic disorder has been challenged by reports of low-grade mucosal inflammation as a relevant pathological substrate in some IBS patients (reviewed in 2). In particular, increased numbers of inflammatory cells and chemical mediators have been identified in mucosa specimens from the ileum and colon of IBS-D apparently not hypercellular on routine histology [29,30]. In agreement with those studies, we have found that the vast majority of jejunal biopsies from IBS-D were informed by the pathologist as within normal limits or showing mild, usually considered as non-specific, mucosal inflammation. Moreover, specific counts of IELs, based on CD3 immunohistochemistry, revealed a 1.5-fold increase in IBS-D jejunum further supporting the presence of low grade epithelial inflammation and making unlikely the existence of more severe inflammatory processes such as lymphocytic enteritis or celiac disease [31]. In addition, the youth of our patients also argues against microscopic colitis as an alternative diagnosis since most of these patients are in the average age of their 70s by the time of diagnosis. Although quantitative differences in inflammatory cells may be present throughout studies, they may be well explained by patient selection, segmental or etiological differences in IBS

subsets [2,29,30]. Whether these findings may serve to differentiate etiological (postinfectious, post stress) variants of IBS or may be related to clinical severity still remains unknown.

Mast cells have been frequently considered in the context of allergic and parasite inflammation but growing and convincing evidence indicates that they also participate in a wide variety of physiological and pathological processes [32] including the regulation of epithelial barrier, mucosal immune function, motility and gut visceral sensitivity [13,33]. Such abnormalities in gut function have been reported in IBS and could be partly responsible for clinical findings in these patients, especially those with predominant diarrhea, since patients with increased intestinal mast cells [34], and up to 70% of those with systemic mastocytosis [35], develop diarrhea and abdominal discomfort.

The quantitative analysis of mucosal mast cells was based on CD117 positive immunohistochemistry which has shown high correlation with tryptase staining, it is not altered by massive degranulation and can be regarded as specific for mast cells in the gut mucosa [36]. Our results indicate that jejunal mastocytosis (>20 MC/hpf) is a constant feature in the mucosa of this selected group of patients with IBS-D. Our IBS-D patient group_was quite homogeneous and could be profiled and well represented by a naive person experiencing active and mostly severe bowel disturbances, not previously diagnosed or treated, and suffering from moderate psychological stress but no coincident allergic disorders. Increased mast cells numbers and mast cells biological products have been previously described in the terminal ileum and proximal and distal colon of IBS patients [17-19,29,30]. Moreover, increased number of mucosal mast cells have been recently described in the duodenal mucosa of patients with diarrhea, with some of them probably belonging to the IBS-D subgroup [37]. We cannot exclude that small bowel mast cell hyperplasia may represent an epiphenomenon linked to low-grade mucosal inflammation since

there have been conflicting reports describing increased or decreased mast cell numbers in the upper small intestine of disorders like chronic urticaria [38], psoriasis [39] or celiac disease [40]. Biopsies of the upper small bowel are often obtained as part of the clinical evaluation of diarrhea and, based on routine histopathology, informed as normal. IBS-D may be easily overlooked by following conventional approach and we suggest that on account of the relative ease of jejunal capsule biopsy sampling and the very high sensitivity and positive predictive values of the CD-117 analysis (over 90% in our study), its validity as complementary criteria for the positive diagnosis of IBS-D deserves further evaluation.

Some studies have shown a potential influence of clinical history or age on mucosal mast cell numbers [17,24,41]. Although our data do not support a correlation between age and gender with mast cell numbers, we acknowledge the relatively low number of participants included as a limitation to properly evaluate this aspect. Some questions remain to be answered such as the influence of the clinical course (active or remission), the length of clinical history or the relationship of clinical severity with mucosal mast cell numbers or mast cell phenotype. Again, further studies are warranted to answer these and other emerging worries.

Tryptase is an abundant specific neutral protease of human mast cells that can be measured in various biological fluids and may serve as a useful marker of mast cell activation [42]. Our study is unique in reporting the association of jejunal mast cell hyperplasia with, only local, *in vivo* mast cell activation, as disclosed by elevated levels of jejunal luminal tryptase but not serum tryptase. Although mast cells are the only significant source of tryptase in the intestinal mucosa no correlation between the number of mucosal mast cells and the levels of luminal tryptase was detected. One plausible explanation for these findings is that mast cell activation and secondary release of tryptase may be not a continuous process. In fact, mast cell activation in IBS seems to be more a piecemeal-like phenomenon

where slow and selective release of mediators occurs and increased luminal release takes place only in specific settings. Indeed, ultrastructural signs of piecemeal degranulation and *in vitro* release of mast cell products such as tryptase and histamine have been shown in colonic and ileal biopsies of IBS [17-19] and in some cases of duodenal samples, in patients with chronic diarrhea [43]. Although tryptase, via activation of protease-activated receptor-2 or other unrelated mechanisms, is a good candidate to explain some of the pro-secretory and pro-inflammatory effects of mast cells [44-45], other mast cell mediators may be also involved.

The mechanisms, mediators and pathways that may account for mast cell activation and hyperplasia in the jejunum of IBS-D remain to be fully characterised. The list of potential candidates is large and growing although the known ability of molecules such as stem cell factor [46], IL-4 [46], transforming growth factor-¤$_1$ [47], corticotropin-releasing hormone [48] and IgE [46] to modulate crucial aspects of human mast cell physiology such as secretory activity, growth and maturation, phenotype or migration could make them initial, but not exclusive, favourites.

We have found that IBS-D patients were suffering from higher levels of psychological stress than the control group. Although we did not find a positive correlation between stress levels and mast cell numbers or tryptase release a suggestive trend was apparent. Anyway, it is well accepted that IBS is a stress-sensitive disorder, where life events are strong predictors of clinical exacerbation [49] and the existence of distorted autonomic patterns along with neuroendocrine abnormalities in the hypothalamic-hypophyseal-adrenal axis [50] seem to be related with changes that result in predominant bowel habit (diarrhea/constipation) and intestinal visceral hyperalgesia [51]. These observations, consistent with the participation of neurohumoral mediators of stress in the initiation and development of such pathophysiological abnormalities [11,52], are being substantiated by experimental and clinical studies showing that both stress and corticotropin-

releasing hormone regulate intestinal epithelial and immune function via mast cell activation [12,15,22,53].

Finally, epidemiologic studies suggest that IBS and functional dyspepsia overlap to a greater extent than would be expected by chance alone [20,21]. In agreement with these proposals, we have found that 70% of our IBS-D fulfilled Rome II criteria for functional dyspepsia. Although extension of mucosal mast cell involvement from the distal colon to the upper gut might be a helpful explanation to understand the frequent overlap, we did not observe any difference in jejunal mast cell numbers between dyspeptic and non-dyspeptic IBS patients. Although not thoroughly evaluated, others have shown that mast cells were increased in the antrum and corpus of patients with Helicobacter pylori-negative functional dyspepsia [54], compared to controls. Thus, another key unanswered question is whether patients with dyspepsia and IBS share_similar pathogenesis and different clinical expressions.

In conclusion, jejunal mast cell hyperplasia and tryptase release may be frequent and useful findings in non treated IBS-D. Their validity as biological markers for IBS-D and usefulness to develop mast-cell related treatment strategies in these patients should be established by further studies.

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CAPÍTULO 2

“CHRONOLOGICAL ASSESSMENT OF MAST CELL-MEDIATED GUT DYSFUNCTION AND MUCOSAL INFLAMMATION IN A RAT MODEL OF CHRONIC PSYCHOSOCIAL STRESS”

INTRODUCTION

The increasing incidence of stress-related morbidity in modern societies is in close dependence with raised people expectations, seemingly endless, as a mandatory toll to overcome everyday life confrontation against ubiquitous environmental, psychosocial, and economic determinants (1). Relevant to digestive disorders, there is now compelling evidence for the modulatory role of physical and psychological stresses, whether acute or chronic, in shaping the clinical course of a number of functional and inflammatory conditions of the gastrointestinal tract (2). In particular, epidemiological, empirical and clinical observations provide valuable support for life stress as a common co-morbid event in the irritable bowel syndrome (IBS), which may strongly influence symptom onset, severity, and persistence in certain IBS subtypes (3-6). Furthermore, intestinal dismotility, visceral hyperalgesia, reactivation of mucosal inflammation, and epithelial dysfunction have been all related to stress episodes in post-infective and diarrhea-predominant IBS (7-9). However, the mechanisms by which stress impacts on IBS pathophysiology remain to be fully established. In this regard, the development of animal models reproducing stress-related gastrointestinal dysfunction has lately evolved as a helpful approach to improve our understanding on IBS pathophysiology (10). Relevant experimental models include studies in naturally or genetically modified stress-sensitive rodent species and the use of paradigms such as neonatal maternal deprivation (11,12), water avoidance stress (13), and intestinal infection (14) or irritation (15,16), in both early life and adulthood. From these models we have learned that single acute or repetitive exposure to homotypic stresses of different nature leads to intestinal pathobiology revealed as increased ion secretion, macromolecular permeability, microscopic inflammation, visceral hypersensitivity, dysmotility, and even bacterial penetration (17-19). In addition, these responses involve dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis and are mediated by stress-related

neuropeptides such as corticotropin-releasing factor (CRF), neural mechanisms and mast cells (20,21), a profile which has been also described in IBS patients (22).

Despite much information has been gathered we still lack detailed information on the time course of specific stress-related mucosal alterations in the small and the large intestine of these animals. Furthermore, the vast majority of laboratory stress protocols applied for the study of intestinal disorders have been criticized for the use of repetitive homotypic stimuli. The predictive validity of those models remains disputed because in socially organized mammals, dominant stressors in natural conditions are frequently heterotypic and complex and best represented by social experiences (23). Crowding stress (CS) is a well-known validated rat model of chronic social stress that might adjust to the type and intensity of stressors experienced by these animals in their natural environment on a daily basis (24) and may also reflect well life stress for humans. Several metabolic, endocrine, and HPA axis disturbances including changes in body temperature, food intake and body weight gain, have been reported in response to CS (25). However, the effect of CS on gastrointestinal function remains largely unexplored. Therefore, the goals of the present study were to determine the ability of long-term CS to provoke functional and structural abnormalities in the rat intestine, to characterize the dynamics of these responses, and to evaluate its potential as a model for the study of IBS pathophysiology.

MATERIAL AND METHODS

Animals

Male Wistar Kyoto rats (175-200 g on arrival, Iffa Credo Laboratories, L'Arbresle, Lyon, France) were maintained on a normal 12:12 h dark:light cycle (0700/1900 h) and provided with food and water *ad libitum*. Rats were allowed for acclimation for 2 weeks before the experimental procedures. Procedures were approved by the Animal Care Committee at Institut de Recerca Vall d'Hebron, and performed in accordance with the European Union Directive for the Protection of Vertebrate Animals used for experimental and other scientific purposes (86/609/EEC).

Stress Protocol

Rats were randomly assigned to one of the following experimental protocols and kept in separate housing areas: CS, in which groups of eight animals were housed together in standard cages (48x23x14 cm) half-filled with bedding for up to 15 consecutive days to increase their social stress; Sham-crowding (SC), in which control rats were housed in pairs for equal time in cages containing hollow plastic cylinders to minimize environmental stress. Experiments were performed between 9'00 to 12'00 AM to control the effect of circadian rhythm.

HPA axis activity

The efficacy of CS and SC to switch on the stress HPA axis circuitry and adaptational changes were assessed by measuring the spontaneous release of plasma corticosterone at days 1, 3, 7, 12 and 15. The residual capacity of the HPA axis to respond to additional stimuli was determined by measuring corticosterone release at day 15, thirty minutes after intraperitoneal (i.p.) administration of vehicle (0.9% NaCl) or CRF (10 μg/kg, Sigma, Barcelona, Spain). Rats were euthanized by decapitation and truncal blood was collected into chilled tubes containing EDTA (1.5

mg/mL, Sarsted, Barcelona, Spain). Plasma corticosterone concentration was measured with an immunoassay kit (IDS Ltd., Boldon, UK).

Assessment of stress responses in the rat intestine

Rats were euthanized at day 1, 3, 7, 12, or 15, and the entire intestine excised. Thereafter, the small intestine and the colon were half-divided into proximal and distal segments, and coded for blind evaluation. Stress-induced intestinal chrono-pathobiology was determined using selected histophysiological indicators.

1. Myeloperoxidase (MPO) activity

We measured MPO activity, an index of neutrophil infiltration, in mucosal scrapings as previously described (Vicario et al., 2005). MPO is expressed as U/g wet tissue.

2. Transmission (TEM) and scanning (SEM) electron microscopy

Transmural intestinal fragments (jejunum, ileum, proximal colon and distal colon) were fixed for 4h in 4% glutaraldehyde in 0.1mol/L sodium cacodylate buffer (pH 7.4), postfixed for 2h in 1% (w/v) osmium tetroxide containing 0.8% (w/v) of potassium hexacyanoferrate, and dehydrated through a graded acetone series. TEM samples were infiltrated in Epon's resin, polymerized, contrasted, and examined using a Hitachi H-7000 microscope at 75Kv equipped with a MegaView III camera (Soft Imaging System). After critical point drying with CO_2, SEM samples were mounted on metallic tubs and coated with gold, examined under a SEM Hitachi S-570 (15kV) device, and images were captured with the Quartz PCI v.5.5. Chemicals (electron microscopy grade) were obtained from Sigma. Images were used to evaluate epithelial damage, with special focus on mitochondrial abnormalities including enlargement, swelling, loss of cristae, and vacuolisation, under a fixed size observation window of 300 μm^2. Intact and damaged mitochondria were quantified

and results expressed as the percentage of intact mitochondria per area in each segment. In addition, we evaluated qualitative differences and changes in cellular density on the luminal surface of intestinal tissues along time.

3. Flow cytometry

We next examined the cellular content in the lumen of the small intestine of SC and CS rats at day 15. After an overnight fast, the entire small intestine (containing the jejunum and the ileum) was excised and gently flushed with 50 mL of RPMI (Gibco, Barcelona, Spain). Luminal content was centrifuged (200 x*g*, 5 min, 4ºC) to eliminate pelleted debris. Cells were purified by gradient centrifugation with Percoll (Amersham Biosciences, Uppsala, Sweden). Cell suspension was washed, resuspended in PBS (pH 7.4) containing 2% (v/v) fetal calf serum and 0.5 g/L sodium azide, and cell counting and viability (>90% in all cases) were determined by trypan blue exclusion (Sigma). Double-colour immunofluorescence was performed by incubating with monoclonal mouse anti-rat antibodies CD45-PE-conjugated and CD3-FITC-conjugated (Becton Dickinson, BD, Barcelona, Spain). Stained cells were analysed using a FACSCalibur flow cytometer (BD) and data were analysed with CellQuest software (BD Biosciences).

4. Numbering mucosal mast cells by immunohistochemistry

Following standard procedures, formalin-fixed, paraffin-embedded tissue fragments were sectioned at 3µm. Mast cells were identified using sheep anti-rat mast cell protease-II (RMCP-II) antibody (1:500; Moredun, Midlothian, UK), following incubation with horse anti-mouse biotinylated IgG (Vector Laboratories, Burlingame, CA, USA). Staining was developed with the Vectastain® kit (Vector Laboratories). Positive cells in the mucosa were counted at high magnification (x400) in 10 to 12 well-oriented

sections/tissue using the CellB Image Acquisition Software (Olympus, Barcelona, Spain), and results are expressed as number of positive cells per mm^2.

5. Assessment of intestinal mast cell activation

We examined TEM micrographs, containing mucosal mast cells (15-25 cells/tissue), for the presence of piecemeal degranulation (loss of intragranular electrodensity without signs of inter-granular or granule-to-cell membrane fusion), as a marker of cell activation (26,27). Degranulation is expressed as the percentage of granules with decreased density per mast cell in each intestinal segment. To further assess mast cell activation, we measured the intestinal content of RMCP-II in mucosal scrapings from the jejunum and the distal colon of SC and CS rats. RMCP-II was quantified with an ELISA Kit (Moredun) and is expressed as ng/mg of wet weight. We also measured its luminal release in the jejunum of SC and CS rats at day 15, using a technique of segmental *in vivo* perfusion. Briefly, non-fasted rats were anesthetized with isofluorane and the intestine exposed through a midline laparotomy. A 15-cm segment of the jejunum was isolated with its neural and blood supply intact, gently rinsed of luminal content, and cannulated at the proximal and distal ends. The segment was reintroduced into the abdominal cavity and the laparotomy closed. The jejunum was perfused with NaCl 0.9% at a constant rate of 0.2 mL/min, for 30 min to allow equilibration, followed by 30 min in which intestinal perfusate was collected by gravity into chilled containers. Intestinal perfusion was also carried out in rats previously treated with the mast cell stabilizer ketotifen (1 mg/kg i.p., at -12h, and at -24h, day 15) or vehicle (0.9% NaCl). Intestinal luminal perfusates were assayed for RMCP-II by an ELISA kit and RMCP-II are expressed as µg/L.

6. Mucosal-to-serosal transport of macromolecules in Ussing chambers

Since it is our interest to study proximal regions of the intestine, segments of the proximal jejunum were obtained after 15 days if stress, stripped of longitudinal

muscle and myenteric plexus, opened along the mesenteric border, and two to four adjacent pieces from each segment mounted in Ussing chambers (Dipl.-Ing. K. Mußler, Scientific Instruments, Aachen, Germany). The chamber exposed 0.67 cm^2 of tissue area to 4 mL of circulating oxygenated Krebs buffer at 37°C. The buffer contained (in mM) 115 NaCl, 1.25 $CaCl_2$, 1.2 $MgCl_2$, 2.0 KH_2PO_4, and 25 $NaHCO_3$ (pH 7.35). The serosal buffer contained 10 mM glucose osmotically balanced by 10 mM mannitol in the mucosal buffer. Fifteen minutes after mounting the tissues, horseradish peroxidase (HRP type VI, Sigma) was added to the luminal buffer at a final concentration of 10^{-5} M. Serosal samples (0.5 mL) were collected at 30 min intervals for 2 h. HRP activity was determined by a modified Worthington method (Maehly et al., 1954). Flux of HRP is expressed as $pmol \cdot h^{-1} \cdot cm^{-2}$.

Statistical analysis

Results are expressed as mean ± S.E.M. Nonparametric or parametric tests were used when appropriate for single (Mann-Whitney *U* test, unpaired Student's *t*-test, and Pearson correlation test) and multiple comparisons (one-way ANOVA followed by post-hoc tests). Significance was set at $p<0.05$.

RESULTS

Crowding stress activates HPA axis

Stressed rats showed higher plasma corticosterone levels than sham-stressed animals from day 1 (CS:223±29 *v* SC:49±7.4 ng/mL; p=0.0007), and up to day 15 (CS:139±23 *v* SC:42±4.5 ng/mL; p=0.0044). Despite some adaptation (38% reduction in corticosterone release) was observed after 15 days of CS (Figure 1A), HPA reactivity to incoming stressors was preserved, as shown by equal corticosterone responses to exogenous CRF challenge than SC rats (Figure 1B).

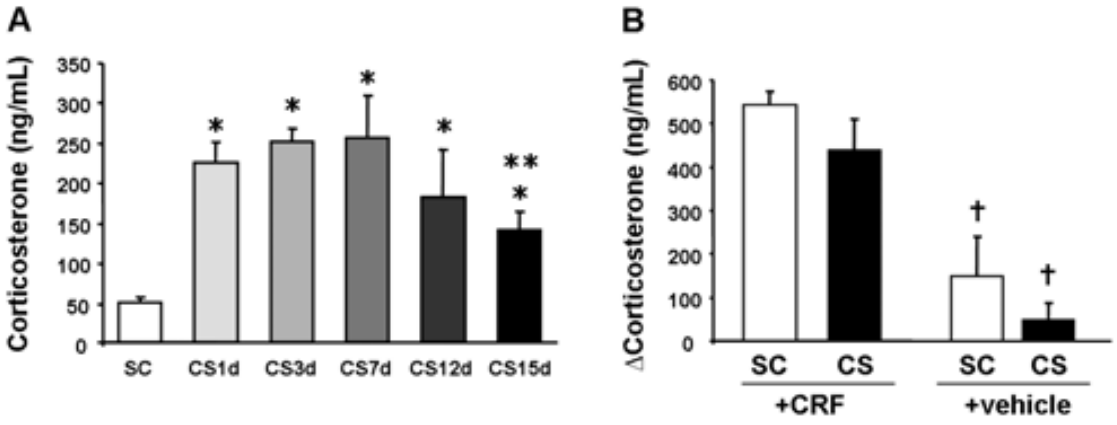

Figure 1. Activation of the hypothalamic-pituitary-adrenal axis in rats exposed to long-term sham-crowding (SC) and crowding stress (CS). A: Spontaneous light-phase release of plasma corticosterone at days 1, 3, 7, 12 and 15; **B**: Increment in plasma corticosterone in response to corticotropin-releasing factor (CRF, 10µg/Kg) or vehicle (0.9% NaCl) i.p. administration at day 15. Values represent the mean+SEM (n=8-16 rats/group; *p<0.05 *v* corresponding SC; **p<0.05 *v* CS at day 1; † p<0.05 *v* CRF).

Crowding stress triggers intestinal inflammation and leukocyte extravasation to the small intestinal surface

While all tissues from SC rats displayed low and stable mucosal MPO activity levels, tissues from CS rats showed mild though significant elevation of mucosal MPO activity. This inflammatory component was evident from day 3 in the ileum and the colon, and from day 7 in the jejunum, throughout day 15 (Figure 2).

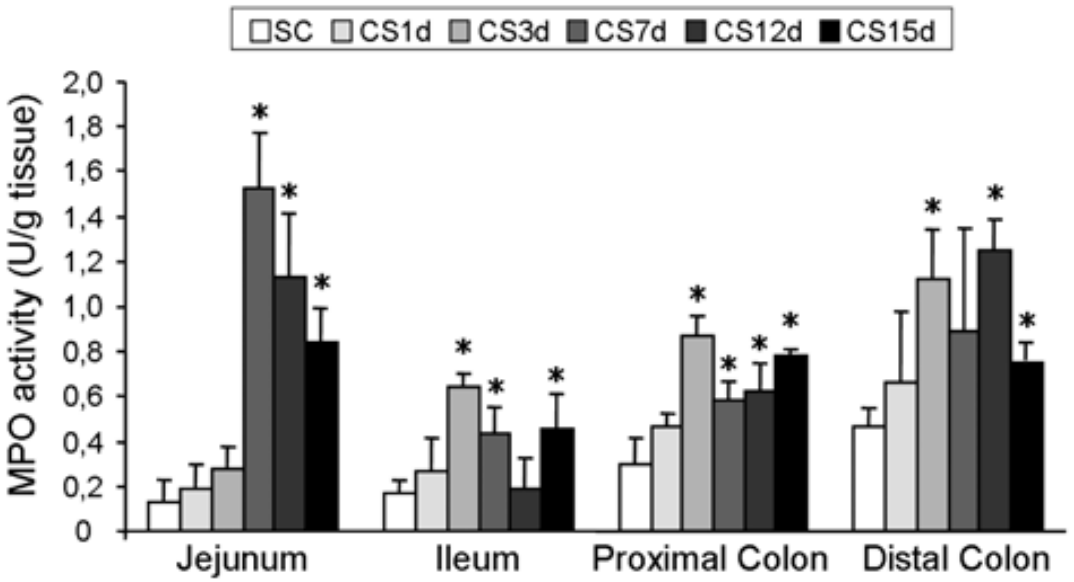

Figure 2. Dynamics in time of mucosal myeloperoxidase (MPO) activity in mucosal scrapings of sham-crowding (SC) and crowding stress (CS) rats. Bars represent the mean+SEM (n=12-16 rats/group; *p<0.05 *v* corresponding SC group).

Ultrastructural evaluation of the intestinal surface of CS rats disclosed a growing accumulation of leukocytes and red blood cells along time only in the small intestine (jejunum and ileum), mostly located within and underneath the mucus layer (Figure 3A). The surface of the colon did not show differences between SC and CS rats, and no sign of altered epithelial morphology or cell extravasation was detected (data not shown). After morphological identification of blood cells on the surface of the small intestine, we next identified leukocytes by immunostaining procedures and confirmed a remarkable over 10-fold increase in total leukocyte counts (CS:585,000±30,000 *v* SC:44,000±15,000; $CD45^+$cells; n=8; p<0.0001), and numbers of both T lymphocytes and other $CD45^+$ non-T cells in the lumen of the small bowel of CS rats, at day 15 (Figure 3B). In addition, stressed rats also displayed higher ratios for $CD45^+CD3^+$ populations, respect to total luminal leukocytes (CS:34±3.1 *v* SC:20±3.2%; n=8; p=0.0072; Figure 3C).

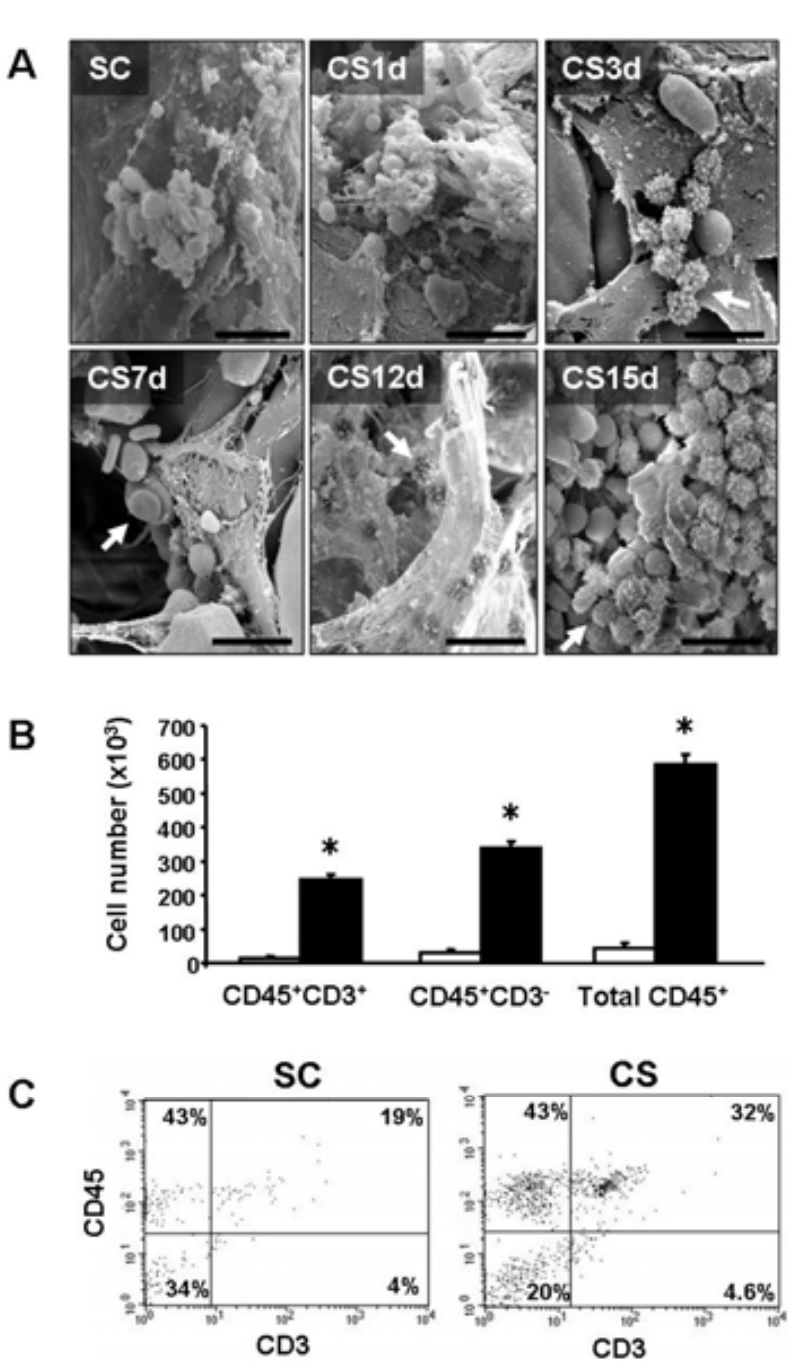

Figure 3. Leukocyte extravasation over time into the small bowel lumen of rats submitted to social stress. A: Representative scanning electron micrographs of the rat jejunal surface in sham-stress (SC) and at different time points during crowding stress (CS) exposure. The density of blood cells increases over time in the surface of the small intestine from CS3d where some cells are detected, through CS15d where extensive areas of the surface are covered by blood cells. Red blood cells and leukocytes are indicated (white arrows). The jejunal surface in SC rats remained as shown throughout time. Bars=15μm in SC and 10μm in CS photographs. B: Cell counts in jejunal washings from SC rats (empty bars) and CS rats (filled bars) at day 15. Bars represent the mean+SEM (n=8 rats/group; **p*<0.0001 *v* SC group). C-Representative dot-plot showing CD45/CD3 double staining of cells present in the small intestinal lumen at day 15.

Crowding stress induces mitochondrial damage

On electron microscopy (magnification 500-800x), the global architecture and integrity of the apical side of the intestinal epithelium was preserved during the stress exposure. On the contrary, ultrastructural assessment (magnification 3000x) of epithelial mitochondria revealed growing signs of organelle damage along time in both enterocytes and colonocytes, but only in CS rats (Figure 4A). Indeed, as compared to SC tissues, the number of intact mitochondria in CS rats was significantly reduced already at day 3 in the colon, while remained unaltered in the small intestine until day 12 (Figure 4B).

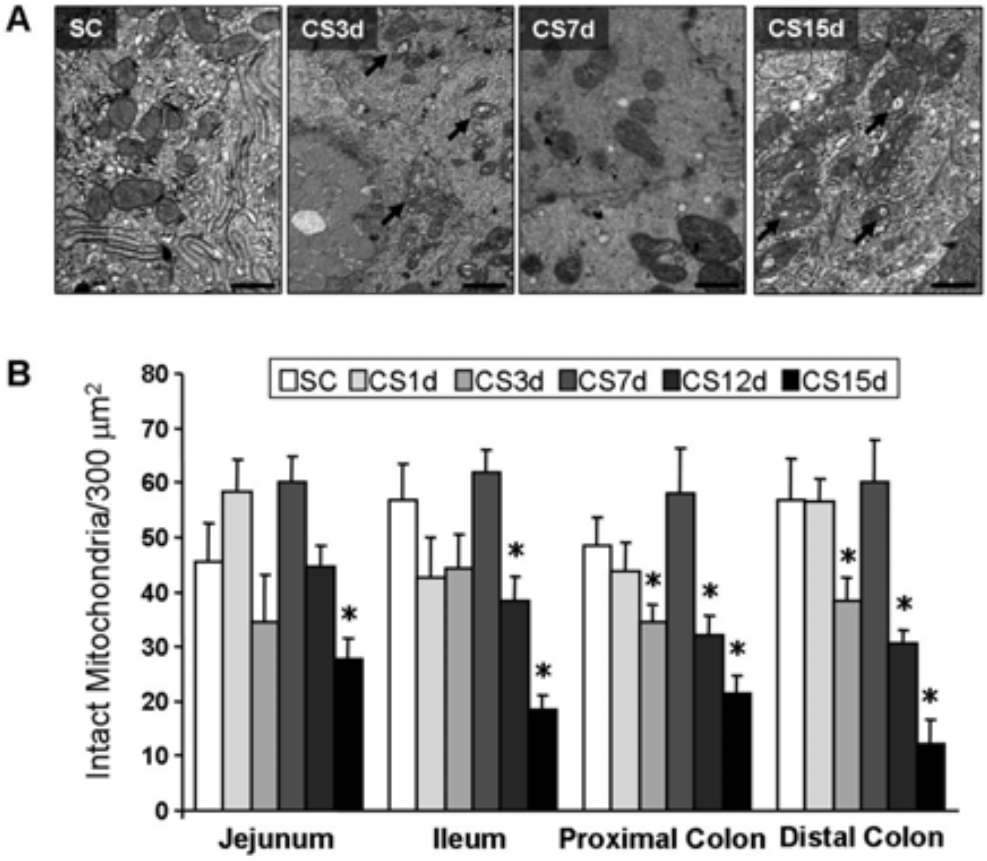

Figure 4. Time-dependent alterations in epithelial mitochondria in the small and large bowel of rats submitted to social stress. A: Representative transmission electron micrographs of mitochondria from jejunal epithelial cells of sham-crowded (SC) and crowding stress (CS) rats at different time points. Epithelial damage is demonstrated by mitochondrial swelling and the loss of cristae (arrows) Bars=1µm in SC, CS7d, CS15d; and 2µm in CS3d photographs. B: Quantification of intact mitochondria in intestinal segments of SC rats (empty bars) and CS rats (filled bars) at different time points. (n=6 rats/group; *p<0.05 *v* corresponding SC group).

Crowding stress induces selective distal colonic mast cell hyperplasia yet extensive mast cell activation across the intestine

As assessed by RMCP-II immunostaining the number of mucosal mast cells remained unchanged throughout time in the small intestine and proximal colon of SC and CS animals. Only in the distal colon of CS rats mast cell numbers significantly increased at days 12 and 15 (day15: CS:68.2±14.7 *v* SC:32.9±4.56 cells/mm^2; n=6; *p*=0.0447; Figures 5A and B).

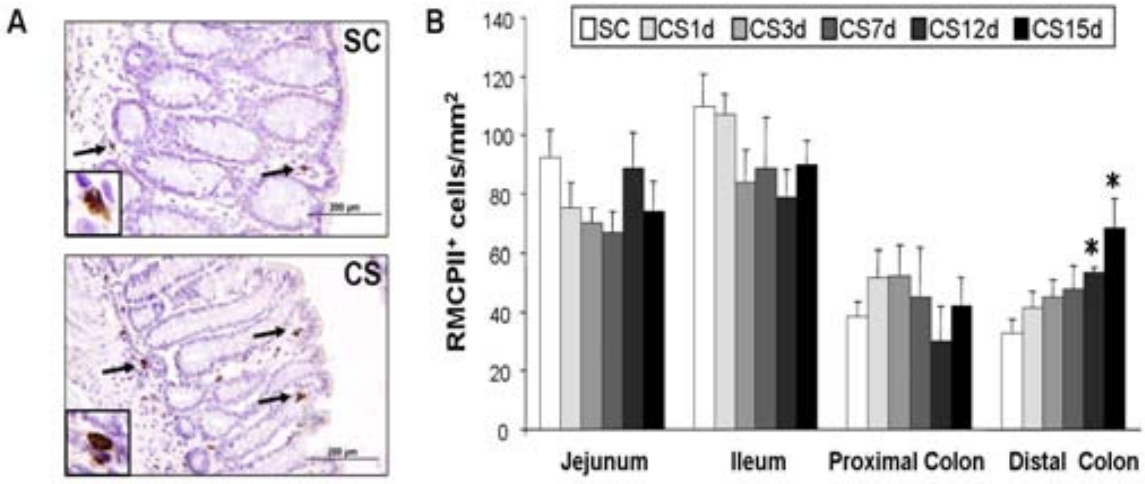

Figure 5. Time-dependent alterations in mucosal mast cell counts in the small and large bowel of rats submitted to social stress. A: Representative photographs of RMCP-II immunostaining in the distal colon of sham-crowded (SC) and crowding-stressed (CS) rats at day 15. Positive cells are located at the crypt base and between cells in the lamina propria along the crypt (arrows). Insert in each picture shows positive staining of a mast cell. B: Quantification of RMCP-II$^+$ cells in the intestinal mucosa of SC and CS rats in all intestinal segments at different time points of the experimental protocol. Bars represent the mean+SEM (n=6 rats/group; *p<0.05 *v* corresponding SC group).

Interestingly, ultrastructural evaluation disclosed increased piecemeal degranulation in mast cells from CS tissues (Figure 6A), that was significantly higher already at day 1 in all intestinal segments, and increased along time to 4-fold in the jejunum (day15: CS:32.4±2.9 *v* SC:7.6±3.1%; n=6; *p*=0.0002), and to 7-fold in the distal colon (day15: CS:41.2±5.6 *v* SC:5.6±2.2%; n=6; *p*=0.0001), compared to respective SC tissues (Figure 6B).

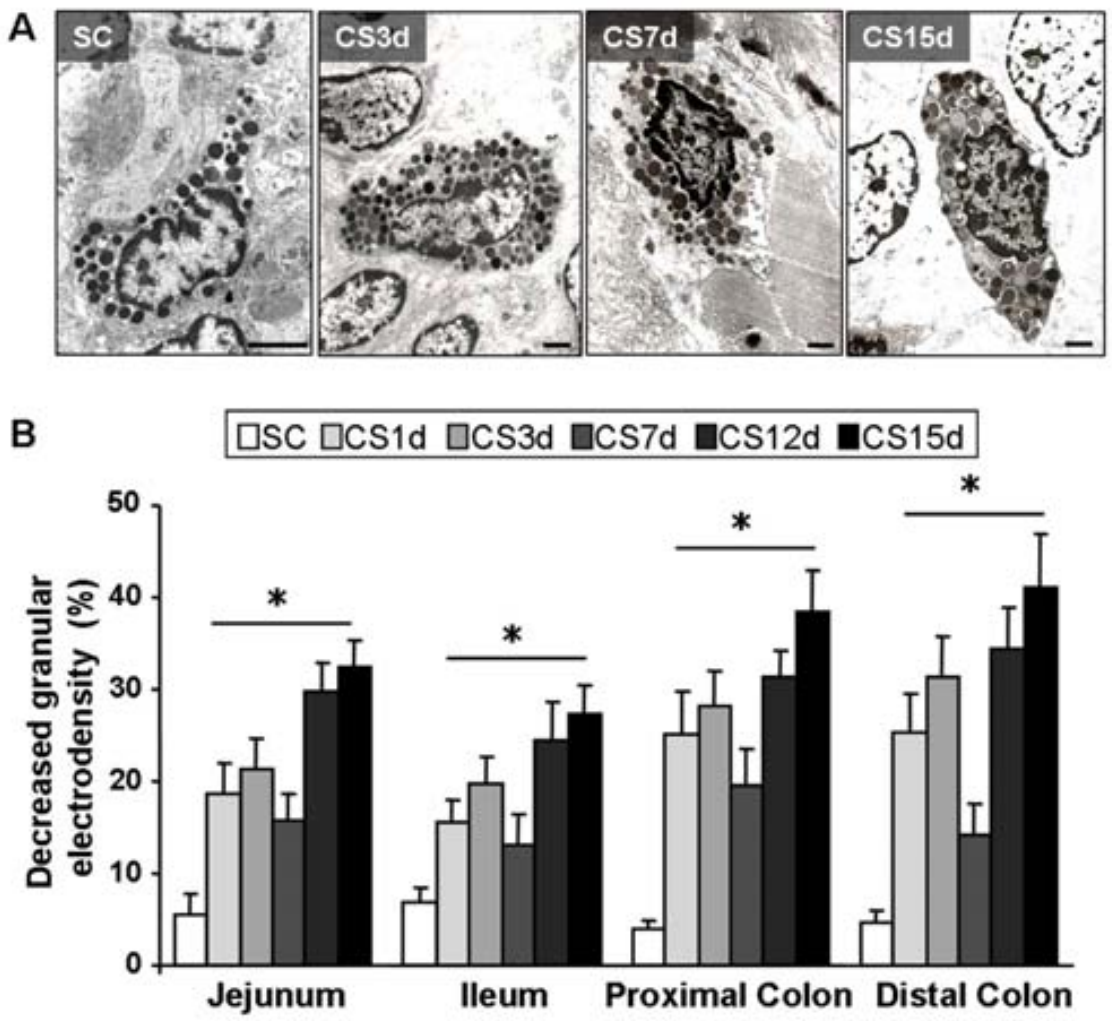

Figure 6. Increasing piecemeal degranulation of mast cells during stress exposure. A: Representative micrographs of mucosal mast cells in the jejunum showing different granular density in sham-crowded (SC) and crowding-stressed (CS) rats at different time points of the experimental protocol. B: Percentage of granules with decreased density in each mast cell (15-25 mast cells/rat). Bars represent the mean+SEM (n=6 rats/group; *p<0.05 *v* corresponding SC group). Bars=2µm in SC, and 1µm in CS photographs.

Consistent with mast cell activation, the mucosal content of RMCP-II was higher in CS rats, both in the jejunum (CS:413±32.4 *v* SC:239±21.2 ng/mg; n=6; p=0.0025) and the distal colon (CS:71.8±11.4 *v* SC:38.5±3.6 ng/mg; n=6; p=0.0190), at day 15 (Figure 7A). Moreover, intestinal perfusion showed the luminal release of RMCP-II to be significantly higher in the jejunum of CS rats (CS:23±1.9 *v* SC:15.7±2.7 µg/L; n=5-6; p<0.0346), and this effect was prevented by ketotifen (Figure 7B). Of importance to understand the underlying mechanism of stress-induced epithelial dysfunction is the

positive correlation between decreased granular density in mast cells and mitochondrial damage in both the jejunum (p=0.003; r=0.7425) and the distal colon (p=0.001; r=0.8626).

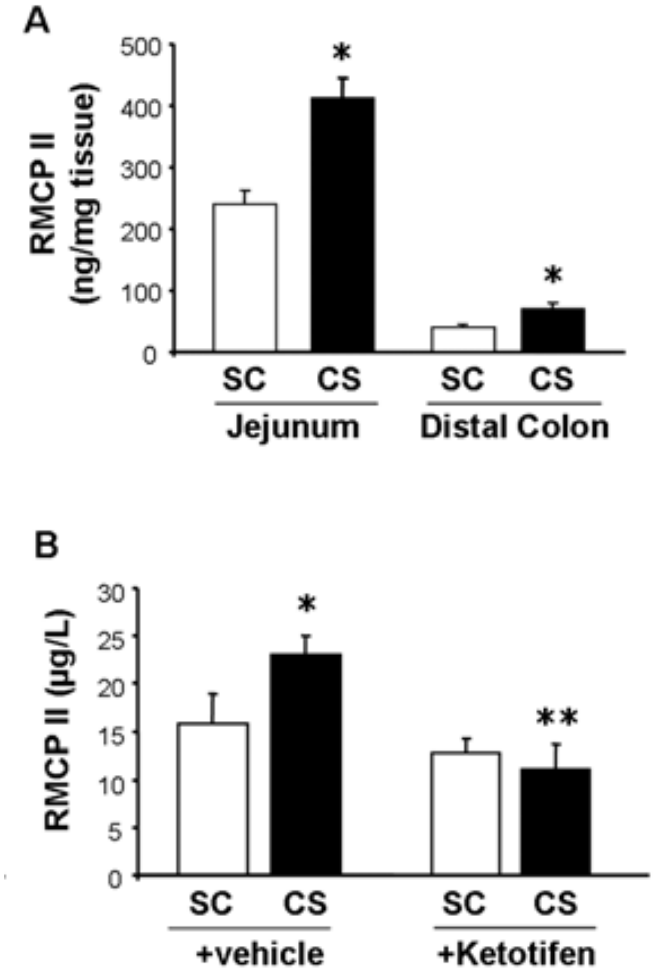

Figure 7. Increased intestinal content of rat mast cell protease (RMCP)-II in rats submitted to social stress. A: Mucosal content of RMCP-II (ng/mg tissue) in sham-crowded (SC) and crowding-stressed (CS) rats at day 15. B: Luminal content of RMCP-II (µg/L) in the jejunum of sham-crowded (SC) and crowding-stressed (CS) rats at day 15, after pretreatment with ketotifen (1 mg/kg i.p., at -24h, and at -12h) or vehicle (0.9% NaCl). Bars represent the mean+SEM (n=6-8 rats/group; *p<0.05 *v* corresponding SC group; **p<0.05 *v* vehicle).

Increased jejunal epithelial permeability by crowding stress is mediated by mast cells.

HRP flux across jejunal tissues was significantly enhanced at day 15 in stressed rats (CS:19±2.9 *v* *SC*:6.3±3.5 pmol·h^{-1}·cm^{-2}; n=5-6; p=0.0237). Not surprisingly, HRP flux correlated with RMCP-II content in the jejunal mucosa (p=0.001; r=0.9176) and with

mast cell degranulation as assessed by TEM (p=0.001; r=0.9003). Notably, pre-treatment with ketotifen abolished HRP flux enhancement, highlighting the role of mast cells in stress-induced barrier dysfunction (Figure 8).

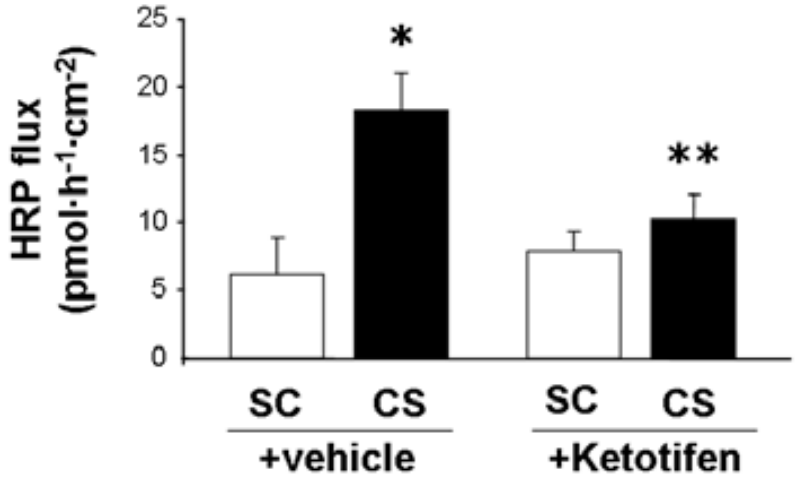

Figure 8. Mast cell-dependent effect of stress on intestinal permeability to macromolecules. HRP flux (pmol·$h^{-1}cm^{-2}$) across the jejunum in sham-crowded (SC) and crowding-stressed (CS) rats and after injection with ketotifen (-24h and -12h; 1 mg/kg i.p.) on day 15 of the experimental protocol. Bars represent the mean+SEM (n=5-6 rats/group; *p<0.05 *v* SC group; **p<0.05 *v* CS group).

DISCUSSION

This study shows the conspicuous impact of chronic psychosocial stress on intestinal function in the rat and the chronology of stress-induced abnormalities. Despite some segmental differences across the intestine, crowding stress acts as a consistent triggering factor for the development of mucosal low-grade inflammation, mitochondrial damage, and barrier dysfunction. Importantly, these stress-induced abnormalities seem to rely on biological mediators released by activated mast cells. Given the resemblance of above findings to those reported in some diarrhea-prone IBS patients, we discuss the potential relevance and translational applicability of this novel experimental model for the research of stress-sensitive intestinal disorders.

Crowding stress is a laboratory model widely used to evaluate the activity of the HPA axis in rodents (28). The establishment and maintenance of social hierarchy in crowded rats implicates competition for resources (space, food and water), and results in strong psychological and physical experiences for all subjects, including dominant and subordinate animals (23). Consistent with prior knowledge in this and other chronic psychosocial stress-based experimental models such as subordinate colony housing (29) or maternal deprivation (30), we found a significant activation of the HPA axis at the onset of CS, manifested by elevated plasma corticosterone levels, which was maintained until the end of the stress protocol. Although not a universal finding in IBS patients, possibly in relation to its clinical heterogeneity, higher baseline levels of free cortisol are not uncommon in IBS (31,32). Activation of HPA axis persisted until the end of crowding, yet adaptation to stress exposure appeared along time, as shown by diminished basal corticosterone levels at day 15 compared to day 1. However, and in agreement with earlier literature (28), CRF-induced corticosterone surge response remained unaffected after 15 days of CS, and equal to control rats. Although not explored by us, crowding reduces CRF-induced ACTH increase and also promotes defective HPA responses to other specific endogenous

stimulators such as vasopressin (28). Anyhow, factors such as the type and intensity of stressor, individual susceptibility, and variations in experimental protocols, may account for controversial reports on HPA responses, either facilitated or attenuated, to novel stressors in animals exposed to chronic stress (29,33-35). Interestingly, several studies indicate that upon stimulation, IBS display normal to exaggerated HPA axis responses, including cortisol and ACTH over release to CRF (7,31), though conflicting results also exist (32,36). Because stress-related HPA dysregulation may aggravate intestinal inflammatory responses (29,37,38), the preservation of CRF sensitivity in CS rats could be of major importance to regulate intestinal homeostasis and to determine the extension of intestinal dysfunction (39-42).

In keeping with past studies in chronic mild-stress models, we did not observe signs of macroscopical inflammation in the small bowel and the colon mucosa during stress exposure. On the contrary, a major finding in our study was the detection of a variety of readouts demonstrating the capacity of CS to initiate and maintain microscopical inflammation and barrier dysfunction across the rat intestine. While the ability of mild chronic stress to provoke mucosal dysfunction in the rodent intestine is well established, the evidence in favour of mild chronic stress as a *per se* inducer of intestinal mucosal inflammation is rather scarce. One first interesting observation in our model was the progressive decrease of intact epithelial mitochondria in all segments of CS rats, as determined by enlargement, swelling and loss of cristae. These morphological alterations, direct evidence of membrane injury and representative of epithelial damage, developed earlier in the large bowel, perhaps indicating a greater susceptibility to chronic psychosocial stress. Interestingly, normal number of intact organelle was observed at day 7, presumably as the result of adaptative mechanisms to the persistence of stress. However we cannot exclude other mechanisms, the epithelial turnover, which takes 5 to 7 days (43), may explain the observed recovery in all intestinal segments. Nevertheless,

persistent psychosocial stress disrupts mitochondrial morphology in intestinal epithelial cells, alterations similar to those reported in other stress protocols such as chronic water avoidance stress (19,26), effect associated with enhanced epithelial permeability (44), via rearrangement of F-actin and tight junction proteins. We ignore but presume, based on our preliminary observations and supported by studies in socially isolated rats (45), that enhanced mitochondrial dysfunction and proapoptotic signalling could be also present. This phenomenon may partly explain the compromised intestinal epithelial cell kinetics disclosed in other rat models of chronic stress (45,46). Accounting for the essential role of these organelles in cellular function, generating more than 90% of the cell's energy requirements through oxidative phosphorylation, and its emerging implication in neuroimmunomodulation (47), the functional consequences derived from CS-mediated mitochondrial damage on intestinal physiology should receive due consideration.

We also found a modest but significant elevation of mucosal MPO activity, indicative of neutrophil infiltration, that begun earlier, at day 3, in the ileum and the colon than in the jejunum, and persisted throughout day 15 in all tissues. Convergent results have been described in the lamina propria of the ileum and colon of rats after long-lasting water avoidance or cold stress (19,48), although MPO activity may normalize after 10 days in models of immobilization stress (49). Despite the known immunomodulatory ability of glucocorticoids, the lack of correlation between time-course levels of circulating corticosterone and MPO activity makes its direct contribution to MPO response in our model unlikely, and suggests the participation of additional mechanisms.

In addition to neutrophil infiltration, we showed significant CS-mediated promotion of mucosal mast cell activation, as denoted by all, ultrastructural signs of piecemeal degranulation from the onset of stress, and increased mucosal and luminal levels of RMCP-II at day 15. Although, elevated counts of intestinal mucosal mast

cells is a common consequence of chronic stress exposure (19,26,37,42,48), we only appreciated the late development of significant mast cell hyperplasia in the distal colon of CS rats. Disparities in numbers of mast cells may depend on various experimental factors and, at the end, of minor functional relevance compared to their state of activation, a quasi-universal effect of chronic stress, as latter discussed. Whether the selective mast cell hyperplasia and the earlier development of mucosal inflammation and mitochondrial damage of the stressed colon reflects its higher susceptibility to psychosocial stress than that of proximal regions of the small intestine, remains to be proved.

We also noticed a novel finding, namely the stress-and time-dependent rise in the density of surface leukocytes and T lymphocytes in the small intestine. These cells were mostly located within and underneath the mucus layer, what taken in conjunction with the preservation of the apical structure of enterocytes, suggests that their presence is a consequence of paracellular migration into the lumen rather than a byproduct of contamination when manipulating the tissue. This phenomenon could be facilitated by the considerable increment in circulating granulocytes (50,51) and the disruption of tight junctions (12) occurring in response to long-term psychosocial and homotypic stress exposure. Moreover, although circulating total lymphocytes are commonly decreased in these models, this may be a reflection of their selective redistribution and concentration into lymphoid organs (52) such as the intestinal mucosa. These cell population adjustments partly rely on stress-related corticosterone and catecholamine release. Notwithstanding, and in view of the diverse receptors for stress mediators found on leukocytes (53), including CRF-R1 and CRF-R2, the participation of additional mechanisms cannot be excluded and remains subject of future research. Noticeably, immune infiltrate is also larger in the intestinal and colonic mucosa of 50% of IBS patients than in healthy controls, and

characterized by increased populations of activated T lymphocytes and mast cells (54,55).

Importantly, our experiments corroborate previous observations in several other models of chronic stress, to show the significant enhancement of macromolecular permeability across the jejunal epithelial barrier in CS rats. Overall, this effect of CS relates to mast cell activation but not mast cell hyperplasia, as inferred from the strong positive correlations between HRP flux and both RMCP-II mucosal content and mast cell piecemeal degranulation, and also by the remarkable ketotifen-mediated decrease in the uptake of HRP. Moreover, the positive correlation attained for piecemeal degranulation of mast cells and mitochondrial damage in both the jejunum and the distal colon highlights the importance of mast cell activation in CS-induced epithelial dysfunction, a consistent observation in models of acute and chronic stress (56). Although not explored by us, CD8+ and CD4+ T cells and neutrophils may also participate, because these immune cells play central roles in the development of intestinal barrier dysfunction after restraint stress, and burn injury, respectively (37).

Several molecules may be involved in mediating the activation of mast cells in response to stress, including CRF, acetylcholine, nerve growth factor, and substance P. Of special significance may be the role of peripheral CRF, released by regional sensory and sympathetic nerves, immune cells, and gut enteroendocrine cells (57,58). Compelling evidence indicates that activation of CRF-R1 is responsible for stress-induced increases in colonic transit, visceral hyperalgesia, and paracellular permeability (58). Alternatively, activation of CRF-R2 is thought to dampen motor and nociceptive actions of CRF-R1 and may be involved in stress-related enhancement of both paracellular and transcellular permeability (59). Notably, recent observations disclose segmental differences in gene and protein expression of CRF receptor subtypes in the colon of WKY rats, both at baseline and after acute

stress exposure (60). Since a role for local CRF signalling in stress-induced alterations in colonic function in WKY rats is also supported (18,60), its contribution to the observed variations in psychosocial stress-induced responses across the intestine and its functional significance remains to be elucidated. Expression of both CRF receptors on mucosal mast cells along with pharmacological blockade or studies in mast cell-deficient rodents suggest a prominent role of CRF-mast cell axis in mediating stress-induced intestinal dysfunction. Moreover, CRF has been recently proved to modulate macromolecular permeability acting via mast cells in the human colon (61), and to contribute to stress-related progression and exacerbation of IBS manifestations by modulating the autonomic and enteric nervous systems as well as the immune function (62).

Finally, we acknowledge that our work raises some intriguing questions that could be matter for future research. Overall, being IBS a stress-sensitive disorder, improved discrimination of resilient and stress-prone individuals may be of great value to unravel the mechanistic basis for disease susceptibility. A seemingly helpful approach for this purpose is to characterize the functional and bio-pathological differences between dominant and subordinate animals in response to chronic psychosocial stress, and hence gaining insight into the role of life stress, whether protective or prejudicial, in intestinal pathophysiology.

In summary, we show the ability of long-term crowding stress to reproduce in part mucosal pathobiological substrate (dominated by mast cell activation and hyperplasia and barrier dysfunction) commonly present in the intestine of certain IBS subgroups (22,54). Although not fully investigated here, previous observations in crowded rats (63) converge with own´s unpublished results to indicate that CS also provokes diarrhea and colonic hypersensitivity to balloon distension in these rats. Considering the strong empirical support for the role of psychosocial events (3,4,5) in the onset of IBS manifestations and its connection to intestinal barrier dysfunction

and mucosal inflammation (64), it appears that our model may be considered suitable for the study of stress-related intestinal disorders and potentially helpful to unravel the complex pathophysiology underlying certain human intestinal disorders, particularly IBS.

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CAPÍTULO 3

“CORTICOTROPIN-RELEASING FACTOR TRIGGERS JEJUNAL MAST CELL ACTIVATION AND LOCAL PROINFLAMMATORY RESPONSES IN DIARREA-PRONE IRRITABLE BOWEL SYNDROME PATIENTS”

INTRODUCTION

Irritable bowel syndrome (IBS), a highly prevalent disorder, is defined by the presence of recurrent abdominal pain and discomfort, associated with disrupted bowel habits (diarrhoea, constipation or an alternation of both) and is associated with an important alteration of the quality of life (1). Several mechanisms have been proposed as the underlying cause of IBS however its etiology is not well understood. Unfortunately, IBS lacks of specific diagnostic markers and remains a rather heterogeneous symptom-based disorder. Psychological factors are known to be key components in the outcome of clinical manifestations of IBS symptoms. Experimental and clinical studies consistently show the association between both, acute and chronic stress with gut dysfunction (2) and identify stress as a permissive factor in the development and/or exacerbation of IBS symptoms.

Compelling experimental evidence demonstrated that corticotropin-releasing factor (CRF) is the main mediator of the neuroendocrine, immunological, autonomic, behavioural and visceral responses to stress (3). CRF has been localized in the hypothalamus and in brainstem and limbic structures, being this pattern of distribution congruent with a role of central CRF in regulating homeostatic and emotional response systems. However, recent studies point to an equal important contribution of the peripheral CRF signalling locally expressed in the gut to the gastrointestinal (GI) stress response (4). Two types of CRF receptors (CRF-R1 and CRF-R2) have been detected in the colon and ileum, where exerts an effect on the regulation of intestinal permeability, ion secretion, motility, inflammation and pain perception (5). In addition, CRF has been shown to enhance transcellular uptake of macromolecules in human colonic mucosa via CRF-R1 and CRF-R2, located on subepithelial mast cells (6).

Stress-induced mucosal barrier dysfunction is critical in the development of intestinal inflammation. Convincing evidence indicates that intestinal mast cells

plays a key role in the regulation of intestinal barrier impairment (7), and both acute and chronic stress activate intestinal mast cells, as demonstrated in humans and rodents (8-10) . An increase in intestinal permeability has been described in patients with diarrhea-prone IBS (IBS-D) (11) and these patients show mucosal mast cell hyperplasia and activation in the jejunum (12). However, the mechanisms responsible for local responses and its contribution to disease are not fully described. Our hypothesis was that systemic CRF mediates intestinal barrier dysfunction involving local immune activation, which differentiates between IBS-D and health

MATERIAL AND METHODS

Participants and clinical assessment

Newly diagnosed diarrhea-predominant IBS patients (IBS-D), selected according to Rome II criteria (13), and healthy volunteers (H), without a history of gastrointestinal complaints, were prospectively recruited from the outpatient gastroenterology clinic at Hospital Vall d'Hebron and by public advertising, respectively. All candidates underwent a full medical history and physical examination. At the time of inclusion IBS-D were experiencing daily, soft to watery stools (Bristol scale (14): 5-7), that varied in number (3 to 10/day) and abdominal pain or discomfort that was relieved with defecation. An allergological evaluation was also performed that includes a battery of skin prick tests (Laboratorios Leti SA, Barcelona, Spain) for 32 common foodstuffs and 24 inhalants using histamine and saline as positive and negative controls, respectively, to rule out food and respiratory allergy. Severity of IBS-D was assessed using a modified visual analogue scale, from 0 (no symptoms) to 10 (maximum), to evaluate the quality of life according to the self-perceived intensity of abdominal pain and diarrhea (15). Reasonable exclusion of functional dyspepsia (16) and other gastrointestinal diseases was achieved in IBS-D patients by means of a broad biochemical and serological profile, including antitransglutaminase, and anti-endomisium antibodies. In addition, upper and lower endoscopy, abdominal sonography and barium studies were performed when considered necessary by the responsible physician. Mastocitosis was also ruled out in all participants by means of a baseline serum tryptase levels and clinical history. Participants with a history confirmed or suspected of infectious gastroenteritis within 12 months prior to the initiation of IBS symptoms were excluded, to keep a homogeneous study population. Participants with major depression or other psychiatric illnesses were also excluded to avoid interferences with HPA axis determinations. None of the participants was allowed to take antihistamines, ketotifen, nedocromil, cromolyn, acetylsalicylates,

NSAIDs, anticholinergics, theophylline, β_2-agonists, codeine or opioid derivatives for at least two weeks prior to jejunal perfusion and the jejunal biopsy. Participants with intake of steroids, immunosuppressants or antidepressants any time within the last 6 months before the study were also excluded to prevent any effect on immune activity or the stress response. Written informed consent was obtained from each participant. The study was approved by the ethics committee of the hospital and by the Spanish Drug Agency (Agencia Españoladel Medicamento, protocol 02-0492) and conducted according to the revised Declaration of Helsinki.

Baseline Stress and Depression Levels

Stress levels were measured using the Spanish version of the Modified Social Readjustment Scale of Holmes-Rahe (17). This validated questionnaire reflects the occurrence of significant life events in the last year and allows stratification of participants as suffering from low (0-150), moderate (151-300) or severe (>300) stress. Depression was evaluated using the Spanish version of the Beck's Depression Inventory (18), and participants classified as suffering from low (10-18), moderate (19-29) or severe (>30) depression. Participants filled in both questionnaires the same day of the experimental procedure.

Experimental design

Two experimental procedures were performed in participants: a jejunal perfusion, within 3 weeks after inclusion, and a jejunal biopsy one month after the perfusion was performed. Experimental procedures were carried out at the same time of the day to avoid interferences due to the circadian rhythm. During the jejunal perfusion, psychological variables, pain perception and autonomic variables were monitored as well as blood and intestinal effluents in order to assess the systemic and the local

response to stress. The jejunal biopsy was collected to determine the inflammatory infiltrate and the expression of CRF receptors.

Jejunal perfusion method

At inclusion, participants were randomly assigned to perform the stress or the sham stress protocol. A modified double lumen closed-segment perfusion technique (19) was used. After an overnight fast, a multichannel tube was introduced orally and placed in the jejunum under fluoroscopic control. The infusion port was opened 5 cm distally to the angle of Treitz and drainage port 20 cm distally to the infusion port. Two additional channels connected to inflatable latex balloons were placed just oral and caudal to the infusion and drainage ports **(Figure 1**).

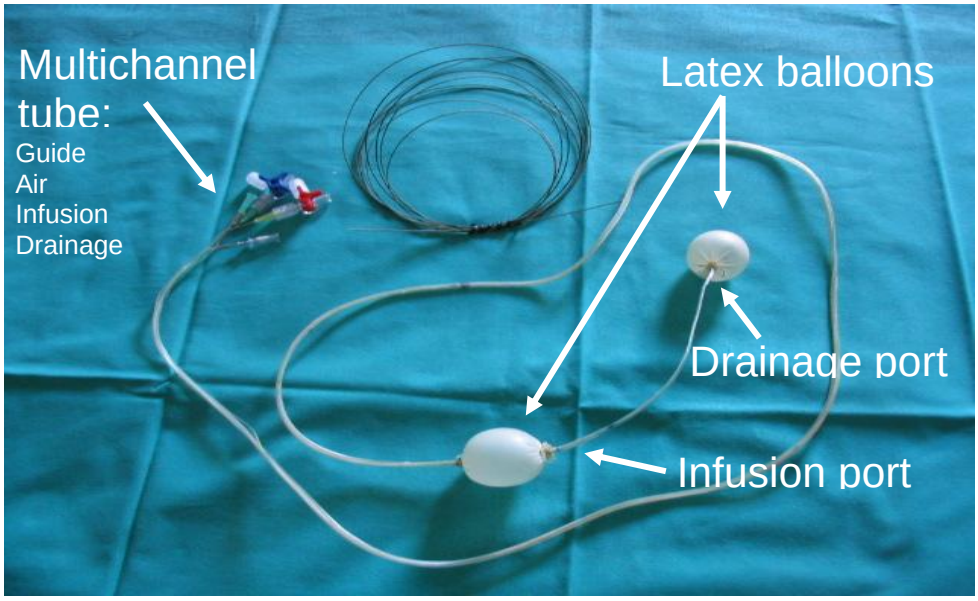

Figure 1: Multichannel tube

When adequate, placement of the tube was firstly achieved and the proximal balloon was inflated to avoid bilio-pancreatic contamination. To wash out residual debris in the lumen, the distal gut was rinsed for 5 minutes. Thereafter, both balloons were inflated under the perception threshold, the segment occluded and perfused at a 5 mL/min rate using a calibrated volumetric pump (Compat enteral; Novartis Nutrition Corporation, Minnesota, USA). An additional gravity drainage port

placed just oral to the proximal balloon prevented accumulation of gastroduodenal and bilio-pancreatic secretions. Maintenance of constant pressure within balloons was monitored by a barostat.

The perfusate was a water-based isosmotic (280mOsm/kg) solution containing mannitol 180mmol/L, xylose 100mmol/L, and polyethylene glycol (PEG) 4000 (5g/L), as a non-absorbable marker, without glucose or electrolytes. The pH was adjusted to 7.8 with NaOH 0.05N and the temperature was 37°C.

Once the multichannel tube was placed in the jejunum and after 30 minutes of equilibration, intestinal effluents were collected into chilled containers at 4°C by siphonage from the drainage port during 90 minutes. Every 15 minutes intestinal effluents were collected and immediately transferred to plastic tubes, snap frozen and stored at -80°C until analyzed. Aprotinin (Sigma Chemical Co, MO, USA) 0.01 ml/mL was added to intestinal samples to minimize proteolysis. Appearance of effluents was checked for the presence of yellow content and samples were analyzed for lipase activity by a colorimetric method (Cobas Mira Plus CC Analyzer, Roche Diagnostics Systems, Barcelona, Spain) to exclude contamination with biliopancreatic secretions. Blood samples were collected in plastic tubes (BD Vacutainer® Plus Plastic K2-EDTA Tubes, NJ, USA), centrifuged and aliquoted for hormonal (ACTH and cortisol) determinations.

1. Induction of experimental stress

a) Stress *protocol*

In a single blinded design, the participants included in the stress protocol received 10 mL of saline e.v. as a placebo after 30 minutes of basal period (**Figure 2**). Acute stress was induced by intravenous administration of 100 μg of human CRF (Corticotropin Releasing Hormone, Ferring GmbH, Kiel, Germany), after 30 min of the basal period and 30 min of the placebo period.

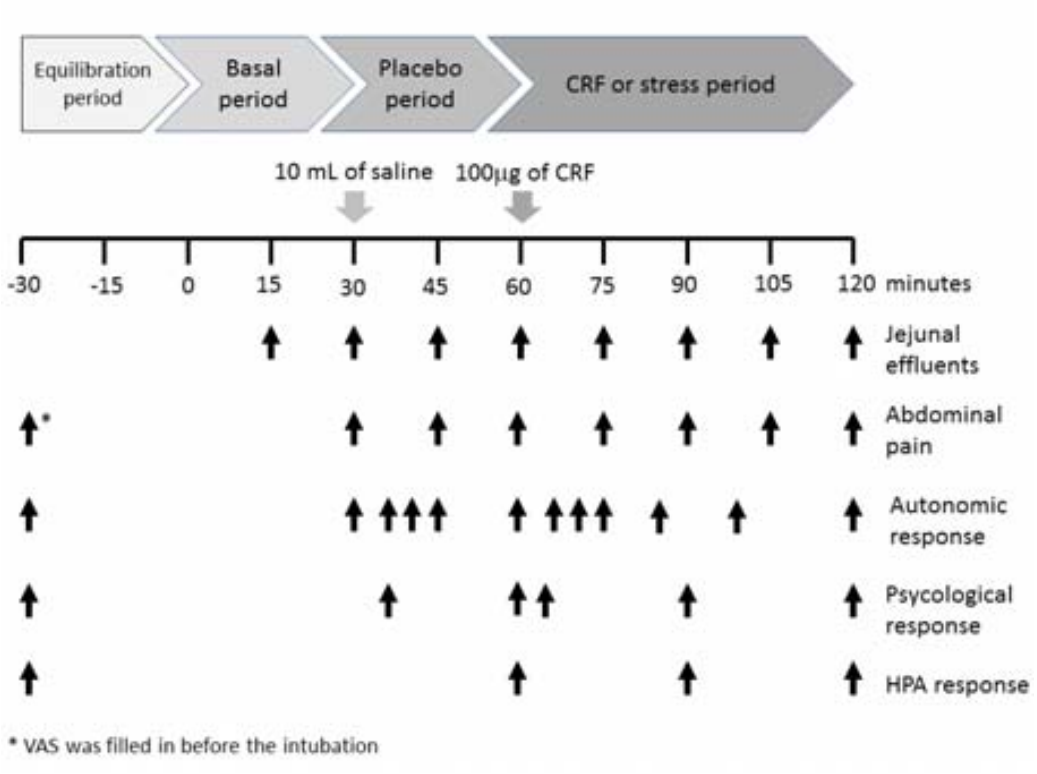

Figure 2: Experimental design
CRF; corticotropin-releasing hormone, VAS; visual analogic scale, HPA; Hypothalamic-pituitary axis.

Systemic, psychological, abdominal and mucosal response was assessed at baseline, after placebo infusion of 5 mL of saline and after the CRF infusion for 30 minutes under basal conditions, for 30 minutes after the placebo bolus and for 60 minutes after CRF infusion. Five mL of blood were obtained from each participant at baseline, at the end of the basal period, at the end of the placebo period and 30 and 60 minutes after CRF administration. Blood samples were centrifuged within 30 minutes (at 3000 rpm for 10 min at 4ºC), except those for hormonal determinations that were kept cooled in an icebox before centrifugation.

b) *Sham Stress protocol*

In addition, to assess whether the changes observed could be related to the intubation itself, we performed the sham stress protocol in a representative number of participants from both groups IBS-D patients and healthy volunteers using the same technique and the same experimental protocol except for the placebo and the

CRF administration. Intestinal effluents and blood samples were collected at the same timepoints. Collection and processing of samples was performed exactly in both groups.

2. Assessment of systemic responses

a) Autonomic response

Autonomic response was measured by monitoring blood pressure and heart rate at baseline and during the study. Systolic and diastolic blood pressures (brachial measurement) and heart rate were recorded with an automatic sphygmomanometer (Omron M4-I, blood pressure monitor; Omron Healthcare, West Sussex, UK). Additional measurements were performed every 5 minutes during the placebo and CRF period.

b) Psychological response

Psychological response to CRF was evaluated by the *Subjective Stress Rating Scale* (20). This questionnaire has been used to assess the level of acute stress experienced by participants during experimental procedures. The questionnaire was completed by participants at the end of the basal period, 5 minutes after the beginning the placebo period, before the CRF administration and 5, 30 and 60 minutes after the CRF infusion.

c) Hypothalamic-pituitary axis response

The concentration of cortisol and adenocorticotropin-releasing hormone (ACTH) in plasma were measured in all participants at baseline and during the experimental protocol (30 min after placebo infusion, 30 min and 1 hour after CRF administration). Blood samples were collected in plastic tubes (BD Vacutainer® Plus Plastic K_2- EDTA Tubes, New Jersey, USA), conserved at 4ºC, and plasma was obtained by centrifugation at 1300 xg for ten minutes and stored at –20ºC until analyzed. Cortisol

and ACTH were measured by a chemiluminescent immunometric assay (Immulite 2000, Cortisol and ACTH tests, EURO/DPC, Glyn Rhonwy, Llanberis, Gwynedd, UK).

3. Assessment of jejunal mucosal activity

Throughout the study, jejunal mucosal activity was determined by measuring epithelial response, mast cell activity and mucosal inflammation. Measurements were performed every 15 minutes during the basal, the placebo and the CRF period. In both protocols (stress and sham stress) the effluents were collected at the same time-points.

a) Epithelial Barrier

Macromolecular permeability and epithelial secretion were determined by measuring the jejunal albumin output (mg/15 min/20 cm) and the net water flux (μl/min/cm), respectively, every 15 minutes during the study protocol. Samples (effluents) were analyzed for the presence of albumin by spectrophotometry in an automatized chemistry analyzer (Olympus AU5400, Olympus, Barcelona, Spain), using a standard curve, and net water flux was calculated by a standardized formula (21) as follows: $Net\ Water\ Flux\ (mL/min) = (V \times PEGp/PEGa) - V$, where V is the infusion rate (5 mL/min), $PEGp$ the concentration of PEG in the perfusion solution, and $PEGa$ the concentration of PEG in the jejunal effluent. PEG concentration was obtained by turbidimetry (22) with the same spectrophotometer and values were interpolated in a standard curve. Samples with a PEG recovery index outside the 90%-110% range or detectable lipase were considered unacceptable and studies with two or more unacceptable periods were excluded.

b) Mast cell activity

Jejunal concentration of the mast cell protease, tryptase in the effluents was assayed by means of a specific fluoroenzyme-immunoassay (FEIA-UniCAP, Thermo-Fisher, Uppsala, Sweden). To determine tryptase in the intestinal lumen, samples

were first lyophilized to increase concentration by a factor of 30 and reconstituted in phosphate buffer saline (PBS)+ 1% bovine serum albumin + multiprotease inhibitor cocktail (dilution 1:100), containing AEBSF 104 mM, aprotinin 0.08 mM, leupeptin 2 mM, bastatin 4 mM, pepstatin 1.5 mM, E-64 1.4 mM (Sigma-Aldrich, St. Louis, MO). Samples were then centrifuged at 1000 x*g* for 10 minutes a t 4˚C, the supernatants were collected and tryptase concentration was determined and expressed as ng/mL after correction according to the concentration factor. Results are expressed in ng/15min/20cm.

c) Mucosal proinflammatory response

Effluent samples were analysed for the presence of the proinflammatory chemokines MIP-1α (Macrophage inflammatory Protein-1α). MIP-1β and RANTES (Regulated on Activation, Normal T cell Expressed and Secreted), using the SearchLight Human Chemokine Array 1 (Pierce Biotechnology, Rockford, U.S.A), a multiplexed sandwich ELISA that generates a chemiluminescent signal that is imaged using a 12-bit cooled CCD camera (Fuji, Japan), to calculate each chemokine concentration, samples were first lyophilized and reconstituted to achieve a 10 fold concentration in PBS + 1% bovine serum albumin + multiprotease inhibitor cocktail (dilution 1:100), containing AEBSF 104 mM, aprotinin 0.08 mM, leupeptin 2 mM, bastatin 4 mM, pepstatin 1.5 mM, E-64 1.4 mM (Sigma-Aldrich, St. Louis, MO). Samples were then centrifuged at 1000 x*g* for 10 minutes at 4˚C, the supernatants were collected and a quantitative measurement was performed. Results are expressed in pg/min/cm.

4. Assessment of abdominal pain perception

A visual analogical scale (VAS) of 10 cm was used to assess abdominal pain experienced by the participants during the perfusion study. The VAS was fulfilled at baseline, after 30 minutes in the basal period, 15 and 30 minutes after the placebo

infusion (45 and 60 min respectively), 15, 30, 45, and 60 minutes after CRF administration (CRF period).

Jejunal biopsy

A jejunal biopsy was performed within the month after the jejunal perfusion. The day of the biopsy all patients were clinically symptomatic. Mucosal biopsies were obtained using a Watson capsule with an attached 3 mm diameter aspiration tube. After an overnight fast, the instrument was orally inserted under fluoroscopic control, between 8-10 am, to the proximal jejunum, 5 to 10 cm distal to the Treitz's angle. A tissue sample was obtained by suction with a 50 mL syringe, and immediately split into two similar pieces with a sterile scalpel. One fragment was fixed in formalin and embedded in paraffin for further microscopic examination. The remaining fragment was placed either in RNAse free tubes containing 500 µL of RNA Later Solution (Ambion, Madrid, Spain) and stored at -80ºC until processed for RNA isolation.

1. Assessment of mucosal inflammation in jejunal biopsies

An experienced pathologist, who was blinded to the clinical diagnosis, examined the biopsy specimens. Following general procedures, jejunal biopsies were stained with hematoxylin and eosin for general histological examination and epithelial morphometry as performed in routine clinical practice. In particular, the presence of eosinophilic infiltration, epithelial abnormalities including villous atrophy and microorganisms were evaluated at x400 magnification.

For immunohistochemistry, paraffin-embedded samples were cut at 5 µm sections, and paraffin was removed with xylene and rehydrated following standart procedures. Endogenous peroxidase activity was blocked with 0.2% hydrogen peroxidase solution and non-specific labelling was blocked with serum blocking

solution. Sections were incubated in complete medium for 1 hour at room temperature with *c-kit* anti-human rabbit polyclonal antibody (CD117, Dako, Carpinteria, CA, USA) at a dilution of 1:50). As a negative control the primary antibody was omitted and replaced with PBS. The reaction was revealed by the avidin-biotin complex peroxidase method (ABC Elite kit, Vector Burlingame, CA, USA) followed by staining with the peroxidase substrate 3.3 diaminobenzidine tetrachloride (DAB; Sigma GmbH, Deisenhofen, Germany). The slides were counterstained with 50% hematoxylin. Human gastrointestinal tumour tissue was used as a positive control for *c-kit* expression. Quantisation of mast cells was performed on immunostained sections with a Leitz microscope (Laborlux S microscope, E. Leitz, Wetzlar, Germany) at x400 magnification. The number of cells stained for *c-kit* were counted in at least 8 contiguous non-overlapping fields at 400x magnification and expressed as number of mast cells per high power field (MC/hpf).

2. Assessment of CRF-R expression in the jejunal mucosa

Biopsies were homogenized in the FastPrep mixer (Bio101) in RLT cell lysis buffer (Qiagen) followed by RNA isolation (RNeasy Mini Kit, Qiagen) and on-column DNase treatment (Qiagen). Complementary DNA synthesis was performed using one microgram of total RNA with the High Capacity Reverse Transcription Reagents Kit (Applied Biosystems). RTQ-PCR was performed on an ABI PRISM® 7500 FAST Sequence Detection System (Applied Biosystems) using validated TaqMan Gene Expression Assays for CRF-R1 (Hs00366363_m1) and CRF-R2 (Hs00266401_m1) and the human 18s (Hs99999901_s1) as endogenous control. Each sample was run in triplicate and data was analyzed by the $\Delta\Delta$CT as described in the User Bulletin #2 from Applied Biosystems (Relative Quantitation of Gene Expression: ABI PRISM® Sequence Detection System: User Bulletin #2; RevB).

Data expression and Statistical Analysis

Data are expressed median [Q1,Q3] unless otherwise stated. Non-parametric tests (Mann-Whitney *U* test, Wilcoxon Signed Ranks test, Fisher's exact test and Spearman's correlation coefficient) were used when appropriate to increase statistical assurance. Net area under the curve over time (AUC) was calculated via the trapezoidal method. Hormonal, autonomic and psychological changes as well as net water flux, albumin and tryptase and chemokines release were compared using a two way repeated-measures analysis of variance (ANOVA) were disease condition (IBS-D) was considered as the between-subjects factor (Group) and changes along the perfusion-time as the within-subject factor (Time). Significance level was established at $p<0.05$. All data were analyzed using a commercial software (SPSS 15.0, SPSS Inc., Illinois, USA).

RESULTS

Participants

Thirty-eight subjects were included, 21 IBS-D patients and 17 H subjects (**Figure 3**). Ten participants (five from each group) were randomly assigned to perform the sham stress protocol. The rest of the participants (28) were submitted to the jejunal perfusion with placebo and CRF administration (16 IBS-D and 12 H). Twelve IBS-D patients and 11 H volunteer completed the study, whereas the other 5 (4 IBS-D and 1 H) were incapable to finish the perfusion study because of bad tolerance to the perfusion procedure (nausea in 1 IBS-D and 1 H, vomiting in 1IBS-D and anxiety in 1 IBS-D) or by contamination of the jejunal samples by biliopancreatic secretions (1 IBS-D). None of the IBS-D patients presented diarrhea during the procedure, despite being symptomatic and the administration of CRF.

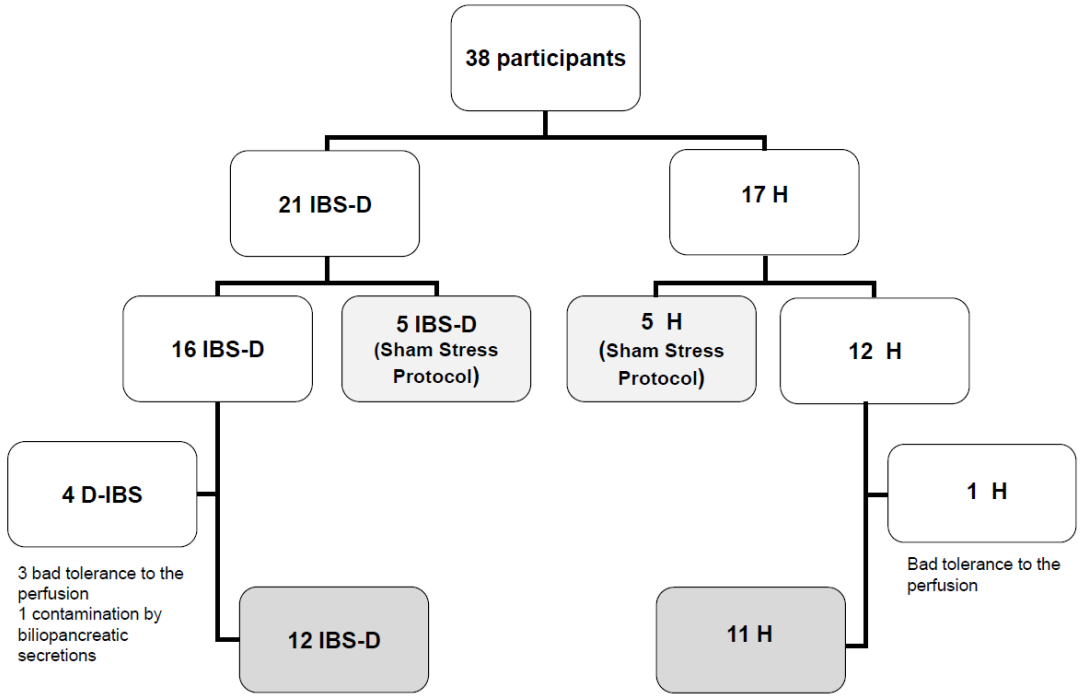

Figure 3: Flow chart of the participants
D-IBS: diarrhea-IBS, H:healthy volunteer.

Baseline stress levels were significantly higher in IBS-D patients compared to H participants. In the stress protocol, IBS-D patients (10/12) most frequently scored as moderate (66.6%) to severe (16%) baseline level of stress whereas the majority of the H participants (9/11) had low levels of stress (82%), and only two had moderate levels of stress (18%). In the IBS-D group, 42% (5/12) did not score for depression, 33% (4/12) showed low depression, and 25% (3/12) were moderately depressed. None of the H volunteers were depressed. In the sham stress protocol, baseline and depression levels of the participants were comparable to those of the IBS-D and H volunteers that performed the study protocol.

Clinical and demographic characteristics, and baseline stress and depression of the participants are shown in **Table I**.

Table I. Clinical and demographic characteristics of the included participants

	Study protocol		*Sham Stress Protocol*	
	HEALTHY	IBS-D	HEALTHY	IBS-D
Number of subjects	11	12	5	5
Gender F:M	5:6	5:7	3:2	3:2
Age (years)	23(20-40)	31(20-59)	28(23-42)	30(21-44)
Symptom duration	NA	8.5 (7-15)	NA	11(8-14)
Severity (0-10)	NA	6.3 (4.3-8.2)	NA	5.9 (3.8-7.9)
Functional dyspepsia	0/11	0/12	0/5	0/5
Holmes-Rahe Scale	79 [49,123]	198 [166,244]*	81[59,136]	201[133,292]*
Beck's questionnaire	1 [0,4]	12 [4,12]*	0 [0,5]	10 [3,10]

F, female; M, male; IBS-D, diarrhea-predominant irritable bowel syndrome; NA, non applicable. Data are expressed as median (range) for age and symptom duration and as median [Q1,Q3] for severity, Holmes-Rahe Scale and Beck's questionnaire. * IBS-D vs healthy, p=0.0023, # IBS-D vs healthy, p=0.0064; ** IBS-D vs H, p=0.03

Systemic response to stress

1. Autonomic response

Heart rate and blood pressure significantly increased during CRF administration as shown by a main effect for heart rate: F (4,72)=6.3; systolic blood pressure: F(4,72)= 20.3; and diastolic blood pressure: F(4,72)= 17.4; ($p<0.0001$ for all). The increment (stress period respect to basal levels) was similar in IBS-D patients than in H volunteers. All parameters returned to baseline 5 minutes after the stress period. No differences were observed in the placebo period. During the Sham Stress protocol, no significant changes were detected nor respect to baseline, neither between IBS-D and H participants (data not shown).

2. Psychological response

At baseline, IBS-D patients showed a higher SSRS score compared to the H group (IBS-D: 5.4[3.6-8.8]; H: 3.6[1.7-4.7]; $p=0.048$). Placebo did no induce any change (IBS-D: 6.1[3.8-8.9]; H: 4.2[2.1-4.9]; $p=0.05$). CRF administration induced a marked raise in the levels of acute stress experienced by participants as shown by a significant main effect for time, [F (6,160)= 4.28; $p<0.001$], with no differences between groups [(F (1,21)=0.97;$p=0.34$)]. Participants submitted to the Sham Stress protocol showed no differences in the SSRS score (data not shown).

3. Hypothalamic-pituitary axis response

At baseline there were no differences between ACTH (IBS-D: 14.9 [11.3-23.1] vs. H: 14.1[9.9-16.5] pg/mL; $p=0.67$) and cortisol concentration (IBS-D: 15.2[12.1-16.6] vs H: 11.5[6.2-14.8] μg/dL; $p=0.08$) in IBS-D patients compared to H volunteers. Both patients and healthy subjects showed a significant hypothalamic-pituitary-axis response to CRF administration: F (3,79)=43.24; $p<0.001$ for ACTH and F(3,79)=38.77; $p<0.001$ for cortisol for time), without differences between groups. Exogenous response to CRF was not influenced by baseline stress levels (Holmes-Rahe) (ACTH (r:-0.13; $p=0.578$) and cortisol (r=-0.001; $p=0.995$). No differences were observed in

both ACTH and cortisol between the participants included in the sham stress protocol (data not shown).

Jejunal local response to stress

1. Epithelial barrier response

Jejunal net water flux at baseline was similar in both groups (IBS-D: 55.35 [28.3-71.01] *vs*. H: 40.85 [21,77-57,14] µL/min/cm; p= ns). Placebo did not induce changes in any group, but the secretory response to exogenous CRF was enhanced in IBS-D patients respect to H volunteers (IBS-D: 74.2 [44.8-91.8] *vs.* H: 43 [32.8-58.9] µL/min/cm; p=0.015) (**figure 4A**). Moreover, there was an increase in the secretory response to CRF that was different in the IBS-D patients as shown by a significant main effect for group (F(1,20)=9,006; p=0.007) and time (F(7,157)=6,372;p<0,001).

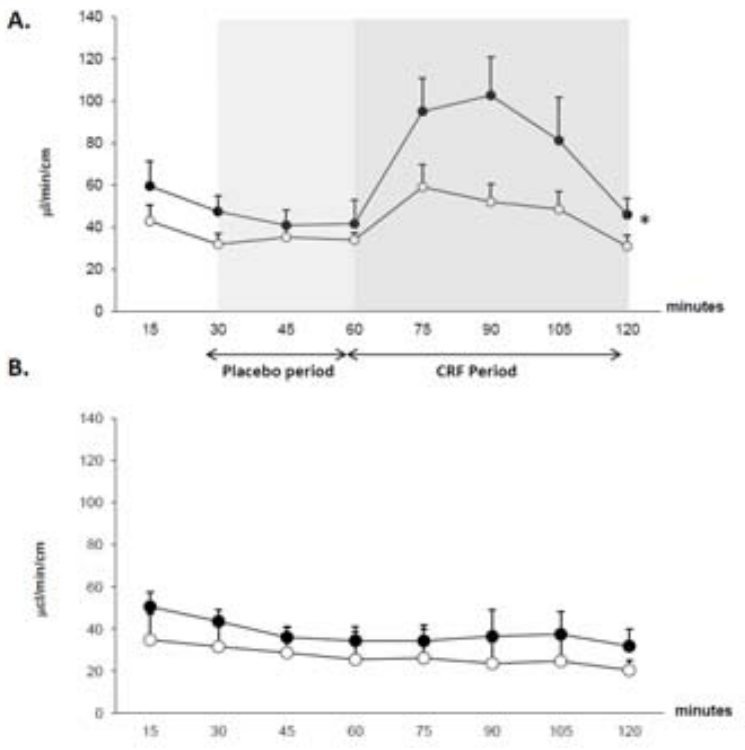

Figure 4- Jejunal net water flux. **A**- In the stress protocol group, exogenous CRF induced a marked increase in the secretory response in IBS-D patients (*p=0.015 IBS-D *vs*. H). **B**. With the Sham stress protocol no differences in net water flux were observed throughout the study. Data are expressed as mean±SEM. Filled dots represents IBS-D patients and open symbols represents H volunteers. Mann-Whitney and Wilcoxon T test for unpaired data.

The total secretory response to acute stress was significantly higher in the IBS-D group than in the H group (IBS-D_{AUC}: 3.85[2.45-4.34] vs. H $_{AUC:}$ 2.08[1.45-2.73] ml/60 min; p=0,0028). In addition, no correlation was found between the baseline stress and the secretory response to stress expressed as net water flux AUC (r=0,38, p=0,07) in any experimental group.

In the Sham Stress protocol (**Figure 4B**) data distribution mimics the same pattern in IBS-D than in H volunteers.

Basal luminal release of albumin was higher in patients than in healthy subjects (IBS-D: 1.29 [0.69-1.52] *vs*. H: 0.57[0.23-0.86] mg/15 min/20 cm; p=0,0037) and kept higher throughout the study. No changes were observed after placebo administration in any group but, interestingly, although CRF increased albumin output in both groups, it was only significant in the IBS-D group (IBS-D: CRF: 2.96[1.78-3.39] *vs*. placebo: 1,32 [0.83-1.49]; p=0,0042; H: CRF: 1.0 [0.34-1.46]; *vs*. placebo: 0.66 [0.16-1.2] mg/15 min/20 cm; p=0,32) (**Figure 5A**). Albumin release in response to CRF was different as shown by a significant main effect for group, F(1,19) = 4.725; p = 0.042 and by time, F (7,155)=5,072; p<0.001. The total response to exogenous CRF was significantly higher in the IBS-D patients (IBS-D $_{AUC}$: 6.44 [3.51-9.96] vs. H $_{AUC}$: 3.03 [1.16-4.61]mg/60 min; p=0,017).

The enhanced macromolecular permeability, expressed as albumin output, did not correlate with the baseline stress levels (r=0,31, p=0,15).

At baseline, IBS-D patients submitted to the sham stress protocol showed higher albumin output than H volunteers, and this difference it was maintained high throughout the study. The kinetics of the sham stress protocol is similar in IBS-D and in H, and no increment was observed during the jejunal perfusion procedure (**figure 5B**)

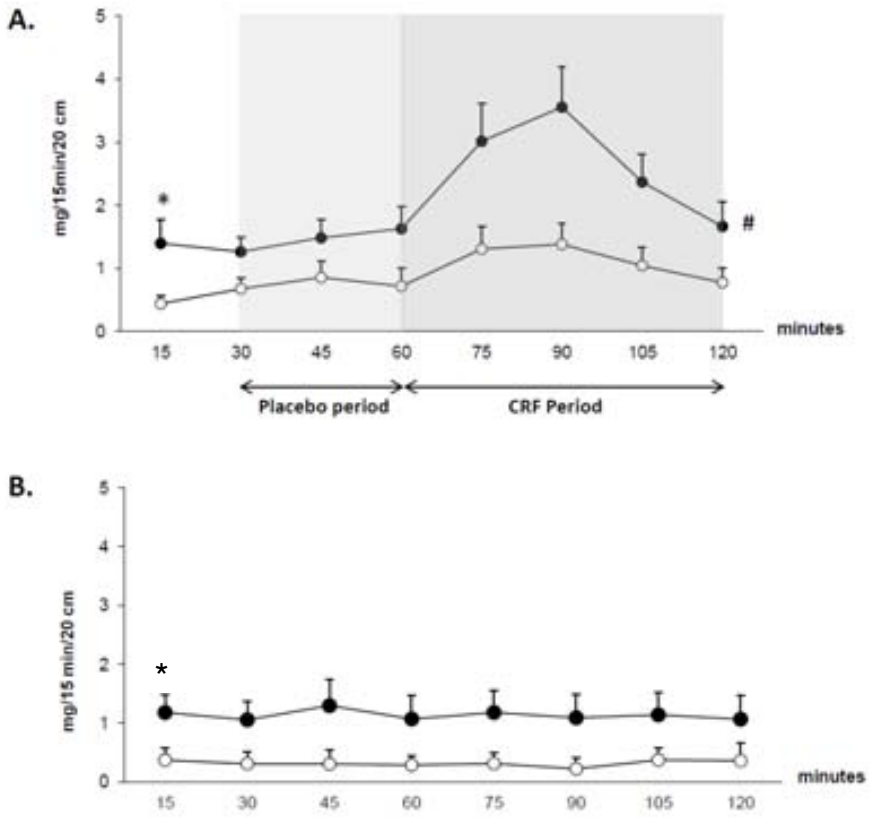

Figure 5. **Albumin release in the jejunal mucosa**. A- Stress protocol: Basal albumin release was higher at baseline in IBS-D patients than in H subjects (*p=0.0037 IBS-D *vs*. H) and remained higher throughout the study. Exogenous CRF increased albumin output in both groups, but it was only significant in IBS-D patients (# p=0.0024 CRF *vs*. Placebo). B. Albumin release in the sham stress protocol. Data are expressed as mean±SEM. Filled dots represents IBS-D patients and open symbols represents healthy volunteers. Mann-Whitney and Wilcoxon T test for unpaired data

2. Mast cell response to stress

Tryptase release in the jejunal lumen was higher at baseline in the IBS-D group than in the H group (IBS-D: 17.15 [8.9-27.63] vs. H: 5.67 [3.5-6.4] ng/15min/20cm; p=0,0008) and remained higher throughout the study. CRF significantly enhanced tryptase jejunal release in both groups (IBS-D: CRF: 95.38 [42.28-143.5] vs. placebo: 30.25 [16.49-35.45] ng/15min/30cm; p=0.0004; and H: CRF: 31.3 [20.84-45.23]*vs*. placebo: 8.22 [3.57-17.55] ng/15min/30cm ;p=0.0004) (**figure 6A**). However, this increase was significantly higher in the IBS-D group compared to the H group (Δ[CRF-

placebo] IBS-D: 63.19 [24.67-113.4] *vs.* H: 21.3[14.44-39.72]; p=0.012). In addition, the total response to CRF in tryptase release was significantly enhanced in the IBS-D group as shown by a larger AUC (IBS-D_{AUC}: 274[104.1-464.5] *vs.* H_{AUC}: 83[38.1-107.7] ng/60min; p=0,0023). Tryptase curves were not influenced by PBS, bovine serum albumin, aprotinin or protease inhibitors (data not shown).

In the participants of the sham stress protocol tryptase release in the jejunal lumen was higher in the IBS-D patients at baseline compated to the healthy subjects and remained enhanced during all the study (**figure 6B**).

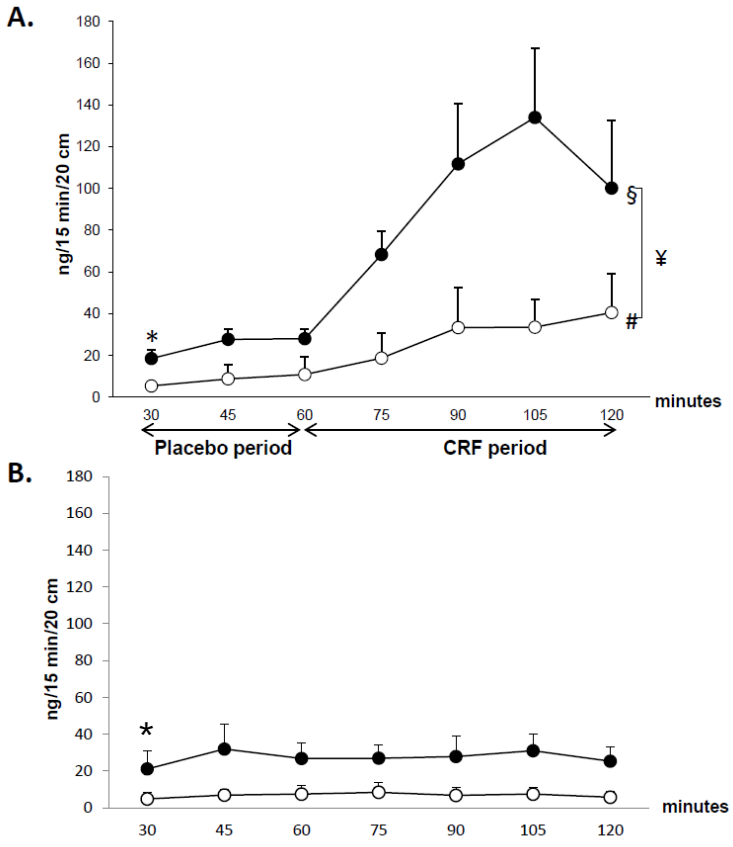

Figure 6: Jejunal luminal release of tryptase. A- Stress protocol: *p=0.0008 IBS-D vs. H at baseline # p=0.0004 CRF vs. placebo in H volunteers; § p=0.0004 CRF vs. placebo in IBS-D; ¥ p=0.012 IBS-D vs. H volunteers. B- Sham stress protocol: * p= Filled dots represents IBS-D patients and open symbols represents healthy volunteers.Data are expressed as mean±SEM. Mann-Whitney and Wilcoxon T test for unpaired data.

Jejunal tryptase release in response to CRF was different as shown by a significant main effect for group, F(1,19) =10,72; p = 0.004 and by time (period), F (6,131-)=10,05; p<0.001 (**Figure 7**).

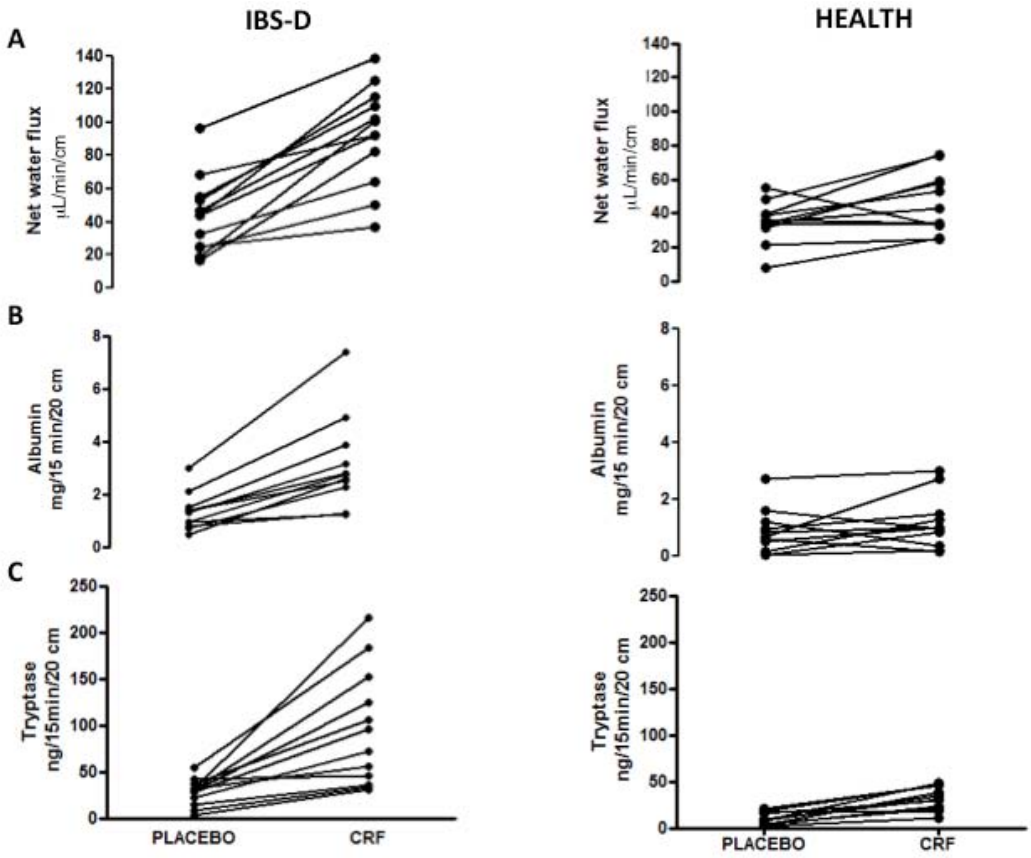

Figure 7: Individual jejunal response to stress in IBS-D patients and in healthy volunteers. A: Net water flux. B: Albumin release. C: Tryptase release. Data represents mean values in each period. Placebo includes the mean of the placebo period (30-60 min) and CRF includes the mean of the CRF period (60-120 minutes)

No sex-related differences were observed in the tryptase response to CRF (Tryptase AUC male 92.2[72.93-304.8] vs female 107.73[38.06-146.86] ng/60min; p=0,72). Similarly to net water flux and albumin output, no correlation was observed between baseline stress levels and tryptase output (r=0,26, p=0,27) However, the total amount of tryptase released in response to CRF, expressed as AUC, positively correlated to the response to acute stress (**figure 8**) in both net water flux (r=0,65, p=0,0008) and albumin output (r=0,64, p=0,0010), suggesting the role of mast cells in

the regulation of the secretory response and in the enhanced permeability observed after CRF administration.

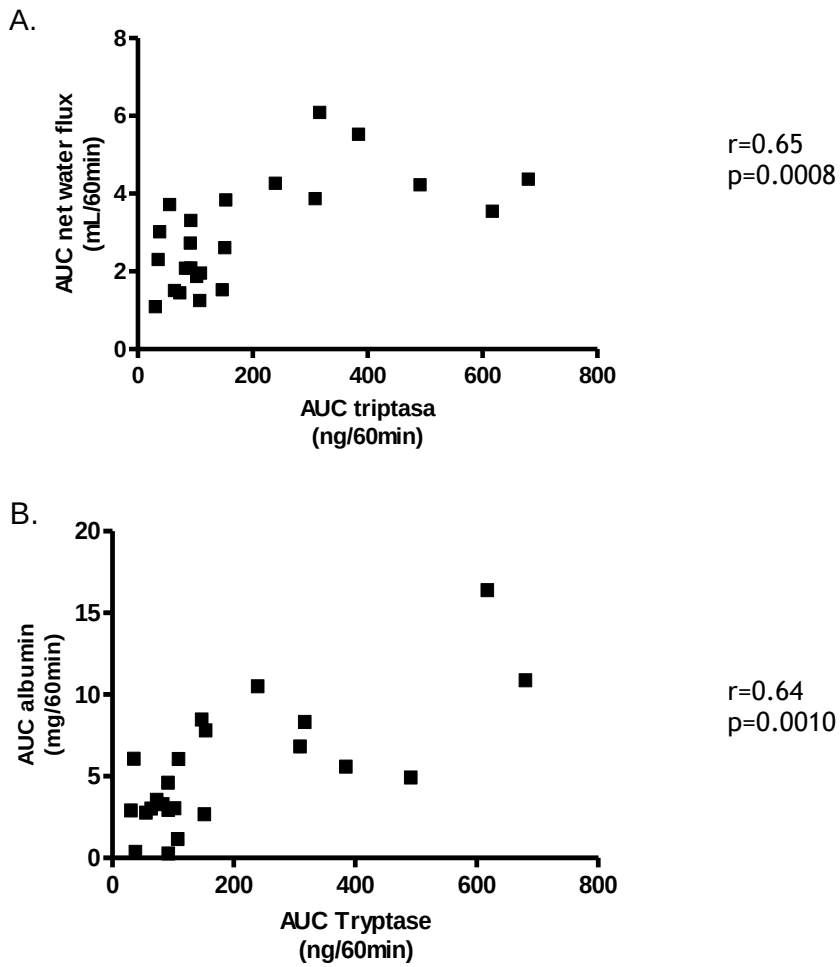

Figure 8. Correlation analysis between tryptase response to stress and jejunal barrier response to stress. Total tryptase released in response to CRF, expressed as AUC, was correlated to total net water flux (A) and albumin (B) released during the acute stress. Spearman Correlation test.

3. Mucosal proinflammatory response to stress

The release of the proinflammatory chemokine MIP-1α in the jejunum, was not different at baseline in the IBS-D group when compared to the H group (IBS-D: 224 [93-1160] *vs.* H: 159[93-253] pg/15min/20cm;p>0,99). MIP-1α output in the jejunal lumen was not affected by placebo in any group (IBS-D placebo *vs* basal p=0,71 and H placebo *vs.* basal p=0,165). However, CRF induced a significant increase only in IBS-D

patients and not in H participants (IBS-D: CRF: 1,008 [649-1,603] vs. placebo: 346[299-691] pg/15min/20cm; p=0,0175; H: CRF: 301 [34-575] *vs.* placebo: 4.5 [4-191];p=0,12). The total response to stress, expressed as AUC was stronger in the IBS-D (IBS-D_{AUC}: 2.12 [1.49-2.67] *vs.* H_{AUC}:0.82 [0.08-1.36] ng/60min;p=0.014). Acute stress induced by exogenous CRF significantly increased MIP-1α release in both groups (F(1,12)=9,187;p=0.009, with a trend in the effect for time (F(6,82)=2.239;p=0.052)

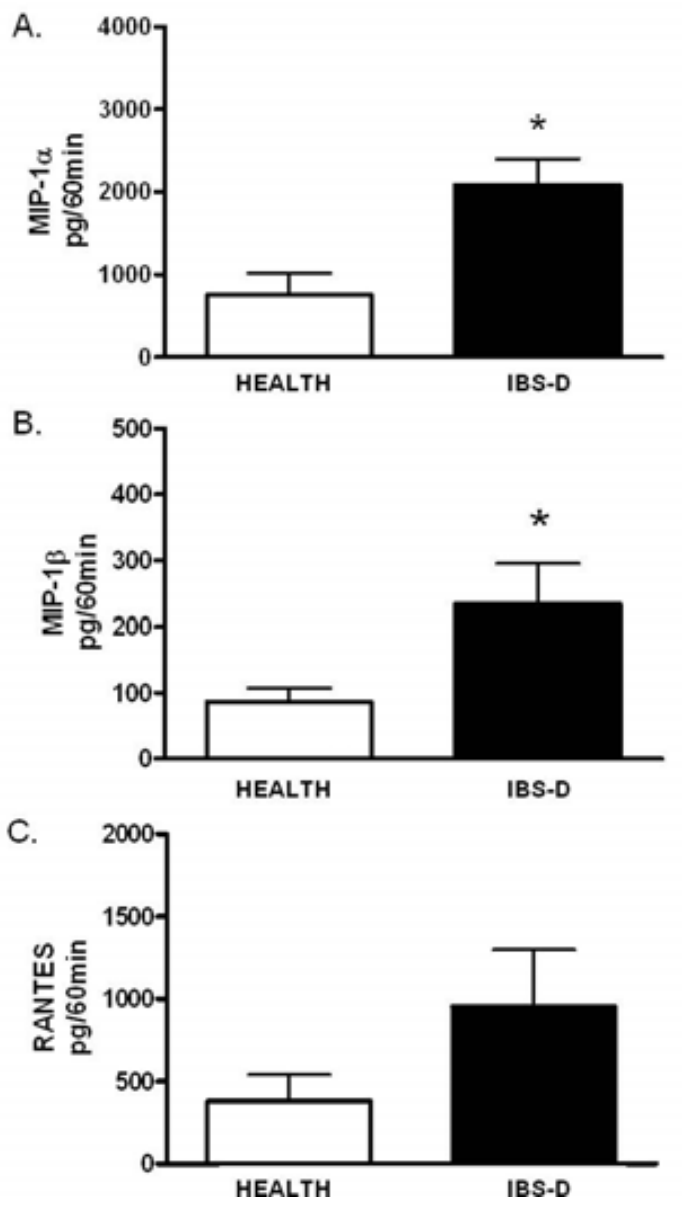

Figure 9: Mucosal proinflammatory total response to stress. A. MIP-1α. **B**. MIP-1β. **C.** RANTES. Data represents the total response to CRF (AUC during 60 min in the CRF period) in the healthy volunterrs and in the IBS-D patients. * p<0.05. Mann-Whitney *U* test

A similar response was observed with MIP-1ß, with no differences in baseline levels in both groups (IBS-D: 28 [25-57] *vs.* H: 23[4-34] pg/15min/20cm; p=0,259) and no changes were induced by placebo. The total response to stress was higher in the IBS-D patients than in the H group (IBS-D_{AU}c: 226.3 [95.5-369.1] *vs.* H_{AUC}: 82.8[35.7-124.5] pg/60min;p=0.036) with a significant increase after CRF administration. However, the 2-way repeated measures ANOVA analysis did not shown differences in the main effect for group and for time (F(1,12)=3.792;p=0.072) for group and F(6,82)=1,735;p=0.12) for time)

In the case of RANTES, another proinflammatory chemokine, baseline concentration was similar in both groups (IBS-D: 334[23-535] *vs.* H: 36[16-90] pg/15min/20cm;p=0.08). The total response to stress was higher in the IBS-D (IBS-D_{AUC}: 917.6 [109.6-1585] *vs.* H_{AUC}: 87.2[62.9-833.7]; p=0.051) with a significant increase after CRF administration in IBS-D patients but not significant. No differences were observed for the main effect for group (F(1,12)=3.458;p=0.085) and for time (F(1.730;p=0.13) in the release of RANTES.

Abdominal visceral perception response to stress

Abdominal pain perception by participants before the introduction of the tube was not different between the experimental groups (IBS-D: 0[0-1] vs H: 0[0-0]; p=0.25). After the basal period, and after one hour of the perfusion, IBS-D patients scored higher abdominal pain than the H group (IBS-D: 3[2-3] vs H:0[0-0]; p=0.0001), and this difference remained higher throughout the study, especially after the CRF administration.In addition, the VAS score during the stress period positively correlated with total tryptase released in response to CRF (AUC tryptase vs AUC abdominal pain: r=0.66; p=0.0006) and with the albumin output (r=0.62; p=0.002) and net water flux (r=0.61; p=0.0019). Correlations between the clinical and the biological parameters are shown in Table II.

Table II: Correlations between clinical and biological variables.

	Net water flux	Albumin	Tryptase
Holmes-Rahe Scale	r=0.38 p=0.08	r=0.31 p=0.15	r=0.20 p=0.41
Abdominal pain	r=0.61 p=0.0019	r=0.62 p=0.002	r=0,66 p=0.0006

Spearman Correlation Test

Jejunal mucosal mast cell counts

According to our previous results[9], mast cells (CD117+, *c-kit* +) were markedly increased in the jejunal mucosa of IBS-D patients compared to the H group (IBS-D: 28.5 [25,3-39,8] *vs.* H: 12.9[8.5-15.7] MC/hpf; p < 0.0001).

Gene expression of CRF receptors in the jejunal mucosa

The expression of CRF-R1 and CRF-R2 in the jejunum was assessed in 12 IBS-D and 10 H volunteers. Q-RT-PCR analysis demonstrated a significant down-regulation of CRF-R1 (p=0.0021) and an up-regulation of CRF-R2 (p=0.0007) (**Figure 10**).

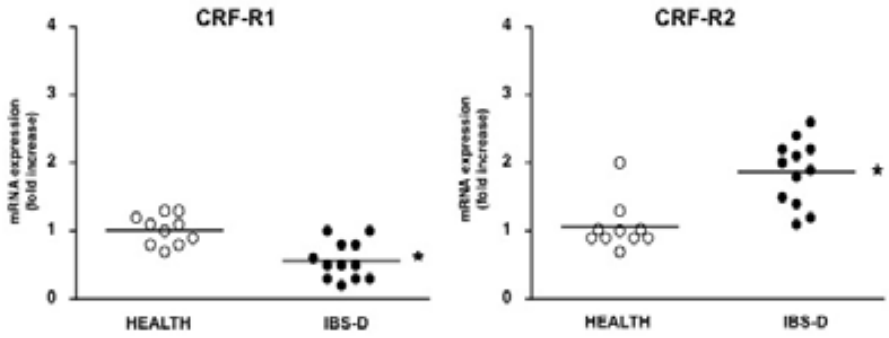

Figure 10. Expression of CRF-R mRNA in the jejunum of IBS-D patients and in healthy volunteers. Healthy volunteers (n=10) are represented as open symbols and IBS-D patients (n=12) as filled symbols. Results are expressed as fold-change respect to the H group *p = 0.021 for CRH-R1 (A), * p=0.007 for CRH-R2 (B).

DISCUSSION

In this study, we have evaluated the effect of acute stress in the jejunal barrier function and its relationship to mast cell activation in IBS-D patients. We have shown that the human jejunum responds to peripheral administration of CRF, the main regulator of the stress response, inducing water secretion and increasing epithelial permeability in parallel to mast cell activation and the induction of a microinflammatory environment. Of importance to IBS-D etiopathogenesis, CRF-induced changes are significantly larger in patients than in health.

Stress has been demonstrated to modify intestinal motility, epithelial secretion, visceral sensitivity, intestinal permeability and local inflammatory responses in the gastrointestinal tract (4,23). Epidemiological, empirical and clinical observations have linked stressful or traumatic life events to the onset of symptoms, severity and persistence in certain IBS subtypes (24,25). About 50% of IBS patients display psychiatric comorbidities, commonly anxiety and depression, and suffer more chronic stress than the healthy population (26). However, the exact mechanism by which stress exerts its effects in the GI tract in IBS remains unclear.

CRF is the principal neuromediator of the stress response. Central nervous system administration in animals mimics the behavioural, autonomic and visceral responses to stress (4,27). Moreover, CRF is considered to be a major mediator of stress responses in the brain-gut-axis (5,28) and several studies point to an equally important contribution of the peripheral CRF signalling locally expressed in the gut to the gastrointestinal response to stress (4,5,29-31). Several reports have demonstrated that exogenous administration of CRF increased colonic motility, visceral perception and anxiety (32,33) more intensively in IBS patients (34,35) than in healthy controls and that these changes were reversed by peripheral administration of α-helical CRF, a non-selective CRF receptor antagonist (35).

Previous reports have demonstrated alterations in HPA axis in IBS-D patients, with higher urine cortisol levels in women with IBS (36), and higher increase in ACTH after CRF infusion with respect to their healthy control counterparts (34,37). In contrast, other studies have described that CRF administration in IBS patients implied blunted plasma ACTH and cortisol concentration suggesting a lower HPA axis activity (38). In our study, HPA response to systemic administration of CRF was increased similarly in the IBS-D patients than in the healthy volunteers. Moreover, we have observed that psychological response to exogenous CRF was similar in both groups. To assess whether the altered response to acute stress in our IBS-D patients was influenced with chronic stress and depression levels we attempted to correlate the HPA axis response with basal levels of stress and depression, and did not observe any association between them. In this study, neither the epithelial barrier nor the mast cell responses were influenced by the basal stress of the participants.

Despite no differences in the HPA axis response to stress between healthy volunteers and IBS-D patients, we have observed higher local intestinal changes secondary to CRF administration. It could be due to the local effect of CRF in the intestinal tract, since CRF receptors are widely distributed not only in the CNS but also in the gastrointestinal tract (5). Nevertheless, to date, the expression of CRF receptors in the small intestine has only been reported in experimental animals, and mainly in the ileum of rodents. In addition, CRF receptors type 1(CRF-R1) have been described in mast cells and macrophages of the lamina *propria* and in submucosal and myenteric neuronal plexus in the human colon (39). CRF-R2 seems to have a fewer expression than CRF-R1 (40) in the human colonic epithelium, and has also been described in sigmoid colon biopsies and in HMC-1 cells, a human mast cell line (6). We have observed a dowregulation of CRF-R1 simultaneously with an upregulation of CRF-R2 in the jejunal mucosa of IBS-D patients. To our knowledge,

the presence of CRF receptors in the human jejunum and its specific function has not been established yet.

While convergent functional evidence accumulates in favour of a major role of peripheral CRF signalling system in the gut response to stress, how and where the peripheral CRF signalling is recruited by stress remains unclear. CRF-R1 is involved in stress-related alterations of viscerosensitivity and colonic motor function whereas CRF-R2 is involved in stress-induced delayed gastric emptying (4,5,28) and may reduce visceral perception (41). CRF-R1 and CRF-R2 receptors have also been implicated in the regulation of ion secretion and in intestinal permeability induced by stress in experimental animals (42-47).

Although assessed in healthy volunteers (6,48), this study provides evidence on the effects of peripheral administration of CRF on epithelial barrier function in IBS-D patients. Based on our results, CRF-R2 seems to be involved in regulation of intestinal permeability in the jejunum in IBS-D patients though, additional experiments using specific antagonists of both CRF-R1 and CRF-R2 are warranted to confirm our findings.

Current conceptual framework states that clinical symptoms of IBS could be associated with structural and functional abnormalities of the mucosal barrier, highlighting the crucial importance of elucidating the contributory role of epithelial barrier defects in the pathogenesis of IBS, along with the low-grade inflammation and the immune activation (49). The alteration in barrier function has been studied *in vivo*, by means of the evaluation of urinary excretion of oral probes, such as lactulose-mannitol (48,50) or ^{51}Cr-EDTA (51) and *in vitro* in colonic biopsies (52) or in fecal supernatants applied to murine colonic strips mounted in Ussing Chambers (53). Moreover, abnormalities have been described at the molecular and structural levels as in the tight junctions in IBS patients, correlating with symptoms of IBS (54,55). Likewise we have observed an increase in macromolecular permeability (blood to

lumen albumin permeability) in IBS-D patients at baseline that was maintained throughout the study and was clearly enhanced by CRF administration. We have also observed an increase in the net water flux in the jejunum in response to CRF only in the IBS-D patients group. Water secretion is mainly driven by active chloride secretion into the intestinal lumen (56) and previous observations consistently showed that acute and chronic stress induced chloride secretion (10,18,57) and increased net water flux (8,18) (i.e. reduced water absorption or heightened secretion) in both animal models and in the human jejunum that were mediated by the parasympathetic nervous system and by tryptase and histamine (8). An osmotic drag can be induced by the increased luminal appearance of large macromolecules, such as albumin, and this can cause an increase in luminal transport. However, no significant correlation was found between albumin output and net water flux all along the present study.

Abdominal pain perceived by participants in response to CRF administration positively correlated with total albumin released in the jejunal lumen, suggesting that the increase in intestinal permeability generated in response to stress is related to abdominal pain, a cardinal symptom of IBS. In addition, the abdominal pain perceived by participants in response to acute stress correlate with mast cell activation.

Most studies designed to assess the pathophysiology of IBS has been performed in the ceacum and the colon, probably due to an easier access to tissue samples in these parts of the intestine. We first demonstrated a mast cell hyperplasia and activation in the jejunum of IBS-D patients (12). With the use of mast cell deficient rats (10,44) or mast cells stabilizers (6,44) it has been shown experimentally that the stress-induced increase in intestinal permeability is dependent on mast cells. Moreover, recently, our group has shown that the alterations in the expression and distribution of tight junctions proteins in the jejunal mucosa of IBS-D patients are

linked to the activation of mast cells and bowel disfunction (59,60). Activation of jejunal mast cells suggests that local mast cell-mediated inflammatory events contribute to functional disturbances and clinical manifestations of IBS-D. With the present study, we wanted to get further insight into the mechanisms of mucosal mast cell activation through acute stress and its contribution to the increase in the intestinal permeability, as distinctive features of IBS-D, and we have proved that the increase in intestinal permeability and the secretory response in the jejunum secondary to CRF administration is correlated with intestinal mast cell activation, suggesting a pivotal role of mast cells in the generation of this stress-induced increase intestinal permeability. Although tryptase release following CRF was also increased in healthy controls it was to a lesser extent than in IBS-D patients. This suggests a regulatory role of MC in the secretory and barrier function in the IBS-D jejunum secondary to stress, that could be mediated by CRF-R2 receptors present in mast cells, mast cells express receptors for CRF (both subtype CRF-R1 and CRF-R2), suggesting that mast cells act as a link between the brain and the peripheral tissues (58,59). Nonetheless, the expression of CRF-R in jejunal mast cells has never been assessed.

We cannot determine whether this increase in permeability by acute stress is related to the fact that the jejunum of IBS-D patients have more mast cells than healthy subjects, as stress is known to promote release of mast cell mediators in the jejunum of healthy volunteers (8) or if the increase in permeability leads to more mast cell activation and hyperplasia. To test that, future studies should be addressed to assess the effect of mast cell stabilizers in the increase in intestinal permeability secondary to stress in IBS-D patients. To our knowledge, although observed in healthy volunteers (48), to date, no study has addressed the potential role of mast cell stabilizers in regulating the stress-induced increase in intestinal permeability in IBS patients.

Our study does not provide final proof of the involvement of CRF in the *in vivo* jejunal mast cell-driven response. In that sense, a clinical assay with pexacerfont, a selective CRF-R1 antagonist (60) has been performed in patients with IBS-D with no significant effects on gastric emptying, colon motility and stool frequency. No human assay with CRF-R2 antagonists has been carried out, and according to our findings, this could potentially be a treatment for IBS-D symptoms, at least, those related with small intestine mast cell activation. On one hand, mast cells can also be activated by other neuropeptides, such as neurotensin or substance P (61,62), or by neurotransmitters, such as acetylcholine (63) and vasoactive intestinal polypeptide (64), and we cannot exclude a mast cell activation by alternative molecules released by CRF receptor-bearing cells. On the other hand, other cells may be also involved in the CRF-induced changes we have observed, since mucosal eosinophils (65,66) and macrophages (31) are shown to be a cellular source of peripheral CRF and express CRF-R in human colon and the jejunum of chronically stress rodents and also involved in the regulation of permeability (66).

In conclusion, our results reinforce, in concordance with our previous findings (12,54,55) that IBS-D extends beyond the colon to also involves the small bowel. Thus, stress via CRF-driven mast cell activation and chemokine release may be a relevant mechanism in the pathophysiology of IBS and in stress-induced IBS associated gut mucosal inflammation.

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DISCUSIÓN GENERAL

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El SII es uno de los trastornos médicos más prevalentes (Lovell *et* Ford, 2012a), con un gran impacto económico, directo e indirecto, debido a su recurrencia y cronicidad (Fortea *et al.*, 2013) para el que existen pocas opciones terapéuticas claramente satisfactorias. El inicio de los síntomas, la gravedad y la persistencia en ciertos subtipos de SII se han vinculado a acontecimientos vitales estresantes o traumáticos. Alrededor del 50 % de los pacientes con SII presenta comorbilidad con trastornos psiquiátricos, comúnmente ansiedad y depresión, y sufren más estrés crónico que la población sana (Bennett *et al.*, 1998; Lydiard *et al.*, 2001; Nichol *et al.*, 2008). Sin embargo, el mecanismo exacto a través el cual el estrés ejerce sus efectos en el tracto gastrointestinal en el SII sigue siendo poco conocido.

A pesar de la elevada prevalencia e importancia de SII no existe un único marcador biológico distintivo, por lo que el diagnóstico se sigue fundamentando en criterios clínicos operativos y en la exclusión de otros trastornos que presenten manifestaciones clínicas comunes con el SII. En los últimos años, la visión convencional del SII como un trastorno funcional, no orgánico, ha sido cuestionada a raíz de observaciones variadas y repetidas de la presencia de un proceso de inflamación mucosa de bajo grado y activación inmunológica, como sustrato patológico relevante en algunos pacientes con SII (Bercik P *et al.*, 2005; Matricon *et al.*, 2012; Sebastián-Domingo, 2013). En particular, un aumento de número de células inflamatorias y de mediadores bioquímicos ha sido identificado en muestras de mucosa procedentes sobre todo del íleon y del colon de SII-D (revisado en Tabla III, Introducción).

Los mecanismos fisiopatológicos concretos del SII se desconocen, pero numerosos estudios han demostrado que, junto con la presencia de una microinflamación de la mucosa intestinal, concurren alteraciones de la motilidad y de la sensibilidad visceral, así como trastornos de la percepción en el sistema

nervioso central, que conforman la teoría de que, al menos algunos subtipos de SII, son consecuencia de un disfunción del eje regulador cerebro-intestino. Además, los pacientes con SII suelen presentar un aumento de la permeabilidad epitelial intestinal (ver tabla II, Introducción) y, recientemente se han descrito alteraciones estructurales y moleculares de la barrera epitelial asociadas, a su vez, con las manifestaciones clínicas y a la activación inmunológica (Zhou *et al*., 2010; Vivinus-Nébot *et al*., 2012: Martinez *et al*., 2012a). Estos defectos parecen ser más pronunciados en pacientes con el subtipo SII-D, poniendo de relieve la importancia crucial de dilucidar el papel participativo de los defectos de la barrera epitelial en la patogénesis del SII, así como la inflamación de bajo grado. Gracias a la investigación en los últimos años se están descubriendo nuevos mecanismos fisiopatológicos que han ayudado a desvelar moléculas potencialmente útiles como marcadores para el diagnostico y también como dianas terapéuticas.

En este contexto, los resultados obtenidos de esta tesis son innovadores e importantes al poner de manifiesto por primera vez que el yeyuno de pacientes con SII tiene no sólo una hiperplasia mastocitaria en la lámina propia, sino que también, y más importante, que existe una activación de éstos. También hemos mostrado que en el yeyuno de pacientes con SII-D existe un aumento de la permeabilidad intestinal, que se manifiesta especialmente después de la activación mastocitaria en respuesta al estrés agudo. En definitiva, y dada la enorme importancia nutricional e inmunológica del intestino delgado, es muy importante reconocer y destacar que este síndrome no afecta solo al intestino grueso, como venía siendo tradicional, sino que también están implicadas las regiones más proximales del tracto gastrointestinal.

En el primer trabajo de esta Tesis hemos evidenciado que el yeyuno de pacientes con SII-D, a pesar de mostrarse normal en la histopatología de rutina, presenta un aumento discreto de linfocitos intraepiteliales CD3+ y un aumento marcado de mastocitos, que es paralelo al aumento de la concentración de triptasa

en la luz yeyunal. Aunque habitualmente los mastocitos se han considerado en el contexto de la inflamación alérgica y en las enfermedades parasitarias, participan en una amplia variedad de procesos fisiológicos y patológicos (Gurish *et* Austen, 2001), incluyendo la regulación del transporte epitelial, de la permeabilidad intestinal, de la motilidad y de la sensibilidad visceral intestinal (Santos *et al.*, 2005; Bischoff, 2009). Tales anormalidades en la función intestinal han sido descritas en el SII y pueden ser en parte responsable de los hallazgos clínicos en estos pacientes, especialmente en aquellos con SII-D, ya que hasta el 59% de pacientes con mastocitosis sistémica presentan manifestaciones gastrointestinales, sobretodo diarrea, sensación de hinchazón y dolor abdominal (Sokol *et al.*, 2013), lo que refuerza el papel del mastocito en la generación de los síntomas del SII. Nosotros determinamos la triptasa sérica basal a todos los participantes incluidos en el capítulo 1 y 2, y se mantuvo dentro de los límites de la normalidad en todos ellos. Aunque una triptasa sérica basal no descarta la presencia de una mastocitosis sistémica, ninguno de los pacientes incluidos presentaba lesiones cutáneas típicas de la enfermedad, como la urticaria pigmentosa o la telangiectasia *macularis eruptiva perstans*, y tampoco presentaban *flushing* ni prurito cutáneo ni ningun otro síntoma característico de la mastocitosis. Aun así, no podemos descartar que algún paciente con SII-D de los estudiados presente en realidad una mastocitosis sistémica indolente o un síndrome de activación mastocitaria (Frieri *et al.*, 2013; Picard *et al.*, 2013), ya que los síntomas a nivel abdominal de ambas entidades son solapables. También descartamos la existencia de alergia no solo a alimentos sino también respiratoria, puesto que se ha descrito una inflamación intestinal en pacientes con asma y rinitis alérgica (Magnusson *et al.*, 2003; Collins *et al.*, 1996; Knutsson *et al.*1993) .

En el primer trabajo, observamos un aumento de la liberación de triptasa a la luz yeyunal de los pacientes con SII-D lo que indica la existencia de una activación mastocitaria local en pacientes con SII-D. El tratamiento con fármacos

estabilizadores de la membrana del mastocito, como el cromoglicato disódico o el ketotifeno (Lobo, Tesis Doctoral, UAB 2013; Klooker *et al.,* 2010) en pacientes con SII se ha asociado a una mejoría de la sintomatología, especialmente del número y consistencia de la diarrea en el SII y de la hipersensibilidad visceral, que no se ha acompaña de la disminución del número de mastocitos. Esto refleja la idea de que probablemente tenemos que darle más importancia al grado de activación celular que al contaje numérico de células en la mucosa del SII. No obstante, es interesante destacar, que la mayoría de publicaciones en esta área estudian la activación mastocitaria usando como referencia la inmunotinción con triptasa, método que creemos subestima el número de mastocitos degranulados ya que los gránulos liberados no se tiñen, o por la liberación de mediadores mastocitarios en el sobrenadante de biopsias incubadas *in vitro* (Barbara et al., 2004; Park *et al.*, 2003; Park *et al.*, 2006; Buhner *et al.*, 2009; Foley *et al.*, 2011; Lee *et al.*, 2013). Nosotros, hemos cuantificado la liberación de triptasa a la luz intestinal *in vivo*, con un método cuantitativo, como es el enzimainmunoensayo, lo que refleja de forma más fehaciente la activación del mastocito en el SII-D en su entorno local. Aunque los mastocitos son la única fuente significativa de triptasa en la mucosa intestinal no hemos detectado ninguna correlación entre el número de mastocitos de la mucosa y los niveles de triptasa luminal. Una explicación plausible para estos resultados es que la activación de los mastocitos y la liberación secundaria de la triptasa pueden ser un proceso no continuo. De hecho, en el colon e íleon de pacientes con SII la activación de los mastocitos en el SII parece ser más de tipo *piecemeal* (fragmentario), donde se produce una liberación lenta y selectiva de mediadores, y el aumento de la liberación luminal sólo tiene lugar en contextos específicos (Park et al., 2003).

Este incremento del número y la activación de los mastocitos en la mucosa intestinal en los pacientes con SII no es un hallazgo casual. Los mastocitos se localizan en la proximidad de los nervios entéricos (Park *et al.*, 2003; Barbara *et al.*,

2004), lo que puede alterar por un lado, la función motora intestinal, y por otro lado, les permite interaccionar bidireccionalmente con las terminaciones nerviosas entéricas y aferentes, quién tiene un papel primordial en el control de las funciones gastrointestinales. Los mastocitos además, tienen una función importante en la regulación de la permeabilidad intestinal así como en la función barrera epitelial (Barbara *et al.*, 2007), por lo que podemos considerar que los mastocitos poseen todas las características para ser los "candidatas ideales" para modular la disfunción intestinal del SII y participar en la traducción de síntomas como dolor abdominal y la alteración del ritmo deposicional.

Tanto el estrés agudo como el estrés crónico aumentan la permeabilidad epitelial a moléculas de pequeño y gran tamaño en el yeyuno y en el colon de roedores (Santos *et al.* 2000, Meddings *et* Wain, 2000; Soderholm *et* Perdue, 2001) con la participación de los mastocitos, el CRF y algunas citocinas, como el IFN-γ, la IL-4 o la quinasa de la cadena ligera de la miosina (Ferrier *et al.*, 2004). Los pacientes con SII-D estudiados en el primer capítulo tenían niveles de estrés basal más elevados que los controles sanos. En nuestro estudio no encontramos una correlación positiva entre los niveles de estrés basal y el número de mastocitos o la liberación de triptasa, aunque observamos una tendencia sugestiva. Si bien no se ha definido claramente si el estrés psicológico *per se* es causa o consecuencia del SII, se acepta que el SII es un trastorno sensible al estrés, donde los acontecimientos de la vida son fuertes predictores de exacerbación clínica (Bennett *et al.*, 1998) y la existencia de patrones distorsionados autonómicos junto con las anomalías neuroendocrinas en el eje HPA (Fukudo *et al.*, 1998; Dinan *et al.*, 2006) parecen estar relacionados con los cambios que se traducen en el hábito intestinal predominante y la hiperalgesia visceral intestinal (Mayer *et al.*, 2001; Tache *et al.*, 2004, Nicholl *et al.*, 2008). Estas observaciones, en relación con la participación de mediadores neurohormonales del estrés en el inicio y desarrollo de tales

anormalidades fisiopatológicas, han sido evaluadas a nivel experimental y clínico en los capítulos 2 y 3, respectivamente.

En este sentido, el objetivo del segundo trabajo fue desarrollar un modelo experimental de estrés crónico que se tradujera en anomalías estructurales y funcionales en el intestino de la rata, y en consonancia con nuestros resultados previos, estudiar la función del mastocito en el desarrollo de estas anomalías y evaluar su potencial como modelo para el estudio de la fisiopatología del SII. Para ello, hemos desarrollado un modelo experimental de estrés por aglomeración en ratas Wistar-Kyoto, una cepa más susceptible al estrés, por ser deficientes en acetilcolinesterasa intestinal. Representa un modelo de estrés psicosocial, en el que el establecimiento de la jerarquía social y la competición por el espacio, la comida o el agua, da lugar a un moderado estrés físico y psicológico, que refleja mejor el estrés social al que se ven sometidas las sociedades actuales y en particular, las de los países civilizados (Tamashiro *et al.*, 2005). El estrés generado por el hacinamiento se considera de intensidad leve o moderada, pero suficiente para activar el eje HPA, como se demuestra en el capítulo 2, en las que las ratas sometidas al estrés por aglomeración presentaron un aumento de la cortiscosterona en plasma en comparación con el grupo control. Este modelo de estrés psicosocial demuestra, por un lado, la presencia de inflamación intestinal, mostrada por el aumento de la actividad mieloperoxidasa en el yeyuno, íleon y colon, el aumento de leucocitos y linfocitos T en la luz intestinal y la hiperplasia de mastocitos, aunque de forma segmentaria en el colon. Además, y de forma similar a las observaciones del capítulo 1, en este trabajo también observamos una mayor activación de los mastocitos en las ratas estresadas, demostrada por la presencia de degranulación tipo *piecemeal* mantenida en el tiempo en todo el intestino como por el aumento de RMCP-II en la mucosa de yeyuno y colon de ratas sometidas a estrés crónico en comparación con el grupo control. Asimismo, realizamos una perfusión segmentaria *in vivo* y observamos

un aumento de la liberación luminal de RMCP-II que fue revertida con el tratamiento con ketotifeno. De forma interesante, en este modelo animal, hemos observado un aumento de la permeabilidad epitelial yeyunal a macromoléculas que es dependiente del mastocito, ya que el ketotifeno reduce remarcablemente la captación de HRP. Este efecto se relaciona con la activación mastocitaria más que con la hiperplasia, como se infiere de la alta correlación que existe entre el flujo a HRP y la degranulación del mastocito y la RMCP-II.

El aumento de leucocitos y linfocitos T de manera proporcional al tiempo y al estrés observado, a la vez que la preservación de la estructura apical de los enterocitos, sugiere una migración paracelular a la luz intestinal. Este fenómeno podría corresponderse al aumento de granulocitos circulantes y la alteración de las uniones estrechas que ocurre como respuesta a un estrés crónico psicosocial por deprivación materna (Söderholm *et al*., 2002). Hemos observado, además, que el hacinamiento produce una alteración de la morfología mitocondrial en las células epiteliales del intestino. Se han descrito alteraciones similares en otros modelos de estrés, como el estrés crónico por evitación de agua (Santos *et al.,* 2001; Söderholm *et al*., 2002), que se asocian a un aumento de la permeabilidad epitelial (Somasundaram *et al.,* 1997). La correlación positiva alcanzada entre la degranulación mastocitaria y el daño mitocondrial en el yeyuno y en el colon refleja más aun la importancia de la activación del mastocito en la disfunción epitelial inducida por el estrés crónico por aglomeración, observación consistente con la de otros modelos de estrés agudo y crónico (Gareau *et al*., 2008). Por tanto, la alteración de la permeabilidad epitelial observada por el estrés crónico en nuestro modelo experimental de estrés queda sustentada por todos estos hallazgos.

De acuerdo con estudios anteriores en modelos de estrés crónico, y como ocurre en los pacientes con SII, en nuestro modelo no hemos observado signos de inflamación macroscópica en la mucosa del intestino delgado y del colon durante la

exposición al estrés. Por el contrario, uno de los principales hallazgos de este trabajo es la demostración de la capacidad del estrés por aglomeración para iniciar y mantener la inflamación microscópica y la disfunción de la barrera a lo largo del intestino de la rata. Estas alteraciones generadas por el estrés parecen depender de mediadores biológicos liberados por los mastocitos activados en la mucosa intestinal.

Las disparidades encontradas en relación a la hiperplasia de mastocitos en los distintos segmentos intestinales estudiados pueden depender de varios factores experimentales y, posiblemente de la menor relevancia funcional en comparación con el grado de activación, un efecto casi universal del estrés crónico. Se desconoce si el desarrollo más precoz de la hiperplasia de mastocitos y el daño mitocondrial en el colon refleja su mayor susceptibilidad para el estrés psicosocial que en las áreas proximales del intestino delgado.

En este modelo de estrés crónico psicosocial nuestro grupo también ha demostrado la presencia de alteraciones de la motilidad y de hipersensibilidad visceral (Vicario *et al.,* 2012), que junto con la microinflamación y la alteración de la función barrera representan los mecanismos fisiopatológicos de la disfunción intestinal existente en el SII y validan el modelo de estrés crónico presentado en esta tesis como modelo experimental para el estudio del SII.

Varias moléculas pueden estar implicadas en la mediación de la activación de los mastocitos en respuesta al estrés, incluyendo el CRF, la acetilcolina, el NGF y la sustancia P. De especial importancia puede ser el papel del CRF periférico, liberado por terminaciones nerviosas sensoriales y simpáticas regionales, células inmunológicas y células enteroendocrinas del intestino (Karalis *et al.*, 1991; Tache *et* Bonaz, 2007). Varios estudios han demostrado que la administración exógena de CRF aumenta la motilidad colónica, la percepción visceral y la ansiedad, más intensamente en pacientes con SII, y estas respuestas se inhiben mediante la administración periférica de CRF(9-41)-α-helicoidal, un antagonista no selectivo del

receptor de CRF (Fukudo *et al.*, 1998; Sagami *et al.*, 2004). Así, como último objetivo de esta tesis, hemos querido estudiar si en el yeyuno de pacientes con SII-D, los mastocitos desempeñan un papel en el aumento de la permeabilidad intestinal inducida por el estrés agudo que contribuya a la fisiopatología de esta enfermedad. Para ello, en el tercer trabajo, realizamos una perfusión yeyunal segmentaria en pacientes con SII-D y en voluntarios sanos. Con ella, hemos demostrado por primera vez que el yeyuno humano responde a la administración periférica de CRF, el principal regulador de la respuesta al estrés, mediante la inducción de la secreción de agua y aumento de la permeabilidad en paralelo a la activación local de los mastocitos y la inducción de un entorno microinflamatorio. De manera importante para la fisiopatología del SII, los cambios inducidos por el CRF son significativamente mayores en los pacientes con SII-D que en los sanos.

El hecho que los cambios yeyunales observados en respuesta a la administración de periférica CRF no se acompañen de una respuesta diferencial de la activación del eje HPA en pacientes con SII-D, sugiere una acción local del CRF, de forma preferente en los pacientes. Se ha establecido que la presencia de receptores de CRF en el tracto gastrointestinal insinúan el papel de las vías periféricas en la regulación de la función gastrointestinal durante la respuesta al estrés (Tache *et* Perdue, 2004; Larrauche *et al.*, 2009). La función de los receptores de CRF en el intestino ha sido establecida mayoritariamente en base a estudios en roedores. En humanos, solo se ha descrito anteriormente la expresión CRF-R1 y en menor cantidad de CRF-R2 en el colon (Muramatsu *et al.*, 2000; Yuan *et al.*, 2007; Wallon *et al.*, 2008; Larrauche *et al.*, 2009), donde los CRF-R1 están relacionados con la actividad motora y la sensibilidad visceral. En animales, ambos receptores se han implicado también en la regulación de la secreción de iones y en el aumento de la permeabilidad intestinal (Saunders *et al.*, 2002; Keita *et al.*, 2010; Santos *et al.*, 200; Yang *et al.*, 2006). En este trabajo hemos descrito por primera vez la presencia

de receptores de CRF en el yeyuno humano. De forma interesante, en el yeyuno de pacientes con SII-D se produce una infraexpresión de CRF-R1 y una supraexpresión de CRF-R2, que es paralela a la permeabilidad intestinal aumentada y a la activación del mastocito, lo que sugiere que los receptores de tipo 2 en el yeyuno humano tienen una función en la regulación de la permeabilidad intestinal. En este estudio, no realizamos estudio inmunohistoquímico para establecer si los receptores de CRF en la biopsia de yeyuno se localizan en los mastocitos o en otras células de la mucosa yeyunal. Sin embargo, recientemente se ha descrito la presencia de CRF-R1 y CRF-R2 en mastocitos de la lámina propia del colon humano (Wallon *et al.*, 2008). Este hallazgo, junto con los resultados obtenidos en estudios realizados en ratas deficientes en mastocitos (Santos *et al.*, 2000, Santos *et al.,* 2008) y con el uso de fármacos estabilizadores del mastocito (Santos *et al.,* 2008; Wallon *et al.*, 2008) y estudios *in vitro* que muestran que el CRF modula la permeabilidad macromolecular a través de los mastocitos en el colon humano sano (Wallon *et al.*, 2008), sugieren un papel prominente de eje CRF-mastocito en la mediación de la disfunción intestinal inducida por el estrés. De todos modos, aunque parece claro que en la respuesta intestinal al estrés tiene un papel predominante el sistema periférico de señalización de CRF, cómo y dónde se recluta ésta sigue sin conocerse.

La activación de los mastocitos yeyunales en respuesta a la administración periférica de CRF indica que los eventos inflamatorios locales mediados por mastocitos pueden contribuir a trastornos funcionales y manifestaciones clínicas de SII-D. Con este último trabajo, hemos querido obtener una mayor comprensión de los mecanismos de activación de los mastocitos de la mucosa secundarios al estrés agudo y su contribución al aumento de la permeabilidad intestinal, como características distintivas de SII-D, y hemos demostrado que el aumento de respuesta secretora y de la permeabilidad en el yeyuno secundaria a la administración de CRF está correlacionada con una activación de los mastocitos intestinal, lo que sugiere un

papel fundamental de los mastocitos en la generación de este aumento de la permeabilidad intestinal inducida por el estrés. Aunque la liberación de triptasa secundaria al CRF también se incrementó en los controles sanos fue en menor medida que en los pacientes de SII-D. Esto sugiere un papel regulador del mastocito en la secreción y la función de la barrera en el yeyuno SII-D secundaria al estrés, que podría estar mediado por los receptores de CRF-R2 presentes en los mastocitos del yeyuno humano. Cabe destacar que los efectos de la administración periférica de CRF en la barrera epitelial en humanos no habían sido establecidos con anterioridad.

Sin embargo, no podemos descartar que estos hallazgos sean debidos a otras vías independientes del CRF, ya que los mastocitos también pueden ser activados por otros neuropéptidos tales como la neurotensina o la sustancia P (Keita *et al.*, 2010), o por neurotransmisores como la acetilcolina o el VIP (Keita *et al.*, 2013). Tampoco podemos excluir una activación de los mastocitos por moléculas alternativas liberadas por otras células portadoras de receptores para la CRF, como el eosinófilo (Wallon *et al.*, 2011) o los macrófagos (Yuan *et al.*, 2012). Para comprobarlo, se deberían realizar futuros estudios usando fármacos estabilizadores del mastocito o con antagonistas del receptor de CRF. Hasta la fecha, en humanos solo se ha realizado un ensayo clínico con un antagonista de CRF-R1 con escasos efectos en los síntomas del SII (Sweetser *et al.*, 2009). No obstante, en base a nuestros resultados, los fármacos antagonistas selectivos del receptor de CRF-R2 podrían tener un efecto potencial en pacientes con SII-D, al menos en la regulación de la permeabilidad intestinal y de la activación mastocitaria.

Estos resultados, refuerzan, en concordancia con los trabajos previos que el SII-D se extiende más allá del colon y también afecta el intestino delgado. La activación de mastocitos secundaria al estrés por CRF y la liberación de quimiocinas puede ser un factor relevante en la fisiopatología de la recaída del SII y de la inflamación intestinal inducida por el estrés. La modulación neurohumoral del

sistema inmunológico entérico proporciona un mecanismo por el cual el estrés puede provocar la inflamación intestinal en el SII.

CONCLUSIONES

CONCLUSIONES

Considerando los resultados de esta Tesis doctoral, podemos concluir que:

1. El Síndrome del Intestino Irritable con predominio de diarrea presenta alteraciones orgánicas y biológicas que se extienden más allá del intestino grueso, demostrando afectación del intestino delgado.

2. La hiperplasia mastocitaria y la activación del mastocito en el yeyuno son características del Síndrome del Intestino Irritable con predominio de diarrea.

3. El estrés crónico por aglomeración en ratas inicia y mantiene la inflamación microscópica y la disfunción de la barrera epitelial a lo largo del intestino y es dependiente de la activación mastocitaria.

4. El estrés crónico por aglomeración en ratas representa un nuevo modelo experimental para el estudio del Síndrome del Intestino Irritable con predominio de diarrea.

5. La administración periférica del factor liberador de corticotropina a pacientes con Síndrome del Intestino Irritable induce mayor activación mastocitaria, activación inflamatoria y disfunción de la barrera epitelial que a sujetos sanos.

6. Este modelo de estrés agudo experimental en humanos puede ser útil y relevante para el estudio de la fisiopatología del Síndrome del Intestino Irritable con predominio de diarrea.

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INDICE