The differentiation and integration of the hippocampal dorsoventral axis are controlled by two nuclear receptor genes

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Abstract

The hippocampus executes crucial functions from declarative memory to adaptive behaviors associated with cognition and emotion. However, the mechanisms of how morphogenesis and functions along the hippocampal dorsoventral axis are differentiated and integrated are still largely unclear. Here, we show that *Nr2f1* and *Nr2f2* genes are distinctively expressed in the dorsal and ventral hippocampus, respectively. The loss of *Nr2f2* results in ectopic CA1/CA3 domains in the ventral hippocampus. The deficiency of *Nr2f1* leads to the failed specification of dorsal CA1, among which there are place cells. The deletion of both *Nr2f* genes causes almost agenesis of the hippocampus with abnormalities of trisynaptic circuit and adult neurogenesis. Moreover, *Nr2f1/2* may cooperate to guarantee appropriate morphogenesis and function of the hippocampus by regulating the *Lhx5-Lhx2* axis. Our findings revealed a novel mechanism that *Nr2f1* and *Nr2f2* converge to govern the differentiation and integration of distinct characteristics of the hippocampus in mice.

Keywords

Memory; Hippocampus; *Nr2f* Gene; Place Cell; Trisynaptic Circuit

Introduction

Memory, including declarative and nondeclarative memory, unifies our mental world to ensure the quality of life for people of all ages, from newborns to elderly individuals (Eichenbaum & Cohen, 2014; Kandel, Dudai, & Mayford, 2014). The pioneering studies of Milner and her colleagues revealed that the hippocampus is required for declarative memory but not nondeclarative memory (Penfield & Milner, 1958; Scoville & Milner, 1957). The discovery of activity-dependent long-term potentiation and place cells provides the neurophysiological basis of hippocampal function (Bliss & Lomo, 1973; O'Keefe & Dostrovsky, 1971). The rodent hippocampus can be divided into the dorsal and ventral domains, corresponding to the posterior and anterior hippocampus in humans, respectively. In recent decades, numerous studies have supported the Moser theory that the hippocampus is a heterogeneous structure with distinct characteristics of gene expression, connectivity, and functions along its dorsoventral axis (Bast, 2007; Fanselow & Dong, 2010; Moser & Moser, 1998; Strange, Witter, Lein, & Moser, 2014). The dorsal hippocampus, which connects and shares similar gene expression with the neocortex (Fanselow & Dong, 2010), serves the “cold” cognitive function associated with declarative memory and spatial navigation. The ventral hippocampus, which connects and generates similar gene expression with the amygdala and hypothalamus (Cenquizca & Swanson, 2007; Kishi et al., 2000; Pitkanen, Pikkarainen, Nurminen, & Ylinen, 2000), corresponds to the “hot” affective states related to emotion and anxiety (Fanselow & Dong, 2010; Tyng, Amin, Saad, & Malik, 2017). Nonetheless, to date, the molecular and cellular mechanisms by which the morphogenesis, connectivity, and functions along the dorsoventral axis of the hippocampus are differentiated and integrated are largely unknown.

The hippocampus, a medial temporal lobe structure in the adult rodent forebrain, originates from the medial pallium (MP) in the medial line of the early dorsal telencephalon. The cortical hem (CH), which is located ventrally to the MP, functions as an organizer for hippocampal

60 development (Hebert & Fishell, 2008; Schuurmans & Guillemot, 2002). It has been
61 demonstrated that both extrinsic signals, such as WNTs and BMPs, and intrinsic factors,
62 including EMX1, EMX2, LEF1, LHX2, and LHX5, are involved in the regulation of early
63 morphogenesis of the hippocampus. As the earliest *Wnt* gene to be exclusively expressed in
64 the cortical hem, *Wnt3a* is required for the genesis of the hippocampus (S. M. Lee, Tole, Grove,
65 & McMahon, 2000); in addition, *Lef1* is downstream of Wnt signaling, and the hippocampus
66 is completely absent in *Lef1*$^{neo/neo}$ null mutant mice (Galceran, Miyashita-Lin, Devaney,
67 Rubenstein, & Grosschedl, 2000). Wnt signaling is essential for early development of the
68 hippocampus. *Emx1* and *Emx2* are mouse homologs of *Drosophila empty spiracles* (Simeone
69 et al., 1992). Interestingly, the dorsal hippocampus is smaller in an *Emx1* null mutant (Yoshida
70 et al., 1997), while *Emx2* is required for the growth of the hippocampus but not for the
71 specification of hippocampal lineages (Tole, Goudreau, Assimacopoulos, & Grove, 2000).
72 Moreover, *Lhx5*, which encodes a LIM homeobox transcription factor and is specifically
73 expressed in the hippocampal primordium, is necessary for the formation of the hippocampus
74 (Zhao et al., 1999). *Lhx2*, encoding another LIM homeobox transcription factor, is required for
75 the development of both the hippocampus and neocortex (Mangale et al., 2008; Monuki, Porter,
76 & Walsh, 2001; Porter et al., 1997). Intriguingly, deficiency of either *Lhx5* or *Lhx2* results in
77 agenesis of the hippocampus, and more particularly, these genes inhibit each other (Hebert &
78 Fishell, 2008; Mangale et al., 2008; Roy, Gonzalez-Gomez, Pierani, Meyer, & Tole, 2014;
79 Zhao et al., 1999), indicating that the *Lhx5* and *Lhx2* genes may generate an essential regulatory
80 axis to ensure the appropriate hippocampal development. Nevertheless, whether there are other
81 intrinsic genes that participate in the regulation of morphogenesis and function of the
82 hippocampus has not been fully elucidated.
83 *Nr2f* genes, including *Nr2f1* and *Nr2f2,* encode two transcription factor proteins belonging to
84 the nuclear receptor superfamily (Yang, Feng, & Tang, 2017). Mutations of *Nr2f1* are highly

85 related to neurodevelopmental disorders (NDD), such as intellectual disability (ID) and autism
86 spectrum disorders (ASD) (Bertacchi et al., 2020; Bosch et al., 2014; Contesse, Ayrault,
87 Mantegazza, Studer, & Deschaux, 2019), and mutations of the *Nr2f2* gene are associated with
88 congenital heart defects (CHD) (Al Turki et al., 2014). By using animal models, our studies
89 and others have demonstrated that *Nr2f* genes participate in the regulation of the development
90 of the central nervous system (Zhang et al., 2020). The *Nr2f1* plays an essential role in the
91 differentiation of cortical excitatory projection neurons and inhibitory interneurons, the
92 development of the dorsal hippocampus, and cortical arealization (Armentano et al., 2007;
93 Bertacchi et al., 2020; Del Pino et al., 2020; J. Feng et al., 2021; Flore et al., 2017; Lodato et
94 al., 2011; C. Zhou et al., 1999; C. Zhou, Tsai, & Tsai, 2001). *Nr2f2* plays a vital role in the
95 development of the amygdala, hypothalamus, and cerebellum (S. Feng et al., 2017; Kim,
96 Takamoto, Yan, Tsai, & Tsai, 2009; Tang, Rubenstein, Tsai, & Tsai, 2012). Nevertheless,
97 whether and how *Nr2f1* and/or *Nr2f2* genes regulate the differentiation and integration of
98 hippocampal morphogenesis, connectivity, and function is still largely unclear.
99 Here, our data show that *Nr2f1* and *Nr2f2* genes are differentially expressed along the
100 dorsoventral axis of the postnatal hippocampus. The loss of *Nr2f2* results in ectopic CA1 and
101 CA3 domains in the ventral hippocampus. In addition, the deficiency of *Nr2f1* leads to not only
102 dysplasia of the dorsal hippocampus but also failed specification and differentiation of the
103 dorsal CA1 pyramidal neuron lineage. Furthermore, the deletion of both genes in the $RX^{Cre/+}$;
104 $Nr2f1^{F/F}$; $Nr2f2^{F/F}$ double mutant mouse causes almost agenesis of the hippocampus,
105 accompanied by compromised specification of the CA1, CA3, and dentate gyrus (DG) domains.
106 The components of the trisynaptic circuit are abnormal in the corresponding single-gene or
107 double-gene mutant model. Moreover, *Nr2f* genes may cooperate to guarantee the appropriate
108 morphogenesis and function of the hippocampus by regulating the *Lhx5-Lhx2* axis.
109

110 **Results**

111 **Differential expression profiles of *Nr2f1* and *Nr2f2* genes along the dorsoventral axis in**
112 **the developing and postnatal hippocampus**

113 To investigate the functions of the *Nr2f1* and *Nr2f2* genes in the hippocampus,
114 immunofluorescence staining was first performed to examine their expression in wild-type
115 mice at postnatal month 1 (1M). In both the coronal and sagittal sections, NR2F1 exhibited a
116 septal/dorsal high-temporal/ventral low expression pattern along the hippocampus (Figure 1Aa,
117 d, c, f, g, j, m, i, l, and o), and its expression is highest in the dorsal CA1 region (Figure 1Aa,
118 d, c, f, j, and l), where place cells are mainly located (O'Keefe & Conway, 1978; O'Keefe &
119 Dostrovsky, 1971), and the dorsal DG, where there are adult neural stem cells (NSCs) (Gould
120 & Cameron, 1996). However, the expression of NR2F2 was high in the temporal/ventral
121 hippocampus but was barely detected in the septal/dorsal part of the hippocampus (Figure 1Ab,
122 c, e, f, h, i, k, l, n, and o). The dorsal-high NR2F1 and ventral-high NR2F2 expression profiles
123 were further verified in the postnatal hippocampi at 1M by western blotting assays (Figure 1Ap,
124 q). At embryonic day 10.5 (E10.5), NR2F1 was detected in the dorsal pallium (DP) laterally
125 and NR2F2 was expressed in the MP and CH medially (Figure 1—figure supplement 1Aa-b).
126 At E11.5 and E12.5, the expression of NR2F2 remained in the CH (Figure 1—figure
127 supplement 1Ac-d, Bb-c). Interestingly, NR2F1 and NR2F2 generated complementary
128 expression patterns in the hippocampal primordium with NR2F1 in the dorsal MP and NR2F2
129 in the ventral CH at E14.5 (Figure 1—figure supplement 1Ba-f). Additionally, septal/dorsal-
130 high NR2F1 and temporal/ventral-high NR2F2 expression patterns were observed at postnatal
131 day 0 (P0) (Figure 1—figure supplement 1Bg-l). The data above revealed that the differential
132 expression patterns of *Nr2f1* and *Nr2f2* genes were generated and maintained along the
133 dorsoventral axis in the early hippocampal primordium, the developing and postnatal

134 hippocampus, indicating that they could play distinct roles in the mediation of the
135 morphogenesis and functions of the hippocampus.
136 Next, to investigate the roles of *Nr2f* genes in the hippocampus, an RX^{Cre} mouse was used to
137 excise the expression of the *Nr2f1* and/or *Nr2f2* genes (Swindell et al., 2006; Tang et al., 2012).
138 The deletion efficiency of RXCre recombinase was verified by immunofluorescence assays.
139 Compared with control mice, either *Nr2f2* or *Nr2f1* could be excised in the postnatal
140 hippocampus of corresponding single-gene mutants at 1M (Figure 1—figure supplement 1Ca-
141 i). In addition, compared with control mice, both the *Nr2f1* and *Nr2f2* genes were almost
142 completely deleted in the hippocampal primordium in mutant mice at E14.5 (Figure 1—figure
143 supplement 1Cj-o). Since the LacZ expression serves as an indicator for the deletion of *Nr2f2*
144 (Swindell et al., 2006; Tang et al., 2012), we performed immunofluorescence staining with
145 antibodies against NR2F2 and LacZ on the sagittal sections of $RX^{Cre/+}$; $Nr2f2^{F/+}$ and $RX^{Cre/+}$;
146 $Nr2f2^{F/F}$ mice at E11.5. NR2F2 was readily detected at the hippocampal primordium of the
147 heterozygous mutant embryo at E11.5 (Figure 1—figure supplement 1Da, c, g); in contrast, the
148 expression of *Nr2f2* was significantly reduced in the homozygous mutant (Figure 1—figure
149 supplement 1Dd, f, j). In addition, compared with the heterozygous mutant embryo (Figure 1—
150 figure supplement 1Db-c, h), the LacZ signals clearly increased in the hippocampal
151 primordium of the homozygous mutant embryo at E11.5 (Figure 1—figure supplement 1De-f,
152 k), suggesting that RX-Cre recombinase can efficiently excise the *Nr2f2* gene in the
153 hippocampal primordium as early as E11.5. Intriguingly, we observed that the expression of
154 *Nr2f1* increased in the caudal hippocampal primordium of the *Nr2f2* homozygous mutant
155 embryo at E11.5 (Figure 1—figure supplement 1Di, l), indicating that similar to the
156 observations in the early optic cup (Tang et al., 2010), *Nr2f1* and *Nr2f2* genes could be partially
157 compensate with each other in the developing hippocampal primordium. All the data above

158 show that RXCre recombinase could efficiently excise *Nr2f1* and/or *Nr2f2* in the early
159 developing and postnatal hippocampus.
160
161 **The *Nr2f2* gene is required for the appropriate morphogenesis of the ventral**
162 **hippocampus but not of the dorsal hippocampus**
163 Given that the *Nr2f2* gene is highly and specifically expressed in the postnatal ventral
164 hippocampus and the CH of the hippocampal primordium (Figure 1 and Figure 1—figure
165 supplement 1), we asked whether the *Nr2f2* gene is required for the appropriate morphogenesis
166 of the hippocampus, particularly the ventral hippocampus. To answer this question, we
167 conducted Nissl staining with samples from the *Nr2f2* single-gene ($RX^{Cre/+}$; $Nr2f2^{F/F}$) knockout
168 mouse model. In coronal sections, compared with the control at 1M, the septal hippocampus
169 was normal; unexpectedly, an ectopic CA-like region was observed medially in the temporal
170 hippocampus in the *Nr2f2* mutant, where the prospective posterior part of the medial
171 amygdaloid (MeP) nucleus was situated, indicated by the star (Figure 1Ba-f). The presence of
172 the ectopic CA-like region in the ventral but not dorsal hippocampus of the mutant was further
173 confirmed by the presence of the prospective MeP and amygdalohippocampal area (AHi) in
174 sagittal sections, as indicated by the star (Figure 1Bg-l). Furthermore, immunofluorescence
175 assays were performed to verify whether specific lineages of the hippocampus were altered
176 with sagittal sections, in which the subregions of both the dorsal and ventral hippocampus were
177 well displayed and distinguished. Ctip2 is a marker for CA1 pyramidal neurons and DG granule
178 neurons, and HuB is a marker for CA3 pyramidal cells (Sugiyama, Osumi, & Katsuyama,
179 2014). The dorsal hippocampus appeared normal in both the control and mutant mice at 1M
180 (Figure 1Ca-l). Nonetheless, compared with the observations in control mice, an ectopic HuB-
181 positive CA3 pyramidal neuron lineage, indicated by the star, and a duplicated Ctip2-positive
182 CA1 pyramidal neuron lineage, indicated by the arrowhead, were observed in the ventral

183 hippocampal area in the mutant (Figure 1Ca-f, m-r), revealing that there were ectopic CA1 and
184 CA3 lineages in the *Nr2f2* mutants.
185 Consistent with the previous report (Leid et al., 2004), the expression of Ctip2 was detected in
186 the amygdala including the AHi and posteromedial cortical amygdaloid nucleus (PMCo); in
187 addition, Ctip2 was also highly expressed in the dorsal part of the MeP (MePD) in the control
188 (Figure 1Cb, n).
189 Intriguingly, compared with the controls at 1M, there were ectopic CA domains in the mutant
190 ventral hippocampus with the expense of the Ctip2 positive AHi and MePD amygdaloid nuclei
191 (Figure 1Ce, q), indicated by the arrows. Clearly, all the data above suggested that the *Nr2f2*
192 gene is necessary to ensure the appropriate morphogenesis of the ventral hippocampus.
193 At early embryonic stages, *Nr2f2* was preferentially expressed in the CH (Figure 1—figure
194 supplement 1Ab, d, 1Bb, e), the organizer of the hippocampus, and at postnatal 1-month-old
195 (1M) stage, *Nr2f2* was also highly expressed in some amygdala nuclei such as the AHi and
196 medial amygdaloid nucleus, which are adjacent to the ventral/temporal hippocampus (Figure
197 1Ae, h, n) (Tang et al., 2012). We would like to investigate the correlation of the CH and/or
198 amygdala anlage with the duplicated ventral hippocampal domains in the *Nr2f2* mutant in detail
199 in our future study. The observations above suggest that *Nr2f2* is not only specifically
200 expressed in the ventral hippocampus but is also required for morphogenesis and probably the
201 function of the ventral hippocampus. Since the ventral hippocampus participates in the
202 regulation of emotion and stress, mutations in the *Nr2f2* gene lead to CHDs, and the formation
203 of the ventral hippocampus is disrupted in *Nr2f2* mutant mice at 1M, we wondered whether
204 CHD patients with *Nr2f2* mutations also exhibit symptoms associated with psychiatric
205 disorders such as depression, anxiety, or schizophrenia.
206

207 **The *Nr2f1* gene is required for the specification and differentiation of the dorsal CA1**
208 **identity**

209 Next, we asked whether the deletion of the *Nr2f1* gene by RX-Cre also affected the
210 development of the hippocampus. Consistent with the previous finding in *Emx1*$^{Cre/+}$; *Nr2f1*$^{F/F}$
211 mutant mice (Flore et al., 2017), it was the septal/dorsal hippocampus, not the temporal/ventral
212 hippocampus, that was specifically shrunken in both coronal (Figure 2Aa-f) and sagittal
213 sections (Figure 2Ag-i) of RX$^{Cre/+}$; *Nr2f1*$^{F/F}$ mutant mice. Then, we asked whether the loss of
214 the *Nr2f1* gene caused abnormal specification and differentiation of hippocampal lineages.
215 Compared with the control mice, *Nr2f1* mutant mice had fewer HuB-positive CA3 pyramidal
216 neurons, as indicated by the star; intriguingly, Ctip2-positive CA1 pyramidal neurons failed to
217 be detected, as indicated by the arrowhead, with Ctip2-positive DG granule neurons unaltered
218 in the dorsal hippocampus (Figure 2Ba-l). The loss of the dorsal CA1 pyramidal neuron identity
219 in mutant mice was further confirmed by Wfs1, another dorsal CA1 pyramidal neuron-specific
220 marker (Takeda et al., 2001) (Figure 2Ca-r). Nonetheless, the HuB-positive and Ctip2-positive
221 lineages were comparable in the ventral hippocampus between the control and mutant mice
222 (Figure 2Ba-f, m-r), even though the low expression of NR2F1 was detected there. Indeed,
223 *Nr2f1* is not only expressed at the highest level in the dorsal CA1 but is also required for the
224 specification and differentiation of dorsal CA1 pyramidal neurons, among which place cells
225 are essential for learning and memory (O'Keefe & Conway, 1978; O'Keefe & Dostrovsky,
226 1971).

227 Given that dysplasia of the dorsal hippocampus was generated in both the *Emx1*^{Cre} and RX^{Cre}
228 models (Flore et al., 2017) (Figure 2Aa-l), we asked whether the development of dorsal CA1
229 pyramidal neurons was also abolished in *Emx1*$^{Cre/+;}$ *Nr2f1*$^{F/F}$ mutant mice. To answer this
230 question, immunofluorescence staining was conducted first. Compared with that in the control
231 mice, the proportions of either the Wfs1- or Ctip2-positive CA1 domain were reduced in the

232 mutant mice at 3M (Figure 2—figure supplement 1Aa-h), indicating that the differentiation of
233 the dorsal CA1 pyramidal neurons was also compromised in the *Emx1*^{Cre} model, although it
234 was less severe than that in the RX^{Cre} model. To make our findings more consistent with
235 previous studies, we further conducted experiments with the *Emx1*^{Cre} model. Afterward, to
236 investigate the fine structure of the dorsal CA1 pyramidal neurons, Golgi staining was
237 performed. Compared with those of control mice, the numbers of secondary dendrites and
238 branch points of both the apical and basal dendrites were significantly reduced in the dorsal
239 CA1 pyramidal neurons of mutant mice at 3M (Figure 2—figure supplement 1Ba-e). Then, the
240 dorsal hippocampus-related spatial learning and memory behavior test, the Morris water maze,
241 was performed (Vorhees & Williams, 2006). Consistent with a previous report (Flore et al.,
242 2017), spatial learning and memory function was significantly impaired in adult *Emx1*$^{Cre/+}$;
243 *Nr2f1*$^{F/F}$ mice, compared with the control mice (Figure 2—figure supplement 1C). The data
244 above suggest that *Nr2f1* is vital for the morphogenesis, lineage specification, and spatial
245 learning and memory of the dorsal hippocampus, and particularly, the compromised dorsal
246 CA1 lineage could contribute to the phenotypes associated with neurodevelopmental disorders,
247 including ID or ASD.
248
249 **The *Nr2f1* and *Nr2f2* genes coordinate to ensure the genesis of the hippocampus**
250 Given that the loss of either *Nr2f1* or *Nr2f2* leads to dysplasia of the dorsal or ventral
251 hippocampus, respectively, we asked whether these genes compensate for each other to
252 regulate the morphogenesis of the hippocampus. To answer this question, the RX$^{Cre/+}$; *Nr2f1*$^{F/F}$;
253 *Nr2f2*$^{F/F}$ double-mutant mouse was generated, and a few homozygous double-gene mutant
254 mice survived for approximately 3 weeks (3W). Nonetheless, the reason for the lethality of the
255 double-mutant mice is still unknown. Nissl staining data showed that compared with that of
256 control mice, the septal hippocampus was severely shrunken, as indicated by the star, and the

257 temporal hippocampus was barely observed in the double-mutant mouse brains (Figure 3Aa-
258 h). Unexpectedly, an ectopic nucleus was observed in the region of the prospective temporal
259 hippocampus, indicated by the arrowhead, in the double-mutant mice (Figure 3Ag-h). In
260 addition, compared with those of controls, the regions with HuB-positive CA3 pyramidal
261 neurons and Ctip2-positive or Prox1-positive DG granule neurons were diminished in the
262 double mutants; in particular, Ctip2-positive dorsal CA1 pyramidal neurons could not be
263 detected in the double mutants (Figure 3Ba-l). Furthermore, compared with the domains of
264 controls, no HuB-positive, Ctip2-positive, or Prox1-positive domains could be detected in the
265 prospective temporal hippocampus in the double mutants (Figure 3Ca-l). The results above
266 suggest that the *Nr2f1* and *Nr2f2* genes coordinate with each other to mediate the appropriate
267 morphogenesis of the entire hippocampus.
268
269 ***Nr2f* genes and adult neurogenesis in the hippocampus**
270 Given that the *Nr2f1* or *Nr2f2* gene was highly expressed in the dorsal or ventral DG,
271 respectively (Figure 1A), and that RX-Cre recombinase could efficiently delete either gene in
272 the DG (Figure 1—figure supplement 1Ca-i), we asked whether the loss of the *Nr2f1* or/and -
273 *Nr2f2* gene in the DG would affect hippocampal adult neurogenesis. To answer this question,
274 the ventral DG, dorsal DG, and septal DG were chosen to perform immunofluorescence assays
275 in the *Nr2f2* mutant, *Nr2f1* mutant, and double mutant models, respectively. Adult NSCs in the
276 subgranular zone (SGZ) of the DG express both GFAP and Nestin, and newborn granule
277 neurons express Dcx (Gao, Arlotta, Macklis, & Chen, 2007). The numbers of NSCs and
278 newborn neurons in the SGZ of the DG were comparable between control mice and either the
279 *Nr2f2* or *Nr2f1* mutant mice (Figure 3—figure supplement 1Aa-p, Ba-h); nonetheless,
280 compared with those of control mice, the numbers of the NSCs and newborn granule neurons
281 in the SGZ of the DG were reduced in the double mutants (Figure 3—figure supplement 1Aq-

282 x, Bi-l), and the reduction in both lineages was significant (Figure 3—figure supplement 1Ca,
283 b). The data above suggest that *Nr2f* genes may coordinate with each other to execute essential
284 functions for appropriate hippocampal adult neurogenesis in the DG.
285
286 **Hippocampal trisynaptic connectivity was impaired in postnatal *Nr2f2* single-, *Nr2f1***
287 **single-, and double-mutant mice at about 1M**
288 Given that dysplasia of the hippocampus was observed in all three mouse models, we asked
289 whether the connectivity of the hippocampal trisynaptic circuit associated with the DG, CA3,
290 and CA1 regions (Amaral, 1993) was normal in these models. To answer this question, the
291 components of the trisynaptic circuit were characterized in the ventral hippocampus of *Nr2f2*
292 mutants, the dorsal hippocampus of *Nr2f1* mutants, and the septal hippocampus of double
293 mutants. Calretinin is a marker of mossy cells, Calbindin is a marker of mossy fibers, and
294 SMI312 is a marker of Schafer collaterals (Flore et al., 2017). Compared with those of controls
295 (Figure 4Aa, b, e, f, i, and j), the numbers of Calretinin-positive mossy cells were reduced,
296 Calbindin-positive mossy fibers were longer but thinner, and SMI312-positive Schafer
297 collaterals were thinner and discontinued in the ventral hippocampus of *Nr2f2* mutants at 1M
298 (Figure 4Ac, d, g, h, k, and l). In addition, similar to the previous report (Flore et al., 2017), the
299 numbers of Calretinin-positive mossy cells were decreased, Calbindin-positive mossy fibers
300 were shorter and thinner, and SMI312-positive Schafer collaterals were barely detected in the
301 dorsal hippocampus of *Nr2f1* mutants (Figure 4Ac, d, g, h, k, and l), compared with those of
302 controls at 1M (Figure 4Ba, b, e, f, i, and j). Moreover, compared with those of control mice
303 (Figure 4Ca, b, e, f, i, and j), the numbers of Calretinin-positive mossy cells were reduced; both
304 Calbindin-positive mossy fibers and SMI312-positive Schafer collaterals were barely detected
305 in the prospective septal hippocampus of the double mutants at 3W (Figure 4Cc, d, g, h, k, and
306 l). Clearly, without both *Nr2f* genes, the connectivity of the hippocampal trisynaptic circuit was

307 abolished more severely. The observations above revealed that the formation of the trisynaptic
308 circuit, which is one of the fundamental characteristics of hippocampal neurophysiology (Basu
309 & Siegelbaum, 2015), was abnormal in all three mouse models, indicating that both the
310 morphology and functions of the hippocampus are most likely compromised in the loss of the
311 *Nr2f1* and/or *Nr2f2* gene.
312
313 **The expression of several essential regulatory genes associated with early hippocampal**
314 **development was abnormal in double mutants**
315 Given that the hippocampus was almost completely diminished in double mutants, we asked
316 how *Nr2f* genes participated in the regulation of the early morphogenesis of the hippocampus.
317 To answer this question, total RNA isolated from the whole telencephalons of control (n=5)
318 and double-mutant-(n=3) embryos at E11.5 was used to generate cDNA, and then real-time
319 quantitative PCR (RT-qPCR) assays were performed. As expected, compared with that of
320 control mice, the expression of *Nr2f1* and *Nr2f2* was reduced significantly in the double mutant
321 mice (Figure 5A). Then, we mainly focused on the intrinsic regulatory networks by analyzing
322 the expression profiles of two groups of transcription factor genes. The *Foxg1*, *Gli3*, *Lhx2*,
323 *Otx1, Otx2*, and *Pax6* genes, which are highly related to the early patterning of the dorsal
324 telencephalon (Hebert & Fishell, 2008), were in the first group; *Axin2*, *Emx1*, *Emx2*, *Lef1*, *Lhx5*,
325 and *Tcf4* genes, which are associated with early hippocampal development (Galceran et al.,
326 2000; Moore & Iulianella, 2021; Tole et al., 2000; Yoshida et al., 1997; Zhao et al., 1999),
327 were in the other group. The expression of the *Foxg1*, *Gli3*, *Lhx2*, *Otx1, Otx2*, and *Pax6* genes
328 was comparable between the controls and double mutants (Figure 5A), indicating that the early
329 patterning of the dorsal telencephalon is largely unaltered. Compared with that of control mice,
330 the expression of the *Axin2*, *Emx2*, *Lef1*, and *Tcf4* genes was normal in the double mutants;
331 interestingly, the expression of the *Emx1* and *Lhx5* transcripts was decreased significantly in

332 the double mutants at E11.5 compared to that in control mice (Figure 5A). Consistent with the
333 downregulated expression of *Lhx5* transcripts in the double mutant, the expression of the Lhx5
334 protein was reduced in the CH in the double mutants at E11.5; moreover, the number of Lhx5-
335 positive Cajal-Retzius cells decreased in the double mutant embryos at E11.5, E13.5 and E14.5
336 (Figure 5Ba-d, a'-d', a''-d'', i-l, i'-l', q-t, q'-t'). The expression of *Lhx2* was expanded
337 ventrally into the choroid plexus in the *Lhx5* null mutant mice (Zhao et al., 1999), indicating
338 that *Lhx5* could inhibit *Lhx2* expression locally. Consistent with RT-qPCR data, the expression
339 of Lhx2 was comparable between the control and double-mutant mice at E11.5 (Figure 5Be-h,
340 e'-h'). Interestingly, the expression of the Lhx2 protein was increased in the hippocampal
341 primordium in the *Nr2f* double-mutant mice at E13.5 and E14.5 (Figure 5Bm-p, m'-p', u-x, u'-
342 x'). The upregulation of Lhx2 expression is most likely associated with the reduced expression
343 of the *Lhx5* gene.
344 Next, we asked whether neural precursor cells (NPCs), intermediate progenitor cells (IPCs),
345 or newborn neurons were affected in the early development of the hippocampus in double-
346 mutant mice. Sox2 is a marker for NPCs, Tbr2 is a marker for IPCs, and NeuroD1 is a marker
347 for newborn neurons (Yu, Marchetto, & Gage, 2014). The expression of Sox2 in the
348 hippocampal regions was comparable between the control and double-mutant mice at E14.5
349 (Figure 5Ca-d, a'-d'), indicating that the generation of NPCs was normal. Nevertheless,
350 compared with the control embryos, the numbers of Tbr2-positive IPCs and NeuroD1-positive
351 newborn neurons were reduced in the double-mutant embryos (Figure 5Ce-l, e'-l'), and the
352 reduction was significant (Figure 5D). Our observations were consistent with previous findings
353 in *Lhx5* null mutant mice that the specification of the hippocampal NPCs was normal, but the
354 later differentiation event was abolished (Zhao et al., 1999). All the data above suggest that
355 *Nr2f* genes may cooperate to ensure the early morphogenesis of the hippocampus by regulating
356 the appropriate expression levels of *Lhx5* and *Lhx2* genes. Nevertheless, we could not exclude

357 other possibilities that *Nr2f* genes could also participate in the modulation of hippocampal
358 development through *Emx1* or other genes.
359
360 **Discussion**
361 In our present study, we observed dorsal-high NR2F1 and ventral-high NR2F2 expression
362 profiles in the postnatal hippocampus. The deletion of the *Nr2f2* gene led to duplicated CA1
363 and CA3 domains of the ventral hippocampus. The loss of *Nr2f1* resulted in the failed
364 specification and differentiation of the dorsal CA1 pyramidal neuron lineage with a diminished
365 dorsal hippocampus. Furthermore, the deficiency of both *Nr2f* genes caused atrophy of almost
366 the entire hippocampus, accompanied by compromised generation of the CA1, CA3, and DG
367 identities. In addition, the dorsal trisynaptic components, ventral trisynaptic components, or
368 entire trisynaptic components were abolished in the corresponding *Nr2f1* gene mutant, *Nr2f2*
369 gene mutant, or *Nr2f1/2* double-gene mutant mice. Moreover, *Nr2f* genes may cooperate to
370 ensure the appropriate morphogenesis and function of the hippocampus by regulating the *Lhx5-*
371 *Lhx2* axis.
372
373 **1. *Nr2f2* governs the distinct characteristics of the ventral hippocampus**
374 Sixty years ago, the pioneering work of Milner and her colleagues discovered the essential role
375 of the hippocampus in declarative memory (Penfield & Milner, 1958; Scoville & Milner, 1957).
376 Recently, accumulating evidence has supported the Moser theory that the hippocampus is a
377 heterogeneous structure with distinct characteristics of gene expression, connectivity, and
378 function along its dorsoventral axis (Bast, 2007; Fanselow & Dong, 2010; Moser & Moser,
379 1998; Strange et al., 2014). The dorsal hippocampus marked in blue, in which gene expression
380 is similar to the neocortex, serves the "cold" cognitive function associated with declarative
381 memory and spatial navigation, and the ventral hippocampus marked in red, in which gene

382 expression is close to the hypothalamus and amygdala, corresponds to the "hot" affective states
383 related to emotion and anxiety (Figure 5—figure supplement 1). The ventral hippocampus
384 generates direct connectivity with the amygdala, hypothalamus, medial prefrontal cortex
385 (mPFC), and olfactory bulb (Cenquizca & Swanson, 2007; Hoover & Vertes, 2007; Kishi et
386 al., 2000; Pitkanen et al., 2000; Roberts et al., 2007). Nonetheless, thus far, the molecular and
387 cellular mechanism of how the morphogenesis, connectivity, and function of the ventral
388 hippocampus is achieved has been largely unclear.
389 *Nr2f2*, a nuclear receptor gene associated with heart disease(Al Turki et al., 2014) (High et al.,
390 2016), was highly and exclusively expressed in the ventral hippocampus in 1-month-old mice
391 and was expressed ventrally in the CH of the hippocampal primordium in mouse embryos
392 (Figure 1, Figure 1—figure supplement 1, Figure 5Ea), indicating that the *Nr2f2* gene may
393 participate in the regulation of the development and function of the ventral hippocampus. First,
394 deficiency of the *Nr2f2* gene led to the duplication of the CA1 and CA3 domains of the ventral
395 hippocampus but not the dorsal hippocampus, which was confirmed both morphologically and
396 molecularly (Figure 1). Second, the formation of the trisynaptic circuit was specifically
397 abolished in the ventral hippocampus of *Nr2f2* mutants (Figure 4), indicating that the
398 intrahippocampal circuit, information transfer, and function of the ventral hippocampus could
399 be compromised. Third, the ventral hippocampus generates neural circuits with the mPFC,
400 amygdala, nucleus accumbens, and hypothalamus, which are associated with
401 anxiety/behavioral inhibition, fear processing, pleasure/reward seeking, and the
402 neuroendocrine system, respectively (Anacker & Hen, 2017; Baik, 2020; Bryant & Barker,
403 2020; Cenquizca & Swanson, 2007; Herman et al., 2016; Kishi et al., 2000; O'Leary & Cryan,
404 2014; Pitkanen et al., 2000). These ventral hippocampal projections may be important for
405 processing information related to emotion and anxiety. Intriguingly, our previous studies
406 revealed that *Nr2f2* is required for the development of the hypothalamus, amygdala, and

407 olfactory bulb (S. Feng et al., 2017; Tang et al., 2012; X. Zhou et al., 2015), all of which
408 generate functional neural circuits with the ventral hippocampus (Fanselow & Dong, 2010).
409 Particularly, both the hypothalamus and amygdala are also diminished in $RX^{Cre/+}$; *Nr2f2*$^{F/F}$
410 mutant mice (S. Feng et al., 2017; Tang et al., 2012), indicating that their interconnectivities
411 with the ventral hippocampus are abnormal. Thus, all the findings above suggest that *Nr2f2* is
412 a novel and essential intrinsic regulator that controls the morphogenesis, connectivity, and
413 function of the ventral hippocampus.
414 Given that mutations of *Nr2f2* are highly associated with CHDs (Al Turki et al., 2014), the
415 expression of *Nr2f2* is also confined to the ventral hippocampus in human embryos (Alzu'bi et
416 al., 2017), and *Nr2f2* gene is required for the distinct characteristics of the ventral hippocampus
417 in mouse (Figure 1, Figure 4), we wondered whether CHD patients carrying mutations of *Nr2f2*
418 also display symptoms of psychiatric disorders, such as depression, anxiety, or schizophrenia,
419 related to the ventral hippocampus. In our future study, we would like to generate
420 hippocampus-specific or hippocampal subdomain-specific conditional knockout models to
421 dissect distinct roles of the *Nr2f2* gene in the hippocampus, particularly in the ventral
422 hippocampus, in detail.
423
424 **2. The *Nr2f1* gene is required for the specification and differentiation of dorsal CA1**
425 **pyramidal neurons**
426 The expression of *Nr2f1*, another orphan nuclear receptor gene associated with
427 neurodevelopmental disorders (Bertacchi et al., 2020; Bosch et al., 2014; Contesse et al., 2019),
428 is high in the dorsal MP of the hippocampal primordium and is higher in the dorsal
429 hippocampus than in the ventral hippocampus (Figure 1, Figure 1—figure supplement 1, Figure
430 5Ea) (Flore et al., 2017). Consistent with previous observations in *Emx1*$^{Cre/+}$; *Nr2f1*$^{F/F}$ mutant
431 mice (Flore et al., 2017), the dorsal hippocampus but not the ventral hippocampus was

432 specifically shrunken in RX$^{Cre/+}$; *Nr2f1*$^{F/F}$ mice (Figure 2, Figure 5—figure supplement 1).
433 *Nr2f1* is expressed at the highest level in dorsal CA1 pyramidal neurons (Figure 1), indicating
434 that the *Nr2f1* gene may play a role in the specification and differentiation of dorsal CA1
435 pyramidal neurons. As expected, the expression of Ctip2 and Wfs1, two markers for dorsal
436 CA1 pyramidal neurons, could not be detected in the prospective dorsal CA1 domain in RX$^{Cre/+}$;
437 *Nr2f1*$^{F/F}$ mutant mice (Figure 2); furthermore, *Emx1*$^{Cre/+}$; *Nr2f1*$^{F/F}$ mutant mice partially
438 phenocopied the compromised development of the dorsal CA1 lineage (Figure 2—figure
439 supplement 1). It seems that the spatiotemporal activity of RX-Cre recombinase is better or
440 broader than that of the Emx1-Cre recombinase during the critical period of the specification
441 of the dorsal CA1 pyramidal neuron identity. In addition, the Golgi staining assay revealed that
442 the development of the dendrites of the dorsal CA1 pyramidal neurons was abnormal (Figure
443 2—figure supplement 1). All the observations above indicate that the *Nr2f1* gene is not only
444 necessary for the morphogenesis of the dorsal hippocampus but is also required for the
445 specification and differentiation of the dorsal CA1 pyramidal neurons, among which there are
446 place cells. The identification of place cells fifty years ago was one of the most important
447 breakthroughs in understanding the role of the hippocampus in memory (O'Keefe &
448 Dostrovsky, 1971). Except for spatial information, place cells in the dorsal CA1 may also
449 encode nonspatial representations, such as time (Eichenbaum, 2017; Lisman et al., 2017).
450 Notably, 95% of patients carrying *Nr2f1* mutations are associated with ID. Here, our
451 observations support the notion that the *Nr2f1* gene is a novel intrinsic regulator that specifies
452 the dorsal CA1 pyramidal cell identity, which will benefit the understanding of both
453 neurophysiological functions of the hippocampus and the etiology of NDD including ID and
454 ASD.
455

456 **3. *Nr2f1* and *Nr2f2* cooperate to ensure the appropriate morphogenesis of the**
457 **hippocampus by regulating the *Lhx5-Lhx2* axis in mice**

458 In wild-type mice, *Nr2f1* and *Nr2f2* genes generated complementary expression profiles in the
459 embryonic hippocampal primordium with *Nr2f1* in the dorsal MP, marked in green; *Nr2f2* in
460 the ventral CH, marked in red (Figure 5Ea, Figure 1—figure supplement 1); and in the postnatal
461 hippocampus with high-NR2F1 expression in the dorsal, marked in green; and high-NR2F2
462 expression in the ventral, marked in red (Figure 5Ea, Figure 1). These findings indicated that
463 *Nr2f* genes may coordinate to regulate hippocampal development. Indeed, as discussed above,
464 the loss of either *Nr2f1* or *Nr2f2* only leads to dysplasia of the dorsal hippocampus (Flore et
465 al., 2017) (Figure 2, Figure 2—figure supplement 1) or ventral hippocampus (Figure 1),
466 respectively; intriguingly, while both genes are efficiently excised by RX-Cre in the
467 hippocampal primordium (Figure 1—figure supplement 1), more severely shrunken
468 hippocampi developed in the 3-week-old double knockout mice (Figure 3). The dosage-
469 dependent severity of hippocampal abnormalities suggested that two nuclear receptor genes,
470 *Nr2f1* and *Nr2f2* could cooperate with each other to execute an essential and intrinsic function
471 in the development of the hippocampus.

472 It is known that both extrinsic signals and intrinsic factors participate in the regulation of the
473 early development of the hippocampus. Notably, mutations of *Wnt3a* and *Lef1* eliminate the
474 entire hippocampus (Galceran et al., 2000; S. M. Lee et al., 2000). Given that the expression
475 of *Axin2*, *Lef1* and *Tcf4*, three Wnt-responsive transcription factor genes, was not altered in the
476 *Nr2f* double mutant (Figure 5A), it is unlikely that abnormal Wnt signaling is the cause of the
477 compromised hippocampus. *Lhx5* is specifically expressed in the hippocampal primordium and
478 is required for the morphogenesis of the hippocampus (Zhao et al., 1999). *Lhx2* is necessary
479 for hippocampal development by repressing cortical hem fate (Mangale et al., 2008; Monuki
480 et al., 2001). Agenesis of the hippocampus is observed in either *Lhx5* or *Lhx2* null mutant mice,

481 and these genes particularly repress each other (Hebert & Fishell, 2008; Mangale et al., 2008;
482 Roy et al., 2014; Zhao et al., 1999), indicating that the proper expression levels of *Lhx5* and
483 *Lhx2* genes are critical to maintain the appropriate development of the hippocampus (Figure
484 5Eb). The transcriptional and protein expression levels of *Lhx5* but not *Lhx2* were first reduced
485 in the hippocampal primordium of *Nr2f* double-mutant mice at E11.5; later, enhanced
486 expression of the Lhx2 protein was detected in the hippocampal primordium of double-mutant
487 mice at E13.5 and E14.5 (Figure 5A-B). Moreover, the number of Lhx5-positive Cajal-Retzius
488 cells was clearly reduced in the double mutant embryos at E11.5, E13.5 and E14.5; consistent
489 with the observations in the *Lhx5* null mutant (Li et al., 2021; Miquelajáuregui et al., 2010),
490 the generation of Sox2-positive hippocampal NPCs was not affected, but the development of
491 Tbr2-positive IPCs and NeuroD1-positive newborn neurons was abnormal in *Nr2f* double-
492 mutant mice (Figure 5). Thus, our findings reveal a novel intrinsic regulatory mechanism that
493 *Nr2f1* and *Nr2f2*, two disease-associated nuclear receptor genes, may cooperate with each other
494 to ensure proper hippocampal morphogenesis by regulating the *Lhx5-Lhx2* axis. Intriguingly,
495 compared with the adult $Emx1^{Cre/+}$; $Nr2f1^{F/F}$ mutant mice, the hippocampus was much smaller
496 in the adult $Emx1^{Cre/+}$; $Nr2f1^{F/F}$; $Nr2f2^{F/F}$ double-gene mutant mice; nevertheless, both the
497 dorsal and ventral hippocampus were readily detected in double-gene mutants with $Emx1^{Cre}$
498 (our unpublished observations). In addition, the discrepancy between the shrunken dorsal
499 hippocampus associated with the loss of *Nr2f1* and the duplicated CA domains of the ventral
500 hippocampus associated with the deficiency of *Nr2f2* suggested that the regulatory network
501 related to *Nr2f* genes during the early morphogenesis of the hippocampus could be much more
502 complicated than suspected and should be investigated in our future study.
503
504 **4. *Nr2f* genes are imperative for the formation of the trisynaptic circuit**

505 The hippocampus and entorhinal cortex (EC) are interconnected through various neural circuits
506 to mediate the flow of the information associated with declarative memory (Basu &
507 Siegelbaum, 2015). Both direct and indirect glutamatergic circuits are involved in the relay of
508 information from the EC to the hippocampal CA1, and the trisynaptic pathway is the most well-
509 characterized indirect circuit. The EC sends sensory signals from association cortices via the
510 perforant path to the DG, then the DG granule cells send excitatory mossy fiber projections to
511 CA3 pyramidal neurons, and CA3 pyramidal neurons project to CA1 via the Schaffer
512 collaterals (H. Lee, GoodSmith, & Knierim, 2020). Consistent with the high expression of
513 NR2F1 in the dorsal hippocampus and NR2F2 in the ventral hippocampus (Figure 1), the dorsal
514 trisynaptic circuit is specifically damaged in *Nr2f1* mutants, as is the ventral trisynaptic circuit
515 in *Nr2f2* mutant mice. Moreover, the hippocampal trisynaptic circuit was almost completely
516 absent in the double mutants (Figure 4, and our unpublished observations). The information
517 transfer associated with the trisynaptic circuits should be abolished particularly in the dorsal
518 and/or the ventral hippocampus in the above corresponding genetic mouse models.
519 Interestingly, *Nr2f1* is also required to specify the medial EC cell fate (J. Feng et al., 2021).
520 Therefore, the impaired formation and function of trisynaptic circuits could be caused by the
521 abnormal development of CA1, CA3, DG or EC lineages. Nonetheless, given that newborn
522 granule neurons are continuously generated in the adult DG to integrate into the existing neural
523 circuits essential for declarative memory (Toda, Parylak, Linker, & Gage, 2019; Tuncdemir,
524 Lacefield, & Hen, 2019) and that hippocampal adult neurogenesis was severely compromised
525 in the *Nr2f* double mutant (Figure 3—figure supplement 1), we could not exclude the
526 possibility that impaired adult neurogenesis may also contribute to the malformation and
527 impaired function of the trisynaptic pathway in double mutants. We would like to investigate
528 what sort of synaptic circuitry is compromised either physiologically or morphologically in the
529 trisynaptic circuit of individual animal model in detail in future studies.

530

531 The hippocampus is heterogeneous along its dorsoventral axis, and either the dorsal or ventral
532 hippocampus generates unique and distinguishable characteristics of gene expression and
533 connectivity, which enable the hippocampus to execute an integrative function from the
534 encoding and retrieval of certain declarative memory to adaptive behaviors. Lesions of the
535 dorsal hippocampus, which are essential for the cognitive process of learning and memory,
536 lead to amnesia and ID; while damage to the ventral hippocampus, which is central for emotion
537 and affection, is highly associated with psychiatric disorders including depression, anxiety, and
538 schizophrenia. Our findings in this study reveal novel intrinsic mechanisms by which two
539 nuclear receptor genes, *Nr2f1* and *Nr2f2*, which are associated with NDD or CHD, converge
540 to govern the differentiation and integration of the hippocampus along the dorsoventral axis
541 morphologically and functionally. Furthermore, our present study provides novel genetic
542 model systems to investigate the crosstalk among the hippocampal complex in gene expression,
543 morphogenesis, cell fate specification and differentiation, connectivity, functions of
544 learning/memory and emotion/anxiety, adaptive behaviors, and the etiology of neurological
545 diseases. Nevertheless, many enigmas, such as whether and how the abnormalities of either the
546 dorsal or ventral hippocampus affect the characteristics of the other, remain unsolved. In
547 addition to the excitatory lineages and circuits, interneurons and inhibitory circuits play vital
548 roles in maintaining the plasticity and functions of the hippocampus. We also wonder whether
549 and how defects in interneurons and inhibitory circuits could contribute to the compromised
550 morphogenesis, connectivity, and functions of the hippocampus and the etiology of psychiatric
551 and neurological conditions including ID, ASD, depression, anxiety, and schizophrenia.

552

553 **Materials and Methods**

554 **Animals**

555 *Nr2f1-floxed* (*Nr2f1*$^{F/F}$) mice, *Nr2f2-floxed* (*Nr2f2*$^{F/F}$) mice, *Emx1*^{Cre} mice and RX^{Cre} mice
556 (Swindell et al., 2006) (PMID: 16850473) used in the study were of the C57B6/129 mixed
557 background. The noon of vaginal plug day was set as the embryonic day 0.5 (E0.5). Only male
558 mice at age of 10 weeks or older were used in the Morris water maze. For other experiments,
559 both male and female mice were used. All animal protocols were approved by the Institutional
560 Animal Care and Use Committee (IACUC) at the Shanghai Institute of Biochemistry and Cell
561 Biology, Chinese Academy of Sciences (Protocols: SIBCB-NAF-14-001-S308-001). All
562 methods were performed in accordance with the relevant guidelines and regulations. Only the
563 littermates were used for the comparison.
564
565 **Nissl staining**
566 We used xylene to dewax paraffin sections, followed by rinsing with 100%, 95%, and 70%
567 ethanol. The slides were stained in 0.1% Cresyl Violet solution for 25 mins. Then the sections
568 were washed quickly in the water and differentiated in 95% ethanol. We used 100% ethanol to
569 dehydrate the slides, followed by rinsing with the xylene solution. Finally, the neutral resin
570 medium was used to mount the slides.
571
572 **Immunohistochemical (IHC) staining**
573 The paraffin sections were dewaxed and rehydrated as described above for Nissl staining. The
574 slides were boiled in 1×antigen retrieval solution (DAKO) under microwave conditions for 15
575 min. After cooled to room temperature (RT), the slides were incubated with 3% H_2O_2 for 30
576 min. Then, the slides were treated with blocking buffer for 60 min at RT and then incubated
577 with the primary antibody in the hybridization buffer (10×diluted blocking buffer) overnight
578 (O/N) at 4 °C. The next day, the tyramide signal amplification kit (TSA) (Invitrogen) was used
579 according to the manufacturer's protocol. After being incubated with 1%TSA blocking buffer,

580 the sections were treated with a biotinylated secondary antibody for 60 min at RT. After being
581 washed with 1×PBS three times, the slides were incubated with 1×HRP-conjugated
582 streptavidin for 1 h. Next, the tyramide working solution was prepared, including the 0.15‰
583 H_2O_2 in distilled water, the 100×diluted tyramide substrate solution (tyramide-488 or tyramide-
584 594), and the amplification buffer. The sections were incubated with the working solution for
585 10 min. Then the slides were counterstained with DAPI and mounted with the antifade
586 mounting medium (Southern Biotech) (S. Feng et al., 2017; Zhang et al., 2020). Finally, the
587 sections were observed and images were captured with a digital fluorescence microscope
588 (Zeiss).
589 The following primary antibodies were used in the study: mouse anti-NR2F1 (1:1000, R&D,
590 Cat # PP-H8132-00), mouse anti-NR2F2 (1:2000, R&D, Cat # PP-H7147-00), rabbit anti-
591 NR2F2 (1:2000, a gift from Dr. Zhenzhong Xu, Zhejiang University, China), rabbit anti-HuB
592 (1:500, Abcam, Cat # ab204991), rat anti-Ctip2 (1:500, Abcam, Cat # ab18465), rabbit anti-
593 Wfs1 (1:500, ProteinTech, Cat # 11558-1-AP), goat anti-Prox1 (1:500, R&D, Cat # AF2727),
594 rabbit anti-Calretinin (1:500, Sigma, Cat # C7479), rabbit anti-Calbindin (1:500, Swant, Cat #
595 CB38), mouse anti-SMI312 (1:200, Covance, Cat # SMI-312R), rabbit anti-Sox2 (1:500,
596 Affinity BioReagents, Cat # PA1-16968), rat anti-Tbr2 (1:500, Thermo Fisher, Cat # 12-4875-
597 82), goat anti-NeuroD1 (1:200, Santa Cruz, Cat # sc-1084), goat anti-Lhx2 (1:200, Santa Cruz,
598 Cat # sc-19344), goat anti-Lhx5 (1:200, R&D, Cat # AF6290), goat anti-β-galactosidase (LacZ)
599 (1:400, Biogenesis, Cat # 4600-1409), mouse anti-GFAP (1:500, Sigma, Cat # G3893), rabbit
600 anti-Nestin (1:200, Santa Cruz, Cat # sc-20978), goat anti-Dcx (1:500, Santa Cruz, Cat # sc-
601 8066). The following secondary antibodies were used in the study: donkey anti-mouse IgG
602 biotin-conjugated (1:400, JacksonImmuno, Cat # 715-065-150), donkey anti-rabbit IgG biotin-
603 conjugated (1:400, JacksonImmuno, Cat # 711-065-152), donkey anti-goat IgG biotin-

604 conjugated (1:400, JacksonImmuno, Cat # 705-066-147), donkey anti-rat IgG biotin-
605 conjugated (1:400, JacksonImmuno, Cat # 712-065-150).
606
607 **Western blotting**
608 We homogenized the isolated dorsal and ventral hippocampus tissues from 1-month-old mice
609 respectively in the RIPA buffer (Applygen) with protease inhibitor cocktail (Sigma) and
610 phosphatase inhibitors (Invitrogen) and then centrifuged at the speed of 12,000 rpm for 30 mins.
611 We collected the supernatants and analyzed the total concentrations by the BCA kit (Applygen).
612 Gradient SDS-PAGE gels were used to separate the same amounts of protein sample (40
613 μg/lane), and then the proteins were transferred to the PVDF membranes (Millipore). After
614 being blocked by the 3% BSA (Sigma) for 2h, the membranes that contained proteins were
615 incubated by primary antibodies at 4 °C O/N. The membranes were rinsed with 1×PBST three
616 times for 10 mins and then treated with biotinylated secondary antibodies for 2h at RT. After
617 washing with 1×PBST, membranes were treated with the HRP-conjugated streptavidin for 1h
618 at RT. Finally, we used the chemiluminescence detection system (Tanon) to detect the bands.
619 The density of the protein band was analyzed by the software Image J.
620 The primary antibodies were used in the experiment as below: mouse anti-NR2F1 (1:2000,
621 R&D, Cat # PP-H8132-00), rabbit anti-NR2F2 (1:3000, a gift from Dr. Zhenzhong Xu,
622 Zhejiang University, China), mouse anti-GAPDH (1:1000, Santa Cruz, Cat # sc-32233). The
623 following secondary antibodies were applied in the study, including goat anti-mouse IgG
624 biotin-conjugated (1:1000, KPL, Cat # 16-18-06), goat anti-rabbit IgG biotin-conjugated
625 (1:1000, KPL, Cat # 16-15-06).
626
627 **Golgi staining**

628 Deep anesthesia was performed before sacrificing the control and mutant mice, and the brains
629 were immediately isolated. The FD rapid GolgiStain kit (FD NeuroTech) was used to process
630 the brain tissue samples, which were immersed in an equal volume of immersion solution
631 mixed with solutions A and B and stored in the dark for two weeks at RT. At least 5mL of
632 immersion solution was used for each cubic meter of the tissue. To achieve the best results, the
633 container of tissue was gently swirled from side to side twice a week during the incubating
634 period. Afterwards, the brain tissue was transferred to solution C in the dark at RT for at least
635 72 hours (up to 1 week). Finally, the tissues were cut into 100 μm thick slices with a cryostat
636 at -20°C to -22°C and transferred to gelatin-coated microscope slides containing solution C
637 using a sample retriever. The slices were dried naturally at RT. The concrete staining procedure
638 of the kit was followed using the manufactory's protocol. Then, the sections were rinsed twice
639 with double-distilled water for 4 minutes each time. The slices were placed in a mixture of one
640 volume of solution D, one volume of solution E, and two volumes of double distilled water for
641 10 minutes and were rinsed twice with distilled water for 4 minutes each time. The sections
642 were later dehydrated in 50%, 75%, and 95% ethanol for 4 minutes respectively. Next, the
643 slices were dehydrated in 100% ethanol 4 times for 4 minutes each time. Finally, the sections
644 were cleared in xylene and mounted with a neutral resin medium.
645
646 **Morris water maze**
647 By recording the time spent by the mice swimming in the water tank and finding the escape
648 platform hidden underwater, and the swimming trajectory, the Morris water maze test can
649 objectively reflect the spatial learning and memory ability of the mice. The test was divided
650 into the control and mutant group with at least eight mice in each group. We poured tap water
651 into the water maze tank and added an appropriate amount of well-mixed, milky white food
652 dye. The height of the liquid level was about 1 cm higher than the escape platform, and the

653 water temperature was kept at about 25±1℃. At the same time, four markers of different shapes
654 were pasted on the four directions of the inner wall above the water tank to distinguish different
655 directions. The Morris water maze test was divided into the training phase and the probe trial
656 phase. The training phase lasted for 6 days, 4 times a day, and the interval between each training
657 was about 30 minutes. During training, the mice were placed into the tank from the entry points
658 of four different quadrants facing the inner wall, and their latency was recorded from the time
659 they entered the water to the time they found a hidden underwater platform and stood on it.
660 After the mouse found the platform, we let it stay on the platform for 10 seconds before
661 removing it. If the mouse failed to discover the platform 60 seconds after entering the water, it
662 was guided to find the platform and left to stay for 10 seconds. Each mouse was placed into
663 the water tank from four water entry points and recorded as one training session. The probe
664 trial was carried out on the seventh day, and the underwater platform was removed. Each
665 experimental mouse was put into the water tank at the same water entry point and allowed to
666 move for 60 seconds. The time that each mouse spent in the quadrant, where the platform was
667 originally placed, was recorded.
668
669 **RNA isolation and quantitative real-time PCR**
670 Total RNAs were prepared from the whole telencephalon of the control (n=5) and double
671 mutant (n=3) mice at E11.5 respectively, with the TRIzol Reagent (Invitrogen) by following
672 the manufactory's protocol. Reverse-transcription PCR and real-time quantitative PCR assays
673 were performed as described previously (Tang et al., 2012). A student's t-test was used to
674 compare the means of the relative mRNA levels between the control group and mutant group.
675 Primer sequences are as follows:
676 *Axin2-f*, 5'-*CTGCTGGTCAGGCAGGAG*-3', *Axin2-r*, 5'-*TGCCAGTTTCTTTGGCTCTT*-3';
677 *Nr2f1-f*, 5'-*CAAAGCCATCGTGCTATTCA*-3', *Nr2f1-r*, 5'-*CCTGCAGGCTTTCGATGT*-3';

678 *Nr2f2-f*, 5'-*CCTCAAAGTGGGCATGAGAC*-3', *Nr2f2-r*, 5'-*TGGGTAGGCTGGGTAGGAG-*
679 3'; *Emx1-f*, 5'-*CTCTCCGAGACGCAGGTG*-3', *Emx1-r,* 5'-*CTCAGACTCCGGCCCTTC*-3';
680 *Emx2-f*, 5'-*CACGCTTTTGAGAAGAACCA*-3', *Emx2-r,* 5'-*GTTCTCCGGTTCTGAAACCA*-3';
681 *Foxg1-f*, 5'-*GAAGGCCTCCACAGAACG*-3', *Foxg1-r*, 5'-*GGCAAGGCATGTAGCAAAAG*-3';
682 *Gli3-f*, 5'-*TGATCCATCTCCTATTCCTCCA*-3', *Gli3-r*, 5'-*TCTGGATACGTCGGGCTACT*-3';
683 *Lef1-f*, 5'-*TCCTGAAATCCCCACCTTCT*-3', *Lef1-r*, 5'-*TGGGATAAACAGGCTGACCT*-3';
684 *Lhx2-f*, 5'-*CAGCTTGCGCAAAAGACC*-3', *Lhx2-r*, 5'-*TAAAAGGTTGCGCCTGAACT*-3';
685 *Lhx5-f*, 5'-*TGTGCAATAAGCAGCTATCCA*-3', *Lhx5-r,* 5'-*CAAACTGCGGTCCGTACA*-3';
686 *Otx1-f*, 5'-*CCAGAGTCCAGAGTCCAGGT*-3', *Otx1-r*, 5'-*CCGGGTTTTCGTTCCATT*-3';
687 *Otx2-f*, 5'-*GGTATGGACTTGCTGCATCC*-3', *Otx2-r*, 5'-*CGAGCTGTGCCCTAGTAAATG*-3';
688 *Pax6-f*, 5'-*GTTCCCTGTCCTGTGGACTC*-3', *Pax6-r*, 5'-*ACCGCCCTTGGTTAAAGTCT*-3';
689 *Tcf4-f*, 5'-*AAATGGCCACTGCTTGATGT*-3', *Tcf4-r*, 5'-*GCACCACCGGTACTTTGTTC*-3'.
690

691 **Quantification and statistical analysis**

692 The number of specified immunofluorescent marker-positive cells was assessed by Image J
693 Cell Counter in full image fields. Three brain sections per mouse were counted for each index.
694 GraphPad Prism 7.0 (GraphPad) was used to perform statistical analysis. The data analysis
695 used one-way analysis of variance (ANOVA), Dunnett's or Tukey's post hoc tests, and
696 student's unpaired t-test. The data were expressed as the mean ± SEM. The data obtained from
697 at least three independent replicates were used for statistical analysis. $P < 0.05$ was considered
698 the significant statistical difference.
699

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Data availability

Numerical data are available in the manuscript and supporting files.

Competing interests

The authors declare no competing interests.

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942 **Figure legends**

943 **Figure 1. Duplicated CA1 and CA3 domains are generated in the ventral hippocampus**
944 **of RX$^{Cre/+}$; *Nr2f2$^{F/F}$* mutant mice.**

945 **A,** The expression of NR2F1 (**a, d, g, j, m**) and NR2F2 (**b, e, h, k, n**) in coronal sections (**a-f**)
946 and sagittal sections (**g-o**) of the hippocampus at postnatal month 1 (1M); representative
947 Western blots and quantitative densitometry data for the expression of NR2F1 and NR2F2 in
948 the dorsal and ventral hippocampus at 1M (**p-q**). **B,** In coronal sections along the rostrocaudal
949 axis (**a-f**) and sagittal sections along the lateral-medial axis (**g-l**) of the hippocampus in mutant
950 mice, compared with that in control mice (**a-c**, **g-i**), the ectopic CA-like structure, indicated by
951 the star, was observed in the ventral region in *Nr2f2* gene mutant (RX$^{Cre/+}$; *Nr2f2$^{F/F}$)* mice at
952 1M (**d-f**, **j-l**). **C,** The expression of HuB and Ctip2 in the corresponding inserted area in **a-f**
953 under a high magnification objective lens at 1M (**g-r**); compared with those of control mice (**a-**
954 **c**, **g-i**, **m-o**), the duplicated HuB-positive CA3 domain, indicated by the star, and Ctip2-positive
955 domains, indicated by the arrowhead, were specifically observed in the ventral hippocampus
956 (**d-f, p-r**) but not in the dorsal hippocampus (**d-f**, **j-l**) of *Nr2f2* mutant mice at 1M; Ctip2
957 positive AHi and MePD amygdaloid nuclei were barely observed in the *Nr2f2* mutant mice,
958 indicated by the arrows, instead of the ectopic CA domains at the prospective amygdaloid
959 regions (**e-f**, **q-r**). Data are expressed as the mean ± SEM. *Student's t test*, $^{*}P<0.05$, $^{**}P<0.01$.
960 AHi, amygdalohippocampal area; AMY, amygdala nuclei; CTX, cortex; dCA1, dorsal CA1;
961 dCA3, dorsal CA3; dDG, dorsal dentate gyrus; dHPC, dorsal hippocampus; MePD,
962 posterodorsal part of the medial amygdaloid nucleus; PMCo, posteromedial cortical
963 amygdaloid nucleus; vCA1, ventral CA1; vCA3, ventral CA3; vDG, ventral dentate gyrus;
964 vHPC, ventral hippocampus. Scale bars, **Aa-c**, **Ad-f**, **Aj-o**, **Cg-r**, 100 μm; **Ag-i**, **Ba-l, Ca-f**,
965 200μm.

966

967 **Figure 1—figure supplement 1. The expression of *Nr2f* genes in the early developing**
968 **hippocampus and different conditional knock mouse models.**
969 **A,** The expression of *Nr2f1* and *Nr2f2* genes in the forebrain at E10.5 (**a-b**) and E11.5 (**c-d**).
970 **B,** The expression of *Nr2f1* and *Nr2f2* genes in the developing hippocampus at E12.5 (**a-c**),
971 E14.5 (**d-f**), and P0 (**g-l**). **C,** Compared with that of control mice (**a-c**), *Nr2f2* is efficiently
972 deleted by RXCre recombinase in the hippocampus of *Nr2f2* mutant mice at 1M (**d-f**); *Nr2f1*
973 is clearly deleted by RXCre recombinase in the hippocampus of *Nr2f1* mutant mice at 1M (**g-**
974 **i**). Compared with that of control mice (**j-l**), NR2F1 and NR2F2 were efficiently deleted by
975 RXCre recombinase at the hippocampal primordium, including the MP and CH, in *Nr2f1/2*
976 double-mutant mice at E14.5 (**m-o**). **D,** Compared with that of the control mice (**a-c, g-h**), the
977 expression of *Nr2f2* was significantly decreased in the hippocampal primordium of the
978 homozygous mutant mice at E11.5; meanwhile, the LacZ signals obviously increased in the
979 *Nr2f2* homozygous mutant mice at E11.5 (**d-f, j-k**). Compared with that of the control mice (**i**),
980 the expression of NR2F1 is activated in the caudal hippocampal primordium of the
981 homozygous mutant mice at E11.5 (**l**). CH, cortical hem; dHPC, dorsal hippocampus; HP,
982 hippocampal primordium; HPC, hippocampus; MP, medial pallium; vHPC, ventral
983 hippocampus. Scale bars, **Aa-d**, **Ba-f**, **Cj-o**, 200 μm; **Bg-l**, **Ca-i**, **Dg-l**, 100 μm; **Da-f**, 250 μm.
984
985 **Figure 2. The specification and differentiation of the dorsal CA1 lineage failed with the**
986 **dysplastic dorsal hippocampus in RX$^{Cre/+}$; *Nr2f1$^{F/F}$* mutant mice.**
987 **A,** In coronal sections along the rostrocaudal axis (**a-f**) and sagittal sections along the lateral-
988 medial axis (**g-l**) of the hippocampus, compared with that of control mice (**a-c**, **g-i**), the dorsal
989 hippocampus was shrunken, indicated by the star, in *Nr2f1* gene mutant (RX$^{Cre/+}$; *Nr2f1$^{F/F}$)*
990 mice at 1M (**d-f**, **j-l**). **B,** The expression of HuB and Ctip2 in the corresponding inserted area
991 in **a-f** under a high magnification objective lens at 1M (**g-r**); compared with that of control

992 mice (**a-c**, **g-i**, **m-o**), the HuB-positive CA3 domain was reduced in the dorsal hippocampus,
993 especially the Ctip2-positive dorsal CA1, which was barely detected in *Nr2f1* mutant mice at
994 1M (**d-f**, **j-l**), while their expression in the ventral hippocampus was comparable between the
995 controls and mutants (**d-f**, **p-r**). **C,** The expression of HuB and Wfs1 in the corresponding
996 inserted area in **a-f** under a high magnification objective lens at 1M (**g-r**); the expression of
997 HuB and the dCA1 marker Wfs1 in the control (**a-c**, **g-i**, **m-o**) and *Nr2f2* mutant mice (**d-f**, **j-l**,
998 **p-r**) at 1M. Wfs1-positive dorsal CA1 could not be detected in *Nr2f1* mutant mice at 1M, as
999 indicated by the arrowhead. dHPC, dorsal hippocampus; HPC, hippocampus; vHPC, ventral
1000 hippocampus. Scale bars, **Aa-l**, **Ba-f**, **Ca-f,** 200 μm; **Bg-r**, **Cg-r,** 100 μm.
1001
1002 **Figure 2—figure supplement 1. Defects in *Emx1*$^{Cre/+}$; *Nr2f1*$^{F/F}$ mutant mice.**
1003 **A,** Immunofluorescence staining data showed that compared with those of control mice (**a, c,**
1004 **e, g**), the proportions of either the Wfs1- or Ctip2-positive dorsal CA1 domain were reduced
1005 in *Emx1*$^{Cre/+}$; *Nr2f1*$^{F/F}$ mutant mice at 3M (**b, d, f, h**). **B,** Golgi staining showed that compared
1006 with those of controls, the numbers of branch points and secondary dendrites of both apical
1007 and basal dendrites were significantly reduced in the dorsal hippocampal CA1 pyramidal
1008 neurons of *Emx1*$^{Cre/+}$; *Nr2f1*$^{F/F}$ mutant mice (**a-c**). **C,** The Morris water maze behavior test
1009 showed that compared with that of controls, spatial learning and memory were significantly
1010 damaged in *Emx1*$^{Cre/+}$; *Nr2f1*$^{F/F}$ mutant mice. Data are expressed as the mean ± SEM. *Student's*
1011 *t test*, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. Scale bars, **Aa-h,** 100 μm; **Ba-b,** 50 μm.
1012
1013 **Figure 3. Defects in the hippocampus in RX$^{Cre/+}$; *Nr2f1*$^{F/F}$; *Nr2f2*$^{F/F}$ double-gene mutant**
1014 **mice.**
1015 **A,** In coronal sections along the rostrocaudal axis, compared with control mice (**a-d**), the
1016 hippocampus was atrophic in RX$^{Cre/+}$; *Nr2f1*$^{F/F}$; *Nr2f2*$^{F/F}$ double mutant mice, indicated by the

1017 star, and an ectopic unknown nucleus was observed in the caudal plates, indicated by the
1018 arrowhead (**e-h**). **B,** Compared with that of control mice (**a-c**, **g-i**), the expression of HuB,
1019 Ctip2, and Prox1 was decreased in the hippocampus of *Nr2f1/2* double-gene mutant mice at 3
1020 weeks postnatal (3W) (**d-f**, **j-l**). **C,** Compared with that of control mice (**a-c**, **g-i**), the expression
1021 of HuB could not be detected in the presumptive CA3 domain, and the expression of Ctip2 or
1022 Prox1 could not be detected in the presumptive DG domain of the prospective ventral
1023 hippocampus of $RX^{Cre/+}$; $Nr2f1^{F/F}$; $Nr2f2^{F/F}$ double mutant mice. Scale bars, **Aa-h**, 200 μm;
1024 **Ba-l**, **Ca-l,** 100 μm.
1025
1026 **Figure 3—figure supplement 1. Adult neurogenesis was abnormal in the hippocampi of**
1027 ***Nr2f1/2* double-gene mutant mice.**
1028 **A,** The expression of GFAP and Nestin, markers of NSCs, in the SGZ of the vDG in control
1029 and *Nr2f2* mutant mice at 1M (**a-h**), in the SGZ of the dDG in control and *Nr2f1* mutant mice
1030 at 1M (**i-p**) and in the SGZ of the DG in control and *Nr2f1/2* double-gene mutant mice at 3W
1031 (**q-x**). **B**, The expression of Dcx, a marker of newborn neurons, in the SGZ of the vDG in
1032 control and *Nr2f2* mutant mice at 1M (**a-d**), in the SGZ of the dDG in control and *Nr2f1* mutant
1033 mice at 1M (**e-h**) and in the SGZ of the DG in control and *Nr2f1/2* double-gene mutant mice
1034 at 3W (**i-l**). **C,** Quantitative analysis of GFAP/Nestin-positive cells (**a**) and Dcx-positive cells
1035 (**b**) in the SGZ of the DG in control and *Nr2f1/2* double-gene mutant mice at 3W. The numbers
1036 of GFAP and Nestin double-positive NSCs and Dcx-positive newborn neurons were
1037 significantly reduced in double-mutant mice. Data are expressed as the mean ± SEM. *Student's*
1038 *t test*, $^{**}P<0.01$, $^{***}P<0.001$. dDG, dorsal DG; NSC, neural stem cell; SGZ, subgranular zone;
1039 vDG, ventral DG. Scale bars, **Aa-x**, 50 μm; **Ba-l**, 100 μm.
1040

1041 **Figure 4. The impairment of hippocampal trisynaptic connectivity in *Nr2f2* single-gene,**
1042 ***Nr2f1* single-gene, and *Nr2f1/2* double-gene mutant mice.**
1043 **A,** The expression of Calretinin, Calbindin, and SMI312 in the ventral hippocampus of the
1044 control (**a-b**, **e-f**, **i-j**) and *Nr2f2* single-gene mutant mice (**c-d**, **g-h**, **k-l**). **B,** The expression of
1045 Calretinin, Calbindin, and SMI312 in the dorsal hippocampus of the control (**a-b**, **e-f**, **i-j**) and
1046 *Nr2f1* single-gene mutant mice (**c-d**, **g-h**, **k-l**). **C,** The expression of Calretinin, Calbindin, and
1047 SMI312 in the hippocampus of the control (**a-b**, **e-f**, **i-j**) and *Nr2f1/2* double-gene mutant mice
1048 (**c-d**, **g-h**, **k-l**). dHPC, dorsal hippocampus; HPC, hippocampus; vHPC, ventral hippocampus.
1049 Scale bars, **Aa-l**, **Ba-l**, **Ca-l**, 100 μm.
1050
1051 **Figure 5. *Nr2f* genes regulate the expression of key genes associated with early**
1052 **hippocampal development.**
1053 **A,** The expression profiles of genes involved in hippocampal development in control and the
1054 double mutant mice at E11.5. **B,** Compared with that of control mice (**a-b**, **a'-b', a''-b'', i-j,**
1055 **i'-j', q-r, q'-r'**), the expression of Lhx5 was reduced in double-mutant mice at E11.5 (**c-d**, **c'-**
1056 **d', c''-d''**), E13.5 (**k-l**, **k'-l'**) and E14.5 (**s-t**, **s'-t'**); the expression of Lhx2 was comparable
1057 between the control and double-mutant mice at E11.5 (**e-h**, **e'-h'**); and compared with that of
1058 control mice (**m-n**, **m'-n'**, **u-v**, **u'-v'**), the expression of Lhx2 was increased in double-mutant
1059 mice at E13.5 (**o-p**, **o'-p'**) and E14.5 (**w-x**, **w'-x'**). **C,** Compared with that of control mice (**a-**
1060 **b**, **a'-b'**), the expression of Sox2 was normal in double-mutant mice at E14.5 (**c-d**, **c'-d'**);
1061 compared with that of control mice (**e-f**, **e'-f'**), the expression of Tbr2 was decreased in *Nr2f*
1062 mutant mice at E14.5 (**g-h**, **g'-h'**); compared with that of control mice (**i-j**, **i'-j'**), the expression
1063 of NeuroD1 was reduced in double-mutant mice at E14.5 (**k-l**, **k'-l'**). **D,** Quantitative analysis
1064 of Tbr2-positive cells and NeuroD1-positive cells in **Ce'-h'** and **Ci'-l'**. **E,** In the hippocampal
1065 primordium of the early embryo, *Nr2f1* is expressed dorsally in the MP, and *Nr2f2* is expressed

1066 ventrally in the CH. In the mature hippocampus, the expression of *Nr2f1* is higher in the dorsal
1067 hippocampus, which is related to spatial learning and memory, and the expression of *Nr2f2* is
1068 mainly in the ventral hippocampus, which is associated with emotion and anxiety (**a**). Our
1069 findings support a novel molecular mechanism by which *Nr2f1* and *Nr2f2* may cooperate to
1070 ensure the appropriate morphogenesis and functions of the hippocampus by modulating the
1071 *Lhx5-Lhx2* axis (**b**). Data are expressed as the mean ± SEM. *Student's t test*, $^{**}P<0.01$. CH,
1072 cortical hem; ChP, choroid plexus; DP, dorsal pallium; HP, hippocampal primordium; MP,
1073 medial pallium. Scale bars, **Ba-x, Ca-l**, 200 μm.
1074
1075 **Figure 5—figure supplement 1. *Nr2f1* and *Nr2f2* genes coordinate to control distinct**
1076 **characteristics of the hippocampus.**
1077 Roles of *Nr2f1* and *Nr2f2* genes in the development and function of the hippocampus and the
1078 association with neurological diseases. *Nr2f1* is required for the morphogenesis of the dorsal
1079 hippocampus and the specification of dorsal CA1 pyramidal neuron lineage, which are
1080 associated with neurodevelopmental disorders, including ID and ASD. *Nr2f2* is required to
1081 prevent the duplication of the CA1 and CA3 lineages of the ventral hippocampus, which may
1082 be related to psychiatric diseases such as depression, anxiety, or schizophrenia. The *Nr2f1* and
1083 *Nr2f2* genes are novel intrinsic regulatory genes, which cooperate with each other to ensure the
1084 early morphogenesis of the hippocampus.
1085

1086 **The information of the source data files**

1087 **Figure 1-source data 1.** The Nissl staining results of the control and $RX^{Cre/+}$; *Nr2f2*$^{F/F}$ mutant
1088 mice at 1M (part 1); the expression of HuB and Ctip2 in the hippocampus of the control and
1089 $RX^{Cre/+}$; *Nr2f2*$^{F/F}$ mutant mice at 1M (part 1).

1090

1091 **Figure 1-source data 2.** The expression of NR2F1 and NR2F2 in coronal sections and sagittal
1092 sections of the mouse brain at 1M (part 1); the expression of HuB and Ctip2 in the hippocampus
1093 of the control and $RX^{Cre/+}$; *Nr2f2*$^{F/F}$ mutant mice at 1M (part 2).

1094

1095 **Figure 1-source data 3.** The expression of NR2F1 and NR2F2 in coronal sections and sagittal
1096 sections of the mouse brain at 1M (part 2).

1097

1098 **Figure 1-source data 4.** The expression of NR2F1 and NR2F2 in sagittal sections of the mouse
1099 brain at 1M (part 1).

1100

1101 **Figure 1-source data 5.** The expression of NR2F1 and NR2F2 in sagittal sections of the mouse
1102 brain at 1M (part 2); the Nissl staining results of the control and $RX^{Cre/+}$; *Nr2f2*$^{F/F}$ mutant mice
1103 at 1M (part 2).

1104

1105 **Figure 1-source data 6.** The expression of NR2F1 and NR2F2 in sagittal sections of the mouse
1106 brain at 1M (part 3); Western blots data for the expression of NR2F1 and NR2F2 in the dorsal
1107 and ventral hippocampus at 1M; the Nissl staining results of the control and $RX^{Cre/+}$; *Nr2f2*$^{F/F}$
1108 mutant mice at 1M (part 3).

1109

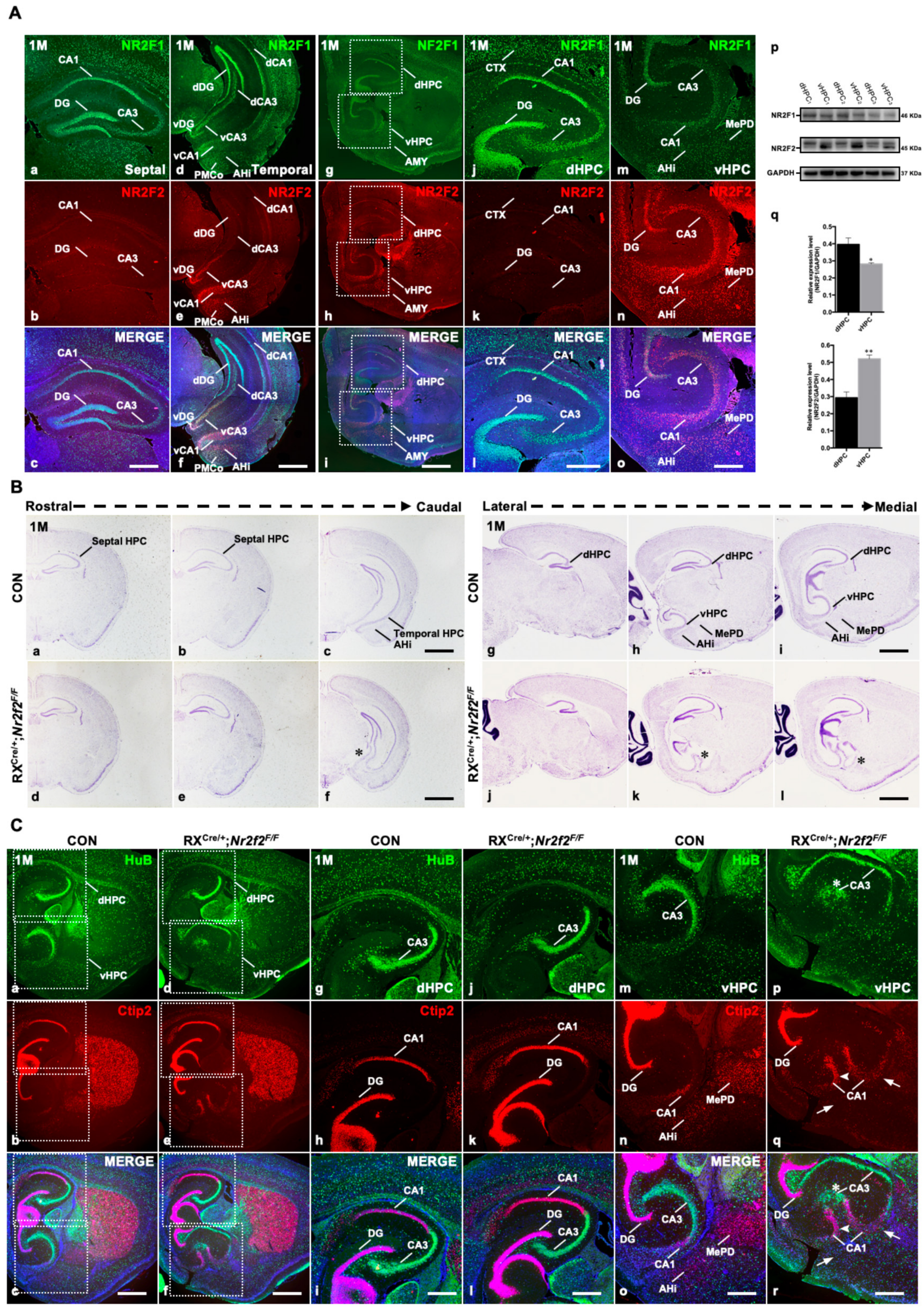
1110 **Figure 1-figure supplement 1-source data 1.** The expression of *Nr2f1* and *Nr2f2* genes in the
1111 developing hippocampus at E12.5; the deletion efficiency of RXCre recombinase in the
1112 hippocampus of $RX^{Cre/+}$; $Nr2f2^{F/F}$, $RX^{Cre/+}$; $Nr2f1^{F/F}$, and $RX^{Cre/+}$; $Nr2f1^{F/F}$; $Nr2f2^{F/F}$ mice (part
1113 1).
1114
1115 **Figure 1-figure supplement 1-source data 2.** The expression of *Nr2f1* and *Nr2f2* genes in the
1116 telencephalon at E10.5, E11.5, E14.5, and P0; the deletion efficiency of RXCre recombinase
1117 in the hippocampus of $RX^{Cre/+}$; $Nr2f2^{F/F}$, $RX^{Cre/+}$; $Nr2f1^{F/F}$, and $RX^{Cre/+}$; $Nr2f1^{F/F}$; $Nr2f2^{F/F}$ mice
1118 (part 2).
1119
1120 **Figure 2-source data 1.** The Nissl staining results of the control and $RX^{Cre/+}$; $Nr2f1^{F/F}$ mutant
1121 mice at 1M (part 1).
1122
1123 **Figure 2-source data 2.** The Nissl staining results of the control and $RX^{Cre/+}$; $Nr2f1^{F/F}$ mutant
1124 mice at 1M (part 2); the expression of HuB and Wfs1 in the hippocampus of the control and
1125 $RX^{Cre/+}$; $Nr2f1^{F/F}$ mutant mice at 1M.
1126
1127 **Figure 2-source data 3.** The expression of HuB and Ctip2 in the hippocampus of the control
1128 and $RX^{Cre/+}$; $Nr2f1^{F/F}$ mutant mice at 1M.
1129
1130 **Figure 2-source data 4.** The expression of HuB, Wfs1, and Ctip2 in the hippocampus of the
1131 control and $RX^{Cre/+}$; $Nr2f1^{F/F}$ mutant mice at 1M.
1132
1133 **Figure 2-figure supplement 1-source data 1.** The expression of Wfs1 and Ctip2 in the dorsal
1134 hippocampus of the control and $Emx1^{Cre/+}$; $Nr2f1^{F/F}$ mutant mice at 3M; Golgi staining results

1135 of the dorsal hippocampal CA1 pyramidal neurons and Morris water maze behavior test data
1136 of the control and *Emx1*$^{Cre/+}$; *Nr2f1*$^{F/F}$ mutant mice.
1137
1138 **Figure 3-source data 1.** The Nissl staining results of the control and RX$^{Cre/+}$; *Nr2f1*$^{F/F}$; *Nr2f2*$^{F/F}$
1139 double-gene mutant mice at 3W.
1140
1141 **Figure 3-source data 2.** The expression of HuB and Ctip2 in the hippocampus of the control
1142 and RX$^{Cre/+}$; *Nr2f1*$^{F/F}$; *Nr2f2*$^{F/F}$ double-gene mutant mice at 3W.
1143
1144 **Figure 3-source data 3.** The expression of HuB and Prox1 in the hippocampus of the control
1145 and RX$^{Cre/+}$; *Nr2f1*$^{F/F}$; *Nr2f2*$^{F/F}$ double-gene mutant mice at 3W.
1146
1147 **Figure 3-source data 4.** The expression of HuB, Ctip2, and Prox1 in the hippocampus of the
1148 control and RX$^{Cre/+}$; *Nr2f1*$^{F/F}$; *Nr2f2*$^{F/F}$ double-gene mutant mice at 3W.
1149
1150 **Figure 3-figure supplement 1-source data 1.** The expression of GFAP, Nestin, and Dcx in
1151 the SGZ of vDG in the control and RX$^{Cre/+}$; *Nr2f2*$^{F/F}$ mutant mice at 1M, in the SGZ of dDG
1152 in the control and RX$^{Cre/+}$; *Nr2f1*$^{F/F}$ mutant mice at 1M, and in the SGZ of DG in the control
1153 and RX$^{Cre/+}$; *Nr2f1*$^{F/F}$; *Nr2f2*$^{F/F}$ double-gene mutant mice at 3W (part 1).
1154
1155 **Figure 3-figure supplement 1-source data 2.** The expression of GFAP, Nestin, and Dcx in
1156 the SGZ of vDG in the control and RX$^{Cre/+}$; *Nr2f2*$^{F/F}$ mutant mice at 1M, in the SGZ of dDG
1157 in the control and RX$^{Cre/+}$; *Nr2f1*$^{F/F}$ mutant mice at 1M, and in the SGZ of DG in the control
1158 and RX$^{Cre/+}$; *Nr2f1*$^{F/F}$; *Nr2f2*$^{F/F}$ double-gene mutant mice at 3W (part 2).
1159

1160 **Figure 3-figure supplement 1-source data 3.** The expression of GFAP and Nestin in the SGZ
1161 of vDG in the control and $RX^{Cre/+}$; *Nr2f2*$^{F/F}$ mutant mice at 1M, in the SGZ of dDG in the
1162 control and $RX^{Cre/+}$; *Nr2f1*$^{F/F}$ mutant mice at 1M, and in the SGZ of DG in the control and
1163 $RX^{Cre/+}$; *Nr2f1*$^{F/F}$; *Nr2f2*$^{F/F}$ double-gene mutant mice at 3W; quantitative analysis of
1164 GFAP/Nestin-positive cells in the SGZ of DG in the control and $RX^{Cre/+}$; *Nr2f1*$^{F/F}$; *Nr2f2*$^{F/F}$
1165 double-gene mutant mice at 3W.
1166
1167 **Figure 3-figure supplement 1-source data 4.** The expression of GFAP, Nestin, and Dcx in
1168 the SGZ of vDG in the control and $RX^{Cre/+}$; *Nr2f2*$^{F/F}$ mutant mice at 1M, in the SGZ of dDG
1169 in the control and $RX^{Cre/+}$; *Nr2f1*$^{F/F}$ mutant mice at 1M, and in the SGZ of DG in the control
1170 and $RX^{Cre/+}$; *Nr2f1*$^{F/F}$; *Nr2f2*$^{F/F}$ double-gene mutant mice at 3W (part 3); quantitative analysis
1171 of Dcx-positive cells in the SGZ of DG in the control and $RX^{Cre/+}$; *Nr2f1*$^{F/F}$; *Nr2f2*$^{F/F}$ double-
1172 gene mutant mice at 3W.
1173
1174 **Figure 4-source data 1.** The expression of Calretinin, Calbindin, and SMI312 in the ventral
1175 hippocampus of the control and $RX^{Cre/+}$; *Nr2f2*$^{F/F}$ single-gene mutant mice at 1M.
1176
1177 **Figure 4-source data 2.** The expression SMI312 in the dorsal hippocampus of the control and
1178 $RX^{Cre/+}$; *Nr2f1*$^{F/F}$ single-gene mutant mice at 1M, and the expression of Calretinin and
1179 Calbindin in the hippocampus of the control and $RX^{Cre/+}$; *Nr2f1*$^{F/F}$; *Nr2f2*$^{F/F}$ double-gene
1180 mutant mice at 3W.
1181
1182 **Figure 4-source data 3.** The expression of Calretinin and Calbindin in the dorsal hippocampus
1183 of the control and $RX^{Cre/+}$; *Nr2f1*$^{F/F}$ single-gene mutant mice at 1M, and the expression of

1184 SMI312 in the hippocampus of the control and $RX^{Cre/+}$; *Nr2f1*$^{F/F}$; *Nr2f2*$^{F/F}$ double-gene mutant
1185 mice at 3W.
1186
1187 **Figure 5-source data 1.** The expression of Lhx5 and Lhx2 in the telencephalon of the control
1188 and $RX^{Cre/+}$; *Nr2f1*$^{F/F}$; *Nr2f2*$^{F/F}$ double-mutant mice at E14.5; the expression of Tbr2 and
1189 NeuroD1 in the telencephalon of the control and $RX^{Cre/+}$; *Nr2f1*$^{F/F}$; *Nr2f2*$^{F/F}$ double-mutant
1190 mice at E14.5 (part 1).
1191
1192 **Figure 5-source data 2.** The expression of Sox2, Tbr2, and NeuroD1 in the telencephalon of
1193 the control and $RX^{Cre/+}$; *Nr2f1*$^{F/F}$; *Nr2f2*$^{F/F}$ double-mutant mice at E14.5.
1194
1195 **Figure 5-source data 3.** The expression profiles of genes involved in the hippocampal
1196 development of the control and double mutant mice at E11.5; the expression of Lhx5 and Lhx2
1197 in the telencephalon of the control and $RX^{Cre/+}$; *Nr2f1*$^{F/F}$; *Nr2f2*$^{F/F}$ double-mutant mice at E11.5
1198 and E13.5; the expression of Tbr2 and NeuroD1 in the telencephalon of the control and $RX^{Cre/+}$;
1199 *Nr2f1*$^{F/F}$; *Nr2f2*$^{F/F}$ double-mutant mice at E14.5 (part 2); quantitative analysis of Tbr2-positive
1200 and NeuroD1-positive cells in the hippocampal primordium of the control and $RX^{Cre/+}$; *Nr2f1*$^{F/F}$;
1201 *Nr2f2*$^{F/F}$ double-mutant mice at E14.5.
1202
1203 **Figure 5-figure supplement 1-source data 1.** Roles of *Nr2f1* and *Nr2f2* genes in the
1204 development and function of the hippocampus and the association with neurological disorders.

Figure 1, Yang et al.



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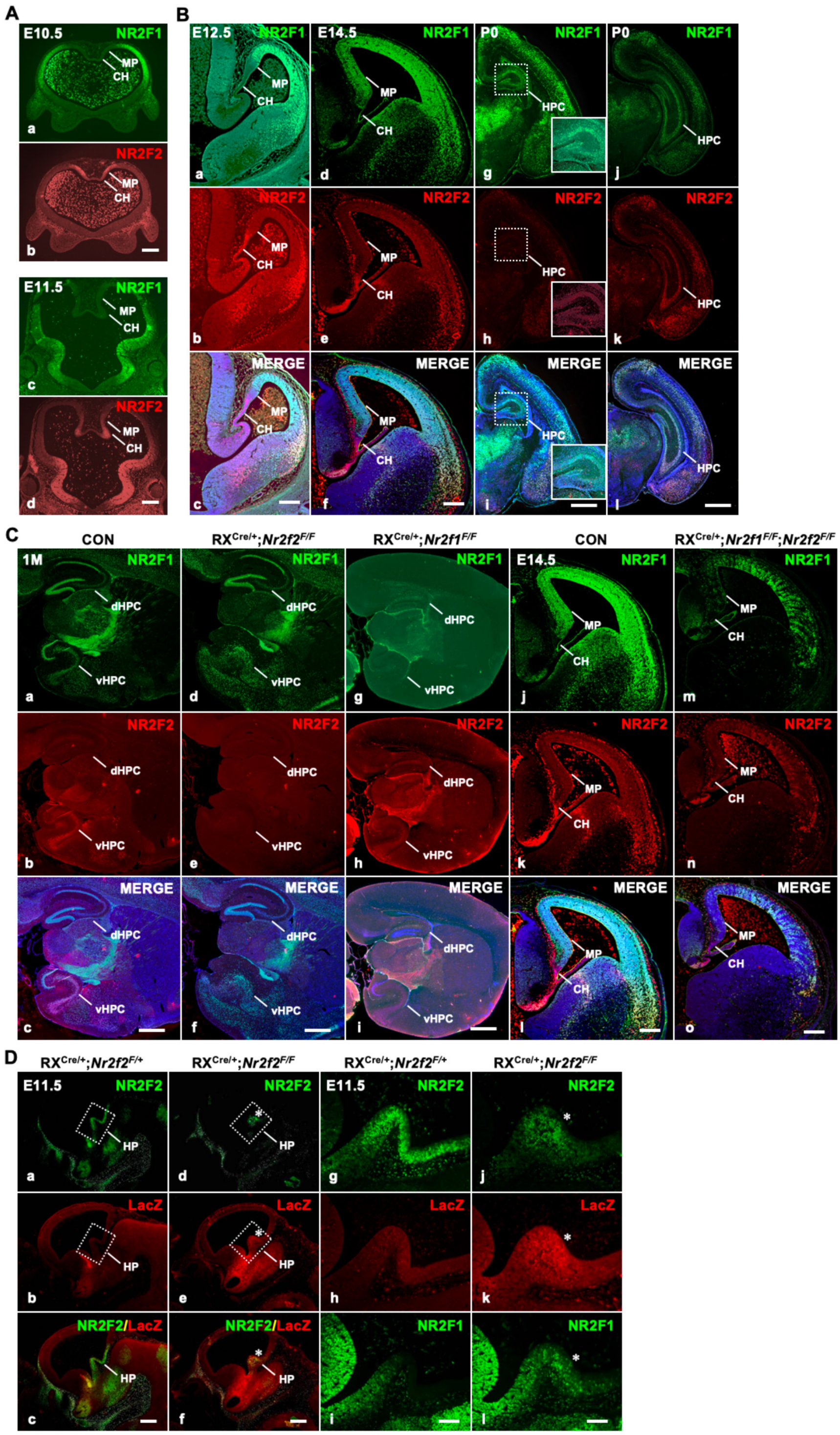
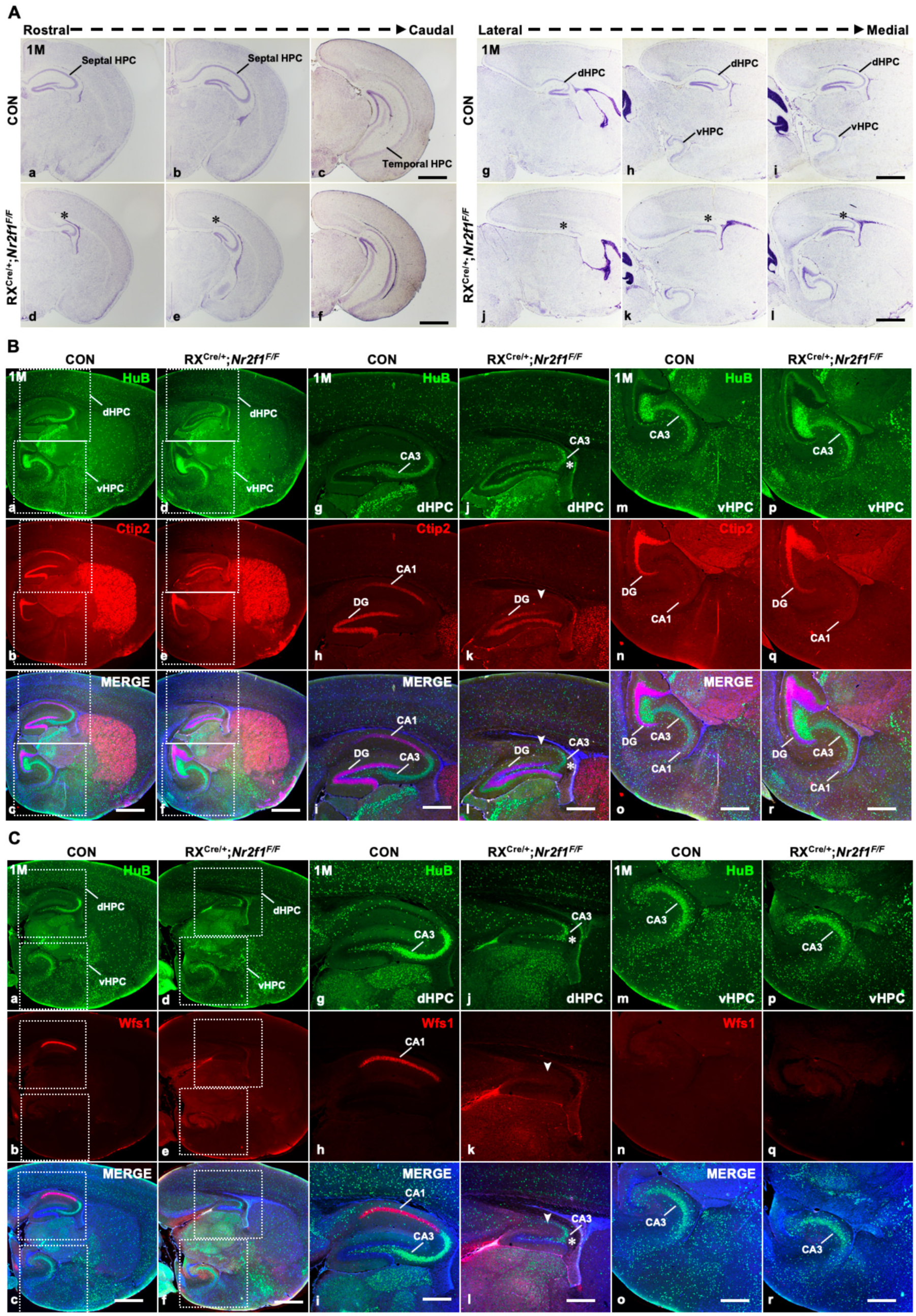


Figure 2, Yang et al.



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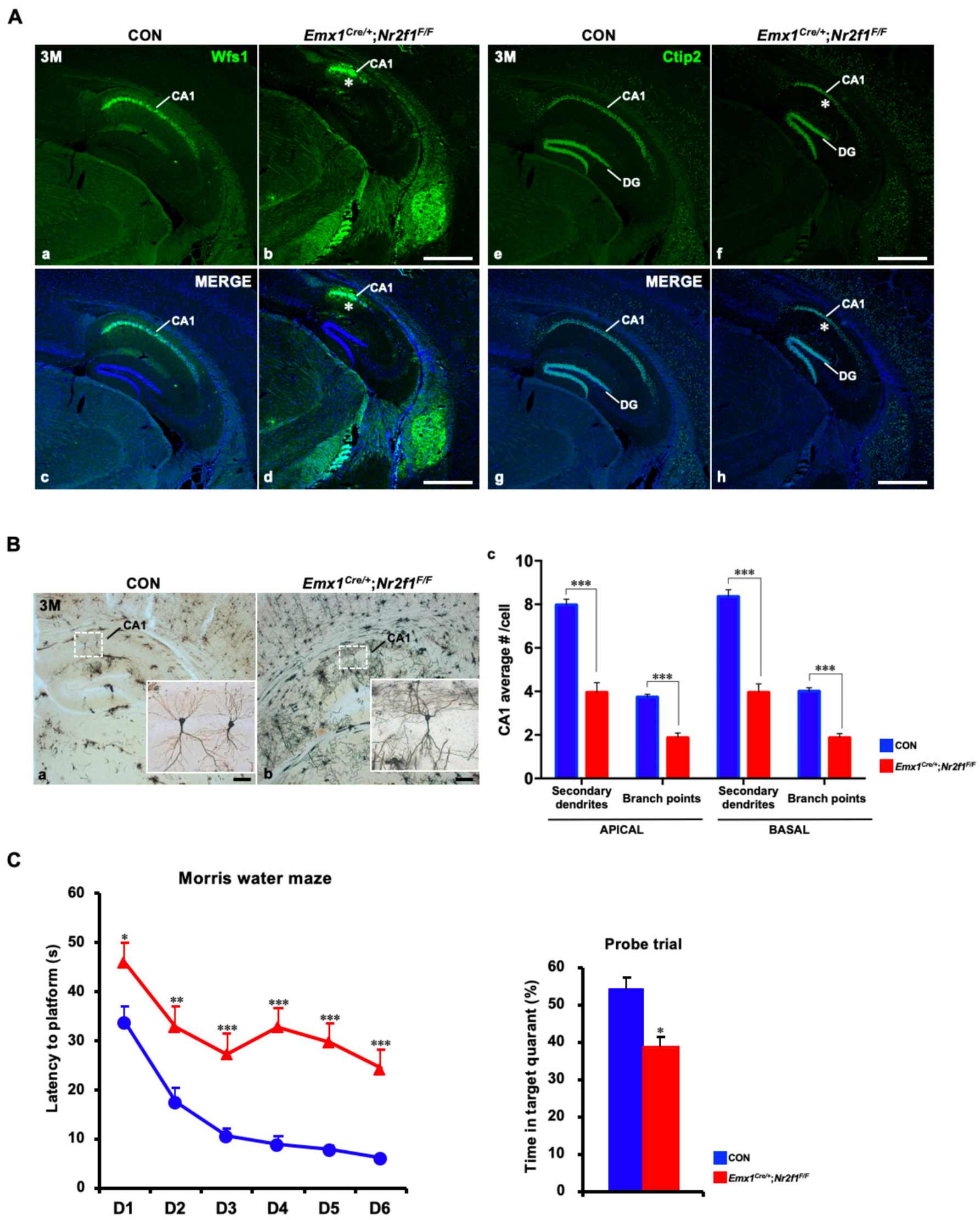

Figure 3, Yang et al.

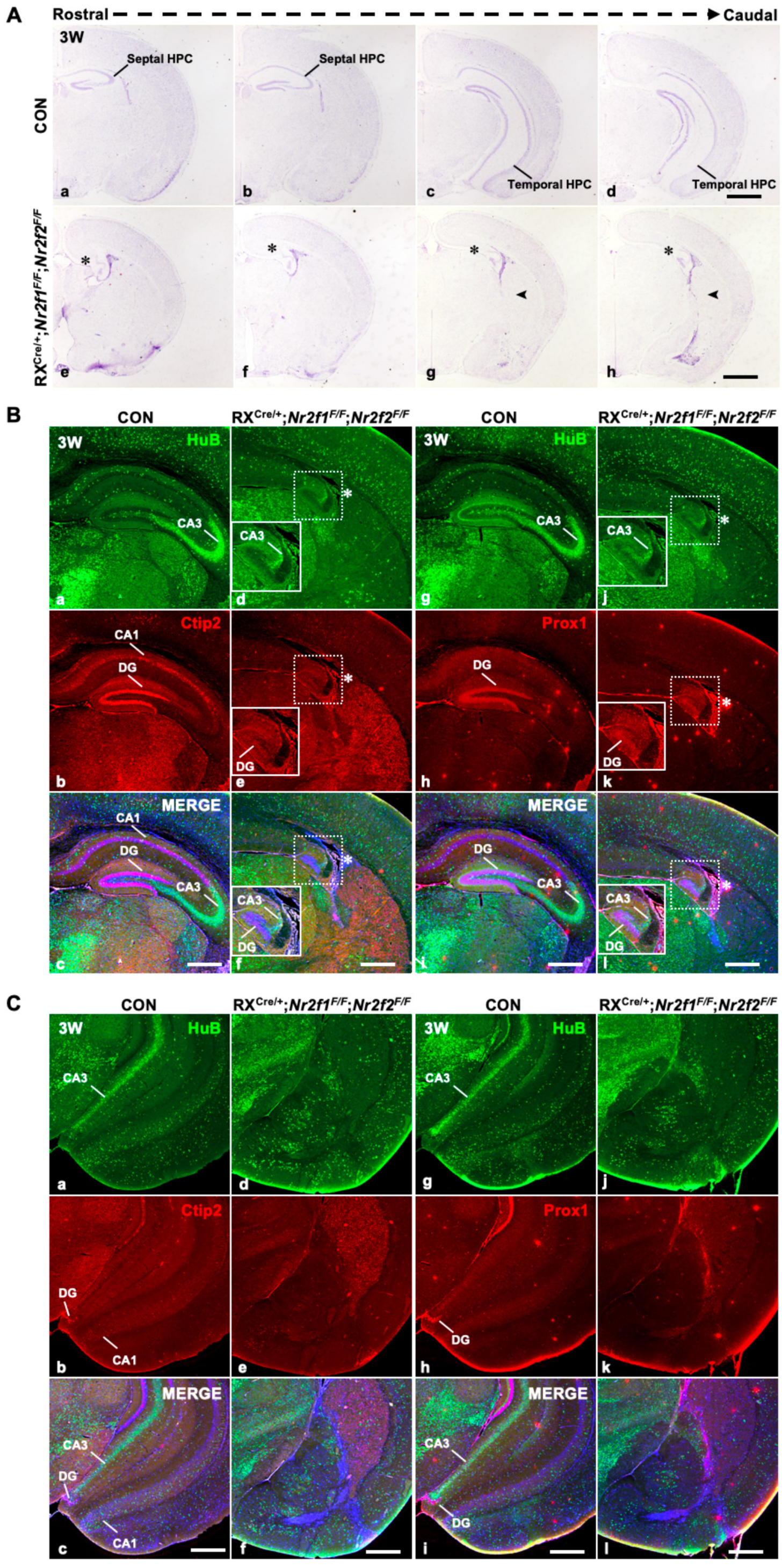

Figure 3—figure supplement 1, Yang et al.

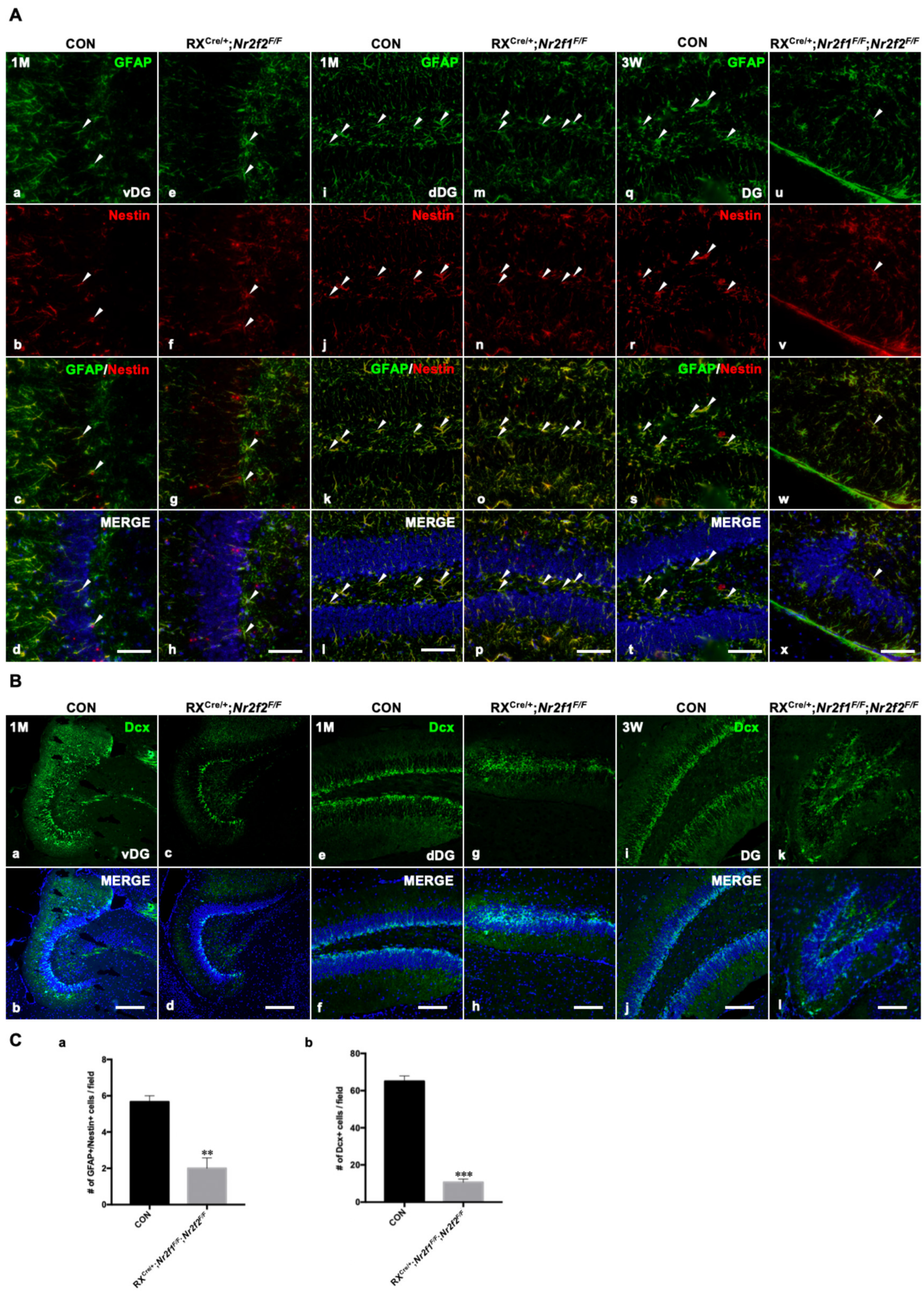

Figure 4, Yang et al.

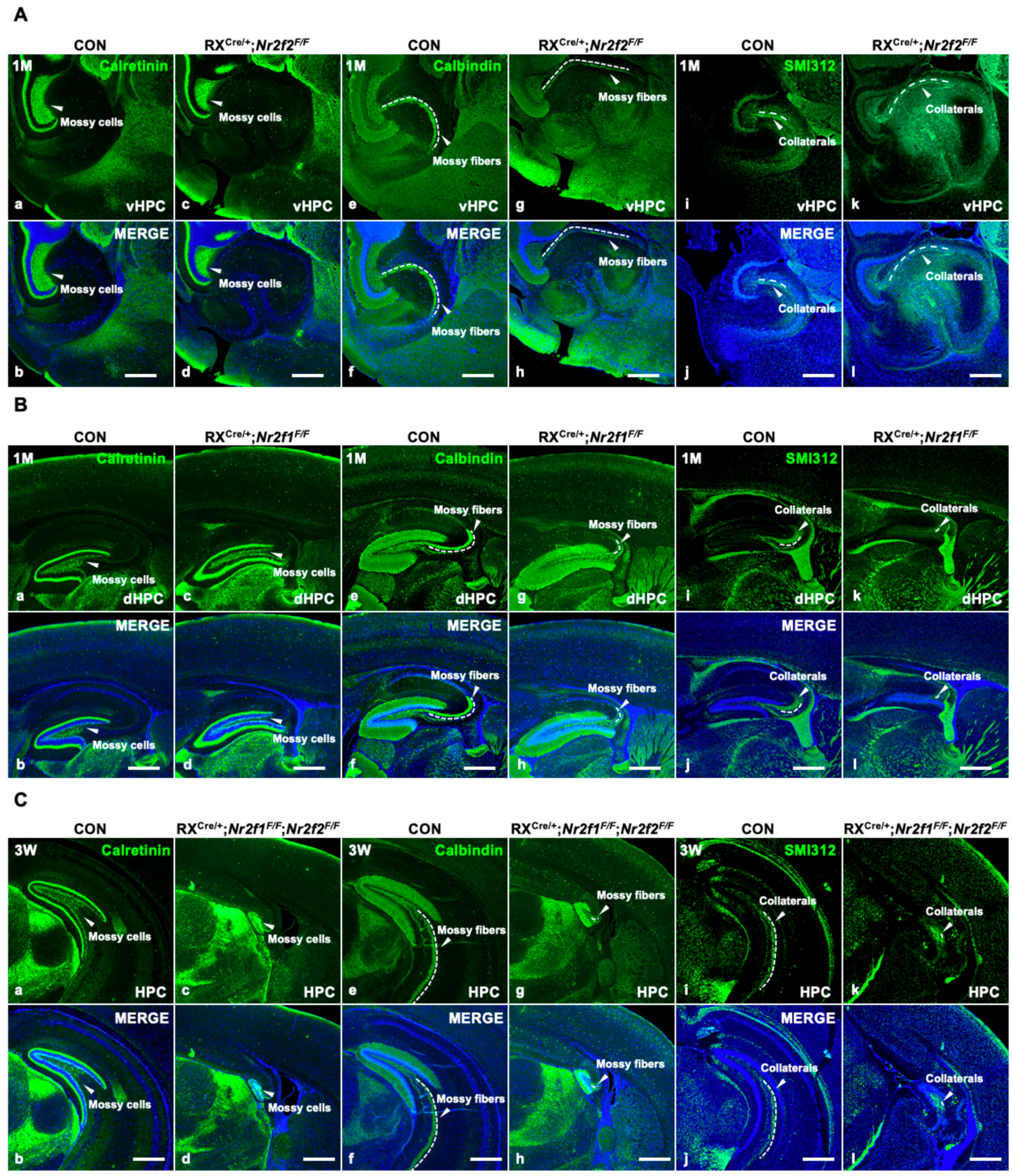

Figure 5, Yang et al.

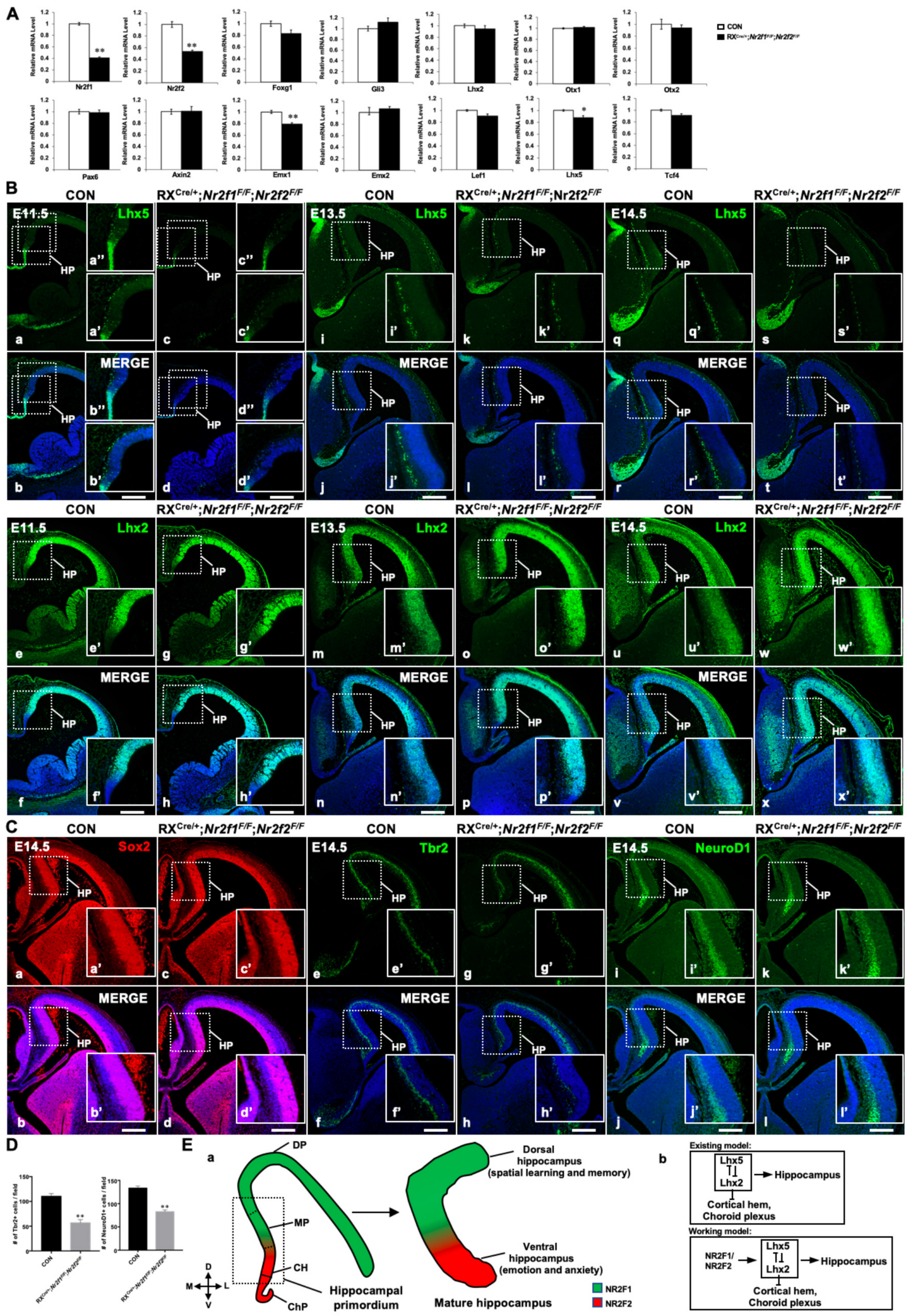
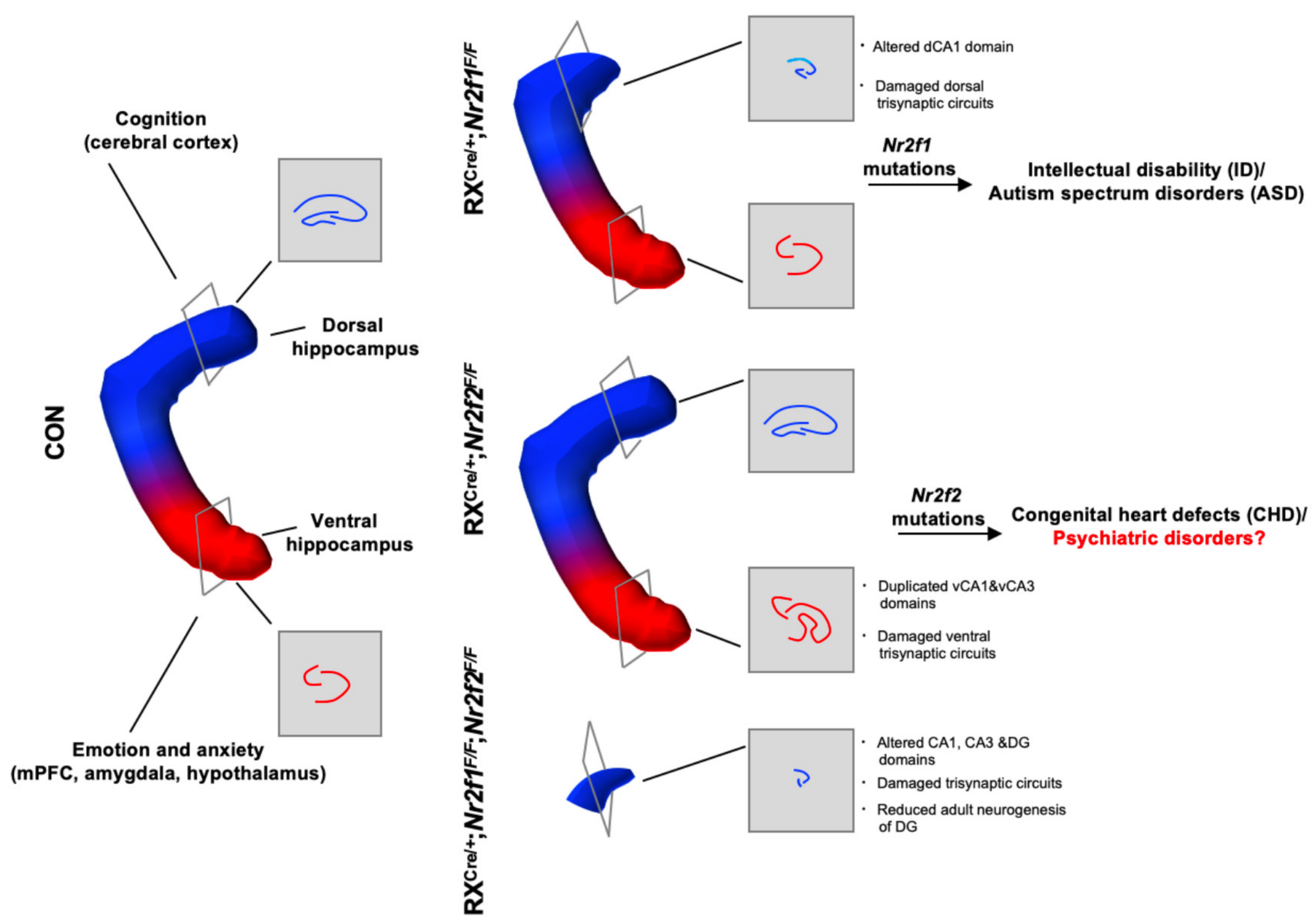
Figure 5—figure supplement 1, Yang et al.