Intraoperative abobotulinumtoxinA alleviates pain after surgery and improves general wellness in a translational animal model

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Abstract

Pain after surgery remains a significant healthcare challenge. Here, activity of abobotulinumtoxinA (aboBoNT-A, Dysport®) was assessed in a post-surgical pain model in pigs. Full-skin-muscle incision and retraction surgery on the lower flank was followed by intradermal injections of either aboBoNT-A (100, 200, or 400 U/pig), vehicle (saline), or wound infiltration of liposomal extended-release bupivacaine. Animals were assessed for mechanical sensitivity, distress behaviors, latency to approach the investigator for 5 days following surgery. Immunohistochemical analyses of total and cleaved synaptosomal-associated protein-25 kD (SNAP-25) and pain-related biomarkers were performed in the aboBoNT-A-400 U and saline groups. At Day 1, partial reversion of mechanical allodynia was achieved in aboBoNT-A groups with full reversion from Day 3. Reduced distress and normalized approaching responses were observed with aboBoNT-A from 6 h post-surgery. Bupivacaine reversed mechanical allodynia for 24 h after surgery but did not affect distress or approaching responses. Cleaved SNAP-25 was absent in the skin and dorsal root ganglia but present in the ipsilateral dorsal horn of aboBoNT-A-injected animals. A decrease in some pain-related biomarkers was seen with aboBoNT-A versus saline. Clinical investigation to support the use of aboBoNT-A as a tolerable and efficacious analgesic drug for post-surgical pain, is warranted.

Introduction

Pain after surgery remains a significant healthcare challenge as a significant number of patients continue to suffer from moderate to severe pain, some of them for a prolonged period [1,2]. Pain after surgery can be accompanied by anxiety, depression, elevated stress, and catastrophizing, which can enhance acute pain after surgery, facilitate its chronicity and interfere with postoperative recovery and rehabilitation. The management of pain after surgery, especially severe pain, continues to rely heavily on opioid drugs, despite associated acute side-effects, slower healing and risk of addiction [3]. There is an urgent need for novel, non-opioid, analgesic drugs that are tolerable and provide effective, prolonged relief from pain after surgery.

Botulinum toxins (BoNTs) are a diverse group of proteins produced by *Clostridium botulinum* and related bacteria [4]. In nature, they are synthesized as a single-chain polypeptide that is modified post-translationally to form two polypeptide chains, a 100-kDa heavy chain (HC) and a 50-kDa light chain (LC), which are connected by a disulfide bond [5]. Upon binding to cell-surface receptors, the molecule is internalized via an endosome, followed by translocation of the LC into the cytosol. The LC cleaves intracellular transport proteins known as SNARE proteins and inhibits cellular secretion from the target cell, to impart inhibitory effects on synaptic transmission [5]. For several decades, BoNT-type A (BoNT-A) preparations have been used in esthetic as well as therapeutic applications, providing effective and long-lasting relief for patients with muscle hyperactivity disorders. More recently, BoNT-A has been approved for chronic migraine paralleled with an accumulated evidence on its efficacy in a range of pain conditions

in the clinic and in animal models [6,7]. Here, the analgesic activity of abobotulinumtoxinA (aboBoNT-A; Dysport®) was evaluated using a model of post-surgical pain in domestic pigs [8].

Post-surgical pain in pigs [8] is a translational animal model based on several considerations. Firstly, there is a remarkable similarity between the pig and the human skin in terms of its structure, innervation, immune response and post-incisional healing [9,10]. Behavioral changes seen in pigs following full-skin and muscle incision and retraction (SMIR) surgery (that mimics operations performed in the clinic), are relevant to evoked pain, non-evoked, resting pain and pain-related anxiety- and depression-like reactivity [8,11]. Mechanical sensitivity and guarding behavior are considered surrogates for evoked, movement-related pain and non-evoked, resting pain, respectively, which are seen in both human patients after surgery [12,13] and in a rodent model [14]. Reduced social interaction, withdrawal when approached as well as reluctance to initiate approach of a caregiver, on the other hand, can be considered as surrogates for post-surgical pain-mediated anxiety or depression seen in human patients [15] and in rodent models of post-surgical pain [16,17].

We hypothesized that intraoperative aboBoNT-A treatment would be tolerable and provide effective and prolonged relief from post-SMIR surgical pain compared with its vehicle (saline) only or infiltrated liposomal bupivacaine (Exparel®; an approved, slow-release anesthetic).

Results

Von Frey test

At baseline, one day before the surgery (Day-1), all animals responded to the WF of 60 g, therefore no animals were excluded from the study. One hour following surgery, WF of the saline group decreased to 1 to 2 g and remained low throughout 5 days following surgery, with slow and mild recovery to 5.0 ± 0.86 g on Day 5 (Fig. 1A).

AboBoNT-A increased WF dose-dependently in comparison to saline treatment (Fig. 1A). Specifically, animals injected with 400 U aboBoNT-A showed a marked and significant reversal of allodynia on day 1, followed by similar effects seen in the 200 U and 100 U aboBoNT-A groups from days 2 and 3, respectively (Fig. 1A). A full recovery to baseline sensitivity following aboBoNT-A treatment was reached 4 to 5 days after injection (Fig. 1A). Liposomal bupivacaine fully reversed mechanical allodynia up to 2 h post-administration, followed by a partial reversal 4 and 6 h post-administration. From Day 1, the WF of bupivacaine-injected animals continued to decline on days 1 and 2 (Fig. 1A). Liposomal bupivacaine was inactive in WF from Day 3 (Fig. 1A).

The number of animals showing WF of ≥ 26g (considered as a normal mechanical sensitivity) was significantly influenced by treatment. Specifically, in the aboBoNT-A 100 U group, only one animal showed these responses on Day 4, followed by three animals by Day 5 (Fig. 2A). In the aboBoNT-A 200 U group, three, five and five animals showed these responses on days 3, 4 and 5, respectively. While one animal

showed this response on Day 1 in the aboBoNT-A 400 U group, all 6 animals did so on days 5 and 6 (Fig. 2A). The opposite pattern was seen in liposomal bupivacaine-injected animals. Specifically, while WF responses of all 6 animals were ≥ 26 g 1 and 2 h after the surgery, only four and two animals showed normal sensitivity at 4 and 6 h after surgery, respectively (Fig. 2A). On Day 1 (24 h after surgery), WF ≥ 26 g was detected in only one animal in the liposomal bupivacaine group, with allodynia detected from day 2 in all six animals (Fig. 2A).

DBS test

At baseline, one day before the surgery (Day-1), animals exhibited no signs of distress. After the SMIR, however, distress behaviors emerged; animals began exhibiting guarding behavior as they protected the site of the incision, moving away when approached by the investigator (category scorings 3 and 4; see Materials and Methods). The DBS of the saline group remained high up to Day 3 after the surgery, followed by a gradual decline on days 4 and 5 (Fig. 1B). Animals injected with aboBoNT-A showed significant and sustained reductions in DBS when compared with the saline group between days 1 and 3 without a clear dose-dependency (Fig. 1B). In contrast to aboBoNT-A, liposomal bupivacaine showed lack of efficacy in the DBS test throughout the study (Fig. 1B).

While the day before the surgery all animals lacked distress, 1h after the surgery distress scores of ≥ 2 predominated in all groups (Fig. 2B). Between 2 and 6h after the surgery, there was a steady increase in the number of animals with no distress in groups treated with aboBoNT-A, but not in those treated with saline or liposomal bupivacaine (Fig. 2B). Starting D1, behavior of virtually every animal treated with aboBoNT-A is normal, while on D1-D3 the vast majority of saline- and liposomal bupivacaine animals continue exhibiting distress corresponding DBS of > 2 (Fig. 2B). On D5 all animals were distress-free (Fig. 2B).

Approaching test

On the day before the surgery (Day − 1), there were no significant differences in approaching time across treatment groups, as mean values of all groups were 60–90 sec across groups (Fig. 1C). On Day 0 at 2 h after surgery, mean approaching time increased similarly across groups to greater than 130 s. At 6 h, animals injected with aboBoNT-A 200 U and 400 U were significantly faster to initiate approach than saline- and liposomal bupivacaine-injected animals (Fig. 1C). On Day 1, animals injected at 200 U aboBoNT-A were significantly faster to approach the investigator than saline- or liposomal bupivacaine-injected ones, while those injected at 400 U were faster than liposomal bupivacaine-injected animals (Fig. 1C). On days 2 and 3, all aboBoNT-A treatment groups exhibited significantly shorter approach latencies in comparison to the saline group (Fig. 1C). On days 4, 5 and 6, approaching latencies of animals injected with aboBoNT-A 200 U remained shorter than those injected with saline (Fig. 1C).

On the day before surgery (Day − 1), all but one animal approached the investigator within 90 s in each group (Fig. 2C). On Day 0 at 2 h after the surgery and treatment, no animals in the aboBoNT-A 200 U, saline- or bupivacaine groups reached this threshold, while one animal and two animals, respectively, injected with aboBoNT-A 400 U and 100 U, did approach within 90 s (Fig. 2C). At 6 h and on days 2 and 3,

between four and six animals injected with aboBoNT-A 200 U and 400 U approached the investigator within 90 s, whereas only one animal per group reached this threshold in the saline- and bupivacaine-injected groups (Fig. 2C). On days 3–6, all but one of 18 animals (94%) injected with aboBoNT-A approached the investigator within 90 s, whereas this number was two to three animals in the saline group (33–50%) and two to four animals in the bupivacaine group (33–67%; Fig. 2C).

Open field test

Locomotor activity in the Open field test was similar at Day 3 across all treatment groups for total distance travelled, the speed of locomutation and as the percentage of time spent in the central zone (Fig. S1 A-C). In the latter measure, there was a trend of a higher proportion of time spent in the center by animals injected with aboBoNT-A 400 U compared with other groups (Fig. S1 C).

Body weight

All groups showed a comparable body weight growth during the study. There was no difference in absolute body weight as well as in percent body weight gain across aboBoNT-A-, saline-, and liposomal bupivacaine-injected groups on days − 5, 0 and 5 (Table S1).

Would inflammation

Daily clinical evaluation of would inflammation showed that swelling was absent in all animals, whereas the levels of redness were rare and mild. There was no effect of treatment on the frequency or intensity of redness across groups (Fig. S2).

Histopathology and IHC analyses

H&E examination of the skin cross-section samples that were collected on Day 6 revealed similarity in wound healing and inflammation in aboBoNT-A- and saline treated animals (Fig. S3). In addition, image analyses of CD31 IHC from the dermis around the incision area revealed similarity in blood micro vessel density (Fig. S4 A-C). The similarity in blood vessel size distribution in aboBoNT-A and saline-treated animals was confirmed by a quantitative analysis (Table S2).

In both aboBoNT-A and saline-treated animals the total SNAP-25 immunolabelling (N-terminal part) was detected in the skin adjacent to the injection site (Fig. 3). Specifically, the total SNAP-25 immunolabelling was detected in nerve endings around small arteries, in arrector pili muscles and in nerves in the deep and superficial dermis (Fig. 3A–C). In contrast, the same area of the skin was devoid of cleaved SNAP-25 in both aboBoNT-A and saline-treated animals (Fig. 3D–F).

Cleaved SNAP-25 was absent in the dorsal horn of the lumbar spinal cord of saline-treated animals, while being abundant in the corresponding area of aboBoNT-A-treated animals (Fig. 4). Specifically, in vehicle-treated animals cleaved SNAP-25 was absent in both the ipsilateral (Fig. 4A, B) and contralateral dorsal horn (Fig. 4C, D) of the lumbar spinal cord. In contrast, intense synaptic- and dendritic-like staining of cleaved SNAP-25 was detected focally in the lateral part of the dorsal horn ipsilateral to the injection (Fig. 4E, F), while mild staining was detected in the lateral part of the dorsal horn contralateral to the injection

(Fig. 4G, H). Quantification of cleaved SNAP-25 in the ipsilateral dorsal horn rostro-caudally in the lumbar spinal cord revealed its high intensity at the L5-L6 sections whereas milder staining was detected in more rostral sections (L1-L2 and L3-L4) of the spinal cord (Fig. 4I).

We found a high level of GFAP staining throughout the lumbar spinal cord of saline-treated animals (Fig 5A, B). Specifically, GFAP staining was found to be diffuse and bilateral, with a particularly high level staining in the upper lamina of the dorsal horn (lamina I and II) and around the central canal (Fig. 5A, B). In comparison to vehicle-treated controls, aboBoNT-A-treated animals showed a marked decrease in GFAP staining which was diffuse and bilateral (Fig. 5C, D). This reduction in GFAP staining was particularly robust in both ipsilateral and contralateral dorsal horns (lamina I and II) and around the central canal of the spinal cord (Fig. 5C, D). Rostro-caudally, reductions of GFAP staining in the lumbar spinal cord were observed in all sections with the highest magnitude in L3-L4 and L5-L6 sections (Fig. 5E).

We found a high level of Iba1 staining in the grey matter of the lumbar spinal cord in animals injected with saline (Fig. 6A, B). Specifically, in these animals Iba1 staining was found to be diffuse and bilateral, with a particularly high level staining in a focal point of the lateral part of the ipsilateral dorsal horn of the L5-L6 spinal cord sections (Fig. 6A, B, arrowheads). In comparison to vehicle-treated controls, aboBoNT-A-treated animals showed a moderate decrease in Iba1 staining which was diffuse and bilateral (Fig 6C, D). A focal point of intense iba1 immunolabelling noted in vehicle-treated animals was fully absent in aboBoNT-A animals (Fig. 6C, D; E). There was no significant difference in Iba1 immunostaining in L1-L4 spinal cord sections between saline and AboBoNT-A injected animals (Fig. 6E).

We detected high level of CGRP staining bilaterally in the upper lamina of the lumbar spinal cord, which was similar in saline- and aboBoNT-A-treated animals (Fig. 7 A,B). We also found a moderate level of SP staining bilaterally in upper lamina of the dorsal horn of the lumbar spinal cord (Fig. 7 C, D). There was no difference in SP staining between aboBoNT-A and saline-treated animals neither in the dorsal horn (Fig. 7 C, D).

In the DRG emerging form the L4-L5-6 levels of the lumbar spinal cord we detected lack of cleaved SNAP-25 in both saline- and aboBoNT-A-treated animals (Fig. 8 A,B). We also detected intense GFAP, Iba-1, CGRP and SP staining in the same DRGs (Fig. 8 C-J). There was no difference in staining of these markers between saline- and aboBoNT-A-treated animals (Fig. 8 C-J).

Discussion

This study demonstrated that intraoperative administration of aboBoNT-A provided effective and prolonged pain relief related to evoked pain, non-evoked pain and pain-associated anxiety- and depression-like reactivity in a translational, post-surgical domestic pig model. We saw a marked post-operative reduction of withdrawal force from 60 g in all healthy animals to less than 2 g in animals after surgery, indicative of allodynia (mechanical hypersensitivity). AboBoNT-A treatment was associated with dose- and time-dependent, enduring reversal of SMIR-induced allodynia from 24 h after administration.

Specifically, a robust, ≥ 30% reversal was seen from days 1, 3 and 4 onwards in animals injected with aboBoNT-A 400 U, 200 U and 100 U, respectively. By Day 5, allodynia of all aboBoNT-A-injected animals approached the pre-operative baseline WF of 60 g. Furthermore, aboBoNT-A dose-dependency in mechanical sensitivity was also detected when assessing the number of animals with normal mechanical sensitivity (i.e. withdrawal responses of 26g or above) compared with those with allodynia (i.e. withdrawal responses less than 26g) per group. The results obtained in pigs confirm and expand an earlier evidence of the analgesic activity of BoNTs on evoked pain-related responses in rodent models of postsurgical pain. For example, an intraplantar administration of 3.5 and 7 U/kg, but not of 1 U/kg of onabotulinumtoxinA (onaBoNT-A) in rats resulted in similar, near-maximal and enduring (up to 10 days) reversal of mechanical hyperalgesia induced by the gastrocnemius muscle cut [18]. Also, onaBoNT-A given via intraplanar or intrathecal routes, 24 or 48 h before the surgery, respectively, reversed allodynia for 5 days in the rat (“Brennan”) model of post-surgical pain [19].

AboBoNT-A treatment was also associated with reduced post-operative distress behaviors in pigs (wound guarding, reduced social interaction and withdrawal when approached) suggesting its efficacy in non-evoked, resting pain. These effects preceded those of mechanical sensitivity, as they were detected 6 h after surgery. The effects of BoNT-A on measures of non-evoked, resting pain have been described in other models of post-surgical pain. For example, in rats, intraplantar or intrathecal injection of onaBoNT-A significantly reduced the cumulative post-surgical pain score – a measure of non-evoked pain based on the position of the operated paw in relation to the cage floor [19]. Also, aboBoNT-A reduced non-evoked postoperative pain and need for rescue analgesia in dogs undergoing radical bilateral mastectomy [20]. Overall, the analgesic efficacy of aboBoNT-A in the post-surgical pain pig model is well aligned with few examples of its use in the clinic. For example, in a retrospective clinical study conducted in patients undergoing mastectomy, 100 U of onaBoNT-A administered via intra-operative muscle infiltration significantly reduced immediate postoperative pain, rescue analgesia and pain associated with the expander breast reconstruction [21]. In a multicenter prospective clinical study conducted in pediatric patients undergoing lower extremity limb lengthening and deformity correction, intramuscular BoNT-A injection immediately before surgery reduced post-operative pain for 4 days, reduced the need for rescue medication and improved quality of life [22].

Here, for the first time, we provide evidence that peripherally administered BoNT-A reduces pain-mediated anxiety- or depression-like reactivity in an animal model. AboBoNT-A effects were seen in some distress behaviors (withdrawal when approached, reduced sociability) as well as in the outcome of the Approaching test. Specifically, intraoperative aboBoNT-A injection reduced approaching responses starting 6 h after treatment, resolving behavioral conflict, normalizing social bonding and enabling further socialization as detected in further shortening of approach latencies in aboBoNT-A-injected animals compared with the saline-injected group.

Considering the importance of social interaction for the animal, behavioral changes observed following aboBoNT-A treatment may be considered a surrogate for improved wellbeing in humans. Supporting

translational validity of these data, there is growing clinical evidence of the antidepressant-like activity of BoNT-A, although whether it is a direct or indirect effect remains to be investigated. For example, in a randomized, double-blind, crossover clinical study and in a subsequent pooled analysis, BoNT-A injected in the forehead region improved symptoms of depression in patients with major depressive disorder [23,24].

Importantly, a recent review of 40,000 post marketing safety reports in the FDA Adverse Event Reporting System showed that patients who received BoNT injections for symptomatic treatment in a range of conditions (spasticity, spasms, hyperhidrosis, migraine) and for esthetics, reported a significantly lower number of depression reports regardless of the injection site, compared with patients undergoing different treatments for the same conditions [25]. These findings suggest that while the facial feedback and emotional proprioception hypothesis of the antidepressant activity of BoNT [26] is plausible, anxiolytic or antidepressant effects of BoNT are mediated by more complex central mechanisms, involving the spinal cord and the brain (see below). We acknowledge that there are several differences in experimental variables between the current translational study and the clinical studies reviewed by Makunts et al. [25]. However, we hypothesize that the neuroanatomical substrate mediating anxiolytic and/or antidepressant-like effects of BoNT-A associated with ongoing pain in pigs as well as those detected in human patients, are fundamentally the same.

All doses of aboBoNT-A (100, 200 and 400 U) were well tolerated; animals showed normal weight gain and locomotor activity, and a complete lack of systemic or local adverse effects. The level of wound inflammation was low and similar across treatment groups and there was no treatment effect on wound healing or microvasculature. Thus, the effects of aboBoNT-A on pain-related behavioral responses described here were not confounded by adverse effects or by differences in would inflammation, healing process or granulation tissue formation, suggesting that aboBoNT-A has a specific analgesic efficacy.

Immunohistochemical detection of cleaved-SNAP-25 was used in the present study as an indirect method to detect the enzymatic activity of aboBoNT-A, in order to identify potential sites of its activity, as performed previously in rodent studies [27–31]. This is the first study where cleaved SNAP-25 was investigated following a specific, intradermal administration of BoNT-A. We harvested the tissue from the experimental animals at the end of the study (Day 6), making it impossible to investigate earlier time points, which is a limitation of HC investigations.

The total, uncleaved SNAP-25 was clearly present in the skin adjacent to the injection area, specifically in nerve endings around small arteries, in arrector pili muscles and in nerves in the deep and superficial dermis in both aboBoNT-A- and saline-injected animals; interestingly, cleaved SNAP-25 was absent from these structures. This suggests that analgesic activity, which was driven by aboBoNT-A, at least near the end of the study, did not require the release of neuromediators in the skin and therefore, was not dependent on peripheral mechanisms. AboBoNT-A enzymatic activity was detected in the central nervous system, as indicated by the presence of cleaved SNAP-25 in the ipsilateral dorsal horns of the lumbar spinal cord of aboBoNT-A-injected animals and absence from these sites in saline-injected controls. Indeed, cleaved SNAP-25 staining, which appears to be synaptic-like and dendritic-like, was observed in

almost all L5-L6 spinal cord sections, which receives the somatosensory innervation from the incision area of the skin and located in the lateral part of the ipsilateral dorsal horn. A minimal cleaved-SNAP-25 staining was noted in the same regions of the contralateral dorsal horn of aboBoNT-A-injected, but not saline-injected animals. The contribution of the injected area in distribution of cleaved-SNAP-25 in the lumbar spinal cord was further supported by its anatomical rostro-caudal distribution, as this was weaker at L1-L2 and L3-L4 levels. Cleaved SNAP-25 staining was absent around motor neurons in ventral horns and in all dorsal root ganglia on both ipsilateral or contralateral sides. This suggests that intradermally injected aboBoNT-A taken up specifically by sensory neuronal terminals around the incision undergoes retrograde axonal transport from the injected dermis to the upper lamina of the dorsal horn via the DRGs [32,33]. In rats, intramuscular injections of a low dose of onaBoNT-A (5U/kg), or high doses of onaBoNT-A administered via intramuscular, subcutaneous or intra-nerve routes results in cleaved SNAP-25 in the ipsilateral dorsal horn of the spinal cord [34].

Spinal microglia and astrocytes have been known to react to a range of peripheral insults, including incision, and are known to contribute to post-surgical pain [35–38]. Pain biomarkers GFAP and Iba1 were assessed as markers of astrocyte [39] and microglial [40,41] activation, respectively. Unilateral administration of intradermal aboBoNT-A injections resulted in a robust bilateral reduction in the GFAP immunolabelling and milder bilateral reduction in the Iba1 immunolabelling. Based on these findings, we hypothesize that the analgesic efficacy of aboBoNT-A in post-surgical pain is mediated, in part, by the reduction of astrocyte and microglial activation in the spinal cord. The effect on GFAP was diffuse in both the white and grey matter, but especially marked in the lamina I and II of the ipsilateral and contralateral dorsal horns and around the central canal. As seen with cleaved SNAP-25, effects of aboBoNT-A on GFAP was characterized with anatomical specificity – robust reduction at the L5-L6 levels, milder reduction at L3-L4 and no effect at L1-L2. These findings confirm and expand earlier findings on bilateral effects of BoNT following unilateral administration. Our own work [42,43] and those by others [44] showed that unilateral injections of aboBoNT resulted in bilateral reduction in mechanical hyperalgesia in rodent models of polyneuropathic pain, mirror pain or bilateral inflammatory hyperalgesia. Similarly, a moderate, bilateral decrease in Iba1 staining was only noted in the L5-L6 grey matter of aboBoNT-A-injected animals in comparison to those in saline-injected controls. The decrease was particularly marked in the lateral part of the ipsilateral dorsal horns compared to those in saline-injected pigs.

We found no difference in CGRP and substance P immunolabelling between aboBoNT-A- and saline-injected animals both in the lumbar spinal cord and DRG. This suggests that analgesic activity of aboBoNT-A does not involve reduction in CGRP or substance P expression in the spinal cord and DRG. These findings are well aligned with earlier evidence that CGRP may not play a significant role in post-operative pain. According to Ishida et al. [45], mice deficient in the α isoform of CGRP and wild-type controls exhibited similar mechanical sensitivity in the von Frey test following plantar incision. Unlike CGRP, there is evidence suggesting the role of substance P in post-surgical pain. For example, mice with a deletion of pre-protachykinin A gene (which codes for substance P) displayed reduced incision-induced mechanical allodynia and heat hyperalgesia in comparison to wild-type controls [38]. Also, according to

Chen et al. [46] SMIR-stimulated increase in SP concentration in DRG can be detected both 14 and 28 days after surgery. Thus, analgesic activity of aboBoNT-A in the pig appears to bypass the spinal substance P. Alternatively, the effect of aboBoNT-A on spinal substance P is present at earlier time-points (before 6 days) than measured in this study. Additional studies are needed to investigate these hypotheses.

It is a limitation of the current study that aboBoNT-A was administered at a single timepoint, intraoperatively, and only using intradermal route of administration. A systematic head to head assessment of subcutaneous, intramuscular and intradermal routes of BoNT-A administration is needed using the current model, in order to optimize the injection conditions before the approach is evaluated in a clinical study. Similarly, the timing of BoNT-A injection may influence the latency of onset of analgesic efficacy of BoNT-A in post-surgical pain. We can speculate that administration of BoNT-A before the surgery will result in a faster onset of its analgesic efficacy. Systematic evaluation of the timing of the injection in the current model will provide another valuable information on optimization of aboBoNT-A injection conditions. Another limitation of the study is that we could only collect the tissue for cleaved SNAP-25 and biomarker analysis on day 6. It is possible that the profiles of cleaved SNAP-25 and pain-related biomarkers is different at earlier time-points.

In conclusion, in a pig model of post-surgical pain, a single intraoperative treatment of aboBoNT-A via intradermal injections resulted in effective and prolonged relief of evoked and non-evoked pain. In addition, effective reduction of pain-associated anxiety/depression-like reactivity was also demonstrated. The analgesic effect of aboBoNT-A does not seem to require peripheral activity and appears to be driven largely by its activity in the spinal cord. These data support further studies using the current model in order to optimize aboBoNT-A injection conditions and subsequent clinical evaluation of aboBoNT-A as a novel option for the effective, multimodal relief of post-surgical pain.

Materials And Methods

Study design

The study was conducted with five treatment groups (N = 30; n = 6 per group). The sample size was decided based on multiple previous studies evaluating efficacy of analgesic drugs in this model [8]. The primary outcome measure that was used to determine the sample size was mechanical sensitivity measured in the von Frey test (see below; [8]). Animals were habituated before surgery (Day 0) as described previously [8]. We performed SMIR instead of a full-skin incision, in order to obtain more pronounced signs of evoked and non-evoked pain (*8*) and to better mimic operations performed in the clinic [11]. Following incision closure immediately after surgery, pigs received i.d. injections of either aboBoNT-A 100, 200 or 400 U/pig saline as a negative control or liposomal extended-release bupivacaine as a positive control. Three animals were housed per pen, and all received the same treatment. All animals were assessed by the same investigator, blinded to treatment allocation throughout the study. Behavioral assessment involved a standard battery of tests which included von Frey (for mechanical

sensitivity), Approaching, and Distress Behavior Score (DBS) tests, performed in the home pen (see 'Assessments' and Table S3). Mechanical sensitivity and guarding behavior are considered surrogates for evoked, movement-related pain and non-evoked, resting pain, respectively, which are seen in both human patients after surgery [12,13] and in a rodent model [14].

The Approaching then the DBS test were performed on all three animals housed in a pen simultaneously, followed by the von Frey test performed on individual animals. Assessments were performed the day before surgery (Day − 1) to obtain a baseline in each measure and on days 0 to 6 (Table S3). On the day of the surgery (Day 0), full and shortened battery (DBS and von Frey testing only) were performed twice each (at 2 h and 6 h, and at 1 h and 4 h, respectively post-surgery; Table S3). From Day 1, behavioral tests were performed in the morning, at approximately 8:30 am (at least 1 h after the morning feed). The Open field test was performed on days − 1 (habituation) and 3. Wound inflammation was evaluated daily. Animals were weighed on days − 5 and 5 at the end of behavioral testing, and before surgery and testing on Day 0. On Day 6 and after the final Approaching test and incision inflammation scoring, animals were euthanized with an intraperitoneal injection of sodium pentobarbitone (> 100 mg/kg). Tissues were then collected from animals in the aboBoNT-A 400 U and saline groups, for immunohistochemical (IHC) analysis. Experimental procedures were reviewed and approved by the MD Biosciences Institutional Animal Care and Use Committee (Ness Ziona, Israel). Studies were performed in full compliance with guidelines of the Israel National Ethics Committee, Committee for Research and Ethical Issues of the International Association for the Study of Pain [47], ARRIVE guidelines and the US National Research Council Guide for the Care and Use of Laboratory Animals.

Animals

Young (8–10 weeks old), male Danish Landrace x Large White cross-bred castrated pigs, were provided by Lahav Labs (Negev, Israel). All animals were drug or test naïve and in good health as confirmed by an attending veterinarian. Animals were acclimated for 5 days before the experiment. Throughout the acclimatization and testing period animals were housed in smooth-walled pens (140 × 240 cm), three per pen and maintained on a 12 h light/dark cycle (lights on from 07:00 to 19:00 h) under a constant temperature (21 ± 2°C) and humidity (55 ± 5%).

Food (Dry Sows; Ct # 5420; Milobar; Oshrat, Israel) was provided twice daily and water *ad libitum*. Animals were provided enrichment to root and chew on, and were given a unique identification ear mark in the form of a four-digit number. Animals weighed between 11 and 13 kg at the start of the habituation period.

Surgery

The anesthesia and the surgery were performed as described previously[8]. In the morning of the day of the surgery (Day 0), pigs were anesthetized with a 5% isoflurane/oxygen (2–3 L/min) mixture with the aid of anesthetic facemask (Stephan Akzent Color, Gackenbach, Germany). The anesthesia permitted relatively quick recovery for the assessment of behavioral responsiveness 1 h after the surgery/treatment. An

incision was made on the left side of the lower back, approximately 3 cm lateral and parallel to the spine (Fig. S4). Before administering the incision, the area of the low back was scrubbed with antiseptic liquid (Polydine® solution, Dr. Fischer laboratories, Bnei Brak, Israel) and wiped with 70% ethanol. A 7-cm long, full skin thickness incision was made, including the fascia and the underlying muscle (gluteus medius muscle) which was retracted to perform the incision. A sterile cover, disinfected with an antiseptic liquid (3% synthomycine), was placed around the incisional area. The fascia was sutured with 3 – 0 vicryl thread, while the skin was sutured with 3 – 0 silk thread (Assault UK Ltd., West Yorkshire, UK) using continuous suturing methods. Following the incision closure, animals received study treatment (described below) and an intramuscular injection of an antibiotic (10% w/v Marbocyl®; Vetoquinol UK Ltd., Buckingham, UK) into the neck. The animals were then returned to their pens for recovery and observation.

Drug administration

AboBoNT-A was provided by Ipsen Limited (Wrexham, UK). Each vial containing 500 U of purified *C. botulinum* type A neurotoxin complex was reconstituted in saline (0.9% sodium chloride) to obtain the final concentration. AboBoNT-A (100, 200 or 400 U/pig) or saline were administered in 2 mL total volume split into 10 intradermal injections (0.2 mL/site) around the incision (Fig. S4) using 30 G needles attached to 1-mL syringes (BD Micro Fine Plus; Becton Dickinson, Plymouth, UK). Liposomal extended-release bupivacaine (EXPAREL®) was provided as an injectable suspension for infiltration (266 mg/20 mL) by Pacira Pharmaceuticals, Inc., (Parsippany, NY, USA). Liposomal bupivacaine (26.6 mg/pig) was infiltrated in the incision as a 2-mL fixed volume using 21-G needle attached to 5-mL syringe.

Assessments

von Frey test

Evaluation of mechanical sensitivity with von Frey filaments was conducted as described previously [8]. Von Frey filaments (Ugo Basile, Gemonio, Italy), which ranged from 1.0 to 60.0 g (Table S4), were applied approximately 0.5 cm proximal to the incision line three times with interval of 5 to 10 seconds. Withdrawal reaction was considered if the animal moved away from the stimulus or twisted its flank; the accompanying vigorous tail wiggling was not considered a withdrawal reaction when independent. The von Frey test was performed while the pigs were fed by a dedicated investigator previously familiarized with the animal. If withdrawal was not achieved, a thicker filament was applied. If withdrawal by the animal from the filament did occur, a thinner filament was applied. Animals were pre-exposed to the von Frey test two days before the surgery (Day – 2) to habituate them to the procedure and baseline WF was recorded on Day – 1. Animals were excluded from the study if their WF responses at baseline were below 26 g.

DBS test

The DBS test was performed to measure non-evoked, resting pain after surgery[8]. Expression of 14 behaviors were monitored, divided into seven categories, which are either specific to post-surgical period (behaviors with the score 1) or exhibited by normal, intact animals (behaviors with the score 0; Table S5). The total DBS is the sum of the scores on each category and can range from 0 (normal) to 7 (very distressed). Assessment of DBSs were not performed in a particular order, but were recorded as exhibited by the animal. The DBS test lasted approximately 3 minutes.

Approaching test

The Approaching test is used to measure non-evoked pain-related anxiety- or depression-like reactivity. The basis of the Approaching test is a social bond that young pigs establish with their caregiver as a result of their daily interactions [48]. When the familiar caregiver enters their home pen, young, habituated animals are quick to approach them; the young pigs are slow to approach unfamiliar individuals. Following SMIR, however, seeking social interaction with the caregiver is replaced with a behavioral conflict between seeking and avoidance responses causing approach latencies to increase. We measured the latency (time in seconds) to approach the investigator entering their home-pen using a cut-off time of 180 s.

Open field test

The Open field test was performed as previously described[49]. Briefly, animals were placed individually into a rectangular arena (2.5 × 4.7 × 1.6 m) and monitored for locomotor activity for 5 minutes with a CCTV camera. The data were analyzed with AnyMaze software (Stoelting Co, Dublin, Ireland). The total distance travelled (m), speed of locomotion (m/s) and the percentage of time spent in the center of the area were quantified.

Wound inflammation

Wound inflammation was scored based on two categories: redness (0- normal; 1- slight redness at the area of the incision; 2- spread redness) and swelling (0, no swelling; 1, slight swelling; 2, pronounced swelling). The final score for each animal is the sum of the scores in each category (max score = 4). The wound inflammation was scored 6 h after the surgery on Day 0 and once daily on days 1 to 6.

Histopathology and Immunohistochemistry

Tissue sampling

The entire cutaneous incision site (9 × 2 × 1 cm; n = 6/group) and the entire lumbar vertebral column (with the spinal cord and DRGs; n = 3/group) were collected from animals injected with 400 U aboBoNT-A or saline. The tissues were fixed in 4% formalin for 48 h. For the skin, a transversal section was made at the middle of the cutaneous incision. The lumbar spinal cord was isolated and separated in 3 equal parts (L1-L2, L3-L4 and L5-L6). Each part was sectioned in about 10 tissue sections of 5 mm each (i.e. 10 spinal cord sections per cassette). Dorsal root ganglia emerging from L4/L5/L6 were sampled on both

sides. All the tissue samples were processed to paraffin blocks. Four micrometer sections were stained with hematoxylin and eosin (H&E) and paraffin slides were produced for immunohistochemical (IHC) analyses.

Wound healing was evaluated by a pathologist (SL) using H&E sections. CD31 (ab28364; Abcam) IHC and image analyses on full tissue sections (Nanozoomer, Hamamatsu, Japan) at the level of the deep granulation tissue (layer 1), superficial granulation tissue (layer 2) and in the surrounding superficial dermis (adjacent to healing part/layer 3; suppl. Figure 3) were used to quantify vessel density (number of blood vessels/mm^2) and size (blood vessel surface area) with the aid of the Object colocalization module (HALO™ system, Indica labs, Albuquerque, NM, USA).

IHC analyses of synaptosomal-associated protein, 25 kD (SNAP-25) and pain-related biomarkers

The IHC analyses were performed using a standard streptavidin–biotin–peroxidase procedure and specific heat mediated antigen retrieval methods. Endogenous peroxidase was blocked for 10 minutes in a 3% hydrogen peroxide solution in a tris-buffered saline (TBS) buffer. The sections were incubated with a primary rabbit polyclonal antibody (EF14007; Ipsen Innovation, Les Ulis, France) which is specific for the BoNT-A-cleaved form of SNAP-25 [50]. After washing the slides with TBS, the sections were incubated with a biotinylated secondary antibody for 10 to 15 minutes (anti-Rabbit IgG). Sections were then washed with TBS and incubated for 30 minutes with an amplification system (streptavidin–biotin–peroxidase, ABC vector) for 30 min. After a wash in TBS, sections were incubated for 5 to 10 minutes with a solution of 0.02% diaminobenzidine containing 0.01% hydrogen peroxide. An antibody directed against the N-terminal part of SNAP-25 (SYSY 111011, aa 20–40) was used in the same condition to detect total SNAP25in tissues. Counterstaining was performed using aqueous hematoxylin. The presence of cleaved SNAP-25 in the spinal cord was quantified by counting the number of cleaved SNAP-25 positive spinal cord sections on each slide.

IHC was also used to investigate the following pain-related markers: glial fibrillary acidic protein (GFAP, Dako Z0334), ionized calcium-binding adaptor protein-1 (IbA1; ab178847; Abcam, Paris, France), substance P (ab67006; Abcam, Paris, France), and calcitonin gene-related peptide (CGRP, C8198 (Sigma Aldrich; Saint Quentin Fallavier, France). GFAP and Iba1 immunostaining were quantified by image analyses (HALO, percentage of positive pixels in the entire ipsilateral dorsal horns (GFAP) or in the focal area in the same dorsal horn (Iba1, Fig. 5, arrowheads). For other markers, i.e. substance P and CGRP, immunostainings were arbitrary quantified as minimal (grade 1), moderate (grade 2) or marked (grade 3). All slides were evaluated by a board-certified veterinary pathologist (SL) using a light microscope (Olympus BX41).

Data analysis

Mechanical sensitivity data (von Frey test) were first transformed to quoted stimulus by a log transformation of the gram units multiplied by 10,000. The analysis includes a full factorial repeated

measure mixed linear model. The fixed effects include treatment, time and their interaction while the random effects were estimated for animal nested in treatment and time. Tukey post hoc test was derived to compare any group at any time with any other (including itself over time). The approaching time was treated as a continuous variable, and therefore, analyzed using a full factorial repeated measure mixed linear model with fixed effects and random coefficients identical to the mechanical sensitivity analysis. Analyses were performed using JMP®, Version 14 (SAS Institute Inc., Cary, NC, USA). The DBS, body weight, incision score and open field test data were analyzed using one-way ANOVA followed by Tukey post-hoc test.

Statistical analysis of the blood vessel density data was completed using repeated measures ANOVA. To compare each layer a Student t-test was used. For the blood vessel size evaluation, vessels were classified in 8 quantiles (from “small –", to “large ++” in saline injected animals). Data from AboBoNT-A injected animals were compared to saline injected animals (controls) using a Fischer exact test for the 3 layers. Two-way ANOVA was used to compare the staining intensity of GFAP and Iba1 in the different levels of the spinal cord on 2 tissue sections per paraffin block (3 animals/group, 6 tissue sections/group).

Declarations

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Competing interests: Sylvie Cornet, Denis Carré, Lorenzo Limana, Laurent Pons, Steven Evans, Stephane Lezmi and Mikhail Kalinichev were employees of at Ipsen at the time the research was conducted

David Castel, Sigal Meilin, and Ron Horn were employees of MD Bioscience

Data and materials availability: The data that support the findings of this study are available from Ipsen but restrictions apply to the availability of these data, which were used under license for the current study, and so are not publicly available. Data are however available from the authors upon reasonable request and with permission of Ipsen.

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Figures

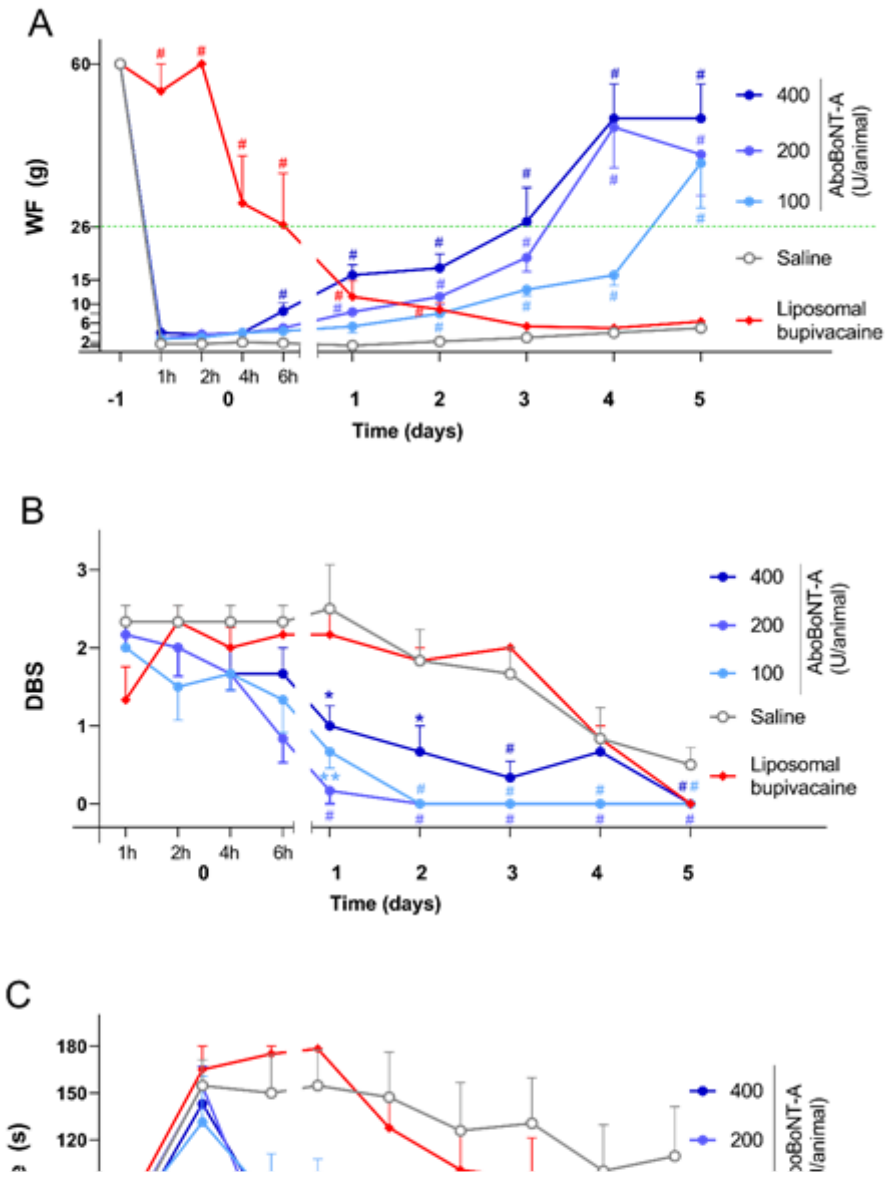

Figure 1

Effects of aboBoNT-A, saline and liposomal bupivacaine on pain-related behavioral responses in pigs. Mechanical sensitivity/withdrawal force (WF; A), DBS (B) and approaching time (C) in animals that received a full-skin and muscle incision and retraction surgery (Day 0), before intraoperative treatment with either aboBoNT-A (100 U, 200 U, or 400 U), saline or liposomal bupivacaine (n=6/group). The green dotted line in panel A at 26g represents the mechanical sensitivity threshold between normal mechanical sensitivity and hypersensitivity/allodynia. *p<0.05, **p<0.01, #p<0.001 vs saline-injected animals.

AboBoNT-A, abobotulinumtoxinA; DBS, distress behavior score; U, unit; WF, withdrawal force.

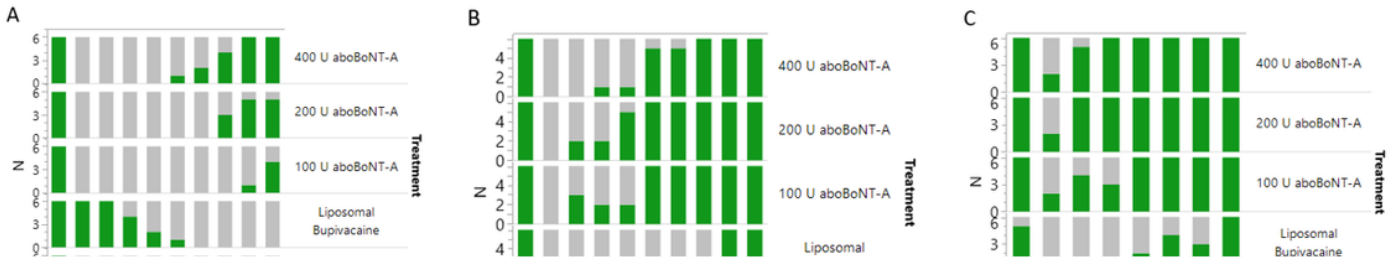

Figure 2

Effects of aboBoNT-A, saline and liposomal bupivacaine on the number of animals exhibiting pain-related behavioral responses versus those with normal responses in each group. Mechanical sensitivity/withdrawal force (WF; A), DBS (B) and approaching time (C) in animals that received a full-skin and muscle incision and retraction surgery (Day 0), before intraoperative treatment with either aboBoNT-A (100 U, 200 U, or 400 U), saline or liposomal bupivacaine (n=6/group). Panel A shows the number of animals (N) with normal mechanical sensitivity (WF $\geq$26g) vs hypersensitivity/allodynia (WF < 26g). Panel B shows the number of animals exhibiting moderate distress (DBS $\geq$ 2) vs those negligible or no distress (DBS < 2). Panel C shows the number of animals that initiated approach in $\leq$90 s or those that took more than 90 s in the Approaching test.

AboBoNT-A, abobotulinumtoxinA; DBS, distress behavior score; U, unit; WF, withdrawal force.

Figure 3

Presence of the total SNAP-25 and absence of cleaved SNAP-25 immunolabelling in the skin of aboBoNT-A-treated animals. Photomicrographs depict the total SNAP-25 immunolabelling in small arteries (A), arrector pili muscles (B), and in nerve fibers of the dermis (C) in a representative animal. Cleaved SNAP-25 immunolabelling was absent in corresponding areas (D, E, F). Scale bars: 50 µm (A, B, D, E); 25 µm (C, F).

AboBoNT-A, abobotulinumtoxinA; U, unit

Figure 4

Assessment of cleaved SNAP-25 immunolabelling in the spinal cord of saline- and aboBoNT-A treated animals. Photomicrographs depict lack of cleaved SNAP-25 immunolabeling in the ipsilateral (A, B) and contralateral (C, D) dorsal horn of the spinal cord in a representative animal treated with vehicle, and presence of cleaved SNAP-25 immunolabeling in the ipsilateral (E, F) and contralateral (G, H) in the dorsal horn of the spinal cord in a representative animal treated with aboBoNT-A. In all aboBoNT-A-treated animals, cleaved SNAP-25 was detected in the lateral part of the ipsilateral dorsal horn (E,G, arrowheads), representing synaptic and dendritic-like staining, whereas in the contralateral dorsal horn (G,H arrowhead) a minimal staining was present in the same location. Quantification of positive spinal cord sections for cleaved SNAP-25 from lumbar (L) sections 1-2, 3-4 and 5-6 from three representative animals treated with saline (blue symbols) and aboBoNT-A (red symbols; I). Size bars: 100 µm (A, C, E, G) or 20 µm (B, D, F, H).

AbobotulinumtoxinA, aboBoNT-A

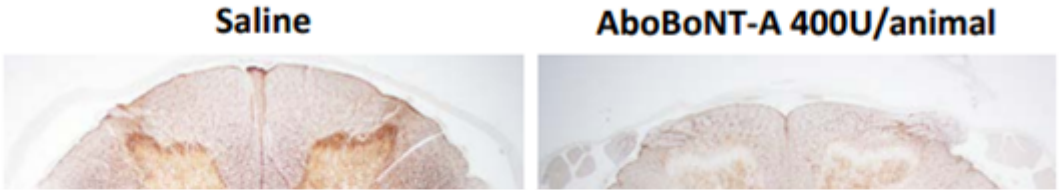

Figure 5

GFAP immunolabelling in the lumbar spinal cord of saline- and aboBoNT-A treated animals. Photomicrographs are taken from L5-L6 sections taken from representative animals treated with either saline (A, B) or 400 U aboBoNT-A (C,D). Scale bar: 1 mm (A, C), 200 µm (B, D). Quantification of GFAP immunobelling in entire ipsilateral dorsal horns from lumbar (L) sections 1-2, 3-4 and 5-6 from animals treated with saline (blue symbols) and aboBoNT-A (red symbols; n=6 tissue section/group; E). *p<0.05, **p<0.01 vs saline-injected animals.

Glial fibrillary acidic protein, GFAP; AbobotulinumtoxinA, aboBoNT-A; Unit, U

Figure 6

Iba1 immunolabelling in the lumbar spinal cord of saline- and aboBoNT-A treated animals. Photomicrographs are taken from L5-L6 sections taken from representative animals treated with either saline (A, B) or 400 U aboBoNT-A (C,D). Quantification of Iba1 immunobelling in the highly positive foci (arrowhead) from lumbar (L) sections 1-2, 3-4 and 5-6 from animals treated with saline (blue symbols) and aboBoNT-A (red symbols; n=6 tissue sections/group; E). Scale bar: 1 mm (A, C), 200 µm (B, D). **p<0.01 vs saline-injected animals.

Ionized calcium-binding adaptor protein-1, Iba; AbobotulinumtoxinA, aboBoNT-A; Unit, U

Figure 7

CGRP and SP immunolabelling in the lumbar spinal cord of saline- and aboBoNT-A-treated animals. Photomicrographs are taken from L5-L6 sections taken from representative animals treated with either saline (A, C) or 400 U aboBoNT-A (B, D). Scale bars: 1 mm

Calcitonin gene-related peptide, CGRP; Substance P, SP; AbobotulinumtoxinA, aboBoNT-A; Unit, U

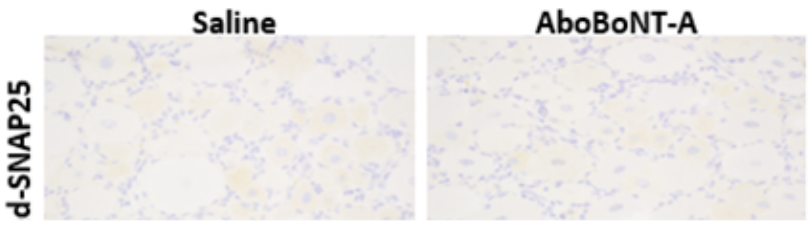

Figure 8

Cleaved SNAP-25, GFAP, Iba-1, CGRP and SP staining in the DRGs of saline- and aboBoNT-A-treated animals. Photomicrographs are taken from ipsilateral DRGs located on L5-L6 sections of the lumbar

spinal cord in representative animals treated with either saline (left column) or 400 U aboBoNT-A (right column). Scale bars: 50 μm.

Glial fibrillary acidic protein, GFAP; Calcitonin gene-related peptide, CGRP; Substance P, SP; Ionized calcium-binding adaptor protein-1, Iba1; AbobotulinumtoxinA, aboBoNT-A; Unit, U

Supplementary Files

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- SupplementaryMaterials.pdf