A juvenile mouse pheromone inhibits sexual behaviour through the vomeronasal system

David M. Ferrero1, Lisa M. Moeller2, Takuya Osakada3, Nao Horio3, Qian Li4, Dheeraj S. Roy1, Annika Cichy2, Marc Spehr2, Kazushige Touhara2,4 & Stephen D. Liberles1

Animals display a repertoire of different social behaviours. Appropriate behavioural responses depend on sensory input received during social interactions. In mice, social behaviour is driven by pheromones, chemical signals that encode information related to age, sex and physiological state1. However, although mice show different social behaviours towards adults, juveniles and neonates, sensory cues that enable specific recognition of juvenile mice are unknown. Here we describe a juvenile pheromone produced by young mice before puberty, termed exocrine-gland secreting peptide 22 (ESP22). ESP22 is secreted from the lacrimal gland and released into tears of 2- to 3-week-old mice. Upon detection, ESP22 activates high-affinity sensory neurons in the vomeronasal organ, and downstream limbic neurons in the medial amygdala. Recombinant ESP22, painted on mice, exerts a powerful inhibitory effect on adult male mating behaviour, which is abolished in knockout mice lacking TRPC2, a key signalling component of the vomeronasal organ3,5. Furthermore, knockout of TRPC2 or loss of ESP22 production results in increased sexual behaviour of adult males towards juveniles, and sexual responses towards ESP22-deficient juveniles are suppressed by ESP22 painting. Thus, we describe a pheromone of sexually immature mice that controls an innate social behaviour, a response pathway through the accessory olfactory system and a new role for vomeronasal organ signalling in inhibiting sexual behaviour towards young. These findings provide a molecular framework for understanding how a sensory system can regulate behaviour.

We developed a genome-based strategy for identifying additional mouse pheromones (Fig. 1a). Chemicals that function as pheromones include urinary volatiles, steroid derivatives and proteins secreted into bodily fluids such as urine, tears and saliva6–7. Several protein pheromones are encoded by large, rapidly evolving gene families, but most pheromone homologues encoded by the mouse genome are of unknown function8–10. We constructed quantitative PCR (qPCR) primers to detect expression of protein pheromones and their homologues, including exocrine gland-secreting peptides (ESPs), androgen-binding proteins (ABPs), major urinary proteins and other lipocalins. Expression levels were quantified in complementary DNA (cDNA) derived from various pheromone-producing tissues obtained from mice of different sexes, ages and physiological states.

Using this strategy, we identified several peptides with striking age-dependent production in the extraorbital lacrimal gland, including ESP22 produced by juveniles, ESP15 and ESP16 produced by adults of both sexes and A2BP27 produced by neonates (Fig. 1b and Extended Data Fig. 1a). We also identified male-enriched peptides of unknown function, including ESP24 and various ABPs. Interestingly, sexually dimorphic production of ESP24 and the male pheromone ESP1 was similar (~500-fold male-enriched), but occurred in different mouse strains (Extended Data Fig. 2b).

Because juvenile pheromones are unknown, we performed additional studies of ESP22. ESP22 was maximally expressed in lacrimal gland between 2 and 3 weeks of age, and decreased sharply after 4 weeks of age, near puberty (Fig. 1b). Quantitative analysis indicated ESP22 expression in lacrimal gland to be similar in male and female juveniles, and approximately 50-fold higher in juveniles than adults (Extended Data Fig. 2d). ESP22 expression was not detected in CDNA derived from 16 other mouse tissues, including other exocrine glands, internal organs and sensory epithelia (Fig. 1c and Extended Data Fig. 2f). In contrast, ABP27 expression was detected in adult salivary gland as well as neonatal lacrimal gland (Extended Data Fig. 2e).

Next, we identified lacrimal gland cell types that expressed ESP22 and other pheromone homologues using RNA in situ hybridization. We found that ESP22 is produced by a subset of lacrimal secretory cells, termed acinar cells (Extended Data Fig. 1c), which release contents into tears, a source of mouse pheromones11. ESP22 expression was detected in juvenile but not adult acinar cells, whereas ESP24 expression was detected only in adult male acinar cells (Fig. 1d). Furthermore, ESP22 was not expressed in castrated and ovariectomized adults, suggesting sex-hormone-independent ESP22 gene regulation (Extended Data Fig. 1b).

To test whether ESP22 protein was secreted into tears by acinar cells, we generated and affinity-purified a polyclonal anti-ESP22 antibody. Western blot analysis using this antibody identified a protein of expected mass (approximately 10 kDa) that was enriched in juvenile tears (Fig. 1e). Concentrations of this protein (3–5 ng μl−1 in juvenile tears, or 300–500 nM) were determined using a standard curve of recombinant ESP22 (Extended Data Fig. 3). Mass spectrometry identified ESP22-derived tryptic peptides in tears of juveniles but not adults, indicating greater than 100-fold enrichment (Fig. 1f), and showed the primary structure of mature ESP22 (amino acids 23–111, Extended Data Fig. 4). Together, these findings indicate that ESP22 is a lacrimal peptide secreted into tears of juvenile mice.

Next, we asked whether ESP22 was detected by the mouse olfactory system. Other protein pheromones, including ESP1, activate basal vomeronasal organ (VNO) sensory neurons11, so we examined electrophysiological responses to ESP22 in the VNO. Recombinant ESP22 was prepared as a fusion protein with maltose binding protein (MBP), which enhanced solubility12. Electrovomeronasogram (EVG) recordings indicated that recombinant ESP22 (200 nM) evoked a negative field potential in the VNO (Fig. 2a), with a sensitivity matching ESP1 responses previously reported with this technique13–14. MBP was not similarly detected, although small EVG responses to MBP were observed at higher concentrations (data not shown). High-affinity responses to ESP22 in the VNO required the iron channel TRPC2 (Fig. 2a), and were not observed in electroolfactogram (EOG) recordings of the main olfactory epithelium (Fig. 2b), which is also important for pheromone-driven social behaviours15–17.

Next, we used extracellular loose-seal recordings to examine ESP22 responses in individual VNO sensory neurons. ESP22 evoked robust and repetitive discharge patterns in 1.3% of basal VNO sensory neurons (5/383), consistent with detection by one or a few VNO receptors.
ESP22 is secreted into juvenile tear fluid. a, Strategy to identify mouse pheromones. Abp-b27 is also known as Sgb2b27. Abp-b2 is also known as Sgb2b2. b, Age-dependent gene expression in lacrimal gland (LG) determined by qPCR (n = 4−12, mean ± s.e.m.). c, Esp22 expression in juvenile and adult tissues determined by PCR with reverse transcription (RT-PCR), olfactory epithelium (OE), olfactory bulb (OB), harderian gland (HG), submaxillary gland (SMG), parotid gland (PG), sublingual gland (SLG). d, Age- and sex-dependent Esp expression in lacrimal gland determined by in situ hybridization. Scale bar, 100 μm. e, Western blot analysis of tears using anti-ESP22 antibody. f, Mass spectrometry analysis of an ESP22-derived tryptic peptide (GIVFNTIK) from tears.

Threshold ESP22 responses observed by single unit extracellular recordings (Fig. 2d) occurred at similar concentrations (20 PM) to threshold ESP1 responses previously measured using genetically encoded calcium indicators18. Most neurons responsive to ESP22 were activated by juvenile tears but not by MBP or adult tears (6/11, Fig. 2e and Extended Data Fig. 5), with neuron viability verified by K+-mediated depolarization. High-affinity ESP22 responses were also recorded in 1−2% of VNO sensory neurons using current-clamp recording techniques and single-neuron calcium imaging (data not shown).

We next identified limbic neurons activated by ESP22 exposure using immunohistochemistry for the neural activity marker cFos in cryosections of adult male mouse brains. ESP22 and juvenile tears (Fig. 2f and Extended Data Fig. 6) induced cFos expression in the medial amygdala (MeA), a region that receives VNO input by way of the accessory olfactory bulb13,19. cFos responses were not observed in Trpc2−/− mice (Fig. 2f), or in other amygdala regions that receive olfactory input (Extended Data Fig. 6). cFos responses were enriched in the postero-ventral MeA (Fig. 2g), which sends projections to hypothalamic areas that control defensive and reproductive responses20,21.

These findings indicate ESP22 to be a juvenile chemosignal that activates a VNO response pathway. However, a role for the VNO in regulating adult–juvenile social interactions is unknown. Trpc2−/− mice provide a valuable tool for VNO loss-of-function studies, and show severe deficits in sex recognition2. Here, we introduced Trpc2+/+ or Trpc2−/− males to juveniles and monitored social behaviour.

Surprisingly, we observed that Trpc2−/− mice displayed a striking increase in sexual behaviour towards prepubescent females (Supplementary Videos 1 and 2). Although Trpc2+/+ mice showed rare mounting attempts towards juvenile females, Trpc2−/− mice showed vigorous mounting behaviour quantified as increases in mean mounting attempts and the percentage of animals mounting in 3 and 10 min, as well as decreases in mounting latency and intermount interval (Fig. 3 and Extended Data Fig. 7). A similar percentage of Trpc2+/+ males showed mounting behaviour by 30 min, but these mounts were rare and did not increase in frequency during the trial duration (Fig. 3d and Extended Data Fig. 7b). In contrast, the sexual behaviour of Trpc2−/− and Trpc2−/− males towards adult females was similar, as reported previously2,2. Trpc2−/−
mice showed sexual behaviour towards juvenile females even when presented simultaneously with adult oestrous females (Extended Data Fig. 7c), and also showed increased sexual behaviour towards juvenile males (Extended Data Fig. 8). On the basis of these findings, VNO signalling normally prevents mating advances towards young, and one mechanism probably involves detection of chemosignals released from juvenile animals.

We reasoned that ESP22 is an excellent candidate to function as such a mating inhibitor based on the timing of its expression, the role of another ESP as a pheromone and the ability of ESP22 to activate both VNO sensory neurons and central limbic regions. ESP22 is juvenile-enriched in several strains of mice, but we identified two strains (C3H and CBA) that lacked juvenile ESP22 expression (Fig. 4a). These mouse strains provided valuable tools for controlling ESP22 levels during social interactions, and we observed increased sexual behaviour of wild-type males towards C3H and CBA juveniles (Fig. 4b).

We asked whether painting recombinant ESP22 onto C3H juveniles blocked male sexual approaches. We observed that males displayed similar levels of sexual behaviour towards unainted, ESP6-painted and MBP-painted C3H juveniles (Fig. 4d). However, males showed a significant reduction in mounting attempts and an increase in mounting latency towards C3H juveniles painted with ESP22 (1 ng). Higher ESP22 amounts (10 μg) caused a striking 70-fold reduction in mounting attempts towards C3H juveniles, with most animals (10/11) failing to show a single mating attempt during the entire 30 min trial (Fig. 4c, d). A dose-dependent analysis indicated that amounts of ESP22 derived from small quantities of juvenile tears (≤200–333 nl) were sufficient for inhibition of adult male sexual behaviour (Fig. 4e). ESP22 was not aversive, as ESP22 painting did not affect social interaction time (Extended

Figure 3 | Trpc2−/− males display increased sexual behaviour towards juveniles. a, Raster plots depicting individual mounting displays of adult Trpc2−/− and Trpc2−/− males (n = 12) towards female juveniles (C57BL/6, 2–3 weeks old) during behavioural testing (30 min). Each tick indicates onset of one mount. b, Quantitative analysis of parameters associated with sexual behaviour towards juvenile and adult females shown by Trpc2−/− and Trpc2−/− males (mean ± s.e.m., *P < 0.05, **P < 0.01, Mann–Whitney U-test).

Figure 4 | ESP22 inhibits male sexual behaviour. a, ESP22 levels in lacrimal gland from mouse strains and ages indicated (n = 5–12, averages ± s.e.m.). b, Sexual behaviour of wild-type males towards juveniles from strains indicated (n = 11 or 12, mean ± s.e.m.). c–g, Raster plots and quantification of sexual behaviour shown by wild-type males (d–f) or Trpc2−/− males (f) towards C3H juveniles (d–f) or C57BL/6 oestrous females (g) painted with ESP6 (10 ng), ESP22 (10 μg or indicated) or MBP (4 mg) (n = 9–12, averages ± s.e.m.). Arrow depicts ESP22 concentration in C57BL/6 juvenile tears. h, Model for ESP22 signalling. *P < 0.05, **P < 0.01, Student’s one-tailed t-test (a), one-tailed (b, d, e, g) or two-way (f) analysis of variance (ANOVA) followed by Tukey’s honestly significant difference (HSD) post hoc tests.
Data Fig. 9). ESP22 also did not inhibit sexual behaviour of Trpc2<sup>−/−</sup> males, consistent with a role for vomeronasal circuits in mediating ESP22 responses (Fig. 4f). Interestingly, Trpc2<sup>−/−</sup> males did not show further increases in sexual behaviour towards C3H juveniles (Fig. 4f), suggesting that C3H juveniles do not release other VNO-dependent mating inhibitors. However, ESP22 did inhibit the sexual behaviour of C3H adult males, which presumably have encountered little or no ESP22 previously (Extended Data Fig. 10). Finally, recombinant ESP22 also decreased sexual behaviour towards adult females in oestrous (Fig. 4g). Lower levels of sexual behaviour persisted towards ESP22-painted oestrous females, suggesting that oestrous females release other signals that counteract ESP22. On the basis of these findings, ESP22 is a juvenile pheromone that blocks sexual behaviour through the vomeronasal system (Fig. 4h).

Behavioural responses to ESP22 differ from responses to other VNO activators, such as pheromones and predator odours that trigger mating, aggression and fear<sup>12,13,22,23</sup>. These findings are consistent with the existence of parallel subcircuits of the accessory olfactory system, which selectively channel sensory inputs to enable proper selection of a behavioural display<sup>20</sup>. Identifying a collection of VNO activators that regulate different instinctive behaviours provides a valuable toolbox to understand how a sensory system controls behaviour.

METHODS SUMMARY

All animal procedures were in compliance with institutional animal care and use committee guidelines. Full details of experimental procedures for qPCR analysis, RNA in situ hybridization, western blot analysis, mass spectrometry; recombinant proteins, electrophysiology, cFos staining, behaviour analysis and statistical analysis are provided in Methods.

Online Content Any additional Methods, Extended Data display items and Source Data are available in the online version of the paper; references unique to these sections appear only in the online paper.

Received 7 December 2012; accepted 16 August 2013.

Published online 2 October 2013.

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Supplementary Information is available in the online version of the paper.

Acknowledgements We thank M. Albers and S. R. Datta for reading the manuscript, J. Yang, C. Mark Fletcher and Y. Tachie-Baffour for experimental assistance, and the Taplin Mass Spectrometry Facility for mass spectrometry analysis. This work was supported by a grant from the National Institutes of Health (to S.D.L., award number R01 DAO10155) and in part by a Grant-in-Aid for Young Scientists (S) from the Japan Society for the Promotion of Science, and by ERATO Touhara Chemosensory Signal Project from the Japan Science and Technology Agency (to K.T.) N.H. is supported by a Grant-in-Aid for JSPS Fellows, M.S. is a Lichtenberg-Professor of the Volkswagen Foundation and D.M.F. is supported by a Boehringer Ingelheim Fonds PhD Fellowship.

Author Contributions D.M.F., S.D.L., M.S. and K.T. conceived the project, designed the experiments and wrote the manuscript. D.M.F. performed molecular biology, biochemistry and behaviour experiments. D.S.R. and Q.L. performed in situ hybridization analysis, L.M.M., A.D. and T.O. N.H. performed electrophysiological analysis, T.O. performed cFos analysis.

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Electrophysiology. EVG and extracellular recordings were performed as described previously with minor modifications. To prevent dialysis of intracellular components, action-potential-driven capacitive currents were recorded in ‘loose-seal’ cell-attached configuration (seal resistance 30–150 MΩ) from vomeronasal sensory neuron somata located deep in the sensory epithelium’s basal layer close to the basement membrane. Spikes were analysed using Igor Pro functions (SpAcAn, G. Dugue and C. Rousseau). Inter-stimulus intervals were 30 s. Neuronal responses were classified according the following criteria: (1) discharge was time-locked to stimulus presentation (responses occurred during and/or up to 3 s after stimulus onset); (2) spike patterns clearly deviated from previous baseline activity (frequency histograms (1 s bin width) were calculated over repeated trials and responses were evaluated according to an $\Delta t = 100 \text{ms}$ (baseline) criterion). MBP and ESP22 evoked TRPC2-independent EVG and EOG responses at approximately 100-fold higher concentrations (data not shown).

**Cfos staining.** Sexually naive males (Japan SLC, C57BL/6, 9–11 weeks old) were housed individually (17 cm × 25 cm Plexiglas test chambers, 12 h light/12 h dark cycle). Stimuli included ESP22 (250 μg), tear fluid (containing 50 μg protein) or MBP (200 μg) in 20 μL Tri-HCl (pH 7.5, 100 μL) transduced onto a piece of cotton (30 mg) and dried in a Speed Vac (3 h). High concentrations of ESP22 were necessarily used for Cfos studies, as this non-volatile stimulus is poorly investigated when presented in isolation. Stimuli were placed on bedding during the dark phase (90 min), and all mice were observed to investigate the stimulus during testing. Mice were then anaesthetized with pentobarbital sodium and perfused quickly. Brains were removed and post-fixed in 4% paraformaldehyde in PBS (3 h, 4 °C) and cryoprotected in 15 and 30% sucrose solutions in PBS (4 °C). Immunochemistry and quantification of cFos-positive nuclei were performed as described previously. MEA regions were defined using annotated anatomical landmarks (Extended Data Fig. 6), comparison with a reference image (Bregma - 1.90 mm), and Lshad staining (Extended Data Fig. 7).

**Behaviour.** Before experiments, sexually naive adult males (2–4 months old, C57BL/6) were maintained under a reverse light cycle for 2 weeks and individually housed for at least 24 h. Behavioural testing occurred in the home cage with the food tray removed more than 3 h after onset of dark phase. A sexually naive male or female (17- to 18-day-old juvenile or adult in oestrus) was introduced to the male, and interaction behaviour was recorded for 30 min using a digital camcorder compatible with low light conditions (Sony). For some experiments, females were painted with swabbing stimul (100 μL) on the back (50 μL), head (25 μL) and anogenital region 24 h before testing. Mounting behaviour was defined when males used both forepaws to copulate onto a female for copulation, and parameters associated with mounting behaviour were analysed using Matlab (Mathworks). In rare cases (<5%), juvenile pups showed stimulus-independent escape behaviour and were excluded from analysis. Animals were randomly assigned to different testing conditions. For Fig. 4c-e, quantification was performed blind to experimental conditions.

**Statistical analyses.** All samples represent biological replicates. Sample sizes for biochemistry, electrophysiology, cFos and behaviour met or exceeded the standards in the field. In Fig. 1b, sample sizes (n) for data points reading left to right are as follows: 12, 7, 7, 8, 6, 13 for ESP22; 12, 7, 8, 6, 12 for ESP4; 8, 5, 7, 6, 12 for ESP15; 8, 10, 8, 10, 8, 4 for ESP16; 10, 8, 6, 10, 6, 5 for ESP22. In Fig. 4a, sample sizes reading left to right are 13, 10, 6, 6, 5, 7 and 5. In Fig. 4b, sample sizes reading left to right are 19, 9, 9, 9 in Fig. 4d, sample sizes reading left to right are 14, 12, 11, 9 and 9 in Fig. 4e, sample sizes reading left to right are 12, 12, 12, 11, 11 and 11 in Fig. 4f, and sample sizes reading left to right are 12, 12, 11, 11 and 11. Categorical data were analysed by a Fisher’s exact test. Other reported P values were calculated using a one-tailed Student’s t-test (qPCR, Cfos, Mann–Whitney U-tests (mouse behaviour) or one- or two-way ANOVA followed by Tukey’s HSD post hoc tests (mouse behaviour), as indicated in the figure legends.
Extended Data Figure 1 | RNA in situ hybridization to characterize expression of Esp genes in the lacrimal gland. a–c, Colorimetric analysis in tissue from animals indicated using cRNA riboprobes for (a) Esp15 and (b) Esp22, and two-colour fluorescence analysis (c) in juvenile lacrimal gland with cRNA riboprobes for Esp22 (red) and a marker for acinal secretory cells, Rab3D (green). cRNA riboprobes for Esp15 are expected to cross-hybridize with Esp16 mRNA. Some images used in b are identical to panels in Fig. 1d, and are included for reference. Dashed boxes (c) indicate regions magnified below. Arrows, acinar cells; arrowheads, ductal cells; scale bars, 100 μm (a, b, c top), 20 μm (c bottom).
Extended Data Figure 2 | qPCR analysis of gene expression. a, *Esp22* qPCR primers specifically detect a plasmid containing cloned *Esp22*, but not plasmids containing other *Esp* genes with greater than 60% identity to *Esp22*. b–f, cDNA was derived from lacrimal gland (b–e), submaxillary gland (e) or other tissues (f) of animals indicated. In f, abundance is calculated by normalization to amounts of *Gapdh*. C57BL/6 mice were used (b–d) unless otherwise indicated (b). Experiments where sex is not indicated involved equal numbers of males and females; olfactory epithelium (OE), olfactory bulb (OB), hardarian gland (HG), submaxillary gland (SMG), parotid gland (PG), sublingual gland (SLG) (*n* = 6–12, averages ± s.e.m., **P < 0.01, two-way ANOVA followed by Tukey’s HSD post hoc tests).
Extended Data Figure 3 | Quantification of protein concentrations in tear fluid by western blot analysis using an anti-ESP22 antibody. a, A standard curve based on signal intensity was generated using different concentrations of recombinant ESP22 (a, left panel; b). The arrow indicates the intensity level of the band in the juvenile tear sample (a, right panel). c, Entire western blot analysis of tear fluid using anti-ESP22 antibody.
Extended Data Figure 4 | ESP22-derived tryptic peptides identified by mass spectrometry. a, The amino-acid sequence of immature ESP22 is depicted, along with a predicted signal peptide and the epitope used for antibody generation. Four tryptic peptides were identified by mass spectrometry (highlighted in red), including one peptide containing the first amino acid after the predicted signal sequence and another containing the encoded carboxy (C)-terminal residue. Trypsin does not efficiently cleave amino (N)-terminal lysines or arginines, consistent with R23 being the first amino acid in mature ESP22. b, Mass spectrum of an high-performance liquid chromatography fraction of juvenile tear fluid showing the ESP22-derived tryptic peptide GIVFNTIK, with sequence identity confirmed by tandem mass spectrometry analysis.
Extended Data Figure 5 | Electrophysiological responses to ESP22 in VNO sensory neurons. 

a, Single-unit extracellular loose-seal recording from a single VNO sensory neuron repeatedly exposed to different stimuli indicates reproducibility of responses. 

b, The percentage of basal VNO sensory neurons responsive to 20 pM ($n = 383$) and 2 nM ($n = 749$) ESP22.
Extended Data Figure 6 | cFos responses to ESP22 in the amygdala.
a, ESP22 and juvenile tear fluid, but not MBP, induce cFos expression in the postero-ventral MeA. Dashed lines and arrows indicate boundaries of MeA regions. b, Similar responses were not observed in other amygdala nuclei that receive olfactory input, including the postero-medial cortical amygdala (PMCo), anterior cortical amygdala (CoA) and postero-lateral cortical amygdala (PLCo) (mean ± s.e.m., n = 3).
Extended Data Figure 7 | Trpc2<sup>−/−</sup> males show increased sexual behaviour towards wild-type juveniles.  

a, b. Histograms of mounts by minute of social interaction and intermount intervals shown towards juveniles by Trpc2<sup>+/+</sup> and Trpc2<sup>−/−</sup> males (sum, n = 12). Inset depicts average intermount intervals (mean ± s.e.m., *P < 0.05, **P < 0.01, Mann–Whitney U-test). c. Analysis of adult male sexual behaviour during simultaneous interaction with juvenile and adult oestrous females. Trpc2<sup>+/+</sup> and Trpc2<sup>−/−</sup> males show similar amounts of sexual behaviour towards adult oestrous females, but Trpc2<sup>−/−</sup> males show increased sexual behaviour towards juveniles (n = 10, averages ± s.e.m., *P < 0.05, **P < 0.01, one-way multivariate ANOVA).
Extended Data Figure 8 | Trpc2<sup>−/−</sup> males show sexual behaviour towards juvenile males. **a**, Raster plots depicting individual mounting displays of adult Trpc2<sup>+/+</sup> and Trpc2<sup>−/−</sup> males towards juvenile males (C57BL/6, postnatal day 17) during social interaction (30 min). Each tick indicates onset of one mount. **b**, Quantitative analysis of parameters associated with sexual behaviour towards juvenile males shown by Trpc2<sup>+/+</sup> and Trpc2<sup>−/−</sup> males (<sup>n</sup> = 11 or 12, averages ± s.e.m., *P < 0.05, **P < 0.01, Mann–Whitney U-test).
Extended Data Figure 9 | ESP22 did not decrease social investigation time.
Wild-type C57BL/6 males were introduced to C3H juvenile females painted with stimuli indicated. Social investigation time of the male was recorded as time spent with the nose in direct contact with the female. These data were extracted from the same experiments reported in Fig. 4c, d, with additional experiments involving TMT (100 μl, 155 mM, n = 11 or 12, averages ± s.e.m., **p < 0.01, one-way ANOVA followed by Tukey’s HSD post hoc tests).
Extended Data Figure 10 | ESP22 (10 μg) inhibits sexual behaviour of C3H males. a, Raster plots of sexual behaviour shown by C3H males towards C3H juvenile females (postnatal day 17) painted with indicated stimuli (30 min social interaction). Each tick indicates onset of one mount. b, Quantitative analysis of parameters associated with sexual behaviour towards juvenile females shown by C3H males (n = 11, averages ± s.e.m., *P < 0.05, **P < 0.01, one-way ANOVA followed by Tukey’s HSD post hoc tests).