INVESTIGATING MIMETICS OF A PEPTIDE DERIVED FROM THE EFFECTOR DOMAIN OF MARCKS AS POSSIBLE THERAPEUTICS FOR SPINAL CORD INJURY

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ABSTRACT

Like other conditions affecting the central nervous system, spinal cord injury (SCI) is difficult to treat with molecular therapies because the blood-brain barrier makes intravenous treatments largely ineffective. For example, a synthetic peptide chain derived from the effector domain (ED) of myristoylated alanine-rich C-kinase substrate (MARCKS) has been found to improve functional recovery after SCI in female mice; however, peptides do not always pass the blood-brain barrier and are easily degraded due to natural proteases and are excreted during kidney filtration. Therefore, the ED peptide cannot access the central nervous system to exhibit its effects if administered intravenously. Instead of injecting the ED peptide into the bloodstream, we propose to find compounds that can pass the blood-brain barrier in place of the ED peptide, improving treatment compatibility. To find such alternatives, we screened compound libraries via competitive enzyme-linked immunosorbent assay (ELISA) and identified five potential ED peptide mimetics—compounds that mimic the structure and function of the ED peptide. We then used another competitive ELISA to verify their structural similarity to the peptide. After performing toxicity tests to determine the appropriate concentrations of the mimetics to use in functional assays, we found that all five mimetics trigger a significant increase in neurite length in neurons from female mice, but not male mice, when compared to the vehicle control solution. Although more functional tests are necessary, these results suggest that these mimetics trigger ED peptide functions and may provide a more efficient treatment alternative for SCI.

1 INTRODUCTION

Spinal cord injury (SCI) is a detrimental condition impacting 39 individuals per million in the USA that results in permanent impairment or loss of motor and neurological function.[5] There is evidence that triggering signal cascades mediates neurite outgrowth, cell migration, and synaptogenesis by targeting function-proactive cell adhesion molecules, leading to an improvement of functional recovery and regeneration after SCI.[11] One such cell adhesion molecule, neural cell adhesion molecule (NCAM) —a cell-surface molecule known to help neurons stick to other cells or the extracellular matrix—binds to extracellular sugar polymer, polysialic acid (PSA) (FIGURE 1).[7] PSA has several different functions in the mammalian nervous system, including cell migration, axon growth, central nervous system plasticity, and regeneration—all of which are helpful in SCI recovery.[2,21] Given the benefits of PSA, we were interested in identifying and characterizing its receptors; of these, we chose to further analyze myristoylated alanine-rich C-kinase substrate (MARCKS) due to its high concentrations in the hippocampus and spinal cord, which could prove helpful in developing treatments targeting SCI.[16]

The effector domain (ED) on MARCKS acts as the binding site for PSA through the cell, regulat-

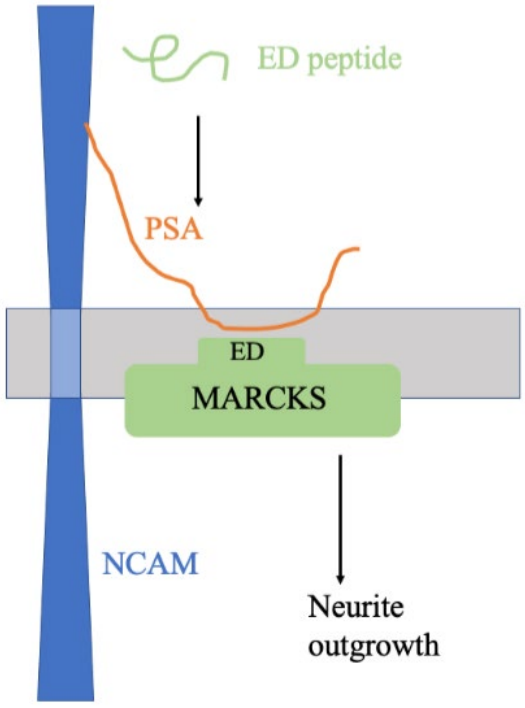

FIGURE 1: SCHEMATIC OF MARCKS-PSA INTERACTION.

Extracellular PSA bound to NCAM binds to the effector domain (ED) of intracellular MARCKS through the cell membrane. When the ED peptide is applied, it competes with endogenous MARCKS and causes PSA to dissociate from MARCKS, therefore initiating a signal cascade that leads to neurite outgrowth.

ing the beneficial effects of PSA (FIGURE 1).[23] A synthetic peptide (ED peptide) containing the same amino acid residues as the ED of MARCKS was previously used to verify the MARCKS-PSA interaction, but it was unexpectedly found to also trigger downstream effects on its own. The ED peptide triggers neurite outgrowth of neurons, increases functional recovery, and enhances phosphorylation levels of MARCKS in neurons and spinal cords in female mice but not male mice.[24]

Although the ED peptide shows promising effects on SCI models, it cannot be administered intravenously because the blood-brain barrier protects the central nervous system from some peptides. To allow for an intravenous treatment option for SCI using the MARCKS-PSA pathway, we are looking for ED peptide mimetics—compounds that mimic the structure and function of the ED peptide—that also cross the blood-brain barrier. We hypothesized compounds that are structurally similar to the ED peptide are also functionally similar to the ED peptide. The current study tests this hypothesis by identifying mimetics based on structural similarity to the ED peptide via biochemical assays, then evaluates their effects on neurons to determine their functional similarity to the ED peptide, including sex-specific outcomes.

2 MATERIALS & METHODS

ELISA SCREENING AND VERIFICATION OF ED PEPTIDE MIMETICS

To identify potential mimetics, we screened for small organic compounds from the National Institute of Health Clinical Collection Libraries 1 and 2 (NIH CCL1 and NIH CCL2) and the Natural Compound Library (NCL) via competitive ELISA (FIGURE 2A). Onto wells of an ELISA plate, we coated 1 µg/mL of MARCKS antibody (MARCKS AB, Sigma Aldrich). As a negative control, we treated two wells with phosphate buffered saline (PBS). Wells were then incubated overnight at 4°C and then washed with PBS to discard any loose antibodies that were not attached to the bottom of the well. Wells were then treated with blocking solution (5% bovine serum albumin [BSA] + 0.1% Triton X-100 in PBS) for one hour at room temperature (RT) to prevent any compounds or reagents from adhering to parts of the well that were not coated with MARCKS AB. After washing, we added individual compounds from the NIH CCL1, NIH CCL2, and NCL to the wells and incubated them at RT for 30 minutes. Biotinylated ED peptide (GenScript) was then mixed with the compounds in each well (final compound concentration = 20 µM; final peptide concentration = 5 µM) for one hour at RT. In this step, compounds compete with the peptide for access to the MARCKS AB, hence the term "competitive ELISA." After washing, wells were treated with streptavidin coupled to horseradish peroxidase (SA-HRP, Jackson ImmunoResearch. Pierce™) at 1:5000 in 5% BSA plus PBS for 30 minutes at RT. Streptavidin has a high affinity to biotin, so SA-HRP specifically binds to the biotinylated peptide. Afterward, wells were washed, then treated with HRP substrate (0.1% O- phenylenediamine dihydrochloride [OPD, Thermo Fisher Scientific] +

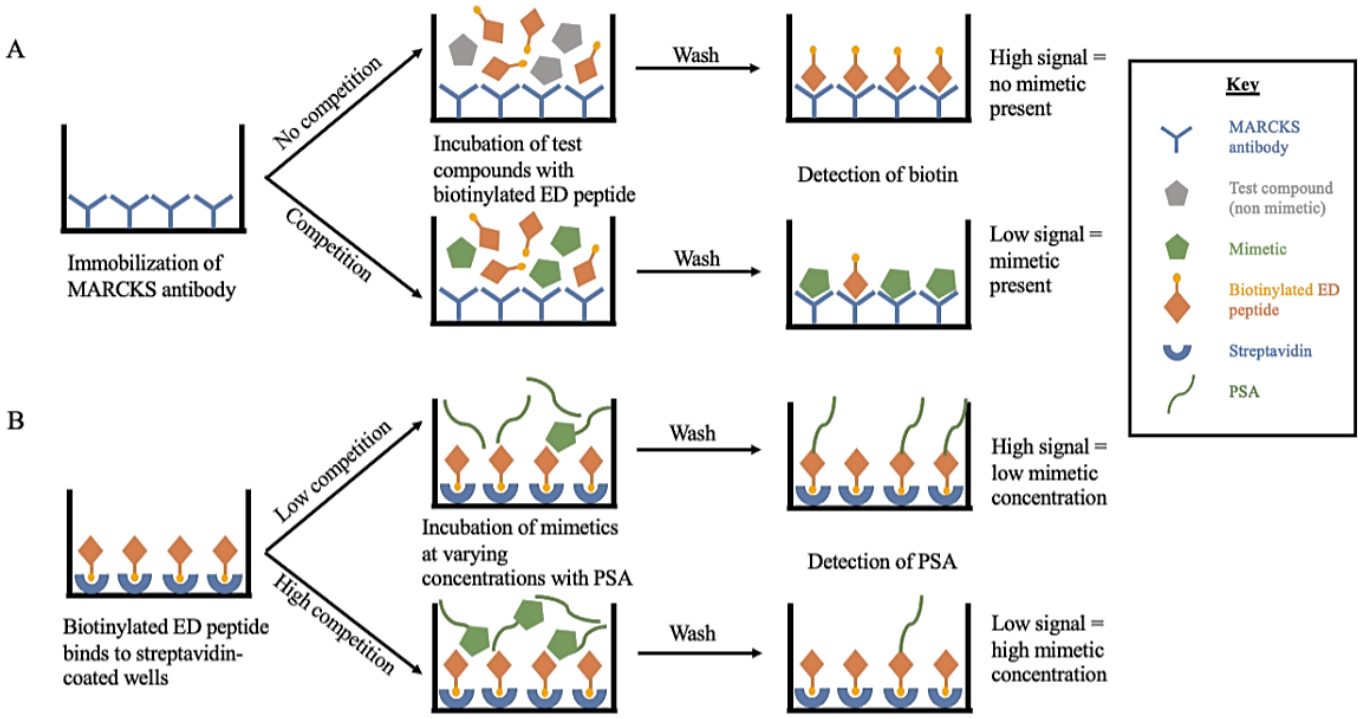

FIGURE 2: SCHEMATIC OF COMPETITIVE ELISAS.

A) *Screening compound libraries for MARCKS ED peptide mimetics. Compounds that are not structurally similar to the ED peptide will not bind to the MARCKS AB, therefore allowing the ED peptide to bind. Conversely, compounds that are structurally similar to the ED peptide will bind to the antibody and block the ED peptide from binding. The peptide is detected via streptavidin bound to horseradish peroxidase and OPD.*

B) *Verifying that mimetics imitate the ED peptide by seeing if they interact with the ED peptide- binding to site on PSA. At high concentrations of the mimetic, more PSA will bind to the compound, leaving less free PSA to bind to the peptide. At low concentrations of the mimetic, more unbound PSA will be able to bind to the immobilized peptide and remain in the wells for detection. PSA is detected via anti-NCAM primary antibody, secondary antibody coupled to HRP, and OPD.*

0.1% hydrogen peroxide [H_2O_2] in OPD buffer). OPD reacts with HRP and H_2O_2 to produce an orange compound; the more peptide, the more SA-HRP, and the more orange the solution appears. Color can be quantified by measuring absorbance at 450 nm with an ELISA reader (EL800; BioTek Instruments) after stopping the reaction at 20 minutes with 2.5 M sulfuric acid.

From this screen, we identified amiodarone, epigallocatechin, nonyloxytryptamine, sertraline, and tegaserod (Sigma Aldrich) as potential mimetics. To verify these compounds, we repeated this procedure except we added varying concentrations (0, 1, 5, 10, 25, 50, 100, 200 μM) of the select compounds from the libraries during the competition step to see if they inhibit the binding of the ED peptide to MARCKS AB in a concentration-dependent manner. Mimetics were prepared by dissolving the powdered form in dimethyl sulfoxide (DMSO) to make 10 mM stocks which were diluted with PBS to the concentrations listed above. Tacrine, a compound found during the screen to not have any effect on MACKS binding to the MARCKS AB, was used as a negative control. Tacrine also binds to an

antibody that activates another cell adhesion molecule, L1; since L1 and MARCKS are unrelated, this offers further reason to use tacrine as a negative control.[11]

To determine if the verified mimetics also inhibit the interaction between the ED peptide to its binding partner PSA, we first incubated varying concentrations (0, 1, 5, 10, 25, 50, 100, 200 μM) of each mimetic with 20 μg/mL of colominic acid (PSA, Sigma Aldrich) at 4°C overnight. Then we coated 5 μM biotinylated ED peptide on a streptavidin-coated plate (Thermo Fisher Scientific) for one hour at RT. Subsequently, unbound ED peptide was washed off with PBS and the mixed solution of PSA and small organic compounds were added to the wells to incubate at RT for one hour (FIGURE 2B). Afterward, the wells were washed and incubated with 1:200 of anti-PSA-NCAM (primary antibody 735, Thermo Fisher Scientific) in PBS for one hour at RT. Subsequently, wells were washed and incubated with 1:1000 of mouse-HRP (secondary antibody) dissolved in blocking solution at RT for 30 minutes. Lastly, wells were washed and treated with HRP substrate. After 10 minutes, we stopped the reaction with sulfuric acid and read absorbance at 450 nm on the ELISA reader.

CEREBELLAR GRANULE CELL CULTURE

Cerebellar granule cells make up the largest population of neurons in the mammalian brain and are relatively simple in structure, making them an ideal model for in vitro neuronal cultures.[13] To obtain these cells, we removed cerebella from 6 to 8-day-old mice that were euthanized in accordance with the IACUC protocol #: 09-051. To begin the culture, 48-well plates are coated with 0.01% Poly-Lysine-L (PLL) for 30-60 minutes in the incubator (37°C). PLL is a chain of synthetic amino acids that helps cells adhere to the bottom of the well-plates. Then, PLL was aspirated off, and wells were washed with sterile distilled water and left to dry. Stocks of medium (containing Neurobasal A, B-27, Penicillin/Streptavidin, BSA, Na-Pyruvate, L-Glutamine, Transferrin holo, Insulin, L-Thyroxine, and Na-Selenite) were prepared, filtered, and warmed in a 37°C water bath before use. Once cerebella were removed using sharp forceps, the meninges and blood vessels were cleaned off. Subsequently, each cerebellum was cut into pieces and put into test tubes of HBSS (Hank's Balanced Salt Solution). HBSS is a buffer that keeps cells in their preferred pH range and helps maintain high viability during short-term incubations. HBSS was then removed via an aspirator, and trypsin was added to the tissue for a 15-minute incubation in a 37°C water bath. Trypsin breaks down adhesive proteins that help cells stick together. Cell cultures require seeding individual cells for optimal imaging and even treatment stimulation. Subsequently, trypsin was carefully aspirated from each test tube, and tissue was then washed with HBSS. DNase was then added to each test tube to digest any DNA that leaked from damaged cells, which can form aggregates and hinder dissociation. Cells were then dissociated via pipette until there were no visible pieces of cerebella left. Dissociated cells were incubated with the DNase solution for 5 minutes in a 37°C water bath and then diluted with HBSS before centrifugation at 200 g for 5 minutes at 4°C. Centrifugation is a separation technique that uses centrifugal force to collect heavier particles in a solution (in this case, the cells) at the bottom of a container, therefore creating a cell pellet. Afterward, HBSS was aspirated off, and the cell pellet was resuspended in a pre-warmed medium. Cell solution was combined with fluorophores (acridine orange and propidium iodide, Nexcelom Bioscience) that stain dead and live cells different colors to count the cells. Initial viability and concentration of live cells were measured using the Cellometer Auto 2000 Cell Viability Counter. Cells from males and females were measured and cultured separately.

TOXICITY TESTS

The cerebellar granule cell culture procedure detailed above was carried out, separating male and female mice. After counting cells, cells from each sex were separately diluted to 500,000 cells/mL with a pre-warmed medium. Cells were seeded into 24 wells (12 for male cells and 12 for female cells) on 0.01% PLL-coated 48-well plates with 250 μL of 500,000 cells/mL solution in each well and maintained at 37°C for 24 hours. Afterward, cerebellar granule cells were incubated with 0.1% DMSO as a

vehicle control, 1, 10, and 100 μM of the mimetic for another 24 hours. For live imaging, propidium iodide and calcein-am solution (each 4 ng/mL) were first added to each well, and the plate was then incubated (37°C) for 20-30 minutes before using the fluorescent microscope. AxioVision was used to control contrast and adjust the clarity of the images. Four images were taken per well. The experiment was performed three times in triplicate for each compound ($n = 36$).

Neurite Outgrowth

The cerebellar granule cell culture procedure detailed above was carried out, subsequently diluting cells from each sex to 100,000 cells/mL with a prewarmed medium. Cells were seeded into 4 wells per treatment (two for males and two for females) on 0.01% PLL-coated 48-well plates. Cells were immediately stimulated with 0.1% DMSO for final concentrations in the wells of 0, 0.1, 1, 10, 100, and 1000 nM of each mimetic diluted in DMSO. One row of wells was left untreated to see if DMSO would affect neurite outgrowth. Plates were then incubated for 24 hours at 37°C. Each well was treated with 2.5% glutaraldehyde to fix cells and left to incubate for 30 minutes at RT. Subsequently, the wells were washed with water. Staining solution (1% toluidine blue O, 1% methylene blue in 1% borax) was added to the wells and left to incubate at RT for 30 minutes. Wells were then washed with water, and the plate was left to dry so images could be taken. Images were taken using AxioVision. Neurites were traced and measured using ImageJ.

Statistical Analysis

Statistical comparisons between groups were performed by one-way ANOVA test using StatView for all experiments because each had more than two experimental groups. Subsequently, Fischer's PLSD post-hoc test is used to determine the statistically significant variance between groups. According to Fischer's PLSD test, asterisks in figures signify statistically significant differences between groups wherein, $* p < 0.05$, $** p < 0.01$, $*** p < 0.001$. Every experiment was performed three independent times and the averages and standard errors of the mean were calculated from all trials on Microsoft Excel. Schematics, graphs, and calculations were done with Microsoft Office.

3 Results

Five Compounds Inhibit the Binding of ED Peptide to the MARCKS Antibody

Our results show that epigallocatechin, amiodarone, sertraline, tegaserod, and nonyloxytryptamine inhibit the binding of the ED peptide to MARCKS AB in a concentration-dependent manner. At 25 μM and higher concentrations, all the mimetics inhibit the ED peptide from binding to the antibody significantly more than our negative control, tacrine, another small organic compound from the NIH CCL1 (Figure 3).

ED Peptide Mimetics Bind to PSA

To further verify the integrity of the structural similarity between the mimetics and the ED peptide, we conducted a competitive ELISA to see if the mimetics could inhibit the binding of PSA to the ED peptide (Figure 2B). From this procedure, we determined that the compounds inhibit the binding of the ED peptide to PSA in a concentration-dependent manner, and all compounds significantly inhibit the binding of PSA to MARCKS at six concentrations or more (Figure 4).

The Five Mimetics are Nontoxic in Cerebellar Granule Cells at 1μM

Before determining if the identified mimetics trigger ED peptide-mediated functions, we determined the cytotoxicity of the mimetics. In all five MARCKS ED peptide mimetics, concentrations of 1 μM are nontoxic for cerebellar granule cells in both sexes. A 10 μM concentration of epigallocatechin gallate, is also nontoxic (Figure 5). We define the toxic range to be any percent viability equal to or less than 50%. There is no apparent sex-specific difference in toxic ranges shown in the data. Based on the data shown in Figure 5, we suggest using a maximum of 1 μM concentration in live functional assays. Therefore, we chose seven concentrations ranging from 0 μM (DMSO) to 1 μM of each compound to stimulate the cerebellar granule cells in our neurite outgrowth assays.

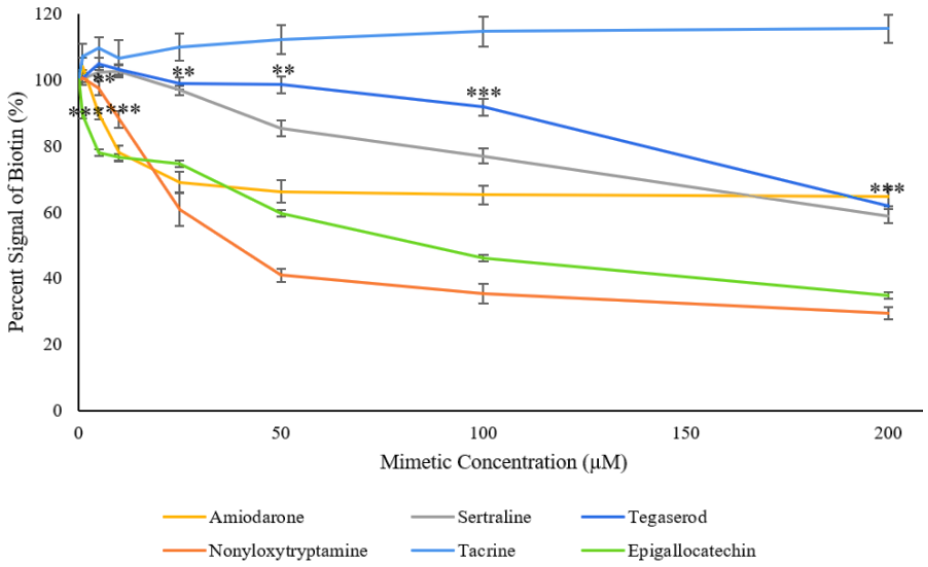

FIGURE 3: VERIFYING POTENTIAL ED PEPTIDE MIMETICS.

Verifying epigallocatechin gallate, amiodarone, sertraline, tegaserod, and 5-nonyloxytryptamine as potential ED peptide mimetics. Error bars indicate standard error of the mean. (Three independent trials were carried out in triplicate, $n = 9$*). Asterisks signify statistically significant differences between the identified mimetics and the negative control tacrine wherein* $* p < 0.05$*,* $** p < 0.01$*,* $*** p < 0.001$*. (One-way ANOVA,* $F = 63.39$*,* $p < 0.0001$*).*

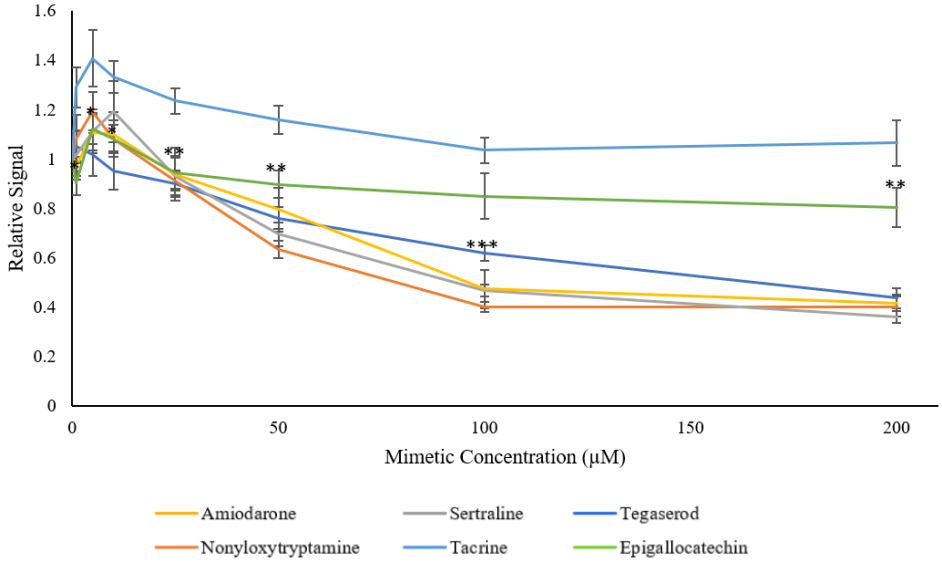

FIGURE 4: DETERMINING IF MIMETICS INHIBIT THE BINDING OF PSA TO ED PEPTIDE.

Determining if epigallocatechin, sertraline, amiodarone, tegaserod, and nonyloxytryptamine inhibit PSA from binding to ED peptide. Error bars indicate standard error of the mean. (Three independent trials were carried out in triplicate, $n = 9$*). Asterisks signify statistically significant differences between the identified mimetics and the negative control tacrine wherein* $* p < 0.05$*,* $** p < 0.01$*,* $*** p < 0.001$ *(One-way ANOVA,* $F = 12.512$*,* $p < 0.0001$*).*

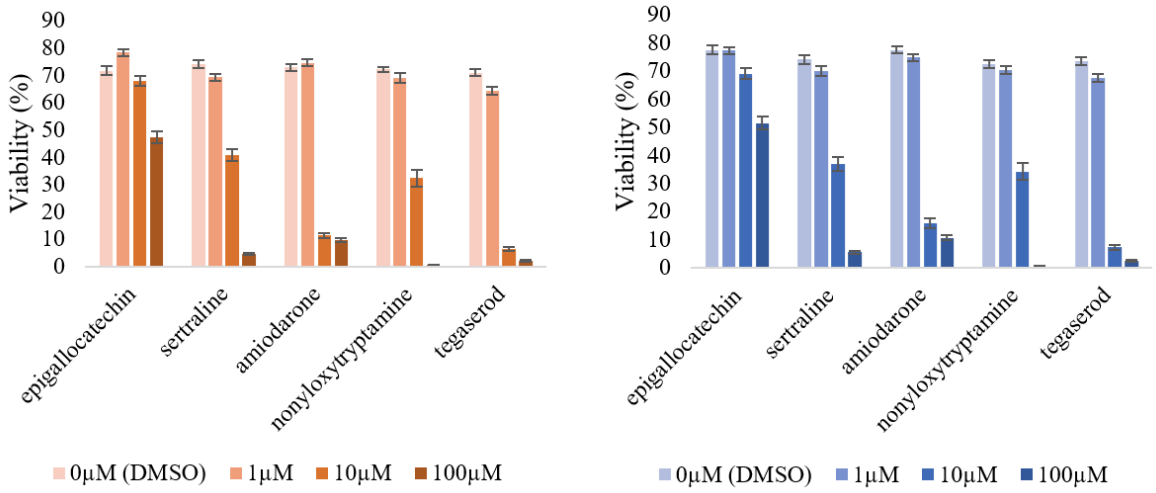

FIGURE 5: MEASURING NEUROTOXICITY OF MARCKS ED PEPTIDE MIMETICS IN CEREBELLAR GRANULE CELLS.

Cerebellar granule cells were dissociated from 6 to 8- day-old mice, separated by sex and seeded onto PLL-coated plates. After 24 hours, cells were treated with DMSO or 1 μM, 10 μM, or 100 μM of each mimetic. Cell viability was measured by propidium iodide and calcein-am staining to take the ratio of live cells to total cells. The bar graphs show the average cell viability and error bars indicate standard error of the mean. (Four images were taken per well. Three independent trials were carried out in triplicate, $n = 36$).

THE FIVE MIMETICS PROMOTE NEURITE OUTGROWTH IN FEMALE CEREBELLAR GRANULE CELLS AT NANOMOLAR CONCENTRATIONS

Our first functional test was to determine whether ED peptide mimetics promote neurite outgrowth in female cerebellar granule cells and not males, as the ED peptide does. All five mimetics significantly stimulated neurite outgrowth at 0.01-1000 nM, except for amiodarone which promoted neurite outgrowth at only 1-1000 nM (FIGURE 6). In addition, neurite outgrowth was observed only in female cerebellar granule cells, supporting the discovery that MARCKS promotes neurite outgrowth in a sex-specific manner. There was no significant difference in neurite length between DMSO-treated and mimetic-treated male cerebellar granule cells (FIGURE 6). Nonyloxytryptamine was the most effective mimetic, promoting the most neurite outgrowth at 10000 nM with an average neurite length of 48.6 μm (SEM 1.85 μm) compared with the vehicle control, DMSO, with an average neurite length of 31.4 μm (SEM 0.77 μm). Epigallocatechin, sertraline, and tegaserod were also effective compounds in promoting neurite outgrowth with average neurite lengths of 46.5 μm (SEM 1.43), 42.2 μm (SEM 1.38), and 41.8 μm (SEM 1.27) at 1000, 100, and 1000 nM, respectively. Amiodarone was the least effective mimetic in promoting neurite outgrowth with an average length of 41.5 μm (SEM 1.36) at 100 nM (FIGURE 6).

4 DISCUSSION

Our results show that nonyloxytryptamine, epigallocatechin, sertraline, tegaserod, and amiodarone mimic both the structure and function of the ED peptide. We determined structural similarity with competitive ELISAs and functional similarities by measuring neurite outgrowth.

The identified ED peptide mimetics may prove to be better treatment options because they are not as easily degraded or blocked by the blood-brain barrier as the ED peptide. Additionally, compounds from the libraries that we screened have a history of use in human clinical trials and SCI studies.

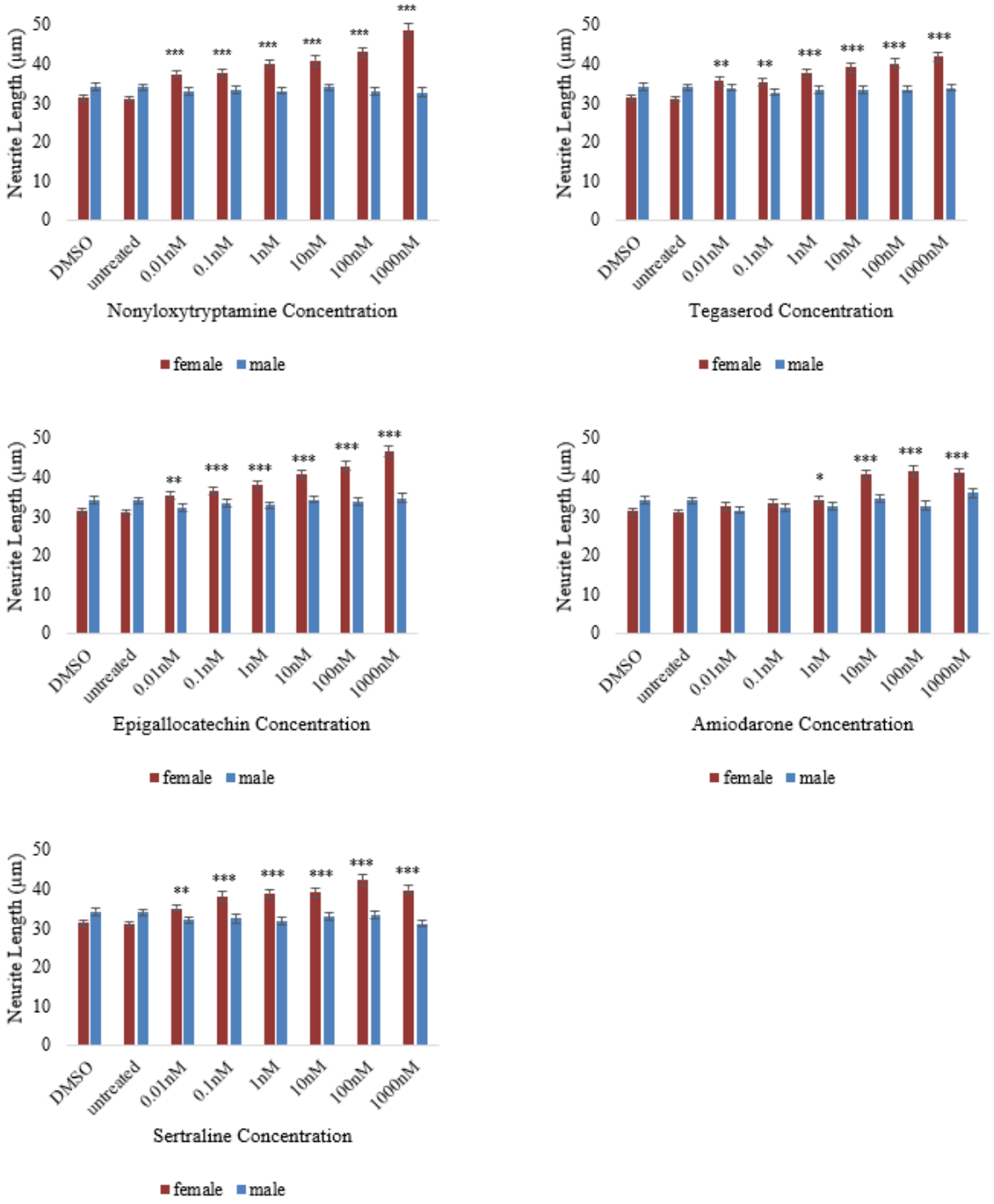

FIGURE 6: MEASURING NEURITE OUTGROWTH OF CEREBELLAR GRANULE CELLS TREATED WITH DIFFERENT CONCENTRATIONS OF ED PEPTIDE MIMETICS.

Cerebellar granule cells were dissociated from 6 to 8- day-old mice, separated by sex and seeded onto PLL-coated plates. Cells were immediately treated with 0.1% of DMSO and each mimetic with final concentrations of 0 nM, 0.1 nM, 1 nM, 10 nM, 100 nM and 1000 nM. The bar graphs show the average neurite length and error bars indicate standard error of the mean (100 neurites were measured in each treatment per trial. Three independent trials were carried, $n = 300$*). Asterisks signify statistically significant differences between the identified mimetics and DMSO wherein* $* p < 0.05$, $** p < 0.01$, $*** p < 0.001$. *(Nonyloxytryptamine, one-way ANOVA,* $F = 21.11$, $p < 0.0001$*; epigallocatechin, one-way ANOVA,* $F = 18.39$, $p < 0.0001$*; sertraline, one-way ANOVA,* $F = 13.064$, $p < 0.0001$*; tegaserod, one-way ANOVA,* $F = 10.789$, $p < 0.0001$*; amiodarone, one-way ANOVA,* $F = 11.968$, $p < 0.0001$*).*

Two of the other verified MARCKS ED peptide mimetics, tegaserod, and nonyloxytryptamine, are also PSA mimetics that facilitate nervous system repair and improve recovery after SCI in mice.[4,15] Epigallocatechin gallate (EGCG) is an active compound found in green tea that has been shown to pass through the blood-brain barrier.[9] In some regards, EGCG appears to be functionally similar to the ED peptide in that EGCG improves functional recovery after chronic and acute SCI and sciatic nerve injury.[12,18,19,25] Additionally, the ED peptide and EGCG increase the phosphorylation levels of MARCKS in neurons.[6,27] EGCG also has neuroprotective, anti-inflammatory, and anti-edema effects after spinal cord and sciatic nerve injury and attenuates neuropathic pain.[1,3,8,14,20,22,26,28]

While we have found that the ED peptide improves functional recovery after SCI in female mice, the molecular mechanisms underlying sex-specific responses remain unclear. Therefore, ED peptide mimetics are not only beneficial in potentially substituting the peptide as a form of treatment but might offer insight into how the ED peptide promotes neurite outgrowth and improves functional recovery in female mice.

In the future, we will test if the mimetics enhance MARCKS phosphorylation levels specifically in female but not male cerebellar granule cells. We predict that the ED peptide mimetics will be effective replacements for the ED peptide in recovery from trauma not only in SCI but also in other model systems of trauma in mammals, fundamentally improving recovery response■

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