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Lambert-Eaton myasthenic syndrome: mouse passive-transfer model illuminates disease pathology and facilitates testing therapeutic leads

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Abstract

Lambert-Eaton myasthenic syndrome (LEMS) is an autoimmune disorder caused by antibodies directed against the voltage-gated calcium channels that provide the calcium ion flux that triggers acetylcholine release at the neuromuscular junction. To study the pathophysiology of LEMS and test candidate therapeutic strategies, a passive-transfer animal model has been developed in mice, which can be created by daily intraperitoneal injections of LEMS patient serum or IgG into mice for 2–4 weeks. Results from studies of the mouse neuromuscular junction have revealed that each synapse has hundreds of transmitter release sites but that the probability for release at each one is likely to be low. LEMS further reduces this low probability such that transmission is no longer effective at triggering a muscle contraction. The LEMS-mediated attack reduces the number of presynaptic calcium channels, disorganizes transmitter release sites, and results in the homeostatic upregulation of other calcium channel types. Symptomatic treatment is focused on increasing the probability of release from dysfunctional release sites. Current treatment uses the potassium channel blocker 3,4-diaminopyridine (DAP) to broaden the presynaptic action potential, providing more time for calcium channels to open. Current research is focused on testing new calcium channel gating modifiers that work synergistically with DAP.

Keywords

Lambert-Eaton myasthenic syndrome; active zone; voltage-gated calcium channels; GV-58

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Competing interests

The authors declare no competing interests.

Lambert-Eaton myasthenic syndrome

Lambert-Eaton myasthenic syndrome (LEMS) is an autoantibody-mediated disorder of the neuromuscular junction that is often associated with small cell lung cancer.¹ LEMS is characterized by a muscle weakness that affects everyday activities and quality of life, with an incidence of about 1 in 100,000. LEMS symptoms have been shown to be due to a decrease in the magnitude of transmitter release from the neuromuscular junction² caused by autoimmune-mediated removal of presynaptic calcium channels.^{3,4} The presence of antibodies to other presynaptic proteins may also contribute to the presynaptic neuromuscular weakness that exists (described in more detail below).⁵⁻⁷ Although motoneurons may attempt to compensate for this autoantibody-mediated attack by upregulating the expression of other types of calcium channels,⁸ overall, LEMS results in decreased presynaptic calcium entry during an action potential.⁹ At synapses, calcium ion flux is nonlinearly related to the amount of transmitter released, so small changes in calcium entry can have large effects on transmitter release.¹⁰

LEMS clinical presentation

LEMS can affect many muscles, but usually it causes weakness primarily in the upper legs and hips, although it can also affect the upper arms and shoulders.^{11,12} Like myasthenia gravis (MG), LEMS may also affect muscles of the eyelids, causing ptosis, and it occasionally affects muscles that move the eyes, resulting in diplopia, but these symptoms are usually mild. Occasionally, there is weakness in the face and sometimes in muscles that allow for chewing, swallowing, and breathing, as may occur in MG. Involvement of the autonomic nervous system is also likely and may cause a dry mouth; sometimes erectile dysfunction is also present in men.^{11,12}

About half of patients with LEMS have or will develop cancer, especially small cell lung cancer. Most of these patients are smokers, and an LEMS diagnosis is followed by a computed tomography (CT) scan of the chest to screen for small cell lung cancer. Follow-up chest CT scans are recommended for up to 5 years to monitor for an underlying cancer. The other half of LEMS patients, who tend to be younger, do not develop cancer.^{11,12}

Independent of association with cancer, the disease progression is the same.¹² Proximal weakness is usually slowly progressive, and weakness and fatigue in LEMS patients can fluctuate daily. In the long term, patients without cancer usually do well with treatment, but most continue to have some symptoms. Patients with cancer can have a more progressive course of the disease.

The normal function of mammalian chemical transmitter release sites

To better understand LEMS disease pathology and to design treatment strategies, it is important to characterize the organization of the transmitter release site and the mechanisms that control chemical transmitter release from the neuromuscular junction. The use of the mouse as a model system has permitted details of neuromuscular synaptic structure and function to be more easily studied. These studies have been useful because the mouse neuromuscular junction has been shown to be similar to the human neuromuscular junction.

¹³ The following details of synaptic structure and function have been elucidated in the mouse model of the neuromuscular synapse.

The adult mouse neuromuscular synapse is characterized by a complex pretzel-shaped endplate region that has been visualized by staining the postsynaptic acetylcholine receptors using fluorescently tagged α -bungarotoxin (Fig. 1A). Specific sites for transmitter release within this structure have been most clearly identified using an antibody to the protein bassoon (Fig. 1A), which is a scaffolding protein involved in organizing the presynaptic cytoskeleton, localizing calcium channels, and synaptic vesicle trafficking selectively at the release site.¹⁴ Each mammalian neuromuscular synapse has about 700 transmitter release sites that can be quantified by the number of bassoon puncta in immunostained preparations (Fig. 1A).¹⁵ Each transmitter release site, or active zone, contains an average of two synaptic vesicles that are associated with release machinery (docked) and ready for release.¹⁶ Despite the presence of 700–1000 active zones, each with two docked release-ready vesicles, only an average of about 70–100 synaptic vesicles are released when an action potential propagates into the motor nerve terminal (Fig. 1C).¹⁷ Therefore, assuming homogeneity, on average, each active zone has a relatively low probability for releasing transmitter (only about 10% of the time).

In considering the mechanisms that are responsible for this low probability of transmitter release from each release site, data from frog neuromuscular synapses leads to the hypothesis that there are few calcium channels in each active zone, and each calcium channel opens with a low probability during the brief (1–2 ms) presynaptic action potential depolarization.^{18–20} Furthermore, even when a calcium channel does open in a presynaptic motor active zone, the probability that this calcium flux triggers vesicle fusion is likely to be very low (about 5%).²⁰ Therefore, if these data translate to mice, there are two low-probability events that control transmitter release from the active zone: the low probability that one of the few channels present opens to conduct calcium ions into the nerve terminal during a single action potential and the low probability that the calcium flux through one open calcium channel will trigger vesicle fusion. In designing treatment strategies for any neuromuscular disease in which it is desirable to increase transmitter release, these two low-probability components are drug targets.

LEMS antigens

LEMS targets the highly ordered active zone proteins that trigger transmitter release and is often associated with small cell lung cancer. In this paraneoplastic form of LEMS, the autoantibody-mediated attack on the motor nerve terminal is thought to occur as a side effect of the immune system targeting small cell lung cancer proteins. Because these tumor cells are of neuroendocrine origin, they express the same types of calcium channels and secretory machinery as nerve terminals. Therefore, although LEMS is generally characterized by autoantibodies to the pore-forming α_1 subunit of P/Q-type calcium channels, LEMS patients may also have antibodies to N-type calcium channels, auxiliary subunits of calcium channels (especially the β subunits), muscarinic receptors, RIM-binding proteins, and synaptotagmin (the calcium sensor that triggers transmitter release), among other proteins.^{4,6,7,21–30} Clinically, patients suspected of having LEMS are tested for antibodies to calcium channels,

and there is considerable variability in these antibody titers across patients (Fig. 2). In fact, about 15% of patients diagnosed with LEMS (by clinical criteria) test negative for antibodies to calcium channels.²⁶ These patients are defined as seronegative LEMS patients and presumably show the same type of muscle weakness of presynaptic origin due to the presence of autoantibodies to other presynaptic proteins.

The LEMS passive-transfer mouse model

To elucidate structural and functional pathology associated with LEMS, an animal model was developed that accurately reflects the disease mechanisms and phenotype. Because LEMS is an antibody-mediated disorder, the injection of human patient antibodies into mice recapitulates the LEMS phenotype in these animals. As discussed below, this animal model has not only been useful in elucidating pathological mechanisms and changes to the neuromuscular junction but has been critical to the development of novel therapeutic leads.³¹

To generate the LEMS passive-transfer mouse model, the following protocol has been shown to be effective.^{8,9,32–38} First, mice are injected intraperitoneally with one sensitizing dose of LEMS patient serum (~ 1–1.5 mL) or purified IgG (~ 10 mg). This is followed 24 h later with a single intraperitoneal injection of cyclophosphamide (300 mg/kg) to suppress the mouse immune response to the patient serum proteins. Then, mice are injected intraperitoneally with 1–1.5 mL LEMS patient serum or plasma (or 10 mg of purified IgG) daily for an additional 14–30 days to generate the LEMS phenotype in mice.

Several laboratories have used this approach to document significant reductions in the magnitude of transmitter release at synapses using electrophysiological recordings from *ex vivo* neuromuscular preparations.^{33–35,37,38} This electrophysiological assay of transmitter release can also reveal the short-term synaptic facilitation that is characteristic of LEMS.³⁸

Disruptions in active zone organization have been documented in LEMS passive-transfer mice using freeze-fracture electron microscopy.^{32,36} Healthy control active zones of the adult mouse neuromuscular junction contain about 20 intramembraneous particles arranged in two double rows (Fig. 3, top left; Fig. 4). These are thought to represent transmembrane proteins and include calcium channels and calcium-activated potassium channels, among others.³⁹ On the basis of calcium imaging data from frog neuromuscular synapses,^{19,20} it is hypothesized that there are relatively few calcium channels among the active zone proteins. If these data translate to mice, there may only be about eight calcium channels in each mouse active zone.

After LEMS passive transfer to the mouse, these normally well-organized active zone proteins become disrupted in their arrangement and reduced in their number (Fig. 4). While control active zones are consistently very tightly organized into two double rows of proteins (with rare exceptions, see Figure 4B and 4C), there is significant variability in the disorganization induced by LEMS passive transfer (Figure 4D and 4E). Within a single motor nerve terminal, some LEMS passive-transfer active zones retain some double-row structure, while others break down into a disorganized cluster.

The reduction in active zone particles in freeze-fracture electron micrographs, coupled with the reduction in calcium-triggered transmitter release (described above), led to the hypothesis that there are fewer calcium channels at neuromuscular synapses of LEMS passive-transfer mice. This hypothesis was supported by extracellular recordings of ionic currents from these nerve endings in which there was a reduction of about 50–60% in total action potential-evoked calcium current in LEMS passive-transfer synapses.⁹ In addition to an overall decrease in total calcium entry, pharmacological studies of LEMS passive-transfer synapses revealed that the calcium channel subtypes present at the neuromuscular junction had changed.^{8,9,40} Instead of transmitter release being entirely controlled by P/Q-type calcium channels (as in healthy adults),⁴¹ mouse LEMS passive-transfer synapses were shown to be only partially controlled by P/Q-type channels; the remainder of transmission is controlled by other types, including L-type channels that usually do not play a significant role in triggering transmitter release at synapses.⁴¹ These changes are interpreted as homeostatic alterations in calcium channel expression as the motor neurons attempt to compensate for the autoantibody-mediated attack by upregulating the expression of other calcium channel types.

Therefore, on the basis of data from the mouse LEMS passive-transfer model, the autoantibody-mediated attack on presynaptic P/Q-type calcium channels results in a decrease in calcium channel number, a disruption in calcium channel organization at the active zone, and an upregulation of other calcium channel types in an attempt to compensate for the disease pathology.

Symptomatic treatment of LEMS

Symptomatic treatment strategies that increase transmitter release have emerged as the primary therapeutic approach and are currently recommended.^{42,43} Most commonly, a potassium channel blocker is used alone; less commonly, it is used in combination with an acetylcholinesterase inhibitor.^{43–47} In most cases, 10–20 mg of the potassium channel blocker 3,4-diaminopyridine (DAP; which increases calcium entry by broadening the action potential depolarization, increasing the time that calcium channels have to activate; see Fig. 5) is administered 3–4 times per day and leads to serum levels of about 1 μM .^{43,45–47} However, DAP has dose-limiting side effects that include seizures, paresthesia, gastric symptoms, difficulty sleeping, fatigue, and deterioration of muscle strength.^{42,43,46,47} The latter two may be due to reported effects on axonal potassium channels that limit firing frequencies⁴⁸ and/or reduction in activity-dependent facilitation caused by DAP.⁴⁹ Thus, the current LEMS treatment approach indirectly increases calcium entry into the nerve terminal, but, owing to dose-dependent limitations used to reduce the risk primarily of seizures, DAP daily doses are kept under 80 mg/day.⁴³ Under these conditions, DAP often does not provide enough symptomatic relief for patients to return to normal activity. In fact, results of a meta-analysis of clinical trials that have been performed using the myasthenia gravis score (QMG) as an endpoint indicated that clinical benefits of DAP alone were modest.⁴⁷ In summary, treatment of LEMS using DAP treatment alone is only modestly effective, and DAP has side effects if higher doses are used in an attempt to improve symptomatic relief. Furthermore, coupling DAP with an acetylcholinesterase inhibitor is not significantly better,^{43,45,47} and there are currently no other effective symptomatic treatment options.

Testing novel therapeutic leads using the LEMS passive-transfer mouse model

Using the mouse passive-transfer model of LEMS, novel treatment strategies can be tested and leads optimized in an iterative fashion. Recently, this passive-transfer model has been used to develop a calcium channel gating modifier (GV-58) that acts as an agonist at the calcium channels that remain in the LEMS neuromuscular junction. GV-58 is a synthetic analog of (*R*)-roscovitine (Fig. 6A). Roscovitine is a trisubstituted purine that is best known as an inhibitor of cyclin-dependent kinases.⁵⁰ Roscovitine is often screened as a racemate, but only (*R*)-roscovitine has calcium channel agonist effects that are independent of cyclin dependent kinases.^{51–53} (*R*)-roscovitine and GV-58 act selectively on P/Q- and N-type calcium channels, slowing their deactivation kinetics (Fig. 6B) due to an increase in the mean open-time of calcium channels.⁵⁴ This effect leads to increased calcium flux and increased transmitter release at neuromuscular and central nervous system synapses.^{51,53} The effect of (*R*)-roscovitine on calcium channels was the motivation to develop new analogs of this drug that lack potent kinase inhibitory effects as potential treatments for motor nerve terminal dysfunction.⁵⁵ GV-58 is an improved (*R*)-roscovitine analog that has the potential to treat neuromuscular weakness, with significantly reduced activity to inhibit cyclin-dependent kinase activity and high calcium channel potency.^{37,55}

When *ex vivo* nerve–muscle preparations are removed from a mouse following a passive-transfer model of LEMS, GV-58 can roughly double the transmitter release from the neuromuscular junction (Fig. 6C, middle red trace). This effect is very similar to the increase in transmitter release caused by a clinically relevant dose of DAP (Fig. 6C, right black traces). Because DAP broadens the action potential and thus increases the probability that calcium channels will open, this symptomatic treatment targets one of the two low-probability events governing transmitter release at mammalian neuromuscular synapses (see above). By holding channels open longer, the gating modifier GV-58 acts to increase the probability that calcium flux through an open calcium channel will trigger vesicle fusion, targeting the second low-probability event at neuromuscular synapses (see above). Furthermore, because GV-58 can only modify open channels, and DAP increases the number of open channels,³⁸ it was hypothesized that these two drugs would work synergistically to increase transmitter release. In fact, when the two drugs were applied together to the *ex vivo* LEMS passive-transfer model mouse neuromuscular junction, the combined drug effect was larger than the sum of each drug effect alone (Fig. 6C, right red trace).

Further testing of GV-58 in preclinical experiments is currently focused on *in vivo* behavioral effects. There are no published reports of behavioral testing in the mouse LEMS passive-transfer model. It seems that the variability in unpublished behavioral effects is due to the variability in the LEMS patient serum used for passive transfer (see Fig. 2). If passive transfer does not reduce transmitter release sufficiently, muscle contractions in the mouse are not significantly affected and do not manifest as behavioral deficits (despite significant electrophysiological effects on endplate potentials measured in *ex vivo* experiments from affected muscles). Therefore, effective behavioral testing in the mouse LEMS passive-

transfer model requires the use of carefully chosen patient serum that will induce very large reductions in transmitter release.

In summary, the mouse neuromuscular junction and associated LEMS passive-transfer model have been very effective animal models that have allowed a productive study of the structure and function of healthy mammalian neuromuscular synapses and the effects of LEMS patient serum. Furthermore, using the mouse LEMS passive-transfer model, novel therapeutic approaches have been tested effectively.

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Figure 1.

Structure and function of the mouse neuromuscular junction. (A) A single mouse neuromuscular junction stained for postsynaptic acetylcholine receptors using Alexa-594–labeled α -bungarotoxin (red) and presynaptic active zones using Alexa-488–labeled antibodies to bassoon (green puncta). One labeled active zone (AZ) is circled in white, and an artist rendering of the active zone is shown in (B). (B) Artist rendering of a single AZ, including two synaptic vesicles docked and ready to fuse (blue spheres), proteins involved in vesicle fusion (brown tube-like structures), presynaptic P/Q-type calcium channels (purple disks), and other active zone proteins (yellow disks). Note the ordered arrangement of active zone proteins into two double rows. (C) Average action potential–triggered endplate potential (EPP) recorded from a single neuromuscular junction (top) and two representative spontaneous miniature EPPs (mEPPs). (C) Diagram of predicted calcium influx into an active zone following a single action potential stimulus. Mammalian active zones contain 2–3 synaptic vesicles (spheres) and a small number of calcium channels (in this case, six are pictured). After action potential stimulations, only one of these channels may open, leading to a local cloud of calcium ion influx. Adapted from Refs. 17 and 56.

Figure 2.

A radioimmune assay for voltage-gated calcium channel (VGCC) antibodies in the serum of LEMS patients. In this plot of a representative sample of 10 patients diagnosed with LEMS (indicated by letter code), serum VGCC antibody titer varies between 0 and 14 fmol/L. Healthy control patients (Cont) have no detectable antibodies to VGCCs. Adapted from Ref. 37.

Figure 3. LEMS patient antibody effects on the mouse neuromuscular synapse. Top: The normally well-organized active zone is disrupted after LEMS passive transfer. Bottom: LEMS passive transfer to mice results in a strong reduction in the recorded endplate potential (EPP). Adapted from Ref. 38.

Figure 4.

Freeze-fracture electron micrographs of active zone structure in control and LEMS passive-transfer mouse neuromuscular junctions. (A) Low-power view of control (left panel) and LEMS passive-transfer (right panel) presynaptic nerve terminal membranes that include several active zones (arrows and arrowheads). (B) High-power view of a control active zone that includes a rare slight disruption (X) in the ordered array of intramembraneous particles (tinted red). (C) Diagrams of the typical organization of two control active zones. Red dots represent hypothesized location of calcium channels based on analogy with published data at the frog neuromuscular junction. (D) High-power view of a LEMS passive-transfer active zone that includes a disrupted array of active zone particles (tinted red). (E) Diagrams that represent the range of organization of active zone particles after LEMS passive transfer (red dots represent hypothesized location of calcium channels). Adapted from Ref. 36.

Figure 5.

The effects of DAP on calcium influx during an action potential can be modeled in cells that express calcium channels *in vitro*. A simulated action potential waveform is used as a voltage command in a voltage clamp recording of calcium current from a cultured neuron. The control action potential (cont; thin line) can be broadened to simulate the effects of DAP (broadened; thick line). The effects of this action potential broadening on calcium current are shown below. The current activated by the broadened action potential waveform is larger and lasts longer. Adapted from Ref. 57.

Figure 6.

GV-58 is a novel calcium channel gating modifier that increases calcium flux during a depolarization and increases transmitter release in a manner that acts synergistically with DAP. (A) Structure of GV-58. (B) Calcium tail current (bottom) elicited by a voltage step (top) in a cultured cell expressing P/Q-type calcium channels. Control calcium currents decay quickly following voltage repolarization (black), but GV-58 significantly prolongs calcium current deactivation, leading to a much larger calcium influx (red). (C) Representative endplate potentials recorded from an *ex vivo* mouse neuromuscular junction. A mouse injected daily with serum from a healthy control patient has neuromuscular EPPs that are of normal large size (left; control serum). After passive transfer of LEMS to a mouse using daily injections of LEMS patient serum, the EPP size is greatly reduced (middle and right; LEMS). Acute exposure of LEMS passive-transfer synapses to 50 μ M GV-58 increases EPP amplitude by about twofold (middle red trace). This is the same effect as can be observed after acute exposure to 1.5 μ M DAP (right black traces). However, using a combination of GV-58 plus DAP leads to a synergistic effect that increases EPP amplitude by more than the sum of both drugs in isolation (right red trace). Adapted from Refs. 37, 38, and 55.