Supplemental Information

Cai et. al.

Table 1S

Choice	Task	Days	Genotype	Test	M±SEM	Statistic	Probability
1 then 2	2NC+	1-3	Separate	2-factor		day x test-geno: F(10, 84) = 1.70	p = 0.10
	2NC-			ANOVA		main effect test-geno: F(5, 84) = 0.99	p = 0.43
	2C+					main effect days: F(2, 84) = 2.5	p = 0.09
	2NC+			t-test	wt: 41.1±6.1 ko: 32.7±5.3	t(11) = 1.01	p = 0.34
	2NC-			t-test	wt: 34.6±3.1 ko: 31.9±3.6	t(14) = 0.51	p = 0. 62
	2C+			t-test	wt: 27.7±4.0 ko: 31.7±3.3	t(17) = 0.79	p = 0. 44
1 then 2	2NC+	15-17	Separate	2-factor		day x test-geno: F(10, 74) = 1.41	p = 0.19
	2NC-			ANOVA		main effect test-geno: F(5, 74) = 1.20	p = 0.12
	2C+					main effect days: F(2, 74) = 0.16	p = 0.16
	2NC+			t-test	wt: 60.7±4.8 ko: 64.9±2.3	t(20) = 0.79	p = 0.44
	2NC-			t-test	wt: 52.8±2.3 ko: 54.1±2.6	t(11) = 0.32	p = 0.76
	2C+			t-test	wt: 64.0±10.1 ko: 63.0±4.2	t(8) = 0.11	p = 0. 91
1 then 3		1-3	Separate	2-factor		day x test-geno: F(10, 84) = 1.29	p = 0.25
				ANOVA		main effect test-geno: F(5, 84) = 0.75	p = 0.59
						main effect days: F(2, 84) = 075	p = 0.48
				t-test	wt: 11.4±2.8 ko: 18.5±2.1	t(2.0) =	p = 0.08
				t-test	wt: 12.5±2.8 ko: 11.4±2.0	t(14) = 0.33	p = 0.74
				t-test	wt: 13.1±4.6 ko: 10.2±3.1	t(17) = 0.54	p = 0.60
		15-17	Separate	2-factor		day x test-geno: F(10, 74) = 0.60	p = 0.81
				ANOVA		main effect test-geno: F5(, 74) = 0.75	p = 0.59
						main effect days: F(2, 74) = 4.4	p = 0.02 *
				t-test	wt: 10.0±1.7 ko: 18.5±2.1	t(20) = 0.30	p = 0. 77
				t-test	wt: 12.5±1.4 ko: 10.0±1.8	t(14) = 0.97	p = 0.35
				t-test	wt: 10.7±4.7 ko: 12.5±2.0	t(8) = 0.40	p = 0.70
2 first	2NC+	1-3	Separate	2-factor		day x test-geno: F(10, 84) = 0.49	p = 0.89
	2NC-			ANOVA		main effect test-geno: F(5, 84) = 1.30	p = 0.28
	2C+					main effect days: F(2, 84) = 0.66	p = 0.52
	2NC+			t-test	wt: 22.6±3.9 ko: 24.4±4.8	t(11) = 0.29	p = 0.78
	2NC-			t-test	wt: 35.1±4.7 ko: 35.0±3.2	t(14) = 0.04	p = 0.97
	2C+			t-test	wt: 28.8±5.3 ko: 32.8±4.7	t(17) = 0.55	p = 0.59
	2NC+	15-17	Separate	2-factor		day x test-geno: F(10, 74) = 0.61	p = 0.80
	2NC-			ANOVA		main effect test-geno: F(5, 74) = 0.67	p = 0.65
	2C+					main effect days: F(2, 74) = 0.04	p = 0.96
	2NC+			t-test	wt: 19.2±2.7 ko: 18.0±1.9	t(20) = 0.38	p = 0.71

	2NC-			t-test	wt: 24.5±2.9 ko: 25.3±2.9	t(14) = 0.19	p = 0.85
	2C+			t-test	wt: 17.6±6.6 ko: 15.3±2.1	t(8) = 0.39	p = 0.71
3 first	2NC+	1-3	Separate	2-factor		day x test-geno: F(10, 84) = 3.30	p = 0.0012 **
	2NC-			ANOVA			p = 0.69
	2C+					main effect test-geno: F(5, 84) = 0.61	p = 0.0.56
						main effect days: F(2, 84) = 0.59	
	2NC+			t-test	wt: 11.4±2.8 ko: 18.5±2.1	t(11) = 2.0	p = 0.08
	2NC-			t-test	wt: 12.5±2.9 ko: 11.4±2.0	t(14) = 0.33	p = 0.74
	2C+			t-test	wt: 13.1±4.6 ko: 10.2±3.1	t(17) = 0.54	p = 0.60
	2NC+	15-17	Separate	2-factor		day x test-geno: F(10, 76) = 1.40	p = 0.20
	2NC-			ANOVA		main effect test-geno: F(5, 76) = 4.20	p = 0.004 **
	2C+						p = 0.32
						main effect days: F(2, 76) = 1.14	
	2NC+			t-test	wt: 10.2±2.3 ko: 7.6±1.9	t(20) = 0.85	p = 0.40
	2NC-			t-test	wt: 12.5±2.1 ko: 17.3±2.4	t(14) = 1.4	p = 0.19
	2C+			t-test	wt: 7.7±1.3 ko: 9.3±1.9	t(8) = 0.61	p = 0.56
1 then 2	2NC+	1-3	Combined	1-factor	37.2±4.1	f(2, 45) = 1.4	p = 0.26
	2NC-			ANOVA	32.9±2.6		
	2C+				30.0±2.5		
	2NC+	4-6	Combined	1-factor	47.0±3.4	f(2, 45) = 5.5	p = 0.007 **
	2NC-			ANOVA	39.2±1.9	2NC- v. 2C+ q = 4.7 p < 0.01 **	
	2C+			(1)	51.5±2.8		
	2NC+	7-9	Combined	1-factor	50.5±2.0	f(2, 45) = 12.4	p < 0.0001
	2NC-			ANOVA	45.1±2.0	2C+ v. 2NC+ q = 4.3 p < 0.05 *	***
	2C+			(1)	60.9±2.7	2C+ v. 2NC- q = 6.9 p < 0.001 ***	
	2NC+	10-12	Combined	1-factor	59.2±3.1	f(2, 45) = 4.4	p = 0.02 *
	2NC-			ANOVA	50.2±2.7	2NC- v. 2C+ q = 4.1 p < 0.05 *	
	2C+			(1)	62.6±3.4		
	2NC+	13-15	Combined	1-factor	63.7±4.0	f(2, 50) = 4.7	p = 0.014 *
	2NC-			ANOVA	49.9±2.4	2NC- v. 2NC+ q = 3.9 p < 0.05 *	
	2C+			(1)	62.8±3.6	2NC- v. 2C+ q = 3.7 p < 0.05 *	
	2NC+	15-17	Combined	1-factor	62.3±2.6	f(2, 42) = 3.01	p = 0.06
	2NC-			ANOVA	53.7±1.9		
	2C+				63.4±4.4		
1 then 3	2NC+	1-3	Combined	1-factor	14.7±2.0	f(2, 45) = 0.60	p = 0.85
	2NC-			ANOVA	11.8±1.6		
	2C+				11.4±2.6		
	2NC+	15-17	Combined	1-factor	9.7±1.1	f(2, 45) = 0.62	p = 0.54
	2NC-			ANOVA	11.0±1.2		
	2C+				11.8±2.1		
2 first	2NC+	1-3	Combined	1-factor	23.4±2.9	f(2, 45) = 3.2	p = 0.05 *
	2NC-			ANOVA	35.1±2.6	2NC+ v. 2NC- q = 3.5 p < 0.05	
	2C+				31.1±3.5		
	2NC+	15-17	Combined	1-factor	18.6±1.6	f(2, 45) = 4.8	p = 0.013 *
	2NC-			ANOVA	25.0±1.9	2NC- v. 2C+ q = 3.9 p < 0.05 *	
	2C+				16.2±2.7		
3 first	2NC+	1-3	Combined	1-factor	24.3±2.8	f(2, 45) = 0.50	p = 0.61
	2NC-			ANOVA	20.2±2.5		
	2C+				22.2±3.2		
	2NC+	15-17	Combined	1-factor	8.9±1.5	f(2, 45) = 5.7	p = 0.006 **
	2NC-			ANOVA	15.5±1.8	2NC- v. 2NC+ q = 4.4 p < 0.01 **	
	2C+				8.6±1.2	2NC- v. 2C+ q = 3.7 p < 0.05 *	

1 then 2	2NC+	1-3 v. 15-17	Combined	t-test	1-3: 37.2±4.1 15-17: 62.8±2.6	t(33) = 5.5	p < 0.0001 ***
	2NC-				1-3: 32.9±2.6 15-17: 53.7±1.9	t(27) = 6.1	p < 0.0001 ***
	2C+				1-3: 30.0±2.5 15-17: 63.4±4.4	t(27) = 7.1	p < 0.0001 ***
1 then 3	2NC+	1-3 v. 15-17	Combined	t-test	1-3: 14.7±2.0 15-17: 9.7±1.1	t(33) = 2.4	p = 0.02 *
	2NC-				1-3: 11.8±1.6 15-17: 11.0±1.2	t(30) = 0.41`	p = 0.68
	2C+				1-3: 11.4±2.6 15-17: 11.8±2.1	t(27) = 0.10	p = 0.92
2 first	2NC+	1-3 v. 15-17	Combined	t-test	1-3: 23.4±2.9 15-17: 18.6±1.6	t(33) = 1.6	p = 0.13
	2NC-				1-3: 35.1±2.6 15-17: 25.0±1.9	t(30) = 3.1	p = 0.004 **
	2C+				1-3: 31.1±3.5 15-17: 16.2±2.7	t(27) = 2.9	p = 0.008 **
3 first	2NC+	1-3 v. 15-17	Combined	t-test	1-3: 24.7±2.8 15-17: 8.9±1.5	t(33) = 5.4	p < 0.0001 ***
	2NC-				1-3: 15.5±1.8 15-17: 4.7±3.1	t(30) = 1.5	p = 0.13
	2C+				1-3: 8.6±1.2 15-17: 13.6±4.6	t(27) = 3.0	p = 0.006 **

Table 2S

1 then 2	2NC+ 2NC- 2C+	4-6	Separate	2-factor ANOVA		day x test-geno: F(10, 84) = 1.5 main effect test-geno: F(5, 84) = 2.6 main effect days: F(2, 84) = 2.9	p = 0.16 p = 0.04 * p = 0.06
	2NC+			t-test	wt: 49.5±5.8 ko: 44.2±3.0	t(11) = 0.77	p = 0.46
	2NC-			t-test	wt: 40.3±3.6 ko: 38.6±2.3	t(14) = 0.42	p = 0.68
	2C+			t-test	wt: 55.0±5.4 ko: 48.9±2.9	t(17) = 1.1	p = 0.30
	2NC+ 2NC- 2C+	7-9	Separate	2-factor ANOVA		day x test-geno: F(10, 84) = 1.1 main effect test-geno: F(5, 84) = 5.1 main effect days: F(2, 84) = 2.7	p = 0.34 p = 0.0010 *** p = 0.08
	2NC+			t-test	wt: 47.6±3.1 ko: 53.9±1.9	t(11) = 1.7	p = 0.13
	2NC-			t-test	wt: 44.0±2.6 ko: 45.8±2.9	t(14) = 0.43	p = 0.67
	2C+			t-test	wt: 61.5±4.4 ko: 60.5±3.6	t(17) = 0.17	p = 0.87
	2NC+ 2NC- 2C+	10-12	Separate	2-factor ANOVA		day x test-geno: F(10, 84) = 1.9 main effect test-geno: F(5, 84) = 2.4 main effect days: F(2, 84) = 0.76	p = 0.053 p = 0.06 p = 0.47
	2NC+			t-test	wt: 56.0±3.9 ko: 63.0±4.8	t(11) = 1.1	p = 0.28
	2NC-			t-test	wt: 47.8±4.4 ko: 51.8±3.6	t(14) = 0.66	p = 0.52
	2C+			t-test	wt: 67.0±4.9 ko: 59.5±4.5	t(17) = 1.1	p = 0.28

	2NC+	13-15	Separate	2-factor		day x test-geno: F(10, 66) = 1.4	p = 0.22
	2NC-			ANOVA		main effect test-geno: F(5, 66) = 2.0	p = 0.11
	2C+					main effect days: F(2, 66) = 4.3	p = 0.02 *
	2NC+			t-test	wt: 57.0±6.8 ko: 70.4±3.2	t(16) = 1.8	p = 0.09
	2NC-			t-test	wt: 54.9±2.5 ko: 46.9±3.2	t(14) = 1.7	p = 0.11
	2C+			t-test	wt: 63.9±6.5 ko: 61.9±4.3	t(17) = 0.26	p = 0.80
1 then 2	2NC+	21-23	Separate	2-factor		day x test-geno: F(2, 14) = 3.4	p = 0.06
				ANOVA		main effect test-geno: F(1, 14) = 0.13	p = 0.73
						main effect days: F(2, 14) = 0.28	p = 0.76
	2C+	21-23	Separate	2-factor		day x test-geno: F(2, 16) = 0.6	p = 0.56
				ANOVA		main effect test-geno: F(1, 16) = 1.1	p = 0.33
						main effect days: F(,) = 2.8	p = 0.09
	2C+	26-28	Separate	2-factor		day x test-geno: F(2, 16) = 1.5	p = 0.24
				ANOVA		main effect test-geno: F(1, 16) = 1.9	p = 0.21
						main effect days: F(2, 16) = 1.5	p = 0.26
	2NC+	15-17 v. 21-23	Combined	t-test	(1): 62.8±2.6 (2): 63.6±2.5	t(29) = 0.19	p = 0.85
	2C+	15-17 v. 21-23		t-test	(1): 63.4±4.4 (2): 72.5±5.1	t(18) = 1.3	p = 0.19
	2C+	21-23 v. 26-28		t-test	(1): 72.5±5.1 (2): 82.2±4.1	t(18) = 1.5	p = 0.16
	2C+	15-17 v. 26-28		t-test	(1): 63.4±4.4 (2): 82.2±4.1	t(18) = 3.1	p = 0.006 **

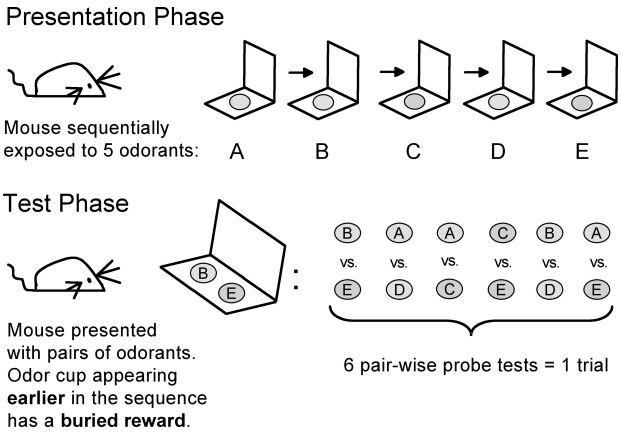


Fig. 1S - Outline of the 5-sequence protocol as adapted from Fortin et al., 2002. See Fig. 1 for cup array and shuffling.

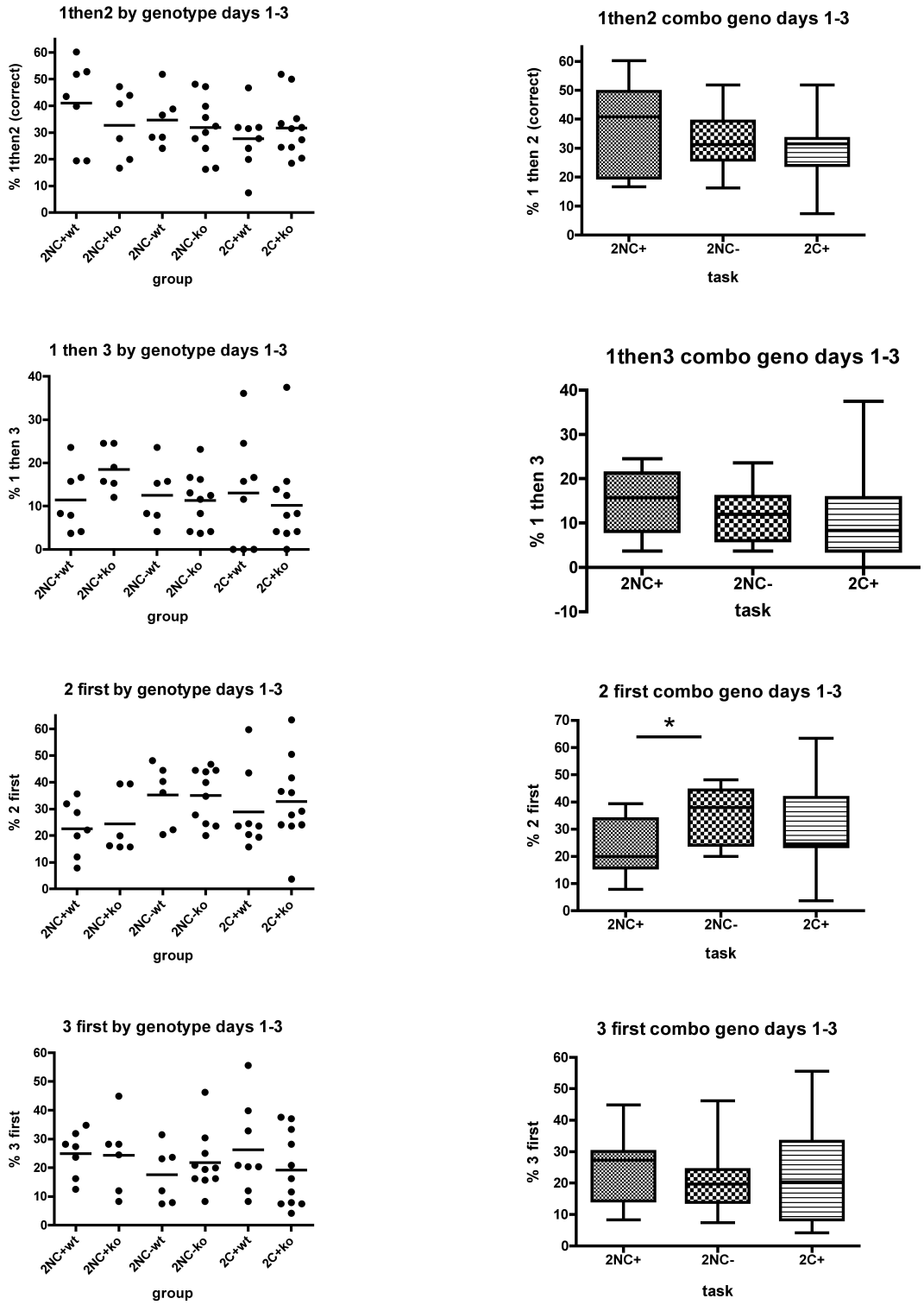
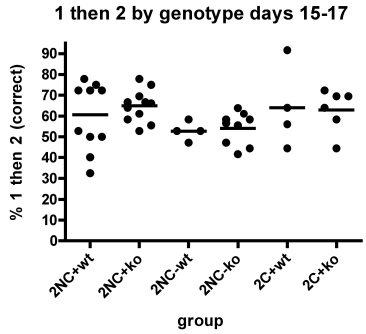
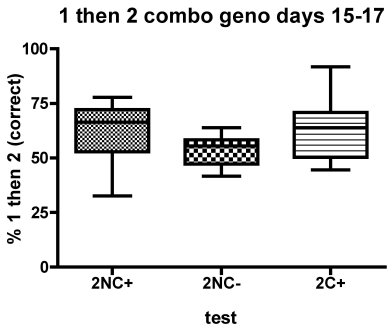
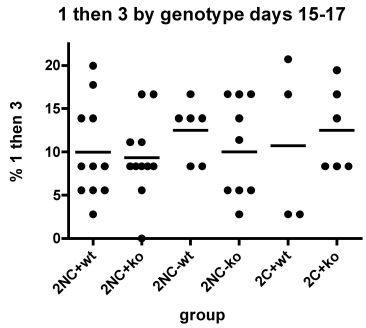
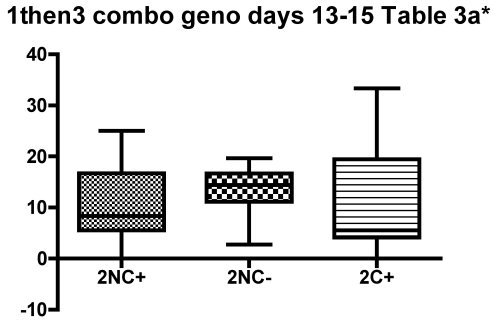


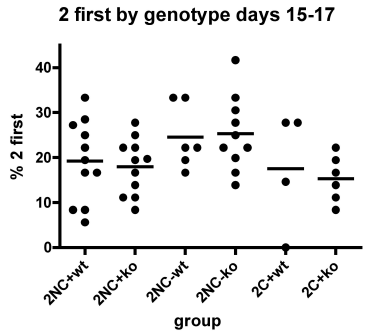
Figure 2S(a). 2-sequence response choices days 1-3
(Left column) Comparison of 2-sequence choices by genotype for each of three tasks (2NC+, 2NC-, 2C+) using one-factor ANOVA with Tukey-Kramer post-hoc analysis. (Right column) 2-sequence choices after grouping of genotypes. "2NC+": odor 2 was not held constant and odor 1 was emphasized by presenting and rewarding it twice; "2NC-": odor 2 was not held constant and odor 1 was not emphasized; "2C+": odor 2 was held constant and odor 1 was emphasized. Means are depicted in scattergrams (left) and medians in box and whisker plots (right).

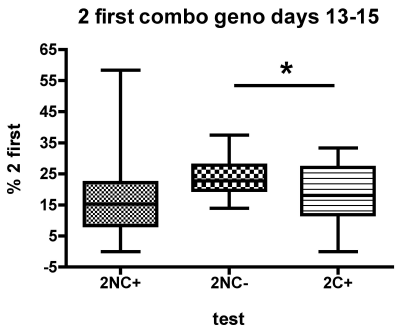


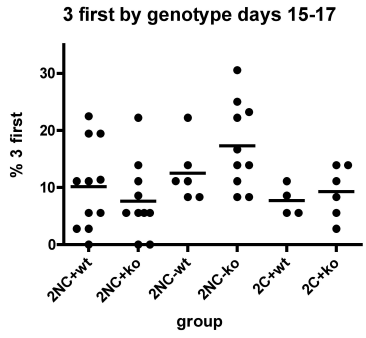












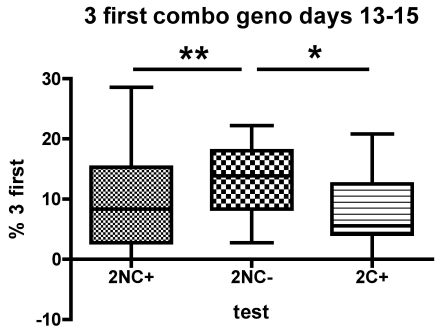


Figure 2S(b) 2-sequence response response choices days 15-17
(Left column) Comparison of 2-sequence choices by genotype for each of three tasks (2NC+, 2NC-, 2C+) using one-factor ANOVA with Tukey-Kramer post-hoc analysis. (Right column) 2-sequence choices after grouping of genotypes. Means are depicted in scattergrams (left) and medians in box and whisker plots (right). Task abbreviations are as described in Fig. 2S1.

Table 3S - Post-block comments by subjects

Subject	Block	Delay	Trial Acquired	Comments
13 yo male	1	10 sec		I didn't see any pattern at all.
	2		2	I found a pattern and you used the same symbols as yesterday. (Q: What was the pattern?) For the row of three, it would always be the first symbol that you had seen. And for the row of two, it would always be the second one you had seen. (Q: Anything else?) No.
	3	30 sec		Same pattern as last time. One time I forgot the block I was looking at.
	4	90 sec		(Q: Since it is obvious this is now a memory test, I want to ask you how you kept the memory of the symbol.) I gave them all names, like 'target', 'hubcap', 'check', square-up', 'square-down'. I said them as I went along in my mind, sort of".
11 yo male	1	10 sec		If I got one correct, they were both on the side. I think the middle wasn't the correct answer.
	2			I noticed that it went in order from far left, middle, to right middle, left, and it keeps going like that.
	3			I think I got both things right when they were in the middle. There was some weird pattern, but I did not understand it.
	4			I don't think it was ever the left thing when the first three pictures appeared. But when two pictures were left, when I guessed the middle of three, I guessed the thing on the right, but it was always wrong.
	5			Once I got the first one right, I guessed things that looked like it. [Still does not understand the boundaries of a trial, i.e. that single images are grouped with the following triplets and doublets.]
	6		1	OK, the correct answer is the first symbol you showed me, For the group of two, it was the second symbol you showed. (Q: How did you realize this?) I have been trying many things – how light or dark the symbol was, or by what the symbol was. I also thought that depending on what the place was. Then I tried this. It was a process of elimination. This is one of the things I thought it could be. It also helped to come up with names for them, like 'snow-flake'.
	7	30 sec		It is the same pattern as last time, except I have to remember them [longer]."
10 yo male	1	10 sec		I don't get it.
	2			I think I know the pattern. [But was unable to state it.]
	3			I'm confused.
	4			I don't know.
	5		8	You have to pick the first one you saw, then pick the second one you saw.
	6			It was easy to remember [the pattern].
19 yo fe-male	1	10 sec	6	The first one that you choose is the first one that pops up in a set. The second one is the second one that I see – you then take away the first choice. [Regarding errors on trials 9 and 12:] I forgot which one came first.

	2			First slide pick the first one which shows up [among the triplet]; second slide pick the second one [among the doublet]. I realized this after the last test.
	3	30 sec		Same thing I noticed the first time I did this.
	4	60 sec		I used the same rule. I repeated the name of the thing to myself, such as 'pie' followed by 'squiggly thing'. [Regarding error on trial 13:] I wasn't paying attention because it was the same thing over and over again.
		90 sec		Subject reported feeling "antsy" and "bored".
41 yo female	1	10 sec		There's a pattern. [But she could not state it explicitly.]
	2		1	First you pick the first symbol. Then the next symbol to choose is the second one you saw during the slides with only one symbol shown.
	3			You have to remember the symbol during the blank period because sometimes it's a long wait.
15 yo female	1	10 sec	X	It's hard to remember because of the blank slides.
	2			I'm trying to remember.
	3			
	4			Drawing a "blank"; "distracted".
	5			When I looked at the screen I went blank. I was thinking of other things.
	6			"Choose the first picture for the triplet." [Correct.] [She said that the second image she was] "picking at random".
45 yo male	1	10 sec	X	I don't know which is correct or incorrect.
	2			I think there is a pattern.
	3			I don't know the pattern.
	4			I just choose the pictures I like.
	5			I still don't know the pattern.
	6			[No comment.]
	7			There are always two that look similar so I pick whichever image doesn't belong or look alike.
45 yo female	1	10 sec	X	What does symbol mean? [Explained. Not a native English speaker.]
	2			Don't know.
	3			Nothing.
	4			[Tried choosing shapes based on their sizes.]
	5			No.
	6			Nothing.

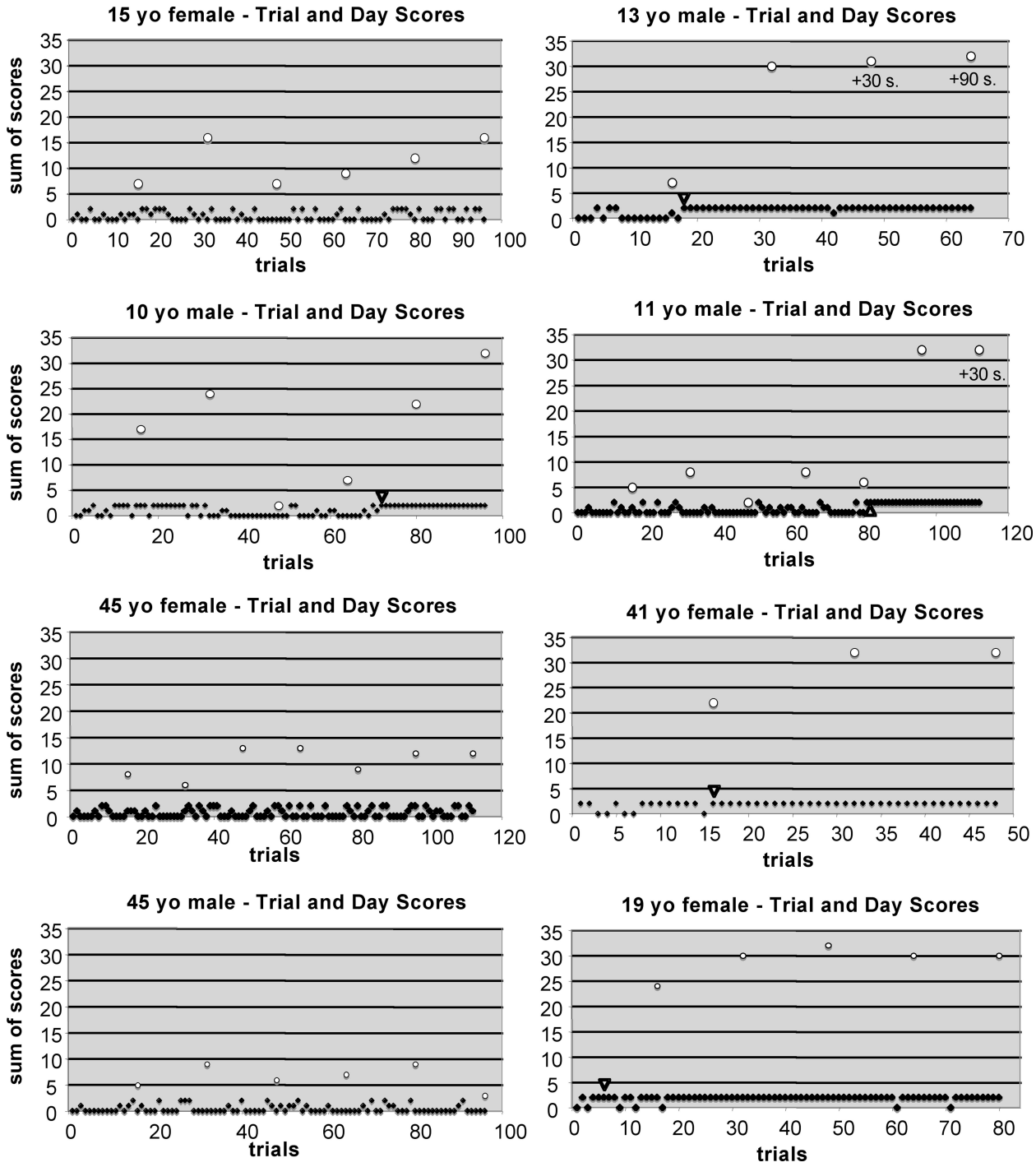


Figure 7S Human 2-sequence learning curves - complete set.
For each trial: 2 points for choice of image 1 then 2 (correct), 1 point for 1 first, 0 points for 2 first or 3 first. "yo" means "years old". Trial performance - black diamonds. Day scores are the sum of trial scores - white circles; 32 points maximum. Trial on which explicit rule acquired - open triangle; 15 yo female, 45 yo male, and 45 yo female did not acquire the rule, so no triangle is present. Delays between exposure and test phases were 10 seconds, except for days showing "+30s" and "+90s" which had delays of 30 and 90 seconds, respectively.

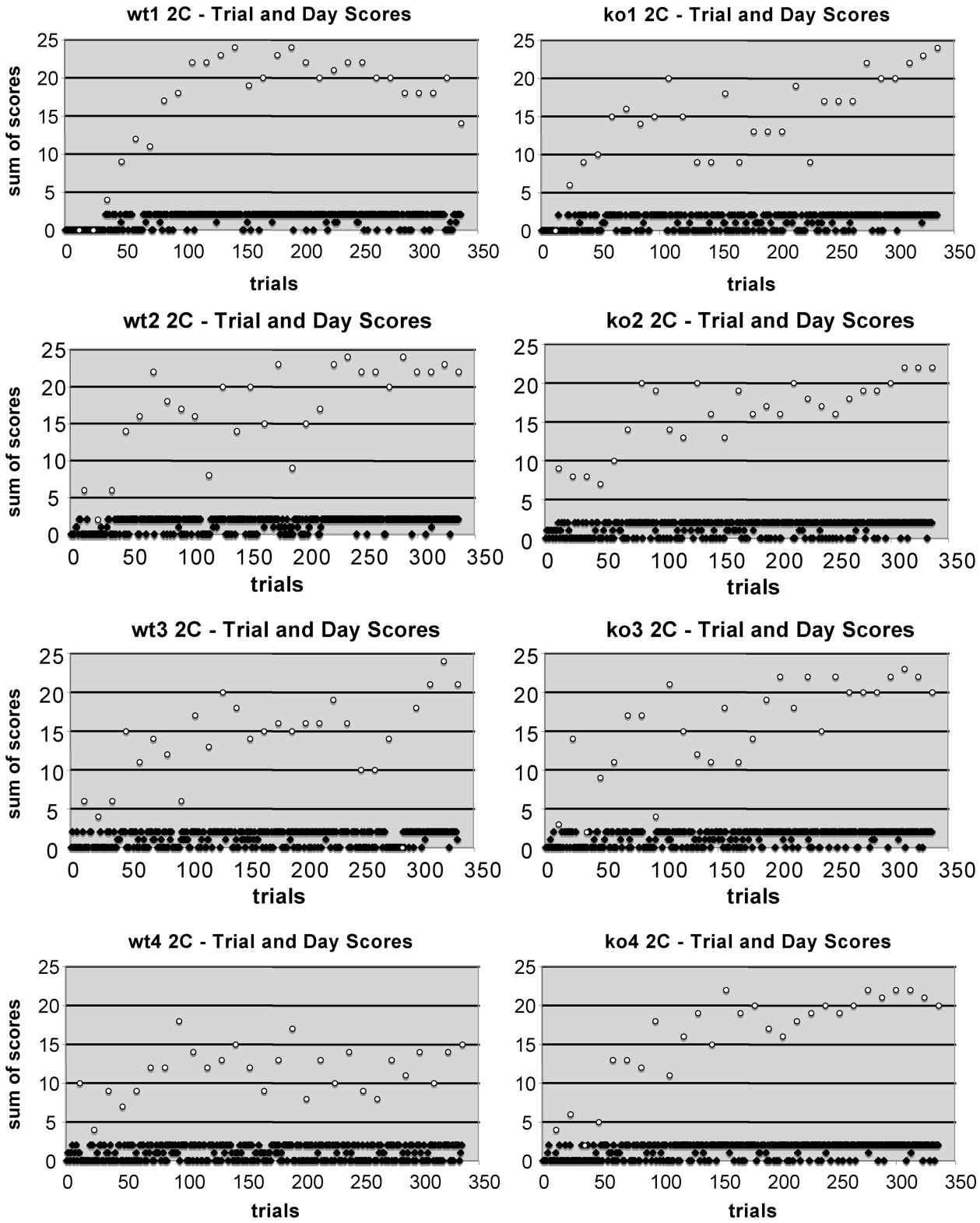


Figure 8S(a) Mouse 2-sequence learning curves

Additional learning curves. For each trial: 2 points for choice of image 1 then 2 (correct), 1 point for 1 first, 0 points for 2 first or 3 first. Trial performance - black diamonds. Day scores are the sum of trial scores - white circles; 24 points maximum per day (2 points x 12 trials/day). Test holds second odor constant ("2C"). Delays between exposure and test phases are approximately 15 seconds.

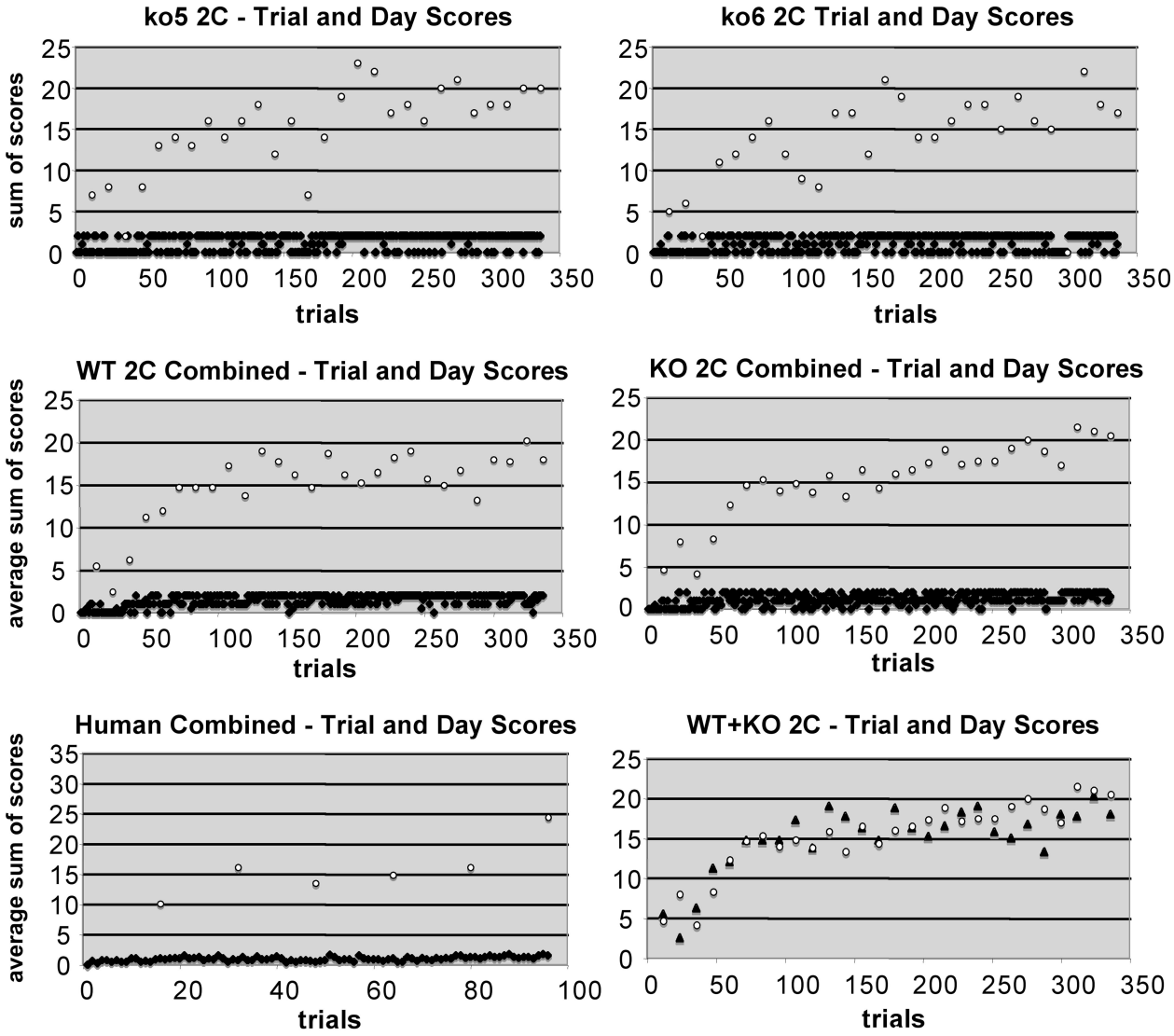


Figure 8S(b) Mouse 2-sequence learning curves

Additional learning curves. All wt scores and all ko (FX) scores are averaged in "WT2C Combined" and "KO2C Combined", respectively. Knockout scores are shown with wild-type scores (black triangles) in WT+KO 2C". All human scores are averaged in "Human Combined".

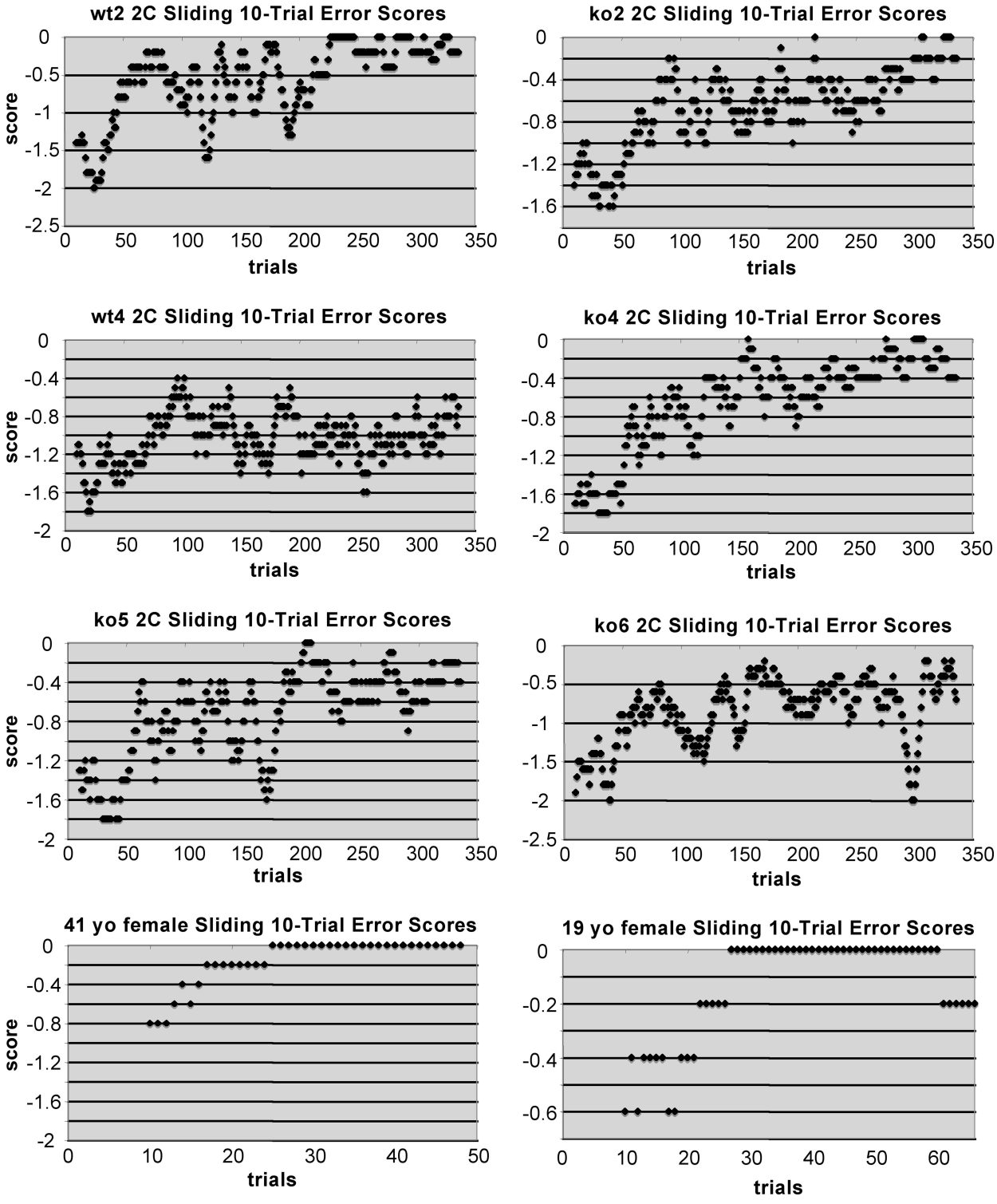


Figure 10S Additional Mouse and Human Sliding Error Score Plots

Error score = (trial points obtained - maximum trial points) Each point represents the average error score of the current trial and the prior nine trials. 41 yo female and 19 yo female acquired the rule on trials 13 and 6, respectively.

Odor Learning and Memory—Template for 2 Sequence Task

No food days 1-3 (to lab on Friday). Shape to dig on day 4 (Monday) + 4 pellets evening day 4

Continue shape to dig and adjust pellet number if necessary (0-6 pellets) for full motivation with stable weight and no starvation (through Sunday)

Begin acquisition (learning) test second Monday (Day 11 of preparation = Day 1 of acquisition).

Day one will include one additional shape as a 13th trial (do not record). Subsequent days will consist of only 12 trials.

Genotype:

Animal ID:	**Day of Testing:**	**Date:**	**Operator Initials:**	**Test Type:**	**Delay Time:**	**Pellets Fed:**

Trial:	**Choice:**	**Shape:**	**1then2**	**1then3**	**2first**	**3first**	**Pinch:**	**Comments:**
1 - 31**2**								
2 - 1**2**3								
3 - **2**31								
4 - 3**2**1								
5 - 13**2**								
6 - **2**13								
7 - **2**31								
8 - 1**2**3								
9 - 31**2**								
10 - 13**2**								
11 - 3**2**1								
12 - **2**13								
Sums:		X						X

Total Num. of Trials:	% 1then2	% 1then3	%2first	%3first

Refresh sand every Monday and Thursday morning before testing

Shaping Schedule-has buried food (is baited): wait for animal to choose correctly:

- D1 Trial 1,4,7,10,13 trial are shapes (every 3rd trial)
- D2 Trial 1,4,7,10
- D3 Trial 1,4,7
- D4 Trial 1,4
- D5 Trial 1
- D6+ no shaping

Choice/Shape coding:

- Place a checkmark in the Choice column if the mouse was given a choice; in the Shape column if that was the case
- Sum only the number of Choices made (a checkmark=a "1")
- If for some reason a trial was not done, leave the Choice box blank and put a "n.d." in the Comments box with the reason why

Negative reinforcement coding:

- Only actual pinches get a checkmark
- Write "T" (threatened) in comments section if no pinch was given
- Mice are only pinched when they stop responding to (retreating from) the sight of the pinchers

Type of test coding:

Components of Test:	*Abbreviations:*
Emphasis v. no Emphasis	Emph v. NonEmph
2 constant v. 2 changing	2C v. 2NC
With punishment v. dropped punishment	P v. nP
Delays	(write in the time)

Tester Instructions

Before the first day's testing, please read the underlined text:

"You are going to see some symbols in a slide presentation.
You will have to make a choice of one symbol when you see more than one.
I will respond correct or incorrect.
You try to make as many correct choices as you can.
You are to say nothing during the test. Simply point to a symbol."

After we are done today, I may ask you some questions."

****

For blank slides, follow the timing on the scoring sheet; count to yourself by mentally saying "one one-thousand, two one-thousand, three one-thousand", then tell the subject: "Please choose a symbol."

****
After the first day's testing, please read the underlined text:

"Did you notice anything about the test?"

(From Willingham et. al., 1989)

Make notes on the front of the scoring sheet quoting (if possible), or paraphrasing if necessary, the comments of the subject as to what, if anything, he or she noticed about the test.

(If a rule were discovered and used successfully, it should be evident in the data. No need to explicitly ask for a pattern if the subject did not explicitly discover it. This would pre vent any further attempt to continue the task.)

Scoring Sheet

Problem	Triplet Order	Triplet Choice*	Correct (1)# Incorrect (0)	Pair Choice	Correct (1) Incorrect (0)
1	3-1-2				
2	1-2-3				
3	2-3-1				
4	3-2-1				
5	1-3-2				
6	2-1-3				
7	2-3-1				
8	1-2-3				
9	3-1-2				
10	1-3-2				
11	3-2-1				
12	2-1-3				
13	1-2-3				
14	1-3-2				
15	2-1-3				
16	2-3-1				
Total					

* Use the designation in the triplet order immediately to the left.
# Based on the rule stated on the back of the score sheet. Under no circumstances let the subject see this rule, or even hint that one exists.

Timing for slide sets 1-7:
1) Single image 1 2 or 3 seconds^
2) Single image 2 2 or 3 seconds
3) Blank 10 seconds = default (the “delay”)
4) Triplet 3 seconds

If correct triplet
5) Blank following 2 seconds
6) Pair 3 seconds

If correct pair
7) Blank following 2 seconds

If incorrect pair

7) Blank following 10 seconds

If incorrect triplet

- S to look up to ceiling

5) Advance to blank slide after pair

- S to look at blank 15 seconds

^ say “one one-thousand, two one-thousand, three one-thousand” to yourself, then say “please choose a symbol”; use 3 seconds if needed by the subject

****

(Back of scoring sheet):

Initial Information – Not to be seen by the Subject

RULE = _____

DELAY _____

Date _____

Test Day Number _____

Block Number _____

Subject Name _____

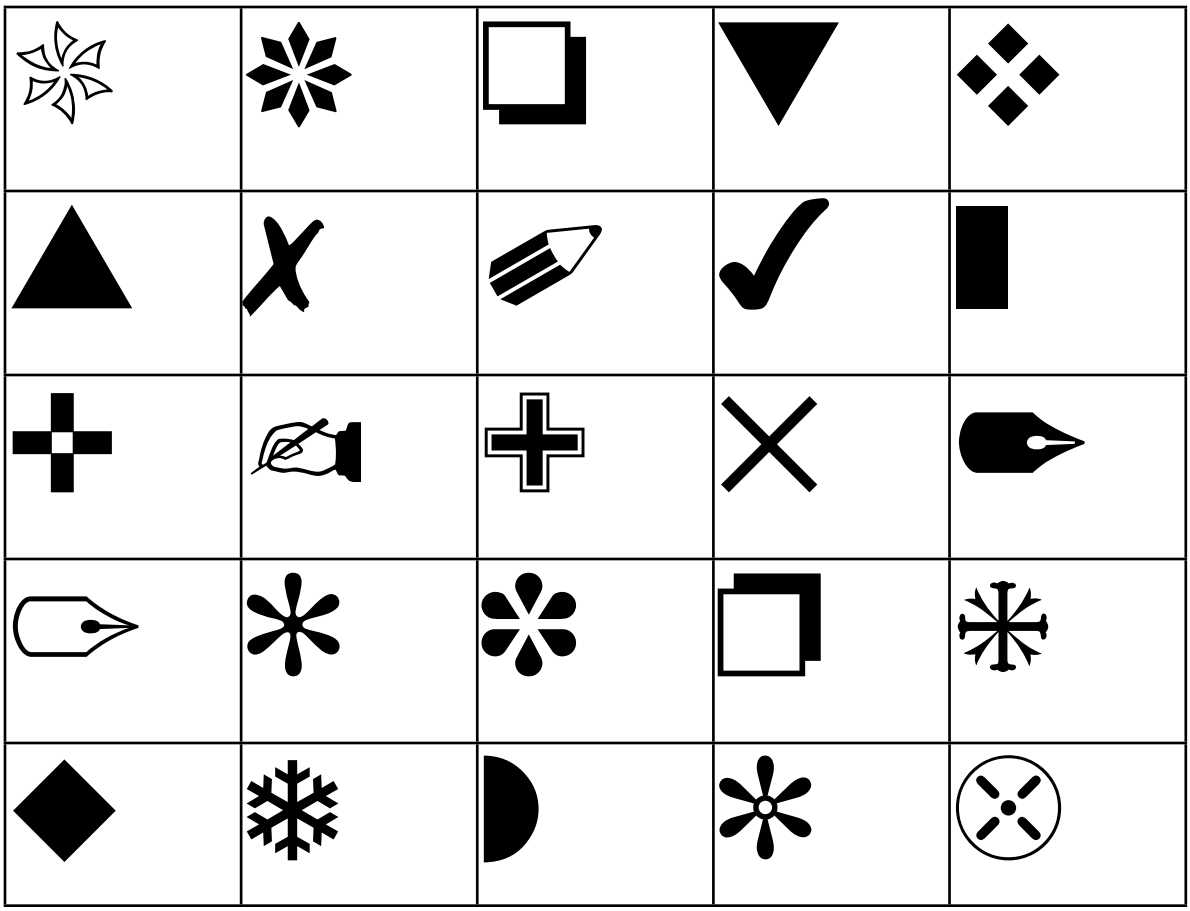
Subject Age/Sex _____

Subject Phone _____

Tester Name _____

Tester Email _____

Symbols for Human 2-sequence testing.



Monotype Sorts, 96 point

Table 4S

subject (A)	significant error change point (B)	rank sum difference (C)	total blocks (D)	statistically near-errorless (E)	perfect blocks (F)	reported insight trial (G)
11yo M+	8 vs 9 *** 8 vs 10 *** 8 vs 11 ***	-56.25 -56.25 -56.25	11	YES 9-11	9-11 (30/30)	81 of 112
15yo F-	none	n.a.	10	NO 1-9 #	none (13/30)	never of 96
13yo M+	2 vs. 3 ** 2 vs. 4 ** 2 vs. 5 * 2 vs. 6 ** 2 vs. 7 ns #	-22.35 -22.35 -19.80 -22.35 -22.35	7	YES 3-7	3-4, 6-7 (46/47)	18 of 64
45yo F-	none	n.a.	12	NO 1-11 #	none (9/30)	never of 112
10yo M+	7 vs 8 * 7 vs 9 ** 7 vs 10 * #	-39.95 -42.80 -42.80	10	YES 8-10	9-10 (25/25)	72 of 96
41yo F+	1 vs. 2 ns 1 vs 3 * 1 vs 4 * 1 vs 5 ns #	-9.600 -9.600 -9.600	5	YES 2-5	4-5 (32/32)	17 of 48
45yo M-	none	n.a.	12	NO 1-11 #	none (2/30)	never of 112
19yo F+	1 vs 2 ns 1 vs 3 ns 1 vs 4 ns	-4.000 -12.00 -12.00	8	YES 1-8	3-6 (70/75)	6 of 80
wt1	None	n.a.	34	6 8-32	12, 14, 15, 19, 32 (20/30)	
ko1	none	n.a.	34	6, 10-11, 15, 21 24-33	28, 30, 32 (29/30)	
wt2	none	n.a.	34	5-11 13-18 21-33	23, 24, 27, 29 (27/30)	
ko2	none	n.a.	34	8-9, 11, 13-15 17-33	33 (27/30)	
wt3	29 vs 30 ns 29 vs 31 * 29 vs 32 ** 29 vs 33 * 29 vs 34 ns	-39.85 -48.60 -43.20 -43.02	34	11, 13-14, 18-24 27-33	32 (27/30)	
wt4	none	n.a.	34	10-11, 14, 19 30, 33	none (17/30)	
ko3	none	n.a.	34	7-8, 11-12, 15 19-33	none (26/30)	

ko4	none	n.a.	34	8-10 12-33	30 (25/30)	
ko5	none	n.a.	34	6-8, 10-13, 16 19-33	none (25/30)	
ko6	30 vs 31 ** 30 vs 32 * 30 vs 33 **	-50.40 -41.25 -46.85	34	9, 14, 16-18, 20 22-28, 32-33	none (21/30)	

Table Legend

Significant changes between block errors assessed by Kruskal-Wallis test followed by Dunn's Multiple Comparison test in groups of 10 (initial) and then 11 blocks, e.g. 1-10, 9-20, etc. All data were also assessed as complete sets, but significance levels are those from the 10-11 block analyses.

For results showing a significant reduction in error median, significance of difference from no error state was assessed by Wilcoxon Signed Rank test.

A – "M" is human male, "F" is human female, "+" means the human subject reported acquiring the correct rule; "-" means they did not do so, "yo" is "years old".

B – Blocks showing significant decrease in consecutive error median (Dunn's Multiple Comparison Test)

C – Difference in rank sum for column B

D – Total blocks

E – Statistically near-errorless performance (by Dunn's Multiple Comparison Test) after change point (B) or start point if no detectable significant error change point

F – Blocks with perfect performance (truly errorless) among those listed in (E). In parentheses are the number of perfect trials out of the trials following the insight trial or the last 30 trials if no reported insight (including for all mice).

G – Trial at which the human subject reported knowing the rule.

- Indicates that the last block trial number (n) too small (i.e. it contained significantly fewer than 10 trials). Humans had 16 trials per day and mice had 12 (see Methods), so often an exact multiple of 10 trials was not performed.

Endnotes (EN)

EN1 - ***Learning sets***

Early learning set experiments required monkeys to learn to choose the rewarded object from a pair presented simultaneously during a trial (Harlow, 1949). When first presented with a new pair of objects, the subject would have to choose one randomly. From the response, either reward or no reward, the subject would presumably be informed as to which object was the rewarded one. Thus, if it had learned to use a "win-stay, lose-shift" strategy (Levine, 1959), the subject would be able to choose the rewarded object in the following trials. In its most stringent sense, achieving a learning set would require that the subject choose the correct object on trial 2 greater than 90% of the time, or "near errorlessly" (Reid and Morris, 1993). Harlow found that rhesus monkeys could, on average, improve their correct choice on trial two from just over 50% to well over 90% (Harlow, 1949). They therefore appeared to have achieved a learning set. What made the concept of learning set interesting to many scientists was that as a potential measure of higher order cognitive function, it could be used to compare intelligence among non-human species (Macphail, 1982; Slotnick et al., 2000; Warren, 1973).

Rats and mice originally performed very poorly on learning set tests when compared with primates, birds and other animals (Warren, 1973). However, rodents have relatively poor visual acuity (Artal et al., 1998), and when tested for visual stimulus discrimination, they also perform poorly compared to birds or primates. When the sensory modality was changed from a visual to an olfactory one, rats and mice performed very well in learning tasks (Jennings and Keefer, 1969; Nigrosh et al., 1975). Indeed, in tests of learning set acquisition, rats were able to obtain the target criterion of performance much more rapidly than monkeys had in earlier experiments,

in which hundreds of trials had been required (Slotnick et al., 2000). Furthermore, it was argued that the ability to achieve learning set within a handful of trials indicated that rats had a special facility for olfactory learning (Eichenbaum and Otto, 1993). However, others have argued that such exceptional performances might be due to "contaminant" cues accessible to the rodent subjects (Reid and Morris, 1993). We have previously provided evidence that, in at least some test formats, such artifacts can produce what appear to be remarkable feats of memory (Katz et al., 2003).

EN2 - ***Effect of developmental stage on learning curves***

Harlow also tested human subjects with the same test apparatus used by his monkeys (Harlow, 1949). The ages of the children he studied ranged from two to five years old. Unfortunately, the capacity for rational, hypothesis-based thought is relatively limited in humans for much of that developmental period, e.g. at least until after the pre-conceptual substage of Piaget, ending at four years of age; information processing related to the use of strategies for memorization is believed to begin at seven to eight years of age (Bernstein et al., 2003). Therefore, differences in the learning curves between animals and more mature humans may have been missed, as the data here suggest.

EN3 – ***Insight effect on learning curves***

The human subjects in these studies who inferred the rule, e.g. "when shown three images simultaneously, chose the image shown two-back first followed by that shown one-back second", did so, as noted in the primary text, by hypothesis testing, i.e. they reported trying vari-

ous possible rules to determine which consistently produced correct responses. The variable choice of hypotheses to test led to another distinguishing feature of the human learning curves: the moment of insight, when the correct rule was obtained and confirmed, was also highly variable as to the trial on which it occurred.

EN4 – *Examples of streak and slump performance by rodents in olfactory learning sets*

The best performing rat in one olfactory learning set study performed 11 of 20 post-training problems with one or no errors; however, the same rat did not meet criterion on 9 of the 20 problems (Slotnick and Katz, 1974). Individual rat performance in another olfactory learning set study also suggested variable performance: "True errorless learning (making no mistakes after the first information trial) occurred in one problem for each of two rats, in 3 problems for 1 rat, and, for the best performer (L14), on 4 of the last 10 problems" (Slotnick et al., 2000). A final example further documented that rats in an olfactory learning set assay "did sometimes perform errorlessly from trial 2 onwards on a given problem, but … such animals always performed less well on later problems" (Reid and Morris, 1993).

EN5 – *Learning set use in mental retardation*

It is conceivable that further increases in olfactory list length might allow some discrimination between FX and wild-type mouse short-term sequence memory, although as previously, these genotypes did not differ in working memory for spatial location of four baited arms of a radial maze. Nonetheless, those with FXS could recall an average of more than three numbers on the K-ABC (forward) number recall subtest, approximately three sequential hand movements,

and touch over three pictures in the order stated by the examiner (Dykens et al., 1987; Hodapp et al., 1992; Kemper et al., 1988). Therefore, the primary importance of the memory assessment in this study is the demonstration that for mice it remains error-prone in a way that is different from that of human subjects who have explicitly acquired the rule of the task.

Nonetheless, essentially all prior learning set (LS) studies on human subjects have used repeated trials of two-object discrimination (with changing positions among right and left, also known as "object-quality discrimination" tests; reviewed in (Kaufman and Prehm, 1966)). In those LS studies, normal subjects outperformed those with mild mental retardation, who in turn outperformed those with more severe mental retardation. Therefore, LS studies in humans have used two-item lists, and performance did correlate with IQ and mental age. Furthermore, as we found with our normal human subjects, acquisition of the two-item learning set by those with mental retardation occurred at quite different trial number for participants, including a substantial proportion who did not appear to significantly improve (Wischner et al., 1962). A learning set examination that included those with superior IQs also showed the influence of mental age and IQ on trial at which LS was acquired, but also indicated that beyond an mental age of 8 or 9, such "win-stay, lose-shift" LS tasks were too simple to provide discrimination (Harter, 1965). In contrast, the 2-sequence task used here may be more comparable to a more complex form of object-quality discrimination learning set known as conditional discrimination. In conditional discriminations, the reward value of a particular object depends on some other associated attribute, such as the color of the background (e.g. tray) on which it was presented. Similarly, the reward value of any particular odor in the 2-sequence task depended on its presentation position. In each case, the test went beyond object(odor)-reward to object(odor)-condition-reward. These

conditional discriminations also effectively contain built-in reversals, as do the 2-sequence tests, e.g. sometimes squares on blue trays (or the odorant rosemary in position 2) can be rewarded and sometimes not.

EN6 - ***Protocol effects on learning and memory tasks***

Of note, aside from strain-dependent effects (e.g. see (Paradee et al., 1999)), replication of some of the subtle differences reported in FX mouse cognitive tests appears to be dependent on aspects of protocol that would not seemingly directly relate to intelligence or memory. For example, it was previously shown that differences between wt and FX mouse performance in a classic 8-arm radial maze (RAM) task were not likely due to a profound learning deficit, since the early "memory" differences could be eliminated by a protocol change ("strong choice") which required the mice to stop to choose a door, rather than allowing impulsive choices early in the task (Yan et al., 2004). Diminished impulse suppression was subsequently shown to have a dramatic effect on the error rate of FX mice in tasks measuring attention (Moon et al., 2006).

The ability of mice to suppress responses to spatial cues might also have a significant effect on performance of mice in olfactory-based matching tasks. To further examine this issue, we have compared wt and FX male mice (on the FVB/NJ strain background beginning at 3 months of age) in an olfactory task we designed for use in an 8-arm radial maze. The testing included both win-stay components and changes of correct odorant ("reversals"). When a single exposure arm was employed followed by the mouse having to choose from among four test arms (see Fig. 12S, below), the FX mice were able to learn as effectively as the wt mice (Fig. 14S). However, when the test was altered such that odorants were first presented from one of four different arms (four exposure doors; Fig. 13S), neither the FX nor the wt mice could perform the task (Fig 15S).

Nor could they successfully perform the task when each of the four exposure arms had the same spice odorant. The four exposure door tests included the mice which had already successfully learned to perform the same task from the single exposure arm. Therefore, it appeared that the mice (wt and FX) were not assessing the odorants marking each test door on each trial, as would be required when the exposure arms were changing on each trial. (FVB/NJ mice are blind as adults, so a visual spatial map would not be produced). However, when the exposure arm was in a fixed position, the mice could learn the relative spatial position of the odor-marked test doors over a number of trials, and thereby improve their performance. In these particular tests, therefore, the wt mice were not obviously more attentive than the FX mice to odorant cues. These results serve to emphasize the significant impact that apparatus design can have on mouse learning performance. Indeed, it should be noted that in some cognitive studies, FX mice apparently outperformed wt mice (e.g. see (Fisch et al., 1999)).

In the work presented here, there was never any observation that FX mice on average performed better or worse than wt mice. However, a recent study of olfactory discrimination learning using a "runway" design did find an elevated error rate by FX mice (Larson et al., 2008). The olfactory runway task required water-restricted mice to choose a water-rewarded scent and avoid an unrewarded one. After obtaining criterion for percent correct choice score over twenty trials, the odor pair would be changed. Therefore, the olfactory runway test was a win-stay, lose-shift task which required mice to learn to stay with the odor providing a reward. A win-stay, lose-shift task for human subjects would generally be considered easier to learn than the more complex, memory intensive task used in this 2-sequence study. This raises the question of why FX mice could learn like wild type in a seemingly more demanding task?

Among the differences in protocol between the runway and present tests were trial number per day (12-13 in this study vs. 40 for the runway task) and housing (singly here vs. 3-4 mice per cage for the runway task). Increased trial number could have led to increased attentional demands with consequent increased FX error rates (Moon et al., 2006). Furthermore, housing differences may have led to motivational variation among the mice. In the studies presented here, daily monitoring of the weight of each food-restricted mouse was very important. Mouse body weights were not perfectly stable on the defined calorie diets used (footnote 1, below), necessitating on going adjustments of calorie intake. As mice gained weight, they were less hunger motivated and often apparently content to "game" the task, e.g. by making a repetitive spatial choice until rewarded. When mice became too low in weight and thereby very hungry, they often became highly agitated and made rapid choices with an elevated error rate. The latter was particularly true for the FX mice. By food or water restricting mice in groups, more dominant mice could obtain more sustenance per day than less dominant mice. As a result, more dominant mice would have less motivation to perform, particularly for tasks with large numbers of trials as in the runway task. Conversely, the least dominant mice can become too agitated from hunger or thirst to perform adequately. Therefore, we do not believe it would have been possible to food restrict our mice in groups and retain a consistent amount of motivation and calmness (ability to attend to the task).

In the olfactory runway studies it was observed that for wild-type and FX mice, "errors were preceded by errors less often than expected" (Larson et al., 2008). This could be consistent with mice losing motivation, e.g. upon receiving a reward, not feeling the need to further attend to the task but rather being willing to make a simple response (in effect waiting) until a particular

port became rewarded again (a reasonable strategy if the subject was not trying to maximize reward intake. See also footnote 2 below.) Another potential sign of elevated agitation or inadequate motivation was that FX mice had 50% more no-response trials than wild type in the runway tests. Therefore, it seems consistent with our observations that inadequate motivation (e.g. from thirst), or possibly elevated agitation from too much thirst, or both, led the mice to olfactory runway sessions with elevated errors (what we here would term "slumps"). Of the two possibilities, our experience would suggest that low water was a greater factor, since we would expect this to produce a more FX-specific effect on agitation, consistent also with observations that FX mice have reduced impulse control (Moon et al., 2006). Therefore, we would predict that protocol changes to address motivation and emotional arousal, e.g. from variable water intake, could reduce the genotypic differences seen. One obvious means to accomplish this would be to singly house the subjects and individually monitor water intake (footnote 3, below).

Nevertheless, the olfactory runway study serves to emphasize that mutation of the *Fmr1* gene can have behavioral consequences in mice. As shown with the "strong choice" 8-arm radial maze task, behavioral differences can be revealed or masked with seemingly minor protocol changes (Yan et al., 2004). However, it is unlikely that elevated arousability, impulsivity, or attention deficit could account for most of the diminished intelligence in humans with mental retardation. We would not expect, for example, a treatment of the ADHD seen in many of those with FXS to provide a substantial cure for the intellectual deficits seen in FXS. Similarly, it is telling that conditions can be found under which FX and wild-type mice can learn to perform a complex cognitive task in a very comparable manner; this is something that is not likely to be readily accomplished in comparing normal and FXS humans, for example in 2-back tests. There-

fore, we believe that the current affirmative olfactory 2-sequence data are supportive of essentially normal implicit learning and memory ability in FX mutant mice.

(1) Use of smaller unit-size, fixed calorie food has been helpful in reducing weight fluctuations (e.g. 0.19 g pellets from Bio-Serve, in addition to 500 mg or 1 g. pellets).

(2) Errors were further suppressed in this study by the use of tail pinch threat/punishment for incorrect responses.

(3) Mice may have an instinct that would induce them to try new food sources after having just obtained sustenance from a particular location. If so, then FX mice may have had a harder time suppressing such an instinct. This would not have been an issue in the current study, as rewarded odors changed on each trial.

EN7 – *Metacognitive deficit in Fragile X Syndrome*

It is clear that conscious strategic thinking is important in human performance of sequence tasks of the type used in this study. Consistent with this, a primary deficit observed in those with FXS is metacognitive, i.e. a deficit in ability to employ more sophisticated strategies to augment basic memory functions (e.g. see Sternberg, 1986). This would not be unexpected, as conscious, spontaneous strategy use may be seriously deficient in those with mental retardation (Brown, 1974; Butterfield and Wambold, 1973; Vakil et al., 1997). Although those with mental retardation were able to benefit from strategies when those were taught (Brown, 1974; Butterfield and Wambold, 1973; Das et al., 1979), transfer of such strategy was generally poor (Ellis et al., 1982; Minsky et al., 1985). Therefore, the poor memory frequently noted in the retarded is

most likely produced not necessarily from a major deficit in short-term memory or from a complete inability to internally rehearse, but rather from a deficit in spontaneous use of internal rehearsal and in the coordination of strategy steps (Butterfield and Wambold, 1973; Conners et al., 1998).

The well-known variation in FXS IQ subtest strengths, (e.g. see Theobald, 1987), may also reflect the degree to which implicit vs. explicit systems are involved. Non-verbal tests such as Block Design (WISC-III) may require more strategic thinking than many verbal tests, which rely on vocabulary, (e.g. the Peabody Picture Vocabulary Test). The real impact of FXS might be better reflected, or at least less variable, in an IQ score comprising measures of metacognitive thinking and strategic problem-solving dependent on explicit learning and memory functions, rather than those based on implicit learning and memory such as vocabulary acquisition. Language acquisition in children is thought to occur primarily by implicit means; clearly this too is not functioning at normal levels in FXS. Nonetheless, language deficits may be expected to have an amplified effect on metacognitive strategic thinking as compared to vocabulary, which is crystallized knowledge based largely on direct associative learning.

EN8 – ***Impact of emphasis and punishment***

Emphasis and punishment were found to have substantial effects on the new 2-sequence task performance. The most simple explanation for the positive effect of emphasis on learning was that it made the emphasized odorant more salient. This is supported by the various ways in which we found that emphasis could be achieved, e.g. by repeating odor two twice or placing additional rewards in one of the odors. Therefore, emphasis is likely a form of von Restorff ef-

fect, in which one item in a list is made more salient, and therefore recalled more effectively, than nonemphasized items residing in the same position. Such effects have been demonstrated frequently in human studies, and have also been reported for rodents (Reed and Richards, 1996).

Dropping punishment after two weeks of training had a dramatic negative effect on performance in the 2-sequence task. It has been shown that mild tail pinch can induce greater food consumption (Levine and Morley, 1982), so pinching may have allowed the mice to consume more rewards. However, the mice were not pinched on most errors (Methods). Instead, it seems more likely that the pinch, or fear of the pinch, increased the mouse's attention to the task. (See also EN5, above.) Controversy exists as the value of aversive stimuli in the Lovaas technique, a form of applied behavior analysis used in the treatment of autism in humans. The data presented here tend to support the view that such approaches may have a beneficial effect on performance, though there remain serious questions as the ethicalness of applying aversive stimuli in human learning.

EN9 – ***Brain anatomical bases for some differences in problem solving among species***

Although rodents have frontal lobes with orbital prefrontal and anterior cingulate cortices corresponding to those of primates, there is evidence to suggest that they do not have a lateral prefrontal cortex in a form recognizable in the primates. This could be relevant here since the lateral prefrontal cortex may be associated with alternative strategy consideration and decision-making (Striedter, 2005). In humans, fMRI indicates that the lateral prefrontal cortex is involved in the maintenance and manipulation of information in working memory, including in n-back sequence tasks (for which FXS subjects show weakness) (Braver et al., 1997; D'Esposito et al.,

1999). Interestingly, neuroimaging during a serial reaction time test showed that the dorsolateral prefrontal cortex of humans became activated as the subjects became aware of the sequence (Grafton et al., 1995). Thus, rodents might have different problem solving options from primates in general, not only because of likely differences in internal labeling abilities relative to humans, but also because of other human or primate-specific brain regions. Even among primates, portions of the prefrontal cortex and networked regions such as the mediodorsal nucleus and pulvinar of the thalamus, appear to have undergone substantial further evolution in humans (reviewed in (Striedter, 2005)).

EN10 – ***Other reasons for relative resistance of mice to cognitive deficits in FXS***

In addition to lack in mice of important brain regions mediating some metacognitive functions in humans, it has been previously proposed that the relative resistance of mice to the negative intellectual effects of FMRP loss could be due to a subtle effect on neural performance that is much less notable in the mouse's significantly smaller neocortex (Yan et al., 2004). Although larger groups of wild-type and FX mice could provide increased statistical power which might reveal a difference in performance in this task, at the very least we can conclude that no major difference is likely, i.e. one comparable in magnitude to that observed in similar tests between normal and FXS humans.

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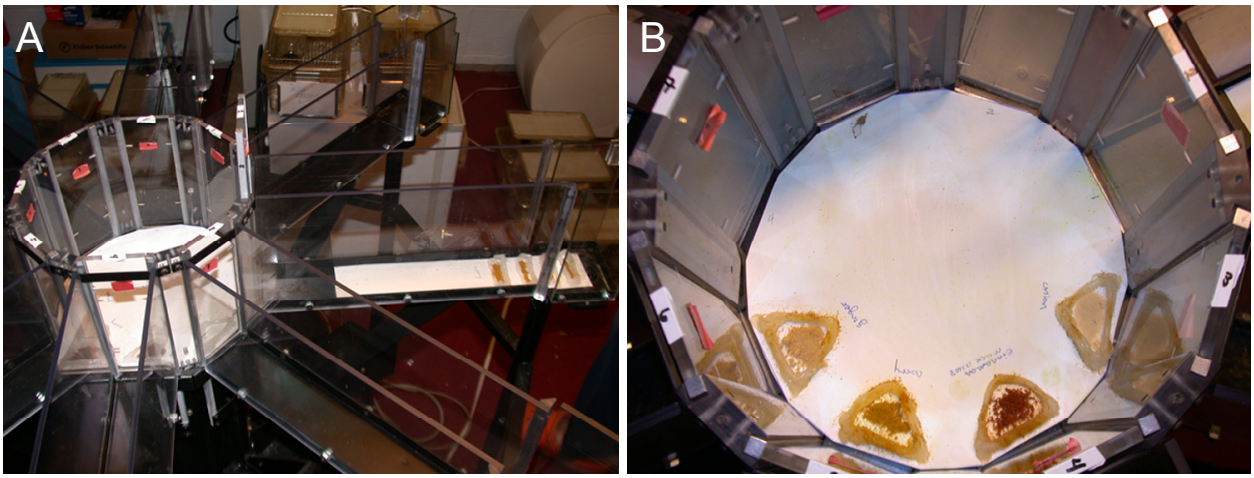

Figure 12S 1-exposure door test
(A) Three month old male FVB/NJ wt and FX mice (n = 7) were food restricted to approximately 80 - 85% free-fed body weight (exact weight range for each mouse was determined by its motivation to perform and lack of agitation - see text) and then shaped to eat sugar treats (TestDiet 5TUT) from the terminal runway cups of an 8-arm radial maze. An exposure arm contained one replaceable “odor card” with one of the four spices on it. After the subject mouse smelled the spice in the exposure arm, the four test doors were opened. (B) The central arena had four test doors with a well in front of each containing a different spice. The mouse had to learn to match the spice in the exposure arm to the same spice in the cup outside of one of the test doors. If the correct arm was entered, the mouse could retrieve a food reward. If an incorrect arm is entered, the mouse received a 30 - 60 second time out in the arm.

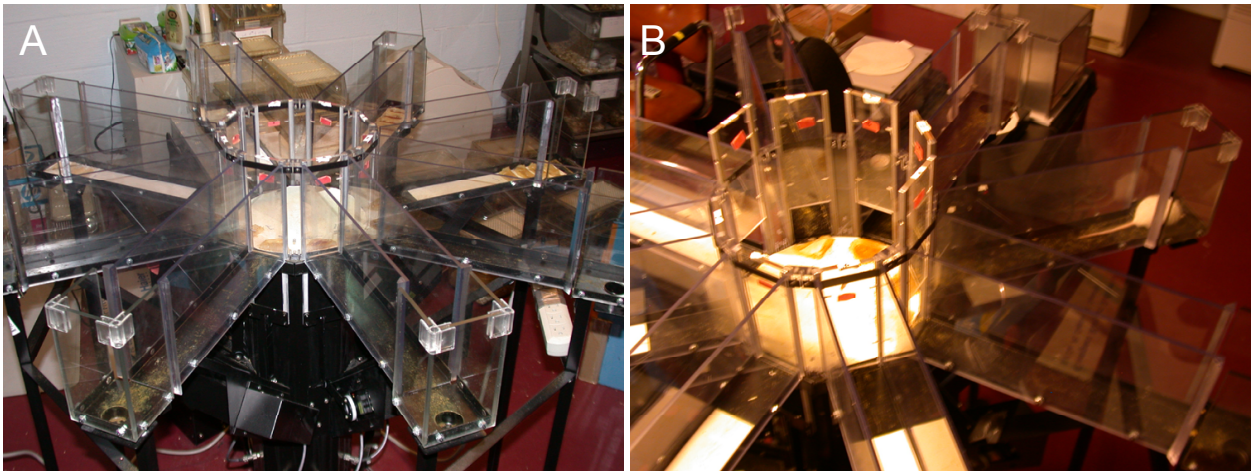

Figure 13S 4-exposure door test
(A) In contrast to the 1-exposure arm test (Figure 12S), four exposure arms were used, (necessitating only a single operator.) Each of the exposure odor arms contained an “odor card” with one of the four spices. After the mouse subject smelled the spice in one exposure arm, the four test doors were opened. (B) The mouse had to learn to match the odor in the exposure arm to the well with the same spice outside one of the test arms. If the correct arm was entered, the operator dropped a food reward into the cup at the end of the arm. If an incorrect arm was entered, the mouse received a 30-60 second time out in the arm. Four passes through a singe sequence of odor cards was used in a single day’s testing. The sequence of odor cards was changed the following day. (Four of the exposure tests during each day, one for each of the spices, was an “example” trial in which only the correct test door would open.)

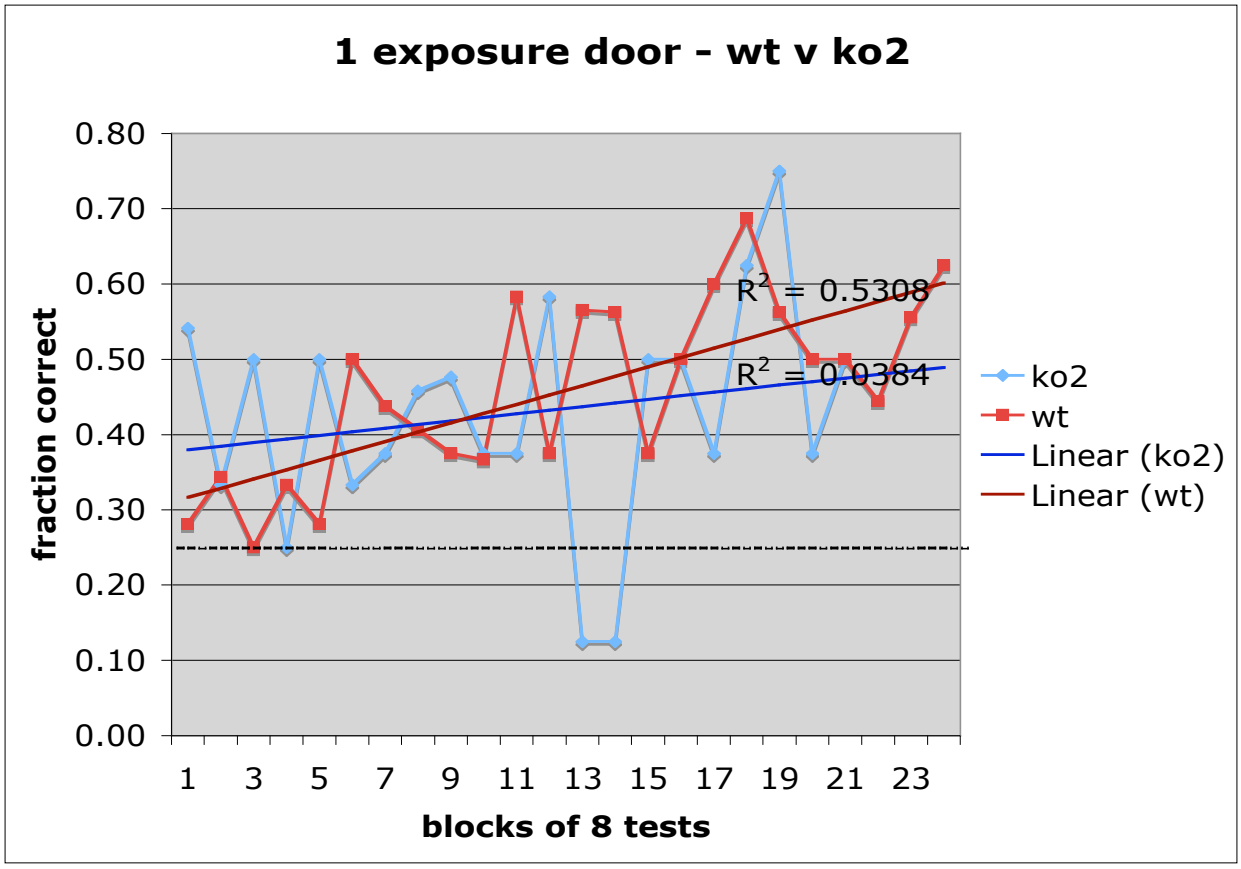

Figure 14S 1-exposure door results
The dashed line indicates the expected random choice percentage correct. Both the wild type (wt) and knockout (ko) were able to improve. No significant difference in performance was found between the genotypes. Therefore, both wt and FX mice can learn to match at least one odor.

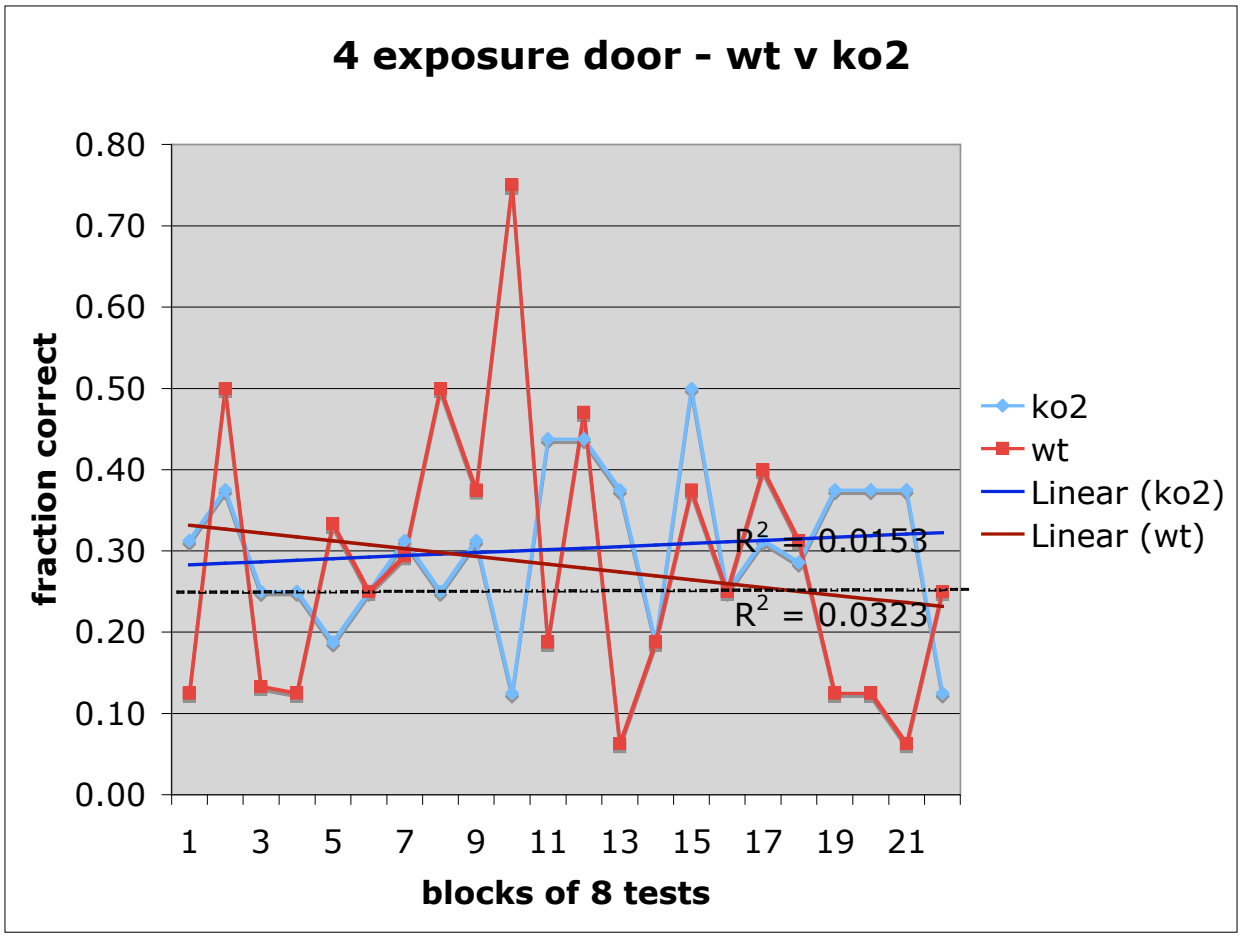

Figure 15S 4-exposure door results
The dashed line indicates the expected random choice percentage correct. Neither wt nor ko2 mice could learn the 4-exposure door test after 184 trials.