Supplementary materials

Tryptophan hydroxylase 2 deficiency alters autism-related behavioural phenotypes in rats

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The attentional set-shifting task (ASST) procedure

The ASST evaluates cognitive flexibility, a component of executive control subserved by the prefrontal cortex (Birrell & Brown, 2000).

As previously described (e.g., Popik & Nikiforuk, 2015; Potasiewicz et al., 2020), testing was conducted in a dimly illuminated (20 Lux) Plexiglas apparatus (length x width x height: 38 x 38 x 17 cm) with the grid floor and wall dividing half of the length of the cage into two sections. During testing, one glass digging pot (internal diameter of 6 cm and a depth of 3 cm) was placed in each section. Each pot was defined by a pair of stimulus cues with two stimulus dimensions (odour and digging medium). To mark each pot with a distinct odour, 5 µl of a flavouring essence (Dr. Oetker[®], Poland or The Body Shop, UK) was applied to a piece of blotting paper fixed to the external rim of the pot immediately before use. A different pot was used for each combination of digging medium and odour; only one odour was ever applied to a given pot. The bait (one-half of a Honey Nut Cheerio, Nestle[®]) was placed at the bottom of the "positive" pot and buried in the digging medium. A small amount of powdered Cheerio was added to the digging media to prevent the rat from trying to detect the buried reward by its smell.

The procedure was conducted over a period of three consecutive days for each rat.



Fig.S.1.1. Plexiglas ASST apparatus.

Day 1. Habituation



Fig.S.1.2. Example of bowl sawdust-filled.

Rats were habituated to the testing area and trained to dig in pots filled with sawdust to retrieve the food reward.

The rats were transported from the housing facility to the testing room, where they were presented with one unscented pot (filled with several pieces of Cheerios) in their home cages. After the rats had eaten the Cheerio from the home cage pot, they were placed in the apparatus and given three trials to retrieve the reward from both sawdust-filled baited pots. With each exposure, the bait was covered with an increasing amount of sawdust. Animals that did not dig for a food reward over 3 consecutive daily sessions were excluded from the experiment.



Fig.S.1.3. Examples of stimuli used in the ASST training phase: (A) Example of odour (i.e. flavour essences) placement and (B) Example of media placed bowls.

Rats were trained on a series of simple discriminations (SDs) to a criterion of six consecutive correct trials.

For these trials, the rats had to learn to associate the food reward with an odour cue (e.g., arrack vs. orange, both pots filled with sawdust) or a digging medium (e.g., plastic balls vs. pebbles, no odour). All rats were trained using the same pairs of stimuli. The positive and negative cues for each rat were presented pseudo-randomly and equally. These training stimuli were not used again in later testing trials.



Fig.S.1.4. Examples of stimuli pairs used in the ASST testing phase.

Rats performed a series of discriminations in a single test session.

The first trial at the beginning of each discrimination phase was a discovery trial, during which the animals were allowed to dig in both bowls; this trial counted toward the six criterion trial. In the subsequent trials, each incorrect choice was recorded as an error. Digging was defined as any distinct displacement of the digging media with either the paw or the nose; the rat could investigate a digging pot by sniffing or touching it without displacing material. Testing was continued at each phase until the rat reached the criterion of six consecutive correct trials, after which testing proceeded to the next phase.



Fig. S.1.5. Diagram of the ASST Testing Phase

In the simple discrimination involving only one stimulus dimension, the pots differed along one of the two dimensions (e.g., digging medium). For the compound discrimination (CD), the second (irrelevant) dimension (i.e., odour) was introduced, but the correct and incorrect exemplars of the relevant dimension remained constant. The exemplars and the relevant dimension were unchanged for the reversal of this discrimination (Rev 1), but the previously correct exemplar was now incorrect, and vice versa. The intra-dimensional (ID) shift was then presented, comprising new exemplars of both the relevant and irrelevant dimensions, with the relevant dimension remaining the same as previously described. The ID discrimination was then reversed (Rev 2) so that the formerly positive exemplar became the negative one. For the extra-dimensional (ED) shift, a new pair of exemplars was again introduced; however, this time, the relevant dimension was also changed. Finally, the last phase was the reversal (Rev 3) of the ED discrimination.

The exemplars were always presented in pairs, and they varied so that only one animal within each treatment group received the same combination. Each exemplar's assignment in a pair as positive or negative at a given phase and the pots' left-right positioning in the test apparatus on each trial were randomized.

Supplement 2.

The effect of the oestrous cycle on females' performance.

Determination of the estrus cycle

Rat's oestrous cycle was divided into 4 phases: proestrus, estrus, metestrus and diestrus. The current stage of the cycle was determined by the results of vaginal cytology. Smear samples were collected 2 hours after the end of the test using sterile cotton-tipped swabs. The swabs were gently and shallowly inserted into the vaginal orifice (approximately 1 cm) to avoid overstimulation that could lead to pseudopregnancy. After gently rotating the swab against the vaginal wall (2-3 twists) to collect vaginal epithelial cells, the samples were prepared on glass slides. Using a light microscope with a 20× objective lens, the oestrous cycle stage was determined based on the appearance and abundance of cells within vaginal samples. When the sample examination showed a low concentration of leukocytes and a high concentration of cells of polygonal shape and small nuclei, the stage was identified as proestrus. Estrus was characterized by the presence of a large number of cells with enucleated and needle-like shapes or clumps of rounded cells with jagged edges. In metestrus, a combination of leukocytes and clusters of rounded, nucleated epithelial cells was observed. Diestrus was identified by a large number of leukocytes and a few small clumps of nucleated epithelial cells (Potasiewicz et al., 2020; Schneider & Popik, 2009).

The effect of the oestrous cycle on social behaviours in female rats

Data on separate behaviours were analyzed by a repeated measure ANOVA with cycle stage (metestrus - diestrus and proestrus - estrus) and genotype as between-subject factors and the type of behaviour as a repeated measure. There were no significant effects of the oestrous cycle and cycle × genotype or cycle × genotype × behaviour interactions (Fig. S.2.1, Table S.2.2). Tukey post hoc test conducted after a significant cycle × behaviour interaction revealed that more sniffing episodes were recorded during the proestrus – estrus phase (p<0.001).

The total number of episodes was subjected to a two-way ANOVA with the cycle stage and genotype as between-subject factors (Table S.2.2).

Fig. S.2.1. The effect of the oestrous cycle on social behaviour in TPH2-WT and TPH2-KO females.

Table S.2.1. Separate behaviors											
	df1 df2 F p										
Genotype (G)	1	48	26.935	< 0.001	0.359						
Cycle phase (C)	1	48	2.715	0.106	0.054						
G×C	1	48	0.202	0.655	0.004						
Behaviour (B)	2.33	111.68	388.605	< 0.001	0.890						
B×G	2.33	111.68	12.159	< 0.001	0.202						
B×C	2.33	111.68	6.106	0.0018	0.113						
B×C×G	2.33	111.68	0.513	0.6275	0.011						



Table S.2.2. Total number of SI episodes											
	df1 df2 F p η_{P^2}										
Genotype (G)	1	48	26.935	< 0.001	0.359						
Cycle phase (C)	1	48	2.715	0.106	0.054						
G×C	1	48	0.202	0.655	0.004						

The effect of the oestrous cycle on females' behaviour in the resident-intruder test

Data were analyzed using a repeated measure ANOVA with the cycle stage (metestrus - diestrus and proestrus - estrus) and genotype as between-subject factors and the type of behaviour (dominant vs. submissive) as a repeated measure. There were no significant effects of the oestrous cycle and cycle × genotype or cycle × genotype × behaviour interactions (Fig. S.2.2, Table S.2.3).



Fig.S.2.2. The effect of the oestrous cycle on females' behaviour in the resident-intruder test.

Table S.2.3. Resident - Intruder											
	df1	df2	F	р	\mathfrak{y}_{p^2}						
Genotype (G)	1	56	11.861	0.001	0.175						
Cycle phase (c)	1	56	0.444	0.508	0.008						
G×C	1	56	0.069	0.793	0.001						
Behaviour (B)	1	56	21.165	< 0.001	0.274						
B×G	1	56	2.468	0.122	0.042						
B×C	1	56	1.048	0.310	0.018						
B×C×G	1	56	2.034	0.159	0.035						

Effect of the oestrous cycle on marble burying activity of female rats.

The percentage of buried marbles (Fig. S.2.3a) and distance travelled (Fig. S.2.3b) were analyzed by a two-way ANOVA with the cycle stage and genotype as between-subject factors.

Fig. S.2.3.	Effect of the oe	strous cycle on ma	rble burying act	ivity in TPH2-V	VT and TPH2-KO females.
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Table S.2.4. marble-burying test											
	Burie	ed mar	Distanc	e traveled	l [m]						
	df1	f1 df2 F p η_{p^2} F p η_{p^2}									
Genotype (G)	1	35	3.344	0.0759	0.087	18.269	< 0.001	0.343			
Cycle (C)	1	35	0.438	0.5124	0.012	0.326	0.572	0.009			
G×C	1	35	0.773	0.3854	0.022	0.077	0.784	0.002			



Effect of the oestrous cycle on ASST performance in female rats

Data were analyzed by a repeated measure ANOVA with cycle stage (metestrus - diestrus and proestrus - estrus) and genotype as between-subject factors and the ASST phase as a repeated measure.

Fig.S.2.4. Effect of the oestrous cycle on ASST performance in TPH2-WT and TPH2-KO females

Table S.2.5. ASST											
Effect	1(1	1(2	TTC			Time/Trial					
Effect	an	a 12	F	p	$\mathfrak{g}_{p^{2}}$	F	р	\mathfrak{y}_{P}^{2}			
Genotype (G)	1	26	0.069	0.794	0.003	16.448	< 0.001	0.387			
Cycle phase (C)	1	26	0.014	0.907	0.001	0.386	0.539	0.015			
G×C	1	26	1.924	0.177	0.069	0.093	0.763	0.004			
ASST phase (P)	3.79	98.49	63.96	< 0.001	0.711	15.539	< 0.001	0.374			
P×G	3.79	98.49	0.731	0.566	0.027	1.329	0.247	0.049			
P×C	3.79	98.49	0.469	0.748	0.018	1.256	0.281	0.046			
P×C×G	3.79	98.49	1.432	0.231	0.052	0.558	0.764	0.021			



TPH2-KO





Details of the ANOVA analysis.

Table S.3.1. Social Interaction (SI) test											
Effect	df1	\mathfrak{y}_{P}^{2}									
Genotype (G)	1	55	17.411	< 0.001	0.240						
Sex (S)	1	55	0.192	0.663	0.003						
G×S	1	55	48.833	< 0.001	0.470						
Behavior (B)	2.75	151.37	607.529	< 0.001	0.917						
BxG	2.75	151.37	18.677	< 0.001	0.254						
B×S	2.75	151.37	21.624	< 0.001	0.282						
B×T×S	2.75	151.37	11.475	< 0.001	0.173						

Table S.3.2. SI: ultrasonic vocalizations																	
Effect df1	df1 df		1(1 1()		USV number			duration	duration			bandwidth			peak frequency		
Effect	an	a12	F	р	\mathfrak{y}_{p^2}	F	р	\mathfrak{y}_{p^2}	F	р	\mathfrak{y}_{p^2}	F	р	\mathfrak{n}_{p^2}			
Genotype (G)	1	55	0.352	0.555	0.006	1.890	0.175	0.033	17.658	< 0.001	0.243	36.612	< 0.001	0.399			
Sex (S)	1	55	20.852	< 0.001	0.275	5.744	0.019	0.094	100.903	< 0.001	0.647	26.357	< 0.001	0.323			
G×S	1	55	30.518	< 0.001	0.358	3.169	0.081	0.054	34.373	< 0.001	0.385	48.030	< 0.001	0.466			

Table S.3.3. Resident-Intruder (R-I) test											
Effect	df1	df2	F	р	\mathfrak{g}_{P}^{2}						
Genotype (G)	1	91	21.074	< 0.001	0.188						
Sex (S)	1	91	1.039	0.311	0.011						
G×S	1	91	0.111	0.739	0.0012						
Behavior (B)	1	91	51.011	< 0.001	0.359						
BxG	1	91	16.594	< 0.001	0.154						
B×S	1	91	3.345	0.071	0.305						
B×T×S	1	91	4.751	0.032	0.049						

Table S.3.4. R-I: ultrasonic vocalizations														
Effect df1	1(1	1()	USV nu	mber		duration			bandwidth			peak frequency		
Effect	an	a12	F	р	\mathfrak{n}_{P}^{2}	F	р	\mathfrak{y}_{p^2}	F	р	\mathfrak{n}_{P}^{2}	F	р	\mathfrak{y}_{p^2}
Genotype (G)	1	91	7.101	0.009	0.072	34.127	< 0.001	0.273	50.482	< 0.001	0.357	0.052	0.820	0.001
Sex (S)	1	91	5.668	0.019	0.059	18.338	< 0.001	0.168	45.549	< 0.001	0.334	6.335	0.014	0.065
G×S	1	91	1.347	0.249	0.015	21.904	< 0.001	0.194	12.484	< 0.001	0.121	0.947	0.333	0.011

Table S.3.5. marble burying test											
			Buried r	narbles	[n]	Distance traveled [m]					
	df1	$f1 df2 F p \eta_{P}^{2} F p \eta_{P}^{2}$									
Genotype (G)	1	66	7.426	0.008	0.101	45.278	< 0.001	0.407			
Sex (S)	1	66	2.498	0.119	0.036	5.378	0.023	0.075			
G×S	1	66	0.359	0.551	0.005	0.044	0.834	0.0001			

Table S.3.6. ASST												
Effect	TTC				Time/Tr	ial						
Effect	df1	df2	F	р	n _{p²}	df1	df2	F	р	ŋ₂²		
Genotype (G)	1	46	6.092	0.017	0.117	1	46	0.017	0.898	0.000		
Sex (S)	1	46	12.931	< 0.001	0.219	1	46	78.445	< 0.001	0.630		
G×S	1	46	4.609	0.037	0.091	1	46	27.667	< 0.001	0.376		
ASST phase (P)	2.6	119.71	91.408	< 0.001	0.665	4.6	211.63	6.987	< 0.001	0.132		
P×G	2.6	119.71	4.016	0.013	0.080	4.6	211.63	2.259	0.055	0.047		
P×S	2.6	119.71	3.745	0.017	0.075	4.6	211.63	8.899	< 0.001	0.162		
P×G×S	2.6	119.71	6.505	< 0.001	0.124	4.6	211.63	1.323	0.259	0.028		

USVs emission during the social interaction and resident-intruder tests.



Fig.S.4.1. 22-kHz (alarm) USV emission in TPH2-WT and TPH2-KO rats during the social interaction (a) and resident-intruder (b) tests. Data are presented as mean ± SEM of the total number of alarm calls. The figure includes only alarm-emitting rats (details in the result description).



Fig. S.4.2. The distribution of 50-kHz USV types emitted during the social interaction (a) and residentintruder (b) tests. Data are presented as the percentage distribution of call categories. Symbols: ****p<0.001, a significant difference between TPH2-WT and TPH2-KO animals in a given sex (planned comparisons).

Table S.4.1. SI: percentage USV distribution								
Effect	df1	df2	F	p	\mathfrak{y}_{p^2}			
call types (C)	1.83	100.38	128.519	< 0.001	0.700			
C×G	1.83	100.38	0.625	0.523	0.011			
C×S	1.83	100.38	72.852	< 0.001	0.569			
C×G×S	1.83	100.38	1.375	0.257	0.024			

Table S.4.2. R-I: percentage USV distribution								
Effect	df1	df2	F	р	$\mathfrak{y}_{p^{2}}$			
call types (C)	1.65	149.83	240.949	< 0.001	0.726			
C×G	1.65	149.83	20.999	< 0.001	0.187			
C×S	1.65	149.83	13.653	< 0.001	0.130			
C×G×S	1.65	149.83	8.004	0.001	0.081			

Table.S.5.1

			RESULTS	
			Male TPK2 KO	Female TPH2 KO
		Social Interaction Test		
Analysed behaviours	Sniffing	the rat sniffs the body of the conspecific	-	\downarrow
	Anogenital sniffing	the rat sniffs the conspecific's anogenital region	-	\downarrow
	Social grooming	the rat licks and chews the conspecific's fur	-	-
	Climbing	the rat climbs over or stands on the conspecific's back	\downarrow	\downarrow
	Following	the rat follows the conspecific	\downarrow	\downarrow
	Aggressive behaviours	fighting	\uparrow	_
'	Copulatory-like behaviours		\uparrow	-
		Resident-Intruder Test		
Analysed behaviours	Dominant behaviours	lateral threatening		÷
		sideway lateral pushing		
		clinch attacking		
		chasing	•	
		standing on top of the supine intruder	I	
		aggressive grooming		
		boxing		
		biting		
	Submissive behaviours	supine postures		
		fleeing behaviour		•
		defensive upright posture	-	
		freezing		

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