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BEHAVIORAL NEUROSCIENCE

Emotions and motivated behavior converge on an amygdala-like structure in the zebrafish

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Abstract

The brain reward circuitry plays a key role in emotional and motivational behaviors, and its dysfunction underlies neuropsychiatric disorders such as schizophrenia, depression and drug addiction. Here, we characterized the neuronal activity pattern induced by acute amphetamine administration and during drug-seeking behavior in the zebrafish, and demonstrate the existence of conserved underlying brain circuitry. Combining quantitative analyses of *cfos* expression with neuronal subtype-specific markers at single-cell resolution, we show that acute D-amphetamine administration leads to both increased neuronal activation and the recruitment of neurons in the medial (Dm) and the lateral (Dl) domains of the adult zebrafish pallium, which contain homologous structures to the mammalian amygdala and hippocampus, respectively. Calbindin-positive and glutamatergic neurons are recruited in Dm, and glutamatergic and γ-aminobutyric acid (GABAergic) neurons in Dl. The drug-activated neurons in Dm and Dl are born at juvenile stage rather than in the embryo or during adulthood. Furthermore, the same territory in Dm is activated during both drug-seeking approach and light avoidance behavior, while these behaviors do not elicit activation in Dl. These data identify the pallial territories involved in acute psychostimulant response and reward formation in the adult zebrafish. They further suggest an evolutionarily conserved function of amygdala-like structures in positive emotions and motivated behavior in zebrafish and mammals.

Introduction

Animals exhibit an astonishing diversity of behaviors, but also a common set of adaptive responses and processes. It is therefore not surprising that basic neural systems mediating core motivational and emotional processes have been evolutionary conserved – with emotions being defined as common central states of neural circuits that assign positive or negative values to a stimulus or experience and give rise to behavioral and/or physiological responses (Kalueff *et al.*, 2012; Kittilsen, 2013; Anderson & Adolphs, 2014). From this point of view, the brain can be considered as a modular system that can increase in complexity by building on and interconnecting to preexisting building blocks (Kelley, 2004). One such evolutionary conserved key element of the brain is the reward system. Drugs of abuse, for example amphetamine, strongly activate the reward system and can trigger strong emotional and motivational responses in most, if not all, vertebrates (Robbins *et al.*, 2008; Sesack & Grace, 2010; O'Connell & Hofmann, 2011).

The zebrafish (*Danio rerio*) has been used to identify and characterize evolutionarily conserved neural systems (Mueller & Wullimann, 2009), and is ideally suited for a genetic and molecular analysis of emotional and motivational behaviors, including the response to drugs of addiction (Norton & Bally-Cuif, 2010; Guo *et al.*, 2012; Klee *et al.*, 2012). In mammals, the amygdala and the hippocampus are part of the mesocorticolimbic reward circuitry. The amygdala plays a pivotal role in mediating negative but also positive emotions, and in conveying value of motivational signals (Paton *et al.*, 2006; Murray, 2007; Morrison & Salzman, 2010; Johansen *et al.*, 2011). It is strongly activated by drugs of abuse and drug-associated cues (Brown *et al.*, 1992; Mead *et al.*, 1999; Buffalari & See, 2010), and plays a role in goal-directed behavior (Parkes & Balleine, 2013; Rhodes & Murray, 2013). The amygdala is reciprocally connected to the hippocampus (Pitkänen *et al.*, 2000; Kishi *et al.*, 2006), which strongly contributes to spatial learning and memory (Scoville & Milner, 1957; Milner, 1972). Molecular genetic, developmental, hodological and behavioral data suggest that the medial (Dm) and the lateral zone of the dorsal telencephalon (Dl) of ray-finned fish likely contain the structures homologous to the mammalian amygdala and hippocampus, respectively (Rodríguez *et al.*, 2002; Portavella *et al.*, 2004; Northcutt, 2006; Martín *et al.*, 2011; Mueller *et al.*, 2011). A precise functional mapping of these territories, however, is still largely missing.

As a first cellular approach towards establishing the neuroanatomical correlates of reward and motivation-based behavior in zebrafish,

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we used here the immediate-early gene (IEG) *cfos* (Clayton, 2000; Lau *et al.*, 2011) to identify the telencephalic regions activated by amphetamine and during drug-induced place preference behavior. We show that Dm (encompassing amygdala-like areas) and Dl (encompassing hippocampus-like areas) are activated by amphetamine, and Dm, but not Dl, during drug-seeking behavior. Our analysis supports the functional conservation of at least two pallial components of the brain reward system in zebrafish, an important emerging model for the molecular genetic dissection of the neural networks underlying emotional and motivational behaviors.

Materials and methods

Fish strain and handling

Wild-type zebrafish (*Danio rerio*) of the AB strain were used in all experiments. Adult zebrafish were maintained and bred following standard procedures (Westerfield, 2000), and in accordance with the institute guidelines for animal welfare.

Molecular cloning of cfos

The primers 5′-ATGATGTTTACCAGCCTTAACGC-3′ and 5′-TCA AAGAGTGAGGAGGGTTGG-3′ were used to amplify *cfos* from a cDNA library constructed from an adult AB wild-type brain, and it was cloned into the StrataClone PCR Cloning Vector *pSC-A* (Agilent Technologies, Santa Clara, CA, USA). The retrieved 1050-base pair *cfos* sequence includes the whole coding region without the 5′- or 3′-untranslated region. Sequencing revealed more than 99% sequence identity with the published zebrafish *cfos* reference sequence (Accession Number NM_205569; Gene ID 394198).

Acute amphetamine and saline injections

Adult zebrafish (4–6 months old; 300–400 mg weight) were first injected intraperitoneally with 110 mM sodium chloride (63.8 mg/kg). This first injection proved necessary to calibrate the system in order to avoid unspecific pain and fear-induced *cfos* expression (not shown). Two hours after this first injection (a time interval in which *cfos* mRNA expression is returned to basal levels; Clayton, 2000), the fish were split into a control and an amphetamine group (four to six fish per group). The control group was again injected with 110 mM sodium chloride; the amphetamine group was injected with 32.61 mM of D-amphetamine hemisulfate salt (60 mg/kg; Sigma-Aldrich A5880) in 110 mM sodium chloride. Thirty minutes after this second injection (at the peak of *cfos* mRNA expression; Clayton, 2000), the fish from the control and amphetamine group were killed immediately by putting them in ice-cold phosphate-buffered saline (PBS), their brains were then dissected and analysed for *cfos* expression.

Conditioned place preference (CPP) behavior assay

The CPP test was performed as previously described (Ninkovic & Bally-Cuif, 2006; Ninkovic *et al.*, 2006; Webb *et al.*, 2009), with the following modifications: (i) the two 'black spots' on the initially non-preferred compartment were omitted; and (ii) the fish were habituated for 3 days (instead of 2 days) to the test environment and their initial place preference was measured on the fourth day using the ZebraLab software (Viewpoint, Lyon, France). Similarly, control fish exhibiting a non-oriented behavior were also habituated to the experimental setup for 3 days and their behavior was tracked on the fourth day. To elicit or monitor a drug-seeking approach or a light-avoidance behavior, fish were repeatedly injected with amphetamine or saline, respectively. Following conditioning, amphetamine-treated fish exhibited a change in place preference of 71.83 ± 5.95%, now spending 76.91 ± 4.94% (n = 6) of their time in the initially non-preferred compartment; as expected, saline-injected fish did not change place preference (change of −0.04 ± 4.05%), spending 78.24 ± 4.80% (n = 6) of their time in the preferred compartment. Control fish were housed in tanks of the same size but with no color cues, and therefore spent equal amounts of their time on either side of the tank (side 1 : 51.5 ± 2.9%; side 2 : 48.5 ± 2.9%). The final behavior testing, which measured place preference after conditioning, was not preceded by amphetamine or saline injection. All the fish were killed 30 min after monitoring, and their brains were dissected and analysed for *cfos* expression.

5′-Bromo-2′-deoxyuridine (BrdU) labeling

Adult (4–6 months old) or juvenile (1 month old) fish were placed for 6 h in tank water containing 1 mM BrdU (Sigma-Aldrich B5002). Population density was controlled (8–10 adult and 15–20 juvenile fish per 500 mL) and the fish were kept in the dark during the application of the chemical.

In situ *hybridization and immunohistochemistry*

In situ hybridization and immunohistochemistry were performed essentially as previously described (Adolf *et al.*, 2006; Brend & Holley, 2009; Lauter *et al.*, 2011), but with modifications (see below) permitting highly sensitive multicolor stainings at single-cell resolution. The following probes were used – *cfos*; *vglut2.2* (courtesy of Rebecca Schmidt, Karlsruhe, Germany); and *gad65/67* (Higashijima *et al.*, 2004). In brief, adult brains were dissected and fixed in 4% paraformaldehyde in PBS overnight at 4 °C. The samples were then dehydrated and incubated with 2% H_2O_2 for 35 min to block endogenous peroxidase activity. After rehydration, the brains were embedded in a bovine serum albumin (Sigma-Aldrich A3912) gelatin type A (Sigma-Aldrich G1819) mixture (Levin, 2004) that contained 6.47% formaldehyde and 0.075% glutaraldehyde. This embedding procedure allowed us to carry out the hybridization of the RNA probes on adult brain sections rather than on whole-mount brains [as it is commonly done in fluorescent *in situ* hybridizations (FISH) in the zebrafish], which, in our experience, greatly increases the sensitivity and signal-to-noise ratio. Subsequently, 50-μm serial sections were cut with a Leica VT1000S microtome. The sections were washed in PBS containing 0.1% Tween-20 (PBST), incubated for 30 min with 10 μg/μL Proteinase K, washed in PBST, refixed for 20 min at room temperature (RT) with 4% paraformaldehyde, washed in PBST, and prehybridized for at least 1 h at 65 °C. Digoxigenin (DIG)-, fluorescein (FLUO)- and dinitrophenol (DNP)-labeled anti-sense RNA probes were hybridized overnight at 65 °C with a concentration of 1 ng/μL as previously described (Lauter *et al.*, 2011). The sections were sequentially incubated with horseradish peroxidase-conjugated anti-FLUO (1 : 500; Roche, Basel, Switzerland), anti-DIG (1 : 500; Roche) and anti-DNP (1 : 500; PerkinElmer, Waltham, MA, USA) antibodies. The RNA probes were sequentially revealed with custom-made FLUO (FITC; 1 : 200; http://www.xenbase.org/other/static/methods/FISH.jsp), tetramethylrhodamine (TAMRA; 1 : 250) and cyanine 5 (Cy5; 1 : 100; PerkinElmer) conjugated tyramide in PBST containing 2% (v/v) dextran sulfate (Sigma-Aldrich D8906) and 0.003% (v/v) H_2O_2 for 35 min at RT.

Immunohistochemistry was performed after *in situ* hybridization. To reveal BrdU, the sections were incubated with 2 M HCl in PBST at 37 °C for 30 min. For other primary antibodies, no pre-treatment was applied. Free-floating sections were washed in PBST, blocked in 10% (v/v) normal goat serum (NGS) in PBST for 1 h at RT and incubated in NGS/PBST at 4 °C overnight with the primary antibodies. Next, sections were washed in PBST, blocked in NGS/PBST for 30 min at RT and incubated in PBST at 4 °C overnight with the secondary antibodies. The sections were washed in PBST, the nuclei counterstained for 10 min at RT with 4',6-diamidino-2-phenylindole (DAPI; 1 µg/µL) and mounted on microscope slides in Vectashield (Vector Laboratories H-100, Burlingame, CA, USA).

Primary antibodies were rat anti-BrdU (1 : 250; Abcam, Cambridge, UK), rabbit anti-calbindin D28k (1 : 500; Swant, Marly, Switzerland), rabbit anti-*cfos* (1 : 2000; sc-52; Santa Cruz Biotechnology, Dallas, TX, USA), human anti-HuC/D (1 : 2000; courtesy of Bernard Zalc, Salpêtrière Hospital, Paris, France), mouse anti-parvalbumin (1 : 500; Merck Millipore, Billerica, MA, USA). Goat antibodies coupled to AlexaFluor dyes (488, 546 or 647; 1 : 1000; Molecular Probes, Invitrogen, Carlsbad, CA, USA) were used as secondary antibodies.

Image acquisition, analysis and cell counting

Images were acquired with a Zeiss LSM 700 confocal microscope using a 20× air (numerical aperture 0.8) or 40× oil-immersion (numerical aperture 1.3) objective with 405, 488, 555 and 639 nm lasers. Images were collected at a size of 512 × 512 pixels and automatically stitched upon acquisition using the 'Tilescan' mode of the ZEISS ZEN software. Confocal data were processed and analysed with VOLOCITY 6.3 (PerkinElmer) software. Cell counting and intensity measurement of single-labeled cells was performed on 42-µm Z-stacks with 2-µm Z-intervals (21 optical sections), and an *X* and *Y* pixel size of 0.391 µm from 50-µm serial sections using VOLOCITY 6.3 (PerkinElmer) software. Colocalization analysis of double-labeled cells was performed manually using VOLOCITY's 6.3 (PerkinElmer) 'point tool'. Intensity measurements of *cfos* expression were normalized using the mean intensity of *cfos* expression per cell of saline-injected control fish. Cell counting and intensity measurements were performed within regions of interest (ROIs) that correspond to the Dm and Dl and the subpallium. Dm, Dl and the subpallium were defined by calbindin and parvalbumin expression and/or DAPI staining. *cfos*-positive and DAPI cells were counted in a ROI measuring 390 µm (width) × 200 µm (height) in Dm, and 200 µm (width) × 390 µm (height) in both Dl and the subpallium (Fig. 3E and F). Data from the coexpression analysis of *cfos* together with the neuronal markers calbindin, *vglut2.2* and *gad65/67* were obtained from a ROI measuring 580 µm (width) × 200 µm (height) in Dm, and 200 µm (width) × 390 µm (height) in both Dl and the subpallium (Figs 4 and 5). In the BrdU time course analyses of juvenile- and adult-born neurons, distance measurements in micrometers were performed within a ROI of 580 µm (width) × 135 µm (height) and 200 µm (width) × 270 µm (height) for Dm and Dl, respectively (Fig. 6). *cfos*-positive cells in fish exhibiting a non-oriented or oriented behavior were counted within a ROI measuring 580 µm (width) × 200 µm (height) in Dm, and 200 µm (width) × 390 µm (height) in both Dl and the subpallium (Fig. 8).

Statistics

All the quantitative data are presented as the mean ± standard error of the mean (SEM). Statistical analyses were performed using unpaired two-tailed Student's *t*-tests (Figs 3E and F, 4E–G, 5A–C″, and 8D) or a one-way ANOVA followed by a post-test for linear trend (Figs 6E and F′, and 7A and B) with Prism 6.0 (GraphPad, La Jolla, CA, USA) software. Differences were considered significant for $P < 0.05$. Graphs were created with Aabel 3.0.6 (Gigawiz, Oklahoma City, OK, USA) and Illustrator 15.0.2 (Adobe, San Jose, CA, USA).

Results

To identify and characterize the territories involved in reward processing in the zebrafish telencephalon, adult fish were injected with the psychostimulant D-amphetamine. Acute administration of D-amphetamine induces a net increase of biogenic amines, notably dopamine, at the synapse, directly activating primary reward centers (Sulzer *et al.*, 2005; Sulzer, 2011). Upon chronic administration in zebrafish (Ninkovic & Bally-Cuif, 2006; Ninkovic *et al.*, 2006; Webb *et al.*, 2009), as in mammals, it triggers a dose-dependent conditioned response measurable in a place preference assay, which fades when the drug-place association is decreased. Neuronal activation in amphetamine- vs. saline-injected control fish was revealed using FISH for the IEG *cfos*, the expression of which reliably highlights neuronal recruitment and plasticity (Knapska *et al.*, 2007). Within the telencephalon, the most striking changes in *cfos* induction were observed at an antero-posterior level slightly rostral to the anterior commissure (Fig. 1) located between cross-sections 85 and 92 according to Wullimann's neuroanatomical atlas (Wullimann

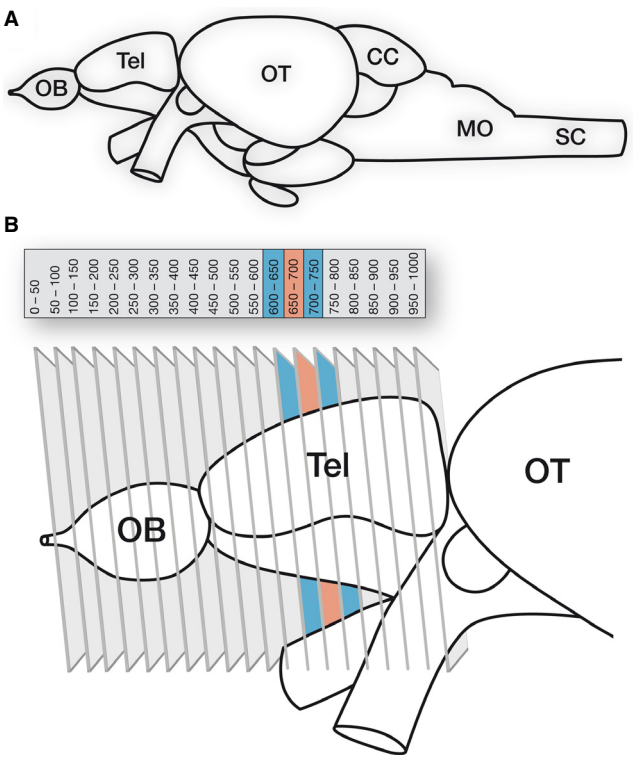


FIG. 1. Schematic overview of the zebrafish brain depicting the ROI. (A) Whole brain. (B) Telencephalon and sectioning scheme. Twenty 50-µm-thick coronal sections were cut from the telencephalon (total antero-posterior coverage – approx. 1 mm). We focused on an area located just rostral to the anterior commissure (red and blue sections). CC, corpus cerebelli; MO, medulla oblongata; OB, olfactory bulb; OT, optic tectum; SC, spinal cord; Tel, telencephalon. Adapted and modified from Wullimann *et al.* (1996) and Wullimann & Mueller (2004).

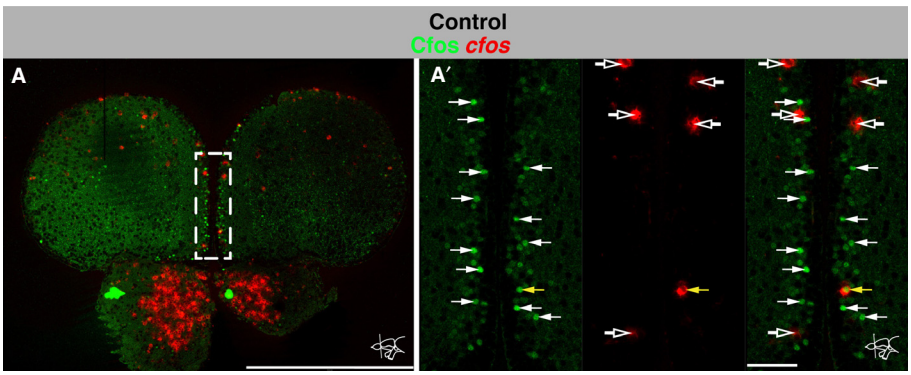
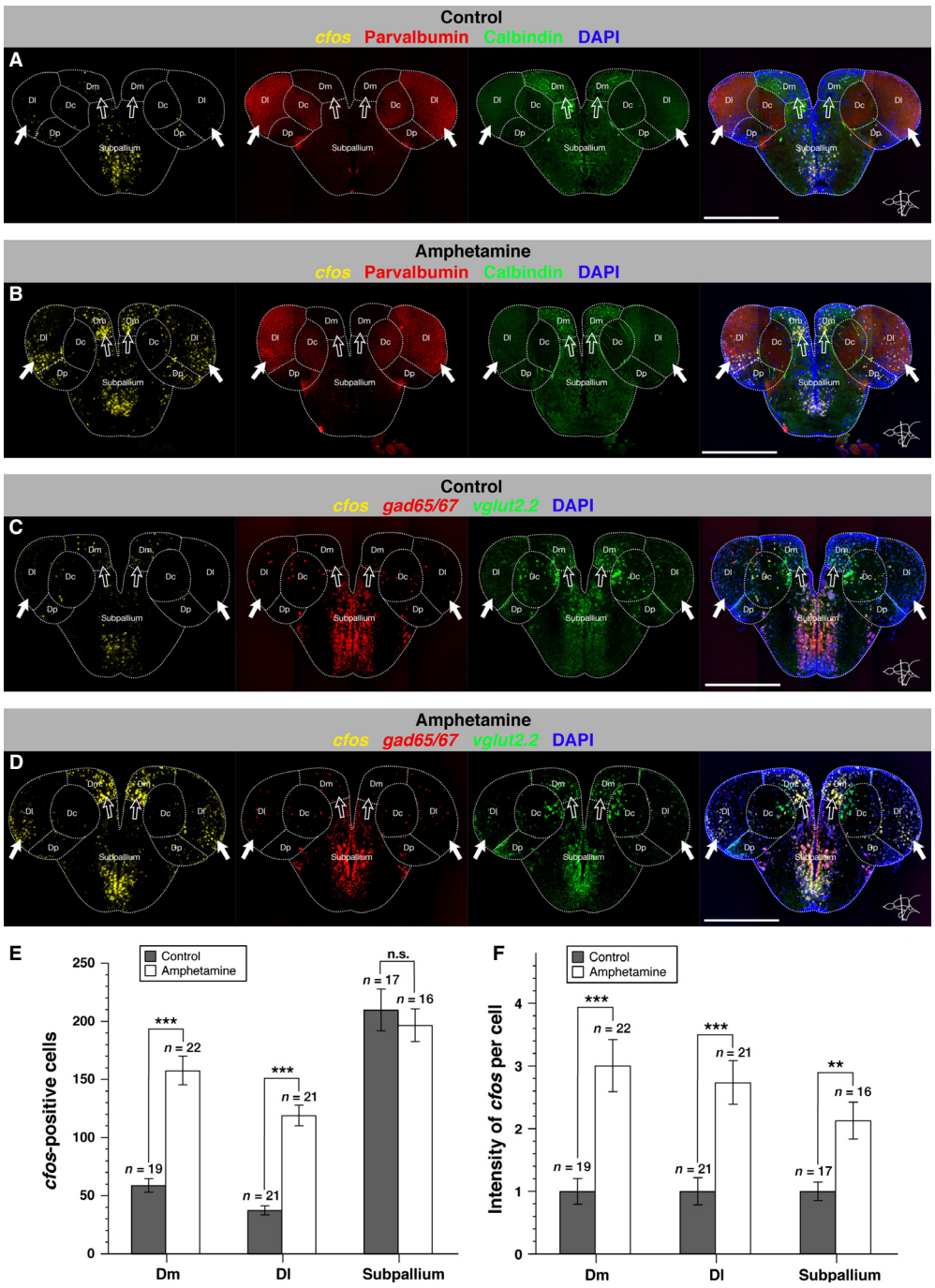


FIG. 2. Non-identical cFos protein and *cfos* mRNA expression in a saline-injected control fish. (A–A′) Single optical section at an anterior level of the brain of a saline-injected control fish depicting cFos protein (immunohistochemistry, green) and *cfos* mRNA (FISH, red). The dashed white box in (A) outlines the region magnified in (A′). Note that a much larger number of cells are positive for cFos protein (solid white arrows) than for *cfos* mRNA (open white arrows), and that only one cell expresses both cFos protein and *cfos* mRNA (solid yellow arrow). Scale bars – 500 μm (A); 50 μm (A′).



et al., 1996). The main telencephalic subdivisions are all present at this level, and are readily identified by a specific DAPI, calbindin D28k and parvalbumin staining pattern (Mueller *et al.*, 2011, and see below).

Dm and Dl nuclei are activated by acute D-amphetamine administration

To identify the most reliable readout for neuronal activation, we first compared the patterns of *cfos* mRNA and cFos protein expression in double-staining experiments in adult fish that were killed within 30 min following brief handling or injection sessions. Combining *in situ* hybridization and immunohistochemistry on single brain sections revealed only partly overlapping expression patterns (Fig. 2). Notably, a number of cells could be observed that were positive for cFos protein but negative for *cfos* mRNA, suggesting protein stability beyond 30 min. We thus chose to monitor *cfos* mRNA as a trustworthy readout of *cfos* induction, and developed a novel protocol for highly sensitive multicolor FISH at single-cell resolution on adult zebrafish brain sections (J.W. von Trotha, in preparation; see Materials and methods).

We observed a strong increase in *cfos* expression following acute D-amphetamine injection in Dm and Dl, hosting the territories homologous to the mammalian amygdala and hippocampus, respectively (Fig. 3A–D). Notably, in Dl, *cfos*-expressing cells were more evenly distributed than in Dm, in which we observed a dense activation pattern in the ventromedial region (Figs 3B and D, and 4B and B′, and D and D′). In Dm, saline-injected control fish expressed *cfos* in 58.74 ± 5.97 SEM cells (n = 19 brains), whereas amphetamine-injected fish expressed *cfos* in 157.60 ± 12.41 SEM cells (n = 22 brains), corresponding to a 2.7-fold increase in the number of activated cells upon drug administration (unpaired two-tailed t-test $t_{39} = 6.83$, $P < 0.0001$; Fig. 3E). In Dl, we found 37.36 ± 3.99 SEM and 119.0 ± 8.88 SEM *cfos*-positive cells in control (n = 21 brains) and amphetamine (n = 21 brains) fish, respectively, which corresponds to a 3.2-fold increase (unpaired two-tailed t-test $t_{40} = 8.39$, $P < 0.0001$; Fig. 3E). In the subpallium, however, we observed similar numbers of *cfos*-positive cells following saline or amphetamine injection [respectively, 209.8 ± 18.12 SEM (n = 17 brains) and 196.6 ± 14.04 SEM (n = 16 brains); unpaired two-tailed t-test $t_{31} = 0.58$, $P = 0.5723$; Fig. 3E], arguing for a drug-specific increase of *cfos* expression in Dm and Dl rather than a general effect of amphetamine injection. We further verified that there was no difference in the total number of DAPI-stained cells in Dm, Dl and the subpallium in amphetamine and control fish (data not shown). In addition to the increase in the number of *cfos*-positive cells following amphetamine injection, the intensity of *cfos* expression per cell may also be modified. To assess this point, we measured the intensity of FISH staining on confocal images. We observed a 3.0-fold ± 0.42 SEM and 2.74-fold ± 0.35 SEM increase in the intensity of *cfos* expression per cell in Dm (unpaired two-tailed t-test $t_{39} = 4.12$, $P = 0.0002$) and Dl (unpaired two-tailed t-test $t_{40} = 4.22$, $P = 0.0001$), respectively (Fig. 3F). In the subpallium, we could also observe a 2.13-fold ± 0.35 SEM increase in the intensity of *cfos* expression per cell in amphetamine-injected fish (unpaired two-tailed t-test $t_{31} = 3.48$, $P = 0.0015$; Fig. 3F). Together, these results show that amphetamine specifically activates Dm (amygdala) and Dl (hippocampus) areas in the adult zebrafish pallium, both by recruiting neurons and by increasing neuronal activation or plasticity. The latter process, also observed in the subpallium, may partially contribute to the neuronal response to amphetamine.

Calbindin and glutamatergic neurons in Dm are activated by acute D-amphetamine administration

To further characterize the drug-activated cells in Dm and Dl, we made use of the neuronal subtype markers calcium-binding protein (CBP) calbindin D28k, *vglut2.2*, a marker of glutamatergic neurons, and *gad65/67*, a marker of γ-aminobutyric acid (GABA)ergic neurons. Expression of these markers was analysed together with *cfos* in triple-fluorescent labeling experiments monitored under confocal microscopy. In the telencephalon, calbindin is mostly expressed in Dm and the subpallium (Figs 3A and B, and 4A, B, A″ and B″). Its expression is complementary to parvalbumin, another CBP, which labels Dl and the central zone of the dorsal telencephalon (Dc; Grandel *et al.*, 2006; Mueller *et al.*, 2011; Figs 3A and B, and 4A′ and B′). *vglut2.2* is strongly expressed throughout the pallium but only weakly in the subpallium, whereas *gad65/67* is strongly expressed in the subpallium, although scattered expression can also be seen in the pallium (Mueller & Guo, 2009; Figs 3C and D, and 4C – D″).

We found that, in amphetamine-injected fish, 25.31 ± 3.59% SEM (n = 5 brains) of the calbindin-positive cells in Dm also expressed *cfos*, which represents a significant, 1.6-fold increase compared with control fish, where only 16.06 ± 1.43% SEM (n = 6 brains) of the calbindin-positive cells expressed *cfos* (unpaired two-tailed t-test $t_9 = 2.56$, $P = 0.0306$; Fig. 4E). The number of calbindin-positive cells in amphetamine and control brains, expectedly, was unchanged (data not shown). In Dm, we further observed a strongly significant, 3.73-fold increase in the proportion of *vglut2.2* neurons expressing *cfos* upon amphetamine treatment (unpaired two-tailed t-test $t_9 = 10.65$, $P < 0.0001$), whereas the proportion of *gad65/67*-positive neurons expressing *cfos* remained unchanged in amphetamine (n = 5 brains) vs. control fish (n = 6 brains; unpaired two-tailed t-test $t_9 = 0.12$, $P = 0.9045$; Fig. 4E). The mean number of cells expressing *vglut2.2* or *gad65/67* in the amphetamine and the control sample was about the same (data not shown). Together, these results indicate that, among the neuronal subtypes tested, acute amphetamine administration recruits neurons within the calbindin-positive and glutamatergic population(s) in Dm.

To determine whether this cell recruitment in Dm preferentially targets a specific neuronal subtype, we determined the percentage of *cfos*-positive cells that also expressed calbindin, *vglut2.2* or *gad65/*

FIG. 3. Dm and Dl nuclei are activated by an acute D-amphetamine treatment. (A and B) Single optical sections of the brain of a saline-injected control fish (A) and an amphetamine-injected fish (B) depicting *cfos* expression (FISH, yellow) together with parvalbumin and calbindin (immunohistochemistry, red and green, respectively), and DAPI (blue) staining. (C and D) Single optical sections of the brain of a saline-injected control fish (C) and an amphetamine-injected fish (D) depicting *cfos* expression (FISH, yellow), *vglut2.2* (FISH, green) and *gad65/67* (FISH, red) expression together with DAPI staining. Note the increase in *cfos* expression in Dm and Dl areas (arrows) in amphetamine- compared with saline-injected control fish. Brain regions are outlined by white dots. Dc, central zone of the dorsal telencephalon; Dl, lateral zone of the dorsal telencephalon; Dm, medial zone of the dorsal telencephalon; Dp, posterior zone of the dorsal telencephalon. Scale bar – 500 μm. (E) Number of *cfos*-positive cells per section in Dm, Dl and the subpallium for control (black bars) and amphetamine (white bars) fish. (F) Quantification of the intensity of *cfos* expression per cell for control (black bars) and amphetamine (white bars) fish. Error bars depict SEM. n is the number of analysed brains. ***$P < 0.001$; **$P < 0.01$; n.s., not significant (unpaired two-tailed Student's t-test).

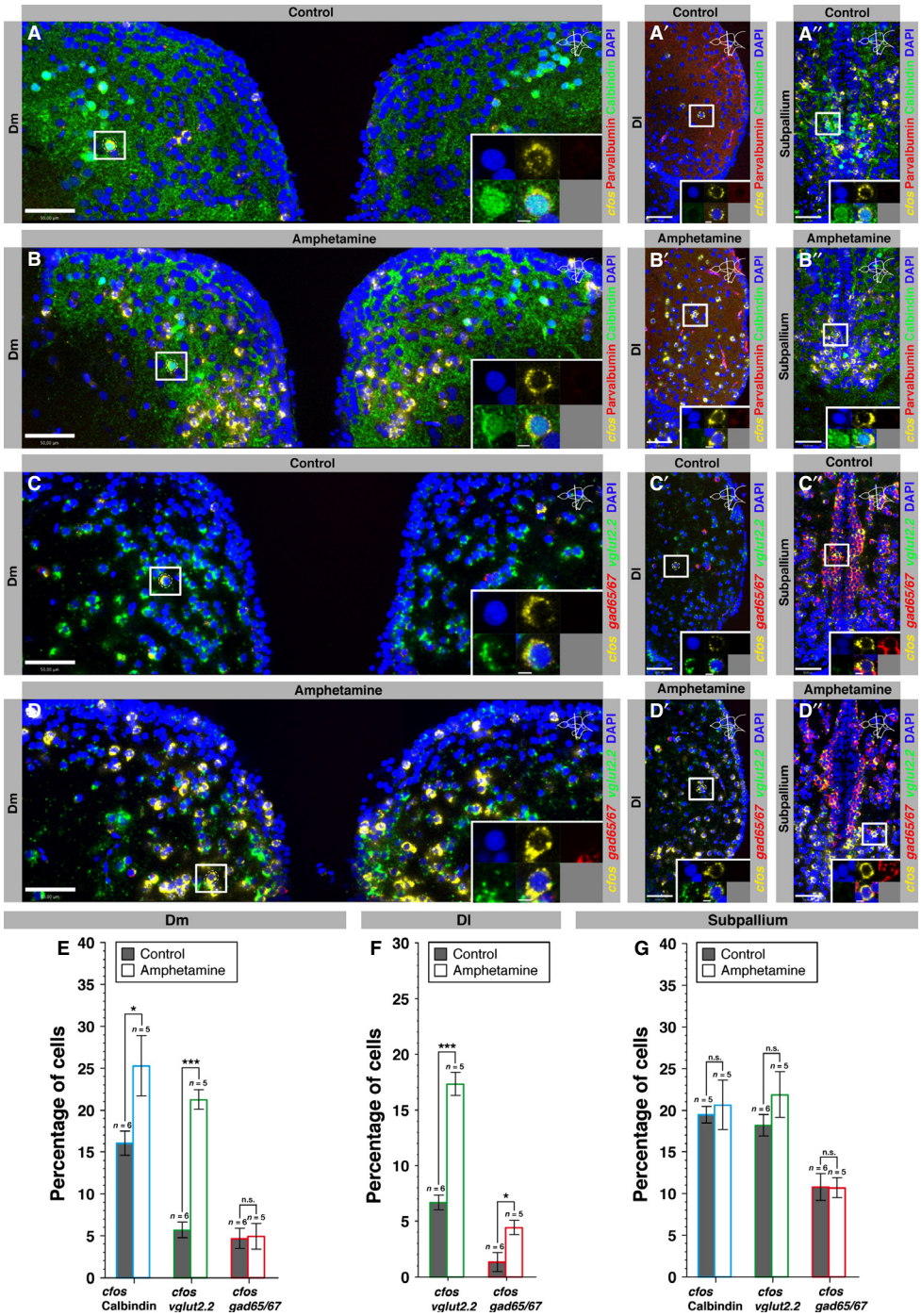


FIG. 4

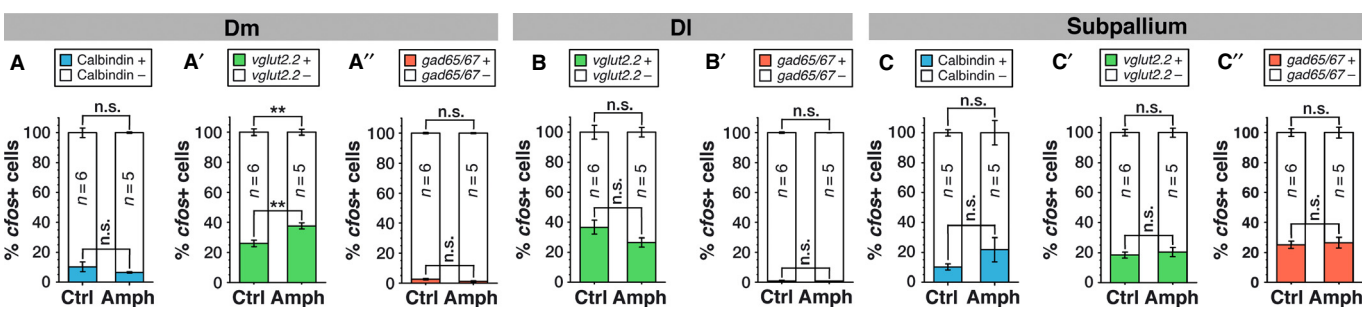


FIG. 5

FIG. 4. Calbindin, glutamatergic and GABAergic neurons in Dm and Dl are recruited by an acute amphetamine treatment. (A–B″) Single optical sections of Dm (A, B), Dl (A′, B′) and the subpallium (A″, B″) of a saline-injected control fish (A–A″) and an amphetamine-injected fish (B–B″) showing *cfos* expression (FISH, yellow) together with a double immunostaining for calbindin (green) and parvalbumin (red), counterstained with DAPI (blue). The insets depict the cells that are boxed in the main frames at a higher magnification. (C–D″) Single optical sections of Dm (C, D), Dl (C′, D′) and the subpallium (C″, D″) of a saline-injected control fish (C–C″) and an amphetamine-injected fish (D–D″) showing, in triple FISH, *cfos* (yellow), *vglut2.2* (green) and *gad65/67* (red) expression, counterstained with DAPI (blue). Scale bars – 50 μm (main frames); 5 μm (insets). (E) Proportion of calbindin-, *vglut2.2*- and *gad65/67*-positive cells that also express *cfos* in Dm in control (black bars) and amphetamine (white bars) fish. (F) Proportion of *vglut2.2*- and *gad65/67*-positive cells that also express *cfos* in Dl in control (black bars) and amphetamine (white bars) fish. (G) Proportion of calbindin-, *vglut2.2*- and *gad65/67*-positive cells that also express *cfos* in the subpallium in control (black bars) and amphetamine (white bars) fish. Error bars depict SEM. *n* is the number of analysed brains. ***$P < 0.001$; *$P < 0.05$; n.s., not significant (unpaired two-tailed Student's *t*-test).

67 in amphetamine compared with saline conditions. We found that the proportion of calbindin- or *gad65/67*-positive neurons was unchanged within the *cfos*-expressing population after amphetamine treatment, but observed a significant increase in the proportion of *vglut2.2*-positive neurons (unpaired two-tailed *t*-test $t_9 = 3.88$, $P = 0.0038$; Fig. 5A – A″). Thus, upon acute amphetamine administration, the pool of active neurons in Dm enlarges while maintaining a constant proportion of calbindin and GABAergic subtypes but disproportionally increasing its recruitment of glutamatergic neurons.

As a control for the specificity of our analysis, we quantified the expression of *cfos* among the different neuronal subtypes in the subpallium. Because this territory primarily responds to acute amphetamine treatment by increasing the intensity of *cfos* expression while maintaining a stable number of *cfos*-positive cells (Fig. 3E and F), we did not expect changes in the subtype-specific expression of *cfos*. Accordingly, we did not observe a difference in the proportion of *cfos*-positive neurons within the calbindin-, *vglut2.2*- or *gad65/67*-positive populations between amphetamine ($n = 5$ brains) and control brains ($n = 5$ brains; Fig. 4G). Likewise, the distribution of *cfos* positivity between the different neuronal subtypes analysed remained unchanged (Fig. 5C – C″).

Glutamatergic and GABAergic neurons in Dl are activated by acute D-amphetamine administration

Neuronal recruitment within the glutamatergic population was similar in Dl to that observed in Dm, with a strongly significant, 2.59-fold increase in the proportion of *vglut2.2*-positive neurons expressing *cfos* in fish that received an injection of the psychostimulant (unpaired two-tailed *t*-test $t_9 = 9.14$, $P < 0.0001$; Fig. 4F). The GABAergic population, however, appeared more robustly recruited than in Dm, with the proportion of *gad65/67*-positive neurons expressing *cfos* enhanced by 3.32-fold (unpaired two-tailed *t*-test $t_9 = 2.83$, $P = 0.0198$; Fig. 4F). There was no difference in the overall number of *vglut2.2* and *gad65/67* cell numbers between amphetamine and control fish (data not shown). In contrast to Dm, there was no preferential activation of glutamatergic or GABAergic neurons in Dl after acute amphetamine administration (Fig. 5B and B′). We conclude that, in Dl, both glutamatergic and GABAergic neurons are activated by amphetamine, with a proportionally similar recruitment within each population.

Juvenile- rather than adult-born neurons respond to acute D-amphetamine in the adult zebrafish

The zebrafish is an excellent system to investigate the function of adult-born neurons, as adult neurogenesis is much more widespread in the zebrafish than in the mammalian brain (Adolf *et al.*, 2006; Grandel *et al.*, 2006; Zupanc & Sîrbulescu, 2011; Kizil *et al.*, 2012; Schmidt *et al.*, 2013). In the adult pallium, neuronal progenitor cells are aligned along the superficially located ventricle. They deposit neurons in the parenchyma in a concentric manner and with little cell mixing as the brain grows (Chapouton *et al.*, 2007; Kaslin *et al.*, 2008; Schmidt *et al.*, 2013). Because newborn neurons have been shown more plastic in the adult rodent hippocampus and olfactory bulb (Schmidt-Hieber *et al.*, 2004; Ge *et al.*, 2007; Nissant *et al.*, 2009; Alonso *et al.*, 2012; Gu *et al.*, 2013), we expected that adult-born neurons would be preferentially recruited when the reward system is stimulated during adulthood.

To test whether adult-born neurons in Dm and Dl respond to acute D-amphetamine administration, 4-month-old fish were given a 6-h BrdU pulse to label proliferating progenitors, and the response of BrdU-labeled neurons to amphetamine was analysed after a 2-month chase. This duration is sufficient for the functional integration of adult-born neurons in the zebrafish pallium (Rothenaigner *et al.*, 2011). Even after this relatively long time interval, however, we found that the BrdU-labeled neurons remained relatively close to the ventricular zone (VZ; about 5–10 and 15–20 μm away in Dm and Dl, respectively) and failed to reach the amphetamine-responsive areas identified above. Accordingly, they were mostly *cfos*-negative (data not shown). This suggested that most of the neurons that respond to amphetamine in Dm and Dl in a middle-aged adult (5–7 months old) are born at juvenile stage rather than at adulthood, although we cannot generally exclude the possibility that some adult-born neurons may at much later stages also participate in the amphetamine response. To determine whether and at what age juvenile-born neurons respond to amphetamine, 1-month-old fish were given a 6-h BrdU pulse, and their brains were subsequently analysed after 3, 4, 5 and 6 months of chase (Fig. 6A–D). We found that, on average, *cfos*-expressing cells were located 135.8 ± 5.66 μm SEM ($n = 2259$ cells) away from the VZ in Dm, and 107.4 ± 5.39 μm SEM away in Dl ($n = 2983$ cells), and that both of these distances remained fairly constant over time (Fig. 6E and E′, blue lines). In contrast, as expected, the BrdU-

FIG. 5. Preferential neuronal recruitment in Dm of the *vglut2.2*-positive neuronal pool after acute amphetamine administration. (A–A″) Percentage of *cfos*-positive cells in Dm that are (A) calbindin-positive (blue bars) or calbindin-negative (white bars), (A′) *vglut2.2*-positive (green bars) or *vglut2.2*-negative (white bars), or (A″) *gad65/67*-positive (red bars) or *gad65/67*-negative (white bars) in control and amphetamine fish. (B–B″) Percentage of *cfos*-positive cells in Dl that are (B) *vlgut2.2*-positive (green bars) or *vglut2.2*-negative (white bars), or (B′) *gad65/67*-positive (red bars) or *gad65/67*-negative (white bars) in control and amphetamine fish. (C–C″) Percentage of *cfos*-positive cells in the subpallium that are (C) calbindin-positive (blue bars) or calbindin-negative (white bars), (C′) *vlgut2.2*-positive (green bars) or *vglut2.2*-negative (white bars), or (C″) *gad65/67*-positive (red bars) or *gad65/67*-negative (white bars) in control and amphetamine fish. Error bars depict SEM. *n* is the number of analysed brains. **$P < 0.01$; n.s., not significant (unpaired two-tailed Student's *t*-test).

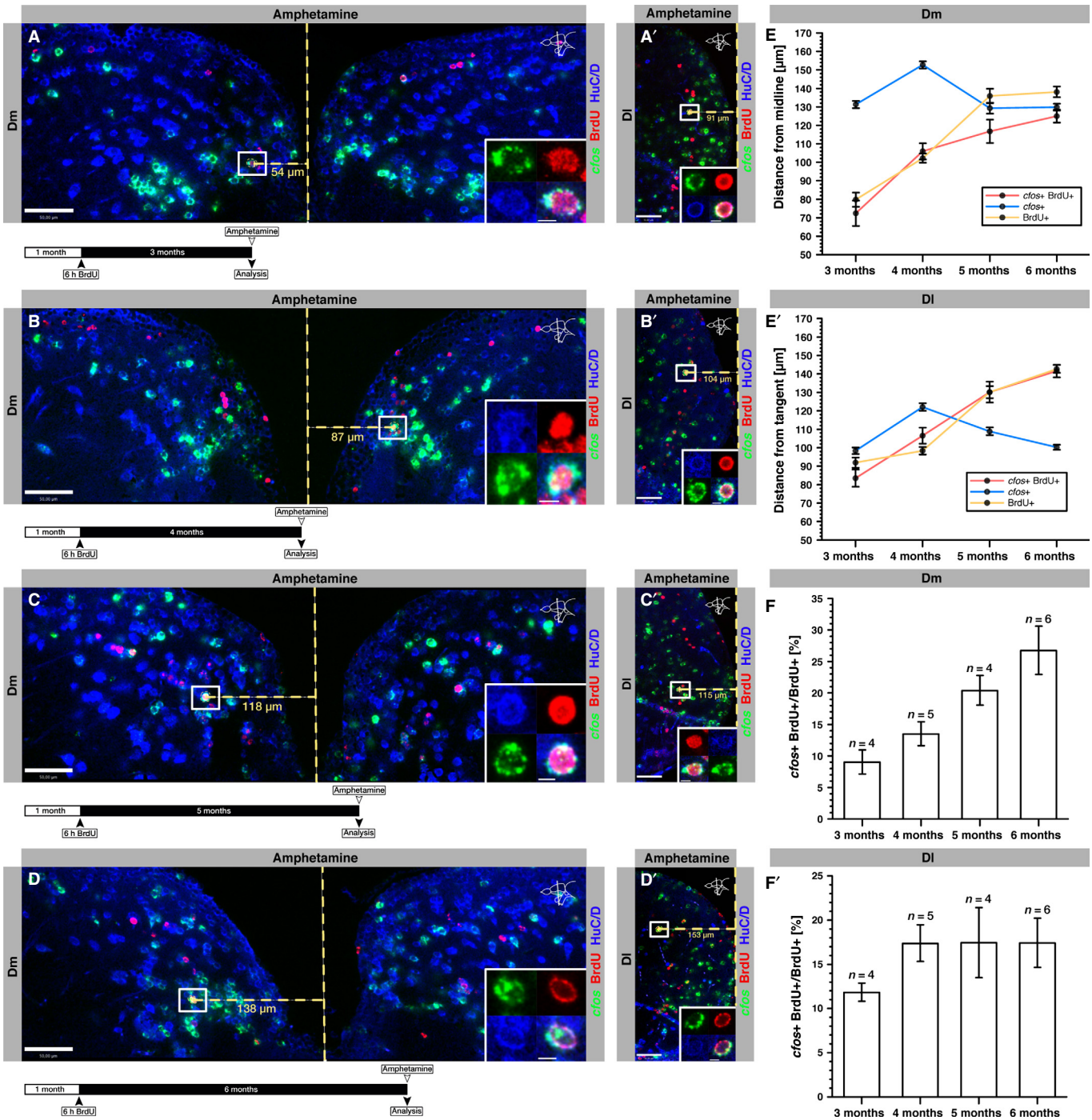


FIG. 6. Juvenile-born neurons respond to acute amphetamine treatment. (A–D′) Single optical sections of Dm (A–D) and Dl (A′–D′) of fish labeled with BrdU at 1 month and analysed after 3 (A, A′), 4 (B, B′), 5 (C, C′) and 6 (D, D′) months within 30 min following acute amphetamine administration. A vertical dashed yellow line represents the midline of Dm (A) or the tangent of Dl (A′), used to measure the distance of BrdU-labeled neurons from the VZ. Length measurements in micrometers are shown for the cell boxed in the main frame beneath the horizontal dashed yellow measurement line. The insets depict the boxed and measured cells at a higher magnification. A schematic design of the experiment is depicted on the lower left beneath each confocal image. Scale bars – 50 µm (main frames); 5 µm (insets). (E) Quantification of the distance of *cfos*-positive (blue line), BrdU-positive (yellow line) and *cfos*/BrdU double-positive cells (red line) from the VZ of Dm after the indicated time points. (E′) Quantification of the distance of *cfos*-positive (blue line), BrdU-positive (yellow line) and *cfos*/BrdU double-positive cells (red line) from the VZ of Dl after the indicated time points. (F, F′) Proportion of BrdU-positive neurons expressing *cfos* in Dm (F) and Dl (F′) at the indicated time points following an administration of BrdU to 1-month-old fish (for absolute values, see Fig. 7). Error bars depict SEM. *n* is the number of analysed brains. (F) one-way ANOVA $F_{3,15} = 7.25$, $P = 0.0004$ post-test for linear trend between 3 and 6 months, highly significant; (F′) one-way ANOVA $F_{3,15} = 0.95$, $P = 0.1830$ post-test for linear trend between 3 and 6 months.

labeled neurons dislocated away from the VZ with increasing time intervals, thereby progressively moving into a field of high *cfos* expression (Fig. 6A–E′, yellow lines). For example, after a 3-month chase, BrdU-positive neurons in Dm were found 79.73 ± 3.84 µm SEM ($n = 83$ cells) away from the VZ, and at 6 months they were located at 138.09 ± 2.93 µm SEM ($n = 297$

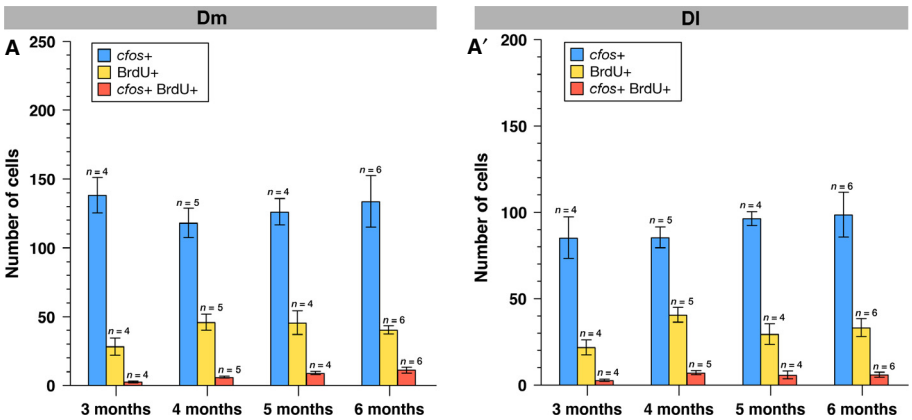


FIG. 7. Time-dependent analysis of the juvenile-labeled BrdU-positive cells and activated neurons following amphetamine treatment. Number of BrdU-positive, *cfos*-positive and BrdU/*cfos*-double-positive neurons in Dm (A) and Dl (A′) at the indicated time points following an administration of BrdU to 1-month-old fish. Error bars depict SEM. *n* is the number of analysed brains.

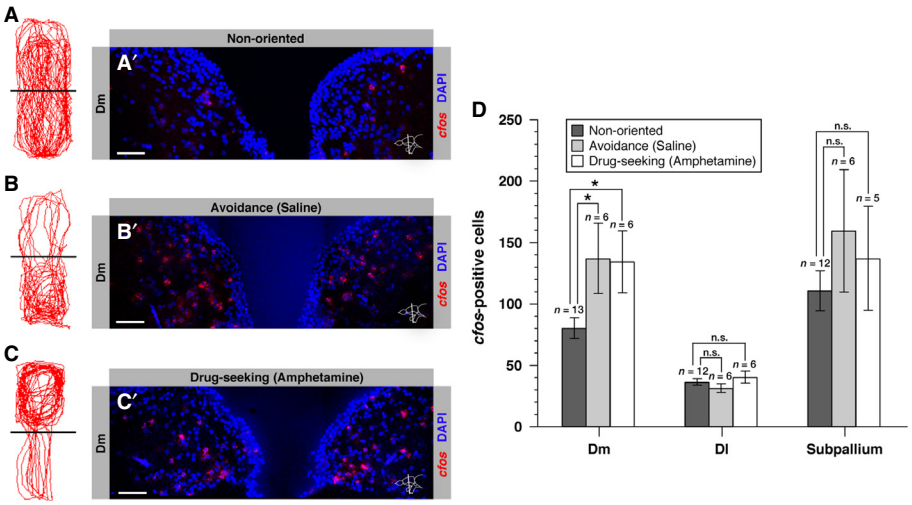


FIG. 8. Dm is activated in both negative and positive emotional contexts. (A–A′) Swimming trajectory of a fish placed in a neutral tank (non-oriented behavior) (A) and its corresponding neuronal activation pattern in Dm analysed via *cfos* expression (A′). (B–B′) Swimming trajectory of a saline-injected fish placed in a visually cued tank (bright – top; dark – bottom) and following an unconditioned light avoidance behavior (B) and its corresponding neuronal activation pattern in Dm analysed via *cfos* expression (B′). (C–C′) Swimming trajectory of an amphetamine-conditioned fish that exhibits a drug-seeking approach behavior (C) and its corresponding neuronal activation pattern in Dm analysed via *cfos* expression (C′). The amphetamine-conditioned fish was placed in the same visually cued tank as in (B) (bright – top; dark – bottom), but after pairing amphetamine with the initially non-preferred bright compartment. (D) Number of *cfos*-positive cells per section in Dm, Dl and the subpallium in control fish that exhibit non-oriented behavior (black bars), an unconditioned avoidance (gray bars) or a drug-seeking behavior (white bars). Error bars depict SEM. *n* is the number of analysed brains. $*P < 0.05$; n.s., not significant (unpaired two-tailed Student's *t*-test).

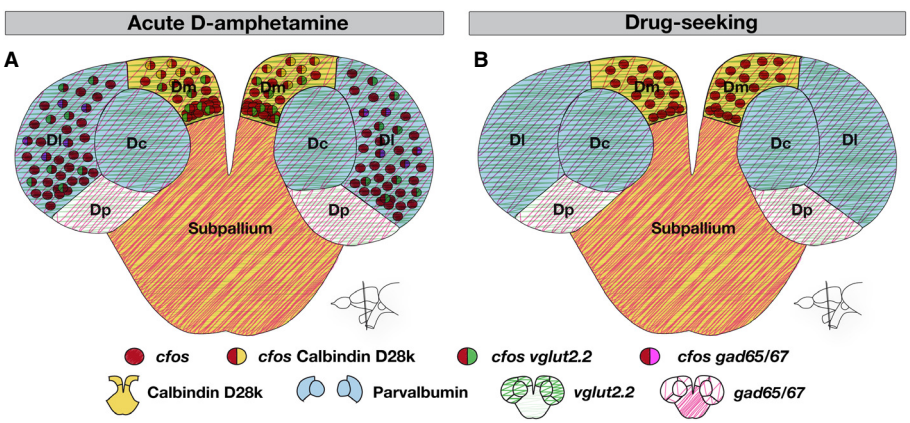


FIG. 9. Schematic visualization of the telencephalic regions in the zebrafish activated by acute amphetamine treatment and during drug-seeking or light avoidance behavior. (A) Neuronal activation visualized by *cfos* expression (red dots) after amphetamine injection. Coexpression of *cfos* together with neuronal markers in their respective expression domains is color-coded. (B) Neuronal activation visualized by *cfos* expression (red dots) during drug-seeking behavior.

cells; Fig. 6A, D and E). Accordingly, the proportion of BrdU-positive neurons that also expressed *cfos* increased from 9.05 ± 1.93% SEM after 3 months to 26.78 ± 3.84% SEM after 6 months (one-way ANOVA $F_{3,15} = 7.25$, $P = 0.0004$ post-test for linear trend; Figs 6F and 7A). In Dl, BrdU-positive neurons were found at 91.90 ± 2.75 μm SEM ($n = 107$ cells) and 142.65 ± 2.11 μm SEM ($n = 381$ cells) from the VZ after 3 and 6 months, respectively (Fig. 6A′, D′ and E′). Thus, the repositioning from superficial towards deeper brain structures occurred slightly faster for newborn neurons in Dl than in Dm and, together with a more proximate field of *cfos* expression, resulted in more and younger drug-activated BrdU/*cfos* double-positive neurons in Dl compared with Dm (Fig. 6E and E′). These results were confirmed when directly quantifying the proportion of BrdU-positive neurons expressing *cfos* – in contrast to the continuous increase seen in Dm, in Dl a maximal proportion of juvenile-born neurons responding to amphetamine was already attained when these neurons were 4 months old (4 months – 17.38 ± 2.07% SEM of *cfos*+ BrdU+ cells; 6 months – 17.42 ± 2.78% SEM of *cfos*+ BrdU+ cells; one-way ANOVA $F_{3,15} = 0.95$, $P = 0.1830$ post-test for linear trend; Figs 6F′ and 7A′). Together, our results suggest that, in the zebrafish pallium, juvenile rather than adult-born neurons respond to amphetamine, and that the neurons born in Dl participate in this response at an earlier age than those born in Dm.

Dm is activated during drug-seeking following conditioning

In ray-finned fish, such as the zebrafish, Dm, but not Dl, was recently shown to be involved in light avoidance (Lau *et al.*, 2011) and taste aversion behavior (Martín *et al.*, 2011). Both studies suggest that Dm plays a similar role to the mammalian amygdala in the negative emotional and motivational context of fear-related behaviors (Johansen *et al.*, 2011). Our results showing a strong activation of Dm in response to acute D-amphetamine administration suggested that Dm may also mediate reward-stimulated behavior, and therefore prompted us to examine whether it may also play a role in motivated behavior. This behavior can be investigated in the zebrafish via a CPP test (Ninkovic & Bally-Cuif, 2006; Webb *et al.*, 2009; Mathur *et al.*, 2011). We used a biased CPP test where adult zebrafish are placed in a tank showing two visually distinct compartments (dark vs. bright), chosen such that fish exhibit an initial preference for one compartment. Thus, saline-injected fish exhibit an avoidance behavior for the initially non-preferred compartment (bright) and do not change their place preference over time (Fig. 8B). In contrast, after a repeated pairing of a drug such as amphetamine with the initially non-preferred compartment, fish exhibit a drug-seeking approach behavior and reverse their place preference from the initially preferred (dark) to the initially non-preferred (bright) compartment (Fig. 8C; for details, see Materials and methods).

To analyse the involvement of Dm neurons in CPP behavior, we therefore compared *cfos* expression in amphetamine-injected fish with control fish placed in a neutral tank that did thus not exhibit a directed behavior (Fig. 8A). The number of *cfos*-positive cells in Dm in fish involved in drug-seeking behavior was 134.30 ± 25.28 SEM ($n = 6$ brains), compared with 80.38 ± 8.51 SEM ($n = 13$ brains) in control fish, representing a 1.7-fold increase (unpaired two-tailed *t*-test $t_{17} = 2.58$, $P = 0.0194$; Fig. 8A′, C′ and D). To compare the activated Dm area with that involved in unconditioned place preference driven by light avoidance, the same analysis was conducted in fish injected with saline and placed in the visually cued experimental tank (Fig. 8B). We observed 1.7 times more *cfos*-positive neurons in Dm in saline fish (137.0 ± 28.56 SEM *cfos*-positive cells, $n = 6$ brains; unpaired two-tailed *t*-test $t_{17} = 2.50$, $P = 0.0229$) compared with control fish (Fig. 8A′, B′ and D), mimicking in location and fold increase the *cfos* response attained in drug-seeking fish. In contrast, we did not observe a difference in the number of *cfos*-positive cells in Dl or the subpallium between fish that exhibited drug-seeking or avoidance behavior and control fish (Fig. 8D).

These results suggest that Dm is similarly activated during motivated behavior (drug-seeking approach) and unconditioned light avoidance behavior. They also highlight commonalities (Dm) and differences (Dl) in the neuronal activity pattern observed following acute amphetamine injection and during drug-seeking behavior. These findings are summarized in Fig. 9.

Discussion

We have used acute injections of the psychostimulant D-amphetamine, together with a quantitative analysis of *cfos* expression and markers of neuronal subtypes, to identify and characterize conserved elements of the brain reward circuitry in the zebrafish telencephalon. This led to the identification of drug-activated territories in Dm and Dl, containing areas homologous to the mammalian amygdala and hippocampus, respectively. We further demonstrated that Dm is similarly recruited in amphetamine-conditioned motivational behavior. Our results suggest an evolutionary conserved function of the amygdala in encoding values and motivational signals, and locate the relevant domain in the zebrafish adult brain to the ventral part of Dm.

Validity of the cfos FISH approach as a read-out of neuronal recruitment or plasticity

cfos expression has been used as an immediate marker of neuronal recruitment and plasticity in a number of brain territories, including the amygdala (Knapska *et al.*, 2007). Compared with other IEGs, its expression is primarily neuronal and tends to be extinguished upon repeated encounter of the same stimulus, such that it better reveals novel neuronal activation/plasticity events (Knapska *et al.*, 2007). Indeed, we noted that a first episode of fish handling or intra-peritoneal injection led to multiple sites of *cfos* induction, which were virtually abolished already after the second handling/injection episode (not shown). We used this property here to buffer the background effects of our amphetamine/saline administration procedure using a pre-handling/injection session, before measuring the specific drug-associated changes in *cfos* expression.

Most studies aiming to provide spatial mapping of neuronal activation relied on the immunodetection of cFos protein (Brown *et al.*, 1992; Mead *et al.*, 1999; Miller & Marshall, 2005; Rademacher *et al.*, 2006) This is largely for practical reasons, as immunostaining allows single-cell resolution and can be easily combined with the co-detection of several other antigens, for example for neuronal subtypes. cFos protein, however, exhibits longer stability than *cfos* mRNA (approximately 2 h vs. 30 min, respectively), which likely resulted in the lasting cFos patterns that we observed (Fig. 2). The new FISH technique that we developed for this study has multiple advantages in this respect – it combines highly sensitive detection of the dynamic *cfos* mRNA expression pattern at single-cell resolution and a quantitative analysis, together with the possible co-detection of other cell type-specific mRNAs or antigens in multicolor stainings. As shown in this study, this new mapping technique proved invaluable for the precise spatiotemporal dissection of the neural circuits underlying drug response and motivated behavior.

Evolutionary conserved function of amygdala nuclei in the processing of emotions

The mammalian amygdala is a heterogeneous collection of nuclei involved in the formation of stimulus-value associations and the storage of the emotional aspects of memories. In mammals, the amygdala is pivotal for the processing of both negative and positive emotions (Phelps & LeDoux, 2005; Murray, 2007; Johansen *et al.*, 2011). It is activated by drugs of abuse or merely the presentation of drug-associated cues, as distinctive neuronal populations assign either a positive or a negative value to a motivational signal (Brown *et al.*, 1992; Carelli *et al.*, 2003; Paton *et al.*, 2006). More recently, it has been shown that the amygdala also integrates spatial and motivational information (Peck *et al.*, 2013). A number of studies specifically implicated the basolateral amygdala (BLA) in these properties, both for positive and aversive learning (Baxter & Murray, 2002; Paton *et al.*, 2006; Shabel & Janak, 2009; Johansen *et al.*, 2011; Tye *et al.*, 2011). The BLA is also involved in processing unconditioned emotional states such as those driving ethological avoidance, for example the avoidance of predators or bright, highly visible places in the wild (reviewed in Knapska *et al.*, 2007). Although amygdala nuclei have been considered homologous between mammals, birds, reptiles and some amphibians, based on comparison of developmental and anatomical data, such similarities are less obvious within the teleost brain (Moreno & González, 2007). Recent studies based on *cfos* detection implicated the Dm subdivision of the zebrafish telencephalon in the innate anxiety-like response displayed in a dark-light box (Lau *et al.*, 2011), and we confirm these results in the present work. Further, Dm lesions were shown to impair the retention of an active avoidance learning response in goldfish (Portavella *et al.*, 2004; Martín *et al.*, 2011). Together, these observations suggest that Dm hosts equivalents to the amygdala nuclei driving avoidance strategies and avoidance learning. Importantly, our results now extend this morpho-functional conservation to the activation by drugs of abuse and to the processing of rewarded learning and positive emotions. They also further circumscribe the relevant amygdala territory to a ventral nucleus of Dm. From our data, it is tempting to speculate that the functional similarities between the BLA and ventral Dm may further extend to goal-directed behavior, adding new evidence for the homology of the two structures. The strict operational definition of goal-directed behavior involves both contingency degradation and instrumental devaluation (reviewed in Mannella *et al.*, 2013). In the CPP test, lowering the association between amphetamine and the initially non-preferred compartment after conditioning decreases the drug-seeking response (Ninkovic & Bally-Cuif, 2006), verifying contingency degradation.

The functional conservation of a BLA-like domain within Dm that we propose here is complemented by neuroanatomical similarities. In agreement with its pallial location and expression of pallial identity markers (reviewed in Wullimann & Mueller, 2004), most neurons in Dm express *vglut2.2*. Only a small fraction expresses *gad65/67*. Similarly, the mammalian BLA is predominantly composed of glutamatergic neurons (80%), with only a minority of GABAergic interneurons (20%; McDonald & Mascagni, 2001; Marek *et al.*, 2013). Both neurotransmitters are involved in BLA functions in mammals, including motivated learning (reviewed in Knapska *et al.*, 2007). Although we have not tested their functional implication in zebrafish, the recruitment of *vglut2.2*-positive neurons in response to amphetamine in Dm is in agreement with a conserved involvement of glutamatergic signaling in the reception or processing of rewarding cues. Although we did observe an increase in the proportion of *gad65/67*-positive neurons expressing *cfos* upon amphetamine administration in Dl, this was not the case in Dm, which may indicate a differential response of GABAergic neurons to amphetamine treatment between the two areas. Finally, neuronal recruitment by amphetamine administration preferentially targets the glutamatergic subtype whereas the proportion of *cfos*-positive GABAergic neurons remains constant, suggesting that this recruitment occurs at the expense of other neurons, possibly producing neuropeptides. These neurons remain to be identified.

The GABAergic and glutamatergic neuronal phenotypes of the mammalian BLA can be subcategorized based on their expression of various CBPs, including parvalbumin, calbindin, calretinin and the calcium-sensitive enzyme calcium/calmodulin-dependent kinase II (Pitkänen & Kemppainen, 2002). Although we have not analysed co-expression in detail, the striking similarity in glutamatergic and GABAergic composition between the BLA and Dm does not extend to the expression of parvalbumin and calbindin. Both CBPs are expressed in the BLA and the hippocampus (Kemppainen & Pitkänen, 2000; McDonald & Mascagni, 2001; Pitkänen & Kemppainen, 2002; Jinno & Kosaka, 2006). In contrast, in the zebrafish telencephalon their expression is largely mutually exclusive – Parvalbumin is expressed in Dl, in which calbindin-positive neurons are only few, if any, but is excluded from Dm, where calbindin-positive neurons are numerous (Mueller *et al.*, 2011; and this study). Although it has been shown that calbindin expression can be affected by amphetamine (Gonçalves *et al.*, 2010), our study is, to the best of our knowledge, the first to report a specific activation of calbindin neurons in an amygdala-like region. Finally, it was shown in primates that distinct populations of amygdala neurons encode positive or negative motivational signals (Paton *et al.*, 2006); whether this is also the case in the zebrafish remains to be determined.

Mapping reward circuitry components in the adult zebrafish brain

Compared with the amygdala, the precise function of the hippocampus in addiction is much less characterized (Robbins *et al.*, 2008; Ricoy & Martinez, 2009; Koob & Volkow, 2010). Thus, it remains to be determined whether the specific activation of glutamatergic and GABAergic neurons that we observed in Dl of the zebrafish pallium also has counterparts in mammals. However, the hippocampus and the amygdala interact substantially (White & McDonald, 1993; Packard *et al.*, 1994; McIntyre *et al.*, 2002; McDonald & Hong, 2013) and this cross-talk is a crucial process in drug addiction, as drugs of abuse simultaneously convey their rewarding properties on both the emotional and memory systems (Phelps, 2004; Robbins *et al.*, 2008; Wells *et al.*, 2011). Although we showed that both Dm and Dl are activated by acute amphetamine injections, only Dm, but not Dl, was activated during drug-seeking behavior. This observation highlights that the direct targets of acute amphetamine administration and motivational processes involve partly distinct neuroanatomical correlates. In addition, it suggests that, following conditioning, CPP does not primarily rely on the processing of spatial information but is triggered instead through stimulus–response associations. Specifically, hippocampal circuits are likely to assess the salience of stimuli based on their novelty (reviewed in Mannella *et al.*, 2013). Hence, hippocampal activation would be expected to vanish after the reiterated association of a constant dose of amphetamine with the same visual cue.

Another key component of the mammalian mesocorticolimbic reward circuitry is the nucleus accumbens (NAc; Koob & Volkow, 2010). A striatal homolog of the mammalian NAc in teleosts remains yet to be identified, even if conserved expression of molecular markers has suggested that the zebrafish subpallium is composed of striatal-, pallidal- and septal-like subdivisions (Ganz *et al.*, 2012). Although we observed the highest number of *cfos*-expressing cells in amphetamine-injected fish in the subpallium, the number of cells recruited after amphetamine treatment was not increased as compared with saline-injected control fish. However, the intensity of *cfos* expression per cell in response to amphetamine was about twofold higher, suggesting that neurons in the subpallium may also participate in the drug response, in addition to those activated in Dm and Dl.

Adult neurogenesis and amphetamine response

The vast neurogenic and regenerative potential of the adult zebrafish brain combined with a molecular genetic amenability and behavioral analyses make this system a prime candidate to decipher the role of adult neurogenesis in drug addiction and emotional and motivational behaviors (Norton & Bally-Cuif, 2010; Zupanc & Sîrbulescu, 2011; Guo *et al.*, 2012; Kizil *et al.*, 2012; Schmidt *et al.*, 2013). Our study, however, did not reveal a strong response of adult-born neurons to amphetamine; instead, it suggests that most drug-activated neurons both in Dm and Dl of a middle-age adult are born at the juvenile rather than the adult stage. Our BrdU analysis in adults allowed tracing only a subset and not all adult-born neurons, and it was restricted to a 2-month tracing interval. Nevertheless, together with our comprehensive juvenile analysis that showed a recruitment of 4.5- and 6-month-old neurons in Dl and Dm, respectively, this suggests that the neuronal response is mediated mostly by early- rather than late-born neurons firmly integrated into their specific networks (Rothenaigner *et al.*, 2011). This may be surprising in two ways. First, adult-born neurons exhibit enhanced synaptic plasticity and contribute to learning and memory processes in rodents (Schmidt-Hieber *et al.*, 2004; Ge *et al.*, 2007; Deng *et al.*, 2010), which would make them prime candidates for recruitment in a behavioral process conditionally induced in the adult. Second, it has been shown that adult hippocampal neurogenesis is affected by drugs of abuse in rodents (Eisch *et al.*, 2000; Eisch & Harburg, 2006; Mandyam *et al.*, 2008), and that impaired neurogenesis increases drug-seeking behavior and the risk to drug relapse (Noonan *et al.*, 2010; Mandyam & Koob, 2012; Recinto *et al.*, 2012; Canales, 2013). Along these lines, we previously documented a link between the abnormal regulation of expression of genes involved in adult neurogenesis and the absence of amphetamine-induced CPP in the zebrafish mutant *no addiction* (Webb *et al.*, 2009). When tools are available to target specific subsets of the pallial and subpallial germinal zones, it will be important to assess the long-term effects of adult neurogenesis manipulations on CPP behavior.

Conclusions

Taken together, our data highlight for the first time that the reward circuitry in zebrafish involves Dm, a territory that shares developmental origin, gene expression and neuronal connections with the mammalian BLA. Like the mammalian BLA, Dm is activated both upon acute administration of the rewarding drug amphetamine and following conditioning during drug-seeking behavior. These findings suggest an evolutionary conserved function of the amygdala in the processing of positive emotions and induction of motivated behavior.

Conflict of interests

The authors declare that no competing interests exist.

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Abbreviations

BLA, basolateral amygdala; BrdU, 5′-bromo-2′-deoxyuridine; CBP, calcium-binding protein; CPP, conditioned place preference; DAPI, 4′,6-diamidino-2-phenylindole; DIG, digoxigenin; Dl, lateral zone of the dorsal telencephalic area; Dm, medial zone of the dorsal telencephalic area; DNP, dinitrophenol; FISH, fluorescent *in situ* hybridization; FLUO, fluorescein; GABA, γ-aminobutyric acid; IEG, immediate-early gene; NAc, nucleus accumbens; NGS, normal goat serum; PBS, phosphate-buffered saline; PBST, PBS containing 0.1% Tween; ROI, region of interest; RT, room temperature; VZ, ventricular zone.

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