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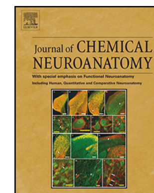

Altered neurochemical levels in the rat brain following chronic nicotine treatment

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ABSTRACT

Converging evidence shows that neurochemical systems are crucial mediators of nicotine dependence. Our present study evaluates the effect of 3-month chronic nicotine treatment on the levels of multiple quaternary ammonium compounds as well as glutamate and gamma aminobutyric acid in the rat prefrontal cortex, dorsal striatum and hypothalamus. We observed a marked decrease of acetylcholine levels in the dorsal striatum (22.88%, $p < 0.01$), reflecting the impact of chronic nicotine in local interneuron circuits. We found decreases of carnitine in the dorsal striatum and prefrontal cortex (19.44%, $p < 0.01$; 13.58%, $p < 0.01$, respectively), but robust enhancements of carnitine in the hypothalamus (26.59%, $p < 0.01$), which may reflect the alterations in food and water intake during chronic nicotine treatment. Finally, we identified an increase of prefrontal cortex glutamate levels (8.05%, $p < 0.05$), supporting previous studies suggesting enhanced prefrontal activity during chronic drug use. Our study shows that quaternary ammonium compounds are regulated in a highly brain region specific manner during chronic nicotine treatment, and provides novel insights into neurochemical regulation during nicotine use.

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Introduction

Nicotine is the main psychoactive component of tobacco and one of the most widely taken drugs of abuse (Laviolette and van der Kooy, 2004). The use of nicotine has a number of psychoactive effects including euphoria, reduced stress, anti-fatigue and enhanced cognitive functions (Benowitz, 2010). However, similar to other psychoactive drugs such as cocaine and alcohol, the repeated use of nicotine results in physical dependence, which is characterized by diverse aversive effects particularly associated with drug withdrawal. By acting on nicotinic acetylcholine receptors (nAChRs), which are distributed at presynaptic and postsynaptic sites in different types of neurons distributed in various brain areas (Albuquerque et al., 2009), nicotine regulates many pathways such as the reward and stress systems (Watkins et al., 2000). Chronic nicotine differentially affects the function of release-regulating nAChR subtypes (Grilli et al., 2005). So far, a growing body of evidence has demonstrated that chronic nicotine administration alters several neurochemical circuits, for example affecting glutamate and gamma aminobutyric acid (GABA) levels in the ventral tegmental area (VTA) (Changeux, 2010), glutamate levels in the prefrontal cortex (Shameem and Patel, 2012). The modulation of GABA release seems to be very important for the action of nicotine (Barik and Wonnacott, 2006; Zhu and Chiappinelli, 2002). Nicotine application also enhances the levels dopamine and opioid peptides in the striatum (Hadjiconstantinou and Neff, 2011), and the levels of several classes of neuropeptides in the hypothalamus (Chen et al., 2007; Lage et al., 2007; Li et al., 2000; Plaza-Zabala et al., 2012). By comparison, there is still much less known about the impact of chronic nicotine on quaternary ammonium substances, such as acetylcholine, choline, carnitine and acetylcarnitine, which are closely linked to cholinergic neuromodulation.

Acetylcholine and choline are both endogenous agonists of nAChRs. Choline is the precursor for intracellular acetylcholine synthesis and also its degeneration product after extracellular release. By binding to the nACHR, they can both compete with nicotine, thus affecting the activation and desensitization of nAChRs during chronic nicotine treatment (Dani and De Biasi, 2001). For example, previous *in vitro* studies in a striatal slice preparation showed that acute nicotine application resulted in the increased release of acetylcholine, while this increase could

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be attenuated if the animal was pre-treated with chronic nicotine (Yu and Wecker, 1994). The attenuation of acetylcholine release may be related to nAChR desensitization or the activation of presynaptic nAChRs on GABAergic interneurons that in turn inhibit activity of cholinergic striatum neurons. In addition to regulating nAChR density and sensitivity, the total amount of intra- and extracellular acetylcholine may also be regulated by chronic nicotine treatment, but such data are currently available neither for acetylcholine nor for choline.

Carnitine and acetylcarnitine are two quaternary ammonium substances that have structural similarities to choline and acetylcholine, respectively. Synthesis of acetylcholine and acetyl-L-carnitine is accomplished by coupled enzyme systems choline acetyltransferase and carnitine acetyltransferase (White and Scates, 1990). Due to this structural similarity, carnitine and acetylcarnitine can have an impact on cholinergic neurotransmission, while also exhibiting neuroprotective properties (Imperato et al., 1989; Picconi et al., 2006; Ricny et al., 1992). Importantly, carnitine and acetylcarnitine are also critical substances involved in fatty acid metabolism (Jones et al., 2010). Fatty acid metabolism is a pathway affected by nicotine treatment, as has been shown in peripheral tissue (Cryer et al., 1976; Hellerstein et al., 1994). Despite the apparent links of carnitine and acetylcarnitine to the cholinergic system, little is known about whether their levels are regulated in the brain during chronic nicotine treatment.

Our study aimed to evaluate the effects of chronic nicotine treatment on the changes of these quaternary neurochemicals as well as glutamate and GABA as reference, which are fundamental excitatory and inhibitory amino acid neurotransmitter in the brain. Based on the types of cholinergic projections, we selected three representative brain areas, namely prefrontal cortex (PFC), dorsal striatum (DS), and hypothalamus (HT), to study the impact of chronic nicotine on the target neurochemicals. PFC receives cholinergic project from basal forebrain, HT receives cholinergic projection from brain stem, while DS has its own cholinergic interneurons in the absence of cholinergic innervations from other brain areas (Woolf, 1997). PFC, DS and HT areas are all important brain areas involved in drug dependence (Koob and Volkow, 2010). We used high accuracy mass spectrometry (MS) to monitor the levels of acetylcholine, choline, carnitine, acetylcarnitine, glutamate and GABA. Differential analysis revealed that each neurochemical was altered in at least one brain region. The results revealed the specific decrease of acetylcholine, carnitine and acetylcarnitine in DS, increase of glutamate and decrease of carnitine in PFC, increase of choline, carnitine and GABA in HT. Our study provides novel insights into the involvement of quaternary ammonium compounds in neurochemical distortions caused by chronic nicotine treatment, advancing our understanding of neurochemical mechanisms of drug addiction.

Materials and methods

Reagents

Choline chloride, acetylcholine chloride, carnitine hydrochloride, glutamic acid, GABA and acetylcarnitine hydrochloride were purchased from Sigma–Aldrich (St. Louis, MO, USA). Choline-d9 chloride (Sigma–Aldrich), acetylcholine-d4 chloride (Medical Isotopes Inc., Pelham, NH, USA), carnitine-d3 hydrochloride, acetylcarnitine-d9 hydrochloride, GABA-d6 and glutmamate-d5 (Cambridge Isotope Laboratories Inc., Andover, MA, USA), were used as internal standards. LC–MS grade formic acid, ammonium hydroxide solution; methanol and acetonitrile were supplied by Sigma–Aldrich. Water was obtained from a GenPure water system (TKA, Niederelbert, Germany).

Animals and sample preparation

Long-Evans rats (*Rattus norvegicus*) were used in this study ($n = 20$). The animals were housed under constant temperature and humidity with free access to food and water. The population was divided into a drug and a control group with 10 animals each. The nicotine administration protocol was adapted from previous research performed on mice or monkeys (Oddo et al., 2005; Quik et al., 2006). Drinking solution for the control group consisted of 1% solution of saccharine and was given to the group for 13 weeks. Animals in the drug group received a gradient dose of nicotine in drinking water that also contained 1% saccharine. Animals were started with 10 μg/ml nicotine for the first week, then 25 μg/ml nicotine for the second and third weeks and finally 50 μg/ml for 10 weeks. This profile was used to adapt animals to the drinking water containing nicotine. Water consumption was monitored on a daily basis for both groups. During the final week, the average water consumption was 23 ml for nicotine group rats, and 35 ml for control rats. Average nicotine intake during the last week of administration was thus 1.5 mg/kg/day. The average weight of nicotine rats at the end of the administration period was 393 g, 11 g less than the average weight of control rats (unpaired *t*-test, $p < 0.01$). The handling of the animals and the experimental procedures were approved by the veterinary office of Fribourg, Switzerland and in full compliance with applicable European Union veterinary directives. Animals were sacrificed by decapitation after anesthetization with ketamine (100 mg/kg, Streuli Pharma AG, Uznach, Switzerland). The three brain areas of interest were afterwards stored at −80 °C prior to dissection. The dissections of HT, DS and PFC were performed carefully from each hemisphere under microscope following their marginal lines according to the adult rat atlas. First the olfactory bulb was removed, then the PFC was cut away by a frontal section about 2 mm from the frontal pole. The rest of the hemisphere was cut in frontal sections using a razor blade. From 1.5 to 2 mm sections, the areas of interest (DS and HT) were trimmed using a miniature scalpel blade. HT including the anterior, tuberal and posterior areas was dissected. The structures were identified on frontal sections based on different colors of the structures (cortex, white matter and nuclei). All the operations were performed on dry-ice cooled glass plate (around −10 °C) to protect the decay of tissues.

Sample pre-treatment

The tissue of each brain area was collected from the left hemispheres. The extraction method has been described elsewhere (Falasca et al., 2012). Briefly, tissue (21 mg) was spiked with 30 μl of a mixed internal standard spiking solution. The tissues were then homogenized using a Precellys 24 homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France), with 120 μl ice-cold acetonitrile containing 0.3% formic acid. The homogenates were centrifuged at 22,000 × *g* for 20 min at 4 °C. The supernatants were collected and filtered by 0.20 μm filter membranes (Millex-LG, Millipore, Billerica, MA, USA). 15.6 μl of the filtered supernatants were diluted with 89.4 μl of 85% acetonitrile containing 0.3% formic acid. The concentrations of acetylcholine-d4, choline-d9, carnitine-d3, acetylcarnitine-d9, GABA-d6 and glutamate-d5 in final tissue extracts were 0.3 μM, 4 μM, 1 μM, 1 μM, 15 μM and 50 μM, respectively.

LC–FT–MS analysis

In this study, we employed a capillary LC system (Eksigent, Dublin, CA, USA) coupled to a LTQ-Orbitrap Discovery mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a nano-electrospray ion source operating in positive

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