Stimulation of dopamine D₁ receptor improves learning capacity in cooperating cleaner fish

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Accurate contextual decision-making strategies are important in social environments. Specific areas in the brain are tasked to process these complex interactions and generate correct follow-up responses. The dorsolateral and dorsomedial parts of the telencephalon in the teleost fish brain are neural substrates modulated by the neurotransmitter dopamine (DA), and are part of an important neural circuitry that drives animal behaviour from the most basic actions such as learning to search for food, to properly choosing partners and managing decisions based on context. The Indo-Pacific cleaner wrasse *Labroides dimidiatus* is a highly social teleost fish species with a complex network of interactions with its ‘client’ reef fish. We asked if changes in DA signalling would affect individual learning ability by presenting cleaner fish two ecologically different tasks that simulated a natural situation requiring accurate decision-making. We demonstrate that there is an involvement of the DA system and D₁ receptor pathways on cleaners’ natural abilities to learn both tasks. Our results add significantly to the growing literature on the physiological mechanisms that underlie and facilitate the expression of cooperative abilities.

1. Introduction

Animals’ individual fitness depends on displaying adaptive social behaviour patterns in a context-appropriate manner [1]. Learning and memory processes rely on stimulus evaluation integrated into adaptive behaviour, and to be adaptive, actions must be rewarding to some extent in order to motivate repetition or, in contrast, detrimental enough to induce avoidance. Thus, learning and memory processes become critical to developing accurate contextual decision-making strategies [1,2]. Experimental data have shown that the evolution of learning and memory systems is more conservative than previously thought, with teleost fish relying on similar neural substrates for learning and memory processes as mammals [3]. In mammals, the hippocampal region is linked to relational memory processes such as spatial cognition and temporal discounting, whereas the amygdala is linked to emotional learning and memory. In teleosts, the dorsolateral (Dl) and dorsomedial (Dm) parts of the telencephalon, putative homologous regions to the mammalian hippocampus and amygdala (respectively), are linked to comparable neuronal processes [1,3–5]. Indeed, similar to mammals, teleost fish are capable of using high-order cognitive capabilities which require complex associative neural mechanisms [1,3–5]. Indeed, similar to mammals, teleost fish are capable of using high-order cognitive capabilities which require complex associative neural mechanisms [1,3–5]. However, the Dm region is essential to emotional learning and memory [5]. These two distinct memory systems in the vertebrate forebrain are thus equally important and potentially complementary in the learning process. Yet, while the neural substrates involved seem to be identified, questions remain regarding how they are modulated by different neurotransmitter circuitries.

The neurotransmitter dopamine (DA) is involved in a variety of neurochemical and neurohormonal actions that include general cognition and reward assessment [7,8] and, more specifically, in stimuli assessment and conditioned reinforcement [9,10]. DA works as a teaching signal that helps to predict future events and facilitates behavioural adjustment and learning. Animals assign
different values to stimuli they receive from the social environment, and the DA neurons signal these occurrences with stronger or weaker phasic bursts indicating positive or negative experiences, respectively [11–13]. In subsequent iterations, DA neurons will signal putative changes in previously acquired information, allowing animals to evaluate changing situations and adapt their behaviour accordingly [14–16]. In addition to response–reward and stimulus–reward associations, DA also has an important role in memory consolidation, facilitating memory traces of different types of tasks and learning, which will occur at different dopaminergic terminal brain regions, such as the hippocampus and amygdala in mammals (Dl and Dm, respectively, for teleosts [17]). Indeed, both Dl and Dm regions are part of an important neural circuitry responsible for reward processing called the mesolimbic reward system, a dopaminergic pathway that is well described in mammals [18,19] and teleosts alike [6,20–23] (for a more comprehensive review of putative homologous brain regions for teleosts, see [24,25]).

Dopaminergic function modulates animal behaviour from the most basic actions such as learning to search for food [26,27], to properly choosing partners and managing decisions based on context [28,29]. In fish, few studies have directly dealt with the role of the DA system on behaviour, which have mostly focused on locomotor activity [30], brain responses to changes in light and hydrostatic pressures [31], feeding behaviour [27], coping with unpredictability [32], and the role of DA in zebrafish learning via the effects of nicotine [33]. Indeed, in zebrafish, researchers have shown that the increase in dopaminergic activity contributed to improved learning and choice accuracy [33]. Here, we asked if changes in DA activity are linked to individual learning ability in the context of cooperative behaviour. We chose the well-known Indo-Pacific bluestreak cleaner wrasse Labroides dimidiatus, to study the effect of dopaminergic manipulation in relation to two tasks with different social–ecological significance. Cleaner wrasses feed exclusively of ectoparasites, diseased tissue, mucus and scales of other visiting reef fish (usually referred as client fish [34–36]). One of the most interesting characteristics of this system is the existence of a conflict of interest between both sides of these partnerships that occurs, because cleaners prefer to eat client mucus, which is detrimental to clients and constitutes cheating [37]. Because clients may respond to cheating cleaners by attacking (punishing), leaving, or simply completely avoiding to interact, cleaners need to learn to adjust their feeding behaviour (to eat against their preference) if they want to motivate clients to visit and to continue to interact [38]. An appropriate example of the level of decision-making demanded of cleaners in natural conditions occurs when two clients seek service simultaneously. In such a situation, cleaners must make a choice regarding which client to inspect first, with the potential consequence that the ignored client may leave [39]. The decision rule in this situation will be to attend first the client less willing to wait, which is usually a visitor species with wider territories that encompass more than one cleaner (these are referred to as choosy clients). The process of choosing the most appropriate client should be a matter of learning by trial and error and later memorizing and recognizing the specificities (visual cues) associated with each visitor species.

This study followed the experimental design of Cardoso et al. [40] in which two plates with different patterns are presented simultaneously; only one of these plates offers a food reward and, when ignored by the cleaner, is immediately removed (representing the real-life situation where when a visitor client is ignored, it swims away [41]). Two tasks were designed that differed in terms of the cues available to identify the correct choice: in the first task, cleaners needed to identify a specific pattern that consistently provided food, whereas in the second task, they had to identify the site where food was consistently provided. Because cleaners eat exclusively off the surface of clients, which vary in terms of parasite or mucus distribution, regardless of location (with clients themselves being mobile), the first task (cue or pattern discrimination) was deemed to be socioecologically more meaningful than the second (side/spatial discrimination). These two different learning situations putatively rely on different neural substrates, with cue learning being Dm dependent and spatial learning being Dl dependent [40]. DA is an appropriate candidate to affect these learning tasks because it is generally linked to the learning process and due to a wide distribution of dopaminergic receptors (D1 and D2) in teleost fish [24] in the forebrain area [24]. Nevertheless, variance in the expression of these receptors in regions of interest for learning (such as Dl and Dm), coupled with the difference in ecological context relevance, may allow alternate effects of DA on both of our tested tasks. We predicted that D1 and D2 antagonists would impair cleaner wrasses’ learning speed, because interference with DA signalling is reported to impair the acquisition of conditioned approach responses [42]. However, D2 antagonists are reported not to have an effect on working memory, which is an important aspect of learning [13]. Overall, we expected that D1 and D2 agonists would improve learning speed, as increasing DA signalling is reported to enhance learning of a conditioned response to visual stimuli [43] and spatial learning [33], and to facilitate working memory [13].

2. Material and methods

(a) Animals and housing

Experiments were conducted at the Oceârio de Lisboa fish housing facilities (Lisbon, Portugal), where we used a total of 10 wild-caught cleaner wrasses originating in the Maldives and directly imported to Portugal by a local distributor. The fish were kept in individual aquaria (100 × 40 × 40 cm), combined in a flow-through system that pumped water from a sump tank (150 × 50 × 40 cm). The nitrate concentration was kept to a minimum (always less than 0.3 mg l⁻¹) and each tank contained an air supply and a commercial aquarium heater (125 W, Eheim, Jäger). Small polyvinyl chloride (PVC) pipes (10–15 cm long; 2.5 cm diameter) were used as shelter. Experiments were conducted between March and October 2013. Cleaner fish were previously taught to feed on mashed shrimp from a Plexiglass plate (white colour), as a way to simulate the wild feeding habit of picking food off their clientele, and took 1–3 days to learn. Different colour patterns were randomly assigned to every Plexiglass plate, and a different pair of plates was used for each treatment. Each individual was used in both experimental tasks, and tested with each of the five treatments.

(b) Experiment 1: cue discrimination task

This experiment consisted of several sessions (up to eight) in which pairs of plates with different patterns were presented to the cleaner fish (electronic supplementary material, figure S1a): one was deemed to be the one from which the cleaner needed to start eating first (correct pattern) and the second that could wait but had no food/reward accessible (incorrect pattern).
All plates were initially introduced to cleaners the day before the start of the experiments, with a small piece of prawn (reward) in the back. Cleaners were submitted to one session per day, on alternate days. Each session was composed of 10 trials, and each individual was subjected to repeated sessions until it was considered to have learned the task at hand. A cleaner was considered to have learned the task if it had performed: three successive sessions in which the individual chose the correct plate in at least seven out of 10 trials; two successive sessions in which the cleaner chose the correct pattern plate at least eight out of 10 trials; or just one session with at least nine out of 10 trials. The first trial began 10 min after application of the treatment and consisted of successive presentation of the patterned colour plates every 10 min until the completion of 10 trials. The ‘correct’ pattern plate had available prawn whilst the ‘incorrect’ pattern plate had inaccessible prawn (covered with transparent tape). The food reward was placed at the back of the plates, so that it did not create any sort of choice bias. Experimental individuals would then have complete access to both plates, but, whereas a correct choice would enable both plates to stay inside the aquarium (electronic supplementary material, figure S1b), an incorrect choice would consequently cause the correct plate (and the only one with accessible food) to be immediately removed, preventing cleaners from eating the food reward (electronic supplementary material, figure S1c). The left–right position of the plates was changed among trials.

(c) Experiment 2: side discrimination task
This task consisted of several sessions, with a similar layout to the previous experimental task, with pairs of plates with colour patterns being displayed to the cleaner fish, each pair associated with a treatment (electronic supplementary material, figure S1a). This task consisted of learning the correct side to start eating from (spatial learning), instead of a correct colour pattern plate (cue learning), which could be inserted in any side of the aquarium (as above). Plates were again initially introduced to cleaners with a small piece of prawn at the back, one plate at a time and one day before the beginning of experiments. Cleaners were (as above) subjected to one session a day, on alternate days, for a maximum of eight sessions. Before each session, individuals were injected with one of five randomly assigned compound treatments (see below). A session would start 10 min after the injection, and plates were displayed in 10-min intervals, for a total of 10 trials per session, until the individual was considered to have learned the task at hand. Each session was composed of 10 trials, and each individual was subjected to repeated sessions until it was considered to have learned the task at hand (for details, see above). The ‘correct’ side was chosen randomly and was maintained between trials and sessions but it changed between treatments. The ‘correct’ side plate had available prawn, whereas the ‘incorrect’ side plate had inaccessible prawn (covered with transparent tape). Experimental individuals would then have complete access to both plates, but, whereas a correct choice would enable both plates to stay inside the aquarium (electronic supplementary material, figure S1b), an incorrect choice would consequently cause the correct plate (and the only one with accessible food) to be immediately removed, preventing cleaners from eating the food item (electronic supplementary material, figure S1c). The left–right position of the plates was changed among trials.

(d) Dopaminergic treatment
Cleaners were weighed before the onset of the experiment, so that injection volume could be adjusted to body weight. The following treatments were used: saline solution for control (0.9% NaCl); a selective D1 receptor agonist SKF-38393 (D047, Sigma) (D1a); a D1 receptor antagonist SCH-23390 (D054, Sigma) (D1ant); a selective D2 and D3 receptor agonist Quinpirole (Q102, Sigma) (D2a); selective D3 receptor agonist Metoclopramide (M0763, Sigma) (D2ant). Injection volumes were always 15 µl per gram of estimated body weight (gbw). This process never exceeded 3 min. Dosages applied were first based on previous studies: 5.0 µg gbw⁻¹ of SKF-38393 [44–46]; 0.5 µg gbw⁻¹ of SCH-23390 [47,48]; 2.0 µg gbw⁻¹ of Quinpirole [49]; and 5.0 µg gbw⁻¹ of metoclopramide [30,50], and later exogenously administered to female cleaner wrasses in the wild (unpublished data, 2012). SKF-38393 is a selective D1 and partial D2 receptor agonist that can simulate DA activity [44]. SCH-23390 is a high-affinity selective D1 receptor agonist with negligible effects on D2 receptors and slight effects on 5-HT2A/C (serotonin) receptors [51]. However, effects on the serotonin systems can be dismissed, because 5-HT2A receptors have not yet been found in fish [52], and the dosage needed to produce effects on 5-HT2C receptors is 10-fold higher than the dosage needed for D1 blockade [53]. Quinpirole is a selective D2 and D3 receptor agonist [54] widely used in a variety of scientific studies related to D2 receptor manipulation. Metoclopramide, commonly known for its anti-emetic effect via the chemoreceptor trigger zone, is a selective D2 receptor antagonist, acting as an inhibitor of DA action [30,55]. Although it also has slight effects on the serotonin system (5-HT3/4), the target receptors have not yet been discovered in the teleost fish brain [52]. Because reward-driven behaviour and decision-making faculties are controlled by central mechanisms (as previously stated), all compounds chosen are reported to be capable of crossing the blood–brain barrier, to ensure that the effects could take place in (but not exclusively on) the central system [56–59].

(e) Statistical analysis
The same cleaners were used in all treatment groups in both tasks. The order in which the treatments were administered was completely random. However, to eliminate the possibility of any behaviour bias owing to treatment sequence order, we first examined if it had an influence on cleaner wrasse learning scores by using a linear-mixed model, with treatment (five levels: D1a, D1an, D2a, D2an, and saline) and treatment sequence (five levels: 1, 2, 3, 4, 5) as fixed factors. Treatment sequence order was not significant in either of the tasks (cue: F₄,₁₄ = 0.39, p = 0.81; side: F₄,₁₇ = 2.59, p = 0.07) and thus it was not included in further comparisons between treatments. Data were analysed using planned comparisons of least-squares means to contrast the effect of each dopaminergic treatment with the reference (saline) group [60]. All statistical tests were two-tailed. Although it would be interesting to compare both tasks, they are not comparable, because the second task relies on reversal learning (cleaners first learned to make a discrimination, such as choosing the correct colour pattern plate and, in the second experiment, they learned to reverse the choice—i.e. to understand that it was the specific site and not the pattern that was important). It also would not be correct to compare both tasks directly owing to potential effects of experimental order (experiment 2 always followed experiment 1).

3. Results
(a) Experiment 1: cue discrimination task
Cleaners injected with the D1 agonist required significantly fewer sessions to learn a cue discrimination task (planned comparisons: D1a versus saline: F₁,₁₉ = 6.69, p = 0.03, figure 1a), but there were no significant effects of the remaining compounds on cleaners’ learning speed (D1ant versus saline: F₁,₁₉ = 2.43, p = 0.15; D2a versus saline: F₁,₁₉ = 0.02, p = 0.90; D2ant versus saline: F₁,₁₉ = 0.22, p = 0.65, figure 1a). A similar pattern was found in the learning curves for each treatment,
comparisons (electronic supplementary material, figure S2b).

Distinct and confirms the pattern found with planned treatments, but the D1a learning curve was still the most significant. To the best of our knowledge, this study provides the first insight into the neural pathways of learning facilitation (via D1 stimulation) underlying the expression of cooperative behaviour in a non-kin-related context.

Systemic pharmacological manipulations of the DA system influenced both cue and spatial learning tasks, which are putatively dependent on Dm and D1 telencephalic regions, respectively, via the increase in D1 receptor stimulation that produced significant learning effects. In fish, both D1 (mostly the D1A subtype) and D2 receptors are widely expressed within the D1 and Dm telencephalic regions [24], with a few variations in other regions of the diencephalon and mesencephalon. However, there are differences in the affinity of DA for each of these receptors: for instance, D2-like receptors have a 10- to 100-fold greater affinity than the D1-like family, with the D1A receptors being reported to have the lowest affinity for DA [61,62]. Moreover, the D1-like receptors are solely found postsynaptically, with the D1A receptors being thought to mediate most D1-like effects in vertebrates, whereas the D2-like receptors are the predominant type of autoreceptor, being found both pre- and postsynaptically [61]. This means that the pathways involved in the function of both receptor subtypes are distinct, with the D2 receptors being able to induce a negative feedback regulation that may inhibit DA neuron firing, synthesis, and release [63], and in that way functioning as a control mechanism, contrary to the direct stimulatory role of the D1 receptors. Moreover, some studies have referred to the opposing and sometimes complementary roles played by the D1 and D2 receptors in animals’ behavioural response modulation [47], which includes some mechanisms related to recognition, memory acquisition, consolidation, and retrieval phases [64].

In response to ecologically relevant cues, DA neurons normally increase rapid firing, which enable the expression of behaviours allowing the successful acquisition of a reward [65]. However, the specific pathways involving learning associations may be diverse. Here, we found that stimulation of the D1 receptors was critical for a significant enhancement of cleaners’ learning abilities. When cleaners encountered an unexpected reward (such as a plate with food), which was linked to either a pattern or a site, activation of the low-affinity D1 receptors triggered goal-directed reward learning and improvement of learning speed. These results are consistent with those found in rats by Hotte et al. [66], in which injections of a D1 receptor agonist, prior to testing trials, facilitated object recognition and localization, and the temporal order of tasks. Indeed, the improvement of cleaners’ learning speed under the influence of D1 receptor stimulation occurred, irrespective of task (cue or spatial), which may mean that both set-ups were equally relevant as predictors of rewards and that D1 receptor stimulation may be similarly relevant to both neural systems (D1 and Dm). However, the amount of relevance linked to any social–ecological context is perhaps more dependent on the magnitude of the prospective reward to be gained (small or large; certain or uncertain) instead of relying on simple cue or pattern discrimination. For example, gaining access to a less frequent fish visitor may preclude a higher reward for cleaners than a typical client inspection. Further

Figure 1. The effect of D1 receptor agonist (D1a), D1 antagonist (D1ant), D2 agonist (D2a), and D2 antagonist (D2ant) on cleaner fish L. dimidiatus learning of: (a) cue discrimination task and (b) side discrimination task. Means are shown ± standard error of the mean (s.e.m.). Probability values refer to planned comparisons of least-squares means effect of each dopaminergic treatment group against the reference (saline) group (*p = 0.03; **p = 0.04; non significant (n.s.) > 0.05). Sample sizes were n = 10, and cleaners had a maximum of eight sessions to learn each task.

where the D1a learning curve was distinct from all other curves (electronic supplementary material, figure S2a).

(b) Experiment 2: side discrimination task
Only cleaners treated with the D1 agonist took significantly fewer sessions to complete this task (D1a versus saline: $F_{1,9} = 5.49, p = 0.04$, figure 1b), whereas no changes in learning speed were found following other treatments (D1ant versus saline: $F_{1,9} = 0.33, p = 0.58$; D2a versus saline: $F_{1,9} = 0.10, p = 0.76$; D2ant versus saline: $F_{1,9} = 1.82, p = 0.21$, figure 1b). Overall, all learning curves were similar among the different treatments, but the D1a learning curve was still the most distinct and confirms the pattern found with planned comparisons (electronic supplementary material, figure S2b).

4. Discussion
Here, we tested the role of the dopaminergic system by manipulating two of the most abundantly expressed DA receptors in vertebrate brains (D1 and D2 receptors) on the ability of the cleaners to solve problems linked to their cooperative context and to two putatively different neural systems. Our results indicate a greater role for D1 receptor pathways than D2 receptor pathways in reward-associative learning. The effect of the D1 receptor stimulation turned out to be significant in both reward-related learning tasks relying on cue or spatial learning, whereas results of D2 receptors stimulation and selective blockade of both D1 and D2 receptors were not statistically significant. To the best of our knowledge, this study provides the first insight into the neural pathways of learning facilitation (via D1 stimulation) underlying the expression of cooperative behaviour in a non-kin-related context.

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testing is thus necessary to determine the scope of D2 receptor activity modulation in helping cleaners make finer judgements concerning not just the gaining of an equally sized and equally probable reward (which was tested here), but also to evaluate the magnitude of reward, the risk involved, and putative changes in conditions.

A potential explanation for the lack of significant effects of D2 receptor stimulation in our study may come from the motivational value of the stimuli: while we tested only for a positive reward-predictive association, some neuronal DA groups respond distinctly to aversive or even to the absence of stimuli [67]. For instance, by using D1 or D2 receptor knock-down mice, Danjo et al. [68] found that a D2-specific receptor pathway was critical for the perception and expression of aversive responses (place aversion). Moreover, in tests performed with wild female cleaner wrasses in the field, the blockade of D1 and D2 receptors seem to increase sensitivity to negative stimuli but with different magnitudes (unpublished data). Future studies should consider testing for the implications of D2 receptor pathways on cleaners’ learning and decision-making in response to aversive stimuli such as client punishment or simple food removal. Furthermore, because D2 autoreceptors require a lower concentration of agonists to be activated than their postsynaptic counterparts, the same agonist can produce different effects across a range of doses (low doses induce behavioural inhibition and higher doses induce activation) [61]. Considering that animal decision-making is thought to be dependent on a fine balance between D1 and D2 receptor activity, further dose ranges should be used to test for the significance of D2 receptor pathways in order to explain the absence of D2 receptor-related effects in our study.

Successful reward-learning association does not necessarily need intact DA transmission, as long as reward cues become effective predictors [69]. Recently, Darvas et al. [70] found that animals are capable of associative learning with as little as 30% of baseline DA levels. Additionally, DA blockade may only impair associative learning in animals with a tendency to learn through a form of stimulus–reward learning where DA-mediated incentive salience is attributed to reward cues [10,71,72], but not of those forms that instead rely on the predictive value of such reward cues [70]. Here, none of the administered antagonists (D1 and D2) delayed the cleaners’ learning ability in either of the two tasks, probably because DA signalling did not decrease to a degree where it would compromise the acquisition of a basal conditioned response. DA is most likely not mediating the motivational properties of reward cues, but rather their predictive properties and continuous updating, which leads to cleaner wrasses learning both tasks at a similar pace as the control (saline) group, even when their DA signalling is selectively inhibited (electronic supplementary material, figure S3).

Laboratory experiments have shown that cleaners are easily able to learn to increase their foraging output, to solve tasks quickly, and even to relearn the tasks when these are reversed [73]. These outstanding learning capabilities, which translate into higher cognitive and cooperative abilities, are sustained by a physiological framework that supports a highly successful adaptation to their naturally complex social environment. We demonstrate that there is involvement of the DA system and D1 receptor pathways on cleaners’ natural abilities to learn. Further research is necessary to test the influence of these same pathways in the development of key learning abilities of other highly social or cooperative species of vertebrates.

**Ethics.** Animal procedures used in this study were approved by the Portuguese Veterinary Office (Direcção Geral de Veterinária, licence no. 0420/000/000/2009).

**Data accessibility.** Supporting behavioural data can be accessed at Dryad: http://dx.doi.org/10.5061/dryad.89gc4.

**Authors’ contributions.** M.C.S. and J.P.M.M. designed the study. J.P.M.M., T.P.S., and M.P. ran experimental procedures. J.P.M.M. analysed the data. M.C.S. and J.P.M.M. wrote the paper. All authors discussed results and commented on the manuscript.

**Competing interests.** The authors declare no competing financial interests.

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**References**


54. Levant B, Grigoriadis DE, DeSouza EB. 1993 [3H]quinpirole binding to putative D1 and D2 dopamine receptors in rat brain and pituitary gland:


Correction to: Stimulation of dopamine D₁ receptor improves learning capacity in cooperating cleaner fish

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We recently discovered an error in the article cited above: we realized that the order of treatments did not seem to be counterbalanced in the cue discrimination task during the first two injection rounds, compared with the side discrimination task that was always properly counterbalanced. The effect of sequence order and treatment was tested and demonstrated to be non-significant to the results (Methods section) but nevertheless, the word random cannot apply to the cue experimental design. While we acknowledge that the cue learning sequence effects could, in theory, have contributed to the D1a significant results, as these fit the side learning results we believe that it is very unlikely that sequence rather than treatment caused the first results (also demonstrated statistically as we mentioned previously).

In addition, from our initial sample (12 cleaners), two individuals were excluded from all analysis (cue and side) because they were not in good health condition during the entire duration of experiment procedures.