Selective Action of Acute Systemic Clozapine on Acetylcholine Release in the Rat Prefrontal Cortex by Reference to the Nucleus Accumbens and Striatum

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ABSTRACT
The effects of i.p. clozapine [0 (n = 6), 5 (n = 5), 10 (n = 5), 20 (n = 9) and 40 (n = 5) mg/kg] on acetylcholine (ACh) release in the prefrontal cortex (PFC), nucleus accumbens (NAC) and striatum (STR) were studied by simultaneous triple microdialysis in freely moving rats. Clozapine dose-responsively increased extracellular ACh in the studied areas. The effect was larger in the PFC. Comparisons of the slopes of the regression equations showed differences between the effects in PFC and nucleus accumbens (t = 4.29; df = 56; P < .001) and PFC and STR (t = 4.56), but not between nucleus accumbens and STR. These differential actions were not artifacts of the simultaneous perfusion because clozapine (20 mg/kg) increased ACh levels during single microdialysis of the PFC (353 ± 72%; n = 5) or STR (168 ± 24%; n = 5), in the same proportion as the respective increases in those areas during the simultaneous triple microdialysis (PFC = 330 ± 41%; STR = 144 ± 18%; n = 9). Local infusion of tetrodotoxin (10 μM) reduced ACh in the areas studied to about 30% of the mean baselines, confirming the neuronal origin of this neurotransmitter. Extrapolation of these results to humans suggests that adequate levels of cholinergic activity in the PFC are required for mental health, and that a similar ACh release in the human PFC by clozapine could be therapeutic. The low impact on striatal ACh could explain the lack of extrapyramidal symptoms by clozapine.

The efficacy of neuroleptics to ameliorate schizophrenia symptoms and the receptor binding and blocking properties of those drugs (Creese et al., 1976; Seeman and Lee, 1975; Snyder et al., 1975) led to the dopaminergic theory of schizophrenia. This theory oriented research toward the study of the involvement of different brain dopaminergic systems in the production of this mental illness and their role in the therapeutic benefits of neuroleptics.

Typical neuroleptics, such as haloperidol, are considered to be therapeutically effective but produce EPS including parkinsonism (rigidity, tremor and akinesia), dystonia and akathisia (Ayd, 1961; Snyder et al., 1974). In animal models they induce catalepsy and antagonize apomorphine and amphetamine induced stereotypies (Szechtman et al., 1988). Conversely, the behavioral profile of atypical neuroleptics does not include catalepsy and they do not antagonize the mentioned stereotypies (Ljungberg and Ungerstedt, 1978, 1983; Moore and Kenyon, 1994; Tschandz and Rebec, 1989). These drugs are also clinically effective and do not produce EPS (Gerlach and Simmelsgard, 1978; Herman and Pleasure, 1983; Rama Rao et al., 1981).

Clozapine can be considered as the best representative of atypical neuroleptics. The neurochemical mechanisms and anatomical substrates that underlie its clinical profile have not been unequivocally determined and remain unclear. Some evidences, gathered from studies on human brains and experimental animal models, strongly suggest that the therapeutic efficacy of clozapine relates to its selective action on the activity of limbic components, particularly the frontal cortex. However, its lack of EPS apparently depends on its neutral activity on the motor mechanisms of the basal ganglia with special reference to the striatum. Long-term treatment with clozapine, for instance, induces depolarization blockade in mesolimbic-mesocortical (A10) but not in nigrostriatal (A9) DA neurons (Chiodo and Bunney, 1983, 1985; White and Wang, 1983). Besides, acute or chronic clozapine administration modifies DA turnover and metabolism preferentially in the PFC as compared to the NAC or STR (Hernandez and Hoebel, 1995; Moghaddam and Bunney, 1990; Pehek and Yamamoto, 1994; Yamamoto and Cooperman, 1994; Youngren et al., 1994). This regional specificity has been related to the high affinity of clozapine for the D4 dopamine receptor subtype, which seems more abundant in the PFC than in the NAC or STR (Lahti et al., 1993; Schwartz

ABBREVIATIONS: Ach, acetylcholine; AChE, acetylcholinesterase; DA, dopamine; D, dopamine receptor; M, muscarinic receptor; 5-HT, serotonin; PFC, prefrontal cortex; NAC, nucleus accumbens; STR, striatum; EPS, extrapyramidal symptoms; TTX, tetrodotoxin
et al., 1993; Seeman, 1992; Seeman et al., 1993; Van Tol et al., 1991).

Interactions between DA and ACh have been described. They have been widely explored in the STR (Consolo et al., 1992, 1987; Damsma et al., 1990a, 1990b; Dawson et al., 1988; De Boer et al., 1990, 1992; Imperato et al., 1993, 1994; Kubota et al., 1987; Stoenf et al., 1992), in the NAC (Russell et al., 1989; Wedzony et al., 1988), and more scarcely in the PFC (Day and Fibiger, 1992, 1993) and the lateral hypothalamus (Parada et al., 1994b). Those interactions might well have something to do with the pharmacological actions of neuroleptics.

The study of the relative impact of neuroleptics on cholinergic systems is important because these drugs are dopamine receptor blockers (Creese et al., 1976; Seeman and Lee, 1975; Snyder et al., 1975), some postsynaptic DA receptors are located on cholinergic neurons (Dawson et al., 1988; Le Moine et al., 1990), ACh has been involved in psychosis and schizophrenia (Abood and Biel, 1962; Singh and Kay, 1985; Yeomans, 1995), and an excessive activation of cholinergic mechanisms in the striatum has been claimed to be responsible for the EPS produced by typical neuroleptics (Bruno et al., 1962; Stoenf et al., 1992).

Our study explored a differential action exerted by acute systemic zuclopenthixol on cholinergic systems within PFC, NAC and STR. This differential action was studied monitoring modifications of ACh levels in samples collected from those areas using simultaneous triple microdialysis. Clozapine preferentially increased ACh in the PFC and had less effect in the NAC and STR. These results reinforce previous suggestions that its therapeutic efficacy is due to a selective action on the frontal cortex and that its lack of EPS has to do with a little effect on the STR.

**Methods**

*Surgery.* Forty-four male Wistar rats weighing 320 to 400 g were individually housed at 18 to 22°C on a 12- to 12-hr light-dark schedule (6:00-18:00) with food and water ad libitum. Under ketamine anesthesia (100 mg/kg) three permanent 21-gauge, stainless steel guide cannulas, 10 mm long were stereotaxically implanted in each rat. The guide shafts were aimed to the PFC, NAC and STR using a single system. The chromatogram for each sample required 1 min for successive samples in each area.

**Acetylcholine assay.** ACh was measured by reverse phase, high performance liquid chromatography with electrochemical detection (HPLC-EC) using a single piston pump and a pulse damper from SSI Co, a 20 μl sample loop and an amperometric detector (EG&G Princeton Applied Research Corp., Princeton, NJ). The mobile phase contained 200 mM potassium phosphate at pH 8.0. ACh was separated on an 8 cm C18 analytical column (Chrompack Inc.,营业收入) and then converted sequentially to betaine and hydrogen peroxide in an enzyme reactor (Chrompack Inc.,营业收入) and finally oxidized by a peroxidase-enzyme reactor (Sigma Chemical Co., St. Louis, MO) containing 0.1 N HCl 1 ml/kg; n = 6) were monitored. The ip injections were performed transiently hand-holding the animals during the injection, and returning them to the experimental cage immediately thereafter. Extracellular ACh monitoring continued for more than 2 hr after each injection. The results of this experiment showed that i.p. clozapine was more effective to increase ACh in the PFC than in the NAC or STR. To confirm that these results were not artifacts due to simultaneous microdialysis in the three studied regions 10 animals bearing the three guide shafts were perfused in the PFC (n = 5) only or in the STR (n = 5) only and ACh changes after 20 mg/kg of i.p. clozapine were assessed. This clozapine dose was selected from the dose-response study of the first experiment and ensured a robust effect on ACh in the PFC. To determine the neuronal origin of extracellular ACh four animals undergoing simultaneous microdialysis in the PFC, NAC and STR received a simultaneous 50-min TTX infusion (10 μM in Ringer’s solution) in each area by reverse microdialysis.

**Statistical analysis.** Absolute basal levels of ACh from PFC (n = 44), NAC (n = 39) and STR (n = 44) dialsates were compared by means of one-way analysis of variance followed by a Student-Newman-Keuls test. Data from each subject in each experimental session were normalized by converting peak heights to percents of the means of the three consecutive baseline samples. The pharmacological effects were assessed in each group and for each area, comparing the ACh levels from the pretreatment samples with the means levels of the samples obtained after drug administration (Clozapine, vehicle or TTX), using one-way analysis of variance for repeated measures followed by mean’s regression coefficient comparisons to detect the points significantly different. The relationship between the clozapine doses and the ACh increases in each brain area were explored with linear regression analysis applied on data from the second postinjec-
tion sample. The statistical significance in the regional differences of the dose-response curves was determined comparing the slopes of the regression lines.

**Histology.** After the experiments and under pentobarbital anesthesia the animals were sacrificed and perfused with formalin through the heart. The brains were fixed in formalin during at least 1 wk and then cut in 40-µm sections and the tracks of the microdialysis probes were localized to identify the perfused areas.

**Results**

**Basal ACh output.** Figure 1 shows the mean (± S.E.M.) absolute basal levels of ACh in the dialisates from each of the three regions studied. The values, neither normalized nor corrected for probe recovery, were 0.84 ± 0.12, 0.57 ± 0.12 and 0.33 ± 0.03 pmol/20 µl for the PFC, NAC and STR, respectively. The one-way analysis of variance combined with the Student-Newman-Keuls test showed that basal ACh was higher in the PFC than in the NAC or STR (df: 2/95; F = 7.32; P < .01). ACh levels in these last subcortical areas were not significantly different.

**Effects of acute systemic clozapine administration on ACh output in the PFC, NAC and STR during simultaneous triple microdialysis: A dose-response study.** Statistical analysis of the data presented in figure 2 showed that the four clozapine doses increased ACh levels in the three regions studied. This effect was apparently more conspicuous in the PFC (top graph) than in the NAC (middle graph) or the STR (bottom graph). In most cases the maximal increases in ACh levels were detected during the second sample postinjection. Therefore data from this point were used for the linear regression analyses exploring the dose-response relationship in each region. The regression lines and equations for the PFC, NAC and STR are shown in figure 3. The correlation was positive and significant for all regions but stronger for PFC (R = .78; P < .0001) than for NAC (R = .46; F = 7.7; P < .01) or STR (R = .6; F = 17.8; P < .005). Statistical comparisons showed that the PFC slope (7.61) was larger than the NAC (2.55) or STR (2.03) slopes at P < .01, and showed no differences between these last two slopes [t = .06; df = 56; ns].

**Effects of acute systemic clozapine administration on ACh output during isolated microdialysis of the PFC and NAC.** Clozapine administered during single microdialysis modified ACh levels after the same regional pattern displayed during the simultaneous triple brain microdialysis. Clozapine administration (20 mg/kg i.p.) produced in the PFC, 50 min later, similar large increases in ACh levels (fig. 4, top graph) when this area was perfused alone (352.8 ± 72.3%), or when it was perfused simultaneously (339.4 ± 41.3%) with the NAC and STR. No differences were evident between the weak effects induced by clozapine on ACh levels.
Effects of local TTX infusion on ACh output during the simultaneous perfusion of the PFC, NAC and STR.

Local TTX infusion by reverse microdialysis drastically reduced ACh levels in all three areas under study (fig. 5). The lowest ACh levels were detected in the second samples collected during the TTX infusions (PFC: 23.9 ± 6.5%; NAC: 21.9 ± 3.7%; STR: 31.4 ± 3.6%; by reference to the corresponding baselines) and levels remained significantly low during the rest of the experiment. The means regression coefficient comparisons that followed the one-way analysis of variance for repeated measures showed that for each area all the infusion and postinfusion data analyzed were significantly lower than their corresponding preinfusion data at $P < 0.0001$ [$PFC$ (df: 3/6, $F = 55.2$); $NAC$ ($F = 28.2$); STR ($F = 33$)].

Figure 6 is a photograph of the histological sections showing the three areas perfused in one rat. The microdialysis probes were located in the PFC at the level of the cingulum regions I and II and the infralimbic cortex. NAC probes were in the posterior medial region of the NAC. Probes in the STR were in the posterior part of the caudate-putamen complex and in close proximity to the lateral border of the globus pallidus.

Discussion

Systemic injections of clozapine differentially affected ACh release in three dopaminergic terminal areas. Nevertheless, some points have to be addressed before discussing the significance of this disparate modification in regional ACh release.

Collected ACh must come from cholinergic terminals located in close proximity to the microdialysis probes. Distant origins seem unlikely since acetylcholine is rapidly metabolized by AChE, which makes it necessary the local infusion of AChE inhibitors to have detectable basal values. Drugs infused through microdialysis probes have a rather restricted local action. Thus, in our experiments neostigmine must have prevented the enzymatic degradation of locally released ACh and TTX must have blocked release from local ACh terminals.

The ACh basal levels were low in the STR and higher in the NAC and PFC. Several authors have reported different ACh basal levels during isolated microdialysis of those areas in two different groups of animals (open circles in both graphs). The arrows mark the injection time. Each graph includes for comparison the results obtained from the same areas with the same clozapine dose and during simultaneous triple microdialysis (solid circles). These data were taken from the main experiment (see top and bottom graphs of fig. 2). The results obtained under both conditions were practically the same.
to alter the pharmacological responsivity of cholinergic neostigmine in the microdialysis perfusate have been shown. However, high concentrations of ACh within the PFC. Nevertheless, the three areas were under the same conditions for they were perfused using the same solution with the same neostigmine concentration. Thus, the differential effects of clozapine could be interpreted as the result of an interaction between specific regional neurochemical systems and the drug’s particular pharmacological profile.

The type of interaction responsible for this differential action is not clear yet, but the possibility that clozapine-induced ACh release might be the consequence of the blockade of DA receptors deserves special attention. At least five different DA receptors subtypes have been recognized (Schwartz et al., 1993; Seeman, 1992), but to date just D3 receptors have been positively identified on ACh neurons in the STR (Dawson et al., 1988; Le Moine et al., 1990). One intriguing feature of the striatal DA-ACh interaction is that DA seems to exert a dual control on ACh neurons. On the one hand, many data support the classical view that DA, acting on D2 receptors, exerts a tonic inhibitory control on striatal ACh neurons (Damsma et al., 1990a; Dawson et al., 1988; De Boer et al., 1990, 1992, 1993; Kubota et al., 1987). On the other hand, new data suggest a facilitation through D1 receptors (Consolo et al., 1987, 1992; Damsma et al., 1990b; Imperato et al., 1993; Imperato et al., 1994). No clear conceptual picture has been elaborated yet to reconcile and explain the opposite effects of the same transmitter on apparently the same target neurons, but it has been proposed that both mechanisms are operative in the tonic regulation of striatal interneurons, and that in physiological conditions D2 inhibition equals or exceeds D1 facilitation on ACh output (DeBoer and Abercrombie, 1996).

Some of the previously mentioned facts are relevant to the present study for clozapine has a very low but equal affinity for D1 and D2, and a high affinity for D3 receptors. Such a pharmacological profile could explain the low impact of this neuroleptic on ACh neurons in the NAC or STR and the selective action on ACh neurons in the PFC.

The D3 receptor was cloned initially from the human brain (Van Tol et al., 1991) where it seems to be unhomogeneously distributed displaying high densities in the frontal cortex and very low densities in other brain areas including the STR (Schwartz et al., 1993; Seeman, 1992). It was recently identified in the rat brain where it seems to follow a similar distribution pattern (O’Malley et al., 1992) as the human D3 receptor. The ACh release induced by clozapine in the DA terminal territories studied, as well as the different magnitudes for that phenomenon, could be explained assuming that cholinergic neurons in the three areas explored express also inhibitory D3 receptors in proportions matching the relative territorial D3 distribution just described.

Our results add to many others supporting the view that clozapine is therapeutically efficacious, without producing EPS, due to a preferential action on the frontal cortex without modifying the activity of motor mechanisms in the STR. Thus, clozapine low liability for EPS might be explained by a low impact on striatal cholinergic neurons, as shown in this report. Our study demonstrates a robust ACh release induced by clozapine in the PFC and adds to several other findings suggesting that this region is a particular target for the therapeutic actions of the drug (Bourdelais and Deutch, 1994; Chiodo and Bunney, 1985; Daly and Moghaddam, 1993; Hernandez and Hoebel, 1995; Moghaddam and Bun-
ney, 1990; Pehek and Yamamoto, 1994; Youngren et al., 1994). It also suggests that low levels of cholinergic activity in the PFC are involved in schizophrenia, and that ACh release in this area relates to the therapeutic efficacy of clozapine. In this regard it is worth mentioning that cholinergic activity somewhere in the brain has been associated with mental health. High doses of antimuscarinic agents administered to normal people have been shown to produce a psychotic state including several kinds of hallucinations and disruptions of thinking, with memory loss and confusion (Abood and Biel, 1962; Fisher, 1991; Singh and Kay, 1985; Yeomans, 1995). Administration of nonselective antimuscarinics to chronic schizophrenic patients exacerbates both positive and negative symptoms (Gershon and Olariu, 1960; Singh and Kay, 1975, 1985). Conversely, improvements in schizophrenia symptoms have been reported after treatments with some cholinergic agents (Abood and Biel, 1962; Peiffer and Jenney, 1957; Singh and Kay, 1985).

In conclusion, our results showed that systemic acute clozapine dose-responsively and -differentially increased extracellular ACh in the PFC and to a lower extent in the NAC and STR. The low impact in the STR was considered as an explanation for clozapine low liability to produce EPS. The larger effect on the PFC could be an indirect index of the clozapine’s therapeutic action and suggests that cortical ACh mechanisms could be involved in schizophrenia.

References


