Inverse oculomotor responses of achiasmatic mice expressing a transfer-defective Vax1 mutant

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23 **ABSTRACT**

24 In animals that exhibit stereoscopic visual responses, the axons of retinal ganglion cells
 25 (RGCs) connect to brain areas bilaterally by forming a commissure called the optic chiasm
 26 (OC). Ventral anterior homeobox 1 (Vax1) contributes to formation of the OC, acting
 27 endogenously in optic pathway cells and exogenously in growing RGC axons. Here, we
 28 generated *Vax1^{AA/AA}* mice expressing the *Vax1^{AA}* mutant, which is selectively incapable of
 29 intercellular transfer. We found that RGC axons cannot take up *Vax1^{AA}* protein from
 30 *Vax1^{AA/AA}* mouse optic stalk (OS) cells, of which maturation is delayed, and fail to access
 31 the midline. Consequently, RGC axons of *Vax1^{AA/AA}* mice connect exclusively to ipsilateral
 32 brain areas, resulting in the loss of stereoscopic vision and the inversed oculomotor
 33 responses. Together, our study provides physiological evidence for the necessity of
 34 intercellular transfer of Vax1 and the importance of the OC in binocular visual responses.

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36

37 **INTRODUCTION**

38 Animals collect visual information via the eyes, where photoreceptors in the retina convert
39 light stimuli to electrochemical signals (Seabrook et al., 2017). These signals are then
40 transmitted to inner retinal neural circuits before being sent to the brain via the axons of
41 retinal ganglion cells (RGCs). RGC axons, which are bundled in the optic nerve, deliver
42 visual information to multiple brain areas, including the dorsal lateral geniculate nucleus
43 (dLGN) of the thalamus, for pattern and color recognition; the superior colliculus (SC) of
44 the midbrain, for oculomotor responses; and the suprachiasmatic nucleus (SCN) of the
45 hypothalamus, for circadian rhythm control (Rusak and Groos, 1982; Seabrook et al., 2017;
46 Zhang et al., 2017).

47 In many binocular animals, RGC axons are not only wired to brain areas on the
48 same (i.e., ipsilateral) side but are also connected to those on the opposite (i.e.,
49 contralateral) side (Herrera et al., 2019; Petros et al., 2008). The population of RGCs
50 whose axons project to contralateral brain areas is variable among vertebrate species.
51 Human RGC axons are split equally to both sides at the midline, whereas all RGC axons
52 in *Xenopus laevis* and zebrafish extend exclusively to the contralateral side across the
53 midline (Herrera et al., 2019; Petros et al., 2008). In mice, the population of RGCs that
54 connects to ipsilateral brain areas is low: ~3% in pigmented mice and ~1% in albino mice
55 (Rice et al., 1995).

56 RGC axons form a midline structure called the optic chiasm (OC), which is located
57 beneath the SCN and splits the axon bundles into ipsilateral and contralateral paths
58 (Herrera et al., 2019; Petros et al., 2008). Pathway selection for RGC axons at the OC is
59 determined by specific guidance cues. For example, ephrin B2 and B3 expressed in radial
60 glia of the ventral hypothalamus (vHT) act through a receptor, EphB1, in RGC axons from
61 the ventral temporal retina to repel the axons from the midline, guiding their growth
62 ipsilaterally (Williams et al., 2003). EphB1 is present in about 50% of human RGCs and ~3%
63 mouse RGCs, but is absent in *Xenopus* and zebrafish RGCs, suggesting a critical role for

64 EphB1 in ipsilateral pathway selection by RGC axons (Herrera et al., 2003; Rebsam et al.,
65 2012). Pathway-selection cues are not only provided by cells located along RGC axon
66 growth tracks, but also by neighboring RGC axons. Sonic hedgehog (Shh), which is
67 expressed in contralaterally projecting RGC axons, also serves as a repulsive cue in the
68 OC by acting on its co-receptor, Boc, which is expressed in RGC axons from the ventral
69 temporal mouse retina (Peng et al., 2018).

70 The cues that guide the majority of mouse RGC axons across the midline, however,
71 have not been identified as clearly as the ipsilateral guidance cues. Binding of vascular
72 endothelial growth factor-a (Vegfa) to its receptor, neuropilin-1 (Nrp1), has been
73 demonstrated to support the growth of RGC axons at the midline (Erskine et al., 2011).
74 Homophilic interactions between neuronal cell adhesion molecule (Nr-CAM) expressed in
75 RGC axons and vHT cells has also been suggested to promote midline crossings of RGC
76 axons (Williams et al., 2006). Nr-CAM also cooperates with plexinA1, a receptor for
77 semaphorin 6D (Sema6D), to support contralateral RGC axon projection (Kuwajima et al.,
78 2012). However, a majority of RGC axons still cross the midline in mice lacking these cues,
79 suggesting the presence of other key regulator(s) of contralateral RGC axon growth.

80 Ventral anterior homeobox 1 (Vax1) is expressed in ventral and medial regions of
81 the vertebrate forebrain (Bertuzzi et al., 1999; Hallonet et al., 1999). The forebrain
82 commissural structures, including the anterior commissure (AC), corpus callosum (CC),
83 hippocampal commissure (HC), and OC, do not form properly in humans and mice having
84 homozygous *VAX1* mutations (Bertuzzi et al., 1999; Mui et al., 2005; Slavotinek et al.,
85 2012). Given the absence of *VAX1* gene expression in commissural neurons, it had been
86 thought that Vax1 functions as a transcription factor that induces axon growth factors in
87 cells located along commissural axon growth tracks. However, it was found that the
88 transcription factor activity of Vax1 is dispensable for the promotion of mouse RGC axon
89 growth (Kim et al., 2014). More surprisingly, Vax1 protein was detected in mouse RGC
90 axons, despite the absence of autonomous *Vax1* gene expression in RGCs (Kim et al.,
91 2014). It was further found that Vax1 protein is transferred from cells along RGC axon

growth tracks and promotes axon growth by stimulating axonal mRNA translation in mouse retinal explants (Kim et al., 2014). However, whether intercellular Vax1 transfer is also critical for the growth of RGC axons *in vivo* has been remained unclear.

Here, we generated *Vax1^{AA/AA}* mice, in which the *Vax1* was replaced with a transfer-defective *Vax1^{AA}* mutant. We found that *Vax1^{AA}* protein was incapable of binding to heparan sulfate proteoglycans (HSPGs) and penetrating RGC axons, resulting in retarded growth of RGC axons. Most of all, the OC was not formed in *Vax1^{AA/AA}* mutant mice, and RGC axons projected exclusively to ipsilateral brain areas. Consequently, *Vax1^{AA/AA}* mice exhibited inversed oculomotor behaviors, resulting from ipsilaterally biased retinogeniculate and retinocollicular connections in opposing to those of the contralateral-dominant connections in *Vax1^{+/+}* mice. Our findings in *Vax1^{AA/AA}* mice, therefore, not only confirm the physiological importance of Vax1 transfer in OC formation but also provide a molecular basis for the oculomotor abnormalities seen in achiasmatic animals.

RESULTS

Identification of a CAG sugar-binding motif in Vax1

Vax1 is secreted from cells that interact with developing mouse RGC axons and enters into the axons. Binding of Vax1 to RGC axons is mediated by heparan sulfate (HS) sugar chains of HSPGs, such as syndecan (Sdc) and glypican (Glp) (Kim et al., 2014). Another homeodomain protein, Otx2 (orthodenticle homeobox 2), has been found to bind chondroitin sulfate (CS) sugar chains of chondroitin sulfate proteoglycan (Beurdeley et al., 2012; Miyata et al., 2012). Binding of Otx2 to CS was mediated by the conserved glycosaminoglycan (GAG) binding motifs, [-X-B-B-X-B-X-] and [-X-B-B-B-X-X-B-X-] (Cardin and Weintraub, 1989).

117 We found mouse Vax1 also contains a GAG-binding motif located at amino acids
 118 100–105 (Figure 1A). To investigate the possibility that Vax1 binds to HS chains through
 119 this putative CAG-binding motif, we replaced lysine and arginine (KR) amino acid residues
 120 in the motif to two alanines (A), yielding Vax1^{AA} (Figure 1A). HeLa cells were transfected
 121 with DNA construct that expresses a mRNA containing Vax1 or mutant Vax1^{AA} together
 122 with enhanced green fluorescent protein (EGFP) but produces EGFP separately from
 123 Vax1 or Vax1^{AA} using an internal ribosome entry site (IRES). The KR-to-AA mutation
 124 suppressed Vax1 transfer from EGFP-positive donor cells to EGFP-negative recipient cells
 125 (Lee et al., 2019) (Figure 1B). However, it did not significantly affect Vax1 transcription
 126 factor activity, assessed by monitoring the expression of a luciferase reporter downstream
 127 of a Vax1 target sequence in the intron 5 of *transcription factor 7-like 2 (Tcf7l2)* (Vacik et
 128 al., 2011) (Figure 1C). This finding contrasts with the reduced transcription factor activity of
 129 the Vax1^{R152S} mutant (Figure 1C), in which arginine 152 (R152) of the DNA binding motif
 130 was mutated to serine (S) (Slavotinek et al., 2012).

131 We found that the level of Vax1^{AA} protein in growth media of the transfected cells
 132 was significantly elevated compared with that of Vax1 (Figure 1D). However, the amount
 133 of Vax1^{AA} in the medium was not further increased by the addition of free heparin, which
 134 competes with HS chains of HSPGs to bind extracellular Vax1 and release it from the cell
 135 surface into the growth medium (Lee et al., 2019) (Figure 1D). The results suggest
 136 reduced affinity of Vax1^{AA} for HS sugars compared with Vax1, together with a result that
 137 shows the impaired interaction between Vax1^{AA} and syndecan-2 (Sdc2) HSPG (Figure 1E).
 138 Consequently, Vax1^{AA} added to growth medium bind or penetrate HeLa cells much less
 139 efficiently than Vax1 (Figure 1F).

140 We further tested whether the KR-to-AA mutation also influences the binding of
 141 Vax1 to RGC axons and subsequent axon growth stimulation. We found that Vax1^{AA}
 142 protein added into the growth medium of mouse retinal explants was not detected in
 143 neurofilament 160 (Nf160)-positive RGC axons nor did it induce axonal growth as
 144 efficiently as Vax1 protein, which penetrated retinal axons and significantly promoted

axonal growth (Figure 1G). We also found that Vax1^{AA} was not transferred to RGC axons from human embryonic kidney 293T cells co-cultured with mouse retinal explants (Figure 1H). Consequently, the lengths of RGC axons extending toward Vax1^{AA}-expressing 293T cells were shorter than those growing towards Vax1-expressing 293T cells. These results suggest that the KR residues are necessary for the binding of Vax1 to HSPGs and subsequent penetration into RGC axons.

151

152 **Generation of Vax1^{AA/AA} mice**

To investigate the consequences of the KR to AA mutation *in vivo*, we introduced the corresponding mutation into the mouse *Vax1* gene and generated Vax1^{AA} mice (Figure S1A and S1B; see Methods for details). Vax1^{AA/AA} homozygous mice were born without any recognizable morphological defects and survived without significant health problems, whereas the mice carry homozygous non-sense mutations (Vax1^{-/-}) or hemizygous KR-to-AA mutation together non-sense mutation (Vax1^{AA/-}) die after birth with the cleft palates that interfere with breathing (Bertuzzi et al., 1999) (Figure S1C – S1E). Noticeably, the incisors grow continuously in approximately a quarter of Vax1^{AA/AA} mice (Figure S1D). The outgrowing incisors make the mice difficult to consume chow and cause a lethality after weaning, unless the outgrown incisors were not cut off regularly (Figure S1E).

Using Vax1^{AA/AA} mouse embryos, we examined whether the mutation affects intercellular transfer of Vax1 *in vivo*. We found Vax1^{AA} was strongly expressed in ventral medial forebrain structures, including the optic stalk (OS) of Vax1^{AA/AA} mouse embryos at 14.5 days post-coitum (dpc; E14.5), an expression pattern similar to that of Vax1 in E14.5 Vax1^{+/+} littermate mice (Figure 1I). However, unlike the distribution of Vax1, which was present in RGC axons as well as OS cells in Vax1^{+/+} mice, Vax1^{AA} was not detectable in the RGC axons of Vax1^{AA/AA} mice (Figure 1I). These results suggest that the KR-to-AA mutation also interferes with the transfer of Vax1 to RGC axons *in vivo*.

171

172 ***Delayed maturation of the OS in $Vax1^{AA/AA}$ mice***

173 A coloboma, the fissures in the ventral eyecup and OS, is observed in the eyes of humans
174 and mice harboring *VAX1* mutations (Bertuzzi et al., 1999; Hallonet et al., 1999; Slavotinek
175 et al., 2012). A coloboma was also present in E14.5 $Vax1^{AA/AA}$ and $Vax1^{-/-}$ mouse eyes
176 (Figure 2A [top row] and 2B). The optic fissures, however, disappeared in $Vax1^{AA/AA}$ mice
177 by E16.5, whereas they remained unclosed in $Vax1^{-/-}$ mice even after birth (Figure 2A
178 [middle row] and 2B). The unclosed OS in E14.5 $Vax1^{AA/AA}$ mice expressed Pax2 (paired
179 homeobox 2), an OS marker, but not Pax6, a retinal marker, similar to the distribution
180 observed in $Vax1^{+/+}$ littermates (Figure 2C [coronal], left and center columns). It contrasts
181 with E14.5 $Vax1^{-/-}$ mice, which co-expressed Pax2 and Pax6 in the OS (Figure 2C
182 [coronal], right column). Therefore, these results suggest that OS fate is specified properly
183 in $Vax1^{AA/AA}$ mice, but not in $Vax1^{-/-}$ mice.

184 Pax2-positive OS cells were clustered separately from RGC axons in E14.5
185 $Vax1^{AA/AA}$ and $Vax1^{-/-}$ mice, whereas they were spread among RGC axons in E14.5
186 $Vax1^{+/+}$ mice (Figure 2C, sagittal). The OS cells in E14.5 $Vax1^{+/+}$ mice expressed an
187 astrocyte precursor cell (APC) marker, S100 calcium-binding protein β (S100 β), but had
188 lost a neuroepithelium marker, E-cadherin (Ecad) (Figure 2D, left column of E14.5 panel).
189 The ventral OS (vOS) cells in E14.5 $Vax1^{AA/AA}$ mice also expressed S100 without Ecad,
190 however their dOS cells co-expressed Ecad and S100 β (Figure 2D, center column of
191 E14.5 panel). However, S100 β was undetectable in E14.5 $Vax1^{-/-}$ mouse vOS cells, which
192 instead expressed the retinal marker Vsx2 (Figure 2D, right column of E14.5 panel). In
193 E16.5 $Vax1^{AA/AA}$ mice, similar to the distribution observed in $Vax1^{+/+}$ littermates, S100 β -
194 positive OS APCs had entirely lost Ecad expression and started to disperse among RGC
195 axons, whereas S100 β -negative OS cells still formed Ecad-positive neuroepithelial
196 clusters in E16.5 $Vax1^{-/-}$ mice (Figure 2D, E16.5 panel). These results suggest that

197 differentiation of APCs from the OS neuroepithelium is impaired in $Vax1^{-/-}$ mice but merely
198 delayed in $Vax1^{AA/AA}$ mice.

199

200 ***Agenesis of the OC in $Vax1^{AA/AA}$ mice***

201 The APCs and RGC axons were segregated from each other throughout the entire OS in
202 E14.5 $Vax1^{AA/AA}$ mice (Figure 3A and 3B, center columns in HT/OS junction panels).
203 These naked RGC axons in $Vax1^{AA/AA}$ mice failed to cross the vHT midline, whereas RGC
204 axons in $Vax1^{+/+}$ littermates were intercalated by Vax1-positive OS cells and formed the
205 OC at the vHT midline (Figure 3A and 3B, left columns in HT/OS junction and OC panels).
206 However, RGC axons were able to form the optic tract (OT) along the lateral wall of post-
207 chiasmatic brain region of E14.5 $Vax1^{AA/AA}$ mice (Figure 3A and 3B, center columns in OT
208 panels). RGC axons of E14.5 $Vax1^{-/-}$ mice also failed to extend to the vHT midline but
209 could form the OT (Figure 3A and 3B, right columns in OC and OT panels), as it was
210 reported previously (Bertuzzi et al., 1999).

211 The vHT of E14.5 $Vax1^{AA/AA}$ and $Vax1^{-/-}$ mice protruded ventrally by virtue of the
212 absence of underlying RGC axons bundles, while the vHT of E14.5 $Vax1^{+/+}$ mice was
213 flattened over RGC axons forming the OC (Figure 3A, OC panel). However, sonic
214 hedgehog (Shh), which is necessary for specification of the vHT and OS (Chiang et al.,
215 1996; Kim and Lemke, 2006), showed a similar expression pattern in the vHTs of E14.5
216 $Vax1^{+/+}$, $Vax1^{AA/AA}$ and $Vax1^{-/-}$ mice (Figure 3C, top row), suggesting that fate specification
217 of the vHT is unaffected in $Vax1^{AA/AA}$ and $Vax1^{-/-}$ mice. The Glial-positive radial glia, which
218 express various RGC axon guidance cues (Herrera et al., 2019; Petros et al., 2008), were
219 also observed in vHT of $Vax1^{AA/AA}$ and $Vax1^{-/-}$ mice, suggesting normal development of
220 vHT radial glia from Nestin-positive vHT neuroepithelium in the mice (Figure 3C, second
221 and third rows).

222 Attractive guidance cues for RGC axons, including Vegfa and Nr-Cam (Erskine et
223 al., 2011; Williams et al., 2006), were expressed properly in the vHT of E14.5 *Vax1^{AA/AA}*
224 and *Vax1^{-/-}* mice, although their expression pattern is not identical to those in the vHT of
225 *Vax1^{+/+}* littermates owing to ventral protrusion of HTs in *Vax1^{AA/AA}* and *Vax1^{-/-}* mice (Figure
226 3D, second and third rows). EphrinB2, which triggers the repulsion of ipsilaterally
227 projecting RGC axons at the vHT midline (Williams et al., 2003), was not observed in the
228 vHT of E14.5 *Vax1^{AA/AA}* and *Vax1^{-/-}* mice (Figure 3D, first row), suggesting the absence of
229 ephrinB2-dependent repulsive signaling to RGC axons. However, EphB1, which binds
230 ephrinB2 to promote retraction of RGC axons, and its upstream transcription factor Zic2
231 were detectable in ventral temporal RGCs of E14.5 *Vax1^{+/+}*, *Vax1^{AA/AA}*, and *Vax1^{-/-}* mice
232 (Herrera et al., 2003; Williams et al., 2003) (Figure 3E, top four rows). Shh, which was
233 identified to repulse adjacent ventral temporal RGC axons from the vHT midline (Peng et
234 al., 2018), was also expressed properly in RGCs (Figure 3E, bottom row). These results
235 suggest that the agenesis of the OC in *Vax1^{AA/AA}* mice did not likely result from the loss of
236 attractive and/or gain of repulsive signals listed above.

237

238 ***Intact transcription factor activity of Vax1^{AA/AA} in vivo***

239 Our results indicate normal specification of OS and vHT but delayed differentiation of OS
240 APC in *Vax1^{AA/AA}* mice, of which RGC axons fail to form the OC (Figure 3F). The delayed
241 maturation of OS cells in *Vax1^{AA/AA}* mice could have resulted from autonomous alteration
242 of gene expression, if the KA-to-AA mutation altered Vax1 transcription factor activity
243 sufficiently to create a difference *in vivo* despite minimally affecting transcription factor
244 activity in cultured cells (Figure 1B). We, thus, compared the activities of Vax1 and Vax1^{AA}
245 *in vivo* by monitoring the expression of reporters driven by *Pax6* α -enhancer, where Vax1
246 binds to suppress the enhancer activity (Mui et al., 2005) (Figure 4A, diagram). We used
247 α -Cre;*R26^{tm11}* mice, which express histone H2B-GFP (H2B-GFP) and membrane bound
248 tdTomato (tdTom*) reporters at *ROSA26* gene locus upon the excision of *loxP-STOP-loxP*

(*LSL*) cassette by Cre recombinase expressed at downstream of *Pax6* α -enhancer (Marquardt et al., 2001; Wang et al., 2019) (Figure 4A, diagram). The H2B-GFP signals were observed only in the retina, but not in the OS, of E12.5 *Vax1*^{+/+} and *Vax1*^{AA/AA} littermate mice (Figure 4A), suggesting that *Vax1* and *Vax1*^{AA} suppressed *Pax6* α -enhancer successfully in the OS.

We also compared the abilities of *Vax1* and *Vax1*^{AA} to bind *Pax6* α -enhancer sequences *in vivo*. DNA fragments isolated from E10.5 mouse heads by chromosome immunoprecipitation (ChIP) using an anti-*Vax1* antibody and used for polymerase chain reaction (PCR) and quantitative PCR (qPCR) detection of *Pax6* α -enhancer sequences in the ChIPed DNA. The results revealed no significant difference in the binding abilities of *Vax1* and *Vax1*^{AA} on the target sequences (Figure 4B and 4C). Therefore, our results suggest that the delayed maturation of the OS in *Vax1*^{AA/AA} mice is unlikely resulted from autonomous alteration of *Vax1*-dependent target gene expression.

262

263 ***Restoration of RGC axon growth in *Vax1*^{AA/AA} mice by external supplementation of*** 264 ***Vax1* protein**

In *Vax1*^{+/+}; α -Cre; *R26*^{tm11} and *Vax1*^{AA/AA}; α -Cre; *R26*^{tm11} mice, we could also visualize RGC axons using tdTom* reporter (Figure 3A, diagram). After comparing the lengths of tdTom*-labeled RGC axons, we found RGC axon extension was less efficient in E12.5 *Vax1*^{AA/AA} mice than that in *Vax1*^{+/+} littermates (Figure 3A, arrowheads). Given the fact that exogenous *Vax1* is capable of stimulating RGC axon growth (Kim et al., 2014), it is possible that the impaired RGC axon growth in *Vax1*^{AA/AA} mice might be related to the absence of *Vax1* in the axons (Figures 1F and 3B). We thus tested whether RGC axon growth in the OS and HT of *Vax1*^{AA/AA} mice could be restored by supplying *Vax1* protein exogenously. To this end, we implanted the third ventricle (V3) of E12.5 *Vax1*^{AA/AA} mouse brain slabs with collagen beads that had been soaked in a solution containing recombinant

275 Flag-Vax1 proteins or Flag-peptides for 12 hours (Figure 4C; see details in Methods). We
276 could detect Flag-Vax1 proteins in L1-CAM-positive RGC axons (Figure 4C, bottom row),
277 which could be also identified by intraocularly-delivered lipophilic Dil fluorescent dye
278 (Figure 4C, diagram). We further found an increase of Dil-labeled axons in the vHT midline
279 of Vax1-implanted slabs in comparing with Flag peptide-implanted slabs (Figure 4C,
280 second row). Some axons grew even farther across the midline, suggesting that
281 exogenous Vax1 is necessary for the extension of RGC axons to form the OC.

282

283 ***Ipsilaterally biased RGC projections in the Vax1^{AA} mouse brain***

284 Using adult Vax1^{AA/AA} mice, we were able to analyze the structures and functions of the
285 mouse visual system, which are difficult to study in Vax1^{-/-} mice because they die
286 perinatally (Bertuzzi et al., 1999). We found no significant difference in brain size and
287 shape between P30 Vax1^{+/+} and Vax1^{AA/AA} littermate mice (Figure 5A and 5C [left column]).
288 The olfactory bulb (OB) of Vax1^{AA/AA} mice also appeared normal (Figure 5C, the image in
289 left bottom corner), although a previous report noted hypoplastic OBs in the few surviving
290 Vax1^{-/-} mice (Soria et al., 2004). Furthermore, Vax1^{AA/AA} mice have only one pituitary gland
291 (data not shown), whereas Vax1^{-/-} mice have an extra pituitary gland by virtue of failure to
292 suppress ectopic pituitary fate specification in the ventral anterior forebrain (Bharti et al.,
293 2011). These results also suggest that Vax1-dependent specification of ventral forebrain
294 structures is not significantly altered in Vax1^{AA/AA} mice.

295 Among the commissures that are reported to be absent in Vax1^{-/-} mice (Bertuzzi et
296 al., 1999), the CC and OC are missing in Vax1^{AA/AA} mice, whereas the AC and HCC are
297 present (Figure 5A and 5C [left column]). The posterior commissure (PC), where the
298 oculomotor nerves (ON) cross the midline (Buettner et al., 2002), was also present and
299 appropriately positioned beneath the SC in Vax1^{AA/AA} mice (Figure 5A, rightmost column).
300 We further visualized the RGC axons of mice using fluorescent dye-conjugated cholera
301 toxin B (CTB) protein (Luppi et al., 1990). The Alexa Fluor 488-conjugated CTB and Alexa

302 Fluor 594-conjugated CTB were injected into the right and left eyes, respectively, of
 303 *Vax1^{+/+}* and *Vax1^{AA/AA}* littermate mice at postnatal day 28 (P28; Figure 5B). Fluorescence
 304 signals emitted by CTB-labeled RGC axons in the mice were then detected at P30 (Figure
 305 5C). Green and red fluorescence signals were predominantly detected in RGC axons that
 306 projected across the midline in *Vax1^{+/+}* mice (Figure 5C, top row). Consequently, the
 307 majority of CTB fluorescence signals were observed in the contralateral SCs of *Vax1^{+/+}*
 308 mice (Figure 5C, third row). In contrast, fluorescence signals of CTB were detected
 309 exclusively in the ipsilateral SCs of *Vax1^{AA/AA}* mice, in which it was not possible to detect
 310 the OC where differentially labeled RGC axons met (Figure 5C, second and bottom rows).

311 We also examined whether ipsilaterally projecting *Vax1^{AA/AA}* mouse RGC axons
 312 were properly connected to the dLGN, a thalamic nucleus that relays visual information
 313 from the retina to the visual cortex (Seabrook et al., 2017). Axons from RGCs in the
 314 ventral-temporal mouse retina project to the ipsilateral dLGN, where the majority of RGC
 315 axons from the contralateral retina are connected (Herrera et al., 2019; Petros et al., 2008).
 316 The minor ipsilateral axon terminals are then segregated from the major contralateral axon
 317 terminals by a retinal activity-dependent refinement process during postnatal development
 318 (Guido, 2018; Huberman et al., 2008). Segregation of binocular RGC axons was clearly
 319 seen in the dLGN of P30 *Vax1^{+/+}* mice (Figure 5D, left two columns). However, RGC
 320 axons in the dLGN of P30 *Vax1^{AA/AA}* mice originated only from the ipsilateral retina, and no
 321 contralateral RGC axons were observed in the dLGN (Figure 5D, right two columns).

322 We further examined whether retinocollicular topographic connectivity was
 323 established properly in the *Vax1^{AA/AA}* mouse SC, which lacks contralateral RGC axon
 324 terminals (Figure 5C, bottom row). Axons from RGCs in the temporal retina are known to
 325 connect to the anterior SC, whereas those from the nasal retina link to the posterior SC
 326 (Lemke and Reber, 2005). In the perpendicular axis, RGC axons from the dorsal retina
 327 arrive at the lateral SC, and those from the ventral retina are wired to the medial SC. Thus,
 328 axons of Pax6 α -Cre-affected RGCs in the ventral and peripheral retina, which express
 329 membrane-localized EGFP (EGFP*) in *ROSA26* gene locus of *R26^{tm4}* Cre reporter mice

(Marquardt et al., 2001; Muzumdar et al., 2007), map to medial and peripheral parts of the SC of P30 $Vax1^{+/+}$ mice (Figure 5E, middle row). This pattern was also observed in the SCs of $Vax1^{AA/AA}$ littermate mice (Figure 5E, bottom row), implying that the retinocollicular topography is established properly in $Vax1^{AA/AA}$ mice.

334

335 ***Light-stimulated retinal and cortical responses in $Vax1^{AA/AA}$ mice***

336 Next, we examined whether visual information can be properly processed in the retina and
 337 delivered to the brain in $Vax1^{AA/AA}$ mice. First, we tested the activities of P45 mouse
 338 retinas using electroretinography (ERG) recordings. The shapes of scotopic ERG a-waves,
 339 which reflect the function of rod photoreceptors, appeared normal in $Vax1^{AA/AA}$ mice,
 340 although the amplitudes of scotopic ERG b-waves, which are generated by bipolar cells
 341 and Müller glia in the inner retina downstream of photoreceptors (Miura et al., 2009), were
 342 decreased (Figure 6A [right column] and 6B). However, the amplitudes of photopic ERG a-
 343 waves, which reflect the activity of cone photoreceptors, were significantly decreased in
 344 $Vax1^{AA/AA}$ mice compared with $Vax1^{+/+}$ littermate mice (Figure 6A [left column] and 6C
 345 [graph in left]), leading to a consequent decrease in amplitudes of photopic ERG b-waves
 346 (Figure 6A [left column] and 6C [graph in right]). The reduced ERG waves in $Vax1^{AA/AA}$
 347 mice might be related to the decrease in S-opsin-positive cone photoreceptors in the
 348 ventral retina (Figure S2C), a feature reminiscent of mouse retina deficient for the related
 349 $Vax2$ gene (Alfano et al., 2011). However, we found no significant differences in other cell
 350 types in the retina and optic nerves in $Vax1^{AA/AA}$ mice compared with $Vax1^{+/+}$ littermates
 351 (Figure S2).

352 We also examined whether visual information received by the retina is delivered to
 353 the cortex via the dLGN in $Vax1^{AA/AA}$ mice. To this end, we recorded electrical activities of
 354 neurons in the monocular zone of the primary visual cortices (V1) in both hemispheres of
 355 mouse brain while giving the visual stimuli only to the left eye. We found that it
 356 predominantly triggered responses of cortical neurons in the right V1 of $Vax1^{+/+}$ mice;

conversely, it mainly activated neurons in the left V1 of *Vax1^{AA/AA}* mice (Figure 6D and 6E). Collectively, these results demonstrate that *Vax1^{AA/AA}* mouse retina senses a light stimulus and send visual information to the ipsilateral dLGN for subsequent delivery of the information to the visual cortex, whereas the information is sent to mainly to the contralateral brain areas in *Vax1^{+/+}* mice.

Reduced visual acuity of *Vax1^{AA/AA}* mice

We next examined whether the ipsilaterally-biased retinogeniculate and retinocollicular pathways influenced the visual responses of *Vax1^{AA/AA}* mice. First, we tested whether the mice could discriminate light and dark spaces. We found that P45 *Vax1^{+/+}* and *Vax1^{AA/AA}* littermate mice generally spent longer periods in the dark chamber than in the illuminated chamber (Figure 6F; Movie S1 and S2). On the contrary, P45 retinal dystrophy 1 (*rd1*) mutant *C3H/HeJ* blind mice, which have a homozygous mutation of *phosphodiesterase 6b* (*Pde6b*; *Pde6b^{rd1/rd1}*) gene (Chang et al., 2002), spent equivalent periods in dark and light chambers (Figure 6F; Movie S3). These results suggest that *Vax1^{AA/AA}* mice discriminate light and dark space as efficiently as *Vax1^{+/+}* mice.

Second, we examined whether mice could recognize images that mimic a looming shadow of predator (Yilmaz and Meister, 2013). P45 mice were placed in a transparent box covered by a computer monitor displaying a black circle that expands by a 20° angle from the mouse head. *Vax1^{+/+}* mice froze or hid under a shelter while the circle in the monitor expanded (Figure 6G; Movie S4). *Vax1^{AA/AA}* mice also exhibited hiding and/or freezing responses; however, their response frequency was significantly lower than that of *Vax1^{+/+}* mice (Figure 6G; Movie S5). *Pde6b^{rd1/rd1}* mice showed no behavioral responses at all (Figure 3D; Movie S6). These results suggest that *Vax1^{AA/AA}* mice could recognize the shadow pattern, but did so less efficiently than *Vax1^{+/+}* mice.

382 Third, we investigated whether mice could discriminate near and far objects by
383 placing them on a transparent plate, half of which was printed with a flannel pattern image
384 (i.e., safe zone) and the other unprinted half extended over a floor with the same flannel
385 pattern (i.e., cliff zone) (Sloane et al., 1978). P45 *Vax1*^{+/+} mice stayed in the safe zone and
386 rarely ventured into the cliff zone (Figure 6H; Movie S7). However, *Vax1*^{AA/AA} littermate
387 mice moved freely between cliff and safe zones (Figure 6H; Movie S8), as did P45
388 *Pde6b*^{rd1/rd1} blind mice (Figure 6H; Movie S9). These results suggest that *Vax1*^{AA/AA} mice
389 have impaired depth perception, a visual capacity that requires bilateral RGC axon
390 projections to ipsilateral and contralateral LGNs (Larsson, 2013; Petros et al., 2008).

391 Last, we assessed the visual acuity of mice by measuring optomotor responses
392 (OMRs) to horizontally moving black and white vertical strips using OptoMotry (Prusky et
393 al., 2004). P45 *Vax1*^{+/+} mice turned their heads in the direction of vertical stripe movement
394 (Figure 6I; Movie S10); however, P45 *Vax1*^{AA/AA} and *Pde6b*^{rd1/rd1} mice failed to show a
395 valid OMR (Figure 6I; Movie S11 and S12). These results suggest that the visual acuity of
396 *Vax1*^{AA/AA} mice is significantly compromised compared with that of *Vax1*^{+/+} mice.

397

398 ***Vax1*^{AA/AA} mice exhibit inverse oculomotor responses**

399 The OMR-based visual acuity test counts head-turn events to moving objects. Therefore,
400 despite having normal visual perception, mice may not respond properly if their oculomotor
401 system is abnormal. Interestingly, *Vax1*^{AA/AA} mice startled in response to the stimuli, but
402 did not show corresponding head-turn behavior (Figure 6I; Movie S8). This contrasted with
403 *Pde6b*^{rd1/rd1} blind mice, which showed no stimulus-dependent responses (Figure 6I; Movie
404 S9). These results suggest that the reduced visual acuity of *Vax1*^{AA/AA} mice might have
405 resulted from an abnormal oculomotor system that cannot trigger proper head-turn
406 responses.

407 Achiasmatic humans and dogs were also previously reported to have reduced
408 visual acuity (Apkarian et al., 1995; Dell'Osso and Williams, 1995; Williams et al., 1994).
409 Interestingly, they commonly exhibited seesaw nystagmus—an out of phase vertical
410 oscillation in the two eyes in the absence of a visual stimulus. We thus tracked eye
411 movements in P45 *Vax1*^{+/+} and *Vax1*^{AA/AA} littermate mice, and found the eyes of *Vax1*^{AA/AA}
412 mice oscillated in an oval track in the absence of visual stimulus (Figure 7A and 7B; Movie
413 S11). The oscillatory cycles of the two eyes were out of phase in the vertical axis but in
414 phase in the horizontal axis (Figure 7A, right column; Movie S11), phenocopying the
415 seesaw nystagmus of achiasmatic humans and dogs. In contrast, the eyes of *Vax1*^{+/+}
416 littermate mice gazed stably (Figure 7A, left column; Movie S10).

417 Spontaneous oscillations of *Vax1*^{AA/AA} mouse eyes were also evident during pupil
418 contraction responses to light illumination (Movie S13). However, the speed of pupil
419 contraction was not significantly different between *Vax1*^{+/+} and *Vax1*^{AA/AA} mice (Figure 7C
420 and 7D; Movie S12 and S13). We also examined pupil contraction after illuminating only
421 one eye with light. Monocular illumination to *Vax1*^{+/+} mice induced immediate contraction
422 of pupils in direct (i.e., stimulated) eyes, followed by contraction of the pupils of consensual
423 (i.e., unstimulated) eyes (Figure 7E and 7F; Movie S14). Interestingly, pupil contraction
424 occurred faster in the consensual eyes of *Vax1*^{AA/AA} mice (Figure 7C; Movie S15),
425 suggesting that pupillary oculomotor outputs are sent in opposite routes in *Vax1*^{AA/AA} mice
426 compared with *Vax1*^{+/+} mice.

427 We also investigated the optokinetic reflex (OKR) of mouse eyes to moving objects
428 (Cahill and Nathans, 2008). *Vax1*^{+/+} mice rotate their eyes correspondingly and periodically
429 in the direction of movement of black and white vertical stripes, which rotate clockwise or
430 counter clockwise (Figure 7G and 7H; Movie S16 and S18); however, they did not rotate
431 their eyes when the stripes converged, diverged, or stopped in the front eye field (Figure
432 7G and 7H; Movie S20, S22, and S24). Interestingly, the eyes of *Vax1*^{AA/AA} mice, which
433 oscillated constitutively in the absence of visual stimulation, stopped moving when the
434 stripes moved clockwise or counterclockwise (Figure 7G and 7H; Movie S17 and S19).

435 These results suggest that *Vax1*^{AA/AA} mice recognize the movement of objects; however,
436 their oculomotor systems do not operate in the same way as they do in *Vax1*^{+/+} mice,
437 which rotate their eyes and heads correspondingly to moving objects.

438

439

440 **DISCUSSION**

441 Many vertebrate organs exhibit bilateral symmetry. However, these paired organs are not
442 simply duplications, but instead are frequently functional complements of each other. For
443 instance, the left hemisphere of the human cerebral cortex houses the language center,
444 whereas the right hemisphere is where pattern recognition occurs (Wolman, 2012). This is
445 demonstrated by the ‘split-brain’ phenomenon, in which an individual whose two cerebral
446 hemispheres are not connected by the CC cannot match words to the corresponding
447 objects. In vertebrate binocular visual systems, bilateral projection of RGC axons at the
448 OC is necessary for the brain to receive visual information coming from the two eyes
449 (Herrera et al., 2019; Petros et al., 2008). Therefore, proper development of the OC is
450 required for stereoscopic vision—achieved by overlapping focal-drifted images from each
451 eye—and the coordination of bilateral oculomotor responses.

452 Agenesis of the OC (AOC) has been reported in various vertebrates, including
453 humans, dogs, and fish (Apkarian et al., 1995; Dell’Osso and Williams, 1995; Karlstrom et
454 al., 1996; Williams et al., 1994). However, the molecular features of AOC have not been
455 identified except in *belladonna* (*bel*) zebrafishes, which carry mutations in the LIM
456 homeobox 2 (*lhx2*) gene (Seth et al., 2006). In the achiasmatic *bel* mutants, ventral
457 diencephalic regions, including the preoptic area (POA), vHT and OS, are not patterned
458 properly by virtue of the failure to express key genes, including *vax2*, *zic2.1*, and *pax2.1*
459 (Seth et al., 2006). However, *Lhx2* and *Pax2* are properly expressed in the *Vax1*^{AA/AA}
460 mouse OS (Figure 2C; *Lhx2* data not shown). *Vax2* and *Zic2* are not present in the OS of

461 *Vax1*^{+/+} and *Vax1*^{AA/AA} mice, but are expressed in the ventral and ventral-temporal retina,
462 respectively (Figure 3D; Vax2 data not shown). Furthermore, the OS was specified
463 properly in *Vax1*^{AA/AA} mice, without the expression of the retinal markers, Pax6 and Vsx2
464 (Figure 2C and 2D). The vHT was also correctly specified in *Vax1*^{AA/AA} mice through
465 appropriate expression of Shh (Figure 3C). Therefore, it is unlikely that AOC in *Vax1*^{AA/AA}
466 mice results from the failure to specify the OS and vHT.

467 Despite normal specification, OS maturation was delayed in *Vax1*^{AA/AA} mice. In
468 these mice, the optic fissures at the OS were closed completely by E16.5, whereas in
469 *Vax1*^{+/+} mice, they had already disappeared by E14.5 and OS cells had spread among
470 RGC axons in a salt-and-pepper pattern (Figure 2C and 2D). The morphology of the E14.5
471 *Vax1*^{AA/AA} mouse OS was largely similar to that of E12.5 *Vax1*^{+/+} mice, whose OS cells still
472 exhibited neuroepithelial characteristics (Figure 2B). The molecular mechanisms
473 underlying the maturation of OS cells still remain largely unknown, although several
474 markers of OS cell lineage have been identified. For instance, Nestin and Ecad are
475 expressed in the OS neuroepithelium; S100β is expressed in OS APCs; and glial fibrillary
476 acidic protein (Gfap) is expressed in OS astrocytes (Tao and Zhang, 2014). These
477 markers, however, are not mutually exclusive. S100β can be detected together with Ecad
478 in OS cells (Figure 2D), suggesting an OS cell transition state between neuroepithelium
479 and APC. As a result, it is difficult to dissect developmental stages of OS cells clearly with
480 the limited information available. Thus, a more comprehensive understanding of OS
481 maturation will require the identification of additional markers that are selectively
482 expressed at specific OS developmental stages.

483 It has been proposed that Vax1 specifies the OS in vertebrates by directly
484 suppressing the expression of a retinal fate determinant Pax6 (Bertuzzi et al., 1999; Mui et
485 al., 2005). Expression of Pax6 in the OS was suppressed properly in *Vax1*^{AA/AA} mice,
486 whereas it was induced ectopically in the *Vax1*^{-/-} mouse OS (Figure 2C). The ability of
487 *Vax1*^{AA} to bind DNA sequences of *Pax6* α-enhancer were not different from that of *Vax1*
488 (Figure 4B), suggesting that *Vax1*^{AA} could regulate target gene expression as efficiently as

489 Vax1. Therefore, Vax1 transcription factor activity might not be crucial for OS maturation,
490 which was delayed in *Vax1^{AA/AA}* mice, although it might be necessary for the expression of
491 other target genes, including gonadotrophin-releasing hormone (GnRH) in the SCN and
492 POA (data not shown).

493 Achiastic animals, including the *Vax1^{AA/AA}* mice reported here, exhibit a seesaw
494 nystagmus characterized by failure to maintain gaze with the eyes at vertical axis
495 (Apkarian et al., 1995; Dell'Osso et al., 1998). The rostral interstitial nucleus of medial
496 longitudinal fasciculus (riMLF) and the interstitial nucleus of Cajal (INC) in the tegmentum
497 of the midbrain has been identified as a key regulatory center for vertical gaze (Buittner et
498 al., 2002; Zee, 1986). riMLF neurons project directly to the oculomotor nucleus (ON) in the
499 midbrain, and indirectly via the INC, to trigger ocular muscle contraction (Buittner et al.,
500 2002). Medial riMLF neurons innervate the third cranial nerve (CN-III; also called the
501 ocular motor nerve) in the ON to stimulate the ipsilateral inferior rectum (IR) muscle, which
502 is responsible for downward eye rotation. They are also wired to the fourth cranial nerve
503 (CN-IV; also called the trochlear nerve) across the PC to stimulate the contralateral
504 superior oblique (SO) muscle, which mediates nasal-downward eye rotation. Efferent
505 projections forming the lateral riMLF reach CN-III to regulate contractions of the ipsilateral
506 inferior oblique (IO) muscle and the contralateral superior rectus (SR) muscle, which
507 mediate temporal-upward and upward rotations, respectively.

508 RGC axons innervate brain areas bilaterally in *Vax1^{+/+}* mice (Figure 5C – 5E).
509 Therefore, SC and riMLF neurons at both sides can be activated even by monocular
510 stimulation, consequently the four ocular muscles—IR, SO, IO and SR—can contract
511 simultaneously to result in vertical gaze. In contrast, the SC receives visual inputs only
512 from ipsilateral eyes in *Vax1^{AA/AA}* mice (Figure 5C). Therefore, signals from the right eye in
513 *Vax1^{AA/AA}* mice might induce contractions of IR and IO in the right eye and SR and SO in
514 the left eye, resulting in nasal-downward pendular motion of the right eye and temporal-
515 upward pendular movement of the left eye (Figure 7A; Figure S3). This vertically out of
516 phase but horizontally in-phase eye movement is then followed by temporal-upward and

nasal-downward pendular motions, completing one oscillation cycle. This alternating eye movement, therefore, suggests that the signals from the two eyes are desynchronized and/or feeding back on each other.

SC neurons are also connected to the paramedian pontine reticular formation (PPRF), a horizontal gaze center in the pons that also receives oculomotor inputs from ipsilateral prefrontal cortex (Buttner-Ennever and Buttner, 1988; Zee, 1986). The PPRF receives inputs mainly from the contralateral SC and delivers them to the neighboring abducens nucleus (ABN). Abducens internuclear neurons (AIN) in the ABN then relay the signals to CN-III across the midline to stimulate the medial rectus (MR) ocular muscle, while abducens motor neurons (AMN) in the ABN connect ipsilaterally to the lateral rectus (LR). Given the binocular nature of efferent nerve fibers of ABN neurons, contractions of the MR in one eye and the LR in the other eye rotate both eyes in the same direction (Figure 7C). Horizontal gaze is therefore achieved when MR and LR in the same eye are activated simultaneously. However, because of the seesaw nystagmus, horizontal eye gaze was not observed in *Vax1^{AA/AA}* mice.

Vax1^{AA/AA} mouse eyes also failed to pursue moving objects (Figure 7G). However, instead of rotating correspondingly in the direction of object movement as *Vax1^{+/+}* mice, the eyes of *Vax1^{AA/AA}* mice frequently stopped oscillating when mice were presented objects moving clockwise or counterclockwise (Figure 7G; Movie S17 and S19). This stimulus-driven ectopic gaze of *Vax1^{AA/AA}* mouse eyes might result from the combinatorial activation of extraocular muscles. As discussed above, the seesaw nystagmus of *Vax1^{AA/AA}* mice is likely driven by alternating activation IR+IO and SR+SO ocular muscles. Therefore, IR+IO-driven nasal (and downward) movement of the right eye could be antagonized by LR-induced temporal movement in response to clockwise movement of the object. At the same time, SR+SO-driven temporal (and upward) movement of the left eye might be antagonized by MR-induced nasal movement. However, given the presence of the respective downward and upward forces after antagonism by the LR and MR, the right

544 eye position is slightly below the center and the left eye position is slightly above the
545 center during the ectopic gaze in responding to clockwise stripe rotation (Figure 7G).

546 The *bel rev* achiasmatic zebrafish model also exhibits nystagmus when facing non-
547 moving stripes (Huang et al., 2006). However, the nystagmus disappears in the dark and
548 after non-patterned illumination of the visual field, which trigger seesaw nystagmus in
549 achiasmatic mammals (Apkarian et al., 1995; Dell'Osso et al., 1998) (Figure 7A).
550 Furthermore, in contrast to the stimulus-driven ectopic gaze in achiasmatic *Vax1^{AA/AA}* mice
551 (Figure 7G), *bel rev* zebrafish exhibit reversed horizontal OKR in response to the rotation
552 of stripes (Neuhauss et al., 1999; Rick et al., 2000). It was proposed that the ipsilateral
553 RGC projection supplies a reversed retinal slip velocity input to the optokinetic system in
554 zebrafish to elicit eye movements that compensate for retinal slip in the wrong direction
555 (Neuhauss et al., 1999; Rick et al., 2000). However, reversed OKR and postural
556 abnormalities have not been reported in humans (Apkarian et al., 1995), dogs (Dell'Osso
557 and Williams, 1995; Hogan and Williams, 1995), or mice (Figure 7G). Therefore, the
558 oculomotor circuits in animals with a non-stereoscopic visual system (like zebrafish) is
559 likely different from those in the stereoscopic visual systems of mammals.

560 The oculomotor circuit also controls bilateral pupillary contraction, which is
561 triggered by RGCs that are wired to the pretectal nucleus (PN) in the midbrain (Szabadi,
562 2018). PN neurons relay these signals to the Edinger-Westphal nucleus (EWN), which
563 projects axons to the ipsilateral CN-III to induce pupillary contraction (Hultborn et al., 1978;
564 Kourouyan and Horton, 1997). It has been suggested that the EWN receives signals from
565 ipsilateral and contralateral PNs to induce bilateral pupillary contraction. However, given
566 the faster pupil contraction of directly stimulated eyes compared with consensual eyes in
567 *Vax1^{+/+}* mice (Figure 7E), the PN might primarily stimulate the contralateral EWN in mice.
568 Consequently, the repeated midline crossings at retina-PN and PN-EWN axes enable the
569 directly stimulated eye to respond faster than the consensual eye. However, an ipsilateral
570 retina-PN connection followed by a contralateral PN-EWN connection might result in an
571 inverse order of pupillary contraction in *Vax1^{AA/AA}* mice (Figure 7E). These results suggest

572 that the operation of the oculomotor system depends on a constant number of midline
573 crossings; therefore, the system cannot function properly if one of those commissures is
574 missing.

575 The axons of SC neurons also project contralaterally to the cervical spinal cord
576 through the tectospinal tract, and can trigger head turns in response to visual stimuli
577 (Gandhi and Katnani, 2011). Therefore, activation of the right SC, which receives a
578 majority of its inputs from the left eye, which captures objects in the left visual field,
579 predominantly contracts the left neck muscle to trigger a leftward head turn in *Vax1^{+/+}* mice.
580 Given the exclusive ipsilateral retinocollicular connection, the spinal outputs in achiasmatic
581 *Vax1^{AA/AA}* mice are likely inverse to those in *Vax1^{+/+}* mice. This might make *Vax1^{AA/AA}* mice
582 turn their heads in the direction opposite the stimulus. *Vax1^{AA/AA}* mice, however, startled
583 instead of turning their heads in the direction opposite the movement of horizontally drifting
584 stripes (Figure 6G). These results suggest that head-turn behavior is not only determined
585 by eye-SC-spinal cord circuits, but is also affected by cortical circuits that regulate activity
586 of the PPRF to pursue the objects (Gandhi and Katnani, 2011).

587

588

589

590 **METHODS**

591 *Mouse strains*

592 *Vax1*^{-/-} and *Pax6* α -*Cre* mice were reported previously (Bertuzzi et al., 1999; Marquardt et
593 al., 2001). *Gt(ROSA)26Sor*<sup>tm4(ACTB-tdTomato,-EGFP)*Luo*/J (*R26*^{tm4}) and *Gt(ROSA)26Sor*^{tm11(CAG-}
594 <sup>tdTomato*, -GFP*)*Nat*/J (*R26*^{tm11}) mouse strains were purchased from Jackson laboratory
595 (Muzumdar et al., 2007; Wang et al., 2019).</sup></sup>

596 *Vax1*^{AA/AA} mice were generated using CRISPR/Cas9 system with a CRISPR RNA
597 (crRNA) and a trans-activating crRNA (tracrRNA) in following procedures. Two crRNAs
598 were designed to target two nearby sites of the second exon of *Vax1* gene, which include
599 DNA sequences encoding K101 and R102 (Figure S1A). The sequences of crRNAs were:
600 #1, 5-TGGATCTGGACCGGCCCAAG-3 and #2, 5-AAGGACGTGCGAGTCCTCTT-3. The
601 synthetic single-stranded DNA oligonucleotide (ssODN) containing missense (KR-to-AA)
602 and synonymous mutations, 5'-
603 TCTCAGAGAGATTGAGCTGCCGAGCCAGCTCGGTTCTCTCCCGGCCCAACACGTATT
604 GGCAACGCTGGAACTCCATCTCCAGCCTGTAGAGCTGCTCAGCTGTAAACGAGGTCC
605 TGGTCGCCGCGGGCCGGTCCAGATCCAAGCCTTTGGGCAGGATGATTTCTCGGATA
606 GACCCCTTGGCATCTAGGAAAGGG-3' (IDT, Inc., USA), was used as a donor DNA. We
607 then injected those crRNA/tracrRNA duplex and ssODN together with Cas9 mRNA
608 (Toolgen, Inc., Seoul, Korea) into the cytoplasm of one cell-stage *C57BL/6J* mouse
609 embryos. To screen the founders carrying the KR-to-AA mutation in the *Vax1* gene, PCR
610 was performed (Figure S1B). The primer sequences to identify the KR-to-AA knock-in
611 allele were: 5'-AGTATGAAGTTAGCCCCCTTGG-3' as a forward primer and 5'-
612 GTAAACGAGGTCCTGGTCGC-3' as a knock-in-specific reverse primer, and those for
613 wild-type allele, 5'-AAGAGGACTCGCACGTCC-3' as a wild-type-specific forward primer
614 and 5'-AGGAAAGGCAAGCTGTCATC-3' as a reverse primer. Then, the genomic regions
615 spanning the mutated second exon of the founder mice were validated by direct-
616 sequencing analysis (Bionics Co., Ltd., Seoul, Korea). We obtained three *Vax1*^{+/AA} male
617 mice after analyzing 83 pups obtained from the 439 injected embryos. The off-springs of

the *Vax1*^{+/AA} mouse were then backcrossed with wild-type *C57BL/6J* mice over 6 generations to eliminate unwanted off-target mutations introduced by CRISPR/Cas9. All experiments were performed according to the Korean Ministry of Food and Drug Safety (MFDS) guidelines for animal research. The protocols were certified by the Institutional Animal Care and Use Committee (IACUC) of KAIST (KA2010-17) and Yonsei University (A-201507-390-01). All mice used in this study were maintained in a specific pathogen-free facility of KAIST Laboratory Animal Resource Center and Yonsei Laboratory Animal Research Center.

Cell and explants culture

Human cervical cancer HeLa cells and human embryonic kidney (HEK) 293T cells were cultured in Dullbeco's Eagle Modified Media (DMEM) supplemented with 10% fetal bovine serum (FBS). For the luciferase assay, HEK293T cells (10^5) were transfected with 1 μ g pCAGIG-V5 vectors encode *Vax1*, *Vax1*^{AA}, or *Vax1*(R152S) cDNA together with pGL3-Tcf7l2-luciferase (0.2 μ g) and pCMV- β -gal (0.2 μ g) reporter constructs. Luciferase activities in the transfected cells were measured at 24h post-transfection, and normalized by β -galactosidase activities to obtain relative luciferase activity of the cells. To test cellular penetration of *Vax1* protein, V5 peptides or V5-*Vax1* proteins, which were purified from HEK293T cells, were added into the growth media (1.5 pmol/ml [final concentration]) of HeLa cells, which express GFP-Sdc2. Distribution of V5 peptides and V5-*Vax1* proteins on cell surface and inside the cells were examined by immunostaining with mouse anti-V5 and chicken anti-GFP antibodies.

Retinal explants prepared as described previously (Kim et al., 2014). Briefly, the retina was prepared from mouse embryos at E13, and mixed with collagen in DMEM with 10% FBS. The retinal explants in collagen were then cultured in Neurobasal medium containing B27 supplement (Invitrogen Inc.) for 48h to allow the axons grow from the explants. 6X-Histidine peptides or *Vax1*-6X-His proteins, which were purified from *E.coli*, were then added into the culture medium (2 pmol/ml [final concentration]) of retinal

646 explants. Alternatively, the retinal explants were placed next to collagen droplets
647 containing HEK293 cells (10^5 cells/droplet), which express Vax1 or Vax1^{AA}. The lengths of
648 retinal axons grown from the explants were measured before and after the treatments to
649 determine axon growth rate.

650 Slab embryo culture with collagen gel was also performed as described previously
651 (Kim et al., 2014). Collagen droplets mixed with Flag peptides (10 μ g/ml) or Flag-Vax1
652 proteins (200 μ g/ml) were prepared and placed into the third ventricle of the slab embryos.
653 The embryos were then incubated for 12 hr at 37°C in a humidified atmosphere
654 supplemented with 7% CO₂.

655

656 *Detection of Vax1 proteins in the growth medium*

657 Heparin (10 mg/ml) was added into growth medium of HEK293T cells (10^7) express V5-
658 Vax1 or V5-Vax1^{AA}. Macromolecules including the proteins and lipids in the growth
659 medium were precipitated by adding trichloroacetic acid (TCA; 20% final). The precipitates
660 were washed with cold acetone three times and dissolved in 2X-SDS sample buffer for
661 SDS-PAGE followed by WB to detect Vax1 proteins released in the growth medium.

662

663 *Immunohistochemistry and in situ RNA hybridization*

664 Distribution of proteins in HeLa and mouse embryonic cells were examined by
665 immunostaining. Cultured cells were fixed in 4% paraformaldehyde (PFA) in phosphate
666 buffered saline (PBS) for 10 mins at 36-h post-transfection. Sections of mouse embryos,
667 eyes, and brain slabs were fixed in 4%PFA/PBS at room temperature for 2 h, and then put
668 in a 20% sucrose/PBS solution at 4°C for 16 h before embedding in OCT (optimal cutting
669 temperature) medium for cryofreezing and cryosection.

670 The cells and sections were incubated in a blocking solution containing 0.2% Triton
671 X-100, 5% normal donkey serum, and 2% bovine serum albumen (BSA) in PBS for 1 h. To
672 stain the proteins in the cells, the samples were incubated with the indicated primary

antibodies in blocking solution without 0.2% Triton X-100 at 4°C for 16 h and then with the appropriate secondary antibodies conjugated with fluorophores. Immunofluorescence was subsequently analyzed using Olympus FV1000 and Zeiss LSM810 confocal microscopes. Antibody information is provided in Table S1.

Distributions of mRNA of interest in the embryonic sections were detected by *in situ* hybridization (ISH) with dioxygenin (DIG)-labeled RNA probes and visualized them by immunostaining with alkaline phosphatase (AP)-conjugated α -DIG followed by AP-mediated colorization, as it was described in a previous report (Kim et al., 2014).

Chromatin immunoprecipitation (ChIP) and PCR

Chromatin immunoprecipitation was done as it was described previously (Mui et al., 2005). E10.5 mouse embryonic heads were isolated and chopped into small pieces in prior to the incubation in 1% formaldehyde in PBS at room temperature for 10 min. The nuclei were isolated for the immunoprecipitation with rabbit anti-Vax1 antibody or pre-immune rabbit IgG. DNA fragments coprecipitated with the antibodies were purified by phenol/chloroform/isoamyl alcohol extraction, and 100 ng of these immunoprecipitated DNAs were used as templates for PCR amplification of the Pax6 α -enhancer.

Electroretinogram (ERG)

Mice were either dark- or light-adapted for 12 h before ERG recording and anesthetized with 2,2,2-tribromoethanol (Sigma). After the pupils of the mice were dilated by 0.5% tropicamide, a gold-plated objective lens was placed on the cornea and silver-embedded needle electrodes were placed at the forehead and tail. The ERG recordings were performed using Micron IV retinal imaging microscope (Phoenix Research Labs) and analyzed by Labscribe ERG software according to the manufacturer's instruction. To obtain scotopic ERG a- and b-waves, a digital bandpass filter ranging from 0.3 to 1,000 Hz

699 and stimulus ranging from -2.2 to $2.2 \log(\text{cd} \cdot \text{s} \cdot \text{m}^{-2})$ were used. To yield photopic ERG a-
700 and b-waves, filter ranging from 2 to 200 Hz and stimulus ranging from 0.4 to $2.2 \log(\text{cd} \cdot \text{s}$
701 $\text{m}^{-2})$ with $1.3 \log(\text{cd} \cdot \text{s} \cdot \text{m}^{-2})$ background were used.

702

703 *In vivo extracellular recording and data analysis*

704 We performed *in vivo* extracellular recordings in the monocular V1 (bregma, -3.50 mm;
705 lateral, 2.50 mm; depth, 0.70 mm) of Vax1^{+/+} and Vax1^{AA/AA} mice. Mice were anesthetized
706 with the urethane (2 g per kg body weight, intraperitoneal injection) and restrained in a
707 custom-designed head-fixed apparatus. A small craniotomy with the diameter of ~ 0.5 mm
708 was made over V1 of the left and the right hemispheres, and we inserted a 32-channel
709 silicon electrode (A1x32-Poly3-10mm-50-177-CM32, Neuronexus) using micro-drive
710 motorized manipulator (Siskiyou). After waiting 20 ~ 30 minutes for stabilization, we
711 started recording visual responses by presenting a full-field flashing light for 5 times to the
712 left eye. The visual stimuli were presented at 10 Hz for 500ms, 5 pulses of 50 ms duration,
713 and total 30 trials through a gamma-corrected monitor. Extracellular signals were filtered
714 between 500~5000 Hz at 30 kHz sampling rate, amplified by miniature digital head-stage
715 (CerePlex μ , Blackrock Microsystems), and saved through data acquisition system
716 (CerePlex Direct, Blackrock Microsystems). We performed spike sorting using the Klusters
717 software (<http://neurosuite.sourceforge.net/>) and further analyzed firing rates of isolated
718 single units using MATLAB. We analyzed the z-score of firing activity in each single unit
719 from -1 to +2 s of the onset of the visual stimuli and plotted peri-stimulus time histogram
720 (PSTH) of the normalized activity. Firing rate change index (FR index) of individual cells
721 was calculated using the following formula:

722
$$\text{FR index} = (\text{mean z-score for 1 s after stimuli onset} - \text{mean z-score for 1 s before stimuli}$$

723
$$\text{onset}) / (\text{mean z-score for 1 s after stimuli onset} + \text{mean z-score for 1 s before stimuli}$$

724
$$\text{onset})$$

725

726 *Light-dark chamber assay*

727 The light-dark test apparatus was composed of light (21cm(width, W) X 29cm(depth, D) X
728 20cm(height, H), 700 lux) and dark (21cm(W) X 13cm(D) X 20cm(H), ~5 lux) chambers.
729 The dark chamber is separated from light chamber by an entrance in the middle wall
730 (5cm(W) X 8cm(H)). Mice were introduced in the light chamber with their heads toward the
731 opposite side of the dark chamber and allowed to freely explore the apparatus for 10 min.
732 Amounts of time spent in the light and dark chambers and number of transitions were
733 analyzed by Ethovision XT10 software (Noldus).

734

735 *Looming assay*

736 Looming test was performed as described previously with some modifications (Yilmaz and
737 Meister, 2013). Briefly, the behavioral arena was prepared with an open-top acryl box
738 (30cm(W) X 30cm(D) X 30cm(H)), which contains a nest in the shape of a triangular prism
739 (10cm(W) X 12cm(D) X 10cm(H)). The looming disk was programmed as a black circle in
740 a gray background, increasing its size from 2 degrees of visual angle to 20 degrees in
741 250 ms and maintained for 250 ms. The pattern was repeatedly presented 10 times with
742 500 ms of interval for each trial.

743

744 *Optomotor response (OMR)*

745 Mouse visual acuity was measured with the OptoMotry system (Cerebral Mechanics) as
746 previously described (Prusky et al., 2004). Mice were adapted to ambient light for 30 mins
747 and then placed on the stimulus platform, which is surrounded by four computer monitors
748 displaying black and white vertical stripe patterns. An event that mice stopped moving and
749 began tracking the stripe movements with reflexive head-turn was counted as a successful
750 visual detection. The detection thresholds were then obtained from the OptoMotry
751 software.

752

753 *Measurements of pupillary contraction and optokinetic response (OKR)*

754 Mouse heads were mounted to a plate and clamped to a holder to prevent head
755 movement during measurement. Images of a mouse eye that show the pupil and the
756 corneal reflection were recorded by CCD camera (120/240 Hz) with Infrared (IR) filter
757 (ISCAN Inc.). To measure the OKR, the head-fixed mice were put in front of the screens
758 that display gray background or black and white vertical stripes (30% contrast) moving at a
759 spatial frequency of 0.2 c/d and angular velocity of 12 d/s. To examine the pupil
760 contraction, the mice were kept in the dark for 30 secs and then exposed to 500 lux of light
761 for 10 secs. The pupil position and diameter were measured by the ISCAN software
762 (ISCAN Inc.).

763

764 *Statistical analyses*

765 Statistical tests were performed using Prism Software (GraphPad; v5.0) measurement
766 tools. All data from statistical analysis are presented as the average $\pm$ STD. Comparison
767 between two groups was done by unpaired Student's t-test, and the differences among
768 multiple groups were determined by analysis of variance (ANOVA) with Tukey's post-test
769 used to determine the significant differences among multiple groups. P-values < 0.01 were
770 considered as statistically significant results.

771

772

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779 Korean Ministry of Agriculture, Food, and Rural Affairs. All of the animals were handled
780 according to approved institutional animal care and use committee (IACUC) protocols
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941 **Figure 1. Identification a CAG sugar binding motif of Vax1.** (A) Consensus amino acid
942 sequences of GAG sugar-binding motifs in mouse Otx2 and Vax1. (B) Expression of V5-
943 Vax1 and EGFP, which are independently translated from a same transcript, was
944 examined by immunostaining of transfected HeLa cells with mouse anti-V5 (red) and
945 chicken anti-GFP (green) antibodies. Arrows point HeLa cells express V5-Vax1 without
946 EGFP, implicating the transfer of V5-Vax1 but not EGFP from V5;EGFP double-positive
947 cells. (C) HEK293T cells were transfected with a DNA vector encodes Vax1,
948 Vax1(R152S), or Vax1(KR/AA) cDNA together with a Vax1 target *Tcf7l2*-luciferase
949 reporter DNA construct. Luciferase activities in the transfected cells were measured at 24-

h post-transfection. The values are average obtained from four independent experiments and error bars denote standard deviations (SD). *p*-values were determined by ANOVA test (*, *p*<0.01; **, *p*<0.005; ***, *p*<0.001; ns, not significantly different). **(D)** V5-tagged Vax1 or Vax1^{AA} proteins were expressed in HEK293T cells, and growth media of the transfected cells were then collected after incubating for 3h in the presence (+) or absence (-) of heparin (10 mg/ml final; see Methods for details). Cell lysates and TCA-precipitated fractions of growth medium were analyzed by 10% SDS-PAGE and subsequent western blotting (WB) with anti-Vax1 antibody (α -Vax1). A graph below the WB data shows the relative intensities of Vax1 bands in the blots. The values are average obtained from four independent experiments and error bars denote SD. **(E)** Interactions of V5-Vax1 and V5-Vax1^{AA} with GFP-Sdc2 in HEK293T cells were assessed by immunoprecipitation (IP) with α -V5 and subsequent WB with α -GFP. Relative amounts of V5-Vax1 and GFP-Sdc2 in the cell lysates were also examined by WB. **(F)** V5-Vax1 or V5-Vax1^{AA} recombinant proteins were added into growth medium of HeLa cells expressing GFP-Sdc2 and incubated for 3 h. Vax1 proteins inside cells and/or at the cell surface were detected by immunostaining with mouse α -V5 (red) and chick α -GFP (green). **(G)** Retinas were isolated from E13.5 mice and cultured as described in Methods. Axonal lengths of retinal explants were measured at 24 h post-culture; then, the explants were treated with 6X-His-tagged recombinant Vax1 or Vax1^{AA} proteins. **(H)** Alternatively, retinal explants were co-cultured with HEK293T cells transfected with pCAGIG (Mock), pCAGIG-V5-Vax1, or pCAGIG-V5-Vax1^{AA}. Axonal lengths were re-measured after 24 h and the explants were immunostained with α -Vax1 (green) and α -NF160 (red). Arrowheads indicate the areas magnified in each inset. The changes in axonal length during the 24-h incubation period were shown in graphs. The values in the graph are averages and error bars denote SDs (n=6). **(I)** Intercellular transfer of Vax1 from E14.5 Vax1^{+/+} and Vax1^{AA/AA} littermate mouse OS cells to RGC axons was determined by immunostaining of the embryonic sections with α -Vax1 (green) and α -NF160 (red). The solid lines in the images indicate the boundary of OS, and the dotted-lines mark the border between the OS and RGC axon bundles.

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Min et al., Fig2

980 **Figure 2. Developmental delay of OS differentiation in *Vax1*^{AA/AA} mice. (A)** Pictures of
 981 mouse eyes (frontal view) with the indicated genotypes are taken at various developmental
 982 stages. Arrowheads point the optic fissures. N, nasal; T, temporal; D, dorsal; V, ventral.
 983 **(B)** Sagittal sections of mouse embryos were stained with hematoxylin & eosin (H&E).
 984 Positions of the sections are indicated by blue plates in the diagram (leftmost column).
 985 APC, astrocyte precursor cell; ODH, optic disc head; OF, optic fissure; dOS, dorsal optic
 986 stalk; vOS, NR, neural retina; RPE, retinal pigment epithelium; ventral optic stalk; vHT,
 987 ventral hypothalamus. Scale bars in the pictures are 100 μm. **(C)** Distributions of Pax6 and
 988 Pax2 in E14.5 mouse embryonic sections were examined by immunostaining. Boxed
 989 areas in top row images of coronal sections are magnified in two bottom rows. **(D)** Sagittal
 990 sections including medial OS of E14.5 and E16.5 mouse embryos were stained with the
 991 antibodies recognized the corresponding markers. APC, astrocyte precursor cell; ONE,
 992 optic neuroepithelium; RPC, retinal progenitor cell.

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Min et al._Fig3

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998 **Figure 3. Agenesis of OC in *Vax1*^{AA/AA} mice.** (A) Coronal sections of E14.5 mouse
 999 embryos were stained with H&E. *, ventral midline of HT equivalent to the position of OC in
 1000 *Vax1*^{+/+} littermates. V3, third ventricle. (B) The sections were also immunostained with the
 1001 antibodies recognize *Vax1* (green) and an RGC axon marker, L1-CAM (L1, red). (C)
 1002 Distributions of *Shh* mRNA in the embryonic sections were detected by *in situ* hybridization
 1003 (ISH). Development of hypothalamic radial glia (RG) from NPC was determined by
 1004 immunostaining for an RG marker Glast (glutamate aspartate transporter 1) and an NPC
 1005 marker Nestin, respectively. (D) Expression of a repulsive guidance cue, ephrinB2, and the
 1006 attractive guidance cues, *Vegfa* and Nr-CAM, for RGC axons in E14.5 mouse vHT was
 1007 examined by ISH (*ephrin B2* and *Vegfa*) and IHC (Nr-CAM). (E) Expressions of retinal
 1008 genes that induce ipsilateral RGC axon projection were examined by ISH (*EphB1* and
 1009 *Shh*) and IHC (*Zic2*). Boxed areas in top row images are magnified in bottom rows. (F)
 1010 Schematic diagrams that show the structures of OS/vHT junctions and RGC axon
 1011 pathways in *Vax1*^{+/+}, *Vax1*^{AA/AA}, and *Vax1*^{-/-} mice. Nas, nasal; Temp, temporal. OS NE, OS
 1012 neuroepithelium.

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Min et al., Fig4

Figure 4. Regulation of RGC axon growth by Vax1 transfer. (A) The activities of a Vax1 target *Pax6* α -enhancer in mouse embryos were measured by detecting H2B-EGFP, of which expression is induced together with membrane bound tdTomato (tdTom*) at *R26^{tm11}* gene locus after α -Cre-dependent excision of *loxP-STOP-loxP* cassette. Arrow heads point the frontlines of the RGC axons. **(B)** Binding of Vax1 and Vax1^{AA} proteins on the *Pax6* α -enhancer was determined by PCR detection of *Pax6* α -enhancer sequences in DNA fragments isolated from E10.5 mouse embryonic cells by ChIP with indicated antibodies (see details in Methods). Input, mouse chromosomal DNA; No Ab, no antibody; Rb IgG, preimmune rabbit IgG; α -Vax1, rabbit anti-Vax1 polyclonal antibody. **(C)** Relative levels of *Pax6* α -enhancer sequences in the ChIPed samples were compared by qPCR. The numbers in y-axis are average $2^{-\Delta Ct}$ values of the samples against critical threshold (Ct) values of Input. Error bars denote SD (n=5). **(D)** Collagen beads soaked with Flag peptides or Vax1-Flag protein were implanted in the third ventricle of E12.5 *Vax1^{AA/AA}* mouse embryonic brain slabs, while Dil fluorescent dye was filled in their right eyes (diagram). Distribution of Dil-labeled RGC axons in the horizontal sections of brain slabs were then visualized after 12h (i.e., E13). The penetration of Vax1-Flag proteins into RGC axons were confirmed by co-immunostaining with anti-Flag and anti-L1-CAM antibodies.

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Min et al., Fig5

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Figure 5. Ipsilaterally-biased RGC axon projection in *Vax1*^{AA/AA} mice. (A) Coronal sections of P30 mouse brain were stained with H&E. The sections containing the indicated commissural structures were identified and shown. AC, anterior commissure; CC, corpus callosum; D3V, dorsal third ventricle; HCC, hypothalamic cell cord; Pb, probus bundle; PC, posterior commissure; SC, superior colliculus. **(B)** Diagram depicts fluorescence labeling of mouse RGCs. **(C)** Alexa488- and Alexa594-labeled CTB proteins were injected into right and left eyes of the mice at P28, respectively. Brains of the CTB-injected mice were isolated at P30 and fluorescent signals emitted from the brains were visualized by Axio Zoom stereoscope (Zeiss; three center columns), and then the fluorescent signals in coronal sections of the brains were detected by FV1000 confocal microscope (Olympus; rightmost column). Bright-field images of the brains were also taken to show the structure of the brains and optic nerves (leftmost column). CX, cerebral cortex; OB, olfactory bulb; *, ventral midline of HT equivalent to the position of OC in *Vax1*^{+/+} littermates. **(D)** Coronal sections of the CTB-injected mouse brains were collected and fluorescent signals in the LGN area were detected. The areas surrounded by dotted lines are dLGN. Contra, contralateral LGN section; Ipsi, ipsilateral LGN section. **(E)** The retinas and brains of P30 *Vax1*^{+/+} and *Vax1*^{AA/AA} littermate mice carrying α -Cre;*R26*^{tm4/+} transgenes, which express membrane-targeted EGFP (EGFP*) at *R26*^{tm4} gene locus upon Cre-dependent excision of *loxP*-*tdTomato**-*loxP* gene cassette, were isolated and visualized. The areas surrounded by dotted lines are SC. PT, pretectum.

Min et al., Fig6

1063 **Figure 6. Reduced visual acuity of *Vax1*^{AA/AA} mice. (A)** Electrophysiological activities of
1064 P45 *Vax1*^{+/+} and *Vax1*^{AA/AA} mouse retinas were examined by ERG (see details in
1065 Methods). The amplitudes of scotopic **(B)** and photopic **(C)** ERG a- and b-waves at 1.6 log
1066 cds/m² condition are presented. Numbers of mice tested are given in the graphs (4
1067 independent litters). **(D)** Light-evoked excitation of cortical neurons in P45 *Vax1*^{+/+} and
1068 *Vax1*^{AA/AA} mouse V1 were measured by silicon multielectrode probes after monocular
1069 illumination (see details in Methods). Responses of neurons in the monocular zones of
1070 ipsilateral (Ipsi) and contralateral (Contra) visual cortices were recorded. Color-coded
1071 heatmap represents average z-scores of spike firing rates. Red indicates increase and
1072 blue indicates decrease of firing rates from the baseline, respectively. **(E)** Mean firing-rate
1073 change index of ipsilateral and contralateral V1 neurons in *Vax1*^{+/+} and *Vax1*^{AA/AA} mice. y-
1074 axis values are mean ± SEM. **(F)** Relative occupancy of light and dark chambers by P45
1075 *Vax1*^{+/+} (WT), *Vax1*^{AA/AA} (AA), and *Pde6b*^{rd1/rd1} (*rd1*) mice for 10-min measurement period
1076 was determined and shown in a graph. **(G)** Escaping responses of the mice, which were
1077 given the expanding black circle on top screens to mimic a looming shadow of predator,
1078 were determined and shown in a graph. **(H)** To determine stereoscopic vision of the mice,
1079 relative occupancy of safe and cliff zones by the mice for 2-min measurement period was
1080 determined and shown in a graph. **(I)** Accuracy of the mice to turn their heads to the
1081 directions where black and white vertical stripes rotate was measured and shown in a
1082 graph (head turn). Startling responses of the mice in response to the stimuli was also
1083 measured (startling). Numbers of the mice tested are given in the graphs in F – H.
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1085

1087 **Figure 7. Inversed oculomotor responses of $Vax1^{AA/AA}$ mice. (A)** Positions of pupil
1088 centers in right and left eyes of head-fixed P45 $Vax1^{+/+}$ and $Vax1^{AA/AA}$ mice were tracked
1089 by the iSCAN rodent eye tracking system while the mice were kept in dark. Relative
1090 positions of pupil centers against the position at time 0 (t_0) were plotted in the
1091 oscillograms. **(B)** Peak positions that the pupil centers moved at vertical and horizontal
1092 axes are measured and the averages are shown in graphs. Error bars are SD (n=6). **(C)**
1093 The P45 mice were adapted in dark for 30 mins and illuminated with room light. **(E)**
1094 Alternatively, the dark-adapted mice were illuminated monocularly with a point light. Pupil
1095 diameters were measured by iSCAN eye tracking system before and after the
1096 illuminations. Relative pupil diameters against t_0 are plotted in oscillograms. **(D and F)**
1097 Average pupil diameters of the mice in dark ($t_0 \sim t_5$) and light ($t_5 \sim t_{14}$) conditions are
1098 measured and shown in graphs. Error bars are SD (n=6 [$Vax1^{+/+}$] and n=7 [$Vax1^{AA/AA}$]). *,
1099 $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$. **(G)** Head-fixed P45 $Vax1^{+/+}$ and $Vax1^{AA/AA}$ mice were
1100 positioned in a chamber surrounded by monitors, which display gray background. Center
1101 positions of the pupils in right and left eyes were marked and then tracked by the iSCAN
1102 rodent eye tracking system while the mice were exposed to the monitors display black and
1103 white vertical stripes (0.2 c/d), which are moving in the indicated directions for 30 seconds.
1104 Relative positions of pupil centers of right eyes against the position at t_0 are plotted in the
1105 oscillograms. Peak positions of the pupil centers between 30 sec and 60 sec at horizontal
1106 **(H)** and vertical **(I)** axes were collected, and the averages are shown in graphs. Error bars
1107 are SD (n=6 [$Vax1^{+/+}$] and n=8 [$Vax1^{AA/AA}$]).
1108

Supplemental Information

Figure S1. Generation of *Vax1*^{AA/AA} mice.

Figure S2. Anatomical features of retina and optic nerve in *Vax1*^{AA/AA} mice.

Figure S3. Spontaneous oscillation of *Vax1*^{AA/AA} mouse eyes.

Table S1. List of antibodies used in this study.

Movie S1. Response of *Vax1*^{+/+} mice to a looming shadow.

Movie S2. Response of *Vax1*^{AA/AA} mice to a looming shadow.

Movie S3. Response of *Pde6b*^{rd1/rd1} mice to a looming shadow.

Movie S4. Cliff assay of *Vax1*^{+/+} mice.

Movie S5. Cliff assay of *Vax1*^{AA/AA} mice.

Movie S6. Cliff assay of *Pde6b*^{rd1/rd1} mice.

Movie S7. Optomotor response of *Vax1*^{+/+} mice.

Movie S8. Optomotor response of *Vax1*^{AA/AA} mice.

Movie S9. Optomotor response of *Pde6b*^{rd1/rd1} mice.

Movie S10. Eye movement of *Vax1*^{+/+} mice in dark.

Movie S11. Eye movement of *Vax1*^{AA/AA} mice in dark.

Movie S12. Pupil contraction of *Vax1*^{+/+} mice after binocular light illumination.

Movie S13. Pupil contraction of *Vax1*^{AA/AA} mice after binocular light illumination.

Movie S14. Pupil contraction of *Vax1*^{+/+} mice after monocular light illumination.

Movie S15. Pupil contraction of *Vax1*^{AA/AA} mice after monocular light illumination.

Movie S16. Eye movement of *Vax1*^{+/+} mice in response to vertical stripes rotating clockwise direction.

Movie S17. Eye movement of *Vax1*^{AA/AA} mice in response to vertical stripes rotating clockwise direction.

Movie S18. Eye movement of *Vax1*^{+/+} mice in response to vertical stripes rotating counter clockwise direction.

Movie S19. Eye movement of *Vax1*^{AA/AA} mice in response to vertical stripes rotating counter clockwise direction.

Movie S20. Eye movement of *Vax1*^{+/+} mice in response to converging vertical stripes.

Movie S21. Eye movement of *Vax1*^{AA/AA} mice in response to converging vertical stripes.

Movie S22. Eye movement of *Vax1*^{+/+} mice in response to diverging vertical stripes.

Movie S23. Eye movement of *Vax1*^{AA/AA} mice in response to diverging vertical stripes.

Movie S24. Eye movement of *Vax1*^{+/+} mice in response to stationary vertical stripes.

Movie S25. Eye movement of *Vax1*^{AA/AA} mice in response to stationary vertical stripes.

Min et al._FigS1

Figure S1. Generation of *Vax1*^{AA/AA} mice. (A) Structure of mouse *Vax1*^{AA} gene (top) and its partial sequences of exon 2 that encode AA replacing KR in the GAG binding motif (bottom). The substituted nucleotides for missense (red, KR to AA) and synonymous (green) mutations are shown. The amino acid sequences from the *Vax1* and the *Vax1*^{AA} alleles are shown at the bottom of each nucleotide sequence. The relative positions of two crRNAs and ssODN are indicated in short blue bars and black bar with asterisk (*), respectively. (B) Top, relative positions of genotyping primers are indicated by arrows. The red asterisks (*) indicate KR to AA mutation. Bottom, PCR genotyping results using genotyping primers. (C) Ventral views of P0 mouse mouths (top) and palates (bottom). Arrows indicate the clefts of lip (top) and palate (bottom), respectively. (D) Ventral views of P30 mouse mouths (top) and palates (bottom). (E) Survival rates of *Vax1*^{AA/AA} mice by the indicated ages in x-axis are provided. Numbers of mice examined were provided in the graph.

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Figure
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immunostained with the antibodies that recognize the indicated marker proteins. Pax2 and Gfap, astrocytes; CD31, endothelial cells; Olig2, oligodendrocytes. Vax1 is not expressed in the adult optic nerves. **(C)** Sections of the eyes of P30 *Vax1*^{+/+} and *Vax1*^{AA/AA} littermate mice were stained by H&E or the antibodies that recognize the indicated marker proteins. Rhodopsin, rod photoreceptors; M-opsin, M-cone photoreceptors; S-opsin, S-cone photoreceptors; Calbindin, horizontal and amacrine cells; Vsx2, bipolar cells; Pax6, amacrine cells; Brn3b, RGCs; Sox9, Müller glia; Gfap, astrocytes.

Figure S3. Spontaneous movement of $Vax1^{AA/AA}$ mouse eyes. Relative positions of the pupils in right and left eyes of head-fixed P45 $Vax1^{+/+}$ and $Vax1^{AA/AA}$ were recorded by the iSCAN rodent eye tracking system at every 8 msec for 10 secs while the mice were kept in dark. The positions were plotted in the graphs. The results show the eyes of $Vax1^{AA/AA}$ rotate spontaneously in an oval track while $Vax1^{+/+}$ mouse eyes keep their positions in dark.

Table S1. Antibodies used in this study

Antigen	Origin	Dilution	Maker	Catalog #
b-actin	Rabbit	1:1000	Santa cruz biotechnology	SC-1616
Brn3b	Goat	1:200	Santa cruz biotechnology	SC-31989
CD31	Hamster	1:200	Millipore	MAB1398Z
E-cad	Mouse	1:200	BD	610181
GFAP	Rabbit	1:200	Abcam	AB48050
GFP	Rabbit	1:200	Abcam	AB290
GFP	Mouse	1:200	Santa cruz biotechnology	SC-9996
Glast	Guinea pig	1:500	Millipore	AB1782
L1-CAM	Rat	1:200	Millipore	MAB5272
M-opsin	Mouse	1:200	Millipore	AB5405
Nestin	Mouse	1:100	Millipore	MAB353
NF160	Mouse	1:50	DSHB	2H3
Nr-CAM	Rabbit	1:200	Abcam	AB24344
Olig2	Rabbit	1:200	Millipore	AB9610
Pax2	Rabbit	1:200	Invitrogen	71-6000
Pax6	Rabbit	1:200	Covance	PRB-278P
Pax6	Mouse	1:200	Santa cruz biotechnology	SC-81649
Rhodopsin	Mouse	1:200	Chemicon	MAB5356
S-opsin	Rabbit	1:200	Millipore	AB5407
S100b	Rabbit	1:500	Abcam	AB52642
Sox9	Rabbit	1:200	Santa cruz biotechnology	SC-20095
Vax1	Rabbit	1:50	Mui et al. (2005)	N.A.
Vsx2	Guinea pig	1:200	gift from Dr. Mi-Ryoung Song (GIST)	N.A.
Zic2	Rabbit	1:200	Millipore	AB15392