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

Jakob William von Trotha,1 Philippe Vernier2 and Laure Bally-Cuif1
1Institute of Neurobiology A. Fessard, Laboratory of Neurobiology and Development, CNRS UPR3294, Team Zebrafish Neurogenetics, Avenue de la Terrasse, bldg 5, F-91198 Gif-sur-Yvette, France
2Institute of Neurobiology A. Fessard, Laboratory of Neurobiology and Development, CNRS UPR3294, Team Development of Neurotransmission, Avenue de la Terrasse, bldg 5, F-91198 Gif-sur-Yvette, France

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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 o-ampetamine 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 DI. The drug-activated neurons in Dm and DI 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 DI. 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 evolutionarily 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 (Kaluduff 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 (DI) 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,
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′-ATGATGGTTACCAGCCCTAGCG-3′ and 5′-TCA AAGGAGGAGGGTTG-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% H2O2 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) H2O2 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/mL) 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 ‘Tilescans’ 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 & Muller, 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 DI nuclei are activated by acute α-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 α-amphetamine injection in Dm and DI, hosting the territories homologous to the mammalian amygdala and hippocampus, respectively (Fig. 3A–D). Notably, in DI, 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 \( t_{99} = 6.83, P < 0.0001; \) Fig. 3E). In DI, 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 \( t_{20} = 8.39, P < 0.0001; \) 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 \( t_{22} = 0.58, P = 0.5723; \) Fig. 3E], arguing for a drug-specific increase of cfos expression in Dm and DI 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, DI 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 \( t_{22} = 4.12, P = 0.0002 \)) and DI (unpaired two-tailed t-test \( t_{22} = 4.22, P = 0.0001 \)), 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 DI (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 α-amphetamine administration**

To further characterize the drug-activated cells in Dm and DI, we made use of the neuronal subtype markers calcium-binding protein (CBP) calbindin D28k, vlglut2.2, 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 DI 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′). vlglut2.2 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 vlglut2.2 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 vlglut2.2 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, vlglut2.2 or gad65/67.

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**Fig. 3.** Dm and DI nuclei are activated by an acute α-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), vlglut2.2 (FISH, green) and gad65/67 (FISH, red) expression together with DAPI staining. Note the increase in cfos expression in Dm and DI areas (arrows) in amphetamine- compared with saline-injected control fish. Brain regions are outlined by white dots. Dc, central zone of the dorsal telencephalon; DI, lateral 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, DI 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).
Acute D-amphetamine administration (Fig. 5B and B′) shows, 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 DI 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).

Glutamatergic and GABAergic neurons in DI are activated by acute Δ-amphetamine administration

Neuronal recruitment within the glutamatergic population was similar in DI 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 t0 = 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 t0 = 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 DI after acute amphetamine administration (Fig. 5B and B′). We conclude that, in DI, 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 Δ-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 & Siribulescu, 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 DI respond to acute Δ-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 DI, 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 DI 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 DI (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-
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 cells).

**Fig. 6.** Juvenile-born neurons respond to acute amphetamine treatment. (A–D′) Single optical sections of Dm (A–D) and DI (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 DI (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. (F) 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 DI after the indicated time points. (F, F′) Proportion of BrdU-positive neurons expressing *cfos* in Dm (F) and DI (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.
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 zebralsh 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 (Martin 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 (Knap'ska 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 (Knap'ska 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 ethical 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; Martin 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 & Martínez, 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 & Shabal, 2008; Ricoy & Martinez, 2009; Koob & Volkow, 2010). Thus, it remains to be determined whether the specific activation of GABAergic and glutamatergic neurons that we observed in Dl of the zebrafish pallium also has counterparts in mammals. However, the hippocampus and the amygdala interact substantially (White & Shabal, 2008; Ricoy & Martinez, 2009; Koob & Volkow, 2010). 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 & Sribulescu, 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 permitted tracing only a subset and not all adult-born neurons, and it allowed tracing only a subset and not all adult-born neurons, and it

**Conclusions**

Taken together, our data highlight for the first time that the reward circuitry in zebrafish involves Dm, a territory that shares development 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-diamindino-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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