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独立行政法人日本学術振興会 理事長 殿

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受 付 番 号 201860736
氏 名 田中真司

（氏名は必ず自署すること）

海外特別研究員としての派遣期間を終了しましたので、下記のとおり報告いたします。
なお、下記及び別紙記載の内容については相違ありません。

記

1. 用務地（派遣先国名）用務地： シャーロッツビル （国名：アメリカ合衆国 ）

2. 研究課題名（和文）※研究課題名は申請時のものと違わないように記載すること。
オプトジェネティクスを用いた炎症反射メカニズムの解明

3. 派遣期間：2019 年 1 月 1 日 ～ 2021 年 3 月 31 日

4. 受入機関名及び部局名
受入機関名： ヴァージニア大学
部局名： Division of Nephrology and CIIR

5. 所期の目的の遂行状況及び成果…書式任意 **書式任意（A4 判相当 3 ページ以上、英語で記入も可）**
（研究・調査実施状況及びその成果の発表・関係学会への参加状況等）
（注）「6. 研究発表」以降については様式 10－別紙 1～4 に記入の上、併せて提出すること。

研究の成果を以下に記載いたします。

Validation of mice for selective vagus efferent versus afferent fiber stimulation.

Since choline acetyltransferase (ChAT) and vesicular-glutamate transporter 2 (Vglut2) are established markers for motor efferent neurons and sensory afferent neurons, respectively, we crossed heterozygous *Chat-ires-Cre* and *Vglut2-ires-Cre* mice with homozygous *Ai32* mice (containing a Cre-dependent channelrhodopsin-2 (ChR2)-enhanced yellow fluorescent protein (eYFP) allele) to produce *Chat-ChR2* (for selective efferent fiber stimulation) and *Vglut2-ChR2* (for selective afferent fiber stimulation) mice and littermate controls (Fig. 1a). ChR2 is a nonselective cation channel that was discovered from the green alga *Chlamydomonas reinhardtii*. It is not expressed in rodent/human tissues and has a unique characteristic that the gate is opened only during blue light application. Thus, blue light can selectively depolarize ChR2-expressing neurons via Na^+ entry, evoking an action potential. We confirmed the selectivity of stimulation (efferent vs. afferent fibers) in *Chat-ChR2* and *Vglut2-ChR2* mice by observing changes in heart rate and respiratory rate during optogenetic vagus nerve stimulation (VNS). Selective stimulation of vagus efferent fibers decreases heart rate without changing respiratory rate since these fibers innervate the sinoatrial and atrioventricular nodes. Optogenetic VNS in *Chat-ChR2* mice resulted in a significant decrease in heart rate as expected (Fig. 1b). In contrast, selective stimulation of vagus afferent fibers decreases respiratory rate, which is known as Hering-Breuer inflation reflex. Optogenetic VNS in *Vglut2-ChR2* mice resulted in a significant decrease in both heart rate and respiratory rate (Fig. 1c); the latter change is probably through vago-vagal reflex, in which vagus efferent fibers are stimulated following the stimulation of vagus afferent fibers. Next we transected the vagus and applied blue laser to the central or distal end of the transected vagus (Fig. 1f, g). In *Chat-ChR2* mice, stimulation

at the distal end decreased heart rate but stimulation at the central end did not change heart rate or respiratory rate (Fig. 1f). In *Vglut2-ChR2* mice, stimulation at the central end decreased both heart rate and respiratory rate whereas stimulation at the distal end had no effects (Fig. 1g). Unchanged heart rate during stimulation at the distal end of the cut vagus in *Vglut2-ChR2* mice excluded the possibility that vagus efferent fibers are stimulated in *Vglut2-ChR2* mice. These results suggest that efferent and afferent fibers are selectively stimulated by optogenetic VNS in *Chat-ChR2* and *Vglut2-ChR2* mice, respectively. Stimulation at different frequencies revealed the frequency response of changes in heart rate and respiratory rate in these mice (Fig. 1d, e). 5 Hz was used in the remainder of the optogenetic VNS experiments since stimulation at 5 Hz produced a small (< 10%) but reliable reduction in heart rate and respiratory rate.

Fig. 1

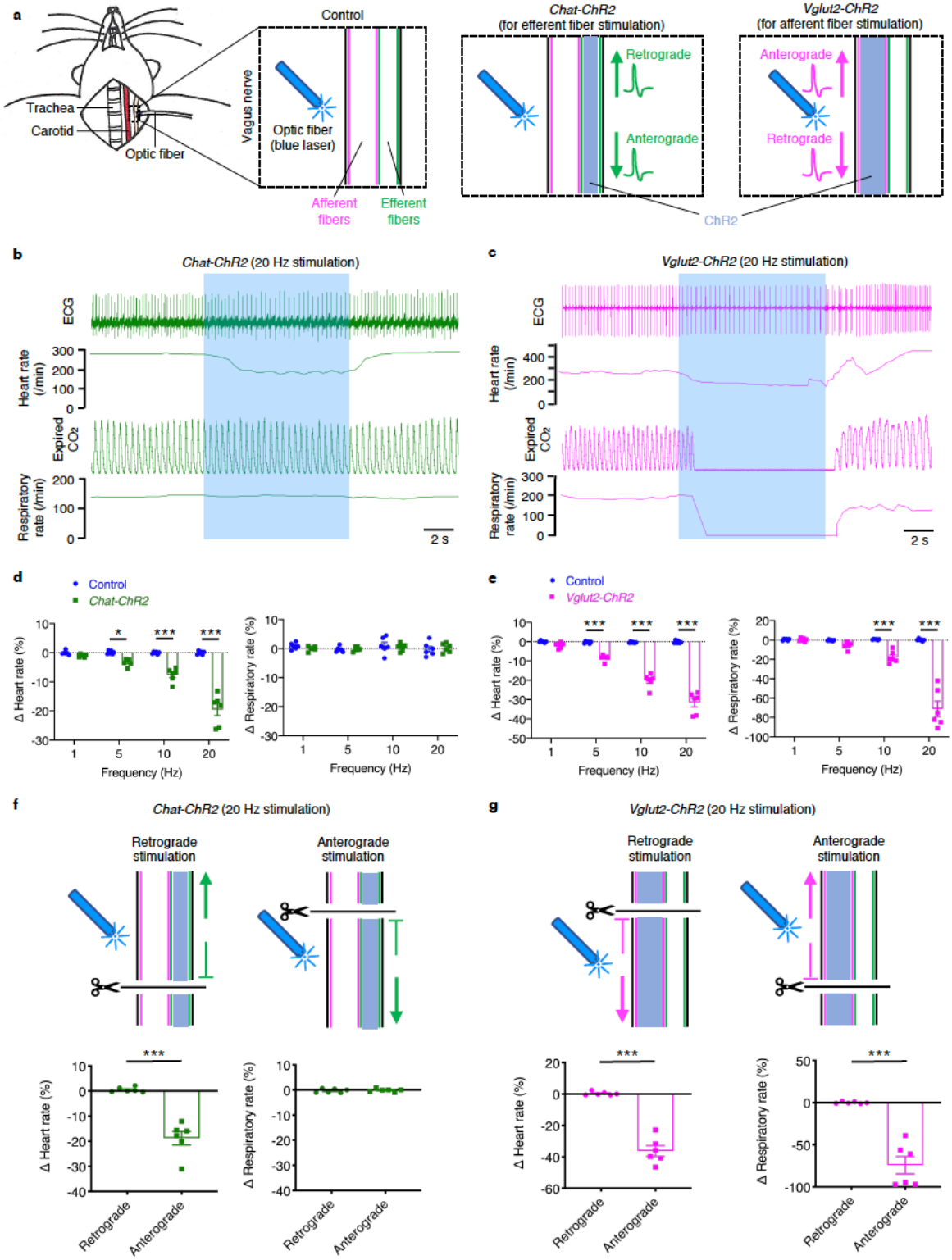

Fig. 1 | Validation of *Chat-ChR2* and *Vglut2-ChR2* mice. a, Cartoon depicting optogenetic vagus nerve

stimulation strategy in *Chat-ChR2* (for selective efferent fiber stimulation) and *Vglut2-ChR2* (for selective afferent fiber stimulation) mice. The left cervical vagus nerve was surgically exposed in anesthetized mice and blue laser was used for stimulation. ChR2 expression in the vagus nerve was limited to efferent and afferent fibers in *Chat-ChR2* and *Vglut2-ChR2* mice, respectively. Action potentials are transmitted in two directions (anterograde and retrograde) in ChR2-expressing fibers. **b** and **c**, Representative measurements of heart rhythm (ECG) and respiratory rhythm (expired CO_2) following optogenetic vagus nerve stimulation (blue shading; 20 Hz, 10 s) in *Chat-ChR2* (**b**) and *Vglut2-ChR2* (**c**) mice. **d** and **e**, Frequency response data summarizing the effects of optogenetic vagus nerve stimulation (10 s) on heart rate and respiratory rate in *Chat-ChR2* (**d**), *Vglut2-ChR2* (**e**), and control (**d**, **e**) mice. **f** and **g**, Changes in heart rate and respiratory rate following optogenetic retrograde versus anterograde vagus nerve stimulation (20 Hz, 10 s) in *Chat-ChR2* (**f**) and *Vglut2-ChR2* (**g**) mice. The left cervical vagus nerve was transected and blue laser was applied to the central or distal end for retrograde or anterograde stimulation. n = 6 in each group (**d**-**g**). Data are represented as mean ± s.e.m. $*P < 0.05$ and $***P < 0.001$ by two-way ANOVA with post hoc Sidak test (**d**, **e**) or unpaired two-sided Student's *t* test (**f**, **g**).

Stimulation of either vagus efferent or afferent fibers in an anterograde manner contributes to the protective effect of VNS against kidney ischemia-reperfusion injury (IRI).

We previously showed that VNS with electrodes at the left cervical vagus nerve protected kidney against IRI (an established mouse model of acute kidney injury). Electrical VNS activates four distinct neural pathways: efferent and afferent fibers in anterograde and retrograde directions. To identify which pathway is critical for the kidney protection, we applied blue laser to the left cervical vagus nerve (optogenetic VNS) 24 h before bilateral kidney IRI in *Chat-ChR2* and *Vglut2-ChR2* mice, and mice were euthanized at 24 h (Fig. 2a). Optogenetic VNS in *Chat-ChR2* mice (Fig. 2b) significantly decreased plasma creatinine (a marker for renal function, Fig. 2c), histological tubular injury (Fig. 2d), and renal *Kim-1* expression (a marker for kidney injury, Fig. 2e), suggesting that vagus efferent fiber stimulation in an anterograde and/or retrograde manner ameliorates kidney IRI. To distinguish retrograde and anterograde stimulation in efferent fibers, we applied bupivacaine (a local anesthetic) directly to the vagus to block nerve conduction, and then applied blue laser to the central (for retrograde stimulation) or distal (for anterograde stimulation) side of the anesthetized area (Fig. 2f). Plasma creatinine data suggested that anterograde but not retrograde stimulation of efferent fibers was protective against kidney IRI (Fig. 2f). We repeated similar experiments using *Vglut2-ChR2* mice to investigate the role of afferent fibers in the kidney protection by VNS (Fig. 2g-k). Interestingly, selective vagus afferent fiber stimulation (Fig. 2g) significantly decreased plasma creatinine (Fig. 2h), histological tubular injury (Fig. 2i), and renal *Kim-1* expression (Fig. 2j) at 24 h after kidney IRI. Furthermore, experiments with nerve conduction blockade by bupivacaine revealed that anterograde but not retrograde stimulation of afferent fibers was protective against kidney IRI (Fig. 2k). Taken together, these findings suggest that two distinct neural pathways (anterograde efferent and afferent fiber stimulation) contribute to the protective effect of electrical VNS against kidney IRI.

Fig. 2

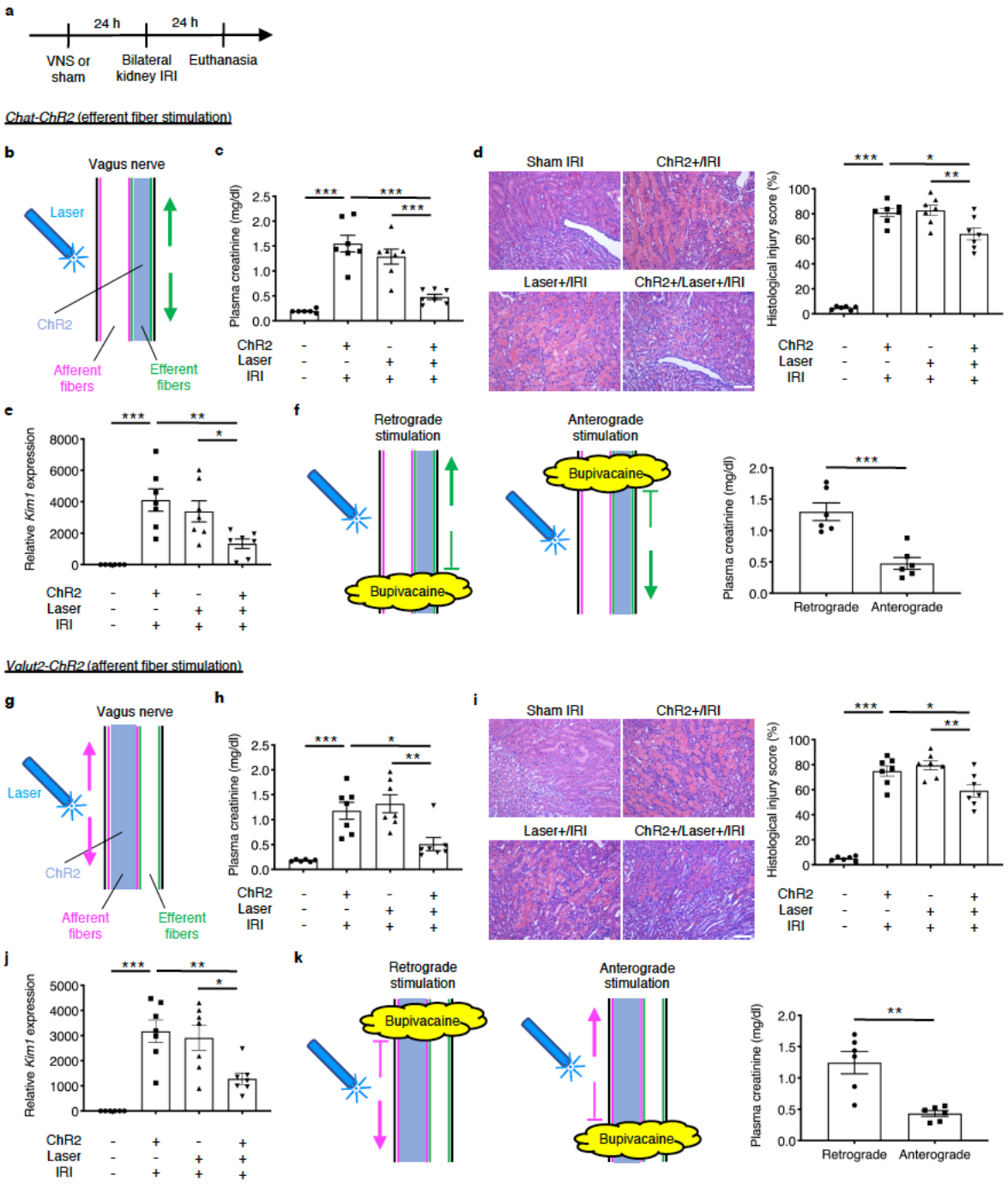

Fig. 2 | Stimulation of either vagus efferent or afferent fibers in an anterograde direction protects kidneys against IRI. a, Timeline of experiments. **b,** Cartoon depicting optogenetic stimulation of cervical vagus nerve in *Chat-ChR2* mice. Note that action potentials are transmitted only in efferent fibers in both anterograde (downward) and retrograde (upward) directions. **c**-**e**, Effect of selective efferent fiber stimulation (5 Hz, 10 min) on plasma creatinine (a representative marker for kidney function) (**c**), acute tubular necrosis score (with representative H&E staining of kidney sections) (**d**), and renal *Kim1* (a representative marker for tubular injury) mRNA (**e**) in *Chat-ChR2* and control mice. **f**, Effect of optogenetic retrograde versus anterograde vagus nerve stimulation (5 Hz, 10 min) on plasma creatinine in *Chat-ChR2* mice with cartoon depicting the strategy. Blue laser was applied to the central (for retrograde stimulation) or distal (for anterograde stimulation) side of the area anesthetized with bupivacaine. **g,** Cartoon depicting optogenetic stimulation of cervical vagus nerve in *Vglut2-ChR2* mice. Note that action potentials are transmitted only in afferent fibers in both anterograde (upward) and retrograde (downward) directions. **h**-**j**, Effect of selective afferent fiber stimulation (5 Hz, 10 min) on plasma creatinine (**h**), acute tubular necrosis score (with representative H&E staining of kidney sections) (**i**), and renal *Kim1* mRNA (**j**) in *Vglut2-ChR2* and control mice. **k**, Effect of optogenetic retrograde versus anterograde vagus nerve

stimulation (5 Hz, 10 min) on plasma creatinine in *Vglut2-ChR2* mice with cartoon depicting the strategy. Blue laser was applied to the distal (for retrograde stimulation) or central (for anterograde stimulation) side of the area anesthetized with bupivacaine. n = 6 in sham IRI groups and n = 7 in IRI groups (**c-e, h-j**), n = 6 in each group in bupivacaine experiments (**f**, **k**). Scale bars, 100 μm. Data are represented as mean ± s.e.m. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ by one-way ANOVA with post hoc Tukey test (**c-e, h-j**) or unpaired two-sided Student's *t* test (**f**, **k**).

We further identified the C1 neurons–sympathetic nervous system–splenic nerve–spleen–kidney axis as the downstream pathway of anterograde vagus afferent fiber stimulation (Fig. 3).

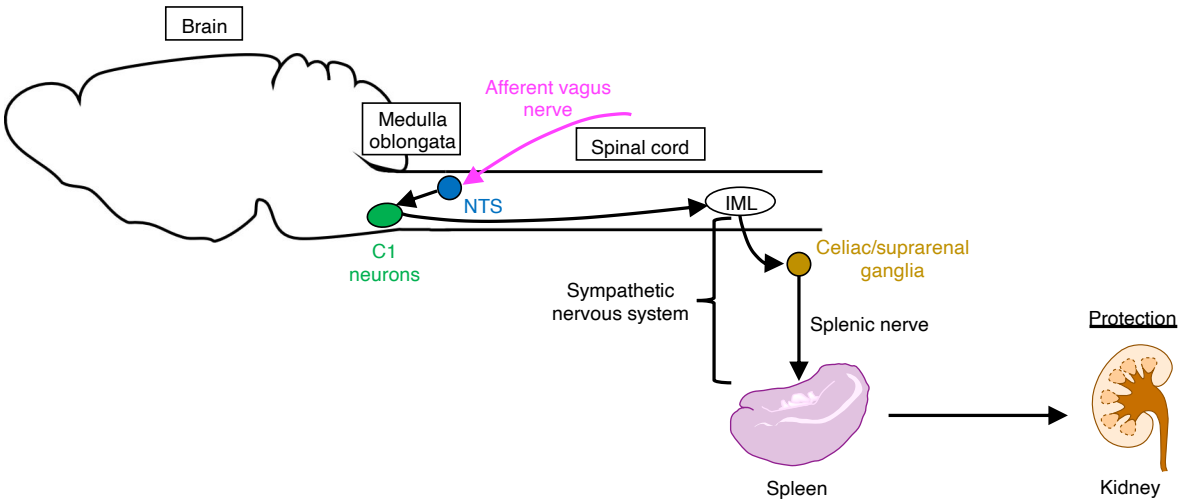

Fig. 3 l A newly discovered neural circuit involved in the kidney protection by vagus afferent fiber stimulation. Parasagittal view of the brain is shown. Stimulation of afferent vagus nerve activates C1 neurons residing in the medulla oblongata through the nucleus tractus solitarius (NTS), which is an integrative center for sensory information from the vagus nerve. The sympathetic nervous system plays a critical role as an efferent pathway from C1 neurons, and the signal is transmitted to the spleen through the splenic nerve, which is predominantly a sympathetic nerve. The signal from the splenic nerve probably alters the phenotype of splenocytes as in the canonical CAP activation, which contributes to the kidney protection against IRI. It remains unclear how these splenocytes with an altered phenotype protect the kidney. IML, the intermediolateral cell column.