Research

Intrahippocampal infusions of anisomycin produce amnesia: Contribution of increased release of norepinephrine, dopamine, and acetylcholine

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Intra-amygdala injections of anisomycin produce large increases in the release of norepinephrine (NE), dopamine (DA), and serotonin in the amygdala. Pretreatment with intra-amygdala injections of the β-adrenergic receptor antagonist propranolol attenuates anisomycin-induced amnesia without reversing the inhibition of protein synthesis, and injections of NE alone produce amnesia. These findings suggest that abnormal neurotransmitter responses may be the basis for amnesia produced by inhibition of protein synthesis. The present experiment extends these findings to the hippocampus and adds acetylcholine (ACh) to the list of neurotransmitters affected by anisomycin. Using in vivo microdialysis at the site of injection, release of NE, DA, and ACh was measured before and after injections of anisomycin into the hippocampus. Anisomycin impaired inhibitory avoidance memory when rats were tested 48 h after training and also produced substantial increases in local release of NE, DA, and ACh. In an additional experiment, pretreatment with intrahippocampal injections of propranolol prior to anisomycin and training significantly attenuated anisomycin-induced amnesia. The disruption of neurotransmitter release patterns at the site of injection appears to contribute significantly to the mechanisms underlying amnesia produced by protein synthesis inhibitors, calling into question the dominant interpretation that the amnesia reflects loss of training-initiated protein synthesis necessary for memory formation. Instead, the findings suggest that proteins needed for memory formation are available prior to an experience, and that post-translational modifications of these proteins may be sufficient to enable the formation of new memories.

A dominant view of the molecular basis for memory is that the formation of long-term memory for an experience depends on de novo protein synthesis initiated by that experience (Davis and Squire 1984; Frey and Morris 1998; Kandel 2001; Dudai 2002; Nader 2003; Alberini 2008). This view is supported by numerous studies showing that drugs that interfere with protein synthesis by inhibiting translational processes near the time of training produce later amnesia.

Despite the centrality of experience-induced protein synthesis in contemporary models of memory formation, the necessity of protein synthesis for memory consolidation and long-term potentiation (LTP) stabilization has been questioned since the beginning of experiments of this type (e.g., Flexner and Goodman 1975; Barraco and Stettner 1976; Flood et al. 1978; Martinez et al. 1981), and continues to be questioned in several recent reviews (Routtenberg and Rekart 2005; Gold 2006, 2008; Radulovic and Tronson 2008; Routtenberg 2008; Rudy 2008). There are many instances of intact memories formed in the presence of extensive inhibition of protein synthesis, and a wide range of behavioral and pharmacological manipulations can rescue memory impaired by protein synthesis inhibitors. For example, amnesia is attenuated in a graded manner by increasing the training trials and foot shock intensity in avoidance tasks (Flood et al. 1975, 1978). Moreover, a wide range of stimulants, such as amphetamine, strychnine, corticosteroids, and caffeine, block amnesia induced by anisomycin (Flood et al. 1978). Like memory, LTP is sometimes insensitive to protein synthesis inhibitors. Simultaneous inhibition of both protein synthesis and degradation does not interfere with induction and maintenance of LTP (Fonseca et al. 2006a). Also, the specific schedule and frequency of test pulses after induction of LTP determine the vulnerability of LTP to anisomycin-induced impairment; anisomycin treatment does not impair LTP unless test pulses at a rate of 1/10 sec were administered during the anisomycin exposure (Fonseca et al. 2006b).

Findings that memory and LTP can survive the inhibition of protein synthesis challenge the necessity of specific training- or stimulation-initiated protein synthesis for memory formation and synaptic plasticity. Several actions of protein synthesis inhibitors offer alternative accounts for amnesia produced by these drugs. These include cell sickness (Rudy et al. 2006; Rudy 2008), activation of protein kinases and superinduction of immediate early genes (Radulovic and Tronson 2008), abnormal neural electrical activity (Agnihotri et al. 2004; Xu et al. 2005), and intrusion of neural "noise" that masks the primary changes representing memory formation (Gold 2006). Neural responses to inhibition of protein synthesis such as these may impair memory either secondary to or independent of interference with protein synthesis.

Another example of the mechanisms by which inhibition of protein synthesis might impair memory is by altering neurotransmitter functions. This possibility was suggested in early studies (e.g., Flexner and Goodman 1975; Quartermain et al. 1977) and has recently been supported by studies of neurotransmitter release at the site of intra-amygdala injections of anisomycin (Canal et al.

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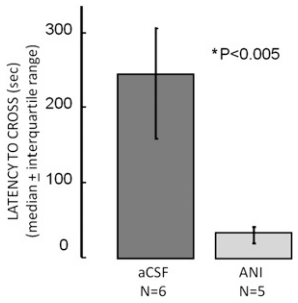

Figure 1. Effects of intrahippocampal injections of anisomycin on memory. Anisomycin injected into ventral hippocampus 20 min before training resulted in significantly lower retention latencies on memory tests 48 h after training.

2007). In addition to impairing later memory after inhibitory avoidance training, pretraining injections of anisomycin into the amygdala produced rapid and dramatic increases in release of norepinephrine (NE), dopamine (DA), and serotonin (5-HT) at the sites of injection. The release of NE and DA then plummeted below baselines from 2 to 6 h after anisomycin injections, recovering within 48 h after anisomycin injection. The possibility that these neurochemical changes contribute to anisomycin-induced amnesia was supported by studies showing attenuation of amnesia in rats pretreated with intra-amygdala injections of the β-adrenergic receptor antagonist propranolol, apparently acting to blunt the effects of the large increases in release of NE after anisomycin injection. In addition, amnesia was produced by injections of high doses of norepinephrine into the amygdala.

In addition to amnesias produced by anisomycin injections into the amygdala, as above, anisomycin also impairs memory when administered to other memory systems, including the hippocampus, where anisomycin impairs inhibitory avoidance memory (Quevedo et al. 1999; Debiec et al. 2002; Milekic et al. 2006). The present study extends the prior findings (Canal et al. 2007) in several respects. Experiments presented here determine whether anisomycin injections into the hippocampus result in changes in release of the catecholamines, NE and DA, at the site of injection, as seen previously in the amygdala. Additionally, the present experiments determine whether intrahippocampal injections of anisomycin result in increased release of acetylcholine, a neurotransmitter not examined in the previous study. To examine parallels with earlier amygdala findings, a further experiment determines whether intrahippocampal pretreatment with propranolol is effective in attenuating anisomycin-induced amnesia.

Results

Pretraining administration of anisomycin into the hippocampus impaired memory

Figure 1 shows the latencies to cross into the shock compartment on memory tests administered 48 h after inhibitory avoidance. The rats had received either anisomycin or aCSF injected into ventral hippocampus 20 min before training. The anisomycin-treated rats exhibited latencies significantly lower than those of aCSF-treated controls (Mann-Whitney *U*-test, $P < 0.005$).

Anisomycin produced large increases in NE, DA, and ACh release in the hippocampus

After anisomycin injections into the hippocampus, release of NE, DA, and ACh increased in the first sample of all rats tested. The values were significantly greater than those in the parallel samples taken from aCSF controls for all three neurotransmitters.

As shown in Figure 2, NE levels increased substantially above baseline (731%) in the first sample (P1) collected after anisomycin injections into the hippocampus ($P < 0.01$). The levels returned to baseline in the subsequent sample (P2: $P > 0.02$ vs. baseline), collected during the second hour after injection. There was a trend toward a decrease below baseline in the next sample (P3: $P < 0.06$ vs. baseline). ANOVAs revealed a significant effect of treatment (anisomycin vs. aCSF) ($F_{(1,45)} = 18.35$, $P < 0.0001$) and of time (i.e., within the anisomycin group) on NE levels ($F_{(4,20)} = 16.17$, $P < 0.00001$).

The pattern of results seen with measurements of DA release was similar to that described above for NE (Fig. 3). DA levels also increased above baseline (544%) in the first sample taken after anisomycin injections into ventral hippocampus during the first post-injection sample (P1) ($P < 0.005$). After the initial surge, DA release returned to baseline levels at P2 and P3 (*P*s > 0.2). Differences in DA release across treatments (anisomycin vs. aCSF) were statistically significant ($F_{(1,45)} = 21.33$, $P < 0.0001$) as were the increases in DA release from baseline after anisomycin injection ($F_{(4,20)} = 9.55$, $P < 0.001$).

ACh release, measured in a separate set of rats, also increased after anisomycin injections. As shown in Figure 4, ACh levels increased significantly above baseline (254%) after anisomycin injection into ventral hippocampus during P1 ($P < 0.005$). Within 30 min, ACh levels returned to baseline and remained there until the end of microdialysis (P2 and P3 values vs. baseline: *P*s > 0.05). The difference in ACh release during P1 across treatments (anisomycin vs. aCSF) was statistically significant ($P < 0.0001$).

Pretreatment with propranolol attenuated anisomycin-induced amnesia

The effects on 48-h memory of pretreatment with propranolol before ANI injections were tested in a separate set of rats (Fig. 5). As shown before, ANI (sal-ANI) exhibited latencies on the memory test that were significantly lower than those of the control groups (*P*s < 0.05). The rats pretreated with propranolol before ANI had latencies that were significantly higher than those of the sal-ANI group, while still significantly lower than those of the controls (*P*s < 0.05).

Discussion

Infusions of anisomycin into the hippocampus resulted in amnesia 48 h later. These findings are consistent with those of many prior studies involving injections of protein synthesis inhibitors

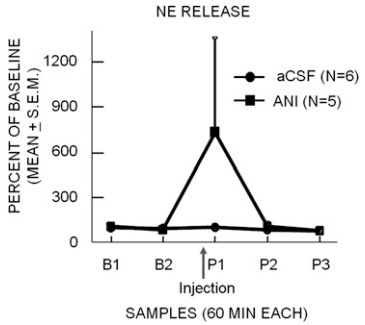

Figure 2. Effects of intrahippocampal injections of anisomycin and aCSF on release of NE. Microdialysis samples were collected every 60 min beginning 2 h before and ending 3 h after injections. Anisomycin and aCSF microinfusions were performed during the first 4 min of P1. Note that NE levels exhibited a large increase in release in the ventral hippocampus immediately after injections of anisomycin, returning to baseline in subsequent samples. (B) Baseline; (P) post-injection.

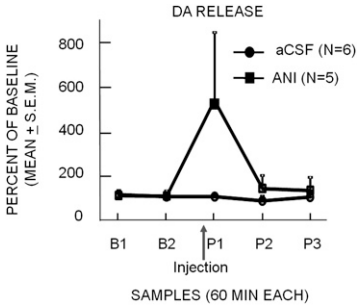

Figure 3. Effects of intrahippocampal injections of anisomycin and aCSF on release of DA. Microdialysis samples were collected every 60 min beginning 2 h before and ending 3 h after injections. Anisomycin and aCSF microinfusions were performed during the first 4 min of P1. Note that DA levels exhibited a large increase in release in ventral hippocampus immediately after injections of anisomycin, returning to baseline in subsequent samples. (B) Baseline; (P) post-injection.

into the hippocampus (e.g., Bourtchouladze et al. 1998; Quevedo et al. 1999, 2004; Taubenfeld et al. 2001; Barriantos et al. 2002; Agnihotri et al. 2004; Artinian et al. 2007), as well as into other brain areas such as the amygdala (Nader et al. 2000; Schafe and LeDoux 2000; Debiec et al. 2002; Duvarci et al. 2005; Parsons et al. 2006; Canal et al. 2007; Milekic et al. 2007), and prefrontal cortex (Santini et al. 2004; Akirav and Maroun 2006; Touzani et al. 2007). These reports are part of a large set of papers showing that direct brain injections of protein synthesis inhibitors produce amnesia for many tasks (cf. Morris et al. 2006; Alberini 2008; Hernandez and Abel 2008; Klann and Sweatt 2008). Findings like these have led to the conclusion that de novo protein synthesis initiated by an experience is an important component of memories formed for that experience. However, other consequences of protein synthesis inhibition may also be important for causing amnesia, including abnormal neurophysiological activity (Agnihotri et al. 2004; Xu et al. 2005), gene superinduction, and apoptosis (cf. Routtenberg and Rekart 2005; Gold 2008; Radulovic and Tronson 2008; Routtenberg 2008; Rudy 2008).

An additional consequence of direct brain infusions of anisomycin is aberrant release of neurotransmitters at the site of injection. The present findings show that intrahippocampal injections of ANI result in large increases in release of NE, DA, and ACh at the site of injection. These results are similar to those seen previously in the amygdala. Intra-amygdala injections of ANI, under conditions that produce amnesia, also result in abnormal increases in release of the neurotransmitters measured, NE, DA, and serotonin (Canal et al. 2007). Thus, the present experiment extends the neurochemical and pharmacological findings to include the hippocampus in addition to the amygdala and also extends the neurotransmitters affected by anisomycin to acetylcholine, in addition to NE, DA, and serotonin.

Thus, it appears that inhibition of protein synthesis results in abnormally high release of several neurotransmitters, an effect that may itself produce amnesia. Studies examining injections of other drugs into the hippocampus often reveal inverted U dose-response functions with impairments evident at high doses (Quevedo et al. 1998; Stefani and Gold 1998; Roozendaal et al. 1999; Goshen et al. 2007; Hein et al. 2007). Therefore, it is likely that the large increases in release of the neurotransmitters measured here, and presumably other neurotransmitters as well, are sufficient to produce amnesia. Supporting this view is evidence that memory deficits of severity comparable to that obtained with anisomycin can be produced by direct brain injections of high doses of norepinephrine (Liang et al. 1990; Canal et al. 2007) and DA (Huber et al. 1989; Morice et al. 2007). Additional support for this view comes from findings in both the previous (Canal et al. 2007) and present experiments showing that ANI-induced amnesia is attenuated by pretreatment with the β-adrenergic receptor antagonist propranolol. The results obtained with propranolol pretreatment add to extensive evidence that a host of pharmacological agents can rescue memory from the effects of protein synthesis inhibitors (cf. Barraco and Stettner 1976; Martinez et al. 1981; Davis and Squire 1984; Routtenberg and Rekart 2005; Gold 2006, 2008). Substantial increases in the release of dopamine and acetylcholine were also seen after anisomycin treatment. Tests of the significance for amnesia of increased release of these and presumably other neurotransmitters to anisomycin-induced amnesia will require additional studies including, for example, receptor blockade, as shown here using propranolol to test the importance of excessive norepinephrine release for amnesia.

The reason that anisomycin results in excessive release of neurotransmitters needs further investigation. One possibility is that the increased neurotransmitter release is a physiological attempt to reinitiate protein synthesis. According to this view, increased neurotransmitter release represents a homeostatic feedback mechanism using cell–cell signaling to engage intracellular signaling cascades to activate transcription and translation mechanisms. Such events do follow inhibition of protein synthesis. ANI treatment results in the activation of mitogen-activated protein kinases and immediate early genes (Radulovic and Tronson 2008). Superinduction of c-fos, c-jun, and egr-1 expression can be seen in vitro within 30–60 min after ANI treatment (Edwards and Mahadevan 1992; Torocsik and Szeberenyi 2000a,b). In addition to viewing the increase in neurotransmitter release as a putative compensatory response, the increase in expression of kinases, transcription factors, and immediate early genes may itself result in delayed rebound effects on translation, perhaps including synthesis of proteins that they themselves may impair synaptic plasticity (Hughes et al. 1997; Routtenberg and Rekart 2005). Still, this scheme does not specify the source of the increase in neurotransmitter release, in particular, whether at the site of injection or whether projected through activation of circuits that eventually project back to the site of inhibition of protein synthesis.

Note that these abnormal neural responses to protein synthesis inhibitors are not necessarily side effects of the drugs that inhibit protein synthesis. Many of these effects of protein synthesis inhibitors may be a direct consequence of depressed protein synthesis per se. In this respect, the extensive convergent evidence showing that different protein synthesis inhibitors impair memory need not support a requirement for training-initiated new protein synthesis in memory formation. Such findings equally

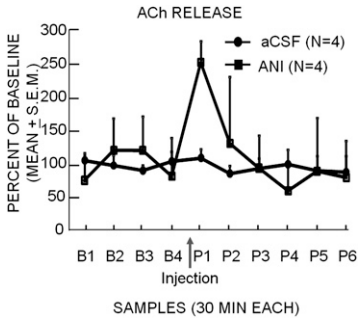

Figure 4. Effects of intrahippocampus injections of anisomycin ($N = 4$) and aCSF ($N = 4$) on release of ACh. Microdialysis samples were collected every 30 min beginning 2 h before and ending 3 h after injections. Note that ACh release increased significantly immediately after injections of anisomycin, returning to baseline in subsequent samples. (B) Baseline; (P) post-injection.

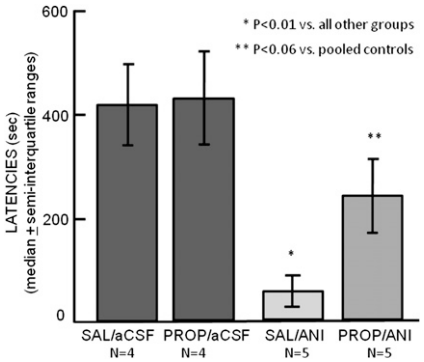

Figure 5. Propranolol attenuation of anisomycin-induced amnesia. Pretreatment with the β-blocker propranolol resulted in significantly higher latencies on the memory test trials in anisomycin-treated rats than the latencies seen after saline was injected prior to anisomycin infusions. The latencies in the sal/ANI group were intermediate to those of amnestic and nonamnestic rats. (SAL) Saline; (aCSF) artificial cerebrospinal fluid; (PROP) propranolol; (ANI) anisomycin.

support the possibility that abnormal neural activity results from inhibition of protein synthesis inhibition and that it is the abnormal neural activity that is responsible for impairments of memory and neural plasticity. Future studies are needed to determine whether other inhibitors of protein synthesis also result in abnormal neurotransmitter release.

In addition to amnesias produced by general protein synthesis inhibitors like ANI, more specific inhibitors of cell signaling mechanisms and transcription factors also impair memory (cf. Izquierdo et al. 2002; Rodrigues et al. 2004; Sharma and Carew 2004; Arnsten et al. 2005; Davis and Laroche 2006; Alberini 2008; Klann and Sweatt 2008). These treatments presumably block more specific programs of gene expression and protein synthesis than the generalist blockade of protein synthesis by ANI. The question arises, however, whether these treatments also have their actions on memory by their intended actions or by resulting in altered neurotransmitter functions that underlie the memory impairments. In a recent study, we found that CREB antisense treatment administered 6 h before training resulted in memory impairments assessed 48 h after training (Canal et al. 2008). The antisense treatment also reduced training-initiated release of NE release; injections of clenbuterol, a β-adrenergic receptor agonist, at the time of training reversed the amnesia. These findings suggest that changes in NE responses to training may contribute to amnesia produced by CREB antisense and raise a cautionary note, not only about general inhibitors of protein synthesis, but also about the interpretations of amnesia after other treatments that selectively block cell and molecular processes thought to participate in memory formation.

In viewing the present findings, it is important to distinguish between problems of interpreting the results obtained with protein synthesis inhibitors and the general issue of the role of protein synthesis in memory formation. The present findings do not challenge the extensive evidence of changes in gene and protein expression patterns after training (cf. Clayton 2000; Levenson and Sweatt 2005; Abraham and Williams 2008; Gold 2008; Klann and Sweatt 2008). These changes are likely to participate in cellular responses important for brain functions including neural memory and plasticity, although the relationship to specific behavioral memories is less clear. However, the present findings suggest that proteins necessary and sufficient for the formation of new memories are available prior to an experience, perhaps ready for post-translational modifications needed for the formation of new memories (Routtenberg and Rekart 2005).

In summary, the present results add to those obtained over the past 30 yr offering the possibility that inhibitors of protein synthesis may induce amnesia by altering neurotransmitter functions rather than, or in addition to, directly interfering with specific protein synthesis needed for the formation of new memories. Additional experiments with other protein synthesis inhibitors, as well as with inhibitors of kinases and transcription factors, are needed to determine the extent to which neurochemical actions mediate the amnesias produced by inhibitors of global protein synthesis and by inhibitors of specific programs of protein synthesis. Beyond that, it will also be important to determine whether these treatments lead to an understanding of the mechanisms of producing amnesia versus revealing the mechanisms of memory formation.

Materials and Methods

Subjects

Male Sprague-Dawley rats (Harlan Laboratories, Oregon barrier), approximately 3 mo old, were housed individually with free access to food and water for at least 1 wk prior to surgery. The rats were maintained on a 12-h light–dark cycle (lights on at 0800). All behavioral procedures were performed between 1000 and 1500. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Illinois, Urbana–Champaign, and were in compliance with the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals.

Surgeries

Rats were anesthetized with isoflurane and then placed in a stereotaxic apparatus with skulls in a horizontal orientation. For the microdialysis experiments, a 23-gauge injector guide cannula (Plastics One) was implanted into one side of the ventral hippocampus, and a 23-gauge microdialysis guide cannula (Bioanalytical Systems Inc.) was implanted into the contralateral ventral hippocampus (coordinates: nosebar: −3.3 mm, AP: − 5.5 mm, ML: ±4.8 mm; DV: −4.8 mm from dura). Skull screws were inserted, and the assemblage was anchored in place with dental cement. Stylets flush with the guide cannulae tips were secured in the cannulae. Beginning 1 wk after surgery, rats were handled for 5 d before further experimental procedures were performed.

Injections and microdialysis procedures

Anisomycin (Sigma) was dissolved in 1 N HCl, brought to pH 7.2 with 1 N NaOH, and to a final concentration of 125 μg/μL with artificial cerebrospinal fluid (aCSF) (128 mM NaCl, 2.5 mM KCl, 1.3 mM $CaCl_2$, 2.1 mM $MgCl_2$, 0.9 mM NaH_2PO_4, 2.0 mM Na_2HPO_4, 1.0 mM dextrose). The dose of anisomycin used for infusions in the microdialysis experiments was 125 μg per side, administered bilaterally in 1 μL. This dose is at or above doses that strongly and quickly inhibit protein synthesis at the infusion site with inhibition lasting at least 6 h (Rosenblum et al. 1993; Canal and Gold 2007; Wanisch and Wotjak 2008). Control rats received microinfusions of aCSF. After injections, the cannulae were left in place for an additional 1 min.

In the microdialysis experiments, rats received bilateral injections of anisomycin, on one side via a 30-gauge standard injection cannula and the other side via the injection port of the combination microdialysis probe/injector cannula (MD-2252, 30 gauge; Bioanalytical Systems Inc.) in which an injection port passes through the microdialysis membrane to the tip of the probe, allowing microinjections during microdialysis. Both the inner injection needle of the dialysis probe and microinfusion cannulae extended 2 mm beyond the tip of the guide cannulae.

Unilateral microdialysis was conducted before, during, and after bilateral microinfusions. Rats were placed in a holding chamber (30 cm long, 30 cm wide, 41 cm deep) with fresh

bedding, food, and water during microdialysis. Dialysis probes were inserted into the microdialysis guide cannulae, and brains were perfused continuously at a rate of 1.0 μL/min with aCSF prepared as above. NE and DA were measured in one set of rats (aCSF, $N = 6$; ANI, $N = 5$) and ACh in a separate set of rats (aCSF, $N = 4$; ANI, $N = 4$). For ACh microdialysis, the perfusate also contained 100 nM neostigmine, an acetylcholinesterase inhibitor included to enable the observation of treatment-related changes in acetylcholine release in the hippocampus (Chang et al. 2006). Temporal resolution for NE and DA microdialysis samples was 1 h (60 μL/sample) and for ACh samples was 30 min (30 μL/sample). To allow equilibration with brain extracellular fluid and to avoid temporary changes in extracellular neurotransmitter levels caused by acute tissue damage (Westerink and Timmerman 1999), the first hour of dialysate was discarded. After collection of baseline samples for 2 h, injections of anisomycin or aCSF were administered bilaterally into the ventral hippocampus over 4 min (0.25 μL/min) via a CMA/100 microinjection pump (Carnegie Medicin). Unilateral microdialysis sampling continued during the injection procedures. Microdialysis continued for 3 h after anisomycin or vehicle injections for NE/DA samples and ACh samples.

In the first behavioral experiment demonstrating amnesia produced by anisomycin injections, anisomycin (125 μg per side; $N = 5$) or aCSF ($N = 6$) were injected bilaterally 20 min prior to training. The injections were made using a CMA/100 microinjections pump at a volume of 1 μL per side delivered over 4 min (0.25 μL/min).

In the second behavioral experiment, testing the efficacy of propranolol in attenuating anisomycin-induced amnesia, anisomycin, propranolol hydrochloride (2.5 μg/side; Sigma-Aldrich), saline, or aCSF was infused bilaterally using injection procedures as above. Rats received intrahippocampal injections of propranolol or saline 30 min before training and ANI or aCSF 20 min before training (sal/aCSF, $N = 4$; propranolol/aCSF, $N = 4$; sal/ANI, $N = 5$; propranolol/ANI, $N = 5$). After each injection, the cannulae were left in place for an additional 1 min.

Inhibitory avoidance training and memory testing

The inhibitory avoidance apparatus consisted of a trough-shaped box (91 cm L × 23 cm W at the top × 7.6 cm W at the bottom × 15.2 cm D). A well-lit, white start compartment chamber (31 cm long) was separated from a dark shock compartment (60 cm long) by a metal divider that could be lowered below the floor. On each of the two days before training, rats were placed in the inhibitory avoidance apparatus for 5 min/day with the divider lowered, at which time they could freely explore the apparatus. The pretraining procedure was used because pre-exposure to the training apparatus increases the sensitivity of inhibitory avoidance memory to manipulations of the hippocampus (Rudy et al. 2002; Huff et al. 2005; Rudy and Matus-Amat 2005; McHugh and Tonegawa 2007).

On the day of training, rats received pretraining injections as described above. During training, rats were placed in the start chamber facing away from the divider, which was lowered 20 sec later. Latency to cross (four paws) into the shock chamber was recorded. After rats crossed into the shock box, the divider was raised, and a shock (0.5 mA/1.5 sec) was delivered through the floor. Rats were removed from the shock chamber 1 min following the shock. Rats were placed back in the start compartment 48 h after training for a memory test. The divider was lowered 20 sec later, and the latency to cross into the shock chamber was recorded as the index of memory, with a 600-sec maximum latency.

NE and DA assay procedures

Samples were assayed for NE and DA concentrations using high-performance liquid chromatography with electrochemical detection (HPLC-ED). Samples were separated by an ODS C18 reverse phase analytical column (HR-80, 3 μm, 100 × 3.2 mm; ESA). The mobile phase contained 75 mM NaH_2PO_4, 1.3 mM SDS, 20 μM EDTA, 12.5% acetonitrile (v/v), 3% methanol (v/v), and 0.02% triethylamine (v/v) (pH 5.6), and was driven by a solvent delivery system (ESA 580 pump) at a rate of 0.6 mL/min. Samples were automatically injected by a Waters 717 plus autoinjector. Electrochemical detection was carried out by an ESA Coulochem III detector with Model 5014B analysis cell. The working potentials were set at −175 mV for electrode I, +200 mV for electrode II, and +300 mV for the guard cell. Injection volume in this experiment was 50 μL. The detection limit of this system was ≈1 pg for each amine. The assay was completed in 25 min.

ACh assay procedures

ACh content in each dialysate sample was assayed by HPLC-ED (Bioanalytical Systems Inc.). The assay system included an ion-exchange microbore analytical column, a microbore ACh/Ch immobilized enzyme reactor (IMER) containing acetylcholinesterase and choline oxidase, an auxiliary electrode with radical flow electrochemical thin-layer cell and thin-layer gasket, a "wired" enzyme electrode (a redox polymer film containing horseradish peroxidase coated on the surface of a 6-mm glassy carbon working electrode), a DA-5 interface between detector and computer, controlling and analyzing software, and a low-dispersion Rheodyne injection valve (model 9725i) with a 10-μL PEEK loop. Stable and relatively pulse-free flow was achieved with a Shimadzu LC-10ADvp pump. The potential held by the working electrode was 100 mV versus an Ag/AgCl reference electrode. The mobile phase contained 50 mM Na_2HPO_4 (pH 8.5) and 0.005% ProClinTM 150 microbiocide. The flow rate was 140 μL/min. The injection volume in this experiment was 6.0 μL. The detection limit was 65 fmol. The assay was completed in 13 min.

Histology

After behavioral testing and microdialysis, rats were deeply anesthetized with sodium pentobarbital and were perfused intracardially with 0.9% saline followed by 4% paraformaldehyde. Brains were removed, post-fixed for ~48 h in 4% paraformaldehyde, and then cryoprotected in 20% glycerol in 0.1 M phosphate buffer for ~24 h. Frozen sections (50 μm) were obtained using a Leica cryostat. The sections were mounted on slides, stained with cresyl violet, and later analyzed for cannulae placements. Only rats with infusion cannula tip placements in the ventral hippocampus were included in the data analysis. An example of a typical cannula placement in the hippocampus is shown in Figure 6.

Statistics

Inhibitory avoidance scores were analyzed with Mann-Whitney *U*-tests (Siegel 1956). Neurochemical data were analyzed with repeated-measures ANOVAs and post hoc *t*-tests using Statview software. Because the means and standard deviations for neurotransmitter concentrations in samples from treated and untreated

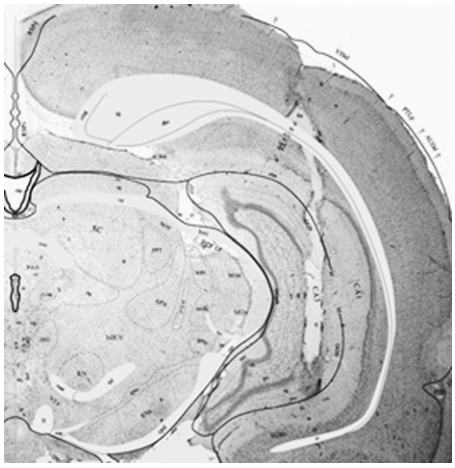

Figure 6. Photomicrograph showing a representative cannula placement in the ventral hippocampus.

groups were extremely different, the data were analyzed using $\log_{10}$ transforms of the values. Scheffé's post hoc *t*-tests were used to compare anisomycin versus vehicle results at each sample time.

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