Molecular mechanisms linking dysautonomia and impaired cardiac contractility in critical illness.

Methods

Patient studies-

The patient cohort used for baroreflex analysis was at UCLH (MREC No: 09/H0805/58; ISRCTN76894700. Patients undergoing major colorectal surgery were screened for the COMPETE-C trial as approved by the Cornwall and Plymouth Research Ethics Committee (Ref: 08/H0203/159) and conducted at Derriford Hospital, Plymouth, UK, between March 2009 and April 2010 (ISRCTN 14680495) (1). The predefined aim of this study was the association between parasympathetic autonomic dysfunction as defined by abnormal heart rate recovery, and clinical outcomes (sepsis, length of hospital stay). To avoid bias, all cardiopulmonary exercise and autonomic analyses were undertaken by investigators blinded to clinical outcomes. All research involving human participants was approved by the Institutional Review Boards as detailed below. Informed consent was obtained and all clinical studies were conducted according to the principles expressed in the Declaration of Helsinki. Three cohorts at two separate centers were studied before planned elective surgery.

Cardiopulmonary exercise testing

At both centers, patients underwent preoperative symptom-limited maximal cardiopulmonary exercise testing (CPET) on a stationary, electronically-braked, cycle ergometer (UCLH: Corival, Lode, Gronigen, Netherlands; Plymouth: Zan, nSpire, Hertford,

UK) with on-line breath-by-breath gas analysis and ECG monitoring. The anerobic threshold (AT), measured as body mass-corrected oxygen consumption ($ml \cdot kg^{-1} \cdot min^{-1}$), was used as a marker of aerobic fitness (2). AT was determined by the modified V slope method and confirmed with the ventilatory equivalents method. To determine parasympathetic (vagal) activity, abnormal heart rate recovery was measured as defined by previous studies that demonstrated the negative prognostic power of a heart rate reduction of ≤12 beats per minute (bpm) at peak exercise to that measured one minute after cessation of exercise. (3-5)

The primary outcome was length of hospital stay stratified according to the presence or absence of heart rate recovery ≤12 bpm (4, 6). Secondary outcome measures were 600-day mortality, major postoperative morbidity as defined by the Clavien-Dindo scale (7), and intraoperative hemodynamics. Full details of the COMPETE-C protocol have been published elsewhere (1). Comparison of intraoperative hemodynamic changes between groups measured by esophageal Doppler flowmetry (CardioQ^{TM}, Deltex Medical, Chichester, UK) was restricted to timepoints free of acute surgical or anesthetic interventions i.e. immediately prior to incision and the last reading at the end of the operation prior to extubation/transfer to a postoperative care facility.

Rodent Experiments

All experiments were performed in accord with the UK Animals (Scientific Procedures) Act (1986), summarized in supplementary Tables 2 and 3, and adhered to ARRIVE guidelines (Supplementary Table 5). Sprague Dawley male juvenile rats (Charles River, Harlow, UK) and

an established NOX2$^{-/-}$ murine colony on a C57BL/6J background (King's College London British Heart Foundation Centre, London, United Kingdom) were used. Animals were maintained under artificial day–night cycles (12 h light–dark cycles; 23±1°C room temperature, controlled environment humidity), received a standard rat diet and water *ad libitum*, and adapted to laboratory conditions for at least 3 days. Surgical procedures were carried out aseptically under local anaesthesia, with body temperature maintained at 37°C.

Sino-aortic denervation

Carotid sinus and aortic nerve denervation (SAD) were performed under isoflurane anesthesia, as described previously (8). Duration of surgery was less than 15 minutes. Sham-operated rats and mice underwent the procedure, but ensuring that nerves were undamaged and left intact. Rats were subsequently housed in individual cages with controlled temperature (23–25°C), 12 hour light-dark cycle and free access to food and tap water. All rodents were analyzed on an intention-to-treat basis, and thus all nerve ablations were considered as having SAD. A clinical severity system was used to score illness severity, as previously described (9).

Statistical methods

Analysis of the Plymouth cohort CPET data, blinded to outcomes, revealed ~35% patients with an abnormal HRR. Values <12 bpm confer a ~2-fold relative risk of adverse outcomes (4-6). Given a median length of stay in COMPETE-C was 8 days (1), we estimated that at least 142 patients would be required to detect a hazard ratio >1.6 for median time to

discharge for AD patients (accrual time 600 days, 80% power at a two-sided significance level of 5%). Rodent experiments were designed in accordance with ARRIVE (Animal in research: reporting *in vivo* experiments) guidelines (Supplementary Table 8). Rodent numbers were minimized on the basis of previous studies showing n=5/6 per genotype is sufficient to reveal biologically relevant differences.(10) Randomization of littermates (which were of mixed genotype for mice) was employed, in accordance with guidelines for cardiovascular research.(11)

Supplementary Table 1. STROBE adherence.

Supplementary Table 2. Rat – experimental summary.

Supplementary Table 3. Mouse – experimental summary.

Supplementary Table 4. Quantitative PCR Primers and references for their use.

Supplementary Table 5. ARRIVE guideline adherence.

Supplementary Table 1: STROBE Statement—Checklist of items that should be included in reports of *cohort studies*

	Item No	Recommendation	
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	Page 1 main text
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	Page 3- 4main text
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	Page 5,6 main text
Objectives	3	State specific objectives, including any prespecified hypotheses	Page 6 main text
Methods			
Study design	4	Present key elements of study design early in the paper	Pages 7-11 main text; suppl material.
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	Pages 7-11 main text; suppl material.
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up	Page 7-11 main text; suppl material
		(*b*) For matched studies, give matching criteria and number of exposed and unexposed	N/A
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	7-11; Page 14 main text; suppl material
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	Pages 6-11 main text; suppl material,

Bias	9	Describe any efforts to address potential sources of bias	Throughout methds 6-11. main text; suppl material
Study size	10	Explain how the study size was arrived at	suppl material
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	Pages 6-11 -main text; Page 6-11 - suppl material
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	Page 11,12, suppl material
		(*b*) Describe any methods used to examine subgroups and interactions	Page 11,12, suppl material
		(*c*) Explain how missing data were addressed	Table 2
		(*d*) If applicable, explain how loss to follow-up was addressed	N/A
		(*e*) Describe any sensitivity analyses	n/a
Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	12-17; suppl
		(b) Give reasons for non-participation at each stage	12-17; suppl
		(c) Consider use of a flow diagram	-
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	12-17; suppl
		(b) Indicate number of participants with missing data for each variable of interest	12-17; suppl
		(c) Summarise follow-up time (eg, average and total amount)	12-17; suppl
Outcome data	15*	Report numbers of outcome events or summary measures over time	12-17; suppl
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make	12-17; suppl

		clear which confounders were adjusted for and why they were included	
		(*b*) Report category boundaries when continuous variables were categorized	95% CI throughout manuscript.
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	RR throughout manuscript.
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	n/a
Discussion			
Key results	18	Summarise key results with reference to study objectives	Page 17, main text
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	Page 17-19, main text
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	Page 20-21, main text
Generalisability	21	Discuss the generalisability (external validity) of the study results	Page 20,21, main text
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	See acknowledgments.

Supplementary Table 2. Summary of rat experimental procedures reported.

Experiment	Figure	Controls	Intervention	Total
Echos 3 week after SAD	Figure 2	4	4	8
Cardiac immunoblots	Figure 2	6	6	12

Supplementary Table 3. Mouse experimental procedures reported.

Experiment	Figure	Controls	Intervention	Total
Echos 26 days after SAD	Figure 3	7	4	8
Cardiac immunoblots	Figure 3	5	5	12

Supplementary Table 4. Quantitative PCR Primers and references for their use.

Gene	Primer sequence	Reference
AT1R	Forward: 5′-CGG CCT TCG GAT AAC ATG AGA-3′ Reverse: 5′-CCT GTC ACT CCA CCT CAA AAC -3′	PMID: 23079082
GRK2	Forward: 5′-CCCTCTCACCATCTCTGAGC-3′ Reverse: 5′-GGTTGGGGAACAAGTAGAA-3′	PMID: 6103237
GRK5	Forward: 5′-CCCTCTCACCATCTCTGAGC-3′ reverse, 5′-GTTGGGGAACAAGTAGAA-3	http://pga.mgh.harvard.edu/primerbank/
Cyclophilin	Forward: 5′-GAGCTGTTTGCAGACAAAGTTC-3′ Reverse: 5′-CCCTGGCACATGAATCCTGG-3′	http://pga.mgh.harvard.edu/primerbank/
HPRT	Forward: 5′-CTCATGGACTGATTATGGACAGGAC-3′ Reverse: 5′-GCAGGTCAGCAAAGAACTTATAGCC-3′	PMID:15040812

Supplementary Table 5. ARRIVE guidelines

	ITEM	RECOMMENDATION	Section/ Paragraph
Title	1	Provide as accurate and concise a description of the content of the article as possible.	Main text page 1
Abstract	2	Provide an accurate summary of the background, research objectives, including details of the species or strain of animal used, key methods, principal findings and conclusions of the study.	Main text 3,4
INTRODUCTION			
Background	3	a. Include sufficient scientific background (including relevant references to previous work) to understand the motivation and context for the study, and explain the experimental approach and rationale. b. Explain how and why the animal species and model being used can address the scientific objectives and, where appropriate, the study's relevance to human biology.	Main text 5,6
Objectives	4	Clearly describe the primary and any secondary objectives of the study, or specific hypotheses being tested.	Main text 6
METHODS			
Ethical statement	5	Indicate the nature of the ethical review permissions, relevant licences (e.g. Animal [Scientific Procedures] Act 1986), and national or institutional guidelines for the care and use of animals, that cover the research.	Main text 7
Study design	6	For each experiment, give brief details of the study design including: a. The number of experimental and control groups. b. Any steps taken to minimise the effects of subjective bias when allocating animals to treatment (e.g. randomisation procedure) and when assessing results (e.g. if done, describe who was blinded and when). c. The experimental unit (e.g. a single animal, group or cage of animals). A time-line diagram or flow chart can be useful to illustrate how complex study designs were carried out.	Suppl Material p7-9
Experimental procedures	7	For each experiment and each experimental group, including controls, provide precise details of all procedures carried out. For example: a. How (e.g. drug formulation and dose, site and route of administration, anaesthesia and analgesia used [including monitoring], surgical procedure, method of euthanasia). Provide details of any specialist equipment used, including supplier(s). b. When (e.g. time of day). c. Where (e.g. home cage, laboratory, water maze). d. Why (e.g. rationale for choice of specific anaesthetic, route of administration, drug dose used).	Suppl Material, p7-9.
Experimental animals	8	a. Provide details of the animals used, including species, strain, sex, developmental stage (e.g. mean or median age plus age range) and weight (e.g. mean or median weight plus weight range). b. Provide further relevant information such as the source of animals, international strain nomenclature, genetic modification status (e.g. knock-out or transgenic), genotype, health/immune status, drug or test naïve, previous procedures, etc.	Suppl Material, p7-9. Main text p7

Housing and husbandry	9	Provide details of: a. Housing (type of facility e.g. specific pathogen free [SPF]; type of cage or housing; bedding material; number of cage companions; tank shape and material etc. for fish). b. Husbandry conditions (e.g. breeding programme, light/dark cycle, temperature, quality of water etc for fish, type of food, access to food and water, environmental enrichment). c. Welfare-related assessments and interventions that were carried out prior to, during, or after the experiment.	Suppl Material, p4-
Sample size	10	a. Specify the total number of animals used in each experiment, and the number of animals in each experimental group. b. Explain how the number of animals was arrived at. Provide details of any sample size calculation used. c. Indicate the number of independent replications of each experiment, if relevant.	. Suppl Material, p8
Allocating animals to experimental groups	11	a. Give full details of how animals were allocated to experimental groups, including randomisation or matching if done. b. Describe the order in which the animals in the different experimental groups were treated and assessed.	Main text p5; suppl Material, p8
Experimental outcomes	12	Clearly define the primary and secondary experimental outcomes assessed (e.g. cell death, molecular markers, behavioural changes).	Suppl Material, p7-9
Statistical methods	13	a. Provide details of the statistical methods used for each analysis. b. Specify the unit of analysis for each dataset (e.g. single animal, group of animals, single neuron). c. Describe any methods used to assess whether the data met the assumptions of the statistical approach.	Suppl Material, p11
RESULTS			
Baseline data	14	For each experimental group, report relevant characteristics and health status of animals (e.g. weight, microbiological status, and drug or test naïve) prior to treatment or testing. (This information can often be tabulated).	Suppl Material
Numbers analysed	15	a. Report the number of animals in each group included in each analysis. Report absolute numbers (e.g. 10/20, not 50%[2]). b. If any animals or data were not included in the analysis, explain why.	Suppl Material
Outcomes and estimation	16	Report the results for each analysis carried out, with a measure of precision (e.g. standard error or confidence interval).	P12-14, main text. Figures 5-9
Adverse events	17	a. Give details of all important adverse events in each experimental group. b. Describe any modifications to the experimental protocols made to reduce adverse events.	P12, main text
DISCUSSION			
Interpretation/ scientific implications	18	a. Interpret the results, taking into account the study objectives and hypotheses, current theory and other relevant studies in the literature. b. Comment on the study limitations including any potential sources of bias, any limitations of the animal model, and the imprecision associated with the results[2]. c. Describe any implications of your experimental methods or findings for the replacement, refinement or reduction (the 3Rs) of the use of animals in research.	P17-21, main text.

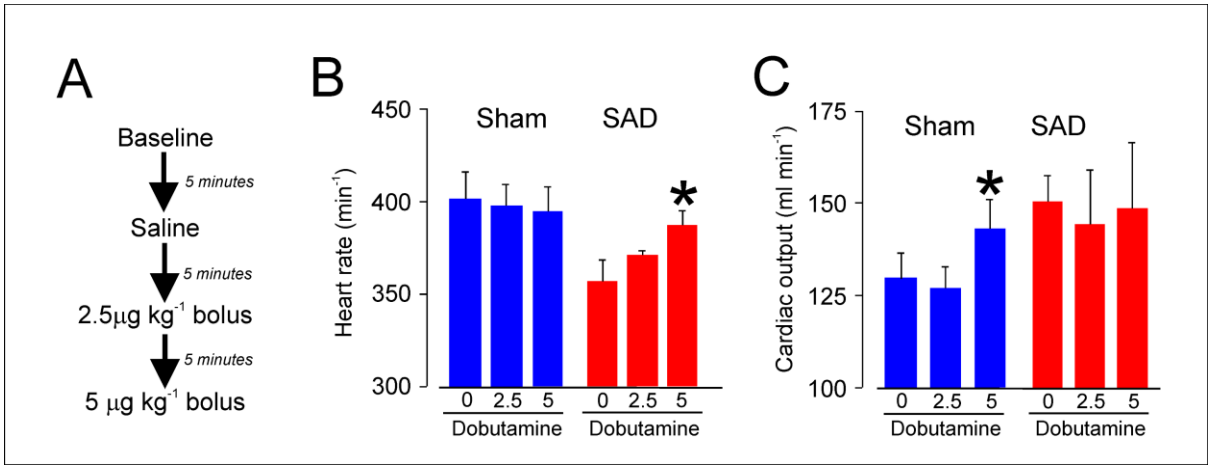
Generalisability/ translation	19	Comment on whether, and how, the findings of this study are likely to translate to other species or systems, including any relevance to human biology.	P19-21, main text.
Funding	20	List all funding sources (including grant number) and the role of the funder(s) in the study.	Noted in acknowled gments

Supplementary Table 6. Clavien-Dindo grading of postoperative complications.

Grades	Definitions
Grade I	Any deviation from the normal postoperative course without the need for pharmacological treatment or surgical, endoscopic and radiological interventions. Acceptable therapeutic regimens are: drugs such as antiemetics, antipyretics, analgesics, diuretics and electrolytes, and physiotherapy.
Grade II	Requiring pharmacological treatment with drugs other than those allowed for grade I complications. Blood transfusions and total parenteral nutrition are also included.
Grade III	Requiring surgical, endoscopic or radiological intervention.
Grade III-a	Intervention not under general anaesthesia
Grade III-b	Intervention under general anaesthesia
Grade IV	Life-threatening complication (including CNS complications: brain haemorrhage, ischaemic stroke, subarachnoid bleeding, but excluding transient ischaemic attacks) requiring IC/ICU
Grade IV-a	Single organ dysfunction (including dialysis)
Grade IV-b	Multi-organ dysfunction

Supplementary Figure 1. Lack of inotropic effect is accompanied by tachycardia in SAD rats.

A. Protocol. B. Heart rate at escalating doses of dobutamine. C. Cardiac output at escalating doses of dobutamine.



Additonal references

1. Challand C, Struthers R, Sneyd JR, Erasmus PD, Mellor N, Hosie KB, Minto G. Randomized controlled trial of intraoperative goal-directed fluid therapy in aerobically fit and unfit patients having major colorectal surgery. *Br J Anaesth* 2012; 108: 53-62.
2. ATS/ACCP Statement on cardiopulmonary exercise testing. *Am J Respir Crit Care Med* 2003; 167: 211-277.
3. Cole CR, Blackstone EH, Pashkow FJ, Snader CE, Lauer MS. Heart-rate recovery immediately after exercise as a predictor of mortality. *The New England journal of medicine* 1999; 341: 1351-1357.
4. Cole CR, Foody JM, Blackstone EH, Lauer MS. Heart rate recovery after submaximal exercise testing as a predictor of mortality in a cardiovascularly healthy cohort. *Ann Intern Med* 2000; 132: 552-555.
5. Jouven X, Empana JP, Schwartz PJ, Desnos M, Courbon D, Ducimetiere P. Heart-rate profile during exercise as a predictor of sudden death. *N Engl J Med* 2005; 352: 1951-1958.
6. Cole CR, Blackstone EH, Pashkow FJ, Snader CE, Lauer MS. Heart-rate recovery immediately after exercise as a predictor of mortality. *N Engl J Med* 1999; 341: 1351-1357.
7. Clavien PA, Strasberg SM. Severity grading of surgical complications. *AnnSurg* 2009; 250: 197-198.
8. Krieger EM. Neurogenic Hypertension in the Rat. *Circ Res* 1964; 15: 511-521.
9. Brealey D, Karyampudi S, Jacques TS, Novelli M, Stidwill R, Taylor V, Smolenski RT, Singer M. Mitochondrial dysfunction in a long-term rodent model of sepsis and organ failure. *AmJPhysiol RegulIntegrComp Physiol* 2004; 286: R491-R497.
10. Murdoch CE, Alom-Ruiz SP, Wang M, Zhang M, Walker S, Yu B, Brewer A, Shah AM. Role of endothelial Nox2 NADPH oxidase in angiotensin II-induced hypertension and vasomotor dysfunction. *Basic research in cardiology* 2011; 106: 527-538.
11. Sullivan LM. Repeated measures. *Circulation* 2008; 117: 1238-1243.