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Midbrain neurons important for the production of mouse ultrasonic vocalizations are not required for distress calls

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Summary

A fundamental feature of vocal communication is that animals produce vocalizations with different acoustic features in different behavioral contexts (i.e., contact calls, territorial calls, courtship calls, etc.). The midbrain periaqueductal gray (PAG) is a key region that regulates vocal production, and artificial activation of the PAG can elicit the production of multiple species-typical vocalization types.[1–9] How PAG circuits are organized to regulate the production of different vocalization types remains unknown. On the one hand, studies have found that partial PAG lesions abolish the production of some vocalization types while leaving others intact,[3,8,10,11] suggesting that different populations of PAG neurons might control the production of different vocalization types. On the other hand, electrophysiological recordings have revealed individual PAG neurons that increase their activity during the production of multiple vocalization types,[12–14] suggesting that some PAG neurons may regulate the production of more than one vocalization type. To test whether a single population of midbrain neurons regulates the production of different vocalization types, we applied intersectional methods to selectively ablate a population of midbrain neurons important for the production of ultrasonic vocalizations (USVs) in mice. We find that although ablation of these PAG-USV neurons blocks USV production in both males and females, these neurons are not required for the production of distress calls. Our findings suggest that distinct populations of midbrain neurons control the production of different vocalization types.

Keywords

vocalization; ultrasonic; periaqueductal gray; caspase

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Author contributions

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P.Z. and K.A.T. designed the experiments. P.Z. conducted the experiments. P.Z., Y.W., Z.H., and K.A.T. analyzed the data. P.Z. and K.A.T. wrote the manuscript, and all authors approved the final version.

Declaration of Interests

The authors declare no competing interests.

Results

In the past, the inability to selectively manipulate vocalization-related PAG neurons made it challenging to determine whether a single population of PAG neurons regulates the production of multiple vocalization types. The recent identification of specialized neurons in the caudolateral PAG whose activity is important for the production of male mouse courtship ultrasonic vocalizations (PAG-USV neurons[15]) opens the door to understanding whether these neurons are also required for the production of other vocalization types. Mice produce USVs during same-sex and opposite-sex social interactions[16–23] and also produce human-audible distress calls (i.e., squeaks) in aversive contexts.[24–26] In the current study, we tested whether selective ablation of PAG-USV neurons blocks the production of squeaks.

Ablation of PAG-USV neurons blocks USV production in both male and female mice

To ablate PAG-USV neurons in male and female mice, we used the TRAP2 activity-dependent labeling strategy.[27,28] Because this approach differs from the activity-dependent labeling method used previously to manipulate PAG-USV neurons,[15] we first confirmed that TRAP2-mediated ablation of PAG-USV neurons blocks USV production. In addition, because previous work tested the effects of PAG-USV manipulations on USV production in males only, here we also tested the effects of PAG-USV ablation on female USV production.

Briefly, the caudal PAG of male and female TRAP2;Ai14 mice was injected bilaterally with a virus driving the Cre-dependent expression of caspase (AAV-FLEX-taCasp3-TEVp; Figure 1A, Figure S1A–C). Mice were subsequently returned to group-housing with their same-sex siblings for 11 days and were single-housed for 3 days prior to the first behavioral measurements to promote high levels of social interaction and USV production.[29] Two weeks following viral injections (day 14), male and female subject mice were given 30-minute social encounters with a novel, group-housed female in their home cage, a context which elicits high rates of USV production in both males and females.[29] Following these social encounters, mice received IP injections of 4-hydroxytamoxifen (4-OHT), which enables the transient expression of Cre recombinase in recently active neurons, hence permitting the expression of caspase in PAG-USV neurons. Ten days later, male and female subjects were given a second 30-minute social encounter with a novel, group-housed female, and rates of USV production and non-vocal social behaviors were compared between the pre-4-OHT and post-4-OHT behavior sessions.

Using this approach, we first measured the effects of PAG-USV ablation on male USV production. Previous studies using microphone arrays to localize and assign USVs to individual mice reported that males produce ~85% of total USVs recorded during male-female interactions.[16,30] Based on these findings, we assumed that the majority of USVs recorded during our male-female interactions were produced by the male. Consistent with previous work,[15] ablation of PAG-USV neurons dramatically reduced USV production in male mice (Figure 1B, black symbols; N = 11 PAG-USV_{casp} males; pre-4-OHT mean USVs = 1582 ± 928; post-4-OHT mean USVs = 53 ± 84; $p < 0.001$, two-way ANOVA with repeated measures on one factor and post-hoc Tukey's HSD tests; see Table S1 for complete statistical details). In contrast to USV production, the amount of time males spent engaged in non-vocal interactions with females was not affected by PAG-USV ablation

(Figure 1C, black symbols; includes both time spent investigating female and time spent mounting female; mean proportion time interacting with female pre-4-OHT = 0.25 ± 0.09; post-4-OHT = 0.26 ± 0.08; p = 0.77, two-way ANOVA with repeated measures on one factor and post-hoc Tukey's HSD tests; see Figure S1D–E for a more detailed consideration of non-vocal behaviors and Videos S1–S2 for examples of PAG-USV_{casp} males engaged in olfactory investigation of female social partners and female urine). In control TRAP2;Ai14 males with GFP expressed unilaterally in PAG-USV neurons, USV rates tended to decline (p = 0.06) and time spent interacting with females declined (p = 0.04; Figure 1B–C, green symbols; N = 12 PAG-USV_{GFP} males; pre-4-OHT mean USVs = 2309 ± 985 USVs; post-4-OHT mean USVs = 1664 ± 787 USVs; pre-4-OHT mean proportion time interacting with female = 0.31 ± 0.09; post-4-OHT mean proportion time interacting with female = 0.24 ± 0.06; see also Figure S1D–E). Given that rates of rates of USV typically correlate with rates of social interaction in male-female interactions,[29] we considered rates of USV production in the two groups of males relative to the amount of time they spent interacting with females (Figure 1D). This analysis revealed that PAG-USV_{GFP} males exhibit a small concomitant decrease in both USV production and social interaction time following 4-OHT treatment (i.e., no change in USVs per second interaction time), while PAG-USV_{casp} males exhibit a selective and robust decrease in USV production following ablation of PAG-USV neurons ($p < 0.001$ for change in USVs per second interaction for PAG-USV_{casp} males; p = 0.81 for difference in PAG-USV_{GFP} males). To more clearly quantify the magnitude of change in USV rates over time for each group, we also calculated the change in USV rates for each male (change in USV rate = total post-4-OHT USVs / total pre-4-OHT USVs). A comparison of these values showed that males that underwent PAG-USV ablation exhibited a much stronger decrease in USV production than control males (mean post-4-OHT / pre-4-OHT USVs for PAG-USV_{casp} males = 0.07 ± 0.12; mean post-4-OHT / pre-4-OHT USVs for PAG-USV_{GFP} males = 0.71 ± 0.32; $p < 0.001$, t-test). We note that these two groups of males were tested during different periods of time (see Table S2 for testing dates of mice in each experimental group) and with different groups of female social partners, raising the possibility that some factor unrelated to the expression of GFP in PAG-USV neurons might account for the small decrease in social motivation in PAG-USV_{GFP} males. Nonetheless, these small changes in behavior are distinct from the selective and robust decrease in USV production observed in the PAG-USV_{casp} males. In summary, we conclude that TRAP2-mediated ablation of PAG-USV neurons reduces USV production in male mice without affecting time spent engaged in social interactions with females, consistent with prior work.[15]

We next measured the effects of PAG-USV ablation on female USV production. Previous studies found that females vocalize at the highest rates during same-sex interactions [17,20,29] and that both females in a pair produce USVs during same-sex interactions.[17] Given these findings, we reasoned that we should see reduced (but non-zero) USV rates during female-female interactions in which one partner has undergone ablation of PAG-USV neurons. In line with this idea, we observed that ablation of PAG-USV neurons tended to reduce USV production during female-female interactions (Figure 1E, black symbols; N = 12 PAG-USV_{casp} females; pre-4-OHT mean USVs =1468 ± 599 USVs; post-4-OHT mean USVs = 579 ± 657; p = 0.06, two-way ANOVA with repeated measures on one factor and

post-hoc Tukey's HSD tests). Rates of USVs did not change over time in pairs containing a PAG-USV$_{GFP}$ female (Figure 1E, green symbols; N = 12 GFP females; pre-4-OHT mean USVs = 2060 ± 873 USVs; post-4-OHT mean USVs = 2410 ± 1273 USVs; p = 0.77). We next reasoned that we should see near-zero USV rates in female-female interactions in which both partners have undergone ablation of PAG-USV neurons. Consistent with this idea, we found that USV rates were near-zero during recordings from pairs of females that had both undergone ablation of PAG-USV neurons (Figure 1G, right column; mean USVs = 49 ± 36; N = 8 trials from pairs made up of N = 6 total PAG-USV$_{casp}$ females). These USV rates were significantly lower than USV rates recorded from control pairs containing 1 PAG-USV$_{GFP}$ female and 1 unmanipulated female (Figure 1G, left column; $p < 0.001$; mixed-model ANOVA with post-hoc Tukey's HSD tests). Ablation of PAG-USV neurons had no effect on the amount of time females spent engaged in non-vocal interactions with female partners (Figure 1F, black symbols; pre-4-OHT mean proportion time interacting with female partner = 0.25 ± 0.05; post-4-OHT = 0.26 ± 0.05), and control PAG-USV$_{GFP}$ females also showed no change in social interaction time following 4-OHT treatment (Figure 1F, green symbols; pre-4-OHT mean proportion time interacting with female partner = 0.24 ± 0.06; post-4-OHT = 0.30 ± 0.10; two-way ANOVA with repeated measures on one factor; $p > 0.05$ for main effects and interaction). In summary, we conclude that ablation of PAG-USV neurons blocks USV production in both female and male mice without affecting time spent engaged in social interactions with female partners.

PAG-USV neurons are not required for the production of squeaks

With these strategies in hand, we next asked whether ablation of PAG-USV neurons in males and females blocks the production of squeaks. The same PAG-USV$_{casp}$ and PAG-USV$_{GFP}$ males and females that were tested for USV production (Figure 1) were subjected to a mild footshock paradigm the day following their second social interaction (day 25, Figure 1A; each mouse received 10 footshocks delivered over 5 minutes, see STAR Methods). We selected a footshock intensity (0.5 mA) that reliably elicited squeaks in control PAG-USV$_{GFP}$ males and females (Figure 2A, right; 116/120 and 119/120 footshocks elicited squeaks in N = 12 control males and N = 12 control females, respectively). In contrast to the pronounced effects of PAG-USV ablation on USV production, the ablation of PAG-USV neurons did not block the production of squeaks, and both male and female PAG-USV$_{casp}$ mice produced squeaks reliably in response to footshock (Figure 2A, left; 120/120 and 120/120 footshocks elicited squeaks in N =12 PAG-USV$_{casp}$ males and N = 12 PAG-USV$_{casp}$ females, respectively; two-way ANOVA, $p > 0.05$ for main effects of sex, group, and interaction). We conclude that the activity of PAG-USV neurons is not required for the production of squeaks.

Although the activity of PAG-USV neurons is not required for the production of squeaks, it remains possible that the activity of these neurons plays a more subtle role in modulating the acoustic features of squeaks. We examined this possibility by quantifying the acoustic features of squeaks produced by PAG-USV$_{casp}$ and PAG-USV$_{GFP}$ mice (see STAR Methods). Although mean squeak duration was greater in females than in males, there was no effect of PAG-USV ablation on squeak duration (Figure 2B; two-way ANOVA, p = 0.002 for main effect of sex, $p > 0.05$ for main effect of group and interaction). Ablation

of PAG-USV neurons also had no effect on the mean dominant frequency of squeaks, and mean dominant frequency did not differ significantly between females and males (Figure 2C; two-way ANOVA, $p > 0.05$ for main effects and interaction). We next considered squeak amplitude. Please note that because the microphone was placed near the footshock chamber, audio clipping occurred during portions of squeaks in many trials (mean proportion of total squeaks affected by clipping by group: PAG-USV$_{casp}$ males = 0.94 ± 0.06, PAG-USV$_{casp}$ females = 0.97 ± 0.03, PAG-USV$_{casp}$ males = 0.99 ± 0.01, PAG-USV$_{casp}$ females = 0.99 ± 0.02; mean proportion clipped per individual squeak by group: PAG-USV$_{casp}$ males = 0.08 ± 0.03, PAG-USV$_{casp}$ females = 0.11 ± 0.05, PAG-USV$_{casp}$ males = 0.13 ± 0.03, PAG-USV$_{casp}$ females = 0.16 ± 0.05). We therefore report mean squeak amplitudes in volts, directly measured as the mean amplitude of the recorded audio waveform. Interestingly, although female mice on average produced louder squeaks than males (Figure 2D; two-way ANOVA, $p = 0.02$ for main effect of sex), overall mice that underwent ablation of PAG-USV neurons produced squeaks that were quieter than those produced by control PAG-USV$_{GFP}$ mice (Figure 2D, $p < 0.001$ for main effect of group; $p = 0.54$ for interaction; see Figure S2A for additional example spectrograms).

One possibility is that although PAG-USV neurons are not required for squeak production, the activity of these neurons regulates the amplitude of non-USV vocalizations. Another possibility is that the effects on squeak amplitude can be attributed to the ablation of non-PAG-USV neurons that increase their activity around the time of 4-OHT treatment. Although behavior sessions are designed to maximize USV production, mice also engage in various non-vocal behaviors and experiences that may increase activity in non-PAG-USV neuronal populations. For example, PAG neurons related to fear responses and nociception may increase their activity as mice are handled by the investigator and receive an IP injection of 4-OHT. To test whether we could recapitulate the effects on squeak amplitude following ablation of PAG neurons that are recruited by any such non-vocal/non-social experiences, we performed control experiments in which caudal PAG neurons were ablated in mice that were placed alone in their homecage inside the recording chamber for 30 minutes and then given an IP injection of 4-OHT (Figure 3A, N = 5 PAG-control$_{casp}$ males). Ablation of PAG-control$_{casp}$ neurons did not reduce rates of USV production (Figure 3B; $p = 0.16$, paired t-test), nor did it affect the acoustic features of USVs (pre-4-OHT mean duration = 47.0 ± 9.1 ms; post-4-OHT mean duration = 49.7 ± 9.0 ms; pre-4-OHT mean dominant frequency = 69.2 ± 4.4 kHz; post-4-OHT mean dominant frequency = 69.8 ± 3.3 kHz; pre-4-OHT mean dB relative to background = 14.9 ± 3.9 dB; post-4-OHT mean dB relative to background = 11.4 ± 1.0 dB; paired t-tests, $p > 0.05$ for all). Similarly, ablation of PAG-control$_{casp}$ neurons did not affect the production of squeaks (Figure 3C; 48/50 footshocks elicited squeaks in N = 5 PAG-control$_{casp}$ males; one-way ANOVA), mean squeak duration (Figure 3D), or mean squeak dominant frequency (Figure 3E; one-way ANOVAs, $p > 0.05$ for all). Notably, PAG-control$_{casp}$ males produced squeaks that were significantly lower in amplitude than squeaks produced by both PAG-USV$_{casp}$ and PAG-USV$_{GFP}$ males (Figure 3F; one-way ANOVA, $p < 0.001$ for all pair-wise post-hoc comparisons). This finding is consistent with the idea that PAG-USV neurons regulate neither the production nor the acoustic features of squeaks, and that reduced squeak amplitude in PAG-USV$_{casp}$ mice can be attributed to the ablation of non-PAG-USV neurons

that increase their activity during or shortly following the behavior session and 4-OHT treatment.

Finally, we tested whether ablation of PAG-USV neurons in female mice affects the production of squeaks produced in a social context, during courtship interactions with males. We found that 9 out of 9 PAG-USV$_{casp}$ females produced squeaks during interactions with males (Figure S2B), indicating that ablation of PAG-USV neurons does not block the production of squeaks in social contexts (mean number of squeaks produced = 106.3 ± 64.5 in N = 9 trials; 596 / 957 squeaks overlapped in time with male USVs). In summary, these results provide strong support for the idea that PAG-USV neurons do not regulate the production of squeaks.

Discussion

In the current study, we confirm previous work showing that the activity of a specialized population of PAG-USV neurons is important for USV production in male mice,[15] and we extend these findings to show that ablation of PAG-USV neurons also impairs USV production in females. In contrast to the robust effects on USV production, ablation of PAG-USV neurons did not block the production of squeaks and did not affect the duration or dominant frequency of squeaks produced by males and females in response to footshock. Although we observed a significant decrease in squeak amplitude following ablation of PAG-USV neurons, these effects were not specific to PAG-USV neuronal ablation and were also observed following ablation of control (non-PAG-USV) neurons in the caudal PAG. The fact that ablation of such control neurons failed to affect rates of USV production or USV acoustic features underscores the selective role of PAG-USV neurons in regulating USV production. Taken together, our findings support the idea that distinct populations of neurons control the production of USVs and squeaks.

The TRAP2 method should in principle allow us to express caspase in caudal PAG neurons that increase their activity in association with either vocal or non-vocal aspects of social interactions. The fact that ablation of male and female PAG-USV neurons robustly reduces USV production but does not decrease social interaction suggests that this region of the PAG may not contain neurons that regulate social interaction or social/sexual motivation more broadly, or at least that neural changes elsewhere can compensate for the ablation of such neurons, leaving social interaction rates unaffected in our post-4-OHT measurements from PAG-USV$_{casp}$ males and females. We favor the interpretation that this region of the PAG does not regulate social or sexual motivation, but additional experiments would be required to distinguish between these possibilities. In further support of the idea that the PAG contains neurons that specifically regulate USV production, a recent study found a transcriptionally-defined Esr1^{+} cluster of PAG neurons that exhibited increased IEG expression following male mating (which is associated with high rates of USV production) and also following production of isolation USVs from mouse pups.[31] Future experiments that employ functional manipulations of activity can test the exciting idea that this transcriptionally-defined cluster represents PAG-USV neurons that control USV production across development.

Given that adult mice often produce USVs in coordination with olfactory investigation of social partners and social cues, one possibility is that the activity-dependent ablation of caudal PAG affected neurons that regulate social sniffing (either PAG-USV neurons themselves or other caudal PAG neurons), and that this in turn contributes to or accounts for disruption of USV production. Because the production of squeaks is not linked to sniffing, ablation of such neurons would not be expected to affect the production of squeaks. Although our experiments cannot definitively rule out this possibility, three lines of evidence suggest that ablation of PAG-USV neurons does not disrupt sniffing. First, PAG-USV_{casp} mice continue to engage in head and body movements associated with anogenital investigation of social partners (Video S1). Second, we include a video of PAG-USV_{casp} males interacting with female urine (Video S2). Although we did not directly record respiration in these sessions, rapid movements of the whiskers, nose, and abdomen typically associated with sniffing can be seen. Finally, in our previous work, optogenetic activation of PAG-USV neurons elicited USV production but did not entrain or alter the ongoing rate of respiration.[15] Nonetheless, a detailed kinematic analysis of respiration following ablation of PAG-USV neurons would be required to fully rule out the possibility that social sniffing is disordered in PAG-USV_{casp} mice.

Neurons important for the production of squeaks remain to be identified. Although we do not know for certain that such neurons reside within the PAG, a wealth of previous work in non-murine species has shown that extensive lesions of the PAG cause complete mutism.[3–5] However, given the well characterized roles of the PAG in regulating fear behaviors and nociception and the fact that mice produce squeaks almost exclusively when in fear or when in pain, the use of activity-dependent labeling approaches to identify such neurons presents a particular challenge. A recent study in mice found that pharmacological inhibition of the dorsolateral PAG abolished the production of squeaks, but the concomitant effects on the expression of fear behaviors make it difficult to know whether this region of the PAG directly or indirectly regulates squeak production.[32] We note, however, that the region of the PAG targeted in that study overlaps with the portion of the PAG manipulated in the current study, raising the intriguing possibility that PAG-USV neurons and neurons that regulate the production of squeaks may reside in similar regions within the PAG. Given the well-known role of the hindbrain nucleus retroambiguus (RAm) and PAG projections to RAm in regulating vocal production,[4,5,15,33–38] an intersectional approach that restricts activity-dependent labeling to Ram-projecting PAG neurons may provide a useful future strategy to identify and characterize midbrain neurons that regulate the production of squeaks. It would be of great interest to characterize the axonal projections of PAG neurons important for squeak production and to compare them to those of PAG-USV neurons,[15] to begin to understand how differences in the activity of midbrain-to-hindbrain circuits underlie the production of distinct vocalization types.

Adult mice produce USVs predominantly during social interactions and in coordination with non-vocal social behaviors, with the highest rates of USVs produced by males and females during interactions with novel female social partners.[29,39] In contrast, male and female mice produce squeaks when in pain and during aversive contexts, and female mice also produce squeaks during social interactions with males.[26,32,40,41] In addition to being produced in relatively non-overlapping behavioral contexts, USVs and squeaks are also produced

via distinct sound production mechanisms (i.e., vibration of the vocal folds to generate squeaks vs. an intralaryngeal whistle mechanism to generate USVs).[42–47] In light of these differences, one interpretation of our results is that while distinct populations of midbrain neurons regulate the production of sonic and ultrasonic vocalizations, a single population may regulate the production of vocalizations types that share a sound production mechanism but differ in their contextual usage. For example, in rats, does a single population of PAG-USV neurons control the production of both 50 kHz and 22 kHz USVs? Another possibility is that distinct populations of midbrain neurons regulate the production of vocalization types that differ in their contextual usage, even if these vocalization types have similar sound production mechanisms. While the current study cannot distinguish between these possibilities, the present findings form an important foundation to understand how midbrain circuits are organized to regulate vocal communication across diverse species with diverse vocal repertoires.

STAR Methods

Resource availability

Lead contact—Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Katherine Tschida (kat227@cornell.edu).

Materials availability—No new materials were generated for this study.

Data and code availability—All source data generated have been deposited in a digital data repository and are publicly available as of the date of publication. All original MATLAB codes have been deposited in a digital data repository and are publicly available as of the date of publication. The DOI is listed in the Key Resources table. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

Experimental model and study participant details

Ethics statement—All experiments and procedures were conducted according to protocols approved by the Cornell University Institutional Animal Care and Use Committee (protocol #2020–001).

Subject details—Male and female TRAP2;Ai14 mice were housed with their siblings and both parents until weaning at postnatal day 21. TRAP;A14 mice were generated by crossing TRAP2 (Jackson Laboratories, 030323) with Ai14 (Jackson Laboratories, 007914). TRAP2;Ai14 males and females were used as subject animals in all experiments, and C57BL/6J females (Jackson Laboratories, 000664) were used as visitors. Mice were kept on a 12h:12h reversed light/dark cycle and given ad libitum food and water for the duration of the experiment.

Method details

Viral injections—The following viruses were used: AAV2/5-ef1alpha-FLEX-taCasp3-TEVp (Addgene, 45580) and AAV2/1-CAG-FLEX-EGFP-WPRE (Addgene, 51502). The

final injection coordinates for caudal PAG were: AP = −4.7 mm, ML = 0.7 mm, DV = 1.75 mm. Viruses were pressure-injected with a Nanoject III (Drummond) at a rate of 5 nL every 20 seconds. A total volume of 250 nl of virus was injected into the PAG (bilaterally for caspase virus, unilaterally for GFP virus).

Drug preparation—4-hydroxytamoxifen (4-OHT, HelloBio, HB6040) was dissolved at 20 mg/mL in ethanol by shaking at 37°C and was then aliquoted (75 uL) and stored at −20°C. Before use, 4-OHT was redissolved in ethanol by shaking at 37°C and filtered corn oil was added (Sigma, C8267, 150 uL). Ethanol was then evaporated by vacuum under centrifugation to give a final concentration of 10 mg/mL, and 4-OHT solution was used on the same day it was prepared.

Study design—To express either caspase or GFP in PAG-USV neurons, adult (>8 weeks) male and female subject mice first received injections of virus (bilateral injections of caspase virus for PAG-USV_{casp} mice, unilateral injections of GFP virus for PAG-USV_{GFP} mice) into the caudal PAG (day 0). Eleven days later, subject mice were single-housed for 3 days. On day 14, subject mice were given 30-minute social interactions with a novel, group-housed female visitor to elicit USV production (see below for details of vocal and non-vocal behavior recording and analysis). Following the social interaction, subject mice received IP injections of 4-OHT (50 mg/kg) to enable expression of viral transgenes in PAG-USV neurons. On day 24, subject mice were given a second 30-minute social encounter with a novel, group-housed female to measure effects of viral expression on USV production and non-vocal social behavior. On day 25, mice were subjected to a mild footshock paradigm to measure effects of viral expression on the production and acoustic features of squeaks.

To express caspase in control (non-PAG-USV) neurons of the caudal PAG, adult male mice first received bilateral injections of caspase virus into the caudal PAG (day 0). Eleven days later, males were single-housed for 3 days. On day 13, males were given 30-minute social interactions with a novel, group-housed female visitor to elicit and measure baseline USV production. On day 14, males in their home cages were placed inside the recording chamber but not given a social partner. After 30 minutes, males received IP injections of 4-OHT (50 mg/kg) to enable expression of caspase in control PAG neurons. On day 24, these PAG-$control_{casp}$ males were given a second 30-minute social encounter with a novel, group-housed female to measure effects of viral expression on USV production and non-vocal social behavior. On day 25, PAG-$control_{casp}$ males were subjected to a mild footshock paradigm to measure effects of viral expression on the production and acoustic features of squeaks.

USV recording and analyses—To elicit USVs, single-housed male and female subject mice were given a 30-minute social experience with a novel, group-housed female visitor in their home cage. Home cages were placed in a sound-attenuating chamber (Med Associates) and fitted with custom lids that allowed microphone access and permitted audio (and video) recordings. USVs were recorded with an ultrasonic microphone (Avisoft, CMPA/CM16), amplified (Presonus TubePreV2), and digitized at 250 kHz (Spike 7, CED). USVs were detected with custom Matlab codes[15] using the following parameters: mean frequency > 45 KHz; spectral purity > 0.3; spectral discontinuity < 1.00; minimum USV duration = 5 ms;

minimum inter-syllable interval = 30 ms). The duration of each USV was calculated as the difference between USV onset and offset. The mean dominant frequency of each USV was determined by calculating the dominant frequency at each time bin of the USV and then averaging across the syllable.[15] The amplitude of each USV was defined as the bandpower from 30–125 kHz, converted to dB and measured relative to periods of quiet background noise recorded in the same trial.[15]

Analyses of non-vocal social behaviors—Non-vocal behavior was recorded in each trial with a webcam (Logitech). BORIS software was used by trained observers to score and record non-vocal behaviors from video recordings of pairs of interacting mice. The following behaviors were recorded: resident-initiated social investigation (sniffing or following), visitor-initiated social investigation, mutual social investigation, and resident-initiated mounting (no instances of visitor-initiated mounting were observed in our dataset).

Footshock delivery—Subject mice were placed in a footshock chamber (Med Associates) inside of a sound attenuating chamber (Med Associates) equipped with an ultrasonic microphone (Avisoft) and a webcam (Logitech). A mild (0.5 mA) shock was delivered once every 30 seconds, for a total of 10 footshocks (trial duration = 5 minutes). Audio and video recordings were conducted as described above for USVs. To avoid potential inhibition of social motivation and USV production during TRAPing social sessions, footshocks were delivered to mice in a single, post-4-OHT session, and a footshock intensity (0.5 mA) was selected that reliably elicited production of squeaks in control animals.

Analysis of squeak acoustic features—A custom Matlab code was used that allowed trained users to manually annotate the onsets and offsets of individuals squeaks from spectrograms created from Spike2 audio recordings. The duration of individual squeaks was then calculated from these annotations. Another custom Matlab code was used to calculate the mean dominant frequency and the mean amplitude of each squeak. Because the microphone was placed near the top of the footshock chamber, audio clipping occurred during portions of squeaks in many trials. We therefore report mean squeak amplitudes in volts, directly measured as the mean amplitude of the audio waveform recorded in Spike2 during manually annotated squeak times.

Post-hoc visualization of viral labeling—Mice were deeply anesthetized with isoflurane and transcardially perfused with ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (4% PFA). Dissected brains were post-fixed overnight in 4% PFA at 4° C, cryoprotected in a 30% sucrose solution in PBS at 4° C for 48 hours, frozen in embedding medium (Surgipath, VWR), and stored at −80°C until sectioning. Brains were cut into 80 um coronal sections on a cryostat, rinsed 3 × 10 minutes in PBS, and processed at 4°C with NeuroTrace (1:500, Invitrogen) in PBS containing 0.3% Triton-X. Tissue sections were rinsed again 3 × 10 minutes in PBS, mounted on slides, and coverslipped with Fluoromount-G (Southern Biotech). After drying, slides were imaged with a 10x objective on a Zeiss LSM900 confocal laser scanning microscope. Because expression of the caspase virus cannot be directly visualized by looking for expression of a fluorescent tag, we took

the following approach to assess the spread of the caspase virus. In TRAP2;Ai14 mice that are treated with 4-OHT following a social encounter, neurons throughout the brain that upregulate Fos during the social encounter will be labeled with tdTomato. Because all TRAPed, caspase-expressing PAG-USV neurons will subsequently be ablated, viral spread was examined by assessing the absence of tdTomato labeling in the PAG of TRAP2;Ai14 mice. As shown in Figure S1A–C, the absence of tdTomato labeling was not restricted to the lateral column of the PAG but was observed also in the dorsal and ventral PAG, over an anterior-to-posterior range that extended throughout much of the caudal PAG (from −4 mm AP to −5 mm AP in most mice, and slightly beyond that AP range in a subset of mice). Absence of tdTomato labeling also typically extended into the tegmentum just lateral to the PAG, and to the region of the superior colliculus dorsal to the PAG injection site. Absence of tdTomato labeling was not observed in the cerebellum, and viral spread extended only minimally into the most medial portion of the parabrachial nucleus (Figure S1C). Expression of the GFP virus in PAG-USV_{GFP} males and females was visualized directly.

Quantification and statistical analysis

To determine whether to use parametric or non-parametric statistical tests for a given comparison, we examined the normality of the residuals for the relevant data distributions (determined by visual inspection of plots of z-scored residuals). Parametric, two-sided statistical comparisons (alpha = 0.05) were used in cases in which residuals did not diverge notably from the 45-degree line of normal distribution. No statistical methods were used to pre-determine sample size. Data analyses were conducted using R and RStudio. Same-sex cages of mice were selected at random for inclusion into experimental groups (PAG-USV_{casp}, PAG-USV_{GFP}, or PAG-$control_{casp}$). Mice were only excluded from analysis in cases in which viral injections were not targeted accurately. See also Table S1 for complete details of the statistical analyses used in this study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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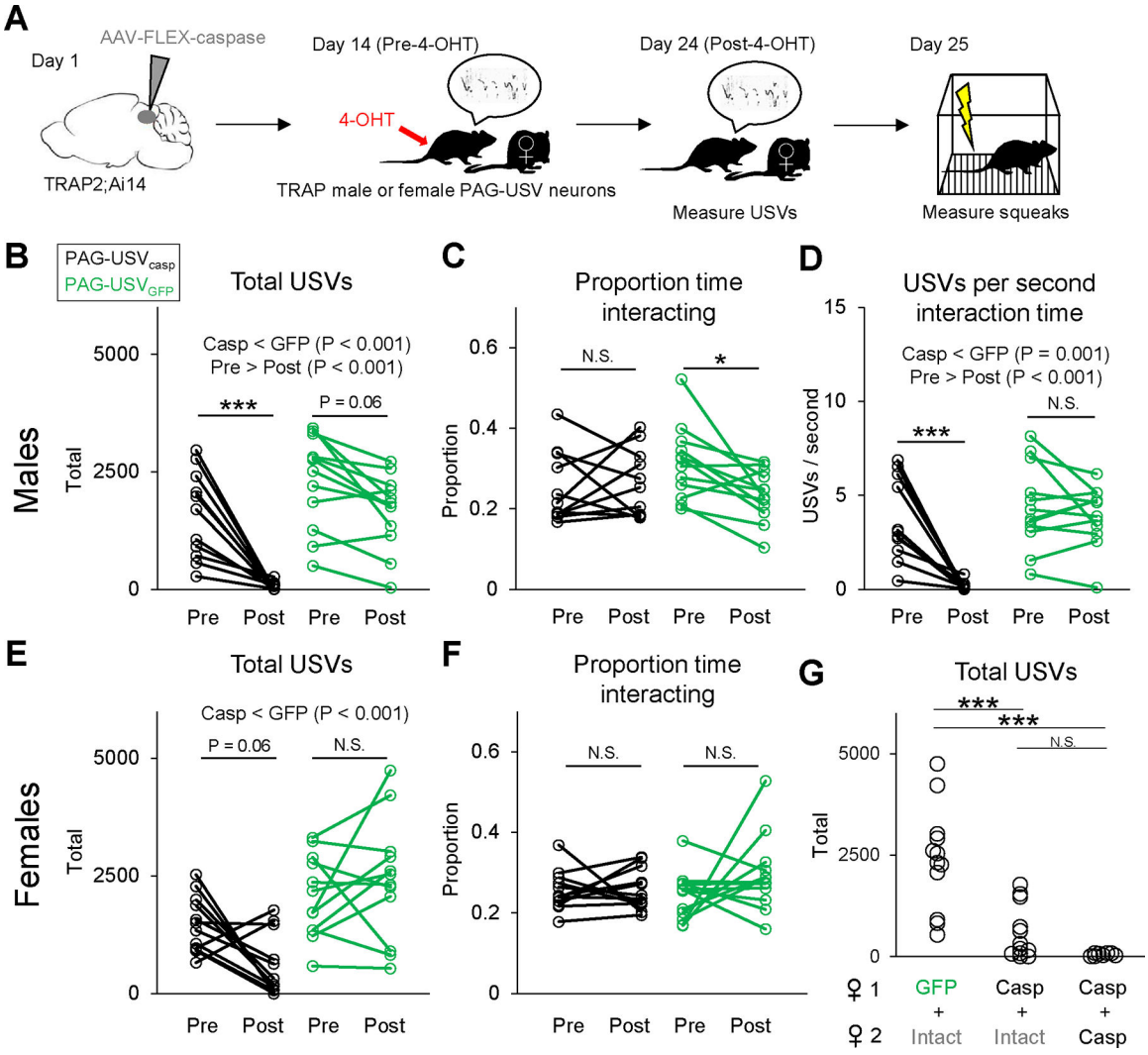

Figure 1. Ablation of PAG-USV neurons blocks USV production in male and female mice.

(A) Schematic shows experimental timeline for TRAP2-mediated ablation of PAG-USV neurons and behavioral measurements. (B) Total USVs produced by experimental (PAG-USV_{casp}, black points, N = 11) and control (PAG-USV_{GFP}, green points, N = 12) male mice during 30-minute social interactions with females are shown before and after 4-OHT treatment. (C) Same as (B), for proportion time subject males spent interacting with females before and after 4-OHT treatment. (D) Same as (B), for number of USVs produced per second of male-initiated social interaction before and after 4-OHT treatment. (E) Total USVs produced by experimental (PAG-USV_{casp}, black points, N = 12) and control (PAG-USV_{GFP}, green points, N = 12) female mice during social interactions with females are shown before and after 4-OHT treatment. (F) Same as (D), for proportion time subject females spent interacting with females before and after 4-OHT treatment. (G) Total USVs are plotted for interactions between pairs of females that included 1 PAG-USV_{GFP} female and 1 intact female (left, N = 12 trials), 1 PAG-USV_{casp} female and 1 intact female (middle, N = 12 trials), and 2 PAG-USV_{casp} females (right, N = 8 trials including round-robin

pairings of N = 6 PAG-USV$_{casp}$ females). Data in left and middle columns are the same data represented in Figure 1D, post-4-OHT. See also Figure S1, Table S2, and Videos S1–S2.

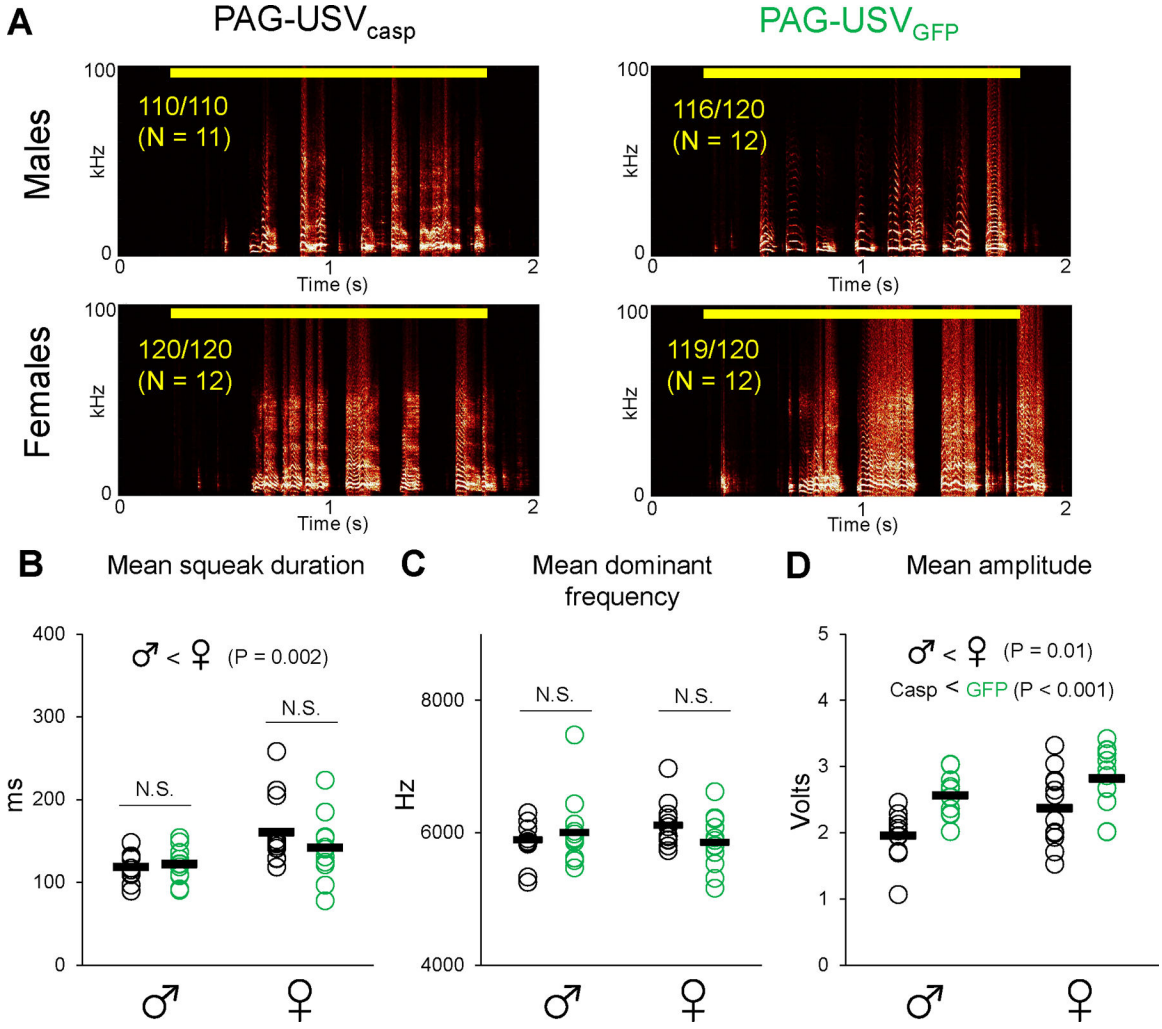

Figure 2. PAG-USV neurons are not required for the production of squeaks.

(A) Representative spectrograms show squeaks produced in response to footshocks for experimental (PAG-USV$_{casp}$) and control (PAG-USV$_{GFP}$) male and female mice. Yellow bar at the top of each spectrogram indicates timing of footshock. Yellow text indicates proportion of total trials across all mice in each group in which footshocks elicited squeaks. (B) Mean squeak duration is plotted for male (left) and female (right) mice from experimental (PAG-USV$_{casp}$) and control (PAG-USV$_{GFP}$) groups. (C) Same as (B), for mean squeak dominant frequency. (D) Same as (B), for mean squeak amplitude. See also Figure S2 and Table S2.

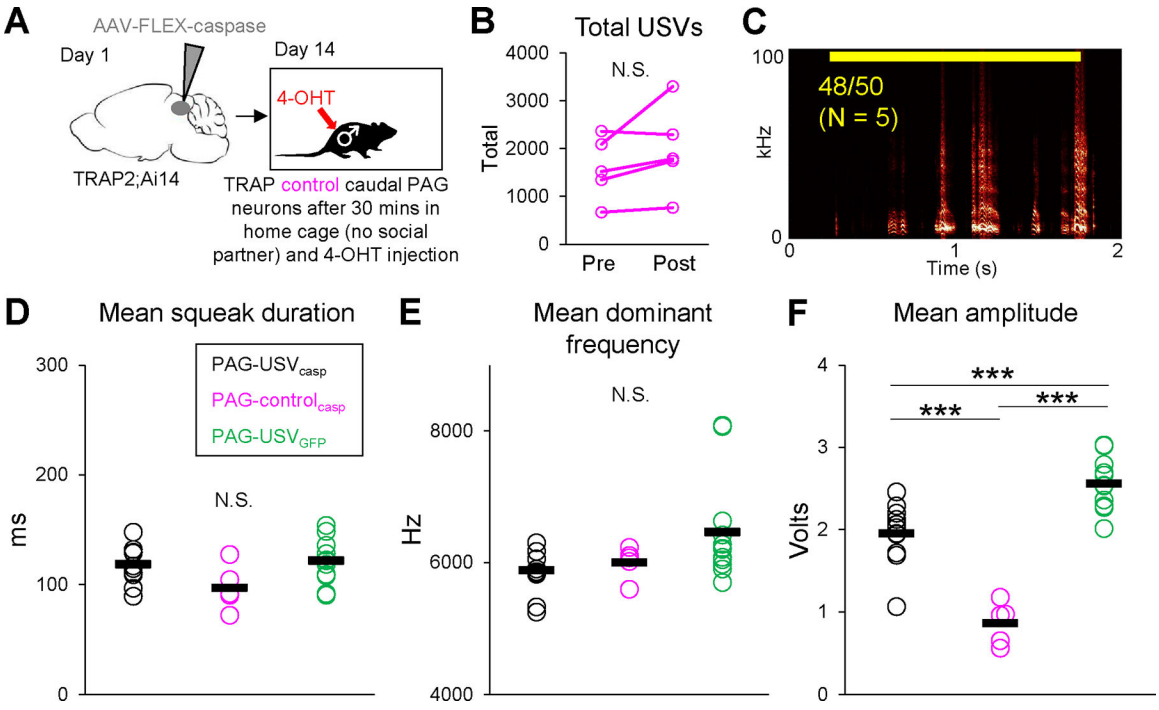

Figure 3. Effects of ablation of control (non-PAG-USV) caudal PAG neurons on production of USVs and squeaks in male mice.

(A) Schematic shows experimental timeline for TRAP2-mediated ablation of control, non-PAG-USV neurons in male mice. (B) Total USVs produced by PAG-control$_{casp}$ males (N = 5) during interactions with females are shown before and after 4-OHT treatment. (C) Representative spectrogram shows squeaks produced by a PAG-control$_{casp}$ male in response to footshock. Yellow bar at the top of the spectrogram indicates timing of footshock. Yellow text indicates proportion of total trials across N = 5 PAG-control$_{casp}$ males in which footshock elicited squeaks. (D) Mean squeak duration is plotted for PAG-USV$_{casp}$ males (black points, N = 11), PAG-control$_{casp}$ males (magenta points, N = 5), and PAG-USV$_{GFP}$ males (green points, N = 12). (E) Same as (D), for mean squeak dominant frequency. (F) Same as (D), for mean squeak amplitude. See also Table S2.

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Neurotrace 435/455 Blue	Thermo Fischer Scientific	CAT#: N21479
Bacterial and Virus Strains		
pAAV-flex-taCasp3-TEVp	Addgene	RRID: Addgene_45580
AAV2/1-pCAG-FLEX-EGFP-WPRE	Addgene	RRID: Addgene_51502
Chemicals, Peptides, and Recombinant Proteins		
4-Hydroxytamoxifen	Hello Bio	HB6040
Deposited Data		
Source data and original MATLAB codes used in analysis	Cornell eCommons	https://doi.org/10.7298/28ys-5n97
Experimental Models: Organisms/Strains		
Fos$^{tm2.1(icre/ERT2)Luo}$/J	Jackson Labs	IMSR_JAX:030323
B6.Cg*Gt(ROSA)26Sor*$^{tm14(CAG-tdTomato)Hze}$/J	Jackson Labs	IMSR_JAX:007914
C57BL/6J	Jackson Labs	IMSR_JAX:000664
Software and Algorithms		
MATLAB	Mathworks	http://www.mathworks.com RRID: SCR_001622
ZEN	Zeiss	https://www.zeiss.com RRID:SCR_013672
Spike2	CED	http://ced.co.uk RRID:SCR_000903
ImageJ	NIH	https://imagej.net/ij/ RRID:SCR_003070
Behavioral Observation Research Interactive Software	Open Behavior	https://github.com/olivierfriard/BORIS RRID:SCR_021509
R Project for Statistical Computing	R Core Team	http://www.r-project.org/ RRID:SCR_001905
R Studio	Posit	https://posit.co/ RRID:SCR_000432