主論文の要約

Differential effects of spinal motor neuron-derived and skeletal muscle-derived Rspo2 on acetylcholine receptor clustering at the neuromuscular junction

（運動神経細胞と筋肉細胞より分泌されるRspo2は神経筋接合部のアセチルコリン受容体の集積に対して異なる役割をもつ）

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【Introduction】

The release of the neurotransmitter acetylcholine (ACh) from the axon terminal of the spinal motor neuron (SMN) activates the postsynaptic acetylcholine receptor (AChR) and elicits an action potential in the target muscle fiber. To ensure efficient neuromuscular signal transmission, dense AChR clusters must be compactly formed against presynaptic nerve terminals in the proper organization. Genetic depletion of the embryonic AChR γ-subunit in muscle fibers markedly decreases staining for AChR clusters with progressive accumulation of synaptic vesicles in the presynaptic terminal, suggesting that embryonic AChR clustering is required for further pre- and postsynaptic development. AChR clustering and formation of the neuromuscular junction (NMJ) are mediated by SMN-derived and muscle-derived molecules. R-spondins (Rspo1-4) play essential roles in development through Wnt signaling pathways in vertebrates. As a member of the Rspo family, Rspo2 binds directly to the leucine-rich-repeat-containing G-protein coupled receptors (Lgr) 4 and 5 to enhance stabilization of Wnt receptors on the cell membrane and to activate Wnt signaling pathways. Rspo2 is implicated in development of the larynx, lung, limb, and trachea in mice. *Rspo2*-deficient mice present with an abnormally short left hindlimb and lack of digits on both forelimbs at embryonic day (E) 18.5, and mice die perinatally due to respiratory distress. In cultured myogenic C2C12 cells, Rspo2 is able to promote muscle differentiation through Wnt/β-catenin signaling. We recently reported by laser capture microdissection of mouse SMNs that Rspo2 is highly expressed in SMNs. Rspo2 is likely to be excreted from the axon terminals of SMNs and is accumulated at the NMJ in wild-type mice. In cultured C2C12 myotubes, Rspo2 induces MuSK phosphorylation and AChR clustering, which requires Wnt ligands but not agrin. Lgr5 is associated with MuSK at the NMJ and serves as a receptor for Rspo2. In *Rspo2*-knockout mice, the number and density of AChRs at the NMJ are reduced with widened synaptic clefts, sparse synaptic vesicles, and markedly reduced frequency of miniature endplate potentials. We assumed that SMN-derived Rspo2 was anchored at the NMJ and promoted AChR clustering as well as NMJ formation, but Rspo2 is also expressed marginally in the diaphragm at E18.5 and in adults. To dissect the differential roles of SMN-derived Rspo2 and muscle-derived Rspo2 on AChR clustering and NMJ formation in mouse embryos, we generated transgenic mice expressing Rspo2 specifically in SMNs or in skeletal muscle on the background of *Rspo2*$^{-/-}$.

【Materials and Methods】

The diaphragms from E18.5 embryos were separated and performed by immunofluorescence. The diaphragms and spinal cords were harvested from E18.5 embryos for RNA extraction and quantitative RT-PCR. The ultrastructure of the left diaphragm was observed by electron microscopy.

【Results】

SMN-specific Rspo2 mitigated abnormal features of the NMJs and AChR clusters observed in *Rspo2*$^{-/-}$ mice including (i) abnormal broadening of enlarged AChR clusters, (ii) three of six abnormal ultrastructural features, and (iii) abnormal expression of nine genes in SMNs and the diaphragm. In contrast, muscle-specific Rspo2 normalized all six abnormal ultrastructural features, but it had no effect on AChR clustering and NMJ formation at the light microscopy level or on abnormal gene expression in SMNs and the diaphragm.

【Discussion】

We rescued the phenotypes of *Rspo2*$^{-/-}$ mice by MCK-promoter-driven muscle-specific Rspo2 and VAChT-promoter-driven SMN-specific Rspo2. Rspo2 plays critical roles in the development of multiple tissues including the craniofacial structures, kidneys, lungs, and limbs in mice and humans. *Rspo2*$^{-/-}$ mice die shortly after birth due to respiratory distress, and they have defects of the left hindlimb and digits of both forearms. Neither muscle-derived Rspo2 nor SMN-derived Rspo2 rescued the perinatal death or limb anomalies. The methods of our NMJ analyses can be divided into four categories: (i) low-magnification analysis of the whole-mount diaphragms, (ii) high-magnification analysis of the NMJ, (iii) ultrastructural analysis of the NMJ, and (iv) gene expression analysis of the diaphragm and spinal cord. In three of the four categories (i, ii, and iv), SMN-derived Rspo2 mitigated aberrant NMJ features in *Rspo2*$^{-/-}$ mice more than muscle-derived Rspo2. Ultrastructural features (iii) were normalized by both SMN-derived and muscle-derived Rspo2, but muscle-derived Rspo2 improved more features than SMN-derived Rspo2. Individual NMJ features can also be divided into three categories by the effects of muscle- and SMN-derived Rspo2: (a) features improved by both muscle-derived and SMN-derived Rspo2, (b) features improved by muscle-derived Rspo2 more than SMN-derived Rspo2, and (c) features improved by SMN-derived Rspo2 more than muscle-derived Rspo2. First, features improved by both muscle-derived and SMN-derived Rspo2 were three of the six ultrastructural parameters. Elevated expression of the *Chat* gene in the spinal cord was also mitigated by both Rspo2's. Second, features improved by muscle-derived Rspo2 were the other three of the six ultrastructural parameters. Third, features improved by SMN-derived Rspo2 included the bandwidth of AChR clusters and the area of AChR clusters. In the diaphragm, elevated expression levels of NMJ-specific genes, target genes of Wnt/β-catenin signaling, and myogenic marker genes were normalized only by SMN-derived Rspo2. In the spinal cord, elevated expression of *Agrn* was normalized only by SMN-derived Rspo2. Thus, SMN-derived Rspo2 improved the NMJ phenotypes more than muscle-derived Rspo2. Higher expression of Rspo2 in the spinal cord compared to the diaphragm at E18.5 in wild-type mice also supports the notion that SMN-derived Rspo2

plays a pivotal role in NMJ development. Rspo2 is a secreted protein, but the distance that Rspo2 may travel in our body remains unknown. Indeed, only SMN-derived Rspo2 over-corrected the bandwidth of AChR clusters, which was determined by branching of motor axons. The reason why only muscle-derived Rspo2, but not SMN-derived Rspo2, normalized three ultrastructural features remains unknown, but muscle-derived and SMN-derived Rspo2 may have different roles during normal development of the NMJ. Alternatively, as Lgr5 is a receptor for Rspo2, and is highly expressed in motor neurons including SMNs, muscle-derived Rspo2 might have worked on Lgr5 expressed in SMNs. As Rspo2 is a secreted protein, both SMN-derived Rpos2 and muscle-derived Rspo2 can reach the target tissues and exert overlapping effects. Identifying the origin of a secreted protein is thus challenging and enigmatic.

【Conclusion】

To summarize, we demonstrate that SMN-derived Rspo2 is required for AChR clustering at the motor endplate and for the NMJ formation, and muscle-derived Rspo2 also plays a substantial role in ultrastructural formation of the NMJ.