Enantiomeric and Monoaminergic Contributions to Methamphetamine's Bidirectional Effects on Fentanyl-Depressed Respiration in Mice

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ABBREVIATIONS:

fentanyl (FENT); saline (SAL); minute volume (MVb); Whole-Body Plethysmography (WBP); opioid-induced respiratory depression (OIRD).

ABSTRACT:

Rationale: Fentanyl remains the primary cause of fatal overdoses, and its co-use with methamphetamine (METH) is a growing concern. We previously demonstrated that racemic METH can either enhance or mitigate opioid-induced respiratory depression (OIRD) dependent upon whether a low or high dose is administered. The optical isomers of METH, dextromethamphetamine (*d*-METH) and levomethamphetamine (*l*-METH), differ substantially in dose expression and thus may differentially contribute to the bidirectional effects of the racemate. Furthermore, it is unknown which of METH's monoamine (MA) receptor mechanisms mediate these respiratory effects. Thus, systematic evaluation of monoamine receptor selective agents may identify treatment targets for OIRD.

Methods: The two optical isomers of METH, *d*-METH and *l*-METH, were tested in adult male mice to determine their effects on basal and fentanyl-depressed minute volume (MVb; i.e., respiratory frequency x tidal volume) using whole-body plethysmography. Next, six selective agonists at MA receptors involved in METH's activity [phenylephrine (PNE; α_1), clonidine (CLON; α_2), SKF-82958 (SKF; D_1), quinpirole (QPR; D_2), 8-OH-DPAT (8-OH; $5HT_{1A}$), and DOI ($5HT_2$)] were singly tested on MVb, and then if stimulatory, in combination with fentanyl.

Results: *d*-METH elevated MVb and *l*-METH decreased MVb. Under fentanyl-depressed conditions, the bidirectional effects of racemic METH were recreated by *d*-METH while *l*-METH significantly exacerbated OIRD at 1.0 and 3.0 mg/kg. MVb was dose-dependently increased by PNE and SKF and decreased by CLON and QPR. Neither 8-OH nor DOI altered basal MVb. Under fentanyl-depressed conditions, SKF transiently elevated MVb, while PNE more persistently increased it. Interestingly, DOI transiently increased depressed MVb, while 8-OH decreased MVb further.

Conclusions: *d*-METH and *l*-METH differentially contribute to the bidirectional respiratory modulation observed with the racemate. Selective activation of MA receptors alters basal respiration and OIRD, with D_1 and α_1 receptors representing potential targets as respiratory stimulants, whereas α_2, D_2, and $5HT_{1A}$ receptors may mediate the exacerbation of OIRD by METH.

1. Introduction

The co-use of fentanyl with stimulants, particularly methamphetamine (METH), has signaled a new emerging fourth phase of the overdose epidemic (Friedman and Shover, 2023). From 2013 to 2019, deaths involving stimulants increased 317% (from 1.2 to 5.0 per 100,000), second only to synthetic opioids over the same period. Notably, overdose due to stimulant and synthetic opioid co-use showed the largest relative increase compared to stimulants, prescription opioids, or heroin alone (Cano and Huang, 2021; Mattson et al., 2021). These observations underscore the need to evaluate how this polydrug abuse affects life-sustaining drug-affected physiological processes such as respiration.

Evidence published in the scientific literature by our laboratory and others (Cruickshank and Dyer, 2009; Elder et al., 2023a; Hassan et al., 2016; Mendelson et al., 2006; Richards et al., 1995) demonstrates that amphetamine-type stimulants such as methamphetamine (METH) affect respiration primarily by increasing ventilatory frequency. Conversely, previously published studies from our laboratory in mice showed that METH's effects on respiration are not entirely stimulatory, exemplified by the presence of mild, yet significant depressant effects on uncompromised "basal" respiration, which are apparent at lower doses than those that produce stimulation (Elder et al., 2023a). A similar pattern was observed when combined with fentanyl, whereby low doses of METH exacerbated opioid-induced respiratory depression (OIRD), but a

high dose reversed OIRD (Elder et al., 2023a). These bidirectional effects of METH on respiratory parameters should be of particular relevance to toxicity caused by nonmedical use because they are induced by doses that would be expected to produce plasma levels similar to those achieved in humans (Mendelson et al., 2006; Ortman et al., 2021; Rauhut and Bialecki, 2011). Both the respiratory stimulant and depressant effects of METH have potential consequences for treating polydrug toxicity and OIRD in that pro-depressant effects may complicate resuscitation following opioid overdose, and stimulatory effects may be exploited for the development of opioid receptor-independent analeptics.

Ample scientific evidence exists detailing the substantial differences in pharmacology that exist between METH's two optical isomers (enantiomers), dextromethamphetamine (*d*-METH) and levomethamphetamine (*l*-METH). Specifically, METH's enantiomers differ greatly in their overall potency, selectivity for releasing primary monoamines, and pharmacokinetic parameters, with *d*-METH exhibiting substantially greater overall potency for monoamine release, selectivity for dopamine (DA) release, and relative effects on serotonin (5-Hydroxytryptamine or 5-HT) compared to *l*-METH, which acts primarily as a selective releaser of norepinephrine (NE) (Kuczenski et al., 1995; Rothman et al., 2001; Rothman and Baumann, 2003). These differences in pharmacology can be seen in Table 1 which includes the inhibitory constants (K_i) and EC_{50} values that represent the potency of the enantiomers of METH to competitively inhibit DAT, NET, and SERT, and to induce monoamine efflux *in vitro*, respectively. The large differences in pharmacology and potency between the two enantiomers lead to marked differences in physiological, subjective, and behavioral effects in both humans and animals that could be hypothesized to extend to respiratory modulation (Mendelson et al., 2006; Nishimura et al., 2017). Evaluating the respiratory effects of METH's individual

enantiomers not only provides a basis for understanding the monoaminergic determinants of respiratory modulation, but is also important for translational validity, as the vast majority of illicit METH consumed globally is in the form of *d*-METH hydrochloride (HCl), while other illicit amphetamines such as MDMA and amphetamine are primarily racemic (Cunningham et al., 2013; Losacker et al., 2021; Wang et al., 2015). Based on the existing evidence, it can be hypothesized that the administration of enantiopure preparations of the two individual isomers would show a separation of the bidirectional effects produced by the racemate into stimulant and depressant effects based on their relative potency to release DA, NE, and 5-HT.

While the findings from experiments with amphetamine-type stimulants demonstrated potentially useful respiratory stimulant effects for the reversal of OIRD, such stimulants are limited in their clinical utility for several reasons. Specifically, amphetamines are themselves drugs of abuse that are highly addictive, cause neurotoxicity, produce respiratory stimulation at potentially unsafe doses, and can result in toxic interactions in combination with mu opioid receptor (MOR) agonists (Ashok et al., 2017; Mark et al., 2004; Volkow et al., 2001). Extrapolating from METH's primary mechanism of action as an indirect agonist of monoamine receptors, it is likely that selective activation of individual monoamine receptor targets differentially mediate its bidirectional effects on respiration. Therefore, it can be hypothesized that if individual DA, NE, and 5-HT receptor subtypes differentially contribute to the stimulant or depressant effects of METH on respiration, selective activation of those receptors would be expected to produce the stimulant or depressant effects.

There were two objectives of the present study. First, we wanted to evaluate whether METH's enantiomers differentially contribute to racemic METH's previously observed bidirectional effects on basal and fentanyl-depressed respiration using whole-body

plethysmography (WBP) methodology utilized in earlier studies by our lab, including the aforementioned experiments with racemic METH and fentanyl (Elder et al., 2023a, 2023b). Should the enantiomers exhibit differential modulation of respiratory parameters, it may provide insight into the physiological targets that mediate the stimulant vs depressant components of the racemate and allow their separation. Secondly, we wanted to assess monoamine receptor selective agonists to determine whether they altered basal respiration. Agonists that effectively elevated basal MVb or were devoid of significant depressant effects were consequentially evaluated for their effects on depressed MVb in subjects pretreated with fentanyl. The results from these studies would provide insight into which mechanism(s) of METH's pharmacology might be involved in toxic vs. potentially therapeutic respiratory outcomes.

	***d*-Methamphetamine**		***l*-Methamphetamine**	
Target	**K_i (nM)**	**EC_{50} (nM)**	**K_i (nM)**	**EC_{50} (nM)**
DAT	114	24.5	4840	416
NET	48	12.3	234	28.5
SERT	2,137	736	14,000	4,640
DAT/NET	2.38	1.99	20.68	14.59
SERT/NET	44.52	59.84	59.83	162.8

Table 1: Pharmacodynamic Profiles of *d*- and *l*-Methamphetamine for Monoamine Release and Reuptake Inhibition *in vitro*. Values given on the left for each enantiomer correspond to reuptake inhibition potency as measured by the inhibition constant (K_i) for each transporter as a concentration in nanomolar (nM). Righthand values for each enantiomer correspond to the EC_{50} values for monoamine release in nanomolar (nM). Values for DAT/NET and SERT/NET rows represent the selectivity of each individual enantiomer for reuptake inhibition or release as a ratio of the identified receptor affinities and EC_{50}'s, respectively. Data adapted from (Rothman and Baumann, 2003).

Phenylephrine		Clonidine		SKF-82958		Quinpirole		8-OH-DPAT		DOI	
Target Receptor	Affinity K_i (nM)	Target Receptor	Affinity K_i (nM)	Target Receptor	Affinity K_i (nM)	Target Receptor	Affinity K_i (nM)	Target Receptor	Affinity K_i (nM)	Target Receptor	Affinity K_i (nM)
α_1	**100 - 370**	α_1	501	**D_1**	**4.56**	D_1	1,000	**$5HT_{1A}$**	**0.65**	$5HT_{1A}$	2,355
α_2	1253 - 1467	**α_2**	**27 - 41**	D_2	264	**D_2**	**47 – 204**	$5HT_7$	39-251	**$5HT_{2A}$**	**0.79 – 14.5**
				D_3	n.d.	**D_3**	**24.35**			**$5HT_{2B}$**	**26.84**
				D_4	n.d.	**D_4**	**52.7**			**$5HT_{2C}$**	**3.01 - 60**

Table 2: Receptor Selectivity and Binding Profiles of Selected Monoamine Agonists. Values given are inhibitory constants (K_i) with units of nanomolar (nM) derived from ligand displacement studies with selected agonists at related receptors. Receptor targets and Ki values in bold correspond to the target of interest. Receptor-agonist pairings for which no reliable data was available are indicated by "n.d.". Data adapted from the following studies (Andersen et al., 1985; Boess and Martin, 1994; Borsini et al., 1995; Boundy et al., 1993; Boyajian and Leslie, 1987; Campiani et al., 1998; Egan et al., 1998; Lawler et al., 1999; Lovenberg et al., 1993; Nelson et al., 1999; Neumeyer et al., 2003; Sokoloff et al., 1990; Sprouse et al., 2004; Van Tol et al., 1991).

2. Materials and Methods

2.1. Materials

d-Methamphetamine hydrochloride [(2*S*)-*N*-methyl-1-phenylpropan-2-amine)] was provided by the National Institute on Drug Abuse (Bethesda, MD, USA) Drug Supply Program. *l*-Methamphetamine hydrochloride (Catalogue # 13998; (2*R*)-*N*-methyl-1-phenylpropan-2-amine; Cayman Chemical, Ann Arbor, MI, USA), Fentanyl citrate (#F3886; *N*-phenyl-*N*-[1-(2-phenylethyl)piperidin-4-yl]propenamide; Sigma-Aldrich, Inc., St. Louis, MO, USA), phenylephrine (#P6126; 3-[(1*R*)-1-hydroxy-2-(methylamino)ethyl]phenol; Sigma-Aldrich, Inc.), clonidine (Catalogue #1140407; N-(2,6-dichlorophenyl)-4,5-dihydro-1H-imidazol-2-amine; U.S. Pharmacopeia (USP, North Bethesda, MD, USA), SKF-82958 (#HY-10435A; 9-chloro-5-

phenyl-3-prop-2-enyl-1,2,4,5-tetrahydro-3-benzazepine-7,8-diol; MedChemExpress LLC, Monmouth Junction, NJ, USA), quinpirole (#Q102; (4aR,8aR)-5-propyl-1,4,4a,6,7,8,8a,9-octahydropyrazolo[3,4-g]quinoline; Sigma-Aldrich, Inc.), 8-OH-DPAT (#H8520; 7-(dipropylamino)-5,6,7,8-tetrahydronaphthalen-1-ol; Sigma-Aldrich, Inc.); and DOI (Catalogue #13885; 1-(4-iodo-2,5-dimethoxyphenyl)propan-2-amine; Cayman Chemical), were obtained commercially. All drugs were prepared in saline, sterilized by filtration through 0.2 μm filtration disks, and administered s.c. at a volume of 10 ml/kg body weight.

2.2. Subjects

Adult male mice [Swiss Webster, CFW(SW), Charles River Laboratories International, Raleigh, NC, USA] weighing approximately 25–50 g at the time of testing were housed four subjects per cage in Association for Assessment and Accreditation of Laboratory Animal Care-accredited facilities. Mice had *ad libitum* access to food (Teklad 7012 Rodent Diet; Envigo, Madison, WI, USA) and tap water. Vivaria were maintained at 22°C ± 2°C and 45%–50% humidity, with lights set to a reverse 12-h light/dark cycle (lights off at 10:00). All tests were conducted on weekdays during the dark period between 11:00 and 17:00 to ensure mice were active (i.e., not asleep). All subjects were acclimated to the vivarium for at least one week before the commencement of studies and were experimentally and drug-naive before testing. Subjects were tested once and were not used for any subsequent tests to preclude drug or testing history effects. All procedures were carried out in accordance with the National Research Council's Guide for Care and Use of Laboratory Animals (2011). This experimental protocol was approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University.

2.3. Apparatus

Mice were tested using whole-body plethysmograph devices (FinePointe WBP Chamber with Halcyon Technology, Data Sciences International, St. Paul, MN, USA) while unrestrained and allowed free movement in individual isolated experimental vessels. Experimental vessels (0.5 L volume with adjustable 0.5L/min room air bias flow) were housed in a laboratory illuminated by custom 660 nM-emitting T8-style ceiling-mounted light tubes each with 96, 0.2-watt Epistar 2835 SMD LEDs (Shenzhen Benwei Electronics Co., Ltd., Longhua District, Shenzhen, China). This wavelength is minimally visible to mice (Peirson et al., 2018) which enabled maintenance of subjects in the dark phase of their activity cycle during testing. These testing conditions have been used previously by our lab to accurately measure the respiratory effects of both stimulant and depressant drugs in mice (Elder et al., 2023a, 2023b). For the experiments described here ambient room air was used for bias flow inputs to experimental vessels rather than the gas mixture of 5% CO_2, 21% O_2 with balanced N_2 used in previous studies. Ambient room air was utilized to create normocapnic conditions for all experiments in order to maximize face validity and increase the translational capacity of results. Respiratory rate (Freq), tidal volume (TVb), and minute volume (MVb) were recorded using software (FinePointe Software Research Suite; Data Sciences International).

2.4. Three-phase WBP Protocol

WBP testing for all treatment conditions was carried out using the three-phase protocol described previously (Elder et al., 2023a) for assessing drug effects on basal and opioid-depressed respiration. The present study consisted of two stages, with the first dedicated to evaluating the differential effects of METH isomers on basal- and fentanyl-depressed MVb, and the second involving the systematic evaluation of monoamine agonists for their ability to affect basal and fentanyl-depressed respiration.

In stage one of this study, the two optical isomers (enantiomers) of METH, *d*-METH and *l*-METH, were tested under basal and fentanyl-depressed conditions following a procedure that was identical to those described in (Elder et al., 2023a) for tests with *d*-amphetamine and racemic METH, aside from the change in gas mixture. Both *d*- and *l*-METH were evaluated under both basal and depressed conditions at the same nominal doses as those used for tests of racemic METH (1.0, 3.0, 10 mg/kg) to evaluate whether the effects of either enantiomer differed from those reported originally with the racemate and to determine their individual contributions to its effects. An additional test with a higher dose of *l*-METH (30 mg/kg) was conducted to evaluate potency differences across an extended dose range. For all experiments under basal conditions, saline was administered prior to Phase II (basal conditions), followed by a dose of either *d*- or *l*-METH as the 'test compound' prior to the start of Phase III. Control groups received three saline injections (vehicle), with one injection administered prior to the initiation of Phases I, II, and III, respectively. Experiments under depressed conditions consisted of treatment with fentanyl (0.3 mg/kg s.c.) administered prior to Phase II. This dose of fentanyl was selected because it consistently produces MVb depression of approximately 50% from baseline at the beginning of Phase III. Additionally, 0.3 mg/kg fentanyl has been employed consistently across studies in our laboratory as the standard dose for tests on fentanyl-depressed conditions because it reproduces fentanyl's bidirectional effects on depressed MVb.

Experiments with monoamine agonists in stage two consisted of an initial evaluation in which six agonists selective at different monoamine receptors involved in METH's activity (Bolme et al., 1974; Corcoran et al., 2014; Desai et al., 2005; Eilam and Szechtman, 1989; Guenther et al., 2009; Jaster et al., 2022; Stone et al., 2014; Zarrindast et al., 2002) were evaluated using the three-phase protocol. The six monoamine agonists chosen to selectively

activate monoamine receptors of interest were phenylephrine (PNE; α_1), clonidine (CLON; α_2), SKF-82958 (SKF; D_1), quinpirole (QPR; D_2), 8-OH-DPAT (8-OH; $5HT_{1A}$), and DOI ($5HT_2$). The affinities of each agonist at their respective receptor targets are given in Table 2. For all tests during an initial evaluation, saline was administered prior to Phase II (basal conditions), followed by a dose of a monoamine agonist as the 'test compound' prior to the start of Phase III. Control groups received three saline injections (vehicle), with one injection administered prior to the initiation of Phases I, II, and III, respectively. Phenylephrine (0.3, 1.0, 10 mg/kg s.c.), clonidine (0.03, 0.1, 1.0 mg/kg s.c.), SKF-82958 (0.1, 0.3, 1.0 mg/kg s.c.), quinpirole (0.3, 1.0, 3.0 mg/kg s.c.), 8-OH-DPAT (0.01, 0.1, 0.3 mg/kg s.c.), and DOI (0.1, 1.0, 3.0 mg/kg s.c.) were screened under basal conditions to determine their ability to elevate eupneic MVb. Results from initial tests of basal respiration were used to identify the maximally effective dose for stimulating MVb if MVb elevation occurred. In the second evaluation stage, doses of each monoamine agonist that produced the greatest elevation of MVb under basal conditions or the highest two doses of inactive compounds were selected for subsequent testing under fentanyl-depressed conditions for their ability to modulate MVb depression. Depressed conditions in this stage consisted of treatment with fentanyl (0.3 mg/kg s.c.) administered prior to Phase II. Additional doses of monoamine agonists were evaluated under fentanyl-depressed conditions if the initial dose produced a significant reversal of depressed MVb in order to determine the dose-responsiveness of MVb elevation. Compounds that decreased basal respiration were excluded from subsequent tests under fentanyl-depressed conditions.

2.5. Statistical Analysis

The primary dependent measure, normalized MVb, was expressed as a percentage of baseline MVb collected during Phase I. Normalized group MVb data from time-course tests were

analyzed using the methodology that has been described in previous publications for determining the effect of treatment conditions (Elder et al., 2023a). Complete reversal of fentanyl respiratory depression was defined as occurring when treatment groups did not differ significantly from vehicle control at a respective time point(s) during Phase III. Partial reversal was defined as an increase in the MVb of a treatment group following the initiation of Phase III but that remained significantly lower than that of vehicle controls. Significant respiratory stimulant effects were considered to occur when a treatment group had MVb values that were significantly greater than fentanyl-treated controls at any time point in Phase III, regardless of level of reversal. Raw MVb values (ml/min) from Phase I were analyzed using one-way ANOVAs to determine if between-group differences existed at baseline, followed by a Holm-Šídák multiple comparisons test comparing all treatment groups if a significant group effect was detected. Area under the curve (AUC) calculations were conducted to summarize the overall influence of treatment on normalized MVb over the entirety of Phase III after agonist administration (t = 20 – 80). AUC data were analyzed via one-way ANOVA, and significant treatment effects were followed by Holm-Šídák multiple comparisons tests to detect differences between individual treatment groups and vehicle or fentanyl-treated controls. All analyses were performed using software (GraphPad Prism 9 for Macintosh; GraphPad Software, San Diego, CA, USA) and statistical significance for all analyses was set at a level of $\alpha = 0.05$.

3. Results

3.1. Differential effects of methamphetamine enantiomers on basal and depressed respiration

The dose-dependent effects of *d*-METH (1.0, 3.0, 10 mg/kg) on basal MVb in subjects who received saline prior to Phase II are shown in Figure 1A. Administration of *d*-METH significantly affected MVb [$F(48, 444) = 10.10$; $p < 0.0001$], producing dose-dependent

elevations of MVb that were significantly ($p<0.05$) greater than saline controls at one or more time points post-administration for all doses tested. All doses of *d*-METH significantly increased MVb compared to saline controls within 10 min of administration, after which MVb in subjects who received intermediate (3.0 mg/kg) and high (10 mg/kg) doses continued to increase, eventually reaching peak values at 60 min post-administration of 144.9% ($p = 0.0020$) and 227.5% ($p = 0.0029$) of baseline, respectively. The results of experiments with *l*-METH (Figure 1B) indicated that treatment significantly affected basal respiration [$F(64, 556) = 7.580$; $p < 0.0001$]. Administration of an intermediate (3.0 mg/kg) dose of *l*-METH under basal conditions significantly decreased MVb to 49.91% of baseline ($t = 30$, $p = 0.0101$) within 10 min post-administration, and MVb values remained significantly depressed relative to saline-treated controls for 20 min until they no longer differed from controls at 35 min post-administration ($t = 55$). At the lowest dose (1.0 mg/kg) of *l*-METH basal MVb was similarly depressed within 15 min of administration to 43.33% of baseline ($t = 35$; $p = 0.0080$) which was significantly lower than controls. Interestingly, administration of a higher dose (10 mg/kg) of *l*-METH did not significantly alter MVb at any time point after administration, neither stimulating nor depressing basal respiration. Therefore, a higher dose, 30 mg/kg, was subsequently tested and produced significant stimulation of a magnitude similar to 3.0 mg/kg *d*-METH but with a more protracted onset, as seen in the 25 min latency to exert significant effects. The results obtained from this follow-up test displayed the dose-dependent transition from depressant effects at low doses (1.0, 3.0 mg/kg) to respiratory stimulant effects at high doses (30 mg/kg) that were similar to the effects of low-moderate doses of *d*-METH.

The effects of *d*-METH on respiration that was depressed by the administration of fentanyl (0.3 mg/kg) are shown in Figure 1C. In fentanyl-pretreated mice, there was a significant

effect of *d*-METH over time [$F(64, 560) = 9.741$; $p < 0.0001$] on respiration. In contrast to the dose-dependent stimulation of MVb observed with *d*-METH under basal conditions, administration of *d*-METH to subjects that were pretreated with fentanyl (0.3 mg/kg) produced bidirectional, dose-dependent effects following a similar pattern as was observed with racemic METH. Specifically, *d*-METH at the lowest dose tested (1.0 mg/kg) had pro-depressant effects, the highest dose (10 mg/kg) had pronounced stimulating effects, and the intermediate dose (3.0 mg/kg) had no significant effect on fentanyl-depressed MVb. The pro-depressant effects of 1.0 mg/kg *d*-METH were characterized by a 30-min increase in the duration of significant depression ($t = 35 – 60$) along with MVb values that were significantly lower than fentanyl-treated controls from 25 – 50 min ($t = 45 – 70$) post-administration. The respiratory stimulant properties of 10 mg/kg *d*-METH became apparent at 15 min post-administration ($t = 35$) when MVb values rose slightly above saline-treated controls (74.82 vs 72.74% of baseline, respectively), constituting a complete reversal of fentanyl-induced depression. Subsequently, MVb values continued to rise throughout the remainder of Phase III in subjects who received 10 mg/kg *d*-METH, finally reaching a peak of 153.3% of baseline at the final observation point ($t = 80$), however this increase did not reach statistical significance compared to saline-treated controls.

The effects of *l*-METH (1.0, 3.0, 10 mg/kg) on fentanyl-depressed respiration are shown in Figure 1D. Analysis of MVb data showed a significant effect of *l*-METH treatment over time [$F(64, 560) = 4.534$; $p < 0.0001$] on fentanyl-depressed respiration. The effects of *l*-METH administration under depressed conditions were consistent with results observed under basal conditions. Specifically, all doses of *l*-METH had pro-depressant effects on MVb that varied according to an inverted-U-shaped dose-response and were greatest after administration of the

intermediate 3.0 mg/kg dose. The pro-depressant effects of *l*-METH on fentanyl-depressed respiration were characterized by increased duration and magnitude of MVb depression beginning 10 – 15 min after administration. MVb in subjects who received 3.0 mg/kg *l*-METH 20 min after 0.3 mg/kg fentanyl varied between 31.81 – 39.15% of baseline after onset (t = 30 – 80) and remained significantly depressed compared to saline controls until the penultimate time point in phase III (t = 75). The highest dose of 10 mg/kg *l*-METH had the least pro-depressant effect on MVb that were characterized by nonsignificant reductions of 8 – 15% of baseline after onset (t = 30 min) compared with fentanyl-treated controls at all time points except for one in which MVb depression was significant (t = 35, p = 0.0259).

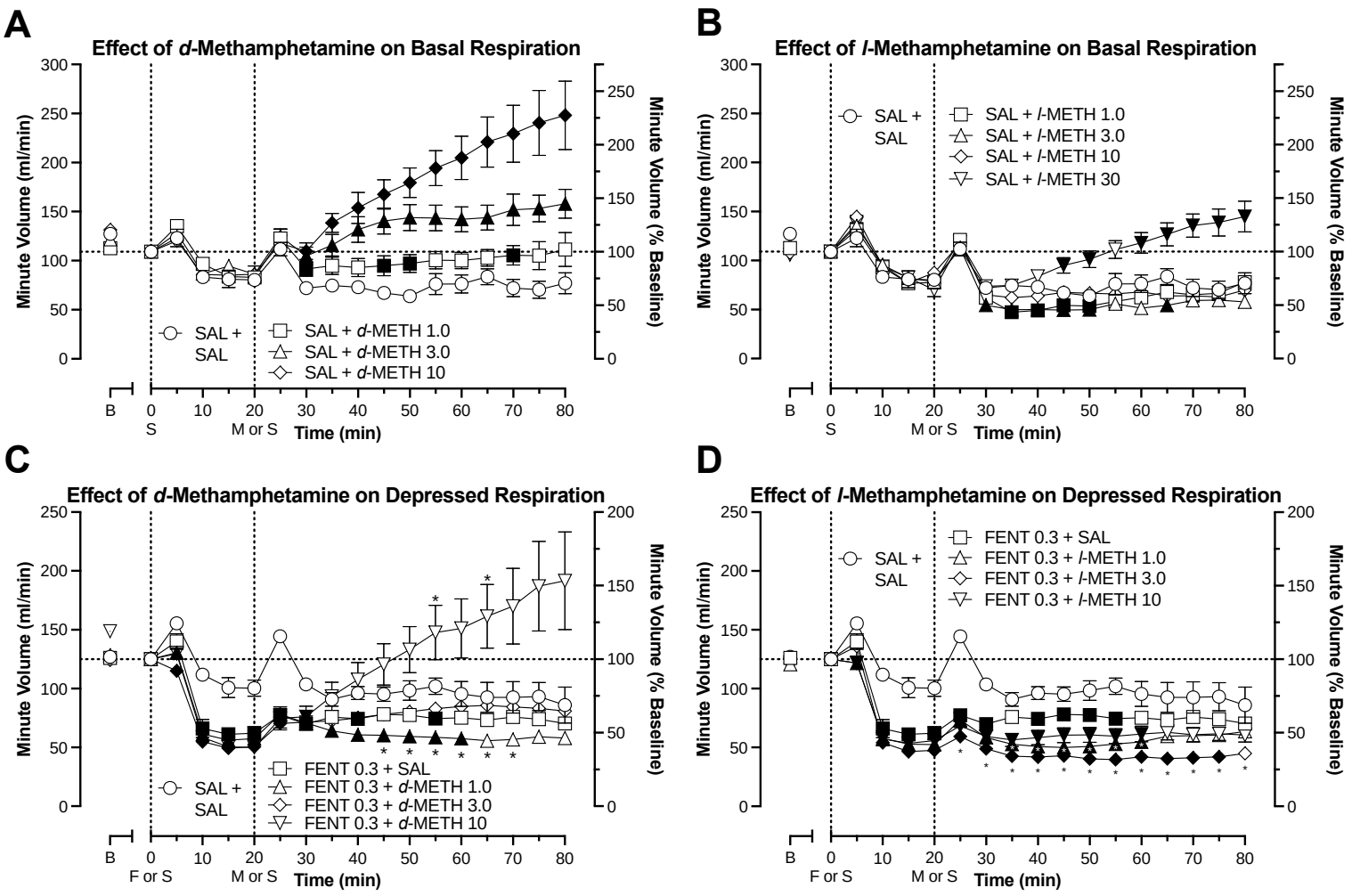


Figure 1: Effect of *d*- and *l*-methamphetamine on basal and fentanyl-depressed minute volume. A) Dose- and time-effects of *d*-methamphetamine (*d*-METH) and B) *l*-methamphetamine (*l*-METH) on basal minute volume following saline (SAL) pretreatment. C) Dose- and time-effects of *d*-METH and D) *l*-METH on depressed minute volume following pretreatment with 0.3 mg/kg fentanyl (FENT). Left ordinate: mean raw MVb (ml/min) indexing values of symbols only during baseline (B) of Phase I. Right ordinate: normalized (percent baseline) MVb indexing values of symbols during the 80-min test session following Phase I baseline. These symbols indicate mean MVb expressed as a percentage of baseline MVb of 8 mice per treatment group. Filled symbols indicate a significant ($p \leq 0.05$) difference from SAL +

SAL treated controls at individual time points. Additional * symbols above (FENT 0.3 + *d*-METH 10), below (FENT 0.3 + *d*-METH 1.0; FENT 0.3 + *l*-METH 3.0) or within (FENT 0.3 + *l*-METH 1.0) specific time points indicate a significant difference at that time point between individual treatment groups and FENT 0.3 + SAL controls of $p \leq 0.05$ according to Holm-Šídák post-hoc comparisons. Abscissa labels: M = *d*- or *l*-METH injection, S = saline injection, F = fentanyl injection. N = 8 per group. No significant differences were detected at baseline across experimental conditions when raw MVb values were compared via one-way ANOVA for experiments presented in panel A) [$F(3, 28) = 1.294$; $p = 0.2958$], panel C) [$F(4, 35) = 1.584$; $p = 0.2002$], or panel D) [$F(4, 35) = 0.2669$; $p = 0.8973$]. Significant differences in raw MVb means were detected at baseline for experimental conditions presented in panel B when compared via one-way ANOVA [$F(4, 35) = 2.697$; $p = 0.0465$], but no significant differences were detected between individual groups by subsequent Holm-Šídák multiple comparisons tests.

3.2. Testing monoamine receptor agonists for their effects on respiration

The results from basal experiments with the selective α_1-receptor agonist phenylephrine and the selective α_2-receptor agonist clonidine are shown in Figure 2A and 2B, respectively. Phenylephrine treatment significantly affected basal MVb over time [$F(48, 448) = 1.847$; $p = 0.0008$] that varied as a function of dose according to an inverted-U-shaped relationship. Administration of low (0.3 mg/kg) and intermediate (1.0 mg/kg) doses of phenylephrine stimulated respiration elevating MVb values above those of controls by 15 min post-administration. However, increases in MVb induced by phenylephrine were small in magnitude and only significantly differed from controls at a single time point ($t = 40$) after administration of 0.3 mg/kg ($p = 0.0043$) and 1.0 mg/kg ($p = 0.0037$). At the highest dose of 10 mg/kg, phenylephrine's effects on MVb depressed MVb values throughout phase III, although MVb

depression was only significant at a single time point (t = 25; p = 0.0353) compared with controls.

Under basal conditions, clonidine treatment significantly [F(48, 448) = 5.550; p < 0.0001] affected MVb over time, producing potent and long-lasting depressant effects at all doses tested (0.03, 0.1, 1.0 mg/kg). The depression of basal MVb following administration of clonidine was rapid and sustained, with decreased onset latency and increased duration of depression as the dose increased. Both intermediate (0.1 mg/kg) and high (1.0 mg/kg) doses of clonidine significantly depressed MVb relative to controls within 5 min and remained significantly depressed thereafter for the entirety of phase III. The magnitude of MVb depression by clonidine was similarly dose-dependent, with maximum depression of 48.3%, 32.1%, and 25.3% of baseline occurring after administration of 0.03, 0.1, and 1.0 mg/kg, respectively.

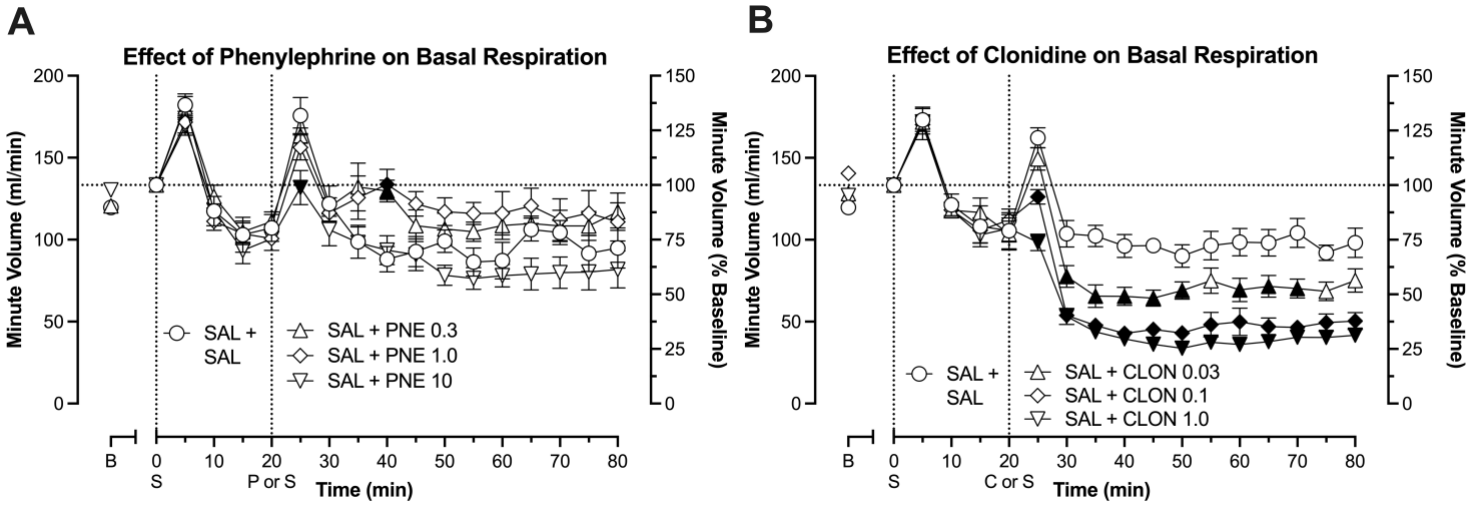


Figure 2: Effects of selective α_1 and α_2 adrenergic receptor agonists on basal respiration. Panel A) Dose- and time-effects of phenylephrine (PNE) and B) clonidine (CLON) on basal minute volume following saline (SAL) pretreatment. Filled symbols indicate a significant ($p \leq 0.05$) difference from SAL + SAL treated controls at individual time points. Abscissa labels: P =

phenylephrine injection, C = clonidine injection, S = saline injection. N = 8 per group. No significant differences were detected at baseline across experimental conditions when raw MVb values were compared via one-way ANOVA for groups in panel A) [$F(3, 28) = 0.7559$; $p = 0.5283$] or B) [$F(3, 28) = 1.325$; $p = 0.2859$]. All other details are the same as in Figure 1.

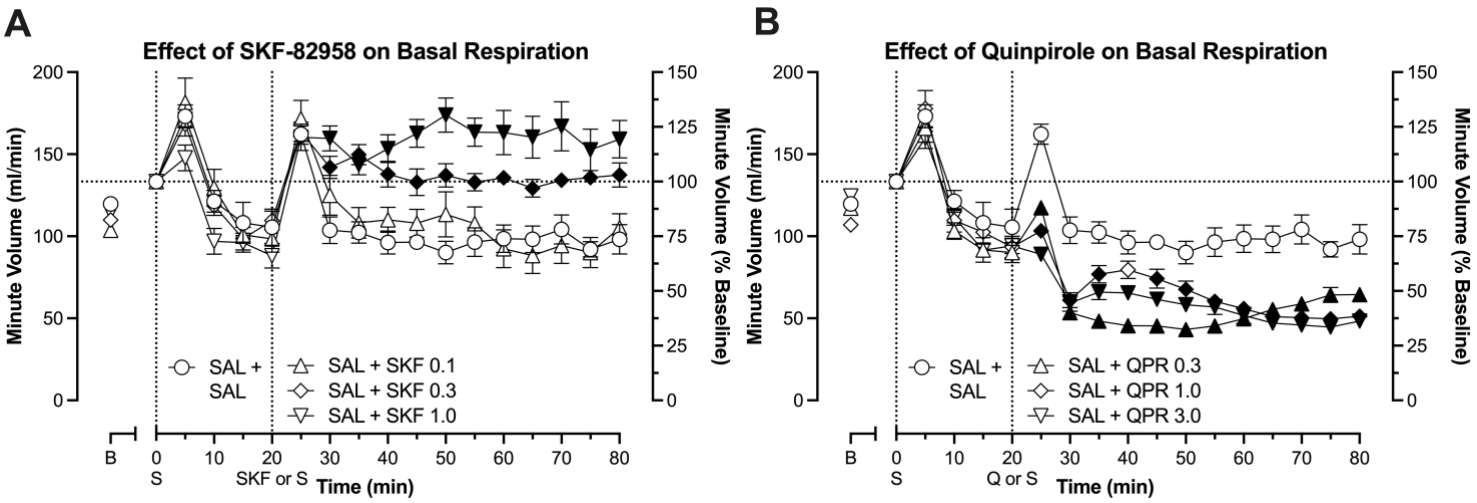
The results from tests under basal conditions with the selective D_1-receptor agonist SKF-82958 and the selective D_2-receptor agonist quinpirole are shown in Figures 3A and 3B, respectively. SKF-82958 treatment had significant effects on basal MVb over time [$F(48, 448) = 8.112$; $p < 0.0001$] that were dose-dependent. Administration of intermediate (0.3 mg/kg) and high (1.0 mg/kg) doses of SKF-82958 significantly stimulated respiration, elevating MVb values above those of controls within 10 min post-administration, after which they remained significantly elevated for the rest of the session. The magnitude of MVb elevation by intermediate and high doses of SKF was dose-dependent, achieving maximum MVb values of 122.8% and 130.4% of baseline after onset ($t \geq 25$), respectively. At the lowest dose of 0.1 mg/kg, SKF-82958's effects on MVb were nonsignificant compared with controls, and produced only modest increases (< 10%) in MVb throughout phase III.

Under basal conditions, quinpirole treatment significantly [$F(48, 448) = 5.861$; $p < 0.0001$] affected MVb over time, producing complex and sustained depressant effects at all doses tested (0.03, 1.0, 3.0 mg/kg). The depression of basal MVb following administration of quinpirole was rapid and significant at all doses. Quinpirole's effects on MVb were characterized by a substantial initial decrease immediately after administration, followed by effect profiles that varied with dose. Both intermediate (1.0 mg/kg) and high (3.0 mg/kg) doses of quinpirole displayed a complex modulation of respiration over the course of phase III characterized by a period of rapid recovery and then gradual reduction in MVb. In comparison, the lowest dose (0.3

mg/kg) displayed a more typical pattern of depression and gradual recovery. Maximum depression to 32.42%, 37.28%, and 33.47% of baseline occurred after administration of 0.3, 1.0, and 3.0 mg/kg, respectively. Time to peak depression differed between the lowest (30 min) and higher two doses (55 min).

Figure 3: Effects of selective dopamine D_1 and D_2 receptor agonists on basal respiration.

Panel A) Dose- and time-effects of SKF-82958 (SKF) and B) quinpirole (QPR) on basal minute



volume following saline (SAL) pretreatment. Filled symbols indicate a significant ($p \leq 0.05$) difference from SAL + SAL treated controls at individual time points. Abscissa labels: SKF = SKF-82958 injection, Q = quinpirole injection, S = saline injection. N = 8 per group. No significant differences were detected at baseline across experimental conditions when raw MVb values were compared via one-way ANOVA for groups in panel A) [$F(3, 28) = 1.199$; $p = 0.3281$] or B) [$F(3, 28) = 1.921$; $p = 0.1491$]. All other details are the same as in Figure 1.

The effects of selective $5HT_{1a}$-receptor agonist 8-OH-DPAT and the selective $5HT_2$-receptor agonist DOI are shown in Figure 4A and 4B, respectively. No significant effect of 8-OH-DPAT treatment on basal MVb over time was detected [$F(48, 448) = 1.269$; $p = 0.1147$]. Minor fluctuations in MVb occurred over the course of phase III, with low and high doses of 8-OH-DPAT tending to lower MVb relative to controls, but post-hoc analyses of differences in MVb by time point could not be completed due to the lack of significant interaction of treatment and time. Conversely, a significant interaction of time and treatment [$F(48, 448) = 1.452$; $p = 0.03$] was detected following administration of DOI (0.1, 1.0, 3.0 mg/kg). Treatment with DOI at intermediate (1.0 mg/kg) and high (3.0 mg/kg) doses affected MVb gradually over phase III and peaked towards the end of the session. DOI at 1.0 mg/kg nonsignificantly increased MVb relative to controls between 40- and 60-min post-administration (t = 60 – 80), while a dose of 3.0 mg/kg tended to decrease MVb to a nonsignificant degree beginning at 25 min post-administration (t = 45).

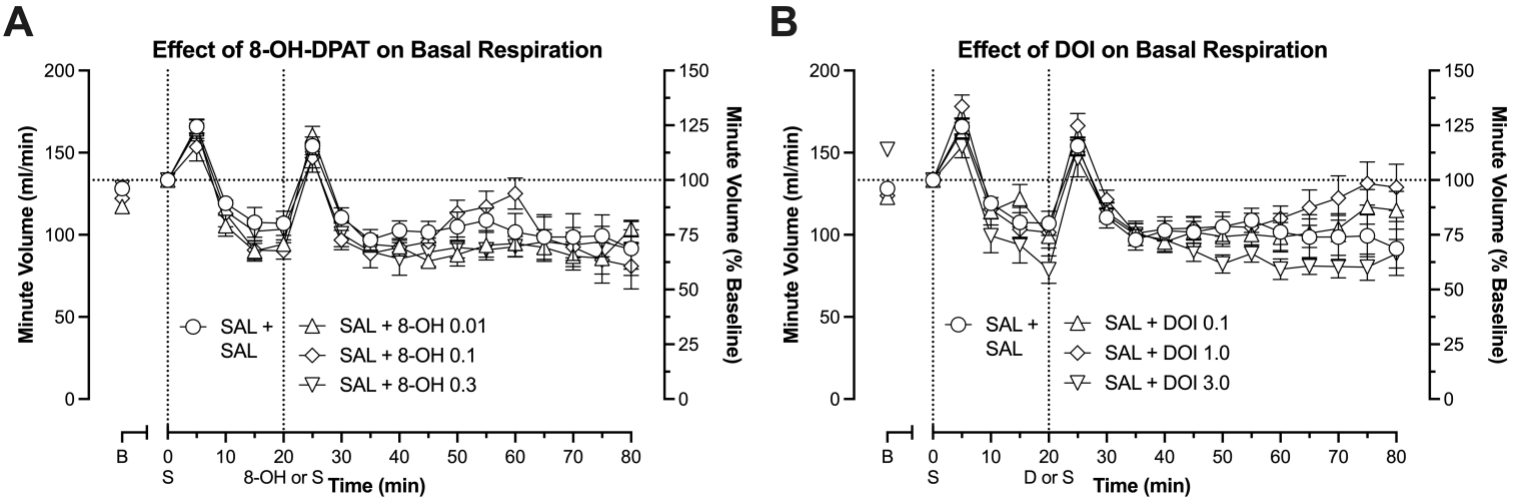
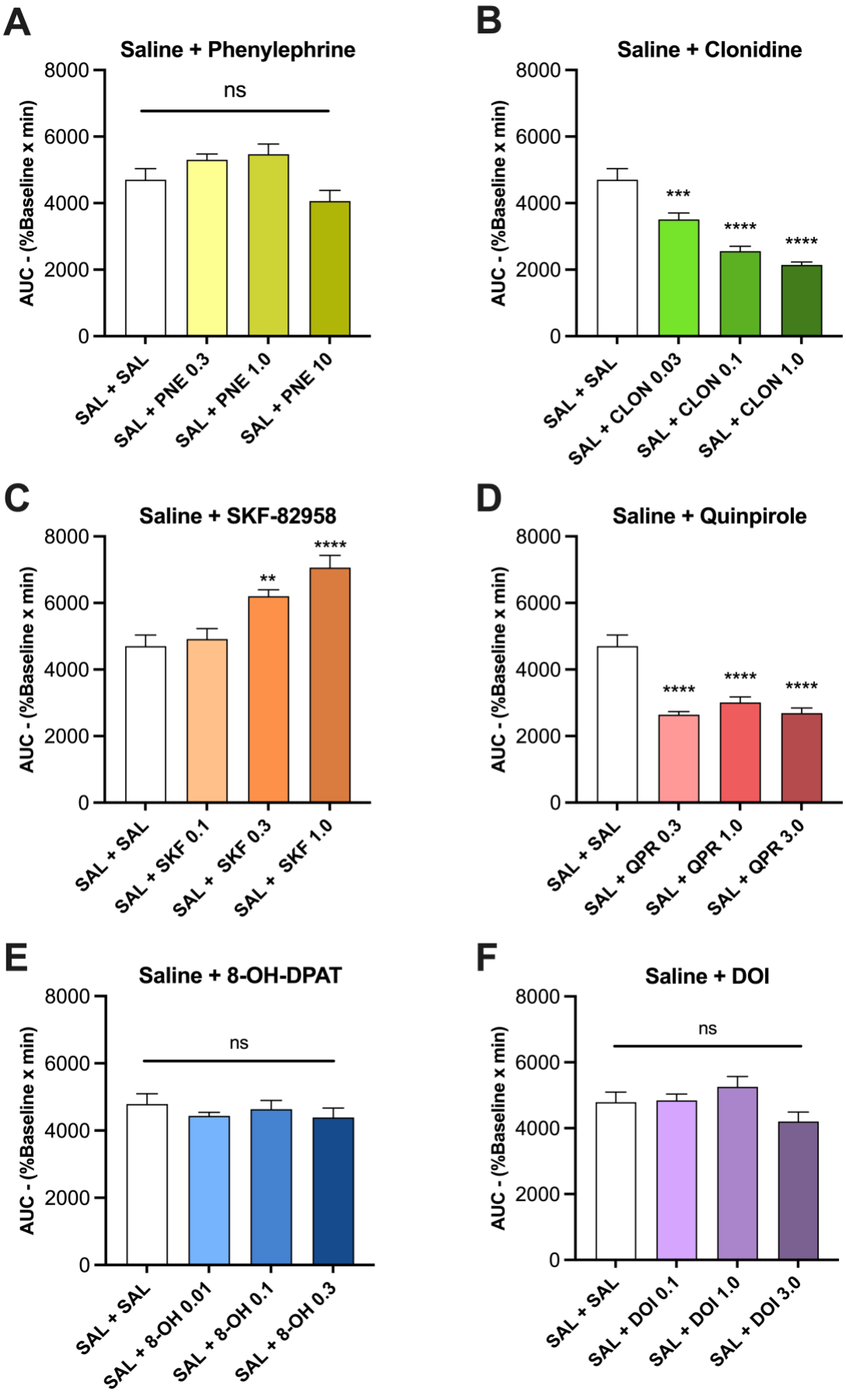


Figure 4: Effects of selective $5HT_{1a}$ and $5HT_2$ serotonin receptor agonists on basal respiration. Panel A) Dose- and time-effects of 8-OH-DPAT (8-OH) and B) DOI (DOI) on basal minute volumefollowing saline (SAL) pretreatment. Filled symbols indicate a significant (p

449 ≤ 0.05) difference from SAL + SAL treated controls at individual time points. Abscissa labels: 8-
450 OH = 8-OH-DPAT injection, D = DOI injection, S = saline injection. N = 8 per group. No
451 significant differences were detected at baseline across experimental conditions when raw MVb
452 values were compared via one-way ANOVA for groups in panel A) [$F(3, 28) = 1.180$; $p = 0.3350$] or B) [$F(3, 28) = 2.694$; $p = 0.0651$]. All other details are the same as in Figure 1.

454 The results of post-hoc analyses of monoamine agonist treatment effects on the basal area
455 under the curve (AUC) of normalized MVb across time throughout phase III are shown in Figure
456 5. AUC analysis of the effect of adrenergic agonist treatments demonstrated that phenylephrine
457 significantly affected MVb AUC [$F(3, 28) = 4.840$; $p = 0.0077$] increasing AUC (non-
458 significantly) relative to saline controls in an inverted-U-shaped dose-response relationship
459 (Figure 5A). Conversely, clonidine had pronounced dose-dependent depressant effects on MVb
460 [$F(3, 28) = 29.23$; $p < 0.0001$] over the course of phase III (Figure 5B) exemplified by significant
461 reductions in AUC relative to controls at all doses ($p \leq 0.0004$). Figures 5C and 5D show that
462 treatment with the selective dopaminergic D_1-like receptor agonist SKF-82958 and the D_2-like
463 receptor agonist quinpirole affected AUC in a subtype-specific manner similar to the adrenergic
464 agonists, whereby SKF-82958 dose-dependently elevated [$F(3, 28) = 13.16$; $p < 0.0001$], and
465 quinpirole decreased [$F(3, 28) = 22.25$; $p < 0.0001$] AUC relative to controls. Maximal effects of
466 SKF-82958 on AUC were seen after treatment with the highest dose (1.0 mg/kg), which
467 significantly increased AUC ($p < 0.0001$) relative to controls. Quinpirole consistently and
468 significantly decreased AUC ($p < 0.0001$) relative to controls. As expected, neither 8-OH-DPAT
469 [$F(3, 28) = 0.5466$, $p = 0.6545$] (Figure 5E) nor DOI [$F(3, 28) = 2.418$, $p = 0.0872$] (Figure 5F)
470 had main effects on AUC nor were any significant changes detected compared to respective
471 saline-treated controls at any dose tested. However, the nonsignificant trends present in basal

time course data were apparent, with DOI tending to increase slightly, and 8-OH-DPAT tending to decrease slightly, basal AUC relative to controls.


A
Saline + Phenylephrine
AUC - (%Baseline x min)
8000
6000
4000
2000
0
ns
SAL + SAL
SAL + PNE 0.3
SAL + PNE 1.0
SAL + PNE 10
B
Saline + Clonidine
AUC - (%Baseline x min)
***
****
****
SAL + SAL
SAL + CLON 0.03
SAL + CLON 0.1
SAL + CLON 1.0
C
Saline + SKF-82958
AUC - (%Baseline x min)
**
****
SAL + SAL
SAL + SKF 0.1
SAL + SKF 0.3
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D
Saline + Quinpirole
AUC - (%Baseline x min)
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****
SAL + SAL
SAL + QPR 0.3
SAL + QPR 1.0
SAL + QPR 3.0
E
Saline + 8-OH-DPAT
AUC - (%Baseline x min)
ns
SAL + SAL
SAL + 8-OH 0.01
SAL + 8-OH 0.1
SAL + 8-OH 0.3
F
Saline + DOI
AUC - (%Baseline x min)
ns
SAL + SAL
SAL + DOI 0.1
SAL + DOI 1.0
SAL + DOI 3.0

Figure 5: Area Under the Curve summary analysis of the effects on Minute Volume during phase III by treatment. Panel A) Dose-effects of phenylephrine (PNE); B) clonidine (CLON); C) SKF-82958 (SKF); D) quinpirole (QPR); E) 8-OH-DPAT (8-OH); and F) DOI on area under the curve (AUC) of normalized minute volume x time in saline (SAL) pretreated subjects during phase III (60 min). Abscissa labels correspond to injections given at t = 0 and t = 20, with saline identified as SAL and numbers corresponding to the dose administered in mg/kg. AUC is given on the ordinate as the product of % baseline x minutes (min). **; ***; **** above bars indicate a significant difference between individual treatment groups and SAL + SAL controls of $p \leq 0.01$; 0.001; 0.0001, respectively, while "ns" above bars indicates nonsignificant differences when analyzed via a one-way ANOVA followed by Holm-Šídák post-hoc comparisons.

3.3. Evaluation of active monoamine agonist effects on fentanyl-depressed respiration

Based on the results obtained under basal conditions, four monoamine agonists were selected for further tests under fentanyl-depressed conditions based on either: 1) their ability to elevate basal MVb at one or more doses (phenylephrine and SKF-82958); or 2) lack of significant depression of basal MVb in conjunction with published evidence supporting efficacy of either target receptor activation or selected agonist under opioid-depressed conditions (8-OH-DPAT and DOI) (Corcoran et al., 2014; Guenther et al., 2009; Lalley et al., 1995; Onimaru et al., 1998; Stettner et al., 2008). The results of experiments with selected agonists under fentanyl-depressed conditions are shown in Figure 6. Administration of phenylephrine at two doses (0.3, 1.0 mg/kg) following pretreatment with fentanyl (0.3 mg/kg) significantly affected MVb, with a main effect of treatment condition x time (Figure 6A; [$F(48, 448) = 2.807$; $p < 0.0001$]). Subjects that received fentanyl (0.3 mg/kg) had significantly ($p < 0.0001$) depressed MVb values relative to saline-treated controls that were between 47.6 and 55.2% of baseline at the time of

phenylephrine administration. Both doses of phenylephrine completely reversed MVb depression within 15 min (t = 35) of administration, at which point phenylephrine-treated groups had MVb values of 66.2 and 63.7% of baseline, respectively. The results of experiments with two doses of SKF-82958 (0.3, 1.0 mg/kg) under fentanyl-depressed conditions are shown in Figure 6B. Analysis showed a significant main effect of treatment condition x time [$F(48, 448) = 3.333$; $p < 0.0001$] on MVb. Administration of both 0.3 mg/kg and 1.0 mg/kg SKF-82958 to fentanyl-pretreated subjects significantly increased MVb relative to fentanyl-treated controls within 10 min (t = 30) but failed to achieve complete reversal. MVb values in subjects treated with 1.0 mg/kg SKF-82958 remained nonsignificantly greater than fentanyl-treated controls for the duration of phase III and were no longer significantly depressed relative to saline-treated controls after 30 min (t = 50).

The results of experiments with the serotonin receptor agonists 8-OH-DPAT and DOI, which lacked significant effects on basal respiration, are shown in Figures 6C and 6D, respectively. There was a main effect of treatment x time on mean MVb values across treatment groups in the analysis of fentanyl + 8-OH-DPAT results [$F(48, 448) = 3.742$; $p < 0.0001$]. Following administration of the higher dose (0.3 mg/kg) of 8-OH-DPAT, fentanyl-induced MVb depression was significantly worsened by 20 min (t = 40), and MVb values remained lower (nonsignificantly) than fentanyl-treated controls for an additional 20 min (t = 60). Conversely, the results presented in Figure 6D show that administration of DOI to fentanyl-depressed subjects slightly elevated MVb values between 5- and 25-min post-administration (t = 25 – 45). Two-way ANOVA demonstrated a main effect of treatment [$F(48, 448) = 4.133$; $p < 0.0001$] on MVb. Subsequent post-hoc comparisons indicated that treatment with 3.0 mg/kg DOI increased

MVb sufficiently to achieve complete reversal at 15 min post-administration (t = 35) that subsided by the next 5-min bin.

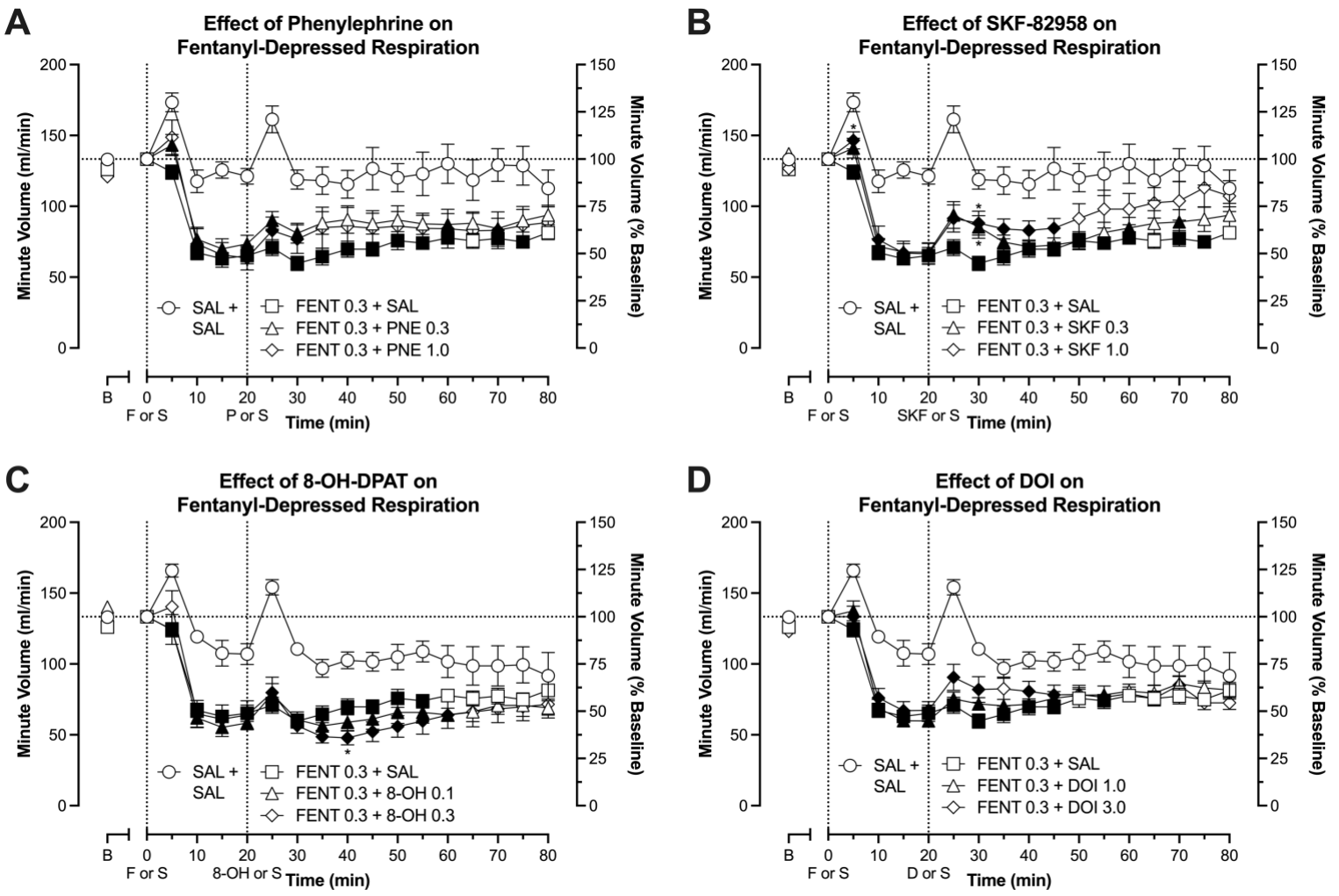

Figure 6: Effects of selected monoamine agonists on fentanyl-depressed respiration. Dose- and time-effects of A) phenylephrine (PNE), B) SKF-82958 (SKF), C) 8-OH-DPAT (8-OH), and D) DOI (DOI) on minute volume (MVb) depressed by pretreatment with 0.3 mg/kg fentanyl (FENT). Abscissa labels: S = saline injection, F = fentanyl injection, PNE = phenylephrine injection, SKF = SKF-82958 injection, 8-OH = 8-OH-DPAT injection, D = DOI injection. N = 8 per group. Additional * symbols above or below specific points indicate a significant difference

at that time point between individual treatment groups and FENT 0.3 + SAL controls of $p \leq 0.05$ according to Holm-Šídák post-hoc comparisons. No significant differences were detected at baseline across experimental conditions when raw MVb values were compared via one-way ANOVA for groups in panel A) [$F(3, 28) = 0.8281$; $p = 0.4896$], panel B) [$F(3, 28) = 0.6345$; $p = 0.599$], panel C) [$F(3, 28) = 1.116$; $p = 0.3592$], or panel D) [$F(3, 28) = 0.6462$; $p = 0.5919$]. All other details are the same as in Figure 1.

3.4. Summary analysis of monoamine agonist effects on fentanyl-depressed respiration

The results of the post-hoc area under the curve (AUC) analysis of normalized MVb x Time during phase III for agonist experiments under fentanyl-depressed conditions are shown in Figure 7. Analysis of AUC data for fentanyl and phenylephrine treatment groups via one-way ANOVA demonstrated a main effect of treatment [$F(3, 28) = 7.637$; $p = 0.0007$] on AUC, and subsequent post-hoc comparisons confirmed that pretreatment with fentanyl (0.3 mg/kg) decreased AUC significantly ($p = 0.0003$) relative to saline-treated controls. Administration of phenylephrine (0.3, 1.0 mg/kg) nonsignificantly increased AUC relative to fentanyl-treated controls, and all treatment groups who received fentanyl had significantly lower AUCs than saline controls (Figure 7A). Similarly, administration of SKF-82958 (0.3, 1.0 mg/kg) to fentanyl-pretreated subjects significantly affected AUC [$F(3, 28) = 8.933$; $p = 0.0003$] over the course of phase III (Figure 7B). Treatment with SKF-82958 dose-dependently increased AUC in fentanyl-pretreated subjects, but post-hoc comparisons indicated that the increases in AUC conferred by SKF-82958 were not significant relative to fentanyl-treated controls. Figure 7C shows the effects of 8-OH-DPAT (0.1, 0.3, mg/kg) on AUC in fentanyl-depressed subjects. 8-OH-DPAT significantly affected AUC with a main effect of treatment [$F(3, 28) = 10.29$; $p < 0.0001$], characterized by dose-dependent reductions in AUC during phase III. However, post-hoc

comparisons indicated that 8-OH-DPAT-mediated decreases in AUC were nonsignificant relative to fentanyl-treated controls. The effects of treatment with DOI on AUC are shown in Figure 7D. Analysis of AUC data from groups that received DOI (1.0 and 3.0 mg/kg) following fentanyl pretreatment demonstrated a significant main effect of treatment [$F(3, 28) = 8.933$; $p = 0.0006$], characterized by small, dose-dependent increases in AUC. However, as with previous agonist treatments, neither dose of DOI significantly increased AUC relative to fentanyl-treated controls, and all fentanyl-pretreated groups had significantly diminished AUCs than saline-treated controls regardless of DOI condition. Finally, Figure 7E shows AUCs for naloxone reversal that were generated from a secondary analysis of data collected in previously published experiments on the reversal of fentanyl-induced respiratory depression (Elder et al., 2023a) to provide an active and commonly used comparator treatment. Analysis of data from treatment groups that received fentanyl (0.3 mg/kg) prior to naloxone (0.1, 1.0, 10 mg/kg) at the start of phase III showed a significant main effect of treatment on AUC [$F(4, 35) = 6.309$; $p = 0.0006$] after naloxone administration. However, post-hoc comparisons demonstrate that despite achieving rapid and complete reversal of fentanyl-induced depression, naloxone did not significantly increase MVb AUC over 60 minutes relative to fentanyl-treated controls.

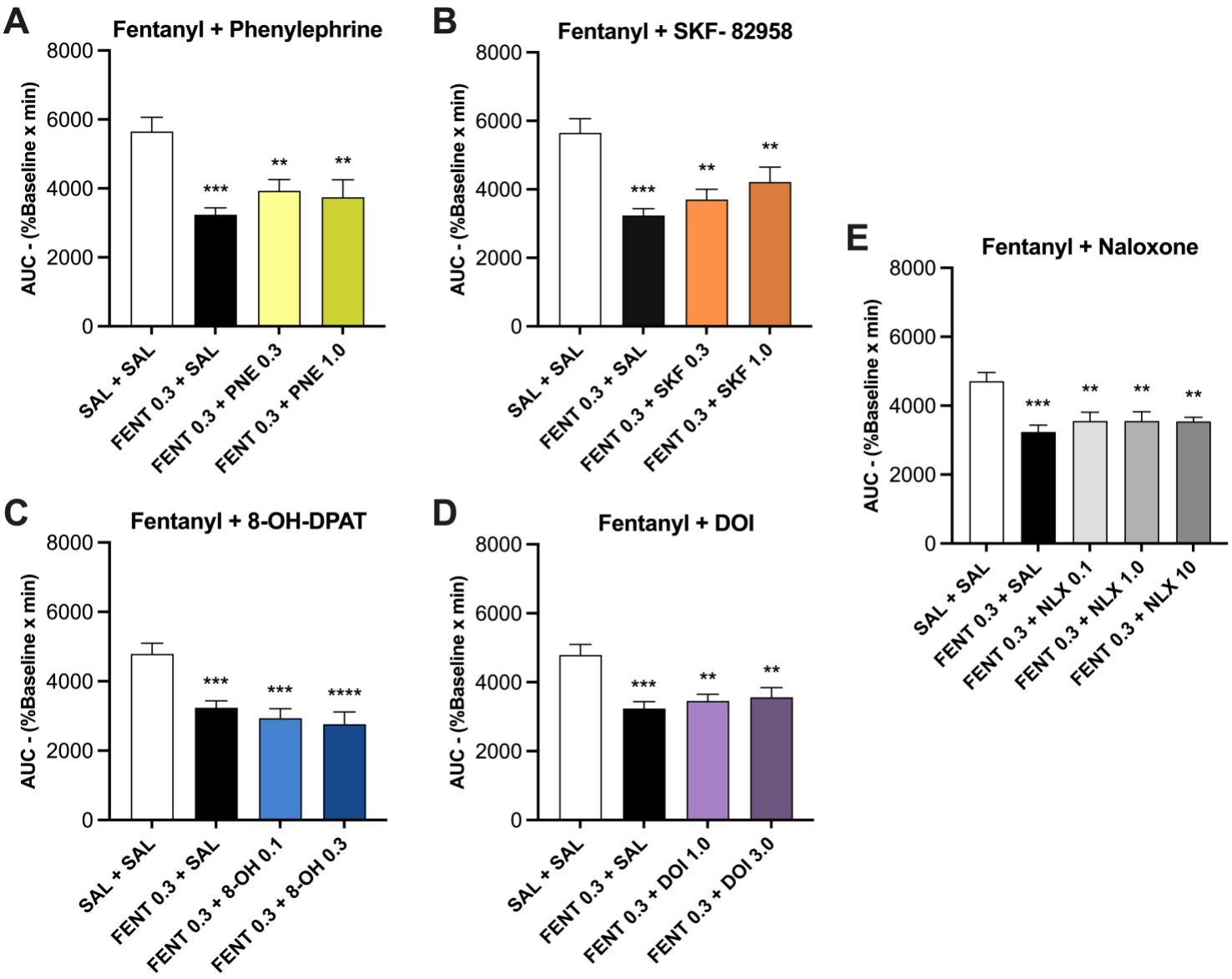

Figure 7: Area Under the Curve summary analysis of the effect on fentanyl-depressed minute volume during phase III by treatment. Dose-effects of A) phenylephrine (PNE), C) SKF-82958 (SKF), D) 8-OH-DPAT (8-OH), E) DOI (DOI), and E) naloxone (NLX) on area under the curve (AUC) of normalized minute volume x time during phase III (60 min) in subjects pretreated with 0.3 mg/kg fentanyl (FENT). Abscissa labels correspond to injections given at time t = 0 + time t = 20, with saline identified as SAL, fentanyl as FENT, and numbers corresponding to the dose administered in mg/kg. AUC is given on the ordinate as the product of

% baseline x minutes (min). **; ***; **** above bars indicate a significant difference between individual treatment groups and SAL + SAL controls of $p \leq 0.01$; 0.001; 0.0001, respectively, when analyzed via a one-way ANOVA. Data for AUC analysis of FENT 0.3 + NLX (0.1, 1.0, 10 mg/kg) was obtained from experiments reported in Elder et al., 2023a.

3.5. Treatment Effects on Frequency and Tidal Volume

The dose-dependent effects of *d*-METH (1.0, 3.0, 10 mg/kg) on basal Freq in subjects who received saline prior to Phase II are shown in Figure 8A. Administration of *d*-METH significantly affected Freq [$F(48, 444) = 8.664$; $p < 0.0001$], producing dose-dependent elevations of Freq that were significantly ($p<0.05$) greater than saline controls for all doses tested. All doses of *d*-METH significantly increased Freq compared to saline controls within 10 min of administration, quickly reaching peak values by 10 – 15 min post-administration, which were maintained throughout the recording period. The dose-related effects of *d*-METH on basal TVb are shown in Figure 8B. Administration of *d*-METH significantly affected TVb [$F(48, 444) = 8.704$; $p < 0.0001$], characterized by transient depression at low doses (1.0 mg/kg) and gradual yet robust increases at the highest dose (10 mg/kg).

The effects of *d*-METH on Freq which were depressed by the administration of fentanyl (0.3 mg/kg) are shown in Figure 8C. In fentanyl-pretreated mice, there was a significant effect of *d*-METH over time [$F(64, 560) = 25.52$; $p < 0.0001$] on Freq. In contrast to the dose-dependent stimulation of Freq observed with *d*-METH under basal conditions, administration of *d*-METH to subjects that were pretreated with fentanyl (0.3 mg/kg) produced bidirectional, dose-dependent effects following a similar pattern as was observed with racemic METH and nearly identical to those observed on MVb with *d*-METH, demonstrating that METH and its enantiomers primarily modulate MVb via alterations in Freq. The effects of *d*-METH on TVb

that was depressed by the administration of fentanyl (0.3 mg/kg) are shown in Figure 8D. In fentanyl-pretreated mice, there was a significant effect of *d*-METH over time [$F(64, 560) = 7.637$; $p < 0.0001$] on TVb, albeit to a lesser degree than Freq. *d*-METH's effects on fentanyl-depressed TVb closely mirrored its effects on depressed Freq, albeit with a greater magnitude of depressant effects and milder stimulant effects at lower and higher doses, respectively. While the relationship between TVb and Freq was similar to what was observed under basal conditions, the balance of their contributions shifted toward TVb-driven depression. At low (1.0 mg/kg) and moderate (3.0 mg/kg) doses, *d*-METH significantly depressed TVb, in contrast to the significant compensatory elevation seen in fentanyl-treated controls who received saline at t = 20. The highest dose of 10 mg/kg *d*-METH had complex effects on TVb, inducing transient significant depression at t = 35 followed by a gradual, nonsignificant increase compared to controls.

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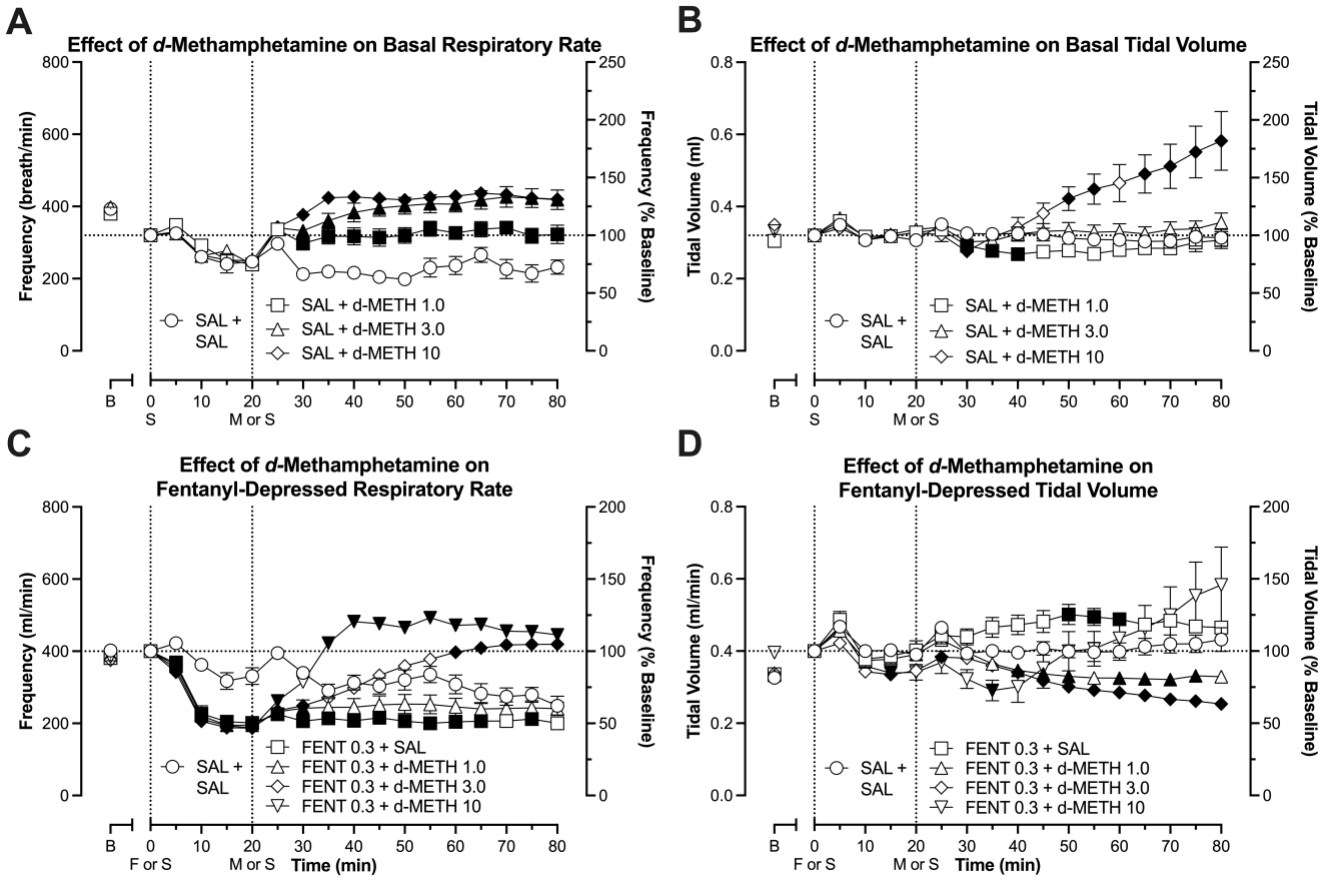

609 **Figure 8: Effects of *d*-METH on basal and fentanyl-depressed breath frequency and tidal**
610 **volume.** Panel A) Dose- and time-effects of *d*-methamphetamine (*d*-METH) on breath frequency
611 (Freq); Panel B) dose- and time-effects of *d*-METH on tidal volume (TVb); C) dose- and time-
612 effects of *d*-METH on fentanyl- (FENT) depressed breath frequency (Freq); D) dose- and time-
613 effects of *d*-METH on FENT-depressed TVb. Left ordinate: mean raw Freq (breaths/min) or
614 TVb indexing values of symbols only during baseline (B) of Phase I. Right ordinate: normalized
615 (percent baseline) Freq or TVb indexing values of symbols during the 80-min test session
616 following Phase I baseline. Symbols indicate mean Freq or TVb expressed as a percentage of
617 baseline Freq or TVb for treatment groups consisting of 8 mice. Filled symbols indicate
618 significant differences compared to the respective Freq or TVb of saline-treated controls at

individual time points ($p \leq 0.05$). Abscissa labels: B = mean baseline Freq or TVb, F, M or S = fentanyl, *d*-METH or saline (SAL) injection, respectively. Legend labels correspond to the dose in mg/kg. All other details are the same as in Figure 1.

The dose-dependent effects of SKF-82958 (1.0, 3.0, 10 mg/kg) on basal Freq in subjects who received saline prior to Phase II are shown in Figure 9A. Administration of SKF-82958 significantly affected Freq over time [$F(48, 444) = 7.861$; $p < 0.0001$], producing dose-dependent elevations of Freq that were significantly greater than saline controls at 0.3 and 1.0 mg/kg. The two highest doses of SKF-82958 significantly increased Freq compared to saline controls within 10 and 15 min of administration, respectively,, representing peak effects maintained throughout the recording period. The dose-related effects of SKF-82958 on basal TVb are shown in Figure 9B. SKF-82958 had a significant effect on TVb over time [$F(48, 444) = 1.999$; $p = 0.0002$] but did not significantly alter TVb at any timepoint relative to saline-treated controls when data were analyzed via post-hoc comparisons.

The dose-dependent effects of clonidine (0.03, 0.1, 1.0 mg/kg) on basal Freq in subjects who received saline prior to Phase II are shown in Figure 9C. Administration of clonidine significantly affected Freq [$F(48, 448) = 4.194$; $p < 0.0001$], producing significant dose-dependent depression of Freq at all doses when compared with saline-treated controls. All doses of clonidine significantly decreased Freq from 5 – 15 min post-administration ($t = 25 – 35$), which represented peak depressant effects that slowly dissipated for 0.03 and 0.1 mg/kg treatments, while the maximal depressant effects of 1.0 mg/kg were maintained throughout the recording period. The dose-related effects of clonidine on basal TVb are shown in Figure 9D. Clonidine had a significant effect on TVb over time [$F(48, 448) = 4.173$; $p < 0.0001$], with post-hoc comparisons showing significant depression by the highest dose (1.0 mg/kg) at two time

points (t = 25 and 40) and significant depression by the intermediate dose (0.1 mg/kg) from 15 – 35 min (t = 35 – 55) compared with saline-treated controls.

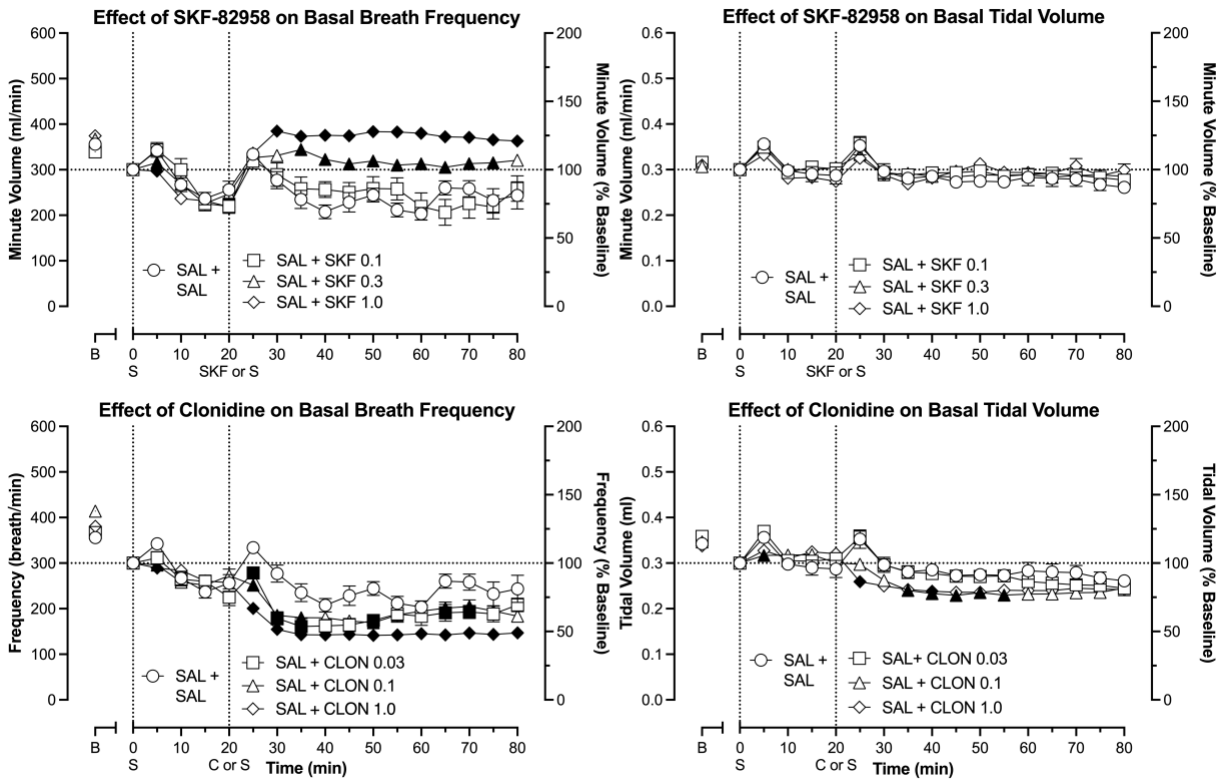

Figure 9: Effects of representative stimulant and depressant agonists on basal and depressed breath frequency and tidal volume. Panel A) Dose- and time-effects of SKF-82958 (SKF) on breath frequency (Freq); Panel B) dose- and time-effects of SKF on tidal volume (TVb); Panel C) dose- and time-effects of clonidine (CLON) on breath frequency (Freq); Panel D) dose- and time-effects of CLON on tidal volume (TVb). Left ordinate: mean raw Freq (breaths/min) or TVb indexing values of symbols only during baseline (B) of Phase I. Right ordinate: normalized (percent baseline) Freq or TVb indexing values of symbols during the 80-min test session following Phase I baseline. Symbols indicate mean Freq or TVb expressed as a percentage of baseline Freq or TVb for treatment groups consisting of 8 mice. Filled symbols

indicate significant differences compared to the respective Freq or TVb of saline-treated controls at individual time points ($p \leq 0.05$). Abscissa labels: B = mean baseline, S, SKF, or C = saline (SAL), SKF-82958, or clonidine injection, respectively. Legend labels correspond to the dose in mg/kg. All other details are the same as in Figure 1.

4. Discussion and Summary

Overall, previously published reports by other laboratories and the results of the present study show that monoamine receptors are: 1) present in brainstem regions relevant to respiration; 2) involved in modulating the activity of respiratory networks; 3) able to be manipulated pharmacologically to alter respiration; and 4) capable of altering OIRD in laboratory animals (Ciarka et al., 2007; Imam et al., 2020; Lalley, 2008; Ramirez et al., 2012; van der Schier et al., 2014). In the first stage of the present study the effects of the two METH enantiomers, *d*- and *l*-METH, on basal and fentanyl-depressed respiration were evaluated to determine their individual contributions to the bidirectional respiratory modulation observed previously with racemic METH. There were two main findings from these experiments. First, *d*- and *l*-METH were shown to have opposing effects on basal respiration, as evidenced by the complete separation of respiratory stimulant and respiratory depressant effects between nominally equal doses of *d*-METH and *l*-METH, respectively (Figures 1A and 1B). Second, experiments that evaluated the two enantiomers under fentanyl-depressed conditions showed a recapitulation of the racemate's bidirectional respiratory effects with *d*-METH, while *l*-METH was shown to significantly exacerbate fentanyl-induced respiratory depression at all doses tested (Figures 1C and 1D).

While the enantiomers tended to modulate fentanyl-depressed respiration in the manner that was hypothesized, the unexpected recapitulation of bidirectional effects in fentanyl-depressed experiments with *d*-METH provides insight into the pharmacological determinants of

METH's respiratory activity and how it may be altered in the presence of fentanyl. While these results support the hypothesis that efficacy for releasing monoamines, more specifically relative efficacy for DA/5HT release, is correlated with respiratory stimulation, it remains to be determined whether such stimulation is the result of a direct effect of METH on monoamine transmission in brainstem respiratory networks or relies on monoamine-induced increases in downstream glutamate transmission to these areas (Fischer et al., 2021). Since amphetamines exert their effects via multiple mechanisms that include TAAR1 activation, their ability to influence synaptic monoamine levels may vary substantially based on the neurophysiology of the CNS regions in question (Abekawa et al., 1994; Stephans and Yamamoto, 1995; Underhill et al., 2019, 2014). Furthermore, since glutamate is known to play a primary role in controlling respiratory network activity, both intrinsically and extrinsically, to carry signals from distal inputs like chemosensors, it is possible that METH modulates glutamatergic inputs to respiratory networks through its effects on monoamines in regions that interact with those projections (Ang et al., 1992; Martelli et al., 2013; Pilowsky et al., 2009). Regardless of how monoamine release by METH specifically leads to increased respiratory output, these results provide compelling evidence that potency and selectivity for monoamine release is a key determinant of the nature of respiratory stimulation, as evidenced by *l*-METH's opposing effects on MVb.

In the second stage of this study, six selective monoamine receptor agonists (phenylephrine, clonidine, SKF-82958, quinpirole, 8-OH-DPAT, and DOI) were initially characterized for their effects on basal respiration to identify receptor-agonist pairings with respiratory stimulant effects. Subsequent experiments evaluated the ability of agonists that did not depress basal respiration to reverse respiratory depression induced by an ED_{50} dose of fentanyl. The results obtained from these experiments provided two primary findings. First,

700 experimental results showed that agonists of excitatory catecholamine receptors, phenylephrine
701 (α_1) and SKF-82958 (D_1), stimulated MVb in a dose-dependent manner under both basal and
702 fentanyl-depressed conditions. Second, agonists of inhibitory catecholamine receptors often
703 associated with presynaptic neurons, clonidine (α_2) and quinpirole (D_2), were shown to depress
704 basal respiration following administration dose-dependently. These results are in line with
705 previously published research demonstrating respiratory stimulant effects of D_1 receptor agonists
706 and the rhythm-enhancing effects of NE inputs to brainstem nuclei (Errchidi et al., 1991, 1990;
707 Lalley, 2008, 2005, 2004). Additionally, these results show a dichotomy between respiratory
708 stimulant effects of post-synaptic receptors and depressant effects of pre-synaptic autoreceptors,
709 further supporting the hypothesis that mild increases in synaptic monoamines induced by low
710 doses of amphetamines may preferentially activate autoreceptors to induce respiratory
711 depression.

712 Although neither of the two serotonin receptor agonists strongly modulated basal or
713 depressed MVb, a similar pattern emerged whereby 8-OH-DPAT ($5HT_{1a}$), an agonist of an
714 inhibitory presynaptic receptor subtype, tended to be pro-depressant, while DOI ($5HT_{2a}$), an
715 agonist of excitatory post-synaptic receptor subtypes, tended to be mildly stimulating. Although
716 ample evidence in the literature supports the respiratory activity of 8-OH-DPAT, many of the
717 studies published on the respiratory effects of 5HT agonists were conducted in neonates and in
718 the context of various existing respiratory pathologies (Bodineau et al., 2004; Guenther et al.,
719 2009; Günther et al., 2006; Mathew, 2011; Stettner et al., 2008; Veasey, 2003). Our results
720 contradict some earlier findings from experiments with morphine, fentanyl, and remifentanil-
721 treated animals that showed $5HT_{1a}$-mediated selective reversal of respiratory depression, but are
722 in line with negative results that have been reported from other preclinical and clinical trials of

723 $5HT_{1a}$ agonists, including buspirone, for the treatment of central apneas (Guenther et al., 2012,
724 2010; Oertel et al., 2007; Ren et al., 2015). Although *in vivo* models have demonstrated
725 respiratory stimulant effects of DOI previously in different contexts (Andrzejewski et al., 2017;
726 Budzinska, 2009), these data represent the first time the stimulant effects of DOI on OIRD have
727 been reported. The limited efficacy of DOI under fentanyl-depressed conditions may be a
728 product of its psychedelic pharmacology, which is known to induce broad enhancements in
729 neuronal network activity and glutamate transmission within the CNS (Inserra et al., 2021;
730 Mason et al., 2020; Nichols, 2016, 2004; Vollenweider and Kometer, 2010). Taken together,
731 these findings indicate that monoaminergic inputs influence respiration, which can be
732 manipulated in either direction using agonists selective for excitatory post-synaptic receptors or
733 inhibitory presynaptic receptors.

734 The primary findings from experiments conducted in stages one and two of this study are
735 complementary and provide insight into the monoaminergic mechanisms that mediate METH's
736 bidirectional effects on respiration. Findings in stage one demonstrated that the enantiomer with
737 the greatest absolute and relative potency for releasing DA, *d*-METH, acted almost entirely as a
738 respiratory stimulant under basal and depressed conditions at the doses tested. Conversely, *l*-
739 METH, a substantially less potent monoamine releaser biased toward NE release, primarily acted
740 as a depressant when tested at the same doses. However, when assayed at a dose beyond those
741 used in experiments with other enantiomeric compositions, 30 mg/kg *l*-METH displayed
742 moderate yet significant stimulant effects similar to those of 3.0 mg/kg *d*-METH or 10 mg/kg
743 racemic METH, thus confirming that *l*-METH also displays dose-related bidirectionality rather
744 than solely dose-dependent depression. These findings demonstrate that the two enantiomers
745 have differential potency as respiratory stimulants, as opposed to exerting opposing influences

on respiration as was originally hypothesized. The respiratory stimulant activity of high-dose *l*-METH may be explained as a consequence of the administration of an adequate dose to produce sufficient release of NE, and possibly DA, to cause a shift in the balance of indirect agonism toward an overall excitatory effect on neurotransmission and respiratory output. This hypothesis could also be extended in the opposite direction for *d*-METH, whereby the administration of lower doses (≤0.3 mg/kg) may engender depressant effects as a function of lesser NE release leading to a shift in the balance toward overall inhibition. When considered alongside the results obtained from experiments of catecholamine receptor agonists, the evidence suggests that increased signaling at excitatory post-synaptic catecholamine receptors may underlie the respiratory stimulant properties of METH and represents a mechanism that could potentially be exploited in the future development of respiratory stimulant therapeutics. Similarly, inhibitory presynaptic catecholamine receptors may be responsible for the respiratory depressant effects of METH and represent a target for medications development efforts to rescue METH-compromised respiration.

Interestingly, enhancement of glutamate may be a shared mechanism among the agonists that produced elevations of fentanyl-depressed respiration as well as the amphetamines (Chen et al., 2006; Kalivas and Duffy, 1995; Kanbayashi et al., 2000; Nishino et al., 1998). A growing body of recent evidence points to the involvement of AMPA receptor activation in the generation and stimulation of respiratory network activity, which could potentially be the downstream effector mediating the effects of excitatory monoamine receptors (Dahan et al., 2018; Imam et al., 2020; Oertel et al., 2010; Ren et al., 2009; van der Schier et al., 2014). In fact, preliminary data collected in early experiments with the α_1 antagonist prazosin, which is thought to decrease glutamatergic transmission from regions such as the hypothalamus, spinal cord and brainstem,

showed dose-dependent depression of MVb following administration (data not shown) (Chen et al., 2006).

Overall, these data demonstrate that activation of monoaminergic receptors can differentially modulate respiration based on the receptor subtype's effect on cellular and network activity. Furthermore, while data from these experiments cannot confirm whether the respiratory effects of METH are mediated by monoamine receptors in the brainstem, similarities between the effects of METH and catecholamine receptor agonists on respiratory frequency suggest they are likely involved. To this point, the findings reported here have identified potential targets for future analeptic development (D_1 & α_1) based on their ability to mitigate OIRD from fentanyl, as well as the receptors that are likely mediators of enhanced OIRD toxicity (D_2 & α_2). . Future studies should be conducted specifically in human subjects to test the cross-species consistency of the bi-directional effects of racemic methamphetamine, and to evaluate the use of monoaminergic analeptics by themselves and in combination with opioid antagonists such as naloxone for their ability to provide rapid and sustained reversal of OIRD, especially in the context of fentanyl.

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