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EFECTO DE ÁCIDO TAUROURSODEOXICÓLICO SOBRE LOS DESÓRDENES CARDIORRESPIRATORIOS EN INSUFICIENCIA CARDIACA

TESIS PARA OPTAR AL GRADO DE
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1. Resumen

La insuficiencia cardiaca (HF) es una de las principales causas de muerte a nivel mundial y su incidencia irá aumentando con el tiempo. Durante la HF, la disfunción del sistema nervioso simpático (SNS) se relaciona con mayores tasas de mortalidad, y al día no se cuentan con estrategias efectivas para el tratamiento de la insuficiencia cardiaca no-isquémica (HFpEF). Se ha identificado que las neuronas catecolaminérgicas del tronco encéfalo en la zona rostral ventrolateral (C1 RVLM) juegan un rol fundamental en el desbalance autonómico y en la disfunción cardiorrespiratoria en la HFpEF. Sin embargo, los mecanismos celulares y moleculares responsables de su sobre-activación durante este síndrome no han sido estudiados con profundidad. Se ha demostrado que la señalización de estrés de retículo (ERS) participa de forma importante en la sobre-activación de núcleos centrales de control del SNS en HF isquémica, sin embargo, se desconoce si existe ERS a nivel central durante la HFpEF. Por lo tanto, el objetivo del presente trabajo de tesis fue determinar la presencia y contribución de la ERS en la RVLM en los desórdenes cardiorrespiratorios y autonómicos en ratas con HFpEF. Se indujo HFpEF de forma quirúrgica en ratas Sprague-Dawley, a las cuales se les administró TUDCA (ácido tauroursodeoxicólico, un inhibidor característico de ERS) de forma intracerebroventricular durante 4 semanas una vez establecida la enfermedad. Comparadas con las ratas HFpEF tratadas con vehículo, la administración de TUDCA en HFpEF (HF+Veh vs. HF+TUDCA, $p<0.05$) previno la hipertrofia cardiaca (HW/BW 4.4±0.3 vs. 4.0±0.1mg/g;) y produjo una marcada restauración de la función diastólica (EDP: 4.9±0.6 vs. 3.7±0.4mmHg). Además, la administración de TUDCA mejoró el control autonómico cardiaco (LF/HF_{HRV} ratio 3.02±0.29 vs. 1.14±0.24), redujo la incidencia de arritmias (141.5±26.7 vs. 35.67±12.5 eventos/h;) y normalizó los desórdenes respiratorios (Apneas: 11.83±2.26 vs. 4.33±1.80 eventos/h). El análisis molecular reveló un aumento en la expresión de biomarcadores de ERS (aumento de 3 veces en la expresión de BiP, CHOP y sXBP1), neuroinflamación (7 veces TNF-α y 3 veces IL-1β) y del Sistema Renina-Angiotensina (6 veces AT1 y 9 veces NOX2) en la RVLM en ratas con HFpEF y su consecuente disminución tras el

tratamiento con TUDCA. En conjunto, los resultados sugieren un efecto beneficioso de TUDCA sobre la fisiopatología de la HFpEF, lo que podría significar una nueva estrategia terapéutica para futuros estudios pilotos.

2. Abstract

Heart failure (HF) is among the major causes of death worldwide and its prevalence is increasing in time. In HF, sympathetic nervous system (SNS) dysfunction is related with increased mortality, and at date no therapeutic strategy is approved for non-ischemic heart failure. Chronic activation of catecholaminergic neurons within the brainstem rostral ventrolateral medulla (RVLM), a major integration site for sympathetic activity regulation, play an essential role on cardiorespiratory alterations during non-ischemic heart failure (HF) pathophysiology. Endoplasmic reticulum stress (ERS) is known to participate in the development and progression of several cardiovascular diseases. Whether ERS in the brain contribute to non-ischemic HF progression/maintenance remains completely unknown. Accordingly, we aimed to determine the presence and contribution of brainstem ERS on cardiovascular and respiratory outcomes in non-ischemic HF rats. Adult male Sprague-Dawley rats underwent volume overload to induce non-ischemic HF. Tauroursodeoxycholic acid (TUDCA), an ERS inhibitor, was intracerebroventricularly delivered for 4 weeks after HF induction to assess the contribution of ERS on cardiorespiratory HF outcomes. Compared to vehicle treated HF rats, TUDCA administration in HF (HF+Veh vs. HF+TUDCA, $p<0.05$) prevented cardiac hypertrophy (HW/BW 4.4±0.3 vs. 4.0±0.1mg/g;) and markedly restored diastolic cardiac function (EDP: 4.9±0.6 vs. 3.7±0.4mmHg). In addition, TUDCA improved cardiac autonomic control (LF/HF_{HRV} ratio 3.02±0.29 vs. 1.14±0.24), reduced the incidence of cardiac arrhythmias (Arrhythmias: 141.5±26.7 vs. 35.67±12.5 events/h;) and normalized breathing disorders (Apneas: 11.83±2.26 vs. 4.33±1.80 events/h). Analysis of brainstem ERS-related genes expression confirmed the presence of ERS (3-fold augment in BiP, CHOP and sXBP1 expression), inflammation (TNF-α, 7-fold, IL-1β, 3-fold)and activation of brain renin-angiotensin signaling pathway (AT1, 6-fold, NOX2, 9-fold) in the RVLM and that TUDCA treatment completely abolished ERS and ERS-downstream targets in the RVLM of HF rats. Together our results support a salutary effect of TUDCA treatment for the control of cardiorespiratory dysfunction in of non-ischemic HF.

1. Índice capítulos

2. Capítulo 1: Introducción, planteamiento del problema, hipótesis, objetivos

Las enfermedades cardiovasculares (CVD, por sus iniciales en inglés) constituyen la principal causa de muerte a nivel mundial (Cannon, 2013), dentro de las cuales, la insuficiencia cardiaca (HF, por sus iniciales en inglés) se posiciona como uno de los problemas de salud pública más importantes, dado que más de un 36% de las muertes reportadas por CVD son debido a HF (Mozaffarian et al., 2016). Esta patología se caracteriza por la disfunción eléctrica y contráctil del músculo cardiaco, disminuyendo el suministro de nutrientes a los tejidos, lo que, sumado a los frecuentes episodios de descompensación, agrava considerablemente la calidad de vida de los pacientes (Hobbs et al., 2002). A pesar de que el cuadro patológico de la HF ha sido documentado desde el año 1550 antes de Cristo (McKee et al., 1971) y que la investigación en CVD durante las últimas décadas ha aumentado más de 4 veces, la incidencia de la HF se ha cuadruplicado en los últimos años, afectando alrededor del 20% de la población mundial sobre los 65 años (Ponikowski et al., 2014), y se estima que su prevalencia aumentará en un 46% para el año 2030 (Heidenreich et al., 2013). Por lo tanto, es crucial contar con una estrategia terapéutica efectiva contra este síndrome, debido al aumento en la esperanza de vida y la tasa de envejecimiento de la población durante los últimos años (INE, 2008). De acuerdo a proyecciones del Instituto Nacional de Estadísticas (INE, 2008), para el año 2030, la HF podría llegar a afectar alrededor de 1,5 millones de chilenos. Además, la HF es una enfermedad con una alta tasa de mortalidad, cercana al 50% dentro de los primeros 4 años del diagnóstico (Swedberg et al., 2005); lo que es especialmente preocupante, ya que la mortalidad de este síndrome ha aumentado de forma sostenida y se pronostica que se duplique para 2030 (Heidenreich et al., 2013). Por lo tanto, es de extrema necesidad conocer los mecanismos celulares y moleculares responsables de la patogénesis de la HF y así establecer nuevas terapias farmacológicas y/o no farmacológicas con el fin de mejorar la expectativa y calidad de vida de los pacientes

Comúnmente, la HF es vista como una etapa terminal de diferentes CVD (van Empel & Brunner-La Rocca, 2015a), sin embargo, su etiología es desconocida, debido a que los pacientes con HF presentan múltiples comorbilidades, ya sea de origen cardiaco o no-cardiaco, lo que complica su diagnóstico y su tratamiento (McMurray et al., 2012). Dependiendo de la cantidad de sangre eyectada por el músculo cardiaco, la HF puede ser clasificada en dos subtipos: HF de fracción de eyección reducida (HFrEF), caracterizada por una disminución en la función sistólica, o HF de fracción de eyección preservada (HFpEF), caracterizada por una disfunción diastólica (van Empel & Brunner-La Rocca, 2015b; Yancy et al., 2013), que presentan cuadros sintomatológicos y tazas de mortalidad similares, pero mecanismos moleculares necesariamente diferentes (van Empel & Brunner-La Rocca, 2015a), dado que los tratamientos que resultan ser efectivos para HFrEF han demostrado no tener efecto alguno en la progresión de pacientes con HFpEF (van Empel & Brunner-La Rocca, 2015a).

Independiente del tipo de insuficiencia cardiaca, ambas, HFpEF y HFrEF, se caracterizan por 4 aspectos principales interrelacionados: (1) disfunción cardiaca (Toledo et al., 2016; Triposkiadis et al., 2009), (2) inflamación crónica a nivel sistémico (Anker & von Haehling, 2004; Ueland et al., 2015), (3) activación crónica del sistema Renina-Angiotensina (RAS, por sus iniciales en inglés) (Sciarretta et al., 2009; Unger & Li, 2004) y (4) desbalance del sistema nervioso autónomo, caracterizado por una sobre-activación del sistema nervioso simpático e inhibición de la rama parasimpática (Andrade et al., 2015; Kishi, 2012). De forma importante, las últimas investigaciones han demostrado que la activación del RAS en centros cerebrales de control autonómico juega un papel fundamental en el deterioro de la función cardíaca, la respuesta inflamatoria y el sistema nervioso simpático (Xu & Li, 2015) y que la ruta de señalización de estrés endoplásmico (ERS, por sus iniciales en inglés) es un punto nodal que comanda las 4 características principales en animales con HF (Wei et al., 2016a). Por lo tanto, nuevas investigaciones son necesarias para posicionar esta ruta de señalización como una nueva terapia contra la HFpEF.

Fisiopatología de la HFpEF

Los principales mecanismos moleculares asociados a la fisiopatología de la HF están señalados en la Tabla 1. Hasta los años 2000, la HFpEF era meramente considerada una etapa temprana del cuadro de la HFrEF (Paulus & Tschope, 2013), dado que los pacientes de HFpEF descompensados evolucionan a HFrEF en etapas más severas y que las pruebas ecocardiográficas para el diagnóstico estaban enfocadas hacia el estudio de la función sistólica y la fracción eyectada (van Empel & Brunner-La Rocca, 2015a). No fue hasta que los especialistas comenzaron a documentar que los tratamientos que mostraban alta efectividad para HFrEF eran inefectivos o empeoraban la progresión de pacientes con HFpEF que la enfermedad fue reconocida como un síndrome diferente de la HFrEF (van Empel & Brunner-La Rocca, 2015a; Yancy et al., 2013). Las comorbilidades más frecuentes en HFrEF tienden a ser cardiacas, como son la hipertensión arterial, enfermedad coronaria e infarto al miocardio (Azad & Lemay, 2014; Mozaffarian et al., 2016), mientras que dentro de las comorbilidades más frecuentes en pacientes con HFpEF tienden a ser no-cardíacas, entre ellas, obesidad, diabetes tipo 2 e insuficiencia renal (Ather et al., 2012). Actualmente, no existen terapias efectivas para la HFpEF (Butler et al., 2014), por lo que es necesario investigar los mecanismos asociados a su progresión.

De forma importante, niveles elevados de citoquinas pro-inflamatorias, sobre-activación del RAS y el desbalance autonómico se asocian con un peor pronóstico y expectativa de vida para el paciente de HF (Anker & von Haehling, 2004; Florea & Cohn, 2014; Fyhrquist et al., 1972). Dicha activación neurohumoral (niveles elevados de catecolaminas y componentes del RAS) es uno de los mecanismos cruciales en la progresión de la HF, y por lo tanto el tratamiento con antagonistas contra el RAS y receptores β-adrenérgicos corresponden al “gold standard” en el tratamiento para la enfermedad (Hartupee & Mann, 2016; Mozaffarian et al., 2016; Willenbrock, et al., 2000).

Tabla 1. Principales mecanismos moleculares y celulares involucrados en la patogénesis de la insuficiencia cardiaca

	Efectos deletéreos a la función cardiaca y mecanismos moleculares asociados
Inflamación crónica	
MCP-1 y VCAM-1	-Reclutan monocitos/macrófagos al miocardio, estableciendo las bases de la inflamación crónica a nivel cardiaco y sistémico en HF (Birdsall et al., 1997; Juncos et al., 2011; Savic-Radojevic et al., 2013).
TNF-α	-Citoquina fundacional que induce la síntesis de citoquinas y quimioquinas de la cascada inflamatoria vía NF-κB y AP-1 (Olsson, 1993) -Promueve la diferenciación de miofibroblastos cardiacos, induciendo fibrosis vía TGF-β (Porter, Turner, O'Regan, & Ball, 2004), alterando la contractilidad y conductividad del corazón (Kawara et al., 2001) -Induce muerte de cardiomiocitos por apoptosis vía TNFR1 (Kubota et al., 2001) -Reduce biodisponibilidad de NO vía estrés oxidativo, produciendo disfunción endotelial y sobrexcitación de neuronas presimpáticas (Goodwin et al., 2007; Guggilam et al., 2011)
IL-1β	-Altera la homeostasis del calcio, disminuyendo la contractilidad del corazón (McTiernan et al., 1997) -Junto con TNF-α, propaga la respuesta inflamatoria vía NF-κB y reduce biodisponibilidad de NO (Bujak & Frangogiannis, 2009) (Ing et al., 1999)
Simpato-excitación	
Norepinefrina (NE)	-Altera la respuesta cronotrópica e inotrópica del corazón por potenciación de corrientes de calcio (Hasking et al., 1986) -Induce vasoconstricción, vía señalización cAMP/PKA, aumentando la presión arterial (Kaye et al., 1994) -Promueve la hipertrofia de cardiomiocitos y la fibrosis cardiaca por la inhibición de la ruta NO-cGMP-PKG (Calderone et al., 1998)
Actividad Simpática	-Aumentada en todos los modelos de HF (Houser et al., 2012), promueve la liberación de NE y la activación de neuronas presimpáticas, creando un estado de hiperactivación crónica en animales y pacientes con HF (Kaye et al., 1994). -Promueve arritmias (Dean & Lab, 1989) y disminuye variabilidad del ritmo cardiaco (May et al., 2013) -Induce la activación del Sistema Renina-Angiotensina (DiBona, 2000)
RAS	
Angiotensina-II	-Potente vasoconstrictor, aumenta la presión arterial de forma crónica (Hall, 1991) -Inducción de arritmias cardiacas por inhibición de corrientes de potasio (Hasenfuss, 1998) -Induce hipertrofia cardiaca en conjunto con citoquinas pro-inflamatorias y estrés oxidativo vía NF-κB y NADPH-oxidasa (Sriramula & Francis, 2015) -Induce apoptosis de cardiomiocitos vía estrés oxidativo (Seshiah et al., 2002) y estrés de retículo endoplásmico (Song et al., 2011) -Promueve simpato-excitación mediante la estimulación de aferentes cardiacas (Allen, 1998)

MCP1, “Monocyte chemotratant protein-1”; VCAM-1 “Vascular Cell Adhesion Molecule-1”; TNF-α, “Tumor Necrosis Factor-1”; TNFR1, “TNF Receptor 1”; IL-1β, “Interleuquina-1β”; AP-1, “Activator Protein-1”; TGF-β, “Transforming Growth Factor-β”; NO, óxido nítrico; PKA, “Protein Kinase A”, cAMP, “cyclic AMP”; cGMP, “cyclic GMP”; PKG, “Protein kinase G”.

Sin embargo, a pesar de que dichas familias de fármacos han demostrado mejorar el estatus clínico y la sobrevida en varios pacientes de HF, la tasa de mortalidad sigue siendo elevada (Heidenreich et al., 2013; Willenbrock et al., 2000), lo que pone en evidencia la falta de estudios que busquen mejorar la comprensión de los mecanismos moleculares precisos responsables de la respuesta neurohumoral. En este sentido, la inflamación crónica se posiciona como un blanco farmacológico interesante, dado que se ha demostrado que la respuesta inflamatoria juega un papel importante en distintas clases de HF (Hofmann & Frantz, 2013; Paulus & Tschope, 2013), lo que ha permitido una mejor comprensión de la etiología de enfermedad, dado que la HF es altamente prevalente en la población mayor (Mozaffarian et al., 2016) y está ampliamente demostrado que la inflamación aumenta con la edad (Franceschi & Campisi, 2014; Maggio et al., 2006; Varadhan et al., 2014). Es más, se ha propuesto un nuevo paradigma, en el cual la inflamación juega un rol crucial en la progresión de la HFpEF (Paulus & Tschope, 2013), dado que comorbilidades no-cardiacas con un cuadro inflamatorio están presentes en todos estos pacientes y, además, tienden a ser mayores que los pacientes de HFrEF (van Empel & Brunner-La Rocca, 2015a). Más aún, la deleción de quimioquinas responsables del inicio de la respuesta inflamatoria previenen la progresión de la HFpEF (Juncos et al., 2011). Sin embargo, a la fecha no existen terapias aprobadas para la HF destinadas hacia inhibición de la respuesta inflamatoria. Cabe mencionar que la contribución de mediadores inflamatorios en la progresión de la HF no está restringida únicamente al corazón y el plasma, sino que también son capaces de inducir simpato-excitación (Guggilam et al., 2007; Wei et al., 2013), y sus niveles se encuentran elevados en áreas de control simpático en animales con HF (Francis et al., 2011; Kang et al., 2011). Interesantemente, investigaciones recientes han demostrado que la señalización de estrés de retículo endoplásmico juega un rol importante en la respuesta inflamatoria y la progresión de la HF (Chen et al., 2016; Wei et al., 2016a), sin embargo, los mecanismos moleculares asociados que los relacionan no han sido estudiados.

Control autonómico en HFpEF

La HF está estrechamente asociada con la disfunción del sistema nervioso autónomo, caracterizada por simpato-excitación y/o una disminución de tono

parasimpático, conduciendo a un empeoramiento del pronóstico de la enfermedad (Florea & Cohn, 2014; Kishi, 2012). Se ha descrito que los pacientes con HFpEF presentan valores elevados en la concentración plasmática de norepinefrina (NE), similares a los que presentan pacientes con HFrEF (Cohn et al., 1984; Kitzman et al., 2002). De hecho, los valores de NE plasmática son comúnmente utilizados como predictor de mortalidad en pacientes con HF (Florea & Cohn, 2014). Se piensa que la simpato-excitación ocurre como un sistema compensatorio frente a una reducción en la fracción de eyección, que estimula las aferentes cardiacas hacia centros de control simpático en el cerebro (Xu & Li, 2015). Sin embargo, la activación crónica del sistema nervioso simpático tiene efectos deletéreos hacia el miocardio, dado que altera la respuesta cronotrópica e inotrópica del corazón (Hasking et al., 1986), aumenta la incidencia de arritmias (Dean & Lab, 1989) y promueve la hipertrofia de cardiomiocitos y la fibrosis cardiaca (Calderone et al., 1998).

El tono simpático es modulado por diferentes centros del cerebro, ubicados en el hipotálamo y el tronco encefálico, sin embargo, la médula rostral ventrolateral (RVLM, por sus iniciales en inglés), es uno de los puntos nodales más importantes para el control simpático, dado que integra diferentes señales provenientes de las demás áreas de control reflejo y autonómico (Guyenet, 2006; Kishi, 2013). La salida simpática es fuertemente dependiente de la actividad de una subpoblación de neuronas llamada C1, que son catecolaminérgicas y usan glutamato como neurotransmisor (Swanson & Sawchenko, 1983), dado que su destrucción inhibe la respuesta simpática a la estimulación eléctrica de diversas aferentes (Madden & Sved, 2003).

La hiperactivación de neuronas de la RVLM representa un suceso importante que contribuye al desarrollo de desbalance autonómico en HF (Del Rio et al., 2006). De forma importante, nuestro laboratorio ha demostrado que la hiperactivación crónica de neuronas C1 se relaciona con el aumento del tono simpático cardiaco y la incidencia de arritmias en ratas con HFpEF (Toledo et al., 2017). Sin embargo, los mecanismos asociados a esta hiperactivación deben ser dilucidados. Se ha propuesto que un mecanismo central en la hiperactivación crónica de neuronas

pre-simpáticas está dado por estrés oxidativo, lo cual podría responder en parte a una respuesta inflamatoria dada la activación del RAS a nivel cerebral (Xu & Li, 2015). De forma importante, nuestro laboratorio ha reportado un aumento en el nivel de expresión de componentes del RAS y producción de especies reactivas del oxígeno (ROS) en la RVLM de ratas con HFpEF (Andrade et al., 2017a; Toledo et al., 2017) y nuestros resultados preliminares demuestran un aumento en la expresión de citoquinas proinflamatorias (TNF-α e IL-1β) en este núcleo de control simpático. Sin embargo, el mecanismo preciso por el cual estrés oxidativo se relaciona con la inflamación y RAS en el RVLM, y particularmente en las neuronas pre-simpaticas, aún no ha sido evidenciado.

Quimiorreflejos y HFpEF

Los quimiorreflejos periféricos y centrales son sistemas fisiológicos que regulan el ritmo ventilatorio y el tono simpático en respuesta a cambios repentinos en las concentraciones de O_2 y CO_2 respectivamente (Guyenet et al., 2010). Pacientes con HF muestran una sensibilidad aumentada de dichos quimiorreflejos, lo cual se relaciona de forma directa con un aumento en la incidencia de desórdenes respiratorios como apneas y patrones ventilatorios irregulares, lo que, a su vez, se relaciona con un peor pronóstico y aumento en las tasas de mortalidad (Toledo et al., 2016), y como grupo hemos demostrado que en HFpEF existe una potenciación selectiva del quimiorreflejo central (Del Rio et al., 2017; Toledo et al., 2017), la cual se relaciona directamente con un aumento en la actividad del sistema nervioso simpático (Toledo et al., 2017). Es más, hemos demostrado recientemente que la destrucción selectiva de las neuronas quimisosensitivas del núcleo retrotrapezoide (RTN, principal quimiorreceptor central (Guyenet et al., 2010)) en ratas con HFpEF restaura los desórdenes respiratorios y el desbalance autonómico (Diaz et al., 2020), lo cual sugiere una fuerte interacción entre las neuronas del RVLM y los quimiorreceptores centrales, dado que, de manera similar, la destrucción de las neuronas C1 RVLM restaura la incidencia de desórdenes respiratorios y la simpato-excitación inducida por hipercapnia en ratas con HFpEF (Andrade et al., 2019; Toledo et al., 2019), sin embargo, los mecanismos moleculares responsables de la sobreactivación de dichas neuronas durante la HFpEF son completamente desconocidos al día de hoy. Considerando

que ambos núcleos se encuentran en directa proximidad (Toledo et al., 2016), podría esperarse que los mecanismos moleculares responsables de la sobre-activación del RVLM pudiesen afectar a su vez a los quimiorreceptores centrales.

Sistema Renina-Angiotensina en HFpEF

El RAS fue primeramente descrito como un mecanismo compensatorio sistémico frente a disminución al flujo sanguíneo y/o la presión arterial. En breve, el RAS comprende la proteólisis de Angiotensinógeno (péptido inactivo secretado de forma constitutiva por el hígado) por la Renina, (enzima que es secretada por el riñón frente a una caída en la presión arterial); el Angiotensinógeno se convierte a Angiotensina-I mediante corte proteolítico, que también es inactivo; el cual es cortado a Angiotensina-II por la enzima convertidora de angiotensina (ECA) de tipo 1 en los órganos blanco (Danser et al., 1995; Ganten et al., 1971; Guazzi et al., 1999), produciendo un potente aumento en la presión arterial mediante la inducción de vasoconstricción, secreción de aldosterona y elevación del tono simpático (Riegger, 1991), mediado por la interacción entre Angiotensina-II y su receptor AT1. En condiciones fisiológicas, la secreción de Renina disminuye frente al aumento en la presión arterial, sin embargo, en HF, el RAS se mantiene activado de forma crónica y persistente, incluso en pacientes y modelos animales que presentan hipertensión (Dostal & Baker, 1999; Ferguson et al., 2001; Manrique et al., 2009; Unger & Li, 2004). La activación crónica del RAS resulta fácil de comprender en HFrEF, donde existe una reducción crónica en el flujo sanguíneo, sin embargo, los componentes del RAS se encuentran aumentados incluso en HFpEF (Suzukiet al., 2004; Yoshimura et al., 2000). El origen de la activación del RAS en HFpEF no ha sido dilucidado, sin embargo, investigaciones recientes sugieren que la respuesta inflamatoria podría contribuir dado que la expresión del receptor AT1 está gobernada por el factor transcripcional NF-κB (Paul et al., 2006; Sciarretta et al., 2009), el factor transcripcional por excelencia activado por citoquinas proinflamatorias (Borish & Steinke, 2003; Olsson, 1993). Interesantemente, se ha descrito que la activación del receptor AT1 activa a su vez al factor NF-κB, creando un sistema de retroalimentación positiva que perpetua la activación crónica del RAS, la inflamación y la hiper-activación de sistema nervioso simpático (Sciarretta et al., 2009; Sumners et al., 1994; Xu & Li, 2015).

Adicionalmente, se ha descrito la existencia de Sistemas Renina-Angiotensina completamente funcionales en áreas cerebrales de control simpático (Paul et al., 2006; Richoux et al., 1988). Múltiples trabajos han demostrado que estos RAS se encuentran sobre-activados en HF y que la inhibición del receptor AT1, NF-κB o anti-oxidantes a nivel central previene la simpato-excitación y mejora la función cardiaca en modelos animales con HFrEF (Guggilam et al., 2011; Kang et al., 2011; Kleiber et al., 2010; Sharma et al., 2017). La sobre-activación de neuronas pre-simpáticas, así como el rol del receptor AT1 en este proceso no ha sido estudiado en HFpEF. A la fecha, se han reportado niveles aumentados de AT1 en el NTS (Shigematsu et al., 2001) y en órganos circumventriculares (Yoshimura et al., 2000), sin embargo, no se han estudiado los efectos del bloqueo del RAS sobre el control autonómico y la función cardiaca en HFpEF. Reportes anteriores de nuestro laboratorio muestran la activación crónica de neuronas del RVLM de ratas con HFpEF (Toledo et al., 2017), y resultados preliminares muestran niveles aumentados de AT1 y NF-kB, sugiriendo que la activación del RAS a nivel de este núcleo de control autonómico juega un rol importante en HFpEF.

El receptor AT1 es expresado en neuronas y astrocitos (Paul et al., 2006; Sumners et al., 1991) y se piensa que estos últimos son la principal fuente de Angiotensina-II en el cerebro (McKinley et al., 2003). De hecho, la ablación astro-específica en HFrEF mejora la función cardiaca, control autonómico y sobrevida de los animales mediante la reducción de la activación de neuronas de la RVLM (Isegawa et al., 2014). Investigaciones recientes han demostrado que en células CATH.a, modelo de neuronas catecolaminérgicas presimpáticas *in vitro*, responden de manera similar a las neuronas C1. De hecho, la estimulación con Angiotensina-II produce aumentos en las especies reactivas de oxígeno, activación de NF-κB (Haack et al., 2010) y síntesis de citoquinas pro-inflamatorias (Agarwalet al., 2013). Esto sugiere que RAS e inflamación podrían participar de la activación de neuronas C1 de la RVLM en ratas con HFpEF. De hecho, resultados previos de nuestro laboratorio muestran niveles aumentados del receptor AT1 de Angiotensina-II y del factor transcripcional NF-κB en la RVLM, lo cual se correlaciona con niveles aumentados del marcador de activación neuronal FosB (Toledo *et al.,* 2017), sugiriendo la

activación crónica del RAS, el cual promovería la activación de neuronas pre-simpáticas en este importante nucleo de control autonómico. Sin embargo, deben realizarse más investigaciones *in vitro* con el fin de dilucidar una relación causal entre la activación neuronal crónica y la activación del RAS.

Señalización de Estrés de Retículo Endoplásmico y HFpEF

La vía de señalización de ERS ha despertado el interés de las últimas investigaciones en HF, dado que su activación puede conducir a: (1) disfunción cardiaca mediante la inducción de muerte por apoptosis de cardiomiocitos (Song et al., 2011) (Chen et al., 2016); (2) fibrosis cardiaca (Groenendyk et al., 2016); (3) aumento de los niveles de NE plasmática y simpato-excitación (Wei et al., 2016a); (4) aumento de la actividad de los RAS a sistémicos y centrales (Wei et al., 2016a) y; (5) reclutamiento de células inflamatorias al miocardio y la consecuente producción de citoquinas vía NF-κB (Jiang et al., 2017). Sin embargo, ningún estudio ha evaluado activación de esta ruta en HFpEF. Más aún, si la activación de ERS en núcleos cerebrales relacionados con el control simpático (i.e. RVLM) en HFpEF y su relación con la progresión de la patología es totalmente desconocido.

La ruta de ERS canónica se activa frente a la acumulación de proteínas mal plegadas en el retículo endoplásmico, mediante la disociación de la chaperona residente BiP (“Binding immunoglobulin Protein”) de las proteínas IRE1 (“Inositol-Requiring Enzyme 1”) y PERK (“Protein kinase RNA-like Endoplasmatic Reticulum Kinase”), las cuales forman homodímeros que se activan por auto-trans-fosforilación (Gardner et al., 2013). La activación de PERK reprime la traducción mediante la fosforilación de eIF2 (“eucariotic iniciation factor-2”), previniendo la acumulación de nuevos agregados, a su vez, promueve sobrevida celular o apoptosis (dependiente de la expresión de CHOP-“CCAAT-enhancer-binding protein Homologous Protein”), de acuerdo al estado metabólico celular (Halliday et al., 2017). La activación de IRE1 permite el splicing del mRNA del factor transcripcional XBP1 (“X-box Binding Protein 1), que induce la expresión de chaperonas que restablecen la proteostasis celular (Gardner et al., 2013). Además, existe una ruta alternativa en la que IRE1 promueve la activación de NF-κB vía JNK (“c-Jun N-terminal Kinase”) (Hasnain et al., 2012), y otra mediada por IKK

(“IκB Kinase”) (Tam et al., 2012). Además, la ruta de ERS puede ser activada por estrés oxidativo, y la activación de esta ruta genera ROS (Malhotra & Kaufman, 2007), los cuales a su vez promueven la activación de JNK y NF-κB, lo que activa la respuesta inflamatoria y la transcripción de componentes del RAS (Chen et al., 2016). Se ha descrito que la señalización Ang-II/AT1 activa ERS (Song et al., 2011; Xu et al., 2009; Young et al., 2012; Young et al., 2015). De forma recíproca, la inducción de ERS a nivel central mediante tunicamicina induce la activación del RAS y la producción de ROS en el RVLM y simpato-excitación (Chao et al., 2013). Sin embargo, las rutas responsables de su activación no se conocen completamente. Se ha propuesto que ROS derivadas de NADPH oxidasa (activada por AT1) induce la activación de PERK e IRE1 (Hasnain et al., 2012), mientras que nuevas investigaciones sugieren que es dependiente de MAP kinasas (Wei et al., 2016b). Sin embargo, la activación de estas quinasas es dependiente de estrés oxidativo (Son et al., 2011), por lo que es posible que las dos señales sean necesarias para activar ERS, sin embargo, dicha hipótesis no ha sido probada aún. Esta evidencia, en conjunto, sugiere a ERS como un nuevo blanco interesante para el tratamiento de HFpEF, dado su activación modularía las respuestas del RAS, inflamación y estrés oxidativo, rutas claves en la hiperactivación de neuronas pre-simpáticas, cruciales en la progresión de HFpEF.

Recientemente, se ha descrito que la infusión de la chaperona química TUDCA (“Tauroursodeoxycholic Acid”) en ratas antes de inducir un infarto agudo al miocardio, mediante ligadura de la arteria coronaria, reduce la disfunción cardiaca, activación de RAS e inflamación en núcleos circunventriculares asociados al control simpático (Wei et al., 2016a), demostrando que la señalización ERS a nivel central contribuiría a la progresión del deterioro de la función cardiaca. Si bien estos resultados son alentadores, no se ha demostrado que TUDCA pueda establecerse como un tratamiento para la HFpEF, principalmente porque su utilización ha sido exclusivamente restringida como agente protector antes del desarrollo de HF, lo cual le resta potencial traslacional. Por lo anterior, y dado que el uso de TUDCA y sus derivados se encuentra actualmente aprobado para el tratamiento de enfermedades no-cardiacas (Vang et al., 2014), es interesante el

estudiar los efectos del tratamiento con TUDCA sobre el control simpático y la función cardiaca una vez desarrollada la patología de la HFpEF. Por otra parte, si bien se ha reportado una fuerte inter-dependencia entre las rutas de ERS y RAS en diversos tejidos (Chen et al., 2016; Xu et al., 2009), aún no se ha descrito si Angiotensina-II induce la activación de ERS a nivel de la RVLM. Resultados previos de nuestro laboratorio muestran que ratas con HFpEF presentan niveles aumentados del receptor AT1, NF-kB y componentes de la ruta ERS como CHOP y la forma procesada de XBP1 (sXBP1). Estos resultados han sido asociados a niveles aumentados de marcadores de activación neuronal como FosB y un aumento en los niveles de estrés oxidativo (Toledo et al., 2017). Experimentos en células CATH.a (modelo de neuronas pre-simpáticas) muestran que la estimulación con Angiotensina-II produce un aumento en la producción de citoquinas pro-inflamatorias, la sobreexpresión de componentes del RAS y producción de especies reactivas del oxígeno (Agarwal et al., 2013; Mitra et al., 2010; Yin et al., 2010). Considerando que tanto Angiotensina-II como las ROS inducen ERS en la RVLM (Chao et al., 2013), y que reportes previos de nuestro grupo muestran que la simpato-excitación en HFpEF se relaciona con un aumento de ROS en la RVLM (Toledo et al., 2017), la cual se asocia a un aumento en la actividad de enzima NADPH-oxidasa 2, un componente río abajo característico del RAS (Andrade et al., 2017b), es altamente posible que exista ERS mediado por Angiotensina-II en la RVLM durante la progresión de la HFpEF, lo cual sería la diana molecular que induciría el aumento en el estrés oxidativo y la inflamación local, resultando en la activación crónica de las neuronas C1, simpato-excitación y deterioro de la función cardiaca en HFpEF.

En resumen, pacientes con insuficiencia cardiaca de fracción de eyección preservada presentan desbalance autonómico y activación crónica del RAS, los cuales participan de forma importante en la progresión de la enfermedad. Reportes de nuestro laboratorio demuestran que la sobre-activación crónica de neuronas pre-simpáticas de la RVLM constituye un mecanismo clave en la simpato-excitación y la incidencia de arritmias en ratas con HFpEF. Investigaciones realizadas en modelos *in vitro* y animales con HFrEF demuestran que la simpato-

excitación en HF es dependiente de la señalización de AT1, que induce estrés oxidativo y la síntesis de citoquinas pro-inflamatorias, promoviendo la sobre-activación neuronal mediante alteraciones en el equilibrio de neurotransmisores en núcleos claves de control simpático como la RVLM. Sin embargo, los mecanismos responsables de la activación neuronal en la RVLM en HFpEF no han sido estudiados con profundidad. Además, investigaciones recientes sugieren que ERS ejerce un rol central en la señalización de RAS, estrés oxidativo e inflamación en HFrEF. El uso de inhibidores de ERS como TUDCA previene la simpato-excitación y disfunción cardiaca en HFrEF, sin embargo, no existen trabajos disponibles en los que se haya investigado la capacidad de esta droga para prevenir la progresión de la enfermedad una vez establecida. Asimismo, se desconoce si la ruta ERS es activa en la RVLM de animales con HFpEF, ni su rol en la progresión de la enfermedad.

HIPOTESIS DE TRABAJO:

"El estrés de retículo endoplásmico en el tronco encefálico promueve la simpato-excitación en ratas con insuficiencia cardiaca con fracción de eyección preservada"

OBJETIVOS:

OBJETIVO GENERAL

Determinar el papel del estrés de retículo en la activación de neuronas pre-simpáticas en la RVLM y su contribución al desbalance autonómico, arritmias y progresión del deterioro de la función cardiaca en ratas con HFpEF.

OBJETIVOS ESPECÍFICOS

Objetivo 1

Determinar la presencia de estrés de retículo endoplásmico en la RVLM de ratas con HFpEF

Racional: Se ha documentado ampliamente la contribución de la señalización de estrés de retículo a nivel cardiaco en HFrEF, sin embargo, los mecanismos moleculares asociados a la activación de esta ruta a nivel central han sido pobremente estudiados y se desconoce su contribución en HFpEF en ambos niveles.

Metodología: Se indujo HFpEF en ratas Sprague-Dawley de forma quirúrgica mediante una fístula aortocaval y se evaluaron los niveles de expresión y activación de marcadores asociados a la respuesta de estrés de retículo (BiP, CHOP, sXBP1), inflamación (TNF-α, IL-1β) y RAS (AT1, NADPH oxidasa, NF-κB) mediante Western blot y RT-qPCR en micropunches de RVLM de ratas HFpEF y pseudo-operadas (Sham). La progresión de la HFpEF se determinó mediante ecocardiografía 4 semanas post-operación y los análisis moleculares fueron realizados a las 8 semanas post-operación.

Objetivo 2

Determinar la contribución del estrés de retículo en la simpato-excitación en ratas con HFpEF

Racional: Se ha documentado que TUDCA previene la activación de RAS y la simpato-excitación administrado de forma crónica previo a la inducción de HFrEF, sin embargo, no se conoce el rol de ERS sobre la simpato-excitación en HfpEF.

Metodología: Se distribuyeron de forma aleatoria ratas Sprague-Dawley en 4 grupos: Sham+vehículo, Sham+TUDCA, HFpEF+vehículo y HFpEF+TUDCA. Se administró TUDCA por vía intracerebroventricular durante 4 semanas en ratas Sham y HFpEF a las 4 semanas post-operación. A las 8 semanas post-fístula u operación Sham (4 semanas post-tratamiento), se evaluó el control simpato-vagal mediante análisis de la variabilidad del ritmo cardiaco a partir de la señal de presión adquirida por telemetría, y se determinaron los niveles de biomarcadores de ERS, RAS y neuroinflamación en micropunches de RVLM, con el fin de

determinar el rol de ERS en la activación neuronal en la RVLM, simpato-excitación en HFpEF.

Objetivo 3

Determinar si la inhibición crónica del estrés de retículo en ratas con HFpEF disminuye la progresión de la patología.

Racional: Se ha demostrado que pacientes y animales con HF presentan desórdenes ventilatorios, hipersibilidad quimiorefleja y desbalance autonómico, sin embargo, no existe ningún estudio señalando el rol de ERS en ninguno de estos parámetros en la progresión de la HfpEF.

Metodología: Se evaluaron parámetros ventilatorios basales, variabilidad ventilatoria y la incidencia de desórdenes ventilatorios (Apneas/hipopneas), así como la sensibilidad quimiorrefleja mediante pletismografía de cuerpo completo y la función cardiaca mediante ecocardiografía en ratas Sham y HFpEF tratadas con TUDCA o vehículo a las 4 semanas (antes de iniciar los tratamientos) y a las 8 semanas post-fístula u operación Sham (4 post-tratamiento) y se evaluó la progresión de la enfermedad mediante los cambios en parámetros ventilatorios basales, sensibilidad quimiorrefleja y parámetros ecocardiográficos basales. A su vez, se medió la función cardiaca en tiempo real de los animales a las 8 semanas mediante la construcción de loops de presión-volumen, con el fin de estudiar el rol de ERS sobre la función cardiaca en HFpEF. A su vez, se determinó el rol de ERS en la arritmogénesis mediante el análisis de eventos arrítmicos a partir de la forma de la onda de la derivada de las señales de presión en los 4 grupos. Asimismo, se determinó el rol de ERS en el acoplamiento cardio-respiatorio mediante el análisis de coherencia entre las señales de ventilación y la presión arterial sistólica así como mediante el análisis de los volúmenes expiratorios.

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3. Capítulo 2: Mecanismos celulares y moleculares asociados a la progresión de la HFpEF

A la fecha no se cuentan con estrategias terapéuticas efectivas para el tratamiento de la HFpEF. Esto es, en gran medida, debido a que existe información limitada en la literatura sobre este síndrome y a que los estudios se han centrado principalmente en el estudio de la insuficiencia cardiaca isquémica (HFrEF). A pesar de ello, grandes avances se han realizado durante los últimos años, y se ha descubierto que las neuronas del tronco encefálico encargadas de controlar la salida simpática hacia el corazón (C1 RVLM) y las neuronas quimiorreceptoras centrales juegan un rol fundamental en el establecimiento del desbalance autonómico y la disfunción cardiorrespiratoria en la insuficiencia cardiaca, sin embargo, los mecanismos celulares y moleculares responsables de la sobre-activación de dichas subpoblaciones neuronales durante la HFpEF son completamente desconocidos. Estudios recientes demuestran que la destrucción de las neuronas C1 RVLM restaura la función cardiaca y autonómica en HFpEF, sin embargo, la contribución directa de las neuronas quimiosensitivas del RTN ha sido pobremente estudiada en el contexto de esta enfermedad.

El segundo capítulo de esta tesis contempla el contexto de los objetivos específicos 1 a 3. Corresponde a 2 artículos científicos: 1. El artículo de la revista American journal of physiology. Lung cellular and molecular physiology (volumen 318) publicado en enero de 2020, titulado “Episodic stimulation of central chemoreceptor neurons elicits disordered breathing and autonomic dysfunction in volume overload heart failure” de **Hugo S. Díaz,** David C. Andrade, Camilo Toledo, Katherin V. Pereyra, Karla G. Schwarz, Esteban Díaz-Jara, Claudia Lucero, Alexis Arce-Álvarez, Harold D. Schultz, Josiane N. Silva, Ana C. Takakura, Thiago S. Moreira, Noah J. Marcus y Rodrigo Del Rio; 2. La revisión sistemática de la revista The Journal of physiology (volumen 598) publicada en enero de 2020, titulada “Neuroinflammation in heart failure: new insights for an old disease” de **H. S. Díaz**, C. Toledo, D. C. Andrade, N. J. Marcus, y R. Del Rio.

En el primer artículo, se estudia el rol de las neuronas quimiosensitivas centrales del núcleo retrotrapezoide (RTN) en el contexto de la fisiopatología de la HFpEF mediante

un paradigma de estimulación repetitiva de los quimiorreflejos centrales mediante hipercapnia. Los principales hallazgos de la publicación son que la estimulación periódica de los quimiorreflejos centrales (EHS) induce desórdenes respiratorios y desbalance autonómico en todos los grupos experimentales, sin embargo, dichos desórdenes fueron mucho más exacerbados en las ratas HFpEF, los cuales disminuyeron marcadamente tras la inyección de la inmunotoxina SSP-SAP, que destruye las neuronas quimiorreceptoras del RTN de forma selectiva. Dichos cambios fueron asociados a una potenciación del quimiorreflejo central en HFpEF, que fue abolida tras la inyección de la toxina. De forma importante, la destrucción de dichas neuronas no sólo previno las alteraciones respiratorias y autonómicas inducidas por EHS, sino que también a nivel basal, las cuales dependen de un acoplamiento entre las neuronas respiratorias (RTN) y las autonómicas (RVLM). Sin embargo, la toxina no tuvo ningún efecto sobre la función cardiaca de los animales en ninguna condición experimental, sugiriendo que la disfunción autonómica y cardiorrespiratoria en HFpEF podría surgir de la interacción entre 2 o más poblaciones neuronales. De hecho, una publicación de nuestro grupo hecha en paralelo demuestra que la destrucción selectiva de las neuronas C1 RVLM restaura la función cardiorrespiratoria y autonómica, pero sin afectar la sensibilidad quimiorrefleja central, sugiriendo una fuerte interacción entre ambas subpoblaciones en la progresión de la enfermedad, al recapitular, de forma selectiva, distintos aspectos fisiopatológicos de la HFpEF. Si bien esta publicación no se relaciona de forma directa con la consecución de los objetivos específicos del trabajo de tesis, éste sienta las bases para la caracterización fisiopatológica del modelo experimental, estableciendo los quimiorreflejos centrales como un nuevo blanco de intervención en el tratamiento de la HFpEF, como se constata en la publicación enviada (detallada en el próximo capítulo).

El segundo artículo corresponde a una revisión sistemática sobre los principales mecanismos celulares y moleculares responsables de la progresión de la HFpEF y HFpEF, así como una comparación de los efectos diferenciales de los mecanismos estudiados en los diferentes síndromes. En dicha revisión se da a conocer la complejidad de los circuitos neuronales responsables de la sobre-activación del sistema nervioso simpático en ambos subtipos de HF y se identifican los principales mecanismos moleculares centrales y periféricos responsables de la progresión de la

enfermedad. Se identifica que en diferentes etiologías de HF existe una sobre-activación selectiva de los RAS centrales en núcleos cerebrales de control del SNS, sin afectar áreas próximas o remotas como la corteza cerebral, la cual se relaciona de forma directa con la disfunción autonómica y cardiaca en HFrEF. Sin embargo, los mecanismos moleculares responsables de la sobreactivación neuronal en HFpEF son completamente desconocidos a la fecha. Nuevas evidencias posicionan a la señalización ERS como un intermediario clave en los efectos fisiopatológicos relacionados con los RAS centrales en diversas patologías cardiacas, por lo que resulta interesante el estudio de esta ruta de señalización en el contexto de la HFpEF.

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RESEARCH ARTICLE

Episodic stimulation of central chemoreceptor neurons elicits disordered breathing and autonomic dysfunction in volume overload heart failure

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Díaz HS, Andrade DC, Toledo C, Pereyra KV, Schwarz KG, Díaz-Jara E, Lucero C, Arce-Álvarez A, Schultz HD, Silva JN, Takakura AC, Moreira TS, Marcus NJ, Del Rio R. Episodic stimulation of central chemoreceptor neurons elicits disordered breathing and autonomic dysfunction in volume overload heart failure. *Am J Physiol Lung Cell Mol Physiol* 318: L27–L40, 2020. First published October 16, 2019; doi:10.1152/ajplung.00007.2019.— Enhanced central chemoreflex (CC) gain is observed in volume overload heart failure (HF) and is correlated with autonomic dysfunction and breathing disorders. The aim of this study was to determine the role of the CC in the development of respiratory and autonomic dysfunction in HF. Volume overload was surgically created to induce HF in male Sprague-Dawley rats. Radiotelemetry transmitters were implanted for continuous monitoring of blood pressure and heart rate. After recovering from surgery, conscious unrestrained rats were exposed to episodic hypercapnic stimulation [EHS; 10 cycles/5 min, inspiratory fraction of carbon dioxide ($F_{I_{CO2}}$) 7%] in a whole body plethysmograph for recording of cardiorespiratory function. To determine the contribution of CC to cardiorespiratory variables, selective ablation of chemoreceptor neurons within the retrotrapezoid nucleus (RTN) was performed via injection of saporin toxin conjugated to substance P (SSP-SAP). Vehicle-treated rats (HFVeh and ShamVeh) were used as controls for SSP-SAP experiments. Sixty minutes post-EHS, minute ventilation was depressed in sham animals relative to HF animals ($\dot{V}_E$: 5.55 2.10 vs. 1.24 1.35 mL/min 100 g, P 0.05; ShamVeh vs. HFVeh). Furthermore, EHS resulted in autonomic imbalance, cardiorespiratory entrainment, and ventilatory disturbances in HFVeh but not ShamVeh rats, and these effects were significantly attenuated by SSP-SAP treatment. Also, the apneahypopnea index (AHI) was significantly lower in HFSSP-SAP rats compared with HFVeh rats (AHI: 5.5 0.8 vs. 14.4 1.3 events/h, HFSSP-SAP vs. HFVeh, respectively, P 0.05). Finally, EHSinduced respiratory-cardiovascular coupling in HF rats depends on RTN chemoreceptor neurons because it was reduced by SSP-SAP treatment. Overall, EHS triggers ventilatory plasticity and elicits cardiorespiratory abnormalities in HF that are largely dependent on RTN chemoreceptor neurons.

breathing disorders; chemoreflex; heart failure; retrotrapezoid nucleus; ventilatory plasticity

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INTRODUCTION

Heart failure (HF) affects more than 20% of the population over 75 yr of age, and its prevalence is expected to double by 2030 (34). Therapeutic management of HF is costly, and prognosis still remains poor as the 5-yr survival rate is ~50% (34). Disordered breathing (3, 11) and autonomic dysfunction (19) are pathophysiological hallmarks of HF, which are associated with deterioration of cardiac function and increased mortality risk (16, 19, 42). Aberrant cardiovascular reflex function, particularly chemoreflexes, is thought to play a crucial role in the development of these autonomic and respiratory disturbances (8, 11, 40). In support of this notion, HF patients display enhanced ventilatory responses to hypoxia and/or hypercapnia (11). Recently, we have shown that volume overload HF rats display enhanced central chemoreflex (CC) gain concomitant with autonomic dysfunction (7, 39). Of note, enhanced central chemoreflex gain was correlated with disordered breathing and augmented sympathetic tone in these animals (39). However, no comprehensive studies addressing a

link between central chemoreceptors and cardiorespiratory alterations in volume overload HF have been conducted.

The neurons that mediate the central chemoreflex (CC) are primarily located in the retrotrapezoid nucleus (RTN) on the ventral medullary surface (14, 26, 40). These neurons respond to changes in CO_2/H (21, 32) and elicit a reflex cardiorespiratory response characterized by increases in ventilation (26) and sympathetic outflow (28). This cardiorespiratory response results from excitatory projections to presympathetic neurons located in the rostral ventrolateral medulla (RVLM) (28, 35), as well as to the respiratory central pattern generator (rCPG) (21, 32). Additionally, activation of central chemoreceptors triggers respiratory synchronous modulation of sympathetic nerve activity, leading to respiratory-sympathetic coupling (13, 14). Ablation of RTN chemoreceptor neurons significantly diminishes the hypercapnic ventilatory response (HCVR) in conscious rats without affecting basal ventilation (26). Considering that volume overload heart failure rats display enhanced central chemoreflex sensitivity, sympathoexcitation, and ventilatory instability, and that the RTN neurons send projections to the RVLM and rCPG, we hypothesized that RTN chemoreceptor neurons play a role in HF pathophysiology.

It has been proposed that episodic stimulation of the central chemoreceptors occurring during apneas leads to exaggerated sympathetic responses (40) and that breathing disorders in patients with HF are a function of chemoreceptor-mediated hyperventilation and subsequent decreases in P_{CO_2} below the apneic threshold (22). Previous work shows that periodic stimulation of CC triggers ventilatory plasticity, characterized by changes in poststimulation normoxic minute ventilation ($\dot{V}_E$) (4, 27). However, the relationship between this phenomenon and disordered breathing patterns is controversial (27), and the effects of episodic stimulation of CC on breathing disorders in HF has not been studied yet. Considering that disordered breathing, sympathoexcitation, and increased HCVR are observed in rats with HF (2, 7, 39, 40), it is plausible that ventilatory plasticity resulting from hypercapnic stimulation contributes to the development and/or exacerbation of these pathophysiological hallmarks. Therefore, in this study we aimed to assess whether ventilatory plasticity, disordered breathing, and autonomic dysfunction were elicited after episodic hypercapnic stimulation in rats with volume overload HF and whether these sequelae were mediated by RTN chemoreceptor neurons.

MATERIALS AND METHODS

Animals. Twenty-four adult male Sprague-Dawley rats (250 12 g) were used for these experiments. Animals were housed in a controlled temperature environment (22–25°C) with a 12-h light-dark cycle and ad libitum access to food and water, in accordance with the guidelines set forth by the American Physiological Society and the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Council of Europe No. 123, Strasbourg 1985). All experimental protocols were approved by the Ethics Committee for Animal Experiments of the Pontificia Universidad Católica de Chile. Experiments were performed 8 wk after induction of HF (Supplemental Fig. S1; all Supplemental Material is available at https://doi.org/10.6084/m9.figshare.9275216.v1). At the end of the experimental protocol, all animals were humanely euthanized with an overdose of anesthesia (pentobarbital sodium 100 mg/kg ip).

Volume overload heart failure model. Rats underwent surgery to produce an arteriovenous (A-V) fistula using the needle technique as previously described (2, 7, 39). Briefly, under anesthesia (Isoflurane: 5% for induction; 1.5% for maintenance balanced with O_2), the inferior vena cava and the abdominal aorta were exposed using a midline incision. Both vessels were clamped caudal to the renal artery and the aortic bifurcation, respectively. The aorta was punctured using an 18-gauge needle and advanced until it perforated the adjacent vena cava. Immediately afterward, a drop of histoacryl glue (BBraun, Germany) was used to seal the aorta at the puncture point. The A-V fistula was confirmed by visualization of bright red arterial blood entering the vena cava through the anastomosis. The peritoneal cavity was closed with absorbable suture (Novosyn 4/0, BBraun, Germany), and the skin was closed with absorbable suture (Novosyn 3/0, BBraun, Germany) and metallic clips (Kent Scientific, Torrington, CT). Postoperative management consisted of administration of 5 mg sc enrofloxacin, 1 mg sc ketoprofen, 5 mL ip saline solution, and 2% topical lidocaine hydrochloride jelly. Sham-operated rats underwent the same anesthesia and surgical procedures without the anastomosis.

Ablation of RTN chemoreceptor neurons. At 4 wk post-HF or Sham surgery, rats were anesthetized (100 mg/kg ketamine and 10 mg/kg xylazine) and fixed to a stereotaxic frame (Supplemental Fig. S1). Bilateral injections of saporin toxin conjugated to substance P (SSPSAP; 0.6 ng/30 nL; Advanced Targeting Systems, San Diego, CA) into the RTN were administered to destroy chemoreceptor neurons, as previously described (10, 31, 37, 38). SSP-SAP dose was selected based on previous studies showing a 50–60% ablation of Phox2bTH neurons (i.e., chemoreceptor units) in the RTN of rats (10, 31, 37, 38). Facial motoneurons, catecholaminergic and serotonergic neurons, and neurons located in the ventral respiratory column caudal to the facial motor nucleus are not affected by these injections (37). Three separate injections of 30 nL of SSP-SAP were placed 2.4 mm caudal to lambda, 1.8 mm lateral to the midline, and 8.5 mm below the dura mater (10, 31, 37, 38) separated by 200 m, using a Hamilton syringe (0.5 L, Sigma, Germany) connected to a 32-gauge injection needle, as previously described (10, 31, 37, 38). Vehicleoperated rats were injected with sterile saline solution. Enrofloxacin (5 mg sc) and ketoprophen (1 mg sc) were administered postsurgery for protection against infection and pain relief, respectively. After surgery, animals were allowed to recover for 2 wk, and physiological experiments were performed 4 wk after SSP-SAP or saline injections (Supplemental Fig. S1).

Echocardiography. At 4 wk post-HF surgery, cardiac function was evaluated under isoflurane anesthesia (5% for induction; 1.5% for maintenance balanced with O_2) using transthoracic echocardiography. M-mode echocardiography was recorded for quantification of cardiac dimensions in the midpapillary muscle region with the parasternal short-axis view using a SonoaceR3 imaging system (Samsung, Korea). Left ventricular end-systolic diameter (LVESd) and left ventricular end-diastolic diameter (LVEDd) were measured using averaged measurements from 3 consecutive cardiac cycles in accordance with guidelines set forth by the American Society of Echocardiography (20). The left ventricular end-systolic volume (LVESv) and left ventricular end-diastolic volume (LVEDv) were calculated using the Teicholz method (2, 39). The following criteria were used for volume overload HF: ejection fraction 50 and end-

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diastolic volume and stroke volume 1.5-fold changes relative to Sham (2, 7, 39). Subsequently, rats were assigned to one of the following experimental groups: Shamvehicle (ShamVeh), ShamSSP-SAP, HFVeh, and HFSSP-SAP (Table 1).

Blood pressure telemetry implantation and assessment. At 7 wk post-HF or Sham surgery, rats were anesthetized with 2% isoflurane in O_2, and a skin incision was made to expose the femoral artery. The tip of a pressure catheter attached to a telemetry transmitter [PA-C40, Data Sciences International (DSI), New Brighton, MN] was guided into the femoral artery, and the transmitter body was placed into a subcutaneous pocket. After surgery the rats received a subcutaneous injection of ketoprofen (1 mg) and enrofloxacin (1 mg). Arterial blood pressure was measured in conscious, freely-moving rats in a whole body plethysmography chamber (Emka Technologies, France) using a radiotelemetry system (DSI). Blood pressure was recorded at a sampling rate of 500 Hz and heart rate was derived from dP/dt of the arterial pressure recordings (5, 7, 39).

Ventilation analyses and episodic chemoreceptor stimulation. Basal ventilation was recorded by unrestrained whole body plethysmography while the rats breathed room air. The input and output flow of the plethysmograph were set to 2.0 L/min (39), and baseline recordings were made for 1 h (prestimulation phase) (8, 15, 24, 39). Respiratory stability at rest was determined by construction of Poincare plots and quantified by analysis of short-term variability (SD1) and long-term variability (SD2) of the breath-to-breath interval variability over 300 consecutive breaths (8, 15, 39). Apneic episodes (cessation of breathing for a duration 3 breathing cycles), hypopnoeas (reductions 50% in V_T amplitude compared with 3 previous normal breaths), sigh frequency (increase 50% in V_T amplitude), and postsigh apneas (cessation of breathing for a duration 3 breathing cycles immediately after the sigh) were averaged during resting breathing, as previously described (8, 15, 39). Apnea, hypopnea, and postsigh apnea duration were quantified as well. Tidal volume (V_T), respiratory frequency (R_f), and minute ventilation (V_E: V_T R_f) were determined by unrestrained whole body plethysmography and analyzed using ECG auto software (Emka Technologies, France) (5, 39). Ten-second segments of stable ventilation (10 2 valid cycles) were used for analysis. Following baseline, animals were subjected to episodic hypercapnic stimulation (EHS) (10 cycles of 7% CO_2/21% O_2 balance N_2, 5 min, spaced by normoxic periods of 5 min). At the termination of EHS, ventilation was recorded under normoxic conditions for 90 min (poststimulation phase) to determine if this paradigm resulted in ventilatory plasticity. Two days before EHS experiments, chemoreflex gain was analyzed by estimating the hypoxic ventilatory response (HVR), calculated by the slope between inspired fraction of oxygen ($F_{I_{O_2}}$) 21% and 10%, and the HCVR, calculated by the slope between inspiratory fraction of carbon dioxide ($F_{I_{CO_2}}$) 0.03% and 7%, as previously described (15, 25, 39). HVR and HCVR were measured during 10-min exposures to either hypoxic or hypercapnic gas challenges. Ventilatory variability as well as apnea incidence were also quantified during the post-EHS phase. All recordings were made at an ambient temperature of 25 2°C, as previously described (39).

Cardiac autonomic function analysis. Cardiac autonomic function was assessed by analysis of heart rate variability (HRV) (2, 7, 8, 15, 39) before and after episodic hypercapnic stimulation. We calculated dP/dt from arterial pressure waveforms to calculate heart rate and applied a Kalman smoothing method before visually inspecting HRV in the time domain. Then, estimations of power spectral density of HRV were obtained for a 10-min window using an autoregressive method after Hann windowing with 50% overlap. Cut-off frequencies were defined as low frequency (0.04–0.6 Hz) and high frequency (0.6–2.4 Hz) (2, 8, 39). Additionally, we used the low frequency-tohigh frequency ratio as an indicator of cardiac autonomic balance. Low frequency and high frequency were expressed as normalized units (n.u.). HRV data analysis was performed using Kubios 3.0.2 software (Finland).

Active expiration analysis. Active expiration was determined as previously described (1, 23). Briefly, 3 segments of respiratory air flow at rest were randomly chosen by a blind operator, and 20 consecutive respiratory cycles were analyzed. Both expiratory time and volume were evaluated. To determine the presence of forced breaths, the expiratory phase was divided into 2 parts: early expiration (E1), corresponding to the initial 50% of the total expiratory time; and late expiration (E2), corresponding to the final 50% of the total expiratory time (1, 23). Increases in the ratio between E2 and E1 expiratory phases (E2/E1) were used as the indicator of active expiration (1). Values of E1 and E2 were obtained by calculations of the area under the first half of the expiratory curve and the area under the second half of the expiratory curve, respectively (1).

Cardiorespiratory coupling analysis. Calculations of coherence between V_T and systolic blood pressure (SBP) signals were assessed before and after episodic hypercapnic stimulation using Matlab software (R2106a version, Natick, MA). Auto- and cross-spectral estimates were computed in 10-min, artifact-free recordings using the Welch's overlapped segment averaging method. A fast Fourier transform (FFT) algorithm was applied to each variable (24). The oscillations in the respiratory signal were taken as the input signal and SBP as the output signal for coherence analysis. The magnitude of the mean square coherence was assessed over a range of 0.1 Hz centered at the frequency of the maximum V_T spectral peak in the lowfrequency domain (i.e., breathing oscillations) (24).

Measurement of arterial blood gases. Arterial blood gases were measured in conscious, freely moving rats (n 4 per group) using a blood gas analyzer (iSTAT1 CG8, Abbott). Under isoflurane (2%),

Table 1. *Echocardiographic parameters at 4 wk post-Sham or HF surgery*

	ShamVeh (n 6)	ShamSSP-SAP (n 6)	HFVeh (n 6)	HFSSP-SAP (n 6)
LVEDV, L	273.70 35.58	300.20 17.90	383.3 27.20*	353.00 10.72
SV, L	208.10 18.78	236.40 14.77	323.40 31.58*	302.00 31.96*
EF, %	77.67 3.53	72.85 1.10	80.10 6.34	81.18 7.07
FS, %	48.23 3.22	43.40 0.96	49.92 7.82	57.68 12.10

Values are mean SE; n 6 rats per group. EF, ejection fraction; FS, fractional shortening; HF, heart failure; LVEDV, left ventricular end-diastolic volume; SSP-SAP, substance P-saporin toxin; SV, stroke volume. One-way ANOVA, followed by Holm-Sidak post hoc analysis. *P 0.05 vs. ShamVeh.

rats were anaesthetized and a vascular access port was placed in the right carotid artery. One week after surgery, animals were put in a whole body plethysmograph and 100 L of blood were withdrawn at baseline and at 60 min post-EHS. Samples were analyzed immediately, and the volume of blood withdrawn was immediately replaced by an equal volume of sterile saline solution (39) (Supplemental Table S1).

Immunofluorescence. After physiological experiments, rats were deeply anesthetized with urethane (1.8 g/kg iv) then perfused through the ascending aorta with 150 mL of PBS (pH 7.4) followed by 4% paraformaldehyde (0.1 M; pH 7.4) (Sigma, Germany). The brain was removed and stored in the perfusion fixative for 24–48 h at 4°C. Using a vibrating microtome, a series of brain coronal sections (40 m) were cut and stored in cryoprotectant solution at 20°C (20% glycerol plus 30% ethylene glycol in 50 mM phosphate buffer, pH 7.4) before histological processing. All histochemical procedures were done using free-floating sections (37). The percentage of neurons eliminated after the injection of the SSP-SAP toxin in the RTN (12.36 to 11.0 to bregma) was determined by immunofluorescence. Tyrosine hydroxylase (TH) was detected using mouse antibody (1: 2,000, Chemicon, Temecula) and Phox2b with a rabbit antibody (1:800, gift from J.-F. Brunet, Ecole Normale Supérieure, Paris, France). These primary antibodies were detected by incubation with appropriate secondary antibodies tagged with fluorescent reporters to reveal TH (goat anti-mouse Alexa 488, Invitrogen, Carlsbad, CA) and Phox2b (donkey anti-rabbit Cy3, Jackson, West Grove, PA). The images were acquired with a high-resolution epifluorescence Leica microscope. The images were quantified using ImageJ software through the content of neurons Phox2b TH (National Institutes of Health, Betheseda, MD). Cells were counted using a computerassisted mapping technique based on the Neurolucida software as previously described (37). The Neurolucida files were exported to the NeuroExplorer software (MicroBright-field, Colchester, VT) to count the various types of neuronal profiles within a defined area. Images were captured with a SensiCam QE 12-bit CCD camera (resolution 1,376 1,040 pixels; Cooke, Auburn Hills, MI). IPLab software (Scanalytics, Rockville, MD) was used for merging of color channels in photographs of dual labeling experiments. Immunofluorescence analyses were performed in the same rats on which physiological experiments were performed.

Data analysis. GraphPad Prism 8.0 statistical software (La Jolla, CA) was used to analyze the data. Normal distribution of the data was assessed with the D'Agostino-Pearson test. The statistical significance of the data with normal distribution was evaluated using the one-way ANOVA or two-way ANOVA parametric test, followed by a Sidak post hoc analysis. Statistical significance of nonnormally distributed data was evaluated using nonparametric one-way ANOVA or twoway ANOVA, followed by a Dunn post hoc analysis. The level of significance was defined as P 0.05. Results were shown as mean SE in text and tables and median range in figures.

RESULTS

SSP-SAP toxin selectively destroys chemoreceptor neurons of the RTN. We used substance P-conjugated saporin toxin (SSP-SAP), which destroys Neurokinin 1 (NK1)-positive neurons (expressed by chemoreceptor neurons within the RTN) (38). SSP-SAP toxin or vehicle were injected bilaterally into the RTN of Sham and HF rats according to stereotaxic coordinates. According to previous evidence, Phox2b is predominantly expressed by CO_2-activated neurons in the RTN (10, 31, 37, 38). Four weeks after treatment, we determined the number of RTN neurons eliminated by SSP-SAP by counting the number of RTN neurons that expressed Phox2b and were devoid of tyrosine-hydroxylase immunoreactivity (Phox2bTH). The percentage of destruction of Phox2bTH neurons was 45 16% and 50 14% in ShamSSP-SAP and HFSSP-SAP rats, respectively, when compared with paired animals that received vehicle injections (Fig. 1). No changes were observed in TH immunoreactivity in the proximity of the

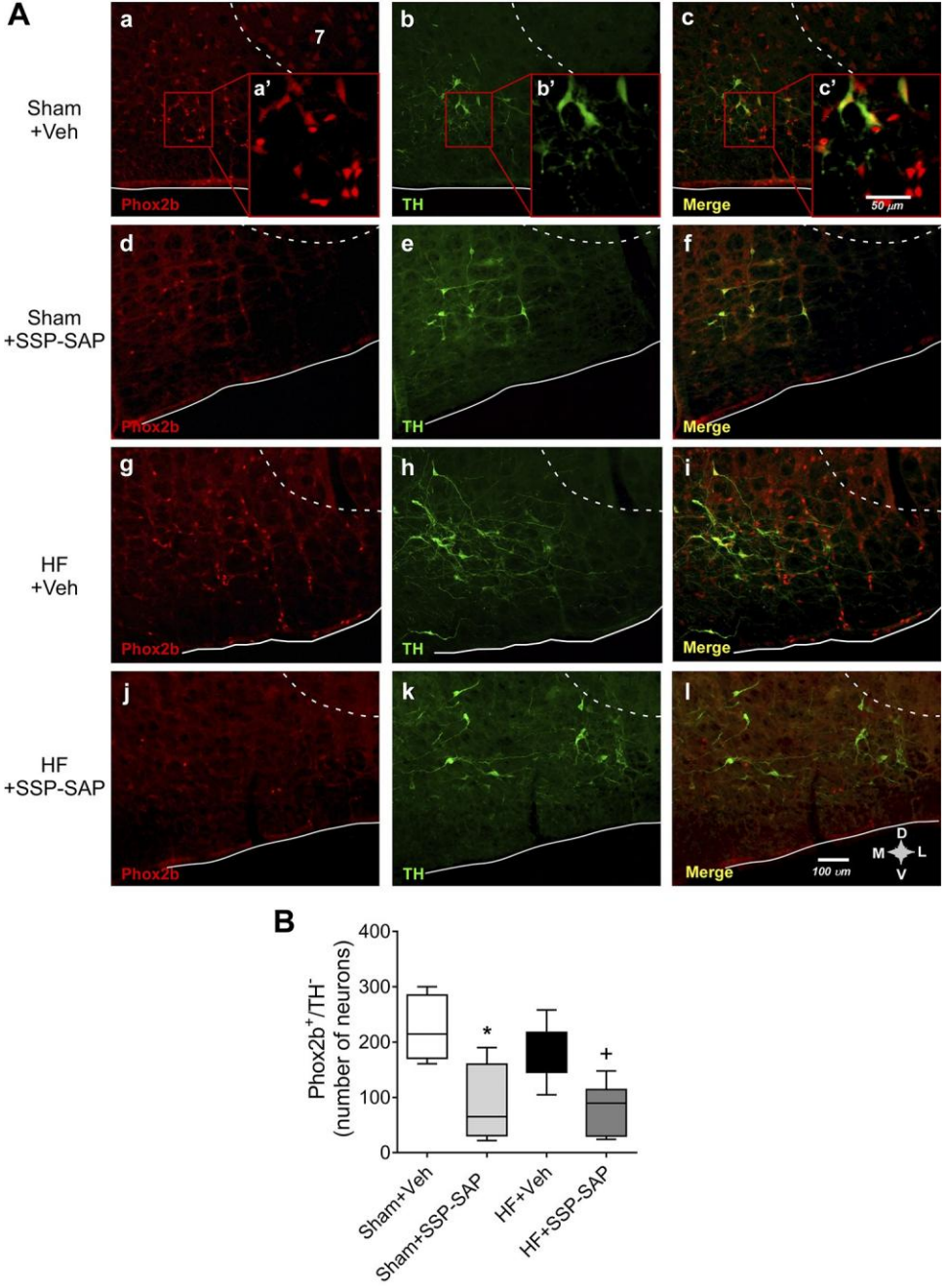

Fig. 1. Substance P-conjugated saporin (SSP-SAP) toxin destroys Phox2b-positive neurons but not tyrosine-hydroxylase (TH)positive neurons within the retrotrapezoid nucleus (RTN). *A*: representative images of histological sections (40 m) of the ventral surface of the brainstem (bregma level: 11.6 mm) of rats receiving SSP-SAP or vehicle (0.9% NaCl) in the RTN. *B*: quantification of the total number of Phox2b TH neurons in Sham and heart failure (HF) rats treated with vehicle or with the SSPSAP toxin. One-way ANOVA followed by the Holm-Sidak post hoc test. Box and whiskers represent median range. **P* 0.05 vs. ShamVeh *P* 0.05 vs. HFVeh; *n* 6 rats per group.

injection site. Also, no changes were observed in Phox2b immunoreactivity in other brainstem respiratory sites (i.e., Facial, pre-Bötzinger, Bötzinger).

Baseline cardiorespiratory parameters after SSP-SAP treatment. Baseline hemodynamic and ventilatory data at rest are shown in Table 2. Compared with ShamVeh and HFVeh animals, we found that SSP-SAP-treated animals showed no significant change in resting mean blood pressure (100.01 4.39 vs. 96.50 4.95 mmHg, ShamVeh vs. ShamSSP-SAP, respectively; 96.01 3.8 vs. 92.01 7.76 mmHg, HFVeh vs. HFSSP-SAP, respectively) or heart rate (298.20 13.63 vs. 299.50 14.69 beats/min, ShamVeh vs. ShamSSP-SAP, respectively; 331.4119.33 vs. 285.40 19.63, HFVeh vs. HFSSP-SAP, respectively). No changes in resting V_T or respiratory rate were found between groups (Table 2). Additionally, no significant changes in baseline hemodynamic parameters were observed after EHS (Supplemental Table S2).

Episodic hypercapnic stimulation triggers ventilatory plasticity in heart failure. EHS resulted in ventilatory long-term depression in Sham rats, whereas this response was absent in HF rats (Fig. 2). Indeed, HF rats showed augmented ventilation until 90 min after EHS. Both $\dot{V}_E$ and R_f values were significantly higher in HFVeh animals compared with ShamVeh animals after EHS (Fig. 2, *C–E*) ($\dot{V}_E$: 5.51 2.10 vs.

1.24 1.35 mL/min 100 g; R_f: 10.4 3.28 vs. 2.55 2.42 breaths/min, respectively). Ablation of RTN neurons blunted EHS-induced changes in ventilation in HF rats (Fig. 2, *A–E*). We did not find significant changes in arterial blood gases before or after EHS in all groups (Supplemental Table S1).

To determine whether there was an association between post-EHS, ventilatory plasticity, and central chemoreflex sensitivity in HF, we measured the HCVR (Fig. 3, *A* and *C*). HCVR was significantly higher in HF rats compared with Sham (5.5 0.4 vs. 4.1 0.1 $\dot{V}_E/F_{I_{CO_2}}$%, respectively), and the enhanced HCVR was reduced by SSP-SAP in HF animals (2.0 0.6 vs. 5.5 0.4 $\dot{V}_E/F_{I_{CO_2}}$%, HFVeh vs. HFSSP-SAP, respectively). We found no significant differences in the HVR in any experimental group (Fig. 3, *B* and *D*).

Ablation of RTN chemoreceptor neurons attenuates disordered breathing in heart failure rats. Rats with HF displayed marked alterations in resting breathing patterns in normoxia (Fig. 4*A*). HFVeh rats showed an increase in the breath-to-breath interval variability compared with ShamVeh animals (Fig. 4, *B–F*) (SD2: 71.5 5.4 vs. 53.2 7.9 ms) and in the coefficient of variation of V_T (12.9 1.7 vs. 8.7 0.6%, respectively) (Fig. 4, *D* and *F*). SSP-SAP treatment attenuated the alterations observed in resting normoxic breathing patterns in HF rats (Fig. 4). Exposure to EHS exacerbated breathing instability in HF rats but had no effect in other groups (Fig. 4). The coefficient of variation of V_T increased 2-fold after EHS compared with baseline (12.9 1.7 vs. 20.3 1.5%, HFVeh pre- vs. post-EHS, P 0.05). SSP-SAP treatment in HF completely abolished the deleterious effects of EHS on breathing stability (Fig. 4).

In addition to abnormalities in ventilatory patterns, we observed a higher incidence of apneas and hypopneas [apnea-hypopnea index, (AHI)] in HF compared with Sham Veh rats (9.0 1.3 vs. 4.7 0.5 events/h, P 0.05, respectively). SSP-SAP treatment reduced AHI in HF rats (9.0 1.3 vs. 4.3 0.8 events/h, HFVeh vs. HFSSPSAP rats, P 0.05), and the EHS-induced increase in AHI in HF rats was prevented by SSP-SAP treatment in HF

(Table 3).

Effects of RTN chemoreceptor neuron ablation on EHSdependent cardiac autonomic imbalance in heart failure. At baseline, HF rats displayed an increase in the low-frequency component of HRV (LF_{HRV}) and a decrease in the highfrequency component of HRV (HF_{HRV}) compared with Sham rats (Fig. 5). These effects were attenuated by ablation of RTN chemoreceptor neurons in HF rats (Fig. 5, *A–E*). After EHS, changes in cardiac autonomic balance were observed in both Sham and HF rats and were characterized by additional increases in the LF_{HRV} component and a decrease in the HF_{HRV} component (Fig. 5, *A–E*). Accordingly, the LF_{HRV}-to-HF_{HRV} ratio, an indirect measure of cardiac autonomic balance, was significantly increased (P 0.05) by EHS in ShamVeh (1.1 0.1 vs. 2.2 0.2, prevs. post-EHS;) and in HFVeh rats (2.0 0.2 vs. 3.6 0.7, pre- vs. post-EHS;); however, the effect of EHS on cardiac autonomic imbalance was larger in HF animals (Fig. 5*E*). Selective ablation of RTN chemoreceptor neurons significantly attenuated the EHS-induced changes in cardiac autonomic balance in HF (Fig. 5).

Active expiration in heart failure rats and effects of RTN chemoreceptor neuron ablation. Compared with Sham rats, HF rats displayed active expiration in normoxia as evidenced by an increase in the early-to-late expiration ratio (E2/E1) (0.89

Table 2. *Effect of substance P-saporin toxin on baseline cardiores*

	ShamVeh (*n* 6)	ShamSSP-SAP (*n* 6)	HFVeh (*n* 6)	HFSSP-SAP (*n* 6)
Hemodynamic				
SBP, mmHg	124.20 4.57	120.20 6.63	114.60 6.62	115.80 9.78
DBP, mmHg	88.01 4.57	84.83 4.14	87.01 2.86	80.01 6.80
MABP, mmHg	100.01 4.39	96.50 4.95	96.01 3.84	92.01 7.76
PP, mmHg	36.01 2.47	34.67 2.75	27.60 4.78	35.01 3.56
HR, beats/min	298.20 13.63	299.50 14.69	331.41 19.33	285.40 19.63
Ventilatory				
V_T, mL/100 g	0.31 0.03	0.25 0.01	0.32 0.02	0.26 0.01
R_f, breaths/min	85.52 2.76	72.58 2.76	85.79 2.70	81.16 2.30
V_E, mL·100 g^{1}·min^{1}	24.65 1.29	19.35 1.03	22.62 1.78	20.26 1.28

Values are mean SE; *n* 6 rats per group. DBP, diastolic blood pressure; HF, heart failure; HR, heart rate; MABP, mean arterial blood pressure; PP, pulse pressure; R_f, respiratory frequency; SBP, systolic blood pressure; SSP-SAP, substance P-saporin toxin; V_E, minute ventilation; V_T, tidal volume. One-way ANOVA followed by the Holm-Sidak post hoc test.

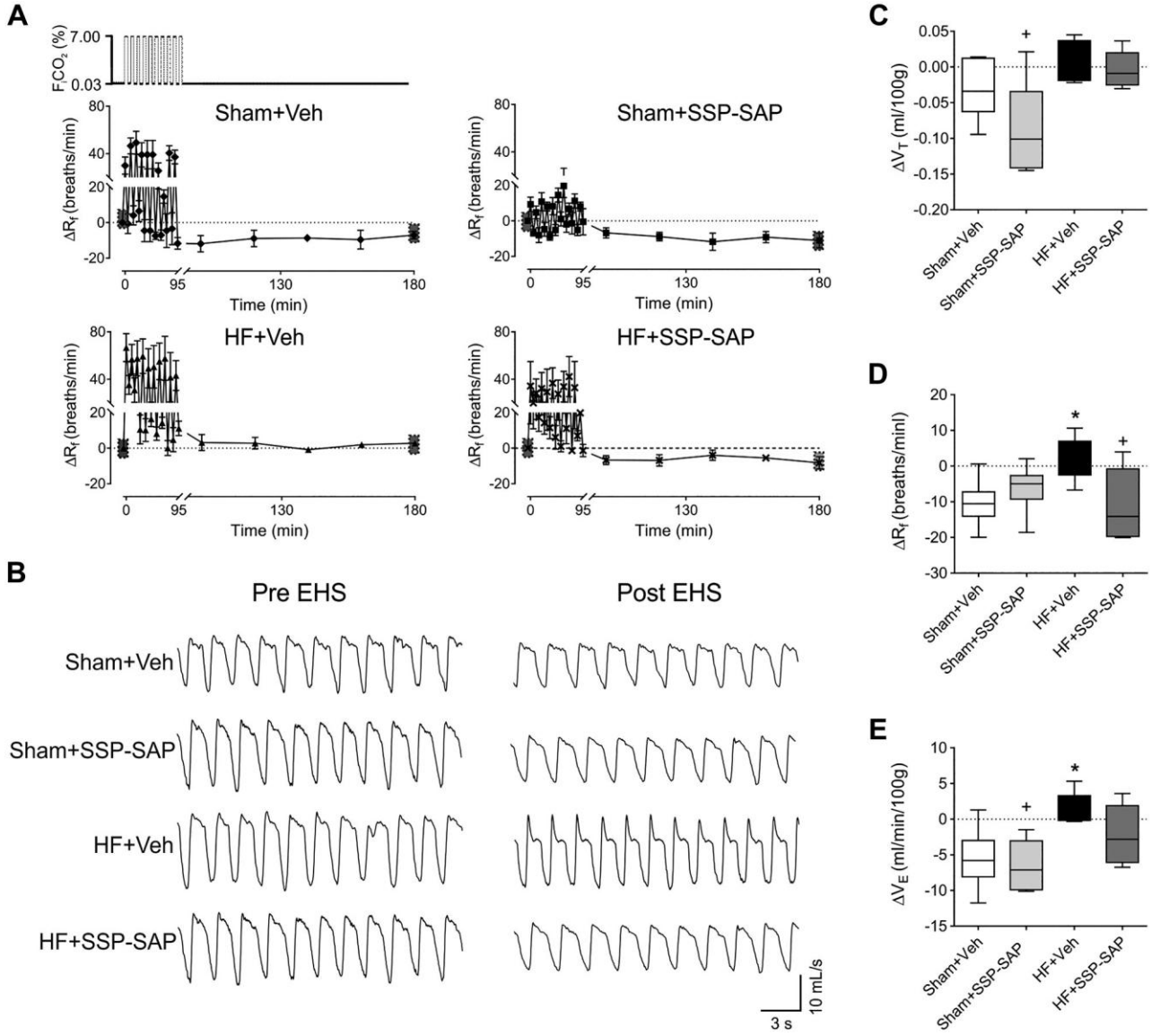

Fig. 2. Ventilatory plasticity induced by episodic hypercapnic stimulation in heart failure (HF) is retrotrapezoid nucleus (RTN) chemoreceptor neuron-dependent. *A*: schematic of episodic hypercapnic stimulation (EHS) paradigm (*top panel*). Effect of EHS on ventilation (R_f) during pre- and post-EHS phases. Note that during the post-EHS phase, ShamVeh rats showed ventilatory depression, and this effect was absent in HFVeh rats. Selective ablation of RTN neurons by substance P-conjugated saporin (SSP-SAP) toxin normalizes the post-EHS ventilatory response in HF rats. *B*: representative traces of ventilation pre- and post-EHS in all experimental groups. *C–E*: summary data of tidal volume (V_T), respiratory frequency (R_f), and minute ventilation (V_E) during post-EHS phase,

respectively. Data represent mean SE (*A*) while box and whiskers represent median range (*C–E*). One-way ANOVA followed by Holm-Sidak post hoc test; *n* 6 rats per group. **P* 0.05 vs. ShamVeh; *P* 0.05 vs. HFVeh.

0.19 vs. 0.68 0.09 HFVeh vs. ShamVeh, respectively, Fig. 6, *A* and *B*). In HF rats, partial elimination of RTN chemoreceptor neurons nearly eliminated active expiration, mainly by decreasing the late expiratory phase without significant changes in the early expiratory phase (E2: 0.05 0.01 vs. 0.08 0.01 mL, HFVeh vs. HFSSP-SAP, respectively). After EHS, active expiration was exacerbated in HF rats, as evidenced by the large increase in E2/E1 (0.89 0.19 vs. 1.24 0.24, HFVeh pre-EHS vs. HFVeh post-EHS, respectively, Fig. 6, *A* and *B*). The effect of EHS on the increase of E2/E1 in HF rats was dependent on the integrity of RTN chemoreceptor neurons because SSP-SAP treatment in HF rats resulted in a significant decrease in E2/E1 (1.24 0.19 vs. 0.55 0.07, HFVeh post-EHS vs. HFSSP-SAP post-EHS, respectively, Fig. 6, *A* and *B*). No significant changes in the total expiratory time were observed in any of the experimental groups (Fig. 6*C*).

Ablation of RTN chemoreceptor neurons attenuates EHSdependent cardiorespiratory coupling in rats with heart failure. Coupling between cardiac autonomic function and ventilation was determined by calculating the coherence between oscillations in the V_T and SBP. Cardiorespiratory coupling was increased (i.e., increased coherence) by EHS only in HF rats (Fig. 7), and SSP-SAP treatment blunted the EHS-induced increase in cardiorespiratory coupling in HF animals (Fig. 7). EHS had no effects on cardiorespiratory coupling in ShamVeh or ShamSSP-SAP-treated rats.

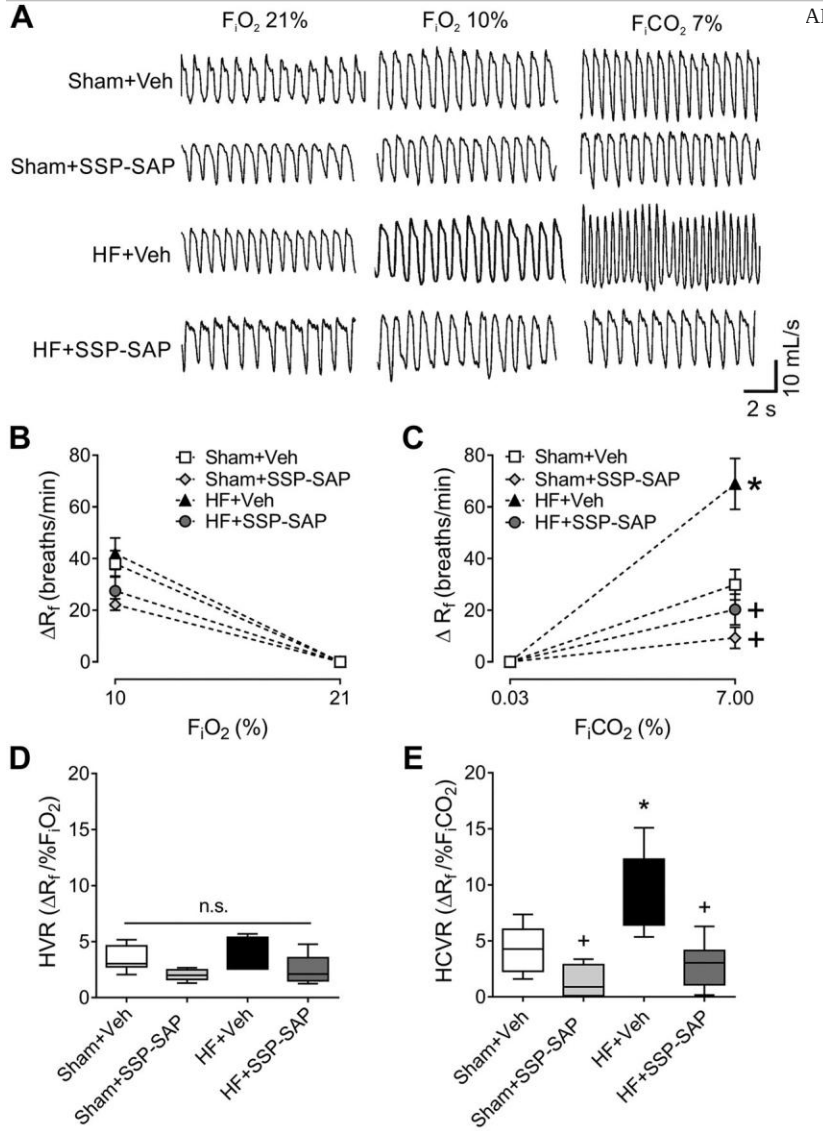

Fig. 3. Selective ablation of retrotrapezoid nucleus (RTN) chemoreceptor neurons blunts the hypercapnic ventilatory response (HCVR). *A*: representative traces of ventilation during normoxia (F_{IO_2} 21%), hypoxia (F_{IO_2} 10%), and hypercapnia ($F_{I_{CO^2}}$ 7%). *B* and *C*: respiratory frequency (R_f) during hypoxia (*B*) and the hypoxic ventilatory response (HVR) (*C*) were not different between groups. *D* and *E*: the respiratory frequency (R_f) during hypercapnia (*D*) and the hypercapnic ventilatory response (HCVR) (*E*) were increased in heart failure (HF) rats and substance P-conjugated saporin (SSP-SAP) toxin reduced both. Data represent mean SE (*B*, *C*), and box and whiskers represent median range (*D*, *E*). One-way ANOVA followed by Holm-Sidak post hoc test; *n* 6 rats per group. **P* 0.05 vs. ShamVeh; *P* 0.05 vs. HFVeh. n.s., not significant.

DISCUSSION

The main aim of this study was to assess whether acute episodic stimulation of chemoreceptors with hypercapnia in the setting of volume overload HF could elicit ventilatory plasticity, leading to the onset of irregular breathing patterns and autonomic imbalance (3, 7, 11, 18). We have previously proposed that periodic stimulation of chemoreflex pathways may play a major role in the maladaptive cardiorespiratory changes associated with HF (40). In this study, we show that episodic stimulation of the central chemoreflex elicits ventilatory long-term depression in normal rats, which was not observed in HFVeh animals. Importantly, this phenomenon was associated with the development of irregular breathing patterns, increased AHI, and exacerbation of cardiac autonomic imbalance in HF rats. Furthermore, our data show that these responses are largely dependent on RTN chemoreceptor neurons, since their selective destruction using SSP-SAP blunted ventilatory changes and EHS-induced breathing/autonomic disturbances in HF animals. Ventilatory long-term depression in response to hypercapnia in healthy rats has previously been reported (4); however, those experiments were performed in anesthetized, vagotomized, and mechanicallyventilated animals (4). This is the first study to address the effects of EHS on ventilatory patterns and autonomic function in unrestrained rats and the first to address these parameters in the setting of volume overload HF. The precise mechanisms responsible for EHS-induced long-term depression of ventilation have not been thoroughly studied; however, our results suggest that central chemoreceptors play a pivotal role in this phenomenon. Indeed, the normal long-term depression of ventilation observed after EHS was absent in HFVeh rats, concomitant with enhanced CC sensitivity. More importantly, ablation of RTN chemoreceptor neurons attenuated abnormal ventilatory responses to EHS in HF rats.

Central chemoreceptors and heart failure. There are numerous putative sites for central chemoreception in the brain; however, the medullary RTN is thought to be a primary region. Chemoreceptor neurons within the RTN are activated by changes in cerebrospinal fluid levels of CO_2/H and send projections to the rCPG (21, 32, 40) and the RVLM (28, 35),

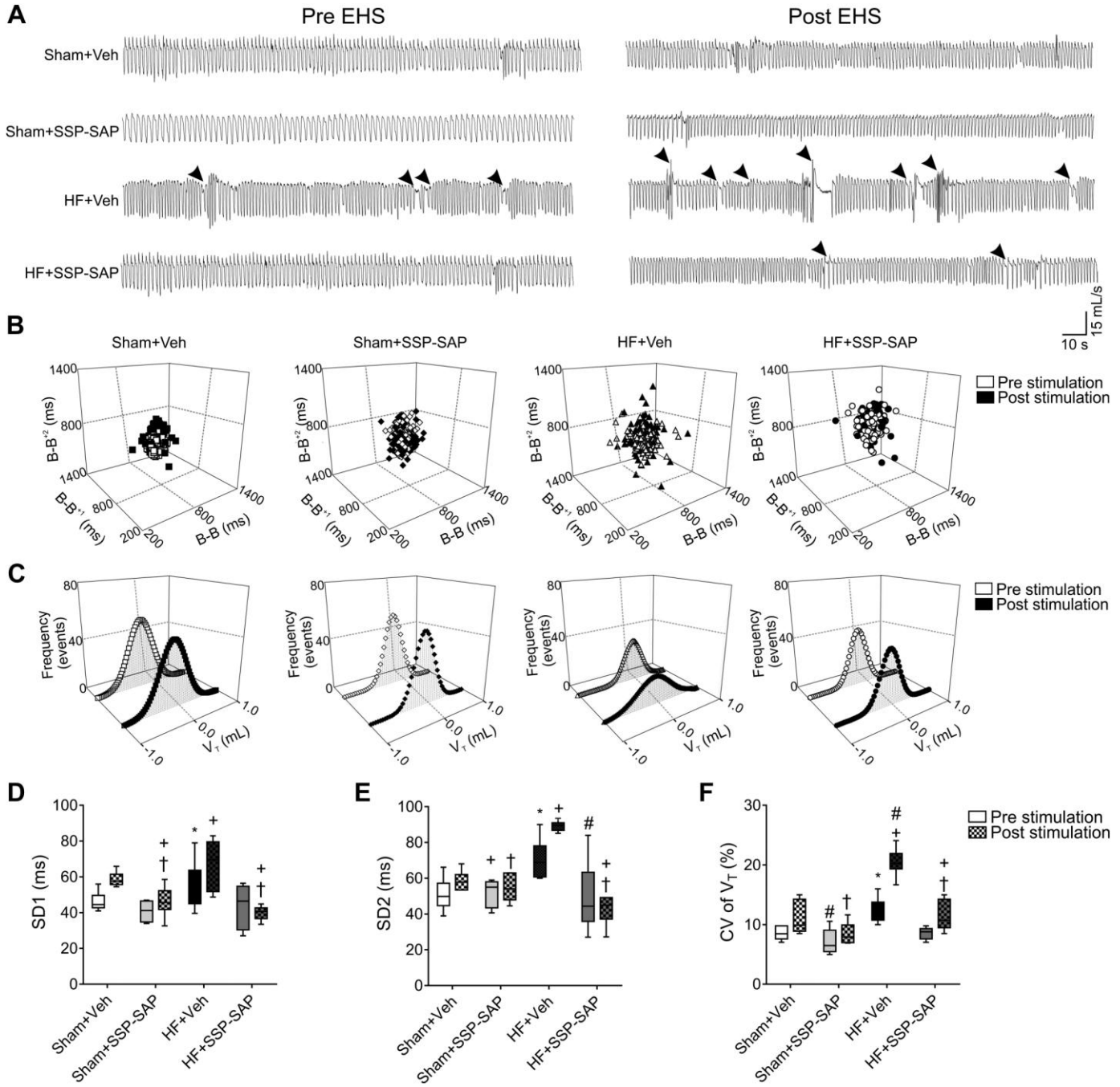

Fig. 4. Selective ablation of retrotrapezoid nucleus (RTN) chemosensory neurons prevents ventilatory disturbances elicited by episodic hypercapnic stimulation. *A*: representative traces of ventilation during pre- and post-episodic hypercapnic stimulation (EHS) phases. Arrows point to disturbances in breathing patterns such as apneas/hypopneas. *B* and *C*: representative Poincaré plots and histograms during pre- and post-EHS phases. *D* and *E*: summary data of short-term (SD1) (*D*) and long-term variability (SD2) (*E*), and coefficient of variation (CV) of tidal volume (V_T) (*F*) during pre- and post-EHS phases. Substance P-conjugated saporin (SSP-SAP) toxin injection in the RTN diminished breath-to-breath and V_T amplitude variability in the post-EHS phase in heart failure (HF) rats. Box and whiskers represent median range. Two-way ANOVA followed by Holm-Sidak post hoc tests; *n* 6 rats per group. **P* 0.05 vs. ShamVeh Pre; #*P* 0.05 vs. HFVeh Pre; *P* 0.05 vs. ShamVeh Post; †*P* 0.05 vs. HFVeh Post.

the latter being considered a major nodal point for integration of sympathetic drive. Upon activation, the RTN initiates reflex increases in respiratory rate and sympathoexcitation (14). Additionally, activation of the RTN induces cardiorespiratory coupling, a phenomenon that has been linked to chronic sympathoexcitation (13). In addition to their contribution to CO_2 homeostasis, there is evidence that central chemoreceptor function is altered in disease states. Indeed, augmented central chemoreflex sensitivity, breathing disturbances, and autonomic dysfunction are commonly observed in patients with HF (11, 19, 29, 40, 42). Clinical studies in HF patients show a positive correlation between CO_2 chemosensitivity and AHI (36), and that hypercapnic stimulation elicits an increase in sympathetic nerve activity in HF patients but not in healthy ones. Further-

Table 3. *Effect of substance P-saporin toxin on breathing disorders following episodic hypercapnic stimulation*

	ShamVeh (*n* 6)		ShamSSP-SAP (*n* 6)		HFVeh (*n* 6)		HFSSP-SAP (*n* 6)	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Spontaneous apnea, events/h	3.4 0.4	4.3 0.4	3.3 0.8	4.0 0.7	5.4 0.9*	7.8 0.9	2.5 1.3	3.7 1.1
Hypopneas, events/h	1.2 0.4	1.6 0.4	0.9 0.2	1.8 0.4	3.7 1.1*	5.8 1.3	1.7 0.4	2.2 0.7†
Apnea/hypopnea index, events/h	4.7 0.5	6.0 1.1	5.0 0.4	6.0 1.1	9.0 1.3*	14.4 1.3#	4.3 0.8#	5.5 0.8†
Spontaneous apnea duration, s	3.2 0.3	3.0 0.2	2.9 0.1	3.0 0.2	3.2 0.2	3.1 0.3	3.5 0.3	4.0 0.6
Hypopneas duration, s	2.9 0.2	3.0 0.1	3.0 0.2	2.5 0.1	2.7 0.1	2.5 0.2	2.5 0.4	2.6 0.2
Sights, events/h	12.8 1.2	13.6 1.1	14.8 2.6	18.5 2.5	16.9 2.8	16.8 2.3	14.0 1.2	14.0 0.6
Post sight apnea, events/h	12.0 0.4	12.1 1.9	12.1 0.4	12.0 1.6	14.0 2.1	13.3 2.7	11.0 0.6	10.0 1.2
Post sight apnea duration, s	4.5 0.2	4.4 0.2	3.9 0.2	4.3 0.1	4.2 0.3	3.8 0.2	4.4 0.2	4.3 0.3†

Values are mean SE; *n* 6 rats per group. AHI, apnoea-hypopnoea index; EHS, episodic hypercapnic stimulation; HF, heart failure; Pre, previous to EHS; Post, 90 min post EHS; SSP-SAP, substance P-saporin toxin. Two-way ANOVA followed by Holm-Sidak post hoc analysis. **P* 0.05 vs. ShamVeh Pre; #*P* 0.05 vs. HFVeh Pre; *P* 0.05 vs. ShamVeh Post; †*P* 0.05 vs. HFVeh Post.

more, our previous studies in animal models of HF strongly support the notion that enhanced central chemoreflex sensitivity contributes to the development of disordered breathing and sympathoexcitation (39). Based on these findings, we hypothesized that acute episodic stimulation of central chemoreceptors, which may occur clinically as a result of disordered breathing patterns in HF, may exacerbate ventilatory instability in part via induction of ventilatory plasticity.

Central chemoreceptors and disordered breathing patterns in heart failure. From a clinical perspective, ~40% of HF patients have central sleep apnea, combined with a higher HCVR (18, 36). The proposed relationship between these two phenomena is a high resting minute-ventilation resulting in CO_2 'wash-out' from central chemosensitive areas and closer resting proximity to the apneic threshold (38). We observed that EHS in HF rats resulted in augmented ventilation, in contrast to Sham rats, in which EHS elicited ventilatory longterm depression. Of note, we found that selective ablation of RTN chemoreceptor neurons using SSP-SAP in HF rats prevented the effects of EHS but did not have a similar effect in Sham rats. In addition, our results show that SSP-SAP injection into the RTN reduced breathing instability at rest in HF rats under baseline conditions. These results suggest a prominent role for RTN chemoreceptor neurons in shaping resting breathing patterns in HF before and after hypercapnic challenges. These findings confirm and extend previous reports showing that RTN chemoreceptor neurons regulate normoxic ventilation (10, 31, 37, 38, 41). Together, these results suggest a crucial role for RTN chemoreceptor neurons in the regulation of breathing patterns in HF, suggesting that RTN neurons are a plausible therapeutic target to reduce resting hyperventilation and decrease AHI in HF. Future studies should focus on the precise mechanisms underlying RTN chemoreceptor neurons and regulation of breathing in HF.

Central chemoreceptors and cardiac autonomic dysfunction in heart failure. Three major brain nuclei have been linked to sympathoexcitation in HF: the hypothalamic paraventricular nucleus (PVN), the nucleus of the solitarii tract (NTS), and the RVLM (19, 42), with the RVLM being the major regulatory area for sympathetic outflow (13, 33). We have previously shown that enhanced central chemoreflex gain is associated with increased Fos B labeling in the RVLM of HF rats, suggesting a link between central chemoreceptors and chronic neuronal activation in presympathetic regions of the brainstem (39). Given that putative connections exist between the RTN and the RVLM (28, 35), we hypothesized that EHS of central chemoreceptors would enhance cardiac sympathetic tone. In this study, EHS increased cardiac sympathetic tone in both healthy and HF conditions, but the effect was larger in rats treated with vehicle compared with those that received SSPSAP injections. Importantly, SSP-SAP injection into the RTN of HF rats attenuated the higher cardiac sympathetic tone elicited by EHS. Furthermore, SSP-SAP not only reduced the EHS-induced sympathetic response in HF, but also normalized baseline cardiac autonomic imbalance (LF_{HRV}-to-HF_{HRV} ratio) before application of EHS. Importantly, SSP-SAP treatment did not affect catecholaminergic neurons surrounding the RTN in either Sham or HF animals, which play an important role in regulating sympathetic outflow to the heart (28, 35); thus, improvements in autonomic control observed in HFSSPSAP are unlikely to be related to an off-target effect of SSP-SAP treatment. Taken together, these results suggest that RTN chemoreceptor neurons play a role in cardiac autonomic regulation in HF, probably by regulating stimulation of presympathetic neurons in the RVLM. Although RTN neurons make synaptic connections with NTS neurons (13, 28, 35), previous studies indicate that acute inhibition of the NTS has no significant effects on sympathetic activity or central chemoreflex function (28). Therefore, a putative RTN-RVLM connection as well as its role in HF progression deserves further investigation.

Active expiration is commonly associated with a concomitant increase in sympathetic outflow (1). Indeed, the presence of both active expiration and sympathoexcitation has been linked to cardiorespiratory dysfunction (30). We found that HF rats displayed active expiration at rest during eupneic conditions, suggesting the recruitment of abdominal muscles during resting breathing. Importantly, it has been shown that stimulation of RTN neurons results in activation of expiratory muscles and active expiration (17). In our studies we found that SSP-SAP treatment abolished active expiration in HF rats in normoxic conditions. These results strongly support the notion that RTN chemoreceptor neurons are required for the maintenance of active expiration in the setting of volume overload HF. Future studies

using EMG recordings are needed to fully assess the presence HF rats. This was dependent on RTN chemoreceptor neurons and contribution of the RTN on active expiration in high-output because SSP-SAP completely eliminated the EHSinduced active HF (17). In addition to our observations of the effects of HF on expiration. Lastly, we showed that EHSinduced active expiration active expiration during resting breathing, we found that EHS occurs in tandem with cardiac autonomic imbalance in HF rats.

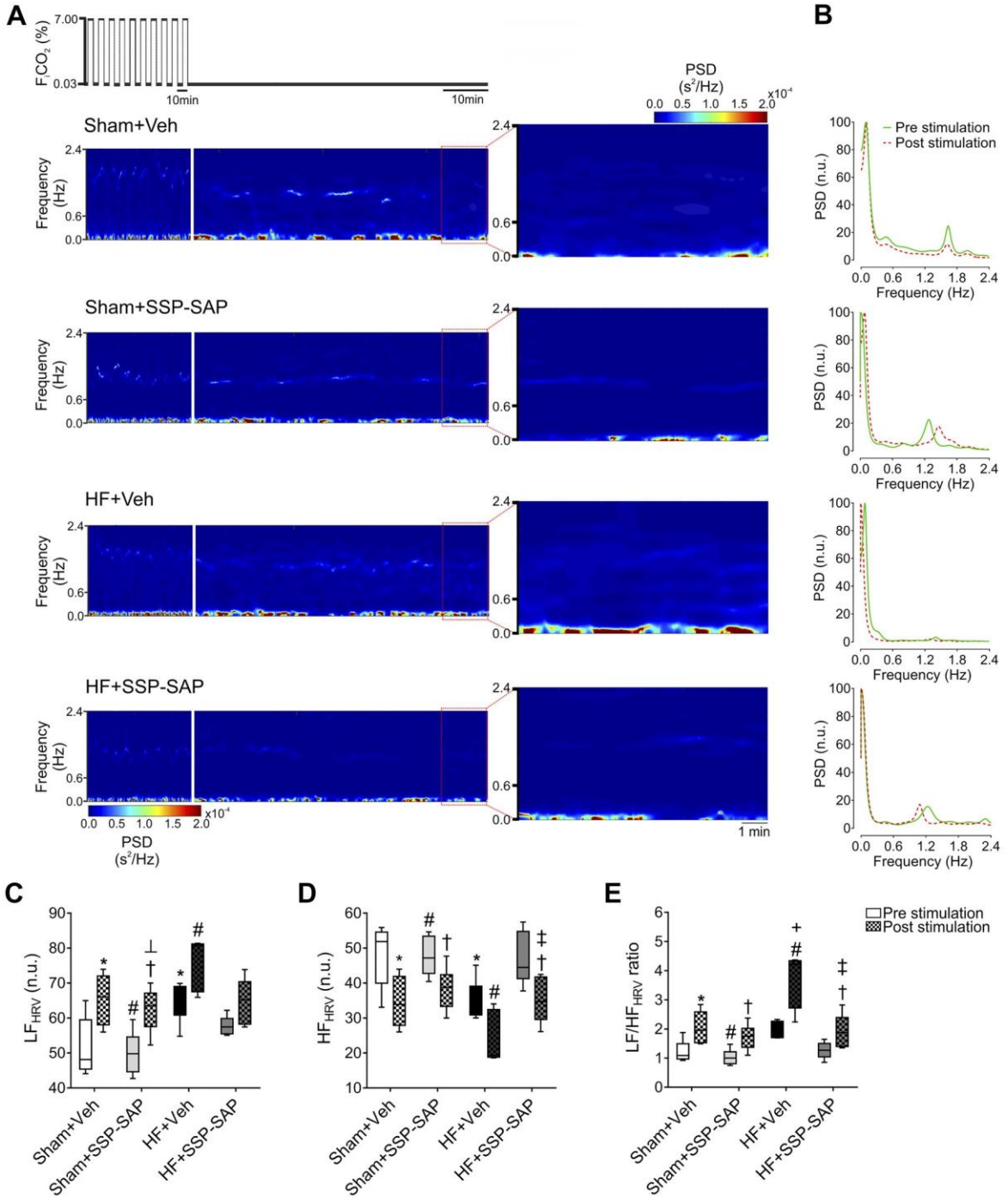

Fig. 5. Ablation of retrotrapezoid nucleus (RTN) chemosensory neurons attenuates autonomic imbalance in heart failure (HF) rats. *A*: representative time-varying heart rate variability (HRV) analysis during pre- and post-episodic hyperapnic stimulation (EHS) period in 1 rat per group. Note that HF rats displayed a marked increase in the low-frequency HRV component (0.04–0.6 Hz), and substance P-conjugated saporin (SSP-SAP) toxin injection in the RTN blunted this enhanced sympathetic response during the post-EHS period. *B*: representative power spectral density (PSD) analysis of HRV during pre- and post-EHS phases. *C–E*: summary data of low frequency (LF) (*C*), high-frequency (HF, 0.6–2.4 Hz) (*D*), and LF/HF ratio (*E*) during pre- and post-EHS phases. Note that the autonomic imbalance during post-EHS phase was blunted by SSP-SAP toxin delivery into the RTN. Box and whiskers represent median range. Two-way ANOVA followed by Holm-Sidak post hoc analysis; *n* 6 rats per group. **P* 0.05 vs. ShamVeh Pre; #*P* 0.05 vs. HFVeh Pre; ⊥*P* 0.05 vs. ShamSSP-SAP Pre; ‡*P* 0.05 vs. HFSSP-SAP Pre; *P* 0.05 vs. ShamVeh Post; †*P* 0.05 vs. HFVeh Post. n.u., normalized units.

induces a further increase in late-expiratory flows in These results suggest that

RTN chemoreceptor neurons may serve as a nodal point for the entrainment of respiratory and cardiovascular function in HF rats. Importantly, alterations in respiratory-cardiovascular coupling has been proposed to underlie the pathophysiology of oscillatory breathing in HF (40). In this study,

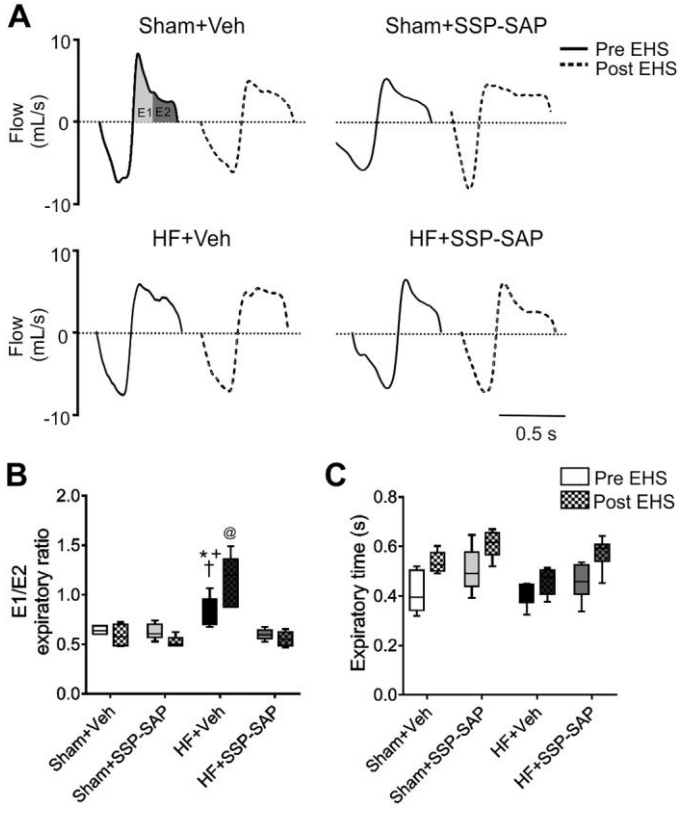

Fig. 6. Episodic hypercapnic stimulation further increases active expiration in heart failure (HF) rats. *A*: representative traces of one breathing cycle pre(continuous traces) and post- (segmented traces)-episodic hypercapnic stimulation (EHS) in 1 ShamVeh rat, 1 ShamSSP-SAP-treated rat, 1 HFVeh rat, and 1 HFSSP-SAP-treated rat. In HF rats, the early expiratory flow (E1) was reduced, whereas the late expiratory flow (E2) was increased compared with Sham rats. *B*: summary data showing E2/E1 ratio. *C*: summary of expiratory time obtained in 20 consecutive respiratory cycles in all groups, preand post-EHS. Box and whiskers represent median range. Two-way ANOVA followed by post hoc analysis of Holm-Sidak; *n* 6 rats per group. **P* 0.05 vs. ShamVeh; *P* 0.05 vs. ShamSSP-SAP; †*P* 0.05 vs. HFSSP-SAP; @*P* 0.05 vs. HFVeh post-EHS.

respiratory-cardiovascular coupling was observed during the onset of breathing disorders, which was partially mediated by RTN chemoreceptor neurons because SSP-SAP decreases respiratory-cardiovascular coupling in HF rats. Nevertheless, we cannot rule out the contribution of sympathoinhibitory reflexes elicited by lung stretch receptors (12) in the genesis of disordered breathing in HF. Further studies should focus on the role of pulmonary receptors in the development of altered breathing patterns and autonomic imbalance in HF.

Strengths and limitations. To date, the vast majority of studies in the field of chemoreflex function and heart failure (HF) pathophysiology have been done in reduced ejection fraction models (35% LVEF) (6). These models are characterized by overt reductions in tissue perfusion, including both central and peripheral chemosensory areas (7, 9, 25). To our knowledge, this is the first study showing that RTN chemoreceptor neurons play a role in cardiorespiratory alterations after EHS in the setting of HF without the confounding effect of chronic reductions in tissue perfusion because previous evidence from our laboratory showed volume overload HF rats displayed no chronic decrease in LVEF at 8 wk post-HF induction (7). From a translational perspective, volume overload HF recapitulates some but not all characteristics of human HF with preserved ejection fraction (43). Although there are obvious differences between the etiology of human HF and the experimental volume overload model used in this study, it is important to note that both preserved ejection fraction HF in humans and experimental volume overload HF have comparable levels of disordered breathing and sympathetic activation. These two important hallmarks of human HF are positively correlated with disease progression and poor prognosis.

Despite the fact that SSP-SAP injections within the RTN region primarily result in the ablation of RTN chemoreceptor neurons (37, 38), some studies have shown off-target effects such as reduced NK1R immunoreactivity in C1 neurons in the vicinity of the injection site (37). However, when administered at a dose that kills 90% of Phox2b RTN neurons, the toxin spares nearby C1 catecholaminergic, serotonergic, and cholinergic neurons (37). However, considering that C1 neurons constitutively express substance P receptors (13), it is plausible that injections of SSP-SAP into the RTN may target C1 neurons as well. Nevertheless, our data showing that Sham SSP-SAP-treated rats display similar levels of cardiac sympathetic outflow compared with ShamVeh rats strongly suggest that there was no physiological effect of SSP-SAP on sympathetic control areas in close proximity to the injection site.

Interestingly, we found that Sham rats continue to display a post-EHS hypoventilation even after ablation of RTN Phox2b neurons. This result strongly suggests that, contrary to what is observed in HF rats, other structures besides the RTN contribute to the hypoventilatory response after EHS in healthy rats. Indeed, raphe adrenergic and serotoninergic neurons have been linked to ventilatory long-term depression in healthy animals (4, 44). Additionally, we wish to acknowledge that peripheral carotid body chemoreceptors, which are able to elicit a chemoreflex response during hypercapnic stimulation, may also play a role in EHS-induced hypoventilation observed in sham animals. With that said, we observed no differences in the HVR between HF and Sham rats. Discrepancies from our previous studies showing a reduction in HVR in HF rats (40) are likely due to the marked difference in the protocol used to assess HVR. In previous studies, we used only 2–3 min of hypoxic gas stimulation (40) compared with the 10-min hypoxic exposure used in the present study. However, it is important to note that we did not observe a potentiation of the HVR in the present or previous studies. Nevertheless, we cannot preclude the possibility that tonic afferent drive from the carotid bodies modulates the central chemoreflex and contributes to altered breathing patterns and cardiac autonomic dysfunction in the setting of volume overload HF. Future studies are needed to precisely define the role of the carotid body on cardiorespiratory adjustments in volume overload HF before and after EHS.

In summary, our results indicate that EHS triggers ventilatory plasticity in healthy rats, characterized by long-term ventilatory depression. Interestingly, this physiological response to EHS was blunted in volume overload HF rats. Furthermore, EHS exacerbates breathing instability and the incidence of breathing

disorders in HF. Additionally, EHS worsens cardiac autonomic imbalance in HF rats. Impor-

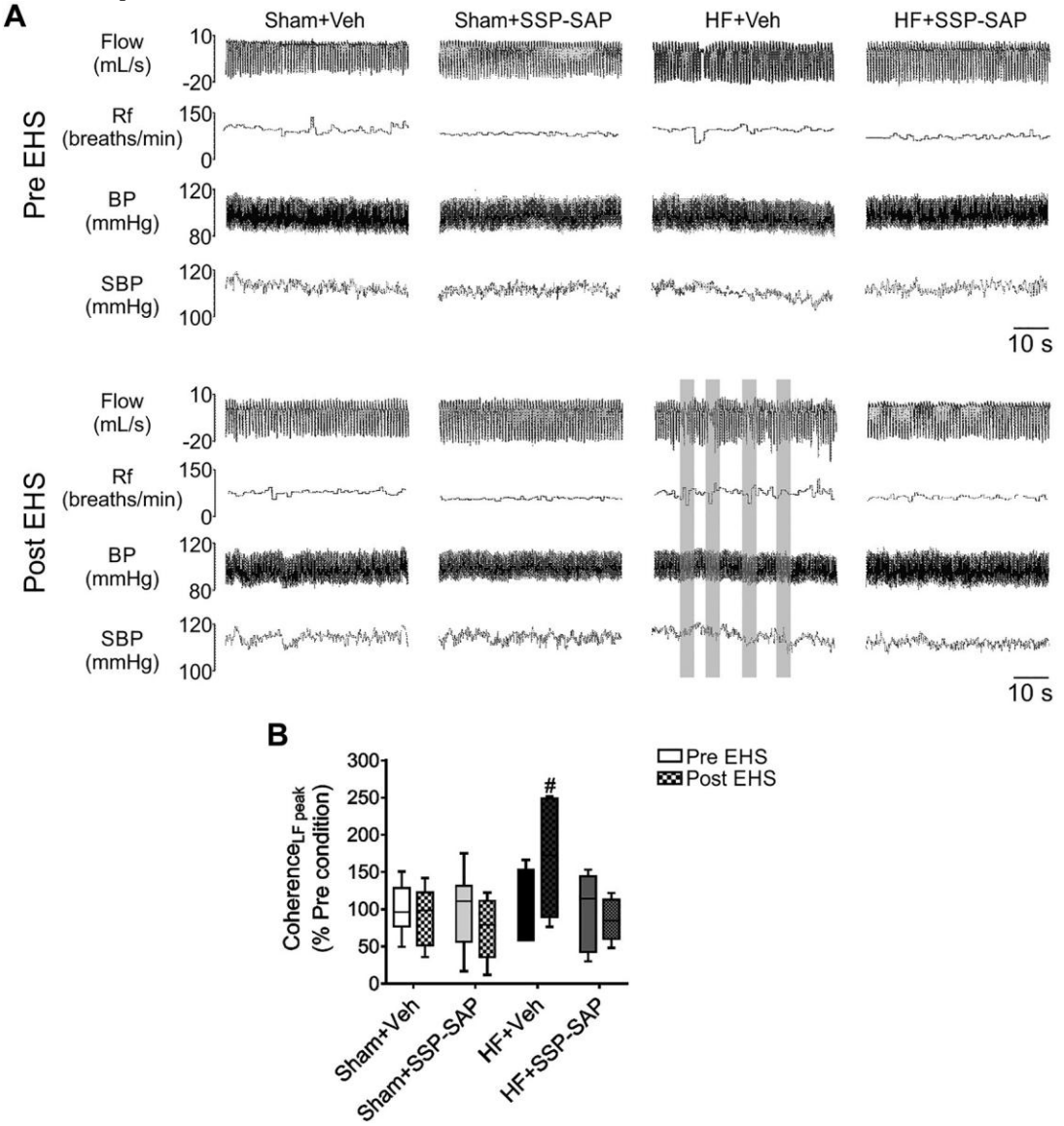

Fig. 7. Episodic hypercapnic stimulation-induced respiratory-cardiovascular coupling in heart failure depends on intact retrotrapezoid nucleus (RTN) chemoreceptor neurons. *A*: representative traces of respiratory flow, respiratory frequency (R_f), blood pressure (BP), and systolic blood pressure (SBP) in 1 rat per group during pre- and post-episodic hypercapnic stimulation (EHS) phase. Segments where coupling between ventilatory and cardiovascular signals was observed are highlighted (gray). *B*: summary data of coherence analysis. Note that following EHS, heart failure (HF) rats displayed an increased coherence between tidal volume (V_T) oscillation and SBP, and this was blunted in HF rats treated with substance P-conjugated saporin (SSP-SAP) toxin. Box and whiskers represent median range. Two-way ANOVA followed by Holm-Sidak post hoc analysis; *n* 6 rats per group. #*P* 0.05 vs. HFVeh Pre-EHS. LF, low frequency.

tantly, we found that RTN chemoreceptor neurons play a seminal role in EHS-induced ventilatory plasticity, disordered breathing, and exacerbation of cardiac autonomic imbalance in rats with volume overload HF because partial ablation of these neurons restores normal cardiorespiratory responses to EHS in HF rats.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

R.D.R. conceived and designed research; H.S.D., D.C.A., C.T., K.V.P., K.G.S., E.D.-J., C.L., A.A.-A., and J.N.S. performed experiments; H.S.D., D.C.A., C.T., K.V.P., K.G.S., E.D.-J., C.L., A.A.-A., and J.N.S. analyzed data; H.S.D., D.C.A., C.T., H.D.S., A.C.T., T.S.M., N.J.M., and R.D.R. interpreted results of experiments; H.S.D., D.C.A., C.T., K.G.S., J.N.S., A.C.T., and T.S.M. prepared figures; H.S.D., D.C.A., C.T., C.L., A.A.-A., H.D.S., N.J.M., and R.D.R. drafted manuscript; H.S.D., D.C.A., C.T., K.V.P., K.G.S., E.D.-J., H.D.S., A.C.T., N.J.M., and R.D.R. edited and revised manuscript; H.S.D., D.C.A., C.T., K.V.P., K.G.S., E.D.-J., C.L., A.A.-A., H.D.S., J.N.S., A.C.T., T.S.M., N.J.M., and R.D.R. approved final version of manuscript.

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TOPICAL REVIEW

Neuroinflammation in heart failure: new insights for an old disease

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Abstract Heart failure (HF) is a complex clinical syndrome affecting roughly 26 million people worldwide. Increased sympathetic drive is a hallmark of HF and is associated with disease progression and higher mortality risk. Several mechanisms contribute to enhanced sympathetic activity in HF, but these pathways are still incompletely understood. Previous work suggests that inflammation and activation of the renin–angiotensin system (RAS) increases sympathetic drive. Importantly, chronic inflammation in several brain regions is commonly observed in aged populations, and a growing body of evidence suggests neuroinflammation plays a crucial role in HF. In animal models of HF, central inhibition of RAS and pro-inflammatory cytokines normalizes sympathetic drive and improves cardiac function. The precise molecular and cellular mechanisms that lead to neuroinflammation and its effect on HF progression remain undetermined. This review summarizes the most recent advances in the field of neuroinflammation and autonomic control in HF. In addition, it focuses on cellular and molecular mediators of neuroinflammation in HF and in particular on brain regions involved in sympathetic control. Finally, we will comment on what is known about neuroinflammation in the context of preserved *vs*. reduced ejection fraction HF.

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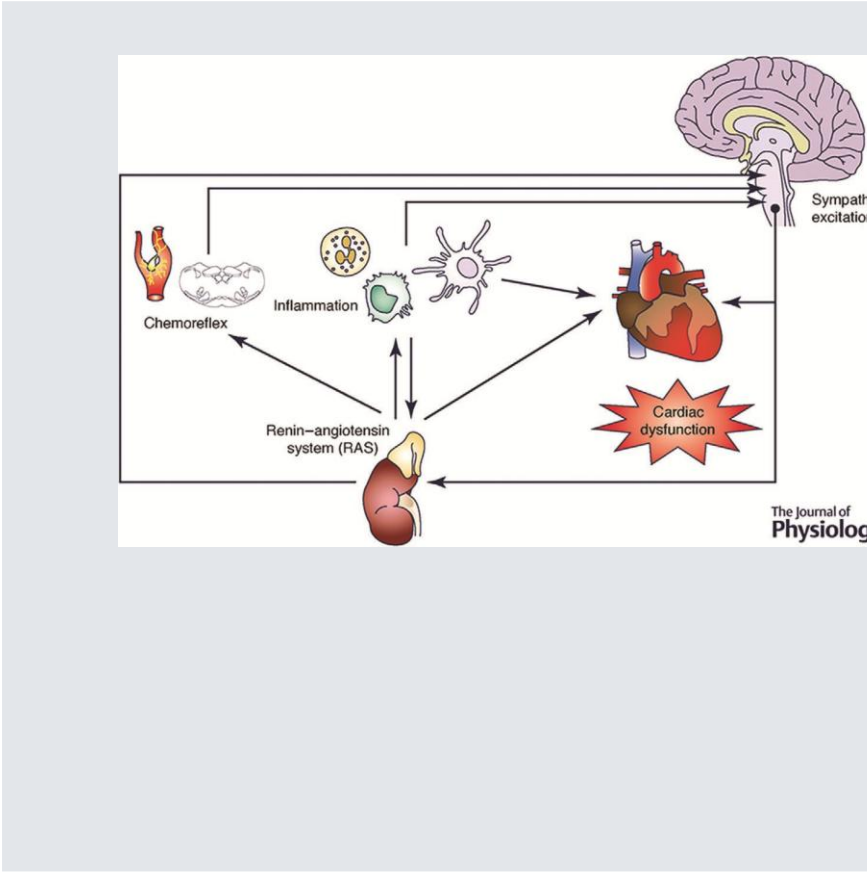

Abstract figure legend Integrative physiology of heart failure progression: Heart failure (HF) is characterized by chronic inflammation, renin–angiotensin system (RAS) overactivity, sympathoexcitation and cardiac dysfunction. Sympathoexcitation and RAS activation occur early in HF progression as a compensatory response to haemodynamic challenges. Chronic activation of these compensatory mechanisms becomes maladaptive and has toxic effects on cardiac muscle leading to electrophysiological abnormalities and initiation of fibrotic processes. RAS activation also enhances chemoreflexsensitivity(furtherexacerbatingsympatheticactivation)andpromotesdiffuseinflammation .Bothsystemic and brain RAS and inflammation feed back to exacerbate sympathoexcitation, generating a vicious cycle that contributes

to further cardiac dysfunction.

Introduction

Cardiovascular diseases (CVDs) are the leading cause of death worldwide (Ponikowski *et al*. 2014), and heart failure (HF), which affects 20% of the population over the age of 65 (Ponikowski *et al*. 2014), specifically, is projected to double in prevalence by 2030 (Heidenreich *et al*. 2013). The increasing prevalence of HF is considered a major public health issue as approximately 36% of CVD deaths are attributed to HF (Mozaffarian *et al*. 2016). HF presentation and aetiology are diverse, but patients are often classified according to their underlying physiological impairment.

HF with preserved ejection fraction (HFpEF) is defined by abnormal/inadequate diastolic function, whereas HF with reduced ejection fraction (HFrEF) is a product of systolic failure. Until recently, HFpEF was erroneously considered to be an early stage of HFrEF (Van Empel & Brunner-La Rocca, 2015*a*); however, therapies shown to be effective for HFrEF are often ineffective or even harmful when used to treat HFpEF (van Empel & Brunner-La Rocca, 2015*a*). Importantly, both HF syndromes are equally prevalent and are associated with high mortality rates (Yancy *et al*. 2013). Both HFpEF and HFrEF are commonly viewed as the terminal stage of various CVDs; however, their respective aetiologies remains unclear, as both are often accompanied by various cardiac and non-cardiac comorbidities (Paulus & Tschope, 2013; Ponikowski *et al*. 2014). The most frequent HFrEF comorbidities are coronary heart disease and myocardial infarction (MI), while the most frequent comorbidities in HFpEF patients are non-cardiac, such as obesity, hypertension, type-2 diabetes and renal failure (Paulus & Tschope, 2013; van Empel & Brunner-La Rocca, 2015*a*; Mozaffarian *et al*. 2016).

HF is characterized by four main hallmarks (Abstract Figure): (i) cardiac dysfunction (Yancy *et al*. 2013; Azad & Lemay, 2014); (ii) systemic inflammation (Rauchhaus *et al*. 2000; Anker & von Haehling, 2004; van Empel & Brunner-La Rocca, 2015*b*); (iii) chronic activation of the renin–angiotensin system (RAS) (Suzuki *et al*. 2004; Sciarretta *et al*. 2009), and (iv) autonomic imbalance, characterized by increased sympathetic activity and parasympathetic withdrawal (Kishi, 2012; Xu & Li, 2015). Figures1–3summarizeputativemechanismscontributing to deterioration of cardiac function in HF, as well as the crosstalk between these pathways. In the early stages of HF, increased RAS and sympathetic nervous system (SNS) activity are thought to act as compensatory mechanisms to counteract myocardial dysfunction; however, their long-term activation has deleterious effects on cardiac function. Chronic increases in noradrenaline (NA) and angiotensin-II (Ang-II; a major RAS effector peptide) increase cardiac chronotropy and inotropy (Hasking *et al*. 1986; Hall, 1991), which in turn may lead to increased arrhythmia susceptibility and contribute to cardiac hypertrophy (Calderone *et al*. 1998; Hasenfuss, 1998; Iravanian & Dudley, 2008).

An important bi-directional relationship exists between RAS and SNS. Augmented sympathetic tone stimulates renin secretion from kidney juxtaglomerular cells, augmenting Ang-II concentrations (DiBona, 2000). In the other direction, Ang-II may modulate SNS activity via its effects in the central nervous system as well as peripheral tissues (Figs 2 and 3). Acute application of Ang-II has been shown to stimulate afferent peripheral chemoreflex activity (Allen, 1998), and previous studies have shown that chronic RAS activation that occurs in experimental HF contributes to heightened chemoreflex

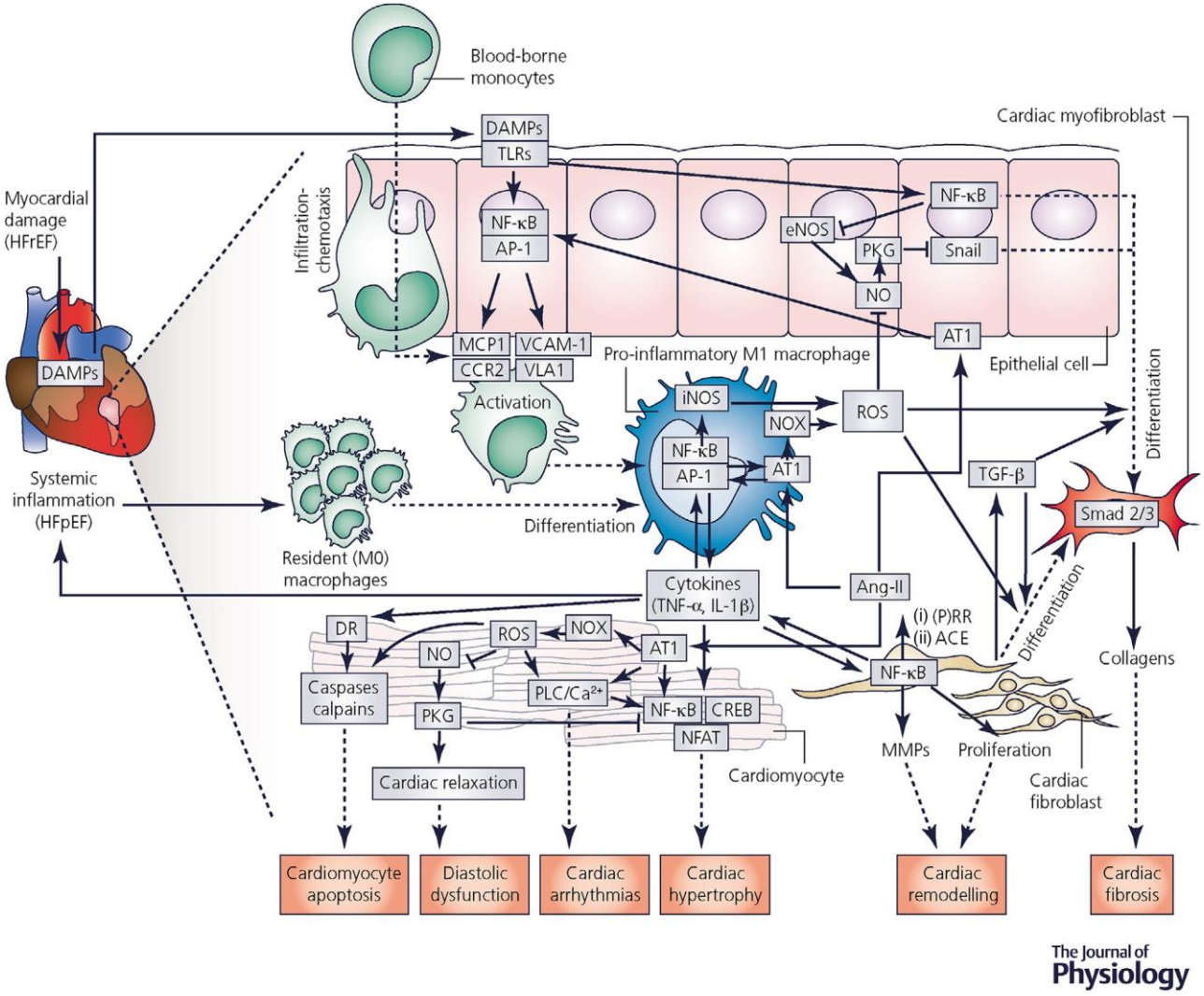

Figure 1. Cardiotoxic effects of inflammation and angiotensin-II in heart failure
Inflammation plays a crucial role in cardiac pathophysiology in both heart failure (HF) syndromes: HF with reduced and preserved ejection fraction (HFrEF and HFpEF, respectively). However, since they have different aetiologies, the primary stimuli responsible for the onset of myocardial inflammation in both syndromes must be different. Indeed, in HFrEF the primary stimulus is likely to be ischaemia. Ischaemia induces cardiomyocyte cell death, leading to liberation of damage-associated molecular patterns (DAMPs), activating toll-like receptors (TLRs) on endothelial cells, which express chemoattractant and adhesion molecules such as monocyte chemoattractant protein-1 (MCP-1) and vascular cell adhesion molecule-1 (VCAM-1) via nuclear factor-κB (NF-κB)–activator protein-1 (AP-1). This recruits blood-borne monocytes/macrophages to the heart and promotes their differentiation to the pro-inflammatory M1 phenotype. M1 macrophages produce large amounts of pro-inflammatory mediators and reactive oxygen species (ROS) and propagate the inflammatory response to other cell types in the heart. In HFpEF, the primary stimulus is inflammation *per se*, which promotes the differentiation of resident resting macrophages (Mφ) to the M1 phenotype. Pro-inflammatory cytokines (i.e. tumour necrosis factor (TNF)-α and interleukin (IL)-1β) augment inducible nitric oxide synthase (iNOS) expression, producing excessive amounts of nitric oxide (NO) and ROS, and altering normal function of endothelial nitric oxide synthase (eNOS). iNOS-derived ROS reacts with NO to form peroxynitrite, reducing NO availability. Decreased protein kinase G (PKG) signalling, promotes endothelial dysfunction to pro-fibrotic cardiac myofibroblasts, by mechanisms dependent on NF-κB, Snail and transforming growth factor β-1 (TGF-β1). Cardiac fibroblasts are the key effectors of the cardiac RAS and have autocrine and paracrine functions which promote cardiac fibrosis. NF-κB activation by pro-inflammatory cytokines promotes cardiac fibroblast proliferation and the expression of (pro)-renin receptor and angiotensin converting enzyme 1 (ACE1), which cleaves, respectively, angiotensinogen to angiotensin-I and then to angiotensin-II (Ang-II), the major RAS bioactive peptide. Ang-II exerts potent pro-oxidant and pro-inflammatory effects in practically all cardiac cell types, by activating NADP(H)-oxidases

(NOX) and NF-κB, respectively. This establishes a positive feedback loop between RAS and inflammation. In cardiac fibroblasts, ROS facilitates TGF-β1-mediated differentiation

drive, which is related to sympathoexcitation (Li *et al*. 2006; Andrade *et al*. 2015; Del Rio *et al*. 2015). In addition to promoting autonomic dysfunction, RAS activation has deleterious effects on cardiac function. Cardiac fibrosis, endothelial dysfunction, left ventricular hypertrophy and cardiomyocyte cell death are all associated with chronic RAS activation (Ing *et al*. 1999; Willenbrock *et al*. 2000; Sciarretta *et al*. 2009; Ueland *et al*. 2015), and have been shown to be dependent on oxidative stress and nuclear factor-κB (NF-κB)/pro-inflammatory signalling (Sriramula & Francis, 2015). Mechanistically, reactive oxygen species (ROS) reduce NO bioavailability and promote NF-κB activation, which in turn results in upregulation of Ang-II type I receptor (AT1) and pro-inflammatory cytokines (Xu & Li, 2015). Also, circulating Ang-II and cytokines can directly increase sympathetic tone by exciting specific circumventricular nuclei (Fig. 3). The combination of these factors contributes to development of a vicious cycle which promotes progressive decline in cardiac function in HF.

Reduced cardiac output and peripheral blood flow in HFrEF triggers activation of RAS as well as carotid body (CB) chemoreceptors, which in turn activate brainstem autonomic control areas (Hall, 1991; Atlas, 2007; Andrade *et al*. 2015). Interestingly, RAS and sympathetic activity are also upregulated in HFpEF animal models, characterized by volume overload in which cardiac output and peripheral blood flow show no major alterations (Yoshimura *et al*. 2000; Suzuki *et al*. 2004). One of the common threads that could play a role in autonomic dysregulation in both HFrEF and HFpEF pathophysiology is the initiation of a diffuse and persistent inflammatory response (Paulus & Tschope, 2013; Weirather & Frantz, 2015). Plasma cytokines such as tumour necrosis factor (TNF)-α and interleukin (IL)-1β can directly induce sympathoexcitation by exciting circumventricular organs (CVOs), like the subfornical organ (SFO) (Wei *et al*. 2013, 2018; Simpson & Ferguson, 2017); or affect autonomic regulation indirectly by driving RAS activation via NF-κB in deeper SNS control areas (Paul *et al*. 2006; Sciarretta *et al*. 2009) (Fig. 3). Chronic RAS activation induces SNS overactivity, which in turn stimulates renin secretion, perpetuating RAS activation, inflammation and sympathoexcitation (Fig. 2). This vicious cycle of RAS activation and sympathoexcitation is associated with increased mortality and poor prognosis in HF patients (Rauchhaus *et al*. 2000; Anker & von Haehling, 2004; Sciarretta *et al*. 2009; Triposkiadis *et al*. 2009).

Current therapies for HF are focused on attenuating the effects of neurohormonal activation (NA and RAS) (Willenbrock *et al*. 2000; Hartupee & Mann, 2017). Despite these drugs being highly effective in improving clinical outcome and survival in some HF patients, others are non-responsive (Willenbrock *et al*. 2000; Heidenreich *et al*. 2013), suggesting that other pathways could be important in HF progression. In this regard, inflammation could be considered as a potential target for HF treatment (Paulus & Tschope, 2013). However, in a worldwide study using specific TNF-α blockers in HFrEF patients, no positive effect was reported, and the study was abandoned (Mann *et al*. 2004). In contrast, treatment with IL-1β inhibitors resulted in improvement of clinical outcomes like exercise capacity in patients with HFpEF (Van Tassell *et al*. 2014), suggesting that inflammation may play differential roles in HFpEF *vs*. HFrEF pathophysiology. Nevertheless, these purported therapeutic benefits need to be assessed in larger populations to establish the importance of this pathway in HFpEF pathophysiology. Importantly, all current treatment strategies for HF are targeted to the heart or plasma, even though RAS and inflammation have also been shown to be upregulated in the brain, notably in key areas for SNS control (Francis *et al*. 2004*a*,*b*; Kang *et al*. 2011; Xu & Li, 2015; Gowrisankar & Clark, 2016). Some studies have shown that neuroinflammation in the brainstem plays a key role in sympathoexcitation and cardiac dysfunction in HF (Cato & Toney, 2005; Guggilam *et al*. 2007). One potential reason why current HF

therapy has limited efficacy is its inability to effectively target inflammation in the brain. Currently, there are no approved therapies for HF targeting inflammation, nor are there RAS inhibitors capable of crossing the blood–brain barrier

to a myofibroblastic phenotype, which in turn results in excessive production of TGF-β1 and matricellular collagens via Smad2/3. NF-κB in cardiac fibroblasts promotes overexpression of matrix metalloproteinases (MMPs), which contribute to deleterious cardiac remodelling. In cardiomyocytes, Ang-II/AT1 signalling activates Gq protein and phospholipase C (PLC), increasing intracellular Ca^{2}_{+} concentrations, promoting cardiac arrhythmias. AT1 activation augments NOX-derived ROS, which reduces cardiomyocyte NO bioavailability by reacting with neuronal nitric oxide synthase (nNOS)-derived NO. This reduces PKG activity, which plays an important role in cardiac relaxation and inhibition of cardiac hypertrophy via inhibition of nuclear factor of activated T-cells (NFAT). As a result, reduced NO bioavailability results in diastolic dysfunction and cardiac hypertrophy in cardiomyocytes. Finally, NF-κB–AP-1-mediated activation of pro-inflammatory cytokines also contributes to cardiac hypertrophy and activation of death receptors (DRs) on cardiac myocytes, thereby promoting expression of caspases and calpains (related to cardiomyocyte apoptosis). Sustained myocardial inflammation and oxidative stress results in cardiomyocyte cell death. The combined influence of these cellular signalling events results in declines in cardiac function. CCR2, chemokine (C-C motif) receptor 2; VLA-1, very late antigen-1 (α1β1 integrin).

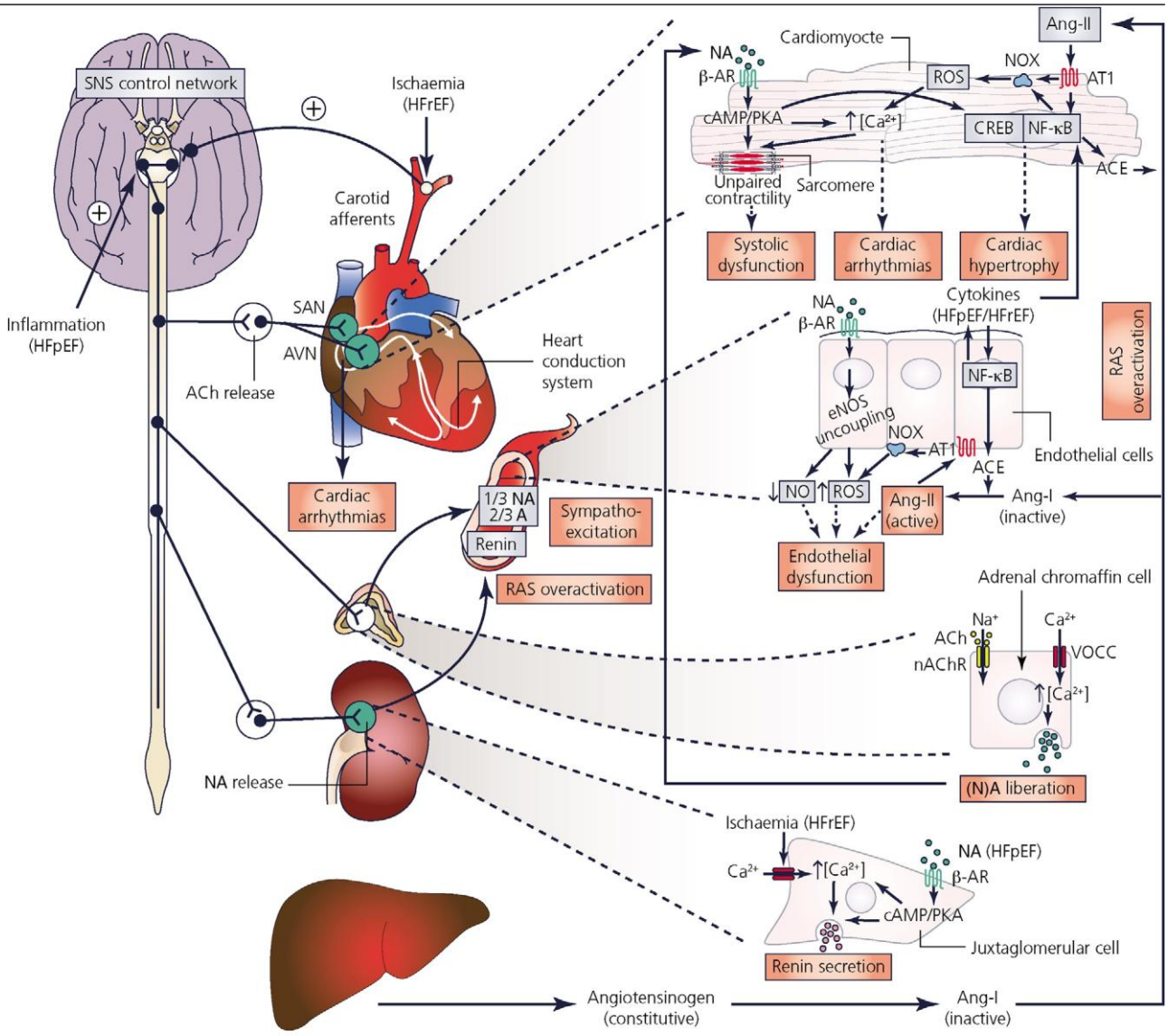

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Figure 2. Multisystemic effects of sympathoexcitation in heart failure: angiotensin and inflammation In HFrEF aberrant cardiovascular reflexes (i.e. carotid body chemoreflex) are promoted by stagnant ischaemia and make a significant contribution to sympathoexcitation, while in HFpEF, blood-borne proinflammatory cytokines contribute to the onset of sympathoexcitation by exciting the neurones residing in sensory circumventricular organs, which subsequently activate those on central sympathetic nervous system (SNS) control nuclei. In both HFrEF and HFpEF, increases in sympathetic outflow result in increased noradrenaline (NA) release from sympathetic ganglia to (i) the heart via the cardiac stellate ganglia, and (ii) the kidney juxtaglomerular cells via the coeliac ganglion. In conjunction with NA, ischaemia promotes renin secretion by activating renal baroreceptors and augmenting intracellular Ca^{2}+ concentration in juxtaglomerular cells. In the bloodstream, renin cleaves angiotensinogen to inactive angiotensin-I (Ang-I), which in turn is cleaved to its active form, Ang-II, by angiotensin converting enzyme (ACE) in local tissues. ACE is induced by pro-inflammatory cytokines and Ang-II itself via NF-κB, thus setting up a feedback loop of persistent RAS activation and consequent inflammation, establishing a vicious cycle of sympathoexcitation. SNS activity in adrenal medullary chromaffin cells activates nicotinic acetylcholine receptors (nAChR) resulting in depolarization of the cell membrane and activating voltage-operated calcium channels (VOCC), promoting (nor)adrenaline ((N)A) secretion into the plasma. Note that adrenals produce both NA and A, while sympathetic ganglia only releases NA. Liberation of NA in the heart conduction system disrupts pacemaker activity, and increases in plasmatic (N)A and Ang-II alter cardiomyocyte contractility and heart architecture, by mechanisms dependent on protein kinase A (PKA), calcium signalling, cAMP response element-binding (CREB), NF-κB and ROS, promoting systolic dysfunction, arrhythmias and

cardiac hypertrophy, which is aggravated by reduced NO bioavailability, which also promotes endothelial dysfunction via β-adrenergic-mediated endothelial nitric oxide synthase (eNOS) uncoupling and NOX-derived ROS. Overall, sympathoexcitation in both HFpEF and HFrEF creates a vicious cycle which is maintained and reinforced by systemic inflammation and chronic RAS activation, promoting, in the long term, toxic effects that results in cardiac function impairment. β-AR, β-adrenergic receptor; AVN, atrioventricular node; NO, nitric oxide; NOX, NAD(P)H oxidase; SAN, sinoatrial node.

(BBB), despite RAS seems to play a fundamental role in neuroinflammation in various CVDs including HF. Thus, reviewing the role of neuroinflammation in HF progression, the molecular and cellular responses involved in onset of neuroinflammation, and the effects on autonomic control and HF pathophysiology will provide insights for new therapeutic approaches.

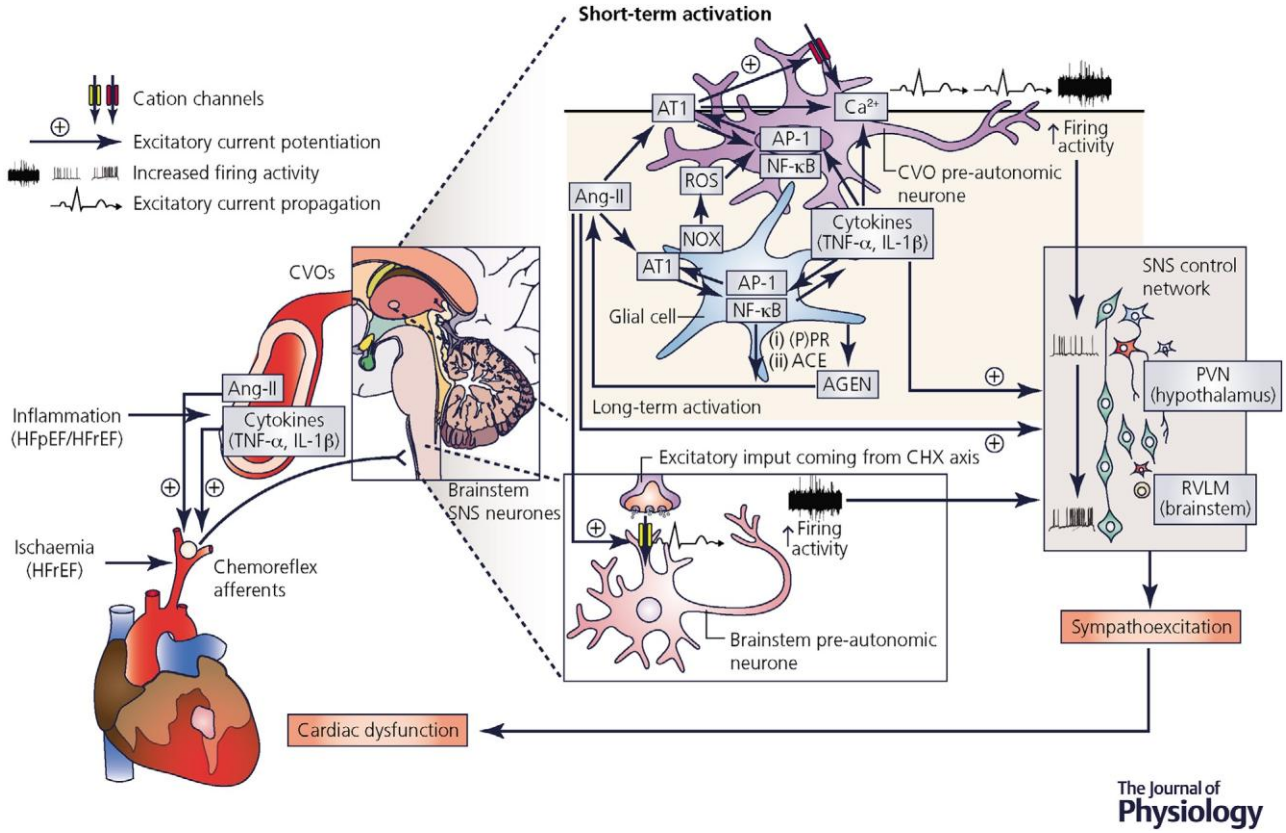

Figure 3. Brain mechanisms of sympathoexcitation in heart failure
HFpEF and HFrEF are characterized by chronic sympathoexcitation, which plays a major role in cardiac function impairment. It is widely accepted that chemoreflex chronic activation constitute a major source of sympathoexcitaton during HFrEF, while in HFpEF, it has been proposed that sympathoexcitation is a consequence of activation of neurones residing in the circumventricular organs (CVOs) in response to blood-borne cytokines and Ang-II, which are synaptically connected to deeper nuclei of SNS control in the brain, transmitting plasma-derived signals within the brain (short-term activation). Activation of chemoreceptors triggers increased firing activity of brainstem SNS control nuclei such as the nucleus of the solitary tract, which in turn activates deeper centres of SNS control network such as the paraventricular nucleus (PVN) and the rostral ventrolateral medulla (RVLM), resulting in increased sympathetic activity. In CVOs two mechanisms of sympathoexcitation can be distinguished in response to systemic inflammation: the short-term activation is mainly mediated by circulating Ang-II and TNF-α, which directly excites CVO neurones by activating Na_+ and Ca^2_+ excitatory currents. On the other hand, long-term activation of these neurones requires *de novo* synthesis of Ang-II by glial cells (specifically astrocytes) from constitutively produced angiotensinogen (AGEN) after NF-κB-dependent expression of (pro-)renin receptor ((P)PR) and angiotensin converting enzyme (ACE) in response to proinflammatory cytokines, Ang-II and ROS, establishing chronic RAS activation and neuroinflammation, since RAS is bidirectionally related to proinflammatory cytokine synthesis (mainly by microglia). Both RAS and neuroinflammation propagate to deeper SNS control areas that are synaptically connected to CVOs, and augment

basal neuronal firing activity and sensitivity, promoting a positive feedback that results in chronic sympathoexcitation and cardiac dysfunction. IL-1β, interleukin-1β; NOX, NAD(P)H oxidase; TNF-α, tumour necrosis factor-α.

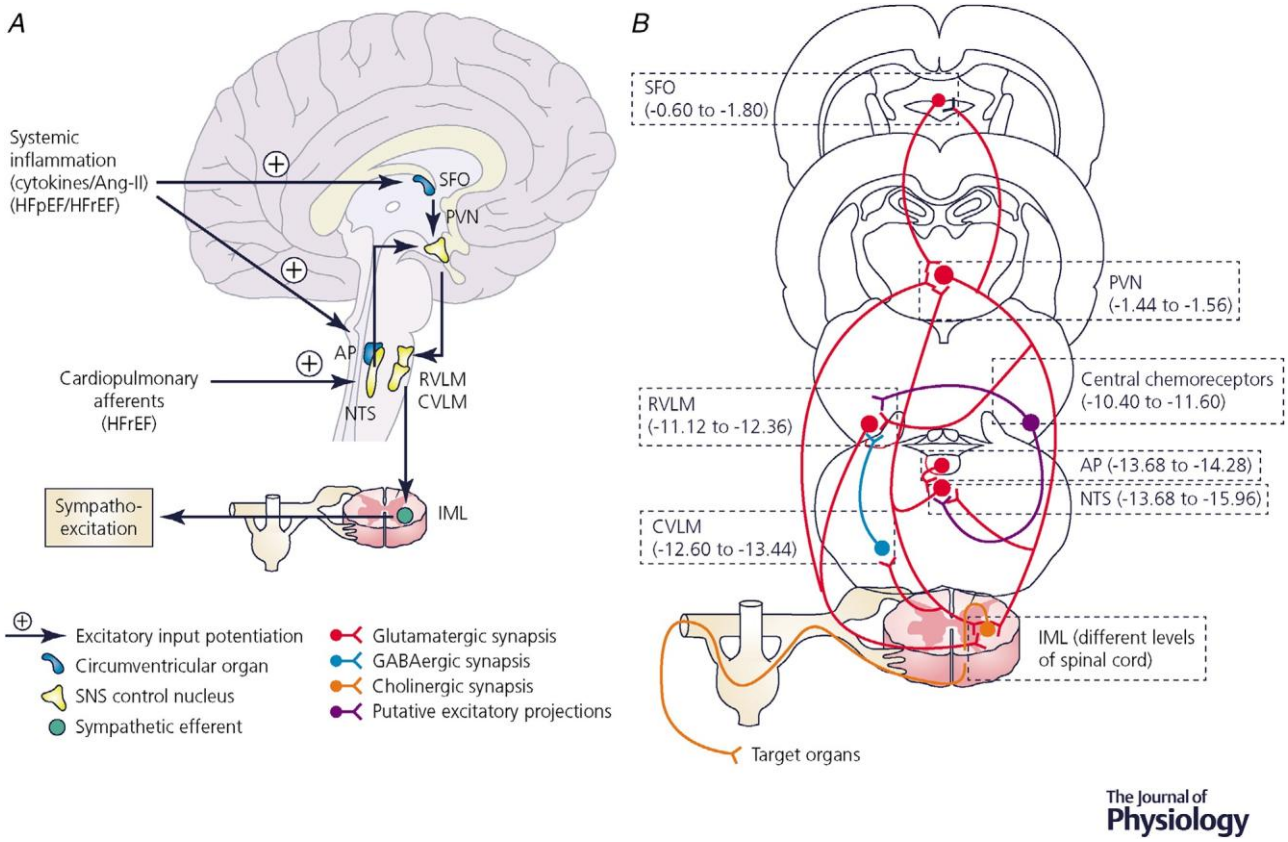

Figure 4. Main areas involved in sympathetic control in heart failure
A, neuronal main structures involved in sympathoexcitation during both aetiologies of HF showing the two major neuronal pathways shown to play crucial roles in HF progression: the NTS–PVN–RVLM axis (activated by chemoand baroreflexes, and presumably the primary source of sympathoexcitation in HFrEF) and the SFO–PVN–RVLM axis (activated by blood-borne cytokines/Ang-II and plausible primary source of sympathoexcitation during HFpEF). The nucleus of the solitary tract (NTS) integrates afferent pathways coming from cardiopulmonary baroreceptors and chemoreceptors and transmits this information to the hypothalamic paraventricular nucleus (PVN) and later, to the rostral ventrolateral medulla (RVLM) in the brainstem, which integrates and process this information and other inputs coming from subfornical organ (SFO) axis to sympathetic pre-ganglionic neurones of the intermediolateral cell column of the spinal cord (IML), which are the source of all sympathetic activity to target organs. *B*, detail of neuroanatomical connections within the SNS control network, as well as stereotaxic coordinates in the rat brain of selected structures according to rat brain atlas (Paxinos, 1980). The NTS is reciprocally connected to pre-sympathetic neurones within the PVN via glutamatergic synapses. Besides, the NTS has direct excitatory connections to the IML and indirect inhibitory connections to the RVLM, sending glutamatergic projections to caudal ventrolateral medulla (CVML) GABAergic neurones. The PVN parvocellular pre-autonomic neurones are reciprocally connected with the NTS and the RVLM and send descending projections to the IML. RVLM neurones receive glutamatergic and GABAergic inputs from the PVN and the CVLM, respectively. Also, it has been proposed that putative connections between RVLM and central chemoreceptors of the retrotrapezoid nucleus (RTN) are important in HF progression, since hypercapnic stimulation induces sympathoexcitation in HF animals and enhanced RTN chemoreflex has been reported in these animals. The RVLM also sends glutamatergic connections to the IML, and enhanced NMDA output currents are key for sympathoexcitation in HF. Sensory CVOs, like the SFO and the area postrema (AP), are highly vascularized organs that lack a normal BBB and sense peripheral signals to modulate CNS activity. SFO is reciprocally connected with the PVN via glutamate and plays an important role in the regulation of sympathetic activity in response to peripheral hormones and inflammation in health and disease. The AP sends excitatory projections to the NTS, regulating sympathetic outflow. However, the role of the AP in HF progression has not been deeply studied. Finally, the IML receives input signals from all these SNS-regulating nuclei at different levels of the spinal cord and sends cholinergic projections to stimulate the sympathetic ganglionic neurones (catecholaminergic) projecting to target organs. Overall all this results in chronic sympatoexcitation during HF progression.

Role of brain autonomic control areas in heart failure progression

Neuroinflammation has been reported in autonomic control areas of the central nervous system (CNS) in HF, without affecting surrounding areas, and there is some evidence suggesting that it plays an important role in cardiac dysfunction in HF (Guggilam *et al.* 2007; Kang *et al.* 2011; Xu & Li, 2015). Therefore, understanding neuronal circuitry in these regions, and the effects of neuroinflammation on neurotransmission is important to the study of HF progression. There are three primary brain nuclei involved in regulation of sympathetic tone: the nucleus of the solitary tract (NTS), the hypothalamic paraventricular nucleus (PVN) and the rostral ventrolateral medulla (RVLM). The intermediolateral column of the spinal cord (IML) receives input signals from SNS-regulating nuclei at different levels of the spinal cord and sends cholinergic projections to stimulate the sympathetic ganglionic neurones (catecholaminergic) projecting to target organs (Elfvin *et al.* 1993) (Figs 4 and 5). Two major neuronal axes have been shown to play crucial roles in HF progression: the NTS–PVN–RVLM axis (activated by chemo- and baroreflexes) and the SFO–PVN–RVLM axis (activated by blood-borne cytokines and Ang-II); since inactivation or ablation these networks prevents neurohumoral and haemodynamic disorders in different HFrEF models (Zhong *et al.* 1992; Patel, 2000; Wei *et al.* 2013; Chen *et al.* 2015).

The nucleus of the solitary tract. The NTS integrates afferent signals coming from cardiopulmonary baroreceptors and chemoreceptors (Gordon & Leone, 1991), and sends glutamatergic projections to nitrergic (nNOS-positive) and GABAergic interneurons surrounding the PVN, as well as to pre-sympathetic neurones within the PVN (Affleck *et al.* 2012). The NTS also has direct excitatory connections to the IML and indirect inhibitory connections to the RVLM via glutamatergic projections to caudal ventrolateral medulla (CVML) GABAergic neurones (Schreihofer & Guyenet, 2002; Guyenet, 2006; Affleck *et al.* 2012) (Fig. 4). Increased chemoreflex activity combined with depressed baroreflex sensitivity has been observed in HF, and both contribute to high sympathetic activity in HFrEF and HFpEF (Del Rio *et al.* 2013; Gronda & Vanoli, 2016; Andrade *et al.* 2017*a*). Given the important role of the NTS in integrating chemoreceptor and baroreceptor input, it is plausible that alterations in NTS function may also contribute to HF progression, but at this point its role is not clear and further research is needed (Toledo *et al.* 2016).

The paraventricular nucleus. The parvocellular pre-autonomic neurones in the PVN contribute to control of SNS activity and are implicated in HF-associated sympathoexcitation (Pyner, 2009). They are reciprocally connected with the NTS (Swanson & Sawchenko, 1983) and the RVLM, and send descending projections to the IML (Kenney *et al.*2003).Theactivity of these neurones is crucially modulated by GABAergic and nitrergic interneurones surrounding the PVN (Biancardi *et al.* 2010). NO tunes the firing activity of PVN neurones that project to the RVLM by potentiating GABAergic presynaptic currents (Li *et al.* 2003*c*). It has been shown that GABA-A receptor and nNOS density in the PVN is decreased in HFrEF (Carillo *et al.* 2012; Sharma *et al.* 2013), which leads to PVN excitation by turning synaptic balance toward glutamatergic transmission (Li *et al.* 2003*b*). In contrast to HFrEF models, no studies have directly addressed neurotransmitter balance in the PVN in HFpEF (Table 1).

The rostral ventral lateral medulla. The RVLM is considered the most important nodal point of the cardiovascular sympathetic control network (Guyenet, 2006), since destruction of RVLM neurones causes sympathetic activity to fall to zero (Pilowsky *et al.* 2009). RVLM neurones receive glutamatergic and GABAergic inputs from the PVN and the CVLM, respectively (Sved *et al.*

2002; Pyner, 2009). NO also modulates basal firing activity of RVLM neurones by enhancing GABAergic currents coming from PVN interneurones (Li *et al.* 2003*c*). In animal models of HF, reduced NO bioavailability associated with oxidative stress in the RVLM is a key mechanism contributing to upregulation of sympathetic tone (Xu & Li; 2015; Kishi, 2013). RVLM neurones are glutamatergic, but there is a subpopulation of C1 cells (50–70%) that are catecholaminergic (Swanson & Sawchenko, 1983). C1 neurones send projections to the PVN to control the hypothalamic–pituitary axis (Guyenet, 2006) and modulate systemic inflammation (Abe *et al.* 2017). In HFrEF patients, higher levels of brain catecholamines have been reported, which are strongly correlated with sympathoexcitation (Lambert *et al.* 1995). RVLM C1 neurones are overactivated in HFrEF rats, and this effect is blunted after CB ablation – the major source of peripheral chemoreception (Del Rio *et al.* 2013) – suggesting that peripheral chemoreceptor afferents contribute to RVLM C1 hyperactivation in HFrEF; however, the peripheral chemoreflex has an apparent minor contribution in experimental HFpEF pathophysiology (Del Rio *et al.* 2017) since the central but not the peripheral chemoreflex directly contributes to autonomic imbalance and cardiac function impairment in HFpEF (Toledo *et al.* 2017), suggesting that the two chemoreflexes play differential roles in distinct HF aetiologies and that the central chemoreflex could be a promising novel target for HFpEF management. Despite the underlying mechanisms of CB chemoreflex over-sensitization in HFrEF being well known, those responsible for central chemoreflex hyperactivity in HFpEF have not been studied yet.

While there are many studies examining the role of these brain nuclei in HFrEF, little is known about the importance of these neural networks in HFpEF (Table 1). We speculate that the RVLM is a key source of sympathetic hyperactivation in HFpEF since increased activity of C1 cells has been reported (Toledo *et al.* 2017), and ablation of RVLM C1 neurones restores autonomic balance, reduces arrhythmia incidence and improves cardiac contractility in HFpEF rats, mitigating disease progression (Andrade *et al.* 2019). Interestingly, augmented RVLM C1 was associated with increased central chemoreflex sensitivity (Toledo *et al.* 2017), and increased sympathetic activity is observed in response to central chemoreflex stimulation, which is dependent on the integrity of central chemosensory neurones inside the retrotrapezoid nucleus (Takakura & Moreira, 2011),

strongly suggesting an important relationship between RVLM C1 cells and central chemoreceptors of the retrotrapezoid nucleus (Fig. 4). Despite it having been proposed that inflammation could play a major role in enhanced chemoreflex sensitivity and sympathoexcitation under pathological conditions (Pena-Ortega, 2019), no˜ direct evidence exists about neuroinflammation in those regions in HFpEF (Table 1), as well as their plausible underlying mechanisms and their importance for HFpEF pathophysiology.

Circumventricular organs. CVOs, like the SFO and the area postrema (AP), are also important in control of SNS in both health and disease (Cottrell & Ferguson, 2004; Llewellyn*etal*.2014). CVOs are highly vascularized organs that lack a normal BBB (Fry & Ferguson, 2007) and sense peripheral signals to modulate CNS activity and hormone release in response to several stimuli (Cottrell & Ferguson, 2004). Activity of the SFO–PVN–RVLM axis is increased in HFrEF (Llewellyn *et al.* 2014) and SFO ablation prevents rises in arterial pressure and heart rate in response to peripheral Ang-II, TNF-α or IL-1β administration in healthy animals (Ferguson & Li, 1996; Ferguson & Bains, 1997; Osborn *et al.* 2012; Wei *et al.* 2013). The SFO could play an important role in autonomic dysregulation in both HFrEF and HFpEF since plasma Ang-II and cytokines are increased in both of these HF populations (Kishi, 2012; van Empel & Brunner-La Rocca, 2015*b*) and they can act as potent activators of the SFO–PVN–RVLM axis. Thus, chronic SFO activation by peripheral stimuli could be a key contributor to sympathoexcitation in both HF subsets. In fact, SFO is a major source for *de novo* synthesis of Ang-II within the brain (Sinnayah *et al.* 2006) and it has

been shown that AT1 knockdown in this nucleus in HFrEF rats reduces the expression of TNF-α, IL-1β, CD68 (a marker of microglial activation) and c-Fos (a marker of neuronal activation) in the PVN, diminishes plasma NA, and slightly restored cardiac function, without affecting plasma cytokines or Ang-II (Yu, 2018). However, the contribution of SFO in HFpEF pathophysiology is completely unknown (Table 1), despite evidence strongly suggesting that it could play a major role in HFpEF pathophysiology. We speculate that in both HF aetiologies, SFO is responsible of the establishment and maintenance of brain RAS overactivity, promoting the chronic activation of PVN and RVLM pre-sympathetic neurones, and consequently, their activation triggers autonomic imbalance and cardiac function impairment. However, the actual contribution of these nuclei to disease pathology and the underlying mechanisms responsible of their chronic activation in both HF subsets remain to be elucidated.

The AP is in the immediate proximity of the NTS and the dorsal motor nucleus of the vagus (Cottrell & Ferguson, 2004). It receives inputs from arterial baroreceptors, the NTS, the PVN and the vagus, and sends projections to the NTS, regulating sympathetic outflow (Ferguson & Bains, 1997). To date, no studies have examined the potential role of the AP in HF progression.

Relationships with parasympathetic nervous system. Brainstem SNS control nuclei also modulate parasympathetic nervous system activity and the neural– inflammatoryreflex (Fig.5). This reflex is characterized by activation of a vagal cholinergic efferent arm by the NTS (Tracey, 2002), or a greater splanchnic catecholaminergic efferent arm (Martelli *et al.* 2016) by SNS preganglionic neurones in response to acute inflammatory challenges. The result of this activation is the inhibition of cytokine synthesis by macrophages of the mononuclear phagocytic system – formerly called the reticuloendothelial system – which includes all resident and circulating macrophagic lineages in the body, including the heart, kidney, brain and spleen (Tracey, 2002). It has been proposed that vagal stimulation could be a novel strategy for HF management (De Ferrari, 2014), as it could: (i) restore autonomic imbalance by activating the parasympathetic arm of the autonomic nervous system and (ii) reduce peripheral inflammation by stimulating the neural inflammatory reflex, potentially resulting in improved clinical outcomes in HF patients. In animal models of HFrEF, vagal nerve stimulation leads to restoration of autonomic balance, decreased RAS activity, reduction in pro-inflammatory cytokine levels, decreased arrhythmia incidence, and reduced mortality (Sabbah *et al.* 2011). Despite promising evidence in animal models of HF, large randomized trials in humans have failed to demonstrate any significant improvement in autonomic imbalance, inflammation, or cardiac function following vagal nerve stimulation (De Ferrari *et al.* 2017).

Even more, the contribution of the neural antiinflammatory reflex in chronic inflammatory diseases such HF has not been comprehensively studied and the major evidence available comes from acute experiments performed in healthy animals (Tracey, 2002; Martelli *et al.* 2016). Recent studies have shown a reciprocal relationship between pre-sympathetic neurone activity and peripheral inflammation. Optogenetic stimulation of C1 neurones or restraint stress prior to acute kidney damage in mice reduced markers of renal inflammation (Abe *et al.* 2017), suggesting that C1 activation could reduce systemic inflammation by activating the neural inflammatory reflex. This finding is contrary to our hypothesis linking activation of SNS to a pro-inflammatory state in both HFrEF and HFpEF since in both cases chronic activation of C1 neurones is observed (DelRio*etal.*2013;Toledo*etal.* 2017) in conjunction with systemic inflammation (Xu & Li, 2015; van Empel & Brunner-La Rocca, 2015*a*). Also, both HF syndromes are accompanied by decreased vagal activity (Gronda & Vanoli, 2016; Andrade *et al.* 2017*a*), which would indicate that the inflammatory reflex should be also depressed in HF. Hence,

available evidence suggests that the actual contribution of this reflex to human and experimental HF pathophysiology is minimum.

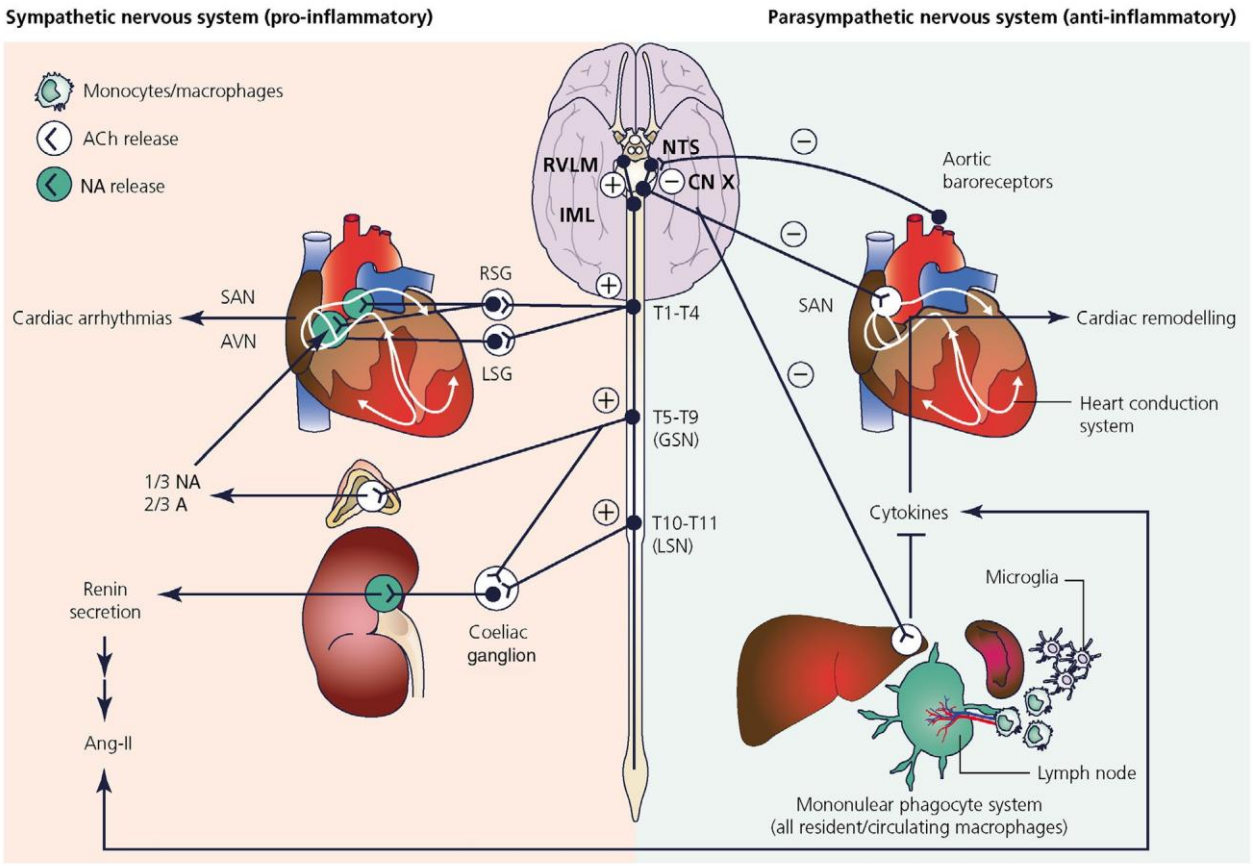

Figure 5. Role of autonomic nervous system in heart failure pathophysiology: pro-inflammatory and anti-inflammatory

Brainstem SNS control nuclei also modulate both sympathetic and parasympathetic nervous system activity. RVLM neurones' chronic hyperactivation during HF results in increased sympathetic activity (left panel). Sympathetic activity is transmitted from brain to target organs through the IML, which sends cholinergic (preganglionic) fibres to different sympathetic ganglia or effector organs from different levels of spinal cord, such as (i) the left and right stellate ganglia (LSG and RSG), liberating noradrenaline (NA) in the heart conduction system, promoting cardiac arrhythmias; (ii) the coeliac ganglion, promoting renin liberation from kidney and persistent RAS activation; (iii) the adrenal gland, which liberates adrenaline (A) and NA, augmenting sympathetic activity. In conjunction, these overall processes result in a vicious cycle of sympathoexcitation and RAS activation, and consequent chronic inflammatory state, worsening cardiac function. On the other hand, the parasympathetic nervous system exerts anti-arrhythmic and anti-inflammatory effects through cholinergic efferent projections to the heart conduction system and other cholinergic projections that regulate the neural inflammatory reflex (right panel), respectively. This reflex is regulated by the NTS and the vagus (CN X), which receive excitatory inputs from aortic baroreceptors and/or are activated in response to acute inflammation, and then inhibit pro-inflammatory cytokine production by mechanisms dependent on acetylcholine (ACh) and α7-nicotinic receptors expressed by the mononuclear phagocyte system (which comprises all blood-borne and resident macrophages). However, during HF, baroreceptor sensitivity and vagal efferent activity are decreased (independent of HF aetiology). This, in junction with persistent inflammation that is already present as a product of HF pathophysiology and increased sympathoexcitation (that indirectly promotes inflammation), results in overcoming the capacity of the neural inflammatory reflex to reduce inflammation. Ang-II, angiotensin II; AVN, atrioventricular node; GSN, greater splanchnic nerve; LSN, lesser splanchnic nerve; NTS, nucleus of the solitary tract; SAN, sinoatrial node.

Table 1. Comparison of overall haemodynamic and neurohumoral responses between HFrEF *vs.* HFpEF

	HFrEF				HFpEF			
Prevalent	Myocardial infarction, coronary artery disease	Diabetes mellitus 2, comorbidities			obesity, age			
Cardiac function								
Ejection fraction	↓				Preserved			
Systolic function	Impaired				Preserved at early stages and impaired at terminal ones			
Diastolic	Preserved at early stages and impaired at terminal ones	Impaired function						
LV Hypertrophy	↑				↑			
Chemoreflex	↑ = drive							
Arrhythmias	↑				↑			
Neurohumoral factors (peripheral)								
SNA*	↑ CSNA		↑ RSNA		↑ CSNA		↑ RSNA	
NA*ǂ	↑				↑			
Cytokines*ǂ↑	↑ Ang-II*ǂ↑	↑ ER Stress*↑	?					
Brain	PVN	RVLM	NTS	CVOs	PVN	RVLM	NTS	CVOs
Humoral								
RAS	↑	↑	↑	↑	↑	↑	↑	↑
Cytokines	↑	↑	?	↑	?	?	?	?
ER stress	↑	?	?	↑	?	?	?	?
Glutamate	↑	↑	↓	?	?	?	?	?
GABA	↓	↓	?	?	?	?	?	? NO
	↓	↓	↓	↓	?	?	?	?
Cellular								
Neurones	↑	↑	↑	↑	?	↑	↑	↑
Astrocytes	↑	↑	↑	?	?	?	?	?
Microglia	↑	↑	↑	↑	?	?	?	?
BBB	↑	↑ impairment						

*Age-related; ǂ, associated with increased mortality. Downward arrows indicate diminished function or blunted cell response while upward ones indicate augmented function or cellular response, and an equals sign indicates no differences compared to control animals or patients. ?, not assessed yet. Ang-II, angiotensin-II; BBB, blood–brain barrier; CSNA, cardiac sympathetic nerve activity; CVOs, circumventricular organs; ER, endoplasmic reticulum; GABA, γ-aminobutyric acid; HFpEF, heart failure with preserved ejection fraction; HFrEF, heart failure with reduced ejection fraction; LV, left ventricle; SNA, sympathetic nerve activity; NA, noradrenaline; NO, nitric oxide; NTS, nucleus of the solitary tract; PVN, hypothalamic paraventricular nucleus; RAS, renin–angiotensin system; RSNA, renal sympathetic nerve activity; RVLM, rostral ventrolateral medulla.

Relationship between neuroinflammation and sympathoexcitation in heart failure

In HF, RAS peptides and pro-inflammatiory cytokines are found in the myocardium, plasma and brain (Szczepanska-Sadowska *et al.* 2010; Xu & Li, 2015). Inflammation triggers activation of neurones, astrocytes and microglia (Kannan *et al.* 1996; Guggilam *et al.* 2007; Hermann & Rogers, 2009), leading to a chronic neuroinflammatory state that triggers sympathoexcitation and contributes to cardiac dysfunction (Rana *et al.* 2010; Potapenko *et al.* 2012); in fact, TNF-α knockout or central TNF-α

inhibition results in a dramatic reduction of mortality after MI in HFrEF mice (Guggilam *et al.* 2008). Central infusion of prostaglandins or cytokines induces sympathoexcitation and pressor responses (Feuerstein *etal.* 1982; Guggilam *et al.*2007), and increased expression of pro-inflammatory cytokines has been observed in HFrEF animals at the level of PVN (Guggilam *et al.* 2008), NTS (Wei *et al.* 2016*b*), RVLM (Francis *et al.* 2004*b*), and CVOs (Wei *et al.* 2016*b*). Accordingly, central cytokine inhibition reduces autonomic dysfunction in HFrEF animals (Guggilam *et al.* 2008; Kang *et al.* 2009, 2011; Dworak *et al.* 2014). Therefore, neuroinflammation plays a key role in HF pathophysiology and understanding its molecular and cellular basis could permit us to develop novel pharmacological strategies for HF management.

Neuroinflammation results from integration of a complex array of signals coming from many cell types present in specific brain areas (Aloisi, 2001; Dong & Benveniste, 2001; Carson *et al.* 2006). Important components of this process include production of inflammatory mediators (TNF-α, IL-1β) and oxidants (superoxide, peroxynitrite) by microglial cells, excessive glutamate and ATP release by astrocytes, and subsequent uncontrolled neuronal hyperactivity (Hauss-Wegrzyniak *et al.* 1998). It is widely accepted that neuroinflammation plays an important role in age-related neurodegenerative diseases such as Alzheimer's and Parkinson's diseases (Becher *et al.* 2016); however, neuroinflammation in CVDs is restricted to autonomic control areas (Rana *et al.* 2010; Dworak *et al.* 2014; Biancardi *et al.* 2016), contrary to Alzheimer's and Parkinson's diseases, on which wide brain areas are affected and massive neuronal death is observed (Hauss-Wegrzyniak *et al.* 1998). In fact, in HFpEF and HFrEF no neuronal death has been observed in those areas shown to be affected by neuroinflammation (Dworak *et al.* 2014; Andrade *et al.* 2019). In CVDs, neuroinflammation induces sympathoexitation by mechanisms dependent on Ang-II/NF-κB signalling, with increased ROS and pro-inflammatory cytokines. Subsequent activation of pre-sympathetic neurones occurs as a result of an imbalance between excitatory and inhibitory inputs in response to neuroinflammatory insults (Fig. 6). The mechanisms underlying this spatially selective neuroinflammation in SNS control nuclei in HF remain to be elucidated.

TNF-α and IL-1β receptors are present throughout the brain (Kinouchi *et al.* 1991; Wei *et al.* 2013), and these cytokines are produced *in situ* mainly by microglia (Aloisi,2001; Rana*etal.*2010; Kapoor*etal.*2016*a*).TNF-α and IL-1β signalling activates NF-κB, promoting cytokine synthesis and AT1 upregulation (Szczepanska-Sadowska *et al.* 2010; Xu & Li, 2015). Cytokine signalling also induces JNK and p38-mitogen-activated protein kinase (MAPK) phosphorylation, which promotes activation of AP-1 proteins (c-Jun, JunD, c-Fos, FosB/Fra1). Indeed, FosB, a classical marker of neuronal activation, is critical for cytokine synthesis in the brain (Nomaru *et al.* 2014) and for peripheral macrophages (Murray & Wynn, 2011). In animal models of HFrEF, neuronal AT1 upregulation in the RVLM is dependent on AP-1 (Lui *et al.* 2006), and augments the expression of catecholamines in RVLM C1-like cells (Swanson *et al.* 1998). This mechanism could explain, at least in part, the association between upregulation of cytokines/RAS and sympathoexcitation.

Pro-inflammatory cytokines also modulate neurotransmission (Fig. 6). TNF-α and IL-1β upregulate AMPA/kainate receptor expression via NF-κB, enhancing excitatory postsynaptic currents (Furukawa & Mattson, 1998). TNF-α also induces endocytosis of GABA-A receptors, diminishing inhibitory postsynaptic currents, and enhancing L-type calcium currents via inositol 1,4,5-trisphosphate signalling (Furukawa & Mattson, 1998), leading to a chronic excitatory state. In addition to modulating synaptic transmission by altering receptor/ion channel expression and function, inflammation can also exert effects on neurotransmission by altering neurotransmitter balance. Previous work shows that excitatory neurotransmitters (i.e. glutamate and catecholamines) are increased and inhibitory ones (i.e. GABA and NO) are decreased in the PVN of

HFrEF rats (Kang *et al.* 2011), and this phenomenon is prevented by central cytokine antagonism or NF-κB blockade (Guggilam *et al.* 2008; Kang *et al.* 2009). Similar alterations in the balance between excitatory and inhibitory neurotransmitters has been reported in the NTS and the RVLM in HFrEF (Patel *et al.* 1996; Wang *et al.* 2009; Kumagai *et al.* 2012), but little is known about this phenomenon in HFpEF (Table 1).

Increases in central TNF-α that may contribute to the alterations outlined above occur rapidly after an insult to the cardiovascular system. Selective increases in TNF-α have been observed in the rat hypothalamus and brainstem within 30 min post-MI (Francis *et al.* 2004*a*,*b*). A second wave of cytokines is detected 3h later and is associated with c-Fos glial activity (Vitkovic *et al.* 2000). Interference with this pro-inflammatory cascade post-MI has the potential to affect both autonomic and cardiac function, but current evidence is equivocal. Continuous intraperitoneal infusion of the cytokine synthesis inhibitor pentoxifylline (PTX) for 4 weeks after MI dramatically prevented TNF-α increase in both plasma and PVN; however, PTX treatment was not able to prevent cardiac dysfunction (Francis *et al.* 2004*a*). In contrast, in another study performed by Kang *et al.*, continuous intracerebroventricular infusion of PTX or etanercept (a TNF-α blocker) prevented the increase in plasma cytokines, reduced the level of Fos related antigens (Fra) immunorectivity and restored neurotransmitter imbalance in the PVN, and improved cardiac function in HFrEF rats (Kang *et al.* 2009). In this same study, intraperitoneal infusion of PTX or etanercept had no effects on brain or plasma cytokines (Kang *et al.* 2009). These conflicting results may be associated with differences in the concentrations of inhibitor used and/or the administration route, but in either case the relationship between neuroinflammation and peripheral inflammation in HF requires further study.

It has been proposed that hyper-activation of nuclei involved in generation/modulation of sympathetic activity *per se* could contribute to neuroinflammation in HF. Indeed, it has been shown that increases in NMDA currents can upregulate TNF-α and IL-1β synthesis in glial cells (Park & Bowers, 2010), and that destroying cardiac sympathetic afferents prevents TNF-α and IL-1β upregulation in the hypothalamus and brainstem post-MI (Francis *et al.* 2004*b*). Together, these results suggest that afferent input from the periphery may precipitate and/or maintain neuroinflammation. The precise stimuli responsible for the onset of neuroinflammation in HF remain to be elucidated and deserve further investigation.

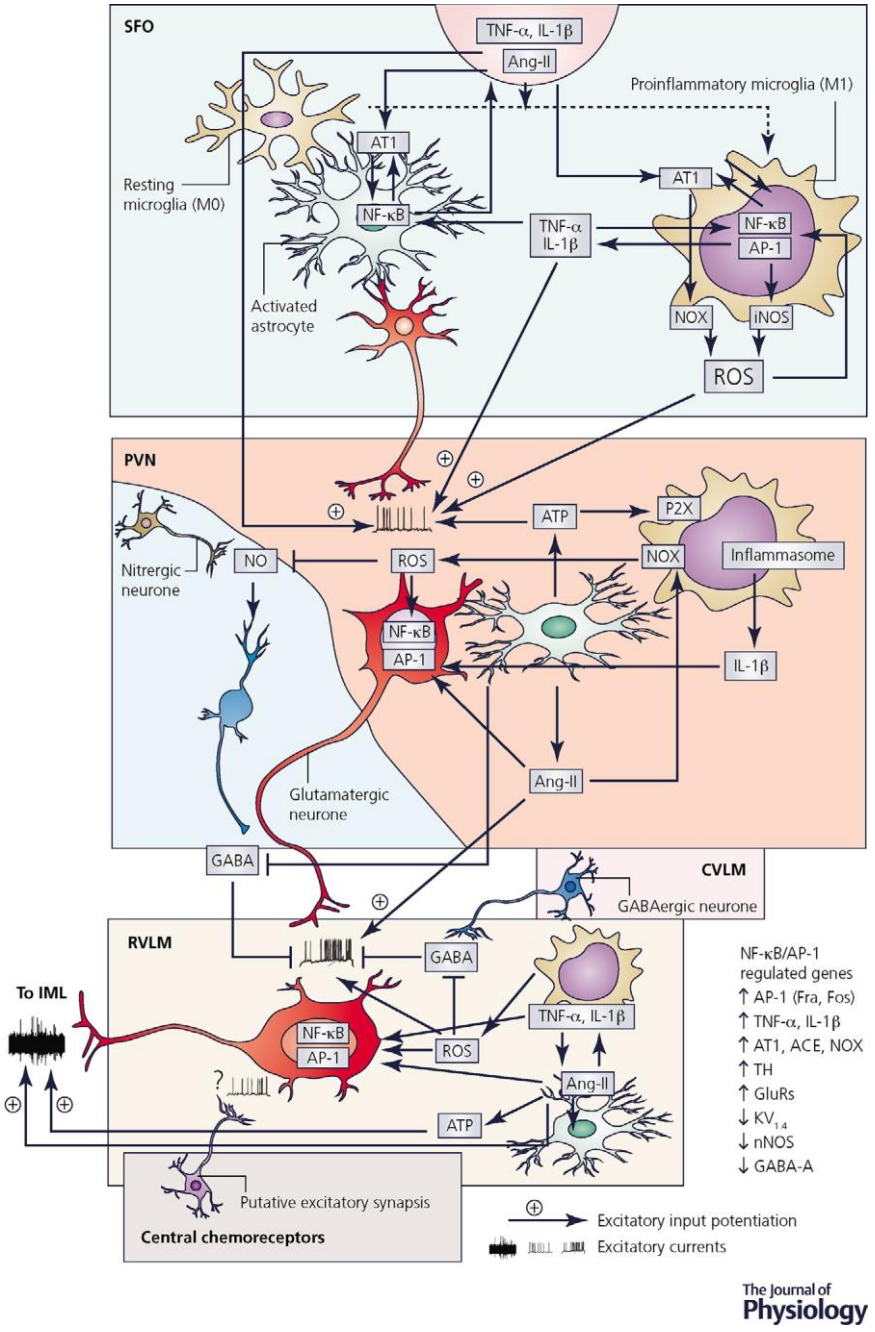

Figure 6. Cellular and molecular mechanisms of neuroinflammation in HF: central role of RAS
Neuroinflammation in HF is restricted to key areas of sympathetic control and results from integration of a complex array of signals coming from many cell types, including (i) production of proinflammatory cytokines and ROS by microglia, (ii) excessive Ang-II and ATP release by astrocytes, and (iii) subsequent chronic pre-sympathetic neuronal activation produced from imbalances between excitatory and inhibitory inputs in response to neuroinflammatory insults. Neuroinflammation induces sympathoexitation by mechanisms dependent on Ang-II/NF-κB signalling, increasing ROS and pro-inflammatory cytokines. Note that pathophysiological mechanisms responsible of inflammation-mediated cardiotoxicity and neuroinflammation are similar, and that neurones, astrocytes and microglia are intimately connected, forming a 'quad-partite' synapse. In CVOs, like the SFO, blood-borne Ang-II and cytokines induce the activation of astrocytes and the differentiation of resting microglia to the proinflammatory M1 phenotype, which produce large amounts of cytokines and ROS, which in turn induce *de novo* production of Ang-II by astrocytes and neurones via NF-κB, perpetuating chronic RAS activation. In this nucleus, TNF-α, ROS and Ang-II augment pre-sympathetic neurone firing, which triggers the activation of deeper nuclei synaptically connected within the SNS control network. The activity of PVN pre-sympathetic neurones is

crucially modulated by GABAergic and nitrergic interneurones surrounding the PVN. NO tunes the firing activity of PVN neurones that project to the RVLM by potentiating GABAergic presynaptic currents. Chronically activated neurones, astrocytes and microglia have been observed in HF within this nucleus, where Ang-II has been shown to potentiate excitatory currents in SFO–PVN and PVN–RVLM glutamatergic (excitatory) synapses and inhibit GABAergic PVN–RVLM currents. Also, microglial ROS diminishes NO bioavailability and activated astrocytes internalize GABA, turning the synaptic balance to glutamatergic transmission. In addition, activated astrocytes secrete large amounts of ATP, which augments neurone firing and induces microglial inflammasome activation by mechanisms dependent on P2X receptors/channels and Ang-II signalling, promoting chronic activation of pre-sympathetic neurones. In the RVLM, similar cellular and molecular mechanisms of neuroinflammation have been observed; however, a simpler scheme is shown to avoid redundancies. Brain RAS signalling within these brain SNS control nuclei perpetuates neuroinflammation and neuronal activation by (i) upregulating AP-1 protein, which promotes neuronal and microglial activation; (ii) augmenting the expression of NOX, AT1 receptor and ACE, maintaining RAS activation (and consequent microglial and astrocytic activation); (iii) augmenting expression of proinflammatory cytokines such as TNF-α and IL-1β, (iv) upregulating genes related to excitatory neurotransmitters such as TH and glutamate receptors; and (v) decreasing expression of inhibitory current-related genes such as those of GluRs and diminishing GABA-A receptor and nNOS density, which leads to an excitatory state, resulting in chronic sympathoexcitation and cardiac function impairment. ACE, angiotensin converting enzyme; Ang-II, angiotensin-II; AP-1, activator protein-1; GluRs, glutamate receptors; iNOS, inducible nitric oxide synthase; IL-1β, interleukin-1β; $KV_{1.4}$, voltage-gated K_+ channel; NOX, NAD(P)H oxidase; nNOS, neuronal nitric oxide synthase; TH, tyrosine hydroxylase; TNF-α, tumour necrosis factor-α

The brain RAS in heart failure

While there are a number of potential insults that could precipitate neuroinflammation in HF, previous studies indicate that activation of tissue-specific RAS in the brain (Ganten*etal*.1971;Richoux*etal*.1988) plays an important role in the brain inflammatory process (Sumners *et al.* 1991; Ferguson & Li, 1996; Ferguson *et al.* 2001; Kumagai *et al.* 2012; Rodriguez-Perez *et al.* 2015; Xu & Li, 2015). All necessary components of a functional RAS have been found in the cerebral cortex, hypothalamus and brainstem (Richoux *et al.* 1988; Paul *et al.* 2006), and high concentrations of ACE have been measured in rat and human cerebrospinal fluid (CSF) (Ito *et al.* 1980; Naruse *et al.* 1985; McKinley *et al.* 1990). Angiotensinogen and ACE levels are elevated in the CSF of hypertensive rats and humans (Ruiz *et al.* 1983; Genain *et al.* 1984), and it has been shown that brain RAS is upregulated in brainstem nuclei involved in sympathetic control in both HFrEF and HFpEF (Riegger, 1991; Yoshimura *et al.* 2000; Shigematsu *et al.* 2001; Francis *et al.* 2004*a*,*b*; Kang *et al.* 2011; Sharma *et al.* 2013; Zera *et al.* 2015).

From a pharmacological perspective, Ang-II exerts neuroexcitatory and pro-inflammatory effects via AT1 receptors, which are antagonized by AT2 receptor activation (Unger, 1999; Abdalla *et al.* 2001). Conversely, Ang-I/III/IV are thought to be inactive, while the special angiotensin peptide Ang(1–7) inhibits Ang-II/AT1 effects through Ang(1-7)/MAS signalling (Santos *et al.* 2000). In this review, we will focus on Ang-II and AT1 receptors as it is widely appreciated that Ang-II/AT1 signalling plays a key role in HF progression at both central and peripheral levels, and because Ang-II competition experiments have demonstrated that 95% of Ang-II binding sites correspond to AT1 in whole brain and hypothalamic–brainstem preparations (Sumners *et al.* 1991).

AT1 is expressed in astrocytes and neurones (Sumners *et al.* 1991; Paul *et al.* 2006), but is believed to be absent in resting microglia. Interestingly, the strongest brain immunoreactivity for AT1 receptors is found in the CVOs, followed by the PVN, the NTS and the RVLM, where astrocytes are thought to produce and deliver Ang-II to neurones (McKinley *et al.* 2003). Angiotensinogen is constitutively produced by astrocytes and then cleaved in the extracellular milieu (Sumners *et al.* 1991). Historically, the enzymes responsible for angiotensinogen processing in the brain were unknown, and it was proposed that Ang-II

production resulted from cleavage of angiotensinogen by non-specific proteases (McKinley *et al.* 2003). More recent studies have demonstrated the existence of (pro)-renin receptors (responsible of angiotensinogen cleavage to Ang-I) in astrocytes (Li *et al.* 2012) and that expression of the gene for ACE (which cleaves Ang-I to Ang-II) is dependent on AT1/NF-κB activation (Liu *et al.* 2006; Gowrisankar & Clark, 2016), confirming the essential role of astrocytes in the control of brain RAS activity. The importance of these central RAS pathways in HF comes from studies showing that astrocyte-specific AT1 ablation significantly improves survival in MI-HF mice by reducing RVLM neuronal activation, sympathoexcitation and left ventricular (LV) remodelling (Isegawa *et al.* 2014).

Ang-II acts as a potent neurotransmitter within the CNS and exerts excitatory actions on many of the autonomic nuclei involved in SNS control (Fig. 6). Ang-II acts in the SFO, AP, NTS, PVN and RVLM (Jeulin & Nicolaidis,

1988; Kumagai *et al.* 2012), by potentiating excitatory currents (Ferguson & Bains, 1997; Wang *et al.* 2004, 2013; Cato & Toney, 2005) and weakening inhibitory ones (Ferguson & Li, 1996; Li *et al.* 2003*a*; Li & Pan, 2005). Outside the CNS, Ang-II alters cardiovascular reflex function by acting on CB glomus cells to increase carotid sinus nerve firing activity (Allen, 1998), and promoting sympathoexcitation in HFrEF (Li *et al.* 2006; Del Rio *et al.* 2013, 2015; Andrade *et al.* 2015). These effects have been shown to be AT1-dependent and AT2-independent, and surprisingly, G-protein-independent (Li & Pan, 2005). Therefore, chronic Ang-II elevation in HF can directly mediate sympathoexcitation by acting as a neurotransmitter, or potentially indirectly by promoting neuroinflammation (Fig. 3).

As PVN, NTS and RVLM neurones are situated within the BBB, it is believed that local Ang-II generated by surrounding astrocytes stimulates these neurones, while SFO and AP neurons can be stimulated by both astrocyte- and plasma-derived Ang-II (Figs 3 and 6). Brain RAS has been widely studied in hypertension and HFrEF, but little is known about its activity in HFpEF (Table 1). In HFpEF rats, endogenous Ang-II excites NTS neurones (Shigematsu *et al.* 2001) and AT1 is upregulated in the SFO, the PVN, and the RVLM (Yoshimura *et al.* 2000). However, the precise stimuli that upregulate brain RAS in both HFrEF and HFpEF are still unclear.

In addition to the important effects of circulating Ang-II on CVOs and synaptically connected nuclei, circulating Ang-II may also potentially affect autonomic nuclei within the brain parenchyma by compromising the integrity of the BBB. It has been reported that peripheral Ang-II gains access to the NTS, the PVN and the RVLM in spontaneously hypertensive rats (SHR) via BBB impairment (Biancardi *et al.* 2016). Ang-II was shown to colocalize with neurones and microglia in the NTS, the PVN and the RVLM, and the AT1 blocker losartan prevented BBB impairment. In this study, the use of the vasodilator hydralazine had no effect on BBB permeability, indicating that BBB disruption is dependent on Ang-II/AT1 signalling (Biancardi *et al.* 2014). This novel mechanism requires further study in the context of HF as plasma levels of Ang-II are similar in hypertensive and HF patients (Fyhrquist*et al.*1972;Roig*et al.*2000)and recent studies have reported increased BBB permeability in HFrEF and HFpEF (Werf *et al.* 2015; Adamski *et al.*

2018).

Inflammatory cells in the brain: astrocytes, microglia and more

Early theories of inflammation-mediated sympathoexcitation in HFrEF centred around cytokine-mediated activation of the hypothalamic–pituitary– adrenal axis (McCann *et al.* 2000). Cytokine synthesis was thought to result from macrophage-derived prostaglandins (Riegger, 1991), which can cross the BBB and induce cytokine synthesis within the PVN (Feuerstein *et al.* 1982). However, studies showing that ablation of perivascular macrophages failed to prevent the increase in TNF-α in plasma and the PVN in HFrEF rats (Yu *et al.* 2010) suggest that the main cytokine source within the CNS may not be perivascular macrophages. Further research confirmed that microglia are primary contributors to hypothalamic TNF-α and IL-1β production in HFrEF (Rana *et al.* 2010), but the mechanisms by which microglia are activated in brain autonomic control areas remain unknown.

Astrocytes. Astrocytes anatomically and functionally connect neuronal synapses with brain blood vessels (Volterra & Meldolesi, 2005) and contact multiple preand postsynaptic neurones, constituting the 'tripartite synapse' (Araque *et al*. 1999). There are at least four astrocyte subsets involved in maintaining brain homeostasis: resting (surveillant), activated, reactive and BBB astrocytes (Khakh & Sofroniew, 2015). Astrocytes actively sense neuronal activity and secrete neuroactive substances, such as glutamate and ATP for maintaining correct synaptic function (Chao *et al*. 1995; Giaume *et al*. 2013; Khakh&Sofroniew,2015). In particular, chronic astrocyte activation results in increased neural activity (Marina*etal*. 2013), suggesting that astrocytes could be important in HF pathophysiology, since increased astrocytic activation has been observed in SNS control areas in HFrEF (Table 1) and in the RVLM glutamate (also secreted by astrocytes) is crucial to autonomic dysfunction associated with HF (Kumagai *et al*. 2012). In fact, chronic activation of RVLM astrocytes is associated with increased mortality in HFrEF animals (Isegawa *et al*. 2014).

In addition to neuronal activity, Ang-II and pro-inflammatory cytokines also activate astrocytes, promoting brain RAS upregulation, synthesis of pro-inflammatory cytokines and astrocyte proliferation (Sumners *et al*. 1994; McKinley *et al*. 2003). Both cytokines and Ang-II activate NF-κB, which promotes ACE transcription (Gowrisankar & Clark, 2016), augmenting local Ang-II levels in HF, which augments neuronal activity and promotes neuroinflammation (Kang *et al*. 2011; Xu & Li, 2015). Therefore, it is plausible that peripheral cytokine signalling through BBB astrocytes and CVOs could induce astrocyte activation, promoting activation of surrounding microglial cells, and then propagate RAS activation and neuroinflammation to deeper nuclei in the SFO–PVN–RVLM network. In fact, activated astrocytes have been found in the PVN and the RVLM in HFrEF rats, and they are associated with neuroinflammation and sympathoexcitation (Marina *et al*. 2013; Kim *et al*. 2015). In the RVLM, optogenetic stimulation of astrocytes induces sympathoexcitation by exciting C1 neurones (Marina *et al*. 2013). In the PVN, activated astrocytes decrease GABA levels and GABA-A receptor expression in HFrEF animals, resulting in chronic activation of pre-sympathetic neurones (Kim *et al*. 2015). After MI, astrocyte-derived ATP is released to the extracellular milieu in the PVN and is taken up by neurones and microglia. This process results in neurone hyper-excitability and neuroinflammation by mechanisms dependent on P2X7 signalling (Carson *et al*. 2006; Du *et al*. 2015). P2X receptors are ATP-gated ion channels that once activated induce membrane depolarization, augmenting neurone firing (Volonte *et al*. 2012) and inflammasome activation (a fundamental step for IL-1β processing in microglia and macrophages) (Aloisi, 2001). In previous studies, selective inhibition of ATP release from RVLM astrocytes diminished sympathoexcitation and cardiac dysfunctioninHFrEFrats(Marina*etal*.2013),and similar results have been reported in HF mice with selective ablation of astrocyte AT1 (Isegawa *et al*. 2014). Taken together, these studies suggest that astrocyte-mediated neuroinflammation could play an important role in HFrEF progression. To date, no studies have addressed astrocytic activation in HFpEF (Table 1).

BBB astrocytes could also play a role in HF progression by modulating BBB integrity. Indeed, BBB impairment in SNS control areas

has been shown in HFrEF rats (Werf *et al.* 2015), and very high AT1 density has been reported in CVO astrocytes (McKinley *et al.* 2003). It is plausible that increases in peripheral Ang-II and cytokines during early stages of HF could activate BBB astrocytes residing in CVOs and disrupt neuro-glio-vascular units. This BBB dysfunction could permit Ang-II to access deeper areas, activating resting astrocytes in SNS control nuclei, propagating neuroinflammation and sympathoexcitation. While this hypothesis has not been specifically tested in HF models, it has been shown that peripherally administered Ang-II induces BBB disruption in mice, by mechanisms dependent on microvascular inflammation and ROS (Zhang*etal*.2010).Furthermore, pro-inflammatory cytokines and ROS induce BBB impairment (Nimmerjahn *et al.* 2005; Abbott *et al.* 2006) by Ang-II/AT1-dependent signalling (Biancardi *et al.* 2016; Biancardi & Stern, 2016). Finally, some studies suggest a role for ATP in BBB dysfunction as well. Suppression of P2X7 (mainly expressed by BBB astrocytes) prevents BBB disruption after cerebral ischaemia via RhoA downregulation (Zhao *et al.* 2016). This finding further underscores a potential link between BBB dysfunction and neuroinflammation, as RhoA/Rho-kinase signalling is implicated in endothelial dysfunction (Shatanawi*etal*.2011) and microglial activation (Rodriguez-Perez *et al.* 2015). In the context of HFpEF, BBB dysfunction has been reported in cortex and pons (Adamsky*etal*.2018); however, this study was mainly focused in cortex areas related to cognitive impairment and no major attention was put in SNS control areas. Future studies are needed to address the contribution of altered BBB integrity to autonomic dysregulation and cardiac dysfunction in HF.

Microglia. Microglia are the resident macrophages of the CNS and play an essential role in regulation of neuroinflammation and neuronal activity (Nimmerjahn *et al.* 2005). Microglial cells develop physical contacts with astrocytes and presynaptic neurones, regulating neurotransmission, forming a 'quad-partite synapse' (Schafer *et al.* 2013). Microglia are present in the six main brain regions (Yang *et al.* 2013), and activated microglia have been observed in key autonomic control areas in hypertension and HF (Rana *et al.* 2010; Biancardi *et al.* 2014; Kapoor *et al.* 2016*a*). Like macrophages, microglia are characterized by three major phenotypes: the resting M0 phenotype and the activated M1 (pro-inflammatory, $CD11b^{High}iNOS^{High}$) and M2 (anti-inflammatory, $CD11b^{High}Arginase1^{High}CD206^{High}$) phenotypes, all of which are CD11b-positve and

Iba-1-positive (Crain *et al.* 2013).

Several reports have suggest that brain RAS contributes to neuroinflammation by promoting the microglial M1 phenotype via NADPH oxidase (Rey *et al.* 2007; Joglar *et al.* 2009), p38-MAPK (Rodriguez-Perez *et al.* 2015) and RhoA/Rho kinase-dependent pathways (Barcia *et al.* 2012). In primary microglia, Ang-II-induced increases in cytokine expression are abolished by the microglial inhibitor minocycline (Shi *et al.* 2014). Furthermore, AT1 is upregulated in activated microglia (Wu *et al.* 2013) and intracerebroventricular infusion of minocycline attenuates cardiac dysfunction and elevated plasma noradrenaline after chronic Ang-II infusion (Shi *et al.* 2010*a*). Finally, in a mouse model of constitutively overactivated brain RAS, inflammatory microglia have been observed in the PVN and the RVLM (Shi *et al.* 2014).

With respect to HF, activated microglia have been shown to colocalize with activated (Fra-positive) neurones in the PVN, the RVLM and the NTS of post-MI rats (Rana *et al.* 2010, Dworak *et al.* 2014). In these studies, microglial activation was not accompanied by neuronal cell death, as neuronal count was similar in the PVN, the RVLM and the NTS in HFrEF and Sham rats (Dworak *et al.*

2014). One explanation for this observation could be that the activated microglia observed could be a mixture of M1/M2 phenotypes, since both are $CD11b^{High}$ and M2 microglia secrete anti-inflammatory molecules that prevent neuronal cell death (Kapoor *et al.* 2016*a*); however, this hypothesis has not been assessed yet. Most research on activated microglia has focused on the M1 phenotypeonly (Kapoor *et al.* 2016*a*) and little is known about M2 microglia activation in brain pre-sympathetic nuclei in HFrEF. It is known that the M2 phenotype attenuates cardiovascular dysfunction and sympathoexcitation associated with intrathecal kainic acid injection in rats (Bhandare *etal.*2015). To date, no studies have addressed the potential role of activated microglia in autonomic and cardiac dysfunction in HFpEF. Future studies should attempt to characterize the microglial profiles in both HFrEF and HFpEF and any potential role they may play in sympathoexcitation and cardiac dysfunction.

While the exact mechanisms by which the microglial phenotype transitions to 'active' are unclear, there is evidence to suggest that this transition takes place in response to augmented astrocytic and neuronal activation (Kapoor *et al.* 2016*a*). Microglia express receptors for several neuroactive substances derived from both astrocytes and neurones (Pocock & Kettenmann, 2007). ATP induces M1 microglial activation via P2X7 receptor-mediated inflammasome activation and IL-1β production (Volonte *et al.* 2012). Catecholamines reduce microglial iNOS expression, TNF-α and IL-6 production through α1- and β1-adrenergic receptors, preventing neuronal cell death (Mori *et al.* 2002). AMPA and group I/II metabotropic glutamate receptors induce the M1 phenotype, while group III receptors induce the M2 phenotype (Domercq *et al.* 2013). While these findings in primary cultured microglia are promising, there is still no direct evidence linking synaptic activity and microglial activation. Further investigation is needed to characterize the microglial phenotypes associated with HF pathophysiology, as well as the stimuli responsible for their activation. We speculate that microglia play an essential role in the onset and maintenance of neuroinflammation in HF in response to rises in RAS activity produced by astrocyte activation, first, at CVOs, and in the following stages, at deeper SNS control areas within the SFO–PVN–RVLM axis, promoting chronic neural and astrocytic activation and cardiac function impairment.

Neurones. Neurones have also been shown to play a role in the neuroinflammatory response (Carson *et al.* 2006). Glutamate stimulation in FosB-null mice results in significant reductions in microglia-mediated cytokine release (Nomaru *et al.* 2014), suggesting that neuronal activation status modulates cytokine production by glial cells. It is worth noting that interpretation of this result is confounded by the fact that FosB is an AP-1 protein that is also responsible for cytokine synthesis by immune cells (Liu *et al.* 2006; Mehta & Griendling, 2007; Nomaru *et al.* 2014; Sriramula & Francis, 2015). In C1 neurone-like cells, Ang-II induces synthesis of pro-inflammatory cytokines (Agarwal *et al.*

2013) and AT1 upregulation (Mitra *et al.* 2010). Also, destruction of sympathetic afferents to the brain prevents increases in pro-inflammatory cytokines in the PVN after MI (Francis*et al.*2004*b*), suggesting that neurones play a role in neuroinflammation and RAS activation in brain SNS control areas during HF. Further study is needed to confirm a role of neuronal activation in neuroinflammation in this disease. The available evidence shows that neuroinflammation in HF is a complex process that integrates cellular and molecular responses, involving at least three different cellular lineages (Fig. 6).

Pro-inflammatory cytokines: from periphery to the brain

Numerous studies have shown that chronic inflammation in HF patients originates from both cardiac and non-cardiac comorbidities. Cardiomyocyte death triggers inflammatory cell activation in HFrEF and HFpEF, and chronic inflammation associated with ageing, hypertension and/or type-II diabetes can also lead to cardiac dysfunction (Anker & von Haehling, 2004; Ueland *et al.* 2015; van Empel & Brunner-La Rocca, 2015*b*). Until 2012 the brain parenchyma was thought to be impermeable to circulating Ang-II and cytokines under physiological conditions due to the BBB (Schelling *et al.* 1976; Yarlagadda *et al.* 2009; Roncevic, 2012). However, several studies suggest that Ang-II and cytokines gain access to the brain via the CVOs (John & Buckingham, 2003; Shi *et al.* 2010*b*; Wei *et al.* 2013) and BBB disruption, as shown in HFrEF and HFpEF (Werf *et al.* 2015; Adamski *et al.* 2018).

It has been shown that haemodynamic changes and increased SNS activity induced by peripherally administered IL-1β and TNF-α were prevented by SFO destruction (Wei *et al.* 2013). Since the authors observed a delayed haemodynamic response after cytokine injections (10–20 min), they suggested that cytokines trigger cellular responses that lead to increased neuronal excitability rather than directly affecting neurotransmission. Recent studies using patch-clamp techniques have shown that TNF-α augments firing rate of SFO neurones by altering the transient Na^+ current (Simpson & Ferguson, 2017). In these studies, 24 h pre-incubation with TNF-α augmented basal firing rate and neuronal excitability of SFO neurones (Simpson & Ferguson, 2017). In another study, TNF-α receptor 1 knock-down in the SFO diminished the levels of c-Fos, RAS components and pro-inflammatory cytokines in the SFO and the PVN, and ameliorated sympathoexcitation and cardiac dysfunction in HFrEF rats (Yu *et al.* 2017). These findings suggest that long-term exposure of CVOs to blood-borne pro-inflammatory cytokines could promote neuroinflammation and RAS activation in sympathetic control nuclei in HF. Previous work has shown that PVN activation by the SFO triggers activation of RVLM pre-ganglionic neurones (Cottrell & Ferguson, 2004; Llewellyn *et al.* 2014), which in turn could mediate the activation of the surrounding glial cells. Indeed, it has recently been shown that blood-borne IL-1β upregulates pro-inflammatory cytokines and RAS in the SFO and the PVN in healthy rats after 2–3 h, and that these changes are attenuated by microinjections of losartan directly into the SFO or after SFO destruction (Wei *et al.* 2018), demonstrating the strong relationship between RAS and neuroinflammation.

In addition, cytokine-induced BBB dysfunction has been proposed as a potential mechanism underlying neuroinflammation in

HF. Serum proteins are found in CSF after MI in rats (Werf *et al.* 2015), and TNF-α administration induces BBB disruption in the ventral brainstem, hippocampus and some cortical areas (Ter Horst *et al.* 1997; Liu *et al.* 2013). These data suggests that chronic exposure to plasma-derived inflammatory mediators could induce BBB disruption and permit the entrance of cytokines and Ang-II to deeper brain areas, promoting neuroinflammation and chronic neural activation in nuclei that regulate SNS activity. Therefore, the role of BBB disruption in HF progression is an area of intense interest, and important studies focused on BBB permeability in HF patients are currently under way.

Neuroinflammation in HF: preserved *vs.* reduced ejection fraction

Table 1 summarizes the main haemodynamic and neurohumoral derangements in HFrEF and HFpEF, as well as the current state of knowledge about cellular and molecular mechanisms of neuroinflammation in key brain autonomic control areas. Neuroinflammation is becoming recognized as an important contributor to CVD pathophysiology (Biancardi & Stern, 2016; Winklewski *et al.* 2016); however, at present, the body of research in this field specific to HF is limited. This is particularly true for HFpEF (Table 1), and to date there are no effective therapies for this HF aetiology. While the exact mechanisms underlying neuroinflammation in HF have not been elucidated, systemic inflammation could play a pivotal role in the neuroinflammatory state, since myocardial and systemic inflammation are common hallmarks in both HFpEF and HFrEF. However, the fact that therapies that are effective against HFrEF are ineffective or even exacerbate HFpEF suggest particular and distinct mechanisms underpinning the aetiology of each (Paulus & Tschope, 2013; van Empel & Brunner-La Rocca, 2015*a*). Nevertheless, as discussed previously, there are common pathophysiological substrates that may contribute to the maintenance and/or development of HF which deserve further investigation.

HFrEF. In HFrEF, since ischaemic cardiac comorbidities are more prevalent in this group (vanEmpel&Brunner-La Rocca, 2015*a*), the primary stimulus for inflammation could be cardiovascular damage *per se*, in which death of cardiomyocytes activates inflammatory cells via toll-like receptors (Fig. 1). The activation of RAS and SNS in response to ischaemia would also act as a secondary stimulus for inflammation in HFrEF, since ischaemia activatesAng-IIsignalling,MCP-1expressionintheheart, and consequent myocardial recruitment of peripheral and resident monocytes/macrophages (Matsuda *et al.* 2015). Plasma Ang-II and cytokines acting at the BBB could activate CVO neurones and consequently autonomic control nuclei in the early stages of HFrEF (Braga *et al.* 2011;Wei*etal*.2013).This process could be potentiated or exacerbated by hyperactive CB chemoreceptors stimulated by ischaemia and Ang-II (Allen, 1998; Del Rio *et al.* 2013; Andrade *et al.* 2015).

In the brain, microglial activation possibly occurs in conjunction with the second wave of cytokines (Vitkovic *et al.* 2000), which then increases cytokine synthesis and Ang-II concentrations, leading to chronically upregulated brain RAS (Paul *et al.* 2006; Shi *et al.* 2010*b*; Xu & Li, 2015). Upregulation of brain RAS leads to sustained activation of

pre-sympathetic neurones by (i) exciting neurones directly (Li *et al*. 2003*a*; Cato & Toney, 2005), (ii) altering neurotransmitter equilibrium via NF-κB (Kang *et al*. 2009; Kang *et al*. 2011; Kim *et al*. 2015) and (iii) reducing NO bioavailability, secondary to increases in microglial ROS production (Guggilam *et al*. 2008, 2011; Dworak *et al*. 2014). Sustained cytokine production may also contribute to neurone hypersensitization (Wei *et al*. 2013) and BBB impairment (Liu *et al*. 2013), allowing the entrance of blood-borne inflammatory factors which potentiate neuroinflammation and sympathoexcitation (Abstract Figure). Persistent sympathoexcitation in HFrEF contributes to further deterioration of cardiac function by altering cardiac contractility and promoting arrhythmias (Dean & Lab, 1989; Del Rio *et al*. 2013), LV hypertrophy (Kishi, 2012; Paulus & Tschope, 2013) and peripheral RAS activation (DiBona, 2000). The combination of sympathoexcitation with cardiac inflammation aggravates LV hypertrophy (Matsuda *et al*. 2015), and promotes sustained myofibroblast activation and development of cardiac fibrosis (Paulus & Tschope, 2013), which in turn alters cardiac contractility and rhythmicity (Sciarretta*etal*. 2009), and worsens cardiac function (Figs 1 and 2).

HFpEF. Inflammation is positively correlated with age (Franceschi *et al*. 2000; Franceschi & Campisi, 2014) and HFpEF patients tend to be older than HFrEF ones (Hummel & Kitzman, 2013), and have a higher prevalence of inflammatory non-cardiac comorbidities (van Empel & Brunner-La Rocca, 2015*a*). Accordingly, it is possible that chronic inflammation leads to monocyte recruitment to the heart (Juncos *et al*. 2011; Paulus & Tschope, 2013), promoting synthesis of cytokines and their release to the plasma (Porter *et al*. 2004). This would lead to peripheral RAS activation as well as brain RAS upregulation via CVO astrocytes and neurones (Sriramula & Francis, 2015). We hypothesize that this would promote neuroinflammation in key autonomic control areas of the brain (Fig. 6). At this point the notion of a neuroinflammatory response in HFpEF remains hypothetical (Table 1), and further investigations are necessary to determine its presence and underlying mechanisms as well as its relevance to sympathoexcitation and cardiac function in HF, despite evidence strongly suggesting that neuroinflammation occurs in HFpEF. Future studies should aim to quantify and compare the levels of cytokines and neurohumoral factors between HFpEF and HFrEF cohorts to get a better understanding of the pathophysiological differences between both syndromes and to identify novel treatment strategies.

HFrEF *vs.* HFpEF. Chemoreflex sensitivity is a novel candidate that may contribute to the observed differences between HFrEF and HFpEF (Andrade *et al*. 2017*a*; Del Rio *et al*. 2017; Toledo *et al*. 2017). The peripheral chemoreflex plays a crucial role in HFrEF pathophysiology, as it is chronically potentiated in HFrEF, and CB ablation or normalization reduces sympathetic activation, disordered breathing (Marcus *et al*. 2014), markers of C1 neuronal activation and cardiac dysfunction, and improves survival (Del Rio *et al*. 2013; Andrade *et al*. 2015). However, this potentiation of the peripheral chemoreflex seems to be unique to HFrEF as is not observed in HFpEF animals (Del Rio *et al*. 2017; Toledo *et al*. 2017; Andrade *et al*. 2019). In fact, central chemoreflex activity has been shown to be enhanced in HFpEF and is associated with sympathoexcitation and cardiac dysfunction

(Toledo *et al.* 2017). While the mechanisms for peripheral chemoreflex hypersensitization have been extensively studied, those responsible for central chemoreflex hypersensitization in HFpEF are unknown. Central chemoreceptive neurones likely send projections to the RVLM, since their stimulation by hypercapnia augments sympathetic activity in healthy animals and humans (Toledo *et al.* 2016), and cardiac dysfunction is aggravated during hypercapnia in HFpEF rats (Toledo *et al.* 2017). Thus, it is plausible that neuroinflammation in the RVLM promotes augmented central chemoreflex drive during HFpEF, since the main central chemoreceptors located in the retrotrapezoid nucleus are considered an extension of the ventrolateral medulla (Toledo*etal.*2016). Indeed, ablation of C1-RVLM neurones in HFpEF rats diminishes both baseline and hypercapnia-induced cardiac dysfunction (Andrade *et al.* 2019), suggesting that central chemoreceptors in HFpEF could play an important role in disease pathophysiology. However, additional evidence is required to determine the significance of this hypothesis as the role of inflammation on central chemoreceptor activity and its consequences for cardiac physiology in HF have not been studied.

Future perspectives

Current therapies for HFpEF are ineffective, underscoring an urgent need for new research to identify pharmacological or non-pharmacological treatment strategies for this disease. Findings showing that blockade of CNS inflammation markedly improves autonomic control, peripheral inflammation and cardiac function in HFrEF animals (Guggilam *et al.* 2008, 2011; Kang *et al.* 2011) suggests that neuroinflammation plays a crucial role in HF pathophysiology. Thus, future strategies for HF treatment should consider targeting neuroinflammation. The most obvious approach to this is RAS blockade in the brain,givenitscrucialroleinHFpathophysiology andthat RVLM RAS is directly related with increased mortality in HFrEF animals (Isegawa *et al.* 2014). In support of this notion, central administration of the AT1 blocker losartan greatly improved cardiac function and autonomic control in HF rats with no major effects on the physiology of healthy animals (Riegger, 1991; Yoshimura *et al.* 2000; Shigematsu *et al.* 2001; Liu *et al.* 2006; Kang *et al.* 2011; Sharma *et al.* 2013). Future preclinical studies should assess the effectiveness and pharmacokinetics of novel AT1 blockers capable of crossing the BBB and their effect on autonomic imbalance, cardiac function and neuroinflammation in HF. In this regard, telmisartan (AT1 blocker) is a promising candidate to treat HF, since it is the only FDA-approved RAS inhibitor capable of crossing the BBB and it is well-tolerated by patients (Ruilope, 2011).Indeed, CVD patients treated with telmisartan show greater improvement in clinical outcomes compared with those treated with other RASi nhibitors (Ruilope,2011).In hypertensive and HF rats, orally administered telmisartan reduces ROS levels in the RVLM and improves cardiac hypertrophy, autonomic imbalance and survival, when compared to candesartan, which is unable to cross the BBB (Kishi *et al.* 2014). Despite the promise of this drug, few studies using telmisartan in HF have been conducted, and the results of those that have are not conclusive (Bohm *et al.* 2017).

Endoplasmic reticulum stress has recently emerged as a potential novel target in HF treatment, as blocking this pathway is beneficial in several cardiac and non-cardiac chronic diseases (Vang *et al.* 2014). In the

setting of HF, tauroursodeoxycholic acid (TUDCA; an endoplasmic reticulum stress inhibitor) prevents cardiac fibrosis (Groenendyk *et al.* 2016), cardiomyocyte apoptosis (Chen *et al.* 2016), brain RAS activation, neuroinflammation and sympathoexcitation in HFrEF animals (Wei*etal*.2016*a*,*b*). TUDCA and its derivatives have been shown to cross the BBB, and are well-tolerated by humans (Vang *et al.* 2014). Further investigation is needed to better understand the mechanism of action of TUDCA in the brain and assess the effects of peripheral administration of this compound on brain and cardiac pathophysiology. Nothing is known about endoplasmic reticulum stress at either central or peripheral levels in HFpEF.

Finally, non-pharmacological treatment strategies such as exercise training may also be considered in the treatment of HFpEF. Recent research has shown that exercise training reduces ROS levels in the RVLM and restores autonomic control and cardiac function in HFpEF (Andrade *et al.* 2017*b*), suggesting its potential as a therapeutic strategy for HFpEF. It is worth noting that a subset of HFpEF patients display exercise intolerance (Paulus & Tschope, 2013; van Empel & Brunner-La Rocca, 2015*a*) and thus would not be well-suited for this therapeutic approach. New studies aiming to elucidate the mechanisms responsible for the beneficial effect of exercise training in HFpEF pathophysiology, as well as those responsible for exercise intolerance, are warranted.

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Additional information

Competing interests

None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

Author contributions

H.S.D., C.T., D.C.A., N.J.M. and R.D.R. contributed to the preparation of the manuscript. All authors have read and approved the final version of this manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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4. Capítulo 3: Efecto de TUDCA sobre la fisiopatología de la HFpEF

Si bien la destrucción selectiva de neuronas C1 RVLM resulta en un rescate de la función autonómica y cardiorrespiratoria en HFpEF, dicha estrategia carece de valor traslacional, por lo que nuevas aproximaciones son necesarias en la búsqueda de nuevas alternativas terapéuticas para este síndrome. Publicaciones previas en HF isquémica demuestran que la administración de TUDCA previo a la inducción quirúrgica de la insuficiencia cardiaca previene el alza en la actividad simpática y el deterioro de la función cardiaca en ratas HFrEF, sin embargo, la capacidad terapéutica de dicha droga una vez establecida la enfermedad, así como su potencial terapéutico en HFpEF no ha sido evaluado. Considerando que la revisión realizada en el capítulo anterior propone a la RVLM como una región clave en el establecimiento de la fisiopatología de la HFpEF y que ERS podría mediar los mecanismos centrales responsables de la disfunción cardiorrespiratoria en este síndrome, se determinó si la administración intracerebroventricular de TUDCA una vez establecida la enfermedad es capaz de detener la progresión de la enfermedad y restaurar la función cardiorrespiratoria en ratas HFpEF.

El tercer capítulo de esta tesis contempla el contexto de los objetivos específicos 1 a 3. Corresponde al artículo enviado a la revista Proceedings of the National Academy of Sciences (PNAS) en abril de 2020, titulado “Brainstem endoplasmic reticulum stress inhibition restores cardiorespiratory and autonomic function in non-ischemic heart failure” de **Hugo S. Díaz**, David C. Andrade, Camilo Toledo, Karla G. Schwarz, Katherin V. Pereyra, Esteban Díaz-Jara y Rodrigo Del Rio. A modo de resumen, se indujo HFpEF de forma quirúrgica en ratas Sprague-Dawley y se les administró TUDCA de forma central durante 4 semanas posterior a la inducción de la enfermedad. Comparadas con las ratas HFpEF tratadas con vehículo, la administración de TUDCA en HFpEF (HF+Veh vs. HF+TUDCA, $p<0.05$) redujo la hipertrofia cardiaca (HW/BW 4.4±0.3 vs. 4.0±0.1mg/g;) y produjo una marcada restauración de la función diastólica, además, la administración de TUDCA mejoró el control autonómico cardiaco, redujo la incidencia de arritmias y normalizó los desórdenes respiratorios. El análisis molecular reveló un aumento en la expresión

de biomarcadores de ERS, neuroinflamación y del Sistema Renina-Angiotensina en la RVLM en ratas con HFpEF y su consecuente disminución tras el tratamiento con TUDCA. En conjunto, los resultados sugieren que el tratamiento con TUDCA previno la progresión de la enfermedad y que su administración podría tener un efecto beneficioso sobre la fisiopatología de la HFpEF, lo que podría significar una nueva estrategia terapéutica para futuros estudios piloto.

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Main Manuscript for

Inhibition of brainstem endoplasmic reticulum stress restores cardiorespiratory and autonomic function in non-ischemic heart failure.

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HSD performed experiments, data analysis and manuscript preparation, DCA performed experimental procedures and revised manuscript, CT performed experimental procedures and manuscript preparation, KGS participated on manuscript redaction and experimental procedures, KVP participated on manuscript redaction, EDJ contributed on manuscript redaction and RDR approved experimental protocols and reviewed manuscript

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Figures 1 to 4

Abstract

Chronic activation of catecholaminergic neurons within the brainstem rostral ventrolateral medulla (RVLM), a major integration site for sympathetic activity regulation, play an essential role on cardiorespiratory alterations during non-ischemic heart failure (HF) pathophysiology. Endoplasmic reticulum stress (ERS) is known to participate in the development and progression of several cardiovascular diseases. Whether ERS in the brain contribute to non-ischemic HF progression/maintenance remains completely unknown. Accordingly, we aimed to determine the presence and contribution of brainstem ERS on cardiovascular and respiratory outcomes in non-ischemic HF rats. Adult male Sprague-Dawley rats underwent volume overload to induce non-ischemic HF. Tauroursodeoxycholic acid (TUDCA), an ERS inhibitor, was intracerebroventricularly delivered for 4 weeks after HF induction to assess the contribution of ERS on cardiorespiratory HF outcomes. Compared to vehicle treated HF rats, TUDCA administration in HF (HF+Veh vs. HF+TUDCA, $p<0.05$) lessened cardiac hypertrophy (HW/BW 4.4±0.3 vs. 4.0±0.1mg/g;) and markedly restored diastolic cardiac function (EDP: 4.9±0.6 vs. 3.7±0.4mmHg). In addition, TUDCA improved cardiac autonomic control (LF_{HRV}/HF_{HRV} ratio 3.02±0.29 vs. 1.14±0.24), reduced the incidence of cardiac arrhythmias (Arrhythmias: 141.5±26.7 vs. 35.67±12.5 events/h;) and normalized breathing disorders (Apneas: 11.83±2.26 vs. 4.33±1.80 events/h). Analysis of brainstem ERS-related genes expression confirmed the presence of ERS, inflammation and activation of brain renin-angiotensin signaling pathway in the RVLM and that TUDCA treatment completely abolished ERS and ERS-downstream targets in the RVLM of HF rats. Together our results support a salutary effect of TUDCA treatment for the control of cardiorespiratory dysfunction in of non-ischemic HF.

Significance Statement

Heart failure (HF) is among the major causes of death worldwide and its prevalence is increasing in time. In HF, sympathetic nervous system (SNS) dysfunction is related with increased mortality, and at date no therapeutic strategy is approved for non-ischemic heart failure. Brainstem catecholaminergic neurons have been identified as pivotal drivers of HF pathophysiology. However, its molecular and/or cellular mechanism underling HF has not been yet elucidated. The present study presents a brainstem pathway associated to endoplasmic reticulum stress (ERS) that may drive the autonomic and respiratory alterations during non-ischemic HF.

Main Text

Introduction

HF is a global pandemic affecting more than 26 million people worldwide (1) and its prevalence is expected to double the next decade (2). Despite the effort made over the past 20 years, no treatment has yet improved survival in HF patients (3). No current therapies are available for non-ischemic (diastolic) HF, which is equally prevalent and lethal as ischemic (systolic) HF (1). In HF, independently of its etiology, sympathoexcitation and respiratory disorders predicts poorer prognosis and higher mortality rates (4-7). We have previously shown that during HF, C1 catecholaminergic neurons of the brainstem rostral ventrolateral medulla (RVLM, a major source of sympathetic drive (5)) are chronically overactivated (8), and their specific ablation restores autonomic control, respiratory pattern and cardiac function (9, 10). However, the molecular mechanisms responsible on RVLM C1 neuron overactivation in non-ischemic HF have not been deeply investigated. Interestingly, increased brain Renin-Angiotensin System (BRAS) activity, in areas that control sympathetic nervous system (SNS), is crucial on both HF syndromes (11-16), by

promoting neuron firing (17, 18), microglial cytokines production (19, 20) and astrocytic Angiotensin-II processing (21, 22), resulting in a feedforward mechanism that perpetuates SNS and cardiorespiratory dysfunction during HF (5, 23).

Endoplasmic reticulum stress (ERS) is strongly related with increased BRAS activity, reactive oxygen species (ROS) and inflammation in brain nuclei that control SNS during cardiovascular disease, causing neuronal chronic activation and autonomic dysfunction (14, 24-26). Angiotensin-II administration mimics hypertension in healthy rats by inducing ERS in the RVLM and other brain areas of SNS control (24, 26). Conversely, ERS induction by tunicamycin upregulates BRAS and ROS in the RVLM, inducing hypertension (26). Tauroursodeoxycholic acid (TUDCA) is the taurine derivate of ursodeoxycholic acid (UDCA, an FDA-approved drug for treatment of liver disease (27)), that has been shown to inhibit ERS. Importantly, brain TUDCA administration, previous to surgical induction of systolic HF, prevents BRAS upregulation in SNS control nuclei and, likely, cardiac and autonomic dysfunction (14). ERS during ischemic HF has been associated with a series of downstream signaling cascades such as the mitogen-activated protein kinases (MAPKs), like the p38- and p44/42-MAPKs (14, 25), which are also characteristic downstream elements of BRAS in HF and connect ERS, BRAS and inflammation via activation of NF-kB (5, 23, 28). However, the expression of ERS biomarkers in the RVLM in both HF subsets has not been assessed yet, neither the ability of TUDCA to restore cardiac function after HF establishment. Considering that C1 RVLM neurons hyperactivation is associated with increased ROS and BRAS downstream elements activity such as NADPH oxidase-2 in non-ischemic HF (8, 29), it is plausible to speculate that ERS in the RVLM could be involved on the progression of this pathology.

Therefore, we addressed if intracerebroventricular administration of TUDCA after surgical induction of non-ischemic HF rats could prevent cardiorespiratory and autonomic dysfunction during disease progression. We found that TUDCA improved cardiac hypertrophy, accompanied with a marked restoration of cardiac diastolic function, autonomic control, arrhythmogenesis, breathing patterns and chemoreflex gain, which were associated with a decreased RVLM expression of ERS markers (BiP (binding immunoglobulin protein, GRP78), CHOP (C/EBP Homologous Protein) and spliced form of XBP1 (x-box binding protein-1)), BRAS downstream elements (AT1 (Angiotensin-II receptor), $gp91^{phox}$ (NADPH oxidase catalytic subunit)), proinflammatory cytokines (TNF-α, IL-1β). Thus, our study uncovers molecular mechanism of ERS in the major region of the brainstem that regulate the sympathetic outflow suggesting potential of TUDCA in treatment of non-ischemic HF.

Results

Intracerebroventricular TUDCA administration prevents cardiac hypertrophy and restores cardiac diastolic function in non-ischemic HF

To investigate the role of ERS on HF progression as well as the therapeutic potential of TUDCA, we surgically induced HF by the arteriovenous fistula technique (8, 9, 29-31) and 4 weeks after HF or Sham surgery, we chronically administered TUDCA (10 µg/day) or Vehicle (Veh, artificial cerebrospinal fluid) intracerebroventricularly (0.25 µl/h for 28 days, Fig. 1A). Echocardiographic data at experimental week 8 (Fig. 1B-H, table S1) show that HF+Veh rats display dilated cardiac chambers compared to Sham rats without change in ejection fraction. In HF rats TUDCA treatment significantly reduced ($p < 0.05$) both left ventricular diastolic volume (EDV: 180.1 ± 28.9 vs. 135.5 180.1 ± 8.0 µl; HF+Veh vs. HF+TUDCA) and restored fractional shortening (FS: 36.0 ± 3.2 vs. 41.3 ± 2.8%; HF+Veh vs. HF+TUDCA), suggesting that TUDCA administration prevented cardiac function deterioration in HF. No deleterious effects in cardiac architecture and cardiac function was found in Sham+TUDCA rats (Fig. 1). Cardiac function analysis (Fig. 1I-M) revealed that TUDCA treatment restored cardiac diastolic function determined by single-beat estimation (Fig. 1J, L), which was accompanied with reduced cardiac hypertrophy index (HW/BW 4.35 ± 0.27 vs. 3.99 ±

0.13mg/g; HF+Veh vs. HF+TUDCA, p<0.05). Importantly, both HF+Veh and HF+TUDCA rats started with the same degree of cardiac dysfunction before osmotic minipump implantations (Table S1). Therefore, these results show that TUDCA rescues cardiac diastolic function and prevents cardiac hypertrophy in non-ischemic HF.

TUDCA treatment restores autonomic control and arrhythmogenesis in HF

Autonomic dysfunction, particularly sympatho-excitation, is directly related with arrhythmogenesis and cardiac function deterioration in HF (5, 7), therefore, we addressed if TUDCA administration could restore autonomic balance in HF rats. In order to test this hypothesis, we estimated cardiac sympathovagal control by spectral analysis of heart rate variability (HRV) from radiotelemetry recordings of blood pressure on awake animals at resting conditions. As shown in Fig. 2, HF+Veh rats displayed a significant increase in the low frequency (LF) and decrease of high frequency (HF) component of HRV compared to Sham rats, and concomitant higher LF_{HRV}/HF_{HRV} ratio, indicative of autonomic imbalance, which was restored after TUDCA administration (LF_{HRV}/HF_{HRV}: 3.02 ± 0.29 vs. 1.14 ± 0.24; HF+Veh vs. HF+TUDCA, p <0.05). As expected, TUDCA administration produced a marked reduction in arrhythmia score in HF rats (141.5 ± 26.7 vs. 35.7 ± 12.5; HF+Veh vs. HF+TUDCA, p <0.05), without affecting healthy rats (Fig. 2B and 2F).

TUDCA normalizes breathing patterns and central chemoreflex sensitivity in HF

Respiratory instability such as apneas and breathing pattern regularity are another important pathophysiological hallmark in HF (6) which was associated to altered chemoreflex gain (6, 8, 30, 31). Therefore, we addressed the ability of TUDCA on the restoration of disordered breathing patterns and chemoreflex sensitivity in HF rats (Fig. 3). As shown in Fig 3A-H and Table S3, HF+Veh compared to control rats displayed breathing patterns irregularity both in frequency and amplitude of ventilation (breath-to-breath SD2: 53.14 ± 2.64 vs. 74.47 ± 4.50; Coefficient of variation (CV) of V_T: 6.74 ± 2.11 vs. 12.68 ± 1.05%; Sham+Veh vs. HF+Veh, p <0.05) and increased apnea/hypopnea index (AHI 4.67 ± 1.31 vs. 11.83 ± 2.26 events/h; Sham+Veh vs. HF+Veh, p <0.05). TUDCA treatment markedly normalized all these parameters (SD2 74.47 ± 4.50 vs. 44.53 ± 1.59; CV of V_T 12.68 ± 1.05 vs. 7.89 ± 0.39%; AHI 11.83 ± 2.26 vs. 4.33 ± 1.80 events/h; HF+Veh vs. HF+TUDCA, p <0.05). No changes in apnea time, sights and post-sight apnea incidence in all experimental conditions were observed Fig. 3H, Table S3. In terms of chemoreflex control, compared to control groups no changes were observed in ventilatory response to hypoxia (HVR), in accordance with previous reports (8, 30, 31), but an exacerbated hypercapnic ventilatory response (HCVR) was observed in HF+Veh rats, which was normalized by TUDCA administration (HCVR: 9.29 ± 1.41 vs. 4.75 ± 0.85 ml/min/F_iCO_2%; HF+Veh vs. HF+TUDCA, p <0.05). TUDCA did not changed ventilatory patterns and chemoreflex gain in Sham rats (Fig 3, Table S3).

TUDCA reduced the levels of ERS and BRAS markers in the RVLM and restored brain-

After physiological experiments, expression of mRNA levels of ERS, BRAS and neuroinflam matory biomarkers were measured by RT-qPCR in RVLM micr opunches (Fig. 4A-J). Compared to Sham rats, HF+Veh rats showed increased expression (p <0.05) of ERS biomarkers BiP, CHOP and sXBP1 (3.2-,3.1- and 4.3-fold increase vs. Sham respectively); BRAS downstream elements AT1 and $gp91^{phox}$ (6.8- and 9.0-fold); and proinflammatory cytokines TNF-α and IL-1β (6.4- and 2.2-fold), which were normalized by TUDCA intracerebroventricular administration.

We have previously shown that central chemoreflex is another major source of sympathoexcitation during non-ischemic HF (8, 30, 31). Chemoreflex-mediated sympathoexcitation is associated with increased cardiovascular-respiratory coupling and active expiration (30), phenomena linked to

sympathoexcitation in cardiovascular disease (32, 33). Therefore, we tested the ability of TUDCA for ameliorate these pathological phenomena in HF rats. We measured expiratory volumes to determine the early-to-late expiration ratio (E2/E1) as subrogated of presence of active expiration (Fig. 4K-M). TUDCA treatment in HF rats reduced forced respiration (E2/E1 ratio 0.94 ± 0.07 vs. 0.74 ± 0.05; HF+Veh vs. HF+TUDCA, $p < 0.05$). Accordingly, coupling between cardiac autonomic function and ventilation was determined by calculating the coherence between oscillations of V_T and the systolic blood pressure (SBP) signals (Fig. 4M). Cardiorespiratory coupling was increased (increased coherence) in HF+Veh and it was significantly lower in HF+TUDCA rats. Even more, we found a strong correlation between coherence and active expiration that was present only in HF+Veh rats (Pearson r 0.814, $p = 0.048$; vs. Pearson r 0.475, $p = 0.341$; HF+Veh vs. HF+TUDCA) (Fig. 4N).

Discussion

Current therapies for non-ischemic HF are limited (34) and the molecular mechanisms responsible on disease progression are poorly understood (5). Sympathoexcitation is related with cardiorespiratory dysfunction and higher mortality in HF (4). SNS activity is controlled by a complex network that comprises several brain areas (5), such as the paraventricular hypothalamic nucleus (12, 13), the nucleus of the solitary tract (11), the RVLM (8-10) and circumventricular organs (25). However, evidence suggest a major role of the RVLM on the progression of non-ischemic HF (5). In fact, we have shown that RVLM C1 neurons are a primary source of sympathoexcitation in HF rats (8), and their specific ablation rescues cardiac, autonomic and respiratory function (9, 10). Also, central chemoreflex is a major contributor on SNS dysfunction during HF, since destruction of central chemoreceptor neurons restores autonomic control and respiratory patterns at rest (30), without affecting cardiac function, suggesting a strong relationship between RVLM C1 and central chemoreceptive neurons on the maintenance of sympathoexcitation during non-ischemic HF. However, the mechanisms responsible of C1 RVLM overactivation, as well as its relationship with central chemoreflex and sympathoexcitation have been not elucidated yet. BRAS at brain SNS control nuclei has been shown to play a pivotal role on HF pathophysiology (5, 11, 16, 21, 23), and ERS is proposed to be a central downstream element on BRAS signaling, mediating ROS and cytokines production, sympathoexcitation and cardiac dysfunction (14, 24, 25). However, the expression of BRAS or ERS markers in the RVLM during HF has not been explored. We show that HF rats express increased levels of ERS biomarkers BiP, CHOP and spliced form of XBP1 (35); the BRAS downstream elements AT1 and $gp91^{phox}$; and the proinflammatory cytokines TNF-α and IL-1β. Namely, intracerebroventricular administration of TUDCA after HF establishment reduced the expression of ERS, BRAS and inflammatory mediators and restored cardiorespiratory and autonomic dysfunction, possibly by reducing chronic hyperactivation of C1 RVLM neurons. Importantly, TUDCA treatment also reduced central chemoreflex gain and cardiorespiratory coupling, suggesting a strong relationship between ERS in the RVLM and central chemoreceptors activity during HF.

ERS occurs in response to different stressors, including BRAS, ROS and proinflammatory cytokines (24, 26, 28, 36), which are upregulated in SNS control areas in non-ischemic HF, as shown in (8, 11, 29). The most characteristic ERS biomarkers are BiP, CHOP and sXBP1, which levels get upregulated (35). ERS during ischemic HF has been associated p38- and p44/42-MAPKs (14, 25), that mediates BRAS and inflammation via NF-kB (5, 23, 28). Despite not being directly assessed, evidence is strongly suggestive that ERS could mediate BRAS inflammatory cascade via MAPKs, as proposed by (14, 25, 28) and shown in Fig. 1A, since Angiotensin-II induces ERS in the RVLM (26) and both ERS and BRAS require MAPKs for inducing inflammation (5, 28). TUDCA did not altered the expression levels of any of studied mRNAs in Shams. Namely, MAPK inhibition ameliorates ERS and BRAS (25) and TUDCA diminishes MAPK signaling and BRAS (14) at SNS control areas during ischemic HF, resulting in reduced sympathoexcitation. However, and despite

the marked effect of TUDCA con mRNA expression of ERS and BRAS biomarkers in the RVLM, we cannot discard the possibility that effects of TUDCA could be mediated by other molecular mechanisms. Further investigations are needed in order to elucidate the exact molecular mechanisms by which TUDCA improves ERS in the RVLM as well as cardiorespiratory dysfunction during HF. Also, additional investigation is needed in order to identify the specific cellular subpopulations responsible on the production of Angiotensin-II, ROS and proinflammatory cytokines in the RVLM and other brain SNS control areas during non-ischemic HF in order to design novel cell-targeted therapies against this syndrome.

Respiratory disorders are another important pathophysiological hallmark in HF (6) and have been related with increased chemoreflex gain (6, 8, 30, 31). In fact, we have shown that in non-ischemic HF, central chemoreflex gain is a major source of sympathoexcitation and ventilatory instability (8, 30). There are numerous putative sites for central chemoreception in the brain, however the main area is the retrotrapezoid nucleus (RTN) (6, 33, 37), which sends projections to the central pattern generator (6, 37) and to the RVLM (32, 38), regulating resting ventilatory patterns and sympathetic tone respectively. Upon activation, the RTN initiates reflex increases in respiratory rate and sympathoexcitation (38). In addition, activation of the RTN induces cardiorespiratory coupling, a phenomenon that has been linked to chronic sympathoexcitation (32, 33). In fact, we have recently shown that increased cardiorespiratory coupling is directly related with increased central chemoreflex sensitivity and abnormal ventilatory patters in HF (30). Active expiration is associated with increases in SNS drive (39) and stimulation of RTN neurons results in activation of expiratory muscles and active expiration (40). In fact, we found a strong correlation between ventilation and SBP signal coherence and E2/E1 expiratory ratio in HF, that was eliminated by TUDCA. Also, we have shown that selective destruction of RTN chemoreceptive neurons eliminates cardiorespiratory coupling and active expiration in non-ischemic HF, with consequent reduction of central chemoreflex gain (30). However, the molecular mechanisms responsible of RTN chemoreflex overactivity in HF have not been elucidated. Despite respiratory disorders and chemoreflex sensitivity are associated with increased BRAS activity in HF (16) and that our results clearly show that TUDCA treatment markedly reduced central chemoreflex gain, cardiorespiratory coupling and active expiration, the mechanisms responsible of RTN overactivation in non-ischemic remain to be elucidated. Considering that the RTN is in the immediate proximity of the RVLM (33, 37), one could expect a similar molecular response as those overserved in the RVLM. However, further investigations are necessary to confirm this hypothesis.

This is the first report showing a potential therapeutic effect of a drug on non-ischemic HF pathophysiology by a novel mechanism that has not been previously studied in this syndrome. Overall, our results show that ERS in the RVLM contributes importantly on sympathoexcitation during non-ischemic HF, promoting cardiac dysfunction, autonomic imbalance, respiratory disorders and neuroinflammation and highly suggest that pharmacological blockade of brain ERS in human HF could result in promissory outcomes.

Methods

Animals

Twenty-two adult male Sprague-Dawley rats (250±12g) were obtained from the Laboratory Animal Facility at the Pontificia Universidad Católica de Chile. Animals were housed with light cycling (12-hour light/dark) under controlled temperature and humidity conditions. Rats had free access to water and standard diet (Prolab RMH3000, LabDiet, USA). The animal welfare guidelines used in this work were in accordance with the American Physiological Society. Protocols were approved by the Ethics Committee for Animal Experiments of the Pontificia Universidad Católica de Chile (protocol ID 170710022). Experiments were performed 8 weeks after HF surgery (Figure 1). At the end of the experimental timeline, all animals were humanely euthanized with an overdose of anaesthesia (sodium pentobarbital 100 mg/kg i.p.).

Volume overload heart failure

Experimental HF was induced by surgical construction of an arteriovenous fistula between the abdominal aorta and inferior vena cava, as previously described (8, 9, 29-31). Under isoflurane anesthesia (5%-1.5%), the vessels were exposed through a midabdominal incision and immediately clamped caudal to the renal artery and to the aortic bifurcation. The aorta was punctured using an 18-gauge needle and advanced until it perforated the adjacent vena cava. Without delay, a drop of histoacryl® glue (Braun, Germany) was used to seal the aorta. The arteriovenous fistula was confirmed by visualization of bright red arterial blood entering the vena cava through the anastomosis. Post-operative administration of 5 mg enrofloxacin (s.c.), 1mg ketoprofen (s.c.), 5 mL saline solution (i.p.) and 2% lidocaine hydrochloride jelly (topical) were made. Sham-operated rats underwent the same surgical procedures without performing the anastomosis.

Echocardiography

Transthoracic M-mode echocardiography was performed using a Mindray Z6 Vet imaging system under anaesthesia (isoflurane 5-1.5%). Left ventricular (LV) remodelling was evaluated at 4 and 8 weeks after HF-surgery. LV end-systolic and diastolic diameters (LVESD and LVEDD, respectively) were measured using averaged measurements from 3 consecutive cardiac cycles. LV end-systolic and diastolic volumes (LVESV and LVEDV, respectively) were calculated using the Teicholz method (8, 9). Stroke volume (SV) is the difference between LVEDV and LVESV. Ejection fraction (EF) was calculated based on the formula: EF=(LVEDV/SV) x100 (%). The following criteria were used for volume overload HF (EF ≥ 50, EDV and SV ≥ 1.5-fold changes relative to Sham) (8, 9). Subsequently, rats were assigned to one of the experimental groups: Sham+vehicle (Sham+Veh, n=6), Sham+TUDCA (n=4), HF+Veh (n=6) and HF+TUDCA (n=6) (Table S1).

Intracerebroventricular delivery of drugs

4 weeks post HF or Sham surgery, rats were anesthetized (Ketamine: 100 mg/kg; and xylazine: 10 mg/kg) and fixed to a stereotaxic frame (Kopf Instruments, USA). Under aseptic conditions, the skull was exposed, and a small hole was drilled to place a 25-gauge guide cannula in the left lateral cerebral ventricle (stereotaxic coordinates from bregma: AP, −1.0 mm; DV, −4.5 mm; and ML, −1.5 mm). The cannula was secured in place with one screws and dental orthodontic resin was applied to the skull surface. A 31-gauge stainless steel cannula was inserted into the guide cannula and advanced 0.5 mm beyond its tip was used for infusion of drug or vehicle (artificial cerebrospinal fluid) into the left lateral cerebral ventricle. Later, an osmotic mini-pump (model 2004, DURECT Corporation, USA) containing TUDCA or Vehicle solution was implanted subcutaneously at the back of the neck and connected to the implanted cannula. TUDCA concentrations were adjusted to deliver 10 µg/day (flow 0.25 µL/h for 28 days). After surgery, rats received a subcutaneous injection of ketoprofen (1 mg) and enrofloxacin (1 mg).

Cardiac function

Animals were anesthetized with α-chloralose and urethane (40 and 800 mg/kg, respectively) and intubated with a 16-gauge cannula. Under binocular magnification, a 2-F pressure-volume (PV) conductance catheter (Millar, SPR-869) was placed into the right carotid artery and advanced to the LV as previously described (8, 9, 29-31). After an equilibration period (25-min), 10–15 successive PV loops were recorded, and LV hemodynamic parameters were calculated. Baseline hemodynamic and load-dependent cardiac function parameters are shown in Table S2. Load-independent cardiac function parameters were determined by calculating the slope of the end-systolic pressure-volume relationship (ESPVR) and the end-diastolic pressure-volume relationship (EDPVR) using single-beat calculations (31). Volumes were calibrated with arterial blood by the cuvette calibration method and intravenous 30% NaCl bolus for determination of parallel conductance (8, 9, 29-31). Data analysis was performed with the PV loop module of LabChart 7.0 software (Table S2)

Blood pressure telemetry

At week 7 post HF and Sham surgery, rats underwent a radio-telemetry device (DSI, USA). implantation surgery under 2 % isoflurane anaesthesia. A skin incision was made to expose the femoral artery. The tip of a pressure transmitter PA-C40 was guided into the femoral artery, and the transmitter was placed into a subcutaneous pocket. Animals received a subcutaneous injection of ketoprofen (1 mg) and enrofloxacin (1 mg) at the end of the operation. Arterial blood pressure was measured in conscious rats in a whole-body plethysmography chamber (Emka Technologies, France) using a radio-telemetry system (DSI, USA). BP was recorded (sampling rate 500 Hz) and heart rate was derived from dP/dt of the arterial pressure recordings (9, 30).

Ventilation analyses

Resting breathing was recorded for 2 hours, followed by 10 min exposures to either hypoxic or hypercapnic gas challenges using unrestrained whole-body plethysmography. Tidal volume (V_T), respiratory frequency (R_f), and minute ventilation (V_E: V_T x R_f) were analysed using ECG auto software (Emka Technologies, France) (8-10). Respiratory stability at rest was determined by creation of Poincare plots and quantified by analysis of SD1 and SD2 of breath-breath interval variability over 300 consecutive breaths (8-10). Apnoeic episodes (cessation of breathing for a duration ≥ 3 breathing cycles), hypopneas (reductions ≥50% in V_T amplitude compared to 3 previous normal breaths), sigh frequency (increase ≥50% in V_T amplitude) and post-sigh apnoea (cessation of breathing for a duration ≥ 3 breathing cycles immediately after the sigh) were scored during resting breathing (table S3), as previously described (8-10). Chemoreflex gain was determined by estimating the hypoxic ventilatory response (HVR), calculated by the slope between F_iO_2 21% and 10% and the hypercapnic ventilatory response (HCVR), calculated by the slope between F_iCO_2 0.03% and 7%, as previously described (8-10).

Cardiac autonomic function

Cardiac autonomic function was evaluated by analysis of heart rate variability (HRV) (9, 30). In order to determine heart rate, dP/dt from arterial pressure waveforms was calculated. A Kalman smoother method analysis was used to visually inspect HRV changes in time-varying domain. Then, estimations of power spectral density (PSD) of HRV was obtained in a 10 min window, using an autoregressive method analysis after Hann windowing with 50% overlap. Cut-off frequencies were defined as: low frequency (LF): 0.04 – 0.6 Hz and high frequency (HF) 0.6 – 2.4 Hz (9, 30). Additionally, we used LF/HF ratio as an indicator of cardiac autonomic balance. LF and HF were expressed as normalized units (n.u.). HRV data analysis was performed using Kubios 3.0.2 software (Finland).

Active expiration analysis

First, 3 segments of respiratory flow at rest were randomly chosen, where 20 consecutive respiratory cycles were visualized (30). Both expiratory time and volume were evaluated. To determine the presence of forced breaths, the expiratory phase was divided into 2 parts: early expiration (E1), corresponding to the initial 50% of the total expiratory time; and late expiration (E2), corresponding to the final 50% of the total expiratory time. Values of E1 and E2 were obtained by calculations of the area under the first half of the expiratory curve and the area under the second half of the expiratory curve, respectively. Increases in the ratio between E2 and E1 expiratory phases (E2/E1) was used as the indicator of active expiration (1).

Cardio-respiratory coupling

Calculations of coherence between V_T and SBP signals were made using Matlab software (R2106a version, Natick, USA). Auto- and cross-spectral estimates were computed in 5 min artefact-free recordings using the Welch's over-lapped segment averaging method. A fast Fourier transform (FFT) algorithm was applied to each variable (30). For coherence analysis, oscillations in the respiratory signal was taken as the input signal and SBP as the output signal. The magnitude of the mean square coherence was assessed over a range of 0.015 Hz centred at the frequency of the maximum V_T spectral peak in the very low frequency domain (0.01 to 0.25 Hz) (ref) (i.e. breathing oscillations).

qPCR

Rat brains were immediately removed after endpoint physiological experiments, frozen in liquid nitrogen and stored at −80°C for subsequent use. Frozen brains were cut into 100-µm coronal sections through the medulla oblongata using a cryostat. RVLM were bilaterally punched using a blunt 18-gauge needle attached to a syringe as previously described (8). RNA isolation and cDNA synthesis were performed using the RNAqueous Micro® (Ambion, USA) and iScript® (Promega) kits respectively, according to manufacturer instructions. RNA purity was assessed by spectrophotometry through the 260/280 ratio (1.88 ± 0.11). Gene expression of the ERS, BRAS and inflammatory markers was assessed by SYBR green chemistry real-time PCR following reverse transcription of total RNA. The nucleotide sequences of the primers are shown in Table S4. Real-time PCR was performed using the ABI prism 7700 Sequence Detection System (Applied Biosystems). β-Actin mRNA was quantified as an internal control for each sample and quantifications were performed using the $2^{-\Delta\Delta CT}$ method.

Data Analysis

Data are presented as mean ± standard error (SEM) in text and tables. Median and interquartile ranges are shown in violin plots. The statistical analysis was made with GraphPad Prism 8.0 statistical software (La Jolla, USA). Normal distribution of the data was assessed with the D'Agostino and Pearson test. Correlations were performed using Pearson analysis. Statistical significance of data with normal distribution was evaluated using one-way ANOVA or two-way ANOVA parametric test, followed by a Sidak post-hoc analysis, as mentioned in text. The level of significance was defined as $P<.05$.

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Figures and Tables

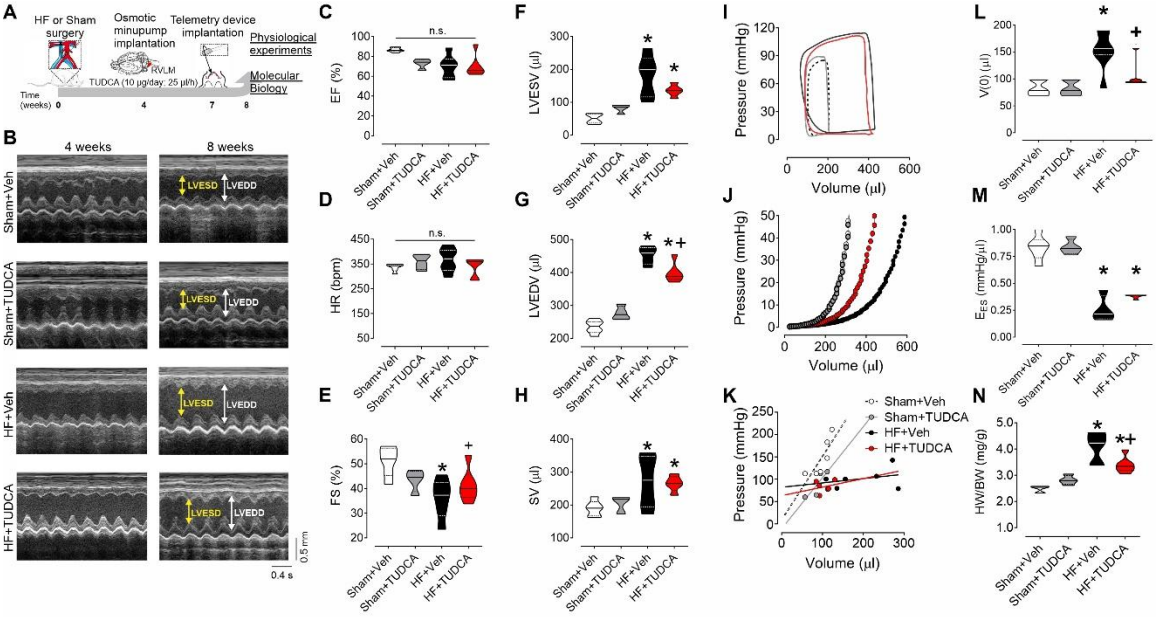

Figure 1. TUDCA treatment improves cardiac function in HF rats. HF was surgically induced by the arteriovenous fistula at week 0, and, 4 weeks after HF or Sham surgery, TUDCA or Vehicle was administered (10 µg/day) intracerebroventricularly (0.25 µl/h for 28 day; A). B: representative echocardiographic recordings of the four groups at 4 and 8 weeks. C-H: Summary data quantification of echocardiographic parameters. I: representative pressure-volume loops, H: exponential curves of diastolic function measured by End-Diastolic Pressure-Volume Relationship (EDPVR); and J: curves of systolic function measured by End-Systolic Pressure-Volume Relationship (ESPVR) from the four groups and their respective quantifications (L, M), estimated from single-beat calculations. Note that HF+TUDCA animals show a markedly restored diastolic function and lesser cardiac hypertrophy (N). B) Summary of the effect of TUDCA treatment on volume at pressure 0 (V0). Violin plots show data as median ± quartiles. n: Sham (6), Sham+TUDCA (4), HFpEF (6), HFpEF+TUDCA (6). One-way ANOVA and Holm-Sidak posthoc analysis *: p<0.05 vs. Sham+Veh; +: p<0.05 vs. HF+Veh

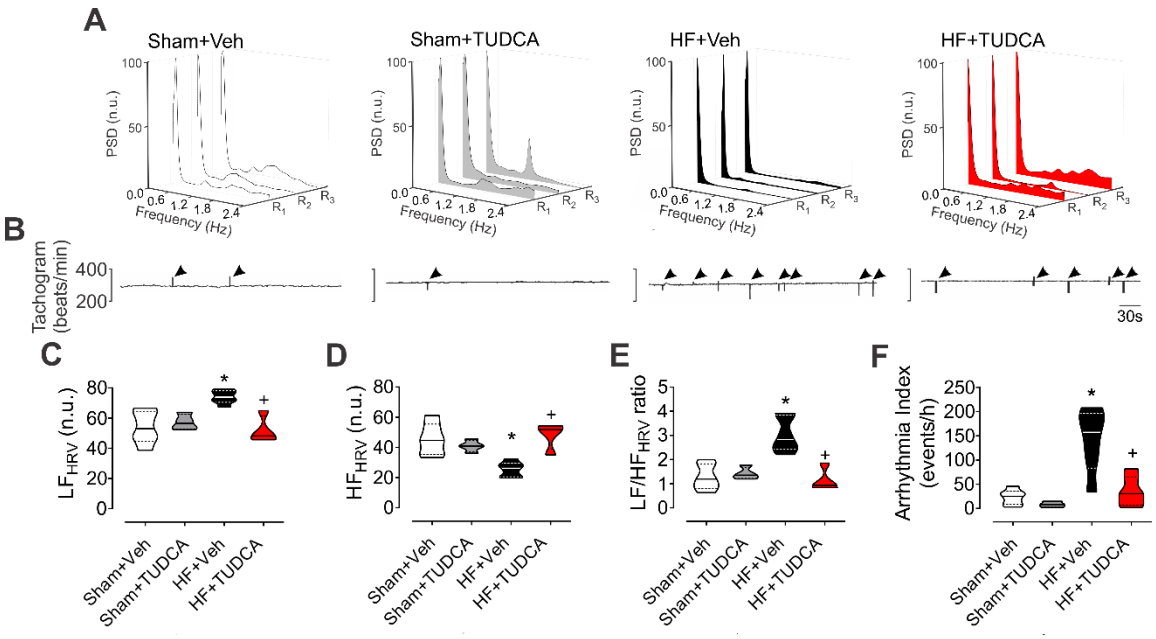

Figure 2. TUDCA treatment restores autonomic control to the heart in HF rats. (A) Representative heart rate variability (HRV) spectra during 10 min at rest all experimental conditions. Note that HF+Veh rats displayed a marked increase in the low frequency HRV component (LF, 0.04-0.6 Hz) and TUDCA infusion blunted this augmented sympathetic drive. (B) Representative 5 minutes of heart rate tachograms of one rat per group. C-E: Summary data of LF, high frequency (HF, 0.6-2.4 Hz) and LF/HF ratio (E). Note that the autonomic imbalance (E) and arrhythmias (F) were blunted by TUDCA administration. Violin plots show data as median ± quartiles. n: Sham (6), Sham+TUDCA (4), HFpEF (6), HFpEF+TUDCA (6). One-way ANOVA and Holm-Sidak posthoc analysis *: p<0.05 vs. Sham+Veh; +: p<0.05 vs. HF+Veh

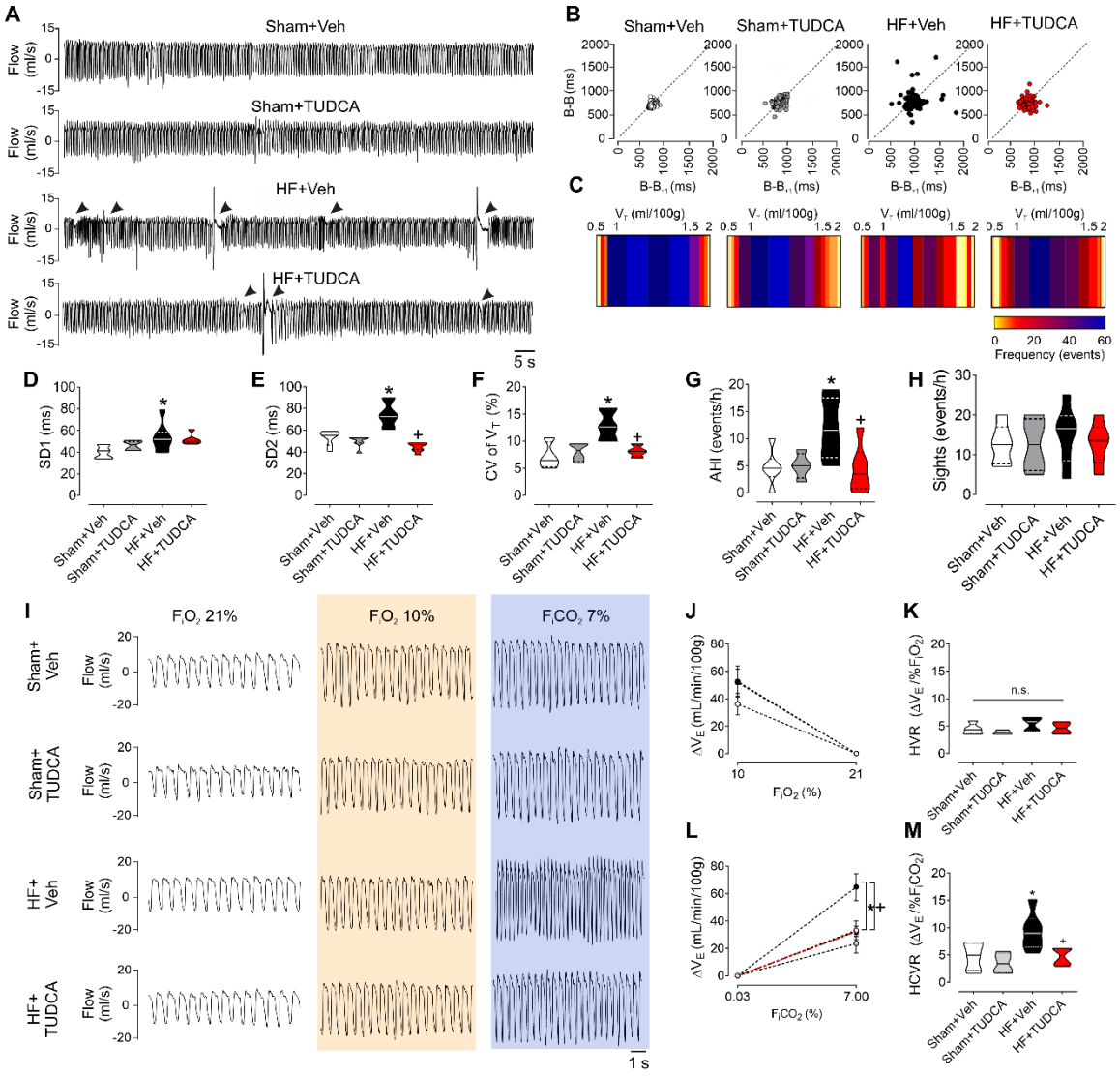

Figure 3. ERS inhibition at brain level blunted ventilatory disturbances in resting breathing in HF rats. A: Representative traces of resting ventilation in the four experimental groups. B: Representative Poincaré plots of breath-to-breath (BB) variability. C: Representative histograms of tidal volume (V_T). D-E: Summary data of short-term variability (SD1) and long-term variability (SD2) of ventilation. F: calculated V_T coefficient of variation (CV of V_T) of ventilation. G-H: quantification of apnoeic events and sights during 1 hour of resting ventilation. I: Representative traces of ventilation during chemoreflex analysis. J-K: quantification of the hypoxic ventilatory response (HVR). L-M: quantification of the hypercapnic ventilatory response (HCVR). Note that TUDCA reduced ventilatory variability, apnoea incidence, and central chemoreflex gain in HF rats, with no major effects on basal ventilation in Shams. Violin plots show data as median ± quartiles. n: Sham (6), Sham+TUDCA (4), HFpEF (6), HFpEF+TUDCA (6). One-way ANOVA and Holm-Sidak posthoc analysis *: p<0.05 vs. Sham+Veh; +: p<0.05 vs. HF+Veh

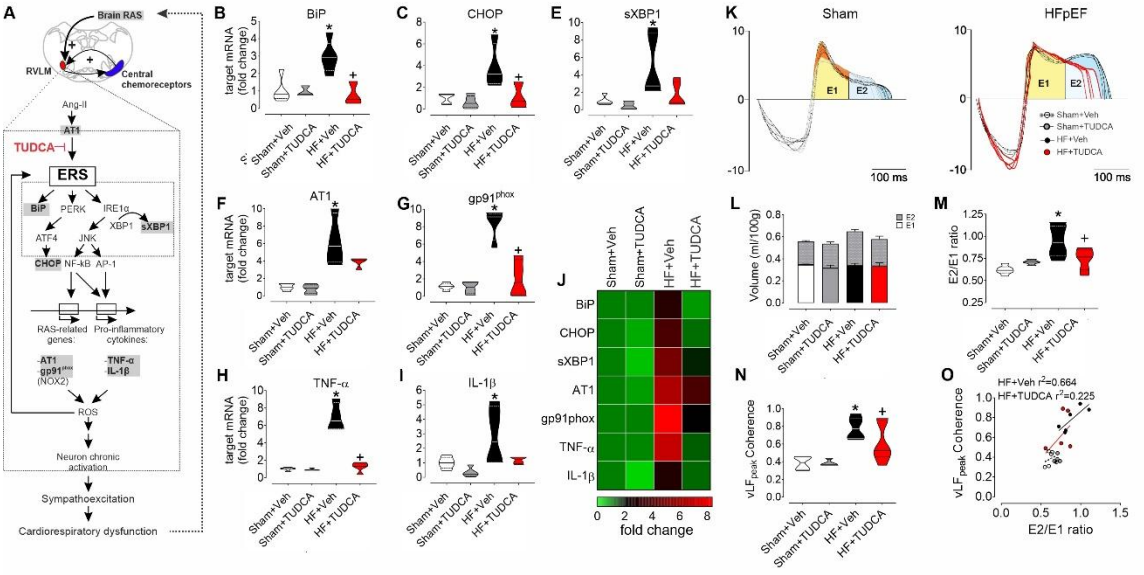

Figure 4. ERS is upregulation in the RVLM and cardiorespiratory coupling is ameliorated by TUDCA in HF. A: Scheme showing main putative the signaling pathways responsible on chronic neuron activation and consequent autonomic imbalance in heart failure. Measured molecular targets are highlighted in figure. B-I: mRNA expression in RVLM micropunches of ERS, BRAS and neuroinflammatory biomarkers and summary data (J). K: representative traces of 5 consecutive ventilations in one rat per group showing the expiratory phases. L: quantification of active expiration. M: quantification of coherence between VT oscillations and systolic blood pressure signals, indicative of cardiorespiratory coupling. Note that coherence values and active expiration strongly correlates only in HF+Veh and this correlation was lost after TUDCA administration (N). Namely, TUDCA reduced RVLM expression of ERS and BRAS biomarkers as well as active expiration and cardiorespiratory coupling. Violin plots show data as median ± quartiles. n: Sham (6), Sham+TUDCA (4), HFpEF (6), HFpEF+TUDCA (6). One-way ANOVA and Holm-Sidak posthoc analysis *: p<0.05 vs. Sham+Veh; +: p<0.05 vs. HF+Veh.

5. Conclusiones generales del trabajo de tesis

Las terapias actuales contra la insuficiencia cardiaca no-isquémica (HFpEF) son limitadas y los mecanismos moleculares responsables de su progresión son escasamente conocidos. La sobre-activación del sistema nervioso simpático (SNS) se relaciona con un peor pronóstico de la enfermedad, disfunción cardiorrespiratoria y mayores tasas de mortalidad, por lo que conocer los mecanismos responsables de este fenómeno nos permitirán comprender de mejor manera la fisiopatología de la enfermedad y así idear nuevas estrategias terapéuticas contra este síndrome. Los circuitos neuronales encargados de la regulación de la actividad del SNS son complejos y comprenden diferentes regiones centrales y periféricas, sin embargo, en HFpEF, son las neuronas C1 RVLM y las quimiorreceptoras del RTN aquellas que juegan un rol más preponderante en el establecimiento de las alteraciones cardiorrespiratorias durante la progresión de la enfermedad. Sin embargo, los mecanismos responsables de su sobre-activación durante la HFpEF se desconocen hasta ahora.

Se ha propuesto que la señalización de ERS a nivel central podría mediar la progresión de la insuficiencia cardiaca, dado que la administración de TUDCA previo a la inducción de HFrEF a nivel central previene la simpato-excitación en animales experimentales, sin embargo, la presencia de ERS en la RVLM en ningún subtipo de HF ha sido evaluado, y el rol de ERS en la progresión de la HFpEF nunca había sido estudiado hasta ahora. Por ende, el presente trabajo de tesis contempló la siguiente hipótesis: “El estrés de retículo endoplásmico en el tronco encefálico promueve la simpato-excitación en ratas con insuficiencia cardiaca con fracción de eyección preservada”

De acuerdo con lo anterior, en el **Objetivo 1: Determinar la presencia de estrés de retículo endoplásmico en la RVLM de ratas con HFpEF**, se evaluaron los niveles de expresión de marcadores carácteristicos asociados a la respuesta de estrés de retículo (BiP, CHOP, sXBP1), inflamación (TNF-α, IL-1β) y RAS (AT1, NADPH oxidasa) RT-qPCR en micropunches de RVLM de ratas HFpEF y pseudo-operadas (Sham) 8 semanas posterior a la operación. Encontramos un aumento significativo en la

expresión de todos los biomarcadores anteriormente mencionados en RVLM de ratas HFpEF en comparación con las Sham (paper 3, figura 4) La progresión de la HFpEF se determinó mediante ecocardiografía 4 semanas post-operación y los análisis moleculares fueron realizados a las 8 semanas post-operación. Estos resultados en conjunto sugieren la activación de las rutas de señalización de RAS y ERS durante HFpEF, al menos en el punto estudiado, por lo que, intervenciones orientadas a inhibir dichas rutas podrían constituir una estrategia terapéutica efectiva en el tratamiento de la enfermedad.

Con respecto al **Objetivo 2: Determinar la contribución del estrés de retículo en la simpato-excitación en ratas con HFpEF**, se administró TUDCA por vía intracerebroventricular durante 4 semanas en ratas Sham y HFpEF a las 4 semanas post-operación (una vez corroborada la inducción de HFpEF mediante ecocardiografía) y se evaluó el tono simpático cardiaco y los niveles de expresión de biomarcadores de ERS, RAS y neuroinflamación en dicho punto experimental (8 semanas desde el inicio de los experimentos, 4 semanas de administración de TUDCA o vehículo). Se encontró que la administración de TUDCA restauró el desbalance autonómico cardiaco en ratas HFpEF, y, consecuentemente los eventos arrítmicos, sin alterar dichos parámetros en ratas Sham (paper 3, figura 2). El análisis molecular reveló que el tratamiento con TUDCA redujo la expresión de los marcadores de ERS, RAS y neuroinflamación estudiados, sin afectar la fisiología de los animales Sham, lo que sugiere que la administración de la droga podría significar una alternativa segura y efectiva para el tratamiento de la HFpEF, sin embargo, se requieren estudios adicionales de función cardiaca y respiratoria con el fin de conocer en profundidad el efecto beneficioso de TUDCA sobre la fisiopatología de la enfermedad.

En el **Objetivo 3: Determinar si la inhibición crónica del estrés de retículo en ratas con HFpEF disminuye la progresión de la patología**, se optó por la misma estrategia experimental que el objetivo anterior, poniendo especial foco en las consecuencias

cardiorrespiratorias de la HFpEF, así como el efecto de TUDCA sobre dichos parámetros.

Uno de los principales hallazgos es que la administración intracerebroventricular de una droga inhibidora de ERS (TUDCA) restaura las alteraciones cardiorrespiratorias de la HFpEF y previene la progresión de la enfermedad. Específicamente, el tratamiento con TUDCA previno la hipertrofia cardiaca, a su vez, restauró la función diastólica (paper 3, figura 1), el desbalance autonómico y la frecuencia de arritmias cardiacas (paper 3, figura 2). A nivel respiratorio, el tratamiento redujo la incidencia de desórdenes ventilatorios y restauró la estabilidad ventilatoria en reposo, así como la sensibilidad quimiorrefleja (paper 3, figura 3), lo cual fue asociado a una disminución en la expresión de biomarcadores de ERS, RAS y neuroinflamación en la RVLM y un acoplamiento cardio-respiratorio en ratas HFpEF (paper 3, figura 4). Dichos resultados son promisorios para la realización de futuros estudios piloto utilizando esta droga en la HFpEF humana, dado que derivados de TUDCA se encuentran aprobados por la FDA, y la seguridad de la droga ha sido comprobada por miles de años de uso en la medicina tradicional china para el tratamiento de las afecciones del hígado.

Sin embargo, y a pesar de que los resultados de la investigación son prometedores, reconocemos que el presente estudio no está exento de limitaciones. En lo que respecta a los mecanismos moleculares propuestos en el trabajo de tesis, se evaluó la expresión de biomarcadores de las rutas de ERS y RAS. Sin embargo, y a pesar de que la evidencia fisiológica es altamente sugerente, la expresión de los biomarcadores estudiados fue analizada en punto final, y la activación de dichas rutas propiamente tal no fue evaluada, y experimentos adicionales son necesarios en orden de establecer una relación causal directa entre los fenómenos observados y las rutas de RAS y ERS. Por ende, el presente estudio no permite determinar el/los mecanismo(s) molecular(es) preciso(s) responsable(s) de los efectos beneficiosos de TUDCA en la fisiopatología de la HFpEF. A pesar de ello, TUDCA es reconocido por ser un inhibidor clásico de ERS, por lo que es imposible descartar que la ERS en el tronco encefálico juegue un rol central en las alteraciones fisiopatológicas observadas (cardiorrespiratorias y autonómicas), dado el marcado efecto beneficioso de la droga en prácticamente toda la

fisiopatología de la enfermedad. Una segunda limitación del presente trabajo corresponde a los mecanismos celulares. Como se expone en la revisión sistemática del capítulo 2, los mecanismos celulares y la circuitería responsable de regular el tono simpático y la función cardiorrespiratoria durante la progresión de la insuficiencia cardiaca son altamente complejos y han sido pobremente estudiados en HFpEF, y si bien el presente trabajo aporta a dilucidar los patrones de expresión alterados de ciertos biomarcadores a nivel de la RVLM, el presente grado de avance de la investigación no da cuenta sobre las subpoblaciones celulares responsables de la producción de los mediadores estudiados, como las citoquinas proinflamatorias, mediadores de ERS y/o los elementos río abajo del RAS estudiados en este trabajo. Por lo tanto, futuras investigaciones son necesarias para establecer las dianas celulares y moleculares responsables de los diferentes aspectos fisiopatológicos de la enfermedad para así diseñar mejores estrategias dirigidas hacia dichos tipo celulares y mecanismos.

6. Anexos

Publicaciones científicas durante periodo de tesis

1. Andrade DC, Arce-Alvarez A, Toledo C, Diaz HS, Lucero C, Schultz HD, Marcus NJ, Del Rio R*. Exercise training improve cardiac autonomic control, cardiac function and arrhythmogenesis in rats with preserved ejection fraction heart failure. J Appl Physiol (1985). 2017. doi: 10.1152/japplphysiol.00189.2017.
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9. Toledo C, Lucero C, Andrade DC, Diaz HS, Schwarz KG, Pereyra KV, Arce-Alvarez A, Lopez NA, Martinez M, Inestrosa NC, Del Rio R*: Cognitive impairment in heart failure is associated with altered Wnt signaling in the hippocampus. Aging 2019 doi: 10.18632/aging.102150.
10. Díaz HS, Toledo C, Andrade DC, Marcus NJ, Del Rio R. Neuroinflammation in heart failure: NEW insights for an old disease. J Physiol. 2019 doi: 10.1113/JP278864.
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12. Toledo C, Andrade DC, Díaz HS, Pereyra KV, Schwarz KG, Díaz-Jara E, Oliveira LM, Takakura AC, Moreira TS, Schultz HD, Marcus NJ, Del Rio R. Rostral ventrolateral medullary catecholaminergic neurones mediate irregular breathing pattern in volume overload heart failure rats. J Physiol. 2019. doi: 10.1113/JP278845.

Presentaciones en congresos

Congresos internacionales

1. Hugo S. Díaz, David C. Andrade; Camilo Toledo; Claudia Lucero; Alexis Arce-Álvarez; Katherin V. Pereyra; Harold D. Schultz; Josiane N. Silva; Ana C. Takakura; Thiago S. Moreira; Noah J. Marcus, Rodrigo Del Rio, Episodic stimulation of central chemoreflex elicits long-term breathing disorders and autonomic imbalance in heart failure rats. European Respiratory Society Congress, Madrid, Spain, 2019

2. Hugo S. Díaz, Rodrigo Del Rio, Brainstem pre-sympathetic neuron controls oscillatory breathing in heart failure rats. European Respiratory Society Congress, Madrid, Spain, 2019
3. H.S. Díaz, D. C. Andrade. C. Toledo. N. Silva, A.C. Takakura, T.S. Moreira, R. Del Rio EPISODIC HYPERCAPNIC STIMULATION OF CENTRAL CHEMORECEPTORS INDUCED RESPIRATORY PLASTICITY AND ELICITS LONG-TERM BREATHING DISORDERS IN HEART FAILURE RATS. 2nd Neural Control of Breathing Symposium, São Paulo, Brazil, 2019
4. Esteban Diaz-Jara, David C. Andrade, Camilo Toledo, Hugo Díaz, Rodrigo Del Rio. EXERCISE TRAINING INTOLERANCE IN HIGH OUTPUT HEART FAILURE IS ASSOCIATED WITH ALTERED CHEMOREFLEX FUNCTION. 2nd Neural Control of Breathing Symposium, São Paulo, Brazil, 2019
5. Rodrigo Del Rio, David C Andrade, Camilo Toledo, Hugo S Diaz, Claudia Lucero, Alexis Arce-Alvarez. Brainstem pre-sympathetic neuron controls oscillatory breathing in heart failure. Experimental Biology, San Diego, California, United States, 2018
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