Indian Journal of Biochemistry & Biophysics
Vol. 60, May 2023, pp. 414-422
DOI: 10.56042/ijbb.v60i5.477

Effects of Morpholino-mediated knockdown of the Angiotensin-converting enzyme 2 (ACE-2) on dopamine Neurogenesis and the Hypothalamic Pituitary-adrenal (HPA) Axis in Larval Zebrafish (Danio rerio)

L Divya Christy, Mir Abbas Raza, Clarita AC & Jayshree Nellore*

Department of Biotechnology, School of Bio and Chemical Engineering, Sathyabama Institute of Science and Technology (Deemed to be University), Jeppiar Nagar, Rajiv Gandhi Salai, Chennai-600 119, Tamil Nadu, India

Received 11 April 2023; *revised 11 June 2023*

While the presence of *ACE2* and its expression in brain structures, along with its recent link with the novel SARS-CoV-2, are known, their impact on the development of the brain and on the activity of the HPA axis in larval zebrafish is not known. Therefore, the primary objective of this study was to determine how silencing the *ACE2* gene affects DA neurogenesis and the endocrine stress response in larval zebrafish (Danio rerio). In this investigation, an *ACE2* AUG morpholino oligonucleotide was introduced by electroporation into the 96 hpf zebrafish in order to silence the expression of the *ACE2* gene using antisense technology. Electroporation of the embryo in 1X PBS on 1.5% Agarose gel with *ACE2* AUG morpholino oligonucleotide was done to transfer it into the embryo. After successful transfer, no visible morphological changes were observed. A significant reduction in *ACE2* gene expression confirms the successful oligonucleotide transfer. Nurr1 expression was significantly reduced as *ACE2* expression was silenced, but there was no significant effect on GCR or CRH expression. Interestingly, a significant increase in locomotor activity was seen after the silencing of *ACE2* expression. Overall, these results facilitate the prediction of possible neurological manifestations of brain *ACE2* dysregulation during and after COVID-19 infection.

Keywords: *ACE2* gene, Dopamine neurogenesis, HPA axis, Zebrafish development, COVID-19 and neurological manifestations

SARS-CoV-2 uses the angiotensin-converting enzyme (*ACE2*) protein, a single-pass type 1 transmembrane receptor, as an assess portal to cells[1]. The expression of *ACE2* was identified in multiple cell types, such as type II alveolar cells of the respiratory system, enterocytes, intestinal epithelial cells, endothelial cells, epithelial cells of the conjunctival epithelium, kidney cells (renal tubules), and certain immune cells, such as alveolar monocytes/macrophages and certain cells of the CNS, including those of the cerebral cortex and brainstem[2-7].

Brain angiotensin (Ang) converting enzyme-2 (ACE2) was discovered two decades ago as a key regulator in the classical axis of the renin-angiotensin system (RAS), a hormonal system that regulates not only systemic vascular resistance to blood flow and blood pressure but also fluid and electrolyte balance[8]. More recently, ACE2 has been shown to play critical roles in neuro-inflammation, gut dysbiosis, and the regulation of stress and anxiety-like behaviors[9]. The acute and chronic neurological manifestations of COVID-19, as well as its potential contribution to long-term adverse cerebrovascular, neuropsychiatric, and neurodegenerative pathologies, are still poorly understood.

Accumulating studies have proved that perinatal exposure to environmental adverse conditions such as toxic compounds, stress, and drug treatments causes anxiety and depression-related behaviors[10]. Such conditions are frequently associated with functional abnormalities in the HPA axis and in the dopamine (DA) system.

The HPA axis is a network of brain and peripheral systems; it contains the hypothalamus, which releases corticotropin-releasing factor (CRF), and projects to the anterior pituitary, which secretes adrenocorticotropic hormone (ACTH). The end product of HPA axis activation (*i.e.*, the release of glucocorticoids by the adrenal gland) serves to alert an organism to environmental and physiological changes and to maintain homeostasis[11]. Brain stem catecholaminergic centers play an important role in the regulation of the HPA axis. However, the DA system is an important afferent modulator of this region as it plays a key role in the response to stress

---

*Correspondence:
E-mail: jayshree.biotech2021@gmail.com

and, in particular, in the pathological response observed in many neurological disorders[12]. The present study examines the poorly understood link between ACE2 expression and DA neurogenesis, CRF, glucocorticoids, and behavior, with a particular focus on early development.

In neurochemistry, transgenic and knockout animal models are invaluable tools to study neurohormonal systems since they reveal the effects of changes in single components of these systems on the whole physiology, as has been exemplified for the RAS[13]. Mice that ubiquitously overexpress ACE2 by germline deletion of a stop-lox cassette result in decreased CRH synthesis and suppress anxiety-like behavior in the elevated plus maze. In the Morris water maze and the Y-maze experiments, ACE2-deficient mice show severe impairment in memory and cognition[14]. Although DA signaling has long been implicated in motor programming, reward-related behaviors, visual and olfactory perceptions, thermoregulation, learning and memory, and emotions, the downstream mediator of ACE2 in the regulation of neurogenesis remains elusive.

The zebrafish (*Danio rerio*) has emerged as a powerful model to study the development of DA systems and their physiology[15,16]. In addition, the early development of an intact DA system during the embryo (*i.e.*, 18 h postfertilization [hpf] to 3 days postfertilization [dpf]) and larval (>3 dpf) stages[17,18] makes the zebrafish a good model for investigating DA neurogenesis. Previous neuroanatomical investigations in zebrafish have demonstrated that the major dopaminergic innervation of the pituitary originates from a subset of neurons (preoptic-hypophyseal DA (POHDA)) in the antero-ventral part of the preoptic area. thus providing morphological support for the DA actions on the anterior pituitary hormone secretion in zebrafish. Expression profiles of several genes (*foxg1a*, *dlx2a*, and *nr4a2a/b*) were different in the POHDA compared with the adjacent suprachiasmatic DA neurons, suggesting that POHDA neurons develop as a distinct DA cell population in the preoptic area[19].

Interestingly, Zebrafish ace2 and RAS signaling are highly conserved in sequence and structure compared to their mammalian counterparts. The localized expression of ACE2 in the anterior neuromasts and the forming olfactory organs was demonstrated in zebrafish during the embryonic (*i.e.*, 17 h postfertilization [hpf]) to larval (>3 dpf) stages, making the zebrafish a good model for investigating ACE2 expression. However, to our knowledge, there is no study investigating the changes in ace2 expression over the development of DA neurons controlling anterior pituitary functions.

Nurr1 is an early mdDA differentiation gene; deletion of this gene results in a delay in mdDA neuronal differentiation, indicating that this gene is involved in the correct specification of DA progenitors[21]. Proliferating matrix zones have been detected within the telencephalon of the zebrafish brain[22]. In light of this, the telencephalon is an appropriate area of the brain on which to focus early DA neurogenesis studies. Furthermore, hypophysiotropic cells in the preoptic area of the telencephalon produce CRF. Thus, the telencephalon is an important region in both the integration of signals to the HPI axis and an area of increased proliferation during early development.

The presence of ACE2 and its expression in brain structures involving cognition, behavior, and locomotion, along with its recent link with the novel SARS-CoV-2, have led to the need for an investigation into its modulatory effects on the HPA axis and Dopamine. In this study, an ACE2 AUG morpholino oligonucleotide was introduced by electroporation into the embryo in order to silence the expression of the ACE2 gene using antisense technology. No visible morphological changes were observed post-transfer, and a significant reduction in ACE2 gene expression confirms the successful oligonucleotide transfer. Nurr1 expression was significantly reduced as ACE2 expression was silenced, but no significant effect on GCR or CRH was observed. Interestingly, a significant increase in locomotor activity was seen after the silencing of ACE2 expression, warranting further investigation and research on the role and effects of ACE2.

Materials and Methods

Zebrafish maintenance and collection of embryos

Wild-type adult (<8 months old) Zebrafish were reared and maintained in a zebrafish housing system at the Sathyabama Institute of Science and Technology under standard laboratory conditions of temperature 28 0.5 °C and pH 7.2+0.2. On a 14:10 Dark/light photoperiod according to the standard breeding protocols[23]. Fertilized Embryos were collected within 0.5 h of spawning, grown in embryo medium, and staged in the range of 24 hpf, 48 hpf, 72 hpf, and 96 hpf using a stereo microscope (ZEISS). Dead embryos were removed.

Experimental treatments

The experimental groups used for the study comprise the following: (i) Control group: Zebrafish Electroporated without ACE2 AUG Morpholino oligonucleotide and only in 1X PBS (ii) Test group: Zebrafish Embryos Electroporated with ACE2 AUG Morpholino oligonucleotide.

Danio rerio angiotensin I converting enzyme 2 (ACE2) AUG Morpholino oligo sequence written from 5' to 3':

ID: NM_001007297.1

Description: Danio rerio angiotensin I converting enzyme 2 (ACE2), mRNA

CDS: [13:2371](+)

CAAGGCTGTGGTC**[(ATG)TGTGCTCGCTG GCTCCTG]**CTTT

Preparation of embryos and description of electroporation experiments

The Petri dish platinum electrodes were used to introduce the ACE2 AUG Morpholino oligonucleotide into the embryos[24] with slight modifications.

1.5% of low-melting Agarose was cast in one of the six wells of the cell culture plate covered with aluminum foil. A well-like depression was made at the center of the gel as it solidified. The agarose gel was then removed from the cell plate and placed on the Petri dish filled with 1X PBS. The PBS was filled such that it did not completely submerge the gel and did not enter the well. To optimize the process and check for its feasibility, the procedure was done multiple times by filling the well with two different concentrations of Rhodamine B (Tetraethyl rhodamine) for each stage. 1 mM and 0.5 mM of Rhodamine B were dissolved in 1.5 mL of 1X PBS, , respectively. The Electric Pulses applied met the following conditions: 20 V, 3 s pulse length, 5 s pulse interval, 3 pulses (The first pulse, or the poring pulse, makes micro-holes in the embryos). The second pulse, or transfer pulse, transfers the Morpholino into the cytoplasm of the embryos. The third pulse, or the polarity-changed transfer pulse, increases the opportunity of introducing Morpholino into the embryos. Embryos were first dechorionated manually, washed three times in E3 medium, and then added to the well (containing Rhodamine B) using a fire-polished Pasteur pipette[25,26].

The electrodes were placed in the Petri dish, submerged in the 1X PBS on either side of the gel. After subjecting the embryos to the Electroporation conditions, the Embryos were then washed four times with E3 Medium and observed immediately for any morphological changes. The Embryos were also checked for their Mortality rate after 24 h. Gene expression analysis was done for both Control groups as well as Zebrafish Embryos Electroporated with ACE2 AUG Morpholino oligonucleotide.

Toxicity assay

The medium was changed at 24 h intervals with fresh E3 medium. Dead Embryos were removed. The mortality rate was recorded, and the Embryos were checked for any visible morphological changes between the Control groups and the test embryos under a stereo zoom microscope.

Locomotor Activity

The swimming activities of the larvae, *i.e.*, 72 hpf and 96 hpf, were evaluated as described previously[23,27]. A petri plate was marked with graph lines of 1 cm distance. Embryos were allowed to swim in the petri plate for 1 min, and the total distance crossed was noted. This was performed for embryos of 72 hpf and 96 hpf embryonic stages for both Control groups that were only electroporated in 1X PBS and Zebrafish Embryos Electroporated with ACE2 AUG Morpholino oligonucleotide. The results were documented and compared.

RNA Isolation

Total RNA was extracted at various developmental stages using the TRIZOL (Invitrogen) reagent. After isolating the total RNA (1000 ng/l) was converted into cDNA by using the cDNA synthesis kit from Applied Biosystems.

Real-Time PCR assay

The fluorescent RT-PCR reaction was carried out in the Applied Biosystem Step One instrument with specific primers targeting ACE2, CRH, GCR ACE2, and NURR1 genes (the primers of the sequence are described in (Table 1). The thermal profile for qRT-PCR used in the current study is listed in (Table 2). Glyceraldehyde Phosphate dehydrogenase was used as an internal control; the assay was performed in duplicate with a 25 μL reaction. Q-PCR data was analyzed quantitatively by using the formation of 2-ΔΔCt[28].

Statistical analysis

Relative quantification of gene expression was calculated after normalizing with the reference genes. The Relative expression was assessed by using $2^{-\Delta Ct}$

Table 1 — Primer Sequences and Annealing temperatures Z: Zebrafish F: Forward R: Reverse Ta: annealing Temperature

Primer Name	Sequence	Primer length	Accession number	Ta (°C)
zcrhf	CACCGCCGTATGAATGA	451	101684	62.2
zcrhr	TTCGCTCGTGGTTACTTC	451	101684	
zgcrf	GACTCCTGAACTTCTGCT	319	567112	55.8
zgcrr	CTCTTCCTCTGACTCCAC	319	567112	
zACE2f	TTCTGCCTTCCGACTTCA	123	000114	60
zACE2r	AGACTTGCCACCTCCATT	123	000114	
nurr1f	CGGGTCGGTTTACTACAAG	111	17586.1	57
nurr1r	TGGTGGAAGTTGTGGAGAG	111	17586.1	

Table 2 — Thermal profile for qRT PCR

Stage	Condition	Time	No. of cycles	Remarks
Stage 1	95°C	5 min	1	Enzyme activation
Stage 2	94°C	30 sec		Denaturation
	Gene specific	30 sec	40	Annealing
	72°C	30 sec		Primer extension
Stage 3	95°C	30 sec		Melt curve
	60-95°C	30 sec	1	
	95°C	30 sec		

formulae. Fold Change after Electroporation with AUG ACE2 Morpholino Oligonucleotide was calculated for 96 hpf by using $2^{-\Delta\Delta Ct}$ formulae (fold change of >1.5 was considered significant). SPSS 15ver software was used to perform one-way ANOVA in order to determine the significance of difference in the studies between the Control groups and Test groups at 96 hpf. $P < 0.05$ was considered significant[29].

Results

Determination of electroporation parameters with rhodamine B

The electroporation technique was optimized to 20 V, 3 s pulse length, 5 s pulse interval, 3 pulses, and a 0.5 mM concentration of Rhodamine B. After optimizing the process, it was done on dechorionated embryos at 72 hpf and repeated on the embryos at 96 hpf with a 0.5 mM concentration of AUG ACE2 Morpholino oligonucleotide.

Figure 1 shows pink pigmentation after electroporation of Rhodamine B. Thus, we confirm that the conditions are suitable to transfer the ACE2 Morpholino oligonucleotide into the Zebrafish embryos.

Morphological Effects

The mortality was evaluated for each stage after electroporation with Rhodamine B (Tables 3 & 4). A 100% mortality rate was observed in both chorionated 24 hpf embryos as well as dechorionated 48 hpf embryos. The lowest mortality rate in Zebrafish electroporated with Rhodamine B was seen in embryos

Fig. 1 — 96 hpf embryo electroporated with Rhodamine B containing a colored spot near the heart region under a stereo zoom microscope (marked in the image)

Table 3 — Mortality Rate of Embryos Electroporated without *ACE2* AUG Morpholino oligonucleotide or Rhodamine B as control at different stages of Embryonic Development

Developmental stage	No. Embryos Electroporated without Morpholino or Rhodamine B	No. Embryos Dead	Mortality Percentage
24 hpf	50	0	0
48 hpf	50	0	0
72 hpf	50	0	0
96 hpf	50	0	0

Table 4 — Mortality Rate data of Embryos Electroporated with Rhodamine B at different stages of Embryonic Development

Developmental stage	No. Embryos Electroporated with Rhodamine B	No. Embryos Dead	Mortality Percentage
24 hpf	20 (both concentrations)	20	100
48 hpf	20 (both concentrations)	20	100
72 hpf	20 (0.5 mM)	2	20
96 hpf	20 (0.5 mM)	0	0

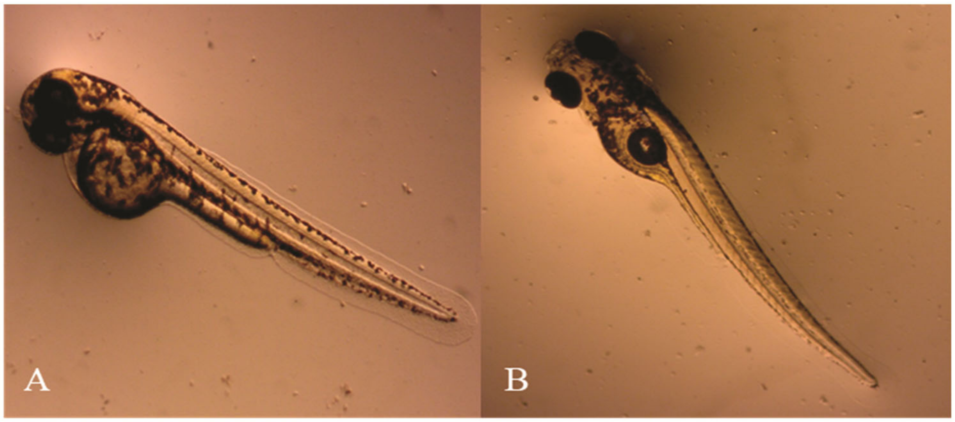

Fig. 2 — Morphological changes in Zebrafish embryo (A) Electroporated without Morpholino *vs.*; and (B) Electroporated with Morpholino at 96 hpf from a Stereo zoom microscope

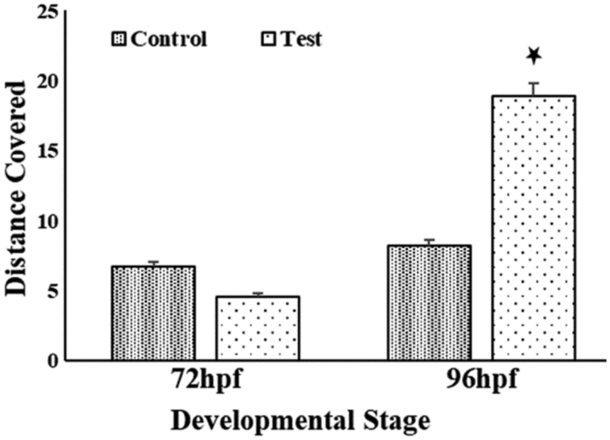

Fig. 3 — Locomotor Activity of Control group Zebrafish Embryos *vs.* Test group (Electroporated with *ACE2* AUG Morpholino oligonucleotide) Zebrafish Embryos in Developmental Stages of 72 hpf and 96 hpf , respectively. *$P < 0.05$ compared to control at 96 hpf, the bar depicts the standard error (SE)

that were induced with Rhodamine B at the 96 hpf embryonic developmental stage. There was no significant effect on morphology after electroporation with Morpholino at 96 hpf compared to controls (Fig. 2).

Locomotor changes

The normal hatching period for Zebrafish Embryos is 48 hpf, but since the mortality rate for those embryos after electroporation was 100%, the locomotor functions of the Zebrafish embryo were recorded for stages 72 hpf and 96 hpf. The data (Fig. 3) shows a 130.48% increase in locomotor activity in the 96 hpf test group of zebrafish embryos when compared to control groups in the same developmental stage, with a p-value of 0.05. The changes in locomotor activity of the control and Test groups were not found to be significant in 72-hpf embryos.

Gene expression studies

Average duplicates were taken from the Ct values for expression of ACE2, GCR, and CRH for the 24 hpf, 48 hpf, 72 hpf, and 96 hpf embryonic developmental stages of control zebrafish embryos as well as the 96 hpf stage of test zebrafish embryos. GAPDH expression as an internal control was also taken in duplicates, and the average was calculated. The ΔCt value was taken for each stage, and then the relative expression was calculated and compared.

Expression studies in controls

Using optimized QPCR conditions, all targeted mRNAs were detected during all developmental stages (Fig. 4A–D). Each of the three genes (*ACE2, GCR, CRH, and NURR1*) showed significant differences in expression over the developmental time course, as determined by ANOVA. Relative expression of each of the three genes was significantly higher in Control zebrafish embryos at 96 hpf than at the other developmental stages (Fig 4A–D).

Effects of *ACE2* AUG Morpholino antisense on gene expression

Relative Expression of ACE2 shows a significant decrease after electroporation with the ACE2 AUG Morpholino oligonucleotide, indicating that the gene has successfully been silenced and its expression has been reduced. A comparison of GCR and CRH Relative expressions between the control and Test Groups shows no significant decrease in relative expression after electroporation with the ACE2 AUG Morpholino oligonucleotide. In contrast, the relative expression of Nurr1 shows a significant decrease between the control and Test Groups in 96-hpf zebrafish embryos (Fig. 5).

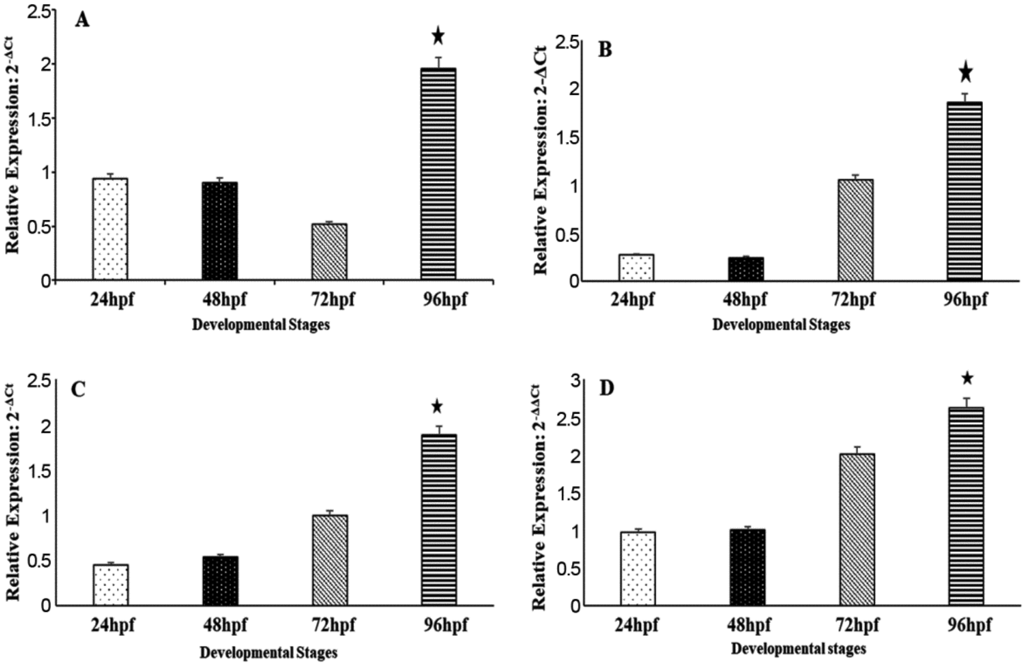

Fig. 4 — Relative expression (2-ΔCt) of (A) *ACE2*; (B) gr; (C) crh; and (D) nurr1 in Control group Zebrafish at 24 hpf, 48 hpf, 72 hpf and 96 hpf developmental stages, *$P< 0.05$ compared to all other stages, the bar depicts Standard error (SE)

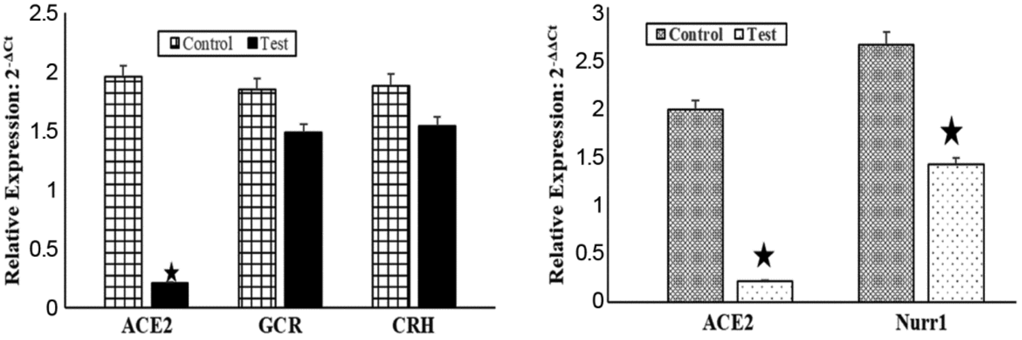

Fig. 5 — (A) Relative expression 2-ΔCt for *ACE2*, gcr and crh at 96 hpf in Control group Zebrafish Embryos *vs*. 96 hpf Zebrafish Embryos electroporated with *ACE2* AUG Morpholino oligonucleotide; and (B) Relative expression 2-ΔCt for *ACE2* and NURR1 at 96 hpf in Control group Zebrafish Embryos *vs*. 96 hpf Zebrafish Embryos electroporated with *ACE2* AUG Morpholino oligonucleotide, *$P< 0.05$ compared to control *ACE2* expression, the bar depicts Standard error (SE)

Table 5 — Fold change for *ACE2, gr, crh* and *nurr1* for 96 hpf zebrafish embryos. All the genes were ranked based on the P value. This table showed the gene expression based on (≥ 1.5 fold-change) criteria and divided into Down-regulated or not regulated. FC Fold Change

Gene	Fold change	P value	
ACE2	3.36	0.05*	Downregulated
GR	1.29	0.078	No change
CRH	1.27	0.09	No change
Nurr1	2.33	0.05*	Downregulated

Table 5 shows the relative quantity or fold change in gene expression of target genes with respect to the control group as calculated for 96 hpf samples. The modest effect of the ACE2 AUG Morpholino oligonucleotide resulted in a significant downregulation of ACE2 (3.36-fold). *NURR1* expression was down-regulated 2.33-fold, and there was no significant effect on both GCR and CRH genes.

Discussion

This study shows that the electroporation technique is an efficient technique for the uptake of antisense ACE2 AUG Morpholino oligonucleotides (morpholinos) in the zebrafish larvae in "knockdown" experiments. Optimizing electrical parameters and concentration could really be fruitful. In an attempt to demonstrate the efficacy of this approach for the analysis of a specific developmental timeframe—that of the initial development of the zebrafish Dopamine and HPA axis—we have done a systematic characterization of the efficiency of *in vivo* electroporation in zebrafish across multiple developmental stages, from 24 to 96 h postfertilization. We show that electroporation is efficient at delivering a 0.5 mM concentration of AUG ACE2 Morpholino oligonucleotide to dechorionated embryos at multiple developmental steps, including 72 or 96 hpf. The electroporation technique was optimized to 20 V, 3 s pulse length, 5 s pulse interval, 3 pulses, and a 0.5 mM concentration of AUG ACE2 Morpholino oligonucleotide. Previous work showed the possibility of transfecting zebrafish embryos with plasmid DNA by using this technique. To our knowledge, the results presented here are the first to introduce AUG ACE2 Morpholino oligonucleotide in zebrafish embryonic tissues using electroporation. This technique, compared with traditional microinjection, can be particularly advantageous for aquatic fish species with fast embryonic development and a predictable spawning habit. In addition, chorion removal is found to be necessary for the access of plasmid DNA[30].

Results of RT-PCR experiments confirm that *ACE2 expression* was significantly higher in Control zebrafish embryos at 96 hpf than at other developmental stages. This result is indicative of the development of the RAAS system as the embryonic developmental stages of the Control Zebrafish embryos progress and the evidence of expression of ACE2 in the embryonic development as early as 96 hpf. Early development of the RAAS system is also seen in human embryonic stages[31].

Expression of *ACE2* was reduced during zebrafish development by introducing *ACE2*-targeting morpholino oligonucleotides into 72-hpf embryos. Lack of functional *ACE2* resulted in a significant effect on behavioral function (increased locomotor activity), but no morphological defects were detected. The increased levels of locomotor activities are attributed to the increased stress and anxiety levels in zebrafish larvae[32]. These locomotor activities depend on the integrity of brain function, nervous system development, and visual pathways[33].

Stressors ranging from mild to severe will stimulate the hypothalamic-pituitary interrenal (HPI) axis in teleost fish[34], which is homologous to the mammalian hypothalamic-pituitary-adrenal (HPA) axis[35]. In our study, the effects of silencing the *ACE2* gene on the HPA axis were analyzed through the quantification of CRH and GR gene expression by RT-PCR, and we found no differences at 96 hpf. This may be because in our experiment, exposure and sampling occurred prior to maturation of the stress (HPI) axis[36].

To identify the effects of ACE2 silencing on DA neurons within the early zebrafish brain, this study assessed the expression of one marker: NURR1. The decreased expression of NURR1 following ACE2 knockdown is particularly intriguing, considering recent clinical studies implicating human NURR1 as a risk factor for neurodevelopmental disorders. This finding is supported by studies in which animals with genetic alterations in ACE2 expression exhibited a distinct pattern of phenotypes, including behavioral dysfunctions, impairments in dopamine synthesis, and neurogenesis[37]. Low levels of dopamine are associated with depression and anxiety[38].

Conclusion

Given the recent publications suggesting that the binding of SARS-CoV-2 with ACE2 and the subsequent downregulation of ACE2 expression in cell membranes may increase SARS-CoV-2 pathogenesis, developing an *in vivo* animal model of ACE2 takes on added importance. The results of the morpholino experiments are the first knockdown phenotypes reported for zebrafish ACE2. One of our major findings, decreased dopamine neurogenesis following *in vivo* ACE2 knockdown, has implications for depression and anxiety. Thus, this study substantiates the biological relationship between the SARS-CoV-2-induced downregulation of ACE2 and the prevalence of depression and anxiety among patients with COVID-19. However, this study acts as an intriguing gateway into the further research required on the modulatory effects of ACE2. If anything, the pandemic is a trigger for the importance of ACE2 and its relationship with the brain and its functions.

Acknowledgement

The authors would like to thank Sathyabama Institute of Science and Technology for providing the opportunity to carry out this research work.

Conflict of interest

All authors declare no conflict of interest.

References

1 Alipoor SD & Mirsaeidi M, SARS-CoV-2 cell entry beyond the *ACE2* receptor. *Mol Biol Rep*, 49 (2022) 10715.

2 Chaudhary S, Rai P, Sesham K, Kumar S, Singh P, Nag TC, Chaudhuri P, Trikha A & Yadav SC, Microscopic imaging of bronchoalveolar fluids of COVID-19 positive intubated patients reveals the different level of SARS-CoV-2 infection on oral squamosal epithelial cells. *Indian J Biochem Biophys*, 58 (2021)

3 Kabbani, Nadine & James L, Olds, Does COVID-19 infect the brain? If so, smokers might be at a higher risk. *Mol Pharmacol*, 97 (2020) 351.

4 Meng-Yuan L, Li L, Zhang Y & Xiao-Sheng W, Expression of the SARS-CoV-2 cell receptor gene *ACE2* in a wide variety of human tissues. *Infect Dis Poverty*, 9 (2020) 23.

5 Li Yan-Chao, Bai Wan-Zhu & Hashikawa T, The neuroinvasive potential of SARS-CoV-2 may play a role in the respiratory failure of COVID-19 patients. *J Med Virol*, 92 (2020) 552.

6 Zhou L, Xu Z, Castiglione GM, Soiberman US, Eberhart CG & Duh EJ, *ACE2* and TMPRSS2 are expressed on the human ocular surface, suggesting susceptibility to SARS-CoV-2 infection. *Ocul Surf*, 18 (2020) 537.

7 Zubair AS, McAlpine LS, Gardin T, Farhadian S, Kuruvilla DE & Spudich S, Neuropathogenesis and neurologic manifestations of the coronaviruses in the age of coronavirus disease 2019: a review. *JAMA Neurol*, 77 (2020) 1018.

8 Ribeiro VT, de Souza LC & Simões e Silva AC, Renin-angiotensin system and Alzheimer's disease pathophysiology: from the potential interactions to therapeutic perspectives. *Protein Pept Lett*, 27 (2020) 484.

9 Angelopoulou E, Karlafti E, Georgakopoulou VE, Papalexis P, Papageorgiou SG, Tegos T & Savopoulos C, Exploring the Role of *ACE2* as a Connecting Link between COVID-19 and Parkinson's Disease. *Life*, 13 (2023) 536.

10 Chen F, Zhou L, Bai Y, Zhou R & Chen L, Hypothalamic-pituitary-adrenal axis hyperactivity accounts for anxiety-and depression-like behaviors in rats perinatally exposed to bisphenol A. *J Biomed Res*, 29 (2015) 250.

11 Hinds JA & Sanchez ER, The Role of the Hypothalamus–Pituitary–Adrenal (HPA) Axis in Test-Induced Anxiety: Assessments, Physiological Responses, and Molecular Details. *Stresses*, 2 (2022) 146.

12 Smith SM & Vale WW, The role of the hypothalamic-pituitary-adrenal axis in neuroendocrine responses to stress. *Dialogues Clin Neurosci*, 8 (2022) 383.

13 Alenina N & Bader M, *ACE2* in brain physiology and pathophysiology: evidence from transgenic animal models. *Neurochem Res*, 44 (2019) 1323.

14 Lanza K, Dias R, Oliveira RMDS, Belotto L, Miranda AS & Simões e Silva AC, Renin-Angiotensin System in Central Nervous System Diseases and its Interaction with COVID-19. *Curr Med Chem*, 28 (2021) 5733.

15 Kareklas K, Teles MC, Nunes AR & Oliveira RF, Social zebrafish: Danio rerio as an emerging model in social neuroendocrinology. *J Neuroendocrinol*, (2023) 1.

16 Fitzgerald JA, Könemann S, Krümpelmann L, Županič A & Berg CV, Approaches to test the neurotoxicity of environmental contaminants in the zebrafish model: from behavior to molecular mechanisms. *Environ Toxicol Chem*, 40 (2021) 989.

17 Doyle JM & Croll RP, A Critical Review of Zebrafish Models of Parkinson's Disease. *Front Pharmacol*, 13 (2022).

18 Wasel O & Freeman JL, Chemical and genetic zebrafish models to define mechanisms of and treatments for dopaminergic neurodegeneration. *Int J Mol Sci*, 21 (2020) 5981.

19 Fontaine R, Affaticati P, Bureau C, Colin I, Demarque M, Dufour S, Vernier P, Yamamoto K & Pasqualini C, Dopaminergic neurons controlling anterior pituitary functions: anatomy and ontogenesis in zebrafish. *Endocrinology*, 156 (2015) 2934.

20 Choi SSA, Chan HH, Chan CM, Wang X, Webb SE, Leung KW, Tsim KWK & Miller AL, Neuromasts and Olfactory Organs of Zebrafish Larvae Represent Possible Sites of SARS-CoV-2 Pseudovirus Host Cell Entry. *J Virol*, 96 (2022) 01418.

21 Mesman S & Smidt MP, Acquisition of the midbrain dopaminergic neuronal identity. *Int J Mol Sci*, 21 (2020) 4638.

22 Ghaddar B, Lübke L, Couret D, Rastegar S & Diotel N, Cellular mechanisms participating in brain repair of adult zebrafish and ma mMals after injury. *Cells*, 10 (2021) 391.

23 Westerfield M, The zebrafish: a guide for the laboratory use of zebrafish (Brachydanio reriro). *Instiute of Neuroscience, University of Oregon*, (1993).

24 Thomas JL, Electroporation, an alternative to biolistics for transfection of Bombyx mori embryos and larval tissues. *J Insect Sci*, 3 (2003) 17.

25 Zhang C, Ren Z & Gong Z, Transgenic expression and genome editing by electroporation of zebrafish embryos. *Marine Biotechnol, 22* (2020) 644.

26 Kaneko T, Reproductive technologies for the generation and maintenance of valuable animal strains. *J Reprod Develop*, 64 (2018) 209.

27 Behra M, Cousin X, Bertrand C, Vonesch JL, Biellmann D, Chatonnet A & Strähle U, Acetylcholinesterase is required for neuronal and muscular development in the zebrafish embryo. *Nat Neurosci*, 5 (2002) 111.

28 Schmittgen TD & Livak KJ, Analyzing real-time PCR data by the comparative CT method. *Nat Protocols*, 3 (2008)1101.

29 Tippabathani J & Nellore J, Temporal changes in key developmental transcription factors in dopamine neurons during MPP$^+$ induced injury and recovery in zebrafish brain. *Indian J Biochem Biophys*, 58 (2021) 45.

30 Zhang C, Ren Z & Gong Z, Transgenic expression and genome editing by electroporation of zebrafish embryos. *Marine Biotechnol*, 22 (2020) 644.

31 Tang J, Liu B, Li N, Zhang M, Li X, Gao Q, Zhou X, Sun M, Xu Z & Lu X, Development of Renin-Angiotensin-Aldosterone and Nitric Oxide System in the Fetus and Neonate. *J Matern -Fetal Neonatal Med*, (Academic Press) 2020, 643.

32 Basnet RM, Zizioli D, Taweedet S, Finazzi D & Memo M, Zebrafish larvae as a behavioral model in neuropharmacology. *Biomedicines*, 7 (2019) 23.

33 Atzei A, Jense I, Zwart EP, Legradi J, Venhuis BJ, van der Ven LTM, Heusinkveld HJ & Hessel EVS, Developmental neurotoxicity of environmentally relevant pharmaceuticals and mixtures thereof in a zebrafish embryo behavioural test. *Int J Environ Res Public Health*, 18 (2021) 6717.

34 Guo H & Dixon B, Understanding acute stress-mediated immunity in teleost fish. *Fish Shellfish Immunol Rep*, 2 (2021) 100010.

35 Norris DO & Hobbs SL, The HPA axis and functions of corticosteroids in fishes. *Fish Endocrinol*, (CRC Press) 2020, 721.

36 Wei P, Zhao F, Zhang X & Ru S, Long-term exposure of zebrafish to bisphenol S impairs stress function of hypothalamic-pituitary-interrenal axis and causes anxiety-like behavioral responses to novelty. *Sci Total Environ*, 716 (2020) 137092.

37 Okechukwu CE, The downregulation of angiotensin-converting enzyme 2 expression may be associated with depression and anxiety among patients with severe acute respiratory syndrome Coronavirus 2. *Curr Med Issue*, 19 (2021) 218.

38 Attademo L & Bernardini F, Are dopamine and serotonin involved in COVID-19 pathophysiology? *Eur J Psychiatr*, 35 (2021) 62.