Effects of antioxidants on nicotine recognition in rats

Introduction:

According to the CDC, nicotine addiction accounts for over 7 million deaths a year worldwide, twice that of narcotics and alcohol combined (11). Most adults need 30 attempts to quit smoking for a year or longer, and this is likely an underestimate (1). The addictive properties of nicotine are thought to involve activation by acetylcholine of brain reward pathways of the Ventral tegmental area and nucleus accumbens (see Figure 1) (8). Drugs like bupropion and varenicline target monoamine and cholinergic nicotinic receptor mechanisms involved in these pathways and have been approved to treat nicotine addiction, although their success has been limited (19). Because of this, novel approaches of treating nicotine addiction are necessary. The goal of current studies will be to evaluate redox signals as a target for development of new interventional approaches to smoking cessation. A variety of approaches have been used to treat addiction. The nicotine patch is an example of a substitution approach for which the goal of treatment is to maintain a sustained low level of the addicting substance for the purpose of reducing craving for the drug (12). Varenicline and bupropion also represent examples of substitution-based approaches targeting a reduction of craving (18). A second approach which could be characterized as an antagonist approach involves preventing activation of neural systems involved in the addicting process (6). The following is a proposal to test blockers of redox signaling as potential treatment medications for addiction using the antagonist approach.

In the current study, we will evaluate the hypothesis that redox signaling-related effects on neurotransmission participate in the subjective effects of nicotine using a drug discrimination paradigm. In this paradigm, rat subjects learn to recognize the effects of a drug and report its presence or absence using behavioral responses emitted to obtain food reward or avoid aversive stimuli (17). If the hypothesis is correct, then interference with redox signals should fully or partially block the nicotine discriminate stimulus effects. Vitamin C, vitamin E, apocynin, and FSNY-1 were chosen as potential antagonists for this study to evaluate multiple sources and targets of reactive oxygen species (ROS). Vitamin C is a cytosolic antioxidant, vitamin E is a membrane antioxidant, apocynin is a NADPH oxidase inhibitor, and FSNY-1 is thought to inhibit hydrogen peroxide and hydroxide radical. As positive controls, the proposed studies will evaluate the nonselective NN receptor antagonist mecamylamine for the ability to antagonize nicotine discrimination (see preliminary data in subsequent sections). The NN receptor antagonist hexamethonium will also be included as a negative control for mecamylamine, because it has same mechanism of action but does not cross the blood brain barrier. It is expected that mecamylamine, but not hexamethonium, will block the discriminative stimulus produced by nicotine.

Specific Aim

Evaluate the potential antagonizing effects of antioxidants on the discriminative stimulus properties of nicotine.

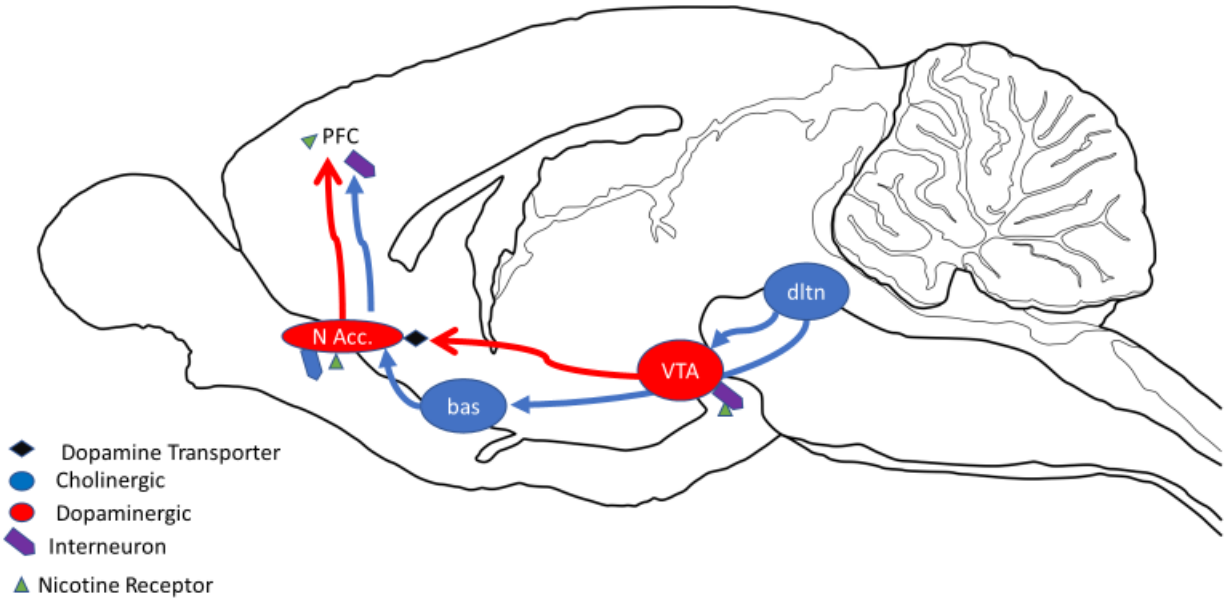

Figure 1. Circuit diagram of nicotinic cholinergic modulation of brain reward pathways. Ventral tegmental area (VTA) is activated in the reward pathway and causes dopamine release to the Nucleus Accumbens (N Acc.) and Prefrontal cortex (PFC). The N Acc processes this information and send it to the PFC. The N Acc is mostly associated with sleep regulation, instrumental and spatial learning, and as a major component in the addiction pathway. The laterodorsal tegmental nuclei (dltn) regulates cholinergic response to VTA and Nucleus Basalis (bas). These cholinergic regions are associated with arousing stimuli and modulating attention. Nicotine receptors exist directly on N Acc and on an interneuron affecting VTA activation and deactivation. The pathway ends on GABA interneurons that regulate the PFC directly.

Significance

According to the CDC, nicotine addiction accounts for over 7 million deaths a year worldwide, twice that of narcotics and alcohol combined, and is expected to rise to 10 million by 2030 (11). Most adults need 30 attempts to quit smoking for a year or longer, and this is likely an underestimate (1). This data suggests that if each attempt is an individual event, there is a 19% - 20% chance of successfully quitting smoking on any given attempt (1). By the age of 40, most smokers have made at least 40 attempts to quit smoking, which shows the timeframe and low probability of success of current smokers. The addictive properties of nicotine are thought to involve activation by acetylcholine of brain reward pathways of the Ventral tegmental area and nucleus accumbens (see Figure 1) (8). Drugs like bupropion and varenicline target monoamine and cholinergic nicotinic receptor mechanisms involved in these pathways and have been

approved to treat nicotine addiction, although their success has been limited. Because of this, novel approaches of treating nicotine addiction are necessary.

Innovation

The current study is innovative because it will evaluate the hypothesis that redox signaling effects, related to neurotransmission, participate in the subjective effects of nicotine using a drug discrimination paradigm. If the hypothesis is correct, then interference with redox signals should fully or partially block the nicotine discriminate stimulus effects. Because the sources of redox signals in the brain have not been well described a comprehensive approach using vitamin C, vitamin E, apocynin, and FSNY-1 will be used as potential antagonists for identifying critical sources and targets of reactive oxygen species (ROS) involved in nicotine addiction. Vitamin C is a cytosolic antioxidant, vitamin E is a membrane antioxidant, apocynin is a NADPH oxidase inhibitor, and FSNY-1 inhibits hydrogen peroxide hydroxide radical. Should an effect be found, these drugs would also give us an idea of the target of action for antioxidants against the addiction pathway, an as yet to be studied approach towards addiction.

Approach

Aim one will be addressed using 32 rats that have received training for nicotine discrimination using a dose of .4 mg/kg. Once that rats have been trained, 4 antioxidant compounds and 2 positive control standards will be evaluated for their ability to antagonize the nicotine discriminative stimulus effect. A within subject experimental design will be used, in which separate groups of 6 to 8 rats will receive up to 6 doses of each antioxidant. It is expected that if redox signals are necessary for identifying nicotine subjective effect, then a dose dependent reduction in nicotine-appropriate responding would occur following most of the antioxidants. Conversely, the absence of any significant effects would suggest that redox signals do not participate as neural mechanisms of nicotine discrimination.

Specific Methods

Following the standard protocol for nicotine discrimination used by the ATDP (8), 32 male Sprague-Dawley (SD) rats (Invigo) will be trained to discriminate nicotine (0.4 mg/kg) from saline using a two-lever choice methodology. Subcutaneous injections of nicotine or vehicle will occur 15 minutes prior to the start of the training session. Food will be available as a reinforcer under a fixed ratio 10 schedule when responding occurs on the injection appropriate lever. All tests will occur in standard, commercially available chambers (Coulbourn Instruments), using 45 mg food pellets (Bioserve) as reinforcers.

Training sessions will occur in a double alternating fashion, and tests will be conducted between pairs of identical training sessions (i.e., between two saline or two nicotine training sessions). Tests will occur only if, in the two preceding training sessions, subjects meet the criteria of emitting 85% of responses on the injection-correct lever for both the first reinforcer

(first fixed ratio) and the total session. Test sessions will last for twenty minutes, or until twenty reinforcers have been obtained. If fewer than 3 rats respond after a dose of antioxidant, then data from that dose will not be used or presented on graphs.

Intraperitoneal injections (1 ml/kg) of vitamin C, apocynin, FSNY-1, hexamethonium, or their vehicle (0.9% saline), or vitamin E and its vehicle (2% methylcellulose), will occur 15 minutes prior to the start of the test session. Subcutaneous injections of the training dose of nicotine will occur 15 min prior to the start of the test session. A starting dose for each test drug will be determined based upon data from experimental literature and locomotor activity studies of the antioxidance performed previously in our laboratory. The starting doses and pretreatment times are shown in the table below. (please make a table 1). The dose ranges to be tested will include doses that are inactive to those that show biological activity as evidenced by a significant decrease in response rate. Mecamylyamine was tested for its ability to block nicotine

Preliminary Studies

Nicotine Discrimination. My current laboratory has previously developed preclinical assays for nicotine drug discrimination and self-administration as part of the National Institute on Drug Abuse (NIDA), Addiction Treatment Discovery Program (ATDP). Approximately 20 novel compounds have been evaluated in these assays against the standards of mecamylamine, bupropion, and varenicline. The drug discrimination studies have provided significant insight into the acute actions of these standard compounds. Fig.2 shows ability of the positive standard compounds to inhibit nicotine's discriminative stimulus effect (upper) or substitute for nicotine (lower panels) in different squads of nicotine-trained SD rats. Only mecamylamine has significant nicotine-blocking action in the discrimination assay, whereas bupropion and varenicline do not prevent correct identification of nicotine when presented acutely. On the other hand, both bupropion and varenicline have a significant nicotine-like action (substitution) when presented to nicotine-trained rats in the absence of nicotine. These results would seem to confirm the suggestion that the latter medications may at least partially mimic the CNS actions of nicotine. This action is not a property of the full nicotine antagonist mecamylamine, which did not substitute for nicotine. Both mecamylamine and bupropion significantly reduced the rate of responding for food reinforcement (data not shown) at doses that antagonized or substituted, predicting significant side effects at their effective doses. Inhibition of responding after varenicline occurred at doses higher than those needed for full substitution, suggesting a more favorable therapeutic window.

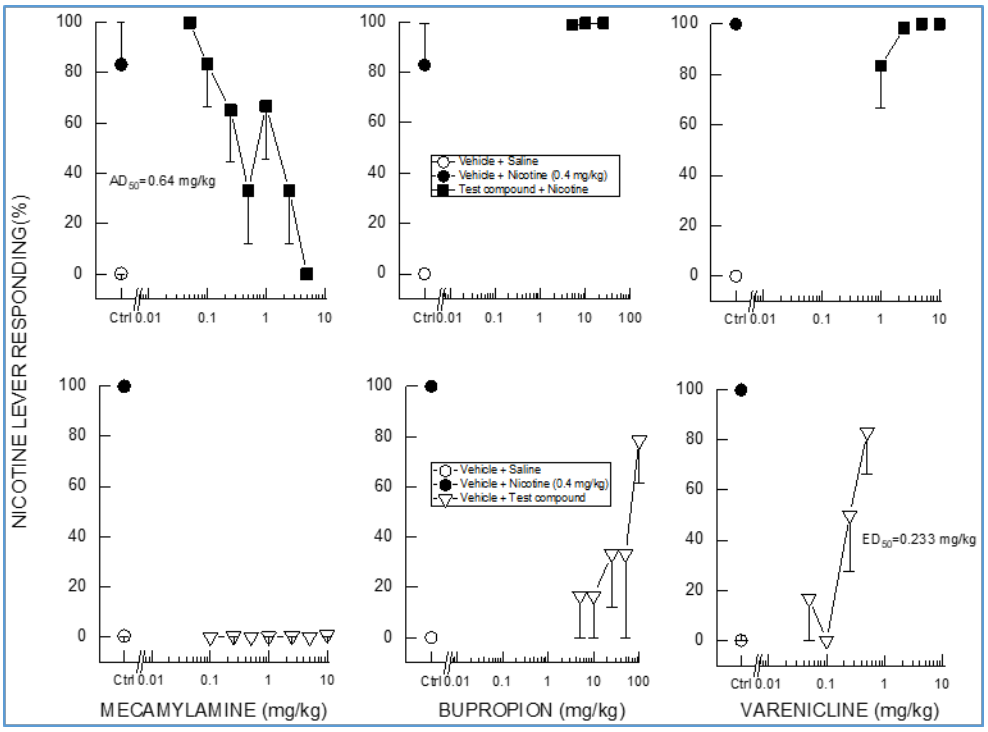

Fig.2. Positive control standards in the drug discrimination assay. Groups of 6 SD rats were trained to discriminate nicotine (0.4 mg/kg s.c.) from saline using an ATDP standard protocol1. Antagonism study data (upper panels left to right) show the % of responses on the nicotine-associated lever (+SEM) during sessions when the trained rats were pretreated with test compound at different doses and subsequently received nicotine prior to testing. Substitution study data (lower panels) show nicotine lever responding following pretreatment with the test compound in the absence of nicotine. Studies of bupropion and varenicline were discontinued after doses that reduced the response rate by 20% or greater (not shown).

Nicotine self-administration. Studies of self-administration confirm the drug discrimination outcomes. Self-administration of drugs by animal subjects is the hallmark preclinical test of potential antagonist or substitution-based medication efficacy. The self-administration assay allows our ATDP site to address the major aspects of nicotine addiction and assess efficacy of potential medications. The test compounds will also be administered to rats trained to lever press for food, to test whether the compounds will also suppress food-maintained behavior (or behavior in general) rather than specifically reducing nicotine self-administration. Our laboratory has completed evaluations for a number of potential smoking-cessation medications. The effects of the positive control standards mecamylamine, bupropion, and varenicline on nicotine intake are shown as a function of dose In Fig.3. Mecamylamine significantly diminished nicotine intake in the same dose range for which it inhibited nicotine discrimination, confirming dependence of nicotine intake on NN receptor stimulation. However, neither varenicline nor bupropion significantly affected responding for nicotine (Fig. 4A) when tested in doses for which there had been partial or full substitution in the discrimination assay. In separate companion studies, bupropion (but not mecamylamine or varenicline), significantly

inhibited responding for food. confirmed that treatment effectively decreased nicotine intake in two subsequent test sessions, suggesting a more subtle, extinction-like action of these standards, consistent with their ability to partially or fully substitute for nicotine. Our Lab has determined that varenicline can inhibit nicotine intake if the rats receive injections during a second and third test session, suggesting that the acute discrimination and self-administration tests are not useful for detecting substitution-based medications.

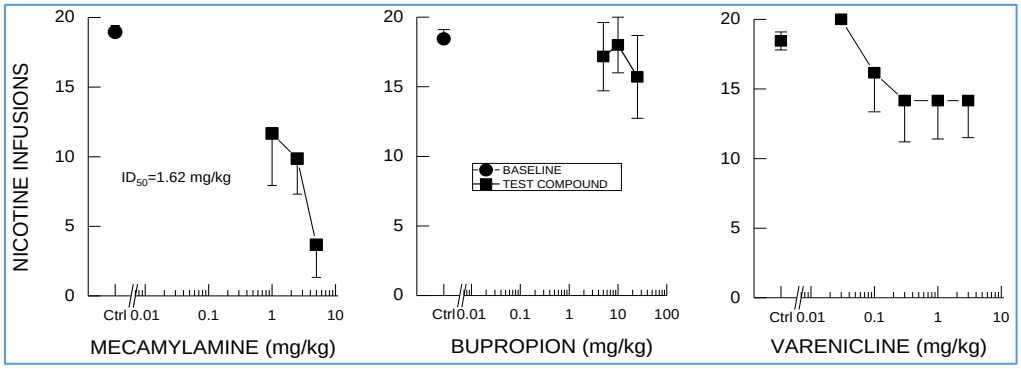

Fig.3. Effect of positive control standards in the rat nicotine self-administration assay. *(Left Panel)* Male Sprague-Dawley rats were trained to self-administer nicotine via a jugular vein catheter (0.03 mg/kg/infusion) using a fixed-ratio 1 (FR1) schedule of reinforcement until stable nicotine intake was obtained. During all sessions, rats were placed in self-administration test chambers and nicotine was available until 20 infusions had been obtained or 2 h had elapsed. Nicotine antagonism test sessions with different doses of mecamylamine, bupropion or varenicline (panels left to right) were conducted after a given rat had demonstrated stable nicotine intake during the two preceding sessions. The mean (baseline) nicotine infusions (+/- SEM) are shown to the left of the axis break, whereas data to the right represent independent groups of 6 rats tested at each dose. *(Right Panel)* Effect of varenicline (1 mg/kg) in a group of 6 rats when injected prior to testing on each of 3 daily sessions. * $p<0.05$ against baseline within RM-ANOVA.

Possible outcomes and interpretation

We will examine the potential outcomes of each of the tested drugs. First, if all the antioxidants have an antagonistic effect then it would be hard to argue against the effect's antioxidants have on ROS in the addiction pathway and their ability to block nicotine. If however, none of the drugs are able to antagonize, then we can assume there may be alternate routes of action for nicotine or the antioxidants do not antagonize each other enough for an observable effect. If vitamin C is effective what would that mean? It would mean that the antioxidants are either directly or indirectly interacting with the cytosolic side of cell membranes and preventing the actions of the nicotine pathway (13). Similarly, apocynin should also be able to antagonize in this situation because it too acts extracellularly as an NADPH oxidase inhibitor (16). In contrast to these two drugs, vitamin E and FSNY-1 would show that the antioxidants were able to act intracellularly to stop the nicotine pathway. Vitamin E acts on intracellular membrane transporters (2) and FNSY-1 acts on hydrogen peroxide hydroxide radicals produced

intracellularly (15). The four of these drugs, should they antagonize, would be able to give an indication as to where the mechanism of action is taking place. Should hexamethonium be able to antagonize, then it should have similar effects to the drug mecamylamine as they have the same mechanism of action (3). Both drugs act on nicotine acetylcholine receptors throughout the nervous system, but hexamethonium cannot enter the blood brain barrier (9). Should any of the tested drugs shows the ability to antagonize, the next step would be to find a dose that blocks but does not produce toxic effects, like mecamylamine does (10).

Potential pitfalls

Because of existing drugs like mecamylamine, we know it is possible to block the effects of nicotine (4). The procedures used, including the nicotine discrimination trials, are well established and should be able to show any effects if any exist. A potential pitfall may be that the drugs do antagonize but at doses that also elicit a toxic effect that masks the antagonization. We also know the effects of antioxidants on ROS and that ROS is a byproduct of the nicotinic addiction pathway (7).

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