Author's response to reviews

Title: Pretreatment with Intrathecal Amitriptyline Potentiates Anti-Hyperalgesic effects of Post-Surgical Systemic Amitriptyline following Spinal Nerve Ligation

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Author's response to reviews: see over
Dear Executive Editor Alam:

My colleagues and I are honored to know that our manuscript entitled “Pretreatment with Intrathecal Amitriptyline Potentiates Anti-Hyperalgesic effects of Post-Surgical Systemic Amitriptyline following Spinal Nerve Ligation” (previous title: "Pretreatment with Intrathecal Amitriptyline Potentiates the Analgesic Effects of the Compound on Axotomy-Induced Neuropathic Pain upon Post-Injury Intra-Abdominal Treatment") (MS: 1682055925443873) has positive responses from the reviewers with valuable comments. The text has been revised with tracked mark.

Following are point-by-point responses to the comments from reviewers.

Responses to the reviewer

- **Q1:** What was the rationale for the doses chosen? 50 mg/kg i.p. twice daily is a very high dose – acute effects occur with doses of 10 mg/kg, and daily doses of <20 mg/kg produce long lasting effects on sensory changes. Also what was the i.t dose in mg? This needs to be stated directly rather than left to the reader to perform calculations.

*Answer:* we appreciate reviewer’s question. You are attentive. The amitriptyline solution was prepared **50mg/ml** (not **50mg/kg**) for intraperitoneal administration. The authors are deeply sorry that the solution was presented in a wrong unit. In the present study, the group P was pretreated with **90 µl** of 7.5 mM amitriptyline intrathecally followed by left L5 SNL, and then treatment with amitriptyline intra-abdominally at **12.5 mg/kg twice daily** for 3 days after nerve injury.

**90 µl** of 7.5 mM: it means
- **1mM:** 1mmole/1L = 313.86mg/1000ml = 0.31386mg/ml
- 7.5 × 0.31386mg/ml = 2.3539mg/ml
- 2.3539mg/ml = 0.2118mg/90 µl
- The intrathecal dose of amitriptyline hydrochloride is about 0.21mg in 90µl.

- **Q2:** Amitriptyline given intrathecal is known to produce neurotoxic effects. The authors must consider and reference this literature

*Answer:* we really appreciate this valuable comment. Therefore, a short paragraph was included in the discussion section with intrathecal amitriptyline induced neurotoxicity. “**Whether intrathecal amitriptyline induces neurotoxicity on CNS is still unknown. There is no direct neural damage in 3% amitriptyline-saline intraspinal administration to dogs, though adhesive**”
arachnoiditis appears [1]. Intrathecal amitriptyline and morphine 15µg/hr administered to (co-infused) rats for 5 days maintains anti-nociceptive effect on morphine tolerance by increase of anti-inflammatory cytokine interleukin-10 expressions [2]. In SD rats, high concentration of amitriptyline infiltrating the sciatic nerve easily damage peripheral nerve fibers, causing direct injury to axons thereby producing Wallerian degeneration of the nerve fibers [3]. Although intrathecal amitriptyline showed no obvious or extended infiltration of inflammatory cells in spinal cord in the present study, based on evidence of related literatures, it is important that low concentrations of amitriptyline be administered either peripherally or centrally to prevent neurotoxicity."

Q3: The paper considers only Na+ channels blocking properties of amitriptyline – it is a complex drug with many actions, and recognition of this complexity of action needs to be made. Can refer to a review on mechanisms of action.

Answer: we appreciate the reviewer’s comments. Administration of amitriptyline to alleviate pain is so perplexity that no single one mechanism is able to fully explanation its actions. However, amitriptyline has been considered as a sodium channel blocker. I have made up the possible effects of amitriptyline through mechanism of

◆ ATP: When A1Rs are present, actions of amitriptyline may, however, partly depend on A1Rs [4]
- Amitriptyline also had no effects on currents evoked by ATP at rat P2X4 receptors, or at rat or human P2X7 receptors [5].
- adenosine A1 receptors are not required in order for amitriptyline to cause antinociception in mice.
◆ serotonin noradrenaline reuptake inhibitors
◆ Excitatory amino acid transporters (EAATs)
-amitriptyline could increase the expression of EAATs which may be one of its mechanisms in the treatment of neuropathic pain [6].
◆ Noradrenaline
-amitriptyline treatment had no effect on the development of mechanical allodynia; interruption of GDNF and BDNF signaling impaired the prevention of hypersensitivity to alphabeta-MeATP/NA.; tricyclic antidepressants given in the perioperative period may be useful in preventing nerve injury-induced sensory changes that contribute to the development of chronic post-surgical neuropathic pain [7]
- low concentrations of amitriptyline suppress the response of human peripheral C-type axons to nicotine by directly inhibiting nAChRs. Blockade of tetrodotoxin-sensitive, voltage-dependent sodium channels does not contribute to this effect [8]

- Amitriptyline inhibited Kv1.1 and Kv7.2/7.3 channels in a concentration-dependent and reversible manner

- The anti-allodynic effect of chronic antidepressant treatment is mediated by a recruitment of the endogenous opioid system acting through delta-opioid receptors [10]

- the potency of peripheral sodium channel blockade for several tricyclic antidepressants and selective serotonin reuptake inhibitors with their therapeutic efficacy by inhibited Na\(_V\)1.7 in a state- and use-dependent manner [11]

- Use-dependent block by amitriptyline was similar in TTXs and Na\(_V\)1.8 channels. Surprisingly, use-dependent block by lidocaine was more pronounced in Na\(_V\)1.8 than in TTXs channels [12]

- Less negative membrane potential and repetitive firing have little effect on tetrodotoxin-resistant Na\(_+\) current amplitude but increase their sensitivity to lidocaine, mexiletine, and amitriptyline so that concentrations after intravenous administration of these drugs can impair channel function [14]

- Amitriptyline and local anesthetics interact with a common binding site. Furthermore, at therapeutic concentrations, the ability of amitriptyline to act as a potent use-dependent blocker of Na\(_+\) channels may, in part, explain its analgesic actions [15]

- It was concluded that amitriptyline blocked the TTX-S and TTX-R of Na\(_+\) channels in rat sensory neurons by modulating the activation and the inactivation kinetics [16]

- sodium channel expression is altered within higher-order spinal sensory neurons after peripheral nerve injury and suggest a link
between misexpression of the Na(v)1.3 sodium channel and central mechanisms that contribute to neuropathic pain after peripheral nerve injury [17]

♦ **GABA (B) receptor**
- amitriptyline and fluoxetine increase the pain threshold to a thermal stimulus, the expression of GABA(B(1)) subunits, and baclofen-stimulated [35S]GTP gamma S binding, a measure of GABA(B) receptor function [18, 19].
- daily administration (10 mg/kg, i.p.) of amitriptyline

♦ **NMDA**
- Amitriptyline reverses hyperalgesia in rats by a mechanism unrelated to monoamine reuptake inhibition, and likely due to NMDA receptor antagonism [20]

**Q4:** The figures require some attention to detail. In the text, the sequence of considering groups is consistently S, L, A then P. The legends in Fig 2 should read in that order from the bottom (it is LSAP – needs to be SLAP). Ditto for Fig. 3 (it is SLPA, needs to be SLAP) - AND the data should be presented in that order, i.e. dark green A group beside the L group. SLAP for Figs 4 and 5 as well.

*Answer:* The group order of the figures have been revised.

**Q5:** Discussion. This was generally not very well written. The first sentence is meaningless and just dangles. Begin with a summary of key and original observations. The authors need to consider differential effects of ami on thermal thresholds (reversal POD4, POD7 and POD14) but only modest effects on pressure thresholds POD4 and POD7. There is a literature on differential effects on ami on thermal vs mechanical thresholds (particularly allodynia determined by von Frey thresholds) and this needs to be meaningfully considered. Ref 12 shows differential long lasting effects on sensory thresholds with a 7 day post surgical regimen of ami – some sort of direct comparison with that other study that used a post-surgical ami regimen needs to be made.

*Answer:* We appreciate comments from reviewer in discussion section. “The first sentence is meaningless and just dangles”. The first sentence was omitted.

Minor edits
- Change Title: Pretreatment with intrathecal amitriptyline potentiates antihyperalgesic, Na+ channel and microglial effects of postsurgical systemic
amitriptyline following spinal nerve ligation (the study does NOT use an
axotomy model; need to be more specific in terms of what was examined)

**Answer:** done. Change to “Pretreatment with Intrathecal Amitriptyline
Potentiates Anti-Hyperalgesic effects of Post-Surgical Systemic Amitriptyline
following Spinal Nerve Ligation”

- POD day numbers can be regular numbers (as in figures) rather than subscripts.
  **Answer:** done

- Fig 2: Fonts need to be consistent, and larger – these will be far too small when
  the figure is reduced. Fig 3 is very nice, but may lose information with
  shrinkage.
  **Answer:** done

- The text needs many minor edits. Here are a few examples, but it is not a
  complete list. The authors should read the final manuscript version carefully for
details.
  **Answer:** done

- Abstract: Do not need to include p values in the abstract. page 4, line 1:
  **Answer:** done. The P values were erased.

- intrathecal pretreatment with amitriptyline potentiates the intra-abdominal
  amitriptyline effect on thermal hypersensitivity, reverses (present tense) Nav1.8
  changes, and attenuates…
  **Answer:** done

- Conclusions: Concomitant …line 7: hypersensitivity; it also decreased activated
  microglia and astrocytes, and restored dysregulated … (DO NOT subscript the
  1.3 and 1.8 – in several places it is fine but it is inconsistently presented)
  **Answer:** done

- Page 5, Line11: axotomy, neurons require less depolarization… line 12: and
  easily fire due to actions… Nav1.7 channels… line 14: injured nerve [11] (wrong
  ref is cited)
  **Answer:** done

- Page 6 Line 1: the mechanism… line 2: is ref 16 correct here? Line 8: In view of
the above reports, we hypothesized…

*Answer:* done; cited ref16 cancelled; and change “we therefore hypothesized” to “In view of the above reports, we hypothesized”

- Page 15, Line 2: Surgery and …line 4: In the S group, ipsi… In the P group, intrathecal…(put the group identity up front as much as possible) Line 11: hypersensitivity responses as indicated by a …

  *Answer:* done

- Page 16, Line 2: Regardless of the treatment with ami,

  *Answer:* done

- Line 9: 3A and 3B, group S vs group L) any vs indicates a comparison and so noneed to state “compare” (here and elsewhere);

  *Answer:* done

- line 12: expression for 3 weeks (not 2 weeks, check the figure)

  *Answer:* done

- Page 17 Line 3: Ami inhibited glial cell activation …line 5: and increased activation was most (the start of the sentence uses the past tense, be consistent)

  *Answer:* done
References


11. Dick IE, Brochu RM, Purohit Y, Kaczorowski GJ, Martin WJ, Priest BT: Sodium channel blockade may contribute to the analgesic efficacy of


