Genetic Identification of AChE as a Positive Modulator of Addiction to the Psychostimulant D-Amphetamine in Zebrafish

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ABSTRACT: **Addiction is a complex maladaptive behavior involving alterations in several neurotransmitter networks. In mammals, psychostimulants trigger elevated extracellular levels of dopamine, which can be modulated by central cholinergic transmission. Which elements of the cholinergic system might be targeted for drug addiction therapies remains unknown. The rewarding properties of drugs of abuse are central for the development of addictive behavior and are most commonly measured by means of the conditioned place preference (CPP) paradigm. We demonstrate here that** **adult zebrafish show robust CPP induced by the psychostimulant D-amphetamine. We further show that this behavior is dramatically reduced upon genetic impairment of acetylcholinesterase (AChE) function in *ache*/+ mutants, without involvement of concomitant defects in exploratory activity, learning, and visual performance. Our observations demonstrate that the cholinergic system modulates drug-induced reward in zebrafish, and identify genetically AChE as a promising target for systemic therapies against addiction to psychostimulants. More generally, they validate the zebrafish model to study the effect of developmental mutations on the molecular neurobiology of addiction in vertebrates.**

INTRODUCTION

Addiction, the uncontrollable compulsion to take drugs in spite of their negative effect on normal brain function, is a widespread and costly brain disorder in modern societies. Yet, its neural mechanisms remain

incompletely understood, and pharmacological treatments, if available, are in most cases ineffective.

The mesocorticolimbic dopamine (DA) system, originating in the midbrain ventral tegmental area (VTA) and innervating the nucleus accumbens (NAc) in the ventral striatum, was directly implicated in the rewarding effects of most drugs of abuse in higher vertebrates (Everitt and Wolf, 2002; Laakso et al., 2002; White, 2002; Wise, 2002). Indeed, psychostimulants increase extracellular levels of DA in the NAc, for example, by blocking the DA transporter (Amara and Sonders, 1998; Jones et al., 1998; Gainetdinov et al., 2002; Saal et al., 2003), thereby affecting the homeostasis of the brain reward system. However, addiction is an intricately complex dysfunction of the reward pathway and likely involves a plethora of neurotransmitter responses, themselves dependent on parameters that can vary upon drug administration, such as progressive neuroadaptative mechanisms. To date, additional monoamine systems (norepinephrine, serotonin) and modulatory pathways [acetylcholine (ACh), GABA, glutamate] have been proposed to contribute to the manifestation of the addictive state, by sharing molecular components with the DA system, by influencing the activity of VTA or NAc neurons (Gaspar et al., 2003; Auclair et al., 2004; Kelley, 2004), or by as yet unresolved mechanisms.

Recent emphasis was placed on the cholinergic system. In several instances, blocking muscarinic cholinergic transmission in rodents increases response to psychostimulants in the self-administration or conditioned place preference (CPP) tests (Gerber et al., 2001; Ichikawa et al., 2002), paradigms classically applied to evaluate addictive behavior. The NAc is densely innervated by cholinergic interneurons; the activity of these neurons is decreased by DA release (Alcantara et al., 2003) and their ablation increases the sensitivity to psychostimulants and the reinforcing effects of cocaine (Hikida et al., 2001). Further, other components of the cholinergic system, like M5 muscarinic receptors on VTA DA neurons or nicotinic ACh receptors on DA terminals in the striatum, are implicated in the regulation of addictive behavior (see Tzschentke, 1998). While most studies agree that cholinergic activity can modulate DA transmission, and thereby the propensity for addiction, a reliable pharmacological target of this system for drug addiction therapy has to be determined. Using pharmacological inhibitors, Hikida et al. (2003) suggested that inhibiting acetylcholinesterase (AChE) activity, which terminates ACh action at the synapse, can block cocaine- and morphine-induced CPP in the mouse. Thus AChE inhibitors might be promising therapeutic agents, although their specificity and range of efficiency remain to be determined.

Here we address the relevance and generality of AChE inhibition on drug-induced behavior. The zebrafish, often proposed as an alternative to mammalian models, is increasingly used as a new genetic system in social behavior studies, because of its amenability to large-scale genetic screens (Gerlai et al., 2000; Darland and Dowling, 2001; Guo, 2004; Lockwood et al., 2004). We demonstrate that adult zebrafish show a clear CPP induced by a widely used psychostimulant, D-amphetamine, and that this effect is significantly attenuated upon genetic impairment of AChE activity in $ache^{sb55}/+$ mutants (Behra et al., 2002). Our results provide the first genetic demonstration that AChE is a promising target for therapeutic approaches to addiction, and validate the zebrafish to study the consequences of developmental mutations and the neuronal pathways underlying this condition in vertebrates. The experimental paradigms developed here are the first sufficiently robust to use the excellent genetic model zebrafish to conclusively screen for mutations affecting reward.

METHODS

Animal Care and Maintenance

Adult zebrafish were kept in the fish facility, as previously described (Kimmel et al., 1995). At least 2 days before each assay, the fish were moved to an isolated room under maintenance conditions and feeding schedule identical to the fish facility (14/10 h day/light cycle, two feedings per day at 8:00 am and 2:00 pm—except in the case of learning tests, see below—water temperature 28°C). Thus, we kept environmental variance at a minimum for all behavioral assays. The AB strain was bred in our fish facility for more than 20 generations. $ache^{sb55}/+$ (Behra et al., 2002), initially on ABO background, were crossed for at least three generations to our AB strain before all experiments. They were identified in random brother-sister crossing, giving rise to 25% $ache^{sb55}/ache^{sb55}$ immobile embryos, as described previously (Behra et al., 2002). All experiments reported in this article were done on 3- to 6-month-old females (however, we observed no difference in the behavior of males compared to females; not shown). Care was taken to always test the same fish at the same time of the day over the successive days of each experiment.

Place Preference (PP) Determination

The testing apparatus was a 3 L, rectangular tank containing 2 L of water and placed in an isolated cabinet with top illumination. The water level was kept to 10 cm from the tank bottom to minimize stress. Distinct visual cues divided the experimental tank into two halves: a dark half colored in brown and a light half colored in white with two frighten-

ing, black circles placed at the bottom of the tank. After an initial introduction in the testing apparatus, each fish was separately accommodated to the new environment for 2 entire days (days 1 and 2) and was afterwards recorded in one 15 min trial on day 3 using Noldus Ethovision v.2.3 system (Noldus Information Technology, Netherlands). The preferred compartment was defined as the compartment in which a fish spent most time on day 3, and the basal level of PP (*PPi*), serving as a basis for all further comparisons, was calculated as the percentage of time that the fish spent in the preferred compartment during the 15 min recording on day 3. *PPi* levels between $ache^{sb55}/+$ and their wild-type siblings were measured in the set-up described above and compared using independent samples Student's *t* test; all graphics were generated using Origin v.7 (OriginLab, Northampton, USA).

D-Amphetamine-Induced CPP

After PP determination (day 3), each fish was weighed (typically, weights varied between 0.5 and 3 g per fish) then intraperitoneally injected with D-amphetamine (40 μg of D-amphetamine;Sigma-Aldrich A0922, Germany) and methylene-blue as a tracer (3 μg per gram of fish in 110 m*M* NaCl, if not otherwise indicated) and immediately confined to the nonpreferred compartment for 45 min (day 4). Restriction to this compartment was achieved using a transparent slider such that visual contact with the preferred compartment was possible, to minimize the difference between the conditioning and the measuring tank. The experimental tank and conditions were otherwise identical to the ones used for PP determination, and each fish was tested alone. After 45 min, the fish was removed from the experimental tank and kept in a 1.5 L tank on a color-neutral background. On day 5, the fish was injected intraperitoneally with a saline solution (3 μg of methylene-blue per gram of fish in 110 m*M* NaCl), then restricted for 45 min into the preferred compartment. Between each injection session, the experimental tank was cleaned with 70% ethanol and rinsed with fish facility water. The D-amphetamine treatment was repeated on days 6 and 8 and the saline treatment on day 7. PP was then measured again on day 9. Repeating the saline treatment on day 9, followed by measurement of PP on day 10, lead to identical results (not shown). Unless otherwise specified, conditioning was estimated as the change in PP before and after treatment, relative to the PP before treatment, as follows: % of change = $100 \times (Ppi - PPf)/PPi$, where % of change is the relative change in PP, *PPi* is the percentage of time spent in the initially preferred compartment (measured at day 3, see above), and *PPf* the percentage of time spent in this same compartment after treatment (measured at day 9 or 10). For percentages outside the range 30–70%, the calculation of % of change was followed by arcsine transformation (as recommended in Hogg, 1995) [Figs. 1(C) and 2(C)]. In all experiments, a comparison with scoring the percentage of change in absolute values (*PPi* − *PPf*) was performed, leading to identical conclusions [see Supplemental Figs.

1(B) and 2, and see Fig. 3(B,C)]. The significance of all comparisons was established using independent samples Student's *t* test and all graphics were generated using Origin v.7 (OriginLab). All manipulations were performed according to authorization N°AkZ 209.1/211-2531-20/02 from the Government of Upper Bavaria and the German Institute for Drug Control (Bundesopiumstelle).

Learning and Memory Tests

Learning and memory tests were conducted in a T-maze, following a procedure modified from Darland and Dowling (2001). The experimental tank was composed of one long (46 cm) and two equally short (30 cm) arms. All arms were 6 cm wide and 15 cm deep with a water level of at least 10 cm. One of the two short arms opened into a deep (20 cm) square tank (23 × 23 cm) with black walls and artificial grass to offer a favorable habitat for the zebrafish. Most of the fish tested spent the majority of their time in this compartment once they found it. Two days before testing, a group of 10 fish was restricted with the transparent slider to the long arm of the T-maze for 1 h per day to accommodate to the testing environment. After accommodation, each fish was placed alone at the beginning of the long arm and the time needed to find the deep compartment was recorded. After reaching the deep compartment, the fish got their daily feeding. Each fish was tested once a day. We considered that a fish had learned the task when its time to find the target compartment varied less than 10% upon consecutive trials. The learning ability of fish with different genotypes was compared using independent samples *t* test and all graphics were generated using Origin v.7 (OriginLab).

Brain D-Amphetamine Levels

The brains were removed, put on ice, weighed, and homogenized in 100 μL of plasma-spiegel solution (0.12% H_3PO_4, pH 3.5 adjusted with 6 *M* NaOH). Amphetamine extraction and determination of amphetamine concentration were carried out according to an HPLC protocol developed by Labmed (Dortmund, Germany).

AChE and ACh Assays

The brains were removed, put on ice, and weighed. AChE activity of brain extracts was determined according to Ellman et al. (1961) and measured as the amount of ACh that is broken down by AChE per gram of brain tissue per time unit. ACh concentration in brain extracts was measured with the Amplex Red Acetylcholine/Acetylcholinesterase Assay Kit (Molecular Probes, Eugene, OR) according to the manufacturer's instructions.

Electroretinography (ERG)

ERGs were performed on adult zebrafish as previously described (Makhankov et al., 2004). Briefly, the recording

electrode was positioned on the surface of the cornea. A chlorodized silver wire was fixed on the opposite nostril as a reference electrode. To ensure oxygenation of the animal, MESAB medium (Sigma–Aldrich, Buchs, Switzerland) was flushed by gravity forces over the gills by a plastic tube inserted into the mouth. The fish were dark-adapted for at least 30 min before mounting the electrode under dim red light. Before exposure to light, they were adapted in complete darkness for at least 15 min. A 1 s light stimulus was chosen with an interstimulus interval of 10 s. Illumination was increased in 1.0 log unit steps over the range from -5 log unit (0.5 cd/m^2) to -1 log unit (5000 cd/m^2). Unattenuated light intensity was measured at the diffuser surface of the light guide over the fish head by a light meter (Tekronix
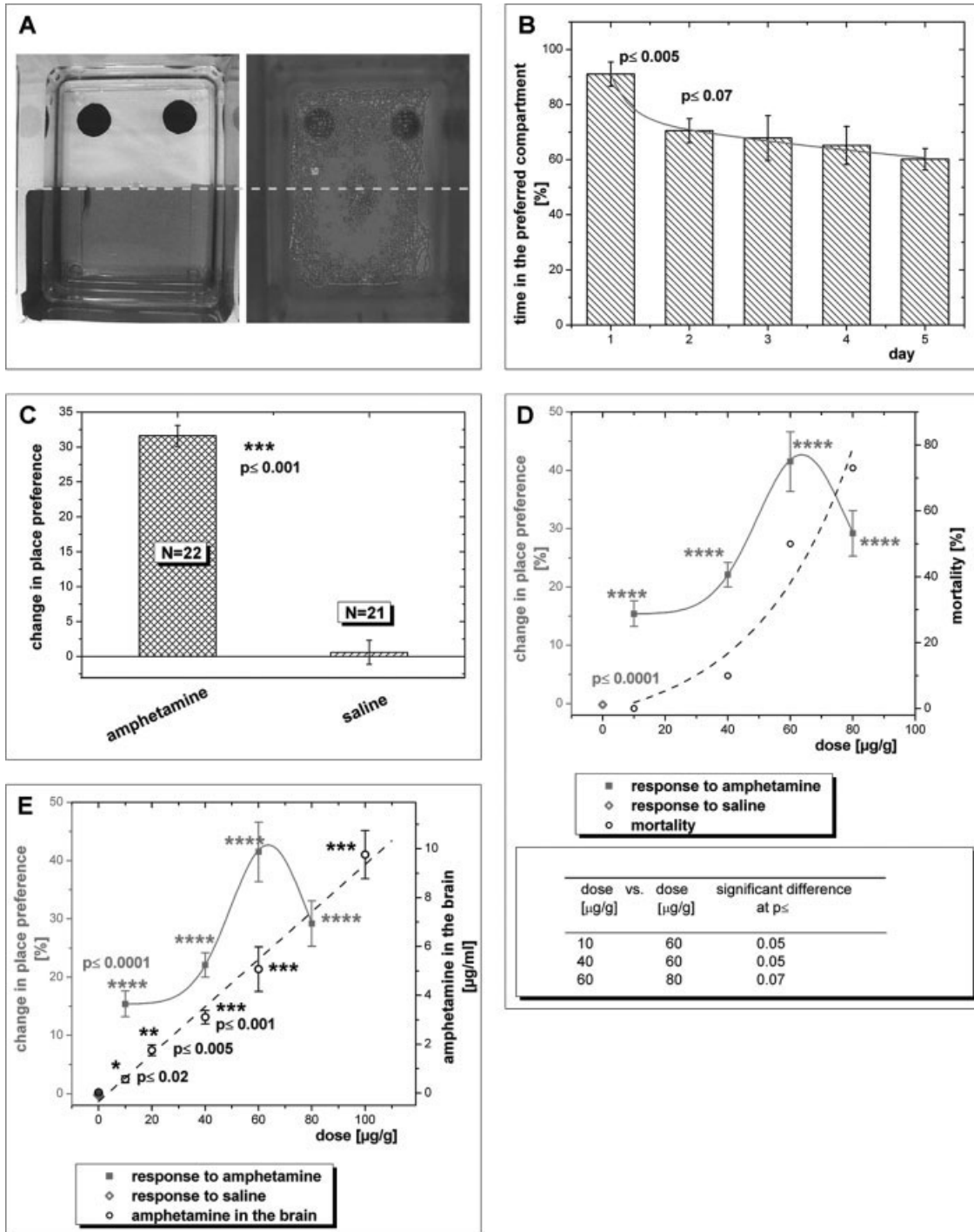

Figure 1

J17; Texas Instruments, USA). ERG responses were averaged three to seven times depending on the signal-to-noise ratio. Statistical analysis was performed using SPSS v.11 (SPSS Inc., Chicago, IL).

RESULTS

Amphetamine-CPP in Zebrafish

Amphetamine can trigger a conditioned change in PP in higher vertebrates, but has only been tested in teleosts (goldfish) in an associative paradigm (Lett and Grant, 1989). Thus we first invested in setting-up a reliable amphetamine-conditioned CPP test in zebrafish. Our test was based on remembering the association of the rewarding effect of amphetamine with an initially nonpreferred environment, recognizable by visual cues. To establish the ideal visual parameters, we built on previous reports demonstrating PP of adult zebrafish for one side of a two-color tank (Serra et al., 1999; Gerlai et al., 2000; Darland and Dowling, 2001).

Reliability and robustness of a CPP test means it must meet a number of requirements. First, before being confronted with the drug, the animal has to habituate to the test conditions until its PP to one side of the tank becomes stable. This level (PP after habituation, or *PPi*) is the only reliable base to measure changes in PP following administration of the drug. Second, a two-color compartment has to be developed where *PPi* is not excessive so as to permit reversal upon adminis-

Figure 1 Establishment of reliable test conditions to measure D-amphetamine-induced reward in zebrafish adults. (A) Place preference measurement set-up (left panel, viewed from top) and representative video-recorded route followed by a wild-type fish in this set-up at day 3 (right panel, example with 58% of the time spent in the brown compartment). (B) Habituation curve. Percentage of time spent in the brown versus the white compartment [see (A)] in 15 min measurements over 5 consecutive days (one trial per day). Each bar represents the average of five fish, with standard errors indicated. The variations in the preference of fish for the brown side between consecutive days were compared using independent samples Student's *t* test. Note that the time spent in the preferred compartment significantly decreases until day 3, after which time it remains stable at a value of 55–70% (until at least day 10, not shown). This initial decrease might reflect increased exploratory activity of the fish as it gets used to the test tank. The percentage of time spent in the preferred compartment at day 3 (PP after habituation, later referred to as *PPi*) is taken as a basis in all subsequent measurements. (C) Change in place preference induced by the intraperitoneal administration of 40 μg/g of D-amphetamine (left) paired with the nonpreferred compartment, compared to the administration of saline (right) in identical conditions. The ''change in place preference'' (*y* axis) is measured as the relative difference in time spent in the preferred (nonamphetamine-paired) compartment before (*PPi*) and after (*PPf*) drug exposure (in percentage of *PPi*). This change was compared between *n* amphetamine-treated and control fish using independent samples Student's *t* test followed by arcsine transformation (Methods), standard errors are indicated [see Supplemental Fig. 1(A), middle and bottom panels, for a survey of the response of individual fish]. Note that amphetamine-injected fish significantly revert their place preference to choose the amphetamine-paired compartment after drug exposure, while fish injected with saline do not revert their preference. Identical conclusions are reached when scoring the absolute difference between *PPi* and *PPf* [see Supplemental Fig. 1(B)]. (D) Change in place preference (*y* axis, left, red curve) and mortality (percentage of fish dying during the procedure, *y* axis, right, dotted black curve) as a function of the dose of amphetamine injected (*x* axis, saline injections indicated as the zero dose). Each point is based on the test of at least 15 fish, with standard errors indicated; statistical significance between the responses at different doses was calculated using independent samples Student's *t* test (see values on the graph and table below). Our experimental conditions produce a dose/change response curve following a Gaussian distribution similar to that observed in mammals (Tzschentke, 1998), where doses above a certain threshold (here 50 μg/g) induce a toxic response. (E) Change in place preference [*y* axis, left, red curve, same as in (D)] and brain amphetamine levels (*y* axis, right, dotted black curve) as a function of the dose of amphetamine injected (*x* axis). Each measurement of brain amphetamine levels is averaged from three fish, with standard errors indicated; statistical significance between the levels of amphetamine in the brain at consecutive doses injected was calculated using independent samples Student's *t* test (see values in black on the graph). Note that the level of amphetamine received in the brain is a linear function of the dose injected within the range of values used in our experiments. Note also that it keeps increasing linearly above the threshold dose inducing maximum CPP, confirming that the decreased CPP above this dose is due to a toxic effect of amphetamine rather than its elimination from the zebrafish organism.
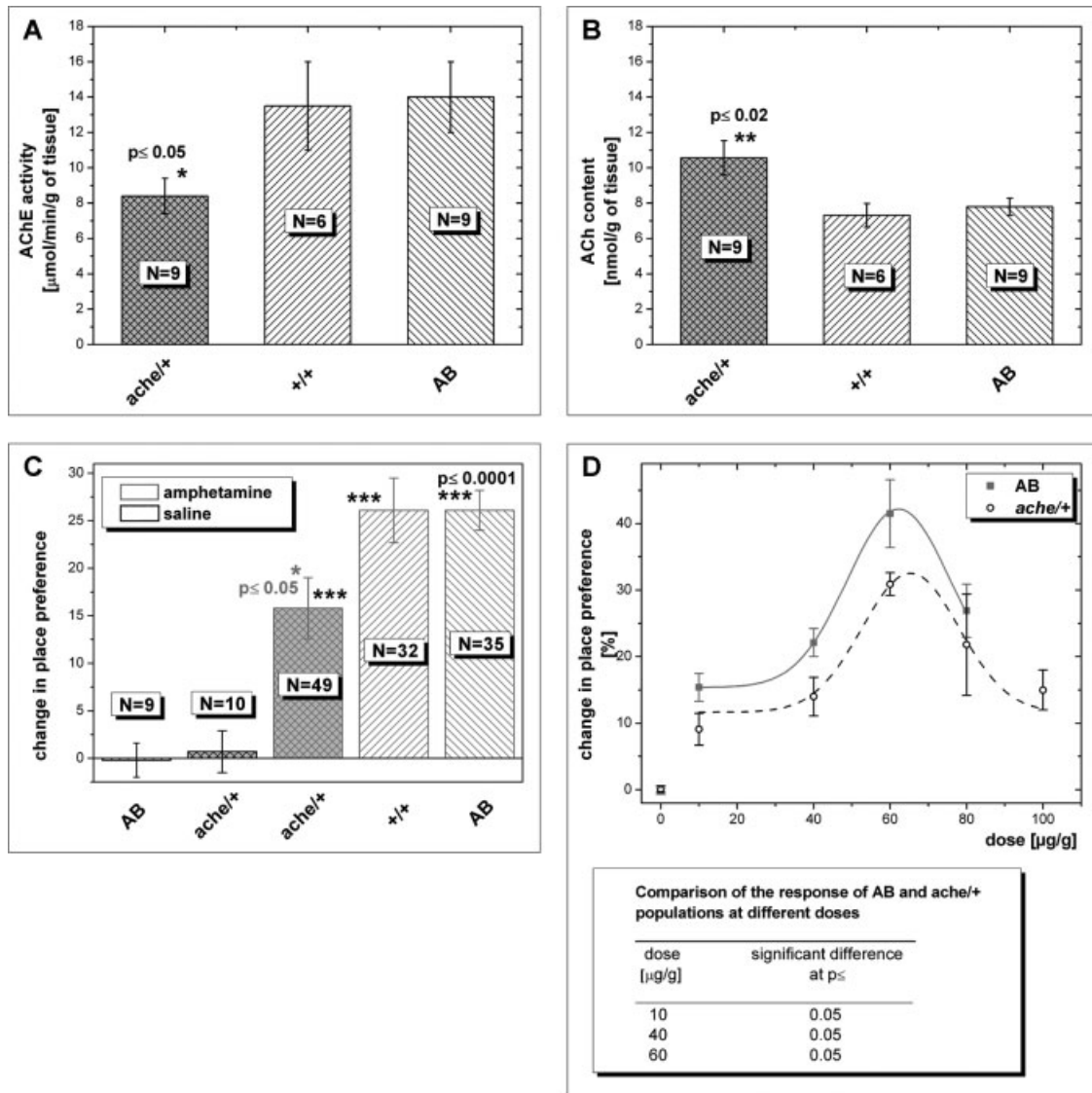
Figure 2 Lowered amphetamine-induced CPP in $ache^{sb55}/+$ heterozygotes. In all cases, fish with different genotypes were compared using independent samples Student's t test, and standard errors are indicated. (A,B) Altered brain levels of AChE activity (A) and ACh (B) in $ache^{sb55}/+$ heterozygotes (left) compared to their wild-type siblings ($+/+$, middle) or AB controls (right). Each value is an average of n fish. Brain levels of AChE are reduced by nearly 50% (A) and brain levels of ACh are increased by 40% (B) in $ache^{sb55}/+$ heterozygotes compared to their wild-type siblings, which do not differ from AB controls. (C) Change in place preference induced by 40 μg/g amphetamine in $ache^{sb55}/+$ heterozygotes (red, left), their wild-type siblings ($+/+$, red, middle), and AB controls (red, right), also compared to the effect of saline injections in $ache^{sb55/+}$ (black, right) and AB (black, left). The change in place preference (y axis) is measured and statistically evaluated as in Figure 1(C), and each value is an average of n fish. Amphetamine-induced change in place preference is significantly decreased (1.6 times) in $ache^{sb55}/+$ heterozygotes compared to their wild-type siblings, which do not differ from AB controls, and saline injections have no effect. Identical conclusions were reached when scoring the change in place preference in absolute values (Supplemental Fig. 2). (D) Compared dose-response curve in $ache^{sb55}/+$ heterozygotes (dotted line) and AB controls (red line). The percentage of change in PP (y axis) is represented as a function of the dose of amphetamine injected (x axis). Each point is the average of at least 15 fish. Statistical significance between the responses of the two genotypes at a given dose was calculated using independent samples Student's t test (standard errors indicated, see significance values on the table below the graph). $ache^{sb55}/+$ heterozygotes responded to amphetamine following a Gaussian curve of similar amplitude to that of wild-type fish but shifted towards lower y values (center position for wild-type fish: 65.3; for $ache^{sb55}/+$ fish: 62.3). Thus, the dose of 40 μg/g amphetamine selected as optimal for wild-type fish is also optimal in $ache^{sb55}/+$ heterozygotes, which responded with lower CPP than wild-type to the complete range of amphetamine doses tested.

tration of an optimal dose of drug. In higher vertebrates, conditions where *PPi* to one side approaches 55–65% work best (Tzschentke, 1998). Third, the dose of drug has to be determined as the best compromise between a high response and an acceptable rate of animal survival, as determined on a dose/response-survival curve. In addition, to make sure that the drug acts via its impairment of brain function, one has to ascertain that the level of drug received in the brain is in proportion to the dose administered.

Because previous studies did not agree on the preference of zebrafish adults for brightness or darkness (Serra et al., 1999; Gerlai et al., 2000; Darland and Dowling, 2001), we first had to find appropriate conditions yielding reliable results. We thus designed a number of two-color 3 L tanks and tested more than 20 wild-type adults in each. We selected a light brown versus white contrast [Fig. 1(A)], where a test on 50 wild-type adults demonstrated that more than 95% of these fish spent between 55 and 70% of their time on the brown side following 2 days of habituation (on days 1 and 2; not shown). We further observed that the percentage of time spent in this preferred compartment remained stable after day 3 [Fig. 1(B)]. We thus measured PP at day 3 as *PPi* and started our conditioning procedure at day 4. In such conditions, more than 90% of the animals tested significantly reverted their PP following three intraperitoneal administrations of 10 μg amphetamine per gram of body weight (on days 3, 5, and 7), each time paired with 45 min in the nonpreferred compartment and separated by daily injections of saline (on days 4 and 6) paired with the preferred compartment [Supplemental Fig. 1(A), top panel]. Control fish injected every day with a saline solution alternatively paired with the white and brown compartments failed to revert their PP [Supplemental Fig. 1(A), bottom panel].

Next, we determined the optimal dose of drug permitting robust CPP at acceptable toxicity. In a dose-response study, we observed that doses of amphetamine below 5 μg/g did not lead to a reproducible change in PP, while doses above 50 μg/g progressively reduced this change due to immobilization and stress of the animal and/or death [Fig. 1(D), each point >15 fish]. Similar observations have been reported in mammals (Tzschentke, 1998). Thus, we selected a dose of 40 μg/g, which triggers robust change in PP but low mortality [Fig. 1(D)]. We further verified that at this value, the dose of amphetamine received in the brain, measured by gas-HPLC on brain extracts within 10 min after injection, was a linear function of the dose injected [Fig. 1(E), each point = 3 fish]. In these conditions and using the experimental paradigm described above, 95% of

wild-type fish significantly reverted their PP when administered with 40 μg/g amphetamine ($n = 22$) [Fig. 1(C)], while 97% of control fish (i.e., injected only with a saline solution) failed to do so [$n = 21$; $p < 0.001$; Fig. 1(C); see also Supplemental Fig. 1].

Reduced Amphetamine-Induced CPP in Zebrafish AChE Mutants

In mammals, lowered brain ACh signaling has been associated with an increased propensity to get addicted to psychostimulants (Gerber et al., 2001; Ichikawa et al., 2002). To determine whether the cholinergic system was involved in a similar regulatory pathway in zebrafish, we measured the sensitivity of zebrafish with genetically impaired ACh metabolism towards the rewarding effects of amphetamine. Zebrafish *ache*[sb55] mutants harbor a point mutation in the AChE-encoding gene, resulting in the production of a nonfunctional AChE enzyme (Behra et al., 2002). Because AChE is the only ACh-degrading enzyme in zebrafish, *ache*[sb55]/*ache*[sb55] homozygous embryos are completely deficient in ACh hydrolysis and die of progressive paralysis at early larval stages (Behra et al., 2002). *ache*[sb55]/+ heterozygotes, however, reach adulthood without obvious locomotor or any morphological defects (not shown; $n > 50$). We found that AChE activity was decreased by nearly 50% in the brains of *ache*[sb55]/+ heterozygotes compared to their wild-type siblings or our AB control strain [Fig. 2(A)] ($n = 9$; $p < 0.05$), resulting in a 1.4-fold increase in their level of brain ACh [Fig. 2(B)] ($n = 9$; $p < 0.02$). Immunohistochemical analyses failed to reveal defects in the location and organization of dopaminergic-, serotonergic-, and cholinesterase-positive brain neuronal clusters in *ache*[sb55]/+ heterozygotes, suggesting that, in spite of their perturbed levels of AChE and ACh, these fish do not suffer from grossly abnormal neuroanatomy (not shown). These results suggest that *ache*[sb55]/+ mutants are a valuable genetic model to test the effects of increased brain levels of ACh.

To measure the impact of lowered AChE activity on drug-induced effects, we assessed the behavior of *ache*[sb55]/+ adults upon administration of 40 μg/g amphetamine in the conditions described above. We found that *ache*[sb55]/+ heterozygotes exhibit a significantly lowered change in PP compared to their wild-type siblings: while the average change in siblings is 24%, it is 14% in *ache*[sb55]/+, representing a 46% reduction in the response [Fig. 2(C), red crossed bar] ($n = 49$; $p < 0.05$; see also Supplemental Fig. 2). Again, siblings did not differ from controls of the
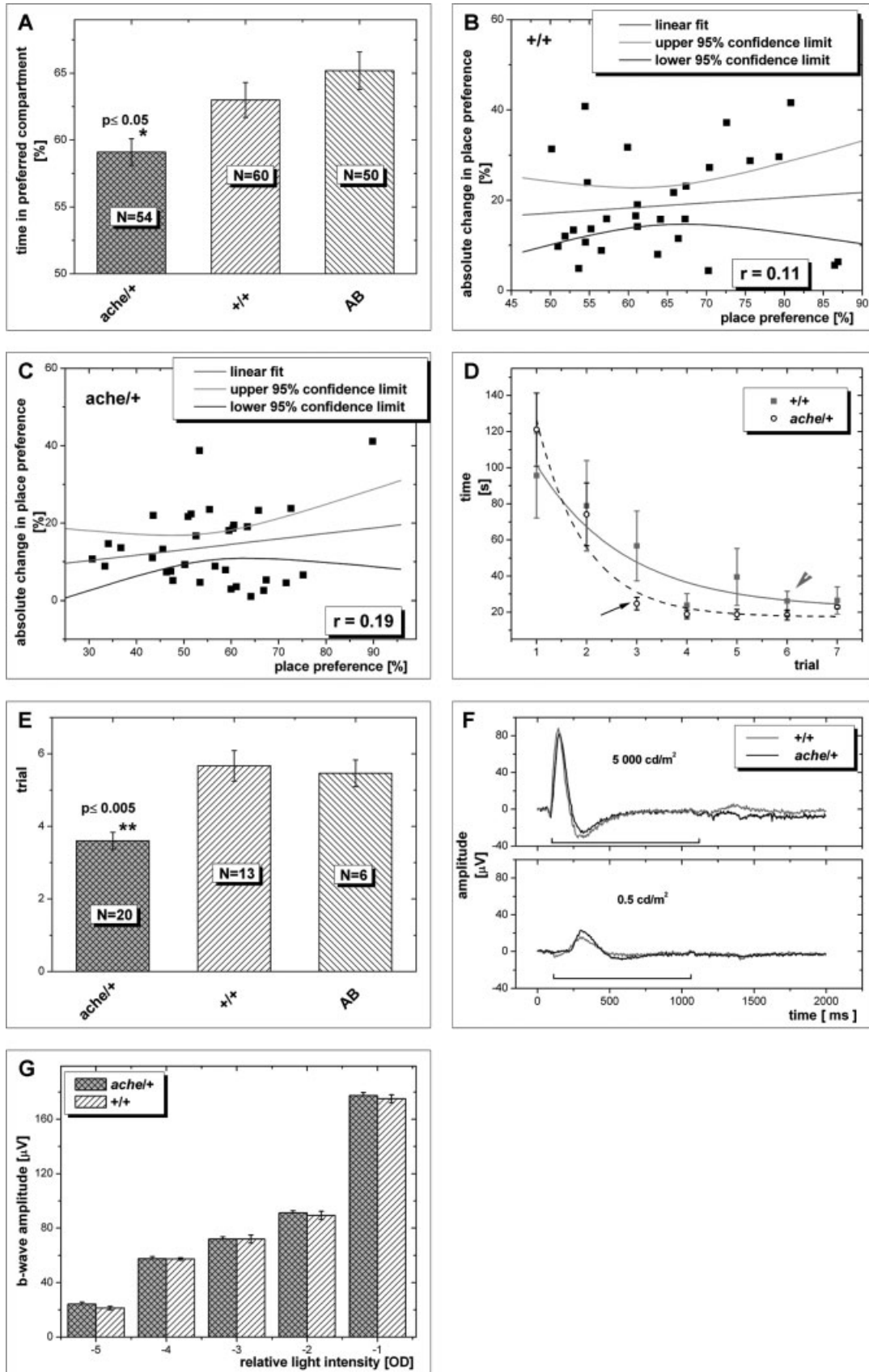
Figure 3

wild-type AB strain [Fig. 2(C), red stippled bars], and *ache*^{sb55}/+ fish injected with saline, like AB fish, failed to modify their PP [Fig. 2(C), black bars]. A dose-response curve confirmed that the dose of amphetamine administered (40 μg/g) was also triggering the most robust rewarding effects tolerable for a reasonable survival rate in *ache*^{sb55}/+ fish [Fig. 2(D); each point > 15 fish; note that *ache*^{sb55}/+ fish respond with a significantly lower CPP than their siblings over a range of doses reaching at least 60 μg/g]. We conclude that the cholinergic system strongly modulates addictive behavior in zebrafish.

Genetic Impairment of AChE Function Is Not Associated with an Abnormally High Initial PP, with Lowered Memory, or with Vision Defects

Three parameters unrelated to reward per se might artificially bias the CPP response: (i) the initial level of PP (a high *PPi* may interfere with conditioning); (ii) the capacity of the animal tested to learn and remember which compartment was associated with the drug; and (iii) the capacity of the animal tested to appreciate the visual cues identifying the drug-paired compartment. To rule out an involvement of these factors on the lowered CPP response of *ache*^{sb55}/+ heterozygotes, we assessed their basal levels of PP, memory, and visual performance.

We used the PP test to measure the *PPi* of *ache*^{sb55}/+ heterozygotes, as described above: after 2 days of habituation, the percentage of time spent in the preferred compartment was recorded. Although, like wild-type fish, most *ache*^{sb55}/+ heterozygotes still preferred the brown side (not shown), we found that their preference for this compartment was significantly decreased compared to that of their wild-type siblings or AB controls [Fig. 3(A); $n = 54$; $p < 0.05$]. Thus, *ache*^{sb55}/+ fish display a relative greater initial preference for the conditioning compartment, making it unlikely that their reduced change in PP following

Figure 3 Lower *PPi*, increased learning potential, and unaltered vision in *ache*^{sb55}/+ adults. In all cases, fish with different genotypes were compared using independent samples Student's *t* test, and standard errors are indicated. (A) Preference of *ache*^{sb55}/+ heterozygotes (left) for one compartment of the test tank, compared to that of their wild-type siblings (middle) or AB controls. The percentage of time spent in the preferred versus nonpreferred compartment of the tank after 2 days of habituation is measured (*PPi*). Bars represent an average result for *n* fish. Note that *PPi* is significantly smaller ($p < 0.05$) in *ache*^{sb55}/+ fish. (B,C) Correlation between the percentage of time spent in the initially preferred compartment before conditioning (*PPi*, *x* axis) and the absolute change in place preference after conditioning (*PPf* − *PPi*, *y* axis) in wild-type (B) and *ache*^{sb55}/+ (C) fish. Each dot represents an individual fish. A linear regression analysis shows that the correlation factor between the absolute change in PP and *PPi* is below 0.2 for both genotypes ($r = 0.17$, $n = 31$, $p < 0.3$ for AB and $r = 0.19$, $n = 33$, $p < 0.3$ for *ache*^{sb55}/+), indicating that these values are not linked. (D,E) Learning capacity of *ache*^{sb55}/+ heterozygotes compared to their wild-type siblings (+/+) and AB controls (AB). (D) Time needed to reach a deep chamber target (*y* axis) as a function of the trial number (one trial per fish per day). Each point is the average of at least 30 fish, with standard error indicated. Both *ache*^{sb55}/+ and +/+ fish decreased their time upon trial, thus progressively learning to find the target (see Supplementary Movies online). Note also that they started from comparably high values (>100 s) and reached a plateau at comparably low values (20 s) after enough trials. Thus our results are not biased by a differential speed of swimming. However, *ache*^{sb55}/+ heterozygotes reached this plateau after trial 3 (arrow) while +/+ siblings needed six trials (arrowhead). (E) Compared number of trials required to learn the position of the target chamber (*y* axis) in the different genotypes; each bar is the average of *n* fish (see bars). We considered that one fish had learned when its time to find the target varied less than 10% upon consecutive trials; the first trial of this plateau was then considered as "learning trial". Note that *ache*^{sb55}/+ heterozygotes (left) learned in significantly less trials than their wild-type siblings (middle) or control fish (right) ($p < 0.05$). (F,G) Functionality of the retinal network of *ache*^{sb55}/+ fish compared to their wild-type siblings (+/+), measured by electroretinography. Typically, the evoked response consisted of an initial negative deflection (a-wave) followed by a large, positive component (b-wave) after applying a light stimulus (Dowling, 1987). (F) Amplitude of the electric response for a stimulation of 5000 cd/m^2 (top) and 0.5 cd/m^2 (bottom) in one fish of each genotype (color-coded). Note that the curves are similar in both genotypes. A range of stimulation values was tested between these extremes and showed a similar response in *ache*^{sb55}/+ and +/+ fish. (G) Compared amplitude of the b-wave response in *ache*^{sb55}/+ fish compared to their wild-type siblings (+/+).

amphetamine administration was due to their difference in *PPi* compared to wild-type fish. In support of this interpretation, we further verified that, for both *ache*$^{sb55/+}$ and wild-type fish, the absolute change in PP following amphetamine administration was unrelated to the initial *PPi* level [Fig. 3(B,C)]: the correlation factor between the absolute change in PP and *PPi* in a linear regression analysis was below 0.2 for both genotypes. These results further support our conclusion that the lowered change in PP of *ache*$^{sb55/+}$ fish upon amphetamine injection was not related to their basal preference levels.

We next assessed the learning capacities of *ache*^{sb55}/+ heterozygotes. We built a simple T-maze assay where the fish had to reach a deep chamber, after which performance they received a food treat (see Methods for details). One trial was performed per fish per day. We observed that the time taken to find the chamber decreased upon trials in both *ache*^{sb55}/+ heterozygotes and wild-type siblings, showing that both populations were capable of learning [Fig. 3(D), and Supplemental Movie 1 on-line]. We considered the learning phase to be over when the time needed to find the compartment changed less than 10% upon consecutive trials. We observed that *ache*^{sb55}/+ fish needed less trials to reach this point than their wild-type siblings or AB fish [Fig. 3(D), arrow and arrowhead, and Fig. 3(E); n = 20; p < 0.005], while the time needed to reach the chamber in the initial trial and after learning was comparable in all cases [Fig. 3(D), see time for trial 3 in *ache*^{sb55}/+, for trial 6 in wild-type, and see Supplemental Movies 2 and 3 online]. The location of the chamber was memorized for over 2 weeks after a pause in testing in both genotypes (not shown). We conclude that, in this test, *ache*^{sb55}/+ fish learn faster than controls. Thus, their reduced CPP is unlikely to result from an inability to learn and remember.

Finally, we assessed the visual performance of *ache*^{sb55}/+ fish by ERG. The ERG b-wave was used to evaluate physiological function *in vivo*. A two-way repeated measure ANOVA indicated that the b-wave amplitude of *ache*^{sb55}/+ heterozygotes was not significantly different from their wild-type siblings [Fig. 3(F,G); two-way ANOVA, n = 3 each; F(df = 2) < 1, p < 0.7]. These ERG tests together with the T-maze assay suggest that vision defects are unlikely to account for the reduced CPP in *ache*^{sb55}/+.

DISCUSSION

The zebrafish behavioral repertoire is complex and is increasingly studied for the modeling of parameters

such as anxiety, addiction, and social interactions (Engeszer et al., 2004). To date, two studies, focusing respectively on ethanol and cocaine, used zebrafish adults to approach the neurogenetics of drug addiction (Gerlai et al., 2000; Darland and Dowling, 2001). Importantly, in contrast to these early works, we failed to reproduce efficient drug administration by dissolving drugs in water (Darland and Dowling, 2001): in such cases, in our hands, the amount of drug reaching the brain was undetectable (as measured by HPLC, see Methods) (not shown). Rather, we successfully used intraperitoneal injections. We also found that a habituation phase of at least 2 days to the test conditions was necessary to obtain reliable assessment of PP and CPP. Finally, our method eliminates scoring behaviors unrelated to the reward pathway per se but rather reflecting changes in the stress status of the tested individual, or impaired drug uptake or transport to the brain. It was crucial, for instance, to verify the amount of drug received by the brain for each subject showing atypical behavior, and to verify the normality of other parameters involved such as memory and vision. In a CPP test using the so-called ''biased'' place conditioning procedure (i.e., with a preexisting bias for one area of the test arena, as we used here), anxiety might also affect place reversal. Here we rule out the influence of such a phenomenon by demonstrating that the *PPi* of *ache*^{sb55}/+ mutants is not greater than that of their siblings [Fig. 3(A)], which should permit conditioning. Finally, because *ache*$^{sb55/+}$ and wild-type fish differ in their initial place preference level (*PPi*), verifying that the *PPi* value does not influence CPP is another important control [Fig. 3(B,C)]. Together, the reliability of our set-up was instrumental in detecting psychostimulant-induced differences in the CPP paradigm between different genetic backgrounds such as *ache*^{sb55}/+ heterozygotes and wild-type. This success demonstrates that zebrafish adults can be used to screen for the effect of developmental mutations on adult reward-related behavior and therefore to identify dominant modulators of behavior related to addiction, as we here identify AChE.

Another major result of our study is the implication of the cholinergic system in the modulation of the rewarding properties of amphetamine, of PP, and of cognitive capacities in zebrafish. Because this parallels the situation in mammals, our results provide the first validation of the zebrafish model for studying the neurotransmitter and molecular pathways that underlie the process of addiction in vertebrates. This result is of high significance given the demonstrated amenability of the zebrafish system to genetic screens and the molecular mapping of mutations, at a level to

date remaining far from reach in other vertebrate models. In a parallel ENU mutant screen (J. Ninkovic and L. Bally-Cuif, unpublished observations), the procedure described in this article allowed scoring, in less than 5 months, of 1128 mutagenized zebrafish genomes and recovered 26 mutants potentially affected in their response to the rewarding effects of amphetamine. Such a forward genetic approach will provide crucial and unbiased information not only on the molecular biology of drug addiction but also on the neuronal and molecular networks underlying natural reward learning and memory in vertebrates.

A number of molecular components of the zebrafish cholinergic system have been identified (Zirger et al., 2003; Williams and Messer, 2004), but, outside of choline acetyltransferase (ChAT; the ACh synthesizing enzyme) and AChE (Clemente et al., 2004; Mueller et al., 2004), their spatial distribution in the adult brain has not been established. The latter reports agree on the presence of ChAT immunoreactive nuclei and fibers in the diencephalon and on AChE activity in most of the forebrain, while Mueller et al. (2004) describe in addition an intense cholinergic innervation and cell bodies in the subpallium (striatum). Because the *ache* mutation is likely to affect ACh amounts at all brain levels, our results do not permit us to point to the specific cholinergic pathways and developmental time points involved in modulating reward, exploratory activity, or learning in zebrafish. These results do not necessarily contrast with previous targeted studies performed in mammals where, although the cholinergic system was initially locally perturbed, for example, by targeted neuron ablation, a widespread adaptative regulation of cholinergic transmission was noted (Kitabatake et al., 2003). In our model however, because the genetic impairment of AChE function operates since the earliest developmental stages, a permanent increase in cholinergic activity operates to modulate behavior. This may suggest a role for desensitization of AChR rather than an acute inhibition of AChE. In mammals, DA terminals projecting to the NAc harbor nAChRs that are highly prone to desensitization. These have been invoked in explaining the gradual decrease of DA release that follows the stabilization of high NAc ACh levels by AChE inhibitors (Zhou et al., 2001). A zebrafish functional equivalent to the NAc remains to be identified, but it is possible that a similar mechanism is at play in the zebrafish subpallium. In addition, in mammals, desensitization of the mAChR present on DA cell bodies projecting to the NAc might also limit the reinforcing effect of amphetamine triggered by ACh on these neurons (Fiorillo and Williams, 2000; Fink-Jensen et al., 2003). It will

be important to determine whether DA neurons of the posterior tuberculum, the likely zebrafish equivalent of the mammalian VTA (Rink and Wullimann, 2001), receive cholinergic innervation via mAChR. The action of cholinergic input on exploratory behavior and learning processes in mammals is believed to rely on different control centers such as the dorsal hippocampus and amygdala, and the ascending basal cholinergic forebrain system, respectively (File et al., 2000; Degroot and Treit, 2002). Recent results suggest that the goldfish medial pallium might contain neurons functionally homologous to the amygdala in an avoidance learning paradigm (Portavella et al., 2004). Direct equivalents of all these centers in zebrafish remain to be identified by projection tracing followed by experimental perturbation and behavioral tests as described in this article. Obviously, our current knowledge of the functionality of the zebrafish adult CNS remains fragmentary, making behavioral phenotypes still more difficult to interpret than in higher vertebrates. The present article, by providing the reliable tools to assess a behavioral function in zebrafish, precisely sets the stage to link the neuroanatomical and neurotransmitter networks with their function in this species.

Although psychostimulants and the modulating effect of the cholinergic system generally affect both reward and locomotor activity, instances of dissociations between these two effects in given paradigms have also been reported (Tzschentke, 1998, and references therein). Strikingly, in our conditions, amphetamine does not modify zebrafish swimming speed (not shown). It is possible that amphetamine is ineffective on locomotor activity in zebrafish, or that our experimental conditions did not permit us to measure this response.

Although the central modulatory role of ACh in the CNS and in particular in the control of central DA transmission is well documented, a universal and directly accessible target of this system for drug addiction therapy has been lacking. Local microinfusions of AChE inhibitors into the hippocampus have been demonstrated to reduce anxiety and to improve memory in mammalian animal models (Degroot et al., 2001; Degroot and Parent, 2001; Degroot and Treit, 2002, 2003), but their effect on addiction has not been tested, and their practicality in humans is questionable. We demonstrate here that lowering the central activity of AChE by two-fold is sufficient to reduce the rewarding effect of amphetamine. Further, the group of $ache^{sb55}/+$ heterozygotes includes a proportion of fish where amphetamine-induced CPP was not only lowered but completely abolished (not shown). In line with our study, a recent report pointed

to the pharmacological inhibition of AChE activity by intraperitoneal injection of donepezil in the mouse as a potential means of decreasing the addictive response elicited by cocaine and morphine (Hikida et al., 2003). It is likely that such injections also globally affect AChE levels, like in zebrafish $ache^{sb55/+}$ mutants, further suggesting that targeting AChE at the organismal level might be effective over a broad range of drugs. Importantly, $ache^{sb55}/+$ zebrafish survived the general modification of AChE and ACh levels over their entire life span without deleterious effects in our housing conditions. In particular, in mixed families of $ache^{sb55}/+$ and $+/+$ siblings raised together from the first days onwards, the ratio of $ache^{sb55}/+$ adults, as well as their size and reproduction rate, was never biased (not shown). Together, our findings suggest that a treatment moderately lowering AChE activity could be envisaged in a systemic manner over an extended period of the individual's life with a significant improvement of his resistance to addiction. The zebrafish model itself might be used to select anti-AChE compounds that exhibit minimal side effects (Behra et al., 2004).

CONCLUSION

Our results show that the rewarding potential of amphetamine has been conserved through vertebrate evolution. They also demonstrate that higher central cholinergic activity in zebrafish is associated with decreased sensitivity towards the addictive properties of amphetamine, increased exploratory activity, and faster learning, demonstrating that the importance of the cholinergic system in modulating these behaviors has also been evolutionarily conserved. Importantly, our results also provide the first genetic arguments supporting manipulations of AChE activity as a promising avenue towards limiting addiction behavior to psychostimulants. Together, and given the ease to produce mutants in zebrafish, our findings set the stage to make the zebrafish a highly attractive model to study these behavioral processes at the neuroanatomical and molecular levels, and in particular to give insight into the molecular neurobiology of drug-induced reward in vertebrates.

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