PIBB 生物化学与生物物理进展
Progress in Biochemistry and Biophysics
2009, 36(4): 410~416
www.pibb.ac.cn

Sec-10 Knockout Increases The Neuroactive-drug Responses Without Affecting Function of The Postsynaptic Ionotropic Receptors in Neuromuscular Junctions*

ZHANG Lei[1)**], LI Jiang-Li[1)**], GAO Shang-Bang[1)**], WU Zheng-Xing[1)], ZHANG Rong-Ying[1)***], XU Tao[1, 2)***]
([1)] *Key Laboratory of Molecular Biophysics, Ministry of Education, Huazhong University of Science and Technology, Wuhan* 430074, China;
[2)] *National Key Laboratory of Biomacromolecules, Institute of Biophysics, The Chinese Academy of Sciences, Beijing* 100101, China)

Abbreviations: KO, knockout; aa, amino acid; NMJs, neuromuscular junctions; mPSCs, miniature postsynaptic currents; ACh, acetylcholine; AChRs, acetylcholine receptors; WT, wild type; GPCRs, G-protein coupled receptors

Abstract Exocyst complex is known to function in the exocytosis network, however, the molecular mechanism is unclear yet. Using UV/trimethylpsoralen mutagenesis, the *sec-10* (one component of the exocyst complex) knockout mutant of *C. elegans* was obtained for the first time. The drug sensitive assays revealed clearly that the *sec-10* gene affected the neural signal transmission, however, the electrophysiological assay showed the function of the ionotropic receptors in the neuromuscular junctions (NMJs) were unaltered compared with the wild type (WT). Thus it was assumed that the *sec-10* gene might not influence the known ionotropic receptors in the NMJs, but some other pathways instead.

Key words exocyst complex, *sec-10*, NMJs, ionotropic receptors
DOI: 10.3724/SP.J.1206.2009.00100

The exocyst complex, also known as the SEC-6－SEC-8 complex, is a multi-protein complex composed of SEC-3, SEC-5, SEC-6, SEC-8, SEC-10, SEC-15, EXO-70 and EXO-84. It is conserved from yeast to mammals[1, 2] and is required for polarized exocytosis. In the budding yeast *Saccharomyces cerevisiae*, this complex directs vectorial targeting of secretory vesicles to sites of rapid membrane expansion [3 ~5]. Similarly, the mammalian exocyst complex has been directly implicated in the targeting of Golgi-derived vesicles to the basolateral membrane of polarized epithelial cells and to the growth cones of differentiating PC12 cells[6].

However, the molecular mechanism underlying the function of the exocyst complex is still unclear. Here, we used the nematode *Caenorhabditis elegans* as a model, because its genome is completely sequenced and many functional genomic tools are available. Using UV/trimethylpsoralen mutagenesis, we obtained a *sec-10* knockout (KO) mutant which was mildly uncoordinated. The Drug sensitivity assays revealed the *sec-10* KO mutant was hypersensitive to the aldicarb, levamisole and nicotine. Our electrophysiological experiments showed no differences of miniature postsynaptic currents (mPSCs) in muscle end-plate caused by spontaneous release of acetylcholine between the *sec-10* mutant and WT, indicating that the amount of neurotransmitter in a single synaptic vesicle and the postsynaptic response to the spontaneous quantal release of acetylcholine were not changed in the mutant. The mechanism underlain needs to be further explored.

*This work was supported by a grant from The National Science Foundation of China (30500117, 30770523).
**These authors contributed equally to this work.
***Corresponding author.
Zhang Rong-Ying. Tel: 86-27-87792337, E-mail: ryzhang@mail.hust.edu.cn
Xu Tao. Tel: 86-10-64888524, E-mail: xutao@ibp.ac.cn
Received: February 23, 2009 Accepted: March 26, 2009

1 Materials and methods

1.1 Strains

Standard methods were used for the maintenance and manipulation of *C. elegans* [7]. *C. elegans* variety Bristol was used as the wild-type strain. The *sec-10* mutant strain containing a 140 bp deletion (eliminating Ⅳ: 7780126～7780265) and 5 bp insertion (gagaa) was obtained by UV/trimethylpsoralen mutagenesis, as described in the *C. elegans* Gene Knockout Protocols from Dr. Michael Koelle's laboratory (http://info.med.yale.edu/mbb/koelle/). The *sec-10* mutant strains were backcrossed 8 times and balanced with JK2958 before the phenotype analysis. Primers used for the PCR screening of the *sec-10* deletion mutation were (5′ cttggctggaatttctcttgcag 3′) and (5′ aattgacattgtgg-ggatactcg 3′).

1.2 Drug sensitivity assays

Acute sensitivities to aldicarb (1 mmol/L; Sigma), levamisole (800 μmol/L; Sigma) and nicotine (30 mmol/L: Sigma) were determined by assaying the time course of the onset of paralysis following acute exposure of a population of animals to these drugs. In each experiment, 25～30 worms were placed on drug plates and prodded every 10 min over a 2 h period to determine if they retained the ability to move, worms that failed to respond at all to this harsh touch were classified as paralyzed.

1.3 Electrophysiology

The worms used for electrophysiological recording were raised at 20℃ under standard laboratory conditions on NGM agar plates cultured with OP50 *Escherichia coli*. All recordings were obtained from the body wall muscles of adult worms. The worm preparation was similar to that previously described by Richmond *et al*[8]. Briefly, an animal was immobilized on a Sylgard-coated glass coverslip by applying a cyanoacrylat adhesive along the dorsal side. A longitudinal incision was made in the dorsolateral region, and after clearing the intestine and gonad, the cuticle flap was folded back and glued to the coverslip. The preparation was then washed briefly (～30 s) with 1 g/L collagenase (Sigma Type Ⅰ) in standard external solution. Borosilicate glass pipettes with a tip resistance of 3～5 MΩ were used as electrodes and the classical whole-cell configuration was obtained by rupturing the patch membrane of a gigaohm seal. The cell was voltage-clamped at –60 mV to record mPSCs and acetylcholine-induced inward currents. Data were sampled at a rate of 10 kHz after filtering at 2.9 kHz.

The recording pipette solution contained (in mmol/L): 115 KCl, 25 KOH, 25 Glutamate, 0.1 $CaCl_2$, 50 HEPES, 5 MgATP, 0.5 Na_2GTP, 1 BAPTA (pH 7.2 with KOH, osmolarity adjusted to 315 mOsm with sucrose). The external solution included (in mmol/L): 150 NaCl, 5 KCl, 1 $CaCl_2$, 4 $MgCl_2$, 10 Glucose, 15 HEPES (pH 7.4 with NaOH, osmolarity adjusted to 330 mOsm with sucrose).

1.4 Data analysis

Data analysis was performed using Igor Pro (Wavemetries, Lake Oswego, OR, USA). Amplitude and frequency of mPSCs were analyzed using MiniAnalysis (Snaptosoft, Decatur, GA). A detection threshold of 5 pA was used in initial automatic analysis, followed by visual inspections to include missed events and to exclude false events resulting form baseline fluctuations. For experiments measuring mPSCs, at least 30～60 s continuous data were used in the analysis. Unless stated otherwise, results were presented as $\bar{x} \pm s$ with the indicated number of experiments. Statistical significance was evaluated by Student's t test or Mann-Whitney rank sum test according to the normality of datum distribution in SigmaStat 3.11 (Systat Software, Inc., Canal Blvd., Suite C Richmond, CA, USA). $P<0.05$ was considered to be statistically significant.

1.5 Plasmid and transgenic strains

The reporter gene P0925 [*sec-10*::*gfp*] construct contains a 5.3 kb fragment upstream of the ATG start codon of *sec-10* that was amplified by PCR using with primers (5′ gaccggatcccctattcactaagtgcgttgagcctac 3′) and (5′ caccggtaccctgtaattatttgttcctaaagcatgaa 3′) and subcloned into the vector pPD95.75 (a gift from A. Fire). The rescue gene P0926 [*sec-10*::*sec-10*] contains the 5.3 kb upstream and a full-length *sec-10* genomic DNA. To make a full-length *sec-10* genomic DNA, we amplified a 3.1 kb fragment by PCR using with primers (5′ ataaggtaccatgagtggtggacaatatgtgacatatgt ac 3′) and (5′ aaaagggcccattttatttacaagaatacccgcaacgg 3′). The resulting fragment was then fused to the P0926 [*sec-10*::*gfp*], using the *Kpn* Ⅰ and *Apa* Ⅰ site to generate the P0926 [*sec-10*::*sec-10*].

The strain HD633 [*sec-10*::*gfp*] was produced by injecting WT with the *sec-10*::*gfp* at 20 mg/L. The strain HD631 [*sec-10*::*sec-10*] was produced by injecting *sec-10* mutant with the *sec-10*::*sec-10* at 20 mg/L, along with the marker plasmid myo-3::T dimer at 50 mg/L.

2 Results

2.1 *Sec-10* is hypersensitive to aldicarb, levamisole and nicotine

Using UV/trimethylpsoralen mutagenesis, we obtained a *sec-10* KO mutant. The 140 bp-deletion and 5bp-insertion (described in **Materials and methods**) introduced a premature stop codon in exon4, resulting in a C-terminal truncated *sec-10* translation product of 457aa compared with the wild-type protein that is 659aa in length. The *sec-10* mutant displayed a weak uncoordinated phenotype. In order to verify if this phenotype was caused by the deletion of *sec-10* gene, we injected the plasmid P0926 [*sec-10*::*sec-10*] into the *sec-10* mutant, and the uncoordinated phenotype was fully rescued, which means the defect was caused by the deletion of the *sec-10* gene. Mutations that exhibit uncoordinated movement are often caused by the defects of the body wall muscles or the nervous system. Using neuroactive drugs, we tested whether *sec-10* mutants display defects in synaptic signaling. First, aldicarb was used to detect neuromuscular signaling in *sec-10* mutant [7, 9, 10]. Aldicarb, the acetylcholine esterase inhibitor, causes accumulation of acetylcholine (ACh) at neuromuscular junctions (NMJs), leading to acute paralysis and subsequent death. *Sec-10* mutants are hypersensitive to aldicarb (Figure 1a). However, this could be induced by two possibilities: the amount of released ACh increases or the sensitivity of the postsynaptic response increases[11].

Therefore, we used two additional drugs, levamisole and nicotine. Levamisole is a potent cholinergic agonist, and is often used as a pesticide. It leads to a hypercontracted paralysis of wild-type nematodes, usually followed by relaxation and death [12]. The *C. elegans* body wall muscle expresses two distinct acetylcholine receptors. One is sensitive to levamisole while the other is sensitive to nicotine[13]. If the defect were due to the enhanced sensitivity of the postsynaptic response, one would expect the mutant is more sensitive in the presence of levamisole and nicotine. Our result showed *sec-10* is hypersensitive to levamisole and nicotine (Figure 1b, c). However, we could not exclude the possibility that the hypersensitivity to aldicarb may also be caused by the elevated release of acetylcholine.

The reporter gene P0925 [*sec-10*::*gfp*] was injected into the WT, and the expression pattern showed that *sec-10* was widely expressed in intestine, pharynx, vulve, gonad sheath cell, hypodermis, coelomocyte, neurons and muscle (data not show), considering the hypersensitivity to the neuroactive drugs, we speculated the *sec-10* might function in the NMJs.

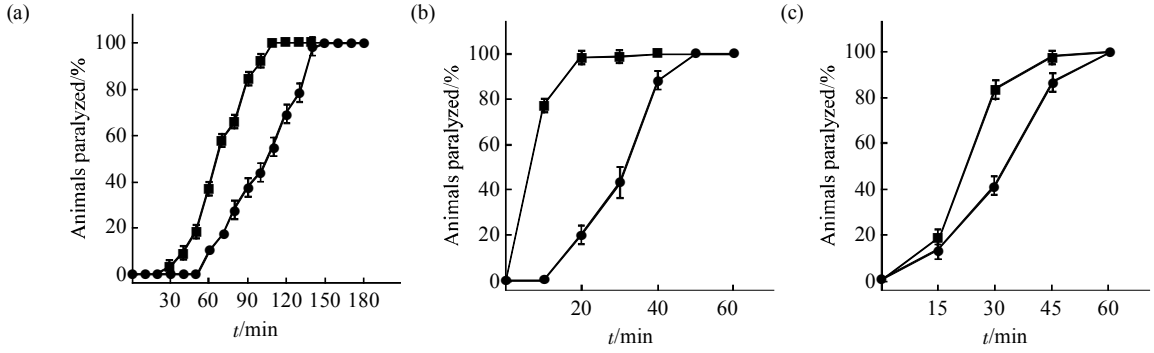

Fig. 1 *Sec-10* mutant showed hypersensitive to aldicarb, levamisole and nicotine

(a) Aldicarb (1 mmol/L)-induced paralysis of adult animals cultivated at 20℃ (WT, n = 3; *sec-10*, n =3). (b) Levamisole (800 μmol/L)-induced paralysis of adult animals cultivated at 20℃ (WT, n = 3; *sec-10*, n =3). (c) Nicotine (30 mmol/L)-induced paralysis of adult animals cultivated at 20℃ (WT, n = 3; *sec-10*, n =3). ●—●: N2; ■—■: *sec-10*.

2.2 The postsynaptic receptors of *sec-10* mutant show normal responses under electrophysiology conditions

To explore whether *sec-10* contributes to the response of the body wall muscles, the *in vivo* electrophysiological assay was further performed by us. The body wall muscle expresses two distinct acetylcholine receptors (AChRs) and one GABA receptor. Using whole-cell voltage-clamp recordings of individual body wall muscles, it is possible to distinguish the two acetylcholine receptors because one is sensitive to levamisole while the other is sensitive to nicotine[14]. Application of acetylcholine in wild-type animals activates both receptors.

Our results showed inward currents induced by acetylcholine showed no significant difference in WT and the *sec-10* mutant (Figure 2a, *A*). The peak amplitude of the *sec-10* mutant was slightly smaller than that of the WT (Figure 2a, *B*), however, the cell capacitance of the *sec-10* mutant was also smaller, compared with the WT. The current density as determined by peak amplitude of response normalized to cell capacitance has no difference between the *sec-10* mutant and WT (Figure 2a, *C*). We also applied levamisole and nicotine. As a whole, the *sec-10* mutant exhibited wild-type responses to ACh (WT, (26.756 4 ± 5.199 63) A/F, n = 13; *sec-10* mutant, (27.663 1 ± 4.714 02) A/F, n = 8, respectively; Figure 2a, *C*). Response to levamisole (WT, (5.831 34 ±0.696 267) A/F, n = 7; *sec-10* mutant, (5.836 95 ± 0.974 317) A/F, n = 7, respectively; Figure 2b, *C*) and nicotine(WT, (18.859 8 ± 2.904 14) A/F, n = 6; *sec-10* mutant, (19.210 8 ± 2.166 35) A/F, n = 6, respectively; Figure 2c, *C*) were also unaffected in *sec-10* compared with the WT.

Besides, the response to muscimol, a GABA agonist, also indicates that the GABA receptor function in the NMJs was not affected (WT,(18.129 6 ± 1.354 4) A/F, n = 8; *sec-10* mutant, (18.812 1 ± 2.010 87) A/F, n = 5, respectively; Figure 2d, *C*).

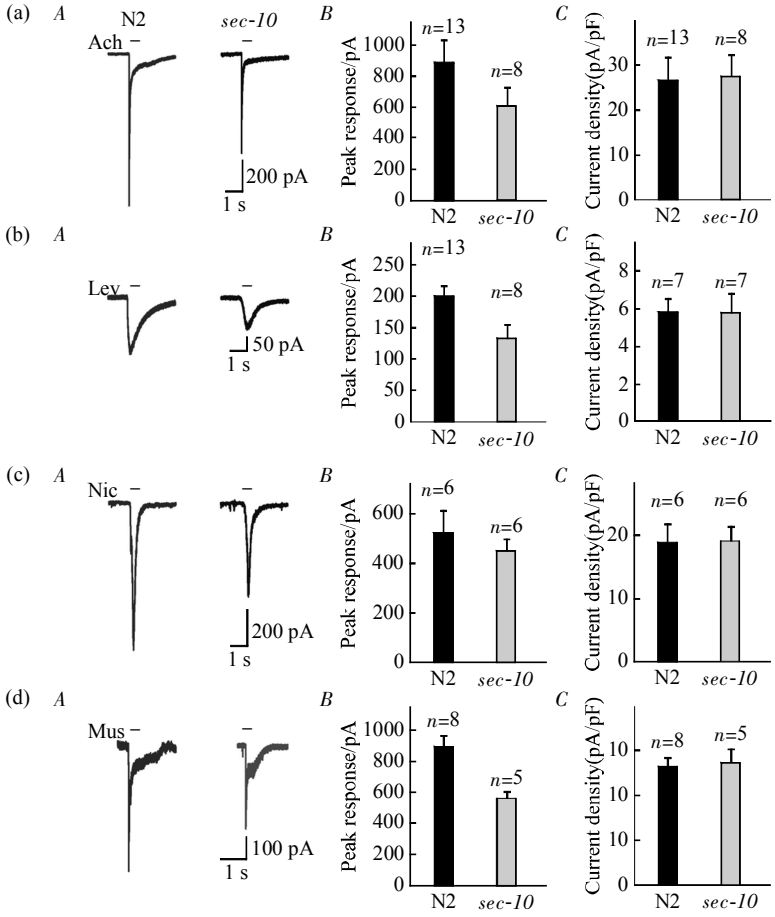

Fig. 2 ***Sec-10*** **mutant did not change the postsynaptic receptors currents in body wall muscle**

(a) *A*: Representative traces showing acetylcholine (0.5 mmol/L)-induced inward currents in WT (WT) and the *sec-10* mutant. *B*: Comparison of the peak amplitudes between WT and *sec-10* mutant. *C*: Comparison of the acetylcholine-induced inward currents density between WT and *sec-10* mutant (WT, n = 13; *sec-10*, n = 11). (b) *A*: Representative traces showing levamisole (0.5 mmol/L)-induced inward currents in WT (WT) and the *sec-10* mutant. *B*: Comparison of the peak amplitudes between WT and *sec-10* mutant. *C*: Comparison of the levamisole-induced inward currents density between WT and *sec-10* mutant (WT, n = 7; *sec-10*, n = 7). (c) *A*: Representative traces showing nicotine (0.5 mmol/L)-induced inward currents in WT (WT) and the *sec-10* mutant. *B*: Comparison of the peak amplitudes between WT and *sec-10* mutant. *C*: Comparison of the nicotine-induced inward currents density between WT and *sec-10* mutant (WT, n = 6; *sec-10*, n = 6). (d) *A*: Representative traces showing muscimol (0.1 mmol/L)-induced inward currents in WT (WT) and the *sec-10* mutant. *B*: Comparison of the peak amplitudes between WT and *sec-10* mutant. *C*: Comparison of the muscimol-induced inward currents density between WT and *sec-10* mutant (WT, n = 8; *sec-10*, n = 5).

We checked the miniature postsynaptic currents (mPSCs) of end-plate at the neuromuscular junctions using electrophysiological methods. We recorded mPSCs at the neuromuscular junctions of the *sec-10*

mutant. In the absence of action potentials or direct nerve stimulation, synaptic vesicles at the presynaptic site may release neurotransmitters spontaneously, causing mPSCs in the postsynaptic cell membrane.

In *sec-10* mutant, the frequency and amplitude of mPSCs were not changed compared with WT(Figure 3a). mPSC amplitude histograms of the WT and the mutant revealed that the distribution of mPSC amplitude has no difference(Figure 3b). Cumulative mPSC amplitude distribution showed that the mPSCs amplitude in the *sec-10* mutant is same as that in the WT. In wild-type preparations, the frequency of mPSCs was (23.239 1 ± 4.988 68) Hz (n = 11). The frequency of mPSCs was not affected in *sec-10* mutants(24.444 3±4.863 59, n= 5) (Figure 3d). The mean amplitude of mPSCs was also not changed in the *sec-10* mutant (WT, (24.221 9 ± 0.155 715) pA, n = 11; *sec-10* mutant, (24.685 5 ± 0.197 208) pA, n = 5, respectively; Figure 3e). Besides, the rise time, decay time and quantal size were not changed in the *sec-10* mutant either (Figure 3f~ h).

These results showed that the mutation of *sec-10* did unlikely change the postsynaptic response mediated by the two acetylcholine receptors (AChRs) and GABA receptors at the body wall muscles as identified by the pharmacological experiments.

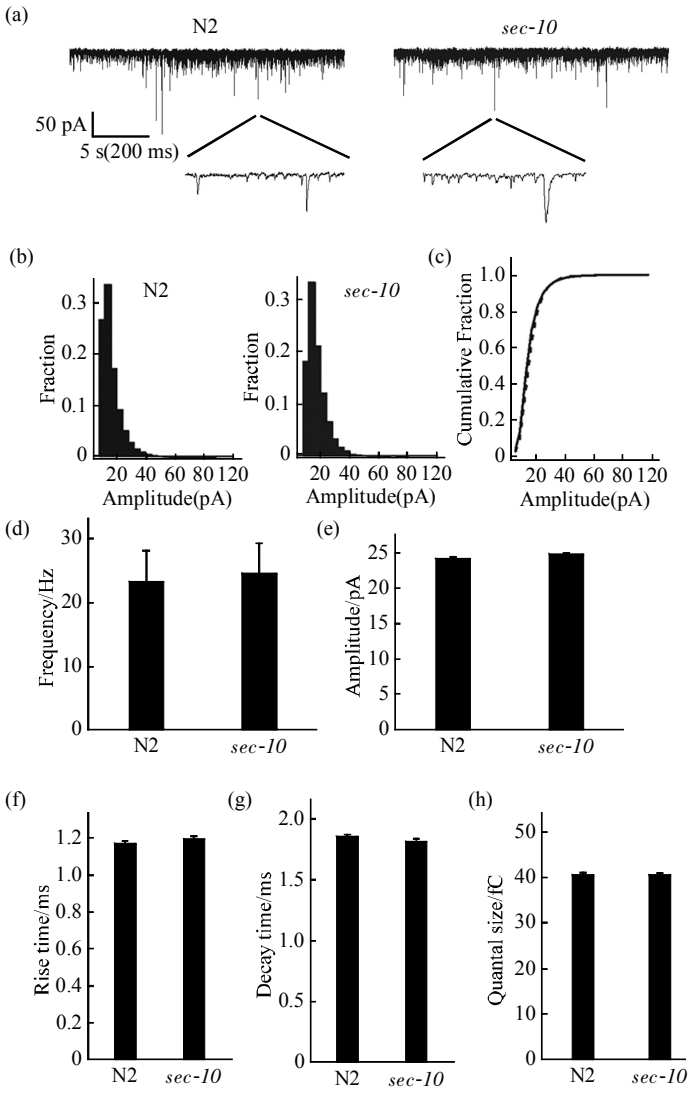

Fig. 3 *Sec-10* mutant did not change the mPSCs at the *C. elegans* neuromuscular junctions

(a) Representative traces of mPSCs of the WT (WT) and the *sec-10* mutant. In WT, mPSCs showed great variability in amplitudes. In *sec-10* mutant, the frequency and amplitudes of mPSCs were not changed compared with WT. A portion of the traces shown above were showing at expanded time scale. (b) mPSC amplitude histograms showed that amplitude mPSCs were not changed in the mutants. (c) Cumulative mPSC amplitude distribution showing that mPSCs have no differences between WT and *sec-10* mutant. — : N2; ····· : *sec-10*. (d) The frequency of mPSCs was not changed in the mutant. (e) The mean amplitude of mPSCs was not changed in the mutant. (f) The rise time was not changed in the mutant. (g) The Decay time was not changed in the mutant. (h) The quantal size was not changed in the mutant. (WT, n = 11; *sec-10*, n = 5).

3 Discussion

The *sec-10* KO accelerated the time course of aldicarb-induced paralysis, which suggested the presynaptic release increases or the sensitivity of postsynaptic response increases. Our data then revealed that the *sec-10* mutant is hypersensitive to levamisole and nicotine, suggesting the postsynaptic sensitivity is likely to be affected. However, the electrophysiological results showed the body wall muscle responded normally to ACh, levamisole and nicotine. The mPSC amplitude was not affected significantly in the *sec-10* mutant, suggesting there was little disruption of postsynaptic ionotropic receptors and the quantal size of neurotransmitters might not be changed. Frequency of mPSC was the same in the wild-type and the *sec-10* mutant, suggesting that presynaptic release probability is normal.

In conclusion, our results suggested that the postsynaptic ionotropic receptors were not affected in this mutant. However, the drug sensitivity assay implied there might be some other pathways influenced by deletion of the *sec-10* gene. For example, the G-protein coupled receptors (GPCRs) could be a candidate, for all known transmitter molecules not only mediate the fast chemical synaptic transmission *via* ionotropic receptors, but also activate a variety of GPCRs[15]. Further work need to be done to clarify the mechanism.

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Sec-10 影响神经肌肉接头突触后敏感性的研究 *

章　雷 [1)**]　李江丽 [1)**]　高尚邦 [1)**]　吴政星 [1)]　张蓉颖 [1)***]　徐　涛 [1,2)***]

([1)]华中科技大学分子生物物理教育部重点实验室，武汉 430074; [2)]中国科学院生物物理研究所，生物大分子国家重点实验室，北京 100101)

摘要　exocyst 复合物在分泌过程中起重要作用．可是分子机理尚未研究透彻．通过 TMP/UV 的基因敲除方法，得到了 exocyst 复合物中一种组分 *sec-10* 的缺陷型线虫．药学结果显示这种线虫神经信号传递存在缺陷，可是电生理的方法证明在神经肌肉接头处的已报道离子型受体和野生型相比并未发生改变．因此猜测，*sec-10* 并未直接影响神经肌肉接头处的离子型受体，而是通过其他途径来行使功能．

关键词　exocyst 复合物，*sec-10*，神经肌肉接头，离子型受体

学科分类号　Q71，Q424　　**DOI:** 10.3724/SP.J.1206.2009.00100

* 国家自然科学基金资助项目(30500117, 30770523).

** 共同第一作者.

*** 通讯联系人.

张蓉颖. Tel: 027-87792337, E-mail: ryzhang@mail.hust.edu.cn

徐涛. Tel: 010-64888524, E-mail: xutao@ibp.ac.cn

收稿日期：2009-02-23，接受日期：2009-03-26