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RESEARCH PAPER

Brain Disposition and Catalepsy After Intranasal Delivery of Loxapine: Role of Metabolism in PK/PD of Intranasal CNS Drugs

Yin Cheong Wong • Zhong Zuo

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ABSTRACT

Purpose To elucidate the role of metabolism in the pharmacokinetics and pharmacodynamics of intranasal loxapine in conscious animals.

Methods At pre-determined time points after intranasal or oral loxapine administration, levels of loxapine, loxapine metabolites, and neurotransmitters in rat brain were quantified after catalepsy assessments (block test and paw test). Cataleptogenicity of loxapine was also compared with its metabolites.

Results Intranasally administered loxapine was efficiently absorbed into systemic circulation followed by entering brain, with t_{max} ≤15 min in all brain regions. Oral route delivered minimal amounts of loxapine to plasma and brain. Brain $AUC_{0-240min}$ values of 7-hydroxy-loxapine were similar after intranasal and oral administration. Intranasal loxapine tended to induce less catalepsy than oral loxapine, although statistical significance was not reached. The catalepsy score was positively and significantly correlated with the striatal concentration of 7-hydroxy-loxapine, but not with loxapine. 7-hydroxy-loxapine was more cataleptogenic than loxapine, while the presence of loxapine tended to reduce rather than intensify 7-hydroxy-loxapine-induced catalepsy. The increases in striatal dopamine turnover were comparable after intranasal and oral loxapine administration.

Conclusions The metabolite 7-hydroxy-loxapine, but not loxapine, was the main contributor to the catalepsy observed after intranasal and oral loxapine treatment. Intranasal route could effectively deliver loxapine to brain.

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Y. C. Wong • Z. Zuo (✉)
School of Pharmacy, Faculty of Medicine
The Chinese University of Hong Kong
Room 801C Lo Kwee-Seong Integrated Biomedical Sciences Building
Shatin, New Territories, Hong Kong
e-mail: joanzuo@cuhk.edu.hk

KEY WORDS antipsychotics · catalepsy · central nervous system · intranasal administration · metabolism

ABBREVIATIONS

3-MT	3-methoxytyramine
5-HIAA	5-hydroxyindole-3-acetic acid
5-HT	Serotonin
7-OH-amoxapine	7-hydroxy-amoxapine
7-OH-loxapine	7-hydroxy-loxapine
8-OH-amoxapine	8-hydroxy-amoxapine
8-OH-loxapine	8-hydroxy-loxapine
AUC	area under the curve
C_{max}	maximum concentration
CNS	central nervous system
CYP	cytochrome P450
DA	dopamine
DOPAC	3,4-dihydroxyphenylacetic acid
HVA	homovanillic acid
IM	intramuscular
IV	intravenous
PD	pharmacodynamics
PK	pharmacokinetics
$t_{1/2}$	half life
t_{max}	time to maximum concentration

INTRODUCTION

Intranasal administration is a promising route for delivering therapeutic compounds, and it remains as an active research area for both systemic and central nervous system (CNS) targeted drug delivery. Compared with the conventional oral route, one of the suggested advantages of intranasal delivery is that drug delivered via nasal route might avoid the first-pass metabolism in gastrointestinal tract and liver. Therefore, metabolite formation after intranasal drug

administration does not receive adequate attentions and investigation on the role of metabolism is scarce. However, our recent review (1) revealed that considerable amounts of metabolites could be present in plasma following intranasal administration of a variety of drugs to humans, and drug metabolism does alter the systemic disposition of intranasal drugs. Moreover, our literature review identified that there is a lack of research on the role of metabolism on the pharmacokinetics (PK) and pharmacodynamics (PD) of intranasally administered CNS drugs. Indeed, metabolism can play vital roles in the therapeutic and/or adverse effects of intranasal drug since it determines the bioavailability of the parent drug and the conversion of drug to active, inactive, or toxic metabolites, which in turn affects the efficacy of the intranasal drug and also the onset and duration of the therapeutic and/or adverse effects (1). Therefore, metabolite formation and subsequent disposition in brain after intranasal drug administration deserves more attention, especially for CNS drugs since many of these drugs give rise to active and/or toxic metabolites in humans (2).

In the present study, loxapine is chosen as the model drug because it possesses favorable physicochemical and therapeutic properties for intranasal delivery and has demonstrated relatively high permeability across the *in vitro* human respiratory epithelium (Calu-3 cell model) according to our screening of 22 antipsychotics (Wong and Zuo, AAPS Annual Meeting 2010 poster presentation). In addition, loxapine is extensively metabolized into several hydroxylated and *N*-desmethylated metabolites in both humans (3) and rats (4) (Fig. 1). These metabolites have different affinities to the dopamine (DA) D_2 receptors (5), which gives rise to different pharmacological activities (2). Changes in the brain disposition of these active metabolites are expected to alter the PD outcomes. Therefore, loxapine seemed to be a suitable drug to demonstrate the role of metabolism in the PK and PD of CNS drugs delivered via nasal route.

Extrapyramidal symptoms are one of the most common and troublesome adverse effects of typical antipsychotics and include a variety of different movement disorders such as parkinsonism (6). Available data suggest that catalepsy is a good predictor of antipsychotics-induced acute parkinsonism (7) and support the predictive validity of rodent catalepsy model for detecting the extrapyramidal symptoms liability of antipsychotics in humans (8). Antagonism of DA D_2 receptors in striatum is the main determinant of both extrapyramidal symptoms in humans and catalepsy in rats (9). Therefore, the disposition of loxapine and its metabolites, which are DA D_2 receptor antagonists with differential affinities, in striatum after intranasal loxapine administration is directly associated with these motor side effects and thus warrants investigation.

Imaging tools such as radioligands and fluorescence measures are common methods utilized in the assessment of intranasal delivery. For instance, previous *in vivo* studies on the intranasal delivery of antipsychotics (10–12) utilized antipsychotics radiolabeled with technetium-99 (^{99m}Tc) to investigate the biodistribution of the intranasally administered drugs. It should be noted that these imaging techniques alone do not confirm the presence of intact, free, or biologically active drug present in the CNS tissues. In addition, they do not distinguish between parent drug, active metabolites, and inert metabolites. Moreover, attaching radioligand to the drug might also alter the physicochemical and pharmacological properties of the drug, which will interfere with the PK and PD outcomes of the experiments. In view of the limitations of these imaging tools, more specific assays of intact drug and its metabolites, such as liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) adopted in the present study (4), could provide more complete, definitive descriptions of the PK profiles of the intranasal drug and the active metabolites formed *in vivo*. Moreover, instead of measuring the whole brain concentration, both the levels of drug and active metabolites in the target brain regions should be determined (e.g. striatum for extrapyramidal side effects of antipsychotics).

Rodents, particularly rats, are the most common laboratory animals used for preclinical PK evaluation of intranasal delivery (13). These animals are usually anesthetized during experiments to facilitate nasal drug administration. In the classic rat model for studying intranasal drug delivery described by Hirai *et al.* (14), nasal surgery was conducted in anesthetized rats to isolate the nasal cavity. In fact, the anesthesia and surgery could affect the PK profiles of the intranasal drugs. Mayor and Illum (15) reported that in the surgery-free rats, the nasal absorption of insulin could be enhanced by anesthesia, and the enhancement effects varied with the type of anesthesia chosen. Yang *et al.* (16) later reported that the nasal surgery performed, in addition to anesthesia, could suppress the systemic clearance of intranasal stavudine. Moreover, PD actions in conscious animals would be expected to be more comparable to effects seen in humans, than would those obtained from animals that have received anesthesia and/or suffered from surgery. To avoid the interferences caused by anesthesia and/or surgery, conscious rats that have not received any anesthesia or surgery would be used in the present PK/PD study.

We hypothesized that different routes of loxapine administration would lead to different dispositions of parent and active metabolites *in vivo*, resulting in differential PD outcomes. The objectives of the present study are to investigate 1) the concentrations of loxapine and its metabolites in different brain regions and plasma after loxapine administration via nasal route and oral route to conscious rats; 2) the PD effects of loxapine on catalepsy behaviors and on CNS neurotransmitters; and 3) the contribution of active metabolites to the PD effects of loxapine.

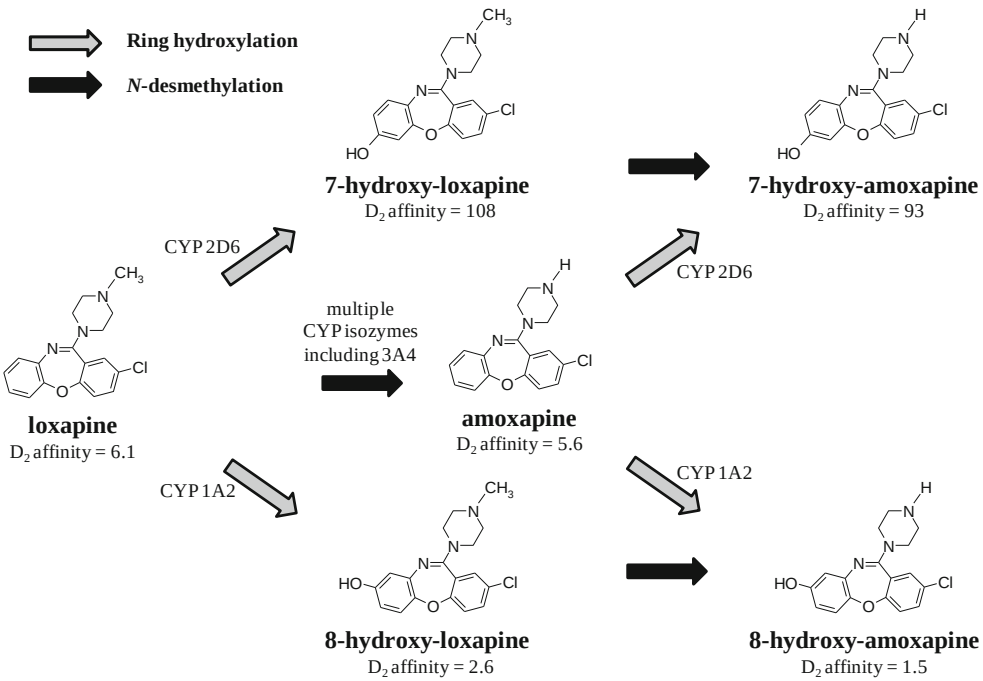

Fig. 1 Summary of metabolic pathways of loxapine and the major CYP isozymes involved. Affinity to human dopamine D_2 receptor was calculated as $10^7 \times 1/K_d$ where K_d = equilibrium dissociation constant in molarity [affinity data from ref. (5)].

MATERIALS AND METHODS

Materials

Loxapine succinate and amoxapine were obtained from Sigma (St. Louis, MO, USA). 7-Hydroxy-loxapine (7-OH-loxapine), 8-hydroxy-loxapine (8-OH-loxapine), 7-hydroxy-amoxapine (7-OH-amoxapine) and 8-hydroxy-amoxapine (8-OH-amoxapine) were purchased from TC Scientific Inc. (Alberta, Canada). Dopamine (DA) hydrochloride, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), serotonin (5-HT) hydrochloride, 5-hydroxyindole-3-acetic acid (5-HIAA), and ephedrine hydrochloride were purchased from Sigma (St. Louis, MO, USA). 3-Methoxytyramine (3-MT) hydrochloride was obtained from International Laboratory (USA). (*E*)-ferulic acid was obtained from Wako Pure Chemical Industries (Japan). Acetic acid was obtained from Rankem (India).

Experimental Procedures

The experimental procedures for the PK/PD study are summarized in Fig. 2, which were consisted of four steps: *(i) drug administration through nasal and oral routes*, *(ii) catalepsy tests*, *(iii) analyses of drug and neurotransmitter levels*, and *(iv) data analyses*. In addition to the PK/PD study, a separate study was conducted to compare the direct cataleptogenic effects of loxapine with its metabolites including 7-OH-loxapine to explore the potential PD mechanisms.

Male Wistar rats (180–230 g) were used in all studies. The study was approved by the Department of Health of Hong Kong SAR and the Animal Ethics Committee, in The Chinese University of Hong Kong.

Drug Ddministration Through Nasal and Oral Routes

In the PK/PD study, four to five rats were used for each time point per treatment group. Nasal loxapine solution was prepared by dissolving 7 mg loxapine succinate in 1 ml of 2.5% (w/v) Solutol® HS 15 (BASF Corporation, NJ, USA) in normal saline and the concentration of loxapine (free base) was 5.2 mg/ml. This concentrated solution was diluted 10-fold with normal saline to achieve a loxapine concentration of 0.52 mg/ml for oral administration. The dose of loxapine was 0.3 mg/kg. All the drug administration procedures and behavioral tests were conducted in conscious rats.

For rats receiving intranasal loxapine (nasal group), an average of 12 μl of 5.2 mg/ml loxapine solution (containing 2.5% (w/v) Solutol® HS 15 in normal saline) was administrated by pipetting in drops, alternating between each naris, within 2 min. The rats were held firmly in upright position with the head held in the vertical position, and the drops were placed onto the naris and naturally sniffed in by the rats. For rats receiving oral loxapine (oral group), around 120 μl of 0.52 mg/ml loxapine solution (containing 0.25% (w/v) Solutol® HS 15 in normal saline) was gavaged to rats. Rats receiving vehicle solutions without loxapine via nasal or oral route served as controls (control group), and their catalepsy behaviors and neurotransmitter levels at different time points after vehicle administration were measured in the

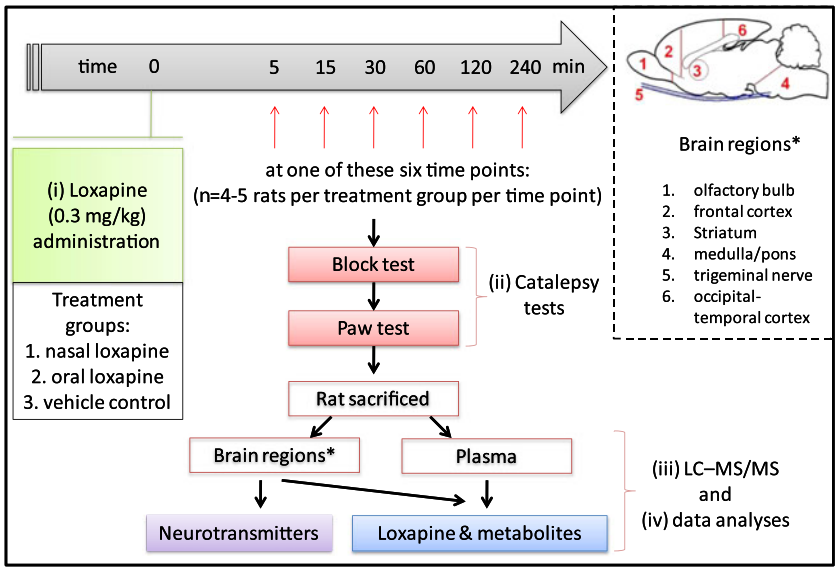

Fig. 2 Experimental procedures for the pharmacokinetic/pharmacodynamic (PK/PD) study.

same way as the loxapine-treated rats. After the dosing, the rats were returned to cage and were kept singly.

Catalepsy Tests

Catalepsy in rodents is a behavioral condition characterized by the maintenance in unnatural posture imposed by experimenters (17,18). At a pre-determined time point after loxapine administration (5, 15, 30, 60, 120, or 240 min), the cataleptic responses of rats from the nasal group and oral group were assessed by classic block test and then by paw test. For the block test (19), rats were placed with their forepaws on the platform (5.5 cm × 5.5 cm) of a 7 cm height block. Time (up to 180 s) was measured until the rat removed both of its forepaws from the block or climbed onto the block. In addition to the block test, the paw test was subsequently conducted in the same rat according to what has been described by Ellenbroek *et al.* (20). Briefly, a plastic box with a height of 20 cm was used. The box had two holes of 4 cm diameter for the forelimbs and two holes of 5 cm for the hindlimbs. The rat was held behind the forelimbs and gently placed in the box. First the hindlimbs were placed in the holes and then the rat was lowered and the forelimbs were positioned in the holes. The forelimb retraction time was defined as the time it took the rat to retract the first forelimb. Likewise, the hindlimb retraction time was defined as the time it took the rat to retract the first hindlimb.

During both the block test and the paw test, three consecutive attempts were made with the longest latency recorded as the catalepsy score. Rats were considered to be "cataleptic" if the measured time was ≥10 s in at least one of the three attempts. The same procedure had been conducted in the rats 30 min before loxapine dosing to get the rats familiar with the test apparatus and the handlings.

Analyses of Drug and Neurotransmitter Levels

After the catalepsy tests at the pre-determined time point (5, 15, 30, 60, 120, or 240 min), the rats were euthanized by carbon dioxide inhalation. Blood was collected by cardiac puncture followed by centrifugation at 15,000 × g for 5 min to obtain the plasma. The whole brain was then removed from skull, quickly rinsed with cold normal saline (4°C) and was wiped by tissue paper to remove excess water. The brain was then dissected into six anatomical regions including 1) olfactory bulb; 2) frontal cortex; 3) striatum; 4) medulla/pons; 5) trigeminal nerve; and 6) occipital-temporal cortex (Fig. 2). The frontal cortex was defined as the cortical tissues anterior to the genu of the corpus callosum. Meninges and blood vessels were removed and each tissue was weighted. The dissection was guided by a rat brain matrix (coronal, 1 mm, Roboz Surgical Instrument Co.) to ensure the accuracy and reproducibility of the dissection process. From each tissue 20 mg was taken for neurotransmitter analysis according to the liquid-liquid extraction method described in the Supplementary Material. Briefly, the brain tissue sample was first homogenized in 0.3% acetic acid in water by an ultrasonic probe (Microson XL-2000, Misonix, USA). Ethyl acetate was then added and after a period (20 min) of liquid-liquid extraction process, the ethyl acetate was evaporated to dryness and the concentrations of acidic neurotransmitters (DOPAC, HVA, and 5-HIAA) were quantified by a LC–MS/MS system operated under negative ionization mode. The basic neurotransmitters (DA, 3-MT, and 5-HT) remained in the aqueous phase were quantified by the same LC–MS/MS system operated under positive ionization mode. The neurotransmitter analysis was conducted on the same day as the

PK/PD study to minimize potential degradation of neurotransmitters.

The remaining brain tissues were frozen at −80°C until drug analysis. The concentrations of loxapine and its metabolites in the brain tissues and plasma were analyzed by the LC–MS/MS method previously developed and validated by our research group (4). Briefly, 1 volume of methanol and 7 volumes of perchloric acid (10% w/w) were added to a brain tissue sample as homogenization medium. The sample was homogenized by an ultrasonic probe (Microson XL-2000, Misonix, USA) for 20 to 30 s. After centrifugation the supernatant of the homogenate was loaded to the solid phase extraction cartridge (Oasis mixed-mode cation-exchange (MCX)) to extract loxapine and its metabolites. The concentrations of loxapine and its metabolites were quantified simultaneously by a LC–MS/MS system operated under positive ionization mode. For plasma samples, after protein precipitation with perchloric acid (10% w/w), they were subjected to the same solid phase extraction procedures and LC–MS/MS analysis. For brain tissue, the lower limits of quantification were 3 ng/g for loxapine and amoxapine and 5 ng/g for the four hydroxylated metabolites of loxapine (7-OH-loxapine, 8-OH-loxapine, 7-OH-amoxapine, and 8-OH-amoxapine). For plasma, the lower limits of quantification were 1 ng/ml for loxapine and amoxapine and 2 ng/ml for the four hydroxylated metabolites.

Data analyses

Brain and plasma PK parameters of loxapine and its metabolites were calculated by WinNonlin (version 2.1, Pharsight) using non-compartmental analysis. Area under the curve (AUC) was calculated using linear/log trapezoidal method. Analyte with a concentration lower than the lower limit of quantification was considered as 0 ng/g (brain tissue) or 0 ng/ml (plasma) in the calculation unless specified otherwise. Brain and plasma drug concentrations were compared between nasal route and oral route by Student's *t*-test. The concentrations of neurotransmitters were compared among nasal group, oral group, and control group by one way ANOVA with post hoc Tukey (when variances were equal according to Levene's test) or Games-Howell test (when variances were not equal according to Levene's test). For catalepsy tests, Mann–Whitney *U*-test was used to compare the catalepsy scores between nasal route and oral route. In all the tables and figures, data are presented as mean ± S.E.

Cataleptogenic Effects of Loxapine and its Metabolites

One day before the catalepsy study, the rats were anesthetized with an intraperitoneal dose of ketamine (100 mg/kg) and xylazine (10 mg/kg) followed by catheterization in the right jugular vein with a polythene tube (Portex fine bore polythene tubing, I.D. 0.5 mm, O.D. 1.0 mm). The rats were allowed to recover from the surgical procedure for around 18 h before the catalepsy study.

Loxapine or its metabolites was first dissolved in DMSO and then diluted to 0.25 ml with normal saline acidified with HCl (pH 5). In the treatment groups, individual compound was separately injected slowly through the catheter over 15 s. The dose was 0.05 mg/kg for each compound. At 10, 20, 30, 60, 120 and 240 min after IV administration, the rat was repeatedly assessed in the block test as described above. Control rats received an IV administration of the vehicle solution via the catheter. An additional treatment group, the "co-administration" group, was included to study the effect of loxapine on 7-OH-loxapine induced catalepsy. Loxapine solution (at dose 0.05 mg/kg) was co-administered with 7-OH-loxappine solution (at dose 0.05 mg/kg) via the jugular vein catheter. Block test was conducted as described above.

At each time point, the catalepsy score of each treatment group was compared with that of control group by Mann–Whitney *U*-test. The catalepsy scores between loxapine group, 7-OH-loxapine group, and co-administration group were compared by Kruskal-Wallis test followed by Mann–Whitney *U*-test. In all test, $p<0.05$ was considered statistically significant. In all the figures, data are presented as mean ± S.E.

RESULTS

Enhanced Brain Uptake of Loxapine After Intranasal Delivery

The PK profiles of loxapine and its metabolite 7-OH-loxapine in different brain regions after loxapine administration via nasal and oral routes are shown in Fig. 3, and the corresponding PK parameters are summarized in Table I. Nasal route could rapidly deliver loxapine to brain with t_{max} less than 15 min in all brain regions. Loxapine level was exceptionally high in the trigeminal nerve at 5 min although it returned to levels comparable to that of other regions after 5 min. This suggests that some of the intranasally applied loxapine could be immediately absorbed by the trigeminal nerve that innervates the respiratory epithelium of the nasal cavity. In general, oral route delivered minimal amount of loxapine to brain and the total brain disposition ($AUC_{0–240min}$) of loxapine to was 22 times (trigeminal nerve and striatum) to 114 times (frontal cortex) lower than that of 7-OH-loxapine.

In contrast to the striking difference in brain levels of loxapine seen between nasal and oral groups, brain levels of 7-OH-loxapine were similar in both groups. The $AUC_{0–240min}$ ratios of 7-OH-loxapine to loxapine in brain were at

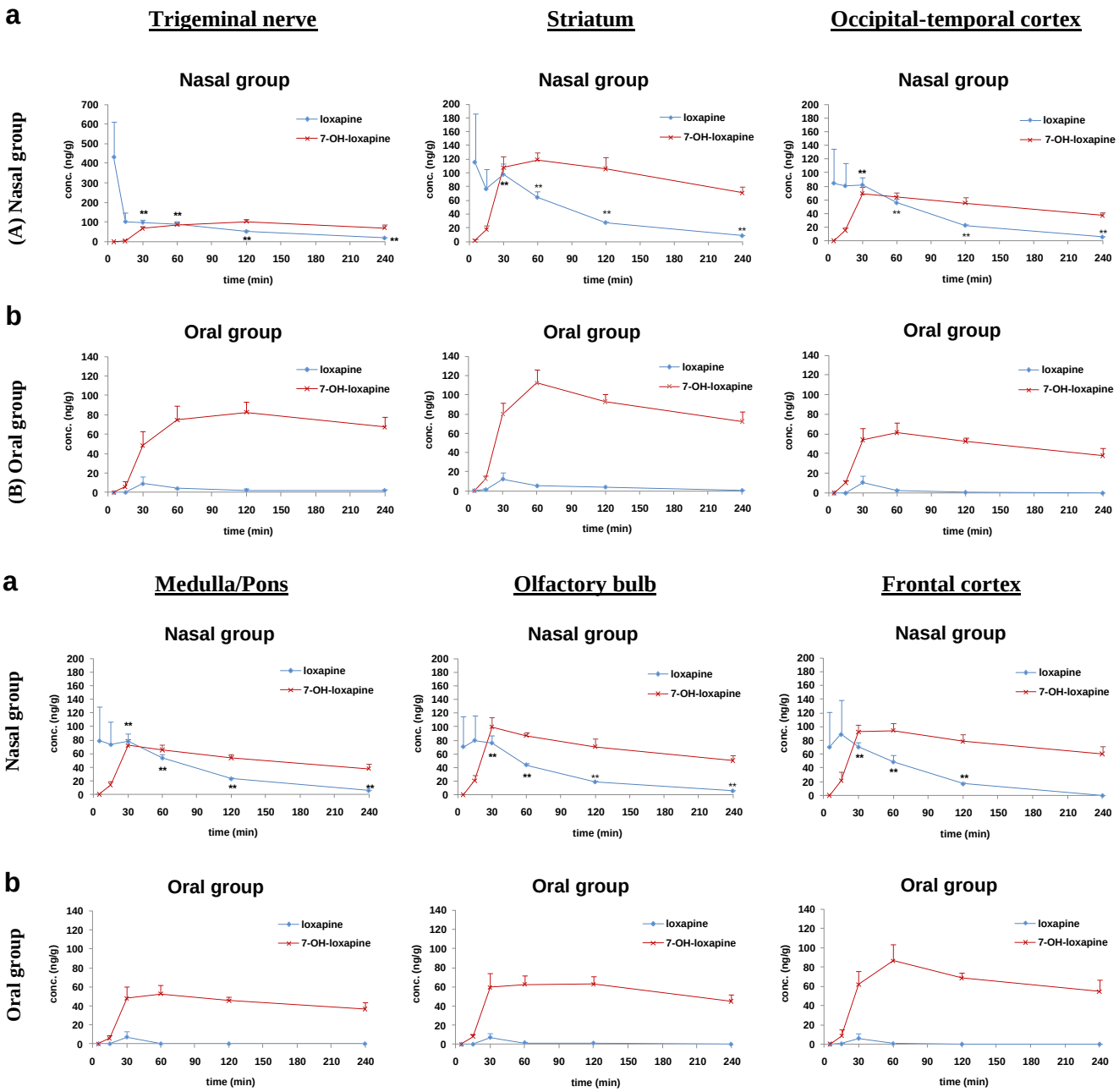

Fig. 3 Pharmacokinetic profiles of loxapine and its metabolite 7-OH-loxapine in different brain regions after loxapine administration (0.3 mg/kg) via (**a**) nasal and (**b**) oral routes ($n = 4$–5 per time point). Note the scales of the vertical axis are 700 ng/g for trigeminal nerve and 200 ng/g for other regions in nasal group, and 140 ng/g for all regions in oral group. Significant difference between nasal and oral groups: * $p < 0.05$, ** $p < 0.005$.

least 10 times lower after intranasal administration (1.9 in average) than after oral administration (48 in average). The levels of 7-OH-loxapine exceeded that of loxapine after 30 to 60 min in nasal group, and the $AUC_{0–240min}$ of 7-OH-loxapine was 19% (trigeminal nerve) to 154% (frontal cortex) higher than that of loxapine.

Other loxapine metabolites (8-OH-loxapine, amoxapine, 7-OH-amoxapine, and 8-OH-amoxapine) were not detected in brain neither after nasal loxapine administration nor oral loxapine administration.

Enhanced Systemic Exposure of Loxapine After Intranasal Delivery

The PK profiles of loxapine and its metabolites in plasma after loxapine administration are shown in Fig. 4,

Table I Brain Pharmacokinetic Parameters of Loxapine and its Metabolite 7-OH-Loxapine After Loxapine Administration (0.3 mg/kg) via Nasal and Oral Routes ($n = 4–5$ Per Time Point)

Brain regions	$AUC_{0-240min}$ (ng•min/g)						C_{max} (ng/g)			
	Nasal			Oral			Nasal		Oral	
	Loxapine	7-OH-loxapine	7-OH-loxapine: loxapine ratio	Loxapine	7-OH-loxapine	7-OH-loxapine: loxapine ratio	Loxapine	7-OH-loxapine	Loxapine	7-OH-loxapine
Trigeminal nerve	15854	18860	1.2	725	15969	22	433	103	9	83
Striatum	9523	21658	2.3	871	19621	23	115	119	12	112
OT cortex	7951	11711	1.5	387	10994	28	85	69	11	61
Medulla/Pons	7729	11761	1.5	160	9818	61	79	72	7	53
Olfactory bulb	6814	15723	2.3	319	12595	40	80	100	7	63
Frontal cortex	6765	17213	2.5	130	14766	114	89	94	6	87
average	9106	16154	1.9	432	13960	48	147	93	9	76

Brain regions	t_{max} (min)				$t_{1/2}$ (min)			
	Nasal		Oral		Nasal		Oral	
	Loxapine	7-OH-loxapine	Loxapine	7-OH-loxapine	Loxapine	7-OH-loxapine	Loxapine	7-OH-loxapine
Trigeminal nerve	5	120	30	120	79	*NA*	120	*NA*
Striatum	5	60	30	60	62	236	64	288
OT cortex	5	30	30	60	52	232	28	264
Medulla/Pons	5	30	30	60	56	225	*NA*	349
Olfactory bulb	15	30	30	60	55	227	34	346
Frontal cortex	15	60	30	60	44	284	*NA*	281
average	8	55	30	70	58	241	62	306

The brain regions are listed according to the disposition ($AUC_{0-240min}$) of loxapine after intranasal loxapine administration

NA not applicable, *OT cortex* occipital-temporal cortex

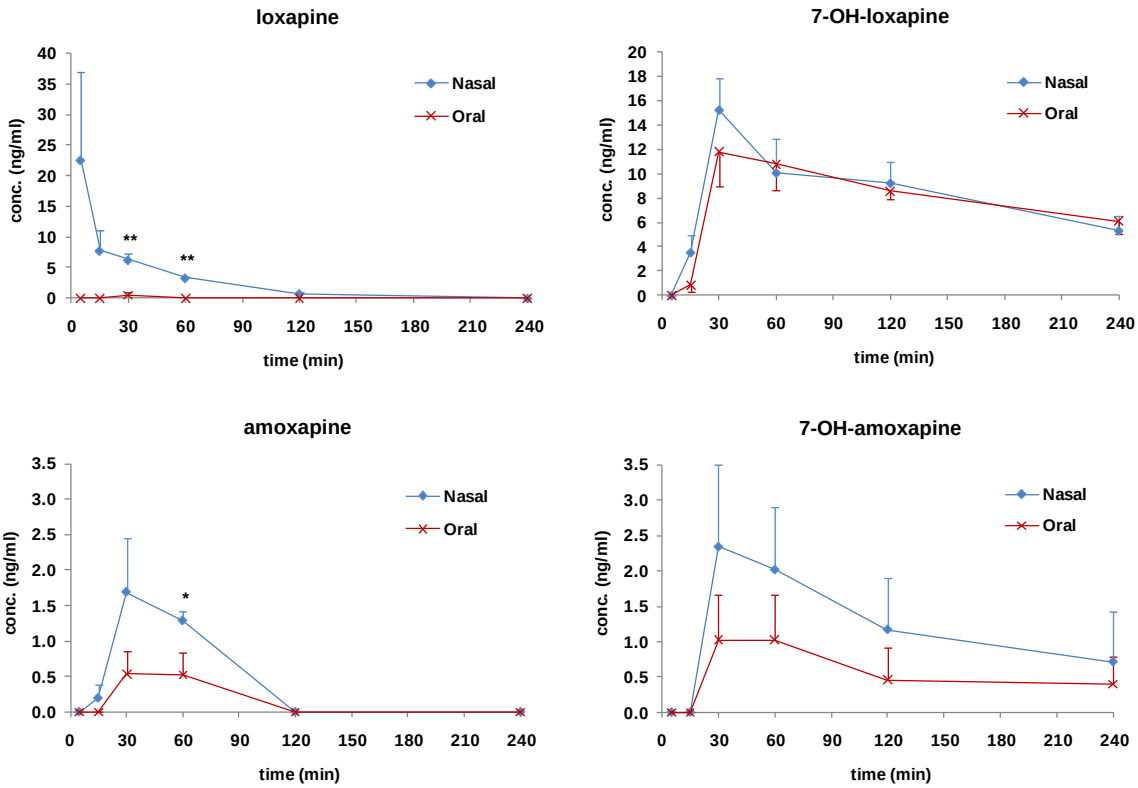

Fig. 4 Pharmacokinetic profiles of loxapine and its metabolites in plasma after loxapine administration (0.3 mg/kg) via nasal and oral routes ($n = 4$–5 per time point). Significant difference between nasal and oral groups: * $p < 0.05$, ** $p < 0.005$.

and the corresponding PK parameters are summarized in Table II. Nasal route rapidly delivered loxapine to systemic circulation. Similar to that observed in brain, oral route resulted in an unquantifiable amount of intact loxapine in plasma, suggesting that substantial amount of oral loxapine was eliminated and/or metabolized before entering the systemic circulation. Nasal and oral routes generated similar amounts of 7-OH-loxapine in plasma, which was consistent with that observed in brain. Nasal route generated slightly more amoxapine and 7-OH-amoxapine than oral route but the levels in both groups were not significantly different at most time points.

Lower Tendency of Catalepsy After Intranasal Delivery

The results of the block test and paw test in rats receiving loxapine through nasal or oral route are shown in Fig. 5. For block test, rats in nasal group and oral group started to develop catalepsy at around 30 min and 60 min, respectively. From 60 min to 240 min, both severity and incidence of catalepsy were higher in oral group than in nasal group, although not reaching statistical significance due to large variation among individual rats. A similar trend was observed in paw test. For both forelimb and hindlimb, catalepsy was observed

Table II Plasma Pharmacokinetic Parameters Loxapine and its Metabolites After Loxapine Administration (0.3 mg/kg) via Nasal and Oral Routes ($n = 4$–5 Per Time Point)

	$AUC_{0-240min}$ (ng•min/ml)		C_{max} (ng/ml)		t_{max} (min)		$t_{1/2}$ (min)	
	Nasal	Oral	Nasal	Oral	Nasal	Oral	Nasal	Oral
Loxapine	603	0	23	0	5	*NA*	35	*NA*
7-OH-loxapine	1919	1933	15	12	30	30	173	206
amoxapine	98	36	2	1	30	30	*NA*	*NA*
7-OH-amoxapine	287	133	2	1	30	30	120	142

NA not applicable

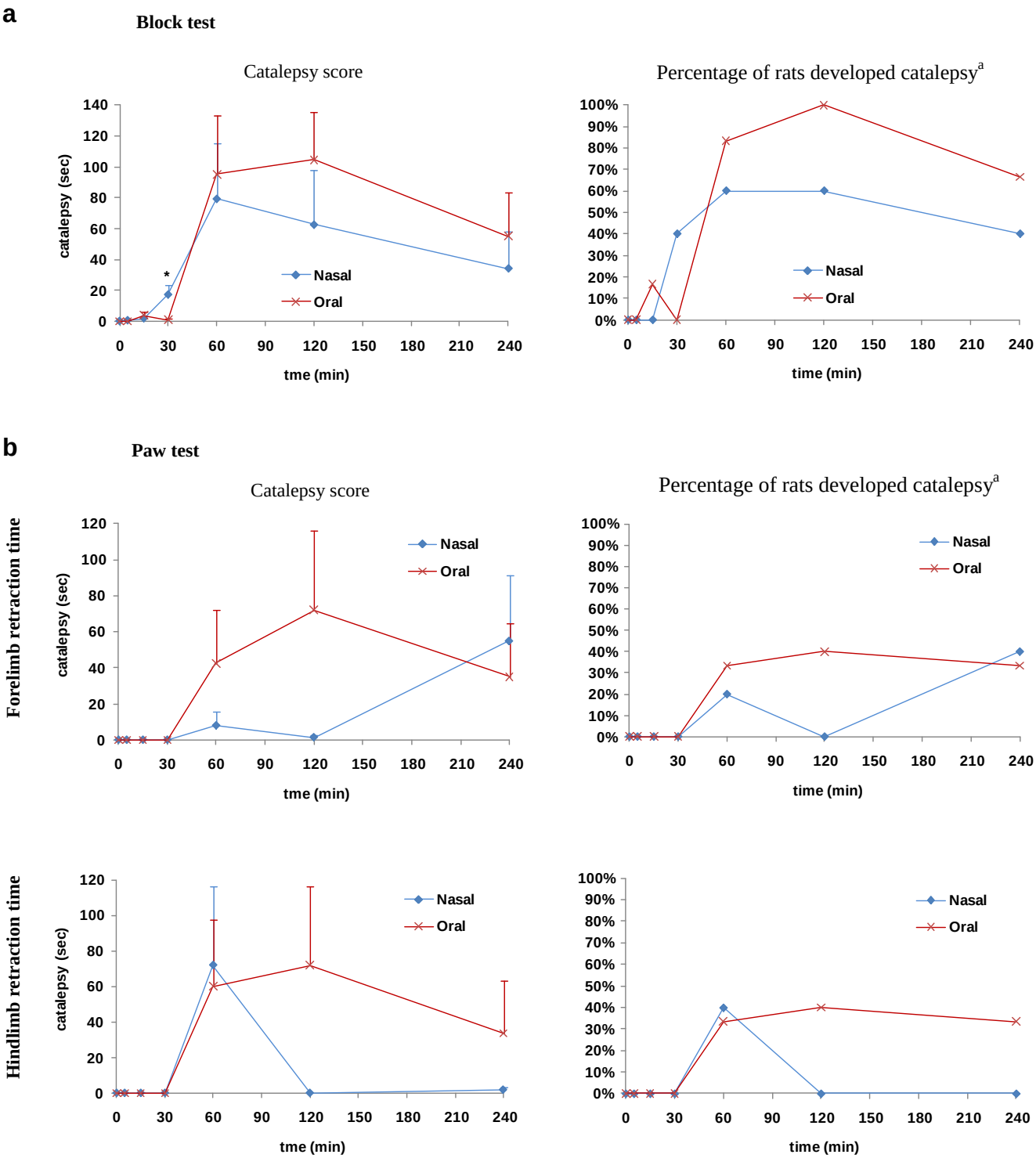

Fig. 5 Cataleptic effects of loxapine (0.3 mg/kg) administered via nasal and oral routes as measured by (**a**) block test and (**b**) paw test ($n = 4$–5 per time point). For each rat, catalepsy tests were conducted at only one time point. Significant difference between nasal and oral groups: * $p < 0.05$ by Mann–Whitney *U*-test. [a] Rats were considered to be "cataleptic" if the measured time was ≥10 s.

starting from 60 min. Both severity and incidence of catalepsy were generally higher in oral group than in nasal group from 60 min to 120 min for forelimb and from 120 min to 240 min for hindlimb, although not reaching statistical significance due to large inter-individual variation.

Most the control rats escaped from the test apparatus within 10 s with no notable catalepsy behaviors being observed. The use of a 10-sec criterion to provide a 'threshold measure' of true catalepsy was also adopted by other studies (such as (18,19,21,22)).

Comparable Effects on Neurotransmitter Levels After Intranasal and Oral Loxapine Administration

The effects of loxapine administration on the levels of DA, 5-HT, and their metabolites HVA, DOPAC, 3-MT, and 5-HIAA in striatum and olfactory bulb are shown in Fig. 6a and b, respectively. Development and validation of the LC–MS/MS method for the quantification of these neurotransmitters are presented in the Supplementary Material.

Concentrations of these six neurotransmitters (DA, 5-HT, HVA, DOPAC, 3-MT, and 5-HIAA) in control group rats (rats receiving vehicle only) were comparable (in the same order of magnitude) with the literature values in normal rats (23,24). Since the levels of these neurotransmitters remained stable after vehicle administration via nasal and oral routes in the control group rats, the concentrations of neurotransmitters at different time points after vehicle administration were pooled in each individual brain region, which served as the "pre-drug" baseline levels of that region. The following discussion on the effects of loxapine refers to

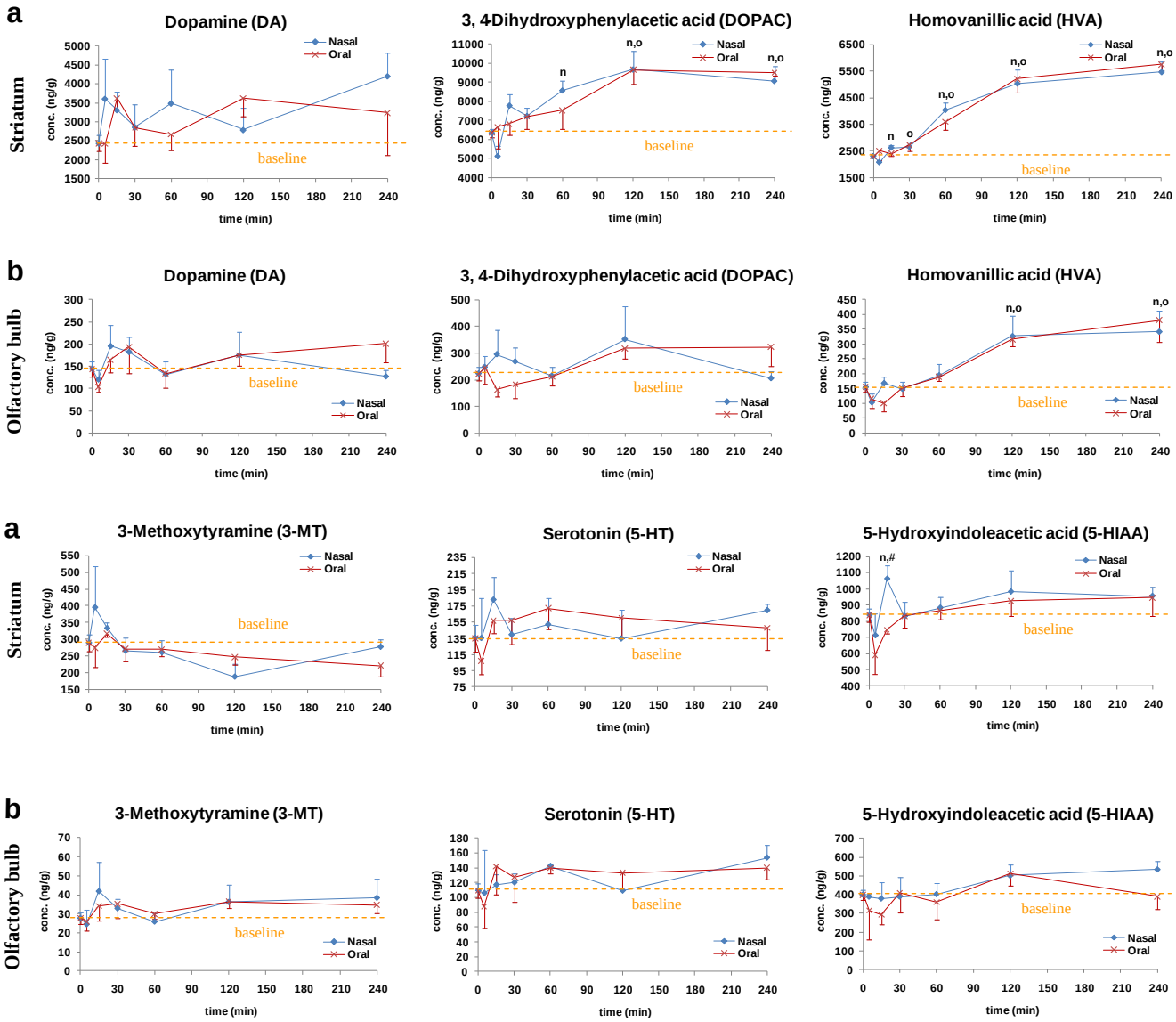

Fig. 6 The effects of loxapine administration (0.3 mg/kg) via nasal and oral routes ($n = 4$–5 per time point) on the levels of dopamine, serotonin, and their metabolites in (**a**) striatum and (**b**) olfactory bulb. Significant difference ($p < 0.05$) between nasal group and baseline (^{n}), between oral group and baseline (^{o}), and between nasal group and oral group ($^{\#}$).

the changes of neurotransmitter concentrations from their corresponding baselines after loxapine administrations.

In striatum, after nasal and oral loxapine administrations, significant changes in neurotransmitter levels were observed in HVA, DOPAC, and 5-HIAA but not in DA, 3-MT, and 5-HT. For both nasal and oral groups, the levels of the DA metabolites HVA and DOPAC increased significantly with time while the levels of DA, 3-MT, and 5-HT did not change significantly from the baseline. The levels of neurotransmitters in striatum were comparable between nasal group and oral group at each time point, and the only significant difference between the nasal and oral groups was observed at 15 min where there was a spike in 5-HIAA level in nasal group.

In olfactory bulb, the trends of alteration in neurotransmitter levels were very similar to that observed in striatum. However, significant changes in neurotransmitter levels were observed only for HVA in both nasal and oral groups but not for other neurotransmitters. The levels of neurotransmitters in olfactory bulb were comparable between nasal group and oral group at each time point.

Greater Cataleptogenic Effect Generated by 7-OH-Loxapine Than Loxapine

The catalepsy induced by loxapine and its five metabolites, namely 7-OH-loxapine, 8-OH-loxapine, amoxapine, 7-OH-amoxapine, and 8-OH-amoxapine, administered via IV bolus injection is shown in Fig. 7. These compounds were administered intravenously to reduce variations in absorption and systemic exposure. At the present dose (0.05 mg/kg), the cataleptogenicity was in the order of 7-OH-loxapine >loxapine >amoxapine ≥7-OH-amoxapine, 8-OH-loxapine, and 8-OH-amoxapine.

The catalepsy in rats co-administered with 7-OH-loxapine (0.05 mg/kg) and loxapine (0.05 mg/kg) via IV injection is shown in Fig. 8. The catalepsy profile of this co-administration group was indeed similar to that of 7-OH-loxapine group (i.e. the score increased to a maximum at 120 min and then decreased). However, the catalepsy scores at every time point tested were lower in co-administration group than that in 7-OH-loxapine group although such

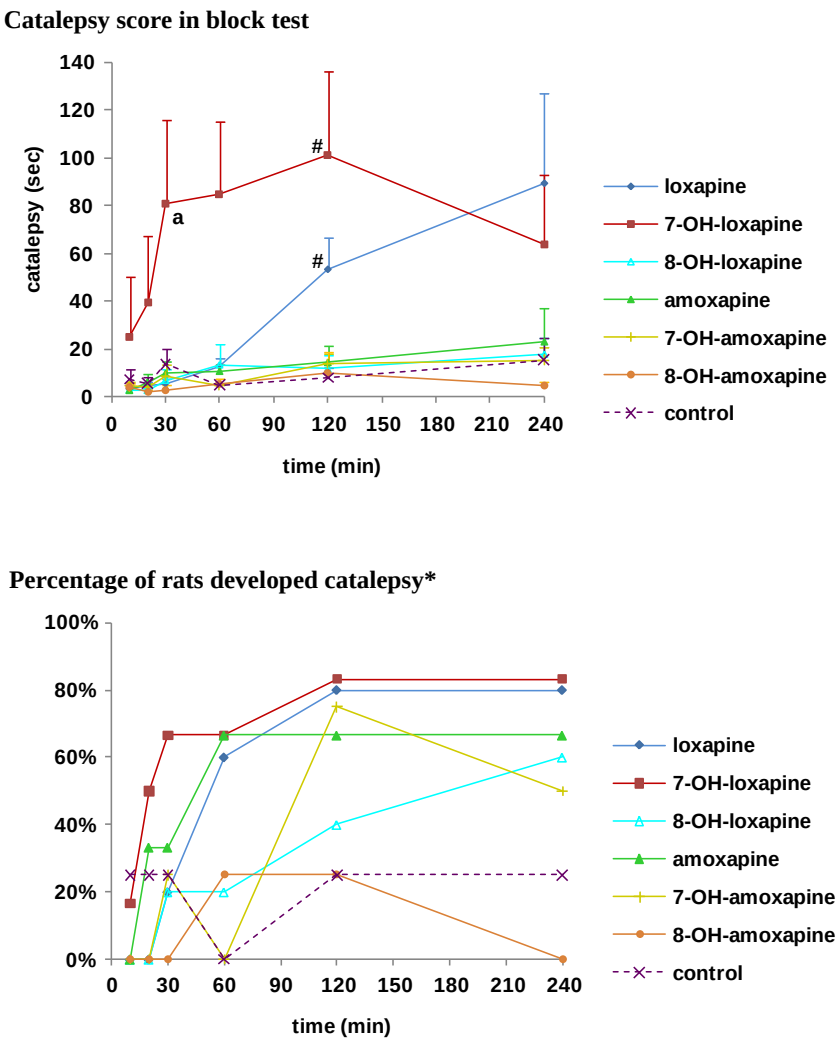

Fig. 7 Comparison of block test cataleptic score (*top*) and percentage of rats developed catalepsy (*bottom*) after IV administration of loxapine and its metabolites (0.05 mg/kg) ($n = 4$–6 per group). Repeated testing on catalepsy at different time points was conducted on the same rat. * Rats were considered to be "cataleptic" if the measured time was ≥10 s. # Significant difference compared with control group in Mann–Whitney *U*-test ($p < 0.05$). [a] Significant difference between 7-OH-loxapine and loxapine groups in Kruskal-Wallis test followed by Mann–Whitney *U*-test ($p < 0.05$).

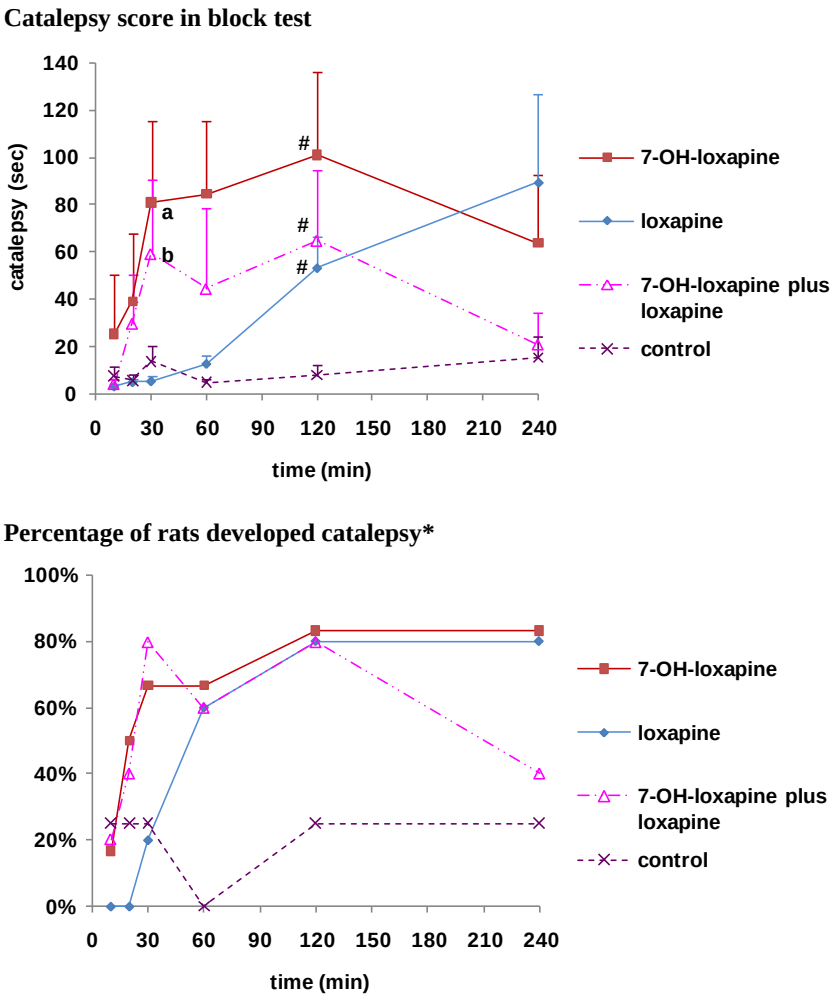

Fig. 8 Block test cataleptic score (*top*) and percentage of rats developed catalepsy (*bottom*) after IV co-administration of 7-OH-loxapine (0.05 mg/kg) and loxapine (0.05 mg/kg) ($n = 4$–6 per group). Repeated testing on catalepsy at different time points was conducted on the same rat. * Rats were considered to be "cataleptic" if the measured time was ≥10 s. # Significant difference compared with control group ($p < 0.05$) in Mann–Whitney *U*-test. [a,b] Significant difference between 7-OH-loxapine and loxapine groups([a]) and between co-administration and loxapine groups([b]) in Kruskal-Wallis test followed by Mann–Whitney *U*-test ($p < 0.05$).

differences did not reach statistical significance. The percentages of rats developed catalepsy were comparable for the co-administration group and 7-OH-loxapine group at all time points except for the 240 min time point when the percentage in the co-administration group (40%) was half of that in the 7-OH-loxapine group (83%). Therefore, the addition of loxapine did not intensify, but rather alleviated the cataleptogenic effect of 7-OH-loxapine.

DISCUSSION

Altered Brain Disposition of Loxapine After Intranasal Loxapine Administration

Conventionally loxapine is administered to patients through oral route (tablet, capsule, or concentrated liquid); however, the oral bioavailability of loxapine is low (33% by comparing with intramuscular (IM) loxapine) (25). Together with our previous PK work (4), the present data suggest that the low bioavailability of oral loxapine is due to extensive first-pass metabolism. A previous study on tritiated amoxapine in rats found that by 288 h after oral administration, 70% of the radioactivity given was excreted into feces, in which the metabolite 7-OH-amoxapine accounted for most of the fecal content while intact amoxapine was not detected in feces (26). Since loxapine and amoxapine share similar metabolic pathways and CYP enzymes (Fig. 1), most of the orally administered loxapine administration might also be eliminated in feces as metabolites, particularly as 7-OH-loxapine. The metabolites in feces could be generated from two sources. First, metabolism by CYPs could occur in the intestine wall. Second, the loxapine absorbed from the intestine would also be subjected to metabolism in liver, and the metabolites formed would be excreted to bile and then feces.

In contrast to oral administration, intranasal administration could efficiently deliver loxapine to brain and blood. The absolute bioavailabilties of intranasal loxapine (*vs* IV loxapine) is ~50% in conscious rat model (Wong and Zuo, AAPS Annual Meeting 2011 poster presentation). Results from the present study suggest that direct nose-to-CNS delivery of loxapine through the olfactory pathway is not evident in the

conscious rats. At each time point, the levels of loxapine in different brain regions were more or less the same. The rostral parts of the brain, which are located near to the nasal cavity (i.e. olfactory bulb and frontal cortex), did not achieve higher loxapine levels than the caudal parts of the brain (occipital-temporal cortex). Therefore, after intranasal administration, loxapine should be rapidly absorbed by the blood vessels within the nasal cavity into systemic circulation. Plasma loxapine then entered the CNS after passing through the blood–brain barrier and homogeneously distributing to different parts of the brain. During systemic circulation, loxapine was also converted to metabolites including 7-OH-loxapine when passing through the liver. The 7-OH-loxapine formed would then be homogeneously distributed to different parts of the brain. The brain to plasma $AUC_{0-240min}$ ratios of 7-OH-loxapine and loxapine were around 8 and 15, respectively, suggesting that these lipophilic molecules were deposited preferentially in brain tissues. This highlights the importance of measuring the brain levels of drug and metabolites when investigating intranasal delivery of CNS drugs since the plasma levels might not reflect their dispositions in the CNS.

In conscious animal models, a certain amount of the intranasally applied drug will inevitably be swallowed into gastrointestinal tract. Since loxapine is almost completely metabolized during first-pass metabolism as discussed above, the swallowed nasal drug would be absorbed into systemic circulation as metabolites. Although the exact amount of nasal loxapine swallowed into gastrointestinal tract was not investigated, the portion of loxapine being swallowed was expected to be low due to the following reasons: 1). Loxapine is a small, lipophilic molecule (log D at pH 7 is 2.04, calculated by ACD/Labs Software V11.02) that is absorbed rapidly within the nasal cavity (plasma t_{max} occurred at around 5 min in the present study). Mechanistic studies suggested that highly permeable drugs were primarily absorbed through the nasal mucosa before it is cleared to the GI tract (27). 2). The volume of loxapine solution applied was relatively small (6 μl for each naris), which helped to minimize the chance of overloading the nasal cavity.

To our knowledge, this is the first study to elucidate the concurrent PK profiles of parent drug and its metabolites in different brain regions after intranasal drug application in conscious animals. Information on metabolite disposition in brain after intranasal drug application is scarce, and most of the few reports are dealing with the disposition of the active moieties in brain after intranasal administration of the prodrugs. Readers are referred to our recent review for a more detailed discussion (1).

Role of Active Metabolite in the Cataleptic Effects of Loxapine

In the present PK/PD study, the parent drug loxapine was barely detectable in brain after oral loxapine administration while considerable amounts of loxapine were found in striatum and other brain regions after intranasal loxapine administration. Although the brain levels of 7-OH-loxapine after oral and intranasal loxapine administrations were very similar, the catalepsy tended to be more severe in rats receiving oral loxapine than that receiving intranasal loxapine. These observations suggest that the catalepsy observed is due to, at least in part, the active metabolite 7-OH-loxapine. Individual IV injections of 7-OH-loxapine and loxapine to rats confirmed that 7-OH-loxapine is indeed more cataleptogenic than loxapine. This is consistent with the higher DA D_2 receptor affinity of 7-OH-loxapine (Fig. 1), and DA D_2 receptor antagonism by antipsychotics in striatum is the most important factor to determine catalepsy in rats and extrapyramidal side effects in humans (9). Moreover, after intranasal and oral loxapine administrations, the concentration of 7-OH-loxapine in striatum was higher than all the other studied brain regions. This observation is in line with the unexceptionally high affinity of 7-OH-loxapine to DA D_2 receptor since striatum is densely populated with dopaminergic neurons and receptors. Nevertheless, the possibility that loxapine itself can induce catalepsy cannot be excluded. Since loxapine will always undergo metabolism to generate the cataleptogenic 7-OH-loxapine, the catalepsy observed after loxapine treatment could be contributed by both loxapine and the 7-OH-loxapine generated *in vivo*.

To compare the overall effect size of loxapine given by different routes of administration, the area under the data of the block test catalepsy score to time curves was calculated by use of the trapezoid rule. The area under the data (score × min) for oral loxapine (0.3 mg/kg, Fig. 5a), nasal loxapine (0.3 mg/kg, Fig. 5a), and IV loxapine (0.05 mg/kg, Fig. 7) were 1.7×10^4, 1.2×10^4, and 1.1×10^4, respectively. By taking the loxapine dose into consideration, IV loxapine would be ~6-fold more cataleptogenic than nasal loxapine and ~4-fold more cataleptogenic than oral loxapine.

In different regions of rat brain, the levels of the cataleptogenic metabolite 7-OH-loxapine were comparable after intranasal and oral loxapine administration. Since the nasal route, but not the oral route, also delivered intact loxapine to brain, the $AUC_{0-240min}$ ratios of 7-OH-loxapine to loxapine in brain were at least 10 times lower after intranasal administration that after oral administration. Paradoxically, intranasal loxapine tended to induce less catalepsy than oral loxapine although the total amount ($AUC_{0-240min}$) of loxapine and 7-OH-loxapine in brain was higher after intranasal loxapine administration. The presence of loxapine might therefore exert an antagonistic effect on 7-OH-loxapine induced catalepsy. This is confirmed by our finding that when IV loxapine was co-administered to rats receiving IV 7-OH-loxapine, it tended to reduce rather than increase the catalepsy induced by IV 7-OH-loxapine. The proposed antagonistic effect of loxapine on 7-OH-loxapine induced catalepsy might

be due to their competition for D_2 receptor binding. Since the D_2 receptor affinity of loxapine is at least several folds lower than that of 7-OH-loxapine, the presence of loxapine, a weaker D_2 ligand, might antagonize the anti-dopaminergic effects including catalepsy induced by 7-OH-loxapine, a stronger D_2 ligand, by competing for receptor binding. In fact, PD interactions between the parent compounds and their metabolites have been reported for several CNS drugs. For instance, both midazolam and its active metabolite 1′-hydroxymidazolam act as full agonists at the GABA-benzodiazepine receptor complex. Midazolam and 1′-hydroxymidazolam compete for the same receptor and the PD interaction between these two substrates has been characterized by a competitive interaction model using quantitative electroencephalogram parameters as a PD endpoint (28). The major metabolite of Δ^1-tetrahydrocannabinol, Δ^1-tetrahydrocannabinol-7-oic acid, was also found to antagonize the cataleptic effects of Δ^1-tetrahydrocannabinol in mice (29).

The relationships between catalepsy score in block test and the striatal concentrations of 7-OH-loxapine, loxapine, and the ratio of 7-OH-loxapine to loxapine in the PK/PD study were computed and are presented in Fig. 9. For both the nasal and oral loxapine groups, there were moderate but statistically significant positive correlations between the catalepsy score and the logarithm of striatal concentration of 7-OH-loxapine, but not loxapine. Slightly better correlations with catalepsy score were noted when the ratios of 7-OH-loxapine to loxapine were evaluated. The results further indicated that the main determinant of catalepsy is 7-OH-loxapine (or the ratio of 7-OH-loxapine to loxapine) but not loxapine itself. Moreover, the correlations between catalepsy scores and the logarithm of striatal concentrations of 7-OH-loxapine and the striatal ratio of 7-OH-loxapine to loxapine were weaker (lower correlation coefficients) in the nasal loxapine group than that in the oral loxapine group. This could probably be due to the presence of considerable amount of loxapine in nasal group, which could interfere with (or specifically, antagonize) the cataleptic effect induced by 7-OH-loxapine as mentioned above. As could be seen in Fig. 9a, rats might demonstrate catalepsy when the striatal concentration of 7-OH-loxapine reached ~50 ng/g.

In humans, 7-OH-loxapine is a substantial metabolite and the systemic disposition ($AUC_{0-\infty}$) of 7-OH-loxapine (104 ng/h/ml) is comparable to that of loxapine (166 ng/h/ml) after oral loxapine ingestion (3). In view of the significant formation of 7-OH-loxapine and its exceptionally high affinity to the D_2 receptors, together with its cataleptogenicity as identified in the present study, it is likely that 7-OH-loxapine would have considerable contribution to the extrapyramidal symptoms in patients receiving loxapine treatment. Moreover, considering the PD interactions between 7-OH-loxapine and loxapine, the ratio of these two species in brain might determine the severity of extrapyramidal symptoms. Strategies that could lower the level of 7-OH-loxapine (or 7-OH-loxapine to loxapine ratio) in brain might help to reduce the extrapyramidal side effects associated with loxapine treatment. Co-administration of loxapine with compounds that selectively inhibit CYP 2D6-mediate formation of 7-OH-loxapine (30) could be a strategy to lower the 7-OH-loxapine level, which warrants further investigation.

Besides 7-OH-loxapine, it is worth discussing the cataleptogenicity of other loxapine metabolites. These metabolites were present in quantifiable amounts in rat brain after intranasal administration of loxapine at a higher dose (1 mg/kg) (Wong and Zuo, AAPS Annual Meeting 2010 poster presentation). In the present study, amoxapine was much less cataleptogenic than loxapine, which is consistent with the literature reports (e.g. (31)). The underlying reason could be that amoxapine dissociates more rapidly from D_2 receptor than loxapine (32). This allows frequent access of endogenous DA to the receptor, which helps to restore DA neurotransmission required for motor function (32). The catalepsy scores achieved by the 8-hydroxylated metabolites (8-OH-loxapine and 8-OH-amoxapine) were just comparable with that of vehicle controls, which is consistent with their relatively low D_2 receptor affinities when compared with that of 7-OH-loxapine and loxapine (Fig. 1). In contrast to the severe catalepsy induced by 7-OH-loxapine, the 7-OH-amoxapine induced catalepsy was rather weak, although these two compounds have comparable D_2 receptor affinities. One possible explanation is that 7-OH-loxapine is more lipophilic than 7-OH-amoxapine (log D at pH 7 are 1.93 and 1.31, respectively, calculated by ACD/Labs Software V11.02). Therefore, 7-OH-loxapine could be more permeable to brain than 7-OH-amoxapine and might have attained higher concentration in the target site striatum while 7-OH-amoxapine might not have approached effective concentrations at the present low dose (0.05 mg/kg).

Since loxapine and its metabolites have appreciable affinities to different DA receptor subtypes and 5-HT receptor subtypes (25), loxapine administration might affect the dopaminergic and serotonergic neurotransmissions in brain which is worth investigating. To our knowledge, the present study represents the first report on the effects of DA antagonists (such as antipsychotics) on brain neurotransmitter levels after intranasal delivery of DA antagonists. Intranasal loxapine increased DA turnovers (i.e. increased formations of DA metabolites HVA and DOPAC) in striatum and, to a lesser extent, olfactory bulb, while no significant change was observed in the levels of 5-HT and its metabolite 5-HIAA. Intranasal loxapine and oral loxapine induced comparable changes in neurotransmitter levels. Further pharmacological investigations are needed to determine whether the effect on neurotransmitter levels differs between intranasal and oral loxapine administration in other brain regions (e.g. frontal cortex) and to elucidate the relationship between changes in neurotransmitters and catalepsy outcomes.

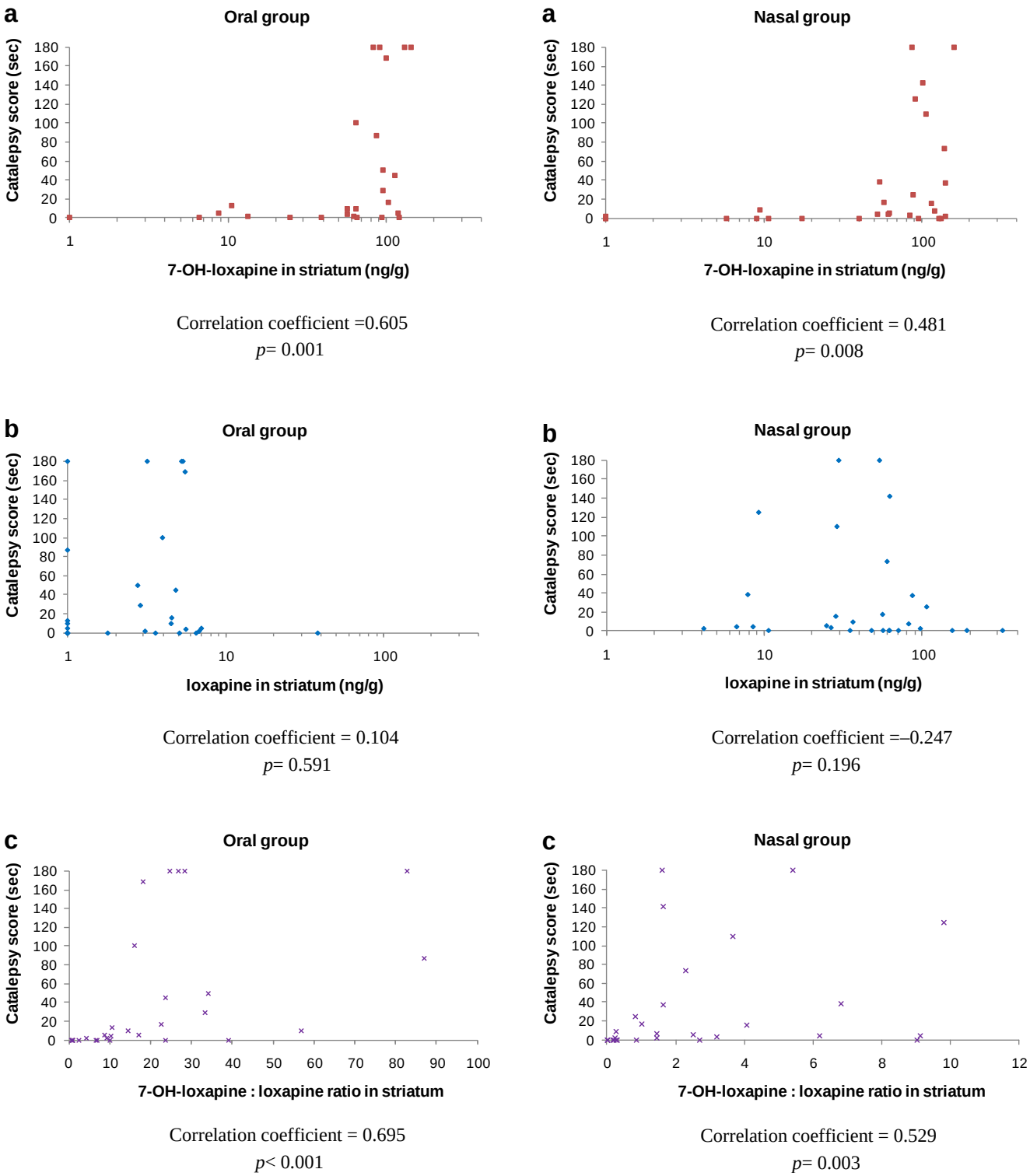

Fig. 9 Correlation between catalepsy score in block test and the logarithm of striatal concentrations of (**a**) 7-OH-loxapine, (**b**) loxapine, and (**c**) striatal ratio of 7-OH-loxapine to loxapine in rats receiving oral (*left panel*) and nasal (*right panel*) loxapine treatment in the PK/PD study ($n = 29$ per group) using the Spearman correlation test. Analyte with a striatal concentration lower than the lower limit of quantification (5 ng/g for 7-OH-loxapine and 3 ng/g for loxapine) was considered as 1 ng/g in the statistical calculations.

Clinical Potential of Intranasal Loxapine

Loxapine is given to patients via oral or, occasionally, via IM routes. Loxapine is extensively metabolized in both humans (3,33) and in rats as observed in our present and previous works (4). The generated metabolites have differential pharmacological activities (2,4). The parent drug loxapine is subjected to several competing routes of metabolism before and after systemic absorption, including ring hydroxylation and *N*-desmethylation (Fig. 1). Therefore, a relatively high degree of both within-subject and between-subject variability is observed when loxapine is administered orally (3,33). IM formulation of loxapine is designed for the rapid management of acute psychotic patients and agitation (34). Unfortunately, clinical studies (35) found that the plasma levels of loxapine achieved by IM loxapine were only slightly higher that achieved by oral loxapine within the initial four hours of loxapine administration. Furthermore, both *in vivo* and *in vitro* studies suggest that the commercial preparation of IM loxapine (Loxitane®) is myotoxic (36) and could lead to skeletal muscle damage and/or pain at injection site. These observations indicate that the PK and PD advantages offered by IM loxapine are indeed minimal. Therefore, it is worth developing a new and safe formulation of loxapine that enables rapid delivery of loxapine.

As suggested by a recent systematic review (37), loxapine is a treatment option in acute situations where rapid tranquillization is needed, and is effective in the management of agitation and aggression of different origins (e.g. (38–41)). In addition to its tranquilizing efficacy in psychiatric conditions, loxapine and its metabolite amoxapine also possess analgesic effects in humans and animals (42–46). Noteworthy is that a low-dose loxapine has been found to be effective for the management of acute migraine in a Phase 2 trial (42). Coincidentally, one of the direct nose-to-CNS pathways is the trigeminal pathway which involves the delivery of the intranasally applied compound to the caudal brain (e.g. medulla and pons) via the trigeminal nerve (47) and, at the same time, the activation of the trigeminovascular system is heavily involved in the genesis of migraine (48). In the present study, the level of loxapine in trigeminal nerve was high immediately after intranasal loxapine administration (Fig. 3). A previous study in rat also found that the intranasally applied lidocaine, a local anesthetic, could target the trigeminal nerve and the connected orofacial structures (49). Therefore, intranasal loxapine might provide an additional benefit of targeting the trigeminal system and might have dual potentials for the treatments of both psychiatric disorders and pain conditions such as headache, which warrants further development. *In vivo* studies in animal disease models including schizophrenia models and migraine models are being undertaken to establish the therapeutic effects of intranasal loxapine.

CONCLUSION

The present study explores the roles of metabolism in the PK and PD of intranasal loxapine. Our studies provide concrete evidence that 7-OH-loxapine, but not the parent loxapine, is the main contributor to the catalepsy observed in rats after intranasal and oral loxapine administrations, and this metabolite is probably the culprit of extrapyramidal side effects observed in patients receiving loxapine. The presence of loxapine might partially reduce the cataleptic effect induced by 7-OH-loxapine, but further mechanistic studies are required to verify this. Compared with the conventional oral route, the nasal route could efficiently deliver loxapine to brain with lower 7-OH-loxapine to loxapine ratio. PK and PD data suggest that the nasal route is a promising alternative for delivering loxapine, and the potential of developing loxapine into intranasal medication warrants further investigation.

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