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Changes in the Hypothalamic Gonadotropin-Releasing Hormone Gene Expression and the Pituitary Luteinizing Hormone Immunoreactivity in Male Rats: Comparison of Clozapine with Typical Antipsychotics

Myeong Ok Kim, Phil Ok Koh, Jin Hyun Kim[1], Ki Myung Chung[1], Sang Soo Kang[1], and Wan Sung Choi[1]*

Department of Biology, College of Natural Sciences and
[1]Department of Anatomy, College of Medicine, Gyeongsang National University, Chinju 660-751, Korea

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Evidence suggested that atypical antipsychotics (APs) such as clozapine show less side effects than those of typical APs such as haloperidol and sulpiride. However, little is known about chronic effects of these drugs on changes in gonadotropin releasing hormone (GnRH) mRNA expression and luteinizing hormone (LH) immunoreactivity. Male rats were divided into water-, haloperidol-, sulpiride-, and clozapine-treated groups, and these drugs were administered orally for 4 weeks. The changes in the expression of GnRH mRNA and the LH immunoreactivity were determined in the hypothalamus and pituitary, respectively, using *in situ* hybridization and immunohistochemistry. GnRH mRNAs were clearly expressed in the water-treated control rats. This was significantly reduced by the chronic treatments with the typical APs, especially with haloperidol, but not with atypical APs clozapine. Likewise, LH immunoreactivity was clearly stained in the control group. While its immunoreativity was significantly reduced by the chronic APs treatments, clozapine treatment showed only slight attenuation. The results show that the atypical APs clozapine has less side effects in the gonadal function than the typical APs haloperidol and the sulpiride. These results suggest that clozapine is a safer drug than the typical APs, at least in the reproductive system.

Antipsychotics (APs) have been the mainstay of treatment for acute and chronic psychoses for the past several decades. While they have clearly advanced the treatment, there have been substantial side effects such as extrapyramidal symptom (EPS), tardive dyskinesia, hyperprolactinemia, and impairment of the gonadal function. Specifically, typical APs, such as haloperidol and sulpiride, have been popularly used in schizophrenic patients. Treatment with typical APs has a major drawback that most patients under chronic medication suffer from extreme movement disorders including muscular rigidity and akinesia. Moreover, prolonged treatment invariably leads to irreversible tardive dyskinesia (Mycek et al., 2000). Clinical evidence indicates that all effective APs have one principal common denominator with regards to their mode of aciton, i.e. they antagonize dopamine (DA) mediated neurotransmission in the brain. This can be achieved by means of blocking various types of DA receptors (Carlsson, 1988). In fact, most of the clinically approved APs currently used seem to share one specific mechanism of action, e.g. they act as DA-D2 receptor antagonists. Yet, whereas most of the classical APs, haloperidol and sulpiride, usually are therapeutically effective at mainly D2-receptor occupancy in the brain, the atypical AP, clozapine, seems more or less effective at D2 receptor occupancy, as judged by studies using position emission tomography (PET) (Farde et al., 1988; Nordstrom et al., 1995). At the same time clozapine causes very few extrapyramidal side effects and actually appears to possess significantly higher efficacy than the classical APs.

It has been well-known that gonadotropin-releasing hormone (GnRH) stimulates secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the pituitary gland (Kala and Kala, 1985). On the other hand, the activity of hypothalamo-pituitary-gonadal axis is known to be regulated by the feedback effect of gonadal steroids as well as by complex interactions between different classical neurotransmitters and neuropeptides in hypothalamus and extrahypothalamic areas (Kala and Kala, 1985; Brann and Malesh, 1991). Role of DA in the regulation of gonadotropin secretion is still controversial. Both stimulatory and inhibitory effects have been reported. For example, an inhibitory dopaminergic

*To whom correspondence should be addressed.
Tel: 82-55-751-8716, Fax: 82-55-759-0779
E-mail: choiws@nongae.gsnu.ac.kr

influence on the LH secretion has been suggested by Fuxe et al. (1976) and Gallo (1980). On the other hand, Li and Pelletier (1992a, b) have studied effects of haloperidol as well as bromocriptine, a DA D2 receptor agonist, on GnRH mRNA levels in rats and found that chronically treated bromocriptine increased, whereas haloperidol decreased the number of silver grains per neuron. Meanwhile, Tortonese and Lincoln (1994) have reported that sulpiride administered to rats for many days induced an immediate and sustained increase in the secretion of LH, whereas bromocriptine was without effect.

Although a study showed that repeated haloperidol administration decreased the GnRH gene expression in the hypothalamus (Li and Pelletier, 1992a), there is no report on chronic effects of atypical APs on the GnRH gene expression or on LH immunoreactivity thus far. From a clinical point of view, it is worth comparing their chronic effects on the GnRH mRNA level and on LH immunoreactivity because of the recently increasing tendency of atypical APs medication in schizophrenic patients. Therefore, in the present study, we determined chronic effects of haloperidol, sulpiride, and clozapine on the expression of GnRH mRNA and LH immunoreactivity in the hypothalamus and the pituitary, respectively. *In situ* hybridization and immunohistochemistry were used.

Materials and Methods

Animals and tissue preparation

Male Spraque-Dawely rats (Animal Breeding Center, Gyeongsang National University) weighing 240-260 g at the start of the experiment were housed under controlled conditions (artificial light from 06:00 to 20:00, 22 ±0.5℃) with free access to food. The rats were allowed to adapt to their new environment for 1 week before the experiments. The antipsychotics used in the present study were clozapine (Research Biochemicals International; RBI) 20; (±)-sulpiride (RBI) 40; haloperidol (RBI) 1 (given in mg/ml). The drugs were initially dissolved in the minimum quantity of 1 M acetic acid and diluted with deionized water. After dilution to an appropriate volume with deionized water, the pH of the solutions was adjusted with 1 M sodium hydroxide to 6.5. Chronic treatments of rats with APs were induced by free access to the water containing the antipsychotics for 4 weeks. The rats in the control group were administered with water alone. In all experiments, five animals per group were used. On the morning after the last day of the treatment, animals were anesthetized with pentobarbital sodium (30 mg/kg, i.p.), and were perfused intracardially with freshly prepared cold 4% paraformaldehyde in 0.1 M phosphate buffer (4% PFA, pH 7.4). The brain tissues were removed, postfixed by immersion in 4% PFA for 48 h and cryoprotected by immersion in 20% sucrose phosphate buffer for 24 h. The tissues were frozen using OCT compound (A.O. Co.), and 10 μm sections were cut on a Leica cryostat (Leica CM3050, Germany). The sections were then saw-mounted on plus charged and RNase free Fisher slides, dryed and stored at -70℃ until use.

In situ hybridization

In situ hybridization was performed as described previously (Angerer et al., 1987). The stored slides were fixed with ice-cold 4% PFA for 10 min and washed with 2x SSC buffer (2x SSC; 0.5 M NaCl; 0.3 M sodium citrate, pH 7.0), pretreated with proteinase K (1 mg/ml, 15 min) and acetylated with acetic acid (0.25%, 10 min). The sections were hybridized with cRNA probes, obtained by antisense or sense transcription of the cDNA clone (a gift from Dr. S. Ojeda, USA) coding for the monkey GnRH subcloned into pGEM Bluscript plasmid. [^{35}S]UTPαS (Amersham) was used for transcription. After digestion of the template cDNAs on DNase I (Amersham), the GnRH cRNA probes were purified on Sephadex G-50 column (Pharmacia RNA grade), eluted with SET buffer (0.1% SDS, 1 mM EDTA, 10 mM Tris, 10 mM DTT) and measured by a liquid scintilation counter (Beckman, USA). Polyacrylamide electrophoresis was performed to confirm the size of the GnRH transcript. The specific activity of the cRNA probes was approximately 1×10^9 cpm/ml. The sections were incubated with prehybridization buffer [50% formamide, 0.6 M NaCl, 10 mM Tris-HCl (pH 7.5), 0.02% Ficoll, 0.02% polyvinylpyrollidine, 0.1% bovine serum albumin, 1 mM EDTA (pH 8.0) and dextran sulfate] for 1 h at 37℃ and then with probe solution [1 $\times 10^5$ cpm/ml of hybridization buffer (prehybridization buffer containing 50 μg/ml of yeast tRNA and 10 mM of DTT additionally] for 24 h at 60℃ in a humidified incubator, according to the routine method of hybridization. After the hybridization, the slides were post-treated with RNase A (50 μg/ml) at 37℃ for 20 min, washed in a 0.1x SSC buffer at 65℃ for 30 min, dehydrated in alcohol solutions with ascending concentrations. The slides were exposed to β-max autoradiography X-ray film (Amersham, Uppsala, Sweden) for 4 d in light-tight cassettes at -70℃. The slides were dipped in NTB2 emulsion (1:1 dilution, Eastman), exposed at 4℃ for 14 d, developed in Kodak D19 developer (1:1 dilution, Eastman Kodak Co.) at 15℃, and stained with cresyl violet. To assess the specificity of the hybridization signal, consecutive sections were alternatively hybridized with sense or antisense probes. Sections were observed under a dark.

Immunohistochemistry

The stored tissue sections on slide were air-dryed and dipped in 0.1 M phosphate buffered saline (PBS, pH 7.4) for 5 min at room temperature. And then, the slides were incubated in 0.3% H_2O_2 for 10 min and rinsed throughly with PBS. Sections were blocked with 1% normal goat serum (NGS) in PBS for 1 h to

eliminate nonspecific binding of primary antibody, and then incubated with the primary antibody to the rat LH (Biogenesis Co.; 1:2000 with 2% NGS in PBS) at 4℃ for 24 h in a humidified chamber. After rinsing with PBS, the sections were incubated with biotin-conjugated goat anti-rabbit IgG (Vectastatin kit; 1:200 in PBS containing 1% NGS) for 1 h, followed by avidin-biotin-peroxidase complex (Vector Laboratories Inc., Burlingame, USA) for 1 h at room temperature. The sections were washed with PBS and incubated in 0.5% 3,3'-diaminobenzidine (DAB)-0.03% hydrogen peroxide in PBS for 3 min. Sections were then washed, dehydrated in serial concentrations of ethanol and coverslipped with a synthetic permounting medium (Fisher, USA). As a negative control, normal serum was applied instead of the primary antibody reaction. Slides were observed under microscope and then photographed. Localization of neurons expressing LH immunoreactivity as well as the counting the number of LH-immunostained neuron were also performed randomly by an observer unaware of the treatment.

Statistics

Statistical analysis was carried out using one-way analysis of variance (ANOVA) and Student's *t*-test.

Results

Effects of chronic administration of water, haloperidol, sulpiride, and clozapine on the hypothalamic GnRH mRNA level

Analysis of the autoradiograms indicated that a strong signal could be obtained after 2 wk of exposure. Hybridization with a sense nucleotide produced no labelling in adjacent sections, suggesting specific signal against GnRH mRNA. In control group, GnRH mRNA was expressed in the preoptic area of the hypothalamus (Figs. 1a and 2a). As shown in Fig. 3, the average number of GnRH containing neurons in the control group

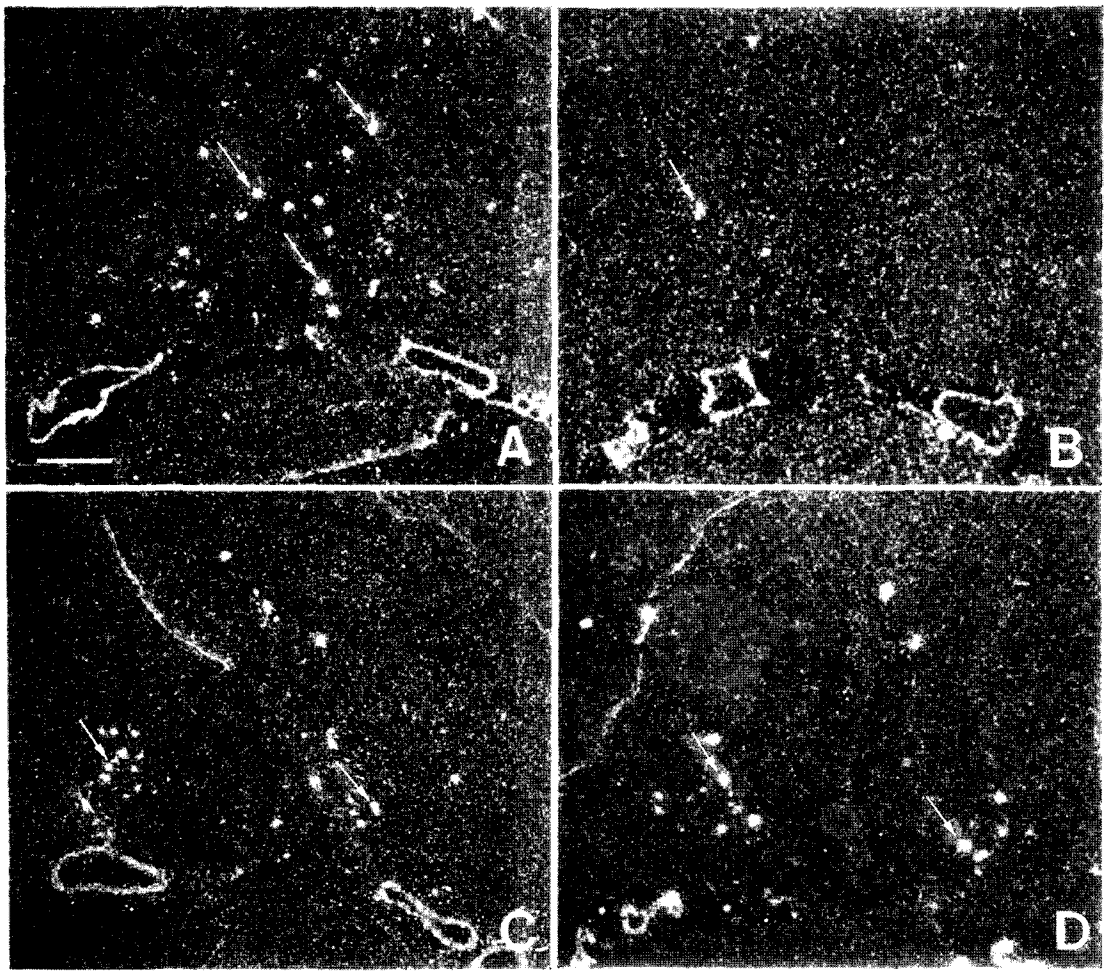

Fig. 1. Dark-field autoradiograms for GnRH mRNA in the chronic antipsychotics-treated rat hypothalamus by *in situ* hybridization. GnRH mRNA containing neurons were localized in the vascular organ of the lamina terminalis (OVLT) of water (A), haloperidol (B), sulpiride (C), and clozapine-treated animals (D). Arrows indicate GnRH mRNA containing cells. Scale bar=250 μm.

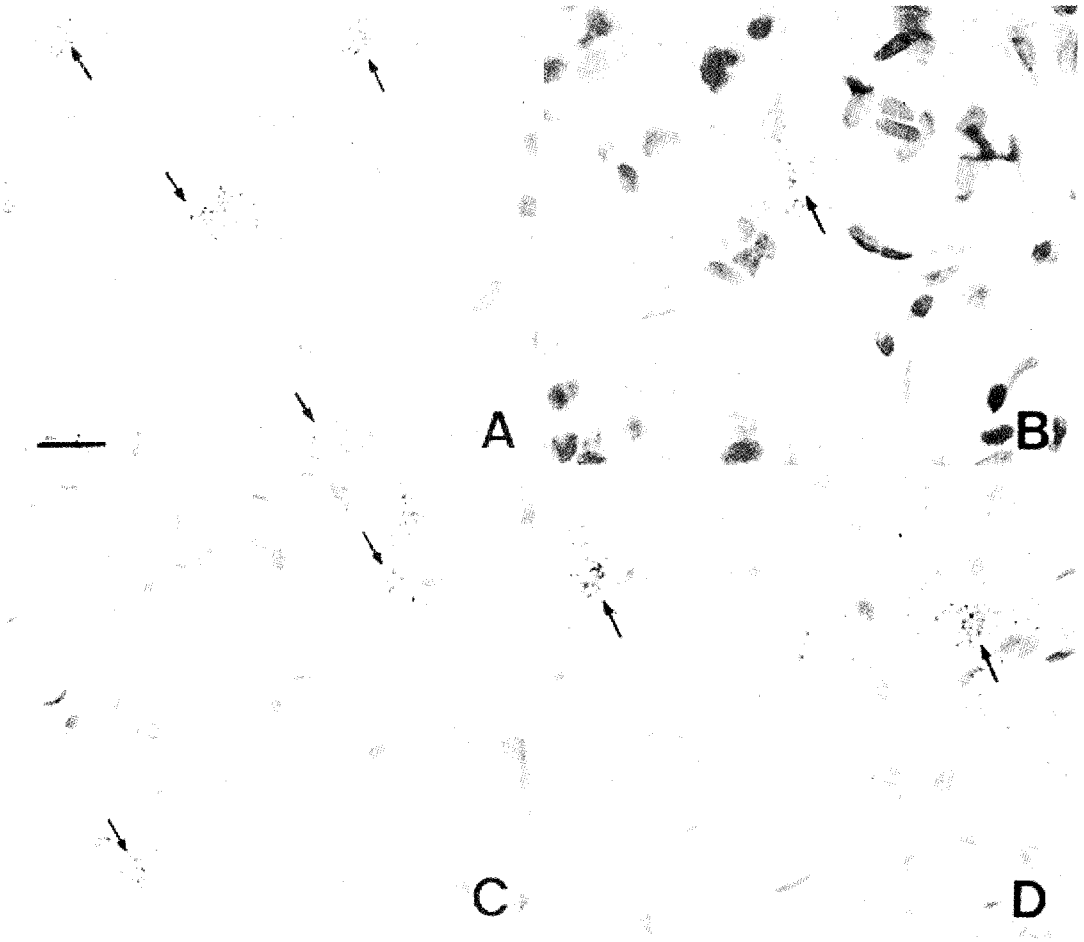

Fig. 2. Bright-field photomicrograms for GnRH mRNAs in the vascular organ of the lamina terminalis (OVLT) of water (A), haloperidol (B), sulpiride (C), and clozapine-treated rats (D). Sections were counterstained by cresyl violet. Arrows indicate GnRH mRNA containing cells. Scale bar=25 μm.

was 28.8 per five sections. On the other hand, chronic treatment with haloperidol, sulpi-ride, and clozapine decreased the mean number of grains overlying labeled neurons by 77.4 ($P < 0.001$), 36.5 ($P < 0.05$), and 20.1% ($P > 0.05$), respectively. Alternatively, when the GnRH mRNA level in each of animal groups was quantita-

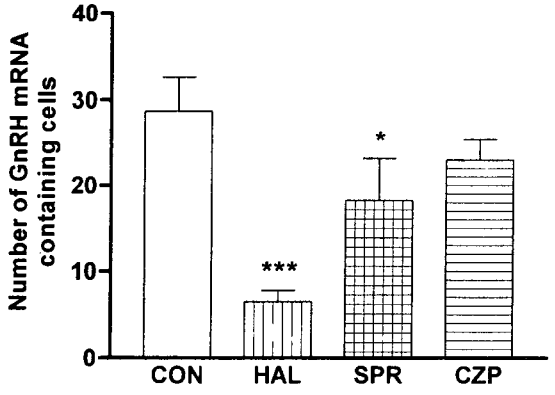

Fig. 3. The number of GnRH mRNA containing neurons in the OVLT of water (A, CON)-, haloperidol (B, HAL)-, sulpiride (C, SPR)-, and clozapine (D, CZP)-treated rats. The vertical bars denote the standard error of the mean. The number of animals used in each group was 5. Twenty-five sections per group were counted. *$P < 0.05$ and ***$P < 0.0001$ *vs* water group.

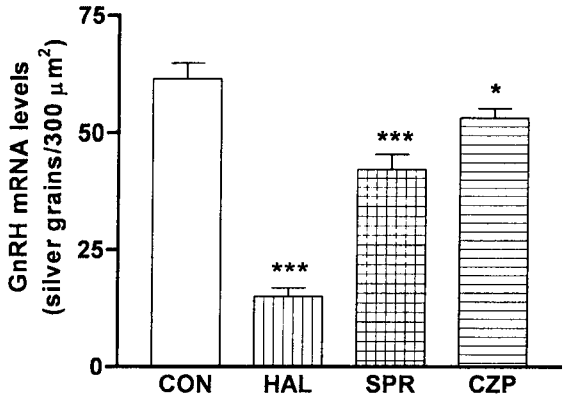

Fig. 4. Quantitative analysis for GnRH mRNA in the OVLT of water (A, CON)-, haloperidol (B, HAL)-, sulzpiride (C, SPR)-, and clozapine (D, CZP)-treated rats. Siver grains for GnRH mRNAs were counted using the densitometric scanning program (NIH program) and data were expressed in the mean (silver grains/ 300 μm_2) S.E.M. The vertical bars denote the standard error of the mean. The number of animals used in each group was 5. Twenty-five sections per group were counted. *$P < 0.05$ and ***$P < 0.0001$ *vs* water group.

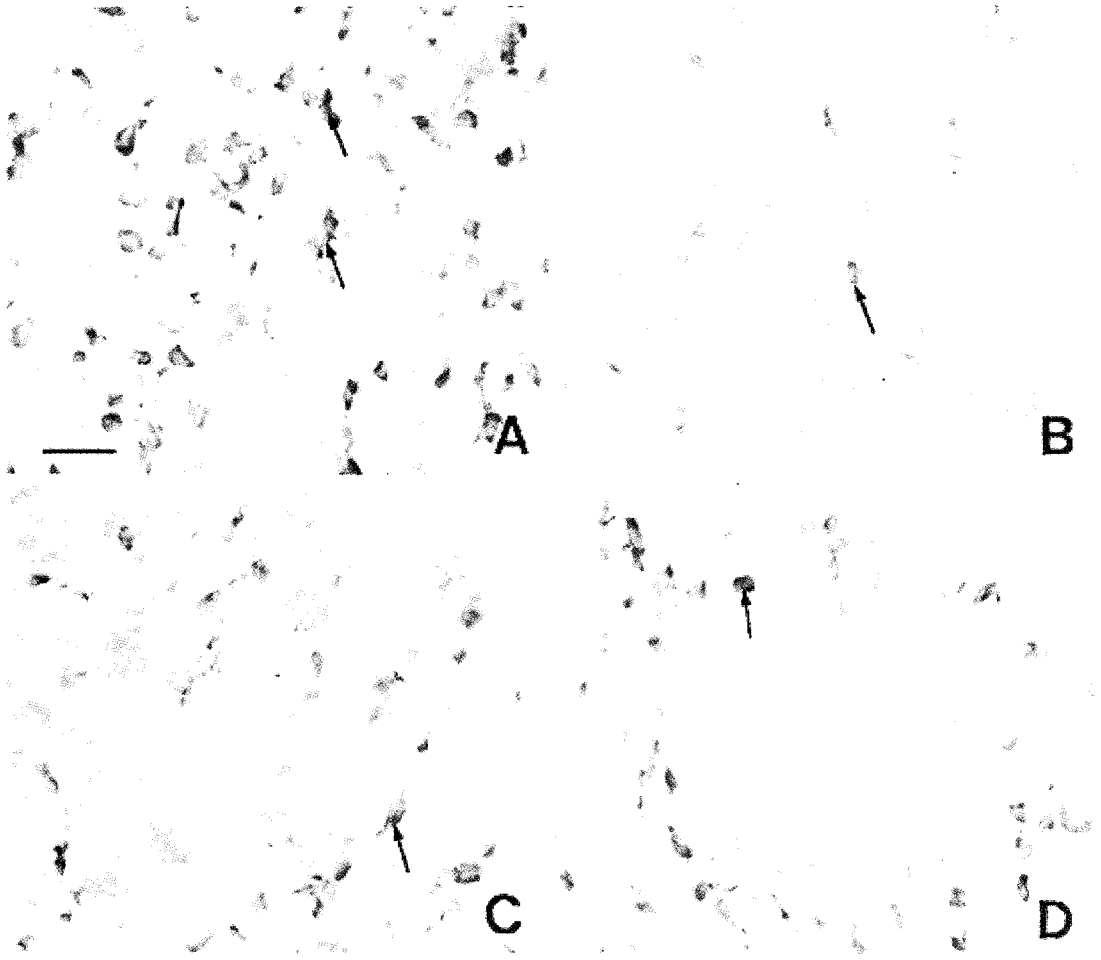

Fig. 5. Representive localization of the luteinizing hormone (LH) immunoreactive cells in the pituitary anterior lobe of water (A)-, haloperidol (B)-, sulpiride (C)-, and clozapine-treated (D) rats. Arrows indicate the cell stained positively for LH antibody. Scale bar=25 μm.

tively analysed using NIH program, their levels were 24.6 ($P < 0.001$), 69.1 ($P < 0.001$), and 86.7% ($P < 0.05$) of control group for haloperidol, sulpiride, and clozapine groups, respectively (Fig. 4).

Effects of chronic administration of water, haloperidol, sulpiride, and clozapine on the pituitary LH immunoreactivity

Using immunohistochemistry, we localized LH-producing cells to the rat pituitary. The absence of immunoreactivity when the primary antibody was omitted suggested that the staining was specific for LH. Many of the LH-immunostained cells were counted in the sections of control group (Fig. 5a).

As shown in Fig. 5b-d, the LH immunoreactivity was significantly reduced by the chronic treatments with the APs. The decreases in LH immunoreactivity were pronounced in the groups treated with the typical APs haloperidol and sulpiride than those with atypical APs clozapine.

Discussion

The new atypical APs have a number of advantages over the typical APs. These include lower rates of neurologic and, for some agents, neuroendocrinologic side effects and improved efficacy in the treatment of negative symptoms (Keck and McElroy, 1997). However, to date, there is no comparison data between the typical and atypical APs on changes in hypothalamic GnRH gene expression and pituitary LH immunoreactivity. To ascertain these unknown points, we used clozapine, haloperidol, and sulpiride as atypical and typical APs, respectively, and compared hypothalamic GnRH gene expression and pituitary LH immunoreactivity. We found that the clear expression of GnRH mRNA in the OVLT of the control group was significantly reduced by the treatments with the typical APs haloperidol and sulpiride, but not by the atypical AP clozapine. In addition, the LH immunoreactivity shown in control group was attenuated significantly by the chronic haloperidol and sulpride treatments, but not by clozapine although the level was still lower than the control group. These

results suggest that the new atypical AP clozapine has an advantage over the typical APs haloperidol and sulpiride in view of the side effects in the gonadal neuroendocrine function.

The mechanisms involved in the decreased GnRH mRNA levels in the OVLT of the hypothalamus in male rats cannot be directly inferred from this study, but several findings suggest that they may be related to APs-induced dopaminergic receptor occupancy. It has been demonstrated that DA could stimulate GnRH release *in vitro* from mediobasal hypothalamus (Schneider and McCann; 1969; Negro-vilar et al., 1979), suggesting that GnRH mRNA levels in the septohypothalamic area are positively modulated by the dopaminergic system. In the present study, chronic treatments with APs significantly reduced the GnRH mRNA expression in the OVLT. These results are in part consistent with the report of Li and Pelletier (1992a) that a 14-day treatment with bromocriptine, a dopamine receptor agonist, increased the number of silver grains, while the same treatment with haloperidol decreased it. The dopaminergic neurons are projected from the anterior hypothalamus, especially to the OVLT, which contain GnRH cell bodies (Bjorklund and Nobin, 1973). Jennes et al. (1983) have reported that dopaminergic neurons might exert direct effects on GnRH neurons since there are contacts between dopaminergic endings and GnRH cell bodies (Jennes et al., 1983). Likewise, our data that chronic treatments with APs attenuate GnRH mRNA expression may be due to a direct blocking effect of dopaminergic neurons on GnRH neurons. However, we could not rule out the alternative possibility that the blockade of DAergic receptor may modulate GnRH neuronal activity at different anatomical levels. Indeed, an inhibitory effect of DA on GnRH release from the hypothalamus has been reported (Owens et al., 1980; Tasaka et al., 1985). Testing of this possibility is left for further study.

In a PET analysis of central dopamine receptor occupancy in patients treated on typical APs and clozapine, schizophrenic patients on conventional doses of various typical APs demonstrated D2 occupancy of 70% to 80% in the basal ganglia. Patients with higher side effects had higher D2 occupancy than patients without side effects. However, the patients treated with clinically effective doses of clozapine showed lower D2 occupancy of 38% to 63%. This finding was thought to correlate with the atypicality of the drug and the low frequency of EPS (Fade et al., 1992). Indeed, atypical APs including clozapine are known to have relatively higher the 5-HT2 affinity than the typical APs and this relatively higher occupancy at the 5-HT2 receptor may provide protection from D2 receptor occupancy (Kapur and Remington, 1996). These clinical studies highly suggest that relatively reduced side effects of clozapine may be due to its higher 5-HT2 occupancy. However, Yamada et al. (1995) observed that 5-HT did not contribute to the changes observed in GnRH and LH release induced by repeated L-DOPA administration in male rats. Further studies are necessary.

In the pituitary, the number of LH immunoreactive cells was also decreased by the chronic treatment with APs. Consistent with our data, Vijayan and McCann (1978) have reported that i.c.v. injection of DA increased LH secretion in female rats. In addition, a prolonged treatment with L-DOPA in male rats induced release of LH from the pituitary (Yamada et al., 1995). Taken together, the decrease in LH immunoreactivity induced by the APs in male rats observed in the present study appears to have been due to a decrease in GnRH release via suppression of dopaminergic systems in the hypothalamic-pituitary axis. On the contrary, there are some reports of an inhibitory action of DA on LH release (Fuxe et al., 1976; Gallo, 1980). This possibility remains to be proven experimentally.

It is also known that hyperprolactinemia can inhibit GnRH or LH release. In the human, traditional and typical APs elevate prolactin levels (Rubin, 1987). In contrast, clozapine dose not cause sustained prolactin elevations (Meltzer et al., 1979). When prolactinemia status is persistent, disruption of normal hormonal production and cyclical secretion of sex steroids (Yazigi et al., 1997; Melmed, 1995; Vance, 1997). Whereas, prolactin levels are lowered, these side effects usually return to normal. In experimental animals, Park and Selmanoff (1991) reported that suppression of LH increase after castration was induced by exogenous administration of prolactin in male rats. In addition, in male rats bearing a prolactin-secreting tumor, serum LH level and LH release from the oituitary in response to GnRH administration were reduced (Hodson et al., 1980). More recently, Yamada et al. (1995) observed that serum prolactin levels were decreased by a prolonged L-DOPA administration in male rats. However, it is left to test if the increase in serum prolactin levels contributed to the decrease in GnRH mRNA level and LH immunoreactivity induced by APs.

In summary, chronic administration of each of the antipsychotics was found to differentially decrease the basal levels of GnRH mRNA expression and LH immunoreactivity, especially in the case of the typical antipsychotics haloperidol and sulpiride. These findings suggest that the atypical APs have less side effects than the typical APs when mediated as a AP.

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