Tyrosine Hydroxylase-Green Fluorescence Protein Transgenic Zebrafish as A Biosensor and Animal Model for Nicotine and Ketamine Drug Effects

SUEN Fung Ki

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Philosophy

Principal Supervisor: Prof. K.K.L. Yung

Hong Kong Baptist University

August 2012
Abstract

Zebrafish has become a common vertebrate model for study of neurogenesis and neurodevelopment. The transparent rapid development and more close relationship to humans than invertebrate models were the leading reasons for using them in neurological research. Recently, zebrafish has been employed as model to study neurological disorders of addictive drugs by analyzing behavior, morphological and neuroregulatory systems. Taking the advantage of transparent body, transfection of green fluorescent protein (GFP) in zebrafish is developed and widely used to label endogenous protein, cells, organs or even organelles.

In the present study, green fluorescent plasmid constructs were produced containing the promoter of tyrosine hydroxylase (TH; a key synthetic enzyme for catecholamines) and GFP. The constructs were microinjected into zebrafish embryonic cells during the one-cell stage. At 3 days post-fertilization (dpf), GFP started to express in olfactory bulb (OB), telencephalon (Tel), posterior tuberculum (TPp), pretectal area (PPv) and periventricular hypothalamus (PTN) of zebrafish. The present results were confirmed by TH immunohistochemical staining and 6-hydroxydopamine (6-OHDA) challenge in the zebrafish with the same developmental stages. This transgenic fish model provided a novel drug response model which can also be used for studying neurological disorders relating to catecholamines in the nervous system.

Nicotine and ketamine used as a drug in present study to alter intrinsic TH level in zebrafish brain. They had different pharmacological mechanisms that inducing stimulative effects by binding to distinct receptor which further activating the synthesis and release of dopamine. First, locomotion assay was examined to study the general excitatory effects of nicotine and ketamine. Locomotion activities were markedly elevated in a wide range of nicotine concentrations and low doses of ketamine treatment. Since increased locomotion activity was due to activation of dopamine release and excitatory synaptic transmission, it implied that TH level was elevated followed by increase of locomotion activity. Second, TH protein level was assessed in Western blot analysis. Same as the above results, TH protein levels were significantly increased followed by a rising concentrations of nicotine and low doses of ketamine treatments. Finally, TH expression was examined in prior established transgenic zebrafish model. Surprisingly, the trend of TH induction was similar to the results in western blotting.
Based on the parallel results in drug response, TH-GFP transgenic zebrafish model is reliable and useful for expressing intrinsic TH level in a more effective way. The effective transgenic model prevents abundant processes in other experimental assays. TH-GFP transgenic zebrafish, as a novel high throughput sensing model, is highly recommended to be used in drug testing.
# Table of Contents

Declartion i
Abstract ii
Acknowledgement iv
Table of Contents v
List of Figures x
List of Abbreviation xiii

## Chapter 1 Literature review 1

1.1 Zebrafish 1
   1.1.1 Introduction 1
   1.1.2 Dopamine 2
   1.1.3 Tyrosine hydroxylase (TH) 3
   1.1.4 Distribution of TH in zebrafish 4
   1.1.5 6-hydroxydopamine (6-OHDA) 6

1.2 Nicotine 8
   1.2.1 Introduction 8
   1.2.2 Structure and Functions 9
      1.2.2.1 Structure 9
      1.2.2.2 Clinical usages 10
         1.2.2.2.1 Nicotine dependence drug 10
         1.2.2.2.2 Antipsychotic drug 10
      1.2.2.3 Physical usage 11
         1.2.2.3.1 Stimulative drug 11
   1.2.3 Pharmacological mechanism 11
      1.2.3.1 A nicotinic acetylcholine (nACh) receptor antagonist 11
   1.2.4 Nicotine effects in humans 13
   1.2.5 Nicotine effects in animals 14

1.3 Ketamine 16
   1.3.1 Introduction 16
   1.3.2 Structure and Functions 17
      1.3.2.1 Structure 17
      1.3.2.2 Clinical usages 18
         1.3.2.2.1 Anesthetic drug 18
         1.3.2.2.2 Anti-depression drug 18
1.3.2.3 Anti-addiction drug 19
1.3.2.3 Illicit abuse 19
1.3.3 Pharmacological mechanism 20
1.3.3.1 A non-competitive NMDA receptor antagonist 20
1.3.3.2 Other receptors agonist 21
1.3.3.3 Other receptors antagonist 21
1.3.4 Ketamine effects in humans 22
1.3.5 Ketamine effects in animals 23

1.4 Objectives of the thesis 25
1.4.1 Production of TH-GFP transgenic zebrafish 25
1.4.2 Acute nicotine treatment in larval zebrafish 25
1.4.3 Acute ketamine treatment in larval zebrafish 26

Chapter 2 Methodology and materials 27
2.1 Animal care and maintenance 27
2.2 Primers design of TH sequence 27
2.3 Polymerase chain reaction (PCR) assay 28
2.4 Electrophoresis 29
2.5 Sequencing 29
2.6 Preparation of plasmid DNA 29
2.7 Bacterial culture 30
2.8 Microinjection 31
2.9 Whole-mount antibody immunofluorescence 32
2.9.1 Single immunofluorescence 32
2.9.2 Double immunofluorescence 33
2.10 6-OHDA treatment 34
2.11 Nicotine and ketamine treatment 34
2.12 Locomotion assay 35
2.13 Western blot analysis 36
2.13.1 Protein extraction 36
2.13.2 Protein quantification and sodium dodecyl sulfate-Polyacrylamide gel electrophoresis (SDS-PAGE) 36
2.13.3 Immunoblotting 37
2.13.4 Stripping 38
2.13.5 Semi-quantitative analysis of Western blot results 38
2.14 Microscopy and imaging 39
2.15 Statistical analysis 39
# Chapter 3 Construction of TH-GFP plasmid

3.1 Introduction 40
3.2 Objectives 42
3.3 Materials and Methods 43
   3.3.1 Primers design of TH1 sequence 43
   3.3.2 PCR assay 43
   3.3.3 Electrophoresis 43
   3.3.4 Sequencing 44
   3.3.5 Preparation of plasmid DNA 44
   3.3.6 Bacterial culture 45
   3.3.7 Microinjection 45
   3.3.8 Whole-mount antibody immunofluorescence 45
   3.3.9 6-OHDA treatment 46
3.4 Results 47
   3.4.1 Cloning of TH1 sequence 47
   3.4.2 Construction of TH-GFP plasmid 47
   3.4.3 Production of transgenic TH-GFP zebrafish 49
   3.4.4 6-OHDA reduced TH expression 51
   3.4.5 Survival differences of transgenic and non-transgenic zebrafish 51
3.5 Discussion 53
   3.5.1 Cloning of TH1 sequence 53
   3.5.2 Autofluorescence of zebrafish 54
   3.5.3 TH-GFP expression in transgenic zebrafish 54
   3.5.4 6-OHDA treatment confirmed the successful transfection of TH-GFP plasmid 56
   3.5.5 Survival difference of transgenic and non-transgenic zebrafish 56
   3.5.6 Transfection efficiency 57
3.6 Conclusion 80

# Chapter 4 Acute nicotine treatments in larval zebrafish

4.1 Introduction 81
4.2 Objectives 83
4.3 Materials and Methods 84
   4.3.1 Nicotine treatment 84
   4.3.2 Locomotion assay 84
   4.3.3 Western blot analysis 85
4.4 Results 86
   4.4.1 Acute nicotine treatments induced aberrant swimming pattern 86
4.4.2 Acute nicotine treatments enhanced locomotion activity dose dependently 87
4.4.3 Acute nicotine treatments increased TH protein level dose dependently 88
4.4.4 Acute nicotine treatments increased TH-GFP expression in transgenic zebrafish dose dependently 88

4.5 Discussion 90
4.5.1 Choice of 5dpf zebrafish in locomotion assay 90
4.5.2 Acute nicotine treatments induced aberrant swimming pattern 90
4.5.3 Enhancement of locomotion activity by nicotine is dose dependent 92
4.5.4 Increase of TH protein level by nicotine is dose dependent 93
4.5.5 Increase of TH-GFP expression in transgenic zebrafish by nicotine is dose dependent 94

4.6 Conclusion 106

Chapter 5 Acute ketamine treatments in larval zebrafish 107
5.1 Introduction 107
5.2 Objectives 109
5.3 Materials and Methods 110
5.3.1 Ketamine treatment 110
5.3.2 Locomotion assay 110
5.3.3 Western blot analysis 111
5.4 Results 112
5.4.1 Acute ketamine treatments induced aberrant swimming pattern 112
5.4.2 Acute ketamine treatments changed locomotion activity 113
5.4.3 Acute ketamine treatments changed TH protein level 114
5.4.4 Acute ketamine treatments changed TH-GFP expression in transgenic zebrafish 114

5.5 Discussion 116
5.5.1 Acute ketamine treatments induced aberrant swimming pattern 116
5.5.2 Acute ketamine treatments changed locomotion activity 117
5.5.3 Acute ketamine treatments changed TH protein level 118
5.5.4 Acute ketamine treatments changed TH-GFP expression in transgenic zebrafish 119

5.6 Conclusion 131
Chapter 6 Summary and Conclusion 132

List of References 138
Appendix I 160
Appendix II 162
Appendix III 163
Appendix IV 164
Curriculum Vitae 168