厚生労働科学研究費補助金

障害者対策総合研究事業

アンチセンスによる筋強直性ジストロフィーの治療の最適化

平成 23 年度　総括・分担研究報告書

主任研究者　石浦　章一

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厚生労働科学研究費補助金（障害者対策研究事業）
総括研究報告書

筋強直性ジストロフィーの治療の最適化

総括研究者　　石浦章一　東京大学大学院 総合文化研究科　教授

研究要旨

筋強直性ジストロフィー１型(DM1)は、多くの遺伝子のスプライシング異常を主徴とする全身性疾患で、筋強直、精巣萎縮、白内障、耐糖能異常などが特徴である。私たちは同病のモデル動物を用いて、筋強直症状を治療すべく、塩素チャネル遺伝子のアンチセンスを効率よく筋肉内に導入するバブル・リポソーム法を採用し、その効果を調べた。その結果、超音波照射によって筋細胞内への導入が促進され、塩素チャネル遺伝子のスプライシングが正常化した。

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A．研究目的

筋強直性ジストロフィー１型(DM1)は、第19染色体にあるDMPK遺伝子の3’非翻訳領域にあるCTGリピートの伸長による疾患である。症状は、筋強直、精神遅滞、精巣萎縮、白内障、耐糖能異常などの全身性で、数多くの遺伝子のスプライシングが異常になって全身症状が出現すると考えられている。本症は我が国の筋ジストロフィーの中では一番多く、筋力低下やミオトニアなどの治療法の開発が望まれ、QOL改善が目標である。

平成23年度は、塩素チャネル遺伝子を指標に、スプライシングを正常化させるアンチセンスを効率良く筋肉に導入する方法の開発に力を注いだ。

すなわち、効率の良いエクソンスキッピングを行わせるために、アンチセンス・モルフォリノオリゴヌクレオチドをモデル動物（CTG リピートを300含むトランスジェニックマウス）に導入した。この時、導入効率を上げるため、バブル・リポソームを用いた。

B．研究方法（倫理面の配慮含む）

塩素チャネルミニ遺伝子（エキソン 6、7a、7でできている短い遺伝子）を用いて、エキソン7a の有無を定量するスプライシングアッセイを行った。エクソン 7a がない成人（正常）型と、エクソン 7a を含む幼若（異常）型の比率は、正常筋では圧倒的に前者が多く、DM 筋では後者が増えている。エクソン 7a0～25 をコードするモルフォリノアンチセンスオリゴを、塩素チャネル・ミニ遺伝子とともに CTG300 を持つトランスジェニックマウス HAS-LR の tibialis anterior (TA) 筋に１回注射した。このときのアンチセンス配列は、エクソン 7a の 0～25 に対するものであり、細胞系では最もエクソン 7a スキッピングが起こりやすくなるものである。最後の注射から２日後に、mRNA を抽出し、スプライシング頻度を測定した。

このとき、モルフォリノオリゴ(10μg)はバブル・リポソームとともに筋注した（全 30μl)。その後、0-3 Wの出力で超音波を 0.5-3 分照射し、筋内にアンチセンスを導入した。

（倫理面への配慮）

今回の実験は、DM 患者生検筋からの mRNA スプライシング異常から派生した研究である。生検筋は、インフォームドコンセントを得て取得し、国立精神･神経医療研究センター倫理委員会で承認を受けたものを用いている。

C．研究結果

バブル・リポソームを用いるデリバリー法は比較的新しく、条件検討が必要であった。本研究で、出力、時間を変化させ、１Wの出力で超音波を１

分間照射が最適条件であることが分かった。

次に、アンチセンスによる内因性の塩素チャネル遺伝子のスプライシング正常化機能をPCR法で定量した。その結果、異常型塩素チャネルのパーセンテージが18%から11%に低下した。これは対照（正常）マウスの10%に近い値であった。またこの時に、筋強直性ジストロフィーでスプライシング異常が指摘されているSERCA1とPdlim3遺伝子の異常型スプライシングの比率は変化させなかった。以上の事実は、アンチセンスが特異的に塩素チャネル遺伝子に作用したことを示していた。

D．考察

昨年までの研究で、塩素チャネルスプライシングを正常化させるアンチセンスの最適配列は決定されていた。今年度は、それを用いて動物の治療実験を行ったもので、新たにドラッグデリバリーについて検討した。その結果、単なる筋注よりも、バブル・リポソームを用いた方がアンチセンス導入の効率が良いことが明らかになった。

ところが1つ問題が出てきた。筋注という物理的刺激によってスプライシングが異常型に変化することも分かった。異常型というのは胎児型アイソフォームであるが、筋注刺激で筋変性を起こし、再生に伴ってタンパク質が幼若型へと変化したと考えられた。この事実から、筋注の回数や方法に改善の余地があることが明らかになった。

E．結論

筋強直性ジストロフィーのモデルであるCTGリピートを300含むトランスジェニックマウスHAS-LRに対して、私たちが新しく開発したアンチセンスモルフォリノオリゴ配列＋バブル・リポソーム法は劇的な治療効果をもたらした。今後は、実際のQOLに必要な筋萎縮を防ぐため、筋力低下に関わる新規遺伝子を同定し、同様にアンチセンス治療を行うことが必要となる。

F．健康危険情報

なし

G．研究発表

1．論文発表

(1) Ishiura, S., Kino, Y., Oma, Y., Sasagawa, N. & Nukina, N. (2011) MBNL proteins regulate alternative splicing of the skeletal muscle chloride channel CLCN1. *In Fifty years of neuromuscular disorder research after discovery of serum creatine kinase as a diagnostic marker of muscular dystrophy.* (Takeda, S. ed.) pp. 18-25, Igaku-shoin, Tokyo, 2011.

(2) Ohsawa, N., Koebis, M., Suo, S., Nishino, I. & Ishiura, S. (2011) Alternative splicing of PDLIM3/ALP, for <alpha>-actinin-associated LIM protein 3, is aberrant in persons with myotonic dystrophy. Biochem. Biophys. Res. Commun. 409, 64-69

(3) Koebis, M., Ohsawa, N., Kino, Y., Sasagawa, N., Nishino, I. & Ishiura, S. (2011) The alternative splicing of myomesin 1 gene is aberrantly regulated in myotonic dystrophy type 1. Genes to Cells, 16, 961-972

H．知的財産権の出願・登録状況

なし

厚生労働科学研究費補助金（障害者対策研究事業）
分担研究報告書

筋強直性ジストロフィーの治療の最適化

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研究要旨

先天性筋強直性ジストロフィーは、タイプ1線維がタイプ2線維より小径（12%以上の差）という病理所見で定義づけられる先天性ミオパチーである先天性筋線維タイプ不均等症（CFTD）類似の所見を呈することがある。従って、CFTDの鑑別診断を十分に検討することが、先天性筋強直性ジストロフィーの正確な診断に繋がる。本年度、乳幼児期に発症しCFTDに類似した筋病理所見を呈する*LMNA*ミオパチーを見出したため、CFTD様の病理所見を呈する例の中に、*LMNA*遺伝子変異による例があるかどうかを検討した。その結果、*LMNA*変異による例の中には筋病理学的にCFTDと鑑別困難な例が存在することを明らかにした。

A．研究目的

我々はこれまでに、先天性筋強直性ジストロフィーが臨床病理学的にミオチュブラーミオパチーや先天性筋線維タイプ不均等症（CFTD）類似の所見を呈することを示してきた。このことは、ミオチュブラーミオパチーやCFTDの鑑別診断を十分に検討することが、先天性筋強直性ジストロフィーの正確な診断に繋がることを示している。本年度は、その内の一つとして、CFTD様の病理所見を呈する例の中に、核膜蛋白質A型ラミンをコードする*LMNA*遺伝子変異による例があるかどうかを検討した。

*LMNA*遺伝子は染色体1q21に存在し、その遺伝子変異は、Emery-Dreifuss型筋ジストロフィー(EDMD)、肢体型筋ジストロフィー1B型(LGMD1B)、先天性筋ジストロフィー(L-CMD)などの原因となる。EDMDは幼小児期に発症する緩徐進行性の筋ジストロフィー、病初期からの関節拘縮、心伝導障害を伴う心筋症を三主徴とする。LGMD1Bは近位筋優位の筋力低下と心伝導障害を伴う心筋症を特徴とするが、関節拘縮は明らかではない。L-CMDは乳児期早期に発症し、重症の筋力低下と筋緊張低下、呼吸筋と頚部筋の筋力低下が目立つ。*LMNA*ミオパチーの筋病理は筋線維の大小不同、壊死再生所見、間質結合組織の増加といった非特異的な筋ジストロフィーの特徴を示し、タイプ1線維、タイプ2線維ともに侵される。

先天性筋線維タイプ不均等症（CFTD）は筋病理所見でタイプ１線維がタイプ２線維より小径(12%以上の差)であることを特徴とする先天性ミオパチーである。臨床的には他の先天性ミオパチーと同様に筋緊張低下と筋力低下があり、多くは嚥下障害、関節拘縮、側弯症、細長い顔、高口蓋等を示す。原因遺伝子として *ACTA1*、*TPM3*、*SEPN1* の３遺伝子が見出されている。

LMNA ミオパチーと CFTD は筋病理学的にも臨床的にも異なっているにも関わらず、今回我々は乳幼児期に発症し CFTD に類似した筋病理所見を呈する *LMNA* ミオパチーを見出したため、臨床・筋病理学的に検討を行った。

B．研究方法（倫理面の配慮含む）

国立精神・神経医療研究センター骨格筋レポジトリーに登録されている症例のうち、筋病理学的に CFTD が示唆された 94 例（CFTD 群）、及び *LMNA* 変異の同定されている 31 例（LMNA 群）を対象とし、臨床病理・遺伝学的解析を行った。

（倫理面への配慮）

今回の実験は、DM 患者生検筋からの mRNA スプライシング異常から派生した研究である。生検筋

は、インフォームドコンセントを得て取得し、国立精神・神経医療研究センター倫理委員会で承認を受けたものを用いている。

C．研究結果

CFTD群のうち2例に*LMNA*遺伝子変異を見出した。一方、LMNA群の5例で筋線維タイプ不均等(FTD)が認められた。成人発症例1例を除く、6例の発症年齢は1歳2ヵ月から5歳（平均2歳8ヵ月）で、高口蓋や呼吸障害はなく、血清CK値は915±698 IU/Lと全例で高値を示した。筋病理所見から計算したfiber size disproportion (%FSD)［(mean type 2 fiber diameter) − (mean type1 fiber diameter) ／mean type 2 fiber diameter ×100］は27.8±17.8%で*ACTA1*及び*TPM3*に遺伝子変異のあるCFTD 9例の%FSD（41.3±13.5%）と比べ、有意差は認めなった。

CFTD群、LMNA群それぞれのタイプ1線維とタイプ2線維の平均径を通常年齢における筋線維径と比較したところ、CFTD群ではタイプ1線維萎縮を、LMNA群ではタイプ2線維肥大を認めた。このことは、両群におけるFTDの成因の違いを示唆した。

D．考察

*LMNA*変異は筋病理所見上、CFTDと鑑別困難な例が存在する。高口蓋や呼吸障害を認めず、高CK血症があり、CFTDに類似した所見を呈する症例においては*LMNA*遺伝子解析も考慮すべきと考える。

E．結論

*LMNA*遺伝子変異におけるFTD所見は、CFTDと異なり、タイプ2線維の肥大による相対的なタイプ1線維萎縮で成り立っていることが示唆された。今後は筋線維肥大に関わるメカニズム等を含め、更なる分子生物学的解析を行うことが必要となる。

F．健康危険情報

なし

G．研究発表

1．論文発表

(1) Keduka E, Hayashi YK, Shalaby S, Mitsuhashi H, Noguchi S, Nonaka I, Nishino I: *In Vivo* Characterization of Mutant Myotilins. Am J Pathol. [Epub ahead of print]

2．学会発表

特になし

H．知的財産権の出願・登録状況

1．特許取得

特になし

2．実用新案登録

特になし

3．その他

特になし

別紙４

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ
Kanno, K., Ishiura S.	Genetic variants of the dopaminergic system in human and model organisms.	Inoue-Murayama, M. et al.	From Genes to Animal Behavior	Springer	New York	2011	227-237
Ishiura S, Kino Y, Oma Y, Sasagawa N, Nukina N.	MBNL proteins regulate alternative splicing of the skeletal muscle chloride channel *CLCN1*	Takeda, S.	Fifty years of neuromuscular disorder research after discovery of serum creatine kinase as a diagnostic marker of muscular dystrophy.	Igaku-shoin	Tokyo	2011	18-25

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
古戎道典、石浦章一	RNA結合タンパク質が引き起こす筋強直性ジストロフィー	医学のあゆみ	238	481-484	2011
Ohsawa, N., Koebis, M., Suo, S., Nishino, I., Ishiura, S.	Alternative splicing of PDLIM3/ALP, for <alpha>-actinin-associated LIM protein 3, is aberrant in persons with myotonic	Biochem. Biophys. Res. Commun.	409	64-69	2011
Koebis, M., Ohsawa, N., Kino, Y., Sasagawa, N., Nishino, I., Ishiura, S.	The alternative splicing of myomesin 1 gene is aberrantly regulated in myotonic dystrophy type 1.	Genes to Cells	16	961-972	2011
Kanno, K., Ishiura, S.	Differential effects of the HESR/HEY transcription factor family on dopamine transporter (*DAT1*) reporter	J. Neurosci. Res.	89	562-575	2011
Yonemura, Y., Futai. E., Yagishita, S., Suo, S., Tomita, T., Iwatsubo, T., Ishiura, S.	Comparison of presenilin 1 and presenilin 2 gamma-secretase activities using a yeast reconstitution system.	J.Biol.Chem.	286	44569-44575	2011
Asai, M, Yagishita, S., Iwata, N., Saido, T.C., Ishiura, S., Maruyama, K.	An alternative metabolic pathway of amyloid precursor protein C-terminal fragments via cathepsin B in a human neuroglioma model.	FASEB J.	25	3720-3730	2011

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Sato, K., Tanabe, C., Yonemura, Y., Watahiki, H., Zhao, Y., Yagishita, S., Ebina, M., Suo, S., Futai, E., Murata, M., Ishiura, S.	Localization of mature neprilysin in lipid rafts.	J.Neurosci.Res.	90	870-877	2012
Oguro, A., Kubota, H., Ishiura, S., Shimizu, M., Atomi, Y.	Protective role of the ubiquitin binding protein Tollip against the toxicity of polyglutamine-expansion proteins.	Neurosci.Lett.	503	234-239	2011
Keduka E, Hayashi YK, Shalaby S, Mitsuhashi H, Noguchi S, Nonaka I, Nishino I.	*In Vivo* Characterization of Mutant Myotilins.	Am J Pathol.	Epub Ahead of Print	Epub Ahead of Print	2012

Ⅳ.研究成果の刊行物・別刷

Chapter 10
Genetic Variants of the Dopaminergic System in Humans and Model Organisms

Kouta Kanno and Shoichi Ishiura

10.1 Introduction

Human personality is shaped by both genetic and environmental factors. Molecular genetics has begun to identify specific genes for quantitative traits. The first candidate genes investigated were components of the monoamine neurotransmitter pathways, such as serotonin and dopamine. The serotonergic system is involved in mood, anxiety, and aggression. Temperamental predisposition and behavior are likely to be influenced by genetic variations of serotonergic genes – i.e., serotonin-metabolizing enzymes, tryptophan hydroxylase and monoamine oxidase (MAO), catechol-*O*-methyltransferase (COMT), 14 kinds of serotonin receptor (5-hydroxytryptamine, or 5HT) and serotonin transporter (SERT).

The dopaminergic system is involved in the brain's reward system and addictive behavior. Human or animal behavior is also influenced by dopaminergic genes such as tyrosine hydroxylase (TH), dopamine receptors (DRD), and dopamine transporter (DAT). Noradrenergic and γ-aminobutyric acid (GABA)ergic genes are also involved in behavior.

It has been reported that single nucleotide polymorphisms (SNPs) and simple microsatellites in and around the coding regions of the dopamine- and serotonin-related genes – e.g., DRD3, DRD4, DAT1 (SLC6A3), TH, COMT, brain-derived neurotrophic factor (BDNF), 5HT2A, MAOA, and SERT (5-HTT, SLC6A4) (D'Souza and Craig 2008) – are important factors in human neuropsychiatric disorders and behavior.

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M. Inoue-Murayama et al. (eds.), *From Genes to Animal Behavior*,
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Here, we primarily focus on the dopaminergic systems and review reports, including our recent studies, on functional DAT1 polymorphisms.

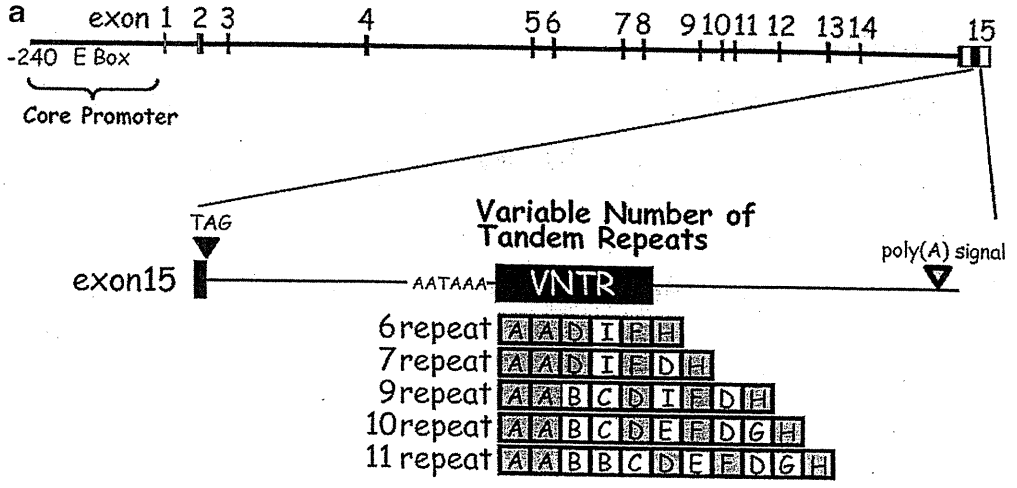
The dopaminergic nervous system plays important roles in regulating locomotion, cognition, reward, addiction, and hormone release (Jackson and Westlind-Danielson 1994; Missale et al. 1998; Bannon et al. 2001; Uhl 2003). Dopamine and its related genes are thought to be involved in neuropsychiatric disorders and behavioral traits. The human dopamine transporter (*DAT1*) gene is involved in many dopamine-related disorders. Levels of DAT are reduced in Parkinson's disease (PD) and elevated in attention deficit hyperactivity disorder (ADHD), Tourette's syndrome, and major depression (Madras et al. 1998; Muller-Vahl et al. 2000; Brunswick et al. 2003; Krause et al. 2003). Additionally, several psychoactive drugs, including cocaine, amphetamine, and methylphenidate, are known to inhibit dopamine reuptake by the DAT protein (Giros et al. 1991, 1992; Giros and Caron 1993; Kilty et al. 1991; Shimada et al. 1991).

10.2 Functional Genetic Polymorphism of *DAT1*: The Variable-Number Tandem Repeat

The dopamine transporter, which is a major tuner of synaptic dopamine levels, is a 620-amino-acid protein belonging to the family of Na^+/Cl^--dependent neurotransmitter transporters with 12 putative transmembrane domains and is located on axon terminals (Uhl 2003). A functional genetic polymorphism exists in part of the 3′-noncoding region included in exon 15 of the *DAT1* gene (Michelhaugh et al. 2001). As shown in Fig. 10.1, this 3′-UTR contains a 40-bp variable-number tandem repeat (VNTR) polymorphism ranging from 3 to 11 repeats, with 9 and 10 repeats being the most common alleles (Vandenbergh et al. 1992; Michelhaugh et al. 2001). We identified 6-, 7-, 9-, 10-, and 11-repeat alleles and their sequences in a Japanese population (Fuke et al. 2005) (Fig. 10.1). The repeats' unit sequences with 9 and 10 repeats were the same as those reported by Mill et al. (2005).

This VNTR polymorphism is known to be associated with many neuropsychiatric disorders such as ADHD, PD, and drug abuse (Cook et al. 1995; Vandenbergh et al. 2000; Ueno 2003; D'Souza and Craig 2008). Many genetic studies have reported significant associations between disorders and addictions with these genotypes. However, discrepancies exist among the studies, although a recent meta-analysis showed a small but significant association between the 10-repeat allele and ADHD (Yang et al. 2007).

If the VNTR is associated with these diseases, what is the mechanism? One possible answer is the different levels of DAT expression among the genotypes. In fact, modified gene expression, depending on the genotype, was observed in vivo (Heinz et al. 2000; Jacobsen et al. 2000; Martinez et al. 2001; Mill et al. 2002; D'Souza and Craig, 2008). We first demonstrated modified gene expression in vitro in Cos-7 cells using the luciferase reporter assay (Fuke et al. 2001), and since then several groups have confirmed the results in mammalian cell lines (Inoue-Murayama et al. 2002;



b

Repeat Units

(A) AGGAGCGTGTCCTATCCCCGGACGCATGCAGGGCCCCCAC
(B) AGGAGCATGTCCTATCCCTGGACGCATGCAGGGCCCCCAC
(C) AGGAGCGTGTACTACCCCAGAACGCATGCAGGGCCCCCAC
(D) AGGAGCGTGTACTACCCCAGGACGCATGCAGGGCCCCCAC
(E) TGGAGCGTGTACTACCCCAGGACGCATGCAGGGCCCCCAC
(F) AGGAGCGTGTCCTATCCCCGGA
CCGGACGCATGCAGGGCCCCCAC
(G) AGGAGCGTGTACTACCCCAGGATGCATGCAGGGCCCCCAC
(H) AGGAGCGTGTACTACCCCAGGACGCATGCAGGGCCCCCAT
(I) TGGAGCGTGTACTACCCCAGGATGCATGCAGGGCCCCCAC

Fig. 10.1 Genomic structure of the *DAT1* gene and allelic variants of variable-number tandem repeat (VNTR) polymorphism in exon 15. (**a**) Coding region (*black box*), noncoding region (*open boxes*), VNTR, and constant repeat units (*gray boxes*) are shown. Exon 15 of the *DAT1* gene contains a stop codon (*black arrowhead*) and polyadenylation signal (*open arrowhead*). Upstream of the VNTR are six nucleotides, AATAAA, that resemble a polyadenylation signal. The allelic variants of VNTR polymorphism indicate repeat units type (*A–I*) in each allele. (**b**) Nucleotide sequence of each unit of VNTR polymorphism in the 3′-UTR of the *DAT1* gene

Miller and Madras 2002; Greenwood and Kelsoe 2003; Mill et al. 2005; VanNess et al. 2005; D'Souza and Craig 2008). However, these studies also generated conflicting results, both in vivo and in vitro.

For example, in in vivo single-photon emission computed tomography (SPECT) studies in the striatum, Jacobsen et al. (2000) reported that DAT availability was higher in the brain of the 9-repeat (r) group than in the 10r group, whereas Heinz et al. (2000) reported that the value in the 10/10r group was higher than that in 10/9r. Martinez et al. (2001), on the other hand, detected no significant difference among genotypes. In a study of postmortem brain tissue, reverse transcription polymerase chain reaction (RT-PCR) evaluation showed that DAT expression in the samples of 10r was higher than that in 9r (Mill et al. 2002; Brookes et al. 2007).

In in vitro studies, possible reasons for these discrepancies include differences in methodology, such as in the cell lines and promoters used in the reporter assay and the location of the 3′ untranslated region (UTR) in the reporter vectors. We observed differing results depending on the cell lines used. The 3′-UTR, including the VNTR, decreased luciferase activity with the DAT1 core promoter in SH-SY5Y,

Neuro2A, and Cos-7 cells but did not do so in HEK293 cells (Fuke et al. 2005). Such differences could be the result of differential expression of regulating factors in each cell, but the molecular and neural bases remain unknown because no factor interacting with the VNTR has yet been characterized, although it is expected that proteins bind to the region (Michelhaugh et al. 2001).

10.3 HESR1: A Protein Binding to the 3′-UTR of DAT

To clarify the molecular mechanism of DAT gene regulation via the VNTR, we screened proteins that bound to the 3′-UTR using a yeast one-hybrid system and identified HESR1 (the hairy/enhancer of split related transcriptional factor 1 with YRPW motif) protein as a *trans*-acting factor through the 3′-UTR of the *DAT1* gene (Fuke et al. 2005). We then showed that HESR1 bound directly to the region by electrophoretic mobility shift assay (EMSA) and repressed expression of the endogenous *DAT1* gene in a mammalian cell line (by RT-PCR assay) (Fuke et al. 2006).

However, it is possible that other factors affect *DAT* gene expression via the VNTR, as it is expected that more than one factor would bind to such a region (Michelhaugh et al. 2001). The HESR family genes – *HESR1*, *HESR2*, *HESR3* – were characterized as a direct transcriptional target of the Notch signaling pathway involved in neural development (Kokubo et al. 1999; Leimeister et al. 1999; Nakagawa et al. 1999, 2000; Henderson et al. 2001; Iso et al. 2001, 2003; Wang et al. 2002; Sakamoto et al. 2003).

The HESR family genes encode a basic helix–loop–helix (bHLH) domain that is essential for DNA binding, an Orange domain, and a YRPW motif. HESR proteins bind to E boxes or N boxes, which are known bHLH-binding consensus sites, and repress expression of target genes (Nakagawa et al. 2000; Iso et al. 2001, 2003). The bHLH domain sequences among the HESR family are highly conserved (Steidl et al. 2000). In fact, human HESR1 and HESR2 (Belandia et al. 2005) and mouse Hesr1 and Hesr2 (Kokubo et al. 2007) repress gene expression at the same genome site in reporter assay systems. Thus, not only HESR1, but also HESR2 and HESR3, may be candidate regulating factors for DAT expression via the VNTR.

10.4 HESR Family Genes: Candidate Regulating Factors for DAT Expression

Recently, we performed luciferase reporter assays to examine whether HESR2 and HESR 3 could affect DAT gene expression via the 3′-UTR including the VNTR region in human neuroblastoma SH-SY5Y cells (Kanno and Ishiura 2009). We found that HESR1 and HESR2 inhibited reporter gene expression via both the core promoter and 3′-UTR, whereas HESR3 enhanced it only via the core promoter. We did not expect the HESR family to affect the core promoter region because HESR1 was

identified as a protein binding to the 3′-UTR, but the core promoter does also contain an E box, known to be a bHLH consensus binding site. Additionally, a functional –67 A/T SNP in this promoter region has been reported to be associated with personality traits such as ADHD and bipolar disorder (Greenwood and Kelsoe 2003; Ohadi et al. 2006, 2007; Shibuya et al. 2009). HESR family proteins may also interact with this SNP. Only HESR3 increased reporter luciferase activity via the DAT core promoter. We also found that HESR1, including the Leu94Met SNP in the second helix of the bHLH domain, lacked inhibitory activity (Fuke et al. 2005). The latest study demonstrated that an SNP transformed HESR1 from an androgen receptor co-repressor to an activator (Villaronga et al. 2009).

Furthermore, HESR1 and HESR2 may differentially alter DAT expression patterns depending on VNTR alleles. Relatively strong inhibition of luciferase activity with 10r was observed with HESR1. In general, our results in these reporter assays showed a tendency for luciferase activity with 9r to be higher than that with 10r, although the difference was not statistically significant, and the highest activity was with 7r. Human HESR2, but not mouse Hesr2, diminished the difference in luciferase activity between 9r and 10r. These findings basically support our idea that different DAT expression levels can be altered by factors in each cell, depending on VNTR alleles. This may explain the discrepancies between the many previous studies described above.

10.5 Behavioral and Neurochemical Aspects of the Hesr Family

We also reported increased expression of the *DAT* gene in the brains of *Hesr1* knockout (KO) mice (Fuke et al. 2006). The KO mice showed decreased spontaneous locomotor activity, reduced exploration of novelty, and enhanced anxiety-like behavior in the open-field test and the elevated plus-maze test (Fuke et al. 2006). This is consistent with our in vitro data because HESR1 is thought to be an inhibitory factor for *DAT*. Additionally, the expression of several dopamine receptor genes, *D1*, *D2*, *D4*, and *D5*, the main targets of synaptic dopamine responsiveness, were enhanced in the *Hesr1* KO mice. Although we did not directly measure synaptic extracellular dopamine levels, decreased activity and increased dopamine transporter and receptors seem to indicate a low synaptic dopamine level in the KO mice. These phenomena are the opposite of those in *DAT* KO mice (Fig. 10.2). Mice lacking the *DAT* gene show decreased intraneural storage of dopamine, spontaneous hyperlocomotion, and down-regulation of several dopamine-related genes, such as dopamine receptor D1 and D2 (Giros et al. 1996; Caine 1998; Jaber et al. 1999; Fauchey et al. 2000; Gainetdinov et al. 2002). This indicates the importance of Hesr1 in the dopaminergic system in vivo.

We also conducted an immunohistochemical analysis to investigate the localization of Hesr family proteins in the mouse midbrain dopaminergic region (Fig. 10.3). Immunostaining for tyrosine hydroxylase (TH), a DA neuron marker, and each Hesr were conducted from the anterior (–3.04 to –3.49 relative to bregma) to the posterior part (–3.94 from bregma) of the midbrain dopaminergic regions: ventral

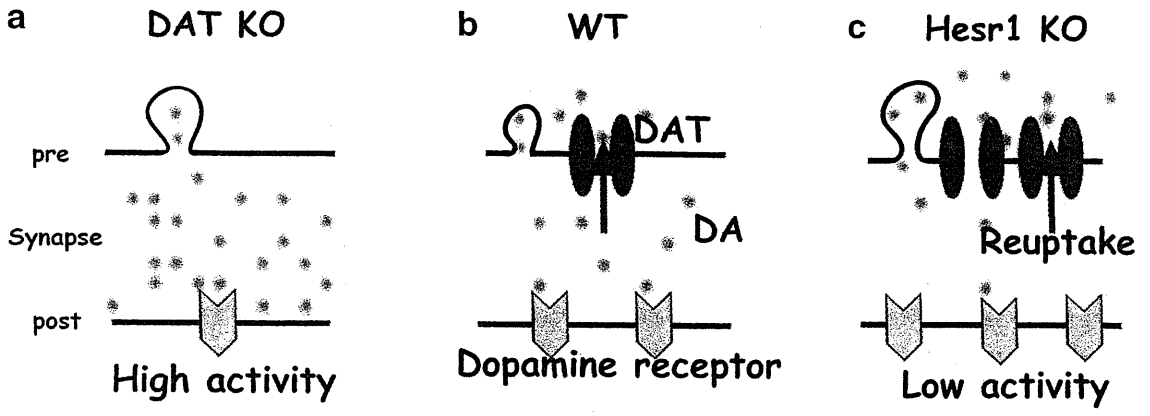


Fig. 10.2 Synapses in *DAT* or *Hesr1* knockout (*KO*) mice. (**a**) *DAT* KO mouse. This indicates increased synaptic extracellular dopamine and decreased dopamine receptors. (**b**) Wild-type mouse. This indicates the normal synaptic state. (**c**) *Hesr1* KO mouse. This indicates possibly decreased synaptic extracellular dopamine and increased dopamine receptors

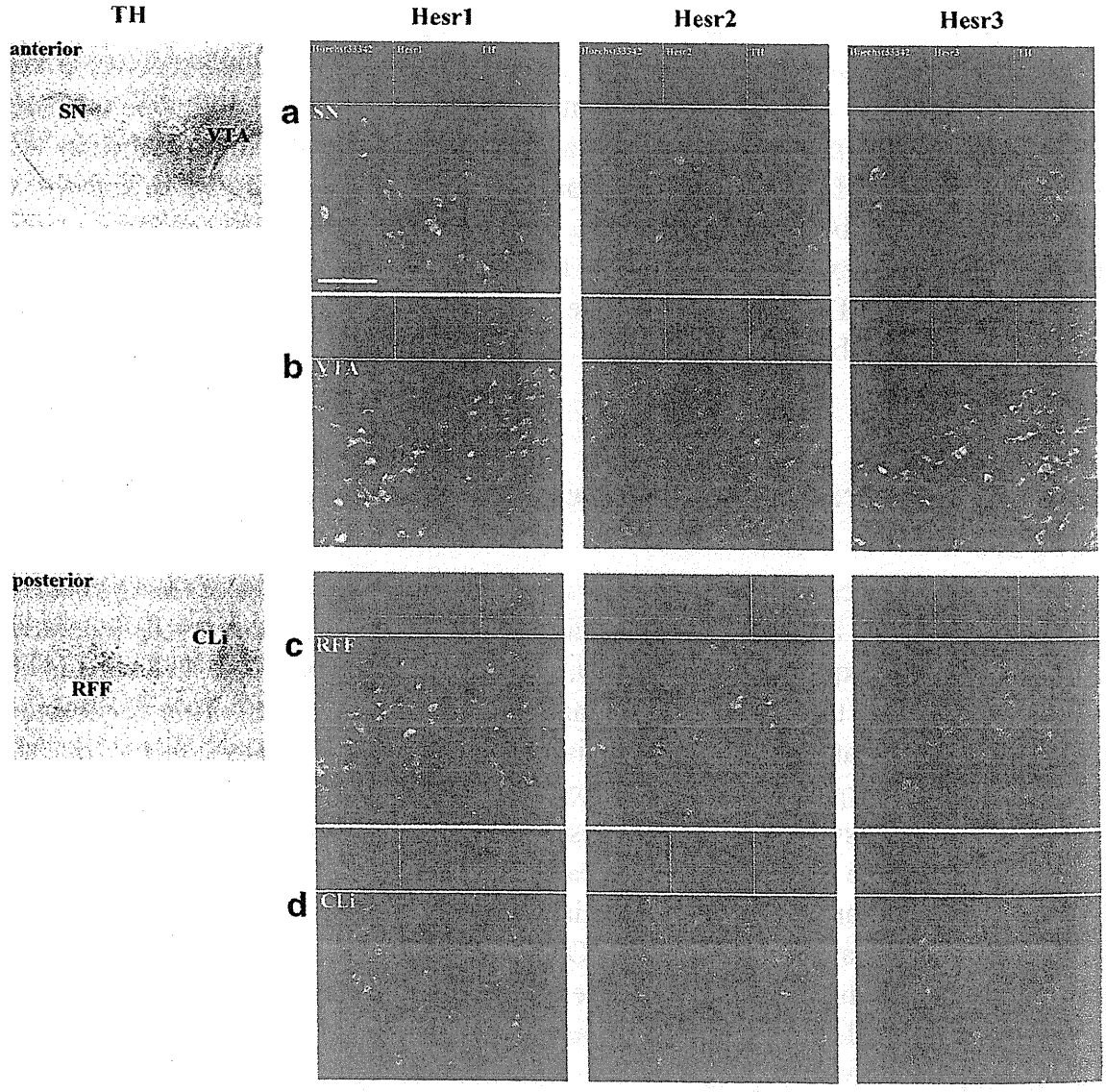


Fig. 10.3 Immunohistochemistry for tyrosine hydroxylase (*TH*) and Hesr family. TH (*green*, Cy2); Hesr1/2 (*red*, Cy3); Hesr3 (*magenta*, Cy3); nucleus (*blue*, Hoechst 33342). *VTA*, ventral tegmental area, *SN*, substantia nigra; *RFF* (RFF/A8), retrorubral field and A8 DA cells; *CLi*, caudal liner nucleus of raphe. *Bars* 500 μm for immunoenzymatic staining for TH; 100 μm for immunofluorescence staining

tegmental area (VTA), substantia nigra (SN), retrorubral field and A8 DA cells (RFF/A8), caudal liner nucleus of raphe (CLi). Each Hesr was expressed in almost all dopaminergic neurons (TH-ir cells) in the mouse midbrain. Thus, Hesr family proteins may affect *DAT* gene expression, as was observed in transfected cells. Further investigation of the in vivo functions of Hesr family members, especially Hesr2 and Hesr3, in the dopaminergic system is needed.

Unique dopamine neurons have recently been found in which DAT expression is relatively low. Lammel et al. (2008) identified a type of dopaminergic neuron within the mesocorticolimbic dopamine system with unconventional fast-firing properties and low DAT/TH mRNA expression ratios that selectively projects to the prefrontal cortex and nucleus accumbens core and medial shell as well as to the basolateral amygdala. Could Hesr family proteins be involved in such a neuron, generating diversity in dopaminergic neurons? Our immunohistochemical study found differential cellular localization between the Hesr family proteins. Hesr1 and Hesr2 were primarily expressed in the nucleus, whereas Hesr3 was cytoplasmic (Fig. 10.3). Additionally, it is possible that cellular localization of Hesr1 is altered depending on the hormonal state (Belandia et al. 2005). A combination of chemical, neuroanatomical, and molecular studies is needed to understand Hesr function in the brain. Such studies may help explain conflicts in the previous in vivo neuroimaging studies (Heinz et al. 2000; Jacobsen et al. 2000; Martinez et al. 2001) and ex vivo RT-PCR analyses (Mill et al. 2002; Brookes et al. 2007).

Although it seems clear from transfection culture studies that the VNTR has a role in regulating DAT1 expression, at the same time, discrepancies have been noted in the differential effects of the various alleles. In the future, an in vivo approach using transgenic mice (e.g., *DAT-9r* or *DAT-10r* knock-in mice) may provide a clearer and more direct approach to characterizing the mechanisms of DAT transcriptional regulation. If such animals are generated, our data from luciferase assays with the mouse Hesr family can add a molecular basis to the research.

Our recent findings of HESR family function regarding *DAT* may suggest new strategies for the treatment of *DAT*-related disorders. Functional VNTR polymorphism also exists in the SERT gene located in intron 2, and two transcription factors, Y box-binding protein 1 (YB-1) and CCTC-binding factor (CTCF), were found to be responsible for the modulation of VNTR function (Klenova et al. 2004). YB-1 and CTCF are targets of lithium (LiCl), a mood stabilizer (Roberts et al. 2007). LiCl modified the levels of CTCF and YB-1 mRNA and protein. HESR proteins may also be a target of drugs.

10.6 Conclusions

Our studies and others indicate that the VNTR in the 3′-UTR of the *DAT* gene affects gene expression. Ex vivo RT-PCR studies and in vivo human neuroimaging studies have demonstrated differential DAT expression depending on the alleles, primarily focusing on 9r and 10r, although the results are conflicting.

More genetic and personality studies combined with neuroimaging should be done to clarify the relation between psychological and neurological states, especially DAT expression levels or function. Further molecular biological studies are also necessary to clarify the mechanism of modification of DAT expression and its signaling pathway, which may also help find new neuropsychological drug targets.

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References

Bannon MJ, Michelhaugh SK, Wang J, Sacchetti P (2001) The human dopamine transporter gene: gene organization, transcriptional regulation, and potential involvement in neuropsychiatric disorder. Eur Neuropsychopharmacol 11:449–455

Belandia B, Powell SM, Garcia-Pedrero JM, Walker MM, Bevan CL, Parker MG (2005) Hey1, a mediator of notch signaling, is an androgen receptor corepressor. Mol Cell Biol 25:1425–1436

Brookes KJ, Neale BM, Sugden K, Khan N, Asherson A, D'Souza UM (2007) Relationship between VNTR polymorphisms of the human dopamine transporter gene and expression in post-mortem midbrain tissue. Am J Med Genet B Neuropsychiatr Genet 144B:1070–1078

Brunswick DJ, Amsterdam JD, Mozley PD, Newberg A (2003) Greater availability of brain dopamine transporters in major depression shown by [99m Tc] TRODAT-1 SPECT imaging. Am J Psychiatry 160:1836–1841

Caine SB (1998) Cocaine abuse: hard knocks for the dopamine hypothesis? Nature Neurosci 1:90–92

Cook EH Jr, Stein MA, Krasowski MD, Cox NJ, Olkon DM, Kieffer JE, Leventhal BL (1995) Association of attention-deficit disorder and dopamine transporter gene. Am J Hum Genet 56:993–998

D'Souza UM, Craig IW (2008) Functional genetic polymorphisms in serotonin and dopamine gene systems and their significance in behavioural disorders. Prog Brain Res 172:73–98

Fauchey V, Jaber M, Caron MG, Bloch B, Moine CL (2000) Differential regulation of the dopamine D1, D2 and D3 receptor gene expression and changes in the phenotype of the striatal neurons in mice lacking the dopamine transporter. Eur J Neurosci 12:19–26

Fuke S, Suo S, Takahashi N, Koike H, Sasagawa N, Ishiura S (2001) The VNTR polymorphism of the human dopamine transporter (DAT1) gene affects gene expression. Pharmacogenomics J 1:152–156

Fuke S, Sasagawa N, Ishiura S (2005) Identification and characterization of the Hesr1/Hey1 as a candidate trans-acting factor on gene expression through the 3′ noncoding polymorphic region of the human dopamine transporter (DAT1) gene. J Biochem 137:205–216

Fuke S, Minami N, Kokubo H, Yoshikawa A, Yasumatsu H, Sasagawa N, Saga Y, Tsukahara T, Ishiura S (2006) Hesr1 knockout mice exhibit behavioral alterations through the dopaminergic nervous system. J Neurosci Res 84:1555–1563

Gainetdinov RR, Sotnikova TD, Caron MG (2002) Monoamine transporter pharmacology and mutant mice. Trends Pharmacol Sci 23:367–373

Giros B, Caron MG (1993) Molecular characterization of the dopamine transporter. Trends Pharmacol Sci 14:43–49

Giros B, el Mestikawy S, Bertrand L, Caron MG (1991) Cloning and functional characterization of a cocaine-sensitive dopamine transporter. FEBS Lett 295:149–154

Giros B, el Mestikawy S, Godinot N, Zheng K, Han H, Yang-Feng T, Caron MG (1992) Cloning, pharmacological characterization, and chromosome assignment of the human dopamine transporter. Mol Pharmacol 42:383–390

Giros B, Jaber M, Jones SR, Wightman RM, Caron MG (1996) Hyperlocomotion and indifference to cocaine and amphetamine in mice lacking the dopamine transporter. Nature 379:606–612

Greenwood TA, Kelsoe JR (2003) Promoter and intronic variants affect the transcriptional regulation of the human dopamine transporter gene. Genomics 82:511–519

Heinz A, Goldman D, Jones DW, Palmour R, Hommer D, Gorey JG, Lee KS, Linnoila M, Weinberger DR (2000) Genotype influences in vivo dopamine transporter availability in human striatum. Neuropsychopharmacology 22:133–139

Henderson AM, Wang SJ, Taylor AC, Aitkenhead M, Hughes CC (2001) The basic helix–loop–helix transcription factor HESR1 regulates endothyelial cell tube formation. J Biol Chem 276:6169–6176

Inoue-Murayama M, Adachi S, Mishima N, Mitani H, Takenaka O, Terao K, Hayasaka I, Ito S, Murayama Y (2002) Variation of variable number of tandem repeat sequences in the 3′-untranslated region of primate dopamine transporter genes that affects reporter gene expression. Neurosci Lett 334:206–210

Iso T, Sartorelli V, Poizat C, Iezzi S, Wu HY, Chung G, Kedes L, Hamamori Y (2001) HERP, a novel heterodimer partner of HES/E (spl) in notch signaling. Mol Cell Biol 21:6080–6089

Iso T, Kedes L, Hamamori Y (2003) HES and HERP families: multiple effectors of the Notch signaling pathway. J Cell Physiol 194:237–255

Jaber M, Dumartin B, Sagne C, Haycock JW, Roubert C, Giros B, Bloch B, Caron MG (1999) Differential regulation of tyrosine hydroxylase in the basal ganglia of mice lacking the dopamine transporter. Eur J Neurosci 11:3499–3511

Jackson DM, Westlind-Danielson A (1994) Dopamine receptors: molecular biology, biochemistry and behavioral aspects. Pharmacol Ther 64:291–336

Jacobsen LK, Staley JK, Zoghbi SS, Seibyl JP, Kosten TR, Innis RB, Gelernter J (2000) Prediction of dopamine transporter binding availability by genotype: a preliminary report. Am J Psychiatry 157:1700–1703

Kanno K, Ishiura S (2009) Function of transcription factor HESR family on dopamine transporter expression via variable number of tandem repeat. Abstract 618.26 from 2009 Society for Neuroscience Annual Meeting, Chicago, IL

Kilty JE, Lorang D, Amara SG (1991) Cloning and expression of a cocaine-sensitive rat dopamine transporter. Science 254:578–579

Klenova E, Scott AC, Roberts J, Shamsuddin S, Lovejoy EA, Bergmann S, Bubb VJ, Royer H-D, Quinn JP (2004) YB-1 and CTCF differentially regulate the 5-HTT polymorphic intron 2 enhancer which predisposes to a variety of neurological disorders. J Neurosci 24:5966–5973

Kokubo H, Lun Y, Johnson RL (1999) Identification and expression of novel family of bHLH cDNAs related to Drosophila hairy and enhancer of split. Biochem Biophys Res Commun 260:459–465

Kokubo H, Tomita-Miyagawa S, Hamada Y, Saga Y (2007) Hesr1 and Hesr2 regulate atrioventricular boundary formation in the developing heart through the repression of Tbx2. Development 134:747–755

Krause KK, Dresel SH, Krause J, Fougere C, Ackenheil M (2003) The dopamine transporter and neuroimaging in attention deficit hyperactivity disorder. Neurosci Biobehav Rev 27:605–613

Lammel S, Hetzel A, Häckel O, Jones I, Liss B, Roeper J (2008) Unique properties of mesoprefrontal neurons within a dual mesocorticolimbic dopamine system. Neuron 57:760–773

Leimeister C, Externbrink A, Klamt B, Gessler M (1999) Hey genes: a novel subfamily of hairy- and enhancer of split related genes specifically expressed during mouse embryogenesis. Mech Dev 85:173–177

Madras BK, Gracz LM, Fahey MA, Elmaleh D, Meltzer PC, Liang AY, Stopa EG, Babich J, Fischman AJ (1998) Altropane, a SPECT or PET imaging probe for dopamine neurons. III. Human dopamine transporter in postmortem normal and Parkinson's diseased brain. Synapse 29:116–127

Martinez D, Gelernter J, Abi-Dargham A, van Dyck CH, Kegeles L, Innis RB, Laruelle M (2001) The variable number of tandem repeats polymorphism of the dopamine transporter gene is not associated with significant change in dopamine transporter phenotype in humans. Neuropsychopharmacology 24:553–560

Michelhaugh SK, Fiskerstrand C, Lovejoy E, Bannon MJ, Quinn JP (2001) The dopamine transporter gene (SLC6A3) variable number of tandem repeats domain enhances transcription in dopamine neurons. J Neurochem 79:1033–1038

Mill J, Asherson P, Browes C, D'Souza U, Craig I (2002) Expression of the dopamine transporter gene is regulated by the 3′ UTR VNTR: evidence from brain and lymphocytes using quantitative RT-PCR. Am J Med Genet B Neuropsychiatr Genet 114B:975–979

Mill J, Asherson P, Craig I, D'Souza UM (2005) Transient expression analysis of allelic variants of a VNTR in the dopamine transporter gene (DAT1). BMC Genet 6:3

Miller GM, Madras BK (2002) Polymorphisms in the 3′-untranslated region of human and monkey dopamine transporter genes affect reporter gene expression. Mol Psychiatry 7:44–55

Missale C, Nash SR, Robinson SW, Jaber M, Caron MG (1998) Dopamine receptors: from structure to function. Physiol Rev 78:189–225

Muller-Vahl KR, Berding G, Brucke T, Kolbe H, Meyer GJ, Hundeshagen H, Dengler R, Knapp WH, Emrich HM (2000) Dopamine transporter binding in Gilles de la Tourette syndrome. J Neurol 247:514–520

Nakagawa O, Nakagawa M, Richardson JA, Olson EN, Srivastava D (1999) HRT1, HRT2, and HRT3: a new subclass of bHLH transcription factors marking specific cardiac, somatic, and pharyngeal arch segments. Dev Biol 216:72–84

Nakagawa O, McFadden DG, Nakagawa M, Yanagisawa H, Hu T, Srivastava D, Olson EN (2000) Members of the HRT family of basic helix–loop–helix proteins act as transcriptional repressors downstream of Notch signaling. Proc Natl Acad Sci USA 97:13655–13660

Ohadi M, Shirazi E, Tehranidoosti M, Moghimi N, Keikhaee MR, Ehssani S et al (2006) Attention-deficit/hyperactivity disorder (ADHD) association with the DAT1 core promoter-67 T allele. Brain Res 1101:1–4

Ohadi M, Keikhaee MR, Javanbakht A, Sargolzaee MR, Robabeh M, Najmabadi H (2007) Gender dimorphism in the DAT1-67 T-allele homozygosity and predisposition to bipolar disorder. Brain Res 1144:142–145

Roberts J, Scott AC, Howard MR, Breen G, Bubb VJ, Klenova E, Quinn JP (2007) Differential regulation of the serotonin transporter gene by lithium is mediated by transcription factors, CCCTC binding protein and Y-box binding protein 1, through the polymorphic intron 2 variable number tandem repeat. J Neurosci 27:2793–2801

Sakamoto M, Hirata H, Ohtsuka T, Bessho Y, Kageyama R (2003) The basic helix–loop–helix genes hesr1/hey1 and hesr2/hey2 regulate maintenance of neural precursor cells in the brain. J Biol Chem 278:44808–44815

Shibuya N, Kamata M, Suzuki A, Matsumoto Y, Goto K, Otani K (2009) The −67 A/T promoter polymorphism in the dopamine transporter gene affects personality traits of Japanese healthy females. Behav Brain Res 203:23–26. doi:10.1016/j.bbr.2009.04.008

Shimada S, Kitayama S, Lin CL, Patel A, Nanthakumar E, Gregor P, Kuhar M, Uhl GR (1991) Cloning and expression of a cocaine-sensitive dopamine transporter complementary DNA. Science 254:576–578

Steidl C, Leimeister C, Klamt B, Maier M, Nanda L, Dixon M, Clarke R, Schmid M, Gessler M (2000) Characterization of the human and mouse HEY1, HEY2, and HEYL genes: cloning, mapping, and mutation screening of a new bHLH gene family. Genomics 66:195–203

Ueno S (2003) Genetic polymorphisms of serotonin and dopamine transporters in mental disorders. J Med Invest 50:25–31

Uhl GR (2003) Dopamine transporter: basic science and human variation of a key molecule for dopaminergic function, locomotion, and Parkinsonism. Mov Disord 18:71–80

Vandenbergh DJ, Persico AM, Hawkins AL, Griffin CA, Li X, Jabs EW, Uhl GR (1992) Human dopamine transporter gene (DAT1) maps to chromosome 5p15.3 and displays a VNTR. Genomics 14:1104–1106

Vandenbergh DJ, Thompson MD, Cook EH, Bendahhou E, Nguyen T, Krasowski MD, Zarrabian D, Comings D, Sellers EM, Tyndale RF, George SR, O'Dowd BF, Uhl GR (2000) Human dopamine transporter gene: coding region conservation among normal, Tourette's disorder, alcohol dependence and attention-deficit hyperactivity disorder populations. Mol Psychiatry 5:283–292