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MORPHOLOGICAL CHANGES IN RAT DOPAMINERGIC NEURONS AFTER CHRONIC HALOPERIDOL TREATMENT: POSSIBLE RELATION TO VACUOUS CHEWING*

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Introduction

Neuroleptic drugs are chronically used in the treatment of schizophrenia and other psychotic disorders and are thought to exert their antipsychotic activity by blocking dopamine receptors (Carlson and Lindquist 1963, Creese et al. 1977). Unfortunately, neuroleptic treatment is associated with acute and delayed motor side effects, including parkinsonism, akathisia and tardive dyskinesia (TD). TD, characterized by involuntary movements predominantly in the orofacial region, develops in up to 20% of patients chronically treated with neuroleptics such as haloperidol (Jeste and Wyatt 1979; Klawans and Rubovits 1972; Tarsy and Baldessarini 1974, 1977). Although the relationship between TD and long-term haloperidol treatment has been established, the pathophysiology of this motor disturbance is still unknown. A peculiar characteristic of this syndrome is that TD generally persists after haloperidol withdrawal and occasionally becomes irreversible, indicating that haloperidol has produced long lasting changes in brain function that are no longer related to the presence of the drug (Meshul et al. 1992, 1994). A relevant contribution to the understanding of human TD has been provided by using animal models of motor impairments. Rats chronically treated with haloperidol develop vacuous chewing movements (VCMs), a syndrome similar to human TD for both time course and signs (Ellison and See 1989; Egan et al. 1996; Waddington 1990, 1997). Moreover, in some rat strains, it has been shown that haloperidol-induced VCMs develop with different frequency, mimicking what has been observed in haloperidol treated patients (Tamminga et al. 1990). So far different hypothesis have been formulated to explain the development of VCMs, including the existence of a dopaminergic receptor supersensitivity (Klawans et al. 1972, 1977; Tarsy and Baldessarini 1974; Burt et al. 1977), a dopamine D1/D2 receptor imbalance (Casey 1995; Waddington 1997; Peackok and Gerlach 1997) and a ?-aminobutiric acid (GABA) deficiency (Fibiger and Lloyd 1984; Gale 1980). All these studies considered VCMs as a consequence of haloperidol mediated receptor blockade and focused their attention on basal ganglia circuits and on the altered neurotransmitter-receptor equilibrium in these areas. Recently, however, the emphasis on TD and VCMs studies has shifted from neurotransmitter models to an approach that considers TD or haloperidol-induced VCMs as consequence of a direct neurotoxic effect mediated by haloperidol itself (De Keyser 1991). The toxicity of haloperidol was first attributed to an increase of free radical byproducts of catecholamine metabolism, a direct consequence of the increased catecholamine turnover mediated by haloperidol (Cadet et al. 1986, Jeste and Wyatt 1981). Both L-dopa and dopamine can undergo auto-oxidation with a consequent increase in oxygen free radicals shown to be cytotoxic to rat primary hippocampal neurons, NCB-20 (Parsons 1985) and C6 cell lines (Behl et al. 1995, Vilner and Bowen 1993) and basal ganglia neurons (Cadet et al. 1986, Lohr 1991). Accordingly, *ex vivo* studies have reported neuropathological alterations in basal ganglia of rats chronically treated with haloperidol. Electron microscopy studies have shown that chronic haloperidol treat-

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ment increases perforated postsynaptic density in the head of caudate nucleus of rats (Meshul et al. 1992) and produces a spine density alteration in rat striatum (Kelley et al. 1997). Significant neuronal loss and glial reactions have been shown in the caudate nucleus of patients who had received chronic neuroleptic treatment (Nielsen and Lyon 1978, Andreassen et al. 1998).

Recently, several studies demonstrated that a haloperidol metabolite haloperidol-pyridinium (HPP+) shared structural similarity and toxic effect with 1-methyl-4-phenylpyridinium (MPP+), the metabolite that mediates the toxicity on nigrostrial dopaminergic neurons induced by administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Fang et al. 1995a, Wright et al. 1998, Petzer et al. 2000). Post-mortem studies have revealed the presence of HPP+ in different brain regions of patients treated with haloperidol (Eyles et al. 1997), and HPP+ was shown to exert a direct toxic effect on dopaminergic mesencephalic neurons in culture (Bloomquist 1994). The possibility that haloperidol metabolites could mimic *in vivo* the toxicity of MPP+, led us to investigate the presence of morphological alterations of dopaminergic neurons after chronic haloperidol treatment and to verify if a correlation existed between any morphological changes and the development of VCMs in rats.

Materials and Methods

Animals

Male Sprague-Dawley albino rats (Charles River) weighting 100-125 g were kept on a 12h/12h dark/light cycle with food and tap water available *ad libitum.* All experimental protocols were approved by the Ethical Committee at the University of Cagliari and performed in strict accordance with the E.C. regulation for care and use of experimental animals (CEE N° 86/609).

Drugs and chemicals

Haloperidol hydrochloride, clozapine and RS 102221 hydrochloride were purchased from Tocris Cookson Ltd. (Avonmouth Bristol, UK). Ritanserin and 3,3'-diaminobenzidine were from Sigma Co. (St. Louis, MO, U.S.A.). Amisulpride mesylate was generously supplied by Sanofi-Synthélabo (Bagneaux, France).

Immunocytochemistry

Rats were anaesthetized with Equithesin (2.5 mg/kg i.p.) and perfused transcardially with 4% paraformaldehyde in 0.1% phosphate buffer (PB), pH 7.4. The brains were subsequently post-fixed in the same fixative for two hours and cryoprotected overnight with a solution of 30% sucrose in 0.1M PB at 4° C. 40µm coronal sections were cut using a cryostat (Leica 3050). Free-floating cytochemical stainings were performed in strictly accordance with the general methodological procedures indicated by Coté et al. (1993).

Immunostaining for tyrosine hydroxylase (TH)

Briefly, after rinsing in phosphate buffered saline with 0.2% Triton X-100 (PBS+T), sections were incubated with 0.3% of H_2O_2 in PBS and, after extensive washing, with a blocking

solution containing 1% BSA and 20% normal goat serum in PBS+T to reduce background. Sections were then incubated overnight at 4° C with an anti-TH antibody (1 : 400 from Chemicon, Temecula, CA, U.S.A.). After rinsing, sections were incubated with a goat anti-mouse biotinylated IgG (1 : 600; Vector, Burlingame, CA, U.S.A.) for one hour, followed by an avidin-biotin complex (ABC) (1 : 500; Vectastain ABC kit from Vector) for an additional hour.

Immunostaining for dopamine transporter (DAT)

Sections were extensively washed in tris buffer saline (TBS) and then incubated in 0.3% H_2O_2 in TBS for 15 min. Subsequently, sections were incubated for 1 hour in 1% BSA and 20% normal goat serum in TBS + 0.3% Triton X100 (TBS+T) and then incubated with an rat anti-DAT antibody (1 : 5000; Chemicon) for two days at 4 °C. After rinsing, a goat anti-rat biotinylated IgG (1 : 800; Vector) was added for 1 hour. Sections were incubated with ABC (1 : 500) for 1 hour.

After ABC incubation, sections for both TH and DAT staining were exposed to 3,3'-diaminobenzidine containing cobalt chloride and nickel ammonium sulfate for 15 min. Immunostaining was developed by adding H_2O_2. Finally, after washing in PBS+T, all sections were mounted on gelatin-coated glass slides, air-dried, dehydrated in ascending concentrations of ethanol, cleared with xylene, and coverslipped with Entellan. The mounted sections were examined under a BX-60 Olympus microscope.

Quantitative analysis of immunostained TH fibers in the striatum and nucleus accumbens

The region of analysis corresponded to bregma coordinates +2.2 to +0.7 mm as indicated in the Paxinos and Watson atlas. The density of the dopaminergic nerve fibers in the striatum and nucleus accumbens, was quantified using an image analysis system (KS 300; Karl Zeiss Vision GmbH, Hallbergmoos, Germany). The nerve fiber density in at least 10 alternated sections, was determined by measuring the percentage of the area occupied by TH-positive fibers respect to standardized area, using an X100 lens and a Zeiss Sound Vision digital microscope video camera. Gray values, measured in TH-negative areas of the sections, were subtracted as background from the resulting binary picture. The analysis procedure was strictly carried out as a blind study.

Cell body size measurement

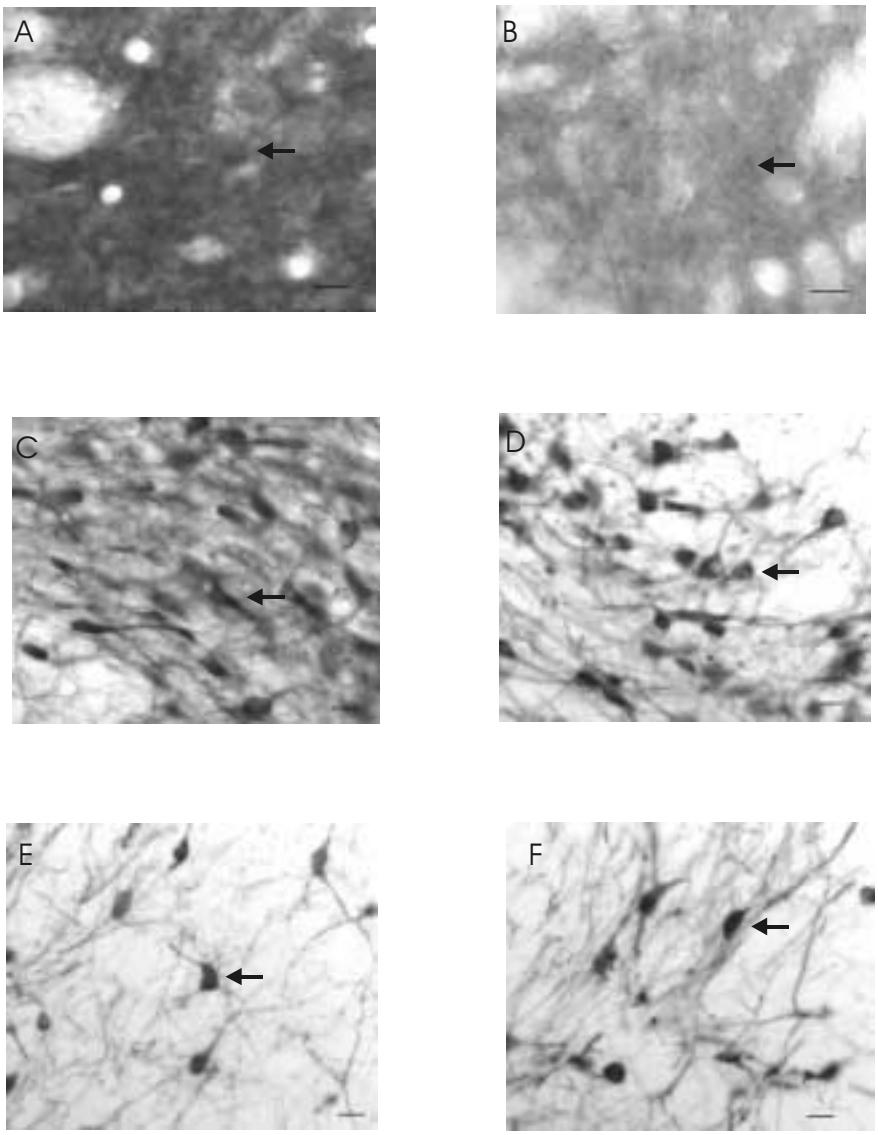
The region of analysis corresponded to bregma coordinates –4.8 to –6.04 mm in order to evaluate the cell body size of substantia nigra and VTA, known to be heavily populated by dopaminergic neurons. For each section, cell body area (μm^2) was determined (40X magnification) for TH-positive cell somata only in those neurons where nuclei could be observed.

Vacuous chewing movements quantification

Vacuous chewing movements were measured 12 hours, 3 days and 3 weeks after the last dose of neuroleptic. Animals were placed individually in a small (30x20x30) plexiglass cage, and were allowed to adapt to the observation cage for a period of 1 hour. In the morning (between

Figura 1

Micrographs showing TH-IM fibers in the striatum of rats treated with vehicle (A) and haloperidol 1 mg/kg (B). Note the dense immunoreactivity in patch-like regions (arrows). C-D: micrographs showing TH positive neurons (arrows) from substantia nigra pars compacta of rats treated with vehicle and haloperidol 1 mg/kg respectively. E-F: TH immunoreactive neurons from substantia nigra pars reticulata (arrows) of rats treated with vehicle and haloperidol 1 mg/kg respectively. Scale bars: (A-B) = 10µm; (C-D-E-F) = 20 µm.



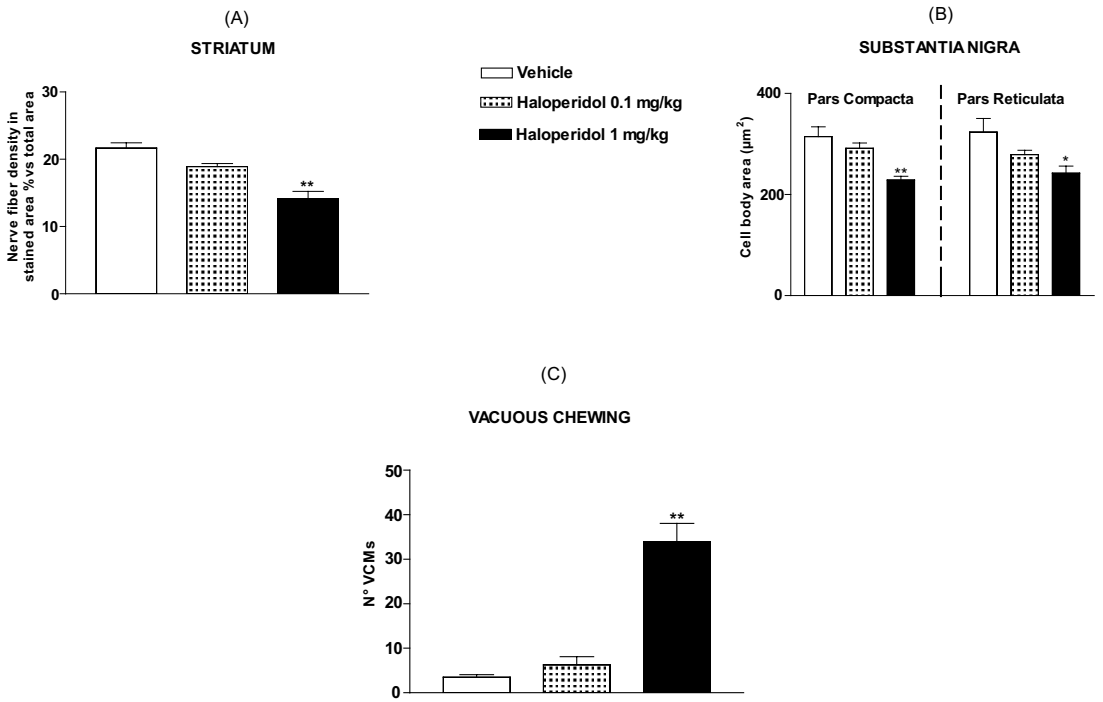
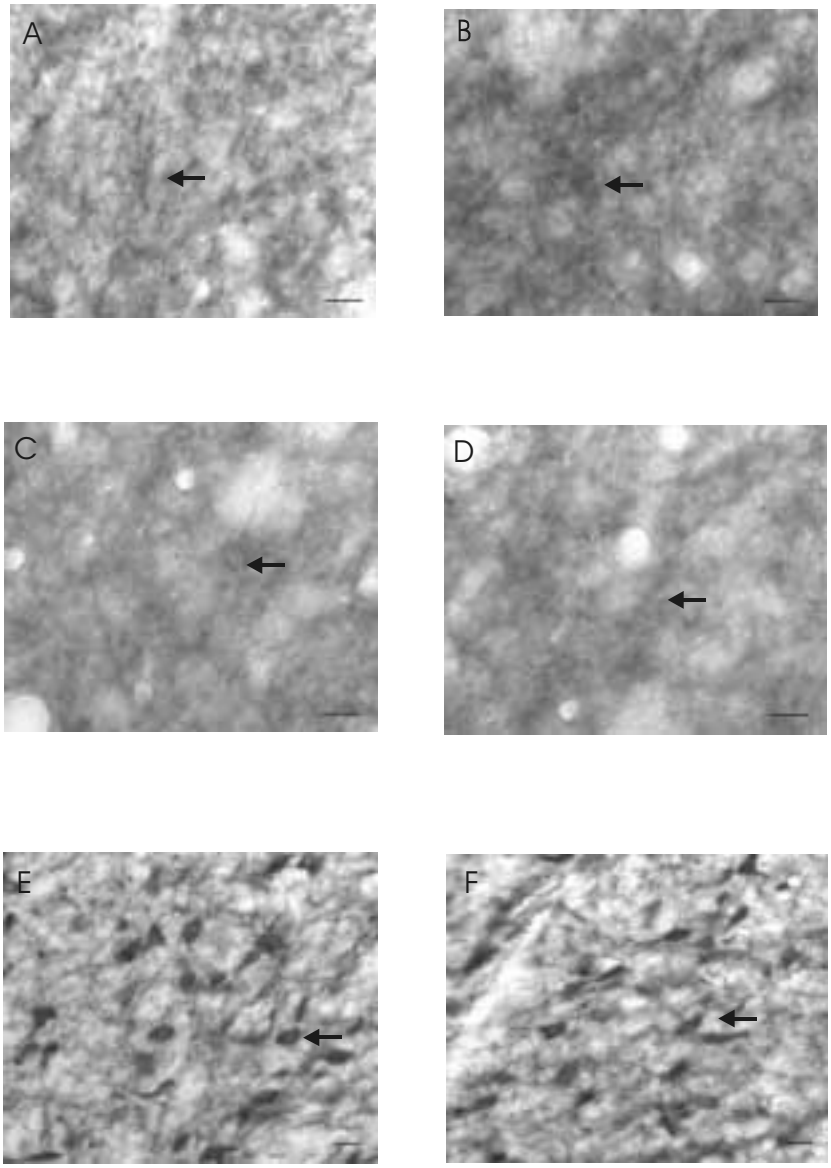


Figura 2

A: histogram showing the TH-IM nerve fiber density in the striatum of rats treated with vehicle, haloperidol 0.1 and 1 mg/kg. A significant reduction in the TH-IM nerve fiber density was found with haloperidol at the dose of 1 mg/kg. B: cell body area of TH-IM neurons in the substantia nigra pars compacta and reticulata. Haloperidol, at the dose of 1 mg/kg, determined a significant reduction of cell body area both in pars compacta and reticulata with respect to vehicle. C: histogram illustrating the number of VCMs in rats treated with vehicle and haloperidol at the dose of 0.1 and 1 mg/kg. This last dose caused a significant increase of VCMs. Statistical significance vs. respective control treated rats has been analyzed using ANOVA test followed by Newman-Keuls post hoc-test (*P<0.05; **P<0.01)

Figura 3

Micrographs of TH-IM fibers in the core of the nucleus accumbens of rats treated with vehicle (A) and haloperidol 1 mg/kg (B). C-D: TH-IM fibers in the shell of the nucleus accumbens of rats treated with vehicle and haloperidol 1 mg/kg respectively. E-F: micrographs showing TH positive neurons (arrows) from VTA of rats treated with vehicle and haloperidol. Scale bars: (A-B-C-D) = 10µm; (E-F) = 20 µm.



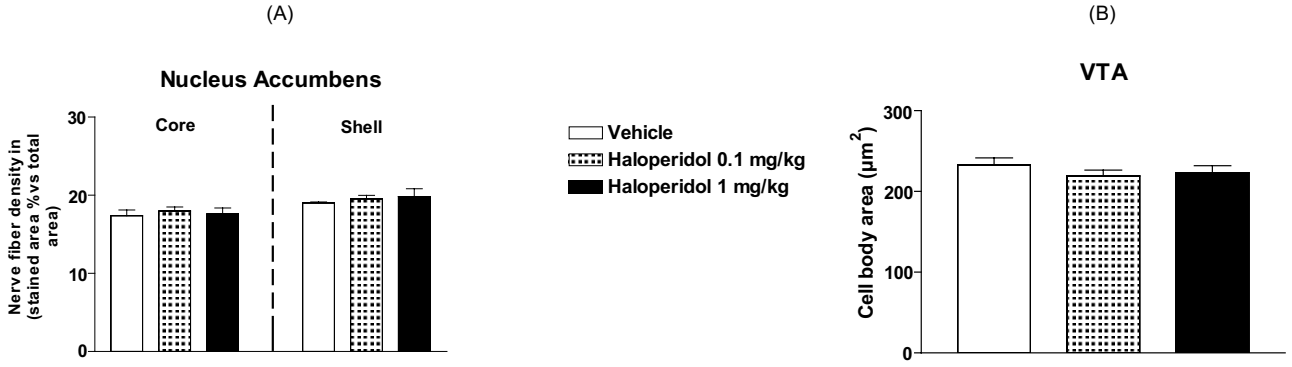
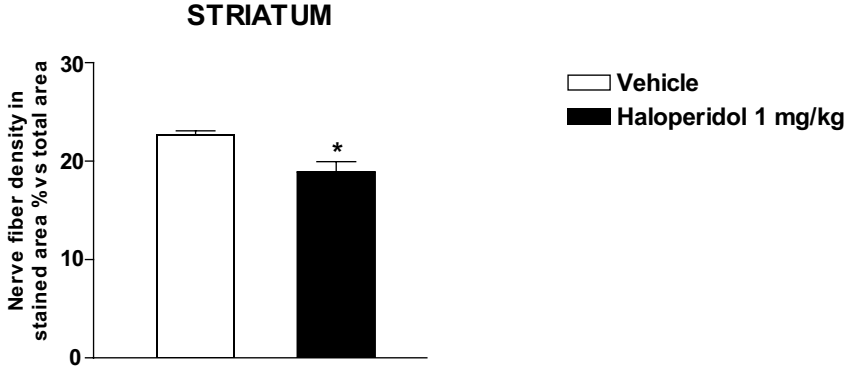


Figura 4

A: Histogram showing the TH-IM nerve fiber density in the nucleus accumbens (core and shell) of rats treated with vehicle, haloperidol 0.1 and 1 mg/kg. No differences were found in TH-IM nerve fiber between treatments. B: cell body area of TH-IM neurons in the VTA. No differences were found between treatments.

Figura 5

DAT-IM nerve fiber density in the striatum of rats treated with vehicle and haloperidol 1 mg/kg. A significant reduction in the DAT-IM nerve fiber density was found with haloperidol at the dose of 1 mg/kg. Statistical significance vs. respective control treated rats has been analyzed using T test followed (*P<0.05; **P<0.01)



9:00 and 11:00 A.M.) vacuous chewing movements were scored during a 30 minutes observation period, and counting of VCMs was stopped whenever the animal began grooming and restarted when grooming stopped (Gunne et al. 1982). For calculation purposes, each burst of jaw tremor was counted if its duration was at least of 3 sec. A vacuous chewing movement consisted of a rapid movement of the jaw, which resembled chewing but did not appear to be directed at any particular stimulus. In all the experiments, the observer was blind to the treatment given to the rats.

Statistical analysis

The statistical significance of the effect of any treatment was evaluated by T test , one-way or two-ways analysis of variance (ANOVA). When a significant ($P < 0.05$) interaction was demonstrated, the Newman-Keuls post-hoc test was used to compare the effect of different drugs.

Results

Correlation of VCMs with morphological changes in rats following 4 weeks of treatment with haloperidol (0.1 mg/kg and 1 mg/kg).

Under high power microscopic lens (100X), a dense immunoreactivity in patch-like regions were observed in the striatum (Fig. 1 A-B). TH-IM neurons were seen throughout the entire substantia nigra pars compacta (Fig. 1 C-D) and reticulata (Fig. 1 E-F). The somatodendritic profile was characterized by large perikarya with dendritic arborization. The comparison of striatal TH-IM fiber density between rats treated respectively with vehicle, haloperidol 0.1 mg/kg and haloperidol 1 mg/kg, displayed a significant difference (one-way ANOVA $F_{2,24} = 23.2$, P

< 0.01). A significantly lower TH-IM fiber density was found in the striatum of rats treated for 4 weeks with haloperidol at the dose of 1 mg/kg vs. vehicle treated rats ($P < 0.01$) (Fig. 2-A). Conversely, haloperidol at dose of 0.1 mg/kg produced only a small reduction of fiber density that was not significant.

The evaluation of cell body size in substantia nigra pars compacta and reticulata, showed significant differences between the three different groups of treatment (one-way ANOVA $F_{2,24} = 13.72$, $P < 0.01$, pars compacta; $F_{2,24} = 6.07$, $P < 0.05$, pars reticulata) (Fig. 2-B). A significant reduction of cell body size in rats treated with haloperidol 1 mg/kg vs. vehicle was found both in pars compacta ($P < 0.01$) and pars reticulata ($P < 0.05$).

After 4 weeks of treatment with vehicle or haloperidol (0.1 or 1 mg/kg), significant differences in VCMs were observed (one-way ANOVA, $F_{2,33} = 39.63$, $P < 0.01$). At the dose of 1 mg/kg of haloperidol, rats showed significantly more VCMs with respect to rats treated with vehicle ($P < 0.01$) (Fig. 2-C).

A dense TH staining was also found in the core (Fig. 3 A-B) and shell of the nucleus accumbens (Fig. 3 C-D). Moreover, a vast number of small and rounded oligodendritic TH-IM dopamine neurons were observed in VTA (Fig. 3 E-F). No differences were found between the treatments in the TH-IM fiber density both in core (one-way ANOVA, $F_{2,24} = 0.21$, $P > 0.05$) and shell of nucleus accumbens (one-way ANOVA, $F_{2,24} = 0.38$, $P > 0.05$) (Fig. 4-A). The analysis of cell body area also revealed no differences between the treatments in the VTA (one-way ANOVA, $F_{2,24} = 0.42$, $P > 0.05$) (Fig. 4B).

The comparative study of striatal dopaminergic fiber density visualized with the antibody against DAT confirmed the reduction of staining in haloperidol treated rats vs. vehicle (T test, $P < 0.05$) (Fig. 5).

Correlation of VCM with morphological changes in rats following 2-3-4 weeks of treatment with haloperidol and withdrawal

Rats treated with haloperidol (1 mg/kg) for 2, 3, 4 weeks and in withdrawal for 3 days and 3 weeks (after 4 weeks haloperidol treatment) showed significant morphological alteration both in striatum (one-way ANOVA, $F_{5,48} = 11.4$, $P < 0.01$) and in nigra pars compacta (one-way ANOVA, $F_{5,48} = 24.3$, $P < 0.01$) and reticulata (one-way ANOVA, $F_{5,48} = 21.16$, $P < 0.01$). In the same experimental groups significant differences in VCMs were observed (two-ways ANOVA test, $F_{1,22} = 698$, $P < 0.001$). Rats treated with haloperidol (1 mg/kg) for 2 weeks, showed no difference in TH-IM striatal fibers (Fig. 6-A) nor in cell body size in substantia nigra pars compacta and reticulata (Fig. 6-B), but displayed significant VCMs increase with respect to vehicle treated rats ($P < 0.05$) (Fig. 6-C). Just after three weeks of treatment, there was a significant decrease of TH-IM fiber density in striatum ($P < 0.05$) (Fig. 6-A), while a light , although no-significant, decrease was found in cell body area of TH-IM neurons in substantia nigra pars compacta and reticulata (Fig. 6-B). At 3 weeks of haloperidol treatment, rats showed a high significant increase of VCMs compared to vehicle treated rats ($P < 0.01$) (Fig. 6-C).

Accordingly to the results showed above, rats treated with haloperidol for 4 weeks, displayed a decrease of TH-IM fiber density in striatum and a cell body shrinkage of TH-IM neurons in substantia nigra pars compacta and reticulata, associated with an increase of VCMs.

The morphological alterations, observed after 4 weeks of haloperidol treatment, persisted in the different areas after 3 days of withdrawal ($P < 0.01$ in each condition vs. vehicle treated rats) (Fig. 6 A-B). Moreover rats still exhibited significantly more VCMs than vehicle treated rats (Fig. 6 C). After 3 weeks of withdrawal, both VCMs and striatal fiber density were completely

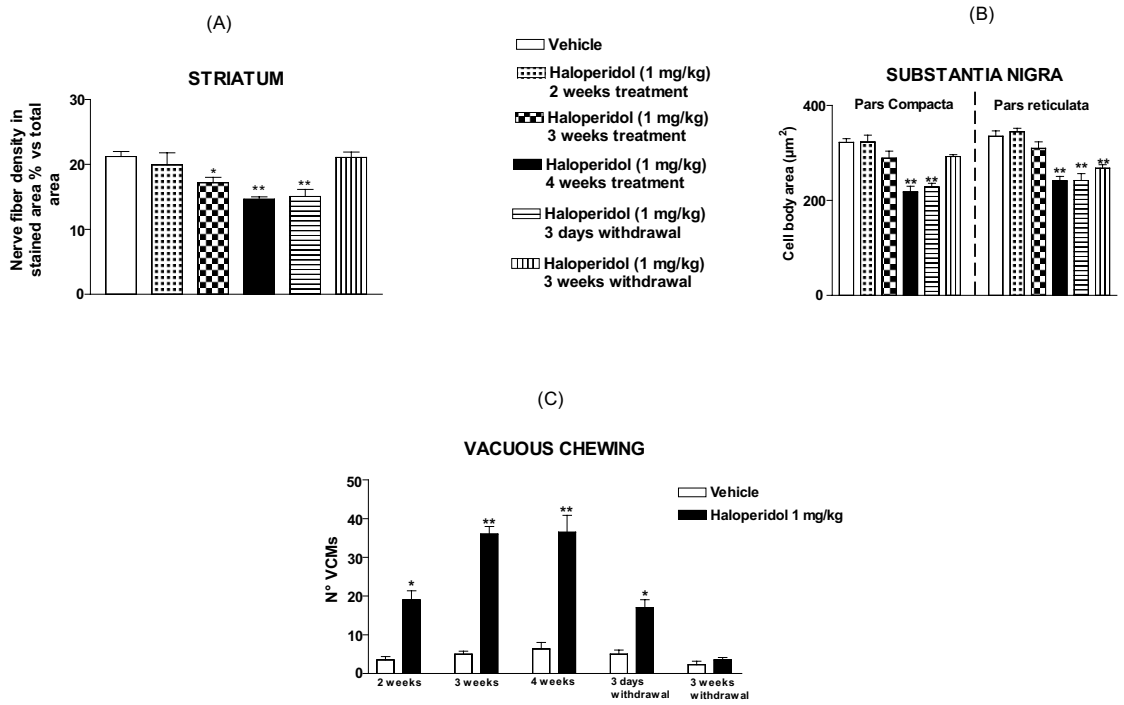


Figura 6

A: Histogram showing the TH-IM nerve fiber density in the striatum of rats treated with vehicle, haloperidol 1 mg/kg for 2, 3, 4 weeks and after 3 days and 3 weeks of withdrawal. A significant reduction in the TH-IM nerve fiber density was observed at 3 and 4 weeks of treatment and this reduction was still present at 3 days of withdrawal. B: cell body area of TH-IM neurons in the substantia nigra pars compacta and reticulata. Haloperidol (1 mg/kg) administered for 3 and 4 weeks and after a withdrawal of 3 days determined a significant reduction of cell body area both in pars compacta and reticulata with respect to vehicle. In pars reticulata the reduction of nerve fiber density persisted at 3 weeks of withdrawal. C: histogram illustrating the number of VCMs in rats treated with vehicle and haloperidol at the dose of 1 mg/kg in the same groups described above. Statistic analysis for morphological study was carried out using one-way ANOVA while vacuous chewing quantification was followed by two-way ANOVA; in both cases Newman-Keuls post hoc test was applied to compare haloperidol treated rats with respect to controls (*P<0.05; **P<0.01)

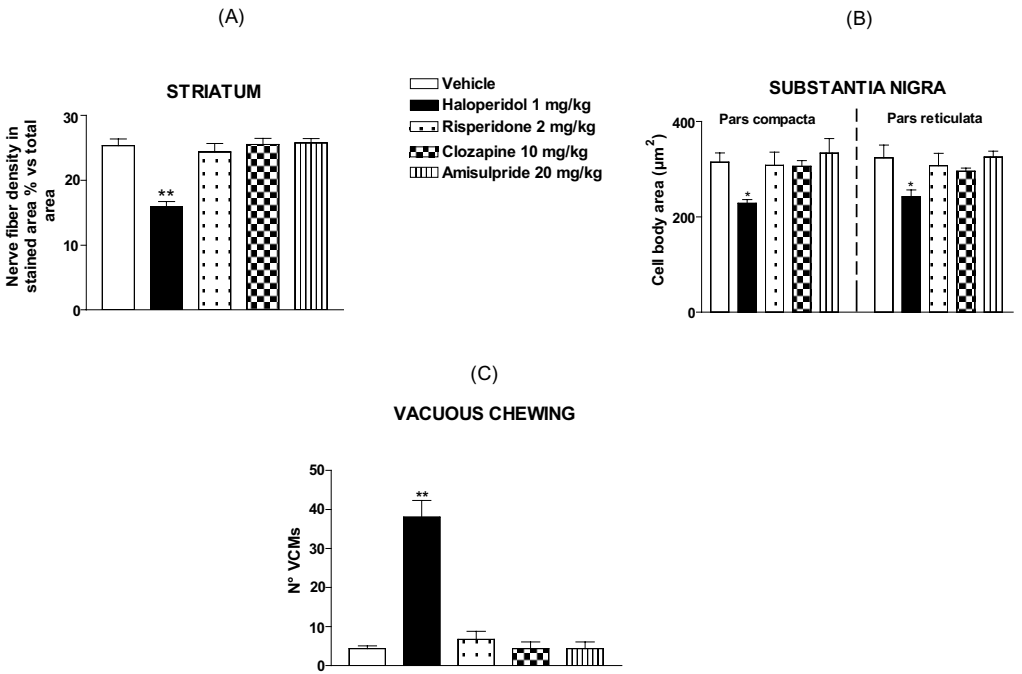


Figura 7

A: Histogram showing the TH-IM nerve fiber density in the striatum of rats treated with vehicle, haloperidol 1mg/kg and atypical neuroleptics risperidone (2 mg/kg), clozapine (10 mg/kg) and amisulpride (20 mg/kg). A significant reduction in the TH-IM nerve fiber density was found with haloperidol at the dose of 1 mg/kg but not with the atypical neuroleptics. B: cell body area of TH-IM neurons in the substantia nigra pars compacta and reticulata. Compare with vehicle, haloperidol determined a significant reduction of cell body area both in pars compacta and reticulata, while atypical neuroleptics had no effect. C: histogram illustrating the number of VCMs in rats treated with vehicle, haloperidol 1 mg/kg and atypical neuroleptics risperidone (2 mg/kg), clozapine (10 mg/kg) and amisulpride (20 mg/kg). Haloperidol caused a significant increase of VCMs, while the atypical neuroleptics didn't cause any change in VCMs compared with vehicle. Statistical significance vs. respective control treated rats has been analyzed using ANOVA test followed by Newman-Keuls post hoc-test (*P<0.05; **P<0.01)

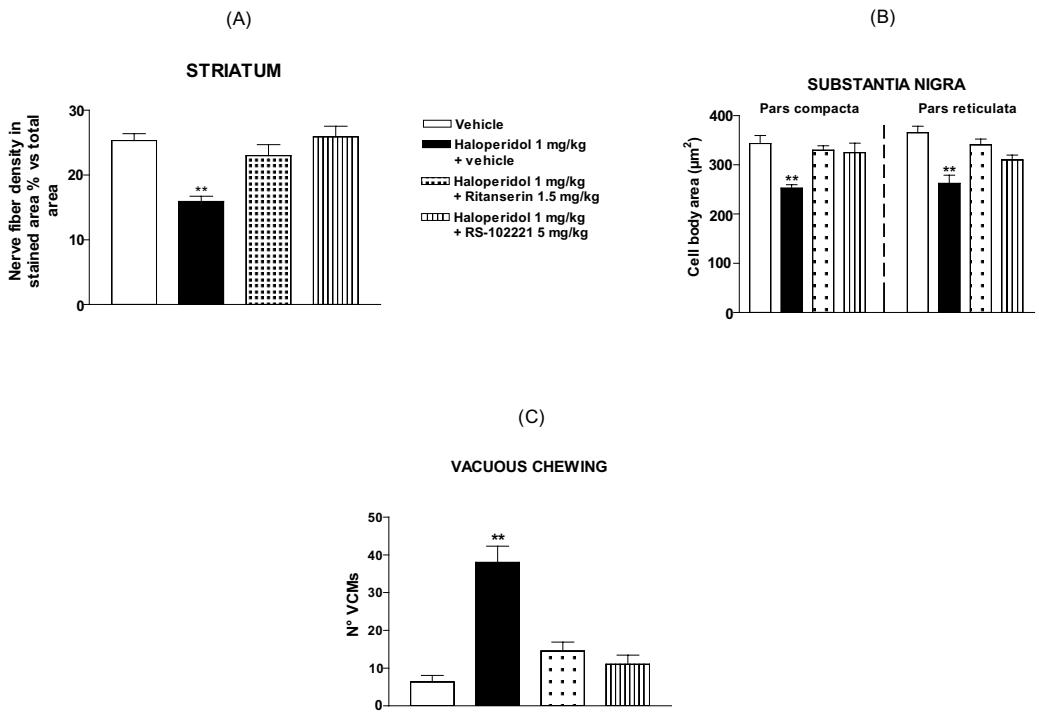


Figura 8

A: Histogram showing the TH-IM nerve fiber density in the striatum rats in co-treatment with haloperidol associated to ritanserin (1.5mg/kg) or RS-102221 (5mg/kg). The co-treatment with ritanserin and RS-102221 reduced the damage induced by haloperidol. B: cell body area of TH-IM neurons in the substantia nigra pars compacta and reticulata. Cell body shrinkage induced by haloperidol was prevented by co-treatment with ritanserin and RS-102221. C: histogram illustrating the number of VCMs in rats co-treated with haloperidol 1mg/kg and ritanserin (1.5mg/kg) or RS-102221 (5mg/kg). The VCMs induced by haloperidol were significantly reduced by ritanserin and RS-102221. Statistical significance vs. respective control treated rats has been analyzed using ANOVA test followed by Newman-Keuls post hoc-test (*P<0.05; **P<0.01)

restored and no differences could be observed with vehicle treated rat levels (Fig. 6 A-C). However in substantia nigra pars reticulata ($P < 0.05$) but not in compacta the shrinkage of the cell body area still persisted after 3 weeks of withdrawal.

Correlation of VCM with morphological changes in rats following 4 weeks of treatment with haloperidol and atypical neuroleptics

A comparison of 4 weeks treatment effects between vehicle, haloperidol (1 mg/kg) and atypical neuroleptics such as risperidone (2 mg/kg), clozapine (10 mg/kg) and amisulpride (20 mg/kg), displayed significant morphological differences between groups in striatum (one-way ANOVA, $F_{4,40} = 18.27$, $P < 0.01$), nigra pars compacta (one-way ANOVA, $F_{4,40} = 4.69$, $P < 0.01$) and nigra pars reticulata (one-way ANOVA, $F_{4,40} = 4.16$, $P < 0.01$) (Fig. 7 A-B). Significant staining reductions were confirmed in rat treated with haloperidol, but not significant differences with vehicle treated rats were found after atypical neuroleptics treatments. In the same manner VCMs increased in haloperidol treated rats but did not change with atypical neuroleptic treatments with respect to the vehicle treated rats (Fig. 7-C).

Correlation of VCM with morphological changes in rats following a co-treatment of haloperidol+ritanserin or RS 102221

Co-treatment of haloperidol (1 mg/kg) with ritanserin (1.5 mg/kg) or RS 102221 for 4 weeks significantly reduced the damage induced by haloperidol in striatum (one-way ANOVA, $F_{3,32} = 11.46$, $P < 0.05$). In fact, after ritanserin and RS 102221 co-treatments, no significant differences in striatal nerve fiber density were found compared with vehicle treated rats (Fig. 8-A). In the substantia nigra, it was found an absence of cell body shrinkage haloperidol-induced after co-treatment of haloperidol with ritanserin or RS 102221 ($P > 0.05$ for both treatments vs. controls). The VCMs induced by haloperidol were significantly reduced by ritanserin and RS 102221 ($P > 0.05$ for both treatments vs. vehicle treated rats).

Discussion

The present results indicated that four weeks treatment with high doses of haloperidol can profoundly affect the morphology of nigrostriatal dopaminergic neurons. The immunocytochemical study showed that haloperidol decreased (by about 30%) the normal density of dopaminergic terminals in striatum and produced a significant reduction of cell body size of dopaminergic neurons in the substantia nigra. The use of two antibodies directed against two different markers (TH and DAT) for dopaminergic fibers indicated that the staining reduction in striatum was likely due to a decrease of dopaminergic fibers rather than to the marker expression levels. Moreover, chronic haloperidol treatment has been reported not to affect TH or DAT expression in the rat striatum (Cottingham et al. 1990 Rivest et al. 1995).

The reversible fiber loss in the striatum associated with the shrinkage of dopaminergic neuron cell bodies in the substantia nigra after chronic treatment with haloperidol, could be interpreted as a sign of functional impairment of the nigrostriatal dopaminergic pathway. Different studies, in fact, reported cell body shrinkage and axonal density reduction in nigrostriatal dopaminergic neurons during neuro-degenerative processes induced by toxic drugs, like 6-

hydroxydopamine (6-OHDA), MPTP and by high concentrations of dopamine (Sherman and Moody 1995, Cochiolo et al. 2000, Simantov et al. 1996), or in pathological states such as Parkinson and Huntington diseases (Waters et al. 1988). Interestingly, haloperidol affected the nigrostriatal but not the meso-limbic dopaminergic neurons, resembling what has been observed in MPTP treated animals (Hung and Lee 1998, Nomoto et al. 2000).

We sustain the possibility that in rats chronic haloperidol could produce a neuronal toxicity similar to that exerted by MPP+ (Bloomquist 1994), by inducing a striatal loss of synaptic density associated with a reduced total number of hyperthrophic mitochondria (Roberts et al. 1995), strongly supporting the hypothesis that HPP+ and MPP+ are inhibitors at complex I of the mitochondrial electron transport chain (Rollema 1994).

The reduction of nigrostriatal dopaminergic axons observed in the present study well correlate with the depletion of striatal dopamine showed by Subramanyam (1990) after intrastriatal administration of HPP+ or MPP+.

In addition, haloperidol and its metabolites have been shown to inhibit monoamino oxidase type B (MAO-B) and by this inhibition to increase dopamine auto-oxidation and the production of free radicals. (Fang 1995b). Free radicals posses many disruptive toxic effects that depend on the detoxifying enzymes profile of the different cells. In this regard, basal ganglia are supposed to be particularly vulnerable to lipid peroxidation induced by free radicals resulting from catecholamine oxidation (Cadet 1988, Lohr 1991). Haloperidol treatment has also been shown to increase glutamate release (De Souza et al. 1999, See and Lynch 1995) and glutamatergic synapses in striatum (Meshul and Tan 1994), suggesting a possible toxicity induced by calcium.

An additional aspect, that could correlate VCMs with the observed dopaminergic neuronal alteration, is the down-regulation of GABA receptor in globus pallidus after chronic haloperidol treatment and after 6-OHDA striatal lesion (Sasaki 1997, Pan et al. 1985). Reduced GABAergic activity has been correlated with enhanced oral activity in neuroleptic-treated animals (Gunne et al. 1984, 1986) and proposed as a pathophysiological hypothesis for TD by mean of a decreased GABA activity in the neuro-anatomical loops of the corticostriatothalamocortical circuit (Fibiger 1984). Very recently, the impairment of the striatal-nigra GABAergic system induced by haloperidol was not found after atypical antipsychotic treatment (Sakai 2001).

All these hypothesis on haloperidol toxicity are not conflicting but rather represent different attempts to define a final common pathway leading to neuronal abnormalities that might underlie the development of TD in human or VCMs in rodents.

In the present study, we found a remarkable temporal correlation between the development of rat VCMs and the appearance of morphological deficits in the nigrostriatal dopaminergic neurons. This correlation was also maintained when the haloperidol treatment was terminated or when rats were co-treated with drugs known to reduce haloperidol-induced VCMs, such as ritanserin (Takeuchi et al. 1998), suggesting a direct possible role of dopaminergic neurons impairment in the physiopathology of VCMs in rats. In agreement with this hypothesis, striatum lesions produced by intracerebral injection of 6-OHDA exerted purposeless chewing in rats (Jicha and Salamone 1991) and increased VCMs in neonatally lesioned rats after haloperidol treatment (Huang et al. 1997), while the administration of reserpine is known to produce oral dyskinesia in rats (Neisewander et al. 1994).

We did not observe any morphological changes or development of VCMs in rats treated with high doses of three unrelated classes of atypical antipsychotics: the dibenzodiazepine clozapine, the benzo-oxazol derivative risperidone and the substituted benzamide amisulpride, indicating that the development of VCMs could not be related to D2 dopaminergic receptors supersensitivity, due to the antagonistic action of the antipsychotics on these receptors.

In spite of the demonstration that chronic haloperidol treatment increases the number of D2

receptors (Burt et al. 1977) and apomorphine-induced motor activity and rearing behavior (Bernardi et al. 1981; Bernardi and Palermo-Neto 1983, 1984), a direct correlation between D2-supersensitivity and VCMs has been often criticized.

The lesion of dopaminergic terminals in striatum, observed in the present study, could add further explanations to the dopamine receptor supersensitivity induced by haloperidol treatments, since 6-OHDA-mediated lesions of the nigrostriatal neurons produce not only a D2 supersensitivity to apomorphine but also a D1/D2 dopamine receptor density imbalance (Kelly et al. 1975, Lange 1990, Narang and Wamsley 1995, Lau and Fung 1986) recently proposed as a substrate for TD (Peacock 1997).

Interestingly, we showed that the 5-HT_{2A-2C} antagonist ritanserin and the 5-HT_{2C} selective antagonist RS102221 not only prevented the VCMs (Takeuchi et al. 1998), induced by haloperidol but also exerted a protective role on the morphological impairments induced by the neuroleptic. Such findings suggest the possibility that 5-HT2 antagonists could protect nigrostriatal neurons from toxic insults but further investigations are necessary to confirm these data and to better address the mechanisms underlying this protection.

In conclusion, the data presented here raise the possibility that structural alterations and damages of dopaminergic neuronal architecture induced by haloperidol, may be involved in the development of oral dyskinesia in rats and possibly in humans. Further studies are needed to identify which neurons are primary involved in the morphological changes observed after chronic haloperidol treatment and to address the clinical relevant question whether longer treatment (i.e. 6-12 months) with smaller doses of haloperidol might produce the same damage and if this damage could permanently affect the morphology of the nigrostriatal dopaminergic system and the behaviors related to its proper function.

Riassunto

Oggetto: il trattamento cronico con aloperidolo induce discinesia tardiva (TD) negli umani e *vacuous chewing movements* (VCMs) nei ratti. Recenti studi suggeriscono che il metabolita dell'aloperidolo pyridinium (HPP+) può esercitare un'azione tossica simile a quella del 1-methyl-4-phenylpyridinium (MPP+), portando allo sviluppo di disturbi motori indotti da aloperidolo. Nel presente lavoro noi abbiamo investigato se un danneggiamento dei neuroni dopaminergici dopo assunzione cronica di aloperidolo potrebbe rappresentare un possibile substrato fisiopatologico di VCMs nei ratti.

Metodo: a questo proposito, sono stati usati diversi trattamenti amntipsicotici per analizzare la correlazione fra lo sviluppo di VCM e le alterazioni morfologiche dei neuroni *tyrosine-hydroxylase-immunostained* (TH-IM).

Quando i ratti venivano trattati con aloperidolo mostravano un aumento significativo di VCM che era sia tempo- (1-4 settimane) che dose (0.1-1 mg/kg) dipendente.

Risultati: l'analisi immunocitochimica ha mostrato un restringimento dei corpi cellulari dei neuroni TH-IM nella pars compacta e reticulata della substantia nigra e una riduzione della densità delle fibre dopaminergiche soltanto nello striato dei ratti trattati con aloperidolo. Non sono state oservate differenze nei neuroni TH-IM dell'area ventro-tegmentale e del nucleus accumbens rispetto ai controlli. L'antipsicotico atipico risperidone (2 mg/kg, i.p.), l'amisulpride (20 mg/kg, i.p.) e la clozapina (10 mg/kg, i.p.) non hanno prodotto alcun cambiamento morfologico nigrostriatale nei VCM in ogni momento della sperimentazione.

La deplezione dei TH-IM e i VCM erano ancora presenti dopo tre giorni di interruzione nei ratti trattati per 4 settimane con la dose più alta di aloperidolo. Sia i cambiamenti morfologici che i correlati comportamentali scomparivano dopo tre settimane di interruzione.

Conclusioni: quattro settimane di co-trattamento con ritanserin (1.5 mg/kg, i.p. due volte al giorno) o

RS 102221 (5 mg/kg, i.p. due volte al giorno) riducevano significativamente i VCM e i cambiamenti morfologici indotti da aloperidolo (1 mg/kg). Questi risultati suggeriscono che l'aloperidolo induce un danneggiamento morfologico dei neuroni dopaminergici nigrostriatali che sembra essere direttamente associato con l'insorgenza, la permanenza e la scomparsa di VCM nei ratti trattati con aloperidolo.

Summary

Keywords: *Antipsychotics – Vacuous chewing – Ritanserin - RS 102221*

Object: Chronic treatment with haloperidol has been shown to induce tardive dyskinesia (TD) in humans and vacuous chewing movements (VCMs) in rats. Recent studies suggest that the pyridinium metabolite of haloperidol (HPP+) may exert a toxic action similar to that of 1-methyl-4-phenylpyridinium (MPP+), leading to the development of haloperidol induced motor disorders. In the present work we investigated if an impairment of dopaminergic neurons after chronic haloperidol could represent a possible physiopathologic substrate of VCMs in rats.

Method: For this purpose, different antipsychotic treatments were used to analyze the correlation between VCMs development and morphological alterations of tyrosine-hydroxylase-immunostained (TH-IM) neurons. When treated with haloperidol rats displayed a significant increase of VCMs that was both time- (1-4 weeks) and dose (0.1-1 mg/kg) dependent.

Results: Immunocytochemical analysis showed a shrinkage of TH-IM cell bodies in substantia nigra pars compacta and reticulata and a depletion of dopaminergic fiber density only in the striatum of haloperidol treated rats. No differences were observed in TH-IM neurons of ventral tegmental area and nucleus accumbens with respect to controls. The atypical antipsychotics risperidone (2 mg/kg, i.p.), amisulpride (20 mg/kg, i.p.) and clozapine (10 mg/kg, i.p.) did not produce any nigrostriatal morphological changes nor VCMs at any time point.

TH-IM depletion and VCMs were still present after three days of withdrawal in rats treated for four weeks with the highest dose of haloperidol. Both the morphological changes and the behavioral correlate disappeared after three weeks of withdrawal.

Conclusions: Four weeks of co-treatment with ritanserin (1.5 mg/kg, i.p. twice a day) or RS 102221 (5 mg/kg, i.p. twice a day) significantly reduced the VCMs and the morphological changes induced by haloperidol (1 mg/kg). These results suggest that haloperidol induces a morphological impairment of the dopaminergic nigrostriatal neurons which appears to be directly associated with the arising, permanency and disappearance of VCMs in haloperidol treated rats.

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