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Brain Levels of the Neurotoxic Pyridinium Metabolite HPP+ and Extrapyramidal Symptoms in Haloperidol-Treated Mice

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Abstract

The typical antipsychotic haloperidol is a highly effective treatment for schizophrenia but its use is limited by a number of serious, and often irreversible, motor side effects. These adverse drug reactions, termed extrapyramidal syndromes (EPS), result from an unknown pathophysiological mechanism. One theory relates to the observation that the haloperidol metabolite HPP+ (4-(4-chlorophenyl)-1-[4-(4-fluorophenyl)-4-oxobutyl]-pyridinium) is structurally similar to MPP+ (1-methyl-4-phenylpyridinium), a neurotoxin responsible for an irreversible neurodegenerative condition similar to Parkinson's disease. To determine whether HPP+ contributes to haloperidol-induced EPS, we measured brain HPP+ and haloperidol levels in strains of mice at high (C57BL/6J and NZO/HILtJ) and low (BALB/cByJ and PWK/PhJ) liability to haloperidol-induced EPS following chronic treatment (7–10 adult male mice per strain). Brain levels of HPP+ and the ratio of HPP+ to haloperidol were not significantly different between the haloperidol-sensitive and haloperidol-resistant strain groups ($P = 0.50$). Within each group, however, strain differences were seen ($P < 0.01$), indicating that genetic variation regulating steady-state HPP+ levels exists. Since the HPP+ levels that we observed in mouse brain overlap the range of those detected in post-mortem human brains following chronic haloperidol treatment, the findings from this study are physiologically relevant to humans. The results suggest that strain differences in steady-state HPP+ levels do not explain sensitivity to haloperidol-induced EPS in the mice we studied.

Keywords

haloperidol; adverse drug reaction; tardive dyskinesia; mouse; HPTP; HPP+

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Conflict of Interest Statement

The authors have no financial interests or potential conflicts of interest to declare.

1. Introduction

First-generation or “typical” antipsychotic drugs are widely used to treat psychotic disorders, but their use is limited by their propensity to cause a number of motor side effects, collectively termed extrapyramidal syndromes (EPS) (Dayalu & Chou 2008, Hsin-tung E and Simpson 2000). After acute treatment, about half of patients experience restlessness, involuntary spasms or muscular rigidity (Leucht *et al.* 2003). With long-term treatment, tardive dyskinesia (TD) develops in about a third of patients (Gerlach & Casey 1988). TD involves repetitive, involuntary and purposeless movements, primarily of the orofacial region (e.g., chewing movements and tongue protrusion) (Crane 1968). TD is often irreversible (Soares-Weiser & Fernandez 2007) and there is currently no proven treatment (Tandon *et al.* 2008). Though a number of theories have been proposed, the precise pathophysiological mechanisms responsible for the development of these adverse drug reactions are not known.

Haloperidol is a prototypical first-generation antipsychotic and is still widely used, despite high liability for EPS. One current theory for the pathophysiological effects of haloperidol relates to its structural similarity with MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), a neurotoxic contaminant produced during the manufacture of the synthetic opioid MPPP (1-methyl-4-phenyl-4-propionoxypiperidine). MPTP is responsible for an irreversible neurodegenerative condition similar to Parkinson's disease (Burkhardt *et al.* 1993), via its pyridinium metabolite MPP+ (1-methyl-4-phenylpyridinium) (Jackson-Lewis & Przedborski 2007). Haloperidol undergoes biotransformation into HPTP (4-(4-chlorophenyl)-1-[4-(4-fluorophenyl)-4-oxobutyl]-1,2,3,6-tetrahydropyridine), which is similar in structure to MPTP. Both haloperidol and HPTP can then be oxidized to a pyridinium metabolite HPP+ (the 4-(4-chlorophenyl)-1-[4-(4-fluorophenyl)-4-oxobutyl]pyridinium species) which is similar in structure and toxicity to MPP+ (Subramanyam *et al.* 1990, Rollema *et al.* 1994, Igarashi *et al.* 1996, Bloomquist *et al.* 1994, Fang *et al.* 1995).

Therefore, it is plausible that HPP+ contributes to the pathophysiological effects of haloperidol via toxicity similar to that of MPP+. Consistent with this notion, HPP+ is found in post-mortem brain tissue, plasma and urine from patients with schizophrenia chronically treated with haloperidol (Eyles *et al.* 1994, Eyles *et al.* 1997, Avent *et al.* 1997, Subramanyam *et al.* 1991).

A positive relationship between the severity of TD and peripheral blood levels of HPP+ has been reported by two studies of a total of 51 haloperidol-treated patients (Iwahashi *et al.* 2001, Ulrich *et al.* 2005). In light of evidence that HPP+ is capable of crossing the blood-brain barrier (owing to its high lipophilicity) (Kawashima *et al.* 2002), it is conceivable that peripherally-derived HPP+ (e.g. from the liver) could be taken up by the brain and induce damage. Alternatively, HPP+ could be produced locally in the brain via a currently unidentified enzymatic mechanism. Regardless of its source, however, HPP+ would need to act in the brain to produce haloperidol-induced motor side effects. Since a large, well-controlled examination of brain HPP+ levels in subjects with and without TD is infeasible, we have investigated this question using a mouse model where we could tightly control drug exposure, environmental factors and collect brain tissue.

Mice chronically treated with haloperidol demonstrate, in a strain-specific manner, many of the motor side effects seen in humans, including vacuous chewing movements (akin to TD), reduced locomotion (hypokinesia) and muscular rigidity (Parkinsonism) (Fujiwara 1992, Waddington *et al.* 1983, Ethier *et al.* 2004). In recent studies we examined susceptibility to haloperidol-induced motor side effects across a panel of 27 inbred mouse strains, finding

that broad-sense heritability for each of these responses exceeded 70% (Crowley et al. 2010), and mapped quantitative trait loci regulating drug response (Crowley *et al.* 2011). To examine whether brain HPP+ levels correlate with EPS susceptibility, we chose two strains that were very sensitive to haloperidol-induced motor side effects (C57BL/6J and NZO/HILtJ) and two resistant strains (BALB/cByJ and PWK/PhJ) from Crowley et al (Crowley et al. 2010). As shown in Figure 1A, a summary of previously published data (Crowley et al. 2010), following chronic treatment with haloperidol (administered via a slow release implant), C57BL/6J and NZO/HILtJ mice show significantly more tardive-dyskinesia-like chewing movements, hypokinesia and Parkinsonism than BALB/cByJ or PWK/PhJ mice (all $P < 0.01$). In the present study, we chronically treated 7–10 adult male mice from each of these strains with haloperidol and measured brain levels of HPP+ and haloperidol.

2. Methods

2.1 Animals

All testing procedures were conducted in strict compliance with the Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of the University of North Carolina. Male mice from 4 inbred strains were obtained from the Jackson Laboratory (Bar Harbor, ME). These included two strains previously shown (Crowley *et al.* 2010) to be highly susceptible to haloperidol-induced vacuous chewing movements (C57BL/6J, N=9, and NZO/HILtJ, N=10) and two strains shown to be largely resistant (BALB/cByJ, N=7, and PWK/PhJ, N=8).

2.2 Antipsychotic exposure

Slow release haloperidol pellets (6.7 mg/kg/day; Innovative Research of America; Sarasota, FL) (Fleischmann *et al.* 2002) were implanted subcutaneously with a trocar under two minutes of isoflurane anesthesia. This method was previously shown to yield human-like steady-state concentrations of haloperidol (4–20 ng/ml) (Hsin-tung E and Simpson 2000) in >95% of mice tested across diverse inbred strains (Crowley et al. 2010), including those examined in this study. Pellets were implanted at 8 weeks of age and mice were sacrificed 30 days later.

2.3 HPP+ and haloperidol levels

Upon sacrifice, whole brain was collected, weighed, snap frozen in liquid nitrogen and stored at −80°C until analysis. Brains were homogenized with 2 mL of 1.15% KCl solution and an internal standard PB226.3 (oxalate salt of 1,2,5-trimethyl-3-(1-methyl-1,2,3,6-tetrahydropyridin-4-yl)-1H-indole) (P. Bissel and N. Castagnoli, Jr., manuscript in preparation) was added to a concentration of 80 ng/mL. For each sample, 0.25 mL of this solution was mixed with 0.5 mL of methanol followed by 30 seconds of vortex and centrifugation at 10,000 rpm for 10 min. The supernatant was diluted with 1mL of 30 mM NH_4OAc followed by clean-up through a solid phase extraction cartridge (Igarashi & Castagnoli 1992). Finally, the eluent was dried under a stream of nitrogen and the residue was re-dissolved in 0.5 or 1 mL of 70/30 H_2O/MeOH containing 0.2% acetic acid.

LC/MS/MS with multiple reaction monitoring was then used to measure levels of haloperidol, HPP+ and PB226.3 (Gorrod & Fang 1993). Standard curves were prepared for both haloperidol (Sigma-Aldrich, St. Louis, MO) and HPP+ (kindly provided by Dr. Kazuo Igarashi). Reversed phase liquid chromatographic separations were performed with an Atlantis C18 (150 × 2.1mm, 5.0 μm dp) column from Waters (Milford, MA) on an Agilent (Wilmington, DE) 1100 series HPLC equipped with a diode array detector (DAD), column heater set at 40°C, and Thermo Survey (San Jose, CA) auto sampler. In each analysis, 20 μL of standards or sample solution (30/70 CH_3OH/H_2O) of each extract was injected onto the

column via the auto-sampler. Mobile phase A consisted of 1% formic acid (FA) and mobile phase B contained 1% (v/v) FA in acetonitrile. The mobile phase was delivered to the HPLC column at a flow rate of 0.2 mL/min and the gradient mobile phase elution program was as follows: (time 0 min: 80/20 % A/B; time 6.5 min: 5/95% A/B; time 9.5 min: 5/95% A/B; time 9.6 min: 80/20% A/B; 14 min: 80/20% A/B). For MS analysis the HPLC column effluent was pumped directly into a Thermo Instrument TSQ triple quadruple mass spectrometer (Thermo Finnigan, San Jose, CA) equipped with an ESI source. The instrument was calibrated with a solution of polytyrosine according to the manufacture's recommendation. Tuning parameters were obtained by teeing the haloperidol, HPP+ and PB226.3 standards (10 ng/μL) at a rate of 10 uL/min into 50/50% A/B of mobile phase at a rate of 0.2 mL/min to achieve reasonable MS sensitivity in Multiple Reaction Monitoring (MRM). MS parameters for detection were: spray voltage (4500 V), sheath gas pressure (15 psi), auxiliary gas pressure (10 psi), capillary temperature (300°C) and polarity (positive). Masses in daltons were: PB226.3 (parent: 255.2, product: 123.0), haloperidol (parent: 376.2, product: 212.0) and HPP+ (parent: 354.0, product: 123.0).

2.4 Statistical analysis

Brain levels of HPP+ and haloperidol were expressed as ng/gram of wet tissue weight. The effect of strain on levels of HPP+, haloperidol and the ratio of HPP+ to haloperidol was assessed by one-way analysis of variance (ANOVA) followed by pairwise testing using the Tukey-Kramer HSD test using JMP software (version 10.0; SAS; Cary, NC). The statistical analysis results were represented as mean ± standard error and a $P < 0.05$ was considered statistically significant.

3. Results and Discussion

As shown in Figure 1A, following chronic haloperidol treatment, C57BL/6J and NZO/HILtJ mice show significantly more extrapyramidal symptoms than BALB/cByJ or PWK/PhJ mice. The top panel indicates that tardive-dyskinesia-like chewing movements develop most profoundly in C57BL/6J mice, followed by NZO/HILtJ, while BALB/cByJ and PWK/PhJ mice remain largely unaffected. The middle panel demonstrates a clear haloperidol-induced reduction of locomotor activity (hypokinesia) in all four strains, with C57BL/6J and NZO/HILtJ mice again the most susceptible. The bottom panel shows that NZO/HILtJ mice are most sensitive to haloperidol-induced Parkinsonian-like rigidity on the inclined screen, followed by C57BL/6J and the two resistant strains. To examine whether brain HPP+ levels correlate with EPS susceptibility, we chronically treated adult male mice from these four strains with haloperidol and measured brain levels of HPP+ and haloperidol. Figure 1B displays the structures of compounds relevant to this experiment.

Figure 1C depicts strain means in the brain concentrations of haloperidol and HPP+. For HPP+, there were significant differences between strains ($F_{3,30}=5.11$, $P=0.006$) with NZO/HILtJ similar to PWK/PhJ and both greater than C57BL/6J and BALB/cByJ. Brain haloperidol concentrations were similar between strains ($P=0.09$). The ratio of brain HPP+ to haloperidol was significantly different between strains ($P=0.002$) with a pattern of mean differences like that for HPP+ (C57BL/6J: 0.20, NZO/HILtJ: 0.42, BALB/cByJ: 0.14, PWK/PhJ: 0.40).

We hypothesized that strains with greater EPS would have higher levels of HPP+. As shown in Figure 1C, brain levels of HPP+ and the ratio of HPP+ to haloperidol were not significantly different between the haloperidol-sensitive and haloperidol-resistant strains. Two strains did possess significantly higher levels of HPP+ and a higher ratio of HPP+ to haloperidol (NZO/HILtJ and PWK/PhJ), but this did not match the strain distribution of haloperidol-induced motor side effects. The HPP+ levels that we observed after chronic

haloperidol treatment (10–60 ng/g of brain tissue) overlap the ranges of those detected in patient brains at post-mortem (Eyles et al. 1997) and in rat brain after acute i.p. injection of 10 mg/kg (Igarashi & Castagnoli 1992, Igarashi *et al.* 1995).

This is the first study to examine brain HPP+ levels in animals at high and low liability to haloperidol-induced EPS. For these four strains, differences in steady-state HPP+ levels do not explain sensitivity to motor side effects. Although our data are not consistent with a simple causal role of HPP+ on EPS in mice, we cannot rule out the possibility that HPP+ is still involved in a more complex manner. For example, it may be possible that a strain like PWK/PhJ, with relatively high levels of HPP+ and low liability to EPS, is superior at neutralizing the free radical and reactive oxygen species thought to underlie the neurotoxic properties of HPP+. The opposite situation could be true for a strain like C57BL/6J, with relatively low levels of HPP+ and high liability to EPS. These ideas could be tested by administering HPP+ itself to mice from these four strains. Furthermore, previous work has demonstrated important differences in the basal dopaminergic systems of inbred mouse strains. Directly relevant to this study is the observation that the EPS-susceptible strain C57BL/6J has a lower density of dopamine receptors in the caudate nucleus (Sahakian *et al.* 1980) and lower dopamine content in the hypothalamus than the EPS-resistant strain BALB/cByJ (George *et al.* 1995). Further complexity could result from the fact that HPP+ can inhibit the presynaptic uptake of dopamine and serotonin (Wright *et al.* 1998) and that brain gene expression among inbred strains is known to differ widely (Letwin *et al.* 2006).

The inclusion of more mouse strains with divergent responses to haloperidol would, of course, provide a more powerful test of the hypothesis examine here. This could be achieved, for example, by sampling more of the 27 strains examined in Crowley et al (Crowley et al. 2010) or by screening other divergent populations such as the Collaborative Cross (Welsh *et al.* 2012) or Diversity Outbred (Churchill *et al.* 2012).

It is also conceivable that HPP+ levels vary across different brain regions and by examining whole brain HPP+ levels important differences were diluted out. Data from the literature are limited in this regard, with one human study reporting modest differences across regions (Eyles et al. 1997) and one rat study reporting 7-fold higher levels in the striatum versus whole brain (Igarashi and Castagnoli 1992). The small size of the mouse brain and trace levels of HPP+ forced us to include whole brain tissue in this study.

In conclusion, this study of haloperidol-sensitive and haloperidol-resistant inbred mouse strains failed to support the suggestion that HPP+-induced neurotoxicity contributes to the extrapyramidal side-effects seen in patients receiving long-term typical antipsychotic therapy.

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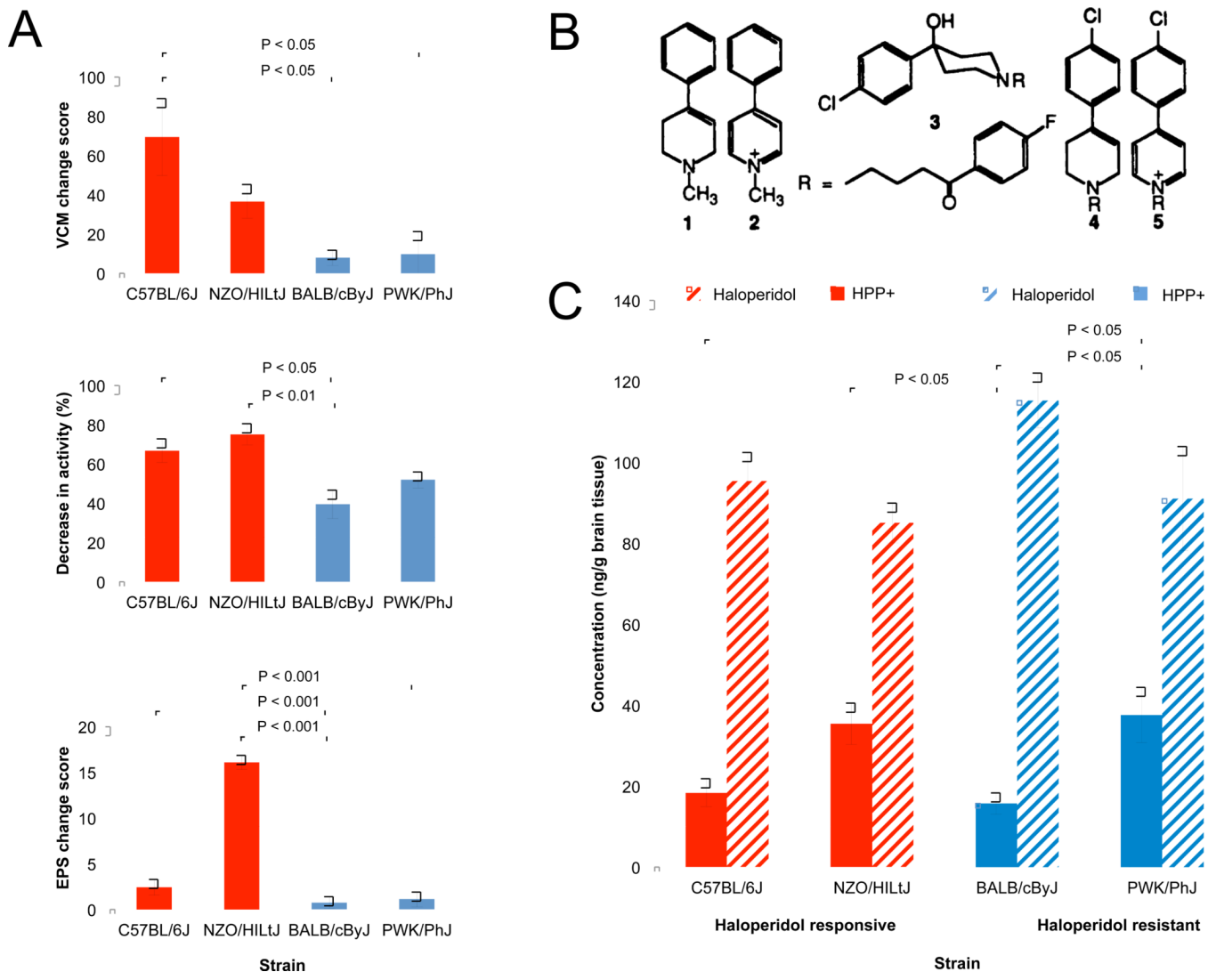

Figure 1.

Haloperidol response and brain HPP+ levels across four inbred mouse strains. **A)** Phenotypic data from Crowley et al (Crowley et al. 2010) demonstrating that, after chronic haloperidol treatment, C57BL/6J and NZO/HILtJ mice show more vacuous chewing movements, a greater decrease in locomotor activity and more rigidity than BALB/cByJ or PWK/PhJ mice. **B)** Structures of compounds discussed in the text. 1: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP); 2: 1-methyl-4-phenylpyridinium species (MPP +); 3: haloperidol (HP); 4: haloperidol tetrahydropyridine derivative (HPTP); 5: haloperidol pyridinium species (HPP+). **C)** Brain HPP+ and haloperidol levels across these four strains. Taken together, the haloperidol responsive strains did not differ from the resistant strains in overall HPP+ or haloperidol levels, or the ratio of HPP+ to haloperidol.