Supplemental Material

Materials and Methods

Subjects

A total of 12 male Sprague Dawley rats were used (4-6 months, Charles River, Kingston, NY). All subjects were individually housed, and had *ad libitum* access to food and water. They were kept on a 12-hr dark/light cycle in a vivarium. Experiments were in strict compliance with the Yale Animal Resource Center guidelines.

Apparatus

Two chambers (H10-11R-TC, Coulbourn Instruments, Allentown, PA) were used for conditioning and testing. Both chambers (designated A and B) were rectangular (27 cm wide × 28 cm deep × 30.5 cm high) and had a standard grid floor made of 16 stainless steel rods. The chambers were individually housed in sound-attenuating isolation cubicles (Coulbourn Instruments, Allentown, PA). The two chambers differed in three respects: chamber A was illuminated by a chamber light while chamber B was not; vinegar was used as an odorant for chamber A while Windex was used for chamber B; and chamber A had a grid floor whereas chamber B had a flat solid floor on the grid floor. An infrared video camera (Circuit Specialists, Mesa, AZ) was mounted on the top of each chamber to record behavior during experiments. The auditory USV CS was presented via a speaker located on a side wall in each chamber. The grid shock US was delivered by a shock generator (ENV-410, MED Associates) and grid scrambler (ENV-412, MED Associates).

Auditory stimulus

A 22 kHz ultrasonic vocalization (USV) was used as an auditory cue. The 22 kHz USV was produced by a naïve rat after giving a 1 sec, 1 mA foot shock in a sound-proof chamber. Emitted calls were detected by a high-frequency microphone (Model 7012, ACO Pacific, Belmont, CA) mounted on the chamber. These calls were amplified by a Tucker-Davis Technologies (TDT, Gainesville, FL) MA2 preamplifier. Then the USV was digitized by an RP2.1 digital signal processor (TDT) at a rate of 100 kHz, and stored using RPvdEX software (TDT). The recorded USV was delivered via the RP2.1 and a high-frequency electrostatic loudspeaker (ES1, TDT). The loudspeaker was driven by an electrostatic loudspeaker driver (ED1, TDT) and the RPvdEX software. Although termed a "22 kHz USV", the actual principal frequency of the USV was 19 kHz. The amplitude of the stimulus was ~65 dBSPL (measured using an Ultraprobe 9000; UE Systems).

Behavioral Procedure

Subjects were briefly handled for 2 min for 5 days (Day 1-5) upon arrival. On day 6, rats were placed in chamber A and received 5 CS-US pairings. Following a 2-min baseline, the 22 kHz USV CS (65 dB, 7.8 s, 19 kHz) was presented and followed by coterminating footshock US (0.5 s, 0.5 mA). Intertrial intervals (ITIs) ranged from 90 s to 150 s with a mean of 117 s. Rats were returned to their cages 1 min after the last CS-US presentation. On days 7 and 8, freezing to the cue and the conditioning context was tested individually on a separate day. The order of the two tests was counterbalanced among subjects. In the cue freezing test, each rat was placed in a novel chamber B in which the CS was continuously presented for 6 min after a 2 min baseline. For the contextual freezing test, rats were returned to the conditioning context (chamber A) and remained in the context for 8 min. All behavioral experiments were video-taped for development and evaluation of an automated freezing recognition analysis.

Data Analysis

All videotaped data were analyzed by the software (written in Delphi 7, Borland Software Corp., Cupertino, CA; see Kholodar-Smith, Boguszewski, and Brown, 2008) and independently scored off-line with a stopwatch by the experimenter. Freezing was measured as a CR. Pearson's linear correlation was used to measure correlations of manual versus machine analysis of freezing behavior during the 6 min of CS presentation or context test.

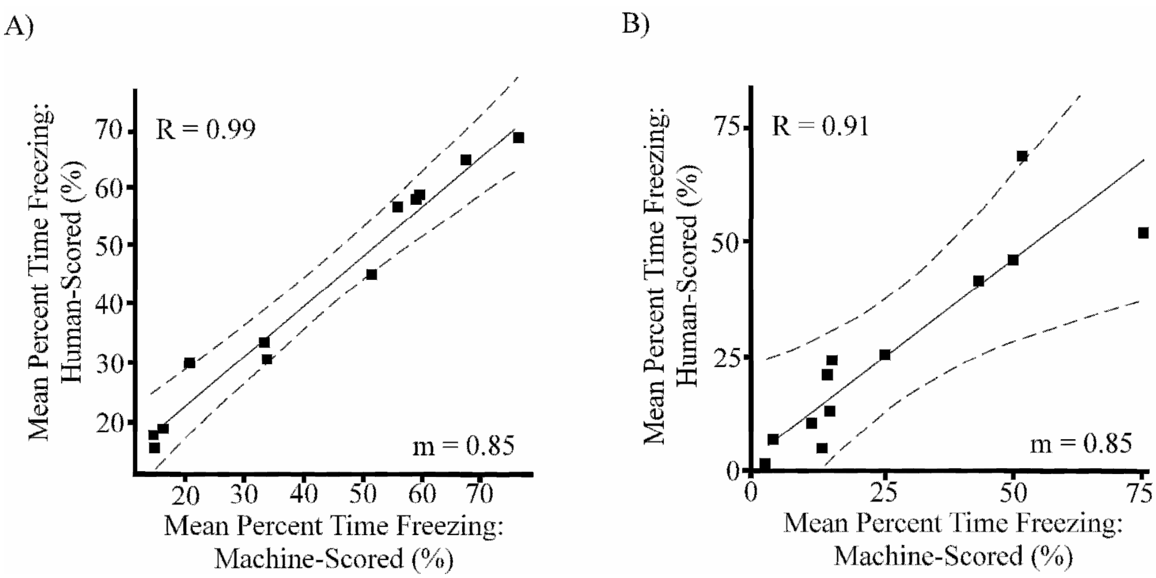
Results

The scatter plots in the Supplemental Figure show the relationship between manual scoring (ordinate) and machine scoring (abscissa) during the cue test (Supplemental Figure A) and the context test (Supplemental Figure B). The straight lines in the figure are the best-fitting regressions. The curved lines are 99.9% confidence intervals on this regression. For cue tests (Supplemental Figure A), the Pearson correlation between automatic and manual measurements was 0.99 ($p < 0.001$). The slope and Y intercept of the best-fitting regression were, respectively, +0.85 and +6.01 s. For the context tests (Supplemental Figure B), the correlation between automatic and manual measurements was 0.91 ($p < 0.001$). The slope and Y intercept were +0.85 and +3.82 s, respectively. In both cases, the machine analysis included slightly more freezing time. The average of these two correlations (0.95) is similar to a previously-reported average correlation between two manual scorings of cue and context conditioning (0.94; Lindquist, Jarrard, and Brown, 2004).

References

Kholodar-Smith, D. B., Boguszewski, P., & Brown, T. H. (2008). Auditory trace fear conditioning requires perirhinal cortex. *Neurobiology of Learning and Memory*. 90(3), 537-543.

Lindquist, D. H., Jarrard, L. E., & Brown, T. H. (2004). Perirhinal cortex supports delay fear conditioning to rat ultrasonic social signals. *Journal of Neuroscience,* 24(14), 3610-3617.



Supplemental Figure. Scatter plots showing correlations between automatic and manual measurements. A) Correlation between automatic and manual scoring of freezing during 6 min of CS presentation. B) Correlation between the two measurements during 6 min of context test. Straight lines represent the best-fitting regressions. Curved lines depict 99.9% confidence intervals.