Non-redundant coding of aversive odours in the main olfactory pathway

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Many species are critically dependent on olfaction for survival. In the main olfactory system of mammals, odours are detected by sensory neurons that express a large repertoire of canonical odorant receptors and a much smaller repertoire of trace amine-associated receptors (TAARs)1–4. Odours are encoded in a combinatorial fashion across glomeruli in the main olfactory bulb, with each glomerulus corresponding to a specific receptor5–7. The degree to which individual receptor genes contribute to odour perception is unclear. Here we show that genetic deletion of the olfactory Taar gene family, or even a single Taar gene (Taar4), eliminates the aversion that mice display to low concentrations of volatile amines and to the odour of predator urine. Our findings identify a role for the TAARs in olfaction, namely, in the high-sensitivity detection of innately aversive odours. In addition, our data reveal that aversive amines are represented in a non-redundant fashion, and that individual main olfactory receptor genes can contribute substantially to odour perception.

There are 15 Taar genes in the mouse, 14 of which are expressed in the main olfactory pathway and serve a chemosensory function2. All of the Taar genes are located in a single gene cluster on mouse chromosome 10 with no interspersed genes2 (Fig. 1a). To determine how the TAARs contribute to odour perception, we used in vivo transgenic recombination to generate a mouse strain (ΔTaar-9) in which all 14 olfactory Taar genes (from Taar2 through to Taar9) are deleted (Fig. 1a). Homozygous ΔTaar-9 mice breed normally, show no apparent health issues or behavioural deficits, and exhibit the same weight and locomotor activity as wild-type littermates (Supplementary Fig. 1).

To examine the functional consequences of removing the TAARs, we performed in vivo optical imaging of odour-evoked responses from glomeruli in the olfactory bulbs of anaesthetized mice. This was done by crossing ΔTaar-9 mice to ‘OMP-spH’ mice in which the genetically encoded activity reporter synaptophluorin (spH) is expressed from the olfactory marker protein (Omp) locus and thus in all glomeruli10. We compared odorant responses in ΔTaar-9 homozygous mice with those in control mice that retain two intact Taar gene clusters.

Glomeruli in the olfactory bulb receive axonal inputs from sensory neurons that express the same odorant receptor or Taar gene, and sensory neurons that express a majority of the TAARs project to a cluster of glomeruli in the dorsal-caudal olfactory bulb11,12 (Fig. 1b). Consistent with our previous observations, low concentrations of structurally diverse amines robustly activated a small subset of dorsal glomeruli in control mice (Fig. 1c–e), and glomeruli with specific response profiles could be recognized across individual animals5,13. All of these high-sensitivity amine responses were abolished in homozygous ΔTaar-9 mice, while responses to non-amine odors persisted (Fig. 1c–e). These results demonstrate that all of the high-sensitivity amine responses derive from glomeruli corresponding to Taar genes.

Urine is a rich source of amines that could be exploited for intra- and interspecies chemical communication. It has been reported that the urine of predator cats contains high concentrations of β-phenylethylamine (PEA), an odorant that specifically activates Taar4 in cultured cells6,14 and Taar4-expressing olfactory sensory neurons15. Using our in vivo imaging method, we observe that TAAR4 glomeruli are activated by PEA and by the volatiles from the urine of an adult puma (Fig. 1e). Responses to puma urine in the dorsal bulb were abolished in homozygous ΔTaar-9 mice (Fig. 1e). Therefore, the most sensitive amine/urine-responsive glomeruli in the dorsal bulb correspond to the TAARs. We note that it is possible that glomeruli outside our imaging area (in the ventral bulb) respond to amines at the concentrations tested.

Many amines share a characteristic, offensive odour. In fact, two primary amines, PEA and isopentylamine (IPA), have been reported to elicit innate aversion in mice2,15,16. We therefore tested whether the amines that activate TAAR glomeruli are aversive and whether the TAARs mediate this aversion. Wild-type, heterozygous and homozygous ΔTaar-9 littermates (n = 504 mice) were tested in a two-chamber place preference assay where they could choose to occupy an odorized or a non-odorized compartment (Fig. 2a). Odorants were diluted in water and presented in partially enclosed dishes so that mice could smell the stimuli without direct contact with the odour source. Under these conditions, all mice strongly avoided the red fox15–17. In contrast, negative control odours, water, ethyl vanillin and peanut butter oil, did not elicit aversion (Fig. 2b).

Using this assay, we observe that wild-type and heterozygous ΔTaar-9 mice exhibit aversion to structurally diverse amines including PEA, IPA, N-methylpiperidine (NMP) and cadaverine (CAD) when tested at multiple concentrations. Notably, the aversion elicited by low concentrations of amines was TAAR-dependent as it was abolished in homozygous ΔTaar-9 mice (Fig. 2c). We note that concentrated amines (100% PEA and 10% IPA), which are highly pungent to humans, were aversive to mice regardless of genotype (Fig. 2c). Thus, mice are averse to certain amines, and aversion to low concentrations of amines is dependent on the TAARs.

To determine whether the TAARs are required for aversion to natural stimuli that contain ethologically relevant concentrations of amines, we tested for avoidance of the odour of predator cat urine, which is enriched in PEA14. Wild-type and heterozygous ΔTaar-9 mice were averse to puma urine. The aversion to urine was abolished in homozygous ΔTaar-9 mice, which lack the olfactory TAARs (Fig. 2b, c). Taken together, the data indicate that the TAAR family is required for innate aversive responses to volatile amines at naturally occurring concentrations.

Next, we examined the functional impact of removing a single Taar gene from the receptor repertoire. To do this, we used a gene-targeted mouse strain (ΔTaar-4-YFP) in which the TAAR4 coding sequence is replaced with that of yellow fluorescent protein (YFP)14 (Fig. 1a). TAAR4 responds selectively and robustly to PEA and urinary volatiles from predator cats when expressed in cultured cells and in native olfactory sensory neurons6,13,14. Using our in vivo imaging assay, we find that low concentrations of PEA or volatiles from puma urine
Figure 1 | Deletion of all olfactory Taar genes abolishes high-sensitivity amine and predator odor response in the dorsal olfactory bulb. a, Diagram of the Taar gene cluster and targeted alleles. Olfactory Taar (black) and non-olfactory Taar1 (white) are shown (polygons reflect gene orientation). T4-RFP: the tau::mCherry marker (red) is inserted downstream of Taar4. ΔT4-YFP: the Taar4 coding sequence is replaced with Venus YFP (yellow). ΔT2-9: all olfactory TAAR genes are deleted and the Taar9 coding sequence is replaced with the odorant receptor S50 or CFp (cyan fluorescent protein; grey box; green triangle indicates loxp site; see Methods). WT, wild type. b, Dorsal view of the olfactory bulbs from a double heterozygous ΔT4-YFP;OMP-spH mouse in which all glomeruli express spH (green) and TAAR glomeruli are labelled (yellow). Anterior is up. Scale bar, 500 μm. c, Imaging of odour evoked activity in the olfactory bulbs of a heterozygous T4-RFP mouse (left panels) and a homozygous ΔT2-9 mouse (right panels). Top panels show resting spH fluorescence. Pseudocoloured panels show fluorescence changes in response to β-phenylethylamine (PEA; 2 mM vapour concentration, v.c.), isopentylamine (IPA; 24 nM v.c.) and cadaverine (CAD; 550 nM v.c.). Data are displayed as ΔF in arbitrary units (see Methods). Maximum response is 8.3% ΔF/F. d, Maximum response projection for amines or non-amines in a heterozygous T4-RFP mouse (left) and a homozygous ΔT2-9 mouse (right). Amine stimuli: PEA (2 mM v.c.), IPA (24 nM v.c.), CAD (550 nM v.c.), N-methylpiperidine (NMP; 7.5 μM v.c.) and trimethylamine (4 μM v.c.). Non-amine stimuli: propyl acetate (19 μM v.c.), phenetole (860 nM v.c.), 2-heptanone (5.3 μM v.c.) and isopropyl tiglate (1.5 μM v.c.). Maximum response, 7.4% ΔF/F. e, Responses to urinary volatiles in heterozygous T4-RFP (top) and homozygous ΔT2-9 (bottom) mice. Locations of red fluorescent protein (RFP)-labelled TAAR4 glomeruli (top left) are indicated (arrowheads). SpH fluorescence is shown in a ΔT2-9 mouse (bottom left). Pseudocoloured panels show responses to puma urine (undiluted headspace vapour), NMP (150 nM v.c.) and propyl acetate (19 μM v.c.). Maximum response, 5.4% ΔF/F.

Figure 2 | Deletion of all olfactory Taar genes abolishes aversion to low concentrations of structurally diverse amines and predator urine. a, Video images of the behavioural testing chamber. Mice move between odorized (top) and non-odorized (bottom) compartments separated by a curtain. Blue traces show the location of the mouse during single three-minute trials. Panels represent three stages of one experiment—habitation to the chamber, exposure to water, and exposure to a test stimulus (odour or water). Aversion index = Timeodour – Timewater. b, Aversion index values for wild-type, heterozygous and homozygous ΔT2-9 cluster deletion mice (key in c). Odorants are 2% trimethylthiazoline (TMT), 0.5% ethyl vanillin (EV) and undiluted peanut butter oil (PB oil). Data are mean ± s.e. (n = 20–25 mice per genotype, per odorant). c, Aversion index values for PEA, IPA, NMP, CAD and puma urine. Concentrations are given as percentage dilution of pure odorant in water. Puma urine was undiluted. Data are mean ± s.e. (n = 20–25 mice per genotype, per odorant). Statistical significances for pairwise comparisons are indicated: a, homozygous mice differ from wild type and heterozygous, b, homozygous mice differ from wild type (P < 0.05, generalized linear mixed model). Wild-type and heterozygous mice did not differ statistically for any odour, and the aversion response did not differ with sex (P = 0.669).
Amines with sex (did not differ statistically for any odour, and the aversion response did not differ ('a', homozygous mice differ from both wild-type and heterozygous mice.

Water. Predator urines were undiluted. Data are mean indicated with arrowheads in subsequent panels. All dorsal TAAR glomeruli are labelled in T4-RFP mice (top left panel, red) and their locations are indicated with arrowheads in subsequent panels. All dorsal TAAR glomeruli, except for those corresponding to the deleted Taar4 gene, are labelled in homozygous ΔT4-YFP mice (top right panel, yellow)11. Pseudocoloured panels show odour-evoked fluorescence changes in response to PEA (2 mM v.c.), IPA (24 mM v.c.), NMP (150 mM v.c.) and puma urine (undiluted headspace vapour). Response maps are displayed as ΔF in arbitrary units (see Methods). Maximum response, 7.5% ΔF/F. A, Aversion index values for wild-type, heterozygous and homozygous ΔT4-YFP mice. Negative values indicate avoidance. Odorants are PEA, IPA, NMP, puma urine and Canadian lynx urine. Concentrations given as percentage dilution of pure odorant in water. Predator urines were undiluted. Data are mean ± s.e. (n = 20–25 mice per genotype, per odourant). Statistical significances for pairwise comparisons: #a, homozygous mice differ from both wild-type and heterozygous mice (P < 0.05; generalized linear mixed model). Wild-type and heterozygous mice did not differ statistically for any odour, and the aversion response did not differ with sex (P = 0.639).

Figure 3 | Deletion of a single Taar gene abolishes aversion to a specific amine and to natural predator odours. a, In vivo imaging of the left caudomedial olfactory bulb in a heterozygous T4-RFP mouse and a homozygous ΔT4-YFP mouse (anterior is up, medial is right). TAAR4 glomeruli are labelled in T4-RFP mice (top left panel, red) and their locations are indicated with arrowheads in subsequent panels. All dorsal TAAR glomeruli, except for those corresponding to the deleted Taar4 gene, are labelled in homozygous ΔT4-YFP mice (top right panel, yellow). Pseudocoloured panels show odour-evoked fluorescence changes in response to PEA (2 mM v.c.), IPA (24 mM v.c.), NMP (150 mM v.c.) and puma urine (undiluted headspace vapour). Response maps are displayed as ΔF in arbitrary units (see Methods). Maximum response, 7.5% ΔF/F. A, Aversion index values for wild-type, heterozygous and homozygous ΔT4-YFP mice. Negative values indicate avoidance. Odorants are PEA, IPA, NMP, puma urine and Canadian lynx urine. Concentrations given as percentage dilution of pure odorant in water. Predator urines were undiluted. Data are mean ± s.e. (n = 20–25 mice per genotype, per odourant). Statistical significances for pairwise comparisons: #a, homozygous mice differ from both wild-type and heterozygous mice (P < 0.05; generalized linear mixed model). Wild-type and heterozygous mice did not differ statistically for any odour, and the aversion response did not differ with sex (P = 0.639).

thresholds of TAAR4 sensory neurons and glomeruli to the aversive predator cue, PEA, are the lowest so far observed in the main olfactory system13.

We note that the TAARs may not function solely as detectors of predator-derived and aversive odours. Although we show that mouse TAARS contribute to the detection and avoidance of several aversive amines, recent data indicate that TAAR5 mediates attraction to its preferred ligand, trimethylamine—a socially relevant metabolite that is enriched in male mouse urine28. However, it should be noted that this amine elicits robust aversion in rats. The TAAR repertoire is also evolutionarily retained in many vertebrate species, including humans19. Apart from their role in aversion or attraction, our view is that the TAARS are retained in many species because they are required more generally for high-sensitivity amine detection. The behavioural response to this input may be context and species specific.

It is generally thought that odour representations in the main olfactory bulb are highly distributed and redundant, with each input channel (glomerulus) making a small contribution to the representation of a given odour21. In this view, single receptor deletions should have little effect at the level of behaviour. Behavioural deficits have been induced by the genetic removal of receptors in specialized olfactory pathways.

Mutant mice lacking a subset of vomeronasal receptors, which map to the accessory olfactory bulb, display deficits in aggression and mating22. Mice lacking the gene for guanylyl cyclase D, which is expressed in sensory neurons that project to atypical necklace glomeruli, show deficits in social transmission of food preference23,24. In contrast, the TAARs are mapped to a subset of the ~2,000 typical main olfactory bulb glomeruli that are thought to represent odors in a combinatorial fashion. In spite of this, removal of even a single TAAR results in a measurable deficit in odour-guided behaviour. Our data suggest that the representations of general odours in the main olfactory system may be less redundant than previously thought, a fact that may shed light on how vertebrates retain large numbers of chemosensory receptor genes over evolutionary time.

METHODS SUMMARY

All procedures were approved by the Northwestern University Animal Care and Use Committee. Generation of the AT2-9CFP, T4-RFP and ΔT4-YFP alleles was previously described11. The TAAR cluster deletion allele AT2-9CFP was similarly generated by in vivo Cre-mediated trans-allelic recombination11,25. Imaging of odour-evoked activity from the olfactory bulbs was performed as previously described with some modifications16,17. Recordings were made from 8 to 12 week-old male and female heterozygous OMP-spH mice that were heterozygous for the T4-RFP allele (n = 13), homozygous for the AT2-9CFP allele (n = 9), or homozygous for the ΔT4-YFP allele (n = 4). Data are expressed as ΔF to account for the properties of the resting spH fluorescence18,19. Response images are averages of two or more trials, or the first trial in cases where significant adaptation was observed.

Aversion was assessed in 5–7 week-old male and female F3 mice from heterozygous crosses. A total of 594 ΔT2-9CFP and 238 ΔT4-YFP mice were used for 1,138 experiments (20–25 mice of each genotype for each odour). The assay was similar to that used in previous studies16,17 and was performed under dim red light. A curtain divided the cage into odorized (1/3) and non-odorized (2/3) chambers. After two days of habituation to the experimental protocol (with no odour), mice were re-habituated to the chamber for 3 min, exposed to a water stimulus for 3 min, and then to the test stimulus for 3 min. The location of the mouse was tracked using Limelight 3.0 software (Actimetrics). The aversion index was calculated as the difference between the times spent in the odorized chamber with an odorant as a stimulus and with water as a stimulus. Data were analysed with a generalized linear mixed model with genotype, odour and sex as fixed factors and a random-subjects factor using SPSS (IBM). Pairwise contrasts were used to compare genotypes for each odour with a least-square-differences adjustment for multiple comparisons.

Full Methods and any associated references are available in the online version of the paper.

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METHODS

Gene targeting. The TAAR cluster deletion allele AT2-9CFP was generated by Cre-mediated trans-allelic recombination in vivo25. We used two targeted alleles that introduce loxP sites into the 5’ and 3’ ends of the cluster—an 1::Taar1-loxP-IRES-td-tau::Cerulean (aT1-YFP) in which a loxP site is inserted just downstream of the Taar1 coding sequence, and Cerulean—Taar9-loxP—CFP in which the Taar9 coding sequence is replaced with that of Cerulean CFP followed by loxP. An HPRT-Cre strain (129S1/Sv-Hprt<sup>m1<sub>Cre</sub>/MmJ; Jax 004302; ref. 27) was used to mediate recombination in aT1-YFP/AT9-CFP compound heterozygotes as described25.

In vivo imaging. For glomerular imaging, mice were anaesthetized with sodium pentobarbital as described31, or with urethane (1 g kg<sup>−1</sup> i.p.; Sigma) and chlorpromazine hydrochloride (10 mg kg<sup>−1</sup>), and given atropine sulphate (5.4 mg kg<sup>−1</sup>; Med-Pharmex). The bone overlying the bulbs was thinned using a dental drill. Glomeruli were imaged using a custom Nikon epifluorescence microscope and a 4× (0.2 NA) objective. Light excitation was provided using a 200 W metal-halide lamp (Prior Scientific) attenuated by neutral density filters and standard filter sets for mCherry (49008; Chroma), YFP (86001 JP3, Chroma), or green fluorescent protein (GFP) (96343, Nikon).

Odorants were applied using a custom-made, flow dilution oflfactometer and controller (LASOM, RPMetrics). Amines were diluted in water and subsequently by flow dilution. Predator urine volatiles were applied from the undiluted headspace concentration. Images were acquired at 25 Hz over 20 s (encompassing a 4 s pre-stimulus period and a 4 s odorant pulse) using a NeuroCCD-SM256 camera and Neuroplex software (RedShirtImaging). Blank trials were subtracted from odor trials before analysis to compensate for photobleaching. Response maps were obtained by subtracting a 3 s temporal average preceding the stimulus from a 3 s temporal average encompassing the response peak. Responses are expressed as ΔF to account for the fact that the background spH fluorescence is not correlated with the pool of indicator that reports neuronal activity10,26. Stimuli were presented during the nocturnal phase.

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Odorants were applied using a custom-made, flow dilution oflfactometer and controller (LASOM, RPMetrics). Amines were diluted in water and subsequently by flow dilution. Predator urine volatiles were applied from the undiluted headspace concentration. Images were acquired at 25 Hz over 20 s (encompassing a 4 s pre-stimulus period and a 4 s odorant pulse) using a NeuroCCD-SM256 camera and Neuroplex software (RedShirtImaging). Blank trials were subtracted from odor trials before analysis to compensate for photobleaching. Response maps were obtained by subtracting a 3 s temporal average preceding the stimulus from a 3 s temporal average encompassing the response peak. Responses are expressed as ΔF to account for the fact that the background spH fluorescence is not correlated with the pool of indicator that reports neuronal activity10,26. Stimuli were presented during the nocturnal phase.

Vapour concentrations were estimated using published vapour pressures (US EPA, Estimation Programs Interface Suite, v 4.0). Vapour concentrations were estimated using published vapour pressures (US EPA, Estimation Programs Interface Suite, v 4.0). Vapour concentrations were estimated using published vapour pressures (US EPA, Estimation Programs Interface Suite, v 4.0). Vapour concentrations were estimated using published vapour pressures (US EPA, Estimation Programs Interface Suite, v 4.0). Vapour concentrations were estimated using published vapour pressures (US EPA, Estimation Programs Interface Suite, v 4.0). Vapour concentrations were estimated using published vapour pressures (US EPA, Estimation Programs Interface Suite, v 4.0).

Behavioral analysis. ΔT4-YFP and ΔT2-9CFP littermates were housed in same-sex groups of 2–5 individuals. All animals were maintained in a reverse 12/12 h light-dark cycle and provided with food and water ad libitum. Cages were changed daily to prevent adaptation to amines that are present in mouse urine. Mice of all genotypes were tested between 5 and 7 weeks of age under low intensity red light during the nocturnal phase.

The experimental protocol consisted of three parts: handling (2 days), pre-trials (2 days) and experimental trials (1–3 days). Handling habituated the mice to the experimenter and consisted of placing each mouse individually onto the experimenter’s cupped, gloved hands for five minutes and allowing them to roam this small area freely. Pre-trials were identical to experimental trials (see below) except that no odorants were used. Pre-trials functioned to eliminate the novelty of the odour delivery and experimental chamber. Experiments were performed in clean, autoclaved 30 × 18 × 12 cm cages. A disposable curtain isolated one-third of the cage with minimal air transfer between sections. This smaller section, or ‘odorized’ compartment, was topped with a thin piece of clear acrylic, which functioned to minimize the loss of odour. Each mouse was introduced to the larger section of the cage and allowed to habituate for 3 min. At the end of this time period, a 3.5 cm covered Petri dish containing 20 µl of water on filter paper was introduced to the odorized compartment. The top of the Petri dish was perforated to allow odorants to escape, but to prevent direct contact with the stimulus. Mice were allowed to interact with this Petri dish for 3 min. The Petri dish was then removed and another identical Petri dish with 20 µl of an odorant on filter paper was added. The experiment was terminated after another 3 min.

The acrylic top was cleaned with 70% isopropyl alcohol and the cages were washed and autoclaved. The mice were video recorded and their location tracked using Limelight 3.0 software (Actimetrics). The aversion index was calculated as the difference between the time spent in the odorized chamber when an odour (or water) was present and the time spent in the odorized chamber when water was present. Data from mice that showed a very strong preference for either chamber (those that spent <2% or >98% of the trial duration in the odorized chamber) during the initial water trial were discarded.

Mice were naive to each stimulus and were tested only once for a given odour. Monomolecular stimuli consisted of three control odorants, water, trimethylthiazoline (2% in water) and a saturated solution of ethyl vanillin (0.5% w/v, in water), as well as 4 amines that activate TAAR glomeruli, β-phenylethylamine (100%, 10%, 0.5% and 0.005%), isopropylamine (10% and 0.5%), N-methylpiperidine (2%) and cadaverine (10%). Complex odorants were undiluted peanut butter oil, puma urine (from Puma concolor) and lynx urine (from Lynx canadensis). Predator urines were collected at the Philadelphia Zoo, shipped frozen and stored at −80 °C. Odour concentrations represent what was placed in the Petri dish and are expressed as percent dilution in water. The saturated vapour concentrations (maximum possible odorant concentration) for the stimuli are as follows: 100% phenylethylamine = 22 µM; 10% isopropylamine = 243 µM; 10% cadaverine = 5 µM; 2% N-methylpiperidine = 30 µM. The actual stimulus concentrations in the odorized chamber are probably much lower than these theoretical maxima.

We note that the concentrations in the behavioural and imaging experiments are difficult to compare given the differences in odour presentation methods.

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