of the cells, we measured the intracellular levels of ATP in the various conditions in which we tested the Ca effect on the K fluxes. The addition of ionophore in the absence of Ca slightly increased the ATP content of the cells in a manner reminiscent of the effect of propranolol[20]. The additional presence of Ca stimulated ATP hydrolysis through the Ca pump and induced a rapid fall in ATP from 1 mM to about 0.55 mM followed by a slower decline, presumably because of the buffering effect of membrane-bound adenylate kinase. These effects were independent of the ionophore concentration, which makes ATP alone an unlikely source of the different kinetic patterns observed in the intact cells. It is also unlikely that the variable Ca sensitivity of the K permeability system results from a non-homogeneous distribution of Ca within the cell since the K fluxes we report here were measured simultaneously in the same cells (see legend to Fig. 1 in ref. 18) where we had previously determined a constant K_m of 1 μM for the Ca activation of the Ca pump at all ionophore concentrations.

We also investigated the effect of ionophore removal on the Ca-dependent K flux. This we could only try in intact red cells loaded with Ca if the Ca pump were inhibited, and for this reason we first depleted the cells of Mg, a necessary internal cofactor for Ca extrusion[21]. Mg extraction and Ca loading were performed in succession by preincubating the cells in the presence of ionophore (10 μmol per l cells) first in a medium containing 2 mM EDTA and no Ca or Mg and then in Ca-containing media in the absence of Mg and chelator. The ionophore was then removed by washing the cells eight times in Ca-free ice-cold solutions at low haematocrits (about 1%). This simple procedure restored the normal Ca impermeability of the intact cell and, with the Ca pump arrested, the cells remained effectively resealed to Ca, losing only about 25% of their original Ca content during the initial washes. These cells also showed a high Ca affinity pattern similar to that of the intact cells at high ionophore concentrations shown in Fig. 4.

The low Ca affinity pattern shown in Fig. 3 has never been observed before. Since the cells were disturbed least in these conditions we feel that the low affinity response is closer to the behaviour of the normal system. The high affinity pattern would then correspond to an altered reactivity to internal Ca which may or may not occur in the normal cell depending on whether the Ca sensitivity of the K permeability mechanism is or is not subject to control by cellular regulatory mechanisms. Since the maximum increase in K permeability induced by Ca is more than three orders of magnitude larger than the "ground" K permeability of the intact red cell, an increase in Ca_i^{2+} even in the micromolar range[22], may suffice to produce a functionally significant increase in K permeability. A relatively small effect could thus account for the Ca-induced hyperpolarisation observed in amphiuma red cells[23] and perhaps also in other cells[5,8].

The heterogeneous behaviour of ATP-depleted red cell populations in relation to the Ca-induced K flux[15,23] can now be interpreted as resulting from a variable Ca affinity of the K gate. This revives the old controversy[10] about whether the depleting procedure accumulates[25] or removes[26] metabolites which control the K permeability of red-cell membranes, but now it can be reformulated more precisely in terms of the control of the Ca sensitivity of the K-gating mechanism.

Since many of the conditions in which the effect of internal Ca on the K permeability is obtained resemble those in which 1,2-diacylglycerol accumulates in the red-cell membrane, as recently shown by Allan and Michell[27], we feel tempted to suggest the possibility that 1,2-diacylglycerol, or a similar metabolite, mediates the Ca effects on the K channel. According to Allan and Michell, the rate of formation of this substance depends on the internal Ca^{2+} concentration, whereas the fast conversion to an inactive phosphatide, by the action of a diacylglycerol kinase, would depend on the intracellular level of ATP. If the Ca effect on the K permeability depends on the level of a certain intermediate, the different Ca sensitivities observed in the present experiments may merely reflect the variation in the relative rates of formation and breakdown of this intermediate in the different conditions.

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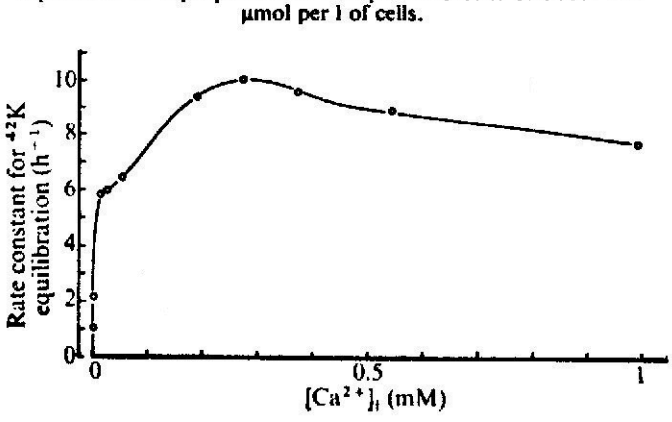
Fig. 4 The rate of ^{42}K equilibration as a function of the internal Ca^{2+} concentration in intact red cells in the presence of a high ionophore concentration. The concentration of ionophore used in this experiment was 1 mg ml^{-1} in the ethanol phase. This is equivalent to a proportion of ionophore to cells of about 100 μmol per l of cells.



Stereospecificity of interaction of neuroleptic drugs with neurotransmitters and correlation with clinical potency

THE molecular mechanism responsible for the therapeutic activity of a drug is often suggested by the biochemical event which correlates best with clinical potency. Thus, if optical or geometrical isomers of a drug have markedly different clinical potencies, the most relevant biochemical effect should also exhibit isomeric specificity.

While numerous biochemical mechanisms have been proposed to explain the antischizophrenic action of neuroleptics, the

ability of these drugs to antagonise behavioural effects of apomorphine and amphetamine in animals correlates best with clinical potency[1]. Since apomorphine and amphetamine-induced behaviours are mediated by brain catecholamines, especially dopamine, the neuroleptics are thought to act by blocking dopamine receptors in the brain. Clinical potencies of phenothiazine neuroleptics correlate reasonably well with their ability to inhibit a dopamine-sensitive adenylate cyclase which is presumably linked to the dopamine receptor[2,3]. Because pharmacologically potent butyrophenones are relatively weak inhibitors of this cyclase and show only a partial correlation with clinical potency[4], however, it has been suggested that these drugs may only indirectly affect dopamine receptors, perhaps by blocking dopamine release[5]. The chemical similarity of butyrophenones to γ-aminobutyric acid (GABA) suggests that these agents might in some way facilitate GABA transmission in the brain[1]. Since inhibitory GABA neuronal pathways may synapse on dopamine cell bodies, facilitation of GABA effects could reduce the firing rate of dopamine neurones resulting in behaviours like those associated with dopamine receptor blockade[6].

In both clinical and animal pharmacological screening, tain neuroleptics display isomeric specificity. α-Flupenthixol and *cis*-thiothixene are much more potent clinically and in animals than their geometrical isomers β-flupenthixol and *trans*-thiothixene respectively[7,8]. Similarly (+)-butaclamol is much more potent than its optical isomer (−)-butaclamol[9]. The relative potencies of butaclamol, flupenthixol and thiothixene isomers upon the dopamine-sensitive adenylate cyclase accord with their isomeric specificity in clinical studies and pharmacological screens[10,11].

Using recently developed binding techniques to study neurotransmitter receptors directly, we have examined critically how reliably stereospecificity and correlations between clinical and biochemical effects can reveal the biochemical basis of neuroleptic drug action. The results suggest that although these criteria are valuable, they can obfuscate rather than clarify drug mechanisms if used incautiously. Of the several biochemical effects of neuroleptics, blockade of dopamine receptors best accounts for therapeutic effects of both butyrophenone and phenothiazine neuroleptics.

Synaptic receptor binding studies were conducted as previously described[12–20]. Briefly, washed crude synaptic membrane or total particulate fractions of various brain regions representing approximately 1 mg of protein were incubated in a uffered solution containing a low concentration of tritiated ligand (1–8 nM) in the presence or absence of various concentrations of the drug under study. After incubation, radioactivity bound to the membranes was separated by filtration or centrifugation, extracted into an appropriate fluor and counted by liquid scintillation spectrometry. The amounts bound in the presence and absence of the drug were compared. The ligands used in this study and the receptor to which each binds is as follows: ^{3}H-γ-aminobutyric acid (^{3}H-GABA), the GABA receptor[12]; ^{3}H-dopamine (^{3}H-DA and ^{3}H-haloperidol (^{3}H-Halo), the dopamine receptor[13,14]; ^{3}H-naloxone (^{3}H-Nal) ^{3}H-dihydromorphine (^{3}H-DHM), the opiate receptor[15]; ^{3}H-dihydroalprenolol (^{3}H-DHA), the β-adrenergic receptor[16,21]; ^{3}H-5-hydroxytryptamine (^{3}H-5-HT) and ^{3}H-lysergic acid diethylamide (^{3}H-LSD), the 5-HT receptor[17,18]; ^{3}H-quinuclidinyl benzilate (^{3}H-QNB), the muscarinic cholinergic receptor[19]; and ^{3}H-strychnine (^{3}H-Stry), the glycine receptor[20].

Receptor binding for each ligand was assayed in the brain region containing the highest density of receptor sites or in whole brain. The highest density of dopamine receptor sites is in the corpus striatum[13,14]. Binding of ^{3}H-strychnine to glycine is most enriched in spinal cord membranes[20]. Receptor binding for ^{3}H-LSD, ^{3}H-5-HT and ^{3}H-DHA is greatest in the cerebral cortex[16–18,21]. Although there exist regional variations in opiate, GABA and muscarinic receptor binding, the characteristics of binding sites do not vary regionally and substantial activity is present in whole brain or forebrain, which were used for assays of these receptors[12,15,19].

Uptake of radioactive GABA by synaptosomes in nuclei-free sucrose homogenates of whole rat brain was assayed as previously described[22].

All radioactive agents were purchased from New England Nuclear, and all other substances were obtained from the company of origin.

There are no marked stereospecific influences of neuroleptics on receptor binding for ^{3}H-GABA, for the opiates ^{3}H-naloxone and ^{3}H-dihydromorphine, for binding of ^{3}H-strychnine to the glycine receptor, for the binding of ^{3}H-quinuclindinyl benzilate (^{3}H-QNB) to the muscarinic cholinergic receptors or for ^{3}H-dihydroalprenolol (^{3}H-DHA) binding to the β-noradrenaline receptor (Table 1). In contrast, the neuroleptic isomers are extremely potent in competing for ^{3}H-haloperidol binding to the dopamine receptor and display striking stereospecificity. (+)-Butaclamol reduces ^{3}H-haloperidol binding 50% at 1 nM and is more than 1,000 times as potent as (−)-butaclamol in eliciting this effect. α-Flupenthixol inhibits ^{3}H-haloperidol binding 50% at 2 nM and is 50 times as potent as β-flupenthixol, while *cis*-thiothixene is 100 times more potent than *trans*-thiothixene in competing for ^{3}H-haloperidol binding. These neuroleptics also demonstrate stereospecificity with respect to ^{3}H-dopamine binding, although they are less potent in competing with this ligand. Thus, while (+)-butaclamol is less than 1% as potent in inhibiting ^{3}H-dopamine as ^{3}H-haloperidol

Table 1 Displacement of various receptor ligands by neuroleptic isomers

Compound	IC_{50} (μM)*									
	^{3}H-DHA[a]	^{3}H-5-HT[b]	^{3}H-LSD[c]	^{3}H-DA[d]	^{3}H-Halo[e]	^{3}H-QNB[f]	^{3}H-Stry[g]	^{3}H-Nal[h]	^{3}H-DHM[i]	^{3}H-GABA[j]
(+)-Butaclamol	N.e.†	1.0	0.05	0.1	0.001	100	N.e.	18	17	N.e.
(−)-Butaclamol	N.e.	8.0	7.0	16.0	1.3	40	N.e.	20	17	N.e.
α-Flupenthixol	N.e.	4.0	0.10	0.2	0.002	0.7	N.e.	55	100	100
β-Flupenthixol	N.e.	60.0	6.0	10.0	0.10	0.9	N.e.	50	100	N.e.
cis-Thiothixene	80	1.0	0.3	0.7	0.003	3.2	50	15	30	50
trans-Thiothixene	80	7.0	1.0	19.0	0.30	2.8	50	15	35	50

*Concentration which inhibits ligand binding 50%.
†No effect at 100 μM.
Four to six concentrations of each drug were evaluated in triplicate to obtain IC_{50} values. Data are the mean of 2–4 determinations which varied less than 20%.
[a]Rat cerebral cortex membranes, ^{3}H-DHA = 1 nM.
[b]Rat cerebral cortex membranes, ^{3}H-5-HT = 7 nM.
[c]Rat cerebral cortex membranes, ^{3}H-LSD = 3 nM.
[d]Calf striatal membranes, ^{3}H-DA = 5 nM.
[e]Calf striatal membranes, ^{3}H-Halo = 2 nM.
[f]Rat forebrain homogenate, ^{3}H-QNB = 1 nM.
[g]Rat spinal cord–brainstem, synaptosomal membranes, ^{3}H-Stry = 2 nM.
[h]Rat forebrain membranes, ^{3}H-Nal = 1 nM.
[i]Rat forebrain membranes, ^{3}H-DHM = 1 nM.
[j]Rat brain synaptosomal membranes, ^{3}H-GABA = 8 nM.

Table 2 Inhibition of GABA uptake into rat brain synaptosomes by neuroleptic drugs

Compound	IC_{50} (μM)*	Average clinical daily dose† (μmol kg)
Butyrophenones		
Fluspirilene	3	0.066
Pimozide	10	0.108
Clofuperol	15	0.077
Benperidol	30	0.060
Bromoperidol	30	0.153
Spiroperidol	40	0.058
Penfluridol	60	0.466
Trifluperidol	65	0.096
Haloperidol	75	0.152
Moperone	100	0.802
Droperidol	200	—
Fluanisone	500	3.44
Azaperone	600	—
Pipamperone	1,000	11.10
Phenothiazines and related agents		
Trifluoperazine	17	0.297
Fluphenazine	18	0.168
Triflupromazine	20	4.59
Chlorpromazine	21	12.00
Promazine	30	33.00
α-Flupenthixol	35	0.099
β-Flupenthixol	35	—
cis-Thiothixene	38	0.393
trans-Thiothixene	45	—

*Concentrations which inhibit 50%.

†For clinical potencies, the midpoint values of daily dose ranges[28-31] were meaned and converted to μmol kg^{-1} assuming a human weight of 70 kg.

Six concentrations of each drug were evaluated in triplicate to obtain IC_{50} values. Data are the mean of three determinations which varied less than 15%.

binding, it is 160 times more potent than (−)-butaclamol in competing for ^{3}H-dopamine binding. Similarly α-flupenthixol and *cis*-thiothixene are less than 1% as potent in inhibiting ^{3}H-dopamine as ^{3}H-haloperidol binding yet they still display stereospecificity with respect to their corresponding isomers. Interestingly flupenthixol and thiothixene are just as potent in competing for ^{3}H-QNB as for ^{3}H-dopamine binding, but they display no stereospecificity in inhibiting ^{3}H-QNB binding.

Relative potencies of numerous neuroleptics as inhibitors of ^{3}H-haloperidol binding to the dopamine receptor correlate well with clinical efficacy[14,23,24]. There is, however, a much weaker correlation of neuroleptic clinical and pharmacological potency with ^{3}H-dopamine binding, in spite of the fact that both dopamine and haloperidol presumably interact with the same receptor. The differences between interactions of ^{3}H-haloperidol and ^{3}H-dopamine with the dopamine receptor seem to stem from their labelling two different states of the dopamine receptor. Agonists are much more potent in competing for ^{3}H-dopamine than ^{3}H-haloperidol binding. Conversely, antagonists have substantially higher affinity for haloperidol than dopamine-binding sites. Thus dopamine and haloperidol respectively label discrete "agonist" and "antagonist" states of the dopamine receptor[14]. This two-state model for the dopamine receptor accords with data supporting such a model for the 5-HT, α-noradrenergic and opiate receptors[25]. One would expect pharmacological activities of antagonists to be related better to their affinities for antagonist than agonist states of the receptor, which explains the better correlation of neuroleptic clinical activity with affinity for haloperidol than for dopamine binding[23].

The binding of ^{3}H-LSD and ^{3}H-5-HT is also inhibited stereospecifically and with considerable potency by these neuroleptics. The neuroleptics display about the same potency in inhibiting ^{3}H-LSD as ^{3}H-dopamine binding as well as a similar degree of stereospecificity. Thus (+)-butaclamol is 140 times as potent as (−)-butaclamol in lowering ^{3}H-LSD binding and is about 160 times as potent as the (−)-isomer in lowering ^{3}H-DHA binding. The neuroleptics are somewhat less potent in lowering the binding of ^{3}H-5-HT, but still display a degree of stereospecificity. The potencies of these neuroleptics in inhibiting ^{3}H-dopamine and ^{3}H-LSD binding are similar to their potencies as inhibitors of the dopamine-sensitive adenylate cyclase[2,3]. In spite of these stereospecific effects, blockade of 5-HT receptors is probably not a major source of neuroleptic clinical efficacy, because relative influences of an extensive series of these drugs on ^{3}H-LSD binding do not correlate with clinical potency[17].

Since the chemical structure of butyrophenones resembles that of the inhibitory neurotransmitter GABA[1], it was also of interest to evaluate the influence of butyrophenones on GABA uptake by synaptosome-enriched brain homogenates. In this type of tissue preparation ^{3}H-GABA is accumulated primarily by nerve terminals[26,27]. The butyrophenones examined are all capable of inhibiting ^{3}H-GABA uptake (Table 2). The most potent, fluspirilene, reduces uptake 50% at 3 μM, similar to its potency in inhibiting ^{3}H-dopamine binding and in inhibiting the dopamine-sensitive adenylate cyclase[2,3]. There is a several hundredfold variation in the relative potencies of the butyrophenones in reducing GABA uptake, and these variations correlate well with the relative clinical potencies of the drugs ($r = 0.86$, $P < 0.001$). If inhibition of GABA uptake were a common mechanism whereby therapeutic effects of all neuroleptics were elicited, then the phenothiazines and related agents should also show a similar correlation between inhibition of GABA uptake and clinical potency. We detect no correlation, however, between the influence of these agents on GABA uptake and their clinical effects. The very potent neuroleptic α-flupenthixol is no more effective in reducing GABA uptake than the weak neuroleptic promazine. Similarly, the potent neuroleptic fluphenazine has about the same potency as the moderately effective neuroleptic chloropromazine.

The most striking finding indicating that the pharmacological activity of neuroleptics related to phenothiazine cannot be attributed to influences on GABA uptake derives from experiments with stereoisomers. No difference is apparent in the potencies of α- and β-flupenthixol in competing for GABA uptake in spite of the fact that essentially all the pharmacological activity resides in the α-isomer with β-flupenthixol being essentially inactive[8]. Similarly, the *cis*- and *trans*-isomers of thiothixene have the same affinities for GABA uptake sites, though only *cis*-thiothixene has clinical and pharmacological activity[7].

It could be argued that the correlation between relative potencies of butyrophenones in inhibiting GABA uptake and their clinical potencies indicates that the therapeutic actions of butyrophenones may be mediated at least in part by inhibition of GABA uptake. Since uptake of GABA into nerve terminals is thought to inactivate synaptically released GABA, such an effect would potentiate the synaptic effects of GABA, which in turn could inhibit the firing of dopamine neurones. Similarly inhibition of opiate receptor binding by butyrophenones but not by phenothiazines correlates well with clinical antischizophrenic activity ($r = 0.78$, $P < 0.01$ with no added Na^+; $r = 0.61$, $P < 0.05$ with 100 mM Na^+)[32]. There is also some correlation between antipsychotic potencies of neuroleptics and inhibition of dopamine release[5]. Before the identification of ^{3}H-haloperidol binding to dopamine receptor sites[14,23,24] these formulations might have represented plausible mechanisms for clinical effects of butyrophenones if not of phenothiazines. But since the butyrophenones are about 100–1,000 times more potent in competing for ^{3}H-haloperidol binding to dopamine receptor sites than in reducing GABA uptake, opiate binding or dopamine release and since inhibition of haloperidol binding predicts phenothiazine as well as butyrophenone activities, a more parsimonious explanation attributes clinical actions of both butyrophenones and phenothiazines to blockade of dopamine receptors[14,23,24].

The correlation between the influences of these drugs on ^{3}H-GABA uptake and clinical potencies emphasises the difficulties inherent in evaluating correlations between bio-

chemical and clinical effects of drugs. Similarly, one should be circumspect about concluding that stereospecificity of biochemical effects "proves" that one is dealing with the pharmacologically relevant site of a drug's action. Thus, if a given behavioural effect in animals is elicited more by (+)-butaclamol than by (−)-butaclamol, it should not automatically be assumed that the behaviour is dopamine mediated, since the drug effect might also involve 5-HT receptors.

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Transmitter metabolism in substantia nigra after inhibition of dopaminergic neurones by butyrolactone

Dopaminergic nigrostriatal neurones have recently been found to contain dopamine (DA) also in their dendrites[1]. Certain observations suggest a release of transmitter from these dendrites: depolarisation by potassium ions induces a release of ^{3}H-DA from incubated tissue slices of rat substantia nigra[2], and stimulation of the median forebrain bundle increases 3,4-dihydroxyphenylacetic acid, a major metabolic of DA[3], in the mesencephalic region containing dopaminergic cell bodies and dendrites[4], as well as in caudate-putamen, the terminal area. These similarities between terminal and somato-dendritic areas point to a similar regulation of DA metabolism and an active role of DA in both parts of the neurone. We have studied this question in a situation where the dopaminergic neurones are inhibited. γ-Butyrolactone (GBL) applied systemically, strongly reduces unit activity of the dopaminergic neurones in zona compacta of substantia nigra[5]. Under these conditions, the changes observed in the somato-dendritic complex differed markedly from those reported for the terminal area[6–13].

In male rats (200–300 g) of a random-bred SIV (Sprague–Dawley–Ivanovas) strain, the somato-dendritic complex was analysed as a whole with biochemical techniques, while DA nerve cell bodies were studied by histochemical microfluorimetry[12]. For biochemical analyses the brains were quickly removed after decapitation, placed on dry ice and covered with carbon dioxide snow. Standardised tissue cylinders (about 1 mg) from substantia nigra were obtained as described in Fig. 1[14]. These cylinders contained the medial two thirds of zona compacta and small amounts of tissue from zona reticulata and lemniscus medialis. Their localisation corresponds to the area of highest dendritic density[1] and to the highest DA concentration in substantia nigra (F. Hefti and W. Lichtensteiger, unpublished). DA concentration was determined in individual tissue cylinders with an enzymatic-isotopic method[15]. The rate of DA synthesis was assessed by measuring accumulation of dopa after inhibition of dopa-decarboxylase with 3-hydroxybenzylhydrazine (NSD 1015, Sandev) with a newly developed enzymatic-isotopic assay[16]. In these conditions dopa accumulated linearly in substantia nigra cylinders up to 60 min (ref. 16).

Both inhibition of dopaminergic cells by GBL[8,9,5] and interruption of impulse flow by axotomy[9,10,13] have been found to lead to an increase in the concentration of dopamine in caudate-putamen. After a single injection of 750 mg kg^{-1} of GBL. DA concentration was greatly increased between 15 and 150 min (ref. 8). We could confirm

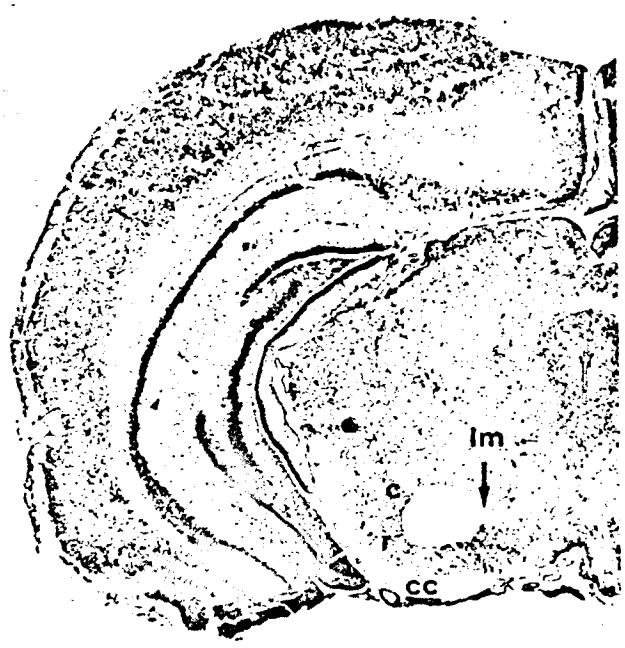

Fig. 1 Localisation of standardised tissue cylinders in substantia nigra. From frozen brain, a 1-mm thick frontal slice was sectioned extending anteroposteriorly from level A 1400 to level A 2400 according to the atlas of Koenig and Klippel[30]. A cylinder of 1-mm diameter was punched out on each side. The figure shows the histological control of such a slice fixed after punching (Toluidine blue staining). c, Zona compacta; r, zona reticulata of substantia nigra; lm, lemniscus medialis; cc, crus cerebri.