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Abstract

Over the past three decades, a large body of evidence has accumulated demonstrating that the neuropeptide arginine vasopressin (AVP) plays a critical role in regulating social behavior. The overwhelming majority of this evidence comes from adults, leaving a gap in our understanding of AVP's role during development. Here, we investigated the effect of chronic AVP deficiency on a suite of juvenile social behaviors using Brattleboro rats, which lack AVP due to a mutation in the Avp gene. Social play behavior, huddling, social investigation & allogrooming, and ultrasonic vocalizations (USVs) of male and female rats homozygous for the Brattleboro mutation (Hom) were compared to their wild type (WT) and heterozygous (Het) littermates during same-sex, same-genotype social interactions. Male and female Hom juveniles exhibited less social play than their Het and WT littermates throughout the rise, peak, and decline of play's developmental profile. Hom juveniles also emitted fewer prosocial 50 kHz USVs, and spectrotemporal characteristics (call frequency and call duration) of individual call types differed from those of WT and Het juveniles. However, huddling behavior was increased in Hom juveniles, and social investigation and 22 kHz USVs did not differ across genotypes demonstrating that not all social interactions were affected in the same manner. Collectively, these data suggest that the Avp gene plays a critical role in juvenile social development.

Significance Statement

Several neurodevelopmental disorders are characterized by deficits in social behaviors, the underlying neurobiology of which is not understood. Arginine vasopressin (AVP) has emerged as a candidate neuropeptide through which two such groups of disorders, autism spectrum

disorders and schizophrenia, might impact social function. Nonetheless, only a few studies have investigated AVP's role in social development. Here, we find that rats with a mutation in the Avp gene exhibit "atypical" juvenile social behaviors and vocal communication. These findings suggest that AVP plays a critical role in the regulation of the quantity, quality, and type of social behaviors expressed during development.

Introduction

Childhood and adolescence are periods of marked social development, when individuals acquire the necessary skills for independence (reviewed in Spear, 2000). The most prominent social behavior of juveniles across many species is social play, where individuals engage in mock fighting behavior (Bekoff and Byers, 1998; Pellis and Pellis, 1998). In rats, social play emerges during the juvenile phase (~18 days of age), peaks during early adolescence (~35 days of age), and declines thereafter (Panksepp, 1981; Pellis and Pellis, 1990). This well-characterized developmental profile makes play ideal for studying juvenile and adolescent social development. Furthermore, play contributes to social and emotional development (Pellegrini, 1988; Vanderschuren et al., 1997; Hol et al., 1999; van den Berg et al., 1999). During social interactions, such as play, rats emit ultrasonic vocalizations (USVs) as a form of affective communication (reviewed in Wöhr and Schwarting, 2013). Calls with frequencies close to 50 kHz are thought to signal positive affect, whereas ~22 kHz calls are thought to signal distress (reviewed in Brudzynski, 2013). Infant rats and mice also emit ~40 kHz calls when separated from their mother (reviewed in Scattoni et al., 2009).

Many neurodevelopmental disorders are characterized by deficits in social behaviors such as play and communication (e.g., autism spectrum disorders, schizophrenia, and attention deficit hyperactivity disorder; Alessandri, 1992; Jones et al., 1994; Jordan, 2003; Scattoni et al., 2009). Uncovering the underlying neurobiology by which neurodevelopmental disorders impact

social function is a difficult task, especially given that the neural mechanisms that regulate "normal" social development are not understood. Here, we focus on the role of arginine vasopressin (AVP) in social development. This peptide is often referred to as a "social neuropeptide" because of its actions on a number of social and antisocial behaviors including pair bonding, parental behaviors, social recognition, flank marking, and aggression (reviewed in Caldwell et al., 2008; Albers, 2012; Bosch and Neumann, 2012). The overwhelming majority of this research has been conducted on adults, but emerging evidence indicates that AVP also influences juvenile social behaviors. The most direct evidence comes from intracranial injections of AVP agonists or antagonists, which impact social play (Cheng and Delville, 2009; Veenema et al., 2013), social recognition (Veenema et al., 2012), and USVs (Lukas and Wöhr, 2015) of juvenile rodents. While these findings provide strong evidence that AVP influences the immediate expression of juvenile social behaviors, the direction of the effects often depends on the age and sex of the subjects, context of the experiment, and brain area injected (Veenema et al., 2012, 2013; Bredewold et al., 2014). Hence, we do not yet understand the role of AVP in social development.

Brattleboro rats provide a unique model to study the effects of lifelong deficits in AVP on social behaviors. These rats have a single base pair deletion in exon 2 of the Avp gene that disrupts the production of AVP (Schmale and Richter, 1984). The behavior of adult homozygous Brattleboro (Hom) rats has been well studied, and deficits have been found in the major functions assigned to AVP including social behaviors such as social recognition/discrimination (Engelmann and Landgraf, 1994; Feifel et al., 2009) and social interactions (Lin et al., 2013). Studies on the behavioral development of Brattleboro rats have been confined to early postnatal life (first 2 weeks of life; e.g., Zelena et al., 2008; Lin et al., 2013). Infant Hom rats exhibit decreased aggregation (Schank, 2009) and emit fewer maternal separation-induced USVs (Varga et al., 2015), suggesting that the development of social

behaviors might be affected by the Brattleboro mutation. Juvenile social development has not been studied in Brattleboro rats.

In the present study, we test the impact of chronic AVP deficiency on juvenile social development by assessing the effects of the Brattleboro mutation on several social behaviors (social play, USVs, huddling, and social investigation & allogrooming). We find that male and female AVP-deficient Hom rats exhibit lower levels of social play at all stages of play development (onset, peak, and decline of play). Juvenile Hom rats also emit fewer 50 kHz USVs with altered spectrotemporal characteristics. Not all social behaviors are affected in the same manner, however, as juvenile Hom rats display more huddling episodes, and social investigation & allogrooming does not differ between genotypes. These data demonstrate that deficits in AVP throughout development impact the quantity and quality of juvenile social interactions and communication.

Methods

Animals and housing conditions

A colony of Brattleboro rats (with Long Evans background) was established in our laboratory from rats purchased from the Rat Resource and Research Center (University of Missouri, Columbia, MO). Brattleboro rats were housed in either opaque plastic cages with Carefresh bedding and wood chips (48 x 27 x 20cm) or ventilated transparent OptiRat plastic cages with Bed-O-Cobs® bedding (35.6 x 48.5 x 21.8cm). For all experiments, the day of birth was considered postnatal day 0 (P0). Room lights were set to a 12h:12h light:dark cycle (lights off at 1700 h ET), and ambient temperature was maintained at 23°C. Food and water were available *ad libitum*. All procedures were in accordance with the Guide for Care and Use of Laboratory

Animals and were approved by the Animal Care and Use Committee at Georgia State University and the University of Massachusetts, Amherst.

Experiment 1: Emergence of play behavior in Brattleboro rats.

Wild type (WT), heterozygous (Het), and homozygous (Hom) Brattleboro offspring were obtained from Het x Het breeding pairs from our colony. Overall, the distribution of genotypes was 1.3:2.0:0.9 (WT:Het:Hom). Each rat pup was tested for play behavior once at P17, P19, P21, or P23. All rats were tested prior to weaning, which occurred at P24. Rats were removed from their litters 2-3 hours before being paired with a similarly treated age-matched, same-genotype, same-sex rat in a clean cage for a social behavior test (as in Panksepp 1981, Paul et al. 2014).

Experiment 2: Social behaviors and ultrasonic vocalizations of juvenile Brattleboro rats.

WT, Het, and Hom Brattleboro offspring were obtained from Het x Het breeding pairs from our colony. Overall, the distribution of genotypes was 0.9:2.0:0.9 (WT:Het:Hom). Rats were weaned at P22, at which point they were housed with an age-matched, same-genotype, same-sex cagemate. At P33 (±2 days) or P43 (±2 days), cagemates were single-housed for ~24 hours before being reunited in a social behavior test the following day.

Social behavior tests

All tests were conducted within the first 2.5 hours of lights off under red light. Animals were paired with an age-matched, same-sex, same-genotype playmate in a fresh cage for 20

minutes, and their behavior was videotaped. In Exp. 2, bedding was removed from the test cage to minimize background noise interference with the ultrasonic recordings. Play Attacks (lunges toward the nape of the playmate's neck), Pins (animal lying in supine position with playmate on top), and Boxing Events (both animals standing on their hind paws and pushing each other with their forepaws), as described in Meaney and Stewart (1981a) and Vanderschuren et al. (1997), were scored by a researcher blind to the treatment conditions using JWatcher software (http://www.jwatcher.ucla.edu/; Exp. 1) or The Observer XT11 (Noldus Information Technolgy Inc., Wageningen, The Netherlands; Exp. 2). In Exp. 2, the number of Social Investigation & Allogrooming events and Huddling Episodes were also scored using The Observer XT11.

Ultrasonic Vocalization Recordings

Vocal emissions were recorded for the duration of the social behavior tests using an UltraSoundGate CM16/CMPA microphone (Avisoft Bioacoustics, Glienicke, Germany) placed just above the testing cage. The microphone was connected to a computer via an Avisoft Bioacoustics UltraSoundGate 116Hb. Acoustic data were recorded with a sampling rate of 250 kHz in 16 bit format, and spectrograms were constructed by fast Fourier transformation (256 FFT length, 100% frame, FlatTop window and 50% time window overlap; SASLab Pro, Avisoft Bioacoustics). All USVs made within the first 10 min of the play behavior trial were manually marked by investigators blind to the age, sex, and genotype of the rats. In order to be marked, calls had to be at least 10 ms in length and distinct calls had to be separated by at least 10 ms. Several call parameters were quantified, including fundamental frequency, duration, and

number of calls emitted. Call frequency (in Hz) was calculated by averaging the fundamental frequency at call onset, call offset, and peak amplitude of the call (Integrated Frequency). A subset (20% random sampling) of the calls were selected and manually classified into the 15 call categories described in Wright et al. (2010).

Genotyping of Brattleboro rats

The Brattleboro mutation is a single base pair deletion in exon 2 of the Avp gene that disrupts processing of the AVP prohormone (Schmale and Richter, 1984). Previous genotyping protocols required DNA sequencing after PCR amplification to detect the single base pair deletion. In the present study, we developed a faster and cheaper method, replacing the sequencing step with a restriction enzyme digest followed by gel electrophoresis. Tail tissue was harvested from rat pups between 8-12 days of age using ice-cold ethanol as a local anesthetic. For animals in Exp. 1, tails were digested at 55°C overnight in 400µl of Tail Lysis Buffer containing 4µl of Proteinase K. DNA was extracted and purified with phenol, chloroform:isoamyl alcohol (24:1), isopropanol, and 70% ethanol. For animals in Exp. 2, the REDExtract-N-Amp^{TM} Tissue PCR Kit (Sigma-Aldrich) was used for tail digestion and DNA extraction. DNA surrounding the base pair deletion was amplified by PCR using the following primers, Forward: GACGAGCTGGGCTGCTTC, Reverse: CCTCAGTCCCCCACTTAGCC. Twenty µl of PCR product was then digested with BcgI restriction endonuclease (New England BioLabs, Ipswich, MA) at 37°C overnight using the following concentrations: 3µl 10X NEBuffer 3, 4µl 10X S-adenosylmethionine, 2µl nuclease-free water, and 1µl BcgI. BcgI recognizes and

cuts only the mutant Brattleboro PCR product, resulting in two DNA fragments of similar size (92 and 97 base pairs). Therefore, samples from WT rats exhibit a single 222bp band after gel electrophoresis, whereas those of Hom rats exhibit a single ~95bp band (the two fragments do not separate on a 2% agarose gel). Samples from Het rats exhibit both WT and Hom bands. To validate this procedure, we confirmed the genotyping results from a subset of samples with the traditional sequencing methodology.

Statistical Analyses

Data were analyzed by three-way ANOVA with genotype, age, and sex as the independent variables. Because the main effect of age was significant for the overwhelming majority of measures (22 of 27), data were also analyzed separately at each age by two-way ANOVA, and this analysis was depicted in all figures. Where the main effect of genotype was significant, and in a few cases where it approached significance (P=0.06), post hoc comparisons between each genotype were assessed using Fisher's PLSD. For a number of comparisons, the data were not normally distributed due to the high number of zero values, and the distribution could not be corrected by using a log(x+0.01) transformation (see Tables 1 – 3). This is to be expected when age groups prior to the onset of the behavior are included in the analysis (in Exp. 1) and when one assesses behaviors not highly expressed during motivated social behavior tests (e.g., distress USVs and huddling in Exp. 2). Although multifactorial ANOVA is robust when the data are not normally distributed, we confirmed significant main effects and post hoc comparisons from non-normally distributed data with the appropriate nonparametric test (Kruskal-Wallis or Mann-Whitney U Tests). For all of these comparisons except post hoc tests assessing age

differences in Experiment 1, the ANOVA and nonparametric tests yielded the same result. For the post hoc tests where the results differed, we reported the nonparametric statistic. Significance was assumed when $P<0.05$.

Results

Experiment 1: Emergence of play behavior in Brattleboro rats.

Consistent with other reports (Panksepp, 1981; Paul et al., 2014), the developmental onset of play occurred around P19-P21 (Fig. 1). Play behavior was virtually absent at P17, increased slightly at P19, and further increased at P21 (main effect of age, $P<0.0001$ for Total Play[a1], Pins[b1], and Pounces[c1]; P17 vs. P19 and P19 vs. P21, $P<0.0001$, for Total Play[a4,a5], Pins[b4,b5], and Play Attacks[c4,c5]; superscripts used here and further in the document indicate rows in Tables 1-3). Play behavior of males and females did not differ (main effect of sex, $P>0.38$ for Total Play[a3], Pins[b3], and Play Attacks[c3]).

Overall, Hom rats played less than their WT and Het littermates (Fig. 1A; Total Play, main effect of genotype, $P<0.04$[a2]; Hom vs. WT[a6] or Het[a7], $P<0.05$). This was due to fewer numbers of Pins (Fig. 1B; main effect of genotype, $P<0.02$[b2]; Hom vs. WT[b6] or Het[b7], $P<0.009$) and Play Attacks: while the main effect of genotype fell short of significance for Play Attacks ($P=0.06$ for ANOVA and $P=0.09$ for Kruskal-Wallis test[c2]), post hoc comparisons indicated that Hom weanlings did exhibit fewer Play Attacks than WT weanlings ($P<0.04$[c6]; not illustrated). Boxing events were rare in all genotypes, with fewer than 0.25 events during the 20-min test for all groups; no Hom pairs exhibited a Boxing event. Decreased Total Play and Pins of Homs were evident at P21 (main effect of genotype, $P<0.003$ for both behaviors[a8,b8]; Hom vs. WT,

P<0.0005 for both behaviors[a9,b9]) and P23, although comparisons for Total Play fell short of significance at P23 (main effect of genotype, P<0.03 for Pins[b12], P=0.06 for Total Play[a12]; Hom vs. WT, P<0.04 for Pins[b13], P=0.09 for Total Play[a13]). Notably, Het animals exhibited less Total Play and fewer Pins than WT animals at P21 (Het vs. WT, P<0.04 for both behaviors[a11,b11], Fisher's PLSD). Genotype did not impact Total Play or Pins at P19 (main effect of genotype, P>0.32 for Total Play[a15] and Pins[b15]), when play was low for all genotypes; levels of play at P17 were too low for statistical analyses.

Experiment 2: Social behaviors and ultrasonic vocalizations of juvenile Brattleboro rats.

Social Behaviors. As for weanling-aged rats, Hom juveniles played less than WT and Het juveniles due to reductions in both Pins and Play Attacks (Figs. 2 & 3B; main effect of genotype, P<0.0001 for Total Play[d2], Pins[e2], and Play Attacks[f2]; Hom vs. WT[d4,e4,f4] or Het[d5,e5,f5], P<0.005 for all three behaviors). Analysis of the temporal profile of Total Play revealed that Hom juveniles played less than WT and Het rats across the entire 20-min test (Fig. 2C,D). Hom juveniles exhibited fewer Total Social Behaviors than WT and Het juveniles (Fig. 3A; main effect of genotype, P<0.0001[g2]; Hom vs. WT[g4] or Het[g5], P<0.0001). Reductions in play and social behaviors of Hom juveniles were evident at both P34 and P44, although the post hoc comparison between WT and Hom groups for Pins at P34 fell short of significance when each age was analyzed separately (Figs. 2 & 3). Social Investigation & Allogrooming did not differ between genotypes (Fig. 3C; main effect of genotype, P>0.51[h2]) and Huddling episodes were increased in Hom juveniles (Fig. 3D; main effect of genotype, P<0.0001[i2]; Hom vs. WT[i4] or Het[i5],

P<0.0001), indicating that not all social behaviors are affected in the same manner by the Brattleboro mutation.

The number of all social behaviors decreased between P34 and P44 (Figs. 2&3; main effect of age, P<0.01 for Total Play[d1], Pins[e1], Play Attacks[f1], Total Social Behaviors[g1], Social Investigation & Allogrooming[h1], and Huddling[i1]). There were no significant sex differences in the number of social behaviors (main effect of sex, P>0.46 for Total Play[d3], Pins[e3], Play Attacks[f3], Total Social Behaviors[g3], Social Investigation & Allogrooming[h3], and Huddling[i3]), except that male Het juveniles exhibited more Pins than female Het juveniles at 44 days of age (Fig. 2B; genotype x sex interaction at P44, P<0.008[e6]; Het male vs. Het female, P<0.002[e7]).

Ultrasonic Vocalizations. Similar to play and overall social behaviors, Hom rats emitted fewer USVs than WT and Het rats (Fig. 4; main effect of genotype, P<0.0001[j1]; Hom vs. WT[j2] or Het[j3], P<0.003) due to a selective reduction in 50 kHz USVs (Fig. 4B,C; 50 kHz USVs: main effect of genotype, P<0.0001[k2]; Hom vs. WT[k3] or Het[k4], P<0.002; 22 kHz USVs: main effect of genotype, P>0.25[l2]). Decreased 50 kHz USVs of Hom rats was evident at both juvenile ages, although the post hoc comparisons between WT and Hom rats fell short of significance when P44 data were analyzed separately (Fig. 4B). Unlike social behaviors, the number of 50 kHz and 22 kHz USVs increased across age (main effect of age, P<0.04 for 50 kHz[k1] and 22 kHz[l1] USVs).

The 50 kHz USV category consists of calls with a broad range of frequencies (30 – 117 kHz in the present study) and spectral-temporal structures (e.g., constant frequency, frequency steps, frequency trills), and it is not known whether these calls are functionally equivalent. To determine which types of 50 kHz USVs were impacted by the Brattleboro mutation, we

277 classified a subset (20%) of each animal's USVs according to the call types proposed by Wright
278 et al. (2010) and assessed the impact of the Brattleboro mutation on the quantity (number) and
279 quality (duration and Integrated Frequency, which was defined as the mean of the call onset,
280 peak amplitude, and call end frequency) of the vocalizations most frequently emitted during
281 social behavior testing.

282 Figure 5 illustrates the percentage of all classified calls, regardless of the genotype of
283 the caller. Most USVs fell within the 50 kHz category, with type 4 calls (flat calls) being the most
284 common (33.3%) followed by type 1 (complex calls, 15.0%), type 10 (trills, 11.1%), type 2
285 (upward-ramp calls, 10.1%), and type 7 calls (step-up calls, 9.7%). The percentage for each of
286 the remaining call types was less than 5%, including 22 kHz USVs (type 15), which comprised
287 2.5% of calls.

288

289 *Quantity of ultrasonic vocalization call types.* In general, juvenile Hom rats emitted fewer calls
290 of each type than their WT and Het littermates, and for most this was due to decreased call
291 number at P34 (Fig. 6A-E). At P34, Hom rats emitted fewer upward-ramp, flat, and step-up
292 calls (Fig. 6B-D; main effect of genotype, $P<0.03$ for each call type^{n2,o2,p2}; Hom vs. WT, $P<0.02$
293 for upward-ramp^{n3} and step-up^{p3} calls; Hom vs. Het, $P<0.02$ for each call type^{n4,o4,p4}), although
294 the comparison between WT and Hom flat calls fell short of significance ($P=0.06^{o3}$). At P44,
295 there were no significant differences between genotypes for these calls (main effect of
296 genotype, $P>0.05$ for upward-ramp^{n5}, flat^{o5}, and step-up^{p5} calls). Trills were reduced in Hom
297 juveniles at both P34 and P44 (Fig. 6E; main effect of genotype, $P<0.01$ for both P34^{q3} and
298 P44^{q6}; Hom vs. WT, $P<0.04$ for P44^{q7}; Hom vs. Het, $P<0.005$ for both P34^{q5} and P44^{q8}),

299 although the difference between Hom and WT rats was not significant at P34 (P=0.10[q4]). When
300 analyzed across both ages, Hom rats emitted fewer complex calls than WT and Het rats (main
301 effect of genotype, P<0.03[m2]; Hom vs. WT [m3]or Het[m4], P<0.04), but these comparisons were not
302 significant when assessed separately for each age (Fig. 6A; main effect of genotype, P>0.15 for
303 P34[m5] and P44[m6]).

304 Upward-ramp, step-up, and trill calls increased from P34 to P44 (Fig. 6B,D,E; main
305 effect of age, P<0.04 for each call type[n1,p1,q1]); complex and flat calls did not differ across age
306 (Fig. 6A,C; main effect of age, P>0.13 for both calls[m1,o1]). Only trills differed between the sexes,
307 with males emitting more than females (Fig. 6E; main effect of sex, P<0.005[q2]).
308
309 *Quality of ultrasonic vocalization call types.* For most USV types, Hom rats emitted calls with a
310 lower integrated frequency, but duration was only altered for step-up calls and trills. Upward-
311 ramp, flat, and step-up calls of Hom rats had a lower integrated frequency than those of WT and
312 Het rats, (main effect of genotype, P<0.02 for each call type[s1,t1,u1]; Hom vs. WT[s2,t2,u2] or Het[s3,t3,u3],
313 P<0.05 for each call type). The age at which these effects were significant depended on the call
314 type (see Fig. 6G-I for details). Complex calls of Hom rats also had a lower integrated
315 frequency than those of Het rats (main effect of genotype, P<0.02[r1]; Hom vs. Het, P<0.006[r3]),
316 but did not differ significantly from WT rats (P=0.10[r2]); Hom and Het differences in complex calls
317 were not significant when P34 and P44 were analyzed separately (Fig. 6F). Trills were the only
318 call type analyzed for which integrated frequency was unaffected by genotype (Fig. 6J; main
319 effect of genotype, P>0.24[v1]).

The duration of USVs also differed by genotype, but only for step-up calls and trills, and only at P44 (Fig. 6K-O). Hom rats emitted longer duration step-up calls than WT and Het rats at P44 (Fig. 6N; main effect of genotype, $P<0.003$ for P44[w1]; Hom vs. WT[w2] or Het[w3], $P<0.02$). For trills, both Hom and Het rats emitted shorter calls than WT rats at P44, and Hom and Het rats did not differ from each other (Fig. 6O; main effect of genotype, $P<0.01$[x1]; Hom[x2] or Het[x3] vs. WT, $P<0.02$; Het vs. Hom, $P>0.26$[x4]).

The quality of USV call types also changed with age. Integrated frequency decreased from P34 to P44 for each call type except trills (Fig. 6F-J; main effect of age, $P<0.05$ for complex[r4], upward-ramp[s4], flat[t4], and step-up[u4] calls; main effect of age, $P>0.18$ for trills[v2]). This reduction in Integrated Frequency was evident in all genotypes; no interaction between age and genotype was found (age x genotype, $p>0.05$ for complex[r5], upward-ramp[s5], flat[t5], and step-up[u5] calls). In addition, the duration of complex, upward-ramp, and flat calls also decreased from P34 to P44 (Fig. 6K-M; main effect of age, $P<0.03$ for each call type[y1,z1,aa1]); the duration of step-up calls and trills did not vary with age (Fig. 6N,O; main effect of age, $P>0.10$ for step-up calls[w4] and trills[x5]).

Discussion

The present study suggests that the Avp gene plays an important role in social development. The Brattleboro mutation, which disrupts the production of AVP, impacted both social behaviors and ultrasonic communication of juvenile rats. Hom rats played less and emitted fewer 50 kHz USVs than their WT and Het littermates. In addition, the spectrotemporal characteristics of USVs emitted by Hom rats differed from that of WT and Het rats. Social deficits, however, were

342 behavior- and USV-specific. Huddling episodes were increased in Hom rats, and Social
343 Investigation & Allogrooming and 22 kHz USVs did not differ across genotypes. Hence, Hom
344 Brattleboro rats are not simply asocial, rather their social behaviors are “atypical” compared to
345 WT and Het rats.

346 Deficits in the social play of Hom rats were evident throughout play’s developmental
347 profile (onset [P21 and P23], peak [P34], and decline [P44]), suggesting that AVP is important
348 for the overall level of play rather than its developmental timing. This is similar to the persistent
349 developmental deficits reported for body and brain weights of Hom Brattleboro rats (reviewed in
350 Boer, 1985), but differs from other measures (e.g., eye opening, ear opening, incisor eruption),
351 which occur earlier in Hom Brattleboro rats (Boer et al., 1980; Zelena et al., 2009). Notably, the
352 greatest deficits in neural development of Hom Brattleboro rats occur in the cerebellum (Boer et
353 al., 1982), a brain region whose development correlates with the ontogeny of play across
354 several species (Byers and Walker, 1995). It has been proposed that play behavior contributes
355 to cerebellar development (Byers and Walker, 1995). Following this logic, it is possible that
356 decreased play of Hom rats contributes to developmental deficits in their cerebellar size and
357 morphology. The reverse, however, is also possible.

358 Adult Het rats exhibit partial reductions in AVP neural mRNA expression and pituitary
359 peptide content (Dorsa and Bottemiller, 1982), and sometimes exhibit behavioral differences
360 from WT rats (Brot et al., 1992). Nonetheless, social behaviors of Het rats in the present study
361 did not differ statistically from those of WT rats except for a transient reduction in social play
362 during play’s developmental onset (at P21). These data raise the possibility of a gene dosage

effect during play's developmental onset. Perhaps the onset of play requires higher levels of AVP than its maintenance or that Het rats have insufficient AVP at this age to stimulate play.

The Brattleboro mutation also affects USVs. Infant Hom Brattleboro pups emit fewer maternal separation-induced 40 kHz USVs (Lin et al., 2013; Varga et al., 2015). Here, we demonstrate that USV deficits of Hom Brattleboro rats persist into the juvenile stage and include prosocial vocalizations. Juvenile Hom rats emitted fewer USVs during the social interaction test due to a selective reduction in 50 kHz calls. Fifty kHz USVs reflect a positive affective state and are considered a form of prosocial communication (reviewed in Brudzynski, 2013; Wöhr and Schwarting, 2013). Fifty kHz calls are emitted during appetitive interactions and in anticipation of reward stimuli such as mating, play, addictive drugs, and "tickling" (Barfield et al., 1979; Knutson et al., 1998, 1999; Panksepp and Burgdorf, 2000; Burgdorf et al., 2008). Furthermore, 50 kHz calls elicit approach behavior (Wöhr and Schwarting, 2007) and "self administration" for their playback (Burgdorf et al., 2008). Hence, decreased 50 kHz USVs in Hom Brattleboro rats may indicate decreased prosocial motivation for, or reward value of, social interactions in AVP-deficient animals.

In contrast, 22 kHz calls are emitted in response to aversive stimuli (e.g., electric shocks, predators, drug withdrawal, and aggressive interactions; Sales, 1972; Tonoue et al., 1986; Cuomo et al., 1988; Blanchard et al., 1991; Vivian and Miczek, 1991; Barros and Miczek, 1996; Covington and Miczek, 2003), are thought to reflect a negative affective state akin to anxiety or distress (reviewed in Brudzynski, 2013; Wöhr and Schwarting, 2013), and are not affected by the Brattleboro mutation (present findings). Hence, AVP-deficiency does not affect all forms of vocal communication, with distress-like calls being particularly independent of AVP status. This

conclusion should be tempered by the low levels of 22 kHz calls for all genotypes in the present experiment, which is consistent with previous studies measuring USVs during prosocial playful interactions (Burgdorf et al., 2006). In addition, while the 22 kHz USVs in the present study were within the frequency range of distress-like USVs, their duration was much shorter than that typically reported: ~24 ms in the current study (see Figure 5G) versus 300-1200 ms in studies investigating USVs in response to aversive stimuli (Tonoue et al., 1986; Brudzynski and Ociepa, 1992). Brudzynski et al. (1993) reported two distinct populations of 22 kHz USVs in response to experimenter handling: short calls of 20 to 300 ms and long calls of 300 to over 2000 ms, with most long calls falling between 500 - 600 ms. The functional significance of these short 22 kHz calls is not known. Therefore, it is possible that the 22 kHz USVs in the present study were not true anxiety or distress-like calls. Future studies are needed to determine whether 22 kHz USVs are altered in Hom Brattleboro rats tested under aversive conditions.

We further analyzed the USVs according to subcategories suggested by Wright et al. (2010) to determine whether the Brattleboro mutation differentially impacted different types of calls, i.e. altered their vocal repertoire. In general, Hom Brattleboro rats emitted fewer of each USV call type analyzed. Reductions were evident in each of the 5 most common call types – Flat calls, Complex calls, Trills, Upward-Ramp calls, and Step-Up calls (all 50 kHz calls). Deficits were most robust for Trills, which were present at both P34 and P44, and least robust for Complex calls, which were not significant when each age was analyzed separately. In addition to the quantity of USVs, the Brattleboro mutation impacted spectrotemporal characteristics of USVs. Flat, Upward-Ramp, and Step-Up calls of Hom rats had lower integrated frequencies. In addition, Step-Up calls were longer and Trills were shorter in Hom

rats. While it is not clear why AVP-deficiency impacts the spectrotemporal quality of USVs in a call-specific manner, it is clear that several USV call types of Hom rats sound different than those of WT and Het rats. It is interesting to speculate that the reduced number and integrated frequency of 50 kHz USVs of Hom rats may contribute to their "atypical" social behaviors. Call frequency is an important feature for USV call structure. Frequency is the dominant feature mice use to discriminate between tone categories (Radziwon and Dent, 2014). In addition, rats will approach a speaker playing 50 kHz calls and tones (Wöhr and Schwarting, 2007). Hence, the decreased integrated frequency of Flat, Upward-Ramp, and Step-Up calls of Hom rats might impact call meaning or appetitive quality. Therefore, the reduced number and frequency of 50 kHz calls might lead to less play by reducing the amount of prosocial stimulation during social interactions. This rationale, however, cannot explain the increased huddling seen in Hom rats. Perhaps the prosocial nature of 50 kHz USVs depends upon the type of social behavior. For example, because 50 kHz USVs stimulate locomotor behavior (Wöhr and Schwarting, 2007), it is possible that they stimulate "active" social behaviors such as play, but inhibit "passive" social behaviors such as huddling. It should be noted that a direct relationship between the number of USVs and play events has not been established, and it is also possible that play triggers USVs. Our data suggest that a simple relationship between play and USVs as a whole is unlikely – in Exp. 2, for example, the number of USVs increased with age whereas the number of play events (and social interactions) decreased with age.

Despite significant interest in adolescent social development, most studies on the development of USVs have focused on infant maternal separation-induced 40 kHz calls. A few reports have found that adult rodents emit more USVs than adolescents during same-sex or

opposite sex interactions (Cherry, 1987; Willey et al., 2009; Willey and Spear, 2012; Kabitzke et al., 2015). Similarly, we found that the number of both 50 kHz and 22 kHz USVs emitted during same-sex juvenile social interactions increase across a short 10-day interval from P34 to P44, which approximates early/mid-adolescence adolescence in rats (Vetter-O'Hagen and Spear, 2012). We further found that the developmental increases in 50 kHz USVs were specific to call type, occurring in Upward-Ramp, Step-Up, and Trill calls, but not Complex or Flat calls. In addition, the spectrotemporal characteristics of several 50 kHz call types changed across these ages: integrated frequency decreased for Complex, Upward-Ramp, Flat, and Step-up Calls and duration decreased for Complex, Upward-Ramp, and Flat calls. These findings raise the possibility that spectrotemporal characteristics of some rat USVs convey age-related information of the caller and thereby influence age-dependent social interactions. For example, perhaps the spectrotemporal characteristics of prepubertal calls elicit less aggression from same-sex adults, whereas those of postpubertal calls may better stimulate sex behaviors in the opposite sex.

Males emit more USVs than females, both as infants in response to maternal separation (Bowers et al., 2013) and as juveniles immediately preceding and following play bouts (Himmler et al., 2014). In the present experiment, we found that the sex difference in the number of USVs during juvenile play is restricted to Trills, again with males emitting more than females. Although Himmler et al. (2014) did not report whether sex differences were present in all or some USV call types, Trills comprised 77% of calls in their analysis. We did not detect any sex differences in the integrated frequency or duration of any call type, including Trills, suggesting that juvenile sex differences in USVs are limited to quantity rather than spectrotemporal quality. With the exception of a single comparison in 44-day-old Het rats (Fig. 4A), we did not detect sex

differences in other social behaviors, including play. Although males are often reported to engage in more rough-and-tumble play than females (Meaney and Stewart, 1981b; Pellis, 2002; Olesen et al., 2005), this sex difference is dependent upon the testing conditions and behaviors measured (Thor and Holloway, 1984; Argue and McCarthy, 2015). The absence of sex differences in play in the present experiments is not surprising as studies testing same-sex pairs of rats after a period of isolation, as we did in the present experiments, generally do not detect sex differences in juvenile social play (Panksepp and Beatty, 1980; Panksepp, 1981; Veenema et al., 2013; Paul et al., 2014).

Currently, we cannot determine whether the altered social behavior and USVs of Brattleboro rats are due to the absence of central or peripheral actions of AVP. Hom Brattleboro rats develop diabetes insipidus due to the absence of AVP-mediated water reabsorption at the level of the kidney (Valtin and Schroeder, 1964), and symptoms are evident before play onset: increased plasma osmolarity is present at 10-14 days of age and polydipsia develops between 15-16 days of age (Dlouhá et al., 1982; Zelena et al., 2009). Nonetheless, acute intracerebroventricular (ICV) injections of a vasopressin 1a receptor (V1aR) antagonist decrease maternal separation-induced 40 kHz USVs of infant rat pups (Winslow and Insel, 1993; Bleickardt et al., 2009), as well as 50 kHz USVs and play behavior of male juvenile rats (Veenema et al., 2013; Lukas and Wöhr, 2015). These findings argue that the altered play and USVs of Brattleboro rats is due to a direct disruption of AVP's actions in the brain rather than a disruption of AVP's peripheral actions or indirect compensatory changes resulting from the absence of AVP during development. Decreased anxiety-like, depressive-like, and maternal behaviors of Brattleboro rats persist after the restoration of AVP's peripheral actions, indicating

that several behavioral abnormalities of Brattleboro rats are due to the loss of AVP's central actions (Fodor et al., 2012; Balázsfi et al., 2015).

We do not yet know which AVP system is responsible for the deficits seen in the Brattleboro rats. The limited available data focus on juvenile social play and do not provide a comprehensive understanding of AVP's regulation of social development. AVP cells in the BNST appear to play an inhibitory, rather than stimulatory, role in juvenile social play, at least in males. During the developmental emergence in weanling-aged rats (P18 - P23), BNST AVP mRNA expression of males correlates negatively with play behavior, whereas in females, BNST AVP mRNA expression is not detectable (Paul et al., 2014). Furthermore, V1aR antagonist injections into the septum, a projection area of BNST AVP cells, increases play behavior of juvenile males, but not females (Veenema et al., 2013). In the same study, ICV V1aR antagonist injections decreased play behavior of male rats but increased play behavior of female rats. AVP mRNA expression in the PVN of male, but not female, rats correlates positively with their play behavior during play's developmental emergence raising the possibility that the PVN is the site of AVP's stimulatory actions, at least in males (Paul et al., 2014). At present, however, it is difficult to incorporate these sex-specific findings with the present results in which "atypical" social behaviors of Hom rats were seen in both sexes (including play deficits). Complicating matters further, play behavior and the effects of pharmacological manipulations of AVP on play can depend upon the context in which the animals are tested (Bredewold et al., 2014). AVP in the PVN regulates the stress axis and autonomic function, both of which could influence social behavior in a context-specific manner through their actions on stress or arousal. Adult Brattleboro rats are less reactive to various stressors (Balázsfi et al.,

2015). If true for juveniles, this decreased stress reactivity could have contributed to the present findings where play was tested in a novel home cage after a period of isolation. Although rats play less after restraint stress (Klein et al., 2010), isolation increases play in rats (Panksepp and Beatty, 1980). Hence it is not clear whether decreased stress reactivity would lead to higher or lower levels of play. The low levels of 22 kHz USVs (and absence of long 22 kHz USVs) across all genotypes suggest that rats were not distressed or anxious in the novel cage during testing. Genotype differences in novel cage exploration at the beginning of the test is unlikely to account for the decreased play as Hom Brattleboro rats exhibited lower levels of play across the entire 20-min test (Fig. 2C,D). More studies are needed to dissect out which AVP systems contribute to social development and how.

Early life experiences can significantly impact behavioral development (Kundakovic and Champagne, 2015), and juvenile social play is particularly sensitive to both prenatal and early postnatal environments (Veenema and Neumann, 2009; Kirsten et al., 2010; Taylor et al., 2012; Karkow and Lucion, 2013), including natural variations in maternal care (Parent and Meaney, 2008). Given that the Brattleboro mutation impacts maternal behaviors (Fodor et al., 2012), care must be taken when designing and interpreting results using this model. Indeed, Hom Brattleboro dams influence several behavioral and physiological characteristics of their offspring (e.g., body weight, brain weight, startle response, and stress reactivity; Snijdewint et al., 1988; Zelena et al., 2003, 2009; Feifel and Priebe, 2007). To minimize the potential impact of the early life environment in the present experiment, we tested Hom, Het, and WT littermates born to Het dams, thereby removing potential prenatal and postnatal confounds of maternal

genotype. Nonetheless, we cannot rule out possible confounds of differential maternal or sibling treatment towards Hom Brattleboro pups.

While it is now clear that AVP influences juvenile social behaviors, we know very little about how AVP acts to regulate social development. This represents a critical gap in our knowledge as altered AVP function has been implicated in neurodevelopmental disorders such as autism spectrum disorders and schizophrenia (Heinrichs et al., 2009; Rubin et al., 2014 and references therein). Notably, Hom Brattleboro rats exhibit behavioral abnormalities associated with schizophrenia and autism spectrum disorders, including decreased social interactions, social cognitive deficits, and attenuated prepulse inhibition, which is consistent with the hypothesis that AVP signaling is disrupted in these disorders (Engelmann and Landgraf, 1994; Feifel and Priebe, 2007; Feifel et al., 2009; Lin et al., 2013). The present findings add social play and vocal communication to this list. Our findings also provide insight into AVP's role in social development. By measuring multiple social behaviors in the same experiment, we were able to demonstrate that chronic disruption of AVP production does not simply decrease overall levels of social behavior, but rather alters the type of social behavior in which the animal expresses, leading to an "atypical" rather than asocial phenotype. Future studies are needed to uncover the neural mechanisms through which AVP influences both normal and disordered social development.

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Figure Legends

Figure 1. Hom Brattleboro weanlings play less than their WT and Het littermates. Number of Total Play Behaviors (A) and Pins (B) of homozygous Brattleboro (Hom), heterozygous Brattleboro (Het), and wild type (WT) rats during a 20-min test at 17 days of age (P17), P19, P21, or P23. Sample sizes are indicated within each bar. Data from each age were obtained from separate cohorts of animals. Genotypes with differing letters differ significantly from each other ($P<0.05$, Fisher's PLSD); where differences approach significance, the p-value is included in parentheses next to the letter representing the appropriate comparison. See Results for ANOVA details.

Figure 2. Social play is decreased in Hom Brattleboro juveniles. Number of Pins (A) and Play Attacks (B) of male and female Hom, Het, and WT rats during a 20-min test at P34 or P44. The temporal profile of play is illustrated in C (for P34) and D (for P44) as the number of Total Play Behaviors binned every 5 min. Sample sizes are indicated within each bar in A and B. Data from each age were obtained from separate cohorts of animals. Genotypes with differing letters differ significantly from each other ($P<0.05$, Fisher's PLSD); where differences approach significance, the p-value is included in parentheses next to the letter representing the appropriate comparison. In panels C and D, significant differences between Hom and WT or Het rats within each bin are indicated by * and #, respectively ($P<0.005$, Fisher's PLSD). See Results for ANOVA details.

Figure 3. Social behavior is altered in Hom Brattleboro juveniles. Number of Total Social Behaviors (A), Total Play Behaviors (B), Social Investigation & Allogrooming Behaviors (C), and Huddling Episodes (D) of Hom, Het, and WT rats during a 20-min test at P34 or P44. Sample sizes are indicated within each bar. Data from each age were obtained from separate cohorts

of animals. Genotypes with differing letters differ significantly from each other (P<0.05, Fisher's PLSD). See Results for ANOVA details.

Figure 4. Hom Brattleboro juveniles emit fewer 50 kHz USVs. Number of all (A), 50kHz (B), and 22 kHz (C) USVs of male and female Hom, Het, and WT rats during the first 10 min of a 20-min test at P34 or P44. Sample sizes are indicated within each bar. Data from each age were obtained from separate cohorts of animals. Genotypes with differing letters differ significantly from each other (P<0.05, Fisher's PLSD); where differences approach significance, the p-value is included in parentheses next to the letter representing the appropriate comparison. See Results for ANOVA details.

Figure 5. Ultrasonic vocalization call types emitted during social behavior testing. Percentage of USV call types (A), as defined in Wright et al. (2010), emitted by male and female Hom, Het, and WT juveniles (P34 or P44) during the first 10 min of a 20-min test; data are combined across sex, genotype, and age. Representative spectrograms of the most common call 50 kHz types [Complex (B), Upward Ramp (C), Flat (D), Step Up (E), and Trill (F)] as well as the 22 kHz call type (G).

Figure 6. The quantity and quality of USV calls is altered in Hom Brattleboro rats. Number (A-E), Integrated Frequency (F-J), and Duration (K-O) of Type 1 (complex), Type 2 (upward-ramp), Type 4 (flat), Type 7 (step-up), and Type 10 (trill) USV calls of male and female Hom, Het, and WT rats during the first 10m in of a 20-min test at P34 or P44. Data from each age were obtained from separate cohorts of animals. Genotypes with differing letters differ significantly from each other (P<0.05, Fisher's PLSD); where differences approach significance, the p-value is included in parentheses next to the letter representing the appropriate comparison. See Results for ANOVA details.

Table 1. Experiment 1 Statistical Analyses

	Data Structure	Dependent Variable	Comparison	Type of Test	P-value	Power
a1	Non-normal Distribution	Total Play	main effect of age	3-way ANOVA, K-W	<0.0001 (ANOVA & K-W)	1.000
a2	Non-normal Distribution		main effect of genotype	3-way ANOVA, K-W	0.0310 (ANOVA), 0.0375 (K-W)	0.649
a3	Non-normal Distribution		main effect of sex	3-way ANOVA, M-W	0.9342 (ANOVA), 0.3852 (M-W)	0.051
a4	Non-normal Distribution		P17 vs. P19	M-W	<0.0001	1.000
a5	Non-normal Distribution		P19 vs. P21	M-W	<0.0001	1.000
a6	Non-normal Distribution		Hom vs. WT	Fisher's PLSD, M-W	0.0024 (Fisher's), 0.0124 (M-W)	0.804
a7	Non-normal Distribution		Hom vs. Het	Fisher's PLSD, M-W	0.0079 (Fisher's), 0.043 (M-W)	0.612
a8	Normal Distribution		main effect of genotype, P21	2-way ANOVA	0.0021	0.918
a9	Normal Distribution		Hom vs. WT, P21	Fisher's PLSD	0.0004	0.934
a10	Normal Distribution		Hom vs. Het, P21	Fisher's PLSD	0.0604	0.610
a11	Normal Distribution		Het vs. WT, P21	Fisher's PLSD	0.0342	0.470
a12	Normal Distribution		main effect of genotype, P23	2-way ANOVA	0.0582	0.550
a13	Normal Distribution		Hom vs. WT, P23	Fisher's PLSD	0.0876	0.526
a14	Normal Distribution		Hom vs. Het, P23	Fisher's PLSD	0.0190	0.604
a15	Normal Distribution		main effect of genotype, P19	2-way ANOVA	0.6717 (ANOVA)	0.110
b1	Non-normal Distribution	Pins	main effect of age	3-way ANOVA, K-W	<0.0001 (ANOVA & K-W)	1.000
b2	Non-normal Distribution		main effect of genotype	3-way ANOVA, K-W	0.0118 (ANOVA), 0.0046 (K-W)	0.773
b3	Non-normal Distribution		main effect of sex	3-way ANOVA, M-W	0.9655 (ANOVA), 0.5681 (M-W)	0.050
b4	Non-normal Distribution		P17 vs. P19	M-W	<0.0001	1.000
b5	Non-normal Distribution		P19 vs. P21	M-W	<0.0001	1.000
b6	Non-normal Distribution		Hom vs. WT	Fisher's PLSD, M-W	0.0003 (Fisher's), 0.0015 (M-W)	0.950
b7	Non-normal Distribution		Hom vs. Het	Fisher's PLSD, M-W	0.0019 (Fisher's), 0.0085 (M-W)	0.797
b8	Normal Distribution		main effect of genotype, P21	2-way ANOVA	0.0015	0.933
b9	Normal Distribution		Hom vs. WT, P21	Fisher's PLSD	0.0003	0.942
b10	Normal Distribution		Hom vs. Het, P21	Fisher's PLSD	0.0436	0.764
b11	Normal Distribution		Het vs. WT, P21	Fisher's PLSD	0.0363	0.436
b12	Normal Distribution		main effect of genotype, P23	2-way ANOVA	0.0280	0.667
b13	Normal Distribution		Hom vs. WT, P23	Fisher's PLSD	0.0377	0.770
b14	Normal Distribution		Hom vs. Het, P23	Fisher's PLSD	0.0099	0.691
b15	Non-normal Distribution		main effect of genotype, P19	2-way ANOVA, K-W	0.3325 (ANOVA), 0.3213 (K-W)	0.230
c1	Non-normal Distribution	Play Attacks	main effect of age	3-way ANOVA, K-W	<0.0001 (ANOVA & K-W)	1.000
c2	Non-normal Distribution		main effect of genotype	3-way ANOVA, K-W	0.0629 (ANOVA), 0.0887 (K-W)	0.537
c3	Non-normal Distribution		main effect of sex	3-way ANOVA, M-W	0.9314 (ANOVA), 0.4056 (M-W)	0.051
c4	Non-normal Distribution		P17 vs. P19	M-W	<0.0001	1.000
c5	Non-normal Distribution		P19 vs. P21	M-W	<0.0001	1.000
c6	Non-normal Distribution		Hom vs. WT	Fisher's PLSD, M-W	0.0086 (Fisher's), 0.0318 (M-W)	0.645

Abbreviations: K-W = Kruskal Wallis test, M-W = Mann-Whitney U test

Table 2. Experiment 2 Statistical Analyses

	Data Structure	Dependent Variable	Comparison	Type of Test	P-value	Power
d1	Normal Distribution	Total Play	main effect of age	3-way ANOVA	<0.0001	0.997
d2	Normal Distribution		main effect of genotype	3-way ANOVA	<0.0001	1.000
d3	Normal Distribution		main effect of sex	3-way ANOVA	0.6259	0.076
d4	Normal Distribution		Hom vs. WT	Fisher's PLSD	<0.0001	1.000
d5	Normal Distribution		Hom vs. Het	Fisher's PLSD	<0.0001	1.000
e1	Non-normal Distribution	Pins	main effect of age	3-way ANOVA, M-W	0.0041 (ANOVA), 0.0002 (M-W)	0.843
e2	Non-normal Distribution		main effect of genotype	3-way ANOVA, K-W	0.0031 (ANOVA), <0.0001 (K-W)	0.890
e3	Non-normal Distribution		main effect of sex	3-way ANOVA, M-W	0.9169 (ANOVA), 0.3571 (M-W)	0.051
e4	Non-normal Distribution		Hom vs. WT	Fisher's PLSD, M-W	0.0048 (Fisher's), <0.0001 (M-W)	0.908
e5	Non-normal Distribution		Hom vs. Het	Fisher's PLSD, M-W	0.0016 (Fisher's), <0.0001 (M-W)	0.903
e6	Non-normal Distribution		genotype x sex, P44	2-way ANOVA	0.0076	0.826
e7	Normal Distribution		Het male vs. Het female, P44	Fisher's PLSD	0.0019	0.921
f1	Normal Distribution	Play Attacks	main effect of age	3-way ANOVA	<0.0001	0.999
f2	Normal Distribution		main effect of genotype	3-way ANOVA	<0.0001	1.000
f3	Normal Distribution		main effect of sex	3-way ANOVA	0.5730	0.085
f4	Normal Distribution		Hom vs. WT	Fisher's PLSD	<0.0001	1.000
f5	Normal Distribution		Hom vs. Het	Fisher's PLSD	<0.0001	1.000
g1	Normal Distribution	Total Social Behaviors	main effect of age	3-way ANOVA	<0.0001	1.000
g2	Normal Distribution		main effect of genotype	3-way ANOVA	<0.0001	1.000
g3	Normal Distribution		main effect of sex	3-way ANOVA	0.8892	0.052
g4	Normal Distribution		Hom vs. WT	Fisher's PLSD	<0.0001	1.000
g5	Normal Distribution		Hom vs. Het	Fisher's PLSD	<0.0001	1.000
h1	Normal Distribution	Social Investigation / Allogrooming	main effect of age	3-way ANOVA	0.0090	0.758
h2	Normal Distribution		main effect of genotype	3-way ANOVA	0.5137	0.156
h3	Normal Distribution		main effect of sex	3-way ANOVA	0.4631	0.109
i1	Non-normal Distribution	Huddling	main effect of age	3-way ANOVA, M-W	<0.0001 (ANOVA), 0.0001 (M-W)	0.999
i2	Non-normal Distribution		main effect of genotype	3-way ANOVA, K-W	<0.0001 (ANOVA), <0.0001 (K-W)	1.000
i3	Non-normal Distribution		main effect of sex	3-way ANOVA, M-W	0.8084 (ANOVA), 0.8321 (M-W)	0.057
i4	Normal Distribution		Hom vs. WT	Fisher's PLSD, M-W	<0.0001 (Fisher's), 0.0003 (M-W)	0.948
i5	Non-normal Distribution		Hom vs. Het	Fisher's PLSD, M-W	<0.0001 (Fisher's), <0.0001 (M-W)	1.000
j1	Normal Distribution	All USVs	main effect of genotype	3-way ANOVA	<0.0001	0.993
j2	Normal Distribution		Hom vs. WT	Fisher's PLSD	0.0020	0.942
j3	Normal Distribution		Hom vs. Het	Fisher's PLSD	<0.0001	0.998
k1	Normal Distribution	50 kHz USVs	main effect of age	3-way ANOVA	0.0380	0.537
k2	Normal Distribution		main effect of genotype	3-way ANOVA	0.0001	0.990
k3	Normal Distribution		Hom vs. WT	Fisher's PLSD	0.0022	0.947
k4	Normal Distribution		Hom vs. Het	Fisher's PLSD	<0.0001	0.996
l1	Non-normal Distribution	22 kHz USVs	main effect of age	3-way ANOVA, M-W	0.0001 (ANOVA), <0.0001 (M-W)	0.986
l2	Non-normal Distribution		main effect of genotype	3-way ANOVA, K-W	0.2541 (ANOVA), 0.2262 (K-W)	0.282

Abbreviations as in Table 1

Table 3. USV Call Type Statistical Analyses

	Data Structure	Dependent Variable	Comparison	Type of Test	Exact P-value	Power
m1	Normal Distribution	Complex calls	main effect of age	3-way ANOVA	0.1349	0.304
m2	Normal Distribution	(Number)	main effect of genotype	3-way ANOVA	0.0258	0.676
m3	Normal Distribution		Hom vs. WT	Fisher's PLSD	0.0303	0.594
m4	Normal Distribution		Hom vs. Het	Fisher's PLSD	0.0116	0.796
m5	Normal Distribution		main effect of genotype, P34	2-way ANOVA	0.1603	0.366
m6	Normal Distribution		main effect of genotype, P44	2-way ANOVA	0.1587	0.367
n1	Normal Distribution	Upward-Ramp calls	main effect of age	3-way ANOVA	0.0028	0.875
n2	Normal Distribution	(Number)	main effect of genotype, P34	2-way ANOVA	0.0185	0.725
n3	Normal Distribution		Hom vs. WT, P34	Fisher's PLSD	0.0112	0.724
n4	Normal Distribution		Hom vs. Het, P34	Fisher's PLSD	0.0163	0.755
n5	Normal Distribution		main effect of genotype, P44	2-way ANOVA	0.6322	0.120
o1	Normal Distribution	Flat calls	main effect of age	3-way ANOVA	0.8419	0.054
o2	Normal Distribution	(Number)	main effect of genotype, P34	2-way ANOVA	0.0270	0.673
o3	Normal Distribution		Hom vs. WT, P34	Fisher's PLSD	0.0574	0.520
o4	Normal Distribution		Hom vs. Het, P34	Fisher's PLSD	0.0056	0.832
o5	Normal Distribution		main effect of genotype, P44	2-way ANOVA	0.5538	0.141
p1	Normal Distribution	Step-Up calls	main effect of age	3-way ANOVA	0.0355	0.549
p2	Normal Distribution	(Number)	main effect of genotype, P34	2-way ANOVA	0.0058	0.850
p3	Normal Distribution		Hom vs. WT, P34	Fisher's PLSD	0.0084	0.857
p4	Normal Distribution		Hom vs. Het, P34	Fisher's PLSD	0.0022	0.918
p5	Normal Distribution		main effect of genotype, P44	2-way ANOVA	0.0543	0.562
q1	Non-normal Distribution	Trills	main effect of age	3-way ANOVA, M-W	0.0144 (ANOVA), 0.0025 (M-W)	0.694
q2	Non-normal Distribution	(Number)	main effect of sex	3-way ANOVA, K-W	0.0046 (ANOVA), <0.0001 (K-W)	0.830
q3	Normal Distribution		main effect of genotype, P34	2-way ANOVA	0.0092	0.807
q4	Normal Distribution		Hom vs. WT, P34	Fisher's PLSD	0.0978	0.789
q5	Normal Distribution		Hom vs. Het, P34	Fisher's PLSD	0.0045	0.746
q6	Normal Distribution		main effect of genotype, P44	2-way ANOVA	0.0009	0.954
q7	Normal Distribution		Hom vs. WT, P44	Fisher's PLSD	0.0300	0.887
q8	Normal Distribution		Hom vs. Het, P44	Fisher's PLSD	0.0005	0.923
r1	Normal Distribution	Complex calls	main effect of genotype	3-way ANOVA	0.0142	0.754
r2	Normal Distribution	(Int. Freq.)	Hom vs. WT	Fisher's PLSD	0.1043	0.414
r3	Normal Distribution		Hom vs. Het	Fisher's PLSD	0.0053	0.703
r4	Normal Distribution		main effect of age	3-way ANOVA	<0.0001	0.999
r5	Normal Distribution		age x sex	3-way ANOVA	0.9431	0.059
s1	Normal Distribution	Upward-Ramp calls	main effect of genotype	3-way ANOVA	0.0002	0.986
s2	Normal Distribution	(Int. Freq.)	Hom vs. WT	Fisher's PLSD	0.0007	0.936
s3	Normal Distribution		Hom vs. Het	Fisher's PLSD	<0.0001	0.990
s4	Normal Distribution		main effect of age	3-way ANOVA	<0.0001	0.994
s5	Normal Distribution		age x sex	3-way ANOVA	0.6940	0.106
t1	Normal Distribution	Flat calls	main effect of genotype	3-way ANOVA	0.0020	0.916
t2	Normal Distribution	(Int. Freq.)	Hom vs. WT	Fisher's PLSD	0.0022	0.930
t3	Normal Distribution		Hom vs. Het	Fisher's PLSD	0.0014	0.825
t4	Normal Distribution		main effect of age	3-way ANOVA	<0.0001	0.999
t5	Normal Distribution		age x sex	3-way ANOVA	0.8255	0.079
u1	Normal Distribution	Step-Up calls	main effect of genotype	3-way ANOVA	0.0186	0.721
u2	Normal Distribution	(Int. Freq.)	Hom vs. WT	Fisher's PLSD	0.0416	0.531
u3	Normal Distribution		Hom vs. Het	Fisher's PLSD	0.0066	0.706
u4	Normal Distribution		main effect of age	3-way ANOVA	0.0416	0.521
u5	Normal Distribution		age x sex	3-way ANOVA	0.0588	0.548
v1	Normal Distribution	Trills	main effect of genotype	3-way ANOVA	0.2452	0.288
v2	Normal Distribution	(Int. Freq.)	main effect of age	3-way ANOVA	0.1801	0.252
w1	Normal Distribution	Step-Up calls	main effect of genotype, P44	2-way ANOVA	0.0026	0.908
w2	Normal Distribution	(Dur.)	Hom vs. WT, P44	Fisher's PLSD	0.0195	0.592
w3	Normal Distribution		Hom vs. Het, P44	Fisher's PLSD	0.0006	0.947
w4	Normal Distribution		main effect of age	3-way ANOVA	0.2480	0.198
x1	Normal Distribution	Trills	main effect of genotype, P44	2-way ANOVA	0.0090	0.810
x2	Normal Distribution	(Dur.)	Hom vs. WT, P44	Fisher's PLSD	0.0040	0.799
x3	Normal Distribution		Het vs. WT, P44	Fisher's PLSD	0.0157	0.659
x4	Normal Distribution		Hom vs. Het, P44	Fisher's PLSD	0.2632	0.213
x5	Normal Distribution		main effect of age	3-way ANOVA	0.1010	0.357
y1	Normal Distribution	Complex calls (Dur.)	main effect of age	3-way ANOVA	0.0210	0.638
z1	Normal Distribution	Upward-Ramp calls (Dur.)	main effect of age	3-way ANOVA	0.0078	0.775
aa1	Normal Distribution	Flat calls (Dur.)	main effect of age	3-way ANOVA	0.0036	0.854

804 Abbreviations as in Table 1

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