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D-cycloserine enhances short-delay, but not long-delay, conditioned taste aversion learning in rats

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A R T I C L E   I N F O

A B S T R A C T

NMDA receptors have been implicated in conditioned taste aversion (CTA), a form of associative learning with the unique temporal characteristic of associating taste and toxic stimuli across very long delays. D-cycloserine (DCS), an NMDA receptor agonist, has been shown to enhance short-delay CTA learning. Here we examined the interaction of DCS with varying temporal parameters of CTA. DCS (15 mg/kg) administered prior to the pairing of 0.125% saccharin and LiCl (38 mM, 12 ml/kg) enhanced CTA when there was a short delay between the taste-toxin pairing (10 min), but not when there was a long delay (45 min). DCS activity remained at effective levels over the long delay, because DCS administered 60 min prior to a short-delay pairing enhanced CTA. The interaction of DCS with the delay between taste stimulus onset and LiCl injection was investigated by administering DCS and then 5 min access to saccharin 45 min prior to a short-delay pairing of saccharin and LiCl. DCS failed to enhance CTA in rats pre-exposed to saccharin, even with a short delay between the second saccharin exposure and LiCl injection. These results suggest that DCS enhancement of CTA is dependent on mechanisms underlying gustatory processing during long-delay taste-toxin associations.

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1. Introduction

Conditioned taste aversion (CTA) is a form of associative learning in which an animal avoids and reacts aversively to a taste such as saccharin (conditioned stimulus, CS) that has been previously paired with a toxin or a malaise-inducing treatment such as LiCl injection (unconditioned stimulus, US). CTA learning is robust in that an animal can form a strong aversion that can last for months (Houpt et al., 1996; Martin and Timmins, 1980; Steinert et al., 1980) after only a single trial of a taste-toxin pairing (Garcia and Koelling, 1967). Whereas most associative learning requires the CS and US to be administered contingently with a delay of seconds or less, CTA learning is unique among Pavlovian learning paradigms because conditioning is supported across long delays (minutes to hours) between taste and toxin (Garcia et al., 1966; Kalat and Rozin, 1973; Revusky and Bedarf, 1967; Smith and Roll, 1967).

NMDA receptor (NR)-mediated neurotransmission has been implicated in many forms of associative learning, such as fear conditioning and eye blink conditioning, as well as CTA learning (for a review, see Jimenez and Tapia, 2004). NRs are ubiquitously distributed throughout the brain and play a major role in neurotransmission associated with development, plasticity, learning and memory, excitotoxicity, and diseases such as schizophrenia (for a review, see Cull-Candy et al., 2001). Various experiments have suggested a critical role for NRs in

CTA protocols in which aversions are induced by pairing novel sweet tastes (saccharin or sucrose solutions) with systemic LiCl injections. NR antagonists such as MK-801, APV, or agmatine administered into the gustatory cortex or amygdala impair CTA learning in rats (Gutierrez et al., 1999; McKay et al., 2002; Tucci et al., 1998). The NR2B subunit is tyrosine-phosphorylated in the gustatory cortex after CTA training, with the level of NR2B phosphorylation matching the novelty and quantity of the taste stimulus (Rosenblum et al., 1997). Lastly, induction of NR-dependent long-term potentiation (LTP) in the gustatory cortex one week before conditioning enhances CTA learning (Escobar and Bermudez-Rattoni, 2000).

Most pharmacological studies of NRs and CTA have focused on the glutamate-binding site of NRs, but NRs require co-agonists to become activated: the neurotransmitter glutamate must bind on the NR2 subunit, while a second agonist, either glycine or D-serine, must bind on the obligatory NR1 subunit (Anson et al., 1998; Johnson and Ascher, 1987). D-serine is likely the principle endogenous agonist at the NR1 glycine-binding site because the distribution of D-serine more closely overlaps with that of NRs in the majority of the brain, including the cortex and amygdala (Mothet et al., 2000; Schell et al., 1995) and D-serine is up to three times more potent at the NR than glycine (Matsui et al., 1995; Priestley et al., 1995; Wolosker et al., 1999).

While it is clear that NRs are critical for learning and memory, accumulating evidence shows that D-serine is also important. D-serine administered systemically immediately after cortical damage attenuates reference memory deficits in rats (Andersen et al., 2003), compensates for NR antagonist-induced learning impairments (Ohno et al., 1994; Steele and Stewart, 1993), and rescues impaired NR-dependent LTP in

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aged rats (Mothet et al., 2006). Similarly, a mouse strain lacking D-amino acid oxidase (DAAO), an endogenous enzyme that metabolizes D-serine, has increased occupancy of the NR1 site due to elevated extracellular D-serine levels. These mice lacking DAAO show improved performance in tests of spatial learning (Almond et al., 2006; Maekawa et al., 2005). Conversely, degradation of D-serine using exogenous DAAO greatly attenuates NR-mediated neurotransmission in hippocampal neurons (Mothet et al., 2000). However, the role of D-serine has not been thoroughly explored in CTA learning.

The drug D-cycloserine (DCS), a potent agonist at the NR1 glycine-binding site, has been shown to compensate for age- or lesion-induced deficits in eye blink conditioning in rabbits (Thompson and Disterhoft, 1997) and maze learning in rats (Aura et al., 1998; Riekkinen et al., 1998; Schuster and Schmidt, 1992; Temple and Hamm, 1996). DCS has also been shown to facilitate learning in normal animals by enhancing acquisition of avoidance learning in mice (Flood et al., 1992), extinction of fear conditioning in rats (Ledgerwood et al., 2005; Walker et al., 2002), acquisition and extinction of eye blink conditioning in rabbits (Thompson et al., 1992; Thompson and Disterhoft, 1997), and maze learning in rats and mice (Lelong et al., 2001; Pussinen et al., 1997; Quartermain et al., 1994).

Consistent with a prior study (Land and Riccio, 1997), our laboratory has shown that systemic DCS (7 or 15 mg/kg) dose-dependently enhanced CTA learning in rats when administered before a saccharin-LiCl pairing (Nunnink et al., 2007). This enhancement by DCS was not due to increased malaise or increased neural responsiveness to the toxin, because DCS administered without LiCl did not produce a CTA, DCS did not increase LiCl-induced "lying-on-belly" behavior, and DCS did not increase LiCl-induced c-Fos in central visceral relays such as the nucleus of the solitary tract, parabrachial nucleus, central nucleus of the amygdala, supraoptic nucleus, and paraventricular nucleus. Furthermore, DCS acted specifically at the NR1 glycine-binding site to enhance CTA because pretreatment with the partial NR1 agonist HA-966 blocked the effect of DCS on CTA learning.

While DCS enhanced CTA under certain conditions, there are many parameters of CTA learning that have not previously been explored in conjunction with DCS. In particular, CTA can be formed with a long interval (minutes to hours) between the CS and US, but the neurochemical events underlying this long-delay learning are unknown. Although there are reports showing no differences between short and long delay CTAs as measured by the aversion magnitude (e.g. 15- and 30-min delays (Schafe et al., 1995) or 30-, 60-, and 90-min delays between the CS and US (Martin and Timmins, 1980)), the possibility remains that different neurochemical events mediate shorter delays and longer delays. There have been very few pharmacological invasions of shorter vs. longer CS–US intervals to test this possibility.

The interaction of endogenous D-serine and NRs is a potential candidate for participation in the long-delay interval. The kinetics of D-serine are suggestive; recent evidence shows that D-serine is the principle agonist at the NR1 site, but it does not normally saturate NRs in vivo (for a review, see Miller, 2004). These low levels of D-serine may play a rate-limiting role in the regulation of NR activation. The majority of brain D-serine is present in glia (Schell et al., 1995) although a small amount is found in neurons as well (Kartvelishvily et al., 2006). Upon activation of non-NMDA glutamate receptors during neurotransmission, such as AMPA receptors (AMPARs), D-serine is synthesized in glia by serine racemase, an enzyme that converts L-serine into D-serine (Kim et al., 2004; Schell et al., 1995; Wolosker et al., 1999). D-serine is released when sodium influx through AMPARs on glia causes sodium-dependent amino acid transporters to act in reverse (Levi and Patrizio, 1992). Thus, NR activation is a coordinated process whereby glutamate released by the presynaptic neuron not only binds to postsynaptic NRs but also causes nearby glia to synthesize and release D-serine, which then permits activation of postsynaptic NRs (Schell et al., 1995, 1997; For review, see Wolosker et al., 2008).

D-serine can also have relatively delayed effects on synaptic function, such as priming the internalization of NRs over a time course of minutes (Nong et al., 2003). Furthermore, the in vivo time course of D-serine release and synaptic build-up after artificial stimulation is slow compared to classical neurotransmitter release. For example, microdialysis at high temporal resolution showed that high potassium stimulation of the intact striatum caused an increase of D-serine levels 24% above baseline within 1 to 2 min (Ciriacks and Bowser, 2004).

Given the ability of CTA to withstand long CS–US delays and the relatively slow time course of D-serine build-up in synapses, activity at the NR1 subunit might contribute to the long intervals in CTA learning. Therefore, as an initial investigation of the role of NRs in the temporal delay characteristics of CTA, we examined the effects of DCS on short- vs. long-delay CTA learning. If endogenous D-serine becomes saturating after some interval, then exogenous DCS may be more potent in enhancing CTA at short-delay intervals. Consistent with this hypothesis, we demonstrate in experiment 1 that DCS enhanced learning when administered before a short-delay (10 min) CTA protocol but had no effect on CTA learning with a long delay (45 min) between the taste and toxin. This is a novel finding of a difference in pharmacological sensitivity between short-delay and long-delay CTA learning.

The remaining experiments were intended to determine if the differential effect of DCS on short- and long-delay learning was specific to the delay interval. Experiment 2 showed that the lack of effect of DCS on long-delay CTA was not due to a degradation of DCS across the long delay, because DCS administered 60 min prior to conditioning still enhanced short-delay CTA. Finally, experiment 3 showed that DCS only enhanced CTA when there was a short period of gustatory processing (10 min) prior to LiCl administration; DCS did not enhance CTA after longer periods of taste exposure (55 min), even if there was a short-delay between the termination of the taste and LiCl administration. These results suggest that DCS enhances CTA learning by interacting with mechanisms underlying the initial taste exposure rather than with mechanisms subserving the association of taste and toxin.

2. General methods

2.1. Subjects

Adult male Sprague–Dawley rats (250–300 g, Charles River Laboratories, Wilmington, MA) were housed individually in polycarbonate cages in a temperature- and humidity-controlled colony room with a light-dark cycle of 12-h light, 12-h dark. All conditioning trials were conducted during the light phase. Rats had free access to pelleted Purina Rat Chow 5001. Eight to ten days before the conditioning day, all rats were placed on a water-restriction schedule. Water was given in one daily access period starting at 3 h per day and gradually decreased to 10 min per day. Following conditioning, ad libitum water was returned to rats overnight. Two-bottle preference tests were begun the day after conditioning. All procedures and experiments were approved by the Florida State University Institutional Animal Care and Use Committee.

2.2. Conditioning procedure

DCS treatment: At varying times as specified below, rats were injected prior to conditioning with either DCS (Sigma-RBI, St. Louis, MO, 15 mg/kg, i.p.) or isotonic saline (1 ml/kg) as a control vehicle. This dose of DCS was chosen based on our previous report because it produced a maximal enhancement of CTA but had no aversive effects on its own (Nunnink et al., 2007). DCS was administered 15 or 60 min prior to conditioning, because a pharmacokinetic study has shown that DCS reaches the brain 15 and 60 min after systemic administration in rats (Baran et al., 1995).

Saccharin CS: Rats were given 10-min access to 0.125% sodium saccharin (saccharin) as the CS. Saccharin consumption during the conditioning procedure for each experiment was measured by weighing the

bottles before and after access. To ensure that rats had sufficient exposure to the CS, rats that did not consume at least 2 g of saccharin were excluded from the studies ($n=8$). (During bottle manipulations there is a possible spillage of ~0.5 g and a certain minimal amount of intake (e.g. >0.5 ml) is required for CTA acquisition (Barker, 1976)). Across all experiments, included rats ($n=147$) drank an average of $10.6±0.3$ g during 10-min novel saccharin access. In experiment 3, rats were given 5-min access to either saccharin or distilled water as a pre-exposure.

LiCl US: At varying times after saccharin access, rats were given injections of either LiCl (Sigma-RBI, St. Louis, MO. 38 mM, 12 ml/kg, i.p., made isotonic with NaCl) or isotonic saline vehicle (12 ml/kg) as the US. This relatively low dose of LiCl was chosen because it induces a significant but submaximal CTA allowing for a DCS-induced increase to be seen (Nachman and Ashe, 1973).

CS–US intervals: The CS–US delay was timed from the start of saccharin access to the time of LiCl injection, with delays of either 10 min (short-delay) or 45 min (long-delay). In the absence of manipulations, we found no difference in CTA magnitude between short- and long-delay saccharin-LiCl conditioning protocols, consistent with some published reports (Martin and Timmins, 1980; Schafe et al., 1995).

The groups were designated as follows: veh/sac/veh groups received a vehicle injection before 10-min saccharin access followed by a vehicle injection. These groups served as controls for repeated injection and were not expected to acquire a CTA; DCS/sac/veh groups received a DCS injection before 10-min saccharin access followed by a vehicle injection. These groups served as DCS-treated controls and were not expected to acquire a CTA because we have shown that DCS alone does not induce CTA (Nunnink et al., 2007); veh/sac/LiCl groups received a vehicle injection before 10-min saccharin access followed by a LiCl injection. These groups served as LiCl-treated controls and were expected to show a moderate baseline CTA; DCS/sac/LiCl groups received a DCS injection before 10-min saccharin access followed by a LiCl injection. These groups served to determine the effects of DCS on CTA.

2.3. Two-bottle preference tests

CTA magnitude and extinction rate were measured by 24-h two-bottle tests that began the day after conditioning and lasted 14 days. Rats were given simultaneous access to bottles of water and saccharin placed side by side. The position of the bottles was alternated each day to detect and eliminate any position bias. Rats showing a significant position bias, as measured by a paired *t*-test, were excluded from the study. Consumption of both water and saccharin was measured daily by weight. The preference score was calculated each day for each rat by dividing the amount of saccharin consumed by the amount of total fluid consumed (saccharin/(water+saccharin)). Because saccharin access during the preference testing was not paired with any aversive stimulus, the preference tests constituted extinction trials. A CTA was considered extinguished when the average preference was not significantly different from the average preference of control rats.

2.4. Statistical analysis

The first day of two-bottle preference tests was taken as a measure of the initial magnitude of CTA. Significant differences were detected by one-way ANOVA for first-day preference scores and two-way ANOVA with one repeated measure (test day) across extinction trials (Statistica) with a significance level of $p<0.05$. Post hoc comparisons were made using the Newman–Keuls multiple comparison test. Data are presented as means with standard error of the mean.

3. Experiments 1a and 1b: effects of DCS on short-delay and long-delay CTA

For the first investigation of the role of NRs in the temporal delay characteristics of CTA, we examined the effects of DCS on short- vs.

long-delay CTA. Systemic DCS was administered before conditioning with either a short delay (10 min) or a long delay (45 min) between the start of saccharin access and LiCl administration.

3.1. Experiment 1a methods: DCS and short-delay CTA

Rats ($n=40$) were placed on a water restriction schedule as described above. On conditioning day, rats were injected with either DCS (15 mg/kg) or saline vehicle (1 ml/kg). Fifteen minutes later, rats were given 10-min access to 0.125% saccharin. At 10 min after the start of saccharin, rats were injected with LiCl (38 mM, 12 ml/kg) or vehicle (12 ml/kg). Thus, there were four groups: veh/sac/veh10 ($n=8$), DCS/sac/veh10 ($n=8$), veh/sac/LiCl10 ($n=12$), and DCS/sac/LiCl10 ($n=11$). (See Fig. 1A for schematic). Two-bottle preference tests began 24 h later. One rat was excluded from the experiments for failing to drink enough on conditioning day (final $n=39$).

3.2. Experiment 1a results: DCS and short-delay CTA

Mean saccharin intake ($10.7±0.7$ g) on conditioning day did not differ among groups. On the first day of two-bottle preference testing, both LiCl-treated groups showed a significant taste aversion compared
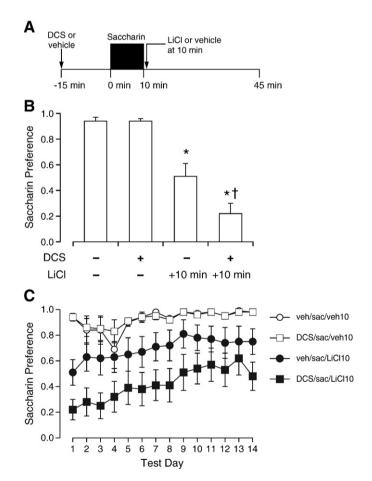

Fig. 1. DCS enhanced short-delay CTA. A. Schematic of experiment 1a. B. CTA magnitude as measured on the first day of 2-bottle preference testing. Both LiCl-treated groups had a significantly lower saccharin preference compared to vehicle-treated controls. DCS/sac/LiCl10 rats had significantly lower saccharin preferences compared to veh/sac/LiCl10 rats. *$p<0.05$ vs. veh/sac/veh10 group. †$p<0.05$ vs. veh/sac/LiCl10 group. C. Extinction of CTA across 14 days of 2-bottle preference testing. Veh/sac/veh10 (white circles) and DCS/sac/veh10 groups (white squares) were not significantly different on any day. Compared to the veh/sac/veh10 group, veh/sac/LiCl10 rats (black circles) showed a significantly lower saccharin preference on days 1 and 6. Saccharin preferences of the DCS/sac/LiCl10 group (black squares) were significantly lower than controls (veh/sac/veh10 and DCS/sac/veh10) on all days and significantly lower than the veh/sac/LiCl10 group for 10 of 14 days (Days 1–9 and 14).

to controls (veh/sac/veh10 and DCS/sac/veh10), as revealed by a one-way ANOVA (F[3,35]=19.05, $p<0.000001$). Further, DCS/sac/LiCl10 showed a significantly lower saccharin preference than veh/sac/LiCl10 on the first day ($p<0.05$). (See Fig. 1B). Thus, DCS enhanced the initial magnitude of a short-delay CTA.

Across 14 days of two-bottle extinction testing, a two-way ANOVA showed significant effects of drug treatment (F[3,35]=6.98, $p<0.005$) and extinction day (F[13,455]=8.64, $p<0.000001$) but no interaction. Controls (veh/sac/veh10 and DCS/sac/veh10) maintained a high intake of saccharin to the near exclusion of water intake and were not significantly different from each other on any day. Veh/sac/LiCl10 rats showed a decreased preference for saccharin overall compared to veh/sac/veh10 controls that was significantly lower on days 1 and 6. Rats that received DCS before a short-delay pairing (DCS/sac/LiCl10) had significantly lower saccharin preferences than controls (veh/sac/veh10 and DCS/sac/veh10) on all days and significantly lower preferences than LiCl-treated controls (veh/sac/LiCl10) for 10 of 14 days (Days 1–9 and 14). (See Fig. 1C). Thus, DCS enhanced the persistence of a short-delay CTA.

3.3. Experiment 1b methods: DCS and long-delay CTA

Rats ($n=50$) were placed on a water restriction schedule as described above. On conditioning day, rats were injected with either DCS (15 mg/kg)

Fig. 2. DCS failed to enhance long-delay CTA. A. Schematic of experiment 1b. B. CTA magnitude as measured on the first day of 2-bottle preference testing. Both LiCl-treated groups had a significantly lower saccharin preference compared to vehicle-treated controls. *$p<0.05$ vs. veh/sac/veh45 group. C. Extinction of CTA across 14 days of 2-bottle preference testing. Veh/sac/veh45 (white circles) and DCS/sac/veh45 groups (white squares) were not significantly different on any day. Compared to the veh/sac/veh45 group, veh/sac/LiCl45 rats (black circles) showed a significantly lower saccharin preference on 12 of 14 days (Days 1–11 and 13). Saccharin preferences of the DCS/sac/LiCl45 group (black squares) were significantly lower than controls (veh/sac/veh45 and DCS/sac/veh45) on the first 13 of 14 days but did not differ from veh/sac/LiCl45 rats on any day.

or saline vehicle (1 ml/kg). Fifteen minutes later, rats were given 10-min access to 0.125% saccharin. At 45 min after the start of saccharin, rats were injected with LiCl (38 mM, 12 ml/kg) or vehicle (12 ml/kg). Thus, there were four groups: veh/sac/veh45 ($n=11$), DCS/sac/veh45 ($n=8$), veh/sac/LiCl45 ($n=12$), and DCS/sac/LiCl ($n=14$). (See Fig. 2A for schematic). Two-bottle preference tests began 24 h later. Two rats were excluded from the experiments for failing to drink enough on conditioning day and three rats were excluded for showing a significant position bias during extinction testing (final $n=45$).

3.4. Experiment 1b results: DCS and long-delay CTA

Mean saccharin intake (11.5±0.6 g) on conditioning day did not differ among groups. On the first day of two-bottle preference testing, both LiCl-treated groups showed a significant taste aversion compared to controls (veh/sac/veh45 and DCS/sac/veh45), as revealed by a one-way ANOVA (F[3,40]=15.59, $p<0.00001$). However, DCS/sac/LiCl45 was not significantly different than veh/sac/LiCl45 on the first day. (See Fig. 2B). Thus, DCS failed to enhance the initial magnitude of a long-delay CTA.

Across 14 days of two-bottle extinction testing, a two-way ANOVA (F[39,520]=2.2, $p<0.0001$) revealed a significant interaction of drug treatment and extinction day. Controls (veh/sac/veh45 and DCS/sac/veh45) maintained a high intake of saccharin to the near exclusion of water intake. Veh/sac/LiCl45 rats showed a significantly decreased preference for saccharin compared to veh/sac/veh45 controls on 12 of 14 days (Days 1–11 and 13). Rats that received DCS before a long-delay pairing (DCS/sac/LiCl45) had significantly lower saccharin preferences than controls (veh/sac/veh45 and DCS/sac/veh45) on the first 13 of 14 days, however, DCS/sac/LiCl45 rats were not significantly different from veh/sac/LiCl45 controls on any day. (See Fig. 2C). Thus, DCS failed to enhance the persistence of a long-delay CTA.

DCS administered before a short-delay pairing enhanced CTA by increasing the magnitude of the aversion and slowing extinction (expt 1a). However, DCS administered before a long-delay pairing failed to enhance the CTA (expt 1b). Experiments 2 and 3 were designed to test if the difference in the effect of DCS on short- vs. long-delay CTA could be explained by a diminished effectiveness of DCS during the long-delay interval, or by an interaction of DCS with the gustatory processing induced by CS exposure.

4. Experiment 2: duration of DCS activity

Experiment 1 showed that DCS enhanced short-delay CTA but not long-delay CTA. During the longer delay (45 min) between the CS and US in experiment 1b, DCS may have become ineffective due to clearance by metabolism or excretion or by a functional reduction in NRs induced by DCS (Nong et al., 2003). There has been one study that measured DCS levels in the plasma and whole brain homogenates after systemic injection of 320 mg/kg DCS in the rat and found an almost two-fold increase in brain tissue from 15 to 60 min (Baran et al., 1995). Thus, it is likely that DCS is still present and increasing in the brain across the 45-min CS–US interval. However, there is not enough known about the pharmacokinetics of DCS to correlate brain levels with functional activity, or to confirm brain levels after the smaller dose employed here. To determine whether DCS was still functionally effective across a delay comparable to the delay in experiment 1b, we administered DCS 60 min before a short-delay CTA.

4.1. Methods

Rats ($n=40$) were placed on a water restriction schedule as described above. On conditioning day, rats were injected with either DCS (15 mg/kg) or vehicle (1 ml/kg). Fifteen or 60 min later, rats were given a short-delay pairing of 10-min access to 0.125% saccharin immediately followed by an injection of LiCl (38 mM, 12 ml/kg) or

saline vehicle (12 ml/kg). Thus, there were four groups: veh/sac/veh (n=7), veh/sac/LiCl (n=9), DCS15/sac/LiCl (n=10), and DCS60/sac/LiCl (n=9). (See Fig. 3A for schematic). Two-bottle preference tests began 24 h later. Two rats were excluded from the experiment for failing to drink enough on conditioning day and three rats were excluded for showing a significant position bias during extinction testing (final n=35).

4.2. Results

Mean saccharin intake (12.0±0.6 g) on conditioning day did not differ among groups. On the first day of two-bottle preference testing, all LiCl-treated groups showed a significant taste aversion compared to controls (veh/sac/veh), as revealed by a one-way ANOVA ($F[3,31]=$ 12.43, $p<0.00005$). Saccharin preferences of rats that received DCS 15 min before conditioning (DCS15/sac/LiCl) were not different from LiCl controls (veh/sac/LiCl) on the first day. Rats that received DCS 60 min before conditioning (DCS60/sac/LiCl), however, showed a significantly lower preference for saccharin on the first day compared to LiCl controls. (See Fig. 3B).
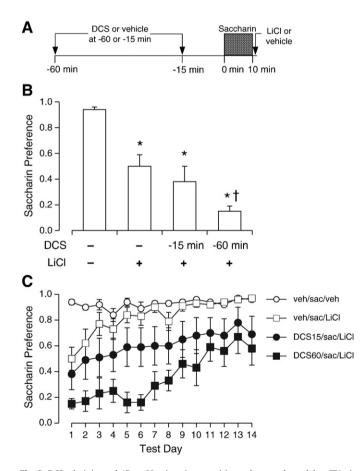

Fig. 3. DCS administered 15 or 60 min prior to pairing enhances short-delay CTA. A. Schematic of experiment 2. B. CTA magnitude as measured on the first day of 2-bottle preference testing. All LiCl-treated groups had a significantly lower saccharin preference compared to vehicle-treated controls. DCS60/sac/LiCl rats pretreated with DCS 60 min prior to conditioning had a significantly lower preference than veh/sac/LiCl rats. *$p<0.05$ vs. veh/sac/veh group, †$p<0.05$ vs veh/sac/LiCl group. C. Extinction of CTA across 14 days of 2-bottle preference testing. Compared to the veh/sac/veh group (white circles), the veh/sac/LiCl (white squares) extinguished by day 2. Saccharin preferences of the DCS15/sac/LiCl group (black circles) were lower than veh/sac/veh on 7 of the first 8 days of testing, but were not different from the veh/sac/LiCl group on any day. Preferences of the DCS60/sac/LiCl group (black squares) were significantly lower than both veh/sac/veh and veh/sac/LiCl groups on all days, and significantly lower than DCS15/sac/LiCl on 5 days (days 2, 3, 5, 6, and 7).

Across 14 days of two-bottle extinction testing, a two-way ANOVA ($F[39,403]=1.77$, $p<0.005$) revealed a significant interaction of drug treatment and extinction day. Controls (veh/sac/veh) maintained a high intake of saccharin to the near exclusion of water intake. LiCl controls (veh/sac/LiCl) showed a significantly decreased preference for saccharin that quickly extinguished by the second day.

The preference scores of rats that received DCS 15 min before conditioning (DCS15/sac/LiCl) were not different from LiCl controls on any day. An enhancement of CTA was seen as slower extinction, however, because DCS-treated rats extinguished only after 7 days vs. 2 days for the veh/sac/LiCl rats. Rats that received DCS 60 min before conditioning (DCS60/sac/LiCl) had significantly lower saccharin preferences than veh/sac/LiCl controls on all days. (See Fig. 3C).

Thus, CTA learning was enhanced when DCS was administered 60 min prior to short-delay conditioning, indicating that DCS was still effective 60 min after systemic administration. Therefore, the failure of DCS to enhance long-delay learning in experiment 1b cannot be due to degradation or diminished responsivity to DCS during the 45-min CS–US interval.

5. Experiment 3: duration of gustatory processing

The failure of DCS to enhance long-delay CTA suggests an interaction between DCS and dynamic processes which occur during the CS–US interval. Little is known about the neurochemical events during this interval. There is behavioral evidence that at some point after a novel taste exposure, learned safety is acquired (e.g. 3.5 h, Kalat and Rozin, 1973), although the neurochemical substrate for learned safety is unknown. Some biochemical events have been characterized after CS exposure, such as the phosphorylation of NR2B subunits and MAP kinase (Berman et al., 1998; Rosenblum et al., 1997). Because DCS enhances CTA without increasing aversive responses to the LiCl US (Nunnink et al., 2007), it is possible that exogenous DCS interacts with gustatory processing.

To test if DCS interacts with gustatory processing induced by CS exposure, we designed a "two-pulse" experiment in order to distinguish the period of gustatory processing from the delay between the termination of the CS and LiCl injection. A saccharin pre-exposure was administered 45 min before a short-delay pairing of saccharin and LiCl. Thus, rats receiving a saccharin pre-exposure had a 55 min period of gustatory processing from the initial taste exposure prior to LiCl administration, but a short-delay (0 min) between the termination of taste exposure and LiCl administration. We choose the timing for the pre-exposure based on the long-delay timing of experiment 1b and evidence that a 55 min pre-exposure is not sufficient to induce learned safety (Kalat and Rozin, 1973).

Experiment 3 had two predicted outcomes: If DCS enhancement is diminished as a consequence of gustatory processing during the long delay after CS exposure (e.g. gustatory modulation of endogenous D-serine or NRs), then the two pulses should model a long-delay (because there is a long interval for processing between the first exposure to saccharin and LiCl injection), and DCS should not enhance the CTA. If, however, DCS enhancement is diminished by some other aspect of the long delay (e.g. diminished ability to associate the toxin with the taste trace), then the two pulses should represent a short-delay (because there is no delay between the second exposure to saccharin and LiCl injection), and the DCS should enhance the CTA.

5.1. Methods

Rats (n=54) were placed on a water restriction schedule as described above. On conditioning day, rats were injected with either DCS (15 mg/kg) or vehicle (1 ml/kg). Fifteen minutes later, rats were given 5-min access to either 0.125% saccharin or water as control. Forty-five minutes after the start of saccharin or water exposure, rats were given a short-delay paring of 10-min access to 0.125% saccharin

immediately followed by an injection of either LiCl (38 mM, 12 ml/kg) or saline vehicle (12 ml/kg). Thus, there were five groups: veh/H20/sac/veh (n=6), veh/H20/sac/LiCl (n=11), veh/sac/sac/LiCl (n=12), DCS/H20/sac/LiCl (n=11), and DCS/sac/sac/LiCl (n=11). (See Fig. 4A for schematic). Two-bottle preference tests began 24 h later. Three rats were excluded from the experiment for failing to drink enough on conditioning day (final n=51).

5.2. Results

A one-way ANOVA showed no significant differences in intake among groups during the 5-min saccharin or water pre-exposure. A one-way ANOVA (F[4,46]=5.29, p<0.01) revealed that mean intakes during the 10-min saccharin access were significantly higher in the saccharin pre-exposed groups compared to the water pre-exposed groups, but there was no significant effect of DCS on saccharin intake. (See Table 1).

On the first day of two-bottle preference testing, all LiCl-treated groups showed a significant taste aversion compared to controls
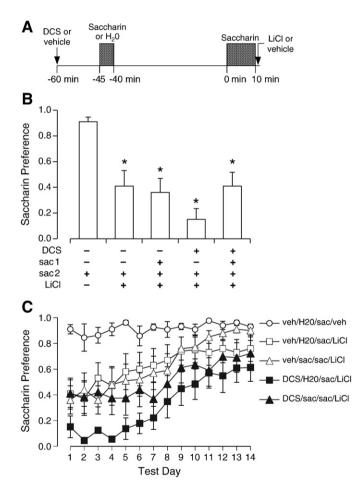
Table 1
Mean±s.e.m. intakes of water and saccharin during pre-exposure and conditioning in Experiment 3

Group	Drug pre-treatment	Pre-exposure solution	5-min pre-exposure intake (g)	10-min Saccharin intake (g)
veh/H$_2$O/sac/veh	Vehicle	H$_2$0	8.0±1.7	7.8±0.9
veh/H$_2$O/sac/LiCl	Vehicle	H$_2$0	7.5±1.8	7.0±0.8
DCS/H$_2$O/sac/LiCl	DCS	H$_2$0	10.2±0.8	7.1±0.6
veh/sac/sac/LiCl	Vehicle	Saccharin	7.9±0.8	11.2±1.1*
DCS/sac/sac/LiCl	DCS	Saccharin	8.4±0.7	10.9±1.1*

Rats were injected with either vehicle or DCS, followed by 5 min pre-exposure to either H$_2$0 or saccharin, followed by 10-min access to saccharin prior to injection with either vehicle or LiCl (See Fig. 4A for timeline). *p<0.05 vs. intake of H$_2$0-pre-exposed groups.

(veh/H2O/sac/veh), as revealed by a one-way ANOVA (F[4,46]=4.75, p<0.05). The saccharin preferences of DCS-treated rats were not different from LiCl control rats on the first day. (See Fig. 4B).

Across 14 days of 2-bottle extinction testing, a two-way ANOVA (F[52,598]=1.53, p<0.05) revealed a significant interaction of drug treatment and extinction day. Controls (veh/H2O/sac/veh) maintained a high intake of saccharin to the near exclusion of water intake. Both LiCl controls were not significantly different from each other on any day, but showed a significantly decreased preference for saccharin that persisted for 4 days (veh/H2O/sac/LiCl) or 6 days (veh/sac/sac/LiCl). Thus, there was no evidence of a "learned safety" effect induced by the initial saccharin exposure.

Rats that received DCS before water and a saccharin-LiCl pairing (DCS/H2O/sac/LiCl) had significantly lower saccharin preferences for 5 or 6 of 14 days compared to veh/sac/sac/LiCl and veh/H2O/sac/LiCl controls, respectively. Thus, DCS administered before a single saccharin exposure followed immediately by LiCl (a short-delay protocol) enhanced CTA but DCS administered before a saccharin pre-exposure and a short-delay protocol did not enhance CTA. Rats that received DCS before a saccharin pre-exposure and a saccharin-LiCl pairing (DCS/sac/sac/LiCl) were not significantly different from LiCl controls on any day. (Fig. 4C).

Therefore, an exposure to saccharin 55 min before LiCl injection was sufficient to diminish the effect of DCS even though LiCl was administered immediately after the second saccharin exposure. This suggests that the failure of DCS to enhance long-delay CTA is due to an interaction with gustatory processing during the CS–US interval, and not some other aspect of the delay.

6. Discussion

6.1. Effects of DCS on short- vs. long-delay CTA

A unique feature of CTA learning is the ability to associate a taste and toxin with a long interstimulus interval. Our laboratory and others have previously reported that DCS enhances CTA, confirming a role for NRs in CTA (Land and Riccio, 1997; Nunnink et al., 2007). Given our findings and the kinetics of D-serine, we hypothesized that endogenous D-serine acting at NR1 subunits plays a more robust role during long CS–US intervals, vs. short CS–US intervals, in CTA learning. Consistent with this hypothesis, experiment 1 showed that DCS only enhanced learning when administered before a short-delay CTA protocol and had no effect on CTA learning when there was a long delay between saccharin access and LiCl administration. The lack of effect of DCS on long-delay CTA learning was not due to a short half-life of DCS (experiment 2) nor the temporal distance between the termination of taste and LiCl injection (experiment 3).

The enhancement of short-delay, but not long-delay, CTA by DCS is novel; no pharmacological treatment has previously distinguished between short-delay and long-delay learning. In some cases, it has been shown that the length of the CS–US interval can impact the magnitude of CTA (Smith and Roll, 1967; Nachman and Jones, 1974). However, the effect of CS–US delay depends on the specific parameters

Fig. 4. Pre-exposure to saccharin blocks DCS enhancement of short-delay CTA. A. Schematic of experiment 3. B. CTA magnitude as measured on the first day of 2-bottle preference testing. All LiCl-treated groups had a significantly lower saccharin preference compared to vehicle-treated controls. *p<0.05 vs veh/H2O/sac/veh group. C. Extinction of CTA across 14 days of 2-bottle preference testing. Compared to the veh/H2O/sac/veh group (white circles), veh/sac/LiCl-treated rats had significantly lower saccharin preferences for 4 days (veh/H2O/sac/LiCl; white squares) or 6 days (veh/sac/sac/LiCl; white triangles). Veh/sac/LiCl-treated rats (veh/H2O/sac/LiCl and veh/sac/sac/LiCl) did not differ on any day. Preferences of the DCS/H2O/sac/LiCl group (black squares) were significantly different from the veh/H2O/sac/veh group for first 12 days, and from veh/sac/LiCl-treated rats for 5 days (veh/sac/sac/LiCl) or 6 days (veh/H2O/sac/LiCl). Although the preferences of the DCS/sac/sac/LiCl group (black triangles) were significantly lower than the veh/H2O/sac/veh group for 9 out of 14 days (days 1–8, 11), they were not different on any day from veh/sac/LiCl-treated rats.

of conditioning (e.g. CS intensity or quality, US toxicity) and testing (e.g. one-bottle vs. two-bottle extinction trials). Using 0.125% saccharin paired with 38 mM, 12 ml/kg LiCl, our laboratory found no differences in CTA with short (10 min) or long-delay (45 min) CS–US intervals in the absence of pharmacological treatment, consistent with other reports showing little qualitative or quantitative difference in magnitude among CTAs of different delays, e.g. with 15- or 30-min delays between CS and US (Schafe et al., 1995) or with 30-, 60-, or 90-min delays between CS and US (Martin and Timmins, 1980). We have ruled out the simplest pharmacokinetic distinction between short- vs. long-delay learning: the half-life of DCS is long enough that it persists throughout the long delay. In fact, DCS administered 60 min prior to saccharin access was more effective than DCS administered 15 min prior to saccharin at enhancing CTA learning. This is consistent with published measures showing that after systemic administration, levels of DCS continually rise in the brain over 60 min (Baran et al., 1995). These results demonstrate that DCS is still active in the central nervous system at least 60–70 min after systemic administration. Therefore, the differing effect of DCS on short- vs. long-delay is not a pharmacological artifact, but rather a nonpharmacological distinction between the mechanisms underlying short- and long-delay CTA learning.

We hypothesized that DCS interacts with processes underlying the novel taste exposure causing a change over the long delay such that DCS no longer enhances the CS–US association. To further elucidate the interaction of DCS with gustatory processing induced by the CS exposure, a "two-pulse" experiment was designed to distinguish the period of gustatory processing from the delay between the termination of the CS and LiCl injection. Consistent with our hypothesis, an exposure to saccharin 55 min before LiCl injection was sufficient to diminish the effect of DCS even though LiCl was administered immediately after the second saccharin exposure. This suggests that the failure of DCS to enhance long-delay CTA is due to an interaction with gustatory processing during the CS–US interval, such as modulation of endogenous D-serine or NRs, and not some other aspect of the delay.

There was variation of CTA magnitude after LiCl treatment among vehicle-treated groups in all four experiments (i.e., veh/sac/LiCl groups in expts 1a, 1b, and 2; veh/H2O/sac/LiCl and veh/sac/sac/LiCl groups in expt 3). However, a two-way ANOVA comparing all 5 vehicle/LiCl groups revealed no significant interaction ($p = 0.22$) or group effect ($p = 0.21$), but showed an expected effect of days ($F[13,663] = 22.91, p < 0.000001$), as all groups showed extinction over 14 days of 2-bottle testing.

6.2. Implications of the short- vs. long-delay distinction

The ability of DCS to enhance short-delay CTA learning is consistent with the effects of DCS on other forms of associative learning such as avoidance learning (Flood et al., 1992) and eye blink conditioning (Thompson et al., 1992; Thompson and Disterhoft, 1997). Unlike these types of learning which require a short CS–US interval (i.e.: ~0.5 s; Kimble, 1961), CTA learning can tolerate long delays (Garcia et al., 1966). Manipulating varying delays in the CTA paradigm allows for the exploration of possible mechanisms unique to either short- or longer-delay learning. The finding that DCS enhances short-delay learning, but not long-delay learning, suggests that each may be mediated by different mechanisms.

Our results suggest that long-delay CTA learning is composed of different phases or involves synaptic or intracellular events that differ over time and vary in their sensitivity to NR agonists. Candidate factors in gustatory circuitry that are correlated with taste exposure and NR sensitivity include phosphorylated NR2B and phosphorylated MAP kinase. After a novel taste exposure, the NR2B subunit is tyrosine-phosphorylated in the gustatory cortex (Rosenblum et al., 1997) and activation of MAP kinase develops in the gustatory cortex and basolateral amygdala (Swank, 2000). MAP kinase activation is NR-dependent because NR antagonists block both the phosphorylation of MAP kinase and the acquisition of a CTA (Berman et al., 2000).

Another possibility is a limiting role for D-serine, an endogenous ligand at the NR1 site. It has been shown that basal levels of D-serine do not saturate NR sites in the gustatory circuitry (for a review, see Miller, 2004) and that D-serine release and synaptic build-up are slow compared to classical neurotransmitter release (Ciriacks and Bowser, 2004; Nong et al., 2003). Thus, there may be limiting amounts of this agonist available for NR activation during short-delay learning, allowing the receptor activity to be increased by exogenous DCS. During the long delay, however, NRs may become saturated with newly synthesized D-serine released from surrounding glia, rendering any exogenous agonists useless for NR-activation enhancement.

Acknowledgments

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