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The Roles of Estrogen, Nitric Oxide, and Dopamine in the Generation of Hyperkinetic Motor Behaviors in Embryonic Zebrafish (*Danio rerio*)

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Abstract

Both estrogen (E2) and nitric oxide (NO) have been shown to affect motor function, in part, through regulation of dopamine (DA) release, transporter function, and the elicitation of neuroprotection/neurodegeneration of healthy neurons, as well as in neurodegenerative conditions such as Parkinson's disease (PD). Currently, the "gold standard" treatment for PD is the use of levodopa (L-DOPA). However, patients who experience long-term L-DOPA and a monamine oxidase inhibitor (MAOI) treatment may develop unwanted side effects such as hyperkinesia which can be exacerbated by female Parkinsonian patients also on E2 replacement therapy. The current study was designed to determine whether embryonic zebrafish treated with either E2 or L-DOPA/MAOI develop a de novo-induced hyperkinetic movement disorder that relies on the NO pathway to elicit this hyperkinetic phenotype. Results from this study indicate that 5 days post-fertilization (dpf), fish treated with an L-DOPA + MAOI co-treatment or E2 elicited the development of a de novo hyperkinetic phenotype. In addition, the de novo L-DOPA + MAOI- and E2-induced hyperkinetic phenotypes are dependent on NO and E2 for its initiation and recovery. In conclusion, these findings point to the central role both NO and E2 play in the facilitation of de novo hyperkinesia.

Keywords: nitric oxide, estrogen, motor dysfunction, dopamine, L-DOPA, monoamine oxidase inhibitor, zebrafish

1. Introduction

Movement disorders are prominent symptoms of a number of neurodegenerative diseases such as Parkinson's disease (PD), Huntington's, and Alzheimer's disorders. For example, PD affects the motor system of the brain due to dopamine (DA) neurotransmitter deficiency. This disease is caused by the systematic degeneration of DA neurons in the basal ganglia of the brain [1]. Those with this movement disorder exhibit tremors, bradykinesia (hypokinesia), rigidity, balance and posture impairment, loss of automatic movements, and speech difficulties. PD affects millions across the world; the European Parkinson's Disease Association states that 6.3 million people have the neurodegenerative disorder globally [2]. Those who suffer with PD are without a cure and must resort to methods of PD treatment for relief. Currently, the "gold standard" treatment for PD is the use of levodopa (L-DOPA). A precursor to dopamine, L-DOPA is a small enough molecule to pass the blood-brain barrier and enter the basal ganglia where it is acted upon by DOPA decarboxylase to create an increase in dopamine levels. As DA neurons degenerate, an influx of dopamine from exogenous L-DOPA reverses the negative effects of PD [3]. In conjunction with L-DOPA, monoamine oxidase inhibitors (MAOI) are used to also increase dopamine levels as a co-treatment by inhibiting the DA-degrading enzyme monoamine oxidase. Thus, inhibiting monoamine oxidase in conjunction with L-DOPA treatment creates higher levels of DA in PD patients to help alleviate their symptoms. However, patients who experience long-term L-DOPA and MAOI treatment may develop unwanted side effects such as hyperkinesia, an increase in muscular activity that may be excessive or abnormal [4].

Previous studies have suggested that estrogen (E2) has neuroprotective effects in DA neurons and can regulate the synthesis of DA as a pro-dopaminergic agent [5]. In addition, studies show that DA neurons of the central nervous system have E2 receptors and the presence of the E2 synthesis enzyme aromatase [5]. It is clear that there is a connection between E2, the central nervous system, and movement disorders like PD. Indeed, premenopausal women are less likely to show PD symptoms with a majority of patients being male and over 60 [6]. Thus, there appears to be a sexual dimorphism between males and females when it comes to PD prevalence [6]. As a result of the hormonal differences, E2 is considered a neuroprotectant molecule, but there is no evidence for a similar role for testosterone [6]. Recently, this effect has been examined in female rats which have been treated with the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) neurotoxin and have shown the ability to resist muscular activity loss compared to males [6]. In addition to being neuroprotective, there is also accumulating evidence that E2 may also cause detrimental effects such as hyperkinetic/chorea/dystonia symptoms in females on postmenopausal replacement therapy after hysterectomy [5]. There is also the recent case of a patient suffering from adult-onset Sydenham's chorea who discontinued E2 replacement therapy and months later these hyperkinetic/chorea symptoms were significantly diminished [7].

Part of the mechanism by which E2 may exert its influence in the nigrostriatal (BG) of PD patients is through its documented influence on nitric oxide (NO) levels through its regulation of the expression of nitric oxide synthase (NOS) [8]. NO, a gas released by the actions

of the NOS enzyme on L-arginine, acts as a signaling molecule with direct actions on existing metabolic pathways, as well as through genomic mechanisms [9, 10]. As a gas, NO can diffuse across cellular membranes without the aid of membrane-bound transport proteins or receptors. NO can interact directly with its end targets either in the cell in which it was synthesized or in surrounding cells. In turn, its actions are precisely controlled due to its very short half-life and restricted diffusion distance [11, 12]. At higher concentrations NO can act as a free radical in some situations or binds to superoxide anion (O_2^-), causing pathological oxidative stress effects [13]. It is under these conditions that NO is thought to play a role in the genesis of such neurological diseases as PD [4]. On the other hand, NO at lower concentrations can act as a cellular protectant through prevention of apoptosis, excitotoxicity, neuronal depolarization, and regulation of the redox state in the mitochondria [14, 15]. In particular, NO has been implicated in the neuromodulation/neuroprotection of DA neurons in the nigrostriatal (BG) pathway associated with either animal models of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) or 6-hydroxydopamine (6OHDA) neurotoxicity that create PD-like symptoms or from PD patient clinical data. NO acting at the cellular level interacts with either its soluble guanylyl cyclase (sGC) receptor molecule to produce cyclic GMP (cGMP) which activates a cascade of cellular enzymes or causes S-nitrosylation of cysteine residues leading to protein conformational changes [16, 17]. These two pathways are referred to as either the NO-sGC-cGMP-dependent or NO-sGC-cGMP-independent pathways, respectively. In the BG, one of the four nitric oxide synthase (NOS) isoforms, neuronal (n)NOS, is believed to act through the NO-sGC-cGMP-dependent pathway which acts to modulate transcription factors, phosphodiesterases, ion-gated channels, or cGMP-dependent protein kinases (PKG), each of which continues to act physiologically in the nervous system [18]. In the BG, NO has been shown to affect DA release, influence transporter function, and elicit neuroprotection of DA neurons [19].

Zebrafish (*Danio rerio*) have been found to be an excellent model for studying motor disorders because they show similar neurological functions that humans possess and can easily demonstrate PD-like symptoms with damage to its basal ganglia-like structures [20]. In turn, a model where BG-like pathways are simpler and the DA neurons fewer in number and easier to visualize and access would be ideal for such studies. The embryonic zebrafish would appear to fit these criteria. The DA system has been well characterized in both embryonic development and in adults. The DA system in zebrafish, which is equivalent to the nigrostriatal pathway in mammals, has been shown to ascend to the subpallium (striatum) from the basal diencephalon [21]. Also, zebrafish embryos and adults respond to the DA neurotoxins MPTP and 6OHDA, as well as to the DA receptor agonists/antagonists in much the same manner as in mammalian models of PD [22–24]. Indeed, there are an increasing number of studies which make a case for the use of zebrafish as a model for the study of movement disorders such as PD [20]. Earlier observations from our laboratory have established a zebrafish locomotor dysfunctional hypokinetic model linked to both E2 and NO deficiency [24]. In our most recent study, it was demonstrated that when NO synthases are inhibited in zebrafish, using nNOSI, a condition called “listless” occurs where the fish lack swimming abilities, are rigid, and have difficulty maintaining balance, similar to human symptoms of PD [25]. Also, co-treatment with either nNOSI or estrogen (E2), an upstream regulator of NO synthase, could rescue fish

from the “listless”/PD-like phenotype caused by exposure to the neurotoxin 6-hydroxydopamine (6 OHDA) [26]. In turn, NO-deprived zebrafish were rescued from the “listless”/PD-like phenotype when co-treated with L-DOPA, a precursor to DA used routinely in PD therapy. Most significantly, NO involvement in the motor homeostasis of the embryonic zebrafish was shown to be expressed through the NO-sGC-cGMP-PKG-dependent pathway [26]. Therefore, initial evidence for NO's E2-linked role in locomotor activities in an embryonic zebrafish model was established.

It is the hypothesis of this study that when embryonic zebrafish are treated with either E2 or L-DOPA/MAOI that a de novo-induced hyperkinetic movement disorder phenotype will be generated. In conclusion, these results establish a rapid turnover zebrafish model for the study of the role of NO-E2-related DA actions in normal and hyperkinetic movement phenotypes.

2. Materials and methods

2.1. Fish preparations

The compound *roy;nacre* double-homozygous mutant zebrafish, named *casper*, were used in this study. Casper shows the effect of combined melanocyte and iridophore loss in which the body of the embryonic and adult fish is largely transparent due to loss of light absorption and reflection [27]. These transgenic fish were obtained from Carolina Biological Supply. All fish were maintained in a basic embryonic rearing solution (ERS) consisting of NaCl, CaCl₂, KCl, and MgSO₄. These necessary ions were dissolved in deionized water containing a 0.05% methylene blue solution, which serves as an antimicrobial agent. All solutions were changed every 24 h, and embryos were incubated at 28°C. All reagents were obtained from Sigma-Aldrich, and solutions were made daily before use unless noted otherwise. Fish treated at 4–6 days post-fertilization (dpf) were allowed to hatch on their own prior to treatment. All procedures were in accordance with NIH guidance for the care and treatment of animals.

2.2. Reagent preparations

2.2.1. E2-related reagents

All E2-related reagents for treating zebrafish have been previously tested in a dose-response paradigm to insure optimal results and proper survival [26]. Based on previous studies, E2 (17 β -estradiol, Sigma) used at 1 and 5 μ M, and initially solubilized in a 100% ethanol stock solution diluted down to the base treatment solution with ERS, ensuring that the ethanol concentration in the final solution was equal to or lower than 0.5%. The control group consisted of ERS salt solution plus 0.5% ethanol. The reagent 4-androstene-3,17-dione (4-OH-A, MW-286.4, Sigma) was used as an aromatase inhibitor (AI) to block the production of E2 from androgens [24, 25, 28]. It was used at 50 μ M and made from a 100% ethanol stock solution diluted down to the base treatment solution with ERS, ensuring that the ethanol concentration in the final solution was equal to or lower than 0.5%.

2.2.2. NO-related reagents

All NO-related reagents for treating zebrafish have been previously tested in a dose-response paradigm to insure optimal results and proper survival. Based on literature review, baseline target concentrations were identified. Proadifen hydrochloride (Sigma) was used as a selective nNOS inhibitor (nNOSI). With ERS as the diluent, fish were tested at 10, 30, and 50 μ M. The 50 μ M concentration provided optimal results in its ability to create the hypokinetic (listless) condition, and this dose was used throughout the current study.

Diethylenetriamine/nitric oxide adduct (DETA-NO, Sigma) was used to provide a slow extended release of exogenous NO as a co-treatment with some of the inhibitors used in the experiments in an effort to show that NO inhibition-mediated symptoms exhibited by fish can be rescued. It was dissolved into ERS resulting in working concentrations of 400–50 μ M with 50 μ M providing the best results.

1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, Sigma) was used as a soluble guanylyl cyclase (sGC) inhibitor which compromises the NO-cGMP-dependent pathway by reducing cGMP production. It was dissolved into a 0.1% DMSO solution and then diluted with ERS to a working concentration of 30 μ M for application. In addition, DTT (dithiothreitol, Sigma) was used as an inhibitor of the NO-cGMP-independent pathway which prevents S-nitrosylation events at a concentration of 100 μ M.

2.2.3. DA-related reagents

The L-DOPA DA precursor L-DOPA ethyl ester (L-3, 4-dihydroxyphenylalanine methyl ester, Sigma) are used at concentrations up to 10 mM, which is the limit of its solubility in the ERS control solution. L-DOPA is acted upon by DOPA decarboxylase to be converted into DA. It was used to elevate the neurotransmitter in deficient fish starting at ranges prescribed previously for zebrafish embryos [29, 30]. The optimal dose was 10 mM and used throughout the current study.

Monoamine oxidase inhibitor (MAOI) is an agent used to manipulate the zebrafish DA neurons by preventing DA degradation at the synapse. The MAOI, L-deprenyl (Sigma), was used at a concentration of 50 μ M to elicit hyperdyskinetic behavior in a co-treatment paradigm with L-DOPA.

A DA receptor antagonist (haloperidol, Sigma) was used at a concentration of 1–50 μ M as prescribed for zebrafish embryos [31] and 1 μ M was found to be optimal.

2.3. Hyperkinesia phenotype protocols

Fish at 5 dpf were co-treated with L-DOPA + MAOI for up to 48 h or E2 alone for 3–6 h to induce a hyperkinetic state. Using this protocol, additional experiments were also designed to determine whether either L-DOPA or MAOI alone could cause the hyperkinetic phenotype. Specifically, fish were treated with either L-DOPA, MAOI, or a L-DOPA + MAOI co-treatment along with the ERS controls. Next, studies were designed to determine if the hyperkinetic

phenotype could be modified changing NO levels in the L-DOPA + MAOI-treated fish. Specifically, the co-treatment (L-DOPA + MAOI) was compared to the L-DOPA + MAOI + nNOSI tri-treatments along with their respective controls. Next, experiments were designed to test recovery of 5 dpf fish after a 40-h treatment with L-DOPA + MAOI which was followed by either ERS, nNOSI, or DETA-NO post-treatment washouts. The third set of experiments looked at the role of E2 in the generation of the hyperkinetic state. Specifically, fish were treated with either E2, at various concentrations, L-DOPA + MAOI, and L-DOPA + MAOI + AI. Similar co-treatment studies were carried out with E2 according to the following protocols: E2 + haloperidol and E2 + nNOSI.

2.4. Data collection

For visual analysis, fish were characterized using a dissecting microscope, as expressing the hyperkinetic dyskinesia phenotype when their swimming behaviors became significantly different from ERS controls. Specifically, the 'hyperkinetic dyskinesia' phenotype was identified as showing rapid, erratic, and brief spurts of swimming movements and was either calculated as a percent of the treated group or by video capture analysis using a Nikon SMZ1500 microscope to measure the number of spontaneously initiated swimming movements per minute. Also, fish were timed (seconds) as to the duration of their startle/escape response to being touched by a probe on the tail region. The percent survival under the various experimental conditions was also determined for both the hyperkinetic treatment conditions.

2.5. Data analysis

Data were analyzed for significant differences either by a z-test for two-population proportions or for multiple proportions using chi-square contingency table test, followed by a Marascuilo's post-hoc analysis. In addition, for timed video capture movements and startle/escape responses, statistical analysis by using either a two-tailed *t*-test or an analysis of variance (ANOVA) one-way paired *t*-test or ANOVA Single Factor tests. For the ANOVA analysis a Tukey post-hoc method was also run to determine significant differences between the various treatment groups. Sample sizes for all separately treated fish were $n = 30$ and all experiments were repeated in triplicate.

3. Results

3.1. L-DOPA + MAOI co-treatment cause the development of a de novo hyperkinetic phenotype in 5 dpf fish

Figure 1A shows the percentage of zebrafish that demonstrated a hyperkinetic phenotype when co-treated with L-DOPA + MAOI over 40 h of treatment compared with ERS controls. These data show that a significant portion of a population exhibited hyperkinesia after 24 h (55%) in the co-treatment and rises to 90% after 40 h ($p < 0.01$) compared to 0% for the ERS

controls. This provides evidence that the co-treatment is an effective combination for inducing the hyperkinetic phenotype compared to ERS controls that demonstrated no hyperkinesia. Specifically, the hyperkinetic fish demonstrated spontaneous swift, erratic, and chorea/catatonic excitement-like movements when compared to controls.

Figure 1B demonstrates that co-treated fish remain stable for the duration of the treatment paradigm with no significant deaths when compared to ERS controls ($p > 0.05$).

Figure 2 shows photomicrographs from video capture of zebrafish fin movements under various treatment conditions. Note that control fish exhibited synchronous and symmetrical adduction (**Figure 2A**) and abduction (**Figure 2B**) of fin positions during movement or at rest. In contrast, the L-DOPA + MAOI co-treated fish show asymmetric and asynchronous adduction and abduction in their pectoral movements (**Figure 2C**). Behaviorally, these fish exhibit a lack of control of swimming movements and chorea/catatonic excitement-like symptoms.

Figure 1. The effects of an L-DOPA + MAOI co-treatment on generating a hyperkinetic motor behavior phenotype in 5 dpf zebrafish evaluated at 24 and 40 h post-treatment. (A) The percentage of hyperkinetic zebrafish induced by co-treatment with L-DOPA + MAOI compared to ERS controls over 24- and 40-h treatment periods shows a significant increase ($*p < 0.01$) in the appearance of the hyperkinetic phenotype when compared to ERS controls. (B) Shows that there was no significant difference between the ERS and co-treatment mortality rates at both 24 and 48 h post-treatment ($p > 0.05$). Bars = $\pm$ SD.

Figure 2. Video capture photomicrographs of 5 dpf fish fin movements 40 h post-treatment. (A and B) Pictures represent ERS control zebrafish pectoral fin synchronous movements in the adduction (A) and abduction (B) states (arrows). (C) Asymmetric pectoral fin movements in response to L-DOPA + MAOI co-treatment. Note that while the left fin is abducted, the right one is adducted (arrows).

3.2. MAOI is more effective than L-DOPA in eliciting the de novo hyperkinetic phenotype in 5 dpf fish

Figure 3 shows the frequency of initiated movements among ERS control, L-DOPA, MAOI, and co-treatment (L-DOPA + MAOI) fish. This experiment tested which of the two DA-related reagents in the co-treatment was more responsible for generating the hyperkinetic phenotype. These data show that MAOI is the primary facilitator of de novo hyperkinesia in the co-treatment when compared to L-DOPA ($p < 0.01$). Specifically, control and L-DOPA frequency of spontaneous movements were not significantly different at 40 h of treatment (41 ± 5 compared to 37 ± 12 times/min, respectively, $p > 0.05$). Similarly, L-DOPA + MAOI and MAOI-only treatments were not significantly different (74 ± 15 compared to 73 ± 12 times/min, $p > 0.05$). However, MAOI treatment was significantly different from L-DOPA treatment (37 ± 12 compared to 73 ± 12

Figure 3. The number of spontaneous movements that 5 dpf zebrafish initiated over a 1-min duration after 40 h under various treatment conditions. Note that ERS controls initiated movements at approximately 41 times/min, L-DOPA treated zebrafish at 37 times/min, MAOI at 74 times/min, and co-treated L-DOPA/MAOI at 73 times/min. Bars = $\pm$ SD. * $p < 0.01$.

times/min, $p < 0.01$). Specifically, both MAOI and the L-DOPA + MAOI co-treatment initiated by approximately twofold the number of spontaneous movements than that of either ERS or L-DOPA alone.

3.3. The de novo L-DOPA + MAOI-induced hyperkinetic phenotype is dependent on NO, E2, and the DA system for its initiation and recovery

Figure 4 shows the effect of nNOSI on the L-DOPA + MAOI-induced hyperkinetic phenotype. During the first 48 h of treatment, ERS controls showed no hyperkinesia; however, L-DOPA + MAOI co-treatment demonstrated 94% hyperkinesia. Note that **Figure 4A** shows that the L-DOPA + MAOI + nNOSI tri-treatment significantly reduced the co-treatment-induced hyperkinesia after 48 h of treatment (43% vs. 94%, respectively, $p < 0.01$). **Figure 4B** shows the duration of zebrafish swimming, post-tail probe, comparing ERS control, L-DOPA + MAOI

Figure 4. The effect of nNOSI on the L-DOPA + MAOI-induced hyperkinetic phenotype at 48 h post-treatment. (A) The tri-treatment (L-DOPA + MAOI + nNOSI) caused a significant reduction ($*p < 0.01$) in the percent of fish demonstrating the tail probe-induced hyperkinetic phenotype when compared to the co-treatment (L-DOPA + MAOI). (B) The duration of zebrafish swimming, post-tail probe, comparing ERS control, L-DOPA + MAOI co-treatment, and L-DOPA + MAOI + nNOSI tri-treatments. Note that the tri-treatment significantly decreased the swim duration ($***p < 0.05$) when compared to the co-treatment values. Also note that two distinct swimming behaviors, rapid followed by twitch-swim, were observed only in co-treated fish in response to probe stimulation and were significantly different from ERS control values and from each other ($*p < 0.05$ rapid swim, and $**p < 0.05$ Twitch-swim). Bars = $\pm$ SD.

co-treatment, and L-DOPA + MAOI + nNOSI tri-treatments. Note that co-treatment escape swimming behaviors demonstrate a rapid swim followed by much longer twitch swim duration. Specifically, the co-treatment rapid swim phenotype had an average swim duration of 5.1 ± 2.5 s followed by a twitch swim duration of 36.2 ± 12.5 s. In contrast, the L-DOPA + MAOI + nNOSI tri-treatment was significantly reduced the swim duration to 1.5 ± 0.2 s when compared to the co-treatment values ($p < 0.01$).

Figure 5. Demonstration of the percentages of 5 dpf fish that recovered after 24 hours of post-treatment (washout) with various treatments from the hyperkinetic state initially induced by the co-treatment of L-DOPA + MAOI. (A) The washout with ERS showed that approximately 80% of the fish recovered when compared to 0% of the non-washed out fish. At this point the ERS washout fish were not significantly different from those of controls ($*p > 0.05$). (B) Recovery rate of hyperkinetic fish in response to ERS, and nNOSI post-treatments (washout). Note that nNOSI post-treatment significantly inhibits the recovery response when compared to either ERS or DETA-NO post-treatments ($*p < 0.01$). Bars = $\pm$ SD.

Figure 5 depicts experiments testing recovery of 5 dpf fish after a 40 hours treatment with L-DOPA + MAOI to induce the hyperkinetic phenotype. Data were collected after a 24 hours post-treatment washout with either ERS or nNOSI. **Figure 5A** shows that fish treated continually with ERS demonstrates normal (non-hyperkinetic) swimming behaviors and the fish that were not washed out with ERS (just kept in the co-treatment) had a 0% recovery rate. However, the fish that were washed out with ERS solution after the initial co-treatment had approximately an 80% recovery back to normal swimming patterns. **Figure 5B** shows that post-treatment washout with nNOSI post-treatment (less than 20%) washouts showed significantly less recovery ($p < 0.01$).

Figure 6 shows what happens to swimming durations when at 5 dpf, zebrafish were treated with different concentrations of E2. At 6 h post-treatment, fish were lightly touched with a probe and their escape response timed (s—seconds) until they stopped. When the ERS control fish were stimulated they swam for 0.5 ± 0.3 s. Fish treated with $1 \mu\text{M}$ E2, responded by swimming 1.2 ± 0.5 s, which was not significantly ($p > 0.05$) different when compared to the ERS controls. However, fish treated with a $5 \mu\text{M}$ E2 dosage, swam at durations significantly longer (4.7 ± 3.9 s, $p < 0.05$) when compared to both the ERS and standard $1 \mu\text{M}$ dosage of E2. Specifically, 100% of the higher dosage $5 \mu\text{M}$ E2 fish exhibited significant hyperkinetic activity. Conversely, fish treated with $10 \mu\text{M}$ E2 were not able to survive the treatment.

When exposed to various treatments with the reagents AI, and L-DOPA + MAOI or L-DOPA + MAOI + AI, 5 dpf fish exhibited several different swimming phenotypes (**Figure 7**). Specifically, fish treated with AI were 67% listlessness, a significantly higher proportion ($p < 0.05$) when compared to the two other group's swimming characteristics. Most significantly, hyperkinesia

Figure 6. Zebrafish at 5 dpf are treated with different concentrations of E2 and at 6 h post-treatment, fish were lightly touched with a probe and their escape response timed (s—seconds) until they stopped swimming. Note that a $5 \mu\text{M}$ E2 concentration caused a significant increase in hyperkinetic swimming activity when compared to a $1 \mu\text{M}$ E2 dose ($*p < 0.05$). A $10 \mu\text{M}$ dose of E2 was found to be toxic. Bars = $\pm$ SD.

Figure 7. Effects of AI treatment on L-DOPA + MAOI-induced hyperkinetic activity in 5 dpf fish. Note that the addition of AI to the tri-treatment (L-DOPA + MAOI + AI) significantly reduced ($*p < 0.01$) the hyperkinetic probe-induced swim time (s—seconds) when compared to that of the co-treatment (L-DOPA + MAOI). Bars = $\pm$ SD.

was seen in approximately 80% of the co-treated (L-DOPA + MAOI) fish, a significant percentage ($p < 0.05$) when compared to negligible percentages in both the AI fish and the tri-treated (or L-DOPA + MAOI + AI) fish (**Figure 7A**). Normal swimming behavior was the dominant phenotype in the tri-treated fish with a significant ($p < 0.05$) proportion of fish exhibiting this behavior. **Figure 7B** shows that AI treated fish exhibited a significantly reduced swimming duration ($p < 0.05$) when compared to both the co-treatment and tri-treatments. The addition of AI to the L-DOPA + MAOI co-treatment significantly reduced the response time of fish exhibiting the hyperkinetic phenotype. Specifically, co-treated fish (L-DOPA + MAOI) swam at 2.9 ± 0.4 s when probed which is much quicker when compared to the AI (0.2 ± 0.4 s) and tri-treated fish (1.0 ± 1.8 s).

Figure 8A shows the effect of HA on E2-induced hyperkinesia. Specifically, the addition of HA to E2 in a co-treatment paradigm (E2 + HA) significantly reduced ($p < 0.05$) the hyperkinetic probe-induced swim time by fourfold from 4.2 ± 3.9 s to 0.5 ± 0.3 s. In addition, the co-treatment values were very similar to those of the ERS (0.4 ± 0.2 s) and HA (0.4 ± 0.2 s)

Figure 8. The effect of HA and nNOSI on E2-induced hyperkinesia. (A) Note that the addition of HA to E2 as a co-treatment significantly reduced ($*p < 0.05$) the hyperkinetic probe-induced swim time (s—seconds) by fourfold when compared to E2 values. Also, the co-treatment reduced the swim time values to approximately those of the ERS and HA controls. (B) Note that the addition of nNOSI to E2 as a co-treatment significantly reduced ($*p < 0.01$) the hyperkinetic probe-induced swim time (s—seconds) by threefold when compared to E2 values. Also, the co-treatment swim time values were not significantly different ($p > 0.05$) from those of the ERS and nNOSI controls. Bars = $\pm$ SD.

control values. **Figure 8B** shows the effect of nNOSI on E2-induced hyperkinesia. Specifically, the addition of nNOSI to E2 in a co-treatment paradigm (E2 + nNOSI) significantly reduced ($p < 0.01$) the hyperkinetic probe-induced swim time by three-fold from 4.39 ± 2.3 s to 1.3 ± 0.5 s. In addition, the co-treatment values were not significantly different ($p > 0.05$) to those of the ERS (0.8 ± 0.3 s) and nNOSI (0.9 ± 0.6 s) control values.

4. Discussion

The goal of this study was to explore the hypothesis that the co-treatment of L-DOPA + MAOI, and E2 by itself will produce a zebrafish model of de novo hyperkinesia which are both

dependent on the NO pathway for its expression. Also, the current study explored the possibility of using nNOSI as a modulating agent to reduce the de novo hyperkinetic dyskinesia phenotype in the zebrafish.

Data from the current study shows that 5 dpf zebrafish exhibited hyperkinesia as early as 24 h after treatment with an L-DOPA + MAOI co-treatment. Specifically, the hyperkinetic fish demonstrated spontaneous swift, erratic, and chorea/catatonic excitement-like movements, as well as, a significant increase in the number of spontaneous movements when compared to controls. This is the first report of de novo L-DOPA + MAOI-induced hyperkinesia in embryonic zebrafish. However, L-DOPA has been shown in older zebrafish larvae to facilitate recovery of swimming speed after treatment with the antipsychotic fluphenazine [29]. In addition, data from the current study also show that MAOI is the primary facilitator of hyperkinesia in the co-treatment when compared to L-DOPA. Specifically, both MAOI and the L-DOPA + MAOI co-treatment initiated by approximately twofold the number of spontaneous movements than that of either ERS or L-DOPA alone. In an interesting corollary to this finding, it was shown in an earlier study that L-DOPA administered to zebrafish reduced the number of neurons in its nigrostriatal-like pathway which was partially rescued by monoamine oxidase inhibition [32]. This study was focused on the possibility that L-DOPA contains a neurotoxic product that may cause oxidative stress to DA neurons. We saw none of these symptoms in our study perhaps due to the fact that our findings were collected over a matter of 1–2 days duration which was not long enough to see these potential side effects. On the other hand, the fact that monoamine oxidase inhibition increased fish motor activity by a post-treatment paradigm is in support of our findings [32].

The current study also reported that the de novo L-DOPA + MAOI-induced hyperkinetic phenotype is dependent on NO for its initiation and recovery. Specifically, the L-DOPA + MAOI + nNOSI tri-treatment significantly reduced the L-DOPA + MAOI co-treatment-induced hyperkinesia. Similar results in earlier studies have shown that in hemiparkinsonian rats nNOSI improves L-DOPA-induced dyskinesia [4]. The findings are also in line with earlier suggestions of the possibility that nNOSI could be used as a therapeutic agent to reduce the dyskinetic side effects of long-term L-DOPA therapy [33]. In turn, current post-treatment studies demonstrated that NO accelerates recovery from the L-DOPA + MAOI-induced hyperkinetic phenotype when compared to ERS controls. In contrast, nNOSI post-treatment significantly reduced the rate of recovery from the hyperkinetic phenotype. These findings are most likely explained by the documented effects of NO on DA dynamics. Specifically, in the BG, NO has been shown to affect DA release, influence transporter function, and elicit neuroprotection of DA neurons [19].

In the present study, it was also determined that E2 can cause a de novo hyperkinetic phenotype in zebrafish. Specifically, a 3–6 h treatment with E2 elicited a tenfold increase in fish swim duration when compared with that of ERS controls. E2 was also found to significantly affect the L-DOPA + MAOI co-treatment-induced de novo hyperkinetic phenotype. Specifically, the addition of AI to the L-DOPA + MAOI co-treatment significantly reduced the response time of fish exhibiting the hyperkinetic phenotype returning them back to control

levels. These data suggest that E2 is linked to the DA system regulating motor activity in the embryonic zebrafish. This finding was further validated in this study by results showing that the DA receptor antagonist, haloperidol (HA), significantly diminished the E2-induced de novo hyperkinetic activity. Specifically, a co-treatment of E2 + HA significantly reduced by fourfold the hyperkinetic phenotype when compared to just an E2 treatment. This evidence leads to the conclusion that the E2-induced hyperkinetic phenotype acts through the DA D1/D2 receptor system. This conclusion is further substantiated by an earlier study that showed that HA significantly reduced the level of larval zebrafish locomotor activity along a similar time line [31]. Furthermore, the effects of E2 on stimulating/regulating DA levels and thus motor activity have been well documented in other animal models. Specifically, it has been shown that E2 influences DA dynamics in the nigrostriatal pathway that is crucial for normal motor function and is the site of PD pathology [5]. In this system, similar to NO, E2 affects the synthesis, release and turnover of DA, as well as DA transporter and receptor expression [5]. E2 derivatives have also been shown to cause hyperactivity in animal models. Specifically, the addition of bisphenol A, a xenoestrogen exhibiting E2-mimicking hormone-like properties, was shown to cause hyperactivity in newborn mice, adult male rats, and larval zebrafish [34–36]. However, the present study reports for the first time a rapid de novo E2-induced hyperkinetic response over just a 3–6 h duration in the embryonic zebrafish. In addition, the current de novo E2-induced hyperkinetic zebrafish model appears to correlate with accumulating evidence that E2 may also cause detrimental effects such as hyperkinetic/chorea/dystonia symptoms in female patients either through postmenopausal replacement therapy or through E2 replacement therapy after hysterectomy [5]. There is also the recent case of a patient suffering from adult-onset Sydenham's chorea who discontinued E2 replacement therapy and months later these hyperkinetic/chorea symptoms were significantly diminished [7].

5. Conclusions

The current study was designed to determine whether embryonic zebrafish treated with either E2 or L-DOPA/MAOI would develop a de novo-induced hyperkinetic movement disorder and that they rely on the NO pathway to elicit this hyperkinetic phenotype. Results from this study indicate that 5 dpf fish treated with an L-DOPA + MAOI co-treatment or E2 elicited the development of a de novo hyperkinetic phenotype. In addition, the de novo L-DOPA + MAOI- and E2-induced hyperkinetic phenotypes are dependent on NO and E2 for its initiation and recovery. In conclusion, these findings point to the central role that both NO and E2 play in the facilitation of de novo hyperkinesia. In turn, the actions of both E2 and L-DOPA + MAOI in the induction of the hyperkinetic phenotype is dependent on the NO pathway and acts through the DA system. Most significantly, nNOSI has the capacity in this model to modulate the de novo hyperkinetic phenotype which suggests the possibility that it may be further tested for its therapeutic value in patients suffering from long-term L-DOPA-induced dyskinetic side effects.

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Conflicts of interest

The authors have no conflicts of interest.

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