Research Article

Chronic Behavioral and Neurochemical Effects of Four Novel *N*-Benzyl-2-phenylethylamine Derivatives Recently Identified as "Psychoactive" in Adult Zebrafish Screens

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ABSTRACT: Potently affecting human and animal brain and behavior, hallucinogenic drugs have recently emerged as potentially promising agents in psychopharmacotherapy. Complementing laboratory rodents, the zebrafish (*Danio rerio*) is a powerful model organism for screening neuroactive drugs, including hallucinogens. Here, we tested four novel *N*-benzyl-2-phenylethylamine (NBPEA) derivatives with 2,4- and 3,4-dimethoxy substitutions in the phenethylamine moiety and the −F, −Cl, and −OCF₃ substitutions in the *ortho* position of the phenyl ring of the *N*-benzyl moiety (34H-NBF, 34H-NBCl, 24H-NBOMe(F), and 34H-NBOMe(F)), assessing their behavioral and neurochemical effects following chronic 14 day treatment in adult zebrafish. While the novel tank test behavioral data indicate anxiolytic-like effects of 24H-NBOMe(F) and 34H-NBOMe-(F), neurochemical analyses reveal reduced brain norepinephrine by all four drugs, and (except 34H-NBCl) - reduced dopamine and serotonin levels. We also found reduced turnover rates for all three brain monoamines but unaltered levels of their respective metabolites. Collectively, these findings further our understanding of complex central behavioral and neurochemical effects of chronically administered novel NBPEAs and highlight the potential of zebrafish as a model for preclinical screening of small psychoactive molecules.

KEYWORDS: zebrafish, behavior, psychopharmacology, novel compounds, in silico drug activity
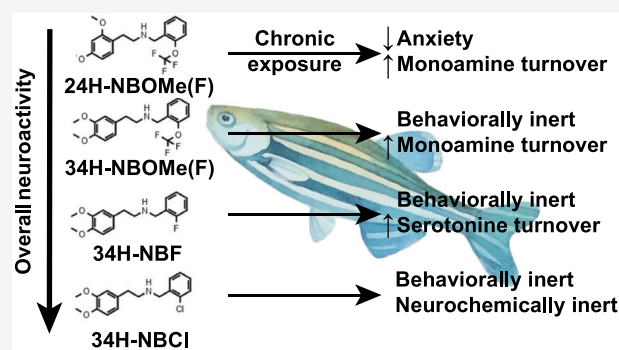
1. INTRODUCTION

Serotonergic psychedelic drugs are powerful hallucinogens that affect human brain function and behavior, causing sensory, emotional, and cognitive changes with altered sociality, ego dissolution, and mystical experiences.[1−4] Psilocybin, lysergic acid diethylamide (LSD), and some other psychedelic drugs have recently shown promise as potential treatments for mood disorders, anxiety, and post-traumatic stress disorder.[2,5−13] Animal studies have also demonstrated cognitive improvement and increased cortical plasticity and neurogenesis in response to these drugs.[14,15]

NBOMes are a relatively new class of synthetic drugs, which are derivatives of phenethylamines with methoxy groups in the second and fifth positions and various lipophilic groups at the fourth position. Representing synthetic psychedelics, 25I-NBOMe and other members of this class have been extensively studied for robust serotonergic activity.[16−19] Like classical hallucinogens, they have strong effects on the central nervous system (CNS), including hallucinations, altered consciousness,

euphoria, and sensory and visual effects.[20,21] However, these drugs can also be toxic and may cause severe side effects and mortality,[22−24] raising concerns over their use and abuse.

Both classical serotonergic psychedelics (e.g., LSD and psilocybin) and NBOMes exert overt CNS effects by activating serotonin 5-HT$_{2A}$ receptors commonly associated with various cognitive, affective (e.g., depression), and psychotic (e.g., schizophrenia) disorders.[25−27] While NBOMes represent highly selective 5-HT$_{2A}$ agonists,[28−32] they also display high affinity for other receptors, such as serotonin 5-HT$_{2C}$, α1 adrenergic, and H1 histamine receptors, but not for dopamine

D1-D3 and trace amine (TAAR) receptors or dopamine, serotonin, and norepinephrine transporters.[29,31,32] In rodents, the activation of 5-HT$_{2A}$ receptors causes a characteristic "head twitch" response, which is typically seen for LSD and psilocybin,[33−36] but is also reported for some NBOMes (e.g., 25H-, 25B-, and 25C-NBOMes).[37−39]

Complementing rodent studies, a small freshwater teleost fish, the zebrafish (*Danio rerio*), is a powerful *in vivo* model system for neuroscience research and CNS drug discovery.[40−42] Zebrafish assays are useful for CNS drug screening because these fish have high genetic and physiological similarity to humans, are cost-effective and easy to keep, have rapid reproduction and development, and are sensitive to a wide range of psychoactive drugs,[43,44] including all major classes of hallucinogens, such as serotonergic psychedelics,[45,46] antiglutamatergic dissociatives,[47] opioidergic agonists,[48−50] and anticholinergic deliriants.[42]

Zebrafish anxiety-like behavior parallels that of other common model species and humans and its bidirectional sensitivity to anxiogenic and anxiolytic factors.[51] For example, anxiogenic-like effects in zebrafish can be induced by exposure to novelty, predators, alarm pheromones, net chasing, overcrowding, and social isolation.[52−54] In contrast, conventional anxiolytics, chronic antidepressants, environmental enrichment, and social interactions are widely known to reduce anxiety-like behavior in zebrafish.[55,56] The novel tank test is a commonly used aquatic assay to assess anxiety-like behavior in adult zebrafish.[57] The test assesses their exploratory activity in novel unfamiliar environments by measuring activity in "aversive" top vs "protective" bottom parts of the apparatus, conceptually similarly to the rodent open field and other novelty-based paradigms.[58] Thus, increased bottom dwelling is associated with an increased anxiety-like state in zebrafish, whereas increased top exploration typically reflects their anxiolytic-like responses.[58]

Conveniently for studying hallucinogenic compounds, the zebrafish serotonergic system exhibits high level of complexity and shares numerous conservative features with mammals in terms of its general neuroanatomy and neurochemistry.[59,60] Indeed, zebrafish possess all seven families of serotonin receptors found in humans and rodents (5-HT$_1$-5-HT$_7$), including those most relevant to hallucinogenic effects, such as 5-HT$_{2A}$, 5-HT$_{2C}$, and 5-HT$_{1A}$.[59] However, most zebrafish serotonin receptor genes exist in duplicates due to an additional round of whole-genome duplication that occurred in teleost fish during evolution.[61] Notably, brain expression profiles obtained for zebrafish orthologues of 5-HT$_{1A}$, 5-HT$_{1B}$, and 5-HT$_{2C}$ receptors reveal a widespread and overlapping distribution.[62] For example, the expression of *htr1aa*, *htr1ab*, and *htr1bd* genes (encoding zebrafish 5-HT$_{1A}$ and 5-HT$_{1B}$ receptors, respectively) has been mapped to the raphe nuclei, thalamus, hypothalamus, posterior tuberculum, cerebellum, optic tectum, reticular formation, and ventral telencephalon.[62] Expression of the zebrafish ortholog of the 5-HT$_{2C}$ receptor gene (*ht2cr*) is observed in the medulla oblongata, hypothalamus, dorsal thalamus, posterior tuberculum, telencephalon, and olfactory bulbs.[63] Importantly, despite some species differences, the expression profiles of these key serotonin receptor genes generally parallel those of their mammalian counterparts.[62,63] Consistent with this, binding of a radio-labeled 5-HT$_{1A}$ agonist [^{3}H]8-OH-DPAT is detected in the hypothalamus, optic tectum, and telencephalon in zebrafish brain slices, with similar density values observed in

the mouse brains under the same protocol.[64] To date, there are no data on expression profiles of other zebrafish serotonin receptor genes as well as on binding affinity of zebrafish serotonin receptors to serotonin and serotonergic drugs. Nevertheless, recent studies with various serotonergic ligands in zebrafish have reported robust and specific responses resembling those observed in mammals, implying sufficient structural and functional similarities of serotonin receptors across taxa.[65−69]

Capitalizing on the growing utility of zebrafish assays as a powerful *in vivo* drug screening platform, we have recently tested a battery of 10 novel *N*-benzyl-2-phenylethylamines (NBPEAs) with the 2,4- or 3,4-dimethoxy substitutions in the phenethylamine moiety and the −OCF$_3$, −F, −Br, and −Cl substitutions in the *ortho* position of the phenyl ring of the *N*-benzyl moiety. Testing their acute behavioral and neurochemical effects, we have previously identified four interesting neuroactive compounds for further analyses[70] (Figure 1),
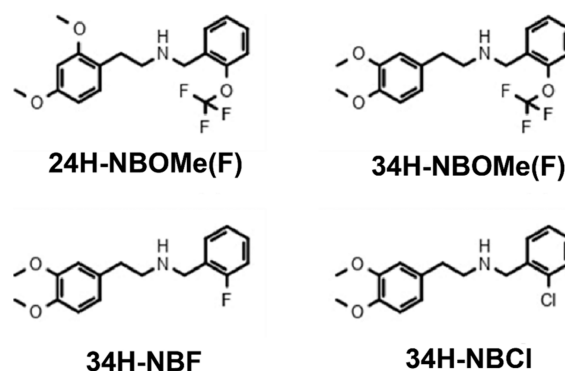
Figure 1. Structural formulas of the four tested NBOMe compounds used in this study (their chemical names were simplified for clarity, as commonly done for NBOMes). Their complete chemical names, according to conventional International Union of Pure and Applied Chemistry (IUPAC) nomenclature, are (*N*-(2-trifluoromethoxybenzyl)-2-(2,4-dimethoxyphenyl)ethylamine (24H-NBOMe(F), *N*-(2-trifluoromethoxybenzyl)-2-(3,4-dimethoxyphenyl)ethylamine 34H-NBOMe(F)), *N*-(2-fluorobenzyl)-2-(3,4-dimethoxyphenyl)-ethylamine (34H-NBF), and *N*-(2-chlorobenzyl)-2-(3,4-dimethoxyphenyl)ethylamine (34-NBCl).

including two compounds with pronounced beneficial (anxiolytic/antidepressant-like) acute behavioral effects with increased brain serotonin and dopamine turnover (*N*-(2-trifluoromethoxybenzyl)-2-(2,4-dimethoxyphenyl)ethylamine, 24H-NBOMe(F)) and *N*-(2-trifluoromethoxybenzyl)-2-(3,4-dimethoxyphenyl)ethylamine, 34H-NBOMe(F)), one compound with anxiogenic-like profile but no overt neurochemical profiles (*N*-(2-fluorobenzyl)-2-(3,4-dimethoxyphenyl)-ethylamine, 34H-NBF) acutely, and a behaviorally inert compound with overt neurochemical activity similar to 24H-NBOMe(F) and 34H-NBOMe(F), *N*-(2-chlorobenzyl)-2-(3,4-dimethoxyphenyl)ethylamine (34H-NBCl). Their ability to cross the zebrafish blood-brain barrier and enter the brain as well as chemical synthesis, chemical structures, and analytical data have already been reported previously.[70−73] Typically, in the 25X-NBOMe conventional nomenclature, the X (i.e., H for hydrogen here) indicates the substitution at position 4 of the phenethylamine moiety.[70,74] However, as 24H- and 34H-isomers carry a methoxy group at this position, they can also be described as 24- and 34-NBOMes, respectively.[70] Here, we will specifically use the latter nomenclature, aiming at a greater

clarity in order to emphasize that no other substitutions (beyond the two methoxy groups) were introduced at the phenyl ring of the phenethylamine moiety (also see similar examples in refs 70, 72, and 75).

Given the growing concerns over synthetic hallucinogenic drug use and abuse, it is critical to understand more fully their long-term chronic use *in vivo*. The present study aims to characterize behavioral and neurochemical profiles of 24H-NBOMe(F), 34H-NBOMe(F), 34H-NBF, and 34H-NBCl following their chronic 14 day exposure to adult zebrafish, probing CNS activity and assessing the potential of these novel NBOMes as putative CNS therapies.

2. RESULTS

2.1. Neurobehavioral Assays. The drugs evoked significant effects on zebrafish anxiety-like behavior and locomotion. The analysis of generalized linear models (GLM) using the Wald chi-square test revealed a significant treatment effect on the time fish spent in the top section of the novel tank test (NTT), immobility duration, and total distance traveled ($p < 0.05$; Table 1). In addition, a significant day effect

Table 1. Significant Factors Affecting Zebrafish Behavior in the Novel Tank Test (NTT) Calculated by the Wald Chi-Square Test for Generalized Linear Models Using Days 1, 3, and 14, Treatment Group (Control, 34H-NBCl, 34H-NBF, 24H-NBOMe(F), and 34H-NBOMe(F)), and Their Interaction Effects as Predictors[a]

factor	$\chi 2$	Df	P
Top duration			
treatment	27.178	4	<0.001
day	2.708	2	0.258
treatment × day	4.274	8	0.832
Distance			
treatment	11.083	4	0.026
day	7.838	2	0.02
treatment × day	4.937	8	0.764
Top entries			
treatment	6.32	4	0.176
day	13.066	2	0.001
treatment × day	7.325	8	0.502
Freezing duration			
treatment	18.571	4	0.001
day	3.919	2	0.141
treatment × day	12.524	8	0.129

[a]χ^2, Wald chi-square test statistics; Df, degrees of freedom; P, probability value. $n = 19-21$ per group.

was found for the total distance and the number of top entries, with no significant day × group interaction effect at any end point ($p > 0.05$; Table 1), suggesting that the drug's behavioral effects were relatively stable over time. Supplementary data for this study are also presenterd in Supplementary Tables S1−S5.

To estimate the overall effect of drug treatment on zebrafish behavior in the novel tank test, we calculated estimated marginal means averaged over the levels of the day factor and performed posthoc comparisons of the drug treatment versus control groups using Dunnett's test (Figure 2 and Tables S3 and S4). Overall, fish exposed to 24H-NBOMe(F) spent significantly more time on top of the tank over the testing days (Dunnett's posthoc multiple comparisons of EMMeans, $p < 0.001$), whereas fish treated with 34H-NBOMe(F) spent

significantly less time immobile over the testing days (Dunnett's posthoc multiple comparisons of EMMeans, $p < 0.05$). In contrast, posthoc comparisons did not reveal significant differences in the total distance traveled and the number of top entries for any of the treatment groups compared to the control group.

2.2. Neurochemical Effects. The high-performance liquid chromatography (HPLC) analyses revealed significant drug effects on brain levels of norepinephrine, dopamine, and serotonin (KW-test, $p < 0.05$; Table S5), with posthoc Dunn's test showing significantly lower levels of all three monoamines in 24H-NBOMe(F), 34H-NBOMe(F), and 34H-NBF ($p < 0.05$ vs control) groups (Figure 3 and Table S5). The effect of the trifluoromethoxy-substituted NBPEA was most potent, with 24H-NBOMe(F) reducing >10-fold the levels of all three monoamines ($p < 0.001$ vs control group, Dunn's test). Interestingly, 34H-NBCl reduced only norepinephrine ($p < 0.05$ vs control group, Dunn's test) but not dopamine or serotonin levels ($p > 0.05$, NS). Similarly, 24H-NBOMe(F), 34H-NBOMe(F), and 34H-NBF also significantly increased serotonin turnover in the brain, assessed as serotonin metabolite (5-hydroxyindoleacetic acid, 5-HIAA) to serotonin ratio ($p < 0.05$), whereas 24H-NBOMe(F) and 34H-NBOMe(F) also increased dopamine turnover, as reflected by altered 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) to dopamine ratios (Figure 4). However, none of these drugs directly affected monoamine metabolites DOPAC, HVA, and 5-HIAA ($p > 0.05$, KW-test; Figure 3 and Table S5).

To complement behavioral and neurochemical analyses of the four NBOMes tested here *in vivo*, we also performed *in silico* estimation of their CNS activity using the PASS Online 2022 version[76,77] that contains data on >5000 drugs and mechanisms of action, yielding 479 psychotropic activities predicted with high accuracy of prediction (Pa = 0.96; Table S1). For 34H-NBF and 34H-NBCl, the database predicted antistress/antineurotic (Pa = 0.601, Pi = 0.034 and Pa = 0.610, Pi = 0.032 activity, respectively) and, for 24H-NBOMe(F) and 34H-NBOMe(F), antineurogenic pain (Pa = 0.573, Pi = 0.009 and Pa = 0.597, Pi = 0.008, respectively), assessed by PharmaExpert software[78] (Table 2).

3. DISCUSSION

To the best of our knowledge, this is the first study utilizing zebrafish for *in vivo* psychopharmacological screening of chronic effects of novel NBPEAs. Studying the four compounds, previously identified as potentially neuroactive acutely, 34H-NBF, 34H-NBCl, 24H-NBOMe(F), and 34H-NBOMe(F), we observed robust anxiolytic-like effects for 24H-NBOMe(F) and, to a lesser extent, 34H-NBOMe(F), accompanied by markedly altered levels of brain monoamines. While 24H-NBOMe(F), 34H-NBOMe(F), and 34H-NBF significantly increased serotonin turnover in the brain, 24H-NBOMe(F) and 34H-NBOMe(F) also increased dopamine turnover without affecting the levels of monoamine metabolites DOPAC, HVA, and 5-HIAA (Figures 3 and 4).

In general, although some NBPEAs show potential medicinal properties, including treating mental disorders,[79−81] biological effects of some positional isomers, such as those with substituents other than the methoxy group in the ortho-position of the phenyl ring, remain poorly understood. Recently, the ability to activate 5-HT$_{2A}$ receptors *in vitro* has been demonstrated for positional isomers of 25H-NBOMe and
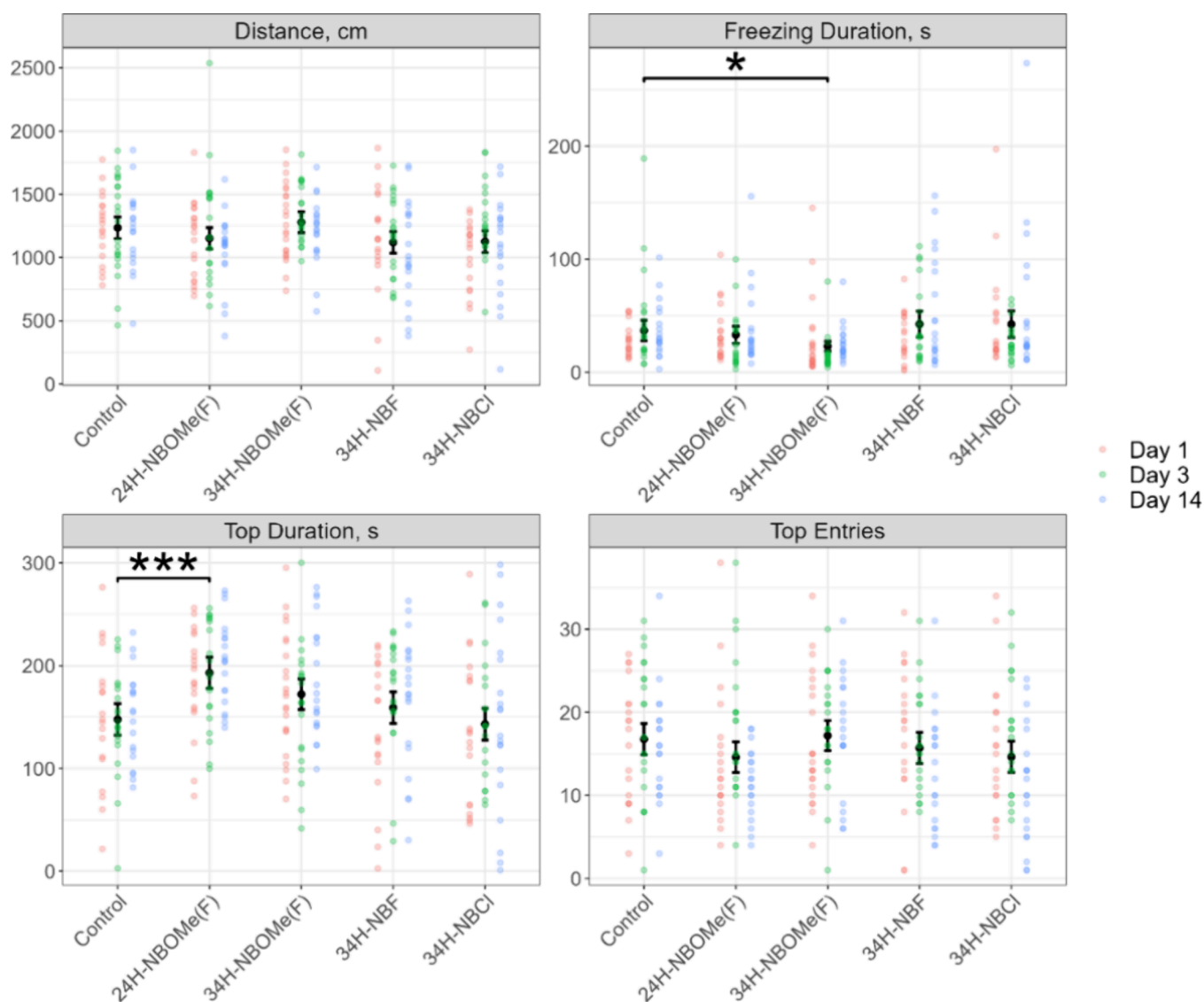
Figure 2. Behavioral effects of the four tested NBOMe drugs in the adult zebrafish novel tank test on days 1, 3, and 14 of treatment. Black dots with error bars represent EMMeans averaged over the levels of "day" factor and the respective confidence intervals. Colored dots represent individual data points acquired on the three different days of testing. ***$p < 0.001$, *$p < 0.05$ (Dunnett's posthoc comparisons of EMMeans), $n = 19-21$ per group. For interpretation of references to color in figure legends please refer to the online version of this article.

25H-NBF,[72,82] including 34H-NBF used in the present study. Interestingly, different positioning of the two methoxy groups in the phenethylamine moiety affects the ability of these drugs to recruit βarr2 and miniGαq in the human embryonic kidney (HEK) 293T cells transfected with the human 5-HT$_{2A}$ receptor.[72,82] Furthermore, for both 25H-NBOMe and 25H-NBF isomers, compounds with 2,4-methoxy substitutions have shown higher potency and efficiency in activation of the human 5-HT$_{2A}$ receptor, compared to the 3,4-substituted isomers,[72,82] collectively suggesting the critical role of the 2-methoxy substitution in NBPEA activity.[83] In contrast, the −NBF compounds are consistently less potent in this activity (compared to their −NBOMe counterparts), supporting the role of the oxygen atom in the 2-substituent benzyl ring as a key determinant of 5-HT2 receptor affinity.[84] On the one hand, drugs with a polar methoxy or hydroxyl group in the ortho-position may display enhanced neurotropic activity,[85] and various positional isomers of 25H-NBOMe also have shown high affinity for human 5-HT$_{2A}$ receptors.[75] Among

various NBPEA derivatives, 24H-NBOMe was the most potent and effective 5-HT$_{2A}$ receptor ligand, as assessed by the recruitment of both βarr2 and miniGαq in the human embryonic kidney (HEK) 293T cells transfected with the corresponding proteins.[75] While 34H-NBOMe has lower potency but relatively high efficacy of binding to this receptor, 25H-NBF is highly affine for it, whereas 34H-NBF has a lower efficacy, compared to classical hallucinogens (e.g., LSD) and 25H-NBF.[75]

As chronic 24H-NBOMe(F) induced strong anxiolytic-like behavior in adult zebrafish, reducing time in top of the novel tank test, 34H-NBOMe(F) was also anxiolytic in the present study, albeit less potent since it only reduced freezing behavior in zebrafish (Figure 2). These behavioral effects of chronic exposure to these drugs are generally in line with acute behavioral effects of 24H-NBOMe(F) and 34H-NBOMe(F), where 24H-NBOMe(F) was anxiolytic at 5 mg/L vs 20 mg/L 34H-NBOMe(F).[70] While behavioral inactivity of 34H-NBCl is seen in both acute[70] and chronic studies (Figure 2), acute
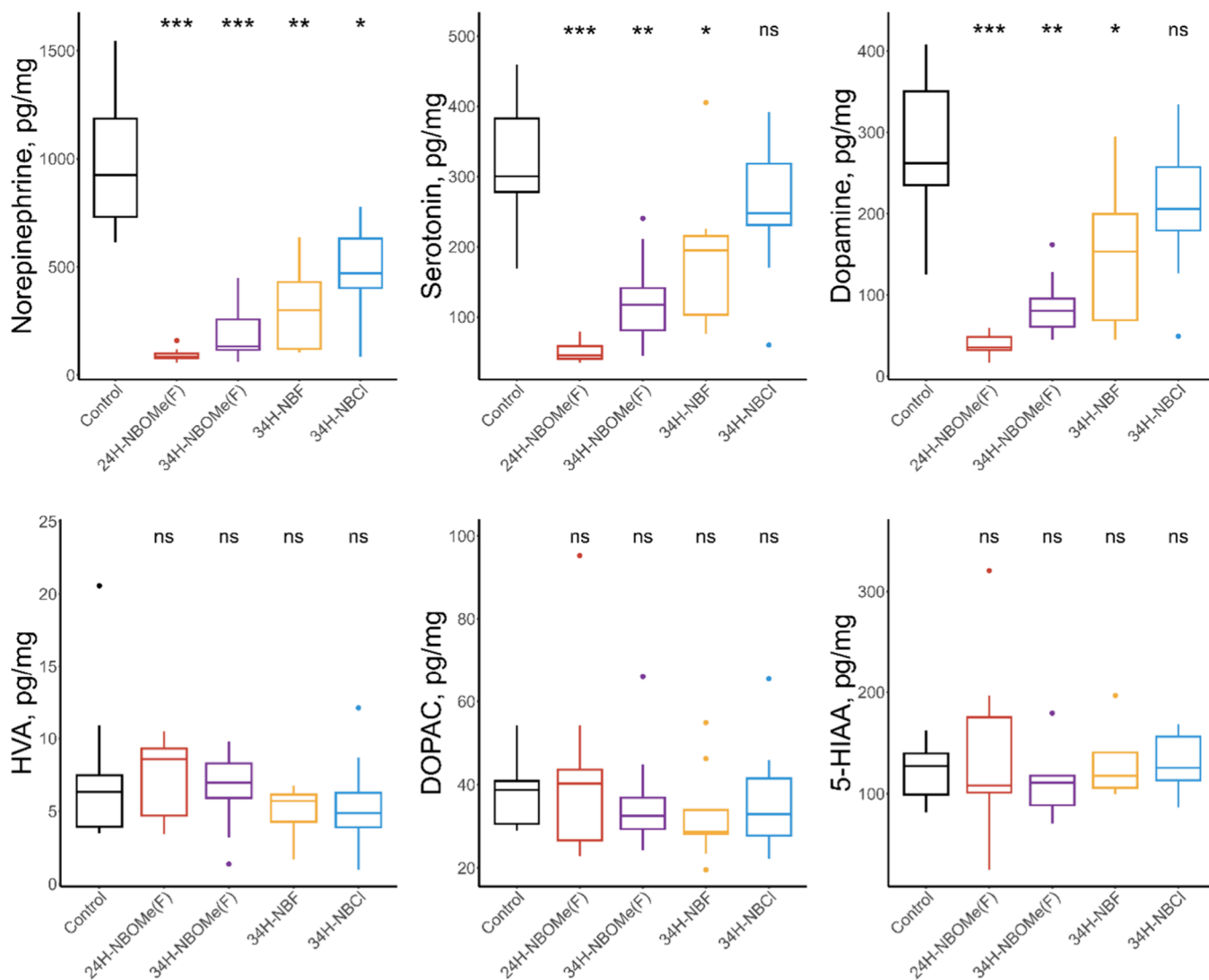
Figure 3. Effects of studied NBOMes on zebrafish brain monoamines and their metabolites, assessed by HPLC. Data are presented as mean ± SD. 5-HT, serotonin; 5-HIAA, 5-hydroxyindoleacetic acid; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, ns, nonsignificant (posthoc Dunn's test for significant Kruskal−Wallis data). $n = 9$−11 per group. For interpretation of references to color in figure legends please refer to the online version of this article.
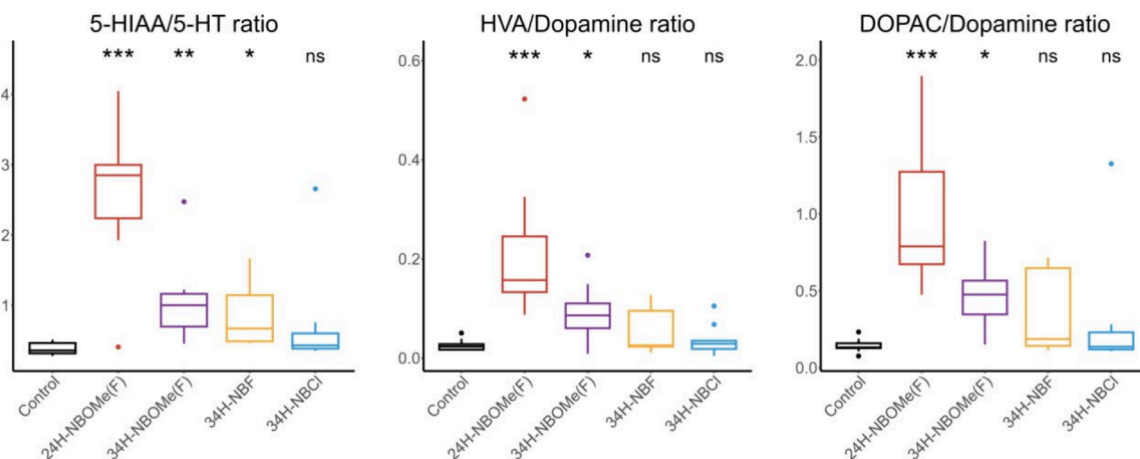
Figure 4. Effects of the four studied NBOMes on zebrafish brain monoamine turnover rates. Data are presented as mean ± SD. 5-HT, serotonin; 5-HIAA, 5-hydroxyindoleacetic acid; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, ns, nonsignificant (posthoc Dunn's test for significant Kruskal−Wallis data). $n = 9$−11 per group.

Table 2. Selected Biological Activities Predicted for the Four NBOMe Compounds the Four Tested NBOMe in the Present Study (Also See Table S2 for Details)[a]

activity	N	compounds
choline oxidase inhibitor	4	**34H-NBCl, 34H-NBF,** 34H-NBOMe(F), 24H-NBOMe(F)
antineurotic	4	**34H-NBCl, 34H-NBF,** 34H-NBOMe(F), 24H-NBOMe(F)
INS expression inhibitor	4	**34H-NBCl,** 34H-NBF, 34H-NBOMe(F), 24H-NBOMe(F)
neurotransmitter antagonist	4	34H-NBF, 34H-NBCl, 34H-NBOMe(F), 24H-NBOMe(F)
nitric-oxide synthase stimulant	4	34H-NBF, 34H-NBCl, 34H-NBOMe(F), 24H-NBOMe(F)
calcium channel activator	4	34H-NBCl, 34H-NBF, 34H-NBOMe(F), 24H-NBOMe(F)
benzodiazepine omega receptor agonist	4	34H-NBCl, 34H-NBF, 24H-NBOMe(F), 34H-NBOMe(F)
serotonin release stimulant	3	**34H-NBF, 34H-NBCl,** 34H-NBOMe(F)
vasodilator, cerebral	3	**34H-NBF,** 34H-NBCl, 34H-NBOMe(F)
neuroprotector	3	24H-NBOMe(F), 34H-NBOMe(F), 34H-NBCl
dopamine release stimulant	3	34H-NBCl, 34H-NBF, 34H-NBOMe(F)
antineurogenic pain	**2**	**34H-NBOMe(F), 24H-NBOMe(F)**
amyloid beta aggregation inhibitor	2	34H-NBOMe(F), 24H-NBOMe(F)
analgesic	2	34H-NBOMe(F), 24H-NBOMe(F)
serotonin uptake stimulant	2	34H-NBCl, 34H-NBF
sigma 1 receptor agonist	2	34H-NBCl, 34H-NBF

[a]N, total number of compounds predicted. Activities and compounds that are predicted with significance (Pa > 0.5) are bolded.

34H-NBF is mildly anxiogenic,[70] unlike its chronic action reported here. Similarly, acute exposure to 24H-NBOMe(F), 34H-NBOMe(F), and 34H-NBCl, but not to 34-NBF, increases the 5-HIAA/serotonin and DOPAC/dopamine ratios, indicating elevated turnover rates of these two key brain monoamines.[70] However, chronic treatment here with all four agents decreased norepinephrine levels and, except 34H-NBCl, dopamine and serotonin levels without altering their respective metabolites (Figures 3 and 4). At the same time, these compounds (except 34H-NBCl) increased serotonin turnover, and both NBOMe(F) compounds (24H-NBOMe(F) and 34H-NBOMe(F)) also increased dopamine turnover, closely resembling the effects of this group on monoamine metabolism observed previously acutely.[70] As numerous studies in rodents show tolerance at the level of behavior and neurotransmitter release following repeated administration of NBOMe drugs,[86–88] our findings in zebrafish seem to contradict these findings suggesting a relatively stable effect of compounds throughout exposure duration (lack of significant treatment × day interaction effect), likely due to species- or compound-related differences.

In general, the potency of reducing monoamine levels and increasing their turnover rates was 24H-NBOMe(F) > 34H-NBOMe(F) > 34H-NBF > 34H-NBCl (Figures 3 and 4). These findings further support the pronounced ability of NBOMe(F) compounds to increase monoamine turnover in the zebrafish brain, necessitating further in-depth studies of these substitutes in other model systems in vivo. Interestingly, for example, neurochemical effects of NBOMe derivatives in rodents are associated with elevated glutamatergic neuro-transmission, which, in turn, has been linked to 5-HT$_{2a}$ receptor hyperactivation.[86,89] While it remains unclear whether

such a regulation loop exists in zebrafish, the overlaps between zebrafish serotonin and glutamate signaling have already been noted.[90,91] Thus, it may be promising to further examine the neurochemical effects of NBPEAs in zebrafish by assessing glutamatergic neurotransmission, such as extracellular gluta-mate levels.

In summary, testing chronic CNS effects of four novel NBPEAs (34H-NBF, 34H-NBCl, 24H-NBOMe(F), and 34H-NBOMe(F)) in zebrafish provided valuable insights into behavioral, neurochemical, and pharmacological effects of these recently synthesized NBPEAs in vivo, supporting some of these compounds as potential antistress agents. Our findings also corroborate the rapidly growing potential of zebrafish as a promising model for preclinical screening of small psychoactive molecules.

4. METHODS

4.1. Materials and Reagents. The synthesis of NBPEAs was performed similarly to ref 70 using 2,4-dimethoxybenzaldehyde (98%), 3,4-dimethoxybenzaldehyde (99%), 2-fluorobenzaldehyde (97%), 2-chlorobenzaldehyde (97%), 2-bromobenzaldehyde (98%), 2-(trifluoro)methoxybenzaldehyde (96%, Alfa Aesar, Kandel, Ger-many), 2-methoxybenzaldehyde (98%), nitromethane (96%), lithium aluminum hydride (95%), sodium borohydride (99%, Acros Organics, Fair Lawn, NJ, USA), glacial acetic acid (≥99%, Russian GOST 61-75), hydrochloric acid (≥99%, Russian GOST 3118-77), sodium chloride (≥99%, Russian GOST 4233-77), ammonium acetate (≥98.5%, Russian GOST 3117-78), and sodium hydroxide (≥99%, Russian GOST 4328-77, Reachem, Ltd., Moscow, Russia), and anhydrous magnesium sulfate (≥99.0%, Bioshop, Canada) was used without further purification. Solvent tetrahydrofuran (99.8%, Tathimprodukt, Ltd., Kazan, Russia) was dried and purified using standard procedures.[92] Organic solvents, such as methylene chloride (≥99%, TU 2631-019-44493179-98), acetone (≥99%, TU 2633-018-44493179-98), propanol-2 (≥99%, TU 2632-181-44493179-14, EKOS-1, Moscow, Russia), diethyl ether (≥99%, TU 2600-001-45286126-11, Medhimprom, Ltd., Moscow, Russia), and methanol (≥99%, Russian GOST 6995-77, Vekton, Ltd., St. Petersburg, Russia), were used without further purification. Flash chromatography was performed using high-purity-grade Merck silica gel (9385, 60 Å, 230−400 mesh, Merck KGaA, Darmstadt, Germany).

Briefly, 24H-NBOMe(F) and 34H-NBOMe(F) were synthesized from substituted phenethylamines and 2-(trifluoromethoxy)-benzaldehyde, 34H-NBF was synthesized from substituted phenethyl-amine and 2-fluorobenzaldehyde, and 34H-NBCl was synthesized from substituted phenethylamine and 2-chlorobenzaldehyde. A mixture of substituted benzaldehyde and CH$_2$Cl$_2$ (40 mL) was slowly added to a mixture of substituted phenethylamine and CH$_2$Cl$_2$ (40 mL) while being cooled to 0 °C and stirred for 4−5 h. The reaction completeness and formation of imines were analyzed using gas chromatography/mass spectrometry (GC-MS). The solvent was then removed, and the remaining substance was dissolved in 80 mL of pure methanol. NaBH$_4$ (0.839 g, 22.08 mmol) was added to the solution over 1 h, and after the reaction completion, the mixture was neutralized with 20 mL of distilled water, and the organic phase was evaporated. The 3M aqueous NaOH was then added, and the product was extracted with CH$_2$Cl$_2$, washed with water, and dried. Hydrochlorides were obtained by adding 5 mL of propan-2-ol saturated with 5 M hydrochloric acid, washed with diethyl ether, and dried. The drugs were purified using flash chromatography on high-purity Merck silica gel (9385, 60 Å, 230−400 mesh), yielding white powder hydrochlorides. Analytical data used in this study was reported previously.[70,73]

4.2. Animals and Housing. Adults (3−5 months), experimen-tally naïve wild-type short-fin zebrafish (1:1 male:female ratio), were obtained from a local distributor (Axolotl, Ltd., St. Petersburg, Russia). The animals were housed for at least 3 weeks in standard conditions in groups of 10−15 fish in 4 L tanks (2.5−3.75 fish/L) at

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the Aquatic Facility of Almazov National Medical Research Center (St. Petersburg, Russia). The tanks utilized ZebTec Active Blue Stands with a Water treatment unit (Tecniplast, West Chester, USA) and were filled with filtered system water maintained at a temperature of 27 ± 0.5 °C and pH 7.4. The illumination in the holding room, set at 950−960 lx, was provided by 18 W fluorescent light tubes following a 12/12 light/dark cycle. The zebrafish were fed twice a day with small food pellets called Neon Micro Granules, suitable for fish measuring 1−2 cm in length (Dajana Pet, Bohuňovice, Czech Republic), according to zebrafish care standards.[93] All of the fish used in the study belonged to the same baseline population. Fish were acclimated for at least 2 weeks prior to the beginning of the experiments. Experimental groups were housed in water containing 0.2 mg/L (*N*-(2-trifluoromethoxybenzyl)-2-(2,4-dimethoxyphenyl)-ethylamine (24H-NBOMe(F), *N*-(2-trifluoromethoxybenzyl)-2-(3,4-dimethoxyphenyl)ethylamine 34H-NBOMe(F)), *N*-(2-fluorobenzyl)-2-(3,4-dimethoxyphenyl)ethylamine (34H-NBF), or *N*-(2-chlorobenzyl)-2-(3,4-dimethoxyphenyl)ethylamine (34-NBCl), whereas the control group was housed in drug-free water. The outbred population selection for this study was based on population validity considerations and their relevance to the present study. Briefly, although genetically controlled models (e.g., inbred zebrafish strains) can be a more reproducible and reliable system for neurogenetics research, studying CNS drug activity, such as in the present study, is meant to parallel testing drugs in "real" human genetically heterogeneous populations.[94] Thus, using outbred zebrafish (such as those selected here) was deemed a more populationally valid and translationally relevant approach for the purpose of this study.

The study experimental design and its description here, as well as data analysis and presentation, adhered to the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines for reporting animal research and PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) guidelines for planning animal research and testing; see the Ethical Confirmation statement for approval and ethical details of animals use in the research. All animals were allocated to the experimental groups randomly using a random number generator (https://www.random.org/).

4.3. Behavioral Testing. Behavioral testing was conducted on specific days following the beginning of treatment, between 11:00 and 14:00 h. Prior to testing, all of the fish were transported from the holding room and allowed to acclimate to the testing room for at least 2 h. Then, fish were individually subjected to the 5 min novel tank test (*n* = 19−21 per group). Upon completion of behavioral testing, the fish were returned to their respective home tanks. The novel tank test was chosen here for its widespread use as a behavioral test that exhibits sensitivity to alterations in anxiety and locomotion in zebrafish, and it was performed following a similar protocol to that described in refs 52 and 95. The apparatus comprised a 2 L rectangular tank made of acrylic, 20 cm high, 20 cm long, and 5 cm wide.[52,55,96−99] The tank was filled with water up to a height of 19 cm and divided into two equal virtual horizontal sections, representing the top and bottom halves of the tank. The front side of the tank was fully transparent to enable animal observation during the test, while the back and lateral sides were nontransparent and white in color, aiming to enhance contrast and minimize external visual cues during behavioral recording.[55,58,99,100] EthoVision XT11.5 software (Noldus IT, Wageningen, Netherlands) was utilized to analyze the recorded videos and assess parameters including mean velocity (cm/s), time spent in the top section (s), and time spent immobile (s). The mean velocity parameter reflects locomotor activity, analogous to rodent tests, while the preference for the top dwelling corresponds to anxiolytic-like behavior, with increased time spent at the top indicating reduced anxiety.[94,101−103]

4.4. Brain Neurochemistry. Since monoamines play a central role in the CNS effects of NBOMes,[28−32,104,105] we analyzed the concentrations of norepinephrine (NE), serotonin (5-HT), dopamine (DA), and their metabolites 5-hydroxyindoleacetic acid (5-HIAA), 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) in whole brains of zebrafish. We also evaluated 5-HIAA/ serotonin, DOPAC/HVA, and DOPAC/dopamine ratios that reflect brain serotonin and dopamine turnover, respectively, using HPLC, similarly to refs 52, 55, 96, 97, 99, and 106.

On day 14, immediately after behavioral testing, the fish (*n* = 9−11) were euthanized in ice-cold water and decapitated and their brains were extracted, dissected on ice, and stored in liquid nitrogen. On the day of analysis, the brain samples were weighed and placed into 10 μL of a 0.1 M perchloric acid solution (Sigma-Aldrich, St. Louis, MO, USA) containing 100 ng/mL 3,4-dihydroxybenzylamine (DHBA, internal standard) per 1 mg of the brain tissue. The samples were sonicated for 10 s at half-power settings followed by centrifugation and filtration through a 0.22 μm Durapore-PVDF centrifuge filter (Merck Millipore, Billerica, MA, USA). HPLC analysis was performed using a CA-5ODS column and an HTEC-500 chromatograph (Eicom, San Diego, CA, USA) equipped with a carbon WE-3 G electrode and a +650 mV applied potential, similar to previous studies.[52,55,96,97,99,106] The mobile phase for chromatography consisted of 0.1 M phosphate buffer, 400 mg/L sodium octyl sulfonate, 50 mg/L ethylenediaminetetraacetic acid (EDTA), and 17% methanol and was adjusted to pH 4.5 using phosphoric acid (all reagents were purchased from Sigma-Aldrich, St. Louis, MO, USA). Concentration data were normalized using the individual DHBA sample concentrations and presented as pg/mg for brain tissue weight.

4.5. Statistical Analyses and Data Handling. The present study utilized GLM to analyze dynamic behavioral changes observed during the NBPEA treatment. GLM is a generalization of regression methods that allows variables to have distributions other than normal, thus making it suitable for non-normal data analyses.[107] GLM distribution and link function were chosen for each model based on the lowest AIC score. For the total distance (cm), top duration (s), and top entries (*n*), the most appropriate choice was Gaussian distribution with "identity" link. Inverse Gaussian distribution (inverse squared link) was chosen for the freezing duration. To analyze the effects of the day, group (treatment), and day × group interaction, we performed the Wald chi-square (χ^2) test (ANOVA Type II) for GLM fits (Table 1). To examine the behavioral effects of the drugs over the testing days, we also performed posthoc pairwise comparisons of estimated marginal means ("EMMeans") on significant GLM/Wald data with Dunnet's *p*-value adjustment for multiple comparisons (Tables S3 and S4). HPLC data on monoamines and their metabolites were analyzed using the Kruskal−Wallis (KW) test followed by posthoc Dunn's test for pairwise comparisons for significant KW data (Table S5). Graphs were constructed using the ggplot2 R package version 3.4.2 (https://ggplot2.tidyverse.org).

4.6. *In Silico* Estimation of the Neuropsychotropic Activities. The PASS (Prediction of Activity Spectra for Substances) software v. 2022 was used for prediction of neuropsychotropic effects and related molecular mechanisms of action for the studied compounds.[76] PASS-2022 predicts more than 8000 pharmacological effects and mechanisms of action based on the structural formula of compounds and[76,77] based on the training set including >1.6 million structures with known biological activity. Multilevel Neighborhoods of Atoms (MNA) descriptors are used by the PASS database for representation of the molecular structure and Bayesian algorithm for revealing structure−activity relationships. The mean accuracy of prediction (AUC) calculated by leave-one-out cross-validation is 0.93. The prediction result is a list of biological activities with probability to be active (Pa) and to be inactive (Pi). The higher the Pa value and the lower the Pi value, the greater the probability of confirming the predicted biological activity in experimental studies. The prediction results were analyzed by PharmaExpert software[78] that includes over 15,000 known mechanism−effect relationships (Table S1).

■ ASSOCIATED CONTENT

Data Availability Statement

The data sets generated and/or analyzed during the present study may be available from the corresponding authors upon a reasonable request.

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