

# Identification of a brain center whose activity discriminates a choice behavior in zebrafish

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The ability to make choices and carry out appropriate actions is critical for individual survival and well-being. Choice behaviors, from hard-wired to experience-dependent, have been observed across the animal kingdom. Although differential engagement of sensory neuronal pathways is a known mechanism, neurobiological substrates in the brain that underlie choice making downstream of sensory perception are not well understood. Here, we report a behavioral paradigm in zebrafish in which a half-light/half-dark visual image evokes an innate choice behavior, light avoidance. Neuronal activity mapping using the immediate early gene *c-fos* reveals the engagement of distinct brain regions, including the medial zone of the dorsal telencephalic region (Dm) and the dorsal nucleus of the ventral telencephalic area (Vd), the teleost anatomical homologs of the mammalian amygdala and striatum, respectively. In animals that were subjected to the identical sensory stimulus but displayed little or no avoidance, strikingly, the Dm and Vd were not engaged, despite similar levels of activation in the brain nuclei involved in visual processing. Based on these findings and previous connectivity data, we propose a neural circuitry model in which the Dm serves as a brain center, the activity of which predicates this choice behavior in zebrafish.

emotion | fear | anxiety | decision-making

Choosing among alternative behaviors, whether they be regulated by external stimuli, internal drives, or cognitive processes, is a fundamental ability that all living animals possess (1–7). How brains make choices has been studied in disciplines ranging from psychology/cognition to neuroethology/behavior (8–10) using, for example, awake behaving monkeys that perform specific tasks or invertebrate animals that choose between swimming or crawling. Despite the significant advancements that these studies have brought to our understanding of behavioral choices or decision making, each system has limitations. The complex brains of primates make it difficult to delineate neural circuitry underlying behavior, whereas the nervous systems of invertebrates are significantly different from those of humans, making it difficult to extrapolate mechanistic findings from these systems directly to mammals. Therefore, animals with intermediate complexity and similarity may provide an interface that could bridge the currently available model systems and help us to understand the molecular and cellular mechanisms underlying behavioral choices and decision making.

Utilization of distinct sensory neuronal subtypes and “gating” out sensory input by presynaptic inhibition are mechanisms at the sensory end to elicit choice behaviors when animals face alternative sensory cues (1, 11, 12). When faced with identical sensory stimuli, the invertebrate medicinal leech employs “group discriminator” neurons to “decide” between crawling and swimming (7). In more complex animals, it is less clear what brain regions might dictate choice making. Here, we report a study of an innate light/dark (L/D) choice behavior in zebrafish, a vertebrate genetic model organism with a brain similar to that of mammals but with significantly less complexity (13, 14). Our findings show that the medial zone of the dorsal telencephalic region (Dm), the teleost anatomical homolog of the mammalian amygdala (14–17), and the

dorsal nucleus of the ventral telencephalic area (Vd), the zebrafish anatomical homolog of the mammalian striatum (18), are differentially activated between animals that display light avoidance and those that do not, whereas the brain nuclei involved in visual processing are similarly activated among these animals. Because Vd is likely downstream of Dm (15), we suggest that Dm serves as an internal center, the activity of which discriminates the outcome of this choice behavior.

## Results and Discussion

Phototaxis, which is a rapid and short-lived (usually reaching completion within a minute) swim toward or away from a light source, has been reported in larval zebrafish (1, 19, 20). L/D choice behavior is similarly light-driven yet distinct in duration (observable within minutes to tens of minutes), and it has been reported in adult zebrafish (13, 21–23). After being individually introduced into a L/D choice chamber (light intensity readings of 300 and 160 Lux, respectively, on each side) (Fig. 1A), zebrafish explore randomly but exhibit a bias by turning away at the border to avoid the more illuminated space (Movie S1). This was measured by a choice index [(% time in dark – % time in light)/100] (Fig. 1B). When the data were presented as a scatter plot (Fig. 1C), a distribution of the choice index was observed, with most animals displaying a positive value, meaning less time spent on the light side. When zebrafish were tested repeatedly in the same chamber (three trials with a 24-h interval between each trial), we did not observe significant habituation or sensitization of the choice behavior (Fig. 1D,  $n = 13$ ;  $P = 0.64$ ), which was consistent with what has been reported recently (24).

This choice behavior was observed in several different genetic backgrounds in both male and female zebrafish and was unaffected by whether the animal was initially introduced into the light side or the dark side or whether it had been raised in dim or bright lighting conditions (Fig. S1A–D). Moreover, either individual- or group-raised animals exhibited similar light avoidance behavior (Fig. S1E). Interestingly, using an L/D choice chamber designed proportionally to the size of larval zebrafish under the same experimental conditions, we found that 1- and 2-wk-old larvae preferred the light environment instead of avoiding it (Fig. S1F). These results indicate that a choice reversal occurs during the development and maturation of zebrafish, with adult zebrafish generally exhibiting a light avoidance behavior. The neural basis of this choice reversal is currently not known and will be the subject of future investigations.

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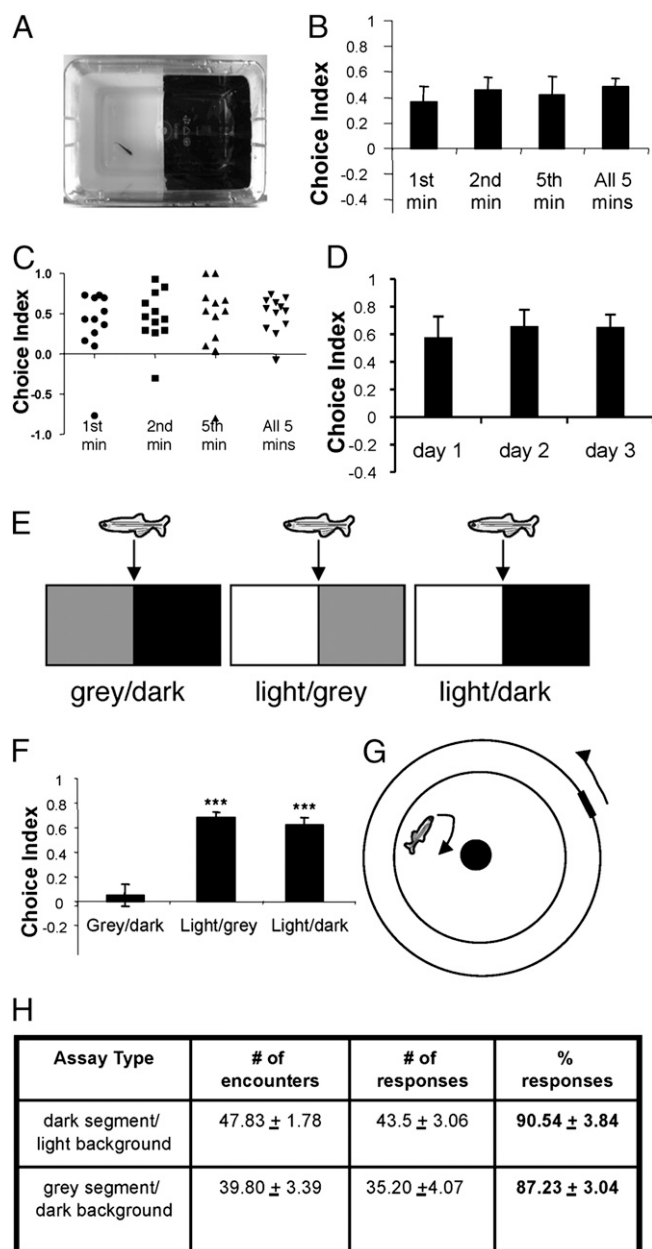
The authors declare no conflict of interest.

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**Fig. 1.** Adult zebrafish display an innate light avoidance behavior. (A) Photograph of the L/D choice chamber. (B) Choice indices in the choice chamber during first, second, fifth, and all 5 min analyzed (a choice index of 1.0 represents 100% time spent on the dark side, and an index of  $-1.0$  represents 100% time spent on the light side) ( $n = 12$  for each group). (C) Scatter plot of B, showing the distribution of choice indices in the tested animals. (D) Choice indices of three trials with a 24-h interval between each trial. The means for choice indices are day 1 ( $0.58 \pm 0.15$ ), day 2 ( $0.66 \pm 0.12$ ), and day 3 ( $0.65 \pm 0.09$ ) ( $n = 13$ ;  $P = 0.64$ , ANOVA followed by the Bonferroni post hoc test). (E) Schemes of different choice chambers. (F) Choice indices in different choice chambers (L/D,  $n = 26$ ; light/grey,  $n = 23$ ; grey/dark,  $n = 24$ ;  $F = 28.55$ ,  $***P < 0.001$  compared with grey/dark, Tukey's test). (G) Scheme of a visual acuity chamber for testing the ability of zebrafish to discriminate between gray and dark. (H) Comparable visual discrimination of gray segment over dark background ( $n = 6$ ) vs. dark segment over light background ( $n = 5$ ), as indicated by comparable % responses ( $F = 0.36$ ,  $P = 0.56$ , Tukey's test).

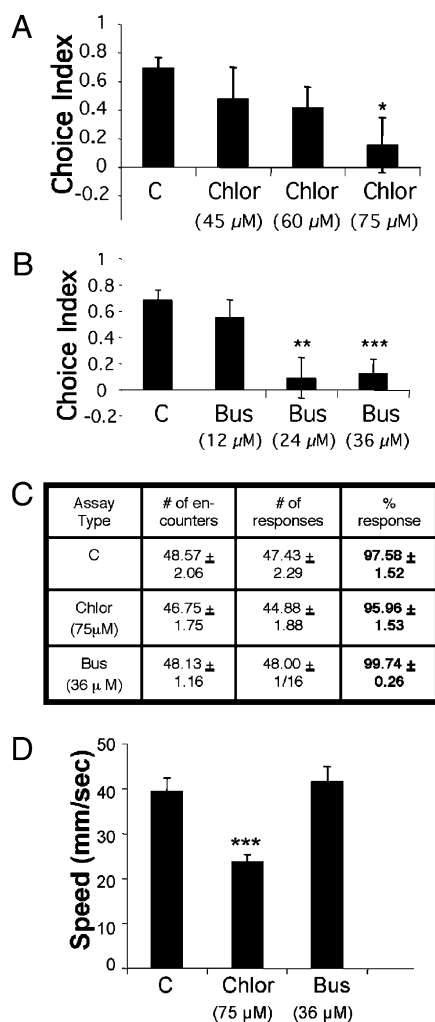
To characterize the sensitivity of the adult choice behavior to lighting conditions further, we introduced adult zebrafish into gray/dark or light/gray chambers (Fig. 1E, the light intensity readings of the light, gray, and dark areas are 300, 200, and 160

Lux, respectively). In the light/gray chamber, zebrafish spent significantly more time on the gray side, similar to their preference in the L/D chamber (Fig. 1F, center bar compared with right bar). In contrast, in the gray/dark chamber, they displayed no preference and spent about equal time on either side (Fig. 1F, left bar). Such lack of preference in the gray/dark chamber is not attributable to a lack of visual discrimination, because zebrafish could visually discriminate gray from dark in a visual acuity test (Fig. 1G and H). We next asked whether increased light intensity would enhance the avoidance behavior. Increasing the ambient light from 300 to 1,000 Lux did not further enhance the avoidance behavior (Fig. S1G). Taken together, these results suggest that adult zebrafish avoid a lit environment when the light intensity is above a certain threshold (at least above 200 Lux).

In rodents, avoidance of a brightly lit environment is sensitive to anxiolytic compounds (25). Similarly, the light avoidance behavior in zebrafish was attenuated by two common anxiolytics (Fig. 2A and B), chlordiazepoxide and buspirone, which enhance GABAergic (26) and monoaminergic (27) signaling, respectively, in mammals and have effects in zebrafish at both behavioral and tissue-binding levels (28, 29) (Fig. S2, chlordiazepoxide had a  $K_i$  of 143 nM; see *SI Materials and Methods*). Because the drugs were administered directly in the tank water and needed to cross multiple barriers (including the absorption and blood-brain barriers) to reach the site of action, a higher concentration than that used during the *in vitro* binding experiment was necessary to achieve an *in vivo* effect. Using the visual acuity assay, we found that neither compound affected visual capability at the highest drug concentrations used in our experiments (Fig. 2C). Although buspirone had no effect on locomotor activity, the highest concentration of chlordiazepoxide (75  $\mu$ M) did reduce locomotor speed (Fig. 2D). Such effects could not account for the reduced avoidance behavior, however, because zebrafish swimming at such a reduced speed could still easily reach either side of the choice chamber. Thus, the light avoidance behavior in adult zebrafish employs GABAergic and monoaminergic substrates, which are likely required in specialized functional brain areas rather than in sensory or motor neurons.

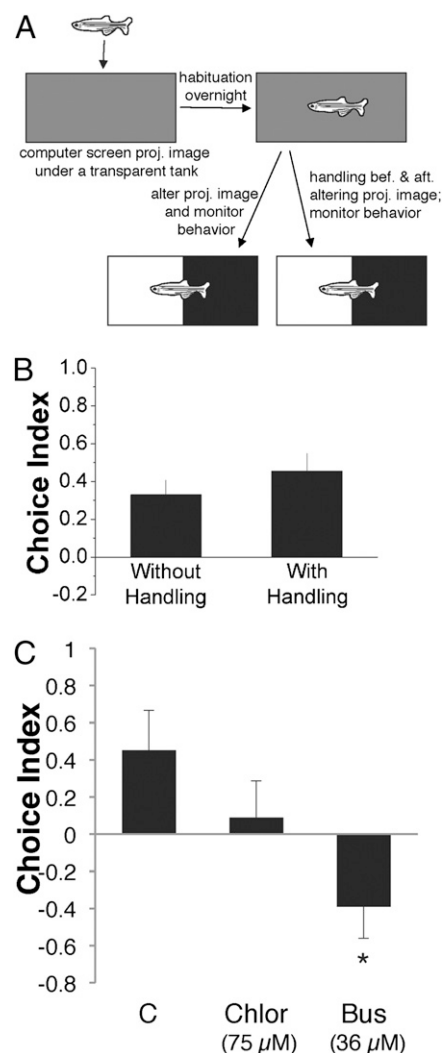
The behavioral paradigm presented in Fig. 1 involved netting zebrafish into a different chamber, a process expected to engage multiple sensory modalities, including visual and mechanosensory as well as stress-modulating pathways, such as the catecholaminergic pathway. These complex polymodal stimuli are difficult to standardize, and therefore are likely to elicit variable patterns of neuronal activity. To obtain consistent neural activity data, we tested whether a unimodal sensory stimulus, (i.e., the L/D visual image) is able to evoke the avoidance behavior. Individual zebrafish were allowed to habituate overnight to a transparent testing chamber atop a computer monitor projecting a brown-colored image that matched the behavioral testing environment. The next day, a computer-generated L/D visual image was remotely projected underneath the tank (Fig. 3A). Animals exposed to such a visual image showed light avoidance behavior (Fig. 3B and Movie S2), which was slightly less intense but, nevertheless, comparable to that of animals that were subjected to both the visual image and handling (netting) ( $P = 0.13$ ). These data suggest that the L/D visual stimulus is sufficient to evoke the avoidance behavior. We next determined whether chlordiazepoxide and buspirone affected this choice behavior evoked solely by the L/D visual stimulus. Whereas chlordiazepoxide did not significantly reduce light avoidance in this setting, strikingly, buspirone-treated animals reversed their choice by showing a preference for the light side (Fig. 3C). Thus, monoaminergic neural substrates are likely to play a central role in regulating the L/D choice.

With a unimodal visual sensory stimulus-evoked behavior, we sought to determine the underlying neural correlates through analysis of behaviorally driven expression of *c-fos*, one of the best-characterized molecular markers for neural activity (30–32).



**Fig. 2.** The light avoidance behavior is attenuated by commonly used anxiolytics. (A) Chlordiazepoxide reduces the light avoidance behavior in a dose-dependent manner. C (control),  $n = 11$ ; Chlor: 45  $\mu$ M,  $n = 11$ ; 60  $\mu$ M,  $n = 11$ ; 75  $\mu$ M,  $n = 12$ ;  $F = 1.65$ ,  $*P < 0.05$  compared with control,  $t$  test. (B) Buspirone reduces the light avoidance behavior in a dose-dependent manner. C,  $n = 11$ ; Bus: 12  $\mu$ M,  $n = 11$ ; 24  $\mu$ M,  $n = 12$ ; 36  $\mu$ M,  $n = 12$ ;  $F = 5.75$ ,  $**P < 0.01$  compared with control,  $***P < 0.001$  compared with control,  $t$  test. (C) Effect of chlordiazepoxide and buspirone on visual acuity at the highest concentration tested. C,  $n = 7$ ; Chlor: 75  $\mu$ M,  $n = 8$ ,  $P = 0.47$  compared with control; Bus: 36  $\mu$ M,  $n = 8$ ,  $P = 0.16$  compared with control,  $t$  test. (D) Effect of chlordiazepoxide and buspirone on swim speed at the highest concentration tested ( $n = 8$  for each group;  $F = 12.56$ ,  $***P < 0.001$  compared with control,  $t$  test). Mean  $\pm$  SEM are shown.

Because *c-fos*-assisted neuronal activity mapping has not been previously carried out in adult zebrafish, we first assessed the basal expression level of *c-fos* in animals that were habituated to their environment (either their natural housing system or the behavioral testing room) for at least 24 h. Little *c-fos* expression was detected in the brain, suggesting low basal expression (Fig. S3A). We next determined the inducibility of *c-fos* in animals subjected to vigorous handling stress (continuous shaking for 30 min in the confinement of a net). Robust induction of *c-fos* was observed in many brain regions, including the hypothalamus (Hy; Fig. S3B), consistent with its known role in teleost stress responses (33). Therefore, *c-fos* in situ hybridization is a suitable method for evaluating stimulus-induced neuronal activity in most, if not all, neurons of the adult zebrafish brain.



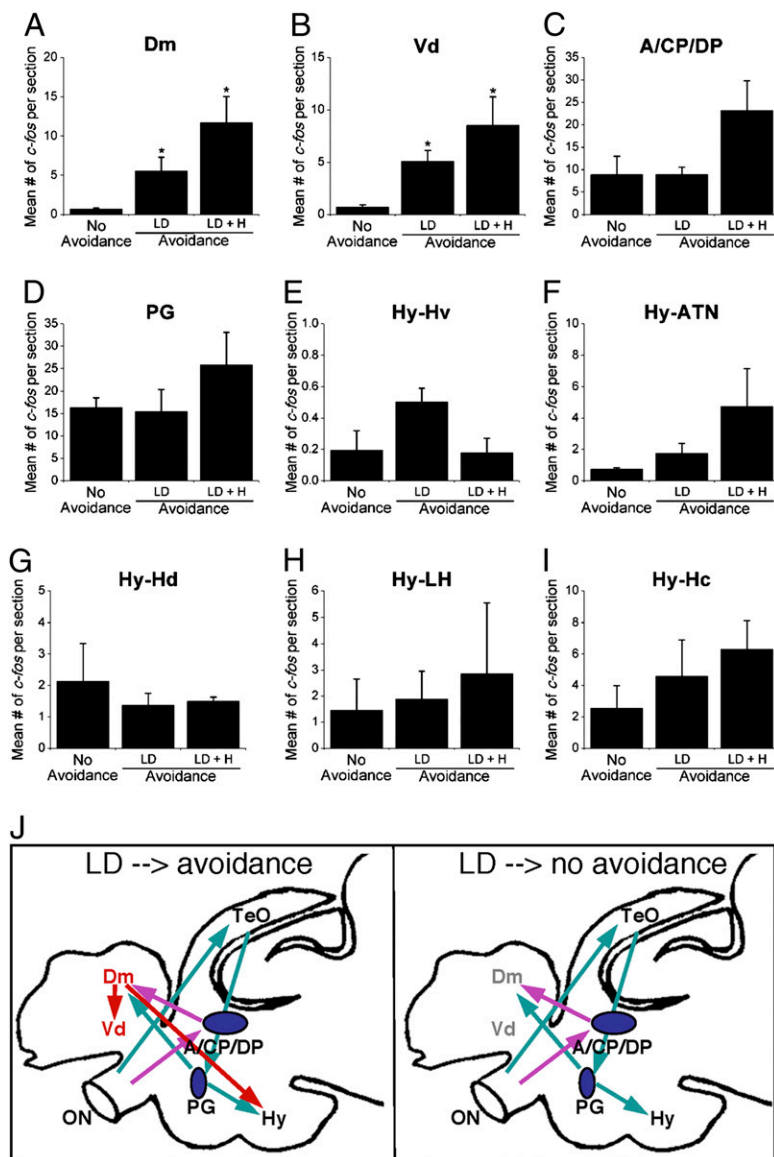
**Fig. 3.** The L/D visual sensory stimulus is sufficient to trigger the avoidance behavior that is reversible by buspirone. (A) Scheme of the experimental design that allows the sensory stimulus of a single modality (visual) to be presented and compared with the effect of visual stimulus plus handling. (B) L/D visual stimulus alone is capable of evoking an avoidance behavior similar to that evoked by L/D + H. Mean  $\pm$  SEM values are shown ( $n = 19$  for each group;  $P = 0.13$ ,  $t$  test). (C) Effect of chlordiazepoxide and buspirone on the choice behavior evoked solely by the L/D visual sensory stimulus. Mean  $\pm$  SEM values are shown ( $n = 8$ ;  $*P < 0.05$ , comparing buspirone-treated with control, ANOVA followed by Dunnett's post hoc test).

During the L/D choice behavioral testing, we noted that a small percentage of animals ( $\sim 10\%$ ), which were siblings of the rest of the animals tested, displayed little avoidance yet had normal visual sensory capacity as assessed by the visual acuity test (Fig. S4A and Movie S2). These nonresponders also did not show significant avoidance behavior when exposed to more intense light (Fig. S4B) or given more time to respond (Fig. S4C). The presence of light-avoidant vs. non-light-avoidant zebrafish thus presented an opportunity to compare the underlying neuronal activity and identify potential neural correlates for the preference behavior. Zebrafish were subjected to the L/D visual stimulus, and their choice behavior was quantified by the choice index. Analyses of *c-fos* expression were carried out in animals that exhibited high avoidance (with choice indices of 0.2 and higher) as well as in animals that displayed little avoidance (with choice indices of 0.02 and lower).

The results of *c-fos* expression are shown in Fig. 4, and the anatomical regions shown in Fig. 4 are schematized in Fig. S5. In







**Fig. 5.** Quantification of *c-fos* expression reveals differential activation of the Dm and Vd in high light-avoidant vs. low light-avoidant animals. Mean number of *c-fos* cells in the DM (A); Vd (B); A, CP, and DP (C); PG (D); ventral hypothalamus (E, Hy-Hv); anterior tuberal nucleus of hypothalamus (F, Hy-ATN); dorsal zone of periventricular hypothalamus (G, Hy-Hd); lateral hypothalamic nucleus (H, Hy-LH); and caudal hypothalamus (I, Hy-Hc). Mean  $\pm$  SEM are shown ( $n = 3$  for each group;  $*P < 0.05$  compared with no avoidance,  $t$  test). (J) Scheme of a core neural circuitry underlying the L/D choice behavior. Details are presented in the text.

sensory stimuli) (7) point to the existence of a brain circuitry that can dictate behavioral choices in the face of identical sensory input potentially to maximize survival and well-being. Here, we show that adult zebrafish display a simple L/D choice behavior to avoid the more illuminated space. It is conceivable that such choice is guided by two opposing instincts: One is to explore the entire space, and the other is to avoid a potentially “dangerous” environment. Using this paradigm, we have shown differential engagement of the Dm and Vd in the light-avoidant and non-light-avoidant animals. Given that the Vd is likely downstream of the Dm, it is plausible that the Dm plays a critical role as a “choice center” in this behavior. Interestingly, lesions of the Dm in the goldfish abrogate a conditioned avoidance behavior (36). Imaging studies in humans uncover a key role for the amygdala, the mammalian counterpart of the Dm, in decision making that comes from value-related predictions (37). Together, these findings suggest a potentially evolutionarily conserved role of the Dm/amygdala in mediating behavioral choices.

We have observed a choice reversal between larval and adult zebrafish, such that adults avoid light, whereas the juveniles prefer light. Furthermore, the light avoidance behavior in adult zebrafish can be reversed to light preference by treatment with buspirone,

a common anxiolytic that targets monoaminergic (27) signaling. Therefore, it would be of great interest to determine in the future whether this developmentally regulated choice reversal is attributable to age-dependent plasticity in monoaminergic neurons.

Three possible scenarios might explain the failure to display an avoidance behavior in some animals that are exposed to the same L/D visual sensory stimulus. First, although the visually related brain regions (e.g., A, CP, DP, PG) appear to be well-activated in these animals, such activation fails to recruit Dm because of insufficient presynaptic input from these sensory-processing brain nuclei to the Dm. Second, these animals may have an intrinsic defect in Dm excitability despite normal input from the A, CP, DP, and PG. Finally, these animals may have insufficient neuromodulatory tone, such as the noradrenergic drive (38), to facilitate Dm recruitment. It is also of great interest to investigate further whether the L/D choice behavior exhibits plasticity in a given animal, a question that we were not able to address in this study because animals had to be killed after a single behavioral readout to perform *c-fos* labeling. Together, the simplicity of this unimodal visual sensory stimulus-evoked L/D choice behavior in a genetically amenable vertebrate affords a great opportunity to explore further how the Dm becomes



differentially activated between light-avoidant and non-light-avoidant fish and how such activation may dictate the animal's choice to avoid the light.

## Materials and Methods

**Experimental Animals.** Adult zebrafish (AB, EK, and Wik) were maintained and bred following standard procedures (39).

**Behavioral Assays.** Animals tested in a given experiment were siblings group-housed (8–12 per housing unit) in identical tanks and tested in identical experimental settings. Light avoidance assays were carried out in either a behavioral chamber (taped half dark and half light) or a transparent chamber with a computer-projected L/D visual image from the bottom. Locomotor activity was assessed by video recording and analyzed by a Dynamic Image Analysis System (Soltec) or by Ethovision (Noldus) and Excel (Microsoft). Visual acuity assay was carried out as previously described (40).

**Pharmacological Study.** Buspirone hydrochloride and chlordiazepoxide hydrochloride were purchased from Sigma-Aldrich. Zebrafish were treated for 1 h, followed by behavioral analyses as described above.

**Statistical Analysis.** The Student's *t* test or one-way ANOVA, followed by post hoc tests (whenever appropriate, either Bonferroni, Dunnett's, or Tukey's test), were used. Data are considered significantly different when *P* < 0.05.

**Analysis of *c-fos*.** The *c-fos* gene sequence information (National Center for Biotechnology Information Gene ID code 394198) was used to prepare the in situ probe. For *c-fos* induction, 30 min after initial exposure to either L/D or L/D + H, zebrafish were killed and their brains were processed for in situ hybridization and counterstained with nuclear fast red. A time frame of 30 min was chosen, based on many previous studies showing the highest expression of *c-fos* mRNA 30 min after behavioral induction of neuronal activities [reviewed in (41) and references therein]. Images were taken with a Zeiss compound microscope. For the quantitative analysis of *c-fos*-positive cells, total numbers of *c-fos*-positive cells were counted in all sections containing the brain regions of interest. The interpretation of neuroanatomy follows the adult zebrafish brain atlas (42). Data were presented as the average number of *c-fos*-positive cells per 30- $\mu$ m section.

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