

## Corrections

### GENETICS

Correction for “Long-range gene regulation links genomic type 2 diabetes and obesity risk regions to *HHEX*, *SOX4*, and *IRX3*,” by Anja Ragvin, Enrico Moro, David Fredman, Pavla Navratilova, Øyvind Drivenes, Pär G. Engström, M. Eva Alonso, Elisa de la Calle Mustienes, José Luis Gómez Skarmeta, Maria J. Tavares, Fernando Casares, Miguel Manzanares, Veronica van Heyningen, Anders Molven, Pål R. Njølstad, Francesco Argenton, Boris Lenhard, and Thomas S. Becker, which appeared in issue 2, January 12, 2010, of *Proc Natl Acad Sci USA* (107:775–780; first published December 22, 2009; 10.1073/pnas.0911591107).

The authors note that, due to a printer’s error, the second affiliation for Anders Molven should instead appear as “Department of Pathology, Haukeland University Hospital, N-5021 Bergen, Norway.” The corrected affiliation line appears below. The online version has been corrected.

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[www.pnas.org/cgi/doi/10.1073/pnas.1101890108](http://www.pnas.org/cgi/doi/10.1073/pnas.1101890108)

### BIOCHEMISTRY

Correction for “In vitro and in vivo reconstitution of the cadherin-catenin-actin complex from *Caenorhabditis elegans*,” by Adam V. Kwiatkowski, Stephanie L. Maiden, Sabine Pokutta, Hee-Jung Choi, Jacqueline M. Benjamin, Allison M. Lynch, W. James Nelson, William I. Weis, and Jeff Hardin, which appeared in issue 33, August 17, 2010, of *Proc Natl Acad Sci USA* (107:14591–14596; first published August 5, 2010; 10.1073/pnas.1007349107).

The authors note that in the Materials and Methods under the section “Actin Pellet Assay,” the 10× stock concentration was listed instead of the 1× final working concentration for the polymerization and reaction buffers. The correct concentrations are as follows: polymerization buffer (20 mM Imidazole pH 7.0, 100 mM KCl, 2 mM MgCl<sub>2</sub>, 0.5 mM ATP, 1 mM EGTA) and reaction buffer (20 mM Imidazole pH 7.0, 150 mM NaCl, 2 mM MgCl<sub>2</sub>, 0.5 mM ATP, 1 mM EGTA, 1 mM DTT).

[www.pnas.org/cgi/doi/10.1073/pnas.1102189108](http://www.pnas.org/cgi/doi/10.1073/pnas.1102189108)

### MEDICAL SCIENCES

Correction for “Prolonged duration local anesthesia with minimal toxicity,” by Hila Epstein-Barash, Iris Shichor, Albert H. Kwon, Sherwood Hall, Michael W. Lawlor, Robert Langer, and Daniel S. Kohane, which appeared in issue 17, April 28, 2009, of *Proc Natl Acad Sci USA* (106:7125–7130; first published April 13, 2009; 10.1073/pnas.0900598106).

The authors note that on page 7129, left column, fourth full paragraph, sentences 3 through 5, “DSPC:DSPG:cholesterol or DMPC:DMPG-cholesterol (molar ratio 3:1:2) were dissolved in *t*-butanol. Dexamethasone was added in some samples before lyophilization. The lyophilized cake was hydrated with 250 mM ammonium sulfate or, in some groups, with 0.1 mg STX, at 55–60 °C.” should instead appear as “DSPC:DSPG:cholesterol or DMPC:DMPG-cholesterol (molar ratio 3:1:2) were dissolved in 9:1 vol/vol chloroform:methanol solution. Dexamethasone was added in some samples. A thin lipid film was obtained by drying the lipid mixture in a rotary evaporator at 60 °C. The pressure in the evaporator was maintained at 480 torr for 20 minutes and then gradually decreased to 20 torr over a period of 30 min. The lipid film was reconstituted in *t*-butanol at 55–60 °C and immediately cooled by immersing in liquid nitrogen. The sample was then transferred to a lyophilizer for 24 h. The lyophilized cake was hydrated with 250 mM ammonium sulfate or, in some groups, with 1.0 mg STX, at 55–60 °C.”

[www.pnas.org/cgi/doi/10.1073/pnas.1102194108](http://www.pnas.org/cgi/doi/10.1073/pnas.1102194108)

# Prolonged duration local anesthesia with minimal toxicity

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Edited by Jerrold Meinwald, Cornell University, Ithaca, NY, and approved March 9, 2009 (received for review January 18, 2009)

**Injectable local anesthetics that would last for many days could have a marked impact on perioperative care and pain management. Formulations have often been limited in duration of action, or by systemic toxicity, local tissue toxicity from local anesthetics, and inflammation. To address those issues, we developed liposomal formulations of saxitoxin (STX), a compound with ultrapotent local anesthetic properties but little or no cytotoxicity. In vitro, the release of bupivacaine and STX from liposomes depended on the lipid composition and on whether dexamethasone was incorporated. In cell culture, bupivacaine, but not STX, was myotoxic (to C2C12 cells) and neurotoxic (to PC12 cells) in a concentration- and time-dependent manner. Liposomal formulations containing combinations of the above compounds produced sciatic nerve blockade lasting up to 7.5 days (with STX + dexamethasone liposomes) in male Sprague-Dawley rats. Systemic toxicity only occurred where high loadings of dexamethasone increased the release of liposomal STX. Mild myotoxicity was only seen in formulations containing bupivacaine. There was no nerve injury on Epon-embedded sections, and these liposomes did not up-regulate the expression of 4 genes associated with nerve injury in the dorsal root ganglia. These results suggest that controlled release of STX and similar compounds can provide very prolonged nerve blocks with minimal systemic and local toxicity.**

liposomes | myotoxicity | neurotoxicity | pain | saxitoxin

The development of local anesthetics to provide prolonged analgesia from a single injection has encountered 3 principal challenges: inadequate duration of action, systemic toxicity, and adverse local tissue reaction. The purpose of this research was to produce a local anesthetic lasting many days without those detrimental sequelae.

A wide variety of controlled-release technologies has been used to extend the duration of nerve block, but most such systems result at best in a several-fold extension of duration compared with unencapsulated drugs. Approaches that encapsulate synergistic drug combinations have achieved nerve blocks lasting many days. For example, coencapsulation of bupivacaine and dexamethasone in polymeric microspheres produced nerve blocks lasting >4 days (1). Coencapsulation of site 1 sodium-channel blockers [tetrodotoxin (TTX), saxitoxin (STX), etc., which block the sodium channel at site 1 on the outer surface] with conventional local anesthetics also greatly prolonged sciatic nerve blockade. Addition of dexamethasone prolonged the sciatic nerve blockade to >9 days in the rat when dexamethasone was added (2). However, tissue reaction to such formulations has been problematic. Conventional local anesthetics are intrinsically myotoxic (3, 4). They are also myotoxic when released from a wide range of delivery systems (3, 5), even when the delivery systems themselves are minimally toxic. The myotoxicity of bupivacaine increases dramatically over extended durations of exposure (3), suggesting that myotoxicity may be an inevitable consequence of sustained release of such compounds. Conventional

local anesthetics are also neurotoxic (6, 7). The presence of particles themselves enhances local anesthetic myotoxicity in vivo (3), and can cause inflammatory responses at the nerve that may considerably outlast the duration of blockade (2, 3, 8). In contrast, site 1 sodium-channel blockers do not cause myo- or neurotoxicity (9, 10), which would make them desirable for an extended release formulation. However, these extremely potent local anesthetics (11), being very hydrophilic, are difficult to encapsulate effectively in polymeric particles, and the systemic toxicity from their initial rapid release is dose-limiting (12, 13). These considerations make the development of particulate systems based entirely on such compounds (i.e., without inclusion of conventional local anesthetics) very difficult.

Here, we developed prolonged duration local anesthetics based entirely on site 1 sodium-channel blockers, the hypothesis being that there would be less harm to local tissue. To allow increased loading without systemic toxicity, we used liposomes, which are biocompatible microscopic lipid vesicles with a bilayer membrane structure that have been used to deliver hydrophilic, amphiphilic, and hydrophobic drugs. In this report, those are STX, bupivacaine, and dexamethasone, respectively. STX was used as the model site 1 sodium-channel blocker due to its greater availability to us; other site 1 sodium-channel blockers may also have been suitable (11). The resulting formulation produced nerve blocks lasting many days in the rat, with minimal local or systemic toxicity and inflammation.

## Results

**Liposomal Formulations.** Liposomes were produced with 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and 2-dimyristoyl-*sn*-glycero-3-phosphoglycerol (DMPG), or 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) and 1,2-distearoyl-*sn*-glycero-3-phosphatidylglycerol (DSPG). Those made with DMPC are referred to as “fluid” liposomes below; those with DSPC as “solid,” based on their phase transition temperatures ( $T_m$ ). Particles were loaded with bupivacaine, STX, and/or dexamethasone (Table S1).

The median volume-weighted diameters of both fluid and solid liposomes were  $\approx 4.0 \mu\text{m}$ , with median zeta potentials of  $\approx -35 \text{ mV}$ , irrespective of drug content (Table S1). The mean encapsulation efficiencies of bupivacaine in solid and fluid liposomes were 64 and 60%, respectively. The liposomal drug

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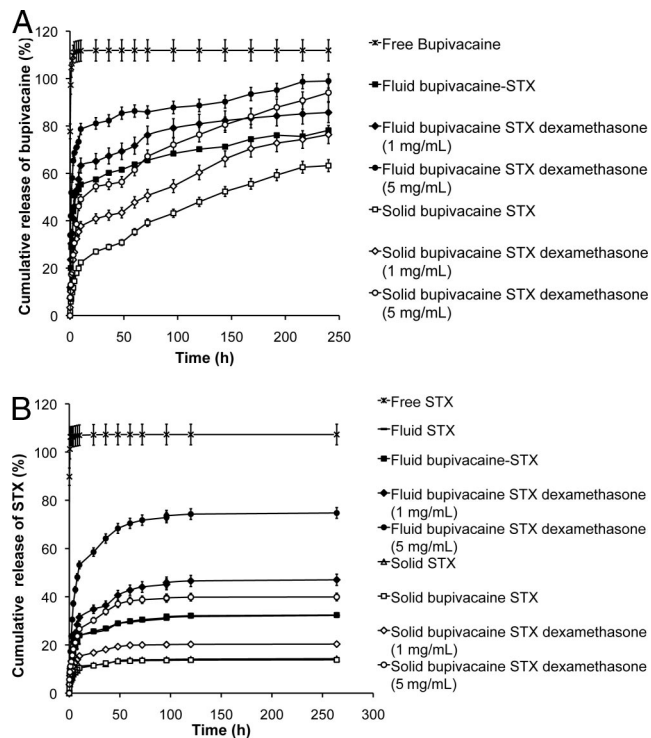
The authors declare no conflict of interest.

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See Commentary on page 6891.

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This article contains supporting information online at [www.pnas.org/cgi/content/full/0900598106/DCSupplemental](http://www.pnas.org/cgi/content/full/0900598106/DCSupplemental).



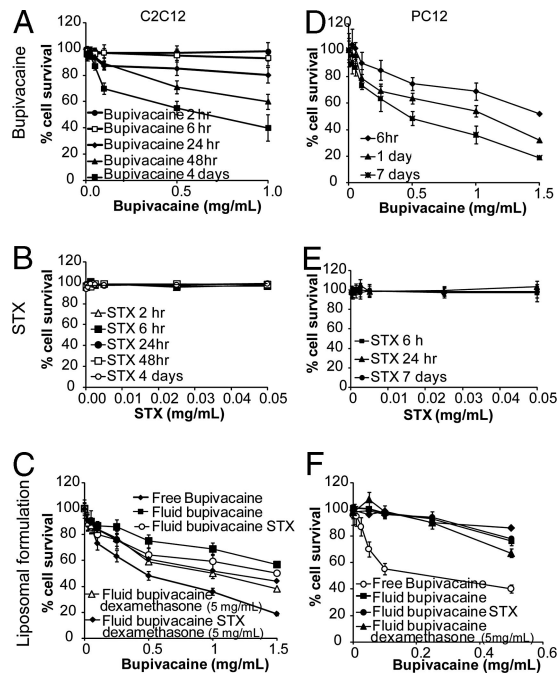
**Fig. 1.** Cumulative release of total encapsulated bupivacaine (A) and STX (B) from liposomes in vitro. Data are means with SDs ( $n = 4$ ). Statistical significance is discussed in the text.

loadings, in mg/mL, were 8.7–10.2 for bupivacaine (i.e.,  $\approx 1\%$  wt/vol), 0.03–0.018 for STX, and 50–60 for lipids.

**In Vitro Drug Release.** To assess the potential of these liposomes to provide sustained nerve blockade, we performed release kinetic studies at 37 °C. All liposome formulations significantly increased the duration of bupivacaine release, compared with free bupivacaine (e.g.,  $P < 0.001$  at 50 h; Fig. 1A). Fluid particles showed faster (e.g.,  $P < 0.05$  at 50 h) release than solid ones. Release of bupivacaine was increased in proportion to the amount of dexamethasone incorporated (e.g.,  $P < 0.05$  when comparing 0 to 5 mg/mL dexamethasone at 50 h). Dexamethasone release was not affected by lipid type (Fig. S1). However, particles with a higher proportion of dexamethasone (5 mg/mL) showed slower release of dexamethasone on a percentage basis, particularly at later time points (e.g.,  $P < 0.05$  when comparing 1 to 5 mg/mL dexamethasone at 50 h). Fluid liposomes showed more rapid release of STX (Fig. 1B) than did solid ones ( $P < 0.05$  at all time points, and  $< 0.001$  after 50 h), and dexamethasone accelerated STX release ( $P < 0.05$ ). These results, which showed sustained release of the compounds of interest for several days, supported their potential to provide prolonged duration local anesthesia.

**Stability.** At 4 °C, both solid and fluid liposomes (containing all compounds, singly and in combination) were stable for 2 weeks: drug leakage was  $< 3\%$  over that period, and there was no significant change in liposome size, zeta potential, and drug to lipid ratio. At 21 °C, particles made with both lipid compositions showed  $> 10\%$  release of bupivacaine in 48 h, i.e., they were not stable. Release of STX and dexamethasone were slower than for bupivacaine, but still higher than at 4 °C.

**Cytotoxicity.** One of the underlying hypotheses of this work was that prolonged exposure to STX would cause less injury to



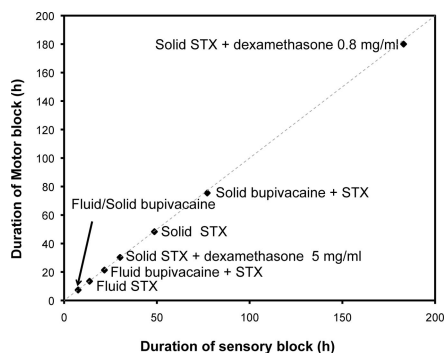
**Fig. 2.** Percentage of C2C12 and PC12 cell survival after exposure to free bupivacaine (A and D), free STX (B and E), and fluid liposomes encapsulating combinations of bupivacaine, dexamethasone and/or STX (C and F). In C and F, data for free bupivacaine are reproduced from A and D for comparison. Data are means with SDs ( $n = 4$ ). For complete particle compositions, see Table S1.

muscle and nerve than bupivacaine. Before animal studies, we investigated this matter in cell culture.

**Myotoxicity.** Myotoxicity was assessed by exposing C2C12 cells for up to 4 days to a range of concentrations of free compounds (bupivacaine, dexamethasone, or STX), or liposomes with varying drug contents and lipids compositions. Myotoxicity of bupivacaine solution increased with concentration and duration of exposure (Fig. 2A) (3). In contrast, free STX (0.005–0.05 mg/mL) was not myotoxic at any of the concentrations or any durations of exposure tested (e.g.,  $P < 0.001$ , 0.01 vs. 0.05 mg/mL at 48 h, and  $P < 0.001$  at 0.1 mg/mL at 6 vs. 24 h; Fig. 2B). This concentration range vastly exceeds that required to achieve effective sciatic nerve blockade (11). Free dexamethasone (0.005–0.5 mg/mL), singly or in combination with the highest STX concentration (0.05 mg/mL), was not myotoxic at up to 2 days (viability  $> 90\%$ ). At day 4, survival was reduced to  $80 \pm 5\%$  ( $P < 0.05$ ) with 0.5 mg/mL free dexamethasone. This reduction in survival did not occur when the dexamethasone was encapsulated in liposomes.

Blank liposomes were not toxic to cells at concentrations from 0.3 to 6 mg/mL of lipids. Further increasing the lipids reduced cell viability [e.g., viability was 50% at 9 mg/mL ( $P < 0.001$ )]. Coadministration of empty liposomes with free drugs did not further reduce cell viability.

Liposomal bupivacaine caused less myotoxicity than the same concentration of free bupivacaine (e.g.,  $P < 0.001$  at 0.5 mg/mL at 4 days; Fig. 2C). Myotoxicity increased with concentration and duration of exposure (Fig. 2C). Liposomes containing STX, dexamethasone, or combinations of both were not myotoxic at any concentration (0–1 mg/mL STX), exposure up to 4 days, or lipid composition. Encapsulation of dexamethasone within bupivacaine liposomes increased toxicity (e.g.,  $P < 0.05$  at 0.5 mg/mL at 4 days; Fig. 2C), possibly due to the more rapid release of bupivacaine (Fig. 1). Addition of STX did not increase the myotoxicity of any liposome formulation tested (Fig. 2C).



**Fig. 3.** Duration of sensory and motor block in animals injected with liposomes containing STX and/or bupivacaine and dexamethasone. Data are means with SDs ( $n = 8$ ). The dotted line denotes identical durations of sensory and motor block.

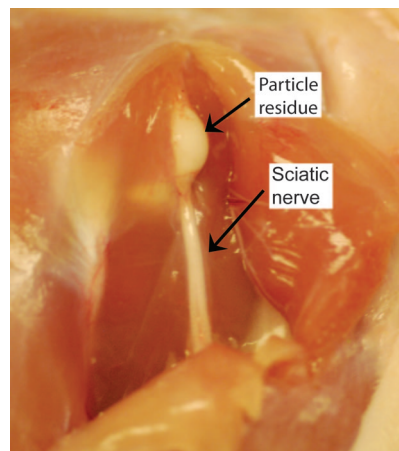
**Neurotoxicity.** Similar studies were performed in PC12 cells (a pheochromocytoma cell line frequently used in neurotoxicity studies) (14). Neurotoxicity of bupivacaine solution increased with concentration and duration of exposure (Fig. 2*D*). Free or encapsulated STX (0.005–0.05 mg/mL) was not neurotoxic at any concentration or duration of exposure (Fig. 2*E*), nor was free or encapsulated dexamethasone (0.005–0.05 mg/mL) for up to 5 days. Free dexamethasone (0.05 mg/mL) showed a 20% decrease in survival at day 7. Blank solid and fluid liposomes were not toxic at concentrations from 0.3 to 9 mg/mL. Incorporation of STX into fluid bupivacaine liposomes did not increase their cytotoxicity (Fig. 2*F*), but incorporation of dexamethasone into bupivacaine liposomes did (e.g.,  $P < 0.01$  at 0.5 mg/mL Fig. 2*F*), possibly due to the more rapid release of bupivacaine (Fig. 1). Similar results were obtained with solid liposomes.

These studies showed that extended exposure to STX-based formulations caused little cytotoxicity, and that therefore the liposomes were good candidates for in vivo use.

**Duration of Nerve Blockade.** To test the ability of liposomal formulations to produce prolonged nerve blockade and systemic toxicity (increases in latency in the un-injected hindlimb, respiratory distress, and death), rats were injected at the sciatic nerve with 0.6 mL of liposome formulations (8 rats per formulation), containing single compounds or combinations. All liposome formulations containing bupivacaine or STX induced motor and sensory nerve block that subsequently reverted to baseline values. Onset of nerve blockade occurred 10–15 min after injection with fluid liposomes, and 0.5–1.5 h after injection with solid liposomes. The durations of sensory and motor blocks are shown in Fig. 3 and were similar in all cases.

A primary goal of this research was to develop injectable formulations that could achieve reliable and prolonged nerve blockade with STX, or at least without bupivacaine. Polymeric microspheres with TTX alone had been ineffective (2). Nerve blockade from fluid liposomes containing STX alone lasted  $\approx 13.5$  h. Solid liposomes containing STX alone produced even longer blocks, lasting 48 h, with no signs of systemic toxicity.

We verified that the drug interactions that extended the duration of block in polymeric particles containing TTX (2) also occurred with STX liposomes. The incorporation of some concentrations of dexamethasone into STX liposomes caused marked systemic toxicity and death, presumably because dexamethasone increased liposome permeability to other compounds (Fig. 1). For example, STX-containing fluid liposomes with 5 mg/mL of dexamethasone were uniformly fatal, but liposomes containing the same quantities of STX or dexamethasone alone



**Fig. 4.** Representative photograph of particle residue (here, with solid STX + dexamethasone liposomes) at the site of injection 4 days after injection. Residue appearance was similar in all particle types. Microscopic appearance is shown in Fig. 5.

were not toxic. In solid liposomes, coencapsulation of dexamethasone at 5 mg/mL reduced the duration of block compared with STX (0.031 mg/mL) liposomes ( $P < 0.01$ ), and 2 of 8 animals died. In contrast, dexamethasone at 0.8 mg/mL led to a marked 3.7-fold increase in the duration of nerve blockade ( $P < 0.001$ ), to 180 h or 7.5 days, with no signs of systemic toxicity.

Coencapsulation of bupivacaine in fluid STX liposomes extended block by 60% to 21.24 h ( $P < 0.001$ ), which was approximately the sum of the block durations of the singly encapsulated compounds (block from bupivacaine fluid liposomes was 7.3 h). In solid liposomes, coencapsulation of bupivacaine increased the block duration of STX particles by 56% ( $P < 0.001$ ), which was more than the sum of the durations of block of the individually encapsulated compounds (block from solid bupivacaine liposomes was 7.4 h.). There were no signs of systemic toxicity from those formulations.

Empty liposomes and dexamethasone liposomes did not produce nerve blockade during serial testing for 24 h. No animals developed autotomy (self-injury) of their hind paws.

**Necropsy and Histology.** To assess tissue reaction, animals from nerve block experiments were killed 4, 14, and 21 days after injection ( $n = 3$  at each time point), if nerve block had resolved. The sciatic nerve and surrounding tissues were harvested, and processed for histology by hematoxylin-eosin staining.

In all cases, liposomes were still seen on gross dissection as a whitish material on the sciatic nerve site at day 4 after injection (Fig. 4). Tissues had a benign appearance, with little matting or apparent inflammation. Microscopic examination of these tissues revealed mild to moderate lymphohistiocytic inflammation along the surface of the tissue at 4 days after injection in all samples (Fig. 5), which dissipated in all cases by 21 days postinjection. Animals injected with bupivacaine liposomes showed a small number of muscle fibers with nuclear centralization (very mild injury); one had a small area of focal fibrosis. In all other cases, the inflammation did not infiltrate muscle and nerve tissue, and there was no evidence of muscle or nerve damage. Individual particles could not be discerned. Because hematoxylin/eosin-stained sections are insensitive for identifying nerve injury, we obtained Epon-embedded sections in 3 animals in each group. Those sections did not reveal nerve injury from any formulation at any time point.

**Real-Time PCR.** To further assess the presence or absence of nerve injury 4 days after injection, we used real time PCR to study the



STX, or the amount of particles delivered, even were we to add epinephrine to the injectate. However, it is possible that, when used in larger animals, such as humans, prolonged blocks will be achieved with little toxicity. This improvement in safety could occur because the systemic toxicity of a given dose of liposomes would be reduced, because the toxic dose scales linearly with volume of distribution (i.e., the mass of the recipient), whereas local effect (e.g., local anesthesia) has a much weaker relationship to body size (32). Therefore, the same formulation might have a better therapeutic index in larger animals. Also, larger animals can be injected with more particle, which can greatly increase the duration of block (1). These issues will have to be confirmed for these formulations in larger animal models. Nonetheless, it is important to recognize the potential of these particles to be systemically toxic.

Another significant advantage of this formulation over the polymeric systems (2) is the very small coefficient of variation in block duration. For example, STX + dexamethasone liposomes gave a block duration of  $180 \pm 4$  h (coefficient of variation = 2.2%). In contrast, 60- $\mu$ m polymeric particles gave a median block duration of 9.25 days, with an interquartile range of 8.3–14.8 days (2). This lower variability may be because the liposomes were a better suspension, without needle-clogging. Reproducibility of block duration is an important clinical performance criterion.

Our data regarding particle stability suggest that these formulations could have storage characteristics suitable for practical use. Obviously, there are many other aspects of formulation and characterization that would have to be addressed before use in humans.

In conclusion, ultralong-acting STX-based liposomal local anesthetics were developed that were biocompatible in terms of myotoxicity, neurotoxicity, inflammation, and systemic toxicity, and did not cause autotomy in an animal model system.

## Methods

**Liposome Preparation.** Liposomes were prepared by modified thin lipid film hydration (33). Lipids were selected to produce relatively fluid (DMPC-DMPG) or solid (DSPC-DSPG) liposomes at 37 °C (phase transition temperatures,  $T_m$ ; DSPC = 56 °C and DMPC = 23 °C). DSPC:DSPG:cholesterol or DMPC:DMPG:cholesterol (molar ratio 3:1:2) were dissolved in *t*-butanol. Dexamethasone was added in some samples before lyophilization. The lyophilized cake was hydrated with 250 mM ammonium sulfate or, in some groups, with 0.1 mg STX, at 55–60 °C. The suspension was homogenized at  $10,000 \times g$  with a 3/8" MiniMicro workhead on a L4RT-A Silverson Laboratory Mixer for 10 min followed by 10 freeze-thaw cycles. Excess free STX was removed by centrifugation ( $4,000 \times g$ , 4 °C for 20 min), and replaced by 2 mL of 30 mg/mL bupivacaine hydrochloride in 20 mM citrate buffer pH 4.0, or with 0.9% saline if bupivacaine was not desired. Liposome suspensions with bupivacaine added were stirred at 50 °C for 4–6 h. Liposome suspensions were dialyzed against 0.9% saline solution in 50-kDa molecular mass cut-off dialysis bags for 48 h. Drug-free liposomes were prepared by the same procedure, omitting the drug.

**Liposome Characterization.** Liposomes were sized with a Beckmann Coulter Counter Multisizer 3. Zeta potentials were measured by using Brookhaven Instruments Corporation ZetaPALS and ZetaPlus software. Liposome drug concentrations were determined after disruption of the liposomes with octyl  $\beta$ -D-glucopyranoside (OGP). Dexamethasone and bupivacaine were quantitated by HPLC (Agilent HPLC 1100 Series system) at 254 and 215 nm, respectively, using methods from the U.S. Pharmacopeia. Determination of STX concentration was based on the method of Bates et al. (34), in which STX is oxidized to fluorescent products. Lipid concentrations were determined by colorimetry by the Bartlett assay (*SI Materials and Methods*) (35).

**In Vitro Drug Release.** One mL of liposomes or compounds in solution were inserted into the lumen of a SpectraPor 1.1 Biotec Dispodialyzer (Spectrum Laboratories) with a 25,000 MW cut-off. The dialysis bag was placed in a test tube with 12 mL PBS and incubated at 37 °C on a tilt-table (Ames Aliquot, Miles). At predetermined intervals, the dialysis bag was transferred to a new

test tube with fresh PBS that was prewarmed to 37 °C. Concentrations of compounds were quantitated as above.

**Stability.** Stability was determined by examining changes in vesicle size, zeta potential, liposome integrity, and drug and lipid leakage (disruption of the membrane) over time at room temperature (21 °C) and 4 °C. At specific time points, 400  $\mu$ L of the liposomal formulation was centrifuged with a Centricon separation filter (30,000 MW Millipore) at  $3,500 \times g$ , for 30 min, at 4 °C. The liposomes were retained in the upper chamber; 100–150  $\mu$ L of the filtrate was recovered from the lower chamber, in which drug and lipid concentrations were determined (*SI Materials and Methods*). Leakage, liposome integrity, size distribution, and zeta potential were evaluated every day for 2 weeks.

**Cell Culture.** C2C12 mouse myoblasts [American Type Culture Collection (ATCC) CRL-1772] were cultured to proliferate in DMEM supplemented with 20% FBS and 1% Penicillin Streptomycin. Cell culture supplies were obtained from Invitrogen, unless otherwise noted. Cells were plated at 50,000 cells per mL in DMEM with 2% horse serum and 1% Penn Strep, and left to differentiate into myotubules for 10–14 days. During differentiation, media were exchanged every 2 to 3 days. Cell viability and proliferation were studied after exposures to liposomes, free drugs, and empty liposomes with free drug for up to 96 h (see below).

PC12 cells (ATCC, CRL-1721) originating from rat adrenal gland pheochromocytoma were grown in 24-well tissue culture dishes (CellBind; Corning) with F-12K (ATCC) supplemented with 12.5% horse serum (Gibco), 2.5% FBS (Gibco), and 1% Penn Strep (Sigma). For neuronal induction (PC12), cells were seeded at a relative low density of  $5 \times 10^4$  cells/cm<sup>2</sup> and 50 ng/mL NGF was added 24 h after seeding. Cell viability and proliferation were evaluated as for C2C12 cells. Experiments with PC12 cells were conducted for up to 7 days.

**Cell Viability Assay.** Cell viability was assessed after adding drug- or particle-containing media by a colorimetric assay (MTT kit, Promega G4100) at selected time points (*SI Materials and Methods*).

**Sciatic Nerve Block and Neurobehavioral Testing.** Animals were cared for in compliance with protocols approved by the Children's Hospital Animal Care and Use Committee, and the Massachusetts Institute of Technology Committee on Animal Care, which conformed to guidelines of the International Association for the Study of Pain (36). Adult male Sprague–Dawley rats (Charles River Laboratories) weighing 320–420 g were housed in groups, in a 6 AM to 6 PM light–dark cycle. Under brief isoflurane-oxygen anesthesia, a 23G needle was introduced postero-medial to the greater trochanter, until bone was contacted, and 0.6 mL of test solution was injected over the sciatic nerve. Thermal nociception was assessed by a modified hotplate test, and motor function via a weight-bearing test (*SI Materials and Methods*) (13, 37).

**Necropsy and Histological Analysis.** Rats were killed by carbon dioxide at 4, 14, and 21 days. The nerve and surrounding tissues were harvested, and histological hematoxylin-eosin sections were produced with standard techniques. Samples for Epon-embedded sections were fixed for 24 h at 24 °C in Karnovsky's KII Solution [2.5% glutaraldehyde, 2.0% paraformaldehyde, 0.025% calcium chloride in 0.1M sodium cacodylate buffer (Aldrich) pH 7.4]. Samples were postfixed in osmium tetroxide, stained with uranyl acetate, dehydrated in graded ethanol solutions, and infiltrated with propylene oxide/Epon mixtures. Subsequently, 1- $\mu$ m sections were cut on an ultramicrotome and stained with toluidine blue. Photomicrographs were obtained by using a Nikon Eclipse 50i microscope with SPOT Insight 4 Meg FW Color Mosaic camera and SPOT 4.5.9.1 software from Diagnostic Instruments.

**Gene Expression and RNA Isolation.** The L4 and L5 dorsal root ganglia were removed on necropsy and immediately frozen in liquid nitrogen. Tissue samples were stored at –80 °C until use. Total RNA was extracted from homogenized DRG samples by acid phenol extraction (TRIzol reagent; Gibco-BRL), and isolated with a Qiagen RNeasy Mini kit column (QIAGEN). The purity and concentration of RNA samples were determined from the absorbencies at 260 and 280 nm, with a NanoDrop 100 spectrophotometer (NanoDrop Technologies).

**Real-Time PCR.** Total DRG RNA samples underwent reverse transcription with SuperScript III (Invitrogen) following the manufacturer's procedure. Real-time PCR for each sample were run in duplicate using 100 ng of cDNA in Taqman gene expression assays (Applied Biosystems) according to the manufacturer's instructions. Real-time PCR was performed using Applied Biosystems Step One equipment and program. The relative amount of specifically amplified cDNA

was calculated using the delta-CT method (38, 39). The Applied Biosystems primers used are as follows: GAPDH: Rn99999916\_S1,  $\beta$ -actin: Rn00667869.m1; Gadd45  $\alpha$ : Rn00577049.m1; ATF3: Rn00563784.m1; Cacna2d1: Rn01442580.m1; Smagp: Rn00788145.g1.

**Statistics.** Data are presented as means  $\pm$  SDs ( $n = 4$  in release kinetics, cell work, and gene expression;  $n = 8$  in neurobehavioral studies). To take multiple

comparisons into account, all statistical comparisons were done with the Tukey–Kramer test, using InStat software (GraphPad).  $P < 0.05$  was considered to denote statistical significance.

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