Author's response to reviews

Title: Sex differences underlying orofacial varicella zoster associated pain in rats

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Author's response to reviews:

Reviewer #1

1) It would be helpful to the reader to have a summary chart of what was done to animals in each group (e.g., behavior, histology, etc). For example in the Immuno-fluorescent staining Results, there is a description that TG were collected from the second and fifth study - a reference chart would help with the significance of the IHC results.

Response: Our goal is to make the experimental design easily interpretable and a summary chart has been added to the manuscript, see Table 1. This table identifies the number of animals used in each group for each experiment. This table is now referenced at the end of the Methods section.

2) The authors addressed the previous comment regarding the high dose of virus (1900 pfu/ul), but it is not clear why/how the low doses were chosen and why have 2 "low" doses. Maybe a comment in the Methods for the rationale of the doses used.

Response: A rational for the doses used in these experiments has been added to the methods section as stated below.

In the methods we have added "Long Evans rats were reported by Envigo to be less docile than Sprague Dawley rats and thus, the Long Evans rats were injected with a lower dose of VZV as

compared to the Sprague Dawley rats in Study #5. This lower dose was expected to compensate for the greater inherent activity of Long Evans rats during the behavioral testing."

In the methods we have also added "The effect of analgesics can be undetectable "swamped out" if the pain response is too great thus, the lowest dose of VZV known to elicit a behavioral response was implemented after administration of gabapentin."

3) Double check that the NiTi wire is 0.16mm and not 0.016"

Response: The NiTi wire is 0.16 mm or 0.006 inches.

4) Reference 29 (Hargreaves et al, Pain 1988) is inappropriate and has nothing to do with operant or orofacial behavioral testing. Comment #4: The Hargreaves paper was removed.

Response: Thank you for your careful review this reference has been removed and citations related to the Ugo Basile chamber have been added.

5) Figure 1 - the viral infection was in the V1/V2 dermatome; however IE62/NeuN staining was present in ~10% of medium V3 and ~10% of small V3 cells. Is there prior evidence that spread within the trigeminal ganglia can occur between divisions once the V1/V2 site is infected?

Response: This is the first evidence for VZV infection of the trigeminal ganglia after injection into the rodent whisker pad. VZV is a human herpes virus one of eight types of herpes viruses. A search of other herpes viral types demonstrated that whisker pad injection of herpes simplex virus type 1 resulted in a small amount of V3 infection with a majority of the infection being present in the V1/V2 region, see Figure 3D from [1]. This additional information as well as a potential explanation has been added to the last paragraph of the discussion.

6) Figure 2 - thermal hyperalgesia is evident for the Poka virus group, developing at 1 week and persisting to day 36 or so, but there appears to be development of thermal hyperalgesia in the control group (MeWo cells) as well up to 1-2 weeks. Can the authors provide discussion about this effect?

Response: Thank you for this observation. The effect was not significant with the power used in these experiments and we did not feel comfortable discussing this result in the paper. After von Frey testing Dr. Kinchington has observed a trend where injection of MeWo cells into the paw

increase the response but these results did not reach significance. This response "trend" in the paw lasted about a week. Dr. Kinchington suspects that this response in the first week is due to a localized peripheral inflammatory response but his work on these peripheral mechanisms is ongoing.

7) Figure 4 - suggestion NOT to show each week of results. From my interpretation, day 7 to day 42 essentially show the same result, with final recovery at day 56. I would recommend reducing the number of graphs (maybe make a Table summarizing results?)

Response: The data shows that within the first five minutes (i.e., five minute bin) there was no significant difference from week to week suggesting that the animals are essentially naïve with respect to the testing after a 7 day inter-testing period. This helps to address comment #2 of Reviewer #2. We believed that the data was best imaged as a graph but we defer to the reviewer and will produce a table if desired.

8) Figure 6 - see comment for figure 4 (consider reducing number of graphs)

Response: We deliberated that the data would be less clear if presented as a table but we defer to the reviewer and will produce a table of the data if desired.

9) Figure 7 - there appear to be floating error bars without corresponding symbols

Response: In the gabapentin graph the error bars are difficult to determine. A colored graph has been added that allows for the error bars to be more easily associated with a particular symbol. Additional graphs can be color coded to enhance the ease of interpretation if difficulty in interpreting the other figures is noted.

10) Not clear why gabapentin was not used in the operant behavioral tests

Response: The operant testing was completed first and took a great deal of time. Second, we observed a robust response with the motivational/affective testing (i.e., PEAP). Our lab decided to use this test for all the following tests because 1) it measured an important part of the pain response (i.e., affective/motivational) and 2) it was easily and completed relatively quickly. The gabapentin experiment was completed later after this decision to use PEAP was made.

Reviewer #2

1) VZV IE62 expression. Given the fact that VZV does not replicated in rats, it is surprising that VZV IE62 expression was detected in cells other than the neurons innervating the whisker pad, i.e., in non-neurons and neurons in V3. Since this study is introducing a novel and exciting model it is important to clearly demonstrate the types and location of cells expressing IE62. Please provide lower power images representing the V3 and V1/V2 tissue sections to clearly demonstrate location of IE62 stained cells. Also speculate in the discussion on how expression of IE62 occurs in neurons that do not innervate the site of injection.

Response: A low power figure has been added to Figure 1 and it appears there is some labeling in the V3 region. This is the first evidence for VZV infection of the trigeminal ganglia after injection into the rodent whisker pad. VZV is a human herpes virus, one of eight types of herpes viruses. A literature search revealed that a similar experiment had been completed with another member of the herpes virus family, notably herpes simples virus type 1. In the last paragraph of the discussion we have added that injection of herpes simplex virus type 1 resulted in a small amount of infection of the V3 region with a majority of the infection being present in the V1/V2 region [1], consistent with our results. Studies of the somatotopic organization of the trigeminal ganglia indicates that there is overlap in the location of neurons within the ganglia that contribute to the V1/V2 and V3 branches [2]. The results could be explained by this overlap; if some of the IE62 positive neurons in the V3 region actually project to the whisker pad then it would be expected that these neurons would become infected by VZV.

2) Place escape/ avoidance paradigm (PEAP). Since this is the primary assay used in these studies and two referenced citations (Baastrup et al 2010, 2011) state that PEAP relies on escape/avoidance learning to a novel aversive environment and can thus only be used once, please assure the reader that successive assays were not influenced by previous testing. Do the experienced injured rats favor the light side of the box in the absence of stimulation? Page 8, line 55. Two of the references cited as using PEAP testing claim that PEAP relies on escape/avoidance learning to a novel aversive environment and can thus only be used once (Baastrup et al, 2011; Baastrup et al, 2010). Is the behavior of the rats influenced by previous sessions such that injured rats would tend to spend more time in the light side of the box even in the absence of stimulation? Page 9 line 24. Were baselines determined prior to noxious stimulation to ensure that no association with dark side of chamber and pain from previous weekly testing? I assume that in the absence of noxious stimulation, there would be no significant differences between in the absence of noxious stimuli?

Response: In pilot studies animals were placed in the box for about 5 minutes without noxious behavior. Animals remained primarily on the dark side of the box and no difference was

observed whether previous testing had been performed and regardless of the treatment. In the results section we now state that during the first 5 minutes there was no significant difference in the time spent on the light side of the box when comparing groups in the first week to subsequent weeks of testing. These results suggest animals do not recall events of a previous week when the 30 minute testing periods are separated by a 7 day period of no testing as described in these studies.

Minor Comments:

3) Format abstract as per Journal instructions

Response: The abstract has the Background, Methods, Results and Conclusions labeled as per journal instructions.

4) Double check references to insure they are relevant. For example, you state on page 7 line 9 that "In the first experiment the animals were trained to obtain reward using the orofacial test chamber from Ugo Basile (Italy) [28, 29]." However, Hargreaves et al (Hargreaves et al, 1988) did not use an orofacial test chamber and the orofacial test chamber used by Neubert et al (Neubert et al, 2005) was not from Ugo Basile. Ugo Basile provides a bibliography of six references for the orofacial stimulation test (Abd-Elsayed et al, 2015; Cha et al, 2012; Prochazkova et al, 2013; Thibault et al, 2016; Zhang et al, 2016; Zuo et al, 2013).

Response: Thank you for this helpful assistance. These corrections have been completed.

5) Page 7 line 33. Define "approximately 300 seconds".

Response: The definition has been more clearly stated as 300 ± 50 seconds.

6) Page 9, line 4. Would face be more accurate than "snout"? Why was the site of VZV injection not tested?

Response: The term snout has been replaced with face. VZV has been tested in the paw by several laboratories but the rational for using the whisker pad was based on the etiology VZV. The whisker pad has a high density of nerve innervation and thus, nerve terminals. VZV infects nerve terminals in the periphery and this high density within the whisker pad was suspected of being optimum for maximizing the infection.

7) Page 9 line 36. Why was the dose of VZV reduced?

Response: A rational for the dose used in this experiment has been added to the methods section as stated “The effect of analgesics can be undetectable “swamped out” if the pain response is too great thus, the lowest dose of VZV known to elicit a behavioral response was implemented after administration of gabapentin.”

8) Page 10 line 19. Why is a coauthor referred to as providing the VZV? Did he provide the VZV for all studies?

Response: Yes Dr. Kinchington provided VZV and Mewo cells for all the experiments.

9) Page 11 line 26. Why would VZV infection be expected to be greater at longer durations?

Response: In previous work VZV infection, as determined by the presence of IE62, showed greater levels of IE62 at 1 month and 18 months post infection versus one week post infection [3] thus, we expected greater VZV infection and neurite retraction in the first and second studies that were of longer duration. This text has been added to the methods section.

10) Results section. Since different doses of VZV were used in these studies, please state the dose in the results section and explain why lower doses were used in later latter experiments.

Response: The doses have been added to the results section and a rational for the doses used in these experiments has been added to the methods section as stated below.

In the methods we have added “Long Evans rats were reported by Envigo to be less docile than Sprague Dawley rats and thus, the Long Evans rats were injected with a lower dose of VZV as compared to the Sprague Dawley rats in Study #5. This lower dose was expected to compensate for the greater inherent activity of Long Evans rats during the behavioral testing.”

In the methods we have also added “The effect of analgesics can be undetectable “swamped out” if the pain response is too great thus, the lowest dose of VZV known to elicit a behavioral response was implemented after administration of gabapentin.”

11) Page 15 lines 19 -24 states that "The response to VZV injection showed a remarkable difference that was significant for 8 weeks in comparison to control (Fig. 4)". Should this be for 7 weeks?

Response: Correct, this change has been made.

12) Figure 1 legend is redundant. Since all data in this figure are from the same animals, state the dose of VZV and the time post injection once and then describe the individual panels.

Response: These changes have been made.

1. Nicoll MP, Proenca JT, Connor V, Efstathiou S: Influence of herpes simplex virus 1 latency-associated transcripts on the establishment and maintenance of latency in the ROSA26R reporter mouse model. Journal of virology 2012, 86(16):8848-8858.

2. Leiser SC, Moxon KA: Relationship between physiological response type (RA and SA) and vibrissal receptive field of neurons within the rat trigeminal ganglion. J Neurophysiol 2006, 95(5):3129-3145.

3. Kennedy PG, Grinfeld E, Bontems S, Sadzot-Delvaux C: Varicella-Zoster virus gene expression in latently infected rat dorsal root ganglia. Virology 2001, 289(2):218-223.