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.

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., 1991; 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; Yee-mans, 1995), and an excessive activation of cholinergic mechanisms in the striatum has been claimed to be responsible for the EPS produced by typical neuroleptics (Brune et al., 1962; Stool et al., 1992).

Our study explored a differential action exerted by acute systemic clozapine 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 special stereotaxic method (Parada et al., 1997). To reduce possible interactions between the PFC and the subcortical nuclei, as a consequence of simultaneous microdialysis in the three regions, the left PFC and right NAC and STR were used. The bregma, the midsagittal sinus and the surface of the skull were the reference points for the rostro-caudal, lateral and ventral stereotaxic coordinates, respectively. In this order and with the incisors bar placed 3.5 mm below rostro-caudal, lateral and ventral stereotaxic coordinates, respectively. No experimental manipulations were performed within at least one week of postsurgical recovery.

Microdialysis. Slim concentric microdialysis probes (Hernandez et al., 1986) were made of silica glass tubing (Polimicro Technologies, Phoenix, AZ) inside a 26-gauge stainless steel tube ending in a tip of cellulose hollow fiber (Spectrum Medical, Laguna Hills, CA) 200 μm in outer diameter, with a 6000 molecular weight cut-off and 4 (PFC and STR) or 3 (NAC) mm in length. The total length of the probes was calculated to allow a 5 mm protrusion from the tip of the guide cannulas. The ACh recovery for these microdialysis probes has been reported to be around 19% (Rada et al., 1993a). At the beginning of one experimental session three probes were connected to the outlets of a triple swivel joint (Meridialysis, Merida, Venezuela) of special design and construction (Parada et al., 1994a). The probes were perfused with Ringer’s solution (135 mM NaCl, 3.7 mM KCl, 1.2 mM CaCl2, 1 mM MgCl2 and 10 mM NaHCO3 at pH 7.4) containing 0.3 μM Neostigmine (Sigma Chemical Co., St. Louis, MO) to prevent the enzymatic ACh degradation and improve its basal recovery. The perfusion Ringer was pumped (World Precise Instruments, Inc SP2101W syringe pump) at 1 μl/min.

Experimental procedures. At the early morning (7:00 A.M.) one awake animal was taken from its home cage and the three microdialysis probes were inserted and secured in place with PE50. The rat was then placed in a circular plastic cage 35 cm in diameter and sample collection started 2 hr later. Samples were simultaneously collected every 25 min from the three brain regions under study. The experimental manipulations were performed after relatively stable baselines of extracellular ACh were obtained. The stability criterion was a variation no greater than 10% of the average of three consecutive samples in each area.

Changes of extracellular ACh after i.p. administration of clozapine [5 (n = 5), 10 (n = 5), 20 (n = 9) or 40 (n = 5) mg/kg/ml] or its vehicle (0.1 N HCl 1 ml/kg; n = 6) were monitored. The i.p. 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.

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.) and then converted sequentially to betaine and hydrogen peroxide in an enzyme reactor (Chrompack Inc. Bromine-Bromine, an irreversible acetylcholinesterase and choline oxidase from Sigma). The resultant hydrogen peroxide was oxidized on a platinum electrode (BAS Inc., West Lafayette, IN) set at 0.5 V with respect to an Ag-AgCl reference electrode (Princeton Applied Res Corp.). The detection limit of this system for ACh was 20 fmol/20 μl standard sample. All samples were analyzed using a single system. The chromatogram for each sample required between 7 and 8 min to be completed and the injection order for the three dialysates obtained during each sampling interval was always PFC, NAC and STR.

Statistical analysis. Absolute basal levels of ACh from PFC (n = 44), NAC (n = 39) and STR (n = 44) dialysates 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 percent 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 mean 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 (df: 1/28; R = .78; F = 41.9; 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) [t = 4.29; df = 56] or STR (2.03) [t = 4.56; df = 56] 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 i.p. administration of different doses of clozapine (5, 10, 20, 40 mg/kg/ml) or its vehicle (HCl 0.1 N). For each dose data were collected from the same animals using simultaneous microdialysis of the three areas explored. Data points are percent values calculated by reference to the appropriate baseline. The arrow on each graph marks the injection time. Clozapine at all doses increased ACh in all regions. This effect was stronger in the PFC than in the NAC or STR, and the maximal effect was evident in the second sample postinjection.

**Fig. 1.** Acetylcholine basal levels (means ± S.E.) obtained during microdialysis in the prefrontal cortex (PFC), nucleus accumbens (NAC) and striatum (STR). PFC and STR columns (n = 44) include data from 40 animals that underwent also simultaneous microdialysis in the other two areas, as well as data from four animals that were perfused just in the PFC or STR. NAC column (n = 40) does not include animals with single perfusion of this structure. Values, not corrected for probe recovery neither normalized, are expressed in pmol/20 µl. PFC showed larger ACh levels than NAC or STR (P < .01) but the difference between the two last regions was not statistically significant.
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 < .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.
The low level of ACh in the STR (0.33 pmol/20 μl) was a striking feature of the present study. Other authors have reported 3 to 14 times larger values (1-4.5 pmol/20 μl) for extracellular ACh during single striatal microdialysis (Ajima et al., 1990; Damsma et al., 1990a; De Boer et al., 1993; Imperato et al., 1994; Robertson et al., 1993). Several methodological differences, including more anterior and lateral anatomical probe placements in the former reports, could explain the higher values.

Whatever the reasons for the differences between the basal ACh release in the STR during different studies, the low basal levels obtained in the striatum during the present one cannot be considered as an artifact of the simultaneous perfusion in the other two areas. Levels were similarly low (0.28 ± 0.01 pmol/20 μl) when the STR was perfused alone. In the same way, basal ACh in the PFC during simultaneous perfusion of all three areas (0.84 pmol/20 μl) was not different from basal levels during the isolated PFC perfusion (1 pmol/20 μl). These considerations suggest that local neostigmine, or the possible local neurochemical depletion during microdialysis of each region, had no detectable influences upon ACh basal levels of the other two areas.

Clozapine differentially and dose-responsively affected ACh release in the three areas examined. Neither the larger impact exerted on cholinergic elements of the PFC, nor the smaller effects on both subcortical nuclei can be explained as artifacts of the simultaneous multiple brain microdialysis or the use of high neostigmine concentrations in the microdialysis perfusate. Against the first possibility it can be argued that clozapine administered during the isolated microdialysis perfusion of the PFC or STR had the same differential effects that were displayed during the simultaneous triple microdialysis, with a similar major impact on extracellular ACh within the PFC. However, high concentrations of neostigmine in the microdialysis perfusate have been shown to alter the pharmacological responsibility of cholinergic neu-
neu, 1990; Pehek and Yamamoto, 1994; Younsgren 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 Oliari, 1960; Singh and Kay, 1975, 1985). Conversely, improvements in schizophrenia symptoms have been reported after treatments with some cholinergic agents (Abood and Biel, 1962; Pfeiffer 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
