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

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Abstract

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 ($bdnf^{-/-}$) showed remarkable learning deficits. Half of the mutants failed a colour discrimination task, whereas the remaining mutants learned the task slowly, taking 3 times longer than control $bdnf^{+/+}$ 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.

Keywords: cognitive evolution; comparative cognition; *Danio rerio*; fish cognition; neurotrophic growth factor; zebrafish model.

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 (Gierszewski et al., 2013; Lucon-Xiccato et al., 2019a), forming long-lasting memories (Triki & Bshary, 2020), flexibly modifying their behaviour (Fuss & Witte, 2019; Lucon-Xiccato & Bisazza, 2014), innovating (Laland & Reader, 1999), using tools (Brown, 2012), solving problems (Mair et al., 2020), inhibiting automatic responses (Lucon-Xiccato et al., 2017), processing numerical information (Bisazza et al., 2010), developing behavioural traditions (Lindeyer & Reader, 2010), and cooperating (Bshary & Grutter, 2006). In many cognitive tasks, fish's performance even exceeds that of many mammalian species (e.g., Bisazza et al., 2014; Lucon-Xiccato et al., 2017).

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 (Bshary & Brown, 2014). 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., Adams & Kafaligonul, 2018; Best & Alderton, 2008; Oliveira, 2013), 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 (Leibrock et al., 1989). At the cellular and molecular level, BDNF contributes to major signalling pathways for neural differentiation, growth, and survival during development (Acheson et al., 1995; Binder & Scharfman, 2004; Ohira et al., 2004; Pencea et al., 2001). Additionally, BDNF displays activational effects that control cognitive output (reviewed in Cunha et al., 2010), such as guiding synaptic plasticity (Briz et al., 2015; Edelmann et al., 2014; Fritsch et al., 2010; Gottschalk et al., 1999; Leal et al., 2017) and modulating neurotransmitter release (Jovanovic et al., 2000; Tyler & Pozzo-Miller, 2001). For instance, the upregulation of BDNF mRNA expression is involved in memory formation (Tokuyama et al., 2000) and in tool-use learning in monkeys (Ishibashi et al., 2002), and in maze learning in rats (Kesslak et al., 1998). Rats' ability to learn can be experimentally impaired with anti-sense BDNF treatment (Mizuno et al., 2000). Similarly, a strain of mutant mice with deletion of one copy of the BDNF gene displayed reduced learning performance (Endres et al., 2012; Linnarsson et al., 1997; Petzold et al., 2015; Psotta et al., 2013). In humans, a genetic polymorphism that affects BDNF secretion impacts memory (Egan et al., 2003) and executive functions (Alfimova et al., 2012; Audiffren et al., 2021; Benzerouk et al., 2013).

BDNF of fish (Bdnf) has high levels of sequence similarity with that of mammals (> 90%) and is expressed in the brain with a similar distribution (Anand & Mondal, 2020; Cacialli et al., 2016; Dalton et al., 2009; Hashimoto & Heinrich, 1997; Nittoli et al., 2018).

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*$^{-/-}$ (null mutant) zebrafish recently generated in our laboratories using the CRISPR/Cas9 genome editing system (D'Agostino et al., 2022) to further characterise the role of Bdnf on cognitive abilities of fish. We analysed the performance of adult *bdnf*$^{-/-}$ zebrafish in a colour discrimination learning task (Gatto et al., 2020), a T-maze task (Miletto Petrazzini et al., 2017), and an inhibitory control task (Lucon-Xiccato & Bertolucci, 2020). Based on the effects of BDNF in mammals (e.g., Audiffren et al., 2021; Ishibashi et al., 2002; Psotta et al., 2013), we hypothesised that natural levels of BDNF predict individual differences in learning and that the *bdnf*$^{-/-}$ zebrafish display impaired cognitive performance compare to control siblings (*bdnf*$^{+/+}$) zebrafish in both tasks. Finally, to elucidate whether the effect of Bdnf on fish cognition is activational or developmental, we observed the learning performance of *bdnf*$^{-/-}$ larvae throughout development and we conducted a rescue experiment using a molecule that mimics BDNF action. If the effects of Bdnf were activational, 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. Materials and methods

(a) Experimental subjects

Overall, the study involved 30 wild-type zebrafish, 184 *bdnf*$^{-/-}$ zebrafish (mutants) and 79 *bdnf*$^{+/+}$ zebrafish (controls). The wild-type zebrafish used to study individual differences belonged to a 500-individuals population kept in the facility of University of Ferrara since 2011. The *bdnf*$^{-/-}$ zebrafish line was generated by clustered regularly interspaced short palindromic repeats (CRISPR)-mediated knockout as described in D'Agostino et al. (2022).

The mutagenesis process generated 25% *bdnf*$^{-/-}$ zebrafish displaying a 40 bp deletion in the exon 2 of the *bdnf* coding sequence impairing all 5 known splicing isoforms in zebrafish. The process also produced 25% individuals with *bdnf*$^{+/+}$ 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 (ESM1, 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 (Lucon-Xiccato et al., 2019b; Santacà et al., 2021). 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 (ESM1, section b). Once the subjects reached the learning criterion of the task, we collected their brain tissues to quantify *bdnf* expression by qPCR (ESM1, 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 (ESM1, section c).

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

In this experiment, we assayed 10 *bdnf*$^{-/-}$ zebrafish and 6 *bdnf*$^{+/+}$ zebrafish with the colour discrimination learning paradigm described in the previous section and in ESM1 (section b). All the subjects were subjected to the reward-colour association. Because some *bdnf*$^{-/-}$ 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*$^{+/+}$ and 12 *bdnf*$^{-/-}$ zebrafish and followed the procedure of Miletto Petrazzini and colleagues (2017). 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 (ESM1, 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*$^{-/-}$ zebrafish and 17 *bdnf*$^{+/+}$ zebrafish, using the paradigm previously adopted in this species (Lucon-Xiccato & Bertolucci, 2020; Lucon-Xiccato et al., 2020). The task measured inhibitory control as ability to withhold foraging behaviour towards live prey that cannot be reached (ESM1, 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.

(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 (Beppi et al., 2021; Faria et al., 2019). We subjected 7-dpf (days post fertilisation) larvae (N = 24 $bdnf^{-/-}$ and 24 $bdnf^{+/+}$) and 21-dpf larvae (N = 20 $bdnf^{-/-}$ and 20 $bdnf^{+/+}$) to a habituation learning task in which they were exposed to 50 vibrational stimulations separated by a 1-s interval (ESM1, 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 (ESM1, section f).

(g) Rescue of learning in $bdnf^{-/-}$ zebrafish

The rescue experiment consisted of assessing the habituation learning performance of $bdnf^{-/-}$ zebrafish exposed to 7,8-dihydroxyflavone hydrate (7,8-DHF), a synthetic molecule that mimics the action of BDNF by activating its TrkB receptor (Daly et al., 2017). A prior study reported that this molecule rescues the behaviour of BDNF-lacking zebrafish (D'Agostino et al., 2022). We performed this experiment in larvae for ethical reasons, using 7-dpf $bdnf^{-/-}$ subjects. Two hours before the habituation learning task, we moved 5 groups of approximately 10 larvae in 5 Petri dishes (N = 49 larvae overall) filled with a solution of

184 2.5% 7,8-DHF and 0.1% solvent (dimethyl sulfoxide: DMSO). Because DMSO could also
185 affect zebrafish behaviour (Christou et al., 2020), we treated 5 groups of control larvae (N =
186 53 control larvae overall) with only this solvent. After the treatment, we assessed the
187 habituation learning performance of the subjects as described in the previous section and in
188 ESM1 (section f).
189
190 (h) Statistical analysis
191 The statistical analysis was performed in R version 3.2.2 (The R Foundation for
192 Statistical Computing, Vienna, Austria, http://www.r-project.org). The tests were two-tailed
193 and α-level for significance was set at P = 0.05.
194 In the first experiment, to study the correlation between levels of BDNF and naturally
195 occurring individual differences in learning, we used Spearman's rank correlation. This non-
196 parametric method allowed us to deal with non-normal data distribution and potential outliers
197 (Schober et al., 2018). The two variables analysed were the index of *bdnf* expression (ESM1,
198 section c) and the number of days necessary to reach criterion in the colour discrimination
199 learning task (ESM1, section b).
200 In the comparison between *bdnf*$^{-/-}$ and *bdnf*$^{+/+}$ zebrafish in the colour discrimination
201 learning and the T-maze task, we performed multiple analysis based on three dependent
202 variables. First, in the colour discrimination task, we analysed the proportion of subjects that
203 reached the learning criterion between *bdnf*$^{-/-}$ versus *bdnf*$^{+/+}$ zebrafish using a Chi-squared
204 test. The Chi-squared analysis was not conducted in the T-maze task because all the subjects
205 reached the learning criterion. Then, for both experiments, we compared the number of days
206 taken by each subject to reach the learning criterion between *bdnf*$^{-/-}$ versus *bdnf*$^{+/+}$ zebrafish.
207 We used Wilcoxon rank sum test as it allowed us to assign the maximum value for the
208 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 this type 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 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 generalised linear mixed-effects models with Poisson error distribution, fitting genotype ($bdnf^{-/-}$ versus $bdnf^{+/+}$) 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 generalised linear model with Poisson error distribution (i.e., a version of the prior model that does 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 (ESM1, section f). We

analysed this index using ANOVAs fitted with genotype (*bdnf*$^{-/-}$ versus *bdnf*$^{+/+}$) 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 ± 4.70 days (mean ± 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 ten (50 %) *bdnf*$^{-/-}$ subjects solved the colour discrimination task within the given time (20 days). Conversely, all the six *bdnf*$^{+/+}$ subjects solved the colour discrimination task. This corresponds to a significantly lower likelihood of learning the colour discrimination for the *bdnf*$^{-/-}$ zebrafish (Chi-squared test: $X^2_1 = 4.364$, $P = 0.037$).

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 ± 7.08 (mean ± standard deviation) for *bdnf*$^{-/-}$ zebrafish. In comparison, *bdnf*$^{+/+}$ zebrafish reached the learning criterion in 3.5 ± 1.64 days. The analysis indicated that *bdnf*$^{-/-}$ zebrafish required a significantly larger number of days to reach the learning criterion compared to *bdnf*$^{+/+}$ zebrafish (Wilcoxon rank sum test: $W = 52$, $P = 0.017$; figure 2b). After removing the data of

the five non-learner *bdnf*$^{-/-}$ subjects, the average number of days to criterion was still more than twice (10.2 ± 7.26) compared to *bdnf*$^{+/+}$ 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*$^{+/+}$ zebrafish and the *bdnf*$^{-/-}$ zebrafish (linear mixed-effects model: $F_{1,154} = 8.429$, P = 0.004). The *bdnf*$^{+/+}$ zebrafish displayed a stepper decrease in the number of errors, and hence greater learning, compared to the *bdnf*$^{-/-}$ zebrafish (figure 2c).

(c) *bdnf* loss 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*$^{+/+}$ 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 (6 versus 12 trials). The repeated measures analysis indicated significant evidence of learning as reduction of number of errors across days in the *bdnf*$^{+/+}$ zebrafish (linear mixed-effects model: $F_{1,24} = 5.835$, P = 0.024), but not in the *bdnf*$^{-/-}$ zebrafish ($F_{1,73} = 2.921$, P = 0.092).

(d) *bdnf* loss 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*$^{-/-}$ zebrafish performed 3.58 ± 5.35 attempts to capture the prey per minute (mean ± standard deviation), whereas the *bdnf*$^{+/+}$ zebrafish scored 2.11 ± 3.39 attacks. A repeated measures analysis on the number of attacks in each minute of the test indicated that the *bdnf*$^{-/-}$ zebrafish displayed a higher number of attempts at the beginning of the experiment (genotype by time interaction: $X^2_1 = 10.204$, 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 ($X^2_1 = 41.812$, P < 0.001), suggesting that *bdnf*$^{+/+}$ zebrafish reduced their attempts more than *bdnf*$^{-/-}$ 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*$^{-/-}$ zebrafish displayed reduced habituation learning compared to the *bdnf*$^{+/+}$ 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 to lack of *bdnf* was constant across development.

(f) BDNF agonist treatment rescues learning in *bdnf*$^{-/-}$ zebrafish

The analysis of the habituation learning index indicated a significant difference between *bdnf*$^{-/-}$ 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*$^{-/-}$ zebrafish (figure 4b).

4. Discussion

The neurotrophin BDNF is a main actor in multiple neural processes in the mammalian brain (Acheson et al., 1995; Briz et al., 2015; Gottschalk et al., 1999; Pencea et al., 2001; Tyler et al., 2001) that determines direct effects on cognitive performance (Cunha et al., 2010; Fritsch et al., 2010; Johnston et al., 1999; Leal et al., 2017). We observed similar effects in teleost fish through a correlational analysis of cognitive individual differences and cognitive phenotyping of a *bdnf*$^{-/-}$ zebrafish (D'Agostino et al., 2022). 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 (Ishibashi et al., 2002; Kesslak et al., 1998; Tokuyama et al., 2000). When we compared the *bdnf*$^{+/+}$ and the *bdnf*$^{-/-}$ zebrafish in the same colour discrimination learning task, we found further evidence of the importance of Bdnf. The *bdnf*$^{-/-}$ 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*$^{+/+}$ zebrafish used as control subjects in this study and the wild-type zebrafish in earlier studies acquired the colour discrimination easily (Gatto et al., 2020; Parker et al., 2012), suggesting that the learning impairment exhibited by the *bdnf*$^{-/-}$ 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*$^{-/-}$ zebrafish learned to find the route towards the exit after seven days of training on average, whereas the *bdnf*$^{+/+}$ zebrafish only took two to three days to achieve the task. Moreover, both *bdnf*$^{-/-}$ and *bdnf*$^{+/+}$ 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*$^{-/-}$ zebrafish.

Finally, we analysed the zebrafish's ability to inhibit a behavioural response (i.e., inhibitory control). Both *bdnf*$^{-/-}$ and *bdnf*$^{+/+}$ zebrafish showed evidence of withholding their foraging behaviour when around prey sealed behind a transparent obstacle. However, inhibition was significantly slower for the *bdnf*$^{-/-}$ subjects. Inhibitory control is considered an executive function, meaning that it is recruited with low specificity (reviewed in Diamond, 2013). 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*$^{-/-}$ 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*$^{-/-}$ and the *bdnf*$^{+/+}$ zebrafish was evident since the early developmental stages, when the brain is at the end of differentiation (Mueller & Wullimann, 2015) and when the post-embryonic *bdnf* expression begins to increase (De Felice et al., 2014). 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

359 administering an artificial molecule that mimics the effect of BDNF instantaneously
360 improves the learning performance of *bdnf*$^{-/-}$ zebrafish. Taken together, results of the two
361 habituation learning experiments suggest that the observed effect of Bdnf on zebrafish
362 cognition is primarily activational.

363 A putative mechanism for the learning deficit displayed by zebrafish with low or no
364 Bdnf is the long-term potentiation (LTP), a form of synaptic plasticity considered a cellular
365 correlate of learning. *In vitro* studies with hippocampal slices showed that exogenous BDNF
366 promotes LTP (Figurov et al., 1996). Moreover, in BDNF mutant mice, cortical and
367 hippocampal LTP were impaired (Bartoletti et al., 2002; Korte et al., 1995; Patterson et al.,
368 1996; Pozzo-Miller et al., 1999). The molecular mechanisms of LTP in zebrafish have
369 received very little attention, but early evidence has suggested similarity with that of
370 mammals (Nam et al., 2004). Therefore, a conservative evolutionary interpretation would be
371 that LTP mediates learning effects of BDNF in zebrafish. Regarding inhibitory control, the
372 mechanisms of BDNF's action are less clear compared to that on learning. One study on mice
373 points towards a signalling pathway involving the receptor TrkB (Besusso et al., 2013),
374 calling for investigations of the same family of receptors in zebrafish brain (Abbate et al.,
375 2014).

376 The results of our study are particularly interesting for comparative research on the
377 evolution of vertebrate cognition. There is evidence that BDNF promotes cognitive
378 performance in humans (Egan et al., 2003), in other primates (Ishibashi et al., 2002;
379 Tokuyama et al., 2000), in rodents (Kesslak et al., 1998; Mizuno et al., 2000), in one bird
380 species (Johnston & Rose, 2001; Johnston et al., 1999), and, with the current study, in a
381 teleost fish. This range of species encompasses all major vertebrate lineages, except for the
382 amphibians. Likewise, across these vertebrate groups, analysis of cognitive performance has
383 revealed substantial similarities (e.g., Bshary & Brown, 2014). 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 a 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*$^{-/-}$ zebrafish was similar at all ages tested. In mammals, BDNF has important developmental roles (Ernfors et al., 1994; Ohira et al., 2004; Pencea et al., 2001), and analysis of *bdnf* expression suggests that the same might occur in zebrafish (De Felice et al., 2014). 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 Tettamanti and colleagues (2010) 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 (Tettamanti et al., 2010). In light of our study's support for

409 similarities in the activational effect, we hypothesis that the diverse evolutionary trajectory
410 might be related to the developmental effects of BDNF.
411 In conclusion, our study suggests that BDNF has an important activational effect on
412 fish cognition that is similar to what has observed in mammals. Once the cellular action of
413 BDNF is fully comprehended, further studies in teleost fish and mammals, as well as in
414 groups related to the transition between fish and tetrapods such as the amphibians, should
415 attempt to understand whether this is due to a cognitive mechanism that is conserved in all
416 vertebrates. Considering that the pathogenesis of several cognitive diseases involves
417 alterations of BDNF levels (Alzheimer's disease: Lee et al., 2005; autism: Armeanu et al.,
418 2017; bipolar disorder: Grande et al., 2010; schizophrenia: Nieto et al., 2013), zebrafish
419 might also help developing new therapeutic strategies based on its similarities with mammals
420 (Cunha et al., 2010; Lu et al., 2014).
421
422 **Ethics**
423 Experiments were conducted in accordance with the ABS/ASAB 'Guidelines for the
424 treatment of animals in behavioural research and teaching' (doi:
425 10.1016/j.anbehav.2019.11.002), European Legislation for the Protection of Animals used for
426 Scientific Purposes (Directive 2010/63/EU), and the law of the country in which they were
427 performed (Italy, D.L. 26/2014). The research project was approved by the Institutional
428 Animal Care and Use Committees of the University of Ferrara (protocol n. TLX_1-2020),
429 and by the Italian Ministry of Health (auth. n. 340/2019-PR). License for fish maintenance
430 and breeding at the University of Ferrara is n. 18/2017-UT.
431
432 **Data accessibility**
433 The datasets supporting this article have been uploaded in ESM2.

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Authors' contributions

T.L.-X.: conceptualization, formal analysis, writing—original draft; G.M.: investigation, methodology, data curation, writing—review and editing; E.G.: investigation, methodology, data curation, formal analysis, writing—review and editing; E.F.: investigation, methodology, data curation, formal analysis, 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.

Competing interests

We declare we have no competing interests.

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Figure captions

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 a transparent glass and had to withhold the capture attempts.

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 represent an individual fish. (b) Number of days required by mutant ($bdnf^{-/-}$) and control ($bdnf^{+/+}$) 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 ($bdnf^{-/-}$ and $bdnf^{+/+}$) divided per each day of training in the colour discrimination task; data points and bars represent means and standard errors, respectively.

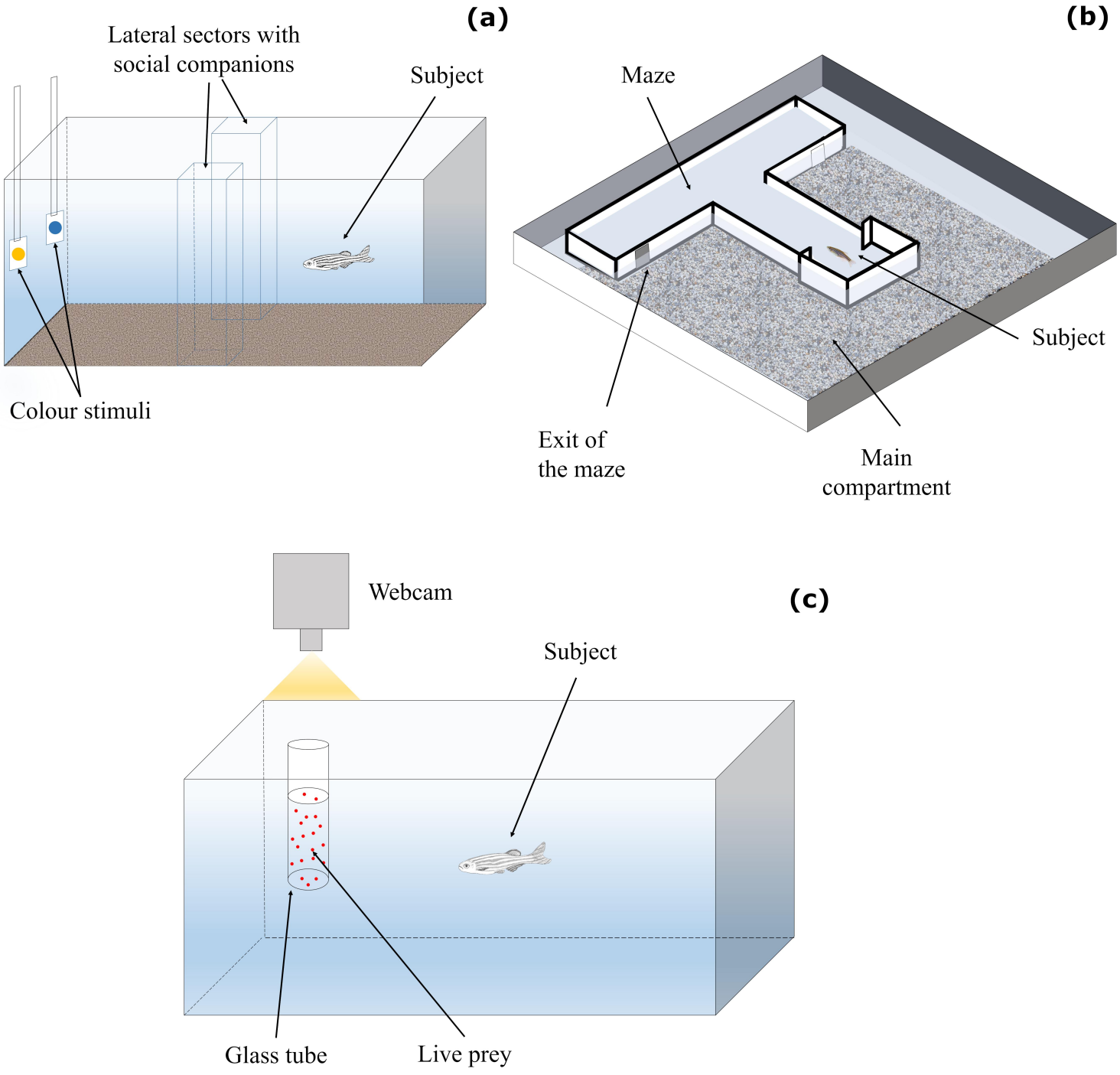
Figure 3

BDNF loss reduced T-maze learning and inhibitory control in zebrafish. (a) Number of days required by mutant ($bdnf^{-/-}$) and control ($bdnf^{+/+}$) 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*$^{-/-}$ and *bdnf*$^{+/+}$) 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*$^{-/-}$ and *bdnf*$^{+/+}$) divided per each minute of the test. Data points and bars represent means and standard errors, respectively.

Figure 4

BDNF loss reduced habituation learning across the entire larval stage but can be rescued with BNDF agonist. (a) Average habituation learning index across the 50 mechanical stimulations of mutant (*bdnf*$^{-/-}$) and control (*bdnf*$^{+/+}$) zebrafish at 7 and 21 dpf; data points and bars represent means and standard errors, respectively. (b) Average habituation learning index of *bdnf*$^{-/-}$ zebrafish exposed to a BDNF agonist (7,8-DHF) and the control solution; data points and bars represent means and standard errors, respectively.


(a)
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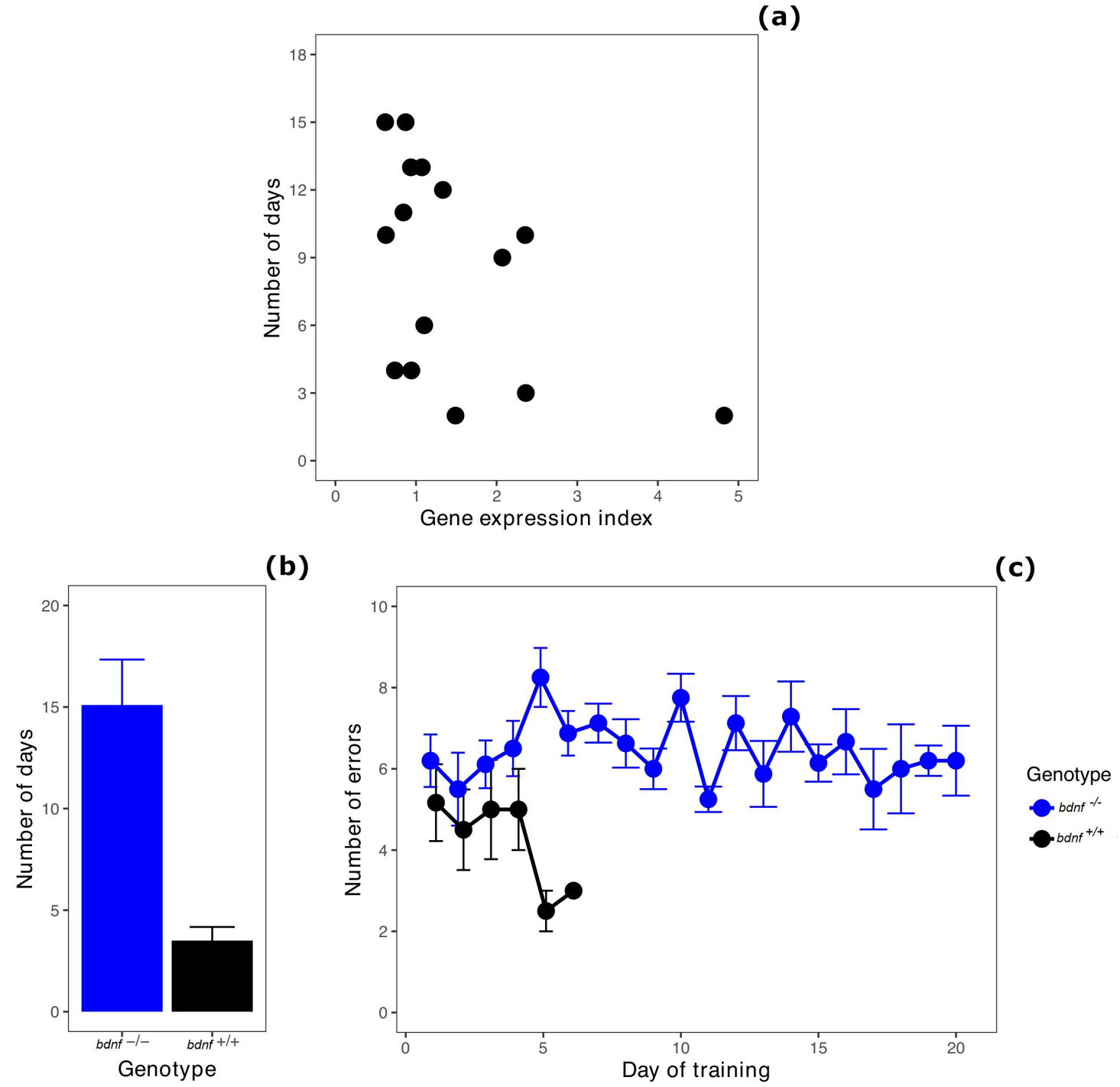
Glass tube
Live prey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Gene expression index
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bdnf +/+
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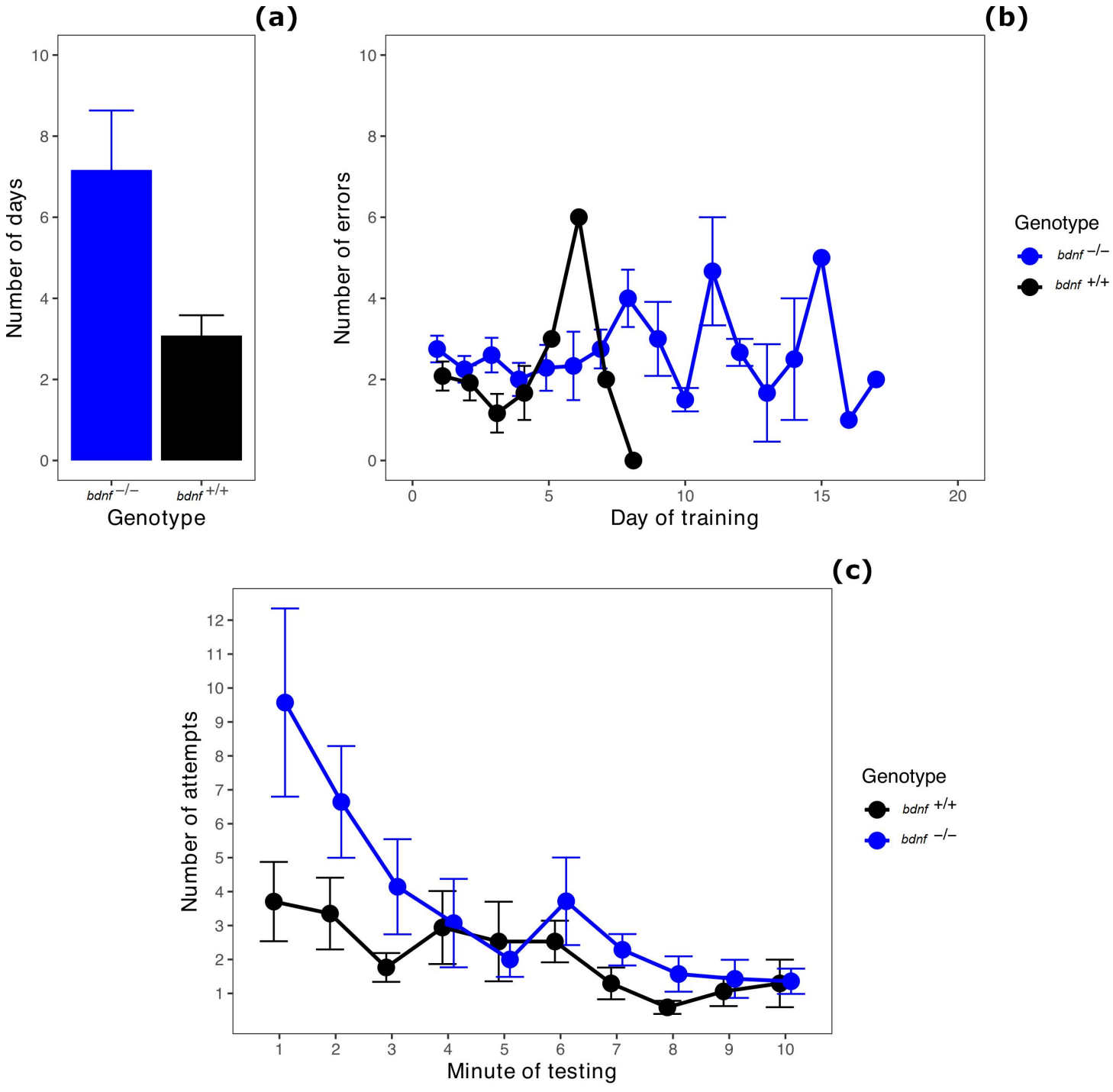
bdnf -/-
bdnf +/+


(a)
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bdnf −/−
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Genotype
(b)
Number of errors
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4
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0
5
10
15
20
Day of training
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bdnf −/−
bdnf +/+
(c)
Number of attempts
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11
10
9
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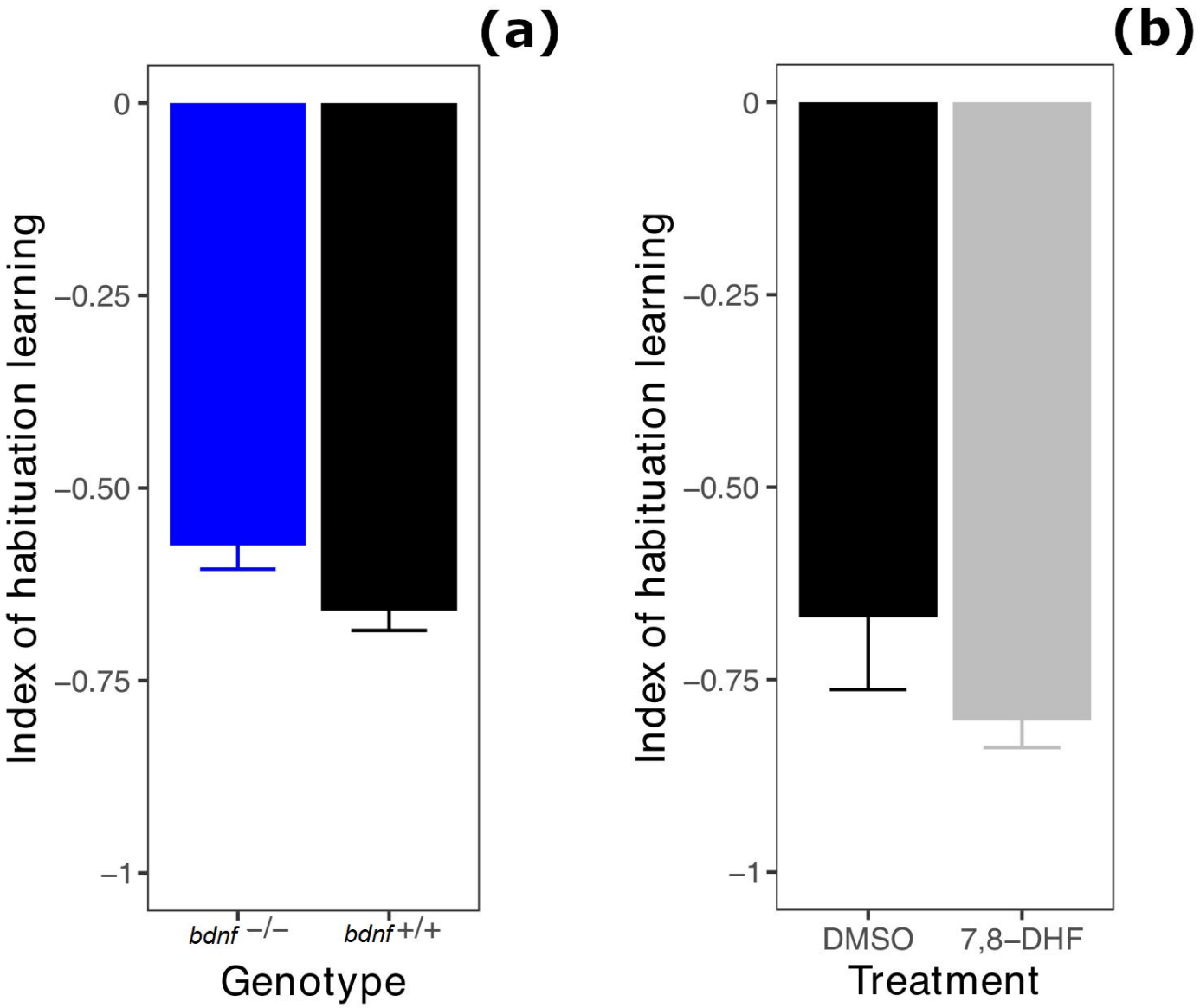
bdnf +/+
bdnf −/−


(a)
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Index of habituation learning
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bdnf +/+
Genotype
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7,8−DHF
Treatment