1   **Distinct cAMP signaling modalities mediate behavioral flexibility and**

2   **consolidation of social status in *Drosophila* aggression.**

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4   Nitin Singh Chouhan, Krithika Mohan and Aurnab Ghose*

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7   Indian Institute of Science Education and Research (IISER) Pune

8   Dr. Homi Bhabha Road, Pune 411 008, INDIA

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15   * Corresponding author:

16   Aurnab Ghose

17   Indian Institute of Science Education and Research (IISER) Pune

18   Dr. Homi Bhabha Road, Pune 411 008, INDIA

19   Email: aurnab@iiserpune.ac.in

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1   **ABSTRACT**

2   Social rituals, like male-male aggression in *Drosophila*, are often stereotyped and its

3   component behavioral patterns modular. The likelihood of transition from one behavioral

4   pattern to another is malleable by experience and confers flexibility to the behavioral

5   repertoire. Experiential modification of innate aggressive behavior in flies alters fighting

6   strategies during fights and establishes dominant-subordinate relationships. Dominance

7   hierarchies resulting from agonistic encounters are consolidated to longer lasting social

8   status-dependent behavioral modifications resulting in a robust loser effect.

9   We show that cAMP dynamics regulated by *Rut* and *Dnc* but not the neuropeptide *Amn*,

10   in specific neuronal groups of the mushroom body and central complex, mediate

11   behavioral plasticity necessary to establish dominant-subordinate relationships. *rut* and

12   *dnc* mutant flies are unable to alter fighting strategies and establish dominance

13   relationships during agonistic interactions. This real time flexibility during a fight is

14   independent of changes in aggression levels. Longer-term consolidation of social status

15   in the form of a loser effect, however, requires additional *Amn* neuropeptide mediated

16   inputs to cAMP signaling and involves a circuit-level association between the α/β and γ

17   neurons of the mushroom body.

18   Our findings implicate distinct modalities of cAMP signaling in mediating plasticity of

19   behavioral patterns in aggressive behavior and in the generation of a temporally stable

20   memory trace that manifests as a loser effect.

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1   INTRODUCTION

2   Aggression is a social behavior involving competitive interactions over resources to

3   ensure reproductive success and survival. Conspecific agonistic interactions may result

4   in dominance hierarchies where those at higher levels have better access to resources.

5   The social ritual of *Drosophila* aggression is modular and comprises of stereotyped

6   behavioral patterns analogous to a sequence of fixed action patterns. These sequences

7   are present in full complexity in socially naive animals and appear to be pre-wired in the

8   nervous system (Chen et al., 2002; Lim et al., 2014; Hoopfer, 2016). However, during

9   aggressive encounters, experiential inputs can alter the likelihood of transitions between

10   specific patterns and lead to experience-dependent plasticity (Yurkovic et al., 2006;

11   Trannoy et al., 2016). Initial agonistic interaction between naïve flies constitutes a

12   conditioning phase where male flies employ a combination of offensive and defensive

13   fighting strategies and display real time, experience-dependent plasticity to establish

14   dominant or subordinate status (Chen et al., 2002; Yurkovic et al., 2006). In flies, the

15   learned subordinate social status is consolidated into a long lasting loser effect,

16   resulting in an increased probability of losing agonistic encounters against familiar as

17   well as unfamiliar opponents (Yurkovic et al., 2006; Trannoy and Kravitz, 2016; Trannoy

18   et al., 2016).

19   Aggression is a complex social behavior influenced by a combinatorial interplay

20   between genetic factors, environmental cues and experience. Population level selection

21   for elevated aggression has implicated several genes, which show significant changes

22   in their expression (Dierick and Greenspan, 2006; Wang et al., 2008; Edwards et al.,

23   2009). However, a single social defeat can lead to the development of a robust loser

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1   effect in such hyperaggressive lines, underscoring the modulatory role of experience in

2   relation to intrinsic abilities (Penn et al., 2010). While regulatory activities of

3   pheromones, neuromodulatory agents and neurotransmitters associated with

4   aggression levels are well documented (Dierick and Greenspan, 2007; Hoyer et al.,

5   2008; Certel et al., 2010; Wang and Anderson, 2010; Wang et al., 2011; Alekseyenko et

6   al., 2013; Alekseyenko et al., 2014; Andrews et al., 2014; Luo et al., 2014; Yuan et al.,

7   2014; Kohl et al., 2015; Hoopfer, 2016), little is known about the neurogenetic

8   underpinnings of the relevant learning and memory components.

9   Social experience significantly alters activity of flies and involves both classical and

10   operant conditioning components (Kamyshev et al., 2002). Experience-dependent

11   behavioral plasticity has also been observed in courtship conditioning assays where

12   males, after an unsuccessful mating experience, modulate their courtship behavior

13   (Siegel and Hall, 1979). Learning and memory in a social environment is likely to be

14   influenced by several factors, including the combinatorial inputs from multiple sensory

15   modalities. Consequently, central integration and interpretation of complex input

16   modalities is likely to involve coordination between multiple neurogenetic circuits.

17   Previous studies have established the centrality of the cyclic adenosine monophosphate

18   (cAMP) pathway in the formation of operant and classical conditioned memories and in

19   integration of sensory inputs (Gailey et al., 1984; Bragina and Kamyshev, 2003; Brembs,

20   2003; Busto et al., 2010). Several mutations associated with defective learning and

21   memory have been mapped to the genetic loci of cAMP pathway components (Dudai et

22   al., 1976; Livingstone et al., 1984; Levin et al., 1992; Feany and Quinn, 1995). In this

23   study, we implicate the cAMP second messenger pathway in behavioral plasticity during

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1   aggressive encounters and in the development of the loser effect. *Rutabaga* (calcium-

2   calmodulin-dependent adenylyl cyclase; *Rut*) and *Dunce* (cAMP phosphodiesterase;

3   *Dnc*) mutants show compromised behavioral flexibility during agonistic encounters and

4   are unable to establish dominance hierarchies. Mutants of the neuropeptide *Amnesiac*

5   (pituitary adenylate cyclase activating polypeptide homologue, PACAP; *Amn*), although

6   competent in modifying behavioral patterns during fights and establishing hierarchies,

7   show no loser effect. These studies demonstrate distinct cAMP signaling modalities in

8   specific neural circuits in mediating behavioral flexibility leading to the establishment of

9   dominance relationships and in the long-term consolidation of social status.

1   **MATERIALS AND METHODS**

2   **Fly stocks and maintenance**

3   Canton S (CS), rut^{2080} and amn^{c651} lines were obtained from Dr R. Strauss, University of

4   Mainz, Germany. dnc^1, rut^{2080};UAS-rut and all Gal4 driver lines were obtained from the

5   stock center at Bloomington, USA. All fly lines were backcrossed for at least nine

6   generations. The autosomes of the X-linked cAMP pathway mutant alleles used were

7   equilibrated to that of the control strain. Stocks were maintained at 25^0C, 60% humidity

8   and a 14h:10h::light:dark cycle on standard food.

9   **Analysis of aggressive behavior**

10   Freshly eclosed flies were isolated and kept in social isolation for a period of 4-5 days

11   before testing. Acrylic paint marks on the upper thoracic region was used to identify

12   individuals. Male – male aggression assays were conducted as described earlier (Chen

13   et al., 2002) with the modification that they were conducted in a six-well chamber. A

14   food cup with yeast paste and a headless female was placed inside a six-well plate

15   chamber. A pair of marked, un-anesthetized, age- and size- matched male flies was

16   introduced into the chamber through gentle aspiration. All fights were conducted at 25^0C

17   and 60% humidity and recorded using a Sony DCR-SR47E/S video camera. Fights

18   between socially naïve flies involved three phases. In the 'fight phase' a pair of naïve

19   flies was allowed to fight for 60 min, this was followed by a 'rest phase' of 60 min where

20   flies were returned to their original food vials and finally a 'test phase' where previously

21   matched flies fought against unfamiliar, naïve opponents for 60 min. SONY PMB

22   software on Windows OS was used for video playback and the fights were manually

23   curated. In all cases the analyzer was blind to the genotype of the fly. Fights were

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1   analyzed on basis of encounters that involved physical interactions and lasted at least 3

2   seconds. Encounters were considered separate if the time interval between them

3   exceeded 2 seconds. Only fights having at least 20 encounters were included in our

4   analysis. Winners and losers were designated based on an ethologically characterized

5   three lunge-three retreat rule (developed in (Yurkovic et al., 2006)). Lunge is defined as

6   a maneuver where a fly rears up on its hind legs and collapses on the opponent (Chen

7   et al., 2002; Zwarts et al., 2012). A retreat occurs when a fly, in response to an offensive

8   action, runs/flies away from the opponent or the food cup (territory) (Chen et al., 2002;

9   Zwarts et al., 2012). Within a trial, a fly was assigned a status of 'winner' if it used three

10   continuous lunges (without any interim retreats) and its opponent executed three

11   corresponding retreats (with no intervening lunges) (Yurkovic et al., 2006). The latter

12   was designated as a 'loser'.

13   We analyzed various parameters to investigate aggression in flies across genotypes.

14   They include: 1. Encounter frequency (encounters per minute measured as total

15   number of encounters divided by total time of fighting); 2. Aggression vigor index (the

16   fraction of time spent fighting in first 10 min from the start of the first encounter); 3.

17   Latency to engage in an encounter (time in seconds from the start of the fight to initiate

18   the first agonistic interaction that lasts for at least 3 seconds). In the second fights, the

19   'Draw' outcomes, where 3 Lunge- 3 Retreat rule was not satisfied, were further divided

20   into three categories based on Penn *et al*. (2010). This categorization was based on

21   usage of lunges and retreats by experienced loser flies against a naïve opponent. 'High

22   intensity' draws consisted of experienced loser flies predominantly using lunges while

23   'low intensity' draws included usage of retreats. Fights in which the flies did not engage

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1 in agonistic interactions were considered as 'no intensity', and categorized as draws. An

2 experienced loser fly may not readily engage in agonistic interactions against naïve

3 opponents. This may preclude escalation during fights and result in higher number of

4 draws. Thus 'high/low intensity' categorization facilitates better assessment of the

5 status-dependent behavioral changes manifested as the loser effect.

6 We devised the loser index in order to assess ability of flies to demonstrate the

7 experience dependent loser effect. The loser index was calculated as a difference

8 between the numbers of encounters lost to the encounters won divided by the total

9 number of encounters in the second fight. Within an encounter, if a fly uses aggressive

10 actions like lunging, boxing (rearing up on hind legs and striking the opponent with

11 forelegs), holding (rearing up on hind legs and holding the other fly's abdomen), chasing

12 (running after the opponent) or fencing (extending its leg forward and pushing the other

13 fly) (Chen et al., 2002; Zwarts et al., 2012) and the other fly responds with a retreat then

14 the former fly is a 'winner' and the latter a 'loser' in that encounter.

15 **Analysis of locomotor behavior**

16 Locomotor behavior was analyzed using a negative geotaxis assay as described before

17 (Ali et al., 2011). A group of ten flies, age and size matched, were introduced into a food

18 vial one day prior to testing. The flies were placed in two head to head joined empty

19 food vials with a distance of 8 cm marked on the lower vial. Following a gentle tap to get

20 all the flies to the base of the vial, the number of flies able to climb above the 8 cm mark

21 in 10 seconds was scored. The experiment was repeated 10 times for each group and

22 the average pass rate calculated. Three experimental replicates were carried out for

23 each genotype.

1 **Statistical analysis**

2 Videotapes were analyzed and each encounter was scored for all fighting strategies and

3 documented on spreadsheets. All statistical analysis was performed using Statistica 8.0

4 and Graphpad Prism 6.0 statistical software. Various statistical tests were employed to

5 facilitate our analysis including Chi-square test, one-factor ANOVA and two-factor

6 repeated measures ANOVA followed by post-hoc Tukey's or Dunnett's multiple

7 comparisons tests. Specific information on statistics used in each experiment is

8 included in the figure legends.

9

1 **RESULTS**

2 **The cAMP pathway mediates establishment of hierarchies within a fight**

3 Pairs of male flies demonstrate stereotyped aggressive behavior towards each other

4 while competing over resources like food or females. These dyadic interactions begin

5 with low aggression maneuvers like wing threat and fencing, which then escalate to high

6 aggression strategies like lunging and tussling. This escalation is a consequence of

7 experience-dependent behavioral transitions, with one fly demonstrating offensive

8 strategies with increasing frequency against an opponent fly employing more and more

9 defensive strategies. These real time behavioral modifications on shorter time scale are

10 stable within a fight and therefore facilitate formation of social dominance hierarchies.

11 We have assigned dominance relationships using a previously developed three lunge –

12 three retreat rule (see Materials and Methods and (Yurkovic et al., 2006)). In wild-type

13 Canton S (CS) flies, 85% of the fights produced dominance relationships (Figure 1A).

14 Prior investigations have implicated the cAMP pathway in the development of learning

15 and memory in multiple conditioning paradigms. cAMP synthesis is regulated by the

16 enzymatic activity of the *Rut* encoded adenylyl cyclase that catalyzes the conversion of

17 ATP to cAMP (Livingstone et al., 1984; Levin et al., 1992). *Rut* has been implicated as a

18 biochemical coincidence detector necessary for the formation of short term memory

19 (STM) in olfactory conditioning assays (Dudai et al., 1988; McGuire et al., 2005). In

20 contrast to CS flies, rut^{2080} mutant flies were unable to form dominance relationships

21 with all their fights ending in draws (Figure 1A; $P < 0.001$). cAMP phosphodiesterase

22 activity encoded by *Dnc* negatively regulates cAMP levels (Davis and Kiger, 1981).

23 Consistent with the deregulation of cAMP dynamics, dnc^1 flies display attenuated

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1 formation of dominance relationships with only 67% of the fights resulting in wins/losses

2 (Figure 1A; $P < 0.01$). The neuropeptide *Amn* is known to stimulate cAMP production

3 via GPCR signaling mediated stimulation of adenylyl cyclase activity (Feany and Quinn,

4 1995). However, *amn^{C651}* mutant flies displayed wild-type levels of dominance

5 hierarchies (Figure 1A; 82% of the fights, $P > 0.05$).

6 For further analysis of experience dependent behavioral modifications during a fight, we

7 focused on lunges and retreats. These strategies are commonly used offensive (lunges)

8 and defensive (retreats) maneuvers and previous work have indicated status dependent

9 modifications in their usage by winner/loser flies (Yurkovic et al., 2006). In line with

10 previous observations, agonistic interactions resulted in an escalation of aggression

11 (Yurkovic et al., 2006; Trannoy et al., 2016). In aggressive encounters between CS flies,

12 the ultimate winners (as established by the 3 lunge-3 retreat rule; see Materials and

13 Methods) progressively increased the deployment of lunges while the losers

14 increasingly adapted to the retreating behavior (Figure 1B, C). In contrast, *rut^{2080}* mutant

15 flies were unable to demonstrate any behavioral transitions pertaining to lunges and

16 retreats (Figure 1B, C; $P < 0.001$). *dnc^{1}* flies were not only inefficient in establishing

17 hierarchies but also displayed compromised behavioral plasticity with significantly

18 reduced ability to modify the frequency of lunges and retreats compared to CS animals

19 (Figure 1B, C; $P < 0.001$). *amn^{C651}* flies, which are competent in establishing hierarchies,

20 were able to modify the usage of offensive and defensive strategies as well as CS flies

21 (Figure 1B, C; $P > 0.05$). These results suggest a correlation between experience

22 dependent behavioral plasticity and formation of dominance hierarchies following

23 aggressive encounters.

11

1   No significant differences in locomotor activity between wild type and mutant flies were

2   found using a negative geotaxis assay (see Materials and Methods), thus ruling out the

3   possibility of motor deficits in these lines (Figure 2A; P > 0.05). Multiple parameters

4   were evaluated to assess the aggression levels of the wild type and mutant lines.

5   Encounter frequency was significantly lower in rut^{2080} mutants compared to CS flies but

6   dnc^1 and amn^{C651} mutants were comparable to wild-type flies (Figure 2B; P_{rut} < 0.05, P_{dnc}

7   and P_{amn} > 0.05). Aggression vigor index was compromised in all the mutant lines

8   compared to CS, but was not significantly different between rut^{2080}, dnc^1 and amn^{C651}

9   flies (Figure 2C; P_{rut} < 0.001, P_{dnc} and P_{amn} < 0.05).

10   While our studies implicate lack of behavioral plasticity in rut^{2080} mutants in establishing

11   dominance hierarchies in rut^{2080} versus rut^{2080} fights, it is also possible that the rut^{2080}

12   flies are unable to execute high intensity maneuvers like lunges. In $rut – rut$ fights,

13   neither opponent can adjust their fighting patterns depending upon experience resulting

14   in a lack of escalation beyond low intensity interactions precluding assessment of

15   behavioral changes in rut^{2080} flies. We therefore analyzed fights between CS and rut^{2080}

16   mutants. CS flies have intact experience dependent behavioral transitions compared to

17   rut^{2080} mutants. Therefore pairing CS vs rut^{2080} may better reveal behavioral escalation

18   during fights. CS vs rut^{2080} fights result in significant dominance hierarchies with 37.5%

19   of fights ending in a win or a loss, though it remains attenuated compared to CS vs CS

20   fights (Figure 3A; P < 0.001). Interestingly, rut^{2080} flies demonstrate increased lunging

21   and retreating behavior compared to their performance in $rut-rut$ fights and also win 9%

22   of the fights against wild-type opponents (Figure 3B, C). rut^{2080} flies, when in a $rut – CS$

23   fight, are capable of executing and modifying the frequency of use of both lunges and

12

1   retreats, although this ability is remains significantly compromised compared to that in

2   CS flies (in CS – CS fights) (Figure 3B, C; $P < 0.001$ for lunges and $P < 0.05$ for

3   retreats). The usage of offensive and defensive strategies for rut^{2080} flies against a wild

4   type opponent are significantly better than *rut-rut* fights (Figure 3B, C; $P < 0.001$ for

5   lunges and $P < 0.01$ for retreats). These results suggest that rut^{2080} flies are capable of

6   executing high intensity maneuvers but are compromised in modifying its frequency

7   during a fight.

8   Furthermore, dnc^{1} mutants are unable to modify fighting strategies as well as CS flies

9   but they are less compromised compared to the rut^{2080} flies in *rut-rut* fights (Figure 1A, B,

10   C). Consistent with the association of ability to modify fighting patterns with the

11   establishment of dominance relationships, *dnc* mutants do establish hierarchies though

12   less efficiently than wild-type CS flies (Figure 1A). Similarly, rut^{2080} flies against wild type

13   opponents demonstrate compromised plasticity and attenuated dominance structures

14   (Figure 3A, B, C). In fact, usage of lunges/retreats by rut^{2080} flies in *rut* – CS fights is

15   comparable to those seen in dnc^{1} (Figure 3B, C; $P > 0.05$ for lunges and retreats).

16   Interestingly, the aggression vigor index is similar for *rut* - CS and *rut* - *rut* fights (Figure

17   3D; $P > 0.05$). This again suggests that inability to develop hierarchical statuses and

18   compromised behavioral plasticity seen in *rut* mutants may be independent of

19   aggressiveness.

20   This series of experiments establish the role of the cAMP signaling in behavioral

21   plasticity underlying modification of fighting strategies during agonistic interactions.

22   Behavioral flexibility, rather than changes in levels of aggression, is correlated with the

23   generation of dominance hierarchies.

1

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

14

1   4A; $P < 0.001$). These experiments implicate the independent involvement of the α/β, γ

2   and α'/β' lobes of the MB together with the F5 neurons of the fan-shaped body (FB) in

3   neuronal processing leading to the formation of dominance. The ellipsoid body (EB) of

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

5   Analysis of behavioral patterns revealed that distinct neuronal circuits mediate *rut*-

6   dependent behavioral plasticity within a fight (Figure 4 and Table 1). Pan-neuronal

7   expression of *Rut* using the *Appl*-Gal4 driver rescued the inability to alter the frequency

8   of use of lunges and retreats seen in rut^{2080} mutants (Figure 4B, C; $P < 0.01$). We also

9   observed a complete rescue of behavioral transitions pertaining lunges and retreats with

10   *Rut* expression limited to the α/β and γ lobe neurons of the mushroom body using the

11   c309 driver (Figure 4D, E; $P < 0.001$). Furthermore, expression of *Rut*, independently in

12   the α/β, α'/β' or γ neurons of the MB or in the FB also rescued the deficit in the

13   progressive increase of lunges and retreats seen in rut^{2080} flies (Figure 4D, E, F, G; $P <$

14   0.01). The ellipsoid body (EB) of the central complex does not appear to be involved in

15   this function as no rescue was observed with the EB only driver (Figure 4F, G; $P > 0.05$).

16   Restricted expression of *Rut* in specific brain regions rescued the behavioral plasticity of

17   lunges/retreats and the ability to establish dominance hierarchies within a fight.

18   To confirm that experience-dependent behavioral flexibility was a major substrate of the

19   rescue and not an indirect effect of changes in other general features of aggression, we

20   evaluated multiple aggression related parameters. As the rescue was strongest

21   (comparable to wild-type) with the *Appl*-Gal4 (pan neuronal) and c309 (α/β and γ

22   neurons of the MB) drivers, these were used to evaluate changes in aggressiveness.

23   However, no change in the frequency of encounters, aggression vigor and latencies to

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1   engage was found (Figure 5A – E; $P > 0.05$). These results suggest that though pan

2   neuronal and α/β and γ drivers fully rescue the ability to alter the intensities of specific

3   fighting patterns during a fight and the establishment of dominance hierarchies, they do

4   not affect the levels of aggression in these animals.

5   Our analysis indicates that in accordance with the multimodal inputs associated with

6   dyadic interactions, multiple neuronal groups in the MB (α/β, α'/β', γ) and CC (FB) are

7   involved in the processing of these inputs that lead to the progressive changes in

8   offensive and defensive strategies within a fight. The rescue experiments also highlight

9   the role of cAMP signaling in behavioral plasticity in aggression to be independent of

10   general changes in aggression levels.

11

12   **cAMP signaling is necessary for the development of the loser effect**

13   To test if aggression-associated hierarchies developed in the first fights influenced the

14   outcomes of subsequent fights, losers from first fights were paired with unfamiliar,

15   socially naïve opponents, after a 60-minute rest period. Dominance relationships were

16   assigned using the three lunge- three retreat rule as in the first fights. Fights that fell

17   under the 'draw' category were further subdivided into three groups. 'High intensity'

18   draws consisted of experienced flies predominantly using lunges in their second fight

19   without satisfying 3 lunge-3 retreat criteria for dominance relationships. Similarly, 'low

20   intensity' draws consisted of experienced flies predominantly using retreats. Fights in

21   which the flies did not engage in agonistic interactions were considered as 'no intensity',

22   and categorized as draws. For statistical analysis, high and low intensity draws were

23   grouped with wins and losses, respectively. Consistent with previous studies (Yurkovic

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1 et al., 2006; Trannoy et al., 2016), wild-type CS loser flies always lost to naïve

2 opponents in their second fights (Figure 6A). This indicates that an experience of social

3 loss results in a strong loser effect in subsequent fights.

4 As rut^{2080} flies did not generate winners or losers in the first fight, both the individuals

5 were considered as experienced and used in second fights. However, as in their first

6 fights, rut^{2080} mutants were unable to form any dominance relationships in their second

7 fights (Figure 6A; $P < 0.001$). dnc^1 also displayed no significant loser effect with 18%

8 losses, 18% wins and 64% draws (Figure 6A; $P < 0.001$).

9 Surprisingly, amn^{C651} flies did not show any loser effect. 50% of analyzed fights resulted

10 in losses and the remaining in wins for losers (Figure 6A; $P < 0.001$). Unlike rut and dnc,

11 amn^{C651} flies develop dominant-subordinate relations as efficiently as CS in their first

12 fights (Figure 1C) but are still unable to consolidate this experience into a loser effect.

13 This suggests a special requirement of Amn gene product in stabilizing the hierarchical

14 structures in flies in the form of a loser effect.

15 A loser index, calculated as the difference between the number of encounters lost and

16 the number of encounters won divided by total number of encounters in the second fight

17 (see Materials and Methods), was used to represent the loser effect as a consequence

18 of past experience. This allowed direct evaluation of experience-dependent alterations

19 in fighting strategy in the losers across genotypes in subsequent fights. CS flies showed

20 a high loser index consistent with their robust loser effect (Figure 6B). In contrast, the

21 amn^{C651} and dnc^1 losers, and the experienced rut^{2080} flies displayed significantly lower

22 loser indices (Figure 6B; $P < 0.001$).

17

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

2 of dominance hierarchies in their first fights, did not display a loser effect. In contrast,

3 amn^{C651}, which could form dominance hierarchies in their first fights, also lacked the

4 loser effect. *Amn* appears to have an independent function from *Rut* and *Dnc,* and is

5 necessary for the stabilization of the social status from the first fight in the form of a

6 loser effect.

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

8 Previous experience of loss results in a robust loser effect in wild-type flies. We

9 investigated the neurogenetic circuitry involved in the development of this effect.

10 In the second fights, both *Appl* and c309 Gal4 driver lines were able to restore the loser

11 effect in rut^{2080} flies to levels statistically indistinguishable from wild-type CS flies (Figure

12 7A, B and Table 1). Pan neuronal (Appl) or combined α/β and γ lobe–specific (c309)

13 expression resulted in most losers losing and a small proportion of non-aggressive

14 draws (Figure 7A; $P < 0.001$). Loser index in these flies was significantly greater than

15 controls (Figure 7B; $P < 0.001$) and comparable to wild-type flies (Figure 7B; $P > 0.05$).

16 Interestingly, expression of UAS-*Rut* limited to individual substructures of the MB and

17 CC was not sufficient to rescue impaired loser effect observed in *rut* flies (Figure 7B;

18 Table 1). Expression in individual neuronal subpopulations using the c739, 201Y, c305a

19 or c205 Gal4 drivers (in the rut^{2080} background), resulted in a higher proportion of non-

20 draw outcomes in losers paired with naïve opponents but failed to show a robust loser

21 effect (Figure 7A; $P < 0.001$). As expected, there was no significant change in the loser

22 index for these flies (Figure 7B; $P > 0.05$). Consistent with compromised learning in the

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1   first fights, expression limited to the EB using the c819 Gal4 driver did not rescue the

2   loser effect (Figure 7; *P* > 0.05).

3   Our results suggest the involvement of the MBs, limited to the combined processing by

4   α/β and γ neurons, in the development of the loser effect.

5

1   **DISCUSSION**

2   Two distinct experience-dependent behavioral modifications are observed in *Drosophila*

3   aggression. The first results in dominance relationships correlated with real time,

4   progressive changes in fighting strategies. The second is the consolidation of the

5   experience of a previous loss to a loser effect in subsequent fights. Our data indicate

6   that these behavioral changes involve specific cAMP-mediated signaling and are

7   processed by overlapping neuronal circuits involving two temporally separated memory

8   traces.

9   *Rut* and *Dnc* enzymes have been implicated in learning and/or STM in multiple

10   paradigms, including classical conditioning and courtship-associated learning and

11   memory (Gailey et al., 1984; Livingstone et al., 1984; Bragina and Kamyshev, 2003;

12   McGuire et al., 2005). *rut* and *dnc* flies do not develop dominance hierarchies as well as

13   CS flies, with all *rut – rut* fights resulting in draws. Compared to CS, both *rut* and *dnc*

14   are unable to display experience-dependent modification of fighting strategies and are

15   deficient in generating losers and winners. *amn* flies, however, modify fighting strategies

16   and establish hierarchal relationships comparable to CS flies. Our results suggest that

17   the plasticity in modifying aggressive strategies during a fight is mediated by cAMP

18   dynamics under the control of *Rut* and *Dnc* gene products (but not *Amn*) and is

19   necessary to establish dominance structures.

20   All three cAMP pathway mutants displayed reduction in aggression vigor as compared

21   to CS. This includes *amn*, which displays normal behavioral flexibility and dominance

22   hierarchies. These results suggest that the level of aggression in flies is independent of

23   their ability to modify behavior in an experience-dependent manner. The latency to

1   engage in fights in cAMP mutants was also comparable to CS. The latter suggest no

2   overt changes in motivational states and support the previous conclusion.

3   Brain region specific expression of the *Rut* gene product in a *rut* mutant background

4   implicates specific neuronal circuits in status-dependent behavioral changes during

5   agonistic encounters. All the drivers that rescue the establishment of dominance also

6   restore the ability to alter the usage of lunges and retreats during a fight.

7   Rescue by Appl or c309 did not alter the encounter frequency, aggression vigor or the

8   latency to engage displayed by *rut* mutants. In line with our analysis of cAMP pathway

9   mutants, these results demonstrate that the rescue of dominance observed is

10   attributable to a restoration of experience-dependent plasticity mediated by *Rut* in

11   specific circuits and not a consequence of modified aggressiveness.

12   Our study suggests a functional role for MBs in learning and memory associated with

13   aggression, consistent with those described in courtship conditioning (Joiner and

14   Griffith, 1999; McBride et al., 1999; Sitnik et al., 2003). Mushroom bodies, which are

15   central to olfactory learning, have been previously correlated with changes in agonistic

16   behavior. *Neuralized* mutants with altered MB organization have been reported to

17   increase aggressiveness when food is limiting (Rollmann et al., 2008). Inhibition of

18   synaptic output from the MB has also been shown to reduce levels of aggression (Liu et

19   al., 2011).

20   Interestingly, not only neuronal groups of the MB (the α/β, γ and α'/β' neurons) were

21   implicated, the FB but not the EB of the central complex was also found to be

22   functionally involved. The FB has been previously reported to mediate visual learning of

23   specific pattern features like "elevation" and "contour orientation" (Liu et al., 2006; Pan

21

1   et al., 2009). A recent study has underscored the importance of CC neurons in

2   aggression by demonstrating the modulation of aggressive behavior by dopaminergic

3   PPM3 neurons that synapse onto the FB (Alekseyenko et al., 2013).

4   The robust loser effect seen in wild-type flies is absent in *rut*, *dnc* and *amn* mutant lines.

5   As in their first fights, all *rut-rut* second fights end in draws. No loser effect was also

6   seen in *dnc* flies. Both *rut* and *dnc* have compromised development of hierarchies

7   suggesting that learning within a fight and establishing dominance relationships are

8   necessary for the development of the loser effect. Interestingly, *amn* flies also lack the

9   loser effect, though they can adjust fighting strategies and develop dominance

10 relationships as well as wild-type flies. The *Amnesiac* gene product appears to mediate

11 the consolidation of the social status acquired during the first fight into the loser effect.

12 Alternatively, *Amn* may be necessary for a distinct memory phase resulting in the loser

13 effect. A similar function for *Amn* has been reported in olfactory conditioning

14 experiments where *amn* mutants fail to develop intermediate-term memory or are

15 unable to consolidate short-term memory into this relatively longer lasting phase (Feany

16 and Quinn, 1995; Yu et al., 2006). *Amn* has also been reported to mediate memory

17 stability in courtship conditioning (Siegel and Hall, 1979). As *Amn* is a secreted

18 neuropeptide, future rescue experiments using the *Amn* receptor will be useful to

19 determine the neural circuits subserving *Amn*-dependent establishment of the loser

20 effect. A less robust, short duration winner effect has been reported in flies (Trannoy

21 and Kravitz, 2016; Trannoy et al., 2016). It remains to be seen if analogous cAMP

22 signaling and circuit features are involved in this phenomenon.

1   Rescue of *rut* simultaneously in the α/β and γ lobe neurons of the MB, not only fully

2   rescues its ability to generate dominance relationships but also the loser effect.

3   However, *rut* expression independently in the α/β or the γ neurons was unable to

4   restore the loser effect, though social hierarchy was rescued. We hypothesize that two

5   distinct *Rut* dependent memory traces facilitate formation of the temporally distinct

6   memory phases. A short-lived engram in distinct MB and CC substructures mediates

7   behavioral plasticity within a fight leading to the formation of dominance relationships.

8   Combinatorial processing between α/β and γ lobe neurons of the MB enable formation

9   of a second, longer lasting memory trace that is necessary for the establishment of the

10 loser effect.

11 Olfactory conditioning studies have suggested that the α/β neurons are indispensable

12 for memory consolidation and retrieval, while odor-shock coincidence detection maps to

13 the γ neurons (Dubnau et al., 2001; Qin et al., 2012; Dubnau and Chiang, 2013). These

14 observations demonstrate recruitment of specific subsets of MB neurons for initial

15 associations and others for memory consolidation suggesting circuit level coordination

16 between MB substructures. Our experiments provide evidence towards such systems

17 level memory consolidation in aggression where an association between α/β and γ

18 lobes of the MB is critical for the establishment of the loser effect. However, existence of

19 two parallel, independent traces with differing kinetics of formation and decay cannot be

20 formally ruled out.

21 Experience-dependent modification of innate behaviors involves multiple components

22 and previous studies have demonstrated the central importance of pheromonal,

23 aminergic and other modulatory activities in aggressive behavior (Chan and Kravitz,

23

1   2007; Dierick and Greenspan, 2007; Hoyer et al., 2008; Dankert et al., 2009; Certel et

2   al., 2010; Wang and Anderson, 2010; Liu et al., 2011; Zwarts et al., 2012; Alekseyenko

3   et al., 2013; Alekseyenko et al., 2014; Andrews et al., 2014; Luo et al., 2014; Yuan et al.,

4   2014; Kohl et al., 2015; Hoopfer, 2016). However, these studies focus on aggression

5   levels and do not directly assess behavioral plasticity and memory components in

6   aggression. A recent study indicated that rut^{2080} and amn^1 are unable to demonstrate

7   dominance hierarchies due to compromised aggression (Trannoy et al., 2016).

8   However, this study did not explore the lack of behavioral flexibility in these mutants. In

9   our study, rut^{2080} has low proclivity to engage in the first 10 min of fights, fail to form

10   dominance relations but are capable of executing high intensity maneuvers. In contrast,

11   amn^{C651} (a functionally strong allele of amn, (Rosay et al., 2001)) showed reduced

12   aggression vigor but was still able to establish dominance hierarchies. Further, rescue

13   of Rut restores dominance patterns but not aggressiveness. Therefore, low aggression

14   is not correlated with the inability to establish winner-loser statuses in a fight.

15   In mammals, the cAMP pathway has been shown to influence aggressive behavior

16   (Breuillaud et al., 2012). cAMP signaling in the basolateral amygdala has also been

17   correlated with memory associated with conditioned defeat, a paradigm analogous to

18   the loser effect (Jasnow et al., 2005; Markham et al., 2010). At the circuit level, recent

19   work in zebrafish has implicated antagonistic regulation by two sub-regions of the dorsal

20   habenula to establish winner-loser status (Chou et al., 2016).

21   Our results implicate sequential recruitment of cAMP signaling components with Rut

22   and Dnc activities required for behavioral flexibility within a fight and, consequently, to

23   establish dominance hierarchies. Behavioral plasticity, rather than aggressiveness, is

24

1   correlated with social status. Establishment of social status facilitates the formation of a

2   more stable and longer lasting memory phase that requires the additional peptidergic

3   activity of the neuropeptide amnesiac (*Amn*). Neuronal circuits subserving aggression

4   associated learning and memory show phasic recruitment. While a short lived *Rut-*

5   dependent trace in multiple MB and CC substructures mediate learning during agnostic

6   encounters, combinatorial processing by both α/β and γ lobe neurons of the MB, is

7   necessary for the development of the longer lasting loser effect.

8   This study provides mechanistic insight into circuit level associations specific to different

9   phases of behavioral plasticity and memory in *Drosophila* aggression and the

10 integration of biochemical signaling at the single neuron level with systems level

11 consolidation.

12

1   **CONFLICT OF INTEREST:** The authors declare no competing financial interests.

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1 **REFERENCES**

2 Alekseyenko OV, Chan YB, Li R, Kravitz EA (2013) Single dopaminergic neurons that
3 modulate aggression in *Drosophila*. Proc Natl Acad Sci USA 110:6151-6156.

4 Alekseyenko OV, Chan YB, Fernandez MP, Bulow T, Pankratz MJ, Kravitz EA (2014)
5 Single serotonergic neurons that modulate aggression in *Drosophila*. Curr Biol
6 24:2700-2707.

7 Ali Y, Escala W, Ruan K, Zhai R (2011) Assaying locomotor, learning, and memory
8 deficits in *Drosophila* models of neurodegeneration. J Vis Exp:e2504.

9 Andrews JC, Fernandez MP, Yu Q, Leary GP, Leung AK, Kavanaugh MP, Kravitz EA,
10 Certel SJ (2014) Octopamine neuromodulation regulates Gr32a-linked
11 aggression and courtship pathways in *Drosophila* males. PLoS Genet
12 10:e1004356.

13 Asahina K, Watanabe K, Duistermars BJ, Hoopfer E, Gonzalez CR, Eyjolfsdottir EA,
14 Perona P, Anderson DJ (2014) Tachykinin-expressing neurons control male-
15 specific aggressive arousal in *Drosophila*. Cell 156:221-235.

16 Aso Y, Grubel K, Busch S, Friedrich AB, Siwanowicz I, Tanimoto H (2009) The
17 mushroom body of adult *Drosophila* characterized by GAL4 drivers. J
18 Neurogenet 23:156-172.

19 Blum AL, Li W, Cressy M, Dubnau J (2009) Short- and long-term memory in *Drosophila*
20 require cAMP signaling in distinct neuron types. Curr Biol 19:1341-1350.

21 Bragina YV, Kamyshev NG (2003) Comparative studies of four *Drosophila* P-insertion
22 mutants with memory defects. Neurosci Behav Physiol 33:73-79.

23 Brembs B (2003) Operant conditioning in invertebrates. Curr Opin Neurobiol 13:710-717.

24 Breuillaud L, Rossetti C, Meylan EM, Merinat C, Halfon O, Magistretti PJ, Cardinaux JR
25 (2012) Deletion of CREB-regulated transcription coactivator 1 induces
26 pathological aggression, depression-related behaviors, and neuroplasticity genes
27 dysregulation in mice. Biol Psychiatry 72:528-536.

28 Busto G, Cervantes-Sandoval I, Davis R (2010) Olfactory learning in *Drosophila*.
29 Physiology 25:338-346.

1 Certel SJ, Leung A, Lin CY, Perez P, Chiang AS, Kravitz EA (2010) Octopamine
2   neuromodulatory effects on a social behavior decision-making network in
3   *Drosophila* males. PLoS One 5:e13248.

4 Chan Y-B, Kravitz E (2007) Specific subgroups of FruM neurons control sexually
5   dimorphic patterns of aggression in *Drosophila melanogaster*. Proc Natl Acad Sci
6   USA 104:19577-19582.

7 Chen S, Lee AY, Bowens NM, Huber R, Kravitz EA (2002) Fighting fruit flies: a model
8   system for the study of aggression. Proc Natl Acad Sci USA 99:5664-5668.

9 Chou MY, Amo R, Kinoshita M, Cherng BW, Shimazaki H, Agetsuma M, Shiraki T, Aoki
10   T, Takahoko M, Yamazaki M, Higashijima S, Okamoto H (2016) Social conflict
11   resolution regulated by two dorsal habenular subregions in zebrafish. Science
12   352:87-90.

13 Dankert H, Wang L, Hoopfer E, Anderson D, Perona P (2009) Automated monitoring
14   and analysis of social behavior in *Drosophila*. Nat Meth 6:297-303.

15 Davis RL, Kiger JA (1981) Dunce mutants of *Drosophila melanogaster*: mutants
16   defective in the cyclic AMP phosphodiesterase enzyme system. J Cell Biol
17   90:101-107.

18 Dierick HA, Greenspan RJ (2006) Molecular analysis of flies selected for aggressive
19   behavior. Nat Genet 38:1023-1031.

20 Dierick HA, Greenspan RJ (2007) Serotonin and neuropeptide F have opposite
21   modulatory effects on fly aggression. Nat Genet 39:678-682.

22 Dubnau J, Chiang AS (2013) Systems memory consolidation in *Drosophila*. Curr Opin
23   Neurobiol 23:84-91.

24 Dubnau J, Grady L, Kitamoto T, Tully T (2001) Disruption of neurotransmission in
25   *Drosophila* mushroom body blocks retrieval but not acquisition of memory.
26   Nature 411:476-480.

27 Dudai Y, Corfas G, Hazvi S (1988) What is the possible contribution of Ca^{2+}-stimulated
28   adenylate cyclase to acquisition, consolidation and retention of an associative
29   olfactory memory in *Drosophila*. J Comp Physiol A 162:101-109.

30 Dudai Y, Jan YN, Byers D, Quinn WG, Benzer S (1976) dunce, a mutant of *Drosophila*
31   deficient in learning. Proc Natl Acad Sci USA 73:1684-1688.

Edwards A, Zwarts L, Yamamoto A, Callaerts P, Mackay T (2009) Mutations in many genes affect aggressive behavior in *Drosophila melanogaster*. BMC biology 7:29.

Feany MB, Quinn WG (1995) A neuropeptide gene defined by the *Drosophila* memory mutant amnesiac. Science 268:869-873.

Gailey D, Jackson F, Siegel R (1984) Conditioning mutations in *Drosophila melanogaster* affect an experience dependent behavioral modification in courting males. Genetics 106:613-623.

Hoopfer ED (2016) Neural control of aggression in *Drosophila*. Curr Opin Neurobiol 38:109-118.

Hoopfer ED, Jung Y, Inagaki HK, Rubin GM, Anderson DJ (2015) P1 interneurons promote a persistent internal state that enhances inter-male aggression in *Drosophila*. Elife 4.

Hoyer SC, Eckart A, Herrel A, Zars T, Fischer SA, Hardie SL, Heisenberg M (2008) Octopamine in male aggression of *Drosophila*. Curr Biol 18:159-167.

Jasnow AM, Shi C, Israel JE, Davis M, Huhman KL (2005) Memory of social defeat is facilitated by cAMP response element-binding protein overexpression in the amygdala. Behav Neurosci 119:1125-1130.

Joiner M, Griffith L (1999) Mapping of the anatomical circuit of CaM kinase-dependent courtship conditioning in *Drosophila*. Learn Mem 6:177-192.

Kamyshev NG, Smirnova GP, Kamysheva EA, Nikiforov ON, Parafenyuk IV, Ponomarenko VV (2002) Plasticity of social behavior in *Drosophila*. Neurosci Behav Physiol 32:401-408.

Kohl J, Huoviala P, Jefferis GS (2015) Pheromone processing in *Drosophila*. Curr Opin Neurobiol 34:149-157.

Levin LR, Han PL, Hwang PM, Feinstein PG, Davis RL, Reed RR (1992) The *Drosophila* learning and memory gene rutabaga encodes a Ca^{2+}/Calmodulin-responsive adenylyl cyclase. Cell 68:479-489.

Lim RS, Eyjolfsdottir E, Shin E, Perona P, Anderson DJ (2014) How food controls aggression in *Drosophila*. PLoS One 9:e105626.

Liu G, Seiler H, Wen A, Zars T, Ito K, Wolf R, Heisenberg M, Liu L (2006) Distinct memory traces for two visual features in the *Drosophila* brain. Nature 439:551-556.

Liu W, Liang X, Gong J, Yang Z, Zhang YH, Zhang JX, Rao Y (2011) Social regulation of aggression by pheromonal activation of Or65a olfactory neurons in *Drosophila*. Nat Neurosci 14:896-902.

Livingstone MS, Sziber PP, Quinn WG (1984) Loss of calcium/calmodulin responsiveness in adenylate cyclase of rutabaga, a *Drosophila* learning mutant. Cell 37:205-215.

Luo J, Lushchak OV, Goergen P, Williams MJ, Nassel DR (2014) *Drosophila* insulin-producing cells are differentially modulated by serotonin and octopamine receptors and affect social behavior. PLoS One 9:e99732.

Markham CM, Taylor SL, Huhman KL (2010) Role of amygdala and hippocampus in the neural circuit subserving conditioned defeat in Syrian hamsters. Learn Mem 17:109-116.

McBride S, Giuliani G, Choi C, Krause P, Correale D, Watson K, Baker G, Siwicki K (1999) Mushroom body ablation impairs short-term memory and long-term memory of courtship conditioning in *Drosophila melanogaster*. Neuron 24:967-977.

McGuire SE, Deshazer M, Davis RL (2005) Thirty years of olfactory learning and memory research in *Drosophila melanogaster*. Prog Neurobiol 76:328-347.

Neuser K, Triphan T, Mronz M, Poeck B, Strauss R (2008) Analysis of a spatial orientation memory in *Drosophila*. Nature 453:1244-1247.

Pan Y, Zhou Y, Guo C, Gong H, Gong Z, Liu L (2009) Differential roles of the fan-shaped body and the ellipsoid body in *Drosophila* visual pattern memory. Learn Mem 16:289-295.

Penn JK, Zito MF, Kravitz EA (2010) A single social defeat reduces aggression in a highly aggressive strain of *Drosophila*. Proc Natl Acad Sci USA 107:12682-12686.

Qin H, Cressy M, Li W, Coravos JS, Izzi SA, Dubnau J (2012) Gamma neurons mediate dopaminergic input during aversive olfactory memory formation in *Drosophila*. Curr Biol 22:608-614.

1 Ramin M, Domocos C, Slawaska-Eng D, Rao Y (2014) Aggression and social
2   experience: genetic analysis of visual circuit activity in the control of
3   aggressiveness in *Drosophila*. Mol Brain 7:55.

4 Rollmann SM, Zwarts L, Edwards AC, Yamamoto A, Callaerts P, Norga K, Mackay TF,
5   Anholt RR (2008) Pleiotropic effects of *Drosophila* neuralized on complex
6   behaviors and brain structure. Genetics 179:1327-1336.

7 Rosay P, Armstrong JD, Wang Z, Kaiser K (2001) Synchronized neural activity in the
8   *Drosophila* memory centers and its modulation by amnesiac. Neuron 30:759-770.

9 Siegel RW, Hall JC (1979) Conditioned responses in courtship behavior of normal and
10   mutant *Drosophila*. Proc Natl Acad Sci USA 76:3430-3434.

11 Sitnik N, Tokmacheva E, Savvateeva-Popova E (2003) The ability of *Drosophila*
12   mutants with defects in the central complex and mushroom bodies to learn and
13   form memories. Neurosci Behav Physiol 33:67-71.

14 Torroja L, Chu H, Kotovsky I, White K (1999) Neuronal overexpression of APPL, the
15   *Drosophila* homologue of the amyloid precursor protein (APP), disrupts axonal
16   transport. Curr Biol 9:489-492.

17 Trannoy S, Kravitz EA (2016) Strategy changes in subsequent fights as consequences
18   of winning and losing in fruit fly fights. Fly 0.

19 Trannoy S, Penn J, Lucey K, Popovic D, Kravitz EA (2016) Short and long-lasting
20   behavioral consequences of agonistic encounters between male *Drosophila*
21   *melanogaster*. Proc Natl Acad Sci USA 113:4818-4823.

22 Wang L, Anderson DJ (2010) Identification of an aggression-promoting pheromone and
23   its receptor neurons in *Drosophila*. Nature 463:227-231.

24 Wang L, Dankert H, Perona P, Anderson D (2008) A common genetic target for
25   environmental and heritable influences on aggressiveness in *Drosophila*. Proc
26   Natl Acad Sci USA 105:5657-5663.

27 Wang L, Han X, Mehren J, Hiroi M, Billeter JC, Miyamoto T, Amrein H, Levine JD,
28   Anderson DJ (2011) Hierarchical chemosensory regulation of male-male social
29   interactions in *Drosophila*. Nat Neurosci 14:757-762.

30 Yoon J, Matsuo E, Yamada D, Mizuno H, Morimoto T, Miyakawa H, Kinoshita S,
31   Ishimoto H, Kamikouchi A (2013) Selectivity and plasticity in a sound-evoked
32   male-male interaction in *Drosophila*. PLoS One 8:e74289.

31

1 Yu D, Akalal DB, Davis RL (2006) *Drosophila* alpha/beta mushroom body neurons form
2   a branch-specific, long-term cellular memory trace after spaced olfactory
3   conditioning. Neuron 52:845-855.

4 Yuan Q, Song Y, Yang CH, Jan LY, Jan YN (2014) Female contact modulates male
5   aggression via a sexually dimorphic GABAergic circuit in *Drosophila*. Nat
6   Neurosci 17:81-88.

7 Yurkovic A, Wang O, Basu AC, Kravitz EA (2006) Learning and memory associated
8   with aggression in *Drosophila melanogaster*. Proc Natl Acad Sci USA 103:17519-
9   17524.

10 Zars T, Wolf R, Davis R, Heisenberg M (2000a) Tissue-specific expression of a type I
11   adenylyl cyclase rescues the rutabaga mutant memory defect: in search of the
12   engram. Learn Mem 7:18-31.

13 Zars T, Fischer M, Schulz R, Heisenberg M (2000b) Localization of a short-term
14   memory in *Drosophila*. Science 288:672-675.

15 Zwarts L, Versteven M, Callaerts P (2012) Genetics and neurobiology of aggression in
16   *Drosophila*. Fly 6:35-48.
17

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

1   average pass rate (One factor ANOVA followed by post hoc Tukey's multiple

2   comparison test, mean ± SEM, n=30). (*B*) Encounters per minute is lower in rut^{2080} flies

3   (*P* < 0.05) but is comparable in amn^{c651} and dnc^1 flies compared to CS flies (*P* > 0.05)

4   (One factor ANOVA followed by post hoc Tukey's multiple comparison test, mean ±

5   SEM n=30). *, indicates comparison with CS. (*C*) Aggression vigor index, defined as the

6   fraction of time spent fighting in first 10 minutes, is significantly lower in rut^{2080} (*P* <

7   0.001), amn^{c651} (*P* < 0.05) and dnc^1 (*P* < 0.05) flies in comparison to CS flies (One

8   factor ANOVA followed by post hoc Tukey's multiple comparison test, mean ± SEM

9   n=30). *, indicates comparison with CS. (*D*) Latency to engage in an encounter is not

10   significantly different in rut^{2080} (*P* > 0.05), dnc^1 (*P* > 0.05) and amn^{c651} (*P* > 0.05) flies in

11   comparison to CS flies. (One factor ANOVA followed by post hoc Tukey's multiple

12   comparison test, mean ± SEM ,n=30). SEM: Standard error of the mean.

13

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

15   opponents.

16   (*A*) The rut^{2080} flies show attenuated dominance structures against a wild type opponent

17   compared to CS v CS fights (*P* < 0.001) but improved hierarchical relationships

18   compared to rut^{2080} v rut^{2080} fights (*P* < 0.01) (Two-tailed Chi-square test; *** *P* < 0.001,

19   ++ *P* < 0.01, n=25). *, indicates comparison with CS and +, with rut^{2080}. (*B*) and (*C*) The

20   rut^{2080} flies against a wild type opponent demonstrate significantly better status-

21   dependent modification of lunges/retreats compared to rut^{2080} v rut^{2080} fights (*P* < 0.001

22   for lunges and *P* < 0.01 for retreats) and is comparable to dnc^1 flies (*P* > 0.05). The

34

1   lunge/retreat trends for rut^{2080} flies in rut^{2080} v CS fights are compromised compared to

2   CS flies ($P < 0.001$ for lunges; $P < 0.05$ for retreats). (Two factor repeated measures

3   ANOVA followed by post hoc Tukey's multiple comparison test, 'P' is the interaction

4   term, mean ± SEM, n=20).

5   Total number of lunges/retreats in every three successive encounters is analyzed and

6   their mean is reported. SEM: Standard error of the mean

7

8   **Figure 4.** Distinct neuronal circuits mediate behavioral plasticity during agnostic

9   encounters.

10   Restricted expression of Rut in specific sub-population of neurons in rut^{2080} flies yields

11   differential rescue of learning impairment in first fights. (*A*) Lack of development of

12   hierarchies in rut^{2080} flies is rescued upon expression of UAS-Rut (in a rut^{2080}

13   background) pan-neuronally (Appl), in the α/β and γ lobes of the MB (c309), α/β lobes of

14   the MB (c739), γ lobes of the MB (201Y), α'/β' lobes of the MB (c305a) and in the F5

15   neurons of the FB. No rescue was seen upon reintroduction of Rut to the EB (c819).

16   (Two-tailed Chi-square test; ** $P < 0.01$, *** $P < 0.001$, +++ $P < 0.001$, n≥15). *, indicates

17   comparison with CS and +, with rut^{2080}; UAS-Rut/+. (*B*) - (*G*) Ability to progressively

18   increase lunges/retreats was rescued (relative to rut^{2080} flies) by expressing Rut either

19   pan-neuronally (Appl; $P < 0.001$ for lunges and retreats) or in α/β and γ (c309; $P <$

20   0.001 for lunges and retreats), α/β alone (c739; $P < 0.001$ for lunges, $P < 0.01$ for

21   retreats), α'/β' (c305a; $P < 0.01$ for lunges, $P < 0.05$ for retreats) alone, γ (201y; $P <$

22   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 +,

1   with rut^{2080}; UAS-*Rut*/+. (*B*) Loser index analysis revealed that pan-neuronal and α/β

2   and γ lobe combined expression is able to rescue the loser effect. All other

3   combinations did not show any rescue. (One factor ANOVA followed by post hoc

4   Tukey's multiple comparison test, *** P < 0.001, $^{+++}$ P < 0.001, mean ± SEM, n≥15).

5   *, indicates comparison with CS and +, with rut^{2080}; UAS-*Rut*/+. SEM: Standard error of
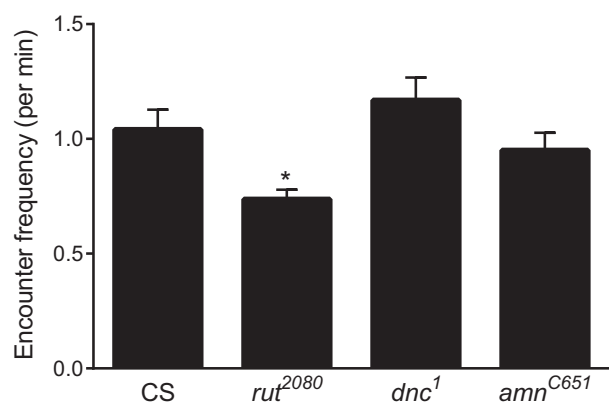
6   the mean.
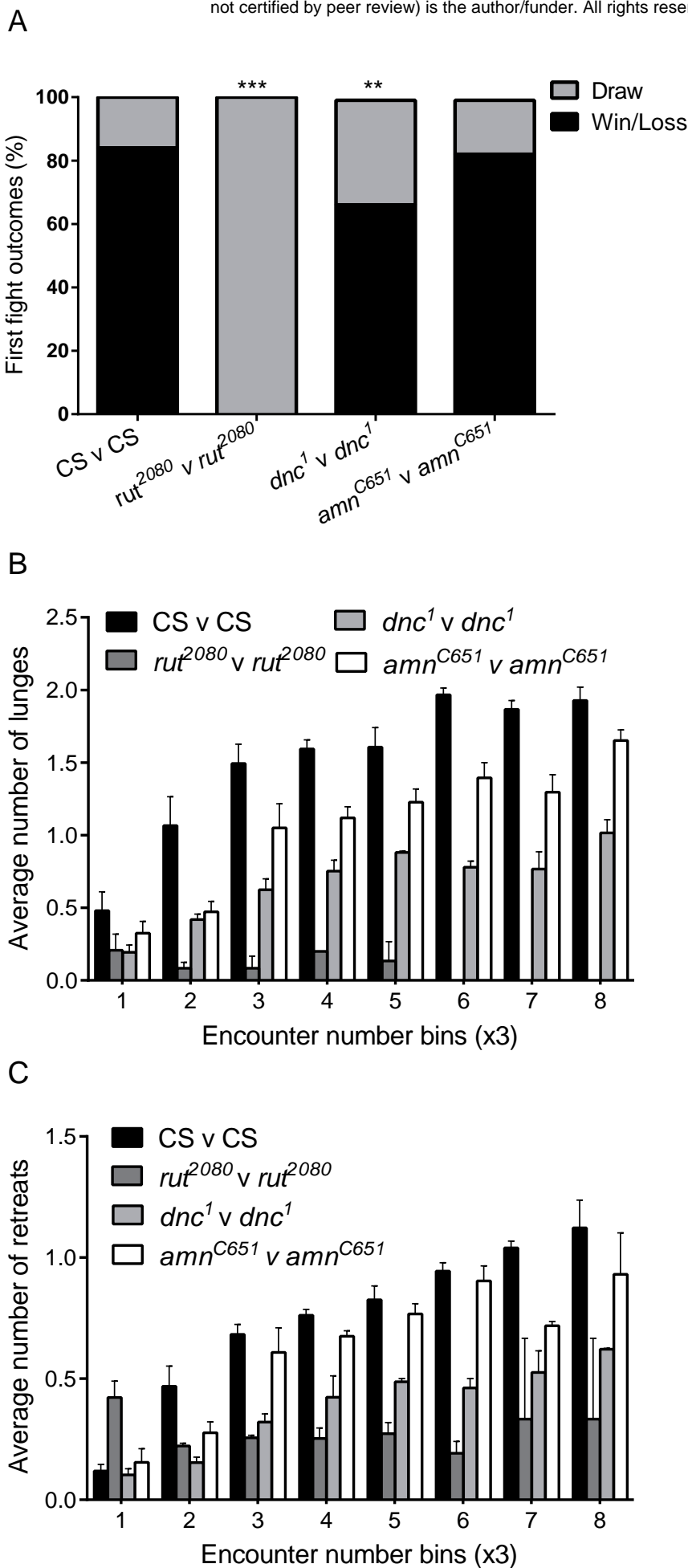
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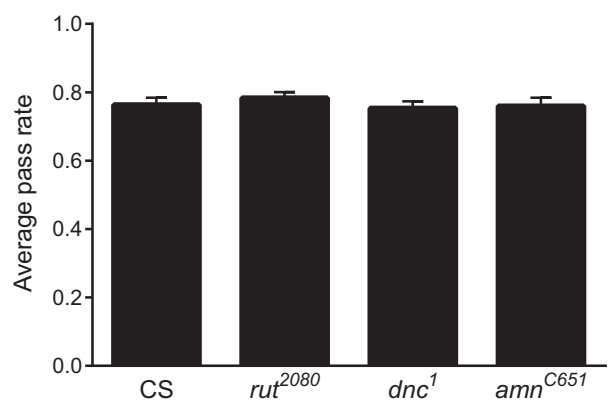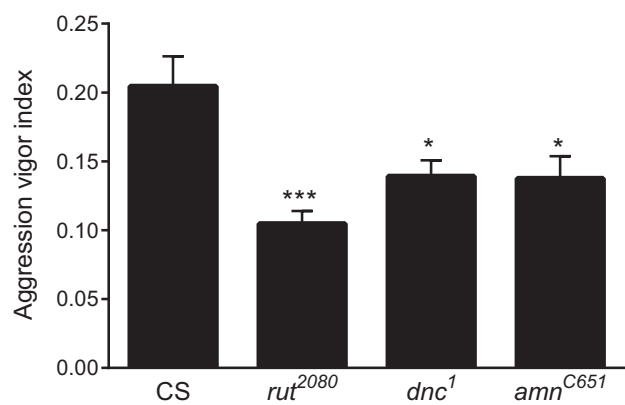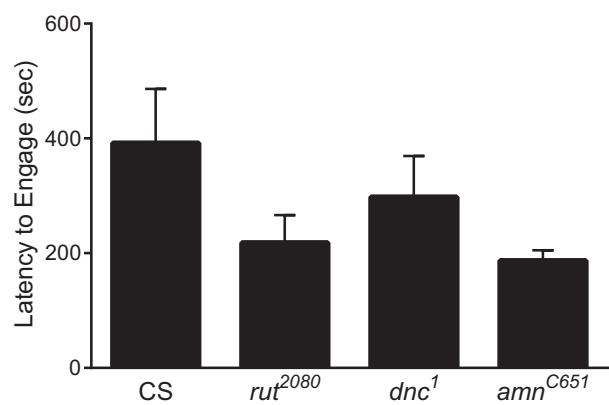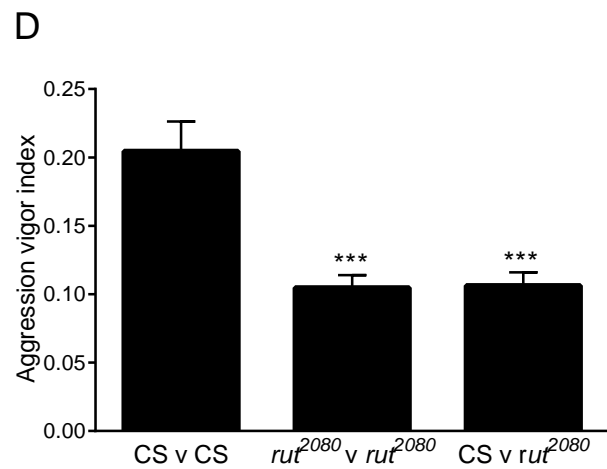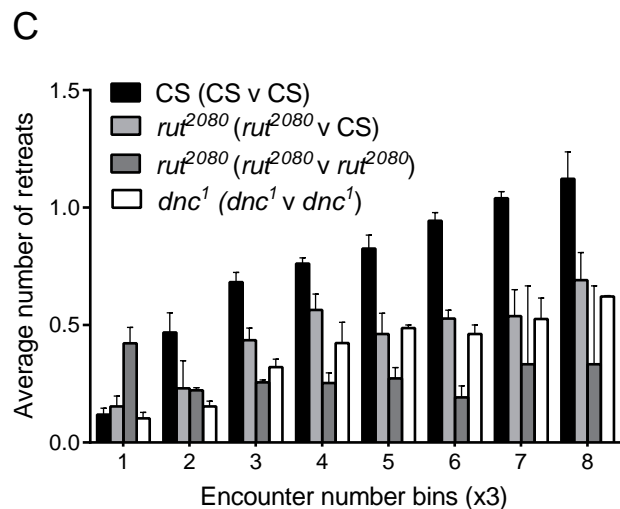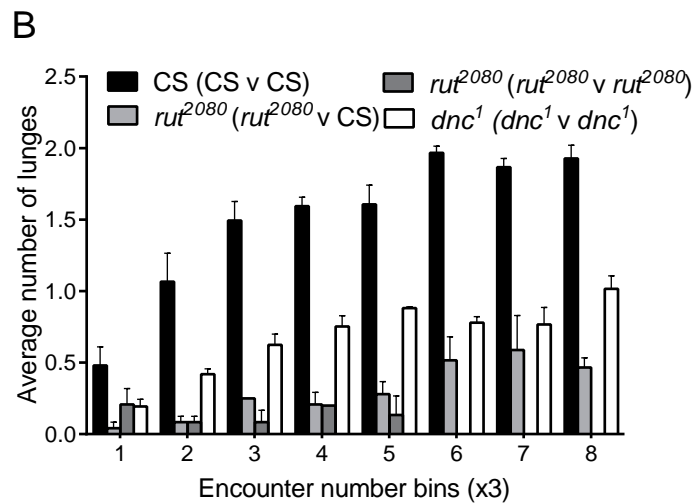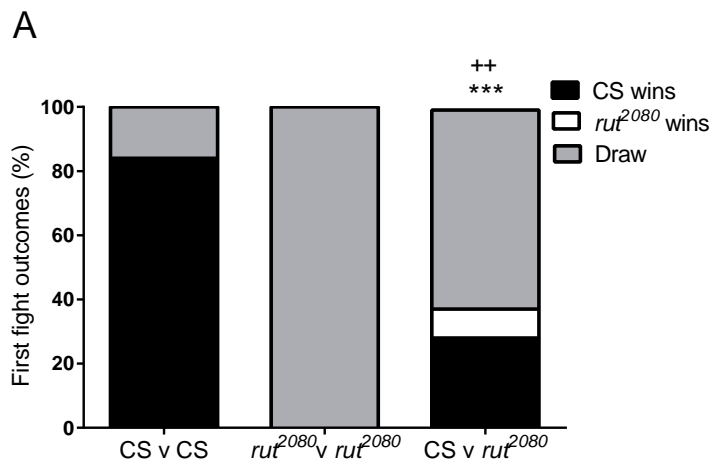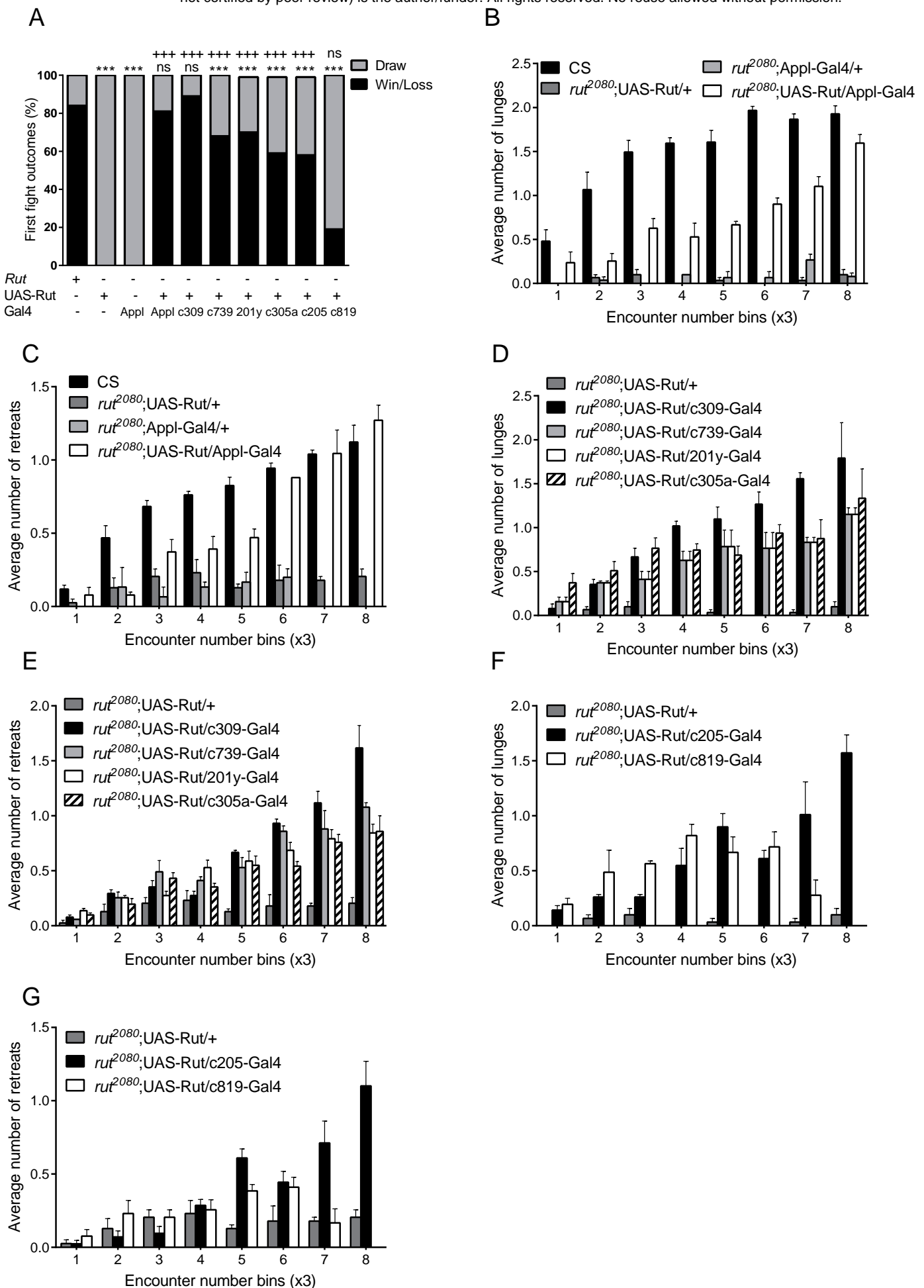
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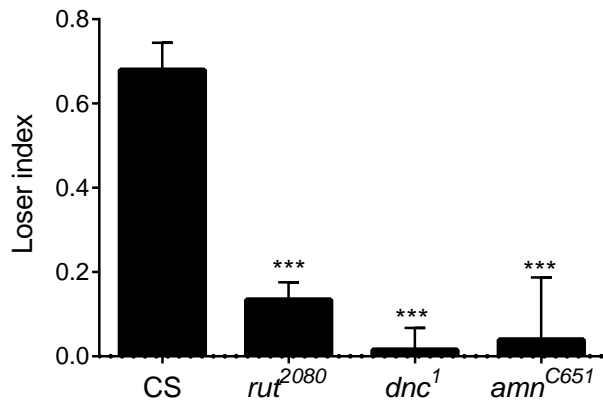
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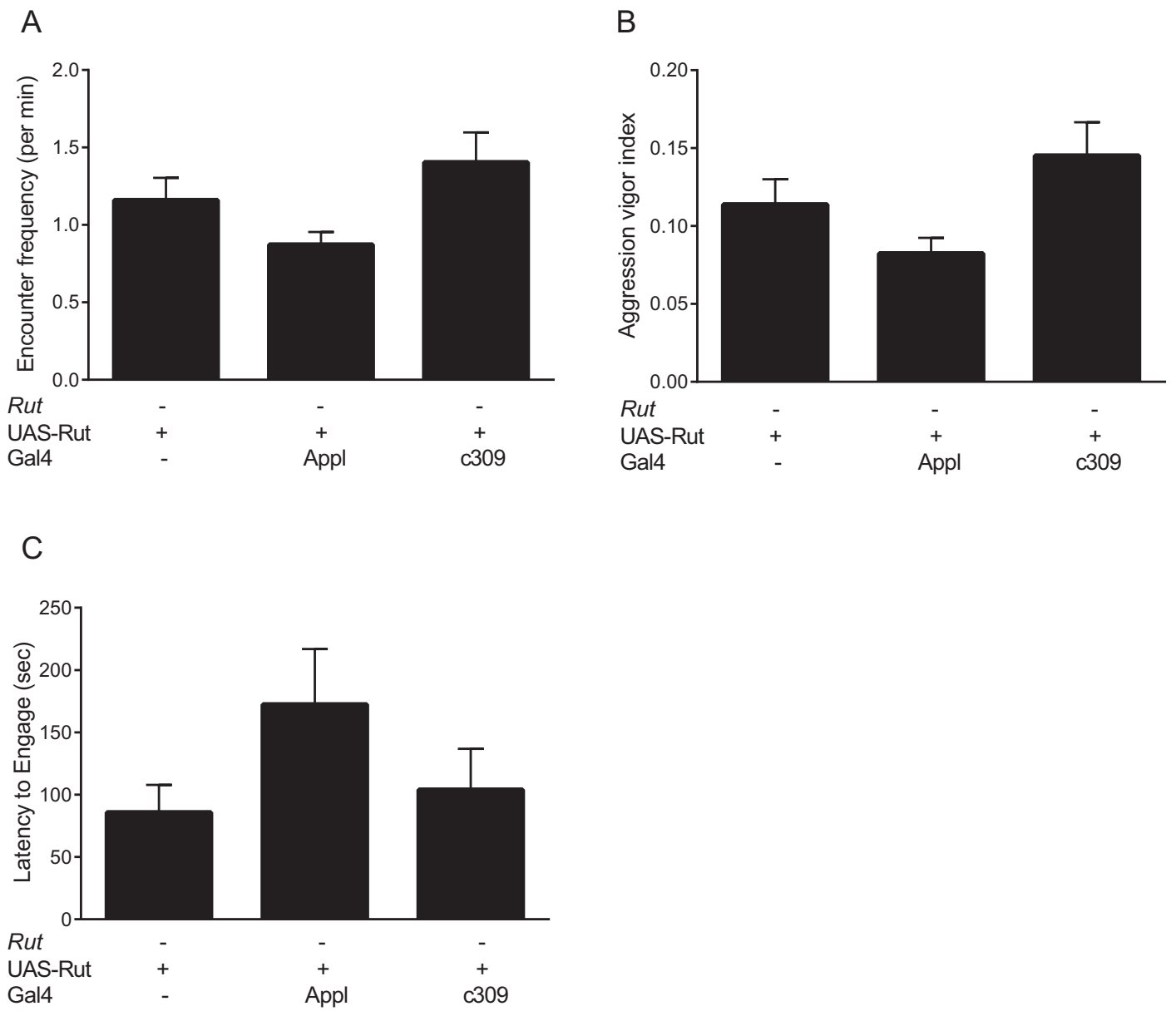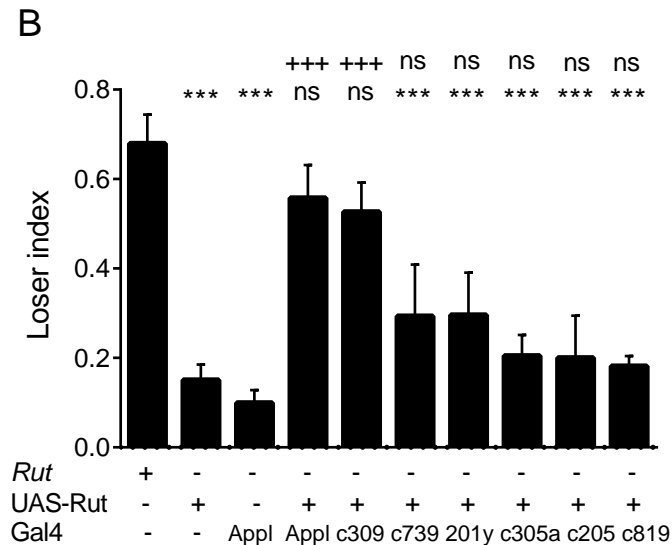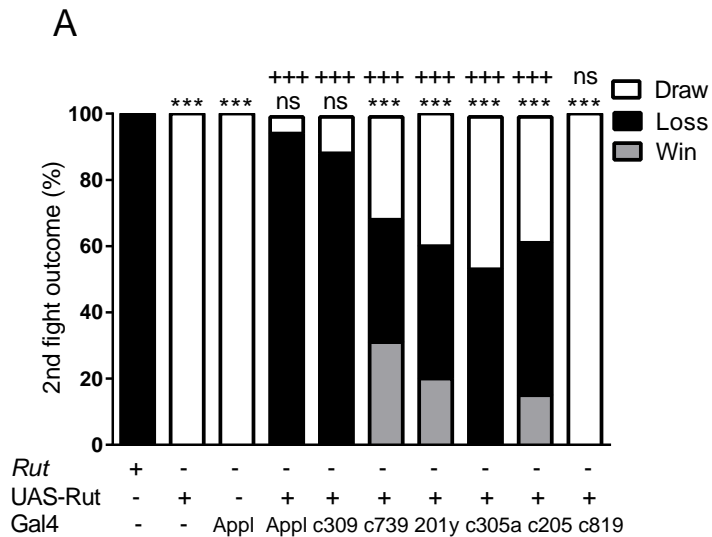
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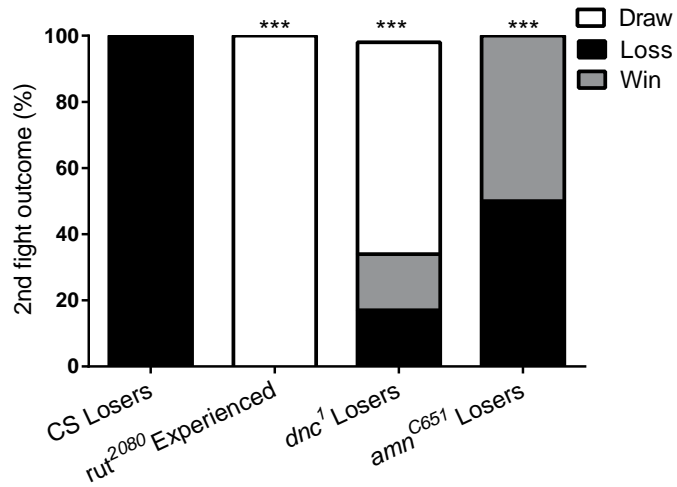
Figure 6

Figure 7

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

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

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