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Behavioral Analysis of Dopaminergic Activation in Zebrafish and Rats Reveals Similar Phenotypes

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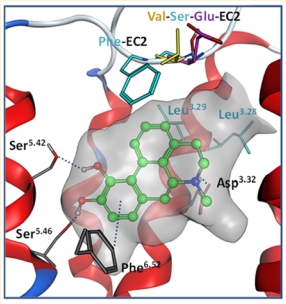
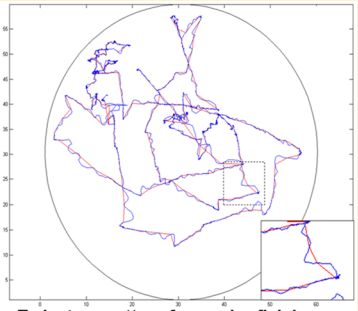
Supporting Information

ABSTRACT: Zebrafish is emerging as a complement to mammals in behavioral studies; however, there is a lack of comparative studies with rodents and humans to establish the zebrafish as a predictive translational model. Here we present a detailed phenotype evaluation of zebrafish larvae, measuring 300−3000 variables and analyzing them using multivariate analysis to identify the most important ones for further evaluations. The dopamine agonist apomorphine has previously been shown to have a complex U-shaped dose−response relationship in the variable distance traveled. In this study, we focused on breaking down distance traveled into more detailed behavioral phenotypes for both zebrafish and rats and identified in the multivariate analysis low and high dose phenotypes with characteristic behavioral features. Further analysis of single parameters also identified an increased activity at the lowest concentration indicative of a U-shaped dose−response. Apomorphine increased the distance of each swim movement (bout) at both high and low doses, but the underlying behavior of this increase is different; at high dose, both bout duration and frequency increased whereas bout max speed was higher at low dose. Larvae also displayed differences in place preference. The low dose phenotype spent more time in the center, indicative of an anxiolytic effect, while the high-dose phenotype had a wall preference. These dose-dependent effects corroborated findings in a parallel rat study and previous observations in humans. The translational value of pharmacological zebrafish studies was further evaluated by comparing the amino acid sequence of the dopamine receptors (D_1−D_4), between zebrafish, rats and humans. Humans and zebrafish share 100% of the amino acids in the binding site for D_1 and D_3 whereas D_2 and D_4 receptors share 85−95%. Molecular modeling of dopamine D_2 and D_4 receptors indicated that nonconserved amino acids have limited influence on important ligand−receptor interactions.



Apomorphine docked to Zebrafish dopamine D_2a-c receptors



Trajectory pattern for a zebrafish larvae at 30 fps (red) and 300 fps (blue)

KEYWORDS: *Zebrafish, dopamine agonist, behavioral model, receptor binding site, apomorphine*

Behavioral models are essential for studying and developing treatments for neurodegenerative and psychiatric disorders. The difficulties in translating early discoveries into new drugs for central nervous system (CNS) disorders have been linked to the use of target-based screening. Phenotypic screenings have been used in the discovery of almost all first-in-class, new molecular entities for CNS indications.[1] This has highlighted the importance of classical pharmacology and the use of in vivo models early in drug discovery. It is therefore essential to develop alternative or complementary animal models that are practical and cost efficient and have a reasonable throughput. Zebrafish, in particular, zebrafish larvae, have attracted attention as an alternative, for carrying out objective behavioral monitoring of relatively large groups.[2−9] The structure and molecular pathways of the CNS are highly conserved between mammals and zebrafish, and a functional nervous system is established after only 4−5 days of embryonic development enabling zebrafish larvae to perform complex behaviors such as swimming and feeding.[10−12] The major dopaminergic pathways in mammals are also represented in the zebrafish brain and homologous receptors for most of the mammalian subtypes have been identified in zebrafish, except for D5.[11−13] Zebrafish and humans share about 70% of the genetic code and the similarity in amino acid sequences of

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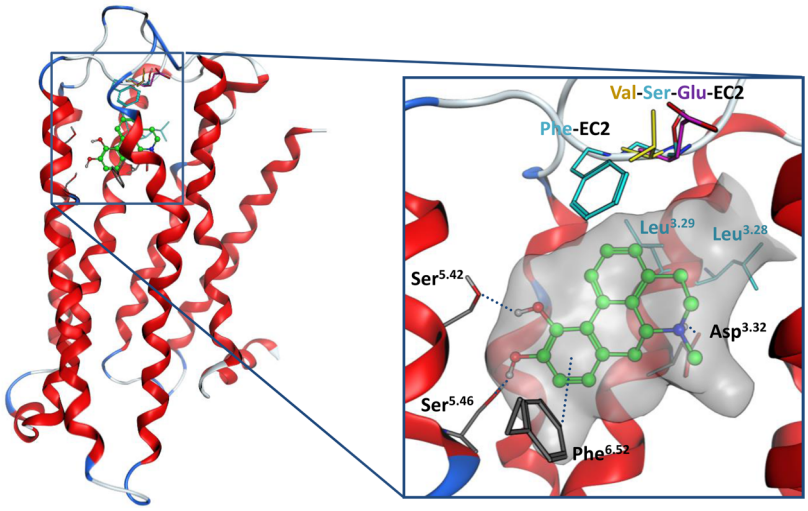

Figure 1. Zebrafish dopamine D_2 receptors (drd2) with nonconserved amino acids shown for each subtype (yellow, drd2a; cyan, drd2b; and purple, drd2c) with its seven transmembrane helixes and close up view of the agonist apomorphine bound in a well-defined binding pocket (gray). The agonist key interactions are highlighted with dotted lines ($Ser^{5.42}$, $Ser^{5.46}$, $Phe^{6.52}$, and $Asp^{3.32}$).

dopamine D_1−D_4 receptors are in the same order (59−72%).[14−16] Thus, it is not surprising that compounds that target the dopamine system show similar effects in zebrafish as in mammals; in general, dopamine agonists increase locomotor activity and antagonists decrease it.[17−20] However, to further predict the translational value of zebrafish in pharmacological studies, it is important to determine how well the ligand binding site of the dopamine receptors are conserved during vertebrate evolution.

Today, most biomedical research is conducted using a single model organism, typically mice or rats. Problems with this approach may arise as rodents and humans differ in several aspects. Finding correlations among phenotypical features in one species does not guarantee translation to other species. Therefore, adding a new species to the mix of comparisons can enhance the ability to identify biological features and mechanisms that are relevant and translate to responses in humans. The combination of multivariate analysis with multiple species could be beneficial for the identification of mechanisms that are common across all the studied species.

We are using a 300 fps camera capable of analyzing up to 192 zebrafish larvae in parallel, pooled into time periods of 1 to 5 min over recording sessions longer than 1 h. In this study, we used 48 larvae in each experiment and the analysis was based on a set of >30 parameters. Most studies to date have evaluated drugs impact on degree of activity, that is, distance traveled, which is a robust variable in an initial drug screen. However, to be able to further differentiate compounds from an activity screen using detailed behaviors, 300 fps was selected to be reasonable both for analyzing activity and for storing data in time periods of 1 h or more. For example, a fast turn in a zebrafish swim bout has a duration of about 10−12 ms, which would be captured in 3−4 frames using 300 fps, while using the typical frame rate of many imaging systems, 30−60 fps, these turns would neither be detected nor classified properly. Based on our research interest in disorders in CNS and dysfunction of the dopaminergic system in particular, we decided to characterize the drug apomorphine, which targets dopamine receptors, and to compare the results with a parallel study in rats and then correlate them to human studies.[21,22] Herein we present data that show how well the binding site of dopamine receptors is conserved between zebrafish, rat, and human and in detail characterize the behavioral response to apomorphine for zebrafish and the correlation to rat and human.

RESULTS AND DISCUSSION

Conservation between Zebrafish, Rat, and Human Binding Sites of Dopamine Receptors. The main target of apomorphine is dopamine receptors, which belong to the G-protein coupled receptors (GPCRs) protein super family. These receptors contain seven transmembrane helices (TM1−7), and the signaling state of each is associated with their active conformations. Studies have pointed to the key role that conformational changes in TM3, TM5, and TM6 have in GPCR activation and the binding of full agonist induces these changes.[33] It has been suggested that two evolutionary conserved motifs (D(E)$R^{3.50}$Y at the intracellular side of TM3 together with CWxx$P^{6.50}$ in TM6) are important for receptor activation of monoaminergic GPCRs.[34] Both these motifs are present in the zebrafish dopamine D_2 receptor. The homology between human and zebrafish dopamine receptors is approximately 70%, but the real meaning of this value is difficult to interpret because it says very little about translation to functionality.[14−16] We therefore compared the amino acid sequences for D_1−D_4 dopamine receptors for humans, rat, and zebrafish. Zebrafish has three subtypes (a−c) of both D_2 and D_4 receptors but only one subtype for D_1 and D_3 receptors. Overall, the agonist key interacting amino acids are conserved across all three species in D_1−D_4 receptors (i.e $Asp^{3.32}$, $Ser^{5.42}$, $Ser^{5.46}$, and $Phe^{6.52}$; see Figure 1s in the Supporting Information). D_1 and D_3 receptors of zebrafish and human have identical amino acid sequences in the binding pockets (20 amino acids within 4 Å from apomorphine in the drd2a_danre homology model), whereas the similarity between rat and human is 95% for D_1 and 100% for D_3 (see Table 3s in the Supporting Information). The homologies between zebrafish and human D_2 and D_4 receptors were in the range 80−95%; subtypes a and c are more conserved than b for both D_2 and D_4 (see Table 3s in the Supporting Information). The corresponding numbers for rat D_2 and D_4 receptors were 100% and 95%, respectively. Three dimensional structure models of all three subtypes (a−c) of zebrafish dopamine D_2 and D_4 receptors

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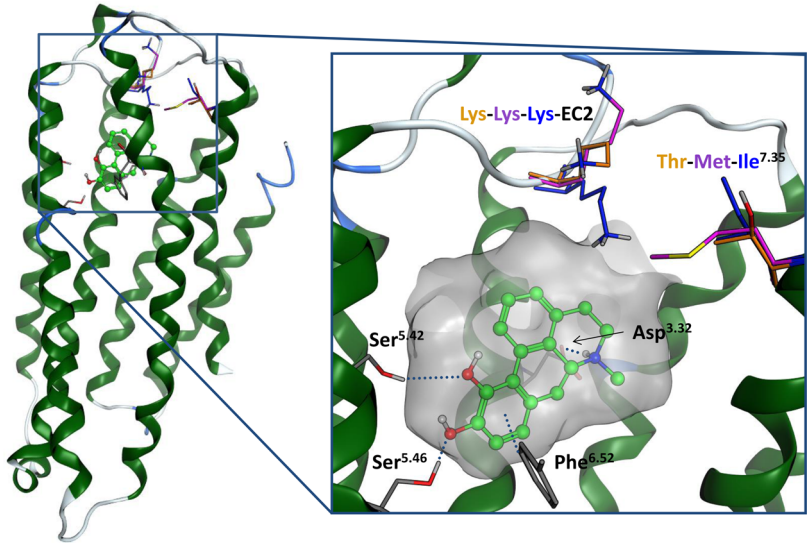

Figure 2. Zebrafish dopamine D_4 receptors (drd4) with nonconserved amino acids shown for each subtype (yellow, drd4a; purple, drd4b; and blue, drd4c) with its seven transmembrane helixes and close up view of the agonist apomorphine bound in a well-defined binding pocket (gray). The agonist key interactions are highlighted by dotted lines ($Ser^{5.42}$, $Ser^{5.46}$, $Phe^{6.52}$, and $Asp^{3.32}$).

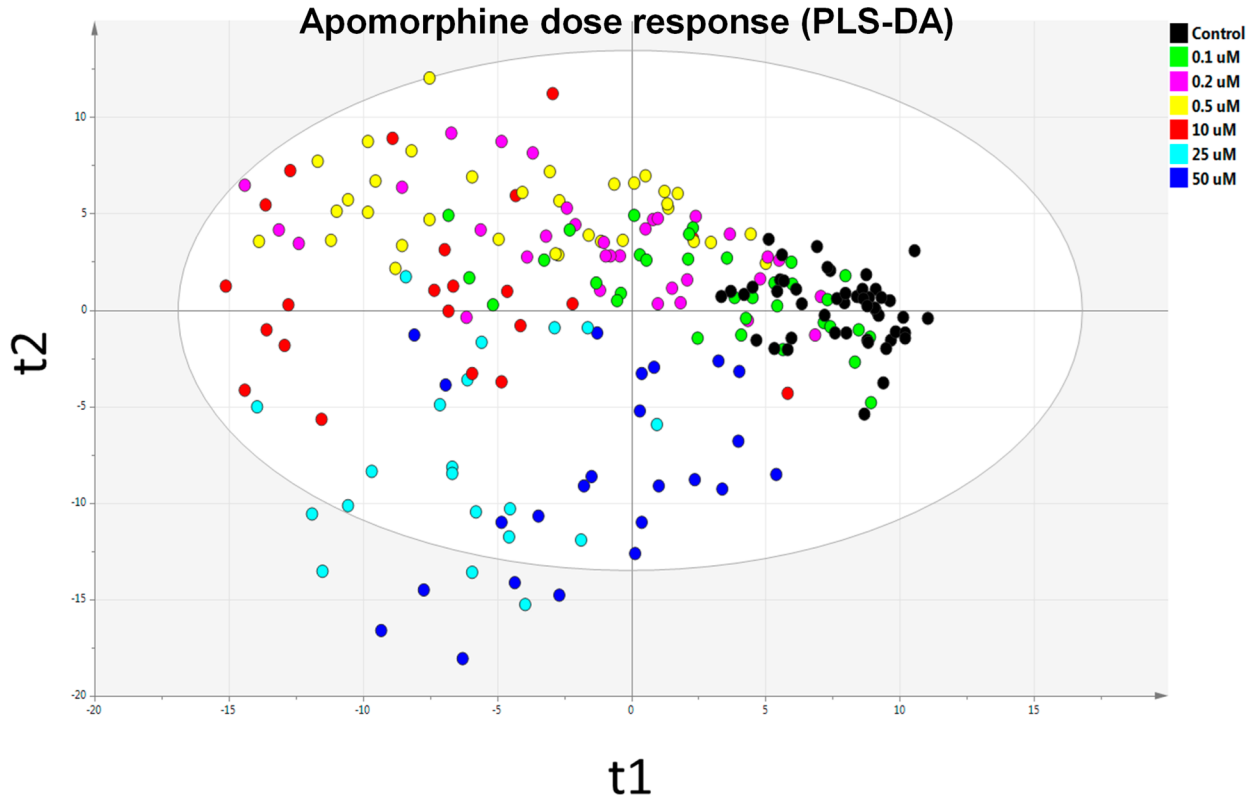

Figure 3. Projections to PLS-DA based on 30 behavioral parameters of larval behavior after dose-dependent exposure to apomorphine (black dots are controls and groups 0.1−50 μM are according to color labels). Analysis identified high-dose (50 μM) and low-dose (0.5 μM) phenotypes. PLS-DA, using discriminant variables denoting treatment group as dependent (Y) variables, and behavioral descriptors collected over 45 min as independent variable block (X). Shown are object scores (t1 vs t2), that is, composite variables representing the *X* variables, for the first two components.

were reconstructed to locate nonconserved amino acids in the ligand binding sites. A homology model complex of zebrafish D_2 receptor subtypes and apomorphine is shown in Figure 1. There is only one nonconserved amino acid between the human D_2 receptor and two of the zebrafish D_2 receptors (a and c) in the binding site. An isoleucine located in the second extracellular loop (EC2) in human is exchanged with a valine in drd2a (yellow) and a glutamic acid in drd2c (purple). The zebrafish D_2 receptor subtype drd2b has four nonconserved amino acids in the binding site. In addition to a serine in the previously mentioned position, there is a phenylalanine in zebrafish and isoleucine in human. There are also two leucines

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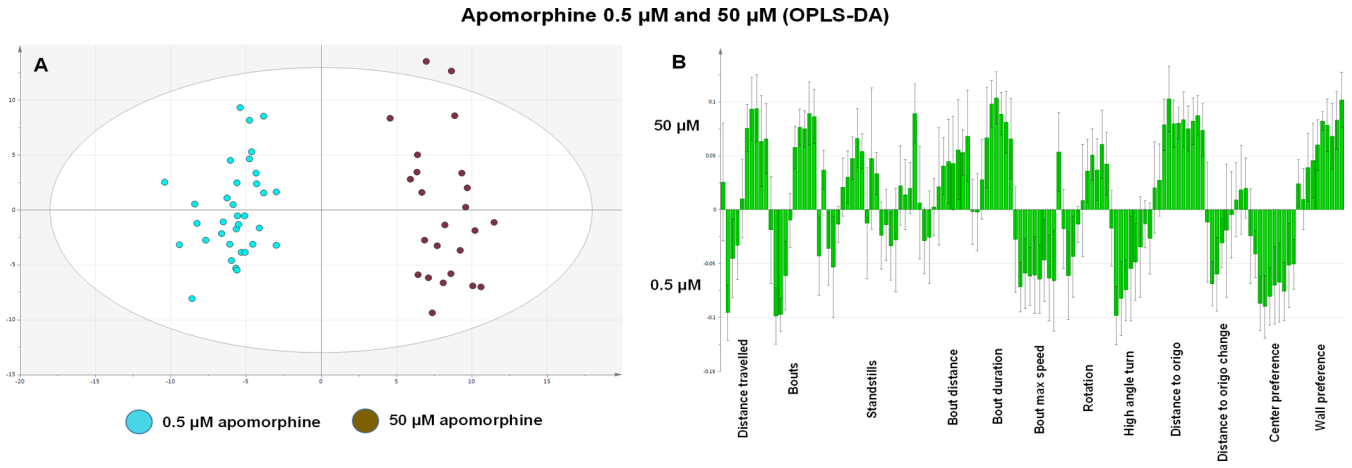

Figure 4. (A) OPLS-DA plot and (B) loading plot identifying parameters separating the groups treated with 50 and 0.5 μM in the multivariate data analysis.

in TM3 (Leu$^{3.28}$ and Leu$^{3.29}$ in drd2b_danre) which are phenylalanine and valine in the human D_2 receptor. Furthermore, the models show that the altered amino acids are in the outer layer of the binding pocket toward the extracellular side and are not directly involved in the ligand−receptor interactions mentioned above.

There are two nonconserved positions in the binding site between the human (drd4_human) and the three subtypes of zebrafish dopamine D_4 receptors (Figure 2). The first is located in the second extracellular loop (EC2) and is an arginine residue in the human receptor, while all zebrafish receptors have the lysine residue in the corresponding position. Also for rat D_4 receptor, the amino acid in this position is not conserved compared to human. Interestingly, zebrafish and human have amino acids (Lys and Arg) with the same type of basic side chain whereas rat instead has a cysteine residue. The second position in the binding site that is nonconserved is positioned on the 4 Å border and is a valine residue in human, while the corresponding residue in the zebrafish receptors is threonine (yellow, drd4a), isoleucine (purple, drd4b), and methionine (blue, drd4c) (Figure 2).

Behavioral Evaluation. Comparing recordings at 30 and 300 fps (Figure 4s), we could confirm that the latter frame rate is necessary to correctly interpret and differentiate between fast transient behavioral features such as turns. This is not to be confused with smoothing used to remove noise or unexplained behaviors. The 30 selected parameters include features of locomotor activities such as distance traveled, number of swim bouts and standstills, and swim bout-specific parameters such as distance, duration, and maximum speed. Other parameters were angular changes and the number of large angle turns, in addition to preference parameters for place, that is, the time spent in respective annulus from the center to periphery and well-sector preferences (complete list with definition in Supporting Information). In addition, combinations of these parameters were also included. These data were evaluated using multivariate analysis and two-way RMANOVA of single parameters. Furthermore, based on the initial study, we performed an expanded analysis of relative frequency of types of turns.[17,35−37]

High- and Low-Dose Phenotypes in Zebrafish Larvae with Discrete Behavioral Profiles Identified by Multivariate Analysis. Initial multivariate data analysis of all seven treatment groups (the two control groups were combined into one before analysis) using projections to latent structures-discriminant analysis (PLS-DA) revealed clear dose-dependent trends when treating zebrafish larvae with high (50, 25, 10 μM) or low doses (0.5, 0.2, 0.1 μM) of apomorphine, as shown in Figure 3.

The reproducibility between experiments was good. The control groups from the high-dose and low-dose experiments overlapped well, although the experiments were performed several months apart (Figure 3). Irons et al., reported an increased distanced moved at 50 μM, and our study verified that this group separates out from both the controls and the other treatment groups.[20] We were also able to identify a low-dose phenotype with maximum separation from the control at 0.5 μM, which verifies that the platform is sensitive enough to pinpoint phenotypes based on modest changes in multiple parameters. The intermediate dose at 10 μM clusters between the low and high treatment groups, suggesting a dose-dependent transition between the two phenotypes (Figure 3).

Next, an orthogonal partial least-squares-discriminant analysis (OPLS-DA) model was created to identify variables separating 0.5 μM and 50 μM doses (Figure 5s, Supporting Information). OPLS-DA facilitates the identification of class-discriminating variables, particularly in a two-class discrimination problem. Variables in the column plot of the predictive loading (refined selection of variables for clarity, Figure 4) that show a positive difference between the 50 and 0.5 μM doses are higher at 50 μM compared to 0.5 μM, while the ones with a negative difference show a decrease at 50 μM compared to 0.5 μM. The uncertainty in each loading value is indicated by 95% confidence intervals estimated with jackknifing. OPLS-DA was used for a detailed analysis of selected single parameters separating high- and low-dose phenotypes. The most significant parameters are listed in Table 1.

Analysis of Single Parameters. A selection of parameters significantly contributing to the difference between larvae treated with high- and low-dose phenotypes was then analyzed in detail (summarized in Table 1). The covariance between some of the included parameters is not corrected for in the single parameter statistical analysis.

In addition to increased distance traveled at high concentration (50 μM) and an opposite effect at low concentration (0.5 μM), there was a trend toward increased distance traveled at the lowest concentration 0.1 μM compared to control confirming previous results by Irons et al. (Figure

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Table 1. Profiling of High- versus Low-Dose Phenotype Comparing Apomorphine Treated and Control Larvae[a]

parameter	trend high-dose phenotype	trend low-dose phenotype
distance traveled	down then up (biphasic)	no effect/down
bouts	down then up (biphasic)	down
bout distance	up	up
bout duration	up	no effect
bout maximum speed	no effect/down	up
center preference (0−R1)	no effect/down	up
wall preference (R3−R4)	up	down
large angle turn (Turn2)	no effect/up	up
distance from center	up	down
Rotation	up	no effect/up
standstill short (5−10 s)	down	down

[a]Trends with increased concentration of apomorphine in each group are reported.

5).[20] Also the number of bouts followed the same trend. Apomorphine induce a U-shaped dose response curve in which larvae treated with 50 and 0.1 μM have a significantly higher distance traveled and number of bouts compared to 0.5 μM in the time period where the most pronounced effect was detected (25−45 min).

In the next section, further detailed analyses of selected parameters over time demonstrate several unique characteristics for the high- and low-dose groups.

High Doses of Apomorphine Increases Number of Spontaneous Bout Initiations Extend Bout Duration and Induce a Wall Place Preference. The high-dose phenotype is characterized by three key features separating it from the control group and the low-dose phenotype. An overall increase in locomotor activity was observed in larvae treated with a high dose of apomorphine (Figure 6A and B). There was a significant effect (RMANOVA) of treatment in distance traveled ($F_{3.81}$ = 4.683, p < 0.05); larvae treated with 50 μM swam significantly longer distances compared to those treated with 10 μM. There was a significant effect of time ($F_{9.729}$ = 7.7024, p < 0.05); the highest dose (50 μM) demonstrated a biphasic response in distance traveled: first, there was a significant reduction compared to the control group during the initial time interval (0−5 min), and then there was a transition to a significant increase wherein the maximum effect was reached after 25−35 min (Figure 6A). The group treated with 25 μM showed a similar biphasic response, although the effect was shifted in time but the intermediate dose group of 10 μM showed instead a decrease in distance traveled during the entire experiment. A similar biphasic response pattern was also seen in the number of bouts and a significant decrease in the number of bouts was observed for all treatment groups compared to the control at 0−10 min, followed by a significant increase for 50 μM compared to 10 μM (Figure 6B).

Bout-specific parameters were also altered after treatment with high doses of apomorphine (Figure 6C−E). The high dose phenotype demonstrated an increased bout duration and there was a significant effect of apomorphine (RMANOVA: $F_{3.68}$ = 26.478, p < 0.0001), wherein the larvae treated with 50 μM had a significantly increased duration per bout compared to other groups (6C). From 15 min and later, the group treated with 50 μM showed significantly higher bout duration than the control, and at 40−45 min, the group treated with 25 μM also exhibited significantly increased bout duration compared to control.

Longer bout durations resulted in an increased distance per bout, and there was significant effect of apomorphine treatment (RMANOVA: $F_{3.66}$ = 14.228, p < 0.0001, Figure 6D), wherein larvae treated with 50 μM exhibited a significantly increased distance traveled per bout compared to other groups (Figure 6D). Based on the increase in duration per bout, the larvae treated with 25 μM also traveled a significantly greater distance per bout compared to the control. The onset was also dose dependent, in that the distance traveled for the 50 μM group was significantly greater than the control group from 15−20 min and the 25 μM group at 40−45 min. In addition, apomorphine had a significant effect (RMANOVA: $F_{3.66}$ = 7.1351, p < 0.001) on the maximum speed per bout (Figure 6E). However, at 50 μM there was no effect but larvae treated with 10 μM had a significantly increased maximum speed per bout compared to the control.

The parameters representing place preference showed distinct differences between the high- and low-dose phenotypes (Figures 6F and 7E). The position of the zebrafish larvae in four areas was analyzed; the center circle (0−R1), the periphery annuli close to the wall (R3−R4), and between (R1−R2) and (R2−R3) (see the Supporting Information for a detailed definition).

Larvae treated with a high dose of apomorphine preferentially performed more swim bouts closer to the wall (R3−R4) (RMANOVA: $F_{3.75}$ = 6.2422, p < 0.001); and the 50 μM group has a significantly higher wall preference compared to the 10 μM group (Figure 6F).

Apomorphine (0.5 μM) Induces a Hypokinetic State with Reduced Motor Coordination and Less Thigmo-

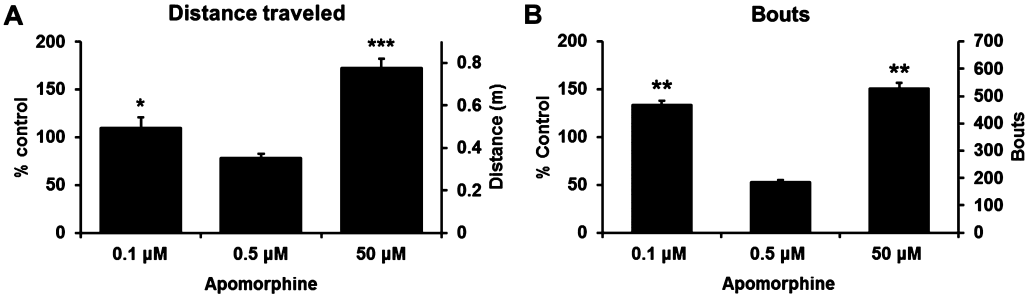

Figure 5. Locomotor activity represented by (A) distance traveled and (B) bouts average over time period of 25−45 min and control group set to 100%, n = 36 (control), 34 (0.1 μM), 35 (0.5 μM), and 24 (50 μM). Statistical analysis was performed using RMANOVA followed by post hoc test (HSD). Significance reported when comparing 50 and 0.1 μM with 0.5 μM groups: *p < 0.05, **p < 0.001, and ***p < 0.0001. Bars represent mean over 20 min ± SEM.

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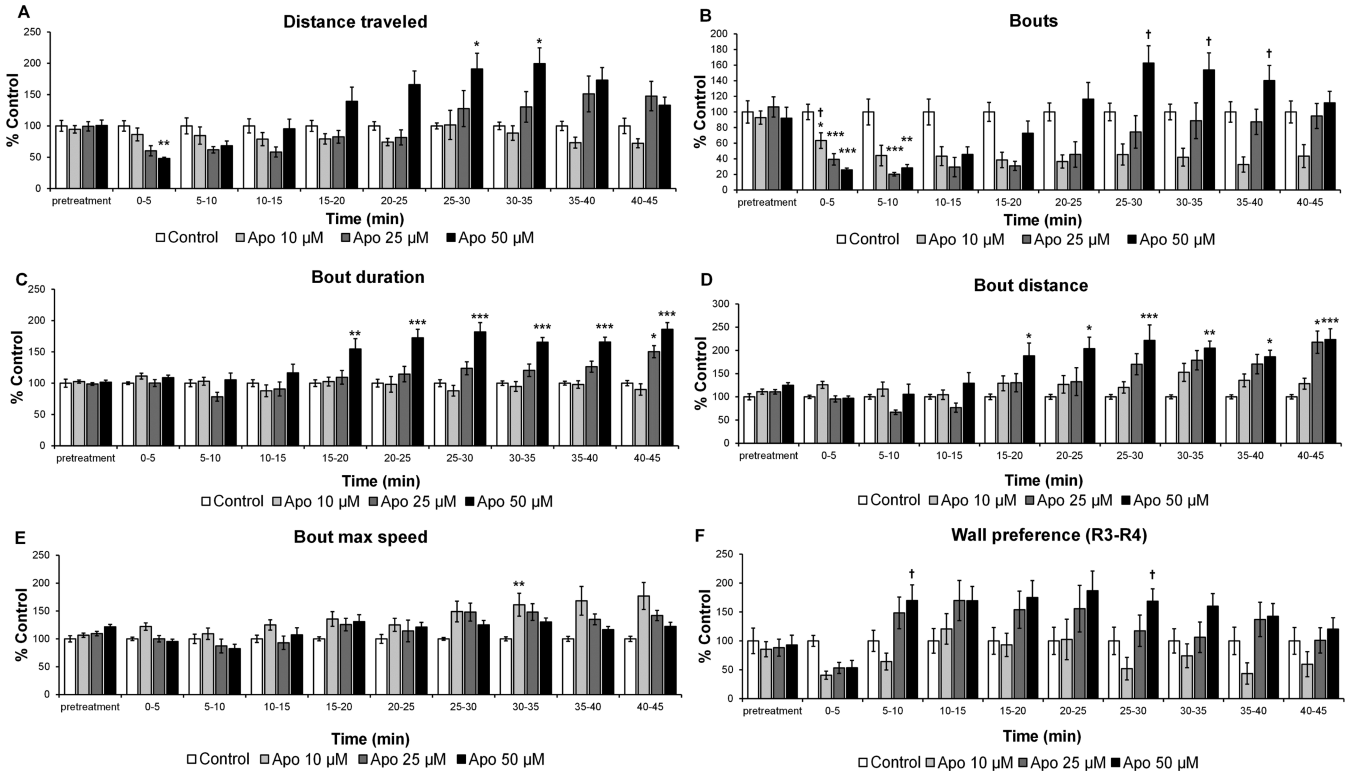

Figure 6. Detailed analysis of parameters (A) distance traveled, (B) bouts, (C) bout duration, (D) bout distance, (E) bout max speed, and wall preference (F) for the high-dose phenotype, n = 24 (control), 24 (50 μM), 21 (10 μM), and 24 (10 μM). Significance reported when comparing treatment with control groups: $^{*}p < 0.05$, $^{**}p < 0.001$, $^{***}p < 0.0001$. Significance reported when comparing 50 with 10 μM: $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.001$, $^{\dagger\dagger\dagger}p < 0.0001$. Bars represent mean over 5 min ± SEM.

taxis. Larvae treated with 0.5 and 0.2 μM apomorphine had a slightly lower activity level compared to the control group (not significant), in line with a possible interaction with presynaptic autoreceptors (Figure 7A and B). However, there was a significant effect in distance traveled in the low-dose group (RMANOVA; $F_{3.128} = 2.79$, $p < 0.05$), wherein distance traveled was significantly higher in the 0.1 μM group compared to the 0.5 μM group (Figure 7A). A similar trend was also observed for the number of bouts, in which a significant effect of apomorphine (RMANOVA: $F_{3.128} = 3.2735$, $p < 0.05$) was detected (Figure 7B). Larvae treated with 0.1 μM had a significantly higher number of bouts compared to 0.5 μM. Further analysis demonstrated a significant apomorphine over time interaction (RMANOVA: $F_{27.1152} = 2.2141$, $p < 0.001$), in which the number of bouts in the group treated with 0.1 μM was significantly higher than in the 0.5 μM group at 25−30 min.

We also identified bout-specific changes in the low-dose group. Similar to the high-dose phenotype bout distance was increased (Figure 7D). Analysis demonstrated that treatment with apomorphine had a significant effect on bout distance (RMANOVA: $F_{3.122} = 14.751$, $p < 0.0001$), wherein all treatment groups showed an increased bout distance compared to the control. There was also a significant interaction effect of apomorphine over time (RMANOVA: $F_{27.1098} = 3.0843$, $p < 0.0001$), in which the peak effect for 0.5 and 0.2 μM treatment groups was between 15−30 min. The onset of apomorphine treatment with 0.5 μM occurred slightly earlier compared to the 50 μM dose, beginning at 5−10 min, but was not significantly different from the control until 15−20 min. In addition, the increase in bout distance compared to control was not as pronounced for 0.5 μM as it was for 50 μM (160% vs 215%). However, the cause of the increased bout distance was different; while the high-dose phenotype performed more bouts with longer durations the low-dose phenotype instead performed fewer bouts but at increased maximum speed per bout (Figure 7C). Statistical analysis demonstrated a significant effect of apomorphine for bout max speed (RMANOVA: $F_{3.122} = 21.606$, $p < 0.0001$), wherein all treatment groups had a higher maximum speed per bout compared to the control. In addition, the maximum speed was significantly higher for the 0.5 μM group than for the 0.1 μM group (HSD; $p < 0.001$). A significant interaction effect of apomorphine over time was found (RMANOVA: $F_{27.1098} = 4.487$, $p < 0.0001$). The onset of increasing maximum speed was at 5−10 min but was not significant compared to control until 10−15 min and later for the 0.5 μM treatment. Larvae treated with 0.2 and 0.1 μM also demonstrated a significantly higher maximum speed compared to control.

The larvae treated with 0.5 μM displayed an inverse and more pronounced place preference compared to those treated with 50 μM (Figure 7E). Statistical analysis (RMANOVA) demonstrated a significant effect of apomorphine on center/periphery 0−R1 ($F_{3.127} = 17.4768$, $p < 0.0001$), and the larvae treated with 0.5 μM performed significantly more bouts in the center area compared to the other groups. This center preference in the low-dose phenotype was confirmed by the significant effect of apomorphine treatment in the center/periphery R3−R4 (RMANOVA: $F_{3.127} = 18.770$, $p < 0.0001$), in which larvae treated with 0.5 μM and 0.2 μM performed significantly fewer bouts close to the wall compared to those treated with 0.1 μM (HSD, $p < 0.0001$, $p < 0.0001$) and control

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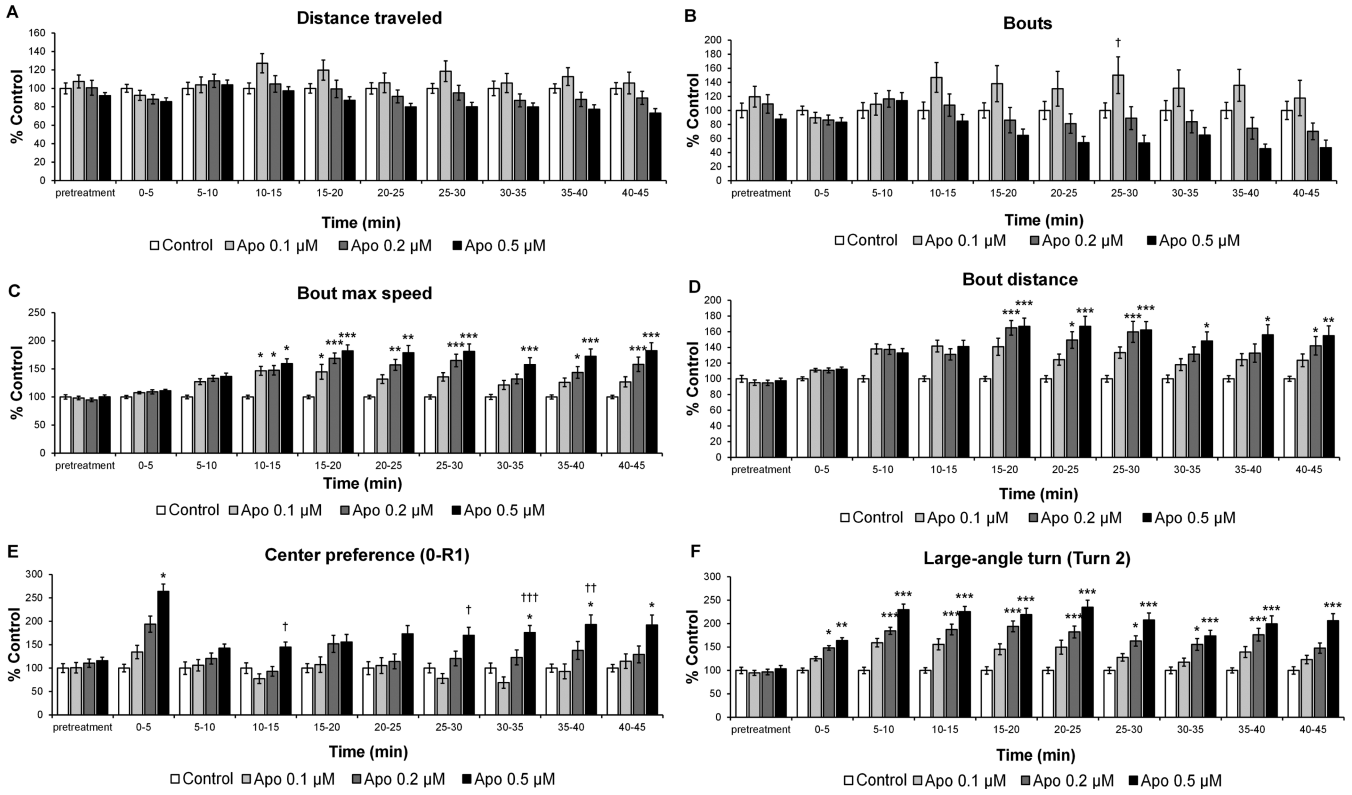

Figure 7. Detailed analysis of parameters (A) distance traveled, (B) bouts, (C) bout duration, (D) bout distance, (E) center preference, and (F) large-angle turns for the low-dose phenotype, n = 36 (control), 35 (0.5 μM), 35 (0.2 μM) and 34 (0.1 μM). Significance reported when comparing treatment with control groups: $^{*}p < 0.05$, $^{**}p < 0.001$, $^{***}p < 0.0001$. Significance reported when comparing 0.5 with 0.1 μM: $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.001$, $^{\dagger\dagger\dagger}p < 0.0001$. Bars represent mean over 5 min ± SEM. There was a significant effect of 0.1 μM apomorphine in bout distance (HSD, $p < 0.05$) and large-angle turns (HSD, $p < 0.001$) compared to control over 45 min but not in individual time intervals.

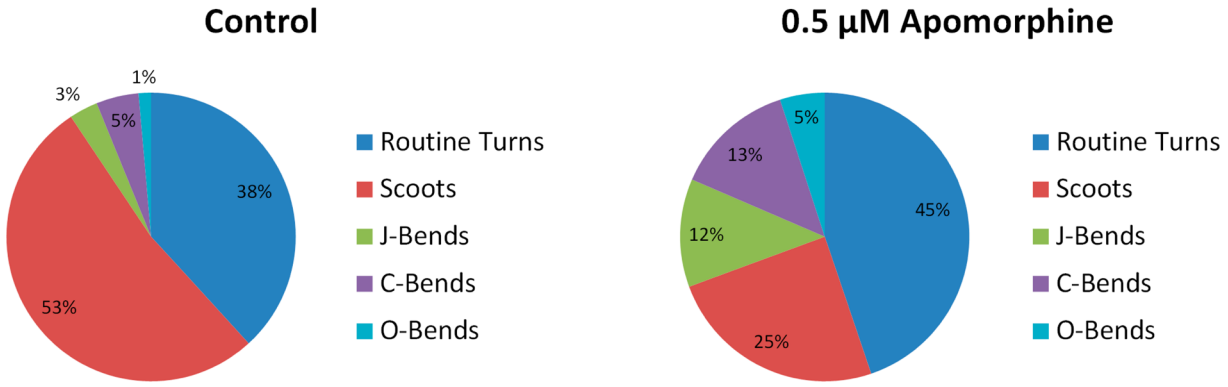

Figure 8. Relative frequency of specific turn types in (A) the control and (B) 0.5 μM treatment group averaged over the whole time period after addition of apomorphine (0−45 min), n = 36 (control), and 35 (0.5 μM).

(HSD, $p < 0.0008$, $p < 0.0001$) (Figure 6s, Supporting Information).

The low-dose phenotype also performed an increased number of large-angle turns (Turn2, Figure 7F). There was a significant effect of apomorphine in Turn2 (RMANOVA, $F_{3.122} = 55.609$, $p < 0.0001$), in which all treatment groups performed significantly more turns greater than 60° compared to the control. Compared to the control groups, the larvae treated with 0.5 and 0.2 μM performed significantly more turns above 60° at several time intervals. At an increased time resolution, we could see that after only 2 min, there was a significant difference between the 0.5 μM and control groups (Figure 7s, Supporting Information). There was also a significant interaction effect of apomorphine over time (RMANOVA: $F_{27.1098} = 3.630$, $p < 0.0001$), and the peak effect for 0.5 μM was after 5−25 min.

We then expanded the analysis and classified specific types of turns (definitions in the Supporting Information).[17,35−37] The low-dose phenotype demonstrated a change in relative frequency of types of turns compared to the control group from primarily scoots to more turns performed with higher velocity and a larger angle change (i.e., J-bends, C-bends, and O-bends) as presented in Figure 8. In addition, the number of turns classified as a startle response increased from 2 to 8%.

Apomorphine Induces High- and Low-Dose Phenotypes in Rats with Dose-Dependent Place Preference. For a translational comparison we correlated the results for the

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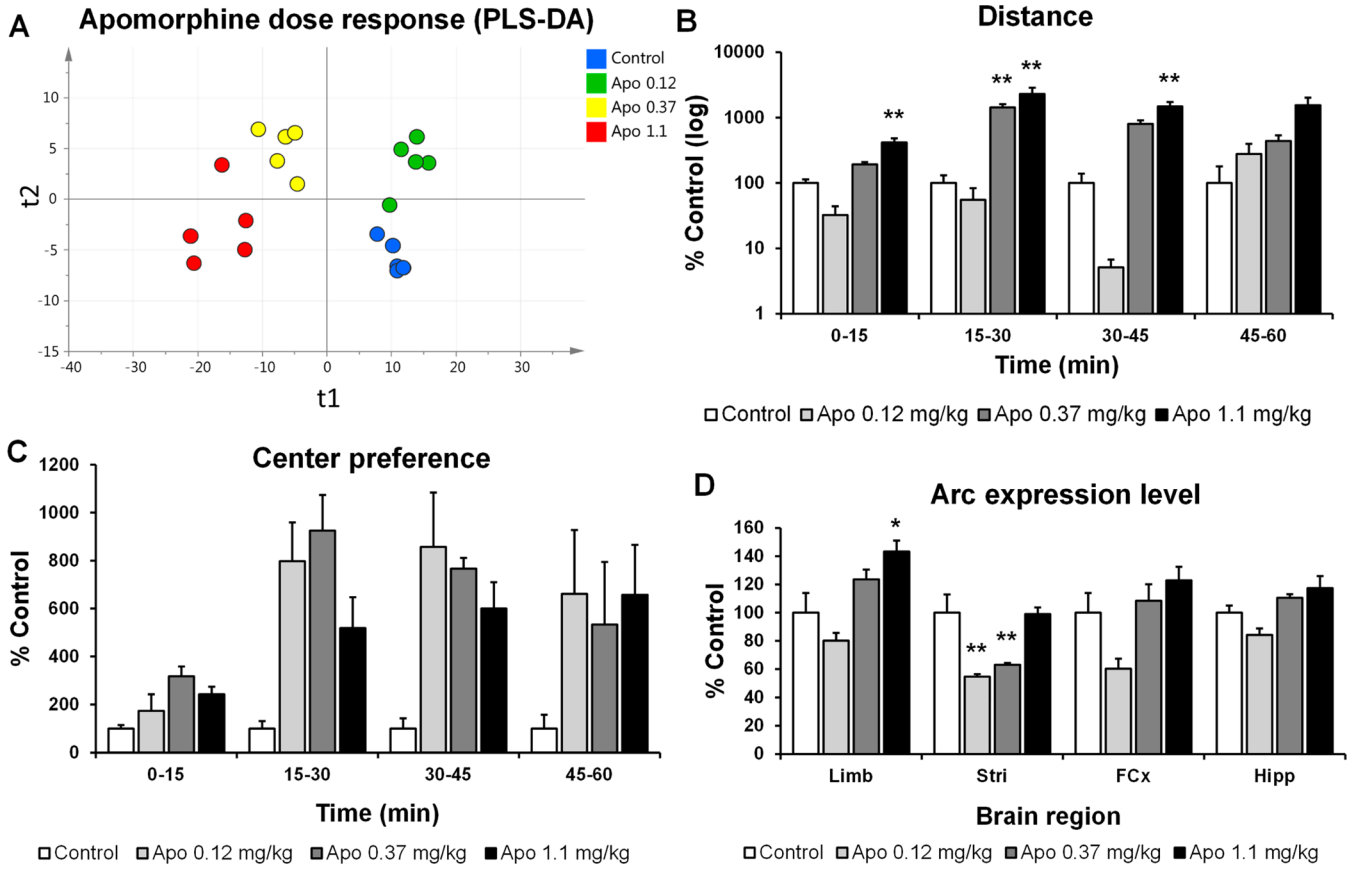

Figure 9. Analysis of behavioral response of rats injected (subcutaneously) with apomorphine. (A) Multivariate analysis of all rat behavioral parameters. PLS-DA, using discriminant variables denoting treatment group as dependent (Y) variables, and behavioral descriptors collected over 60 min as independent variable block (X). Shown are object scores (t1 vs t2), that is, composite variables representing the X variables, for the first two components, In terms of underlying variables, briefly, a location toward the left corresponds to increased activity, and a location upward corresponds to increased center preference in this projection. Single parameters highlighting characteristic behavioral responses to apomorphine: (B) distance and (C) center preference. (D) Neural activity represented by arc expression level in four brain areas. Samples are taken 1 h postapomorphine administration. (Limb, limbic system; Stri, striatum; FCx, frontal cortex; Hipp, hippocampus), n = 5 per group. Significance reported when comparing treatment with control groups: *p < 0.05, **p < 0.001, ***p < 0.0001. Bars represent mean over 15 min ± SEM in (B) and (C). Bars represent mean after 60 min ± SEM in (D).

zebrafish larvae with a parallel study in rats. The behavioral responses of rats (5 in each group, 20 in total) subcutaneously injected with apomorphine were analyzed in an analogous way as the zebrafish larvae data, first by multivariate data analysis (PLS-DA), followed by single-parameter analysis of distance traveled, starts/stops, and center preference using two-way RMANOVA. Similar to zebrafish, the high dose (1.1 mg/kg) and the low dose (0.12 mg/kg) apomorphine groups separate out from control group in the PLS-DA (Figure 9A). The intermediate dose (0.37 mg/kg) group cluster in between high and low dose groups analog to zebrafish larval groups given 25 and 10 μM apomorphine, respectively (Figure 3). In the single parameter analysis, locomotor activity in term of distance traveled increased as expected corroborating previous studies (Figure 9B).[38,39] There was a significant effect of apomorphine on distance ($F_{3.16}$ = 31.337, p < 0.0001), wherein rats treated with 1.1 and 0.37 mg/kg had a significantly increased distance traveled compared to the 0.12 mg/kg and control groups (Figure 9B). Also, the neural activity, represented by Arc expression level, was significantly higher in the limbic region at 1.1 mg/kg compared to control (Figure 9D). In contrast, treatment with 0.12 mg/kg resulted in a trend toward a reduction in the first three time periods (0−45 min) in distance traveled. Likewise, the neural activity was reduced at 0.12 mg/kg compared to control in all brain areas (significantly in striatum). As demonstrated in Figures 9C, rats injected with apomorphine also spend significantly more time in the center of the arena compared to the control group (RMANOVA: $F_{3.16}$ = 9.7924, p < 0.001). The rats treated with intermediate and low doses of apomorphine (0.37 mg/kg, HSD; p < 0.001 and 0.12 mg/kg, HSD; p < 0.05) exhibited the highest center preference, and these rats spent more time in the center compared to the highest dose (1.1 mg/kg, HSD; p < 0.05). However, there was no significance in individual time intervals (Figure 9C). This finding supports the results from the PLS-DA plot wherein low and intermediate groups cluster on the upper half of the plot.

Zebrafish is attracting attention in behavior studies and correlations of drug-induced behaviors with mammalian species have been presented. In our studies of dopamine pharmacology in zebrafish, we have started to analyze whether the current dopamine receptor ligands can bind in the same way across species, and weather detailed behavioral phenotypes correlate with rats and human. The homology of the amino acid sequence of dopamine receptors between zebrafish, rats, and humans was analyzed with special focus on the ligand binding

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site. The dopamine receptors with less than 100% homology were further evaluated by molecular modeling in a complex with the drug apomorphine. The functional effects of apomorphine in rats and zebrafish were analyzed using detailed behavior changes as a readout. Overall, the alignment analysis of amino acid sequence in dopamine receptors D_1−D_4 indicates a highly conserved ligand binding site between zebrafish, rat, and human, more conserved when comparing the whole receptor (59−72% versus 80−100%). We then built a 3D structure model of the zebrafish dopamine D_2 receptor focusing on the ligand binding site. From this model, we can conclude that D_2 receptors in humans and zebrafish most likely bind apomorphine in the same way. In fact, the receptor D_1 in zebrafish has a better amino acid homology in the binding site with humans (100%) than rats (90%) have. The D_1 receptor similarity also highlights the importance of a third species, as the rat−human correlation is not always the best correlation.

Furthermore, we performed a functional analysis of how high and low doses of apomorphine affected behavior in zebrafish and rats. Apomorphine has been studied before in zebrafish larvae, however; herein the methodology makes it possible to dissect the behavior to a detail level more useful for correlative analysis of the underlying mechanisms.

In the multivariate data analysis, the high-dose and low-dose groups separated out from control groups for both zebrafish larvae and rats. These analyses filtered out several parameters that were selectively altered in the high- and low-dose group.

Further analysis of single parameters demonstrated that apomorphine induced gross locomotor changes compared to control in zebrafish larvae, characterized by increased number of bouts and distance traveled at 50 μM and the opposite effect at 0.5 μM. Also in the present rat study, the two highest doses of apomorphine (0.37 and 1.1 mg/kg) increased the distance traveled and 0.12 mg/kg instead lead to hypoactivity confirming that apomorphine dose dependently induce similar phenotypes in zebrafish and rat. These data may reflect the interaction of apomorphine with pre- and postsynaptic receptors according to the hypothesis that the presynaptic receptors located on dopamine neurons function as autoreceptors.[40−42] Stimulation of presynaptic receptors inhibits dopaminergic output, whereas increases in apomorphine concentration in larvae lead to interaction with the postsynaptic receptors which induces the observed hyperlocomotion. Apomorphine is known to display a biphasic dose−response curve in mammals, and a low dose has been demonstrated to have deactivating effects, similar to dopamine antagonists in mammals inducing sedation and increased periods of sleep in humans.[41,43,44] Also in rats, the neural activity (here measured indirectly through arc expression level) was altered at high and low doses of apomorphine in line with the behavioral data. However, analysis of single parameters also revealed that the lowest concentration of apomorphine (0.1 μM) increased locomotor activity in zebrafish significantly compared to 0.5 μM but not in comparison to the control group. There seems to be a U-shaped dose−response curve in zebrafish that confirms previous results.[20] This paradoxical activation at 0.1 μM is difficult to explain; however, apomorphine is unselective and interacts with all dopamine receptors as well as serotonergic and adrenergic receptors.[45−47] One might speculate that as the presynaptic dopamine stimulation weakens, the effect of other receptors where apomorphine possesses high affinity, such as D_4 and 5HT2c, gradually become more important. Antagonism of 5HT2c has been reported to increase locomotor activity.[48,49]

Previous studies as well as this one have demonstrated that exposure to high doses of apomorphine is accompanied by an increase in distance traveled; however, we demonstrate that this increase in locomotor activity is related to a combination of increased numbers of spontaneous initiation of bouts and longer bout durations (which leads to increased bout distances). A larval swim bout is characterized by a discrete number of tail beats followed by a glide period (e.g., beat and glide principle). Analysis of our video recordings revealed that the increased bout duration was characterized by a higher frequency of bouts with repetitive beatings without any stationary phase in between. Since our definition of a bout is based on movement between two stand-stills, these types of repetitive tail beatings were counted as one bout. Locomotion in larvae is regulated through the interplay between number of bout initiations, duration of bouts, and tail-beat frequency and amplitude. In a recent study by Severi et al, experiments with optomotor stimulation showed that larvae first extended bouts by using repetitive tail beats but then also increased the tail-beat frequency with increasing speed.[50] The larvae also performed more bouts as the speed increased. They found that different neurons in the medial longitudinal fasciculus nucleus (nMLF) seem to contribute to the kinematic control of bout duration and tail-beat frequency separately, as indicated by electrode stimulation and ablation experiments. Therefore, dopamine stimulation and inhibition might modulate bout initiation, bout duration, and tail-beat frequency and amplitude separately in zebrafish larvae. We have seen in an ongoing study that the extended bout duration in the high-dose phenotype can be selectively normalized with an intermediate dose of haloperidol in favor of the increased frequency of bout initiations. High dose of haloperidol normalize both bout duration and increased frequency of bout initiations (unpublished experiments). There are significant tyrosine hydroxylase and serotonin immunoreactive projections in the close vicinity of neurons in the nMLF, although there is no evidence of direct connections.[51] Moreover, apomorphine interacts unselectively with dopamine receptors and probably modulates the balance between the direct (D_1 receptors) and indirect pathways (D_2 receptors) controlling motor output through basal ganglia.[52,53] Recent studies support the existence of basal ganglia in all vertebrates including zebrafish.[54,55]

Comparing these data with the human response reveals striking relationships (Figure 8s, Supporting Information). Subcutaneous administration of apomorphine (Apokyn) comparable to the high doses in this study rapidly increases motor activity and is used in Parkinson's patients to switch from an off to an on state, relieving slow movement, problem with movement initiation, and muscle stiffness.[56]

Opposite to the effect of high doses of apomorphine, low doses (0.5 μM) generate hypolocomotion in zebrafish larvae similar to the human response.[43] Further analysis of zebrafish larvae revealed that although fewer bouts were carried out, those executed were performed with increased velocity and larger angle changes, indicative of an increased intensity (tail-beat frequency and amplitude) and a larger initial body bending. Zebrafish display a limited repertoire of turns, which are situation and stress level dependent. For example, under normal conditions, most movements are scoots, that is, short distance movement forward with low speed and angle change. However, other turns come into use when the larvae hunt for

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ACS Chem. Neurosci. 2016, 7, 633−646

prey (J-bends) or escape from a predator (C- and O-bends).[17,37,57] These turns are characterized by increased speed and larger angle change from the initial facing direction. Assuming the autoreceptor hypothesis, low-dose apomorphine preferentially stimulates presynaptic receptors, which leads to a reduction of dopamine synthesis and release. This is functionally equivalent to the effect of a dopamine antagonist, and apomorphine at a low dose has also been reported to exert transient antipsychotic effects in schizophrenic patients.[58,59] However, dopamine antagonists frequently cause reversible drug-induced movement disorders or motor side effects such as dystonia, drug-induced parkinsonism, and akathisia in humans.[60] Although the larvae were habituated and all experiments were performed in a dark, nonstressful environment, drug treated larvae still displayed an increased relative frequency of high-velocity large-angle change turns compared to the control. This abnormal behavior supports the hypothesis that intermediate/low-dose apomorphine induces a state in zebrafish larvae characterized by fewer bout initiations and reduced motor control, resembling a parkinsonistic state in mammals. Altogether, all components of the larval methods of controlling initiation of movement and swim speed using a dynamic combination of bout frequency, bout duration, and tail-beat frequency and amplitude are dose dependently affected by apomorphine.

The behavioral analysis also showed a difference in place preference between the low- and high-dose phenotypes. There was a clear wall preference for larvae at 50 μM and an opposite center preference at 0.5 μM. The place preference for the intermediate dose of 10 μM was more closely associated with the low-dose phenotype at 0.5 μM, whereas the larvae treated with 0.1 μM demonstrated a phenotype more closely resembling the one displayed by larvae treated with 50 μM. This further supports the findings regarding locomotor activity and bout characteristics and confirms the phenotypical similarities between larvae treated with 50 and 0.1 μM. Also in the rat study, low and intermediate doses of apomorphine induced the highest center preference compared to control. Place preference is highly useful and important in behavioral analysis. An increased place preference in the open field (i.e., center of well) normally indicates alleviated anxiety and increased exploratory behavior. Several studies have demonstrated that adult and larval zebrafish display a natural edge preference (thigmotaxis) to avoid predators, which can be reinforced by anxiogenic drugs (caffeine) and relieved by anxiolytic drugs (diazepam), equivalent to rodents' and humans' responses to these drugs.[61,62] Low-dose apomorphine treatment relieves withdrawal-induced anxiety and has an anticonflict effect in rats, which supports the anxiolytic effect we see in zebrafish larvae and rats.[63,64]

In conclusion, the present study demonstrated that the combination of large numbers of parameters and MVA is a powerful tool to identify detailed behavioral changes useful for drug profiling and mechanistic conclusions. In this study, we show that apomorphine dose dependently has an effect on zebrafish larvae bout initiations, bout duration, bout max speed, turn profile, and place preference. Comparisons demonstrate that zebrafish larvae and rats share detailed behavioral phenotypes and that these phenotypes resemble responses reported for humans. This study also establishes that the amino acid sequence and three-dimensional structure of the binding site of dopamine receptors are well conserved between zebrafish, rats, and humans. Taken together, these results further support zebrafish larvae, rats, and humans sharing important functions of the dopamine system, enabling the use of zebrafish larvae as an alternative or complement to mammals for pharmacological studies focusing on the dopamine system.

METHODS

Animal Ethics. This study was conducted in accordance with the national legislation of Sweden and the European Community guidelines for animal studies. All procedures were approved by the ethical committee in Malmö-Lund (permit, M116-12, Zebrafish), or the animal ethics committee in Gothenburg (rat study, permit 325/08).

Fish Maintenance. The zebrafish larvae used in this study were from intercrosses of the wild-type AB strain. Embryos were collected and raised in a 14:10 h light/dark cycle at 28.5 °C on Petri dishes containing E3 embryo medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, and 0.33 mM $MgSO_4$) in an incubator to 5 days postfertilization (dpf). At the age of 5 dpf, the larvae were transferred into 0.8 L aquaria and placed in an Aquaneering, Inc. (San Diego, CA) recirculating system held at 26 ± 1.5 °C where feeding was initiated. Larvae were fed with a commercial larval diet, ZM000 (ZM Fish Food & Equipment, Winchester, U.K.), three times daily until the age of 10 dpf. Behavioral experiments were conducted at 10 dpf. The age of the zebrafish larvae was chosen to ensure a developed blood-brain barrier, which is an important feature when evaluating potential pharmaceuticals for CNS diseases.

Pharmacology. Apomorphine (A4393, Sigma-Aldrich, St. Louis, MO) was dissolved in 100% dimethyl sulfoxide to generate a stock solution (10 mM). The stock solution was then diluted in E3 medium to 2.5 mM, 1.25 mM, and 500 μM (high), or 25 μM, 10 μM, and 5 μM (low).

Experimental Setup and Video Recordings. *Fish.* Each experiment was performed using 48 zebrafish larvae in two 24-well microtiter plates (Cat. No. 303002; Porvair Sciences, Leatherhead, U.K.) that were milled to a depth of 9 mm to reduce shadow artifacts. The microtiter plates had white walls to increase the contrast between the larvae and the background, and to prevent larvae in adjacent wells from acting as visual stimuli. The behavioral setup consisted of a 300 fps digital camera (Genie HM640, Teledyne DALSA, Waterloo, Canada) connected to a computer with video recording software (CamExpert v7.00.00.0912, Teledyne DALSA, Waterloo, Canada; LabVIEW 2011 v11.0, National Instruments, Austin, TX). To maintain the environment in the wells at 28 °C, the microtiter plates were placed parallel to each other in a water bath containing a temperature control unit (Neoheater 25 W thermostat, AQUAEL, Warsaw, Poland). The microtiter plates were placed on a light box containing LED strips (SMD5050 flexible infrared 850 nm trichip). For a schematic image of the experimental setup, see Figure S4 in Anderson et al.[23]

At 10 dpf, the larvae were transferred to the microtiter plates containing 1 mL of E3 medium. All individuals were observed for abnormal swimming behavior and body deformities prior to the experiments. Damaged individuals were removed and replaced. Zebrafish larvae were analyzed in eight treatment groups, divided into a high-dose treatment paradigm (control high, 10, 25, and 50 μM) and a low-dose paradigm (control low, 0.1, 0.2, and 0.5 μM). Each experiment included all doses in a treatment paradigm, n = 12 larvae/dose. The high-dose paradigm was performed in duplicate and the low-dose paradigm in triplicate, using a total of 240 zebrafish larvae.

The data set was generated by automated imaging analysis of video recordings of zebrafish larvae (10 dpf) treated with apomorphine.

The experiments were performed in darkness. The zebrafish larvae were habituated for 45 min before the experiment. The experiment was video recorded for 50 min (5 min pretreatment + 45 min after addition of apomorphine in the medium or a medium with 0.1% dimethyl sulfoxide (DMSO; 20 μL per well) in 5 min time intervals to handle the large amount of data (5 min of recording generates 27.5 GB of video data).

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ACS Chem. Neurosci. 2016, 7, 633−646

Imaging analysis of the video files was performed after the recording using a MATLAB software program originally developed for tracking rats and marmosets.[24,25] The position and angular information from the tracking was then used to calculate 30 parameters (list and definition in the Supporting Information).

Rat. Male Sprague−Dawley rats from B&K Scanbur were used for locomotor studies. Rats weighed 160−180 g at the time of arrival and 220−270 g at the time of the locomotor assessment. They were housed five animals per cage with the lights on between 06:00 h and 18:00 h.

Apomorphine was dissolved in physiological saline (0.9% w/v NaCl) and injected subcutaneously in a volume of 5 mL/kg, 5 min before the initiation of the locomotor recording. Control rats received saline.

Locomotor activity recordings were performed using a Digiscan animal activity monitor (model RZYCCM, Omnitech Electronics, Columbus, MD) placed in sound- and light-attenuating boxes. Each activity monitor was equipped with three rows of infrared photo sensors, each row consisting of 16 sensors. Two rows were placed along the front and the side of the floor (90° angle), and the last row was placed 10 cm above the floor to measure vertical activity. The activity chambers were connected to an analyzer system (Omnitech Electronics, Columbus, MD). The animal's location (center of gravity and vertical position) was sampled at frequencies up to 25 Hz. Recordings were analyzed offline, and parameters including distance traveled, vertical activity, sinuosity of the path traveled, number of starts and stops, time spent in motion, time spent in the middle, velocity, and acceleration were subsequently calculated. All parameters were calculated at different sampling frequencies from 25 to 0.25 Hz. Data were pooled into 15 min periods. All experiments were carried out in accordance with the European Community guidelines for animal studies and with the approval of local animal ethics committees.

Arc Assay-mRNA Assessment. Locomotor activity was recorded for 60 min post administration of apomorphine; thereafter, rats were killed by decapitation. The brains were removed and dissected rapidly on chilled Petri dishes to obtain samples including the striatum, the hippocampus, the limbic region (containing the nucleus accumbens, most parts of the olfactory tubercle, ventral pallidum, and amygdala), and the frontal cortex. Brain samples were frozen on dry ice and stored at −80 °C before subsequent messenger ribonucleic acid (mRNA) analysis. Real-time PCR was performed on tissue homogenates. Total RNA was prepared using the guanidine isothiocyanate method.[26] RNA pellets were dissolved in Milli-Q (MQ) water and stored at −80 °C. The sample concentration was determined spectrophotometrically. Reversed transcription was performed by using a ThermoScript kit (Invitrogen). One microgram of total RNA was reverse transcribed with 25 pmol oligo(dT), 62.5 ng of random hexamers, 7.5 U Thermoscript RT, 10 U RNaseOut, 2 μL of 5×cDNA synthesis buffer, 1 mM deoxynucleotide (dNTP), and 0.05 M dithiothreitol (DTT), adjusted to 10 μL with diethylpyrocarbonate (DEPC)-treated water. cDNA was diluted 40 times and stored at −20 °C.

For real-time polymerase chain reaction (PCR) measurements, 0.7 μL of the cDNA reaction was amplified in a 25 μL reaction mixture containing 1 × PCR buffer, 0.2 mM dNTP, 3.7 mM $MgCl_2$, 0.15 mM SYBR green, 0.4 μM of each primer, and 1 U Taq polymerase. Real-time PCR was performed on an Icycler (Biorad) using the following settings for all genes: 60 s preincubation at 95 °C followed by 40 cycles of denaturation at 95° for 20 s, annealing at 56 °C for 20 s, and elongation at 72 °C for 30s.

The primer sequences were as follows:

Hypoxantine phosphoribosyl transferase (HPRT) (accession number AF001282): Sense: 5′-GGC CAG ACT TGT TGG ATT TG-3′. Antisense: 5′-CCG CTG TCT TTT AGG CTT TG-3′.

Cyclophilin A (accession number M19533): Sense: 5′-GTC TCT TTT CGC CGC TTG CT-3′. Antisense: 5′-TCT GCT GTC TTT GGA ACT TTG TCT G-3′.

Activity-regulated gene (Arc) (accession number U19866): Sense: 5′- GTC CCA GAT CCA GAA CCA CA-3′. Antisense: 5′- CCT CCT CAG CGT CCA CAT AC-3′.

Initial DNA amounts were quantified using a standard curve constructed for every gene using six serial 4-fold dilutions of purified PCR products. The quantity of Arc mRNA was normalized to the geometric mean of the two reference genes (HPRT and cyclophilin A). Correct PCR products were confirmed by agarose gel electrophoresis (2%). PCR products were purified using a PCR purification kit from Qiagen (Valencia, CA) All genes were sequenced at MWG-Biotech (Ebersberg, Germany) and analyzed routinely by melting curve analysis.

Statistical Analysis. *Zebrafish.* Multivariate data analyses were performed using SIMCA-P+, v.13 (Umetrics AB), principal component analysis (PCA), projections to latent structures-discriminant analysis (PLS-DA), and orthogonal partial least-squares-discriminant analysis (OPLS-DA). To identify outliers, we conducted PCA analysis of the different treatment groups. We identified 22 larvae (of 240 total) that were outside of the 99% confidence interval, which corresponds to <10%; these were evenly spread among the treatment groups and no specific localization of the well in the plate was observed. These larvae were removed from the analysis. In addition, we filtered from the data set extreme values (specified for each parameter to values $\geq \mu + 5\sigma$) that were generated in the tracking process (confirmed by analyzing video recordings overlaid with fish tracking marker). These extreme values are the result of errors in the tracking technology.

Further statistical analyses were performed with Statistica v12 (StatSoft, Tulsa, OK) using a two-way repeated measurements analysis of variance (RMANOVA) with treatment and experimental replica as categorical predictors. Tukey's post hoc tests (HSD) were used for further analysis of statistical differences. Significance when comparing treatment with control groups is reported in the figures in this paper as $^{*}p < 0.05$, $^{**}p < 0.001$, and $^{***}p < 0.0001$. The symbol † is used to report significance, comparing the highest dose (50 and 0.5 μM) and lowest dose (10 and 0.1 μM). Bars represent the mean over 5 min ± standard error of the mean (SEM) of two (high) or three (low) experiments normalized to the control set as 100%. Corrections were not made for multiple comparisons and dependent outcome variables.

Rat. Multivariate analysis of rat behavioral data was carried out by means of PLS-DA, using treatment groups as discriminant variables (control, apomorphine 0.12 mg/kg, apomorphine 0.37 mg/kg, and apomorphine 1.1 mg/kg). Behavioral variables, constituting an independent variables block of 11 variables × 7 sampling frequencies × 4 time intervals, for a total of 308 variables, were scaled to zero mean and unit variance (see list of parameters in Ponten et al).[27] Statistical significance was evaluated by cross-validation (Simca version 13.0.3.0, Umetrics AB).

Further statistical analyses were performed with Statistica v12 (StatSoft, Tulsa, OK) using a two-way (RMANOVA) with treatment as categorical predictor. Tukey's post hoc tests (HSD) were used for further analysis of statistical differences. Significance when comparing treatment with control groups is reported in the figures in this paper as $^{*}p < 0.05$, $^{**}p < 0.001$, and $^{***}p < 0.0001$.

Arc mRNA levels were analyzed using one-way ANOVA followed by post hoc Dunnet's test versus saline treated controls (SPSS version 22, IBM).

Sequence Alignment and 3D Reconstruction Models of Zebrafish Dopamine D_2 and D_4 Receptors. A multiple sequence alignment of relevant dopamine receptors as well as the sequences for the templates was performed using Clustal Ω.[28] The alignment was carefully checked and adjusted in order to improve the final homology models of the dopamine receptors (Figure 1s, Table 1s−3s, Supporting Information). Three dimensional (3D) structure models of the different subtypes (a−c) of the zebrafish dopamine D_2 and D_4 receptors were constructed from turkey adrenergic β_1 crystal structure (pdb code: 4BVN)[29] and for the second extracellular loop (EC2) from human dopamine D_3 (pdb code: 3PBL).[29] The force field used for the receptor modeling in this study was Amber99[30] with Born solvation,[31] as implemented in the MOE software (2014.0901; Chemical Computing Group Inc.). Apomorphine was present in the binding site during the refinement stages in the homology modeling procedure, so the final conformation was optimized for agonist interactions. In the

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ACS Chem. Neurosci. 2016, 7, 633−646

modeling procedure, 20 models are independently generated whereupon all side chain conformations are sampled three times for each model. This gives rise to at total of 60 models which are manually inspected with focus on the agonist binding site. The structural properties are evaluated, with respect to bond lengths, angles, rotamer quality, and atom clashes (not shown), and the Ramanchandran plots are shown in the Supporting Information (Figure 2s).

The reason for using the adrenergic β_1 structure as the major template structure is the better resolution relative to dopamine D_3 and difficulties in achieving the important agonist $\pi-\pi$-interaction between the Phe$^{6.52}$ and the aromatic catechol ring in apomorphine (for indexing method, see Ballesteros and Weinstein).[32] The dopamine D_3 crystal structure has an equal number of amino acids between TM5 and a conserved cysteine in EC2 which connect the loop with TM3 (EC2-SS-TM3) and is therefore more suitable as a template for modeling in that particular part. Restraints are introduced in the joint regions between the different templates and are removed by a series of restrained energy minimizations.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschemneuro.6b00014.

Alignment plots and sequence tables for dopamine receptors for zebrafish, rat and human; definitions of behavioral parameters for zebrafish; trajectory plot comparing 30 and 300 camera frame rate; OPLS-DA plot including all parameters; wall preference plot for the low dose group; Turn2 plot; and plot of distance traveled/locomotor activity in zebrafish, rats, and humans (PDF)

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Study conception and design: F.E. and R.O. Acquisition of zebrafish data: F.E. Acquisition of rat data: P.S. and S.W. Software development: F.E., P.P., C.W., J.K., and R.O. Computer model: M.M. Analysis and interpretation of data: F.E., M.Å.A., and R.O. Drafting of manuscript: F.E. Critical revision: F.E., M.Å.A., C.W., J.K., M.M., P.P., P.S., S.W., and R.O.

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Notes

The authors declare no competing financial interest.

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