Cardiotoxic effects of methamphetamine associated with electrophysiological and epigenetic aberrations in Zebrafish

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Abstract

Long-term methamphetamine abuse damages functional and molecular changes in the brain that causes chronic and relapsing disease. In this study, we sought to investigate a relationship between cardiotoxicity and arrhythmia with associated methamphetamine abuse in zebrafish to identify and to understand the adverse cardiac symptoms associated with methamphetamine as well as to assess the applicability of zebrafish as an appropriate model for cardiac-related drug screening studies. Over a two-week duration, zebrafish were first treated with various concentrations of methamphetamine, ranging from 0 to 50 μM. Immediately after treatment, zebrafish underwent electrocardiogram (ECG) measurement for electrophysiological analysis. Results show that a higher incidence of increased heart rate over the duration of the experiment, corroborating with results from previous human case studies involving methamphetamine users. However, abnormalities commonly cited in those same case studies, such as prolongation of QTc, were not significantly presented in obtained ECG recordings. We have also conducted genetic, epigenetic, and histochemical analysis in an attempt to understand the cardiotoxic effects of methamphetamine on zebrafish cardiac function. These results suggested myocardial damage and decrease in gene expression associated with normal physiological function. Finally, this manuscript provides an analysis into potential reasons for the apparent discrepancies in our data with prior research as well as a future outlook of zebrafish cardiotoxic drug screening studies.

Introduction

Cardiotoxicity is one of the most adverse consequences of methamphetamine (Meth) abuse, leading to a notable increase of morbidity and mortality (1). Cardiovascular complications are the second leading cause of death in methamphetamine abusers. Cardiotoxicity can appear early in the course of the drug use and can cause numerous significant effects, such as

pulmonary hypertension, atherosclerosis, cardiac arrhythmias, acute coronary syndrome, and other associated cardiomyopathies (2). Furthermore, a Meth 'binge' study to determine long-term effects discovered that Meth decreased the sensitivity of nervous and cardiovascular physiology through successive treatments, implying the potential remodeling of physiological responses through chronic Meth abuse (3). However, data on the mechanism of the appearance of cardiac dysfunction during drug abuse and the susceptibility of long-term development of cardiotoxicity are limited. Therefore, better understanding of Meth abuse and early diagnosis of pre-clinical cardiac dysfunction in zebrafish, a relevant model for human cardiac studies, is essential in order to understand methamphetamine-induced cardiac toxicity.

Methamphetamines are sympathomimetic amines with a range of adverse effects upon multiple organ systems. Based around a phenylethylamine core, Methamphetamine and its analog, *d*-amphetamine, have high affinity with transporters associated with catecholamine signaling, significantly increasing the number of neurotransmitters such as dopamine and norepinephrine (1). Unlike methamphetamine, *d*-amphetamine has been prescribed as medication to treat neurological disorders such as attention deficit hyperactivity disorder (ADHD) and narcolepsy (4). A possible reason for limited legal Meth use is the addition of the *N*-methyl group compared to amphetamine, which has been shown to confer better penetration through the blood-brain barrier for Meth, leading to stronger and more addictive responses (5). Meth has been shown to induce heightened catecholamine response by promoting catecholamine release, preventing their reuptake, and destabilizing their levels (6, 7). Thus, Meth is responsible for numerous neurotoxic symptoms (7). Given the elucidation of the direct mechanism of Meth on neurological response, the major focus in researching treatments for Meth-related abuse has been associated with neurological modulation. Therefore, less attention was given to researching the direct mechanism of methamphetamine in other physiological systems, such as the cardiovascular system.

Despite the prevailing issue of Meth abuse, studies have shown that cardiac pathology induced by Meth can be attenuated and even reversed through the discontinuation of Meth use and the initiation of subsequent treatment (8). A study in rats regarding the administration of Meth and eventual withdrawal revealed that the rats were able to recover from myocardial pathologic symptoms such as atrophy, fibrosis, and edema starting from 3 weeks after discontinued Meth administration (9). A human case study indicated that attenuation of Meth use and subsequent therapy led to recovery from ventricular hypertrophy and ECG ST deviations (10). Evidence of recovery from Meth abuse has given promise to future potential treatments, but it also necessitates the research to understand the specific mechanism behind Meth-induced cardiovascular pathologies.

Although current research has not fully proven whether the single ventricular heart of zebrafish is comparable to the more complex ventricular conduction system found in higher vertebrates, zebrafish have been proposed as a versatile model system for researching biological applications due similarities with humans pertaining to cardiac physiology. Zebrafish has also emerged as a high-throughput and low-cost animal research model that has been used for phenotype-driven drug screenings for new insights into chemical toxicity due to similar drug metabolism and genetic homology (11). Regarding electrocardiography, previous research in our lab with *in vivo* surface electrocardiography for adult zebrafish was reported with a remarkable similarity used for studies of irregular heartbeats and QT prolongation (12). As such, *in vivo* ECG for adult zebrafish would be a powerful tool for cross-sectional and longitudinal studies for Meth-induced cardiac toxicity. In this study, we attempt to demonstrate the potential of zebrafish ECG in the diagnosis of Meth-induced cardiotoxicity. Developing techniques allow for the accurate and reproducible detection of irregular arrhythmic symptoms, enabling early ECG-based detection of cardiac dysfunction for drug abusers, further defining ECG as a suitable benchmark test for the extent of Meth-induced cardiotoxicity.

Materials and Methods

Zebrafish Husbandry and Experimental Preparation

Wild-type zebrafish were housed in a custom built circulating fish rack system. The water environment was maintained at 28˚ C, ~pH 7.0, and was checked at least once daily. The system is equipped with four filters, a UV, carbon, and two particle filters. Zebrafish were kept under the 14:10 hour light/dark cycle. All zebrafish used in this study were wild-type strain obtained from UCLA and were approximately 1 year old at the onset of the experiment. Prior to methamphetamine treatment, zebrafish underwent open chest surgery to improve subsequent ECG signal acquisition. Under a stereo microscope, scales (above the coelomic cavity and the posterior site above the tail) were removed with forceps, exposing flesh, to allow more direct electrode contact. A small incision, ~2-3 mm, was made on the ventral surface of the fish above the heart. The incision cut through the chest wall and the heart was visible afterward. The fish were recovered in fresh fish water for a few minutes. The incision was not closed via suture, staple, clips, or glue as chest wall and scales have been observed to regrow within 4 weeks. Fish will regrow scales and recover fully within 4 weeks. The 4 zebrafish groups were then housed in separate tanks throughout the duration of the study. Fish were checked every day until the experiment has concluded, to see whether the animal was slow or showed some abnormal activities (*i.e.*, erratic swimming, strained breathing, bloating). In those cases, the fish would be euthanized in accordance to IACUC guidelines.

Drug preparation and Methamphetamine Treatment

The various concentrations (0, 25, 40, and 50 μM) of methamphetamine solutions for treatment were prepared by mixing the specified amount of methamphetamine stock into water obtained from the fish rack system on the day of treatment. The methamphetamine stock (1 mg/mL) was obtained from Sigma-Aldrich (MDL MFCD00056130). The solution (~10 mL) was then placed in a small custom PDMS chamber suitable for housing one zebrafish. Each zebrafish was treated in the designated concentration of methamphetamine solution for 20 minutes before ECG recording.

ECG recording procedure and Instrumental Setup

ECG was obtained using the instrumental setup depicted in **Fig 1a**. Prior to placing the fish in the designated zebrafish station, the fish was first anesthetized in tricaine (200 mg/L) for approximately 3 minutes until the fish became unresponsive to external stimulus. Then, the fish was placed ventral side up in a precut crevice in the middle of the sponge. The sponge was then placed on a glass platform with the pin electrodes as positioned in **Fig 1a**, where the green working electrode was placed near the open incision on the chest while the yellow reference electrode was placed near the lower abdomen. Each fish underwent recording for approximately 1 minute before placing in regular fish water for recovery from anesthesia. Treatment concentrations and durations were approved by University of California-Irvine's IACUC.

Fig 1. Zebrafish ECG Setup. (a) The figure on the left shows the general layout of zebrafish and electrodes during ECG recording. Inlet figure displays the actual setup in lab. The working electrode is shown in green and contacts the chest cavity. The reference electrode is shown in yellow and contacts near the tail. This electrode setup connects with instruments outlined in the block diagram on the right, where signals are processed and displayed on the laptop. (b) Representations of ECG signal processing with labeled waveforms utilizing custom Matlab software. The top displays the orange raw signal, while the bottom displays the blue processed signal.

The components of instrumental setup consisted of the following (**Fig 1a**). The pin electrodes were derived from isolating ends of jumper wires (Austor Part AMA-18-580) and stripping the outer insulator layer of the wire about 2 cm from the metal tip of the wires. The exposed copper wires underneath were then soldered to improve signal integrity and electrode longevity. The pin electrodes were attached to alligator clips at the end of a cable wire leading to the differential AC amplifier (A-M Systems Model 1700). The cable wire was wrapped with aluminum foil and grounded to reduce external electrical noise. The signal from the zebrafish underwent 10000x amplification before undergoing digitalization with the data acquisition box (National Instruments Model USB-6001). The signal was then transmitted to a laptop (Dell Latitude E5470), where the processed ECG signal was displayed using the LabView interface. Examples of ECG recordings are shown in **Fig 1b**.

ECG data collection and analysis

ECG signals were saved as a text file using the LabView interface. The ECG was subsequently analyzed using a custom-designed MATLAB software to determine the positions of ECG waves for each recording. Briefly, the MATLAB m-file first loaded and plotted the ECG data from the output text file from LabView. The m-file then determined locations of peaks corresponding to R waves via local maximum detection. After a denoising step, the P, Q, S, and

T waves were then determined based on predetermined parameters and deviations from the R waves. Finally, relevant statistics such as the QTc duration based on the Bazett formula and heart rate (HR) were calculated and tabulated in an output Excel file corresponding to each ECG data file. Prior to subsequent analysis, each data file was reviewed for accurate ECG wave detection by determining that the locations of P, Q, R, S, and T waves depicted by the software conform to the corresponding peaks/troughs on the ECG.

Bazett Formula: $QTc = \frac{QT\ Interval}{\sqrt{RR\ Interval}}$

RT-PCR Analysis

Zebrafish were euthanized and homogenized. RNA was successfully isolated following the TRIzol® reagent protocol (Invitrogen). Subsequent first strand synthesis of cDNA was conducted following the protocol of M-MLV reverse transcriptase (Promega). qPCR was conducted with SYBR-Green reagent and analyzed with the Quantstudio Real-Time PCR System. DNA primers used for qPCR are shown in **Table 1**.

Table 1. List of Primers used for RT-PCR

Name	Accession No. (NCBI)	Product size (bp)	Primer Sequence
MBD3	NM_212769.1	120	F: 5′-GTCCAACAGGGAAGAAG-3′ R: 5′-GGCAGAGACGTGTTCAG-3′
MBD2	NM_212768.1	105	F: 5′-GAGCTCAGCAGCTAGC-3′ R: 5′-CCGTCCTCTTCTTCTC-3′

MBD1	BC124166.1	102	F: 5′-GAAGGTGAAGATGC-3′ R: 5′-TTCAGCTTCTTCAC-3′
PRMT4	NM_001007183.2	98	F: 5′-GAAGCTGCACTGGT-3′ R: 5′-GTGGAACTCGTATC-3′
NNMT	NM_001372046.1	105	F: 5′-GACATCGGCTCTGGC-3′ R: 5′-CAGCACCTGCTTGAC-3′
LCMT1	NM_001004645.2	105	F: 5′-CACCTGAGATCAACAG-3′ R: 5′-GGTTTCGATAGTGGAG-3′
EHMT2	NM_001113615.1	120	F: 5′-GAGACAGAGCCGCAGC-3′ R: 5′-GAGACTGTCTGCTGCT-3′
EHMT1b	XM_021468789.1	109	F: 5′-GCCAAGGACCAAGTC-3′ R: 5′-CGTGCACGATGTACTG-3′
COMTA	NM_001030157.2	112	F: 5′-CACGATGTGGTCCATC-3′ R: 5′-GCGATGACGCTCTCTG-3′
DMNT3a	NM_001328167.1	103	F: 5′-CACTCCACGCTGGAC-3′ R: 5′-TCGTCGCACTTCTCC-3′
DMNT3b	NM_001020476.2	98	F: 5′-GAAGCTCGTGATGGTC-3′ R: 5′-TGTCAGACTCTTCTC-3′
DMNT1	NM_131189.2	110	F: 5′-GCCTGAAGCTGAGAATGC-3′ R: 5′-CTCATCGGTTAACATCTC-3′
KCNH2	NM_001044931.1	103	F: 5′-CACCTGGGACTGGGTGATC-3′ R: 5′-TGTGGAAATTCAGCACAAT-3′
KCNQ1	NM_001123242.2	112	F: 5′-GATGCCGGAGTAAATATGT-3′

			R: 5′-GGACTCCTCTGACAGCAG-3′
SCN5A	NM_198056.3	110	F: 5′-GGAACTGGCTGGACTTTAG-3′ R: 5′-CTGGATCAGGGCCCCCACG-3′

Protein Extraction and Dot Blot Analysis

After treatment of zebrafish with aforementioned methamphetamine concentrations (25, 40, and 50 μM), the zebrafish were euthanized and homogenized. Their proteins were extracted with the TRIzol® reagent and protocol (Invitrogen). In brief, whole zebrafish (~400 mg.) are gently pipetted in a 1.5 mL Eppendorf (EP) tube. The zebrafish tissues were homogenized by liquid nitrogen before being incubated in 1 mL TRIzol® on ice for 5 min. The extract was centrifuged at 13,000 rpm at 4°C for 45 min and the supernatant was collected into a fresh 1.5 mL EP tube. 200 μL of Chloroform was added and mixed thoroughly, left the tube on ice for 5 min, and centrifuged at 13,000 rpm at 4°C for 15 min. After removing aqueous phase for RNA and precipitating DNA by ethanol, proteins were precipitated by adding 1.5 mL isopropanol, mix thoroughly and incubate for 10 min at room temperature. The extract was centrifuged at 12,000 rpm at 4° for 10 min, supernatant was discarded. 2 mL of 0.3 M guanidine hydrochloride solution was added and incubate for 20 min at room temperature. The sample was centrifuged at 12,000 rpm at 4°C for 5 min, discard the supernatant. The protein pellet was washed with 2 mL of ethanol and dried for 5 min. The protein pellet was resolubilized with 1mL of 1% SDS solution, the supernatant was collected after centrifuge at 12,000 rpm at 4°C for 10 min, ready to use for subsequent protein analysis. Extracted proteins were dissolved in 1% SDS. The protein solution was then dropped on a nitrocellulose membrane (GE Healthcare Protran® BA85) to form 2 μL and 5 μL dots for each concentration group. After 1 hour of incubation, the membrane was then blocked with TBST including 1% BSA for 1 hour, followed by an overnight incubation with the

186 anti-methamphetamine antibody (mouse, 10M25A Fitzgerald). After washing the membrane with
187 TBST, the membrane was then incubated with HRP conjugated anti-mouse IgG (R&D Systems)
188 for 4 hours. After additional washing, the membrane was then incubated in Pierce™ ECL
189 Western Blotting Substrate (Thermo Scientific) and imaged for chemiluminescence.

Histochemical Staining with Masson's Trichrome Stain

After treatment of methamphetamine, zebrafish hearts were isolated as described in previous literature (13). Briefly, the fish were anesthetized by tricaine before undergoing chest incisions. The heart was then located and excised, which were then placed in a solution of perfusion buffer (10 mM HEPES, 30 mM taurine, 5.5 mM glucose and 10 mM BDM in 1x PBS solution). After excision of all hearts, they were subsequently fixed by 4% formaldehyde and cryosectioned with a cryostat. The tissue slices were placed onto frosted microscope slides (Thermofisher Scientific) and underwent Masson's Trichrome staining (following the provided protocol from American Master Tech, TKMTR2). The slides were observed under the microscope for subsequent analysis of collagen and myocardium distribution for cardiac injury analysis.

Antibodies for Immunostaining and ELISA Analysis

Protein extraction by TRIzol® reagent was conducted prior to ELISA. Heart tissue samples were fixed and sectioned with the aforementioned procedure prior to immunostaining. The antibodies used for immunostaining and ELISA were mouse anti-methamphetamine antibody (mouse, 10M25A Fitzgerald), anti-MBD2 (ab45027; Abcam), anti-DNMT1 antibody (mouse, ab13537; Abcam) and the secondary antibodies used were goat anti-mouse IgG-HRP (ab6789; Abcam), goat anti-mouse IgG H&L (Alexa Fluor® 488) (ab150113, Abcam).

Statistical analysis

All parameters derived from data analysis (*i.e.,* QTc, HR) were averaged within each experimental group. Statistical significance was determined by the one-way ANOVA test between experimental groups with significance level $p<0.05$.

Results

Significant Dose-Response Increase in Heart Rate but not in QTc from Methamphetamine Treatment

Zebrafish has been a favorable model for drug screening experiments, including methamphetamine. Indeed, most studies regarding zebrafish methamphetamine treatment concern the neuropsychological effects of methamphetamine, owing to the ability of methamphetamine to enhance catecholamine neurotransmitters (14, 15). Studies of zebrafish cardiotoxicity in response to methamphetamine administration are scarcer. Fang *et al.* conducted a methamphetamine cardiotoxicity study by treating 48 hpf (hours post fertilization) zebrafish embryos with up to 24 hours of methamphetamine administration, documenting decreased heart rate and abnormal morphological changes such as edema and hemorrhage (16). To this end, we sought to verify and expand on their results through our methodology in ECG acquisition, as arrhythmia was a prominent cardiotoxic symptom in methamphetamine abusers (1). Our lab previously developed modalities in acquiring ECG from adult zebrafish and successfully identified abnormal ECG characteristics (12). Using an ECG setup designed in our lab (**Fig 1**), we acquired ECG signals over a two week period. Each experimental group is treated with the designated methamphetamine concentration for 20 minutes for 3 instances per week,

and their ECG was subsequently acquired after treatment. The signals were then processed to eliminate external noise, and parameters such as heart rate and QTc were then quantified. **Fig 2a** comprises representative ECG figures obtained from our ECG system, including ECG diagrams obtained before methamphetamine administration as well as 7 days and 14 days after first administration. **Fig 2b** is a representation of a pathogenic progression of ECG signals due to methamphetamine treatment. The evident increase of heart rate and sporadic signs of QTc prolongation were seen after the first and second weeks of treatment. Through our analysis of ECG recordings of zebrafish treated at varying concentrations of methamphetamine over the period of 2 weeks, a significant increase in heart rate was noted for all zebrafish groups treated with methamphetamine compared to zebrafish not treated with methamphetamine (**Fig 2a**), which is consistent with previous studies of methamphetamine users displaying monomorphic ventricular tachycardia (17). However, our data indicated that significant QTc prolongation did not occur due to methamphetamine treatment (**Fig 3**). This seemingly contradicts with results obtained from previous human medical case studies, as those cases reported significant signs of QTc prolongation in methamphetamine users (18, 19). Comparison between heart rate and QTc for individual measurements (**Fig 3a**) revealed that there was no significant correlation between heart rate and QTc. Furthermore, heart rate tended to display a wider range of values than for QTc across treatment groups, supporting the previous analysis that methamphetamine induces a more significant change in heart rate than in QTc. Heart rate values for each treatment group were more delineated, as values from the control group were colocalized at the lower range of heart rates (mean = 87.4 bpm), whereas values from the 50 μM treatment group were colocalized at the higher range (mean = 94.6 bpm). QTc results did not display the same discrimination as in heart rate, as values were congregated regardless of experimental group. This was corroborated by measuring the variation in heart rate in each group, as the variations

remain relatively similar regardless of experimental treatment. While some outliers populated the high extreme values of QTc, the majority of QTc values were congregated.

Lack of significant differences in ECG Characteristics between Treatment Groups

While significant differences can be found for heart rate between zebrafish treated and not treated with methamphetamine, other characteristics, including ST interval and RR interval, do not show significant correlation. According to the odds analysis shown in **Fig 3d**, only QTc, heart rate, and RTc have shown significant differences between methamphetamine-treated and untreated groups (OR > 2.5). A closer inspection of the odds ratios indicate that the significance was fairly weak for RTc and QTc, which was corroborated by T-tests conducted above. Other parameters that were studied, such as ST interval, have an odds ratio below the significant value.

Fig 2. Analysis of ECG Before and After Treatment. (a) This representative figure indicates the ECG signals before treatment, labeled as 'Baseline', as well as signals obtained at 7 and 14 days after the onset of methamphetamine treatment. ECG waveforms were labeled for the initial ECG wave. The progression of ECG signals depict an increase in heart rate after onset of treatment, with sporadic cases of QTc prolongation. (b) Representation of average QTc measured for each experimental group across the duration of treatment, indicating an increase of QTc, but at an insignificant level.

Fig 3. Comparison of QTc and HR across Experimental Groups for Significance. Correlations of heart rate and QTc (a) and correlation of RR interval and QTc (b), indicating a general positive correlation between heart rate and QTc. (c) Correlation of baseline QTc values obtained before methamphetamine treatment with peak QTc measurements obtained during the course of the treatment, suggesting a general prolongation of QTc. (d) Odds analysis of ECG parameters, indicating the likelihood of significant changes before and after methamphetamine treatment. As shown, RTc, QTc, and heart rate indicate significant changes with OR>2.5.

Sporadic dysregulation of ST interval and other ECG characteristics after Methamphetamine Treatment

While certain ECG parameters do not display significance between treatment groups, an analysis into the variability of these parameters revealed interesting results. Notably, the variability in ST interval increases at higher concentrations (**Fig 4**). While the mean ST interval calculated was not significantly different at all methamphetamine concentrations, the sporadic presence of increasing ST interval duration as methamphetamine concentration increases indicated the propensity of ST interval to vary after methamphetamine dosage. This pattern was not seen in QTc and heart rate, as the variation of both parameters remain similar throughout all treatment groups. Interestingly, RTc also displayed a similar variation to ST, but it would seem that the difference in RTc would be attributed to ST. While the significance of a prolonged ST interval is not well understood, a previous manuscript documented that ST interval prolongation could be associated with left ventricular hypertrophy (20). This could be explained by realizing that the increase in ventricular volume potentially increases the distance that the Purkinje fibers have to cover for both ventricular depolarization and repolarization, increasing the ST interval.

Fig 4. Analysis of specific ECG parameters between experimental groups. Aggregate measurements were plotted for each experimental group for comparative analysis. Based on significance testing, heart rate exhibited significant increases across all experimental groups, supporting that methamphetamine induces increased heart rate in zebrafish. Other parameters (QTc, RTc, and ST) displayed little to no significance across experimental groups. * indicates $p < 0.05$. *ns* indicates no significance.

Ion Channel Dysfunction Associated with Methamphetamine Treatment

While we did not observe significant changes in ECG signals between methamphetamine treated and untreated groups aside from heart rate, we wanted to determine if methamphetamine has induced cardiotoxic damage in cardiac tissue. Through our dot blot

analysis to detect methamphetamine within zebrafish tissue, we were able to determine that methamphetamine does retain within zebrafish after treatment in a dose-response manner (**Fig 5f**). We chose to investigate and genetic and histological consequences of methamphetamine treatment in zebrafish. To analyze molecular roles of methamphetamine in inducing cardiotoxicity, we conducted RT-PCR of several genes associated with ion channels. These potassium and sodium ion channel genes (*KCNH2*, *KCNQ1*, *SCN5A*) have been selected due to their arrhythmogenic property seen in arrhythmic disorders (21). While the potassium ion channel genes *KCNH2* and *KCNQ1* indicated no significant expression changes, *SCN5A* displayed significant upregulation in the methamphetamine treated groups compared to the untreated group (**Fig. 5a**). *SCN5A* corresponds to the sodium channel protein $Na_v1.5$. Through previous studies involving the characterization of gain-of-function *SCN5A* mutations, upregulation of the sodium ion channel is known to be a factor in several arrhythmic diseases, including Brugada and long QT syndromes, as well as cardiovascular diseases such as cardiac hypertrophy and heart failure (22, 23). Therefore, methamphetamine treatment had been demonstrated to potentially induce arrhythmogenic consequences directly due to dysregulation of certain ion channel expressions and functions.

Fig. 5. Genetic Analysis of Methamphetamine Treatment on Zebrafish. (a) The degree of expression of genetic and epigenetic genes in comparison between experimental groups. Results indicate that *MBD1-3*, *NNMT*, *EHMT1*, *DNMT1*, and *DNMT3A-B* exhibit the greatest progressive fold change in expression as methamphetamine dosage treatment increases. *SCN5A*, a sodium channel gene common in arrhythmic syndromes, has also been upregulated by methamphetamine treatment. (b-d) Quantification of expression changes based on functionality of epigenetic proteins (categorized as DNA methylation, histone methylation and acetylation, and methyl-binding proteins). The protein expression across all categories is increased in methamphetamine treated group (25 μM), with the difference being significant in DNA methylation proteins and methyl-binding proteins. (e) Comparison of protein expression of MBD2, a methyl-binding protein, producing the most pronounced difference in expression between control and 25 μM treated group. (f) Dot blot diagram of methamphetamine detection retained in zebrafish tissue, indicating that methamphetamine does retain in zebrafish tissue in a dose-dependent response. (g) Representative trichrome-stained heart sections from zebrafish treated at various concentrations of methamphetamine. * indicates significance with $p < 0.05$. *ns* indicates no significance.

Transcriptional repression as an epigenetic change due to methamphetamine treatment

To further analyze the genetic consequences of methamphetamine treatment, we determined the expression changes of epigenetic factors in zebrafish after methamphetamine treatment. From our analysis, the most noteworthy result shown was the expression change in *MBD2*. Zebrafish treated with 25 μM methamphetamine resulted in an approximately 3-fold change in *MBD2* compared to those in the control group (**Fig 5e**). *MBD2* corresponds a transcriptional repressor that specifically binds to CpG sites formed from DNA methylation, so the upregulation of *MBD2* is an implication of long-term repression of genes. Analysis of other potential epigenetic factors potentially affected by methamphetamine treatment yielded similar results. In the diagram depicted in **Fig 5a**, the factors that have underwent a significant fold change (by at least 2 fold) include *MBD1-3*, *NNMT*, *EHMT1*, *DNMT1*, and *DNMT3A-B*. These factors are mainly involved in the various forms of methylation, including DNA methylation and histone methylation. These results were also supported through our quantification of the expression of DNA methylation, histone modifications, and methyl-binding proteins between the control group and the 25 μM treatment group (**Fig 5b-d**). As demonstrated in a previous study analyzing epigenetic influences in *SCN5A* expression, *SCN5A* dysregulation was associated with epigenetic regulations (24). Thus, the upregulation of DNA methylation could potentially be linked with the dysregulation of pertinent ion channel genes such as *SCN5A*. Interestingly, our results yielded a significant difference between the control and treatment groups for DNA methylation and methyl-binding proteins, but not for histone modifications. Histone modifications are generally associated with short-term, reversible forms of genetic repression, whereas DNA methylation is correlated with long-term and stable genetic repression (25). Masson's trichrome stains were performed in cardiac cryosections in which myocardial fibrosis are indicated in blue at 40 and 50

μM of Meth, compared to that of control (0 μM) and 25 μM of Meth (**Fig. 5h**). These results were also corroborated through our histological analysis, by specific antibodies in fluorescent immunohistochemistry (**Fig 6**), further supporting that methamphetamine induces an upregulation in both DNA methylation and subsequent transcriptional repression.

Discussion

The methamphetamine epidemic continues to fester worldwide, and cardiovascular diseases are the leading cause of death for methamphetamine abusers. Utilizing animal models to study cardiovascular associated mechanisms could be critical in devising treatments for methamphetamine associated diseases. The zebrafish is an excellent model for drug screening

Fig 6. Representative Immunostaining of Methamphetamine and Epigenetic Proteins. (a) Brightfield image of zebrafish heart tissue. (b-d) Immunostaining of methamphetamine (b), DMNT (c), and MBD (d) within heart tissue of methamphetamine treatment group. Antibodies were conjugated with Alexa Fluor 488 to indicate presence of proteins with green fluorescence. Magnification of images 40X

studies due to high fecundity, low maintenance, and similar genetic homology to that in humans. As the zebrafish model is constantly evolving, studies have continued to delineate the applicability of the zebrafish in human medical research. During the initial conception of this study, we have sought to 1. Establish the zebrafish model as an adequate model of drug screening for cardiotoxic effects, and 2. Elucidate the role of methamphetamine in inducing long-term cardiotoxic effects. Utilizing our custom built zebrafish ECG acquisition system in our lab, we were able to acquire ECG from zebrafish during the two-week treatment period with methamphetamine. Based on our results, we determined that heart rate has increased significantly during the two-week period due to methamphetamine treatment through comparison

346 of heart rate between zebrafish treated with methamphetamine and zebrafish that were treated
347 in normal fish water. Additionally, we conducted an analysis on possible gene targets of
348 methamphetamine that are associated with cardiotoxicity and discovered that *SCN5A*, a cardiac
349 sodium ion channel gene commonly associated with arrhythmogenic syndromes, was
350 significantly upregulated due to methamphetamine treatment. Previous studies have shown that
351 deregulation of these genes have resulted in deterioration in cardiac physiology, including the
352 presence of prolonged QTc (26). These results were consistent with previous human case
353 studies (1). Additionally, we have conducted some experiments to analyze possible
354 methamphetamine-induced epigenetic changes, and results indicated that methamphetamine
355 intake was correlated with an increased amount of DNA methylation and associated gene
356 repression. Similar results have been documented for methamphetamine-induced addiction
357 studies in other animal models (27, 28). While hypermethylation could possibly be associated
358 with the upregulation of ion channel genes and the subsequent dysregulation of cardiac rhythm,
359 the exact mechanism linking these epigenetic and physiological changes remains unknown. In
360 the future, experiments should be conducted to determine the gene targets of hypermethylation.
361 Nevertheless, our results have indicated the propensity of methamphetamine to induce
362 cardiotoxicity in zebrafish through genetic and epigenetic means.

363 While some associations have been determined between methamphetamine treatment
364 and pathologic symptoms shown on ECG in zebrafish, the significance is not very evident. For
365 example, there is currently no explanation on why a dose-response increase in
366 methamphetamine was not seen in certain relevant parameters in ECG. While the dose-
367 response was evident when measuring heart rate, the determination of significance in other
368 parameters was less apparent, such as the presence of QTc changes in response to variations
369 of treated methamphetamine concentrations. QTc prolongation has long been regarded as a

prominent symptom in methamphetamine users, and in some isolated cases in this study, QTc prolongation can be seen on ECG signals. However, statistical analysis indicated that there were no significant QTc changes due to methamphetamine treatment. Despite the lack of significance, methamphetamine was confirmed to successfully retain within the zebrafish after treatment, as verified by our dot blot experiment. Methamphetamine retention in zebrafish was also demonstrated in a previous zebrafish study (29). We surmise that methamphetamine might pose an antagonizing interaction with tricaine, the anesthetic agent used to acquire ECG. Both methamphetamine and tricaine have opposing mechanisms, as methamphetamine is a stimulant while tricaine is an established anesthetic, known for preventing action potential firing by blocking voltage-gated sodium channels (30). Tricaine has also been shown to prolong the Q-T interval as well, which could also confound zebrafish ECG results (31). Most zebrafish methamphetamine studies focused on the neuropsychological effects of methamphetamine, which were conducted using behavioral tests and without the need for tricaine (32). Therefore, future improvements should be implemented to reduce the effect of tricaine for zebrafish cardiotoxic studies, as tricaine could introduce confounding circumstances, especially when testing psychostimulants on zebrafish. Future studies should also seek to explain the effect of these chemical entities on ion channel function through analysis of sodium and calcium transients for zebrafish. These future experiments would determine the mechanism of methamphetamine in inducing cardiotoxicity as well as bolster the use of zebrafish as a suitable model for cardiotoxic studies.

Additionally, future directions would also include linking neurotransmitter response to cardiovascular abnormalities in zebrafish as well as an investigation on ion channel function in the heart after methamphetamine administration. The zebrafish model has already been utilized in numerous methamphetamine studies, mostly related to behavioral studies due to

methamphetamine's known ability to disrupt dopamine release and reuptake, thus increasing dopamine expression (33). Therefore, it would be intriguing to understand the role of dopamine in methamphetamine-induced cardiotoxicity, as it would explain whether methamphetamine-induced cardiotoxicity is caused by dopamine or through a direct effect from methamphetamine. One consequence of dopamine response is the change in ion channel expression. Studies have shown the modulation of L-type calcium channels by methamphetamine, but it is not fully understood whether methamphetamine alters calcium channel function directly or via dopamine (28, 34, 35). Overall, more future studies should be planned to elucidate the mechanism of methamphetamine-induced cardiotoxicity.

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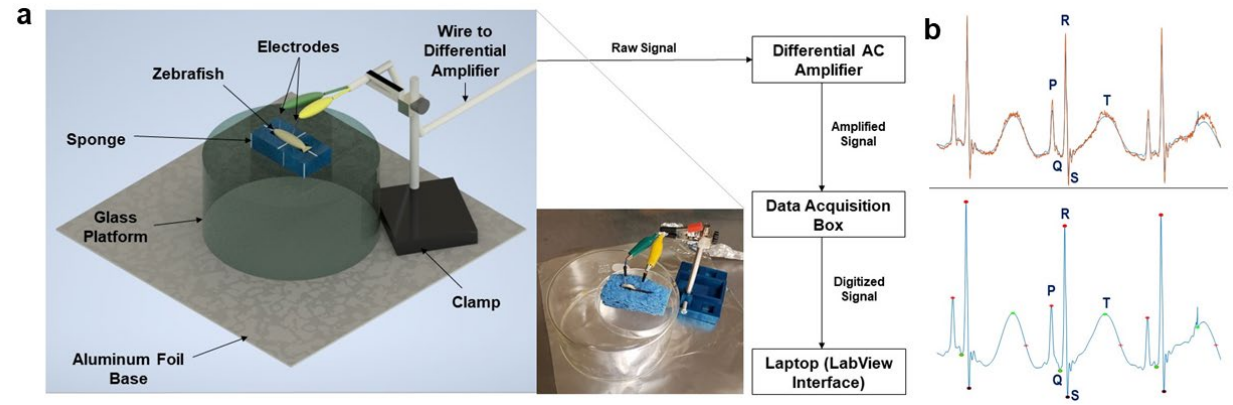

Fig. 1. Zebrafish ECG Setup. (a) The figure on the left shows the general layout of zebrafish and electrodes during ECG recording. Inlet figure displays the actual setup in lab. The working electrode is shown in green and contacts the chest cavity. The reference electrode is shown in yellow and contacts near the tail. This electrode setup connects with instruments outlined in the block diagram on the right, where signals are processed and displayed on the laptop. (b) Representations of ECG signal processing with labeled waveforms utilizing custom Matlab software. The top displays the orange raw signal, while the bottom displays the blue processed signal.

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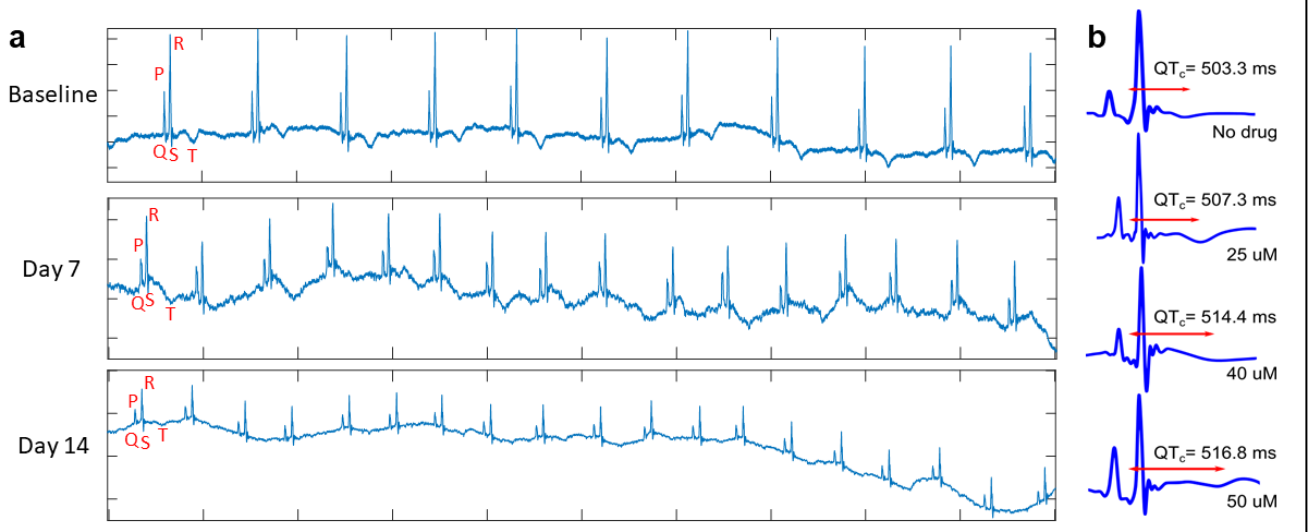

Fig. 2. Analysis of ECG Before and After Treatment. (a) This representative figure indicates the ECG signals before treatment, labeled as 'Baseline', as well as signals obtained at 7 and 14 days after the onset of methamphetamine treatment. ECG waveforms were labeled for the initial ECG wave. The progression of ECG signals depict an increase in heart rate after onset of treatment, with sporadic cases of QTc prolongation. (b) Representation of average QTc measured for each experimental group across the duration of treatment, indicating an increase of QTc, but at an insignificant level.

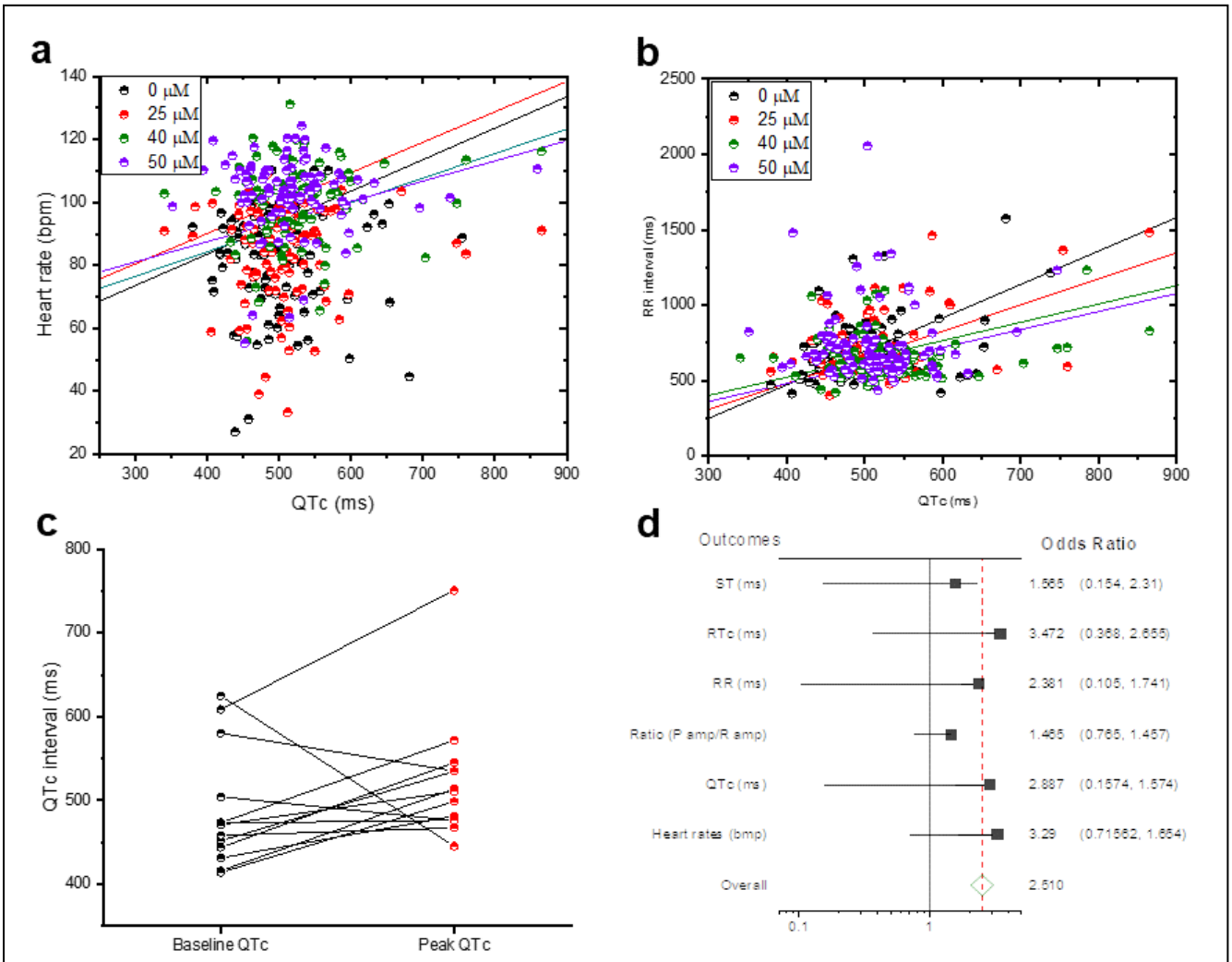

Fig. 3. Comparison of QTc and HR across Experimental Groups for Significance. Correlations of heart rate and QTc (a) and correlation of RR interval and QTc (b), indicating a general positive correlation between heart rate and QTc. (c) Correlation of baseline QTc values obtained before methamphetamine treatment with peak QTc measurements obtained during the course of the treatment, suggesting a general prolongation of QTc. (d) Odds analysis of ECG parameters, indicating the likelihood of significant changes before and after methamphetamine treatment. As shown, RTc, QTc, and heart rate indicate significant changes with OR>2.5.

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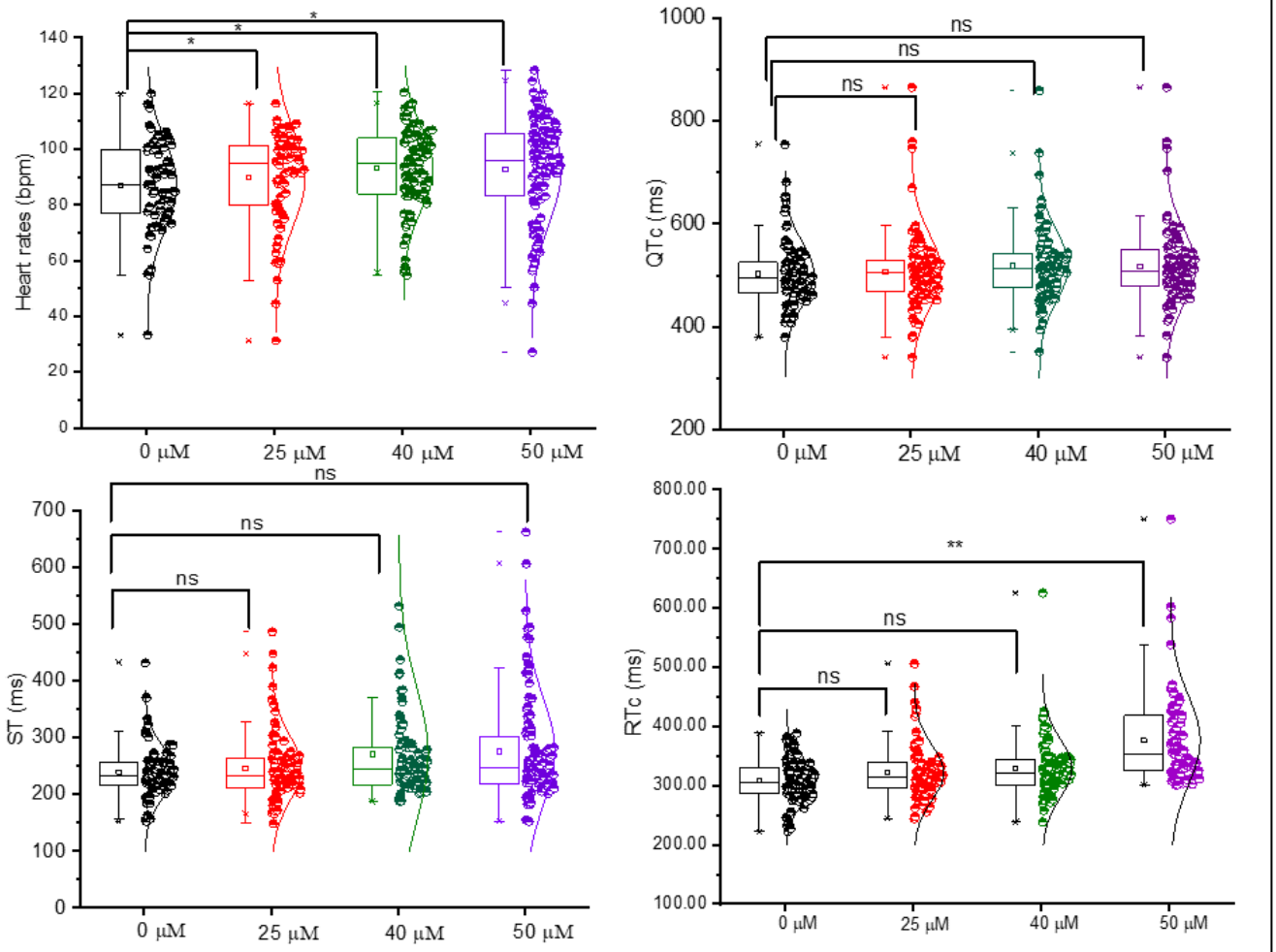

Fig. 4. Analysis of specific ECG parameters between experimental groups. Aggregate measurements were plotted for each experimental group for comparative analysis. Based on significance testing, heart rate exhibited significant increases across all experimental groups, supporting that methamphetamine induces increased heart rate in zebrafish. Other parameters (QTc, RTc, and ST) displayed little to no significance across experimental groups. * indicates $p < 0.05$. *ns* indicates no significance.

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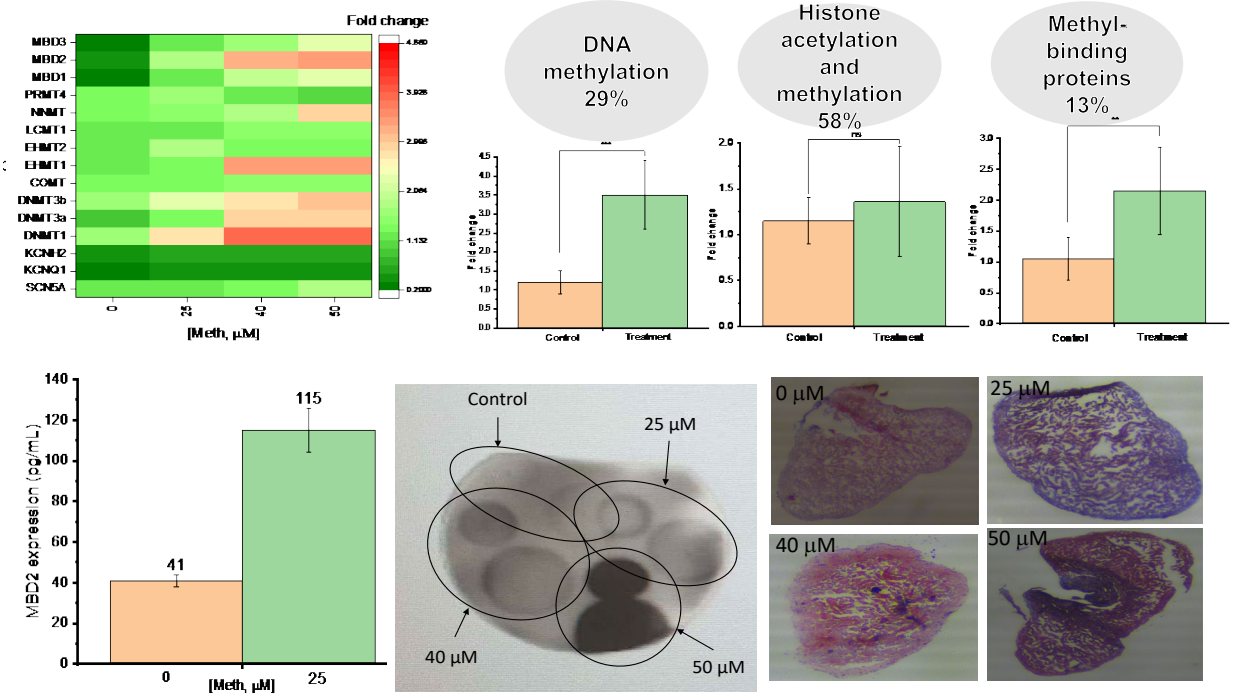

Fig. 5. Genetic Analysis of Methamphetamine Treatment on Zebrafish. (a) The degree of expression of genetic and epigenetic genes in comparison between experimental groups. Results indicate that *MBD1-3*, *NNMT*, *EHMT1*, *DNMT1*, and *DNMT3A-B* exhibit the greatest progressive fold change in expression as methamphetamine dosage treatment increases. *SCN5A*, a sodium channel gene common in arrhythmic syndromes, has also been upregulated by methamphetamine treatment. (b-d) Quantification of expression changes based on functionality of epigenetic proteins (categorized as DNA methylation, histone methylation and acetylation, and methyl-binding proteins). The protein expression across all categories is increased in methamphetamine treated group (25 µM), with the difference being significant in DNA methylation proteins and methyl-binding proteins. (e) Comparison of protein expression of MBD2, a methyl-binding protein, producing the most pronounced difference in expression between control and 25 µM treated group. (f) Dot blot diagram of methamphetamine detection retained in zebrafish tissue, indicating that methamphetamine does retain in zebrafish tissue in a dose-dependent response. (g) Representative trichrome-stained heart sections from zebrafish treated at various concentrations of methamphetamine. * indicates significant with $p < 0.05$. *ns* indicates no significance.

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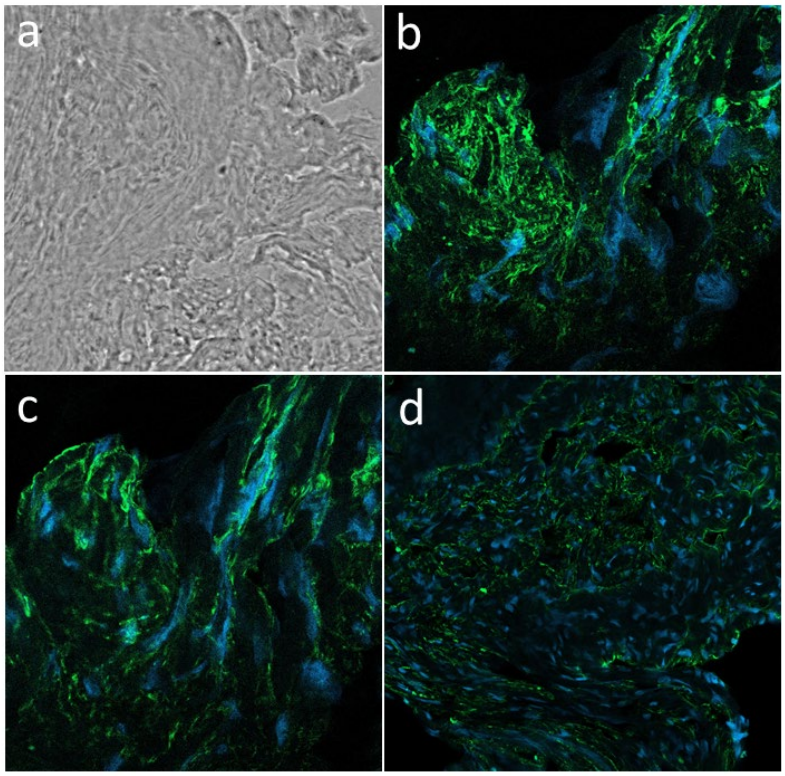

Fig. 6. Representative Immunostaining of Methamphetamine and Epigenetic Proteins. (a) Brightfield image of zebrafish heart tissue. (b-d) Immunostaining of methamphetamine (b), DMNT (c), and MBD (d) within heart tissue of methamphetamine treatment group. Antibodies were conjugated with Alexa Fluor 488 to indicate presence of proteins with green fluorescence. Magnification 40X

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