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Genetic approaches to the investigation of serotonergic neuron functions in animals

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The serotonergic system is one of the most important neurotransmitter systems that take part in the regulation of vital CNS functions. The understanding of its mechanisms will help scientists create new therapeutic approaches to the treatment of mental and neurodegenerative diseases and find out how this neurotransmitter system interacts with other parts of the brain and regulates their activity. Since the serotonergic system anatomy and functionality are heterogeneous and complex, the best tools for studying them are based on manipulation of individual types of neurons without affecting neurons of other neurotransmitter systems. The selective cell control is possible due to the genetic determinism of their functions. Proteins that determine the uniqueness of the cell type are expressed under the regulation of cell-specific promoters. By using promoters that are specific for genes of the serotonin system, one can control the expression of a gene of interest in serotonergic neurons. Here we review approaches based on such promoters. The genetic models to be discussed in the article have already shed the light on the role of the serotonergic system in modulating behavior and processing sensory information. In particular, genetic knockouts of serotonin genes *sert*, *pet1*, and *tph2* promoted the determination of their contribution to the development and functioning of the brain. In addition, the review describes inducible models that allow gene expression to be controlled at various developmental stages. Finally, the application of these genetic approaches in optogenetics and chemogenetics provided a new resource for studying the functions, discharge activity, and signal transduction of serotonergic neurons. Nevertheless, the advantages and limitations of the discussed genetic approaches should be taken into consideration in the course of creating models of pathological conditions and developing pharmacological treatments for their correction.

Key words: serotonergic neurons; genetic models; viral transduction; optogenetics; chemogenetics.

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Генетические подходы к изучению функций серотонинергических нейронов у животных

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Серотонинергическая система, которая принимает участие в регуляции большинства функций ЦНС, является одной из важнейших нейротрансмиттерных систем. Патогенез многих психических и нейродегенеративных заболеваний включает нарушения в функционировании этой системы. Понимание механизмов ее работы поможет не только разработать новые терапевтические подходы к лечению, но и установить, как эта нейротрансмиттерная система взаимодействует с другими отделами мозга, регулируя их деятельность. Ввиду сложности и гетерогенности анатомо-функционального устройства серотонинергической системы, в настоящее время лучшими инструментами для ее изучения являются методы, основанные на манипулировании отдельными типами нейронов и не затрагивающие нейроны других нейротрансмиттерных систем. Такое избирательное управление клетками возможно за счет генетической детерминированности их функций. Белки, обусловливающие уникальность клеточного типа, экспрессируются в нем под регуляцией клеточно-специфичных промоторов. С использованием промоторов, специфичных для генов серотониновой системы, возможно управление экспрессией гена интереса в серотонинергических нейронах. В обзоре рассмотрены подходы с применением таких промоторов. Генетические модели, созданные при помощи описанных подходов, используются для установления роли серотонинергической системы в модулировании поведения и обработке сенсорной информации. В частности, генетические нокауты по серотониновым генам *sert*, *pet1* и *tph2* помогли выяснить вклад

этих генов в формирование и функционирование головного мозга. Кроме того, описываются индуцибельные модели, которые позволили управлять экспрессией генов на различных стадиях онтогенеза. И наконец, приведены примеры достижений в применении этих генетических подходов в оптогенетике и хемогенетике, которые предоставили новый ресурс для изучения функций, разрядной активности и сигнальной трансдукции серотонинергических нейронов. При создании моделей патологических состояний и разработке фармакологических средств их коррекции на основе рассмотренных генетических подходов необходимо учитывать, что каждый из них имеет свои достоинства и ограничения, и выбирать наиболее подходящий из них.
Ключевые слова: серотонинергические нейроны; генетические модели; вирусная трансдукция; оптогенетика; хемогенетика.

Introduction

The serotonergic neurotransmitter system is involved in the regulation of many physiological functions, such as pain sensitivity, the sleep/wake cycle (Whitney et al., 2016), nutritional and reproductive behavior, cognitive functions, and mood (Wong-Lin et al., 2017). It also modulates the functions of the olfactory system (Carlson et al., 2016). Many clinical and preclinical studies emphasize the role of serotonin in the pathogenesis of various mental disorders (Vadodaria et al., 2018).

Serotonin synthesis in the CNS is performed by serotonergic neurons located in brainstem nuclei, the ventromedial part of the medulla oblongata, and the reticular formation of the pons (Baker et al., 1991). The projections of these neurons are widely distributed in the brain and spinal cord. The relatively small number of serotonin neurons and the scattered distribution of their axons complicate the task of studying their functions. In addition, although serotonin neurons are anatomically clustered, their groups are not homogeneous in their electrophysiological properties and usually consist of several subpopulations, which are extensively studied (Ren et al., 2018). Thus, electrophysiological studies carried out by introducing an electrode into raphe nuclei do not provide an adequate understanding of the functions of individual cell populations (Calizo et al., 2011). Although works conducted using pharmacological approaches have made a significant contribution to understanding serotonin neurotransmission, they also have limitations: peripheral effects, the inability to control certain types of neurons, and, in some cases, unclear mechanisms of action (Choi et al., 2004). Therefore, nowadays the attention of researchers is focused on genetic tools that make it possible to manipulate certain types of neurons without affecting neurons of other neurotransmitter systems.

Gene promoters used for transgene expression in serotonergic neurons

Expression of a gene of interest in cells of a certain type is supported by tissue-specific promoters. For expression of target genes in serotonergic neurons, the *tph2*, *pet-1*, and *sert* promoters are used (Hainer et al., 2016). The advantage of the *tph2* gene (neuronal tryptophan hydroxylase), the key enzyme in serotonin biosynthesis, is that it is highly expressed exclusively in serotonergic neurons within the brain, unlike the *tph1* gene, which is hardly expressed in the CNS (except for pineal gland cells, in which it serves as an intermediate in melatonin synthesis) (Patel et al., 2004). The *tph2* promoter contains a NRSE silencer, which prevents transcription of this gene in non-neuronal cells (Patel et al., 2007), and binding sites for the Pet-1 transcription factor (Liu et al., 2010), which directs the development of serotonergic neurons during ontogeny and plays an important role in maintaining the serotonergic phenotype in adult neurons (Liu et al., 2010; Fernandez et al., 2017). Due to the fact that Pet-1 itself is a marker of serotonergic neurons, its gene promoter (or, specifically, its enhancer (Scott et al., 2005)) is also considered tissue-specific for them (Deneris, 2011). Among others, Pet-1 controls the expression of *sert* (or *slc6a4*), a serotonin transporter gene, whose promoter is also used in studies of serotonergic neurons (Hainer et al., 2016). However, unlike Pet-1 or Tph2, *sert* is expressed not only in serotonin neurons but also in astrocytic glia (Blakely, 2001) and can be synthesized in neurons of the prefrontal cortex, thalamus, and retina during the development of the nervous system (Gaspar et al., 2003).

In addition to aforementioned genes, those for aromatic L-amino acid decarboxylase (*aadc* or *ddc*), vesicular transporter of monoamines (*slc18a2* or *vmat2*), monoamine oxidase A and B (*maoa* and *maob*), 5-HT1a/b-autoreceptors (*htr1a* and *htr1b*) are vigorously involved in the functioning of the serotonergic neuron, and so are genes encoding enzymes for the synthesis and reduction of tetrahydrobiopterin (BH4) (Müller, Jacobs, 2010). However, the endogenous promoters of these genes are either weak or not specific enough to be used for gene manipulation in serotonergic neurons (Deneris, Wyler, 2012).

Transgenic animal models for examining serotonergic neurotransmission

Transgenic animals are widely used for studying the serotonergic system. Animal models are used to clarify the role of specific genes in the development and functioning at different levels of organization: from a single cell to the whole body. The most common genetic models are knockout, knock-in, and BAC-based animals. A bacterial artificial chromosome (BAC) is a DNA construct whose length can exceed 350 thousand base pairs (Shizuya et al., 1992), and this feature makes it applicable for large-insert cloning of genes or their promoter regions (Zhuang et al., 2005). In this case, transgenesis is carried out by introducing BAC into the zygote pronucleus (Richardson-Jones et al., 2010). Genetic knockout and knock-in are usually obtained in two steps: first, the gene of interest is inserted into the embryonic stem cells using homology-directed repair (To get knockout, the sequence replaces the gene of interest.) and then the modified cells are introduced into the blastocyst. Adult chimeric animals are mated with wild-type animals, and their heterozygous offspring are selected (Zhuang et al., 2005). These offspring are also crossed among themselves and animals carrying the insertion in the

homozygous state are selected from the third generation (Richardson-Jones et al., 2011).

To examine the serotonergic system with above approaches, different lines of transgenic animals have been generated and most of them are based on the Cre-LoxP or Flp-FRT recombinations. Cre and Flp recombinases recognize the LoxP and FRT sequences, respectively, and catalyze the recombination of the flanked DNA fragment to remove or invert it. The flanked region of DNA can be a gene or a stop cassette in front of the gene, so its removal turns the expression off or on (Muñoz-Jiménez et al., 2017). Based on this recombination, several lines of transgenic animals have been raised for targeting the serotonin system, where Cre recombinase expresses under the control of a specific promoter (*sert*, *pet-1*, or *tph2*) (Hainer et al., 2016). These animals are used for obtaining genetically modified animals with conditional knockout (Liu et al., 2010) and conditional induction of the serotonin system genes (Piszczek et al., 2013) to study the contribution of particular genes (Features and design of these and other genetic models are described in the Table.) For example, the same approach proves the necessity of Pet-1 for not only launching the serotonin program in neurons but also maintaining the serotonergic phenotype in the future (Liu et al., 2010).

Moreover, use of both Cre and Flp recombinases allows studying individual neuron subtypes in the serotonin system, as well as its development, by analyzing the descendants of certain rhombomeres (Kim et al., 2009). There are also many other methods to produce transgenic animals without recombinases. An example is the CRISPR/Cas9 technology, by which the first transgenic pigs with *tph2* knockout were obtained (Li et al., 2017).

Despite the undeniable contribution of genetically modified animals with constitutive changes in gene expression described previously, their main disadvantage is that they are unfit for studying the effects of a single alteration in the neurotransmitter system, because during the development of the nervous system they may experience a compensatory effect of other neurotransmitter systems, as well as aberrations in neurons in general (Hainer et al., 2016).

Inducible expression models

Inducible genetic models permit one not only to avoid adaptive effects but also to study the development of the serotonergic system in ontogeny. One of the widely used systems for gaining spatial and temporal control over transgene expression is the inducible system Cre-ERT2. Cre-ERT2 recombinase is a chimeric protein constructed by fusion of Cre-recombinase and a mutant human estrogen receptor form, which binds the synthetic ligand tamoxifen (TMX) instead of estradiol. After being activated by tamoxifen treatment, chimeric recombinase passes through the nuclear membrane into the nucleus, and induces flanked region recombination, resulting in temporal control of the gene of interest (Kristianto et al., 2017).

Another way to control transgene expression without using Cre recombinases involves ligand-inducible systems, such as Tet-ON/Tet-OFF. Administration of tetracycline-like compounds causes changes in the tetracycline-dependent transactivator (rtTA or tTA) conformation. Depending on the type of system, this protein becomes able to bind (Tet-ON) or not to bind (Tet-OFF) the tetO sequence, thereby initiating or blocking transgene expression, respectively (Das et al., 2016). Besides, there is a modification of this system in which a gene under control of a tissue-specific promoter encodes a chimeric tetracycline-dependent transactivator protein fused with the KRAB domain, a strong repressor. The resulting chimeric protein strongly suppresses the expression of the gene of interest by binding to the tetO upstream of it (Richardson-Jones et al., 2011). Several genetic models based on the Tet-OFF and Tet-ON systems have been produced for examining the serotonergic system (Weber et al., 2012; Donaldson et al., 2014; Hilber et al., 2015). For example, the effect of reduced expression of autoreceptors 5-HT1A on anxiety behavior in adulthood has been discovered (Donaldson et al., 2014). However, unlike Cre activated recombinases, the effect of the tetracycline-inducible system is reversible, because it does not change the DNA sequence, and this feature expands the range of possible applications of this system. The disadvantage of this method is that doxycycline (the most stable and common tetracycline-like compound) has its own effects on the animal behavior and the expression of some genes (Shishkina et al., 2017), which impede the interpretation of behavioral test results.

Viral vectors

Viral vectors are another promising approach for studying the serotonergic system. They are stereotaxically delivered to the raphe nuclei at a certain developmental stage and do not affect foregoing ones. Many studies have been conducted to apply the viral vector approach to transgenic animals. Most of them are based on Cre/LoxP recombination (Tye, Deisseroth, 2012; Verheij et al., 2018), so that vectors should ensure the expression of their transgene in cells where Cre recombinase is synthesized. Nowadays, there are over 250 Cre transgenic mouse lines available as part of "Gene Expression Nervous System Atlas" project in collaboration with the Intramural Research Program of the National Institute of Mental Health (http://www.gensat.org) (Gong et al., 2007) and from commercial repositories such as the Jackson Lab (http://jaxmice.jax.org).

Along with that, efforts are being made to create viral vectors specifically expressing a transgene in the serotonergic system not only in transgenic animals but also in wild-type animals or selection lines. Benzekhroufa et al. (2009) designed a vector for serotonin neurons where a two-step transcription amplification system (TSTA) was used to increase the expression of the gene of interest. In this system, the enhancement of the *tph2* promoter fragment was achieved by adding the UAS enhancer and the GAL4-p65 chimeric *cis*-regulator, as well as many variations of the UAS enhancer cassettes to the promoter. This approach allows achieving high and specific transgene expression in serotonin neurons. Based on these constructs, lentiviral and adeno-associated viral vectors were created for serotonin neuron targeting (Benzekhroufa et al., 2009). Moreover, these vectors were improved by optimizing the IRES site and adding the WPRE sequence to increase the expression level of the genes delivered by the retroviral vectors (Nishitani et al., 2019).

Another interesting approach takes advantage of viral delivery and RNA interference. The interaction of small interfering RNAs with the mRNA of the target gene leads to the mRNA destruction, thus preventing its translation and inhibiting the

Genetic models used to study the serotonergic system

Genetic model	Model design	Model features	Example
Transgenic (BAC)	Injection of a genetic construct with a gene of interest under the control of a cell-specific promoter into an animal pronucleus	Genetic construct persists in all cells lifelong, not reversible, can be transmitted to offspring	Zhao et al., 2011
Targeted viral transduction	Stereotaxic injection of a genetic construct with a gene of interest under the control of a cell-specific promoter in the region of interest	Genetic construct is stereotaxically delivered in the area of interest and can provide inducible and reversible specific expression of the gene of interest	Benzekhroufa et al., 2009
Knockout	An existing gene is replaced or disrupted with an artificial piece of DNA (STOP cassette or a new gene)	The gene is absent from all cells of the body throughout life	Mosienko et al., 2012
Conditional knockout	An animal bearing the Cre gene downstream (and under the specific control) of the cell-specific promoter and an animal bearing the LoxP gene are crossed	Gene expression is absent only from CRE-expressing cells	Liu et al., 2010
Conditional induction	An animal bearing the Cre gene downstream of the cell-specific promoter and an animal bearing a STOP sequence flanked by LoxP are crossed	Gene expression is observed only in CRE-expressing cells	Piszczek et al., 2013
Inducible knockout	An animal bearing the gene for inducible CRE-ERT2 recombinase downstream of the cell-specific promoter and an animal bearing a gene of interest flanked by LoxP are crossed	Gene expression is absent only from CRE-expressing cells after tamoxifen treatment. The system provides time- and space-specific irreversible lack of gene expression	Liu et al., 2010
Inducible transgenic	An animal bearing the tetO sequence upstream of a gene of interest and an animal bearing a gene for tetracycline transactivator (tTA or rtTA) protein downstream of the cell-specific promoter are crossed	Modulation of gene expression occurs only in cells expressing tTA or rtTA after treatment with tetracycline-like compounds. The system provides modulation of temporal- and space-specific reversible gene expression	Hilber et al., 2005
Inducible suppression	An animal bearing the tetO sequence upstream of a gene of interest and an animal bearing a gene for tetracycline transactivator (tTA or rtTA) protein fused with KRAB downstream of the cell-specific promoter are crossed	Modulation of gene expression occurs only in cells expressing tTA-KRAB or rtTA-KRAB after tetracycline-like compounds treatment. The system provides modulation of time- and space-specific reversible gene expression	Richardson-Jones et al., 2011
Knock-in	The insertion of a gene or promoter sequence before the gene of interest	The expression of the endogenous gene of interest is preserved	Sachs et al., 2013
Intersectional transgenic	An animal bearing sequences flanked by loxP and FRT (LoxP - STOP - LoxP - FRT - gene 1 - STOP2 - FRT - gene 2) and an animal bearing Cre and Flp recombinases under the specific control of two different cell-specific promotors are crossed	Gene expression depends on the expression patterns of Flp and Cre recombinases; suitable to study different subtypes of neurons in neurotransmitter systems	Kim et al., 2009
shRNA suppression	Injection of short hairpin RNA (shRNA), which depletes target gene expression, into the region of interest in the brain	The expression of the endogenous gene of interest is suppressed for a short period (several days after a single injection)	Verheij et al., 2018

synthesis of the encoded protein (Rao et al., 2009). Thereby, miRNAs control gene expression not at the transcriptional level as in the above systems but immediately after it. In addition, the effect of a single injection of siRNA lasts only for a few days (Albert et al., 2014). For viral targeting, a functional miRNA analogue, small hairpin RNA (shRNA), is used (Rao et al., 2009). The main feature of this system is its targeting. A miRNA can work only in those cells where the mRNA of its specific gene is synthesized. However, this is the main obstacle for its use: the gene of interest must be endogenous and tissue-specific. This method is also applicable to studies of the serotonergic system (Gautier et al., 2017; Verheij et al., 2018). For example, when examining the effects of *tph2* gene expression suppressing in bulbospinal serotonin neurons, their role in modulating the perception of neuropathic and inflammatory pain was shown (Gautier et al., 2017).

In general, the delivery of DNA and/or RNA using recombinant viral particles holds promise not only in the study of serotonergic neurons but also in medicine, in particular, in genetic therapy. However, it has its drawbacks. For example, the organism may develop an immune response, resulting in the loss in delivery effectiveness with repeated injections (Lukashev, Zamyatnin, 2016). Another problem is the stereotaxic method of delivery of the virus. Invasive intervention injures the brain, which may adversely affect the research results.

Optogenetic and chemogenetic approaches

The methods described above for achieving cell-specific gene expression are used in optogenetics and chemogenetics. Both approaches permit one to control a certain type of neurons in a selective manner to study their functions, firing activity, and signal transduction. In optogenetics, cell-specific promoters are used to govern the expression of genes for light-activated ion channels or pumps genes in the neuron type of interest. Optogenetic proteins change the permeability of the cell membrane for certain ions, which makes it possible to control the discharge activity of the cell with high temporal resolution (Lammel et al., 2016). For optogenetic studies of serotonergic neurons, a BAC line of transgenic Tph2-ChR2 (H134R)-EYFP mice has been developed, in which the transcription of membrane-depolarizing Channelrhodopsin-2 (ChR2) is controlled by the *tph2* promoter (Zhao et al., 2011). The same promoter has been used to create mice in which the ChR2 (C128S) gene is located after the tetracycline-dependent operator (tetO), while tTA is under the control of the *tph2* promoter. By using this line of mice with inducible expression of ChR2 in serotonin neurons, the contribution of these cells to the modulation of anxiety-like behavior and patience to wait for a delayed reward was studied (Miyazaki et al., 2014; Ohmura et al., 2014).

There is one more general approach that does not require the creation of a separate line of animals for the expression of each opsin type in target cells. It is the use of lines expressing Cre recombinase under the control of the *sert* or *pet-1* promoter. In this approach opsins are delivered to neurons by transduction with adeno-associated viruses. The vector contains the opsin gene in the inverted orientation. The gene is flanked by DIO (Double-floxed Inverted Orientation) on both sides and located downstream from the strong neuronal promoter *hSyn*. As an example, Sert-Cre mice were injected with viral vectors containing depolarizing Channelrhodopsin (AAV-hSyn-DIO-ChR2) and hyperpolarizing halorhodopsin (AAV-hSyn-DIO-NpHR) to elucidate the role of the serotonergic system in social deficit in the mouse model of autism (Walsh et al., 2018). Other studies using the same approach to deliver ChR2 to serotonergic neurons of the dorsal raphe nucleus revealed the roles of these cells in suppressing spontaneous discharge activity in olfactory neurons (Lottem et al., 2016), in the neurological response to the expected reward (Li et al., 2016), and in suppressing spontaneous locomotor activity in the open field and reducing the speed of movement (Correia et al., 2017). In addition to the Sert-Cre line, where the *sert* promoter is used to achieve specific expression of Channelrhodopsin in serotonin neurons, the similar Pet1-Cre line is used in optogenetic studies (Challis et al., 2014; Liu et al., 2014; Luo et al., 2017).

To deliver optogenetic proteins to serotonergic neurons of animals that had not been subjected to genetic modifications, lentiviral vectors based on the rat and mouse *tph2* promoters were designed. They made it possible to study the differences in the depressive-like and anxious behavior of these species, stimulating the neurons of the dorsal raphe nucleus (Nishitani et al., 2019).

In chemogenetics, receptors designed to interact with chemical ligands that are inert in the body are used. Nowadays, there are a number of receptors associated with G-proteins called DREADD (Designer Receptors Exclusively Activated by Designer Drugs), which have been obtained by modification of human muscarinic receptors. These receptors no longer respond to acetylcholine nor to any other endogenous molecule, but they bind to clozapine N-oxide (CNO) instead (Alexander et al., 2009). For example, one of the most commonly used DREADDs, hM3Dq, binding to CNO, triggers an intracellular cascade via Gq protein and activation of phospholipase C, which leads to depolarization of neurons and increases their discharge activity (Urban, Roth, 2015). Another chemogenetic receptor, hM4Di, inhibits the discharge activity of neurons through Gi protein (Zhu, Roth, 2014).

Currently, several options are available to achieve the expression of DREADD in genetically determined cells. There are lines of genetically engineered mice in which hM3Dq expression is controlled by the Tet-OFF system (Alexander et al., 2009; Garner et al., 2012) or Cre-Lox recombination (Teissier et al., 2015). In addition, a growing number of promoters are being used in many viral vectors for cell-specific expression of DREADDs, including modified herpes simplex viruses (Ferguson et al., 2011), adeno-associated viruses (Zhu et al., 2014; Scofield et al., 2015) and lentiviruses (Mahler et al., 2014; Vazey, Aston-Jones, 2014).

Numerous studies using chemogenetic approaches were conducted to perceive the functions of serotonergic neurons and their projections. In such experiments mice of the Sert-Cre line are injected with adeno-associated viruses containing the sequence hM4Di or hM3Dq (AAV-hSyn-DIO-hM4Di/hM3Dq) to obtain its expression in the serotonergic neurons (Urban et al., 2016; Fernandez et al., 2017; Singh et al., 2017). For example, it was found that serotonergic projections from the median raphe are essential for regulating the memory of objects and the synaptic plasticity of the hippocampus (Fernandez et al., 2017). In another study, pet1-Cre mice were similarly used to activate and inhibit serotonergic neurons in order to show that serotonergic neurons in the medial raphe nucleus play a key role in regulating anxiety- and depression-like behavior (Li et al., 2018).

Conclusion

Serotonergic neurons form an intricate and extensive network of axon projections throughout the brain. The main task of analyzing these neuronal circuits is to understand how serotonergic networks are related to the numerous functions of this neurotransmitter system. Several new techniques for manipulating subpopulations of serotonergic neurons have been designed in recent years: various lines of animals have been created using the double recombination strategy to achieve transgene expression exclusively in serotonergic neurons. Inducible systems for temporal control of gene expression,

DREADDs and optogenetics, were also successfully used to investigate serotonergic neurotransmission. Depending on the goals of the experiments, the researchers choose which of the available variants of gene expression regulation to use, because each of them has its own advantages and limitations. It is important to note that these models are not completely free of nonspecific effects. Adequate controls should be included in the experimental design for the most accurate and informative interpretation of the results. Nevertheless, the tools and methods depicted in this review, both individually and in combinations, open up new possibilities for the study of the serotonergic neurotransmitter system.

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