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Effects of nicotine and chlorisondamine on cerebral glucose utilization in immobilized and freely-moving rats

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1 Chlorisondamine blocks central nicotinic receptors for many weeks *via* an unknown mechanism. Intracerebroventricular administration of [^{3}H]-chlorisondamine in rats results in an anatomically restricted and persistent intracellular accumulation of radioactivity. The initial aim of the present study was to test whether nicotinic receptor antagonism by chlorisondamine is also anatomically restricted.

2 Male adult rats were pretreated several times with nicotine to avoid the disruptive effects of the drug seen in drug-naïve animals. They then received chlorisondamine (10 μg i.c.v.) or saline, and local cerebral glucose utilization (LCGU) was measured 4 weeks later after acute nicotine (0.4 mg kg^{-1} s.c.) or saline administration. During testing, rats were partially immobilized. Nicotine significantly increased LCGU in the anteroventral thalamus and in superior colliculus. Chlorisondamine completely blocked the first of these effects. Chlorisondamine significantly reduced LCGU in the lateral habenula, substantia nigra pars compacta, ventral tegmental area, and cerebellar granular layer.

3 The second experiment was of similar design, but the rats were not pre-exposed to nicotine, and were tested whilst freely-moving. Acute nicotine significantly increased LCGU in anteroventral thalamus, superior colliculus, medial habenula and dorsal lateral geniculate. Overall, however, nicotine significantly decreased LCGU. Most or all of the central effects of nicotine on LCGU were reversed by chlorisondamine given 4 weeks beforehand.

4 These findings suggest that chlorisondamine blocks nicotinic effects widely within the brain. They also indicate that in freely-moving rats, nicotine can reduce or stimulate cerebral glucose utilization, depending on the brain area.

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Abbreviations: CHL, chlorisondamine; 2-DG, 2-deoxyglucose; LCGU, local cerebral glucose utilization

Introduction

Chlorisondamine (CHL) is a bisquaternary nicotinic receptor blocker (Grimson *et al*., 1955; Plummer *et al*., 1955; Schneider & Moore, 1955; Stone *et al*., 1958). After systemic administration, it blocks ganglionic nicotinic receptors only temporarily (Grimson *et al*., 1955; Plummer *et al*., 1955; Schneider & Moore, 1955; Clarke *et al*., 1994), and its effect on nicotinic receptors of the neuromuscular junction is negligible (Grimson *et al*., 1955; Plummer *et al*., 1955). However, when administered intracerebroventricularly or in a high systemic dose, CHL exerts a remarkably persistent (several weeks) block of central nicotinic responses, examined either behaviourally or *in vitro* (Clarke, 1984; Reavill *et al*., 1986; Fudala & Iwamoto, 1987; Kumar *et al*., 1987; Mundy & Iwamoto, 1988; Jaffe, 1990; Corrigall *et al*., 1992; Brioni *et al*., 1993; Clarke *et al*., 1994; Decker *et al*., 1994; El-Bizri & Clarke, 1994a,b; El-Bizri *et al*., 1995; Reuben *et al*., 1998).

The mechanism by which CHL exerts its long-lasting blockade of nicotinic receptors in the CNS remains elusive (Clarke *et al*., 1994; El-Bizri & Clarke, 1994a–c; Reuben *et al*., 1998). However, we have observed that the i.c.v. administration of [^{3}H]-CHL to rats results in a persistent and selective accumulation of radioactivity in the substantia nigra pars compacta, ventral tegmental area, dorsal raphé, locus coeruleus and in the granular layer of the cerebellum (El-Bizri *et al*., 1995; Reuben *et al*., 1998). In these areas, appreciable radioactivity was still present 12 weeks after [^{3}H]-CHL administration. These findings, together with evidence for intraneuronal retrograde transport, led us to propose that the intracellular accumulation of CHL (or a pharmacologically-active metabolite) may, by some unknown mechanism, underlie its long-lasting blockade of nicotinic receptors (El-Bizri *et al*., 1995).

The initial aim of the present study was therefore to determine whether CHL produces a persistent blockade of central nicotinic receptors *in vivo* only in regions where the drug (or metabolite) is accumulated in high concentrations. To achieve this, local cerebral glucose utilization (LCGU) was quantified using the [^{14}C]-2-deoxy-D-glucose ([^{14}C]-2-DG) method of Sokoloff *et al*. (1977). LCGU is reported to be increased in a number of brain nuclei after subcutaneous or intraperitoneal administration of behaviourally-relevant doses of nicotine (London *et al*., 1985; 1988a,b; McNamara *et al*., 1990). Most of these effects appeared to be blocked by mecamylamine, a centrally-acting nicotinic antagonist (London *et al*., 1988a,b). Importantly, the anatomical pattern of activation matches quite closely the distribution of [^{3}H]-nicotine binding sites (Clarke *et al*., 1985; London *et al*., 1985b), suggesting that most increases in LCGU in response to nicotine result from a drug action which is both local and mediated by $\alpha4\beta2$ nAChRs (Whiting & Lindstrom, 1987; Zoli *et al*., 1998; Marubio *et al*., 1999).

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The primary purpose of this study was therefore to test the prediction that CHL would block the stimulatory effect of nicotine on LCGU only in regions where CHL is accumulated in high amounts. The first experiment, however, revealed unexpectedly modest nicotine-associated increases in LCGU. A second experiment was then performed using a procedure which permitted LCGU determinations in freely-moving animals.

Methods

Subjects

Male Sprague-Dawley rats (Charles River, St. Constant, Quebec, Canada), weighing 150–175 g were maintained on a 12/12 h light-dark cycle. Food and water were available *ad libitum*. Animals were allowed 4–6 days for acclimatization before use. The protocol for these experiments was approved by the University Animal Care Committee.

Design of experiments

Both experiments comprised the same 2×2 design in which rats were randomly allocated to four groups. Depending on group, each rat received an i.c.v. infusion of CHL (10 μg) or saline, followed 24–32 days later by an s.c. challenge with nicotine (0.4 mg kg^{-1}) or saline. Each group comprised eight rats (Experiment 1) or eight to nine rats (Experiment 2). In Experiment 1, rats were initially rendered tolerant to the behaviourally disruptive effects of nicotine by receiving injections of nicotine (0.4 mg kg^{-1} s.c., as base) in their home cages, twice daily for three consecutive days (Louis & Clarke, 1998). Intracerebroventricular infusions were given 1 week later. In Experiment 2, rats were not pretreated with subchronic nicotine.

Drugs

Drugs were as follows: 2-[1-^{14}C]-deoxy-D-glucose ([^{14}C]-2-DG) (specific activity: 51–57 mCi mmol^{-1}; New England Nuclear, Boston, MA, U.S.A.), buprenorphine hydrochloride (Temgesic; Reckitt & Colman Pharmaceuticals, Hull, England), ketamine hydrochloride and xylazine hydrochloride (Research Biochemicals International, Natick, MA, U.S.A.), (−)-nicotine hydrogen tartrate salt (Sigma Chemical Co., St. Louis, MO, U.S.A.), and Na pentobarbital (MTC Pharmaceuticals, Cambridge, Ontario, Canada). Chlorisondamine dichloride (CHL) was a gift of Novartis (Summit, NJ, U.S.A.). All drugs, except for buprenorphine hydrochloride and [^{14}C]-2-DG (which were in aqueous solution), were dissolved in 0.9% saline solution (1 ml kg^{-1} s.c.). (−)-Nicotine hydrogen tartrate solution was neutralized to pH 7.2 with NaOH. Doses of CHL and (−)-nicotine hydrogen tartrate refer to the free base of the compound; doses of buprenorphine hydrochloride, ketamine hydrochloride and xylazine hydrochloride are expressed as the salts.

Administration of CHL

Chlorisondamine and saline were administered by stereotaxic infusion, essentially as previously described (El-Bizri *et al*., 1995). In every pair of days, one rat from each group was operated on, in random order. Rats were anaesthetized with ketamine hydrochloride (125 mg kg^{-1} i.p.) and xylazine hydrochloride (10 mg kg^{-1} i.p.) in Experiment 1 and with Na pentobarbital (60 mg kg^{-1} i.p.) in Experiment 2. The appropriate level of anaesthesia was verified by lack of withdrawal response to a foot pinch. Rats were then mounted in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, U.S.A.). They received bilateral i.c.v. infusions of either CHL (5 μg base per side in 2 μl 0.9% saline) or vehicle *via* stainless steel 30-gauge cannulae connected, *via* PE 10 tubing, to a syringe pump (Sage model 341B; Orion Research Incorporated, Boston, MA, U.S.A.). The stereotaxic coordinates of the cannulae tips were: A_{Bregma} +0.5, $L_{IA} \pm 1.0$/$L_{Bregma} \pm 1.0$, V_{Bregma} −4.6, with the toothbar set 3.5 mm below the ear bars. These coordinates were verified by infusion of China ink (Staedtler, Germany) in additional rats. The infusion rate was 1.1–1.6 μl min^{-1}. Cannulae were retracted 5 min after the end of the infusion. The scalp was sutured and treated with 4% furazolidone antibacterial powder (Topazone; Austin Laboratories, Joliette, Quebec, Canada), and the analgesic buprenorphine hydrochloride (0.1 mg kg^{-1} s.c.) was given.

In each experiment, the presence of long-term CNS block by CHL was confirmed in additional, weight-matched but drug-naïve rats which received comparable infusions of the same solution of CHL or saline (n=4–5). These subjects were tested 4 weeks later for the presence or absence of prostration and ataxia in the first 10 min following an injection of nicotine (1 mg kg^{-1} s.c.) as previously described (El-Bizri *et al*., 1995).

Procedure for [^{14}C]-2-DG infusion

Rats were prepared for [^{14}C]-2-DG infusion as follows. Each day, two rats were implanted with indwelling catheters (PE 50 tubing, 22.5 cm in length) in the left femoral artery and vein under halothane anaesthesia. The level of anaesthesia was ascertained by foot pinch. A sterile topical anaesthetic (2% lidocaine chloride jelly U.S.P.; Xylocaine, Astra Pharma Inc., Mississauga, Ontario, Canada) was applied to the open wound before suturing.

In Experiment 1, the Sokoloff method was employed (Sokoloff *et al*., 1977). Each rat was partially immobilized in a plaster cast that covered the lower half of their body so that their tail, hindlimbs, and the catheters protruded outside the cast. With the rat in a horizontal position, the cast was taped to a lead brick, and the hindlimbs and tail were loosely taped to the brick to prevent escape from the cast. The rats were allowed 2 h to recover from anaesthesia in a ventilated Styrofoam container (to attenuate sound) before drug treatments. The experiment was performed in a room with minimal ambient sound. Throughout the surgery and during the 2 h recovery period, body temperature was maintained at 37°C±1°C with a homeothermic blanket control unit (Harvard Apparatus Canada, Saint-Laurent, Quebec, Canada).

For Experiment 2, a new method was developed that enabled LCGU determinations using freely-moving animals. The arterial and venous catheters were threaded subcutaneously through a small incision at the nape of the neck; 30 cm of each catheter was external to the animal. They were filled with heparinized saline (200 U ml^{-1}) and plugged with 1 cm lengths of stainless steel wire. Rats were permitted to recover for 4–5 h in their home cage with *ad libitum* access to food and water, and were placed in the Styrofoam container 15 min before drug challenge. Body temperature was not monitored.

At the end of the recovery period in both experiments, rats were injected in the flank with nicotine (0.4 mg kg^{-1} s.c.) or 0.9% saline, followed 2 min later by the i.v. infusion of [^{14}C]-2-DG. This 2 min interval was chosen in order to maximize the effect of nicotine on LCGU, which is both time- and dose-dependent (London *et al.*, 1988a).

Determination of local cerebral glucose utilization

LCGU was measured according to the autoradiographic [^{14}C]-2-DG method of Sokoloff *et al.* (1977). The radiotracer [^{14}C]-2-DG was infused *via* the intravenous catheter over 2 s at a dose of 60 μCi kg^{-1}. Following the infusion of the radiotracer, arterial blood samples were collected at 1, 2, 3, 5, 10, 15, 30 and 45 min (Experiment 1) or at 1, 3, 7, 15, 30 and 45 min (Experiment 2). Samples were of 150 μl except at 45 min, when 500 μl was collected. Samples were centrifuged at 12,000 *g* for 5 min to isolate the plasma. The plasma samples were immediately stored at -80°C for subsequent measurement of [^{14}C]-2-DG (Wallac 1410 liquid scintillation counter, LKB, Sweden). Glucose concentrations were determined in 5 μl aliquots of plasma using a colorimetric assay (Glucose 100 Trinder single reagent system, Sigma Chemical Co., St. Louis, MO, U.S.A.).

In Experiment 1, the following additional parameters were measured 30 min before, and again 30 min after the infusion of [^{14}C]-2-DG: mean blood pressure, heart rate, arterial blood pH, arterial pCO_2, arterial pO_2, and hematocrit. Blood pressure was measured with a BP-1 pressure monitor equipped with a blood pressure transducer (World Precision Instruments, Inc., Sarasota, FL, U.S.A.). Heart rate was calculated from blood pressure fluctuations recorded on a chart recorder (Spectra-Source, Inc., San José, CA, U.S.A.). Arterial blood pH, pCO_2, and pO_2 were determined using a Corning 170 pH/Blood Gas Analyser (Ciba-Corning Canada Inc., Markham, Ontario, Canada). In Experiment 2, vital signs were not monitored since the animals were freely moving; only hematocrit was measured.

Forty-five minutes after infusion of the radiotracer, the rats were injected intravenously with general anaesthetic and decapitated 1 min later. Brains were removed immediately after death and immersed in isopentane for 30 s at -50°C and stored at -80°C. Coronal brain sections (20 μm thick) were cut at -16°C in a cryostat (Frigocut model 2700; Reichert-Jung, Nubloch, Germany). Three adjacent sections were taken at each of seven anterior-posterior levels (10.6, 7.6, 5.2, 4.45, 3.4, 1.2 and -1.0 mm from interaural zero). Brain sections were thaw-mounted onto gelatin-coated glass microscope slides and dried on a slide warmer at 50°C for less than 1 min. They were then placed tightly against [^{14}C]-sensitive film (Kodak Biomax MR-2, Amersham, Oakville, Ontario, Canada) along with Carbon-14 standards (tissue equivalent values: 31 – 1017 nCi g^{-1} wet tissue; American Radiolabeled Chemicals, Inc., St. Louis, MO, U.S.A.) for 1 week at room temperature. Films were processed at room temperature in Kodak D19 developer and fixed in Kodak rapid fixer (without hardener). The same brain sections were then Nissl-stained with thionin, digitally aligned with the corresponding film image, and compared with the atlas of Paxinos & Watson (1986), to identify the brain regions of interest. Rates of LCGU were determined, using a computer-assisted imaging device (MCID-M4; Imaging Research Inc., St. Catherine's, Ontario, Canada), from autoradiographic measures of tissue radioactivity, the time course of [^{14}C]-2-DG in arterial plasma, and the arterial glucose concentration (Sokoloff *et al.*, 1977).

Data analysis

Statistics were performed using commercial software (Systat, Evanston, IL, U.S.A.). Probability values are for two-tailed tests. Two-way ANOVA was used to examine the main effects and interaction between the two between-subject factors, CHLORIS (i.e., CHL *vs* saline pretreatment) and NIC (i.e., nicotine *vs* saline treatment). Where the CHL × NIC interaction term was not statistically significant, the main effects of nicotine or CHL are given; where the interaction was significant, the effects of nicotine alone or CHL alone were determined by independent Student's *t*-tests.

A restricted number of brain areas was sampled in order to reduce the possibility of type I (false positive) statistical errors. Most brain areas selected for sampling have been shown to be stimulated by an acute systemic injection of nicotine (see Discussion). Table 1 lists the brain regions and corresponding atlas levels for which LCGU was determined.

Results

In both experiments, behavioural tests of central nicotinic blockade demonstrated that the chlorisondamine-pretreated rats were completely protected from nicotine-induced prostration and ataxia.

Experiment 1 Local cerebral glucose utilization in immobilized rats

In order to facilitate comparisons across brain areas and experiments, LCGU data are shown as a percentage of the saline/saline group (Figure 1). The absolute values for this group are given in Table 1. Nicotine significantly increased LCGU in only two of the brain regions sampled: the anteroventral thalamic nucleus (t = 5.71, d.f. 14, $P<0.0001$) and the superior colliculus (superior grey layer) (F = 30.7, d.f. 1, 28, $P<0.0001$) (Figure 1). In other brain regions, nicotine appeared to have little or no effect (Figure 1). In the anteroventral thalamus, there was a significant CHL × NIC interaction (F = 5.62, d.f. 1, 28, $P<0.025$), and the effect of nicotine was completely blocked (Figure 1). In the superior colliculus, CHL appeared to reduce the nicotine effect, but the CHL × NIC interaction did not reach significance (F = 3.36, d.f. 1, 28, $P<0.08$) (Figure 1).

CHL significantly reduced LCGU in the lateral habenula (F = 6.25, d.f. 1, 28, $P<0.05$), substantia nigra pars compacta (F = 5.17, d.f. 1, 28, $P<0.05$), ventral tegmental area (F = 6.21, d.f. 1, 28, $P<0.05$), granular layer of the cerebellum (F = 4.47, d.f. 1, 28, $P<0.05$), and superior colliculus (F = 8.32, d.f. 1, 28, $P<0.01$). In the first four areas, the depressant effect of CHL appeared to occur whether nicotine was present or absent. In the superior colliculus, however, CHL, in the absence of nicotine, did not exert a discernible depressant effect.

A global measure of LCGU was obtained by determining the mean value of LCGU across entire brain sections at all six brain levels sampled in each rat; LCGU values were weighted by cross-sectional area (Figure 1, lower right panel). ANOVA revealed no significant main effects of nicotine or CHL and no significant interaction ($P>0.4$ for each).

Table 2 shows blood pressure, heart rate, acid-base status, hematocrit, and glucose levels for the four groups of rats. Neither CHL nor nicotine had any significant effect on any of these physiological and biochemical parameters. In addition, CHL pretreatment had no effect on rat weight gain up to the day of the [^{14}C]-2-DG infusion (t = 0.65, d.f. 30, $P>0.5$). The mean ± s.e.mean weight gain of the i.c.v. saline-pretreated rats and the i.c.v. CHL-pretreated rats was 157 ± 6 g and 162 ± 5 g, respectively.

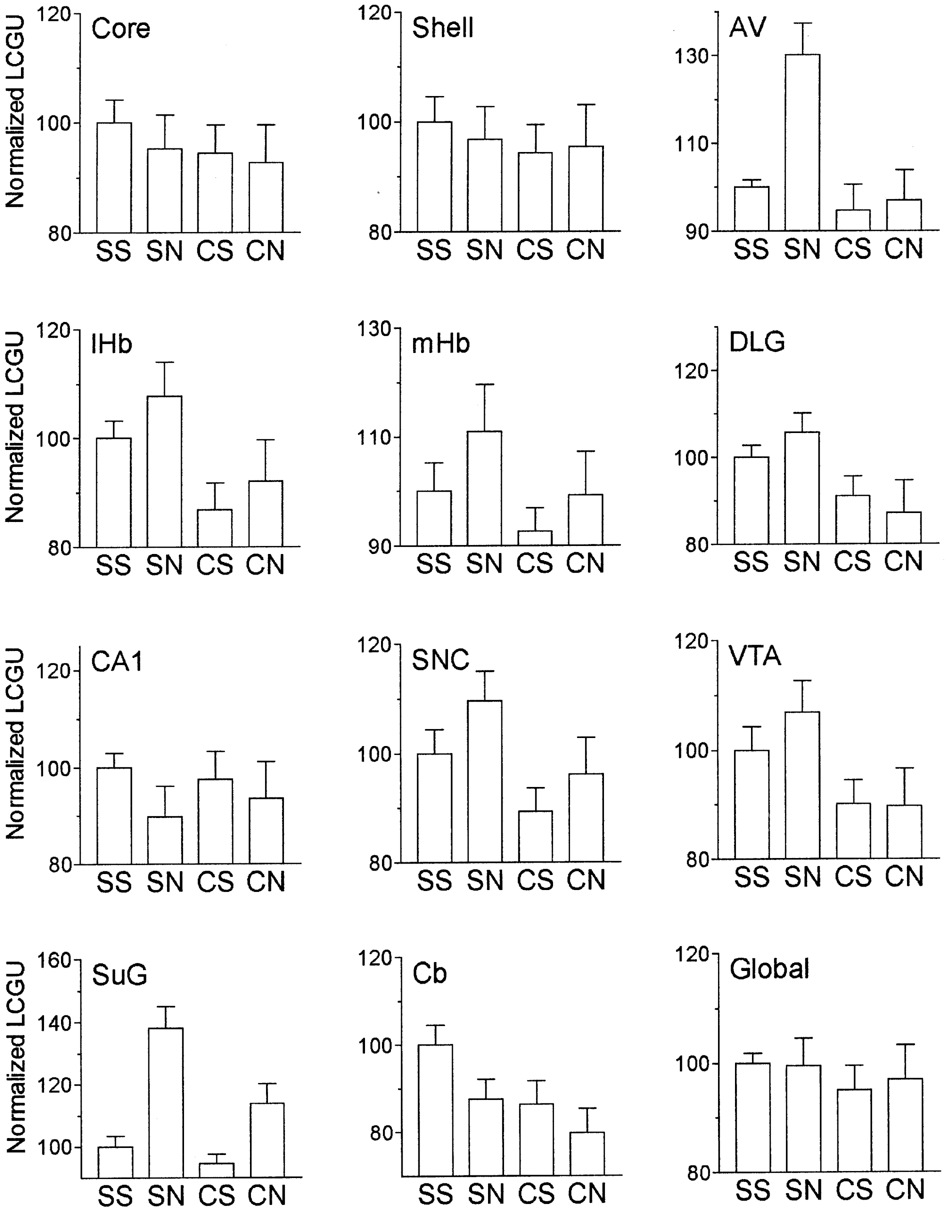

Figure 1 Effects of chlorisondamine (CHL) and nicotine on local cerebral glucose utilization (LCGU) in immobilized rats. Data are from Experiment 1. Rats received six spaced doses of nicotine 0.4 mg kg^{-1} s.c. and were subsequently pretreated with CHL 10 μg i.c.v. (or saline). LCGU was determined 4 weeks later, after injection of nicotine 0.4 mg kg^{-1} s.c. (or saline). The vertical axis represents the mean (±s.e.mean) LCGU, calculated as a percentage of the saline/saline group. Anatomical abbreviations: core, nucleus accumbens core; AV, anteroventral thalamic nucleus; CA1, field CA1 of hippocampus; Cb, granular layer of cerebellum; DLG, dorsal lateral geniculate nucleus; LHb, lateral habenula; MHb, medial habenula; shell, nucleus accumbens shell; SNC, substantia nigra pars compacta; SuG, superficial grey layer of the superior colliculus; VTA, ventral tegmental area. Nicotine significantly increased LCGU only in AV and SuG; a statistically significant CHL × NIC interaction occurred only in AV. CHL significantly lowered LCGU in lHb, SNC, VTA, SuG, and Cb (ANOVA main effect).

Experiment 2 Local cerebral glucose utilization in freely-moving rats

Both increases and decreases in LCGU were seen in response to nicotine (Figure 2). Increases occurred in four brain areas: anteroventral thalamus (F=6.69, d.f. 1, 31, $P<0.025$), superior colliculus (F=23.6, d.f. 1, 31, $P<0.0001$), medial habenula (F=4.32, d.f. 1, 31, $P<0.05$) and dorsal lateral geniculate nucleus (F=5.76, d.f. 1, 31, $P<0.05$). In all four areas (including anteroventral thalamus), the CHL × NIC interaction term was nonsignificant (F<0.4, d.f. 1, 31, $P>0.5$ for each).

In four other areas, nicotine decreased LCGU in the absence of CHL (Figure 2): nucleus accumbens core and shell, lateral habenula and hippocampal CA1 region (t=2.38–2.57, d.f. 15, $P<0.05$ for each). In each of these areas, CHL blocked the inhibitory effect of nicotine, and the CHL × NIC interaction was significant: core (F=8.21, d.f. 1, 31, $P<0.01$), shell (F=8.37, d.f. 1, 31, $P<0.01$), lateral habenula (F=5.38, d.f. 1, 31, $P<0.05$), and CA1 (F=6.78, d.f. 1, 31, $P<0.025$). In these areas, and in the cerebellar granule cell layer, there was a hint of a stimulatory effect of nicotine in the presence of CHL. CHL alone exerted no significant effects in this experiment.

Table 1 Local cerebral glucose utilization (mean ± s.e.mean) for saline/saline control groups in Experiments 1 and 2

Brain region	*AP level**	*Experiment 1* (μmol 100 g^{-1} min^{-1})	*Experiment 2* (μmol 100 g^{-1} min^{-1})
Core	10.6	80.3 ± 3.3	89.7 ± 2.8
Shell	10.6	80.2 ± 3.7	84.7 ± 2.0
AV	7.6	93.1 ± 1.5	113.2 ± 5.9
LHb	5.2	98.3 ± 3.2	108.3 ± 3.3
MHb	5.2	73.7 ± 3.9	90.6 ± 3.9
CA1	4.45	74.5 ± 2.2	67.0 ± 2.6
DLG	4.45	85.7 ± 2.4	89.8 ± 2.9
SNC	3.4	74.4 ± 3.3	86.1 ± 2.7
VTA	3.4	72.0 ± 3.1	80.5 ± 2.5
SuG	3.0	83.9 ± 2.8	89.3 ± 5.4
Cb	−1.0	72.9 ± 3.3	98.3 ± 4.1

*Millimetres anterior to interaural zero (Paxinos & Watson 1986). Abbreviations: core, nucleus accumbens core; AV, anteroventral thalamic nucleus; CA1, field CA1 of hippocampus; Cb, granula layer of cerebellum; DLG, dorsal lateral geniculate nucleus; LHb, lateral habenula; MHb, medial habenula; shell, nucleus accumbens shell; SNC, substantia nigra pars compacta; SuG, superficial grey layer of the superior colliculus, VTA, ventral tegmental area. n = eight rats per group in each experiment.

Table 2 Physiological and biochemical parameters (mean ± s.e.mean. $n = 8-9$)

	Sal/Sal	*Sal/Nic*	*CHL/Sal*	*CHL/Nic*
Experiment 1				
Blood Pressure (mm Hg)	118 ± 3	117 ± 1	118 ± 2	119 ± 2
Heart rate (beats min^{-1})	430 ± 19	441 ± 13	427 ± 13	422 ± 13
pH	7.45 ± 0.01	7.45 ± 0.01	7.46 ± 0.01	7.48 ± 0.03
pCO_2 (mm Hg)	40.5 ± 1.5	40.5 ± 1.1	39.7 ± 0.9	40.7 ± 1.4
pO_2 (mm Hg)	85.5 ± 1.3	88.9 ± 3.3	84.6 ± 1.1	83.8 ± 2.8
Hematocrit (%)	51.9 ± 0.6	51.4 ± 0.7	51.5 ± 0.9	51.1 ± 1.0
Glucose (mg dl^{-1})	198 ± 5	212 ± 8	204 ± 9	188 ± 12
Experiment 2				
Hematocrit (%)	54.4 ± 0.4	55.3 ± 0.3	55.1 ± 0.9	56.8 ± 0.8
Glucose (mg dl^{-1})*	165 ± 5	182 ± 8	167 ± 5	168 ± 3
Glucose (mg dl^{-1})+	159 ± 7	187 ± 9	164 ± 7	165 ± 3

*Mean of samples taken throughout the [^{14}C]-2-DG procedure.
+Mean of samples taken at 7, 15 and 30 min.

A global measure of LCGU was obtained as in Experiment 1 (Figure 2, lower right panel). ANOVA revealed a significant CHL × NIC interaction (F = 7.34, d.f. 1, 31, $P < 0.025$). Nicotine alone decreased LCGU by 11% (t = 2.82, d.f. 15, $P < 0.025$). In the presence of CHL, nicotine tended to increase LCGU but this was not significant (t = 1.72, d.f. 16, $P > 0.1$). CHL alone had no significant effect ($P > 0.4$).

Table 2 shows plasma glucose concentrations and hematocrit for the four groups of rats. Nicotine affected glucose levels in a time-dependent manner (NIC × TIME: F = 3.22, d.f. 5, 155, $P < 0.01$), reflecting an increase occurring at the 7, 15 and 30 min collection times. Within this time frame, the nicotine effect appeared to be blocked by CHL pretreatment (Table 2), but the CHL × NIC interaction term was not significant (F = 4.00, d.f. 1, 31, $P = 0.054$). CHL pretreatment had no effect on hematocrit or on weight gain up to the day of the [^{14}C]-2-DG infusion. The mean ± s.e.mean weight gain of the i.c.v. saline-pretreated rats and the i.c.v. CHL-pretreated rats was 152 ± 11 g and 145 ± 8 g, respectively (t = 0.60, d.f. 32, $P > 0.5$).

Discussion

The main findings of this study are as follows. In immobilized, nicotine-experienced rats, nicotine increased LCGU in only two areas sampled. In freely-moving, drug-naïve animals, nicotine increased LCGU in several brain regions, but the predominant effect of nicotine was inhibitory. Pretreatment with CHL blocked some but not all of the effects of nicotine. CHL appeared to reduce LCGU independently of nicotine in certain brain regions; such an effect was only seen in the immobilized preparation.

Effects of nicotine alone

In our first experiment, nicotine significantly increased local cerebral glucose utilization (LCGU) only in the anteroventral thalamic nucleus and superficial grey layer of the superior colliculus. The responses in these two areas (30 and 38% increase, respectively) were considerably smaller than those seen in most previous studies (35–92% and 41–116%, respectively) (London *et al*., 1985; 1988a,b; 1990; McNamara *et al*., 1990). In addition, we found no clear effect in several areas (medial habenula, dorsal lateral geniculate, substantia nigra pars compacta, and ventral tegmental area) where appreciable increases (typically 30% or more) were reported previously after acute s.c. or i.p. administration of nicotine (London *et al*., 1985; 1988a,b; 1990; McNamara *et al*., 1990). Effects of such magnitude would have been readily detectable, given the within-group variances and relatively large sample sizes of the present study.

The modest effects of nicotine seen in the first experiment were unexpected, particularly since we used the same general method and a dose comparable to that in published studies. In attempting to account for this discrepancy, the potential contribution of two factors could not be excluded: nicotine pre-exposure and stress. In the first experiment, rats were given several spaced injections of nicotine at least 4 weeks in advance of testing, in order to produce long-term tolerance to the behavioural disruption (e.g. ataxia) seen in drug-naïve animals (Stolerman *et al*., 1973; Clarke, 1987). Such spaced dosing with nicotine results in little if any persistent receptor alteration (Ksir *et al*., 1985), and tolerance appears restricted to the disruptive effects of the drug (Clarke, 1987). In contrast, reports of tolerance occurring to the LCGU-increasing effects of nicotine are likely due to short-term receptor desensitization caused by the appreciable plasma nicotine concentrations remaining at the time of testing (Grunwald *et al*., 1988; London *et al*., 1990; Schröck & Kuschinsky, 1991).

The [^{14}C]-2-DG procedure of Sokoloff *et al*. (1977) is associated with several potent stressors (anaesthesia, surgery, restraint), which are likely to stimulate secretion of corticosteroids (Axelrod & Reisine, 1984). Administration of exogenous corticosterone to mice has been shown to reduce sensitivity to nicotine in a number of behavioural and physiological tests, possibly through a direct action at the receptor (Pauly *et al*., 1992). Possibly, the rats in Experiment 1 were inadvertently more stressed than those in other studies where larger nicotine effects were obtained. In these studies, corticosteroid levels

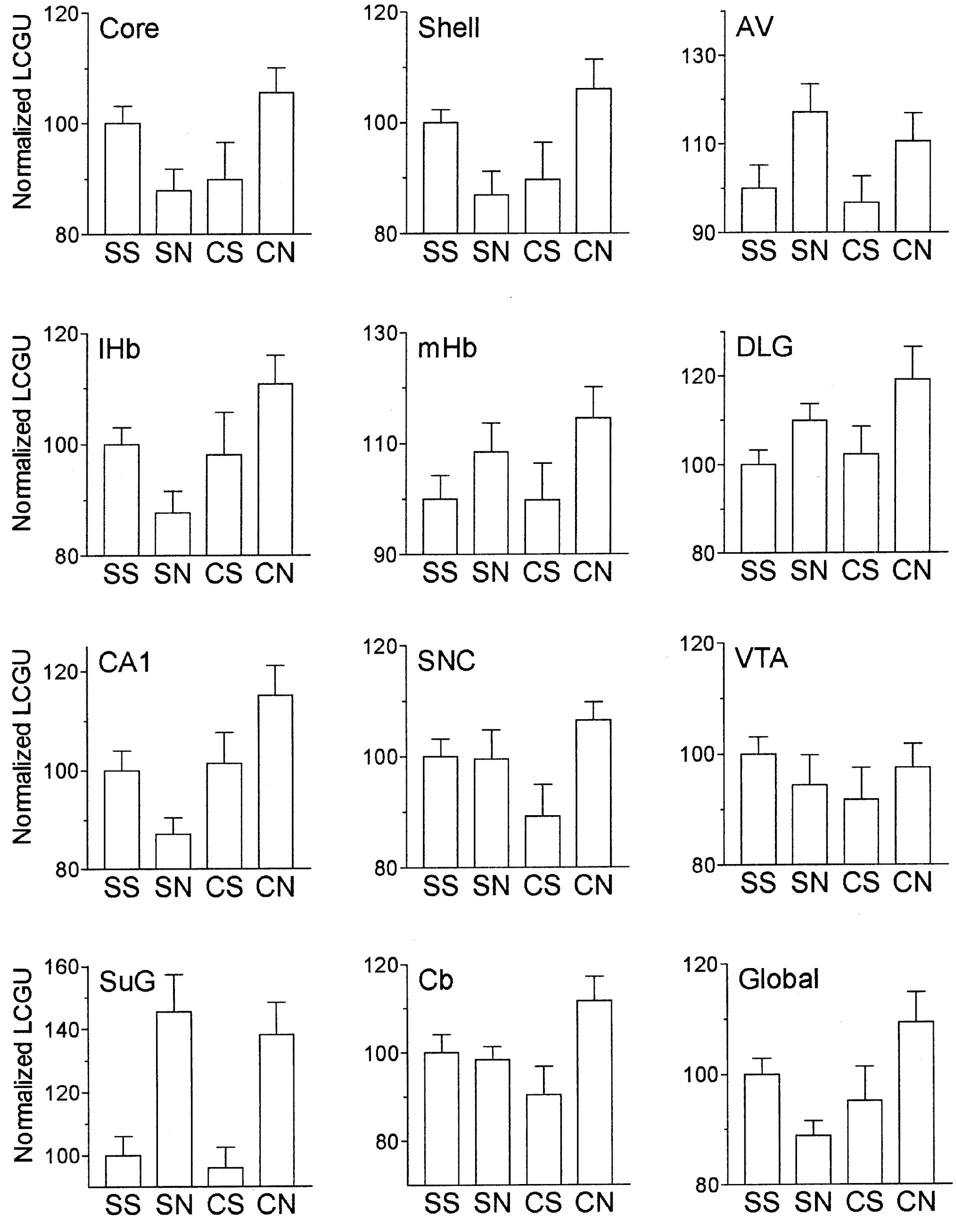

Figure 2 Effects of chlorisondamine (CHL) and nicotine on local cerebral glucose utilization (LCGU) in freely-moving rats. Data are from Experiment 2. Rats were pretreated with CHL 10 μg i.c.v. (or saline). LCGU was determined 24–32 days later, after injection of nicotine 0.4 mg kg^{-1} s.c. (or saline). The vertical axis represents the mean ($\pm$s.e.mean) LCGU, calculated as a percentage of the saline/saline group. For abbreviations, see Figure 1. Nicotine significantly increased LCGU in AV, MHb, DLG and SuG (ANOVA main effect). Nicotine alone significantly decreased LCGU in core, shell, lHb, CA1, and in the global measure. A statistically significant CHL × NIC interaction occurred in core, shell, lHb, CA1, Cb, and in the global measure. Thus, only the inhibitory effect of nicotine was significantly countered by CHL.

were not reported, but plasma glucose levels, where measured, were somewhat lower (London *et al.*, 1990; McNamara *et al.*, 1990).

In an attempt to reduce stress levels, measurement of LCGU was subsequently undertaken in freely-moving rats. Although several methods exist for measuring LCGU in untethered rats, each has potential drawbacks. One such method employs intraperitoneal administration of [^{14}C]-2-DG, which eliminates surgery but yields only semiquantitative estimates of LCGU (Meibach *et al.*, 1980). Another method is fully quantitative but requires blood samples to be taken close to the rat's snout, which provides a potential source of stress (Crane & Porrino, 1989). The method we have developed is quantitative and avoids blood sampling in proximity to the animal. To reduce stress further, we attempted to maximize the recovery period between catheterization and testing; the practical limit was about 6 h due to blood clotting in the arterial line. The lower plasma glucose levels recorded in the second experiment suggest that the rats may have been less stressed than in the first experiment; this result accords with a previous comparison of immobilized *vs* unrestrained rats (Crane & Porrino, 1989).

The modifications introduced in the second experiment did not lead to an enhancement of the stimulatory effects of nicotine on LCGU. Indeed, this experiment revealed a widespread inhibitory effect of nicotine on LCGU. This finding stands in striking contrast to previous studies employing acute i.p. or s.c. administration to drug-naïve but immobilized rats, where no decrements have been reported in any brain area (London *et al.*, 1985; 1988a,b; 1990; McNamara *et al*., 1990). The present study differed from the abovementioned studies not only in the use of freely-moving animals, but also in the rat strain used (Sprague-Dawley *vs* Fisher 344). However, it seems unlikely that rat strain plays a critical role, since large LCGU increases (and no reductions) have been seen in response to nicotine in Sprague-Dawley rats (Grunwald *et al*., 1987).

The effects of nicotine on LCGU in freely-moving rats have been the subject of one previous report (Pontieri *et al*., 1996). This study also used Sprague-Dawley rats. No inhibitory effects were observed, but out of 29 areas sampled, intravenously administered nicotine (0.05 mg kg^{-1}) significantly increased LCGU only in the nucleus accumbens shell. In this region, as in several others, we observed a decrease in LCGU. Factors that may help to explain these differences include dose, route of administration (Porrino, 1993), duration or type of anaesthesia, and possibly stress engendered by blood collection close to the animal in the intravenous nicotine study (Crane & Porrino, 1989).

Nicotinic antagonism by chlorisondamine

In previous reports, elevations of LCGU resulting from systemic administration of nicotine have largely matched the pattern of [^{3}H]-nicotine labelling (Grunwald *et al*., 1987; London *et al*., 1988a,b; McNamara *et al*., 1990), and on this basis it has been suggested that nicotinic stimulation of LCGU occurs *via* a local action in most brain areas. Furthermore, since [^{3}H]-nicotine labelling largely corresponds to $\alpha 4$ $\beta 2$ nAChRs (Whiting & Lindstrom, 1987; Zoli *et al*., 1998; Marubio *et al*., 1999), it is likely that the nicotine enhancement of LCGU is mediated primarily by this receptor subtype.

The clearest block by CHL occurred in the anteroventral thalamus (Experiment 1). It is likely that nicotine acted directly in this nucleus, since it contains a high expression of messenger RNA coding for the $\alpha 4$ and $\beta 2$ nicotinic receptor subunits (Wada *et al*., 1989) and displays very high densities of nicotinic receptor binding (Clarke *et al*., 1985; Swanson *et al*., 1987; Happe *et al*., 1994). Significantly, the anteroventral thalamus does not retain high amounts of radioactivity after administration of [^{3}H]-CHL (El-Bizri *et al*., 1995). Taken together, these observations imply that if intracellular accumulation of CHL (or a metabolite) has any role to play in the extended nicotinic blockade, either conspicuously high levels of accumulation are not required, or residual tritium levels do not reliably indicate tissue concentrations of the active compound(s).

In the superior colliculus, CHL pretreatment did not completely block the nicotine-associated LCGU elevation. Although this finding might suggest that antagonism by CHL persists in some brain areas more than others, this conclusion is not inescapable since the effect of systemic nicotine in the superior colliculus may be at least partly indirect. Thus, nAChRs are located on retinal ganglion cells as well as on their terminals within the superior colliculus (Swanson *et al*., 1987; Prusky & Cynader, 1988). Moreover, the elevation of LCGU following systemic nicotine is abolished by enucleation (London *et al*., 1988b), and local stimulation of retinal nAChRs has been shown to increase LCGU in the superior colliculus (Pazdernik *et al*., 1982). The incomplete antagonism observed in the superior colliculus also suggests that CHL does not dampen LCGU elevations *via* some more general, non-nicotinic mechanism.

The mechanism underlying the widespread LCGU-decreasing effects of nicotine seen in Experiment 2 is unclear. It should be noted that inhibition of LCGU need not imply neuronal inhibition, since the energetics requirements of synaptic hyperpolarization and excitation appear similar (Dewar & McCulloch, 1992). It is likely that the nicotine-induced reduction of LCGU was mediated by nAChR stimulation rather than desensitization, since the effect tended to be reversed, and not mimicked, by CHL. There was no clear regional concordance with either [^{3}H]-nicotine or [^{125}I]-α-bungarotoxin labelling (Clarke *et al*., 1985), believed to represent the predominant nAChR subtypes in rat brain. This suggests that a different nAChR subtype may be involved or that the effects are not local. The finding that CHL blocked the inhibitory effect of nicotine on the 'global' LCGU measure (Experiment 2) again suggests that antagonism is not restricted to the few neuronal populations that show strikingly high radiolabelling for several weeks after administration of [^{3}H]-CHL.

Effects of chlorisondamine in the absence of nicotine

In the first experiment, CHL reduced LCGU in a number of brain areas, further indicating that this drug may exert widespread effects. To date, CHL appears selectively nicotinic in its actions at concentrations attained *in vivo* (Clarke *et al*., 1994). If CHL were to act principally by blocking nicotinic receptors, it would tend to target sites possessing a high tonic level of nicotinic cholinergic transmission. Although present evidence for central nicotinic neurotransmission is fragmentary (Clarke, 1993), candidate sites include the anteroventral thalamus, medial habenula, dorsal lateral geniculate, substantia nigra pars compacta, and ventral tegmental area. In some of these structures, a significant depressant effect of CHL was observed. However, depressant effects were also seen in other structures (lateral habenula, superior colliculus, and cerebellum), where present evidence does not favour nicotinic cholinergic transmission (Clarke, 1993). Future studies may clarify whether these changes represent the direct or indirect consequences of nicotinic receptor blockade, or whether they reflect other, non-nicotinic effects of the drug.

Conclusions

Nicotine, given in a behaviourally active dose, does not always produce marked increases in LCGU in rats. In freely-moving animals, the predominant effect may be inhibitory, as reported in preliminary studies in human subjects (London, 1995). The persistent *in vivo* effects of chlorisondamine appear widely distributed within the brain, suggesting that high levels of intracellular accumulation may not be required for the long-term nicotinic blockade.

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