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Persistent nicotinic blockade by chlorisondamine of noradrenergic neurons in rat brain and cultured PC12 cells

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1 Chlorisondamine (CHL) blocks behavioural responses to nicotine for several weeks or months in rats. Persistent blockade has also been demonstrated *ex vivo*, in assays of nicotine-evoked striatal dopamine release. Central administration of [^{3}H]-CHL leads to long-term retention of radiolabel in nigrostriatal dopaminergic neurons and in few other cell groups. We investigated whether an analogous blockade also occurs in noradrenergic neurons in the brain and in cultured pheochromocytoma (PC12) cells, which have a similar noradrenergic phenotype.

2 Administration of CHL (10 mg kg^{-1} s.c. or 10 μg i.c.v.), 21 days prior, resulted in a near-total block of nicotine-evoked release of hippocampal [^{3}H]-noradrenaline ([^{3}H]-NA) from superfused rat synaptosomes; NMDA-evoked [^{3}H]-NA release was unaffected.

3 Three weeks after administration of [^{3}H]-CHL (10 μg i.c.v.), preferential accumulation of radiolabel was observed in the locus coeruleus, which provides the entire noradrenergic innervation to hippocampus, as well as in previously noted structures.

4 In rat pheochromocytoma (PC12) cells, nicotine evoked [^{3}H]-NA release (EC_{50} approximately 30 μM). This effect was blocked by co-incubation with mecamylamine (10 μM) or CHL (1 μM) but was not affected by α-bungarotoxin. As in the hippocampus, the nicotinic agonist cytisine was at least as efficacious as nicotine.

5 Acute exposure of PC12 cells to CHL 10 or 100 μM (but not 1 μM), followed by 90 min wash-out, almost completely blocked release evoked by 30 μM nicotine. More prolonged (24 h) exposure to CHL 100 μM (but not 1 or 10 μM), followed by 3 days of wash-out, partially inhibited release evoked by nicotine, leaving responses to high K^+ unchanged. A significant (30%) reduction was also seen 5 days after exposure.

6 We conclude that persistent nicotinic blockade by CHL is neither restricted to mesostriatal dopamine neurons, nor to the CNS, nor to neurons possessing the same nicotinic receptor pharmacology. In addition, the persistent blockade does not appear to result from an acute blocking action, but may be dependent upon intracellular accumulation of the antagonist.

Keywords: Nicotine; noradrenaline; dopamine; nicotinic receptors; synaptosomes; striatum; hippocampus; chlorisondamine; PC12 cells; glutamate receptors

Introduction

The ganglion blocker chlorisondamine (CHL) (Plummer *et al*., 1955) is a bisquaternary nicotinic antagonist that exerts a remarkably persistent nicotinic blockade within the CNS (Clarke & Kumar, 1983). This effect persists for several weeks or more with little or no sign of recovery, and can be produced after either a low intracerebral dose or by a high systemic dose (Clarke & Kumar, 1983; Clarke, 1984; Reavill *et al*., 1986; Kumar *et al*., 1987; Mundy & Iwamoto, 1988; Corrigall *et al*., 1992; Clarke *et al*., 1994a; Decker *et al*., 1994; El-Bizri & Clarke, 1994b,c; El-Bizri *et al*., 1995). The CNS blockade resulting from a single systemic dose far outlasts blockade of ganglionic transmission (Clarke *et al*., 1994a). However, recent preliminary evidence suggests that CHL, given in divided doses, can produce a partial nicotinic antagonism in the periphery lasting for several days (Strong *et al*., 1997).

The mechanism underlying the persistent *in vivo* blockade is unknown, but several possibilities have been examined in brain tissue. A neurodegenerative effect appears unlikely, on the basis of silver staining (Clarke *et al*., 1994a). CHL does not appear to alter the density of nicotinic cholinoceptors (nAChRs) in rat brain after a period of chronic blockade, as assessed by binding of [^{3}H]-nicotine and [^{125}I]-α-bungarotoxin (El-Bizri & Clarke, 1994c). The long-term block may depend in part on retention by the blood brain barrier, but physical trapping of this kind cannot provide a complete explanation, since *in vivo* treatment with CHL results in a persistent *ex vivo* blockade that resists prolonged washing (El-Bizri & Clarke, 1994b).

We have hypothesized that the long-term block that follows the administration of CHL depends on intracellular trapping of drug or of a metabolite (El-Bizri *et al*., 1995). This suggestion was based on the observation that intracerebroventricular administration of [^{3}H]-CHL leads to a long-lasting and regionally selective accumulation of radiolabel in rat brain (El-Bizri *et al*., 1995). Accumulation persisted for at least 3 months, and appeared to be intraneuronal. Particularly high concentrations were noted in presumed dopaminergic cell bodies within the substantia nigra pars compacta and ventral tegmental area, in the dorsal raphé nucleus, and in the cerebellar granular layer.

To date, *ex vivo* nicotinic blockade by CHL has been identified only within the nigrostriatal dopamine system (El-Bizri & Clarke, 1994b; El-Bizri *et al*., 1995). Thus, in striatal synaptosomes, release of [^{3}H]-dopamine can be evoked by stimulation of presynaptic nAChRs, and this response is abolished by *in vivo* administration of CHL, even when the antagonist is given several weeks before sacrifice (El-Bizri &

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Clarke, 1994b). We now report an analogous blockade of presynaptic nAChRs within the noradrenergic projection to the hippocampus.

This finding has permitted us to address the following issues. (1) Is the long-term blockade by CHL restricted to a particular nAChR subtype? The nicotinic cholinoceptors controlling noradrenaline release from hippocampal synaptosomes differ pharmacologically from those controlling striatal dopamine release (Clarke & Reuben, 1996; Kulak *et al*., 1997). (2) Is the persistent *ex vivo* blockade in this neuronal population accompanied by evidence of intracellular accumulation of drug? (3) Is the persistent blockade selective for nAChRs ? The only non-nicotinic action of CHL identified thus far is a weak antagonism of NMDA-type glutamate receptors, seen after acute *in vitro* exposure (Clarke *et al*., 1994a). The possibility of a persistent *in vivo* blockade of NMDA-type receptors has not previously been investigated. Therefore, responses to NMDA were tested *ex vivo* in hippocampal synaptosomes (Pittaluga & Raiteri, 1990) during the persistent phase of blockade. (4) Is the persistent blockade by CHL unique to the brain, and is it a consequence of acute blockade ? These questions were addressed in rat pheochromocytoma (PC12) cells.

Methods

Male Sprague-Dawley rats (Charles River, St. Constant, Quebec, Canada), weighing 200–250 g, were maintained on a 12/12 h light-dark cycle. Rats were housed four per cage, and food and water were available *ad libitum*. Subjects were allowed to accommodate to the housing conditions for 4 days after arrival, and were drug-naive prior to testing.

Intracerebroventricular administration of chlorisondamine

The procedure for administering CHL and [^{3}H]-CHL was identical. Rats were anaesthetized with ketamine hydrochloride (125 mg kg^{-1} i.p.) and xylazine hydrochloride (10 mg kg^{-1} i.p.). The level of anaesthesia was ascertained by foot pinch. They were mounted in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, U.S.A.) and received bilateral i.c.v. infusions of either CHL (5 μg base per side in 2 μl 0.9% sterile saline) or vehicle alone. Infusions were given at 1.6 μl min^{-1}, *via* stainless steel 30-gauge cannulae connected by PE 10 tubing to a syringe pump (Sage model 341B; Orion Research, Boston, MA, U.S.A) ($n=16$ rats per group).

Stereotaxic coordinates were: 0.5 mm anterior to bregma, 1.0 mm lateral to the midline derived from skull landmarks and the ear bars, and 4.6 mm posterior to bregma; the toothbar was set 3.5 mm below the ear bars. Cannulae were retracted 5 min after the end of the infusion. The scalp was sutured and treated with 4% antibacterial powder (Topazone; Austin Laboratories, Joliette, Quebec, Canada), and the analgesic buprenorphine hydrochloride (0.05 mg kg^{-1} s.c.) was given. Subjects were sacrificed 3 weeks after surgery.

[^{3}H]-chlorisondamine autoradiography and tyrosine hydroxylase immunohistochemistry

Following administration of [^{3}H]-CHL, radiolabel was visualized autoradiographically, as previously described (El-Bizri *et al*., 1995). In brief, coronal brain sections (20 μm thick) were cryostat-cut, mounted on glass microscope slides, dried, and exposed for 11 days to tritium-sensitive film along with tritium standards (^{3}H-micro-scales: equivalent to 0.07–33.4 nCi mg^{-1} tissue; Amersham). In the present study, sections were taken at 0.5 mm intervals through the substantia nigra pars compacta/ventral tegmental area, and at 0.2 mm intervals starting anterior to the locus coeruleus (LC) and ending posterior to it. Autoradiographs were visualized and quantitated with an MCID M4 microcomputer-based system (Imaging Research, St. Catherines, Ontario, Canada).

It was originally intended to identify the LC by superimposing autoradiographic images with images of the same sections processed for tyrosine hydroxylase immunohistochemistry. However, pilot studies showed the autoradiographic and immunostaining procedures to be incompatible. Instead, brain structures were identified by superimposing the film images with images of the corresponding Nissl-stained sections.

In order to aid identification of the LC in Nissl-stained sections, the brains from two additional weight-matched rats were processed both for tyrosine hydroxylase immunostaining and for Nissl staining, performed in adjacent sections taken through the entire rostrocaudal extent of the LC. Briefly, rats were deeply anaesthetized with Na pentobarbitone prior to fixation by intracardial perfusion with 0.1 M phosphate buffered saline (PBS) containing 4% paraformaldehyde and 2% picric acid. Brains were removed and post-fixed in the same solution for 2 days at 4°C followed by 1 day immersion in 15% sucrose buffered solution, prior to cryostat sectioning. Freely floating coronal sections (40 μm thick) were immersed in 0.1 M PBS containing 0.2% Triton X-100 and 0.3% H_2O_2 for 30 min at room temperature. The primary antibody (mouse anti-tyrosine hydroxylase monoclonal antibody, Incstar Corp., Stillwater, MN, U.S.A.) was applied overnight at 4°C at 1:2500 dilution. Staining was obtained by reacting avidin-biotinylated horseradish peroxidase complexes with 0.02% diaminobenzidine and 0.01% H_2O_2, using a commercially-supplied kit (Vectastain ABC kit, Burlingane, CA, U.S.A.). The sections were mounted on gelatin-coated slides, and adjacent sections were Nissl-stained with cresyl violet.

Autoradiographs were quantitated in the several brain nuclei that showed high retention of tritium (Watson and Paxinos atlas level in parentheses): ventral tegmental area and substantia nigra pars compacta (both at IA 3.7), LC and granular layer of midline cerebellum (both IA-0.68). For comparison, three 'background' areas were also sampled: a 1 mm^2 box located 1 mm dorsal to the substantia nigra, a 1×4 mm horizontal box located 1 mm ventral to the LC, and the middle cerebellar peduncle (IA-0.68).

Dopamine and noradrenaline release from superfused synaptosomes

Methods for synaptosomal preparation and measurement of transmitter release were virtually identical to those described in detail elsewhere (Clarke *et al*., 1994b). In each assay, crude synaptosomal (P2) fraction was prepared from dissected striata and/or hippocampus (approximately 140 and 150 mg per rat, respectively). Where no *in vivo* pretreatment was given, brain tissue was pooled from four rats per assay. The P2 fractions were resuspended (140–150 mg wet weight of original tissue ml^{-1}) in superfusion buffer (SB) composed of the following (in mM concentrations): NaCl, 128; KCl, 2.4; $CaCl_2$, 3.2; KH_2PO_4, 1.2; $MgSO_4$, 1.2; HEPES, 25; (+)-glucose, 10; (+)-ascorbic acid, 1 and pargyline, 0.01 at pH 7.5. These synaptosomal preparations were incubated with 0.1–0.2 μM [^{3}H]-dopamine

([^{3}H]-DA; striatum) or 0.2 μM [^{3}H]-noradrenaline ([^{3}H]-NA; hippocampus) at 37°C for 10 min.

The superfusion apparatus contained 32 identical channels, each comprising a small polypropylene retention chamber, through which superfusate was pumped at 0.4 ml min^{-1}. Each experiment comprised one or more assays. In each assay, data were collected simultaneously from all 32 channels. At the start of each assay, 100 μl of the synaptosomal suspension was introduced to each superfusion chamber. During the next 35 min, synaptosomes were superfused with superfusion buffer (SB) alone or with SB containing antagonist, as appropriate. Next, 12 samples per channel were collected in consecutive 1 min intervals: after a 5 min baseline collection period, a 1 min (0.4 ml) pulse of releasing drug or SB (prepared with or without antagonist as appropriate) was given. Finally, the filters holding the synaptosomes were removed in order to measure residual radioactivity (Wallac 1410 liquid scintillation counter, LKB, Sweden). Each assay incorporated control (SB only) channels, and tissues and treatment conditions were counterbalanced across channels and assays. In striatal and hippocampal synaptosomes preloaded with [^{3}H]-DA or [^{3}H]-NA, tritium release largely corresponds to unmetabolized transmitter (Rapier *et al*., 1988; Pittaluga & Raiteri, 1992). Therefore, in the present study, we refer to DA or NA release, as appropriate.

For each channel, the release occurring in each 1 min collection period was calculated as a percentage of basal release, determined from a 5 min baseline. For each individual channel, drug-evoked release was taken as the peak value occurring among the next three or four fractions. This measure of release is likely to be less affected than the time-averaged drug effect ('area under the curve') by receptor desensitization that occurs within the first minutes of drug administration (Rapier *et al*., 1988; Grady *et al*., 1994), although in practice, similar results have been obtained by both measures (data not shown).

Noradrenaline release from PC12 cells

Rat pheochromocytoma (PC12) cells were obtained from Dr D.K. Berg (University of California, San Diego,U.S.A.). They were of the recently characterized PC12-B variant reported to express nAChRs exclusively insensitive to α-bungarotoxin (Blumenthal *et al*., 1997). The cells were grown in 250 ml flasks (Primaria Falcon 3824) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The culture medium, which was changed every 3 days, comprised Dulbecco's Modified Eagle's Medium (DMEM) containing a high glucose concentration (4.5 g l^{-1}), with added heat-inactivated 10% fetal calf serum and 5% horse serum, 50 u ml^{-1} penicillin and 50 μg ml^{-1} streptomycin. Cells were passaged when 80% confluent.

Prior to a release assay, cells were plated in 24-well tissue culture plates (Falcon 3047) pre-coated with poly-D-lysine at a density of 50,000 cells per well, and maintained in the culture medium supplemented with 50 ng ml^{-1} 2.5 S nerve growth factor for a period of 4–8 days.

On the test day, fresh culture medium (0.3 ml per well) was substituted, now including 0.05 μM [^{3}H]-NA and 1 mM ascorbic acid, and the cells were returned to the incubator for 1 h. All subsequent steps were conducted at room temperature. Medium was removed and each well was washed several times (three rinses of 1 ml each for 10 min, then five rinses of 0.5 ml each for 2 min). The wash medium was HEPES-buffered Kreb's Ringer saline, composed as follows (in mM concentrations): NaCl, 125; KCl, 4.8; $CaCl_2$, 2.6; KH_2PO_4, 1.2; $MgSO_4$, 1.2; HEPES, 25; (+)-glucose, 5.6; (+)-ascorbic acid, 1 at pH 7.4 (Greene & Rein, 1977). Collection periods, each of 2 min duration, were initiated immediately: baseline (one to four periods), agonist challenge (one period), and post-drug (two or three periods). Cells were then lysed with 0.4 M perchloric acid to determine residual [^{3}H]-NA content.

For each incubation well, the release occurring in each collection period was calculated as a percentage of basal release, which was determined from one or more collection periods occurring immediately prior to agonist administration. Agonist-evoked release was typically confined to the challenge period and one subsequent period; it was therefore expressed as the mean release (as a percentage of basal) occurring in the these two periods.

Drugs

Chemicals and suppliers were as follows: 3,4-[ring-2,5,6-^{3}H]-dopamine (60 Ci mmol^{-1}) and levo-[ring-2,5,6-^{3}H]-noradrenaline (55 Ci mmol^{-1}) and [^{3}H]-chlorisondamine chloride (specific activity 3.0 Ci mmol^{-1}) (New England Nuclear, Boston, MA, U.S.A.), l-nicotine hydrogen tartrate, cytisine, poly-D-lysine and N-methyl-D-aspartate (Sigma Chemical Corp., St. Louis, MO, U.S.A.), R(−)-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (D-CPP), strychnine HCl, pargyline HCl, tetrodotoxin and veratridine (Research Biochemicals Inc., Natick, MA, U.S.A.), glycine (BDH, Toronto, Ontario, Canada), DMEM (GIBCO BRL, Canadian Life Technologies, Burlington, Ontario, Canada), 2.5 S nerve growth factor (Prince Laboratories, Toronto, Ontario, Canada). The following were generous gifts: chlorisondamine chloride (CHL) (Ciba-Geigy, Summit, NJ, U.S.A.) and [^{3}H]-CHL (New England Nuclear). Other chemicals and reagents were purchased from commercial sources. For superfusion, drugs were dissolved in superfusion buffer (SB).

Data analysis

Drug effects were examined by analysis of variance, using commercial software (Systat, Evanston, IL, U.S.A.). Multiple comparisons with a single control group were made with Dunnett's *t*-test (Dunnett, 1955); other multiple comparisons were made by Student's *t*-test with Bonferroni's correction (Glantz, 1992). Probability values are two-tailed.

Results

Persistence of chlorisondamine blockade ex vivo *in hippocampal synaptosomes*

Rats were randomly allocated to three groups, and received either CHL 10 μg i.c.v. or CHL 10 mg kg^{-1} s.c. or saline s.c. ($n=5$ per group). Three weeks later, nicotine-evoked release of hippocampal [^{3}H]-NA and of striatal [^{3}H]-DA release was tested *ex vivo*. One rat from each pretreatment group contributed tissue to each assay (two to three channels per condition per rat). Synaptosomes were challenged acutely with a supramaximal concentration of nicotine (100 μM) or with superfusion buffer (SB).

In the absence of CHL, nicotine increased [^{3}H]-NA and [^{3}H]-DA release to similar extents (Figure 1). Pretreatment with CHL, three weeks previously, antagonized both effects. After s.c. CHL, a small residual response was seen, whereas after i.c.v. CHL, blockade was virtually complete.

NMDA-evoked NA release in hippocampal synaptosomes

Channels to be tested with NMDA were superfused with Mg^{2+}-free buffer in which an equivalent molar concentration of Na^{+} was substituted. The Mg^{2+}-free buffer also contained the NMDA co-agonist glycine (10 μM) together with strychnine (1 mM) in order to block inhibitory glycine receptors. NMDA (10–320 mM; given in the same buffer) increased [^{3}H]-NA release in a concentration-dependent manner, but the effect was weak, especially compared to that obtained in parallel by nicotine 100 μM (Table 1).

The releasing action of NMDA 100 μM was abolished by the NMDA receptor antagonist MK-801 (1 μM), and reduced by inclusion of Mg^{2+} 1.2 mM (Table 2). Since MK-801 antagonizes both NMDA and nAChRs (Ramoa *et al*., 1990; Clarke & Reuben, 1995), the competitive NMDA receptor blocker, CPP, was also tested. CPP 5 μM significantly reduced the response to NMDA 100 μM but did not alter the response to nicotine 100 μM (Table 2). Basal release was not significantly affected by MK-801 (4% decrease, $P>0.3$), was reduced by Mg^{2+} (by 12%, $P<0.005$), and was increased by CPP (by 16%, $P<0.0005$).

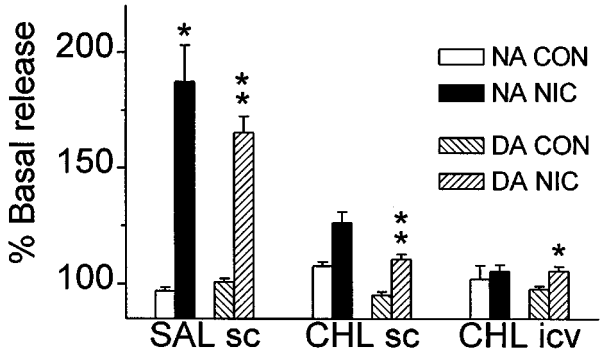

Figure 1 Effects of *in vivo* treatment with chlorisondamine on *ex vivo* [^{3}H]-NA and [^{3}H]-DA release evoked by nicotine. Rats received either saline s.c. (SAL) or CHL 10 mg kg^{-1} s.c. or CHL 10 μg i.c.v. ($n=5$ per group). Three weeks later, synaptosomes were prepared from the hippocampus and striatum of each rat, and loaded respectively with [^{3}H]-NA and [^{3}H]-DA. Release was evoked by a 1 min pulse of nicotine 100 μM (NIC) or of superfusion buffer alone (CON). The vertical axis represents the mean ($\pm$s.e.mean) peak release, calculated as a percentage of basal (i.e. pre-agonist) release ($n=5$ rats). *$P<0.02$, **$P<0.01$ nicotine *vs* control (Bonferroni *t*-test). Basal release of [^{3}H]-NA and [^{3}H]-DA did not significantly differ across pretreatment groups (main effect of CHL: $F<1.0$, *d.f.* 2, 12); pooled across groups, mean$\pm$s.e.mean values for [^{3}H]-NA and [^{3}H]-DA basal release were 1060$\pm$30 and 2230$\pm$110 d.p.m. min^{-1}, respectively.

Chlorisondamine blockade ex vivo *and NMDA-evoked NA release*

Rats were randomly allocated to receive either CHL 10 mg kg^{-1} s.c. or saline s.c. ($n=8$ per group). One week later, drug-evoked [^{3}H]-NA and [^{3}H]-DA release was tested *ex vivo*. Two rats from each pretreatment group were tested in each assay (two channels per condition per rat).

Hippocampal synaptosomes were challenged with nicotine 100 μM or NMDA 100 μM (in Mg^{2+}-free SB) or with SB. Striatal synaptosomes were challenged only with nicotine 100 μM. In saline pretreated rats, nicotine clearly increased release of both [^{3}H]-NA and [^{3}H]-DA (Figure 2). In these animals, the NMDA effect was small but significant ($t=4.59$, *d.f.* 7, $P<0.005$). *In vivo* pretreatment with CHL greatly inhibited nicotine-evoked release of [^{3}H]-NA and [^{3}H]-DA (Bonferroni *t*-test: $P<0.001$ for both; Figure 2), but did not significantly affect NMDA-evoked release ($P>0.9$).

Table 1 [^{3}H]-NA release evoked by N-methyl-D-aspartate (Experiment 2)

Drug	*SB*	*NMDA*	*NMDA*	*NMDA*	*NMDA*	*Nicotine*
Conc (μM)	0	10	32	100	320	100
Peak effect	97$\pm$2	101$\pm$2	*107$\pm$2	**114$\pm$3	**118$\pm$3	**180$\pm$18
n (channels)	10	11	11	11	10	5

Drug challenge comprised a pulse of superfusion buffer (SB) or NMDA or nicotine. Evoked release is expressed as mean$\pm$s.e.mean peak effect, as a percentage of basal release. *$P<0.05$, **$P<0.0001$ *vs* superfusion buffer (Dunnett's *t*-test).

Table 2 Antagonism of N-methyl-D-aspartate-evoked [^{3}H]-NA release (Experiment 2)

Pretreatment	*SB*	*SB*	*MK-801* μM	*Mg*$^{2+}$ 1.2 mM
challenge	*SB*	*NMDA* 100 μM	*NMDA* 100 μM	*NMDA* 100 μM
Peak effect	103$\pm$3	*121$\pm$4	$^{++}$101$\pm$2	$^{++}$108$\pm$2
n (channels)	12	11	10	12

Pretreatment	*SB*	*SB*	*CPP* 5 μM	*SB*	*CPP* 5 μM
challenge	*SB*	*NMDA* 100 μM	*NMDA* 100 μM	*Nic* 100 μM	*Nic* 100 μM
Peak effect	100$\pm$2	*115$\pm$2	*$^{+}$109$\pm$2	*210$\pm$12	*209$\pm$7
n (channels)	13	17	17	13	14

Drug challenge comprised a pulse of superfusion buffer (SB), NMDA or nicotine. Evoked release is expressed as mean$\pm$s.e.mean peak effect, as a percentage of basal release. *$P<0.005$ *vs* SB/SB alone, $^{+}P<0.05$, $^{++}P<0.01$ for agonist alone *vs* agonist plus antagonist (Tukey's test).

Distribution of radiolabel after in vivo *[^{3}H]-chlorisondamine administration*

Three rats received bilateral infusions of [^{3}H]-CHL into the lateral ventricles (5 μg in 2 μl per side) and were sacrificed 3 weeks later for autoradiographic visualization of radiolabel. Tyrosine hydroxylase immunostaining was performed in two additional rats; comparison with adjacent Nissl-stained sections confirmed that the mesencephalic trigeminal nucleus (Me5), which is prominent in Nissl-stained sections, lies immediately lateral to the locus coeruleus (LC) and is virtually coextensive with it in the anteroposterior dimension (Paxinos & Watson, 1986). This close relationship aided identification of the LC in Nissl-stained sections.

Computer-assisted registration of the histological sections and autoradiographs revealed that the entire LC was selectively labelled (Figure 3). Quantitation further revealed that the concentrations of tritium in the LC were comparable to other previously identified areas (substantia nigra pars compacta – SNC, ventral tegmental area – VTA, and granular layer of cerebellum). Mean $\pm$ s.e.mean values ($n=3$ rats) were as follows (pmol mg^{-1} wet tissue equivalent values with reference to [^{3}H]-CHL): 15.6 ± 2.0 (LC), 19.7 ± 1.8 (SNC), 11.8 ± 0.1 (VTA), and 14.6 ± 2.3 (granular cerebellum). These values were much greater than in neighbouring regions: 1.8 ± 0.4 (grey matter ventral to LC), 2.5 ± 0.5 (grey matter dorsal to SNC), and 0.7 ± 0.1 (white matter - middle cerebellar peduncle).

Concentration-dependent effects of nicotine and cytisine on [^{3}H]-NA release from PC12 cells

Nicotine (10, 30, 100, 300 and 600 μM) evoked a concentration-dependent increase in [^{3}H]-NA release from PC12 cells. The results of two pooled assays are shown in Figure 4a. Half-maximal and maximal release occurred at approximately 15 μM and 100 μM, respectively. A third assay (not shown) yielded a smaller maximal effect (210% of basal) but similar potency; the apparent down-turn at the highest concentration (600 μM) was not confirmed. The nicotinic agonist cytisine (3, 10, 30 and 100 μM) also increased [^{3}H]-NA release in a concentration-dependent manner, with a potency similar to that of nicotine (Figure 4b). The maximal effect of cytisine within the concentration range tested was at least as great as that of a high concentration of nicotine (100 μM) tested in parallel.

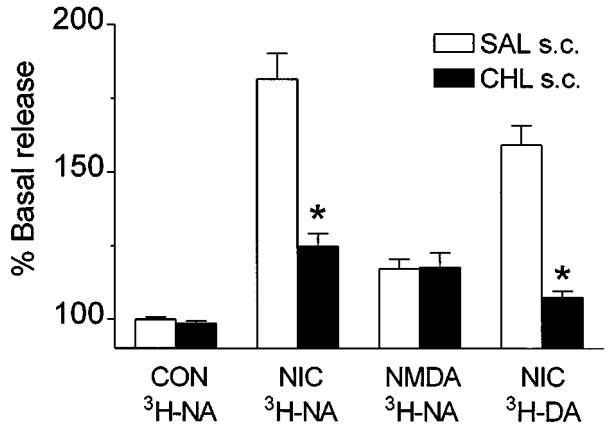

Figure 2 Effects of *in vivo* treatment with chlorisondamine on *ex vivo* responses to nicotine and N-methyl-D-aspartate (NMDA). Rats received either saline s.c. or CHL 10 mg kg^{-1} s.c. ($n=8$ per group). One week later, synaptosomes were prepared from the hippocampus and striatum of each rat, and loaded respectively with [^{3}H]-NA and [^{3}H]-DA. Hippocampal synaptosomes were challenged with superfusion buffer (CON) or nicotine 100 μM or NMDA 100 μM (in Mg^{2+}-free SB). Striatal synaptosomes were challenged only with nicotine 100 μM. The vertical axis represents the mean ($\times$ s.e.mean) peak release, calculated as a percentage of basal (i.e. pre-agonist) release ($n=8$ rats). *$P<0.001$ CHL *vs* control (Bonferroni *t*-test). CHL pretreatment did not significantly alter basal release of [^{3}H]-NA (saline *vs* CHL: 996×109 *vs* 1192×100 d.p.m. min^{-1}, $t=1.33$, *d.f.* 14) or [^{3}H]-DA (2125×126 *vs* 1796×101 d.p.m. min^{-1}, $t=2.00$, *d.f.* 14).

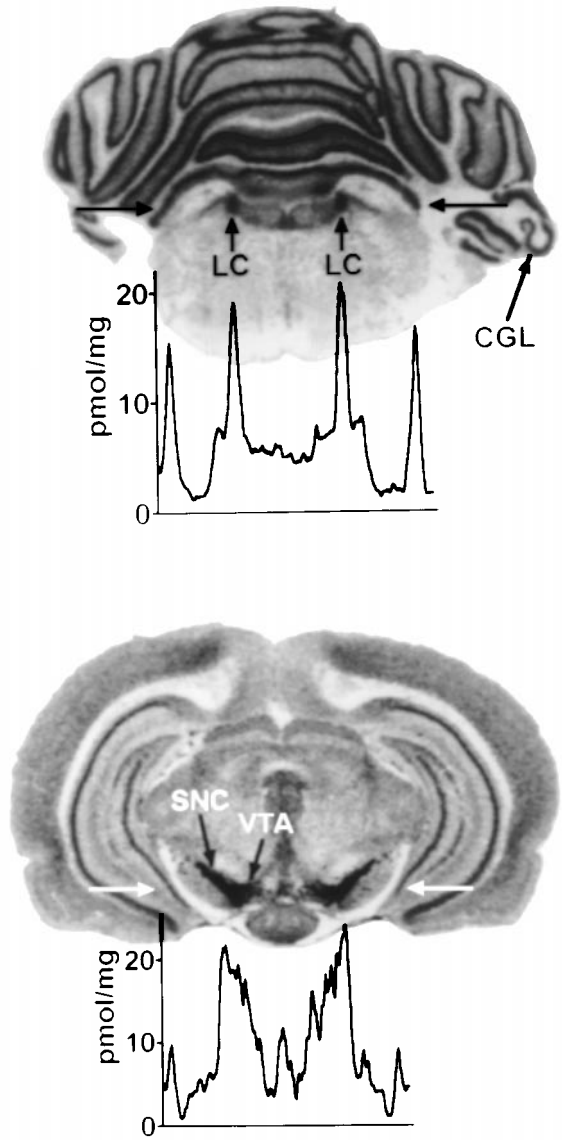

Figure 3 Autoradiographs prepared from two coronal brain sections of a representative rat that received bilateral infusions of [^{3}H]-chlorisondamine (10 μg), 3 weeks prior to sacrifice. The images were computer-generated with reference to tritium-impregnated standards. The upper image is of a section that includes the locus coeruleus (LC), and shows a preferential retention of radiolabel in this nucleus as well as in the granular layer of the cerebellum (CGL). The inset graph shows the density profile (pmol mg^{-1} wet tissue equivalent values with reference to [^{3}H]-CHL) measured along a line between the two horizontal arrows. The lower image and inset are from a section taken through the substantia nigra pars compacta (SNC) and ventral tegmental area (VTA), showing preferential radiolabelling in these two dopamine cell body regions.

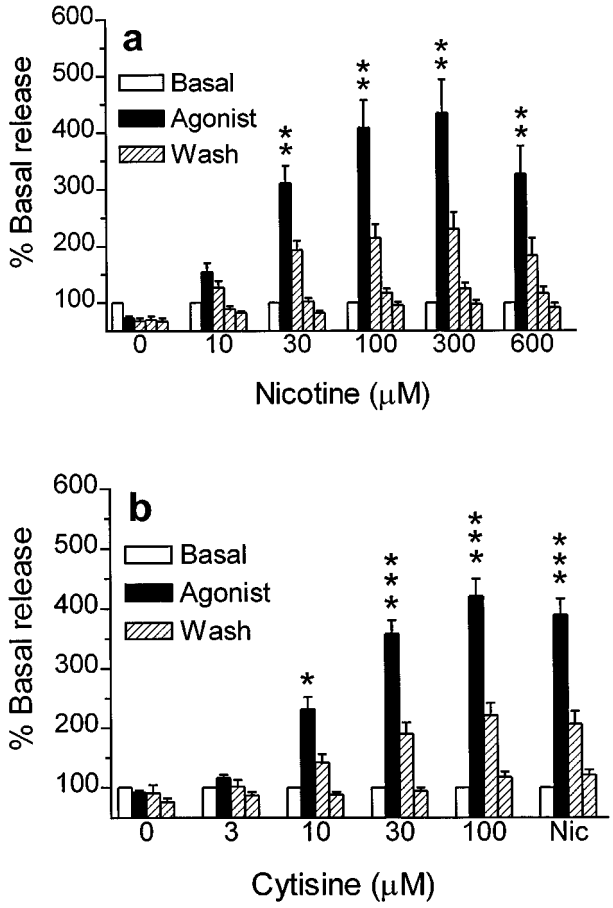

Figure 4 [^{3}H]-NA release from rat PC12 cells evoked by nicotine (a) and cytisine (b). Cells were incubated with [^{3}H]-NA and rinsed. Subsequent collection periods, each of 2 min duration, were as follows: basal (one period), agonist challenge (one period), and post-agonist wash (two or three periods). Basal release (mean ± s.e.mean) was 869 ± 25 d.p.m. 2 min for nicotine and 924 ± 76 d.p.m./2 min for cytisine. The vertical axis represents the mean (± s.e.mean) release, calculated as a percentage of basal release ($n = 12$ wells pooled from two assays). The effects of cytisine were directly compared to that of a high concentration of nicotine (Nic: 100 μM) tested in parallel. $*P < 0.01$, $**P < 0.001$, $***P < 0.0001$ *vs* no agonist (Dunnett's test).

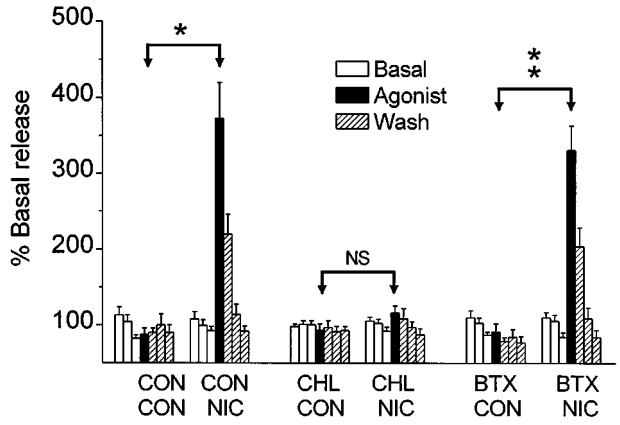

Figure 5 Acute blockade of nicotine-evoked [^{3}H]-NA release by co-incubation with chlorisondamine and α-bungarotoxin in PC12 cells. Cells were incubated with [^{3}H]-NA and rinsed, then exposed to CHL 1 μM and α-bungarotoxin 0.3 mM (BTX) or buffer (CON) for 50 min prior to and during the challenge with nicotine 30 μM or buffer. Subsequent collection periods, each of 2 min duration, were as follows: basal (three periods), agonist challenge (one period), and post-agonist wash (two periods). The vertical axis represents the mean (× s.e.mean) release, calculated as a percentage of basal release ($n = 8$ wells). CHL exerted a complete block, whereas α-bungarotoxin, tested in parallel, was ineffective. $*P < 0.005$, $**P < 0.0001$, NS not significant (Bonferroni *t*-test). Neither antagonist significantly affected basal release ($F = 2.31$, *d.f.* 2,45); mean ± s.e. mean values for control, CHL, and α-bungarotoxin channels were, respectively: 1083 ± 39, 944 ± 42 and 1086 ± 65 d.p.m./2 min.

Acute antagonism by nicotinic antagonists in PC12 cells

To test whether nicotine-evoked NA release from PC12 cells was mediated by nAChRs, cells were challenged with nicotine 100 μM or buffer in the presence or absence of the nicotinic antagonist mecamylamine (10 μM). Mecamylamine, where given, was present throughout the assay, starting 2 min before the agonist challenge. Mecamylamine completely blocked nicotine-evoked [^{3}H]-NA release. Thus, in the absence of mecamylamine, nicotine increased release (mean ± s.e.mean above basal: 246 ± 18% *vs* 110 ± 17% for buffer alone), whereas in the presence of mecamylamine, equivalent values were 85 ± 5% (nicotine) and 111 ± 22% (buffer). Basal release appeared reduced by mecamylamine (787 ± 71 *vs* 670 ± 72 DPM/2 min), but this difference was not significant ($t = 1.25$, *d.f.* 22, $P > 0.2$).

Subsequently, acute antagonism by CHL (1 mM) and α-bungarotoxin (0.3 μM) was tested in parallel (Figure 5). The antagonists were present for 50 min prior to and during the challenge with nicotine 30 μM or buffer. CHL exerted a complete block, whereas α-bungarotoxin was ineffective.

Short-term and long-term persistence of antagonism by chlorisondamine in PC12 cells

In the first test of residual antagonism, PC12 cells were challenged with nicotine approximately 2 h after a relatively brief (1 h) exposure to CHL. Thus, cells were exposed to CHL (1, 10, or 100 μM) or medium alone, followed by two rinses prior to incubation for 1 h in [^{3}H]-NA. After further rinses, basal release was collected and the cells were challenged with nicotine 30 μM or buffer alone. Nicotine-evoked release was almost abolished by the higher concentrations of CHL (10 and 100 μM) but was not significantly reduced by CHL 1 μM (Figure 6a).

The possibility of a more persistent antagonism was tested using the same concentrations of CHL. Cells were exposed to CHL or medium alone for a longer period (24 h) and were challenged 3 days later with nicotine 30 μM or buffer alone. The results of five assays were pooled. Significant nicotinic blockade was observed, but it was incomplete (48% reduction) and was associated with only the highest concentration of CHL (100 μM) (Figure 6b). This high concentration was used in subsequent experiments.

Time course of residual nicotinic block after exposure to chlorisondamine

To investigate how rapidly this residual blockade was lost, the response to nicotine 30 μM was tested at 0, 1 or 2 days after 24 h of exposure to CHL 100 μM or medium alone. Substantial but incomplete blockade was seen when cells were tested on the day of CHL withdrawal (Figure 7). On the following 2 days, however, no significant blockade occurred, even when these data were pooled (unprotected *t*-test: $t = 1.71$, *d.f.* 34, $P > 0.09$).

These results, taken with those of the previous experiment, suggested that blockade might wane in the first two days following CHL exposure and subsequently re-emerge. To test this, the response to nicotine 30 μM was determined at 0, 2 and 5 days after exposure to CHL (100 μM). This experiment was repeated twice. It was found that control responses to nicotine alone varied in magnitude between assays. Accordingly, data from each well were normalized with reference to the mean values of buffer alone (0%) and nicotine alone (100%) obtained in the same assay. CHL pretreatment resulted in significant antagonism at all three times after CHL exposure, with no evidence of a biphasic effect. The percentage reduction was: $50.5 \pm 11.7\%$ at 0 days after CHL (Bonferroni t-test $P<0.001$), $27.8 \pm 10.1\%$ at 2 days after CHL ($P<0.05$), and $31.2 \pm 9.4\%$ at 5 days after CHL ($P<0.01$). As in previous experiments, CHL pretreatment did not significantly alter basal release (CHL *vs* medium alone: 507 ± 15 *vs* 533 ± 12 d.p.m./2 min; $F=0.17$, *d.f.* 1,237).

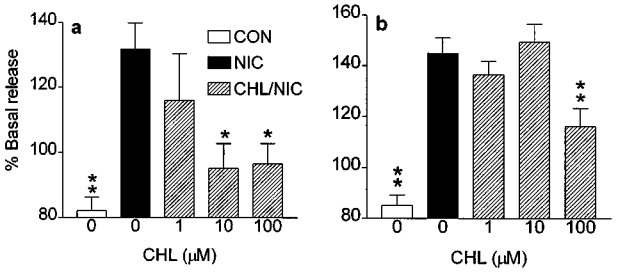

Figure 6 Acute (a) and subchronic (b) antagonism of nicotine-evoked [^{3}H]-NA release by chlorisondamine in PC12 cells. In the acute CHL experiment, cells were exposed to CHL or medium alone (CON) for 1 h prior to loading with [^{3}H]-NA and acute challenge comprising nicotine 30 μM (NIC) or buffer (CON). In the subchronic experiment, cells were exposed to CHL or buffer alone for 24 h, and were maintained in CHL-free medium for 3 days prior to testing. The vertical axis represents the mean ($\pm$s.e.mean) release, pooled across the agonist period and the period following it, calculated as a percentage of basal release. The acute CHL experiment represents the results of three pooled assays ($n=10-12$ wells except nicotine alone $n=23$). Basal release was not significantly altered by acute pretreatment with CHL (main effect: $F=0.93$, *d.f.* 3, 64); mean $\pm$s.e.mean values for the 0, 1, 10 and 100 μM groups were, respectively: 444 ± 26, 533 ± 65, 490 ± 44 and 507 ± 59 d.p.m./2 min. In the subchronic experiment, the results of five assays were combined ($n=26-29$ wells except nicotine alone $n=46$). Basal release was not significantly altered by subchronic CHL (main effect: $F=0.29$, *d.f.* 3,149); mean $\pm$s.e.mean values for the 0, 1, 10 and 100 μM groups were, respectively: 633 ± 58, 681 ± 105, 548 ± 88, and 641 ± 127 d.p.m./2 min. $^*P<0.02$, $^{**}P<0.005$ *vs* nicotine alone (Dunnett's t-test).

Pharmacological selectivity of persistent antagonism by chlorisondamine

To test whether the persistent antagonism by CHL was selectively nicotinic, responses to nicotine (30 μM) and high K^+ (56 mM) were directly compared. Cells were treated for 24 h with CHL 100 μM or medium alone and were challenged 2 days later. In this experiment, the period of CHL exposure was terminated by a 10 min incubation during which fresh medium containing CHL (100 μM) and nicotine 30 μM was substituted. This extra step served to investigate whether a more profound nicotinic block could be obtained by stimulating the nAChR ionophore to open and thus promote a use-dependent block as demonstrated in other tissues (Alkadhi & McIsaac, 1974; El-Bizri & Clarke, 1994a). CHL produced a 50% reduction in nicotine-evoked NA release but did not alter release evoked by high K^+ ($t=0.68$, *d.f.* 57, $P>0.5$) (Figure 8).

Discussion

The main findings of this study are as follows. A single administration of CHL, either systemic or intracerebroventricular, resulted in a long-term and selectively nicotinic block in hippocampal synaptosomes. This blockade was accompanied by an apparent accumulation of drug (or metabolite) in

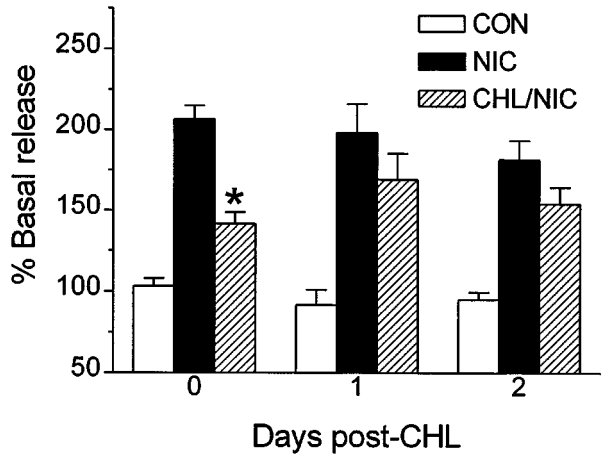

Figure 7 Time course of recovery from blockade by chlorisondamine in PC12 cells. Cells were exposed to CHL or medium alone for 24 h. Subsequently, they were either tested with nicotine 30 μM (NIC) or buffer (CON) directly after loading with [^{3}H]-NA or were maintained in CHL-free medium for 1 or 2 days prior to testing. The vertical axis represents the mean ($\pm$s.e.mean) release, pooled across the agonist period and the period following it, calculated as a percentage of basal release ($n=6$ wells except nicotine alone $n=12$). Basal release was unaffected by CHL pretreatment (CHL *vs* medium alone: 502 ± 24 *vs* 506 ± 14 d.p.m./2 min). $^*P<0.0001$ *vs* nicotine alone (Dunnett's t-test).

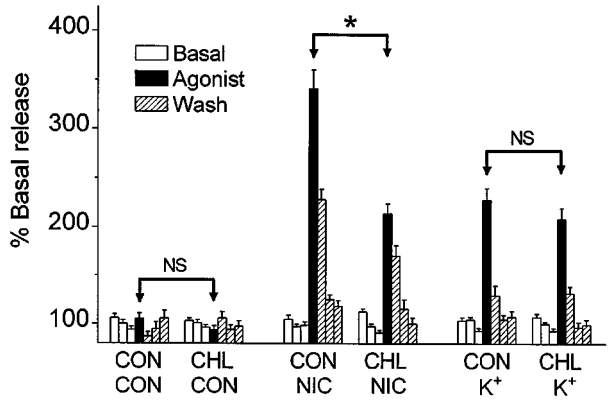

Figure 8 Pharmacologically selective antagonism by chlorisondamine in PC12 cells. Cells were exposed for 24 h to CHL 100 μM or medium (CON). CHL exposure was terminated after a 10 min co-incubation with nicotine 30 μM. Cells were challenged 2 days later with nicotine 30 μM (NIC) or K^+ 56 mM (K^+) or buffer (CON). The vertical axis represents the mean ($\pm$s.e.mean) release, calculated as a percentage of basal release ($n=29-30$ wells). Basal release was unaffected by CHL pretreatment (CHL *vs* medium alone: 996 ± 39 *vs* 1040 ± 42 d.p.m./2 min). $^*P<0.0001$, NS not significant (Bonferroni t-test).

the corresponding neuronal cell bodies within the LC. A form of persistent nicotinic block by CHL was also identified for the first time in cell culture. These findings illuminate several issues.

Is the persistent blockade by chlorisondamine unique to the brain?

CHL persistently blocks a number of central actions of nicotine, as seen in behavioural and other tests (see Introduction). Previously, we have observed only transient effects in the periphery following administration of CHL in a high systemic dose (10 mg kg^{-1} s.c.) that results in central blockade (Clarke *et al*., 1994a). In this study, outward signs of ganglion blockade waned within 24 h, and electrophysiological experiments that were conducted 12 days after CHL administration revealed no residual antagonist action in isolated ganglia. These findings suggested that the long-term block might be unique to the CNS, perhaps reflecting the existence of diffusional barriers and/or metabolic processes (El-Bizri & Clarke, 1994b; El-Bizri *et al*., 1995).

The present study provides the first evidence that administration of CHL can lead to a persistent nicotinic antagonism after *in vitro* administration of CHL. This occurred in cultured cells that are of peripheral, ganglion-like origin (Greene & Tischler, 1976). The blockade was not as complete as in our brain assays, but was nevertheless maintained during several days after drug administration. A preliminary report suggests that a form of persistent ganglionic antagonism can also be obtained *in vivo*, after several administrations of a low dose (0.1 mg kg^{-1} s.c.) of CHL. As in PC12 cells (present study), this antagonism was only partial (Strong *et al*., 1997). However, it appears that the *in vitro* concentration of CHL (100 μM) required to obtain a persistent effect in PC12 cells is approximately 100 fold higher than that required *in vivo*. Thus, whether the same mechanism is involved in unclear.

Is the persistent blockade selective for nicotinic receptors?

CHL acts in a selectively nicotinic fashion both *in vitro* (El-Bizri & Clarke, 1994a,b) and *in vivo* (Clarke, 1984; Reavill *et al*., 1986; Kumar *et al*., 1987; Corrigall *et al*., 1992). The only non-nicotinic action of CHL reported to date was a weak antagonism (IC_{50} 70 μM) at NMDA-type glutamate receptors (Clarke *et al*., 1994a). However, this was observed after acute *in vitro* exposure to CHL, which results in a form of nicotinic blockade that, unlike its *ex vivo* counterpart, is readily reversed by wash-out (El-Bizri & Clarke, 1994b). *In vivo* administration of CHL did not result in *ex vivo* blockade of NMDA-evoked NA release. Hence, it is unlikely that CHL administration results in a long-term *in vivo* blockade of NMDA glutamate receptors, unless this antagonism dissipates *ex vivo* prior to challenge with agonist. Thus, evidence to date, albeit incomplete, suggests that CHL exerts minimal non-nicotinic actions when given in doses used to produce the long-term blockade.

Pharmacological selectivity was also observed in PC12 cells (final experiment), insofar as persistent antagonism by CHL did not extend to NA release evoked by high K^+. These observations do not exclude the possibility that CHL exerts non-nicotinic actions in PC12 cells but they do suggest that both *in vivo* and in cultured cells, the persistent blockade of nicotine-evoked NA release is mediated directly at nAChRs rather than at a subsequent step in the stimulus-secretion pathway. The same conclusion has been drawn from analogous studies of nicotine-evoked DA release (El-Bizri & Clarke, 1994b).

Is the persistent blockade selective for a particular nAChR subtype?

Pharmacological comparisons have indicated that the nAChRs subtypes mediating nicotine-evoked release of striatal [^{3}H]-DA and hippocampal [^{3}H]-NA differ (Clarke & Reuben, 1996; Kulak *et al*., 1997). The finding that both types of nicotinic effect are blocked *ex vivo* by CHL (El-Bizri & Clarke, 1994b; present study) indicates that the persistent blockade associated with this antagonist is not specific to a single nAChR subtype.

The identity of the receptor isoforms that are blocked by CHL in these assays is unknown. At present, the assignment of receptor subtypes to particular nicotinic actions is fraught with uncertainty (for discussion see Clarke & Reuben, 1996; Kulak *et al*., 1997; Sivilotti *et al*., 1997). Modulation of DA release has been tentatively attributed to a subtype that includes both α3 and α4 subunits (Grady *et al*., 1994), and more recently, to two or more different subtypes of nAChR, a minority of which possess an α3/β2 subunit interface (Kulak *et al*., 1997). In contrast, hippocampal NA release may be modulated by nAChRs that contain α3 and β4 subunits (Clarke & Reuben, 1996; Kulak *et al*., 1997). However, these proposals ignore the possible contribution of α6 and β3 subunits, which on recent evidence are expressed at high levels in both DA and NA neurons (Le Novère *et al*., 1996).

The characterization of nAChR subtypes in PC12 cells is complicated by the existence of variant cell lines (Blumenthal *et al*., 1997). The variant (PC12-B) chosen for the present study expresses α3, α5, α7, β2, β3 and β4 subunits, as determined both by levels of transcript and protein (Rogers *et al*., 1992; Blumenthal *et al*., 1997). Other subunits are either undetectable (α2, α4) (Rogers *et al*., 1992) or have not been reported on (α6). The subunit composition of functional nAChRs has not been elucidated, but it is known that α7 protein is only weakly expressed and has not been found to contribute significantly to nicotinic responses (Blumenthal *et al*., 1997). The present observation that nicotine-evoked NA release from PC12-B cells was insensitive to blockade by α-bungarotoxin confirms the lack of involvement of α7-containing nAChRs. A detailed pharmacological characterization of PC12-B cells has yet to be done. However, these cells were reported to be responsive to cytisine (Rogers *et al*., 1992), and we show here that this agonist is of similar potency and at least as efficacious as nicotine in evoking NA release from PC12 cells. These characteristics suggest that the nAChRs in question contain α3 and β4 subunits, and perhaps α5 and β2 subunits as well (Luetje & Patrick, 1991; Papke & Heinemann, 1994; Wong *et al*., 1995; Wang *et al*., 1996; Colquhoun & Patrick, 1997). Hence, on present evidence, nAChRs mediating NA release in hippocampal synaptosomes and PC12-B cells may be of the same subtype.

Is the persistent ex vivo *blockade in CNS noradrenergic neurons accompanied by evidence of intracellular accumulation of drug?*

As the present study confirms, central administration of [^{3}H]-CHL results in a highly persistent and anatomically heterogeneous pattern of radiolabelling in rat brain. Radiolabel is at least partly intracellular, and is present in concentrations several orders of magnitude higher than that of known nAChRs (El-Bizri *et al*., 1995). These characteristics

led us to hypothesize that CHL (or a metabolite) exerts its long-term block of nAChRs by some mechanism dependent upon intraneuronal accumulation. Preferential radiolabelling of the LC was not identified in our previous study (El-Bizri *et al*., 1995), probably because coronal brain sections were taken at rather wide (1 mm) intervals. More detailed re-analysis now reveals that the LC retains levels of tritium comparable to those found in the substantia nigra pars compacta and ventral tegmental area. Since LC neurons are virtually all noradrenergic and provide the entire noradrenergic innervation of the hippocampus (Aston-Jones *et al*., 1995), the occurrence of *ex vivo* blockade in hippocampal synaptosomes is consistent with the hypothesis that intraneuronal accumulation underlies long-term blockade.

Possible mechanisms underlying the persistent antagonism by chlorisondamine

The interstitial concentrations of CHL that are required to produce the persistent central blockade *in vivo* have been estimated to be similar to those that produce acute blockade *in vitro* (Clarke *et al*., 1994a). However, a notable feature of the *ex vivo* form of block associated with CHL is that it does not recover upon extended (90 min) wash-out. This sets it apart from the blockade seen after acute *in vitro* exposure; numerous possibilities could account for this difference (see El-Bizri & Clarke, 1994b).

Several observations indicate that the initial blockade produced by CHL in PC12 cells is not sufficient to produce the long-term blockade. Thus, a high concentration of CHL (100 μM for 24 h) was required to produce a long-term nicotinic blockade. In contrast, a much lower concentration (1 μM) was completely effective when co-applied acutely with nicotine (present study), and an IC_{50} as low as 0.02 μM has previously been reported in PC12 cells (Daly *et al*., 1991). Since acute antagonism by CHL is use-dependent (Alkadhi & McIsaac, 1974; El-Bizri & Clarke, 1994a) and thus presumably dependent upon nAChR channel openings, it was conceivable that even a high concentration of CHL, given in the absence of agonist, might fail to produce an appreciable acute block. However, this is refuted by two observations. First, considerable antagonism was still evident 2 h after agonist-free exposure to a lower concentration of CHL for a shorter time. Second, co-administration of CHL and nicotine did not dramatically augment the long-term blockade compared to administration of CHL alone.

What other mechanisms might underlie the persistent *in vitro* antagonism, given the requirement for high extracellular concentrations of CHL? Conceivably, CHL may deeply but slowly penetrate the nAChR-associated ionophore from the medium, creating an irreversible block. Such a block would be terminated by receptor turnover. The nAChR turnover rate in PC12 cells has been estimated to be 12 h (Kemp & Edge, 1987), but this figure would no doubt depend on culture conditions and the variant cell line under study. Alternatively, high concentrations of CHL may be required to permit sufficient amounts to penetrate the cell, thereby producing a persistent blockade *via* some intracellular mechanism as yet unknown. In this context, it is interesting that certain cationic ligand gated channels can be blocked by cytosolic polyamines, which like CHL, carry more than one positive charge (Bowie & Mayer, 1995).

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