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Supporting Online Material for

Empathy and Pro-Social Behavior in Rats

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Materials and Methods

Subjects

Sprague-Dawley rats (Charles River, Portage, MI) 3-6 months old (270-380g), were housed in pairs ("cagemates") with *ad libitum* access to chow and water in a 12:12 light-dark cycle. In each experiment, animals of the same age were used. The rats' tails were color- marked for individual identification. Animals were allowed at least 2 weeks to acclimate to the housing environment and to establish familiarity with their cagemate. As performance in this paradigm is very sensitive to stress, animals were extensively handled and habituated to the test arenas for four days prior to the experiment. During habituation, rats were brought into the testing room, allowed at least 10 min in their home cages, after which they were handled (5, 10, 15, 15 min per cage on days 1, 2, 3, 4 respectively) and placed in the arena together for 60 min. After each habituation session, rats were returned to their home cages in their home rooms. The rats were weighed regularly throughout the testing period; no animal lost weight during the experiment. All sessions were run during the rats' light cycle. For each experiment, order of testing was counterbalanced between sessions, to control for effects of time of day on behavior.

Set up

Plexiglas arenas (50x50 cm) were used during the testing sessions. A CCD monochrome camera (KPC-S500 *P3*, KT&C Co, Seoul, Korea) was placed above each arena. The cameras were linked via a video card (GV-650, Geovision, Irvine, CA) to a dedicated PC. Ultrasonic vocalizations emitted by the rats at a frequency of ~23 kHz were recorded using a single bat detector (Mini-2 heterodyne detector, Summit, Birmingham, England). Recordings were only used from sessions in which all arenas tested rats in the same experimental condition.

Restrainers

A Plexiglas rodent restrainer (25 by 8.75 by 7.5 cm, Harvard Apparatus, Holliston, MA) was placed in the center of the arena and its location was held constant by small plastic wedges on the arena floor. The restrainer was large enough to allow the trapped rat to move and turn around. The body of the restrainer had several small slits and the back end had a large slit, allowing for olfactory and tactile communication between rats. At the other end, a customized door had two panes that were attached with three screws, and a pole (5 cm) supporting two weights (25 g each). The weights were included in order to facilitate the door falling off to the side once the free rat pushed on the door. The door could be opened only from the outside. The free rat could open the door from the top, from the side, or by pushing up on the door with its snout (Fig. 1A). In stages 2 and 3 of the separated experiment, three weights were used to facilitate rats' learning to open the door sideways. Furthermore, a rubber cap covered the weights to soften the sound that occurred when the door fell over.

Procedures

Initially, rats were tested for 90 min. In these sessions, if the free rat had not opened the restrainer door within 60 min, the investigator opened the restrainer door halfway, to a 45° angle, greatly facilitating door-opening by either rat. Rats remained together in the arena for another 30 min. However, we observed that the activity of most free rats was concentrated in the first 30 min of testing. Therefore, we reduced subsequent sessions to

60 min in length, including 40 min before halfway door-opening and 20 min after (table S1). After 40-60 min respectively, if the free rat had not opened the restrainer door, the investigator opened the restrainer door halfway, to a 45° angle, greatly facilitating door-opening by either rat.

After several test sessions, some trapped rats began to open the door from within the restrainer. When this occurred, the trapped rat was placed back in the restrainer, and a Plexiglas blocker was inserted, preventing his access to the door. When the free rat opened the door, the blocker was removed, allowing the free rat to exit the restrainer. The blocker was then used for that rat on all following test days. Two pairs were removed from the analysis because the trapped rat learned to open the door before we started using the blocker.

After each session, the arena and restrainer were washed with 1% acetic acid, rinsed with water, and washed again with surface cleaner and water. Testing was repeated for 12 days. Rats were placed in the same arena for each session. In order to allow analysis of the rat's movements by video tracking, the rat's head was marked just below the ear line with either a black felt sticker or sharpie mark of approximately the same size. Although rats of different conditions were often tested in different arenas at the same time, females and males were never tested together.

In the separated experiment, two arenas were joined together, and the restrainer was fitted to a hole in the divider between the arenas, so that the restrainer opened into the second arena (Fig. 4). In the separated cagemate condition, the restrainer contained a trapped cagemate and in the separated empty condition, the restrainer was empty. All other details were identical to the trapped condition.

In the chocolate cagemate condition, two restrainers were placed in the arena (Fig. 4): one restrainer contained 5 chocolate chips (Nestlé® Toll House, milk chocolate) and the other contained the trapped cagemate. In the chocolate empty condition, one restrainer contained 5 chocolate chips and the other one was empty. All other details were identical to the trapped condition.

<u>Statistical analysis</u>

Differences in door-opening latencies were examined with a repeated measure ANOVA, with day as the repeated variable. To analyze activity data, we conducted a mixed model analysis (MMA) with an autoregressive covariance structure, and "day" as the repeated measure (for distance from wall and persistence ratio calculations) or "day" and "minute" (for activity calculations). Fischer PLSD was conducted for all post-hoc analyses. A chi square analysis was used to analyze the proportion of openers and non-openers. In all cases, $\alpha < 0.05$ was used as criterion for significance. Door-opening latencies are displayed using the median since door-opening was not a normally distributed variable. Most rats either opened the door within 10 min of the start of the experiment or not at all. Statistical comparison were conducted using SPSS (PASW 18).

<u>Measuring location and movement in the arena</u>

The free tracking software ImageJ (v1.44e, NIH, USA) was used to convert the black marker on the rat’s head into x,y coordinates denoting the rat’s location at each frame at a rate of 0.5 FPS. These data were then used to calculate movement velocity (termed activity). Other indices (time away from wall and persistence ratio) were calculated using the free SEE software *(28)*, developed specifically for the analysis of rodent movement in an arena.

Measuring opening style

Each door-opening was classified to indicate how the door was opened. Three types of opening were observed: 1) rats opened the door by nudging it up with their head from the front of the restrainer ("head"); 2) rats opened the door by leaning on the heavy side of the door ("side"); or 3) rats opened the door by standing on top of the door ("top").

Measuring freezing time

The duration of freezing after door-opening was measured for free rats in the trapped cagemate condition. Freezing was defined as no movement at all and ended as soon as the rat moved any body part. Freezing time was measured by independent observers who were blind to the hypothesis.

Door-opening latencies

Time to door-opening was calculated as the minute when the restrainer door was opened minus the start time. Latencies to door-opening are shown as percentages of the total session time since some animals were tested in long (90 min) sessions and others in short (60 min) sessions. Regardless of session length, no rats tested in the empty condition and most rats tested in the trapped cagemate condition opened the door (table S1).

Boldness measurements

Boldness was measured as the latency from opening the homecage lid halfway to the time that the rat approached the edge of the cage, reared up, and placed its paws on the ledge. This measurement was recorded 3 times for each rat in every cage, once on the first 3 days of habituation. The shortest latency recorded for each animal was averaged for the trapped condition. This latency was shorter for rats who became openers, and their cagemates, than for those who remained non-openers and their cagemates (fig. S1).

Selection of the free rat

In order to minimize the variance due to the hierarchical position of marked rats within the pair, the rat with the shortest boldness latency from each pair was chosen to be the free rat. As such, the shortest time to edge was shorter for free rats than for trapped rats by design (fig. S1).

Definition of non-openers

Rats who did not open the restrainer on at least 2 of the last 3 days of the paradigm (days 10-12) were termed non-openers.

Alarm call sampling

Audio files were collected from all sessions where only one condition was tested (26 rats in the trapped condition, 16 rats in the empty condition, and 8 rats in the object condition). All audio files collected on days 1-3 of male rats in the trapped condition were analyzed for the presence of alarm calls. In addition, audio samples (n=272, 5 min duration) were randomly chosen from all 12 days of the trapped, object and empty conditions and were similarly analyzed. Data from the 2+empty condition could not be analyzed do to a technical error. Judges blind to the experimental condition used the freeware Audacity 1.3 to locate potential alarm calls (~23 kHz), and listened to each candidate segment to verify which of these segments indeed contained alarm calls. Each file was then categorized as either containing alarm calls or not containing alarm calls. The proportion of samples from each experimental condition that contained alarm calls was then calculated. Finally, for alarm calls recorded on day 1, the synchronized video tape was used to determine if the caller was a trapped or free rat.

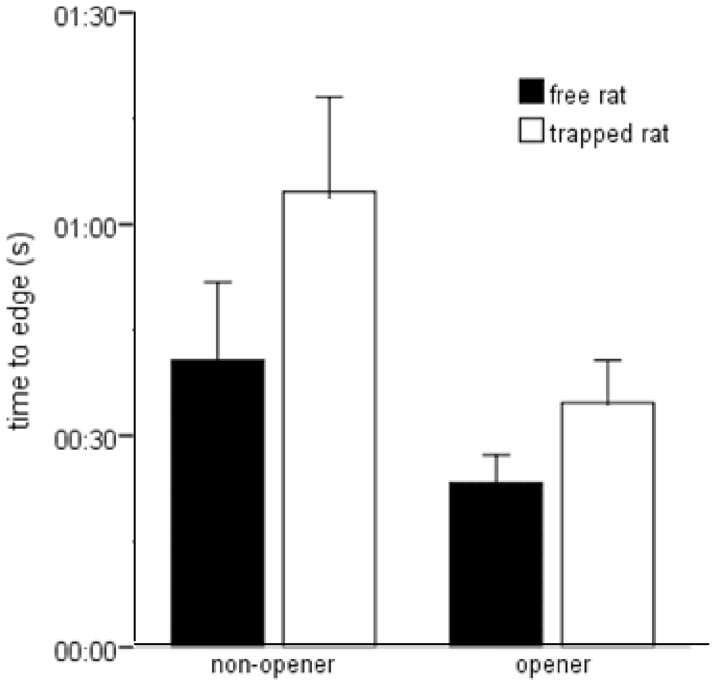

Fig. S1.
The mean boldness latency (±SEM) for rats was measured as the time to approach the edge of an opened cage, rear up, and place the paws on the edge. Post-hoc analysis revealed that rats in the trapped condition (n=30) that became non-openers (n=7) showed a longer latency to approach the edge than rats that learned to open the door (n=23), suggesting that non-openers were more inhibited by trait. The interaction was not significant. Interestingly, both animals in a cage which ultimately proved to contain an opener showed shorter latencies than the animals in cages which ultimately proved to contain a non-opener.

Table S1.
The number of rats that were tested in 90 min (long) and 60 min (short) sessions is listed for each condition with the number of rats that were openers in each group is noted in parentheses. All females were run in short sessions.

Condition	Long	Short	Grand total
2+empty	0	12 (0)	12
empty	8 (0)	12 (0)	20
object	0	8	8
trapped	13 (12)	17 (11)	30
separated cagemate	0	12 (9)	12
chocolate trapped	0	9 (9)	9
chocolate empty	0	6 (6)	6
Total	21	76	97

Movie S1

Five minutes of activity from representative rats in the object, empty and trapped conditions are shown. All movies are sped up by 6 times.

Movie S2

Learning to open the door. Activity of the same free rat on days 1, 5, and 12 of the paradigm is shown. On day 1, the rat does not open the restrainer and 5 min of activity is shown at 20 times real time speed. On day 5, the rat opens the restrainer door for the first time 25 min into the session. Note the momentary startle at the door falling and the extended interaction with the liberated rat. On day 12, the rat opens the restrainer within the first minute. There is no startle and much less interaction between the two rats. Activity from days 5 and 12 are shown at real time.

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