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RESEARCH ARTICLE

Opposing effects of dopamine on agonistic behaviour in crayfish

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ABSTRACT

The effects of dopamine on the agonistic behaviour of crayfish were analysed. When dopamine concentrations of 1 μmol l^{-1} were injected into large crayfish, individuals were beaten by smaller opponents, despite their physical advantage. Injection of 10 μmol l^{-1} dopamine into small animals increased their rate of winning against larger opponents. Injection of a D1 receptor antagonist prohibited the onset of a 'loser' effect in subordinate animals, suggesting that the inhibitory effect of dopamine on larger animals is mediated by D1 receptors. Similarly, injection of a D2 receptor antagonist prohibited the onset of a 'winner' effect in dominant animals, suggesting that the facilitating effect of dopamine on small animals is mediated by D2 receptors. Since the inhibitory effect of 1 μmol l^{-1} dopamine was similar to that seen with 1 μmol l^{-1} octopamine and the facilitating effect of 10 μmol l^{-1} dopamine was similar to that of 1 μmol l^{-1} serotonin, functional interactions among dopamine, octopamine and serotonin were analyzed by co-injection of amines with their receptor antagonists in various combinations. The inhibitory effect of 1 μmol l^{-1} dopamine disappeared when administered with D1 receptor antagonist, but remained when combined with octopamine receptor antagonist. Octopamine effects disappeared when administered with either D1 receptor antagonist or octopamine receptor antagonist, suggesting that the dopamine system is downstream of octopamine. The facilitating effect of 10 μmol l^{-1} dopamine disappeared when combined with serotonin 5HT1 receptor antagonist or D2 receptor antagonist. Serotonin effects also disappeared when combined with D2 receptor antagonist, suggesting that dopamine and serotonin activate each other through parallel pathways.

KEY WORDS: Social status, Serotonin, Octopamine, Modulation, Interplay of biogenic amines

INTRODUCTION

Biogenic amines can modulate feeding and sexual, postural and aggressive behaviours in both vertebrate and invertebrate animals (for reviews, see Weiger, 1997; Kravitz, 2000). Aggression levels, in particular, can be controlled by biogenic amines. In vertebrates, dopamine promotes aggressive behaviour in fish (Maler and Ellis, 1987; Winberg and Nilsson, 1992), lizards (Höglund et al., 2005) and rats (van Erp Annemoon and Miczek, 2000), while serotonin can reduce aggression in fish (Winberg et al., 1992), lizards (Larson and Summers, 2001) and rodents (Saudou et al., 1994; de Boer and Koolhaas, 2005). Some researchers, however, report that serotonin promotes aggression in birds (Shea et al., 1991; Buchanan et al., 1994), dogs (Badino et al., 2004) and rodents (Saudou et al., 1994; Takahashi et al., 2010). In insects, octopamine increases aggressive motivation in crickets (Stevenson et al., 2000; Rillich et al., 2011), whereas serotonin enhances aggression in *Drosophila* (Alekseyenko et al., 2010, 2013, 2014). In crustaceans, serotonin can increase aggressive motivation, while octopamine and tyramine reduce aggressiveness (Antonsen and Paul, 1997, 2001; Huber, 2005; Huber et al., 1997, 2001; Momohara et al., 2013, 2016, 2018; Bacqué-Cazenave et al., 2018, 2019; Bergman and Moore, 2020). Studies also indicate that serotonin and dopamine can mediate aggressive behaviour in ants (Szczuka et al., 2013), and a single dopaminergic neurone can modulate aggression in *Drosophila* (Alekseyenko et al., 2013), while fighting behaviour in hermit crabs can increase serotonin and decrease dopamine (Briffa and Elwood, 2007). Despite these findings, the role of dopamine in crayfish agonistic behaviours is still unclear. In this study, we examined the effect of dopamine on agonistic bouts in crayfish using pharmacological behavioural analyses and found that dopamine has serotonin-like and octopamine-like opposing actions relative to dose.

Evidence of functional interplays between biogenic amines has been reported in mammalian and invertebrate learning and memory processes (Wong et al., 1995; Sasaki-Adams and Kelly, 2001). In *Drosophila*, for example, octopamine-dependent reinforcement requires interactions with dopamine neurones that control appetitive motivation (Burke et al., 2012). Furthermore, serotonergic neurones control the activity of dopaminergic neurones in long-term memory formation (Kasture et al., 2018). However, there have been no attempts to clarify the functional interplay of biogenic amines in agonistic behaviours. We therefore examined both dopamine–octopamine and dopamine–serotonin interactions via co-injection of receptor antagonists in various combinations.

MATERIALS AND METHODS

Animals

Adult male crayfish *Procambarus clarkii* (Girard 1852) (6–9 cm body length from rostrum to telson) were used in all experiments. Individuals were purchased from a commercial supplier in Okayama, Japan, and maintained individually in separate 19×33×15 cm (width×length×height) opaque containers filled with water to a depth of 10 cm under a 12 h:12 h light:dark photoperiod cycle for at least 3 weeks. Each crayfish was fed equal amounts of small food pellets (Kyorinn, Japan) once a week. Experimental trials were performed in dim light conditions at a room temperature of approximately 23°C. Crayfish with damaged legs or that had moulted within the week before the experiments were not used in this study.

Pairing

Two crayfish with a length difference of 3–7% were selected and paired in a new 26×38×24 cm (width×length×height) opaque container filled about halfway with water. Prior to each pairing, an opaque plastic barrier was placed in the centre of the tank, separating it into two areas. A single crayfish was placed on each side of this

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barrier and allowed to acclimate for at least 10 min before the divider was removed. The agonistic bouts were recorded for 45 min using a video camera (Victor GZ-MG330-S, Japan) mounted on a tripod above the container. Crayfish behaviour was analysed frame-by-frame to construct an ethogram for every second of the encounter. After releasing the divider, fighting was recorded as soon as one crayfish approached and made contact with its opponent. The winner–loser relationship was determined after 15–30 min, a period that included several fights. Change in orientation from approach to attack was an evident characteristic of a dominant crayfish (Watanabe et al., 2016) and submissive crayfish retreated or tailflipped following an attack by a dominant crayfish and engaged in no further fights (Sato and Nagayama, 2012). After 45 min of pairing, dominant and subordinate crayfish were re-isolated separately in the stock container for a second pairing session the following day. The video tapes were analyzed by individuals who were blind to the drug(s) that had been administered.

Drug injection

Dopamine hydrochloride (DA), serotonin creatinine sulphate monohydrate (5HT), (±)-octopamine hydrochloride (OA), and their receptor antagonists methylergonovine maleate salt (methergine) as the non-specific dopamine receptor antagonist, R(+)-SCH-23390 hydrochloride (SCH23390) as the dopamine D1 receptor antagonist, chlorpromazine hydrochloride (chlorpromazine) as the dopamine D2 receptor antagonist, WAY-100635 maleate salt (WAY100635) as the serotonin 5HT1 receptor antagonist, and epinastine hydrochloride (epinastine) as the octopamine receptor antagonist were obtained from Sigma-Aldrich (St Louis, MO, USA) and dissolved in physiological saline (van Harreveld, 1936) to make up the required concentrations prior to each experiment. The concentration of each drug was determined from Shiratori et al. (2017) for DA, SCH23390 and chlorpromazine; from Momohara et al. (2016) for 5HT, OA and WAY100635; and from Momohara et al. (2018) for epinastine. The concentration of methergine was determined by our preliminary observation that 10 was the maximum concentration that resulted in no postural or behavioural changes in the animals. Drugs (1 ml in volume) were injected using a 27 gauge (20 mm) needle into the pericardial sinus from the dorsal carapace within the caudal third of the pericard, to avoid damaging the underlying heart. This injection point was determined as in Alcaro et al. (2011). The carapace expanded fully after injection of drugs into the pericardial cavity, then extra fluid leaked out immediately through the gills, returning the carapace to its original shape within 1 or 2 min.

Effect of dopamine application to naive animals

Two naive animals with a length difference of 3–7% were paired. Naive crayfish were defined as isolated newcomers with no previous pairing in the past 3 weeks. Larger or smaller naive animals were injected with either physiological saline or 0.5, 1, 2, 5 or 10 µmol l^{-1} dopamine 15 min prior to pairings against untreated small or large naive opponents, respectively. The winning rate of drug-treated animals was compared with that of saline-injected animals as controls. Number of pairings, size of animals and concentration of drugs are summarized in Table S1.

Effect of dopamine receptor antagonist on dominant or subordinate animals

Two naive animals with similar body length were paired to establish winner and loser relationships, then re-isolated individually in separate containers overnight. The following day, dominant animals were paired with larger naive animals, and subordinate animals were paired with smaller naive opponents. Dominant or subordinate animals were injected with physiological saline, 10 µmol l^{-1} methergine, 10 µmol l^{-1} SCH23390 or 10 µmol l^{-1} chlorpromazine immediately after establishing the winner and loser relationship. The winning rates of drug-treated animals were compared with untreated dominant or subordinate animals (controls). Number of pairings, size of animals and concentration of drugs are summarized in Table S2.

Interplay between dopamine and octopamine

Dopamine (1 µmol l^{-1}) or octopamine (1 µmol l^{-1}) with or without co-injection of either dopamine or octopamine receptor antagonists (10 µmol l^{-1} SCH23390 or 12.5 µmol l^{-1} epinastine, respectively) were injected into naive large animals 15 min prior to pairings with smaller naive opponents. The winning rates of animals co-injected with the receptor antagonists were compared with that of large animals injected with only dopamine or octopamine (controls). Number of pairings, size of animals and concentration of drugs are summarized in Table S3. One pairing between 1 µmol l^{-1} dopamine-injected large naive animal and untreated small naive animal was added for analyses of experiment 3 to prevent the usage of the same data from experiment 1.

Interplay between dopamine and serotonin

Dopamine (10 µmol l^{-1}) or serotonin (1 µmol l^{-1}) with or without co-injection of either dopamine or serotonin receptor antagonists (10 µmol l^{-1} chlorpromazine or 50 µmol l^{-1} WAY100635, respectively) were injected into naive small animals 15 min prior to pairings with larger naive animals. The winning rates of animals co-injected with the receptor antagonists were compared with that of small animals solely injected with dopamine or serotonin as controls. Number of pairings, size of animals and concentration of drugs are summarized in Table S4. One pairing between 10 µmol l^{-1} dopamine-injected naive small animal and untreated naive large animal was added for analyses of experiment 4 to prevent the usage of the same data from experiment 1.

Statistical analyses

The win rate was determined by the number of animals that won pairings / total number of pairings. The win rate within paired animals was analysed by binomial tests. Multiple comparisons of the differences in win rates among drug-treated groups compared with controls were performed using Fisher's exact test after a Bonferroni correction was applied to the alpha, with resulting significance levels of 0.001 (0.05/5), 0.0125 (0.05/4), 0.0166 (0.05/3) and 0.025 (0.05/2). The time in which the dominant–subordinate relationship was determined was analysed using a survival log rank test. Multiple comparisons of the number of fights and average duration of individual fights were analysed using ANOVA on Ranks and a Student's *t*-test if data were normally distributed, or a Mann–Whitney rank sum test if not after a Bonferroni correction was applied to the alpha, with resulting significance levels of 0.001 (0.05/5), 0.0125 (0.05/4), 0.0166 (0.05/3) and 0.025 (0.05/2). Differences in the number of attacks and retreats/tailflips between paired animals were compared using a Mann–Whitney Rank Sum Test. All statistical analyses were carried out using SigmaPlot v14.

RESULTS

Effect of dopamine injection on naive animals

When large and small naive crayfish with a length difference of 3–7% were paired, 25 large animals out of 32 pairings won, while small animals won only 5 pairings. Large animals had a win rate of

Journal of Experimental Biology (2021) 224, jeb242057. doi:10.1242/jeb.242057

78% and were deemed more likely to win (P=0.021; binomial test). When physiological saline was injected into large animals prior to pairings with small animals, 15 large animals won in 19 pairings (79%). Saline-injected large animals were also more likely to win (P=0.019; binomial test) and no statistical difference was found when comparing these with untreated large animals. Instead of saline, 0.5, 1, 2, 5 or 10 µmol l^{-1} dopamine was then injected into large animals 10 min prior to pairings with small animals (Fig. 1A). For 0.5 µmol l^{-1} dopamine injections into large animals, the win rate reduced to 40% (n=4 out of 10 pairings). Injection of 1 µmol l^{-1} dopamine further reduced their win rate to 17%; only two large animals won in 12 pairings with small opponents. Small opponents were more likely to win (P=0.0386; binomial test). The win rate of large animals increased to 40% (n=4 out of 10 pairings) for 2 µmol l^{-1} dopamine, 70% (n=7 out of 10 pairings) for 5 µmol l^{-1} dopamine, and 82% (n=9 out of 11 pairings) for the 10 µmol l^{-1} dopamine injections. Statistically, the win rate of 1 µmol l^{-1} dopamine-injected large animals was significantly lower than saline-injected controls (P=0.0003; Fisher's exact test after Bonferroni correction was applied to the alpha and the significance level set to 0.01=0.05/5).

Similarly to the results for non-treated small animals, the win rate of saline-injected small animals was also low (21%: n=4 out of 19 pairings). Larger opponents were more likely to win in these pairings (P=0.0192; binomial test). Fig. 1B shows the win rate of small animals with and without dopamine injections. The win rate of the 1 µmol l^{-1} dopamine-injected small animals was still low at 20% (n=1 out of 5 pairings). A 5 µmol l^{-1} dopamine injection increased the win rate to 40% (n=4 out of 10 pairings). Small animals were more likely to win when 10 µmol l^{-1} dopamine was injected (P=0.0386; binomial test). Ten small animals won in 12 pairings, with a win rate of 83%, which was statistically higher than saline-injected small animals (P=0.0001; Fisher's exact test after a Bonferroni correction was applied to the alpha resulting in a significance level set to 0.0167=0.05/3).

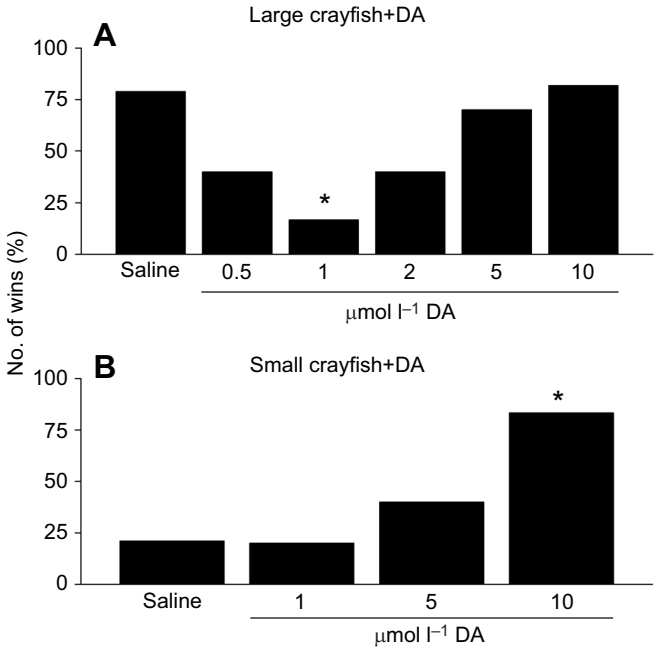

Fig. 1. Effect of dopamine on percentage win rate in encounters between small and large crayfish. Saline or 0.5, 1, 2, 5 or 10 µmol l^{-1} dopamine (DA) was injected into (A) naive large animals 15 min prior to pairings with naive small opponents or (B) naive small animals 15 min prior to pairings with naive large opponents, and their percentage win rate was plotted. Asterisks indicate significant difference in percentage of wins compared with saline-injected animals as controls using Fisher's exact test after applying a Bonferroni correction to the alpha, leading to a significance level P=0.0083 in A and P=0.0125 in B.

Any physical advantage of the large animals was lost by injection of low concentration of dopamine since 1 µmol l^{-1} dopamine-injected large animals were frequently beaten by small opponents. On the other hand, injection of high concentration of dopamine facilitated aggressive motivation since 10 µmol l^{-1} dopamine-injected small animals frequently won against larger opponents. Accordingly, aggressive motivations of the treated animals were estimated by an 'aggression index', which was determined by the total number of attacks/total number of both retreats and tailflips. The number of attacks of both the dopamine-injected large animals and non-treated small opponents (Fig. 2A) and that of both the dopamine-injected small animals and non-treated large opponents were compared (Fig. 2B). Furthermore, the total number of both retreats and tailflips in pairing animals was also compared (Fig. 2C,D). Saline-injected large animals showed more attacks than their small opponents (Fig. 2A) and the total number of retreats and tailflips was lower than that of small opponents (Fig. 2C). There was statistically no significant difference in the number of attacks (P=0.076; Mann–Whitney Rank Sum Test), but there was a significant difference in the number of retreats and tailflips (P=0.019; Mann–Whitney Rank Sum Test). The number of attacks in the large animals decreased when dopamine of 0.5 or 1 µmol l^{-1} was injected, and, at the same time, the number of retreats and tailflips increased (Fig. 2A,C). In 1 µmol l^{-1} dopamine-injected large animals, the number of attacks was statistically significantly lower than that of the small opponents (P=0.038; Mann–Whitney Rank Sum Test) and the number of retreats and tailflips was significantly higher than that of small opponents (P=0.023; Mann–Whitney Rank Sum Test). By contrast, when 10 µmol l^{-1} dopamine was injected, the number of attacks of both the large and small animals increased significantly in comparison to that of the opponents (P=0.012 between 10 µmol l^{-1} dopamine-injected large animals and small opponents in Fig. 2A, and P=0.010 between 10 µmol l^{-1} dopamine-injected small animals and large opponents in Fig. 2B; Mann–Whitney Rank Sum Test). Furthermore, the number of retreats and tailflips of the opponents against 10 µmol l^{-1} dopamine-injected animals increased significantly (P=0.006 between 10 µmol l^{-1} dopamine-injected large animals and small opponents in Fig. 2C, and P=0.018 between 10 µmol l^{-1} dopamine-injected small animals and large opponents in Fig. 2D; Mann–Whitney Rank Sum Test). Furthermore, in the pairings between 5 µmol l^{-1} dopamine-injected large animals and non-treated small opponents, the number of retreats and tailflips of the dopamine-injected large animals was lower than that of the small opponents (P=0.017; Mann–Whitney Rank Sum Test). The aggression index of saline-injected naive large animals was 4.2 (Fig. 2E) while that of saline-injected naive small animals was 0.2 (Fig. 2F). In dopamine-injected animals, the aggression index of low concentration dopamine-injected animals was frequently lower than 1.0. The aggression index of naive large animals was 0.3 after 0.5 µmol l^{-1} dopamine injection, 0.06 with 1 µmol l^{-1} dopamine injection and 0.9 for 2 µmol l^{-1} dopamine injection (Fig. 2E). Furthermore, the aggression index of naive small animals was 0.4 when 1 µmol l^{-1} dopamine was injected (Fig. 2F). On the other hand, injection of a higher concentration of dopamine increased the aggression index in the large as well as small animals: 2.3 in naive large animals after 5 µmol l^{-1} dopamine injection and 10.9 with 10 µmol l^{-1} dopamine injection (Fig. 2E); and 1.2 in naive small

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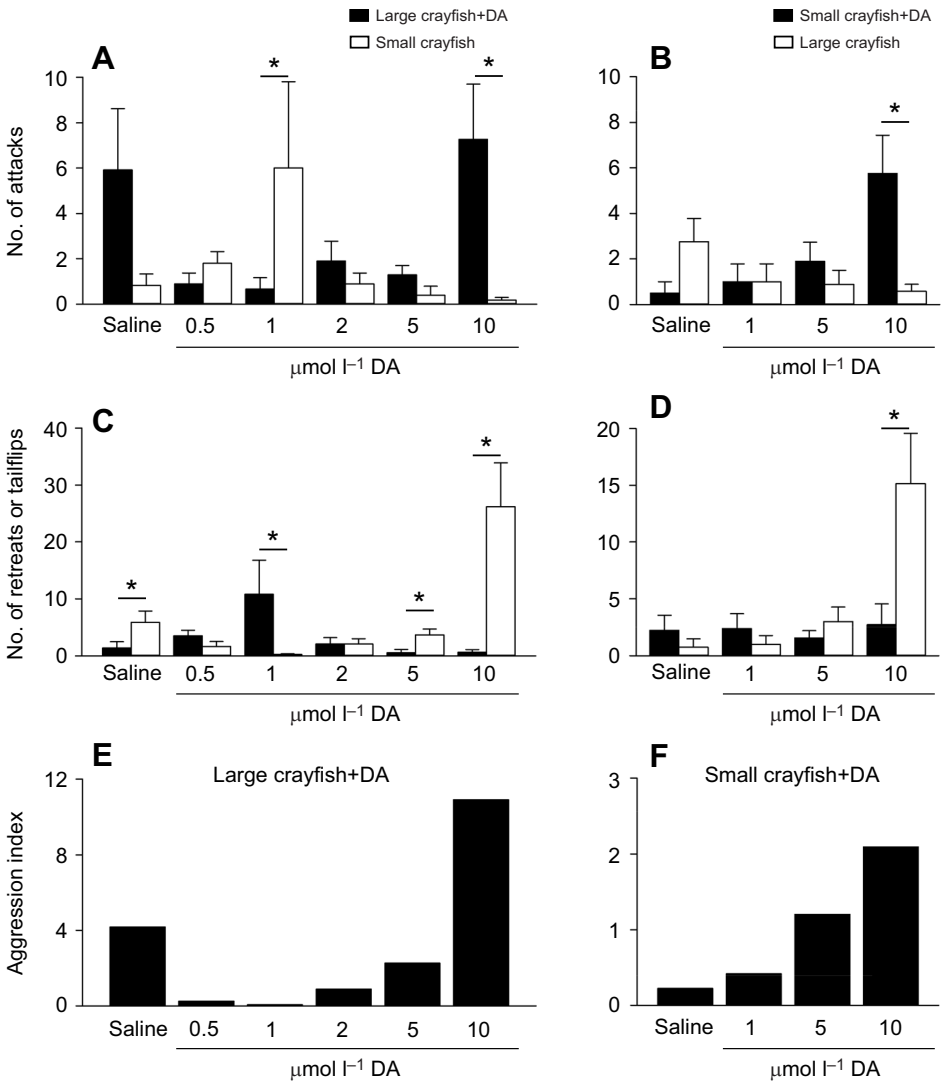

Fig. 2. Comparison of agonistic behaviour in pairings between dopamine-injected crayfish and their opponents. Number of attacks (A) and retreats (C) made by dopamine-injected naive large animals (filled bars) and opponent naive small animals (open bars). Number of attacks (B) and retreats (C) made by dopamine-injected naive small animals (filled bars) and opponent naive large animals (open bars). Asterisks indicate significant difference (P<0.05) between dopamine-injected animals and their untreated opponents using Mann–Whitney Rank Sum Test. Aggression index of (E) dopamine-injected naive large animals that were paired with naive small animals or (F) dopamine-injected naive small animals that were paired with naive large animals. Aggression index was determined as the total number of attacks/the total number of retreats or tailflips.

animals with 5 μmol l^{-1} dopamine injection and 2.1 for 10 μmol l^{-1} dopamine injection (Fig. 2F).

In the pairings between naive large and small animals, the time in which the dominant–subordinate relationship was determined was variable from 4 min to 38 min, with average time of 16 ±2.5 min (n=30; mean±s.d.). In saline-injected large and small animals (controls), dominant–subordinate relationships were also determined within ∼16 min (Fig. 3A,B). Although the decision time to establish the dominant–subordinate relationship was about 15 min in 10 μmol l^{-1} dopamine-injected large animals (Fig. 3A) and about 16 min in 10 μmol l^{-1} dopamine-injected small animals (Fig. 3B), dopamine of lower concentration tended to lead to a longer decision time of 20–25 min (Fig. 3A,B). There were, however, no statistically significant differences from saline-injected animals (P=0.480 in naive large group and P=0.826 in naive small groups; Survival log rank test). The number of fights during agonistic bouts was plotted for dopamine-injected large animals (Fig. 3C) and small animals (Fig. 3D) and in both, the number of fights in 1 μmol l^{-1} dopamine-injected animals was lowest, but no significant difference was found by multiple comparison (P=0.018 against saline injected animals in naive large animals: Student's t-test after a Bonferroni correction was applied to the alpha resulting in a significance level set to 0.010=0.05/5, and P=0.190 in naive small animals; Mann–Whitney Rank Sum Test). In 10 μmol l^{-1} dopamine-injected naive large animals, the average duration of individual fights was longer than that of saline-injected animals (Fig. 3E). Multiple comparisons showed, however, no statistically significant difference (P=0.026; Mann–Whitney rank sum test after a Bonferroni correction was applied to the alpha, resulting in a significance level set to 0.01). The average duration of individual fights in the naive small animals with 1 μmol l^{-1} dopamine injection decreased significantly from that of saline-injected animals (P=0.0160; Student's t-test after a Bonferroni correction was applied to the alpha resulting in a significance level set to 0.0167=0.05/3) (Fig. 3F).

Effect of dopamine receptor antagonists on winner and loser effects

After acquiring their dominant status, dominant small animals frequently won against larger naive opponents, a phenomenon known as the winner effect. In 26 pairings between dominant small and naive large animals, 18 dominant small animals won, resulting in a win rate of 70% (Fig. 4A). Physiological saline with or without a particular dopamine receptor antagonist was injected immediately after animals acquired their dominant state, after which they were paired with naive large opponents the following day to confirm the

Journal of Experimental Biology (2021) 224, jeb242057. doi:10.1242/jeb.242057

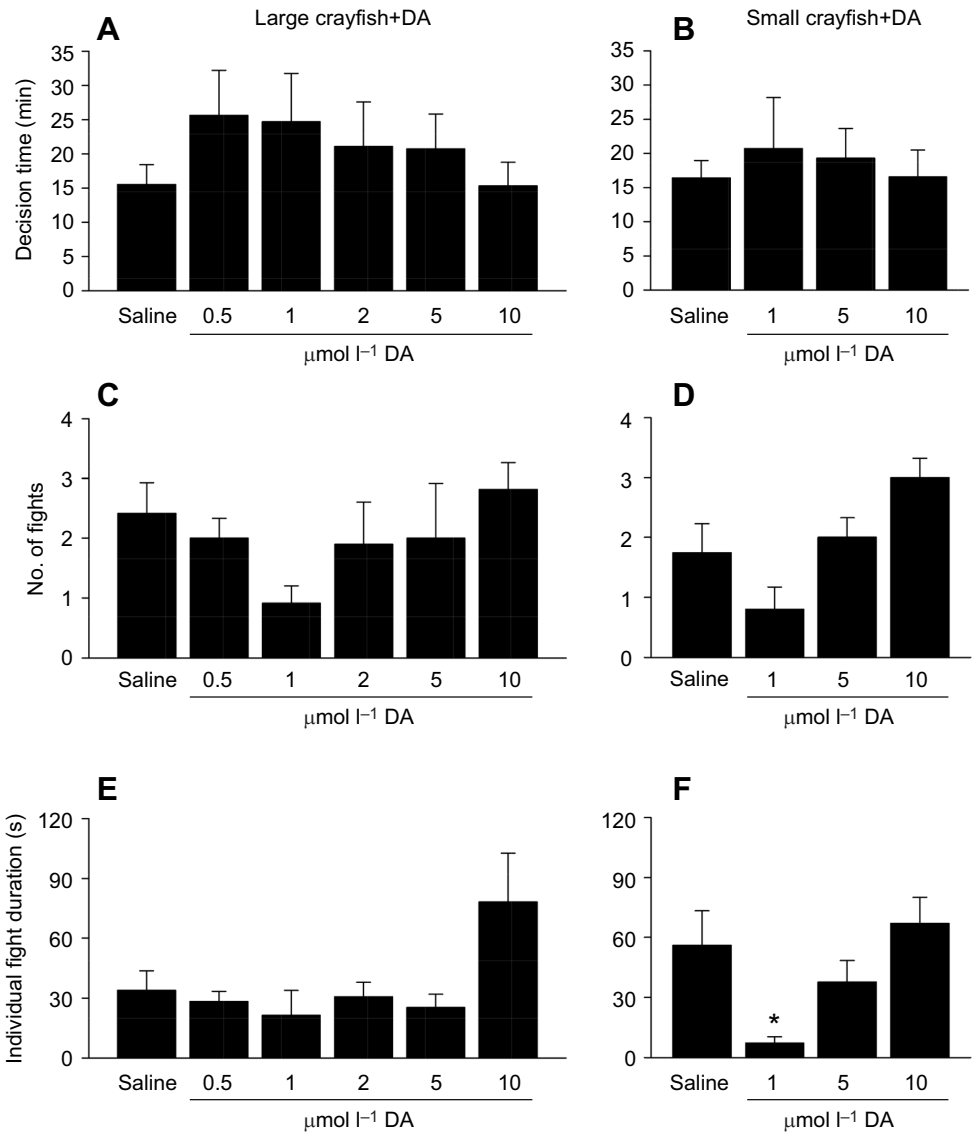

Fig. 3. Effect of dopamine injection on agonistic bouts between large and small crayfish. The time in which the dominant–subordinate relationship was determined when different concentrations of dopamine (DA) were injected into (A) naive large animals and (B) naive small animals. The number of fights of pairings between (C) dopamine-injected naive large and untreated naive small animals or (D) dopamine-injected naive small and untreated naive large animals for 45 min agonistic bouts. Mean duration of individual fights of pairings between (E) dopamine-injected naive large and untreated naive small animals or (F) dopamine-injected naive small and untreated naive large animals for 45 min agonistic bouts. Asterisk indicates significant difference from saline-injected animals as controls using Mann–Whitney Rank Sum Test after applying a Bonferroni correction to the alpha, leading to a significance level set to 0.0166.

establishment of the winner effect (Fig. 4A). Saline alone injected into dominant small animals led to a win rate of 73% (n=11 out of 15 pairings). The win rate of dominant small animals was reduced considerably when the non-specific dopamine receptor antagonist methergine or the D2 receptor antagonist chlorpromazine were injected. In both treatments, the win rate of dominant small animals was 20% (n=2 out of 10 pairings), which was statistically lower than in controls (P=0.0113; Fisher's exact test after a Bonferroni correction to the alpha set the significance level to 0.0125). In contrast, injection of the D1 receptor antagonist SCH23390 showed no significant impact. Eight dominant small animals won in 10 pairings with naive large opponents (P=0.6895; Fisher's exact test).

After losing in previous agonistic encounters, subordinate large animals were beaten by naive small opponents (Fig. 4B), a phenomenon known as the loser effect. In 17 pairings, subordinate large animals won only once. The win rate was 6% and naive small opponents were more likely to win (P=0.0003; binomial test). The win rate of subordinate large animals was still low at 8% (n=1 out of 13 pairings) following physiological saline injection and the tendency for naive small opponents to win was significant (P=0.0034; binomial test). In methergine-injected subordinate large animals, the win rate increased to 60% (n=6 out of 10 pairings). SCH23390 injection in subordinate animals also showed a win a rate of 60% (n=6 out of 10 pairings). Conversely, the win rate of subordinate animals was 9% (n=1 out of 11 pairings) following injection of chlorpromazine. The win rates of both methergine- and SCH23390-injected animals were statistically higher than in controls (P=0.0042; Fisher's exact test after a Bonferroni correction to the alpha set the significance level to 0.0125), whereas win rates after chlorpromazine injection did not differ from controls (P=1; Fisher's exact test).

The aggression index of dominant small animals with no treatment or saline injection was 3.5 (17 attacks/ 4.8 retreats or tailflips) and 4.6 (18.2/4), respectively (Fig. 4C). Aggressive motivation was high and enough to win against larger opponents owing to the achievement of the winner effect. The aggression index of D1 receptor antagonist SCH23390-injected animals was 2.1 (2.6 /1.2), while that of non-specific DA receptor antagonist methergine-injected animals was 0.3 (7.6 / 21.8) and D2 receptor antagonist chlorpromazine-injected animals was 0.1 (0.7/6.9) (Fig. 4C). Subordinate animals without drug treatment or with saline injection had a very low aggression index value of 0.05 (1.1/23 in subordinate large animals and 1.3/25.7 in saline-injected subordinate large animals) (Fig. 4D). Although they had a physical advantage, they were beaten by small opponents owing to

Journal of Experimental Biology (2021) 224, jeb242057. doi:10.1242/jeb.242057

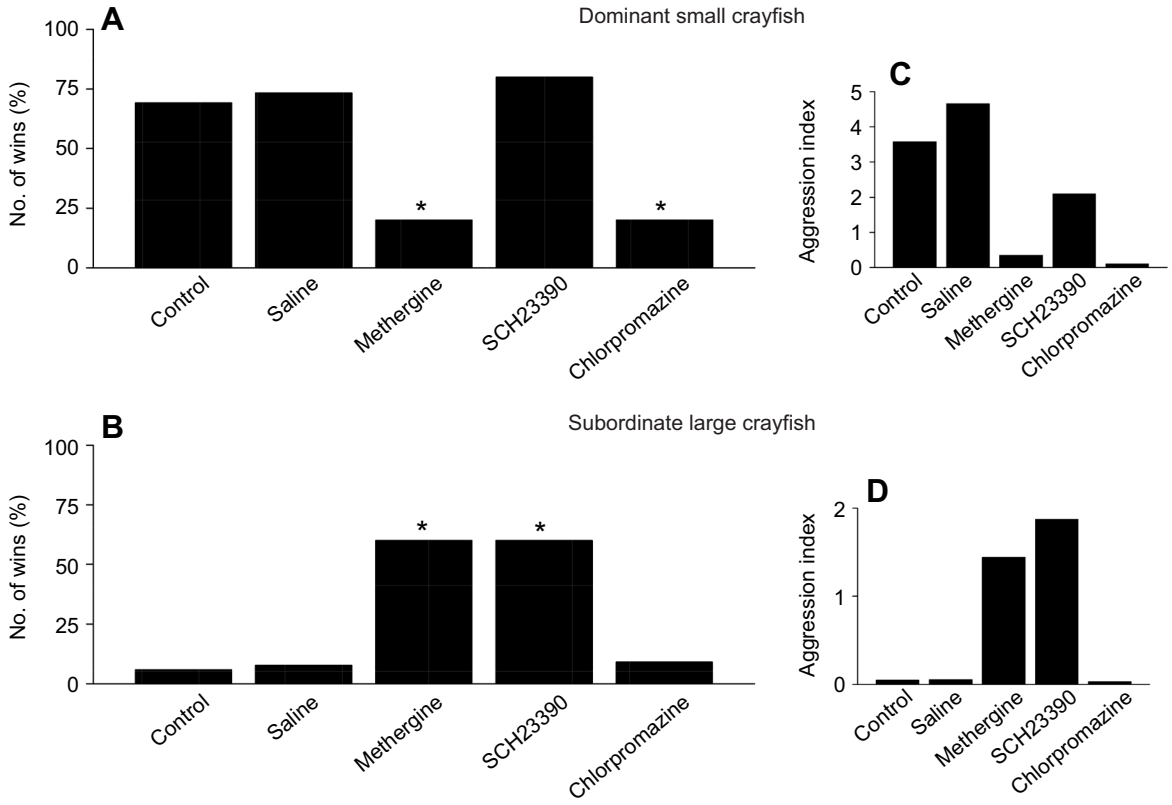

Fig. 4. Percentage of winning bouts in dominant small and subordinate large crayfish. Saline or one of three dopamine receptor antagonists were injected into (A) dominant small animals or (B) subordinate large animals immediately after establishing the winner–loser relationship, after which they were paired with naive large or small opponents, respectively, the following day. Asterisks indicate significant differences in win rates compared with controls using Fisher's exact test after applying a Bonferroni correction to the alpha, leading to a significance level set to 0.0125. Aggression index of dopamine receptor antagonist-injected (C) dominant small animals paired with naive large animals or (D) subordinate large animals paired with naive small animals. Methergine (=Methylergonovine) is a non-specific DA receptor antagonist, SCH23390 is a DA1 receptor antagonist and chlorpromazine is a D2 receptor antagonist. All controls are uninjected animals.

their low aggression index (Fig. 4B). The aggression index of chlorpromazine-injected subordinate animals was also 0.03 (0.2/6.5) while that of methergine-injected animals was 1.4 (2.6 /1.8) and SCH23390-injected animals was 1.9 (1.7/0.9).

The time in which the dominant–subordinate relationship was formed was similar in the dominant animals with or without drug treatments at 11–18 min (P=0.703; survival log rank test) (Fig. 5A). By contrast, decision time was significantly longer when dopamine receptor antagonists were injected into subordinate large animals (P<0.001; survival log rank test) (Fig. 5B). The number of fights and the average duration of individual fights in the dominant small animals (Fig. 5C) and those of subordinate large animals were plotted (Fig. 5D). The number of fights tended to decrease in the drug-treated animals of both dominant and subordinate status, but statistically, no significant differences were found among them by multiple comparisons (P=0.042 in methergine-injected animals, P=0.028 in SCH23390-injected animals and P=0.017 in chlorpromazine-injected animals against controls; Mann–Whitney rank sum test after a Bonferroni correction to the alpha set the significance level to 0.0125, and P=0.118 among subordinate large animals; ANOVA on Ranks). The average duration of individual fights was similar in all groups (P=0.365 in dominant small animals and P=0.531 in subordinate large animals; ANOVA on Ranks).

Interplay between dopamine and octopamine

As shown in Fig. 1A, 1 µmol l^{-1} dopamine-injected naive large animals were frequently beaten by naive small opponents. A similar inhibitory effect was previously observed when octopamine was injected into large crayfish (Momohara et al., 2013). We examined the interactions between dopamine and octopamine via co-injection of dopamine (Fig. 6A) or octopamine (Fig. 6B) with dopamine and octopamine receptor antagonists. To prevent the usage of same data of 1 µmol l^{-1} dopamine injection from Fig. 1A, we added one new pairing to the analysis. The win rate was reduced from 17% (Fig. 1A) to 15% (n=2 out of 13 pairings) in Fig. 5A. Co-injection of the D1 receptor antagonist SCH23390 with 1 µmol l^{-1} dopamine into large animals increased their win rate in pairings with small opponents (n=9 out of 13 pairings), which was statistically higher than solely dopamine-injected controls (n=2 out of 13 pairings) (P=0.0154; Fisher's exact test after a Bonferroni correction was applied to the alpha, setting the significance level to 0.025=0.05/2). The win rate of large animals after co-injection of 1 µmol l^{-1} dopamine and the octopamine receptor antagonist epinastine was 27% (n=3 out of 11 pairings), which was not statistically different from controls (P=0.6299; Fisher's exact test).

The win rate of large animals following injection of 1 µmol l^{-1} octopamine only was 22% (n=4 out of 14 pairings), with small opponents more likely to win (P=0.0309; binomial test). Co-injection of the octopamine receptor antagonist epinastine with octopamine into large animals increased their win rate to 83% (n=5 out of 6 pairings). The win rate of large animals following co-injection of octopamine with SCH23390 also increased to 67% (n=8 out of 12 pairings). The win rate of octopamine-injected large animals with co-injection of epinastine or SCH23390 was

Journal of Experimental Biology (2021) 224, jeb242057. doi:10.1242/jeb.242057

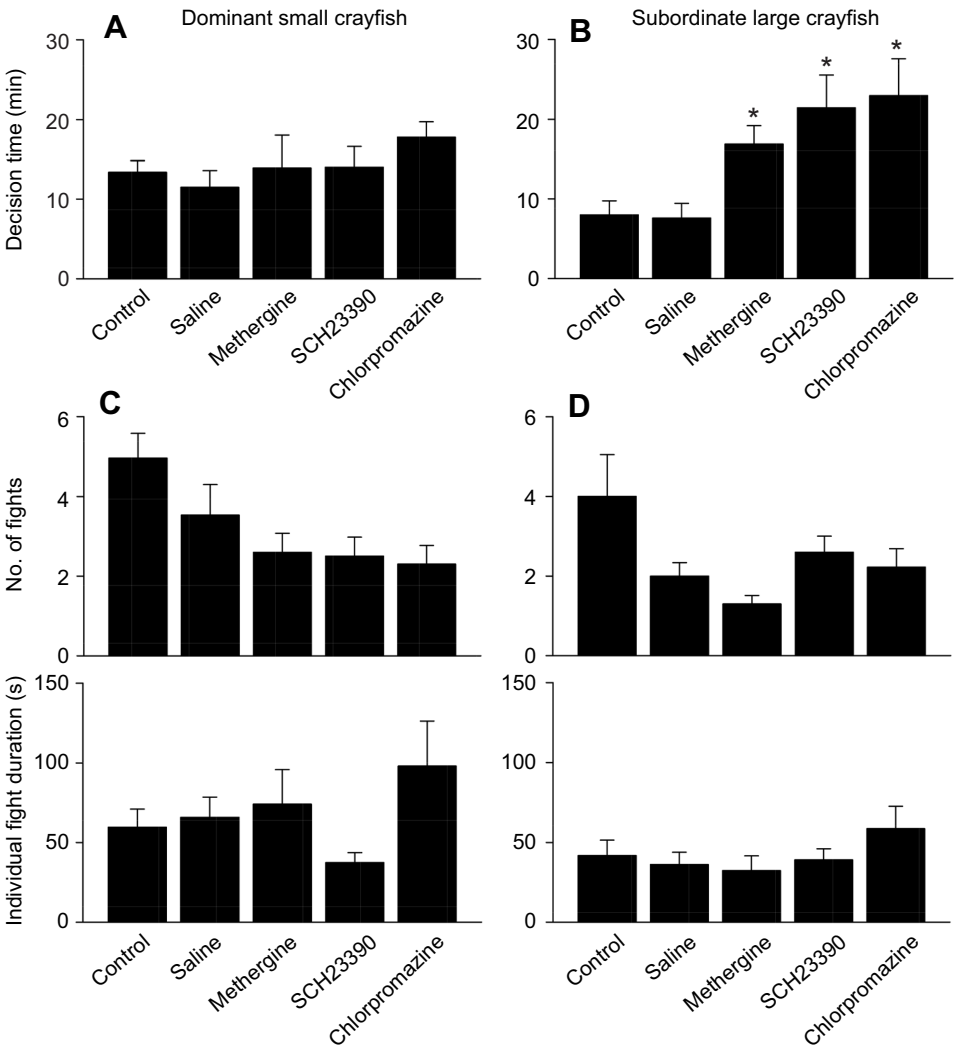

Fig. 5. Comparison of agonistic behavioural acts of dopamine receptor antagonist-injected dominant and subordinate crayfish. The time within which the dominant–subordinate relationship was determined when various dopamine receptor antagonists were injected into (A) dominant small (dom S) animals or (B) subordinate large animals. Asterisks indicate significant difference (P<0.05) from controls using survival log rank test. The number of fights (top panels) and the average duration of individual fights (bottom panels) of the pairings between dopamine receptor antagonist-injected (C) dominant small animals and naive large animals or (D) submissive large animals and naive small animals for 45 min agonistic bouts.

statistically higher than controls (P=0.0147 with co-injection of epinastine and P=0.0243 with co-injection of SCH23390; Fisher's exact test after a Bonferroni correction to the alpha set the significance level to 0.025).

The aggression index of 1 µmol l^{-1} dopamine-injected naive large animals was 0.06 (0.6/10) (Fig. 6C) and that of co-injection of epinastine with 1 µmol l^{-1} dopamine was 0.2 (0.7/4.1). After co-injection of 1 µmol l^{-1} dopamine with SCH23390, the aggression index increased to 3.6 (3.6/1.0) suggesting that SCH23390 blocked the inhibitory action of 1 µmol l^{-1} dopamine. The aggression index of 1 µmol l^{-1} octopamine-injected naive large animals was 0.1 (0.5/4.2) (Fig. 6D). The index increased to 2.5 (2.5/1.0) after co-injection of octopamine with epinastine and also increased to 2.6 (3.1/1.2) after co-injection of octopamine with SCH23390 (Fig. 6D).

As shown in Fig. 7A, the time in which social hierarchy was established in 1 µmol l^{-1} dopamine-injected animals was similar with and without co-injection of antagonists (P=0.189; survival log rank test). Furthermore, the time to form hierarchy in 1 µmol l^{-1} octopamine-injected animals was also similar with and without co-injection of antagonists (P=0.268; survival log rank test) (Fig. 7B). The number of fights and the average duration of individual fights in the naive large animals after co-injection of 1 µmol l^{-1} dopamine with SCH23390 was higher than those of controls (P<0.001 for fight number and P=0.001 in individual fight duration; Mann–Whitney rank sum test after a Bonferroni correction was applied to the alpha resulting in a significance level set to 0.025) (Fig. 7C). In 1 µmol l^{-1} octopamine-injected naive large animals (Fig. 7D), the number of fights tended to decrease when receptor antagonists were injected simultaneously, but no significant differences were found in comparison to levels in controls (P=0.063; ANOVA on Ranks). No statistical difference was also found for the average duration of individual fights when comparing with controls (P=0.054; ANOVA on Ranks).

Interplay between dopamine and serotonin

Injections of either 1 µmol l^{-1} serotonin or 10 µmol l^{-1} dopamine into naive small animals increased their win rate in pairings with naive large animals. To prevent the use of same data for 10 µmol l^{-1} dopamine injection as in Fig. 1B, we added one new pairing. The win rate of 10 µmol l^{-1} dopamine-injected small animals was 85% (n=11 out of 13 pairings), which was slightly increased from 83% in Fig. 1A. The win rate decreased to 17% with co-injection of the D2 receptor antagonist chlorpromazine (n=1 out of 6 pairings) and 30% (n=3 out of 10 pairings) with co-injection of the serotonin 5HT1 receptor antagonist WAY100635 (Fig. 8A). After co-injection of chlorpromazine and WAY100635, the win rate of small animals was statistically lower than in controls (P=0.0095 with co-injection of

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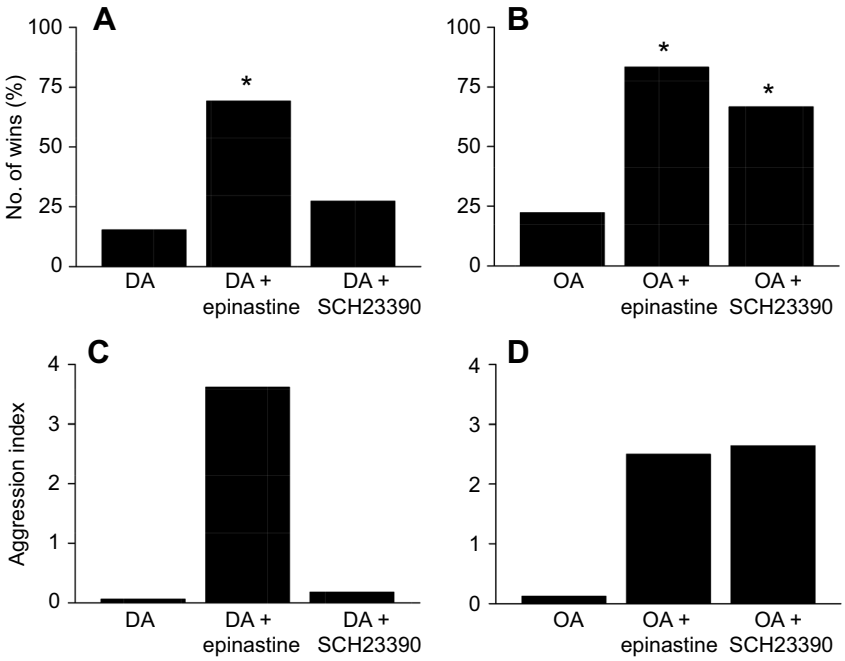

Fig. 6. Percentage of winning bouts in dopamine- or octopamine-injected naive large animals. Win rate of naive large animals injected with (A) 1 µmol l^{-1} dopamine (DA) or (B) 1 µmol l^{-1} octopamine (OA) with or without co-injection of dopamine or octopamine receptor antagonist when paired with naive small opponents. Asterisks indicate significant differences in win rates compared with DA or OA controls using Fisher's exact test after a Bonferroni correction was applied to the alpha, setting the significance level to 0.025. Aggression index of naive large animals injected with (C) 1 µmol l^{-1} dopamine or (D) 1 µmol l^{-1} octopamine with and without receptor antagonist. SCH23390 is a DA1 receptor antagonist and epinastine is an OA receptor antagonist.

chlorpromazine and P=0.0131 with co-injection of WAY100635; Fisher's exact test after a Bonferroni correction to the alpha set the significance level to 0.025).

In pairings with naive large animals, injection of 1 µmol l^{-1} serotonin into naive small animals increased their win rate to 67% (n=12 out of 18 pairings). Their win rate reduced to 33% (n=4 out of 12 pairings) when WAY100635 was injected simultaneously, and decreased to 10% with co-injection of chlorpromazine (Fig. 8B). Statistically, the win rate of small animals with co-injection of serotonin and chlorpromazine was lower than that of controls (P=0.0060; Fisher's exact test after a Bonferroni correction to the alpha set the significance level to 0.025), whereas the win rate with WAY100635 co-injection did not differ significantly from controls (P=0.1349; Fisher's exact test).

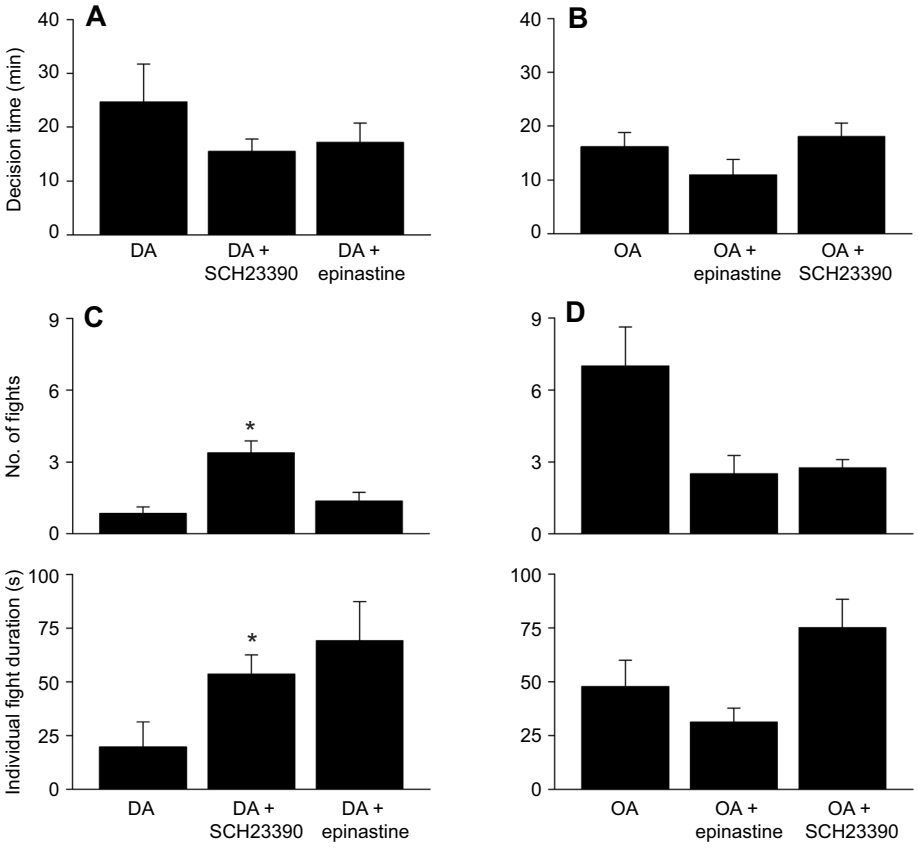

Fig. 7. Comparison of agonistic behavioural acts of dopamine- or octopamine-injected naive large animals with or without co-injection of receptor antagonists. (A) The time in which the dominant–subordinate relationship was determined when (A) 1 µmol l^{-1} dopamine or (B) 1 µmol l^{-1} octopamine was injected with or without DA or OA receptor antagonist into naive large animals. The number of fights (top panels) and the average duration of individual fights (bottom panels) of pairings between naive large animals injected with (C) dopamine or (D) octopamine with or without receptor antagonist and naive small opponents for 45 min agonistic bouts. Asterisks indicate significant difference (P<0.05) from controls using Mann–Whitney rank sum test after a Bonferroni correction was applied to the alpha, setting the significance level to 0.025.

Journal of Experimental Biology (2021) 224, jeb242057. doi:10.1242/jeb.242057

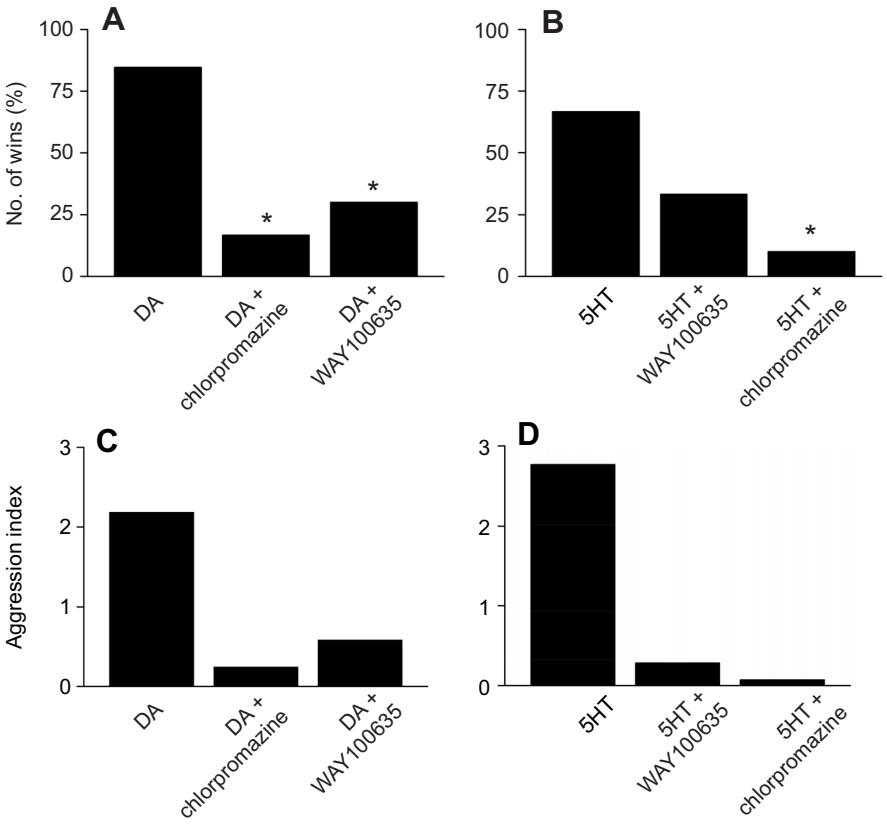

Fig. 8. Percentage of winning bouts in dopamine- or serotonin-injected naive small animals. Win rate of naive small animals injected with (A) 10 µmol l^{-1} dopamine (DA) or (B) 1 µmol l^{-1} serotonin (5HT) with or without co-injection of dopamine or serotonin receptor antagonist when paired with naive large opponents. Asterisks indicate significant differences in win rates compared with DA or 5HT controls using Fisher's exact test after a Bonferroni correction was applied to the alpha, setting the significance level to 0.025. Aggression index of naive small animals injected with (C) 10 µmol l^{-1} dopamine or (D) 1 µmol l^{-1} serotonin with and without receptor antagonist. chlorpromazine is a D2 receptor antagonist and WAY100635 is a 5HT1 receptor antagonist.

Fig. 8C,D shows the aggression index of 10 µmol l^{-1} dopamine-injected and 1 µmol l^{-1} serotonin-injected naive small animals, respectively. The aggression index of 10 µmol l^{-1} dopamine only injected animals was 2.2 (5.5/2.5), which decreased to 0.2 (1.2/4.8) by co-injection of D2 receptor antagonist chlorpromazine and to 0.6 (1.8/3.1) with co-injection of the 5HT1 receptor antagonist WAY100635 (Fig. 8C). Similarly, the aggression index of 1 µmol l^{-1} serotonin only injected animals was 2.8 (7.9/2.9) while after agonistic co-injection of WAY100635 or chlorpromazine it reduced to 0.3 (0.9/3.3) and 0.07 (0.3/4.3), respectively.

As shown in Fig. 9A, the time within which a dominant and subordinate relationship was established in 10 µmol l^{-1} dopamine-injected animals was similar with or without co-injection of antagonists (P=0.880; survival log rank test). By contrast, the decision time after co-injection of WAY100635 became shorter than that of 1 µmol l^{-1} serotonin-injected control animals (P=0.008; survival log rank test) (Fig. 9B). The number of fights and the average duration of individual fights in 10 µmol l^{-1} dopamine-injected small animals was similar regardless of whether antagonists were co-injected (P=0.189 in the number and P=0.239 in the duration; ANOVA on Ranks) (Fig. 9C). The average duration of individual fights in 1 µmol l^{-1} serotonin-injected small animals with and without antagonists were similar statistically (P=0.223; ANOVA on Ranks) while the number of fights decreased after co-injection of both 5HT1 and D2 receptor antagonists (Fig. 9D). The number of fights was statistically significantly lower with chlorpromazine (P=0.017; Mann–Whitney rank sum test after a Bonferroni correction to the alpha set the significance level to 0.025) and almost reached significance with WAY100635 (P=0.026: Mann–Whitney Rank Sum Test after a Bonferroni correction to the alpha set the significance level to 0.025).

DISCUSSION

We showed that large crayfish injected with 1 µmol l^{-1} dopamine prior to pairings were frequently beaten by smaller opponents despite their physical advantage. Furthermore, their aggression index was less than 1. Conversely, despite a physical disadvantage, 10 µmol l^{-1} dopamine-injected small crayfish increased their win rate and aggression index in pairings with large opponents. Furthermore, injection of 1 µmol l^{-1} dopamine induced the extension of decision time and the decrease in the number and duration of fights while 10 µmol l^{-1} dopamine increased the number and duration of fights. Monoamine crosstalk experiments suggested that different concentrations of dopamine had opposing effects and this may have implications for the interactions octopamine and serotonin with dopamine.

Dose-dependent opposing modulatory effects of dopamine

Synergistic and opposing effects of biogenic amines relative to dose concentrations have been shown in many animals. For example, low concentrations of serotonin enhance the synaptic response of LG interneurones in crayfish, but high concentrations of serotonin show an opposing inhibitory effect (Teshiba et al., 2001). In crayfish, injecting a low concentration of serotonin elicits anxiety-like reactions, while injecting a high concentration increases aggressive motivation (Kamada and Nagayama, 2021). In Chinese mitten crabs, injecting a low concentration of serotonin promotes agonistic behaviour, while a high concentration reduces it (Pang et al., 2019). Furthermore, in the lobster stomatogastric pyloric circuit, dopamine and octopamine can independently induce different motor patterns at different concentrations (Flamm and Harris-Warrick, 1986). In honeybee virgin queens, the dopamine antagonist *cis*-flupenthixol decreases fighting ability at low concentrations, while increasing it at high concentrations (Farkhary et al., 2017). Two types of

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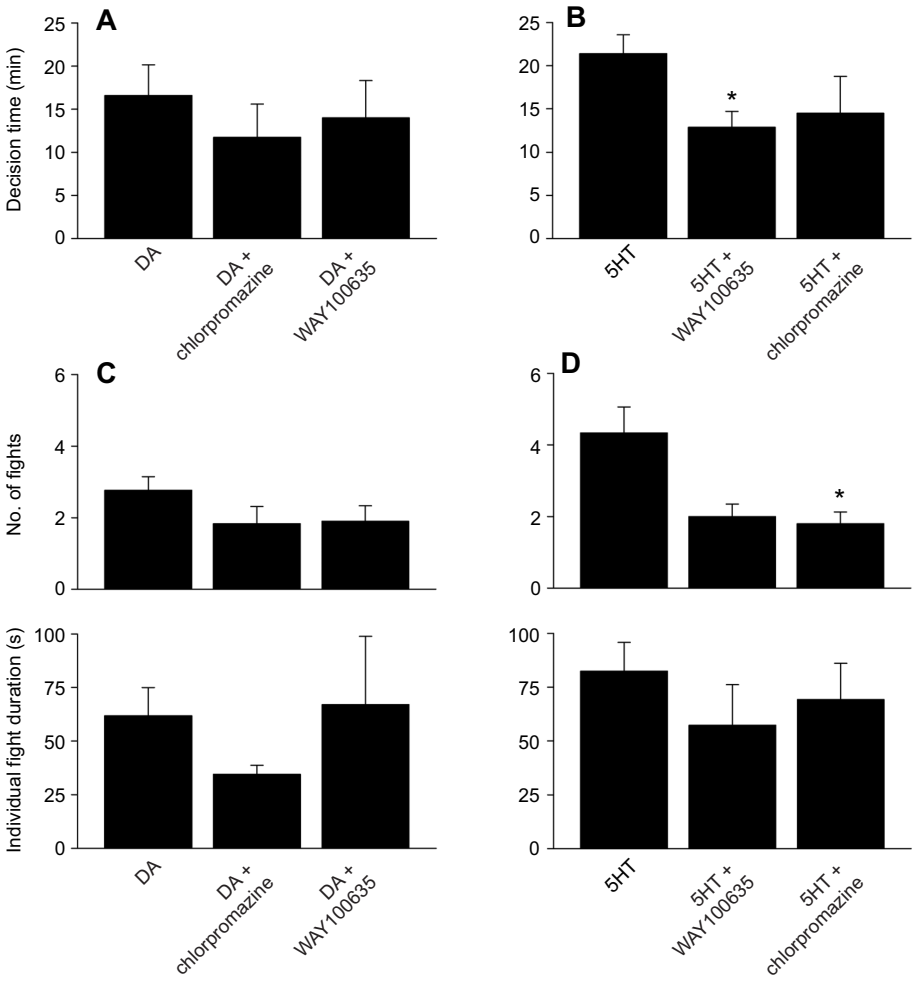

Fig. 9. Comparison of agonistic behavioural acts of dopamine- or serotonin-injected naive small animals with and without co-injection of receptor antagonist. (A) The time in which the dominant–subordinate relationship was determined when (A) 10 µmol l^{-1} dopamine or (B) 1 µmol l^{-1} serotonin was injected with or without DA or 5HT receptor antagonist into naive small animals. Asterisk indicates significant difference (P<0.05) from non-antagonist injected controls using survival log rank test. The number of fights (top panels) and the average duration of individual fights (bottom panels) of pairings between naive small animals injected with (C) dopamine or (D) serotonin, with or without receptor antagonist, and large opponents for 45 min agonistic bouts. Asterisks indicate significant difference (P<0.05) from controls using Mann–Whitney rank sum test after a Bonferroni correction was applied to the alpha, setting the significance level to 0.025.

anatomically distinct dopaminergic neurones provide appetitive and aversive learning in *Drosophila* (Waddell, 2013) and convey positive and negative motivational signals in monkeys (Matsumoto and Hikosaka, 2009).

Opposing functions of two dopamine receptors

Previous winning or losing experiences affect future fight outcomes, which is known as the winner or loser effect (Hsu et al., 2006). In crayfish agonistic bouts, the winner effect is defined by reduced cAMP levels mediated by serotonin 5HT1 receptors, while the loser effect is defined by an octopamine-mediated increase in cAMP levels (Momohara et al., 2016). In *Drosophila*, furthermore, different subtypes of the same amine can have different actions in the modulation of aggressive behaviours (Johnson et al., 2009). Dopamine receptors are classified into five subtypes in vertebrates (Missale et al., 1998) and two receptors, D1 and D2, have been characterised in crustaceans (Clark et al., 2008). The crustacean D1 receptor induces an increase in intracellular cAMP, while the D2 receptor can inhibit adenylyl cyclase, leading to reduced cAMP levels (Clark and Baro, 2006, 2007). Unfortunately, the selectivity of the employed D1 and D2 antagonists have been established in mammals, but not fully in invertebrates. In this study, however, injection of the dopamine D2 receptor antagonist chlorpromazine immediately after animals acquired their dominant state prevented achievement of the winner effect so that they were beaten by naive large opponents with a physical advantage on the following day. Conversely, the D1 receptor antagonist SCH23390 prevented formation of the loser effect but chlorpromazine did not. Therefore, DA antagonists used in this study are most likely acting selectively and our results are consistent with previous findings (Momohara et al., 2016), whereby D1 receptors were able to achieve the loser effect by increasing cAMP levels while D2 receptors mediated the winner effect by decreasing cAMP activity. Similar selective actions of the drugs on different DA receptors are reported in the marbled crayfish (Shiratori et al., 2017). Thus, dopamine, like serotonin and octopamine, regulates the elevation or reduction of cAMP levels to modulate aggressive motivation in crayfish. Further molecular analyses of crayfish dopamine receptors would help to elucidate the selective actions of dopamine receptors directly.

Since 1 µmol l^{-1} dopamine showed a similar effect to octopamine, while 10 µmol l^{-1} dopamine had effects similar to serotonin, it would be reasonable to assume that 1 µmol l^{-1} dopamine activates D1 receptors preferentially, while D2 receptors are strongly activated by 10 µmol l^{-1} dopamine. If the binding affinity of D2 receptors to dopamine is somewhat weaker than D1 receptors, sufficient binding may require high concentrations of dopamine, otherwise, the expression of D1 and D2 receptors may be biased. In the rat prefrontal cortex, the expression of D1 receptor mRNA is higher than D2 receptor mRNA (Santana et al., 2000). Serotonin 5HT1α and 2β receptors have been identified in crayfish (Spitzer et al., 2008) and 5HT1α receptor mRNA expression in dominant animals is higher than that in subordinate animals (Spitzer et al., 2005). Currently, it is not known whether the binding affinity

and/or expression of crayfish D1 and D2 receptors differ. Further quantitative molecular and pharmacological analyses are necessary to explore this point and clarify our conclusion.

Functional interplay between dopamine and other biogenic amines

Using a gas chromatography/mass spectrometry system, Sheddon et al. (2000) analysed serotonin, dopamine and octopamine concentrations in blood before and after agonistic behaviour of shore crabs, and showed a link between the relative concentrations of these amines and fighting ability. Furthermore, recent high performance liquid chromatography (HPLC) analyses suggest that the level of serotonin in the central nervous system increases in dominant crayfish (Bacqué-Cazenave et al., 2017; Momohara et al., 2018). Amine release and their functional interplay could be essential in regulating aggressive motivation in crayfish. Crosstalk among dopaminergic, serotonergic and octopaminergic systems can modulate behavioural outputs in animals. Interactions between dopamine and serotonin are found in both mammals and invertebrates (Wong et al., 1995; Sasaki-Adams and Kelly, 2001; Daw et al., 2002; Ishii et al., 2015; Kasture et al., 2018; Rillich and Stevenson, 2018). For example, a pair of serotonergic projection neurones in *Drosophila* trigger the oscillatory activity of dopamine neurones that mediates long-term olfactory memory formation in the mushroom body (Scheunemann et al., 2018). In rats, the activity of dopaminergic neurones in the ventral tegmental area is under the excitatory control of serotonin $5HT_{2A}$ receptors in the medial prefrontal cortex (Bortolozzi et al., 2005). In *Drosophila*, dopamine modulates the connectivity of serotonergic neurones to their target neurones in the mushroom body (Niens et al., 2017). Dopamine shows two distinct effects – blocking and enhancement – on serotonergic facilitation of synaptic response in crayfish LG interneurones, which depend on the duration of activation time of the dopaminergic pathways (Titlow, 2010). In the former two cases, serotonin modulates downstream dopaminergic neurones to increase dopamine release, while dopamine modulates downstream serotonergic pathways in the latter two cases. Moreover, octopamine-dependent memory formation in *Drosophila* requires signalling via dopamine neurones (Burke et al., 2012). Octopamine triggers an increase in intracellular calcium in dopamine neurones that controls appetitive motivation. In our study, the inhibitory effect of 1 µmol l^{-1} dopamine was maintained after co-injection of the octopamine receptor antagonist epinastine, whereas octopaminergic inhibition disappeared with co-injection of the dopamine D1 receptor antagonist SCH23390. These results strongly suggest that the dopaminergic system is downstream and that octopamine induces dopaminergic inhibition during agonistic behaviour. Conversely, the facilitating effect of 10 µmol l^{-1} dopamine disappeared after co-injection of the serotonin 5HT1 receptor antagonist WAY100635, while serotonergic facilitation was disturbed by co-injection of the dopamine D2 receptor antagonist chlorpromazine. Therefore, the dopaminergic and serotonergic systems could be activated in parallel with mutual, interacting pathways.

In crustaceans, biogenic amines function mainly as neurotransmitters and neuromodulators in the nervous system (Sneddon et al., 2000) and the distribution of amine-containing neurones has been mapped in the central nervous system (for review, see Beltz, 1999). In insects, the suboesophageal ganglion is known to be the integration centre for both courtship and aggressive behaviours (Zhou et al., 2008; Maeda et al., 2014; Tran et al., 2014). Cell bodies of many dopaminergic, serotonergic and octopaminergic neurones are positioned in the suboesophageal ganglion and their axons project anteriorly and posteriorly in the central nervous system of crayfish and lobsters (Beltz and Kravitz, 1983; Schneider et al., 1993; Tierney et al., 2003). Therefore, the suboesophageal ganglion could be a centre for orchestrating functional interplay among these amines. We observed previously that serotonin levels in the suboesophageal ganglion are much higher in dominant crayfish after winning (Momohara et al., 2018) and dopamine levels of dominant animals are higher than those of subordinate animals (K.I., personal observation). Further analyses are necessary to clarify the pathways and interactions of these biogenic amines using pharmacological and physiological techniques.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: T.N.; Methodology: K.I., T.N.; Investigation: K.I.; Writing - original draft: K.I., T.N.; Writing - review & editing: K.I., T.N.; Supervision: T.N.

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