

RESEARCH ARTICLE

cAMP signaling mediates behavioral flexibility and consolidation of social status in *Drosophila* aggression

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ABSTRACT

Social rituals, such as male–male aggression in *Drosophila*, are often stereotyped and the component behavioral patterns modular. The likelihood of transition from one behavioral pattern to another is malleable by experience and confers flexibility to the behavioral repertoire. Experience-dependent modification of innate aggressive behavior in flies alters fighting strategies during fights and establishes dominant–subordinate relationships. Dominance hierarchies resulting from agonistic encounters are consolidated to longer-lasting, social-status-dependent behavioral modifications, resulting in a robust loser effect. We showed that cAMP dynamics regulated by the calcium–calmodulin-dependent adenylyl cyclase, *Rut*, and the cAMP phosphodiesterase, *Dnc*, but not the *Amn* gene product, in specific neuronal groups of the mushroom body and central complex, mediate behavioral plasticity necessary to establish dominant–subordinate relationships. *rut* and *dnc* mutant flies were unable to alter fighting strategies and establish dominance relationships during agonistic interactions. This real-time flexibility during a fight was independent of changes in aggression levels. Longer-term consolidation of social status in the form of a loser effect, however, required additional *Amn*-dependent inputs to cAMP signaling and involved a circuit-level association between the α/β and γ neurons of the mushroom body. Our findings implicate cAMP signaling in mediating the plasticity of behavioral patterns in aggressive behavior and in the generation of a temporally stable memory trace that manifests as a loser effect.

KEY WORDS: Dominance hierarchy, Learning and memory, cAMP signaling, Experience-dependent plasticity, Neural circuits

INTRODUCTION

Aggression is a social behavior involving competitive interactions over resources to ensure reproductive success and survival. Conspecific agonistic interactions may result in dominance hierarchies, where those at higher levels have better access to resources. The social ritual of *Drosophila* aggression is modular and comprises stereotyped behavioral patterns analogous to a sequence of fixed action patterns. These sequences are present in full complexity in socially naive animals and appear to be pre-wired in the nervous system (Chen et al., 2002; Hoopfer, 2016; Lim et al., 2014). However, during aggressive encounters, experience can alter the likelihood of transitions between specific patterns and lead to experience-dependent plasticity (Trannoy et al., 2016; Yurkovic

et al., 2006). Initial agonistic interaction between naive flies constitutes a conditioning phase where male flies employ a combination of offensive and defensive fighting strategies and display real-time, experience-dependent plasticity to establish dominant or subordinate status (Chen et al., 2002; Yurkovic et al., 2006). In flies, the learned subordinate social status is consolidated into a long-lasting loser effect, resulting in an increased probability of losing agonistic encounters against familiar as well as unfamiliar opponents (Trannoy and Kravitz, 2017; Trannoy et al., 2016; Yurkovic et al., 2006).

Aggression is a complex social behavior influenced by a combinatorial interplay between genetic factors, environmental cues and experience. Population-level selection for elevated aggression has implicated several genes, which show significant changes in their expression (Dierick and Greenspan, 2006; Edwards et al., 2009; Wang et al., 2008). However, a single social defeat can lead to the development of a robust loser effect in such hyperaggressive lines, underscoring the modulatory role of experience in relation to intrinsic abilities (Penn et al., 2010). While regulatory activities of pheromones, neuromodulatory agents and neurotransmitters associated with aggression levels are well documented (Alekseyenko et al., 2013, 2014; Andrews et al., 2014; Certel et al., 2010; Dierick and Greenspan, 2007; Hoopfer, 2016; Hoyer et al., 2008; Kohl et al., 2015; Luo et al., 2014; Wang and Anderson, 2010; Wang et al., 2011; Yuan et al., 2014), little is known about the neurogenetic underpinnings of the relevant learning and memory components.

Social experience significantly alters the activity of flies and involves both classical and operant conditioning components (Kamyshev et al., 2002). Experience-dependent behavioral plasticity has also been observed in courtship conditioning assays where males, after an unsuccessful mating experience, modulate their courtship behavior (Siegel and Hall, 1979). Learning and memory in a social environment are likely to be influenced by several factors, including the combinatorial inputs from multiple sensory modalities. Consequently, central integration and interpretation of complex input modalities are likely to involve coordination between multiple neurogenetic circuits.

Previous studies have established the centrality of the cAMP pathway in the formation of operant and classical conditioned memories and in the integration of sensory inputs (Bragina and Kamyshev, 2003; Brembs, 2003; Busto et al., 2010; Gailey et al., 1984). Several mutations associated with defective learning and memory have been mapped to the genetic loci of cAMP pathway components (Dudai et al., 1976; Feany and Quinn, 1995; Levin et al., 1992; Livingstone et al., 1984). In this study, we implicate the cAMP second messenger pathway in behavioral plasticity during aggressive encounters and in the development of the loser effect. *rutabaga* (calcium–calmodulin-dependent adenylyl cyclase; *Rut*) and *dunce* (cAMP phosphodiesterase; *Dnc*) mutants showed compromised behavioral flexibility during agonistic encounters

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and were unable to establish dominance hierarchies. *ammesiac* (predicted to code for three putative neuropeptides; Amn) mutants, although competent in modifying behavioral patterns during fights and establishing hierarchies, showed no loser effect. This study demonstrates that *Rut* in specific neural circuits mediates behavioral flexibility leading to the establishment of dominance relationships and in the long-term consolidation of social status.

MATERIALS AND METHODS

Fly stocks and maintenance

Canton-S (CS), *rut*²⁰⁸⁰ and *amn*^{c651} lines were obtained from Professor R. Strauss, University of Mainz, Germany. *dnc*¹, *rut*²⁰⁸⁰, *UAS-Rut* and all Gal4 driver lines were obtained from Bloomington Stock Center, Bloomington, IN, USA. All fly lines were backcrossed for at least nine generations. The autosomes of the X-linked cAMP pathway mutant alleles used were equilibrated to that of the control strain. Stocks were maintained at 25°C, 60% humidity and a 14 h:10 h light:dark cycle on standard food.

Analysis of aggressive behavior

Freshly eclosed flies were isolated and kept in social isolation for a period of 4–5 days before testing. Acrylic paint marks on the upper thoracic region were used to identify individuals. Male–male aggression assays were conducted as described previously (Chen et al., 2002) with the modification that they were conducted in a six-well chamber. All experiments were conducted between Zeitgeber Time (ZT) 0 and ZT 6 unless otherwise specified. A food cup with yeast paste and a headless female was placed inside a six-well plate chamber. A pair of marked, un-anesthetized, age- and size-matched male flies was introduced into the chamber through gentle aspiration. All fights were conducted at 25°C and 60% humidity and recorded using a Sony DCR-SR47E/S video camera (Sony Corporation, Minato, Tokyo, Japan) at 50 Hz. Fights between socially naive flies involved three phases. In the ‘fight phase’ a pair of naive flies was allowed to fight for 60 min, this was followed by a ‘rest phase’ of 60 min where flies were returned to their original food vials, and finally a ‘test phase’ where previously matched flies fought against unfamiliar, naive opponents for 60 min. SONY PMB software on Windows OS (Microsoft Corporation, Redmond, WA, USA) was used for video playback and the fights were manually curated. In all cases, the analyzer was blind to the genotype of the fly. Fights were analyzed on basis of encounters that involved physical interactions and lasted at least 3 s. Encounters were considered separate if the time interval between them exceeded 2 s. Winners and losers were designated based on an ethologically characterized three-lunge/three-retreat rule [developed in Yurkovic et al. (2006)]. Lunge is defined as a maneuver where a fly rears up on its hind legs and collapses on the opponent (Chen et al., 2002; Zwarts et al., 2012). A retreat occurs when a fly, in response to an offensive action, runs/flyes away from the opponent or the food cup (territory) (Chen et al., 2002; Zwarts et al., 2012). Within a trial, a fly was assigned a status of ‘winner’ if it used three continuous lunges (without any interim retreats) and its opponent executed three corresponding retreats (with no intervening lunges) (Yurkovic et al., 2006). The latter was designated as a ‘loser’.

We analyzed various parameters to investigate aggression in flies across genotypes. They included: (1) encounter frequency (encounters per minute measured as the total number of encounters divided by total time of fighting); (2) aggression vigor index (the fraction of time spent fighting in the first 10 min following the commencement of the first encounter); and (3) latency to engage in an encounter (time in seconds from the start of the fight

to initiate the first agonistic interaction that lasts for at least 3 s). In the second fights, the ‘draw’ outcomes, where the three-lunge/three-retreat rule was not satisfied, were further divided into three categories based on Penn et al. (2010). This categorization was based on usage of lunges and retreats by experienced loser flies against a naive opponent. ‘High-intensity’ draws consisted of experienced loser flies predominantly using lunges whereas ‘low-intensity’ draws included usage of retreats. Fights in which the flies did not engage in agonistic interactions were considered as ‘no intensity’ and categorized as draws. An experienced loser fly may not readily engage in agonistic interactions against naive opponents. This may preclude escalation during fights and result in a higher number of draws. Thus, ‘high/low-intensity’ categorization facilitates better assessment of the status-dependent behavioral changes manifested as the loser effect.

We devised the loser index in order to assess the ability of flies to demonstrate the experience-dependent loser effect. The loser index was calculated as the difference between the number of encounters lost and the number of encounters won divided by the total number of encounters in the second fight. Within an encounter, if a fly used aggressive actions such as lunging, tussling (tugging and pulling the body of the opponent), boxing (rearing up on its hind legs and striking the opponent with its forelegs), holding (rearing up on its hind legs and holding the other fly’s abdomen), chasing (running after the opponent) or fencing (extending its leg forward and pushing the other fly) (Chen et al., 2002; Zwarts et al., 2012) and the other fly responded with a retreat, then the former fly is a ‘winner’ and the latter a ‘loser’ in that encounter.

Analysis of locomotor behavior

Locomotor behavior was analyzed using a negative geotaxis assay as described previously (Ali et al., 2011). A group of 10 flies, age- and size-matched, was introduced into a food vial one day prior to testing. The flies were placed in two vertically joined empty food vials with a distance of 8 cm marked on the lower vial. Following a gentle tap to get all the flies to the base of the vial, the number of flies able to climb above the 8 cm mark in 10 s was scored. The experiment was repeated 10 times for each group and the average pass rate was calculated. Three experimental replicates were carried out for each genotype.

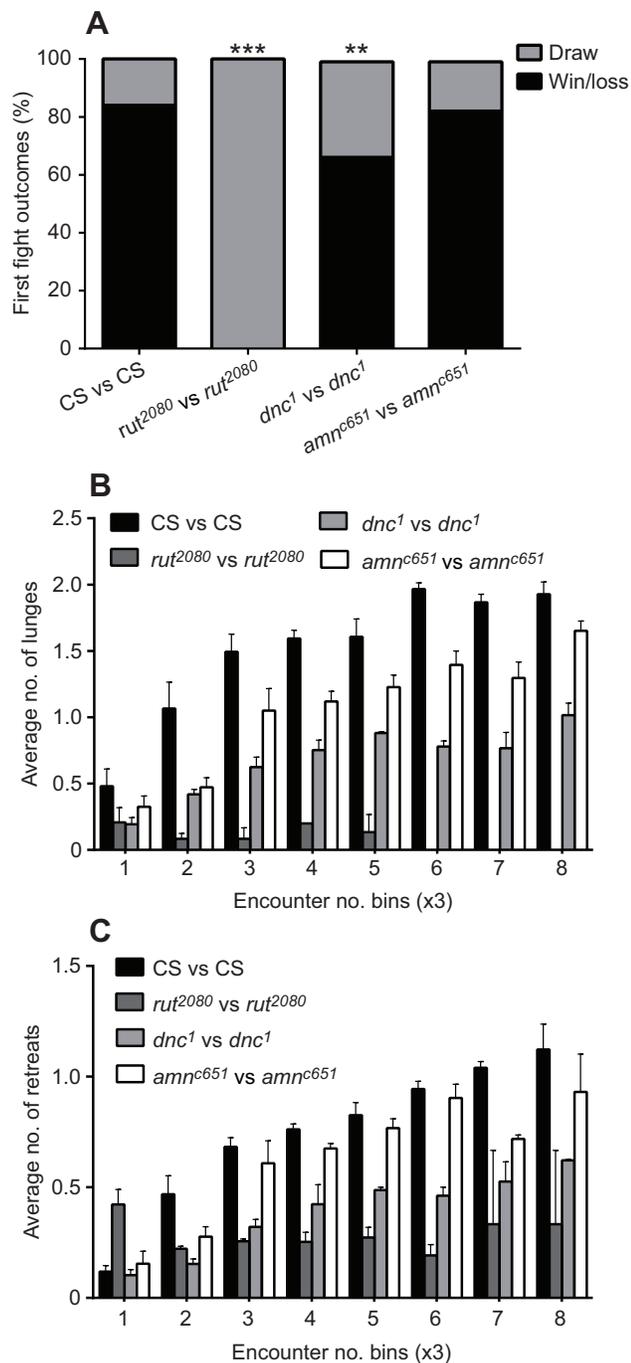
Statistical analysis

Videotapes were analyzed and each encounter was scored for all fighting strategies and documented on spreadsheets. All statistical analysis was performed using GraphPad Prism 6.0 (Graphpad Software, Inc., La Jolla, CA, USA) statistical software. Various statistical tests were employed to facilitate our analysis, including χ^2 test, one-factor ANOVA and two-factor repeated-measures ANOVA followed by *post hoc* Tukey’s or Dunnett’s multiple comparisons tests. Specific information on statistics used in each experiment is included in the figure legends.

RESULTS

The cAMP pathway mediates the establishment of hierarchies within a fight

Pairs of male flies demonstrate stereotyped aggressive behavior towards each other while competing for resources such as food or females. These dyadic interactions begin with low aggression maneuvers such as wing threat and fencing, which then escalate to high aggression strategies such as lunging and tussling. This escalation is a consequence of experience-dependent behavioral transitions, with one fly demonstrating offensive strategies with



increasing frequency against an opponent fly employing more and more defensive strategies. These real-time behavioral modifications on shorter timescales are stable within a fight and facilitate the formation of social dominance hierarchies. We have assigned dominance relationships using a previously developed three-lunge/three-retreat rule [see the Materials and Methods section and Yurkovic et al. (2006)]. In wild-type (WT) CS flies, 85% of the fights produced dominance relationships (Fig. 1A). Prior investigations have implicated the cAMP pathway in the development of learning and memory in multiple conditioning paradigms. cAMP synthesis is regulated by the enzymatic activity of the *Rut*-encoded adenylyl cyclase that catalyzes the conversion of ATP to cAMP (Levin et al., 1992; Livingstone et al., 1984). *Rut* has

Fig. 1. cAMP signaling is required for establishing dominance relationships. (A) Canton-S (CS) and *amn^{c651}* flies form stable dominance relationships (win/loss) in 85% and 82% in their first fights, respectively. *rut²⁰⁸⁰* displayed no dominance structures whereas *dnc¹* showed dominance in 67% of their fights (two-tailed χ^2 test; ** $P < 0.01$, *** $P < 0.001$, $N = 30$). Asterisks indicate comparison with CS. (B) CS winner flies progressively increased lunging. A similar trend was seen with *amn^{c651}* flies ($P > 0.05$). In contrast, *rut²⁰⁸⁰* ($P < 0.001$) and *dnc¹* ($P < 0.01$) flies demonstrated significantly deficient lunging trends (two-factor repeated-measures ANOVA followed by *post hoc* Tukey's multiple comparison test; 'P' is the interaction term, means \pm s.e.m., $N = 30$). (C) Both CS and *amn^{c651}* loser flies ($P > 0.05$) showed progressive increase in the number of retreats whereas this was significantly compromised in *rut²⁰⁸⁰* ($P < 0.001$) and *dnc¹* ($P < 0.01$) flies. *dnc¹* mutants, although compromised in modifying fighting strategies compared with CS flies, demonstrated better trends compared with *rut²⁰⁸⁰* mutants ($P < 0.001$ for lunges; $P < 0.01$ for retreats) (two-factor repeated-measures ANOVA followed by *post hoc* Tukey's multiple comparison test; 'P' is the interaction term, means \pm s.e.m., $N = 30$). Total number of lunges/retreats in every three successive encounters was analyzed and their mean is reported.

been implicated as a biochemical coincidence detector necessary for the formation of short-term memory (STM) in olfactory conditioning assays (Dudai et al., 1988; McGuire et al., 2005). In contrast to CS flies, *rut²⁰⁸⁰* mutant flies were unable to form dominance relationships, with all their fights ending in draws (Fig. 1A; $P < 0.001$). cAMP phosphodiesterase activity encoded by *Dnc* negatively regulates cAMP levels (Davis and Kiger, 1981). Consistent with the deregulation of cAMP dynamics, *dnc¹* flies displayed attenuated formation of dominance relationships with only 67% of the fights resulting in wins/losses (Fig. 1A; $P < 0.01$). The *Amn* gene product is thought to encode for neuropeptide(s) and is known to modulate cAMP signaling (Feany and Quinn, 1995; Moore et al., 1998). However, *amn^{c651}* mutant flies displayed WT levels of dominance hierarchies (Fig. 1A; 82% of the fights, $P > 0.05$).

For further analysis of experience-dependent behavioral modifications during a fight, we focused on lunges and retreats. These strategies are commonly used offensive (lunges) and defensive (retreats) maneuvers and previous work has indicated status-dependent modifications in their usage by winner/loser flies (Yurkovic et al., 2006). Behavioral modulation was assessed by scoring numbers of lunges/retreats in the first 25 encounters in a fight. In line with previous observations, agonistic interactions resulted in an escalation of aggression (Trannoy et al., 2016; Yurkovic et al., 2006). In aggressive encounters between CS flies, the ultimate winners (as established by the three-lunge/three-retreat rule; see the Materials and Methods section) progressively increased the deployment of lunges whereas the losers increasingly adapted to the retreating behavior (Fig. 1B,C). In contrast, *rut²⁰⁸⁰* mutant flies were unable to demonstrate any behavioral transitions pertaining to lunges and retreats (Fig. 1B,C; $P < 0.001$). *dnc¹* flies were not only inefficient in establishing hierarchies but also displayed compromised behavioral plasticity with significantly reduced ability to modify the frequency of lunges and retreats compared with CS animals (Fig. 1B,C; $P < 0.001$). *amn^{c651}* flies, which are competent in establishing hierarchies, were able to modify the usage of offensive and defensive strategies as well as CS flies (Fig. 1B,C; $P > 0.05$). These results suggest a correlation between experience-dependent behavioral plasticity and the formation of dominance hierarchies following aggressive encounters.

No significant differences in locomotor activity between WT and mutant flies were found using a negative geotaxis assay (see the Materials and Methods section), thus ruling out the possibility of overt motor deficits in these lines (Fig. 2A; $P > 0.05$). Multiple parameters were evaluated to assess the aggression levels of the WT and mutant lines. Encounter frequency was significantly lower in

rut²⁰⁸⁰ mutants compared with CS flies but *dnc¹* and *amn^{c651}* mutants were comparable to WT flies (Fig. 2B; $P_{rut} < 0.05$, P_{dnc} and $P_{amn} > 0.05$). Aggression vigor index was compromised in all the mutant lines compared with CS but was not significantly different between *rut²⁰⁸⁰*, *dnc¹* and *amn^{c651}* flies (Fig. 2C; $P_{rut} < 0.001$, P_{dnc} and $P_{amn} < 0.05$).

While our studies implicate lack of behavioral plasticity in *rut²⁰⁸⁰* mutants in establishing dominance hierarchies in *rut²⁰⁸⁰* versus *rut²⁰⁸⁰* fights, it is also possible that the *rut²⁰⁸⁰* flies are unable to execute high-intensity maneuvers such as lunges. In *rut–rut* fights, neither opponent could adjust their fighting patterns depending upon experience resulting in a lack of escalation beyond low-intensity interactions precluding assessment of inherent behavioral changes in *rut²⁰⁸⁰* flies. CS flies have intact experience-dependent behavioral transitions compared with *rut²⁰⁸⁰* mutants. Therefore, pairing CS versus *rut²⁰⁸⁰* may better reveal behavioral escalation during fights. CS versus *rut²⁰⁸⁰* fights resulted in significant dominance hierarchies with 37.5% of fights ending in a win or a loss, although it remains attenuated compared with CS versus CS fights (Fig. 3A; $P < 0.001$). Interestingly, *rut²⁰⁸⁰* flies demonstrated increased lunging and retreating behavior compared with their performance in *rut–rut* fights and also won 9% of the fights against WT opponents (Fig. 3B, C). *rut²⁰⁸⁰* flies, when in a *rut–CS* fight, were capable of executing and modifying the frequency of use of both lunges and retreats, although this ability remained significantly compromised compared with that in CS flies (in CS–CS fights) (Fig. 3B,C; $P < 0.001$ for lunges and $P < 0.05$ for retreats). The usage of offensive and defensive

strategies for *rut²⁰⁸⁰* flies against a WT opponent was significantly better than *rut–rut* fights (Fig. 3B,C; $P < 0.001$ for lunges and $P < 0.01$ for retreats). These results suggest that *rut²⁰⁸⁰* flies are capable of executing high-intensity maneuvers but are compromised in modifying its frequency as well as CS flies during a fight.

Furthermore, *dnc¹* mutants were unable to modify fighting strategies as well as CS flies but they were less compromised compared with the *rut²⁰⁸⁰* flies in *rut–rut* fights (Fig. 1A–C). Consistent with the association of ability to modify fighting patterns with the establishment of dominance relationships, *dnc* mutants do establish hierarchies, although less efficiently than WT CS flies (Fig. 1A). Similarly, *rut²⁰⁸⁰* flies against WT opponents demonstrated compromised plasticity and attenuated dominance structures (Fig. 3A–C). In fact, usage of lunges/retreats by *rut²⁰⁸⁰* flies in *rut–CS* fights was comparable to those seen in *dnc¹* (Fig. 3B,C; $P > 0.05$ for lunges and retreats).

Interestingly, the aggression vigor index was similar for *rut–CS* and *rut–rut* fights (Fig. 3D; $P > 0.05$).

This series of experiments established the role of the cAMP signaling in behavioral plasticity underlying the modification of fighting strategies during agonistic interactions. Behavioral flexibility, rather than changes in levels of aggression, is correlated with the generation of dominance hierarchies.

Specific neural circuits are recruited in cAMP-mediated behavioral plasticity

We next asked which components of the *Drosophila* brain were involved in the status-dependent modifications of aggressive

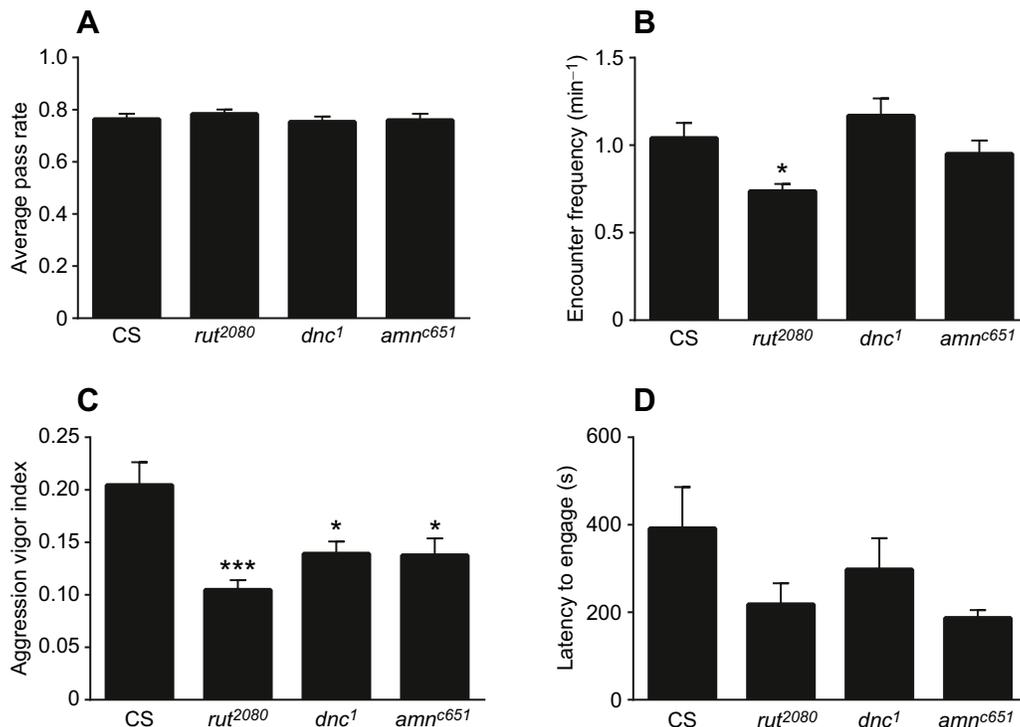


Fig. 2. Aggression levels in cAMP pathway mutant flies. (A) Locomotor activity in flies was analyzed using a negative geotaxis assay. No significant differences were observed between Canton-S (CS) and cAMP pathway mutants in their average pass rate (one-factor ANOVA followed by *post hoc* Tukey's multiple comparison test, means \pm s.e.m., $N=30$). (B) Encounters per minute was lower in *rut²⁰⁸⁰* flies ($P < 0.05$) but was comparable in *amn^{c651}* and *dnc¹* flies compared with CS flies ($P > 0.05$) (one-factor ANOVA followed by *post hoc* Tukey's multiple comparison test, means \pm s.e.m., $N=30$). Asterisks indicate comparison with CS. (C) Aggression vigor index, defined as the fraction of time spent fighting in the first 10 min, was significantly lower in *rut²⁰⁸⁰* ($P < 0.001$), *amn^{c651}* ($P < 0.05$) and *dnc¹* ($P < 0.05$) flies in comparison with CS flies (one-factor ANOVA followed by *post hoc* Tukey's multiple comparison test, means \pm s.e.m., $N=30$). Asterisks indicate comparison with CS. (D) Latency to engage in an encounter was not significantly different in *rut²⁰⁸⁰* ($P > 0.05$), *dnc¹* ($P > 0.05$) and *amn^{c651}* ($P > 0.05$) flies in comparison with CS flies (one-factor ANOVA followed by *post hoc* Tukey's multiple comparison test, means \pm s.e.m., $N=30$). Level of significance in all figures is as follows: * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

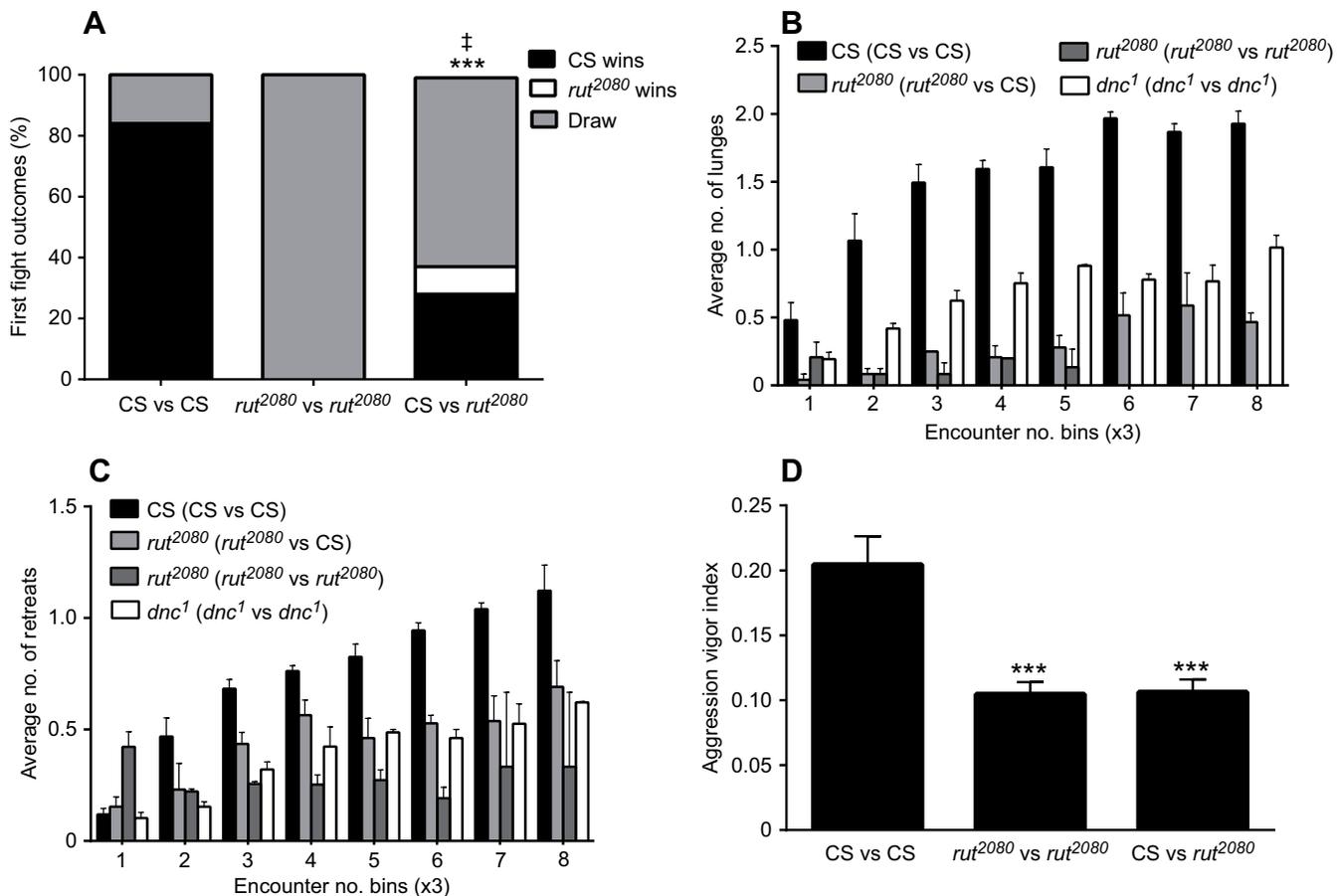


Fig. 3. *rut2080* flies display improved lunging/retreating behavior against wild-type (WT) opponents. (A) The *rut2080* flies showed attenuated dominance structures against a WT opponent compared with CS versus CS fights ($P < 0.001$) but improved hierarchical relationships compared with *rut2080* versus *rut2080* fights ($P < 0.01$) (two-tailed χ^2 test; $***P < 0.001$, $††P < 0.01$, $N = 25$). Asterisks indicate comparison with CS and double daggers indicate comparison with *rut2080*. (B,C) The *rut2080* flies against a WT opponent demonstrated significantly better status-dependent modification of lunges/retreats compared with *rut2080* versus *rut2080* fights ($P < 0.001$ for lunges and $P < 0.01$ for retreats) and was comparable to *dnc1* flies ($P > 0.05$). The lunge/retreat trends for *rut2080* flies in *rut2080* versus CS fights are compromised compared with CS flies ($P < 0.001$ for lunges; $P < 0.05$ for retreats) (two-factor repeated-measures ANOVA followed by *post hoc* Tukey's multiple comparison test; 'P' is the interaction term, means \pm s.e.m., $N = 20$). (D) Aggression vigor index was significantly lower in flies engaged in *rut2080* versus *rut2080* ($P < 0.001$) and CS versus *rut2080* ($P < 0.001$) fights in comparison with flies in CS versus CS fights (one-factor ANOVA followed by *post hoc* Tukey's multiple comparison test, means \pm s.e.m., $N = 30$). Asterisks indicate comparison with flies in CS versus CS fights. The data set for control CS flies, which were trained at the same time of day as experimental lines, is the same as shown in Figs 1 and 2. Total number of lunges/retreats in every three successive encounters is analyzed and their mean is reported.

strategies and formation of dominance relationships. As multiple sensory modalities are likely to be involved in aggressive encounters, a complex pattern of recruitment of neuronal circuits may underlie the integration of these sensory inputs (Asahina et al., 2014; Hoopfer, 2016; Hoopfer et al., 2015; Lim et al., 2014; Ramin et al., 2014; Wang and Anderson, 2010; Wang et al., 2011; Yoon et al., 2013). To test this, we used the UAS-Gal4 binary system to express the *Rut* gene product in restricted neuronal populations in a *rut2080* mutant background. We chose well-characterized Gal4 drivers that have restricted expression in defined neuronal populations of the mushroom body (MB) and central complex (CC) of the fly brain as these regions are strongly implicated as central integrators in multiple behavioral paradigms (Table 1) (Aso et al., 2009; Blum et al., 2009; Joiner and Griffith, 1999; Neuser et al., 2008; Pan et al., 2009; Torroja et al., 1999; Zars et al., 2000a, b). All experiments were conducted at the same time of the day. The genetic rescue and the control experiments were done in parallel. Pan-neuronal expression of *Rut* using the Appl-Gal4 driver completely rescued the inability to form dominance relationships seen in *rut2080* mutants (Fig. 4A; $P < 0.001$). Similar restoration of

dominance hierarchies to WT levels was seen with expression limited to the α/β and γ lobe neurons of the MB using the c309 driver (Fig. 4A; $P < 0.001$). Next, we tested a panel of Gal4 drivers expressing *Rut* in different subpopulations of neurons in the MB and the CC of *rut2080* flies (Table 1). UAS-*Rut* driven by Gal4 drivers c739, 201Y, c305a and c205, but not c819, partially rescued the inability of *rut2080* to form hierarchical relationships (Fig. 4A; $P < 0.001$). These experiments implicate the independent involvement of the α/β , γ and α'/β' lobes of the MB together with the F5 neurons of the fan-shaped body (FB) in neuronal processing leading to the formation of dominance. The ellipsoid body (EB) of the CC did not appear to be involved in this function (Fig. 4A; $P > 0.05$).

Analysis of behavioral patterns revealed that distinct neuronal circuits mediate *rut*-dependent behavioral plasticity within a fight (Fig. 4 and Table 1). Pan-neuronal expression of *Rut* using the Appl-Gal4 driver rescued the inability to alter the frequency of use of lunges and retreats seen in *rut2080* mutants (Fig. 4B,C; $P < 0.01$). We also observed a complete rescue of behavioral transitions pertaining lunges and retreats with *Rut* expression limited to the α/β and γ lobe

Table 1. Expression patterns of Gal4 drivers used and rescue results

Gal4 drivers	Expression pattern	Rescue of learning (first fight)	Rescue of loser effect (second fight)
Appl	Pan-neuronal (Neuser et al., 2008; Torroja et al., 1999)	Winners: Y* Losers: Y*	Y*
c309	α/β and γ lobes of the MB (Aso et al., 2009; Blum et al., 2009; Joiner and Griffith, 1999)	Winners: Y* Losers: Y*	Y*
c739	α/β lobes of the MB (Aso et al., 2009; Blum et al., 2009; Joiner and Griffith, 1999; Zars et al., 2000b)	Winners: Y* Losers: Y*	N
201Y	γ lobes and α/β lobes (sparse) of the MB (Aso et al., 2009; Blum et al., 2009; Joiner and Griffith, 1999; Zars et al., 2000a)	Winners: Y \ddagger Losers: Y*	N
c305a	50% of α'/β' lobes of the MB; weak expression in AL and EB (Aso et al., 2009; Blum et al., 2009)	Winners: Y \ddagger Losers: Y*	N
c205	F5 neurons of the FB (Liu et al., 2006; Pan et al., 2009)	Winners: Y* Losers: Y*	N
c819	R2/R4m neurons of the EB (Liu et al., 2006; Pan et al., 2009)	Winners: N Losers: N	N

Y, yes; N, no; MB, mushroom bodies; EB, ellipsoid body; FB, fan-shaped body; AL, antennal lobes.

* $P < 0.001$; $\ddagger P < 0.01$. Indices were compared between *rut*²⁰⁸⁰/Y; UAS-Rut/+ and *rut*²⁰⁸⁰/Y; UAS-Rut/Gal4.

neurons of the MB using the c309 driver (Fig. 4D,E; $P < 0.001$). Furthermore, expression of *Rut*, independently in the α/β , α'/β' or γ neurons of the MB or in the FB also rescued the deficit in the progressive increased number of lunges and retreats seen in *rut*²⁰⁸⁰ flies (Fig. 4D–G; $P < 0.01$). The EB of the CC did not appear to be involved in this function as no rescue was observed with the EB only driver (Fig. 4F,G; $P > 0.05$).

Restricted expression of *Rut* in specific brain regions rescued the behavioral plasticity of lunges/retreats and the ability to establish dominance hierarchies within a fight.

To confirm that experience-dependent behavioral flexibility was a major substrate of the rescue and not an indirect effect of changes in other general features of aggression, we evaluated multiple aggression-related parameters. As the rescue was strongest (comparable to WT) with the Appl-Gal4 (pan-neuronal) and c309 (α/β and γ neurons of the MB) drivers, these were used to evaluate changes in aggressiveness. However, no change in the frequency of encounters, aggression vigor and latencies to engage was found (Fig. 5A–C; $P > 0.05$). These results suggest that although pan-neuronal and α/β and γ drivers fully rescue the ability to alter the intensities of specific fighting patterns during a fight and the establishment of dominance hierarchies, they do not affect the levels of aggression in these animals.

Our analysis indicates that, in accordance with the multimodal inputs associated with dyadic interactions, multiple neuronal groups in the MB (α/β , α'/β' , γ) and CC (FB) are involved in the processing of these inputs that lead to the progressive changes in offensive and defensive strategies within a fight. The rescue experiments also highlight the role of cAMP signaling in behavioral plasticity in aggression to be independent of general changes in aggression levels.

cAMP signaling is necessary for the development of the loser effect

To test if aggression-associated hierarchies developed in the first fights influenced the outcomes of subsequent fights, losers from first fights were paired with unfamiliar, socially naive opponents after a 60 min rest period. Dominance relationships were assigned using the three-lunge/three-retreat rule as in the first fights. Fights that fell under the ‘draw’ category were further subdivided into three groups. ‘High-intensity’ draws consisted of experienced flies predominantly using lunges in their second fight without

satisfying the three-lunge/three-retreat criteria for dominance relationships. Similarly, ‘low-intensity’ draws consisted of experienced flies predominantly using retreats. Fights in which the flies did not engage in agonistic interactions were considered as ‘no intensity’ and were categorized as draws. For statistical analysis, high- and low-intensity draws were grouped with wins and losses, respectively. Consistent with previous studies (Trannoy et al., 2016; Yurkovic et al., 2006), WT CS loser flies always lost to naive opponents in their second fights (Fig. 6A). This indicates that an experience of social loss results in a strong loser effect in subsequent fights.

As *rut*²⁰⁸⁰ flies did not generate winners or losers in the first fight, both the individuals were considered as experienced and used in second fights. However, as in their first fights, *rut*²⁰⁸⁰ mutants were unable to form any dominance relationships in their second fights (Fig. 6A; $P < 0.001$). *dnc*¹ mutants also displayed no significant loser effect with 18% losses, 18% wins and 64% draws (Fig. 6A; $P < 0.001$).

Surprisingly, *amn*^{c651} flies did not show any loser effect. Fifty per cent of analyzed second fights resulted in losses and the remaining in wins for losers (Fig. 6A; $P < 0.001$). Unlike *rut* and *dnc*, *amn*^{c651} flies developed dominant–subordinate relationships as efficiently as CS flies in their first fights (Fig. 1C) but were still unable to consolidate this experience into a loser effect. This suggests a special requirement of Amn gene product in stabilizing the hierarchical structures in flies in the form of a loser effect.

A loser index, calculated as the difference between the number of encounters lost and the number of encounters won divided by the total number of encounters in the second fight (see Materials and methods), was used to represent the loser effect as a consequence of past experience. This allowed direct evaluation of experience-dependent alterations in fighting strategy in the losers across genotypes in subsequent fights. CS flies showed a high loser index consistent with their robust loser effect (Fig. 6B). In contrast, the *amn*^{c651} and *dnc*¹ losers and the experienced *rut*²⁰⁸⁰ flies displayed significantly lower loser indices (Fig. 6B; $P < 0.001$).

These results show that *dnc*¹ and *rut*²⁰⁸⁰ flies, which displayed compromised development of dominance hierarchies in their first fights, did not display a loser effect. In contrast, *amn*^{c651}, which could form dominance hierarchies in their first fights, also lacked the loser effect. *Amn* appears to have an independent function from *Rut* and *Dnc* and is necessary for the stabilization of the social status from the first fight in the form of a loser effect.

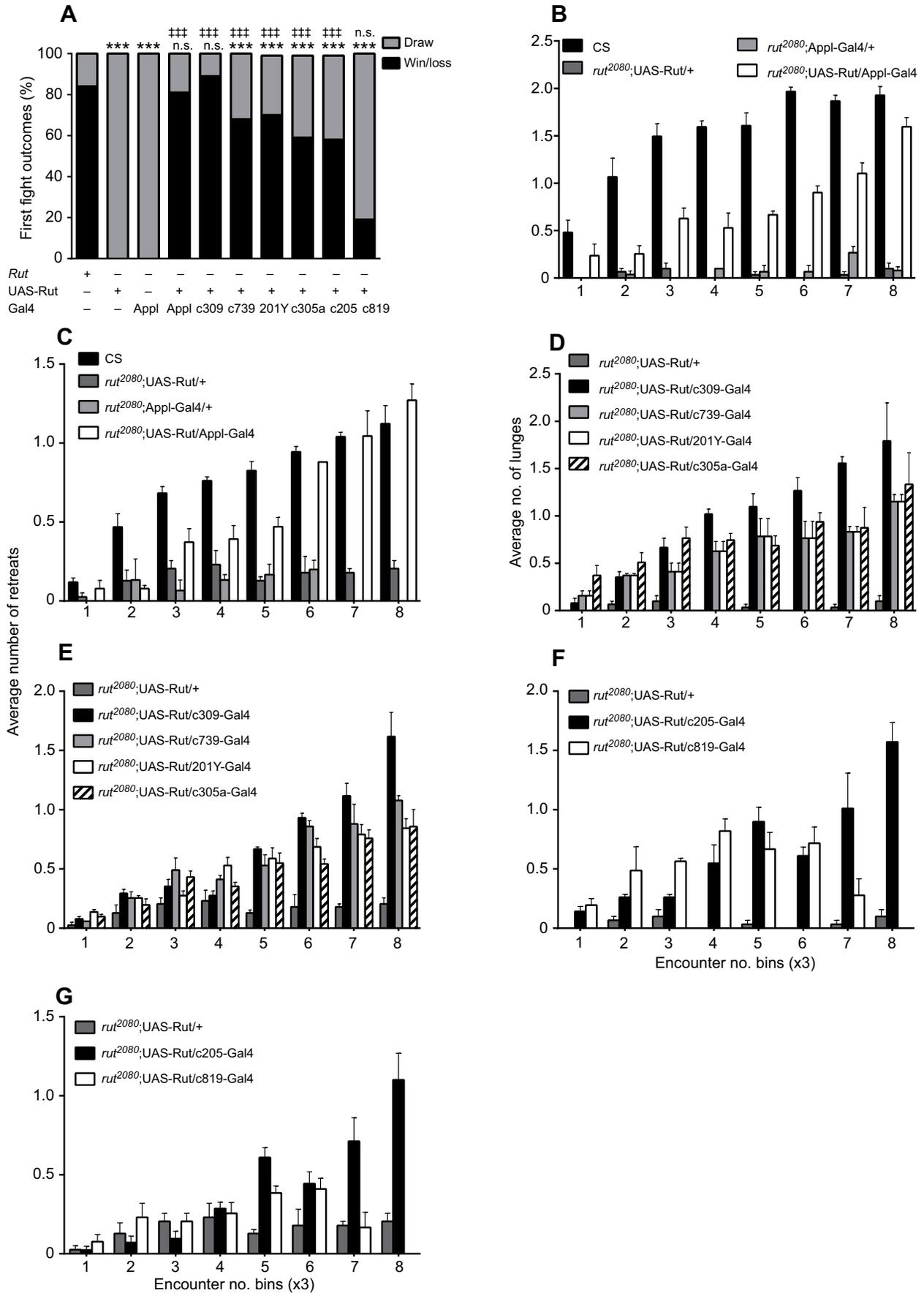


Fig. 4. See next page for legend.

Fig. 4. Distinct neuronal circuits mediate behavioral plasticity during agnostic encounters. Restricted expression of *Rut* in specific subpopulations of neurons in *rut*²⁰⁸⁰ flies yields differential rescue of learning impairment in first fights. (A) Lack of development of hierarchies in *rut*²⁰⁸⁰ flies was rescued upon expression of UAS-*Rut* (in a *rut*²⁰⁸⁰ background) pan-neuronally (Appl) in the α/β and γ lobes of the mushroom body (MB) (c309), α/β lobes of the MB (c739), γ lobes of the MB (201Y), α'/β' lobes of the MB (c305a) and in the F5 neurons of the fan-shaped body (FB) (c205). No rescue was seen upon reintroduction of *Rut* to the ellipsoid body (EB) (c819) (two-tailed χ^2 test; ** $P < 0.01$, *** $P < 0.001$, ††† $P < 0.001$, $N \geq 15$). Asterisks indicate comparison with CS and double daggers indicate comparison with *rut*²⁰⁸⁰;UAS-*Rut*/. (B–G) Ability to progressively increase the number of lunges/retreats was rescued (relative to *rut*²⁰⁸⁰ flies) by expressing *Rut* either pan-neuronally (Appl; $P < 0.001$ for lunges and retreats) or in α/β and γ (c309; $P < 0.001$ for lunges and retreats), α/β alone (c739; $P < 0.001$ for lunges, $P < 0.01$ for retreats), α'/β' (c305a; $P < 0.01$ for lunges, $P < 0.05$ for retreats) alone, γ (201Y; $P < 0.001$ for lunges, $P < 0.05$ for retreats) alone and in the FB (c205; $P < 0.001$ for lunges, $P < 0.01$ for retreats). EB (c819; $P > 0.05$ for lunges and retreats) expression did not show any rescue. B and C are comparisons of Appl-Gal4 with CS and no Gal4 control, D and E are comparisons of MB restricted Gal4 drivers (c309, c739, 201Y and c305a) with no Gal4 control, and F and G are comparisons of central complex restricted Gal4 drivers (c205 and c819) with no Gal4 control (two-factor repeated-measures ANOVA followed by *post hoc* Tukey's multiple comparison test; 'P' is the interaction term, means \pm s.e.m., $N \geq 15$). The data set for control CS flies, which were trained at the same time of day as experimental lines, is the same as shown in Fig. 1. Total number of lunges/retreats in every three successive encounters is analyzed and their mean is reported. n.s., not significant.

α/β and γ lobes of the MB cooperate to mediate cAMP-dependent loser effect

Previous experience of loss results in a robust loser effect in WT flies. We investigated the neurogenetic circuitry involved in the development of this effect.

In the second fights, both Appl and c309 Gal4 driver lines were able to restore the loser effect in *rut*²⁰⁸⁰ flies to levels statistically indistinguishable from WT CS flies (Fig. 7A,B and Table 1). Pan-neuronal (Appl) or combined α/β and γ lobe-specific (c309) expression resulted in most losers losing and a small proportion of non-aggressive draws (Fig. 7A; $P < 0.001$). Loser index in these flies was significantly greater than controls (Fig. 7B; $P < 0.001$) and comparable to WT flies (Fig. 7B; $P > 0.05$).

Interestingly, expression of UAS-*Rut* limited to individual substructures of the MB and CC was not sufficient to rescue the impaired loser effect observed in *rut* flies (Fig. 7B, Table 1). Expression in individual neuronal subpopulations using the c739, 201Y, c305a or c205 Gal4 drivers (in the *rut*²⁰⁸⁰ background) resulted in a higher proportion of non-draw outcomes in losers paired with naive opponents but failed to show a robust loser effect (Fig. 7A; $P < 0.001$). As expected, there was no significant change in the loser index for these flies (Fig. 7B; $P > 0.05$). Consistent with compromised learning in the first fights, expression limited to the EB using the c819 Gal4 driver did not rescue the loser effect (Fig. 7; $P > 0.05$).

Our results implicate combined processing by the α/β and γ neurons of the MBs in the development of the loser effect.

DISCUSSION

Two distinct experience-dependent behavioral modifications are observed in *Drosophila* aggression. The first results in dominance relationships correlated with real-time, progressive changes in fighting strategies. The second is the consolidation of the experience of a previous loss to a loser effect in subsequent fights. Our data indicate that these behavioral changes involve specific cAMP-mediated signaling and are processed by overlapping neuronal circuits involving two temporally separated memory traces.

Rut and *Dnc* enzymes have been implicated in learning and/or STM in multiple paradigms, including classical conditioning and courtship-associated learning and memory (Bragina and Kamyshev, 2003; Gailey et al., 1984; Livingstone et al., 1984; McGuire et al., 2005). *rut* and *dnc* flies do not develop dominance hierarchies as well as CS flies, with all *rut*–*rut* fights resulting in draws. Compared with CS, both *rut* and *dnc* are unable to display experience-dependent modification of fighting strategies and are deficient in generating losers and winners.

rut and *dnc* mutant flies are deficient in memory formation in various behavioral assays (Dudai et al., 1976; Gailey et al., 1984; Livingstone et al., 1984; McGuire et al., 2005). In olfactory conditioning, both *rut* and *dnc* mutants demonstrate rapid memory degradation within an hour of training but has no impact on later memory (Chen et al., 1986; Levin et al., 1992; Tamura et al., 2003). Also, *rut* mutant flies were unable to demonstrate robust 30 min memory after an hour of training in a courtship conditioning paradigm (Ishimoto et al., 2013). Therefore, the inability of *rut* and *dnc* mutants to alter behavioral states might be due to poor memory acquisition pertaining winner–loser status, which results in a lack of hierarchical relationships in a fight.

The *Amn* gene product encodes for three putative neuropeptides (Feany and Quinn, 1995; Moore et al., 1998). Genetic and biochemical studies have implicated the *Amn* locus in cAMP dynamics (Feany and Quinn, 1995; Moore et al., 1998; Bhattacharya et al., 2004). It is assumed, from analogous data in other organisms, that the *Amn* gene product(s) activate adenylyl cyclase activity via GPCR signaling (Kandel and Abel, 1995; McGuire et al., 2005). However, there is no direct evidence of the *Amn* gene products being secreted and acting as a classical neuropeptide via GPCR activation. In our experiments, *amn* flies are as competent in modifying fighting strategies and establishing hierarchical relationships as CS flies but are unable to retain the loser status in subsequent fights. These results suggest that the plasticity in modifying aggressive strategies during a fight is mediated by cAMP dynamics under the control of *Rut* and *Dnc* gene products (but not *Amn*) and is necessary to establish dominance structures.

All three cAMP pathway mutants displayed a reduction in aggression vigor as compared with CS. This includes *amn*, which displayed normal behavioral flexibility and dominance hierarchies. These results suggest that the level of aggression in flies is independent of their ability to modify behavior in an experience-dependent manner. The latency to engage in fights in cAMP mutants was also comparable to CS. The latter suggests no overt changes in motivational states and support the previous conclusion.

Brain region-specific expression of the *Rut* gene product in a *rut* mutant background implicates specific neuronal circuits in status-dependent behavioral changes during agonistic encounters. All the drivers that rescue the establishment of dominance also restore the ability to alter the usage of lunges and retreats during a fight.

Rescue by Appl or c309 did not alter the encounter frequency, aggression vigor or the latency to engage displayed by *rut* mutants. In line with our analysis of cAMP pathway mutants, these results demonstrate that the rescue of dominance is attributable to a restoration of experience-dependent plasticity mediated by *Rut* in specific circuits and not a consequence of modified aggressiveness.

Our study suggests a functional role for MBs and CC in learning and memory associated with aggression, consistent with those described in courtship conditioning (Joiner and Griffith, 1999; McBride et al., 1999; Sitnik et al., 2003). MBs, which are central to olfactory learning, have been previously correlated with changes in

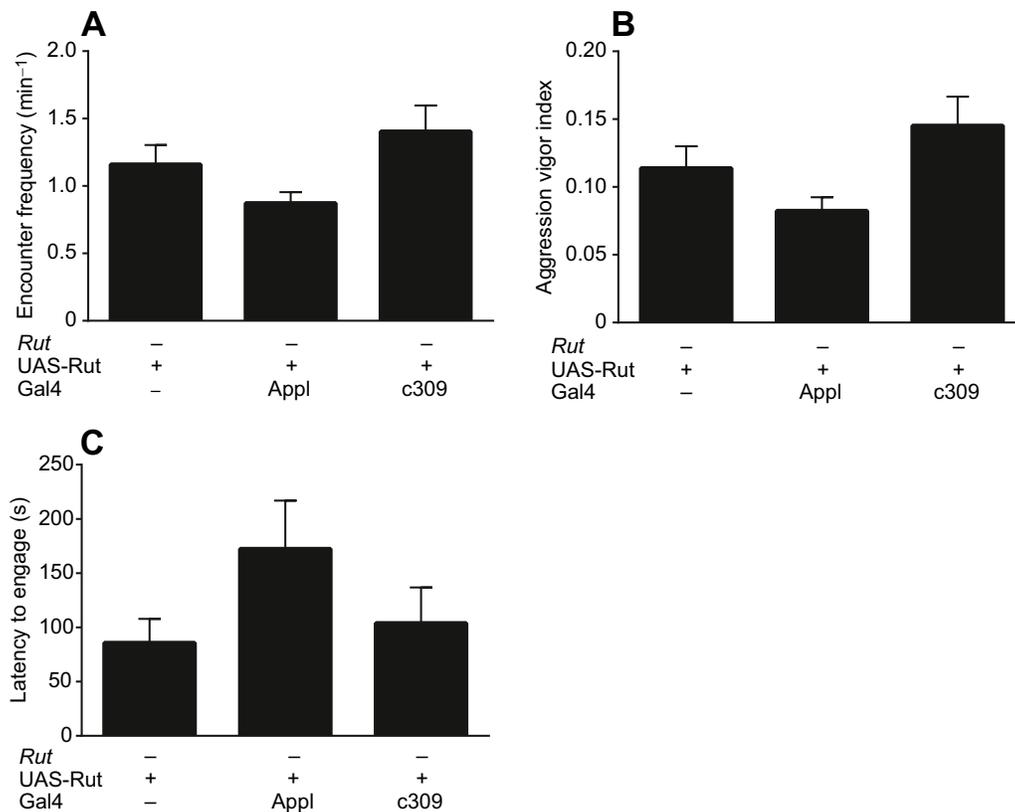


Fig. 5. Aggression levels are unchanged upon rescue of aggression-associated learning. (A) In comparison with *rut*²⁰⁸⁰;UAS-Rut/+ control flies, *Rut* expression in α/β and γ lobes of the mushroom body (c309; $P>0.05$) or pan-neuronally (Appl; $P>0.05$) resulted in a comparable number of encounter frequencies (one-factor ANOVA followed by *post hoc* Dunnett's multiple comparison test, means \pm s.e.m., $N=20$). (B) There was no significant improvement, as compared with *rut*²⁰⁸⁰;UAS-Rut/+ control flies, in aggression vigor index in response to *Rut* expression either pan-neuronally or in the α/β and γ lobes ($P>0.05$; one-factor ANOVA followed by *post hoc* Dunnett's multiple comparison test, means \pm s.e.m., $N=20$). (C) Latency to engage in an encounter ($P>0.05$) remained comparable upon *Rut* expression using either Appl or c309 Gal4 driver lines in comparison with *rut*²⁰⁸⁰;UAS-Rut/+ control flies (one-factor ANOVA followed by *post hoc* Dunnett's multiple comparison test, means \pm s.e.m., $N=20$).

agonistic behavior. *Neuralized* mutants with altered MB organization have been reported to increase aggressiveness when food is limiting (Rollmann et al., 2008). Inhibition of synaptic output from the MB has also been shown to reduce levels of aggression (Liu et al., 2011).

Interestingly, not only were the neuronal groups of the MB (the α/β , γ and α'/β' neurons) implicated, but the F5 neurons of the FB, but not the EB of the CC, were also found to be functionally involved. The FB has been previously reported to mediate visual learning of specific pattern features (Liu et al., 2006; Pan et al., 2009). A recent

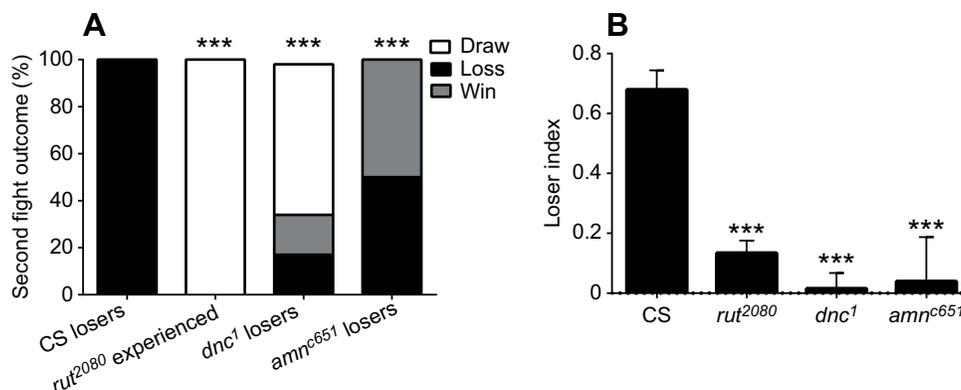


Fig. 6. cAMP signaling is necessary for developing loser mentality. (A) Canton-S (CS) losers lost all their second fights against naive opponents whereas *amn*⁶⁵¹ flies lost and won equal number of fights. *dnc*¹ flies showed weak dominance relationships and their interactions largely ended in draws, again suggestive of memory defects. All previously experienced *rut*²⁰⁸⁰ flies engaged in draws (two-tailed χ^2 test; $***P<0.001$, $N\geq 20$). Asterisks indicate comparison with CS. (B) Loser index assessed the experience-dependent modulation of fighting strategies of experienced flies in their second fights against a naive opponent. *rut*²⁰⁸⁰, *dnc*¹ and *amn*⁶⁵¹ flies all showed severely compromised loser indices compared with CS (one-factor ANOVA followed by *post hoc* Dunnett's multiple comparison test, $***P<0.001$, means \pm s.e.m., $N\geq 20$). Asterisks indicate comparison with CS.

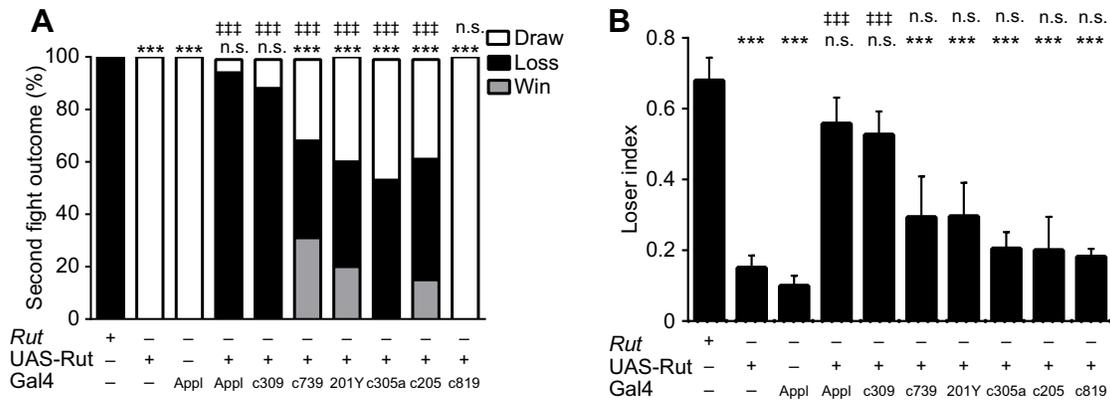


Fig. 7. Recruitment of specific neuronal circuits for the development of the loser effect. (A) Rescue experiments expressing *Rut* (in a *rut*²⁰⁸⁰ background) either pan-neuronally (Appl) or in the α/β and γ lobes of the mushroom body (c309) showed a strong recovery of the loser effect. Expression in the α/β lobes (c739), γ lobes (201Y), α'/β' lobes (c305a) and in the fan-shaped body (c205) of *rut* mutants resulted in partial recovery of dominance relationships. No rescue was seen upon the reintroduction of *Rut* in the ellipsoid body (c819) of *rut*²⁰⁸⁰ (two-tailed χ^2 test; *** P <0.001, ††† P <0.001, N ≥15). Asterisks indicate comparison with CS and double daggers indicate comparison with *rut*²⁰⁸⁰;UAS-Rut/+. (B) Loser index analysis revealed that pan-neuronal and α/β and γ lobe combined expression was able to rescue the loser effect. All other combinations did not show any rescue (one-factor ANOVA followed by *post hoc* Tukey's multiple comparison test, *** P <0.001, ††† P <0.001, means±s.e.m., N ≥15). Asterisks indicate comparison with CS and double daggers indicate comparison with *rut*²⁰⁸⁰;UAS-Rut/+. The data set for control Canton-S flies, which were trained at the same time of day as experimental lines, is the same as shown in Fig. 6. n.s., not significant.

study has underscored the importance of the CC in aggression by demonstrating the modulation of aggressive behavior by dopaminergic PPM3 neurons that synapse onto the FB (Alekseyenko et al., 2013). While there is no evidence of direct anatomical connectivity between the MB and the F5 neurons of the FB (Li et al., 2009; Young and Armstrong, 2010), it is possible that they are functionally linked by neuromodulatory dopaminergic signaling.

In the courtship suppression paradigm, multiple biochemically and anatomically distinct neural circuits have been implicated (Joiner and Griffith, 2000). Our observations in aggression-associated behaviors are analogous and possibly reflect the integration of multiple sensory inputs contributing to the establishment of dominance hierarchies.

The reduced aggression seen in cAMP pathway mutants could be due to alteration in the production of aggression-associated signal(s) and/or their perception. While this possibility awaits systematic investigation, the rescue of behavioral flexibility without altering aggressiveness seen in our experiments suggests that the two may be separable. It is also possible that the observed behavioral deficits originate from neurodevelopmental functions of these genes. Our study does not explicitly rule this out, although adult-specific expression of *Rut* is sufficient to rescue learning and memory defects in other behavioral paradigms (Mao et al., 2004; McGuire et al., 2003; Putz and Heisenberg, 2002; Tan et al., 2010). Future studies will need to address this question specifically in the context of aggression.

The robust loser effect seen in WT flies was absent in *rut*, *dnc* and *amn* mutant lines. As in their first fights, all *rut*–*rut* second fights ended in draws. No loser effect was also seen in *dnc* flies. Both *rut* and *dnc* have compromised development of hierarchies, suggesting that learning within a fight and establishing dominance relationships are necessary for the development of the loser effect. Interestingly, *amn* flies also lacked the loser effect, although they could adjust fighting strategies and develop dominance relationships as well as WT flies. The *Amn* gene product appears to mediate the consolidation of the social status acquired during the first fight into the loser effect. Alternatively, *Amn* may be necessary for a distinct memory phase resulting in the loser effect. A similar function for

Amn has been reported in olfactory conditioning experiments where *amn* mutants fail to develop intermediate-term memory or are unable to consolidate STM into this relatively longer-lasting phase (Feany and Quinn, 1995; Yu et al., 2006). *Amn* has also been reported to mediate memory stability in courtship conditioning (Siegel and Hall, 1979). Our findings support this model as *amn* flies do establish winner–loser relationships but fail to consolidate these dominance hierarchies. If the *Amn* gene product functions as a secreted neuropeptide, future rescue experiments involving the cognate receptor(s) will be necessary to determine the neural circuits subserving *Amn*-dependent establishment of the loser effect. A less robust, short-duration winner effect has been recently reported in flies (Trannoy and Kravitz, 2017; Trannoy et al., 2016). It remains to be seen if cAMP signaling and circuit features analogous to our observations mediate this phenomenon.

Rescue of *rut* simultaneously in the α/β and γ lobe neurons of the MB not only fully rescues its ability to generate dominance relationships but also the loser effect. However, *rut* expression independently in the α/β or the γ neurons was unable to restore the loser effect, although social hierarchy was rescued. We hypothesize that two distinct *Rut*-dependent memory traces facilitate the formation of the temporally distinct memory phases. A short-lived engram in distinct MB and CC substructures mediates behavioral plasticity within a fight leading to the formation of dominance relationships. Combinatorial processing between α/β and γ lobe neurons of the MB enable the formation of a second, longer-lasting memory trace that is necessary for the establishment of the loser effect.

Olfactory conditioning studies have suggested that the α/β neurons are indispensable for memory consolidation and retrieval, whereas odor–shock coincidence detection maps to the γ neurons (Dubnau and Chiang, 2013; Dubnau et al., 2001; Qin et al., 2012). These observations demonstrate recruitment of specific subsets of MB neurons for initial associations and others for memory consolidation suggesting circuit-level coordination between MB substructures. Our experiments provide evidence towards such systems-level memory consolidation in aggression where an association between α/β and γ lobes of the MB is crucial for the establishment of the loser effect. However, the existence of two

parallel, independent traces with differing kinetics of formation and decay cannot be formally ruled out.

Experience-dependent modification of innate behaviors involves multiple components and previous studies have demonstrated the central importance of pheromonal, aminergic and other modulatory activities in aggressive behavior (Aleksyenko et al., 2013, 2014; Andrews et al., 2014; Certel et al., 2010; Chan and Kravitz, 2007; Dankert et al., 2009; Dierick and Greenspan, 2007; Hoopfer, 2016; Hoyer et al., 2008; Kohl et al., 2015; Liu et al., 2011; Luo et al., 2014; Wang and Anderson, 2010; Yuan et al., 2014; Zwarts et al., 2012). However, these studies focus on aggression levels and do not directly assess behavioral plasticity and memory components in aggression. A recent study indicated that *rut²⁰⁸⁰* and *amn¹* are unable to demonstrate dominance hierarchies due to compromised aggression (Trannoy et al., 2016). However, this study did not explore the lack of behavioral flexibility in these mutants. In our study, *rut²⁰⁸⁰* has low proclivity to engage in the first 10 min of fights, fail to form dominance relationships but are capable of executing high-intensity maneuvers. In contrast, *amn^{c651}* [a functionally strong allele of *amn* (Rosay et al., 2001)] showed reduced aggression vigor but was still able to establish dominance hierarchies. Further, the rescue of *Rut* restored dominance patterns but not aggressiveness. Therefore, low aggression is not correlated with the inability to establish winner–loser statuses in a fight.

cAMP signaling appears to have a conserved role in establishing dominance relationships across phyla. As observed previously in the olfactory conditioning and courtship paradigms, *rut* and *dnc* mutants display similar behavioral deficits in aggression-associated behavioral flexibility despite having opposing biochemical functions. *Amn* activity is also thought to increase cAMP levels (perhaps with different kinetics than *Rut*). Thus, cAMP levels and fine-tuning of cAMP levels may encode signal integration by the cAMP second messenger system. In crayfish, accumulation of cAMP is necessary to form the loser effect whereas a reduction in cAMP levels is associated with the winner effect (Momohara et al., 2016). Biogenic amines have been associated with aggression and winner–loser effects in animals including flies (Aleksyenko et al., 2013, 2014; Andrews et al., 2014; Hoyer et al., 2008; Zhou and Rao, 2008), crayfish (Momohara et al., 2016; Panksepp, 2003), crickets (Rillich and Stevenson, 2014, 2015; Stevenson et al., 2005) and mammals (de Almeida et al., 2005). Aminergic signaling commonly impinges on cAMP/Protein Kinase A pathways implicating the cAMP signaling as a central effector of neuromodulation by biogenic amines (Neckameyer and Leal, 2017). In mammals, the cAMP pathway has been shown to influence aggressive behavior (Breuillaud et al., 2012). cAMP signaling in the basolateral amygdala has also been correlated with memory associated with conditioned defeat, a paradigm analogous to the loser effect (Jasnow et al., 2005; Markham et al., 2010). At the circuit level, recent work in zebrafish has implicated antagonistic regulation by two sub-regions of the dorsal habenula to establish winner/loser status (Chou et al., 2016).

Our results implicate sequential recruitment of cAMP signaling components with *Rut* and *Dnc* activities required for behavioral flexibility within a fight and, consequently, to establish dominance hierarchies. Behavioral plasticity, rather than aggressiveness, is correlated with social status. Establishment of social status facilitates the formation of a more stable and longer-lasting memory phase that requires the additional involvement of the *Amn* gene product. Neuronal circuits subserving aggression associated learning and memory show phasic recruitment. While a short-lived *Rut*-dependent trace in multiple MB and CC substructures mediate

learning during agonistic encounters, combinatorial processing by both α/β and γ lobe neurons of the MB is necessary for the development of the longer-lasting loser effect.

This study provides mechanistic insight into circuit-level associations specific to different phases of behavioral plasticity and memory in *Drosophila* aggression and the integration of biochemical signaling at the single-neuron-level with systems-level consolidation.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: N.S.C., A.G.; Methodology: N.S.C.; Formal analysis: N.S.C.; Investigation: N.S.C., K.M.; Resources: A.G.; Writing – original draft: N.S.C., A.G.; Writing – review & editing: N.S.C., A.G.; Supervision: A.G.; Project administration: A.G.; Funding acquisition: A.G.

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