Interstial cells of Cajal integrate excitatory and inhibitory neurotransmission with intestinal slow-wave activity

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The enteric nervous system contains excitatory and inhibitory neurons, which control contraction and relaxation of smooth muscle cells as well as gastrointestinal motor activity. Little is known about the exact cellular mechanisms of neuronal signal transduction to smooth muscle cells in the gut. Here we generate a c-KitCreERT² knock-in allele to target a distinct population of pacemaker cells called interstitial cells of Cajal. By genetic loss-of-function studies, we show that interstitial cells of Cajal, which generate spontaneous electrical slow waves and thus rhythmic contractions of the smooth musculature, are essential for transmission of signals from enteric neurons to gastrointestinal smooth muscle cells. Interstitial cells of Cajal, therefore, integrate excitatory and inhibitory neurotransmission with slow-wave activity to orchestrate peristaltic motor activity of the gut. Impairment of the function of interstitial cells of Cajal causes severe gastrointestinal motor disorders. The results of our study show at the genetic level that these disorders are not only due to loss of slow-wave activity but also due to disturbed neurotransmission.
The enteric nervous system contains two major classes of neurons: (1) excitatory neurons, which constitute the majority of neurons that make up functional circuits and are responsible for generating excitatory output, and (2) inhibitory neurons, which provide a rich variety of inhibitions that shape the output of functional circuits. Proper functioning of gastrointestinal (GI) motility critically depends on the interplay between excitatory and inhibitory enteric neurons, and on signalling to the smooth musculature.

Interstitial cells of Cajal (ICC) form a cellular network that is embedded in the musculature of the GI tract. Previous studies provided clear evidence that ICC generate a rhythmic pacemaker current, which manifests itself as slow waves in the membrane potential of smooth muscle cells, resulting in rhythmic bowel contractions. However, a role of ICC in the transmission of excitatory and inhibitory signals from enteric neurons to smooth muscle cells is highly controversial and remains an outstanding question. This is mainly because of the general lack of models and systems to study ICC function in adult animals. In addition, ICC integrate and mediate excitatory and inhibitory neurotransmission in the gut. These data provide the first in vivo genetic evidence that ICC integrate intestinal pacemaker activity with enteric neurotransmission to control and coordinate digestive activity in adult animals. As impaired ICC function causes severe GI motor disorders, we provide genetic evidence that this is at least, in part, due to altered enteric neurotransmission.

Results

Time-specific genetic targeting of ICC in adult mice. To test the hypothesis that enteric neurons signal via ICC to smooth muscle cells, it is imperative to target ICC in the gut. To achieve this, we took advantage of the exquisite fidelity of the inducible site-specific Cre/loxP recombination system. We generated a CreERT2 allele as a knock-in at the endogenous c-Kit locus (c-KitCreERT2/+ mouse line; Supplementary Fig. S1a-d). In this mouse line, Cre can be activated by tamoxifen (TAM) administration at defined time points in the c-Kit lineage during embryogenesis and in adult animals. c-Kit is a receptor tyrosine kinase, which is specifically expressed in ICC in the gut musculature (Fig. 1a), and c-Kit serves as the only well-established marker for ICC.

By crossing c-KitCreERT2/+ mice with the switchable double-fluorescent R26mT-mG/+ reporter mouse line, we obtained c-KitCreERT2/+R26mT-mG/+ mice (Fig. 1b), in which TAM-activatable CreERT2 was specifically expressed in ICC as manifested by enhanced green fluorescent protein expression of c-Kit-positive cells after TAM administration (Fig. 1c, d and Supplementary Fig. S2). Quantification of enhanced green fluorescent protein/c-Kit double-positive cells revealed a TAM-induced recombination efficiency of ICC in the small and large intestine of >90% (Fig. 1e).

Acute ICC depletion disturbs GI motility. To gain insights into the specific role of ICC for enteric neurotransmission, we crossed...
c-KitCreERT2/+ mice with conditional LSL-R26DTA/+ animals (Fig. 2a)20, which carry a latent diphteria toxin A (DTA) expression cassette. After 3 days of TAM treatment, we observed depletion of more than 50% of the ICC network in the gut (Fig. 2b, c). The ICC depletion resulted in a significantly disturbed GI motility with an increased total GI transit time of more than 5 h as measured by carmine red staining (Fig. 2d). Besides ICC, mast cells express c-Kit in the GI tract21. To exclude a role of mast cell depletion in our model system, we performed adaptive bone marrow transfer from wild-type (WT) animals into c-KitCreERT2/+;LSL-R26DTA/+ mice. Thereby, we were able to reconstitute these animals with WT mast cells (Supplementary Fig. S3). To explore whether mast cells modulate GI motility, we treated c-KitCreERT2/+;LSL-R26DTA/+ mice, repopulated with WT mast cells, with TAM. Three days later, all animals showed a similar increased GI passage time as observed in TAM-treated c-KitCreERT2/+;LSL-R26DTA/+ animals (Fig. 2e). Thus, depletion of mast cells does not modulate GI motility in our experimental setup.

**ICC ablation disrupts intestinal slow-wave activity.** To determine the role of ICC for GI motility in distinct functional regions of the GI tract, we analysed gastric emptying and small bowel passage by fluorescence measurements and colonic motility, using the well-established colon bead expulsion test. The ICC depletion

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**Figure 2 | Depletion of ICC results in disturbed GI motility.** (a) Genetic strategy used to deplete ICC in adult animals by conditional expression of DTA. (b) Representative confocal microscopic images of cryostat sections (left panels) and whole-mounts (right panels) from c-Kit (red)-stained colon of vehicle (veh.; upper panels, -TAM)- and TAM (lower panels, +TAM)-treated c-KitCreERT2/+;LSL-R26DTA/+ mice. Nuclei were counterstained with TOPRO-3 (blue); scale bars, 50 μm. (c) Quantification of ICC in small intestine (left panel) and colon (right panel) after 3 days of +TAM or veh. treatment of indicated genotypes (n = 3 per group; 30 representative fields of view per mouse; ***P < 0.001; Students t-test). (d) Extension of whole GI transit time after 3 days of +TAM or veh. treatment of the indicated genotypes (***P < 0.001; Students t-test). GI transit time was measured before and after treatment of each animal. (e) Extension of whole GI transit time of WT bone marrow (BM)-transplanted c-KitCreERT2/+;LSL-R26DTA/+ and control mice treated with +TAM (N.S., not significant; **P < 0.01; Students t-test). All graphs represent mean ± s.e.m.
resulted in delayed gastric emptying and an extended small and large bowel passage time (Fig. 3a–d). This is consistent with observations in W/W\textsuperscript{v} animals carrying a hypomorphic c-Kit allele in the germline\textsuperscript{47,22}. These animals have a reduced ICC number and display a severely disturbed GI motility. Similar observations were made using c-Kit-blocking antibodies\textsuperscript{33,24}.

To determine whether disturbed GI motility in TAM-treated c-Kit\textsuperscript{CreERT2/+/LSL-R26DTA/} animals is due to disrupted slow-wave activity, we measured the electrical activity and contraction of the small intestinal smooth musculature (Fig. 3e–i). Control animals displayed typical ongoing rhythmic contractions, which are due to slow-wave-type electrical activity, whereas TAM-treated c-Kit\textsuperscript{CreERT2/+/LSL-R26DTA/} animals showed dysrhythmia due to uncoordinated spontaneous contractions and lack of slow-wave-type electrical activity (Fig. 3e–i and Supplementary Fig. 5a) as demonstrated before in W/W\textsuperscript{v} animals\textsuperscript{22}. This validates an essential role of ICC for the generation of slow waves also in acute ICC-depleted adult animals.

**ICC depletion blocks excitatory enteric neurotransmission.**

There has been a long standing controversy whether ICC have a role in the regulation of excitatory and inhibitory neurotransmission, besides generating slow waves\textsuperscript{8–10,25}. In small intestinal circular smooth muscle cells, ongoing slow waves with a frequency of \( \sim 30–35 \) cycles per min (Fig. 3e,g) typically mask neuronal-induced changes of electrical activity after electrical field stimulation (EFS; Supplementary Fig. S4b). ICC-depleted animals lack slow-wave-type electrical activity in the small intestine; however, we did not observe EFS-induced changes of the membrane potential in circular smooth muscle cells (Supplementary Fig. S4b). Notably, we were not able to induce excitatory or inhibitory junction potentials (IJPs). ICC-depleted animals showed a marked and statistically significant depolarized resting membrane potential (RMP) in small intestinal circular smooth muscle cells (RMP c-Kit\textsuperscript{CreERT2/+/LSL-R26DTA/+/TAM: −52.9 ± 3.7 mV versus RMP c-Kit\textsuperscript{CreERT2/+/TAM: −62.4 ± 3.0 mV; mean ± s.e.m; \( n = 6 \) animals per genotype; \( P < 0.001 \), Students \( t \)-test) as demonstrated before in W/W\textsuperscript{v} animals\textsuperscript{22}. This finding argues for a denervation of the smooth musculature from enteric neural input due to ICC depletion and is consistent with previous reports showing a depolarized RMP in accordance with previous reports showing a depolarized RMP in ICC-depleted animals\textsuperscript{22}.

In contrast to small intestine and in accordance with previous studies\textsuperscript{27,28}, we did not observe slow-wave-type electrical activity and rhythmic contractions of colonic circular smooth muscle cells (Fig. 3i–k and Supplementary Fig. S4c). Therefore, intracellular recordings in the colon can be used to reliably study EFS-induced neurotransmission. To investigate excitatory intestinal neurotransmission in acute ICC-depleted adult animals, we measured neuronal-induced colonic contractions after EFS. Importantly, we observed a nearly complete blockade of excitatory neurotransmission as shown by strongly reduced contraction in response to EFS after ICC depletion (Fig. 3j). In line, ICC depletion completely abolished EFS-induced excitatory junction potentials (EJP) without affecting the IJP (Fig. 3k).

These data provide the first genetic in vivo evidence for an essential role of ICC in excitatory neurotransmission to smooth muscle cells in acute ICC-depleted adult animals and support findings in W/W\textsuperscript{v} germline mutant mice that display reduced excitatory neurotransmission and a hypotensive lower oesophageal and pyloric sphincter\textsuperscript{12,13,16,29,30}.

**Deletion of Prkg1 blocks inhibitory neurotransmission.**

To test whether ICC also modulate inhibitory neurotransmission to orchestrate GI motility, we deleted \( c\)-GMP-dependent protein kinase \( I \) (Prkg1) in ICC of adult animals by TAM administration using floxed \( Prkg1 \) mice (\( Prkg1^{f/f} \)). Prkg1 is a central mediator of the nonadrenergic, noncholinergic (NANC) neurotransmitters nitric oxide (NO) and carbon monoxide (CO), which are generated in enteric neurons by neuronal NO synthase (nNOS) and haem oxygenase, respectively\textsuperscript{31,32}. Neuronal-derived NO and CO induces smooth muscle relaxation in the GI tract via activation of \( Prkg1^{32–34} \). Accordingly, global \( Prkg1^{-/-} \) deficient mice (termed \( Prkg1 \) knock out (KO)) show a lack of NO-dependent smooth muscle relaxation associated with severe GI dysfunction\textsuperscript{31}.

Interestingly, inducible smooth muscle cell-specific deletion of \( Prkg1 \) in adult animals does not phenocopy global \( Prkg1 \) deficiency in the gut, indicating a possible role for an additional cell type in neuronal-induced smooth muscle relaxation. ICCs express the \( \beta \)-isoform of \( Prkg1 \) (termed \( PKG1 \beta \)) in the small and large intestine (Fig. 4a and Supplementary Fig. S5b). TAM administration to c-Kit\textsuperscript{CreERT2/+/Prkg1^{f/f}} animals resulted in recombination of the \( Prkg1 \) locus and loss of \( Prkg1 \) expression in only \( \sim 40\% \) of all ICC (Fig. 4c–e). Nevertheless, we observed a profound GI motor dysfunction in mice with a partial deletion of \( Prkg1 \) in the ICC network, as evidenced by an increased GI transit time of \( \sim 2 \) h (Fig. 4f). It is well established that the network
structure of ICC is essential for generation and propagation of slow waves as demonstrated in Fig. 3 (ref. 5). Here we show, in addition, that nitrergic neurotransmission via Prkg1 critically depends on the integrity of the ICC network. This might be also relevant for human GI motility disorders, where partial interruption of the ICC network has been described\(^\text{13}\). Importantly, slow-wave amplitude and rhythmicity was unaffected by Prkg1 deletion, as evidenced by normal ileal contractions (Supplementary Fig. S5c-f). However, we observed a moderate but significant change in the contraction frequency in TAM-treated c-Kit\(^{CreERT2/+}\);Prkg1\(^{f/f}\) animals (Supplementary Fig. S5e). This points to an additional, previously unrecognized role of Prkg1 in the modulation of slow-wave activity \textit{in vivo}.

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**Figure Legends**

**a** Stomach

**b** Small intestine

**c** Colon expulsion

**e** Ctrl (c-Kit\(^{CreERT2/+}\) + TAM

**f** Rhythmicity

**g** Frequency

**h** Amplitude

**i** Ctrl (c-Kit\(^{CreERT2/+}\) + TAM

**j** Ctrl (c-Kit\(^{CreERT2/+}\) + TAM

**k** Ctrl (c-Kit\(^{CreERT2/+}\) + TAM

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*Amplitude (s.d)*

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Intracellular recordings in colonic circular smooth muscle cells showed that ICC-specific deletion of Prkg1 (Supplementary Fig. S5a) abolished specifically the NO-dependent nitrergic slow component of the IJP (sIJP) under NANC conditions (Fig. 4g and Supplementary Fig. S5g). This effect is again not mediated by mast cells as they lack expression of Prkg1 (Supplementary Fig. S5a). Addition of the NOS inhibitor L-Nω-nitroarginine blocked specifically the sIJP in control animals (c-KitCreERT2+/Prkg1f/), thus validating our results (Supplementary Fig. S5g). As expected, deletion of the NO-target Prkg1 in ICC showed no effect...
on ATP-dependent purinergic inhibitory neurotransmission, which is responsible for the fast and larger component of the IJP (fIJP; Fig. 4g and Supplementary Fig. S5g).

Discussion

The mechanisms by which enteric neurons transmit their signals to regulate smooth muscle activity are of outstanding importance for basic and clinical science. The classical view of enteric neurotransmission to the smooth musculature is that enteric neurons release excitatory or inhibitory neurotransmitter that act directly on smooth muscle cells, thereby causing contraction or relaxation of the gut30.

Functional analysis of ICC has provided crucial insights into the generation of pacemaking currents and slow-wave activity in the gut in health and disease31,32,36,37. However, because of the general lack of genetic studies targeting ICC or signalling pathways in ICC, it is still highly controversial whether ICC are involved in enteric neurotransmission and lineage tracing of ICC (Prkg1 in vivo) allows for the first time spatio-temporal genetic manipulation of a specific signalling pathway in ICC (targeting the ATG start codon of Prkg1 in vivo). Therefore, neuronally released NO can still bind and activate the NO receptor soluble guanylyl cyclase with a subsequent increase of cGMP levels in Prkg1-deficient ICC; however, downstream NO–cGMP signalling is blocked by inactivation of the cGMP effector Prkg1. Therefore, it is plausible that Prkg1 deletion in ICC, but not ICC depletion, inhibits sIJP and inhibitory neurotransmission from enteric neurons to smooth muscle cells. Whether the purinergic ATP-dependent fIJP is regulated via ICC remains an outstanding question, which has to be addressed by conditional loss-of-function experiments in the future. Data from W/Wv mice indicate that the purinergic fIJP potential is not altered by the loss of ICC subpopulations31.

Taken together, our results may explain and resolve the apparently contradictory findings in W/Wv and nNOS KO models, which are the basis of the long-lasting controversy on how NO released from enteric neurons relaxes the smooth musculature9,12,29,30,42.

The data presented in this manuscript demonstrate clearly that our novel Cre/loxP-based mouse model has several advantages compared with currently used animal models to study ICC in vivo. Time-specific acute interference with ICC function in adult animals in a cell-specific fashion, using the TAM-activatable Cre/loxP system, precludes adaptive and compensatory mechanisms or developmental defects of ICC and other cell types as the cause of the observed effects9,11. Importantly, by using adoptive bone marrow transfer experiments we can exclude a role of mast cells in our study, which have been suggested to have an important role in GI motility43. Therefore, the results of our study provide the first in vivo genetic evidence that acute impairment of ICC function results in a loss of excitatory and inhibitory neurotransmission in adult mice, and indicate that ICC link slow-wave activity with enteric neurotransmission in vivo.

These findings support the concept that the core units that control GI motility are made up of nerves, ICC and smooth muscle cells44. In contrast to the central nervous system, enteric nerves utilize a second layer of regulation and integration to shape the functional output of nerve activity. This argues for a pivotal role of ICC in the organization and control of coordinated gut peristalsis beyond their role as GI pacemaker cells.

Impairment of the ICC function causes severe GI motor disorders, which are believed to be due to the loss of slow-wave activity4,7,17. As ICC integrate slow-wave activity and enteric neurotransmission, the results of our study support the conclusion that these disorders are at least, in part, due to impaired neurotransmission.

Methods

Materials. All cell culture reagents were obtained from Invitrogen (Groningen, The Netherlands). Primers were made by MWG (Ebersberg, Germany) and restriction endonucleases were obtained from New England Biolabs (Mannheim, Germany). The E. coli strains TOP10 and Sbl5 (Invitrogen) were used for transformation and plasmid amplification. TAM-free base, Peanut oil, Atropine, Guanethidine, Nifedipine and L-N5'-Nitroarginine were purchased from Sigma (Deisenhofen, Germany). Indocyanine green was purchased from Pulsion Medical Systems (Munich, Germany).

Generation of the c-KiCreERT2 mouse line. Targeting the c-Kit locus by a knock-in strategy of CreERT2 into the ATG start codon of c-Kit was performed as previously described for the Rosa26 locus45. A targeting vector containing an Frt-site-flanked neomycin resistance cassette 3' of the CreERT2 (ref. 46) expression cassette was generated by standard cloning procedures (Supplementary Fig. S1a). The linearized targeting vector was electroporated into 129S6 embryonic stem (ES) cells. ES cells were selected with 200 μg/ml−1 G418, and appropriately targeted
clones were identified by PCR. Correct recombination and single-copy insertion was verified by Southern blot analysis with 32P-labelled 5′- and 3′-external probe and internal probe, respectively; for the neomycin resistance cassette (Supplementary Fig. S1b). Twelve per cent of the analysed ES cell clones showed correct homologous recombination, and two clones (number 2 and number 5 in Supplementary Fig. S1b) were injected into C57BL/6 Blcystostats (Polygen, Rümlach, Switzerland). Germline transmission was achieved in two out of two clones, harbouring the targeted allele. A three-primer PCR strategy (Supplementary Fig. S1c) was used to genotype animals.

**Mouse strains.** R26V;Prkg1f/−/− (ref. 19), LSL-R26V;Prkg1f/−/− (ref. 20), conditional Prkg1f/−/− (ref. 47) and global Prkg1 KO (ref. 47) mice have been described previously. The tissues were fixed for 1 h at RT, mast cells were stained with avidin–Texas Red (1:500; Invitrogen) and nuclei were stained with TOPRO-3-iodide (1:1,000; Invitrogen) for 2 h in the dark at RT with secondary antibodies. Nuclear staining was achieved using a Zeiss OPMI 1-FC stereomicroscope (Olympus, Hamburg, Germany) equipped with a Confocal laser-scanning microscopy (Polygen, Rümlach, Switzerland). Confocal laser-scanning microscopy (Zeiss, Oberkochen, Germany) was performed using a Leica TCS SPE SPS DMI 6000 CS microscope equipped with a × 20/0.7 optical section thickness 3 μm) and a × 40/1.25 (optical section thickness 1.5 μm) oil-immersion objective (Leica, Heidelberg, Germany). Images (single optical sections and z-stacks, z-step size 0.5 μm) with a frame size of 1,024 × 1,024 pixels and an image size of 225 × 225 μm (∼450 × 450 μm objective, Zeiss) at 387.5 × 387.5 μm (∼10/0.25 objective, Leica) and 775 × 775 μm (∼20/0.7 objective, Leica) were collected. Images were merged and converted with ZEN 2009 (Zeiss) software, respectively.

**Immunofluorescence staining.** Analysis of frozen sections was performed using murine tissue specimens fixed in 4% buffered formalin for 2 h, dehydrated in grade methanol and then rehydrated in 1% buffered formalin at 4°C for 30% sucrose overnight and embedded in Miles Tissue-Tek (Sakura, Torrance, CA) before being rapidly frozen in liquid nitrogen. Frozen sections (20–30 μm thick) were obtained using a cryostat (Microm HM 560, Thermo Scientific, Schwerte, Germany). Briefly, slides were placed on room temperature (RT), fixed in 4% buffered formalin for 1 min, washed twice in PBS and incubated for 1 h in PBS containing 3% (w/v) bovine serum albumin (BSA), 1% (v/v) Triton X-100 and 1% (w/v) saponin. Slides were incubated for at least 48 h at 4°C with primary antibodies c-Kit (M14) (goat, 1:100; sc-1494, Santa Cruz Biotechnology, Santa Cruz, CA) and PKGβ1 (PKGβ1) (rabbit, 1:100)10. After rinsing, slides were incubated in the dark with secondary antibodies Alexa Fluor 594 donkey anti-goat or Alexa Fluor 488 chicken anti-rabbit (both 1:200; Invitrogen). The same protocol was used for double immunofluorescence staining. Nuclei were stained with TOPRO-3-iodide (1:1,000; Invitrogen) for 2 h in the dark at RT. After three rinses in PBS, slides were mounted with Vectashield Mounting Medium (Vector Laboratories, Burlington, CA). For counting ICC, transversal sections of animals of different genotypes and at different fields of view were analysed using the Zeiss LSM 510 microscope with the ×40/1.3 oil-immersion objective, and at least 2 different frozen sections (30 μm).

For whole-mount preparations of colon and ileum, tissue specimens were flushed with PBS, opened along the mesenteric border, stretched out and mucosal side were pinned up in Sylgard-coated petri dishes, and full-thickness mucosal specimens stained with primary antibodies (Supplementary Fig. S1c) was used to genotype animals. A three-primer PCR strategy (Supplementary Fig. S1c) was used to genotype animals.

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**Intracellular recordings.** Tissue preparation for electrophysiological experiments and intracellular recordings of smooth muscle cells of the circular muscle layer of mouse proximal colon and ileum were performed exactly as described previously21–23. Nifedipine (1 μM) was present throughout all experiments. Neurons were stimulated with single pulses (15 V, 0.3 ms duration) via a platinum electrode, arranged perpendicularly to the circular muscle layer, and connected to a Grass SI1 stimulator via a stimulus isolation unit (Grass SIU59, Grass Instruments, Quincy, MA). EFS of the neurons caused changes in the RMP of the smooth muscle cells, and the responses were recorded against a ‘ground’ Ag-AgCl electrode placed in the bath medium. Evoked electrical events were amplified (DUP 733 microelectrode amplifier, World Precision Instruments, Sarasota, FL) and digitized with an analogue-to-digital converter (SCB 68 interface, National Instruments, Austin, TX). Permanent recordings of membrane potentials were made using the LABVIEW 5.0 programme (National Instruments, Munich, Germany).

**For NANC measurements, 1 μM atropine and 1 μM guanethidine (both from Sigma) were used. The inhibitor of NOS L-NAME was added in a final concentration of 10 μM. Cells from three animals per genotype were analysed in all experiments.**

**Statistical analyses.** Comparisons among data sets were made with analysis of variance, followed by Students t-test. A Bonferroni correction of the P values was performed for multiple testing. Values of P < 0.05 or less were considered to be statistically significant.

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Author contributions

B.S. and D.S. designed research; S.K., B.S., A.K., A.S., M.R. and D.S. performed research; R.F. and F.H. contributed new reagents/analytic tools; S.K., B.S., A.K., A.S., J.M.V., M.S., R.R., M.A.S., R.M.S., G.S. and D.S. analyzed data; and D.S. wrote the paper. All authors discussed the results and commented on the manuscript.

Additional information

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

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