

Molecular and cellular determinants of motor asymmetry in zebrafish

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1 **Abstract**

2

3 Asymmetries in motor behavior, such as human hand preference, are observed throughout bilateria.
4 However, neural substrates and developmental signaling pathways that impose underlying functional
5 lateralization on a broadly symmetric nervous system are unknown. Here we report that in the absence
6 of over-riding visual information, zebrafish larvae show intrinsic lateralized motor behavior that is
7 mediated by a cluster of 60 posterior tuberculum (PT) neurons in the forebrain. PT neurons impose
8 motor bias via a projection through the epithalamic commissure to the habenula. Acquisition of
9 left/right identity is disrupted by heterozygous mutations in *mosaic eyes* and *mindbomb*, genes that
10 regulate Notch signaling. These results define the neuronal substrate for motor asymmetry in a
11 vertebrate and support the idea that developmental pathways that establish visceral asymmetries also
12 govern acquisition of left/right identity.

13

14

15 **Introduction**

16

17 In many bilaterian organisms the nervous system shows striking structural and functional lateralization,
18 and many species show prominent asymmetric motor behaviors such as hand or paw preference
19 (Rogers, 2009). However, attempts to link neuroanatomic and motor asymmetries have yielded
20 contradictory and inconclusive results in humans (Good et al., 2001; Guadalupe et al., 2014; Sun et al.,
21 2012). In other species, despite correlations between asymmetric properties of the nervous system and
22 lateralized behavior, causal relationships have not been established (Davison et al., 2009; Gutierrez-
23 Ibanez et al., 2011; Jozet-Alves et al., 2012; Lee et al., 2017). In the absence of a compelling neuronal

24 basis, it has been difficult to resolve molecular determinants that drive the development of lateralized
25 behavior.

26

27 Research to date has produced limited insight into molecular mechanisms that establish motor
28 asymmetries. Consistent with early theories that outlined a role for single genes of large effect, twin
29 studies on the heritability of handedness in humans revealed an important genetic component (Annett,
30 1972; McManus, 1985; Medland et al., 2006). However, genome-wide genetic studies failed to uncover
31 loci with large contributions (Armour et al., 2014; Eriksson et al., 2010; Somers et al., 2015).

32 Behavioral hand-preference is established as early as 10 weeks of gestation in humans and left/right
33 asymmetries in gene expression within the spinal cord have been identified at this stage, suggesting a
34 role for genes that pattern the nervous system (Hepper et al., 1998; Ocklenburg et al., 2017).

35 Abnormalities in neuroanatomical asymmetry and handedness are associated with schizophrenia and
36 other neurodevelopmental disorders, however without knowledge of the underlying neuroanatomical
37 substrates or developmental pathways these findings are difficult to interpret (Dean et al., 2016;
38 Markou et al., 2017; Sommer et al., 2001).

39

40 In the absence of a clear structural basis for a vertebrate motor asymmetry, many studies have instead
41 focused on molecular genetic pathways that govern the development of brain asymmetries. In
42 particular, work in zebrafish has outlined molecular genetic pathways that govern asymmetric
43 morphogenesis and gene expression within the dorsal diencephalon. In zebrafish, as in many other
44 vertebrates, the habenula shows a pronounced hemispheric asymmetry with well characterized
45 differences in the size, composition and connectivity of subnuclei on the left and right sides (Aizawa et
46 al., 2007, 2005; Amo et al., 2010; Concha et al., 2000; Gamse et al., 2005). Moreover, functional
47 differences between hemispheres are also apparent, with olfactory cues preferentially activating the

48 right habenula and visual stimuli driving responses in the left habenula (Cheng et al., 2017; Dreosti et
49 al., 2014; Krishnan et al., 2014; Zhang et al., 2017). Behaviorally, the habenula in fish has been
50 implicated in social conflict, anxiety and avoidance-learning (Amo et al., 2014; Chou et al., 2016;
51 Facchin et al., 2015; Lupton et al., 2017) and the left-habenula in specifically in attenuating fear and
52 driving light-preference behavior (Duboue et al., 2017). However, to date, assays reporting individual
53 lateralized motor behavior in zebrafish have proven to difficult to reproduce, precluding efforts to
54 resolve underlying asymmetries in brain structure (Barth et al., 2005; Facchin et al., 2009).

55

56 Here we report that individual larval zebrafish show a consistent motor asymmetry across multiple
57 behavioral assays when tested in the absence of visual stimuli. Motor identity is maintained by a cluster
58 of 60 neurons in the rostral lobe of the posterior tuberculum that project to the habenula nuclei, ablation
59 of which also degrades motor bias. Finally, we demonstrate that lateralized behavior is disrupted by
60 haploinsufficient mutations in genes that regulate Notch signaling, supporting the idea that the same
61 pathway that establishes visceral asymmetries during development also governs acquisition of
62 individual left/right motor identity.

63

64 **Results**

65

66 *Larval zebrafish show persistent individual lateralized behavior in locomotor trajectories*

67

68 After loss of illumination, 6 days post-fertilization (dpf) larval zebrafish initiate a circular swimming
69 behavior in which they repeatedly perform same-direction turn maneuvers in a restricted spatial area
70 (Figure 1A)(Horstick et al., 2017). Same-direction turn movements were sustained in individual larvae
71 for two minutes (Figure 1B), however across the population there was no net tendency for larvae to

72 preferentially circle to the left or right (mean Net Turn Angle (NTA) = -60.8 ± 84.2 , one-sample t-test
73 against 0, $p=0.24$; Figure S1A). However, we asked whether individual larvae show lateralized
74 behavior — preferentially swimming in a left or right direction — or randomly select a circling
75 direction on each event. We therefore probed individuals with a series of four light-off trials, each
76 separated by several minutes of illumination. Larvae that circled in a rightward direction on the first
77 trial, showed a significant tendency to circle rightward on subsequent trials, and similarly, left-circling
78 larvae continued to show a leftward bias (repeated measures ANOVA, main effect of trial 1 direction
79 $F_{(1,65)}=22.20$, $p<0.001$; Figure 1C). Lateralized behavior was not observed when larvae were tested
80 under constant illumination, as groups initially classified as left/right based on the first trial did not
81 show differences in mean direction on subsequent trials ($F_{(1,61)}=1.08$, $p=0.30$; Figure 1D). Directional
82 bias on dark trials manifest as a 69.6% probability for larvae to circle in the same direction as on the
83 first trial (match index, $p < 0.0004$ compared to 50% probability ; Figure 1E). Circular swimming is
84 primarily driven by routine-turn maneuvers (Horstick et al., 2017). We therefore used high-speed video
85 recordings and kinematic analysis to directly measure routine-turn direction across a series of four light
86 off trials (Figure 1F). Again, we noted that turn direction on trials 2-4 was significantly correlated with
87 turn direction on the first trial (Figure S1B). Next, we calculated the percentage of routine-turns made
88 in a rightward direction on each trial, then used the mean of the four trials to represent each larva's
89 direction preference. In the dark, the distribution of direction preferences strongly deviated from the
90 expected distribution: 41% of larvae (37/89) produced fewer than than 24% or greater than 76%
91 rightward turns, whereas if turn-direction was random on each trial, only 10% of larvae would have
92 shown this level of bias (Monte Carlo simulations, $p < 0.0001$; Figure 1G). In contrast, under constant
93 illumination the distribution of routine-turn direction bias was similar to the expected distribution
94 ($p=0.119$; Figure 1H). These results reveal that zebrafish larvae raised in the same environment

95 stochastically acquire a left/right directional bias in motor behavior that is manifest when tested under
96 dark conditions.

97

98 A form of unstable lateralized eye-use behavior has been reported in zebrafish, with individuals
99 switching eye-preference over several minutes (Andrew et al., 2009). In contrast we found that turn-
100 bias during dark-induced circling behavior was sustained for at least 45 min (Figure S1C). Next, we
101 tested individuals at 6 dpf to establish their left/right motor identity, then returned them to an incubator
102 overnight. The next day, we recorded responses to a second set of four light-off stimuli. Turn direction
103 bias for individual larvae was significantly correlated between days (Spearman $\rho=0.58$, $p=0.0009$;
104 Figure 1I-J, S1D) and also sustained in larvae that were raised for 4 days between tests ($\rho=0.39$,
105 $p=0.0003$; Figure 1J, S1F). Decomposing turn bias into its magnitude and direction components
106 revealed that overall left/right direction preference was maintained (d6 to d7, Mann-Whitney U
107 $p=0.012$; d6 to d10, $p=0.00039$; Figure 1J) although the strength of the preference was not correlated
108 across days (d6 to d7, $\rho=-0.02$, $p=0.9$; d6 to d10, $\rho=0.054$, $p=0.7$; Figure S1E,G). Thus left/right
109 motor identity is sustained in individual larvae for several days. In birds stable perceptual asymmetries
110 are conferred by visual experience during embryonic development (Rogers, 1982). In contrast, dark-
111 reared zebrafish larvae showed normal lateralized behavior, excluding an instructive role for visual
112 experience in the acquisition of motor identity (Figure S1H). Together, these data provide compelling
113 evidence that zebrafish larvae show a robust motor asymmetry, manifest as persistent individual
114 differences in the direction of circling behavior after loss of illumination. Behavioral asymmetry in
115 zebrafish is of moderate strength: larvae initiate movement in their preferred direction on around 70%
116 of trials. Similar numbers of larvae are left and right-biased, with no apparent population-level
117 asymmetry.

118

119 Next we asked whether motor bias is also present in responses to other stimuli. Zebrafish show positive
120 phototaxis, and select and swim toward one of two simultaneously illuminated regions (Burgess et al.,
121 2010). We first classified larvae as left or right biased during dark-induced circular swimming, then
122 used a closed-loop system to present freely swimming larvae with two identical target spots on the left
123 and right (Figure 2A,B). Target choice was positively correlated with circular swimming direction
124 (Figure 2C). We also tested whether lateralized motor behavior occurred during responses to a non-
125 visual stimulus. In zebrafish, intense auditory stimuli elicit escape responses that are initiated with a
126 rapid bend to either the left or right, raising the possibility that startle direction might show a motor
127 asymmetry. We again pre-classified larvae using dark-induced circular swimming, then measured
128 startle direction. Under constant illumination, escape response direction showed little or no correlation
129 with circular swim direction (Figure 2D, yellow background). However auditory stimuli that were
130 presented in the dark evoked startle responses whose direction showed a significant match to the
131 direction of circular swimming (Figure 2D, grey background). Thus, motor bias was apparent in three
132 different assays, consistent with individual larvae having an intrinsic left/right motor identity.

133

134 In all three assays, motor asymmetry was primarily manifest in the dark, suggesting that that visual
135 cues over-ride intrinsic bias. We tested this idea using *ato7* mutants which completely lack projections
136 from the retina to the brain. Even without retinal signaling, zebrafish respond to changes in whole-field
137 illumination via deep brain photoreceptors (Fernandes et al., 2012; Horstick et al., 2017; Kokel et al.,
138 2010). Thus on trials 2-4 of the dark-induced circling assay, both *ato7* mutants and siblings
139 demonstrated a significant tendency to swim in the same direction as in trial 1 (Figure 2E, grey bars).
140 However, unlike wildtype sibling larvae, *ato7* mutants also showed a weak but significant motor
141 asymmetry under illuminated conditions ($p=0.003$; Figure 2E, yellow bars). Next, we used acute
142 unilateral enucleation to control the source of visual information. After acute unilateral enucleation,

143 circling was performed in the direction contralateral to the intact eye on all four trials (Figure 2F).
144 Together, these results support the idea that visual information, when present, predominates over
145 intrinsic motor asymmetry.

146

147 *Neurons in the posterior tuberculum maintain left/right motor identity*

148

149 We next set out to identify the underlying neuronal substrates for left/right bias. Whereas the parapineal
150 and habenula show consistent population-wise anatomical asymmetries, no brain regions are known in
151 zebrafish to have stochastic hemispheric differences in size or function that would be consistent with
152 individual left/right motor bias (Concha et al., 2000; Gamse et al., 2003). We therefore performed a
153 circuit-breaking screen to identify neuronal substrates, crossing Gal4 lines that label distinct brain
154 structures to a UAS:epNTR-TagRFP reporter for cell-specific ablation using nitroreductase (Figure 3A)
155 (Horstick et al., 2014; Pisharath et al., 2007). After lesioning the labeled population of neurons in each
156 line, we then tested whether individual motor bias remained. Larvae showed a decrease in motor bias
157 after ablation of neurons in two Gal4 lines: *y279* and *y375* (Figure 3B). Reduced motor bias was
158 specific, as ablation did not affect the total amount of turning behavior or baseline locomotion (Figure
159 S2A). Finally, the chemogenetic ablation protocol itself did not disrupt motor bias as ablation of
160 neurons in orthopedia driver line *otpb.A:Gal4* had no effect (Figure 3B). Thus the *y279* and *y375* Gal4
161 lines label neurons that are critical for individual motor bias.

162

163 *y279* and *y375* each express Gal4 in neurons distributed in multiple brain regions (Figure 3C,D). We
164 reasoned that clusters labeled by both lines would be strong candidates for driving lateralized behavior.
165 Because both lines express the same reporter, we could not directly identify co-labeled neurons, but
166 instead compared co-registered whole-brain images of each line (Marquart et al., 2015). Salient virtual

167 co-expression was present in a bilateral cluster of neurons in the posterior tuberculum (PT) (Figure 3E,
168 S2B-E). Cell counts established that each PT hemisphere comprised 28.3 ± 7.2 $y279^+$ cells (mean \pm
169 standard deviation, N=28 larvae, Figure 3F), but did not reveal differences between hemispheres in left
170 and right biased larvae (Figure S2F). We assessed whether the PT clusters were required for left/right
171 bias by using focal laser ablation of $y279$ neurons. As a control we also ablated the caudal
172 hypothalamus (Hc) which is strongly labeled in $y279$. Ablation of the Hc had no effect whereas
173 bilateral ablation of the rostral PT cluster strongly reduced motor bias, consistent with a loss of
174 left/right identity (Figure 3G, S3A). Conversely, after unilateral PT ablations 76% of larvae (38/50)
175 circled in the direction ipsilateral to the intact PT during dark trials, with no effect during baseline
176 illumination (Figure 3H, S3B-C). The posterior tuberculum is a ventral region of the diencephalon that
177 includes identified classes of dopaminergic (DA) neurons (Rink and Guo, 2004; Tay et al., 2011).
178 However, the cluster of PT neurons labeled in $y279$ and $y375$ is situated rostral and dorsal to PT DA
179 neurons (Figure S2B). Moreover *otpa* mutants, which lack key classes of DA neurons, and *otpb.A:Gal4*
180 ablations retained robust left/right bias (Figures 3B ; S3D). It is therefore unlikely that PT
181 dopaminergic function contributes to motor asymmetry. These results confirm that a cluster of neurons
182 in a rostral lobe of the posterior tuberculum impose left/right bias on motor responses in zebrafish.

183

184 *PT neurons show persistent activity after loss of illumination*

185

186 We next examined whether PT neurons are active after loss of illumination by imaging changes in
187 GCaMP6s fluorescence in $y279$ -*Gal4*, *UAS:GCaMP6s* larvae. Because we previously found that photic
188 stimulation acutely terminates localized circling behavior (Horstick et al., 2017), we used an infra-red
189 laser for two-photon excitation of GCaMP6s during these recordings. We attempted to simultaneously
190 record tail movements, but found that persistent motor responses to the loss of illumination were not

191 apparent in immobilized larvae. Nevertheless we reasoned that even if the motor behavior was not
192 preserved, PT neurons might still respond to loss of illumination. Indeed, 16.2% of PT neurons (37/228
193 from 6 larvae) responded to the light off stimulus (Figure 4A-B). PT neuron activity peaked shortly
194 after loss of illumination and remained elevated during the 57s recording interval, consistent with the
195 time-frame of circling behavior (Figure 4C). An additional 3.9% of PT neurons (9/228) were active
196 upon the restoration of illumination. However no significant correlations were observed between the
197 left/right identity of individual larvae and peak GCaMP activation or position of light OFF responsive
198 neurons within the PT (Figure 4D,E). These results establish that a subset of PT neurons fire in
199 response to changes in illumination and remain active on a time scale consistent with the duration of
200 motor asymmetry during dark-induced circling behavior.

201

202 *Projections from the PT to habenula are essential for motor bias*

203

204 The epithalamic commissure (Ec) that runs between the habenula hemispheres is labeled in *y279-Gal4*,
205 *UAS:Kaede* larvae (Figure 5A). As *y279* also labels neurons in the habenula — particularly in the left
206 habenula — we initially suspected that this commissure was formed by *y279* habenula neurons.
207 However when performing confocal scans to verify laser ablations, we noticed that fluorescently
208 labeled fibers were absent in bilateral PT lesioned larvae, raising the possibility that commissural fibers
209 originate in the PT (Figure 5B). To test this, we unilaterally photoconverted Kaede in the PT in *y279-*
210 *Gal4, UAS:Kaede* larvae. Photoconverted Kaede labeled neurites that emerged dorsally from each PT
211 hemisphere and projected to the ipsilateral habenula. Within the habenula these fibers turned and
212 crossed to the contralateral side through the epithalamic commissure (Figure 5C,D). PT projections
213 terminated in the neuropil region of both habenula hemispheres. No salient differences in the PT-
214 habenula projection were observed for left or right-biased larvae, however this observation raised the

215 possibility that the PT controls motor bias via signaling to the habenula. We tested this by laser ablation
216 of $\gamma 279$ habenula neurons (Figure S3A). Bilateral ablation of $\gamma 279$ habenula neurons abolished
217 left/right bias without affecting total turning (Figure 5E-F). Moreover after unilateral habenula ablation
218 motor responses were strongly lateralized during dark-induced circling behavior, without inducing
219 motor bias under baseline illumination (Figures 5G ; S3C). In contrast to PT lesions, total turning
220 behavior did not increase after unilateral habenula ablation (Figure S3E). Intriguingly, selective lesion
221 of the epithalamic commissure also induced a strong motor asymmetry that was similar to ablation of
222 the right-habenula, such that larvae became left-biased only under dark conditions (Figure 5G,H,
223 S3C,F). Together, the loss of motor bias after bilateral ablation of either the PT or habenula, induction
224 of left/right lateralized behavior after unilateral ablations and presence of a direct connection between
225 these areas, argues that the PT-habenula pathway imposes left/right bias on motor responses.

226

227 *Haploinsufficient notch pathway mutations disrupt lateralized behavior*

228

229 Many species, including zebrafish, show a stereotyped asymmetric development of specific brain
230 nuclei and placement of viscera. Genes involved in this process have been relatively well characterized
231 (Concha et al., 2000; Lopes et al., 2010; Yan et al., 1999). However much less is known about genetic
232 pathways that lead to the stochastic acquisition of left or right motor asymmetry. During the course of
233 our studies, we isolated a background mutation ($\gamma 606$) in our wildtype stock that weakened motor bias
234 during circling behavior. In affected clutches, 25% of embryos showed a 'curly-up' phenotype that
235 severely effected tail morphology and precluded behavioral testing (Figure 6A). We speculated that the
236 morphological abnormality represented a homozygous phenotype, with loss of motor asymmetry
237 present in morphologically normal heterozygous larvae (Figure 6B). RNAseq-based bulk segregant
238 mapping linked the mutation to a six Mbp interval on chromosome 9 and revealed a 7.7-fold reduction

239 in the expression of the gene *epb4115* in this interval in *y606* mutants compared to siblings (Figure
240 S4A-B) (Miller et al., 2013). No RNAseq reads mapped to the first two exons, and we were not able to
241 amplify these exons from mutants using genomic PCR (Figure S4C). Consistent with this, genomic
242 PCR revealed a 4.4 kb deletion that excises exons 1 and 2 of *epb4115*, eliminating the transcription and
243 translation start sites (Figure S4D). *Epb4115* is the mutated gene in the *mosaic eyes* (*moe^{b476}*) mutant
244 that shows a similar curly-up phenotype (Jensen et al., 2001) and *moe^{b476}* failed to complement *y606*,
245 confirming that *y606* is a new allele of *mosaic eyes*.

246

247 We incrossed *moe^{y606}* heterozygous adults and tested morphologically normal siblings of curly-up
248 mutants in the dark-induced circling assay, then performed post-hoc genotyping to distinguish wildtype
249 and heterozygous larvae. Wildtype larvae showed normal motor asymmetry, however heterozygous
250 *moe^{y606}* larvae showed a significant reduction in left/right-bias (Figure 6C). Other motor parameters and
251 asymmetric development of the epithalamus and viscera were unaffected in heterozygotes (Figure
252 6E,F). To corroborate these findings we used *moe^{b476}*, an independent allele that we confirmed
253 eliminates the entire *epb4115* gene (Figure S5A-C). Heterozygous mutations in *moe^{b476}* also disrupted
254 motor bias during circling behavior (Figure 6D). These results demonstrate that acquisition of left/right
255 identity in zebrafish requires two functional alleles of *epb4115* in zebrafish.

256

257 *Epb4115* regulates the timing of neurogenesis, interacting with proteins in the *Notch* signaling pathway
258 (Matsuda et al., 2016; Ohata et al., 2011). Notch regulates *Nodal*, the central gene that drives the
259 development of molecular and anatomical left-right asymmetry (Krebs et al., 2003; Raya et al., 2003).
260 We therefore reasoned that other heterozygous mutations in Notch signaling proteins might perturb
261 left/right bias and tested larvae with heterozygous mutations in *mindbomb* (*mib*), an E3 ubiquitin ligase
262 that is essential for notch signaling (Itoh et al., 2003). Indeed, *mib* heterozygotes showed a severe loss

263 of left/right-bias during dark-induced circling (Figure 6G) without perturbing the total amount of
264 turning (Figure 6H). Together, these findings reveal that acquisition of left/right motor identity is
265 disrupted by mutations in genes that regulate Notch signaling levels during embryonic development.

266

267 **Discussion**

268

269 Here, we reveal that in the absence of visual information, zebrafish manifest a durable left/right motor
270 bias that is driven by neurons in the posterior tuberculum. Lateralized motor behavior occurs both
271 during spontaneous movement, and in response to visual and auditory cues. Zebrafish do not show a
272 population-level bias, unlike in humans where 90% of the population favor the right hand (Corballis,
273 2003). Rather, similar to motor asymmetries in many other species, lateralized behavior is manifest as
274 an individual bias to execute movements in a preferred direction on ~70% of trials. Left/right identity is
275 maintained over at least several days despite handling and changes in the environment. Bilateral
276 clusters of ~30 genetically identified neurons per hemisphere in the rostral lobe of the posterior
277 tuberculum are essential for the expression of this motor asymmetry. This conclusion is supported by
278 loss of lateralized behavior after chemogenetic ablation of rostral PT neurons using two transgenic
279 Gal4 lines that have minimal overlap elsewhere in the brain, and direct laser photoablation of the PT. In
280 addition, unilateral laser ablation of PT neurons was sufficient to create a population of fish whose
281 response direction was heavily biased to the side of the intact PT. Critically, after rostral PT lesions,
282 larvae continued to show persistent turning to one side after loss of illumination, but circling behavior
283 was initiated in a random direction on each trial rather than in a preferred direction. It is therefore
284 unlikely that the rostral PT is part of the sensory pathway that initiates dark-induced circling, but rather
285 suggests that the PT is a locus that maintains left/right identity and imposes this bias on motor
286 responses. Finally, neurons in the rostral PT show sustained activity after loss of illumination,

287 consistent with the duration of lateralized circling movements. Together, these findings confirm that the
288 rostral posterior tuberculum drives lateralized behavior in zebrafish and define a specific neuronal
289 substrate for a lateralized motor behavior in a vertebrate.

290

291 *A PT-habenula circuit directs motor asymmetry*

292

293 Our findings suggest a model in which bilateral PT-habenula units compete to strengthen, but not
294 directly drive ipsilateral dark-induced circling behavior (Figure 7). In individual larvae, one hemisphere
295 predominates, providing a greater drive to premotor circuits. How left/right identity is encoded in the
296 PT-habenula unit is unclear: we did not detect differences in the number of neurons or light-off activity
297 between hemispheres that correlated with motor bias. The strong effect of unilateral PT ablations
298 indicate that the intact hemisphere, even if it was not previously dominant, was capable of imposing
299 ipsilateral motor bias, suggesting that dominance is unlikely to reflect a unique quality present in only
300 one hemisphere. However, after unilateral PT ablation, not only was dark-induced motor behavior
301 strongly lateralized, but net turning also increased, consistent with the idea that in the absence of
302 competition, there is greater drive biasing ipsilateral premotor circuits. Although we are not aware of
303 evidence for a direct connection between PT clusters we found that PT neurons project to and
304 commissurally within the habenula, concordant with a previous report (Hendricks and Jesuthasan,
305 2007). Thus competition between PT clusters may take place within the habenula. In keeping with this
306 idea, ablation of *y279* habenula neurons disrupted lateralized responses similar to PT ablation.
307 However, ablation of the habenula commissure yielded a population of larvae with left-biased
308 responses, similar to ablation of either right habenula or PT, possibly suggesting that the left habenula
309 predominates in the absence of communication. Thus, although our findings do not resolve the precise

310 mechanism by which the PT directs motor asymmetry, they demonstrate that a PT-habenula pathway is
311 an essential substrate for lateralized behavior in zebrafish.

312

313 *Genetic control of individual left/right identity*

314

315 The last two decades has produced significant progress in understanding the morphogenetic processes
316 that lead to consistent left-right asymmetries within the nervous system (Roberson and Halpern, 2018).
317 In contrast, much less is known about how stochastic individual patterns of left/right identity are
318 established. In birds, asymmetric sensory experience during a critical period in early development
319 imprints hemispheric dominance for visual feature processing (Koshihara et al., 2002; Rogers, 1982). In
320 contrast, we found that dark-reared larvae and *atoh7* mutants which lack central projections of retinal
321 ganglion cells at all stages maintained individual motor lateralization indicating that neither
322 spontaneous retinal activity nor asymmetric visual experience are required for individual bias to
323 emerge. Further, as in mice and *Drosophila*, selective breeding experiments excluded the possibility
324 that a heritable factor dictates left/right preference (Figure S6)(Buchanan et al., 2015; Collins, 1969).
325 Most likely, directional bias is stochastically determined during embryogenesis by either a specific
326 symmetry breaking event or by natural variability in development. While the nature of this process is
327 not yet known, recent genome-wide association studies have provided tantalizing hints that molecular
328 pathways involved in the development of visceral asymmetries also contribute to the establishment of
329 human handedness (Brandler et al., 2013). Consistent with this idea, we found that left/right motor
330 identity was disrupted by heterozygous mutations in *epb41l5*, a gene required for left-right patterning
331 in mice (Lee et al., 2010). *Epb41l5* is a membrane adapter protein (Matsuda et al., 2016; Ohata et al.,
332 2011) that interacts with *mind-bomb*, a ubiquitin-ligase required for Notch signaling during
333 neurogenesis (Itoh et al., 2003). Accordingly we found that heterozygous mutations in *mind-bomb*, a

334 ubiquitin-ligase required for notch signaling, also disrupted lateralized behavior. Surprisingly,
335 behavioral changes were seen in haploinsufficient mutants suggesting that acquisition of left/right
336 identity is highly sensitive to notch signaling levels. Indeed in mice, haploinsufficient mutations of the
337 notch ligand *delta-like 1* lead to a reduction in dopaminergic neuron differentiation, and in zebrafish
338 manipulations that augment or disrupt notch signaling during habenula neurogenesis can shift the
339 production of lateral or medial neurons and thereby isomerize the habenulae (Aizawa et al., 2007;
340 Trujillo-Paredes et al., 2016). Intriguingly, *notch1a* has a similar temporal profile of expression in the
341 developing PT raising the possibility that heterochronic neurogenesis of PT neuron subtypes is
342 involved in acquisition of left/right identity (Mueller and Wullmann, 2005). Thus although we did not
343 observe changes in the number of *y279* expressing neurons in the left or right PT hemisphere in
344 *epb4115* heterozygous mutants (Figure S7), future studies may reveal alterations in the differentiation
345 of neuronal cell types associated with loss of left/right identity.

346

347 *Neural correlates of motor asymmetries*

348

349 Cerebral asymmetries have been proposed to increase information processing power through
350 hemispheric specialization (Vallortigara and Rogers, 2005), or, to reflect space constraints in the brain
351 that necessitate partitioning processing functions between hemispheres (Barneoud and Van der Loos,
352 1993). Indeed, the left and right habenula nuclei in zebrafish are activated by different sensory
353 modalities and have separable roles in behavior (Cheng et al., 2017; Dreosti et al., 2014; Duboue et al.,
354 2017; Krishnan et al., 2014; Zhang et al., 2017), and in *C elegans* and *Drosophila* brain asymmetries
355 have been linked to sensory processing and memory formation respectively (Pascual et al., 2004;
356 Pierce-Shimomura et al., 2001; Wes and Bargmann, 2001). Asymmetries in sensory processing areas in
357 birds are correlated with lateralized control of behavior (Rogers, 1990). Similarly, it seems natural to

358 postulate that asymmetric structural or functional properties of the nervous system also underlie motor
359 asymmetries. However, evidence for this idea is surprisingly elusive: motor lateralization has only
360 rarely been correlated with an underlying cerebral asymmetry, and causal relationships have yet to be
361 established (Davison et al., 2009; Gutierrez-Ibanez et al., 2011; Jozet-Alves et al., 2012; Lee et al.,
362 2017). The strongest relationship to date has been described in the pond snail *Lymnaea stagnalis* where
363 the direction of coiling behavior by males during mating almost completely matches the side on which
364 two central ganglia are fused (Davison et al., 2009).

365

366 Even less clear is the nature of the ethological advantage conferred by motor asymmetries. An enduring
367 argument is that the hemispheres of an ideal perfectly symmetrical nervous system can not discriminate
368 bilaterally symmetrical sensory stimuli and would therefore reach a stalemate in selecting a left or right
369 motor response to a non-directional cue (Webster, 1977). Intrinsic neural asymmetries may therefore
370 facilitate motor responses to stimuli with ambivalent directional information, avoiding simultaneous
371 initiation of left/right responses, or reactions that are delayed due to difficulty selecting a response
372 direction. Indeed, during dark-induced circular swimming, larvae must initiate movement by
373 contracting muscles on one side. In the light, even small asymmetries in the visual environment may
374 over-ride innate bias, explaining why we do not observe motor asymmetry under illuminated
375 conditions. This idea is also consistent with our finding that *atoh7* mutants, which are blind, show
376 motor bias under both light and dark conditions; in mutants visual stimuli do not reach the brain to
377 over-ride innate direction preference.

378

379 Motor asymmetries such as human handedness are among the most pervasive and salient forms of
380 individual variation. Moreover, variation in motor asymmetries is linked to inter-individual differences
381 in personality, cognitive processing and risk for neurodevelopmental disorders (Markou et al., 2017;

382 Sommer et al., 2001). Yet, attempts to understand how such motor asymmetries are stochastically
383 generated during development, or even their structural or functional basis in the brain, have yielded
384 limited insight. In particular, efforts to study the development and basis for human handedness have
385 been hampered by difficulty in identifying a neural substrate, and inability to perform experimental
386 manipulations. Our identification of a specific neuronal substrate for asymmetric motor behavior in
387 zebrafish opens up a new model for understanding how functional lateralization emerges and is
388 maintained in the nervous system. Moreover our data also suggests that acquisition of left/right identity
389 is sensitive to specific levels of *Notch* pathway activity. These results therefore set the stage to uncover
390 precisely how motor bias is established during development and is maintained by structural or
391 functional asymmetries within the nervous system.

392

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394

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400 National Institutes of Health, Bethesda, MD.

401

402 **Author Contributions**

403

404 EJH and HAB conceived the experiments and wrote the manuscript. EJH, YB and HAB performed
405 experiments. All authors approved the final manuscript.

406

407 **Declarations of Interests**

408

409 The authors declare no competing interests.

410

411 **Figure legends**

412

413 **Figure 1: Individual larvae show persistent motor asymmetry during dark-induced circling**
414 **behavior.**

415

416 A. Schematic of setup for path trajectory analysis (left). Swim trajectories for single larvae during
417 baseline illumination and immediately after the loss of light (yellow and grey backgrounds
418 respectively). Color scale represents time (seconds). Arrowheads along path line represent orientation
419 of larva. Scale bar 20 mm.

420

421 B. Net turn angle over 30 s intervals for larvae before and after the loss of illumination (indicated by
422 top horizontal bars). Individuals were classified as either right (cyan, N=25) or left biased (grey, N=34)
423 based on the first 30 s interval after the loss of illumination. * $p < 0.05$, t-test between groups.

424

425 C. Net Turn Angle for larvae over four 30 s light off trials. Horizontal bars at top indicate baseline
426 illumination (yellow) and light-off (black) periods. Open circles in the first trial (time 0) represent
427 individual NTA for all larvae tested and were used to classify larvae as right (cyan, N=34) or left-biased
428 (black, N=34). Subsequent points represent mean and standard deviation for left/right groups on trials
429 2-4. * $p < 0.05$, t-test between groups.

430

431 D. As for (C) during constant illumination (right, N=29 ; left, N=35).

432

433 E. Match Index during baseline illumination (yellow, N=64) and after the loss of illumination (grey,
434 N=68). * $p < 0.05$, Mann-Whitney U test and (circled) 1-sample permutation test to 0.5.

435

436 F. Visually isolated chamber for high-speed recordings that enable kinematic analysis of routine turns
437 during circling behavior. Right: Time lapse montage over 10 s interval following loss of illumination.
438 Color indicates time.

439

440 G-H. Histogram for percentage of turns executed in a rightward direction (mean of four 10 s trials)
441 after loss of illumination (G, grey, N=89 larvae) or during constant illumination (H, yellow, N=39). Red
442 line represents expected distribution from Monte Carlo simulation assuming larvae have no directional
443 bias.

444

445 I. Rightward turn preference over 24 hour interval. Larvae were classified as right (cyan, N=14) or left
446 (black, N=12) biased by four repeated dark recordings at 6 dpf. In this analysis, larvae with less than
447 33% of turns in a rightward direction were classified as left-biased, and those with greater than 66%
448 classified as right-biased. At 7 dpf % rightward turn use was measured in L/R classified groups. Dotted
449 red lines at 50% indicates no bias.* $p < 0.05$ between groups.

450

451 J. Percentage of trials that had a net rightward bias for larvae tested at 7 or 10 dpf (N=30, 52
452 respectively), after being classified as left or right-biased at 6 dpf. * $p < 0.05$, Mann-Whitney U test.

453

454

455 **Figure 2: Motor asymmetry is correlated across multiple behavioral tasks**

456

457 A. Experimental paradigm: larvae were are classified as left or right biased at 6 dpf based on their
458 circling response after loss of illumination (over 4 trials). The following day, the same larvae were
459 tested in either a two-target phototaxis assay or acoustic startle assay.

460

461 B. Representative path trajectories of individual right (cyan) and left (grey) classified larvae
462 (arrowhead) presented with two symmetric light spots following the loss of illumination.

463

464 C. Percent of trials on which larvae turned toward the right spot, for larvae classified at 6 dpf as left-
465 biased (grey, N=12) and right-biased (cyan bar, N=13). Each larva performed 4 trials, with individual
466 trials excluded if the larvae was adjacent to the edge of the arena when the phototaxis spots were
467 presented. * $p < 0.05$, Mann-Whitney U test.

468

469 D. Percentage of startle responses made in a rightward direction for larvae classified as left (grey) and
470 right (cyan) biased. Larvae were tested either in the dark (grey background) or the light (yellow
471 background). As acoustic stimuli elicit either short or long latency C-starts (SLC, LLC) that are
472 mediated by different circuits, response types were analyzed separately. Red diamond indicates mean.
473 Dark LLC responses: left N=19, right N=20. Dark SLC responses: left N=12, right N=14 ; Light LLC
474 responses: left N=27, right N=23 ; Light SLC responses: left N=18, right N=28. * $p < 0.05$, Mann-
475 Whitney U test.

476

477 E. Match index for *atoh7* mutants and siblings during baseline illumination (sib, N=45 ; mutant, N=57 ;

478 yellow bars) and dark responses (sib, N=51 ; mutant, N=58 ; grey bars). * $p < 0.05$, one-sample
479 permutation test to 0.5.

480

481 F. Net turn angle for each of four trials after unilateral enucleation of the left (grey, N=4) or right (cyan,
482 N=6) eye. Dotted red line shows random output. * $p < 0.05$ between groups.

483

484

485 **Figure 3: Neurons in the posterior tuberculum maintain left/right identity**

486

487 A. Chemogenetic ablation screen: transgenic lines with restricted Gal4 patterns were crossed to a
488 UAS:epNTR-RFP reporter. Both epNTR-RFP+ and non-fluorescent siblings (as controls) were treated
489 with metronidazole before testing for motor asymmetry under light and dark conditions.

490

491 B. Match index for drug treated controls (*y279* N=54 ; *y375* N=43 ; *otpbA* N=35) and following genetic
492 ablation (*y279* N=58 ; *y375* N=37 ; *otpbA* N=46) during paired baseline (yellow) and dark (grey)
493 responses.

494

495 C-D. Whole brain dorsal Zebrafish Brain Browser (ZBB) projections for *y279* (D), *y375* (E). Color is
496 depth scale.

497

498 E. Computed intersect *y279* and *y375* expression patterns. Arrowhead indicates PT.

499

500 F. Dorsal confocal projection through the rostral PT in *y279-Gal4*, *UAS:Kaede* (green) crossed to
501 *vglut2a:dsRed* (red). Scale bar 20 μ m.

502

503 G. Match index in unablated controls (N=45) and after bilateral laser ablation of the PT (N=17) and Hc
504 (N=19) during baseline (yellow) and on dark trials (grey). * $p < 0.05$, Mann-Whitney U test.

505

506 H. Net turn angle (mean on trials 1-4) for left PT hemisphere (grey, N=27) and right PT hemisphere
507 (cyan, N=23) ablations. Hc unilateral ablations (right bars) (left hemisphere grey, N=24 ; right
508 hemisphere cyan, N=22). * $p < 0.05$, t-test.

509

510

511 **Figure 4: Neurons in the posterior tuberculum respond to changes in illumination.**

512

513 A. Raster plot of mean calcium responses (mean of 3 trials) from GCaMP6s expressing PT neurons.
514 Color scale denotes standardized change in fluorescence intensity ($\Delta F/F$). Illumination conditions as
515 indicated on x-axis (Light ON, orange ; dark, grey). Only a subset of no response neurons are included.

516

517 B. Location of light OFF responsive neurons in the rostral PT. Scale bar indicates fluorescence ($\Delta F/F$)
518 change over baseline.

519

520 C. Mean and standard error for response of light OFF (green, N=37) and light ON (purple, N=9)
521 responsive neurons.

522

523 D. Peak change in fluorescence for neurons that respond to light OFF in the left (PTL) and right (PTR)
524 PT for larvae classified as left (red) and right (blue) motor biased.

525

526 E. Location of light OFF responsive neurons within the PT for larvae behaviorally identified as left
527 (red) or right (blue) biased.

528

529

530 **Figure 5: A projection from posterior tuberculum to habenula drives motor asymmetry**

531

532 A-B. 3D rendering of *y279-Gal4, UAS:Kaede* expression in the habenula at 7 dpf in intact larvae (A)
533 and after bilateral ablation of *y279* expressing PT neurons (B). Arrow indicates epithalamic
534 commissure (Ec) present in intact larvae that is lost after bilateral PT ablation. Scale bar 20 μ m.

535

536 C-D. Confocal projections of *y279-Gal4;UAS:Kaede* expressing larvae following unilateral focal
537 photoconversion of Kaede in the left (C) or right (D) rostral PT. Photoconverted Kaede in the red
538 channel is saturated to facilitate visualization of PT projections. Scale bar 20 μ m.

539

540 E. Net turn angle (mean of trials 2-4) for intact control larvae (N=47) and after bilateral ablation of
541 *y279* expressing habenula neurons (N=22). Larvae were classified as left-biased (grey) or right-biased
542 (cyan) based on trial 1 (T1). * $p < 0.05$, t-test.

543

544 F. Total amount of turning for larvae in (E) during locomotion under baseline illumination (yellow) or
545 dark-induced circling behavior (grey).

546

547 G. Net turn angle (mean of trials 1-4) in non-ablated controls (white, N=47) and following unilateral
548 ablation of the left (grey, N=33), right (cyan, N=28) habenula nuclei or following laser section of the
549 epithalamic commissure (Ec) (N=24). * $p < 0.05$ t-test. circled * $p < 0.05$, one-sample t-test to 0.

550

551 H. Representative confocal scan showing *y279* labeled epithalamic commissure (Ec) (arrow) in a
552 control larva (top). Following ablation commissure is absent (bottom). Scale bar 20 μ m.

553

554

555

556 **Figure 6: Left/right identity is disrupted by mutations in genes that regulate Notch signaling**

557

558 A-B. Curly-up morphology in 2 dpf *y606* mutants (A) and sibling larvae (B).

559

560 C. Net Turn Angle for *moe^{y606}* heterozygous (left, +/-) and wildtype (right, +/+) sibling larvae over four
561 30 s light off trials. Open circles in the first trial (time 0) represent individual NTA for all larvae tested
562 and were used to classify larvae as right (cyan, Het N=34 ; WT N=20) or left-biased (black, Het N=27 ;
563 WT N=20). Subsequent points represent mean for left/right groups on trials 2-4.

564

565 D. Same analysis as in (C) for *moe^{b476}* allele showing right (cyan, Het N=11 ; WT N=15) or left-biased
566 (black, Het N=25 ; WT N=9) larvae. * $p < 0.05$ between groups in C-D.

567

568 E. Absolute Net Turn Angle for *moe^{y606}* heterozygous (N=47) and wildtype sibling (N=40) larvae
569 averaged over 4 trials for baseline illumination (yellow bar) and light off (grey bar) trials.

570

571 F. Habenula and heart placement in wildtype sibling and *moe^{y606}* heterozygous larvae. Left: Percentage
572 of larvae with larger habenula hemisphere on each side (N=28, 33 for wildtype, hets). Right:

573 Percentage of embryos with heart positioned on the left, right or midline (N=28,34 for wildtype, hets).

574

575 G-H. Match index (F) and total turning (G) for wildtype sibling and *mib^{ta52b}* heterozygous larvae

576 (N=31,27 respectively), during baseline (yellow) and dark (grey) responses. * $p < 0.05$, Mann-Whitney

577 U test.

578

579

580

581 **Figure 7: Motor asymmetry in zebrafish**

582

583 A. For a right biased larva, the dominant right PT-habenula pathway imposes right motor bias by

584 modulating symmetric visual drive and suppressing the left PT-habenula pathway.

585

586 B. Competition between PT-habenula units is eliminated after unilateral ablations, strengthening motor

587 bias.

588

589 C. Asymmetric visual input after unilateral enucleation overrides PT-habenula modulation of motor

590 output.

591

592 D. Symmetric visual stimuli, after bilateral ablation of PT-habenula units eliminates motor bias leading

593 to randomized turn direction.

594

595

596 **Methods**

597

598 *Zebrafish*

599

600 Adult zebrafish (*Danio rerio*) were maintained with a Tubingen long fin strain background. All animal
601 care and experimental procedures were approved by the NICHD animal care and use committee.
602 Experiments were performed on larvae in the first 10 days post fertilization (dpf), before sex
603 differentiation. Larval zebrafish were raised on 14/10 h light/dark cycle at 28 °C, at a maximum density
604 of 20 in 10 mL E3h medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, 0.33 mM MgSO₄, 1.5 mM
605 HEPES, pH 7.3). For dark-rearing at 3 hours post-fertilization embryos were sorted into 60 mm Petri
606 dishes at a stocking density of 15 larva per 10 mL medium and placed into a dark box till 5 dpf. At 5
607 dpf the media was replaced and larvae maintained under normal light cycles till testing at 6 dpf. For
608 controls, siblings were raised in parallel under the same conditions except with exposure to normal
609 14/10 light cycles.

610

611 Transgenic lines used were enhancer traps *y279-Gal4* and *y375-Gal4* (Marquart et al., 2015),
612 *Tg(UAS:epNTR-tagRFP)y268* (Tabor et al., 2014), *Tg(UAS:Kaede)s1999t* (Davison et al., 2007),
613 *Tg(atox7:GFP)rw021* (Masai et al., 2003), *Tg(otpb.A:Gal4-myl7:GFP)zc67* (Fujimoto et al., 2011) and
614 *TgBAC(vglut2a[slc17a6b]:loxP-mCherry-loxP-Gal4ff)nns21* (Satou et al., 2013). Mutant lines:
615 *otpa^{m866}* (Fernandes et al., 2012). *moe^{b476}* and *mib^{ta52b}* were kind gifts of Ajay Chitnis (Jensen et al.,
616 2001; Schier et al., 1996). Gal4 lines are available from the Zebrafish International Resource Center
617 (<http://zebrafish.org>).

618

619 *Behavior tracking and analysis*

620

621 Behavioral tests were performed on 6 and 7 dpf larvae except as noted. Infrared illumination
622 (CMVision Supplies, 850 nm) was used to monitor larvae with camera lenses fitted with IR-longpass
623 filters to exclude visible light. Visible illumination ($40 \mu\text{W}/\text{cm}^2$ measured using a radiometer
624 (International Light Technologies)), was provided by white LEDs positioned over the recording
625 chamber (Thorlabs). Testing areas were maintained at 26-28°C and larvae were adapted to the
626 recording environment for 30 min prior to starting experiments. We used DAQtimer event control
627 software to coordinate illumination conditions and recordings (Yokogawa et al., 2012). When
628 experiments required confocal brain imaging and behavioral analysis, larvae were raised in medium
629 containing 200 μM PTU starting at 1 dpf. PTU was removed at least 24 h prior to behavioral
630 recordings.

631

632 *Trajectory Analysis:* Path trajectory recordings were performed and analyzed as previously described
633 (Horstick et al., 2017): images were captured with a uEye IDS1545LE-M CMOS camera (1st Vision)
634 and larvae tracked in real-time using DAQtimer. Individual larvae were placed into a 120 x 120 mm
635 arena. Each individual was tracked for 30 s after loss of illumination over 4 successive trials separated
636 by 3 min baseline illumination (with the same protocol used for constant illumination trials except the
637 light remained on throughout). Where behavioral experiments were coupled with manipulations
638 (chemogenetic or laser ablation, genetic mutations) baseline controls were obtained by recording the
639 30s illuminated interval prior to the light off interval except as otherwise noted. We used three
640 measures to characterize directional bias in path trajectories: net turn angle (NTA), Absolute NTA and
641 Match Index. **NTA:** the sum total of all leftward (- degrees) and rightward (+ degrees) path direction
642 changes over a 30 s recording interval. Thus, individuals that changed direction equally to the left and
643 right would have an NTA of 0, indicating no net directional preference. **Absolute NTA:** as for the NTA
644 but taking the absolute value of each path direction change before summation. The absolute NTA

645 measures the total amount of turning behavior during a 30 s interval. **Match Index:** Used for
646 experiments where larvae were subjected to four trials, the Match Index is the fraction of trials 2-4
647 where the NTA had the same sign as on the first trial. We excluded trials where we obtained less than
648 10 s of data for an individual. A MI of 1.0 indicates that a larvae turned in the same direction on trials
649 2-4, as on trial 1, whereas a MI of 0.33 would occur if only one of trials 2-4 were performed in the
650 same direction as on trial 1.

651

652 *Kinematic analysis:* To measure routine turn initiation larvae were recorded using a high-speed camera
653 (DRS Lightning RDT/1; DEL Imaging) at 1000 Hz with off-line analysis of video images using Flote
654 (Harold A Burgess and Granato, 2007). Larvae were tested in a 58 x 58 mm arena with IR illumination
655 and recordings were triggered when larvae entered a centrally placed 15 x 15 mm ROI using DAQtimer
656 software and a uEye camera. To minimize environmental visual cues that might influence trajectories,
657 the arena was completely enclosed with a diffuser underneath the arena and a fitted lid above the arena
658 holding a IR long-pass filter. As for trajectory analysis, we performed 4 recordings (10 s duration) after
659 loss of illumination separated by 3 min of baseline illumination. Control experiments were the same,
660 but without the dark periods. We analyzed only larvae that executed at least 3 routine turns on each of
661 the 4 trials.

662

663 *Startle direction assay:* Acoustic startle tests were performed as previously described (Harold A.
664 Burgess and Granato, 2007). To determine if startle direction bias correlated with dark-circling
665 direction bias, larvae were classified as left/right at 6 dpf using trajectory analysis over 4 trials. At 7 dpf
666 larvae were placed into a 3 x 3 grid of 1 x 1 cm wells. For trials in the dark, larvae were tested with 16
667 repeats of an auditory/vibrational stimulus (~ 18 dB relative to 1 m/s^2), occurring 10 s after loss of
668 illumination. Each repeat was separated by 2 min constant illumination. Trials in the light were the

669 same, without the 10 s dark period. Only larvae performing at least 4 long or short latency startle
670 responses were analyzed. For analysis we compared the percentage of short and long latency C-starts
671 performed in a rightward direction for each pre-classified group of larvae.

672

673 *Two-target phototaxis assay:* For the two-target phototaxis assay, we first identified the left/right
674 identity of larvae at 6 dpf using trajectory analysis over 4 dark trials. Larvae with consistent directional
675 responses to all 4 trials were then retained for testing the next day. At 7 dpf, these larvae were
676 individually placed into a 58 x 58 mm arena and given 5 min to adapt to baseline illumination (20
677 $\mu\text{W}/\text{cm}^2$). After adaption a 15 x 15 mm ROI at the center of the arena was monitored by DAQtimer.
678 Once the larva entered the ROI, full field illumination was extinguished and real-time tracking of the
679 position and orientation of the larva was started. After 3 seconds, two equal intensity light spots (20
680 $\mu\text{W}/\text{cm}^2$, 6 mm radius) were positioned 10 mm from the head of the larva at 55° from its current
681 orientation. Light spots were projected onto the base of the arena (AAXA P2 Pico Projector). At the
682 same time, a high-speed camera was triggered to capture the response of the larva to the phototaxis
683 stimuli. Each larva was tested four times with 5 min constant illumination between trials. Trials were
684 excluded from analysis if a phototaxis target was obscured by the environment perimeter or mis-
685 positioned due to sudden movement of the larva, and only larvae with at least 2 trials were analyzed.

686

687 *Multi-day bias persistence tests:* (1) For testing individual motor asymmetry over 24 h we first
688 classified individuals as left or right biased at 6 dpf using kinematic routine turn analysis as described
689 above. Classification was determined using the directional preference on the first trial only. Larvae
690 were individually housed and returned to the incubator overnight. We then re-tested larvae at 7 dpf,
691 using the same assay. (2) To determine if motor asymmetry persisted over longer timelines, larvae were
692 tested at 6 dpf using path trajectory analysis over 4 dark trials. Individuals performing same-direction

693 circling for all 4 trials were then individually housed, and starting at 7 dpf fed daily (AP100
694 larvae dry food, Zeigler) with the media partially replaced 3 hours after each feeding. No food was
695 given on the day of testing. At 10 dpf larvae were retested using the same assay.

696

697 *Heritability analysis:* We selectively raised larvae classified as left or right-biased over two
698 generations. In each generation parents were incrossed and larvae were tested using kinematic analysis
699 (four repeated trials). To enrich for individuals with consistent motor asymmetry, we raised only larvae
700 that (1) had a percentage rightward turn use in the top or bottom quartile of responses and (2)
701 performed all four trials in the same direction. For each generation, left/right classified adults were
702 incrossed as groups and a minimum of 3 independent clutches combined for analysis.

703

704 *Calcium imaging*

705

706 We co-injected 50 ng of *UAS:nls-GCaMP6s* construct (derived from a previously published plasmid
707 *UAS:nls-GCaMP6s-2a-nls-dsRed* (Tabor et al., 2018)) with 80 ng toll RNA into one cell stage
708 *y279:Gal4* embryos, and raised in medium containing 200 μ M PTU. At 6 dpf larvae were mounted in
709 2% low melting temp agarose and imaged using a 20x immersion objective on an upright Leica TCS-
710 SP5II microscope. To avoid visual stimulation during GCaMP imaging we used a 2-photon Spectra-
711 Physics MaiTai DeepSee laser tuned to 950 nm and installed a 620 nm LED (All Electronics) on the
712 stage to provide visual stimulation at 80 μ W/cm². We performed three trials per larva separated by 3
713 minutes of constant red light illumination, with each trial consisting of 60 s light, 60 s dark, 60 s light.
714 We captured a single plane through the PT using 16x line averaging of GCaMP fluorescence at
715 approximately 0.96 Hz. After imaging, larvae were recovered from the agarose and placed in fresh E3h
716 media overnight. At 7 dpf we used trajectory analysis of dark-induced circling to determine left/right

717 identity. We analyzed fluorescence intensity changes by manually outlining an ROI around each neuron
718 in a maximum projection of the time series data, and calculated $(F_t - F_0)/F_b$ ($\Delta F/F$) where F_0 was the
719 mean fluorescence intensity during the first baseline period. Neurons with a $\Delta F/F$ greater than 3S over
720 three successive timepoints were classified as responders, where S was the standard deviation of $\Delta F/F$
721 during the first baseline period.

722

723 *Ablations*

724

725 *Genetic ablations:* Nitroreductase ablations were performed as previously described (Horstick et al.,
726 2016). To maximize ablation efficiency Gal4 lines were crossed to *UAS:epNTR-tagRFP* and RFP (+)
727 embryos were raised to maturity. Carriers were in-crossed and embryos were sorted using
728 epifluorescence at 2-3 dpf into NTR (+) red fluorescence or NTR (-) groups. Each group was treated
729 with 7.5 mM metronidazole in E3h media from 3 to 5 dpf. Metronidazole media was refreshed after 24
730 h. At 5 dpf larvae were returned to fresh E3h media and allowed to recover overnight. At 6 dpf
731 genetically ablated larvae were tested and a subset imaged on a confocal microscope to validate
732 ablation efficacy.

733

734 *Laser ablations:* We immobilized *y279:Gal4;UAS:Kaede* larvae with tricaine, and performed laser
735 ablation using a Spectra-Physics MaiTai DeepSee laser (tuned to 800 nm, beam power 2 W) on an
736 upright Leica TCS-SP5II microscope with a 20x immersion objective. For ablation we set the ROI to 1-
737 2 neurons per field of view. Efficient ablation was accompanied by the acute and transient development
738 of a bubble. Larvae were then removed from agar and returned to fresh E3h. Controls were
739 anesthetized and mounted for the same duration as ablated larvae. We performed path trajectory
740 analysis during four dark-induced circling trials per larva. For bilaterally ablated larvae, we classified

741 left/right identity using the first trial, then calculated the mean net turn angle for trials 2-4. For
742 unilaterally ablated larvae, where we aimed to see if ablation imposed a pattern of lateralized behavior,
743 we took the mean net turn angle for all four trials. After behavioral analysis we imaged the ablated
744 region to assess ablation efficiency and analyzed only larvae with near complete absence of targeted
745 neurons (~50% of ablated individuals).

746

747 *Genetic mapping*

748

749 To map the *moe*^{v606} mutation, we formed mutant and sibling pools, each consisting of 75 embryos that
750 were derived from a single clutch. We Trizol-extracted total mRNA, purified on a Qiagen RNA mini-
751 cleanup column and sequenced samples using single end 100 bp reads using an Illumina HiSeq (100 M
752 reads per sample). We then used RNAmapper in Galaxy (galaxyproject.org) to perform bulk segregant
753 analysis, identifying a critical region on chromosome 9 and a 7.7-fold reduction in expression of
754 *epb41l5* within that interval. We noted that no reads were present in exon 1 or 2 of *epb41l5*; these
755 exons amplified correctly from sibling genomic DNA but not from mutant DNA (exon 1 primers: 5'-
756 TCCACTTTTGGGGATTACG, 5'-ATTCAATGGCGGAGCAATAC; exon 2 primers: 5'-
757 GGCCATTGACAGTAGTGTGG, 5'-TGACAAGACGCTGAACAAGC) suggesting the presence of a
758 deletion. We then used PCR to assess the presence of genomic DNA in mutants in intervals across a 20
759 kbp candidate region from between exon 3 of *epb41l5* to the 3'UTR of the neighboring gene *ptpn4a*.
760 This narrowed the candidate region to 7 kb allowing us to design primers to amplify and sequence
761 across the deletion, revealing loss of genomic DNA in mutants between bases chr9:213682 to 218117
762 (GRCz11, chr9_KZ114909v1_alt). In complementation testing with *moe*^{b476}, we recovered the curly-up
763 phenotype in 50/192 (26%) embryos, confirming that this was the causative mutation. Sequencing of
764 the PCR product that spanned the deletion enabled us to design a genotyping protocol to distinguish

765 wildtype and heterozygous larvae using 3 primers (5'-CTACCTGAACAACTCAATCCAGTC, 5'-
766 AACCATATAAAATGAGCGTCTCT and 5'-TCATTTTGAAATGCCTGCAA). These primers
767 amplify a single 296 bp band from the wildtype locus, and a 333 bp band from embryos with the
768 deletion.

769

770 Previous work established the presence of a large deletion in *moe*^{b476} but did not define its precise
771 boundaries, precluding genotyping of heterozygotes (Jensen et al., 2001). To map the *moe*^{b476} deletion
772 we used whole-genome shotgun sequencing with 100 bp paired-end reads. After mapping reads to
773 GRCz11 with bowtie2, we focused on a large read-depleted region on chr9 covering *epb4115*.
774 IGVviewer revealed 5 reads where the paired end reads spanned the read-depleted region. PCR across
775 the putative deletion using primers 5-AAACTGCATAAGTGCCTCACC and 5-
776 GAGACATCGATTCCGCTTTG amplified a ~600 bp band. Sequencing of this band produced the
777 expected genomic sequence at each end and demonstrated that the deletion in *moe*^{b476} spans
778 chr9:28835868 to 29370835, completely deleting *epb4115*, *ptpn4a*, *tmem177* and *pth2r*, and exons from
779 *zgc:91818* and *hs6st3b*. We then used these PCR primers to genotype *moe*^{b476} outcrosses because only
780 heterozygous larvae yielded the 600 bp band.

781

782 *Genotyping*: After behavioral analysis we genotyped *moe*^{y606} and *moe*^{b476} larvae as described above.
783 *mib*^{ta52b} larvae were genotyped by PCR amplification across the mutation (primers: 5'
784 GGTGTGTCTGGATCGTCTGAAGAAC ; 5' GATGGATGTGGTAACTGATGACTC, product size
785 194 bp). To discriminate wildtype from heterozygous larvae PCR products were digested with the
786 restriction enzyme NlaIII (New England Biolabs) which digests wildtype but not mutant sequence (WT
787 155 bp ; Het 155, 194 bp ; Mutant 194 bp). For experiments with *otpa* mutants, we compared the
788 'cousin' offspring of wildtype (+/+) and mutant (-/-) sibling parents.

789

790 *Photoconversion*

791

792 We characterized projections from *y279-Gal4, UAS:Kaede* expressing rostral PT neurons by focal
793 photoconversion of Kaede from green to red fluorescence in one PT hemisphere. At 2 dpf PT neurons
794 constitute a salient bilateral cluster in the ventral diencephalon. To photoconvert Kaede, larvae were
795 mounted in 2% low melting temperature agarose on an upright Leica TCS SP5 II confocal microscope.
796 We used a 25x immersion objective and set the field of view to comprise 1-2 Kaede expressing PT
797 neurons, imaged with a 488 nm laser. For photoconversion, we performed a single scan of the field of
798 view with 8 frame averaging using a 405 nm laser at 5% power. Conversion of Kaede to the red
799 fluorescent state was confirmed by imaging with a 568 nm laser. After photoconversion embryos were
800 removed from agarose and maintained in E3h until 5 or 6 dpf for imaging the PT and projections to the
801 habenula.

802

803 *Neuron Counts*

804

805 We counted *y279:Gal4, UAS:Kaede* expressing neurons in the rostral PT in larvae imaged at 6 dpf on
806 an upright Leica TCS SP5 II confocal using the 488 nm laser, a 25x immersion objective and 3x zoom.
807 Images were imported into Imaris for counting. After imaging, larvae were extracted from the agar,
808 individually maintained in 6 well plates for 24 h, then tested at 7 dpf to determine left/right identity
809 using path trajectory analysis of dark-induced circling behavior.

810

811 *Statistical analysis*

812

813 Analysis was performed in IDL (Harris), RStudio (Mathworks) and Gnumeric
814 (<http://projects.gnome.org/gnumeric/>). Data in figures and text are means \pm SEM except where
815 otherwise noted. All t-tests were 2-sided. N reported in figure legends. Box plots show median and
816 quartiles with whiskers indicating 10-90%. Normality was determined by the Shapiro-Francia test.
817 Analysis of non-normal data sets was performed using the Mann-Whitney U-test or for one-sample
818 comparisons to a given number, through Monte Carlo testing (see below).

819

820 To analyze whether distributions of turn direction deviated from that which would be expected if larvae
821 did not show direction bias, we used Monte Carlo simulations rather than comparison to a binomial
822 distribution because the direction of sequential turns are not independent (Chen and Engert, 2014;
823 Horstick et al., 2017). In each simulation, we used the same number of larvae as in the experimental
824 data (i.e., 89 and 39 respectively for light-off and baseline conditions), and the same number of routine-
825 turns produced on each of the four trials for each larva. As sequential routine-turns show a statistically
826 significant likelihood to be executed in the same direction we simulated routine-turn direction across
827 events on a given trial by randomly selecting a turn-direction for the first event, then then using the
828 previously measured 'lock index' to weight a random decision on whether each subsequent turn would
829 be in the same or reverse direction. We used lock indices of 62.8 and 13.2 for light-off and baseline
830 simulations respectively (corresponding to 81.4% and 56.6% chance of executing sequential same-
831 direction routine-turns)(Horstick et al., 2017). The expected histograms of %rightward turns in Figure 1
832 are derived from 10,000 simulations. In the simulation data, 10% of larvae showed less than 23% right-
833 ward turns or greater than 76% right-ward turns during light-off conditions (i.e., a modal value of 9 of
834 the 89 larvae), whereas in our data, 37 of the 89 larvae exceeded these thresholds. This proportion was
835 greater than all 10,000 simulations, hence we assign a p-value of < 0.0001 . In comparison, under
836 baseline conditions, simulations 10% of larvae showed less than 31% rightward turns or greater than

837 68% right-ward turns during light-off conditions, and in our data 7 of the 39 larvae exceeded these
838 thresholds. However, this proportion was exceeded by 1195 simulations, hence we assign a p-value
839 0.119. We used a similar procedure to compare the means of larval Match Indices to 0.5, by ranking the
840 difference between the mean of actual match indices in experimental data, with means derived from
841 100,000 simulations using the same number of larvae and trials per larvae under the null hypothesis
842 assumption that larvae had a 50% (i.e. random) probability of matching the trial 1 direction on each of
843 trials 2-4.

844

845 *Data and Software Availability*

846

847 Further information and requests for image datasets and analysis software should be directed to Harold
848 Burgess (burgessha@mail.nih.gov) or Eric Horstick (eric.horstick@nih.gov)

849

850

851 **References**

852

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doi:10.1016/j.neuron.2017.01.011

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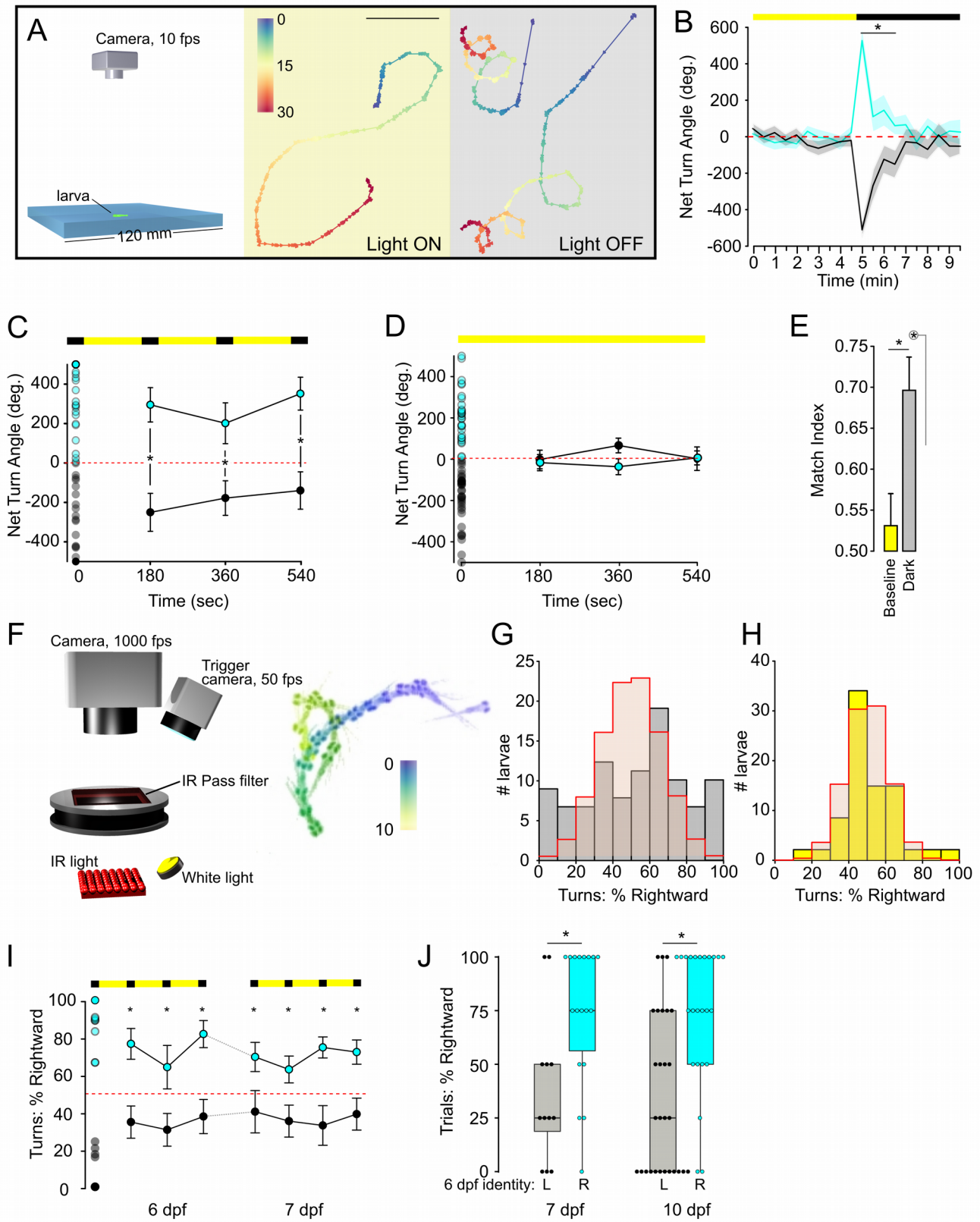


Figure 1

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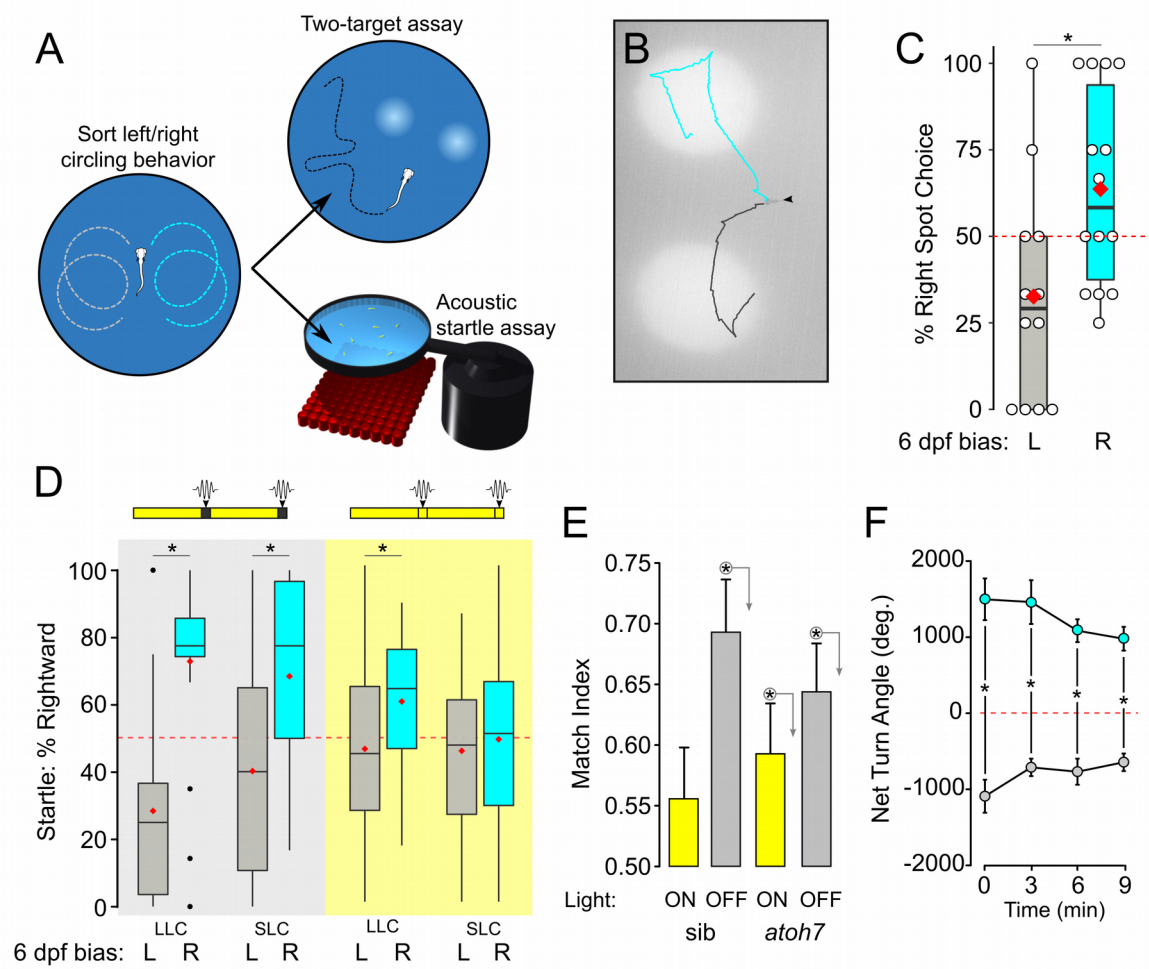


Figure 2

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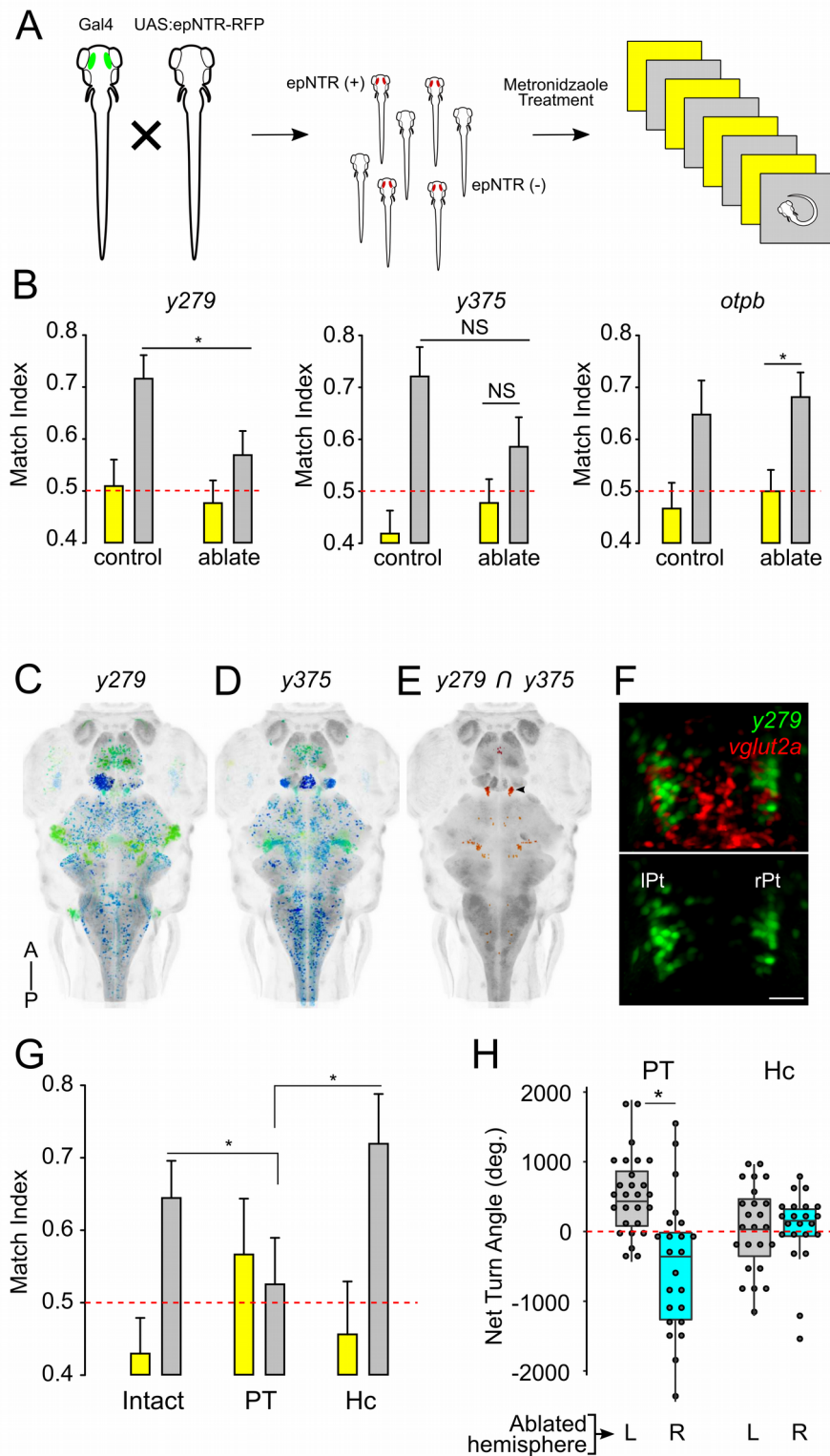


Figure 3

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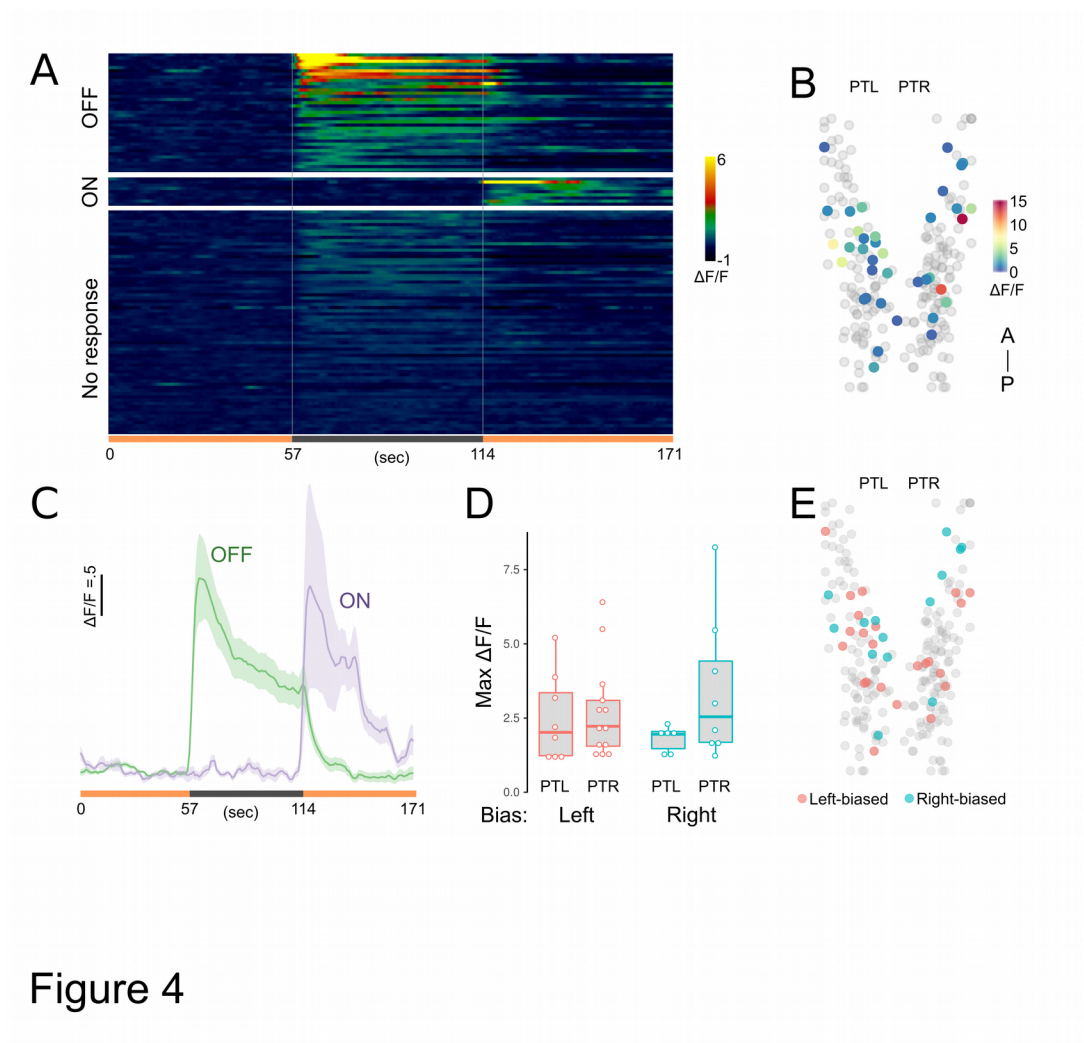


Figure 4

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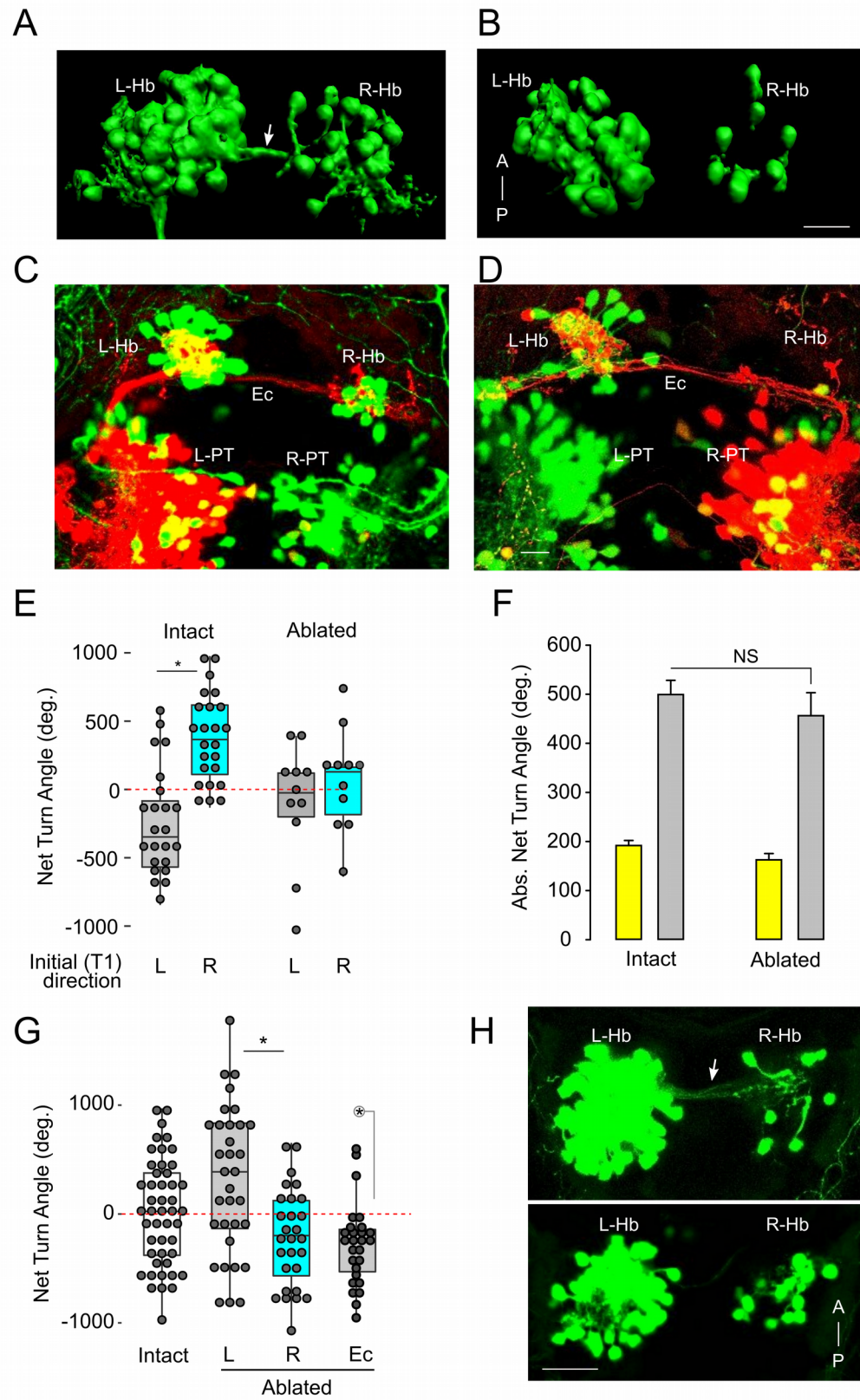


Figure 5

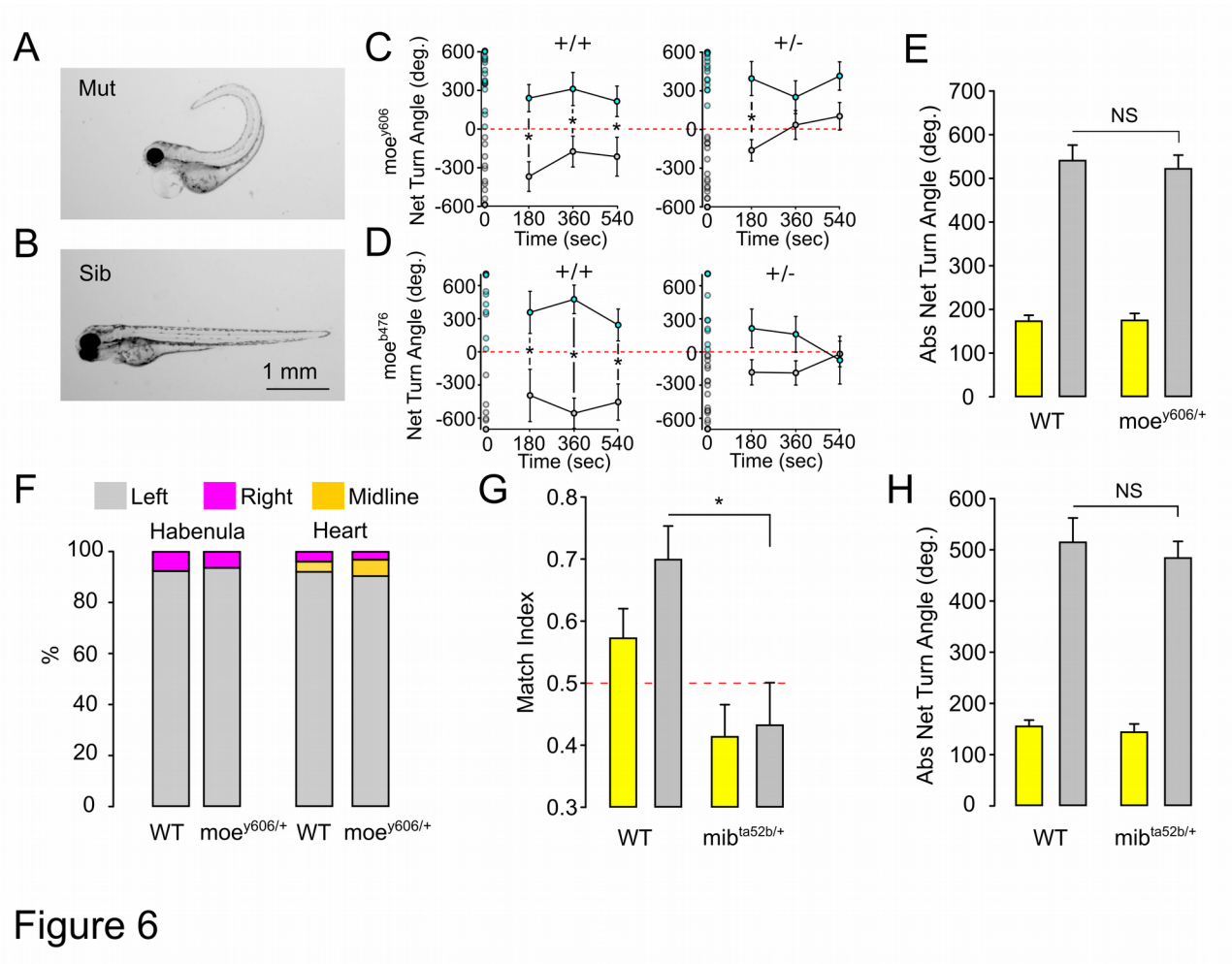


Figure 6

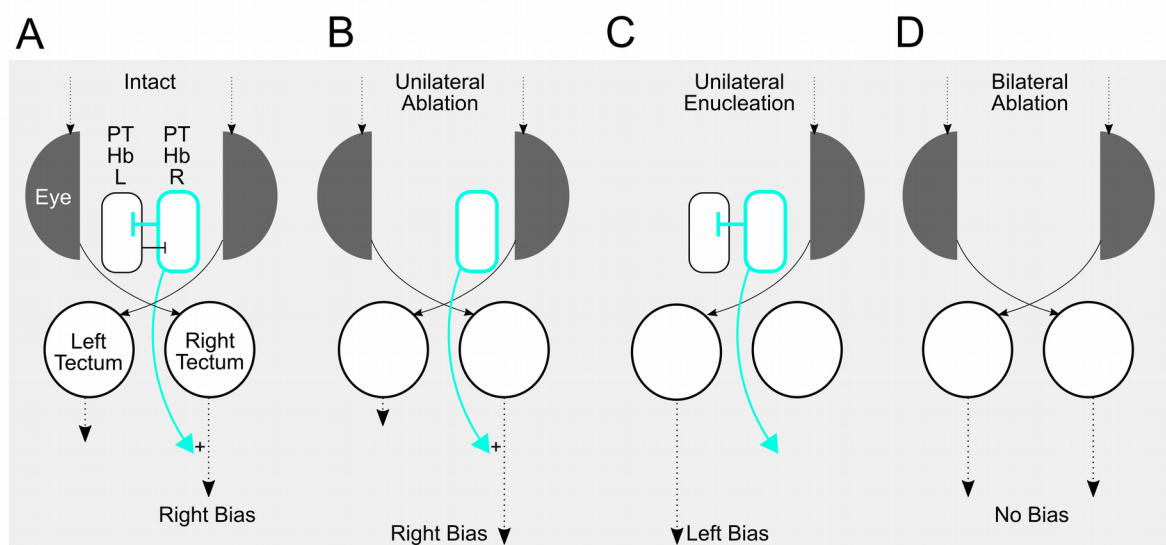


Figure 7