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ANALYSIS OF MOTOR FUNCTION MODULATED BY CHOLINERGIC NEURONS IN PLANARIAN *DUGESIA JAPONICA*

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Abstract—Recent studies of the freshwater planarian *Dugesia japonica* have revealed fundamental mechanisms and unique aspects of neuroscience and neuroregeneration. Here, we identified the gene for planarian choline acetyltransferase (*Djchat*), which is essential for acetylcholine (ACh) biosynthesis. Immunofluorescence studies using anti-*Dugesia japonica* ChAT (DjChAT) antibody revealed that cholinergic neurons are widely distributed in the planarian nervous system, including the brain, ventral nerve cords, optic nerves, and pharyngeal nerve plexus. In order to investigate the function of cholinergic neurons in planarians, we used both pharmacological and RNA interference (RNAi) approaches. Administration of physostigmine (an acetylcholinesterase inhibitor) clearly elevated the amount of ACh, and then induced sudden muscle contraction behavior in a concentration-dependent manner. In addition, we found that pretreatment with tubocurarine (a muscle nicotinic ACh receptor antagonist) or atropine (a non-selective muscarinic ACh receptor antagonist), but not pretreatment with mecamylamine (a neural nicotinic ACh receptor antagonist), significantly extended the latency time for physostigmine-induced contraction behavior, suggesting that muscle nicotinic ACh receptors and muscarinic ACh receptors contribute to physostigmine-induced contraction behavior. We also confirmed that ACh biosynthesis ability and DjChAT-immunoreactivity were eliminated in *Djchat(RNAi)* planarians. Moreover, the decrease of the level of ACh induced by *Djchat(RNAi)* caused extension of the latency time for contraction behavior. Our findings support the possibility that the cholinergic functions of planarians are similar to those of vertebrates, suggesting that planarians are simple but useful model organisms for getting insight into the cholinergic nervous system in higher animals.

Key words: cholinergic neurons, physostigmine, neural network, muscle contraction behavior, neuroregeneration, planarian.

Freshwater planarians are simple but useful organisms for understanding stem cell regulatory systems and neuroregeneration. The planarian CNS is composed of the brain in the anterior part of the body, and a pair of longitudinal ventral nerve cords (VNCs) along the ventral side of the body. The brain is composed of two lobes, and forms an inverted U-shaped structure with nine pairs of lateral branches (Agata et al., 1998; Okamoto et al., 2005). Planarians possess powerful regenerative ability, which is derived from pluripotent stem cells (Agata and Watanabe, 1999; Agata, 2003; Reddien and Sánchez Alvarado, 2004; Reddien et al., 2005; Newmark and Sánchez Alvarado, 2002). The regulation of the plasticity and heterogeneity of stem cells enables the regeneration of complete animals from even very small fragments (Sánchez Alvarado, 2006; Pellettieri and Sánchez Alvarado, 2007; Eisenhoffer et al., 2008). Especially, the stem cell regulatory system underlying planarian brain regeneration has revealed interesting aspects, suggesting that these processes can be divided into at least five steps (Agata and Umesono, 2008). The genes that are expressed in the sequential steps underlying planarian brain regeneration are similar to those expressed during the course of mammalian brain development (Cebrià et al., 2002a; Nakazawa et al., 2003; Inoue et al., 2007; Yazawa et al., 2009).

Recently, we identified in planarians several neurotransmitters that are known to be present in vertebrates, and determined their neural networks by immunofluorescence analysis using specific antibodies (Nishimura et al., 2007a,b, 2008a,b). Several neuroreceptor genes were also identified, and ligand identification and expression analysis were carried out (Saitoh et al., 1996; Nishimura et al., 2009). Accordingly, it is now possible to begin to analyze neural functions from both the presynaptic and postsynaptic points of view. Moreover, since planarians show specific behavioral patterns in response to treatment with pharmacological drugs, some analytical methods have been established to simply but quantitatively evaluate such behavioral changes (Kitamura et al., 2003; Raffa and Desai, 2005; Pagán et al., 2008). In addition, computer-based analytical methods have also begun to be established, allowing us to conduct behavioral analyses with high resolution (Inoue et al., 2004; Hicks et al., 2006). Based on these advances, planarians have provided many unique insights into neuroscience (Nishimura et al., 2008c; Cebrià, 2007, 2008).

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Abbreviations: ACh, acetylcholine; AChE, acetylcholinesterase; AP, alkaline phosphatase; BCIP, 5-bromo-4-chloro-3-indolyl-phosphate; ChAT, choline acetyltransferase; CrAT, carnitine acyltransferase; DIG, digoxigenin; dsRNA, double-stranded RNA; EHC, ethyl homocholine; EST, expressed sequence tag; GAD, glutamic acid decarboxylase; HPLC, high-performance liquid chromatography; mAChRs, muscarinic acetylcholine receptors; MHC, myosin heavy chain; nAChRs, nicotinic acetylcholine receptors; NBT, 4-nitro blue tetrazolium chloride; PVDF, polyvinylidene difluoride; RNAi, RNA, interference; SDS, sodium dodecyl sulfate; SYT, synaptotagmin; TBH, tyramine β-hydroxylase; TH, tyrosine hydroxylase; TPH, tryptophan hydroxylase; VNC, ventral nerve cord; 5'RACE, 5'rapid amplification of cDNA ends.

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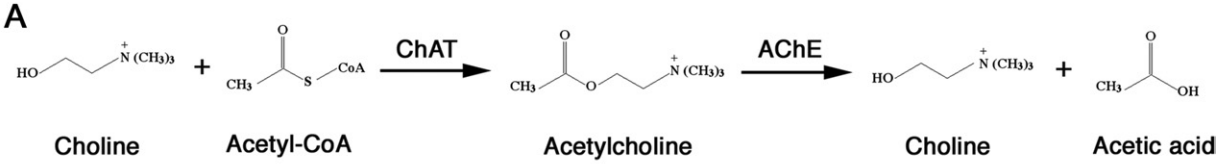
Acetylcholine (ACh) acts as an excitatory neurotransmitter in various organisms. ACh is synthesized by choline acetyltransferase (ChAT; EC2.3.1.6) from choline in cholinergic nerve terminals (Fig. 1A) (Oda, 1999). ACh is then hydrolyzed by acetylcholinesterase (AChE; EC 3.1.1.7) after being released into the synaptic cleft, and then taken up by cholinergic nerve terminals by a high-affinity choline transporter, and used to resynthesize ACh by ChAT (Haga, 1971; Misawa et al., 2001). Previous reports indicated that cholinergic drugs induce specific behavioral changes in planarians (Carolei et al., 1975; Buttarelli et al., 2000; Pagán et al., 2009). In addition, it has been reported

A



B

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D.japonica          1 ------------------------------------------------------------MAHPVREYVRRISSQNLEQYPEWDLSKPLPK    31
C.elegans           1 ------------------------------------------------------------MEKEKVDELPPNDNWYETA...    22
D.melanogaster      1 MASNEASTSAAGSGPESAALFSKLRSFSIGSGPNSPQRVVSNLRGFLTHRLSNITPSDTGWKDSILSIPKKWLSTA.SVD.FGFPDT...    90
M.musculus          1 ------------------------------------------------------------MPILEKVPPKMPV.ASSCEEVLD...    26
H.sapiens           1 ------------------------------------------------------------MAAKT.SSEE.-G...    15

D.japonica         32 LPVPNLKQTLVRYLDL--VAPVVSLEDYEKTKNLVQEFGKPNGIGEILQAKLAENSKSKENWVADLWLEDMYLKNSLALPINSSPGMVFP   119
C.elegans          23 P...S.EA..D...EYAA.VA.GQKASLAT.HDAAHK.-VRQA--TP..EQ.L.IAEKSP..ATKF..PE..MRVRMPT.V..N..YI..   109
D.melanogaster     91 V...A.DE.MAD.IRA--LE.ITTPAQL.R..E.IRQ.SA.Q...AR.HQY.LDKREAED..AYYY..NE..MDIRIP.....N......   178
M.musculus         27 ....P.Q...AT..QC--MQHL.PE.QFR.SQAI.KR..A.G.L..T..E..L.RQEKTA...SEY..N....N.R....V....AVI.A   114
H.sapiens          16 ....P.Q...AT..QC--MRHL..E.QFR.SQAI..Q..A.G.L..T..Q..L.RQEKTA...SEY..N....N.R....V....AVI.A   103

D.japonica        120 RQHFQSEKEQLRFAANLIAGILDYKTILDVRGLPVDRATHNRKGQPFCMEQYYRLFSSYRYPGITKDE-LITNINRDPFDHEHIIVACNG   208
C.elegans         110 KVK.ETKEDHIKYT.L.TR.L.E..NLI.TKQVCREKS.GAQ.L.-M.....D.VL.C..E..VGE.T-Q.-RKQKTNDGN..VL.M.RN   196
D.melanogaster    179 PRR.KTVHDVAH...R.LD...SHREM..SGE..LE..ASRE.N..L..A.....LG.C.R..VKQ.SQFLPSRE.LNDEDR.VV.I.RN   268
M.musculus        115 .....DTND......S..S.V.S..AL..SQSI.T.W.KGQLS....L..K..........L..H.Q.T-.VAQKSSIMPEP..V....CN   203
H.sapiens         104 ....PGTDD......S..S.V.S..AL..SHSI.T.C.KGQLS...L..K...G......L..H.Q.T-.VAQNSSIMPEP..V....CN   192

D.japonica        209 QFFVLEITTSAD-RPSEEDLYNQLRRIVRLAENEKTE-EVVGILTALPRSKWASVRQHLLLDSINKKSMEMIEKSIFILCIDKSVPVTFN   296
C.elegans         197 .T.L.HSRINGA-LV.YA.VEY..AQ.EEISKINQNNTANI.ASGVG..DNA.LFW.DM.TVEQ.S..Y.WVKSAL.VV.L.MED.IDYG   285
D.melanogaster    269 .MYCVVLQA.DRGKL..SEIAS.ILYVLSD.PCLPAKPVP..L...E...T..RD.EM.QE.ER.QRNL.L..TAQVV..L.EPLAGN..   358
M.musculus        204 .....DVVINFR-.L..G..FT...K..KM.S..DERLPPI.L..SDG..E..KA.TV..K..T.RD.LD...RC.CLV.L.--G.----   286
H.sapiens         193 .....DVVINFR-.L..G..FT...K..KM.S..DERLPPI.L..SDG..E..EA.TV.VK..T.RD.LD...RC.CLV.L.--A.----   275

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D.japonica        297 HSE----SVELTKVGIRDNVNCALQLLHGMGSEFNGANRWYDKTMQFVISSDGNCGLNYEHSVAEGIAVIRLIEHILDYMEEI-KSR--R   379
C.elegans         286 KND----TMSIS-EKEKEF.ARGYST.T.H..SKF.L.....A.I.L.V..S.VN..CI...T....VI.NMA.TAIR.AQKYF..KMVW   370
D.melanogaster    359 ARGFTGATPTVHRA.D..ET.M.HEMI..G...Y.SG...F.....LI.CT..TW..C....CS.....VQ.L.K.YKKI..H-PDE-DN   446
M.musculus        287 --G----TGD.S------DTHR.......G.CSL.........SL...VGR..T..VVC...PFD..VLVQCT..L.KH.MTG-NKKLV.   363
H.sapiens         276 --G----G...S------DTHR.......G.YSK.........SL...VGR..T..VVC...PFD..VLVQCT..L.KH.TQS-SRKLI.   352

D.japonica        380 LTRLSSVCDLAPPK-KLEWKIDKYVSEMISEAKQEIKNLADNLDIYIL-RYQK-F-GRE--FPKTVNMSPDSFIQLALQLTHYKIHKCLV   463
C.elegans         371 ND-VRN.H-PKSLTWHFSENSRNILKKQAEVFDELANE.--E.EVL.FNEFG.DSI-KN------WRV...G....IM..A...T.GH..   449
D.melanogaster    447 GLPQHHLPPPERLEWHVGPQLQLRFAQASKSVDKC.DD.--DFYV.RYQS.G.T.I-KS-C-----QV...VY.......A...LYGR..   527
M.musculus        364 ADSV.ELPAPRRLRW.CSPETQGHLASSAEKLQRIV...--DFIV.KFDN.G.T.IKKQKC-------...G...V....AY.RLYQR..   444
H.sapiens         353 ADSV.ELPAPRRLRW.CSPE.QGHLASSAEKLQRIV...--DFIV.KFDN.G.T.IKKQKC-------...A...V....AF.RL.RR..   433

D.japonica        464 PTYESASIRRFSLGRVDNIRAASIPALKWCTAM-NGDIPST--------DDE----------K--II-LLRSAMEWQTKVMLDTILGHGV   531
C.elegans         450 S......V...GA........NTQE..E.V...ASKKESKERKLELFKKAVLKQVKVTLENISGYGVDNHLC.LFCLAREREE.TGEDIP   539
D.melanogaster    528 A......T...LH....C.....TE..E.AK..CQ.EGANVPLESDREDEE.SRKVKFSIYS.DHLRE.F.C.VAR..E..VKN...N.I   617
M.musculus        445 ..........QE.......S.TPE..AFVQ..-TDHKAAVL-------AS.----------.--LQ-..QR.IQA..EYTVMA.T.MAI   513
H.sapiens         434 ..........QE.......S.TPE..AFVR.V-TDHKAAVP-------AS.----------.--LL-..KD.IRA..AYTVMA.T.MAI   502

D.japonica        532 DNHLLGLKEAAVELNIEIPELFKDKTYLESNRFRLSTSQVPTLKDAFMCYGAVVEHGYGAAYNPHQNYIVVAISCWCNCPDTRADVFAQK   621
C.elegans         540 SLF.DP.WSEVMRFPLSTSQVTTSLDIPDCYLTYGAVVRDGYGCPYNIQPDR.IFAPTAFRSD.RTDLQHFKK.LAGAMR.VKELLSN--   627
D.melanogaster    618 .IP....R..SI.VTG.MH.....ES.II.QC.L......ACST.S..G..P.TPR...CS....PEQ..FCV.AFYS.E..S.SRY.KS   707
M.musculus        514 .....A.R.L.RD.CK.P..M.M.E...M....I........TMEM.C...P..PN....C...QPEA.TFC..SFHG.KE.SSVE..EA   603
H.sapiens         503 .....A.R.L.RAMCK.L..M.M.E...M....V........TTEM.C...P..PN....C...QPET.LFC..SFHS.KE.SSSK..KA   592

D.japonica        622 LKESFDEMRILLTSNLELAKNRKAARIQEIIMEESCRSENLLNHTFEAKTANDIGNIKLHE                                682
C.elegans         627 ------------------------------------------------------------                                627
D.melanogaster    708 .QD.L.I..D..QN------------------------------------------------------                        721
M.musculus        604 VGA.LVD..D.CS.RQPAEGKPPT.KERARGPTKPSNLDYSH-------------------                                645
H.sapiens         593 VE..LID..D.CSLLPPTESKPL.TKEKATRPSQGHQP----------------------                                630
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Fig. 1. (A) ACh biosynthetic pathway. ChAT, choline acetyltransferase; AChE, acetylcholinesterase. (B) Multiple alignment of the predicted amino acid sequence of DjChAT with ChAT of *C. elegans* (AAA53660), *D. melanogaster* (AAA28406), *M. musculus* (NP_034021), and *H. sapiens* (NP_066265). Conserved amino acids are indicated by dots. Gaps are indicated by hyphens. Asterisks indicate the EHS motif.