A one-week behavioural protocol and derived variables describing a repertoire of innate and learned behaviours in 129S5 and C57BL/6J mice.

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Overview

The Genes to Cognition (G2C) Program was established as an integrative neuroscience program linking mouse genes to cognition and disease (www.genes2cognition.org)[1,2]. The G2C program established a standardised battery of behavioral tests, used to measure components of the behavioural repertoire in over 2700 mice in 60 mutant lines. Most of these lines were made in either 129S5 or C57BL/6J mouse genetic backgrounds, which differ in wildtype behaviour [3-7]. Behavioural testing on groups of approximately 40 mice was carried out over five days (Monday to Friday) in five standardized artificial environments (Figure 1) that address innate and learned responses. The perception of the features of these environments trigger responses affecting locomotion, exploration and motor coordination, which were detected using video and other tracking devices linked to specialised software. A total of 105 highly redundant measures were made on each mouse from which 16 highly differentiated variables (Table 1) were chosen or derived based on minimal correlation between variables in wildtype mice and maximal similarity between score distributions of 129S5 and C57BL/6J wildtype mice (Figure 2). To gauge the similarity of 129S5 and C57BL/6J mice with respect to each variable, behaviour scores of wildtype mice of each background were z-score transformed; this was done separately in 129S5 and C57BL/6J mice. This document reports the behavioral experimental protocols and the use of wildtype data to derive the set of 16 differentiated variables. The full dataset and analysis will be reported elsewhere (all the authors, manuscript in preparation).

Day 1 – Elevated plus maze (EPM)

The elevated plus maze test, which has historically been used as a test of anxiety [8], was the first test in the behavioural protocol. The apparatus was a plus-shaped maze (Figure 1a) with infrared illumination available from Tracksys (Nottingham, UK) with two exposed arms 45 cm above the ground and two arms protected with walls. The maze was monitored by an IR-filtered digital camera (Tracksys, Nottingham, UK) controlled by Mediacruise software, version 2.24.000. Analysis was carried out using Noldus Ethovision software, version 3.1.16 (Tracksys, Nottingham, UK). Mouse identification was by microchip. Mice were run on the maze for five minutes in a darkened room illuminated by red light, one mouse per maze, two mazes per room, with the experimenter remaining in the room. After each run, the maze was wiped down with ethanol wipes.

Five variables were selected for analysis in the EPM: A) EPM total distance, the total distance (cm) travelled in any arm or central zone of the EPM; B) EPM max speed, the maximum speed (cm/s) travelled in any arm or central zone of the EPM; C) EPM % time in open, the percentage of time in the open or closed arms of the EPM spent in open arms, a classical measure of lack of anxiety; D) EPM time in centre, the total time (s) spent in the central zone of the EPM; and E) EPM max speed, open vs closed, the difference between the maximum speed (cm/s) observed in the open arms and the closed arms of the EPM. Speeds in the open arm are generally lower, as expected. Z-score distributions of these measures in wildtype mice, computed for comparison of 129S5 and C57BL/6J mice, are shown in Figure 2b-f.

Day 2, AM – Open field (OF)

The open field test, which has been used as a test of physical activity and exploratory behaviour [9,10], was conducted on the morning of the second day of the behavioural protocol. The apparatus (Figure 1b) was a white, matte-finish plastic 75 cm by 75 cm box with 42 cm

walls, placed on an infrared bed to permit recording of animal behaviour by infrared-filtered cameras (all available from Tracksys, Nottingham, UK). Mouse identification was by microchip. Animals explored the box for five minutes, and their behaviour was analysed by Noldus Ethovision software, version 3.1.16 (Tracksys, Nottingham, UK). After each run, the apparatus was wiped down with ethanol wipes. The variables are described in the following section.

Day 2, PM – Novel object exploration (NOE)

The novel object exploration task was carried out in the afternoon on the second day of the protocol. The same open field apparatus was used, but included an unopened aluminium 355ml soft drink can placed in the centre of the box (Figure 1c). The animal was allowed to explore the box with the object for five minutes, and behaviour was recorded with the same devices as were used in the open field assay. Results from this assay and the open field were combined. Locomotion was measured as the total distance travelled during the open field and novel object exploration assays and denoted 'OF, NOE total distance'. To increase similarity of behavioural scores between 129S5 and C57BL/6J mice, behavioural scores were log10 transformed before z-score transformation.

To measure a mouse's response to the change in environment from the OF to NOE, we chose the difference between the distance travelled in the novel object exploration and distance travelled in the open field, denoted 'NOE vs OF distance travelled'. Z-score distributions of these measures in wildtype mice, computed for comparison of 129S5 and C57BL/6J mice, are shown in Figure 2g-h.

Day 3 – Rotarod (RR)

The rotarod test, which has historically been used as a test of motor coordination [11], was conducted on the third day of the behaviour protocol on an accelerating rotarod apparatus (EZ-ROD, version 2.12, Accusan Instruments, Columbus, Ohio, USA; Figure 1d). The spindle was of 3.0 cm diameter and was set 35 cm above the bottom of the apparatus. The spindle began rotating at 10 revolutions per minute (RPM) and accelerated to 48 RPM over the course of five minutes. A mouse's fall broke an infrared beam and triggered a switch monitored by a computer. Accompanying software recorded the mouse's latency to fall and the maximum spindle speed attained. Each mouse underwent eight trials in the morning and eight in the afternoon. After each mouse, the apparatus was wiped down with ethanol wipes.

Measures for each mouse's innate motor coordination, motor learning, and motor memory were derived as shown in Figure 3. Two linear models were fit, one to a mouse's latency to fall during the eight trials in the morning session, and the other to the eight trials in the afternoon session. Naïve performance, denoted 'RR naive fall time', was computed as the fitted value of motor performance in the second trial in the morning session. Motor learning, denoted 'RR learning', was measured as the slope of the linear model during the morning session. Motor memory, denoted 'RR memory', was measured as the difference between the fitted midpoint of the afternoon session and the fitted midpoint of the morning session. This model of naive performance, learning, and memory has the following properties in wildtype mice: 1) learning is not correlated with naive performance; and 2) the measure of memory is positively correlated with the measure of learning and more modestly with the measure of naive performance. RR naive fall time was log10 transformed to increase similarity between the 129S5 and C57BL/6J score distributions. Z-score transformations of these measures in wildtype mice, computed for comparison of 129S5 and C57BL/6J mice, are shown in Figure 2i-k.

Days 4-5 – Classical conditioning

Classical conditioning training was conducted on the fourth day of the protocol using an operant box (Coulbourn Instruments, Whitehall, PA, USA; Figure 1e). After two minutes of habituation, a 300 Hz tone at 83-86 dB was played for 30 seconds, co-terminating with a 2-second scrambled shock in the grid floor at 0.45 mA under control of Acctimetrics FreezeFrame software (Coulbourn Instruments, Whitehall, PA, USA). Two more tone-shock pairings were presented at 100-second intervals. The mouse's behaviour was recorded by an overhead video camera and freezing behaviour was detected by Acctimetrics FreezeView software, version 2 (Coulbourn Instruments, Whitehall, PA, USA). A screen grab depicting the training protocol is shown in the upper panel of Figure 4a. Testing was performed on the fifth day of the protocol in the same boxes; mice were placed in the operant box for three minutes, after which the tone was played for two minutes (Figure 4a, lower panel). Freezing was recorded in 30-second time bins.

Six variables related to learning and memory were measured in this assay. Explanations of the parameters and illustrations depicting how the calculations were done are shown in Figure 4b-d. We noted that the way in which the two acquisition (learning) variables were measured was not strictly independent. Not only were they mathematically dependent on the same raw data, it was conjectured that increases in the tone response might in part be driven by general increases in freezing that were due to contextual learning alone. Furthermore, contextual conditioning is known to be hippocampus dependent, whereas cued conditioning is not. To detect separable aspects of learning, a linear regression model was constructed with the tone effect (Learning, tone effect; LRN_tone) as the dependent variable and the general increase in freezing in successive trials (Learning, trial effect; LRN_trial) as the independent variable. Sample R code for this operation is:

```
LRN_tone = lm(LRN_tone ~ LRN_trial)$residuals
```

The linear dependence between the two was subtracted. This was done separately for mice on the C57BL/6J background and on the 129S5 background.

Similar to the manner in which acquisition (learning) variables were not independent, cued memory responses, 'Cued memory, mean' (CU_mean) and 'Cued memory, change' (CU_change), were not strictly independent of the contextual response, (Contextual memory, mean; CT_mean). Furthermore, the temporal evolution of the cued response (Cued memory, change; CU_change) was expected to be correlated with the mean cued response, (Cued memory, mean; CU_mean). To derive a measure of the temporal evolution of the cued response independent of the context effect and the mean cued effect, linear dependencies of CU_change on CT_mean and CU_mean were subtracted, again using linear models. This was done separately for C57BL/6J mice and 129S5 mice. Sample R code for this operation is:

```
CU_change = lm(CU_change ~ CT_mean + CU_mean)$residuals
```

Secondly, dependence of the mean cued effect, CU_mean, on the context effect, CT_mean, was subtracted similarly, using R code:

```
CU_mean = lm(CU_mean ~ CT_mean)$residuals
```

This was done separately for C57BL/6J mice and 129S5 mice.

Although data confounding was handled separately in task acquisition and testing, it was gratifying to note that as a result, the now-uncorrelated variables related to task

acquisition each correlate similarly to, and therefore are similarly predictive of, the two main components of memory. That is, the trial effect during task acquisition (Learning, trial effect; LRN_trial) is now exclusively predictive of the contextual memory variable (Contextual memory, mean; CT_mean). The tone effect during task acquisition (Learning, tone effect; LRN_tone) is now exclusively predictive of the cued memory effect (Cued memory, mean; CU_mean). In other words, the mathematical operations presented here discover separable aspects of task acquisition that separately predict two aspects of memory. A caveat that must be observed, however, is that these mathematical operations are only robust when based on data from many mice, whereas similar calculations on cohort sizes such as 20 mutants and 20 wildtypes are likely to suffer from over-fitting artefacts.

The six variables related to classical conditioning in wildtype mice were z-score transformed separately in 129S5 and C57BL/6J mice for comparison between these strains. Z-score distributions of all measures in wildtype mice are shown in Figure 2l-q.

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Conflict of interest

The authors declare no financial conflicts of interest.

Table 1 – 16 differentiated behaviour variables

Assay	Variable name	Description
Elevated plus maze (EPM), 5 min	EPM total distance	Total distance (cm) travelled in any arm or central zone of the EPM
	EPM max speed	Maximum speed (cm/s) travelled in any arm or central zone of the EPM
	EPM % time in open	Percentage of time in the open or closed arms of the EPM spent in open arms
	EPM time in centre	Total time (s) spent in the central zone of the EPM
	EPM max speed, open vs closed	Difference between the maximum speed (cm/s) observed in the open arms and the closed arms of the EPM
Open field (OF) & novel object exploration (NOE), 5 min	OF, NOE total distance	Total distance travelled (log10 cm) during initial exposure to the open field and in presence of the novel object
	NOE vs OF distance travelled	Difference in distance travelled (cm) in presence of the novel object and during initial exposure to open field
Rotating rod (RR)	RR naive fall time	Fall time on accelerating rotarod (log10 s), naive performance in session 1
	RR learning	Learning on rotarod, measured as increase in fall time per trial (s/trial) in session 1
	RR memory	Memory on rotarod, measured as excess fall time at middle of session 2 relative to middle of session 1
Classical conditioning training/acquisition	Learning, trial effect	Learning, measured as extra % time freezing before third trial compared to % time freezing before first trial
	Learning, tone effect	Learning, measured as increase in % time freezing due to third tone compared to increase in % time freezing due to first tone
Classical conditioning context memory	Contextual memory, mean	Contextual memory, measured as difference in % time freezing during first 120 s re-exposure to the box compared to first 120 s in the box on previous day
	Contextual memory, change	Contextual memory, measured as increase in % time spent freezing from first time bin of 30 s to fourth bin of 30 s during 120 s re-exposure to the box
Classical conditioning cue memory	Cued memory, mean	Cued memory, measured as increase in % time spent freezing during 120 s of tone re-exposure compared to increase in % time spent freezing during initial tone on previous day
	Cued memory, change	Cued memory, measured as increase in % time spent freezing from first time bin of 30 s to fourth bin of 30 s during 120 s re-exposure to the tone

Figures

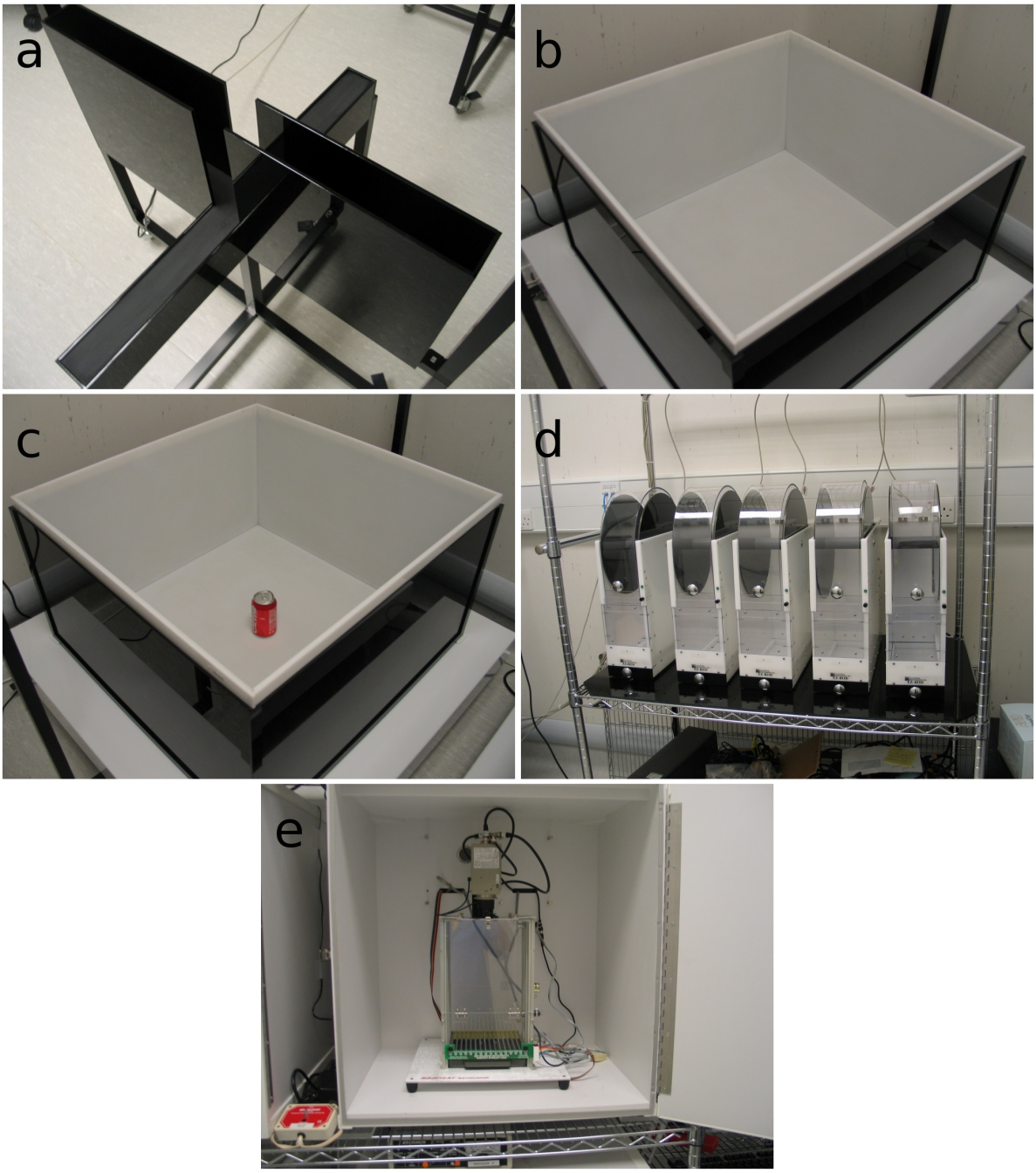

Figure 1 – Apparatus used in behaviour experiments. a) Elevated plus maze. b) Open field. c) Novel object exploration apparatus. d) Five rotarods. e) Classical conditioning apparatus in isolation box.

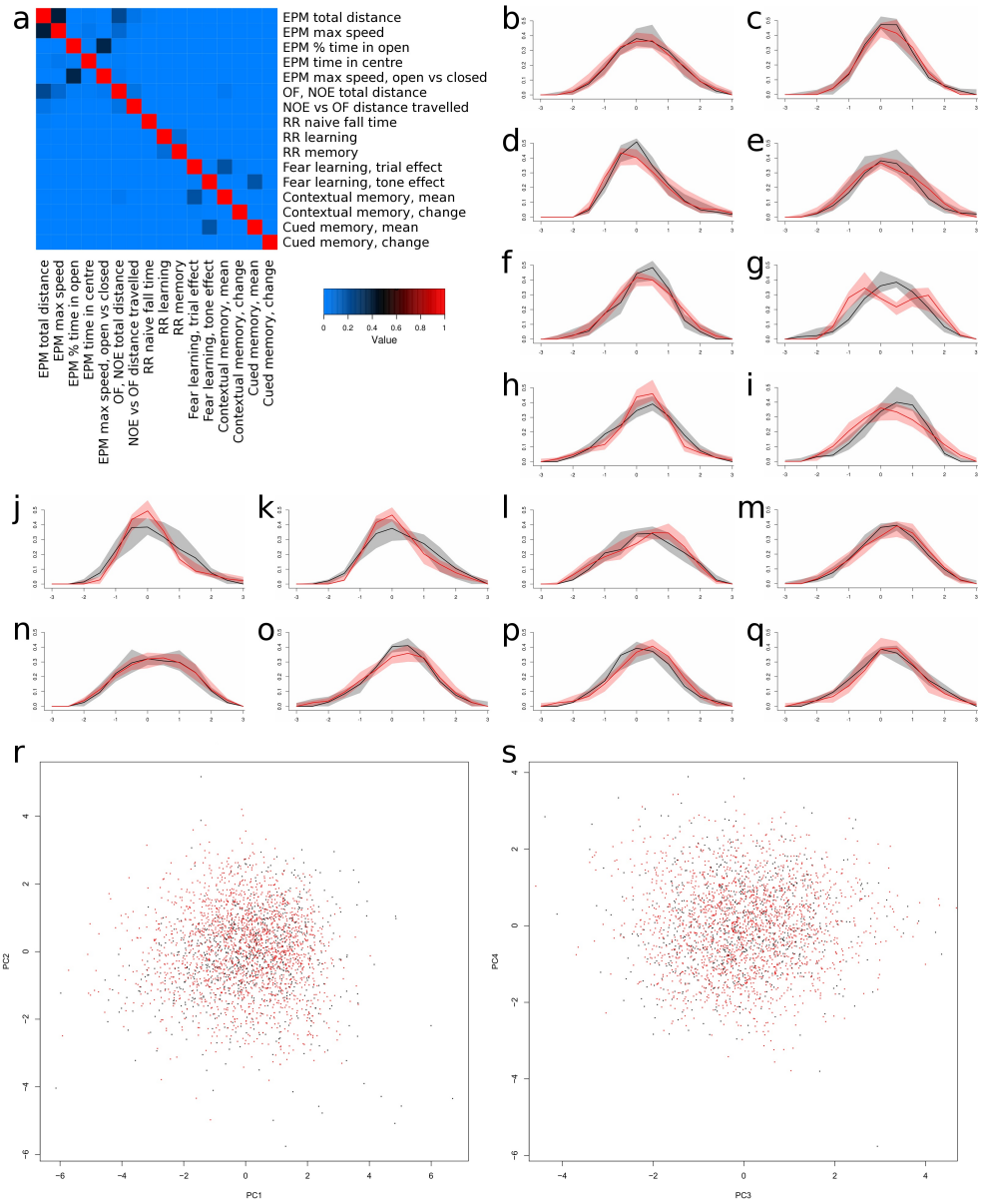

Figure 2 – Correlation of the 16 behaviour variables and distributions of z-score transformed behaviour scores in wildtype animals. a) Pearson R^2 correlations between the 16 differentiated variables in all wildtype mice. b-q) Similarity of the distributions of the behaviour scores of the 16 variables in two wildtype strains (129S5 background, red, and C57BL/6J background, black; X-axes, z-score coordinates; Y-axes, frequency). Lines of mice with 20 or more wildtypes are represented, and z-score distribution for a given mouse line was loess-smoothed with linear fit and Gaussian weights across five data points. Shaded areas represent interquartile range amongst lines. b) EPM total distance. c) EPM max speed. d) EPM % time in open. e) EPM time in centre. f) EPM max speed, open vs closed. g) OF, NOE total distance, log10 transformed. h) NOE vs OF distance travelled, the difference in distance travelled between the OF and NOE assays. i) RR naive fall time. j) RR learning (in the morning session). k) RR memory, (fall time in the middle of the afternoon session, compared to fall time in the middle of the morning session). l) Learning, trial effect. m) Learning, tone effect. n) Contextual memory, mean. o) Contextual memory, change. p) Cued memory, mean. q) Cued memory, change. r-s) Principal component analysis of z-score transformed behaviour scores for the 16 variables in 129S5 (red) and C57BL/6J (black) mice, showing similarity. r) PC2 vs. PC1. s) PC4 vs. PC3.

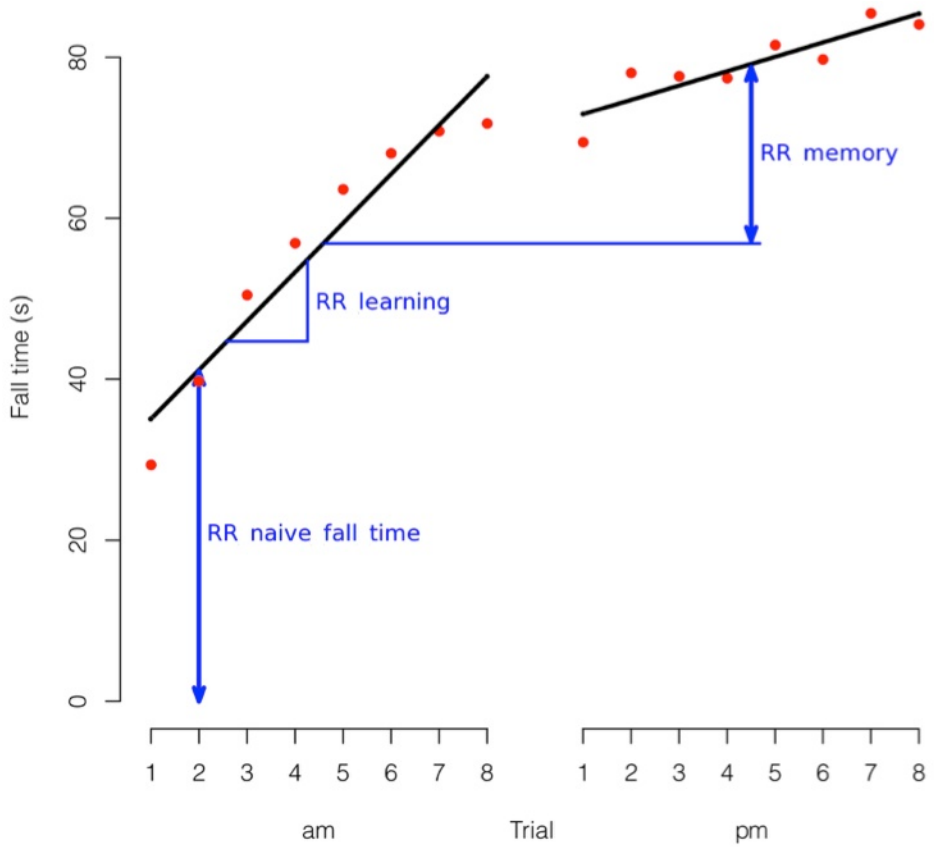

Figure 3 – Derivation of three measures (blue) of mouse rotarod performance. Linear models (black lines) were fitted to morning and afternoon data (red points). RR naive fall time was defined as the second fitted value of the linear model fitted to morning session data. RR learning was defined as the slope of the same linear model. RR memory was defined as the difference between the midpoints of the morning and afternoon linear models.

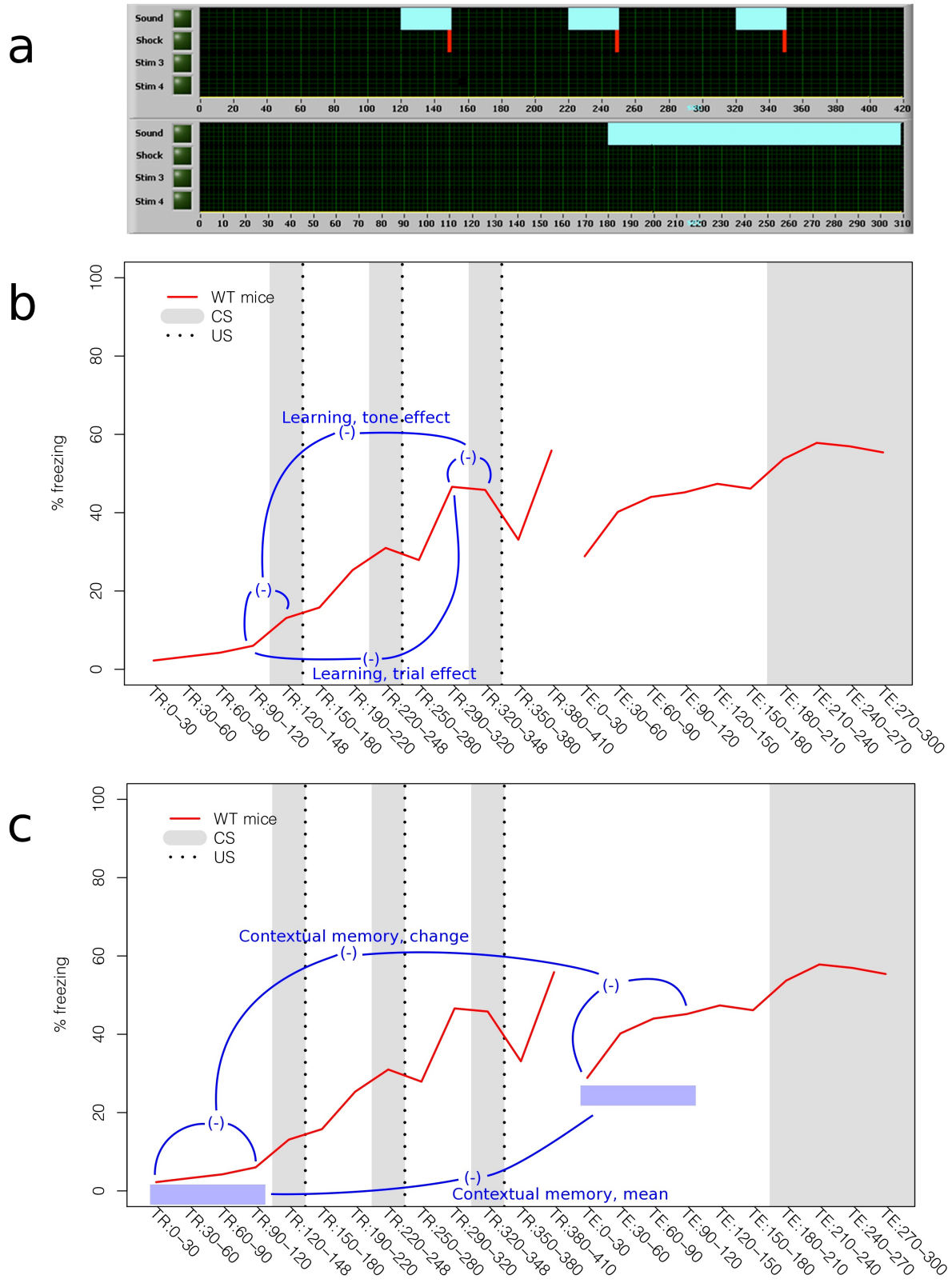

Figure 4, continued on next page.

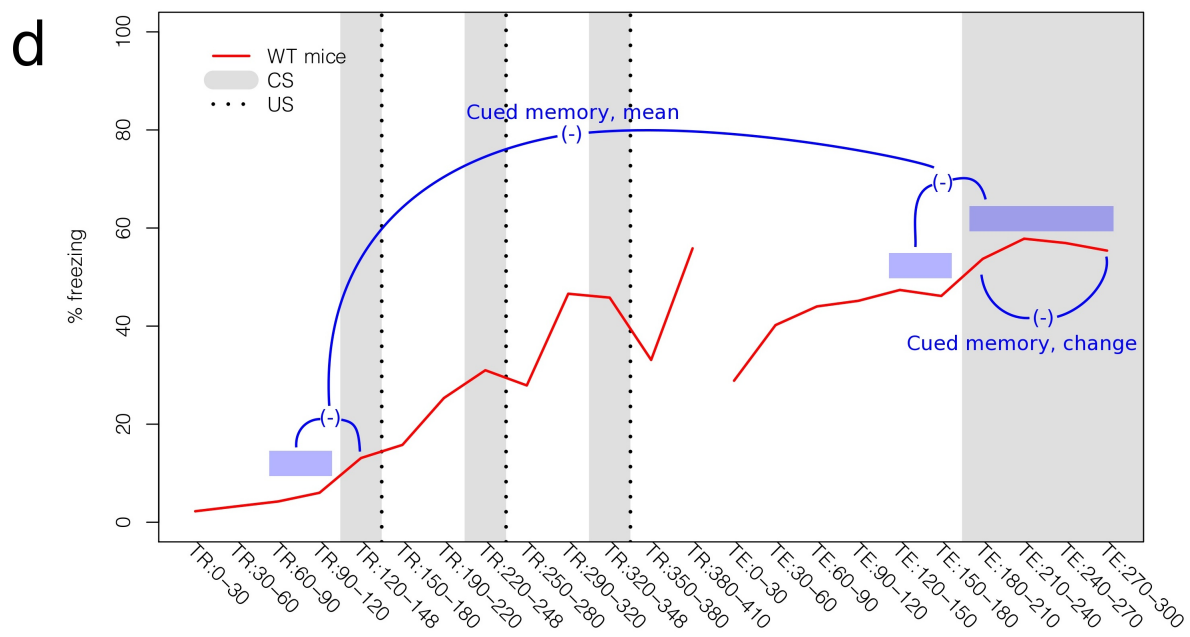

Figure 4 – Derivation of six measures (blue) in classical conditioning protocol. a) Protocols illustrated in partial screen-grabs from FreezeFrame software. Tones (CS) indicated by blue-green boxes and footshocks (US) indicated by narrow red boxes. Time in seconds indicated beneath each panel. Upper panel, training protocol, carried out on day 4. Lower panel, testing protocol, carried out on day 5. Times shown in seconds. b-d) Derivation of six variables. Time bins (horizontal axes) are indicated as TR on day 4 (training day) or TE on day 5 (testing day), followed by time in seconds. Time bins are 28 or 30 seconds long. Red trace represents average wildtype C57BL/6J freezing in response to protocol. Blue shaded regions represent averaging of freezing behaviour across multiple time bins. Blue connecting lines with '(-)' indicate differences in freezing behaviour between indicated time bins. b) Derivation of learning parameters. c) Derivation of contextual memory variables. d) Derivation of cued memory variables.

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