A molecular dissociation between cued and contextual appetitive learning

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In appetitive Pavlovian learning, animals learn to associate discrete cues or environmental contexts with rewarding outcomes, and these cues and/or contexts can potentiate an ongoing instrumental response for reward. Although anatomical substrates underlying cued and contextual learning have been proposed, it remains unknown whether specific molecular signaling pathways within the striatum underlie one form of learning or the other. Here, we show that while the striatum-enriched isoform of adenylyl cyclase (ACS) is required for cued appetitive Pavlovian learning, it is not required for contextual appetitive learning. Mice lacking ACS (ACSKO) could not learn an appetitive Pavlovian learning task in which a discrete signal light predicted reward delivery, yet they could form associations between context and either natural or drug reward, which could in turn elicit Pavlovian approach behavior. However, unlike wild-type (WT) mice, ACSKO mice could not use these Pavlovian conditioned stimuli to potentiate ongoing instrumental behavior in a Pavlovian-to-instrumental transfer paradigm. These data suggest that ACS is specifically required for learning associations between discrete cues and outcomes in which the temporal relationship between conditioned stimulus (CS) and unconditioned stimulus (US) is essential, while alternative signaling mechanisms may underlie the formation of associations between context and reward. In addition, loss of ACS compromises the ability of both contextual and discrete cues to modulate instrumental behavior.

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2003; Kheirbek et al. 2008, 2009), and genetic deletion of AC5 (AC5KO) severely compromises DA’s ability to modulate cAMP levels in the striatum (Iwamoto et al. 2003). Previous studies have shown that AC5KO mice were severely impaired in acquisition of a cued appetitive Pavlovian learning task, while formation of action–outcome contingencies in instrumental learning was intact (Kheirbek et al. 2008). Yet, it remains unknown whether the cAMP pathway in the striatum underlies all forms of appetitive Pavlovian learning, or how it contributes to the ability of Pavlovian cues to modulate instrumental behavior.

In this study, we asked if genetic deletion of AC5 selectively impairs cued or contextual appetitive learning. In addition, we tested whether loss of AC5 affects the ability of conditioned cues or contexts to modulate instrumental behavior. Our data indicate that although loss of AC5 abolishes cued appetitive learning, contextual learning is spared. Although contextual stimuli could elicit approach behavior in AC5KO mice, they could not potentiate an ongoing instrumental response, highlighting the importance of this isoform of AC in Pavlovian–instrumental interactions.

Results

AC5KO mice are impaired in discrete cue appetitive conditioning

Previously, we have shown that AC5KO mice lack the ability to use a discrete auditory Pavlovian CS to predict reward delivery (Kheirbek et al. 2008). To extend these findings, we tested whether AC5KO mice could use short, explicit visual cues to predict reward delivery. Mice were presented with either a 10-sec illumination of the left signal light and extension of the left lever (CS+) or a discrete CS consisting of a cued appetitive Pavlovian task, while for-

| stimulus effect | F(1,10)  | P = 0.97 | session × stimulus interaction | F(11,110) | P = 0.23 | day × genotype interaction | F(11,110) | P = 0.48 |
|----------------|---------|----------|-------------------------------|-----------|----------|----------------------------|-----------|----------|
| ANOVA, stimulus effect | F(1,10)  | P = 0.97 | session × stimulus interaction | F(11,110) | P = 0.23 | day × genotype interaction | F(11,110) | P = 0.48 |
| Normal appetitive conditioning to a long CS in AC5KO mice |

In the conditioning paradigm described above, there existed a tight temporal relation between CS and US, as reward was delivered immediately after CS presentation. As AC5KO mice could not distribute head entry responses to CS offset and reward delivery like WT mice, we next asked whether AC5KO mice could learn a task where there was not a strict temporal predictability of the US. To test this, we used mice using a 2-min CS where reward was delivered at variable time points during CS presentation. Recent studies have suggested that in appetitive Pavlovian learning using a long cue CS rather than a discrete, short cue, CS can modulate different motivational and learning processes and engage different neurobiological substrates (Crombag et al. 2009). To test this, we used 2-min illumination of the testing chamber (dark→light) as a long cue CS for reward availability. Mice were tested for 14 d, four trials a day with each trial consisting of chamber illumination for 2 min (house lights and both signal lights), during which three sucrose pellets were delivered.
on a variable interval (VI) 15-sec schedule of reinforcement. Thus, unlike the discrete cue conditioning procedure, where pellets were delivered immediately after onset of CS in a temporally precise fashion, in this contextual learning task the illumination of the chamber indicated a reward rich context. Head entries into the feeder were measured in the 2 min before CS onset (pre-CS) and during CS presentation. Both AC5KO and WT mice increased their CS head entries across days, suggesting both genotypes could learn the context–US association (acinar effect $F_{(1,14)} = 0.224, P = 0.64$; session effect $F_{(1,13)} = 19.9, P < 0.0001$; session × genotype interaction $F_{(13,182)} = 0.133, P = 0.19$; Fig. 2A). In addition, both groups learned to reduce their pre-CS head entries as they learned the task ($F_{(1,14)} = 2.1$ genotype effect $P = 0.16$; session effect $F_{(1,13)} = 14.3, P < 0.0001$; session × genotype interaction $F_{(13,182)} = 1.7, P = 0.06$; Fig. 2B). These data suggest AC5KO mice can form normal Pavlovian associations to long CSs.

**Normal appetitive contextual learning in AC5KO mice**

We have construed the long stimulus as contextual due to the lack of a precise temporal relationship between the cue and the outcome(s), indicating an increased density of reward without specific temporal information about when reward will be delivered (Crombag et al. 2008). However, context is more broadly viewed as a configural set of cues that together comprise a composite cue indicating increased availability of reward, again without specific temporal information. These data indicate that
Impaired Pavlovian-to-instrumental transfer in AC5KO mice

As AC5KO mice were able to learn context–reward associations, we next asked if a contextual cue previously paired with reward could increase the rate of ongoing instrumental responding for the same reward. In this Pavlovian–instrumental transfer (PIT) paradigm, mice that were trained in the 2-min chamber illumination paradigm described above were trained to acquire a novel lever press response to receive a sucrose reward. Mice acquired the lever press response at a FI 20 sec (F20) schedule of reinforcement and then were trained for 2 d on random interval (RI) 30 and 3 d on a RI60 schedule of reinforcement. As we have previously reported (Kheirbek et al. 2008), lever press rate in AC5KO mice did not significantly differ from WT littermates on the last day of training (AC5KO 1.5 LP/min ± 0.46 SD, WT 1.8 LP/min ± 0.8, genotype effect \(F_{1,14} = -0.81, P = 0.43\)). Twenty-four hours after the last day of RI60, mice were tested for PIT. Mice were given eight presentations of the 2 min CS, with a 2 min ITI. Transfer was measured as a change in response rate to the CS from the pre-CS period (Yin et al. 2006). Interestingly, while WT mice increased their responding during CS presentation, AC5KO mice decreased their responding, suggesting a deficit in PIT in AC5KO mice (CS−pre-CS, single sample t-test, hypothesized mean = 0, WT \(t_{16} = 2.78, P = 0.03\); AC5KO \(t_{7} = 2.8, P = 0.02\); ANOVA \(F_{1,13} = 15.8, \text{cue} \times \text{genotype effect} P = 0.0016; \) Fig. 4A). This difference in responding in AC5KO mice was not due to an increase in conditioned approach during CS presentation compared with that of WT, as cued head entries did not differ between the genotypes (ANOVA, genotype effect \(F_{1,13} = 0.75, P = 0.4\); genotype × cue interaction \(F_{1,13} = 0.06, P = 0.81; \) Fig. 4B). This suggests impaired ability of a previously trained CS to stimulate ongoing lever pressing for reward in AC5KO mice. That the CS decreased responding in AC5KO mice is similar to...
our earlier studies in which we found that in WT mice, a CS decreased responding before Pavlovian training but increased responding after Pavlovian training (Sanders et al. 2007).

Discussion

The results presented here indicate that contextual and cued appetitive learning does not share the same molecular substrates within the striatum. Genetic deletion of ACS, the primary link between DA and cAMP in the striatum, selectively abolishes cued appetitive learning, while leaving contextual learning intact. In addition, deletion of ACS disrupts the ability of Pavlovian stimuli, whether they are contextual or cued, to modulate instrumental behavior, suggesting a shared neural substrate between cued Pavlovian learning and Pavlovian–instrumental interactions.

The anatomical substrates underlying cued and contextual fear conditioning have been studied extensively (Phillips and LeDoux 1992; Fanselow 2000; LeDoux 2000). Lesion and inactivation studies have indicated that the hippocampus and amygdala play a significant role in contextual fear learning, while the amygdala is essential for cued fear learning (Phillips and LeDoux 1992). Recently, a similar dissociation has been proposed for cued versus contextual appetitive learning. Using a modified Y-maze procedure, these studies showed that lesions of this hippocampus rendered rats unable to learn to associate a discrete stimulus with a US (Ito et al. 2005, 2006). In addition, it has been demonstrated that connections between the hippocampus and the NAc shell form a brain circuit that underlies this form of contextual appetitive learning (Ito et al. 2008). It has also been shown that lesions of the core of the NAc inhibit discrete cue Pavlovian learning (Parkinson et al. 1999). As the NAc receives convergent input from amygdala and the hippocampus (Groenewegen et al. 1999; French and Totterdell 2002, 2003), it is possible that information about the nature of the reward-related cues carried by each of these inputs could be processed differently in the NAc.

In addition to the afferent inputs from the amygdala and the hippocampus, the NAc also receives DA input from the ventral tegmental area, which is directly modulated by reward-related cues during the learning process (Schultz 1998a) and modulates plasticity in striatal regions (Reynolds and Wickens 2002). Thus, the NAc provides an attractive target for study of molecular mechanisms that may be differentially modulated by either contextual or discrete conditioned cues. Here we show that ACS, an enzyme expressed throughout the NAc that regulates DA-mediated cAMP production (Iwamoto et al. 2003), is not required for contextual appetitive learning but is required for discrete cue learning. Short, discrete visual cues that immediately preceded reward delivery were unable to elicit conditioned approach behavior in ACSKO mice. Yet, ACSKO mice could learn that contexts were paired with rewards, and in the case of conditioned place preference for drug reward, preference for reward-related context could be detected 4 wk following conditioning. These findings imply that, at the molecular level, discrete and contextual cues are processed differently within the NAc. Although it is likely that loss of ACS within the NAc accounts for these deficits, it should be noted that in a recent report, very low levels of ACS were detected in cortical and hippocampal regions (Kheirbek et al. 2009). Future studies using more elegant, tissue-specific, genetic designs will be required to rule out the contribution of ACS in these regions to discrete cue appetitive learning.

The finding that ACSKO mice lack of PIT suggests shared molecular substrates for discrete cue Pavlovian learning and PIT. A number of anatomical structures have been proposed to underlie PIT, including the ventral tegmental area, amygdala, and the shell of the NAc, a region with high ACS expression (Corbit et al. 2001, 2007). This is supported by data suggesting that increasing local DA concentrations in the shell of the NAc can potentiate PIT (Wyvell and Berridge 2000). Thus, as hypothesized above for discrete cue appetitive Pavlovian learning, ACS may be required for processing of the DA signal associated with the motivational effects of reward-related stimuli on instrumental behavior. Alternatively, the houselight cue in the PIT paradigm may have both contextual and discrete cue properties. The contextual aspect signals a reward-rich environment, giving rise to increased exploratory approach behavior, which is spared in the ACSKO. Though the contingency is less precise, the same cue does contain temporal information that during the next 2 min pellets will be available. This temporal predictive association, impaired in the ACSKO, may be critical for PIT.

In addition to dissociating two forms of appetitive Pavlovian learning, these results may provide insight into how the temporal profile of DA release may underlie these two forms of learning. In vivo, it is known that DA neurons exhibit two modes of firing: tonic and phasic (Grace and Bunney 1984a,b; Overton and Clark 1997). Recordings of DA cell activity and measurements of DA release indicate that conditioned discrete cues elicit phasic activity in DA neurons leading to a phasic increase in DA in terminal regions (Schultz 1998b; Day et al. 2007). The regulatory properties of ACS suggest it may be crucial for processing the phasic DA signal. ACS is negatively regulated by protein kinase A (Iwami et al. 1995), a direct downstream target of activation of ACS, providing a short negative feedback loop constraining cAMP production. Thus, one would expect that very robust stimulation would be required to overcome this constraint. Phasic DA activation by discrete cues could fulfill this role, preferentially activating the lower affinity and extrasynaptically located D1 receptors (Richfield et al. 1989; Gonon 1997). Consistent with this, D1 receptor activation has been shown to be required for initial acquisition of a Pavlovian approach response to a discrete cue (Eyny and Horvitz 2003), and electrophysiological studies indicate that long-term potentiation of glutamatergic inputs in the striatum require D1 receptor activation (Reynolds et al. 2001). Applying this framework, we hypothesize that during acquisition of discrete cue appetitive Pavlovian conditioning, presentation of the US causes phasic release of DA, strengthening those inputs activated by the discrete stimulus that preceded the US. High levels of DA will activate D1 type receptors, stimulating ACS and increasing cAMP levels permissive for LTP induction. In the absence of ACS, D1 receptor stimulation is decoupled from cAMP, blocking the ability of DA to strengthen appropriate synapses, thus weakening the discrete CS–US association.
Therefore, the data presented here suggest that a different mechanism may underlie contextual appetitive learning in the striatum. Although blockade of DA receptors inhibits contextual learning (Acquas et al. 1989; Ettenberg 1989), the underlying specific DA receptor signaling mechanisms remain unknown. It is possible that other AC isoforms with low expression in the striatum mediate contextual learning, while the role of ACS is specific for discrete cue conditioning. This is supported by data indicating that global inhibition of PKA in the NAc inhibits acquisition of CPP for amphetamine (Gerdjikov et al. 2007). In addition, genetic deletion of AC1 and AC8 has been reported to decrease locomotor sensitization to cocaine (DiRocco et al. 2009). However, it is also possible that contextual learning, while being DA-dependent, is not mediated by the phasic DA signal but by increases in tonic levels of DA activating different downstream signaling pathways independent of AC and cAMP. Non-CAMP-dependent signaling pathways have been described that mediate some DA-dependent behaviors, including the β-arrestin 2-mediated pathway, which is a slower pathway than the cAMP pathway (Beaulieu et al. 2005), making it an attractive candidate for this form of learning. Alternatively, D1-type receptors can signal via a phospholipase-C (PLC)-dependent mechanism (Yu et al. 1996; Jin et al. 2003), and recent studies using optogenetic techniques suggest that stimulation of PLC-dependent pathways are sufficient for supporting contextual appetitive conditioning (Airan et al. 2009).

Taken together, our data suggest a very specific discrete cue Pavlovian learning deficit rather than a general deficit in reinforcement learning in AC5KO mice, as mice were able to learn a contextual conditioning task and an action–outcome association-mediated lever pressing task. Therefore, phasic DA signaling via the cAMP pathway may not be required for all forms of reinforcement learning. Future experiments elucidating the non-AC5-dependent signals that contribute to acquisition of action–outcome contingencies and contextual appetitive Pavlovian learning will provide insight into the molecular mechanisms of these forms of learning.

Materials and Methods

Mice

AC5KO mice, generated as previously described (Iwamoto et al. 2003), were backcrossed to C57BL/6 for eight generations. All mice tested were 8–12 wk of age. All animals were group-housed (four to five per /cage) in a temperature- and humidity-controlled barrier facility, with lights on/off at 06:00/18:00 h. All testing was conducted during the light phase. With the exception of conditioned place preference for drug reward, all mice were food restricted for behavioral experiments. Mice were fed regular chow ad libitum for 2 h each day following testing. All experiments were approved by the Institutional Animal Care and Use Committee of the University of Chicago.

Behavioral procedures

Cued appetitive conditioning

Cued appetitive conditioning was conducted in mouse operant conditioning chambers that have two retractable levers, a house light, two signal lights above levers, a signal light, a nosepoke hole on the back wall, and a feeder with photobeam (Med Associates). Naïve, food-restricted mice were placed in the chamber, and sessions began with the onset of the fan. Each session consisted of 40 trials, 20 CS+ and 20 CS− in random order, with a VI 60-sec ITI. CS+ trials consisted of 10-sec illumination of the left signal light and extension of the left lever. After 10 sec, the signal light was turned off and the lever retracted, and a single sucrose pellet was delivered. In CS− trials, the right signal light was illuminated and right lever was extended for 10 sec, and no reward was delivered. Conditioned responses were measured as head entries into the feeder.

Contextual appetitive conditioning for sucrose

Naïve, food-restricted mice were placed in operant conditioning chambers, and sessions began with the onset of the fan, with the houselight off. The contextual CS was a 2-min presentation of the houselight and both signal lights. Each session consisted of four contextual CS presentations delivered at VI 5 min. During contextual CS presentation, three rewards were delivered at a VI 15-sec schedule of reinforcement during CS presentation, excluding the first 7 sec and last 30 sec of CS presentation to reduce the likelihood that mice would form associations between CS onset or offset with reward delivery. Head entries into the feeder were measured during the CS presentation and 2 min preceding CS presentation.

Conditioned place preference for cocaine

Tests were conducted as previously described (Beeler et al. 2009). Briefly, mice were placed in a conditioned place preference apparatus (MedAssociates) which consisted of two separate chambers with a removable gate for access to both sides of the chamber. The flooring differed on either side of the chamber, with wire mesh on one side and bars on the other. Mice were provided with five conditioning sessions for each chamber, alternating between saline and cocaine (20 mg/kg) for a total of 10 sessions. Mice were counterbalanced to eliminate any chamber bias. After training, mice were tested for preference by removing the gate between the chambers and allowing exploration of both sides drug-free for 30 min.

Pavlovian-to–instrumental transfer

After contextual appetitive conditioning for sucrose, mice were trained to acquire a lever press response for sucrose at a FI20 schedule of reinforcement. When mice reached learning criterion of 30 rewards in a 1-h session, they were trained for 2 d at RI30 and 2 d at RI60. Twenty-four hours after the last day of RI60 training, mice were trained for PIT. After an 8-min extinction period to reduce responding, mice were presented with eight 2-min CS presentations with a 2-min ITI. Transfer was calculated as the difference in responding between CS and pre-CS (Yin et al. 2006).

Second-order conditioning

Naïve, food-restricted mice were placed in operant conditioning chambers and trained to press a lever for sucrose pellets at FI20. During training, each rewarded lever press was followed by a 1-sec presentation of the signal light above the lever, followed by reward delivery. After mice reached learning criterion of 30 rewards in a 1-h session, mice were trained for one session at each of the following schedules: FI40, FI60, FI90, FI120, and FI150, followed by 10 d at FI180 to acquire stable responding and the association between the CS and reward delivery. Twenty-four hours after the last FI180 session, mice were given a test session for second-order responding where the CS was given after each lever press, and the reward given at an FI180 schedule (FR1CS/FI180). Lever press response rates were analyzed for the first 15 min of the session and compared with the first 15 min of the FI180 session to control for extinction of the CS–US contingency.

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