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Toxicogenomic analysis of a sustained release local anesthetic delivery system

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Abstract

Concerns over neurotoxicity have impeded the development of sustained release formulations providing prolonged duration local anesthesia (PDLA) from a single injection, for which there is an urgent clinical need. Here, we have used toxicogenomics to investigate whether nerve injury occurred during week-long continuous sciatic nerve blockade by microspheres containing bupivacaine, tetrodotoxin, and dexamethasone (TBD). Animals treated with amitriptyline solution (our positive control for local anesthetic-associated nerve injury) developed irreversible nerve blockade, had severely abnormal nerve histology, and the expression of hundreds of genes was altered in the dorsal root ganglia at 4 and 7 days after injection. In marked contrast, TBD-treated nerves reverted to normal function, were normal histologically and there were changes in the expression of a small number of genes. Toxicogenomic studies have great potential in delineating patterns of gene expression associated with specific patterns of tissue injury (e.g. amitriptyline neurotoxicity), and in identifying related changes in gene expression upon exposure to a drug, biomaterial, or drug delivery system.

Keywords

Toxicogenomics; drug delivery; biocompatibility; prolonged duration local anesthesia; neurotoxicity; nerve injury

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1. Introduction

Drug delivery systems can provide a wide range of therapeutic benefits, including extended duration of effect, steady therapeutic levels, maintenance of high local but low systemic drug levels, and many others. However, tissue reaction to the drug delivery vehicle, and local effects of the sustained release of drugs can have adverse local effects, which can severely limit clinical use [1]. This problem has been seen with particular clarity in attempts to develop sustained-release systems providing prolonged duration local anesthetics (PDLA) for days or weeks from a single injection [2–5]. Such formulations could potentially revolutionize pain management. Perioperative medicine would benefit from prolonged local analgesia, while patients with chronic pain - such as cancer pain – could obtain prolonged relief without having to take systemic medications with their host of complications including toxicity, dependence, and clouding of the sensorium.

The principal obstacle to the development of PDLA has been concern over neurotoxicity. The prolonged proximity of inflammation from drug delivery systems [6, 7] and the intrinsic neurotoxicity of local anesthetics [8] could harm nerve tissue. The impact of potential neurotoxicity has already been seen in a formulation that provided several days of nerve blockade [2] and was approaching commercialization: polymeric microspheres containing 70–75% (w/w) bupivacaine and 0.05% (w/w) dexamethasone [2]. After initial reassuring animal data [9], detailed animal studies during product development showed severe nerve pathology and human subjects experienced neurological symptoms [10]. Subsequently, the Investigational New Drug (IND) application for that formulation was withdrawn. Currently, conventional wisdom suggests caution regarding the use of such devices near major nerves [4]. Although new formulations are being developed to provide very prolonged nerve blockade [5, 11–13] concerns still exist regarding the potential toxicity of those formulations and others.

These difficulties highlight the need to develop methods of detecting toxicity at an early stage in the development of these and other drug delivery systems [1]. Here we employed toxicogenomics to supplement neurobehavioral and morphological studies to study nerve injury of a PDLA formulation, specifically injectable poly(lactic-co-glycolic) acid (PLGA)-based polymeric microspheres containing 50% bupivacaine, 0.05% tetrodotoxin (TTX) and 0.05% dexamethasone (TBD) [3]. Genomic methodologies have been used to assess toxicity in the early stages of drug discovery [14], with the intent of improving safety [15] and to select candidate molecules earlier in the process [16]. Toxigenomics are apposite to the study of the biocompatibility of implanted or injected drug delivery systems [1] which often maintain high local tissue drug concentrations, and can cause a highly variable tissue response [6, 17].

2. Materials and methods

2.1. Materials

TTX stock solutions were made by dissolving 1 mg TTX in citrate buffer (Sigma) in 1 ml water. Bupivacaine hydrochloride (Sigma Chemical Co., St. Louis, MO) was converted to the free base by alkaline precipitation and filtration. Carboxymethylcellulose sodium, Tween 80 (polyoxyethylene sorbitan) and dexamethasone were obtained from Sigma Chemical Co, Epinephrine from American Regent Laboratories, Inc. (Shirley, NY). Poly (lactic-co-glycolic) acid (lactic:glycolic 65:35, MW 120,000) (PLGA) was obtained from Lake Shore (Birmingham, AL), poly (vinyl alcohol) (88% hydrolyzed, MW 20,000) from Polysciences (Warrington, PA).

2.2. Preparation of anesthetic microspheres

PLGA microspheres containing 50% (w/w) bupivacaine, 0.05% (w/w) dexamethasone and 0.05% (w/w) TTX were prepared as previously described [3], using a double emulsion method. That formulation is abbreviated as "TBD" in this report. In brief, bupivacaine free base and dexamethasone were dissolved together with the polymer in methylene chloride and methanol (84:16). An aqueous solution of TTX was sonicated into that mixture, forming the primary emulsion. That emulsion was poured into a polyvinyl alcohol solution, and the secondary emulsion was formed by homogenization. The organic solvent was removed by rotary evaporation and microspheres were isolated by wet sieving, washed by repeated centrifugation, then lyophilized to dryness. The procedure for making blank particles was the same except that no active agents were introduced and there was no sonication step.

2.3. Sciatic nerve blockade

Adult male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 350 – 420 g were housed in groups, in a 6 AM - 6 PM light-dark cycle. Under brief isoflurane-oxygen anesthesia, a 20G needle was introduced postero-medial to the greater trochanter, until bone was contacted, and 0.6 ml of test solution was injected. 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 IASP guidelines [41].

Sciatic nerve injections were performed with either 75 mg of blank microparticles suspended in 80 mM amitriptyline, or 75 mg of TBD microparticles suspended in 1% carboxymethylcellulose [3]. The injectate also included 10 μg/ml epinephrine. Thermal nociception was assessed by a modified hotplate test as previously described [42, 43].

Hind paws were exposed in sequence (left then right) to a 56°C hot plate (Model 39D Hot Plate Analgesia Meter, IITC Inc., Woodland Hills, CA). The time (latency) until paw withdrawal was measured by a stopwatch. (Thermal latency in the un-injected leg was a control for systemic effects of the injected agents.) If the animal did not remove its paw from the hot plate within 12 seconds, it was removed by the experimenter to avoid injury to the animal or the development of hyperalgesia. The experimenter was blinded as to what treatment specific rats were receiving.

2.4. Tissue collection and staining

Animals were euthanized with carbon dioxide for tissue sampling at predetermined time intervals following the injection of the test formulations. The sciatic nerve was exposed and the L4 and L5 dorsal root ganglia (DRG) were identified, removed and immediately frozen in liquid nitrogen. DRG samples were stored at −80 °C until RNA was isolated. The sciatic nerves were removed and fixed for 24 hrs at 24 °C in Karnovsky's KII Solution (2.5 % glutaraldehyde, 2.0 % paraformaldehyde; Electron Microscopy Supplies; Hatfield, PA), 0.025 % calcium chloride in a 0.1M sodium cacodylated buffer pH 7.4 (Aldrich; St. Louis, MO). Samples were post-fixed in osmium tetroxide, stained with uranyl acetate, then dehydrated in graded ethanol solutions and infiltrated with propylene oxide/Epon mixtures. Subsequently, 1 μm sections were stained with toluidine blue and studied by light microscopy (N=3 for each group).

2.5. RNA isolation

Total RNA was extracted from homogenized DRG samples using an acid phenol extraction (TRIzol reagent; Gibco-BRL, CA). Samples were purified on a Qiagen RNeasy Mini kit column (QIAGEN, USA). The purity and concentration of RNA samples were determined

from the absorbencies at 260 and 280 nm, with a NanoDrop 100 spectrophotometer (NanoDrop Technologies, Wilmington, Del).

RNA quality was evaluated using the Agilent 2100 Bioanalyzer. Five micrograms of RNA was used for gene expression experiments.

2.6. DNA microarray and analysis of data

Affymetrix GeneChip Rat Genome 430_2 Array labeling, hybridization, and scanning were performed according to the manufacturer's instructions. To map probes on the Affymetrix Mouse 430_2 array to Refseq transcripts, we used custom Chip Definition Files (CDFs) [44]. Refseq transcript expression levels were then calculated using Robust Multi-Array (RMA) normalization of microarrays (in the Bioconductor R environment) using default settings.

2.7. Functional classification

We used the Database for Annotation, Visualization and Integrated Discovery (DAVID) (http://david.abcc.ncifcrf.gov/tools.jsp) to identify enriched biological themes, particularly Gene Ontology (GO) terms, in our lists of genes, as described [45]. Gene enrichment analysis provides p-values that reflect a relative enrichment of various biological themes within those gene lists. We considered a p-value < 0.005 to represent enrichment in the annotation categories.

2.8. Real Time PCR

Total DRG RNA samples underwent reverse transcription with SuperScript III (Invitrogen) following the manufacturer's procedure. Real-time PCR reactions for each sample were run in duplicate using ~100ng of cDNA with Taqman gene expression assays (Applied Biosystems). Real-time PCR was performed using Applied Biosystems' Step One equipment and program. The relative amount of specifically amplified cDNA was calculated using the comparative cycle threshold (delta-CT) method [46].

The Applied Biosystems primers used are as follows: GAPDH: Rn99999916_S1, β-actin: Rn00667869_m1; Gadd45a: Rn00577049_m1; Atf3: Rn00563784_m1; Cacna2d1: Rn01442580_m1; Smagp: Rn00788145_g1.

2.9. Statistical analysis

DNA microarrays: We identified statistically significant changes in gene expression differences with Significance Analysis of Microarrays (SAM) [47].

Real time PCR and neurobehavioral testing: Data are expressed as mean ± s.d. For neurobehavioral tests differences between groups were compared using a two-tailed student t-test. For real time PCR groups were compared using the Tukey-Kramer test, to take multiple comparisons into account, using InStat software (GraphPad, La Jolla, CA). A *P*-value <0.05 was considered to denote statistical significance for all analyses.

3. Results

Particles were produced as a fine white powder, with yields in the 50% range by weight. Volume-weighted particle diameters were 56 ± 7.2 μm. Animals were injected once at the sciatic nerve with blank PLGA particles ("blank" group), TBD ("TBD" group) or amitriptyline ("amitriptyline" group) formulations. (See rationale for the various groups in their respective subsections.) Blank particles were suspended in the amitriptyline solution so that polymeric particles would be present in all groups.

Animal in all groups underwent neurobehavioral testing until the time of necropsy at 4 and 7 days. Those dates were selected to be within the 9-day duration of block from TBD particles [3]. Animals injected with blank particles or with saline alone ("saline" group) did not develop neurobehavioral deficits at any time. Animals injected with TBD or with amitriptyline developed sensory and motor blockade: a thermal latency of 12 seconds and weight bearing ≤ 50g. This level of nerve blockade lasted until the time of necropsy in the animals sacrificed at 4 and 7 days except in approximately 10% of TBD-treated rats, where nerve blockade recurred ("recurrent group") after an initial recovery (Supplementary Fig S1). In all groups, complete resolution of nerve blockade occurred, except for the amitriptyline group where normal nerve function never returned.

At necropsy, nerves were harvested for histology and the dorsal root ganglia (DRGs) were collected for mRNA extraction and gene analyses. The DRGs are the sites of the sensory cell nuclei of the sciatic nerve. As DRGs are remote from the tissue reaction and inflammation at the site of injection [3] they are not directly affected by them, but injury to the nerves themselves will affect gene expression in the DRGs [18–20]. The mRNA from DRGs was analyzed using Affymetrix rat oligonucleotide arrays.

3.1. Blank PLGA microspheres at 4 and 7 days

PLGA is a biodegradable polymer which has been used extensively in drug delivery, including for delivery of local anesthetics [2, 3, 21, 22]. Microscopy of Epon-embedded sections of the sciatic nerve following injection of PLGA blank particles showed no evidence of neurotoxicity (Fig. 1A). The mRNA of DRGs in this group were analyzed 4 and 7 days after injection using Affymetrix rat oligonucleotide arrays. At both time points, the expression of the vast majority of the ~18,000 genes on the array was unchanged compared to saline-treated controls. When data were stratified so that only genes with at least two-fold change in expression and a low false discovery threshold (with $q<0.01$) were considered, only 1 gene was up-regulated at day 4, and 8 genes at day 7, and no genes were down-regulated in the PLGA blank group (Table 1). None of the established markers for nerve injury were affected. (The changes in gene expression related to nerve injury have been studied extensively [18, 19, 23], and sensitive markers for nerve injury have been identified, particularly activating transcription factor 3 (Atf3) [24, 25] and the growth arrest and DNA-damage-inducible protein 45 alpha (Gadd45a) [20]. Atf3 and Gadd45a are not or minimally expressed in the DRGs of uninjured sciatic nerves [20, 24], but are greatly expressed after axotomy. Such molecules are considered "markers" of nerve injury as opposed to simply molecules that are up-regulated after nerve injury.) Of the affected genes (Table 2), only neuropeptide Y (Npy) has been previously shown to be up-regulated in nerve injury [26, 27].

3.2. Amitriptyline at 4 and 7 days

The purpose of the amitriptyline group was to ensure a relevant positive control specifically for local anesthetic-related nerve injury. Amitriptyline has a structure and mechanism of action similar to that of bupivacaine [28], is itself a local anesthetic, and could be expected to share mechanisms of toxicity. The high concentration of amitriptyline used here reproducibly causes severe nerve injury [29].

On necropsy, tissue and nerve in the amitriptyline group were grossly inflamed. Microscopy of Epon-embedded sections of the sciatic nerve 4 and 7 days after injection of amitriptyline showed severe axonal injury with decreased nerve fiber density, an increased number of macrophages, and myelin debris (including myelin ovoids) consistent with axonal injury and subsequent Wallerian degeneration (Fig. 1B).

After 4 and 7 days of continuous nerve blockade, the expression of the majority of the ~18,000 genes on the array was unchanged compared to saline-treated controls (Fig. 2). This distribution of genes is reflected by the clustering of the vast majority of the genes along the line of identity.

When data were stratified so that only genes with at least a two-fold change in expression and a low false discovery threshold (with $q<0.05$) were considered, 175 genes were up-regulated and 154 genes were down-regulated in the amitriptyline group after four days of continuous blockade (Table 1). After seven days of blockade, 345 genes were up-regulated and 353 genes were down-regulated in the amitriptyline group, using the same criteria (Table 1). Those numbers correspond to 1.8 % and 3.8 % of the total genes examined, at 4 and 7 days respectively. These percentages are comparable to those reported to be affected ≥ 2-fold in a nerve transaction (axotomy) model, using gene expression arrays [19].

3.2.1. Up-regulated genes—When the stringency of the false discovery rate was increased to a q-value <0.01 in the amitriptyline group, 32 genes had a two-fold or greater increase in expression at 4 days, and 172 genes at 7 days (Table 1). The most highly up-regulated genes at 7 days were also increased at 4 days (Table 3A). The higher q values at 4 days for many genes may be due to inter-animal differences in the onset of nerve injury.

Many of the genes that were up-regulated (6 to 106-fold) in the amitriptyline group (Table 3A) are known to be up-regulated in models of peripheral nerve injury [18, 26, 30], including the established nerve injury markers Atf3 [24, 25] and Gadd45a [20]. Other genes known to be up-regulated following nerve damage that were up-regulated in the amitriptyline group were galanin (Gal), small proline-rich protein 1a (Sprr1a), Npy, and calcium channel voltage dependent alpha2/delta subunit 1 (Cacna2d1), arginase 1(Arg1), and Sox11. Regenerating islet-derived 3 beta (Reg3b), is a member of the Pap gene family, of which some members are up-regulated by nerve injury [31].

3.2.2. Down-regulated genes—Genes that were down-regulated were decreased at both 4 and 7 days, with lower q values at 7 days (Fig. 3, Table 2). Many of the genes that were down-regulated (4 to 8-fold), such as voltage gated K+ channels (Kcns1, Kcns3, Kcna4) [19, 26], glutamate receptor (Grik 1) and noggin (Nog), are known to be down-regulated following nerve injury [19] as part of nerve regeneration [18, 19, 26].

3.2.3. Functional classification—We performed a functional classification analysis for the genes that were markedly affected 7 days after amitriptyline treatment (172 up-regulated, 270 down-regulated; Table 1), using functional annotation analysis (http://david.abcc.ncifcrf.gov/tools.jsp). Up-regulated genes belonged to different functional groups than down-regulated genes (Supplementary Fig S2). The trends are consistent with previous findings following nerve transection [19].

3.3. TBD at 4 and 7 days

TBD particles were used here because a) they produced nine days of sciatic nerve blockade in the rat – the longest duration reported so far from a single injection. TTX is an ultra potent local anesthetic marine toxin which is not myotoxic or neurotoxic [32]; it greatly prolongs nerve block from bupivacaine [33] without increasing cytotoxicity [34]. We have previously studied particles containing TTX, bupivacaine and/or dexamethasone singly and in pairs, as well as the unencapsulated drugs; they were not used here as they produced nerve blocks lasting a day and a half or less [3] and therefore would not address our interest in PDLA.

Sciatic nerves from TBD-treated rats appeared normal at all time points, with adjacent pockets of particulate residue surrounded by moderate inflammation. However, there was no evidence of neurotoxicity (Fig. 1B) at 4 or 7 days (N=3 at each time point). Animals from the recurrent group were culled for histology 4 days after recurrence of block was documented.

As with the other animals in the TBD group, no evidence of nerve injury was observed by microscopy of Epon-embedded sections. As in the amitriptyline group, the majority of the ~18,000 genes were unchanged compared to saline-treated controls (Fig. 2) in the TBD groups (including the recurrent group; Supplementary material Fig. S3). However, whereas hundreds of genes were up- or down-regulated in the amitriptyline group, there was no significant gene up-regulation and very few down-regulated genes in the TBD groups compared to control animals, at both 4 and 7 days, and none in the recurrent group (Table 1). The lower number of genes affected in the TBD groups compared to the PLGA blank group may be related to the effect of nerve blockade, which can delay (but does not eliminate) nerve injury [35], and /or the anti-inflammatory effects of dexamethasone.

Five genes in the TBD groups were slightly down-regulated (Table 2) - a markedly lesser effect than in the amitriptyline groups in the number of genes affected and the magnitude of the decrease (Tables 1 and 3) - but none of those genes are associated with peripheral nerve stress or injury. Cytochromes P3a9 (Cyp3a9) is in the cytochrome P450 superfamily of heme-containing mono-oxygenases that metabolize xenobiotics and endogenous compounds including fatty acids and neurotransmitters [36]. Ectonucleoside triphosphate diphosphorhydrolase 4 (Entpd4) modulates ATP-induced norepinephrine exocytosis in nerve [37] and Protein Kinase C beta I (Prkcb1) is protective to the neural elements of the inner ear [38]. The functions of RGD1304982 and LOC500118 are unknown.

3.4. Quantitative validation

Real-time quantitative PCR confirmed the expression profiles of four genes associated with nerve injury that were shown to be markedly up-regulated by the gene array data (Gadd45a, Atf3, SmagP and Cacna2d1) in amitriptyline but not TBD groups. β-actin was used as an unaffected control gene. Gene expression was normalized to GAPDH, and expressed as a ratio to expression in saline-treated animals (Fig. 3).

4. Discussion

This study highlights the potential usefulness of genomic methodologies in the safety screening of biomaterials and drug delivery systems early in the preclinical development process, in a manner analogous to the use of toxicogenomics in the safety evaluation in drug discovery [14]. We have used that technology to address a major issue in the application of sustained release technology to PDLA.

It was reasonable, given the neurobehavioral and histological abnormalities noted [3], and the difficulties encountered with some prolonged duration local anesthetics [1, 10], to hypothesize that the TBD particles could cause nerve injury. However, analysis of the gene array data at up to 7 days suggested that microsphere-based PDLA lasting at least 1 week can be achieved without nerve injury that could be detected by the sensitive measures used here, even with a delivery system that causes perineural inflammation [3], and compounds (in this case bupivacaine) that are known to be neurotoxic [7, 39]. Nerve injury was not seen at 4 days – when the inflammation would be acute, consisting of macrophages and neutrophils – nor at 7 days, when it would be transitioning to a chronic response with lymphocytes, macrophages, and giant foreign body cells [40].

Although gene arrays undoubtedly increase our ability to screen for injury-related signals, we are still confronted by the limitations of our understanding of biological processes and their consequences [1]. Nonetheless, findings from such screens could be useful in that they could show whether the nerve phenotype has been affected by the formulation, and provide warning that there might be a problem, or that a precondition now exists that would enable toxicity if a second circumstance were to occur. (Note, for example, that while PLGA microspheres [40] and unencapsulated bupivacaine solution [34] injected separately do not cause muscle injury, the two together do [7].) Thus for example, our results do not rule out the possibility that PDLA could be neurotoxic if the concentration of bupivacaine were higher (as it was in the withdrawn IND [10]), more particles were injected, there was an impediment to drug clearance from the site of injection, as might occur in patients with poor peripheral circulation, or there was a pre-existing neuropathy. To avoid local anesthetic-related neurotoxicity, it may be wiser simply to avoid amino-ester and amino-amide-type local anesthetics altogether and only use non-neurotoxic local anesthetics, such as the saxitoxins [5].

Just as it is difficult to make definitive statements regarding some gene expression changes, it is not clear whether it is appropriate to conclude that the recurrent blocks seen here (Suppl. Fig. 1) are not a *forme fruste* of nerve injury on the strength of the fact that there were no (or very few) associated changes in gene expression that we could detect with block recurrence. Our results also do not rule out the possibility that peripheral nerve injury occurred but that its effects in the DRGs were masked by nerve blockade [35], or that changes occurred in the spinal cord or higher in the central nervous system that were not studied here.

Absolute safety is difficult to prove without a biomarker for safety. Toxicogenomic approaches, like all others, can only point to the presence of gene expression patterns associated with toxicity. At the point of clinical care, the patient's own disease states or genetic predispositions may cause an adverse reaction to a formulation that was considered safe in the general population. At the very least, toxicogenomics could provide a rich source of leads for further studies of mechanisms of toxicity that could eventually yield relevant injury markers and point to potential cures. Assuming a meaningful positive control for the anticipated type of injury (in our case, the amitriptyline model) the markers identified by toxicogenomics might facilitate the development of simple and cost-effective screening tools for the toxicity of specific therapies. Eventually, the combination of toxicogenomic information for a given therapy with the genomic profile of individual patients may allow the prediction of potential problems, thus enhancing the safety of drug delivery systems.

5. Conclusions

Toxicogenomic analysis of DRGs in animals receiving PDLA lasting one week did not detect alterations in gene expression consistent with nerve injury; there had been concern that there might be such injury based on the presence of neurobehavioral abnormalities and nearby inflammation and muscle injury. These findings are encouraging regarding the safety of PDLA. Toxicogenomic studies have great potential in delineating patterns of gene expression associated with specific patterns of tissue injury (e.g. as done here with amitriptyline neurotoxicity), and in identifying related changes in gene expression upon exposure to a drug, biomaterial, or drug delivery system. The use of such approaches may accelerate the development and improve the safety of drug delivery systems."

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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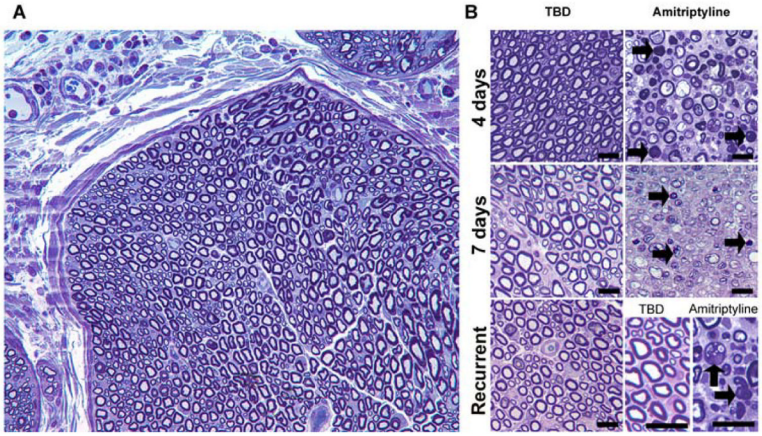

Figure 1.

Representative microscopy of Epon-embedded nerves (400X magnification; scale bar = 25 μm). A. PLGA blank group. B. Animals in the amitriptyline and TBD groups who had sustained nerve blockade, 4 and 7 days after injection (N = 3 per group per time point). In the TBD group (including those with recurrent nerve blockade), nerves appeared completely normal, while nerves in the amitriptyline group showed widespread fiber degeneration with myelin ovoids (arrows). Lower right panel demonstrates high power magnification of representative samples of TBD and amitryptyline treated nerves.

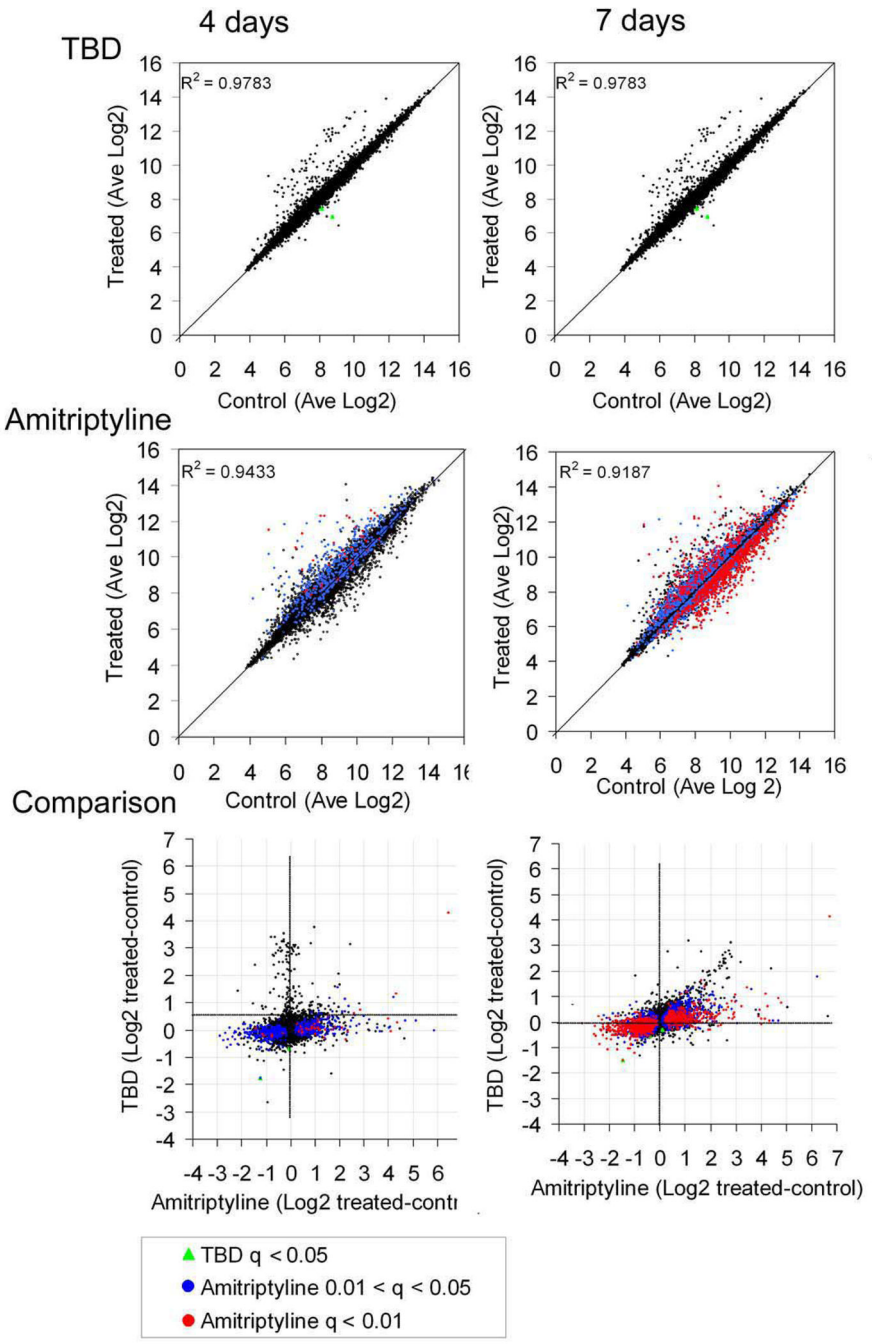

Figure 2.

Changes in gene expression of the TBD and amitriptyline groups. Top four panels: Changes in expression in experimental groups compared to the saline control group. Data points are the log2 of the average of the expression data for each gene. Point colors denote changes in gene expression by group and by q values. The diagonal line through each plot is the line of identity (i.e. where gene expression in the experimental group would be the same as in the saline group). The R^2 values for each data set highlight that the vast majority of genes expressed in the dorsal root ganglia were unaffected by the treatments. N=3 for each group. Bottom panels: Changes in expression in experimental groups compared to each other. Units on the axes are the difference between the experimental groups and saline, expressed as the

log2 of the average of the expression data for each gene. The dotted cross goes through the origin, where there is no change in gene expression.

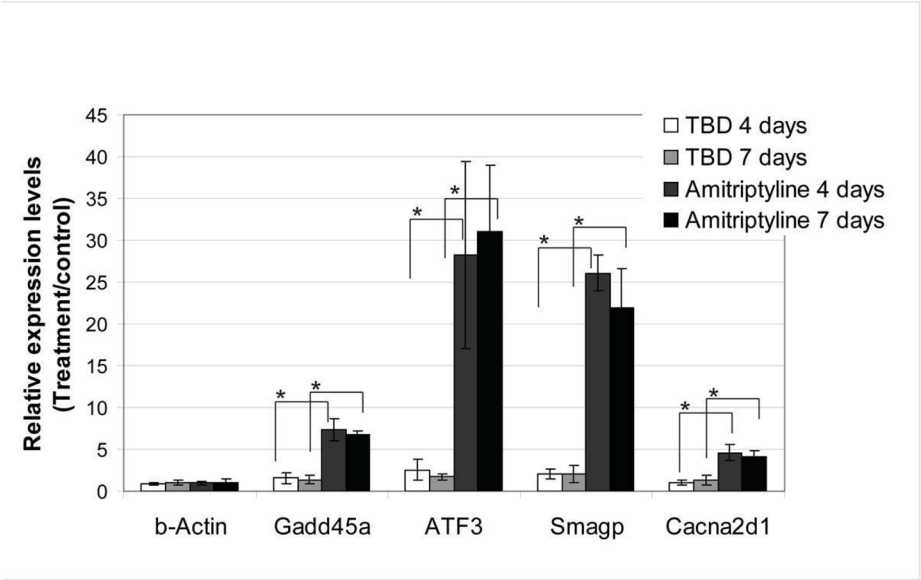

Figure 3.

Real time PCR analysis for the expression of four genes associated with nerve injury, and one (β-actin) that is not. Gene expression is normalized to GAPDH, at 4 (N = 3 per group) or 7 (N = 4) days in the amitriptyline and TBD groups and compared to saline treated animals. Data are means ± standard deviations. Statistical differences between groups were assessed by the Tukey-Kramer test. Asterisk denotes $p < 0.01$ in the comparison between amitriptyline and TBD groups at the same time point.

Table 1

Summary of changes in gene expression

	q ≤ 0.05					q ≤ 0.01				
	Up-regulation		Down-regulation			Up-regulation		Down-regulation		
Magnitude of change (Δ)*	Δ ≥ 2	2 ≥ Δ > 1	Δ ≥ 2	2 ≥ Δ > 1	Percentage of total genes affected‡	Δ ≥ 2	2 ≥ Δ > 1	Δ ≥ 2	2 ≥ Δ > 1	Percentage of total genes affected‡
PLGA alone 4 Days	1	1	0	0	0.01%	1	1	0	0	0.01%
PLGA alone 7 days	50	117	0	0	0.93%	8	17	0	0	0.14%
Amitriptyline 4 days	175	295	154	293	5%	32	23	3	4	0.3%
TBD 4 days	0	0	1	1	0.01%	0	0	1	1	0.01%
TBD 4 days recurrent†	0	0	0	0	0%	0	0	0	0	0%
Amitriptyline 7 days	345	1,300	353	2,786	27%	172	294	270	994	10%
TBD 7 days	0	0	1	3	0.02%	0	0	1	3	0.02%

Data are numbers of genes affected out of 18,000 examined in DRGs of treated animals.

*Change in gene expression compared to saline-treated controls.

†Animals who developed recurrence of nerve block after it had initially resolved.

‡Sum of the up-and down-regulated genes as a percentage of the 18,000 genes examined.

Table 2

Most highly affected genes 7 days after PLGA microsphere injection[§]

Gene ID	Gene symbol	Multiple of change
XM_001076464	Fam111a f	5.76
XM_001068837	Clec1b	5.09
NM_012614	NPY	3.84
NM_012774	Gpc3	3.75
NM_013104	Igfbp6	3.53
NM_001007729	Pf4	3.43
XM_001071146	Dpt	3.17
NM_019143	Fn1	2.41

Data are multiples of change in gene expression in relation to saline-treated controls.

N= 3 animals for each group.

[§]The 8 most up- and down-regulated genes with q<0.01 in animals injected with blank PLGA microspheres.

Table 3A

The most affected genes from amitriptyline and TBD treatments
Most highly affected genes following amitriptyline treatment and corresponding changes in TBD

Up-regulation				
Gene ID	Gene Symbol	Multiple of change following treatment		
		amitriptyline		TBD
		7 days	4 days	
NM_053289	Reg3b	106	87	0**
NM_017134	Arg1	28	17¶	0**
NM_033237	Gal	25	25¶	0**
XM_001081348	Stac2 SH3	20	16¶	0**
NM_012912	ATF3	19	20	0**
NM_057144	Csrp3	18	18.3¶	0**
XM_001056859	Sprr1a	18	13¶	0**
NM_012614	NPY	16	13¶	0**
NM_031659	Tgm1	16	17	0**
NM_182817	Smagp	15	16	0**
XM_001077321	Rasd1	15	20	0**
\NM_00100884	RT1-DA	11	5‖	0**
NM_053349	SOX11	9	9¶	0**
NM_001008515	C1qa	8	5¶	0**
NM_013069	Cd74	8	3‖	0**
XM_001080226	Flrt3	8	8¶	0**
XM_001054004	Sema6a	8	7¶	0**
NM_021776	Ecel	7	6.5¶	0**
NM_013220	Ankrd1	7	9‖	0**
NM_001008524	C1qc	7	4¶	0**
NM_080776	Mmp16	7	5	0**
NM_024127	Gadd45a	6	7	0**
NM_012919	cacna2d1	6	6	0**
XM_001067575	Stmn4	6	5.5¶	0**
NM_001008884	RT1-Db1	6	0	0**

Down-regulation				
Gene ID	Gene Symbol	Multiple of change following treatment		
		amitriptyline		TBD
		7 days	4 days	
NM_052805	Chrna3	8	7¶	0**
XM_001066816	Nmb	6	5¶	0**
XM_001064329	Hoxd1	6	6¶	0**
NM_181370	Hs3st2	6	5¶	0**
XM_001081231	Nog	5	6¶	0**
NM_080688	Plcd4	5	6¶	0**
NM_053954	Kcns1	5	6.5¶	0**
NM_019265	Scn11a	5	5¶	0**
XM_001071922	Ntng1	5	4.5¶	0**
NM_017241	Grik1	5	5¶	0**
XM_001080134	Tmem16c	5	5¶	0**
NM_153734	Cst12	5	4‖	0**
NM_031778	Kcns3	5	4‖	0**
NM_012971	Kcna4	5	4‖	0**
NM_001004263	Itgb6	4	5¶	0**
NM_033352	Abcd2	4	4¶	0**
NM_016996	Casr	4	2¶	0**
XM_001080468	Ppapdc1a	4	3¶	0**
NM_138613	Zfp179	4	4¶	0**
NM_022605	Hpse	4	3‖	0**
XM_001065506	Sorl1	4	3	0**
NM_012852	Htr1d 5	4	6¶	0**
NM_183326	Gabra1	4	3¶	0**
XM_001055608	RGD1565362	4	3¶	0**
XM_001070003	Cpne4	4	4 ¶	0 **

Data are multiples of change in gene expression in relation to saline-treated controls. N= 3 animals for each group.

§ The 25 most up- and down-regulated genes with q<0.01 (unless otherwise indicated) following treatments with amitriptyline, with corresponding change in TBD groups.

¶ $0.01 \leq q \leq 0.05$

‖ $0.05 < q \leq 0.07$

** Data from all TBD treatments (including 4 days, 7 days and the recurrent block group).

†† Genes that were affected in TBD treatments are compared to the effect that was found in the amitriptyline groups.

Table 3B

The most affected genes from amitriptyline and TBD treatments

All changes in gene expression from TBD treatment and corresponding changes from amitriptyline treatment[††]

Down-regulation					
Gene ID	Gene Symbol	Multiple of change following treatment			
		amitriptyline		TBD	
		7 days	4 days	7 days	4 days
XM_001068395	RGD1304982	2¶	3	3	3
NM_147206	Cyp3a9	1.2‖	0	0	1.6
NM_001025771	LOC500118	1.3	1.4‖	1.5	0
XM_001069087	Entpd4	2.3	2¶	1.3	0
NM_012713	Prkcb1	0	0	1.2	0

Data are multiples of change in gene expression in relation to saline-treated controls. N= 3 animals for each group.

[§]The 25 most up- and down-regulated genes with q<0.01 (unless otherwise indicated) following treatments with amitriptyline, with corresponding change in TBD groups.

¶$0.01 \leq q \leq 0.05$

‖$0.05 < q \leq 0.07$

[**]Data from all TBD treatments (including 4 days, 7 days and the recurrent block group).

[††]Genes that were affected in TBD treatments are compared to the effect that was found in the amitriptyline groups.