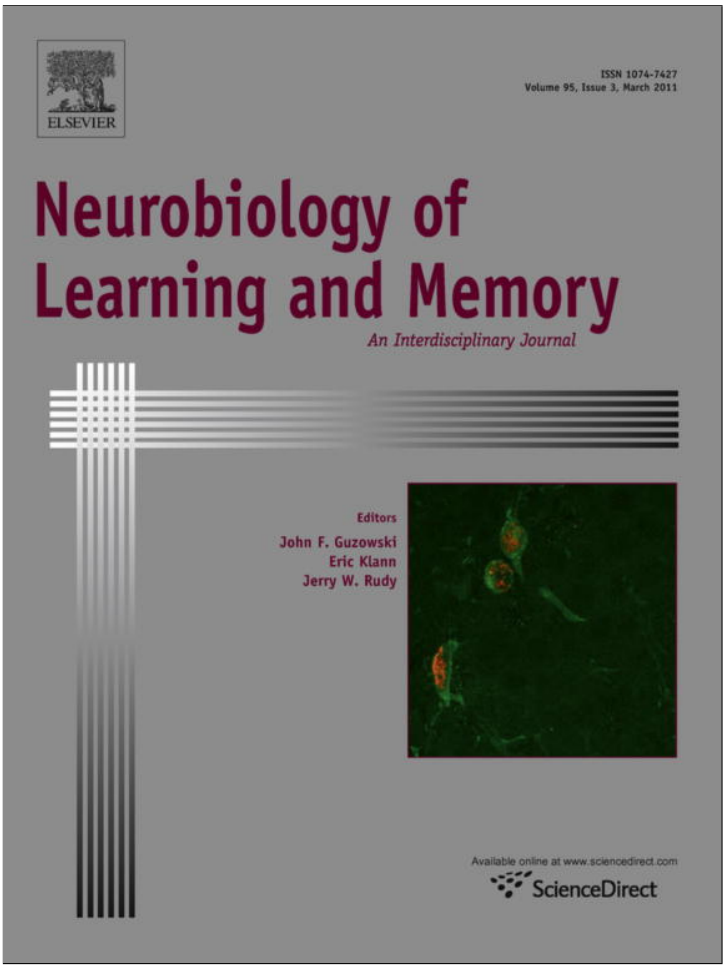

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Mammalian intermediate-term memory: New findings in neonate rat

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ABSTRACT

The ability of anisomycin, a translation inhibitor, and actinomycin, a transcription inhibitor to disrupt a cAMP/PKA-dependent odor preference memory in neonate rat was examined. Previous reports in invertebrates had described a novel translation-dependent intermediate-term memory dissected with these inhibitors, but similar effects have not been reported in mammalian memory systems. When anisomycin was infused into the olfactory bulb after the pairing of peppermint odor and the β-adrenoceptor agonist isoproterenol (2 mg/kg), short-term memory (1 or 3 h) was intact, but intermediate (5 h) and long-term (24 h) memory was disrupted. When actinomycin was infused, only long-term memory was disrupted. This pattern of results is consistent with that reported in invertebrates for intermediate-term memory and led us to try a lower level of the unconditioned stimulus (isoproterenol) to isolate intermediate-term memory from long-term memory. Pups given a dose of 1.5 mg/kg isoproterenol paired with peppermint odor showed memory for peppermint 5 h, but not 24 h, after training. These observations in the rat pup olfactory system parallel short-, intermediate- and long-term memory characteristics previously described in invertebrates. Odor preference memory in neonate rodents offers a tool to increase our understanding of the properties and mechanisms of multi-phasic memory in mammals.

1. Introduction

In 1995 Kandel's group, investigating cAMP/PKA-dependent synaptic sensitization in *Aplysia* cell cultures (Ghirardi, Montarolo, & Kandel, 1995), reported a novel intermediate phase of sensitization that was blocked by disruption of protein translation using anisomycin, but that was not blocked by disruption of protein transcription using actinomycin D. This intermediate form could be selectively demonstrated using serotonin concentrations intermediate between lower concentrations which elicited short term sensitization, insensitive to any disruption of protein synthesis and higher concentrations which induced long-term sensitization (24 h) that could be disrupted by both the transcription inhibitor actinomycin D and the translation inhibitor, anisomycin. Subsequently, behavioral sensitization with three distinct time phases (short-term memory, STM; intermediate-term memory, ITM; long-term memory, LTM) was demonstrated in intact Aplysia, with ITM outlasting STM by more than an hour (Sutton, Masters, Bagnall, & Carew, 2001). The same unconditioned stimulus protocols delivered in a reduced preparation showed ITM was sensitive to inhibition of protein translation, but not protein transcription (Parvez, Rosenegger, Martens, Orr, & Lukowiak, 2006b). These were the first mechanistic studies of multi-phasic memory. A protein synthesis-independent ITM has also been described in *Aplysia* and is distinguished, in part, from translation-dependent ITM by dependence on protein kinase C rather than protein kinase A (Sutton, Bagnall, Sharma, Shobe, & Carew, 2004).

Associative memory also exhibits a protein translation-dependent ITM. This has been characterized in some detail using operant conditioning in the pond snail (Lymnea) (Parvez, Moisseev, & Lukowiak, 2006a). Following 30 min of training an operant ITM is seen at 3 h, which is blocked by the translation blocker anisomycin, but not the transcription blocker actinomycin D. With 1 h of training, memory is seen at longer intervals, up to and including 24 h, and this LTM is blocked by both translation and transcription inhibitors of protein synthesis (Sangha, Scheibenstock, McComb, & Lukowiak, 2003). When the soma critical for inducing pond snail LTM is removed, operant ITM is still observed, providing additional evidence that translation in neurites provides the support for

Abbreviations: AMPA, α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate; BDNF, brain derived neurotrophic factor; cAMP, cyclic adenosine monophosphate; CAMKII, calcium (Ca2+)/calmodulin-dependent protein kinase II; EPAC, exchange protein directly activated by cAMP; Iso, isoproterenol; ITM, intermediate-term memory; LTM, long-term memory; RNA, messenger ribonucleic acids; NMDA, *N*-methyl-D-aspartic acid; PKA, protein kinase A; PND, postnatal day; STM, short-term memory.

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ITM (Scheibenstock, Krygier, Haque, Syed, & Lukowiak, 2002). ITM has also been reported in a classical conditioning paradigm using light as the conditioned stimulus (CS) in Hermissenda. Again this ITM is translation, but not transcription, dependent as revealed by selective protein synthesis inhibitors (Crow, Xue-Bian, & Siddiqi, 1999).

The discovery of protein synthesis machinery and mRNAs near synaptic sites (Steward & Levy, 1982), which are regulated, as first proposed, by synaptic activation (for a recent review see (Bramham & Wells (2007)) and behavioral work with learning and memory in mutant mice with impaired local mRNA targeting (Miller et al., 2002) point to a role for local protein translation in mammalian memory. However, translation-dependent ITM has not yet been described in vertebrates. Translation-dependent ITM in rodents has been assumed in a novel object recognition paradigm, but has not been tested with a translation inhibitor (Taglialatela, Hogan, Zhang, & Dineley, 2009).

The neonate rat shows both short-term and long-term odor preference memory initiated by activation of the cAMP/PKA cascade. Neonate rat odor preference learning depends on the activation of β-adrenoceptors in the olfactory bulb (Harley, Darby-King, McCann, & McLean, 2006; Sullivan, Stackenwalt, Nasr, Lemon, & Wilson, 2000; Sullivan & Wilson, 1992). Activation of β-adrenoceptors (as the unconditioned stimulus, US), paired with a novel odor (as the conditioned stimulus, CS), is both necessary and sufficient for the expression of a preference for the odor 24 h later (Sullivan, McGaugh, & Leon, 1991). We (Langdon, Harley, & McLean, 1997; Rumsey, Darby-King, Harley, & McLean, 2001; Yuan, Harley, Darby-King, Neve, & McLean, 2003) and others (Moriceau & Sullivan, 2004) have shown that manipulations limited to the olfactory bulb are sufficient to induce or prevent early odor preference learning.

In the present experiments, intrabulbar infusions of protein synthesis inhibitors for translation (anisomycin) and transcription (actinomycin) given immediately after training were used to explore the protein synthesis requirements of 3 h, 5 h and 24 h odor preference memory in rat pups. We also varied the dose of the US (isoproterenol, Iso) to probe the induction of ITM without LTM in this model.

2. Material and methods

2.1. Subjects

Subjects were Sprague–Dawley pups. Litter effects were controlled by using one male and one female for a training condition within a litter. Animals were maintained on a 12 h light/dark cycle with food and water available *ad libitum*. All experiments were approved by the Institutional Animal Care Committee of Memorial University and followed the standards of the Canadian Council on Animal Care.

2.2. Surgery

On postnatal day (PND) 5, pups were anesthetized by hypothermia and the skull over the olfactory bulbs exposed. A small plastic screw (Small Parts Inc., USA) was glued upside down caudally and two holes 2 mm apart were drilled over the central portion of the bulbs. A pair of 23 gauge stainless steel guide cannulae, 6 mm long, was lowered until it rested on the surface of the olfactory bulbs. Dental acrylic secured the cannula assembly to the plastic screw. A biting deterrent gel (Four Paws Ltd., NY) was applied to the sutured skin to minimize maternal disturbance. Pups were returned to the dam within 30 min from the start of surgery.

2.3. Training

On PND six pups were removed from the dam to receive a 50 μl subcutaneous injection of either sterile saline or 1, 1.5 or 2 mg/kg Iso (Sigma Chem) and then returned to the home cage for 30 min. The pup was then placed into a clean weigh boat for 10 min away from the dam. Next, it was placed in a polycarbonate cage (30 cm × 19 cm × 13 cm) containing peppermint-scented bedding (0.3 ml peppermint extract in 500 ml fresh bedding) for 10 min in a separate room maintained at 27 °C. In many experiments, the 10 min odor exposure was followed by olfactory bulb infusion of saline, anisomycin, or actinomycin-D as described below. After infusion, the pup was returned to the dam.

2.4. Macromolecular synthesis inhibitor infusions

Insect pins blocking the 23 gauge guide cannula were replaced with 7 mm long 30 gauge stainless steel tubes attached to PE 20 polyethylene tubing and a 10 μl Hamilton syringe secured in a syringe pump (Razel Instruments). Bilateral infusion of 1.25 μl of a blocker into each bulb was made over a two min period either immediately after training (anisomycin, actinomycin D) or 1 or 3 h after training (anisomycin). The infusion cannulae were left in place for an additional 2 min to allow the solutions to diffuse throughout the olfactory bulbs.

Anisomycin (100 μg/μl, Alexis Biochemicals, San Diego, USA) was prepared by mixing 3.2 mg with 14.8 μl of phosphate buffered saline (PBS), followed by 1.15 μl of equimolar HCl and an additional 14.8 μl of PBS. The pH was readjusted to 7.4. Prior to infusion, frozen aliquots of anisomycin were thawed on ice and sonicated for 20 min to ensure the compound was dissolved and mixed. The choice of infusion concentration was based on the work of Schafe and LeDoux (Schafe & LeDoux, 2000). Their work had shown a similar concentration injected in a smaller volume and targeting the lateral amygdala blocked fear memory when given immediately after a single auditory fear conditioning trial. However, with their concentration, Schafe and LeDoux also reported failure of learned freezing at 1 h, but not at 4 h. They attributed this early failure to protein synthesis-dependent impairment of sensory processing at 1 h. Thus, there was some concern here that impairment of olfactory function might compromise the separation of STM and LTM effect in the rat pup. However rat pups infused with anisomycin in the olfactory bulbs immediately after learning exhibited intact STM, allaying these concerns.

A careful investigation of protein synthesis inhibition with an intrahippocampal dose of 62.5 μg/.5 μl anisomycin (Wanisch & Wotjak, 2008) provides evidence of more than 90% inhibition of protein synthesis 30 min after infusion with significant depression of protein synthesis continuing for 6 h and recovery to control levels by 9 h.

Actinomycin-D (2.5 μg/μl, Alexis Biochemicals, San Diego, USA) was prepared fresh by dissolving 1 mg of actinomycin in 200 μl of sterile saline and 200 μl of 50% dimethyl sulfoxide followed by 30 min sonication to dissolve and mix the compound prior to infusion. This is similar to the dose (5 μg/.8 μl) used by Yang and Lu (Yang & Lu, 2005) in the amygdala which blocked d-cycloserine facilitation of extinction and had also been shown to block fear conditioning memory at 24 h (Lin, Yeh, Lu, & Gean, 2003), but not extinction itself (Lin et al., 2003; Yang & Lu, 2005). Otani et al. (Otani, Marshall, Tate, Goddard, & Abraham, 1989) had shown that 5 μg/μl actinomycin D infused at 2 μl/min in the lateral ventricle produces 95% inhibition of RNA synthesis in the dorsal dentate gyrus measured at the end of 30 min, the earliest time point assessed. A drawback of microgram per microliter concentrations of actinomycin-D is the possibility of toxic effects at 24 h (e.g. see (Rizzuto & Gambetti, 1976); using 10 μg). However, these

effects are not typically seen at early time points and for the current study it was important to ensure substantial suppression of RNA synthesis in the first hours after training. Proven nontoxic nanogram per microliter doses of actinomycin-D produce only ~40% suppression of RNA synthesis 2 h after injection, the earliest time point tested (Bailey, Kim, Sun, Thompson, & Helmstetter, 1999). Finally, in the Yang and Lu study with 5 μg/.8 μl infusions, rats exhibit normal extinction levels 24 and 48 h after actinomycin D and normal potentiation of extinction by cycloserine after an actinomycin D infusion that blocked earlier cycloserine potentiation suggesting no permanent loss of function due to their concentration, which is somewhat higher than that used in the present study.

2.5. Testing

Pups were tested 1, 3, 5 or 24 h after odor preference training. Pups from each litter were only tested at one time point and training groups were run one at a time. Pups were given an odor preference test in a quiet room at 27 °C. A stainless steel testing box (36 × 20 × 18 cm) with a mesh bottom was centered over two trays. The trays were 2 cm apart, creating a neutral zone in the center. One tray contained 500 ml of fresh bedding while the other contained 500 ml of peppermint-scented bedding prepared at the same concentration used during training. The tester was blind to the previous training procedure given to the pup.

Each pup underwent five one minute trials, starting in the neutral zone and alternately facing towards or away from the tester with each subsequent trial. When the pup's snout and one paw moved from the neutral zone to either the peppermint or control zone, a timer for that side was started. Pups were given thirty second rest periods between trials. Summation of the time spent over the peppermint side divided by the total time active (time spent over peppermint plus time spent over control) gave the percent time over peppermint. Thus, preference for the conditioned odor was measured by the percent of time spent by the pup over the peppermint odor.

2.6. Statistics

One-way ANOVAs were carried out on each experiment comparing all groups. When ANOVAs were significant, as they were for all experiments reported here, Dunnett's post-hoc comparisons were carried out to test two things: (1) Were the non-learning and learning control groups significantly different, with the latter showing an odor preference? and (2) Was the targeted experimental group different from either the learning (Figs. 1–3) or non-learning (Fig. 4) control groups?

3. Results

3.1. Immediate post-training anisomycin

Interference with protein-translation by intrabulbar anisomycin infusion immediately after the 10 min training trial blocked expression of ITM and LTM, but not STM. Memory was tested at four time points with separate groups of pups: 1 h, 3 h, 5 h and 24 h (see Fig. 1 for percent time spent by pups over peppermint odor in memory tests). Odor preference memory in the anisomycin-infused groups was normal when tested 1 h or 3 h after training. ANOVAs for short-term memory tests were: 1 h groups, $F_{[3,\ 14]} = 18.91$, $p < .0001$, and 3 h groups, $F_{[3,\ 24]} = 10.95$, $p < .0001$. The non-learning control group (saline + saline infusion) in the 1 h and 3 h tests was significantly different ($p < .05$) from the learning control group (2 mg/kg Iso + saline infusion). Finally, pups given odor and a 2 mg/kg Iso injection + anisomycin were not significantly different from the learning group (odor and 2 mg/kg Iso + saline, $p > .05$) in the 1 h and 3 h tests. This result also suggests odor sensing itself was not impaired by anisomycin.

Odor preference memory was blocked in the immediate anisomycin-infused groups tested at 5 h or 24 h after training (Fig. 1). ANOVAs for longer term memory tests were: 5 h group, $F_{[3,\ 26]} = 23.55$, $p < .0001$, and 24 h group, $F_{[3,\ 14]} = 17.59$, $p < .0001$. Again, the non-learning control pups spent significantly less time over the peppermint than the learning control pups ($p < .01$). However, now the 2 mg/kg Iso injection + anisomycin infusion condition differed significantly from the learning control ($p < .01$) and did not show evidence of odor preference. Odor preference memories appear to switch from not requiring post-training olfactory bulb protein translation for memories of 3 h or less to requiring post-training translation for memories of 5 h or longer.

3.2. Anisomycin 1 h or 3 h post-training

Interference with protein-translation by intrabulbar anisomycin infusion beginning 1 h or 3 h after training indicates the requirement of 24 h term memory for bulbar protein synthesis is diminished 1 h after training and no longer present at 3 h (Fig. 2). The inhibition of protein synthesis in the olfactory bulbs of pups at 1 h post-training (Fig. 2A) produced odor preferences at 24 h (~57% time over peppermint) that fell between those of learning (Iso + saline, 75% time over peppermint) and non-learning controls (saline + saline ~24%, saline + anisomycin ~32% time over peppermint) ($F_{[3,\ 20]} = 6.16$, $p < .005$). Four of the 2 mg/Iso + anisomyin pups spent 68–89% of the test time over the conditioned odor, a normal memory result, while three pups spent 4–44% of the time over the conditioned odor, a range characteristic of non-learning. This suggests 1 h after training is near the limit of the interval in which new protein synthesis (via translation) must occur for 24 h memory.

When anisomycin was infused 3 h after training (Fig. 2B), odor preference memory was normal in the group receiving the protein synthesis inhibitor ($F_{[3,\ 12]} = 25.33$, $p < .0001$). The 2 mg/kg Iso + saline learning group (spending ~75% time over peppermint) differed significantly from the saline + saline non-learning control group (spending ~20% time over peppermint, while the 2 mg/kg Iso injection + anisomycin experimental group (spending ~66% time over peppermint) did not differ from the learning group. The non-learning control group for anisomycin (saline + ansiomycin spending ~27% time over peppermint) did differ from the learning group as expected (non-trained pups do not like the smell of peppermint). This suggests the proteins required for LTM are synthesized prior to 3 h after training. It also argues that the earlier lack of 24 h memory with immediate post-training anisomycin was not due to an odor detection impairment caused by a delayed effect of anisomycin.

3.3. Immediate post-training actinomycin D

Interference with protein transcription by intrabulbar actinomycin D infusion immediately after the 10 min training trial prevented expression of LTM, but not ITM. Transcription (Fig. 3A) is not required for 5 h ITM, ANOVA ($F_{[3,\ 20]} = 13.13$, $p < .0001$). The Iso + actinomycin group was not significantly different from the learning (Iso + saline) group when pups were tested for odor preference 5 h after training. These results imply that memory seen 5 h after training can be supported through the translation of existing mRNA within the olfactory bulb. It is also demonstrates that this dose of actinomycin D did not prevent normal olfactory discrimination at 5 h after delivery.

The inhibition of transcription through olfactory bulb infusions of actinomycin D immediately after training did prevent 24 h

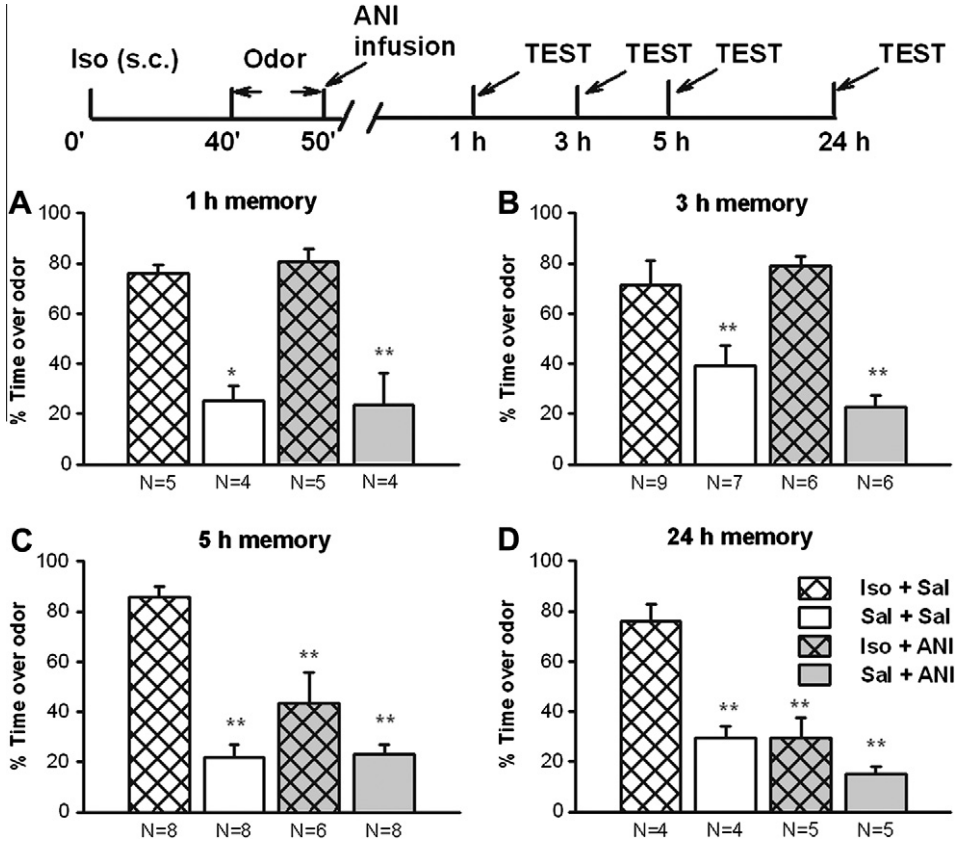

Fig. 1. Behavioral results showing the effect of translation block immediately after odor preference training on subsequent odor memory of rat pups at subsequent testing times. A time-line for the injection, odor training, infusion and testing is given above the graphs. Pups were given systemic (s.c.) injection of isoproterenol (Iso, 2 mg/kg) or saline 40 min before training and intrabulbar infusion of anisomycin (ANI) or saline (Sal) immediately after training. Memory was examined: (A) 1 h, (B) 3 h, (C) 5 h or (D) 24 h after training. Each pup was tested only once in order to avoid possible extinction effects. Each graph was analyzed by one-way ANOVA. Post-hoc analysis was by Dunnett's comparison to the learning controls (2 mg/kg Iso + odor). *, $p < .05$; **, $p < .01$.

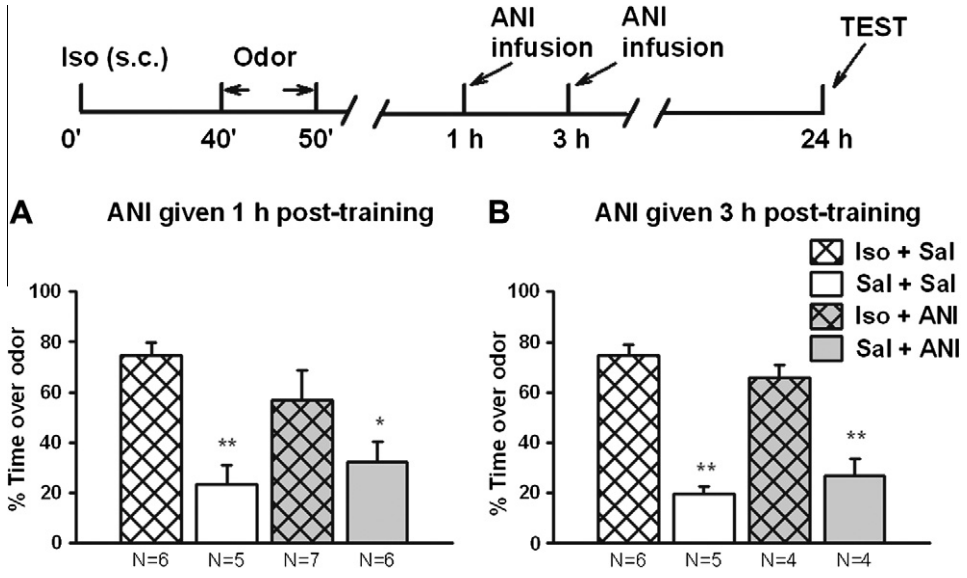

Fig. 2. Effect of delaying translation block on memory. A time-line for the injection of US injection, odor training, infusion of anisomycin (ANI) and test time is given above the graph. Each pup was tested only once. (A) Delaying anisomycin infusion to 1 h post-training resulted in a non-significant effect on memory. (B) Delay of anisomycin infusion until 3 h post-training had no effect on 24 h memory of the peppermint (CS) odor. Each graph was analyzed by one-way ANOVA. Post-hoc analysis was by Dunnett's comparison to the learning controls (2 mg/kg Iso + odor). These data suggest that most critical translation is completed by 1 h after the commencement of memory consolidation. The data also suggest that anisomycin infusion had no long lasting or non-specific detrimental effect on memory. Post-hoc comparisons are against the learning control (2 mg/kg Iso + odor). *, $p < .05$; **, $p < .01$.

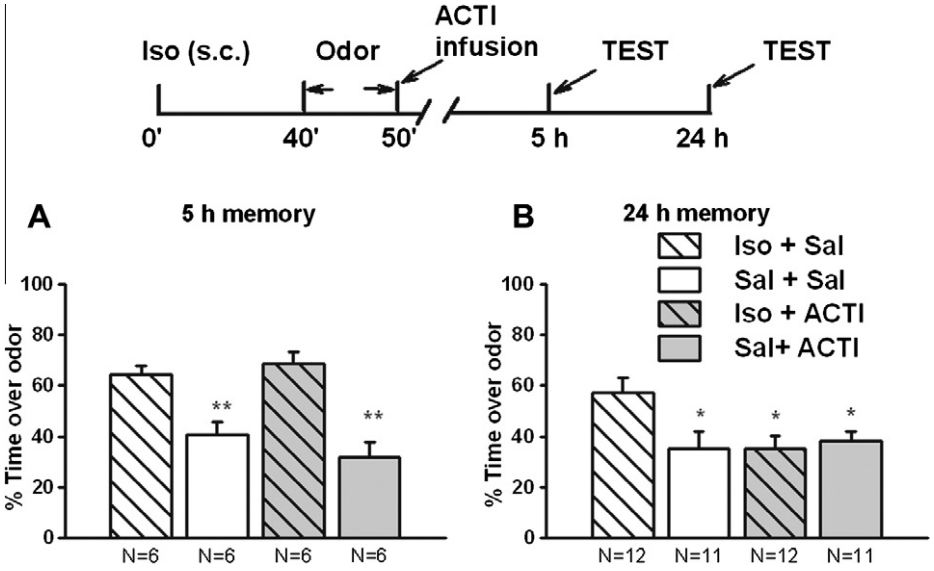

Fig. 3. The effect on memory by infusing a transcription blocker immediately after odor preference training was examined at 5 h (A) or 24 h (B) post-training. The time-line of training, transcription blocker infusion and odor preference testing is given above the graph. Each pup was tested only once. Infusion of actinomycin (ACTI) had no effect on 5 h memory (A) but significantly blocked 24 h odor preference memory (B). Each graph was analyzed by one-way ANOVA. Post-hoc analysis was by Dunnett's comparison to the learning controls (2 mg/kg Iso + odor). *, $p < .05$; **, $p < .01$. Abbreviations: Iso, isoproterenol; Sal, saline.

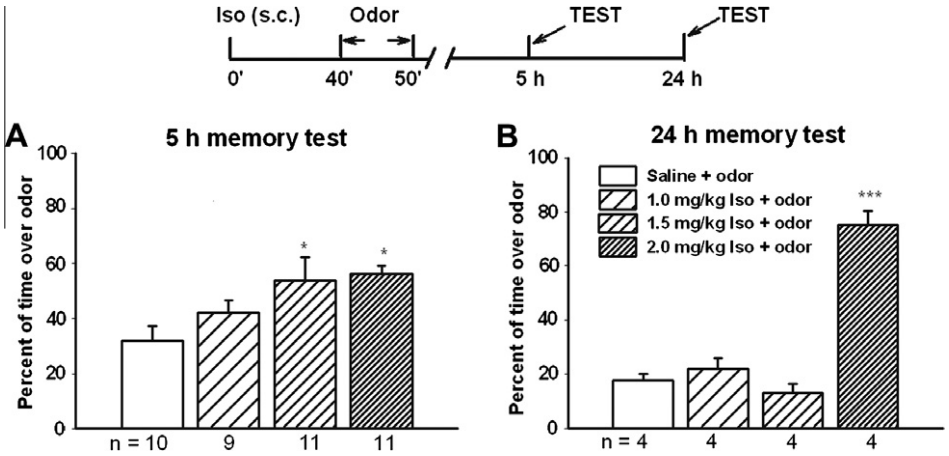

Fig. 4. The effect on ITM (5 h) and LTM (24 h) of pups given variable doses of the unconditioned stimulus, isoproterenol (Iso). The time-line of training, odor preference testing and testing is given above the graph. Each pup was tested only once. (A) Testing pups 5 h following paired CS (odor) and US (1.5, or 2 mg/kg Iso) resulted in significant odor preference compared to pups given odor only. (B) In contrast, the 1.5 mg/kg US dose was ineffective in enabling 24 h LTM while the 2 mg/kg dose was effective. Each graph was analyzed by one-way ANOVA. Post-hoc analysis was by Dunnett's comparison to the non-learning control group (odor only) *, $p < .05$; ***, $p < .001$.

memory (Fig. 3B) with the ANOVA showing a significant group effect ($F_{[3, 42]} = 3.75$, $p < .05$). All conditions examined (saline + saline, Iso + actinomycin, or saline + actinomycin) differed significantly from the learning control ($p < .05$). Twenty-four hour odor preference memory requires transcription as well as translation (anisomycin experiments).

3.4. An intermediate unconditioned stimulus elicits intermediate-term memory

Varying the strength of the unconditioned stimulus resulted in a dose-dependent length of memory for the conditioned odor. One-way ANOVA revealed a group effect for odor preference memory for both 5 h ($F_{[3, 40]} = 3.82$, $p < .05$) and 24 h ($F_{[3, 15]} = 53.32$, $p < .0001$) memory. Post-hoc Dunnett's tests revealed that a dose of 1.5 mg/kg Iso and 2.0 mg/kg Iso produced odor preferences significantly different from the non-learning control (Fig. 4A) at 5 h, while only the 2 mg/kg Iso dose induced an odor preference memory at 24 h (Fig. 4B).

4. Discussion

These results are consistent with the hypothesis that a protein translation-dependent intermediate-term memory exists in a cAMP/PKA-dependent mammalian learning model, paralleling what was described first in invertebrates. Translation-dependent, but not transcription-dependent, cAMP-initiated long-term synaptic plasticity has also been identified in mammalian hippocampus. The activation of β-adrenoceptors in CA1 induces a translation-dependent long-term potentiation mediated by an exchange protein activated by cAMP (EPAC) rather than by the cAMP/PKA cascade (Gelinas et al., 2008). A translation-dependent long-term

10 minTraining	0 min post	1 h post	3 h post	5 h post	24 h post
Odor + 2 mg/kg Iso	anisomycin		STM present	ITM absent	LTM absent
Odor + 2mg/kg Iso		anisomycin			LTM partial
Odor + 2mg/kg Iso			anisomycin		LTM present
Odor + 2mg/kg Iso	actinomycin			ITM present	LTM absent
Odor + 1.5mg/kg Iso				ITM present	LTM absent

Fig. 5. The summary table shows the current evidence for mammalian translation-dependent intermediate odor preference memory (ITM), which is absent following immediate post-training bulbar infusion of the translation inhibitor, anisomycin. Long-term memory (LTM) depends on protein synthesis within the first hour since partial LTM is seen with anisomycin infusion at that interval and LTM is intact with anisomycin infusion 3 h post-training. An immediate infusion of the transcription inhibitor actinomycin, does not alter ITM, but LTM is absent, demonstrating the distinction between translation-dependent ITM and transcription-dependent LTM in this model. Finally a weaker unconditioned stimulus, 1.5 mg/kg Iso can induce an ITM at 5 h but is insufficient to initiate an LTM at 24 h.

depression has also been reported in CA1 (Manahan-Vaughan, Kulla, & Frey, 2000). Whether EPAC (as in the Gelinas experiments) or PKA (as in the earlier *Aplysia* experiments) plays the pivotal role in the present ITM remains to be investigated.

The ability to elicit an ITM, without an LTM, shown here with an intermediate dose of isoproterenol, also replicates earlier findings in the invertebrate models (Lukowiak, Adatia, Krygier, & Syed, 2000; Sutton, Ide, Masters, & Carew, 2002). Since we can now elicit an ITM in isolation, it will be possible to probe whether or not ITM can be boosted to LTM (for example by 24 h spaced presentations) suggesting a serial component to this multi-phasic memory (as in the pond snail (Parvez, Stewart, Sangha, & Lukowiak, 2005) or whether the two processes operate in parallel (see (Izquierdo et al., 2002).

Recently, systemic anisomycin given to 6 day rat pups immediately after an hour of odor + mild shock pairings (14 total), which also produces an odor preference, was shown to prevent memory at 24 h as seen here (Languille, Richer, & Hars, 2009), but anisomycin did not affect preference memory at 4 h (see also (Schafe & LeDoux, 2000) for a similar effect with fear conditioning). The choice of a 5 h testing point in the present study may have been fortuitous for detecting translation-dependent odor preference memory since translation dependence was not seen at 3 h, while 4 h was not tested. Nonetheless, the odor preference training paradigm used here and that used by Languille et al. differ in other outcomes. The memory they induce with repeated shock lasts at least 6 days since pups tested at 12 days show memory, while our single 10 min odor pairing no longer influences odor preference 48 h after training unless steps are taken to boost memory (Christie-Fougere, Darby-King, Harley, & McLean, 2009; McLean, Darby-King, & Harley, 2005). Additionally, the odor preference Languille et al. induce reverses to an odor aversion by day 12. The avoidance memory seen with shock pairings at day 12 was also blocked by anisomycin immediately after training on day 6 suggesting both the appetitive and aversive memory are part of the same process (Languille et al., 2009). Extending the present odor + Iso (or tactile stimulation) training using spaced trials to induce a multi-week memory does not lead to reversal of the original odor preference (Sullivan, Wilson, & Leon, 1989; Woo & Leon, 1987).

Temporally, the important translation events for LTM here are confined to $\sim$ 1 h after training since anisomycin given at 1 h post-training had mixed effects on LTM; with a subgroup of pups failing to learn, while others were successful. There was no significant effect of anisomycin given at 3 h on LTM (see also Languille et al. (2009) as discussed earlier). A similar short time window for 'consolidation' using anisomycin has been reported in pond snail (Fulton, Kemenes, Andrew, & Benjamin, 2005). In conditioned taste aversion learning in 3 day old rat pups, anisomycin prevents 24 h memory when given 1 h, but not 6 h, after training (Gruest, Richer, & Hars, 2004). Interestingly, in rat pups, the 'consolidation' window for conditioned taste aversions shows a developmental progressive decrease in the temporal window for anisomycin interference. While effective 1 h after training on day 3, it is only disruptive immediately after training on day 10, and by day 18, even with immediate injection, anisomycin disruption of LTM is only partial (Languille, Gruest, Richer, & Hars, 2008). Thus, translation-dependent protein synthesis may occur quite rapidly with learning in adult rodents and the very brief time window for disruption could account for the failure to observe mammalian translation-dependent ITM previously.

We set out, initially, to characterize the role of protein synthesis in our memory model. Our results appear consistent with a canonical view of that role, i.e. a role in LTM but not STM, and with the update that there is a translation-, but not transcription-dependent, ITM (see Fig. 5 for a summary diagram). However, there is currently a controversy about whether or not protein synthesis has any unique role in learning and memory (for a recent review see (Gold, 2008). The antibiotic tools used each have other effects that could provide alternate explanations of their actions.

Of particular importance for our rat pup model is evidence that local infusion of anisomycin in the amygdala causes a dramatic increase in monoamines including norepinephrine, followed by a marked reduction, and that infusing norepinephrine prior to an inhibitory learning task produces the same amnesia (at 48 h) seen with pretraining anisomycin (Canal, Chang, & Gold, 2007). The memory block by anisomycin can be reduced by propranolol implicating β-adrenoceptor over-activation in the amnesia (Canal et al., 2007). A β-adrenoceptor agonist, isoproterenol, is the US in our rat pup odor preference learning and varying dosages produce an inverted U curve for effectiveness in inducing learning. When isoproterenol is given at doses higher than the optimal 2 mg/kg (e.g., 4 or 6 mg/k), LTM is not seen (Langdon et al., 1997). Over-activation of β-adrenoceptors by anisomycin could account for a loss of LTM. However, as seen here, and in Languille et al. (2009) (and in other fear conditioning models, e.g., Schafe & LeDoux, 2000), anisomycin does not disrupt STM. We tested STM with a 6 mg/kg Iso US that would not produce LTM. Unlike the pattern of results with anisomycin, this high dose of a β-adrenoceptor agonist prevented STM (see supplementary results, Fig. 1). It appears unlikely that anisomycin is acting solely via over-activation of β-adrenoceptors in our model.

This is only one of a number of alternative explanations e.g., electrophysiological changes (Sharma, 2010), activation of apoptotic cascades (see for review (Alberini, 2008) and/or necrotic tissue effects, (Rizzuto & Gambetti, 1976)), which might explain differences in the temporal effects of the two antibiotics used here, rather than their selective roles in blocking translation and transcription. A conclusive demonstration of selective translational and transcriptional dependence of learning events would require ruling out alternative explanations. We suggest, however, as proposed by others ((Rudy, 2008), see also (Rosenegger, Wright,

& Lukowiak, 2010)), that a proteonomic approach in our model will be more fruitful in illuminating the biology of memory than further explorations of antibiotic effects. Translation-dependent memory events should be associated with changes in the production and/or levels of proteins produced by pre-existing mRNAs. The antibiotic data here suggest such proteins occur in the rat pup olfactory bulb and the narrow time window (~1 h) for the putative critical translation events provides a critical temporal target for analysis.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.nlm.2011.01.012.

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