Gender-specific behavioral features of juvenile and adult haploinsufficient *Scn2a*$^{+/-}$ female mice, model of Autism Spectrum Disorder.

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Abstract.

Variants of the *SCN2A* gene, encoding the $Na_V1.2$ sodium channel, cause a spectrum of neurodevelopmental and epileptic disorders, and are among those that show the strongest association with Autism Spectrum Disorder (ASD). ASD has a male-bias prevalence, but several studies have proposed that female prevalence may be underestimated due to different symptomatic expression compared with males. However, it is unclear whether this is related to actual different pathological features or to greater masking abilities in females.

Studies on *Scn2a*$^{+/-}$ mice, a model of *SCN2A* haploinsufficiency and ASD, have shown an age-dependent ASD-like phenotype attenuated at adulthood in males. However, little is known about the behavioral features of *Scn2a*$^{+/-}$ female mice. We performed a battery of behavioral tests that are relevant for assessing ASD-like features, investigating juvenile and adult *Scn2a*$^{+/-}$ female mice.

Our results demonstrate that female *Scn2a*$^{+/-}$ mice exhibit an overall milder phenotype than males, showing increased sociability and increased risk taking in juveniles, hyper-reactivity to cold stimuli in adults, altered decision-making related behaviors in both. Thus, this is consistent with the male-bias prevalence of ASD and the existence of different ASD phenotypic features in males and females. Both genders should be investigated in studies of mouse models of ASD.

Introduction.

Autism Spectrum Disorder (ASD) is a group of neurodevelopmental dysfunctions with onset in early childhood, characterized by social interaction and communication deficits, restricted and repetitive behaviors, reduced interests, sensory abnormalities and cognitive alterations[1–3]. The refinement of diagnostic criteria and the early assessment of patients have led, over the last few decades, to a significant increase in the number of cases identified, with ASD affecting 1 in 44 children in the United States, across all ethnic and socio-economic groups[4]. Notably, ASD shows a male-oriented imbalance, with a male/female ratio between 3 and 4 in children[3,5,6]. However, it has been shown that women may express ASD symptoms differently than men[7,8]. It has been proposed that this differential manifestation of ASD symptoms in females makes their initial identification more difficult, leading to a delayed or incorrect diagnosis and an underestimation of ASD cases in females[3,9–11]. However, it's unclear whether this gender difference in ASD phenotype is related to actual differences in pathological features and whether it has actual biological causes. In fact, ASD masking behaviors (mimicry, mirroring of peer behavior, camouflage, etc.) have been shown to be more prominent in females and could attenuate/modulate the symptoms[9,12–15], which nonetheless may be similar to those of males in their basic features.

Although detailed pathological mechanisms of ASD are not clear yet, both genetic and environmental factors play a role in ASD etiology and it has been proposed that an imbalance between excitation and inhibition in brain circuits may be involved[2,16,17]. Among the genetic causes of ASD, a relevant target is the *SCN2A* gene, encoding the $Na_v1.2$ voltage-dependent sodium channel. *SCN2A* genetic variants cause a wide spectrum of neurodevelopmental and epileptic disorders, and they are among the variants that show the strongest association with ASD[18–20]. It has been proposed that *SCN2A* variants causing ASD lead to $Na_v1.2$ loss of function (LoF) and haploinsufficiency[18,20,21]. *Scn2a* heterozygous knock-out (*Scn2a*$^{+/-}$) mice[22], which model *SCN2A* haploinsufficiency, show an age-dependent ASD phenotype attenuated in adulthood, associated with mild absence-like seizures and memory impairments[23–26]. Some of the phenotypic features observed in *Scn2a*$^{+/-}$ mice are also present in other mouse models of *Scn2a* haploinsufficiency and autism[27–29]. Interestingly, increased sociability and reduced anxiety-like behavior are the most recurrent behavioral features observed in *Scn2a* mouse models[26,28–30]. Additionally, different types of stereotyped repetitive behaviors have been reported[23,26,28,31].

Historically, behavioral studies were performed using mainly male rodents to avoid interferences with estrus cycle and female hormonal modulations occurring during the tests. Also with regard to *Scn2a* mouse models, only a few studies have evaluated some behavioral features of females, without carrying out extensive characterizations[27–29,31]. Here, we performed on female *Scn2a*$^{+/-}$ mice a battery of behavioral tests relevant to the assessment of ASD-like features. To evaluate the developmental

70 characteristics of the phenotype, we studied *Scn2a*$^{+/-}$ and wild type (WT) littermate female mice in the
71 juvenile (post-natal day, P, P21-44) and the adult (>P60) period, and compared these results with those
72 that we already obtained with *Scn2a*$^{+/-}$ male mice[23].

Material and methods

Animals.

The generation and the genotyping of *Scn2a*$^{+/-}$ mice have been described by Planells-Cases et al.[22]. The original heterozygous breeders were provided by Dr. E. Glasscok of Louisiana State University Health Sciences Center (Shreveport). Heterozygous (HTZ) *Scn2a*$^{+/-}$ and control *Scn2a*$^{+/+}$ littermate mice were generated from crosses between HTZ males and wild-type (WT) females on a C57Bl6/J background (Charles River, France). All mice were housed in a controlled environment (22–24°C, 40–50% humidity) under a 12h dark/light cycle with access to food and water ad libitum. After weaning (post-natal days (P) 21–23), mice were group-housed with same sex littermates of mixed genotype (n=3–5/cage) in standard polycarbonate cages containing plastic houses, wood sticks and nesting material. In this study we performed an extensive behavioral characterization of female mice and selected tests on male mice. Behavioral tests were performed sequentially in juvenile (between P21 and P44, corresponding to the pre-pubescent, P21–34, and pubescent, P35–44, periods of adolescence [32,33] and adult (between P60 and P95) female mice. For all experiments, the animals were habituated to the testing room at least 30 min before the beginning of the tests. The animals were submitted to the behavioral tasks in the following order: Elevated Plus Maze (EPM), openfield, 3-chamber social interaction, Y-maze, grooming, olfaction, marble burying, novel object recognition, rotarod, hot and cold plate. All the tests were conducted on the same mice, from juvenile to adulthood stages, except the hot and cold plate tests that have been performed on distinct groups of mice to avoid cross influence of potential skin damages induced by the first plate tests. A total of 5 litters have been used to assess the behavioral phenotype of female *Scn2a*$^{+/-}$ mice (for a total of 21 WT and 23 HTZ mice) and 2 litters for testing male *Scn2a*$^{+/-}$ mice (for a total of 27 WT and 28 HTZ). Mice were randomized (https://www.randomizer.org/); experiments and analysis were performed blind to genotype by two experimenters (W.M. and M.S.). All procedures were performed in accordance with the European Council Directive (2010/63/EU) and approved by the local ethics committee (Comité Institutionnel d'Éthique Pour l'Animal de Laboratoire (CIEPAL)-Azur, https://ciepal-azur.unice.fr/) and the French Ministry of Research (http://www.enseignementsup-recherche.gouv.fr/, APAFIS#2023020813272228).

Open field test.

The subject mouse was allowed to explore a white wooden box (40x40x40cm) for 30 min under anxiogenic lighting (300 Lux). The apparatus is virtually divided in 3 zones: periphery (5 cm to the edges), center (20x20cm) and intermediate zone. Distance travelled and time spent in periphery and center zones was assessed by automatic tracking the animals with the ANY-maze software (Stoelting, Europe, Dublin, Ireland). To evaluate anxiety-like behavior, percent of time spent in the center versus

107 the periphery was calculated. Entries into a zone were automatically measured, considering an entry
108 when 90% of the body was in the zone and an exit when at least 50% was out.

109 **3-chamber sociability test.**

110 The 3-chamber test is used to assess sociability and social memory in mice. It was performed in both
111 juvenile (P23-28) and adult (>P70) females under low light (30 Lux). The apparatus is a grey rectangle
112 plastic box divided in three zones of equal size (30x35x35cm) separated by transparent walls with 2
113 openings. The two lateral chambers contain a pierced (ø10mm holes) transparent cylinder (8cm
114 diameter) which allows physical contacts between mice. This test consists of 3 phases of 10 minutes:
115 Habituation, Sociability and Social Memory[34,35]. Briefly, the subject mouse was initially allowed to
116 freely explore the apparatus for 10 min during the habituation phase. Then, it was placed in the center
117 zone while a stranger mouse (named "unfamiliar", S1) was placed in one of the two empty cylinders
118 (in the side less preferred in the habituation phase) and allowed to explore again for 10min. In the
119 third phase, the tested mouse was placed again in the center zone and a second new unfamiliar mouse
120 was added in the other cylinder (named "unfamiliar", S2, whereas the previous one is considered in
121 this phase as "familiar"); the tested mouse was then allowed to explore for 10 more minutes. Tracking
122 of movements and assessment of the time spent in each chamber was done with ANY-maze. Close
123 interactions with empty cylinders, unfamiliar or familiar mice (time spent sniffing closely) were
124 manually assessed and the total time in close interaction calculated (Unfamiliar + Empty, T_{S1+E} or
125 Familiar + Unfamiliar, T_{S1+S2}). Social Discrimination Index (SDI) and Social Memory Index (SMI) were
126 calculated as: SDI= $(T_{S1}-T_E)/T_{S1+E}$; SMI=$(T_{S2}-T_{S1})/T_{S1+S2}$. A positive index is a sign of social preference or
127 valid social memory, as the tested mouse spent more time with the social or unfamiliar stimulus,
128 respectively. Negative index indicates aversive behavior towards the social stimulus. A score of 0
129 indicates no preference or no memory of the previously encountered stimulus mouse. Entry into a
130 zone was automatically calculated if 90% of the body was in the zone and an exit if at least 50% was
131 outside.

132 **Olfactory discrimination.**

133 Olfactory discrimination between social and non-social odors, which could interfere with the social
134 behaviors measured in mice, was assessed as previously described with minor modifications[23,36].
135 Squares of filter paper (5×5 cm) were dampened with water (neutral control) or coffee (non-social
136 aversive odor) (approximately 0.5ml). Social odors (attractive) were obtained placing filter paper
137 squares for 3 days in cages housing non familiar juvenile or adult females. Mice were placed in clean
138 plexiglas cages without bedding for 10 min of habituation. Then, filter papers were introduced
139 sequentially (water, coffee, social odor) for 3min each, spaced by 1min interval. Olfactory preference

140 was assessed as the difference between the time spent interacting with (sniffing) social or non-social
141 odors and that spent interacting with (sniffing) the paper dampened with water.

142 **Rotarod.**

143 Motor coordination, balance and motor learning were evaluated using an accelerating rotarod (Bioseb-
144 *In vivo* Research Instruments, Vitrolles, France)[37]. Mice were habituated to the procedure before the
145 test phase: each mouse was trained until able to stay 1min on the circular rod at a constant speed of
146 4rpm. Mice were then placed on the rod that accelerated from 4 to 40 rpm over 5min. Mice were
147 tested with three trials a day (inter-trial interval of 30 min) for 2 consecutive days. Fall latency from
148 the rotating rod was automatically recorded; mice that fell before 15 seconds were put back on the
149 rod and retested.

150 **Marble burying test.**

151 We used the marble burying (MB) test to evaluate motor stereotypies and repetitive behaviors[38]. The
152 apparatus consists of a transparent plastic arena (30×30×30cm) filled with 5 cm of clean bedding
153 (poplar wood chips, average particle size 3.5–4.5 mm; Lignocel Select, SAFE, France) on which 20
154 marbles are arranged (5x4 rows). The tested mouse was allowed to explore the apparatus for 20 min
155 and the number of marbles buried every 5 min was manually scored by the experimenter. A marble
156 was considered buried if more than 50% was buried in the bedding, not counting again the same
157 marble if buried several times.

158 **Grooming.**

159 We performed the self-grooming test in an open field (40x40x40 cm) arena to further study repetitive
160 behaviors. The mouse is placed in the open field in low lighting (30 Lux – non anxiogenic) for 20 min.
161 After 10 min of habituation, the time spent spontaneously self-grooming was measured manually.

162 **Elevated Plus Maze.**

163 We used the Elevated Plus Maze (EPM) test to investigate anxiety-like behavior. The apparatus consists
164 of a grey plastic "plus-shaped" cross (25×5×2.5 cm) elevated 1 meter above the ground, with 2 open
165 arms (anxiogenic zone) and 2 enclosed arms (non-anxiogenic zone), separated by a small center zone
166 (neutral zone). The tested mouse was allowed to freely explore the maze for 5 min. Normally mice
167 spend more time in non-anxiogenic zones than in the anxiogenic zones, and modifications of anxiety
168 levels can modulate this parameter. We scored automatically with the ANY-maze tracking software
169 distance, speed, time spent and number of entries in each zone, considering 90% and 50% of the body
170 for entry and exit parameters, respectively.

171 **Hot and Cold Plate tests.**

172 Thermal sensory processing was evaluated by the hot and cold static plate tests in distinct cohorts. The
173 experimental protocol was as in (Corder et al. 2017; Eaton et al. 2021), with little modifications. Briefly,
174 the tested mouse was placed on a 30°c plate (Bioseb-*In vivo* Research Instruments, Vitrolles, France)
175 for a 5 min habituation phase. After 30 min, the mouse was placed back on the plate, which was heated
176 to 52°C or cooled to 7.5 °C, the latency to the first sign of discomfort was manually scored and the
177 mouse removed from the plate. Signs of discomfort taken into account were hind paw lifting off the
178 plate (>2 sec), shaking, licking, or jumping on the plate. If no sign of discomfort was detected after a
179 cut-off time of 45s (hot plate) or 1 min (cold plate), the mouse was removed from the device to avoid
180 skin injuries. The analysis was performed on video recordings from the top.

181 **Novel Object Recognition Test (NORT).**

182 This memory test based on the spontaneous tendency of rodents to explore novelty has been
183 performed as previously described[39] in an open-field apparatus slightly illuminated (30 lux). During the
184 phase of habituation (Day 1), mice were allowed to freely explore the arena for 10min. On the
185 experimental day (Day 2), mice were submitted to two trials with an inter-trial interval of 1hour. During
186 the first trial (acquisition phase), the tested mouse was placed in the open-field in the presence of two
187 identical objects (wavy transparent glass square saltshakers) and allowed to freely explore for 10min.
188 During the second trial (retention phase), one of the objects was replaced by a novel object (opaque
189 smooth black round ceramic cup) and the mouse was allowed to explore both objects (novel and
190 familiar) for 5min. Mice that did not explore the objects for a minimum of 15s were excluded (juveniles:
191 3WT and 1 HTZ; adults: 3WT and 4HTZ). The pairs of objects used were different in shape, color and
192 texture, but had similar sizes. The two objects were placed symmetrically in the center of the arena
193 about 6 cm away from the wall. The location of the novel object was counterbalanced across animals
194 (right or left side). Recognition memory was evaluated using a discrimination index (DI), quantified as
195 the difference between the time spent exploring the novel object and the familiar object, divided by
196 the total time of exploration of both objects and expressed as percentage.

197 **Y maze test.**

198 Spatial working memory was assessed quantifying the spontaneous alterations in a symmetrical grey
199 plastic Y maze (30×6×15cm), in which the tested mouse was placed at the end of one arm and allowed
200 to freely explore the maze for 8min[40,41]. The sequence and the number of arm entries were
201 automatically quantified with the ANY-maze software, considering 99% of the body for entry and 50%
202 for exit. An alternation was defined as consecutive entries into the three different arms (A, B or C) on
203 overlapping triplet sets (ex: ABCABBACAB=5 alternations). The spontaneous alternation score (%) was
204 calculated as the ratio of actual (total alternations) to possible (total arm entries-2) alternations,
205 expressed as percentage. The percentage of time spent in the center zone while moving between two

206 arms (considered as a decision-making parameter) and the total distance travelled were also
207 automatically evaluated.

208 **Statistical analysis.**

209 Data were analyzed for normality (Shapiro-Wilk test) and equal variances using GraphPad Prism 10.0.3
210 (GraphPad Software, San Diego, California USA). When data were normally distributed, comparisons
211 between genotypes in young or adult mice were evaluated using unpaired Student's t-test or Welch's
212 t-test (when variances were different); the Mann-Whitney test was used for non-normally distributed
213 data. When appropriate, a two-way (repeated) measures ANOVA followed by Sidak's multiple
214 comparison post-hoc test was used. One sample Wilcoxon t-tests were used to compare the
215 discrimination index with a 0% chance level in the novel object recognition test and the 3-chamber
216 sociability test. The results are displayed as mean±S.E.M. We considered the differences as statistically
217 significant when $p \leq 0.05$, * indicates a significant difference between genotypes and # indicates a
218 significant difference in comparisons within subjects (olfaction test) or with chance level. We
219 considered borderline significance (strong trend) when p-values were between 0.05 and 0.09. plots
220 were generated with GraphPad Prism 10.0.3 or Excel.
221

222 **Results.**

223 **Female *Scn2a*$^{+/-}$ mice show normal locomotor activity and no anxiety-like behavior in the openfield.**
224 We initially assessed locomotor activity because it is an important feature that can influence the
225 outcome of numerous behavioral tests. We quantified it in the highly illuminated arena of the open
226 field apparatus during 30min-long sessions, considering the whole arena, the periphery or the center
227 (Figure 1A). The locomotor activity showed no difference between juvenile female *Scn2a*$^{+/-}$ (HTZ) mice
228 and WT female littermates (Figure 1B), in the whole arena (66.9±2.4m distance traveled for HTZ,
229 67.3±5.1m for WT; Mann-Whitney test: U=201, p=0.3509, NS), the periphery (30.3±2.7m HTZ;
230 31.2±1.2m WT; Mann-Whitney test U= 183, p=0.17, NS) or the center (11.5±1.1m HTZ; 11.1±0.8m WT;
231 Student's t-test Center: t(42)=0,3447, p= 0,73, NS). We obtained similar results for adult females
232 (Figure 1D) in the whole arena (58.4±3.9m HTZ, 63.4±4.7m WT, Student's t test: t(36)=0,8240, p=0.41,
233 NS), the periphery (29.7±1.8m HTZ; 32.3±2.6m WT, Student's t test: t(36)=0,8683, p=0.40, NS) or the
234 center (7.3±0.7m HTZ; 8.0±0.7m WT; Student's t test, t(36)=0.6330, p=0.53, NS). Moreover, we
235 evaluated the anxiety behavior by analyzing the percentage of time spent in the center zone of the
236 open field arena (Figure 1C-E), which showed no difference, neither for juvenile females (13.9m3±1.9%
237 HTZ, 12.9m1±1.6% WT; Mann-Whitney test: U=239.5, p=0,97, NS) nor for adult females (6.3±1.0% HTZ,
238 7.4±0.8% WT, Student's t test: t(36)=0,8176, p=0.4190, NS). Therefore, *Scn2a*$^{+/-}$ female mice show
239 normal exploratory and locomotor activity and no anxiety-like behavior across development.

240

241 ***Scn2a*$^{+/-}$ female mice show abnormal sociability but no alteration of social memory in the 3-chamber**
242 **test.**

243 Abnormality in social interactions is a major clinical feature in ASD. The 3-chamber test (three
244 connected chambers, in which transparent holed cylinders are placed in the two opposite side
245 chambers) is used to evaluate sociability in rodents, exploiting their natural preference for social
246 stimuli and social novelty. Sociability and social memory are assessed by the time spent in close
247 interaction with the two cylinders (empty vs. unfamiliar mouse or familiar mouse vs. unfamiliar mouse,
248 respectively) (Figure 2A-B). Sociability and social memory indexes are calculated by subtracting the
249 time spent in interaction with the empty cylinder or the familiar mouse cylinder, respectively, to the
250 time spent in interaction with the unfamiliar mouse cylinder, and by dividing it by the total time spent
251 in close interaction with both cylinders. We tested juvenile and adult females and settled as exclusion
252 criterion a minimum of 40-second of total interaction with both cylinders. Two WT and one HTZ
253 juvenile mice and one WT adult mouse were excluded from the analysis because of this criterion.
254 For juvenile females, we observed a significant increase in the time spent in close interaction with the
255 stimulus mouse compared to the empty cylinder for both genotypes in the sociability phase, but the
256 increase was higher for HTZ mice (33.3±4.1s WT empty, 80.0±7.5s WT with unfamiliar, 39.6±4.2s HTZ

257 with empty, 136.5±14.2s HTZ with unfamiliar; two-way ANOVA, F (1, 52) = 53,20, P<0,0001 for stimulus
258 effect, F(1.52)=10.16, p=0.0024 for genotype effect and F(1.52)=6.478, p=0.0139 for genotype x
259 stimulus interaction; Sidak's post-hoc test: WT empty vs unfamiliar p=0.018, HTZ empty vs unfamiliar
260 p<0.0001; WT unfamiliar vs HTZ unfamiliar p=0.0009) (Figure 2C, left). Consistently, the sociability
261 index was significantly higher than chance (index = 0) level for both genotypes (0.58±0.04 HTZ
262 0.41±0.05 WT One-sample t-test, t(10)=8.132, p<0.0001, for WT, t(15)=14.79, p<0.0001 for HTZ), but
263 higher for HTZ females compared to controls (Student's t test, t(25)=2.527, p=0.018) (Figure 2C, right).
264 In the social memory phase, both HTZ and WT juvenile female mice exhibited a social preference for
265 the unfamiliar mouse (cylinder effect) and, differently than for the sociability phase, there was no
266 significant difference between genotypes in the time spent in close interaction with the unfamiliar
267 mouse (98.9±10.9s HTZ with unfamiliar; 41.1±6.1s HTZ with familiar ; 87.6±13.7s WT unfamiliar ;
268 32.2±3.8s WT familiar; two-way ANOVA, F(1.52)=34.54, p<0.0001 for cylinder effect, F(1.52)=1.107,
269 p=0.2976 for genotype effect, NS and F(1.52)=0.01615, p=0.9 for genotype x cylinder interaction)
270 (Figure 2D, left). Consistently, the social memory index was different from to chance level for both
271 groups (0.42±0.06 HTZ; 0.42±0.07 WT; one-sample t-test: t(10)=5.918, p=0.0001 for WT compared to
272 chance level, t(16)=6.779, p<0.0001 for HTZ compared to chance level), but not significantly different
273 between HTZ and controls (Mann-Whitney, U=88.50, p=0.8262) (Figure 2D, right).
274 The results that we obtained for the sociability phase of adult females are similar to those of juvenile
275 females. We observed a significant increase in the time spent in close interaction with the stimulus
276 mouse compared to the empty cylinder for both genotypes, but the increase was higher for HTZ mice
277 (42.2±4.3s HTZ with empty; 110.8±8.4s HTZ with unfamiliar; 42.5±3.7s WT with empty; 76.8±4.8s WT
278 with unfamiliar; two-way ANOVA, F(1.44)=84.78, p<0.0001 for stimulus effect, F(1.44)=9.031,
279 p=0.0044 for genotype effect and F(1.44)=9.420, p=0.0037 for genotype x stimulus interaction; Sidak's
280 post-hoc test: WT empty vs unfamiliar p=0.0005, HTZ empty vs unfamiliar p<0.0001 WT unfamiliar vs
281 HTZ unfamiliar p=0.0006) (Figure 2E, left). The sociability index was significantly higher than chance
282 level for both genotypes (0.44±0.05 HTZ; 0.29±0.06 WT; one-sample t-test t(11)=5.163, p<0.0003 for
283 WT, t(11)=8.939, p<0.0001 for HTZ), but higher for HTZ females compared to controls (Student's t test:
284 t(22)=2,115, p=0.0460) (Figure 2E, right). The multiple comparison of the time spent in close
285 interaction during the social memory phase showed a significant preference for the new unfamiliar
286 mouse compared to the familiar one in HTZ adult females, whereas WT females showed a trend toward
287 preference for the new unfamiliar mouse (100.1±10.0s HTZ with unfamiliar; 60.1±6.6s HTZ with
288 familiar; 62.3±5.2s WT with unfamiliar; 41.5±3.5s WT with familiar; two-way ANOVA, F(1.44)=20.19,
289 p<0.0001 for cylinder effect, F(1.44)=17.47, p=0.0001 for genotype effect and F(1.44)=2.016, p=0.1627
290 for genotype x cylinder interaction; Sidak's post-hoc test: WT familiar vs unfamiliar p=0.19, HTZ familiar
291 vs unfamiliar p=0.0008, WT unfamiliar vs HTZ unfamiliar p=0.0016) (Figure 2F, left). Notably, the social

292 memory index was significantly different than the chance level for both groups (0.24±0.06 HTZ;
293 0.20±0.04 WT; one-sample t-test t(11)=4.651, p=0.0007 for WT, t(11)=3.781, p=0.003 for HTZ) and
294 there was no significant difference between the index of WT and HTZ females (Student's t-test,
295 t(22)=0.5906, p=0.56) (Figure 2E, right). Consistent with the trend observed in the multiple comparison
296 test (Figure 2F, left), the comparison of the interaction time of WT mice (familiar vs new unfamiliar
297 mouse) using the paired Student's t-test showed a significant preference for the new unfamiliar
298 mouse, as expected for WT mice (t(22)=3.288, p=0.0034).
299 Therefore, both juvenile and adult female *Scn2a*$^{+/-}$ mice show hyper-sociability, but no alteration in
300 social memory.
301
302 **Olfactory discrimination is functional in *Scn2a*$^{+/-}$ female mice at both ages.**
303 We assessed olfactory preference and social odor discrimination of *Scn2a*$^{+/-}$ female mice, which may
304 be involved in the differences that we observed in sociability. Notably, $Na_V1.2$ is expressed also in
305 olfactory granule cells and it has been proposed that it could drive rapid-odor discrimination[42]. Thus,
306 we tested the ability of females to discriminate aversive (coffee) and attractive (social) odors, using
307 the same protocol that we previously used for *Scn2a*$^{+/-}$ male mice (quantified as the difference between
308 the time mice spent interacting with social or non-social odor cues and that spent interacting with
309 paper dampened with water)[23] (Figure 3A).
310 The analysis of the data revealed a significant difference in exploration duration of social vs coffee cues
311 but no inter-genotype effect (two-way ANOVA, juvenile: 58.24±8.6s HTZ social; -3.12±0.8s HTZ coffee;
312 46.24±1.0s WT social; -3.90±1.0s WT coffee; F(1.50)=141.6, <0.0001 for odor effect, F(1.50)=1.845,
313 p=0.1805 for genotype effect, NS and F(1.50)=1.438, p=0.2361 genotype x odor interaction, NS, Figure
314 3B ; adult: 23.6±3.0s HTZ social; -2.8±0.9s HTZ coffee; 21.7±2.9s WT social; -3.2±2.3s WT
315 coffee; F(1.48)=114.4, p<0.0001 for odor effect, F(1.48)=0.2223, p=0.64 for genotype effect and
316 F(1.48)=0.1032, p=0.75 for genotype x odor interaction, Figure 3D). Post-hoc analysis demonstrated
317 that the comparison between coffee and social cues are significantly different for genotype at both
318 ages (Sidak's multiple comparison, juvenile (both genotypes): p<0.0001 and adult (both genotypes):
319 p<0.0001, shown as # in the figure). The total time exploring all cues (water + coffee + social) was used
320 as a marker of interest and exploration and showed no statistical difference between genotypes at
321 both ages (juvenile: 75.6±8.4s HTZ; 67.6±5.1s WT; Mann-Whitney, U=79, p=0.58, Figure 3C; adult:
322 49.6±4.9s HTZ; 57.0±6.2s WT; Student's t-test, t(24)=0.9536, p=0.35, Figure 3E).
323 Thus, we observed that both juvenile and adult female mice of both genotypes are able to perform
324 olfactory discrimination equally well. These results show that olfactory discrimination is functional in
325 *Scn2a*$^{+/-}$ female mice and therefore is not involved in the altered sociability.
326

327 ***Scn2a*$^{+/-}$ females do not show clear motor stereotypies, although juvenile females perform better on**
328 **an acquired Rotarod task.**
329 Motor stereotypies associated with restricted interests are among the main diagnostic characteristics
330 in ASD. To investigate these features in *Scn2a*$^{+/-}$ female mice, we quantified innate (self-grooming,
331 marble burying) and acquired (Rotarod) motor behaviors (Figure 4A).
332 Neither juvenile nor adult HTZ females showed alterations in the amount of time spent self-grooming
333 (juvenile: 37.3±6.1s HTZ; 46.1±11.0s WT; Mann-Whitney, U=200, p>0.9999, NS, Figure 4B ; adult:
334 28.2±3.7s HTZ; 24.8±3.6s WT; Student's t-test: t(23)=0.6509, p=0.52, NS, Figure 4E) or in the number
335 of marbles buried (juvenile: 4.9±.1.1 HTZ; 2.6±0.6 WT; Mann-Whitney, U=107.5, p=0,1330, NS,
336 Figure 4C ; adult: 7.2±1.2 HTZ; 5.2±1.3 WT; Student's t-test: t(26)=1.127, p=0.27, NS, Figure 4F). These
337 results indicate that *Scn2a*$^{+/-}$ female mice do not display deficits in these innate motor behaviors.
338 A more pronounced amelioration in performance and faster motor learning in the rotarod test
339 compared to control mice are features considered as stereotypic motor routines in ASD mouse
340 models[43]. To evaluate the motor behavior acquired during the rotarod test, we measured the latency
341 before falling off the rotating and accelerating rod of the apparatus in two daily three-trial sessions.
342 We initially compared the two genotypes including all the trials, and we observed a strong trend
343 towards increased latency in juvenile *Scn2a*$^{+/-}$ female mice (two-way repeated measure ANOVA on
344 both days, F(4.177, 154.5)=8.796, p<0.0001 for trial effect, F(1.37)=3.895, p=0.056 for genotype effect
345 and F(5.185)=0.4196, p=0.83 for genotype x trial interaction), but no difference in adult mice (two-way
346 repeated measure ANOVA on both days, F(3.460, 86.51)=4.052, p=0.0068 for trial effect,
347 F(1.25)=0.9008, p=0.35 for genotype effect and F(5.125)=0.51, p=0.7693 for genotype trial
348 interaction). Thus, we performed comparisons for juvenile mice by considering the two days
349 separately. We found a significant genotype effect for day 1 (two-way repeated measure ANOVA on
350 Day 1, F(1.760, 65.12)=10.31 p=0.0002 for trial effect, F(1.37)=5.378, p=0.026 for genotype effect and
351 F(2.74)=0.4599, p=0.6331 for genotype x trial interaction, Figure 4D) but not for day 2 (two-way
352 repeated measure ANOVA on Day 2, F(1.982, 73.32)=0.7268, p=0.48, NS for trial effect, F(1.37)=1.860,
353 p=0.1809, NS for genotype effect and F(2.74)=0.3749, p=0.69, NS for genotype x trial interaction,
354 Figure 4G). These results show that juvenile *Scn2a*$^{+/-}$ female mice can perform better on the rotarod
355 than their WT littermates before training, although after training the difference was attenuated. Thus,
356 there were no clear motor stereotypies.
357
358 **Sensitivity to cold, but not hot, stimuli is increased in both juvenile and adult male and in adult**
359 **female *Scn2a*$^{+/-}$ mice.**
360 ASD patients often show hyper- or hypo-reactivity to sensory (thermal, auditory, visual or tactile)
361 stimuli. Moreover, a recent work has shown that a gene-trap mouse model bearing ~75% reduction of

362 *Scn2a* expression has an abnormal reactivity to extreme temperatures[27]. We did not investigate these
363 features in our previous study on male *Scn2a*$^{+/-}$ mice[23]. Therefore, we evaluated here the reactivity to
364 thermal stimuli in juvenile and adult *Scn2a*$^{+/-}$ mice of both sexes, performing the hot (52°c) and cold
365 (7.5°c) plate tests (Figure5A & F). We considered withdrawal (shaking, licking, or lifting) of the hind
366 paws or escaping as markers of thermal discomfort[44], and selected a cut-off time of 45s for the hot
367 plate and 60s for the cold plate tests to avoid skin lesions.
368 In the hotplate test, we observed no differences in the latency of reaction to the thermal stimulus
369 between HTZ and WT littermates at both ages, neither in females (juvenile 7.5±0.6s HTZ; 7.8±0.5s WT;
370 Student's t-test, t(25)=0.4541, p=0.65, NS ; adult 7.9±0.8s HTZ; 8.7±0.8s WT: Student's t-test,
371 t(25)=0.7302, p=0.47, NS, Figure5B & C) nor in males (juvenile 7.7±0.5s HTZ; 7.2±0.8s WT; Student's t-
372 test: t(25)=0.5433, p=0.59, NS; adult 6.0±0.7s HTZ; 5.3±0.4s WT; Student's t-test: t(25)=0.8448, p=0.41,
373 NS, Figure5D & E).
374 The cold plate test revealed a significant difference between genotypes for males at both ages and in
375 adult females. In fact, adult HTZ females showed about 20% reduction in the latency to the first sign
376 of discomfort compared to WT littermates (juvenile 59.5±0.5s HTZ, 60s WT: one-sample Wilcoxon test
377 for HTZ because all WT reached the cut-off time, 60s, p>0.99, NS; adult 45.4±3.7s HTZ; 55.2±2.8s WT;
378 Mann-Whitney, U=69, p=0.0364 Figure5G & H). Both juvenile and adult HTZ males showed around 50%
379 reduction in the latency to the first sign of discomfort compared to their WT littermates (juvenile
380 28.7±3.8s HTZ; 55.4±3.1s WT; Mann-Whitney: U=16, p<0.0001; adult 36.2±5.7s HTZ; 59.1±0.5s WT;
381 Mann-Whitney: U=37.5, p=0.014, Figure5I & J).
382 Thus, male and female *Scn2a*$^{+/-}$ mice had normal heat sensitivity at both juvenile and adult ages,
383 whereas male *Scn2a*$^{+/-}$ mice at both ages and adult *Scn2a*$^{+/-}$ female mice show hyper-reactivity to cold
384 stimuli.
385
386 **_Scn2a_$^{+/-}$ female mice display normal recognition memory but altered spatial working memory and**
387 **decision-making.**
388 Cognitive defects are frequently present in ASD patients and mouse models, including memory deficits.
389 We assessed recognition memory with the novel object recognition test (NORT) and spatial working
390 memory with the Y maze test, as these tests often disclose cognitive dysfunctions in ASD mouse models
391 (Figure 6A-D).
392 We found no significant differences in the discrimination index of the NORT for both juvenile
393 (0.43±0.05 HTZ; 0.30±0.05 WT; Student's t-test: t(22)=1.825, p=0.825, NS, Figure 6B) and adult females
394 (0.29±0.08 HTZ; 0.22±0.05 WT; Student's t-test: t(15)=0.7086, p=0.4894, Figure 6C), which is consistent
395 with normal recognition memory.

396 For the Y maze test, we first investigated the parameters that are usually used to quantify locomotor
397 behavior in this test. Both juvenile and adult HTZ females showed a decrease in the total distance
398 traveled (juvenile: 15.1±1.3m HTZ; 21.5±1.6m WT; t(26)=3.081, Student's t-test: p=0.0048, Figure 6E;
399 adult: 11.5±0.8m HTZ; 19.2±1.3m WT; Student's t-test: t(34)=5.210, p<0.0001, Figure 6I) and a
400 decrease in the total number of entries in the three arms of the maze (juvenile: 36.1±3.3 HTZ; 52.8±4.1
401 WT; Student's t-test: t(26)=3.176, p=0.0038, Figure 6F; adult: 30.8±2.2 HTZ; 47.7±2.8 WT; Student's t-
402 test: t(34)=4.852, p<0.0001, Figure 6J). This result was initially surprising, because we did not find
403 modifications of locomotor activity in other tests. To shed more light on this issue, we quantified the
404 average time that mice spent in the center zone before entering a novel arm, which may be interpreted
405 as a measure of the decision-making duration in the maze[45]. We observed about two-fold increase in
406 the time spent in the center in HTZ females compared to WT littermates at both ages ((juvenile:
407 5.7±0.6s HTZ; 3.0±0.3s WT; Student's t-test: t(26)=4.113, p=0.0003, Figure 6G; adult: 5.2±0.6s HTZ;
408 2.9±0.3s WT; Student's t-test: t(34)=3.140, p=0.0035, Figure 6K). Finally, we identified in adult HTZ
409 females, but not in juvenile ones, a little but significant reduction of the percentage of spontaneous
410 alternations compared to WT littermates (juvenile: 51.9±3.3% HTZ; 51.7±2.8% WT; Student's t-test:
411 t(26)=0.04132, p=0.97, NS, Figure 6H; adult: 47.3±1.6% HTZ; 52.9±1.9% WT; Student's t-test:
412 t(34)=2.236, p=0.032, Figure 6L).
413 Hence, *Scn2a*$^{+/-}$ female mice have normal recognition memory at both ages, but show reduced
414 exploratory behavior in a decision-making context, along with a slight reduction in performance in a
415 spatial working memory task in adult females.
416
417 **Juvenile *Scn2a*$^{+/-}$ mice have reduced anxiety-like behavior in the Elevated Plus Maze test.**
418 Anxiety is a comorbidity often reported in ASD patients. We studied anxiety-like behavior in both
419 juvenile and adult female mice in the Elevated Plus Maze (EPM) test: a cross-shaped maze elevated
420 from the ground with two closed (safe) arms and two open (anxiogenic) arms. Anxiety-like behavior is
421 determined evaluating the number of entries and fraction of time spent in the open arms (Figure7A).
422 We initially evaluated global locomotion and exploration in the EPM by quantifying the total distance
423 traveled, which was not different compared to control littermates (juvenile: 10.3±0.7m HTZ;
424 11.1±0.9m WT; Mann-Whitney test, U=115, p=0.69, NS, Figure 7C; adult: 9.3±0.5m HTZ; 9.3±0.5m WT;
425 Student's t-test: t(33)=0.0042, p=0.99, NS, Figure 7F). In juvenile females, we observed a significant
426 increase in the percentage of entries in the open arms (54.3±2.2% HTZ; 44.1±2.4% WT; Student's t-
427 test, t(30)=3.080, p=0.0044, Figure 7D) and in the percentage of time spent in the open arms
428 (53.1±3.1% HTZ; 35.1±3.1% WT; Student's t-test, t(30)=4.055, p=0.0003, Figure 7G). In adult females,
429 we observed strong trends towards an increase of the percentage of entries in the open arms
430 (50.7±3.5% HTZ; 43.7±1.9% WT; Student's t-test with Welch correction, t(23.81)=1.757, p=0.0918,

431 Figure 7E) and in the percentage of time spent in the open arms (44.4±5.6% HTZ; 31.8±2.8% WT;
432 Student's t-test with Welch correction, t(22.36)=2.005, p=0.0572, Figure 7H). Thus, juvenile *Scn2a*$^{+/-}$
433 female mice showed reduced anxiety in the EPM, but normal locomotor and exploratory features.
434 Adult *Scn2a*$^{+/-}$ female mice showed a reduction of the severity of this dysfunction, as we previously
435 observed for male mice[23].

436 **Discussion.**

437 We performed an extended characterization of the behavioral dysfunctions in female *Scn2a*$^{+/-}$ mice,
438 for which little information is available. Because ASD is a neurodevelopmental disorder with onset in
439 childhood, we investigated both juvenile (P21-42) and adult mice (>P70), as we have already done for
440 male mice[23]. Some recent studies of mouse models of *SCN2A* genetic variants have included female
441 mice in their investigations[27,29], but they have not performed a global behavioral assessment and they
442 analyzed only late developmental stages.

443 Dysfunction of social behaviors is among the core symptoms of ASD. We observed that *Scn2a*$^{+/-}$ female
444 mice display an increased sociability in the 3-chamber test both at juvenile and adult ages, without
445 alterations of social memory and without modifications of olfactory discrimination of social or non-
446 social cues. Increased sociability in the 3-chamber test and in direct social interaction was previously
447 reported in *Scn2a*$^{+/-}$ adult male mice[26,30], as well as in adult male mice of other models of *SCN2A*
448 haploinsufficiency and ASD-like phenotypes[28,29]. Interestingly, it has been proposed that the hyper-
449 social phenotype observed in *Scn2a*$^{\Delta 1898/+}$ mouse model could model the inappropriate social approach
450 behavior reported in the Simons Foundation database for ASD patients with *SCN2A* variants[29]. In our
451 previous study of *Scn2a*$^{+/-}$ male mice, we have observed decreased social communication by ultra-sonic
452 vocalizations (USVs) at both young and adult age[23], which is considered an autistic-like trait. We have
453 not evaluated USVs in *Scn2a*$^{+/-}$ female mice, because females generate much less USVs than males[46–
454 48], complicating the quantification. In particular, in a male-female encounter context, which we have
455 used for adult *Scn2a*$^{+/-}$ male mice[23], USVs are produced by males[49,50].

456 Restricted, stereotyped and repetitive behaviors are other core symptoms of ASD. We assessed the
457 presence of innate motor stereotypies in *Scn2a*$^{+/-}$ female mice by measuring the amount of time spent
458 self-grooming in an open field arena and the number of marbles buried, which were increased in
459 *Scn2a*$^{+/-}$ males[23,51]. Instead, in *Scn2a*$^{+/-}$ female mice we have not observed any differences in these tests
460 compared to WT. Moreover, we found that *Scn2a*$^{+/-}$ female mice do not show a more pronounced
461 acquisition of repetitive motor routines evaluated with the rotarod test, feature that we instead
462 observed in *Scn2a*$^{+/-}$ male mice[23]. Interestingly, this gender difference was observed also in the
463 *Scn2a*^{K1422E} model of *Scn2a* haploinsufficiency and ASD-like phenotype[28]. Overall, our results do not
464 show acquired or innate motor stereotypies in *Scn2a*$^{+/-}$ female mice. Moreover, we did not observe
465 alterations of exploratory and locomotor behavior in the open field test, indicating that *Scn2a*$^{+/-}$ female
466 mice do not show general hyperactivity, which is a frequent comorbidity of ASD patients and mouse
467 models.

468 Abnormalities in sensory processing are further features of ASD phenotypes. Because in our previous
469 study of male *Scn2a*$^{+/-}$ mice we have not investigated these features[23], we have here directly compared

470 the response to thermal stimuli in male and female *Scn2a*$^{+/-}$ mice. Both juvenile and adult male *Scn2a*$^{+/-}$
471 mice showed hyper-reactivity to cold stimuli, whereas female *Scn2a*$^{+/-}$ mice showed hyper-reactivity
472 only in adulthood, and neither males nor females showed altered response to heat. This is consistent
473 with a milder phenotype in females. Notably, a recent study showed hyper-reactivity to both cold and
474 hot stimuli in gene-trap *Scn2a*$^{gtKO/gtKO}$ mice[27], which may be caused by the larger reduction of *Scn2a*
475 expression in that model compared to haploinsufficient *Scn2a*$^{+/-}$ mice.
476 Increased anxiety is a frequent comorbidity in ASD patients and mouse models. We observed instead
477 an apparent decrease in anxiety-like phenotype: more entries and more time spent in the open
478 anxiogenic areas of the elevated plus maze apparatus in juvenile *Scn2a*$^{+/-}$ female mice and a trend
479 towards an increase in adults. Interestingly, these results are consistent with the ones previously
480 observed in *Scn2a*$^{+/-}$ male[23,26] and female mice[31]. Moreover, decreased anxiety has also been identified
481 in other ASD mouse models, such as *Scn2a*^{K1422E} [28], *Shank3b*$^{-/-}$, *Caspr2*$^{-/-}$ and 16p11.2 mice[52]. However,
482 this may be related to a dysfunctional risk-assessment behavior, which would lead to comparable
483 exploration of risky and non-risky areas, as already proposed for other ASD models[52]. Notably,
484 decreased risk-assessment and impulsivity have been observed also in ASD patients, leading in some
485 cases to life threatening behaviors[53–56].
486 Cognitive functions are often impaired in ASD patients and mouse models. Similar to social memory
487 discussed above, neither juvenile nor adult *Scn2a*$^{+/-}$ female mice showed dysfunctions of recognition
488 memory in the NORT. Thus, unlike in males, we found an intact learning and memory storage in *Scn2a*$^{+/-}$
489 female mice, in both short (10min in the 3-chambers test) and long (1h in the NORT) periods of time.
490 However, in the Y maze test, we found a mild decrease of spatial working memory (spontaneous
491 alternations) in adult females, which seems gender-specific, since *Scn2a*$^{+/-}$ males show a normal spatial
492 working memory both at young[23] and adult[23,31] age. In this test, we also observed an impairment in
493 exploratory parameters (distance travelled and total arms entries) in both juvenile and adult females.
494 The difference seen in spontaneous alternations cannot be explained by a reduction in exploratory
495 behavior, because there is no correlation between the number of arm entries and the percentage of
496 spontaneous alternations[57]. Notably, the decreased distance travelled and exploration in the Y maze
497 are not caused by a locomotor deficit, which was not observed in other tests. Evaluating this issue, we
498 found in *Scn2a*$^{+/-}$ females a 2-fold increase in the time spent in the center of the maze at both ages,
499 which resembled to a "hesitation" of the animal to choose where to go. We interpreted this behavioral
500 abnormality as altered decision-making. In fact, it has been proposed that, in maze tasks involving arm
501 choices, rodents show pauses in choice-related points that are related to the decision-making process,
502 called vicarious trial-and-error[45]. The observed behavior of *Scn2a*$^{+/-}$ female mice in the Y-maze test was
503 consistent with a longer decision-making time, which was followed by a relatively high percentage of

correct choices in alternation but led to a decrease in the distance travelled and in the total arms entries. This feature is consistent with the atypical decision-making of ASD patients in choice tests[58]. Therefore, we observed in *Scn2a*$^{+/-}$ female mice a reduction in anxiety-like behaviors in juveniles, hypersensitivity to cold stimuli and a slight reduction in spatial working memory in adulthood, as well as social behavior abnormalities and decision-making deficits at both ages (Table).

In our previous study[23] (Table), we observed in *Scn2a*$^{+/-}$ male mice a prominent ASD-like phenotype in juveniles, comprising social communication deficits (but without interaction or olfactory discrimination deficits), innate (increased marbles buried and time spent self-grooming) and acquired (increased exercise-induced latency to fall from the rotarod) stereotyped behavior, decreased anxiety-like and depressive-like behaviors associated to normal locomotion, functional spatial working and long-term memories but decreased object recognition memory. In adult *Scn2a*$^{+/-}$ male mice, the only behavioral abnormality we identified was a decreased social communication.

Other studies have investigated behavioral features of *Scn2a* models. A study reported in *Scn2a*$^{+/-}$ adult male mice a delayed learning in spatial working memory tasks[24], which could be related to the working memory defect we observed in adult females in the Y-maze test. Another study reported in adult *Scn2a*$^{+/-}$ male mice novelty-induced hyperactivity in the openfield, decreased social approach in the resident-intruder test, increased dominance in the tube test, conflicting results in depressive-like behavior in the force swim test and the tail suspension test, enhanced fear learning and decreased extinction[26]. A further study reported very mild phenotypes in adult *Scn2a*$^{+/-}$ mice, both males and females, with mild non-significant trends toward deficits in learning and social behavior and significant effects only for decreased social memory and increased risk taking in females[31]. A study of another *Scn2a* knock-out model (deletion of exons 4-6) identified in adult male mice altered spatial learning and fear extinction abnormalities associated to enhanced direct social interaction but no social communication or motor stereotypies[30]. In hypomorphic *Scn2a*$^{\Delta 1898/+}$ adult mice it has been recently reported in adult males but not females an increased sociability in the 3-chambers test[29], which resembles the increased sociability that we observed here in female *Scn2a*$^{+/-}$mice. Thus, there is some inter-laboratory variability in the results of behavioral studies, which may be related to specific breeding, handling and experimental design conditions and that complicates the comparison of detailed features. However, a general finding is that adult *Scn2a* loss-of-function models have relatively mild behavioral phenotypes.

Overall, our results are consistent with the literature, showing that both male and female adult *Scn2a*$^{+/-}$ mice have mild phenotypes. Additionally, we have shown that, differently than *Scn2a*$^{+/-}$ male mice, also in the juvenile period *Scn2a*$^{+/-}$ female mice have a milder phenotype, whose features are therefore less markedly reduced in adulthood. Moreover, we have observed some gender-specific features.

538 Interestingly, a milder phenotype of females compared to males has also been observed in other ASD
539 mouse models[59,60].
540 As already outlined in the introduction, it has been shown that ASD female patients can express
541 symptoms differently than men and have more prominent masking behaviors[9,12–15], leading to a
542 delayed or incorrect diagnosis and an underestimation of ASD cases in [9–11]. Our data obtained in
543 *Scn2a*$^{+/-}$ mice are consistent with biological mechanisms for the specific features of ASD in females.
544 There is little information in the literature about the detailed phenotype of ASD patients carrying
545 *SCN2A* variants, in particular regarding gender-related features. The few female cases described in
546 some details presented with developmental and epileptic encephalopathies or other
547 neurodevelopmental disorders[61–64], consistent with a more difficult identification of female ASD
548 phenotypes. Notably, although overall mild, we have found some robust behavioral features in *Scn2a*$^{+/-}$
549 mice.
550 In conclusion, our study identifies for the first time the global behavioral phenotype of juvenile and
551 adult *Scn2a*$^{+/-}$ female mice and compares it with that of *Scn2a*$^{+/-}$ male mice. The specific features and
552 the relatively mild phenotype of *Scn2a*$^{+/-}$ female mice highlight the importance of including females in
553 the study of animal models and developing gender-specific clinical assessments for ASD patients.
554

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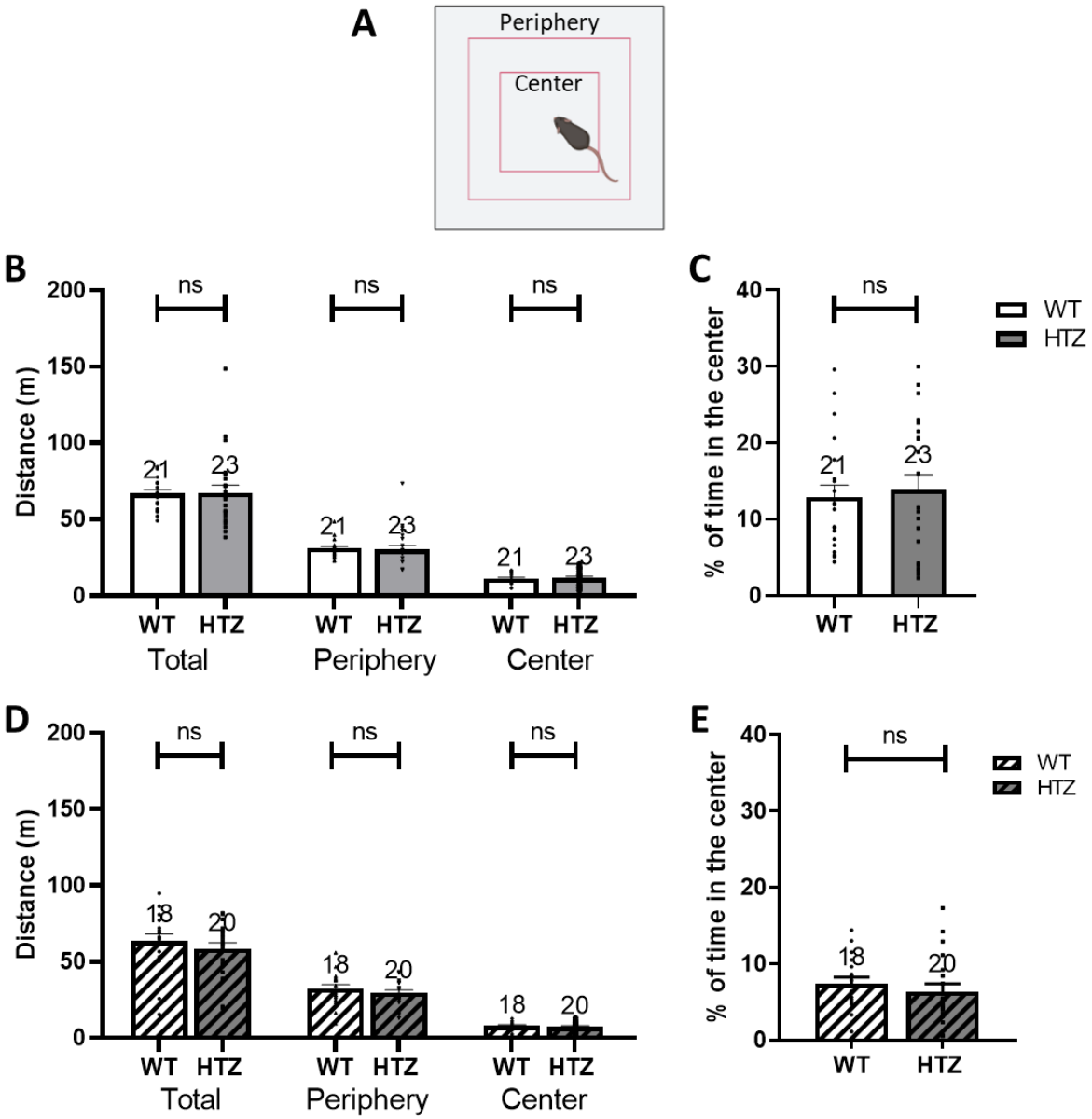
Author Contributions.

I.L. and M.M. conceived the project. W.M. and M.S. performed the experiments and analyzed the data. W.M. wrote an initial draft of the paper. M.S, I.L. and M.M critically revised the manuscript. All authors approved the final version of the manuscript.

Data Availability Statement

Data are available on request.

577 **Figures.**



578

579 **Figure 1: Evaluation of locomotor activity in juvenile and adult *Scn2a*$^{+/-}$ (HTZ) female mice.**

580 **A:** Schematic illustration of the apparatus, which for the analysis was virtually divided in zones: center,
581 intermediary and periphery. **B-C:** Locomotor activity in 30min (total, periphery and center) (B) and
582 percent time spent in the center of the open field in 30min (C) for juvenile female mice. **D-E:** Locomotor
583 activity in 30min (total, periphery and center) (D) and percent time spent in the center of the open
584 field in 30min (E) for adult female mice. ns=non-significant (Mann-Whitney or Student's t-test); the
585 number of mice (N) is indicated above the bars. White: WT, Grey: HTZ. No Pattern, juvenile; striped,
586 adult.

587

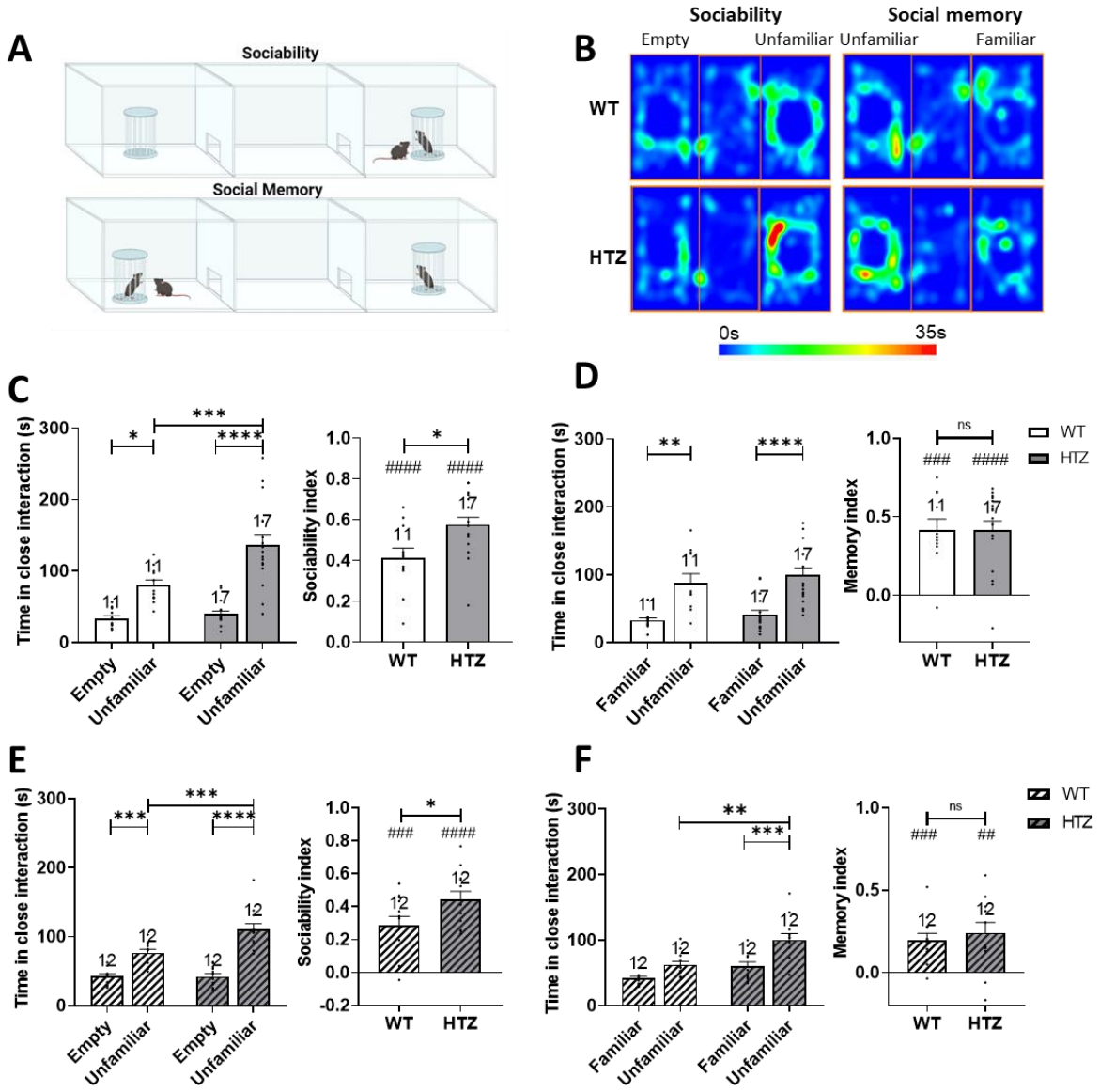


Figure 2: Evaluation of sociability in juvenile and adult *Scn2a*$^{+/-}$ (HTZ) female mice.

A: Schematic diagram of the 3-Chamber Sociability and Social Memory tests. **B:** Representative heatmap of the time spent by WT and HTZ mice in different areas of the apparatus during the Sociability and Social Memory phases. The red areas indicate the locations where the test mouse spent the maximum amount of time. **C-D:** Results in juvenile HTZ female mice for the sociability and social memory phases of the test: (C) Time in close interaction with the empty cylinder or the unfamiliar mouse (Left) and sociability index (Right) in juvenile female mice. (D) Time in close interaction with the familiar or the new unfamiliar mouse (Left) and social memory index (Right) in juvenile female mice. **E-F:** Results for adult HTZ female mice for sociability or social memory phases of the test. (E) Time in close interaction with the empty cylinder or the unfamiliar mouse (Left) and sociability index (Right) in adult female mice. (F) Time in close interaction with the familiar or the new unfamiliar mouse (Left) and social memory index (Right) in adult female mice. Statistical analysis: Two-way ANOVA with Sidak's post-hoc test for multiple comparison test, Mann-Whitney or Student's t-test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. One-sample t-test for comparison to chance level #p<0.05, ##p<0.01, ###p<0.001, ####p<0.0001. The number of mice (N) is indicated above the bars. White: WT, Grey: HTZ. No Pattern, juvenile; striped, adult.

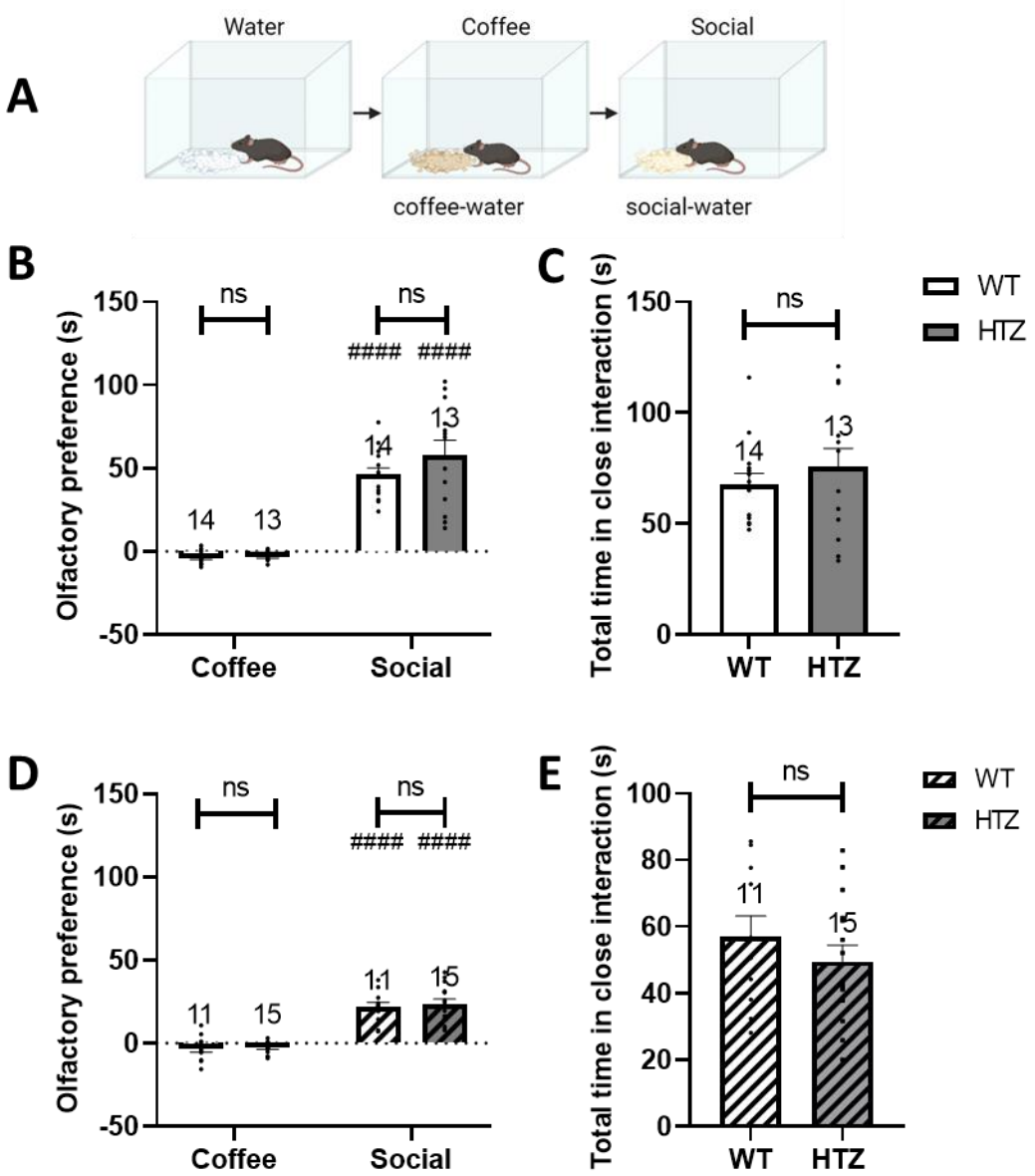


Figure 3: Evaluation of olfactory preference in juvenile and adult *Scn2a*$^{+/-}$ (HTZ) female mice.

A: Schematic illustration of the olfactory test divided in 3 phases: exploration of paper dampened in water, coffee (aversive odor) and social odors (appetitive odor). Scores are calculated as time spent exploring coffee or social odors minus the time spent exploring water. **B-C:** Olfactory preference in juvenile female mice and total time spent in interaction. **D-E**: Olfactory preference in adult female mice and total time spent in interaction. Statistical analysis: 2 Way Anova, significant odor effect ****p<0.0001 (B-D), Mann-Whitney test: ns (C) and Student's t-test: ns (E). Post-hoc analysis: Sidak's multiple comparison test, #### p<0.0001, comparison between coffee and social odors, comparison between genotype: ns. The number of mice (N) is indicated above the bars. White: WT, Grey: HTZ, No pattern: Juvenile, Striped: Adult.

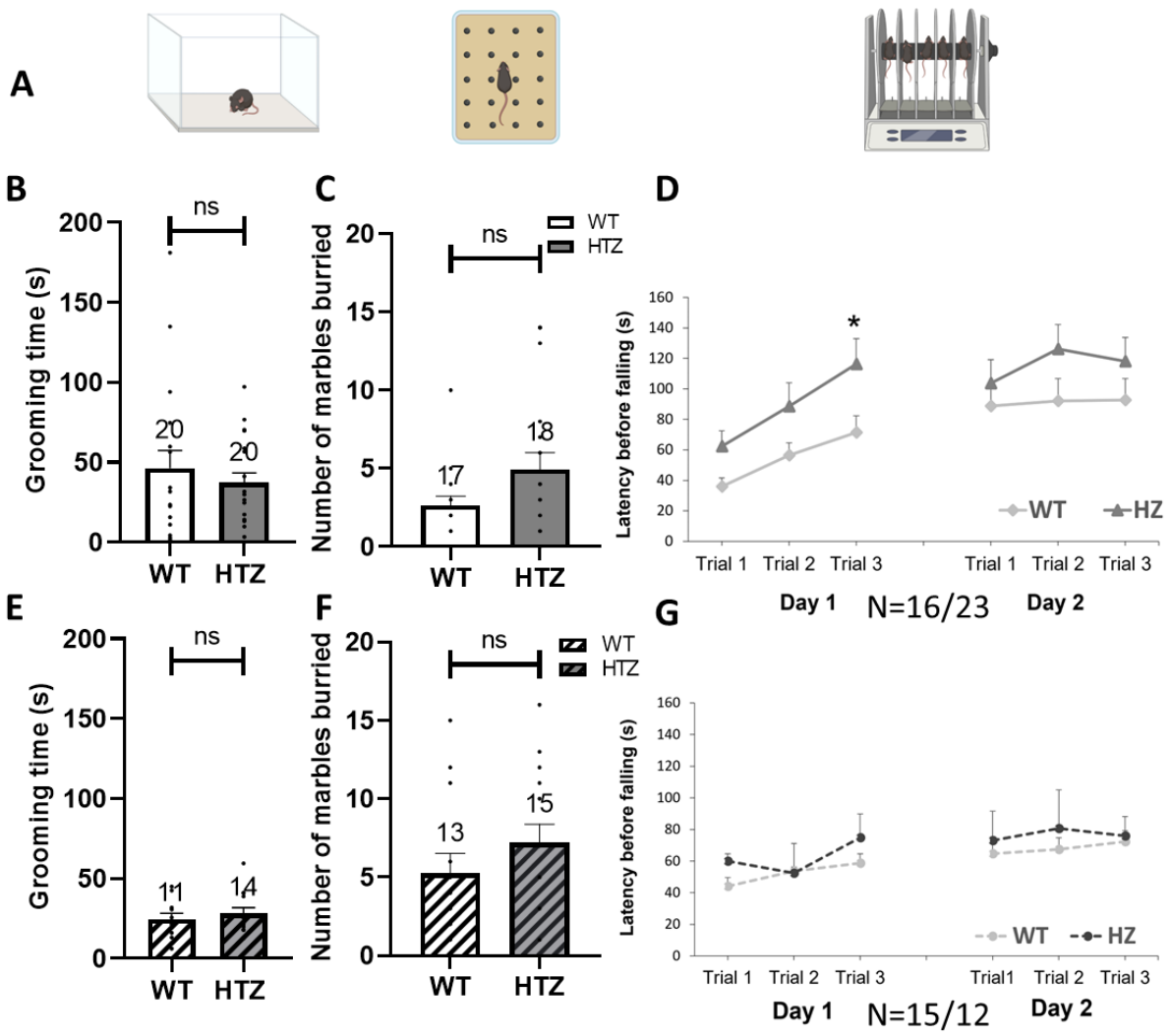
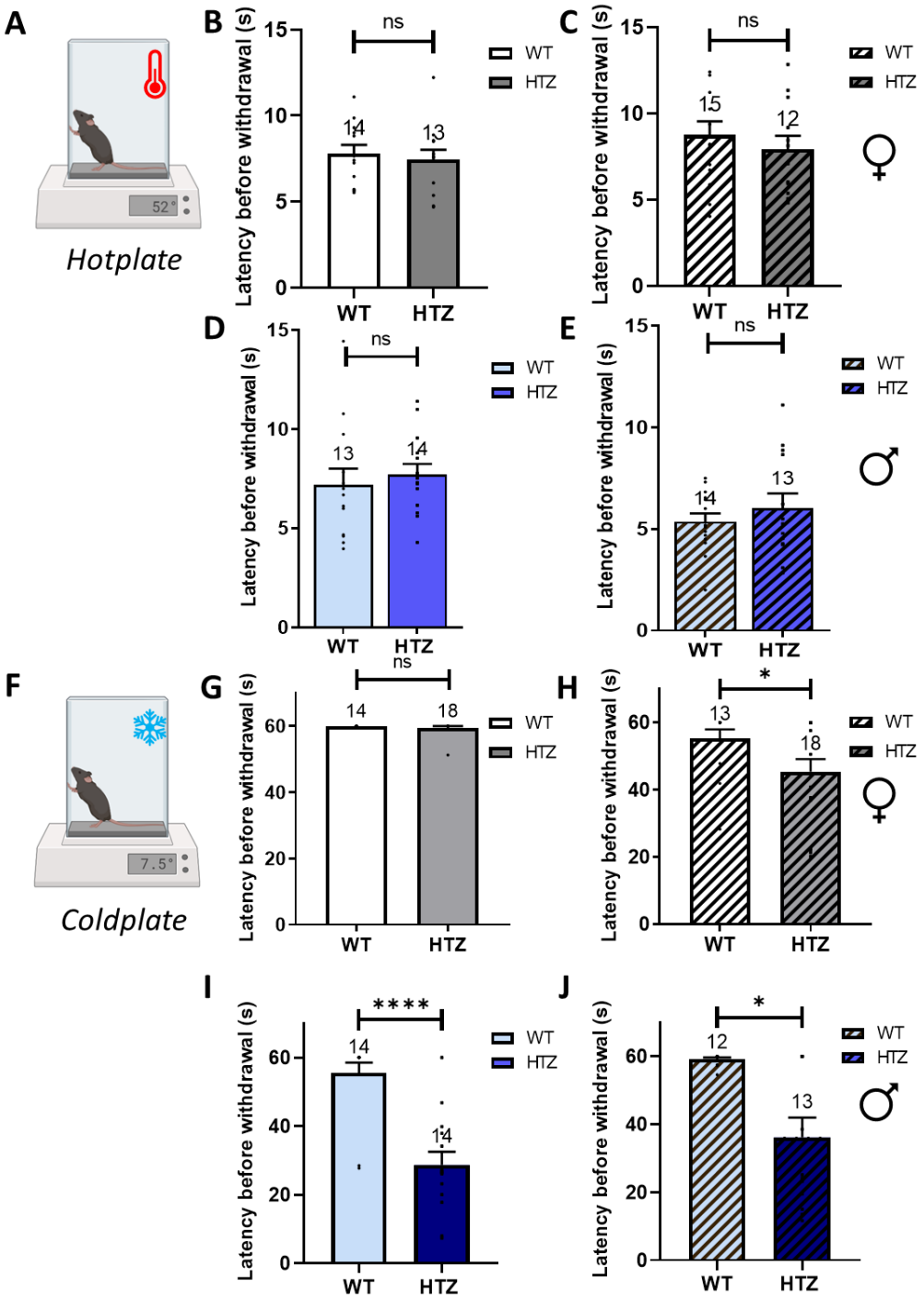


Figure 4: Evaluation of motor stereotypies in juvenile and adult *Scn2a*$^{+/-}$ (HTZ) female mice.

A: Schematic illustrations of grooming test, marble burying and rotarod apparatuses. **B-D:** Evaluation of innate and acquired motor stereotypies in juvenile female HTZ mice. (B) Time spent self-grooming. (C) Number of marbles buried. (D) Latency to fall from the rotarod, 3 trials per day on 2 days. **E-G:** Evaluation of innate and acquired motor stereotypies in adult female HTZ mice. (E) Time spent grooming. (F) Number of marbles buried. (G) Latency to fall from the rotarod, 3 trials per day on 2 days. Statistical analysis: Mann-Whitney or t-test for grooming and marble burying tests and 2 Way Repeated Measure Anova for rotarod analysis *p<0.05 compared to WT, ns: non-significant. In D the difference is significant considering the two days separately. The number of mice (N) is indicated above the bars or below the plots. White: WT, Grey: HTZ, No Pattern: Juvenile, Striped: Adult.



628

629 **Figure 5: Evaluation of thermal sensitivity in juvenile and adult *Scn2a*$^{+/-}$ (HTZ) mice.**

630 **A;F:** Schematic representation of the Hotplate (top, 52°c, 45s cut-off time) and Coldplate (bottom,
631 7.5°c, 1min cut-off time) test protocols. **B-E:** Heat sensitivity in female (B-C) and male (D-E) mice, left
632 column: juveniles and right column: adults. **G-J**: Cold sensitivity in female (G-H) and male (I-J) mice, left
633 column: juveniles and right column: adults. Statistical analysis: One sample Wilcoxon test p>0.9999
634 (juvenile female), and Mann-Whitney or Student's t-test. *p<0.05 compared to WT, ****p<0.0001, ns:
635 non-significant. The number of mice (N) is indicated above the bars. White or light blue: WT (female
636 or male respectively), grey or dark blue: HTZ (female or male respectively), No pattern: Juvenile,
637 Striped: Adult.

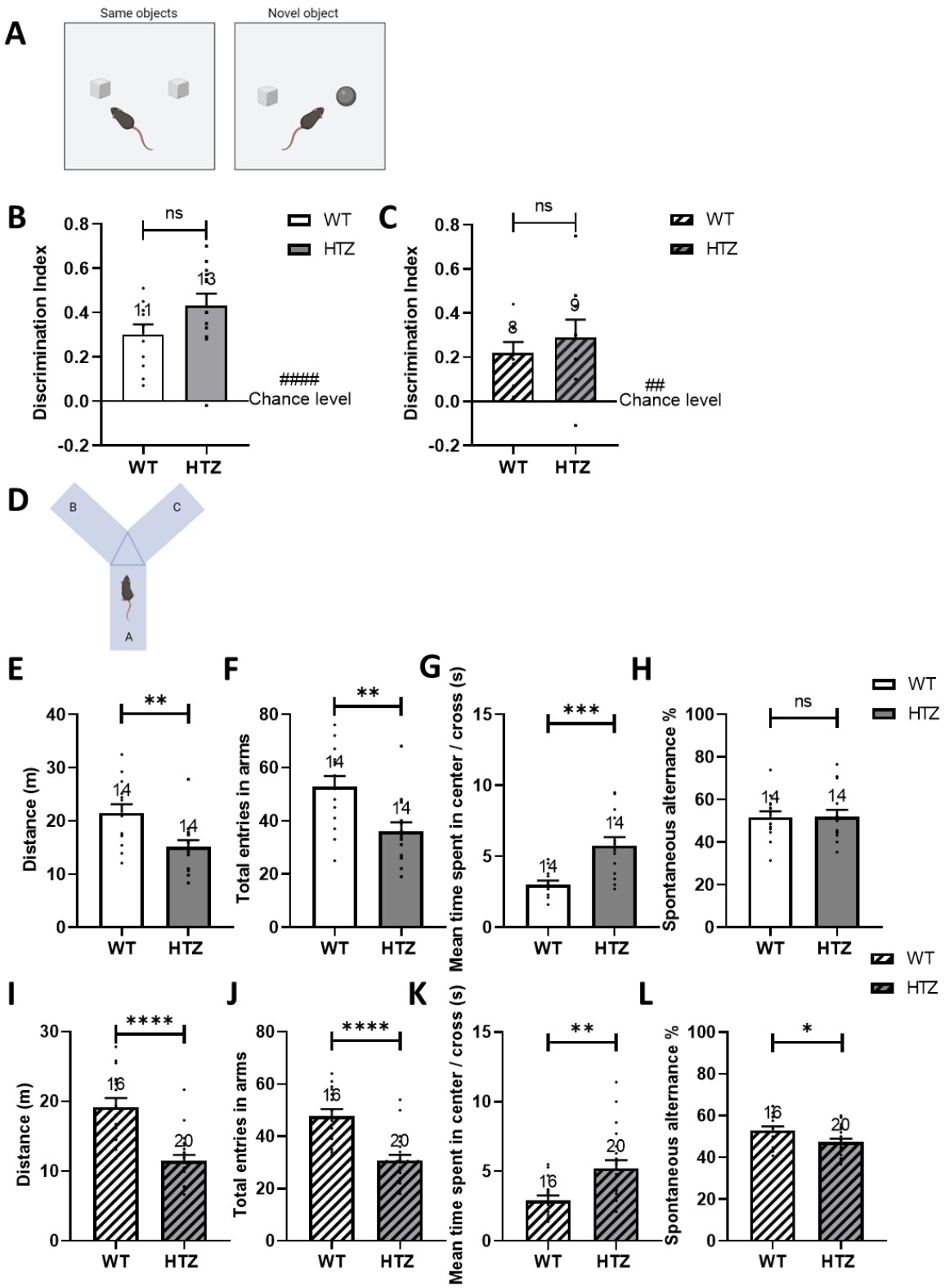


Figure 6: Evaluation of cognitive function in juvenile and adult female *Scn2a*$^{+/-}$ (HTZ) mice.

A: Schematic representation of the Novel Object Recognition Test (NORT) protocol divided in 2 phases: Learning phase with 2 identical objects and Object Recognition phase with a familiar and a new unfamiliar object. **B-C:** Discrimination index of the NORT for juvenile (B) and adult (C) female mice. **D:** Schematic representation of the Y maze apparatus. Quantifications for juvenile (E-H) and adult (I-L)

female mice. **E-G:** Exploration parameters in the Y maze apparatus: Distance (E), total entries in the arms (F) and mean time spent in the center per cross (G) in juvenile female mice. **H:** Percent spontaneous alternation in juvenile female mice. **I-K:** Exploration parameters in the Y maze apparatus: Distance (I), total entries in the arms (J) and mean time spent in the center per cross (K) in adult female mice. **L:** Percent spontaneous alternation in adult female mice. Statistical analysis: Student's t-test. *$p<0.05$ compared to WT, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$, ns: non-significant. The number of mice (N) is indicated above the bars. White: WT, Grey: HTZ, No pattern: Juvenile, Striped: Adult.

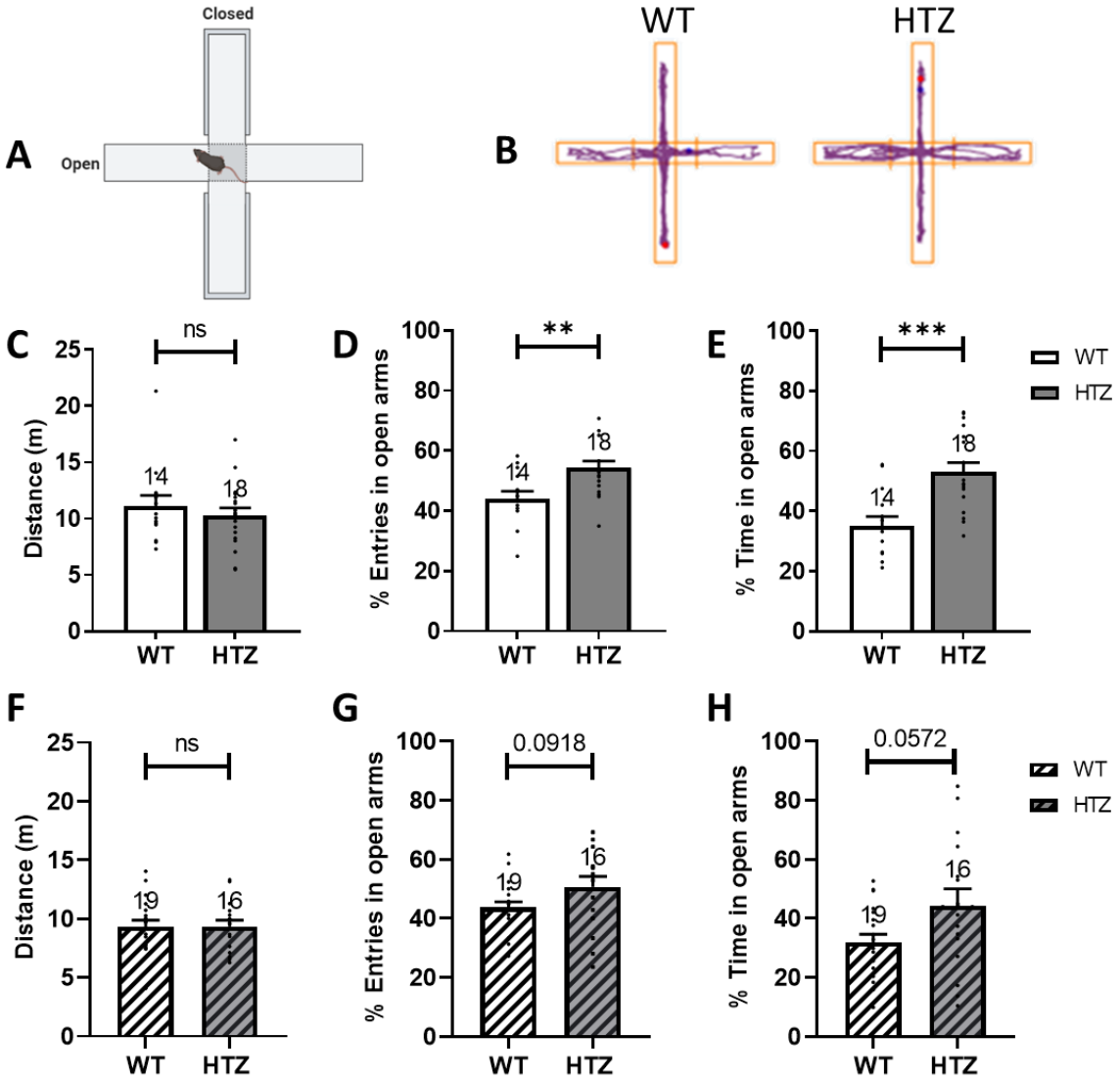


Figure 7: Evaluation of anxiety-like behavior in juvenile and adult *Scn2a*$^{+/-}$ (HTZ) female mice.

A: Schematic representation of the Elevated Plus-Maze (EPM) test. **B:** Representative trackplots of WT and HTZ female. **C-E:** Results in juvenile female mice: Distance in the maze (C), percent of entries (D) and time (E) spent in open arms of the EPM compared to total open + close arms entries or time. **F-H:** Results in adult female mice: Distance in the maze (F), percent of entries (G) or time (H) spent in open arms of the EPM compared to total open + close arms entries or time. Statistical analysis: Mann-Whitney or Student's t-test (with Welch's correction for G-H). *p<0.05 compared to WT, **p<0.01, ***p<0.001, ns: non-significant. The number of mice (N) is indicated above the bars. White: WT, Grey: HTZ, Empty: Juvenile and Striped: Adult.

MALES			FEMALES	
Juvenile	**Adult**		**Juvenile**	**Adult**
-Decreased social communication *-Motor stereotypies (innate and acquired)* *-Memory impairements* *-Increased risk-taking* *-Hyper-reactivity to cold stimuli*	*-Decreased social communication* *-Hyper-reactivity to cold stimuli*	**VS**	*-Increased social interactions* *-Increased risk-taking* *-Altered decision-making*	*-Increased social interactions* *-Mild memory impairements* *-Trend towards increased risk-taking* *-Hyper-reactivity to cold stimuli* *-Altered decision-making*

Table: Comparison of behavioral abnormalities between *Scn2a*$^{+/-}$ male and female mice at juvenile and adult age that we observed in our studies (this report and [23])

References:

1. American Psychiatric Association. *Diagnostic and statistical manual of mental disorders. 5th ed.*
2. Rylaarsdam, L. & Guemez-Gamboa, A. Genetic Causes and Modifiers of Autism Spectrum Disorder. *Front. Cell. Neurosci.* **13**, (2019).
3. Lai, M.-C., Lombardo, M. V. & Baron-Cohen, S. Autism. *Lancet Lond. Engl.* **383**, 896–910 (2014).
4. Maenner, M. J. Prevalence and Characteristics of Autism Spectrum Disorder Among Children Aged 8 Years — Autism and Developmental Disabilities Monitoring Network, 11 Sites, United States, 2018. *MMWR Surveill. Summ.* **70**, (2021).
5. Fombonne, E. Epidemiological surveys of autism and other pervasive developmental disorders: an update. *J. Autism Dev. Disord.* **33**, 365–382 (2003).
6. Loomes, R., Hull, L. & Mandy, W. P. L. What Is the Male-to-Female Ratio in Autism Spectrum Disorder? A Systematic Review and Meta-Analysis. *J. Am. Acad. Child Adolesc. Psychiatry* **56**, 466–474 (2017).
7. Kirkovski, M., Enticott, P. G. & Fitzgerald, P. B. A review of the role of female gender in autism spectrum disorders. *J. Autism Dev. Disord.* **43**, 2584–2603 (2013).
8. Van Wijngaarden-Cremers, P. J. M. *et al.* Gender and age differences in the core triad of impairments in autism spectrum disorders: a systematic review and meta-analysis. *J. Autism Dev. Disord.* **44**, 627–635 (2014).
9. Hull, L. *et al.* “Putting on My Best Normal”: Social Camouflaging in Adults with Autism Spectrum Conditions. *J. Autism Dev. Disord.* **47**, 2519–2534 (2017).
10. Kočovská, E. *et al.* The Rising Prevalence of Autism: A Prospective Longitudinal Study in the Faroe Islands. *J. Autism Dev. Disord.* **42**, 1959–1966 (2012).
11. Lai, M.-C. & Szatmari, P. Sex and gender impacts on the behavioural presentation and recognition of autism. *Curr. Opin. Psychiatry* **33**, 117–123 (2020).
12. Gould, J. & Ashton-Smith, J. Missed diagnosis or misdiagnosis? Girls and women on the autism spectrum. *Good Autism Pract. GAP* **12**, 34–41 (2011).

692 13. Lai, M.-C. *et al.* Quantifying and exploring camouflaging in men and women with autism. *Autism*
693 *Int. J. Res. Pract.* **21**, 690–702 (2017).
694 14. McQuaid, G. A., Lee, N. R. & Wallace, G. L. Camouflaging in autism spectrum disorder: Examining
695 the roles of sex, gender identity, and diagnostic timing. *Autism Int. J. Res. Pract.* **26**, 552–559
696 (2022).
697 15. Rynkiewicz, A. *et al.* An investigation of the 'female camouflage effect' in autism using a
698 computerized ADOS-2 and a test of sex/gender differences. *Mol. Autism* **7**, 10 (2016).
699 16. Lord, C. *et al.* Autism spectrum disorder. *Nat. Rev. Dis. Primer* **6**, 1–23 (2020).
700 17. Sohal, V. S. & Rubenstein, J. L. R. Excitation-inhibition balance as a framework for investigating
701 mechanisms in neuropsychiatric disorders. *Mol. Psychiatry* **24**, 1248–1257 (2019).
702 18. Mantegazza, M., Cestèle, S. & Catterall, W. A. Sodium channelopathies of skeletal muscle and
703 brain. *Physiol. Rev.* **101**, 1633–1689 (2021).
704 19. Rusina, E., Simonti, M., Duprat, F., Cestèle, S. & Mantegazza, M. Voltage-gated sodium channels in
705 genetic epilepsy: up and down of excitability. *J. Neurochem.* **n/a**,.
706 20. Sanders, S. J. *et al.* Progress in Understanding and Treating SCN2A-Mediated Disorders. *Trends*
707 *Neurosci.* **41**, 442–456 (2018).
708 21. Ben-Shalom, R. *et al.* Opposing Effects on NaV1.2 Function Underlie Differences Between SCN2A
709 Variants Observed in Individuals With Autism Spectrum Disorder or Infantile Seizures. *Biol.*
710 *Psychiatry* **82**, 224–232 (2017).
711 22. Planells-Cases, R. *et al.* Neuronal death and perinatal lethality in voltage-gated sodium channel
712 alpha(II)-deficient mice. *Biophys. J.* **78**, 2878–2891 (2000).
713 23. Léna, I. & Mantegazza, M. NaV1.2 haploinsufficiency in Scn2a knock-out mice causes an autistic-
714 like phenotype attenuated with age. *Sci. Rep.* **9**, 12886 (2019).
715 24. Middleton, S. J. *et al.* Altered hippocampal replay is associated with memory impairment in mice
716 heterozygous for the Scn2a gene. *Nat. Neurosci.* **21**, 996–1003 (2018).

717 25. Ogiwara, I. *et al.* Nav1.2 haplodeficiency in excitatory neurons causes absence-like seizures in
718 mice. *Commun. Biol.* **1**, (2018).
719 26. Tatsukawa, T. *et al.* Scn2a haploinsufficient mice display a spectrum of phenotypes affecting
720 anxiety, sociability, memory flexibility and ampakine CX516 rescues their hyperactivity. *Mol.*
721 *Autism* **10**, 15 (2019).
722 27. Eaton, M. *et al.* Generation and basic characterization of a gene-trap knockout mouse model of
723 Scn2a with a substantial reduction of voltage-gated sodium channel Nav 1.2 expression. *Genes*
724 *Brain Behav.* **20**, e12725 (2021).
725 28. Echevarria-Cooper, D. M. *et al.* Cellular and behavioral effects of altered NaV1.2 sodium channel
726 ion permeability in Scn2aK1422E mice. *Hum. Mol. Genet.* ddac087 (2022)
727 doi:10.1093/hmg/ddac087.
728 29. Wang, H.-G. *et al.* Scn2a severe hypomorphic mutation decreases excitatory synaptic input and
729 causes autism-associated behaviors. *JCI Insight* **6**, 150698 (2021).
730 30. Shin, W. *et al.* Scn2a Haploinsufficiency in Mice Suppresses Hippocampal Neuronal Excitability,
731 Excitatory Synaptic Drive, and Long-Term Potentiation, and Spatial Learning and Memory. *Front.*
732 *Mol. Neurosci.* **12**, 145 (2019).
733 31. Spratt, P. W. E. *et al.* The Autism-Associated Gene Scn2a Contributes to Dendritic Excitability and
734 Synaptic Function in the Prefrontal Cortex. *Neuron* **103**, 673-685.e5 (2019).
735 32. Lee, T. T.-Y. & Gorzalka, B. B. Timing is everything: evidence for a role of corticolimbic
736 endocannabinoids in modulating hypothalamic-pituitary-adrenal axis activity across
737 developmental periods. *Neuroscience* **204**, 17–30 (2012).
738 33. Brust, V., Schindler, P. M. & Lewejohann, L. Lifetime development of behavioural phenotype in the
739 house mouse (Mus musculus). *Front. Zool.* **12**, S17 (2015).
740 34. Crawley, J. N. Designing mouse behavioral tasks relevant to autistic-like behaviors. *Ment. Retard.*
741 *Dev. Disabil. Res. Rev.* **10**, 248–258 (2004).

742 35. Moy, S. S. *et al.* Sociability and preference for social novelty in five inbred strains: an approach to
743 assess autistic-like behavior in mice. *Genes Brain Behav.* **3**, 287–302 (2004).
744 36. Witt, R. M., Galligan, M. M., Despinoy, J. R. & Segal, R. Olfactory behavioral testing in the adult
745 mouse. *J. Vis. Exp. JoVE* 949 (2009) doi:10.3791/949.
746 37. Brielmaier, J. *et al.* Autism-relevant social abnormalities and cognitive deficits in engrailed-2
747 knockout mice. *PloS One* **7**, e40914 (2012).
748 38. Thomas, A. *et al.* Marble burying reflects a repetitive and perseverative behavior more than
749 novelty-induced anxiety. *Psychopharmacology (Berl.)* **204**, 361–373 (2009).
750 39. Antunes, M. & Biala, G. The novel object recognition memory: neurobiology, test procedure, and
751 its modifications. *Cogn. Process.* **13**, 93–110 (2012).
752 40. Hamadate, N. *et al.* Liposome-Encapsulated Hemoglobin Ameliorates Impairment of Fear Memory
753 and Hippocampal Dysfunction After Cerebral Ischemia in Rats. *J. Pharmacol. Sci.* **114**, 409–419
754 (2010).
755 41. Ohmura, Y. *et al.* Disruption of model-based decision making by silencing of serotonin neurons in
756 the dorsal raphe nucleus. *Curr. Biol.* **31**, 2446-2454.e5 (2021).
757 42. Nunes. Axonal sodium channel NaV1.2 drives granule cell dendritic GABA release and rapid odor
758 discrimination. *PLoS biology* (2018).
759 43. Rothwell, P. E. *et al.* Autism-associated neuroligin-3 mutations commonly impair striatal circuits
760 to boost repetitive behaviors. *Cell* **158**, 198–212 (2014).
761 44. Corder, G. *et al.* Loss of μ opioid receptor signaling in nociceptors, but not microglia, abrogates
762 morphine tolerance without disrupting analgesia. *Nat. Med.* **23**, 164–173 (2017).
763 45. Redish, A. D. Vicarious trial and error. *Nat. Rev. Neurosci.* **17**, 147–159 (2016).
764 46. Sewell), G. D. S. (née. Ultrasound and mating behaviour in rodents with some observations on
765 other behavioural situations. *J. Zool.* **168**, 149–164 (1972).
766 47. White, N. R., Prasad, M., Barfield, R. J. & Nyby, J. G. 40- and 70-kHz vocalizations of mice (Mus
767 musculus) during copulation. *Physiol. Behav.* **63**, 467–473 (1998).

768 48. Whitney, G., Coble, J. R., Stockton, M. D. & Tilson, E. F. Ultrasonic emissions: do they facilitate
769 courtship of mice. *J. Comp. Physiol. Psychol.* **84**, 445–452 (1973).
770 49. Hanson, J. L. & Hurley, L. M. Female Presence and Estrous State Influence Mouse Ultrasonic
771 Courtship Vocalizations. *PLOS ONE* **7**, e40782 (2012).
772 50. Peleh, T., Eltokhi, A. & Pitzer, C. Longitudinal analysis of ultrasonic vocalizations in mice from
773 infancy to adolescence: Insights into the vocal repertoire of three wild-type strains in two different
774 social contexts. *PloS One* **14**, e0220238 (2019).
775 51. Indumathy, J., Pruitt, A., Gautier, N. M., Crane, K. & Glasscock, E. Kv1.1 deficiency alters repetitive
776 and social behaviors in mice and rescues autistic-like behaviors due to Scn2a haploinsufficiency.
777 *Brain Behav.* **11**, e02041 (2021).
778 52. Oron, O. *et al.* Gene network analysis reveals a role for striatal glutamatergic receptors in
779 dysregulated risk-assessment behavior of autism mouse models. *Transl. Psychiatry* **9**, 257 (2019).
780 53. Bornovalova, M. A. *et al.* Risk taking differences on a behavioral task as a function of potential
781 reward/loss magnitude and individual differences in impulsivity and sensation seeking. *Pharmacol.*
782 *Biochem. Behav.* **93**, 258–262 (2009).
783 54. Guan, J. & Li, G. Injury Mortality in Individuals With Autism. *Am. J. Public Health* **107**, 791–793
784 (2017).
785 55. Moeller, F. G., Barratt, E. S., Dougherty, D. M., Schmitz, J. M. & Swann, A. C. Psychiatric aspects of
786 impulsivity. *Am. J. Psychiatry* **158**, 1783–1793 (2001).
787 56. South, M., Dana, J., White, S. E. & Crowley, M. J. Failure is not an option: Risk-taking is moderated
788 by anxiety and also by cognitive ability in children and adolescents diagnosed with an autism
789 spectrum disorder. *J. Autism Dev. Disord.* **41**, 55–65 (2011).
790 57. Miedel, C. J., Patton, J. M., Miedel, A. N., Miedel, E. S. & Levenson, J. M. Assessment of
791 Spontaneous Alternation, Novel Object Recognition and Limb Clasping in Transgenic Mouse
792 Models of Amyloid-β and Tau Neuropathology. *JoVE J. Vis. Exp.* e55523 (2017) doi:10.3791/55523.

793 58. Mosner, M. G. *et al.* Vicarious Effort-Based Decision-Making in Autism Spectrum Disorders. *J.*
794 *Autism Dev. Disord.* **47**, 2992–3006 (2017).
795 59. Jeon, S. J. *et al.* Sex-specific Behavioral Features of Rodent Models of Autism Spectrum Disorder.
796 *Exp. Neurobiol.* **27**, 321–343 (2018).
797 60. Schaafsma, S. M. *et al.* Sex-specific gene–environment interactions underlying ASD-like behaviors.
798 *Proc. Natl. Acad. Sci.* **114**, 1383–1388 (2017).
799 61. D'Gama, A. M. *et al.* Targeted DNA Sequencing from Autism Spectrum Disorder Brains Implicates
800 Multiple Genetic Mechanisms. *Neuron* **88**, 910–917 (2015).
801 62. Fromer, M. *et al.* De novo mutations in schizophrenia implicate synaptic networks. *Nature* **506**,
802 179–184 (2014).
803 63. Suddaby, J. S., Silver, J. & So, J. Understanding the schizophrenia phenotype in the first patient
804 with the full SCN2A phenotypic spectrum. *Psychiatr. Genet.* (2019)
805 doi:10.1097/YPG.0000000000000219.
806 64. Yamamoto, T. *et al.* Genomic backgrounds of Japanese patients with undiagnosed
807 neurodevelopmental disorders. *Brain Dev.* **41**, 776–782 (2019).
808