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Intraneuronal accumulation and persistence of radiolabel in rat brain following *in vivo* administration of [^{3}H]-chlorisondamine

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1 Chlorisondamine (CHL), a bisquaternary amine, produces a remarkably long-lasting blockade of central responses to nicotine. The mechanism underlying this blockade is not known. The main aim of this study was to test for possible accumulation of [^{3}H]-CHL in rat brain during the period of chronic blockade.

2 Rats received CHL, either systemically (10 mg kg^{-1}) or centrally (10 μg i.c.v.). Seven days later, striatal synaptosomes prepared from these animals were tested for nicotine-induced [^{3}H]-dopamine release. This experiment showed that i.c.v. administration of CHL was as effective as systemic administration in producing *ex vivo* blockade of central nicotinic receptors.

3 Rats received bilateral i.c.v. infusions of [^{3}H]-CHL (10 μg) and radioactivity was subsequently quantified in dissected cerebral cortex, striatum, hippocampus, midbrain and cerebellum. Radiolabel was detected at all three survival times (1, 7, and 21 days). Regional heterogeneity was apparent at 7 and 21 days survival. Radiolabel was almost exclusively confined to the insoluble subcellular fraction in all areas sampled.

4 The anatomical distribution of radiolabel was also visualized in brain sections. Rats received bilateral i.c.v. infusions of [^{3}H]-CHL (10 μg) and were killed at 1, 7, 21 or 84 days. Immediately before they were killed, all rats were tested behaviourally, and central nicotinic blockade was demonstrated at 1, 7 and 21 days; partial recovery was observed at 84 days. Particularly at longer survival times, tritium was found to be heavily concentrated in the substantia nigra pars compacta, ventral tegmental area, dorsal raphé nucleus, and the granular layer of the cerebellum.

5 The possibility of retrograde axonal transport of radiolabel was then examined. Rats received a unilateral intrastriatal infusion of [^{3}H]-CHL (0.34 or 0.034 μg) one week before they were killed. Autoradiographic labelling was largely confined to the site of infusion and to the ipsilateral substantia nigra pars compacta and dorsal raphé nucleus.

6 Thus, after i.c.v. administration, CHL (and/or centrally-formed derivatives) is initially widely distributed within the brain and is then selectively retained within a few brain areas. A persistent accumulation occurs within putative dopaminergic and 5-hydroxytryptaminergic neurones, at least partly through uptake by terminals and/or axons followed by retrograde transport. This persistent and anatomically-selective intraneuronal accumulation possibly underlies the long-term central nicotinic blockade associated with chlorisondamine.

Keywords: Chlorisondamine; [^{3}H]-chlorisondamine; dopamine; nicotine; transmitter release; autoradiography; subcellular fractions; retrograde transport

Introduction

Chlorisondamine (CHL) is a nicotinic antagonist at autonomic ganglia (Grimson *et al.*, 1955; Plummer *et al.*, 1955; Schneider & Moore, 1955). Because of its bisquaternary structure, CHL does not readily cross the blood brain barrier (Clarke, 1984). Systemic administration of CHL produces a transient ganglionic blockade (Grimson *et al.*, 1955; Plummer *et al.*, 1955; Schneider & Moore, 1955; Clarke *et al.*, 1994). In contrast, a single administration of the drug, given either centrally or in a large systemic dose, results in a blockade of central nicotinic responses that can persist for several weeks (Clarke & Kumar, 1983; Clarke, 1984; Reavill *et al.*, 1986; Fudala & Iwamoto, 1987; Kumar *et al.*, 1987; Mundy & Iwamoto, 1988; Corrigall *et al.*, 1992; Decker *et al.*, 1994; El-Bizri & Clarke, 1994b).

The mechanism underlying this remarkably persistent antagonism of central nicotinic responses is not known. Blockade does not appear to result from a neurotoxic effect, since CHL does not cause neuronal degeneration (Clarke *et al.*, 1994). In addition, the chronic effect of CHL on central nicotinic responses in not accompanied by changes in the density of [^{3}H]-nicotine or [^{125}I]-α-bungarotoxin binding to rat brain regional homogenates (Clarke *et al.*, 1994; Decker *et al.*, 1994; El-Bizri & Clarke, 1994a). CHL does not compete for high-affinity nicotinic agonist binding to rat brain membranes, indicating a noncompetitive action (Clarke *et al.*, 1984). Consistent with this observation, CHL exerts an insurmountable and use-dependent blockade in the striatum (El-Bizri & Clarke, 1994b, c). Thus, CHL may act as a channel blocker at brain nicotinic receptors, as proposed in the periphery (Traber *et al.*, 1967; Alkadhi & McIsaac, 1974) and at non-mammalian nicotinic receptors (Lingle 1983a,b; Neely & Lingle, 1986).

Nicotinic blockade associated with CHL can persist *ex vivo*, as shown in superfused synaptosomes prepared from rats that received the drug as long as 12 weeks before they were killed (El-Bizri & Clarke, 1994b). Hence, long-term nicotinic antagonism cannot be explained solely by physical trapping of CHL, or of a derivative, by the blood-brain barrier. The mechanisms underlying nicotinic antagonism produced by *in vivo* and *in vitro* administration of CHL appear to differ, in that only the latter was reversed by prolonged wash-out (El-Bizri & Clarke, 1994b).

Thus, although CHL has fallen out of use as an antihypertensive drug, it produces a remarkably persistent blockade of central nicotinic responses that may represent a novel drug action. The first aim of the present study was to examine whether *ex vivo* nicotinic blockade could be obtained after intracerebroventricular as well as systemic administration of

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CHL. The second aim was to examine the possible retention and regional localization of radiolabel in the brain, following administration of [^{3}H]-CHL by the intracerebroventricular route.

Methods

Drug-naïve male Sprague-Dawley rats (Charles River, St. Constant, Quebec, Canada), weighing 200–250 g, were housed four per cage and maintained on a 12/12 h light-dark cycle. Subjects were allowed 3 days for acclimatization before use, and were randomly allocated to the different treatment groups. Food and water were available *ad libitum*.

Dopamine release from superfused synaptosomes

The synaptosomal preparation has been described previously (El-Bizri & Clarke, 1994c). Briefly, rat striata were dissected and homogenized in 20 vol of ice-cold 0.32 M sucrose/5 mM HEPES, and centrifuged at 1000 *g* for 10 min; the pellet was re-centrifuged at 12 000 *g* for 20 min at 4°C. The final pellet, consisting of the crude synaptosomal (P2) fraction, was resuspended in buffer (mM concentrations: NaCl 128, KCl 2.4, $CaCl_2$ 3.2, KH_2PO_4 1.2, $MgSO_4$ 0.6, HEPES 25; D-glucose 10, L-ascorbic acid 1 and pargyline 0.1, adjusted with NaOH to pH 7.5). The synaptosomal preparation was incubated with [^{3}H]-dopaine (0.12 μM) for 10 min at 37°C, then centrifuged at 1000 *g* for 5 min at RT. The pellet was gently resuspended in buffer (5 ml g^{-1} of wet tissue weight).

The 32-channel superfusion apparatus and procedure have been previously described (El-Bizri & Clarke, 1994c). In brief, the synaptosomes were retained in chambers through which buffer was continuously pumped (0.4 ml min^{-1}). Following a 25 min wash period, 18 samples were collected in consecutive 1 min intervals. The first 6 samples provided a baseline, and nicotine (or buffer) was then given in a 1 min pulse upstream of the chambers. Samples were collected into polypropylene minivials (Sarstedt, Montreal) containing 2 ml scintillation fluid (Hi-Safe III, Fisher Scientific, Montreal, Canada); radioactivity was measured in a liquid scintillation counter (Wallac 1410, LKB, Sweden).

Tests of central nicotinic blockade

Rats received an injection of nicotine ditartrate (1 mg kg^{-1}, s.c. dose as base) and were observed continuously during a 10 min period for the presence or absence of prostration and ataxia. Weight-matched drug-naïve rats served as positive controls ($n=2$ rats per survival time).

Administration of [^{3}H]-chlorisondamine

Rats were anaesthetized with Na pentobarbitone (55 mg kg^{-1}, i.p.). In Experiment 1, rats were mounted in a stereotaxic apparatus and received bilateral infusions of CHL (5 μg CHL per side given in 5 μl 0.9% saline) or 0.9% saline (5 μl per side) into the lateral ventricles ($n=4$ rats per group), via stainless steel 30 gauge cannulae. Stereotaxic coordinates were derived from Bregma: A +0.5, L±1.0, V−4.6, with the tooth bar set 3.5 mm below the ear bars. CHL or saline was given over a period of 5 min per side, and the cannulae were slowly removed after an additional minute. The wound was then sutured with silk thread. In Experiments 2 and 3 ($n=9$ and 16 rats, respectively), the same coordinates were used as in Experiment 1, but rats received bilateral infusions of [^{3}H]-chlorisondamine chloride ([^{3}H]-CHL: 5 μg CHL per side; equivalent to 35.2 μCi or 11.7 nmol CHL per side). [^{3}H]-CHL was given in 7.2 μl saline per side, over a period of 6 to 7 min, and 1 min later the cannulae were slowly removed.

In Experiment 4, [^{3}H]-CHL was infused unilaterally into the left caudate-putamen (CP) ($n=5$ rats). Stereotaxic coordinates were derived from Bregma: A+0.5, L+2.4, V−5.0. The infusion contained 0.34 μg ($n=3$ rats) or 0.034 μg ($n=2$ rats) [^{3}H]-CHL in 0.5 μl saline. Each infusion was given over a period of 5 min, with an additional 5 min before the cannula was slowly removed.

Preparation of subcellular fractions of brain tissue (Experiment 2)

After decapitation, 1–2 ml of blood was immediately collected from each rat and transferred to polypropylene vials (20 ml; Fisher Scientific, Montreal, Canada) containing scintillation cocktail (4 ml per ml of collected fraction) (BSA-NA: Amersham, Montreal, Canada). Cerebral cortex (CC), hippocampus (HC), striatum (ST), midbrain (MB) and cerebellum (CM) of each rat were immediately dissected in ice-cold 0.32 M sucrose/5 mM HEPES at pH 7.5, weighed and homogenized in 10 vol of sucrose/HEPES solution (12 up and down strokes at 850 r.p.m., in a 0.25 mm clearance glass Teflon homogenizer). The homogenates were centrifuged at 1000 *g* for 10 min at 4°C. The pellet, (P1 fraction) was collected and the supernatant (S1) fraction was re-centrifuged at 12 000 *g* for 20 min at 4°C. The supernatant (S2) was collected (see below) and the pellet, consisting of the crude synaptosomal fraction (P2), was resuspended in ice-cold 5 mM HEPES at pH 7.5 (3 ml for cerebral cortex, midbrain or cerebellum; 2 ml for striatum or hippocampus), incubated for 30 min at 4°C, then re-centrifuged at 52 000 *g* for 60 min at 4°C. The final supernatant (S3) and pellet (P3) were both collected. The soluble fractions (S2 and S3) were separately transferred to polypropylene vials containing scintillation cocktail. P1 and P3 fractions were vortexed with tissue solubilizer (1 ml per 100–150 mg of original tissue weight) (NCS: Amersham, Montreal, Canada) and incubated at 37°C for 20 h. Glacial acetic acid (30 μl per 1 ml solubilizer) was then added, and finally scintillation cocktail (4 ml per 1 ml solubilizer) (BSA-NA: Amersham, Montreal, Canada) was added and vortexed prior to liquid scintillation counting.

The amount of protein present in the P1 and P3 fractions were determined in drug-naïve rats, with a commercial kit (Bio-Rad Laboratories Ltd, Mississauga, Ontario, Canada), based on the method of Bradford (1976).

Autoradiography of brain sections (Experiments 3 and 4)

Brains were immediately removed, immersed in isopentane for 30 s at −50°C and stored at −40°C. Coronal brain sections (20 μm thick) were cut at −16°C in a cryostat (Frigocut model 2700; Reichert-Jung, Nubloch, Germany). In Experiment 2 and Experiment 3, three adjacent sections were taken at each of 15 anterior-posterior brain levels, 1 mm apart, starting at 11.7 mm anterior to interaural zero (Paxinos & Watson, 1986). In Experiment 4, additional sections were taken 0.5 mm apart in the vicinity of the substantia nigra. Brain sections were thaw-mounted on pre-cleaned gelatin-coated glass microscope slides, rapidly dried at 50°C, stored at 4°C, and within 24 h were juxtaposed tightly against tritium-sensitive film (Hyperfilm-^{3}H; Amersham, Oakville, Ontario, Canada) at room temperature. Tritium standards (^{3}H-micro-scales; tissue equivalent values approximately 1–32 nCi mg^{-1} tissue; Amersham) were included with each film. The duration of film exposure was 2, 3, 8 and 51 days, respectively, for the 1, 7, 21 and 84 day survival groups. Films were processed in Kodak D19 developer at 22°C for 5 min, and fixed in Kodak rapid fixer (without hardener) for 5 min. Brain autoradiographs were visualized with an MC1D M1 microcomputer-based system (Imaging Research, St. Catherine, Ontario, Canada). Brain structures were identified with reference to the stereotaxic atlas of Paxinos & Watson (1986), and by superimposing the film images with those of the corresponding Nissel-stained sections.

Drugs

Chlorisondamine chloride (CHL) was a gift of Ciba-Geigy (Summit, NJ, U.S.A.). [^{3}H]-chlorisondamine chloride ([^{3}H]-

CHL) (specific activity 3.0 Ci mmol^{-1}; purity 98%) was prepared by reacting CHL with gaseous tritium (Wilzbach, 1957). Radiolabeling was undertaken at New England Nuclear (Boston, MA, U.S.A.). [^{3}H]-CHL was dried from ethanol in a nitrogen atmosphere at 4°C, redissolved in 0.9% saline solution, divided into aliquots and stored at −40°C. [8-^{3}H]-dopamine (specific activity 20–40 Ci mmol^{-1}) was obtained from New England Nuclear (Boston, MA, U.S.A.). (−)-Nicotine di-(+)-tartrate was purchased from Sigma Chemical Co (St. Louis, MO, U.S.A.), and was dissolved in 0.9% saline, then neutralized to pH 7.3 with NaOH. For the *in vitro* assay, nicotine ditartrate was dissolved in buffer. Na pentobarbitone was obtained from MTC Canada Packers Inc. (Cambridge, Ontario, Canada) and pargyline hydrochloride was purchased from Research Biochemicals Inc. (Natick, MA, U.S.A.). Other chemicals and reagents were from commerical sources.

Data analysis

In Experiment 1, basal release was defined as the mean tritium released (d.p.m. min^{-1}) over the six samples collected prior to the nicotine (or buffer) challenge. Release in each 1 min period was then calculated as a percentage of basal release; evoked release was taken as the peak value that occurred in the first three collections following the drug administration (El-Bizri & Clarke, 1994c). Since dopamine represents the major constituent of nicotine-evoked tritium release from synaptosomes preloaded with [^{3}H]-dopamine (Rapier *et al.*, 1988), tritium released will be referred to as [^{3}H]-dopamine release.

In Experiment 2, the regional distribution of tritium in the brain was assessed by analysis of variance, using commerical software (Systat, Evanston, IL, U.S.A.), with a single within-subject factor (AREA).

Results

Experiment 1: Persistence of chlorisondamine blockade ex vivo*: systemic vs central administration*

Rats were randomly allocated to three treatment groups ($n=4$ per group) that received either systemic CHL (10 mg kg^{-1}, s.c.), central CHL (10 μg, i.c.v.), or vehicle (5 μl saline, i.c.v.). Each rat was killed 7 days later, and its striatal synaptosomes were tested with nicotine (2 superfusion channels per rat) and buffer (2 superfusion channels per rat). Synaptosomal preparations of various treatment groups were tested in parallel and in a counterbalanced manner. Synaptosomes prepared from the control group released [^{3}H]-dopamine in response to 1 μM nicotine; in contrast, synaptosomal preparations from the two groups of rats pretreated with CHL failed to do so (Figure 1).

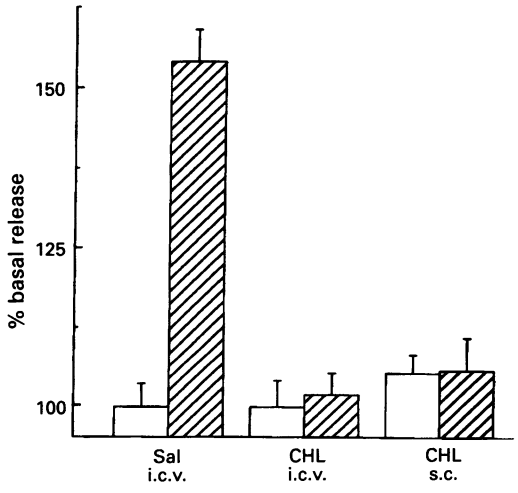

Figure 1 Effects of systemic or central chlorisondamine (CHL) pretreatment on nicotine-induced [^{3}H]-dopamine release from rat striatal synaptosomes. Rats received a single pretreatment with CHL (10 mg kg^{-1}. s.c.) or CHL (10 μg i.c.v.) or saline (Sal, 5 μl i.c.v.), and were permitted to survive for 7 days. Synaptosomes were challenged with a 1 min pulse of (−)-nicotine (1 μM) (hatched columns) or SB (open columns). The vertical axis represents the mean (±s.e.mean) peak release, calculated as a percentage of basal release ($n=4$ rats per group).

Experiment 2: Subcellular distribution of radiolabel in dissected brain regions following i.c.v. infusion of [^{3}H]-chlorisondamine

Rats were killed 1, 7 or 21 days after bilateral infusion of [^{3}H]-chlorisondamine into the lateral ventricles ($n=3$ rats per group), and dissected brain regions were taken for subcellular fractionation. Tritium was almost exclusively confined to the P1 and P3 subcellular fractions of all brain regions sampled. The P1 and P3 fractions contained approximately equal amounts of protein, and together represented approximately 13% of the protein contained in the original wet tissue. The regional distribution of tritium appeared somewhat uniform at 1 day survival in both fractions (AREA: $P>0.5$, for each) (Figure 2a, Figure 3a) but was clearly regionally heterogeneous at 7 days (AREA: $P<0.05$, for each fraction) (Figure 2b, Figure 3b) and 21 days survival (AREA: $P<0.0005$, for each fraction) (Figure 2c, Figure 3c). In every brain region sampled, tritium levels declined over successive survival times. Eventually, highest concentrations were found in the cerebellum (Figure 2c; Figure 3c). The soluble fractions (S2 and S3) contained minimal levels of radioactivity (Table 1), which did not appear to mirror tritium levels detected in P1 and P3

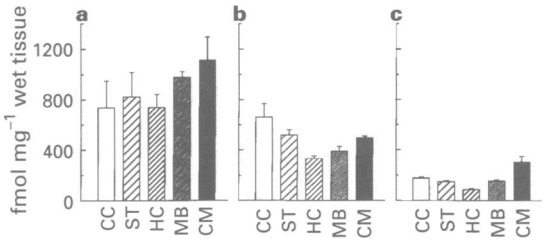

Figure 2 Radiolabel recovered in P1 subcellular fraction of dissected brain regions. Rats received bilateral i.c.v. infusion of [^{3}H]-chlorisondamine (10 μg per rat; $n=3$ rats per survival group) and were permitted to survive for 1 (a), 7 (b) or 21 (c) days. Cerebral cortex (CC), hippocampus (HC), striatum (ST), midbrain (MB) and cerebellum (CM). The vertical axis represents the mean (±s.e.mean) of tritium recovered, converted to fmol equivalent values with reference to [^{3}H]-chlorisondamine ($n=3$ rats per survival time).

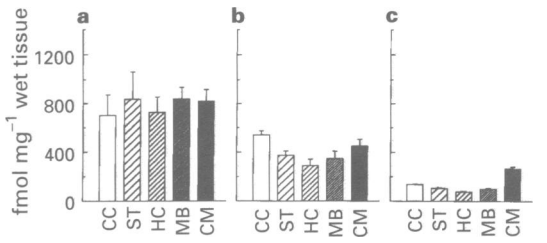

Figure 3 Radiolabel recovered in P3 subcellular fraction of dissected brain regions. Rats received bilateral i.c.v. infusion of [^{3}H]-chlorisondamine (10 μg per rat; $n=3$ rats per survival group) and were permitted to survive for 1 (a), 7 (b) or 21 (c) days. Cerebral cortex (CC), hippocampus (HC), striatum (ST), midbrain (MB) and cerebellum (CM). The vertical axis represents the mean (±s.e.mean) of tritium recovered, converted to fmol equivalent values with reference to [^{3}H]-chlorisondamine ($n=3$ rats per survival time).

Table 1 Tritium detected in the soluble subcellular fractions (S2 and S3) of brain tissue homogenates

		S2-Fraction	
Survival	*1 day*	*7 days*	*21 days*
CC	0.54 ± 0.11	0.34 ± 0.19	0.26 ± 0.07
ST	1.71 ± 0.12	0.17 ± 0.15	0.34 ± 0.18
HC	1.63 ± 0.48	0.32 ± 0.16	0.90 ± 0.20
MB	0.82 ± 0.04	0.43 ± 0.06	0.21 ± 0.04
		S3-Fraction	
Survival	*1 day*	*7 days*	*21 days*
CC	0.17 ± 0.10	0.19 ± 0.09	0.30 ± 0.05
ST	0.31 ± 0.05	0.31 ± 0.05	0.28 ± 0.15
HC	0.26 ± 0.13	0.27 ± 0.14	0.33 ± 0.17
MB	0.07 ± 0.07	0.39 ± 0.27	0.14 ± 0.07
CM	0.27 ± 0.14	0.51 ± 0.14	0.37 ± 0.19

Rats received bilateral infusions of [^{3}H]-chlorisondamine (10 μg per rat) and were permitted 1, 7 or 21 days survival. CC, cerebral cortex; ST, striatum; HC, hippocampus; MB, midbrain; CM, cerebellum. *Mean (± s.e.mean) tritium detected (fmol chlorisondamine equivalent per mg original tissue) ($n = 3$ rats per group).

fractions. Tritium was not detected in the blood samples at any survival time. On the assumption that the label remains attached to the parent compound, and becomes uniformly distributed within the entire CNS, the residual amount of [^{3}H]-CHL in the brain at 1, 7, and 21 days survival would have been approximately 10.0, 6.0, and 2.0% of the initial amount of [^{3}H]-CHL administered, respectively.

Experiment 3: Autoradiographic distribution of radiolabel following bilateral i.c.v. infusion of [^{3}H]-chlorisondamine

Rats received bilateral infusion of [^{3}H]-chlorisondamine into the lateral ventricles and were permitted 1, 7, 21, or 84 days survival ($n = 4$ rats per survival time). Before they were killed, tests of central nicotinic blockade showed that the 1, 7 and 21 days survival groups were completely protected against nicotine-induced prostration and ataxia. However, partial recovery was seen in the 84 days survival group; these rats showed signs of nicotine-induced ataxia and tail rigidity.

Tritium was detected in the autoradiographs at all survival times. At all survival times (1–84 days), radioactivity was highly concentrated in the substantia nigra pars compacta (SNC), ventral tegmental area (VTA), dorsal raphé nucleus

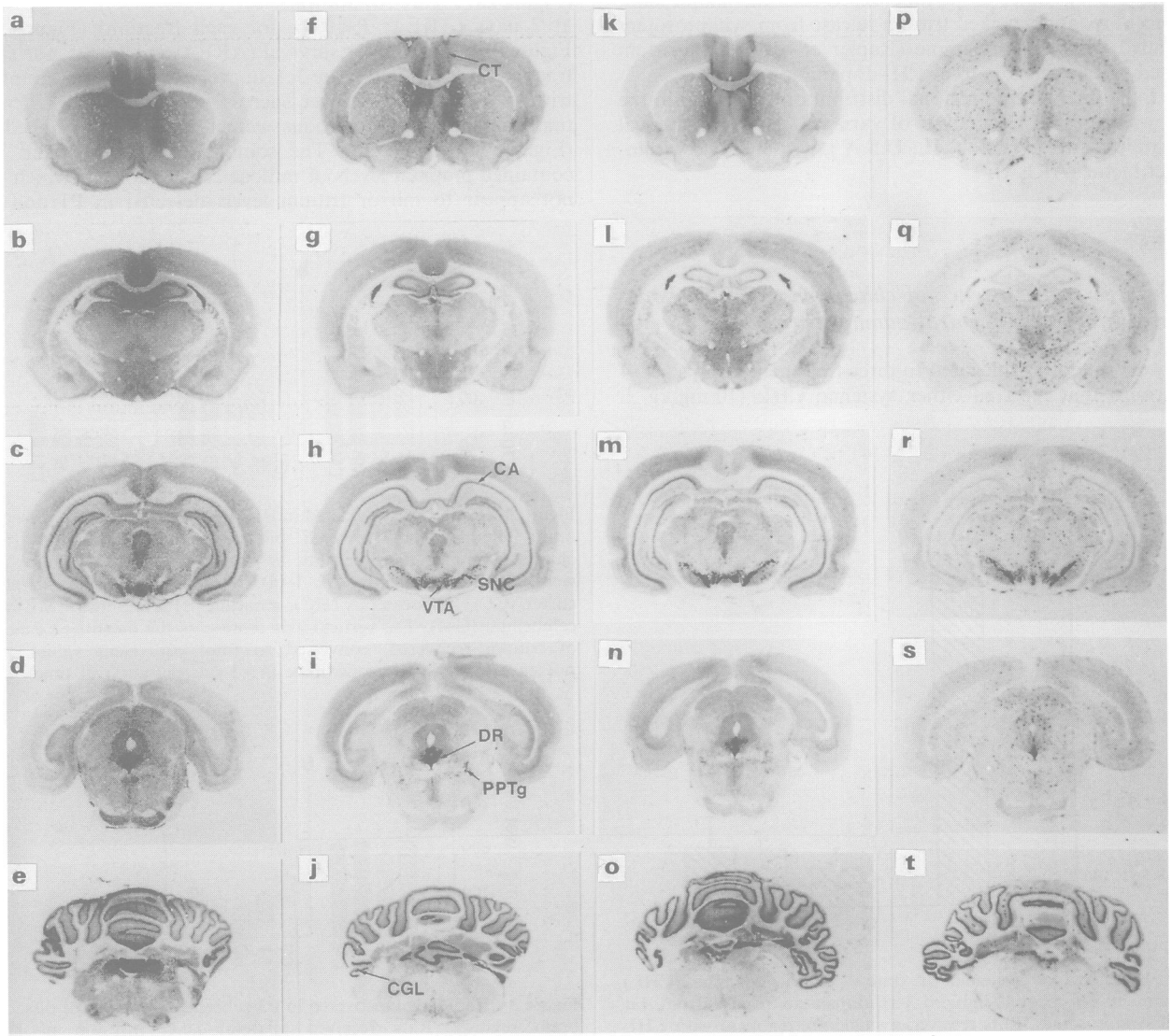

Figure 4 Representative autoradiographs prepared from rats that received bilateral i.c.v. infusions of [^{3}H]-chlorisondamine (10 μg CHL). Rats were allowed to survive for 1 day (a–e), 7 days (f–j), 21 days (k–o) or 84 days (p–t). Longer film exposure times were used for longer survival times, in order to compensate for the decline in radiolabel. For each survival time, the photomicrographs correspond to sections taken at (top to bottom): 9.7, 6.7, 3.7, 2.7 and −1.3 mm anterior to interaural zero. CT, cannula tract; CGL, cerebellar granular cell layer; DR, dorsal raphé nucleus; CA, hippocampal pyramidal layer; PPTg, pedunculopontine tegmental nucleus; SNC, substantia nigra pars compacta; SNR, substantia nigra reticulata; VTA, ventral tegmental area.

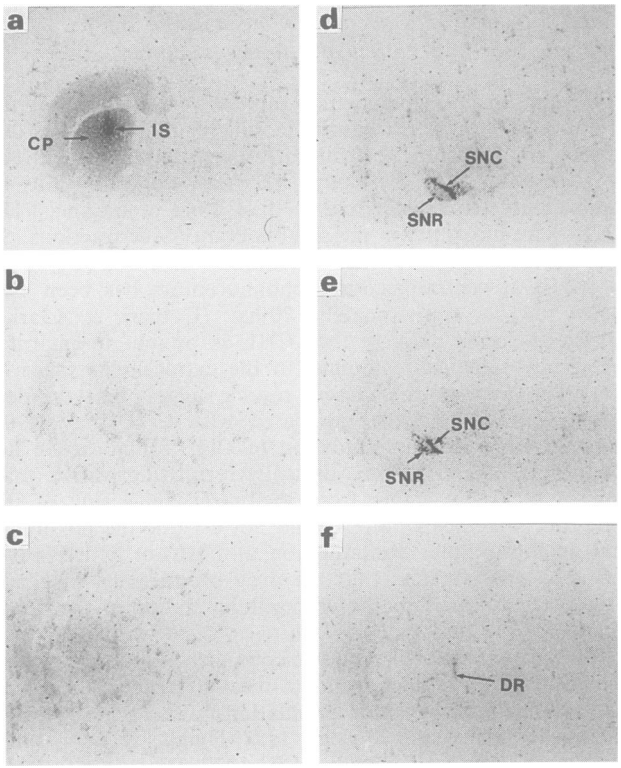

Figure 5 Representative autoradiographs prepared from a rat that received a unilateral infusion of [^{3}H]-chlorisondamine (0.034 μg CHL) into the left caudate-putamen, one week before it was killed. Panels (a–f) correspond to sections taken at: 9.7, 6.7, 4.7, 4.2, 3.2 and 1.7 mm anterior to interaural zero. CP, caudate-putamen; CT, cannula tract; DR, dorsal raphé nucleus; SNC, substantia nigra pars compacta; SNR, substantia nigra reticulata.

(DR), and in the cerebellar granular layer (CGL) (Figure 4). At 1, 7, and 21 days survival, the cannulae tracts were heavily labelled and moderate signal was also seen in the hippocampal pyramidal cell layer (CA), dentate gyrus (DG) and the pedunculopontine tegmental nucleus (PPTg) (Figure 4). At 84 days survival, small, scattered concentrations of label were found in all autoradiographs (Figure 4; panels p to t). These spots may represent individual cells or groups of cells, rendered detectable by the long film exposure used for this survival group.

Experiment 4: Autoradiographic distribution of radiolabel following unilateral intrastriatal infusion of [^{3}H]-chlorisondamine

Following intrastriatal infusion of the higher dose of [^{3}H]-CHL (0.34 μg; $n=3$ rats) one week before the rats were killed, tritium was detected mainly in the ipsilateral CP, ipsilateral SNC and ipsilateral DR; some label was also detected in the contralateral SNC and in the ipsilateral SNR. In the forebrain, labelling was detected in the ipsilateral cerebral cortex, possibly resulting from diffusion from the injection site. In addition, faint labelling was detected in the cerebellum. After infusion of the lower dose of [^{3}H]-CHL (0.034 μg; $n=2$ rats), tritium was detected at the injection site within the CP, in the ipsilateral SNC, and to a lesser extent in the ipsilateral DR; some label was again detected in the contralateral SNC (Figure 5).

Discussion

Following the intracerebroventricular infusion of [^{3}H]-CHL, tritium was detected in the CNS for at least 84 days. It is not yet clear whether this radiolabel represents CHL, or a metabolite of CHL. To our knowledge, there is no published information on the pharmacokinetics of CHL, either in the periphery or in the CNS. Other ganglion blockers, where studied, have been found to be excreted unmetabolized after systemic injection (Mason, 1980). Moreover, some quaternary nicotinic antagonists, when given centrally, were actively transported out of the CNS and were not metabolized (Schanker *et al.*, 1962). Thus, given the present state of knowledge, the possibility cannot be excluded that CHL may be transformed into an active derivative in the CNS. To our knowledge, there are no reports to suggest that ganglion blockers other than chlorisondamine are accumulated centrally.

We have previously shown that central nicotinic blockade resulting from systemic administration of CHL (10 mg kg^{-1}) persists *ex vivo* (El-Bizri & Clarke, 1994c). Experiment 1 demonsrated that *ex vivo* blockade could also be obtained after intracerebroventricular administration of CHL (10 μg). This finding is consistent with the results of behavioural experiments (Clarke & Kumar, 1983; Clarke, 1984). Doses of CHL sufficient to block central actions of nicotine (5–10 μg, i.c.v.) would be ineffective if given systemically. Therefore, if the persistent nicotinic effect associated with CHL results from biotransformation of the drug, the active derivative(s) is most likely to be formed centrally.

Following intracerebroventricular infusion of [^{3}H]-CHL, radiolabel was largely confined to the insoluble P1 and P3 subcellular fractions. This finding suggests that the label either forms a stable bond or is trapped within a compartment that is not disrupted by hypo-osmotic lysis.

Is the radiolabel associated with nicotinic receptors in the brain?

The pattern of autoradiographic labelling, following administration of [^{3}H]-CHL, did not correspond to the distribution of any nicotinic receptor detected thus far with available radioligands (Hunt & Schmidt, 1978; Clarke *et al.*, 1984; London *et al.*, 1985; Schulz *et al.*, 1991). For example, dense labelling was not detected in the interpeduncular nucleus, medial habenula, or thalamus which possess dense [^{3}H]-nicotine labelling, or in the dorsal tegmental nucleus, which is rich in [^{125}I]-αBTX binding. The lack of anatomical correlation is not surprising in view of the high levels of radiolabel present. Thus, the concentration of radiolabel occurring in the most densely labelled regions, as estimated from the tritium standards, exceeded 10 pmol mg^{-1} wet tissue; this figure far exceeds the estimated density of any known nicotinic cholinoceptors in the brain (Happe *et al.*, 1994; London *et al.*, 1985).

To what extent is the chlorisondamine-associated in vivo *blockade reversible?*

In a number of behavioural tests conducted in rats, CHL administration resulted in a central nicotinic blockade lasting for three to five weeks with no sign of recovery (Clarke & Kumar, 1983; Clarke, 1984; Reavill *et al.*, 1986; Fudala & Iwamoto, 1987; Kumar *et al.*, 1987; Mundy & Iwamoto, 1988; Corrigall *et al.*, 1992). Recently, however, Decker *et al.*, (1994) reported that locomotor responses to nicotine recovered six weeks after CHL administration. The present work (Experiment 3) provides evidence for partial recovery from CHL-induced nicotinic blockade at 12 weeks survival. In contrast, at the same time interval, we have observed complete blockade of nicotine-induced dopamine release from striatal synaptosomes (El-Bizri & Clarke, 1994b). Why certain nicotinic responses should recover, or recover faster than other responses, is not yet clear.

Cellular uptake and retrograde transport of chlorisondamine by certain neuronal pathways

The accumulation of label in certain nuclei (SNC, VTA, DR) remote from the site of i.c.v. administration (Experiment 4) suggested that the radiolabel might undergo axonal transport.

Subsequently, intrastriatal infusion of [^{3}H]-CHL was found to label strongly the ipsilateral SNC and to a lesser extent the DR. A consideration of the CP afferents and efferents supports the possibility of retrograde but not anterograde transport:

(1) The SNC receives little, if any, monosynaptic input from the caudate-putamen (CP) (Ribak *et al.*, 1976, 1980; Gerfen *et al.*, 1982), whereas it provides a rich, primarily ipsilateral (Fass & Butcher, 1981) innervation to the CP (Dahlstrom & Fuxe, 1964; Moore & Bloom, 1978). It is likely that the cells labelled within the SNC (Experiment 4) are largely or exclusively dopaminergic, since the nigrostriatal pathway is approximately 95% dopaminergic (van der Kooy & Hattori, 1980). The sporadic clusters of label detected in the ipsilateral SNR may also be attributable to scattered dopaminergic cell bodies which also project to the CP (Gerfen *et al.*, 1987). In contrast, the diffuse labelling detected in the SNR may well be associated with dopaminergic dendrites projecting ventrally from the SNC (Gerfen *et al.*, 1987).

(2) The CP also receives projections from the ipsilateral DR (van der Kooy & Hattori, 1980). Although the DR contains 5-hydroxytryptaminergic and non-5-hydroxytryptaminergic neurones, those innervating the CP are mostly (66 to 80%) 5-hydroxytryptaminergic (Van der Kar & Lorens, 1979; Steinbusch *et al.*, 1980).

Detectable retrograde labelling did not occur within all neuronal pathways. For example, intrastriatal infusion of the [^{3}H]-CHL at a dose that resulted in clear labelling in the SN, did not label certain other areas projecting to the CP, such as the cerebral cortex (Gerfen, 1984; Donoghue & Herkenham, 1986). Conversely, the accumulation of label may not always be caused by retrograde transport. For example, when the higher dose of [^{3}H]-CHL was infused into the CP, label was detected in the cerebellum even though this structure does not directly project to the CP. Thus, it appears that more than one mechanism may be involved in the retention of radiolabel. It is also noteworthy that certain neuronal populations appeared to lose radiolabel at a faster rate than others (e.g. CA vs VTA).

The intracellular retention of quaternary compounds is not an unknown phenomenon. For example, at the neuromuscular junction, [^{3}H]-decamethonium was reported to be taken up by a saturable, possibly carrier-mediated mechanism, and then trapped in the muscle fibres (Creese & England, 1970; Creese & MacLagan, 1970). In other experiments, 1 day following intrastriatal injection of [^{3}H]-1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine ([^{3}H]-MPTP) to rats, label was taken up by nerve terminals, retrogradely transported, and accumulated mainly within the SNC and DR (Campbell *et al.*, 1990). Furthermore, [^{14}C]-1-methyl-4-phenylpyridium ([^{14}C]-MPP$^+$), when administered into the carotid artery in monkeys, was also taken up by nigrostriatal nerve terminals and retrogradely transported (Herkenham *et al.*, 1991). However, the accumulation of label was within the axons and, to a lesser extent, in the cell body region; the retention of [^{14}C]-MPP$^+$ was not detected beyond 10 days.

Could intraneuronal accumulation underlie the persistent nicotinic blocking action of chlorisondamine?

The results from Experiment 3 raise the possibility that the prolonged blockade of central nicotinic cholinoceptors is due to the persistence of high concentrations of antagonist (CHL or a derivative) in the brain. Very low concentrations of radioactivity (equivalent to less than 2 nM of original [^{3}H]-CHL) were measured in the soluble brain tissue fractions (S2 and S3) after [^{3}H]-CHL administration. Since the IC_{50} value for CHL at central nicotinic cholinoceptors has been estimated to be approximately 70 nM (El-Bizri & Clarke, 1994c), it seems unlikely that CHL is present in pharmocologically significant amounts in the extracellular space of the brain. Instead, the present results suggest that the persistent *in vivo* blockade associated with CHL may result from intraneuronal accumulation of CHL (or an active derivative). Thus, CHL, or a centrally formed derivative, may exert a block by acting from the intracellular side of the receptor. Nonetheless, one should not exclude the possibility that an intracellular accumulation of CHL, or a derivative, serves as a depot, and that the drug produces its blockade after being released to the extracellular milieu in the direct vicinity of the neurone.

The turnover rate of central nicotinic cholinoceptors is not known. In the periphery, and in receptors expressed in cell cultures, nicotinic receptor populations possess half-lives of 12 days or less (Kemp & Edge, 1987; Higgins & Berg, 1988; Avila *et al.*, 1989; Fumagalli *et al.*, 1990; Peng *et al.*, 1994). Thus, it is likely that *in vivo*, CNS nicotinic blockade by CHL persists over many cycles of receptor synthesis and degradation.

If the proposed intraneuronal mechanism is correct, the prolonged *in vivo* blockade by CHL may be particularly marked within neuronal populations that preferentially accumulate the drug. Evidence to date is limited, but appears consistent with this notion. Thus, the locomotor stimulant and reinforcing actions of nicotine, which appear to be mediated by activation of the mesolimbic dopamine system (Clarke *et al.*, 1988; Corrigall *et al.*, 1992), are both blocked persistently by CHL. In addition, there is evidence that nicotine-induced prostration results, at least partly, from an action within the cerebellum (Maiti *et al.*, 1986). If the long-term blockade exerted by this drug is indeed anatomically selective, chlorisondamine may serve as a useful tool, in protecting certain neuronal populations from activation by nicotine.

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