Post-synaptic specialization of the neuromuscular junction: junctional folds formation, function, and disorders

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Abstract
Post-synaptic specialization is critical to the neurotransmitter release and action potential conduction. The neuromuscular junctions (NMJs) are the synapses between the motor neurons and muscle cells and have a more specialized post-synaptic membrane than synapses in the central nervous system (CNS). The sarcolemma within NMJ folded to form some invagination portions called junctional folds (JFs), and they have important roles in maintaining the post-synaptic membrane structure. The NMJ formation and the acetylcholine receptor (AChR) clustering signal pathway have been extensively studied and reviewed. Although it has been suggested that JFs are related to maintaining the safety factor of neurotransmitter release, the formation mechanism and function of JFs are still unclear. This review will focus on the JFs about evolution, formation, function, and disorders. Anticipate understanding of where they are coming from and where we will study in the future.

Keywords: Post-synaptic specialization, Neuromuscular junction, Junctional folds, Development, Formation mechanism, Function, Disease

Introduction
Chemical synapses enable cells communication via neurotransmitters release and induce the chemical signal pathway active in the receptor cells [1, 2]. The neuromuscular junction (NMJ) is a typical chemical synapse established between the lower motor neurons in the spinal cord and the skeletal muscle fibers. It comprises three essential elements: presynaptic motor nerve terminal; perisynaptic Schwann cells (SCs); and the specialization post-synaptic plasma membrane of the muscle fiber [3–6]. In the past, most of our understanding of synapse formation and receptor clustering comes from these researches at NMJ. For example, the conclusion that NMJ was a site of neurotransmission between separate cells claimed by Kühne was earlier than the neuron doctrine. And the ‘vesicular hypothesis’ proposed by Jose del Castillo and Katz explained how acetylcholine (Ach) is released by quantal from nerve terminals [5].

The significant difference between the NMJ and neuron-neuron synapses is located in the synaptic cleft and the post-synaptic membrane. The gap of NMJ in mammals is width from 50 to 100 nm, and the basal lamina (BL) is located in the middle of the cleft [7]. While the width of synapse in CNS is about 20–30 nm, and there is no BL existing [8]. Face the active zones (AZs) of the pre-synaptic axon terminal is the primary branch of JFs. At the shoulder areas of JFs crests, AChRs are concentrated at a high concentration of 10,000/um² with 1000X highly than the extrasynaptic areas. At the center-most part of JFs crests, the adhesion molecule integrin α7β1 is anchored to laminin α4 of the BL and is crucial for the positioning of AZs of pre-synaptic axon terminal [9]. At the valleys of the branch of the JFs, voltage-gated sodium...
channels (VGSCs) are concentrated and are responsible for the action potential (AP) in the muscle cells. Nowadays, the signaling pathway of acetylcholine receptor (AChR) clustering on the NMJ has been studied well and reviewed [3, 6, 10, 11]. However, the function and formation mechanism of junctional folds (JFs) are unclear.

During the development of the mouse NMJ, the topography of JFs was under dramatic changes in the Z dimension in the first two weeks after birth [12]. At the E14 stage, BL is already present outside the sarcolemma before the motor nerve innervates. The sarcolemma is flattening without any depression, and the AChRs are pre-clustering in the post membrane (Fig. 1A). At birth, the NMJs are immature with simple round or oval depressions, and primitive synaptic troughs were smooth cup-like depressions 5–6 μm in diameter. Few pit-like or elongated oval invaginations and coated or uncoated caveolae were found beneath these depressions areas, indicating different section forms of the incipient JFs (Fig. 1B). At this stage, though the AZs were preceded the formation of JFs, they were not aligned with the JFs [13]. In the first 5 days, incipient JFs are still few and are primarily pit-like or elongated oval, but they are starting to invaginate into the sarcoplasmic with a depth less than 400 nm (Fig. 1C). Accompany excessive nerves were eliminated on the NMJ, low and narrow sarcoplasmic ridges occur in the cup-like depressions [14]. However, whether JFs are only present in those axons that will be preserved, or those axons that possess FJs will be more resistant to be pruned, requires further investigation in the future. At the P14, those low sarcoplasmic ridges were upheaved and matured into sarcoplasmic protrusions. Though the number of JFs has increased by about 18 folds, they were still predominantly pit-like and arranged in a row in a
side-by-side fashion. The depth of JFs has also elonged and extended to a maximum of about 800 nm (Fig. 1D). At the P28, though few pits still coexisted in a small proportion, the JFs form was mostly slit-like shape [14], and all JFs are directly opposed to AZs of motor terminals (Fig. 1E). The entire post-synaptic apparatus was raised above the surface of the muscle fiber, which gave rise to the term “endplate” [3]. The length of specialized thickened membrane increased 1.5- to 1.8 fold, and the total number of endplate receptors increased over 30 folds [15].

The evolution of junctional folds
NMJ in the lower invertebrate animals

Though protosynaptic proteins were first to emerge in the Fungi and Spongia at 1220 to 766 million years ago (Mya) [16], the recognizable nervous system was first found in the Ctenophore and Cnidarian (Fig. 2). Ctenophores may be the first branch of the animal lineage, and little is known about the physiology of the ctenophore NMJ. Glutamate is the only well-validated neurotransmitter in the NMJ of this animal. In the Cnidarian, synapses contained naked axons and muscle fibers separated by a 13–25 nm gap containing intra-cleft filaments. Though vesicles in the axon terminals suggested that neuropeptides had evolved as neurotransmitters, it lacks the postsynaptic specializations of higher animals [17]. Unlike the vertebrates, synaptic transmission usually travels in bidirectional in the Ctenophore and Cnidarian [18].

In the planarian, the simplest animal that possesses a simple brain has the movement ability to prey. Muscle was under the skin and constituted the dermo-muscular sac [19], it extended sarcomplasmic processes to the nerves, which were named sarconeuronal junction. Their vesicles in the motor neuron axons are of two main types: small clear vesicles measuring 20 to 40 nm and larger osmiophilic granule-containing vesicles measuring 40 to 80 nm. In the protocoelom animals, such as C. elegans [20] and Ascaris lumbricoides [21], body muscles project processes to form the motor neural junction directly with the nerve ring. The nerve fiber contains clusters of vesicles and giant mitochondria in this synapse, and BL was found in the 50 nm synaptic cleft. Interestingly, although nematodes are more distantly related to vertebrates, they both use Ach as their neurotransmitter at the NMJ.

In the Annelida, the segmental nerves of Lumbricus Terrestris send axons into the body wall muscle layers and establish an 85–120 nm wide cleft that contains basement membrane material. As glial cells are not abundant in earthworm nerves, the smaller nerve branches and NMJs consequently are composed entirely of unmyelinated axons and the adjacent muscle cells [22].

In the Mollusca (Aplysia), glial tissue often remains at the site of NMJ to cap the axon, and it enables axons by a single layer in the anterior aorta nerve. Besides, the synaptic cleft is widened, and a clear BL could be found. Type I is innervated by cholinergic axons to mediate inhibition, type II is innervated by serotoninergic axons to mediate excitatory effects, and type III is innervated by glycnergic terminals for modulating excitatory inputs. In most of the three NMJs, caveolae under the postjunctional membrane were very common. Especially in the type III NMJ, strings of caveolae seem to be formed by successive invaginations of the sarcolemma, which seem like the JFs shape. However, as BL did not extend into these caveolae, that means it is not a really JFs [23]. In the Octopus Vulgaris, though the pre-synaptic and postsynaptic membranes at the junction area were thickened, no special folding of the subsynaptic membrane has been found [24].

In Arthropods, glial cells and BL were preserved in their NMJ. The main characteristic of arthropods NMJ is the axon terminal inserted into the muscle membrane and enlarged in a bots manner. In those aquatic arthropods, such as crayfish [25] and blue crab [26], terminal axons make synaptic contact with the muscle granular sarcoplasm. In some flying insects, such as Sphinx ligustri larvae [27], Wasp [28], Cicada [29] and Drosophila [30], the NMJ in the high-frequency muscle fibers shows postsynaptic membranes form laminated stacks and tubules. It occasionally continuity with the circumfibrillar endoplasmic reticulum and the plasma membrane, which is designated post-synaptic subsynaptic reticulum (SSR). What are the roles of SSR surrounding the pre-synaptic bouton is not clear now; it is believed to add to the electrical capacity of the NMJ or maybe is the site of the oscillating mechanism in high-frequency muscle fibers [29]. In most arthropods, glutamate is the excitatory neuromuscular transmitter, while GABA is the main inhibitory neuromuscular transmitter.

JFs emerges in Chaetognaths and Chordata

Chaetognaths (arrow worms) are enigmatic zooplankton whose phylogenetic position remains elusive because they display dual morphological characters of protostomia [31] and deuterostomia [32]. In the skeletal muscles of the head, a type of muscle fibers that give rise to protrusions to cross the basement membrane and contact nerves. In these NMJs, the most striking feature is the development of JFs, which seemed to increase the post-junctional membrane’s surface. The synaptic cleft is about 50 nm in width and contains a dense material that is continuous with BL out of the NMJ, and the SCs partially ensheathe the nerve endings and face the muscle fiber [32]. However, the synaptic transmitters are
currently unknown due to the lack of physiological data in *Chaetognathis* muscles or nerves.

In the **Chordata**, a typical muscular structure in the notochordal lamellae of *Amphioxus* has displayed the classic characteristics of periodic cross-striation. Histological results showed that acetylcholinesterase was found above this muscular structure, indicating that it was innervated by cholinergic motor neurons. Under the electron microscope, post-junctional membrane folds were found in these muscle fibers, suggesting the notochord of *Amphioxus* is a highly specialized hydrokeleton that supplied enough stiffer for its movement [33]. The above results showed that JFs appeared at least 500 Mya on earth.
Matued JFs were found in vertebrate animals
ACh is the principal neurotransmitter at the vertebrate NMJ. In the electric organ of Torpedo marmorata [34], sparsely JFs show deep folds into the sarcolemma; however, complex subjunction folds have not been found. In the Scyliorhinus canicula, varying JFs are located in the muscle fiber membrane under the axon terminal. Especially in the fast-twitch white muscle fibers, the number of JFs was more numerous, and the depth of JFs was more than that of the red fiber. The multiple foldings of the subjunction membrane were found, which indicate that completely mature JFs were established [35]. Besides, numerous, deep, often branched post-synaptic membrane infoldings were also found in adult Lampetra fluviatilis L [36].

In some lower teleost fish, such as Clupea sprattus L., C. harengus L., C. pilchardus L., and Ameiurus nebulosus L., JFs are less complex, and there are no subjunctional folds [37]. In Puffer fish (Tetraodon stendachnieri) [38], Hippocampus hudsonius [39], Snake fish [40], and Carassius [41], the post-synaptic membrane still without the deep infoldings characteristic of other twitch muscle fibers. While in other higher teleost, such as conger eel and sturgeon Acipenser hueri, complex JFs upon the myoseptal end of the white muscle fiber has been found [42]. Interestingly, the lungfish (Lepidosiren paradoxa) is the ancient ancestor of amphibians in the present world, and it has subjunction folds in white myotomal muscle fiber [43].

Interestingly, JF was not found in the larva stage of Rana temporaria L., Ambystoma tigrinum, and Xenopus. But more prominent in adults amphibians (Triturus helveticus) [37], ileofibularis muscle of the turtle (Trionyx sinensis) [44], and the transversus abdominis muscle in the garter snake [45]. In alligator mississippiensis, the pupillae muscle has only a few JFs [46]. In the avians and birds, post-synaptic foldings were shown in posterior latissimus dorsi, while not in the anterior latissimus dorsi muscle [47, 48].

The JFs structure of mammals is the most complex in the animal kingdom. Such as rodents [49], canine [50], primates [51], and even in the neonatal calves [52]. Additionally, in the pectoral muscles of bats (Rhinolophus ferrumequinum Nippon), mitochondria-moderate fibers have well-development JFs [53]. In the white fibers of bottle-nose dolphins, marked branching of JFs also extended deep into the sarcoplasm [54]. Interestingly, even in cultured muscle cells [55], self-organizing 3D organoids of humans [56, 57], and the space mammal animals [58], the structure of JFs is still be found. These results (Fig. 2) indicate that although JFs have species differences among mammals, they are not deviation in microgravity environments. However, what is the evolutionary function of JF? Broadly speaking, high densities of VGSC located in highly folded postsynaptic membranes can amplify the effects of neurotransmitter transmission. As an extreme example, the NMJ of humans performs a ‘nummular’ morphology and occupies the smallest ratio of the muscle fiber’s surface. But it has the largest folding index than fish, frogs, snakes, and mice, which ensures the effective neuromuscular transmission [59].

The mechanism of JFs formation
The mechanism of NMJ formation is very unclear right now. Consider that JFs have emerged after BL and glial cells in evolution. All the factors from axon terminals, SCs, and BL can influence the formation of JFs.

Signals from the axon terminal are vital for JFs initiated
Innervation by motor neurons is essential for the formation of JFs [60]. Denervation induces the JFs absent at E18.5 [61]. In the choline acetyltransferase (ChAT) null mutation, synaptic transmission is entirely and specifically blocked, the nerve terminals were extensively differentiated, and the AZs density was unaltered. However, synaptic maturation was delayed at subsequent stages, and the number of JFs was decreased by 60% at E18.5 [60]. Neuronal activation is necessary to promote Wnt secretion [62], and it is dependent on the Wnt ligand secretion mediator (Wls) [63]. Knockout the Wls in motoneurons, while not in the SC and muscle cells, induced NMJs to become unstable and JFs reduced. R-spondin 2 (Rspo2) is expressed highly in spinal motor neurons (SMNs) and can enhance Wnt receptors’ stabilization to activate Wnt/β-catenin signaling pathways. In Rspo2-knockout mice, the numbers of AZs and postsynaptic folds are decreased, resulting in the frequency of miniature endplate potentials (mEPP) being markedly reduced, and the mutant mice died shortly after birth due to respiratory distress. However, overexpressing Rspo2 in the SMNs did not increase the numbers of AZs and JFs. In contrast, overexpressed Rspo2 in the skeletal muscle, over-correcting all abnormal features of the post-synaptic region observed in Rspo2-knockout mice [6].

Munc is essential for the synaptic vesicle priming at AZs, the fusion-competent synaptic vesicles were decreased in Munc13-1/2-DKO mice. Interestingly, the JFs invaginations are no deep as the normal mice, and it is small and shallow at the post-synaptic membranes of NMJs [64]. However, axons innervated are unnecessary for JFs maintenance. In the diaphragm of rats, ‘empty’ folds are still found at 4–6 months after the phrenic nerve section, indicating that JFs are very stable once the muscle fibers are denervated [65].

Especial the deep primary synaptic grooves and the secondary synaptic clefts were both present 12 weeks after denervated [66]. Furthermore, after...
denervation of the rat soleus muscle at P2, those denervated muscle fibers could still be programmed to form folds and showed marked JFs at P5. At P15, the endplates stopped JFs formation, and the post membrane was completely smooth [67].

SC indirectly affects the formation of JFs

The myelin sheath of Schwann cells is very beneficial for action potential conduction and neurotransmitter release. In the erbb2-deficient mice, SCs were absent in the phrenic nerve, and the NMJs were dysfunctional, resulting in the mutant mice dying at birth. Immunological examination shows that the post-synaptic membrane of the mutants lacked JFs at E18.5, while the JFs in control mice were significant [68]. In the erbb2/4 double knockout mice, nerve terminal endings appeared normal while the JFs were scarce or even lacking, and the number of fold openings per synaptic contact length was decreased in mutant mice [69]. In the conditional knockout model, ablated SCs at E15.5 did not influence the vesicles and AZs in the axon terminal, the synaptic width, and the AChR cluster’s density. But the amplitude of mEPPs and JFs depth were reduced [70]. However, acutely killing the perisynaptic SCs in adult mice with anti-disialoside antibodies has no significant deleterious influence on JFs. Moreover, though AChE was absent in the Colq−/− mice, syn −/−/ mice, synaptic activity decreased. Rescue β2 expression in muscle restored NMJ architecture and improved the weight and lifespan [81].

The major secreted heparan sulfate proteoglycans (HSPGs) in the BL are Agrin and Perlecan [82, 83]. Loss of agrin is lethal to the mice as the acetylcholine receptor could not cluster by the musk pathway, and the JFs was disappeared in the knockout animals [84]. Perlecan is also rich within the basement membrane surrounding skeletal muscle fibers, and it is the acceptor for collagen-tailed AChE that binds to the synaptic BL. In the perlecan-null mice, the AChR cluster and prominent markers of the post-synaptic JFs were normal, while the AChE clustering was utterly absent. Thus perlecan seems not to affect the organization and localization of the post-synaptic membrane [85]. Similar results were also found in the AChE −/− mouse, that JFs were well-formed [86]. However, though AChE was absent in the Colq−/− mutant mice, some subsynaptic cytoplasm appeared necrotic, the JFs were utterly reduced, and most mutants died before they reached maturity [87].

Matrix metalloproteinases are the critical regulators of the extracellular matrix. Secretion of pro-MMP3 is produced by the skeletal muscle and the SC and is concentrated at NMJ extracellular matrix through a hemopexin domain. In the MMP3 null mutant mice, the agrin immunofluorescence was significantly increased at NMJ, and the AChR aggregates were increased. Electron microscopy revealed no difference in the pre-synaptic nerve terminal, while the number and length of JFs were dramatically increased. As in atypical locations, the JFs extent is adjacent to the subsynaptic nuclei of the muscle cell [88]. Notably, tissue inhibitors of matrix metalloproteinases (TIMPs) are the special inhibitor that binds to the regulated the active of MMP3. The synaptic activity regulates TIMP release, that synaptic activity decrease will increase the TIMP release [89].

Fgf18 is expressed in the motor neuron and is also localized at the BL of NMJs. In the Fgf18−/− mice, synaptophysin-positive areas decreased to one-third of the wild-type level. In the post-synaptic membrane, simplified endplates with litter AChR positive areas and significantly fewer JFs [90]. Collagen IV (α2, α3, α6 chains) and collagen XIII are concentrated at mouse NMJs. They are required for synaptic maintenance [91], but not crucial to the JFs formation. Although the number and depth of JFs were not analyzed in collagen XIII mutant mice, some
“naked” post-synaptic without axon terminal innervated, and others post-synaptic covered by SC processes were found [92]. Collagen XIII overexpression mice did not show any defect in the amplitude and frequency of mEPP, and the JFs number seems no different [93].

In addition, molecules involved in AChR clustering also affect the formation of JFs. In the ε AChR mutant mice, the AChR could not transform to the adult type, and the synaptic could not maintain the highly organized structure of the neuromuscular endplates. The post-synaptic membrane was flattened and without the characteristic JFs at P60, which induced those mutant mice always to die prematurely [94]. Conditional ablated the Lrp4 gene in the adult mice induced the AChR clusters fragmentation and also caused the JFs ablation [95].

**Invagination mechanism of JFs formation.**

What is the mechanism of JFs formation is very little known. A widespread explanation is that the newly formed membrane during muscle fiber growth is inserted into the post-synaptic membrane opposite the AZ, which then induces intercalary expansion of the post-synaptic membrane and finally induces the post-synaptic membrane to fold into the cytoplasm (Fig. 3A) [10]. The main feature of this theory is that JF must be formed on the opposite side of AZ. Typically, however, JF is not the first development on the site opposite AZ (Fig. 3B) [14]. And some caveolae composed of BL under the post membrane may be the cross-section of incipient JFs [96]. This mechanical explanation could not be enough to explain the formation of JFs. Especially, this hypothesis could not explain how the subjunctional folds are formed. Contrary to this exocytosis hypothesis, an invagination mechanism (Fig. 3C) will be more reasonable. As noticed that BL in most omega-shaped coated caveolae under the cell surface is continued with the extra BL (Fig. 3B), which means this shallow depression was under invaginating into the cytoplasm [96].

The key question to understanding the invagination mechanism is what mechanism pulls the post-membrane...
inside. In evolution, the first sodium-selective channels may have appeared in extant cnidarians at the 700 Mya [97], and it evolves ankyrin binding in Amphioxus about 520 Mya [98]. Interestingly, JFs first emerge in Chaetognaths and Amphioxus at a similar period (about 500 Mya). JFs formation may be sharing the common mechanism that exists throughout the CNS for clustering VGSCs at a high density [99–102]. It was reported that β-spectrin binding to ankyrin maintains the high local density of VGSCs in the Ranvier node and the axon hillocks of CNS. In Drosophila, TEM results show that the density of SSR was significantly decreased in β-Spectrin mutant [103]. Ablating a single exon in the spectrin repeat region of the Kalrn gene also decreased the JFs fold density in KalSRKO/KO mice [104]. Recently, mice with ankyrins-deficient muscles only lost NMJ Nav1.4 performed significantly less voluntarily movement and fatigued more quickly [105]. Besides, the postsynaptic intermediate filament network under ankyrin proteins is vital to the structural integrity of NMJs. Conditional knockout plectin in muscle cells shows severe muscle weakness and scattered few JFs (mostly curved and disoriented) [106]. In addition, AnkG and AnkR appeared to be present exclusively in the troughs of JFs [107, 108]. Whether ankyrins coupled with the VSGC to Spectrin/Plectin are involved in initiating JFs invagination needs more research in the future.

The function of JFs
As described above, two facts need to be burned into our minds. First, JFs widely exist in different species of fish, amphibians, reptiles, and mammals, which means it is a choice of the convergent evolution. Second, there are so many species on the earth without JFs still thrive well, and even mutant mice that lack the JFs only produce weakness while not vital immediately after birth [81]. Though some morphology hints indicated that JFs contained BL deeply insect into the skeletal cells could retain the spatial location nerve terminal atop the muscle cell no matter the muscle cell is twitching or not [89]. However, what is the function of JFs? There is no clear conclusion yet.

The safety factor of neuromuscular transmission
To understand the role of JFs, we should revisit the progress of how the nerve AP induced the skeletal contraction in more detail. In the beginning, AP arriving at the pre-synaptic nerve terminal usually activated approximately 5% of AZs and released approximately 60–80 vesicles from the docked vesicles pool (approximately 1200~1600 docked vesicles in the adult mouse NMJs) [109]. As the pulse of ACh crosses the cleft at a very high concentration (about 10mM), it will arrive at the post-junctional critical area within 15 usec by free diffusion and not degradation by AChE on its path [110]. Once the ACh binds to the ligate gate ion channel of AChRs on the sarcolemma, it induces the channel pores to open, and non-specific conducts for Na+ accompanied by some Ca2+ influx and K+ efflux [111]. Generally, a single quantal content activated about ~0.3 um2 critical area of AChR in the post-junctional area generating a minEPPs about 0.5 ~ 1 mV in amplitude. In mouse and rat NMJs, the quantal content is about 40~100, while the humans usually are about 20, so the activation of endplate areas occurs at <10% of the total NMJ areas. The number of minEPPs sum to generate an EPP of about 20–35 mV in rat and mouse NMJ, which is larger than the minimally needed to trigger the sodium channel open in the depths of JFs (about 10~12mv). Once the mount of VSGCs was opened in the bottom JFs and the post membrane depolarization reached the threshold, an AP was initiated successfully. AP travels bilaterally on the muscle fiber membrane and then invades the T-tubular system to trigger the muscle fiber contraction [110]. The efficiency of neuromuscular transmission largely depends on the safety factor (SF), defined by the EPP divided by the minimum amplitude of the initiated threshold minus the resting potential. SF is varied considerably among mammal species [112, 113]. In human NMJs, the SF is about 2, while the mouse and rat NMJs range from 1.8 to 6. This SF existing could ensure that the EPP amplitudes remain suprathreshold after a series of nerve activities; even the quantal content of ACh tends to decrease during intense muscle contract. In other words, it means that SF makes muscles less prone to fatigue during continuous exercise [111]. The NMJs of extraocular muscles, which have less prominent JFs and a reduced SF, make them more susceptible to developing myasthenic weakness than fast-twitch skeletal muscle fibers [114].

A “saltatory conduction” model of AP spreading during the JFs
As described above, a single nerve impulse active a small critical area of the total NMJ (less than 10%). However, the details of AP spreading during the JFs were not mentioned before [102]. To simplify our model, we assume that a single AP induced by several ACh vesicles quanta was formed on the crest of one JFs (Fig. 4A). AP arrives at the axon terminal in the normal NMJ, inducing the VGCCs open and ACh to be released into the synaptic cleft. Then, AChRs of “critical areas” are activated, and EPP was initiated attributed to net sodium ion inflow. Then, the EPP is conveyed to the valleys of JFs and induced AP formation by sodium ion inflow, which will depolarize the post-membrane of nearby JFs. Then, the VGSCs of neighborhood JFs were initiated to new AP
by a positive feedback pathway. During the AP transmission between the JFs, the cleft between the JFs and axon terminal should be insulation as AChR are ligand gating while not voltage gating channel [102, 115]. If VGSCs are deficient on the JFs (Fig. 4B), the AP of the nerve terminal induced more VGCCs open and more ACh vesicles quanta release, active more extensive areas of “critical areas” to produce a higher EPP for inducing enough muscle contraction. Stimulation of continuous neural signals causes VGCCs overload in the pre-synaptic membrane, resulting in the muscle being unresponsive to AP, and more accessible to fatigue. These explained that though the loss of JFs did not induce animal death immediately, muscle fatigue might be the common characteristic of JFs insufficient [105]. However, the specific function of JFs needs more detailed research to do in the future.

The delicate structure of JFs ensures the possibility of saltatory conduction

During the progress of the AP spread within NMJ, JFs may have effects in several ways. Firstly, as highly aggregated AChRs are located at the shoulder of JFs, the constant quanital of AChR release will induce more AChR channels to open and increase the EPP’s size. Moreover, the density of VGSCs is only located at the trough of the secondary post-synaptic folds, which will increase the membrane excitability and reduces the AP threshold. Both of them ultimately result in increased SF magnitude [59, 116]. Secondly, the narrow cytoplasmatic space of JFs forms a high resistance pathway for the EPP induced current, and more VGSCs concentrated in the trough of JFs increased the maximum sodium channels conductances, both resulting in the current being easier to convey toward the depths of the secondary synaptic folds [99]. Thirdly, in the cleft between the crest of JF and the pre-synaptic membrane, integrins on the post-synaptic sarcolemma could bridge a physical connection to the axon terminal through the BL. This physical connection will be analogous to the membrane diffusion barrier that exists (paranodal axoglial junction) in the node of Ranvier, that borders the AChR and VGSCs laterally diffusion [117]. Last but most important, as the AChR channel is a ligand gating channel but not voltage-gated, and a large amount of BL exists in the cleft space on the top of JFs, this space will be an electrically insulated area for the AP transmission [115].

Benefit from this delicate structure, slight structural variation in synapses could not induce observable current transfer defect because the high density of post-synaptic Nav1.4 in the JFs has compensation roles [105].

![Diagram of AP conduction within the NMJ.](image)

**Fig. 4** Diagram of AP conduction within the NMJ. **A** In the normal NMJ, (1) a nerve AP arrives at the nerve terminal and produces rapid depolarization, (2) VGCCs opening and Ca$^{2+}$ entry, (3) the transmission vesicles fusion to the pre-membrane and release ACh into the cleft, (4) ACh bind to their post-synaptic receptors and generate a localized EPP at the crest of JFs, (5) EPP arrive the valleys of JFs and produces rapid depolarization, (6) VGSCs opening and generate of a muscle AP, (7) VGSCs on the neighbor JFs induced opening by voltage gating, resulting saltatory conduction happening, (8) Orthogonally aligned JFs propagate the AP along the long axis and drive the muscle fiber contraction. **B** In VGSCs deficient JFs, muscle AP is not initiated by the Na$^+$ inflow of VGSCs. To spread the AP throughout the whole NMJ region, it needs to activate more VGCCs and release more ACh vesicle quanta. Figures C and D are the plane views of A and B, respectively.
In the Lambert-Eaton myasthenic syndrome, the P/Q-type VGCC of the motor neuron terminal was attacked by autoantibodies, and the ACh released was reduced. Ultrastructure results show that JFs were deeper than the normal group, which means more VGSCs will be concentrated in the deep of JFs [118]. However, in the homozygous SCN4A mutation (p.R1454W) patient, the probability of muscle AP initiation and propagation would be reduced as all sodium channels are mutated, which leads to fatigable muscle weakness [115, 119]. Recently, perfect research shows that only loss of Nav1.4 in the NMJ induces the mice to perform voluntary movement reduce and fatigued more quickly, while no defects of NMJ morphology and muscle strength [105]. We risked an interesting guess that JFs may have disappeared in this animal model.

**JFs disorders in NMJ related diseases**

Defects in JFs are a common phenotype of many diseases that affect the NMJ, such as Myasthenia gravis (MG), Muscular dystrophy (MD), Amyotrophic lateral sclerosis (ALS), and aging [9, 120].

MG is an autoimmune syndrome in which neuromuscular transmission fails as NMJ maintenance signals are attacked by autoantibodies [121]. In the early onset stage of this MG, autoimmune antibodies in situ masking the membrane receptors induced the AChRs internalization and depression at the crest [122]. In the moderate and severe group, followed by systematic destruction, the receptor-containing crests of the JFs were destructed, and the JFs were degenerated, which resulted in the post-synaptic membrane was simplification [123]. In LG2 agrin mutation-induced MG, biopsy results from the right deltoid muscle showed that the JFs were simplified, and the diameter of the primary and secondary synaptic clefts was increased [124]. In Lrp4 antibody positive Myasthenia, most endplates had poor development, and JFs degenerated in some appeared denuded of nerve terminals [125]. In Musk inject rat, the sarclemma of these NMJs is markedly simplified with sparse synaptic folds, and the number of secondary endplate folds per length of the primary cleft was significantly reduced [126]. SEM results from MuSK-injected mouse also showed the subneural apparatus lost the labyrinthine structures, and the number of slit-like JFs was markedly decreased [127, 128]. In the Rapsyn Mutations induced congenital myasthenic syndrome of mice and patients, the JFs structure had poor development with few numbers and more short [129, 130]. Especially the secondary clefts did not establish continuity with the primary folds [131], [132].

MD is a group of numerous genetic diseases characterized by progressive weakness and skeletal muscle degeneration. Dystrophin-associated protein complex (DAPC) is crucial for the integrity of sarcomeres and prevents its fragile from contraction-induced injuries [133]. Dystrophin deficiency causes symptoms similar to Duchenne muscular dystrophy (DMD) [134]. This dystrophin mutant mouse has a similar life span to wild-type mice, and the pre-synaptic component of nerve terminals and vesicle density had no significant differences. But the number and depth of JFs were reduced to 50% of normal [135]. The length of subjunctional folds was decreased significantly in the fast and slow fiber of MDX mice [136]. Utrophin is homologous of dystrophin and concentrates in the NMJ areas, and is thought to play roles in promoting post-synaptic membrane invagination. Utlrn-/− mutants induced the numbers of JFs significantly reduced, which is similar to Duchenne and Becker muscular dystrophy [137]. Especially in the severely muscular dystrophy model of MDXurtn-/− double mice, the numbers of JFs are notably absent [138, 139]. Syntrophins associate directly with the dystrophin protein family (utrophin, dystrophin, and dystrobrevin). Though the number of postjunctional folds was not reduced in the α-Syn-/- mice, their JFs displayed minor organization and had fewer openings to the synaptic cleft [108]. Biglycan is an extracellular matrix protein that regulates the dystrophin/utrophin protein complex localization. Several defects of NMJ are observed after P35, including increased segmentation of NMJ, the presence of perijunctional folds, and focal misalignment of AChRs and AChE [140]. In addition, as a laminin receptor on the posterior membrane, integrin α7 knockout mice lose post-synaptic JF and exhibit muscular dystrophic myopathy [76].

ALS is characterized by an adult-onset progressive of motor neurons death, and the patients often have atrophy and death within five years from diagnosis [141]. The JFs length of the outer compartment of TA was shorter in the SOD1 mutant ALS mice [142], and the number of JFs was often missed in the gastrocnemius muscle of Fus-/- mouse at E18.5 [143]. Besides, in a mouse model of spinal muscular atrophy of SMAD 7 mutant mice, the JFs are almost wholly lacking [144]. Interestingly, junctional folds length decreased are more susceptible in fast muscles than the slow muscles in the early stages of ALS mice and patients [142]. Consider that JFs in fast fiber were deeper and more numerous than those in slow fiber, while the mitochondria in fast fiber were less than in slow fiber. And noticed that NMJ alterations were the early onset of the clinical symptoms [145], a dying-back dismantling plays a crucial role in starting ALS [120, 141]. Whether JFs function plays an important role in the onset of ALS requires more research in the future.

Although the elderly show some common features of ALS patients, such as nerve terminal denervation and...
Our investigation of JFs in aging rodents and human NMJs revealed an increase in complexity. Comparing the invasion of SC processes into the synaptic cleft, unopposed junctional folds to the AZs were prevalent [146]. JFs degeneration was also observed in aging mice and patients [51, 147, 148].

Whether defects of JFs play a vital role in these diseases has not been investigated. However, two key questions need to be explored in each condition. Whether the JF phenotype is earlier than the NMJ phenotype? And does affecting the structural stability of JF produce these disease-related phenotypes? However, as the manipulation methods to regulate the JFs were verypoor, the exact role of JFs during these diseases is not well understood [149].

Conclusions
The JFs were first observed in the Chaetognaths (arrow worms) at 500 Mya, and it was extinguished at a wide range of species, indicating that JFs were formed by convergent evolution. Though some cetaceans and bats live in the microgravity environment, their JFs structure did not devolve, suggesting that JFs were vital to animal survival on the earth. Considering that the past hypothesis of vesicle release cannot explain the development of JF, we propose an invagination mechanism in which the JFs were pulled into the cytoplasm by intrinsic or extrinsic mechanisms. Also, we give a concept that the VGSCs concentrated at the bottom of JFs participate in the spread of AP from the critical areas to the whole NMJ areas by a positive feedback way like the saltatory conduction in the Ranvier node, which ensures that the SF magnitude remains constant under physiological conditions. Understanding the formation mechanism of JFs will provide a clear direction for our future research.

Abbreviations
ACH: Acetylcholine; AChE: Acetylcholinesterase; AChR: Acetylcholine receptor; ALS: Amyotrophic lateral sclerosis; AP: Action potential; AZs: Active zones; BL: Basal lamina; ChAT: Choline acetyltransferase; CNS: Central nervous system; DAPC: Dystrophin-associated protein complex; DMD: Duchenne muscular dystrophy; EPP: Endplate potentials; HSPGs: Heparan sulfate proteoglycans; JM: Junctional folds; Lgr5: Leucine-rich-repeat-containing G-protein coupled receptors; MD: Muscular dystrophy; mEPP: Miniature endplate potentials; MG: Myasthenia gravis; Mya: Million years ago; NCAM: Neural cell adhesion molecule; NMJs: Neuromuscular junctions; Rspo2: R-spondin 2; SEM: Scanning electron microscopy; SF: Safety factor; SC: Schwann cell; SMNs: Spinal motor neurons; TEM: Transmission electron microscope; TIMPs: Tissue inhibitors of metalloproteinases; VGCCs: Voltage-gated calcium channels; VGSCs: Voltage-gated sodium channels; Wls: Wnt ligand secretion mediator.

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Author contributions
SZ produced all of the figures and wrote the manuscript. BXP edited and revised the manuscript. All authors read and approved the final manuscript.

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The authors declare that they have no competing interests.

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