Medial Amygdala Arginine Vasopressin Neurons Regulate Innate Aversion to Cat Odors in Male Mice

Wen Han Tong Samira Abdulai-Saiku Ajai Vyas

School of Biological Sciences, Nanyang Technological University, Singapore, Singapore

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Defensive behaviors · Extended amygdala · Innate fear · Medial amygdala · Nonapeptides · Reproductive behaviors · Vasotocin · Sexual selection

Abstract

Aversion to environmental cues of predators is an integral part of defensive behaviors in many prey animals. It enhances their survival and probability of future reproduction. At the same time, animals cannot be maximally defended because imperatives of defense usually trade-off with behaviors required for sexual reproduction like display of dominance and production of sexual pheromones. Here, we approach this trade-off through the lens of arginine vasopressin (AVP) neurons within the posterodorsal medial amygdala (MePD) of mice. This neuronal population is known to be involved in sexual behaviors like approach to sexually salient cues. We show that chemogenetic partial ablation of this neuronal population increases aversion to predator odors. Moreover, overexpression of AVP within this population is sufficient to reduce aversion to predator odors. The loss of fear of the predator odor occurs in parallel with increased recruitment of AVP neurons within the MePD. These obser-

vations suggest that AVP neurons in the medial aspect of the extended amygdala are a proximate locus for the reduction in innate fear during life stages dominated by reproductive efforts.

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Introduction

Reproduction and defense are 2 primary pursuits for animals. These pursuits are guided by behaviors like sexual investment, aversion to cues of predator presence, and the accompanying changes in their motivational states. Many of these behaviors are innate in the sense that their expression is independent of individual experience. And yet, these innate behaviors are continually modulated by the incipient environment of the animals. The living environment of animals is typically replete with uncertainties [1]. The availability of reproductive opportunities cannot be predicted with precision. Probabilities of exposure to predators are likewise variable. These uncertainties require animals to make continuous calibration of innate behaviors as a function of environmental cues. One of the most crucial adjustments in this regard is to reduce

defensive behaviors when reproductive opportunities are present [2–7]. Successful reproduction involves behaviors that are not commensurate with maximal defense. In many species, males must produce publicly broadcasted sexual advertisements that can be perceived by both the intended audience of females and the unintended audience of predators. Males must actively pursue females and compete with other male suitors. These behaviors require at least a temporary suspension of defensive behaviors against predators. The trade-off between predation and reproduction has been widely studied in ecology. However, the neural mechanisms that permit such behavioral trade-off to exist through a reciprocal inversion between reproduction and defense remain currently unknown.

The medial amygdala is an essential node within the social brain network inside the vertebrate brain [8, 9]. It is likely that the medial amygdala plays a vital role in the process of reciprocal interaction between reproductive and defensive behaviors. The medial amygdala receives information about the olfactory environment through converging inputs initiating from main and accessory olfactory systems [10, 11]. Thus, the medial amygdala has access to environmental variability through chemosensory information about the presence of predators or reproductive opportunities. It projects to brain circuits involved in motivation and to effector modules within the hypothalamus [12–19]. These efferent connections provide a pathway for the medial amygdala to coordinate downstream reproductive or defensive behaviors. Anatomically, the posterior aspect of the medial amygdala exhibits broad functional segregation. The posterodorsal medial amygdala (MePD) is chiefly involved in response to cues of reproductive opportunities, and the posteroventral medial amygdala responds robustly to cues of predator presence [20–25]. Lesion studies also support the essential role of the posterodorsal and posteroventral medial amygdala in reproduction and defense, respectively [26, 27]. Thus, environmental cues with contrasting behavioral implications are processed in adjacency within the posterior medial amygdala.

Rodent MePD is enriched in neurons that synthesize the nonapeptide arginine vasopressin (AVP) [15, 28–30]. This extrahypothalamic pool of AVP neurons is preferentially found in males and is dependent on androgens [30–33]. The location within MePD, in addition to sexual dimorphism and androgen responsivity, suggests that MePD-AVP+ neurons are an important component in the mediation of reproductively motivated behaviors. This is supported by the observation that MePD-AVP+ neurons exhibit robust activity when male rodents are ex-

posed to the smell of estrus females or after copulation [34–38]. These observations are congruent with experiments in birds, where analogous vasotocin neurons within the medial aspect of the extended amygdala are recruited during behaviors that are shaped by sexual selection [39–42]. For example, territorial behaviors by male birds when expressed in the context of reproduction involve vasotocin neurons, while the same behavior in the context of nonreproductive resource defense does not involve this neuronal population.

Thus, reproductive and defensive behaviors represent a reciprocal trade-off. The medial amygdala, specifically the posterior parts of the medial amygdala, contains 2 subdivisions that are essential for reproductive and defensive behaviors, and it contains neurons (MePD-AVP+) that are responsive to gonadal hormones that initiate and sustain reproductive behaviors.

In this context, our aim here is to delineate the role of MePD-AVP+ neurons in the mediation of innate fear to predator odor. We ask if overexpression of AVP within MePD-AVP+ neurons increases their recruitment during exposure to predator odor, and if such a manipulation concomitantly reduces fear. We also ask if partial ablation of MePD-AVP+ neurons reduces the recruitment of remaining AVP neurons concomitant to increased fear of predator odors.

Materials and Methods

Animals

A transgenic mice line expressing Cre recombinase from AVP promotor was acquired (JAX stock 023530; Jackson Laboratory; background strain C57BL/6J [43]). Heterozygote animals from this line were crossed with wild-type C57BL/6 animals procured from a local vivarium (InVivos, Singapore). Littermates were genotyped after cessation of all imaging and behavioral experiments by sequencing PCR amplification products of genomic DNA obtained from tail snips and primers suggested by the commercial supplier.

All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the Nanyang Technological University. Only male animals were used in these experiments in view of gender dimorphism inherent in the medial amygdala AVP system. Animals were maintained on a 12-h:12-h day-night cycle with ad libitum access to food and water (lights on at 07:00 h; 4–5 animals/cage).

Viral Vectors

Adeno-associated viral vectors were used to deliver genes for either diphtheria toxin receptor (DTR) or AVP within the MePD. A freely available viral backbone, pAAV-EF1 α -DIO-mCherry-WPRE (AddGene), was used. The vector contains EF1 α promoter and double inverted loxP elements flanking complementary DNA sequences in inverted orientation and a WPRE element for ex-

pression optimization. The vector used for AVP overexpression allowed AVP expression from a robust EF1 α promoter after a recombination event dependent on the presence of Cre recombinase (pAAV-EF1 α -DIO-AVP-IRES-hrGFP-WPRE). An IRES gene was inserted between AVP (gene ID: NM-016992) and GFP coding sequences to allow co-expression of AVP and GFP upon Cre recombination. The forward and reverse primers for the entire PCR fragment contained *NheI* (GCTAGC) and *Ascl* (GGC-GCGCC) restriction sites. The resulting PCR amplicon was digested with *NheI* and *Ascl* restriction enzymes and cloned into the pAAV-EF1 α -DIO-mCherry backbone between the inverted terminal repeats. The AVP ablation vector, pAAV-EF1 α -DIO-DTR-IRES-hrGFP-WPRE, was created using the same techniques (gene ID: AK222688.1). This vector allowed expression of DTR in the presence of Cre recombinase. Viral particles were packaged and synthesized by VectorBuilder (Cyagen Biosciences) at a concentration of $>10^{11}$ viral particles/mL. These AAV viral particles expressed cargo when transduced in human embryonic kidney 293T cells expressing Cre recombinase (online suppl. Figure 1; see www.karger.com/doi/10.1159/000508862 for all online suppl. material).

Timeline of Experiments

Animals were more than 7 weeks old at the start of the experiment. We did not carry out genotyping of littermates at the start of the experiment, expecting a mendelian equivalence between animals with or without Cre recombinase. This ensured that experimenters were blind to the experimental groups until all behavioral and immunohistological imaging experiments were finished. Littermates were randomly assigned to receive 1 of the 2 viral vectors used in the study. Viral vectors were delivered at 7–8 weeks of age through stereotaxic injections targeted at MePD.

Mice were left to recover for 4 weeks before subsequent experiments. For animals infused with a vector containing DTR, 2 doses of diphtheria toxin were administered 2 and 3 weeks after surgery (Sigma-Aldrich D0564; 50 μ g/kg body weight i.p., dissolved in buffered saline at 10 μ g/mL) [44, 45].

Aversion to bobcat urine was quantified 4 weeks after stereotaxic injection of viral vectors. Animals were sacrificed 2 h after exposure to bobcat urine during the aversion assay. Harvested brains were then used to quantify neurons expressing AVP and Fos (an immediate-early gene marker for recent neuronal activity). Tails were harvested during terminal dissection and used for genotyping the animals after data used for statistical analysis had been obtained.

Surgical Methods

The following coordinates were used to position the infusion at the MePD: anteroposterior: -1.70 , mediolateral: ± 2.00 , and dorsoventral: -5.00 from bregma [46]. Prior to all stereotaxic surgeries, mice were anesthetized with 2–3% isoflurane, placed on an isothermal heating pad, and the head was mounted in a stereotaxic frame (Leica Biosystems Vernier Stereotaxic w/Manual Fine Drive Stereotaxic Instrument for Small Animals). Ophthalmic ointment was used to cover the eyes. The scalp was sterilized, and the skull was exposed. Small bilateral holes were drilled into the cranium. Three microliters of AAV viral particles were infused bilaterally into the MePD at an injection rate of 0.3 μ L/min and a total volume of 3 μ L via a 26-gauge stainless steel cannula. The cannula was lowered to the targeted MePD site and left in place for

1 min before and 2 min after injection to prevent any back pressure from forming during the AAV viral particle infusion. Special care was taken to remove the dura so as not to clog the cannula during the infusion.

Diphtheria toxin was purchased from Sigma-Aldrich (D0564) as lyophilized powder, reconstituted in sterile phosphate-buffered saline (PBS) at 1 mg/mL, and aliquots were stored at -80°C . Two weeks after viral delivery, the working stock was prepared freshly by diluting the stored toxin 100 times in sterile PBS to a final concentration of 10 μ g/mL. The toxin was administered intraperitoneally (50 μ g/kg body weight) to animals assigned to the DTR experiment. The second round of a single dose of the toxin was administered the following week.

Behavioral Measurements

The experiments were conducted in a rectangular arena (46 $\times$ 9 cm), which was divided into 2 opposing and identical sections. Mice were first habituated to the testing arena for 3 consecutive days for 1,200 s in the absence of bobcat urine (09:00–14:00 h). During the last of the 3 habituations, mice were additionally exposed to a novel odor (vanilla extract, 2 mL; dilution 1:4). The following day, aversion to bobcat urine was quantified by measuring occupancy in the bisect containing 1 mL of undiluted bobcat urine (100% pure PredatorPee[®] bobcat urine from PredatorPee). Bobcat urine was pipetted onto and absorbed by a piece of c-Fold tissue paper. This bobcat odor paper was then clipped on the far end of the rectangular arena. The bobcat odor paper was changed and freshly prepared for each round of testing. After each trial, the arena was thoroughly cleaned with F-10 and 70% ethanol to prevent lingering odor caused by the mouse's urine and waste products. A 20-min interval was given between trials to ensure all odor has dissipated (trial duration = 1,200 s).

Immunohistological Staining

The product of immediate-early gene *cFos* was used as a proxy for recent neuronal activity (Fos). Animals were returned to their home cage and were not disturbed for 2 h after exposure to bobcat urine during the behavioral assay – this period allowed time for transcription, translation, and intranuclear localization of Fos. Animals were deeply anesthetized with isoflurane. Transcardial perfusion was performed with 50 mL PBS followed by 50 mL of 4% paraformaldehyde dissolved in PBS. Brains were harvested and postfixed in 4% paraformaldehyde overnight at 4°C . They were then transferred to 30% (wt/vol) sucrose for at least 24 h or until they sank to the bottom. Serial 40- μ m-thick coronal sections through the medial amygdala were collected via a Leica CM1950 cryostat at -22°C , and free-floating sections were stored at -20°C in antifreeze solution (2 parts PBS, 1 part ethylene glycol, 1 part glycerol). For antibody staining, sections were first washed in 1 $\times$ PBS (3 $\times$ 10 min) before subjected to blocking with PBS containing 5% (vol/vol) goat serum overnight at 4°C . Free-floating brain sections were then incubated in a cocktail of primary antibodies in 0.5% Triton X-100 and 1 $\times$ TBS for 72 h at 4°C : guinea pig anti-AVP (T-5048; Peninsula Laboratories, 1:1,500), rabbit anti-Fos (ABE457; Merck Millipore, 1:10,000) followed by washing in 1 $\times$ PBS for 6 $\times$ 10 min. Free-floating brain sections were then incubated in a cocktail of secondary antibodies in 0.5% Triton X-100 and 1 $\times$ TBS for 2 h at room temperature: Alexa Fluor 488 AffiniPure donkey anti-guinea pig (706-545-148; Jackson Immuno-research Laboratories, 1:1,000), Dylight 549 goat anti-rabbit

Fig. 1. Representative images showing immunofluorescences for arginine vasopressin (AVP), Fos (a marker for recent neuronal activity), and nuclear boundaries (DAPI). Animals were surgically infused with viral vectors containing cargos of diphtheria toxin

receptor (DTR) or AVP overexpression (OE) within the posterodorsal medial amygdala. The expression of cargos was dependent on the availability of Cre recombinase in the genome of animals. Examples from Cre- to Cre+ are depicted. Scale bars, 10 μ m.

(DI-5149; Vector Laboratories, 1:1,000), followed by washing in $1 \times$ PBS for 6×10 min. Sections were then mounted on Superfrost slides (Fisher Scientific, USA) and completely air-dried. This was followed by mounting with coverslips using Gold Antifade Mountant with DAPI (P36931; Life Technologies).

Imaging

We centered slides on MePD using the DAPI signal as a guide at low magnification. Three stacks within the MePD were then chosen randomly for the image capture at higher magnification for the analysis. Images were captured with the Carl Zeiss LSM 710 laser scanning confocal microscope using a $\times 40$ objective lens with a $\times 1.2$ digital magnification. Sections were optically sliced at 1- μ m

intervals. All DAPI-positive nuclei were counted (866 ± 23 DAPI+ cells, coefficient of variation = 15%). All neurons showing positive immunoreactivity for AVP, or Fos, or colabeled neurons with both AVP and Fos, were counted (Fig. 1). Caution was taken not to duplicate neuron counts across different optical slices. Scores were cumulated per animal across 3 image stacks, leading to total AVP+ (i.e., cells with only AVP+ identities + colabeled neurons), total Fos+ (i.e., cells with only Fos+ identities + colabeled neurons), colabeled neurons with both AVP+ and Fos+, and the total number of DAPI-positive nuclei. Cumulated numbers were then expressed as the percentage of DAPI-positive nuclei and then statistically analyzed. The experimenters remained blind to the experimental treatment groups while scoring.

Table 1. Effect size and type 1 error probabilities for imaging and behavioral endpoints

Endpoint	Interaction	DTR vector ²		OE vector ³	
		<i>p</i> value ⁴	Hedges' <i>g</i> ⁵	<i>p</i> value ⁴	Hedges' <i>g</i> ⁵
Percent AVP+ in MePD	$F_{(1,26)} = 63.63$; $p < 0.001$	<0.0001	4.4	0.0026	1.7
Percent Fos+ in MePD	$F_{(1,26)} = 30.79$; $p < 0.001$	0.0389	1.0	<0.0001	3.4
Percent colabeled in MePD	$F_{(1,26)} = 50.48$; $p < 0.001$	<0.0001	1.9	<0.0001	4.0
Time spent in bobcat bisect	$F_{(1,26)} = 7.88$; $p = 0.007$	0.0527	0.8	0.0527	0.9

¹ *F*, *df*, and *p* values for interaction obtained during ANOVA for 2 between-subject sources of variance (Cre genotype and viral vector).

² Cre− > Cre+ for all intergroup comparisons when infused with vector containing diphtheria toxin receptor.

³ Cre− < Cre+ for all intergroup comparisons when infused with overexpression vector.

⁴ Probability of type 1 error for comparison between Cre− and Cre+ littermates.

⁵ Standardized effect size. 1 *g* = an intergroup difference of 1 SD.

Analysis and Statistics

Each individual animal was used as a biological replicate. The number of animals is depicted in each of the figures, along with raw values. Statistical analysis was carried out using GraphPad Prism 7.0 software.

All endpoints were tested for normality of the distribution using the Shapiro-Wilk test [47]. Several imaging and behavioral endpoints significantly departed from normality. For consistency, all data were rank transformed before subsequent analysis [48], and normality of the transformed data was reconfirmed. Figures represent both transformed and nontransformed data. Probabilities of type 1 error were consistent regardless of whether rank transformation was used. Outliers were examined using the ROUT method with a maximum false discovery rate set at 1% [49]. No outliers were found in the rank-transformed data.

A two-way analysis of variance was conducted with genotype (presence or absence of Cre) and vector (experimentally infused with a viral vector containing either floxed DTR or floxed AVP) as between-subject sources of variance. All endpoints exhibited significant interaction between genotype and experimental infusion. We further conducted 2 post hoc comparisons using Fisher's LSD test (animals with or without Cre, infused with viral vector containing DTR, and animals with or without Cre, infused with viral vector containing AVP overexpression, OE). We avoided nonorthogonal omnibus post hoc comparisons to avoid inflation of significant values associated with multiple testing [50]. The *t*_{df} values and associated *p* values for individual comparisons are stated within the Results and in the Figures.

The magnitude of intergroup differences was computed using Hedges' unbiased *g* (the difference between 2 means divided by the pooled SD for the data) [51]. This is a standardized measure of effect size independent of mean quantities, reported in the Results and in Table 1 along with 95% CIs. A *g* of 1 indicates intergroup differences equal to 1 SD.

Results

Briefly, we manipulated those MePD neurons that have an endogenous AVP identity (MePD-AVP+ neurons) using viral vectors containing cargos with Cre-dependent expression. Animals containing a gene for Cre recombinase (Cre+) were compared with corresponding control littermates without Cre (Cre−). We strove to ablate MePD-AVP neurons using a vector containing DTR and overexpressing AVP in this neuronal population with a vector containing AVP gene driven from a robust promoter (OE).

Experimental Manipulation of MePD-AVP+ Neurons

Analysis of variance (ANOVA) was conducted with vector (DTR or OE) and genetic background of recipient animals (Cre+ or Cre−) designated as between-subject sources of variance (Fig. 2). Rank-transformed data were used. ANOVA revealed statistically significant interaction between vectors and Cre genotype ($F_{[1,26]} = 63.6$, $p < 0.001$). The interaction accounted for 59% of the total variance in the design ($\Delta\bar{x} \pm 95\%$ CI 26.6 ± 6.9 ranks). The main effect of vectors was significant ($F_{[1,26]} = 5.0$, $p = 0.034$). The main effect of Cre genotypes was also significant ($F_{[1,26]} = 9.6$, $p = 0.005$).

We used 2 comparisons to analyze the effects of DTR and OE treatments on immunohistologically detected MePD-AVP+ neurons, comparing Cre+ and Cre− littermates in each instance using Fisher's LSD test. Expres-

Fig. 2. Effects of experimental manipulations on arginine vasopressin (AVP) neurons within the posterodorsal medial amygdala (MePD) after partial ablation using diphtheria toxin receptor (DTR; **a**) or overexpression (OE; **b**). The number of AVP+ neurons relative to total neurons counted is depicted on the ordinates after rank transformation. Violin plots in these panels depict me-

dians and interquartile ranges along with raw values for all data points (n underneath the abscissa). Solid lines represent mean rank for that treatment. **c** Mean intergroup differences due to Cre (i.e., mean difference between Cre+ and Cre- animals for DTR and OE, respectively) along with the probability of type 1 error and 95% CIs for rank-transformed data.

sion of DTR and subsequent administration of diphtheria toxin resulted in a robust ablation of MePD-AVP+ neurons ($t_{26} = 8.1$, $p < 0.001$; $\Delta\bar{x} = 18.5 \pm 2.3$ ranks). The number of AVP+ neurons in all DTR animals (expressed as a percentage of total cells counted) was lower than the minima of non-DTR controls (Hedges' $g = 4.4$). In contrast, Cre+ animals receiving OE vector exhibited a substantial increase in immunohistologically revealed MePD-AVP+ neurons compared to Cre- littermates ($t_{26} = 3.3$, $p = 0.003$; $\Delta\bar{x} = 8.1 \pm 2.4$ ranks). Only 2 of 7 control animals exhibited more neurons with detectable AVP compared to the minima of Cre+ littermates (Hedges' $g = 1.7$). Thus, DTR caused a partial ablation, and OE increased the number of MePD-AVP+ neurons that could be visualized above the detection threshold of the staining.

Online supplementary Figure 2 depicts nontransformed data in addition to associated p values for Shapiro-Wilk tests and probabilities of type 1 error for MePD-AVP+ neurons. One of the 4 experimental groups exhibited nonnormal distribution; thus, nonparametric statistics was used to compare intergroup differences. Expression of DTR significantly reduced and AVP overexpression increased the frequency of MePD-AVP+ neurons, congruent with rank-transformed analysis depicted in the preceding paragraphs. Infusion of DTR within MePD did not significantly alter the number of AVP+ neurons in the paraventricular nucleus of the hypothalamus ($t_{14} = 0.7$, $p = 0.497$; $\Delta\bar{x} = 2.8 \pm 4.0\%$), demonstrating that the effects were restricted to the effects of viral vector specific to the infusion site and did not generalize to other sites with endogenous AVP expression.

Fig. 3. Effects of experimental manipulations on posterodorsal medial amygdala (MePD) neurons showing a marker for recent neuronal activity during cat odor exposure (Fos). Data are presented for partial ablation using diphtheria toxin receptor (DTR; **a**) or overexpression (OE; **b**). The number of Fos-positive neurons relative to total neurons counted is depicted on the ordinate after rank transformation. Violin plots in these panels depict medians and

interquartile ranges along with raw values for all data points (n underneath the abscissa). Solid lines represent mean rank for that treatment. **c** Mean intergroup differences due to Cre (i.e., mean difference between Cre+ and Cre- animals for DTR and OE, respectively) along with the probability of type 1 error and 95% CIs for rank-transformed data.

Change in MePD AVP+ Neurons Altered Recent Neuronal Activity in Response to Cat Odor

The presence of the intranuclear product of immediate-early gene cFos was used as a proxy for recent neuronal activity. Animals were exposed to cat urine, and recent neuronal activity was later inferred by counting Fos+ MePD neurons normalized to the total number of neurons imaged. ANOVA was conducted using rank-transformed data (Fig. 3; 2 between-subject sources of variance: DTR or OE, and Cre+ or Cre-). ANOVA revealed statistically significant interaction between vectors and Cre genotype ($F_{[1, 26]} = 30.8$, $p < 0.001$). The interaction accounted for 47% of the total variance in the design ($\Delta\bar{x} = 23.8 \pm 8.8$ ranks). The main effect of vectors was

not significant ($F_{[1, 26]} = 3.7$, $p = 0.064$). The main effect of Cre genotypes was significant ($F_{[1, 26]} = 6.6$, $p = 0.016$).

Expression of DTR in AVP+ neurons resulted in significant reduction in immunohistologically revealed MePD Fos+ neurons compared to respective Cre- controls ($t_{26} = 2.2$, $p = 0.039$; $\Delta\bar{x} = 6.4 \pm 2.9$ ranks). The number of Fos+ neurons in all except 1 non-DTR animal was higher than the median of DTR animals (Hedges' $g = 1.0$). A more robust response was found when comparing OE animals with respective controls. Overexpression of AVP elicited a large increase in Fos+ neuron ($t_{26} = 5.6$, $p < 0.001$; $\Delta\bar{x} = 17.4 \pm 3.1$ ranks). All animals in the OE groups exhibited a greater number of Fos+ neurons than non-OE Cre- controls (Hedges' $g = 3.4$).

Fig. 4. Effects of experimental manipulations on posterodorsal medial amygdala (MePD) neurons colabeled with AVP and Fos in response to cat odor exposure. Data are presented for partial ablation using diphtheria toxin receptor (DTR; **a**) or overexpression (OE; **b**). The number of colabeled neurons relative to total neurons counted is depicted on the ordinate after rank transformation. Vio-

lin plots in these panels depict medians and interquartile ranges along with raw values for all data points (n underneath the abscissa). Solid lines represent mean rank for that treatment. **c** Mean intergroup differences due to Cre (i.e., mean difference between Cre+ and Cre- animals for DTR and OE, respectively) along with the probability of type 1 error and 95% CIs for rank-transformed data.

Online supplementary Figure 3 depicts nontransformed data in addition to associated p values for Shapiro-Wilk tests and probabilities of type 1 error for MePD-Fos+ neurons. None of the experimental groups exhibited statistically significant departure from normality of the distribution; thus, parametric statistics was used to compare intergroup differences. Expression of DTR significantly reduced and AVP overexpression increased the frequency of MePD-Fos+ neurons, congruent with rank-transformed analysis depicted in the preceding paragraphs.

Ablation and Overexpression of AVP Divergently Affected the Recruitment of MePD-AVP+ Neurons

We further quantified the number of cells showing the colabeling of AVP and Fos. A two-way ANOVA was con-

ducted using rank-transformed data (Fig. 4). ANOVA revealed statistically significant interaction between vectors and Cre genotype ($F_{[1, 26]} = 50.5, p < 0.001$). The interaction accounted for 41% of the total variance in the design ($\Delta\bar{x} = 22.3 \pm 6.5$ ranks). The main effect of vectors was significant ($F_{[1, 26]} = 45.3, p < 0.001$). The main effect of Cre genotypes was not significant ($F_{[1, 26]} = 0.1, p = 0.732$).

Expression of DTR in AVP+ neurons resulted in a large reduction in colabeled MePD neurons in Cre+ animals compared to respective controls ($t_{26} = 4.9, p < 0.001$; $\Delta\bar{x} = 10.6 \pm 2.1$ ranks). The number of colabeled neurons in all DTR-Cre- animals was higher than the median of DTR-Cre+ animals (Hedges' $g = 1.9$). Congruently, overexpression of AVP elicited a huge increase in colabeled MePD neurons ($t_{26} = 5.1, p < 0.001$; $\Delta\bar{x} = 11.7 \pm 2.3$ ranks).

Fig. 5. Stochastic expectation and observed frequency of colabeled neurons across experimental groups. Data are presented for partial ablation using diphtheria toxin receptor (DTR; **a, b**) or overexpression (OE; **c, d**) for animals without (**a, c**) or with Cre (**b, d**). Expected and observed frequencies for individual animals are depicted using interconnected lines.

All animals in the OE groups exhibited a greater number of colabeled neurons than non-OE controls (Hedges' $g = 4.0$).

Online supplementary Figure 4 depicts nontransformed data in addition to associated p values for Shapiro-Wilk tests and probabilities of type 1 error for colabeled neurons. None of the experimental groups exhibited statistically significant departure from normality of the distribution; thus, parametric statistics was used to compare intergroup differences. Expression of DTR significantly reduced and AVP overexpression increased the frequency of colabeled neurons, congruent with rank-transformed analysis depicted in the preceding paragraphs.

Effects of AVP overexpression were especially robust when compared to partial ablation. For example, more

than a third of AVP+ neurons ($35.3 \pm 3.1\%$) exhibited colabeled Fos expression in the OE group compared to 11.5% (± 1.8) AVP+ neurons in the respective controls (Student t test; $t_{12} = 6.61$, $p < 0.001$). Similar comparison for DTR-Cre+ and respective control animals was not statistically significant ($t_{14} = 0.51$, $p = 0.619$).

To analyze if the number of colabeled neurons was reflective of the stochastic chance of encountering a Fos+ and AVP+ neuron, we calculated the expected frequency of colabeling for each animal as the mathematical product of AVP and Fos frequencies. Expected and observed frequencies for each experimental group were rank transformed. The expected frequency was then compared to the observed frequency of colabeling using paired t test (Fig. 5). Animals in the OE-Cre+ group exhibited colabeling that was significantly higher than the

Fig. 6. Effects of experimental manipulations on the aversion to predator odor. Data are presented for partial ablation using diphtheria toxin receptor (DTR; **a**) or overexpression (OE; **b**). Time spent in the vicinity of bobcat urine is depicted on the ordinate after rank transformation. Violin plots in these panels depict me-

dians and interquartile ranges along with raw values for all data points (n underneath the abscissa). Solid lines represent mean rank for that treatment. **c** Mean intergroup difference for DTR and OE along with the probability of type 1 error and 95% CIs for rank-transformed data.

stochastic expectation ($t_6 = 6.39$, $p < 0.001$). In contrast, the difference between expected and observed colabeling frequencies was not different in OE-Cre- animals ($t_6 = 0.58$, $p > 0.58$). This pattern was reversed during the analysis of DTR-Cre- and DTR-Cre+ animals. Observed frequency in DTR-Cre- animals was significantly lower than probabilistic expectation ($t_7 = 4.71$, $p = 0.002$). Animals in DTR-Cre+ animals did not exhibit significant departure between observed and expected frequencies ($t_7 = 1.05$, $p > 0.30$).

Online supplementary Figure 5 depicts non transformed data in addition to associated p values for Shapiro-Wilk tests and probabilities of type 1 error for expected and observed frequencies. None of the observed or expected frequencies across 4 experimental groups departed from a normal distribution in a statistically significant manner. Thus, parametric statistics was used to

compare intergroup differences, leading to observations congruent with rank-transformed analysis depicted in the preceding paragraphs.

Change in MePD AVP+ Neurons Altered Innate Aversion to a Predator Odor

A nonparametric test revealed that animals in both experimental groups treated with DTR vector (Cre+ and Cre-) showed a significant aversion to bobcat urine (Wilcoxon signed rank test, against the theoretical median of 600 s; $p \leq 0.001$; median = 70 s for DTR-Cre- and 20 s for DTR-Cre+). Animals in the OE-Cre- group showed a significant aversion to bobcat urine (Wilcoxon signed rank test, against the theoretical median of 600 s; $p < 0.001$; median = 40 s). In contrast, animals in the OE-Cre+ group did not show statistically significant aversion to bobcat urine ($p = 0.078$; median = 248 s).

Fig. 7. Correlation between the percentage of posterodorsal medial amygdala neurons colabeled with arginine vasopressin and Fos and time spent near cat odor. Rank-transformed data were used. A linear regression trend along with 95% CI bounds is also depicted.

We measured innate aversion by quantifying time spent by animals near bobcat urine placed in the terminus of a rectangular arena. Two-way ANOVA (Fig. 6) revealed statistically significant interaction between vectors and Cre genotype ($F_{[1, 43]} = 7.9, p = 0.007$). The interaction accounted for 15% of the total variance in the design ($\Delta\bar{x} = 22.3 \pm 16$ ranks). The main effect of vectors was not significant ($F_{[1, 43]} = 0.1, p = 803$). The main effect of Cre genotypes was not significant ($F_{[1, 43]} = 1.3, p = 0.268$).

Expression of DTR in AVP+ neurons resulted in a large reduction in time spent near predator odor in Cre+ animals compared to respective controls ($t_{43} = 2.0, p = 0.05$; $\Delta\bar{x} = 10.1 \pm 5.1$ ranks). Ranks for all except 3 DTR-Cre- animals were higher than the mean of all DTR-Cre+ animals (Hedges' $g = 0.8$). Congruently, overexpression of AVP elicited a huge decrease in aversion to bobcat odor ($t_{43} = 2.0, p = 0.05$; $\Delta\bar{x} = 12.1 \pm 6.1$ ranks). All except 1 animal in the OE-Cre+ group exhibited ranks higher than the mean of corresponding OE-Cre- controls (Hedges' $g = 0.9$).

Online supplementary Figure 6 depicts nontransformed data in addition to associated p values for Shapiro-Wilk tests and probabilities of type 1 error for time spent near predator odor. Three out of 4 experimental groups exhibited statistically significant departure from normal distribution; thus, nonparametric statistics was used to compare intergroup differences. Expression of DTR significantly decreased and AVP overexpression increased time spent near predator odor, congruent with rank-transformed analysis depicted in the preceding paragraphs.

Amount of time spent near bobcat odor by OE-Cre- and OE-Cre+ animals was positively correlated with the frequency of colabeled MePD neurons (Fig. 7; Spearman $r = 0.77$, 95% confidence interval = 0.39–0.93; $p = 0.002$; 14 XY pairs). Such correlation was absent in DTR-Cre- and DTR-Cre+ animals (Spearman $r = 0.04$, 95% confidence interval = -0.48 to 0.53; $p = 0.897$; 16 XY pairs).

Discussion

Results in this report suggest that MePD-AVP+ neurons inversely regulate defensive response to a predator odor. Experimental manipulation that raises the amount of AVP within MePD-AVP+ neurons leads to preferential recruitment of these neurons during cat odor exposure and reflects lower aversion. The preferential recruitment in this case is evidenced by the extent of colabeling that surpasses the probabilities calculated based on random chance and on individual frequencies of AVP and Fos-labeled cells. Congruently, experimentally achieved partial ablation of MePD-AVP+ neurons causes greater aversion to cat odors. We will first contextualize the numerical magnitude of these observations. We will then interpret these results in light of known functions of the MePD and male gonadal hormones. We will then provide some testable hypotheses to further confirm or reject our interpretation.

Table 1 summarizes effect sizes observed in the present experiments along with corresponding probabilities of type 1 error. There are 2 principal ways to contextualize

the magnitude of these effect sizes. The first avenue is the conventional frame of reference that defines effect sizes >0.8 as being “large” [52]. At this arbitrary number, $\sim 79\%$ of the treated population is expected to reside above the mean of the control population, and there is $\sim 71\%$ chance that an animal picked at random from the treated group will have a higher rank than an animal picked at random from the corresponding control group. All reported effects in Table 1 either meet or exceed this conventional criterion for the large effect size. The second avenue is to compare the effect size with prior reports that examine the same neuronal population or the same behavioral phenotype. MePD-AVP+ neurons were earlier reported to be preferentially recruited during exposure to the odor of estrus females in rats [37]. The estimated effect size for this effect is ≈ 2.2 , which compares very well with g of 4.0 or 1.9 observed in the present report for overexpression and ablation, respectively. Interestingly, the effect we observe in response to AVP overexpression is quantitatively large and in the same direction as expected in response to sexual experiences rather than cat odor exposure being used in the experiment. Similarly, infection with the protozoan parasite *Toxoplasma gondii* is known to cause a reduction in aversion to predator odor similar to the effects of AVP overexpression observed in this report [53]. The effect sizes for these experiments are estimated to be 0.7 [53], which again compares favorably with g of 0.7–0.8 observed for the behavioral effects in this report. Thus, partial ablation of MePD-AVP+ neurons robustly increases innate aversion to cat odor. Similarly, AVP overexpression in the same neuronal population robustly decreases innate aversion and congruently causes markedly increased recruitment of MePD-AVP+ neurons in response to cat odor exposure.

We did not measure sociosexual behaviors per se in the present report. Yet, multiple strands of evidence suggest that MePD-AVP+ neurons are involved in male sexual behaviors, especially the appetitive aspects of reproduction. Adult male rats have a MePD that is almost double the volume of the female nucleus [54]. Sexual dimorphism in MePD is similar to the bed nucleus of the stria terminalis, an analogous structure within the medial aspect of the extended amygdala. Such sexual dimorphism requires the continual presence of testosterone because castration renormalizes male MePD volume to the level seen in females, and exogenous testosterone supplement reinstates the volume to the precastration level [32]. Similarly, castrated animals no longer exhibit the presence of AVP-containing axons projecting from the MePD and bed nucleus of stria terminalis, while testosterone supple-

mentation reinstates AVP in these efferents [55]. In rats, testosterone creates an epigenetic change within the bed nucleus of stria terminalis in the form of DNA hypomethylation on the AVP promotor [33]. Similarly, AVP promotor in mice MePD undergoes DNA hypomethylation in response to testosterone [56]. These observations provide a molecular link between male gonadal hormones and ongoing plasticity in adult MePD-AVP+ neurons. Placement of testosterone within MePD reconstitutes sexual motivation in castrated animals [2, 57], further supporting the notion that testosterone effects of male sexual behavior requires intact MePD.

MePD-AVP+ efferents project to brain regions that are involved in the regulation of motivational states [13, 15]. For example, AVP+ fibers from the MePD and bed nucleus of stria terminalis are found in the lateral septum, an essential regulator of the mesolimbic dopamine system [17]. The functional significance of AVP projections within the lateral septum is supported by observations that infusion of AVP within this brain region rescues progesterone-induced impairment of social recognition in male rats [14, 58]. These fibers are also seen in the ventral pallidum, a part of the mesolimbic system, where antagonism of the AVP receptor reduces sociosexual behaviors [19]. Thus, MePD-AVP+ neurons have appropriate axonal projections that can reliably alter male-specific behaviors.

Castrated male mice show less innate aversion to bobcat odors when they are supplemented with exogenous testosterone [55]. It is likely that the effects of testosterone on fear flows through its effects on MePD-AVP+ neurons. This is supported by the observation that experimentally creating DNA hypomethylation within rat MePD, akin to the effects exerted by testosterone, reduces defensive behaviors [59]. Individual rats that show proactive investigatory forays towards cat fur also show concomitant DNA hypomethylation of AVP promotor within MePD [60]. These observations suggest that testosterone-responsive MePD-AVP plasticity has behavioral consequences for defensive behaviors apart from the better-studied role of this neuronal population in male-specific reproduction. In this report, experimental treatment that increased recruitment of MePD-AVP+ neurons reduced innate aversion to cat odor, and treatment that reduced the total amount of MePD-AVP+ neurons increased aversion to cat odor. Cat odors are believed to be processed primarily by the posteroventral medial amygdala [20, 21, 61]. Ablation of this subnucleus, for example, reduces aversion to cat odors [27]. In contrast, MePD is thought to be involved in sociosexual

behaviors. The data in the present report suggest that the dichotomy between posteroventral and posterodorsal aspects of the medial amygdala is, in fact, not absolute. While neuronal activity within the posteroventral medial amygdala is needed for defensive behaviors, neuronal activity within the MePD suppresses aversion to predator cues.

The present observations suggest that increasing the amount of AVP in MePD neurons that endogenously express AVP results in an increase in the activation of these neurons upon predator odor exposure. AVP in the brain binds to both its canonical receptor (AVP1aR) and oxytocin receptor (OXTR) [62]. AVP and oxytocin contain 7 similar out of 9 constituent amino acids. AVP1aR and OXTR, similarly, show high sequence homology [63], with AVP exhibiting the same binding affinity to both of these receptors [62]. The effects of AVP on rodent social behaviors involve both AVP1aR and OXTR [64]. Thus, the effects of AVP overexpression or ablation can flow from brain regions that express either of these 2 receptors [65, 66]. In adult mice, the posterior medial amygdala contains copious amounts of AVP-immunoreactive fibers [13, 17, 67] but sparse AVP1aR density [68, 69]. This brain region also shows low numbers of oxytocin-containing fibers in male mice, with dense OXTR distribution [70–72]. Furthermore, castration simultaneously reduces the amount of AVP transcription [56] and OXTR binding density [73] within the medial amygdala, while leaving hypothalamic sources of the AVP intact. These observations suggest that the effects of AVP in MePD might result from the interaction of AVP-containing axons and OXTR present in the vicinity.

An interesting corollary to the present observation can also be found during infection of rats by the protozoan parasite *T. gondii*. The complex life cycle of this parasite requires the predation of chronically infected animals by cats in order for its gametogenesis [74]. Several experiments have shown that *T. gondii* infection reduced the innate aversion of infected rats to cat odors [53, 75]. This likely increases predation rates and hence transmission of the parasite; however, direct evidence of increased predation is lacking. What is interesting in the present backdrop is the mechanism by which innate aversion is reduced. Infected animals show higher transcription of AVP within MePD [59]. In addition, MePD-AVP+ neurons in the infected animals show evidence of recent neuronal activity when rats are exposed to cat odor [59] in parallel to a greater than expected chance of colabeling between AVP and immediate-early gene markers of recent activity. This phenomenon is concomitant with in-

creased testosterone production in the infected rats [76] and establishment of behaviors that are testosterone dependent [77, 78] – thus stressing the underlying role of interactions between testosterone, the MePD-AVP+ system, and its efferents in the regulation of defensive behaviors. While the *T. gondii* model points towards a possibility that MePD-AVP+ neurons might be involved in the reduction of defensive behavior towards predators, this model stops short of tearing apart the causal aspects. It is not possible to separate out a host of inflammatory and neural changes that co-occur during a chronic infection. Present observations go one step further from correlations described in the *T. gondii* model and show that an experimental increase in MePD-AVP is sufficient to reduce innate aversion.

The emerging narrative then is that MePD-AVP neurons inversely titrate defense to predation in addition to engaging in sociosexual behaviors. Imperatives of sex and defense are typically contrary to each other [2–7]. Maximal defense from predators precludes investment in sexual advertisements and sexual pursuits. Thus, it is plausible that behaviors that underpin sexual behavior could be syndromic with the reduction in antipredator behaviors. Reproduction could be favored over the defense in ecologies where payoff from current reproduction outweighs opportunity costs in terms of survival and residual reproduction [79–82]. We posit that the MePD-AVP system is a proximate link in the negotiation of such conditional calibration of defense in the face of current reproduction. The medial amygdala is the first site of convergence for olfactory information from the main and accessory olfactory system. This suggests that the medial amygdala has access to information about the availability of sexual opportunities in the external environment [10, 11]. The medial amygdala, specifically AVP neurons within MePD, is also responsive to testosterone and its metabolites [2, 15, 28, 30–33]. This responsiveness takes the form of DNA hypomethylation in the AVP promoter [33]. This epigenetic change provides the medial amygdala further information about the internal metabolic milieu and the possibility of a metabolic reserve that would be presumably needed for investment in sexual pursuits. The confluence of information about external and internal cues pertaining to the possibility of current reproduction might allow MePD-AVP neurons to initiate a behavioral sequela characterized by reduced defense and increased reproduction. In other words, the MePD-AVP system acts as an arbitrator for the trade-off between current and residual reproduction in male mice.

We provide a proximate justification for the hypothesis described in the previous paragraph. Partial ablation of the MePD-AVP system increased fear of cat odors, and MePD-AVP overexpression reduced the same fear. This leads to at least 2 testable predictions for future experiments. If MePD-AVP is involved in trade-offs between current and residual reproduction, then the manipulation of this system should not change defense response to predators where such a trade-off is not ecologically relevant. This can be tested, firstly, by comparing a semelparous species with phylogenetically related iteroparous species. Our hypothesis will be supported if overexpression of AVP (or its analogous peptide, vasotocin, in birds) reduces defensive behavior in the iteroparous but not semelparous counterpart. It can also be tested, secondly, by comparing a monogamous species with reduced sexual selection with phylogenetically related polygynandrous species. Our hypothesis will be supported if overexpression of MePD-AVP leads to loss of defensive behaviors in polygynandrous species but not the monogamous counterpart.

In conclusion, we provide evidence that experimental manipulation of a nonapeptide neuronal population, known to have a role in sexual behaviors, can concomitantly regulate innate aversion to predator cues in mice. We posit that this phenomenon reflects trade-offs inherent in investments between sexual reproduction and defense.

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Statement of Ethics

All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the Nanyang Technological University.

Conflict of Interest Statement

Authors declare the absence of any competing interests.

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Author Contributions

W.H.T. carried out the experiments, performed the data analysis, and prepared the manuscript; S.A.-S. carried out the experiments; A.V. performed the data analysis, conceived the idea, supervised the research, and prepared the manuscript. All authors approved the final manuscript.

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