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BEHAVIORAL DEPRESSION IN THE SWIM TEST CAUSES A BIPHASIC, LONG-LASTING CHANGE IN ACCUMBENS ACETYLCHOLINE RELEASE, WITH PARTIAL COMPENSATION BY ACETYLCHOLINESTERASE AND MUSCARINIC-1 RECEPTORS

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Abstract—The nucleus accumbens may play a role in acquisition and expression of behavioral depression as measured using the inescapable swim test. Previous work shows that a local injection of a cholinergic muscarinic-1 receptor agonist increases immobility and a specific muscarinic-1 antagonist acts as an antidepressant-like drug by increasing swimming escape efforts. The present study used microdialysis to monitor extracellular acetylcholine levels in the accumbens, fluorescent labeled toxins to monitor changes in acetylcholinesterase and muscarinic-1 receptors, and semiquantitative-polymerase chain reaction to detect changes in gene expression for the muscarinic-1 receptor. Microdialysis showed that acetylcholine levels did not change while an animal was swimming; however, a significant transient decrease occurred when the rat was returned to the dialysis cage, followed by a long-lasting increase that reached a maximum three hours after the test. Acetylcholine levels stayed high even 24 h after the initial test as evidenced by a significant elevation in basal level prior to the second swim. This increase in neurotransmitter may have been partially compensated by a significant increase in the degradative enzyme, acetylcholinesterase, and by a decrease in muscarinic-1 receptors and their gene expression. These results further demonstrate the importance of accumbens cholinergic function in the appearance of a depression-like state.

Key words: microdialysis, behavioral depression, Porsolt test, muscarinic receptor, acetylcholinesterase, rat.

Depressive disorders in humans are characterized by depressed mood, irritability, low self-esteem and feelings of hopelessness (American Psychiatric Association, 2000). Depression is a psychiatric disease that is difficult to study neurochemically because few, if any, of the animal models completely mimic human depression. However, the inescapable swim test (Porsolt test) is a behavioral test for rodents used in the pharmaceutical industry as a predictor of antidepressant drug efficacy (Lucki, 1997). Typically animals placed in a swim tank for a second time become more quickly immobile, which is considered a low motivational state that can be reversed by antidepressant treatment (Porsolt, 1979; Janowsky and Overstreet, 1995; Lucki, 1997).

Unspecified central cholinergic systems have long been recognized to be involved in depressive disorders. Stimulating central cholinergic transmission with systemic acetylcholine (ACh) agonists or acetylcholinesterase (AChE) inhibitors can cause severely depressed mood, dysphoria, and behavioral withdrawal (Janowsky and Risch, 1985; Dilsaver, 1986; Mearns et al., 1994). Depressed humans are supersensitive to cholinergic challenges (Janowsky and Risch, 1985; Dilsaver, 1986). Furthermore, the Flinders sensitive rat, which meets criteria as an animal model of depressive disorder, was bred for increased muscarinic cholinergic sensitivity (Daws and Overstreet, 1999). However, the brain regions involved in this cholinergic function were unknown.

The nucleus accumbens (NAc) is a brain site that is well known for its possible role in various aspects of reinforcement, such as reward, incentive motivation and stimulus salience (Koob, 1992; Berridge and Robinson, 1998; Hoebel et al., 1999; Wise, 2004), but it has also been suggested to modulate negative states. For instance, local injection of methylnaloxonium into the NAc of morphine-dependent rats was sufficient to induce a conditioned place aversion (Stinus et al., 1990), and local neostigmine in the NAc caused a conditioned taste aversion (Taylor et al., 1992). With these two treatments there is an increase of extracellular ACh in the accumbens (Rada et al., 1991, 1996); therefore we were led to hypothesize that high levels of extracellular ACh, relative to dopamine (DA), might play a role in the aversive state (Rada and Hoebel, 2001). In support of this idea, we find that ACh is released in the NAc by a conditioned aversive taste, aversive brain stimulation and withdrawal from morphine, nicotine, diazepam, alcohol and sugar (Rada et al., 1991, 2001, 2004; Mark et al., 1995; Rada and Hoebel, 2001, 2005; Colantuoni et al., 2002).

This cholinergic system in the NAc also seems to be involved in depression-like behavior following the forced swim test. Local application of a muscarinic agonist increases immobility during the swim test, and a relatively

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Abbreviations: ACh, acetylcholine; AChE, acetylcholinesterase; B, Bregma; CREB, cAMP-response-element-binding protein; DA, dopamine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; M1-R, muscarinic-1 receptor; NAc, nucleus accumbens; PBS, phosphate-buffered saline; PCR, polymerase chain reaction.

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specific M1-antagonist acts as an antidepressant-like drug (Chau et al., 1999, 2001b). Little is known of the effects of the swim test on ACh release in the NAc, or subsequent adaptations in expression of muscarinic receptors in behaviorally depressed animals. It was hypothesized that ACh levels would be higher during the second day of swimming when the animals were behaviorally depressed, and that the degradative enzyme for ACh would be up-regulated, and the gene for muscarinic receptors would be down-regulated in response to the excessive ACh. These adaptations were, indeed, found, but they are clearly not sufficient to prevent the animal from acting depressed in accord with the elevated synaptic ACh.

EXPERIMENTAL PROCEDURES

Subjects and surgery

Male, Sprague–Dawley rats (290–350 g; bred at the University of los Andes, Mérida, Venezuela and Princeton University, Department of Psychology, Princeton, NJ, USA) were housed individually in wire cages with food and water *ad libitum* on a reversed 12-h light/dark schedule. For the microdialysis study, rats were anesthetized with a combination of xylazine (10 mg/kg, i.p.) and ketamine (80 mg/kg, i.p.). Bilateral stainless steel guide shafts (21 gauge) were stereotaxically implanted for the shell of the NAc. Coordinates for the guide shaft were anterior to Bregma (B) +1.2 mm, ventral to the level surface of the skull (V) 4 mm, and lateral to midsagittal sinus (L) 0.8 mm (Paxinos and Watson, 2005). Microdialysis probes extended 5 mm beyond the guide shaft. Animals recovered for at least 7 days before testing. All experimental protocols followed the National Institutes of Health guidelines for the care and use of laboratory animals and were approved by the University of Los Andes Research Council and Princeton University Animal Care and Use Committee. All efforts were made to minimize the number of animals used and their suffering.

Swim test

All tests were conducted using a reverse day/night cycle, during the first 6 h of the rats' dark period. On day 1, each subject ($N=8$) was placed in an opaque cylindrical water tank for 10 min (tank diameter 27 cm; height 42 cm; water depth 33 cm changed for each rat; temperature of 25–30 °C). The next day (24 h later) subjects were placed in the water tank again for 10 min, and the length of time they swam during this 10 min period was recorded. "Swimming" was defined by escape behaviors (i.e. diving, rigorous paddling with all four legs, circling the tank, and clambering at the tank walls). "Immobility" was scored as floating and treading water just enough to keep the nose above water. To minimize the influence of experimenter presence on swimming, experimenters stood back from the container, and their shadows were not apparent from the inside of the container.

Tail-pinch

A separate group of rats ($N=6$) was stressed by tail-pinch on two successive days for comparison with the swimming test. Rats were tail-pinched with a rubber-covered forceps placed 3 cm from the proximal end of the tail with enough pressure to cause locomotion but without any signs of pain. Pressure was applied periodically for a total 30 s over the course of 10 min while the animal moved around the cage. Microdialysis samples were collected every 10 min in the exactly the same way as during the swimming tests.

Microdialysis and high performance liquid chromatography for ACh

Microdialysis probes were constructed of fused silica capillary tubing inside stainless steel tubing (26 gauge), with an exposed cellulose membrane (6000 molecular weight cutoff) 0.2 mm OD and 2 mm long (Hernandez et al., 1986; Mark et al., 1991). Probes were inserted and cemented in place 16 h prior to the first collection by briefly anesthetizing the animal with Metofane (methoxyfluorane; 3% for induction and 0.5% for maintenance). Probes were perfused with a modified Ringer (142 mM NaCl, 3.9 mM KCl, 1.2 mM $CaCl_2$, 1.0 mM $MgCl_2$, 1.35 mM Na_2HPO_4, 0.3 mM NaH_2PO_4, pH 7.3) at 0.5 μl/min overnight and 1.0 μl/min starting 3 h before and during sample collection. A low dose of neostigmine (0.3 μM; Sigma, St. Louis, MO, USA) was added to the perfusate to prevent AChE from degrading the ACh. Dialysate was collected every 10 min. A stable ACh baseline prior to the swim test consisted of at least six consecutive 10-min samples within 10% of the mean.

Dialysis samples were collected while the rat was unrestrained in a free-standing 26×26 inch cage during the 1 h baseline (six samples), then in the tank during the 10-min swimming test (one sample). Rats were returned immediately to the dialysis cage where 10-min samples were collected throughout another 60 min, and finally single 10-min samples were collected once each hour for the next 6 h. Rats were left in the dialysis cage overnight with the flow rate set at 0.5 μl/min until next morning when the same procedure as on day 1 was performed again (day 2).

ACh was separated by reverse phase, high performance liquid chromatography using a 10 cm C18 analytical column (Varian Assoc., Lexington, MA, USA). ACh was hydrolyzed to betaine and hydrogen peroxide by an immobilized enzyme reactor treated with AChE and choline oxidase (Sigma Co.). The resulting hydrogen peroxide was detected electrochemically (HPLC Model 400 EG&G, Princeton Applied Research, Princeton, NJ, USA) to yield a measure of ACh.

At the end of the microdialysis experiments, rats were deeply anesthetized with sodium pentobarbital for intracardiac perfusion with isotonic saline, followed by 10% formalin. Brains were sectioned at 40 μm and examined microscopically to discern the microdialysis probe tracks, which were drawn on stereotaxic atlas plates.

AChE and M1 muscarinic receptors labeling

Three new groups of rats were used. The first experimental group ($N=3$) was killed immediately after the first 10-min forced swimming experience, and the second group ($N=5$) immediately following the second swimming experience. A third group of age and weight-matched control rats ($N=5$) had no swimming experience. All were deeply anesthetized with xylazine (10 mg/kg, i.p.) and ketamine (80 mg/kg, i.p.) and perfused intracardially with 150 ml of Ringer's solution followed by 100 ml ice-cold 4% paraformaldehyde in phosphate buffered saline (PBS: 137 mM NaCl; 2.7 mM kCl; 5 mM Na_2HPO_4; 1.7 mM KH_2PO_4, pH 7.4). Brains were removed and immersed in the same fixative for 16 h at 4 °C, followed by sequential immersion in 7, 15 and 30% sucrose for 12 h each. Cryostat coronal sections of 20 μm were made between coordinates B 2.7–B 0.7 mm. Free-floating sections were incubated overnight at 4 °C with either: a) fasciculin toxin coupled to Alexa-488 (1:2000) in PBS, or b) biotinylated MT-3 toxin (1:500) in PBS. Sections were incubated for 1 h at room temperature with streptavidin conjugated with Alexa-488 or Alexa-684 (1:500) or rhodaminated goat anti-rabbit IgG (1:200). Nuclei were stained with Propidium Iodide Red (1:1500; all from Molecular Probes Co., Eugene, OR, USA). Samples were mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA).

AChE was labeled by means of the toxin fasciculin coupled to Alexa-488 and visualized using the 488 nm excitation line of the