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.
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 \((F_{1,3,30} = 19.95, P < 0.001\) 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 \(\sim 30\) 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 \(\sim 2\) h after the beginning of training. These results suggest neurotrophin signaling is required for conditioning beginning \(\sim 1\) 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 \((F_{1,159} = 4.50, P < 0.01\) for the effect of test). Paired training produced an increase in the response to the CS compared to either the pretest or unpaired training \((F_{1,445} = 18.54, P < 0.001\) 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 \((F = 7.76, P < 0.01\) overall, 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 (Oz 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 \(\sim 2\) h after the beginning of training. These results suggest that transcription and DNA methylation are also required for conditioning beginning \(\sim 1\) 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
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 responsiveness 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...
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 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 ($F_{(5,35)} = 32.03$, $P < 0.001$ for the effect of test). Compared to that no-shock control, tail shock produced an increase in the response to siphon stimulation that 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 (Stresmann 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 support 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.
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. SB1). 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. SB2). 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”) (Nairn 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 (Nairn 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. SC1). 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. SC2). 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 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 ($F(2,16)=10.26, P<0.001$ 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. SA). Decitabine blocked sensitization on the
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 Ca2+, 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 Ca2+, and Hebbian potentiation due to Ca2+ 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 (Lin et al. 2014, 2015, 2016), but seems to contradict the dogma that only long-term
and not intermediate-term memory and synaptic plasticity involve transcriptions and gene regulations (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 it 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/mm², 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 Clamptft 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 2.5mA, 1 sec duration to the tail.

The preparation was tested for at least 1 day before the training of (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) (all from Tocris Bioscience), 5-aza-cytidine (75 μM, Sigma-Aldrich), 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 QiAamp Micro genomic DNA isolation kit (Cat # 56304, Qiagen) and the RNAeasyMicro 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 SN 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.

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