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Prolonged nerve blockade delays the onset of neuropathic pain

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Aberrant neuronal activity in injured peripheral nerves is believed to be an important factor in the development of neuropathic pain. Pharmacological blockade of that activity has been shown to mitigate the onset of associated molecular events in the nervous system. However, results in preventing onset of pain behaviors by providing prolonged nerve blockade have been mixed. Furthermore, the experimental techniques used to date to provide that blockade were limited in clinical potential in that they would require surgical implantation. To address these issues, we have used liposomes (SDLs) containing saxitoxin (STX), a site 1 sodium channel blocker, and the glucocorticoid agonist dexamethasone to provide nerve blocks lasting ~1 wk from a single injection. This formulation is easily injected percutaneously. Animals undergoing spared nerve injury (SNI) developed mechanical allodynia in 1 wk; nerve blockade with a single dose of SDLs (duration of block 6.9 ± 1.2 d) delayed the onset of allodynia by 2 d. Treatment with three sequential SDL injections resulting in a nerve block duration of 18.1 ± 3.4 d delayed the onset of allodynia by 1 mo. This very prolonged blockade decreased activation of astrocytes in the lumbar dorsal horn of the spinal cord due to SNI. Changes in expression of injury-related genes due to SNI in the dorsal root ganglia were not affected by SDLs. These findings suggest that formulations of this kind, which could be easy to apply clinically, can mitigate the development of neuropathic pain.

conduction block | drug delivery | hyperalgesia | prolonged anesthesia

Neuropathic pain is caused by a primary lesion or dysfunction in the nervous system (1); nerve trauma is a common cause (in addition to many medical conditions) (2). The prevalence of chronic neuropathic pain cases is ~3.75 million in the United States alone (3), and the management of such cases is a significant burden in health care spending (4). Despite availability of several pharmacological therapies, a significant number of patients have unsatisfactory pain control, and/or experience undesirable side effects from existing pain medication, making it imperative to examine new therapeutic strategies to prevent chronic neuropathic pain.

One major approach that has been investigated is the blockade of the enhanced neuronal activity from injured primary afferents that contributes to the development of neuropathic pain from nerve injury (5–7). It has been argued that excitatory neurotransmitters such as glutamate, released in response to injury-induced aberrant neuronal impulses, lead to calcium-mediated excitotoxicity and subsequent cellular injury at the level of the dorsal root ganglion (DRG) and spinal cord (8–10). It is clear that the inhibition of injury-induced neuronal discharges can mitigate the subsequent development of pain behavior (11, 12).

However, research in this area has encountered two major difficulties. First, the methods developed to provide prolonged blockade to date—a continuous infusion of tetrodotoxin from an osmotic pump (13), a depot of bupivacaine free base (14), and a bupivacaine–dexamethasone microspheres contained in fibrin glue, loaded within a silicone tube (15)—would be difficult to implement clinically, as they would require surgical implantation of a device on the nerve. Second, attempts to block neuropathic pain

by using those devices to inhibit injury-induced neuronal discharges for ~1 wk have had mixed results, even though such blockade resulted in a mitigation, or at least a delaying, of molecular events associated with the development of neuropathic pain (13, 16, 17). A continuous infusion of tetrodotoxin from an osmotic pump or a depot of bupivacaine free base were able to inhibit the onset of pain behaviors (13) after nerve injury. In contrast, bupivacaine–dexamethasone microspheres contained in fibrin glue, loaded within a silicone tube, had no effect on the development of pain behaviors (15). There is no obvious explanation for these discrepancies, but the reports used different drugs [with different effects on ion channels (13, 18, 19) and on local tissue reaction (20)], and sustained release formulations (also with potentially different effects on tissue reaction; ref. 21).

Here, we have used liposomes containing the site 1 sodium channel blocker saxitoxin (STX) with dexamethasone (SDLs) (22) as the means of providing prolonged nerve blockade to address the crucially important issue of the ability of prolonged duration local anesthesia to mitigate the onset of neuropathic pain. STX is a potent local anesthetic that acts at site 1 of the voltage-sensitive sodium channel (23); dexamethasone has been shown to prolong the duration of nerve blockade in several sustained release formulations (24–27), although the mechanism is not understood. SDLs are easy to administer percutaneously, and single injections produce nerve blocks lasting ~1 wk in the rat (26). The SDLs are advantageous in that STX (and the SDLs) does not cause myo- or neurotoxicity (26). This lack of toxicity is in contrast to the encapsulated amino-amide local anesthetics used in previous attempts to mitigate neuropathic pain (15); those compounds are myo- and neurotoxic as free drugs (28, 29) and, when encapsulated, potentially cause local tissue injury (21, 30, 31). To gauge the impact of prolonged blockade with SDLs on neuropathic pain, we have studied their effect on the development of allodynia, and on biological events in the dorsal root ganglia and spinal cord, in the spared nerve injury (SNI) model in the rat.

Results

Sciatic Nerve Blockade with SDLs. Multilamellar/multivesicular SDLs 5.4 ± 1.4 μ m in diameter were prepared using the thin-lipid film technique as reported (26) (Fig. S1 A and B). Injection of 0.3 mL of the SDL formulation at the sciatic nerve increased hind-paw thermal latency in the injected limb for 5–6 d (Fig. S1C). Liposomes made in exactly the same way with (dexamethasone and citrate buffer, pH 4.5, but no STX) had no effect on withdrawal latency.

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The authors declare no conflict of interest.

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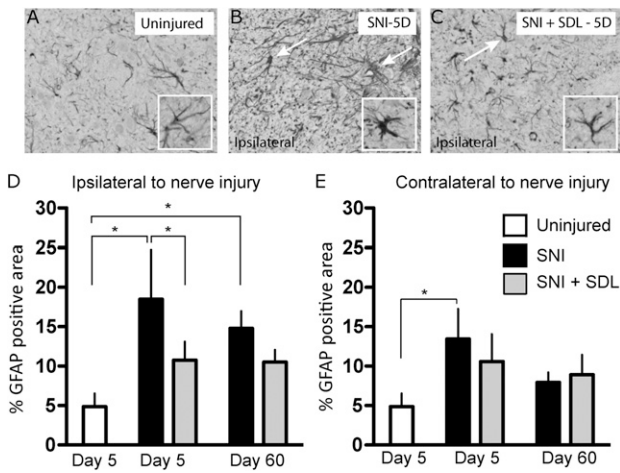


Fig. 4. Prolonged duration nerve block attenuates SNI-induced astrocyte (GFAP) activation. Representative images of GFAP-positive cells (white arrows) in the L4, L5 dorsal horn of spinal cord sections from uninjured (A), SNI-treated (B), and SNI + SDL-treated (C) animals. (Insets) Representative pictures depicting astrocyte morphology in each group. Tissues were harvested 5 d after nerve injury and/or first SDL administration. Quantitative analysis of GFAP staining in dorsal horn ipsilateral to injury (D) and contralateral to injury (E) is expressed as the percentage of area that is GFAP positive. Data are means \pm SD, * P < 0.05, n = 5 rats per group.

astrocytes) in the pain signal processing area of the dorsal horn of the lumbar spinal cord, 5 and 60 d after SNI. Those time points were chosen to represent acute and chronic responses to nerve injury respectively. GFAP-positive glial cells from the dorsal horn of uninjured naïve rats appeared star shaped with thin processes (Fig. 4A Inset), whereas those from dorsal horns ipsilateral to nerve injury, 5 d after SNI exhibited a stubby morphology with thick processes suggestive of astrocyte activation (32) (Fig. 4B Inset). At the same time point, astrocyte morphology in SNI animals treated with SDLs was similar to that from uninjured naïve animals (Fig. 4C Inset).

The average total area covered by GFAP-positive cells (33) was increased at 5 and 60 d in the ipsilateral dorsal horn after SNI, compared with uninjured naïve rats (Fig. 4D, P < 0.05, n = 5). In contrast, SDL-treated SNI animals at days 5 and 60 exhibited ipsilateral GFAP staining that was similar in extent (P > 0.05) to the saline-treated naïve group, but significantly lower (P < 0.05) than that observed in the untreated SNI group (Fig. 4D).

GFAP staining in the contralateral dorsal horn of SNI animals that did not receive SDLs was increased (P < 0.05) compared with uninjured animals at day 5 but not at day 60 (Fig. 4E). There was no such increase in the GFAP-positive area in SDL-treated animals.

Effect of 18-d Nerve Block on Nerve Injury-Induced Gene Expression Changes in the DRG. In addition to astrocyte activation in the CNS, gene expression changes in the cell bodies of peripheral afferent neurons are consistently observed in nerve-injured neuropathic pain models (34). To assess whether prolonged conduction block (18.1 \pm 3.4 d in these experiments) of afferent neurons can alter injury-dependent changes in gene expression, we used a customized RT² Profiler PCR array to profile genes whose expression is known to be altered in the DRG of SNI animals (35, 36), with or without SDL treatment at day 5 and 60. Gene expression in all experimental groups was studied as a multiple of the expression in uninjured, unblocked animals.

SDL treatment alone (in uninjured animals) did not induce an increase in the expression of any genes examined in ipsilateral DRGs on day 5 (Table 1). SNI increased the expression of

Table 1. Effect of 18-d nerve block on the expression of selected genes related to nerve injury in the L4, L5 DRG

	Ppia	Cacna2d1	Htr3a	Scn11a	Npy	Gal	Vip	C1qb	Jun	RT1Db1	Gadd45a	Atf3
Ipsilateral												
5 d												
SDLs	0.8 \pm 0.02	0.5 \pm 0.06	0.5 \pm 0.08	0.4 \pm 0.13	0.5 \pm 0.44	0.7 \pm 0.11	0.2 \pm 0.16	1.2 \pm 0.33	0.7 \pm 0.12	0.6 \pm 0.12	0.8 \pm 0.13	0.8 \pm 0.31
SNI	0.9 \pm 0.09	3.2 \pm 0.51	0.4 \pm 0.02	0.4 \pm 0.11	38.4 \pm 12.8	45.3 \pm 14.1	144.4 \pm 42.4	7.0 \pm 1.32	2.8 \pm 0.26	3.7 \pm 1.24	3.9 \pm 0.64	37.2 \pm 4.14
SDLs + SNI	0.9 \pm 0.05	2.2 \pm 1.04	0.3 \pm 0.14	0.2 \pm 0.16	47.8 \pm 20.3	34.2 \pm 16.7	115.6 \pm 44.7	5.5 \pm 2.29	2.0 \pm 0.74	2.0 \pm 0.87	4.0 \pm 1.22	26.2 \pm 10.78
60 d												
SNI	0.9 \pm 0.09	3.3 \pm 0.83	0.8 \pm 0.27	0.8 \pm 0.17	19.6 \pm 6.03	10.2 \pm 5.60	202.2 \pm 113.1	4.3 \pm 2.65	2.6 \pm 0.49	3.34 \pm 1.83	2.6 \pm 0.66	19.8 \pm 7.32
SDLs + SNI	1.1 \pm 0.05	1.9 \pm 0.78	0.5 \pm 0.15	0.4 \pm 0.24	48.7 \pm 10.2*	13.6 \pm 0.96	155.6 \pm 49.0	3.8 \pm 1.40	2.1 \pm 0.61	3.3 \pm 1.43	3.1 \pm 0.23	17.7 \pm 4.92
Contralateral												
5 d												
SDLs	0.8 \pm 0.04	0.5 \pm 0.20	0.5 \pm 0.15	0.5 \pm 0.17	0.9 \pm 0.84	0.6 \pm 0.11	0.5 \pm 0.42	1.7 \pm 0.69	0.7 \pm 0.26	1.0 \pm 0.38	0.7 \pm 0.08	0.4 \pm 0.21
SNI	0.9 \pm 0.07	0.6 \pm 0.16	0.7 \pm 0.18	0.5 \pm 0.20	0.4 \pm 0.20	0.7 \pm 0.17	0.4 \pm 0.35	1.0 \pm 0.28	0.7 \pm 0.21	0.8 \pm 0.11	0.7 \pm 0.03	0.6 \pm 0.12
SDLs + SNI	0.9 \pm 0.06	0.7 \pm 0.05	0.7 \pm 0.06	0.6 \pm 0.08	1.3 \pm 0.80	1.1 \pm 0.12	0.7 \pm 0.26	1.5 \pm 0.46	0.6 \pm 0.10	0.9 \pm 0.21	0.6 \pm 0.08	0.9 \pm 0.19
60 d												
SNI	0.9 \pm 0.10	1.1 \pm 0.18	1.0 \pm 0.10	0.9 \pm 0.27	1.3 \pm 0.51	0.7 \pm 0.16	3.2 \pm 3.90	1.6 \pm 0.66	1.1 \pm 0.25	1.1 \pm 0.21	0.9 \pm 0.07	1.9 \pm 0.59
SDLs + SNI	0.9 \pm 0.09	0.1 \pm 0.05*	0.2 \pm 0.02*	0.07 \pm 0.02*	1.5 \pm 1.50	0.3 \pm 0.09*	0.2 \pm 0.09*	0.4 \pm 0.21	0.13 \pm 0.05*	0.6 \pm 0.24	0.7 \pm 0.05	0.1 \pm 0.04*

Data are means \pm SD of mRNA fold changes over expression levels in pristine animals (n = 5 rats per group). * P < 0.05 in comparison of SDL-treated SNI group (SDLs + SNI) and untreated SNI group by one-way ANOVA with Bonferroni post hoc comparison. Decreases in mRNA expression levels (numbers < 1, being the inverse of the fold decrease) are shaded, whereas increases are unshaded. Gene abbreviations: Ppia (control), Cyclophilin A; Cacna2d1, Calcium channel α 2 δ 1; Htr3a, 5-hydroxytryptamine receptor 3a; Scn11a, Sodium channel IX α ; Npy, Neuropeptide Y; Gal, Galanin prepropeptide; Vip, Vasoactive intestinal peptide; C1qb, Complement component 1q β chain; Jun, Jun oncogene; RT1Db1, RT1 class II, locus Db1; Gadd45a, Growth arrest and DNA damage-inducible α ; Atf3, activating transcription factor 3.

several injury-related genes in ipsilateral DRG's at 5 and 60 d, whereas treatment with SDLs did not prevent SNI-induced increase in expression of most genes at either time point.

Contralateral DRGs harvested from SNI animals showed no changes in gene expression after 5 and 60 d. However, at 60 d, DRGs harvested from SDL-treated SNI rats exhibited down-regulation of many genes tested (Table 1) ($P < 0.05$) compared with SNI animals that did not receive SDL.

Discussion

Aberrant spontaneous activity in the sensory afferents is consistently observed in animal models of painful neuropathy (37, 38). Peripheral nerve associated injury-induced discharges are thought to be responsible for irreversible changes in the central nervous system (8), leading to maintenance of chronic pain. Previous efforts to mitigate the development of neuropathic pain by prolonged neural blockade have had conflicting results. Bupivacaine-dexamethasone particles contained within a silicone tube provided nerve blockade lasting 8 d, but did not impact the development of allodynia or hyperalgesia in SNI (15) even though the same formulation was shown to prevent the rise in p38 mitogen-activated protein kinase seen in spinal microglia after SNI (17) and prevented the induction of apoptosis in the dorsal horn (16). In contrast, mitigation of pain behavior and reduced injury-induced glial cell activation in the spinal cord was seen following ~1-wk block with bupivacaine free base powder or a tetrodotoxin infusion (13, 14). Different parts of our results were in agreement with both reports. Blockade with a single dose of SDL, resulting in 7-d block, did not prevent allodynia. However, 18-d blockade with repeated dosing resulted in a 4-wk delay in the onset of allodynia. Furthermore, spinal astrocyte activation, a facilitator of central sensitization in chronic neuropathic pain models (34, 39), was markedly decreased in SDL-treated SNI animals; this reduction was still in effect 60 d after SNI, 42 d after nerve block wore off. These results are consistent with the view that very prolonged nerve blockade could mitigate the development of neuropathic pain, and that such could be provided by an easily injectable sustained release system.

There has been interest for several decades in using drug delivery technologies to affect neuronal function locally, in the central (40) and peripheral nervous systems (31), particularly in the development of prolonged duration local anesthetics. A wide range of devices have been used for the latter application (24, 26, 27, 30, 41–45). Few have achieved blocks lasting even a few days (24, 25). Those that did often caused severe difficulties with local inflammation and/or tissue toxicity (21), particularly myo- and neurotoxicity (21, 25). SDLs produce prolonged nerve blockade (~1 wk from a single injection) with only mild inflammation and without evidence of myo- or neurotoxicity (26).

One possible explanation for the ability of SDLs to mitigate the development of mechanical allodynia where the bupivacaine-dexamethasone particles had failed is that the latter were neurotoxic and injured the nerves during blockade. That explanation is not consistent with the reports that nerve block lasting ~1 wk did not mitigate the development of allodynia whether achieved by the bupivacaine-dexamethasone particles (15) or the SDLs, whereas bupivacaine free base powder (presumably more tissue-toxic) was able to mitigate pain behavior (13). Duration of block seems to be a key factor, as seen by the fact that repeated administration of SDL did impact the onset of allodynia. The repeated administration of SDL may be blocking injury-induced late-phase electrical discharges (46) that are important for pain maintenance. However, duration may not be the only factor. Whereas 1 wk of nerve block with SDLs did not mitigate pain behavior, a similar duration of block with tetrodotoxin or bupivacaine free base has been reported to do so (13). The question arises why the SDLs had less effect on

the development of allodynia than was reported with some other approaches (13). One possibility is that nerve blockade may not have been as consistently dense with the SDLs as was in some other reports (13). It is also possible that although dexamethasone prolongs nerve blockade, it also exacerbates the development of neuropathic pain; this would also explain why bupivacaine free base prevented neuropathic pain (13, 14) but bupivacaine-dexamethasone particles did not (15).

Some studies demonstrate the consistent onset of contralateral hyperalgesia in nerve-injured animal models of neuropathic pain (47–51), whereas others report a mild or no hyperalgesic response (52, 53). Here, we observed transient contralateral hyperalgesia in SNI animals that was both slower in onset and lower in intensity compared with its ipsilateral counterpart. At the spinal cord level, astrocyte activation was increased in the contralateral dorsal horn, but it was milder compared with activation in the ipsilateral dorsal horn. The mechanism for contralateral pain is largely unknown, but altered spinal processing of sensory signals has been proposed (54). Mirror image pain, a well documented feature of human causalgia, has been equated with experimental contralateral hyperalgesia (49); the findings of this study raise the possibility of using prolonged ipsilateral nerve conduction block in preventing mirrored pain syndromes.

Nerve injury-induced gene expression changes in the DRG cell soma have been well documented, with the majority of injury-related genes changing expression 3 d after nerve injury (35) and remaining altered even after 13 d (36). In the present study, both 7- and 18-d nerve block failed to prevent SNI-induced gene expression changes in the ipsilateral L4, L5 DRG, even though nerve block mitigated the development of allodynia. These findings raise the possibility that activity-dependent changes in gene expression are not by themselves sufficient to cause neuropathic pain. In the contralateral DRGs, prolonged nerve block induced a significant decrease in expression in 7 of 11 genes, 2 mo after nerve injury. The significance of that finding is unclear; as noted above, contralateral pain following nerve injury is not well understood (nor universally reported) at this time.

Ultra-long-duration nerve block obtained by repeated administration of SDLs delays nerve injury-induced tactile hypersensitivity and attenuates the central astrocyte activation response. There would appear to be many important factors with potential impact on the effectiveness of formulations in mitigating pain behaviors, including type of drug, type of formulation, and duration and intensity of nerve blockade. SDLs provide an approach that is potentially clinically practical for preventing neuropathic pain conditions.

Materials and Methods

Liposome Preparation. Liposomes were produced by the thin lipid film technique, using 1,2-distearoyl-sn-glycero-3 phosphocholine (DSPC) and 1,2-distearoyl-sn-glycero-3-phosphatidylglycerol (DSPG) (Genzyme) as described (26). Size of liposomes was determined using a multisizer 3 Coulter Counter (Beckman Coulter). Intraliposome STX content was determined after separating the lipid fraction using the Bligh and Dryer method (55). Determination of STX content was based on a previously published method by Bates et al. (56). STX was received as a generous gift from Sherwood Hall (Food and Drug Administration, College Park, MD).

Nerve Conduction Studies. For assessing the effect of SDLs on nerve function, we measured evoked compound muscle action potential latencies and amplitudes from the sciatic nerve (57) at the peak of nerve block (day 4 after SDL treatment, as characterized by the thermal paw withdrawal test). Rats were weighed daily and their body weight monitored (Fig. S2).

Detailed protocols for nerve conduction studies, immunohistochemistry, PCR array, spared nerve injury, behavioral tests for nociception, and histological and statistical methods are described in *SI Materials and Methods*.

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