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Anisomycin infusions in the parabrachial nucleus and taste neophobia

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Abstract

To investigate whether *de novo* protein synthesis in the parabrachial nucleus (PBN) is required for recovery from taste neophobia, anisomycin (a protein synthesis inhibitor) was infused immediately after consumption of a novel saccharin solution (Experiment 1). Unexpectedly, this PBN treatment caused a reduction in saccharin intake. In addition, we found that the anisomycin-induced suppression of tastant intake was attenuated by prior intra-PBN infusions of lidocaine (Experiment 2). This pattern of results raises concerns about using anisomycin to investigate memory consolidation processes in the PBN. Thus, a different manipulation may be needed to examine the nature of the neuroplastic changes that occur in the PBN during taste memory formation.

Keywords

Taste neophobia; Memory consolidation; *de novo* protein synthesis; Parabrachial nucleus; Rat

1. Introduction

The reluctance to consume a novel, and therefore potentially dangerous, food is termed taste neophobia (Barnett, 1956, 1958; Best & Barker, 1977; Domjan, 1977; Lin, Amodeo, Arthurs, & Reilly, 2012). If no aversive post-ingestive consequences ensue, this neophobic reaction habituates as indicated by increased consumption until asymptote is achieved. On the other hand, if consumption of the new food is followed by gastrointestinal illness, the food is avoided on subsequent encounters. That is, a conditioned taste aversion (CTA) develops, in which the taste of the food (i.e., conditioned stimulus; CS) is associated with the aversive consequences (i.e., unconditioned stimulus; US; Garcia & Ervin, 1968; for recent reviews see Reilly & Schachtman, 2009). In each case, a memory of the taste (and its post-oral effect) is formed and this knowledge modulates subsequent intake of that food.

According to contemporary views of memory formation, the memory is stabilized over time through consolidation, a process that strengthens the initially labile trace via *de novo* protein synthesis (Davis & Squire, 1984; McGaugh, 2000; Nader, 2003; but see Gold, 2008). Taste memory is no exception and, regardless of whether it is safe (i.e., recovery from neophobia) or unsafe (i.e., CTA), requires consolidation in order to be permanently stored in long-term

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memory. For example, intracerebroventricular infusions of cycloheximide (a protein synthesis inhibitor) administered prior to lithium chloride (LiCl; a nausea-inducing agent) intoxication abolished CTA acquisition (Haupt & Berlin, 1999; Serova et al., 1995). Similarly, formation of a safe taste memory requires *de novo* protein synthesis. In this case, intracranial infusion of anisomycin into the nucleus accumbens (Pedroza-Llinás, Ramírez-Lugo, Guzmán-Ramos, Zavala-Vega, & Bermúdez-Rattoni, 2009) or gustatory cortex (Rodríguez-Ortiz, De la Cruz, Gutiérrez, & Bermudez-Rattoni, 2005) after exposure to a novel tastant prevented the habituation of taste neophobia. That is, the anisomycin-treated rats showed no recovery from neophobia when they were presented with the same taste again (i.e., the rats behaved as if the taste were novel even though they had prior experience with it). This pattern of results supports the hypothesis that *de novo* protein synthesis is required for a taste memory to be consolidated and stored in long-term memory.

The parabrachial nucleus (PBN), located in the dorsolateral pons, is thought to have a critical role in CTA acquisition (see Reilly, 1999, for a review). Rats with PBN lesions fail to show any signs of aversion to a taste after repeated taste-illness pairings (DiLorenzo, 1988; Flynn, Grill, Schulkin, & Norgren, 1991; Grigson, Reilly, Shimura, & Norgren, 1998; Ivanova, & Bures, 1990; Reilly, Grigson, & Norgren, 1993; Spector, Norgren, & Grill, 1992; Yamamoto, & Fujimoto, 1991). Given that gustatory and visceral information converge in the PBN (Herbert, Moga, & Saper, 1990; Norgren & Leonard, 1971, 1973), the prevailing interpretation of the CTA acquisition deficit found in rats with PBN lesions involves a disruption of associative learning processes. That is, the PBN is thought to be the critical site for the plastic changes that underlie memory of the taste-illness association. An alternative interpretation, derived from the finding that decerebrate rats are incapable of CTA acquisition (Grill & Norgren, 1978), is that the PBN merely functions as a conduit through which information is relayed to forebrain structures (as yet unidentified) where the taste memory is stored (for further discussion see Reilly, 2009).

A straightforward method to unravel the nature of the involvement of the PBN (associative memory storage or information relay) is to examine the influence of intra-PBN infusions of a protein synthesis inhibitor (e.g., anisomycin) on CTA acquisition. However, proper interpretation of the results of such an experiment requires that it first be established that the PBN has no involvement in the processing of taste information. To the best of our knowledge no study has examined the effects of protein synthesis inhibition in the PBN on taste processing. This concern prompted the current study in which we sought to determine whether anisomycin infused into the PBN after exposure to a novel tastant (0.15% saccharin) influences subsequent performance directed at that tastant. If neurons in the PBN have a role in consolidating taste memory, anisomycin infusions might be expected to prevent the habituation of taste neophobia. On the other hand, if such processing occurs outside the PBN then we expect that anisomycin infusions have little influence on the recovery from taste neophobia.

As revealed in Experiment 1, intra-PBN infusions of anisomycin did not attenuate recovery from neophobia. Rather, and completely unexpectedly, these infusions significantly reduced saccharin consumption. That is, anisomycin infused into the PBN appeared to induce a CTA to the saccharin tastant. There is, to our knowledge, no evidence suggesting that protein synthesis inhibition produces CTA-like effects. Therefore, we hypothesized that this taste suppression might be a consequence of an effect of anisomycin on synaptic transmission (i.e., a transient, extraordinary large spike in neurotransmitter release at the infusion site; Canal, Chang, & Gold, 2007; Qi & Gold, 2009). Given that lidocaine, a local anesthetic, can attenuate this anisomycin-induced increase in release of neurotransmitters without influencing the effects on protein synthesis inhibition (Sadowski, Canal, & Gold, 2011), in Experiment 2 we sought to determine whether lidocaine, infused into the PBN before intra-

PBN anisomycin administration, would negate the anisomycin-induced taste suppression found in Experiment 1. Thus, we examined whether lidocaine influences anisomycin-induced taste suppression and this is best achieved by using a tastant that supports high intake on Trial 1. Therefore 0.1 M NaCl, a stimulus supporting little taste neophobia, was used so that any attenuating effect of lidocaine would not be compromised by a floor effect on intake of anisomycin-infused animals. The concentration of anisomycin (62.5 ug/0.5 ul) was the same as that employed by Sadowski et al. (2011) whereas lidocaine (5 ug/0.5 ul) was used at half the effective concentration from that study.

2. Material and methods

2.1. Animals

Twenty-nine naïve Sprague-Dawley rats (Charles River Laboratory, Wilmington, MA) served as the subjects. After arrival in the laboratory, the rats were housed individually in plastic cages (26.5 × 50 × 20 cm) in a temperature controlled (21°C) room with 12-12 light-dark cycle (lights on at 7:30 am) and given *ad libitum* food and water until the experiment started, at which time water access was restricted to 30 min/day (see below for details). The University of Illinois at Chicago Animal Care Committee approved all procedures employed in the present research. At all times, rats were treated according to guidelines recommended by the American Psychological Association (1996) and the National and the Institutes of Health (1996).

2.2. Surgery

A total of 20 rats received bilateral cannula implantations prior to behavioral testing. The rats were first anesthetized with intraperitoneal sodium pentobarbital (~65 mg/kg) and then fixed in a stereotaxic instrument (David Kopf Instruments; Tujunga, CA) with blunt earbars. A midline incision was made to expose the skull and a trephine hole (diameter ~3 mm) was drilled above the PBN in each hemisphere. A stainless steel guide cannula (33 gauge; Plastics One, Roanoke, VA) was inserted with the tip positioned 2 mm above the center of each PBN at the following coordinates: AP: -10.9 mm, ML: ±2 mm, DV: -4.6 mm. The cannulas were implanted at a 20-degree angle to avoid damaging the transverse sinus. Throughout the surgical procedure, body temperature was monitored with a rectal thermometer and maintained at ~37°C with a heating pad (Harvard Apparatus, Holliston, MA). The rats in the non-surgical control group (Group CON; n = 9) were given pentobarbital injections but no surgery. The rats were returned to their home cages after recovery from anesthesia.

2.3. Apparatus

All behavioral testing occurred in the home cages. Fluids were presented in calibrated bottles each fitted with a silicone stopper and a steel sipper tube. Fluid intake was monitored to the nearest 0.5 ml.

2.4. Experiment 1: Intra-PBN anisomycin and taste neophobia

After recovery from surgery, rats were placed on a deprivation schedule of 15-min water access in the morning followed 4 hours later by a second 15-min period of water access. When water intake stabilized, the rats with cannulas were divided into two groups based on the infusions to be given. Group SAL (n = 9) served as a second control group and received intracranial infusions of physiological saline whereas Group ANI (n = 11) received intracranial infusions of anisomycin (62.5 ug/0.5 ul; Sigma-Aldrich Co., St. Louis, MO). The neophobia trials (given in lieu of morning water access) were conducted in a 3-day cycle with a taste trial day followed by two water days. Each taste trial involved 15-min access to 0.15% saccharin followed by bilateral intra-PBN infusions of saline vehicle

(Group SAL), or anisomycin (Group ANI), or no infusions for Group CON ($n = 9$). All infusions were 0.5 μ l/side administered at a rate of 0.3 μ l/min.

2.5. Experiment 2: Effect of lidocaine on anisomycin-induced tastant suppression

After completion of Experiment 1, the rats continued on the same fluid deprivation schedule of 15-min water access each morning and afternoon. The procedure of Experiment 2 was identical to that of Experiment 1, except 0.1 M NaCl was used as the tastant and two intra-PBN infusions were administered, the first immediately following removal of the stimulus bottle, the second 10 min later. In this experiment, rats were divided into four groups based on the drugs to be infused. Although the composition of the non-surgical control group (Group CON; $n = 9$) remained as in Experiment 1, the animals with implanted cannulas (one rat was dropped due to a clogged cannula) were counterbalanced for prior experimental conditions and divided into three groups: Group LIDO/SAL ($n = 6$) received intracranial infusions of lidocaine HCl (5 μ g/0.5 μ l); APP Pharmaceuticals, Schaumburg, IL) followed by physiological saline; Group SAL/ANI ($n = 5$) received saline then anisomycin (62.5 μ g/0.5 μ l); and Group LIDO/ANI ($n = 8$) were infused with lidocaine and anisomycin. As in Experiment 1, all infusions were 0.5 μ l/side at infusing rate of 0.3 μ l/min.

2.6. Histology

At the end of all experimentation, Chicago Blue dye (0.5 μ l) was infused into the PBN in each hemisphere. Thereafter, the rats were overdosed with sodium pentobarbital (100 mg/ml) and perfused transcardially. The extracted brains were stored in 4% buffered formalin and then 20% sucrose solution each for 2 days. The brains were sectioned at 50 μ m on a cryostat. The slices were stained with cresyl violet. The placements of guide cannula and the diffusion area of Chicago Blue were then examined with a light microscope (Zeiss Axioskop 40).

2.7. Data analysis

For the behavioral results of each experiment, a two-way analysis of variance (ANOVA) with Group as the between subject variable and Trial as the within-subject variable was conducted to analyze taste trial data. A significant main effect or interaction was followed by post hoc comparisons (simple main effect with adjusted error term taken from the overall ANOVA). The p -value was set at 0.05. All the analyses were conducted with the help of Statistica software (6.0; StatSoft, Tulsa, OK).

3. Results

3.1. Histology

Schematic representations of the cannulas placement are depicted in Figure 1. The histological analysis revealed that there were two rats that had misplaced guide cannulas, and thus their behavioral data were excluded from statistical analysis. The final numbers of subjects for each group were: Experiment 1, Group CON, $n = 9$; Group SAL, $n = 9$; Group ANI, $n = 9$ and Experiment 2, Group CON, $n = 9$; Group LIDO/SAL, $n = 5$; Group SAL/ANI, $n = 4$, Group LIDO/ANI, $n = 8$.

3.2. Experiment 1: Intra-PBN anisomycin and taste neophobia

Saccharin intake data for the three groups (CON, SAL and ANI) used in Experiment 1 are summarized in Figure 2. Inspection of the figure shows that each group consumed similar amounts of the novel tastant on Trial 1. Relative to Groups CON and SAL which each increased intake across subsequent trials, Group ANI decreased saccharin intake across successive trials. Confirming these observations, a two-way ANOVA revealed a significant

main effect of Group, $F(2,24) = 29.92$, $p < 0.05$, a significant main effect of Trial, $F(3,72) = 10.48$, $p < 0.05$, and, more importantly, a significant Group $\times$ Trial interaction, $F(6,72) = 23.76$, $p < 0.05$. Post hoc comparisons revealed no group differences on Trial 1. Furthermore, Groups CON and SAL each increased their intake from Trial 1 to Trial 2 ($ps < 0.05$) and, having reached asymptote, maintained the same level of intake on Trials 2, 3 and 4 ($ps > 0.05$). Group ANI, on the other hand, showed a decrease in intake from Trial 1 to Trial 2 ($p < 0.05$) and although intake was lower across each successive trial (in fact, 6 rats drank 0.5 ml or less on Trial 3, while 7 rats drank 0.5 ml or less on Trial 4), the between-trials differences were not significant ($ps > 0.05$). Could this unexpected suppression by anisomycin be due to a general reduction in fluid consumption? If this were the case, then water intake, like saccharin consumption, should decline across the course of the experiment. Water intake data from the day before each taste trial are summarized in Table 1. Analysis revealed no main effect of Group ($F < 1$) and no Group $\times$ Trial interaction ($F < 1$). Thus, we conclude that intra-PBN infusions of anisomycin may have aversive US properties, which suppressed intake of the saccharin tastant.

3.3. Experiment 2: Effect of lidocaine on anisomycin-induced tastant suppression

The NaCl intake data from the three trials of Experiment 2 are shown in Figure 3. As summarized therein, Groups CON and LIDO/SAL consumed comparable, unchanging high amount of NaCl on each trial whereas Groups SAL/ANI and LIDO/ANI suppressed intake across trials at different rates. These impressions were confirmed with statistical analysis which found a significant main effect of Group, $F(3,22) = 5.00$, $p < 0.01$, a significant main effect of Trial, $F(2,44) = 16.05$, $p < 0.001$, and a significant Group $\times$ Trial interaction, $F(6,44) = 4.57$, $p < 0.01$. Post hoc comparisons revealed that all groups consumed the same amount of NaCl on Trial 1 ($ps > 0.05$). Furthermore, there were no differences in intake between Group CON and Group LIDO/SAL on each of the three trials ($ps > 0.05$). Replicating the finding in Experiment 1, Group SAL/ANI showed a significant decrease between Trial 1 and Trial 2 ($p < 0.05$) but the decline in intake from Trial 2 to Trial 3 did not achieve significance ($p = 0.08$). Regarding Group LIDO/ANI, a significant decrease in intake was found between Trial 2 and Trial 1 ($p < .05$) but not between Trials 3 and 2 ($p > 0.05$). Moreover, Group LIDO/ANI consumed significantly more NaCl than Group SAL/ANI on Trials 2 and 3 ($ps < 0.01$). As in Experiment 1, there were no group differences in water intake on the day before each taste trial ($ps > .15$; Table 1); the behavioral effects of anisomycin or lidocaine cannot, therefore, be attributed to a general influence of these drugs on fluid consumption. Overall, the results indicate that lidocaine attenuated the aversive effects induced by intra-PBN infusions of anisomycin.

4. Discussion

This work was undertaken to determine if protein synthesis in the PBN has a role in taste perception. We expected to obtain a null result which would then allow us to investigate the role of protein synthesis in the PBN during the acquisition of a taste-illness association. However, the present research produced results that were entirely unexpected. That is, we found that intra-PBN infusions of the protein synthesis inhibitor anisomycin given after exposure to a novel taste solution caused rats to avoid that tastant as if they had developed a CTA. Furthermore, this anisomycin-induced CTA-like effect was attenuated by prior intra-PBN infusions of lidocaine, which itself had little influence on taste consumption. How are these results to be explained?

In addition to inhibiting *de novo* protein synthesis, anisomycin has been found to influence neurotransmitter release at the site of infusion. Canal et al. (2007) found that intra-amygdala injections of anisomycin caused massive release of dopamine, norepinephrine, and serotonin. When infused into the hippocampus, anisomycin also induced a spike of

acetylcholine release accompanied with the biogenic amines (e.g., dopamine and norepinephrine; Qi & Gold, 2009). Moreover, this induced neurotransmitter release was not just a by-product of anisomycin. Instead, it may contribute to the memory loss found in anisomycin treated rats. For example, Sadowski et al. (2011) found that pre-treatment with lidocaine attenuated anisomycin-induced amnesia in an inhibitory avoidance task. With the use of both *in vivo* microdialysis and c-Fos immunohistochemistry, Sadowski et al. further demonstrated that lidocaine selectively reduced anisomycin-induced neurotransmitter release and had no influence on protein synthesis inhibition. This later finding is important because it not only implies that anisomycin-induced amnesia, at least partially, results from abnormal neurotransmitter release but it also casts some doubts on the requirement of new protein synthesis for learning (see Gold, 2008, for a review). The fact that the neurotransmitter release evoked by anisomycin is large enough to influence behavioral performance prompts us to consider whether this abnormal release of neurotransmitters might explain the unexpected CTA after anisomycin infusions in the current study. That pre-treatment with lidocaine reduced anisomycin-induced taste suppression in Experiment 2 suggests that this is likely the case.

Among the various neurotransmitters influenced by protein synthesis inhibitors, serotonin is particularly important to current interests because of its critical involvement in CTA formation. For example, manipulations altering serotonin transmission modulate the strength of conditioned aversion induced with LiCl (e.g., Wegener, Smith, & Rosenberg, 1997). Furthermore, increased serotonin levels in the central nervous system have been found to induce a conditioned aversion to a tastant (Willner et al., 1986). This central CTA-inducing effect is particularly relevant given the recent finding that intraperitoneal injections of LiCl significantly increase serotonin concentration in the extracellular space within the PBN (Petr, Jiri, & Karel, 2006). Together, these findings suggest that an increase in serotonergic activity itself is sufficient to induce CTAs. If this analysis is correct, then the CTA-like effect found in Experiment 1 should be attenuated when the evoked release of neurotransmitters by anisomycin is reduced. This is exactly what was happened in Experiment 2, in which the rats given sequential intra-PBN infusions of lidocaine and anisomycin showed less taste aversion than the rats receiving only anisomycin.

If the present results do reflect the influence of anisomycin-induced neurotransmitter release in the PBN then a number of additional questions are raised. For example, was the release of neurotransmitters the only event triggered by anisomycin or did protein synthesis inhibition also occur in the PBN? Is there a, presumably lower, dose of anisomycin that might have no effect on neurotransmitter release that would allow us to pursue our initial interest in the role of the PBN in associative learning? Would infusions of a higher dose of anisomycin into the PBN result in a stronger CTA? Indeed, is the suppression of tastant intake by intra-PBN anisomycin due to the acquisition of a CTA? One straightforward way to investigate this latter question would be to determine whether taste palatability also declined across trials (e.g., Arthurs, Lin, Amodeo, & Reilly, 2012; Lin, Arthurs, Amodeo, & Reilly, 2012). Furthermore, is the induction of a CTA specific to anisomycin or a general feature of protein synthesis inhibitors (which could be tested using cycloheximide or rapamycin to block protein synthesis)? Was the CTA obtained in the present study caused by anisomycin-induced release of serotonin? This question might be investigated by examining the influence of intra-PBN infusions of a serotonergic receptor agonist in the present design. Would temporary inactivation of the PBN by, say, infusions of GABA agonists (e.g., muscimol and baclofen) instead of anisomycin produce the same results? Finally, the current design targeted the whole PBN as the area of interest. However, the PBN is not a homogeneous structure. Instead, it can be divided into medial (gustatory) and lateral (viscerosensory) regions. This anatomical organization prompts questions about the specific locus of action of the anisomycin in the present study.

Irrespective of the answers to the foregoing questions, the present results may provide a way to resolve a long-standing issue of fundamental importance to our understanding of the neural substrates of CTA learning. In an influential article, Grill and Norgren (1978) reported that decerebrate rats were incapable of acquiring CTAs. This finding was widely interpreted as definitive evidence that CTA was a forebrain function because, clearly, the isolated brainstem could not support such learning. However, despite over thirty years of searching, the critical forebrain structure (or structures) for CTA learning has not, to our knowledge, yielded to research (see Reilly, 2009, for a review). An alternative view, as noted in the Introduction, is that the PBN (specifically the medial region) is critically involved in the integrative mechanism that link the taste CS with aversive US. These competing analyses anticipate very different effects of infusions of anisomycin into the PBN of the decerebrate rat after exposure to a novel tastant. That is, whereas the former view predicts that no CTA would be obtained the latter account expects that the decerebrate rat would develop a CTA.

5. Conclusion

To the best of our knowledge, the current study was the first attempt to investigate whether protein synthesis inhibition in the PBN mediates taste memory. Unexpectedly, this pharmacological manipulation, selectively targeted at the PBN, resulted in the acquisition of a CTA to the tastant that preceded the anisomycin infusions. While the nature of the underlying processes engaged (or disengaged) by anisomycin is not clear, this finding, nonetheless, provides further evidence for a critical role of the PBN in taste aversion learning.

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We examined the effect of intra-PBN infusions of anisomycin on taste neophobia. Contrary to expectations, anisomycin suppressed intake of the saccharin tastant. Pre-treatment with lidocaine attenuated the taste suppressing effect of anisomycin.

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Figure 1. Placement of cannula tips (black solid circles) in the PBN for all rats included in the behavioral analyses are shown in a series of schematics (8.76, 9.00, 9.24, and 9.48 mm posterior to bregma) adapted with permission from Paxinos and Watson (2005).

Figure 2.

Experiment 1: Mean ($\pm$ SEM) 0.15% saccharin intake (ml) across the four taste trials in the non-surgical control group (CON) and groups that received intra-PBN infusions of either saline (SAL) or anisomycin (ANI) after each taste trial.

Figure 3.

Experiment 2: Mean ($\pm$ SEM) 0.1 M sodium chloride intake (ml) across three taste trials in the non-surgical control group (CON) and groups that received sequential intra-PBN infusions of lidocaine and saline (LIDO/SAL), saline and anisomycin (Group SAL/ANI), or lidocaine and anisomycin (LIDO/ANI) after each taste trial.

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Table 1
Mean ($\pm$ SE) intake (ml) during the water trials 24 hr prior to each taste trial in Experiments 1 and 2.

Experiment	Group	Trial 1	Trial 2	Trial 3	Trial 4
Experiment 1	CON	14.89 (0.93)	14.22 (1.02)	15.72 (1.01)	15.39 (1.40)
	SAL	17.50 (1.19)	15.61 (1.17)	17.56 (1.32)	15.33 (2.23)
	ANI	16.00 (0.83)	14.17 (1.73)	16.33 (0.87)	14.28 (0.92)
Experiment 2	CON	14.83 (1.21)	13.44 (1.31)	15.39 (1.61)	
	LIDO/SAL	15.40 (2.73)	13.50 (2.86)	15.20 (2.42)	
	SAL/ANI	15.13 (2.45)	14.63 (2.10)	12.88 (3.38)	
	LIDO/ANI	17.81 (0.82)	14.50 (1.77)	14.56 (1.50)	