Distinct cAMP signaling modalities mediate behavioral flexibility and consolidation of social status in *Drosophila* aggression.

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ABSTRACT

Social rituals, like male-male aggression in *Drosophila*, are often stereotyped and its 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. Experiential 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 show that cAMP dynamics regulated by *Rut* and *Dnc* but not the neuropeptide *Amn*, 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 are unable to alter fighting strategies and establish dominance relationships during agonistic interactions. This real time flexibility during a fight is independent of changes in aggression levels. Longer-term consolidation of social status in the form of a loser effect, however, requires additional *Amn* neuropeptide mediated inputs to cAMP signaling and involves a circuit-level association between the α/β and γ neurons of the mushroom body.

Our findings implicate distinct modalities of cAMP signaling in mediating plasticity of behavioral patterns in aggressive behavior and in the generation of a temporally stable memory trace that manifests as a loser effect.

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 of 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; Lim et al., 2014; Hoopfer, 2016). However, during aggressive encounters, experiential inputs can alter the likelihood of transitions between specific patterns and lead to experience-dependent plasticity (Yurkovic et al., 2006; Trannoy et al., 2016). Initial agonistic interaction between naïve 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 (Yurkovic et al., 2006; Trannoy and Kravitz, 2016; Trannoy et al., 2016).

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; Wang et al., 2008; Edwards et al., 2009). 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 (Dierick and Greenspan, 2007; Hoyer et al., 2008; Certel et al., 2010; Wang and Anderson, 2010; Wang et al., 2011; Alekseyenko et al., 2013; Alekseyenko et al., 2014; Andrews et al., 2014; Luo et al., 2014; Yuan et al., 2014; Kohl et al., 2015; Hoopfer, 2016), little is known about the neurogenetic underpinnings of the relevant learning and memory components.

Social experience significantly alters 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 is likely to be influenced by several factors, including the combinatorial inputs from multiple sensory modalities. Consequently, central integration and interpretation of complex input modalities is likely to involve coordination between multiple neurogenetic circuits.

Previous studies have established the centrality of the cyclic adenosine monophosphate (cAMP) pathway in the formation of operant and classical conditioned memories and in integration of sensory inputs (Gailey et al., 1984; Bragina and Kamyshev, 2003; Brembs, 2003; Busto et al., 2010). Several mutations associated with defective learning and memory have been mapped to the genetic loci of cAMP pathway components (Dudai et al., 1976; Livingstone et al., 1984; Levin et al., 1992; Feany and Quinn, 1995). 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 show compromised behavioral flexibility during agonistic encounters and are unable to establish dominance hierarchies. Mutants of the neuropeptide *Amnesiac* (pituitary adenylate cyclase activating polypeptide homologue, PACAP; *Amn*), although competent in modifying behavioral patterns during fights and establishing hierarchies, show no loser effect. These studies demonstrate distinct cAMP signaling modalities in specific neural circuits in mediating 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 Dr R. Strauss, University of Mainz, Germany. *dnc*^{1}, *rut*^{2080}*;UAS-rut* and all Gal4 driver lines were obtained from the stock center at Bloomington, 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^{0}C, 60% humidity and a 14h:10h::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 was used to identify individuals. Male – male aggression assays were conducted as described earlier (Chen et al., 2002) with the modification that they were conducted in a six-well chamber. 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^{0}C and 60% humidity and recorded using a Sony DCR-SR47E/S video camera. Fights between socially naïve flies involved three phases. In the ‘fight phase’ a pair of naïve 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, naïve opponents for 60 min. SONY PMB software on Windows OS 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 seconds. Encounters were considered separate if the time interval between them exceeded 2 seconds. Only fights having at least 20 encounters were included in our analysis. 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/flies 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 include: 1. Encounter frequency (encounters per minute measured as total number of encounters divided by total time of fighting); 2. Aggression vigor index (the fraction of time spent fighting in first 10 min from the start of the first encounter); 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 seconds). In the second fights, the 'Draw' outcomes, where 3 Lunge- 3 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 naïve opponent. 'High intensity' draws consisted of experienced loser flies predominantly using lunges while '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 naïve opponents. This may preclude escalation during fights and result in 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 ability of flies to demonstrate the experience dependent loser effect. The loser index was calculated as a difference between the numbers of encounters lost to the encounters won divided by the total number of encounters in the second fight. Within an encounter, if a fly uses aggressive actions like lunging, boxing (rearing up on hind legs and striking the opponent with forelegs), holding (rearing up on 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 responds 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 before (Ali et al., 2011). A group of ten flies, age and size matched, were introduced into a food vial one day prior to testing. The flies were placed in two head to head 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 seconds was scored. The experiment was repeated 10 times for each group and the average pass rate 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 Statistica 8.0 and Graphpad Prism 6.0 statistical software. Various statistical tests were employed to facilitate our analysis including Chi-square 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 establishment of hierarchies within a fight

Pairs of male flies demonstrate stereotyped aggressive behavior towards each other while competing over resources like food or females. These dyadic interactions begin with low aggression maneuvers like wing threat and fencing, which then escalate to high aggression strategies like 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 time scale are stable within a fight and therefore facilitate formation of social dominance hierarchies. We have assigned dominance relationships using a previously developed three lunge – three retreat rule (see Materials and Methods and (Yurkovic et al., 2006)). In wild-type Canton S (CS) flies, 85% of the fights produced dominance relationships (Figure 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 (Livingstone et al., 1984; Levin et al., 1992). *Rut* has 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, rut^{2080} mutant flies were unable to form dominance relationships with all their fights ending in draws (Figure 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, dnc^{1} flies display attenuated

formation of dominance relationships with only 67% of the fights resulting in wins/losses (Figure 1A; $P < 0.01$). The neuropeptide *Amn* is known to stimulate cAMP production via GPCR signaling mediated stimulation of adenylyl cyclase activity (Feany and Quinn, 1995). However, amn^{C651} mutant flies displayed wild-type levels of dominance hierarchies (Figure 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 have indicated status dependent modifications in their usage by winner/loser flies (Yurkovic et al., 2006). In line with previous observations, agonistic interactions resulted in an escalation of aggression (Yurkovic et al., 2006; Trannoy et al., 2016). In aggressive encounters between CS flies, the ultimate winners (as established by the 3 lunge-3 retreat rule; see Materials and Methods) progressively increased the deployment of lunges while the losers increasingly adapted to the retreating behavior (Figure 1B, C). In contrast, rut^{2080} mutant flies were unable to demonstrate any behavioral transitions pertaining to lunges and retreats (Figure 1B, C; $P < 0.001$). dnc^{1} 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 to CS animals (Figure 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 (Figure 1B, C; $P > 0.05$). These results suggest a correlation between experience dependent behavioral plasticity and formation of dominance hierarchies following aggressive encounters.

No significant differences in locomotor activity between wild type and mutant flies were found using a negative geotaxis assay (see Materials and Methods), thus ruling out the possibility of motor deficits in these lines (Figure 2A; $P > 0.05$). Multiple parameters were evaluated to assess the aggression levels of the wild type and mutant lines. Encounter frequency was significantly lower in *rut*^{2080} mutants compared to CS flies but *dnc*^{1} and *amn*^{C651} mutants were comparable to wild-type flies (Figure 2B; $P_{rut} < 0.05$, P_{dnc} and $P_{amn} > 0.05$). Aggression vigor index was compromised in all the mutant lines compared to CS, but was not significantly different between *rut*^{2080}, *dnc*^{1} and *amn*^{C651} flies (Figure 2C; $P_{rut} < 0.001$, P_{dnc} and $P_{amn} < 0.05$).

While our studies implicate lack of behavioral plasticity in *rut*^{2080} mutants in establishing dominance hierarchies in *rut*^{2080} versus *rut*^{2080} fights, it is also possible that the *rut*^{2080} flies are unable to execute high intensity maneuvers like lunges. In *rut* – *rut* fights, neither opponent can adjust their fighting patterns depending upon experience resulting in a lack of escalation beyond low intensity interactions precluding assessment of behavioral changes in *rut*^{2080} flies. We therefore analyzed fights between CS and *rut*^{2080} mutants. CS flies have intact experience dependent behavioral transitions compared to *rut*^{2080} mutants. Therefore pairing CS vs *rut*^{2080} may better reveal behavioral escalation during fights. CS vs *rut*^{2080} fights result in significant dominance hierarchies with 37.5% of fights ending in a win or a loss, though it remains attenuated compared to CS vs CS fights (Figure 3A; $P < 0.001$). Interestingly, *rut*^{2080} flies demonstrate increased lunging and retreating behavior compared to their performance in *rut-rut* fights and also win 9% of the fights against wild-type opponents (Figure 3B, C). *rut*^{2080} flies, when in a *rut* – CS fight, are capable of executing and modifying the frequency of use of both lunges and

retreats, although this ability is remains significantly compromised compared to that in CS flies (in CS – CS fights) (Figure 3B, C; $P < 0.001$ for lunges and $P < 0.05$ for retreats). The usage of offensive and defensive strategies for *rut*^{2080} flies against a wild type opponent are significantly better than *rut*-*rut* fights (Figure 3B, C; $P < 0.001$ for lunges and $P < 0.01$ for retreats). These results suggest that *rut*^{2080} flies are capable of executing high intensity maneuvers but are compromised in modifying its frequency during a fight.

Furthermore, *dnc*^{1} mutants are unable to modify fighting strategies as well as CS flies but they are less compromised compared to the *rut*^{2080} flies in *rut*-*rut* fights (Figure 1A, B, C). Consistent with the association of ability to modify fighting patterns with the establishment of dominance relationships, *dnc* mutants do establish hierarchies though less efficiently than wild-type CS flies (Figure 1A). Similarly, *rut*^{2080} flies against wild type opponents demonstrate compromised plasticity and attenuated dominance structures (Figure 3A, B, C). In fact, usage of lunges/retreats by *rut*^{2080} flies in *rut* – CS fights is comparable to those seen in *dnc*^{1} (Figure 3B, C; $P > 0.05$ for lunges and retreats).

Interestingly, the aggression vigor index is similar for *rut* - CS and *rut* - *rut* fights (Figure 3D; $P > 0.05$). This again suggests that inability to develop hierarchical statuses and compromised behavioral plasticity seen in *rut* mutants may be independent of aggressiveness.

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

1

2 **Specific neural circuits are recruited in cAMP-mediated behavioral plasticity**

3 We next asked which components of the *Drosophila* brain were involved in the status-
4 dependent modifications of aggressive strategies and formation of dominance
5 relationships. As multiple sensory modalities are likely to be involved in aggressive
6 encounters, a complex pattern of recruitment of neuronal circuits may underlie the
7 integration of these sensory inputs (Wang and Anderson, 2010; Wang et al., 2011;
8 Yoon et al., 2013; Asahina et al., 2014; Lim et al., 2014; Ramin et al., 2014; Hoopfer et
9 al., 2015; Hoopfer, 2016). To test this, we used the UAS-Gal4 binary system to express
10 the *Rut* gene product in restricted neuronal populations in a rut^{2080} mutant background.
11 We chose well-characterized Gal4 drivers that have restricted expression in defined
12 neuronal populations of the mushroom body and central complex of the fly brain as
13 these regions are strongly implicated as central integrators in multiple behavioral
14 paradigms (Table 1) (Joiner and Griffith, 1999; Torroja et al., 1999; Zars et al., 2000b;
15 Zars et al., 2000a; Neuser et al., 2008; Aso et al., 2009; Blum et al., 2009; Pan et al.,
16 2009). Pan-neuronal expression of *Rut* using the *Appl*-Gal4 driver completely rescued
17 the inability to form dominance relationships seen in rut^{2080} mutants (Figure 4A; $P <$
18 0.001). Similar restoration of dominance hierarchies to wild-type levels was seen with
19 expression limited to the α/β and γ lobe neurons of the mushroom body using the c309
20 driver (Figure 4A; $P < 0.001$). Next we tested a panel of Gal4 drivers expressing *Rut* in
21 different subpopulations of neurons in the MB and the central complex of rut^{2080} flies
22 (Table 1). UAS-Rutabaga driven by Gal4 drivers c739, 201Y, c305a and c205, but not
23 c819, partially rescued the inability of rut^{2080} to form hierarchical relationships (Figure

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 central complex does not appear to be involved in this function (Figure 4A; $P > 0.05$).

Analysis of behavioral patterns revealed that distinct neuronal circuits mediate *rut*-dependent behavioral plasticity within a fight (Figure 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 rut^{2080} mutants (Figure 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 neurons of the mushroom body using the c309 driver (Figure 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 increase of lunges and retreats seen in rut^{2080} flies (Figure 4D, E, F, G; $P < 0.01$). The ellipsoid body (EB) of the central complex does not appear to be involved in this function as no rescue was observed with the EB only driver (Figure 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 wild-type) 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 (Figure 5A – E; $P > 0.05$). These results suggest that though 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 naïve opponents, after a 60-minute 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 3 lunge-3 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 categorized as draws. For statistical analysis, high and low intensity draws were grouped with wins and losses, respectively. Consistent with previous studies (Yurkovic

et al., 2006; Trannoy et al., 2016), wild-type CS loser flies always lost to naïve opponents in their second fights (Figure 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 (Figure 6A; $P < 0.001$). *dnc*^{1} also displayed no significant loser effect with 18% losses, 18% wins and 64% draws (Figure 6A; $P < 0.001$).

Surprisingly, *amn*^{C651} flies did not show any loser effect. 50% of analyzed fights resulted in losses and the remaining in wins for losers (Figure 6A; $P < 0.001$). Unlike *rut* and *dnc*, *amn*^{C651} flies develop dominant-subordinate relations as efficiently as CS in their first fights (Figure 1C) but are 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 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 (Figure 6B). In contrast, the *amn*^{C651} and *dnc*^{1} losers, and the experienced *rut*^{2080} flies displayed significantly lower loser indices (Figure 6B; $P < 0.001$).

These results show that *dnc*^{1} and *rut*^{2080} flies, which display 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.

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 wild-type 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 wild-type CS flies (Figure 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 (Figure 7A; $P < 0.001$). Loser index in these flies was significantly greater than controls (Figure 7B; $P < 0.001$) and comparable to wild-type flies (Figure 7B; $P > 0.05$).

Interestingly, expression of UAS-*Rut* limited to individual substructures of the MB and CC was not sufficient to rescue impaired loser effect observed in *rut* flies (Figure 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 naïve opponents but failed to show a robust loser effect (Figure 7A; $P < 0.001$). As expected, there was no significant change in the loser index for these flies (Figure 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 (Figure 7; $P > 0.05$).

Our results suggest the involvement of the MBs, limited to the combined processing by α/β and γ neurons, 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 (Gailey et al., 1984; Livingstone et al., 1984; Bragina and Kamyshev, 2003; 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 to CS, both *rut* and *dnc* are unable to display experience-dependent modification of fighting strategies and are deficient in generating losers and winners. *amn* flies, however, modify fighting strategies and establish hierarchal relationships comparable to CS flies. Our 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 reduction in aggression vigor as compared to CS. This includes *amn*, which displays 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 suggest 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 observed 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 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). Mushroom bodies, which are central to olfactory learning, have been previously correlated with changes in 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 neuronal groups of the MB (the α/β, γ and α'/β' neurons) were implicated, the FB but not the EB of the central complex was also found to be functionally involved. The FB has been previously reported to mediate visual learning of specific pattern features like “elevation” and “contour orientation” (Liu et al., 2006; Pan

et al., 2009). A recent study has underscored the importance of CC neurons in aggression by demonstrating the modulation of aggressive behavior by dopaminergic PPM3 neurons that synapse onto the FB (Alekseyenko et al., 2013).

The robust loser effect seen in wild-type flies is absent in *rut*, *dnc* and *amn* mutant lines. As in their first fights, all *rut*-*rut* second fights end 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 lack the loser effect, though they can adjust fighting strategies and develop dominance relationships as well as wild-type flies. The *Amnesiac* 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 short-term memory 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). As *Amn* is a secreted neuropeptide, future rescue experiments using the *Amn* receptor will be useful to determine the neural circuits subserving *Amn*-dependent establishment of the loser effect. A less robust, short duration winner effect has been reported in flies (Trannoy and Kravitz, 2016; Trannoy et al., 2016). It remains to be seen if analogous cAMP signaling and circuit features are involved in 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, though social hierarchy was rescued. We hypothesize that two distinct *Rut* dependent memory traces facilitate 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 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, while odor-shock coincidence detection maps to the γ neurons (Dubnau et al., 2001; Qin et al., 2012; Dubnau and Chiang, 2013). 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 critical for the establishment of the loser effect. However, 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 (Chan and Kravitz,

2007; Dierick and Greenspan, 2007; Hoyer et al., 2008; Dankert et al., 2009; Certel et al., 2010; Wang and Anderson, 2010; Liu et al., 2011; Zwarts et al., 2012; Alekseyenko et al., 2013; Alekseyenko et al., 2014; Andrews et al., 2014; Luo et al., 2014; Yuan et al., 2014; Kohl et al., 2015; Hoopfer, 2016). However, these studies focus on aggression levels and do not directly assess behavioral plasticity and memory components in aggression. A recent study indicated that *rut*^{2080} and *amn*^{1} 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, *rut*^{2080} has low proclivity to engage in the first 10 min of fights, fail to form dominance relations 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, rescue of *Rut* restores dominance patterns but not aggressiveness. Therefore, low aggression is not correlated with the inability to establish winner-loser statuses in a fight.

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 peptidergic activity of the neuropeptide amnesiac (*Amn*). 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 agnostic 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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1 **FIGURE LEGENDS**

2 **Figure 1.** cAMP signaling is required for establishing dominance relationships.

3 (*A*) CS and *amn*^{C651} flies form stable dominance relationships (win/loss) in 85% and
4 82% in their first fights, respectively. *rut*^{2080} displayed no dominance structures while
5 *dnc*^{1} showed dominance in 67% of their fights. (Two-tailed Chi-square test; ** $P < 0.01$,
6 *** $P < 0.001$, n=30). *, indicates comparison with CS. (*B*) CS winner flies progressively
7 increase lunging. A similar trend was seen with *amn*^{C651}flies ($P > 0.05$). In contrast,
8 *rut*^{2080} ($P < 0.001$) and *dnc*^{1} ($P < 0.01$) flies demonstrated significantly deficient lunging
9 trends. (Two factor repeated measures ANOVA followed by post hoc Tukey's multiple
10 comparison test, *'P'* is the interaction term, mean ± SEM, n=30). (*C*) Both CS and
11 *amn*^{C651}loser flies ($P > 0.05$) showed progressive increase in retreats while this was
12 significantly compromised in *rut*^{2080} ($P < 0.001$) and *dnc*^{1} ($P < 0.01$).

13 *dnc*^{1} mutants, although compromised in modifying fighting strategies compared to CS
14 flies, demonstrate better trends compared to *rut*^{2080} mutants ($P < 0.001$ for lunges; $P <$
15 0.01 for retreats) (Two factor repeated measures ANOVA followed by post hoc Tukey's
16 multiple comparison test, *'P'* is the interaction term, mean ± SEM, n=30).

17 Total number of lunges/retreats in every three successive encounters is analyzed and
18 their mean is reported. SEM: Standard error of the mean.

19

20 **Figure 2.** Aggression levels in cAMP pathway mutant flies.

21 (*A*) Locomotor activity in flies was analyzed using negative geotaxis assay. No
22 significant differences were observed between CS and cAMP pathway mutants in their

average pass rate (One factor ANOVA followed by post hoc Tukey's multiple comparison test, mean ± SEM, n=30). (*B*) Encounters per minute is lower in *rut*^{2080} flies ($P < 0.05$) but is comparable in *amn*^{c651} and *dnc*^{1} flies compared to CS flies ($P > 0.05$) (One factor ANOVA followed by post hoc Tukey's multiple comparison test, mean ± SEM n=30). *, indicates comparison with CS. (*C*) Aggression vigor index, defined as the fraction of time spent fighting in first 10 minutes, is significantly lower in *rut*^{2080} ($P < 0.001$), *amn*^{c651} ($P < 0.05$) and *dnc*^{1} ($P < 0.05$) flies in comparison to CS flies (One factor ANOVA followed by post hoc Tukey's multiple comparison test, mean ± SEM n=30). *, indicates comparison with CS. (*D*) Latency to engage in an encounter is not significantly different in *rut*^{2080} ($P > 0.05$), *dnc*^{1} ($P > 0.05$) and *amn*^{c651} ($P > 0.05$) flies in comparison to CS flies. (One factor ANOVA followed by post hoc Tukey's multiple comparison test, mean ± SEM ,n=30). SEM: Standard error of the mean.

Figure 3. *rut*^{2080} flies display improved lunging/retreating behavior against wild type opponents.

(*A*) The *rut*^{2080} flies show attenuated dominance structures against a wild type opponent compared to CS v CS fights ($P < 0.001$) but improved hierarchical relationships compared to *rut*^{2080} v *rut*^{2080} fights ($P < 0.01$) (Two-tailed Chi-square test; *** $P < 0.001$, $^{++}$ $P < 0.01$, n=25). *, indicates comparison with CS and +, with *rut*^{2080}. (*B*) and (*C*) The *rut*^{2080} flies against a wild type opponent demonstrate significantly better status-dependent modification of lunges/retreats compared to *rut*^{2080} v *rut*^{2080} fights ($P < 0.001$ for lunges and $P < 0.01$ for retreats) and is comparable to *dnc*^{1} flies ($P > 0.05$). The

lunge/retreat trends for *rut*^{2080} flies in *rut*^{2080} v CS fights are compromised compared to 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, mean ± SEM, n=20).

Total number of lunges/retreats in every three successive encounters is analyzed and their mean is reported. SEM: Standard error of the mean

Figure 4. Distinct neuronal circuits mediate behavioral plasticity during agnostic encounters.

Restricted expression of *Rut* in specific sub-population 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 is rescued upon expression of UAS-*Rut* (in a *rut*^{2080} background) pan-neuronally (Appl), in the α/β and γ lobes of the 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 FB. No rescue was seen upon reintroduction of *Rut* to the EB (c819). (Two-tailed Chi-square test; ** $P < 0.01$, *** $P < 0.001$, $^{+++}$ $P < 0.001$, n≥15). *, indicates comparison with CS and +, with *rut*^{2080}; UAS-*Rut*/+. (*B*) - (*G*) Ability to progressively increase 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,

1 $P < 0.01$ for retreats). EB (c819; $P > 0.05$ for lunges and retreats) expression did not
2 show any rescue. (*B*) and (*C*) are comparisons of *Appl*-Gal4 with CS and no Gal4
3 control, (*D*) and (*E*) are comparisons of MB restricted Gal4 drivers (c309, c739, 201y
4 and c305a) with no Gal4 control and (*F*) and (*G*) comparisons are of central complex
5 restricted Gal4 drivers (c205 and c819) with no Gal4 control (Two factor repeated
6 measures ANOVA followed by post hoc Tukey's multiple comparison test, *'P'* is the
7 interaction term, mean ± SEM, n≥15).

8 Total number of lunges/retreats in every three successive encounters is analyzed and
9 their mean is reported. SEM: Standard error of the mean

10

11 **Figure 5.** Aggression levels are unchanged upon rescue of aggression-associated
12 learning.

13 (*A*) In comparison to rut^{2080};UAS-Rut/+ control flies, *Rut* expression in α/β and γ lobes of
14 the MB (c309; $P > 0.05$) or pan-neuronally (Appl; $P > 0.05$) resulted in a comparable
15 number of encounter frequencies (One factor ANOVA followed by post hoc Dunnett's
16 multiple comparison test, mean ± SEM, n=20). (*B*) There is no significant improvement,
17 as compared to rut^{2080};UAS-Rut/+ control flies, in aggression vigor index in response to
18 *Rut* expression either pan-neuronally or in the α/β and γ lobes ($P > 0.05$; One factor
19 ANOVA followed by post hoc Dunnett's multiple comparison test, mean ± SEM, n=20).
20 (*C*) Latency to engage in an encounter ($P > 0.05$) remains comparable upon *Rut*
21 expression using either Appl or c309 Gal4 driver lines in comparison to rut^{2080};UAS-
22 Rut/+ control flies (One factor ANOVA followed by post hoc Dunnett's multiple
23 comparison test, mean ± SEM, n=20).

1 SEM: Standard error of the mean.

2

3 **Figure 6.** cAMP signaling is necessary for developing loser mentality.

4 (*A*) CS losers lost all their second fights against naïve opponents while *amn*^{C651} flies lost
5 and won equal number of fights. *dnc*^{1} flies show weak dominance relationships and
6 their interactions largely ended in draws, again suggestive of memory defects. All
7 previously experienced *rut*^{2080} flies engaged in draws. (Two-tailed Chi-square test; *** P
8 < 0.001, n≥20). *, indicates comparison with CS. (*B*) Loser index assesses the
9 experience dependent modulation of fighting strategies of experienced flies in their
10 second fights against a naïve opponent. *rut*^{2080}, *dnc*^{1} and *amn*^{C651} flies, all showed
11 severely compromised loser indices compared to CS. (One factor ANOVA followed by
12 post hoc Dunnett's multiple comparison test, *** P < 0.001, mean ± SEM, n≥20). *,
13 indicates comparison with CS.

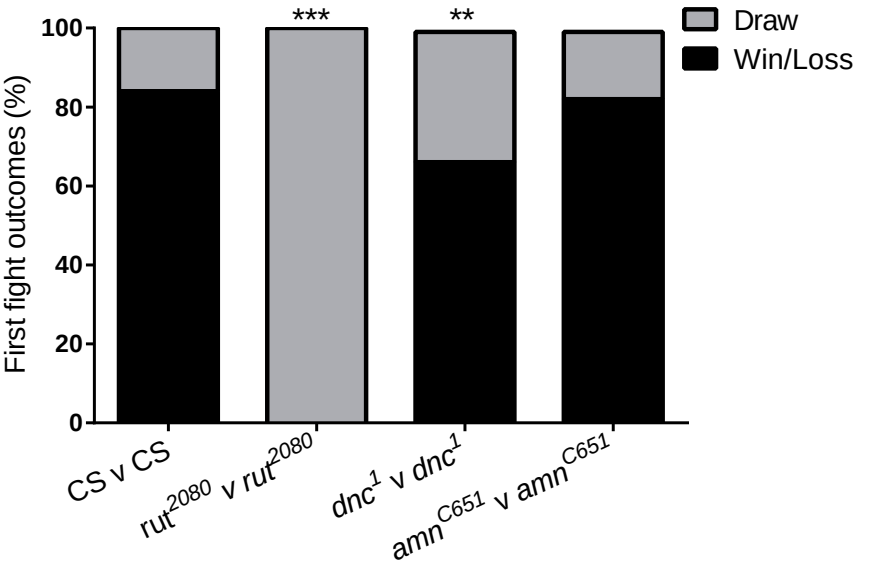
14

15 **Figure 7**. Recruitment of specific neuronal circuits for the development of the loser
16 effect.

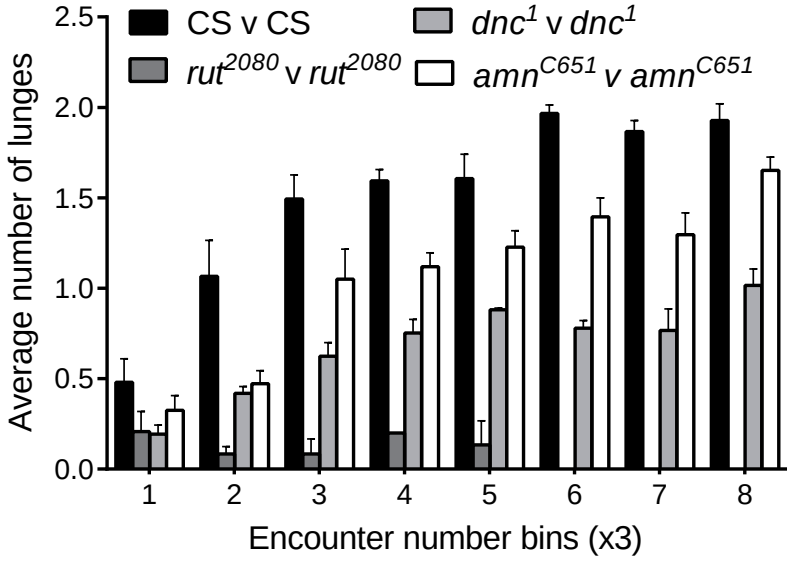
17 (*A*) Rescue experiments expressing *Rut* (in a *rut*^{2080} background) either pan-neuronally
18 (Appl) or in the α/β and γ lobes of the MB (c309) showed a strong recovery of the loser
19 effect. Expression in the α/β lobes (c739), γ lobes (201Y), α'/β' lobes (c305a) and in the
20 FB of *rut* mutants resulted in partial recovery of dominance relationships. No rescue
21 was seen upon the reintroduction of *Rut* in the EB (c819) of *rut*^{2080}. (Two-tailed Chi-
22 square test;*** P < 0.001, $^{+++}$ P < 0.001 n≥15). *, indicates comparison with CS and +,

with *rut*^{2080}; UAS-*Rut*/+. (*B*) Loser index analysis revealed that pan-neuronal and α/β and γ lobe combined expression is 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, *** $P < 0.001$, $^{+++}$ $P < 0.001$, mean ± SEM, n≥15). *, indicates comparison with CS and +, with *rut*^{2080}; UAS-*Rut*/+. SEM: Standard error of the mean.

A


***
**
Draw
Win/Loss
First fight outcomes (%)
100
80
60
40
20
0
CS v CS
rut2080 v rut2080
dnc1 v dnc1
amnC651 v amnC651

B


CS v CS
rut2080 v rut2080
dnc1 v dnc1
amnC651 v amnC651
Average number of lunges
2.5
2.0
1.5
1.0
0.5
0.0
1
2
3
4
5
6
7
8
Encounter number bins (x3)

C

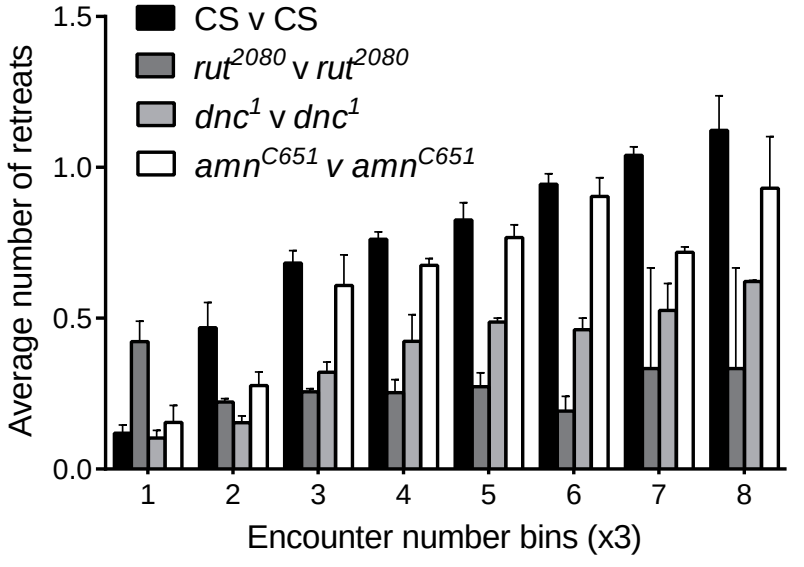

CS v CS
rut2080 v rut2080
dnc1 v dnc1
amnC651 v amnC651
Average number of retreats
1.5
1.0
0.5
0.0
1
2
3
4
5
6
7
8
Encounter number bins (x3)

Figure 1

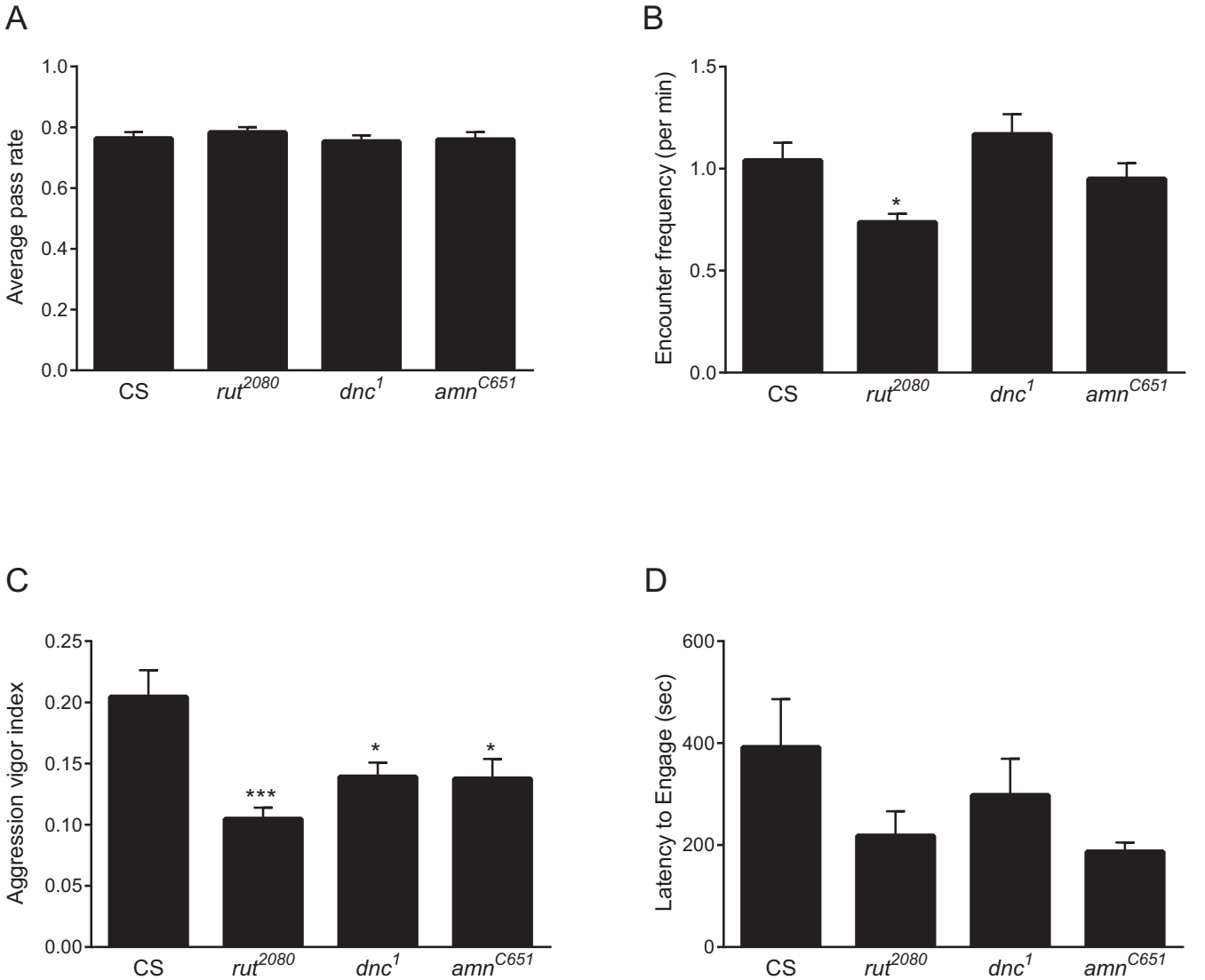

A
Average pass rate
1.0
0.8
0.6
0.4
0.2
0.0
CS
rut2080
dnc1
amnC651
B
Encounter frequency (per min)
1.5
1.0
0.5
0.0
*
CS
rut2080
dnc1
amnC651
C
Aggression vigor index
0.25
0.20
0.15
0.10
0.05
0.00
***
*
*
CS
rut2080
dnc1
amnC651
D
Latency to Engage (sec)
600
400
200
0
CS
rut2080
dnc1
amnC651

Figure 2

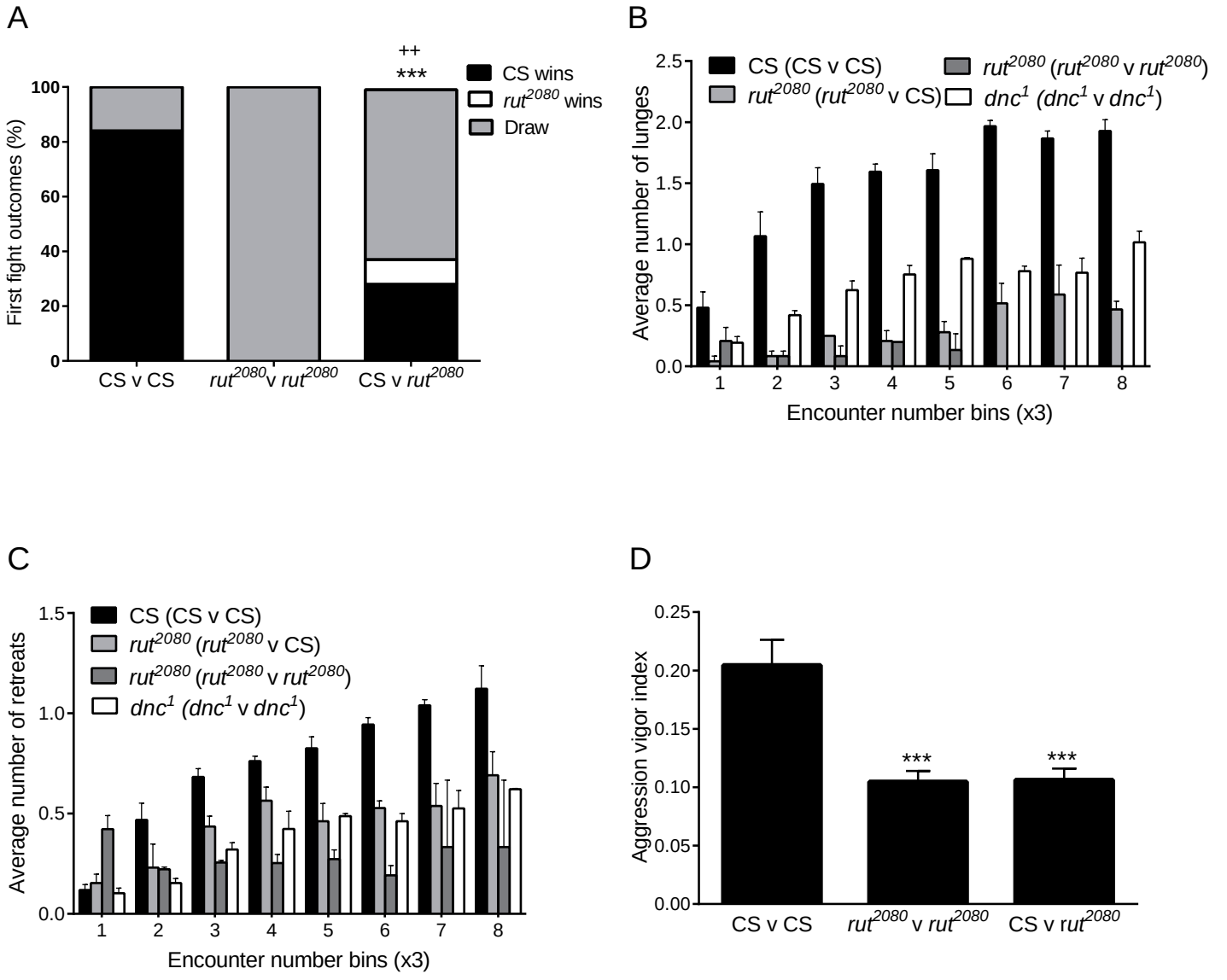

A
First fight outcomes (%)
100
80
60
40
20
0
++
***
CS wins
rut2080 wins
Draw
CS v CS
rut2080 v rut2080
CS v rut2080
B
Average number of lunges
2.5
2.0
1.5
1.0
0.5
0.0
CS (CS v CS)
rut2080 (rut2080 v CS)
rut2080 (rut2080 v rut2080)
dnc1 (dnc1 v dnc1)
1
2
3
4
5
6
7
8
Encounter number bins (x3)
C
Average number of retreats
1.5
1.0
0.5
0.0
CS (CS v CS)
rut2080 (rut2080 v CS)
rut2080 (rut2080 v rut2080)
dnc1 (dnc1 v dnc1)
1
2
3
4
5
6
7
8
Encounter number bins (x3)
D
Aggression vigor index
0.25
0.20
0.15
0.10
0.05
0.00
***
***
CS v CS
rut2080 v rut2080
CS v rut2080

Figure 3

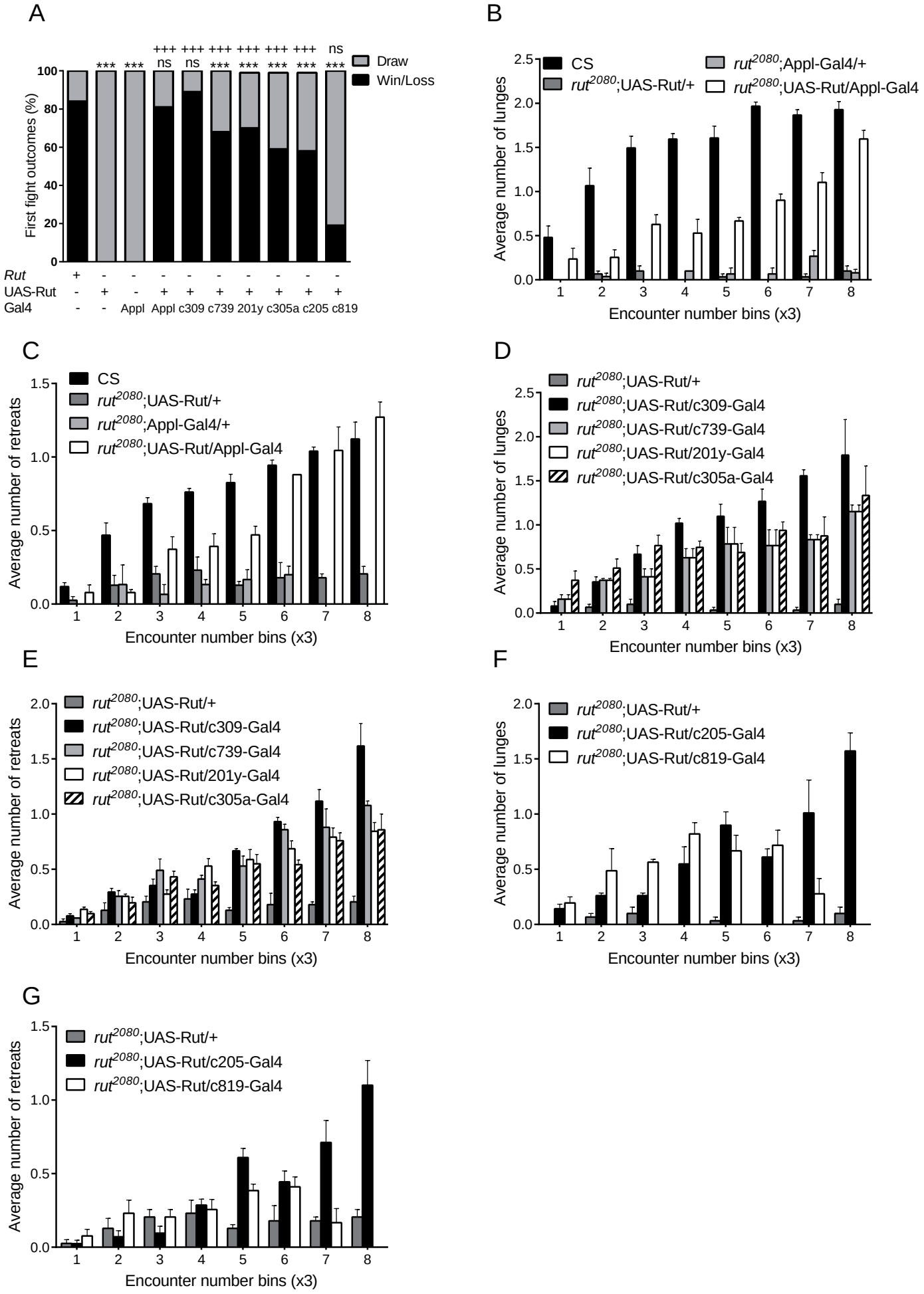

A
First fight outcomes (%)
Draw
Win/Loss
Rut
UAS-Rut
Gal4
Appl
Appl
c309
c739
201y
c305a
c205
c819
B
Average number of lunges
CS
rut2080;UAS-Rut/+
rut2080;Appl-Gal4/+
rut2080;UAS-Rut/Appl-Gal4
Encounter number bins (x3)
C
Average number of retreats
CS
rut2080;UAS-Rut/+
rut2080;Appl-Gal4/+
rut2080;UAS-Rut/Appl-Gal4
Encounter number bins (x3)
D
Average number of lunges
rut2080;UAS-Rut/+
rut2080;UAS-Rut/c309-Gal4
rut2080;UAS-Rut/c739-Gal4
rut2080;UAS-Rut/201y-Gal4
rut2080;UAS-Rut/c305a-Gal4
Encounter number bins (x3)
E
Average number of retreats
rut2080;UAS-Rut/+
rut2080;UAS-Rut/c309-Gal4
rut2080;UAS-Rut/c739-Gal4
rut2080;UAS-Rut/201y-Gal4
rut2080;UAS-Rut/c305a-Gal4
Encounter number bins (x3)
F
Average number of lunges
rut2080;UAS-Rut/+
rut2080;UAS-Rut/c205-Gal4
rut2080;UAS-Rut/c819-Gal4
Encounter number bins (x3)
G
Average number of retreats
rut2080;UAS-Rut/+
rut2080;UAS-Rut/c205-Gal4
rut2080;UAS-Rut/c819-Gal4
Encounter number bins (x3)

Figure 4

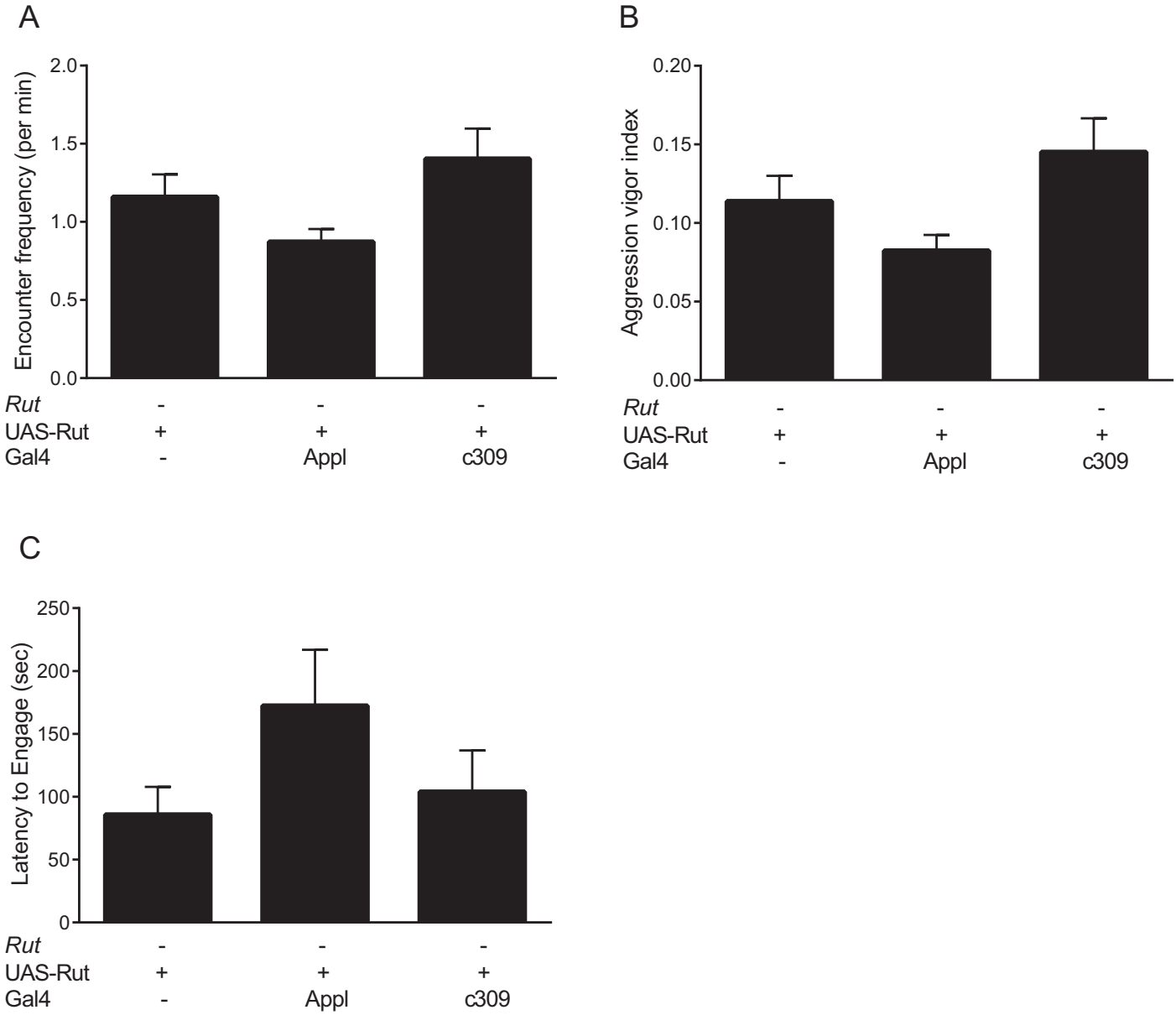

A
Encounter frequency (per min)
2.0
1.5
1.0
0.5
0.0
Rut - - -
UAS-Rut + + +
Gal4 - Appl c309
B
Aggression vigor index
0.20
0.15
0.10
0.05
0.00
Rut - - -
UAS-Rut + + +
Gal4 - Appl c309
C
Latency to Engage (sec)
250
200
150
100
50
0
Rut - - -
UAS-Rut + + +
Gal4 - Appl c309

Figure 5

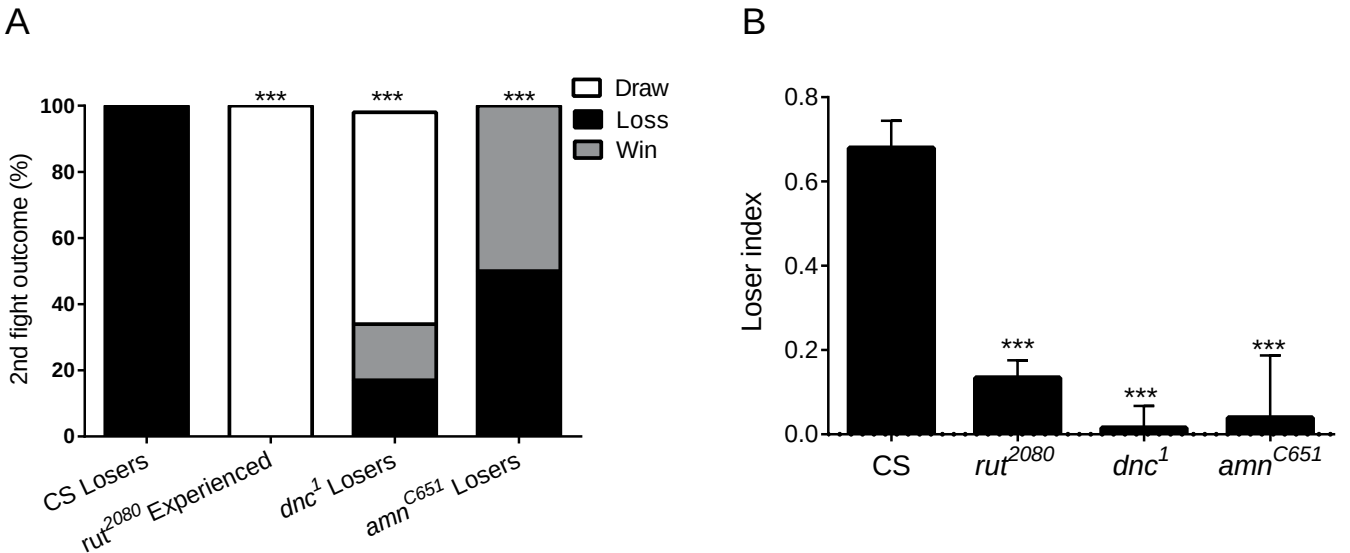

A
B
Draw
Loss
Win
2nd fight outcome (%)
100
80
60
40
20
0
***
***
***
CS Losers
rut2080 Experienced
dnc1 Losers
amnC651 Losers
Loser index
0.8
0.6
0.4
0.2
0.0
***
***
***
CS
rut2080
dnc1
amnC651

Figure 6

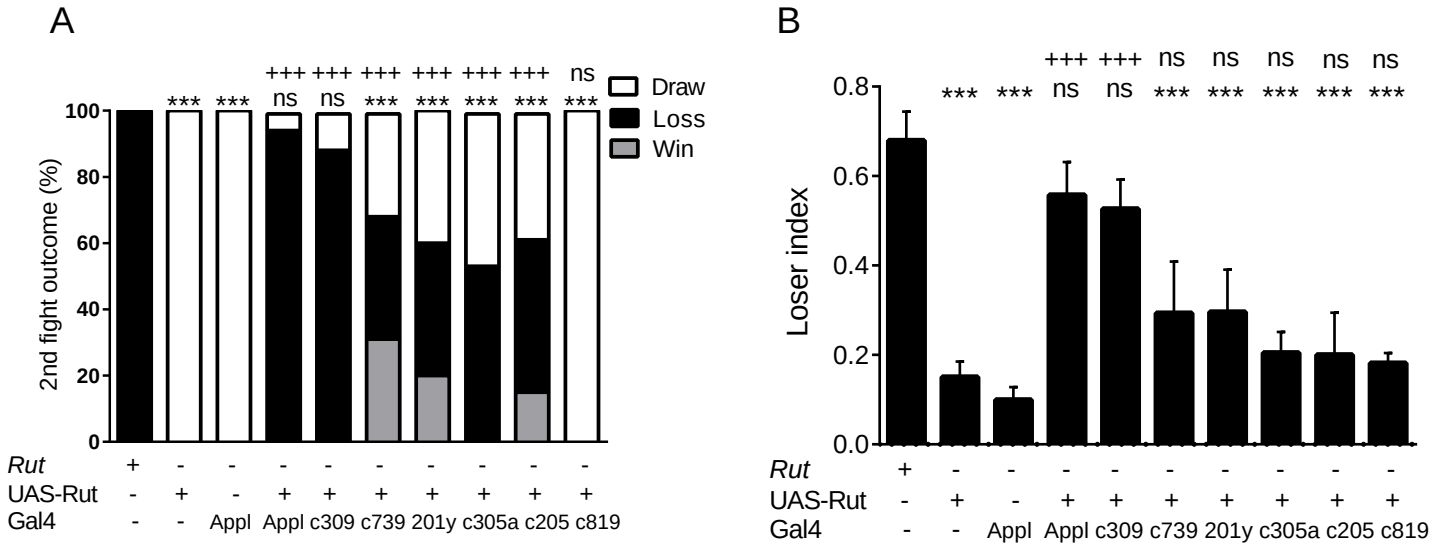

A
B
2nd fight outcome (%)
Draw
Loss
Win
Loser index
Rut
UAS-Rut
Gal4
Appl
c309
c739
201y
c305a
c205
c819

Figure 7

1 **Table 1: Expression patterns of Gal4 drivers used and rescue results**

Gal4 drivers	Expression pattern	Rescue of dominance relationships (1st fight)	Rescue of loser effect (2nd fight)
Appl	Pan-neuronal (Torroja et al., 1999; Neuser et al., 2008)	Winners: Y* Losers: Y*	Y*
c309	αβ and γ lobes of the MB (Joiner and Griffith, 1999; Aso et al., 2009; Blum et al., 2009)	Winners: Y* Losers: Y*	Y*
c739	αβ lobes of the MB (Joiner and Griffith, 1999; Zars et al., 2000a; Aso et al., 2009; Blum et al., 2009)	Winners: Y* Losers: Y*	N
201Y	γ lobes and αβ lobes (sparse) of the MB (Joiner and Griffith, 1999; Zars et al., 2000b; Aso et al., 2009; Blum et al., 2009)	Winners: Y† 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† 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

2

3 Y: yes; N: no; MB: Mushroom bodies; EB: Ellipsoid body; FB: Fan shaped body; AL:
4 Antennal lobes

5 (*, *P<0.001*; †, *P<0.01*; Indices were compared between rut^{2080}/Y; UAS-rut/+ and
6 rut^{2080}/Y; UAS-rut/Gal 4).