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The roles of RNA synthesis and protein translation during reconsolidation of passive-avoidance learning in the day-old chick

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ABSTRACT

This series of experiments investigated the role of protein translation and RNA synthesis on consolidation and reconsolidation of passive avoidance learning (PAL) in day-old chicks. Although it is well established that protein translation is required after a reminder, there are conflicting reports in the literature concerning the requirement for RNA synthesis at this time. Day-old male New Hampshire × White Leghorn chicks were trained on a single trial passive avoidance task. The results confirmed the requirement for protein translation during reconsolidation with memory deficits induced by anisomycin (ANI) (10 mg/kg) detected at 60 min post-reminder. It was also established that RNA synthesis was required for consolidation of PAL through inhibition by 5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) (0.075 μg/kg), administered at or after training. The same dose of DRB was also found to inhibit memory post-reactivation. However injections were required before the reminder trial and memory deficits were evident by 60 min, consistent with that found for ANI post-reminder. As with ANI, the DRB-induced memory deficit post-reminder was also transient, and resolved by 24 h post-reminder. For both reconsolidation drug studies, the memory deficit was wholly dependent on the memory being reactivated by a reminder-trial. The study highlights an important role for RNA synthesis following memory reactivation.

1. Introduction

It is well established that RNA synthesis and protein translation is required for the consolidation of a new memory trace following learning (Bailey et al., 1999; Davis and Squire, 1984; Sangha et al., 2003b). Recent research also suggests that protein translation is again required after retrieving a memory, a process termed "reconsolidation". Protein translation inhibitors have been shown to impair memory following reactivation trials in rats (Bernardi et al., 2007; Debiec et al., 2002; Milekic and Alberini, 2002; Nader et al., 2000), mice (Judge and Quartermain, 1982), chicken (Anokhin et al., 2002), snails (Child et al., 2003), crab (Pedreira et al., 2002) and medaka fish (Eisenberg et al., 2003).

Although, the nature of the observed memory deficit can vary according to the age of the memory, the length of the reactivation trial and the strength of the original trace (Suzuki et al., 2004), it is generally agreed that protein translation is required to restabilise the memory trace after activation (however see Cammarota et al., 2004; Lattal and Abel, 2001; Rodriguez-Ortiz et al., 2005 for exceptions). However, there is debate within the literature about the requirement for RNA synthesis activity during reconsolidation. Studies have reported that the transcription factor, c-AMP response element-binding protein (CREB), is required for reconsolidation post-reminder in mice (Kida et al., 2002). A more recent study has shown that mRNA synthesis inhibition impairs fear conditioning when injected into the lateral amygdala of rats. The effect was present when injections were administered in association with training and with reminder trials, indicating effects on both consolidation and reconsolidation processes (Duvarci et al., 2008). Another transcription factor, *Zif268*, has also been shown to be involved in reconsolidation, but not consolidation (Lee et al., 2004). Additionally, RNA synthesis and protein translation inhibitors induced reconsolidation deficits post-reminder in the sea slug *Hermissenda* (Child et al., 2003). Finally, the RNA synthesis inhibitor, actinomycin-D (ACT-D), impaired reconsolidation in the snail *Lymnaea stagnalis* (Sangha et al., 2003a). The authors of this study extended their findings by showing that ablation of the neuron's soma also impaired reconsolidation. This suggests that, for *Lymnaea*, RNA synthesis and altered gene activity in the soma is a necessary component of reconsolidation.

In contrast to these studies, many others have reported no effect from RNA synthesis inhibitors post-reminder, prompting the argument that proteins translated in the dendrites may be sufficient to support reconsolidation. Parsons et al. (2006) administered anisomycin (ANI) (protein translation inhibitor), ACT-D or 5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) (RNA synthesis inhibitors) after initial learning and after memory reactivation. All three compounds induced deficits in memory consolidation. However ANI was the only compound that disrupted memory recall after a reminder. Another study examined the effect of 1-β-D-arabinofuranosylcytosine triphosphate (ara-CTP), a compound that blocks DNA recombination and

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replication, on reconsolidation. The authors confirmed the impairment induced by ANI reported in other studies, but failed to show a memory impairment post-reactivation with application of ara-CTP (Colon-Cesario et al., 2006). Finally, it has been reported that the transcription factor, CCAAT enhancer binding protein β (C/EBPβ), is required for consolidation but not reconsolidation in the hippocampus (Taubenfeld et al., 2001).

As with the studies reported in other species, protein translation is a requirement for reconsolidation of passive avoidance learning (PAL) in the day-old chick (Anokhin et al., 2002; Litvin and Anokhin, 2000). Both ANI and cycloheximide (CXM), when injected just prior to a reminder trial, induce memory reconsolidation deficits by 60 min following the reminder. To date, RNA synthesis inhibitors have not been examined in this species or using this paradigm. However, one study has suggested indirectly that RNA synthesis is not required for reconsolidation of PAL. These authors examined the effect of Colchicine on PAL after a reminder trial. Colchicine acts to block the axonal transport of proteins from the soma to the dendrite, and transiently disrupts consolidation of PAL, which requires RNA synthesis and protein translation in the cell soma. Colchicine, administered 15 min after a reminder, showed no subsequent effect on memory reconsolidation (Mileusnic et al., 2005). This suggests that only local synthesis at the dendrite is required to stabilise a memory trace post-reactivation under these experimental procedures.

The current study examined the effect of DRB and ANI on reconsolidation following a reminder trial in the day-old chick. DRB has been shown to potently prevent the increase in both messenger RNA (mRNA) and heterogenous nuclear RNA in chick embryo (Granick, 1975). DRB has also been used recently to examine the effect of inhibiting RNA synthesis in the auditory thalamus during memory consolidation of fear learning in rats (Apergis-Schoute et al., 2005) and appears to have less neurotoxic effects compared to other inhibitors such as ACT-D (Wetzel et al., 1976). ANI was also examined post-reminder to confirm the findings of other laboratories, which utilise slightly different experimental protocols (Gibbs et al., 2008).

2. Method

2.1. Animals and experimental housing

Day-old New Hampshire × White Leghorn chickens (*Gallus Domesticus*) were obtained from a local hatchery on the morning of each experiment. Cockerels were always employed as they are excess to food production of this egg laying strain. The chicks were housed in pairs to eliminate the confound of stress caused by social isolation (Andrew, 1991). One chick from the pair was marked with a black marker to assist with identification and recording. Wooden boxes (20 × 25 × 20 cm) were maintained at a temperature of between 26 and 29 °C by a single 25 W white incandescent bulb. Chick mash was made available *ad libitum*, and water was provided when the chicks were kept for more than 24 h.

2.2. Drug preparation and administration

ANI and DRB were administered intracranially into the forebrain using a Hamilton repeated dispensing syringe. A plastic stopper regulated the injection depth to 3.5 mm. The target injection region was the intermediate medial mesopallium (IMM; Reiner et al., 2004), and the location of the injection site was determined using bony landmarks on the skull (Gibbs et al., 2003). Doses of ANI and DRB were prepared in saline or dimethyl sulfoxide (DMSO) respectively to a total injection volume of 10 µl per hemisphere. Control animals received saline or DMSO. The experimenter was blind to the pharmacological treatment of each group and the codes were not broken until after the behavioural data had been collected. Drugs were obtained from Sigma Chemicals (Sydney, Australia).

2.3. Procedure

All procedures were approved by the La Trobe University Animal Ethics committee (AEC07/39(P)) and all efforts were made to minimise suffering in accordance with ethical guidelines. Chicks were trained on a modified version of the single-trial passive avoidance task (Crowe and Hale, 2002). The task involved four components: pretraining, training, reminder and retention.

2.3.1. Pretraining

Pretraining of the chicks occurred in two phases. A chrome bead (2 mm diameter) coated in water was presented to each chick for approximately 10 s to encourage the natural tendency of the birds to peck at bright, rapidly moving objects. The procedure was repeated 20 min later to ensure optimal conditions for training. A water coated red bead (4 mm diameter) was then presented to the chicks, again for 10 s, with the number of pecks to this bead recorded using a behavioural event recorder connected to an on-line computer. The number of pecks at this bead acted as the chick's baseline level of pecking.

2.3.2. Training: experimental group

Upon completion of the pre-training phase, the experimental chicks were trained to avoid a red bead visually identical to the one used in the pre-training trial, but which was coated in concentrated (i.e. 100%) methyl anthranilate (MeA). Chicks that pecked at the aversive bead showed a disgust reaction that included behaviours such as beak wiping, head shaking and distress calls, clearly indicating exposure to an aversive experience.

2.3.3. Control group

Upon completion of the pretraining phase, the control chicks were trained on a water coated red bead, visually identical to the stimulus used in the pretraining trial, to control for any effects of the drug not related to memory processes. This is particularly important when avoidance ratios are used as the dependent variable because if the drug affects pecking rates, for example through sedation, the chicks will appear to be avoiding the bead but the 'avoidance' would be independent of memory processes.

Pecks to both the MeA and the water-coated training beads were counted on a behavioural event recorder connected to an on-line computer, consistent with other laboratories using this task (Gibbs et al., 2008). Chicks that failed to peck at either of the training beads within a 10-second period in either the MeA or the water training conditions were excluded from later analysis as they were deemed not to have learned the task. Although each group initially contained 20 chicks, approximately 10% of the sample was excluded on this basis in a non-dose dependent manner, consistent with previous research (see Gibbs et al., 2008 for review). Specific group sizes for each data point are indicated within the figures.

2.3.4. Reminder (reactivation) trial

Where appropriate, memory reconsolidation for the learned stimulus was activated by reminder trials that involved the presentation of a visually identical dry red bead to that used in training which was exposed for approximately 10 s (see Summers et al., 2003 for a full description of this method). Possible lateralisation effects were avoided by ensuring that the bead was seen with both eyes. Chicks were not permitted to peck at this bead, thus avoiding the possibility of a new trace (i.e. the association between the bead and the absence of its reinforcement properties, or an extinction trial) being initiated. With the presentation of the reminder stimulus, chicks reacted with distress behaviour, indicating at a behavioural observation level that the presentation of the dry bead was a sufficient stimulus to reactivate the memory for the original learned experience.

2.3.5. Retention trial

Retention for the task was measured by presenting the chicks with a dry red bead for a period of 10 s at various times following training and counting the number of pecks at the bead.

2.4. Statistical analysis

For all experiments the dependent variable employed was an avoidance ratio (AR), calculated as the number of pecks at the red pretraining bead divided by the number of pecks at the red test bead plus the number of pecks at the red pretraining bead (AR = peck pre/peck pre + peck test). It should be noted that some other studies using this paradigm and species use a discrimination ratio (DR) as the raw statistic, which proposes to measure discriminative learning. The DR compares pecks to the red test bead with pecks to a non-aversive blue bead, also presented at test. The current study employed an AR as previous research in our laboratory has demonstrated that generalised avoidance to the non-aversive bead occurs if the test trial is presented close to the training trial. Both the AR and the DR use the same scale where a low ratio (i.e. 0.5) is taken to indicate lack of recall of the training and a high ratio (i.e. 1.0) indicates complete avoidance or discriminative learning. Analysis was undertaken by univariate ANOVA with post hoc tests. Relevant post hoc tests were conducted in some cases even despite the absence of significant main effects, consistent with the position advocated by (Howell, 1992 pg. 338). All data were analysed using the SPSS software package.

3. Results

3.1. Experimental series 1: protein synthesis translation is required for reconsolidation of PAL

Experimental series 1 was conducted to confirm that the protein translation inhibitor, ANI, impaired reconsolidation of PAL as reported in previous research (Anokhin et al., 2002). This was done because comparisons across laboratories have suggested differences in results with apparently only minor changes in experimental protocol (Gibbs et al., 2008). A dose response study examined retention of chicks administered saline or various doses of ANI (5, 10, 20, 30 μg/kg) immediately after a reminder trial presented at 120 min following training. This reminder time was chosen as it is considered far enough away from the original learned experience as to not interfere with the earlier labile phases of memory consolidation (Summers et al., 2003). Retention was tested at 180 min following the reminder trial (see Fig. 1a).

Univariate ANOVA revealed overall group differences between ANI and saline treated chicks ($F(4, 81) = 3.094$, $p = 0.02$, $\eta^2 = 0.138$). Post hoc Dunnett's t tests indicated that the memory retention of chicks that received a dose of 10 μg/kg ANI were different from those that were administered saline ($p = 0.007$).

A time of injection study was completed to determine the duration of drug sensitivity associated with the reminder trial. Chicks received saline or ANI (10 μg/kg) at various times before and after the reminder trial (−15, −5, 0, +5 min), which was presented at 120 min. Retention was tested at 180 min post-reminder (see Fig. 1b).

Two-way ANOVA revealed a significant main effect for drug ($F(1,143) = 17.067$, $p < 0.005$, $\eta^2 = 0.112$) and a significant main effect for inject time ($F(3,143) = 3.222$, $p = 0.025$, $\eta^2 = 0.066$). The interaction effect was non-significant ($F(3,143) = 2.339$, $p = 0.076$, $\eta^2 = 0.049$). Post hoc Tukey comparisons were used to examine differences between the injection times. This demonstrated that the chicks administered ANI immediately after training were significantly different from the saline chicks at this time ($p < 0.005$). No other injection times showed significant effects between ANI- and saline-treated chicks.

It was also of interest to determine if the reconsolidation deficit observed was dependent upon the time of reminder-trial. Chicks were given saline or ANI immediately after a reminder trial that was presented at various times post-training (10, 40, 90,120 or 1440 min). Retention was tested at 180 min post reminder (see Fig. 1c).

A two-way ANOVA revealed a significant main effect for drug ($F(1,167) = 8.62$, $p = 0.004$, $\eta^2 = 0.052$), a significant main effect for reminder time ($F(4,167) = 4.25$, $p = 0.003$, $\eta^2 = 0.097$) and a significant interaction effect ($F(4,167) = 2.784$, $p = 0.029$, $\eta^2 = 0.066$). Post hoc Tukey comparisons indicated that the ANI chicks were different from saline chicks with reminders at 90 and 120 min ($ps < 0.002$). No other comparison was significant.

To explore the parameters of the ANI-induced memory deficit following the reminder trial, retention levels were measured post-reminder. Chicks were administered ANI or saline immediately after the reminder trial presented at 120 min post-training. Retention was tested at various reminder-test intervals (10, 30, 60, 120, 180 min and 24 h) (see Fig. 1d).

A two-way ANOVA revealed a significant main effect for drug ($F(1,204) = 15.421$, $p = 0.000$, $\eta^2 = 0.074$). The main effects for reminder-test interval ($F(5,204) = 1.969$, $p = 0.085$, $\eta^2 = 0.049$) and the interaction effect ($F(5,204) = 1.454$, $p = 0.207$, $\eta^2 = 0.036$) were non-significant. Simple main effects analysis indicated significant differences between the groups when tested at 60 ($p = 0.014$), 120 ($p = 0.01$) and 180 min ($p = 0.002$) post-reminder.

As an additional control for the presentation of the reminder trial, chicks were administered ANI or saline under the outlined experimental protocol, but without the reminder presentation. Retention was measured at 180 min post-injection (see Fig. 2).

A two-way ANOVA revealed a significant main effect for reminder presentation ($F(1,67) = 6.689$, $p = 0.012$, $\eta^2 = 0.095$) and a significant main effect for drug ($F(1,67) = 4.202$, $p = 0.044$, $\eta^2 = 0.062$). The interaction effect was also significant ($F(1,67) = 8.392$, $p = 0.005$, $\eta^2 = 0.116$). Post hoc independent samples t-test between ANI- and saline-treated chicks with or without a reminder trial indicated significant effects only when a reminder was present ($t(23.017) = 2.851$, $p = 0.009$), confirming that the effect was contingent upon the reminder.

Each experiment in the series was repeated using water trained drug-treated controls to determine any extraneous effects of ANI on avoidance ratios not related to memory impairment. No significant effects were detected between ANI- and saline-treated chicks at the specified dose ($F(1,34) = 0.101$, $p = 0.753$, $\eta^2 = 0.003$), at any time of injection ($F(3,93) = 0.382$, $p = 0.766$, $\eta^2 = 0.013$), time of reminder presentation ($F(3,129) = 1.619$, $p = 0.189$, $\eta^2 = 0.038$) or reminder-test interval ($F(4,125) = 0.413$, $p = 0.799$, $\eta^2 = 0.014$).

3.2. Experimental series 2: protein synthesis transcription is required for consolidation and reconsolidation of PAL

3.2.1. Consolidation

The effect of RNA synthesis inhibitors, such as DRB, has not been previously examined in the day-old chick. Therefore, we wanted to first confirm that RNA synthesis was required for consolidation of PAL. We thus conducted a dose response study using various doses of DRB (0.025, 0.05, 0.075, 0.1 μg/kg), or DMSO, administered directly after training on a 100% MeA aversive bead. The selected dose range was guided by previous rat studies examining the effect of DRB on memory formation (Apergis-Schoute et al., 2005; Parsons et al., 2006). Retention was tested at 180 min post-training (see Fig. 3a).

Univariate ANOVA revealed a significant effect for group ($F(4,80) = 3.046$, $p = 0.022$, $\eta^2 = 0.138$). Post hoc Dunnett's t-test indicated that the chicks that received 0.075 μg/kg were significantly different ($p = 0.008$) from those that received DMSO.

A time of injection study was completed to investigate if there was a limited period of drug sensitivity associated with the training trial. Chicks received DMSO or DRB (0.075 μg/kg) at various times before

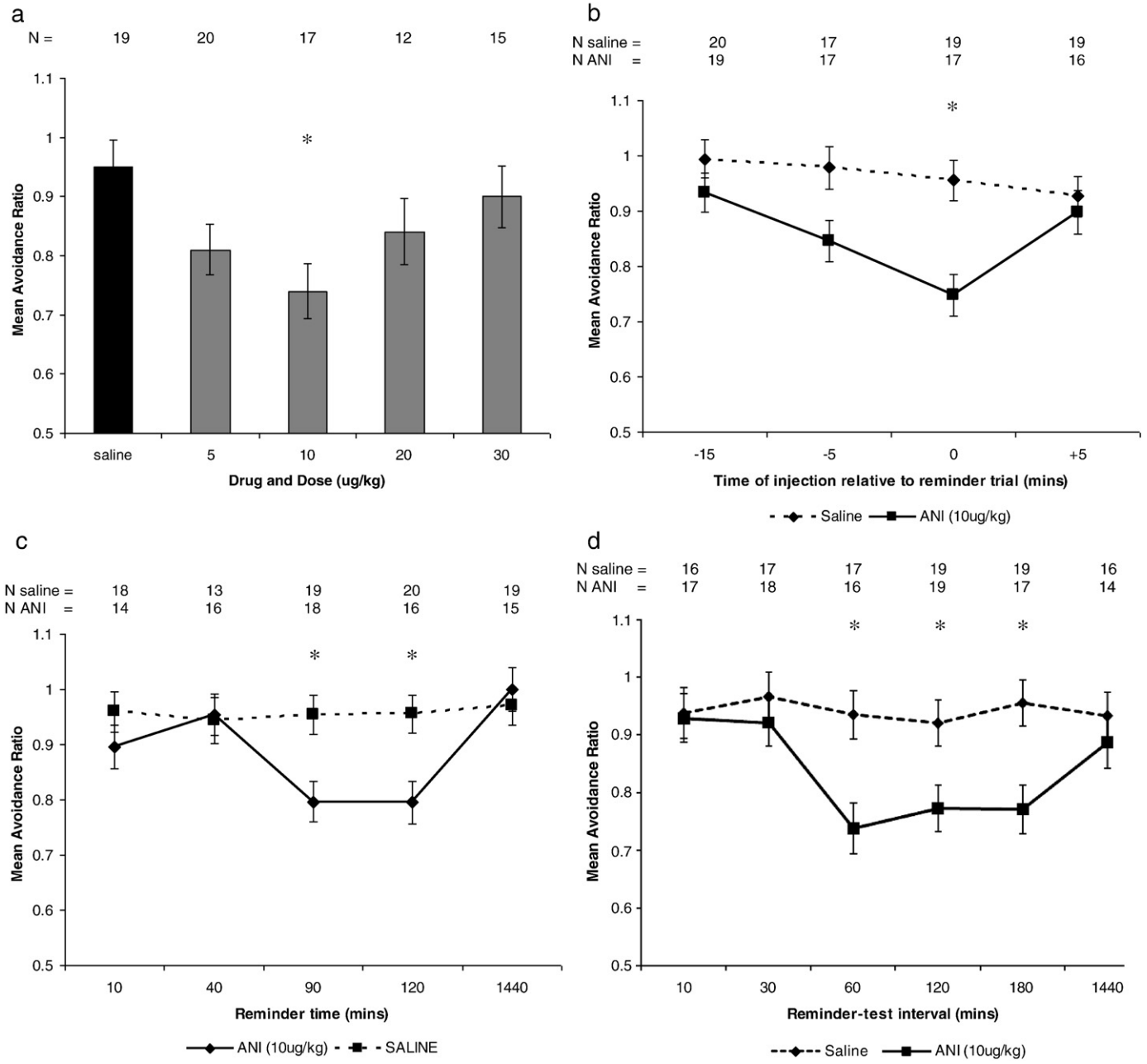

Fig. 1. Mean avoidance ratios of separate groups of chicks administered ANI or saline in association with memory reactivation. Dose response curve (a), time of injection relative to reminder presentation (b), effect of varying the time of reminder presentation (c) and retention levels at various reminder-test intervals (d) (100% MeA) (±SEM) (<0.05).

and after training (−30, −15, −5, 0, +5, +15 and +30 min). Retention was tested at 180 min post-training (see Fig. 3b).

A two-way ANOVA revealed a significant main effect for drug ($F(19.845)$, $p<0.005$, $\eta^2=0.083$). The main effect for time of injection ($F(6,231)=1.893$, $p=0.083$, $\eta^2=0.05$) and the interaction effect ($F(6,231)=1.243$, $p=0.285$, $\eta^2=0.033$) were not significant. However, post hoc simple main effects analysis revealed significant differences between DRB and DMSO treated chicks when injections were administered immediately ($p=0.001$) and 5 min ($p=0.005$) after training. An injection time of immediately following training was chosen for subsequent experiments.

To explore the parameters of the DRB-induced memory deficit, chicks were administered DRB (0.075 μg/kg) or DMSO immediately after training and retention was tested at various times following training (10, 30, 60, 90, 120, 180 min and 24 h) (see Fig. 3c).

A two-way ANOVA revealed a significant main effect for drug ($F(1,230)=28.588$, $p=0.000$, $\eta^2=0.116$). The main effect for training-test interval ($F(6,230)=1.306$, $p=0.256$, $\eta^2=0.35$) and the interaction effect were non-significant ($F(6,230)=1.305$, $p=0.256$, $\eta^2=0.035$). Simple main effects analysis revealed significant differences between DMSO- and DRB-treated chicks when tested at 90 ($p=0.009$), 120 ($p=0.003$), 180 min ($p=0.001$) and 24 h ($p=0.01$) post training.

Each experiment in the series was repeated using water-trained, drug-treated controls to determine any extraneous effects of DRB on avoidance ratios not related to memory impairment. No significant effects were detected between DMSO- and DRB-treated chicks across the dose range ($F(3,49)=1.574$, $p=0.208$, $\eta^2=0.093$), at any time of injection ($F(4,102)=1.623$, $p=0.175$, $\eta^2=0.065$) or at any training-test interval ($F(3,93)=0.08$, $p=0.971$, $\eta^2=0.003$).

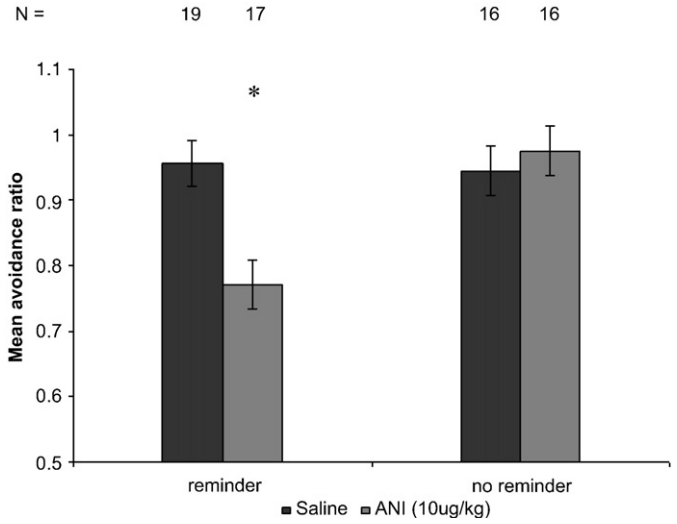

Fig. 2. Mean avoidance ratios of chicks that received either ANI or saline at 120 min post-training (100% MeA) in the presence or absence of a reminder trial. Retention was tested at 180 min post-reminder trial (±SEM) (<0.05).

3.3. Reconsolidation

It was also of interest to determine if DRB would affect memory retention if given in association with a reminder trial, as it is currently unclear if RNA synthesis is required for reconsolidation. Chicks were administered the same dose range of DRB (0.025, 0.05, 0.075 or 0.1 μg/kg) (Apergis-Schoute et al., 2005; Parsons et al., 2006), or DMSO immediately after a reminder trial, consistent with the time of administration used in the consolidation experiments. The reminder trial was presented at 120 min post-training and retention was measured 180 min after the reminder. Univariate ANOVA revealed no significant differences between DMSO- and DRB-treated chicks at any of the doses measured ($F(4,168)=1.552$, $p=0.19$, $\eta^2=0.036$) (data not shown).

This may indicate that RNA synthesis is not required after a reminder. However, it was also possible that the time of administration post-reminder varied from that post-training, and injecting DRB immediately after the reminder, was not sufficient to fully characterise this process. To explore this possibility, various doses of DRB (0.025, 0.05, 0.075 or 0.1 μg/kg) or DMSO were injected 5 min prior to the reminder trial which was again presented at 120 min. Retention was tested at 180 min post-reminder (see Fig. 4a).

Using this protocol, univariate ANOVA revealed a significant main effect for group ($F(4,83)=3.827$, $p=0.007$, $\eta^2=0.162$). Post hoc Dunnett's t tests indicated that the memory retention of chicks that received a dose of 0.075 μg/kg DRB were significantly different from those that were administered DMSO ($p=0.005$).

A time of injection study was completed to determine if there was a limited period of drug sensitivity associated with the reminder trial. Chicks received DMSO or DRB (0.075 μg/kg) at various times before and after the reminder trial (−30, −15, −5, 0, +5, +15 and +30 min), which was presented at 120 min. Retention was tested at 180 min post-reminder (see Fig. 4b).

A two-way ANOVA revealed a significant main effect for drug ($F(1,239)=12.195$, $p=0.001$, $\eta^2=0.051$). The main effect for injection ($F(6,239)=1.147$, $p=0.336$, $\eta^2=0.03$) and the interaction effect ($F(6,239)=1.883$, $p=0.085$, $\eta^2=0.048$) were non-significant. Post hoc Tukey comparisons were used to examine differences between DRB and DMSO treated chicks at each injection time. They showed that the chicks receiving an injection 5 min before the reminder were significantly different from all other injection times.

It was of interest to determine if the reconsolidation deficit observed was dependent on the time of reminder-trial. Chicks were given DMSO or DRB 5 min before a reminder trial that was presented at various times post-training (10, 40, 90, 120, 180 or 1440 min). Retention was tested at 180 min post reminder (see Fig. 4c).

A two-way ANOVA revealed a significant main effect for drug ($F(1,208)=11.041$, $p=0.001$, $\eta^2=0.053$) and a significant interaction

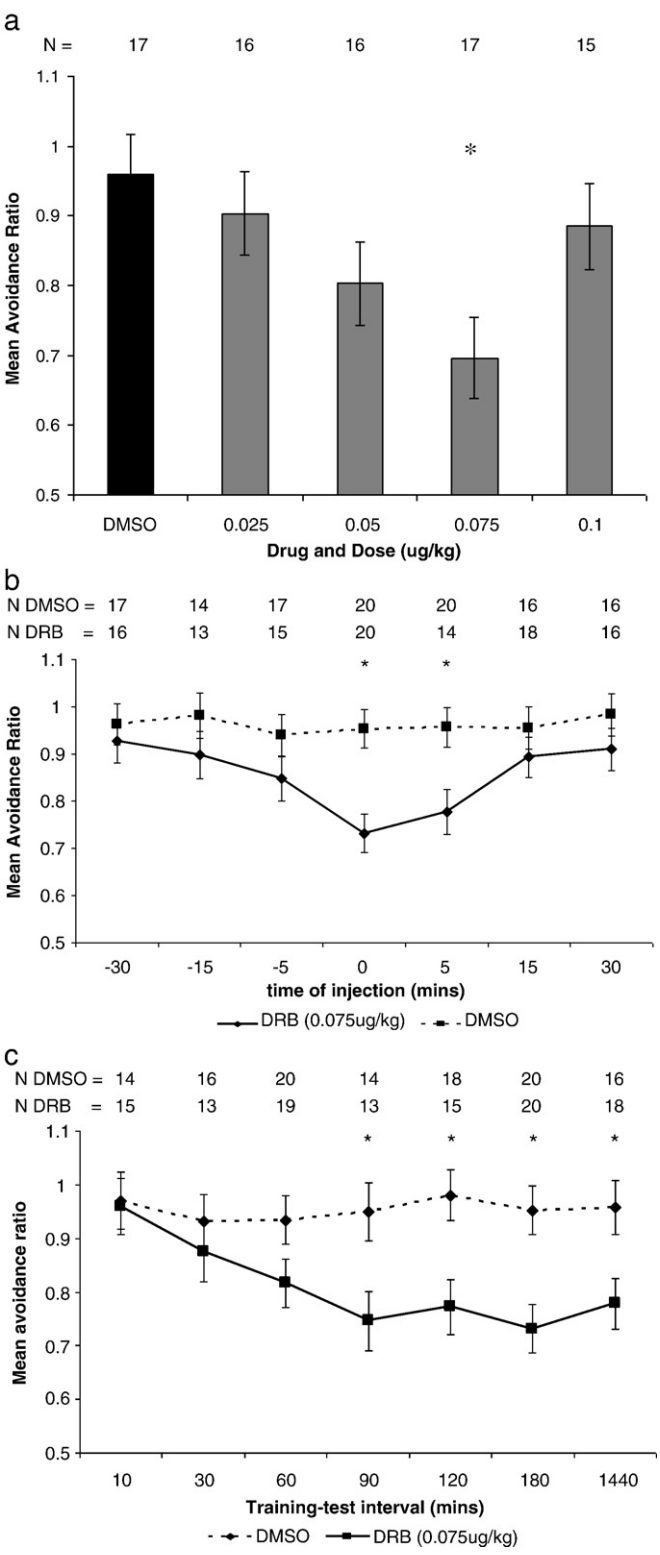

Fig. 3. Mean avoidance ratios of separate groups of chicks administered DRB or DMSO in association with training. Dose response curve (a), time of injection relative to training (b), and retention levels at various training-test intervals (c) (100% MeA) (±SEM) (<0.05).

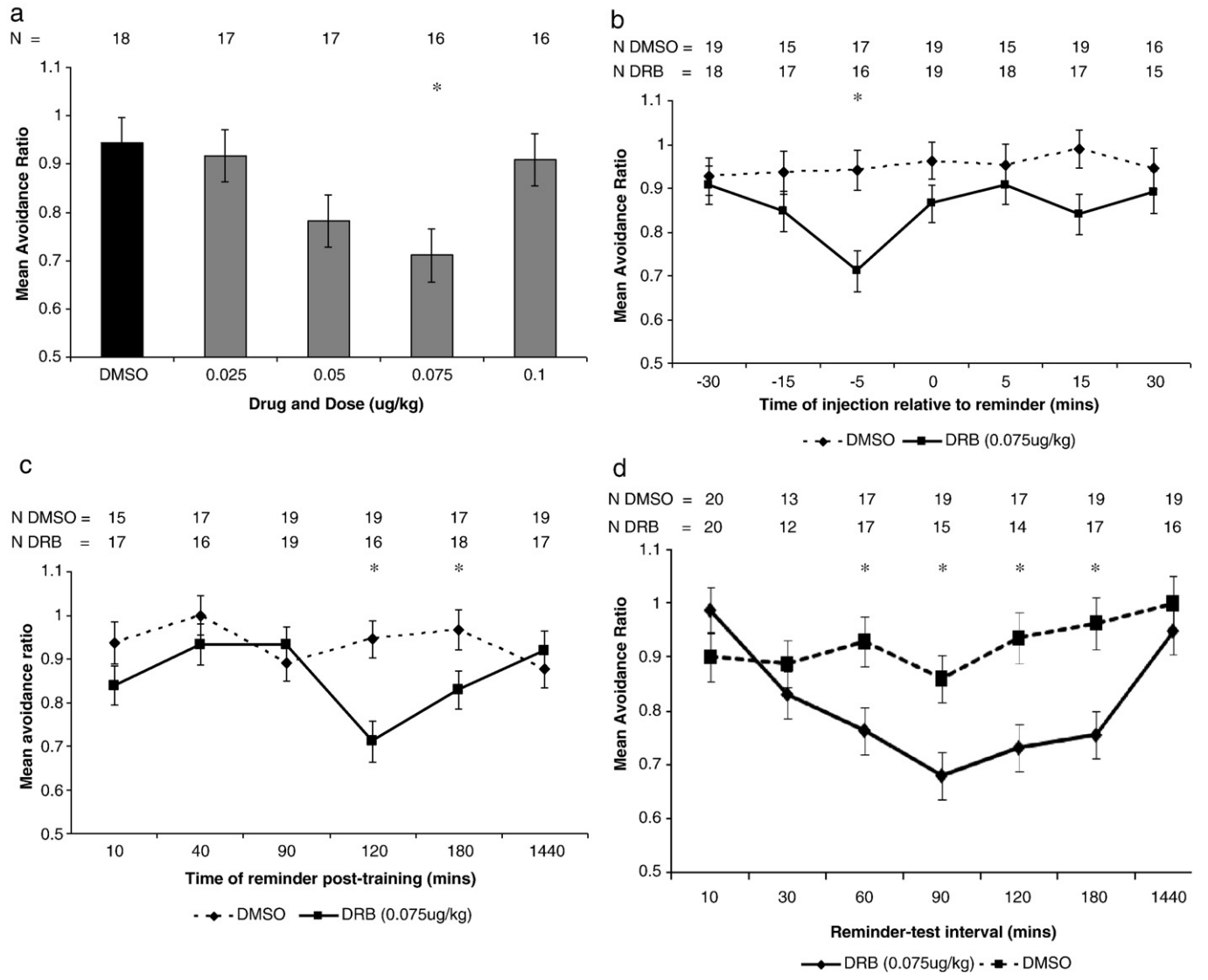

Fig. 4. Mean avoidance ratios of separate groups of chicks administered DRB or DMSO in association with memory reactivation. Dose response curve (a), time of injection relative to reminder presentation (b), effect of varying the time of reminder presentation (c) and retention levels at various reminder-test intervals (d) (100% MeA) (±SEM) (<0.05).

effect ($F(5,208) = 2.914$, $p = 0.015$, $\eta^2 = 0.069$). The main effect for reminder time was non-significant ($F(5,208) = 1.418$, $p = 0.219$, $\eta^2 = 0.035$). Post hoc simple main effects analysis revealed significant differences in retention of DRB and DMSO treated chicks when reminders were administered at 120 ($p = 0.001$) and 180 ($p = 0.029$) min post-training.

To explore the parameters of the DRB-induced memory deficit following the reminder trial, retention was measured at various intervals post-reminder. Chicks were administered DRB or DMSO 5 min before a reminder trial presented at 120 min post-training. Retention was tested at various reminder-test intervals (10, 30, 60, 90, 120, 180 min and 24 h) (see Fig. 4d).

A two-way ANOVA revealed significant main effects for drug ($F(1,234) = 18.553$, $p = 0.000$, $\eta^2 = 0.077$) and test interval ($F(6,234) = 4.318$, $p = 0.000$, $\eta^2 = 0.105$). The interaction effect was also significant ($F(6,234) = 2.846$, $p = 0.001$, $\eta^2 = 0.072$). Post hoc simple main effects analysis revealed significant differences between DMSO- and DRB-treated chicks when retention was measured at 60 ($p = 0.015$), 90 ($p = 0.008$), 120 ($p = 0.004$) and 180 ($p = 0.002$) min post reminder.

As an additional control for the presentation of the reminder trial, chicks were administered either DRB or DMSO under the outlined experimental protocol, but with no reminder trial at 120 min. Retention was measured at 185 min post-injection (see Fig. 5).

A two-way ANOVA revealed a significant main effect for the reminder presentation ($F(1,68) = 4.242$, $p = 0.043$, $\eta^2 = 0.061$) and a highly significant interaction effect ($F(1,68) = 13.657$, $p < 0.0005$, $\eta^2 = 0.174$). The main effect for drug although not significant, demonstrated a very strong trend ($F(1,68) = 3.815$, $p = 0.055$, $\eta^2 = 0.055$). Post hoc independent samples *t*-test between DRB- and DMSO-treated chicks with or without a reminder trial indicated significant effects only when a reminder was presented ($t(17.126) = 3.396$, $p = 0.003$). This indicates that the observed deficit was directly contingent upon the reminder presentation.

Each experiment in the series was repeated using water trained, drug-treated controls. This manoeuvre was employed to determine if there were any extraneous effects of DRB on avoidance ratios not related to memory impairment. For example, if the drug induced sedation, this would appear as a high avoidance (i.e. lack of pecking) but be unrelated to memory processes. No significant differences were detected across the dose range ($F(3,49) = 1.574$, $p = 0.208$, $\eta^2 = 0.093$), at any time of injection ($F(2,69) = 0.685$, $p > 0.05$, $\eta^2 = 0.021$), with all times of reminder presentation ($F(2,72) = 0.516$, $p > 0.05$,

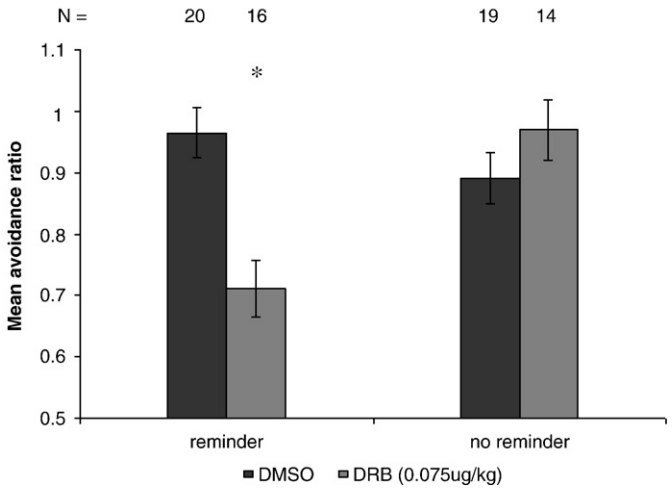

Fig. 5. Mean avoidance ratios of chicks that received either DRB or DMSO at 115 min post-training (100% MeA) in the presence or absence of a reminder trial. Retention was tested at 180 min post-reminder trial (±SEM) (*$p<0.05$).

$\eta^2 = 0.015$) or at any reminder-test interval ($F(4,102) = 0.836$, $p > 0.05$, $\eta^2 = 0.035$). This indicates that any memory deficits detected were due to effects on memory related process rather than to any non-specific effects of the drug.

4. Discussion

The results from this study confirm that protein translation is required for reconsolidation of PAL in the day-old chick. The results also indicated that RNA synthesis, through inhibition by DRB, is required for both the consolidation and the reconsolidation of PAL. The parameters of the DRB-induced memory deficit differed dependent upon whether the drug was administered around the time of training or whether it was administered in association with the reminder trial. Injections of DRB were required immediately or 5 min after training to inhibit consolidation, compared to 5 min before the reminder presentation in order to induce reconsolidation deficits. The retention functions following both training and reminder were also slightly different. Whereas a memory deficit was evident by 90 min after training, significant differences were evident between DMSO- and DRB-treated chicks at the earlier time of 60 min post-reminder. Additionally, the memory deficit persisted until 24 h post-training but had resolved by this time post-reminder. The deficit observed after the reminder trial was directly contingent upon the reminder presentation, as without the reminder trial, no memory disruption was observed. These results strengthen the proposition arising from the rodent literature that RNA synthesis is required following memory reactivation.

Experimental series 1 investigated the findings arising from other laboratories that ANI induced memory deficits in the day-old chick post-reminder. This series was completed because a recent review has suggested that even apparently small procedural differences in the PAL paradigm can produce significant differences in experimental outcomes (Gibbs et al., 2008). A dose of 10 µg/kg ANI impaired memory reconsolidation when injected immediately following the reminder trial. This dose was consistent with other studies that have examined ANI post-reactivation in the day-old chick (Anokhin et al., 2002; Salinska et al., 2004).

A specific window of drug sensitivity around the time of the reminder trial was observed, which was different to that seen around the training. When ANI was administered around the time of training, injections were effective from 30 min prior to 30 min after training (Anokhin et al., 2002; Gibbs and Ng, 1977). In contrast, when ANI was administered around the time of reminder, injections were required either 5 min before, or immediately after the reminder trial. The observed narrow window of drug sensitivity supports other studies examining reconsolidation in the chick (Sherry and Crowe, 2008; Summers et al., 2003).

The current data also demonstrated a specific window of sensitivity for the time of reminder presentation. Deficits were detected only when reminders were presented at 90 or 120 min post-reminder. A reminder time of 120 min post-training was thus employed in the subsequent experiments. This reminder time is commonly used in experiments examining reconsolidation in the day-old chick as it is considered far enough away from the training itself so as to not interfere with the early labile stages of memory (see Summers et al., 2003 for review). Additionally, although it is clear that further changes occur in the circuitry beyond 60 min post-training, it is generally conceded that the majority of the earlier transitory changes have occurred by this time point (see Rose, 2004 for review).

With injections immediately after a reminder presented at 120 min post-training, retention deficits were evident by 60 min, but had resolved by 24 h post reminder. Although other studies have measured retention levels at different reminder-test intervals, the transient deficit observed is consistent with other studies examining memory retention following reminder treatments in the day-old chick (Anokhin et al., 2002; Salinska et al., 2004; Sherry et al., 2005). The ANI-induced memory deficit was dependent upon the presence of the reminder, as without the reminder memory was unaffected when measured at 180 min post-training.

The second series of experiments examined the effect of the RNA synthesis inhibitor, DRB, on memory consolidation and reconsolidation of PAL. A dose of 0.075 µg/kg DRB was found to inhibit memory retention when administered close to the time of training. This dose is consistent with the effective doses demonstrated to disrupt consolidation in the amygdala of the adult rat (Apergis-Schoute et al., 2005; Parsons et al., 2006). The dose response curve showed the characteristic U-shaped curve of increasing effectiveness of higher doses until a reversing trend (Andrew, 1991). The time of injection was also consistent with other rodent studies examining this process (Igaz et al., 2002). Investigating retention at various times after training indicated that chicks receiving DRB were significantly different from control animals by 90 min post-training. The memory deficit persisted until at least 24 h, indicating a permanent memory deficit.

The same dose of DRB (0.075 µg/kg) was observed to inhibit reconsolidation of PAL, however injections were required 5 min prior to the reminder trial which was presented at 120 min. Some difficulties are inherent with using injections prior to the learning or re-learning trial. For example, administering drugs prior to the reminder trial may reduce alertness for the event or the injection may affect initial consolidation of the trace. Several control measures supported the decision to use an injection time of 5 min before the reminder in subsequent experiments, including the absence of effect on memory retention when no reminder trial was presented. This result suggests that: 1) no deficit was observed when no reminder was present because the memory was not reactivated; therefore no reconsolidation phase could be initiated, and 2) administration of the drug at this time had no effect on the formation of the initial trace. If it did, a deficit would be observed regardless of whether the memory was reactivated with a reminder because the original 'copy' of the memory would be compromised. The protocol of administering the drug 5 min before the reminder to target reconsolidation processes independent of consolidation mechanisms is thus justifiable.

Varying the time of reminder revealed significant effects with reminders at 120 and 180 min post-training. This was later than when the reminder trial was administered in association with ANI, with differences between the agents detected at the reminder times of 90 and 120 min. When DRB was administered after a reminder trial at 120 min, memory deficits were detected at 60 min, but had resolved by 24 h post-reminder. This is in contrast with when DRB was injected

post-training. Under this protocol, deficits were detected later, at 90 min, but persisted beyond 24 h. These data are consistent with other studies that have shown transient memory deficits following reminder treatments in the day-old chick (Anokhin et al., 2002; Salinska et al., 2004; Sherry et al., 2005).

In the rodent literature, a transient memory deficit following reactivation is normally interpreted to be due to impaired retrieval mechanisms, rather than to impaired reconsolidation (Nader, 2003). However, intact retrieval mechanisms were observed in the current study as the birds exhibited avoidance and distressed behaviours with the presentation of the reminder stimulus. Some authors have suggested that rather than the original trace being activated with retrieval, a representative trace, or a transient replica of the original that is activated to control and guide behaviour, is triggered (Sara, 2000; Summers et al., 2003). This would allow the organism to use this information without running the risk of permanently erasing the original trace. This proposition is consistent with the observation of a transient memory deficit, as the information contained in the original trace can resume behavioural control after the impaired phase of reconsolidation has resolved.

The issue of extinction can also be addressed with the observation of transiency. In the learning literature, extinction is a process initiated when a conditioned stimulus is presented without its reinforcing properties (i.e. the aversive stimulus). A reminder trial is therefore essentially an extinction trial [although a number of researchers argue that the two processes can be experimentally separated depending on the length of the reminder trial (Eisenberg et al., 2003; Pedreira and Maldonado, 2003)]. The PAL paradigm protects against the confounder of extinction by not allowing the chicks to peck at the reminder bead. Therefore, no new learning process could be initiated. Secondly, if extinction was being initiated, the retention function should appear the same as the consolidation retention function. The results of the current study thus indicate that inhibition of consolidation by DRB is not identical to its effects on reconsolidation.

All of the data points in the current series of experiments were repeated using drug-treated, water-trained birds. This manoeuvre was necessary to control for the non-specific effects of the drug unrelated to memory processes. For example, if the drugs affected the birds ability to produce a pecking response (i.e. through motor inhibition) this would present as a high avoidance ratio but be independent of memory-related processes. For all points examined in the current study, the avoidance ratios of the water-trained birds were low. This indicates that in chicks trained on the aversive bead, no obvious non-memory related effects were impacting upon the bird's ability to respond.

The current study demonstrates that the consolidation, and more importantly the reconsolidation, of PAL requires RNA synthesis. This finding concurs with other studies examining reminder treatments in the mouse (Guzowski, 2002; Kida et al., 2002), sea slug (Child et al., 2003) and snail (Sangha et al., 2003a). The studies using the snail have also demonstrated that ablation of the soma, where RNA synthesis occurs, inhibited reconsolidation. In contrast a similar study with chicks used pharmacological blockade of axonal protein transport from the soma to the dendrite, but found no effect on reconsolidation (Mileusnic et al., 2005). However, the latter authors injected colchicine 15 min after the reminder treatment, perhaps too late to inhibit reconsolidation processes considering that injections were required prior to the reminder in the current study.

The potential mechanism of RNA synthesis post-reactivation of PAL remains speculative. Summers et al. (1996) have suggested that two phases of reconsolidation are present following reminder. The first is rapidly induced and short acting leading to initial destabilisation of the trace. The second is induced later and is longer lasting possibly providing a mechanism whereby the underlying trace may be modified with newly arising information (Summers et al., 1995, 1997). It is feasible to suggest that this modification may involve additional RNA synthesis and structural protein changes that cement the new information into long-term memory. The current study supports this proposition with the demonstration that RNA synthesis is required following reminder. Additionally, the phase of memory inhibited by DRB appears similar to Summers and et al. (2003) proposed second phase of reconsolidation in that it is induced later, and is longer lasting, than the earlier transitory phase.

An earlier study conducted in our laboratory has suggested a possible process leading to RNA synthesis transcription, induced from activation of the dopamine D1 receptor. We found that inhibiting the D1 receptor with SCH23390 post reminder induced retention deficits by 60 min (Sherry et al., 2005), consistent with the DRB induced memory impairment seen in the current study. Stimulation of the D1 receptor activates the enzyme adenylyl cyclase, which subsequently increases the levels of intracellular cyclic AMP (cAMP) between 30 and 60 min post PAL (Brown, 1984; Palermo-Neto, 1997). As a consequence of this increase, cAMP-dependent protein kinase A (PKA) is activated. It is suggested that PKA may then act to phosphorylate both CREB (Bacskai et al., 1993) and dopamine and adenosine 3′5′ monophosphate-regulated phosphoprotein (DARPP-32) (Liu & Graybiel, 1996). When activated, DARPP-32 inhibits protein phosphatase 1 (PP-1) (Greengard et al., 1999). This compound dephosphorylates CREB (Liu & Graybiel, 1996); therefore inhibition of PP-1 by DARPP-32 acts to prolong the activity of CREB by preventing dephosphorylation.

Studies are currently underway in our laboratory investigating the phosphorylation of DARPP-32 after training and reminder treatments. Additionally, we also hope to investigate the activation of the immediate early genes during reconsolidation. Although it is clear that RNA synthesis is required post-reminder, it is possible that not all genes are newly transcribed, but only a subset of them (von Hertzen and Giese, 2005). This could provide a possible explanation of differences in the operating parameters of consolidation and reconsolidation observed in the current study.

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