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Haploinsufficiency of SIX3 Abolishes Male Reproductive Behavior through Disrupted Olfactory Development, and Impairs Female Fertility through Disrupted GnRH Neuron Migration

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Abstract

Mating behavior in males and females is dependent on olfactory cues processed through both the main olfactory epithelium (MOE) and the vomeronasal organ (VNO). Signaling through the MOE is critical for the initiation of male mating behavior, and the loss of MOE signaling severely compromises this comportment. Here, we demonstrate that dosage of the homeodomain gene *Six3* affects the degree of development of MOE but not the VNO. Anomalous MOE development in *Six3* heterozygote mice leads to hyposmia, specifically disrupting male mounting behavior by impairing detection of volatile female estrus pheromones. *Six3* is highly expressed in the MOE, main olfactory bulb (MOB), and hypothalamus; all regions essential in the proper migration of the gonadotropin-releasing hormone (GnRH) neurons, a key reproductive neuronal population that migrates along olfactory axons from the developing nose into the brain. Interestingly, we find that the reduction in *Six3* expression in *Six3* heterozygote mice compromises development of the MOE and MOB, resulting in mis-migration of GnRH neurons due to improper olfactory axon targeting. This reduction in the hypothalamic GnRH neuron population, by 45% in adulthood, leads to female subfertility, but does not impact male hormone levels, suggesting that male infertility is not related to GnRH neuron numbers, but exclusively linked to abnormal olfaction. We here determine that *Six3* is haploinsufficient for MOE development, GnRH neuron migration, and fertility, and represents a novel candidate gene for Kallmann syndrome, a form of inherited infertility.

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AUTHOR CONTRIBUTIONS

E.C.P., H.M.H., E.L.S., M.R.G. and P.L.M. designed the experiments; E.C.P., H.M.H., and E.L.S performed the experiments; E.C.P., H.M.H., E.L.S., and P.L.M. analyzed the data; E.C.P., H.M.H., E.L.S., M.R.G., and P.L.M. discussed the results; and E.C.P., H.M.H., E.L.S., M.R.G., and P.L.M. wrote the manuscript.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest: The authors declare that they have no conflict of interest.

All animal procedures were performed in accordance with the UCSD Institutional Animal Care and Use Committee regulations.

Keywords

Kallmann Syndrome; GnRH Neuron Migration; Olfactory Development; Anosmia; Reproductive Behavior

Introduction

Aggression, mating behavior, and fear responses depend on an intact olfactory circuit in mammals [1]. Olfaction is required to distinguish sex, social, and reproductive status, as well as to express sexual behavior and induce neuroendocrine responses necessary for reproduction [2–5]. In rodents, these odors are processed via two anatomically and functionally separate sets of neurons localized in the main olfactory epithelium (MOE) and the vomeronasal organ (VNO) [6]. In the MOE, complex chemosignals are detected and differentiated by sensory neurons that project to glomeruli in the main olfactory bulb (MOB) [6]. It is believed that MOE neurons respond mainly to volatile odorants in the environment, whereas VNO neurons respond mainly to non-volatile pheromones, with some overlap between the functions of these anatomically distinct organs [7, 6]. Interestingly, volatile odorants that cue mating behavior in mice are processed by the MOE [8–12], and exclusive impairment of the MOE results in disrupted male reproductive behavior due to the inability to recognize estrus females [13, 10, 14]. Although humans do not need their sense of smell for reproduction, olfaction is an essential part of our everyday lives, and it is clear that proper development of the olfactory system is required for normal sexual maturation [15].

The development of olfactory neurons is closely linked to the development of the key reproductive neurons, gonadotropin-releasing hormone (GnRH) neurons [16–18, 14, 19, 20]. GnRH neurons originate in the primordial MOE (the olfactory placode), and migrate along olfactory nerves into the forebrain [18, 21, 22]. Due to the topographical and migratory link between the development of the olfactory system and GnRH neurons, it is not surprising that their fates are tied together and that both are compromised in the developmental disorder termed Kallmann syndrome [23, 24]. Kallmann syndrome is a rare genetic disorder of complex and heterogeneous genetic etiology, leading to various degrees of subfertility, including complete infertility (hypogonadal hypogonadism), along with anosmia/hyposmia in humans [24, 25]. Genetic analysis in familial cases of Kallmann syndrome has demonstrated that there are multiple genes involved in the process of regulating GnRH and olfactory neuron migration, including compound heterozygosity in patients [26, 27]. Despite recent advances, ~68% of the cases of Kallmann syndrome are of an unknown genetic origin, thus identification of new genes of interest in this condition is critical [28, 27].

Several newly discovered genes [29, 30] and haploinsufficiencies [31, 32] that alter GnRH neurons in development in mouse models are candidates for hypogonadal hypogonadism without anosmia. One potential candidate for Kallmann syndrome is the homeodomain gene *Six3*, which is highly expressed in the MOE, MOB, and hypothalamus, areas essential for maintaining reproduction and olfaction (www.brain-map.org, consulted Feb 2017). Due to early postnatal lethality of *Six3* knock-out (KO) mice [33], they have only been studied during early embryogenesis, with a focus on craniofacial and eye development [34, 35].

Haploinsufficiency of *Six3* has been recently studied and shown to produce a phenotype in holoprosencephaly; demonstrating that dosage of SIX3 can be directly proportional to the severity of a phenotype [36–38]. Here, we present the first study of the fertility of adult *Six3* heterozygote (HET) mice, finding that *Six3* haploinsufficiency alters male fertility, with various degrees of subfertility to infertility attributed to disrupted development of the MOE producing an incapacity to express normal sexual behavior.

Materials and Methods

Mouse lines and animal housing

All animal procedures were performed in accordance with the University of California, San Diego, Institutional Animal Care and Use Committee regulations.

Mice were group-housed with ~4 to a cage on a 12-hour light, 12-hour dark cycle (on 6:00 A.M., off 6:00 P.M.), with *ad libitum* chow and water. All mice were kept on a C57BL/6J mouse background. *Six3*-flox mice were provided by Dr. Guillermo Oliver [33]. *Six3* KO mice were created by crossing *Six3*-flox with a Zp3-Cre mouse [39] (https://www.jax.org/strain/006888), allowing germ-line recombination. *Six3*-flox mice were crossed with *GnRH*-Cre [40] mice to create $Six3^{flox/flox}$:$GnRH^{cre}$ mice homozygous for the *Six3* deletion within GnRH neurons. The mice were killed by a CO_2 or isoflurane (Vet One) overdose. Unless specified, all animals used were ~3 months of age.

Behavioral experiments

All behavioral tests were performed during the first three hours of the dark phase using red light illumination. The experimenter was blind to the genotype of the subjects. Before each assay, the mice were habituated for 1 hour in a new cage with fresh bedding and no food or water. All females presented to males in behavioral tests were virgin ovariectomized and primed with 1 μg estradiol benzoate diluted in sesame oil at 9 A.M. the day before testing and 500 μg progesterone diluted in sesame oil at 2 P.M. on the day of testing.

Collection of tissue and histology

Diestrus ovaries, brains, olfactory bulbs, embryos, noses, ovaries, and testes were fixed overnight (~16 h) at 4°C in freshly made mixture of 6:3:1 absolute alcohol: 37% formaldehyde (Fisher F79-4): glacial acetic acid. Tissues were paraffin embedded and serially sectioned at 10 μm. Ovaries, testes, brains, and noses were stained with hematoxylin and eosin (H&E; Sigma-Aldrich). In ovaries, histology was examined and the number of corpora lutea in a single ovary per mouse was recorded blindly.

Immunohistochemistry

Adult brains, olfactory bulbs, and embryos were sectioned at 10 μm. Immunohistochemistry (IHC) was performed as described previously [41]. Sections were immunostained with rabbit anti-GnRH (1:1000; Novus Biologicals, catalog number: NB300-506, RRID:AB_2110266), anti-peripherin (1:200; Abcam, catalog number: ab4573, RRID:AB_2171346), or rabbit anti-OMP (1:100; Santa Cruz Biotechnology, catalog number: sc-67219, RRID:AB_2158005). To increase the visibility of immunostained

neurons, adjustments of brightness, contrast, and color balance were done with Image J (National Institutes of Health, Health, Bethesda) and applied to the entire image. Peripherin quantification was conducted by counting all fibers within the MOE/MOB region in the HET and WT mice. Such analysis could not be performed on the KO mice, as all the fibers were contained in one large mass and could not be individually quantified. Counterstaining was performed using vector Hematoxylin counterstain (H-3401) for 15 seconds, followed by running tap water for two minutes. Slides were then incubated in bluing solution for one minute and then rinsed in distilled H_2O.

GnRH neuron counting and OMP staining were conducted throughout the entire embryonic head, and throughout the adult brain beginning with the front of the olfactory bulb (bregma 4.46) to bregma −2.80 [42]. Each slide was collected and evaluated for GnRH and Olfactory Marker Protein (OMP) staining in a blinded manner using a Nikon Eclipse E400 microscope. Although all collected sections were assessed for staining, neuroanatomical landmarks were used to identify the regions of interest (ROI) as depicted in the corresponding figures. Slides were coded to blind the researcher to treatment group during analysis. In KO mice where recognizable structures were absent, images were taken in locations corresponding to where structures of interest are normally seen. Adult brains analyzed for GnRH include equal numbers of both male and female subjects, although a sex difference in GnRH neurons has not been recorded to our knowledge. Embryos were not sexed.

Lineage tracing

For lineage tracing, *Rosa*^{RFP} reporter mice (https://www.jax.org/strain/007908) were used [43] and mated to *Six3* HET and *GnRH*^{cre} mice to create the *Six3*^{HET}:*Rosa*^{RFP}:*GnRH*^{cre} line. IHC was performed with the anti-RFP antibody (1:1000, Abcam, catalog number: ab62341, RRID: AB_945213).

GnRH-pituitary stimulation tests

For two weeks prior to the hormonal challenge, mice were adapted to handling stress such that they would be unaffected by stress during serial sampling. Baseline tail blood was collected from male and female metestrus/diestrus WT and *Six3* HET littermates. Ten minutes after receiving an IP injection of 1 μg/kg GnRH (Sigma #L7134) diluted in physiological serum, tail blood was collected again. For kisspeptin challenge, 20 minutes after *ip* injection of 30 nmoles of kisspeptin (Tocris #4243) diluted in physiological serum was injected, tail blood was collected again. The total volume of blood collected did not exceed 100 μL. Blood was collected between 11:00 AM and 12:00 PM and was allowed to clot for 1 hour at room temperature. Blood was then centrifuged for 15 min at 2600 X g. Serum was collected and stored at −20°C before Luminex analysis was used to measure LH. The LH assay detection limit was 0.24 ng/mL, inter-assay CV was 15.2 and intra-assay CV was 11.5. This experiment was conducted as previously described [30].

Sperm motility and total sperm count

Epididymides were collected in M2 media at room temperature (Sigma-Aldrich). One epididymis was cut in half and sperm were gently expelled by manual pressure. The

numbers of motile and immotile sperm were counted in a hemocytometer 15 minutes after sperm were expelled. To immobilize motile sperm for a total sperm count, the hemocytometer was placed for 5 minutes on a 55°C heat block. The second epididymis was chopped into small pieces and left 30 minutes at room temperature in M2 media. The solution was filtered through a 70 μm filter (Falcon), and the sperm were diluted in PBS before counting the total number of sperm heads in one epididymis. This experiment was performed as described previously [31].

Vaginal plug formation, mating assay, generation of timed embryos, and estrous cycling

To monitor plug formation, a WT or *Six3* HET female mouse was housed with either a WT or *Six3* HET male mouse at 12 weeks of age and plug formation was monitored for ten consecutive days. Embryos were obtained using timed matings in which one male and one female mouse were housed together, and vaginal plug formation was monitored. If a plug was present, the day was noted as day 0.5 of pregnancy and used to collect embryos for timed mating at embryonic day e13.5 or e17.5. A second cohort of virgin *Six3* WT and HET mice were housed in pairs, and the number of litters born and the number of pups per litter were recorded over a period of 14 weeks. Mice were assessed for estrous cyclicity as previously described [31]. To assess estrous cyclicity, vaginal smears were performed daily between 9:00 and 10:00 A.M. on 120-day-old mice by vaginal lavage as described previously [31]. A cycle was determined by counting the days from one estrus period to the next estrus period.

Determination of day of vaginal opening and preputial separation

Starting at 21 days-of-age, *Six3* WT and HET mice were inspected daily and pubertal onset was determined by the age at vaginal opening in females or by preputial separation in males as previously described [31].

Mounting assay

Male mice were habituated to new cages for 1 hour. Ovariectomized and estrogen primed female mice were then introduced to WT and HET male mice of 16 weeks of age. This test was conducted three times with only the third trial quantified and reported. Trials were conducted one week apart from each other, and the same estrogen primed females were used in all assays. Pairs were videotaped and behavior quantitated in a blind manner with incidences of mounting being recorded over a period of 15 minutes. Some of the same males were used in the mounting assay, the buried food test, and the habituation-dishabituation experiments.

Buried food test

A buried food test was conducted to check for gross malfunction of the main olfactory system as described previously [44]. All mice were food-deprived overnight (18 hrs). A small piece of mouse chow was buried (~3 cm deep) at a random location in a clean mouse cage containing fresh bedding. One mouse was placed in the cage and timed for the latency to find the mouse chow during a period of up to 15 minutes. Mice were videotaped and behavior quantitated in a blind manner.

Sugar water test

Six3 HET and *Six3* WT mice were housed under normal conditions, except a second water bottle was placed on the cage that contained 4% dissolved sugar. Prior to beginning testing, mice were habituated to the presence of two drinking bottles (one containing 4% sucrose and the other water) for 3 days in their home cage. Following this acclimation, mice had the free choice of either drinking the 4% sucrose solution or plain water for a period of 4 days. The location of the bottles was switched each day to avoid a confounding factor of cage-side preference. The water bottles were weighed at the beginning of the study and at the end, the change in weight was reported.

Wheel-running behavior

To determine if *Six3* HET mice had normal activity on running wheels, *Six3* HET and WT male mice were housed individually in cages with running wheels. Food and water was available *ad libitum* during the entire experiment. After 1 week acclimation to the polypropylene cages (17.8 × 25.4 × 15.2 cm) containing a metal running wheel (11.4 cm diameter), locomotor activity rhythms were monitored with a Vitalview data collection system (Version 4.2, Minimitter, Bend OR) that compiled in 6 min bins the number of electrical closures triggered by half wheel rotations. Running wheel activity was monitored for 2 weeks on a 12h Light:12h Dark cycle. Cage changes were scheduled at 3-week intervals. Wheel-running activity was analyzed using ClockLab Analysis (ActiMetrics Software).

Territorial marking assay

Six3 HET and *Six3* WT male mice were placed in a clean empty cage to habituate for 1 hour. After habituation, the bottom of the cage was covered with Whatman paper for 15 minutes to record a baseline for normal urination. This paper was then removed, and the bottom of the cage was covered with a new piece of Whatman paper with 60 μL of estrus female mouse urine placed in the center of the paper. Male mice were allowed to mark the paper for 15 minutes. The Whatman papers were then dried overnight and sprayed with 0.2% ninhydrin diluted in 100% EtOH (Sigma) and air dried until urine spots developed a purple color. Papers were photographed and images were converted to binary and the number of urine marks was determined using Image J software. The number of marks elicited by sensing estrus female urine was determined by subtracting the number of urine marks in baseline markings. This experiment was conducted as previously described [45].

Male countermarking behavior against a strange male's urine

Six3 HET and WT males, who had been singly housed and who were sexually inexperienced, were placed in a cage lined with Whatman paper for 30 min. Fifty μL of male urine pooled from 5 adult male mice was placed in the center of the Whatman paper. The Whatman papers were then dried overnight and sprayed with 0.2% ninhydrin diluted in 100% EtOH (Sigma) and air dried until urine spots developed a purple color. Papers were photographed and images were converted to binary and the number of urine marks was determined using Image J software. The number of marks elicited by sensing male urine was determined by subtracting the number of urine marks in baseline markings. Urine was

collected from male mice by holding mice by the scruff of the neck over a piece of clean parafilm.

Urine collection

Urine was collected from stimulator estrus female mice by holding mice by the scruff of the neck over a piece of clean parafilm. Female stimulator mice were prepared by ovariectomizing female mice and inserting a low-dose estrogen pellet of 0.05 cm diameter subcutaneously. On the day of urine collection, stimulator females were injected with 1 μg of estradiol benzoate diluted in sesame oil 24 hours before collection, followed by 500 μg of progesterone diluted in sesame oil at 2 P.M. Urine was collected at 6 P.M. Urine from 5 different mice of each sex was pooled into separate male and female urine collections on the day of testing. This experiment was performed as described previously [46, 7].

Urine preference test

Preference for investigating the urine of one mouse sex over the other was assessed in a 5 min test during which male and estrus female urine were presented simultaneously on pieces of filter paper. The odor presentations were attached to weigh boats that were placed at opposite ends inside a cage with either a *Six3* HET or WT male mouse. Tests were videotaped and seconds spent investigating the urine stimulus were quantitated in a blind manner. This experiment was conducted as described previously [47].

c-Fos immunohistochemistry

Prior to experimentation, males were isolated from female scent in a room separate from other mice for at least 2 days. On the day of the experiment, male mice were singly housed in a clean cage in a quiet room for three hours prior to female exposure. The male mice were then exposed (at 6:00 PM under dim red light) to urine (60 μL) collected from hormonally primed estrus stimulator female mice. The urine was placed just out of reach of the mice to prevent direct contact. After 90 minutes, males were killed, and brains were collected in fixative. Sections were stained with c-Fos antibody (Santa Cruz Biotechnology; catalog number sc-52 RRID:AB_10160513; 1:1000). Neuroanatomical landmarks were used to identify the counting region as depicted in the corresponding figure. Quantification was performed on biological replicates consisting of c-Fos nuclei within the defined region from a minimum of three unilateral sections. C-Fos-positive cells were quantified by an experimenter blinded to the treatment group. While the c-fos IHC produced a range of staining intensity, only those cells that were darkly stained were included in the quantification. The numbers from each biological replicate were then averaged across all the animals in that group. Slides were coded to blind the researcher to treatment group during analysis.

Resident–intruder aggression assay

Male mutant and control mice were isolated at 9–11 weeks of age for a period of 2 weeks before testing. Mice were singly housed in cages with bedding. Testing lasted 15 min, and began when a group-housed, sexually inexperienced, adult WT “intruder” male was placed in the home cage of the test mouse, whose bedding had not been changed for 4 days.

Aggressive behavior was defined as biting, chasing, or wrestling. Cumulative attack duration, and number of attacks were video recorded, then quantitated by a blinded assessment. This experiment was performed as described previously [48, 6].

Testosterone replacement assay

Male mice were gonadectomized, given a testosterone pellet, and left to recover for 1 week before any behavioral tests were conducted. Pellets made of tubing with an inner diameter of 1.02 mm and an outer diameter of 2.16 mm filled for 1.2 cm with testosterone (Sigma T1500-1G) and inserted into the gonadectomy incision site of *Six3* HET and WT mice. These implants have been shown previously to produce elevated physiological levels of T (11.1 ± 0.8 ng/mL) [49].

Volatile and non-volatile odor detection

To assess the ability of *Six3* HET mice to detect volatile and non-volatile odors, and to discern these odors from each other, we used a home-cage habituation-dishabituation test, as described previously [47, 44]. In these tests, the mice become habituated to a urinary scent after three trials, and are then dishabituated when a new odorant is presented.

Six3 HET and WT males were tested in three different paradigms. First males were presented with (1) volatile/non-contact stimuli, (2) non-volatile and volatile/contact stimuli, and (3) non-volatile (MUPs)/contact stimuli. *Six3* HET and WT males were presented with 20 μL of an odorant sample (either water or urine) pipetted onto a piece of Whatman paper taped to a plastic weigh boat. In tests (1) and (2), mice were presented with deionized water for two minutes three times in a row with one-minute intervals in between, followed by three 2-minute presentations of male urine and finally by three 2-minute presentations of a female estrus urine. In test (3), mice were presented with deionized water for two minutes three times in a row with one-minute intervals in between, followed by three 2-minute presentations of MUPs. Tests were videotaped and quantitated by an experimenter blinded to treatment group. The number of seconds that the mice stretched upwards to smell the filter paper containing the stimulus and the number of seconds mice touched their nose to the odorant stimulus were recorded. If the mouse does not increase their interest in the new urinary stimulus, they cannot differentiate between the two stimuli. If the interest in the odorant is similar to baseline water, then the mouse cannot detect the stimulus.

For each day of testing, one-half of the subjects in the *Six3* HET and WT groups were presented with the male urinary stimulus followed by the estrus female urinary stimulus, and the other half were presented with the same urinary odors but in the reverse order. Subsequently, on a second day of testing, all of the subjects were re-exposed to the same odors but in the opposite order from their first day of exposure. The order of presentation of urine had no effect on the results of these trials; thus, we analyze and present here data from the trials where male urine was presented first followed by female urine. Tests were videotaped and behavior quantitated in a blind manner. Within groups, statistical analysis compared (1) the difference between the number of seconds that subjects spent investigating the third water stimulus versus the male urinary odorant presented, and (2) the difference

between the number of seconds spent investigating the third male urinary stimulus versus the first presentation of the estrus female urinary stimulus.

In experiment (1), male *Six3* HET mice were tested for their ability to detect and distinguish exclusively volatile odors. Volatile odors are airborne and do not need to be directly contacted to be sensed, as opposed to non-volatile odor components. Thus, to separate the volatile odors from the non-volatile, a cage lid with holes to let volatile odors pass through was placed on top of the cage, and the weigh boat was placed on this cage top such that only volatile odorants were available at body level, with subjects having no physical access to the stimulus. As the mice could not contact the stimulus, they could not detect non-volatile odorant components. The number of seconds spent investigating the stimulus by stretching up to reach the weigh boat on top of the cage was quantitated. In experiment (3), mice were tested for their ability to detect non-volatile odorants exclusively by presenting mice with MUPs.

Female odor detection was assessed with four separate odor presentations given consecutively for two minutes separated by one-minute intervals. First, water was given on Whatman paper taped to a weigh boat placed inside of the cage, to record a baseline for interest in an odorless presentation. Then male urine was presented inside of the cage where mice could contact the stimulus. The third presentation was water once more presented outside of the cage where the mice could not physically touch the stimulus but could stretch up and smell it, to record a baseline for investigation of odors presented in this new location. The final stimulus given was male urine on top of the cage, to test for volatile odor detection.

Collection of MUPs

Experiment was conducted as previously described [48]. In brief, urine was collected fresh from group-housed adult C57BL/6J male mice, from multiple cages and combined. To fractionate the MUPs, urine was applied to Amicon Ultra Centrifugal Filters (10,000 MWCO regenerated cellulose, Millipore) and centrifuged at 6000 × g, in a table-top centrifuge at room temperature for 15 min. The first effluent collected was considered the LMW fraction. The HMW fraction was washed by adding sterile 1X PBS (equal to starting urine volume), and centrifuged five times at 6000 × g at room temperature for 15 min. The washed protein fraction was then diluted to starting urine volume with 1X PBS.

Statistical analysis

All the statistical analysis was performed using R data analysis software. A P-value of <0.05 was considered statistically significant. For all experiments, data are expressed as the mean ± SEM. Unpaired two-tailed t-tests are used in all cases, except in cases where data was abnormal, or the variances were unequal, where the Wilcoxon rank sum tests were used. Additionally, in several cases, Two-way ANOVA with Bonferroni post-hoc was used to analyze data. Power analyses were performed before experiments to determine n values. Experimental groups were defined by genotype and thus no sorting mechanism was used. n values stated for each experiment report the number of mice in each group.

Results

Loss of one allele of *Six3* is sufficient to cause subfertility

To test the hypothesis that *Six3* heterozygosity affects reproductive competence, a 120-day fertility assay was performed. We found that both *Six3* HET males and females took longer to generate their first litter when paired with wild-type (WT) mates (Fig. 1a). This subfertility was amplified when *Six3* HET males and females were paired together, in which no litters were sired over the 120-day test period (Fig. 1a, b). Furthermore, *Six3* HET males fathered significantly fewer litters than controls (Fig. 1b). Litter size was not significantly different between genotypes in either sex (WT: males 8.3 ± 2.4, HET: males 7.0 ± 1.0 pups/litter; WT: females 8.3 ± 2.4, HET: females 6.9 ± 2.3 pups/litter; Students t-test, $p>0.05$). As reproductive competence relies on successful completion of puberty, we determined whether the subfertility was due to delayed pubertal onset. Both *Six3* HET males and females had normal pubertal onset as determined by preputial separation in the male and vaginal opening in the female, two external markers of pubertal onset in males and females, respectively (preputial separation WT: 26.7 ± 0.7 days, HET: 26.8 ± 0.8; vaginal opening WT: 27 ± 0.7, HET: 26.7 ± 1.0 days; Students t-test, $p>0.05$). Thus, the subfertility was not due to a delay in pubertal onset. Instead, the female delay to first litter was associated with significantly increased time to vaginal plug by a WT male (Fig. 1c), although all of these females plugged before the end of the 10-day assay, suggesting prolonged estrous cycles. Indeed, *Six3* HET females had prolonged estrous cycles (Fig. 1d–g). Interestingly, we noted a heterogeneous impact of *Six3* haploinsufficiency on estrous cyclicity wherein five *Six3* HET females had prolonged estrous cycles (Fig. 1f), one was in persistent estrus (data not shown), and one spent 80% of the time in persistent metestrus (Fig. 1g). As expected, based on the normal litter size, *Six3* HET females had a similar number of ovarian corpora lutea, a marker of successful ovulation, compared to controls (Fig. 1h, i).

To discern whether the deficiency in *Six3* HET mating efficiency was due to a defect in spermatogenesis, sperm analysis was conducted. Examination in the *Six3* HET mice showed that total sperm count (Fig. 1j) and motility (Fig. 1k) were not significantly different between *Six3* HET and WT males, and that testis morphology was normal (Fig. 1l). To further ascertain the cause for the significantly reduced fertility of the *Six3* HET males, we evaluated their ability to produce a vaginal plug in female mice. *Six3* HET male mice plugged significantly fewer females compared to WT mice, with three of five pairs of mice never plugging over the 10-day assay (Fig. 1c). This effect was exacerbated in *Six3* HET/HET matings in which only one out of four pairs of mice provided a plug during the assay.

Defective GnRH neuron migration in *Six3* HET mice is associated with absence of olfactory fibers

Fertility depends on correct maturation, localization and function of GnRH neurons. Even a modest decrease in the number of GnRH neurons is sufficient to engender irregular or lengthened estrous cycles. To determine if the subfertility of *Six3* HET mice originated at the level of GnRH neurons, we performed GnRH immunohistochemistry and counted GnRH neurons. Adult male and female *Six3* HET mice had ~45% fewer GnRH-immunoreactive

neurons in the hypothalamus with ~10% of the total GnRH neuronal population halted along their migratory route through the olfactory bulb, indicating a defect in migration (Fig. 2a, b). The total number of GnRH neurons in *Six3* HET brains was equivalent between sexes. To determine if the abnormal GnRH neuron migration arose during development, we counted GnRH neurons at e13.5 and e17.5. Despite the total number of GnRH neurons in the entire head being normal at both ages (Fig. 2c–f), the number of GnRH neurons was significantly higher in the nose and lower in the hypothalamus in *Six3* HET than WT (Fig. 2c–f). At e13.5, a time point when we were also able to collect *Six3* KO embryos, we found that KO heads were misshapen and most brain structures (including the MOB) unrecognizable, as has been shown previously [33]. In these embryos, all the GnRH neurons were found in one large tangled group in the region normally occupied by the MOE (Fig. 2e, f). This remarkable mass of GnRH neurons was observed in each of the KO mouse heads. The immunostaining was very specific, with no off-target staining observed (Fig. 2g), as has been previously reported [30]. Thus, in *Six3* HET and KO embryos, GnRH neurons are inhibited in their migration from the nasal region. In addition, the migration of GnRH neurons is more severely disrupted in *Six3* KO than in *Six3* HET mice indicating a dose-dependence of *Six3*.

In embryos late in development, at e17.5, GnRH neurons were found delayed in anterior portions of the migratory pathway. Additionally, in the adult *Six3* HET mouse, GnRH neurons were found delayed in their migratory pathway, and more GnRH neurons were seen in the olfactory bulb. However, it is possible that there is a population of GnRH neurons in the brain that stop expressing GnRH in late development, and therefore is undetectable using anti-GnRH antibody IHC. To exclude this possibility, we created a $Six3^{HET}$:$Rosa^{RFP}$:GnRHcre mouse in which GnRH neurons are labeled with RFP for the life of the neuron regardless of the expression of GnRH. Therefore, if there are GnRH neurons in the brain, that are no longer expressing GnRH, they will be detectable using IHC for RFP. Six3 HET brains were assessed for RFP-positive neurons. In counting the anti-rfp IHC, a ~40% reduction in the number of GnRH neurons was observed in the HET mice as compared to WT mice (the same result that was found using the GnRH antibody) (Fig. 2h, i). Therefore, there was no detection of GnRH neurons that were no longer expressing GnRH. Thus, the source for the loss of hypothalamic GnRH neurons seen previously is solely due to mis-migration.

To investigate whether the abnormal GnRH neuron migration was due to effects within the GnRH neuron, a separate mouse model was used, termed $Six3^{flox/flox}$:$GnRH^{cre}$, where *Six3* is deleted within GnRH neurons. GnRH neuron numbers were assessed in this model and surprisingly GnRH neurons migrated normally, but the total numbers were increased by ~30% in both the adult mouse (Fig. 3a) and in e17.5 embryos (Fig. 3b). This indicates that *Six3* does not act within the GnRH neuron to inhibit the migration of this neuronal population. As expected, the increased number of GnRH neurons did not affect male or female gonadal function as evidenced by normal total amount of sperm and sperm motility in the male (Fig. 3c, d), and comparable number of corpora lutea and estrous cycle length of females (Fig. 3e, f). Thus, the source of impaired GnRH neuron migration stems from the role of *Six3* outside of the GnRH neuron along the migratory pathway.

While gonadal function was unaffected by the loss of *Six3*, we next sought to determine whether pituitary function was altered by the loss of *Six3*. Since GnRH numbers were strongly reduced in *Six3* HET animals, we sought to determine whether pituitary function was intact, as the secretion of GnRH can impact pituitary development and function. Fertility depends on sufficient GnRH stimulation of pituitary gonadotropes to secrete luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which are required for normal gonadal function. Given the decrease in hypothalamic GnRH neurons in *Six3* HET mice, we measured the extent to which the reduction of hypothalamic GnRH neurons affects the reproductive physiology of the severely subfertile male mice. First, we analyzed the ability of the pituitary to respond to endogenous GnRH by measuring the basal level of LH in *Six3* HET males. There was no significant difference in basal LH levels (WT: 2015 ± 793, HET: 1568 ± 612 pg/ml; n= 5, Students t-test, $p>0.05$), or in the capacity of the pituitary to respond to a GnRH challenge, as evaluated by the fold change in LH (Fold change WT: 1.78 ± 0.3, HET: 2.10 ± 0.25; n= 5, Students t-test, $p>0.05$) between *Six3* WT and HET mice. Male fertility, particularly the secretion of LH, is very sensitive to appropriate GnRH levels. To test whether the number of GnRH neurons in *Six3* HET mice is sufficient to generate a LH response, *Six3* HET and WT males were injected with Kisspeptin, a stimulator of GnRH neurons, and the fold change of LH from baseline was determined. Again, no significant difference between genotypes was observed (Fold change WT: 1.16 ± 0.24, HET: 1.80 ± 0.37, n=5, Students t-test, $p>0.05$).

Six3 HET males lack normal mating behavior due to anosmia

Given intact spermatogenesis, the presence of corpora lutea, and normal hormone levels in *Six3* HET mice, it does not appear that the loss of GnRH neurons is sufficient to produce the subfertility observed in these male mice. Therefore, we sought to investigate mating behavior. Male mouse sexual behavior relies on a functional olfactory system and the capacity to detect pheromones [9, 50, 51, 47, 52, 53]. To determine whether the deficit in plugging was due to altered sexual behavior, we determined whether a WT female could elicit sexual behavior in *Six3* HET males. When male mice were presented with an estrogen primed female (ovariectomized and primed with progesterone and estrogen injections), WT males mounted 9 times more frequently than *Six3* HETs (Fig. 4a). Mounting behavior is preceded by chemoinvestigation, wherein males detect female pheromones. Interestingly, *Six3* HET males spent significantly less time chemoinvestigating females than WT males, suggesting a defect in the odor processing in the *Six3* HET males (Fig. 4b). In consideration of the clear alteration of reproductive behavior, pheromone driven aggressive behavior was assessed. Interestingly, *Six3* HET males responded normally to the pheromones of WT male intruders as evidenced by their number of attacks toward the intruder male (Fig. 4c) and the total duration of the attacks (Fig. 4d). Mating behavior follows cues detected primarily by the MOE, while male-male aggressive behavior is driven by detection of pheromones in the VNO [53]. To further assess the functionality of the VNO, territorial countermarking behavior against a strange male's urine was tested. This VNO-mediated behavior was tested by counting urine marking in response to the presentation of male urine. The number of urine marks made was not significantly different between *Six3* HET and WT mice (Fig. 4e, f). These data indicate that the VNO is functional in the *Six3* HET male, while the MOE is impaired. In addition to pheromones, mating behavior is also driven by neural circuits that

regulate sexual motivation [53]. To determine if the lack of mounting behavior was due to a defect in motivation-regulated behavior, mice were tested for their preference of sugar versus water, a test that is an indicator of anhedonia. Anhedonia is the inability to find pleasure in in an activity that would carry hedonic value in a normal situation [54]. Both WT and *Six3* HET mice displayed a preference for the sugar water over unsweetened water, showing that the neuronal motivation circuitry is intact (Fig. 4g), as is the sense of taste. This test also demonstrates that the sweet taste receptors of mice are intact, as identified by the preference towards the sweetened drink. In line with this, we found that *Six3* HET males had normal levels of running wheel activity (Fig. 4h), a rewarding behavior, which also requires normal locomotor function [55]. Additionally, as expected, activity onset of wheel running in normal light:dark conditions (12h light: 12h dark) was normal for *Six3* HET male mice, indicating normal eye development and sensitivity to light (WT: 23.9 ± 0.11, HET: 24.03 ± 0.014; n=5, Student's t-test, t(8)=1.16, p= 0.279)

To further determine whether the deficiency in normal reproductive behavior of *Six3* HET males was due to altered testosterone, we gonadectomized *Six3* HET male mice and inserted a testosterone pellet. Sexual behavior was then reassessed with no change in the discrepancy in mounting behavior (WT: 130 ± 25, HET: 30 ± 18 mounts; n=3, Students t-test, t(4)=8.56, p=0.0001) and chemoinvestigation (WT: 30 ± 8, HET: 4 ± 7 seconds; n=3, Students t-test, t(4)=7.84, p=0.0007) seen between *Six3* HET and WT male mice. The impaired response of *Six3* HET male mice to estrus females was not altered when testosterone levels were equalized between the WT and HET mice; thus, the abnormal mating behavior of *Six3* HET is not due to a paucity of testosterone.

Abnormal MOE development leads to an inability of *Six3* HET males to detect and respond to volatile odors

The normal motivational behavior displayed in the sucrose preference test and running-wheel activity indicate that the absence of mounting behavior in *Six3* HET males is caused by a defect outside of the motivational circuit. To discern whether the altered mating behavior could be engendered by an olfactory deficit, we evaluated general olfaction using the buried food test [44]. Indeed, the *Six3* HET males and females were unable to locate a buried food stimulus during the 15-minute assay (Fig. 5a, b). Odor processing occurs via two morphologically distinct circuits: the MOE and the VNO. The MOE neurons respond mainly to volatile odorants (including those that cue mating behavior) in the environment and VNO neurons respond mainly to non-volatile pheromones detected through direct contact with the odor; however, the VNO can also bind volatile odorants [48, 6]. To determine whether *Six3* HET mice can detect and discriminate volatile and non-volatile odors, habituation/dishabituation tests were performed using intact male and estrus female urinary odorants (Fig. 5c). To test the ability of *Six3* HET mice to detect and differentiate smells, we measured significant increases in investigation times (dishabituation) between (1) the third presentation of water and the initial presentation of male urinary stimulus and (2) the third presentation of male urine and the first presentation of estrus female urine in *Six3* WT and HET males. The first test conducted provided mice with both volatile and nonvolatile odorants with presentation of odors inside the cage. Both WT and *Six3* HET males could detect and differentiate between non-volatile/volatile urine components of male

and estrus female urine (Fig. 5d). However, when the odor stimuli were moved outside of the cage (therefore only volatile odorants would be available to mice), *Six3* HET males were unable to detect the male urine or estrus female urine (Fig. 5e). This locates the olfactory deficit to the MOE. However, to provide further evidence that the VNO is functional, non-volatile odorants were exclusively presented to *Six3* HET and WT mice by using MUPs. MUPs are processed by the VNO and relay information about the donor animal [12]. To test VNO function, three presentations of MUPs were given to HET and WT mice following three presentations of water. Both *Six3* HET and WT mice detected these components, indicating proper VNO function (Fig. 5f).

The ability of *Six3* HET females to detect volatile odorants processed by the MOE was assessed in a similar experiment. The same result was obtained in this experiment as was seen in the *Six3* HET males, with *Six3* HET females being able to detect non-volatile male urine components, but not volatile male urine (Fig. 5g). Female *Six3* HET mice were not able to detect either male or female urine presentation (Fig. 5g). This test was similar in principle to the test conducted on male *Six3* HET mice (Fig. 5c–e), but the methodology was simplified as the mating defect observed in *Six3* HET females was not as severe as that observed in the *Six3* HET males.

Given the inability of *Six3* HET males to detect volatile female urine components, the response of *Six3* HET male mice to estrus female urine was assessed. When presented with estrus female urine, male *Six3* HET mice did not exhibit normal territorial marking, making significantly fewer urine spots around the estrus female urine than the WT mice (Fig. 6a, b). The abnormal response to female estrus cues in *Six3* HET males was confirmed in a separate experiment, where *Six3* HET males were presented with both estrus female and male urine simultaneously within the cage. *Six3* HET mice, unlike the WT mice, did not preferentially sniff estrus female urine over male urine (Fig. 6c).

To determine how *Six3* haploinsufficiency disrupted MOE-processed olfaction, we used immunohistochemistry for olfactory marker protein (OMP), which specifically localizes in the primary neurons of the olfactory system of vertebrates. A total loss of OMP staining of the MOE (Fig. 7a) and MOB (Fig. 7b) was observed in the *Six3* HET mice at e13.5, e17.5, and p56. In the e13.5 *Six3* KO embryos, morphologically recognizable olfactory structures were not detectable; however, a bundle of neurons and axonal fibers with OMP immunoreactive neurons was detected in the region where the MOE normally exists (Fig. 7a). In contrast, OMP staining was preserved in the VNO of *Six3* HET and WT mice (Fig. 7c), supporting the normal non-volatile olfaction in these mice.

Olfaction requires olfactory fibers from the olfactory bulb projecting to the MOE. These olfactory fibers are also responsible for guiding GnRH neuron migration from the VNO into the forebrain during development. Peripherin, an intermediate filament protein can be used as a marker for these fibers [56]. Alteration of these fibers, including mistargeting or absence of olfactory axons along the migratory path of GnRH neurons, could be causing the defects in GnRH neuron migration observed in the *Six3* KO and *Six3* HET mice (Fig. 2). To determine the destiny and localization of peripherin-expressing olfactory fibers, we stained for peripherin in *Six3* WT, HET and KO mice at e13.5. Whereas peripherin fibers appeared

to be normal in the *Six3* WT, *Six3* KO mice displayed a complete absence of peripherin fibers in the upper olfactory system, and *Six3* HET mice displayed a decrease in the number of projections reaching the MOB (Fig. 7d, e). The only peripherin fibers of the *Six3* KO lead into the mass of neurons where GnRH- and OMP-immunopositive neurons were previously observed (Fig. 2b and 7d).

To confirm that odor processing fails to occur in *Six3* HET mice, immunostaining for c-Fos, a marker of neuronal activation, was conducted after exposure to female mouse urine or water. c-Fos activation was absent from the target region of MOE neurons, the mitral layer of the dorsal MOB (Fig. 8a, b). Thus, the neuronal circuits that process olfactory cues from female estrus urine are not activated in *Six3* HET males.

Discussion

Disrupted volatile olfaction leads to infertility of *Six3* HET males

Loss of olfactory processing in the MOE has been shown to disrupt male sexual behavior in various studies [10, 7, 57]. Here, we describe for the first time a role for the homeodomain protein *Six3* in MOE development. The impact of *Six3* loss on MOE development is dosage sensitive (haploinsufficient), in that the effect of *Six3* deficiency was significantly more dramatic in *Six3* KO than HET mice. In *Six3* KO embryos olfactory structures failed to develop into a morphologically recognizable form, and OMP-immunoreactive neurons were found in a cluster halted off their normal migratory pathway. This is markedly different from the *Six3* HET mice where all olfactory structures were morphologically recognizable, although developmentally impaired with loss of OSNs and loss of neuronal activation within the MOB. IHC for cFos was conducted, revealing that some error in MOS development leads to the loss of neuronal activity, which would normally produce male sexual behavior in response to estrus cues. Loss of this neuronal activation results in the reduction in fertility seen in the *Six3* Het males. To specifically determine which set of neurons in the olfactory system is resulting in this impairment, neuron activation would need to be tested.

The loss of olfactory processing due to developmental errors has been described in several mouse lines including *Pax*-6 *SeyNeu*/*SeyNeu* and *Dlx5* KO [17], showing that when cells from the olfactory epithelium do not migrate properly or innervate the brain, the olfactory bulb can still develop. To elicit appropriate sexual behavior in rodents, correct processing of olfactory cues, via two key olfactory circuits, the MOE/MOB and VNO/Accessory Olfactory Bulb (AOB), are required. The MOE is known to process volatile odors, while the VNO is responsible for transmitting signals about water-soluble non-volatile compounds mediating innate behaviors [58]. Volatile odorants processed in the MOE and the efferent signals that they cue have been strongly implicated in the initiation of male sexual behavior [59]. The *Six3* HET males displayed a severe impairment in their ability to detect volatile odors, explaining their reproductive incompetence. The reduced mounting in response to estrus female cues offers an explanation for the finding that *Six3* HET males were unable to father litters during the fertility assay. Other mouse models with deficits in the MOE have shown similar sexual impairments, such as the *Cnga2* mice [57] and *Ac3* null male mice [9]. Similar to the *Six3* HET mice, both the *Cnga2* and *Ac3* null males had an intact functional VNO but failed to mount female mice. The impaired preference for the scents of opposite-

sex urine observed in *Six3* HET mice was also observed in the *Cnga1* and *Ac3* nulls, and MOE-ablated mice, supporting our conclusion that *Six3* HET males do not mount due to an incapacity to process volatile odors in the MOE [52]. The presence of aggressive behavior, and countermarking behavior is further evidence of the proper functioning of the VNO. While aggressive behavior is thought to be regulated by both MOE and VNO circuits [57], there is evidence that under loss of MOE function, the VNO compensates to produce normal aggressive behavior. Thus, even under loss of MOE function, as is observed in the *Six3* HET mice, aggressive behavior can be mediated through the VNO to produce a normal response [59]. Indeed, the VNO developed normally and was functional in *Six3* HET males, allowing the VNO to support normal aggressive response of *Six3* HET males towards WT intruders, and allowing the detection of MUPs. We speculate that deletion of *Six3* in adulthood might also impact function of the olfactory system, as *Six3* is a transcription factor and is expressed in the nose into adulthood. However, at this time, we have only identified the role of *Six3* in olfaction when heterozygous during development and in adulthood, not in adulthood alone. In conclusion, reduction in *Six3* expression in *Six3* heterozygote mice compromises development of the MOE and MOB, resulting in mismigration of GnRH neurons due to improper olfactory axon targeting. In addition, the impairment in development leads to an incapacity of the males to smell estrous females, and thus impairs their normal male sexual behaviors.

Six3 haploinsufficiency impairs GnRH neuron migration

Fertility depends on correct generation and migration of GnRH neurons. Numerous genes have been jointly implicated in the migration of GnRH neurons and olfactory neurons, both of which migrate along axons of the terminal olfactory pathways [17]. Mis-migration of GnRH neurons, in association with anosmia, gives rise to Kallmann syndrome, an infertility syndrome commonly due to the interruption of axonal guidance molecules that control development of the olfactory system [52]. While there is a clear GnRH neuron migratory defect observed in the *Six3* HET mice, the reduction of hypothalamic GnRH neurons was not sufficient to produce either hypogonadism or hypogonadotropism. This is consistent with past findings stating that only 12% of GnRH neurons are required for pulsatile gonadotropin secretion and proper stimulation of the gonads [60]. Therefore, the *Six3* HET males are subfertile due to defects in olfactory development producing altered mating behavior, rather than being due to insufficient GnRH secretion.

Our data support that the inability of a proportion of GnRH neurons to reach their appropriate destinations in the *Six3* HET mice is associated with the loss or mistargeting of olfactory peripherin fibers. *Six3* HET mice show a reduction in the peripherin fibers targeting the MOB, associated with a reduction in the number of GnRH neurons, while *Six3* KO mice show a complete absence of olfactory fibers extending from the MOE to the MOB, giving rise to a bundle of GnRH neurons mislocalized in the MOE. A similar tangled mass of olfactory and GnRH neurons was observed in *Sox10* KO mice [61], a gene identified as causing Kallmann syndrome [17]. Indeed, defects in MOB formation, such as those seen in the *Six3* KO mouse, can directly affect the ability of the olfactory fibers to connect to the brain, which explains the loss of olfactovomeronasal axons in this mouse, accompanied by a loss of GnRH neuron migration [17]. Data from the $Six3^{flox/flox}$:$GnRH^{cre}$ mouse indicate

that the reduction in the number of GnRH neurons found in the adult hypothalamus is mediated through SIX3 actions external to the GnRH neurons themselves, and localized in cells along their migratory route. This is apparent because deletion of *Six3* exclusively within the GnRH neuron in the *Six3*$^{flox/flox}$*:GnRH*^{cre} mouse does not reduce the number of GnRH neurons found in the adult hypothalamus. Instead, the increase in the number of GnRH neurons observed in this mouse model is likely due either to improved survival of migrating neurons, increased GnRH neuron proliferation, maintained expression of GnRH in neurons which lose GnRH expression in late development, or reduced death of GnRH neurons. Interestingly, *Six3* is not the only protein that has been found to be a repressor of GnRH. MSX1 has been shown to be a repressor of GnRH promoter expression through binding to homeodomain elements within the GnRH regulatory region [62]. Therefore, MSX1 KO mice, like the *Six3*$^{flox/flox}$*:GnRH*^{cre} mice, show an increase in the number of GnRH expressing neurons within the hypothalamus [62].

Importantly, the *Six3* HET mouse is, to our knowledge, the first example of a gene that in the heterozygous state gives rise to a fully penetrant phenotype in reproduction. Thus, this may be a candidate gene for Kallmann's syndrome. While *Six3* haploinsufficiency alone is sufficient to cause subfertility, Kallmann syndrome can arise from haploinsufficiency of several genes cumulatively producing complete infertility. Other genetic mutations affecting central components of the olfactory system, such as *Prokr2, Pax6, Chd7, Fgf8/Fgfr1, Prok2, Sox10,* and *Sema3A*, have been identified as causes of Kallmann Syndrome or idiopathic hypogonadal hypogonadism in various mouse models [17, 63–65]. Cases of haploinsufficiencies resulting in compromised fertility and hyposmia/anosmia have thus far been identified in both rodents and humans [47, 66, 32, 67, 31].

Sex differences in subfertility/infertility of *Six3* HET mice

Despite comparable neuroanatomy in *Six3* HET males and females, males were more severely subfertile than females. Extended fertility assays revealed that the *Six3* HET males could plug a few WT females, albeit at a very reduced rate compared to WT mice. A potential explanation for the rare ability of the *Six3* HET males to mount and produce litters despite their loss of MOE input, is that it is possible that some of the *Six3* HET males were able to use tactile, visual, or auditory cues to trigger mating behavior; however, it does not necessary follow that *Six3* HET males can discern ovulating females [9]. Our data support that the more severe reproductive impairment of the *Six3* HET males is caused by impaired MOE development and function, a structure demonstrated to be key in male, but not female sexual behavior [59]. In contrast, female sexual behavior relies on the accessory olfactory system, regulating female sexual receptivity in the form of lordosis behavior [46, 7]. Although there are some cases in which MOE lesions produced alterations in female mating behavior [46, 68], in the majority of cases, female mating behavior is markedly less disrupted than male mating behavior.

A similar differential impact on male and female fertility by gene deletions affecting anosmia and the number of GnRH neurons was observed in studies of the *B3gnt1* KO mice, a gene involved in the formation of axonal connections. *B3gnt1* KO females were fertile, whereas the *B3gnt1* KO males were unable to sire litters at a normal rate despite having

normal reproductive organs [9]. Additionally, they displayed impaired sexual response to females, and olfactory neuron loss [9]. While fertility in *Six3* HET females was not as severely disrupted as it was in the males, they remained subfertile as evidenced by a delay in being plugged, a reduced number of litters and a delay to the first litter during the fertility assay. The decrease in litters mothered correlated with increased estrous cycle length. This agrees with other studies, in which it has been shown that a decreased number of GnRH neurons in the hypothalamus can impact female fertility more severely than male fertility and often is associated with longer and irregular estrous cycles [60, 69, 31]. We believe the prolonged and irregular estrous cycles to be the cause of reduced fertility, as opposed to a behavioral deficit in mating behavior [31]. Mating behavior in females has repeatedly shown to be more strongly regulated by the VNO (a structure that was unaltered in the *Six3* HET mice) than the MOE [59]. While the reason behind the subfertility/infertility of *Six3* HET males and females differs, both sexes lose their ability to smell, and have a reduction in the number of GnRH neurons.

The findings presented here reveal that SIX3 dosage is essential in the proper development of the MOE and MOB olfactory structures. Furthermore, both alleles of *Six3* are necessary for the proper migration of GnRH neurons and the detection of volatile odors. It is possible that SIX3 functions within the nose, in similar capacity to its role in the eye, by regulating the balance in proliferation and differentiation of olfactory structures [70]. These findings have broader implications for human health, as expanding the knowledge basis of the mechanism through which *Six3* regulates neuronal development will provide insight into the diseases engendered by mutations in *Six3*, such as schizencephaly and holoprosencephaly [70]. In conclusion, our study is the first to address the impact of *Six3* haploinsufficiency in adulthood and demonstrates *Six3* to be a key transcription factor in the development of the MOE, olfaction, GnRH neuron migration, and normal fertility, remarkably producing anosmia and subfertility/infertility in the heterozygous state.

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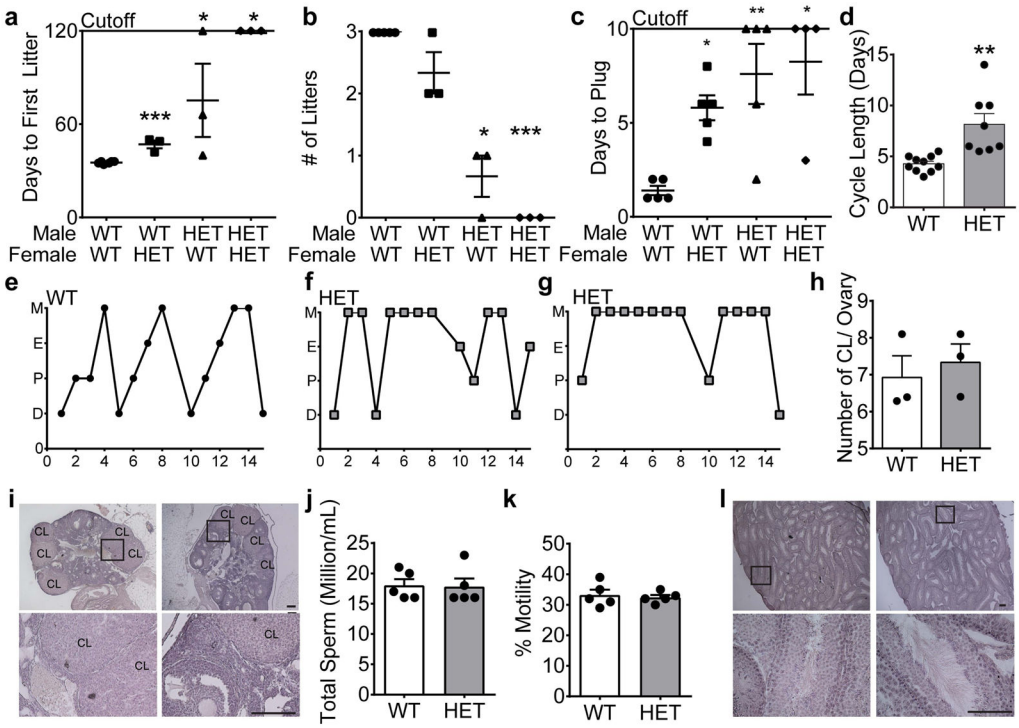

Fig. 1.

Six3 HET mice are subfertile. **a,** Days until first litter (Mann Whitney test as compared to WTxWT, n=3–5) (WTxHET, p=0.0003), (HETxWT, p= 0.024), (HETxHET, p= 0.024). **b,** Average number of litters in a 120-day mating assay (Mann-Whitney test as compared to WTxWT, n=3–5) (WTxHET, p= 1.07), (HETxWT, p=0.018), (HETxHET, p= 0.018). **c,** Days until WT or HET males create a vaginal plug in WT or HET females during a 10-day assay (Mann-Whitney test as compared to WTxWT, n=4–5), (WTxHET, p=0.008), (HETxWT, p=0.024), (HETxHET, p=0.008). **d,** Length of estrous cycles monitored daily in *Six3* HET and WT females over 15 days (Student's t-test, p=0.001, t(16)=3.93, n=8–10). M, metestrus; E, estrus; P, proestrus; D, diestrus. Representative cycles are shown for **e,** WT female, **f,** and **g,** *Six3* HET females. **h,** Number of corpora lutea (Student's t-test, p= 0.574, t(4)= 0.529, n=3) and **i,** pictures of corpora lutea (CL) in *Six3* HET and WT ovaries, n=3. **j,** Total number (Student's t-test, p=0.910, t(8)= 0.117, n=5), and **k,** percent motile sperm (Student's t-test, p=0.688, t(8)= 0.416, n=5) in *Six3* HET and WT mice. **l,** Representative images of testes from *Six3* HET and WT mice, n=3. Boxes on images indicate where the higher magnification images were taken. *p<0.05, **p<0.005, ***p<0.001. Scale bars, 2 mm, 20 μm.

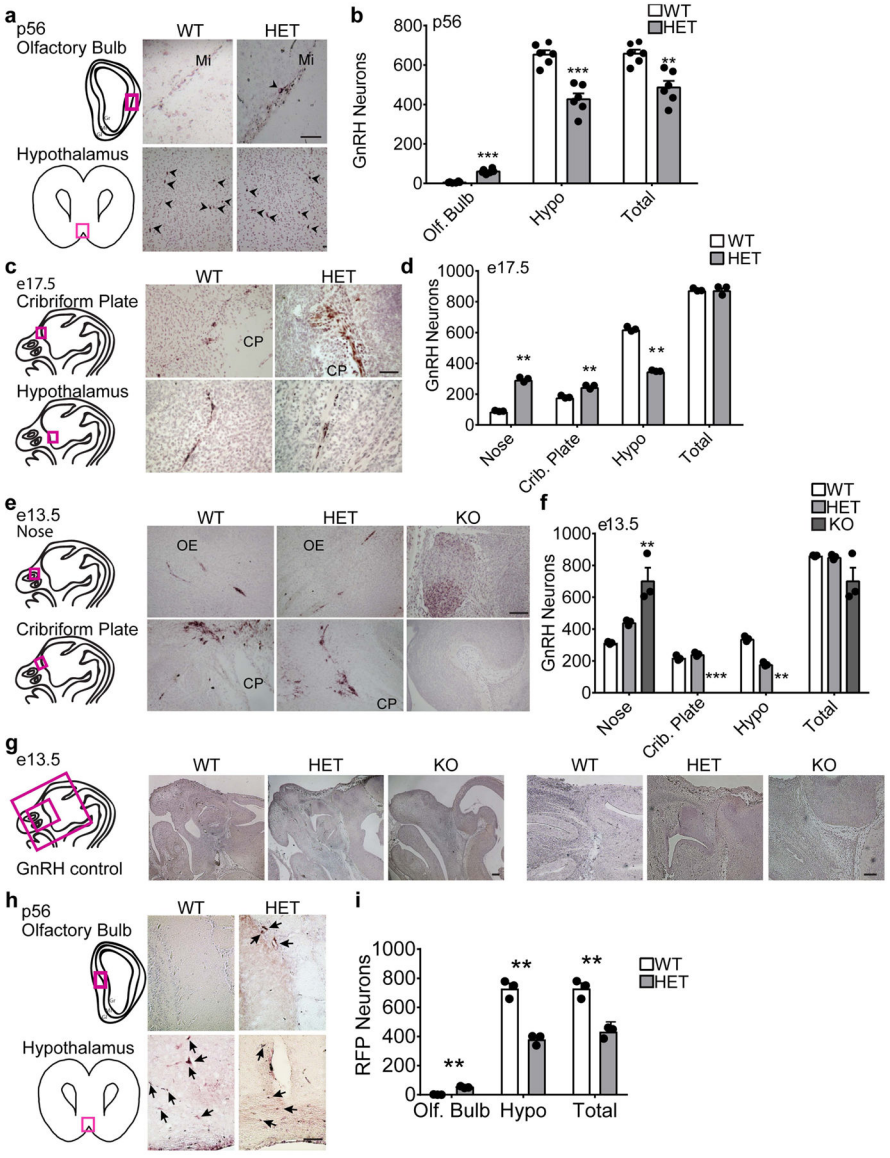

Fig. 2.

Six3 HET and KO mice showed delayed migration of GnRH neurons. *Six3* HET and WT mice were processed for IHC staining for GnRH. **a, c,** and **e,** Representative images of staining in *Six3* WT and HET mice are shown with boxed areas on the drawings of the sagittal mouse head sections to the left of the panels indicating where the (*a*, *c*, and *e*) images were taken. **b,** GnRH neurons at p56 (Student's t-test, n=6 (3 females, 3 males)). Counting was conducted throughout the adult brain beginning with the front of the olfactory bulb to bregma −2.80. (olf. bulb, p= 0.00004, t(6)=11.1), (crib. plate, p=0.0001, t(9)=−6.17), (total, p=0.002, t(8)=−4.34). **d,** GnRH neurons at e17.5 (Student's t-test, n=3). Counting was conducted throughout the entire embryonic head (nose, p=0.001, t(2)=−22.4; crib. plate, p=0.003, t(4)=−7.40; hypo, p=0.0004, t(2)=31.1; total, p=0.973, t(2)=−0.03). **f,** GnRH neurons at e13.5 (Student's t-test, n=3; nose, p=0.002, t(3)=−12.2; crib. plate, p=0.116 t(4)= −2.06; hypo, p=0.0002, t(4)=13.6; total, p=0.488, t(2)=0.821). **g,** Controls for GnRH

staining specificity, images are from e13.5 mice processes for anti-GnRH IHC. **h** and **i,** lineage tracing, IHC for RFP in *Six3*^{HET}:*Rosa*^{RFP}:*GnRH*^{cre} adult mice. RFP marks all GnRH neurons for the duration of the life of the neuron regardless of GnRH expression, to determine presence of GnRH neurons lacking GnRH expression. **i**, Number of RFP-labelled GnRH neurons in *Six3*^{HET}:*Rosa*^{RFP}:*GnRH*^{cre} adult mice. (Student's t-test, n=3; olf. bulb, p=0.004, t(2)=−14.0; hypo, p=0.003, t(3)=8.41; total, p=0.004, t(3)=6.87). Arrows indicate RFP neurons. Mi, mitral layer; CP, cribriform plate; OE, olfactory epithelium. **p<0.005, ***p<0.001. Scale bars, 100 μm (a, c, e, h), 2 mm (g left), 200 μm (g right).

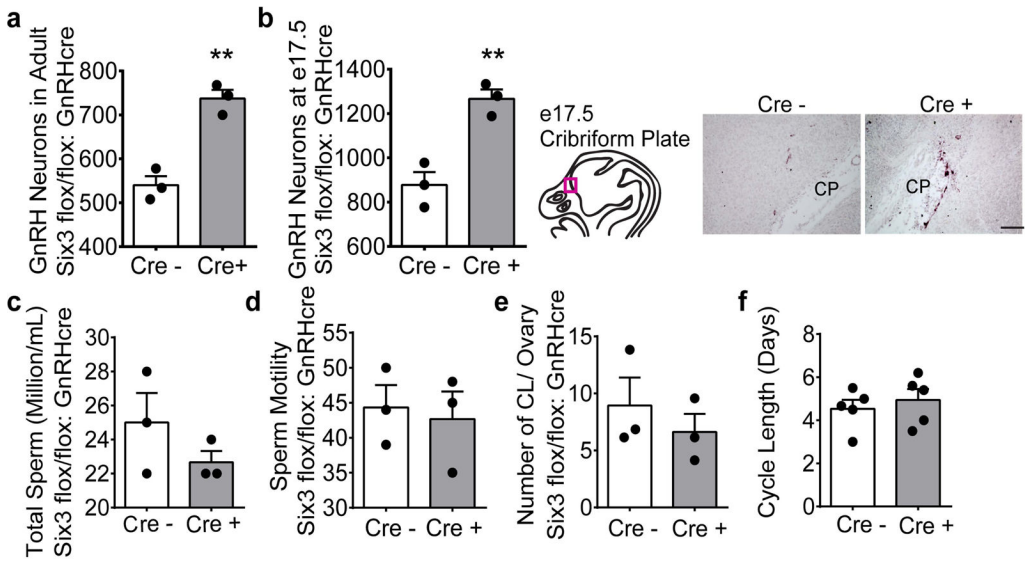

Fig. 3.

Six3$^{flox/flox}$*:GnRH*^{cre} mice have increased numbers of GnRH neurons and normal gonadal function. **a,** and **b** and In the *Six3*$^{flox/flox}$*: GnRH*^{cre} mice, the number of GnRH neurons at (*a*) p56 (Students t-test, p=0.0023, t(4)=6.91, n=3) and (*b*) at e17.5 (Students t-test, p= 0.006, t(4)=5.42, n=3) with representative images from e17.5 of anti-GnRH IHC antibody staining. **d,** Total number (Student's t-test, p=0.277, t(4) =1.26, n=3) and **e,** percent motility (Student's t-test, p=0.758, t(4)=0.330, n=3) of sperm. **f,** Number of corpora lutea in control and *Six3*$^{flox/flox}$*:GnRH*^{cre} mouse ovaries (Student's t-test, p=0.471, t(4)=0.796, n=3). **g,** Length of estrous cycles monitored daily in control and *Six3*$^{flox/flox}$*:GnRH*^{cre} mice (Student's t-test, p=0.471, t(8)=0.616, n=5). CP, cribriform plate. **p<0.005. Scale bars, 100 μm.

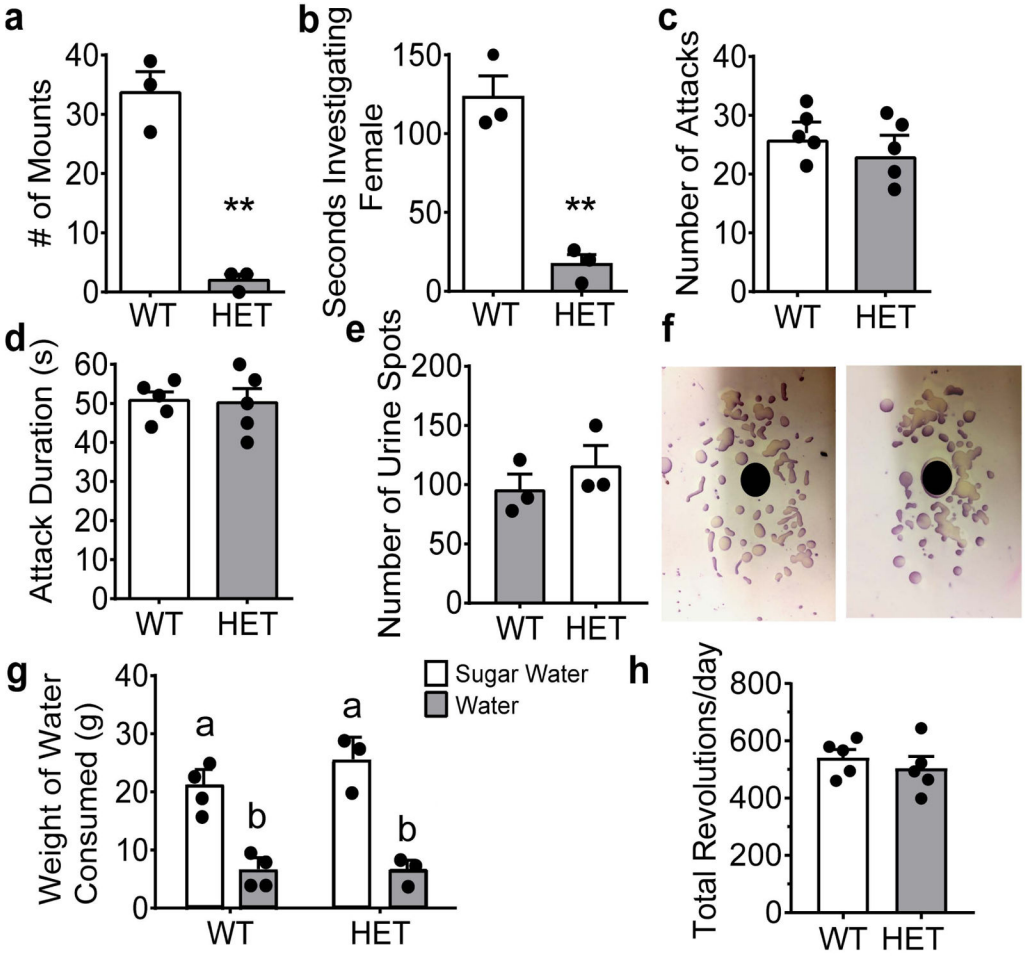

Fig. 4.

Six3 HET males do not respond normally to estrus female cues, but maintain normal aggression patterns towards male intruders. **a,** *Six3* HET males mounting of estrus females, assay was 15 min (Student's t-test, p=0.001, t(4)=8.64, n=3). **b,** Male chemoinvestigation of estrus females, assay was five min (Student's t-test, p=0.002, t(4)=7.09, n=3). **c,** and **d,** Male aggression towards WT intruder males over 15 min as measured in (*c*) number of attacks (Student's t-test, p=0.387, t(8)=0.918, n=5) and (*d*) duration of attacks (Student's t-test, p=0.890, t(8)=0.143, n=5). **e**, Quantification of Territorial marking on Whatman paper **f**, in response to 60 μL pooled male urine (Student's t-test, p=0.392, t(4)=0.959, n=3). **g,** Anhedonia of *Six3* HET and WT mice measured by their intake of sugar water versus unsweetened water (different letters indicate statistical difference by Two-way ANOVA followed by Tukey post hoc, n= 3–4). **h**, Running wheel activity in 12h light:12h dark conditions for 1 week of *Six3* WT and HET male mice. **p<0.01.

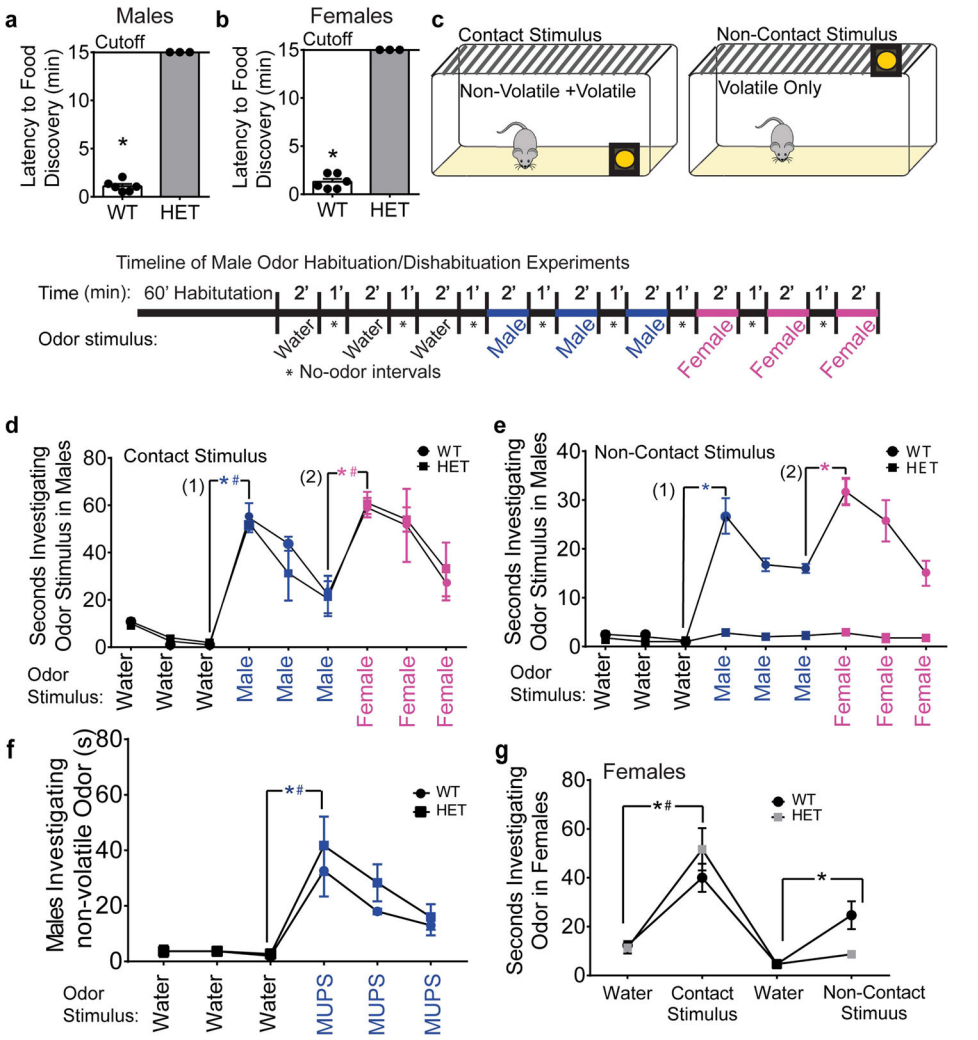

Fig. 5.

Six3 HET mice are unable to detect volatile odors. **a** and **b,** Identification of anosmia using a buried food test (*a*) male (Mann-Whitney, p=0.024, n=3–6) and (b) female (Mann Whitney, p=0.025, n=3–6). **c,** Schematic and time line for the habituation/dishabituation tests conducted to discern the ability to detect and discriminate odors. **d, e,** and **f,** Habituation/dishabituation tests were conducted to discern the ability to detect and discriminate odors. Results from males (*e* and *f*) and females (*g*). Odor placement was used to separate volatile and non-volatile odor components. Mice must be able to physically contact the odor stimulus to detect non-volatile odor components. Thus, when the odor was placed outside of the cage (*f* and *g*), only volatile odors were detectable. We compared the difference between (1) the number of seconds that subjects spent investigating the third water stimulus versus the first urinary odorant presented and (2) the difference between the number of seconds spent investigating the third presentation of the first urinary stimulus versus the first presentation of the second urinary stimulus. **d,** Contact stimulus detection in male mice (Mann-Whitney, n=4; water/male urine: WT, p=0.006; HET, p=0.009; male urine/female urine: WT, p=0.013; HET, p=0.024). **e,** Non-contact stimulus detection in male mice (Mann-

Whitney, n=4; water/male urine: WT, p=0.005; HET, p=0.523; male urine/female urine: WT, p=0.015; HET, p=0.264). **f**, VNO function was tested more directly using three odor presentations of MUPs after three presentations of water (Student's t-test, n=3; WT: p=0.028, t(2)=−5.61; HET: p=0.023, t(2)=−6.41). **g,** Odor investigation in female mice. Comparison of water to non-volatile/volatile odor (contact odor stimulus) (Two-way ANOVA followed by Tukey post hoc, n=3). In graphs with overlapping lines, * indicates WT mice and # indicates HET. $^{*,\#}p<0.05$.

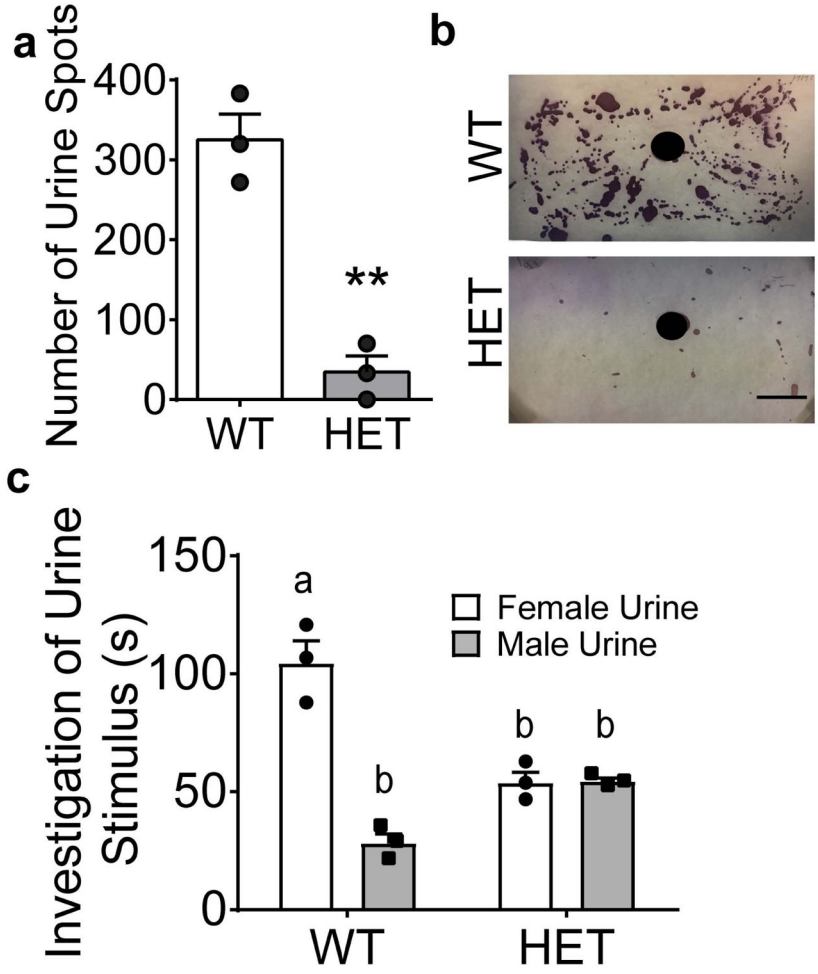

Fig. 6.

Six3 HET mice respond abnormally to estrus female cues. **a,** Territorial marking in response to female estrus urine assessed by placing 60 μL of estrus female urine in the center of Whatman paper (black circle) and quantifying the number of urine spots made by *Six3* WT and HET mice after imaging these pieces of paper **b,** and subtracting a baseline sample with no odor exposure (Student's t-test, p=0.002, t(4)= 7.66, n=3). **c,** Urine preference test with *Six3* HET and WT mice presented with male urine and female estrus urine simultaneously. The amount of time mice spent investigating each odorant was measured over 5 minutes (different letters indicate statistical difference by Two-way ANOVA followed by Tukey post hoc, n=3). **p<0.01. Scale bars, 2.54 cm.

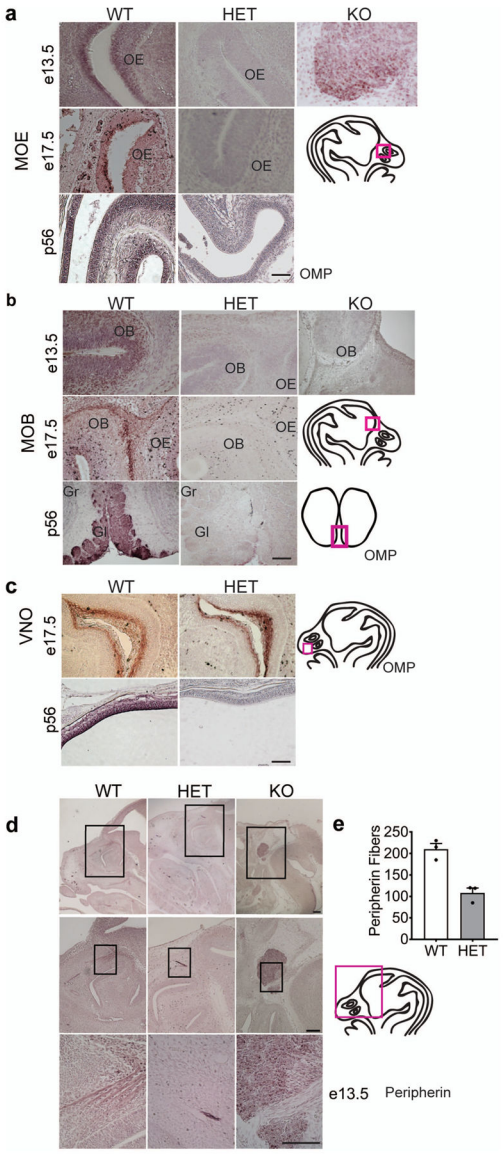

Fig. 7.

Six3 HET mice lack olfactory neurons. **a, b,** and **c,** Olfactory Marker Protein (OMP) IHC to mark all mature olfactory sensory neurons (OSNs) of *Six3* WT, HET and KO mice at e13.5, 17.5, and p56 in (a) the MOE, (*b*) the MOB, and (*c*) the VNO, n=3. Boxes on drawings of the brain to the right of representative IHC images indicate where the pictures were taken. **d,** IHC for Peripherin-positive axons in the olfactory system at e13.5 in *Six3* WT, HET, and KO mice, n=3. **e,** quantification of peripherin fibers in *Six3* WT and HET embryos (Student's t-test, p=0.004, t(4)=5.82, n=3). Boxes on drawings of adult brain (*a*) or embryo head (*b, c*) indicate where the representative images were taken. OB, olfactory bulb; OE, olfactory epithelium; Gr, granular layer; Gl, glomerular layer. Scale bars, 100 μm (a, b, c), panel d, 2 mm, 10 μm, 100 μm.

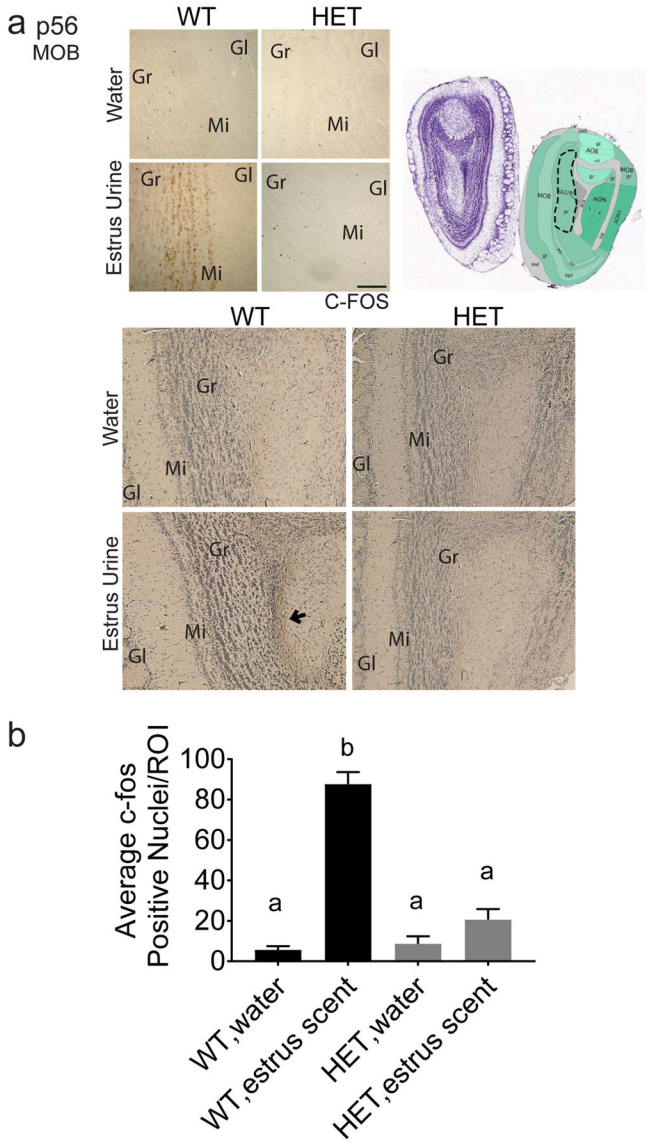

Fig. 8.

Six3 HET mice lack MOB neuron activation in response to estrus scent. **a,** Dotted outline indicates quantified portion. Images obtained from the Allen Institute web site, Allen Mouse Brain Atlas, p56 coronal image 20, (http://mouse.brain-map.org). IHC for c-Fos performed to identify regions in the MOB activated by the female urinary volatile odors presented to *Six3* WT and HET male mice. Water was used as a negative control. Images are taken from the glomerular layer of the MOB, n=3. **c,** Quantification of ROI depicted in (b). Quantification was performed on biological replicates consisting of c-Fos positive nuclei within the defined region from a minimum of three unilateral sections. c-Fos-positive cells were quantified by an experimenter blinded to the treatment group. The numbers from each biological replicate were then averaged across all the animals in that group (different letters indicate statistical difference by two-way ANOVA followed by Tukey post hoc). Boxes on images of brains indicate where the higher magnification images below were taken. Gr,

granular layer; Gl, glomerular layer; Mi, Mitral layer. Image reproduced with permission from the Allen Institute, Image credit: Allen Institute. Scale bar, 200 μm.