ประโยชน์ของการใช้ยีนสารพิษของโรคคอตีบ ในการวิจัยทางประสาทศาสตร์

พูลพล ผดุงชัยโชติ

บทคัดย่อ

ในช่วงทศวรรษที่ผ่านมา เทคโนโลยีที่ก้าวหน้าด้านชีววิทยาระดับโมเลกุลได้ช่วยให้นักวิจัยพัฒนาการจัดลำดับสารพันธุกรรมในสิ่งมีชีวิตหลายชนิด ความสำเร็จที่สำคัญนี้ช่วยให้เข้าใจการทำงานของโปรตีนซึ่งสะท้อนบทบาทของยีนในสิ่งมีชีวิตเหล่านั้น การวิจัยทางพันธุวิศวกรรมในสัตว์ โดยเฉพาะในหนูทดลอง (mice) ทำให้สามารถตรวจสอบยีนจำเพาะที่สนใจเพื่อศึกษาค้นคว้าเกี่ยวกับโรคทางพันธุกรรมทั้งหลาย รวมทั้งการทำงานที่ผิดปกติของระบบประสาทในมนุษย์ การผลิตหนูทดลองที่ขาดยีนจำเพาะซึ่งควบคุมการทำงานของระบบประสาท มีประโยชน์ในการศึกษาการทำงานของระบบประสาทและการแสดงออกของพฤติกรรม ดังเช่นการผลิตหนูทดลองที่แสดงอาการของโรคทางระบบประสาทแบบ extrapyramidal โดยวิธีการใส่ยีนสารพิษของโรคคอตีบเข้าไปในตำแหน่งที่ควบคุมการสร้างเซลล์ประสาทที่มีตัวรับของสารโดปามีน D1 ในยีนของหนู ก่อให้เกิดประโยชน์อย่างมหาศาลในการศึกษาบทบาทของตัวรับของสารโดปามีน D1 และการแสดงออกของพฤติกรรมที่เกี่ยวข้องกับสารโดปามีน

คำสำคัญ: ยีนสารพิษของโรคคอตีบ, สารโดปามีน, พันธุวิศวกรรม

ภาควิชากายวิภาคศาสตร์ คณะแพทยศาสตร์ มหาวิทยาลัยศรีนครินทรวิโรฒ

*ติดต่อได้ที่ poolpolp@hotmail.com, โทรศัพท์ 0 2260 2233 ต่อ 4506, โทรสาร 0 2260 1532

The advantages of employing a diphtheria toxin gene in Neuroscience research

Poolpol Padungchaichot

Abstract

In the last decade, advanced technologies in molecular biology have allowed researchers to modify the genetic composition of many organisms. These progress achievements have greatly enhanced our understanding of the protein functions that mirror the role of genes in organisms. Further experiments in genetic engineering in animals, particularly in mice, have explored genes of interest in order to investigate genetic diseases, including neurophysiological defects in humans. The generation of mice, which lack the particular gene of interest that controls the function of the nervous system, has been useful in studying neurophysiology and behaviour. The recent generating a murine model of extrapyramidal disease by the targeted expression of a diphtheria toxin gene to D1-dopamine (DA) receptor positive neurons offers a powerful opportunity to evaluate the roles of these receptors in DA-mediated behaviors.

Key words: diphtheria toxin gene, dopamine, genetic engineering

Department of Anatomy. Faculty of Medicine. Srinakharinwirot University

*Correspondence to poolpolp@hotmail.com, Tel. 0 2260 2233 ext. 4506, Fax. 0 2260 1532

Review Article

Introduction

Neurodegenerative diseases involving the central nervous system (CNS) dopaminergic system are common in neurological practice. The neurotransmitter dopamine (DA) is thought to play a critical role in a host of physiological neural processes such as motor control, cognition, emotion, reward, and neuroendocrine and cardiovascular regulation[1]. Abnormal dopaminergic neurotransmission has been implicated in several pathological conditions such as schizophrenia[2], Parkinson's disease[3], Gilles de la Tourette's syndrome[4], Huntington's disease (HD)[5], and in movement disorders such as tardive dyskinesia which are known to complicate chronic neuroleptic treatment[6,7].

Idiopathic Parkinson's disease (IPD) is defined by loss of nigrostriatal dopaminergic neurons resulting in the presynaptic breakdown of dopaminergic transmission. Although the brain changes seen early in neurodegenerative diseases may be specific and limited to defined neuronal populations, the changes become widespread with disease progression and ultimately may involve distant neuronal populations. In HD for example, the cell loss is confined to enkephalin positive striatopallidal spiny projection neurons early in the disease but eventually there is marked atrophy involving the entire cortex and basal ganglia. Two hypotheses can be used to account for the ultimate widespread cell loss in neurodegenerative Parkinsonian syndromes (NPS) and HD. The first suggested that a defined insult directly causes the death of all cells, albeit at a different rate, while the second views the primary damage as limited to a small number of neurons with the later extensive destruction representing a transsynaptic or cascade effect. The tools are now available to establish a model of genetically regulated striatal cell death, and the latter can be used to explore the mechanism of secondary or transsynaptic damage within the CNS. Such a model may be useful in understanding the mechanisms of CNS neurodegeneration.

Toxin-mediated targeted cell ablation *in vivo*

An advanced step in transgenic and gene-targeting technology is the availability of cell-specific promoters that can direct expression of toxic products to defined cell populations. The achievement of inserting genes encoding toxic products, which can destroy specific cell populations, directly into the genome by a homologous recombination strategy in embryonic stem (ES) cells, has increased the understanding of cell-cell interactions during embryonic development, and in mature animals. Common toxic mechanisms, which have been used in a large number of cell ablation paradigms, include the Diphtheria toxin protein or Herpes simplex virus-1 thymidine kinase (*tk*), in the presence of nucleoside analogues that inhibit DNA replication.

Gene directed enzyme prodrug paradigms involve the tissue-specific expression of a gene encoding an enzyme that converts an inactive prodrug into a cell toxin. These paradigms have been widely used to establish models of human diseases and to study cell-cell interactions *in vivo*. The enzyme is harmless to cells when innocuous to tissues. It can, however, convert the prodrug into an active toxin which becomes an efficient cell killer, but one that is nevertheless, ineffective on neighbouring cells. Borrelli *et al.*[8] first described the thymidine kinase cell obliteration system and later generated transgenic mice carrying the *tk* gene under the control of the rat growth hormone promoter[9]. Transgenic mice treated with the prodrug, 1-(2-deoxy-2-fluoro-beta-D-arabino-furanosyl)-5-iodouracil (FIAU), were growth retarded and free of both growth hormone and prolactin producing cells in the anterior pituitary gland since FIAU is believed to result in cell death by serving as a defective nucleotide analogue which is incorporated into cellular DNA during replication causing chain termination. However, the theoretical lack of toxicity of prodrug phosphorylation products in established post-mitotic cells expressing the *tk* transgene, and the problem of sterility in transgenic males[10-12] have limitated the widespread applicability of the *tk* transgene approach.

Cell ablation by targeted expression of the diphtheria toxin

The wild-type diphtheria toxin is composed of two protein subunits, A and B. The B subunit is required for movement of subunit A across plasma membranes. The use of only the A subunit restricts the activity of the toxin to the cells in which it is synthesized since, without the B chain, it cannot enter neighboring cells. Many investigators have exploited the transgenic approach to direct the expression of the diphtheria toxin-A chain (DTA) gene using tissue specific regulatory regions[13-16] and have shown that specific cell ablation during development can be a powerful tool for exploring the role played by cell-cell interactions in tissue formation. The DTA gene encodes an adenosine diphosphate (ADP) ribosyltransferase that catalyses the ADP-ribosylation of Elongation Factor-2[17]. This alteration results in an inhibition of protein synthesis and subsequent cell death[14].

The attenuated form of the DTA gene (*tox-176*) has been estimated to be 30-fold less potent than wild-type DTA gene[18] making it necessary for this protein to be produced at high levels to kill cells in which it is expressed. Several transgenic studies have documented that cells expressing low levels of the gene are able to survive albeit with impaired function[15,19-21]. Ross *et al.*[21] generated transgenic mice that demonstrated the reduction of adiposity via the targeted expression of *tox-176* to adipose tissue, using the adipocyte P2 promoter. Those transgenic mice with high levels of toxin expression developed chylous

ascites and died soon after birth while those expressing lower levels of the transgene, had a normal amount of adipose tissue and survived to adulthood; however, they showed a complete resistance to chemically induced obesity suggesting that the *tox-176* gene product was able to specifically perturb cell function without inevitable cell death.

The *Cre/Lox* recombination system

Cre is a member of the integrase family (*Int*) of recombinases[22] and has been shown to perform efficient recombination in specific recognition sequences called LoxP sites in eukaryotic cells[23]. The 34 bp LoxP site consists of two 13 bp inverted repeats, binding sites for the *Cre* protein, and an 8 bp asymmetric core region where recombination occurs, and which is responsible for the directionality of the site[23]. The *Cre* recombinase introduced as a transgene can efficiently excise DNA bracketed by LoxP sites from the chromosome when LoxP sites are oriented in the same direction[24].

The *Cre/LoxP* recombination system[23-26] can be exploited to avoid the need to generate new transgenics for analysis in cases where transgene expression has devastating consequencies on survival or fertility of the animals. The *Cre/LoxP* recombination system permits the targeted deletion of a gene from a specific cell type at a predetermined stage of development. In this approach, conventional targeting techniques are employed to replace the normal gene by one in which a functionally important exon is flanked by LoxP sites so that *Cre*-mediated recombination directed by a specific promoter results in gene inactivation.

Murine model of extrapyramidal disease by the targeted expression of a diphtheria toxin gene to D1-dopamine receptor positive neurons

In recent years, five subtypes of DA receptors have been cloned[27-33]. These five subtypes are subdivided into two classes, referred to as the D1-like subclass (D1 and D5, also called $D1_A$ and $D1_B$ respectively in the rodent system), and the D2-like subclass (D2, D3, and D4)[1,29]. Despite this remarkable diversity, the pharmacological and functional properties of the five receptor subtypes have confirmed the initial hypothesis that DA receptors can either stimulate (D1 and D5) or inhibit (D2, D3 and D4) adenylyl cyclase activity through binding to specific G-proteins[34].

In the study by Drago *et al.*[35], the *Cre/Lox* technology was used to create mice with impaired protein synthesis and therefore death of D1 dopamine receptor (D1R) positive population. A translationally silenced single copy of the attenuated DTA (*tox-176*)[18] was inserted into the D1R gene

locus by the method of homologous recombination in ES cells. Chimeric mice were derived by injection of targeted ES cells into host blastocysts. Breeding of male chimeras with CD1 strain females generated heterozygotes. In heterozygotes, only striatal neurons expressing the D1R would be potential primary targets of the *tox-176* protein. The generation of a mouse line in which the inserted *tox-176* gene is functionally inactivated, and its subsequent expression, involved a strategy similar to one which had been shown to work in the lens[24]. The NEO cassette, required for antibiotic selection of targeted ES cell clones, together with the DNA sequence known to inhibit downstream translation (STOP), was inserted at 5′ end of the *tox-176* initiation codon. The NEO/STOP cassette was flanked by LoxP sites[23-24,36]. Mutant mice producing the *tox-176* gene product selectively in D1R positive cells were generated by mating transgenic mice homozygous for the *Cre* transgene[36] with phenotypically normal knockin mice carrying the dormant *tox-176* gene[35]. As the *Cre* transgene in this experiment was under the control of the Adenovirus *EIIa* promoter, *Cre* expression occurred in very early embryogenesis.

The movement disorder displayed by the *tox-176* mutant mice is dramatic and consists of bradykinesia, dystonia, myoclonus, and postural instability[35]. Bradykinesia, dystonia, and postural instability are seen in untreated idiopathic Parkinson's disease and Parkinsonian syndromes, but myoclonus is more typical of the spectrum of hyperkinetic involuntary movements seen in HD[37] and only rarely seen in neurodegenerative Parkinsonian syndromes[38].

The death of *tox-176* mutant mice was attributed to a combination of hypoxia and malnutrition. The eating disorder was consistent with the role played by the D1R in reward pathways[39-40] and the respiratory abnormality suggestive of a major role of DA in central respiratory control. Although brainstem D2 dopamine receptors (D2Rs) are thought to be of prime importance in the control of respiration[41], the periodic breathing seen in mutant mice is not surprising given the expression of D1R in the brainstem during embryogenesis[42]. The neuropathological changes of apoptosis and reactive gliosis described in this study[35], although characteristic of human neurodegenerative CNS disease[43-44], are unique in a transgenic model.

Brain DA receptor and neuropeptide expression pattern is complex in the CNS. Based on the dual pathway model of basal ganglia circuitry[45-46], which proposes D1R and D2R segregation[47], *tox-176* mutant mice lack D1R-positive striatonigral projection neurons but have an intact D2R-positive striatopallidal pathway. In addition, the neuropeptides substance P and dynorphin (DYN) in the mutant brain were absent with preservation of enkephalin (ENK) expression.

The advantages of a toxin gene targeting strategy

There are several advantages in using a toxin gene targeting strategy as described. Firstly, the nontargeted D1R allele would be predicted to functionally compensate in heterozygous knockout animals containing the dormant *tox-176* gene allowing these mice to have a normal phenotype. Secondly, DNA excision by the *Cre* recombinase enzyme would result in the *tox-176* gene replacing the D1R gene-coding region. All potential regulatory elements at this locus, both 5′ and 3′ to the coding region, would remain undisturbed. Given that the *EIIa* promoter is activated at the fertilized oocyte stage of development, the *Cre* enzyme should excise the NEO/STOP sequence from the genome in cells that would later contribute to all somatic tissues. The exact time and place of *tox-176* expression would, however, be dictated by the surrounding D1 regulatory regions. A major advantage of this approach is that it makes no assumptions about the chromosomal location of the D1 regulatory elements. The *tox-176* gene expression would therefore be expected to mirror the distribution of the dopamine D1 gene transcripts. This is a very important point as a D1-specific promoter proven to work in transgenic mice does not exist. Thirdly, the *tox-176* gene product is substantially less active than the wild type protein and there is also evidence that, its expression, although interfering with cell functioning, is not invariably associated with cell death[15,21]. The fact that the *tox-176* gene is present in a single copy would also be an advantage. This is important, as low level D1 expression occurs in renal proximal convoluted tubules[48], and may also occur in murine parathyroids[49]. Clearly, the value of the animals as a model of neurodegenerative extrapyramidal disease would be greatest if there were chronic low-level injury to the striatal neurons without the presence of metabolic problems (i.e. due to renal or parathyroid diseases) to complicate the analysis. Finally, this approach allows one to establish and expand a dormant heterozygote line. *Tox-176* expressing mice required for analysis could thereafter be produced by timed matings.

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