C1 neurons mediate a stress-induced anti-inflammatory reflex in mice

Chikara Abe1,3,4, Tsuyoshi Inoue2,4, Mabel A Inglis1, Kenneth E Viar1, Liping Huang2, Hong Ye2, Diane L Rosin1, Ruth L Stornetta1, Mark D Okusa2 & Patrice G Guyenet1

C1 neurons, located in the medulla oblongata, mediate adaptive autonomic responses to physical stressors (for example, hypotension, hemorrhage and presence of lipopolysaccharides). We describe here a powerful anti-inflammatory effect of restraint stress, mediated by C1 neurons: protection against renal ischemia-reperfusion injury. Restraint stress or optogenetic C1 neuron (C1) stimulation (10 min) protected mice from ischemia-reperfusion injury (IRI). The protection was reproduced by injecting splenic T cells that had been preincubated with noradrenaline or splenocytes harvested from stressed mice. Stress-induced IRI protection was absent in Chrm7 knockout (a7nACHR−/−) mice and greatly reduced by destroying or transiently inhibiting C1. The protection conferred by C1 stimulation was eliminated by splenectomy, ganglionic-blocker administration or β2-adrenergic receptor blockade. Although C1 stimulation elevated plasma corticosterone and increased both vagal and sympathetic nerve activity, C1- mediated IRI protection persisted after subdiaphragmatic vagotomy or corticosterone receptor blockade. Overall, acute stress attenuated IRI by activating a cholinergic, predominantly sympathetic, anti-inflammatory pathway. C1s were necessary and sufficient to mediate this effect.

Activation of the immune system and leukocyte infiltration leads to tissue and organ injury in both acute conditions, such as IRI1, and chronic conditions, such as rheumatoid arthritis2. The cholinergic anti-inflammatory pathway (CAP) has been defined as the efferent arm of a vagal reflex that limits the duration and intensity of the inflammatory response2–6. The CAP is elicited by electrical stimulation of the vagus nerve or by noninnvasive ultrasound treatment7, procedures that have beneficial effects in a number of inflammatory models, including renal IRI2,8. The CAP is mediated by the spleen and its noradrenergic innervation9. The intrasplenic pathway includes activation of β2-adrenergic receptors expressed by choline acetyltransferase-positive (ChAT+) splenic memory T cells (CD4+CD44highCD62Llow)10,11, acetylcholine (ACh) release by these leukocytes and activation of α7 nicotinic receptors (α7nACHRs) on adjacent macrophages, leading to reduced production of proinflammatory cytokines12. A parallel sympathetic anti-inflammatory reflex, also converging on the spleen, contributes to the immunosuppressive effect of spinal injury13 or anesthesia14.

Of note, stimulating the central or the distal end of a divided cervical vagus nerve provides equal protection against renal IRI in mice8. Moreover, the renal protection conferred by stimulating the central end of the vagus nerve persists even if the stimulus is delivered while the contralateral vagus nerve is blocked8. Altogether, this type of evidence suggests that anti-inflammatory reflexes are not exclusively vagovagal and that, in some cases, the neural component of the CAP may rely on a canonical sympathetic route (i.e., splenic noradrenergic neurons activated by cholinergic preganglionic neurons residing in the spinal cord)15.

Inflammatory reflexes are almost certainly regulated by the CNS, because the CAP is activated by intracerebral administration of drugs2,16,17 and brain death unleashes peripheral inflammation18. Identifying and, ultimately, stimulating the CNS pathways that control the CAP could be beneficial in the management of chronic inflammatory diseases, spinal injury and organ transplantation2,13,18–20.

The autonomic nervous system, notably its sympathetic division, is mobilized by physical and psychological stressors21–23. A subset of these autonomic responses (especially cardiovascular) is mediated or facilitated by C1s, a group of glutamatergic, catecholaminergic and peptidergic neurons that reside in the medullary reticular formation21–23. Acute stress also mobilizes the immune system, as evidenced by rapid reductions in thymus and spleen volume and increased leukocyte flux in blood24. Though better known for their role in controlling sympathetic efferents, C1s also innervate the dorsal motor nucleus of the vagus and the paraventricular nucleus of the hypothalamus25,26. In addition, subsets of C1s are strongly activated by circulating interleukin-1 and lipopolysaccharide, as well as by footshock21. Therefore, we asked whether acute stress reduces tissue injury by activating the CAP, and we tested whether C1s are a critical node for central regulation of this neuroimmune anti-inflammatory reflex.

RESULTS
Restraint stress protects the kidneys from IRI
Most experiments were conducted in dopamine β-hydroxylase-hemizygous DβhCre/0 (hereafter DBH-cre) mice. We first examined whether a brief period of restraint stress provided protection in
an IRI model and whether the CAP contributed to the protective action. We chose to examine renal IRI, evoked by 26 min of ischemia (bilateral clamping of the renal arteries and veins) and followed by 24 h of tissue reperfusion, because renal damage can be precisely quantified by three complementary measures: plasma creatinine (a measure of kidney function), kidney $\text{Kim1}$ mRNA in DBH-cre mice and (d) acute tubular necrosis in DBH-cre mice ($n = 6$ mice per group). One-way ANOVA with Tukey-Kramer test (b; $F_{2,15} = 35.64, P < 0.0001$) or Kruskal-Wallis with Steel-Dwass test (c; $H = 14.36, P < 0.0001$; and d; $H = 14.36, P < 0.0001$); restraint $- IR -$: *$P < 0.05$, **$P < 0.001$ restraint $- IR +$ and restraint $+ IR +$ vs. restraint $- IR -$; $P < 0.05$, †$P < 0.001$ restraint $+ IR -$ vs. restraint $+ IR +$.

(e) Restraint stress protects wild-type mice ($\alpha$7WT) against IRI but had no effect in $\alpha$7nAChR$^{-/-}$ mice ($\alpha$7KO): two-way ANOVA with Tukey-Kramer test; $F_{1,18} = 37.03, P < 0.0001$. ***$P < 0.001$ restraint $+ IR +$ in $\alpha$7WT vs. restraint $- IR +$ in $\alpha$7WT, restraint $+ IR -$ in $\alpha$7KO and restraint $+ IR +$ in $\alpha$7KO; NS, not significant. Error bars represent s.e.m. When no error bar is shown, this is because the data were not normally distributed and a non-parametric test was used.

Figure 1 Restraint stress protects against renal IRI. (a) Timeline of experiments. (b–e) Effect of prior restraint stress on (b) plasma creatinine in DBH-cre mice, (c) $\text{Kim1}$ mRNA in DBH-cre mice and (d) acute tubular necrosis in DBH-cre mice ($n = 6$ mice per group). One-way ANOVA with Tukey-Kramer test (b; $F_{2,15} = 35.64, P < 0.0001$) or Kruskal-Wallis with Steel-Dwass test (c; $H = 14.36, P < 0.0001$) and (d) $H = 14.36, P < 0.0001$; restraint $- IR -$: *$P < 0.05$, **$P < 0.001$ restraint $- IR +$ and restraint $+ IR +$ vs. restraint $- IR -$; $P < 0.05$, †$P < 0.001$ restraint $+ IR -$ vs. restraint $+ IR +$.

A 10-min period of physical restraint provided strong protection from kidney injury evoked by renal ischemia-reperfusion (IR) 24 h later (Fig. 1a), as shown by lower plasma creatinine (0.44 ± 0.18 versus 1.38 ± 0.08 mg per dl; $n = 6$ mice per group; Fig. 1b), reduced $\text{Kim1/Gapdh}$ mRNA ratio (Fig. 1c) and lesser degree of tubular necrosis (Fig. 1d and Supplementary Fig. 1) compared to unstressed mice. Of note, stress did not totally protect the mice against IRI: all three markers of injury were significantly higher in stressed mice subjected to renal IR than in DBH-cre mice subjected neither to stress nor renal IR (Fig. 1b–d).

The CAP requires $\alpha$7nAChRs that are expressed by splenic macrophages and, possibly, by the noradrenergic innervation of the spleen. As an initial test of whether restraint stress protects against renal IRI by activating this pathway, we examined whether restraint protected the kidneys in $\alpha$7nAChR$^{-/-}$ ($\alpha$7KO) mice. Restraint stress did not ameliorate the renal IRI in these mice, but it effectively protected genetically matched controls ($\alpha$7WT), as evidenced by a greatly reduced plasma creatinine level 24 h after renal IR (Fig. 1e).

Seeking further evidence that restraint stress protects mice from kidney IRI by activating the CAP, we first examined whether splenocytes harvested from stressed C57BL/6 mice conferred protection against renal IRI to unstressed mice of the same strain (Fig. 2a). Injection of splenocytes protected unstressed recipient mice from renal IRI in a dose-dependent fashion regardless of the donor, but protection required significantly fewer cells when the splenocytes originated from stressed mice (Fig. 2b). Activation of splenic T-lymphocytes by noradrenaline is a critical stage of the CAP. We therefore tested whether administration of CD4 T-lymphocytes harvested from the spleen of unstressed C57BL/6 mice and incubated with noradrenaline in vitro would protect unstressed mice from renal IRI (Fig. 2c). This was indeed the case (Fig. 2d).

In brief, a short period of physical restraint (10 min) protects the kidneys from IRI inflicted 24 h later. The same degree of protection was observed in two strains of mice (DBH-cre and $\alpha$7WT). The protection conferred by stress was absent in $\alpha$7KO mice; it was transferable to naive mice by injecting splenocytes harvested from stressed mice or by injecting splenic CD4 T cells harvested from naive mice and exposed to noradrenaline in vitro. These results suggest that restraint stress, like vagus nerve stimulation and noninvasive ultrasound, protected mice from renal IRI by activating the CAP.

Selective optogenetic stimulation of C1 neurons protects the kidneys from IRI

C1s are activated by physical stresses such as hypoxia, hemorrhage, hypoglycemia and infection and, as a group, C1s heavily innervate both sympathetic and vagal preganglionic neurons as well as the paraventricular nucleus of the hypothalamus. If activated, each of these CNS output pathways could potentially elicit anti-inflammatory effects. Accordingly, we first asked whether selective stimulation of C1s also protects mice against renal IRI. Five to six weeks after injecting a FLEX (flip-excision) channelrhodopsin2 (ChR2)-mCherry vector (AAV2-DIO-EF1a-ChR2[H134R]-mCherry, hereafter AAV2-ChR2-mCherry) into the left rostral ventrolateral medulla oblongata (RVLM) of DBH-cre mice, Cre-mediated recombination and ChR2 expression was confined to tyrosine hydroxylase-immunoreactive (TH+) neurons located within the ventrolateral medulla, 6.1–7.2 mm caudal to bregma (Fig. 3b and Supplementary Fig. 2a). In mice, most (>95%) TH+ neurons located within this region of the medulla oblongata contain phenylethanolamine N-methyltransferase (PNMT) transcripts; therefore, most of the ChR2+ neurons were, by definition, C1 adrenergic cells. Five to six weeks after injection of the control vector into the left medulla oblongata (AAV2-DIO-EF1a-mCherry, hereafter DIO-mCherry), mCherry immunoreactivity was also confined to TH+ neurons located in the RVLM (results not shown).

Unilateral optogenetic stimulation of C1s was performed in unanesthetized mice 24 h before renal IRI (Fig. 3a; optrode placements illustrated in Fig. 3b and Supplementary Fig. 2b). In anesthetized mice, each light pulse evoked a single action potential in C1s. The stimulation parameters (5 Hz, 10-ms pulses) were selected to produce a small submaximal respiratory stimulation (Supplementary Fig. 2c–f and Supplementary Table 1). C1 stimulation was maintained for 10 min in order to match the duration of the restraint stress. C1 stimulation reduced behavioral activity and locomotion while eliciting a regular, slightly increased breathing rate (Supplementary Fig. 3). These effects ended upon cessation of the stimulus (Supplementary Fig. 3). Respiratory stimulation and reduced behavioral activity were caused by C1 activation and not by the light itself because they were undetectable in the six DBH-cre mice that had received injections of control vector (DIO-mCherry; results not shown).

Plasma creatinine, $\text{Kim1/Gapdh}$ mRNA ratio and tubular necrosis were low in mice subjected to sham surgery and, as expected for renal IRI, these parameters were elevated 24 h after renal IRI (Fig. 3c–e and Supplementary Fig. 4). C1 stimulation greatly reduced all three indices of renal pathology elicited by IR (Fig. 3c–e and Supplementary Fig. 4). By contrast, blue laser illumination of the
ventrolateral medulla of DBH-cre mice that had received the control vector AAV2-mCherry had no protective effect (Fig. 3c–e and Supplementary Fig. 4). In short: mild, selective, optogenetic stimulation of C1 neurons resulted in behavioral quiescence, increased breathing slightly and protected against renal IRI inflected 24 h later.

Renal IRI protection by restraint stress requires C1 neurons

Next, we tested whether the beneficial effect of restraint stress would be attenuated in mice in which the C1s were inhibited during the stress period. We transduced C1s to express Gi-coupled designer receptors exclusively activated by designer drugs (DREADDs) by injecting AAV2-DIO-hSyn-hm4D(Gi)-mCherry bilaterally into the RVLM of DBH-cre mice. When activated, this receptor inhibits neurons, such as C1s, that express inwardly rectifying potassium channels.

We also examined whether the protective effect of restraint stress would be attenuated if C1s were selectively destroyed. To that end, we injected AAV2-DIO-taCas3-TEVe, a FLEX caspase-expressing AAV2 vector, bilaterally into the RVLM of DBH-cre mice. Destruction of C1s eliminated the protective effect of restraint stress (Fig. 4c).

Corticosterone release does not account for the protective effect of C1 stimulation against renal IRI

Stress activates corticosterone synthesis and release (for example, ref. 34). A subset of C1s innervate the hypothalamic paraventricular nucleus and likely contribute to the activation of the corticotropin releasing factor (CRF)–adrenocorticotropic hormone (ACTH)–corticosterone cascade. Corticosterone has anti-inflammatory effects that could conceivably explain the reduced renal damage.
Figure 4 C1 neurons mediate the protective effect of restraint stress against renal IRI. (a) mCherry (magenta) and TH (green) immunoreactivity in the left medulla oblongata of a DBH-cre mouse 6 weeks after stereotaxic microinjection of AAV2-DIO-hSyn-hm4D(Gi)-mCherry DREADD (transverse section; scale bar, 100 µm). (b) Rostrocaudal distribution (mm caudal to bregma) of mCherry and TH immunoreactivities in the left medulla oblongata of a DBH-cre mouse 6 weeks after injection of AAV2-DIO-taCasp3-TEVp (AAV2-caspase). After AAV2-caspase treatment, C1s in the rostral ventrolateral medulla (RVLM) are undetectable (left and lower middle panels) but other catecholaminergic neurons (dorsal medulla, DMM, and locus coeruleus, LC) are intact (top middle and top right panels). ChAT+ neurons are unaffected by AAV2-caspase treatment regardless of location (left and bottom middle). Scale bars, 500 µm (left) or 100 µm (four right panels). Amb, nucleus ambiguus. (d) Rostrocaudal distribution of TH-immunoreactive RVLM neurons in control DBH-cre mice (n = 7) vs. caspase-treated DBH-cre mice (n = 10). Lesions were bilateral; cells were counted on one side only. Two-way ANOVA with Tukey–Kramer test; F_{12,180} = 25.99, P < 0.0001. (e) The protective effect of restraint stress against renal IRI was attenuated by inhibiting (DREADD) or lesioning (caspase) the C1 neurons (n = 6 DBH-cre mice per group). CNO, clozapine N-oxide (3 mg/kg); vehicle, saline. One-way ANOVA with Tukey–Kramer test; F_{5,30} = 14.11, P < 0.0001. ***P < 0.001 restraint×DREADD + vehicle + restraint×IR+ vs. restraint×IR−; and 1P < 0.05, 11P < 0.01 and 111P < 0.001 DREADD + CNO + restraint×IR− vs. caspase + restraint×IR+ and caspase + restraint×IR+ vs. restraint×IR− and DREADD + vehicle + restraint×IR−. NS, not significant. Error bars represent s.e.m.

Figure 5 Corticosterone is released by C1 stimulation and restraint stress but plays no detectable role in protecting kidneys from renal IRI. (a) Restraint stress increased plasma corticosterone in DBH-cre mice (unpaired t-test; t_{10} = 4.991). ***P < 0.001 restraint× vs. restraint−. (b) C1 optogenetic stimulation (AAV2-DIO-EE1α-ChR2-mCherry, ChR2) increased plasma corticosterone in DBH-cre mice, whereas laser light alone was ineffective in DBH-cre mice injected with control vector (AAV2-DIO-EE1α-mCherry, mCherry). Laser, 5 Hz for 10 min. One-way ANOVA with Tukey–Kramer test; F_{2,21} = 6.959, *P < 0.05. for ChR2 + laser+ vs. ChR2 + laser− and mCherry + laser+. (c) Mifepristone (30 mg/kg) did not alter the protective effect of C1 stimulation against renal IRI in DBH-cre mice (n = 6 mice group; unpaired t-test; t_{10} = 0.495, P = 0.6313). NS, not significant. Error bars represent s.e.m.

C1 neuron stimulation activates the autonomic nervous system

As shown before, optogenetic stimulation of C1 in anesthetized DBH-cre mice (10 ms pulses, 5–20 Hz, 10-s trains) produced a robust and sustained frequency-dependent increase in multifiber efferent vagus nerve activity on the ipsilateral side (Fig. 6a). We add here that contralateral efferent vagus nerve activity was also increased by stimulating C1 at 2.5 Hz, albeit to a lesser degree (Fig. 6b). These results accord with the existence of a dominantly ipsilateral monosynaptic excitatory connection between C1 and the dorsal motor nucleus of the vagus. For technical reasons, sympathetic nerve activity was recorded from a renal nerve rather than a splenic nerve. Low-frequency stimulation of C1 (1 Hz) evoked a robust phasic response in the renal sympathetic nerve (onset latency: 59 ± 4.7 ms; peak latency: 102.8 ± 11.7 ms; Fig. 6c). Trains of stimuli delivered at 5–20 Hz also increased mean renal sympathetic nerve activity (sham versus 20 Hz, P = 0.005; 5 Hz versus 20 Hz, P = 0.0164; Fig. 6d). These observations agree with and extend prior evidence that C1s are excitatory and directly innervate both sympathetic and vagal parasympathetic preganglionic neurons.

Figure 6a C1 neuron stimulation activates the autonomic nervous system

Contribution of spine and autonomic nervous system to the protective effect of C1 stimulation against renal IRI

In order to test whether C1 activation protects mice against renal IRI by activating the autonomic nervous system, we administered a ganglionic blocker, hexamethonium, 30 min before optogenetically stimulating C1 (Fig. 7a). These experiments were performed in DBH-cre mice that had received unilateral injections of following restraint stress and/or C1 stimulation. Both restraint stress (10 min) and optogenetic activation of C1s (10 min at 5 Hz) increased plasma corticosterone in unanesthetized DBH-cre mice, although the hormonal rise elicited by C1 stimulation was smaller than that elicited by restraint (Fig. 5a,b). The plasma corticosterone surge elicited by C1 stimulation was not caused by handling the animals nor by the light itself, because brainstem illumination did not change the corticosterone level in mice that had received injections of control vector AAV2-mCherry (Fig. 5b).

In order to test whether corticosterone mediates the renal IRI protection elicited by C1 stimulation, we administered the corticosterone antagonist mifepristone to DBH-cre mice before stimulating C1s optogenetically. Mifepristone did not reduce the renal protective effect, suggesting that the beneficial effect of C1 stimulation against renal IRI is not mediated by the rise in plasma corticosterone (Fig. 5c).
AAV2-ChR2-mCherry 5 weeks prior. Hexamethonium greatly attenuated the renal protection elicited by C1 stimulation (Fig. 7a); thus activation of vagal and/or sympathetic efferents was required for C1 to protect against renal IRI.

The spleen is a critical component of the CAP3, and electrical stimulation of the vagus nerve protects the kidneys from IRI only if the spleen is intact8. Splenectomy, performed 7 d before stimulating C1, also eliminated the protective effect of C1 stimulation against renal IRI (Fig. 7b). This result, combined with the fact that C1 stimulation increases vagal and sympathetic efferent activity and the efficacy of hexamethonium, suggests that C1 stimulation, like restraint stress, protected the kidneys by activating the CAP3. The CAP requires activation of β2-adrenergic receptors expressed by splenic T cells13. Accordingly, we tested whether β2-adrenergic receptor blockade would prevent the protective effect of C1 stimulation against renal IRI (Fig. 7a). Butoxamine, a β2-selective antagonist that blocks the CAP in mice13, eliminated the protective effect of C1 stimulation (Fig. 7a). We also tested labetalol, a nonselective β1-, β2- and α1-adrenergic receptor blocker in humans. However, this agent was ineffective (results not shown), perhaps reflecting weak blockade of β2-receptors in mice or some consequence of its additional actions on β1- and/or α1-receptors in the CNS or elsewhere.

Both vagal and sympathetic preganglionic neurons appear capable of triggering the CAP3. In order to identify which division of the autonomic nervous system mediates the renal IRI protection elicited by C1 stimulation we performed subdiaphragmatic vagal denervations (Fig. 7c). First, we tested the efficacy of the surgical procedure by injecting the retrogradely transported dye Fluoro-Gold intraperitoneally. As expected, in sham-operated mice, the dye was abundantly present in the dorsal motor nucleus of the vagus (Supplementary Fig. 5). By contrast, Fluoro-Gold was absent from this nucleus in mice in which vagus nerve branches had been cut immediately below the diaphragm (n = 3 in each group; Supplementary Fig. 5). We then determined whether the renal protection produced by C1 stimulation was attenuated in mice with subdiaphragmatic vagal denervation; it was not (n = 6 mice per group; Fig. 7c). Thus, the subdiaphragmatic vagus nerve was not required for C1 stimulation to activate the CAP and protect the kidneys from IRI. Since protection was greatly reduced by ganglionic blockade but unaffected by mifepristone, C1 most likely activated the CAP via a sympathetic route.

**Restraint stress and C1 stimulation produce opposite effects on arterial pressure and heart rate**

Given that restraint stress and C1 stimulation were equally effective at protecting the kidneys against IRI, we wondered whether these two stimuli produced similar hemodynamic effects and whether the latter could conceivably account for the protection. Telemetric probes were implanted to record pulsatile arterial pressure (AP) from a carotid artery and the heart rate (HR) was ultimately derived from these recordings. As expected, restraint stress elevated AP (from 97.4±1 mmHg) and HR (from 243.4±17.6 to 593.6±18.2 bpm; n = 4; Fig. 8a–c). These changes persisted for at least another 10 min after the mice were returned to their home cage. By contrast, C1 stimulation at 5 Hz for 10 min lowered AP (from 110.1±2 to 92.2±5.5 mmHg) and HR (from 593.5±22.6 to 461.3±24 bpm; n = 4; Fig. 8d–f), and these parameters returned to control less than a minute after the end of the stimulus (Fig. 8d–f).
In short, C1 stimulation produced a cardiovascular response usually associated with freezing behavior (reduced locomotion, decreased HR) whereas restraint stress produced a pattern more reminiscent of fight or flight behavior (increased HR and arterial pressure)\(^\text{36}\). Both responses are associated with a reduction in renal and splanchnic blood flow\(^\text{36}\), suggesting that C1 stimulation and restraint stress could perhaps have protected the kidneys against IRI via ischemic preconditioning\(^\text{37}\). In order to test the plausibility of such interpretation, we determined whether 10 min of C1 stimulation in a conscious mouse produced a measurable increase in the renal level of hypoxia-inducible factor 1a (Hif1a) transcripts. The renal Hif1a/Gapdh mRNA ratio was unchanged by C1 stimulation, but subjecting the kidneys of anesthetized mice to a short period of ischemia (10 min), which did not produce any detectable renal damage, increased this ratio significantly (no IRI versus IRI: \(P = 0.043\); C1 vs. IRI: \(P = 0.025\); Supplementary Fig. 6). In short, we found no evidence that C1 stimulation for 10 min produced renal ischemia.

**DISCUSSION**

This study demonstrated that acute restraint stress protected the kidneys from IRI and that C1 neurons were necessary and sufficient for this effect to occur. Restraint stress and C1 stimulation produced an equivalent degree of protection against renal IRI by three different measures: plasma creatinine, *Kim1* transcripts and histological analysis of renal tubular damage. Like the protection against renal IRI elicited by ultrasound or vagal efferent stimulation\(^\text{8,27}\), the beneficial effect of restraint stress or C1 stimulation was mediated by the splenic CAP. The protection elicited by C1 stimulation, though mediated by the autonomic nervous system, did not require the subdiaphragmatic vagus nerve. We conclude that C1 neurons could be a nodal point of the neuroimmune reflex mediated via the CAP and suggest that this anti-inflammatory pathway can be activated predominantly via a sympathetic rather than vagal preganglionic efferent pathway.

**Mechanism of injury protection by stress and the C1 neurons**

C1 stimulation elevated plasma corticosterone, albeit to a lesser extent than restraint. Yet this hormone did not contribute detectably to the...
VOL. 20 | NO. 5 | MAY 2017

C1 cells: beyond cardiovascular homeostasis

C1 neurons are glutamatergic and catecholaminergic, and they differentially express several neuropeptides (for example, neuropeptide Y, substance P, enkephalin). Subsets of these neurons are believed to operate as a switchboard for eliciting behaviorally appropriate patterns of autonomic responses. Only a third of the C1s innervate sympathetic preganglionic neurons, which contribute to blood pressure and glucose homeostasis via sympathetic reflexes that are predominantly organized at lower levels of the neuraxis. In addition to their homeostatic function, C1s also have an allostatic role (arousal from sleep, freezing behavior, cardiorespiratory adjustments), which is revealed when they are selectively activated (see ref. 45 and present results). One interpretation of the present study is that a subset of C1s, currently unidentified, regulates the immune system during psychological and possibly other forms of stress. The predominantly sympathetic activation route of the anti-inflammatory splenic mechanism elicited by C1 stimulation suggests that this subset of C1s could be bulbohypothalamic. However, this argument is not compelling. Bulbohypothalamic-C1s also target a common set of CNS structures that are implicated in autonomic regulations (dorsal vagal complex, parabrachial nuclei, periaqueductal gray matter, locus coeruleus and other noradrenergic neurons); activation of any one of these structures could ultimately increase sympathetic efferent activity and activate the CAP via descending pathways other than the bulbohypothalamic C1s. C1s belong to the lower brainstem reticular core and respond to a wide variety of stimuli; for example, they are excited by restraint stress and by somatic as well as by visceral, notably vagal, afferents. This vagal sensory input could explain why activating the central end of a divided vagal nerve also protects the kidneys from IRI. This broad set of inputs, combined with the ability of C1 to activate parasympathetic and sympathetic efferents, may explain why the CAP can be activated by triggers as diverse as stimulation of vagal afferents or efferents, stimulation of the auricular nerve, stress and electroacupuncture. In other words, CAP activation may be one of a constellation of stereotyped effects (arousal, cardiorespiratory stimulation) elicited or facilitated by C1 in response to internal or external ‘danger’ signals. Intense or artificially synchronized afferent-fiber activity, such as that produced by repetitive electrical stimulation of the vagal nerve or subsets of somatic afferents (acupuncture), may be such a trigger.

In conclusion, C1 mediated the protective effect of stress against renal IRI by activating the CAP. In this particular case the CAP seems to have been activated via a sympathetic rather than vagal route. This study also provides proof of principle that localized brain stimulation can produce beneficial anti-inflammatory effects.
METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

ACKNOWLEDGMENTS

Editorial comments by D.A. Bayliss (University of Virginia, Pharmacology Department) are gratefully acknowledged. We thank the University of Virginia Research Histology Core for their assistance in preparation of histology slides. Research reported in this publication was supported by the National Heart, Lung, and Blood Institute of the NIH under award numbers ROIHL028785 and ROIHL074011 (to P.G.G.), by the National Institute of Diabetes and Digestive and Kidney Diseases of the National Institutes of Health (NIH) under award numbers R01DK085259 and R01DK063234 (to M.D.O.) and by two Japan Society for the Promotion of Science Postdoctoral Fellowships for Overseas Researchers (awarded separately to C.A. and T.I.). The stereology data described here was performed with an MBF Bioscience and Zeiss microscope system for stereology and tissue morphology funded by National Institutes of Health grant 1S10RR026799-01 (to M.D.O.). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

AUTHOR CONTRIBUTIONS

C.A., T.I., D.L.R., R.L.S., M.D.O. and P.G.G. designed research studies; C.A., T.I., M.A.I., K.E.V., L.H. and H.Y. conducted experiments and acquired and analyzed the data; and C.A., T.I., D.L.R., R.L.S., M.D.O. and P.G.G. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Reprints and permissions information is available online at http://www.nature.com/reprints/index.html.

1. Li, L. et al. IL-17 produced by neutrophils regulates IFN-gamma-mediated neutrophil migration in mouse kidney ischemia-reperfusion injury. J. Clin. Invest. 120, 331–342 (2010).
2. Pavlov, V.A. & Tracey, K.J. Neural circuitry and immunity. Immunol. Rev. 263, 38–57 (2015).
3. Olofsson, P.S., Rosas-Ballina, M., Levine, Y.A. & Tracey, K.J. Rethinking inflammation: neural circuits in the regulation of immunity. Immunol. Rev. 248, 188–204 (2012).
4. Yamakawa, K. et al. Electrical vagus nerve stimulation attenuates systemic inflammation and improves survival in a rat heartsteal model. PLoS One 8, e56728 (2013).
5. Jiang, Y. et al. Vagus nerve stimulation attenuates cerebral ischemia and reperfusion injury via endogenous cholinergic pathway in rat. PLoS One 9, e103242 (2014).
6. Kato, N. et al. Differential regulation of TNF receptors by vagal nerve stimulation protects heart against acute ischemic injury. J. Mol. Cell. Cardiol. 49, 234–244 (2010).
7. Gigliotti, J.C. et al. Ultrasound modulates the splenic immune response axis in attenuating AKI. J. Am. Soc. Nephrol. 26, 2470–2481 (2015).
8. Inoue, M., Uejima, T., Ueno-Nakamura, Y., Niehaus, J., Popovich, P.G. & Yoshida, Y. Silencing spinal interneurons inhibits immune suppressive autonomic reflexes caused by spinal cord injury. Nat. Neurosci. 19, 784–787 (2016).
9. Rosas-Ballina, M. et al. Acetylcholine-synthesizing T cells relay neural signals in a vagus nerve circuit. Science 334, 98–101 (2011).
10. Ueno, M., Ueno-Nakamura, Y., Niehaus, J., Popovich, P.G. & Yoshida, Y. Silencing spinal interneurons inhibits immune suppressive autonomic reflexes caused by spinal cord injury. Nat. Neurosci. 19, 784–787 (2016).
11. Martelli, D., Yao, S.T., McKinley, M.J. & McAllan, R.M. Reflex control of inflammation by sympathetic nerves, not the vagus. J. Physiol. (Lond.) 592, 1677–1686 (2014).
12. Martelli, D., McKinley, M.J. & McAllan, R.M. The cholinergic anti-inflammatory pathway: a critical review. Auton. Neurosci. 182, 65–69 (2014).
13. Bemik, T.R. et al. Pharmacological stimulation of the cholinergic anti-inflammatory pathway. J. Exp. Med. 195, 781–788 (2002).
14. Pavlov, V.A. et al. Central muscarinic cholinergic regulation of the systemic inflammatory response during endotoxemia. Proc. Natl. Acad. Sci. USA 103, 5219–5223 (2006).
15. Popovich, P.G. et al. Cholinergic antiinflammatory action is effective in endotoxemic animals. J. Auton. Nerv. Syst. 252, R1356–R1369 (2013).
16. DePuy, S.D. et al. Glutamatergic neurotransmission between the C1 neurons and the parasympathetic preganglionic neurons of the dorsal motor nucleus of the vagus. J. Neurosci. 33, 1486–1497 (2013).
17. Olofsson, P.S. The role of the vagus nerve in sepsis and inflammation. Front. Immunol. 5, 133 (2014).
18. Tucker, D.C., Saper, C.B., Ruggiero, D.A. & Reis, D.J. Organization of central sympathetic and adrenergic pathways: I. Relationships of ventrolateral medullary projections to the central nervous system innervation of the rat spleen using viral transneuronal tracing. J. Comp. Neurol. 386, R135–R152 (1997).
19. Reardon, S. et al. Central chemoreceptors and the ventilatory response. Respir. Physiol. 150, 1–13 (2005).
20. Goto, M. et al. Modulation of peripheral sympathetic nerve activity during endotoxia in rats. J. Auton. Nerv. Syst. 170, 28–34 (2011).
ONLINE METHODS

Animals. Animal use was in accordance with the NIH Guide for the Care and Use of Laboratory Animals and was approved by the University of Virginia Animal Care and Use Committee. Dhb-Cre<sup>lox/lox</sup> mice were obtained from the Mutant Mouse Regional Resource Center at the University of California, Davis, CA (Tg(DH1-cre)KH212Gsat/Mmc3; stock no. 032081-UCD). Dhb-Cre<sup>lox/lox</sup> mice were maintained as hemizygous (Cre<sup>0</sup>) on a C57BL/6J background. A total of 182 Dhb-Cre<sup>lox/lox</sup> male mice (DBH-cre), aged between 10 and 14 weeks, were used for these experiments. Chrna7<sup>−/−</sup> (referred to as α7KO) mice (B6.129S7-Chrna7tm1Bay/1c) were obtained from Jackson Laboratories, and WT (Chrna7<sup>+/+</sup>) progeny were used as controls in this experiment (n = 12 α7WT mice and n = 6 α7KO mice). For the adoptive transfer (n = 56) and Fluorogold (FG, n = 6 experiments), we used WT NCI C57BL/6 mice that were purchased from Charles River Laboratories.

Viral vectors. AAV-DIO-EF1α-ChrR2(H134R)-mCherry serotype 2 (AAV2-Chr2-mCherry), AAV-DIO-EF1α-mCherry serotype 2 (AAV2-mCherry), AAV2-DIO-taCasp3-TEVp and AAV-DIO-hSyn-hm4D(Gi)-mCherry were purchased from the University of North Carolina vector core (the first two constructs courtesy of K. Deisseroth (Stanford University), third construct courtesy of N. Shah (University of San Francisco), fourth construct courtesy of B. Roth (University of North Carolina)). AAV2-Chr2-mCherry expresses an enhanced version of the photosensitive cationic channel Chr2 (Chr2(H134R)) fused to the fluorescent reporter mCherry. In these vectors, the Chr2-mCherry (AAV2-Chr2-mCherry), mCherry (AAV2-mCherry), taCasp3-TEVp and hm4D(Gi)-mCherry sequences are flanked by the same double-lox sites (LoxP and lox2272).

Injections of viral vector into the rostral ventrolateral medulla (RVLM) and optical fiber placement. AAV2-Chr2-mCherry or AAV2-mCherry was injected unilaterally into the left RVLM; this was followed by placement of an optical fiber. AAV2-DIO-taCas3-TEVp and AAV2-DIO-hSyn-hm4D(Gi)-mCherry were injected bilaterally into the RVLM; optical fibers were not installed in these mice. The injections were made under aseptic conditions in mice anesthetized with a mixture of ketamine (100 mg/kg) and dexmedetomidine (0.2 mg/kg, i.p.). Depth of anesthesia was deemed sufficient if the corneal and hind paw withdrawal reflexes were absent. Additional anesthetic was administered as necessary (10% of the original dose, i.p.). Body temperature was maintained at 37.0 ± 0.5 °C with a servo-controlled temperature pad (TC-1000; CWE). The mandibular branch of the facial nerve was revealed through a small skin incision (left side or both sides) with a Kopf 1730 stereotaxic apparatus adapted for mouse stereotaxic injections (ear vector, three 140-nL injections were made 300 µm above the injection site (on both sides as required) for later electrical stimulation. The mice were then placed prone on a Kopf 1730 stereotoxic apparatus adapted for mouse stereotoxic injections (ear bar adapter, model EB-5N, Narishige Scientific Instrument Lab; bite bar, Model 926 mouse adapter set at ~2 mm; David Kopf Instruments). The injection vector was loaded into a glass pipette with a 1.2-mm internal diameter, broken to a 25-µm tip (external diameter) and introduced into the brain through a 1.5-mm-diameter hole that was drilled into the occipital plate caudal to the parieto-occipital suture on the left side (or on both sides). The facial nerve was stimulated (0.1 ms, 1–300 µA, 1 Hz) to evoke antidromic field potentials within the facial motor nucleus 29. The light meter (Thorlabs), and the laser setting required to deliver 10 mW was recorded. This setting was later used during the in vivo experiments. The stereotoxic procedure used for bilateral injection of AAV2-DIO-taCas3-TEVp or AAV2-DIO-hSyn-hm4D(Gi)-mCherry into the RVLM was the same except that no optical fiber was inserted.

Mice received postoperative boluses of atipamezole (α<sub>2</sub>-adrenergic antagonist, 2 mg/kg, s.c.), ampicillin (125 mg/kg, i.p.) and ketoprofen (4 mg/kg, s.c.). Ampicillin and ketoprofen were readministered 24 h postoperatively. Mice were housed in the University of Virginia vivarium for at least 4 weeks after virus injection. Mice were maintained in groups of 3–5 per cage on a 12:12-h light-dark cycle. During this time, mice gained weight normally and appeared unremarked by the implanted optical fiber. These mice were randomly divided into the various treatment groups.

Whole-body plethysmography. Optogenetic stimulation of C1 produces a frequency-dependent increase in breathing frequency and amplitude 48. Breathing patterns evoked by brief periods of C1 photostimulation (10 s) were therefore routinely measured in conscious mice to ensure that C1s were effectively transduced to express Chr2 and that the optical fiber was correctly implanted. Breathing was measured in conscious mice using unrestrained whole-body plethysmography (EMKA Technologies), as described previously 26. The chamber was continuously flushed with dry, room-temperature air (23–26 °C) delivered at 0.5 standardized liters per min. Fluctuations in chamber pressure were amplified (x500) and acquired at 1 kHz with Spike 2 software (v7.06; CED). Respiratory frequency (fR; breaths/min) was measured during periods of behavioral quiescence. The measurements were made between 10:00 a.m. and 4:00 p.m. Mice were placed in the plethysmography chamber and habituated to chamber conditions for 2–3 h. At this point, mice were briefly anesthetized with isoflurane while the connection between the implanted fiber optic and the laser was established. Recordings were initiated at least 30 min after the mice had regained consciousness and only during periods of behavioral quiescence. To evaluate the effects of photostimulation on the inhalation, 10- s trains of light pulses (duration, 10 ms) were delivered at various frequencies (5, 10, 15 and 20 Hz). The minority of DBH-cre mice that had received injections of AAV2-Chr2-mCherry and did not have a detectable breathing stimulation at 5 Hz were not included in the experimental group (10 of 182). These mice were used as a control group (no laser group in renal IRI or corticosterone experiment).

Splenectomy. DBH-cre mice (n = 12) were anesthetized with an i.p. injection of ketamine (120 mg/kg) and xylazine (12 mg/kg). Depth of anesthesia was assessed by absence of the corneal and hind paw withdrawal reflexes. Additional anesthetic was administered as necessary (10% of the original dose, i.p.). Body temperature was maintained at 37.0 ± 0.5 °C with a servo-controlled temperature pad (TC-1000; CWE). A small flank incision was performed through which the splenic vasculature was ligated and the spleen removed. Mice received boluses of ketoprofen (4 mg/kg, s.c.) immediately after surgery and again 24 h later. These mice were allowed to recover for 7 d before renal IR.

Subdiaphragmatic vagotomy. Subdiaphragmatic vagotomy (sVNX) was performed in 6 DBH-cre mice that had received injections of AAV2-Chr2-mCherry into the RVLM one week before testing the effect of C1 stimulation on renal IRI. The mice were anesthetized with an i.p. injection of ketamine (120 mg/kg) and xylazine (12 mg/kg). Depth of anesthesia was assessed by absence of the corneal and hind paw withdrawal reflexes. Additional anesthetic was administered as necessary (10% of the original dose, i.p.). Body temperature was maintained at 37.0 ± 0.5 °C with a servo-controlled temperature pad (TC-1000; CWE). After middle upper laparotomy, the stomach was gently manipulated to expose the esophagus. Then, both anterior and posterior trunks of the vagal nerves were identified between the diaphragm and the gastric cardia and then transected. In the sham-operated animals (10 DBH-cre mice), the abdominal vagal nerves were similarly exposed but not cut. Mice received boluses of ampicillin (125 mg/kg, i.p.) and ketoprofen (4 mg/kg, s.c.) immediately after surgery and again 24 h later. Six additional mice (C57BL/6) were used to test the efficacy of the denervation procedure. Three mice were subjected to sVNX and the rest underwent sham surgery, as described above. Seven d after surgery the mice were given an i.p. injection of 200 µl of 1% Fluorogold. After 4 d, the mice were deeply anesthetized and perfused. Brains were removed and postfixed in 4% paraformaldehyde for up to 3 d, and then all brains were sectioned as described below.

Arterial pressure and heart rate measurement in conscious mice subjected to C1 stimulation or restraint stress. The cardiorespiratory effects (AP, HR, breathing parameters) produced by unilateral optogenetic stimulation of C1s were examined in 10 DBH-cre mice prepared as described above. These mice were implanted with a radio-telemetry probe (PA-C10; Data Sciences International)
3 weeks after injection of AAV2-Chr2-mCherry into the RVLM to measure AP (right common carotid artery). A separate cohort of 4 DBH-cre mice was used to measure the effect of 10 min of restraint stress (method described below) on AP and HR; breathing was not measured in these mice. For arterial pressure probe implantation, mice were anesthetized with an i.p. injection of ketamine (120 mg/kg) and xylazine (12 mg/kg), and their body temperature kept at 37.0 ± 0.5 °C. Depth of anesthesia was assessed by absence of the corneal and hindpaw withdrawal reflexes. Additional anesthetic was administered as necessary (10% of the original dose, i.p.). AP and HR were measured a minimum of 1 week after arterial probe implantation using a PhysioTel Receiver (RLA 1020; Data Science International). Mean AP and HR were calculated from pulsatile AP recordings based on values calibrated before implantation of the telemetry probe. For optogenetic experiments only, the mice were placed in the plethysmography chamber in order to record respiratory parameters along with pulsatile AP. Breathing parameters (IR, Vt, MV) were extracted as described earlier.

In one set of 6 DBH-cre mice, we examined the effects of brief (10-s) periods of C1 photostimulation delivered at various frequencies (5, 10, 15 and 20 Hz) during behavioral quiescence. This experiment showed that 5-Hz stimulation produced a reliable and submaximal breathing stimulation. On that basis, 5 Hz was selected to examine the effects of longer periods of stimulation on renal IRI produced a reliable and submaximal breathing stimulation. On that basis, 5 Hz was selected to examine the effects of longer periods of stimulation on renal IRI (10 min). The effect of 10 min of C1 stimulation at 5 Hz on AP, HR and breathing was examined in 4 DBH-cre mice. The effect of 10 min of restraint stress (method described below) on AP and HR was evaluated in a separate cohort of 4 DBH-cre mice.

Nerve recordings in urethane-anesthetized mice. These experiments on anesthetized mice were designed to examine the consequences of C1 optogenetic stimulation on the sympathetic and parasympathetic efferent activities. Vagus nerve activity (VNA) evoked by optogenetic stimulation of C1 cells at various frequencies was recorded in 12 urethane-anesthetized DBH-cre mice (1.6 g/kg urethane i.p. given as a 20% w/v solution). These mice had received injections of AAV2-Chr2-mCherry 4–5 weeks prior and also had an implanted radio-telemetry probe for blood pressure measurement. Depth of anesthesia was assessed by absence of the corneal and hindpaw withdrawal reflexes. Additional anesthetic was administered as necessary (10% of the original dose, i.p.). Body temperature was maintained at 37.0 ± 0.5 °C with a servo-controlled temperature pad (TC-1000; CWE). Following induction of anesthesia, the preimplanted optical fiber was connected to the laser, and mice were placed supine in a stereotaxic frame. A tracheotomy was performed, and mice were mechanically ventilated with pure oxygen (170–220 rpm at 7–8 µL/min; MiniVent type 845; Hugo-Sachs Elektronik). In 6 DBH-cre mice, we recorded from the left vagus nerve and the left renal sympathetic nerve simultaneously. In the other 6 mice, we recorded from the right vagus nerve only (the sympathetic nerve was not recorded). To record VNA, a –15-mm segment of the cervical vagus nerve (left or right) was dissected free of the carotid artery and the cervical sympathetic chain, and the peripheral end of the isolated vagus nerve segment was crushed. The contralateral vagus nerve was always left intact. A renal sympathetic nerve bundle was isolated through a left flank incision. The vagus nerve was placed on a bipolar stainless steel wire electrode (AS633; Cooner Wire) that was positioned rostral to the crush in order to measure multifiber efferent VNA. The renal nerve bundle was placed uncut on identical bipolar stainless steel electrodes. Electrodes and nerves were then embedded in silicone (Kwik-Sil, WPI). Next, the adequacy of anesthesia was rechecked, and the muscle relaxant agent vecuronium was administered (0.1 mg/kg, i.p.). After this point, the adequacy of the anesthesia was determined by the absence of change in AP and HR in response to a firm hindpaw pinch. Physiological signals were filtered and amplified (AP, 10–1,000 Hz, ×1,000; vagus nerve, 30–3,000 Hz, ×10,000; renal nerve: 30–1,000 Hz, ×10,000; BMA-400 amplifier, CWE). The analog signals were digitized (Micro3 1401; CED) and processed using Spike 2 software (v7.06; CED). VNA was high-pass filtered (100 Hz; transition, 50 Hz) when the signal contained a substantial electrocardiogram contamination. The final signal processing consisted of half-wave rectification and averaging. AP and HR were recorded as described above. At the end of the experiment, the vagus nerve was cut on the cephalic side of the recording electrode to measure the electrical noise and, if applicable, the ganglionic blocker hexamethonium bromide was administered (30 mg/kg, i.m.) to eliminate renal sympathetic nerve activity (RSNA) and record the portion of the signal representing the electrical noise. The background noise was systematically subtracted and the balance was taken as efferent VNA or RSNA. These multifiber recordings were subject to considerable interanimal variation in amplitude because of differences in electrode configuration, current leak via extracellular fluid and other factors.

The effect of trains of C1 stimulation (10 s, 5–20 Hz) on VNA and RSNA was therefore normalized by measuring the ratio between the mean nerve activity present while the 10-s stimulus was being applied and the resting activity present during the 10 s preceding the stimulus (both values background subtracted). The nerve activity evoked by low-frequency stimulation of C1 (1 Hz) was determined by constructing peristimulus waveform averages of the half-wave rectified signal (200 sweeps). The amplitude of the peak evoked response was expressed as a percent of the prestimulus value of the nerve activity after background noise subtraction. Sham C1 photostimulation (dummy) was done by setting the laser output to 0 mW.

Optogenetic activation of C1 and renal IRI. The effect of C1 stimulation on renal IRI was examined in DBH-cre mice that had previously received injections of AAV2-Chr2-mCherry or AAV2-mCherry into the left RVLM. These experiments required 90 mice. The breakdown of the various experimental groups and treatments (laser light, drugs, vector) is shown in Supplementary Table 1 for added clarity. All the mice included in Supplementary Table 1 were subjected to bilateral renal IRI the day after the treatment indicated in the table. Each experiment also included 12 mice that were not subjected to renal IRI (not shown on the table) in order to ascertain that their creatinine was appropriately low. Every mouse also carried an implanted optical fiber. Photostimulation of C1s (DBH-cre mice with injections of AAV2-Chr2-mCherry) or sham stimulation (same trains of laser light delivered to DBH-cre mice injected with AAV2-mCherry) was done while the mice were confined to a plethysmography chamber. The mice were placed in one of these chambers for 2 h twice a week for 2 weeks in order to habituate them to this environment and thus minimize the associated stress. Laser light produced no effect of any kind (cardiorespiratory, behavior, corticosterone) in DBH-cre mice injected with control vector (Chr2-mCherry), and therefore we presume that the light activated C1s only when these neurons expressed Chr2.

The mice were briefly anesthetized with isoflurane while the connection between the implanted fiber optics and the laser was established. Drugs were given i.p. 30 min later (30 mg/kg hexamethonium bromide, a ganglionic blocker, n = 6; 30 mg/kg labelatal, a nonselective β1- and β2-adrenergic receptor antagonist, n = 6; 15 mg/kg butoxamine, a selective β2-adrenergic receptor antagonist, n = 6; or 30 mg/kg mifepristone, a corticosterone receptor antagonist, n = 6). A group of control mice received the vehicle of each drug to dissolve the drugs (0.9% NaCl solution i.p. for all drugs except mifepristone; 100% dimethylsulfoxide (DMSO) was used as a control for mifepristone; n = 6). Thirty min after drug or vehicle administration, light pulses (10 ms duration, 5 Hz) were delivered for 10 min. Control mice were briefly anesthetized with isoflurane but photo-stimulation was not applied.

Twenty-four h after applying the light pulses to the RVLM, mice were anesthesitized with i.p. injections of ketamine (120 mg/kg) and xylazine (12 mg/kg), and bilateral flank incisions were made to access the kidneys. Renal IRI, as previously described8, was produced by clamping the renal pedicles for exactly 26 min in 88 DBH-cre mice. The clamps were then removed and the wound sutured after resorption of blood flow was visually observed. Kidneys were allowed to reperfuse for a period of 24 h. This surgical procedure was conducted blind, i.e., the person performing the renal clamp had no knowledge of which combination of drug and optogenetic protocol the mice had been subjected to one day earlier. Mice received ketoprofen (4 mg/kg) and ampicillin (125 mg/kg) after surgery. Twenty-four h after renal IR, the mice were euthanized with an overdose of ketamine and xylazine, and blood (400–600 µL) was collected from the orbital sinus. The kidneys were harvested for histology and extraction of RNA for Kim1 transcript measurement. Finally, the descending aorta was clamped, the animal was perfused through the heart with 4% paraformaldehyde and the brain was collected for histology (see sections on renal and brain histology for details).

Effect of 10-min C1 stimulation or 10-min renal ischemia on renal Hif1a mRNA. These experiments were done in order to determine whether 10 min of C1 stimulation in a conscious mouse produced a measurable increase in the renal level of Hif1a transcripts indicative of a possibly severe hypoperfusion. The experiments used three groups of mice. Group 1 consisted of unanesthetized...
DBH-cre mice with AAV2-ChR2-mCherry injections and a preimplanted optical fiber; C1s were not stimulated in this group (no light was applied). Group 2 comprised identically prepared DBH-cre unanesthetized mice, in which C1s were optogenetically stimulated for 10 min using our standard parameters (10-ms pulses, 5 Hz). Group 3 mice were anesthetized with an i.p. injection of ketamine (120 mg/kg) and xylazine (12 mg/kg) and subjected to 10 min of complete bilateral renal ischemia. The kidneys were harvested 10 min after cessation of C1 stimulation (group 2), sham stimulation (group 1, no light applied) or ischemia (group 3). Groups 1 and 2 were anesthetized with an i.p. injection of ketamine (120 mg/kg) and xylazine (12 mg/kg) before harvesting the kidneys.

Effect of restraint stress on renal IRI. The effect of restraint stress on renal IRI was also examined in DBH-cre mice. A subset of DBH-cre mice had previously received bilateral microinjections of AAV2-DIO-hSyn-hm4D(Gi)-mCherry (DREADD, n = 12) or AAV2-DIO-taCasp3-TEVp (caspase, n = 12) into the RVLM. A total of 48 mice was required for these experiments. The breakdown of the various experimental groups (mouse strain, vector and drug) is shown in Supplementary Table 2. All the mice shown in Supplementary Table 2 were subjected to 26-min renal ischemia followed by 24 h of reperfusion.

For the DREADD experiments, the receptor agonist CNO (Sigma-Aldrich, 3 mg/kg, n = 6) or its vehicle (saline; n = 6) was injected 30 min before restraint stress. For restraint stress, each mouse was placed in an adequately ventilated 50-mL conical plastic tube (Corning Inc.) for 10 min (n = 36 DBH-cre). They could rotate from a prone to supine position and back again but not turn head to tail. Nonrestrained mice were left undisturbed in their home cages (n = 12 DBH-cre). DBH-cre mice with bilateral injections of AAV2-DIO-taCasp3-TEVp were either subjected to restraint stress or left in their home cage.

Twenty-four h after restraint stress, mice were anesthetized with an i.p. injection of ketamine (120 mg/kg) and xylazine (12 mg/kg), and bilateral renal clamping was performed for 26 min as described in the previous section (n = 48 DBH-cre mice). Mice received ketoprofen (4 mg/kg) and ampicillin (125 mg/kg) after surgery. Twenty-four h after renal IR, the animals were euthanized with an over dose of ketamine (120 mg/kg) and xylazine (12 mg/kg) and the blood was collected from the orbital sinus for creatinine measurement. Next, we harvested the kidneys for histology and Kim1 mRNA measurement, and the mice were perfusion-fixed with paraformaldehyde. Finally the brains were removed for histology.

Effect of C1 stimulation or restraint stress on plasma corticosterone. The effect of C1 stimulation on plasma corticosterone and related controls was examined in DBH-cre mice that had previously received injections of AAV2-ChR2-mCherry or AAV2-mCherry into the left RVLM. Every mouse also carried an implanted optical fiber. Four weeks after this procedure, the DBH-cre mice that had received injections of AAV2-ChR2-mCherry were placed in a plethysmography chamber and short trains of light pulses (10 s, 5–20 Hz) were applied to ascertain that breathing was stimulated, thereby indicating that C1s were properly activated. During the next 2 weeks, each mouse was habituated to the plethysmography cage for 2 h twice a week. The actual experiment was conducted on week 7. Mice were briefly anesthetized with isoflurane while the connection between the implanted fiber optic and the laser delivery system was established, and 10-ms light pulses were delivered for 10 min at 5 Hz. This procedure was carried out in 9 DBH-cre mice. In order to control for the effect of the light itself and for any untoward effect of the vector, we performed two control experiments. For the first control experiment we used 9 DBH-cre mice with AAV2-ChR2-mCherry. Following connection of the optical fiber with the laser and placement of the mice in the customary environment (plethysmography chamber), no light was applied. The second control experiment was done in mice that had been injected with control virus (AAV2-mCherry). These mice (6 DBH-cre) received the same light pulses (5 Hz, 10 ms, 10 min) as the experimental group while in the same environment. All these experiments were conducted between 1 and 5 p.m.

For restraint stress, each mouse (DBH-cre) was placed in an adequately ventilated 50-mL conical plastic tube (Corning Inc.) for 10 min. The mice could rotate from a prone to supine position and back again but could not turn head to tail. Six DBH-cre mice were subjected to this treatment. Control mice (6 DBH-cre) were left undisturbed in their home cages. These experiments were also performed between 1 and 5 p.m.

Mice were returned to their original cages after restraint stress or delivery of laser light. Sixty min later, the mice were quickly anesthetized with isoflurane and blood was immediately collected from the orbital sinus. Plasma corticosterone concentration was subsequently measured with an enzyme-linked immunosorbent assay (Enzo Life Sciences).

Brain histology. Following completion of the in vivo experiments, mice were euthanized with an overdose of ketamine and xylazine and perfused transcardially with 50 mL of heparinized saline followed by 100 mL of freshly prepared 4% paraformaldehyde in 100 mL sodium phosphate buffer (pH 7.4). Brains were extracted and postfixed at 4 °C for 24–48 h in the same fixative. Transverse sections (30(30µm thick) were cut on a vibrating microtome and stored in a cryoprotectant solution (20% glycerol plus 30% ethylene glycol in 50 mM phosphate buffer, pH 7.4) at −20 °C.

Immunohistochemical procedures and microscopy were performed as previously described23. The following antibodies were used: mCherry protein was detected with anti-DsRed (rabbit polyclonal, 1:500; Clontech #632496; Clontech Laboratories) followed by Cy3agged anti-rabbit IgG (1:200, Jackson ImmunoResearch Laboratories); tyrosine hydroxylase (TH) was detected with a sheep (1:1,000; Millipore #AB1542; EMD Millipore) or chicken (1:1,000; lot number TH1205; Aves labs) polyclonal antibody followed by Alexa Fluor-488-tagged donkey anti-sheep or Alexa Fluor-488-tagged donkey anti-chicken antibody (1:200; Jackson ImmunoResearch Laboratories). ChAT was detected with a goat polyclonal antibody (1:100; Millipore #AB414P; EMD Millipore) followed by Cy3-tagged donkey anti-goat (1:200; Jackson ImmunoResearch Laboratories).

Brain sections were examined under brightfield and epifluorescence illumination with a Zeiss AxioImager Z1 microscope equipped with a computer-controlled stage and Neurolucida software (v10; MBF Bioscience). Cell counts were performed in a 1-in-3 series of sections that were kept in correct sequential order. Sections from different mice were aligned according to their distance from a reference transverse plane identified as 6.48 mm caudal to bregma, after Paxinos and Franklin31. The plane of this section intersects the most caudal portion of the facial motor nucleus. Images were obtained with a Zeiss MRc camera as TIFF files (1,388 × 1,040 pixels) and imported into Canvas software (v10; ACD Systems) for the composition of figures. Output levels were adjusted to include all information-containing pixels. Balance and contrast were adjusted to reflect true rendering as much as possible. No other image retouching was performed.

Adoptive transfer of splenocytes from stressed to naive mice. Spleens were harvested from donor mice (WT C57BL/6 mice) 24 h after restraint stress (10 min). Single-cell suspensions were generated by passing whole spleen through 40-µm filters into PBS. The cell pellet was collected by centrifugation (500g for 5 min) and then treated with red blood cell lysis buffer (BioLegend) for 3 min according to the manufacturer’s protocol, with some modifications. After cell lysis, the samples were centrifuged and the resulting cell pellet was diluted, and 1 × 10^5, 5 × 10^4 or 1 × 10^5 cells were injected into each recipient mouse (WT C57BL/6 mice) via tail vein 24 h before IR.

Transfer of noradrenaline-preincubated T cells to naive mice. CD4+ T cells were isolated from the spleen using the Dynabeads Untouched Mouse CD4 Cells Kit (Thermo Fisher Scientific Inc.) after splenocytes were obtained from unstressed WT C57BL/6 mice (the details of isolating splenocytes are described above). Isolated CD4+ T cells were treated with noradrenaline (Sigma-Aldrich, 10 µM) for 30 min at 37 °C. After washing the cells with PBS, CD4+ T cells were resuspended in PBS and 1 × 10^5 cells (200 µL) were injected into recipient mice (naive WT C57BL/6 mice) via tail vein 24 h before IR.

Renal histology. The capsule of the harvested kidney was removed. A center transverse section was cut and placed in 4% PPF (1% paraformaldehyde, 1.4% NaCl, 0.2% sodium periodate in 0.1 M sodium phosphate buffer, pH 7.4) for 24 h and then stored in 70% EtOH until paraffin embedding (UVA Research Histology Core). Paraffin sections (5 µm) were cut and stained with hematoxylin and eosin (H&E). The sections were viewed by light microscopy (Zeiss AxiosImager Z1/ Apotome microscope, Carl Zeiss Microscopy). Photographs were taken with an AxioCam MRc camera (Zeiss) and brightness/contrast and white balance adjustments were made using StereoInvestigator software (MBF Bioscience).
The extent of acute tubular necrosis was assessed in an unbiased, systematic manner using design-based stereology to achieve a statistically accurate random sampling of kidney sections and yielding the percentage of total area of the section occupied by injured tubules as previously described. The investigator was blinded to the experimental identity of the sections. Sections were imaged by using a Zeiss Axio Imager Z1/Apoptome Microscope fitted with motorized focus drives and motorized XYZ microscope stage and integrated to a workstation running StereoInvestigator software (MBF Bioscience). The area fraction fractionator probe was used for stereological analysis of the fractional area of the section occupied by tubular necrosis. The following parameters were defined: counting frame, 400 × 400 μm; sample grid, 800 × 800 μm; grid spacing, 85 μm. These values were determined empirically to ensure that adequate numbers of sample sites were visited and adequate numbers of markers (indicating injured tubules) were acquired, in keeping with accepted counting rules for stereology. Injured tubules were identified based on the presence of cast formation, tubule dilation and/or tubular epithelial denudation. A total of 290 ± 7.6 (mean ± s.e.m.) grid sites were evaluated per section.

**Plasma creatinine measurement.** Plasma was prepared by centrifuging heparinized blood at 7,000g for 5 min. Plasma creatinine (mg/dL) was determined with an enzymatic method with minor modifications from the manufacturer’s protocol (twice the volume of sample and standard, using twofold serial dilution of the calibrator (standard) provided in the kit; Diazyme Laboratories) that we have validated using LC-MS.

**Real-time PCR measurements of Kim1, Gapdh and Hif1α mRNA in mouse kidneys.** Renal mRNA was isolated by following the ethanol-precipitation method, and RNA concentration was determined based on a spectrophotometric determination of a 260/280 ratio. cDNA was generated from the resultant tissue RNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) as described by the manufacturer. Resultant cDNA was then used to determine relative mRNA expression of *Kim1*, *Hif1α* and *Gapdh* using the iTAC Universal SYBR Green Supermix (Bio-Rad). Primers used were as follows:

- **Kim1** (forward): ACA TAT CGT GGA ATC ACA ACG AC, (reverse): ACT GCT CTT CTG ATA GGT GAC A
- **Gapdh** (forward) ACG GCA AAT TCA ACG GCA CAG TCA, (reverse): TGG GGG CAT CGG CAG AAG G; and
- **Hif1α** (forward): ACC TTC ATC GGA AAC TCC AAA G, (reverse): CTG TTA GCC TGG GAA AAG TTA GG.

**Statistical analyses.** Based on our previous study, we justified a sample size of 5 mice (Supplementary Methods Checklist). All data sets were tested for normality using the D’Agostino–Pearson omnibus normality test or Kolmogorov–Smirnov test, and then equal variances were tested using the Brown-Forsythe test. If the criteria of normality and equal variance were satisfied, we evaluated statistical significance using one- or two-way ANOVA followed by the Tukey–Kramer test. When the criteria of normality and equal variance were not met, we used a nonparametric statistical test (Kruskal-Wallis followed by Steel-Dwass test for multiple comparisons between groups). All values are expressed as means ± s.e.m. and statistical significance is set at *P* < 0.05.

**Data availability.** The data that support the findings of this study are available from the corresponding author upon reasonable request.

51. Paxinos, G. & Franklin, K.B.J. *The Mouse Brain in Stereotaxic Coordinates* (Academic Press, 2013).