Supporting Information

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SI Results

Sciatic Nerve Blockade with SDLs. Multilamellar SDLs (Fig. S1*A*) produced as described in *SI Materials and Methods* were ~5.4 ± 1.4 μm in diameter (Fig. S1*B*), with an intraliposome STX content (Fig. S1*B*) of ~26 μg/mL (i.e., a loading efficiency of 10%). Administration of 0.3 mL of the liposome formulation at the sciatic nerve produced increased hind-paw thermal withdrawal latency in the injected limb ($P < 0.05$, $n = 5$, compared with the uninjected limb; Fig. S1*C*). The time to onset of block in the injected limb varied between 1–2 h, and lasted 5.7 ± 1.0 d. The uninjected limb demonstrated slight increase in thermal withdrawal latency within the first 24 h, but never reached the withdrawal latency threshold of 7s used here to define nerve block. After day 1, the uninjected limb demonstrated withdrawal latency similar to pretreatment values. Liposomes with only dexamethasone but no STX, (replaced with citrate buffer, pH 4.5) had no effect on withdrawal latency.

Motor strength (Fig. S1*D*), as tested by the hind-limb extensor postural thrust test, demonstrated weakness in the injected limb within 1–2 h after SDL administration ($P < 0.05$, $n = 5$, compared with values in the uninjected limb), whereas the uninjected limb showed no signs of motor weakness. Complete motor strength recovery in the injected limb occurred 6.5 ± 0.5 d after treatment ($P > 0.05$ in comparison with 5.7 ± 1.0-d sensory block).

Transmission electron microscopy (TEM; Fig. S1 *E* and *F*) of sciatic nerves harvested 7 d after SDL treatment showed no sign of injury (i.e., exhibited normal histological morphology).

Effect of SDL Administration on Body Weight Gain in SNI Rats. The total body weight of SDL-treated rats decreased by 5–6% for 2–3 d subsequent to each dose of liposome administration, followed by recovery (Fig. S2). Decrease in weight did not occur in SNI animals not treated with SDLs. About 5% of SNI rats in both SDL-treated and untreated groups demonstrated autotomy (mutilation of the foot) within 2–3 wk of SNI/SDL treatment, and were removed from the study.

SI Materials and Methods

Animal Care. This study was conducted using protocols approved by the Committee for Animal Care at the Massachusetts Institute of Technology in accordance with International Association for the Study of Pain guidelines (1). Adult male Sprague–Dawley rats (Charles River Laboratories) weighing 400–425 g were housed in pairs, allowed standard rat diet and water ad libitum, and maintained on a 7:00 AM–7:00 PM light/dark cycle. Animals were randomly divided into groups that received liposome treatment, nerve-injury surgery, or both. Rats were euthanized by carbon dioxide inhalation, either on day 5 or day 60 after liposome treatment and/or nerve-injury surgery.

SNI. Rats were anesthetized using 2% isoflurane with 98% (vol/vol) oxygen inhalation dispensed through an anesthesia manifold. A 3- to 4-cm skin incision was applied from the left greater trochanter to the knee joint. The muscle layers were separated to expose the sciatic nerve. The trifurcation of the sciatic nerve was identified and the common peroneal and tibial branches exposed and ligated with 5–0 silk suture, while special care was taken to avoid damage to the sural nerve. Approximately 2 mm of the nerve segments below the ligature was transected from both the branches. Immediately following nerve-injury surgery, rats in the liposome treatment group received 0.3 mL of the SDLs as a nerve block injection ipsilateral to the SNI procedure. Animals in the remaining groups did not receive any other forms of treatment. All nerve block injections were performed using a 23-gauge needle. The needle was introduced postero-medial to the greater trochanter, pointing in an antero-medial direction. On contact with bone, the needle was withdrawn ~1 mm, and 0.3 mL of liposome formulation was injected.

Behavioral Tests for Tactile and Thermal Responsiveness. To determine pain sensitivity to tactile stimulus, hind-limb tactile responsiveness to von Frey filaments was measured on all animals at least two times a week for 60 d. As described (2), following acclimatization to an enclosure with wire mesh bottom, calibrated Semmes Weinstein monofilaments (Stoelting) were applied to the lateral plantar surface of rats. Filaments were applied individually to each hind limb for a period of 8 s, with sufficient force to cause slight buckling of the monofilament. Filament strengths ranged from 0.41 g to 15.1 g. The pattern of filament selection was based on the Up–Down method of Dixon (3), where testing began with a 2-g filament followed by a lower or higher weight filament depending on a positive or negative paw response, respectively. A reflexive paw withdrawal or licking of the feet within the 8-s stimulus duration was considered a positive response. Filament mass required to elicit a 50% positive response rate was defined as the withdrawal threshold and calculated as described (3). The investigator remained blinded to the groups during the period of testing.

To determine the duration of SDL-induced nerve blocks, behavioral response to thermal stimulus (hind-paw thermal latency) was evaluated using a modified hotplate, as reported (4). In brief, towel restrained rats were held over a 56 °C preheated hot plate (IITC), and the plantar surface of the hind-paws sequentially placed on the heated plate. The time required for the animal to retract its foot was recorded as the thermal latency. Animals that did not withdraw their foot after 12 s were removed from the hot plate to prevent heat-mediated injury. A withdrawal response of more than 6 s (50% of maximal withdrawal latency) was considered a nerve block. Measurements were repeated three times with a 10-s pause between each test. It must be noted that, despite sciatic nerve injury and/or SDL treatment, animals still retain the ability to withdraw their foot, because the femoral nerve remains unaffected.

To test motor blockade in SDL-treated naive animals, the extensor postural thrust of each animal was tested by sequentially placing each hind paw on a digital weighing scale and measuring the maximum amount of weight the animal could bear without its ankle touching the weighing surface. The duration of motor block was defined as the time required for weight bearing returning halfway to normal from maximal block (< 20 g of weight borne) (4).

Nerve Conduction Studies. Briefly, rats were anesthetized by inhalation of 2% isoflurane in oxygen, and body temperature was maintained with the aid of a heating pad. Amplitude and latencies obtained from the direct motor response (M wave) and the monosynaptically evoked H reflex (H wave) were recorded by stimulating the sciatic nerve at the sciatic notch (hip) and the tibial nerve at the ankle. Stimulations were performed via pin electrodes using a supramaximal square pulse (24.9 mA, 0.05 ms, 1 Hz), and the recording electrode was placed in the plantaris muscle of the feet. A reference electrode was placed in the middle digit, and the ground electrode was placed in the inner thigh. Evoked responses were recorded using the Nemus Myto plus EMG system (EB Neuro). Conduction in the proximal and distal

segments of the nerve was evaluated by measuring the M and H wave latencies.

PCR Array. To evaluate possible SDL-induced gene expression changes in SNI animals, we performed real-time PCR on several well-characterized nerve injury-related genes (Table 1) using the RT^2 Profiler PCR array (SABioscience). Animals were euthanized for tissue harvest either 5 d or 60 d after SNI and/or STX-liposome treatment. Ipsilateral and contralateral L4, L5 dorsal root ganglion (DRG) neurons were harvested from naïve, SNI and SDL-treated SNI animals ($n = 4$, naïve and $n = 5$, SNI/ SDL/ SNI +SDL). Total RNA from DRG's was extracted using the RNeasy Mini kit (SABioscience) according to the manufacturer's protocol. An on-column DNA digestion step was included to remove possible DNase contamination during RNA purification. RNA purity was confirmed using a bioanalyser (Agilent Technologies). The RT^2 First strand kit (SABioscience) was used to convert RNA to cDNA, as per the manufacturer's directions. cDNA from individual samples were added to the RT^2 SYBR Green qPCR Master Mix (SABioscience) and loaded into 384-well array plates that were precoated with customized gene-specific primers sets. Each array plate housed 16 primer sets, including five house-keeping genes. Real-time PCR was performed on the Light Cycler 480 II Real-Time PCR machine (Roche Applied Science), and data were analyzed using the $\Delta\Delta C_T$ method via an online PCR array data analysis web portal (www.SABioscience.com/pcrarrydataanalysis.php.) All gene expression changes were normalized to a control house-keeping gene (Hprt1) and expressed as fold change compared with naïve controls.

Histology. To determine possible effects of SDL on astrocyte activation in SNI animals, we performed immuno-histochemical analysis on L4, L5 spinal cord sections from rats at PO day 5 and day 60. Animals were euthanized by CO_2 inhalation, and spinal cords were harvested and fixed in 4% (wt/vol) paraformaldehyde. Following tissue fixation and dehydration, spinal cords were embedded in paraffin blocks and 5-μm thin slices were cut on a microtome. A total of eight spinal cord sections per animal were stained for glial fibrillary acidic protein (GFAP, a marker for astrocytes) using polyclonal rabbit anti GFAP (DAKO) in a Thermo Autostainer 360 machine, and slides were developed with Thermo Ultra DAB.

High-resolution 2D images were acquired using an FSX-100 microscope (Olympus). All investigators were blinded during image acquisition and analysis. Four images from the outer dorsal horn, covering the medial, central and lateral areas on either side of the spinal cord section were acquired, with each image area being fixed at 220 μm × 165 μm. Using ImageJ (NIH) image analysis software, all acquired images were converted to gray scale, followed by binary contrast enhancement. A gray scale cut off threshold was set using control spinal-cord sections, such that the thin processes of GFAP-positive cells were just visible. All particles in the specified image area were measured and the total percent area of GFAP positive signal calculated.

Statistics. All data are expressed as the mean ± SD of N observations. Statistical significance between nonparametric data obtained from neurobehavioral studies was tested using two-way ANOVA with Dunnet post hoc test, whereas histological image analysis and gene expression analysis was tested using one-way ANOVA with Bonferroni multiple comparison post hoc test. In each case, $P < 0.05$ was considered statistically significant. All data analysis was performed using Graphpad Prism version 5.0 for Mac OSX statistical software.

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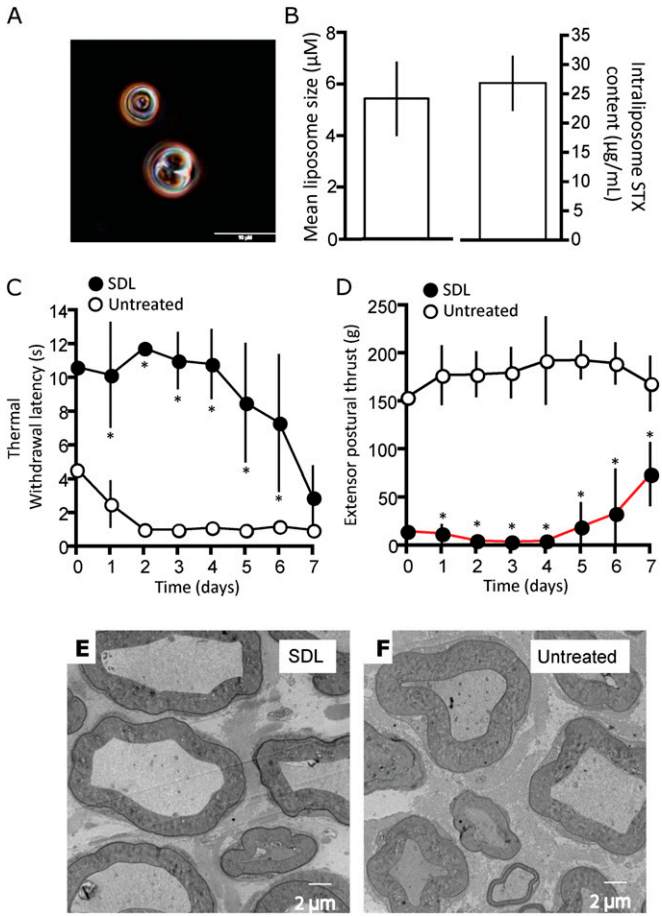

Fig. S1. SDLs produce long-duration nerve blocks without tissue toxicity. (*A* and *B*) Phase contrast image of SDLs (*A*) and mean liposome size and STX content (*B*) from three separately produced liposome batches. (*C* and *D*) Thermal latency (*C*) and extensor postural thrust measurements (*D*) from rats ($n = 5$) that were administered a single injection of SDLs at the sciatic nerve on day 0; measurements in the injected and contralateral (uninjected) extremities are shown. (*E* and *F*) Representative transmission electron microscopy images of sections of sciatic nerve at the injection site (*E*) and the contralateral side (*F*). Data are means ± SD, $*P < 0.05$, $n = 5$.

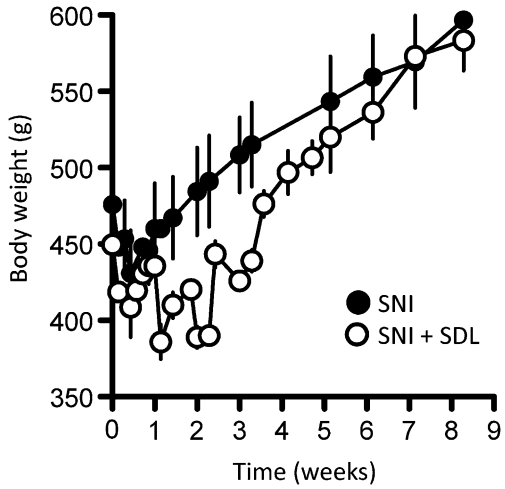

Fig. S2. SDL administration produces transient loss in body weight. Body weight was measured on a daily basis. Data are means ± SD.