The Calmodulin-interacting peptide Pcp4a regulates feeding state-dependent behavioral choice in zebrafish

Highlights

- Food intake regulates production of the intracellular peptide Pcp4a in the brain
- Pcp4a modulates behavioral choice in an approach/avoidance assay
- Pcp4a controls visual size tuning of pcp4a+ neurons in the tectum
- The dopaminergic system modulates visual response properties of tectal pcp4a+ neurons

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In brief

Zaupa et al. identify the intracellular peptide Pcp4a as a mediator of the modulatory action of feeding state on behavioral choice. Food intake represses expression of pcp4a via activation of dopamine D2 receptors, leading to disinhibition of CaMKII and possibly increased CaMKII-dependent weakening of responses to small visual stimuli.
The Calmodulin-interacting peptide Pcp4a regulates feeding state-dependent behavioral choice in zebrafish

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https://doi.org/10.1016/j.neuron.2024.01.001

SUMMARY

Animals constantly need to judge the valence of an object in their environment: is it potential food or a threat? The brain makes fundamental decisions on the appropriate behavioral strategy by integrating external information from sensory organs and internal signals related to physiological needs. For example, a hungry animal may take more risks than a satiated one when deciding to approach or avoid an object. Using a proteomic profiling approach, we identified the Calmodulin-interacting peptide Pcp4a as a key regulator of foraging-related decisions. Food intake reduced abundance of protein and mRNA of \textit{pcp4a} via dopamine D2-like receptor-mediated repression of adenylate cyclase. Accordingly, deleting the \textit{pcp4a} gene made zebrafish larvae more risk averse in a binary decision assay. Strikingly, neurons in the tectum became less responsive to prey-like visual stimuli in \textit{pcp4a} mutants, thus biasing the behavior toward avoidance. This study pinpoints a molecular mechanism modulating behavioral choice according to internal state.

INTRODUCTION

To live and thrive in complex environments, animals and humans have to make decisions to select optimal behaviors in response to external stimuli and internal needs. Internal states have a strong influence on decision-making.\textsuperscript{1–4} For example, hunger can increase the propensity to take risks and to tolerate aversive stimuli.\textsuperscript{5–10} Moreover, metabolic state can modulate processing of sensory cues influencing decision-making. In rodents, non-human primates, and humans, hunger enhances responses to images of food in several cortical areas involved in visual processing and in structures of the limbic system such as the amygdala.\textsuperscript{11–16} Modulation of sensory processing may represent an efficient way to bias behavioral selection processes, which need to be fast for ensuring an animal’s survival, such as deciding to escape or not from potential danger.\textsuperscript{16}

While some progress has been made in identifying the brain regions and neuronal circuits mediating the effect of internal states on decision-making,\textsuperscript{17,23} the molecular mechanisms orchestrating the actions of these circuits are still largely unknown.\textsuperscript{17} Indeed, although several neurotransmitter systems have been implicated in modulating decision-making,\textsuperscript{17,21} little is known about the downstream molecular machinery. This current situation is in stark contrast to the very detailed knowledge of molecular events regulating other cognitive functions such as learning and memory.\textsuperscript{22}

Here, we used zebrafish larva as a model organism to identify molecular regulators of action selection. Zebrafish larvae display several well-characterized motor behaviors, some of which are modulated by internal states.\textsuperscript{23} For example, they approach small moving visual stimuli simulating natural prey, such as rotifers and paramecia,\textsuperscript{24–26} and avoid larger ones resembling approaching predators.\textsuperscript{27,28} While stimulus size is an important factor dictating whether a fish will approach or avoid a visual stimulus,\textsuperscript{24,26,29} internal states also play a crucial role in this decision. Indeed, we previously found that feeding state in zebrafish is a strong regulator of behavioral choice.\textsuperscript{30} Food-deprived fish take more risks when deciding to approach or avoid ambiguous prey-sized visual stimuli, compared with fed animals. Moreover, small visual stimuli resembling prey are overrepresented in the tectum of food-deprived fish, a midbrain region critically involved in regulating prey capture and avoidance behaviors in zebrafish.\textsuperscript{27,28,31–33} Now we have identified the intracellular peptide Pcp4a as an essential
component of the molecular machinery regulating neuronal changes underlying feeding-state-dependent behavioral choice in zebrafish.

Pcp4a is homologous to the mammalian peptide PCP4 (also known as PEP-19), which inhibits the function of Calmodulin,34,35 a calcium-binding protein regulating neuronal excitability and synaptic plasticity.36,37 Accordingly, mice lacking PCP4 have altered cerebellar synaptic plasticity.38 We found that food intake represses expression of pcp4a in the brain. Correspondingly, food-deprived pcp4a mutant fish behave similarly to fed ones in an approach/avoidance decision assay, and their tectal neurons are less responsive to prey-like visual stimuli, like in fed fish. We also found that expression of pcp4a is repressed by the dopaminergic system through dopamine D2-like receptor-mediated inhibition of adenylyl cyclase. Fittingly, activation of D2-like receptors induces changes of visual size representation in the tectum similar to the ones present in pcp4a mutants. Together, our findings point to Pcp4a as a critical regulator of neuronal physiology underlying modulation of behavioral choice guided by internal states.

RESULTS

Food intake regulates transcription of pcp4a in the brain
To understand how feeding state regulates behavioral selection at the molecular level, we performed mass spectrometry analysis to compare the proteomes of food-deprived and fed larvae. With this approach, we identified 166 proteins displaying statistically significant differential abundance in fed versus food-deprived 7 days post-fertilization (dpf) wild-type (WT) larvae (Figure 1A; Table S1). These proteins are involved in several biological processes, including metabolic functions, interaction with nucleic acids, signal transduction, endocytosis/exocytosis, and regulation of cell cycle (Table S1). When choosing candidates for functional validation, we decided to focus on proteins known to regulate neuronal excitability and/or synaptic function and to be expressed in the zebrafish tectum. An outstanding candidate fulfilling these criteria was the peptide Pcp4a, which was 35% less abundant in fed compared with food-deprived fish (Figure 1B; Table S1). Since PCP4 was shown to regulate synaptic plasticity and potentially neuronal excitability via modulation of Calmodulin’s activity,34,35,38 and considering the fact that pcp4a is expressed in the tectum and few other regions of the central nervous system of zebrafish larvae39 (Figures 1C and S1), we decided to further explore a potential role of Pcp4a in regulating behavioral choice according to feeding state. We first tested if regulation of Pcp4a abundance by feeding state happens specifically in the brain. To this end, we measured amounts of pcp4a’s mRNA in the brains of 7 dpf food-deprived and fed larvae using real-time quantitative reverse-transcription polymerase chain reaction (RT-qPCR). In agreement with our mass spectrometry results, we found that pcp4a expression was lower in fed fish than in unfed ones (Figure 1D).

Together, these results indicate that food intake reduces abundance of Pcp4a in the brain, through a mechanism regulating transcription of its mRNA.

Lack of Pcp4a alters behavioral choice
We next tested whether Pcp4a could influence behavioral choice, by mutating the pcp4a locus using the CRISPR-Cas9 technique. We identified a mutation leading to a reading frame-shift and formation of an early stop codon before the sequence coding for the Calmodulin-binding domain (Figure 2A).

We employed an approach/avoidance decision assay in which a larva is confronted with visual stimuli shown from below in the form of small or large black circles on a white background, simulating a prey or a predator, respectively.29,30 (Figure 2B). We used the valence index to quantify the tendency of larvae to approach (positive valence) or avoid (negative valence) a stimulus of a certain size29,30 (Figure 2C). The valence index ranges from −1 (if only avoidances are performed) to +1 (if only approaches are performed), and it is equal to zero when larvae pursue or avoid visual stimuli with a 50% probability. It is important to note that in this assay the small visual stimuli, although resembling potential food, are not perfect imitations of prey and may be perceived ambiguously by fish. Indeed, this type of stimuli is able to induce both approaches and avoidances.29,30 This characteristic makes this assay ideal for testing modulatory effects on behavioral choice. We previously found that fed larvae have a more negative valence index for small circles, compared with food-deprived ones, suggesting a more conservative strategy when interacting with approaching visual stimuli.30 Since amounts of Pcp4a are lower in fed larvae (Figures 1B and 1D), we reasoned that if Pcp4a is involved in regulating approach/avoidance decisions, food-deprived pcp4a<sup>md78/md78</sup> fish may phenocopy fed larvae. Indeed, we observed that 7 dpf pcp4a<sup>md78/md78</sup> fish displayed a more negative valence index for small visual stimuli, compared with control food-deprived WTs (Figure 2C). On the other hand, we did not detect differences of valence index between fed pcp4a<sup>md78/md78</sup> and fed WT fish (Figure S2A), suggesting that mutating pcp4a does not enhance the effect of food intake in the decision assay. Unfed pcp4a mutants displayed increased probability of avoidance of small circles and same approach probability, compared with unfed WTs (Figure 2D), indicating that the reduction of valence index in the mutants is due to an increased propensity to avoid visual stimuli.

To rule out the possibility that the pcp4a<sup>md78</sup> mutation could lead to generalized problems in sensory-motor transformations, we also measured the probability of a fish to react to visual stimuli in the approach/avoidance decision assay, using the activity index (Figure 2E), which ranges from 0 (no interactions with stimuli) to 1 (when fish react to stimuli by approaching or avoiding them 100% of times). We found that pcp4a<sup>md78/md78</sup> fish, either fed or unfed, had unaltered probability of interaction with the visual stimuli, compared with WT controls (Figures 2E and S2B). Moreover, pcp4a<sup>md78/md78</sup> larvae did not display reduction of numbers of active interactions (Figure S2D), nor did they display impairment of spontaneous locomotion (Figures 2F and S2C) and optomotor response (Figures S2E–S2G), compared with WT fish. These results suggest that pcp4a<sup>md78/md78</sup> fish do not have generalized visual processing or motor impairments.

Taken together, these data indicate that Pcp4a regulates a decision-making process strongly modulated by feeding state.
Pcp4a modulates responses to visual stimuli in the tectum

We previously showed that food intake shifts population responses of periventricular neurons (PVNs) in the tectum toward large (predator-like) visual stimuli at the expense of responses to small (prey-like) stimuli.\textsuperscript{30} Since pcp4a is expressed by a fraction of PVNs at 7 dpf (Figures 1C and 3E), we hypothesized that their activity may be modulated by feeding state. To prove this, we performed calcium imaging in the tectum of 7 dpf \textit{elavl3:H2B-GCaMP6s} larvae in which the majority of neurons in the brain contain the nucleus-targeted, genetically encoded calcium indicator GCaMP6s\textsuperscript{40} (Figure 3A). To identify pcp4a-positive (\textit{pcp4a}+) neurons we performed post-mortem fluorescence in situ hybridization chain reaction (HCR)\textsuperscript{41} after \textit{in vivo} calcium imaging to detect localization of \textit{pcp4a}'s mRNA. We first recorded responses of tectal PVNs to black circles of various sizes presented to the larvae on a small screen positioned on one side of the fish (Figure 3B). We quantified the size tuning of each neuron with a weighted sum of visual stimulus sizes (measured as degree of visual angle) eliciting a neuronal response, with weights corresponding to normalized ΔF/F values of each response, which we termed weighted mean response (WMR) angle (see Filosa et al.\textsuperscript{30} and STAR Methods for a detailed explanation). We then fixed the larvae, performed \textit{in situ} HCR to detect mRNA expression changes in fed and not-fed larvae (Figure 1B). We observed a decrease in \textit{pcp4a} mRNA expression in fed larvae (Figure 1D) which was confirmed by quantitative real-time PCR (qPCR) (Figure 2A). To test whether this change in mRNA expression is translated into a change in neuronal activity, we measured neuronal responses to visual stimuli in fed and not-fed larvae (Figure 3B). We found that food intake shifts population responses of PVNs in the tectum toward large (predator-like) visual stimuli at the expense of responses to small (prey-like) stimuli, similar to our previous findings. We further showed that \textit{pcp4a} expression is modulated by feeding state, with a decrease in \textit{pcp4a} mRNA expression in fed larvae (Figure 1D). This suggests that \textit{pcp4a} may play a role in modulating neuronal responses to visual stimuli in the tectum, and that its expression is regulated by feeding state.
pcp4a's mRNA (which was detectable also in pcp4amd78/md78 larvae, likely due to lack or incomplete mutation-induced degradation of the transcript, allowing us to classify PVNs as pcp4a+ and pcp4a− also in pcp4amd78/md78 fish), and finally aligned the images of in vivo calcium imaging with the ones of the stained fish (using the in vivo GCaMP6s signal and the residual signal of GCaMP6s in the fixed larvae for the alignment) to classify each neuron responding to visual stimuli as pcp4a+ or pcp4a− (Figures 3B, 3C, and S3; STAR Methods, for a detailed explanation). In accord with our hypothesis, we found that the population response profile of pcp4a+ neurons was shifted toward larger circle sizes in fed larvae, compared with food-deprived ones.

Figure 2. Mutating pcp4a alters decision to approach or avoid small visual stimuli
(A) Schematic representation of the strategy for mutating the pcp4a gene using the CRISPR-Cas9 technique. The truncated protein, if produced, would lack the Calmodulin-interacting domain (red box in the wild-type protein sequence).
(B) Scheme of the behavioral setup for recording approach/avoidance decisions of zebrafish larvae.
(C) Graph depicting average valence indexes for different sizes of visual stimuli of 7 dpf unfed pcp4a+/+ and pcp4amd78/md78 larvae. Approach and avoidance probabilities were calculated as (approaches/[approaches + avoidances + neutral interactions]) or (avoidances/[approaches + avoidances + neutral interactions]), respectively. For approach probability, p = 0.7 (1/14), p = 0.9 (3/14), and p = 1.0 (5/14). For avoidance probability, p = 0.02 (1/14), p = 0.2 (3/14), and p = 0.04 (5/14).
(D) Graphs showing approach (top) or avoidance (bottom) probability for small visual stimuli in 7 dpf unfed pcp4a+/+ and pcp4amd78/md78 larvae. Approach and avoidance probabilities were calculated as (approaches/[approaches + avoidances + neutral interactions]) or (avoidances/[approaches + avoidances + neutral interactions]), respectively. For approach probability, p = 0.7 (1/14), p = 0.9 (3/14), and p = 1.0 (5/14). For avoidance probability, p = 0.02 (1/14), p = 0.2 (3/14), and p = 0.04 (5/14).
(E) Graph depicting average activity indexes for different sizes of visual stimuli of 7 dpf unfed pcp4a+/+ and pcp4amd78/md78 larvae. In (C)–(E), npcp4a+/+ = 22 larvae, npcp4amd78/md78 = 18 larvae. *p < 0.05; n.s., not significant; two-tailed t test with Bonferroni-Holm correction.
(F) Bar graph showing average spontaneous locomotion of 7 dpf unfed pcp4a+/+ and pcp4amd78/md78 larvae. npcp4a+/+ = 27 larvae, npcp4amd78/md78 = 19 larvae. n.s., not significant; two-tailed t test. Data in (C)–(F) are shown as mean ± standard error of the mean.
See also Figure S2.
A. \( \text{elavl3:H2B-GCaMP6s} \)

B. Live \( \text{Ca}^{2+} \) imaging

C. \( \text{pcp4a} \) in situ HCR

D. \( \text{pcp4a}^+ \) PVNs

E. \( \% \text{pcp4a}^+ \) PVNs

F. \( \% \text{pcp4a}^- \) PVNs

G. \( \% \text{SINs} \)

H. \( \text{elavl3:H2B-GCaMP6s} \)

I. \( \% \text{pcp4a}^+ \) SINs

J. \( \% \text{pcp4a}^- \) SINs

K. \( \% \text{pcp4a}^+ \) PVNs

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A similar shift was observed also in pcp4a−/− neurons (Figure S4A), indicating that the effect of food intake is not restricted only to PVNs expressing pcp4a. The percentage of pcp4a+ tectal PVNs and their fraction responding to the visual stimuli were not altered by feeding state (Figures 3E and S4B), indicating that food intake does not control differentiation of pcp4a+ PVNs or their general responsiveness to visual stimuli. Since abundance of Pcp4a is reduced by food intake, we proceeded to test whether mutating pcp4a in food-deprived fish would phenocopy the effect of feeding on representation of visual stimuli in the tectum, by performing calcium imaging of 7 dpf food-deprived elavl3:H2B-GCaMP6s; pcp4a+/−/md78/md78 and control food-deprived elavl3:H2B-GCaMP6s; pcp4a+/+ fish. In accord with this hypothesis, we found that in homozygous pcp4a mutants, the response profile was shifted toward large visual stimuli (Figure 3F). Interestingly, this effect was mainly limited to pcp4a+ neurons since the response profile of pcp4a−/− neurons was negligibly altered by the mutation in the pcp4a locus (Figure 3G). PVNs in fed pcp4a+/+/md78/md78 fish also displayed a small shift of responses toward large visual stimuli, compared with PVNs in fed pcp4a+/+ larvae (Figure S4C), suggesting that the reduction of Pcp4a abundance that we observed in fed fish does not have a saturation effect on modulation of visual response properties of pcp4a+ PVNs. However, this residual effect is likely too small to have an impact on behavior (Figure S2A). The percentage of pcp4a+ PVNs responsive to visual stimuli were similar in pcp4a+/+/md78/md78 and pcp4a+/− larvae (Figure S4D), suggesting that the pcp4a+/− mutation does not alter in a generalized way the physiology of PVNs.

Superficial interneurons (SINs) present at the outer edge of the tectal neuropil were shown to control size selectivity of tectal PVNs.22,23 Interestingly, we found that a small percentage of SINs express pcp4a (Figures 3H and 3I). We therefore wondered whether size tuning of SINs is altered by the pcp4a+/− mutation. However, we found that size tuning (Figure 3J) and percentage of SINs responsive to visual stimuli (Figure S4E) were not significantly different in pcp4a+/− fish, compared with pcp4a+/+ controls.

Since pcp4a is expressed in the retina,39 including in a small population of retinal ganglion cells (RGCs; Figures S1C and S1D), to rule out a role of Pcp4a in regulating responses of RGCs to the visual stimuli utilized in our experiments, we performed calcium imaging of axons of RGCs in the tectal neuropil of food-deprived 7 dpf atoh7:gai4; UAS:GCaMP6s; pcp4a+/md78/md78 and atoh7:gai4; UAS:GCaMP6s; pcp4a+/− larvae (Figure S4F). pcp4a+/−/md78/md78 fish did not display significant alterations of responses to the visual stimuli in the axons of RGCs (Figures S4G and S4H). These data, together with the fact that size tuning of SINs and pcp4a−/− tectal PVNs were not altered in mutant fish, suggest that Pcp4a regulates size tuning in PVNs in a cell-autonomous way.

It was shown that the mammalian PCP4, by interacting with Calmodulin, can inhibit the enzyme Ca2+/calmodulin-dependent kinase II (CaMKII), an important regulator of synaptic function and plasticity.37,44 We therefore hypothesized that CaMKII activity could be elevated in pcp4a mutant fish, and inhibiting CaMKII could revert the shift toward large visual stimuli observed in mutant fish. To test this hypothesis, we used the CaMKII inhibitor KN-93.45 Application of KN-93 to 7 dpf unfed elavl3:H2B-GCaMP6s; pcp4a+/−/md78/md78 fish abolished the shift of visual size responses in pcp4a+ PVNs observed in elavl3:H2B-GCaMP6s; pcp4a+/−/md78/md78 larvae treated with a control solution (Figure 3K). KN-93 did not induce a shift of responses toward smaller visual stimuli in WT fish (Figure 3K), suggesting that the amount of Pcp4a present in PVNs of unfed fish is sufficient to fully inhibit the CaMKII-mediated effect on size tuning modulation.

The serotonergic and dopaminergic systems control expression of pcp4a

We next wanted to identify neurotransmitter systems able to regulate expression of pcp4a. We first focused our attention on the serotonergic system, as we previously showed that it regulates approach/avoidance decisions and filtering of visual stimuli in the tectum.33 Since we found that serotonergic neurons in the raphe nucleus are more active in food-deprived larvae and that enhancing serotonergic transmission in fed fish phenocopies
Pretectum

A. Serotonin

B. Dopamine (non-selective)

C. Dopamine (D1-selective)

D. Dopamine (D2-selective)

E. Acetylcholine

F. Pretectum

G. th:gal4-VP16; UAS:EGFP-CAAX

H. IERK pERK

I. Pretectum

J. DC2

K. DC4/5

L. EGFP drd2a pcp4a Merge
ev3:gal4; UAS:EGFP

drd2b pcp4a Merge

M. % of pcp4a+ PVNs also drd2a+ or drd2b+

N. pcp4a mRNA fold change

O. pcp4a mRNA fold change

(legend on next page)
the effect of food deprivation, we tested whether increasing serotonergic transmission with the selective serotonin reuptake inhibitor fluoxetine in fed larvae could lead to changes of pcp4a's expression. After treating 7 dpf fed fish for 4 h with fluoxetine, we performed RT-qPCR with samples obtained from their dissected brains. In accord with our hypothesis, we found that pcp4a was upregulated by fluoxetine (Figure 4A). However, the increase of pcp4a's expression was relatively small. We therefore wondered if other neurotransmitters could also contribute to regulate transcription of pcp4a. We decided to interfere with dopaminergic transmission because it was also shown to modulate decision-making. Since several studies showed that in mammals food intake induces release of dopamine, and considering the fact that we observed reduced expression of pcp4a in fed fish, we tested whether activating dopaminergic transmission in food-deprived larvae could reduce expression of pcp4a. Interestingly, treating 7 dpf food-deprived WT larvae with the non-subtype-selective dopamine receptor agonist apomorphine decreased expression of pcp4a (Figure 4B). Dopamine receptors are divided into two groups: D1-like (D1 and D5 subtypes) and D2-like (D2, D3, and D4 subtypes). To identify the type of receptor mediating the effect of dopamine on pcp4a's expression, we treated 7 dpf food-deprived WT larvae with either the D1-like-selective agonist SKF-38393 or the D2-like-selective agonist quinpirole. Quinpirole, but not SKF-38393, was able to reduce expression of pcp4a (Figures 4C and 4D), indicating that D2-like, but not D1-like, receptors activate a signaling cascade leading to repression of pcp4a's expression. We also tested whether cholinergic transmission could regulate expression of pcp4a. However, we found that enhancing cholinergic transmission with the cholinesterase inhibitor donepezil in food-deprived WT larvae did not induce changes of pcp4a's mRNA level (Figure 4E).

Since we observed that dopaminergic signaling is able to inhibit transcription of pcp4a—the same effect induced by feeding—we next tested whether food intake is capable of activating dopaminergic neurons in the zebrafish brain, which are subdivided in several clusters. 

Several dopaminergic neurons, including the ones in the pretectum and in the DC1, DC2, DC4, and DC5 clusters, project axons to the tectum. It is therefore possible that dopaminergic transmission via D2-like receptors could directly control expression of pcp4a in tectal PVNs. In support of this hypothesis, we found that a fraction of pcp4a+ PVNs express the two zebrafish D2-receptor-coding paralogous genes drd2a and drd2b (Figures 4L and 4M). We next investigated how dopaminergic transmission could alter expression of pcp4a. Activation of D2-like receptors leads to inhibition of cyclic AMP (cAMP) production by the enzyme adenylyl cyclase, and subsequent repression of target genes expression via the protein kinase A (PKA)-cAMP response element-binding protein (CREB) pathway. We therefore reasoned that if pcp4a's expression is repressed by D2 receptors through inhibition of this pathway, increasing amounts of intracellular cAMP should induce expression of pcp4a. Indeed, we found that treating WT zebrafish larvae for 1 h with the adenylyl cyclase activator forskolin increased pcp4a's expression (Figure 4N). Moreover, as further confirmation that adenylyl cyclase acts downstream of D2-type receptors to regulate expression of pcp4a, we observed that forskolin could rescue quinpirole-induced repression of the gene's expression (Figure 4O).

These data suggest that food intake in zebrafish activates a subset of dopaminergic neurons, which represses expression of pcp4a through D2-like receptor-mediated inhibition of cAMP production. Concurrently, feeding also reduces serotonergic transmission, inhibiting its effect on induction of pcp4a's transcription.
Figure 5. Activation of dopamine D2-like receptors modulates visual size representation in the tectum

(A) Images showing part of the tectum of a food-deprived 7 dpf elavl3:H2B-GCaMP6s larva before and after treatment with quinpirole. Scale bars, 50 μm.
(B and C) Graphs depicting cumulative percentages of WMR angles of pcp4a+ (B) or pcp4a− (C) PVNs in unfed 7 dpf elavl3:H2B-GCaMP6s larvae before and after 3-h treatment with quinpirole or in control unfed 7 dpf elavl3:H2B-GCaMP6s fish not treated with quinpirole but kept in agarose for the same duration of the drug treatment. n = 5 fish per group.
(D) Confocal images showing localization of pcp4a, vglut2, and gad1b mRNA in tectal PVNs in 7 dpf elavl3:gal4, UAS:EGFP larvae. Scale bars, 10 μm.
(E) Bar graph showing percentages of glutamatergic (vglut2+) or GABAergic (gad1b+) pcp4a+ PVNs. n = 6 larvae per group.
(F and G) Graphs showing cumulative percentages of WMR angles of pcp4a+ and gad1b+ (F) or pcp4a+ and gad1b− (G) PVNs in unfed 7 dpf elavl3:H2B-GCaMP6s larvae before and after 3-h treatment with quinpirole or in control unfed 7 dpf elavl3:H2B-GCaMP6s fish not treated with quinpirole, imaged in two consecutive sessions 3 h apart. Data in these graphs are subsets of the data shown in (B). n = 4 fish per group. ***p < 0.001; n.s., not significant; two-way ANOVA. Data are shown as mean ± standard error of the mean.

See also Figure S6.
Figure 6. Dopamine D2-like receptor signaling modifies visual size tuning of gad1b+ and gad1b–pcp4a+ PVNs

(A) Bar graph depicting percentages of pcp4a+/gad1b+ PVNs, imaged in two sessions 3 h apart, classified as persistent, lost, and gained in quinpirole-treated and untreated (control) 7 dpf elavl3:H2B-GCaMP6s larvae. n = 4 fish per group.

(B) Graph showing tuning size (WMR angle) of persistent pcp4a+/gad1b+ PVNs before and after quinpirole treatment. n = 24 neurons from 4 larvae.

(C) Graph displaying percentages of persistent pcp4a+/gad1b+ PVNs changing response type in the two imaging sessions. Small, neurons responding to circles \( \leq 5 \); large, neurons responding to circles \( \geq 10 \); dual, neurons responding to both types of stimuli. n = 4 larvae per group.

(D) Graph showing percentages of persistent, lost, and gained pcp4a+/gad1b– PVNs in quinpirole-treated and untreated (control) larvae. n = 4 fish per group.

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Dopaminergic transmission modifies size tuning of tectal pcp4a+ PVNs

We previously showed that serotonergic transmission, which is enhanced by food deprivation, modifies population responses of tectal PVNs by inducing activation of neurons responsive to small-sized visual stimuli. We therefore asked whether activation of dopamine D2-like receptors could also change visual size representation in the tectum. We performed an experiment in which we first recorded responses of tectal PVNs to visual stimuli of different sizes, using calcium imaging in 7 dpf food-deprived elav13:H2B-GCaMP6s fish. We then treated the larvae, still embedded in agarose, with the dopamine D2-like receptor agonist quinpirole for 3 h. Afterward, we performed calcium imaging again to monitor responses to visual stimuli of the PVNs imaged during the first session. Finally, we identified pcp4a+ neurons with post-mortem in situ HCR and alignment of the in vivo and post-mortem image stacks. With this strategy, we were able to directly compare size tuning of individual PVNs before and after treatment with quinpirole (Figures 5A, S6A, and S6B). We identified three major classes of neurons based on their responses in the two imaging sessions: persistent (responding to visual stimuli of any size in both sessions), lost (responding only in the first session to stimuli of any size), and gained (responding only in the second session to stimuli of any size). Interestingly, we found that quinpirole shifted the response profile of pcp4a+ PVNs toward large sizes of the visual stimuli (Figure 5B), similar to the change caused by the pcp4a-md78 mutation (Figure 3F) or by food intake (Figure 3D). In contrast, treatment with quinpirole did not have a major impact on responses to visual stimuli in pcp4a– PVNs (Figure 5C). Moreover, we did not detect changes of size tuning between the two imaging sessions in PVNs of fish not treated with quinpirole (Figure 5B). Interestingly, we found that treating unfed pcp4a-md78/md78; elav13:H2B-GCaMP6s larvae with quinpirole did not alter responses to visual stimuli in pcp4a+ PVNs (Figure S6C). These data suggest that activation of dopamine D2-like receptors alters size tuning of pcp4a+ tectal PVNs, mainly through the downstream action of Pcp4a.

To better understand how D2-like receptor signaling modifies size representation in the tectum, we identified the neurotransmitter phenotype of pcp4a+ PVNs. Using in situ HCR to detect mRNAs coding for pcp4a, vglut2 (a glutamatergic marker), and gad1b (a GABAergic marker), we found that the majority (75.1% ± 2.3%) of pcp4a+ PVNs in the tectum are glutamatergic, while a smaller population (36.2% ± 0.6%) is GABAergic (75.1% ± 2.3%) of gad1b (a GABAergic marker). We therefore asked whether activation of dopaminergic tectal PVNs by inducing activation of neurons responsive to large visual stimuli in fish treated with quinpirole and untreated controls. nLost-Control = 55 neurons from 4 larvae, nLost-Quinpirole = 76 neurons from 4 larvae, nGained-Control = 82 neurons from 4 larvae, nGained-Quinpirole = 34 neurons from 4 larvae. *p = 0.03; n.s., not significant; nested two-way ANOVA.

For the calcium imaging experiment in which responses of PVNs were recorded before and after treatment with quinpirole (Figures 5B and 5C), we used the pcp4a-md78/md78; gad1b+/+ subpopulation. In pcp4a+/+ PVNs, we did not observe a net change of number of neurons of the persistent, lost, and gained types after quinpirole treatment, compared with untreated controls (Figure 6A). However, we found that quinpirole shifted the size tuning of the persistent neurons by making them more responsive to large stimuli than small ones (Figure 6B). This change was largely due to dual-responding neurons, which were activated by both small (≤5") and large (≥10") circles, becoming responsive only to large stimuli (Figure 6C). We did not detect alterations of size tuning in control untreated fish during the two imaging sessions (Figure S6D). In the pcp4a+/+ PVNs — subpopulation, we observed a net loss of visual-responsive neurons due to increased numbers of lost PVNs and decreased numbers of gained ones in the tecta of quinpirole-treated fish, compared with untreated controls (Figure 6D). While size tuning of lost PVNs did not differ between quinpirole-treated and untreated fish (Figure 6E), gained neurons were, on average, tuned to larger visual stimuli (Figure 6F). In addition, we observed that tuning of the persistent type of neurons was also shifted toward larger stimuli by quinpirole (Figure 6G), but not in control untreated larvae (Figure S6E). Similar to what we observed in the pcp4a+/+ PVNs, also in pcp4a+/+ neurons this shift was largely due to suppression of responses to small stimuli in dually tuned neurons, which became activated only by large visual stimuli (Figure 6H).

Taken together, these results suggest that activation of dopamine D2-like receptors alters visual stimulus size representation in both glutamatergic and GABAergic pcp4a+ neurons, largely by suppressing activation of dually tuned PVNs by small visual stimuli.

DISCUSSION

While molecular events regulating some cognitive functions such as learning and memory are known in great detail, little is known about biochemical pathways responsible for behavioral choice. Studies in invertebrates have identified several mechanisms that regulate feeding state-dependent behavioral choice in zebrafish. Neuron (2024). https://doi.org/10.1016/j.neuron.2024.01.001
neurotransmitters and neuropeptides involved in decision-making. A number of these neurotransmitters were shown to play crucial roles also in decision-making in rodents, and possibly humans. These findings point to the existence of fundamental, evolutionarily conserved, mechanisms regulating behavioral choice. However, the molecular events downstream of the neurotransmitter receptors have remained largely elusive.

Recently, robust behavioral assays have been used to quantitatively analyze behavioral choice in zebrafish. With these assays, neuronal circuits underlying evidence accumulation leading to action selection, initiation of alternative escape strategies in response to acoustic/vibrational stimuli, and choice of approach or avoidance of visual stimuli have been revealed.

Now, our work identified the Calmodulin-interacting peptide Pcp4a as a key regulator of behavioral choice in zebrafish. We found that abundance of Pcp4a in the brain is regulated by feeding state. Food intake represses expression of pcp4a in the brain, and mutating pcp4a induces food-deprived fish to behave like fed ones by making them take less risks in an approach/avoidance decision assay. This finding pinpoints the identity of an intracellular regulator of behavioral selection in a vertebrate and may pave the way to a better understanding of the molecular basis of action selection also in mammals.

Pcp4a is expressed in a subpopulation of neurons in the tectum, a brain region homologous to the mammalian superior colliculus, centrally involved in regulating prey capture and avoidance of threats. Tectal PVNs display several types of visual size selectivity, with some of them tuned specifically to either small or large stimuli, whereas others respond to a wide range of stimulus sizes. Tectal PVNs that are activated by both small and large visual stimuli, whereas others respond to a wide range of stimulus sizes. Tectal PVNs that are activated by both small and large visual stimuli, whereas others respond to a wide range of stimulus sizes. Tectal PVNs that are activated by both small and large visual stimuli, whereas others respond to a wide range of stimulus sizes. Tectal PVNs are involved in regulating size representation in the tectum.

Accordingly, we found that food intake activates several groups of dopaminergic neurons in the zebrafish brain. These results agree with previous findings in rodents and humans showing that food ingestion induces activation of the dopaminergic system, and they further expand our understanding of the role of dopamine in decision-making. Our data showed that two D2 receptors, drd2a and drd2b, are expressed by tectal PVNs, suggesting that dopamine could act directly on these neurons to regulate expression of pcp4a. In support of this hypothesis, we showed that dopamine could act directly on these neurons to regulate expression of pcp4a. In support of this hypothesis, we showed that dopamine could act directly on these neurons to regulate expression of pcp4a. In support of this hypothesis, we showed that dopamine could act directly on these neurons to regulate expression of pcp4a.

Pcp4a acts most likely in a cell-autonomous way in regulating responses of PVNs to visual stimuli, since we found that the effect of mutating pcp4a is mostly limited to neurons expressing the gene. In support of this hypothesis, we found that activity of SINs, which were shown to be involved in regulating size filtering of visual information, and responses to visual stimuli in RGC axons were not altered in pcp4a mutants. However, we found that a very small population of RGCs expresses pcp4a. Considering the low number of these cells, it is possible that we could not detect Pcp4a-mediated modulation of their activity when we analyzed visual response properties of the whole population of RGCs, and we cannot completely rule out their potential involvement in modulating size tuning of tectal PVNs.

In pcp4a mutants, responses of pcp4a+ PVNs to small visual stimuli are reduced, suggesting that Pcp4a selectively enhances this type of responses in the tectum. There are two main ways by which Pcp4a could induce this size-specific modulation: by promoting activation of neurons responding only to small stimuli or by selectively enhancing responses to small stimuli in dually tuned neurons. Interestingly, we found that the major effect of activating dopaminergic D2-like receptors, which represses expression of pcp4a, was to suppress responses to small visual stimuli in dually tuned pcp4a+ PVNs, which are the most abundant population of pcp4a+ PVNs in the tectum.

We found that enhancing serotonergic transmission with fluoxetine increases expression of pcp4a. This action of serotonin is in agreement with our previous results showing that food deprivation in zebrafish activates serotonergic neurons in the raphe nucleus and that enhancing serotonergic transmission increases responses to small visual stimuli in tectal PVNs. However, the effect of fluoxetine on pcp4a’s expression was relatively subtle, suggesting that more than one neurotransmitter system contribute to regulate transcription of pcp4a. Indeed, we showed that activating dopamine D2-like receptors represses transcription of pcp4a and reduces responses of tectal pcp4a+ PVNs to small visual stimuli, a phenotype that is also present in homozygous pcp4a mutants.

We found that enhancing serotonergic transmission in-deprivation in zebrafish activates serotonergic neurons in the raphe nucleus and that enhancing serotonergic transmission in-deprivation in zebrafish activates serotonergic neurons in the raphe nucleus and that enhancing serotonergic transmission in-deprivation in zebrafish activates serotonergic neurons in the raphe nucleus.
observed that feeding state is capable of altering visual responses in both pcp4a+ and pcp4a− neurons, suggesting that multiple circuit mechanisms for modulating foraging behavior may exist in the tectum, likely involving diverse types of neurons and multiple modulatory systems, including the dopaminergic and serotonergic ones.30

How could modification of size tuning in dually tuned pcp4a+ PVNs induce changes of approach/avoidance behavior? It is possible that they could integrate information about both potential preys and predators and act at a critical circuit interface of sensory and motor networks, where they could be optimal targets of neuromodulatory circuits activated by feeding state for biasing motor outputs toward approach or avoidance, depending on the physiological state of the animal. Such a role at a critical circuit node could also explain their relative low number in the tectum (circa 3% of tectal PVNs are both pcp4a+ and drd2a/b+, according to our estimation). The SINs, another small population of tectal neurons, regulate filtering of visual size information entering the tectum.46,47 The pcp4a+ PVNs identified by us may act in a similar fashion at the output stage. The fact that activation of D2-like receptors affects size tuning of both GABAergic and non-GABAergic dually tuned pcp4a+ PVNs suggests that multiple subtypes of neurons may be involved.

It is possible that Pcp4a regulates postsynaptic activity in these neurons in a synapse-specific manner. This specificity could be achieved, for example, by localizing Pcp4a or dopamine receptors, or both, to synapses receiving inputs conveying information about small stimuli. Alternatively, the activation mode of different synapses could lead to different dynamics of Pcp4a-mediated suppression of CaMKII activity, which we have shown to be important for mediating the action of Pcp4a on modulation of size tuning, in line with a previous in vitro study showing that inhibition of CaMKII by mammalian PCP4 depends on the type of intracellular calcium mobilization.34 Our finding that Pcp4a modulates size tuning of PVNs via CaMKII indicates that its molecular mode of action is conserved across vertebrates, and it also points to a previously unknown role of this molecular interaction in modulating sensory-motor transformations.

STAR METHODS

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QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.neuron.2024.01.001.

ACKNOWLEDGMENTS

We would like to thank Anne Banerjee and the staff of the Zebrafish Facility and the Advanced Light Microscopy Technology Platform at the Max Delbrück Center for Molecular Medicine for technical support. We are also thankful to Marco dal Maschio for advice on analysis of calcium imaging data. This work was supported by the Helmholtz Association of German Research Centers, the Max Planck Society, and by grants from the German Research Foundation (Deutsche Forschungsgemeinschaft, project ID: FI 2339/1-1, and SFB 870 – Assembly and Function of Neural Circuits, TP16).

AUTHOR CONTRIBUTIONS

A.F. conceived and designed all experiments, some of which were started in H.B.’s laboratory and were continued in his own laboratory; provided supervision; administered the project; and performed data analyses. S.S. and H.B. provided supervision. M.Z. and N.N. designed and performed experiments and analyzed data. A.S. analyzed data. A.F. and M.Z. wrote the manuscript, with contributions by H.B. All authors commented on the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: March 1, 2023
Revised: October 6, 2023
Accepted: January 2, 2024
Published: January 30, 2024

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Neuron 112, 1–15, April 3, 2024 13


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## STAR METHODS

### KEY RESOURCES TABLE

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### Critical commercial assays

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### Experimental models: Organisms/strains

| Zebrafish: Tg(elavl3:H2B-GCaMP6s)^{65} | Freeman et al. | ZFIN: ZDB-ALT-141023-2 |
| Zebrafish: Tg(elavl3:Gal4-VP16)^{45P} | Kimura et al. | ZFIN: ZDB-ALT-090116-2 |
| Zebrafish: Tg(14XUAS:Gal4CaMP6s)^{mpm10f6} | Tiele et al. | ZFIN: ZDB-ALT-140811-3 |
| Zebrafish: Tg(-7atoth7:Gal4-VP16)^{1992t} | Del Bene et al. | ZFIN: ZDB-ALT-110912-2 |
| Zebrafish: Tg(5XUAS:EGFP)^{hkuasg9b} | Asakawa et al. | ZFIN: ZDB-ALT-080528-1 |

(Continued on next page)
Lead contact
Further information and requests for resources and reagents should be directed to the lead contact, Alessandro Filosa (alessandro.filosa@mdc-berlin.de).

Materials availability
The pcp4a<sup>md78</sup> zebrafish line is available upon request.

Data and code availability
- Mass spectrometry data have been deposited at MassIVE and are publicly available as of the date of publication. The accession number is listed in the key resources table. Other data reported in this paper will be shared by the lead contact upon request.
The Calmodulin-interacting peptide Pcp4a regulates feeding state-dependent behavioral choice in zebrafish, Neuron (2024), https://doi.org/10.1016/j.neuron.2024.01.001

Please cite this article in press as: Zaupa et al., The Calmodulin-interacting peptide Pcp4a regulates feeding state-dependent behavioral choice in zebrafish, Neuron (2024), https://doi.org/10.1016/j.neuron.2024.01.001

- All original code has been deposited at Zenodo and is publicly available as of the date of publication. DOIs are listed in the key resources table.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Zebrafish lines and maintenance
Zebrafish were kept under standard conditions at 28.5°C on a 14 hr/10 hr light/dark cycle. Embryos and larvae were kept in Danieau’s medium (58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO4, 0.6 mM Ca(NO3)2, 5 mM HEPES, pH adjusted to 7) at a density of approximately 40 fish in a 90 mm plastic Petri dish. Unless otherwise stated, experiments were performed at 7 dpf. All animal procedures were conducted in accordance with institutional (Max Delbrück Center for Molecular Medicine, and Max Planck Institute for Biological Intelligence), State (LAGeSo Berlin, and Regierung Oberbayern), and German ethical and animal welfare guidelines and regulations, and according to protocols approved by LAGESo. Larvae were euthanized, following anesthesia with tricaine, using hypothermic exposure (ice bath) for 20 minutes. Sex of zebrafish cannot be determined at the developmental stages considered in this study. Animals were randomly assigned to experimental groups. The following previously established transgenic lines were used in this study: Tg[elavl3:H2B-GCaMP6s]Tg53, Tg[elavl3:Ga4]nn7Tg67, Tg[5XUAS:EGFP]Tg67, Tg[UAS:EGFP-CAAX]Tg67, Tg[atoh7:Gah]Tg1992Tg, Tg[SXUS:EGFP]Tg1233, Tg[th:Ga4-VP16]Tg1230 Tg[UAS:EGFP-CAAX]Tg67.

The pcp4a	extsuperscript{md78} mutant line was generated using the CRISPR/Cas9 technique. A guide RNA (5’-GGAAGCATCAAACCCTTCTG-3’) targeting exon 2 of pcp4a was synthesized following an established protocol. The sgRNA (100 ng/μl) and Cas9 protein (600 ng/μl) were injected in wild type embryos at one-cell stage. Founders were selected for an 8 bp deletion leading to a frameshift mutation and formation of a premature stop codon in exon 3.

For genotyping fish, the genomic region containing the pcp4a	extsuperscript{md78} mutation was amplified by PCR using the primers 5’-GAAAA CAGACATCCCGCTGTG-3’ and 5’-CCCCCACAAATCCAAAGACGTG-3’, which in wild types produce a 632 bp DNA fragment. After digestion with the restriction enzyme DdeI (New England BioLabs, Cat# R0175L), the DNA fragment amplified from the wild-type allele produces 2 bands of 484 bp and 148 bp. Since the mutant allele is missing the DdeI restriction site, an uncut band of 624 bp is produced.

METHOD DETAILS

Feeding protocol
At 5 dpf, siblings were split in 90 mm Petri dishes with Danieau's medium at approximately 40 fish per dish. Larvae in the fed groups were fed one time at 5 dpf and two times per day from 6 dpf with dry food (SDS-100, Special Diets Services). The medium was changed prior to each feeding. Fish in the food-deprived groups did not receive food, and the medium was changed with the same timing and frequency as in the fed groups. Prior to experiments, gut content was inspected under a stereomicroscope to ensure that fish in the fed groups ingested food.

Mass spectrometry
To obtain each sample for mass spectrometry analysis, we pooled ten 7 dpf larvae (either fed or not fed). After euthanasia, larvae were transferred into 1.5 ml microcentrifuge tubes. Water was removed from the tubes, and the larvae were fast-frozen with liquid nitrogen and stored at -80°C. After thawing them on ice, the samples were re-suspended in 20 μl of 6 M guanidium hydrochloride in 25 mM Tris buffer containing 10 mM tris(2-carboxyethyl)phosphine (TCEP) and 40 mM chloroacetamide, pH 8.0. The samples were then heated at 95°C for two minutes and sonicated using a Bioruptor sonicator (Diagenode) for ten minutes at maximum power for ten cycles. The heating and sonication steps were repeated twice to ensure complete lysis of the samples. Afterward, the samples were incubated for 30 minutes at 37°C, diluted in 200 μl of 20 mM ammonium bicarbonate solution, and then incubated with 1 μg of Trypsin (Promega) for overnight digestion. The digested peptides were purified on a 3 plug C18 StageTip (ThermoFisher) and loaded into a Q Exactive orbitrap mass spectrometer (ThermoFisher).

Raw data were processed in Maxquant version 2.2.0.0	extsuperscript{64} using UniProt fasta database from Danio rerio. Database searching was performed with initial mass deviation of 7 ppm and all identifications were filtered at 1 % for protein and peptide level. Match between proteins was enabled and label-free quantification was performed using the MaxLFQ algorithm. Protein groups file was then analyzed using Perseus 2.0.7.0 to perform two sample t tests. Differences of protein amounts were considered significant if the False Discovery Rate (FDR) was lower than 0.05. Protein functions indicated in Table S1 were obtained from UniProt (www.uniprot.org) and the Zebrafish Information Network (https://zfin.org).

Behavioral assays
The behavioral setup was positioned on a vibration isolation table and shielded from external illumination. The light source was a computer screen placed underneath the recording arena. Fish were imaged using a high-speed camera (Ximea) placed above the chambers containing the larvae.
Spontaneous locomotion was first measured. One hour prior to the experiment, fish were transferred to a 12-wells plate containing 2 ml of Danieau’s medium in each well, which was then placed in the behavior-recording setup 10 minutes before the start of the experiment. Spontaneous locomotion was then imaged for 10 minutes at 40 Hz. The software Ethovision XT 8.5 was then used to track the fish trajectories and calculate the total distance travelled by each larva.

To measure behavioral choice, a previously established visual size discrimination assay was used. Individual larvae were placed in custom-made transparent plastic chambers (100 X 12 mm) containing 4 ml of Danieau’s medium, and black circles of different sizes (1° to 30° of visual angle) moving at a constant speed of 33 °/s were shown on a display with white background underneath the fish. Visual stimuli were generated using PsychoPy v2.0 with a custom-written script. Circle sizes were calculated as the degrees of the visual field, occupied by the stimulus positioned right below a larva. Since the fish were freely moving, the entire set of stimuli was repeated five times, with two-minutes intervals between each set to maximize the number of stimulus-larva interactions. Locomotor responses to the visual stimuli were imaged at 60 Hz, and subsequently manually scored as approaches (if the larvae swim toward an approaching visual stimulus with at least one swimming bout), avoidances (if the larvae swim away from an approaching stimulus with at least one swimming bout), or neutral interactions (when the larvae swim neither toward to or away from stimuli) by an expert investigator blind to the identity of the experimental groups.

To quantify the tendency of fish to approach or avoid circles, a valence index [(approaches − avoidances) / (approaches + avoidances)] was used, which is equal to 1 if 100% of the interactions are approaches, and to −1 if they are 100% avoidances. To quantify the general efficiency of larva-stimulus interactions, an activity index [(approaches + avoidances) / (approaches + avoidances + neutral interactions)] was calculated. The activity index is equal to 0 if 100% of the interactions are neutral, and to 1 if none of them are neutral.

The optomotor response assay was performed on 5 dpf head-fixed larvae. Larvae were mounted in a 6 cm petri dish in 2% low-melting point agarose and covered with Danieau’s buffer. The agarose was cut below the swimming bladder to free the tail. Black and white gratings with bars of different size (1° to 10° of visual angle) moving forward at a constant speed of 60 °/s were displayed on the screen underneath the fish, and the optomotor response of the fish was recorded for 30 seconds. Larvae were illuminated from above with a custom-built infrared LED ring. An infrared filter (780 nm LP, Thorlabs, Cat#FG7806S) was placed in front of the camera to block visible light coming from the screen displaying moving bars. Locomotor responses to the moving bars were imaged at 250 Hz. Amplitude and duration of swimming bouts were manually measured using ImageJ. Attempts of the fish to free itself from the agarose (“struggles”) were distinguished from swimming bouts based on the tail angle (> 90°) and were not included in the analysis.

qRT–PCR

After euthanizing the larvae, their brains were manually dissected using fine dissection forceps (Dumont) in a 35 mm Petri dish containing ice-cold PBS, which bottom was coated with 5% agarose in PBS. Ten brains were pooled for each sample. For the group treated with forskolin, and the related control group, ten whole 5 dpf larvae were pooled for each sample. Samples were mechanically homogenized in Trizol and stored at −80 °C. Total RNA was extracted by chloroform purification followed by isopropanol precipitation. DNase I treatment was used to prevent genomic DNA contamination, and RNA quality was checked using a Nanodrop spectrophotometer (Eppendorf). cDNA was synthesized using the Superscript III First-Strand Synthesis kit (Life Technologies, Cat# 18080051) using oligo(dT) primers, and RNase H treatment was used to destroy the RNA template.

qRT–PCR was performed using Luna Universal qPCR Master Mix (New England BioLabs, Cat# M3003L) in a StepOnePlus Real-Time PCR System (Applied Biosystem). pcp4a’s mRNA was amplified using the primers 5’-TCAGGGTGACACACCCCCATC-3’ and 5’-ATCCCCCTGCCCTAAATGTG-3’. Each biological replica was run in triplicate, 10 ng of cDNA were used for each reaction. The housekeeping β-actin mRNA was used as endogenous control (using the primers 5’-GCTCCGTATGCCCTGCTGT-3’ and 5’-AAGTC CAGACGGAGGTG-3’ for its amplification). For each biological replica, two pools of brains or larvae from the same clutch of fish were collected, one to be subjected to experimental treatment and one as a control for normalization of gene expression using the 2^−ΔΔCt method. Different biological replicas were obtained from different clutches.

In situ hybridization

In situ hybridization to detect pcp4a, vglut2, gad1b, drd2a, and drd2b transcripts was performed using third-generation in situ HCR v.3.0.11 All the probes and fluorophore-conjugated hairpins were purchased from Molecular Instruments Inc. vglut2 probes recognized both zebrafish vglut2 paralogs slc17a6a and slc17a6b. In situ HCR v.3.0 was performed following the manufacturer’s protocol, taking care of keeping the fish always in the dark to preserve endogenous fluorescence signal. Briefly, larvae were fixed overnight in 4% PFA in PBS and then incubated in 100% methanol at -20 °C for at least 10 min. After rehydration in PBT (PBS + 0.1% Tween20), larvae were permeabilized with protease K (10 μg/ml) for 15-30 min, followed by postfixation in 4% PFA. Larvae were then incubated in probe hybridization buffer (Molecular Instruments Inc.) with 4 nM of each probe at 37 °C overnight. After washes at 37 °C, larvae were incubated overnight in amplification buffer (Molecular Instruments Inc.) with 30 nM of each fluorophore-conjugated hairpin at room temperature. After washing in 5X SSCT, larvae were immediately mounted in 1.5% low-melting-point agarose and imaged with a Leica DM6 CFS confocal microscope with a 25X water-immersion objective (HC Fluotar L 25X/0.95, Leica Microsystems). Volume rendering of confocal stacks of the tectum and mapping of position of pcp4a+ PVNs was performed with the software Imaris.
Immunohistochemistry

Immunohistochemistry was performed following an established protocol.72 Larvae were fixed overnight in 4% PFA in PBST (PBS with 0.3% Triton) at 4°C. They were then washed in PBST, and incubated in 150 mM Tris-HCl (pH = 9) for 5 minutes at room temperature, followed by 15 minutes at 70°C. Afterward, larvae were incubated in Trypsin EDTA (Sigma, Cat# T4299, diluted 1:20 in PBST) for 40 minutes on ice. After 1 hour of blocking at room temperature in blocking solution (5% goat serum, 1% BSA, 1% DMSO in PBST), fish were incubated with primary antibodies, each diluted 1:500 in blocking solution for 96 hours at 4°C. Larvae were then washed in PBST, incubated in blocking solution for 1 hour at room temperature, and then with Alexa Fluor secondary antibodies, each diluted 1:300 in blocking solution for 48 hours at 4°C. Primary antibodies against the following antigens were used: GFP (Invitrogen, Cat# A10262), total ERK (ERK; Cell Signaling, Cat# 4696), phosphorylated ERK (pERK; Cell Signaling, Cat# 4370). The following Alexa Fluor secondary antibodies were used: z-chicken-AF488 (ThermoFisher, Cat# A11039), z-mouse-AF547 (Cell Signaling, Cat# 4410S), z-rabbit-AF555 (Cell Signaling, Cat# 4413S). Images of the samples were acquired using a Leica DMi6 CFS confocal microscope with a 25X water-immersion objective (HC Fluotar L 25X/0.95, Leica Microsystems). All larvae were imaged using constant acquisition settings. GFP-positive cells in th:gal4-VP16; UAS:EGFP-CAAX larvae were manually selected to measure the fluorescent intensities of pERK and tERK immunostainings using ImageJ. The cumulative percentage of pERK/tERK values was calculated for each larva and then averaged across fish.

Pharmacology

Wild-type clutchmate larvae were equally split into two different Petri dish just before drug application. The following treatments were used: 1.5 μM fluoxetine hydrochloride (Sigma, Cat# PHR1394) for 4 hours, 10 μM donepezil hydrochloride (Fisher, Cat# 45805001) with 0.1% DMSO for 24 hours, 50 μM apomorphine hydrochloride (AbCam, Cat# ab269887) with 0.1% DMSO for 2 hours, 50 μM SKF-38393 hydrochloride (MedChem Express, Cat# HY-12520A) with 0.1% DMSO for 4 hours, 16.7 μM quinpirole hydrochloride (Sigma, Cat# Q102) with 0.1% DMSO for 3 hours, 10 μM forskolin (Sigma, Cat# F6886) with 0.1% DMSO for 1 hour. Control groups were incubated in Danieau’s medium with 0.1% DMSO, with the exception of the control group for fluoxetine treatment, which was incubated only in Danieau’s medium.

For the treatment combining quinpirole with forskolin, fish were first incubated in 16.7 μM quinpirole hydrochloride with 0.1% DMSO. After two hours, 10 μM forskolin with 0.1% DMSO was added to the medium. Fish were incubated in both drugs for one additional hour.

Calcium imaging

Fish used for Ca2+ imaging were homozygous mitfa<sup>u2w2</sup> mutants lacking skin melanophores.73 Larvae were mounted in 1% low-melting-point agarose containing the myorelaxant pancuronium bromide (0.3 mg/ml, Sigma, Cat# P1918) to minimize fish movement, in a petri dish filled with Danieau’s medium. Black circles of different sizes (1° to 30° of visual angle), moving at a constant speed (4.4°/s) on a grey background, were displayed on a microdisplay (Kopin) on one side of the fish. Responses to the visual stimuli were imaged in the contralateral side of the tectum using a confocal microscope (Zeiss LSM 880 NLO) equipped with a 20X water-immersion objective (W Plan Achromat 20X/1.0 DIC VIS-IR, Zeiss). Time series were acquired at 4 Hz with a pixel size of 1 μm<sup>2</sup>. Three to four z-planes were acquired for each fish at different depths in the tectum.

Raw images were first x-y motion corrected using the NoRMCorre algorithm in Matlab.74 Region of interests (ROIs) corresponding to single neurons were drawn manually. GCaMP6s fluorescence intensity was normalized as

\[
F = \frac{F - F_0}{F_0}
\]

where \( F \) is the fluorescence at each time point and \( F_0 \) is the average baseline fluorescence (10 frames preceding the stimulus presentation). A neuron was considered responding to a stimulus if the calcium peak was higher than two standard deviations of the baseline. The size tuning of neurons was expressed as WMR angles, calculated as weighted sums of visual stimulus sizes, to which neuronal responses were non-zero:

\[
WMRangle = \sum_{i=1}^{n} w_i \cdot x_i
\]

where \( x_i \) are the angular sizes of the visual stimuli and \( w_i \) are the weights calculated as

\[
w_i = \left( \frac{\Delta F}{F_0} \right)_i \left/ \left[ \sum_{i=1}^{n} \left( \frac{\Delta F}{F_0} \right)_i \right] \right.
\]

To analyze responses to visual stimuli in the tectal neuropil, a pixel-wise analysis based on a regression model was performed.30,75 A linear regression model composed of six independent variables corresponding to the individual responses for the different visual stimulus sizes, and by a constant term, was used to represent the temporal series for each pixel. The regressor functions were obtained from the convolution of the waveforms of the stimulus presence with a GCaMP6s kernel, whose \( t_{pew} = 1.8\) s was based on the coefficient of determination R<sup>2</sup>. The distributions of T scores for different sizes of the stimuli were averaged across different trials (Figure S4H). Corrected distributions were obtained by subtracting a term equal to 3 SD, accounting for changes in fluorescence in absence of stimulation. The number of pixels activated by the presentation of visual stimuli (Figure S4G) was quantified by calculating the integrals of the corrected distributions.
Neurons expressing pcp4a were identified post mortem using an approach similar to the MultiMAP method. After recording activity of tectal neurons with Ca²⁺ imaging in a selected set of z-planes, a stack of images of the whole tectum contralateral to the eye receiving visual stimulation was acquired with a 1 μm z-step. Minimal x-y shifts between the same plane in the Ca²⁺ imaging time-lapse and in the stack were manually corrected in ImageJ using the ‘translate’ function to ensure accurate cell identification. Immediately after imaging, the fish were euthanized and fixed in 4% PFA, and in situ HCR was performed to detect pcp4a’s mRNA using the protocol described above. Then a stack of images of the same tectum with the residual GCaMP6s fluorescence and the pcp4a in situ HCR signal was acquired with 1 μm z-step. The two stacks from each larva were then aligned with each other, using the GCaMP6s fluorescence in the two data sets to guide the alignment, with the CMTK plugin in Fiji. The z-planes containing the neurons whose activity was recorded during the in vivo calcium imaging session were identified in the registered stack and the accuracy of the registration was checked manually. Neurons were considered positive for pcp4a if the in situ signal occupied at least 50% of the perimeter of the nuclei labeled by H2B-GCaMP6s. In case of uncertainty, z-planes below and above the imaging plane were checked in the registered stack.

For the experiments testing the effect of dopaminergic D2-like receptor signaling on the activity of pcp4a+ PVNs, responses to visual stimuli were first recorded using in vivo calcium imaging as described above. The fish, still embedded in agarose, were then incubated for 3 hours in Danieau’s medium containing 16.7 μM quinpirole hydrochloride (Sigma, Cat# Q102) or in Danieau’s medium as a control. Larvae were then transferred again under the microscope, with the drug still in the solution, and responses to visual stimuli were recorded a second time from the same z-planes containing the PVNs imaged during the first session. Post mortem identification of pcp4a+ neurons and analyses of GCaMP6s signal were then performed as described above.

For the experiment testing the effect of CaMKII inhibition on the activity of pcp4a+ PVNs, fish were first treated with the CaMKII inhibitor KN93 (5 μM, Adooq Bioscience, Cat#A13276-5) with 0.1% DMSO or Danieau’s medium with 0.1% DMSO for 3 hours prior to experiment. Responses to visual stimuli were recorded using calcium imaging as described above with the drug still in the solution.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Statistical significance was determined using one-sample t tests, two-tailed Student’s t tests, two-way ANOVA, and nested two-way ANOVA in GraphPad Prism (GraphPad, version 9). Normal distribution of data was verified with the Shapiro-Wilk test before performing t tests or ANOVA. P values from multiple Student’s t tests were corrected using the Bonferroni-Holm method. Statistical tests were considered significant if p < 0.05.