Intermediate-term memory in *Aplysia* involves neurotrophin signaling, transcription, and DNA methylation

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Long-term but not short-term memory and synaptic plasticity in many brain areas require neurotrophin signaling, transcription, and epigenetic mechanisms including DNA methylation. However, it has been difficult to relate these cellular mechanisms directly to behavior because of the immense complexity of the mammalian brain. To address that problem, we and others have examined numerically simpler systems such as the hermaphroditic marine mollusk *Aplysia californica*. As a further simplification, we have used a semi-intact preparation of the *Aplysia* siphon withdrawal reflex in which it is possible to relate cellular plasticity directly to behavioral learning. We find that inhibitors of neurotrophin signaling, transcription, and DNA methylation block sensitization and classical conditioning beginning ∼1 h after the start of training, which is in the time range of an intermediate-term stage of plasticity that combines elements of short- and long-term plasticity and may form a bridge between them. Injection of decitabine (an inhibitor of DNA methylation that may have other actions in these experiments) into an LE sensory neuron blocks the neural correlates of conditioning in the same time range. In addition, we found that both DNA and RNA methylation in the abdominal ganglion are correlated with learning in the same preparations. These results begin to suggest the functions and integration of these different molecular mechanisms during behavioral learning.

Long-term but not short-term memory and synaptic plasticity in many brain areas require neurotrophin signaling, transcription, and epigenetic mechanisms including DNA methylation (e.g., Day et al. 2013; Lu et al. 2014; Maddox et al. 2014; Halder et al. 2016; Duke et al. 2017; Mitre et al. 2017). However, it has been difficult to relate these cellular mechanisms directly to behavior because of the immense complexity of the mammalian brain. One approach to that problem is to examine numerically simpler systems such as the marine mollusk *Aplysia californica*. Several early studies suggested that, as in mammals, long-term memory and long-term facilitation in *Aplysia* involve BDNF-like neurotrophins and Trk-like receptors (Purcell et al. 2003; Ormond et al. 2004; Sharma et al. 2006; Pu et al. 2014), and more recently true *Aplysia* orthologs of BDNF and TrkB have been described and shown to be required for long-term facilitation (Kassabov et al. 2013). In mammals BDNF transcription is regulated in an activity-dependent manner by epigenetic mechanisms including DNA methylation (West et al. 2014; Karpova 2014), and BDNF can in turn regulate transcription (Bambah-Mukku et al. 2014). Transcription is also required for long-term plasticity in *Aplysia* (Montarolo et al. 1986) and DNA methylation plays an important role as well (e.g., Rajasethupathy et al. 2012; Lukowiak et al. 2014; Hawkins et al. 2015; Pearce et al. 2017).

However, these studies have generally examined either behavioral learning or cellular mechanisms but not both at once, and therefore have not been able to specify the functional contributions and integration of the different cellular mechanisms at the behavioral level. To bridge that gap, we have been using a semi-intact preparation of the *Aplysia* siphon withdrawal reflex in which it is possible to relate cellular plasticity directly to behavioral learning (Fig. 1A,B; Antonov et al. 1999, 2001, 2003, 2007, 2010; Hawkins et al. 2015; Yang et al. 2015). That preparation undergoes several simple forms of learning including nonassociative forms such as habituation and sensitization, and associative forms such as classical conditioning (Antonov et al. 1999, 2001) that are qualitatively and quantitatively similar to learning in the intact animal (Carew et al. 1981; Hawkins et al. 1998). The reflex in this preparation is partly monosynaptic—LE siphon sensory neurons (SNs) make direct synaptic connections with LFS siphon motor neurons (MNs), and intermediate-term sensitization and conditioning have been found to involve multiple mechanisms of plasticity at those synapses (Antonov et al. 1999, 2001, 2003, 2007, 2010; Hawkins et al. 2015; Yang et al. 2015).

We have now used the semi-intact preparation to examine the roles of additional molecular mechanisms that are thought to contribute to long-term plasticity. We find that inhibitors of neurotrophin signaling, transcription, and DNA methylation block sensitization and classical conditioning beginning ∼1 h after the start of training, which is in the time range of an intermediate-term stage of plasticity that combines elements of both short-term plasticity including covalent modifications and long-term plasticity

A

B

Sensitization

PreTest   PostTest

Stim
Siphon
ITI=15 min

Tail Shock

Conditioning

Paired

Test   Pre   T1   T2   T3   Post
CS
US

Unpaired

Test   Pre   T1   T2   T3   Post
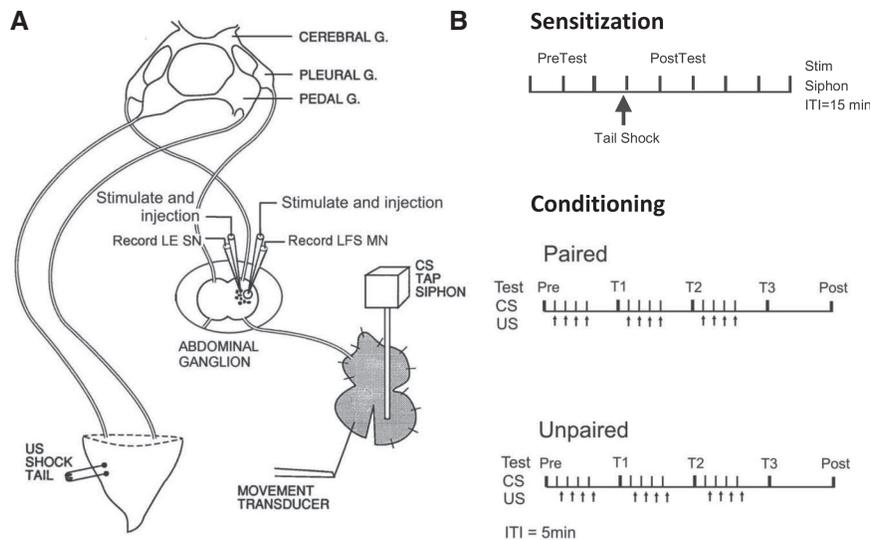CS
US

ITI = 5min

Figure 1. The preparation (*A*) and behavioral protocols (*B*) for sensitization and conditioning. See Materials and Methods for details. The large arrow indicates a train of four shocks, the small arrows indicate single shocks, and the vertical bars indicate siphon taps.

including translation, and may form a bridge between them (Hawkins et al. 2013). Injection of decitabine (an inhibitor of DNA methylation that may have other actions in these experiments) into an LE SN blocks the neural correlates of conditioning in the same time range. In addition, we found that both DNA and RNA methylation in the abdominal ganglion are correlated with learning in the same preparations. These results begin to suggest the functions and integration of these different molecular mechanisms during behavioral learning.

Results

Conditioning is blocked by inhibitors of neurotrophin signaling, transcription, and DNA methylation

During classical conditioning, paired training, in which a siphon tap conditioned stimulus (CS) precedes the tail shock unconditioned stimulus (US) by 0.5 sec, produces a greater increase in subsequent siphon withdrawal than unpaired training with a 2.5 min interval (Antonov et al. 2001). To examine the role of neurotrophin signaling in conditioning we compared changes in the withdrawal reflex in groups that received either paired or unpaired training while the abdominal ganglion was bathed in either the neurotrophin scavenger Trk-Fc or IgG control. In the control group unpaired training produced a decrease in the response to the CS due to habituation ($F_{(3,36)} = 19.95$, $P < 0.001$ for the effect of test). Paired training produced an increase in the response to the CS compared to either the pretest or unpaired training on T1–T3 and the post-test ($F_{(1,24)} = 25.55$, $P < 0.001$ for the effect of pairing on the post-test and $P < 0.001$ overall), demonstrating conditioning (Fig. 2A,B). Trk-Fc significantly reduced the conditioning ($F = 5.27$, $P < 0.05$ for the interaction of drug and pairing on the post-test). As controls, Trk-Fc did not have significant effects on the amplitude of the initial response to either the CS or US or on unpaired training. Trk-Fc also did not reduce conditioning on the first test (T1), which is ~30 min after the beginning of training ($F = 5.52$, $P < 0.05$ for paired versus unpaired). However, it started to have effects by T2 and had significant effects on T3 and the post-test, which is ~2 h after the beginning of training. These results

suggest neurotrophin signaling is required for conditioning beginning ~1 h after the start of training.

Neurotrophin signaling is often regulated by transcription and epigenetic mechanisms or vice versa (Bambah-Mukku et al. 2014; Karpova 2014; West et al. 2014). To examine the role of transcription in conditioning we bathed the abdominal ganglion in either ASW, DMSO, DRB, or Actinomycin D. There was no significant difference in the results with ASW or DMSO, which have therefore been pooled in the control group. In that group unpaired training produced a decrease in the response to the CS due to habituation ($F_{(3,159)} = 4.50$, $P < 0.01$ for the effect of test). Paired training produced an increase in the response to the CS compared to either the pretest or unpaired training ($F_{(1,484)} = 18.54$, $P < 0.001$ for the effect of pairing on the post-test and $P < 0.001$ overall), replicating classical conditioning (Fig. 2C). The reversible transcription inhibitor DRB reduced the conditioning ($F = 7.68$, $P < 0.01$ for the interaction of drug and pairing on the post-test). Similarly, the irreversible transcription inhibitor actinomycin D also significantly reduced the conditioning ($F = 7.76$, $P < 0.01$, not shown). The results with these two different types of transcription inhibitors were not significantly different from each other, suggesting that the results are not due to possible side effects of the drugs. As further controls, neither drug had significant effects on the amplitude of the initial response to either the CS or US. In addition, neither drug had a significant effect on unpaired training.

We next examined the role of an epigenetic mechanism, DNA methylation in conditioning. RG108, which is a small molecule that blocks the active site of DNA methyl transferase, significantly reduced the conditioning ($F = 3.48$, $P < 0.05$ one-tail for the interaction of drug and pairing on the post-test). Similarly, decitabine, which is a cytosine analog that can be incorporated into DNA and block DNA methyl transferase, also reduced the conditioning ($F = 5.46$, $P < 0.05$ on the post-test, not shown). However, decitabine may not have acted by blocking DNA methylation in these experiments because it normally takes many hours to be incorporated into DNA (Öz et al. 2014), and its mechanism of action is unclear. As controls, neither drug had a significant effect on the initial responses to the CS or US or on unpaired training, although there was a trend for both to reduce the decrease in withdrawal due to habituation during unpaired training.

Like Trk-Fc (Fig. 2B), neither DRB nor RG108 reduced the difference between paired and unpaired training on the first test (T1), which is approximately 30 min after the beginning of training ($F = 4.38$, $P < 0.05$ and $F = 3.04$, $P < 0.05$ one-tail for paired versus unpaired). However, they both started to have effects by T2 and had significant effects on T3 and the post-test, which is ~2 h after the beginning of training. These results suggest that transcription and DNA methylation are also required for conditioning beginning ~1 h after the start of training.

Neural correlates of conditioning in the LE SN are blocked by intracellular injection of decitabine

Although the inhibitors were restricted to the abdominal ganglion in these experiments, they might have acted at sites other than the LE-LFS synapses. To analyze the site of action in more detail, we
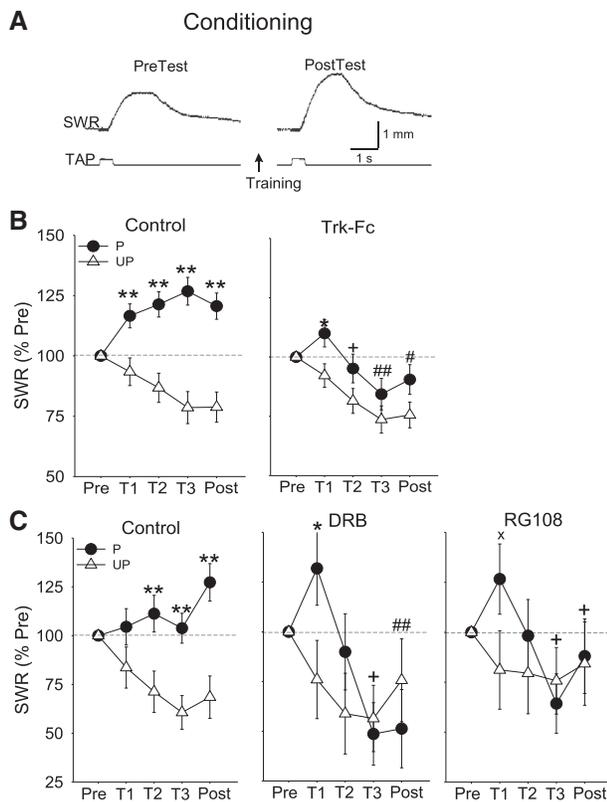
Figure 2. Inhibitors of neurotrophin signaling, transcription, and DNA methylation block conditioning in the siphon-withdrawal preparation. (*A*) Examples of siphon withdrawal (SWR) before (PreTest) and 45 min after (PostTest) conditioning. (*B*) The average amplitude of siphon withdrawal on each test in groups that received paired or unpaired training with the abdominal ganglion bathed in either IgG (*n* = 8 paired and 6 unpaired) or Trk-Fc (*n* = 6 and 8). There was a significant overall drug × pairing × test interaction ($F_{(3,72)}$ = 3.47, P < 0.05). Responses have been normalized to the value on the pretest in each experiment. The average pretest value was 2.0 ± 0.1 mm for IgG and 1.8 ± 0.1 mm for Trk-Fc and the average response to the first tail shock US was 6.4 ± 0.4 mm for IgG and 7.1 ± 0.3 mm for Trk-Fc, not significantly different. (*C*) Average siphon withdrawal on each test in groups that received paired or unpaired training with the abdominal ganglion bathed in either normal seawater or DMSO (Control, *n* = 37 paired and 29 unpaired), DRB (*n* = 9 and 8), Actinomycin D (*n* = 10 and 7, not shown), RG108 (*n* = 10 and 8), or decitabine (*n* = 7 and 6, not shown). There was a significant overall drug × pairing × test interaction ($F_{(12,363)}$ = 2.57, P < 0.01). The average pretest value was 3.1 ± 0.1 mm for control, 3.0 ± 0.3 mm for DRB, 3.5 ± 0.2 mm for Actinomycin D, 3.4 ± 0.2 mm for RG108, and 2.9 ± 0.3 mm for decitabine, and the average response to the first tail shock US was 5.1 ± 0.5 mm, 5.7 ± 0.3 mm, 6.1 ± 0.4 mm, 5.8 ± 0.8 mm, and 5.6 ± 0.6 mm, not significantly different in one-way ANOVAs. In this and subsequent figures error bars indicate SEMs, x = P < 0.05 one-tail, (*) P < 0.05, (**) P < 0.01 for the difference between the trained and control groups, and + = P < 0.05 one-tail, (#) P < 0.05, (##) P < 0.01 for the drug × training interaction.

recorded intracellularly from an identified LE siphon SN and an LFS siphon MN simultaneously with behavioral conditioning and injected either decitabine or vehicle into the SN (Fig. 3A,B). Decitabine is not membrane permeable but rather is taken up by active transport (Ueda et al. 2015), so it should not leak out of the cell. In each experiment, we first confirmed that both neurons contributed to the behavior: the LE SN was activated by the siphon tap CS, and stimulation of the LFS MN produced measurable siphon withdrawal. In vehicle control experiments unpaired training produced a decrease in siphon withdrawal in response to the CS due to habituation ($F_{(3,12)}$ = 9.20, P < 0.01 for the effect of

test), which is caused in part by a decrease in the amplitude of the monosynaptic EPSP from the LE SN to the LFS MN due to homosynaptic depression (F = 13.40, P < 0.001). Paired training produced greater increases than unpaired training in siphon withdrawal ($F_{(1,32)}$ = 18.45, P < 0.001 on the post-test), evoked LFS firing (F = 4.08, P < 0.05 one-tail), evoked LE firing (F = 55.72, P < 0.001), and the EPSP (F = 20.90, P < 0.001). Furthermore, there was a significant within-groups correlation between the increases in the EPSP and siphon withdrawal (r = 0.66, P < 0.05), and the effect of pairing on siphon withdrawal was greatly reduced when that correlation was taken into account in an analysis of covariance. These results replicate those of our previous studies (Antonov et al. 2001, 2003, 2007) and suggest that pairing-specific increases in the monosynaptic SN–MN EPSP make an important contribution to the increase in siphon withdrawal during conditioning. In addition, they demonstrate pairing-specific changes in the evoked firing of the LE SNs that contribute as well. In a few preliminary experiments (see Fig. 3A) we reimpaled the neurons after 24 h and found that all of these effects are maintained for that long, suggesting that our training protocol produces long-term as well as intermediate-term conditioning and that they may involve similar cellular mechanisms.

Injecting decitabine (5 mM in the electrode) into a single LE SN did not have significant effects on either siphon withdrawal or evoked LFS firing, presumably because the withdrawal response in this preparation is mediated by five to eight LE SNs (Byrne et al. 1974; Hickie et al. 1997). This result demonstrates that the preparations were otherwise healthy and capable of conditioning. However, injecting decitabine into an LE neuron abolished the pairing specific increase in evoked firing of that neuron (F = 24.25, P < 0.001 for the interaction of drug and pairing on the post-test). As controls, injecting decitabine did not have a significant effect on the pretest response or on unpaired training. Decitabine also reduced the increase in the EPSP from the LE neuron to an LFS neuron (F = 2.89, P < 0.05 one-tail for the interaction on the post-test). These results suggest that decitabine blocked mechanisms in the presynaptic SNs that contribute to an increase in excitability and firing of those neurons as well as an increase in the EPSP, both of which contribute to the increase in siphon withdrawal following conditioning (Antonov et al. 2001, 2003, 2007).

Intermediate-term sensitization is blocked by Trk-Fc, DRB, and cytosine analogs

In these experiments an inhibitor of neurotrophin signaling blocked conditioning in an intermediate-term time range, similar to the effect of the inhibitor on synaptic facilitation in vitro (Jin et al. 2014, 2015, 2016). However, because only long-term and not intermediate-term plasticity is thought to involve transcription and gene regulation (e.g., Sutton et al. 2001; Kim et al. 2003) we were surprised that inhibitors of RNA synthesis and DNA methylation also blocked conditioning in the intermediate-term range, and that the time courses of those effects were very similar for each of the different types of inhibitors (Fig. 2B,C). Because most previous experiments had used a sensitization protocol (e.g., Montarolo et al. 1986; Rajasethupathy et al. 2012; Kassabov et al. 2013; Pearce et al. 2017) we thought that there might be something unusual about classical conditioning, which had not previously been explored. We therefore next examined the effects of Trk-Fc, DRB, and decitabine on intermediate-term sensitization.

During sensitization, tail shock produces enhancement of responses to subsequent siphon stimulation (Antonov et al. 2010). To examine the role of neurotrophin signaling in sensitization, we compared changes in the withdrawal reflex in groups that received either no shock (control) or a train of four tail shocks while
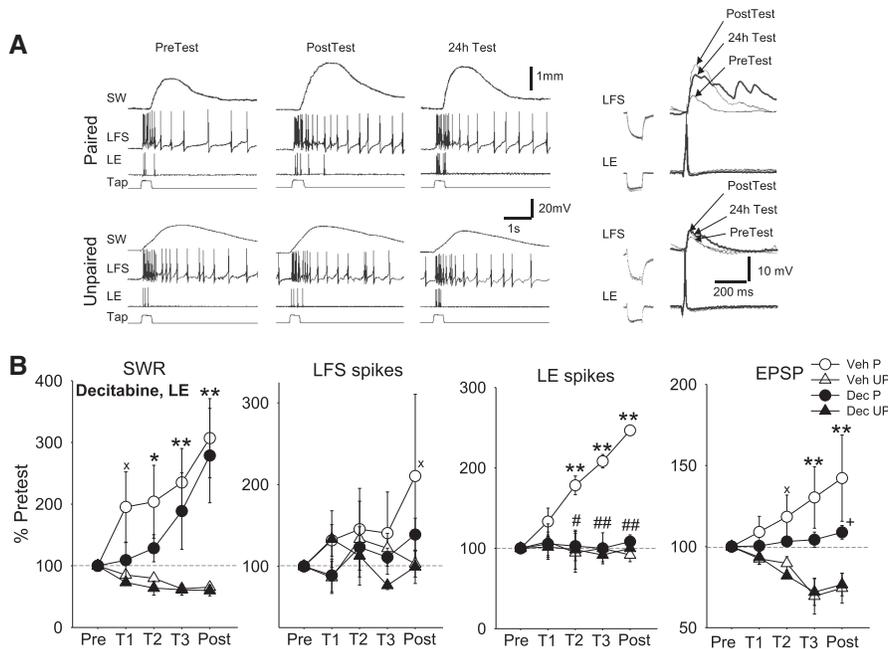
Figure 3. Intracellular injection of decitabine into the LE SN blocks neural correlates of conditioning. (*A*) Examples of siphon withdrawal (SW), evoked firing of an LFS siphon MN and an LE siphon SN, the membrane resistance of the neurons, and the monosynaptic EPSP from the LE neuron to the LFS neuron on the pretest, the post-test, and a 24 h test following paired or unpaired training in a control experiment. (*B*) Average results on each test from experiments like the one shown in *A* with paired or unpaired training following injection of vehicle or decitabine into the LE neuron ($n = 3$ per group). There were significant overall effects of pairing for siphon withdrawal ($F_{(1,8)} = 14.72$, $P < 0.01$), LE spikes ($F = 55.71$, $P < 0.001$), and the EPSP ($F = 12.25$, $P < 0.01$) and a significant overall drug × pairing interaction for LE spikes ($F = 42.57$, $P < 0.001$). The data have been normalized to the value on the pretest in each experiment. The average pretest value for siphon withdrawal was 2.5 ± 0.8 mm for control and 1.9 ± 0.5 mm for decitabine, for evoked LFS firing 6.0 ± 0.3 spikes for control and 5.7 ± 0.8 spikes for decitabine, for evoked LE firing 3.3 ± 0.5 spikes for control and 4.0 ± 0.6 spikes for decitabine, and for the amplitude of the EPSP 8.4 ± 0.9 mV for control and 5.6 ± 0.9 mV for decitabine, and the average siphon withdrawal in response to the first tail shock was 4.9 ± 0.3 mm for control and 4.2 ± 0.3 mm for decitabine, not significantly different.

the abdominal ganglion was bathed with either IgG or Trk-Fc. In the control group repeated siphon stimulation without tail shock produced a gradual decrease in responding, demonstrating habituation ($F_{(5,100)} = 32.03$, $P < 0.001$ for the effect of test). Compared to that no-shock control, tail shock produced an increase in the response to siphon stimulation that lasted more than 77.5 min ($F_{(1,39)} = 16.30$, $P < 0.001$ for the effect of shock overall and $P < 0.01$ on each post-test after the first), demonstrating intermediate-term sensitization (Fig. 4A,B). Trk-Fc did not block sensitization on the first two post-tests ($F = 3.73$, $P < 0.05$ one-tail for the effect of shock on the second post-test), but did significantly reduce it after that ($F = 4.36$, $P < 0.05$ for the interaction of drug and shock on the fourth post-test and $F = 3.02$, $P < 0.05$ one-tail overall). As controls, Trk-Fc did not have significant effects on the amplitude of the initial response to either the siphon tap or tail shock, or on the no-shock (habituation) group. These results suggest that neurotrophin signaling is required for intermediate-term sensitization beginning ~30 min after the shock.

We next compared changes in the withdrawal reflex while the abdominal ganglion was bathed in either ASW, DMSO, DRB, or decitabine. There was no significant difference in the results with ASW or DMSO, which have therefore been pooled in the control group. In that group repeated siphon stimulation without tail shock produced a gradual decrease in responding ($F_{(5,120)} = 9.94$, $P < 0.001$ for the effect of test), replicating habituation. Compared to that no-shock control, tail shock produced an increase in the

response to siphon stimulation that peaked on the second post-test 17.5 min after the shock and lasted more than 77.5 min ($F_{(1,255)} = 22.73$, $P < 0.001$ for the effect of shock overall and $P < 0.001$ on each post-test after the first), replicating intermediate-term sensitization (Fig. 4C).

The reversible transcription inhibitor DRB did not block short-term sensitization on the first two post-tests ($F = 8.92$, $P < 0.01$ for the effect of shock on the second post-test), but did significantly reduce it after that ($F = 10.78$, $P < 0.001$ for the interaction of drug and shock on the third post-test and $F = 4.57$, $P < 0.05$ overall). Similarly, decitabine did not block sensitization on the first two post-tests ($F = 8.23$, $P < 0.01$ for the effect of shock on the second post-test), but did significantly reduce it after that ($F = 8.07$, $P < 0.01$ for the interaction of drug and shock on the third post-test and $F = 5.04$, $P < 0.05$ overall). As controls, neither drug had significant effects on the amplitude of the initial response to either the siphon tap or tail shock or on the no-shock group, although there was a trend for decitabine to reduce habituation in that group.

We also obtained similar results with another cytosine analog, 5-aza-cytidine, which is thought to have basically the same mode of action as decitabine (Stresemann and Lyko 2008) with the same caveats in these experiments. In the control group repeated siphon stimulation without tail shock produced a gradual decrease in responding ($F_{(5,45)} = 13.47$, $P < 0.001$ for the effect of test) replicating habituation, and tail shock produced an increase in the response to siphon stimulation that peaked on the second post-test 17.5 min after the shock and lasted more than 77.5 min ($F_{(1,21)} = 37.17$, $P < 0.01$ for the effect of shock overall and $P < 0.01$ on each post-test after the first), replicating intermediate-term sensitization (not shown). 5-aza-cytidine did not block short-term sensitization on the first three post-tests ($F = 11.15$, $P < 0.01$ for the effect of shock on the third post-test), but did significantly reduce it after that ($F = 4.66$, $P < 0.05$ for the interaction of drug and shock on the fourth post-test and $F = 3.04$, $P < 0.05$ one-tail overall). As controls, 5-aza-cytidine did not have significant effects on the amplitude of the initial response to either the siphon tap or tail shock or on the no-shock group.

These results suggest that, as in conditioning, inhibitors of neurotrophin signaling and transcription and also cytosine analogs do not block short-term sensitization, but they block intermediate-term sensitization beginning ~30 min after the shock.

The change in siphon withdrawal is correlated with DNA methylation in the abdominal ganglion

As another way to examine the role of DNA methylation in intermediate-term learning and to investigate the mode of action of decitabine, we measured the level of DNA methylation in the abdominal ganglion following behavioral training. These experiments ended after three (instead of six) post-tests, which was the earliest test at which decitabine blocked sensitization in the
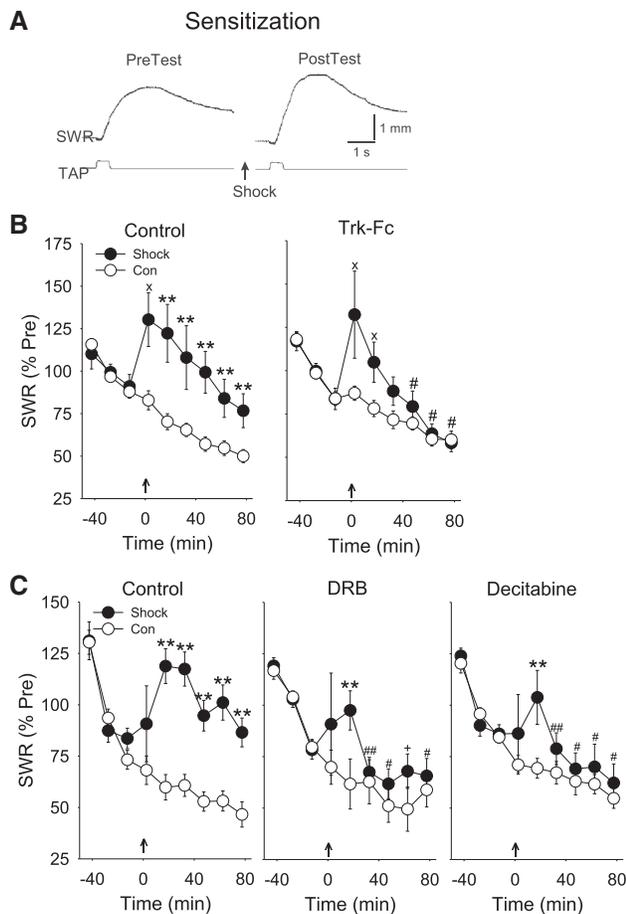
Figure 4. Trk-Fc, DRB, and cytosine analogs block intermediate-term sensitization in the siphon-withdrawal preparation. (*A*) Examples of siphon withdrawal before (PreTest) and after (PostTest) tail shock (sensitization). (*B*) Average siphon withdrawal on each test in groups that received tail shock or no-shock control with the abdominal ganglion bathed in either IgG (*n* = 8 shock and 12 no-shock) or Trk-Fc (*n* = 13 and 10). The average pretest value was 2.2 ± 0.1 mm for IgG and 2.3 ± 0.2 mm for Trk-Fc and the average response to the tail shock was 7.8 ± 0.5 mm for IgG and 8.1 ± 0.2 mm for Trk-Fc, not significantly different. (*C*) Average siphon withdrawal on each test in groups that received tail shock or no-shock control with the abdominal ganglion bathed in either ASW or DMSO (Control, *n* = 12 shock and 11 no-shock), DRB (*n* = 9 and 8), or decitabine (*n* = 9 and 8). There was a significant overall drug × shock interaction ($F_{(2,51)}$ = 5.02, *P* < 0.01). Responses have been normalized to the average value on the three pretests in each experiment. The average pretest value was 2.6 ± 0.2 mm for control, 2.9 ± 0.1 mm for DRB, and 3.1 ± 0.2 mm for decitabine, and the average response to the tail shock was 7.2 ± 0.5 mm for control, 7.0 ± 0.6 mm for DRB, and 8.3 ± 0.9 mm for decitabine, not significantly different. We also obtained similar results with another cytosine analog, 5-aza-cytidine (control *n* = 11 shock and 8 no-shock, 5-aza-cytidine *n* = 3 and 3, not shown). There was a significant overall drug × shock interaction ($F_{(1,21)}$ = 3.04, *P* < 0.05 one-tail). The average pretest value was 2.7 ± 0.2 mm for control and 2.4 ± 0.1 mm for 5-aza-cytidine and the average response to the tail shock was 8.3 ± 0.6 mm for control and 6.8 ± 0.6 mm for 5-aza-cytidine, not significantly different.

experiments shown in Figure 4. The behavioral results replicated those in Figure 4: in the control group repeated siphon stimulation without tail shock produced a gradual decrease in responding ($F_{(2,36)}$ = 10.26, *P* < 0.001 for the effect of test), and tail shock produced an increase in the response to siphon stimulation that peaked on the second post-test ($F_{(1,37)}$ = 13.77, *P* < 0.001 for the effect of shock overall) (Fig. 5A). Decitabine blocked sensitization on the

third post-test (*F* = 4.65, *P* < 0.05 for the interaction of drug and shock). In addition decitabine reduced habituation in the no shock groups ($F_{(1,18)}$ = 5.18, *P* < 0.05), which is also apparent in Figure 4C.

We then removed the ganglion, placed it in ice cold ethanol, and froze it for subsequent assays of DNA 5mC methylation as a percent of total DNA (Fig. 5B1). The DNA was ∼3% 5mC methylated under control (baseline) conditions. There were trends for decitabine to reduce constitutive DNA methylation and for shock to increase it. However, the decitabine effect was not significant, and the overall shock effect was only marginally significant ($F_{(1,3)}$ = 7.90, *P* < 0.05 one-tail in an ANCOVA with the change in siphon withdrawal as the covariate) and decitabine did not block it ($F_{[1,3)}$ = 8.08, *P* < 0.05 one-tail versus no shock). These results do not support an important role of DNA methylation in the behavior, and also suggest that decitabine did not act by inhibiting it. However, those negative conclusions are weakened by the large variability in these experiments, caused in part because they involve between-subjects comparisons. Consistent with that idea, in within-subjects comparisons in the ANCOVA there was a significant negative correlation between DNA methylation and the change in siphon withdrawal overall (*r* = −0.88, *P* < 0.05), suggesting that the DNA methylation and behavior are related (Fig. 5B2). Surprisingly, however, that correlation was similar in each group and not just in the sensitization (control, shock) group. That result again does not support a specific role of DNA methylation in sensitization, but rather suggests that it is involved in something shared by all the groups. One possibility is habituation, which occurs in each group and could indirectly affect sensitization in so far as that involves reversal of habituation (Hawkins et al. 1998, 2006).

The change in siphon withdrawal is also correlated with RNA methylation in the abdominal ganglion

A few recent papers suggest that epitranscriptomic mechanisms including RNA methylation could also play an important role (Widagdo et al. 2016; Li et al. 2017; Walters et al. 2017). RNA has even more different kinds of modifications than DNA including several methylation sites (the "epitranscriptome") (Nainar et al. 2016; Roundtree et al. 2017), and a growing body of evidence suggests that RNA as well as DNA methylation contributes to a variety of functions including neuronal and behavioral plasticity (Nainar et al. 2016; Widagdo et al. 2016; Walters et al. 2017; Merkurjev et al. 2018). In addition, RNA-seq or transcriptome profiling of single identified neurons in Aplysia has shown that they express enzymes that control RNA methylation (writers, readers, and erasers) similar to those found in mammals (Kohn et al. 2016), so it is possible to study the role of RNA methylation in this simple system as well.

In the absence of a good inhibitor of RNA methylation we addressed that question by examining RNA 5mC methylation as a percent of total RNA following behavioral training in some of the experiments shown in Figure 5A. Like DNA, the RNA was ∼3% 5mC methylated under control conditions (Fig. 5C1). There were trends for shock and decitabine to affect RNA methylation, but they were highly variable and not significant. However, there was a significant positive within-subjects correlation between RNA methylation and the change in siphon withdrawal overall (*r* = 0.36, *P* < 0.05), which was similar in each group except decitabine, control (Fig. 5C2). These results indicate that behavioral learning is correlated with RNA as well as DNA methylation, consistent with the idea that both mechanisms may be involved.

Discussion

We have examined mechanisms of intermediate-term learning in a semi-intact preparation of the *Aplysia* siphon withdrawal
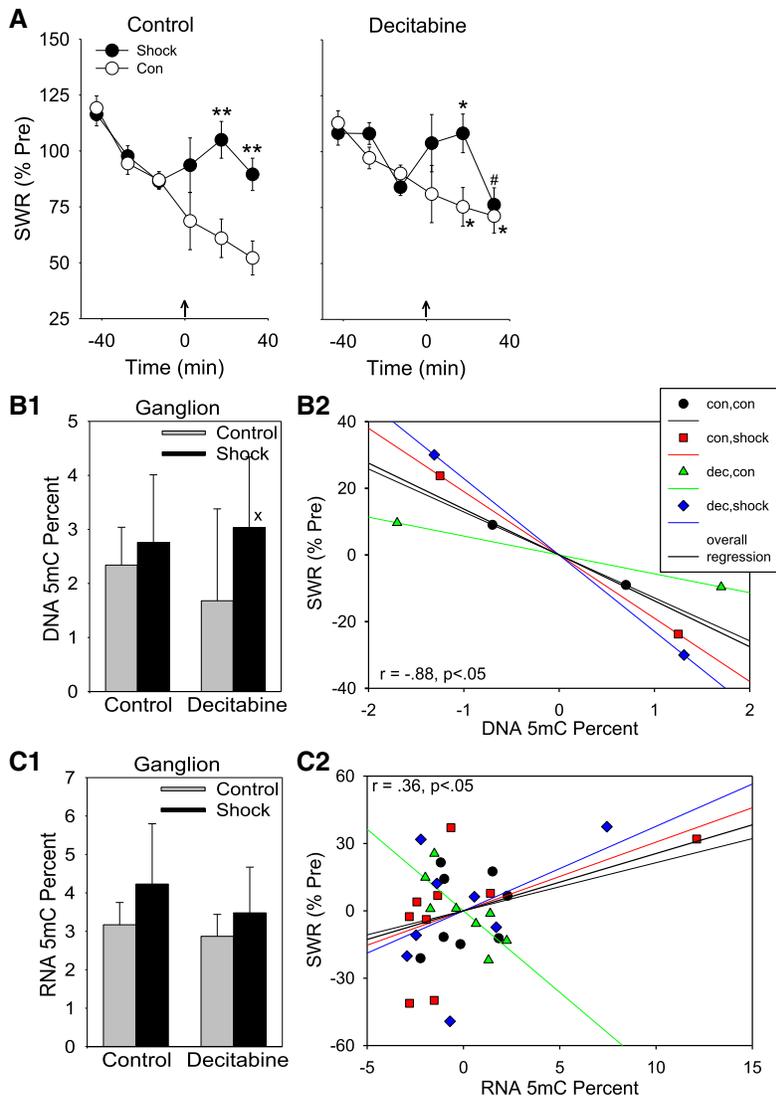
Figure 5. Behavioral learning is correlated with DNA and RNA methylation in the abdominal ganglion. (A) Average siphon withdrawal on each test in groups that received tail shock or no-shock control with the abdominal ganglion bathed in either normal seawater (control, $n = 11$ shock and 10 no-shock) or decitabine ($n = 10$ and 10). There was a significant overall effect of shock ($F_{(1,37)} = 16.58$, $P < 0.001$). Responses have been normalized to the average value on the three pretests in each experiment. The average pretest value was 2.3 ± 0.2 mm for control and 2.7 ± 0.2 mm for decitabine and the average response to the tail shock was 7.8 ± 0.4 mm for control and 7.4 ± 0.5 mm for decitabine, not significantly different. (B) DNA methylation in the abdominal ganglion assayed immediately after the last test in some of the experiments shown in A. (B1) Average 5mC DNA as a percent of total DNA in the different groups (control, $n = 2$ shock and 2 no-shock, decitabine, $n = 2$ shock and 2 no-shock). (B2) Within-groups correlation between the change in siphon withdrawal and 5mC DNA as a percent of total DNA in the abdominal ganglion assayed immediately after the last test in some of the experiments shown in A. There was a negative linear regression in each group and overall ($r = -0.88$, $P < 0.05$) in an ANCOVA. To illustrate the within-groups correlation graphically the mean of each group is set to zero and the groups are pooled, so the correlation reflects the relation independent of group. (C) RNA methylation in experiments similar to those shown in B. (C1) Average 5mC RNA as a percent of total RNA in the different groups (control, $n = 9$ shock and 8 no-shock, decitabine, $n = 8$ shock and 8 no-shock). (C2) Within-groups correlation between the change in siphon withdrawal and 5mC RNA as a percent of total RNA in the abdominal ganglion assayed immediately after the last test in some of the experiments shown in A. There was a positive linear regression in each group (except decitabine, control) and overall ($r = 0.36$, $P < 0.05$).

reflex. The preparation has several advantages for studying cellular and molecular mechanisms of learning. It is possible to restrict drugs to the abdominal ganglion, which mediates the reflex, in behavioral pharmacology experiments. More importantly, it is also possible to record the activity of single identified

neurons in the neural circuit and their synaptic connections, and inject drugs or genetic constructs to study cellular mechanisms during behavioral learning, which is not currently possible in any other preparation.

Previous experiments using this preparation have found that intermediate-term sensitization involves heterosynaptic facilitation by presynaptic cAMP and PKA, which causes an increase in excitability and transmitter release, and postsynaptic Ca^{2+}, which can cause membrane insertion of AMPA-like receptors (Antonov et al. 1999, 2010). Classical conditioning involves two associative mechanisms that interact: activity-dependent facilitation due to priming of the presynaptic adenylyl cyclase by Ca^{2+}, and Hebbian potentiation due to Ca^{2+} influx through postsynaptic NMDA-like receptor channels (Antonov et al. 2003; Yang et al. 2015). These mechanisms all contribute in the intermediate-term time range, and a number of other mechanisms have been identified that also contribute to either short-term or intermediate-term learning or both in this preparation (Antonov et al. 2003, 2007, 2010; Yang et al. 2015). In preliminary experiments we have found that long-term (24 h) conditioning involves the same cellular mechanisms as intermediate-term (Hawkins et al. 2015). However, long-term plasticity in other *Aplysia* preparations is thought to involve additional molecular mechanisms including neurotrophin signaling, transcription, and DNA methylation (Montarolo et al. 1986; Rajasethupathy et al. 2012; Kassabov et al. 2013; Pearce et al. 2017).

We have now examined the roles of those molecular mechanisms in intermediate-term learning in the semi-intact preparation. In behavioral pharmacology experiments we have found that sensitization and classical conditioning are blocked by inhibitors of neurotrophin signaling (Trk-Fc), transcription (DRB and Actinomycin D), and DNA methylation (RG108 and decitabine, although decitabine may act by some other mechanism in these experiments). Furthermore, intracellular injection of decitabine into a single identified neuron in the neural circuit for the behavior blocked cellular changes that are known to underlie the conditioning. Decitabine also consistently reduced habituation, which was not significantly reduced by other inhibitors (see also Antonov et al. 2010).

The inhibitors blocked sensitization and conditioning beginning ~1 h after the start of training, which is in the intermediate-term time range. This time course is similar to that of neurotrophin signaling during synaptic plasticity in vitro (Jin et al. 2014, 2015, 2016), but seems to contradict the dogma that only long-term

and not intermediate-term memory and synaptic plasticity involve transcription and gene regulation (e.g., Sutton et al. 2001; Kim et al. 2003). Our experiments were most similar to those of Sutton et al. (2001), who found that intermediate-term sensitization in a semi-intact preparation of the tail-elicited siphon withdrawal reflex was not blocked by an inhibitor of transcription. However, their experiment had many parametric differences from ours, including delivering five tail shocks spaced over 1 h and applying the inhibitor to a different ganglion. The paper that first described intermediate-term plasticity (Ghirardi et al. 1995) showed that the effect of inhibitors depends not only on the time since training but also on other parameters including the concentration of serotonin (5HT), and that plasticity at the same time (e.g., 30 min) could involve either covalent modifications, translation, or transcription depending on the 5HT concentration. Thus, learning in our preparation may be more similar to facilitation in vitro with a relatively high (100 nM–10 μM) concentration of 5HT.

In addition we found that for sensitization and conditioning, inhibitors of neurotrophin signaling, transcription, and DNA methylation all blocked learning with almost identical time courses. Because those mechanisms can act in series with a lag at each transition, one might expect that blocking the upstream mechanisms would take longer to affect behavior. There are several possible explanations: (1) The inhibitors may block constitutive processes that are necessary to initiate intermediate- or long-term plasticity. (2) The different mechanisms may interact. For example, inhibiting DNA methylation may block RNA synthesis (or vice versa) indirectly by affecting the production of molecules that are necessary for it such as RNA polymerase. In addition, (3) transcription factors including CREB and epigenetic mechanisms including DNA methylation can regulate the expression of neurotrophins such as BDNF (Karpova 2014; West et al. 2014) and BDNF can in turn stimulate CREB-dependent transcription (Bambah-Mukku et al. 2014). Thus, neurotrophin signaling may regulate transcription or DNA methylation and they may in turn regulate neurotrophin signaling, potentially creating a feedback loop so that inhibiting any of them has a similar effect on behavior.

To begin to test these possibilities and to examine the role of RNA as well as DNA methylation more generally, we measured 5mC methylation of DNA or RNA in the abdominal ganglion at the end of some of the behavioral pharmacology experiments. This allowed us to examine the correlations between DNA or RNA methylation and the change in siphon withdrawal within the same preparations. There was a significant negative within-subjects correlation between DNA methylation and the change in siphon withdrawal, and a significant positive within-subjects correlation between RNA methylation and the change in siphon withdrawal. These correlations might be due to causal effects in either direction or common inputs. They were observed for all groups (except RNA methylation with decitabine, no shock), suggesting that they are not specific for sensitization but rather involve something that is shared by those groups. One possibility is habituation due to the test stimulation in each group, which could also affect sensitization in so far as that involves reversal of habituation (Hawkins et al. 1998, 2006). Our results suggest that DNA methylation is positively correlated with the decrease in siphon withdrawal during habituation and RNA methylation is negatively correlated with it, perhaps because RNA methylation inhibits DNA methylation (for example by inhibiting DNA methyltransferases) or vice versa. RNA methylation similarly inhibits an epigenetic mechanism (histone modifications controlling transcription) in mammalian cells (Wang et al. 2018).

These ideas are based on data that involve several simplifications, even in this simple system: we measured methylation in the entire ganglion and not just the relevant neurons, we measured only 5mC methylation and not the many other modifications of DNA or RNA including 6mA methylation, and we did not control the many other pathways that also affect the overall level of methylation. Nonetheless, our results indicate that learning is correlated with both DNA and RNA methylation that may contribute to it, and begin to suggest possible functions of those mechanisms and how they may be integrated at the behavioral level.

Materials and Methods

The behavioral and electrophysiological methods were similar to those we have described previously (Antonov et al. 1999, 2001, 2003, 2007, 2010; Yang et al. 2015). Briefly, the siphon, tail, and central nervous system of hermaphroditic *Aplysia californica* (100–150 g) were dissected and pinned to the floor of a recording chamber filled with circulating, aerated artificial seawater (ASW) at room temperature (Fig. 1A). The siphon was partially split, and one-half was left unpinned. A controlled force stimulator was used to deliver taps of ~20 g/mm^2, 500 msec duration to the pinned half, and withdrawal of the other half was recorded with a low mass isotonic movement transducer attached to the siphon with a silk suture. The peak amplitude of withdrawal was measured using a Digidata 1200 interface to a microcomputer, Clampex version 8.2.0.235, and Clampfit version 8.2.0.235, which were also used to control the stimulation. A fixed capillary electrode was used to deliver AC electric shocks of 25mA, 1 sec duration to the tail.

The preparation was rested for at least 1 h before the beginning of training (Fig. 1B). In sensitization experiments the reflex was tested nine times at 15 min intervals and a train of four shocks (with a 2 sec interval between shocks) was delivered to the tail 2.5 min before the fourth test (first post-test). In test-alone control experiments, the shock was omitted. In conditioning experiments there were three blocks of four training trials each, with a 5 min interval between trials in a block and a 20 min rest between blocks. The response to the siphon tap CS was measured in a pretest 5 min before the first block (Pre), in test trials 15 min after each block (T1-T3), and in a final post-test 45 min after the last block (Post). During paired training, the CS began 0.5 sec before the US (a single tail shock) on each trial. During unpaired training, the interstimulus interval was 2.5 min. Animals were randomly assigned to the training conditions. Experiments were continued only if the siphon withdrawal was between 0.5 and 4.5 mm on the first test, and greater than 3 mm in response to the shock.

In pharmacological experiments, the abdominal ganglion was surrounded by a circular well with the nerves led through a Vaseline seal, so that the ganglion could be bathed in a different solution than the rest of the preparation. Recombinant human TrkB-Fc (250–500 μg/mL, R&D Systems), Actinomycin D (50 μg/mL), DRB (300 μM), RG108 (200 μM), 5-aza-2′-deoxycytidine (decitabine, 75 μM), 5-aza-cytidine (75 μM, all from Tocris Bioscience), or ASW or DMSO (0.05%–0.2%) control was applied for 30 min before and during the experiments. Trk-Fc was applied following a brief rinse with 1% BSA. Actinomycin D, DRB, and RG108 were initially dissolved in DMSO, and then diluted to their final concentrations in ASW immediately before use. Total DNA and RNA were extracted using the QIAmp Micro genomic DNA isolation kit (Cat # 56304, Qiagen) and the RNAqueous-Micro Kit (Cat # 1931, Ambion/Life Technologies) as described previously (Moroz and Kohn 2013) following treatment with RNAse or DNAse, respectively. Global 5-methylcytosine (5-mC) levels were measured using the MethylFlash Global Methylation (5-mC) ELISA Easy Kit (Colorimetric) (Catalog # P-1030, EpiGentek) according to the manufacturer's specifications.

In electrophysiological experiments, the abdominal ganglion was partially desheathed and an LFS siphon MN was impaled with either a single- or double-barrel microelectrode (7–15 Mohm) containing 2.5 M KCl, and an LE siphon SN was impaled with an electrode containing 0.8 M KCl, 0.1% fast green, and 5 mM decitabine or nothing else (vehicle). On each test trial, we measured siphon withdrawal and evoked firing of the LE and LFS neurons in response to the siphon tap and the monosynaptic EPSP produced in the LFS neuron by intracellular stimulation of the LE neuron. In some experiments, we pressure injected decitabine into the LE

neuron 30 min before the start of the experiment. In interleaved control experiments, we injected vehicle.

Most experiments had a full factorial design with two between subjects factors (drug and training condition) and one within subjects factor (test). Accordingly, the data were analyzed with a three-way ANOVA or ANCOVA with one repeated measure (test), followed by planned comparisons of the difference between the training conditions and the drug × training interaction overall, and then at each test to define the time courses of those effects, using Statistica version 13.3. The significance at each time point is indicated in the figures, but only representative time points (routinely including the post-test in conditioning experiments) are described in the Results.

Acknowledgments

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