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MPTP induces neurodegeneration by modulating dopaminergic activity in catfish brain

Mamta Sajwan-Khatri *, Balasubramanian Senthilkumaran *

Department of Animal Biology, School of Life Sciences, University of Hyderabad, P.O. Central University, Hyderabad 500046, Telangana, India

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ABSTRACT

Tyrosine hydroxylase (Th) is an allosteric rate-limiting enzyme in catecholamine (CA) biosynthesis. The CAs, dopamine (DA), norepinephrine (NE), and epinephrine are important neurotransmitters wherein DA contributes a key role in the central nervous system of vertebrates. The present study evaluated DA and Th's significance in DA-ergic activity and neurodegeneration upon 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) exposure in catfish. Further, the expression of certain brain-and ovary-related genes measured through qPCR were downregulated upon MPTP treatment which is in accordance with the decreased levels of L-Dopa, DA, and NE levels estimated through HPLC-ECD. Additionally, TEM analysis depicted structural disarray of brain upon MPTP exposure and also decreased serum levels of testosterone, 11-ketotestosterone, and estradiol-17β. MPTP treatment, *in vitro*, using primary brain cell culture resulted in diminished cell viability and increased ROS levels leading to elevated apoptotic cells significantly. Consequently, the study highlights the MPTP-induced neurodegeneration of the Th and DA-ergic activity in corroboration with female brain-related genes downregulation, also gonadal function as evidenced by depleted sex steroids level and low expression of ovary-related genes.

1. Introduction

The rate-limiting allosteric enzyme tyrosine hydroxylase (Th) regulates the catecholamines (CA) biosynthesis such as dopamine (DA), norepinephrine (NE), and epinephrine in vertebrates. Th is an utmost meticulously modulated enzyme in the central nervous system (CNS) of vertebrates (Kumer and Vrana, 1996; Sabban and Kvetňanský, 2001) which is also involved in the modulation of gonadotropin-releasing hormone (GnRH) and release of gonadotropin (GTHs) in teleost (Goos et al., 1999; Popesku et al., 2008). DA is a key neurotransmitter that likely influences the hypothalamo-hypophyseal-gonadal (HHG) axis and primarily regulates the release of the GTHs (Dufour et al., 2005). In teleost dopaminergic (DA-ergic) regulation of GnRH1 neurons modulates reproduction through DA type-2-like-receptors partly by influencing the activity of neurons and neurological diseases (Bryant et al., 2016). Robust compensating processes occur after prolonged stress with neurotoxin treatment by maintaining adequate levels of CA through Th up-regulation by modulating the synthesis of CA levels (Xing et al., 2017). Neurotoxin 1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine (MPTP), caused Parkinson's disease (PD) and other neurological diseases in all animals including humans. It has been broadly used to create PD models in zebrafish and goldfish, together with other neurodegenerative stress induction in catfish (Anichtchik et al., 2004; Bretaud et al., 2004; McKinley et al., 2005; Wen et al., 2008; Mamta and Senthilkumaran, 2018). In goldfish, MPTP's intraperitoneal (IP) administration has led to a selective loss of DA neurons inducing parkinsonian syndrome (Xing et al., 2017), causing neurodegeneration. In addition, DA neurons are destroyed by neurotoxins, and numerous sensory deficits and cognitive were noticed in which the recovery rate was very low in animal models (Zigmond et al., 1990; Robinson et al., 1990). An earlier report, in this line, suggested that a single low dose (20 mg/kg) of MPTP caused DA neurons damage, determined by the single IP injection effect on DA neurons also labelled by neuron Nissl+ and Th which depicted a significant loss in the substantia nigra neurons in mice (Alam et al., 2017). In earlier studies, significant glial cell activation and glial cell line-derived neurotrophic factor (GDNF) *via* its receptor *GFRα-1* instigated damage of DA neurons with low activity in the MPTP-induced brain of catfish (Mamta and Senthilkumaran, 2018) and other PD models (Yokoyama et al., 2011; Blesa et al., 2012). The expression level of *th* gene was up-regulated by GDNF, which also promoted the *th* gene transcription and stability of mRNA (Xiao et al., 2002). In teleost models, the precise functions of the activation of glial cells and DA-ergic

* Corresponding authors.
E-mail addresses: mamta@uohyd.ac.in (M. Sajwan-Khatri), bsksl@uohyd.ac.in (B. Senthilkumaran).

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system including DA neuronal regulation have not yet been well understood. Therefore, direct brain injection of neurotoxin, MPTP through the pineal window was used to deplete Th and DA activity in 100 days post-hatch (dph) in catfish in this work. The catfish at 100 dph was used in this study as most of the development pertaining to sex differentiation gets completed while brain organization and gonadal development are progressive. Catfish are sturdy to withstand the cranial injections of drugs like MPTP under mild anaesthesia to induce neurodegeneration (Senthilkumaran et al., 2015; Mamta and Senthilkumaran, 2018). Due to this, catfish are very useful animal models for brain neurodegeneration research when compared to common model systems. Furthermore, tracking DA-ergic activity post-MPTP administration was established previously (Mamta and Senthilkumaran, 2018). It is also important to understand the novel regulatory mechanism which might help to elucidate the role of Th implicating DA synthesis in midbrain neurons under normal and neurodegenerative stress conditions. To assess the possible effects on the Th vis-à-vis DA-ergic system activity, upon neurotoxin MPTP exposure, the present analysis provides a basis to understand the significance of Th, in light of changes in the DA-ergic system after neurodegenerative stress, together with its functional implication by considering various brain and gonad-related transcription factors/genes. Subsequently, this study was extended to examine the cytotoxic effects of MPTP on CAs levels, more importantly, to know the interactions of Th and DA-ergic system also its implication on neurodegeneration.

2. Methodological approaches

2.1. Animal sampling and treatment

Animal model *Clarias batrachus* now known as *C. magur* is an annual breeder and commonly knowns as the Asian catfish or air-breathing which undergoes a cyclical pattern of reproduction. *C. magur* in diverse age groups were reared in freshwater tanks under ambient photothermal conditions. *In vitro* fertilization was performed to acquire different age groups of catfish hatchlings during the breeding season using mature male and gravid female, which was intraperitoneally injected with human chorionic gonadotropin (500 IU/100 g body weight). The fertilized embryos were transferred to other small glass tanks with filtered water and an aerator. Usually, catfish embryos take 24 h to hatch and can survive for 1–2 days without feed by utilizing the yolk sac for nutrition. Later, live tubeworms were fed to the hatchlings for 3 months. Later, the fingerlings were fed dried or live worms in addition to food pellets, *ad libitum* and catfish takes about a year to mature which marks the beginning of the reproductive cycle. Catfish (female) at 100 dph were chosen for MPTP treatment in this study mostly but males were also chosen for sex steroids estimation after MPTP treatment. Neurotoxin, MPTP was freshly prepared and injected directly into the fish brain (female) through the pineal window with different concentrations of 5, 10 50, 100, and 150 μg/μl for 7 days and divided into experimental control ($n = 5$) and treated groups into separate glass tanks (50 liter) fitted with water aeration system. After MPTP treatment, female brain and ovary were collected, which were further used for total RNA extraction, *in vivo* expression analysis, and different CAs quantification, whereas sex steroids measurements were done in both sexes independently. Interestingly, the difference in the gonads (morphological) occurs around 50 dph age in catfish (Raghuveer et al., 2011a; Mamta et al., 2014). The Institutional Animal Ethics Committee (Inst. Reg. No.151/1999 dt.22.07.1999, CPCSEA, University of Hyderabad) guidelines were followed to perform all the experiments and sacrificial procedures.

2.2. Quantitative real-time PCR (qRT-PCR)

Brain (female) and ovary-related genes outlined below (see 2.3) were chosen in this study. Expression analysis was performed by qRT-PCR using SYBR green detection method after performing first-strand cDNA synthesis using total RNA, isolated from tissues as described earlier (Mamta and Senthilkumaran, 2018). RNA purity was measured using a NanoDrop spectrophotometer (Technologies and Wilmington, ND-2000, USA) followed by the RNA integrity testing in agarose gel. Later, random hexamers were used for the reverse transcription by Thermo Scientific Inc., verso® reverse transcriptase with total RNA (1 μg), isolated with TRI-reagent® (Sigma) and DNase I treatment to eliminate the genomic DNA as per the manufacturer's protocol for first strand cDNA synthesis. Additionally, both qRT-PCR primers or at least one of those was designed at the exon-exon boundary to prevent any amplification from genomic DNA in qPCR. The reaction was performed in triplicate in MicroAmp® 96-well plates in an applied Biosystems thermal cycler using qPCR primers (Table 1). Later, to check the amplicon specificity analysis of the melting curve was performed, the value of cycle threshold (Ct) was measured from the qPCR amplification and normalized against the expression reference gene, *18S rRNA* expression to generate a ΔCt value (Ct of target gene - Ct of reference gene).

2.3. Expression of female brain and ovary-related genes after MPTP exposure

Expression levels of several brain-related genes such as *glial cell line-derived neurotrophic factor receptor α-1(gfrα-1), catfish gonadotropin-releasing hormone* (*cfGnRH1), brain aromatase* (*cyp19a1b*), *3β-hydroxysteroid dehydrogenase (hsd3b)*, *tryptophan hydroxylase2* (*tph2*), tyrosine hydroxylase (*th*) and *LH-b/GTH-II* (*lh-β*) and gonad related genes *i.e.*, *ovarian aromatase (cyp19a1a), paired box gene 2 (pax2), SRY-related high mobility group box (sox 9), forkhead box L2 (foxl2), adrenal 4 binding protein/Steroidogenic factor-1 (ad4bp/sf-1)* were quantified after neurotoxin MPTP exposure or saline control using specific RT primers.

2.4. In vivo and in vitro, neurotoxin MPTP treatment

Neurotoxin MPTP was used from the laboratories of Hychem

Table 1
List of Primers used for qRT-PCR.

	Primer name with gene symbol	Nucleotide sequence (5′- 3′)	GenBank Accession number
Brain related genes	*gfrα-1 fw*	GGTGAAGCAGCCTTTAGCAAGG	KY553234
	gfrα-1 Rv	CAGAAAAGCATGCCGTAGCTGTGC	
	cfGnRH1 fw	AGCGTGCCGTGATGCAGGAG	X78049
	cfGnRH1 Rv	TCTCTCCCAGCGACAGGCGT	
	cyp19a1b fw	CCAGGTCCATACTGGTTACTG CTG	GU220076
	cyp19a1b Rv	CACAGCAGATGACTTGCTTAG	
	hsd3b fw	GAGGTAAATGTGAAAGGTACCAA	HQ680983
	hsd3b Rv	TAGTACACAGTGTCCTCATGG	
	tph fw	CAGTTCTCACAGGAGATTGGCC	GU290195
	tph Rv	CGAAACTCTCTGAGACAAAGT	
	Th fw	CCAGAGCCAGACTGCTGTCACGAG	KF739410
	Th Rv	CACCGTACGCCTTCACTGTTC	
	Lhβ fw	CACCGTACGCCTTCACTGTTC	X97761.1
	Lhβ Rv	CGGACACTGCTTCACCAAGGAACC	
	18S rRNA fw	GCTACCACATCCAAGGAAGGCAGC	AB105163
	18S rRNA Rv	CGGCTGCTGGCACCAGACTTG	
	cyp19a1a fw	AGGTCCCTGGTTTTGTCTG	GU220075
	cyp19a1a Rv	TGCAGATGGCCTGCTGAGG	
	pax2 fw	ATCCTGGGCAGGTACTATGA	KU301794
	pax2 Rv	GGATCTCCCACGCGAACAT	
ovary related genes	*sox9b fw*	GAGACCCAGTCAGGCCACAG	HM149259
	sox9b Rv	GAGACCCAGTCAGGCCACAG	
	foxl2 fw	CATGGCTATACGCGACAGCTC	HM149259
	foxl2 Rv	CCAGTAGTTCCCCTTCCTCTC	
	ad4bp/sf-1 fw	GGAGACAAGGTGTCCGGGTATCACT	KU312054
	ad4bp/sf-1 Rv	TCATTCGATCGGCCCTCACAGCCTC	

(Catalogue No. 28289–54-5). The fish ($n = 5$) at 100 dph, were injected neurotoxin, MPTP (*in vivo*) right into the brain *via* a pineal window in five different groups with different concentrations, and saline was used for the control group. Dose-responses analysis was performed to know the MPTP effect, *in vitro* using a primary brain cell culture on cell viability by mitochondrial membrane potential (MMP) and 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as explained below.

2.5. CAs level after MPTP exposure by high-performance liquid chromatography-electrochemical detection (HPLC-ECD)

Measurement of brain CA levels (L- Dopa, DA, NE) after neurotoxin, MPTP treatment in comparison with controls were done by high-performance liquid chromatography-electrochemical detection (HPLC-ECD) as defined earlier (Nagao and Tanimura, 1988; Mamta and Senthilkumaran, 2018).

2.6. TEM analysis

TEM analysis was performed for control and MPTP-treated samples (50 μg/μl and 150 μg/μl). Brain (pituitary) samples were fixed in glutaraldehyde 2.5% prepared in phosphate-buffered (PB) 0.1 M, pH 7.2 then incubated for 24 h at 4 °C. Later, each sample was washed thrice in 0.1 M phosphate-buffered saline (PBS) for 45 mins. Further, 1% aqueous osmium tetroxide was used for fixation for 2 h followed by rinsing with deionized water 6 times each, embedded and infiltrated in Araldite 6005 resin after dehydration in graded series of alcohol. The treated samples were polymerized completely and incubated for 72 h at 80 °C. Appropriate thickness sections were made using a Leica, ultramicrotome, saturated aqueous uranyl acetate, mounted Cu grids were used for staining and Reynolds' lead citrate was counterstained. Microphotographs of TEM were acquired with an FEI Technai (G2 S-Twin instrument) at an acceleration voltage of 200 kV. TEM analysis was done at Acharya N G Ranga Agricultural University, Hyderabad.

2.7. Primary brain cell culture

Primary brain cell cultures were prepared by following the protocol of Eguchi and Yamaguchi (2009) with minor modifications. Fish (female) at 100 dph was dissected to take out the brain and washed thrice with PBS to remove any blood. The brain was minced precisely into small pieces using scissors and the blunt end of the syringe, for suspension in L-15 medium, resuspended with 0.5% trypsin (Sigma, 1 ml) with collagenase type lV (Sigma) 0.8%, DNase (Sigma) 0.0015% followed by 30 min incubation at RT. Later, tissues (minced) were filtered by a 40 μm corning strainer (Sigma), then collected cells and spun at 100 *g* for 5 min. Then, a 2 ml medium mixture of Dulbecco's modified eagle medium (Sigma), FBS 10%, antibiotic and antimycotic agents were cells used for resuspension. Approximately 35,000 cells/well, cells were seeded and then incubated at 30 °C for 24 h. To check the viability, primary brain cells were observed using SRL, Giemsa fluorescent dye. This method is used for the subsequent experiments related to primary brain cell culture. All the primary cell culture-based experiments mentioned below were done in six wells per treatment/concentration and repeated thrice.

2.8. Mitochondrial function assay

Cell Proliferation the Cell Titer 96®AQueous One Solution (Alexandria NSW) was used to analyze mitochondrial function 24 h, after adding increasing doses of MPTP to the cell culture plate (96 well). Further, the cell culture was incubated in 5% CO2 at 30 °C for 48 h, and one solution reagent (20 μl) was added. The color developed after 3 h of incubation, was measured as absorbance at 490 nm standard microplate reader (Bio-Rad). It measures the quantity of formazan products in the culture. The percentage of cell viability related to control was expressed as a nontreated cells percentage.

2.9. Cytotoxic analysis: cell viability assay

The neurotoxin MPTP with different concentrations was exposed to primary brain cell culture and then incubated in 5% CO_2 at 30 °C for 48 h. MTT 5 mg/ml (Sigma) was added to the individual treated group, and further incubated for 4–5 h. Later, the plate was read at 570 nm in a Bio-Rad microplate reader, this experiment MTT assay was done in triplicate.

2.10. Membrane permeability: apoptosis assay

To determine the cellular apoptosis or necrosis levels through apoptosis assay (Molecular Probes, 1,597,071) following the method defined by the manufacturer supplied with propidium iodide/ YO-PRO®-1. After 48 h of MPTP treatment, the cells were washed with 1.5–2 ml of PBS containing propidium iodide, 2.5 μl and YO-PRO®-1dye and incubated for 30 mins on ice and further analyzed with a cell analyzer (BD LSR Fortessa™ BD Biosciences).

2.11. BrdU DNA proliferation test

Control, MPTP with three selected different doses (10 μg/μl, 50 μg/μl, 150 μg/μl) were exposed to primary brain cell culture and then incubated for 72 h in 5% CO_2 at 30 °C using microtiter plates (96 well). Later, 10 mM BrdU (Novus biologicals) was added and kept for 25–30 min followed by anti-BrdU was added and kept for incubation 60 min at RT. Chemiluminol was added after three washing cycles and further, measured at 570 nm integration time at 1000 ms and 130 gain, the results were quantified as relative fluorescence units.

2.12. Reactive oxygen species (ROS) generation in vitro

ROS production after MPTP exposure was assessed using a primary brain cell culture dichlorodihydro fluorescein diacetate oxidation (DCFH-DA,) which was measured with a multimode fluorescence reader (USA) as a method defined (Barzegar and Moosavi-Movahedi, 2011). Cells per well ~5000 were seeded and further incubated in a 5% CO_2 incubator at 30 °C for 48 h. The neurotoxin MPTP was exposed to the cells along with 25 mM dye DCFH-DA for 60 mins, then, formed ROS were measured at 523 nm fluorescence intensity excited at 502 nm. All experiments were executed in triplicates to calculate the experimental error.

2.13. In vitro, MMP generation

To measure MMP level upon MPTP treatment, the assay was carried out using primary brain cell culture. Also added lipophilic cationic dye JC-1 (Thermo Fisher Scientific, Cat. No: T3168) which specifically enters the mitochondria and changes the color from red to green, when decreased membrane potential. In a 96-well plate, ~5000 cells were seeded along with MPTP for 48 h. For control, untreated cells were used and positive control as camptothecin (1 mM) followed by PBS wash then incubated in 10 mM of JC-1 dye at 30 °C for 15–30 min, finally estimated with cell analyzer (Biosciences).

2.14. Enzyme immunoassay (EIA)

The levels of sex steroid serum T, 11-KT, and E_2 in control and MPTP-treated samples (50 μg/μl and 150 μg/μl) were measured using the EIA kit (Cayman) as described in the manufacturer's protocol by manufacturers. The assay variations (intra and inter) were measured, and validation was performed using the previously described method (Swapna et al., 2006; Mamta et al., 2020).

2.15. Statistical approach

The expression of data as mean ± standard deviation/error of the mean (SEM). The test was assessed by ANOVA, one-way analysis of variance, and the student-Newman–Keuls' (SNK) *post hoc* test, Student's *t*-test, and probability of $P < 0.05$ was measured as statistically significant using Graph Pad Prism 6.0 software (USA).

3. Results

3.1. In vivo, expression of brain and ovary-related genes after neurotoxin MPTP treatment

The expression levels of brain-related genes, *gfrα-1, cfGnRH1, cyp19a1b, hsd3b, tph2, th* and *lh-β,* and gonad-related genes *i.e., cyp19a1a, pax2, ad4bp/sf-1, sox9b, foxl2* were quantified after 7 days of MPTP treatment in comparison with saline control. Results showed that the brain-related genes *i.e., th, tph2, cfGnRH1, cyp19a1b, lh-β* were significantly ($P < 0.05$) downregulated, however, *hsd3b* showed only a moderate reduction in the treatment group as compared to the control (Fig. 1A). However, certain ovary-related transcripts like *cyp19a1a, pax2, sox9b,* and *foxl2* levels showed a highly significant reduction ($P < 0.01$) in the MPTP-treated group when compared to the respective controls. Additionally, *ad4bp/sf*-1 showed only a moderate reduction ($P < 0.05$) as compared to the control group (Fig. 1B).

3.2. CAs level variations after MPTP exposure: HPLC-ECD

The CAs levels, L-Dopa, DA, and NE exhibited a significant ($P < 0.05$) decrease in brain with increased doses (50 μg/μl, and 150 μg/μl) of MPTP treated group (Fig. 2). However, at lower concentration (10 μg/μl) of MPTP treatment, no significant changes were evident (Fig. 2).

3.3. TEM analysis of control and MPTP treated brain

TEM analysis of the treated brain revealed the disorganization of structure in neuronal brain cells as compared to the control brain. Diverse structural degeneration was seen after the treatment of neurotoxin MPTP with two doses of 50 μg/μl and 150 μg/μl. Different-shaped neuronal cells degenerated nuclear membranes, and deformed cell bodies were also seen. Certain convolutions were also seen in the membrane distortion and the effect seems high as the dose increased (Fig. 3).

3.4. In vivo th expression upon MPTP effect

The expression of Th in brain exhibited a dose-related reduction significantly ($P < 0.05$) upon MPTP treatment *in vivo*, at higher doses (50 to 150 μg/μl) as compared to the control (Fig. 4A).

3.5. Cell viability assay

Relative cell viability was seen with the increasing doses 5 μg/μl, 10 μg/μl, 50 μg/μl, 100 μg/μl, and 150 μg/μl of MPTP exposure to primary cells of brain (Fig. 4B). The cell morphology examination of cultures showed regular adherent nature during the treatment time. However, in the treatment group of 50 μg/μl, cells showed clumping, however, adherence loss, contraction and cell clumping, were found to be higher at concentrations of MPTP (microphotographs not shown). Moreover, discrete structural changes throughout the treatment signified the existence of programmed cell death.

3.6. Flow cytometric analysis: Primary brain cell culture exposed to MPTP

To examine the induced MPTP cellular toxicity in treated cells and control of primary brain cell culture, YO-PRO-1 and PI dyes were added to analyze differences in apoptotic and normal cells (Fig. 5). Resulting in the percentage of the apoptotic cells increased with increasing doses of MPTP exposure (Fig. 5) as compared to the control group (low right quadrant). The cells appeared YO-PRO-1 positively stained, representing the early apoptotic stage shown in the right quadrant (lower), and signified late apoptotic stage cells were shown when stained with both YO-PRO-1 and PI dye (upper right quadrant). Though, the estimation of the deceased cell population from the left upper quadrant.

3.7. BrdU DNA proliferation, in vitro

DNA synthesis was evaluated using BrdU assay in control and MPTP treated primary brain cells. All MPTP treated samples showed a decrease in newly synthesized DNA compared to the control samples (Fig. 6).

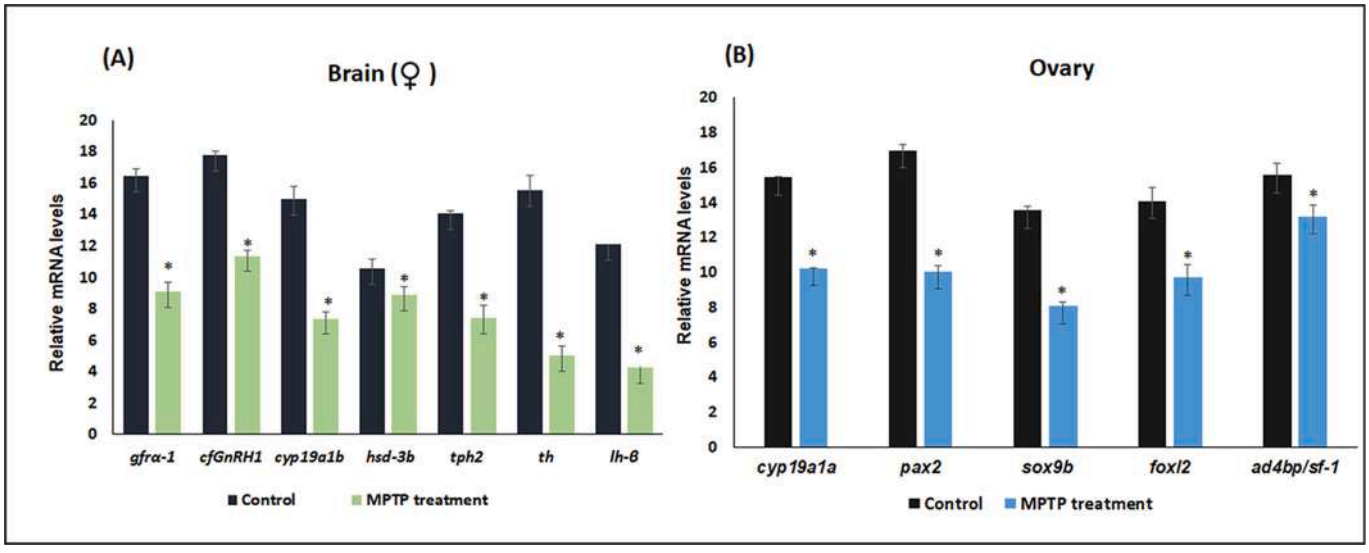

Fig. 1. A and B. Quantitative real-time PCR expression analysis of certain female brain and ovary related genes between MPTP treatment (150 μg/μl) and control in catfish brain. Data ($n = 5$) were expressed as mean ± SEM (*$P < 0.05$; one-way ANOVA on ranks followed by SNK test).

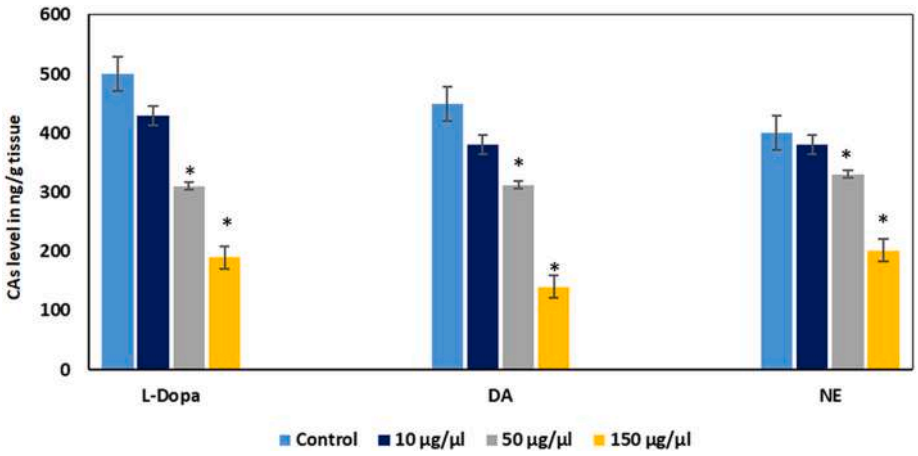

Fig. 2. HPLC-ECD quantitative measurement of catecholamines (CA) levels, such as L-Dopa, DA and NE following neurotoxin MPTP treatment in catfish with three different concentrations 10 µg/µl, 50 µg/ µl and 150 µg/ µl. L-Dopa, DA and NE levels with respective controls. Data (n = 5) were expressed as mean ± SEM (*P < 0.05; one-way ANOVA on ranks followed by SNK test). Abbreviation: L-Dopa- L-3,4-dihydroxyphenylalanine, DA-dopamine and NE- norepinephrine.

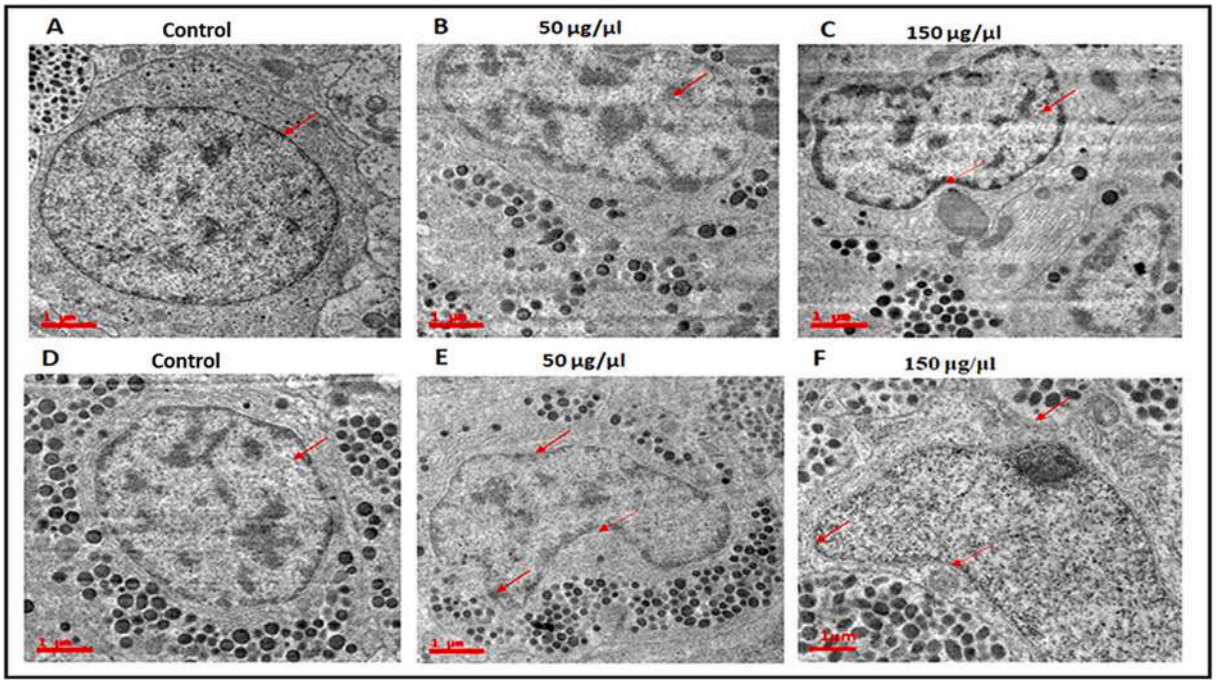

Fig. 3. TEM analysis of female brain after MPTP treatment with two different doses 50 and 150 µg/µl. A and D showing control brain neuronal cells, B and E showing changes in brain neuronal cells at 50 µg/µl, C and F showing disorganization and disarray of neuronal cells at 150 µg/µl.

3.8. Measurement of ROS after MPTP exposure, in vitro

ROS levels following MPTP treatment elevated ($P < 0.05$) significantly with increasing doses (50 µg/µl and 150 µg/µl) when compared to the control (Fig. 7A).

3.9. Estimation of MMP levels in primary brain cell culture treated, in vitro

MMP levels were reduced significantly ($P < 0.05$) in neurotoxin MPTP treated as compared to the control samples (Fig. 7B).

3.10. Estimation of T, 11-KT, and E_2 serum levels after MPTP treatment

The levels of T and 11-KT decreased ($P < 0.05$) significantly in males after MPTP treatment and the reduction was high in 150 µg/µl MPTP dose (Fig. 8A and B). Similarly, in females, the level of E_2 decreased significantly ($P < 0.05$), and the impact seems higher with the elevated dose of MPTP (Fig. 8C).

4. Discussion

The current study evaluated the specific neurotoxic effects of MPTP on Th and DA-ergic system/activity along with various transcription factors implicating its impact on female brain and ovary-related genes, as well as sex steroids leading to neurodegeneration. Prominent expression/levels of *th* and CAs (Mamta et al., 2014) in the female brain along with the serotonergic system (Sudhakumari et al., 2010; Raghuveer et al., 2011b) authenticated that Tph and Th vis-à vis 5-HT and CA might have a significant role in "brain sex differentiation" and also have direct/indirect effects on gonads (Senthilkumaran et al., 2015). The present work focused to impart neurodegeneration through

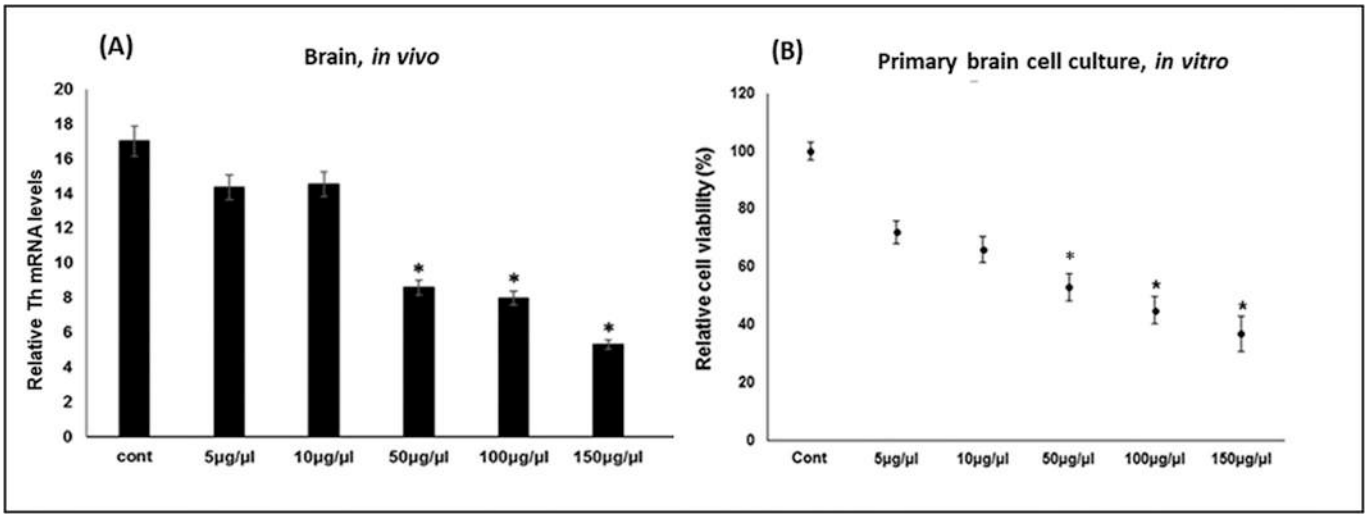

Fig. 4. Cytotoxicity analysis in catfish female brain and primary brain cell culture exposed to MPTP with increasing doses, 5 µg/µl, 10 µg/µl, 50 µg/µl, 100 µg/µl, and 150 µg/µl *in vivo* and *in vitro*. (A), *in vivo* qPCR analysis showed expression of *th* between control and MPTP treated brain and (B), *in vitro* by MTT assay. Data (n = 5) were expressed as mean ± SEM (*P < 0.05; one-way ANOVA on ranks followed by SNK test) Abbreviation: cont-control.

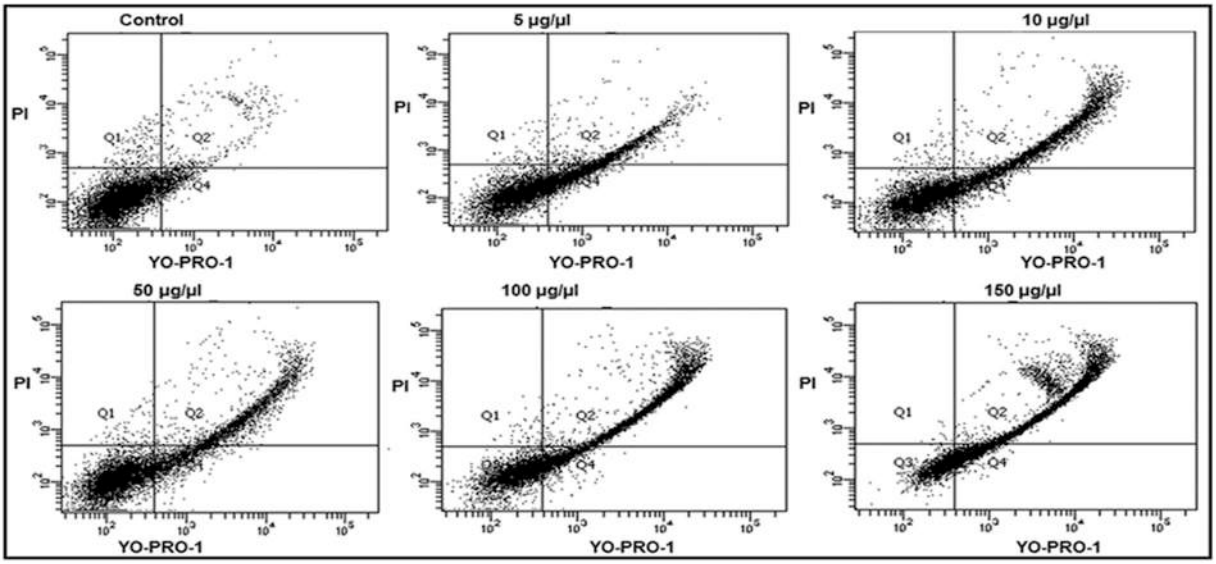

Fig. 5. Flow cytometry analysis of primary brain cell culture, *in vitro* exposed to increasing concentrations of neurotoxin MPTP *i.e.*, 5 µg/µl, 10 µg/µl, 50 µg/µl, 100 µg/µl, and 150 µg/µl and evaluated using YO-PRO-1 and PI labelled dye. All cells in the figures stained with YO-PRO-1 represented early apoptotic stage (lower right quadrant) and cells stained with both YO-PRO-1 and PI (upper right quadrant) denoted late apoptotic stage.

MPTP treatment in catfish to delineate the role of CA-ergic system with an aim to generate a neurodegenerative animal model in lower vertebrates. The exposure of neurotoxin, MPTP reduced the expression of female brain transcripts, *gfrα-1, cfGnRH1, cyp19a1b, hsd3b, tph2* and *th* as well as ovary-related genes like *cyp19a1a, pax2, ad4bp/sf-1, sox9b* and *foxl2*. In addition, low CA (L-Dopa, DA, NE) levels in brain were also evident collaboratively, serum levels of sex steroids (T, 11-KT, E_2) decreased considerably after MPTP treatment. Earlier studies in teleosts exposed to sex steroids altered feedback mechanisms (Habibi et al., 1989; Senthilkumaran and Joy, 1995) by modulating the CA-ergic system. The present study focused on female brain as well as ovary since CA-ergic activity is high in developing catfish (Mamta et al., 2014; Senthilkumaran et al., 2015). It is also appropriate to use female as high levels of CA and its related genes with intense feedback interaction are evident (Senthilkumaran and Joy, 1995; Mamta et al., 2014; Senthilkumaran et al., 2015). In addition, the release of DA affects the expression of *cyp19a1b* in glial cells *via* DA1 receptor which showed inhibitory regulation in cell differentiation and proliferation through transcription factor facilitated pathways, triggered by neurosteroids in goldfish (Xing et al., 2016). Furthermore, neurotransmitters and neurosteroids provide neuroprotective effects and play a significant role in the potential treatment of neurodegenerative diseases in mammals (Mendell and MacLusky, 2018). Previous reports in zebrafish indicated the toxic effect of MPTP explicitly on diencephalic Th+ neurons (Bretaud et al., 2004; McKinley et al., 2005; Wen et al., 2008) and DA-ergic neurons and noradrenergic fibers in medaka brain (Matsui et al., 2009). In contrast, other reports on zebrafish and goldfish showed a reduction of DA-ergic and noradrenergic neurons after MPTP treatment (Pollard et al., 1992; Anichtchik et al., 2004). In animal models, striatal DA depletion is reduced by estrogen partly (Ramirez et al., 2003) preventing the damage of TH immunoreactivity (Shughrue, 2004; Ookubo et al., 2008) following neurotoxicity in mice. In mammals, neurotoxin MPTP substances showed a higher level of glial fibrillary acidic protein (GFAP) which is measured in astrogliosis, and GFAP-positive astrocytes marker

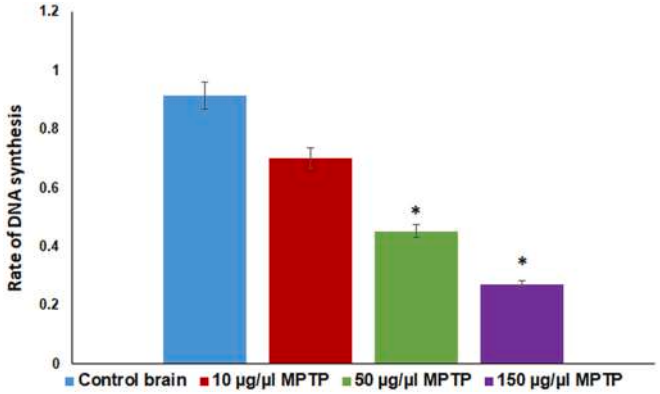

Fig. 6. Histogram representing BrdU incorporation-based proliferation analysis of different MPTP exposed groups *i.e.*, 10 µg/µl, 50 µg/µl, 150 µg/µl and control group in catfish. Data (n = 5) were expressed as mean ± SEM (*P < 0.05; one-way ANOVA on ranks followed by SNK test).

protein apparently in the midbrain and striatum (Sampath and Janardhanam, 2013). Further, a previous study revealed significant reduction in CAs, L-Dopa, DA, and NE in brain confirming the influence of MPTP on CA biosynthesis, therefore, the association of Th and CAs, precisely DA seems important in vertebrates including catfish (Popesku et al., 2012; Mamta and Senthilkumaran, 2018). In goldfish, highest reduction of forebrain DA content and significant decrease of DA-ergic marker, Th were observed after MPTP injection (Pollard et al., 1992; Hibbert et al., 2004). Furthermore, ultrastructure analysis of pituitary using TEM revealed the disorganization of structure in neuronal brain cells and abnormal structural changes in mice after MPTP treatment (Zhang et al., 2017). In mice, MPTP-induction showed enlarged neuronal fibers in the striatum, as well as midbrain exhibiting irregular dense filamentous material and the structure of autophagic with irregular multivesicular bodies, was also observed (Sampath and Janardhanam, 2013). Accordingly, in the present study ultrastructural changes in the brain, especially, neuronal cell disorganization with membrane convolutions were evident in catfish. Thus, MPTP-induced neurodegeneration can be developed in catfish to evaluate neurological diseases as a lower vertebrate model to progressively proceed into mammalian models, if necessary. In fish primary brain cell culture *in vitro*, the exposure of MPTP exhibited lower cell viability and increased toxicity resulting in increased percentage of necrotic cells. Previous studies have reported that neurotoxin MPTP destroyed DA neurons leading to motor deficits and reproductive complications in goldfish (Delgado and Schmachtenberg, 2011; Zupanc and Sîrbulescu, 2011; Venables et al., 2018). In line with this, a decline in newly synthesized DNA was observed after neurotoxicity as evident through BrdU assay in the present study. Likewise, Ampatzis and Dermon (2007) also revealed decreased BrdU-labelled cells post-MPTP injury in the zebrafish cerebellum at 24 h. Further, based on the study, the positively labelled BrdU cells likely label proliferative cells in the cellular division have also ensued. However, in goldfish, BrdU-labelled cells increased substantially 1.9-fold at 4 days following MPTP treatment, but at 7 days no significant differences were detected comparatively with controls which specify that goldfish have a speed retrieval starting at 4 days from MPTP toxicity (Venables et al., 2018). Besides, catfish primary brain cell cultures exposed to MPTP showed increased ROS levels and decreased MMP specifying the neurotoxic effects of MPTP leading to neurodegeneration. Brain glial cells produce paired-like homeodomain transcription factor 3, dopamine transporter *slc6a3,* and *GDNF-Gfrα-1*, these genes/factors regulate the neuronal survival, and DA-ergic system and reduce Th neuronal apoptosis in teleosts (Lin et al., 1993; Yasuhara and Shingo, 2007; Pellegrini et al., 2007; Lykissas et al., 2007; Xing et al., 2017; Mamta and Senthilkumaran, 2018). Comprehensive analysis from this study indicated that the MPTP treatment, *in vivo,* downregulated the brain and ovary-related genes, further affecting Th and DA-ergic activity which are well correlated with reduced CA levels. Ultrastructural changes in brain, as well as primary brain cell culture exposed to MPTP *in vitro*, confirm our findings. Additionally, a reduction in sex steroids (T, 11-KT, and E_2) levels potentially affected gonads which might, indirectly target brain function, and cause neurodegeneration. To further validate the progressive neurodegeneration, specific behavioural tests in catfish may substantiate our experimental findings.

5. Conclusion

In summary, the present study showed that neurotoxin, MPTP specifically affected DA-ergic neurons in the female brain and induced Th and DA activity depletion, which caused neurodegeneration and partial impairment of reproduction in catfish as evidenced by decreased levels of sex steroids and expression of certain ovary related genes. Furthermore, decreased expression levels of certain brain-related genes after MPTP treatment *in vivo* revealed the regulatory influence of Th in coordination with DA-ergic activity. MPTP treatment, *in vitro* also exhibited declined cell viability, MMP, DNA damage, and increased ROS

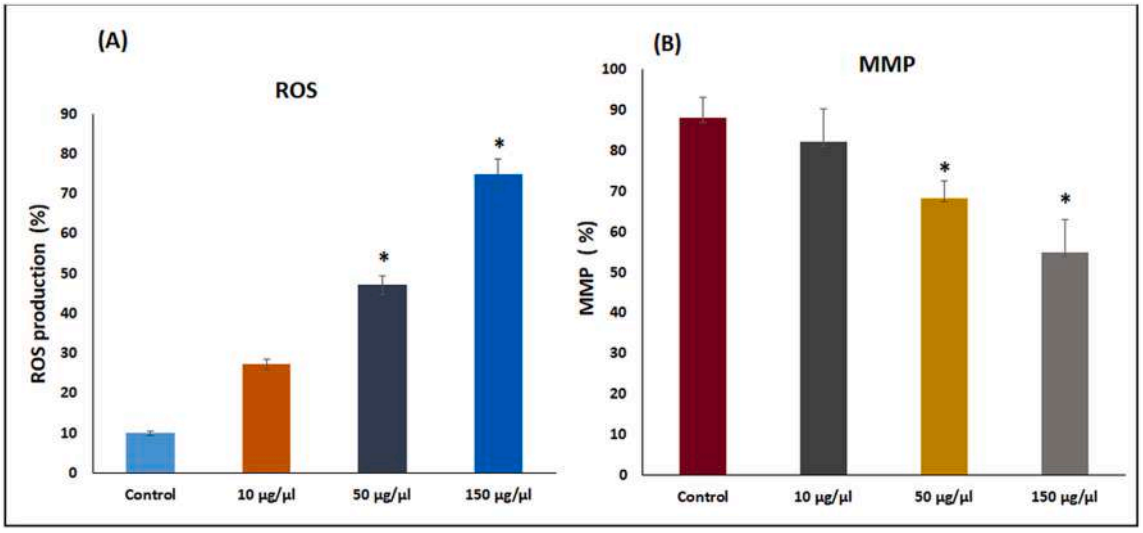

Fig. 7. Percentage of (A) ROS production and (B) MMP collapse was calculated between the control and MPTP treated groups (10 µg/µl, 50 µg/µl, 150 µg/µl) of primary brain cell culture, *in vitro,* after 48 h. Data (n = 5) were expressed as mean ± SEM (*P < 0.05; one-way ANOVA on ranks followed by SNK test).

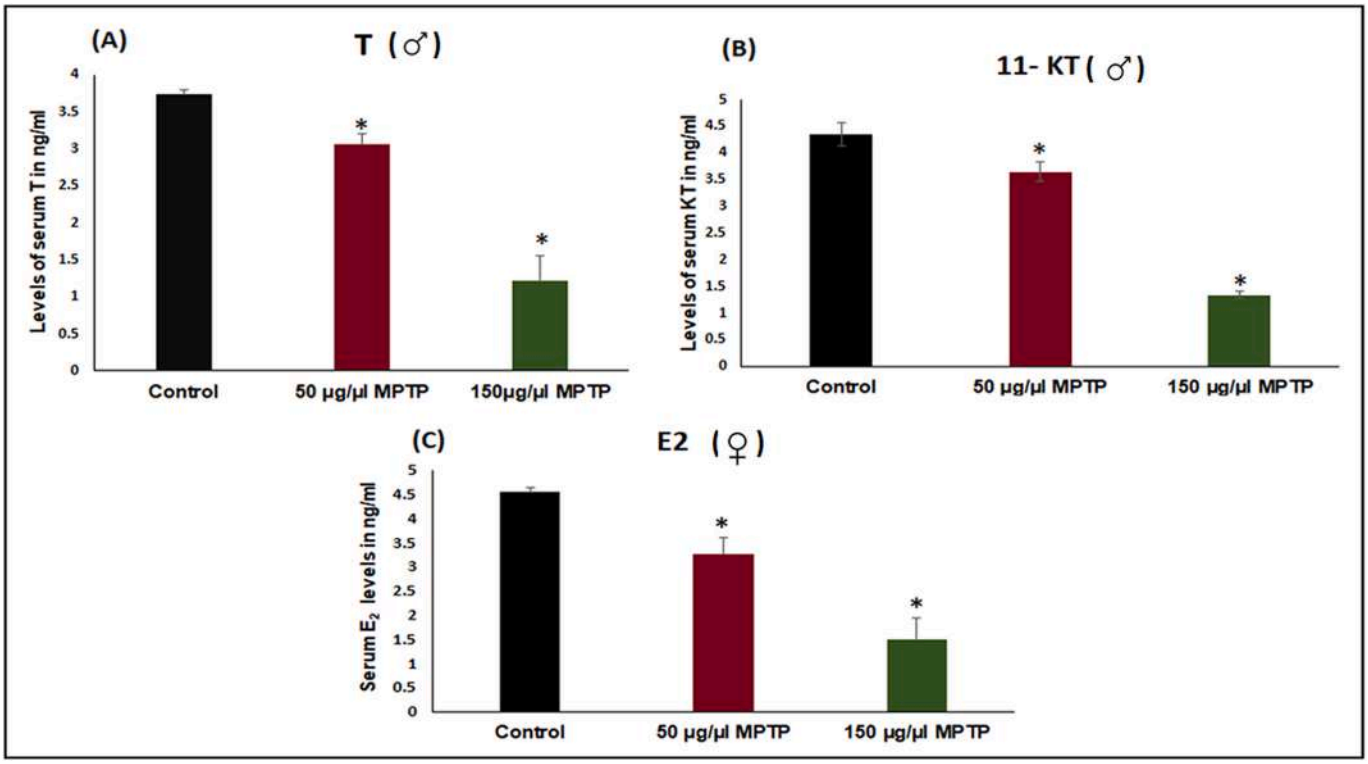

Fig. 8. Serum levels of (A) testosterone (T), (B) 11-ketotestosterone (11-KT), and (C) 17β -estradiol (E_2) after neurotoxin MPTP treatment with different doses, 50 µg/µl and 150 µg/µl in comparison with control. Data (n = 5) were expressed as mean ± SEM (*P < 0.05; one-way ANOVA on ranks followed by SNK test.

possibly depicting neurodegeneration. Additionally, significant reduction in L-Dopa, DA and NE levels were observed along with female brain ultrastructural changes. These findings possibly indicate the application of MPTP treatment to generate a neurodegeneration model in catfish in addition to the primary validation of significant role of Th with DA-ergic system for brain neuronal function in teleost.

CRediT authorship contribution statement

Mamta Sajwan-Khatri: Formal analysis, Validation, Methodology, Data curation, Conceptualization, Investigation, Visualization, Software, Funding acquisition, Writing - original draft, Writing - review & editing. **Balasubramanian Senthilkumaran:** Writing – original draft, Writing – review & editing, Conceptualization, Investigation, Validation, Methodology, Supervision, Funding acquisition, Resources.

Declaration of Competing Interest

The authors declare no conflict of interest. The authors alone are responsible for the content and writing of the paper.

Data availability

The authors are unable or have chosen not to specify which data has been used.

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Research report

GDNF family receptor α-1 in catfish: Possible implication to brain dopaminergic activity

Sajwan-Khatri Mamta, Balasubramanian Senthilkumaran*

Department of Animal Biology, School of Life Sciences, University of Hyderabad, P.O. Central University, Hyderabad, 500046, Telangana, India

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ABSTRACT

Glial cell line-derived neurotrophic factor (GDNF) is a potent trophic factor that preferentially binds to GDNF family receptor α-1 (GFRα-1) by regulating dopaminergic (DA-ergic) neurons in brain. Present study aimed to evaluate the significance of *GFRα-1* expression during early brain development in catfish. Initially, the full-length cDNA of *GFRα-1* was cloned from adult brain which showed high homology with other vertebrate counterparts. Quantitative PCR analysis of tissue distribution revealed ubiquitous expression of *GFRα-1* in the tissues analyzed with high levels in female brain and ovary. Significant high expression was evident in brain at 75 and 100 days post hatch females than the respective age-match males. Expression of *GFRα-1* was high in brain during the spawning phase when compared to other reproductive phases. Localization of *GFRα-1* revealed its presence in preoptic area-hypothalamus which correlated well with the expression profile in discrete areas of brain in adult catfish. Transient silencing of *GFRα-1* through siRNA lowered expression levels of *GFRα-1*, which further down regulated the expression of certain brain-specific genes. Expression of *GFRα-1* in brain declined significantly upon treatment with the 1-methyl-1,2,3,6-tetrahydropyridine causing neurodegeneration which further correlated with catecholamines (CA), L-3,4-dihydroxyphenylalanine, DA and norepinephrine levels. Taken together, GFRα-1 plausibly entrains gonadotropin-releasing hormone and gonadotropin axis either directly or indirectly, at least by partially targeting CA-ergic activity.

1. Introduction

Glial cell line-derived neurotrophic factor (GDNF) is a potent survival factor for many central and peripheral neurons, including dopaminergic (DA-ergic) neurons. GDNF signals preferentially through GPI-anchored receptor, GDNF family receptors-α having stronger binding activity over GFRα-1 (Airaksinen et al., 2006; Trupp et al., 1998). GDNF-GFRα-1 complexes recruit the tyrosine kinase transmembrane protein to execute differentiation of DA-ergic neurons in brain(Naughton et al., 2006; Durbec et al., 1996). The expression of GFRα-1in certain areas of the brain where neurons responsive to GDNF family members reside have been analyzed in mammals (Quartu et al., 2007; Matsuo et al., 2000) and less studied in lower vertebrates including teleosts (Shepherd et al., 2001; Lucini et al., 2010; Lucini et al., 2011). Teleosts serve as an excellent animal model to study neurodegeneration partially due to the abundance of neuronal precursor cells such as glial cells (Zupanc and Clint, 2003). Prominently, glial cells are the most abundant cell type in the teleost brain, which have crucial roles in neuroendocrine systems (Barry et al., 2014; Xing et al., 2014) and also produce neurosteroids (Pellegrini et al., 2007), such as estradiol-17β (E_2) and neurotrophic factor, GDNF (Xing et al., 2016). Infact, co-localization of E_2 receptors (ER) and DA-neurons reported earlier in teleosts indicating a possible interaction of DA-ergic neurons with neurosteroids (Dufour et al., 2010). Studies on ER knockout mice revealed a significant loss of DA neurons which supports the contention that ER mediated mechanisms involve neuroprotective actions (Wang et al., 2001). E_2 also protect the brain against toxicity induced by excitatory neurotransmitters, oxidative stress and neurotoxins (Shahrokhi et al., 2012). Earlier report using *Gfra1* knock-out mice showed loss of motoneurons in

Abbreviations: aa, amino acid; CA, catecholamines; cfGnRH, catfish gonadotropin regulating hormone; cyp19a1, aromatase; Cont, control; DA-ergic, dopaminergic; DIG, digoxigenin; Dph, days post hatch; E_2, estradiol-17β; ER, E_2 receptor; F, female; GDNF, Glial cell line-derived neurotrophic factor; GFRα-1, GDNF family receptor α-1; h11b, 11 β hydroxylase; HPLC-ECD, high performance liquid chromatography-electrochemical detection; hsd3b, 3β-hydroxysteroid dehydrogenase; L-DOPA, L-3,4-dihydroxyphenylalanine; M, male; MO, medulla oblongata; MPTP, 1-methyl-1,2,3,6-tetrahydropyridine; NE, norepinephrine; ORF, open reading frame; PBS, phosphate buffered saline; PCA, perchloric acid; PEI, polyethylenimine; POA-HYP, preoptic area-hypothalamus; PT, pituitary; RACE, rapid amplification cDNA ends; RET, receptor-mediated tyrosine kinase; RT, room temperature; siRNAs, Small interfering RNAs; TBS, tris-buffered saline; TEL + OB, telencephalon + olfactory bulb; TH, thalamus; th, tyrosine hydroxylase; tph, tryptophan hydroxylase

* Corresponding author.
E-mail address: bsksl@uohyd.ernet.in (B. Senthilkumaran).

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lumbar spinal cord (Moore et al., 1996; Cacalano et al., 1998), which showed the requirement of *Gfra1* for survival signalling by GDNF in spinal motoneurons. Furthermore, prominent motoneuron groups that strongly express *Gfra1* were lost in the *Gfra1* mutant, and hence, *GFRa1* is critical for the survival of neurons during development.

GFRα-1 enhances the survival and differentiation of DA neurons, and decreases apoptosis of damaged DA neurons (Yasuhara et al., 2007) thereby exhibiting neurorestorative and neuroprotective actions (Wissel et al., 2006). It is also well known that the enzyme cyp19a1b, (brain aromatase) catalyzes the conversion of androgens to estrogens (Rasheeda et al., 2010) and found to be expressed in glial cells of adult fish brain (Diotel et al., 2010; Forlano et al., 2001). Previous studies in teleosts revealed a feedback mechanism exerted by gonadal steroids (Habibi et al., 1989; Senthilkumaran and Joy, 1995) by targeting brain through catecholaminergic (CA-ergic) system. In goldfish brain, DA released from DA-ergic neurons affects cyp19a1b expression in glial cells, through DA receptor (Xing et al., 2016) which has an inhibitory role in glial cell proliferation and differentiation through transcription factor-mediated path ways elicited by neurosteroids. It remains to be seen whether GFRα-1 target neurosteroids by regulating DA in brain. Considering this, in the present study an attempt was made to damage DA-ergic neurons using neurotoxin 1-methyl-1,2,3,6-tetrahydropyridine (MPTP) that is known to cause loss of DA-ergic neurons in fishes (McKinley et al., 2005) by selectively targeting DA-ergic neurons after systemic administration (Weinreb and Youdim, 2007). This sort of approach will provide a basis to understand the molecular mechanisms of GFRα-1 in DA regulation vis-à-vis neurosteroids release at the level of brain. Among monoamines, serotonin (5-HT) and norepinephrine (NE) have a stimulatory influence on gonadotropin-releasing hormone (GnRH) and gonadotropin (GTH) release while DA inhibits the same (Peter et al., 1991; Goos et al., 1999; Senthilkumaran et al., 2001). Considering these findings, the impact of DA on neurosteroids vis-à-vis GFRα-1's action is an important topic of analysis in teleosts. The air-breathing catfish, *Clarias gariepinus* follows a seasonal reproductive cycle with recrudescence where the brain under goes physiological cyclicity in accordance to gonadal changes and returns to spawning stage as season approaches. This unique characteristic feature facilitates present work to comprehend at the transcript levels of various genes that play crucial roles in neuroendocrine control of reproduction and neural plasticity focusing on the interaction of GFRα-1 on DA-ergic system. Secondly, teleost (catfish) will be an ideal experimental model as distinct changes of mono aminergic system drive GnRH-GTH axis to entrain reproductive cycle.

Main objective of the present study is to investigate the involvement of GFRα-1 in the neuroendocrine control of reproduction through DA-ergic system. Hence to implicate *GFRα-1* as an intermediate between GDNF-DA-ergic system, GFRα-1 was cloned and the ontogenic expression was analyzed in the brain of catfish, *C. gariepinus*. The impact of transient silencing of *GFRα-1, in vivo* through siRNA complexed with polyethylenimine (PEI) in brain was analysed in catfish to understand its functional significance by analysing various brain-specific genes. Further, to examine the neurotoxin effects of MPTP, catecholamines (CA) levels were measured in brain to understand the interaction of *GFRα-1* and DA-ergic activity.

2. Materials and methods

2.1. Animal and sampling

The air-breathing catfish, *C. gariepinus*, bred and reared as per the method described earlier (Raghuveer et al., 2011), were used for the present study. Soon after hatching, the fingerlings were retained in plastic tubs with continuous aeration under ambient photothermal conditions. Live tube worms *(Tubifex tubifex)* were fed for catfish hatchlings *ad libitum* till adulthood. Annual reproductive cycle of catfish is divided into preparatory, pre-spawning, spawning and post-spawning/regressed phases. Commercial pelleted fish feed, was given *ad libitum* to adult catfish (~1-year-old) and reared in the outdoor tanks in ambient photothermal conditions. Fish sampling was done by following the general guidelines of the Institutional Animal Ethics Committee, University of Hyderabad (Reg./No./151/1999 dt. 22.07.1999). Animals were briefly anesthetized with 100 mg/L of ethyl 3-aminobenzoate methane sulfonate (MS-222; Sigma; St. Louis, MO, USA) in mild ice-cold water and, samples were dissected out on ice and stored briefly at −80 °C until analysis for various parameters.

2.2. Cloning of GFRα-1 from catfish brain

Degenerate primers were designed for *GFRα-1* by using the nucleotide information available in NCBI database. Total RNA was prepared using TRI reagent® by following the manufacturer's protocol (Sigma). The quality and quantity of total RNA was analyzed using Nanodrop spectrophotometer (ND-2000, Nano Drop Technologies and Wilmington, DE, USA). About 500 ng of total RNA was used for reverse transcription by following the protocol of verso® cDNA synthesis kit (Thermo Scientific Inc., Waltham, MA, USA). PCR amplification was accomplished with Taq 2X master mix (New England Biolabs Inc., Ipswich, MA) using degenerate primers as per subsequent conditions: initial step of 94 °C (2 min), 94 °C (1 min), 53 °C (1 min), 72 °C (1 min), for 35 cycles and final extension at 72 °C (10 min). The PCR amplicon was gel purified and ligated in pGEM®-T easy vector (Promega, Madison, WI, USA) and sequenced bidirectionally. Gene specific primers were then designed for 5′ and 3′ rapid amplification cDNA ends (RACE) using SMARTer™ RACE cDNA amplification kit (Clontech, Mountain View, CA, USA). The amplicons were cloned into pGEM®-T vector and nucleotide information was obtained through bidirectional DNA sequencing. Both partial and RACE derived nucleotide sequences were aligned through Lasergene software (DNASTAR, Madison, WI, USA) to deduce the full-length cDNA.

2.3. Sequence and phylogenetic analysis

The deduced amino acid (aa) sequences of cloned GFRα-1 of catfish and other vertebrates from GenBank were used for the Clustal Omega alignment and the phylogenetic tree was constructed using neighbor-joining method. The GenBank accession numbers of *GFRα-1* sequences used are as follows. *Ictalurus punctatus* (XM_017482772), *Danio rerio*_a (NM_131730), *D. rerio*_b (NM_131731.1), *Oreochromis mossambicus* (KR779759.1), *Takifugu rubripes* (XM_003961583.2), *Labeo rohita* (HM130051.2), *Salmo salar* (LOC106577126), *Bubalus bubalis* (XM_006071676), *Oryzias latipes* (XM_004080268.2), *Mus musculus* (NM_010279), *C. gariepinus* (KY553234). Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) multiple alignment tool was used for the construction of phylogenetic tree by neighbor-joining method and expressed using Jalview 2.8 and TreeView 1.6.6 software.

2.4. Quantitative real-time PCR (qRT-PCR)

Expression analysis for all the genes were determined through qPCR using SYBR green detection method. Total RNA isolation and first strand cDNA synthesis for all the samples were done as described above. The purity of RNA was checked using a Nano Drop spectrophotometer (ND-2000, NanoDrop Technologies and Wilmington, DE, USA) and the integrity of RNA was confirmed by running the sample in a formaldehyde agarose gel. Random hexamers were utilized for the reverse transcription using verso® reverse transcriptase (Thermo Scientific Inc., Waltham, MA, USA) with 1 μg of brain total RNA isolated from brain using TRI-reagent® (Sigma) as per the manufacturer's protocol followed by DNase I treatment to eliminate the genomic DNA. In addition, one of the qRT-PCR primers chosen were at exon–exon junction. The reaction was done in triplicate using qPCR primers in MicroAmp® 96-Well plates with SYBR green master mix in a 7500-fast thermal cycler (Applied Biosystems, Foster City, CA, USA) as per the

Table 1
List of primers used for the study for cloning and qPCR analysis.

Primer name with gene symbol	Nucleotide sequence (5′–3′)	GenBank Accession number	Purpose
GFRα-1 Dg Fw	GAG GAR MTSAWYTTYMGVTAY TGYAGYGG	KY553234	Cloning of partial cDNAs
GFRα-1 Dg Rv	CADS CAC AYY KYY KRG CRG AAT GCT		
GFRα-1 5P	GCACCAGCAGGGTGTCCACCACTGAAG		RACE
GFRα-1 5N	CTGCCTAAATGCAGCTAAGGCCTGCAATCTG		
GFRα-1 3P	GGTCTCGCCTGGCCGATTTCTTTACCA		
GFRα-1 3N	GGGAAGCCCGAGTGTGACAAGTTCTCA		
GFRα-1 RT Fw	GGTGAAGCAGCCTTTAGCAAGG		qPCR
GFRα-1 RT Rv	CAGAAAAGCATGCCGTAGCTGTGC		
Cyp11a1 Fw	TCAACCAAGCGGACCACTGT	KF739411	qPCR
Cyp11a1 Rv	TCAGGATGCCGTGCCAACTC		
hb11 Fw	GGCAGTGGAGCGAATGCTGAA	HQ680986	qPCR
hb11 Rv	GCACCCCGGGGAACCTTGAGC		
18S rRNA Fw	GCTACCACATCCAAGGAAGGCAGC	AB105163	qPCR
18S rRNA Rv	CGGCTGCTGGCACCAGACTTG		
tph Fw	CAGTTCTCACAGGAGATTGGCC	GU290195	qPCR
tph Rv	CGAAACTCTCTGAGACAAAGT		
th Fw	CCAGAGCCAGACTGCTGTCACGAG	KF739410	qPCR
th Rv	CACCGTACGCCTTCACTGTTC		
Cf GnRH Fw	AGCGTGCCGTGATGCAGGAG	X78049	qPCR
Cf GnRH Rv	TCTCTCCCAGCGACAGGCGT		
hsd3b Fw	GAGGTAAATGTGAAAGGTACCAA	HQ680983	qPCR
hsd3b Rv	TAGTACACAGTGTCCTCATGG		
β-actin F	GCCCATCTATATGAAGGTTATG	EU527190.2	qPCR
β-actin R	CTCCATACCCAGGAAAGATGGCT		

manufacturer's universal thermal cycling conditions. After performing melting-curve analysis to check the amplicon specificity, cycle threshold (Ct) value was calculated from the exponential phase of PCR amplification and the gene expression was normalized against the expression of *18S rRNA* (as reference gene) to generate a ΔCt value (Ct of target gene – Ct of reference gene). Relative expressions of the genes were calculated using $2^{-\Delta Ct}$method.

2.5. Tissue distribution and differential expression of GFRα-1 in adult catfish brain

qRT-PCR was performed to analyze the tissue distribution of *GFRα-1* in adult male and female catfish using the *GFRα-1* specific primers (listed in Table 1). Total RNA was extracted from different tissues (gill, spleen, heart, muscle, kidney, liver, ovary, testis and brain of male and female) of adult catfish using the method described above. Catfish at different age groups [0, 10, 20, 30, 40, 50, 75, 100, 150, 200, and 250 days post hatch (dph)] were collected (5 fish brains were pooled till 100 dph and taken as one biological sample, n = 5 at 0 dph head was taken) for ontogeny analysis (n = 5). Morphological distinction of the gonad occurs in catfish around 50 dph and hence sexing of brain was done from that stage for ontogeny studies. All the samples were stored briefly at −80 °C before being used for total RNA preparation and cDNA synthesis as described earlier.

2.6. Reproductive phase expression

Since catfish is an annual breeder, brain from different reproductive phases were dissected as described earlier and stored to analyze the expression pattern of *GFRα-1*. Total RNA was prepared from each sample (n = 5) followed by cDNA synthesis was done as explained before. Further qPCR was performed in triplicate and the relative expression was determined.

2.7. Expression of GFRα-1 in different regions of the adult female brain

GFRα-1 mRNA levels were analyzed in different regions of the adult female brain such as telencephalon + olfactory bulb (TEL + OB), pituitary (PT), preoptic area-hypothalamus (POA-HYP), thalamus (TH) and medulla oblongata (MO), after dissecting those out as per Raghuveer et al. (2011).

2.8. In situ hybridization (ISH)

Localization of the transcripts of *GFRα-1* in adult catfish brain was done by using ISH, as per the method described earlier (Rajakumar and Senthilkumaran, 2014). Fixation and sectioning of adult brain was done as explained before (Sudhakumari et al., 2017). Based on the sequence information of pGEM-T easy- *GFRα-1* cDNA either T7 or SP6 RNA polymerase was used for sense and antisense 'cRNA' probe preparation using digoxigenin (DIG; Roche Diagnostics GmbH, Mannheim, Germany), 2 µg of digested plasmid was used as a template and RNAase inhibitor (20U) were added then reaction were incubated at 37 °C for 2 h in a thermocycle (ABI Prism 2720 thermocycler) based on *in vitro* transcription. The sections were treated and fixed using phosphate buffered saline (PBS) with Tween 20 in diethyl pyrocarbonate treated water (0.1% v/v), proteinase K and paraformaldehyde (PFA). About 1 µl of purified sense and antisense cRNA probes were diluted in 200 µl of hybridization buffer and heat denatured at 80 °C for 5 min. The mixture was then added onto the sections and kept overnight at 50 °C in a sterile RNase free incubator. Slides were washed with wash and blocking buffer solution (Roche). Anti-DIG-ALP antibody fab fragments (Roche Cat. No. 1109324910) were added onto the sections and kept at 4 °C overnight. Slides were washed with DIG washing buffer (Roche) and incubated further with detection buffer (Roche). The sections were color developed using BCIP-NBT (Roche) as substrates while nuclear red was used as acounter stain. After color development, the slides were washed and dehydrated using a graded ethanol series then mounted using DPX mountant. Finally, photomicrographs were taken in CX41 Olympus microscope (Olympus corporation, Tokyo, Japan) with Q capture pro 6 software-controlled micropublisher 3.3 RTV-CCD camera (Quantitative Imaging corporation, BC, Canada).

2.9. Western blot analysis

Western blot analysis was performed to detect GFRα-1 protein using GFRα-1/GFRα polyclonal antibody (Life Span Biosciences, Cat. No. LS-C294196/70558; Seattle, WA, USA) raised against the conserved N-

terminal regions of human GFRα-1 which showed 85% homology with the conserved domain region of catfish GFRα-1. Tissue homogenate prepared from adult catfish brain and muscles (negative control) by homogenization with ice cold 1 M Tris, pH 7.4, 150 mM NaCl, 1 mM DTT, and protease inhibitor cocktail. The mixture was centrifuged to 10,000 × *g* at 4 °C to isolate supernatant and later quantification of protein was determined using Bradford method. About 50 µg of tissue homogenate protein was run onto a 12% SDS-polyacrylamide gel electrophoresis. Separated proteins were then transferred onto a nitrocellulose membrane (Pall Life sciences, Port Washington, NY, USA) and the bands were visualized by ponceau S staining. Further, the membrane was blocked with 5% skimmed milk in Tris-buffered saline (TBS) for 1 h at room temperature (RT). After several washes with TBS containing 0.1% Tween 20, membrane was incubated with the respective antibody (1: 1000) dilution for overnight at 4 °C. After washing steps, secondary antibody of goat anti-rabbit IgG coated with HRP was added (1: 5000; Merk Bangalore Genei, Bengaluru, India) and the bands were developed by treating with BCIP-NBT (Roche, Roche Diagnostics GmbH, Mannheim, Germany) as substrates.

2.10. Immunohistochemistry (IHC)

Adult catfish brain was dissected to localize GFRα-1 protein as per the procedure described (Rajakumar and Senthilkumaran, 2014). In brief, the tissues were dissected and fixed in 4% PFA in PBS for overnight at 4 °C. It was then washed with PBS several times before being embedded using cryomedium. Brain embedded in cryoblock was cut at 7 µm thickness onto Poly-L-Lysine coated glass slide using cryostat (Leica CM1850, Leica Microsystems). The sections were hydrated using PBS and later blocked with 10% normal goat serum (Merck Bangalore Genei) for 1 h. Polyclonal antibody of GFRα-1 (1:1000) and pre-adsorbed antibody with excess GFRα-1 antigen (for negative control) was added on the sections and kept overnight at 4 °C. Then, the sections were washed thrice with PBS and incubated with HRP-conjugated secondary antibody (1: 5000; Merck Bangalore Genei) for 1 h at RT. Sections were later treated with ABC reagent (avidin-biotinylated horseradish peroxidase complex) supplied in VECTASTAIN® Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) for 30 min. The slides were washed with PBS and developed using commercially supplied 3′, 3′-diaminobenzidine as chromogen and H_2O_2 (Vector Laboratories) as a substrate for horseradish peroxidase. The color development was stopped by washing the sections with PBS and counter stained with hematoxylin (Qualigens fine chemicals, Mumbai, India), followed by dehydration using a graded ethanol series and finally mounted using DPX mountant. All the photomicrographs were taken using CX41 Olympus microscope (Olympus corporation, Tokyo, Japan) fitted with Q capture pro 6 software-controlled micropublisher 3.3 RTV-CCD camera.

2.11. GFRα-1-siRNA PEI mediated transfection, in vivo

Small interfering RNAs (siRNAs) was commercially synthesized using ~500 bp length of *GFRα-1* gene cloned from catfish brain. Experiment was carried out with custom made *GFRα-1* siRNA (5′-UUGGUACAGAUUAUCACUGUUGUAGCU-3′) designed (Sigma) from catfish *GFRα-1* nucleotide sequence. About 2 µg of siRNA was taken and combined with branched PEI (MW 25 kDa, Sigma) dissolved in sterile HEPES-NaCl pH 7.4, constructed on the protocol explained earlier (Höbel and Aigner, 2010, 2013). The mixture was kept for 20 min at RT to form siRNA-PEI complex. Fish were anesthetized with 100 mg/L of MS 222 (Sigma) in mild ice-cold water following the guidelines of institutional animal ethical committee. The mixture was injected (n = 5) directly into the brain through pineal window followed by topical application of denture adhesive powder was applied gently to cure the injected area. Scrambled (control) siRNA (MISSION siRNA Fluorescent Universal Negative Control #1, Cat. No. SIC007 conjugated with 6-FAM, Sigma) was used for control groups. All the injected catfishes were maintained with continuous aeration and replenishment of fresh water daily for 3 days. Administration of siRNA-PEI complex has been done and validated earlier for catfish (Sudhakumari et al., 2017). The samples were immediately frozen using liquid nitrogen and stored briefly at −80 °C for further analysis.

Expression analysis was performed with gene specific primers (see Table 1) of several genes such as tryptophan hydroxylase (*tph*), tyrosine hydroxylase (*th*), catfish gonadotropin-releasing hormone (*cfGnRH*), 3β-hydroxysteroid dehydrogenase (*hsd3b*), 11*β*-hydroxylases (*h11b*), aromatase (*cyp19a1b*) and β-actin,and relative expression was calculated using $2^{-\Delta Ct}$ method. Relative expression of *GFRα-1* transcripts was calculated in PEI alone and control scrambled siRNA treated catfish brain to check the off-target effects, if any.

2.12. MPTP treatment, in vivo

MPTP purchased from Hychem laboratories (Catalogue No. 28289-54-5) was used for understanding its effect on DA-ergic system vis-à-vis on GFRα-1. Dose-response study was performed to understand the effect of neurotoxin MPTP, *in vivo*. The prespawning fish (n = 5) were injected intraperitoneally (IP) with five different concentrations of the MPTP 5, 10, 50, 100 and 150 µg once and then maintained for 5 days, however, the control group received saline injection (vehicle). All the injected fishes were maintained in different tanks each holding 50 l capacity of fresh water. This experiment was done to understand the significance of GFRα-1 considering the changes in DA-ergic system.

2.13. Quantification CA levels in the brain after the exposure of neurotoxin, MPTP using high performance liquid chromatography-electrochemical detection (HPLC-ECD)

Brain CA levels were measured by HPLC-ECD as described previously (Nagao and Tanimura, 1988). Brain tissues were dissected after MPTP exposure and quickly frozen on dry ice and weighed (n = 5). The brain tissues were homogenized by ultrasonic disintegration (Bronson Sonifer 450, Danbury, CT, USA) 0.1 M perchloric acid (PCA) containing 0.1 mM EDTA, after centrifugation at 10,000*g* for 20 min at 4 °C, the clear supernatants were filtered through a 0.45 µm filter (Millex-HV, Millipore Corporation, Billerica, MA, USA). 20 µl of filtered sample was injected onto the C-18 reversed-phase column. The mobile phase contained 20% (vol/vol), methanol, 0.06 M sodium acetate/0.02 M citric acid buffer, 0.035% (wt/vol.) heptasulfonic acid and 0.1 mM EDTA, pH 3.92. The column temperature (17–30 °C) and flow rate (1 ml/min) was adjusted by HPLC-ECD (Waters, Milford, MA, USA) system (Murai et al., 1988). The working standard solutions were prepared in 0.1 M PCA containing 0.1 mM EDTA and stored at −80 °C. The 20 µl injection contained 20 pmol L-3,4-dihydroxyphenylalanine (L-DOPA), 30 pmol DA and 50 pmol NE. Elution peaks were recognized by associating the retention time of each peak in the sample solution to the peak in the standard solution (Murai et al., 1988). The levels of CA were expressed as nanograms per gram of wet tissue weight.

2.14. Statistical analysis

The data of qPCR analysis were expressed as mean ± standard error of mean (SEM). All the data passed homogeneity and normality testswere evaluated by one-way analysis of variance (ANOVA) followed by Student-Newman–Keuls'(SNK) post hoc test, Student's *t*-test and a probability of $P < 0.05$ was considered statistically significant. All the statistical analysis was performed using Sigma Plot 11.0 software (Systat Software Inc., Chicago, IL, USA).

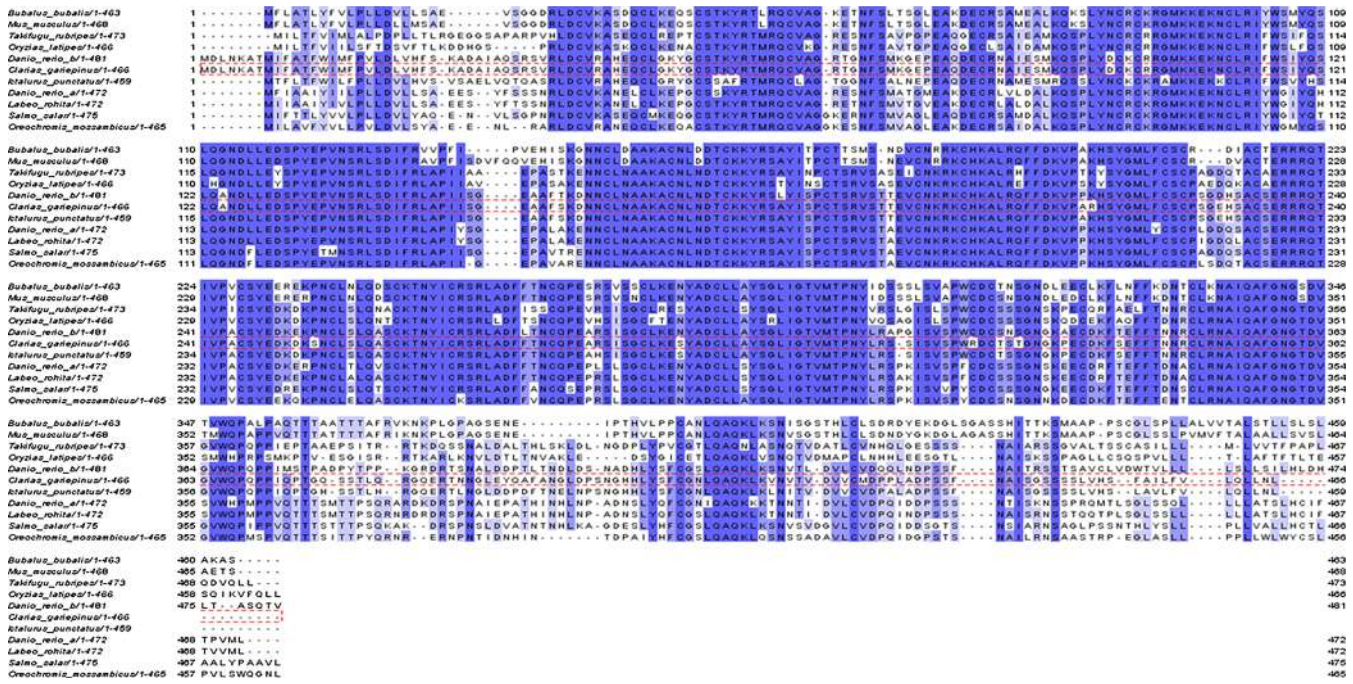

Fig. 1. Multiple sequence alignment of catfish GFRα-1 with deduced amino acid sequences of GFRα-1 of other vertebrates. Protein-BLAST of catfish GFRα-1 showed high homology with other sequences in GFRα-1 domain regions. GenBank accession numbers used for different GFRα- were followed *Ictalurus punctatus* (XM_017482772), *C. gariepinus* (KY553234), *Danio rerio*_a (NM_131730), *D. rerio*_b (NM_131731.1), *Oreochromis mossambicus* (KR779759.1), *Takifugu rubripes* (XM_003961583.2), *Labeo rohita* (HM130051.2), *Salmo salar* (LOC106577126), *Bubalus bubalis* (XM_006071676), *Oryzias latipes* (XM_004080268.2), *Mus musculus* (NM_010279).

3. Results

3.1. Molecular cloning of GFRα-1

Partial cDNA of 244 bp was cloned from adult catfish brain by RT-PCR. cDNA fragments of 5′ and 3′ ends of *GFRα-1* was obtained from brain by following RACE strategies. The nucleotide sequences of partial and RACE cDNAs were aligned using Lasergene software to generate complete cDNA fragment 1401 bp of *GFRα-1*. The open reading frame (ORF) of *GFRα-1* encodes a putative protein of about 467 aa. All the nucleotide sequence information of the genes was submitted to NCBI GenBank and the primers used for cloning were specified in Table 1, the nucleotide sequence of *GFRα-1* of catfish was submitted to GenBank having accession no. KY553234. Multiple sequence alignment (Fig. 1) was performed using Clustal Omega by aligning the catfish *GFRα-1* deduced aa sequence with its vertebrate counter parts to check. Homology of the deduced aa from catfish *GFRα-1* ORF was compared with its counterparts from other vertebrates. To further clarify its classification, phylogenetic analysis was performed (Fig. 2) using neighbor joining method software which revealed that the catfish, *C. gariepinus GFRα-1* showed more homology with *GFRα-1* of *I. punctatus* and *GFRα-1b* of *D. rerio* and formed a separate clade.

3.2. Tissue distribution and brain ontogeny expression analysis by qRT-PCR

The expression of *GFRα-1* were ubiquitous in the tissues (n = 5) analyzed, however, significant ($P < 0.05$) high expression was evident in female brain (Fig. 3A). Moderate transcript levels were detected in male brain and ovary. Low level of expression was seen in testis, gills, spleen, heart and intestine (Fig. 3A). In ontogeny analysis, expression of *GFRα-1*was significantly ($P < 0.05$) high in the developing female brain, at 75 and 100 dph while there were no significant differences in other stages analyzed (Fig. 3B).

3.3. Expression of GFRα-1 during reproductive phases

Phase-wise expression analysis of *GFRα-1* showed elevated levels in the spawning phase when compared to other reproductive phases (Fig. 4).

3.4. Expression of GFRα-1 mRNA in different regions of adult female brain

Expression pattern of *GFRα-1* was quantified in five different regions of adult female brain (TEL +OB, POA + HYP, PT, TH and MO), wherein *GFRα-1* mRNA level was higher in POA + HYP when compared to other regions of brain (Fig. 5).

3.5. ISH of GFRα-1 mRNA in catfish brain

Localization of *GFRα-1* mRNA in catfish brain revealed positive signals in the POA-HYP (Fig. 6A–C), while the sense probe showed no signal confirming the specificity (Fig. 6D) of the reaction.

3.6. Immunolocalization and western blot analysis of GFRα-1 in catfish brain

Immunoreactivity of GFRα-1 was observed in POA-HYP of adult brain (Fig. 7A–C) confirming the antibody specificity. Negative control (pre-adsorbed antibody) of GFRα-1 counter stained with haematoxylin displayed no immunoreactivity in the catfish brain (Fig. 7D). Further, validation of catfish GFRα-1 antibody was tested using western blotting. A sharp band of GFRα-1, ~50 kDa, was observed in the brain-protein gel-segregation which is equivalent to the size of deduced catfish GFRα-1 peptide. Upon transient silencing of *GFRα-1* using siRNA in brain, the levels GFRα-1 decreased considerably (Fig. 8C). Both these results validate the specificity of antibody used in IHC.

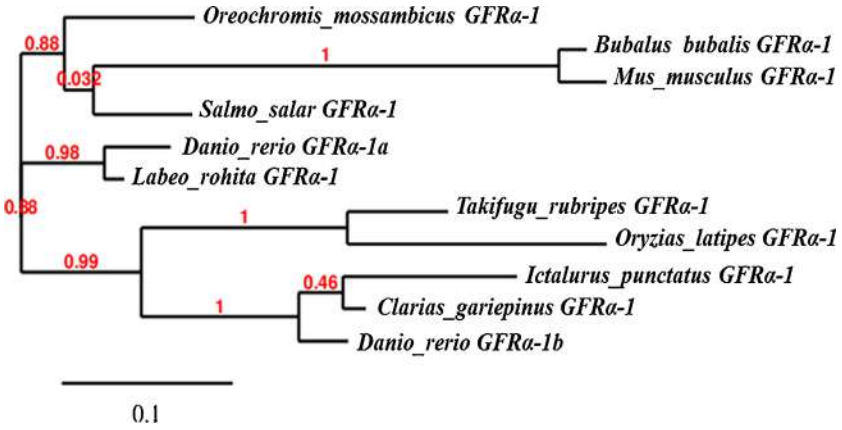

Fig. 2. Phylogenetic tree showing the evolutionary status of catfish GFRα-1. The phylogenetic analysis was performed using the neighbor-joining method and a bootstrap analysis with 1000 replicates was used to assess the strength of the nodes in the tree. The phylogenetic tree was generated using the TreeView software.

3.7. *In vivo transfection of GFRα-1-siRNA in catfish brain*

To check the efficiency of *GFRα-1-siRNA*-PEI complex administration, control groups PEI and control scrambled siRNA (n = 5) were injected separately (Fig. 8A). After the treatment, GFRα-1 mRNA was quantified and compared with respective control groups. PEI alone and control scrambled siRNA injected fish did not show any significant differences in the expression of *GFRα-1*. These results specify that injection of PEI and control scrambled siRNA independently (n = 5) did not affect the expression of genes analyzed including the levels of *GFRα-1*(Fig. 8A). But, *GFRα-1*-siRNA-PEI complex injected brain showed significant ($P < 0.05$) decrease in the expression of *GFRα-1* when compared to all the other groups (Fig. 8A) confirming that *GFRα-1*-siRNA-PEI complex transiently silenced the expression of *GFRα-1* ($P < 0.05$). Further, expression analysis on certain brain-specific genes such as *tph*, *th*, *cfGnRH*, *hsd3b*, *hb11* and *cyp19a1b* (Fig. 8B) revealed significant ($P < 0.05$) reduction when compared to the control groups and unaffected β-actin gene indicating the impact of *GFRα-1* transient gene silencing.

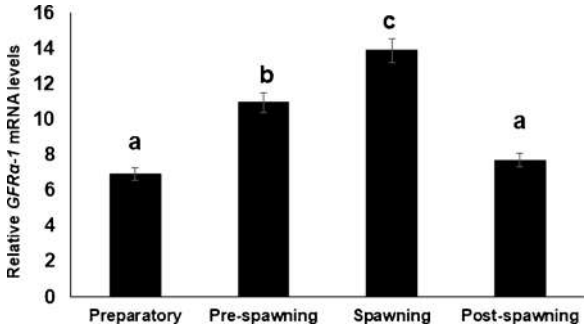

Fig. 4. qRT-PCR analysis of *GFRα-1* expression during reproductive phases in adult female brain. Means with different alphabets differs significantly while means with similar alphabet did not have any significance. The relative expression was normalized with *18S rRNA* and the values were calculated using$2^{-\Delta Ct}$method. Data (n = 5) were expressed as mean ± SEM (* $P < 0.05$; one –way ANOVA followed by SNK test).

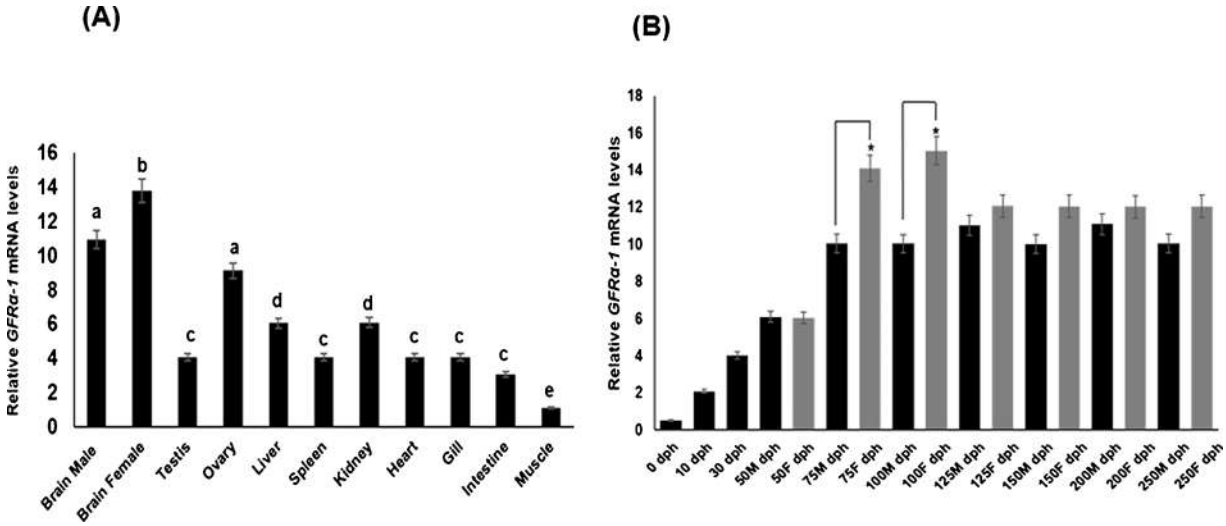

Fig. 3. Tissue distribution (A) and ontogeny (B) of *GFRα-1* expression in the catfish brain. The relative expression was normalized with *18S rRNA* and the values were calculated using $2^{-\Delta Ct}$ method. (A) Data (n = 5) were expressed as mean ± SEM (*$P < 0.05$; one-way ANOVA followed by SNK test). Means with different alphabets differs significantly while means with similar alphabets did not have any significance. (B) Data (n = 5) were expressed as mean ± SEM (*$P < 0.05$ Student's *t*-test). Abbreviation: M-male; F-female; dph- day post hatch.

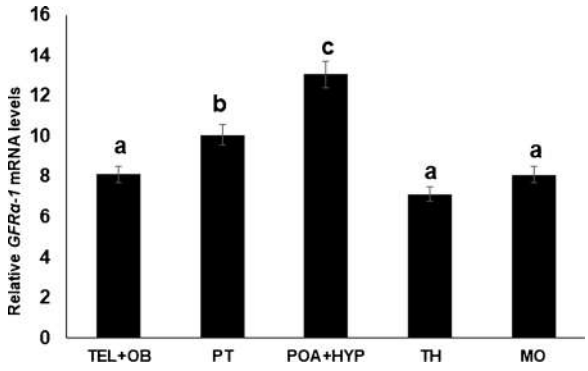

Fig. 5. qRT-PCR analysis of *GFRα-1* expression patterns in different regions of the adult female brain. The relative expression was normalized with *18S rRNA* and the values were calculated using $2^{-\Delta Ct}$method. Data (n = 5) were expressed as mean ± SEM (*P < 0.05; one-way ANOVA followed by SNK test). Means with different alphabets differs significantly while means with similar alphabets did not have any significance. Abbreviation: TEL + OB- telencephalon + olfactory bulb, PT- pituitary, POA-HYP- preoptic area-hypothalamus, TH-thalamus and MO- medulla oblongata.

3.8. Effect of MPTP on catfish brain, in vivo

MPTP treatment, *in vivo* in catfish brain showed dose related decrease in the expression of *GFRα-1* (P < 0.05) when compared to control (Fig. 9).

3.9. Changes in the level of CA in catfish brain after the exposure of MPTP

The levels of CA were significantly (P < 0.05) lower in the MPTP-treated brain when compared with the control group (Fig. 10). The levels of L-DOPA were significantly reduced (P < 0.05) at higher concentration of MPTP treatment when compared to control brain. However, no significant difference was observed at lower concentration of MPTP (Fig. 10A). Similarly, the levels of DA and NE were significantly decreased (P < 0.05) in the brain at two different concentrations as compared to the control groups (Fig. 10B, C).

4. Discussion

In the current study, full-length cDNA of neurotrophic family receptor, *GFRα-1* was cloned from catfish brain using degenerate primers and RACE. Tissue distribution and ontogeny expression analysis revealed high expression of *GFRα-1* in the developing and female adult brain, indicating a plausible role for *GFRα-1* in the brain development of catfish. Expression pattern of *GFRα-1* was in accordance with the transcript changes of *th* and DA levels in the female brain reported earlier (Mamta et al., 2014). Clustal Omega alignment showed its homology with the conserved signature GDNF superfamily domain of other vertebrates including teleosts. Transcript variants *GFRα-1a, GFRα-1b* and *GFRα-2* have been reported in other teleostsean member, *D. rerio* by using RACE (Shepherd et al., 2004). However, our cloning strategies such as degenerate RT-PCR, RACE and genomic PCR with the primers designed onto two neighboring highly conserved exons flanking a variable intron employed in the present study resulted in a single transcript of *GFRα-1* in catfish. The phylogenetic analysis of *GFRα-1* revealed that *GFRα-1* of *C. gariepinus* showed high homology with *GFRα-1* of *I. punctatus* and *GFRα-1b* of *D. rerio* forming a separate clade (Fig. 2). Expression analysis revealed higher *GFRα-1* mRNA levels in the female brain and ovary with moderate levels in several other tissues and male brain. GDNF receptor complex expressed very early in the development of enteric nervous system which has been shown to promote survival, proliferation, differentiation in *D. rerio* (Lucini et al., 2010). In mammals, *GFRα-1* mRNA was predominantly expressed in the developing and adult DA-ergic neurons (Nosrat et al., 1997) to assume a role for *GFRα-1* in brain development perhaps by targeting DA-ergic activity. In catfish, ontogeny analysis of *GFRα-1* in brain suggested that this gene might be essential for early stage of brain development vis-à-vis gonadal development. As per our earlier studies, Tph and Th vis-à-vis 5-HT and CA might have an important role in gonadal differentiation either directly or indirectly (Raghuveer et al., 2011; Mamta et al., 2014). In addition, higher expression of *GFRα-1* in spawning phases of catfish suggested that gene might have role in GnRH-GTH axis. However, the levels of GTHs showed seasonal variation warranting their regulation (Joy et al., 2000; Acharjee et al., 2015) through GnRH cyclicity (Senthilkumaran et al., 1999). Therefore, it can be presumed that

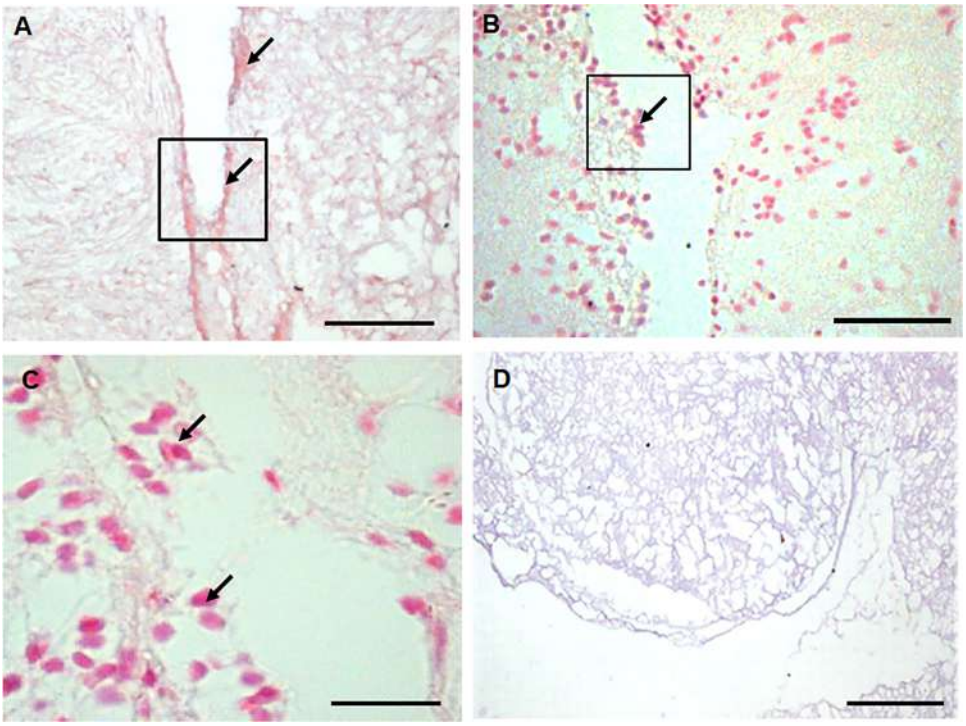

Fig. 6. *In situ* hybridization of *GFRα-1* in POA-HYP regions of adult catfish brain. Antisense probe in POA-HYP (A,B,C) and Sense probe in POA-HYP (D), Scale bar indicates: A and D:50 µm;B: 25 µm; and C: 10 µm. Note: No signal was detected in sense probe as against the antisense probe. Arrows indicates presence of transcript localization.

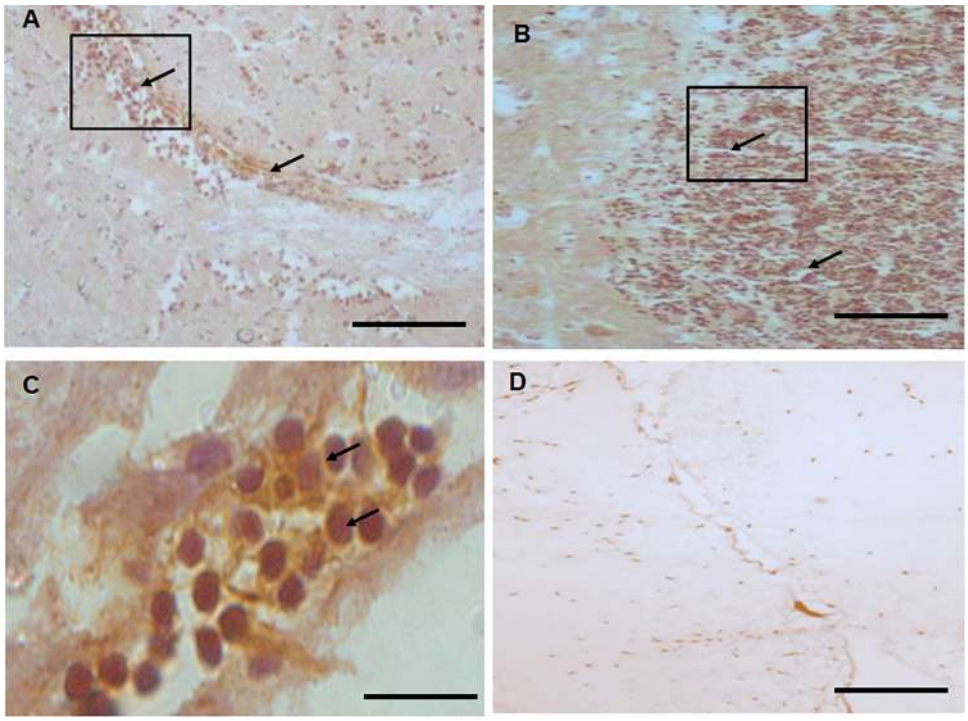

Fig. 7. Immunohistochemistry of GFRα-1 protein in POA-HYP regions of adult catfish brain. GFRα-1 immunoreactivity was observed in POA-HYP(A–C) while preabsorption antibody treatment showed no immunoreactivity (D). Black arrow showing immunoreactive cells in POA-HYP regions. Scale bar indicates: A: 50 µm; B: 25 µm; C:10 µm; D:120 µm. Abbreviation: TEL + OB- telencephalon + olfactory bulb, PT-pituitary, POA-HYP- preoptic area-hypothalamus, TH- thalamus and MO- medulla oblongata.

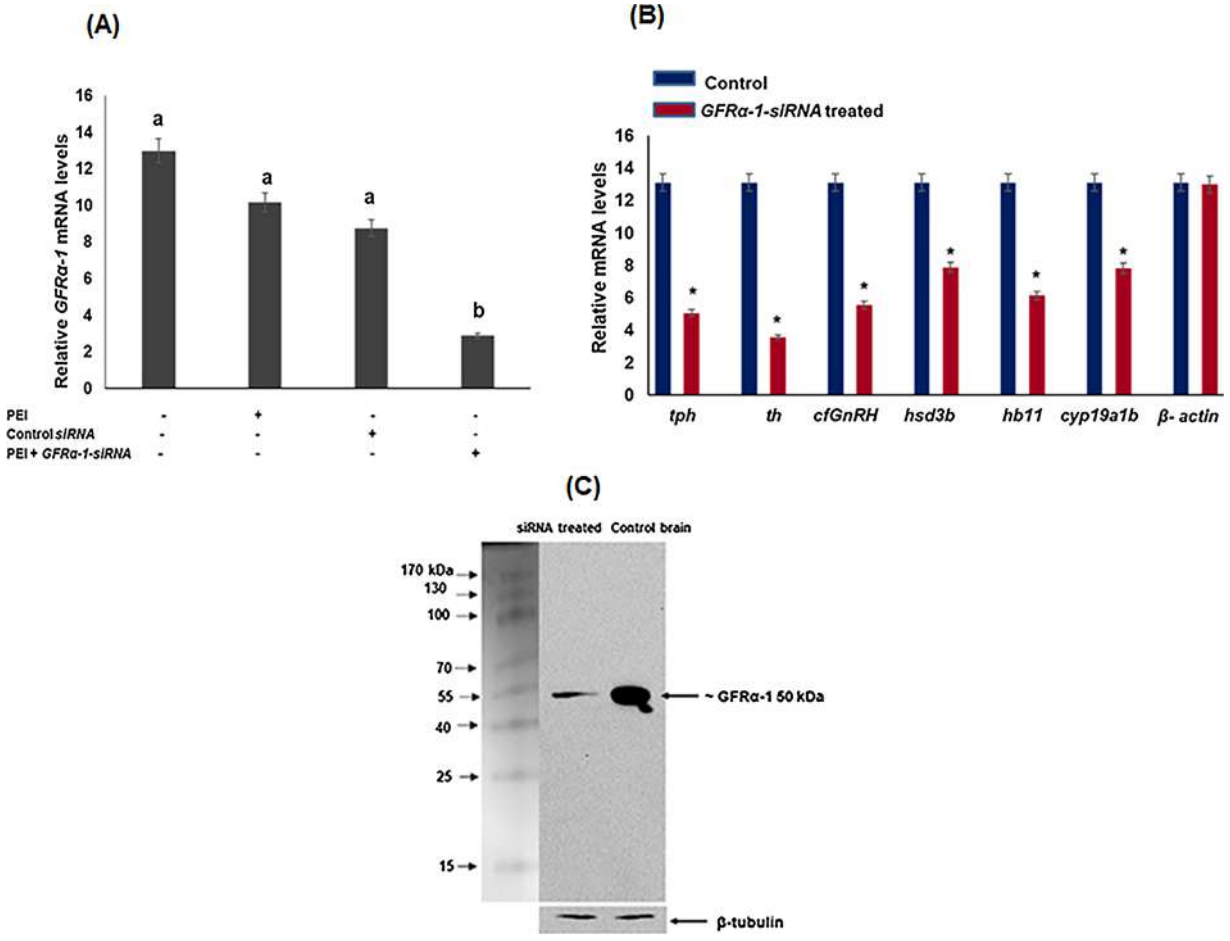

Fig. 8. *In vivo* PEI mediated *GFRα-1*-siRNA transfection in adult catfish. The transcript of *GFRα-1* were quantified and compared (A) between PEI alone, scrambled siRNA (control) and *GFRα-1*-siRNA (treated) group. Data (n = 5) were expressed. as mean ± SEM (*P < 0.05; one-way ANOVA followed by SNK test). Expressions of certain brain-specific genes (B) were observed after transfection with GFRα-*1*-siRNA. Data (n = 5) were expressed as mean ± SEM (*P < 0.05 Student's *t*-test). Validation of western blot (C) showed a sharp band ~50 kDa size while upon siRNA treatment the intensity of band is diminished.

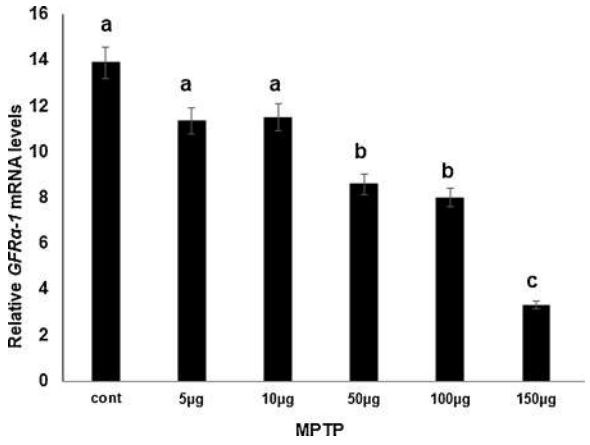

Fig. 9. qPCR analysis showed expression of *GFRα-1* between control and MPTP treated group, *in vivo*. The relative expression was normalized with *18S rRNA* and the values were calculated using comparative $2^{-\Delta Ct}$ method. Data (n = 5) were expressed as mean ± SEM (*P < 0.05; one-way ANOVA followed by SNK test). Means with different alphabets differs significantly while means with similar alphabets did not have any significance. Abbreviation: cont-control.

GFRα-1 might regulate DA to control GnRH-GTH axis on gonads (Peter et al., 1991) through DA-ergic activity. Over expression of *GFRα-1* may also favour DA inhibitory tone either directly or indirectly to regulate GnRH-GTH axis which is well demonstrated in teleosts (Dufour et al., 2010; Peter et al., 1991; Goos et al., 1999; Senthilkumaran and Joy, 1996).

Localization of *GFRα-1* mRNA showed its predominant expression in POA-HYP. By and large, these results creditably support vital significance for GFRα-1 in brain. Earlier study reported intense GFRα-1 protein and high mRNA expression in different regions of brain and its rolein the central nervous system in adult *D. rerio* (Lucini et al., 2010). Interesting part of the present study is to analyze the regulatory role of *GFRα-1* and its function by GFRα-1 siRNA transient silencing *in vivo*, through brain targeted injection wherein delivery of *GFRα-1*-siRNA-PEI complex decreased the expression of *GFRα-1* in catfish brain. After silencing *GFRα-1*, protein levels and the expression of various brain-specific genes i.e. *tph, th*, cf*GnRH*, *hsd3b,h11b, cyp19a1b* were down-regulated and incidentally these genes are implicated to "brain sex differentiation" in catfish (Senthilkumaran et al., 2015). The outcome of the study demonstrated that the silencing of *GFRα-1* act as foremost target to regulate the expression of genes at the level of brain and for the first time that signalling through a neurotrophic factor receptor absolutely required for the brain has been shown. However, it is important to know how GFRα-1 regulate brain related genes as the present study demonstrated an effect, yet, exact mode of action is not clear. Considering these reports low expression of *th* and *tph* in brain after *GFRα-1* silencing indicate a probable effect on CA-ergic and 5-HT-ergic neurons, respectively. This contention needs to be validated further.

Our findings showed possible effects of *GFRα-1* transient knock-down on CA-ergic neurons and reduction of Th levels, the rate limiting enzyme of CA biosynthesis. In view of this, it is important to analyze the relationship between *GFRα-1* and DA-ergic activity. Towards this, present study was extended to analyze the impact of neurotoxin, MPTP on DA levels, *in vivo*. MPTP treatment reduced *GFRα-1* expression and CA levels after neurotoxin post injection in catfish brain and causes cellular toxicity to target DA-ergic system. Based on this, we showed that catfish DA-ergic neurons are susceptible to neurotoxicity after MPTP

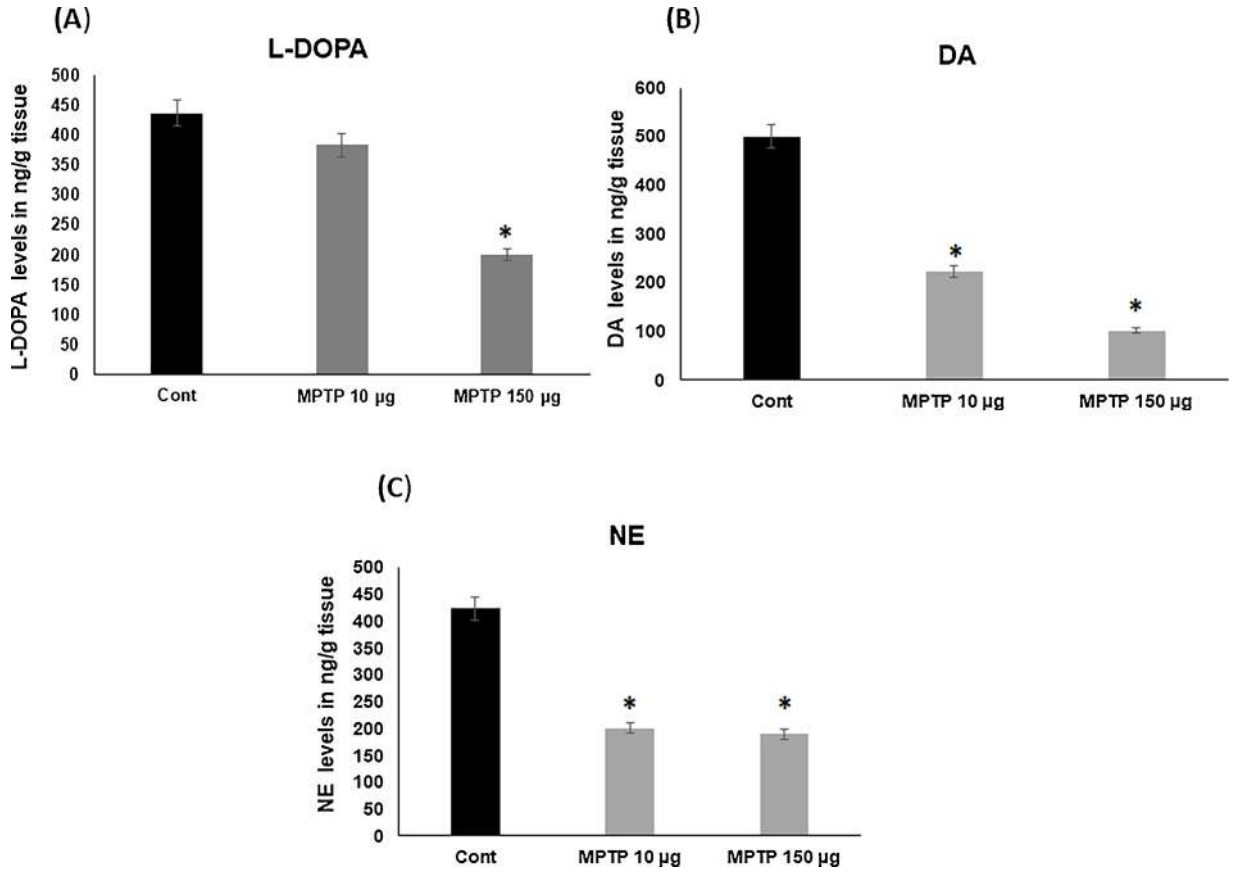

Fig. 10. Quantitative estimation of catecholamines (CA), such as L-DOPA, DA and NE after the exposure of MPTP with two different concentrations 10 μg and 150 μg. L-DOPA (A), DA (B) and NE levels (C) with respective controls using HPLC-ECD. Data (n = 5) were expressed as mean ± SEM (*P < 0.05; Student's *t*-test). Abbreviation: cont-control, L-DOPA- L-3,4-dihydroxyphenylalanine, DA-dopamine and NE- norepinephrine.

treatment. This contention is supported by the reports that identified significant glial cell activation associated with the loss of DA-ergic neurons in MPTP-induced Parkinson disease models (Yokoyama et al., 2010) and causing damage to DA neurons in teleosts (Xing et al., 2016). However, the exact role of activated glial cells in DA neurodegeneration and regeneration remains to be seen. In goldfish, DA neurodegeneration starts after neurotoxin post injection in the HYP (Popesku et al., 2012), though recovery of DA and NE levels was observed after MPTP exposure. In the present study, the effect of MPTP was observed 5 days after an IP injection, however it was not extended to analyze any retrieval of *GFRα-1* and CA levels in the catfish brain. Certainly, in goldfish (Popesku et al., 2012) 6 days after MPTP exposure, DA levels were shown to be decreased by about 70% in the HYP and Tel. Unlike mammals, DA neurons in teleosts might regenerate following injection with MPTP (Poli et al., 1992; Pollard et al., 1992). In addition, high expression of *cyp19a1b* implicating endogenous E_2 having an important role in glial cells reaction in response to the neurotoxin MPTP (Xing et al., 2016) and the production of neurotrophic factors and genes involved in DA neuron development under the modulation of E_2 especially after damage. The involvement of *GFRα-1*, based on its impact on CA levels needs to be considered as the inhibitory tone of DA seems important for GnRH-GTH release during recrudescence (Peter et al., 1991; Senthilkumaran and Joy, 1995).

The GDNF acts solely via GFRα-1 receptor to regulate DA-ergic neuron. *GFRα-1* siRNA silencing led to reduction *th* expression wherein both DA and NE might have been subdued due to fact that Th being rate limiting enzyme of CA biosynthesis. Hence, low level of NE might have reduced GnRH rather than reduction in DA. During recrudescence, reduction in NE levels might influence GnRH-GTH axis as NE plays a crucial role in gonadal recrudescence in seasonally reproducing teleosts (Senthilkumaran and Joy, 1995; Goos et al., 1999). MPTP is a well-known blocker of DA-ergic neuron and hence, reduction in GFRα-1 expression was seen, which is in accordance with the report of Popesku et al. (2012). Further, reduction in DA and NE levels confirms that MPTP did have an impact on CA biosynthesis. Hence, association of GFRα-1and CA in particular DA is evident. In addition, GFRα-1 expression in accordance to CA levels may have role for early brain development, plausibly through DA-ergic activity, either directly or indirectly.

5. Conclusion

In conclusion, abundance of GFRα-1 mRNA and protein expression in the brain seems to suggest asignificant role for this correlate perhaps through GDNF signalling in the brain of catfish, *C. gariepinus*. In addition, tissue distribution and ontogeny analysis showed high expression in developing (female) brain. Localization of GFRα-1 protein by IHC and mRNA by ISH in the POA-HYP of brain indicates plausible significance in relation to GnRH-GTH axis in catfish. Transient silencing of *GFRα-1*-siRNA and MPTP neurotoxin treatment indicate an important role for *GFRα-1* in coordination with DA-ergic activity. Decreased expression of certain brain-specific genes after *GFRα-1*-siRNA transient silencing revealed the regulatory influence of *GFRα-1* on brain more importantly on GnRH-GTH, either indirectly or directly.

Conflict of interest

The authors declare no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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