Brain Research Bulletin 140 (2018) 270–280

Contents lists available at ScienceDirect

Brain Research Bulletin

journal homepage: www.elsevier.com/locate/brainresbull

Research report

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

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ARTICLE INFO

Keywords:
GFRα-1
GDNF signalling
Brain development
MPTP
Teleosts

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) neuronsin 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-tetrahydropyridinecausing 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 axiseither 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 inmammals (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 neuronswhich 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 lumbar spinal cord (Moore et al., 1996; Cacalano et al.,

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

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https://doi.org/10.1016/j.brainresbull.2018.05.010
Received 4 December 2017; Received in revised form 28 February 2018; Accepted 8 May 2018
Available online 31 May 2018

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-1enhances 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-tetra-hydropyridine (MPTP)that is known to cause loss ofDA-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-visneurosteroids release at the level of brain. Among monoamines, serotonin (5-HT) and norepinephrine (NE) have a stimulatory influence ongonadotropin-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