European Journal of Physical and Rehabilitation Medicine
EDIZIONI MINERVA MEDICA

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European Journal of Physical and Rehabilitation Medicine 2017 Mar 06
DOI: 10.23736/S1973-9087.17.04663-9

Article type: Special Article

INNERVATION ZONE TARGETED BOTULINUM TOXIN INJECTIONS

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Conflict of interest: None declared.

ABSTRACT

Muscle overactivity (spasticity, dystonia or spasm) seen in certain neuromuscular disorders has been effectively treated with intramuscular injection of botulinum neurotoxins (BoTXs). Since they act in the nerve terminals, the toxin must be transported to the neuromuscular junctions which are generally clustered in one or more restricted areas (innervations zone(s)) in a skeletal muscle. The innervation zone targeted BoTX injections using guidance is highly recommended to achieve an optimal therapeutic goal with lower doses and fewer side effects. Hence, detection of the injection sites should be based on the knowledge about the localization of the innervation zone and the transport mechanism of BoTX in skeletal muscle.

In this paper, we discuss the relevant muscle architecture and physical principles as regards BoTX distribution during muscle overactivity management.

KEY WORDS: Spasticity, dystonia, innervation zone, neuromuscular junction, botulinum toxin

INTRODUCTION

Botulinum neurotoxins (BoTXs) are used to treat muscle over/hyperactivity (spasticity, dystonia or spasm) seen in certain neuromuscular disorders. They act in the cytosol of the motor nerve terminal via inhibiting acetylcholine (Ach) release from the presynaptic membrane of the neuromuscular junction (NMJ) (1). BoTX does not enter the nerve cytosol through the plasma membrane, but is only internalized by receptor mediated endocytosis at unmyelinated nerve terminals forming presynaptic membranes (2). Therefore, the effect of BoTX injection on muscle hyperactivity largely depends on the amount of toxin transported to NMJs. Since NMJs in a muscle are generally clustered in one or more restricted areas which are defined as innervation zone(s) (IZs), BoTX injection targeting this specific region is highly recommended to achieve an optimal therapeutic goal with lower doses and fewer side effects (3,4). Hence, the knowledge about the localization of IZ and the transport mechanism of BoTX in a muscle is crucial to determine the injection site(s). It should also be kept in mind that the structural and architectural features of a muscle largely determine the characteristics of IZ and BoTX transport.

All BoTX injections are performed by manual placement or various guidance methods like electrical stimulation (ES), electromyography (EMG) or ultrasound (US). Moreover, instrumented guidance of injection is strongly recommended for the treatment of spasticity and focal dystonia in both adults and children (5).

In this review, we discuss the muscle structure and architecture, localization of the IZ, transport mechanism of BoTX, and injection sites and techniques. Accordingly, we aim to provide a more rational basis for BoTX injections as regards safety and effectivity.

Skeletal muscle structure and architecture

Skeletal muscle is a composite tissue formed by striated muscle fibers (cells) and interstitial tissue containing extracellular matrix (ECM). The basic structure and composition of ECM is formed by structural fibers (collagen and elastin) and ground substance molecules (e.g. proteogylcans, glycosaminoglycans) as well as water, electrolytes, nutrients and some plasma proteins (6). Connective tissue that surrounds individual muscle fibers, bundles of muscle fibers (fascicles) and collection of fascicles (muscle) is defined as endomysium, perimysium and epimysium, respectively (7). The composition, amount and morphology of the intramuscular connective tissue show considerable variations between skeletal muscles having different functions (8). For instance, the total content of collagen fibers, which are the most abundant type of connective tissue structure, may vary from 1% to 15% of dry weight of the animal muscle (8).

Skeletal muscle architecture is defined as “the arrangement of muscle fibers relative to the axis of force generation.” The angle between the direction of muscle fibers and the force axis is called as “pennation angle” (Fig. 1). Muscle fascicles extend between tendons, aponeuroses and/or bone surfaces. Muscle fibers within the fascicle elongate parallel to the connective tissue surrounding the fascicle. Therefore, fiber arrangement in a muscle can be ascertained via assessing the fascicle tracking with certain imaging techniques e.g. diffusion tensor imaging (DTI) and US (9). Arrangement of muscle fiber in a fascicle vary among different skeletal muscles. Some muscle fibers may not span throughout but may end at any place within the fascicle. In these fascicles, fibers are serially connected to each other (non-spanning fibers) (10). On the other hand, fibers extending along the entire fascicle are defined as spanning fibers (Fig. 2). The type of muscle fiber array in fascicles and arrangement of fascicles in a muscle are substantial determinants on the distribution of NMJs. It should also be kept in mind that the architectural features are not unique and show

considerable changes between the neuromuscular compartments throughout the muscle (11).

NMJs, its distribution and IZ(s) in the skeletal muscles

NMJs

During its course in a skeletal muscle, a motor neuron axon gives rise to terminal branches that end on the individual fiber. Before positioning over a particular region on the muscle fiber membrane (end plate region), terminal branches lose their myelin sheath distally and divide into small swellings -called as synaptic boutons. They form a cluster of NMJs located on the end plate region (Fig. 3). Membrane of a synaptic bouton (presynaptic membrane), postsynaptic muscle fiber membrane and the synaptic cleft in between constitute a NMJ (end plate) (12). Ach receptors reside on invaginated postsynaptic membranes on muscle fibers. Basal lamina within the synaptic cleft contains Ach esterase which is an enzyme rapidly hydrolyzing Ach (13) (Fig. 4).

Number, localization and morphology (size and shape) of the end plates in muscle fibers, size of the nerve terminal as well as the pattern of neurotransmission in NMJs vary among different fiber types in vertebrates (14). NMJs of the white muscle fiber are well developed and complicated, having larger end plates than the red ones (14). Furthermore, nerve terminals innervating the white muscle fibers have numerous boutons, whereas red fibers have very few of them (15). However, slow and fast fibers of different muscles may have same size and similar features for NMJs. Oki et al. reported that the NMJs of slow fibers of soleus muscle and fast fibers of extensor digitorum longus muscle have some differences, but not as much as the difference between the different fiber types within the same muscle (14,16). These structural changes of NMJs within and between skeletal muscles should be kept in mind when deciding on the optimal dosage of BoTX per muscle and evaluating the

therapeutic effect. It was shown that the effects of BoTx-A induced muscle fiber paralysis on the mechanical properties of skeletal muscle are not uniform; but that they depend on stimulation frequency and muscle length. Muscle weakness was found to be greater at decreased frequency of stimulation and with shorter muscle length (17).

Continuous structural remodeling -expansion/regression and sprouting/retraction- constantly undergoes in a dynamic equilibrium in the adult NMJs (14). Eighty percent of NMJs in a muscle may show remodeling during a 90-day period. Denervation or synaptic blockade of NMJs via toxin injection results in a shift in this remodeling. Blockade of synaptic transmission results in a significant change not only in postsynaptic but also in presynaptic component of the NMJs. After the blockade, substantial degree of nerve terminal outgrowth and arborizations has been detected after 5-6 days of inactivity. These presynaptic changes are accompanied by the spread of existing receptors and the insertion of newly synthesized receptors in the postsynaptic membrane (18). In addition to these regenerative changes, degenerative changes such as decrease in the number and depth of secondary postsynaptic folds were also detected (19). It was also found that these structural changes occurred faster in the soleus than extensor digitorum longus muscle. This may cause variations in the duration of BoTX effects on distinct skeletal muscles.

Innervation zone(s) of the skeletal muscles

Innervation zone (IZ) may be defined as the three-dimensional band-like portion of the skeletal muscle in which NMJs densely exist. IZ of some skeletal muscles have been determined by using certain techniques. Some of those target the structural neuromuscular components as terminal nerve branches, Ach esterase in the synaptic cleft and Ach receptors on the postsynaptic membrane. The others use the electrophysiological properties of neuromuscular innervations.

Intramuscular nerve distribution of the skeletal muscles is investigated using cadaver dissection and Sihler's staining techniques. Using microscopic dissections of muscles, it is difficult to distinguish the terminal nerve branches from the vascular structures and collagen bands, and to trace a terminal branch during its course. On the other hand, Sihler's staining, a whole mount nerve staining technique, demonstrates the precise intramuscular nerve branching and distribution pattern in their three-dimensional position. All nerve branches are stained dark blue or purple and can be visible to their terminals while non-nervous tissue is rendered transparent. Myelin sheath surrounding the nerve fibers is the sole structure of the neural tissue that is stained by hematoxylin component of Sihler's stain. Myelin amount of the nerve fiber reduces towards the end of the terminal branches and disappears before dividing into synaptic boutons. Although terminal ends of the nerve branches are stained weakly and the NMJ cannot be observed, Sihler's staining is the most commonly used technique which indirectly determines the IZ of the skeletal muscles via detecting the area of the most densely localized intramuscular nerve arborization (20-22). It is also used to detect neuromuscular compartments which have distinct IZs in a skeletal muscle. As a disadvantage, intramuscular nerve branching and distribution may be illustrated incompletely in the large and thick muscles (23).

The IZ of a muscle can also be detected histochemically via staining the Ach esterase enzymes in the synaptic clefts of the NMJs. Longitudinal cryosections of a muscle (20 µm) are stained for Ach esterase and then marked as dots. Using computer analysis, these two-dimensional distribution of dots are reconstructed to three-dimensional volumes (24,25). However, Ach esterase activity does not solely exist in the NMJs, but also at the ends of the muscle fibers (26). It is detected that the labeling ability of the enzyme in NMJs is 14 times greater than that in non-junctional regions (27). Therefore, the NMJs are the predominant

source of Ach esterase in muscles. Enzymatic activity at musculotendinous junction has the form of cups cuffing the ends of the muscle fibers (26). This feature also makes this technique to be useful in detecting fascicles including non-spanning muscle fibers in a skeletal muscle (Fig. 2).

We suggest that an anatomical method may also be used to estimate the IZ of a muscle based on the fact that the NMJs are located at a relatively small area (end plate region) near the mid-portions of the muscle fibers (26). As such, three-dimensional localization of NMJs in a muscle may be determined by detecting the course and the length of the muscle fibers. As muscle fibers lie parallel to the connective tissue surrounding the fascicle, their course can be ascertained via scanning the fascicle tracking. On the other hand, midpoints of spanning fibers are possibly located equidistant from the ends of the fascicle because their lengths are similar to the fascicle length. Cluster of these IZs of the fascicles forms the IZ of the muscle distributed in a band-like area. The shape of this zone is determined by three-dimensional architecture of the muscle fibers. For instance, biceps brachii muscle contains spanning fibers which show a substantially symmetric curvilinear course. In accordance with its fiber architecture, it has a reverse V-shaped band like IZ - 5 to 10 mm wide and 4-6 cm long- at nearly the half way between the proximal and distal myotendinous junctions (28). However, fascicle length cannot be considered equal to the fiber length in fascicles containing non-spanning fibers. Fascicles including serially connected fibers do not have a single band-like IZ, but NMJs are scattered through the fascicle consistent with the mid-portions of the intrafascicularly terminating fibers. For instance, gracilis and sartorius muscles consisting of non-spanning fibers do not have a band-like IZ but NMJs disperse through a considerable volume of these muscles. It is detected that the

range of fascicle lengths in muscles with one IZ varies between 32 and 130 mm in humans (29).

Fascicle architecture (course and length) is not unique throughout the muscle and may show significant complexity between the neuromuscular compartments (11,30). Skeletal muscle compartments having different structural and architectural features may also show distinct functional properties and can be activated independently according to the desired task (31) (Fig. 5). When evaluating the NMJ distribution of a muscle, different compartments should be taken into account. Each compartment of a muscle having spanning fibers would have distinct band-like IZs. For example, semitendinosus muscle has upper and lower parts connected by a tendinous inscription. Each part has muscle fascicles arranged in a parallel array and fascicles of each part consist of fibers spanning through. Hence, semitendinosus muscle reveals two IZs consistent with its architecture (29,32) (Fig. 6).

Muscle fascicle length and the three-dimensional arrangement can be determined with cadaver studies and certain imaging techniques such as DTI and US (9,33). However, these techniques have lack of ability to detect the fiber length in the related fascicle. Hence, muscle architecture may only be useful to estimate the IZ if the fascicle has spanning fibers. Fiber arrangement in a fascicle can be determined by applying Ach staining in the skeletal muscles (29). On the other hand, we may assume that the fascicles whose lengths are less than 130 mm have a single band-like IZ (29). As mentioned above, cluster of these zones will form three-dimensional arrangement of IZ of a skeletal muscle. To our knowledge, there is yet no study concerning this topic.

In the superficially located muscles, IZ can be determined by using multichannel superficial EMG (sEMG) signals (34). A linear or 2D electrode array is placed on the skin over

the muscle of interest. Each electrode detects monopolar electrical signals which are generated in the end plate regions (IZ of the muscle fibers) and propagate to the opposite directions towards the ends of the muscle fibers (13,35). The amplified difference of two monopolar signals detected by a pair of electrodes is called as differential EMG signal. If IZ is located between two consecutive electrodes, amplitude of the differential EMG signal generated becomes significantly smaller due to cancellation of the action potentials travelling in opposite directions. Furthermore, surface electrodes should be placed along the direction of the muscle fibers for optimal recording as well. Therefore, this technique can only be used reliably for detecting the IZ of the superficial muscles whose fibers are parallel to the skin (i.e. fusiform muscles like biceps brachii or pennate ones like vastus medialis). Since muscle fiber architecture is not similar for each/every muscle and might show significant complexity between neuromuscular compartments, the distribution of IZ of the deep compartments of a muscle cannot be evaluated using this technique.

Most of the skeletal muscles which are commonly affected in neuromuscular disorders and needed to be treated with BoTX are investigated to detect their IZ using the aforementioned techniques (20,21,28,34). In these studies, the location of the IZ is generally measured according to the bony landmarks whose positions do not change with the movement of the related joint segments. However, IZ is found to be shifted proximally about 3 cm with the flexion of certain joints in the upper and lower extremities (36,37). Isometric contraction may also cause proximal shift in the IZ up to 2.4 cm in biceps brachii muscle (38). This could be explained by the shortening of the muscle fibers towards the stationary segment of the joint and/or lengthening of the distal tendon due to the increasing forces. In some patients, spasticity or dystonia does not allow the physician to place the extremity in the anatomical position in which the IZ is actually detected. As such, we suggest that BoTX

injections to muscles crossing the joints with flexion contracture should be applied more proximally than their determined IZ.

Transport of BoTX in muscle tissue

The BoTX vials comprise a BoTX component, and excipients including lactose, sucrose, and albumin. BoTX component (150-450 kDA) consists of BoTX and non-toxic complexing proteins. Two BoTX molecules constitute a dimer with a molecular weight of 600 or 900 kDA. Protein content also differs between the BoTX serotypes.

Transport of the BoTX in the muscle tissue is ensured by intramuscular injection of BoTX diluted with 0.9% NACI/H_2O (saline). Injection is performed by syringes with certain needle gauges. As biophysical terms, BoTX molecules are solute particles, saline is solvent and force applied on plunger top of the syringe is the pressure source. Transport of BoTX molecules from the barrel of syringe to NMJs in the muscle tissue is possibly based on the transport phenomenon related with fluid and particles. Therefore, before discussing about the transport of BoTX in the muscle, we will briefly give some information about biophysical fundamentals of material (e.g. BoNT molecules and saline) transport in a medium (e.g. muscle tissue).

Fundamentals of transport

Transport of matter is caused by the gradient which is often referred as the driving force. Among those, pressure gradient causes fluid flow (hydraulic flow) and concentration gradient causes diffusion of the particles. In a three-dimensional medium, transport takes place through each coordinates, *x*, *y* or *z* (39).

In fluid flow, molecules are moved via a streaming process caused by an external force. Flow may be undertaken in a restricted volume (e.g. artery, tube) or in an infinite

medium. Fluid flow (hydraulic conductivity) is inversely proportional to the fluid viscosity in case of flow in infinite medium. But in the tube shape restricted volume, it is additionally proportional to fourth degree of radius of the pipe (40). On the other hand, when the water is at rest (not flowing), there is also movement of solute molecules resulting from bombardment of much smaller solvent atoms. If the concentration of the particles is not uniform, there will be more particles wandering from a region of high concentration to one of low concentration than vice versa. Hence, molecules of specific components will travel down their concentration gradient until the gradient is removed or neutralized. This motion is called diffusion. Diffusion is inversely proportional to the particle radius and fluid viscosity (39,40).

Uniformity of the medium in which transport takes place may not be constant and changes considerably according to direction. In isotropic medium, which have uniformity in all orientations, physical and chemical properties are independent of direction; whereas in anisotropic medium, properties depend on the direction determined by the structural features (41). Anisotropy is sometimes used to describe the medium whose properties vary systematically depending on the direction. Skeletal muscles are composed of elongated muscle fibers arranged in parallel array and intercellular space (interstitium) between muscle fibers including connective tissue and certain cells. Therefore, muscle can be accepted as anisotropic medium whose transport properties may change with each direction.

Flow and diffusion in tissue

Hydraulic flow of isotonic saline through interstitium has been evaluated in some tissues. Combined interactive effects of collagen fibrils, glycosaminoglycans and proteogylican core proteins residing in interstitium are found to be responsible for the low interstitial conductivity. Especially the fractional volume of collagen fibrils spanning the

intercellular space offer high resistance to hydraulic flow (42). Moreover, tortuous arrangement of collagen fibrils also reduces hydraulic flow via increasing path length. Because of cell membrane's low hydraulic conductivity, cells act as relatively impervious obstacles to flow (42-44). It is stated that cells contribute very little to tissue resistance to hydraulic flow even in high cellular tissues (e.g. synovium with cell volume 80%) (42).

We may extrapolate the hydraulic conductivity of skeletal muscle tissue from the above quoted information, although it has not been investigated so far. Muscle is a highly cellular tissue with low interstitial volume resembling synovium. Since cells contribute very little to tissue resistance, the main determinant of the fluid flow through the muscle tissue seems to be dense fibrous structures and ground substance molecules in its interstitium. Moreover, hydraulic conductivity of a tissue is not fixed quantity, but increases with hydration and pressure gradient which is the driving force for fluid flow (42).

In addition to fluid flow, particles are also transported with diffusion in ECM which is a typical heterogeneous environment. It is suggested that biomolecules in the ECM interact nonspecifically with collagen fibers via van der Waals forces and then diffuse through the collagen fibers similar to diffusion in a viscous fluid. As in hydraulic flow, diffusion of biomolecules in ECM is considerably affected by highly condensed collagen fibers surrounding the cells. Rearrangement and condensation of collagen fibers are also important (45).

Transport of BoTX within the muscle is probably based on two phenomena; fluid flow and diffusion. During injection, the solution (BoTX and saline) passes through the needle of the syringe and then flows into the muscle tissue. This flow depends on the resistance in the needle and the pressure differences between the syringe and the muscle tissue. Resistance is directly proportional to viscosity of the solution, needle length, and inversely proportional

to fourth degree of the radius (gauge) of the needle (40). Different protein contents in the BoTX may change the viscosity of the solution. Pressure differences probably depend on the force applied on plunger top and the muscle structure which may alter with certain neuromuscular disorders like spasticity. Hence, toxin type, needle gauge and length, pressure applied on the plunger and the muscle structure may have some effects on the flow of BoTX through the muscle.

Hydraulic flow and diffusion of BoTX in the muscle tissue should be considered as regards the three-dimensional architecture and structural features of muscles. Intercellular spaces between the muscle fibers are oriented according to the three-dimensional elongation of muscle fibers. BoTX dimers seem not to be able to travel through the water channels in the muscle fiber membranes, although some amount of saline probably diffuses from the intercellular space into the muscle cell (46). As such, we may conclude that fluid flow and diffusion substantially take place through the interstitium along the collagen fibers coursing nearly parallel to the muscle fibers. Since skeletal muscles have distinct architectural features, transport of BoTX seems to vary between the muscles. Moreover, significant differences between the connective tissue contents of ECM may also change the fractional volume of collagen fibers which has significant effect on fluid flow (8,42). As mentioned before, hydraulic conductivity of a tissue is not a fixed quantity, but increases with hydration and pressure gradient (42). Accordingly, dilution rate, pressure of the injected BoTX solution and the structural changes in the muscle due to spasticity also seem to be important factors on flow of BoTX with saline.

Diffusion of BoTX is also affected by dense connective tissue in the ECM of a muscle. Moreover, it is inversely proportional to the particle radius and fluid viscosity (40). Protein

content of BoTX components differs between the BoTX serotypes (47). This difference may affect tissue diffusion via changing the molecular radius of BoTX component.

Intramuscular transport of BoTX was investigated via measuring neural cell adhesion molecule expression in muscle tissue, force production, muscle fiber diameter variability and Ach staining, and peak amplitude of compound muscle action potential (48-50). It was reported that BoTX largely remained close to the injection site, and exhibited limited distribution in the adjacent muscles. However, measurable weakness of non-injected neighboring muscle was also detected. In an animal study, distribution of BoTX was detected up to 4.5 cm from the injection site (51). In none of these studies, three-dimensional architecture of muscle was not taken into consideration. In a recent human study, transport of BoTX in a normal and spastic biceps brachii muscle was evaluated on sagittal plain with DTI. Extracellular distribution of saline largely took place longitudinally and parallel to the muscle fibers in the shape of long and thin hyperintense layers. While longitudinal distributions were 5.3 cm and 3.2 cm in healthy and spastic biceps brachii muscles respectively; vertical distributions perpendicular to the muscle fibers were restricted to 0.5 cm for healthy and 0.7 cm in spastic muscles. It is well known that IZ of a muscle is placed on three dimensions. Therefore, the distribution of BoTX solution perpendicular to muscle fibers on coronal plain should have also been evaluated in that study. Considering the biceps brachii muscle structure, distribution of BoTX solution perpendicular to muscle fibers on coronal plain should be similar to that in the sagittal plain. As mentioned above, skeletal muscles show distinct features about their architectures and connective tissue contents around the muscle fibers. In most of the skeletal muscles, muscle fiber elongations are not parallel to the muscle orientation and make different angulations with three planes. Therefore, it should be kept in mind that the transport of BoTX in a muscle is dependent on

its three dimensional architecture. On the other hand, differences between connective tissue content probably result in distinct rates of transport among skeletal muscles. In the aforementioned study, fluid flow in biceps brachii muscle was evaluated and presumed that BoTX was transported with the saline. In addition to the fluid flow, BoTX was also transported with diffusion. Therefore, distributions of BoTX in skeletal muscle would probably be greater than those mentioned above.

BoTX injection methods and sites

Since BoTX can only be taken into the nerve terminals at the NMJs, IZ targeted BoTX injections are highly recommended to achieve an optimal therapeutic goal with lower doses and fewer side effects (3,4,52). It has been reported that BoTX injection 1 cm away from the end plate zone (in biceps brachii muscle) yielded an almost 50% reduction in its effect (3).

The BoTX injections are generally applied by palpation and relay on superficial anatomical landmarks (especially for superficial and large muscles). However, this injection technique is blind and sizes of the muscles change with age, gender, body composition and with neuromuscular disorders. In addition, spasticity-related musculoskeletal deformities may change the relationship between the superficial anatomical landmarks and the muscles. For that reason, injections need to be performed using certain techniques (e.g. EMG, ES and US) not only for targeting the IZ but also for injecting to correct muscles while protecting nearby neurovascular structures.

The BoTX injection sites in the muscle have been identified via electrophysiological techniques. Injection can be applied where the EMG activity is the highest. Charts illustrating standard needle EMG localization sites are used for this purpose. However, higher EMG activity may not always be consistent with the localization of IZ in the muscle. Further, some

muscles have more than one IZ. Another EMG guided technique is based on searching for end plate activity (seashell murmur). Detecting end plate potentials may be useful only if the muscle architecture, i.e. the distribution of IZ, is well known.

Some authors use motor point charts for BoTX injections. Motor (entry) point is defined as the area (on or within the muscle) where muscle contraction can be elicited via stimulating with a minimal intensity and short duration of ES. Motor point(s) of a muscle can be localized by superficial or indwelling electrodes by stimulating extra- or intramuscular motor nerve branches. Motor point localization and its relationship with intramuscular nerve ramification are not unique and differ according to the muscle type (53). Moreover, it is stated that there may be considerable differences between motor points and IZ of a muscle (54).

The US-guided BoTX injections seem to be superior to EMG in terms of locating the targeted muscle and avoiding injury to the neurovascular structures. On the other hand, EMG has an advantage in the diagnostic stage via recording abnormal electrical signals of dystonia or spasticity which can be useful to detect the abnormal muscles. Therefore, we recommend that intramuscular BoTX injections should be performed with the guidance of EMG or US to the IZ which have been already localized in the previous literature for commonly injected muscles.

In conclusion, BoTX transport is probably dependent on toxin type, dosage, dilution rate, needle gauge and length, pressure applied on the plunger, and the architectural and structural features of the skeletal muscle. Since IZ targeted injection is highly recommended to achieve an optimal therapeutic goal with lower doses and fewer side effects; transport of the BoTX should be restricted to the muscle portion in which IZs reside. BoTX injection must also be applied to proper muscles while avoiding any damage to the neurovascular

structures. In this sense, illustrations for IZ and guidance (US and/or EMG) are definitely needed.

Funding: None

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FIGURE LEGENDS:

Figure 1. Muscle architecture of a pennate muscle (i.e, gastrocnemius). Teta shows the pennate angle. *A_{GC}, aponeurosis of gastrocnemius muscle; A_S, aponeurosis of soleus muscle; T, tendon.*

Figure 2. Muscle fiber types in a fascicle; non-spanning **(A)** and spanning **(B).** Acetylcholine esterase activity in the neuromuscular junctions (NMJs) and at the ends of the muscle fibers.

Figure 3. Muscle fiber innervation.

Figure 4. Neuromuscular junction. *Ach, acetylcholine.*

Figure 5. Cross-sectional drawing at the proximal 40% of the calf illustrating soleus muscle's anterior and posterior compartments. *F, fibula; T, tibia.*

Figure 6. Schematic drawing of the innervation zones in semitendinosus muscle.