

GABA_A α Subunit Control of Hyperactive Behavior in Developing Zebrafish

Wayne Barnaby^{*‡}, Hanna E. Dorman Barclay[‡], Akanksha Nagarkar[‡], Matthew Perkins[§],

Gregory Teicher^{†‡}, Josef G. Trapani^{*§}, and Gerald B. Downes^{*†‡**}

*Neuroscience and Behavior Graduate Program, †Molecular and Cellular Biology Graduate Program, ‡Biology Department, University of Massachusetts, Amherst, MA 01003

§Biology Department and Neuroscience Program, Amherst College, Amherst, MA 01002

** Author for correspondence:

Gerald B. Downes

611 North Pleasant St., Morrill Science Center, Building 4 North

Amherst MA 01002

Email: gbdownes@umass.edu

Telephone: 413-545-1266

1 **ABSTRACT**

2 GABA_A receptors mediate rapid responses to the neurotransmitter GABA and are
3 robust regulators of the brain and spinal cord neural networks that control locomotor
4 behaviors, such as walking and swimming. In developing zebrafish, gross pharmacological
5 blockade of these receptors causes hyperactive swimming, which has been embraced as
6 an epilepsy model. Although GABA_A receptors are important to control locomotor behavior,
7 the large number of subunits and homeostatic compensatory mechanisms have
8 challenged efforts to determine subunit-selective roles. To address this issue, we mutated
9 each of the eight zebrafish GABA_A α subunit genes individually and in pairs using a
10 CRISPR-Cas9 somatic inactivation approach, then we examined the swimming behavior of
11 the mutants at two developmental stages. We found that disrupting the expression of
12 specific pairs of subunits resulted in different abnormalities in swimming behavior at the
13 first development stage. Mutation of $\alpha 4$ and $\alpha 5$ selectively resulted in longer duration
14 swimming episodes, mutations in $\alpha 3$ and $\alpha 4$ selectively caused excess, large-amplitude
15 body flexions (C-bends), and mutation of $\alpha 3$ and $\alpha 5$ resulted in increases in both of these
16 measures of hyperactivity. At the later stage of development, hyperactive phenotypes were
17 nearly absent, suggesting that homeostatic compensation was able to overcome the
18 disruption of even multiple subunits. Taken together, our results identify subunit-selective
19 roles for GABA_A $\alpha 3$, $\alpha 4$, and $\alpha 5$ in regulating locomotion. Given that these subunits exhibit
20 spatially restricted expression patterns, these results provide a foundation to identify
21 neurons and GABAergic networks that control discrete aspects of locomotor behavior.

22 INTRODUCTION

23 Neural networks in the vertebrate hindbrain and spinal cord rely upon a balance of
24 excitatory and inhibitory neurotransmitter systems to orchestrate locomotion. Classically
25 inhibitory, the neurotransmitter Gamma-AminoButyric Acid (GABA) is recognized as a key
26 regulator of these circuits. GABA exerts its effects through two different classes of
27 receptors, GABA_A and GABA_B. GABA_B receptors are G-protein coupled receptors, while
28 GABA_A receptors are ligand-gated ion channels that generate rapid responses to GABA. In
29 mammalian systems, GABA_A receptors exhibit remarkable diversity, with each receptor
30 thought to form a heteropentamer containing various combinations of 19 different subunits:
31 α 1-6, β 1-3, γ 1-3, δ , ϵ , π , θ and ρ 1-3 (Sieghart and Sperk 2002; Simon et al. 2004; Chua
32 and Chebib 2017). Each subunit is encoded by a discrete gene that is spatially and
33 developmentally regulated to generate distinct, but sometimes overlapping, expression
34 patterns (Laurie, Seeburg, et al. 1992; Laurie, Wisden, et al. 1992; Wisden et al. 1992).
35 Several receptor subunits confer distinct biophysical and pharmacological properties,
36 localize to synaptic or extrasynaptic sites, interact with specific cytoplasmic proteins, and
37 contribute to different neuronal networks (Farrant and Nusser 2005; Jacob et al. 2008;
38 Fritschy and Panzanelli 2014). While this incredible receptor heterogeneity is not fully
39 understood, it could provide the opportunity to better understand, or even manipulate,
40 distinct neuronal networks.

41 Several studies have used pharmacological blockade of GABA_A receptors to reveal
42 the central roles that these receptors play in regulating the initiation, rhythmicity, frequency
43 and duration of locomotor network output from the spinal cord. These studies have been
44 performed in a variety of vertebrate systems. For example, in neonatal mice, application of
45 GABA_A receptor antagonists to the spinal cord has been shown to cause inappropriate,
46 bilateral discharges and regulate the onset and duration of rhythmic activity (Hinckley et al.

47 2005). In lamprey, application of GABA_A receptor antagonists to spinal cord networks
48 increased the frequency of locomotor bursts and disrupted lengthwise coordination
49 (Schmitt et al. 2004). In *Xenopus* tadpoles, GABA_A receptor mediated inhibition has been
50 found to play both a tonic role in regulating the responsiveness to touch stimuli and a
51 phasic role that stops swimming (Perrins et al. 2002; T. D. Lambert et al. 2004; Thomas D.
52 Lambert et al. 2004). Although these and several other studies illustrate the importance of
53 GABA_A receptors in controlling locomotor networks, antagonists that block the majority of
54 receptor isoforms were used, therefore these studies are less informative about the roles
55 of specific subunits.

56 Genetic inactivation of GABA_A receptor subunits in mice has had only limited
57 success in identifying subunits required to mediate locomotor behavior. Although several
58 of the 19 subunits exhibit robust expression in portions of the brain and spinal cord that
59 mediate locomotion, few gene deletions have been found to cause abnormal locomotor
60 behavior (Rudolph and Möhler 2004; Vicini and Ortinski 2004; Smith and Rudolph 2012).
61 Ablation of some GABA_A receptor genes has been shown to cause changes in the
62 expression of other subunits, which suggests that homeostatic adaptations may explain at
63 least some of the deletions that show no or only subtle locomotor phenotypes (Peng et al.
64 2002; Kralic et al. 2006; Zeller et al. 2008; Panzanelli et al. 2011; Zhou et al. 2013; Fritschy
65 and Panzanelli 2014).

66 Early larval-stage zebrafish, ~2-10 days post-fertilization, are a leading model for
67 locomotor neural network analysis, and GABA_A receptors regulate locomotion in this
68 system. Bath application of GABA_A receptor antagonists, such as pentylenetetrazole
69 (PTZ), induces hyperactive swimming, meaning a dramatic increase which is also
70 recognized as an epileptic seizure model (Baraban et al. 2005; Baxendale et al. 2012; Cho
71 et al. 2020). Zebrafish harbor an array of GABA_A subunits similar to mammals, with 23

72 identified subunits (Cocco et al. 2017; Monesson-Olson et al. 2018), however few mutants
73 have been identified as important for locomotion. At 5 days post-fertilization (dpf), loss-of-
74 function mutations in the broadly expressed $\gamma 2$ subunit were reported to elicit hyperactive
75 swimming that is behaviorally similar to PTZ exposure (Liao et al. 2019). At 7 dpf, loss-of-
76 function mutations in $\beta 3$, which is also widely expressed, caused subtle increases in
77 spontaneous swimming (Yang et al. 2019). Although loss-of-function mutations in $\alpha 1$ were
78 found to cause hyperactive swimming at 5 weeks post-fertilization, this behavior was not
79 observed during larval stages (Samarut et al. 2018). Since GABA_A α subunits help form
80 the GABA binding site, they are thought to be obligatory receptor components (Phulera et
81 al. 2018; Zhu et al. 2018; Lavery et al. 2019; Masiulis et al. 2019). Thus, it is surprising
82 that α subunits have not been identified as important for regulation of embryonic or larval
83 locomotor behavior. It is possible that, as in mammals, homeostatic compensation is able
84 to conceal subtype-selective roles. Disrupting multiple subunits simultaneously could
85 evade these mechanisms and reveal α subunits that are required to regulate swimming
86 behavior.

87 Here, we used an F0 CRISPR-Cas9, somatic mutation approach to screen the
88 locomotor phenotypes of mutants in each of the eight α subunits both individually and in
89 combination at two different developmental stages: 48 and 96 hours post fertilization (hpf).
90 We found that disrupting select pairs of α subunits causes different types of hyperactive
91 behavior at 48 hpf, which then decreased or was absent by 96 hpf. The absence of
92 hyperactive behavior by 96 hpf was confirmed in F2 germline mutants and,
93 correspondingly, electrophysiological recordings revealed brain activity indistinguishable
94 from wild-type controls. These findings illustrate subunit selective-roles of GABA_A receptor
95 α subunits in regulating locomotor behavior which, given their restricted expression

96 patterns in larvae, serve as entry points to reveal cellular and circuit mechanisms that
97 enable GABA to control locomotion.

98

99 **MATERIALS AND METHODS**

100 ***Zebrafish maintenance and breeding***

101 Adult zebrafish were maintained according to standard procedures, with the
102 zebrafish facility on a 14-hour light/10-hour dark cycle. Embryos and larvae were kept at
103 28.5°C in E3 media and staged according to morphological criteria (Kimmel et al. 1995;
104 Parichy et al. 2009). All genetic manipulations and behavioral experiments were performed
105 using a Tübingen (Tü) or Tüpfel longfin (TL) genetic background. All animal procedures for
106 this study were approved by the University of Massachusetts Amherst or the Amherst
107 College Institutional Animal Care and Use Committee (IACUC). Amherst College
108 assurance number 3925-1 with the Office of Laboratory Animal Welfare.

109

110 ***Guide and Cas9 RNA preparation and microinjection***

111 Single guide RNAs were designed using the online tool, ChopChop v.2 (Labun et
112 al. 2019) (Supplemental Table). ChopChop selects target sites for CRISPR-Cas9 using the
113 NGG motif and ranks them based on efficiency (Montague et al. 2014). For each gene,
114 two neighboring targets were selected with the high efficiency within exons that would
115 disrupt all known splice-variants as assessed in ENSEMBL. CRISPR-Somatic Tissue
116 Activity Tests were used to assess mutational efficiency for both targets of a selected
117 gene, and the target that yielded better results was used. In some cases, neither of the
118 initial targets was effectively mutated so two additional targets were selected.

119 Template DNA for gRNA synthesis was generated using the PCR based method
120 described in (Shah et al. 2016). For *in vitro* transcription, we first generated an HPLC-

121 purified scaffolding primer (5'-
122 GATCCGCACCGACTCGGTCCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAA
123 CTTGCTATTTCTAGCTCTAAAAC-3'), which was common to all gRNA templateless PCR
124 reactions. Next, for each target, we synthesized a unique primer sequence that contained
125 a 5' T7 binding site, the 20 nucleotide specific target (Supplemental Table), and a 3' 20
126 nucleotide site of scaffolding homology (5'- AATTAATACGACTCACTATA-[20 nucleotide
127 Target Sequence]-GTTTTAGAGCTAGAAATAGC-3'). The PCR reaction contained: 0.4
128 units of Phusion High-Fidelity DNA Polymerase (New England Biolabs, M0530S), 13.4 μ L
129 ddH₂O, 1 μ L target specific primer (10 μ M, IDT), 1 μ L scaffolding primer (10 μ M, IDT), 4 μ L
130 5X Phusion HF, and 0.4 μ L dNTPs (10mM). Reactions were run in a thermocycler (BioRad)
131 using the following conditions: 98°C for 30 seconds then 40 cycles of 98°C for 10 seconds,
132 60°C for 10 seconds, 72°C for 15 seconds, which was followed by 72°C for 10 min. The
133 PCR reaction was purified using the QIAGEN MinElute kit and used as a template for *in*
134 *vitro* transcription reactions. Using 0.5-1 ug of purified PCR product, gRNAs were
135 generated using the MEGAscript T7 Transcription kit (Thermofisher), purified via lithium
136 chloride precipitation, and verified using a TAE denaturing gel. A nanodrop spectrometer
137 was used to determine gRNA concentrations, which were then diluted to 200ng/ μ L in
138 RNase free water.

139 Cas9 mRNA was synthesized similar to Shah *et al.*, 2016, but with the following
140 changes. Purification of the linearized plasmid was performed using the E.Z.N.A. Cycle
141 Pure Kit (Omega Bio-Tek), while purification of the Cas9 mRNA was by lithium chloride
142 precipitation. Cas9 mRNA was diluted to 1200ng/ μ L in RNase free water.

143 Microinjections were performed at the 1-4 cell stage into the yolk of embryonic
144 zebrafish. The injection cocktail contained: 2 μ L gRNA at 200ng/ μ L, 2 μ L Cas9 mRNA at
145 1,200ng/ μ L, 1 μ L stop cassette (Gagnon et al. 2014) at 10 μ M, 2 μ L 0.05% Phenol Red, 4 μ L
146 RNase free water. Approximately 500 pL was injected per embryo. Mock injections,

147 containing all cocktail ingredients except gRNAs, were not observed to have a significant
148 effect compared to uninjected siblings, so uninjected sibling animals were used as the
149 controls for most experiments.

150

151 ***Tyrosinase Pigmentation Analysis***

152 24 hours after injection, injected embryos were dechorionated using forceps and
153 screened for morphological abnormalities using a dissecting scope (Zeiss).
154 Morphologically abnormal fish were excluded from further analysis. At 48 hours and 96
155 hours post-fertilization (hpf), larvae were anesthetized using 0.04% MS-222 (Tricaine) and
156 pictures were taken using a Stemi 305 (Zeiss). All images were captured with the same
157 dimensions (1280 x 960), resolution (72 x 72), lighting conditions and exposure times.
158 Images were analyzed using a custom Python script (source code available upon request).
159 Briefly, a threshold value was empirically selected based on its ability to most optimally
160 segment pigmentation from other parts of the fish and the background across all images.
161 The number of pixels greater than this threshold were summed and reported for each
162 image. The same threshold value was applied to all images.

163

164 ***Behavioral Analysis***

165 Behavioral analysis was performed in a double-blind fashion. At 24hpf, injected
166 embryos had their chorions removed and were subject to morphological screening. At
167 48hpf, we examined escape responses to touch responses similar to as described in
168 (Friedrich et al. 2012; McKeown et al. 2012). Briefly, light touch was applied to the head
169 using a 3.22/0.16g of force von Frey filament. Swimming responses were captured using a
170 high-speed digital camera (XStream 1024, IDT Vision) mounted to a 35 mm lens (Nikon) at
171 a frame rate of 250 Hz. The head-to-tail angle for each frame of the response was

172 measured using custom software written in MATLAB (source code available upon
173 request). C-bends were defined as any body flexion over 110 degrees, while escape
174 response duration was defined as beginning the frame before initial movement was
175 observed and ending at the last frame of detected movement (McKeown et al. 2012).

176

177 ***CRISPR-Somatic Tissue Activity Tests (STAT)***

178 CRISPR-STAT analysis was performed similar to as described by Carrington et al.
179 2015 (Carrington et al. 2015). Primer pairs were designed to flank the targeting gRNA
180 sites as determined in ChopChop span target sequences (Supplemental Table). Forward
181 primers were tagged with a 6-FAM dye (Integrated DNA Technologies) which allowed for
182 visualization in fragment analysis. Reverse primers were tagged with a PIG-tail adapter
183 (5'-GTGTCTT-3') to reduce stutter (Blake et al., 2015). DNA was extracted from 6 embryos
184 using the Extract-N-Amp Tissue PCR Kit (Millipore-Sigma) and amplified using Amplitaq
185 Gold Taq Polymerase (ThermoFisher). PCR conditions followed those suggested by the
186 taq polymerase manufacturer. DNA was diluted 1:20 in ddH₂O and ran on an AB3730xl
187 DNA Analyzer (Genewiz, South Plainfield, NJ). Results were analyzed using Geneious
188 Software (Biomatters, Inc). Peaks were defined as signals exceeding 1,000 Relative
189 Fluorescent Units (RFU). A gene target was considered successfully mutated if the peak
190 $(\sum \frac{\# \text{ of } F0 \text{ peaks}}{\# \text{ of mock injected peaks}})$ ratio was 2 or above.

191

192 ***Gabra3 MUTANT LINES***

193 To generate *gabra3* mutant lines, CRISPR-Cas9 injected animals were raised to
194 adulthood. CRISPR-STAT analysis was used to identify mosaic animals and these animals

195 were crossed to a wild-type strain (TLF). Two *gabra3* mutant alleles were identified in the
196 F1 animals, a 7-base pair deletion (bp), *uma500*, and an 18-bp insertion, *uma501*.

197

198 ***Local Field Potential (LFP) Recording***

199 Local field potential (LFP) recordings were obtained from zebrafish larvae at 96 hpf
200 using a technique similar to that in Liu and Baraban, 2019 . Prior to each recording, larvae
201 were paralyzed by immersion for 30-60 minutes in α -Bungarotoxin (125 μ M in
202 dH20Invitrogen, Waltham MA) and were subsequently embedded dorsal side up in 2%
203 low-melting-point agarose in extracellular solution (130 mM NaCl, 10 mM HEPES, 2 mM
204 KCl, 2 mM CaCl₂, 1 mM MgCl₂, pH 7.8). In some experiments, the convulsant agent PTZ
205 (10 mM) was applied to induce ictal-like brain activity. For each recording, a glass
206 microelectrode (6-12 M Ω) was filled with extracellular solution and inserted under visual
207 guidance into the optic tectum. Local field potentials were recorded at 100X gain in current
208 clamp mode using a Sutter Double IPA amplifier (Sutter Instruments, Novato CA). Voltage
209 signals were low-pass filtered at 500 Hz-1 kHz and digitized at 5-10 kHz using SutterPatch
210 Software (Sutter Instruments, Novato CA). Following acquisition, voltage traces were
211 analyzed for ictal-like activity using NeuroMatic software (Rothman and Silver 2018) and
212 the number of ictal-like events in the first 30 minutes of recording was assessed. For PTZ
213 treated fish, one hour was allocated for wash-on and only the first 30 minutes of recording
214 after this wash-on period was analyzed.

215

216 ***Statistics and Analysis***

217 To determine significant differences the following statistical tests and software were
218 used. Welch's t-test and Ordinary 1-way ANOVA were used where appropriate. When t-
219 tests were applied, F tests were used to compare variance. When ANOVAs were applied,

220 multiple comparison tests were used where test groups were compared against wildtype
221 controls. A Dunnett test was used to correct against familywise error. Figures, plots and
222 statistical testing were performed using Prism (GraphPad Software).

223

224 **RESULTS**

225 ***High-efficiency F0 somatic gene targeting using CRISPR-Cas9***

226 To identify GABA_A α subunits that control larval escape behavior we sought an
227 approach to rapidly screen through different loss-of-function mutant combinations. Injecting
228 zebrafish embryos with a cocktail containing Cas9-encoding and guide RNAs (gRNAs) has
229 been shown to mutate target genes with enough efficiency to cause biallelic disruption
230 (Shah et al. 2015; Varshney et al. 2015; Wu et al. 2018). Phenotypes can often be
231 observed in these F0 somatic mutants, which can save a great deal of time compared to
232 analysis of F2 germline homozygous mutants. A disadvantage of this approach is that F0
233 somatic mutants are genetic mosaics, with different cells harboring different indels in the
234 target gene, which may yield weaker phenotypes compared to those found in germline
235 mutants. To confirm the efficiency of disrupting gene function using this approach, we
236 targeted the tyrosinase (*tyr*) gene, similar to previous studies (Wu et al. 2018). Tyrosinase
237 is essential for producing melanin and its disruption provides an easily observable loss of
238 pigmentation. Phenylthiourea (PTU) is routinely used in zebrafish research to suppress
239 melanin synthesis and it provides an effective method to evaluate CRISPR knockdown of
240 *tyr*. We observed that injection of Cas9 and gRNAs targeting the *tyr* gene led to a
241 substantial and persistent reduction in pigmentation in 48 and 96 hpf larvae, although not
242 all melanin synthesis was eliminated compared to PTU treated controls (Supplementary
243 Figure 1A, B). Correspondingly, PCR analysis of the somatic mutants through CRISPR-
244 Somatic Tissue Activity Tests (STAT), indicated that a variety of mutations were induced in

245 the *tyr* gene (Supplementary Figure 1C). Taken together, these results confirm that F0
246 somatic mutants provide a rapid and effective means to screen gene function.

247

248 ***GABA_A Receptors Control Early Larval Swimming Behavior***

249 Although PTZ is known to elicit hyperactive swimming responses in fish older than 5
250 dpf, its effect on earlier larval stages is less clear. We focused behavioral analysis on 48
251 and 96 hpf larvae since these time points present the opportunity to analyze a more
252 nascent zebrafish nervous system and the early stages of GABA_A receptor control of
253 locomotion. At 48 hpf, larvae are newly hatched and demonstrate burst swimming
254 behavior, while 96 hpf larvae exhibit more mature swimming patterns (Brustein et al. 2003;
255 McKeown et al. 2009; Roussel et al. 2020). Because acoustic and light responses are
256 either absent or less robust at these early developmental stages, light touch was used to
257 induce escape response swimming behavior. At both 48 and 96 hpf, larvae respond to light
258 touch to the head with a well-characterized C-start, which consists of an initial C-shaped
259 body bend to reorient the animal away from the touch stimulus, followed by lower-
260 amplitude body undulations that propel it several body lengths away (Eaton et al. 1977;
261 Granato et al. 1996; O'Malley et al. 1996; Eaton et al. 2001; Kohashi et al. 2012). After
262 PTZ exposure, hyperactive behavior is observed, and two prominent aspects of swimming
263 performance are altered similar to older fish: larvae performed longer duration escape
264 responses and these responses are interspersed with multiple C-shaped body bends
265 (Figure 1; Supplementary Movies 1 and 2). These results indicate that GABA_A receptors
266 are essential to regulate swimming behavior during escape responses in early larval
267 zebrafish, as has been previously shown for later stages of development.

268

269

270 ***Mutation of pairs of α subunits induces hyperactive behavior at 48 hpf***

271 To identify GABA_A α subunits that control locomotion, we generated F0 somatic
272 mutations in each of the 8 subunits individually and in all pairwise combinations, then we
273 analyzed touch-evoked behavior, focusing on escape response duration and body-bend
274 amplitude (Figure 2A). Of the 36 conditions examined, no individually mutated α subunit
275 gave rise to abnormal response durations at 48 hpf, however mutating pairs $\alpha 3/\alpha 5$ or
276 $\alpha 4/\alpha 5$ resulted in increases in swimming times (Figure 2B). Sibling controls exhibited an
277 average swimming duration of 0.53 ± 0.03 seconds ($n=256$). In contrast, larvae with
278 mutations in $\alpha 3/\alpha 5$ and $\alpha 4/\alpha 5$ responded with an average of 1.27 ± 0.33 seconds ($n=18$)
279 and 1.69 ± 0.49 seconds ($n=18$), respectively (Figures 2C, 2D, Supplementary Movies, 3,
280 4).

281 Mutations in $\alpha 3$, $\alpha 4$, and $\alpha 5$ also caused hyperactive increases in the number of
282 C-shaped body bends at 48 hpf. Wild-type larvae exhibited an average of 1.37 ± 0.04
283 ($n=383$) C-bends per escape response, while mutations in $\alpha 3/\alpha 4$ and $\alpha 3/\alpha 5$ performed an
284 average of 5.32 ± 1.89 ($n=18$) and 3.88 ± 1.01 ($n=18$) C-bends per escape response,
285 respectively (Figure 3A, B). Notably, mutations in pair $\alpha 3/\alpha 4$ increased the number of C-
286 bends per escape response without causing significantly longer swimming durations, while
287 mutations in pair $\alpha 4/\alpha 5$ caused an increase in swimming durations without a significant
288 increase in C-bends. No mutant pairs without $\alpha 3$ were found to increase the number of
289 high-amplitude body bends. Similarly, no mutant pairs without $\alpha 5$ were found to increase
290 swimming durations. These observations suggest that $\alpha 3$ might play a dominant role in
291 controlling the number of C-bends during an escape response, while $\alpha 5$ predominantly
292 regulates swimming durations.

293

294

295 ***Hyperactive phenotypes at 48 hpf are absent or attenuated by 96 hpf***

296 The hyperactive phenotypes observed at 48 hpf were absent or greatly reduced by
297 96 hpf. Mutations in pair $\alpha 3/\alpha 5$ resulted in significantly longer duration swimming episodes
298 at 48 hpf, however at 96 hpf the swimming behavior of these same animals was
299 indistinguishable from controls (Figure 4A, B). Mutations in pair $\alpha 4/\alpha 5$ did result in longer
300 duration swimming responses at both 48 and 96 hpf, however, at the later time point, the
301 difference compared to controls was far less (a difference of 1.18 ± 0.15 (n=18) seconds at
302 48 hpf and 0.41 ± 0.07 (n=18) seconds at 96 hpf compared to controls). No other mutations
303 caused increased swimming durations. Similarly, although mutations in pairs $\alpha 3/\alpha 4$ or
304 $\alpha 3/\alpha 5$ induced excess C-bends at 48 hpf, neither these nor any other mutations were
305 found to cause significantly greater large-amplitude body flexions at 96 hpf (Figure 4C,D).

306

307 ***$\alpha 3$ F2 germline mutants confirm that hyperactive phenotypes at 48 hpf are reduced***
308 ***by 96 hpf***

309 Previous studies have suggested that phenotypes observed in F0 somatic mutants
310 can weaken during development, possibly due to the effects of mosaicism (ref). To
311 address whether this mechanism could explain the reduction in hyperactive phenotypes at
312 96 hpf, we generated $\alpha 3$ germline mutants (Figure 5A). In line with $\alpha 3$ selectively
313 controlling high-amplitude body bends but not swimming duration, $\alpha 3$ germline mutants
314 demonstrated an increase in the number of C-bends without a significant increase in
315 response times at 48 hpf (Figure 5B). The $\alpha 3$ germline mutant phenotype was more robust
316 than the $\alpha 3$ F0 somatic phenotype, likely due to the mosaicism of somatic mutants. Similar
317 to our observations using somatic mutants, at 96 hpf the number of C-bends were
318 attenuated and not significantly different from sibling controls ($p = 0.3879$). These data

319 indicate that the absent or weaker phenotypes observed in the F0 somatic mutants at 96
320 hpf are not due to mosaicism.

321 Given that the behavior of $\alpha 3$ mutants was indistinguishable from wild-type controls
322 at 96 hpf, we next asked whether local field potential recordings from the larval brain
323 (Figure 5C) would provide a more sensitive measure of phenotype abnormalities than our
324 behavioral assay. Consistent with previous findings (Liu and Baraban, 2019), LFP
325 recordings over a period of 30 minutes revealed frequent large and abnormal discharges
326 of activity at 96 hpf when PTZ was applied. However, $\alpha 3$ germline mutants and controls
327 had neuronal activities that were indistinguishable from one another, consistent with their
328 functionally normal swimming behaviors (Figure 5D).

329

330 **DISCUSSION**

331 In this study, we performed an F0 somatic mutant screen to identify GABA_A α
332 subunits that regulate hyperactive swimming during early larval stages of zebrafish
333 development. We presented evidence that combinations of $\alpha 3$, $\alpha 4$ and $\alpha 5$ have selective
334 roles in mediating different aspects of hyperactive behavior at 48 hpf. F0 somatic
335 mutations in pairs $\alpha 3/\alpha 4$ significantly increased the number of high amplitude body bends,
336 while mutations in pairs $\alpha 3/\alpha 5$ significantly increased swimming duration, and mutation of
337 pairs $\alpha 3/\alpha 5$ caused significant increases in both parameters. We found that hyperactivity
338 caused by somatic disruption of GABA_A α subunits is ostensibly reduced by 96 hpf, a
339 result confirmed using germline $\alpha 3$ mutants using both behavioral and electrophysiological
340 assays. Taken together, these data lay a foundation to investigate how GABA_A receptors
341 establish and maintain control of escape behavior at neuronal and circuit levels.

342

343 ***GABA_A Receptor Subunits Likely Control Escape Behavior Through Different***
344 ***Cellular Mechanisms***

345 The GABA_A α subunits α 3, α 4, and α 5 regulate escape behavior at 48 hpf, however
346 the cellular mechanisms through which they exert their effects are not yet clear. The
347 zebrafish hindbrain, in particular the Mauthner Cell and its homologs, play a central and
348 well-studied role in C-starts, and these cells are regulated by GABA (Triller et al. 1997;
349 Korn and Faber 2005; Burgess and Granato 2007; Kohashi et al. 2012; Roy and Ali 2014;
350 Liu and Baraban 2019). α 3, α 4, and α 5 are all expressed in discrete populations of
351 hindbrain cells by 48 hpf, so it is possible their reduced expression dysregulates hindbrain
352 circuits to generate hyperactive behavior (Monesson-Olson et al. 2018). Alternatively, each
353 of these subunits is also expressed in distinct cell types in the spinal cord, so it is also
354 possible that spinal cord GABA_A α subunit disruption elicits hyperactive behavior. In future
355 studies it will be interesting to determine the relative contribution of the hindbrain versus
356 the spinal cord in generating the abnormal behaviors caused by reduced GABA_A receptor
357 function.

358 Whether through the hindbrain or spinal cord, α 3, α 4, and α 5 likely control escape
359 behavior through different neurons or subcellular mechanisms. In both the hindbrain and
360 spinal cord at 48 hpf, α 3 and α 5 are expressed in overlapping domains, raising the
361 possibility they are expressed in at least some of the same cells (Monesson-Olson et al.
362 2018). In both structures, α 4 seems to be expressed in cells distinct from α 3 and α 5,
363 therefore its effects could be mediated by different neurons.

364 Even when expressed in the same cells, α 3, α 4, and α 5 are probably expressed in
365 different subcellular domains. In mammalian neurons, GABA_A receptors have been shown
366 to cluster in either synaptic or extrasynaptic domains to mediate phasic or tonic inhibition
367 (Farrant and Nusser 2005; Jacob et al. 2008; Fritschy and Panzanelli 2014). α 3-containing

368 receptors are enriched at synapses, to mediate phasic inhibition, while $\alpha 4$ and $\alpha 5$ -
369 containing receptors are predominantly extrasynaptic, to provide tonic inhibition. Although
370 the subcellular localizations of these subunits have not been directly investigated in
371 zebrafish, the high degree of amino acid sequence similarity suggests that their subcellular
372 distributions are likely conserved, which would localize $\alpha 3$ towards synapses while $\alpha 4$ and
373 $\alpha 5$ would be found in mainly extrasynaptic domains. High resolution expression analysis
374 will be required to determine if this is the case.

375 We did not find significant effects in response to somatic mutation of $\alpha 1$, $\alpha 2a$, $\alpha 2b$,
376 $\alpha 6a$ or $\alpha 6b$, however these subunits cannot be entirely ruled out from playing regulatory
377 roles in controlling zebrafish escape behavior. F0 somatic mutations reduce, but do not
378 eliminate expression, so it is possible that further reducing the expression of these
379 subunits could reveal locomotor phenotypes. Additionally, our screen focused on two
380 behavioral parameters at two developmental stages. Examining additional developmental
381 stages, responses to other sensory stimuli, or other parameters, such as body bend
382 frequency or frequency variability, could reveal roles for these subunits in controlling
383 escape behavior.

384

385 ***Zebrafish Exhibit Robust Homeostatic Mechanisms Across Development***

386 The hyperactive phenotypes observed at 48 hpf were all absent or greatly reduced
387 by 96 hpf. This result was surprising since PTZ readily elicits hyperactive behavior at all
388 time points after 48 hpf, demonstrating that GABA_A receptors play critical roles in
389 regulating locomotion across a wide variety of developmental stages. A likely explanation
390 is that zebrafish employ robust homeostatic compensation. The mechanisms that underlie
391 this compensation are probably multifaceted. The teleost lineage has undergone genome
392 duplication such that there are many duplicated genes in zebrafish, including several

393 GABA_A receptor subunits (Amores et al. 1998; Postlethwait et al. 1998; Monesson-Olson
394 et al. 2018). The expression of homologous genes or simply genes with similar sequence
395 motifs can be recruited through transcriptional adaptation, which is thought to be triggered
396 by nonsense mediated decay (El-Brolosy et al. 2019). Mutations that cause frame-shifts
397 and premature stop codons can cause nonsense mediated decay, therefore many of the
398 mutations generated in this study almost certainly induced transcriptional adaptation. Pairs
399 of α subunits were mutated to uncover adaptations within the α subunit subfamily, however
400 transcriptional responses could involve other GABA_A receptor subunits. At the network
401 level, neurons could switch the neurotransmitter they release or entire circuits could be
402 reconfigured to maintain excitation-inhibition balance as has been observed in developing
403 frogs and some α subunit knock-out mice, respectively (Schneider Gasser et al. 2007;
404 Panzanelli et al. 2011). Our results here identify a narrow window between 48 and 96 hpf
405 across which robust adaptations occur in developing zebrafish. The short time period,
406 relatively small nervous system, ex-utero development, genetic resources, and high-
407 resolution brain and spinal cord atlases make larval zebrafish an outstanding system to
408 further investigate the homeostatic mechanisms activated by GABA_A receptor mutation.

409

410 ***Zebrafish $\alpha 3$ Mutants as a Possible Epilepsy Model***

411 In addition to their roles in modulating locomotion, GABA_A receptors are widely
412 viewed as central factors in the development, progression, and treatment of epilepsy
413 syndromes (Olsen and Avoli 1997; Treiman 2001; Cherubini 2012; Walker and Kullmann
414 2012). Abnormalities in GABA_A receptor inhibition are observed in genetic and acquired
415 epilepsies, drugs that block these receptors, like PTZ, cause seizures, and drugs that
416 enhance GABA_A receptor inhibition are potent anticonvulsants. In zebrafish, PTZ
417 application is an established seizure model, and loss-of-function mutations in $\gamma 2$ and $\beta 3$

418 cause larval hyperactive behavior and/or neuronal activity that model the epilepsies
419 caused by mutations in their human orthologs (Baraban et al. 2005; Baxendale et al. 2012;
420 Liao et al. 2019; Yang et al. 2019; Cho et al. 2020). Similarly, mutations in zebrafish $\alpha 1$ cause
421 light-triggered, hyperactive behavior in juvenile fish (older than ~5 weeks) that seems to
422 model epilepsy caused by reduced $\alpha 1$ function (Samarut et al. 2018). Here, we showed
423 that mutation of $\alpha 3$ causes hyperactive behavior in larval zebrafish. Human loss-of-function
424 variants in $\alpha 3$ cause a rare, severe epileptic encephalopathy, raising the possibility that
425 zebrafish $\alpha 3$ mutants at least partially model this disorder (Niturad et al. 2017). It is not yet
426 clear whether the zebrafish hyperactive swimming observed at 48 hpf is due to brain or
427 spinal cord mechanisms but, regardless, it is relatively simple to screen the phenotype
428 caused by $\alpha 3$ disruption. Given the proven effectiveness of larval zebrafish for high-
429 throughput small molecule screens, $\alpha 3$ mutants could be a new and useful resource to
430 identify novel anti-epileptic drugs (Griffin et al. 2017; Lam and Peterson 2019; Griffin et al.
431 2020; Patton et al. 2021 Jun 11).

432

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443 **FIGURE LEGENDS**

444 **Figure 1. Pentylentetrazole (PTZ) exposure induces hyperactive swimming in early**
445 **larval zebrafish.** PTZ is a potent GABA_A receptor antagonist. Bath application of 10 mM
446 PTZ to both 48 and 96 hpf zebrafish caused an increase in (A) swim duration shown in
447 seconds and (B) C-bends, defined as large-amplitude body bends over 110°. Box plots are
448 representing the 25% and 75% quartiles with the median represented by a horizontal black
449 line and the mean represented by a black plus sign within the box. Tukey's whiskers were
450 used. $n=20$ and 39 for wild type and PTZ treated larvae, respectively, ** $P<0.01$,
451 *** $P<0.001$, **** $P<0.0001$ using unpaired Welch's t-tests.

452

453 **Figure 2. An F0 somatic mutation screen of GABA_A receptor α subunits identifies**
454 **mutant combinations that show increased swimming durations at 48 hpf.** (A)
455 Overview of the GABA_AR α subunit screen. Different colored boxes represent different
456 aspects of the screen. (B) Heat matrix of the 36 single and double F0 somatic mutant
457 conditions indicating mean startle response durations. The heatbar (*right*) indicates the
458 average swim length. The box at lower right shows mock or uninjected controls. Ordinary
459 one-way ANOVA revealed significant differences in swimming durations according to
460 knock-down target, (N=995 larvae total, 612 mutants with 13-26 larvae per condition, 383
461 wild-type siblings; $F(36,958) = 5.316$, $p<0.0001$). A Dunnett's post-hoc test revealed
462 significant pairwise differences between $\alpha 3/\alpha 5$ compared to wild-type and $\alpha 4/\alpha 5$ compared
463 to wild-type (*white asterisks*), with those conditions exhibiting average swim durations of
464 1.27 ± 0.33 seconds ($n=18$) and 1.69 ± 0.49 seconds, respectively. (C) Boxplots of $\alpha 3/\alpha 5$
465 and $\alpha 4/\alpha 5$ somatic mutant swimming durations show the increased swimming durations
466 compared to controls. *** $P<0.001$, **** $P<0.0001$ using Dunnett's multiple comparison test.

467 (D) Traces of representative escape responses for wild-type, $\alpha 3/\alpha 5$ and $\alpha 4/\alpha 5$ somatic
468 mutants. The color spectrum of each trace indicates the beginning (*red*) and end (*blue*) of
469 the response, and *white circles* represent the location of C bends. The videos used to
470 generate these traces are provided in the Supplementary Data.

471

472 **Figure 3. Somatic mutation of pairs $\alpha 3/\alpha 4$ or $\alpha 3/\alpha 5$ causes an increased number of**
473 **large-amplitude body bends at 48 hpf.** (A) Heat matrix of the α subunit single and
474 double somatic mutant conditions indicating mean numbers of C-bends per response. The
475 heatbar (*right*) indicates the average swim length. The box at lower right shows mock or
476 uninjected controls. Ordinary one-way ANOVA revealed significant differences in large-
477 amplitude body bends according to knock-down target, ($F(36, 834)=4.221, p<0.0001$). A
478 Dunnett's post-hoc test revealed significant pairwise differences between $\alpha 3/\alpha 4$ compared
479 to wild-type and $\alpha 3/\alpha 5$ compared to wild-type (*white asterisks*), with those conditions
480 exhibiting average C-bend per response of 5.32 and 3.88, respectively, compared to 1.37.
481 (B) Boxplots of $\alpha 3/\alpha 4$ and $\alpha 3/\alpha 5$ somatic mutant C-bends show the increased number of
482 large amplitude body bends per swimming episode compared to sibling controls.
483 *** $P<0.001$, **** $P<0.0001$ using Dunnett's multiple comparison test. (C) Traces of
484 representative escape responses for wild-type, $\alpha 3/\alpha 4$ and $\alpha 3/\alpha 5$. The color spectrum of
485 each trace indicates the beginning (*red*) and end (*blue*) of the response, and *white circles*
486 represent the location of C bends. The videos used to generate these traces are provided
487 in the Supplementary Data.

488

489

490 **Figure 4. Only $\alpha 4/\alpha 5$ somatic mutants continue to exhibit a hyperactive phenotype at**
491 **96 hpf.** (A) Heat matrix of the single and double F0 somatic mutant conditions showing
492 evoked swimming response durations at 96 hpf. The heatbar (*right*) indicates the average
493 swim length. The box at lower right shows mock or uninjected controls. Ordinary one-way
494 ANOVA revealed a significant difference in swimming duration dependent upon knock-
495 down target ($F(36,754) = 2.914, p < 0.0001$). A Dunnett's post-hoc test revealed only a
496 significant pairwise difference between $\alpha 4/\alpha 5$ (*white asterisk*) and wild-type. (B) A boxplot
497 of $\alpha 3/\alpha 5$, $\alpha 4/\alpha 5$ mutant pairs and controls. Both mutant conditions showed increased
498 swimming durations at 48 hpf, however only the $\alpha 4/\alpha 5$ pair was statistically significant at
499 96 hpf. Although significant, the $\alpha 4/\alpha 5$ swimming duration is reduced compared to 48 hpf.
500 **** $P < 0.01$** using Dunnett's multiple comparison test. (C) Heat matrix of C bends at 96 hpf.
501 No significant differences were detected. (D) Box plots show that conditions that
502 demonstrated increased C bends at 48 hpf were not significantly elevated at 96 hpf.

503

504 **Figure 5. $\alpha 3$ F2 germline mutants confirm the $\alpha 3$ G0 somatic mutant phenotype.**

505 (A) A schematic of the $\alpha 3$ protein is shown based upon (Macdonald et al. 2010). The four
506 transmembrane domains are indicated, along with the location of the zebrafish (*Danio*
507 *rerio*) and human (*Homo sapiens*) $\alpha 3$ mutations. (B) Box plots for $\alpha 3$ trans-heterozygous
508 mutants and siblings swimming duration in seconds (*left*) and C-bends per response (*right*)
509 at both 48 and 96 hpf. Unpaired Student's t-test indicated that $\alpha 3$ mutants exhibit
510 significantly more C-bends at 48 hpf but not at 96 hpf ******= $p < 0.0001$** . $\alpha 3$ mutant swimming
511 durations were not significantly greater than sibling controls at either time point. (C)
512 Schematic of LFP recording setup from a 96 hpf larvae. (D) LFP traces (*left*) from wild-type

513 (n=3), siblings (n=7), PTZ treated wild-type (n=3), $\alpha 3$ heterozygotes (n=6), and $\alpha 3$ trans-
514 heterozygous mutants (n=3). Ictal-like activity was only detected in PTZ treated fish
515 compared to wild-type (*right*), as revealed by ordinary one-way ANOVA with Dunnet's
516 post-hoc test, ****= $p < 0.0001$.

517

518 **Supplementary Figure 1. CRISPR-Cas 9 targeting of *tyr* confirms high-efficiency**

519 **gene targeting.** (A) Wild-type uninjected (*left*) and fish in which *tyr* gRNA and Cas9
520 encoding RNA were injected (*right*) at 48 (*top*) and 96 hpf (*bottom*). The fish in which the
521 *tyr* gene was targeted show reductions in melanophores. (B) Bar graph showing a pixel
522 density analysis of uninjected (n=21), *tyr* CRISPR-injected (n=36), or PTU-treated larvae
523 (n=20). *tyr* CRISPR-injected larvae show reduced pixel density at both developmental
524 ages, indicating effective gene knock-down. Each group measured against pixels detected
525 above an arbitrary threshold determined *a priori*. Ordinary One-Way ANOVA was used
526 ****= $p < 0.0001$. (C) Fragment Analysis of WT (*top*) or *tyr* injected embryos (*bottom*). While
527 PCR analysis of wild-type larvae reveals a single peak of ~278 base pairs, PCR analysis
528 across this same region of *tyr*-CRISPR-injected larvae shows several peaks, indicating the
529 presence of indels in the target region.

530

531 **Supplementary Movie 1. Representative movie of a wild-type larva touch response**

532 **at 48 hpf.** The video was recorded at 250 frames/second (1.18 seconds in length) and was
533 used to generate the trajectory trace shown in Figure 2D. Wild-type larvae respond to
534 touch by performing a C-bend followed by rhythmic, smaller amplitude body bends to
535 propel the animal away from the touch stimulus.

536 **Supplementary Movie 2. Representative movie of an $\alpha 3/\alpha 5$ G0 somatic mutant at 48**
537 **hpf.** The video was recorded at 250 frames/second (5.48 seconds in length) and was used
538 to generate the trajectory trace shown in Figure 2D. $\alpha 3/\alpha 5$ mutants exhibit both increased
539 swimming durations and C-bends per response.

540
541 **Supplementary Movie 3. Representative movie of an $\alpha 4/\alpha 5$ G0 somatic mutant at 48**
542 **hpf.** The video was recorded at 250 frames/second (3.93 seconds in length) and was used
543 to generate the trajectory trace shown in Figure 2D. $\alpha 4/\alpha 5$ mutants exhibit increased
544 swimming durations without a statistically significant increase in the number of C-bends.

545
546 **Supplementary Movie 4. Representative movie of an $\alpha 3/\alpha 4$ G0 somatic mutant at 48**
547 **hpf.** The video was recorded at 250 frames/second (0.99 seconds in length) and was used
548 to generate the trajectory trace shown in Figure 3C. $\alpha 3/\alpha 4$ mutants exhibit increased C-
549 bends without a statistically significant increase in length of response durations.

550

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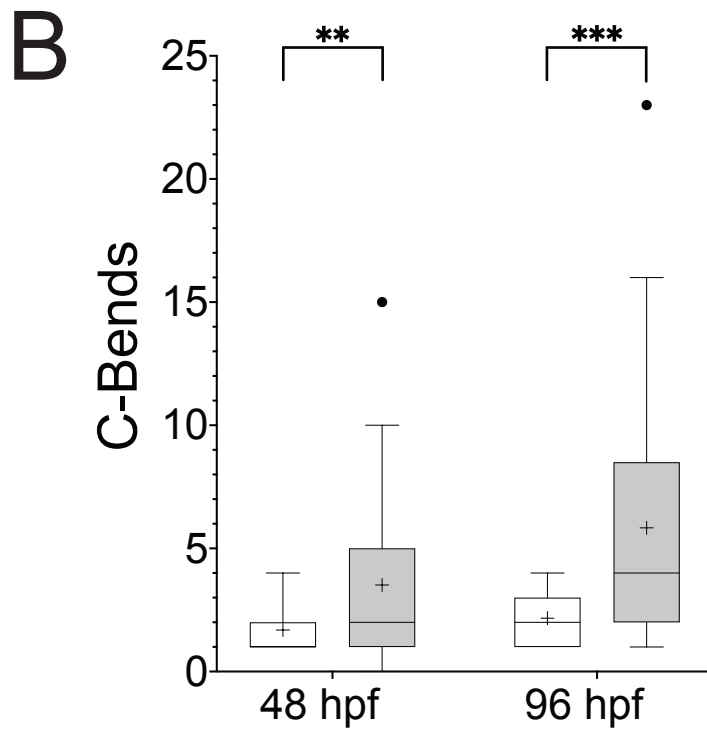
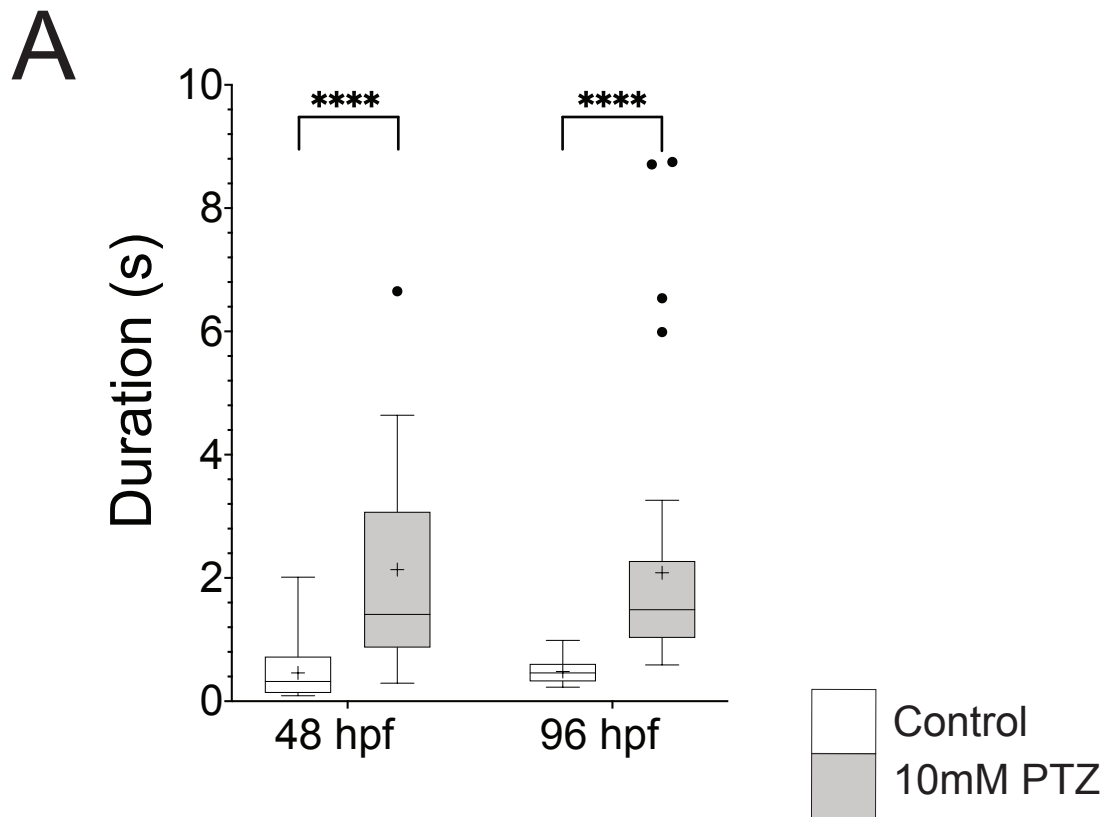
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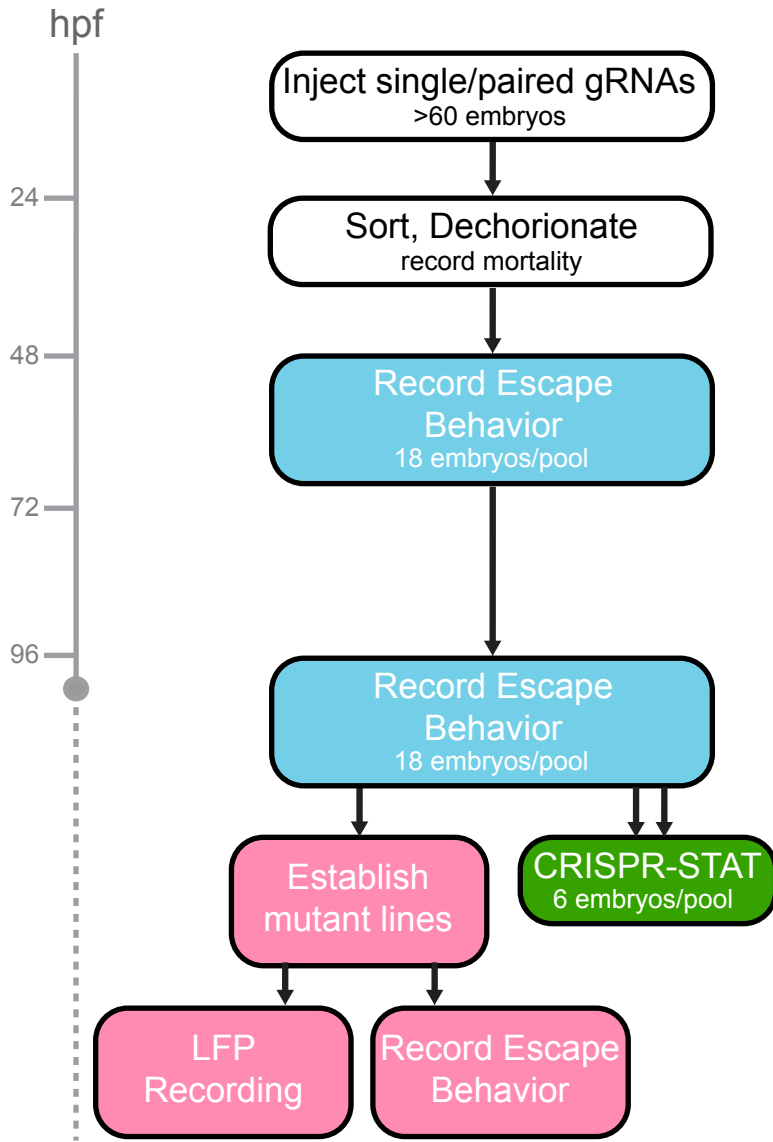
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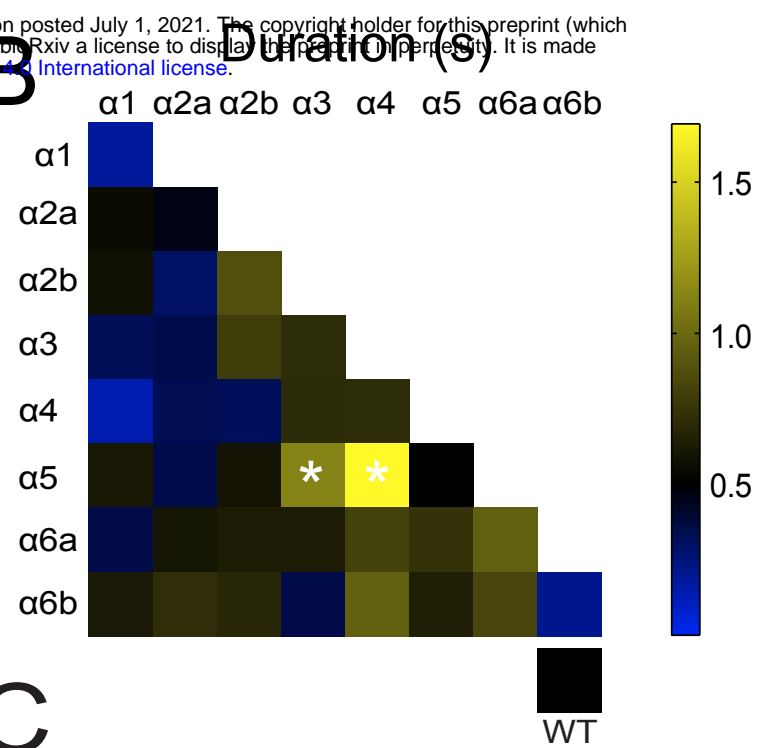
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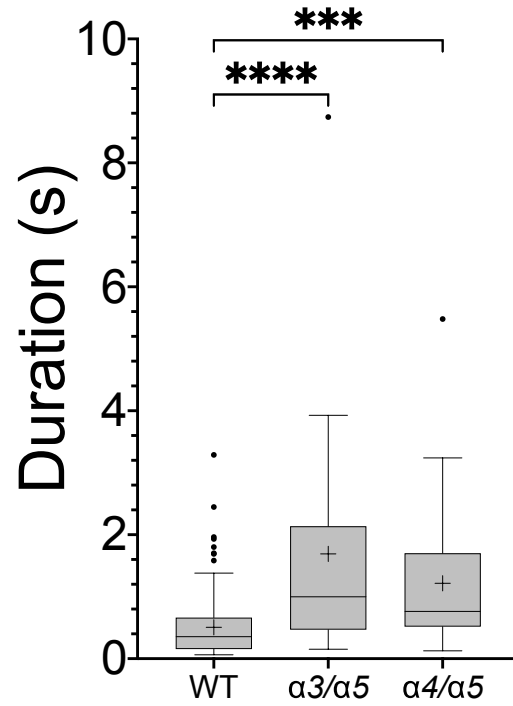
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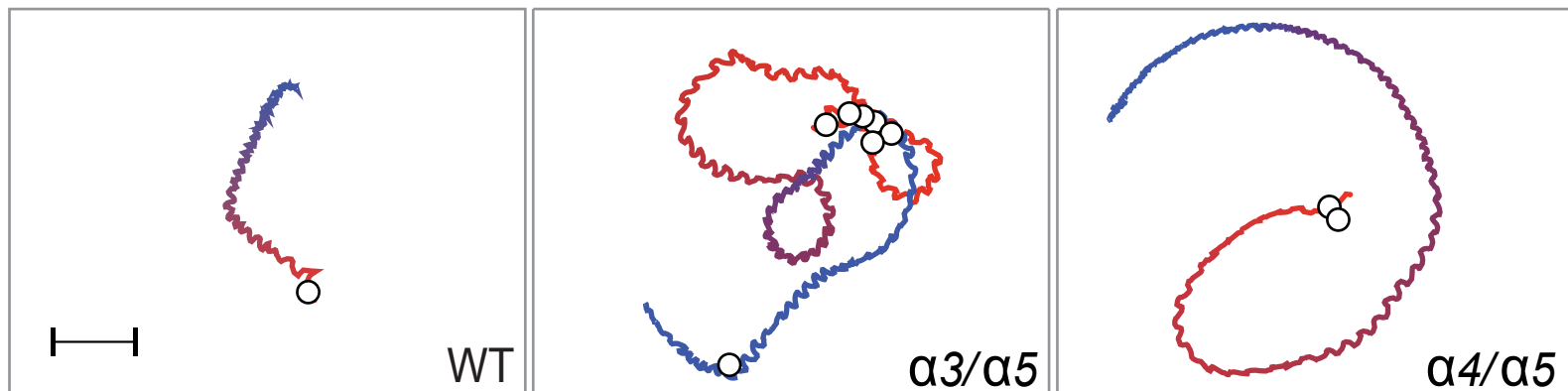
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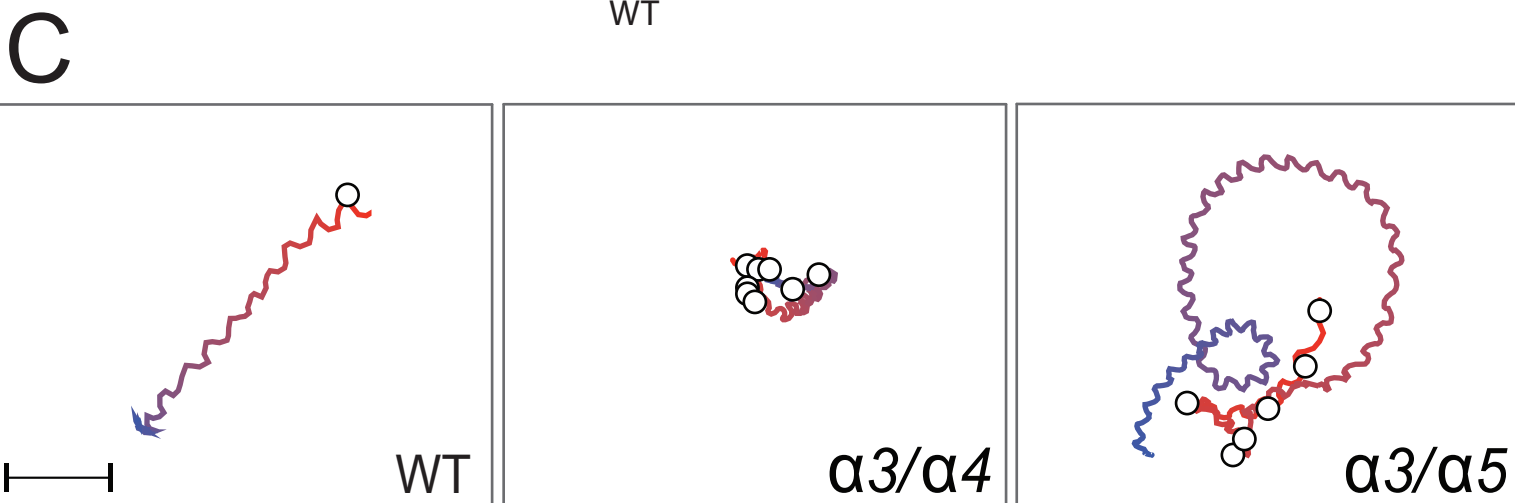
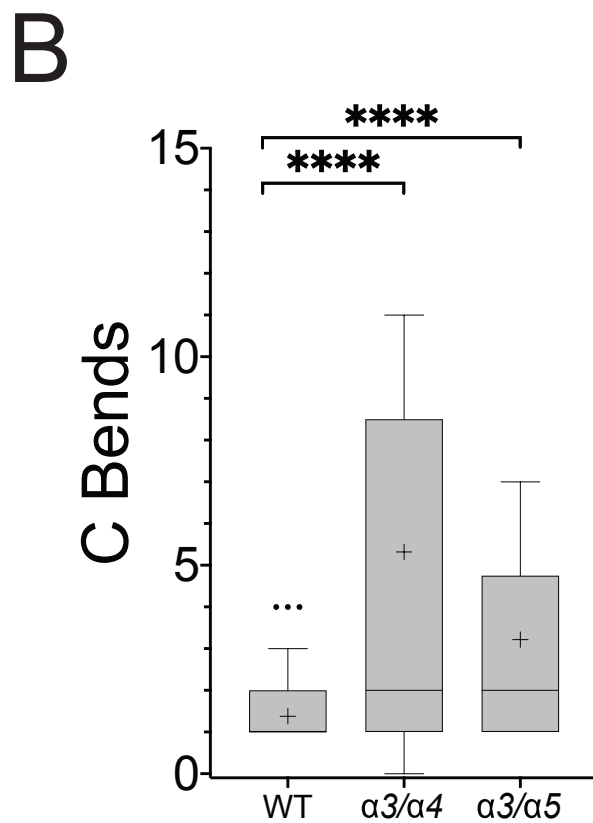
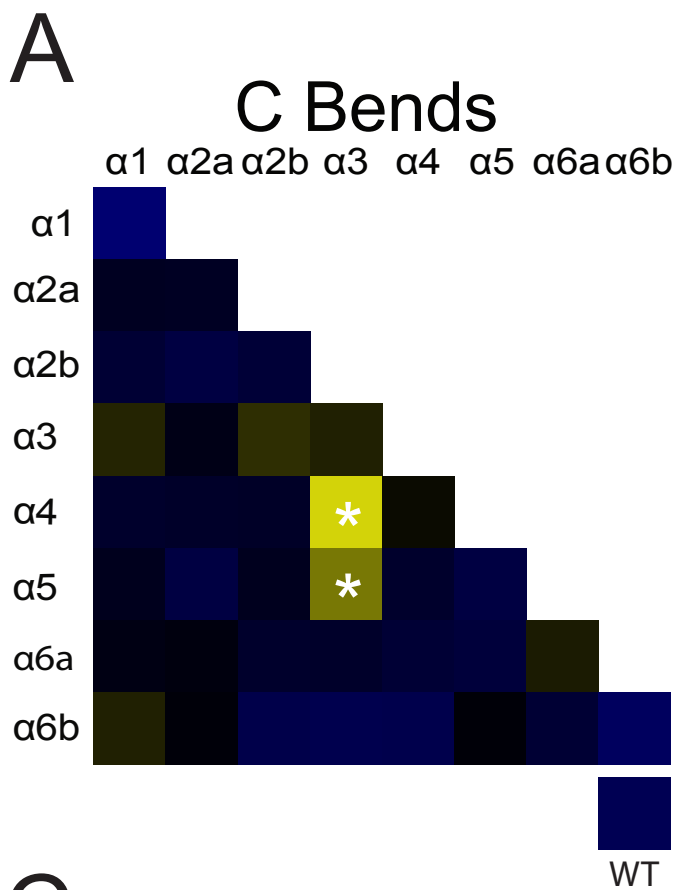


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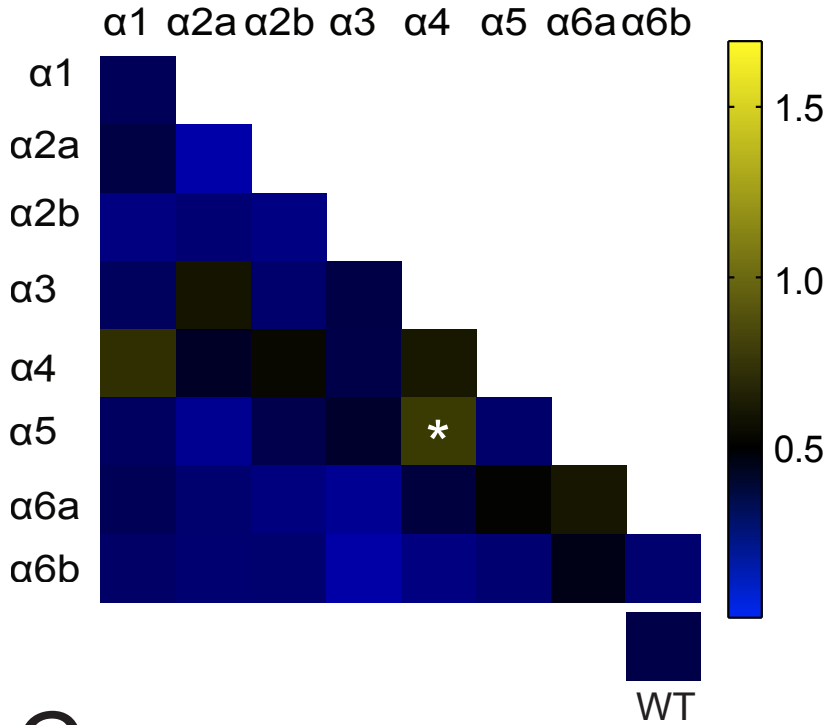
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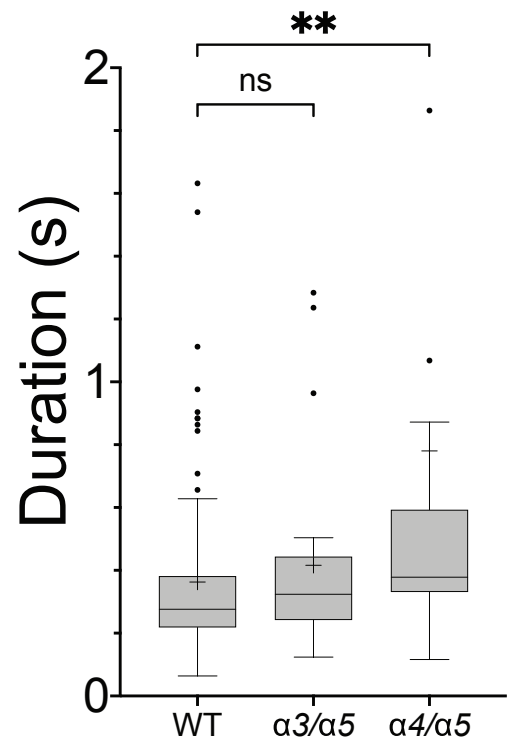


A

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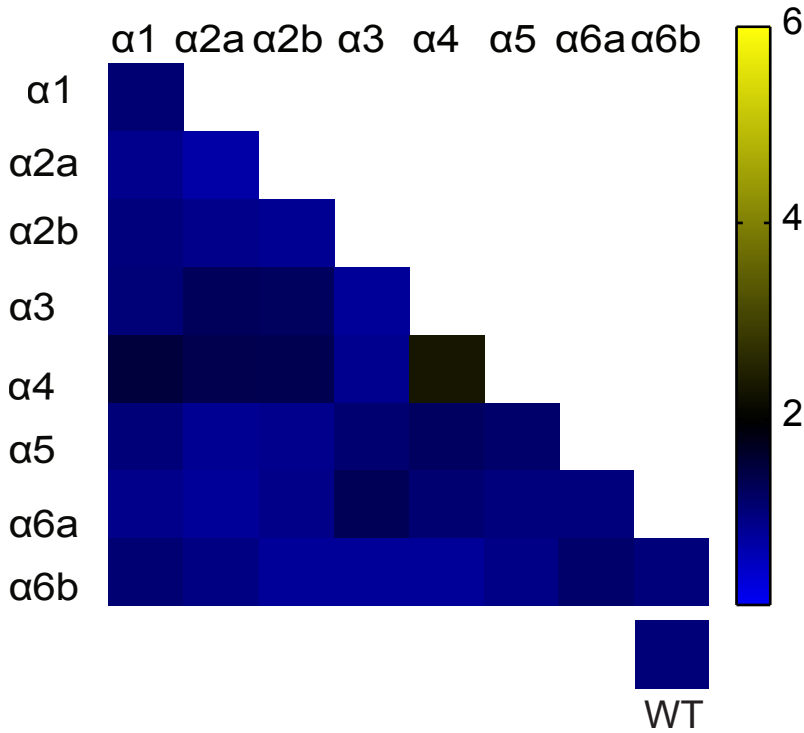


B



C

C Bends



D

