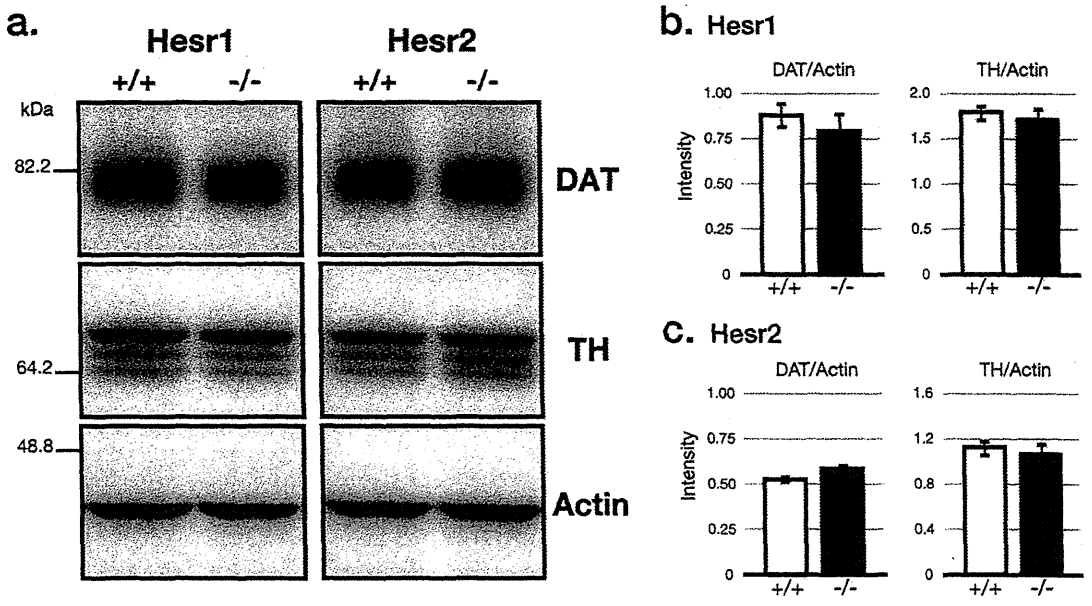

Fig. 3. Western blot analyses of DAT, TH, and actin in the forebrain of wild-type and knockout mice. **a:** Images of immunoblotting for dopamine transporter (DAT; upper panels), tyrosine hydroxylase (TH; middle panels), and actin (lower panels) in wild-type (+/+) and knockout (KO; −/−) mice. **b:** Quantitative Western blotting data in Hesr1 wild-type and KO mice (left panels in a). **c:** Quantitative Western blotting data in Hesr2 wild-type and KO mice (right panels in a). There was no statistically significant difference between wild-type and KO animals. Values are means ± SEM. The numbers of mice used were as follows: Hesr1(+/+), n = 5; Hesr1(−/−), n = 4; Hesr2(+/+), n = 5; Hesr2(−/−), n = 5.

KO lines. In Hesr1 KO mice, DAT mRNA decreased by 33% compared with the wild type.

DAT and TH Expression: Western Blotting

Western blotting analysis was conducted to investigate protein levels of DAT and TH (Fig. 3). As shown in Figure 3b,c, there was a slight reduction in DAT and TH expression in Hesr1 but not Hesr2 KO mice compared with the wild type. However, this difference between genotypes was not statistically significant.

Effect of a Dopamine Agonist on PPI

To determine whether the dopaminergic system was involved in the enhanced PPI observed in Hesr1 KO mice, we investigated the effects of a dopamine agonist on PPI in the Hesr1 line (Fig. 4), because dopamine hyperactivity has been postulated as a possible cause of PPI deficits in schizophrenic patients (Geyer et al., 2001). The generalized linear model test indicated significant effects of dose (saline or 1 or 5 mg/kg of apomorphine) on habituation ($\chi^2 = 23.69$, $P < 0.0001$), startle response ($\chi^2 = 27.63$, $P < 0.0001$), and

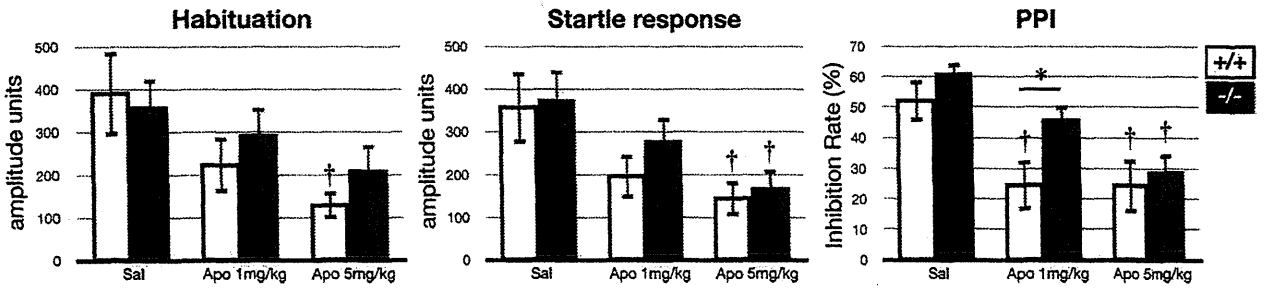

Fig. 4. Effects of the dopamine D1 and D2 receptor agonist apomorphine on PPI in Hesr1 KO mice. An individual mouse was injected with saline (Sal) or 1 or 5 mg/kg pomorphine (Apo) 15 min before measurement of prepulse inhibition (PPI). Average values of the startle response in habituation trials (left panel) and pulse-only trials (middle panel) and of PPI (right panel) are presented. The generalized linear model test indicated significant effects of drug dose (saline and 1 or 5 mg/kg of apomorphine) in all panels. An effect of genotype was detected in only the PPI data. Values represent means ± SEM. *$P < 0.05$, KO (−/−) vs. wild-type (+/+) mice (Student's *t*-test); †$P < 0.05$ vs. saline of the same genotype (Dunnett's method). The numbers of animals used were as follows: Hesr1(+/+), n = 11; Hesr1(−/−), n = 12.

PPI ($\chi^2 = 41.69$, $P < 0.0001$). An effect of genotype was detected only in PPI ($\chi^2 = 10.61$, $P = 0.0011$). There was no significant interaction between dose and genotype in any index (habituation, startle response, and PPI). In the habituation and pulse-only (startle response) trials, post hoc tests indicated that the values of the 5 mg/kg apomorphine condition in wild-type mice were significantly lower than those of saline in the same genotype ($P < 0.05$). In the startle response, the value of the 5 mg/kg apomorphine condition in KO mice was significantly lower than that of saline in the same genotype ($P < 0.05$). The PPI value in the saline group was significantly higher than that of 1 and 5 mg/kg apomorphine in wild-type mice ($P < 0.05$). On the other hand, the PPI value of saline was significantly higher than that of only apomorphine 5 mg/kg in KO mice. Furthermore, when comparing genotypes, the post hoc tests also revealed that the PPI value of KO mice was higher than that of wild-type mice only at the 1 mg/kg dose of apomorphine ($P < 0.05$).

DISCUSSION

Basic Properties of Hesr1 and Hesr2 KO Mice

In the behavioral test battery, no apparent deficit in motor or spontaneous activity was observed in the novel cage, home cage, open field, or rota-rod tests (Table I), although the body weights of both KO mice were significantly lower than those of the wild-type mice, and lower sensitivity to pain in the hotplate test was observed in the Hesr2 KO mice compared with the wild type. Thus, the KO mice did not show any difficulties caused by deficits in motor or spontaneous activity for measurement of emotional behaviors.

In our previous study (Fuke et al., 2006), Hesr1 KO mice exhibited low spontaneous locomotion (open field test) and anxiety-like behavior (open field and elevated plus maze tests). However, in this study, we could not replicate those data. Possible reasons include that the mice after weaning were kept in a different facility and were investigated by a different experimenter. Furthermore, the mice were back-crossed to C57BL/6J for generations. The difference in genetic background may be a cause of the difference in behavioral phenotype in this study compared with previous results. Another possible reason for this may be differences in the apparatuses. Specifically, the open field box was wider and the observation time was shorter. Also, the elevated plus maze floor was 17 cm higher and the open arm was longer and narrower. Thus, the conditions were relatively more severe than in previous studies. The scores of locomotion and time in the open arm (an indicator of anxiety) in both the wild-type and the KO mice were too low to detect any differences. However, when a 24-hr temporal pattern of home cage activity was analyzed in the Hesr1 line (1-hr bin), the generalized linear model test indicated a significant main effect of genotype; Hesr1 KO mouse activity was relatively lower (see Supp. Info. Fig. 1). Furthermore, when the generalized linear model test was applied to the temporal data of total distance in the open field test and time in the light area of the light–dark box test (Supp. Info. Figs. 2, 3), a significant main effect of genotype in the Hesr1 line was observed. The post hoc tests also indicated a time-point-specific significance between genotypes: the distance value in Hesr1 KO mice was lower and time in the light area was shorter than in the wild-type mice at certain time points. These differences in spontaneous activity and anxiety-like behavior were slight, but we believe that our previous data were in part replicated.

Enhanced PPI and Differing Sensitivity to Dopamine Agonist in Hesr1 KO Mice

In the presence of prepulse, it is known that the startle response to strong sensory stimuli is inhibited compared with the response in the absence of prepulse (Geyer et al., 2001). That is, the prepulse makes the animals less reactive to a stimulus applied immediately after the prepulse. It has also been reported that schizophrenia patients exhibit lower PPI, suggesting that they may have deficits in an operational measure of sensorimotor gating (Geyer et al., 2001). D2 receptor antagonists, one type of medication used to treat schizophrenia, enhance PPI, whereas apomorphine, a D1 and D2 receptor agonist, reduces PPI (Geyer et al., 2001). Here, we demonstrated that PPI was enhanced in Hesr1 KO mice (Fig. 1) and that PPI was reduced following apomorphine injection in a dose-dependent manner, as reported previously (Geyer et al., 2001). Furthermore, we found that Hesr1 KO mice showed a lower sensitivity to apomorphine: Hesr1 KO mice required a higher dose of apomorphine to reduce PPI compared with the wild type, without any differences in startle response (Fig. 4). These findings suggest that the enhanced PPI resulted from dopaminergic alterations in Hesr1 KO mice, particularly the altered sensitivity to dopamine or an agonist. Thus, Hesr1 is likely involved in the function of the dopaminergic system.

There are, however, confusing results in Figures 1 and 4. A genotype difference of PPI in the Hesr1 KO line was significant in Figure 1, but there was no difference in the saline condition in Figure 4. We cannot explain these discrepancies, but prior experience with other behavioral battery tests or injection stress could affect the results to a certain extent. Although there was no difference in the saline condition (Fig. 4), PPI in the Hesr1 KO mice differed in Figure 1 (*t*-test), and the generalized linear model also indicated a genotype difference (Fig. 4). Furthermore, the value of PPI in Hesr1 KO mice was approximately twofold higher than that of the wild type in the apomorphine 1 mg/kg condition, suggesting that Hesr1 KO mice exhibit relatively high PPI at least under specific conditions.

DAT Expression in KO Mice and Molecular Contribution of Hesr1 to PPI

Here we demonstrate the involvement of Hesr1 in the sensorimotor gating system, possibly accompanied

by dopaminergic modulation. As reported from our previous study (Kanno and Ishiura, 2011), each Hesr was expressed in dopaminergic neurons throughout the SN and VTA. This suggests that the HESR family can influence DAT expression in dopaminergic neurons in vivo, as observed in our previous culture studies (Kanno and Ishiura, 2011). In fact, DAT mRNA was significantly higher in Hesr1 KO than in wild-type mice at postnatal day 0 (Fuke et al., 2006). These data seem reasonable, in that our previous studies demonstrated the inhibitory effect of HESR1 on a DAT reporter gene in mammalian cell lines (Fuke et al., 2005; Kanno and Ishiura, 2011). Additionally, it has been reported that PPI is lower in DAT KO mice (Geyer et al., 2001). This is the phenotype opposite to that of the Hesr1 KO mice, and, if the DAT levels are higher in adult Hesr1 KO mice, then the molecular dynamics are correlated with the phenotype. However, in this study, DAT and TH proteins, the expression levels of which are thought to reflect the amounts of dopamine innervation or enzymatic activity, were comparable between wild-type and KO mice of both Hesr strains. Moreover, the DAT mRNA level in adult Hesr1 KO mice was actually lower than in the wild type, contrary to our expectations.

These results are puzzling, and further investigations will provide possible explanations. Many environmental and pharmacological manipulations during the developmental stages have been reported to affect PPI (Geyer et al., 2001), suggesting that upregulated DAT in the developmental phase (Fuke et al., 2006) could alter some of the neuronal substrates that affect PPI. In fact, HESR family members have been described in developmental signaling (Dahlqvist et al., 2003; Takizawa et al., 2003; Zavadil et al., 2004) and in the differentiation and maintenance of the dopaminergic nervous system (Stull et al., 2001; Farkas et al., 2003; Sanchez-Capelo et al., 2003). Thus, the physiological functions of the HESRs should be further investigated, focusing on target genes other than DAT or dopamine-related genes because many HESR target genes exist (Fischer and Gessler, 2007).

HESRs had not been reported in clinical studies of psychiatric or developmental disorders, but recent studies have suggested involvement of HESR1 in such disorders, sometimes interacting with other factors, as described below. HESR1 was reportedly upregulated in cell lines derived from the patients of autism-spectrum disorder (Seno et al., 2011). We previously demonstrated that HESR1 with a naturally occurring nonsynonymous SNP at codon 94 (Lue94Met, SNP ID rs11553421) in the HLH domain did not have the ability to repress DAT reporter gene expression (Fuke et al., 2005). Additionally, this SNP converts HESR1 from an androgen receptor corepressor to a coactivator and abolishes HESR1-mediated activation of p53 (Villaronga et al., 2010), which has been reported as a schizophrenia susceptibility gene (Allen et al., 2008). The VNTRs of DAT1 (Cook et al., 1995) and DAT expression level (Krause et al., 2003) are associated with ADHD, features of which are shared with autism-spectrum disorder to a certain degree (Rommelse et al., 2010). Therefore, HESRs may be involved in psychiatric disorders, developmental delay, and some behavioral traits.

CONCLUSIONS

The present study demonstrates that the lack of Hesr1 leads to an alteration in sensitivity to dopamine accompanied by enhanced PPI. This suggests that expression of Hesr1 could influence sensorimotor gating at the physiological level. The functional relationship between HESRs and other target genes involved in sensorimotor gating should be investigated further.

ACKNOWLEDGMENTS

The authors declare no conflicts of interest. We thank Dr. Yumiko Saga (Division of Mammalian Development, National Institute of Genetics) for her kind support.

REFERENCES

Allen NC, Bagade S, McQueen MB, Ioannidis JP, Kavvoura FK, Khoury MJ, Tanzi RE, Bertram L. 2008. Systematic meta-analyses and field synopsis of genetic association studies in schizophrenia: the SzGene database. Nat Genet 40:827–834.

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

Cook EH, 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.

Crawley J, Goodwin FK. 1980. Preliminary report of a simple animal behavior model for the anxiolytic effects of benzodiazepines. Pharmacol Biochem Behav 13:167–170.

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.

Dahlqvist C, Blokzijl A, Chapman G, Falk A, Dannaeus K, Ibanez CF, Lendahl U. 2003. Functional notch signaling is required for BMP4-induced inhibition of myogenic differentiation. Development 130: 6089–6099.

Deacon RM. 2013. Measuring motor coordination in mice. J Vis Exp 75.

Farkas LM, Dunker N, Roussa E, Unsicker K, Krieglstein K. 2003. Transforming growth factor-betas are essential for the development of midbrain dopaminergic neurons in vitro and in vivo. J Neurosci 23: 5178–5186.

Fischer A, Gessler M. 2007. Delta-Notch–and then? Protein interactions and proposed modes of repression by Hes and Hey bHLH factors. Nucleic Acids Res 35:4583–4596.

Franklin KBJ, Paxinos G. 2008. The mouse brain in stereotaxic coordinates. Amsterdam: Elsevier/Academic Press.

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′ non-coding 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.

Geyer MA, Krebs-Thomson K, Braff DL, Swerdlow NR. 2001. Pharmacological studies of prepulse inhibition models of sensorimotor gating deficits in schizophrenia: a decade in review. Psychopharmacology 156:117–154.

Greenwood TA, Kelsoe JR. 2003. Promoter and intronic variants affect the transcriptional regulation of the human dopamine transporter gene. Genomics 82:511–520.

Heinz A, Goldman D, Jones DW, Palmour R, Hommer D, Gorey JG, Lee KS, Linnoila M, Winberger 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 CCW. 2001. The basic helix-loop-helix transcription factor HESR1 regulates endothelial 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.

Jackson DM, Westlinddanielsson A. 1994. Dopamine receptors: molecular biology, biochemistory and behavioral aspects. Pharmacol Ther 64: 291–370.

Jacobsen LK, Staley JK, Zoghbi S, 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.

Jones BJ, Roberts DJ. 1968. The quantitative measurement of motor inco-ordination in naïve mice using an accelerating rotarod. J Pharm Pharmacol 20:302–304.

Kanno K, Ishiura S. 2011. Differential effects of the HESR/HEY transcription factor family on dopamine transporter reporter gene expression via variable number of tandem repeats. J Neurosci Res 89:562–575.

Koide T, Moriwaki K, Ikeda K, Niki H, Shiroishi T. 2000. Multi-phenotype behavioral characterization of inbred strains derived from wild stocks of Mus musculus. Mamm Genome 11:664–670.

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

Kokubo H, Miyagawa-Tomita S, Nakazawa M, Saga Y, Johnson RL. 2005. Mouse hesr1 and hesr2 genes are redundantly required to mediate Notch signaling in the developing cardiovascular system. Dev Biol 278: 301–309.

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

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.

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.

Miczek KA, O'Donnell JM. 1978. Intruder-evoked aggression in isolated and nonisolated mice: effects of psychomotor stimulants and L-dopa. Psychopharmacology 57:47–55.

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.

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.

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, somitic, and pharyngeal arch segments. Dev Biol 216:72–84.

Nishi A, Ishii A, Takahashi A, Shiroishi T, Koide T. 2010. QTL analysis of measures of mouse home cage activity using B6/MSM consomic strains. Mamm Genome 21:477–485.

Pellow S, Chopin P, File SE, Briley M. 1985. Validation of open:closed arm entries in an elevated plus maze as a measure of anxiety in the rat. J Neurosci Methods 14:149–167.

Rommelse NN, Franke B, Geurts HM, Hartman CA, Buitelaar JK. 2010. Shared heritability of attention-deficit/hyperactivity disorder and autism spectrum disorder. Eur Child Adolesc Psychiatry 19:281–295.

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.

Sanchez-Capelo A, Colin P, Guibert B, Biguet NF, Mallet J. 2003. Transforming growth factor beta 1 overexpression in the nigrostriatal system increases the dopaminergic deficit of MPTP mice. Mol Cell Neurosci 23:614–625.

Seno MM, Hu P, Gwadry FG, Pinto D, Marshall CR, Casallo G, Scherer SW. 2011. Gene and miRNA expression profiles in autism spectrum disorders. Brain Res 1380:85–97.

Stull ND, Jung JW, Iacovitti L. 2001. Induction of a dopaminergic phenotype in cultured striatal neurons by bone morphogenetic proteins. Brain Res Dev Brain Res 130:91–98.

Takahashi A, Kato K, Makino J, Shiroishi T, Koide T. 2006. Multivariate analysis of temporal descriptions of open field behavior in wild-derived mouse strains. Behav Genet 36:763–774.

Takahashi A, Nishi A, Ishii A, Shiroishi T, Koide T. 2008. Systematic analysis of emotionality in consomic mouse strains established from C57BL/6J and wild-derived MSM/Ms. Genes Brain Behav 7:849–858.

Takizawa T, Ochiai W, Nakashima K, Taga T. 2003. Enhanced gene activation by Notch and BMP signaling cross-talk. Nucleic Acids Res 31:5723–5731.

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

Ueno S, Nakamura M, Mikami M, Kondoh K, Ishiguro H, Arinami T, Komiyama T, Mitsushio H, Sano A, Tanabe H. 1999. Identification of a novel polymorphism of the human dopamine transporter (DAT1) gene and the significant association with alcoholism. Mol Psychiatry 4: 552–557.

Uhl GR. 2003. Dopamine transporter: Basic science and human variation of a key molecule for dopaminergic function, locomotion, and parkinsonism. Mov Disord 18:S71–S80.

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.

VanNess SH, Owens MJ, Kilts CD. 2005. The variable number of tandem repeats element in DATI regulates in vitro dopamine transporter density. BMC Genet 6.

Villaronga MA, Lavery DN, Bevan CL, Llanos S, Belandia B. 2010. HEY1 Leu94Met gene polymorphism dramatically modifies its biological functions. Oncogene 29:411–420.

Wang WL, Campos AH, Prince CZ, Mou YS, Pollman MJ. 2002. Coordinate Notch3-hairy-related transcription factor pathway regulation in response to arterial injury—mediator role of platelet-derived growth factor and ERK. J Biol Chem 277:23165–23171.

Zavadil J, Cermak L, Soto-Nieves N, Bottinger EP. 2004. Integration of TGF-beta/Smad and Jagged1/Notch signalling in epithelial-to-mesenchymal transition. EMBO J 23:1155–1165.

Journal of Neuroscience Research

Journal of Neuroscience Research 92:671–678 (2014)

Nonredundant Function of Two Highly Homologous Octopamine Receptors in Food-Deprivation-Mediated Signaling in *Caenorhabditis elegans*

Midori Yoshida,[1] Eitaro Oami,[2] Min Wang,[3] Shoichi Ishiura,[1,2] and Satoshi Suo[2]*

[1]Department of Biological Sciences, Graduate School of Science, University of Tokyo, Tokyo, Japan
[2]Department of Life Sciences, Graduate School of Arts and Sciences, University of Tokyo, Tokyo, Japan
[3]College of Arts and Sciences, University of Tokyo, Tokyo, Japan

It is common for neurotransmitters to possess multiple receptors that couple to the same intracellular signaling molecules. This study analyzes two highly homologous G-protein-coupled octopamine receptors using the model animal *Caenorhabditis elegans*. In *C. elegans*, the amine neurotransmitter octopamine induces activation of cAMP response element-binding protein (CREB) in the cholinergic SIA neurons in the absence of food through activation of the Gq-coupled octopamine receptor SER-3 in these neurons. We also analyzed another Gq-coupled octopamine receptor, SER-6, that is highly homologous to SER-3. As seen in *ser-3* deletion mutants, octopamine- and food-deprivation-mediated CREB activation was decreased in *ser-6* deletion mutants compared with wild-type animals, suggesting that both SER-3 and SER-6 are required for signal transduction. Cell-specific expression of SER-6 in the SIA neurons was sufficient to restore CREB activation in the *ser-6* mutants, indicating that SER-6, like SER-3, functions in these neurons. Taken together, these results demonstrate that two similar G-protein-coupled receptors, SER-3 and SER-6, function in the same cells in a nonredundant manner.

Key words: G-protein-coupled receptor; CREB; octopamine; *C. elegans*; food deprivation

Amine neurotransmitters, such as dopamine, noradrenaline, and serotonin, signal primarily through G-protein-coupled receptors (GPCRs). Each neurotransmitter is capable of binding multiple receptors, which in turn couple different G proteins, allowing a single neurotransmitter to activate multiple intracellular signaling pathways. In many cases, multiple receptors bind to the same neurotransmitter and activate the same intracellular signaling cascades. The α_1-adrenergic receptors, for example, consist of three subtypes, α_{1a}, α_{1b}, and α_{1d}. All three receptors bind to both adrenaline and noradrenaline, couple to G protein Gq, and induce activation of phospholipase C. The physiological significance of having multiple receptors with the same function is not well understood. Studies in receptor-knockout mice suggest that these receptors may not be entirely redundant, in part because expression of each receptor is restricted to distinct cell types (Chen and Minneman, 2005).

Recent studies have shown that GPCRs are capable of regulating each other through the formation of heterodimers in vivo and in doing so acquire new functions (Gupta et al., 2010; Pei et al., 2010; He et al., 2011). Functionally similar receptors have been shown to form heterodimers when expressed heterologously in cultured cells, suggesting that these types of receptors can work cooperatively. For example, the α_{1b}-adrenergic receptor facilitates internalization of the α_{1a}-adrenergic receptor by forming a hetero-oligomer, without affecting the pharmacology or signaling of either receptor (Stanasila et al., 2003). Similarly, the α_{1b}-adrenergic receptor is capable of binding the α_{1d}-adrenergic receptor, facilitating its expression on the surface of the cell (Hague et al., 2004). This heterodimer behaves as a single functional entity with increased signaling (Hague et al., 2006). Together, these interactions suggest that similar receptors may perform nonredundant functions when expressed in the same cell. This study analyzes two homologous receptors, SER-3 and SER-6, which likely couple to the same G protein signaling in the model organism *Caenorhabditis elegans*.

Amine neurotransmitters regulate activation of cAMP response element-binding protein (CREB) in *C. elegans* (Suo et al., 2006, 2009). CREB is a transcription factor that plays essential roles in a variety of biological

Contract grant sponsor: KAKENHI, Contract grant number: 23115705 (to S.S.); Contract grant number: 23700439 (to S.S.); Contract grant sponsor: Ministry of Health, Labor and Welfare, Japan, Contract grant number: 23-5 (to S.I.).

*Correspondence to: Satoshi Suo, Komaba 3-8-1, Meguro-ku, Tokyo, 153-8902, Japan. E-mail: suo@bio.c.u-tokyo.ac.jp

Received 20 July 2013; Revised 30 October 2013; Accepted 17 November 2013

Published online 21 January 2014 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/jnr.23345

processes (Lonze and Ginty, 2002; Johannessen et al., 2004). It binds to specific DNA sequences called cAMP response elements (CRE) and regulates expression of its target genes upon phosphorylation (Mayr and Montminy, 2001). Using a reporter for CREB activation, we previously found that CREB is activated in the cholinergic SIA neurons in the absence of food (Suo et al., 2006). This signaling is mediated by the amine neurotransmitter octopamine, which is considered to be the biological equivalent of mammalian noradrenaline (Roeder, 1999), because food-deprivation-mediated CREB activation was decreased in the octopamine-deficient mutant *tbh-1* and CREB can be activated by addition of exogenous octopamine. SER-3, a putative Gq-coupled octopamine receptor, and EGL-30, an α subunit of Gq, function in the SIA neurons to induce CREB activation. Furthermore, this octopamine signaling is suppressed by dopamine through activation of the dopamine receptors DOP-2 and DOP-3 (Suo et al., 2009).

In addition to SER-3, *C. elegans* has another putative octopamine receptor, SER-6, that is highly homologous to SER-3. SER-6 has been shown to bind octopamine and is believed to couple Gq because of its ability to activate inward currents upon octopamine treatment when heterologously expressed in *Xenopus* oocytes, which presumably is mediated by endogenous Ca^{2+}-gated chloride channels (Mills et al., 2012). In this study, we show that SER-6 is involved in octopamine-mediated CREB activation and functions in SIA neurons, similarly to SER-3. Interestingly, loss of either SER-3 or SER-6 leads to diminished signaling, indicating that both receptors are required for normal signaling. These two similar octopamine receptors are therefore working in the same cells and function in a nonredundant manner in vivo.

MATERIALS AND METHODS

Strains

Culturing and genetic manipulation of *C. elegans* were performed as described previously (Brenner, 1974). The alleles used in this study were as follows: *ser-3(ad1774)* I (Suo et al., 2006), *ser-6(tm2104)* IV and *ser-6(tm2146)* IV (gifts from the National BioResource Project [NBRP], Ministry of Education, Culture, Sports, Science and Technology [MEXT], Tokyo, Japan), *octr-1(ok371)X* (Wragg et al., 2007), *tyra-3(ok325)X* (Wragg et al., 2007), *unc-64(e246) III* (Brenner, 1974), *tbh-1(ok1196)* (Suo et al., 2006), and *tzIs3[cre::gfp; lin-15(+)* (Kimura et al., 2002). All mutants used in the CREB activity assay carry *cre::gfp* reporter. These mutants were generated by mating *tzIs3* males with other mutants. The resulting genotypes were confirmed by PCR. *tbh-1(ok1196);tzIs3*, *ser-3(ad1774);tzIs3*, and *unc-64(e246)III;tzIs3* were constructed previously (Suo et al., 2006).

Cloning of *ser-6*

Total *C. elegans* RNA was extracted from all stages of a wild-type Bristol N2 strain using Trizol reagent (Gibco BRL, Rockville, MD). The cDNA of SER-6 was synthesized using a gene-specific primer (5′-TACATACAATTGAATTTCAG-3′) and the Prime Script 1st strand cDNA synthesis kit (TaKaRa). PCR was carried out with a SER-6 reverse primer (5′-GAA CAATTATTACTGAACTGC-3′) and an SL1 primer (5′-GGTTTAATTACCCAAGTTTGAG-3′) matching the 5′-trans-spliced leader sequence found on *C. elegans* RNAs (Blaxter and Liu, 1996) using PfuUltra High-Fidelity DNA Polymerase (Stratagene, La Jolla, CA). The resulting PCR product was cloned into pCR-Blunt (Invitrogen, Carlsbad, CA) and sequenced.

Phylogenetic Analysis

The amino acid sequences of SER-6 and other biogenic amine receptors of human and invertebrates were aligned with ClustalW (DNA Databank of Japan), using relatively well-conserved regions excluding the N terminus, second extracellular loop, third intracellular loop, and the C terminus of these receptors. The phylogenic tree was drawn with PHYLIP by the Fitch-Margoliash method and visualized with TreeView.

Analyses of CRE-Mediated Gene Expression

CREB activation assays were performed as described previously (Suo et al., 2006, 2009). Briefly, animals carrying *cre::gfp* were synchronized by a hypochlorite treatment, and the resulting eggs were placed on NGM plates seeded with *Escherichia coli* OP50 (Brenner, 1974). Animals were incubated for 2 days at 20°C, transferred to new NGM plates, and incubated for an additional 24 hr. Animals were then transferred onto assay plates and incubated for 4 hr at 20°C. Each assay plate contained 1.7% AgarNoble (BD Diagnostics, San Jose, CA) with or without 3 mg/ml octopamine-hydrochloride (Sigma-Aldrich, St. Louis, MO), with bacterial food spread on its surface. For food-depletion assays, synchronized animals were incubated on NGM plates seeded with or without OP50 at 20°C for 6 hr. For soaking assays, synchronized animals were incubated for 4 hr at 20°C on 60-mm NGM plates seeded with bacterial food and overlaid with ~5 ml water. After incubation, animals were collected in M9 buffer (Brenner, 1974) containing 50 mM NaN_3 and mounted on glass slides. The number of SIA neurons expressing green fluorescent protein (GFP) was counted for each animal using a fluorescence microscope (Olympus BX53) to quantify CREB activation. All counting was performed by an experimenter blinded to the genotype and incubation conditions of the animals. Statistical significance was evaluated by an analysis of variance followed by a Tukey-Kramer multiple-comparisons test in GraphPad Prism. Images of animals were obtained with the fluorescence microscope.

Analyses of *ser-6* Expression Patterns

The transcriptional reporter fusion gene *ser-6::gfp* was generated using the fusion PCR method as described elsewhere (Hobert, 2002) using the primers Y54fusionA (5′-GTTAA GCTCCTCGAACTTTCGG-3′), Y54fusionB (5′-AGTCGA CCTGCAGGCATGCAAGCTGCCCAGCGTCAGTGATA GC-3′), Y54fusionE (5′-CTCTCAAACTTTCCGGCGC-3′), fusionD (5′-AAGGGCCCGTACGGCCGACTAGTAGG-3′), fusionF (5′-GGAAACAGTTATGTTTGGTATATTGGG-3′), and fusionC (5′-AGCTTGCATGCCTGCAGGTCGACT-3′). The region corresponding to 5.0-kb upstream and a part of

exon 1 of *ser-6* gene was amplified with the primers Y54fusionA and Y54fusionB by LA Taq (TaKaRa) using genomic DNA as the template. The resulting PCR product was fused to 2–1876 of pPD95.75. *ser-6::gfp* was injected into N2 wild-type animals together with *ceh-17::dsred* (Pujol et al., 2000; Suo et al., 2006), *tbh-1::dsred* (Alkema et al., 2005; Suo et al., 2006), pBluescript (Stratagene), and the transformation marker pRF4, which contains the dominant roller mutation *rol-6(su1006)* (Kramer et al., 1990), as described by Mello et al. (1991). Concentrations of the injected plasmids were 30, 10, 10, 30, and 20 ng/μl, respectively. Images of transformants were obtained with a confocal laser microscope (Leica inverted microscope DMI6000 B).

Cell-Specific Rescue of *ser-6*

To express *ser-6* in the SIA neurons, cDNA of *ser-6* was fused to the *ceh-17* promoter, which induces gene expression in only the SIA and ALA neurons. The coding sequence of *ser-6* was amplified with the corresponding forward (5′-TTCGCC ACCGGTAAAAATGATTTTGCTATC-3′) and reverse (5′-AAATAAGCGGCCGCTCAAAATTTTGGCTTC-3′) primers by PfuUltra High-Fidelity DNA Polymerase (Stratagene) using subcloned *ser-6* cDNA as the template. The PCR product was digested with the restriction enzymes *Age*I and *Not*I and cloned into *Age*I- and *Not*I-digested *ceh-17::dop-2l* (Suo et al., 2009) to obtain *ceh-17::ser-6*. *ceh-17::ser-6* was then injected into *ser-6(tm2104);tzIs3* together with the transformation marker *lin-44::gfp* (Murakami et al., 2001) and pBluescript (Stratagene). The concentrations of the injected *ceh-17::ser-6*, *lin-44::gfp*, and pBluescript were 10, 20, and 70 ng/μl, respectively. Animals carrying *lin-44::gfp*, reflected by expression of GFP in the tail hypodermis, were analyzed in the rescue experiments.

Generation of Heterozygous Mutants and Overexpression of *ser-3* and *ser-6*

To generate heterozygous mutant animals, *ser-3(ad1774);ser-6(tm2104);tzIs3* males, *unc-64(e246)III;tzIs3* hermaphrodites, *ser-3(ad1774);unc-64(e246)III;tzIs3* hermaphrodites, or *ser-6(tm2104);unc-64(e246)III;tzIs3* hermaphrodites were mated before each assay. *unc-64* homozygous animals exhibit an uncoordinated phenotype (Unc; Brenner, 1974). Only non-Unc F1 animals were tested, because Unc animals result from self-fertilization.

To obtain strains that overexpress SER-6 in the SIA neurons, *ceh-17::ser-6* was injected into *ser-3(ad1774);tzIs3*, together with *lin-44::gfp* and pBluescript (Stratagene). The concentrations of the injected expression plasmids, *lin-44::gfp*, and pBluescript were 10, 10, and 80 ng/μl, respectively. CREB activation was analyzed using transformants that express GFP in the tail hypodermis.

To obtain strains that overexpress SER-3 in the SIA neurons, the *ceh-17::ser-3* fusion construct (Suo et al., 2006) was injected into *ser-3(ad1774);tzIs3* together with *lin-44::gfp* and pBluescript (Stratagene). The concentrations of the injected expression plasmids, *lin-44::gfp*, and pBluescript were 10, 10, and 80 ng/μl, respectively. The transformant was then mated with *tzIs3* males, and the sibling *tzIs3* animals carrying the *ceh-17::ser-3* fusion construct were mated with *ser-6(tm2104);tzIs3* males to obtain *ser-6(tm2104);tzIs3* carrying the *ceh-17::ser-3* fusion gene.

RESULTS

SER-6 Is Highly Homologous to the Gq-Coupled Octopamine Receptor SER-3

SER-6 was identified as an amine neurotransmitter receptor by comparing the amino acid sequences of amine receptors between human and *C. elegans* (Chase et al., 2004). Srinivasan et al. (2008) showed that *ser-6* deletion mutants have a defect in serotonin-induced reduction of fat storage. Furthermore, Mills et al. (2012) showed that SER-6 is required for octopamine-mediated alteration of octanol sensitivity. SER-6 has also been shown to function as an octopamine receptor and possibly couple to the Gq signal pathway by an electrophysiological experiment using *Xenopus* oocyte heterologously expressing SER-6 (Mills et al., 2012).

We cloned cDNA of *ser-6* and compared the amino acid sequence of SER-6 with that of SER-3 (Fig. 1A,B). SER-3 is likely a Gq-coupled octopamine receptor and increases intracellular Ca^{2+} concentration in response to 10 nM octopamine when expressed in HEK293 cells (Petrascheck et al., 2007). As expected, SER-3 and SER-6 were highly homologous. The phylogenic tree including human and invertebrate amine receptors (Fig. 1C) shows that SER-6 is homologous to other Gq-coupled octopamine receptors of invertebrates, including SER-3 and insect octopamine receptors AmOAMB and DmOAMB (Han et al., 1998; Grohmann et al., 2003). Among mammalian amine receptors, SER-6 was most closely related to the human α_1-adrenergic receptors, which are also Gq-coupled receptors.

SER-6 Is Involved in Octopamine-Dependent CREB Activation in the SIA Neurons

In *C. elegans*, CREB activation can be detected by fluorescence in animals carrying a *cre::gfp* reporter, in which CRE is fused to a GFP sequence (Kimura et al., 2002). Using this reporter, we have shown that food deprivation induces CREB activation in the SIA neurons (Suo et al., 2006, 2009). This response appears to be mediated through octopamine, because exogenously applied octopamine similarly activates CREB in the SIA neurons, and mutants in the *tbh-1* gene, which encodes a tyramine β-hydroxylase required for octopamine synthesis (Alkema et al., 2005), exhibit decreased response to food deprivation. SER-3 has been shown to function in the SIA neurons to transmit octopamine signaling through EGL-30, the α subunit of Gq. Here, we determined whether SER-6 is also involved in this CREB activation.

Animals carrying *cre::gfp* were exposed to 3 mg/ml octopamine for 4 hr or deprived of food for 6 hr. The number of SIA neurons in each animal expressing GFP was then counted to quantify CREB activation. Wild-type animals exhibited significant GFP expression in the SIA neurons following octopamine treatment or food deprivation (Fig. 2B,E). *C. elegans* has four SIA neurons

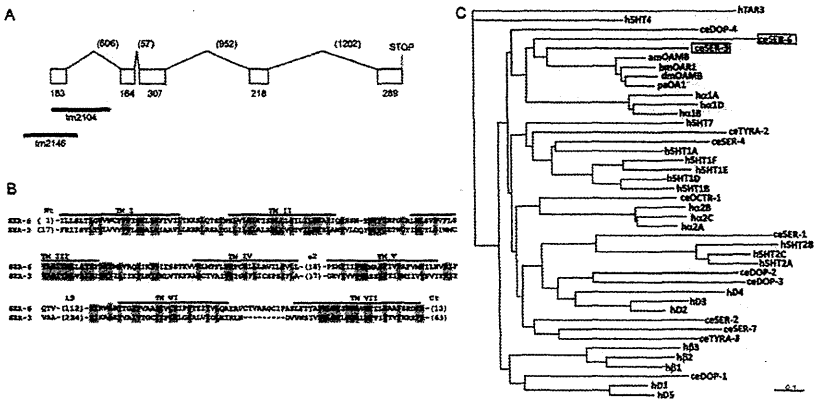

Fig. 1. Gene structure of *ser-6* and comparison between SER-6 and other amine receptors. *ser-6* cDNA was cloned, and the structure of this gene was identified. Black bars indicate the region deleted in the *tm2104* and *tm2146* alleles (**A**). The amino acid sequence of SER-6 was aligned with SER-3 (**B**). Predicted transmembrane (TMs) regions are overscored. Amino acid residues conserved between SER-6 and SER-3 are indicated by gray shading. Numbers in parentheses represent the number of amino acids not shown in the figure. According to the phylogenic tree of SER-6 and other biogenic amine receptors of human and invertebrates, SER-3 and SER-6 are highly homologous (**C**). The amino acid sequences of each receptor were aligned with ClustalW using relatively conserved regions, excluding the N terminus, second extracellular loop, third intracellular loop, and C terminus. The phylogenic tree was calculated by using the PHYLIP package and the Fitch-Margoliash method. Receptor sequences used and the GenBank accession numbers are as follows: *C. elegans* octopamine receptors (ceSER-3, NP491954; ceOCTR-1, CCD83472.1), *C. elegans* dopamine receptors (ceDOP-1, CCD68411.1; ceDOP-2, CBY85347.1; ceDOP-3, NP_001024907.2l ceDOP-4, CCD65696.1), *C. elegans* tyramine receptors (ceTYRA-2, CCD83463.1; ceTYRA-3, CCD83479.1; ceSER-2, NP_001024335.1), *C. elegans* serotonin receptors (ceSER-1, CCD63419.1; ceSER-4, CCD73768.1; ceSER-7, CCD83456.1), insect α-adrenergic-like octopamine receptors (dmOAMB, AAC17442; amOAMB, CAD67999; paOA1, AAP93817.1; bmOAR1, NP_
001091748.1), human dopamine receptors (hD1, P21728; hD2, P14416; hD3, P35462; hD4, P21917; hD5, P21918), human serotonin receptors (h5HT1a, I38209; h5HT1b, JN0268; h5HT1d, A53279; h5HT1e, A45260; h5HT1f, A47321; h5HT2a, A43956; h5HT2b, S43687; h5HT2c, JS0616; h5HT4, Q13639; h5HT7, A48881), and human adrenergic receptors (hα1A, NP000671; hα1B, NP000670; hα1D, NP000669; hα2A, A34169; hα2B, A37223; hα2C, A31237; hβ1, QRHUB1; hβ2, QRHUB2; hβ3, QRHUB3). A human trace amine receptor 3 (hTAR3, AAO24660) was used as an out group. bm, *Bombyx mori*; pa, *Periplaneta americana*; dm, *Drosophila melanogaster*; am, *Apis mellifera*.

(SIADL, SIADR, SIAVL, and SIAVR) and there was no apparent difference in GFP expression rates of these four neurons. As reported previously, this CRE-mediated gene expression was dependent on SER-3, with *ser-3* mutants showing decreased responses to exogenous octopamine and food deprivation (Fig. 2F). Next, we examined two deletion alleles of *ser-6*, *tm2104* and *tm2146*, and found that octopamine-mediated GFP expression was decreased in both mutants (Fig. 2D,G,H). These results suggest that SER-6 is also required for octopamine-dependent CREB activation in the SIA neurons. CREB activation levels induced by food deprivation were also decreased in *ser-6* animals (Fig. 2G,H), suggesting that SER-6 is involved in food deprivation-induced CREB activation in the SIA neurons.

The response to food deprivation was significantly attenuated in octopamine-deficient *tbh-1* mutants (Fig. 2J). However, a small response was observed, consistent with previous reports (Suo et al., 2006), suggesting that the response to food deprivation is partially octopamine independent. The level of CREB activation observed in the *ser-3* mutants in the absence of food was similar to that of *tbh-1*. We also analyzed *tbh-1;ser-3* double mutants and found that *tbh-1;ser-3* responded to food deprivation slightly more strongly than *ser-3* and *tbh-1* single mutants (Fig. 2K). The reason for this increase is unknown. However, because CREB activation was not decreased by the *tbh-1* mutation in the double mutants, it is likely that the CREB activity observed in the *ser-3* mutants is octopamine independent. In contrast, the level of CREB activation in the *ser-6* mutants was higher than that in the *tbh-1* mutants, and the level of CREB activation in the *tbh-1;ser-6* mutants was similar to that in the *tbh-1* mutants (Fig. 2L). These results suggest that some octopamine-dependent signaling is occurring in the absence of *ser-6*. These experiments were repeated in *ser-3;ser-6* double mutants, and their responses to exogenous octopamine and food deprivation were similar to those of the *ser-3* mutants (Fig. 2I).

CREB is activated in the SIA neurons when animals are soaked in water, and this soaking response is independent of octopamine (Suo et al., 2006). *ser-6* mutants responded normally to soaking (Fig. 2G), exhibiting robust activation of CREB. This result confirms that the SIA neurons are present in *ser-6* mutants and that CREB can be activated in these neurons under certain conditions. Reduced octopamine-mediated CREB activation seen in the *ser-6* mutants is therefore not the result of abnormal development of SIA neurons.

In addition to SER-3 and SER-6, the *C. elegans* genome contains another octopamine receptor, OCTR-

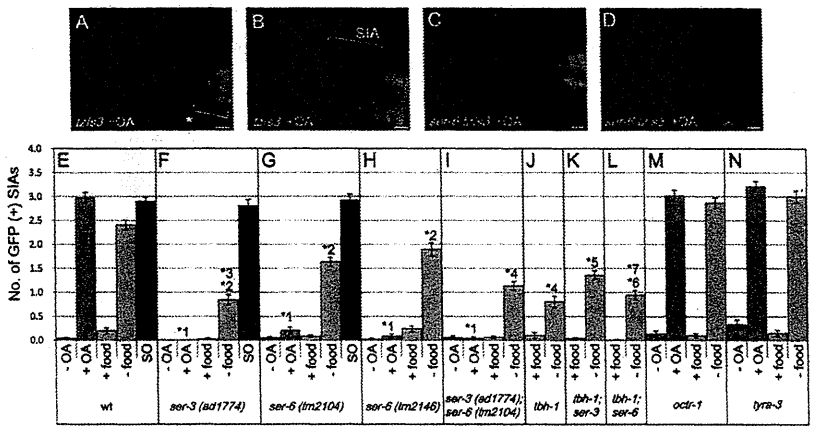

Fig. 2. Octopamine- and food deprivation-induced CREB activation in the SIA neurons. Animals carrying *cre::gfp* were cultured on agar plates containing 0 (**A,C**) or 3 mg/ml (**B, D**) octopamine. Fluorescent images were obtained from wild-type background animals (A,B) and *ser-6(tm2104)* mutants (C,D) after 4 hr of incubation. GFP expression was induced by exogenous octopamine in the SIA neurons of wild-type but not *ser-6* mutants. The bracket marked with an asterisk indicates autofluorescence of the intestine. Wild-type, *ser-3(ad1774), ser-6(tm2104), ser-6(tm2146), ser-3(ad1774);ser-6(tm2104), tbh-1(ok1196), tbh-1(ok1196);ser-3(ad1774), tbh-1(ok1196);ser-6(tm2104), octr-1(ok371),* and *tyra-3(ok325)* mutants carrying *cre::gfp* were incubated on plates containing 0 or 3 mg/ml octopamine (OA) for 4 hr, incubated on NGM plates with or without food for 6 hr, or soaked in water (SO) in the presence of food for 4 hr. The number of GFP-expressing SIA neurons per animal was then determined (**E–N**). Error bars indicate the standard errors of the mean. At least 53 animals were tested. *1$P < 0.001$ (Tukey–Kramer multiple-comparisons test) compared with +OA of wild-type animals. *2$P < 0.001$ compared with −food of wild-type animals. *3$P < 0.001$ compared with −food of *ser-6(tm2104)* mutants. *4$P > 0.05$ compared with −food of *ser-3* mutants. *5$P < 0.001$ compared with −food of *tbh-1* mutants and *ser-3* mutants. *6$P > 0.05$ compared with −food of *tbh-1* mutants. *7$P < 0.001$ compared with −food of *ser-6(tm2104)* mutants. Scale bars = 10 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

1, as well as the tyramine receptor TYRA-3, which has been shown to bind octopamine, albeit weakly (Wragg et al., 2007). We therefore investigated whether OCTR-1 and TYRA-3 are involved in octopamine-mediated CREB activation. The *octr-1* and *tyra-3* mutants responded normally to exogenous octopamine and food deprivation, suggesting that these receptors are not involved in the octopamine-mediated CREB activation seen in the SIA neurons (Fig. 2M,N).

SER-6 Functions in the SIA Neurons to Activate CREB

The observation that octopamine-induced CREB activation was reduced in both *ser-3* and *ser-6* single mutants indicates that both SER-3 and SER-6 are required for CREB activation. Furthermore, the observation that the response to food deprivation in *ser-3;ser-6* double mutants was not smaller than that of either *ser-3* or *ser-6* single mutants also suggests that SER-3 and SER-6 are not redundant. One possibility is that they function in different neurons. Notably, it has been shown that both SER-3 and SER-6 are required for regulation of octanol sensitivity by octopamine and that they function in different neurons for this regulation (Mills et al., 2012). Another possibility is that SER-3 and SER-6 function in the same (SIA) neurons and there may be some interaction at the molecular level. It has been previously reported that ser-6 is expressed in a subset of head and tail neurons (Srinivasan et al., 2008). However, it has not been determined whether *ser-6* is expressed in the SIA neurons. We generated a *ser-6::gfp* reporter fusion gene in which 5 kb of upstream sequence plus a portion of exon 1 are fused to the *gfp* gene. This fusion gene was coinjected along with the *ceh-17::dsred* reporter. The *ceh-17* promoter was used because it induces gene expression in only the four SIA neurons and one additional neuron (the ALA neuron; Pujol et al., 2000). The *ceh-17::dsred* reporter therefore labels the SIA neurons with DsRed expression. A *tbh-1::dsred* reporter construct was also introduced to label the octopaminergic RIC neurons. In these transformants, GFP expression was observed in multiple neurons, with GFP colocalizing with DsRed (Fig. 3), suggesting that *ser-6* is expressed in both the SIA and the RIC neurons.

To determine whether SER-6 functions in the SIA neurons, we performed a cell-specific rescue experiment. We introduced the *ceh-17::ser-6* fusion construct, in which the *ceh-17* promoter was fused to SER-6 cDNA, into *ser-6(tm2104)* mutant animals. These transformants should express SER-6 in only the SIA and ALA neurons. As shown in Figure 4, the transgenic animals responded to exogenous octopamine as robustly as did the wild-type animals, suggesting that expression of SER-6 in the SIA neurons is sufficient to restore CREB activation upon octopamine. CREB activation of the transformants in response to food deprivation was not significantly different from that of the wild-type animals, also suggesting that SER-6 functions in the SIA neurons for food-deprivation response. However, there was no significant difference between CREB activation levels for food deprivation of

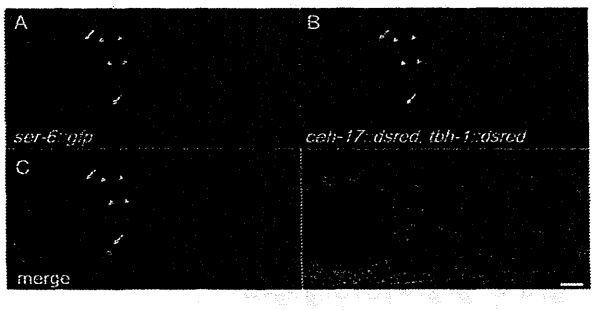

Fig. 3. Expression pattern of *ser-6*. Fluorescent (**A–C**) and corresponding differential interference contrast (**D**) images were obtained from N2 animals carrying the *ser-6::gfp*, *ceh-17::dsred*, and *tbh-1::dsred* constructs. The SIA- and RIC-neuron-specific promoters, *ceh-17* and *tbh-1*, respectively, were used to label the SIA and RIC neurons with DsRed. Merged images show the colocalization of GFP and DsRed. Arrowheads indicate SIA neurons. Arrows indicate RIC neurons. Scale bar = 10 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

ser-6 animals and the transformants. Therefore, it remains possible that *ser-6* also functions in other cells.

Both SER-3 and SER-6 Are Required for Normal CREB Activation in SIA Neurons

The present results suggest that SER-3 and SER-6 function in the same cells and that both of these receptors are required for normal signaling, despite having similar functions. One explanation for the decreased CREB activation seen in *ser-3* and *ser-6* single mutants is a decrease in the total number of octopamine receptors. A specific level of octopamine receptor may be required for normal signaling, and removal of either of these two genes results in an insufficient quantity of octopamine receptors. To address this possibility, we assayed CREB activation in double heterozygous *ser-3/+;ser-6/+* animals. The double heterozygous animals responded slightly more weakly to exogenous octopamine treatment than wild-type animals (Fig. 5B). However, the response of the double heterozygous animals was much stronger than that of the *ser-3* or *ser-6* single mutants, which was essentially zero (Figs. 2F–H, 5B). This result suggests that having both *ser-3* and *ser-6* is important for CREB activation rather than the quantity of octopamine receptor genes. The response to food deprivation was not different between *ser-3/+;ser-6/+* double heterozygous animals and wild-type animals (Fig. 5A,B). Furthermore, we analyzed the *ser-3/ser-3;ser-6/+* and *ser-3/+;ser-6/ser-6* heterozygous animals and found that *ser-3/ser-3;ser-6/+* were similar to *ser-3* single mutants ($P > 0.05$; Figs. 2F, 5C) and that *ser-3/+;ser-6/ser-6* were similar to *ser-6* single mutants ($P > 0.05$; Figs. 2G, 5D) with respect to their response to food deprivation. These results suggest that removing one copy of the *ser-3* or *ser-6* gene has little effect on the response to food deprivation, which further supports the idea that normal CREB activation requires the existence of both octopamine receptors rather than just a specific quantity of receptor.

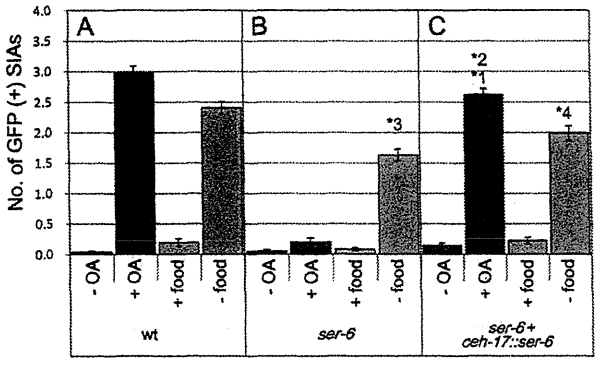

Fig. 4. SIA-neuron-specific rescue of the *ser-6* CREB activation phenotype. The transgenes *ceh-17::ser-6 and lin-44::gfp* were introduced into a *ser-6(tm2104)* mutant carrying *cre::gfp*. The *ceh-17* promoter induces gene expression in only the SIA and ALA neurons. The *lin-44::gfp* construct was used as a cotransformation marker. Transformants were incubated on plates containing 0 or 3 mg/ml octopamine for 4 hr or on NGM plates with or without food for 6 hr (**C**). At least 72 animals were tested. Error bars indicate the standard errors of the mean. CREB activity in wild-type animals and *ser-6(tm2104)* mutants shown in Figure 2E,G is reprinted (**A,B**). *1$P < 0.001$ (Tukey–Kramer multiple-comparisons test) compared with +OA of *ser-6* mutants. *2$P > 0.05$ compared with +OA of wild-type animals. *3$P < 0.001$ compared with −food of wild-type animals. *4$P > 0.05$ compared with −food of wild-type animals.

To address the effect of the gene dosage further, we next assessed CREB activation in animals overexpressing either SER-3 or SER-6. SER-3 was overexpressed in the SIA neurons of the *ser-6* deletion mutant using the *ceh-17::ser-3* fusion construct, and SER-6 was overexpressed in the SIA neurons of *ser-3* deletion mutant using the *ceh-17::ser-6* fusion construct. These animals therefore lacked either SER-6 or SER-3 but overexpressed the other receptor in the SIA neurons, in addition to endogenous expression. It has been shown that multiple copies (typically over 100 copies) of genes are retained in transgenic animals when transformed by injection (Fire et al., 1991). In *ser-3* mutants overexpressing SER-6, CREB activation induced by exogenous octopamine or food deprivation was similar to that for *ser-3* deletion mutants alone ($P > 0.05$; Figs. 2F, 5E). This result suggests that SER-6 alone cannot induce activation of CREB, even when SER-6 is overexpressed. In *ser-6* mutants overexpressing SER-3, some spontaneous CREB activation was observed on the control plates that did not contain octopamine but did contain food (Fig. 5F, first bar). However, this activation was not seen on NGM plates containing food (Fig. 5F, third bar); the cause of this difference is unknown. One possible explanation is that, because control plates for octopamine treatment contained less salts and peptone than NGM plates, these compounds, or the difference in the condition of the bacteria growing on these plates, might have affected CREB activation in this strain. Nonetheless, a moderate increase in CREB activation was observed upon exogenous octopamine treatment in the *ser-3*-overexpressing animals (Fig. 5F), suggesting that SER-3 can partially respond to exogenous

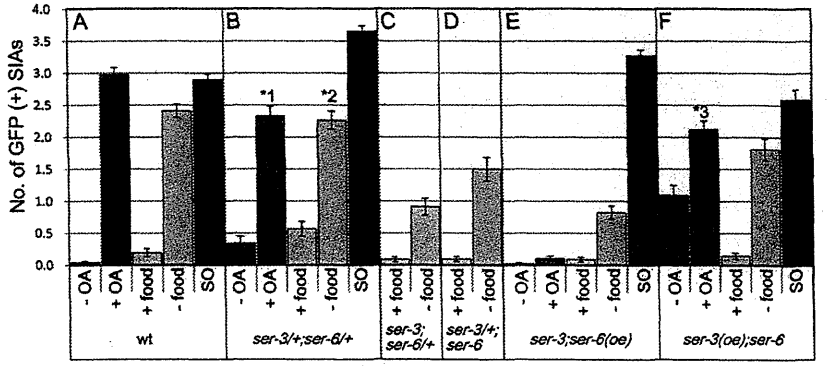

Fig. 5. Octopamine- and food deprivation-mediated CREB activity in heterozygous and overexpressing animals. Double heterozygous animals (**B**), *ser-3/ser-3;ser-6/+* animals (**C**), *ser-3/+;ser-6/ser-6* animals (**D**), *ser-6*-overexpressing animals (E), and *ser-3*-overexpressing animals (F) carrying *cre::gfp* were incubated on plates containing 0 or 3 mg/ml octopamine for 4 hr, incubated on NGM plates with or without food for 6 hr, or soaked in water (SO) in the presence of food for 4 hr. The number of GFP-expressing SIA neurons per animal was then determined. At least 43 animals were tested. Error bars indicate standard errors of the mean. CREB activity in wild-type animals shown in Figure 2E is reprinted (**A**). $*1P < 0.01$ (Tukey–Kramer multiple-comparisons test) compared with +OA of wild-type animals. $*2P > 0.05$ compared with −food of wild-type animals. $*3P < 0.001$ compared with -OA of *ser-3(oe);ser-6* animals.

octopamine without SER-6 when overexpressed. In contrast, the level of CREB activation induced by food deprivation in *ser-3*-overexpressing animals was not different from that of *ser-6* mutants ($P > 0.05$). Collectively, these results suggest that both *ser-3* and *ser-6* are required for full activation of CREB regardless of their quantity and that *ser-3* but not *ser-6* can partially function by itself only when it is overexpressed.

DISCUSSION

It is common for neurotransmitters to possess multiple receptors that couple to the same intracellular signaling. When expressed in a heterologous system, such functionally similar receptors function in a nonredundant manner through receptor–receptor interactions. This study analyzed two homologous octopamine receptors of *C. elegans*, SER-3 and SER-6, which have been shown to couple to the same class of G proteins (Petrascheck et al., 2007; Mills et al., 2012). These receptors were both required for octopamine-mediated CREB activation in the SIA neurons. Cell-specific rescue experiments revealed that SER-6, like SER-3, functions in the SIA neurons, indicating that these receptors function in the same cells. These results suggest that SER-3 and SER-6 act together to transmit octopamine signaling in the SIA neurons.

Using SER-3- and SER-6-overexpressing animals, we further demonstrated that both SER-3 and SER-6 are required for normal CREB activation by octopamine; overexpression of one receptor in the absence of the other could not fully restore normal CREB activation. *ser-3*-overexpressing animals did respond to exogenous octopamine in the absence of *ser-6*, although the response was much weaker than that in the wild-type animals. In contrast, SER-6 could not activate CREB without SER-3 even when overexpressed. These results indicate that, when overexpressed, SER-3 can partially bypass the requirement for SER-6. In addition, CREB activation by food deprivation was stronger in *ser-6* mutants than in *ser-3* mutants or *ser-3;ser-6* double mutants (Fig. 2), suggesting that SER-3 can also partially activate CREB without SER-6 in this condition. One possible mechanism in which SER-3 and SER-6 function cooperatively is that SER-6 functions in part to assist the function of SER-3 by controlling the quantity of functional SER-3. Another possibility is that SER-3 and SER-6 form a dimer and that the heterodimer transmits stronger signals than monomers or homodimers. It has been shown that structurally similar GPCRs can form heterodimers and that dimerization affects their membrane expression as well as their signaling strength (Stanasila et al., 2003; Hague et al., 2004, 2006). It also remains possible that, even though SER-3 and SER-6 are structurally similar, they transmit different intracellular signals in vivo and these signals converge to activate CREB fully. Further efforts, including expression of SER-3 and SER-6 in a heterologous expression system, would be required to elucidate the precise mechanisms by which these receptors function cooperatively.

We found that SER-3 and SER-6 are coexpressed in the SIA neurons. Although both SER-3 and SER-6 are also expressed in other neurons, the expression patterns of these receptors overlap only partially. Neurons expressing only SER-3 or SER-6 are unlikely to be able to respond to octopamine stimulation by fully activating CREB, unlike the SIA neurons. It therefore is possible that, by utilizing multiple functionally similar receptors differentially expressed across several cells types, the nervous system diversifies its sensitivity to neurotransmitters, allowing for more complex neuronal regulation.

ACKNOWLEDGMENTS

We thank Drs. Yuichi Iino and Masahiro Tomioka (University of Tokyo) for their help with confocal

microscopy. *ser-6* Mutants were provided by the National BioResource Project, Ministry of Education, Culture, Sports, Science and Technology. Some strains were provided by the CGC, which is funded by the NIH Office of Research Infrastructure Programs (P40 OD010440).

REFERENCES

Alkema MJ, Hunter-Ensor M, Ringstad N, Horvitz HR. 2005. Tyramine Functions independently of octopamine in the *Caenorhabditis elegans* nervous system. Neuron 46:247–260.

Blaxter M, Liu L. 1996. Nematode spliced leaders—ubiquity, evolution and utility. Int J Parasitol 26:1025–1033.

Brenner S. 1974. The genetics of *Caenorhabditis elegans*. Genetics 77:71–94.

Chase DL, Pepper JS, Koelle MR. 2004. Mechanism of extrasynaptic dopamine signaling in *Caenorhabditis elegans*. Nat Neurosci 7:1096–1103.

Chen ZJ, Minneman KP. 2005. Recent progress in α1-adrenergic receptor research. Acta Pharmacol Sin 26:1281–1287.

Fire A, Albertson D, Harrison SW, Moerman DG. 1991. Production of antisense RNA leads to effective and specific inhibition of gene expression in *C. elegans* muscle. Development 113:503–514.

Grohmann L, Blenau W, Erber J, Ebert PR, Strunker T, Baumann A. 2003. Molecular and functional characterization of an octopamine receptor from honeybee (*Apis mellifera*) brain. J Neurochem 86:725–735.

Gupta A, Mulder J, Gomes I, Rozenfeld R, Bushlin I, Ong E, Lim M, Maillet E, Junek M, Cahill CM, Harkany T, Devi LA. 2010. Increased abundance of opioid receptor heteromers after chronic morphine administration. Sci Signaling 3:ra54.

Hague C, Uberti MA, Chen Z, Hall RA, Minneman KP. 2004. Cell surface expression of $\alpha 1_d$-adrenergic receptors is controlled by heterodimerization with $\alpha 1_b$-adrenergic receptors. J Biol Chem 279:15541–15549.

Hague C, Lee SE, Chen Z, Prinster SC, Hall RA, Minneman KP. 2006. Heterodimers of $\alpha 1_b$- and $\alpha 1_d$-adrenergic receptors form a single functional entity. Mol Pharmacol 69:45–55.

Han KA, Millar NS, Davis RL. 1998. A novel octopamine receptor with preferential expression in *Drosophila* mushroom bodies. J Neurosci 18:3650–3658.

He SQ, Zhang ZN, Guan JS, Liu HR, Zhao B, Wang HB, Li Q, Yang H, Luo J, Li ZY, Wang Q, Lu YJ, Bao L, Zhang X. 2011. Facilitation of μ-opioid receptor activity by preventing δ-opioid receptor-mediated codegradation. Neuron 69:120–131.

Hobert O. 2002. PCR fusion-based approach to create reporter gene constructs for expression analysis in transgenic *C. elegans*. Biotechniques 32:728–730.

Johannessen M, Delghandi MP, Moens U. 2004. What turns CREB on? Cell Signal 16:1211–1227.

Kimura Y, Corcoran EE, Eto K, Gengyo-Ando K, Muramatsu MA, Kobayashi R, Freedman JH, Mitani S, Hagiwara M, Means AR, Tokumitsu H. 2002. A CaMK cascade activates CRE-mediated transcription in neurons of *Caenorhabditis elegans*. EMBO Rep 3:962–966.

Kramer JM, French RP, Park EC, Johnson JJ. 1990. The *Caenorhabditis elegans rol-6* gene, which interacts with the *sqt-1* collagen gene to determine organismal morphology, encodes a collagen. Mol Cell Biol 10:2081–2089.

Lonze BE, Ginty DD. 2002. Function and regulation of CREB family transcription factors in the nervous system. Neuron 35:605–623.

Mayr B, Montminy M. 2001. Transcriptional regulation by the phosphorylation-dependent factor CREB. Nat Rev Mol Cell Biol 2:599–609.

Mello CC, Kramer JM, Stinchcomb D, Ambros V. 1991. Efficient gene transfer in *C. elegans*: extrachromosomal maintenance and integration of transforming sequences. EMBO J 10:3959–3970.

Mills H, Wragg R, Hapiak V, Castelletto M, Zahratka J, Harris G, Summers P, Korchnak A, Law W, Bamber B, Komuniecki R. 2012. Monoamines and neuropeptides interact to inhibit aversive behaviour in *Caenorhabditis elegans*. EMBO J 31:667–678.

Murakami M, Koga M, Ohshima Y. 2001. DAF-7/TGF-β expression required for the normal larval development in *C. elegans* is controlled by a presumed guanylyl cyclase DAF-11. Mech Dev 109:27–35.

Pei L, Li S, Wang M, Diwan M, Anisman H, Fletcher PJ, Nobrega JN, Liu F. 2010. Uncoupling the dopamine D1-D2 receptor complex exerts antidepressant-like effects. Nat Med 16:1393–1395.

Petrascheck M, Ye X, Buck LB. 2007. An antidepressant that extends lifespan in adult *Caenorhabditis elegans*. Nature 450:553–556.

Pujol N, Torregrossa P, Ewbank JJ, Brunet JF. 2000. The homeodomain protein CePHOX2/CEH-17 controls antero-posterior axonal growth in *C. elegans*. Development 127:3361–3371.

Roeder T. 1999. Octopamine in invertebrates. Prog Neurobiol 59:533–561.

Srinivasan S, Sadegh L, Elle IC, Christensen AG, Faergeman NJ, Ashrafi K. 2008. Serotonin regulates *C. elegans* fat and feeding through independent molecular mechanisms. Cell Metab 7:533–544.

Stanasila L, Perez JB, Vogel H, Cotecchia S. 2003. Oligomerization of the $\alpha 1_a$- and $\alpha 1_b$-adrenergic receptor subtypes. Potential implications in receptor internalization. J Biol Chem 278:40239–40251.

Suo S, Kimura Y, Van Tol HH. 2006. Starvation induces cAMP response element-binding protein-dependent gene expression through octopamine-Gq signaling in *Caenorhabditis elegans*. J Neurosci 26:10082–10090.

Suo S, Culotti JG, Van Tol HH. 2009. Dopamine counteracts octopamine signalling in a neural circuit mediating food response in *C. elegans*. EMBO J 28:2437–2448.

Wragg RT, Hapiak V, Miller SB, Harris GP, Gray J, Komuniecki PR, Komuniecki RW. 2007. Tyramine and octopamine independently inhibit serotonin-stimulated aversive behaviors in *Caenorhabditis elegans* through two novel amine receptors. J Neurosci 27:13402–13412.

特集　遺伝子検査による早期診断

疾患

筋強直性ジストロフィー

大澤奈摘　趙　一夢　石浦章一

はじめに

筋強直性ジストロフィー（Myotonic Dystrophy：DM）は，日本では10万人あたり5～7名が，世界的には10万人あたり12名程が発症しており，成人の筋ジストロフィーの中で最も多くの患者を有する。DMは常染色体優性遺伝することが知られており，症状としては筋萎縮に伴う筋力低下，筋強直といった骨格筋の症状のほかに，心伝導障害に伴う不整脈，白内障，インスリン抵抗性などの内分泌異常，脱毛，免疫系異常，中枢神経系異常など，多系統にわたってみられるのが特徴であり，患者によってみられる症状も多彩である。発症年齢は先天性から80歳と幅広く，先天性では上記の症状のほかに，呼吸不全や知的発達遅滞などがみられより重篤で，母親から遺伝することが多い[1]。

責任遺伝子

DMには責任遺伝子の異なる二つの型が存在しており，DM1とDM2と呼ばれている。DMの多くはDM1であり，DM2は日本で1家系のみ認められている。DM1の責任遺伝子は1992年にポジショナルクローニング法によって同定され，第19番染色体長腕（19q13.3）にある*DMPK*（DM protein kinase）であり，プロテインキナーゼと相同性が高い遺伝子であった[2]。このDMPK遺伝子の3'非翻訳領域（3' UTR）にあるCTG繰り返し配列（リピート配列）が，通常5～38リピートのところ，患者では50～3,000以上に伸長しており，このリピート数が多いほど症状は重篤化し，1,000回以上で先天性DMとなる。このリピート配列は世代を経るごとに伸長し，症状がより重篤に，発症も低年齢化する表現促進現象がみられる。しかし必ずしも親より伸長したリピート配列が子に遺伝するわけではなく，体細胞においてもリピート数のモザイク性が確認されている。DMでは長いリピート配列が母親から子へと遺伝することが多いが，他のリピート病であるハンチントン病は父親から遺伝しやすい。

1994年に従来のDM患者の中から，DMPK遺伝子は正常だがDMの症状を呈する患者群が確認されてDM2と区別された。2001年にその責任遺伝子が第3番染色体長腕（3q21）にある*ZNF9*（zinc finger 9）/*CNBP*（CCHC-type zinc finger, nucleic acid binding protein）と同定され，そのイントロン1にあるCCTGリピート配列が患者では異常に伸長していた[3]。通常は10～27リピートだが，患者では75～10,000以上の伸長が認められ，4塩基のリピート病として初の所見であった。しかし，DM1と異なりDM2はそのリピート数と症状の重篤度の相関がみられず，リピートが長くとも先天性の症状を示す患者はいない。

発症の分子機構

翻訳されないリピート配列はRNAレベルで毒性を持ち，症状を呈することがモデルマウスや患者の細胞を用いたさまざまな研究からわかってきた[4]。患者では，MBNL1（muscleblind-like splicing regulator 1）の機能低下，CELF1（CUGBP, Elav-like family member 1）の活性化という，二つ

おおさわ なつみ，ちょう いいむん，いしうら しょういち
東京大学大学院総合文化研究科
〒153-8902　東京都目黒区駒場3-8-1
E-mail address：cishiura@mail.ecc.u-tokyo.ac.jp

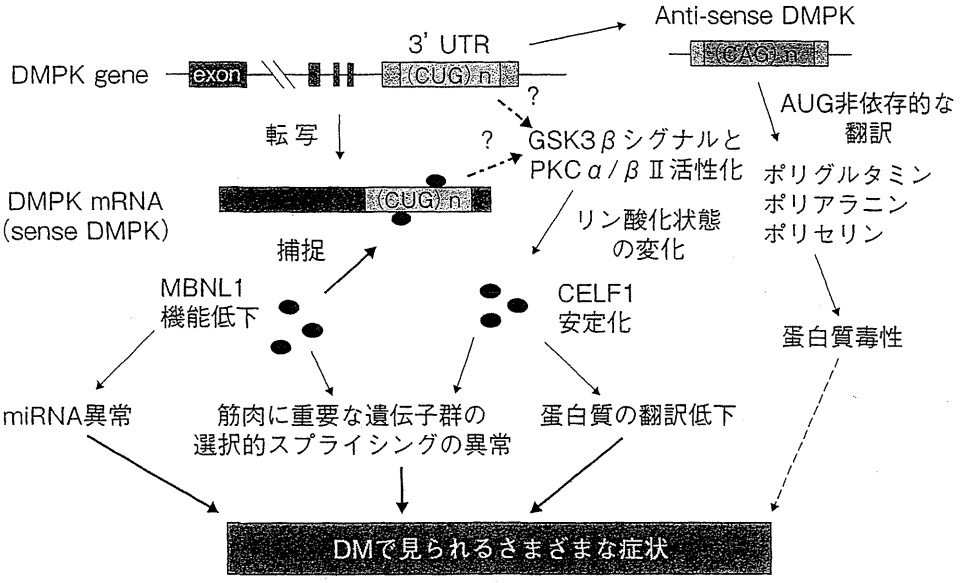

図　DM1 発症の分子機構

のRNA結合蛋白質のバランスが崩れており，筋肉に重要な遺伝子の選択的スプライシングやmRNAの翻訳，miRNAの異常などがみられる(図)。MBNL1は伸長したリピートRNAに捕捉され，核内に凝集されることでその機能が低下する[5]。CELF1はリピートRNAによってGSK3βやPKCのシグナルが活性化され，CELF1のリン酸化状態が変化し安定化することでその活性が上昇する[6]。ただ，リピートRNAによってどのようにGSK3βやPKCのシグナルが活性するかはわかっていない。また，リピートのアンチセンス鎖からAUG非依存的にポリグルタミン，ポリアラニンなどがDM患者の細胞で翻訳され凝集していることから，その蛋白質毒性が発症に関与している可能性もある[7]。

現在，根本的な治療法は未だ確立されていないが，リピート配列からMBNL1を遊離させるための低分子化合物やアンチセンスオリゴヌクレオチドの探索，CELF1のリン酸化状態を正常に保つためのGSK3βやPKCの阻害剤の探索が行われており，少しずつ治療法の糸口がみえつつある。

周産期における筋強直性ジストロフィー

軽症のDMは，日常生活に支障がなく，患者自身が無自覚である場合も多い。しかし世代を経て重症化する本疾患の特徴から，遺伝子検査による発症前診断や出生前診断が重要視される。

DMは遺伝子変異が常染色体上にあるため，両親のいずれかが患者の場合，性別に関係なく50%の確率で子どもに遺伝する。しかしながら，父親由来の事例は稀であり，本疾患の男性生殖能への影響が疑われている。母親が患者の場合，妊娠中の血中プロゲステロンの増加による筋症状の悪化を契機に発見されることが多い。妊娠30週前後より羊水過多，胎動微弱，母体の筋力低下，切迫早産などの症状が現れる。切迫早産に対する塩酸リトドリンの点滴投与は筋症状の悪化，横紋筋融解症を引き起こすことがあるため，本疾患が疑われる場合は注意が必要である。また，分娩時は母親の筋症状悪化が予想されるため，時期・方法の検討が望まれる[8~12]。

先天性筋強直性ジストロフィー（CDM）

子どもがCDMの場合，成人型とは大きく異なった症状がみられる。典型的な成人型の症状であるミオトニアを示さないことが多く，代わりに全身の筋緊張低下に起因する嚥下・哺乳障害，呼吸不全，足・脊椎等における変形が認められる。これらの症状は胎児期から存在し，羊水嚥下障害による羊水過多，筋肉の発育不全による胎動微弱が引き起こされる。出生後は，全身の筋緊張低下がぐにゃぐにゃ児(floppy infant)の症状として現れる。嚥下・哺乳障害，呼吸不全は，出生後直ちに人工呼吸管理や経管栄養による適切な治療を必

要とする場合も多い。しかしながら，適切な治療を受けても生後18カ月までに25％が死亡する。一方で，それ以降は出生時にみられた症状が改善し，半数が成人する。

CDMの患者は成長過程において二相性の症状を示す。出生時にみられた全身性の筋緊張低下は徐々に改善し，正常より遅れはとるが，筋肉は発達し，坐定・独歩等の獲得が期待できる。代わりに，著明な知的障害・表情筋の筋力低下が問題となる。表情筋の筋力低下は，不明瞭な構音・鼻声によって言語障害をもたらし，知的障害の評価に影響を及ぼす。同時に知的障害による早口・小声によって，言語障害が深刻化し，コミュニケーション能力に著しい障害をきたす。このほか，第二相では，成人のDMの基本症状であるミオトニアが学童期から出現し，思春期以降は白内障・不整脈等の症状も現れる。

筋強直性ジストロフィーにおける遺伝子検査と早期診断

本疾患の遺伝子検査は比較的簡便で，正確であり確定診断となる。診断者の末梢血液を用い，責任遺伝子のCTG反復配列を標的とし，その伸長をサザンブロット法やPCR法によって直接診断を下すことが可能である。サザンブロット法では1,000回前後の長い反復配列を検出できるが，それより短い反復配列では，患者と正常人との判別が困難となる。PCR法では50回以上の反復配列はCG含量の高さ故に増幅されない。そこで，両者を組み合わせた診断法が用いられている。

DMは前述の通り，軽症の場合は罹患に気づかず，健常人とほとんど変わらない生活を送る。しかし，本遺伝病は代を経て病状が深刻化するため，ことに周産期管理においては専門医の診察が必須となる。

本症と診断された家族がいる場合の発症前診断や出生前診断，また出生後の遺伝子診断は，医学的対処を考慮すれば非常に重要な意義を持つ。特に出生時での緊急対応等は，事前に十分な準備期間，専門機関への相談がなければ間に合わない場合もある。また，母子ともに本症と診断される場合，患者への負担は精神面・肉体面の双方で大きくなることも特筆すべきであろう。

おわりに

ほかの遺伝性疾患同様，出生前診断に関しては命の選択につながり，特に本症にあっては成人まで成長する場合がある点も十分に考慮すべきである。また，発症前診断などにおいても，診断された場合の本症への理解，本人・家族・知人の反応等を事前に遺伝相談などによって熟慮された後に，患者本人によって診断受診の可否の意思決定がなされるべきである。

文献

1) Harper PS：Myotonic dystrophy, WB Saunders, London, 2000
2) Mahadevan M, Tsilfidis C, Sabourin L, et al：Myotonic dystrophy mutation：an unstable CTG repeat in the 3'untranslated region of the gene. Science **255**：1253–1255, 1992
3) Liquori CL, Ricker K, Moseley ML, et al：Myotonic dystrophy type 2 caused by a CCTG expansion in intron 1 of ZNF9. Science **293**（5531）：864–867, 2001
4) Mankodi A, Logigian E, Callahan, et al：Myotonic dystrophy in transgenic mice expressing an expanded CUG repeat. Sciense **289**：1769–1772, 2000
5) Miller JW, Urbinati CR, Teng-Umnuay P, et al：Recruitment of human muscleblind protein to (CUG) n expressions associated with myotonic dystrophy. EMBO **19**：4439–4448, 2000
6) Jone K, Wei C, Iakova P, et al：GSK3β mediates muscle pathology in myotonic dystrophy. J Clinic Invest **122**：4461–4472, 2012
7) Zu T, Gibbens B, Doty NS, et al：Not-ATG-initiated translation directed by microsatellite. PNAS **208**：260–265, 2011
8) 吉田朱美，前田洋一，佐原雅代，他：筋緊張性ジストロフィー合併妊娠を経験して．産婦の実際**54**：659–664，2005
9) Reardon W, Newcomb R, Fenton I, et al：The natural history on congenital myotonic dystrophy：motarity and long term clinical aspects. Arch Dis Chiled **68**：177–181, 1993
10) 川井 充：筋強直性ジストロフィーの治療とケア，医学書院，東京，2000
11) 厚生労働省：筋強直性ジストロフィーにおける診療・治療マニュアル，厚生労働省精神・神経疾患研究委託費筋ジストロフィー治療のエビデンス構築に関する臨床研究班筋強直性ジストロフィープロジェクト，2008
12) Harper PS：Myotonic Dystrophy（The Facts），Oxford University Press, 2009

BRAIN and NERVE 66(3)：259-264, 2014

総説

筋強直性ジストロフィー研究の現在

道一歩　石浦章一

筋強直性ジストロフィーは，筋症状を主とする遺伝性・進行性多臓器疾患である。責任遺伝子の異なるI型・II型があり，日本での罹患率は10万人に約5人である。発症機構は，異常伸長した非翻訳領域におけるCTG，CCTG反復配列の転写によるRNA毒性である。確立した根本的な治療法はまだない。ここでは，近年進んできたRNA毒性に対する研究を踏まえ，探索中の新規治療法について概説する。

筋強直性ジストロフィー，RNA毒性，MBNL1，CELF1，スプライシング異常

はじめに

筋強直性ジストロフィー（myotonic dystrophy：DM）は，進行性の筋症状を主とする全身性の疾患である。その症状はさまざまで，主症状である筋緊張（ミオトニア，筋収縮後の弛緩障害），進行性筋萎縮による筋力低下以外にも，白内障，耐糖能障害や心伝導障害，内分泌・免疫系異常，知能障害，中枢神経障害（性格変化・認知障害・過眠）など多岐にわたる。筋病理では中心核，筋線維の小径化（タイプ1，赤筋に顕著）などがみられる。患者は特徴的な筋萎縮によって斧様顔貌を呈する。DMの罹患率は日本では10万人に約5人で，これは成人型筋ジストロフィーで最多である。DMはさらにその責任遺伝子の違いから，DM1とDM2に分けられるが，いずれも孤発例の報告はなく，家族性遺伝により発症する。

DM1では，第19番染色体長腕（19q13.3）に位置する*DMPK*（dystrophia myotonica protein kinase）遺伝子の3′末端非翻訳領域（3′-UTR）内のCTG反復配列に異常伸長がみられ，通常5～35回の反復が50回から数千回前後まで増大する。この反復回数の増大に比例して病状は深刻化し，発症年齢も若齢化する。また，世代を経るごとに反復回数の増大がみられ，1,000回以上の患者では非常に重篤な症状を示す先天性DM（CDM）が認められる。

DM2では，第3番染色体長腕（3q21.3）の*CNBP*/*ZNF9*遺伝子のイントロン1に存在するCCTG反復配列の異常伸長がみられる。健常者での反復回数は26回以下だが，DM2患者では数千回に増大する。DM1同様，優性遺伝し，筋力低下やミオトニア，筋症状以外の症状が認められる。しかし，DM1とは異なり，異常伸長の程度と症状の重篤度に明白な相関はない。また，DM2は総じて軽症であり，先天性発症の報告もない。

DM患者は，軽症であれば健常人と変わらない生活を送るが，罹患の有無は，末梢血を用いた遺伝子検査により比較的簡便に確定診断することができる。しかし，その治療は対症療法に限定されており，根本的な治療法はまだない。

I．筋強直性ジストロフィー発症の分子機構

異常伸長したCTG，CCTG反復配列はRNAに転写され，CUG，CCUG反復配列となる。いずれの場合も非翻訳領域に存在することから，DMPKやCNBP蛋白質は正常である。ただ，責任遺伝子自身やその近傍遺

東京大学大学院総合文化研究科広域科学専攻生命環境科学系（〒153-8902　東京都目黒区駒場3-8-1）
*〔連絡先〕cishiura@mail.ecc.u-tokyo.ac.jp

1881-6096/14/¥500/論文/JCOPY

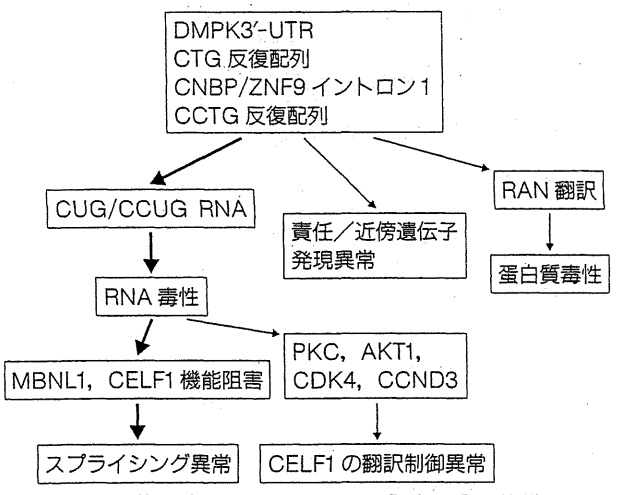

Fig.　筋強直性ジストロフィー発症の分子機構

伝子の発現量は変化するという報告がある[1,2]。しかし，DM の発症機構で重要な役割を果たすのは，変異 RNA そのものである。変異 RNA は，その反復配列の存在により，以下の 2 つの機構によって細胞内秩序を乱し，RNA 毒性を示す（Fig.）。

1 つ目は ATG 非依存的な翻訳（repeat-associated non-ATG translation：RAN 翻訳）によるアンチセンス鎖からのポリグルタミンペプチドの産生である[3]。症状との関連は不明だが，そのほかのポリアミノ酸病のように蛋白質毒性を示す可能性が考えられている。

2 つ目は親和性の高い RNA 結合蛋白質の捕捉による正常機能の阻害である。現在，この機構が最も重要な発症機構だと考えられている。変異 RNA は凝集しやすく，二本鎖となり，核内で安定な構造である foci を形成する[4,5]。このため分解されにくく，核内に蓄積し，以下に示す RNA 結合蛋白質に影響を及ぼす。MBNL1（muscle blind-like protein 1）と CELF1（CUGBP/Elav-like family member 1）はリピート RNA に結合する 2 つの代表的な RNA 結合蛋白質である。核内において，MBNL1 と CELF1 はそれぞれ成人型，幼若型スプライシングを促進するという，スプライシング制御因子として相反する機能を持つ。

DM において，MBNL1 は，二本鎖化した変異 RNA に捕捉され，foci と共局在する。そのため，スプライシングに必要な MBNL1 が減少し，選択的スプライシング異常が引き起こされる[6]。

一方 CELF1 は，選択的スプライシング以外にも RNA 安定性・翻訳の各段階を制御する多機能蛋白質である。すなわち，スプライシング異常以外の機構でも DM の症状に寄与すると考えられている。反復配列存在下で CELF1 は超リン酸化し，安定化することが知られている[7]。ところが，302 番目のセリン残基（S302）においては脱リン酸化が促進され，それに付随する機能が損なわれていることも明らかとなった[8]。これらのリン酸化制御には，PKC（protein kinase C）[9]，AKT1（v-akt murine thymoma viral oncogene homolog 1），CDK4（cyclin-dependent kinase 4），CCND3（cyclin D3）[8]，GSK3β（glycogen synthase kinase 3 beta）[10] などの関与が報告されているが，超リン酸化と呼ばれる CELF1 の詳細なリン酸化プロファイルや上記各因子のリン酸化制御における役割などは不明な点が多い。

II．発症機構と症状との関連

DM では，RNA 毒性によって MBNL1 や CELF1 の機能阻害が引き起こされ，さまざまな遺伝子のスプライシングや発現量に異常が生じる。では，生じた異常はいかにその下流経路に影響して数々の症状に至るのか。

筋症状が特徴的な DM では，筋機能に関連する遺伝子のスプライシング異常が特に多く報告されている。筋肉の緊張や弛緩に重要な細胞内カルシウムイオン濃度調節を行う SERCA1（sarcoplasmic/endoplasmic reticulum Ca^{2+} ATP-ase 1），RyR1（ryanodine receptor 1）[11]，$Ca_V1.1$（L-type Ca^{2+} channel and voltage sensor 1.1）[12]，T 管形成に関与する BIN1（bridging integrator 1）[13]，Z 線に局在する LDB3（LIM domain binding 3）[14]，先天性ミオトニアの原因遺伝子である *CLCN1*（chloride channel, voltage-sensitive 1）[15] などである。*SERCA1* や *RyR1* のスプライシング異常と呼応するかのように，DM 患者では細胞内カルシウムイオン濃度の上昇が報告されており[16]，遺伝子の発現や蛋白質の活性化に異常が生じていると考えられるが，具体的な症状への関連づけは行われていない。$Ca_V1.1$ に関しては筋肉の緊張と弛緩で重要な役割を果たし，ミオトニアへの関与[17]，さらにそのスプライシング異常と筋力低下の程度が相関することが報告された[12]。BIN1 の異常は T 管形成異常による筋肉の発達異常を引き起こし，筋力低下につながるといわれている[13]。CLCN1 は電位依存性クロライドチャネルで，その機能欠損はミオトニアを引き起こす[15]。

その他の症状に関しては，インスリン受容体のスプライシング異常が糖耐能異常に寄与すること[7]，SIX5 の発現量低下が白内障に寄与するらしいこと[18] など数例を除いて，詳細は依然として不明である。

また，MBNL1 や CELF1，あるいはその両方によっ

てスプライシング制御されている遺伝子は少なくとも30以上報告されているが，症状との関連づけがなされたものはわずかである。

Ⅲ．治療法に関する研究

1．変異RNAの分解・無毒化

DMの根本治療となるのは，反復配列の転写産物によるRNA毒性の除去である。2009年，米国のThorntonらのグループが『Science』誌に発表したCUG反復配列を標的としたアンチセンスオリゴヌクレオチド（AON）を用いた研究は直接これを目的とするものであった[19]。彼らはAONのCAG25をHSA^{LR}マウス（DMモデルマウス）に投与すると，異常伸長したCUG-RNAにAONが結合し，Mbnl1との相互作用が阻害されることを発見した。さらに変異RNA自身の凝集体であるfociも除去された。しかしその効果は限定的で，さらにAONの分解されやすさから変異RNAの分解促進には至らなかった。

2012年に同グループから新しい報告が『Nature』誌になされ，今度はAONの両側に2′-O-methoxyethyl（MOE）修飾を施して生体内での安定性を上げ，さらにRNase Hを活性化するために中央に無修飾の塩基を加えたMOE gapmersの開発を発表した[20]。このMOE gapmersをモデルマウスに導入したところ，変異RNAは分解され，fociは除去され，MBNL1の捕捉も解除されたために，*Serca1*，*LDB3*，*Clcn1*などのスプライシングは改善された。効果は症状面でも顕著に現れ，ミオトニアの改善，中心核の減少，筋線維萎縮の阻止が認められた。

さらにスプライシング異常だけでなく，筋トランスクリプトーム全体においても，野生型に近いmRNAの転写が確認された。この際，副作用は認められなかった。また，MOE gapmersは生体内で安定に作用し，導入1年後においても50%の活性が残存したとしている。ヒトに対しても，ヒトDMPK-CUG800を発現するマウスで同様の実験を行い，成果を得ている。

このほかにもAONを用いたスプライシング改善の研究は数多く行われており，AONの長さや修飾が検討されている[21]。しかし，AONを使用した実際の治療は，導入方法や副作用などの検討を要し，実用化までには相当の時間を要すると予想される。日本では2013年に国立精神・神経医療研究センターと日本新薬が協力してデュシェンヌ型筋ジストロフィー（DMと異なり，平均寿命が30歳前後の遺伝病，ジストロフィン遺伝子の異常による：DMD）の治療薬が開発され，臨床試験が始まったばかりである（開発番号：NS-065/NCNP-01）。

2．MBNL1の活性を上げ，CELF1の活性を下げる

直接変異RNAを除去する以外にも，その毒性が影響する経路を阻害することは治療に役立つ。MBNL1は前述のとおり，DM患者では活性が著しく損なわれているスプライシング制御因子である。また，CELF1は異常に活性化されている蛋白質である。この2つのRNA結合蛋白質の活性を正常化させることは治療につながる。

MBNL1は変異RNAによって核内に捕捉されているため，正常な働きが阻害されている。そこで，MBNL1の絶対量の増大，あるいは変異RNAとの結合阻害をすることで機能回復につながると考えられる。実際，アデノ随伴ウイルス（AAV）によるMBNL1の過剰発現は，細胞内に十分なMBNL1機能回復をもたらし，変異RNAの引き起こす異常を改善した[14]。AAVは，DMD患者では遺伝子導入の臨床研究で一定の成果をあげている（NCT00428935）。したがって，DMでも同じような研究結果を期待できると考えられる。なお，DM2に関してもMBNL1の活性低下によるスプライシング異常がみられることから，DM1と同様の治療効果が期待される。このほか，MBNL1を変異RNAから引き離す薬剤は既に有用なもの（pentamidine）が見つかっており，fociの除去，スプライシング異常の改善も認められた[22]。

CELF1はその機能の多様さから活性に関する評価が複雑である。リン酸化されることでCELF1の機能が変化することは知られていたが，近年，リン酸化部位によって制御されるCELF1の機能が異なることがわかってきた。

まず，変異RNAによるPKC経路を介したCELF1の超リン酸化はスプライシング異常を引き起こす[9]。次に，AKTは28番目のセリン残基をリン酸化し，核・細胞質でのCELF1分布を制御して特定のmRNAへの親和性を向上させる[8,23]。

一方，CCND3とCDK4はS302をリン酸化し，CELF1の翻訳制御活性に影響する[23]。具体的には，S302がリン酸化されると，CELF1はeIF2α（eukaryotic initiation translation factor 2α）に結合し，複合体として働くことでmRNAの翻訳を促進する。これによって翻訳制御されている遺伝子には筋肉の発達に関与するものが多いため（cyclin-dependent kinase in-

hibitor, p21, cyclin D1, myocyte enhancer factor 2A など），CELF1 の翻訳制御活性も，そのスプライシング制御活性同様，DM の治療に寄与することが期待される。

DM では，変異 RNA によって GSK3β が活性化されている（RAN 翻訳によって産生されたホモアミノ酸ペプチドによるものだという報告[3]もあるが詳細は不明）。このため，GSK3β によって CCND3 の 283 番目のスレオニン残基におけるリン酸化が促進されており，その結果，CCND3 の分解が促進され，減少していることが認められた。これによって，CELF1 の S302 における脱リン酸化が促進され，その翻訳制御活性が損なわれている[24]。

以上のような機構から，CELF1 の超リン酸化を阻害し，S302 におけるリン酸化のみ促進する治療が望まれる。現在，CELF1 の超リン酸化は PKC 阻害剤である Ro 31-8220[25]，S302 におけるリン酸化は GSK3β の阻害剤である TDZD-8 が有効なこと[24]がわかっている。

3．症状への対症治療

1）ミオトニア

ミオトニアは DM の特徴的な症状の 1 つで，いったん収縮した筋肉が弛緩するまでに時間がかかる，という症状である。CDM の患者では出生直後にみられることはないが，成長に伴い症状を呈す。症状自体に深刻さはないが，ミオトニアによる長期の筋緊張状態は細胞内カルシウムイオン濃度を上げ，筋線維に損傷を与える可能性がある。

ミオトニアはいくつかの薬剤によって改善することができる。ナトリウムチャネルの阻害薬や三環系抗うつ薬，タウリンなどである。特に抗不整脈薬であるメキシレチンは第Ⅱ相（NCT01406873），メキシレチンより安価で副作用が少ないとされる抗てんかん薬のラモトリギンは第Ⅲ相（NCT01939561）まで臨床試験が進んでおり，いずれも良好な治療効果を得ている。

2）筋萎縮による筋力低下

進行性の筋萎縮によって引き起こされる筋力低下は DM のもう 1 つの主症状である。しかし，他の筋疾患に多くみられる顕著な筋肉の壊死や線維化はみられない。そのかわり，蛋白質の合成が減少し，筋線維の小径化が認められており[26,27]，これは，筋同化が阻害されていることを意味する。

そこで，筋同化刺激を与えることで筋萎縮の進行を遅らせることができると考えられ，性ステロイドホルモンであるデヒドロエピアンドロステロン（DHEA）が注目された。しかし，第Ⅱ/Ⅲ相の臨床試験で DHEA を患者に経口投与した結果では，期待していた効果は得られていない（NCT00167609）。

一方，最も筋同化作用の強いインスリン様成長因子 1（IGF-1）を用いた臨床実験も行われている。リコンビナントのヒト IGF-1（rhIGF-1）の半減期が短いため，米国の Moxley のグループは，リコンビナントのヒト IGF1 結合蛋白 3（rhIGFBP3）と複合体を形成させた rhIGF1:rhIGFBP3 を DM 患者に投与した。結果，除脂肪体重の増加と代謝の改善が認められたが，筋力と機能の改善はみられなかった（NCT00233519，第Ⅱ相）。

3）その他の症状

DM では呼吸障害などによって日中の過度の眠気が引き起こされる場合がある。2012 年，一般的なナルコレプシーの治療薬であるメチルフェニデートの投与により症状が改善したと報告された（NCT01421992）。

おわりに

DM はその責任遺伝子が同定されてから約 20 年しか経っていないが，既に AON を用いた根本的な治療法の道筋が見え始めた。また，AON を用いた治療は，異常な mRNA，ひいてはそれがコードする異常蛋白質の発現を阻害することから，DM に限らず，その他多くの遺伝病でも有効な治療法となりうることが予想される。

とはいえ，DM の発症機構に関する知見は，いまだ不十分である。例えば，DM2 では，MBNL1 の活性と *CLCN1* のスプライシング異常は DM1 と同等であるにもかかわらず，そのミオトニアは非常に軽症で済んでいる。これはスプライシング異常以外の発症機構（ミオトニアに対する）が存在することを意味する。また，*MEF2A* や *MEF2C* などのスプライシング異常に関しては，DM 特異的ではなく，他の神経筋疾患でも認められることから，いくつかのスプライシング異常が二次的である可能性も示唆されている。さらに，DM でのスプライシング異常の特徴は，幼若型が多いことが認められる。しかし，幼若型スプライシングがいかに成人型へ切り替え制御されるのか，その全貌は謎に包まれたままである。

文　献

1）Furling D, Lam le T, Agbulut O, Butler-Browne GS, Morris GE: Ghanges in myotonic dystrophy protein kinase levels and muscle development in congenital myotonic dystrophy. Am J Pathol **162**: 1001-1009, 2003