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THE EFFECTS OF PRE-TEST SOCIAL DEPRIVATION ON A NATURAL REWARD INCENTIVE TEST AND CONCOMITANT 50 KHZ ULTRASONIC VOCALIZATION PRODUCTION IN ADOLESCENT AND ADULT MALE SPRAGUE-DAWLEY RATS

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Abstract

In rats, ultrasonic vocalizations (USVs) are often used as a measure of affect, with 22 kHz USVs reflecting negative affect and 50 kHz USVs reflecting positive affect. This study used a modified reward approach task to assess behavioral approach and concomitant USV production toward either a food or social stimulus following varied amounts of pre-test social deprivation in both adolescent and adult rats. Adolescents spent significantly more time investigating the social stimulus than adults, although in the presence of the social stimulus isolate housed adults emitted more 50 kHz USVs than isolate housed adolescents. Isolate housed adults emitted more USVs to a social stimulus than adults that received less pre-test social isolation, a social deprivation effect not evident in the social approach data or any measure when animals were tested with a food stimulus. When tested with a food stimulus, adults emitted more 50 kHz USVs than adolescents, although they spent the same (or even less) time investigating that stimulus than did adolescents. Together, these data demonstrate that 50 kHz USV production and time spent investigating a rewarding stimulus are clearly dissociable and hence seemingly measure different states.

Keywords

adolescent; ontogeny; ultrasonic vocalization; reward; social; rat

1 Introduction

Ultrasonic vocalizations (USVs) have been used as a measure of affect, with calls in the 22 kHz range thought to reflect negative affect and calls in the 50 kHz range used as an index

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of positive affect [1]. Calls centered around 22 kHz typically last around 300 - 3,000 ms and are often seen in response to predator odor, foot shock, and aggressive encounters with a conspecific [2-6]. In contrast, USVs centered around 50 kHz (ranging from 40-75 kHz) are shorter in duration (30-80 ms), and are emitted during copulation and other social interactions, in response to self-administered or experimenter administered drugs, and in expectation of various rewards [7-12]. These 50 kHz USVs have been postulated to serve as an index of positive affective states in rats, given that they are associated with dopamine activity in reward-related brain areas [8, 13-15] and can be inhibited by aversive stimuli [1, 11, 13]. 50 kHz USVs encompass a number of different call types [16] with frequency modulated (FM) calls in particular thought to measure hedonic value and flat calls thought to serve as social signals [17].

Emission of 50 kHz USVs have been shown to increase in anticipation of, or in the presence of a social partner in both adolescent [11, 18] and adult rats [18-20], although few studies have directly compared social USV production across ages [12, 21]. In such ontogenetic studies, adolescents have surprisingly been found to emit fewer 50 kHz USVs in anticipation of a social interaction [21], and during social interactions [12] relative to adults, even though they often engage in higher levels of peer-directed social behaviors, especially play behavior [12, 22-25] and show greater conditioned place preference (CPP) for a social stimulus than adults [26]. The underlying cause of age-related differences in time spent in peer-directed social interactions or preference for a place that had previously been paired with a social stimulus is currently unclear. One might assume that spending more time interacting with a social stimulus would indicate an increase in the hedonic value (pleasure experienced) of the interaction; however age-related differences in 50 kHz USV production in the presence of or in anticipation of a social stimulus could suggest otherwise. In order to further investigate this apparent dissociation of age-related differences between behavioral measures and vocalization measures, this experiment focused on the approach to two natural rewards (social and food stimuli) and the USVs produced in the presence of these stimuli. Utilizing an approach paradigm that requires no prior experience with the task and that also prevents the “consumption” of rewards should allow for a clearer analysis of the relationship between the incentive value of rewards and USV production at different ages. To the extent that approach behavior reflects reward seeking, hence serving as an indirect measure of the incentive value of a stimulus [27, 28], it would be expected that 50 kHz USV production would show similar effects inasmuch as it is thought to be a measure of positive affect and reward seeking [17].

This experiment used a modified version of the natural reward incentive test originally developed by Nocjar & Panksepp [27, 28] as an index of social motivation in adolescent and adult rats. The testing apparatus consists of an open field outfitted with stimulus chambers containing screened windows in two opposite corners of the open field that provide an opportunity for animals to see, smell, and approach a target rewarding stimulus without physically contacting it. In order to confirm the ability of this paradigm to measure the incentive salience of a stimulus for both adolescent and adult animals, the motivation for social interactions was manipulated by varying pre-test social deprivation. Social isolation has been shown to have powerful effects on social motivation, with amount of pre-test social isolation increasing social behaviors, but having little impact on non-social behaviors [29] and increasing USV production to a social stimulus [20]. In the present study, subjects were exposed to varying amounts of social isolation prior to an approach test involving either a food reward (chocolate chips) or a social reward. To the extent that this reward incentive test reliably measures social motivation, increased social deprivation should decrease latency to approach a social stimulus, increase time spent investigating that stimulus, and increase USV production in the test situation without influencing the behavior or USV production of animals tested with the non-social (food) stimulus. Based on previous findings of greater

social CPP in adolescents than adults [26], adolescents would be expected to spend more time investigating the social stimuli than adults. To the extent that USVs emitted in the choice situation reflect incentive salience of the stimuli, any deprivation or age-related increase in choice should be accompanied by increases in 50 kHz production.

2 Methods

2.1 Subjects

A total of 151 Sprague-Dawley male rats bred and reared in our colony at Binghamton University were used in this study (103 used as experimental subjects and 48 as stimulus animals [see below]). The day after birth all litters were culled to 8–10 pups (6 males and 4 females when possible) and housed with their mother in standard breeding cages. At weaning on postnatal day (P) 21, animals to be tested as adolescents were housed with a same-age, same-sex non-littermate assigned to the same test condition or were isolate housed. Animals assigned to be tested as adults were pair-housed with a same-sex littermate at weaning, and rehoused with a same aged, non-littermate male assigned to the same test condition or isolate housed on P63–64. By isolate housing or rehousing both adolescent and adult subjects with non-littermates beginning 11–12 days prior to onset of the testing procedures, time from rehousing to test was controlled across age while allowing pair-housed cage-mates to be assigned to the same experimental treatment without confounding data with litter effects [30]. All animals were housed in a temperature-controlled (20–22° C) vivarium on a 14-/10-hour light/dark cycle (lights on at 0700) with ad libitum access to tap water and food (Purina Rat Chow, Lowell, MA). Animals were treated in accordance with guidelines established by the National Institutes of Health using protocols approved by the Binghamton University Institutional Animal Care and Use Committee.

2.2 Apparatus

Each reward approach testing apparatus consisted of a custom built open arena (45 × 45 × 30 cm for adolescents and 60 × 60 × 30 cm for adults) with triangular reward proximity boxes (13.5 × 13.5 × 18.75 cm for adolescents and 18 × 18 × 25 cm for adults) placed diagonally across opposite corners of the arena. The apparatuses were made of black Lexan with wire mesh screens (17.8 × 6.4 cm for both ages) installed in the wall of the reward proximity boxes abutting the open arena to allow for visual and olfactory access of the test animal to the reward stimuli without physical contact. Clean aspen shavings were used to coat the floor of the arena given that it has been shown that 50 kHz USV production is greatly decreased in the absence of bedding [31]. No shavings were used in the reward chambers in an attempt to decrease the contribution of stimulus animal vocalizations to the USV count. During testing sessions, behaviors were recorded by a video camera for later behavioral scoring. After each testing session the shavings were removed and the open field was wiped clean with a 6% hydrogen peroxide solution.

Ultrasonic vocalizations were measured using Avisoft UltraSoundGate CM16 microphones, recorded by an UltraSoundGate 416–200 recording device, digitized by Avisoft-Recorder USG, and stored as .wav-files for later analysis (Avisoft Bioacoustics, Berlin, Germany). The microphones were placed on the top edge of the reward chamber and pointed downward into the open arena. The microphones are sensitive to frequency ranges between 10 and 125 kHz with a high sensitivity at 50 kHz and moderate directional properties. Sound analysis was performed using Avisoft-SASLab Pro software (Avisoft Bioacoustics, Berlin, Germany) which converts sound files to spectrograms for manual visual counts of calls. A sound was considered to be a 50 kHz USV if it fell between 35 and 80 kHz with a duration of 30–80 ms. Within this range, the 50 kHz calls were further divided into frequency modulated (FM) or flat calls as described by Burgdorf and Panksepp [32]. Calls were classified as FM if they

contained any frequency changes (increases, decreases, or “trills”) with calls displaying a near-constant frequency classified as flat calls. Any calls which contained both flat and FM components were classified as FM. Calls were considered in the 22 kHz category if they fell between 18 and 32 kHz with a duration of 300-3000 ms. Juvenile rats have been shown to spend more time with a high vocalizing partner than a low vocalizing partner [33], and hence stimulus animals were not devocalized. Consequently, in the animals tested with a social stimulus, USVs recorded during testing reflect both the stimulus and experimental animals.

2.3 Procedure

The design of this study was a 2 (age: adolescent, adult) $\times$ 2 (test stimulus: social, food) $\times$ 3 (pre-test social isolation: 25 min, 3 hours, chronically isolate housed) factorial, with 8-11 subjects placed into each group. All testing was conducted between 1300 and 1700 hours under low light conditions (8-10 lux). Animals used as social stimuli were of the same age and sex as the experimental subjects, and were housed in the same manner and exposed to the same amount of pre-test social deprivation as their experimental counterpart. Animals were not screened for spontaneous USV production. Beginning on P32 or P75-76 and continuing for 3 days, all experimental animals were separated from their cage-mate with a wire mesh divider for 30 min, during which each animal was given 4 chocolate chips to consume in order to eliminate any potential neophobia to this novel food stimulus. All animals readily consumed the chocolate chips by the last pre-exposure day. On the day prior to testing (P35 or P77-78), all experimental animals were placed individually in the testing arena with both stimulus chambers empty for 30 min to habituate them to the testing situation, given that rats typically do not direct social behavior towards a social stimulus until they have investigated their new environment [29]. On test day, each pair-housed subject was socially isolated in a holding cage for either 25 min or 3 hrs prior to testing. Isolate housed animals were placed in similar holding cages for 25 min prior to testing. Each experimental animal was then placed for 10 min in the testing arena containing either a non-familiar conspecific or four chocolate chips in one stimulus chamber, while the other chamber remained empty. The test sessions were videotaped and USVs recorded for later analysis.

On test day, latency to approach the stimulus chamber and time spent investigating (nose within 1 cm of the mesh screen) the stimulus and empty chambers were measured.

2.4 Data Analyses

Time spent investigating each chamber and 50 kHz USV production were analyzed using repeated measures analyses of variance (ANOVAs), with chamber (reward/empty) or call type (FM/flat) serving as within-subjects factors and age and pre-test isolation serving as between-subjects measures. Latency to approach the reward stimulus was analyzed using a factorial ANOVA with age and pre-test isolation serving as between-subjects measures. Because USVs recorded in the socially tested groups represent the sum of the USVs produced by both the test and stimulus animals whereas those in the food tested group represent USVs emitted by a single animal, separate ANOVAs were conducted for the socially-tested and food-tested groups for all measures. Pairwise planned comparisons were made using relatively liberal Fisher’s LSD tests to determine the locus of significant main effects and interactions of ANOVAs where 3 orthogonal factors were analyzed to avoid inflating the possibility of type 2 errors [34]. 2 factor ANOVAs were further analyzed with Bonferroni’s test to minimize inflating type 1 errors. For each stimulus group, Pearson R correlations were conducted to compare USV production with behavioral measures. Results were considered significant if $p < 0.05$.

Prior to these analyses, all data were subjected to Levene's test for homogeneity of variance and were assessed for outliers. Subjects were removed as outliers if their total 50 kHz USV production or behavioral scores were more than 2 standard deviations above or below the group mean. A total of 6 animals were removed as outliers, with no more than 2 animals removed from any test group and final sample sizes of 7-10 subjects per group. All measures satisfied homogeneity of variance assumptions except for USV production, likely due to the dramatic increase in USV production in the socially isolated animals. USVs were subjected to a square root transformation (SQRT) to improve variance homogeneity. USV data from one animal was lost due to a technical issue.

3 Results

3.1 Social Stimulus

When tested with a social stimulus, there was a significant effect of pre-test deprivation on USV production, $F(2,40) = 11.54$, $p < 0.001$, tempered by an interaction with age, $F(2,40) = 85.67$, $p < 0.05$, in which isolate housed adults emitted significantly more 50 kHz USVs than adolescents and the 3 hr and 25 min isolated adults (Fig 1 top panels). There were no main effects or interactions involving call type.

A 2 age $\times$ 2 chamber ANOVA of seconds spent investigating each chamber revealed significant main effects of age, $F(1,41) = 13.10$, $p < 0.001$, and chamber, $F(1,41) = 368.05$, $p < 0.001$, tempered by an interaction of these variables, $F(1,41) = 10.25$, $p < 0.01$. Exploration of this interaction revealed that whereas there were no differences in time spent investigating the empty chamber between ages, adolescents spent significantly more time investigating the reward chamber than adults (see Fig 1 bottom panels).

There were no significant main effects or interactions in the ANOVA of latency to approach the social stimulus, with both adolescents and adults approaching the social stimulus relatively quickly (adolescents: 16.71 ± 3.20 ; adults: 15.10 ± 3.24).

3.2 Food Stimulus

Analysis of USV production to a food stimulus revealed a significant main effect of age, $F(1,42) = 8.82$, $p < 0.01$, with adults exhibiting notably more USVs under these testing circumstances than adolescents. There were no significant main effects or interactions involving pre-test social deprivation or call type on USV production (Fig 2 top panels).

Repeated measures ANOVA of the seconds spent investigating each chamber found a main effect of chamber, $F(1,42) = 68.22$, $p < 0.001$, with animals at both ages spending significantly more time investigating the food chamber (62.13 ± 4.60 sec) than the empty chamber (25.74 ± 2.30) (see Fig 2, bottom panels).

There were no significant effects with regard to the latency to approach a food stimulus, with average approach latencies of 29.40 ± 8.38 sec for adolescents and 21.38 ± 8.74 sec for adults.

3.3 Correlations of USVs and behaviors

In animals tested with a social stimulus, FM 50 kHz USV production was modestly but significantly correlated with time spent investigating the social stimulus ($r=0.30$), whereas among animals tested with the food stimulus, 50 kHz USV production was not significantly correlated with any behavioral measure.

4 Discussion

This study used behavioral approach and 50 kHz USV production to examine the hedonic value of social and non-social rewards in adolescent and adult rats. All animals spent significantly more time investigating the social or food stimulus than the empty chamber, indicating that the experimental animals found these rewards motivating. Adolescents spent more time investigating the social stimulus than did adults, whereas there was no significant age difference for the food chamber. When tested with a social stimulus, adult rats that were isolate-housed prior to testing produced more 50 kHz USVs than adolescents or than adults that were socially isolated for 25 min or 3. Amount of social isolation, however, did not significantly affect time spent investigating the social stimulus, nor did it influence 50 kHz USV production to or time spent investigating the food stimulus at either age.

All animals spent significantly more time investigating the social stimulus than the empty chamber, which supports previous findings utilizing a CPP test in which both adolescent [26, 35-37] and adult animals [26, 36] showed a significant place preference for a social stimulus. Both adolescent and adult animals have also shown anticipatory hyperactivity for a social stimulus in a Pavlovian conditioning task, a response thought to reflect high motivational properties of social interactions [36]. In the present study, adolescents spent significantly more time investigating, and showed a greater preference for the social stimulus than adults. This is reminiscent of social CPP tests in which social interactions were found to be rewarding at both ages, although the social interest was more pronounced in adolescents than adults [26, 36]. Collectively, these data support the conclusion that adolescents attribute more incentive value to social interactions than adults.

Animals at both ages also spent significantly more time investigating the food reward. These findings are seemingly inconsistent with other work reporting that non-food-deprived animals did not show CPP for sucrose pellets [38]. The differing nature of these findings may reflect a difference in the motivational properties indexed when using approach to a reward stimulus versus preference for a location in which the food had previously been located. Interestingly, it has also been shown that mildly food deprived adults, but not adolescents, express a CPP for Fruit Loops [39], whereas in the present study, if anything, it was the adolescents who trended towards higher food preference than adults. This may reflect age-related motivational differences between food deprived and non-deprived animals, or differences between tests of motivation which utilize reward approach (the present study) versus those that depend on prior conditioning of a location preference (CPP studies).

The lack of pre-test social deprivation effects on time spent investigating the social stimulus are inconsistent with prior work assessing the effects of isolation on social reward using a CPP test in which CPP for a social stimulus was enhanced by isolate housing in both adolescent and adult animals [26]. The lack of increases in social investigation in the present study may be due to the inability to actually interact with the social stimulus – which may have masked rewarding behavioral consequences of direct interactions with the social stimulus. In the present study, it is also possible that the length of the test session may have masked social isolation effects, i.e. – the subjects may realize early on in the test session that they are unable to access the social stimulus and therefore lose interest. In general, these animals spend a great deal of time attempting to chew on and get their paws through the mesh screen in order to physically contact the partner animal. Experimental animals may find this situation frustrating due to their inability to contact the stimulus animal. In subsequent work utilizing a similar testing procedure, ethanol-induced increases in social investigation were only observed during the first five min of testing and were not evident thereafter [40].

Few studies have directly compared USV production between adolescent and adult animals [12, 21], with much of the USV literature to date testing either adolescent animals [11, 41], adults [9, 42-44], or even both ages without addressing possible age differences in USV production [18, 20]. In the present study isolate housed adults emitted more 50 kHz USVs in the presence of a social stimulus than their adolescent counterparts, and also emitted more 50 kHz USVs than adolescents when tested with a food stimulus. As the spectrograms were scored manually, it is not possible that adolescents were simply emitting USVs at a different frequency – spectrograms ranged from 10 to 100 kHz and all USVs in this range were screened. In prior work comparing 50 kHz USV production across ages, we found that adults emit more 50 kHz USVs during social interactions (although they interact less socially) than do adolescents [12]. Adults were also shown to exhibit anticipatory increases in 50 kHz USV production to a social stimulus whereas adolescents did not [21]. The greater 50 kHz USV production by adults than adolescents in these studies is reminiscent of the present data. There has been little research to date directly comparing 50 kHz USV production across ages, however a previous study from our lab has shown that under some circumstances adolescents emit as many, if not more 50 kHz USVs. In that study, animals were placed alone in the testing chamber for 5 min before being allowed to interact with a conspecific for 10 min. By repeating this across test days, we were able to see anticipatory increases in 50 kHz USV production. On the first test day, there were no age differences in 50 kHz USV production. It was also found that adolescents emitted more 50 kHz USVs during the first min of the anticipatory period than the adults [45]. Further research is necessary to assess age differences in 50 kHz USV production to various rewarding stimuli.

Social isolation greatly increased 50 kHz USV production to a social stimulus in adults when compared to 25 min or 3 hour socially deprived animals. Isolate housed adults have also been shown to emit high levels of 50 kHz USVs during social interactions, and emit more 50 kHz USVs in a chamber that had previously been paired with conspecific exposure than a non-paired chamber [19], although there were no comparisons to non-isolated animals. These effects of social isolation on 50 kHz USVs in the present study were not seen in the presence of a food stimulus, indicating that isolation-induced alterations in 50 kHz USV production are stimulus- and age-specific. Previously rats have been shown to increase 50 kHz USV production in anticipation for a food reward, so it is not simply the case that subjects do not exhibit significant 50 kHz USV production for a food stimulus [42]. The large social-isolation-induced increases in 50 kHz USV production seen in adults in the present study, however, were not accompanied by isolation-induced increases in social investigation, raising the question of whether 50 kHz USVs or social approach reflect the incentive salience of the reward in this situation. USVs have been found to serve as social signals [46] and in this situation it is possible that increased 50 kHz USV production may simply reflect increased social communication from the isolate-housed adults. FM calls have been suggested to reflect hedonic value with flat calls serving as a social signal [17]. In the present study there were surprisingly no differences between call types in any test group, suggesting that these calls may not reflect hedonic value and social signals under all testing circumstances. In the present study isolated adults emitted more 50 kHz USVs than non-isolated adults, but only when tested with a social stimulus. This indicates that the deprivation effect seen here is stimulus specific, indicating that it is not simply a response to heightened anxiety or stress. It is presently unclear what USVs are an indication of, however it does appear that it is not necessarily a good measure of affect in some situations.

The findings of this study clearly show that 50 kHz USV production and time spent investigating a rewarding stimulus are not measuring the same aspects of the incentive salience of a reward and may have different underlying causes. Adolescents spent significantly more time investigating the social stimulus than adults whereas only chronically isolated adults emitted more 50 kHz USVs than adolescents. When tested with

social stimuli, 50 kHz USV production was substantially increased in isolate housed adults; however the same pre-test social isolation effects were not seen in the analysis of time spent investigating the social stimuli. In testing involving a food stimulus, adults emitted significantly more 50 kHz USVs than adolescents, whereas there were no age differences in time spent investigating the food stimulus. Correlational analyses likewise revealed minimal relationships between 50 kHz USV production and time spent investigating either the social or food stimulus. These findings are somewhat inconsistent with a study of adolescent rats isolate housed since weaning conducted by Burgdorf et al., [20] where reliable correlations were observed between frequency modulated 50 kHz USV production and “the rewarding value of the vocalization inducing stimulus” (as measured by CPP or latency to approach the stimulus), however, FM calls were correlated negatively with some measures of play, and positively with some measures of aggression in this same study, suggesting these calls may not be a simple reflection of positive affect. .

A number of recent studies analyzing 50 kHz USV production and behaviors following drug administration in adult animals have found dissociations between behavioral measures of affective state and USV production similar to those observed in the present study. Following doses of morphine which had been found to be reinforcing, rats showed increased locomotor activity but no changes (and often a decrease) in 50 kHz USV production [47-50]. In a study by Wright et al., [51] rats administered morphine developed a CPP for the drug, although these animals failed to exhibit conditioned USV production. Similarly, rats did not show increased production of 50 kHz USVs following administration of rewarding doses of MDMA or nicotine [49, 50].

Collectively, such data in conjunction with the current findings provide compelling evidence that 50 kHz USVs and behavioral measures typically thought to reflect the rewarding value of stimuli do not measure the same aspects of incentive salience, positive affect [20], or reward seeking [17]. Further research is necessary to interpret the motivational and affective significance of 50 kHz USV production as distinct from behavioral measures of approach or preference. In such work, an ontogenetic approach may prove useful when attempting to parse out the significance of these different indices of motivation and affect and their neural regulation.

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Highlights

There were no effects of social isolation on investigation of a food or social stimulus.

Isolation increased 50 kHz USV production in to social but not food stimulus at either age.

Adolescents investigated a social stimulus more than adults with no differences in USVs.

Adults USV more to food than adolescents, no differences in investigation of food.

There was a dissociation between investigation of a rewarding stimulus and USV production.

Fig. 1.

Top Panels: Mean square root of 50 kHz FM and flat USV production to a social stimulus across social isolation groups in adolescents (left) and adults (right). + indicates adolescents emitted less 50 kHz USVs than adults. Δ indicates isolate housed adults emitted more 50 kHz USVs than 25 min or 3 hr isolated adults.

Bottom Panels: Mean seconds spent investigating the social and empty chambers across social isolation groups in adolescents (left) and adults (right). Vertical bars indicate SEM.

Fig. 2.
 Top Panels: Mean square root of 50 kHz FM and flat USV production to a food stimulus across social isolation groups in adolescents (left) and adults (right).
 Bottom Panels: Mean seconds spent investigating the food and empty chambers across social isolation groups in adolescents (left) and adults (right). Vertical bars indicate SEM.