

## Research



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# Individual differences and knockout in zebrafish reveal similar cognitive effects of BDNF between teleosts and mammals

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The remarkable similarities in cognitive performance between teleosts and mammals suggest that the underlying cognitive mechanisms might also be similar in these two groups. We tested this hypothesis by assessing the effects of the brain-derived neurotrophic factor (BDNF), which is critical for mammalian cognitive functioning, on fish's cognitive abilities. We found that individual differences in zebrafish's learning abilities were positively correlated with *bdnf* expression. Moreover, a CRISPR/Cas9 mutant zebrafish line that lacks the BDNF gene (*bdnf*<sup>-/-</sup>) showed remarkable learning deficits. Half of the mutants failed a colour discrimination task, whereas the remaining mutants learned the task slowly, taking three times longer than control *bdnf*<sup>+/+</sup> zebrafish. The mutants also took twice as long to acquire a T-maze task compared to control zebrafish and showed difficulties exerting inhibitory control. An analysis of habituation learning revealed that cognitive impairment in mutants emerges early during development, but could be rescued with a synthetic BDNF agonist. Overall, our study indicates that BDNF has a similar activational effect on cognitive performance in zebrafish and in mammals, supporting the idea that its function is conserved in vertebrates.

## 1. Background

According to a growing number of studies, teleost fish display a level of cognitive sophistication that is similar to mammals. Fish are capable of advanced and extremely rapid learning [1,2], forming long-lasting memories [3], flexibly modifying their behaviour [4,5], innovating [6], using tools [7], solving problems [8], inhibiting automatic responses [9], processing numerical information [10], developing behavioural traditions [11] and cooperating [12]. In many cognitive tasks, fish's performance even exceeds that of many mammalian species (e.g. [9,13]).

The similar cognitive performances observed in fish and mammals might be due to the convergent evolution of cognitive abilities, although most species belonging to these two clades have evolved in very different environments (aquatic versus terrestrial) likely under different selective pressures. Alternatively, the similar cognitive performances might derive from a cognitive toolbox shared between fish and tetrapods that was inherited from a common ancestor [14]. These hypotheses are difficult to analyse exclusively looking at the cognitive performance because similar performances in two species may actually derive from different cognitive processes. This calls for studies that also address the underlying neurobiological mechanisms. If cognitive performance is phenotypically similar between two clades because of homology, we expect to observe also similarities in the mechanisms. While numerous studies have shown similarities in the physiological and molecular mechanisms of fish and mammalian brains (e.g. [15–17]), it is less understood whether these similar neurobiological mechanisms translate into similar effects in cognitive performance.

In this study, we tested whether a molecule critically involved in determining the cognitive abilities of mammals, the brain-derived neurotrophic factor (BDNF), also determines the cognitive abilities of teleost fish. BDNF is a secreted protein belonging to the neurotrophic growth factor family that exhibits high levels of expression in mammalian brain tissues [18]. At the cellular and molecular level, BDNF contributes to major signalling pathways for neural differentiation, growth and survival during development [19–22]. Additionally, BDNF displays activation effects that control cognitive output (reviewed in [23]), such as guiding synaptic plasticity [24–28] and modulating neurotransmitter release [29,30]. For instance, the upregulation of BDNF mRNA expression is involved in memory formation [31] and in tool-use learning in monkeys [32], and in maze learning in rats [33]. Rats' ability to learn can be experimentally impaired with antisense BDNF treatment [34]. Similarly, a strain of mutant mice with deletion of one copy of the BDNF gene displayed reduced learning performance [35–38]. In humans, a genetic polymorphism that affects BDNF secretion impacts memory [39] and executive functions [40–42].

BDNF of fish (*Bdnf*) has high levels of sequence similarity with that of mammals (greater than 90%) and is expressed in the brain with a similar distribution [43–47]. Therefore, we asked whether *Bdnf* impacts fish cognitive performance. First, we focused on natural variation of *bdnf* expression in zebrafish (*Danio rerio*) and tested whether it was associated with individuals' learning performance. Afterwards, we exploited a *bdnf*<sup>-/-</sup> (null mutant) zebrafish recently generated in our laboratories using the CRISPR/Cas9 genome editing system [48] to further characterize the role of *Bdnf* on cognitive abilities of fish. We analysed the performance of adult *bdnf*<sup>-/-</sup> zebrafish in a colour discrimination learning task [49], a T-maze task [50] and an inhibitory control task [51]. Based on the effects of BDNF in mammals (e.g. [32,38,41]), we hypothesized that natural levels of BDNF predict individual differences in learning and that the *bdnf*<sup>-/-</sup> zebrafish display impaired cognitive performance compare to control siblings (*bdnf*<sup>+/+</sup>) zebrafish. Finally, to elucidate whether the effect of *Bdnf* on fish cognition is activation or developmental, we observed the learning performance of *bdnf*<sup>-/-</sup> larvae throughout development and we conducted a rescue experiment using a molecule that mimics BDNF action. If the effects of *Bdnf* were activation, we expected them to be present since early ontogeny, to not vary during development, and to be rescued by administration of the BDNF agonist.

## 2. Material and methods

### (a) Experimental subjects

Overall, the study involved 30 wild-type zebrafish, 184 *bdnf*<sup>-/-</sup> zebrafish (mutants) and 79 *bdnf*<sup>+/+</sup> zebrafish (controls). The wild-type zebrafish used to study individual differences belonged to a population of 500 individuals kept in the facility of University of Ferrara since 2011. The *bdnf*<sup>-/-</sup> zebrafish line was generated by clustered regularly interspaced short palindromic repeats (CRISPR)-mediated knockout as described in [48]. The mutagenesis process generated 25% *bdnf*<sup>-/-</sup> zebrafish displaying a 40 bp deletion in the exon 2 of the *bdnf* coding sequence impairing all five known splicing isoforms in zebrafish. The process also produced 25% individuals with *bdnf*<sup>+/+</sup> genotype, which were used as sibling controls in the experiments of the present study. All the subjects were maintained under standard laboratory conditions before the experiments (electronic supplementary material, S1, section a).

### (b) Effect of individual differences in *bdnf* expression on learning

To investigate individual variation in cognition and *bdnf* expression, we first measured the learning abilities of 15 zebrafish (hereafter 'experimental subjects') in a colour discrimination task [52,53]. In a series of trials, the subjects were exposed to two colour stimuli, yellow versus blue (figure 1a). They had to learn to approach a predetermined colour to obtain a food reward (electronic supplementary material, S1, section b). Once the subjects reached the learning criterion of the task, we collected their brain tissues to quantify *bdnf* expression by qPCR (electronic supplementary material, S1, section c). Considering that each experimental individual learned the task with different speed, they differed for the exposure to non-cognitive factors such as the stimuli, the testing apparatus and the procedure, potentially resulting in alterations of *bdnf* expression that were not related to learning. To control for these confounding factors, we also quantified *bdnf* expression in a control group of 15 zebrafish (hereafter 'control subjects'). Each control subject was tested alongside an experimental subject, and underwent the same procedure minus the association between the food reward and the colour stimulus, preventing the chance of learning. With this procedure, each control subject had the same experience of the corresponding experimental subject in terms of exposure to the stimuli, number of trials and time spent in the apparatus. We used the control subjects to calculate an index of *bdnf* expression corrected for non-cognitive factors for the experimental subjects (electronic supplementary material, S1, section c).

### (c) Effect of *bdnf* loss on colour discrimination learning

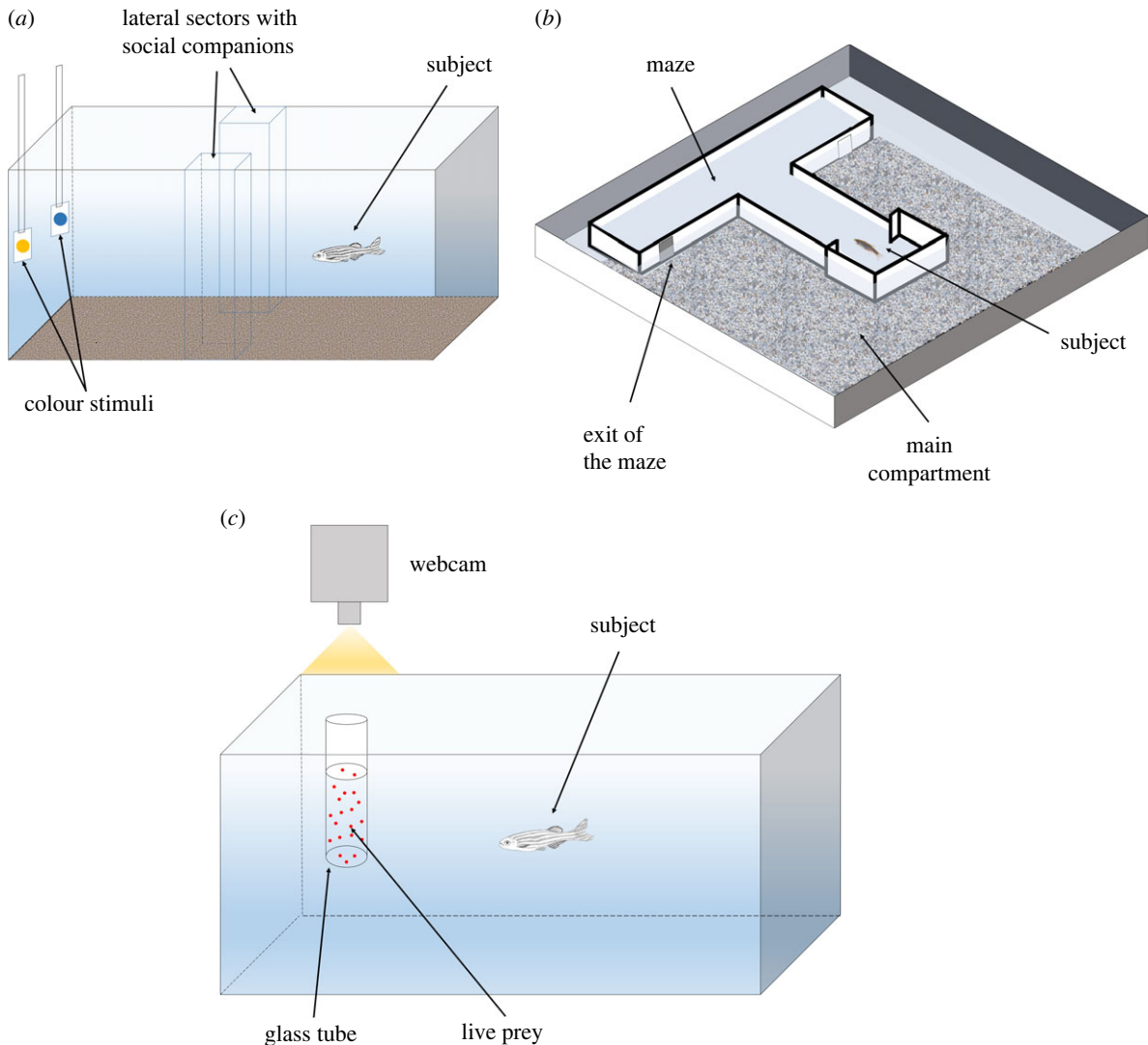
In this experiment, we assayed 10 *bdnf*<sup>-/-</sup> zebrafish and 6 *bdnf*<sup>+/+</sup> zebrafish with the colour discrimination learning paradigm described in the previous section and in electronic supplementary material, S1 (section b). All the subjects were subjected to the reward–colour association. Because some *bdnf*<sup>-/-</sup> subjects showed difficulties in reaching the learning criterion, we added an additional rule: if a subject did not reach the criterion within 20 days of the test phase, the subject was considered unable to learn the task and the experiment was terminated.

### (d) Effect of *bdnf* loss on T-maze learning

The T-maze task involved 12 *bdnf*<sup>+/+</sup> and 12 *bdnf*<sup>-/-</sup> zebrafish and followed the procedure of Miletto Petrazzini *et al.* [50]. In a series of trials, the subject had to learn to choose the predetermined arm of the maze (figure 1b), either the left or the right, to return to its home aquarium (electronic supplementary material, S1, section d). The two arms of the maze were virtually identical, requiring the subject to base its choice on the experience accumulated in the previous trials and in particular on egocentric information (left or right turning direction). We scored the first arm entered by the zebrafish as a measure of its choice and a criterion based on the colour discrimination task as indication of learning.

### (e) Effect of *bdnf* loss on inhibitory control

We assayed inhibitory control in 16 *bdnf*<sup>-/-</sup> zebrafish and 17 *bdnf*<sup>+/+</sup> zebrafish, using the paradigm previously adopted in this species [51,54]. The task measured inhibitory control as ability to withhold foraging behaviour toward live prey that cannot be reached (electronic supplementary material, S1, section e). As the prey, we used *Artemia salina*, normally provided as food in the facility, enclosed inside a transparent glass tube (figure 1c). Subjects were expected to initially attempt to capture the prey and then to inhibit this behaviour after experiencing the transparent obstacle.



**Figure 1.** Experimental apparatuses. (a) Lateral view of the apparatus for the colour discrimination learning task; in a series of trials, the subjects had to select a predetermined colour between two options to obtain a food reward. (b) T-maze used to assess spatial learning; the subjects had to learn to choose a predetermined arm to return to their home aquarium. (c) Lateral view of the apparatus for the inhibitory task; the subject was presented with an unreachable live prey sealed in transparent glass and had to withhold the capture attempts. (Online version in colour.)

### (f) Effect of *bdnf* loss across development

To study the cognitive consequences of *bdnf* loss during the early development of zebrafish, we focussed on habituation learning. This is a simple form of non-associative learning exhibited by larval zebrafish that permits individuals to reduce a response to repeated stimulations [55,56]. We subjected 7-dpf (days post fertilization) larvae ( $N=24$  *bdnf*<sup>-/-</sup> and 24 *bdnf*<sup>+/+</sup>) and 21-dpf larvae ( $N=20$  *bdnf*<sup>-/-</sup> and 20 *bdnf*<sup>+/+</sup>) to a habituation learning task in which they were exposed to 50 vibrational stimulations separated by a 1-s interval (electronic supplementary material, S1, section f). Each stimulation normally produces a startle response in the fish (i.e. high activity). However, due to habituation learning, the startle response was expected to decrease across the repeated stimulations. Therefore, we computed a habituation learning index based on the average change in activity in the series of stimulations with respect to the response to the first stimulation (electronic supplementary material, S1, section f).

### (g) Rescue of learning in *bdnf*<sup>-/-</sup> zebrafish

The rescue experiment consisted of assessing the habituation learning performance of *bdnf*<sup>-/-</sup> zebrafish exposed to 7,8-dihydroxyflavone hydrate (7,8-DHF), a synthetic molecule that mimics the action of BDNF by activating its TrkB receptor [57].

A prior study reported that this molecule rescues the behaviour of BDNF-lacking zebrafish [48]. We performed this experiment in larvae for ethical reasons, using 7-dpf *bdnf*<sup>-/-</sup> subjects. Two hours before the habituation learning task, we moved five groups of approximately 10 larvae in five Petri dishes ( $N=49$  larvae overall) filled with a solution of 2.5% 7,8-DHF and 0.1% solvent (dimethyl sulfoxide: DMSO). Because DMSO could also affect zebrafish behaviour [58], we treated five groups of control larvae ( $N=53$  control larvae overall) with only this solvent. After the treatment, we assessed the habituation learning performance of the subjects as described in the previous section and in electronic supplementary material, S1 (section f).

### (h) Statistical analysis

The statistical analysis was performed in R v. 3.2.2 (The R Foundation for Statistical Computing, Vienna, Austria, <http://www.r-project.org>). The tests were two-tailed and  $\alpha$ -level for significance was set at  $p=0.05$ .

In the first experiment, to study the correlation between levels of BDNF and naturally occurring individual differences in learning, we used Spearman's rank correlation. This non-parametric method allowed us to deal with non-normal data distribution and potential outliers [59]. The two variables analysed were the index of *bdnf* expression (electronic supplementary material, S1,

section c) and the number of days necessary to reach criterion in the colour discrimination learning task (electronic supplementary material, S1, section b).

In the comparison between *bdnf*<sup>-/-</sup> and *bdnf*<sup>+/+</sup> zebrafish in the colour discrimination learning and the T-maze task, we performed multiple analysis based on three dependent variables. First, in the colour discrimination task, we analysed the proportion of subjects that reached the learning criterion between *bdnf*<sup>-/-</sup> versus *bdnf*<sup>+/+</sup> zebrafish using a chi-squared test. The chi-squared analysis was not conducted in the T-maze task because all the subjects reached the learning criterion. Then, for both experiments, we compared the number of days taken by each subject to reach the learning criterion between *bdnf*<sup>-/-</sup> versus *bdnf*<sup>+/+</sup> zebrafish. We used Wilcoxon rank sum test as it allowed us to assign the maximum value for the variable (20 days) to the subjects that did not reach the learning criterion of the colour discrimination task. Last, we analysed the number of errors committed by each subject in each day of training. This variable has a repeated measures structure and a different length across subjects. We, therefore, used linear mixed-effects models (*lme* R function) that can handle these types of data. The linear mixed-effects models included day of training as fixed effects and subject ID as random effect. The number of errors in the T-maze task was log transformed to deal with right-skewed distribution.

In the inhibitory control task, we removed one mutant subject that did not approach the prey. We first compared the behaviour of the zebrafish with the two genotypes in the pre-test phase using Wilcoxon's rank sum test. In particular, we focused on the number of pre-test trials in which the fish approached the Pasteur pipette with the food, which was considered as an indication of willingness to feed. In the subsequent analysis of the test phase, we initially compared the minute in which the fish attempted to capture the prey for the first time using Wilcoxon rank sum test, as an indication of motivation to feed. Then, we analysed the main variable of the test phase (i.e. the number of attacks displayed by each subject in each minute of the experiment), which consisted of repeated measures and followed the Poisson distribution. We applied generalized linear mixed-effects models with Poisson error distribution, fitting genotype (*bdnf*<sup>-/-</sup> versus *bdnf*<sup>+/+</sup>) and minute of the experiment as fixed effects and subject ID as random effect. As we were particularly interested in how the fish interact with the prey after experiencing the transparent barrier, we then conducted a subsequent analysis that compares the fish with the two genotypes in the first minute of the experiment using generalized linear model with Poisson error distribution (i.e. a version of the prior model that does not include repeated measures variables).

In the two habituation learning experiments, the dependent variable was the habituation learning index describing the average change in activity (distance moved) of each subject with the respect to the response to the first stimulation (electronic supplementary material, S1, section f). We analysed this index using ANOVAs fitted with genotype (*bdnf*<sup>-/-</sup> versus *bdnf*<sup>+/+</sup>) and age (7 or 21 dpf) for the first habituation learning experiment. For the second habituation learning experiment, we used Wilcoxon rank sum test to assess the effect of the treatment (7,8-DHF versus DMSO).

### 3. Results

#### (a) Individual differences in learning positively correlate with *bdnf* expression

All the subjects trained to select the target colour reached the learning criterion of the task within  $8.60 \pm 4.70$  days (mean  $\pm$  standard deviation). When we tested for a relationship between zebrafish's learning performance and the index of

*bdnf* expression in the brain, we found a significant negative correlation (Spearman's rank correlation:  $\rho = -0.561$ ,  $p = 0.029$ ). This indicates that experimental subjects with higher *bdnf* expression learned faster the colour discrimination task (figure 2a).

#### (b) *bdnf* loss impairs colour discrimination learning

Five out of 10 (50%) *bdnf*<sup>-/-</sup> subjects solved the colour discrimination task within the given time (20 days). Conversely, all the six *bdnf*<sup>+/+</sup> subjects solved the colour discrimination task. This corresponds to a significantly lower likelihood of learning the colour discrimination for the *bdnf*<sup>-/-</sup> zebrafish (chi-squared test:  $\chi^2_1 = 4.364$ ,  $p = 0.037$ ).

Assigning the maximum value (20 days) to subjects that did not reach the learning criterion, the number of days necessary to acquire the colour discrimination was  $15.1 \pm 7.08$  (mean  $\pm$  standard deviation) for *bdnf*<sup>-/-</sup> zebrafish. In comparison, *bdnf*<sup>+/+</sup> zebrafish reached the learning criterion in  $3.5 \pm 1.64$  days. The analysis indicated that *bdnf*<sup>-/-</sup> zebrafish required a significantly larger number of days to reach the learning criterion compared to *bdnf*<sup>+/+</sup> zebrafish (Wilcoxon rank sum test:  $W = 52$ ,  $p = 0.017$ ; figure 2b). After removing the data of the five non-learner *bdnf*<sup>-/-</sup> subjects, the average number of days to criterion was still more than twice ( $10.2 \pm 7.26$ ) compared to *bdnf*<sup>+/+</sup> zebrafish, although the difference was not significant perhaps due to the reduction in sample size ( $W = 22$ ,  $p = 0.227$ ).

A repeated measures analysis on the number of errors committed by each subject in each day of training indicated a significantly different trend in the *bdnf*<sup>+/+</sup> zebrafish and the *bdnf*<sup>-/-</sup> zebrafish (linear mixed-effects model:  $F_{1,154} = 8.429$ ,  $p = 0.004$ ). The *bdnf*<sup>+/+</sup> zebrafish displayed a stepper decrease in the number of errors, and hence greater learning, compared to the *bdnf*<sup>-/-</sup> zebrafish (figure 2c).

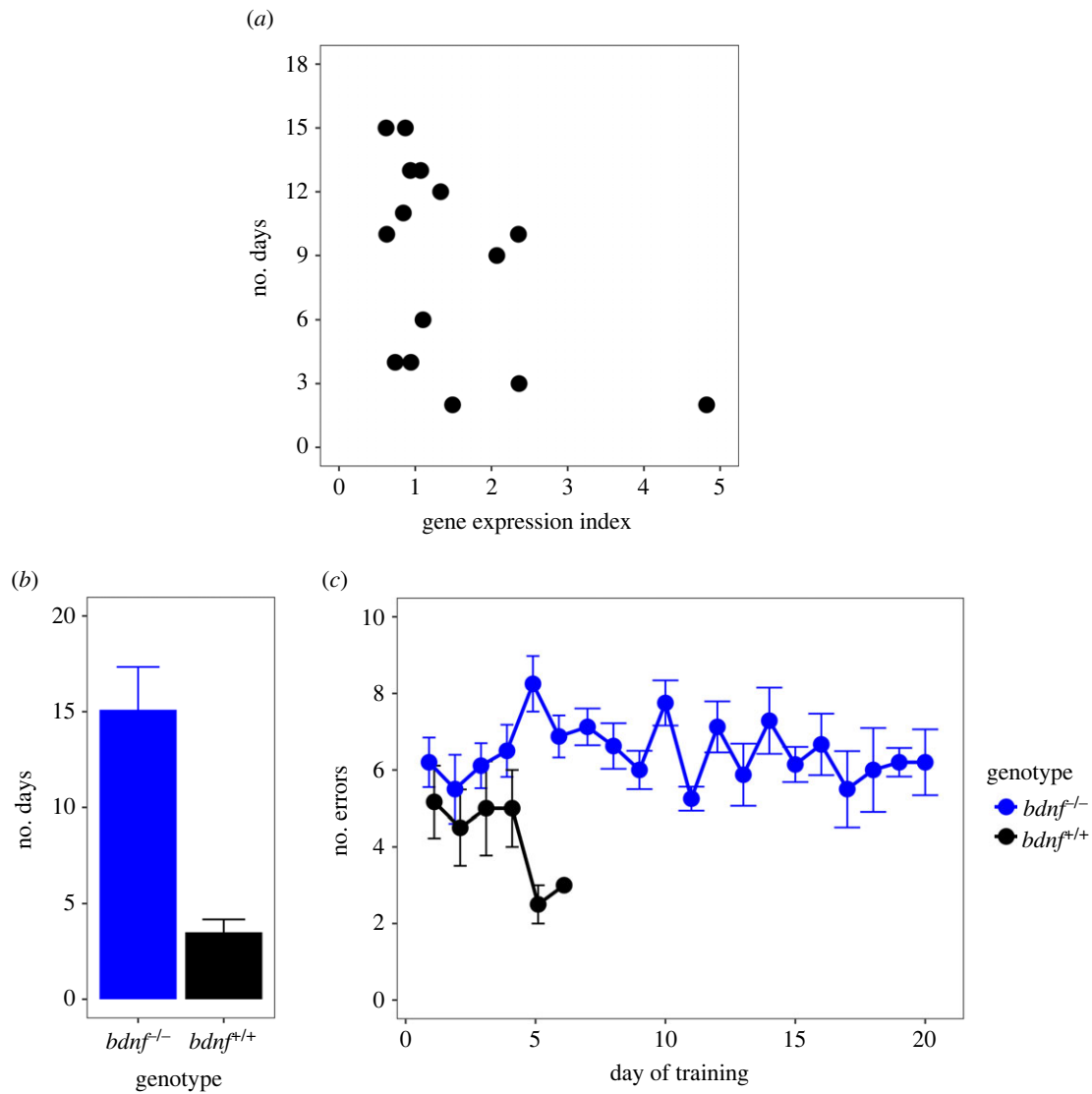
#### (c) Loss of *bdnf* impairs T-maze learning

All the subjects reached the learning criterion, demonstrating the ability to solve the discrimination involved in the T-maze task. However, there was a significant difference between the two genotypes in the number of days necessary to reach the criterion (Wilcoxon rank sum test:  $W = 114.5$ ,  $p = 0.011$ ), evidencing faster learning in the *bdnf*<sup>+/+</sup> zebrafish (figure 3a).

Considering the number of errors per day, the pattern was less clear compared to the colour discrimination learning experiment (figure 3b), possibly due to the lower number of trials administered per day (six versus 12 trials). The repeated measures analysis indicated significant evidence of learning as reduction of number of errors across days in the *bdnf*<sup>+/+</sup> zebrafish (linear mixed-effects model:  $F_{1,24} = 5.835$ ,  $p = 0.024$ ), but not in the *bdnf*<sup>-/-</sup> zebrafish ( $F_{1,73} = 2.921$ ,  $p = 0.092$ ).

#### (d) Loss of *bdnf* reduces inhibitory control

We found no difference in the willingness to feed between the two genotypes in the pre-test phase (Wilcoxon rank sum test:  $W = 122.5$ ,  $p = 0.857$ ) nor differences in the time to approach the prey in the test phase ( $W = 104.05$ ,  $p = 0.420$ ). In the inhibitory task, *bdnf*<sup>-/-</sup> zebrafish performed  $3.58 \pm 5.35$  attempts to capture the prey per minute (mean  $\pm$  standard deviation), whereas the *bdnf*<sup>+/+</sup> zebrafish scored  $2.11 \pm 3.39$  attacks. A repeated measures analysis on the number of attacks in each minute of the test indicated that the *bdnf*<sup>-/-</sup> zebrafish



**Figure 2.** Individual differences in BDNF levels and *bdnf*-loss affected colour discrimination learning performance in zebrafish. (a) Scatter plot of the number of days necessary to reach the colour discrimination learning criterion and the index of *bdnf* expression in the brain of wild-type zebrafish; each data point represents an individual fish. (b) Number of days required by mutant (*bdnf<sup>-/-</sup>*) and control (*bdnf<sup>+/+</sup>*) zebrafish to reach the learning criterion in the colour discrimination task; data points and bars represent means and standard errors, respectively; all the subjects are included in the plot, with the maximum value (20 days) assigned to the subjects that did not reach the learning criterion. (c) Number of errors (choices of the incorrect colour) made by zebrafish of the two genotypes (*bdnf<sup>-/-</sup>* and *bdnf<sup>+/+</sup>*) divided per each day of training in the colour discrimination task; data points and bars represent means and standard errors, respectively. (Online version in colour.)

displayed a higher number of attempts at the beginning of the experiment (genotype by time interaction:  $\chi^2_1 = 10.204$ ,  $p = 0.001$ ; figure 3c). The difference between the two genotypes in the number of attacks was already evident in the first minute of the test ( $\chi^2_1 = 41.812$ ,  $p < 0.001$ ), suggesting that *bdnf<sup>+/+</sup>* zebrafish reduced their attempts more than *bdnf<sup>-/-</sup>* zebrafish immediately after experiencing the transparency for the first time.

### (e) The effects of *bdnf* loss are similar across development

The analysis of the habituation learning index indicated a significant difference between the two genotypes ( $F_{1,84} = 5.573$ ,  $p = 0.021$ ), due to the fact that the *bdnf<sup>-/-</sup>* zebrafish displayed reduced habituation learning compared to the *bdnf<sup>+/+</sup>* zebrafish (figure 4a). The main effect of age was also significant ( $F_{1,84} = 24.511$ ,  $p < 0.001$ ). However, the interaction between age and genotype was not significant ( $F_{1,84} = 0.517$ ,  $p = 0.474$ ),

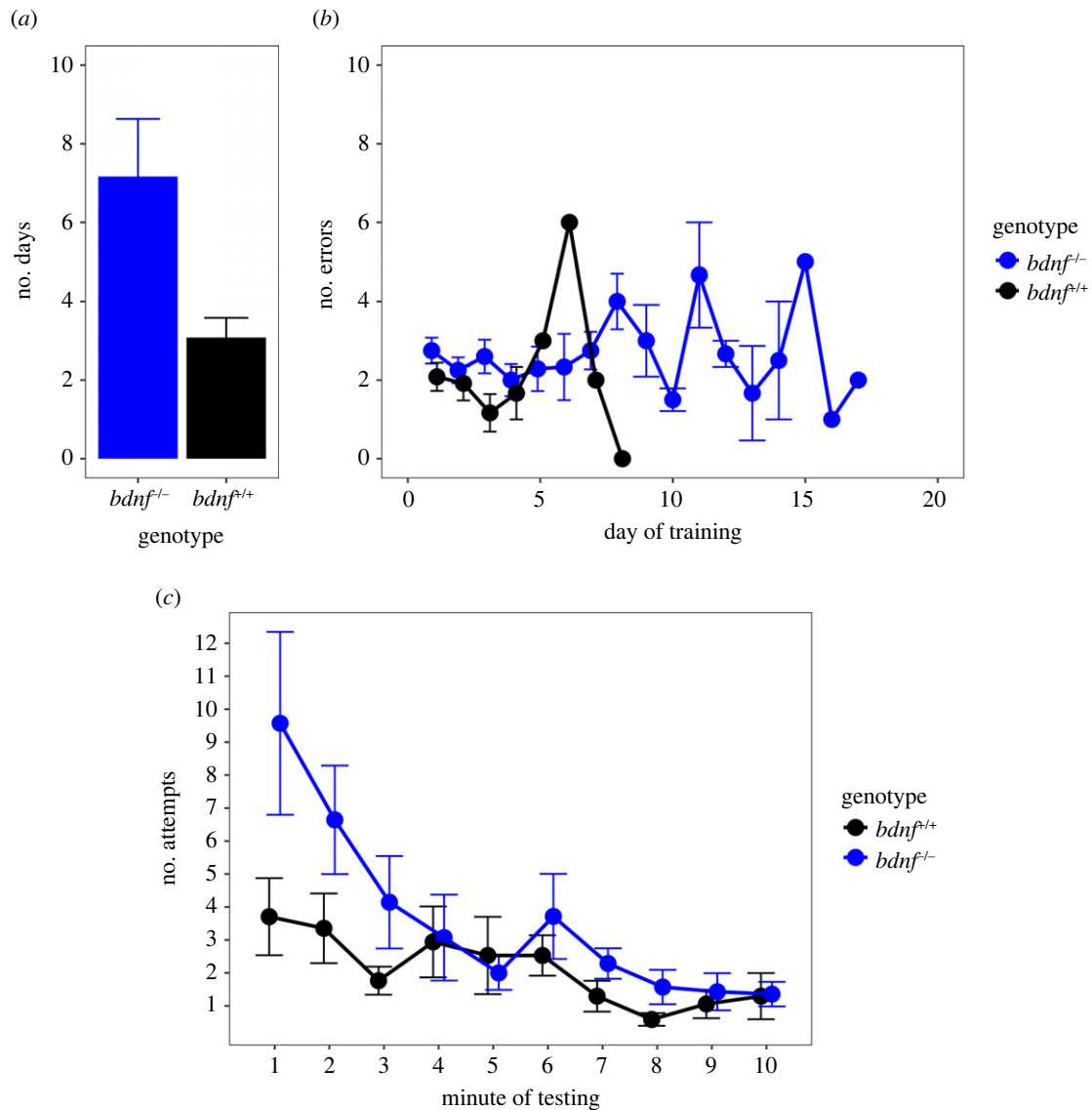
indicating that the reduced habituation learning performance associated with lack of *bdnf* was constant across development.

### (f) BDNF agonist treatment rescues learning in *bdnf<sup>-/-</sup>* zebrafish

The analysis of the habituation learning index indicated a significant difference between *bdnf<sup>-/-</sup>* zebrafish exposed to the treatment with 7,8-DHF and those exposed to solvent as control (Wilcoxon rank sum test:  $W = 1702$ ,  $p = 0.007$ ). The molecule simulating the action of BDNF increased habituation learning performance in *bdnf<sup>-/-</sup>* zebrafish (figure 4b).

## 4. Discussion

The neurotrophin BDNF is a main actor in multiple neural processes in the mammalian brain [19,22,24,27,30] that determines direct effects on cognitive performance [23,26,28,60].



**Figure 3.** *bdnf* loss reduced T-maze learning and inhibitory control in zebrafish. (a) Number of days required by mutant (*bdnf*<sup>-/-</sup>) and control (*bdnf*<sup>+/+</sup>) zebrafish to reach the criterion in the T-maze task; data points and bars represent means and standard errors, respectively; all the subjects are included in the plot. (b) Number of errors (choices of the incorrect arm) made by zebrafish of the two genotypes (*bdnf*<sup>-/-</sup> and *bdnf*<sup>+/+</sup>) divided per each day of training in the T-maze task; data points and bars represent means and standard errors, respectively. (c) Number of attempts to capture the prey made by zebrafish of the two genotypes (*bdnf*<sup>-/-</sup> and *bdnf*<sup>+/+</sup>) divided per each minute of the test. Data points and bars represent means and standard errors, respectively. (Online version in colour.)

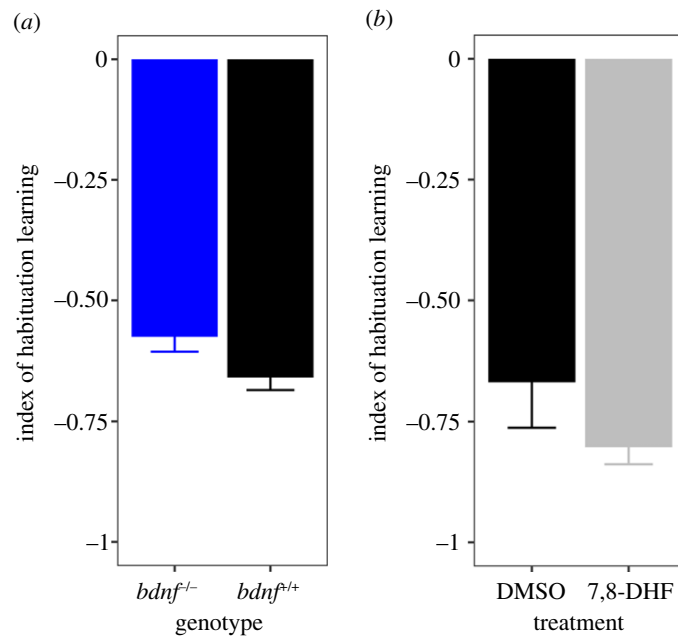
We observed similar effects in teleost fish through a correlational analysis of cognitive individual differences and cognitive phenotyping of a *bdnf*<sup>-/-</sup> zebrafish [48]. Our findings indicate that Bdnf (the fish protein homologous of BDNF) improves colour discrimination learning, maze learning, habituation learning and inhibitory control abilities in fish via activational effects.

In our first experiment, we used qPCR to measure *bdnf* expression in brain tissues of individual zebrafish that learned a colour discrimination task. Individuals that learned the discrimination quickly had higher *bdnf* expression level, similarly to what observed in humans, other monkeys and rats [31–33]. When we compared the *bdnf*<sup>+/+</sup> and the *bdnf*<sup>-/-</sup> zebrafish in the same colour discrimination learning task, we found further evidence of the importance of Bdnf. The *bdnf*<sup>-/-</sup> zebrafish's learning was slow, at the point that half of the subjects did not reach the learning criterion within the training period (20 days). Notably, the *bdnf*<sup>+/+</sup> zebrafish used as control subjects in this study and the wild-type zebrafish in earlier studies

acquired the colour discrimination easily [49,61], suggesting that the learning impairment exhibited by the *bdnf*<sup>-/-</sup> zebrafish was not trivial.

Further evidence that Bdnf impacts zebrafish's learning abilities emerged in two other tasks. In the T-maze task, the *bdnf*<sup>-/-</sup> zebrafish learned to find the route towards the exit after 7 days of training on average, whereas the *bdnf*<sup>+/+</sup> zebrafish only took 2–3 days to achieve the task. Moreover, both *bdnf*<sup>-/-</sup> and *bdnf*<sup>+/+</sup> zebrafish larvae demonstrated habituation learning, a simple form of non-associative learning that reduces an individual's response to repeated stimulations. However, the speed of response reduction, and therefore the speed of habituation learning, was lower for the *bdnf*<sup>-/-</sup> zebrafish.

Finally, we analysed the zebrafish's ability to inhibit a behavioural response (i.e. inhibitory control). Both *bdnf*<sup>-/-</sup> and *bdnf*<sup>+/+</sup> zebrafish showed evidence of withholding their foraging behaviour when around prey sealed behind a transparent obstacle. However, inhibition was significantly slower



**Figure 4.** *bdnf* loss reduced habituation learning across the entire larval stage but was rescued with BDNF agonist. (a) Average habituation learning index across the 50 mechanical stimulations of mutant (*bdnf*<sup>-/-</sup>) and control (*bdnf*<sup>+/+</sup>) zebrafish at 7 and 21 dpf; data points and bars represent means and standard errors, respectively. (b) Average habituation learning index of *bdnf*<sup>-/-</sup> zebrafish exposed to a BDNF agonist (7,8-DHF) and the control solution; data points and bars represent means and standard errors, respectively. (Online version in colour.)

for the *bdnf*<sup>-/-</sup> subjects. Inhibitory control is considered an executive function, meaning that it is recruited with low specificity (reviewed in [62]). For instance, an executive function might be equally involved when an animal chooses between spatial routes, interacts with conspecifics, or hides from predators, whereas a specific function such as spatial memory is likely to only be involved when the animal achieves specific tasks (e.g. storing spatial information). Therefore, the *bdnf*<sup>-/-</sup> zebrafish's low inhibitory control is expected to have widespread effects on their cognitive phenotype.

While the aforementioned results clearly indicate that Bdnf improves cognitive performance in zebrafish, the mechanisms could be related to two different types of effect, especially considering the experiments on knockout zebrafish. The presence/amount of BDNF in the brain during the completion of a task could directly improve cognitive performance (activational effect) or its presence/amount during the development could determine a brain phenotype with improved cognition. Our findings provide support for the former interpretation. The habituation learning difference between the *bdnf*<sup>-/-</sup> and the *bdnf*<sup>+/+</sup> zebrafish was evident since the early developmental stages, when the brain is at the end of differentiation [63] and when the post-embryonic *bdnf* expression begins to increase [64]. The learning difference was also consistent until the end of larval development. Although not conclusive, this trend seems incompatible with a marked developmental effect of BDNF on zebrafish learning. Critically, we found that administering an artificial molecule that mimics the effect of BDNF instantaneously improves the learning performance of *bdnf*<sup>-/-</sup> zebrafish. Taken together, results of the two habituation learning experiments suggest that the observed effect of Bdnf on zebrafish cognition is primarily activational.

A putative mechanism for the learning deficit displayed by zebrafish with low or no Bdnf is the long-term potentiation (LTP), a form of synaptic plasticity considered a cellular correlate of learning. *In vitro* studies with hippocampal slices showed that exogenous BDNF promotes LTP [65]. Moreover,

in BDNF mutant mice, cortical and hippocampal LTP were impaired [66–69]. The molecular mechanisms of LTP in zebrafish have received little attention, but early evidence has suggested similarity with that of mammals [70]. Therefore, a conservative evolutionary interpretation would be that LTP mediates learning effects of BDNF in zebrafish. Regarding inhibitory control, the mechanisms of BDNF's action are less clear compared to that on learning. One study on mice points towards a signalling pathway involving the receptor TrkB [71], calling for investigations of the same family of receptors in zebrafish brain [72].

The results of our study are particularly interesting for comparative research on the evolution of vertebrate cognition. There is evidence that BDNF promotes cognitive performance in humans [39], in other primates [31,32], in rodents [33,34], in one bird species [60,73], and, with the current study, in a teleost fish. This range of species encompasses all major vertebrate lineages, except for the amphibians. Likewise, across these vertebrate groups, analysis of cognitive performance has revealed substantial similarities (e.g. [14]). Theoretically, both convergent evolution and common ancestry could explain this pattern of results. The convergent evolution hypothesis would require many evolutionary steps because it assumes the independent appearance of a cognitive function that determines similar cognitive performance and a BDNF-based mechanisms that affects such cognitive function in each vertebrate group. The likelihood of this scenario increases if we assume that a fundamental constraint that tends to canalise evolution towards the use of BDNF as the molecule controlling cognition is present. However, given our current knowledge, the homology hypothesis offers a simpler explanation: all vertebrates potentially inherited from the common ancestor a core cognitive tool box that determines cognitive performance based on the same mechanisms (e.g. BDNF action). Further research efforts to analyse cognitive mechanisms and underrepresented vertebrate groups such as the amphibians are required to clarify this aspect of vertebrate evolution. This is

currently constrained because the tools used to study cognitive mechanisms, such as the mutagenesis in zebrafish, are often not available outside a few model species, and because the mechanisms of BDNF have been mostly inferred from *in vitro* experiments.

It is worth noting that we did not find developmental effects of Bdnf in zebrafish: the learning deficit of *bdnf*<sup>-/-</sup> zebrafish was similar at all ages tested. In mammals, BDNF has important developmental roles [21,22,74], and analysis of *bdnf* expression suggests that the same might occur in zebrafish [64]. It is possible that Bdnf has a developmental role in zebrafish inherent to functions different from those investigated in our study. However, a phylogenetic analysis conducted by Tetamanti and colleagues [75] revealed a diverse evolutionary trajectory due to positive selection between the BDNF genes of mammals and other vertebrates, and higher mutation rates in teleosts. Moreover, structural alignments indicated that teleost's BDNF diverge from that of amniotes [75]. In the light of our study's support for similarities in the activational effect, we hypothesize that the diverse evolutionary trajectory might be related to the developmental effects of BDNF.

In conclusion, our study suggests that BDNF has an important activational effect on fish cognition that is similar to what has observed in mammals. Once the cellular action of BDNF is fully comprehended, further studies in teleost fish and mammals, as well as in groups related to the transition between fish and tetrapods such as the amphibians, should attempt to understand whether this is due to a cognitive mechanism that is conserved in all vertebrates. Considering that the pathogenesis of several cognitive diseases involves alterations of BDNF levels (Alzheimer's disease: [76]; autism: [77]; bipolar

disorder: [78]; schizophrenia: [79]), zebrafish might also help developing new therapeutic strategies based on its similarities with mammals [23,80].

**Ethics.** Experiments were conducted in accordance with the ABS/ASAB 'Guidelines for the treatment of animals in behavioural research and teaching' (doi:10.1016/j.anbehav.2019.11.002), European Legislation for the Protection of Animals used for Scientific Purposes (Directive 2010/63/EU), and the law of the country in which they were performed (Italy, D.L. 26/2014). The research project was approved by the Institutional Animal Care and Use Committees of the University of Ferrara (protocol no. TLX\_1-2020), and by the Italian Ministry of Health (auth. no. 340/2019-PR). License for fish maintenance and breeding at the University of Ferrara is no. 18/2017-UT.

**Data accessibility.** The datasets supporting this article are provided in electronic supplementary material, S2 [81].

**Authors' contributions.** T.L.-X.: conceptualization, formal analysis, writing—original draft; G.M.: data curation, investigation, methodology, writing—review and editing; E.G.: data curation, formal analysis, investigation, methodology, writing—review and editing; E.F.: data curation, formal analysis, investigation, methodology, writing—review and editing; S.D.: conceptualization, writing—review and editing; C.B.: conceptualization, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

**Conflict of interest declaration.** We declare we have no competing interests.

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