

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

Structural Bases of Atypical Whisker Responses in a Mouse Model of CDKL5 Deficiency Disorder

This is the author's manuscript

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/1725223> since 2023-12-13T14:57:48Z

Published version:

DOI:10.1016/j.neuroscience.2019.08.033

Terms of use:

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)

1 **STRUCTURAL BASES OF ATYPICAL WHISKER RESPONSES IN A MOUSE**
2 **MODEL OF CDKL5 DEFICIENCY DISORDER**

3
4 R. Pizzo¹, A. Lamarca^{1,a}, M. Sassoè-Pognetto^{1,2}, M. Giustetto^{1,2}

5
6 ¹Department of Neuroscience, University of Turin, Corso Massimo D'Azeglio 52, 10126 Turin,
7 Italy

8 ²National Institute of Neuroscience-Italy, Corso Massimo D'Azeglio 52, 10126 Turin, Italy

9 ^a Present address: Cell Adhesion Unit, San Raffaele Scientific Institute, Via Olgettina 60, 20132
10 Milan, Italy

11
12 Corresponding author contact information:

13 Prof. Maurizio Giustetto

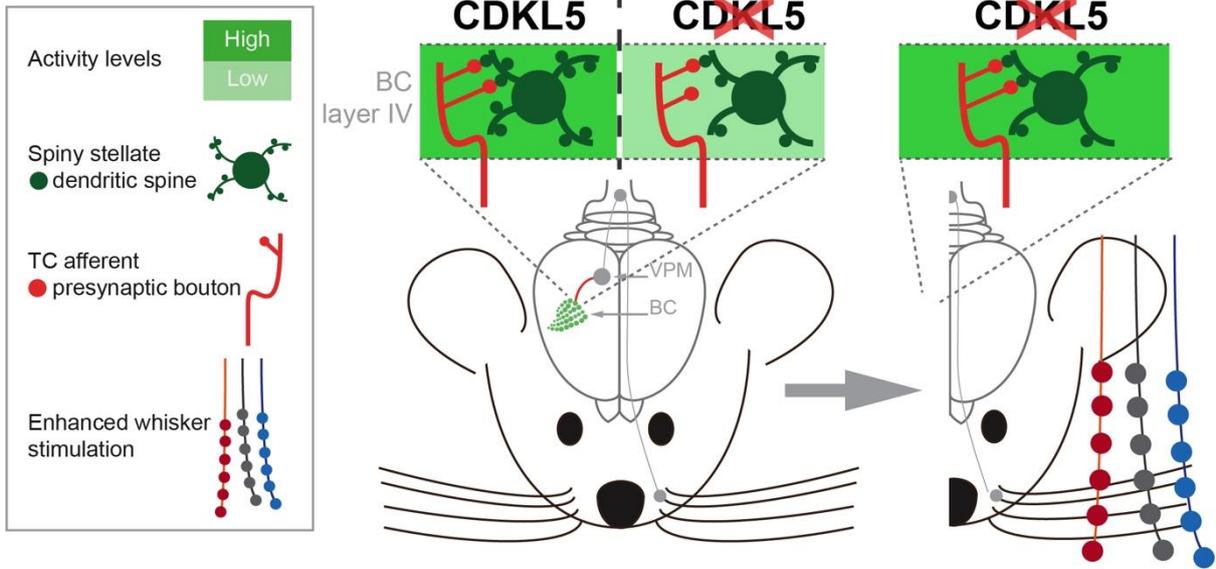
14 Dipartimento di Neuroscienze, Università di Torino

15 Corso M. D'Azeglio 52, 10126 TORINO (Italy).

16 Email: maurizio.giustetto@unito.it

19 **Graphical Abstract**

20



21

22

23

24

25 **Highlights**

26

- 27 • CDKL5 deficiency disrupts the synaptic organization of thalamo-cortical (TC) and
- 28 cortico-cortical (CC) connections in the barrel cortex (BC)
- 29 • CDKL5 deficiency leads to BC hypoactivation
- 30 • CDKL5 deficiency causes atypical whisker-mediated behavioural responses
- 31 • CDKL5 deficiency does not prevent TC circuitry to undergo experience-dependent
- 32 structural plasticity
- 33 • Enhanced sensory stimulation restores cortical connectivity, BC activation levels and
- 34 whisker-related behavioural responses
- 35

36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56

ABSTRACT

Mutations in the *CDKL5* (cyclin-dependent kinase-like 5) gene cause CDKL5 Deficiency Disorder (CDD), a severe neurodevelopmental syndrome where patients exhibit early-onset seizures, intellectual disability, stereotypies, limited or absent speech, autism-like symptoms and sensory impairments. Mounting evidences indicate that disrupted sensory perception and processing represent core signs also in mouse models of CDD, however we have very limited knowledge on their underlying causes. In this study, we investigated how CDKL5 deficiency affects synaptic organization and experience-dependent plasticity in the thalamo-cortical (TC) pathway carrying whisker-related tactile information to the barrel cortex (BC). By using synapse-specific antibodies and confocal microscopy, we found that Cdkl5-KO mice display a lower density of TC synapses in the BC that was paralleled by a reduction of cortico-cortical (CC) connections compared to wild-type mice. These synaptic defects were accompanied by reduced BC activation, as shown by a robust decrease of c-fos immunostaining, and atypical behavioural responses to whisker-mediated tactile stimulation. Notably, a two-day paradigm of enriched whisker stimulation rescued both number and configuration of TC and CC synapses in Cdkl5-KO mice, and restored cortical activity as well as behavioural responses to control levels. Our findings disclose an important role of CDKL5 in controlling the organization and experience-induced modifications of excitatory connections in the BC and indicate how mutations of CDKL5 produce failures in higher-order processing of somatosensory stimuli.

Keywords

Rett Syndrome, barrel cortex, thalamo-cortical, synaptic plasticity.

59 INTRODUCTION

60

61 *De novo* mutations of the Cyclin-dependent kinase-like 5 (*CDKL5*) gene lead to a rare X-linked
62 genetic disorder (Weaving et al., 2004). *CDKL5* deficiency disorder (CDD) patients exhibit
63 among a broad spectrum of clinical signs severe deficits in motor coordination, abnormalities
64 in tactile and visual perception, and autistic traits. In CDD individuals, defective sensory
65 processing in primary cortical areas is thought to be involved in the loss of purposeful hands
66 movement and the progressive appearance of dyspraxia or apraxia, until walking is completely
67 prevented (Bahi-Buisson and Bienvenu, 2011). Sensory defects such visual and auditory
68 impairments were recently revealed in CDD mouse models (Wang et al., 2012; Amendola et
69 al., 2014; Trazzi et al., 2016; Mazziotti et al., 2017). Moreover, CDD mouse models exhibit
70 autistic-like features such as profoundly altered social interaction (Wang et al., 2012; Jhang et
71 al., 2017; Yennewar et al., 2019).

72 *CDKL5* mouse models offer a valuable opportunity to assess the cellular and molecular
73 mechanisms underlying abnormal computation of sensory inputs in CDD (Wang et al., 2012;
74 Amendola et al., 2014; Trazzi et al., 2016; Mazziotti et al., 2017). *CDKL5* is a serine/threonine
75 kinase that is highly expressed in the central nervous system. *CDKL5* localizes both in the
76 cytoplasm and nucleus in a brain region and development-dependent fashion (Hector et al.,
77 2016). *CDKL5* was also found to be present in excitatory postsynaptic structures, where it
78 regulates dendritic spine maturation and growth, and controls excitatory synaptic function
79 (Della Sala et al., 2016; Ricciardi et al., 2012). Synaptic localization of *CDKL5* is mediated by
80 its interaction with the palmitoylated form of postsynaptic density protein 95 (PSD-95) (Zhu
81 et al., 2013; Zhang et al., 2014). Our recent investigations in *CDKL5*-KO mice have disclosed
82 that *CDKL5* controls the molecular organization of excitatory synapses, the turnover of
83 dendritic spines, and the excitatory-inhibitory balance of intrinsic circuits in somatosensory
84 and visual cortical areas as well as the establishment of parvalbumin-expressing interneurons
85 (PV⁺ INs) assembly (Della Sala et al., 2016; Pizzo et al., 2016).

86 Nevertheless, whilst we have started to elucidate how *CDKL5* regulates the molecular
87 composition and structure of cortical synapses, no data are yet available about the role of
88 *CDKL5* either in the organization of thalamocortical (TC) projections, which deliver incoming
89 sensory stimuli to the cortex (Petersen, 2007), or in experience-dependent synaptic plasticity
90 *in vivo*. This information is particularly important as an emerging view suggests that altered
91 encoding of sensory inputs during development is not only critical for sensory-motor responses,
92 but also underlies anxiety and atypical social behavior (Orefice et al., 2016). Notably, mounting
93 evidences posit that autistic features may stem from impaired sensory processing (Robertson

94 and Baron-Cohen, 2017). The vast majority of autistic patients, including individuals affected
95 by fragile X syndrome (FXS), a syndromic form of autism-spectrum disorder (ASD), reported
96 abnormal sensory perception (Sinclair et al., 2015; Robertson and Baron-Cohen, 2017).
97 Therefore, in the present study, we first aimed at addressing how CDKL5 regulates the TC
98 connectivity in the barrel cortex (BC) of the mouse brain which relays tactile information from
99 the whiskers to layer IV for further processing (Feldmeyer et al., 2013). Furthermore, since
100 sensory experience shapes and optimizes neural circuits information processing by promoting
101 structural and functional changes (Holtmaat and Svoboda, 2009), we investigated CDKL5
102 involvement in sensory-induced TC plasticity. Importantly, previous studies have revealed that
103 whisker-to-BC pathway can undergo experience-induced plasticity also in adulthood (Fox et
104 al., 2002; Feldman and Brecht 2005; Yang et al., 2009; Yu et al., 2012; Chung et al., 2017).
105 Finally by employing a whisker specific behavioural test, we assessed that the lack of CDKL5
106 also impact tactile sensory responses.

107

108 **EXPERIMENTAL PROCEDURES**

109

110 **Animals**

111 Animal care and handling throughout the experimental procedures were conducted in
112 accordance with European Community Council Directive 86/609/EEC for care and use of
113 experimental animals, with protocols approved by the Italian Minister for Scientific Research
114 (Authorization number 175/2015-PR) and the Bioethics Committee of the University of Torino.
115 Animal suffering was minimized, as was the number of animals used. Mice for testing were
116 obtained by crossing $Cdk15^{-/+}$ females with $Cdk15^{-/y}$ males and $Cdk15^{-/+}$ females with $Cdk15^{+/y}$
117 males (Amendola et al., 2014). Littermate controls were used for all the experiments. After
118 weaning, mice were housed three to five per cage on a 12 h light/dark cycle (lights on at 7:00
119 h) in a temperature-controlled environment ($21 \pm 2^\circ\text{C}$) with food and water provided *ad libitum*.
120 Six-week old male $Cdk15^{-/y}$ mice and wild-type (WT) littermates were used throughout the
121 study. Mice allocation to experimental groups was randomized by assigning random numbers
122 to animals. Mice were included in the experimental groups only if displayed normal body
123 weight and coat appearance indicating overall good health.

124

125 **Sensory enrichment**

126 Sensory enrichment was performed by placing the mice for two days in standard cages
127 containing strings of plastic beads hanging from the top. The positions of bead strings were
128 changed daily. Animals were free to navigate in the cage through the strings of beads which
129 passively stimulated animals' whiskers. Animals housed both in the sensory enriched and
130 standard environment were provided with food and water *ad libitum* (Yang et al., 2009).

131

132 **Immunofluorescence**

133 Animals were anesthetized with an intraperitoneal injection of Zoletil/Xylazine (Sigma-
134 Aldrich) and transcardially perfused, first with ~ 10 ml PBS and then with ice-cold
135 paraformaldehyde [4% in 0.1 M phosphate buffer (PB), pH 7.4]. After perfusion, the brains
136 were then dissected and kept in the same fixative solution overnight at 4°C . Afterwards, brains
137 were cryoprotected by immersion in 10, 20, and 30% sucrose-PB solutions, cut in $30\ \mu\text{m}$
138 sections with a cryostat and stored at -20°C in a solution containing 30% ethylene glycol and
139 25% glycerol until use. For immunofluorescence processing, after several PBS rinses
140 cryosections were kept in a solution containing 0.05% Triton X-100 and 10% normal donkey
141 serum (NDS) in PBS for 1 h, followed by overnight incubation at 25°C with the appropriate
142 primary antibodies (anti-c-fos, 1:500, # 2250S, Cell Signaling Technology; anti-pan-Homer,

143 1:500, # 160-103, Synaptic Systems; anti-VGAT, 1:500, # 131-002, Synaptic Systems; anti-
144 VGluT1, 1:5000, # 5905, Millipore; anti-VGluT2 1:2000, # 2251, Millipore). Antibodies were
145 diluted in PBS with 3% NDS and 0.05 Triton X-100. The following day, the sections were
146 washed and incubated with suitable fluorescent secondary antibodies (1:1000; Jackson
147 ImmunoResearch, West Grove, PA, USA) followed by NeuroTrace (1:500, N-21480,
148 Molecular Probes, Eugene, USA) when appropriate. After several PBS rinses, the sections were
149 mounted on gelatin-coated glass slides and coverslipped with Dako fluorescence mounting
150 medium (Dako Italia, Milan, Italy).

151

152 **Immunofluorescence image analysis**

153 All analyses were carried out by an investigator who was blind to the genotype and
154 environmental exposure. The location of the BC was identified using standard indications: 1.1
155 mm posterior to the Bregma and 3.4 mm lateral from the midline (Yang et al., 2009), and
156 cortical layers were identified as in Tomassy et al. (2014). Synaptic puncta in the neuropil were
157 analyzed in 5-10 mice per group as indicated for each experimental dataset in the results section.
158 Stacks of 6 optical sections (0.5 μm Z-step size) were acquired from layer IV with a laser
159 scanning confocal microscope (LSM5 Pascal; Zeiss, Germany) using a 100 \times objective (1.4
160 numerical aperture) and the pinhole set at 1 Airy unit. Synaptic puncta were quantified with the
161 “Multi Points Measure Tool” of Imaris Software (Bitplane, Switzerland). Fluorescent puncta
162 were considered for analysis if they were present in at least two consecutive optical sections,
163 and dots smaller than $0.1 \times 0.1 \mu\text{m}$ in the x-y axes were excluded (Luikart et al., 2005). Co-
164 apposition between postsynaptic Homer⁺ puncta and VGluT1⁺ or VGluT2⁺ axon terminals was
165 assessed by visual inspection in the three orthogonal planes with an Imaris-dedicated tool.
166 Puncta were considered as co-apposed (“synaptic” appositions) when no black pixels were
167 detected between pre- and postsynaptic signals (Morello et al., 2018).

168 To quantify inhibitory synapses on the soma of pyramidal cells, the number of VGAT⁺ boutons
169 outlining the profile of identified pyramidal neurons was divided by the perimeter length
170 measured in NeuroTrace-stained pyramidal cells using ImageJ software.

171 For the analysis of c-Fos⁺ cell density, confocal images of the BC were acquired in at least 3
172 corresponding coronal brain sections from at least 6 animals per group with a 20 \times objective
173 using a 1- μm Z-step. Digital boxes spanning from the pial surface to the corpus callosum were
174 superimposed at matched locations on each coronal section of the BC, and divided into 10
175 equally sized sampling areas (bins; layer I: bin 1; layer II/III: bins 2–3; layer IV: bins 4–5; layer
176 V: bins 6–7; layer VI: bins 8–10). Immunopositive cells were manually counted in each bin.

177

178 **Whisker Nuisance Task (WNT)**

179 Test was conducted as in McNamara et al. (2010) and Chelini et al. (2019) with few
180 modifications. The test was performed at the end of the exposure to the either standard or
181 enriched environment (Fig. 4A). On the five days before the test, animals were let to familiarize
182 with handling of the experimenter and with the empty test cage (33,1 × 15,9 × 13,2 cm)
183 (experimental cage) for one hour each day. Habituation to the novel cage was promoted by
184 placing a small amount of the home-cage bedding overnight: bedding was removed before
185 introducing the mouse into the experimental cage. The same 1h habituation to the test cage and
186 experimenter was performed on the test day prior to the whisker stimulation. The test consisted
187 in a continuous touch of the whiskers with the wooden stick (bilateral stimulation) for three
188 consecutive sessions of 5 minutes (15 min in total) separated by a 1 min pause. The actual
189 stimulation was preceded by a 5 min-sham stimulation where the stick was introduced in the
190 test cage but no contact with the animal's whiskers or body occurred. During the test sessions,
191 animals were scored according to McNamara et al. (2010) and Chelini et al. (2019) scale with
192 few modifications. We monitored 5 different parameters: fearful behaviour, stance, evasion,
193 response to stick and grooming, which were classified from 0 to 2 according to the response (0
194 = absent/typical, 1 = present/light response and 2 = profound/accentuated response). Compared
195 to the scale published by McNamara et al. (2010) three categories were omitted: whisker
196 position and whisking response were not evaluated as they could not be reliably scored in
197 preliminary observations; breathing behaviour (hyperventilation) was also excluded as recent
198 studies revealed basal respiratory abnormalities in Cdk15 mutant mice (Lo Martire et al., 2017;
199 Lee et al., 2018) which might affect breathing responses to whiskers stimulation.

200

201 **Statistical analysis**

202 All data are reported as mean ± SEM, with *n* indicating the number of mice. Statistical analysis
203 was performed using Prism software (Graphpad, La Jolla, CA, USA). Puncta density, c-Fos⁺
204 cells density and behavioural data were analysed using 2-way analysis of variance (ANOVA)
205 with genotype (Cdk15^{+/y}, Cdk15^{-/y}) and environment (SE, EE) as fixed factors and mouse as
206 random factor followed by Fisher's LSD *post hoc* test. The analysis of the temporal progression
207 of WN scores was performed by using 2-way ANOVA with repeated measurements (RM).
208 Mean ± SEM and p values for each analysis are reported in Table 1. From all analyses, outliers
209 values were excluded according to the "identify outliers" function (Method: ROUT, Q = 1%)
210 present in Prism software (Graphpad, La Jolla, CA, USA). Power analysis of the statistical tests
211 was performed using G_Power 3.1.9.2 (RRID:SCR_013726). The power of the statistical tests
212 is reported in Table 2.

213 RESULTS

214

215 **CDKL5 deficiency does not affect the number of axon terminals layer IV of the BC.**

216 To investigate thalamic inputs to the BC, we used an antiserum against the vesicular glutamate
217 transporter 2 (VGluT2), which labels selectively thalamic axon terminals in layer IV (Nahmani
218 & Erisir 2005; Bopp et al., 2017). This analysis revealed no differences of VGluT2⁺ puncta
219 density between WT and mutant mice under non-stimulated conditions (SE) (SE-Cdk15^{+y} vs
220 SE-Cdk15^{-y} $p = 0.53$; $n = 8$). Notably, two days of enriched sensory experience (EE) elicited a
221 similar robust increase in the density of VGluT2⁺ synaptic terminals in both WT and KO mice
222 (Environment, $F_{(1,28)} = 12.26$, $p < 0.01$; SE-Cdk15^{+y} vs EE-Cdk15^{+y} $p < 0.05$; SE-Cdk15^{-y} vs
223 EE-Cdk15^{-y} $p < 0.05$; EE-Cdk15^{+y} vs. EE-Cdk15^{-y} $p = 0.55$; $n = 8$) (Fig. 1A, B). Thus, these
224 experiments indicate that the deletion of Cdk15 does not affect experience-induced changes in
225 the number of thalamic afferents in the BC.

226 Cortical neurons residing in layer IV, besides being strongly driven by thalamic inputs, receive
227 excitatory synaptic inputs from neighbouring cortical cells (Harris and Mrsic-Flogel 2013;
228 Schoonover et al., 2014). This local circuitry processes and integrates both horizontal and top-
229 down sensory information within a functional column and between neighbouring barrels
230 (Schubert et al., 2003). To investigate intracortical inputs in layer IV, we used an antibody
231 against the vesicular glutamate transporter 1 (VGluT1), that labels selectively intracortical
232 glutamatergic presynapses (Fremeau et al., 2014). We first quantified the overall density of
233 VGluT1⁺ boutons and found no differences between WT and Cdk15 mutants under standard
234 conditions (SE-Cdk15^{+y} vs SE-Cdk15^{-y} $p = 0.89$; $n = 4-5$) and after EE (EE-Cdk15^{+y} vs EE-
235 Cdk15^{-y} $p = 0.18$; $n = 4-5$) (Fig. 1C, D). In contrast to what we found for thalamic afferents, EE
236 did not produce any changes in the density of VGluT1⁺ intracortical presynapses in both
237 genotypes (SE-Cdk15^{+y} vs. EE-Cdk15^{+y} $p = 0.74$; SE-Cdk15^{-y} vs. EE-Cdk15^{-y} $p = 0.22$). Thus,
238 EE increases the density of TC afferents in both WT and Cdk15 mutant animals but does not
239 affect intracortical presynapses.

240

241 **The pre- post-synaptic configuration is atypical in Cdk15^{-y} mice and is restored by** 242 **enriched sensory experience.**

243 We next analysed the apposition of VGluT2 with Homer to evaluate whether thalamic afferents
244 establish correct contacts with postsynaptic targets in layer IV of the BC. Previous studies have
245 demonstrated that Homer is a reliable postsynaptic marker of axospinous contacts (Meyer et
246 al., 2014) indicating that a Homer⁺ immunopunctum faithfully identifies a dendritic spine.
247 Surprisingly, we found that under normal conditions the density of VGluT2-Homer appositions

248 was significantly lower in Cdk15-KO mice when compared to WT littermates (SE-Cdk15^{+/-} vs
249 SE-Cdk15^{-/-} $p < 0.05$; $n = 6$) (Fig. 2A, B). In fact, VGluT2⁺ terminals lacking an identifiable
250 postsynaptic partner were observed in both genotypes but were significantly more abundant in
251 CDKL5 mutants (SE-Cdk15^{+/-} vs SE-Cdk15^{-/-} $p < 0.05$; $n = 6$) (Fig. 2C). Moreover, Cdk15-KO
252 mice exhibited a lower density of afferent terminals establishing multiple contacts with Homer⁺
253 spines (SE-Cdk15^{+/-} vs SE-Cdk15^{-/-} $p < 0.05$; $n = 6$) (Fig. 2C). These observations indicate that
254 the formation and/or stabilization of axospinous TC synapses in layer IV is affected by deletion
255 of Cdk15.

256 We then investigated the effects of EE on TC axospinous appositions. In WT mice, EE led to
257 an increase in the density of VGluT2⁺ terminals contacting Homer⁺ spines (SE-Cdk15^{+/-} vs EE-
258 Cdk15^{+/-} $p < 0.05$; $n = 6$) (Fig. 2B). However, the percentage of terminals lacking a Homer⁺
259 spine as well as that of terminals contacting multiple Homer⁺ spines remained unchanged
260 (VGluT2⁺ with 0 Homer⁺: SE-Cdk15^{+/-} vs EE-Cdk15^{+/-} $p = 0.57$; VGluT2⁺ with 2 Homer⁺: SE-
261 Cdk15^{+/-} vs EE-Cdk15^{+/-} $p = 0.48$, $n = 6$) (Fig. 2C). These data suggest that in WT animals EE
262 causes an overall increase in the number of thalamo-cortical synapses, without altering the
263 configuration of axo-spinous contacts. In contrast, EE promoted a clear remodelling of TC
264 connections in mutant mice. In fact, not only the density of VGluT2-Homer appositions
265 increased to WT levels after EE (SE-Cdk15^{-/-} vs EE-Cdk15^{-/-} $p < 0.05$; EE-Cdk15^{+/-} vs EE-
266 Cdk15^{-/-} $p = 0.08$, $n = 6$) (Fig. 2B), but this was also accompanied by a significant decrease in
267 the percentage of terminals without a Homer⁺ spine (SE-Cdk15^{-/-} vs EE-Cdk15^{-/-} $p < 0.05$) (Fig.
268 2C). These data indicate that sensory stimulation triggers the remodeling of TC connections in
269 Cdk15^{-/-} mice, restoring a synaptic configuration similar to WT condition.

270 Next, we examined the organization of CC connections by assessing the number of VGluT1-
271 Homer appositions. As for the TC contacts, the organization of CC synapses was altered in
272 Cdk15 mutants, both under SE and after EE. Under basal conditions the density of VGluT1-
273 Homer appositions was significantly lower in Cdk15-KO mice when compared to WT
274 littermates (SE-Cdk15^{+/-} vs SE-Cdk15^{-/-} $p < 0.05$; $n = 5$) (Fig. 2D, E). Accordingly, Cdk15
275 mutants showed a higher percentage of terminals without a Homer⁺ spine (SE-Cdk15^{+/-} vs SE-
276 Cdk15^{-/-} $p < 0.01$) as well as a lower percentage of terminals contacting two or more spines (SE-
277 Cdk15^{+/-} vs SE-Cdk15^{-/-} $p < 0.05$) (Fig. 2F). Moreover, whereas EE did not promote any change
278 in the density or configuration of axo-spinous synapses in WT mice (SE-Cdk15^{+/-} vs EE-
279 Cdk15^{+/-} $p = 0.42$; $n = 5$) (Fig. 2E, F), whiskers stimulation produced a profound effect on
280 synaptic organization in the mutants, normalizing both density (SE-Cdk15^{-/-} vs EE-Cdk15^{-/-} $p <$
281 0.05 ; $n = 5$) (Fig. 2E) and configuration (VGluT2⁺ with 0 Homer⁺: EE-Cdk15^{+/-} vs EE-Cdk15^{-/-}
282 $p = 0.35$; Fig. 2F) of VGluT1⁺-Homer⁺ axo-spinous synapses.

283 Altogether, these data indicate that sensory stimulation promotes plasticity of both TC and CC
284 synapses in layer IV of Cdk15 mutants, restoring a connectivity configuration similar to control
285 levels.

286

287 **Lack of CDKL5 does not affect the number of inhibitory synapses in layer IV of the BC.**

288 Manipulation of whisker activity was reported to lead to functional rearrangements of inhibitory
289 circuits in layer IV of the BC, including modifications in the number of GABAergic synapses
290 targeting excitatory neurons (Jiao et al., 2006; Knott et al., 2002) as well as in the intrinsic
291 properties of interneurons (Sun, 2009). Despite these previous observations, we estimated that
292 the density of GABAergic synapses targeting the cell body of pyramidal neurons was similar
293 in Cdk15-KO and WT mice and was not influenced by EE (Genotype, $F_{(1,17)} = 12.26$, $p = 0.86$;
294 environmental exposure, $p = 0.43$; genotype x environmental exposure, $p = 0.47$; $n = 5$) (Fig.
295 3A, B). Moreover, there were no differences in the density of inhibitory synapses between WT
296 and KO mice also in the neuropil of layer IV both under SE (SE-Cdk15^{+y} vs SE-Cdk15^{-y} $p =$
297 0.70 ; $n = 5$) (Fig. 3C, D) and after EE (SE-Cdk15^{+y} vs EE-Cdk15^{+y} $p = 0.77$; SE-Cdk15^{-y} vs
298 EE-Cdk15^{-y} $p = 0.87$; EE-Cdk15^{+y} vs EE-Cdk15^{-y} $p = 0.63$; $n = 5$). Thus, these data show that
299 deletion of CDKL5 does not induce major basal or activity-dependent structural rearrangements
300 of GABAergic circuitry in layer IV of BC.

301

302 **Cdk15-KO mice show abnormal behavioural responses to whisker stimulation.**

303 To investigate the functional impact of the synaptic defects revealed in the BC of Cdk15-KO
304 mice, we probed mice with a behavioural paradigm, the WNT (McNamara et al., 2010; Learoyd
305 et al., 2012; Fontes-Dutra et al 2018; Chelini et al, 2019), designed to test whisker-related
306 somatosensory responses in mice. During the three 5-min sessions of whisker stimulation, 5
307 different behaviours were scored (see Materials and Methods). In the SE exposed animals no
308 differences between genotypes were detected during the sham session, when the wooden stick
309 was presented in close proximity of the animal's head without any tactile contact (SE-Cdk15^{+y}
310 vs SE-Cdk15^{-y} $p = 0.21$; $n = 9$). Moreover, no differences in the total WN scores were found
311 between WT and mutant mice (SE-Cdk15^{+y} vs SE-Cdk15^{-y} $p = 0.21$; $n = 9$) (Fig. 4B).
312 Intriguingly, when analyzed the temporal progression of the scores across trials, we found that
313 KO mice exhibited statistically significant lower scores only in the first trial (SE-Cdk15^{+y} vs
314 SE-Cdk15^{-y} $p < 0.01$; $n = 9$) (Fig. 4D), whilst no difference was not detected in trials 2 and 3
315 (Trial 2: SE-Cdk15^{+y} vs SE-Cdk15^{-y} $p = 0.60$; Trial 3: SE-Cdk15^{+y} vs SE-Cdk15^{-y} $p = 0.63$; n
316 $= 9$). This was due to a significant reduction of the overall responses shown by the WT mice
317 across trials (SE-Cdk15^{+y}, Trial 1 vs Trial 3, $p < 0.05$; $n = 9$), whereas in Cdk15 mutants WN

318 scores were unchanged (SE-Cdk15^{-y}, Trial 1 vs Trial 3, $p = 0.07$; $n = 9$). The exposure to EE
319 did not produce changes in the overall WN score both in WT (SE-Cdk15^{+y} vs EE-Cdk15^{+y} $p =$
320 0.18 ; $n = 9$) and mutant mice (SE-Cdk15^{-y} vs EE-Cdk15^{-y} $p = 0.32$; $n = 9$) (Fig. 4B), a trend
321 that was maintained in the analysis across trials (SE-Cdk15^{+y} vs EE-Cdk15^{+y}, Trail 1 $p = 0.96$;
322 Trail 2 $p = 0.25$; Trail 3 $p = 0.08$; SE-Cdk15^{-y} vs EE-Cdk15^{-y} Trail 1: $p = 0.89$; Trail 2 $p = 0.51$;
323 Trail 3 $p = 0.11$; $n = 9$) (Fig. 4C). However, the reduction of responses from the first to the
324 third trial shown by SE WT was not detected in EE WT mice (EE-Cdk15^{+y}, Trial 1 vs Trial 3,
325 $p = 0.86$; $n = 9$) (Fig. 4E). Interestingly, the EE-KO mice exhibited an inter-trial increase of the
326 responses (EE-Cdk15^{-y}: Trial 1 vs Trial 3 $p < 0.05$; $n = 9$) (Fig. 4F). Finally, we individually
327 analyzed the responses for the five parameters used to calculate the overall WN score.
328 Interestingly, we found that under basal conditions Cdk15 mutant mice displayed reduced
329 values for both freezing behaviour (SE-Cdk15^{+y} vs SE-Cdk15^{-y} $p < 0.05$; $n = 9$) (Fig. 4H) and
330 stance (SE-Cdk15^{+y} vs SE-Cdk15^{-y} $p < 0.001$) (Fig. 4I), while scores for both stick responses
331 (SE-Cdk15^{+y} vs SE-Cdk15^{-y} $p < 0.01$) (Fig. 4J) and grooming were increased (SE-Cdk15^{+y} vs
332 SE-Cdk15^{-y} $p < 0.05$) (Fig. 4L) compared to WT animals. No differences were detected for
333 evasion scores (SE-Cdk15^{+y} vs SE-Cdk15^{-y} $p = 0.29$) (Fig. 4K). EE did not induce major
334 changes in the WT mice (freezing: SE-Cdk15^{+y} vs EE-Cdk15^{+y} $p = 0.09$; stance: SE-Cdk15^{+y}
335 vs EE-Cdk15^{+y} $p = 0.39$; stick response: SE-Cdk15^{+y} vs EE-Cdk15^{+y} $p = 0.48$; grooming: SE-
336 Cdk15^{+y} vs EE-Cdk15^{+y} $p = 0.13$; $n = 9$) (Fig. 4H-L) beside a reduction in the evasion levels
337 (evasion: SE-Cdk15^{+y} vs EE-Cdk15^{+y} $p < 0.05$). On the other hand, EE led to robust changes
338 in the KO animals behaviours: indeed, EE suppressed almost all behavioural abnormalities
339 shown by SE mutants, except grooming (freezing: SE-Cdk15^{-y} vs EE-Cdk15^{-y} $p < 0.01$; stance:
340 SE-Cdk15^{-y} vs EE-Cdk15^{-y} $p < 0.01$; stick response: SE-Cdk15^{-y} vs EE-Cdk15^{-y} $p < 0.05$;
341 grooming: SE-Cdk15^{-y} vs EE-Cdk15^{-y} $p = 0.16$; $n = 9$) (Fig. 4H-L).

342

343 **The activation of c-Fos is reduced in the BC of Cdk15-KO mice following whisker** 344 **stimulation.**

345 It has been previously reported that whisker tactile stimulation can produce an increase of c-
346 Fos expression, an established marker of neuronal activity, in the BC (Filipkowski et al., 2000;
347 Lecrux et al., 2017; Chelini et al., 2019). To assess tactile-induced neuronal activity in the BC,
348 we investigated the levels of expression of c-Fos 2 hours after the end of WNT administration.
349 Two-way analysis revealed a significant Genotype x Environment effect (two-way ANOVA,
350 Genotype x Environment, $F_{(1,22)} = 9.47$, $p < 0.01$; Genotype, $F_{(1,22)} = 0.84$, $p = 0.37$;
351 Environment, $F_{(1,22)} = 0.01$, $p = 0.91$; $n = 6-8$). Fisher's LSD post-hoc test showed a reduced

352 expression of c-Fos in BC layer IV of Cdk15-KO mice compared to WT animals under SE (SE-
353 Cdk15^{+y} vs SE-Cdk15^{-y} $p < 0.05$; $n = 6$) (Fig. 5A, B), indicating that loss of CDKL5 produces
354 an impairment in stimuli-induced neuronal activation. Importantly, exposing mutants to EE
355 increased c-Fos immunoreactivity in the BC (layer IV: SE-Cdk15^{-y} vs EE-Cdk15^{-y} $p < 0.001$;
356 $n = 6-8$), to levels similar to SE-WT mice. Conversely, EE-WT mice showed a robust reduction
357 in c-Fos⁺ cells in layers II-III compared to SE animals (SE-Cdk15^{+y} vs EE-Cdk15^{+y} $p < 0.05$;
358 $n = 6$) and IV (SE-Cdk15^{+y} vs EE-Cdk15^{+y} $p < 0.01$; $n = 6$).

359 Overall, the present data suggests that the defective connectivity due to CDKL5 loss might
360 underlie an abnormal cortical delivery and processing of tactile stimuli and, importantly, that
361 enhancement of sensory experience might have, to some extent, beneficial effects on both
362 cortical circuitry organization and responsiveness.

363

364 **DISCUSSION**

365 In this study, we investigated the synaptic organization of BC circuits underlying tactile sensory
366 processing in a mouse model of CDD where we analysed structural and behavioural plasticity
367 produced by prolonged sensory stimulation. Our investigation has several major findings.

368 First, the absence of CDKL5 leads to altered numerosity and configuration of both TC and CC
369 synapses as illustrated by a higher percentage of solitary presynaptic terminals, even though the
370 number of both thalamic afferents targeting layer IV neurons and CC excitatory terminals was
371 preserved. Second, these synaptic abnormalities were associated with both reduced neuronal
372 activation and altered whisker-stimulation mediated behavioural responses. Third, we
373 demonstrated that a two-day long sensory stimulation promoted structural synaptic plasticity in
374 both WT and mutant mice, restoring in Cdkl5-KOs the density and configuration of VGluT2⁺
375 and VGluT1⁺ axospinous synapses, neuronal activation and whisker-stimulation related
376 behavioural responses.

377

378 The normal density of VGluT2⁺ and VGluT1⁺ terminals in cortical layer IV that we report in
379 adult mutant mice suggests that CDKL5 deficiency has no detrimental impact on the ability of
380 thalamic and cortical afferents to reach their targets. Rather, the reduced density of axo-spinous
381 contacts in layer IV points out that CDKL5 may be involved in the stabilization phase of
382 excitatory synapses by regulating the postsynaptic molecular mechanisms responsible for spine
383 maturation and trans-synaptic stabilization. Accordingly, previously published data indicate
384 that, in the somatosensory cortex of Cdkl5-KO mice, dendritic spines show reduced spine-head
385 size together with abnormally increased turnover (Della Sala et al., 2016), pointing to a reduced
386 ability of forming stable synapses. There are several molecular mechanisms that can explain
387 how loss of CDKL5 produces these synaptic alterations. One of the first identified CDKL5
388 interactors is PSD-95 (Zhu et al., 2013), a postsynaptic scaffolding protein that stabilizes newly-
389 formed synaptic contacts (Taft and Turrigiano, 2014) by regulating molecular interactions at
390 both the postsynaptic (Béique et al., 2006; Chen et al., 2011; Ehrlich et al., 2007; El-Husseini
391 et al., 2000) and presynaptic compartment (Futai et al., 2007; Hruska et al., 2015). Deletion of
392 CDKL5 was reported to reduce the synaptic expression of PSD-95 in the neocortex (Della Sala
393 et al., 2016; Pizzo et al., 2016) and to prevent the phosphorylation of the synaptic adhesion
394 molecule NGL-1, thus leading to defects in synapse maturation and stabilization (Ricciardi et
395 al., 2012). Accordingly, the reduced connectivity with Homer⁺ structures, which represent
396 structurally and functionally mature spines, in the BC of Cdkl5 mutants further supports a
397 primary role of CDKL5 in the anatomical and functional integrity of dendritic spines and is

398 suggestive of an excessive number of immature and/or silent - i.e.: with impaired
399 neurotransmission capabilities - synaptic contacts.

400 Recent findings indicate that CDKL5 may regulate dendritic spines not only by interacting with
401 PDS-95 but also by directly binding with microtubule-associated proteins such as the IQ Motif
402 Containing GTPase Activating Protein 1 (IQGAP1) (Barbiero et al., 2017) and MAP1S, EB2
403 and ARHGEF2 (Baltussen et al., 2018). Furthermore, CDKL5 might regulate dendritic spines
404 composition and function by controlling the transcriptional levels of key components of
405 dendritic spines. Indeed, Trazzi et al. (2016) reported that CDKL5 phosphorylates HDAC4 thus
406 preventing HDAC4 translocation into the nucleus and promoting the expression of genes
407 essential for synaptic transmission and information processing such as CamKII α and SNAP25
408 (Sando III et al., 2012).

409 Our anatomical findings suggest that the delivery of sensory inputs to the cortex might be less
410 efficient in Cdk15 mutants. Indeed, WNT-induced cortical activation was profoundly reduced
411 in Cdk15 KO mice as well as their behavioural responses to whisker stimulation. Reduced
412 cortical responsiveness to visual and auditory stimuli were previously reported in Cdk15
413 mutants (Mazziotti et al., 2017; Wang et al., 2012). Remarkably, several animal models of ASD,
414 such as fragile X mental retardation protein 1 (Fmr1), Engrailed-2 (En2), and (Mecp2) mutant
415 mice, exhibit abnormal sensitivity to somatosensory stimuli (Chelini et al., 2018; He et al.,
416 2017; Orefice et al., 2016; Zhang et al., 2014), together with functional connectivity defects in
417 sensory brain areas (Chelini et al., 2018; Haberl et al., 2015; Lee et al., 2017; Zerbi et al., 2018).

418 The analysis of WNT parameters further revealed that the altered scores obtained by Cdk15 KO
419 mice were mainly due to lower values for freezing and stance, suggestive of reduced fear. This
420 idea is supported by the deficits in a fear-conditioning paradigm shown by Cdk15 null mice
421 (Vigli et al., 2018, Wang et al., 2012). As a recent study on a mouse model of ASD revealed
422 that impaired BC connectivity is paralleled by disruption of tactile stimuli processing, amygdala
423 activation and fear behaviour (Chelini et al., 2019), we hypothesise that similar abnormalities
424 may underlie fear reduction in Cdk15 KO mice. In contrast, Cdk15 mutants exhibited higher
425 scores for stick response and grooming. Many evidences have pointed out that increased
426 grooming activity may reflect an atypical activation of the striatum (Kalueff et al., 2016) which
427 integrates sensory inputs, including tactile stimuli, processed in the BC (Reig and Silberberg,
428 2014). Given that the striatum and the neocortex are directly and indirectly interconnected in
429 movement-control networks, it is tempting to speculate that increased grooming shown by
430 CDKL5 mutants is produced by altered activity of the striatum or of other brain regions
431 functionally connected with the BC. Moreover, the analysis of the temporal progression of
432 WNT scores revealed, in agreement with a previous study (Chelini et al., 2019), that WT mice

433 exhibited a reduction in the WNT scores across trials, indicating that mice tend to habituate to
434 repeated whisker stimulations. In contrast, mutants did not show any habituation suggesting
435 that Cdkl5 is important for adaptation processes. A similar atypical habituation to whisker
436 stimulation was shown by Fmrp KO mice (He et al., 2017), suggesting that sensory impairments
437 associated with both FXS and CDD may stem from common mechanisms. One possibility is
438 that atypical habituation shown by Cdkl5 mutants originates from abnormal sensorimotor
439 gating in response to tactile inputs. This hypothesis is supported by deficits in prepulse
440 inhibition (PPI) of startle reflex in response to acoustic stimuli displayed by Cdkl5-null mice
441 (Vigli et al., 2018). Intriguingly, atypical sensorimotor gating in a tactile prepulse inhibition
442 test was revealed also in other models of ASD such as Mecp2, Fmr1 and Gabrb3 mutants,
443 further supporting the concept that impairments in mechanisms underlying cortical processing
444 of sensory information may contribute to ASD disorders (Orefice et al., 2016; Zhang et al.,
445 2014).

446 Besides impairments affecting glutamatergic connectivity, atypical processing and integration
447 of sensory inputs in Cdkl5-KO mice can derive from changes in the GABAergic system
448 (Griffen and Maffei, 2014). However, in our settings, no apparent defects were detected in
449 GABAergic circuits in Cdkl5-KO and WT. This is somehow surprising given our previous
450 evidences in primary visual cortex, where deficiency of CDKL5 caused an increased density of
451 VGAT⁺ puncta, including those establishing perisomatic synapses (Pizzo et al., 2016). One
452 possible explanation for these discrepancies is that the impact of Cdkl5 deficiency on
453 GABAergic circuitry is layer and region specific. A similar region-specific alteration of
454 GABAergic circuitry has been reported in Mecp2-KO mice. In this mouse model, a robust
455 enhancement of GABAergic transmission and connectivity was observed in S1 (Dani et al.,
456 2005) and V1 (Durand et al., 2012), leading to a shift in the balance between excitation and
457 inhibition in favour of the latter and to reduced cortical activity; on the other hand, no effects
458 of MeCP2 deletion on inhibitory synaptic currents were found in the medial prefrontal cortex
459 (Sceniak et al., 2016). Therefore CDKL5, like Mecp2, may differentially regulate the
460 organization of GABAergic systems, leading to different functional outcomes in distinct neural
461 circuits.

462 TC projections are very plastic in adulthood and can undergo extensive experience-dependent
463 rewiring (Oberlaender et al., 2012; Yu et al., 2012). For instance, prolonged whiskers
464 stimulation was proven to increase axospinous synapses in BC layer IV (Knott et al., 2002),
465 whereas whiskers trimming induced a reduction in the overall density of TC synapses in adult
466 rat barrel cortex (Wimmer et al., 2010; Oberlaender et al., 2012). In agreement with these
467 observations, we found that EE produced in WT mice an increase in the density of TC synapses.

468 In contrast, we did not detect any changes in CC glutamatergic synapses in EE WT animals.
469 Notably, manipulations of sensory inputs may modify the strength of excitatory connections by
470 adjusting the size of axonal inputs, dendritic spines and interposed contact zones, without
471 affecting synapse number (Cheetham et al., 2007; 2014). Interestingly, the EE-induced increase
472 of TC connections was paralleled by a striking reduction in cortical activity as revealed by the
473 c-Fos staining. We speculate that this effect may arise from sensory adaptation mechanisms
474 (Whitmire and Stanley, 2016). Indeed, prolonged whisker stimulation was shown to decrease
475 neuronal responsivity of layer IV neurons to whisker deflection (Chung et al., 2002; Knott et
476 al., 2002; Khatri et al., 2004; Katz et al., 2006; Quairiaux et al., 2007). Neuronal adaptation to
477 repeated sensory stimulation occurs across all sensory modalities and governs crucial processes
478 such as stimulus detection and discriminability (Castro-Alamancos, 2004; Ollerenshaw et al.,
479 2014). Notably, EE did not promote major changes in WNT-related behaviours indicating no
480 EE-mediated alterations in tactile stimuli processing. However, in contrast to SE-WT animals,
481 no behavioural habituation during WNT was found in the EE-WT mice. We suspect that the
482 increased tactile stimuli experienced during the 2-day long exposure to an enriched environment
483 promoted behavioural habituation to whisker-mediated sensory inputs, thus reducing the
484 adaptation extent potentially elicited by WNT. The decrease in the evasion scores, suggestive
485 of reduced flight responses, supports this notion.

486 Importantly, we showed that stimulation-induced synaptic plasticity was retained in Cdk15
487 mutant mice, even though it was qualitatively different from that occurring in WT mice. Indeed,
488 EE was able to induce not only an increase in the number and a reorganization of TC synapses
489 but also an additional restructuring of intracortical connectivity, leading to a restoration of CC
490 connections to WT levels. Furthermore, exposure to EE re-established neuronal activity levels
491 in BC of mutants. These pieces of evidence are particularly important as they suggest that
492 sensory stimulation might elicit a more general reorganization of circuitry in the mutants,
493 possibly leading to the alleviation of phenotypic abnormalities. Indeed, EE exposure caused a
494 correction in most of the behavioural WTN parameters which were altered in Cdk15-KO mice.
495 Accordingly, the enhancement of sensory experience was proven to be beneficial for both the
496 synaptic and behavioural deficits affecting mouse models of pervasive neurodevelopmental
497 disorders, such as *Mecp2* and *FMR1* KO mice (Kerr et al., 2010; Kondo et al., 2008; Lonetti et
498 al., 2010; Restivo et al., 2005).

499 Overall, our present findings disclose for the first time a primary role of CDKL5 in mechanisms
500 involved in the formation and maintenance of cortical circuitry underlying tactile sensory
501 information processing. Together with our behavioural analyses, showing atypical whisker-
502 mediated responses, these data suggest that sensory defects may underlie the appearance of

503 ASD traits in CDD. Moreover, our data posit that affected cortical circuits can effectively
504 undergo structural remodelling: importantly, the re-establishment of correct cortical circuitry
505 by sensory enhancement, paralleled by improvements in behavioural responses to sensory
506 stimuli, strongly supports the use of enhanced sensory stimulation as a therapeutic strategy for
507 CCD patients.

508

509 **ACKNOWLEDGEMENTS**

510 The authors are grateful to Dr. Francesco Libera and Prof. Yuri Bozzi for precious technical
511 assistance and fruitful advices. The work was funded by L'Albero di Greta [Albero di Greta-
512 Giustetto2018], Fondazione Telethon [GGP15098B], International Foundation for CDKL5
513 Research [IFCR-Giustetto2019] and Association Française du Syndrome de Rett [ASFR-
514 Giustetto 2017].

515

516 **REFERENCES**

- 517 Amendola E, Zhan Y, Mattucci C, Castroflorio E, Calcagno E, Fuchs C, Lonetti G, Silingardi
518 D, Vyssotski AL, Farley D, Ciani E, Pizzorusso T, Giustetto M, Gross CT (2014) Mapping
519 pathological phenotypes in a mouse model of CDKL5 disorder. *PLoS One* 9 (5): e91613.
- 520 Bahi-Buisson N, Bienvenu T (2012) CDKL5-related disorders: from clinical description to
521 molecular genetics. *Mol Syndromol.* 2 (3-5): 137-152.
- 522 Baltussen LL, Negraes PD, Silvestre M, Claxton S, Moeskops M, Christodoulou E, Flynn HR,
523 Snijders AP, Muotri AR, Ultanir SK (2018) Chemical genetic identification of CDKL5
524 substrates reveals its role in neuronal microtubule dynamics. *EMBO J.* 37 (24). pii: e99763.
- 525 Barbiero I, Peroni D, Tramarin M, Chandola C, Rusconi L, Landsberger N, Kilstrup-Nielsen
526 C (2017) The neurosteroid pregnenolone reverts microtubule derangement induced by the loss
527 of a functional CDKL5-IQGAP1 complex. *Hum Mol Genet.* 26 (18): 3520-3530.
- 528 Bèïque JC, Lin DT, Kang MG, Aizawa H, Takamiya K, Huganir RL (2006) Synapse-specific
529 regulation of AMPA receptor function by PSD-95. *Proc Natl Acad Sci U S A.* 103 (51): 19535-
530 40.
- 531 Bopp R, Holler-Rickauer S, Martin KA, Schuhknecht GF (2017) An ultrastructural study of
532 the thalamic input to layer 4 of primary motor and primary somatosensory cortex in the mouse.
533 *J Neurosci.* 37 (9): 2435-2448.
- 534 Castro-Alamancos MA (2004) Dynamics of sensory thalamocortical synaptic networks during
535 information processing states. *Prog Neurobiol.* 74 (4): 213-47.
- 536 Cheetham CE, Barnes SJ, Albieri G, Knott GW, Finnerty GT (2014) Pansynaptic enlargement
537 at adult cortical connections strengthened by experience. *Cereb Cortex.* 24 (2): 521-31.
- 538 Cheetham CE, Hammond MS, Edwards CE, Finnerty GT (2007) Sensory experience alters
539 cortical connectivity and synaptic function site specifically. *J Neurosci.* 27 (13): 3456-65.
- 540 Chelini G, Zerbi V, Cimino L, Grigoli A, Markicevic M, Libera F, Robbiati S, Gadler M,
541 Bronzoni S, Miorelli S, Galbusera A, Gozzi A, Casarosa S, Provenzano G, Bozzi Y (2019)
542 Aberrant somatosensory processing and connectivity in mice lacking *Engrailed-2*. *J Neurosci.*
543 39 (8): 1525-1538

544 Chen X, Nelson CD, Li X, Winters CA, Azzam R, Sousa AA, Leapman RD, Gainer H, Sheng
545 M, Reese TS (2011) PSD-95 is required to sustain the molecular organization of the
546 postsynaptic density. *J Neurosci.* 31 (17): 6329-38.

547 Chung S, Jeong JH, Ko S, Yu X, Kim Y2, Isaac JT4, Koretsky AP (2017) Peripheral sensory
548 deprivation restores critical-period-like plasticity to adult somatosensory thalamocortical
549 inputs. *Cell Rep.* 19 (13): 2707-2717.

550 Chung S, Li X, Nelson SB (2002) Short-term depression at thalamocortical synapses
551 contributes to rapid adaptation of cortical sensory responses in vivo. *Neuron.* 34 (3): 437-46.

552 Dani VS, Chang Q, Maffei A, Turrigiano GG, Jaenisch R, Nelson SB (2005) Reduced cortical
553 activity due to a shift in the balance between excitation and inhibition in a mouse model of Rett
554 syndrome. *Proc Natl Acad Sci U S A.* 102 (35): 12560-5.

555 Della Sala G, Putignano E, Chelini G, Melani R, Calcagno E, Ratto GM, Amendola E, Gross
556 CT, Giustetto M, Pizzorusso T (2016) Dendritic spine instability in a mouse model of CDKL5
557 disorder is rescued by insulin-like growth factor 1. *Biol Psychiatry* 80 (4): 302-11.

558 Durand S, Patrizi A, Quast KB, Hachigian L, Pavlyuk R, Saxena A, Carninci P, Hensch TK,
559 Fagiolini M (2012) NMDA receptor regulation prevents regression of visual cortical function
560 in the absence of *Mecp2*. *Neuron* 76 (6): 1078-90.

561 Ehrlich I, Klein M, Rumpel S, Malinow R (2007) PSD-95 is required for activity-driven
562 synapse stabilization. *Proc Natl Acad Sci U S A.* 104 (10): 4176-81.

563 El-Husseini AE, Schnell E, Chetkovich DM, Nicoll RA, Brecht DS (2000) PSD-95
564 involvement in maturation of excitatory synapses. *Science* 290 (5495): 1364-8.

565 Feldman DE, Brecht M (2015) Map plasticity in somatosensory cortex. *Science* 310 (5749):
566 810-5.

567 Feldmeyer D, Brecht M, Helmchen F, Petersen CC, Poulet JF, Staiger JF, Luhmann HJ,
568 Schwarz C (2013) Barrel cortex function. *Prog Neurobiol.* 103: 3-27.

569 Filipkowski RK, Rydz M, Berdel B, Morys J, Kaczmarek L (2000) Tactile experience induces
570 c-fos expression in rat barrel cortex. *Learn Mem.* 7 (2): 116-22.

571 Fontes-Dutra M, Santos-Terra J, Deckmann I, Brum Schwingel G, Della-Flora Nunes G,
572 Hirsch MM, Bauer-Negrini G, Riesgo RS, Bambini-Júnior V, Hedin-Pereira C, Gottfried C
573 (2018) Resveratrol prevents cellular and behavioral sensory alterations in the animal model of
574 autism induced by valproic acid. *Front Synaptic Neurosci.* 10:9.

575 Fox K (2002) Anatomical pathways and molecular mechanisms for plasticity in the barrel
576 cortex. *Neuroscience* 111 (4): 799-814.

577 Fremeau RT Jr, Kam K, Qureshi T, Johnson J, Copenhagen DR, Storm-Mathisen J, Chaudhry
578 FA, Nicoll RA, Edwards RH (2004) Vesicular glutamate transporters 1 and 2 target to
579 functionally distinct synaptic release sites. *Science* 304 (5678): 1815-9.

580 Futai K, Kim MJ, Hashikawa T, Scheiffele P, Sheng M, Hayashi Y (2007) Retrograde
581 modulation of presynaptic release probability through signaling mediated by PSD-95-
582 neuroligin. *Nat Neurosci.* 10 (2): 186-95.

583 Griffen TC, Maffei A (2014) GABAergic synapses: their plasticity and role in sensory cortex.
584 *Front Cell Neurosci.* 8:91.

585 Haberl MG, Zerbi V, Veltien A, Ginger M, Heerschap A, Frick A (2015) Structural-functional
586 connectivity deficits of neocortical circuits in the *Fmr1*-/*y* mouse model of autism. *Sci Adv.* 1
587 (10): e1500775.

588 Harris KD, Mrsic-Flogel TD (2013) Cortical connectivity and sensory coding. *Nature* 503
589 (7474): 51-8.

590 Hector RD, Dando O, Landsberger N, Kilstrup-Nielsen C, Kind PC, Bailey ME, Cobb SR
591 (2016) Characterisation of CDKL5 transcript isoforms in human and mouse. *PLoS One.* 11 (6):
592 e0157758.

593 He CX, Cantu DA, Mantri SS, Zeiger WA, Goel A, Portera-Cailliau C (2017) Tactile
594 defensiveness and impaired adaptation of neuronal activity in the *Fmr1* knock-out mouse model
595 of autism. *J Neurosci.* 37 (27): 6475-6487.

596 Holtmaat A and Svoboda K (2009) Experience-dependent structural synaptic plasticity in the
597 mammalian brain. *Nat Rev Neurosci.* 10 (9): 647-58.

598 Hruska M, Henderson NT, Xia NL, Le Marchand SJ, Dalva MB (2015) Anchoring and

599 synaptic stability of PSD-95 is driven by ephrin-B3. *Nat Neurosci.* 18 (11): 1594-605.

600 Kalueff AV, Stewart AM, Song C, Berridge KC, Graybiel AM, Fentress JC (2016)
601 Neurobiology of rodent self-grooming and its value for translational neuroscience. *Nat Rev*
602 *Neurosci.* 17 (1): 45-59.

603 Katz Y, Heiss JE, Lampl I (2006) Cross-whisker adaptation of neurons in the rat barrel cortex.
604 *J Neurosci.* 26 (51): 13363-72.

605 Kerr B, Silva PA, Walz K, Young JI (2010) Unconventional transcriptional response to
606 environmental enrichment in a mouse model of Rett syndrome. *PLoS One* 5 (7): e11534.

607 Khatri V, Hartings JA, Simons DJ (2004) Adaptation in thalamic barreloid and cortical barrel
608 neurons to periodic whisker deflections varying in frequency and velocity. *J Neurophysiol.* 92
609 (6): 3244-54.

610 Knott GW, Quairiaux C, Genoud C, Welker E (2002) Formation of dendritic spines with
611 GABAergic synapses induced by whisker stimulation in adult mice. *Neuron* 34 (2): 265-73.

612 Kondo M, Gray LJ, Pelka GJ, Christodoulou J, Tam PP, Hannan AJ (2008) Environmental
613 enrichment ameliorates a motor coordination deficit in a mouse model of Rett syndrome--
614 *Mecp2* gene dosage effects and BDNF expression. *Eur J Neurosci.* 27 (12): 3342-50.

615 Jhang CL, Huang TN, Hsueh YP, Liao W (2017) Mice lacking cyclin-dependent kinase-like
616 5 manifest autistic and ADHD-like behaviors. *Hum Mol Genet.* 26 (20): 3922-3934.

617 Jiao Y, Zhang C, Yanagawa Y, Sun QQ (2006) Major effects of sensory experiences on the
618 neocortical inhibitory circuits. *J Neurosci.* 26 (34): 8691-701.

619 Learoyd AE, Lifshitz J (2012) Comparison of rat sensory behavioral tasks to detect
620 somatosensory morbidity after diffuse brain-injury. *Behav Brain Res* 226:197-204.

621 Lecrux C, Sandoe CH, Neupane S, Kropf P, Toussay X, Tong XK, Lacalle-Aurioles M,
622 Shmuel A, Hamel E (2017) Impact of altered cholinergic tones on the neurovascular coupling
623 response to whisker stimulation. *J Neurosci.* 37 (6): 1518-1531.

624 Lee LJ, Tsytsarev V, Erzurumlu RS (2017) Structural and functional differences in the barrel
625 cortex of *Mecp2* null mice. *J Comp Neurol.* 525 (18): 3951-3961.

626 Lee KZ, Liao W (2018) Loss of CDKL5 disrupts respiratory function in mice. *Respir Physiol*
627 *Neurobiol.* 248: 48-54.

628 Lo Martire V, Alvente S, Bastianini S, Berteotti C, Silvani A, Valli A, Viggiano R, Ciani E,
629 Zoccoli G (2017) CDKL5 deficiency entails sleep apneas in mice. *J. Sleep Res.* 26, 495-497.

630 Lonetti G, Angelucci A, Morando L, Boggio EM, Giustetto M, Pizzorusso T (2010) Early
631 environmental enrichment moderates the behavioral and synaptic phenotype of MeCP2 null
632 mice. *Biol Psychiatry* 67 (7): 657-65.

633 Luikart BW, Nef S, Virmani T, Lush ME, Liu Y, Kavalali ET, Parada LF (2005) TrkB has a
634 cell-autonomous role in the establishment of hippocampal Schaffer collateral synapses. *J*
635 *Neurosci.* 25 (15): 3774-86.

636 Mazziotti R, Lupori L, Sagona G, Gennaro M, Della Sala G, Putignano E, Pizzorusso T (2017)
637 Searching for biomarkers of CDKL5 disorder: early-onset visual impairment in CDKL5 mutant
638 mice. *Hum Mol Genet.* 26 (12): 2290-2298.

639 McNamara KC, Lisembee AM, Lifshitz J (2010) The whisker nuisance task identifies a late-
640 onset, persistent sensory sensitivity in diffuse brain- injured rats. *J Neurotrauma* 27: 695-706.

641 Meyer D, Bonhoeffer T, Scheuss V (2014) Balance and stability of synaptic structures during
642 synaptic plasticity. *Neuron* 82 (2): 430-43.

643 Morello N, Schina R, Pilotto F, Phillips M, Melani R, Plicato O, Pizzorusso T, Pozzo-Miller
644 L, Giustetto M (2018) Loss of Mecp2 causes atypical synaptic and molecular plasticity of
645 parvalbumin-expressing interneurons reflecting Rett Syndrome-like sensorimotor defects.
646 *eNeuro.* 5 (5). pii: ENEURO.0086-18.2018.

647 Nahmani M, Erisir A (2005) VGluT2 immunocytochemistry identifies thalamocortical terminals
648 in layer 4 of adult and developing visual cortex. *J Comp Neurol.* 484 (4): 458-73.

649 Oberlaender M, Ramirez A, Bruno RM (2012) Sensory experience restructures
650 thalamocortical axons during adulthood. *Neuron.* 74 (4): 648-55.

651 Ollerenshaw DR, Zheng HJV, Millard DC, Wang Q, Stanley GB (2014) The adaptive trade-
652 off between detection and discrimination in cortical representations and behavior. *Neuron.* 81
653 (5): 1152-1164.

654 Orefice LL, Zimmerman AL, Chirila AM, Sleboda SJ, Head JP, Ginty DD (2016) Peripheral
655 mechanosensory neuron dysfunction underlies tactile and behavioral deficits in mouse models
656 of ASDs. *Cell* 166 (2): 299-313.

657 Petersen CC (2007) The functional organization of the barrel cortex. *Neuron*. 56 (2): 339-55.

658 Pizzo R, Gurgone A, Castroflorio E, Amendola E, Gross C, Sassoè-Pognetto M, Giustetto M
659 (2016) Lack of CDKL5 disrupts the organization of excitatory and inhibitory synapses and
660 parvalbumin interneurons in the primary visual cortex. *Front Cell Neurosci*. 10:261.

661 Quairiaux C, Armstrong-James M, Welker E (2007) Modified sensory processing in the barrel
662 cortex of the adult mouse after chronic whisker stimulation. *J Neurophysiol*. 97 (3): 2130-47.

663 Reig R, Silberberg G (2014) Multisensory integration in the mouse striatum. *Neuron*. 83 (5):
664 1200-12.

665 Restivo L, Ferrari F, Passino E, Sgobio C, Bock J, Oostra BA, Bagni C, Ammassari-Teule M
666 (2005) Enriched environment promotes behavioral and morphological recovery in a mouse
667 model for the fragile X syndrome. *Proc Natl Acad Sci U S A*. 102 (32): 11557-62.

668 Ricciardi S, Ungaro F, Hambrock M, Rademacher N, Stefanelli G, Brambilla D, Sessa A,
669 Magagnotti C, Bachi A, Giarda E, Verpelli C, Kilstrup-Nielsen C, Sala C, Kalscheuer VM,
670 Broccoli V (2012) CDKL5 ensures excitatory synapse stability by reinforcing NGL-1-PSD95
671 interaction in the postsynaptic compartment and is impaired in patient iPSC-derived neurons.
672 *Nat Cell Biol*. 14 (9): 911-23.

673 Robertson CE and Baron-Cohen S (2017) Sensory perception in autism. *Nat Rev Neurosci*.
674 18 (11): 671-684.

675 Sando R 3rd, Gounko N, Pieraut S, Liao L, Yates J 3rd, Maximov A (2012) HDAC4 governs
676 a transcriptional program essential for synaptic plasticity and memory. *Cell*. 151 (4): 821-834.

677 Sceniak MP, Lang M, Enomoto AC, James Howell C, Hermes DJ, Katz DM (2016)
678 Mechanisms of functional hypoconnectivity in the medial prefrontal cortex of *Mecp2* null mice.
679 *Cereb Cortex*. 26 (5): 1938-56.

680 Schubert D, Kötter R, Zilles K, Luhmann HJ, Staiger JF (2003) Cell type-specific circuits of
681 cortical layer IV spiny neurons. *J. Neurosci*. 23 (7): 2961-70.

682 Schoonover CE, Tapia JC, Schilling VC, Wimmer V, Blazeski R, Zhang W, Mason CA,
683 Bruno RM (2014) Comparative strength and dendritic organization of thalamocortical and
684 corticocortical synapses onto excitatory layer 4 neurons. *J Neurosci.* 34 (20): 6746-58.

685 Sinclair D, Oranje B, Razak KA, Siegel SJ, Schmid S (2017) Sensory processing in autism
686 spectrum disorders and Fragile X syndrome-From the clinic to animal models. *Neurosci*
687 *Biobehav Rev.* 76 (Pt B): 235-253.

688 Sun QQ (2009) Experience-dependent intrinsic plasticity in interneurons of barrel cortex layer
689 IV. *J Neurophysiol.* 102 (5): 2955-73.

690 Taft CE, Turrigiano GG (2013) PSD-95 promotes the stabilization of young synaptic contacts.
691 *Philos Trans R Soc Lond B Biol Sci.* 369 (1633): 20130134.

692 Tomassy GS, Morello N, Calcagno E, Giustetto M (2014) Developmental abnormalities of
693 cortical interneurons precede symptoms onset in a mouse model of Rett syndrome. *Neurochem.*
694 131 (1): 115-27.

695 Trazzi S, De Franceschi M, Fuchs C, Bastianini S, Viggiano R, Lupori L, Mazziotti R, Medici
696 G, Lo Martire V, Ren E, Rimondini R, Zoccoli G, Bartesaghi R, Pizzorusso T, Ciani E (2018)
697 CDKL5 protein substitution therapy rescues neurological phenotypes of a mouse model of
698 CDKL5 disorder. *Hum Mol Genet.* 27 (9): 1572-1592.

699 Trazzi S, Fuchs C, Viggiano R, De Franceschi M, Valli E, Jedynek P, Hansen FK, Perini G,
700 Rimondini R, Kurz T, Bartesaghi R, Ciani E (2016) HDAC4: a key factor underlying brain
701 developmental alterations in CDKL5 disorder. *Hum Mol Genet.* 25 (18): 3887-3907.

702 Vigli D, Rusconi L, Valenti D, La Montanara P, Cosentino L, Lacivita E, Leopoldo M,
703 Amendola E, Gross C, Landsberger N, Laviola G, Kilstrup-Nielsen C, Vacca RA, De Filippis
704 B (2018) Rescue of prepulse inhibition deficit and brain mitochondrial dysfunction by
705 pharmacological stimulation of the central serotonin receptor 7 in a mouse model of CDKL5
706 Deficiency Disorder. *Neuropharmacology.* 144:104-114.

707 Wang IT, Allen M, Goffin D, Zhu X, Fairless AH, Brodtkin ES, Siegel SJ, Marsh ED, Blendy
708 JA, Zhou Z (2012) Loss of CDKL5 disrupts kinome profile and event-related potentials leading
709 to autistic-like phenotypes in mice. *Proc Natl Acad Sci U S A* 109 (52): 21516-21.

710 Weaving LS, Christodoulou J, Williamson SL, Friend KL, McKenzie OL, Archer H, Evans J,

711 Clarke A, Pelka GJ, Tam PP, Watson C, Lahooti H, Ellaway CJ, Bennetts B, Leonard H, Gécz
712 J (2004) Mutations of CDKL5 cause a severe neurodevelopmental disorder with infantile
713 spasms and mental retardation. *Am J Hum Genet.* 75 (6): 1079-93.

714 Whitmire CJ, Stanley GB (2016) Rapid sensory adaptation redux: a circuit perspective.
715 *Neuron.* 92 (2): 298-315.

716 Wimmer VC, Broser PJ, Kuner T, Bruno RM (2010) Experience-induced plasticity of
717 thalamocortical axons in both juveniles and adults. *J Comp Neurol.* 518 (22): 4629-48.

718 Yang G, Pan F, Gan WB (2009) Stably maintained dendritic spines are associated with
719 lifelong memories. *Nature* 462 (7275): 920-4.

720 Yennawar M, White RS, Jensen FE (2019) AMPA receptor dysregulation and therapeutic
721 interventions in a mouse model of CDKL5 Deficiency Disorder. *J Neurosci.* pii: 2041-18.

722 Yu X, Chung S, Chen DY, Wang S, Dodd SJ, Walters JR, Isaac JT, Koretsky AP (2012)
723 Thalamocortical inputs show post-critical-period plasticity. *Neuron.* 74 (4): 731-42.

724 Zerbi V, Ielacqua GD, Markicevic M, Haberl MG, Ellisman MH, A-Bhaskaran A, Frick A,
725 Rudin M, Wenderoth N (2018) Dysfunctional autism risk genes cause circuit-specific
726 connectivity deficits with distinct developmental trajectories. *Cereb Cortex.* 28 (7): 2495-2506.

727 Zhang Y, Bonnan A, Bony G, Ferezou I, Pietropaolo S, Ginger M, Sans N, Rossier J, Oostra
728 B, LeMasson G, Frick A (2014) Dendritic channelopathies contribute to neocortical and sensory
729 hyperexcitability in *Fmr1(-/y)* mice. *Nat Neurosci.* 17 (12): 1701-9.

730 Zhang Y, Matt L, Patriarchi T, Malik ZA, Chowdhury D, Park DK, Renieri A, Ames JB, Hell
731 JW (2014) Capping of the N-terminus of PSD-95 by calmodulin triggers its postsynaptic
732 release. *EMBO J.* 33 (12): 1341-53.

733 Zhu YC, Li D, Wang L, Lu B, Zheng J, Zhao SL, Zeng R, Xiong ZQ (2013) Palmitoylation-
734 dependent CDKL5-PSD-95 interaction regulates synaptic targeting of CDKL5 and dendritic
735 spine development. *Proc Natl Acad Sci U S A* 110 (22): 9118-23.

736

737 **FIGURE LEGENDS**

738 **Figure 1 EE promotes an enhancement of thalamo-cortical connectivity.**

739 **A**, Representative micrographs acquired in the neuropil of BC layer IV, illustrating VGluT2-
740 positive immunofluorescence (red) and NeuroTrace fluorescent Nissl labeling (green) from
741 Cdk15^{+/-} and Cdk15^{-/-} mice exposed to standard (SE) and enriched environment (EE) (scale bar
742 5 μm). **B**, Quantitative analysis of the density of VGluT2-positive puncta shows a significant
743 increase in both WT and Cdk15^{-/-} mice exposed to EE compared to corresponding SE groups (n
744 = 8). **C**, Representative confocal micrographs of BC layer IV neuropil showing
745 immunofluorescence staining for VGluT1 from Cdk15^{+/-} and Cdk15^{-/-} mice kept in standard
746 (SE) and enriched environment (EE) (scale bar 5 μm). **D**, Quantification of the density of
747 VGluT1⁺ puncta revealed no overall changes in the number of intra-cortical excitatory terminals
748 between WT and Cdk15 mutants both under standard and enriched conditions (n = 4-5).
749 Statistical analyses: two-way ANOVA followed by Post hoc Fisher's LSD test, * p < 0.05.

750

751 **Figure 2 EE corrects the thalamo-cortical and the intra-cortical connectivity defects in**
752 **Cdk15^{-/-} mice.**

753 **A**, Representative confocal micrographs of BC layer IV neuropil showing immunofluorescence
754 staining for VGluT2 (red) and Homer (green) from Cdk15^{+/-} and Cdk15^{-/-} mice kept in standard
755 (SE) and enriched environment (EE) (scale bar 5 μm). **B**, Quantitative analysis of the number
756 of VGluT2⁺ terminals juxtaposed to Homer⁺ puncta indicates that EE corrects the reduced
757 VGluT2-Homer apposition index observed in Cdk15^{-/-} mice under standard conditions (n = 6).
758 **C**, Distribution of VGluT2⁺ terminals based on the number of juxtaposed Homer⁺ puncta. Note
759 that under SE KO mice exhibit a higher percentage of solitary VGluT2⁺ terminals (i.e. not
760 facing Homer⁺ puncta; indicated by white arrowheads in **A**), as well as a lower percentage of
761 VGluT2⁺ puncta with two Homer⁺ partners, whereas these differences are not observed
762 following EE (n = 6). **D**, Representative confocal micrographs of BC layer IV neuropil showing
763 VGluT1 (red) and Homer (green) puncta from Cdk15^{+/-} and Cdk15^{-/-} mice housed in standard
764 (SE) and enriched environment (EE) (scale bar 5 μm). **E**, The analysis of the number of
765 VGluT1-Homer appositions shows that EE exposure restores the reduction in cortico-cortical
766 synapses found in Cdk15^{-/-} mice under SE (n = 5). **F**, The distribution analysis of VGluT1⁺
767 terminals, based on the number of juxtaposed Homer⁺ puncta, revealed that SE-mutant mice
768 display a higher percentage of VGluT1⁺ terminals lacking a Homer⁺ postsynaptic partner as
769 well as a lower percentage of VGluT1⁺ puncta with two Homer⁺ counterparts; these differences

770 were abolished by EE (n = 5). Statistical analyses: two-way ANOVA followed by Post hoc
771 Fisher's LSD test, * p < 0.05, ** p < 0.01.

772

773 **Figure 3 No effects of EE on inhibitory GABAergic connectivity in layer IV of the BC.**

774 **A**, Examples of VGAT⁺ puncta contacting the somata of layer IV pyramidal neurons in BC
775 from WT and Cdk15-KO mice (scale bar 5 μm). **B**, Quantitative analysis shows no changes in
776 the density of inhibitory varicosities between WT and mutant mice (n = 5). **C, D**, Representative
777 images acquired in the neuropil of BC layer IV depicting VGAT⁺ staining (**C**) (scale bar 5 μm)
778 and quantitative analysis (**D**) showing no differences in the density of the GABAergic terminals
779 irrespective of both genotype and environmental exposure (n = 5). Statistical analysis: two-way
780 ANOVA followed by Post hoc Fisher's LSD test.

781

782 **Figure 4 Cdk15-KO mice exhibit abnormal responses to repeated whisker stimulation.**

783 **A**, Experimental timeline of the whisker nuisance task (WNT). **B**, Graph showing the total WN
784 scores across trials (n = 9; statistical analysis: two-way ANOVA followed by Post hoc Fisher's
785 LSD test, ** p < 0.01). **C-G**, WN scores recorded for each animal during trials (**C**) and average
786 WN scores assigned to animals during trials (**D-G**) (n = 9; statistical analysis: two-way ANOVA
787 with RM followed by Post hoc Fisher's LSD test, * p < 0.05, ** p < 0.01 between groups (**C-**
788 **G**) and trial 1 vs trial 3 within groups (**D-G**). **H-L**, Mean score across trials for each of the five
789 behavioural parameters (fearful behaviour, stance, response to stick, evasion, grooming)
790 monitored during the WNT (n = 9; statistical analysis: two-way ANOVA followed by Post hoc
791 Fisher's LSD test, * p < 0.05, ** p < 0.01, *** p < 0.001).

792

793 **Figure 5 Cdk15-KO mice exhibit reduced cortical activation to repeated whisker**
794 **stimulation.**

795 **A, B**, Representative examples of c-Fos staining in BC of WT and mutant mice housed in
796 standard and enriched environment (scale bar 50 μm) (**A**) and quantification of the density of
797 c-Fos⁺ cells (**B**) (n = 6-8; statistical analysis: two-way ANOVA followed by Post hoc Fisher's
798 LSD test, * p < 0.05, ** p < 0.01, *** p < 0.001).

799

800 **Table 1. Mean ± SEM values for each statistical analysis.**

Figure		SE		EE		
		Cdk15 ^{+/-y}	Cdk15 ^{-/-y}	Cdk15 ^{+/-y}	Cdk15 ^{-/-y}	
Fig 1B	VGluT2 ⁺ puncta density (puncta/ μm^2)	0.123 ± 0.01	0.11 ± 0.01	0.174 ± 0.02	0.162 ± 0.02	
Fig 1D	VGluT1 ⁺ puncta density (puncta/ μm^2)	0.136 ± 0.01	0.134 ± 0.00	0.131 ± 0.01	0.149 ± 0.01	
Fig 2B	VGluT2 ⁺ - Homer ⁺ appositions density (puncta/ μm^2)	0.117 ± 0.01	0.093 ± 0.01	0.139 ± 0.01	0.120 ± 0.01	
Fig 2C	VGluT2 ⁺ puncta distribution (%)	0 Homer ⁺	26.50 ± 2.12	35 ± 0.84	25 ± 3.74	27 ± 1.38
		1 Homer ⁺	40.67 ± 0.99	42 ± 0.84	39.17 ± 2.64	43.20 ± 1.16
		2 Homer ⁺	27 ± 2.52	20 ± 0.95	28.83 ± 2.59	23.60 ± 0.4
		>2 Homer ⁺	5.67 ± 0.8	3.3 ± 1.2	6.83 ± 1.89	6 ± 1.05
Fig 2E	VGluT1 ⁺ - Homer ⁺ appositions density (puncta/ μm^2)	0.133 ± 0.02	0.10 ± 0.01	0.12 ± 0.01	0.13 ± 0.01	
Fig 2F	VGluT1 ⁺ puncta distribution (%)	0 Homer ⁺	24.51 ± 3.53	36.94 ± 2.84	26.08 ± 1.39	29.96 ± 2.82
		1 Homer ⁺	54.26 ± 3.07	52.17 ± 2.33	56.14 ± 4.99	51.09 ± 1.76
		≥2 Homer ⁺	21.22 ± 3.64	10.9 ± 2.36	17.79 ± 4.38	18.95 ± 2.20
Fig 3B	VGAT ⁺ puncta density on pyramidal neurons (puncta/ μm)	0.54 ± 0.02	0.52 ± 0.02	0.50 ± 0.03	0.52 ± 0.01	
Fig 3D	VGAT ⁺ puncta density (puncta/ μm^2)	0.13 ± 0.00	0.13 ± 0.00	0.13 ± 0.00	0.13 ± 0.00	
Fig 4B	WNT score	Across trials	3.75 ± 0.27	3.38 ± 0.14	4.16 ± 0.20	3.73 ± 0.14
Fig 4C-G		Trial 1	4.19 ± 0.27	3.09 ± 0.19	4.17 ± 0.21	3.14 ± 0.21
		Trial 2	3.75 ± 0.43	3.54 ± 0.17	4.25 ± 0.33	3.77 ± 0.22
		Trial 3	3.31 ± 0.37	3.50 ± 0.19	4.08 ± 0.44	4.04 ± 0.26
Fig 4H	Fearful behaviour	0.50 ± 0.11	0.11 ± 0.05	0.89 ± 0.21	0.59 ± 0.16	
Fig 4J	Stance	1.45 ± 0.19	0.58 ± 0.14	1.69 ± 0.12	1.27 ± 0.18	
Fig 4J	Stick Response	0.62 ± 0.14	1.54 ± 0.17	0.86 ± 0.26	0.91 ± 0.22	
Fig 4K	Evasion	1.07 ± 0.18	0.71 ± 0.19	0.57 ± 0.26	0.39 ± 0.18	
Fig 4L	Grooming	0.10 ± 0.07	0.44 ± 0.10	0.33 ± 0.12	0.49 ± 0.11	
Fig 5B	cFos ⁺ cells density (cells/ mm^2)	Layer I	145.7 ± 72.44	44.32 ± 25.52	34.89 ± 7.37	64.69 ± 14.10
		II-III	470.4 ± 142.9	304.9 ± 69.15	178.0 ± 23.87	450.9 ± 63.85
		IV	598.8 ± 157.1	299.1 ± 77.42	166.7 ± 45.72	812.6 ± 183.3
		V	284.1 ± 97.47	147.0 ± 49.15	103.2 ± 11.67	314.9 ± 50.20
		VI	342.4 ± 75.32	242.6 ± 81.03	172.8 ± 34.82	499.5 ± 81.23
		I-VI	368.3 ± 102.5	207.6 ± 52.35	131.1 ± 20.12	428.5 ± 72.10

802

803 **Table 2. Statistical tests and statistical power yielded**

804

Figure		Statistical Analysis		Power
Fig 1B	VGluT2 ⁺ puncta density	Two-way ANOVA		Genotype (G) 0,14 Environment Exposure (E) 0,95 G x E 0,05
Fig 1D	VGluT1 ⁺ puncta density	Two-way ANOVA		G 0,15; E 0,1; G x E 0,21
Fig 2B	VGluT2 ⁺ - Homer ⁺ appositions density	Two-way ANOVA		G 0,84; E 0,93; G x E 0,06
Fig 2C	VGluT2 ⁺ puncta distribution	Two-way ANOVA	0 Homer ⁺ 1 Homer ⁺ 2 Homer ⁺ >2 Homer ⁺	G 0,6; E 0,51; G x E 0,28 G 0,38; E 0,05; G x E 0,13 G 0,87; E 0,28; G x E 0,07 G 0,25; E 0,34; G x E 0,1
Fig 2E	VGluT1 ⁺ - Homer ⁺ appositions density	Two-way ANOVA		G 0,16; E 0,18; G x E 0,65
Fig 2F	VGluT1 ⁺ puncta distribution	Two-way ANOVA	0 Homer ⁺ 1 Homer ⁺ ≥2 Homer ⁺	G 0,86; E 0,17; G x E 0,36 G 0,23; E 0,05; G x E 0,08 G 0,35; E 0,12; G x E 0,5
Fig 3B	VGAT ⁺ puncta density on pyramidal neurons	Two-way ANOVA		G 0,05; E 0,14; G x E 0,12
Fig 3D	VGAT ⁺ puncta density	Two-way ANOVA		G 0,1; E 0,06; G x E 0,05
Fig 4B	WNT score	Two-way ANOVA		G 0,57; E 0,49; G x E 0,08
Fig 4C-G	WNT score during trials	Two-way ANOVA with RM		G 0,87; E 0,73; G x E 0,19; Trials (T) 0,14; T x G 0,66 T x E 0,36; TxGxE 0,08
Fig 4H	Fearful behaviour	Two-way ANOVA		G 0,73; E 0,9; G x E 0,06
Fig 4I	Stance	Two-way ANOVA		G 0,99; E 0,8; G x E 0,27
Fig 4J	Stick response	Two-way ANOVA		G 0,65; E 0,16; G x E 0,56
Fig 4K	Evasion	Two-way ANOVA		G 0,13; E 0,8; G x E 0,2
Fig 4L	Grooming	Two-way ANOVA		G 0,48; E 0,32; G x E 0,19
Fig 4N	cFos ⁺ cells density	Two-way ANOVA	Layer I II-III IV V VI I-VI	G 0,17; E 0,25; G x E 0,45 G 0,1; E 0,14; G x E 0,45 G 0,21; E 0,06; G x E 0,88 G 0,1; E 0,05; G x E 0,84 G 0,33; E 0,09; G x E 0,82 G 0,16; E 0,05; G x E 0,89

805

Figure 1

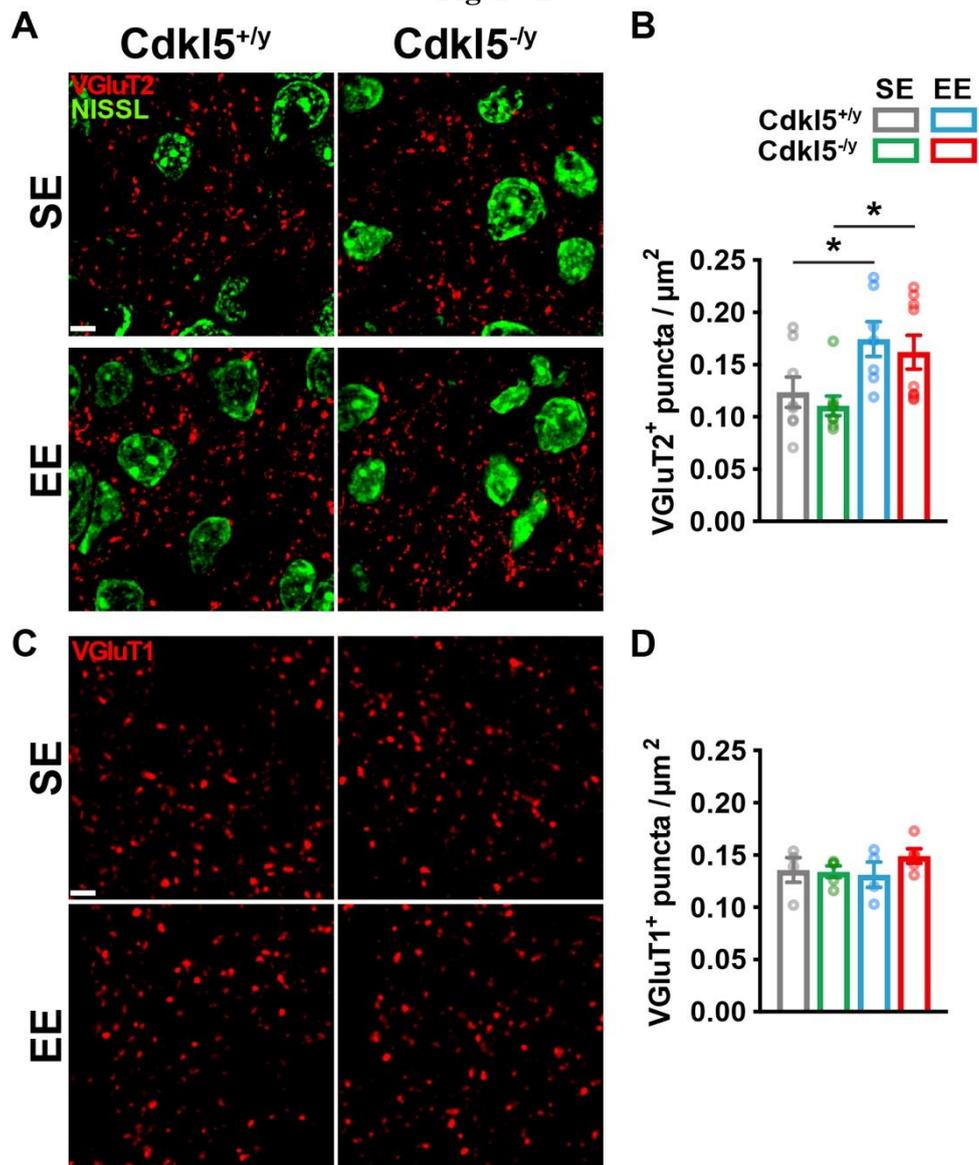


Figure 2

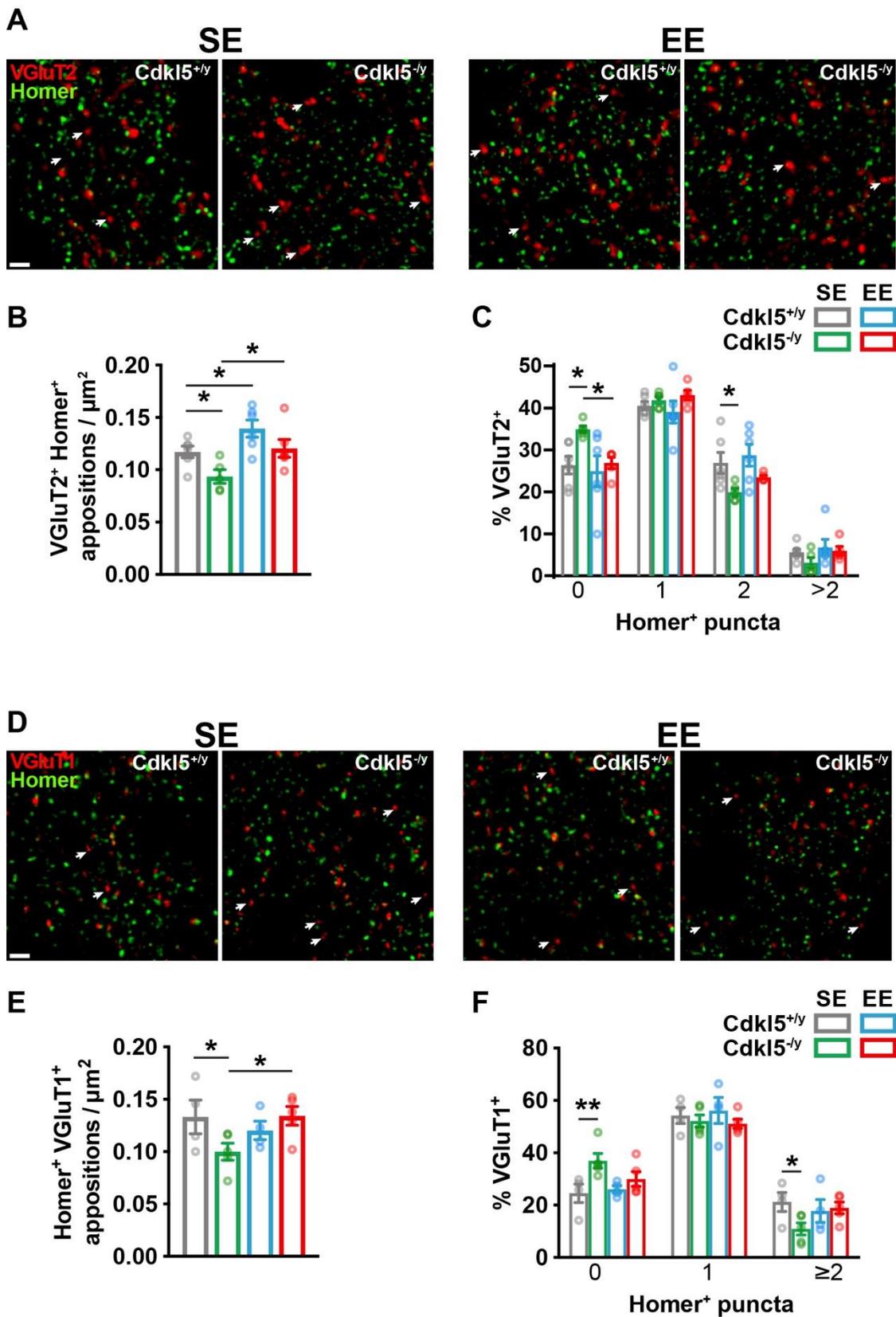


Figure 3

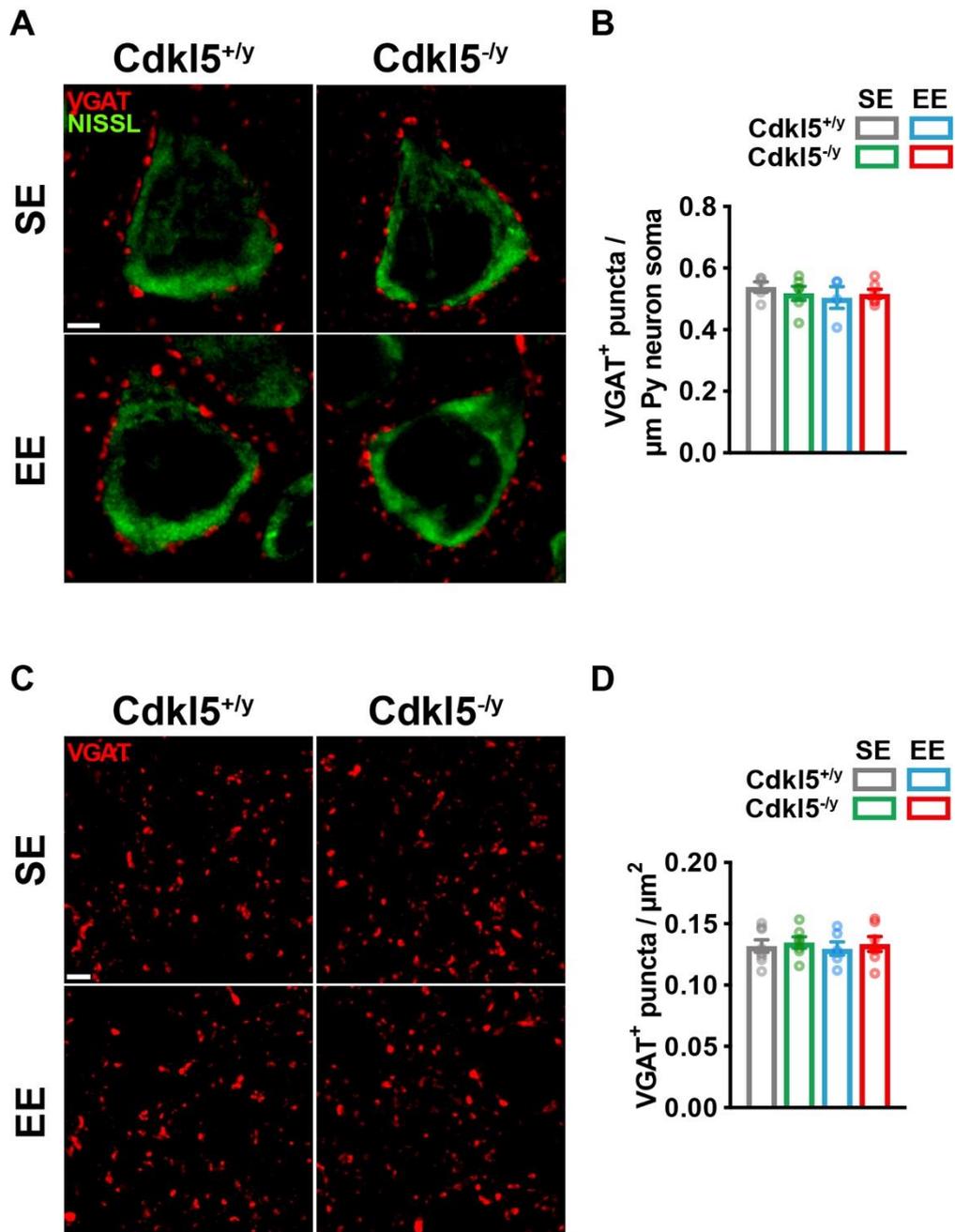
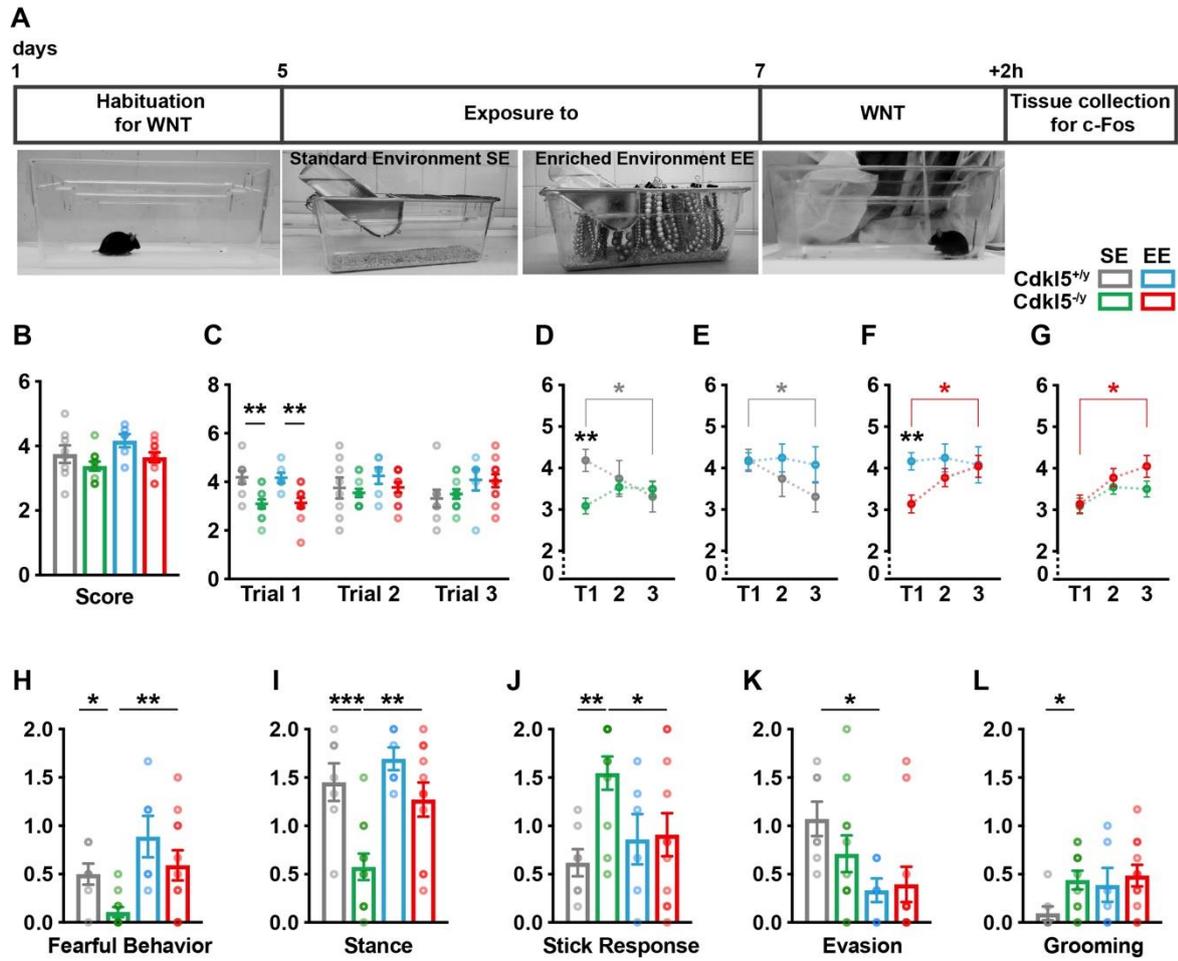
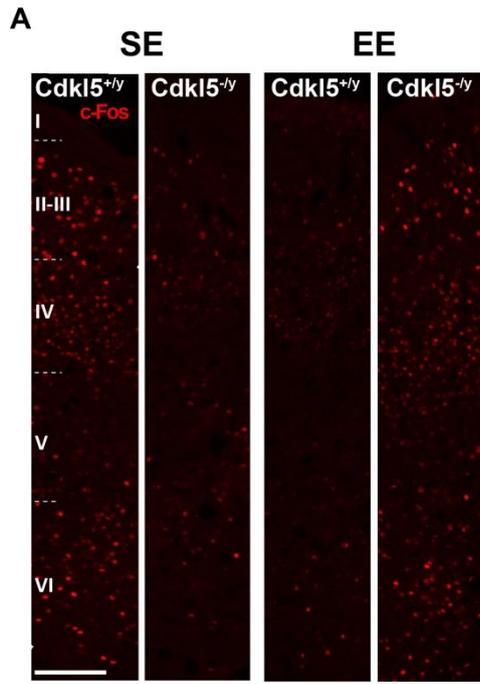


Figure 4



818

Figure 5



819

820

B

