

# Functional identification of an aggression locus in the mouse hypothalamus

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**Electrical stimulation of certain hypothalamic regions in cats and rodents can elicit attack behaviour, but the exact location of relevant cells within these regions, their requirement for naturally occurring aggression and their relationship to mating circuits have not been clear. Genetic methods for neural circuit manipulation in mice provide a potentially powerful approach to this problem, but brain-stimulation-evoked aggression has never been demonstrated in this species. Here we show that optogenetic, but not electrical, stimulation of neurons in the ventromedial hypothalamus, ventrolateral subdivision (VMHvl) causes male mice to attack both females and inanimate objects, as well as males. Pharmacogenetic silencing of VMHvl reversibly inhibits inter-male aggression. Immediate early gene analysis and single unit recordings from VMHvl during social interactions reveal overlapping but distinct neuronal subpopulations involved in fighting and mating. Neurons activated during attack are inhibited during mating, suggesting a potential neural substrate for competition between these opponent social behaviours.**

A central problem in neuroscience is to understand how instinctive behaviours<sup>1</sup>, such as aggression, are encoded in the brain. Classic experiments in cats have demonstrated that attack behaviour can be evoked by electrical stimulation of the hypothalamus<sup>2,3</sup>. However, the precise location of the relevant neurons, and their relationship to circuits for other instinctive social behaviours, such as mating, remain unclear. Studies in the rat have identified a broadly distributed 'hypothalamic attack area' (HAA)<sup>4–8</sup> that partially overlaps several anatomic nuclei<sup>9</sup>. In contrast, neurons involved in predator defence and mating seem to respect the boundaries of specific, and complementary, hypothalamic nuclei<sup>10,11</sup>. How aggression circuits are related to these two homologically distinct behavioural subsystems<sup>9,10</sup> remains poorly understood (but see ref. 12). Immediate early gene (IEG) mapping experiments have suggested that aggression and mating involve similar limbic structures<sup>13–15</sup>, but whether this reflects the involvement of the same or different cells within these structures is not clear.

We have investigated the localization of hypothalamic neurons involved in aggression, and their relationship to neurons involved in mating, in the male mouse. Using a combination of genetically based functional manipulations and electrophysiological methods, we identify an aggression locus within the ventrolateral subdivision of VMH (VMHvl)<sup>9</sup>. Surprisingly, this structure also contains distinct neurons active during male-female mating. Many neurons activated during aggressive encounters are inhibited during mating. These data indicate a close neuroanatomical relationship between aggression and reproductive circuits, and a potential neural substrate for competition between these social behaviours<sup>1</sup>.

## Results

### Intermingled mating and fighting neurons

We first employed conventional non-isotopic analysis of *c-fos* (also known as *Fos*) induction, a surrogate marker of neuronal excitation<sup>16</sup>, to map activity during offensive aggression in the resident-intruder test<sup>17</sup>. For comparison, we performed a similar analysis during mating with females. Mating and fighting induced *c-fos* mRNA in the medial amygdala, medial hypothalamus and bed nucleus of the stria terminalis (BNST;

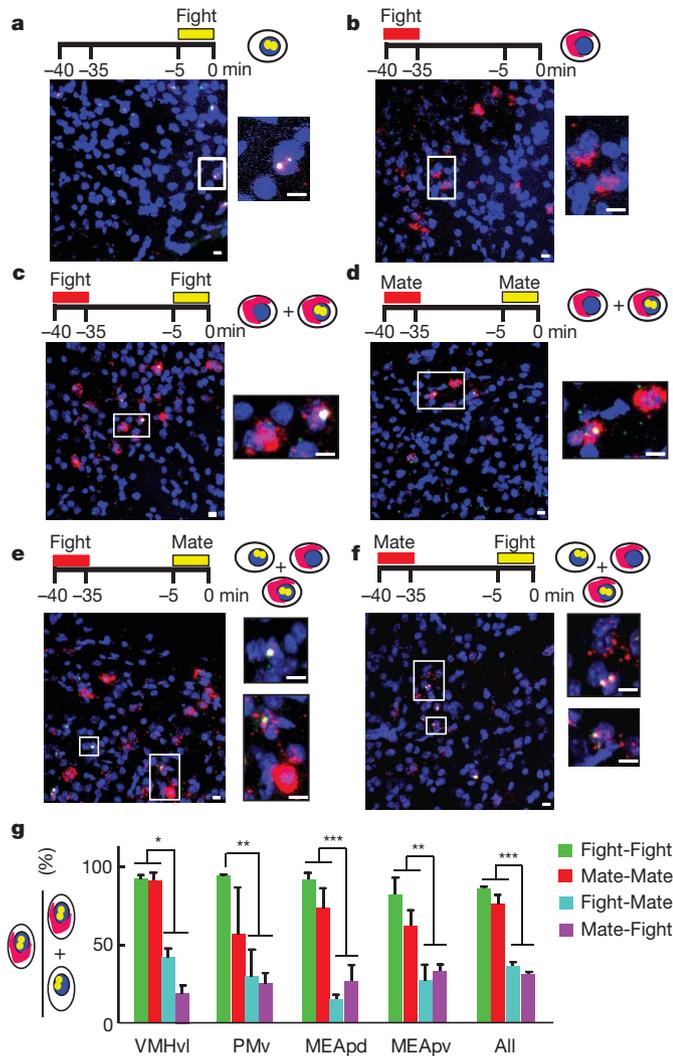
Supplementary Fig. 1), as described previously in rats and hamsters<sup>13,15</sup>, but not in the anterior hypothalamic nucleus (AHN) which has been implicated in aggression by many studies<sup>18,19</sup> (reviewed in ref. 20). Whereas the pattern of mating versus fighting-induced *c-fos* was similar in most structures, such between-animal comparisons do not distinguish whether these social behaviours activate the same or different neurons.

To address this issue, we adapted a method, called cellular compartment analysis of temporal activity by fluorescent *in situ* hybridization (catFISH)<sup>21,22</sup> to compare *c-fos* expression induced during two consecutive behavioural episodes in the same animal (Figs 1a–f). We examined four limbic regions (VMHvl, ventral premammillary nucleus (PMv), medial amygdala posterodorsal (MEApd) and posteroventral (MEApv)) that showed strong *c-fos* induction in single-labelling experiments (Supplementary Fig. 1). Animals killed immediately after 5 min of fighting had almost exclusively nuclear *c-fos* transcripts, whereas those killed 35 min after fighting had essentially only cytoplasmic transcripts (Supplementary Fig. 2). In animals that engaged in two successive episodes of the same behaviour separated by 30 min, most cells expressing nuclear *c-fos* transcripts also expressed cytoplasmic *c-fos* mRNA (Fig. 1c, d, g and Supplementary Fig. 3, green and red bars), indicating activation during both behavioural episodes. By contrast, in animals that sequentially engaged in two different behaviours, only 20–30% of cells with nuclear *c-fos* RNA also expressed cytoplasmic *c-fos* transcripts (Fig. 1e–g and Supplementary Fig. 3, blue and magenta bars). (Nevertheless, the overlap between nuclear and cytoplasmic *c-fos* hybridization was slightly greater than expected by chance even when the two sequential behaviours were different (Supplementary Fig. 4)). These results indicate, first, that the same neurons are likely to be recruited during two successive episodes of mating or fighting, even though such neurons are relatively sparse (Supplementary Fig. 5, <12% of total cells *c-fos*<sup>+</sup>); and second, that mating and fighting may recruit overlapping but distinct sets of neurons in these brain regions.

### Chronic recording from the VMHvl

To gain further insight into the relationship between neurons active during mating and fighting, we performed chronic single-unit recordings

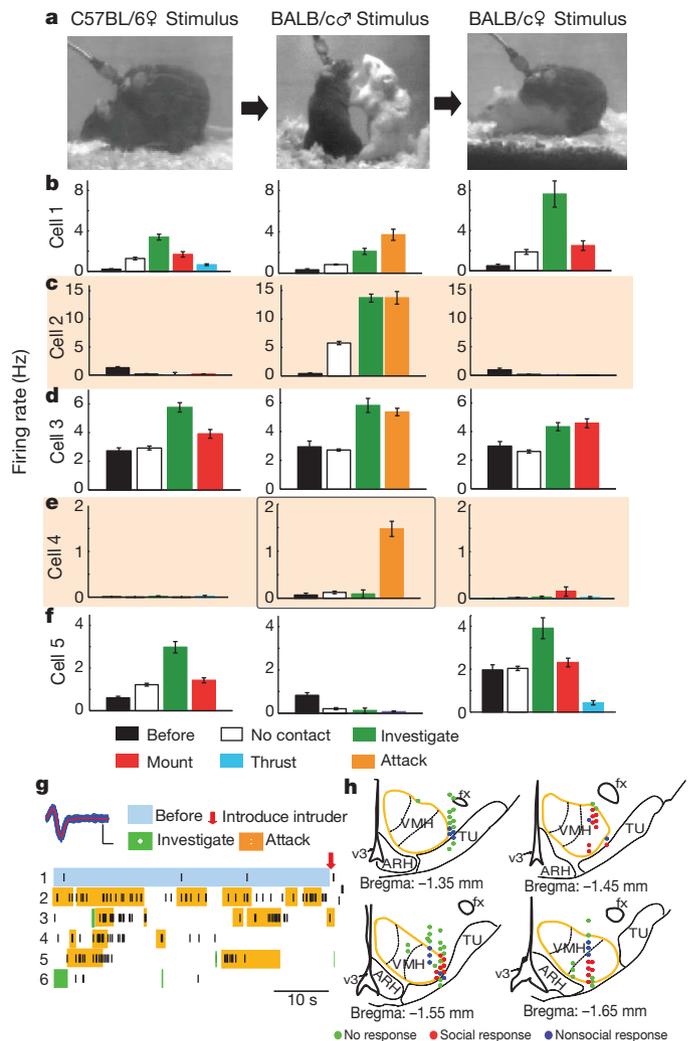
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**Figure 1** | Fos catFISH analysis of cell activation during fighting versus mating. **a–f**, *c-fos* expression patterns following single (**a**, **b**) or two sequential (**c–f**) social interactions. Boxed areas are enlarged to right of each panel. Blue, Topro-3 nuclear counterstain. Red, *c-fos* cytoplasmic transcripts (cRNA probe); yellow dots, nuclear *c-fos* transcripts (red cRNA plus green intron probe signals). Scale bars, 10  $\mu$ m. **g**, Percentage of total cells expressing *c-fos* after the 2nd behaviour (nuclear signal) that also expressed *c-fos* after the 1st behaviour (nuclear + cytoplasmic signal) (one-way ANOVA with Bonferroni correction). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

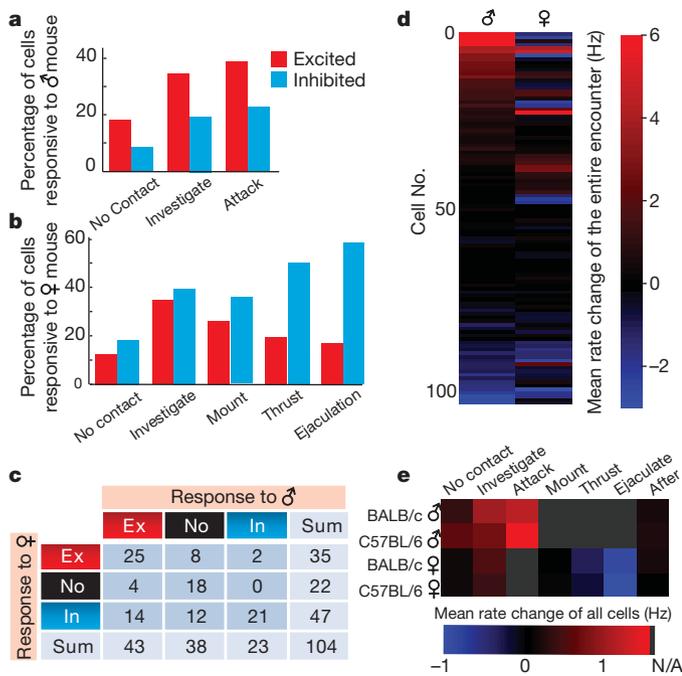
in awake, behaving male mice using a 16-wire electrode bundle<sup>23</sup> (see Methods). We selected VMHvl for these studies, because it showed preferential *c-fos* induction after fighting versus mating (Supplementary Fig. 5; aggression-induced *c-fos* in VMHvl was further confirmed by double-labelling for *c-fos* and *vglut2*, a glutamate transporter enriched in VMH; Supplementary Fig. 6), and because it overlaps partially with the rat HAA<sup>7</sup>. Recording from VMHvl is challenging because of its deep location, and small size; in only 5 of 30 implanted animals were all 16 electrode tracks confined to VMHvl (Supplementary Fig. 7). Neurons excited during social behaviours (Fig. 2h, red dots) were rarely found among the 25 mistargeted animals. We recorded successfully from 104 well-isolated cells in the five VMHvl-targeted animals. By holding the same cell during alternating, sequential exposures to female and male stimulus animals (Fig. 2a and Supplementary Fig. 8), we could distinguish whether the unit was activated by males and/or females (see Methods for unit isolation criteria).

Neuronal activity patterns in VMHvl during social encounters showed diverse temporal dynamics and sex-selectivity (Figs 2, 3 and Supplementary Figs 8 and 9). Spontaneous firing rates before introduction



**Figure 2** | Response patterns of a VMHvl neuron during social encounters. **a**, Video frames taken from consecutive trials with intruder animals of the indicated sex and strain. **b–f**, Average firing rate (over 0.5 s bins;  $\pm$  s.e.m.) during indicated behavioural episodes (manually annotated, frame-by-frame) from five exemplar cells. ‘Before,’ before introducing stimulus animal; ‘No contact,’ periods during encounter without physical contact between intruder and resident. **g**, Recordings from the cell in **e**, middle. Blue trace, superimposed individual spikes; red line, average spike shape. Scale bars, 200  $\mu$ V, 200  $\mu$ s. Raster plots illustrate 300 s of continuous recording. Coloured shading and arrow mark manually annotated behavioural episodes. **h**, Schematics indicating cell response type at each recording site from Bregma level  $-1.35$  mm to  $-1.65$  mm. Anatomical structures based on Allen Brain Atlas ([www.brain-map.org](http://www.brain-map.org)). fx, fornix; ARH, arcuate nucleus; v3, third ventricle; TU, tuberal nucleus.

of the stimulus animal were typically low (median = 1.1 Hz, range 0–12.7 Hz) and rarely increased during home cage behaviours (that is, grooming); some cells were completely silent until the stimulus animal was presented. Spiking activity was correlated with behaviour by computer-assisted manual annotation of videotape (see Methods). Over 50% (53/104) of recorded cells increased their firing rate during at least one behavioural episode of a social encounter (Fig. 3c). A large fraction (41%; 43/104) of VMHvl cells showed increased firing during an encounter with a male stimulus animal, and on average spiking activity increased with escalation of the encounter, independent of intruder strain (Fig. 3e). In many cases (19/43) this increase began as soon as the intruder male was introduced, and continued as the social encounter progressed, whereas in a comparable number increased firing was observed only during close investigation and subsequent attack (Fig. 2b, d, middle plots, Fig. 3a and Supplementary Movie 1).



**Figure 3 | Summary of cell responses in VMHvl during mating and fighting.** **a, b**, Percentage of cells excited (red) or inhibited (blue) during encounters with male (**a**) or female (**b**) mice. **c**, Numbers of cells exhibiting statistically significant changes in firing rate (see Methods) towards males or females. **d**, Firing rate changes for all 104 recorded cells, averaged over entire encounter with males or females. **e**, Firing rate changes averaged over all 104 recorded cells, during various behavioural episodes. Grey, behaviour not applicable (N/A) to the stimulus animal.

Strikingly, a small subset of cells activated in male-male encounters (12%; 5/43) was excited exclusively during attack (Fig. 2e, middle plot, and Fig. 2g).

In contrast, during encounters with females, spiking activity in VMHvl tended to increase only transiently during the initial investigative phase, and subsequently declined as mating progressed (Fig. 3e). Among 35 cells that were excited during female investigation, almost two-thirds (23/35) decreased their firing during subsequent mounting (Fig. 2b and f, left, right, and Fig. 3b), and seven were suppressed (below their baseline firing rate) during thrust and ejaculation (Fig. 2f, right, and Supplementary Fig. 9c, d). Almost half (25/53) of all cells activated during social encounters were excited by both males and females, although most of the largest increases in activity were in sex-specific cells (see Supplementary Footnote 1 and Supplementary Fig. 10). Furthermore, most of this overlap was transitory, occurring during the initial stages of the social encounter and diminishing as the interaction progressed to the consummatory phase of attack or copulation. The observation of partially overlapping populations of male and female excited cells in VMHvl qualitatively confirms the results of our *c-fos* catFISH studies (Supplementary Fig. 10 and Supplementary Footnote 1). However, the evolving segregation of the two populations as the social encounters progressed was not anticipated by the IEG analysis, due to its insufficient temporal resolution.

Our electrophysiological recordings also showed that the majority (14/18) of male excited cells were actively suppressed (below their baseline firing rates) during encounters with females (Figs 2c and 3c, d, Supplementary Fig. 8a, c, e, g and Supplementary Movie 2). Most (86%; 12/14) of those cells, moreover, responded to male intruders before any physical contact. This observation suggests that cells excited during the initiation of an aggressive encounter are selectively suppressed during interactions with a female. In contrast, of the 10 cells selectively excited by females, only two were actively suppressed during

a male-male encounter (Fig. 2f, middle). This asymmetry in sex-specific inhibitory responses indicates that suppression of fighting-related neurons during mating is more pronounced than the converse.

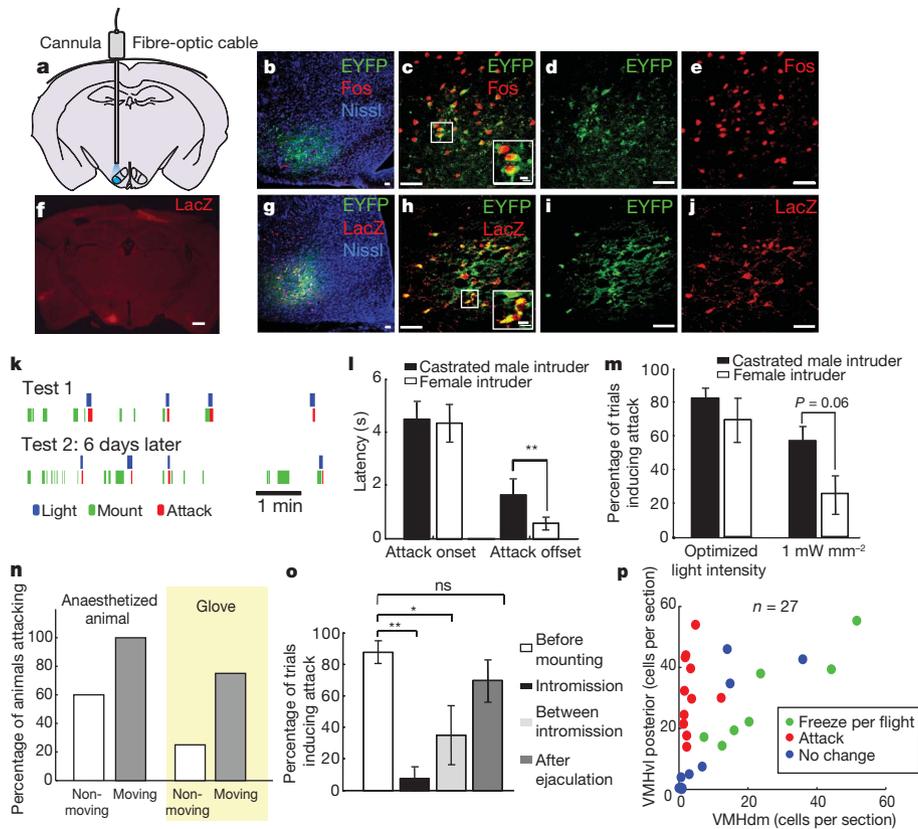
### Optogenetic stimulation induced attack

We next tested whether functional manipulations of VMHvl would affect mating or fighting. Although VMHvl overlaps the rat HAA<sup>7,8,24</sup>, extensive attempts to elicit attack by conventional electrical stimulation of this region in mice were unsuccessful (see Supplementary Footnote 2 and Supplementary Fig. 11). As an alternative, therefore, we expressed channelrhodopsin-2 (ChR2) in VMHvl neurons unilaterally, using stereotactic co-injection of adeno-associated viral vectors (AAV2) expressing Cre recombinase and a Cre-dependent form of ChR2 fused with enhanced yellow fluorescent protein (ChR2-EYFP)<sup>25,26</sup>, and selectively illuminated cells in this region using an implanted fibre-optic cable<sup>27</sup> (Fig. 4a). Because AAV2 infects neurons preferentially<sup>28</sup> (Supplementary Fig. 12) and does not retrogradely infect cells from their axons or nerve terminals<sup>29</sup>, only neurons whose cell bodies are local to the injection site express ChR2 (Supplementary Footnote 3). Optrode recording in anaesthetized animals confirmed that ChR2-expressing cells in VMH can be driven to fire with high temporal precision (Supplementary Fig. 13). Consistent with this result, *c-fos* could be strongly induced in VMHvl on the infected, but not the contralateral control side after repeated blue light stimulation in awake behaving animals (Figs 4b–e).

Optogenetic stimulation of VMHvl in the absence of an intruder did not obviously alter behaviour, except for an occasional increase in exploratory activity. In contrast, in the presence of an intruder, illumination elicited a rapid onset of coordinated and directed attack, often towards the intruder's back (Supplementary Movie 3, see Methods for more detailed behavioural description). Importantly, whereas male mice rarely spontaneously attack females or castrated males, 11/16 ChR2-expressing males exhibited attack towards such intruder animals, within 4–5 s after the onset of illumination (Fig. 4l), over multiple trials (Fig. 4k, Test 1, blue bars). In 9/11 animals, attack was induced during a second test session 1–6 days later (Fig. 4k, Trial 2). Animals with low infection (<10 cells per section,  $N = 4$ ) or animals injected with saline during the surgery ( $N = 4$ ) showed no obvious behavioural changes during light stimulation.

Interestingly, upon illumination offset test animals ceased attack towards females significantly faster than towards castrated males (Fig. 4l, Attack offset). Furthermore, when low intensity ( $1 \text{ mW mm}^{-2}$ ) light was used, castrated males were attacked more readily than females (Fig. 4m). We also tested whether illumination could induce attack towards anaesthetized intruders or inanimate objects. Six of 10 animals attacked stationary anaesthetized animals upon illumination; all test animals attacked if the anaesthetized intruders were artificially moved (Fig. 4n). Two of 8 test animals attacked a stationary inflated glove, while 6/8 animals attacked if the glove was moved (Fig. 4n and Supplementary Movie 4).

Histological analysis showed that when the majority of infected cells was located in VMHvl, light stimulation effectively induced attack (red circles in Fig. 4p). In contrast, freezing and flight were observed when VMHdm and VMHc were infected to an equal or greater extent (green circles in Fig. 4p)<sup>30</sup>. Infection in other regions, such as VMHvl anterior, the lateral hypothalamic area (LHA) and tuberal nucleus (TU) was not associated with illumination-induced behavioural changes (Supplementary Figs 14 and 15, Supplementary Footnote 4). To test more directly whether neurons in regions of the HAA<sup>8</sup> surrounding VMHvl are sufficient to induce aggression, we deliberately infected such regions with AAV2-ChR2. No attack could be induced by light stimulation in such animals ( $N = 5$ ). Strikingly, in cases where the AAV2-ChR2 spread into VMHvl, attack was induced ( $N = 3$ ) (Supplementary Fig. 16). These data indicate that neurons located within VMHvl, but not in adjacent regions, have a key role in mouse aggression.



**Figure 4 | Optogenetic activation of VMHvl elicits attack in mice.**

**a**, Schematic illustrating optic fibre placement; VMHvl shaded in blue. **b–e**, Fos induction (red) in EF1 $\alpha$ ::ChR2–EYFP-expressing (green) cells at 1 h post-illumination. Fos<sup>+</sup> cells outside EYFP<sup>+</sup> region may be synaptic targets of ChR2-activated cells. Blue, fluorescent Nissl stain. **f**, LacZ expression identifies infected cell bodies (red). Scale bar, 500  $\mu$ m. **g–j**, LacZ expression (red) and native ChR2–EYFP fluorescence (green) largely overlaps. Boxed areas in **c**, **h** enlarged at lower right. Scale bars in **b–e**, **g–j**, 50  $\mu$ m or 10  $\mu$ m (insets). **k**, Raster plots illustrating behavioural episodes (legend below) in a ChR2-expressing male paired with a female in two consecutive tests. **l**, Attack onset/offset latencies (relative to initiation versus termination of illumination) towards indicated intruders,  $**P < 0.01$ . **m**, Efficacy of light-stimulated attack.

The observations that overall activity in VMHvl decreases during male–female mating (Fig. 3e), and that many male excited cells are inhibited by females (Fig. 3d), indicated that a progressive inhibition of attack neurons occurs as mating progresses towards its consummatory phase. To test this, we stimulated VMHvl during encounters with females, before mounting, during intromission, between intromissions and after ejaculation. When illumination was delivered before mounting, attack towards the female was elicited in over 80% of trials at light intensities between 1 and 2  $\text{mW mm}^{-2}$ , in all seven tested animals (Fig. 4o, white bar). But during intromission, the same light intensity was often ineffective, even with extended stimulation (Fig. 4o, black bar). Increasing the light intensity fourfold elicited female-direct attack during intromission in five of seven animals, but with increased latency (Supplementary Fig. 17). Between intromissions, attack was evoked in 30% of cases. Strikingly, following ejaculation the frequency of illumination-evoked attack recovered to pre-mounting levels (Fig. 4o, dark grey bar). Thus mating exerts an increasingly strong suppression of optogenetically stimulated attack, as the encounter progresses towards its consummatory phase.

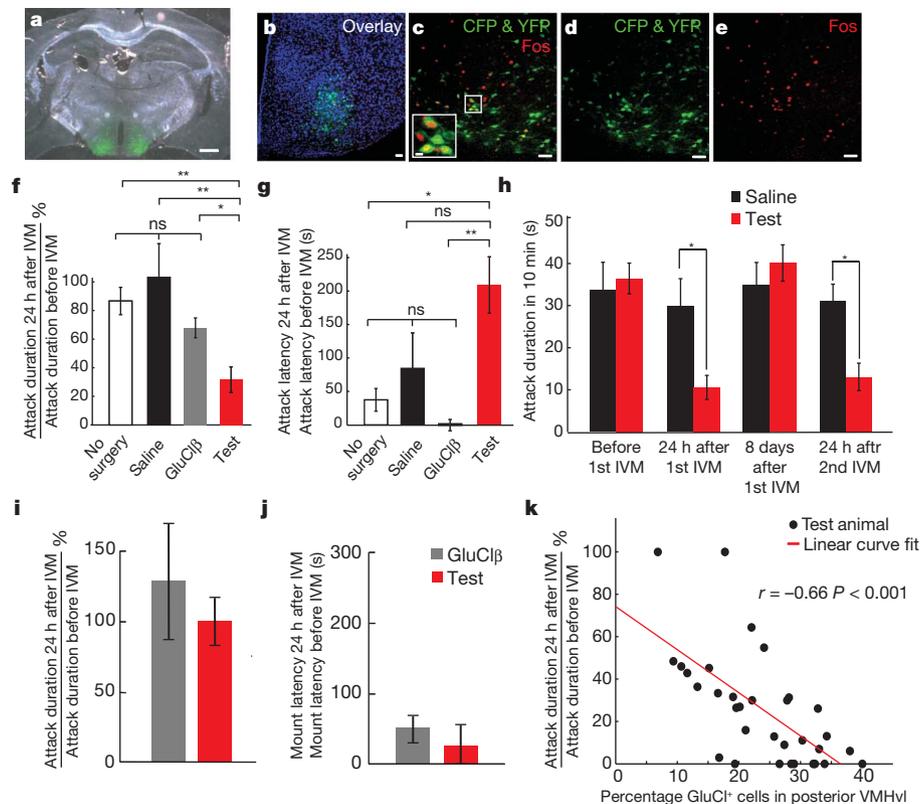
### Mouse aggression requires VMHvl activity

Whether neurons that mediate brain-stimulation-evoked attack are also required for naturally occurring aggression has been controversial. Electrolytic lesions of VMH in rats and mice have yielded seemingly contradictory results<sup>31,32</sup>, and this method destroys axons-of-passage

‘Optimized light intensity’, laser power yielding average maximal response in each animal (range: 1–3.3  $\text{mW mm}^{-2}$ ). 1  $\text{mW mm}^{-2}$ , average response obtained at this power (*t*-test,  $P = 0.06$ ). **n**, Percentage of animals attacking moving versus non-moving anaesthetized animals or inflated glove (yellow shading). **o**, Percentage of trials inducing attacks towards female during successive stages of mating.  $*P < 0.05$ ,  $**P < 0.01$  (one-way ANOVA with Bonferroni correction). ns, not significant. **p**, Distribution of infected cells in each animal, plotted as cells per section in VMHvl posterior portion versus that in (VMHdm + VMHc) region. Colour code indicates whether illumination induced freeze/flight (green), attack (red) or no change in behaviour (blue). See also Supplementary Footnote 4 for further statistical analysis.

as well as cell bodies. There is little evidence that local chemical inhibition of neuronal activity in the rat HAA reduces aggression (although inhibition<sup>33</sup> or killing<sup>34</sup> of Substance P receptor-expressing neurons attenuates ‘hard biting’ behaviour). We therefore asked whether reversible genetic suppression of electrical excitability in VMHvl neurons inhibits attack behaviour. To do this, we used separate AAV2 vectors to co-express two subunits ( $\alpha$  and  $\beta$ ) comprising a *Caenorhabditis elegans* ivermectin (IVM)-gated chloride channel (GluCl $\alpha\beta$ )<sup>35</sup>, which has been mutated to eliminate glutamate sensitivity<sup>36</sup>. Upon IVM binding, this heteropentameric channel prevents action potential firing by hyperpolarizing the membrane<sup>28,35</sup>.

Three weeks after viral injection, animals were administered IVM intraperitoneally 24 h before testing<sup>28</sup>. The experimental group ( $N = 33$ ) showed a decrease in the total attack duration, and an increase in the latency to the first attack, in comparison to saline-injected or GluCl $\beta$ -only injected controls (Figs 5f, g; see Methods). Furthermore, 25% of the experimental animals failed to initiate any attack during the post-IVM test. Experimental animals performed similarly in the rotarod assay before and after IVM administration, indicating no change in motor coordination or fatigue (Supplementary Fig. 18). Eight days after the IVM injection, the aggression level of the test group recovered to the pre-IVM level and could be suppressed again by a second IVM injection (Fig. 5h). Immunohistochemical analysis (Fig. 5a, d) indicated a reverse correlation between the suppression of aggression and the percentage of GluCl-expressing cells in



**Figure 5 | Reversible inhibition of natural aggression by genetic silencing of VMHvl** **a**, Anti-GFP antibody staining (green) in mice bilaterally infected with AAV2-GluCl $\alpha$  + AAV2-GluCl $\beta$ . Scale bar, 500  $\mu$ m. **b–e**, Overlap between GluCl-expressing (green) and Fos-expressing (red) cells, 1 h after fighting. Blue, Topro-3 nuclear stain. Inset in **c** represents boxed area. Yellow cells are double-labelled. Scale bars, 50  $\mu$ m or 10  $\mu$ m (inset). **f, g**, Percent change in cumulative attack duration (**f**) and latency (**g**) during a 600 s resident-intruder trial before versus 24 h after IVM injection. Test, GluCl virus-injected animals ( $n = 33$ ) (red bar). Control, no surgery (white bar,  $n = 12$ ), saline (black bar,  $n = 6$ ) or GluCl $\beta$  virus-injected animals ( $n = 12$ , grey bar) (\*\* $P < 0.01$ , \* $P < 0.05$ ,

*t*-test). **h**, Cumulative attack duration during repeated cycles of IVM injection or washout (\* $P < 0.05$ , Bonferroni after tests of two-way ANOVA with repeated measures). Test, GluCl virus-injected animals ( $n = 12$ ); Control, saline ( $n = 6$ ). **i, j**, Percent change in mount duration (**i**) or latency (**j**) in test ( $n = 12$ ) versus control (GluCl $\beta$  virus injected,  $n = 12$ ) males paired with females. **k**, Percentage of infected cells in posterior portion of VMHvl (Bregma  $-1.4$ – $1.8$  mm) plotted against extent of aggression suppression after IVM injection. The Pearson correlation coefficient is significantly higher than 0 ( $P < 0.001$ ). See Supplementary Fig. 19 for further analysis.

the posterior half of VMHvl (Bregma level  $-1.4$ – $1.75$  mm; Fig. 5k). No such correlation was found in VMHvl anterior (Bregma level  $-1.15$ – $1.4$  mm) or in other regions surrounding VMHvl (Supplementary Fig. 19). Double-label immunostaining for GFP and Fos in animals killed 1 h after an aggressive interaction (following IVM washout) indicated that viral infection overlapped the population activated during fighting ((GFP $^+$  Fos $^+$ )/total Fos $^+$  > 50%;  $n = 4$ ; Fig. 5b–e). These data indicate that genetic silencing of neurons in VMHvl can reversibly inhibit aggressive behaviour. In GluCl-expressing males paired with females, no change in mounting duration or latency to the first mount was observed after IVM injection (Figs 5i, j). Because the overall level of neuronal activity in VMHvl is normally suppressed during the consummatory phase of mating (Fig. 3e), it is not surprising that further inhibition of activity failed to impair such behaviour.

## Discussion

Using genetically based manipulations in mice, we show that neurons necessary and sufficient for offensive aggression are localized within a small subdivision of VMH. The more diffuse HAA identified in rats<sup>6,24</sup> may reflect a species difference, or the fact that electrical stimulation mapping<sup>37</sup> activates both axons-of-passage and neuronal somata, whereas our manipulations are restricted to the latter. Our *in vivo* recordings indicate that some neurons in VMHvl are activated by intruder conspecifics before physical contact. This suggests a function in olfactory coding, perhaps related to sex discrimination. However, optogenetic stimulation of VMHvl evoked aggressive behaviour

towards an inanimate object, arguing for a causal role in the motivation or drive to attack. We suggest that VMHvl has a key role in sensori-motor transformations and/or the encoding of motivational states<sup>9</sup> underlying aggression. The relationship of the aggression circuits within VMHvl to those involved in defensive<sup>12,20,38</sup> or maternal<sup>139,40</sup> aggression remains to be investigated.

Whereas VMH is well established to have a key role in female reproductive behaviour<sup>41,42</sup>, it has not traditionally been considered as a key node in male mating circuitry<sup>11</sup> (but see ref. 13). We have identified cells within the VMHvl of males that are activated during male-female mating, and which are mostly distinct from those activated during fighting. The role of these neurons is not yet clear, because our functional manipulations did not perturb mating behaviour. One possibility is that these female-activated neurons serve to inhibit aggression during mating. Consistent with this idea, many male-activated units were actively inhibited by females, and a higher intensity of illumination was required to evoke attack towards a female during mating encounters. These data identify a neural correlate of competitive interactions between fighting and mating<sup>1</sup>. Whether this competition originates in VMHvl, or is controlled by descending inputs to this nucleus<sup>43</sup>, awaits further investigation.

## METHODS SUMMARY

Sexually experienced C57BL/6N male mice, singly housed on a reverse light-dark cycle, were used. Resident-intruder assays were designed to maximize offensive aggression by the resident; no attacks were initiated by the intruder under our conditions. For *in situ* hybridization, animals were killed 30 min after a 10 min

standard resident-intruder assay and processed as described<sup>44</sup>. For Fos catFISH experiments, animals experienced two 5-min behavioural episodes 30 min apart, and were killed immediately after the second episode. An intronic *c-fos* probe and a *c-fos* cRNA probe were combined to detect nuclear *c-fos* primary transcripts. For chronic recording, a movable bundle of sixteen 13- $\mu$ m tungsten microwires was implanted, and 2 weeks allowed for recovery. On recording days, a flexible cable was attached to the microdrive and connected to a commutator. Recordings were performed in the animals' home cage. Female and male mice were introduced for approximately 10 min per session. Spiking activity and behaviour were synchronously recorded. Data analysis, including behavioural annotation of videotapes, was performed using custom software written in Matlab. For Chr2 experiments, 150 nl of a 4:2:1 mixture of an AAV2 Cre inducible EF1 $\alpha$ ::Chr2 (ref. 26), AAV2 CMV::CRE and AAV2 CMV::LacZ with a similar final titre ( $8 \times 10^{11}$  p.f.u. ml<sup>-1</sup>) was stereotactically injected unilaterally. After 2 weeks of recovery, light pulses (20 ms, 20 Hz, 1–4 mW mm<sup>-2</sup>) were delivered to activate the targeted region for 2–20 s in the presence of various stimuli. For GluCl inactivation experiments, animals in the experimental group (bilateral injection of 10<sup>9</sup> viral particles per side of AAV2 expressing cyan-fluorescent-protein- and yellow-fluorescent-protein-tagged GluCl $\alpha$  and GluCl $\beta$ , respectively, under the control of the CAG promoter (CAG::GluCl $\alpha$ -CFP and CAG::GluCl $\beta$ -YFP)), and each of the three control groups (no surgery, saline or GluCl $\beta$  bilaterally injected) were tested three times in the resident-intruder assay to establish a stable aggression baseline. After the third test, IVM (1%, 5 mg kg<sup>-1</sup>) was injected intraperitoneally and the animals were tested again 24 h and 8 days later.

**Full Methods** and any associated references are available in the online version of the paper at [www.nature.com/nature](http://www.nature.com/nature).

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**Supplementary Information** is linked to the online version of the paper at [www.nature.com/nature](http://www.nature.com/nature).

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## METHODS

**Behavioural tests.** All test animals used in this study were adult proven breeder C57BL/6 male mice (Charles River Laboratory). They were singly housed under a reversed light-dark cycle for at least 1 week before the test. The care and experimental manipulation of the animals were carried out in accordance with the NIH guidelines and approved by the Caltech Institutional Animal Care and Use Committee. For resident-intruder assays, C57BL/6 males were allowed to interact with BALB/c males for 10 min. All intruder mice were group housed, and had similar body weight as the test mice. All resident animals included in the study initiated all the attacks and showed no submissive postures during the aggression test. For mating tests, the residents were allowed to interact with sexually receptive BALB/c and C57BL/6 females for 10 min. Females were screened for receptivity by pairing with a singly housed C57BL/6 male mouse briefly before each test.

**In situ hybridization.** Brains from mice killed 30 min after performing either the 10 min resident-intruder or mating tests were analysed for expression of *c-fos* mRNA throughout the forebrain, using non isotopic *in situ* hybridization on 120 µm thick sections. Details of the procedure have been described previously<sup>44</sup>. For fos catFISH experiments, animals experienced two consecutive 5 min fighting or mating episodes 30 min apart, and were killed immediately after the second episode. Since *c-fos* transcripts are detected only in the nucleus within the first 5 min following induction, and are completely translocated to the cytoplasm as processed mRNA after 35 min, the sub-cellular location of *c-fos* allows one to distinguish neurons activated by a single stimulus from those successively activated by both stimuli: only in the latter case will transcripts be present in both the nucleus and cytoplasm. We used an intronic *fos* probe with a different fluorescent colour label, in addition to the *fos* cRNA probe, which allowed us to more easily differentiate nuclear from cytoplasmic FISH signals. The *c-fos* transcript distribution pattern was examined using both colour combinations (*fos* cRNA probe in green and *fos* intronic probe in red, or vice versa) and no difference was observed. See Supplementary Methods for more detailed dFISH procedure and microscopic analysis.

**Electrophysiological recording.** A bundle of sixteen tungsten microwires (13 µm diameter each, California Fine Wire) attached to a mechanical microdrive was implanted in one hemisphere and secured with bone screws and dental acrylic during stereotaxic surgery. The drive was a miniaturized version of an original design described elsewhere<sup>45</sup>. Two weeks after initial implantation, and on days of recording, a flexible cable was attached to the microdrive and connected to a torqueless, feedback-controlled commutator (Tucker Davis Technology). During recording sessions, the test animals were allowed to stay in their home cage and interact with the stimulus animals freely. Female or male mice were introduced into the test arena for approximately 10 min. A given type of stimulus (for example, a male mouse) was presented on multiple occasions, to examine the reproducibility of a response. All recordings were carried out in subdued light with infrared illumination. A commercial recording system was used for data acquisition (Tucker Davis Technology). Digital infrared video recordings of animal behaviour from both side and top view were simultaneously streamed to a hard disk at 640 × 480 pixel resolution at 25 frames per second (Streampix, Norpix). Each video frame acquisition was triggered by a TTL pulse from the recording setup to achieve synchronization between the video and the electrophysiological recording. Spikes of individual neurons were sorted using commercial software (OpenSorter, Tuck Davis Technology), based on principal component analysis. Unit isolation was verified using autocorrelation histograms. To ensure that single units were isolated, and that the same units were recorded in the presence of sequentially presented male or female stimulus animals, we imposed four criteria to select cells for subsequent statistical analysis. First, the cells had to have a signal/noise ratio >3; second, the spike shape had to be stable throughout the recording; third, the response had to be repeatable during multiple trials; fourth, the percentage of spikes occurring with inter-spike intervals (ISIs) <3 ms (the typical refractory period for a neuron) in a continuous recording sequence had to be <0.1%. Of the cells included in the analysis, 74 out of 104 had all of their ISIs ≥ 3 ms. After each recording session, the microwire bundle was advanced 70 µm, by adjusting a 00-90 screw on the drive by a quarter of a turn. After 5 to 10 recording sessions, which typically were distributed over 2 to 3 months, animals were euthanized and the location of the recording electrodes verified histologically.

**Behavioural annotation and statistical analysis of firing rate changes.** Custom software written in Matlab was used to facilitate manual annotation of mouse behaviour from videotaped recording sessions. Annotations were made using side- and top-view videos played simultaneously. A total of ~1,000 10 min videos were carefully analysed on a frame-by-frame basis. The behavioural results were then correlated with the electrophysiology to obtain histograms of firing rates during various behavioural episodes. Firing rates for each unit were averaged in 0.5 s bins, and the mean firing rate during each behavioural episode (for example, 'Investigation') was compared to the baseline firing rate (that is, before introduction of the test animals) using Kruskal–Wallis one-way analysis of variance by

ranks (with *P* value 0.01), followed by a pairwise test for significance with Tukey–Kramer correction for multiple comparisons, to determine whether there was any statistically significant change in activity during a given episode. If the same stimulus was tested multiple times, only repeatable responses were regarded as positive.

**ChR2 viral activation.** The Cre-inducible EF1α::ChR2–EYFP construct was the gift of K. Deisseroth and was described earlier<sup>46</sup>. Because ChR2 is a membrane protein expressed mainly in axons, we co-injected an AAV2 CMV::LacZ virus to facilitate the quantification and anatomic localization of infected cells. AAV2 CMV::CRE and AAV2 CMV::LacZ viruses were purchased from Vectorbiolabs. AAV2 CRE inducible EF1α::ChR2–EYFP virus was prepared by the Harvard Vector Core Facility. The AAV2-ChR2, AAV2-CRE and AAV2-LacZ viruses were mixed in a 4:2:1 volume ratio to reach a similar final titer ( $8 \times 10^{11}$  pfu/mL). A total of 0.15 µl of the mixed virus suspension (approximately  $1.2 \times 10^8$  particles) was injected unilaterally over a period of 5 min using a fine glass capillary (Nanoject II, Drummond Scientific). After injection, a 24 gauge cannula (Plastics One) was inserted and secured to a depth of approximately 0.6 mm above the target region (Metabond, Parkell). After 2 weeks recovery, and on test days, a 200 µm multimode optical fibre (Thorlabs) was inserted into the cannula and secured with an internal cannula adaptor and a cap (Plastics One). The tip of the fibre was cut flat to the bottom of the implanted cannula. Blue (473 nm) light was delivered in 20 ms pulses at 20 Hz, at final output powers ranging from 1 to 4 mW mm<sup>-2</sup> (CrystalLaser). Each light stimulation episode lasted from 2 to 20 s, depending on the behavioural responses. Initial tests using various frequencies indicated that 10–15 Hz was the minimal frequency necessary to induce a behavioural response. All animals were tested twice with 1 to 6 days between tests. Light-induced attack typically includes the following steps: the stimulated animal approaches the intruder from a distance, bites the intruder's back repeatedly, then either stops abruptly upon the cessation of light stimulation and moves away, or stops biting gradually after several rounds of attack. Light-induced escape behaviour typically includes the following steps: the stimulated animal makes a quick movement towards the corner of the arena. If the animal is engaged in other behaviours such as fighting or mating, it stops those behaviours and moves to a corner of the cage. Typically, the animal will stay in the corner and maintain the same posture for the remainder of the stimulation period.

One hour before sacrificing the animal, a train of light (10 s on and 10 s off, 20 ms, 20 Hz × 20) was delivered to induce Fos expression in the absence of any target animal. A total of 28 animals were implanted and tested. Twenty seven animals were processed for histological analysis and were included in the scatter plot of Fig. 4p. To quantify the extent of infection, we counted all the LacZ<sup>+</sup> cells in various regions and calculated the number of LacZ<sup>+</sup> cells in VMHvl posterior, VMHvl anterior, VMHdm + VMHc, LH and TU for each section. Fluorescent Nissl or NeuN staining was used to determine the boundaries of different VMH subdivisions. In cases where the boundary was hard to determine precisely, we delineated VMHvl as extending from the ventral pole of VMH approximately 1/3 of the way along the dorso-ventral and medio-lateral axes.

**GluCl viral inactivation.** Animals in the experimental group (*n* = 33) were stereotaxically injected bilaterally with a total of 0.9 µl AAV2-GluClα and AAV2-GluClβ, each under the control of the CAG promoter-enhancer, in a 1:1 mixture (approximately 10<sup>9</sup> particles), using a glass capillary attached to an auto nanolitre injector (Drummond). The viral constructs have been described previously<sup>28</sup>. One control group received no surgery (*n* = 12), a second and third control group received either saline (*n* = 6) or AAV2-GluClβ (*n* = 12) during the surgery. After 2 weeks of recovery, the aggression level of the animal was evaluated using a 10 min resident-intruder assay three times on different days. After the third test, a 1% sterile solution of Ivermectin (Phoenectin, AmTech) was injected intraperitoneally at 5 mg kg<sup>-1</sup> animal body weight. The animal were then tested again 24 h and 8 days later. The effect of IVM typically wears off completely by 8 days<sup>28</sup> and any behavioural change is expected to be reversed at that time point. The mating test was performed using a similar procedure, except that a receptive female mouse was used as the stimulus animal. The rotarod assay was performed as described previously<sup>47</sup>. The animal was exposed to a 10 min resident-intruder assay 1 h before sacrifice to induce Fos expression. The brains were then harvested for histological analysis.

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