Evaluation of c-Fos protein expression and NADPH-d reactivity in Neurobion pretreated rat model of inflammatory pain

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Research Article

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Abstract

B-vitamins have been evaluated as a useful adjuvant therapy to treat pain. In spite of clinical and experimental evidence indicating the analgesic effect of B-vitamins, few studies have investigated their effect on aspects of the inflammatory pain response. In the present study, we investigated the analgesic effect of chronic application of B-complex vitamins (Neurobion) using an inflammatory experimental pain model in rats. Nociceptive behavioral responses were evaluated in male Wistar rats after plantar injection of formalin, comparing groups with (TG) and without (CG) Neurobion pretreatment. In addition, neuronal activity in the central pain pathway was evaluated using c-Fos immunohistochemical reactivity and NADPH-d histochemistry. A highly significant reduction of painful behaviors such as licking and flinching were observed in TG, especially during the secondary phase of the formalin test compared to CG. Results suggest that long-term pre-treatment using Neurobion can have a beneficial effect in reducing the chronic phase of pain. In addition, we observed a downregulation of c-Fos and NADPH-d in dorsal spinal neurons, suggesting that the antinociceptive effect induced by Neurobion could be due to a suppression of nociceptive transmission at the spinal level, particularly in the afferent regions of the dorsal spinal horn, which these neurons utilizing nitric oxide at least as one of their pain neurotransmitters.

Introduction

B vitamins should be consumed daily as part of our dietary intake due to their critical and wide-ranging utility in the body, particularly considering that they are not stored by our body. The deficiency of these vitamins can cause pathophysiological conditions including epilepsy, carpal tunnel syndrome, chronic pain, sleep disorders, and autoimmune disorders. In addition to treating deficiencies in the body, B-vitamins appear to engender analgesic effects in doses higher than the daily requirement. This suggests their usefulness in the treatment of pathophysiological pain disorders. Since impressive clinical results were first observed in the 1950s, researchers have maintained a focus on the pain-relieving effects of B vitamins. The anti-inflammatory effects of B-vitamins have also been broadly studied in animal models, including mechanical allodynia and neuropathic pain.

As well, it has been assumed that the synergistic effect of vitamin B1, B6, and B12 (as B-complex) could be more effective in reducing pain, with no risk of side-effects even at the high doses of consumption. Although multiple mechanisms have been suggested to explain the analgesic and anti-inflammatory effects of B-complex vitamins, few investigations have been reported on the effects of B-complex vitamins on analgesia. Fewer studies have investigated their effects on the different aspects of the inflammatory pain response, and the exact pain reduction mechanism caused by B vitamins remains unknown.

The formalin test in rodents is extensively used to induce and model acute and chronic inflammatory pain. It has been reported that formalin injection is a valid and reliable model that is highly sensitive in the testing of varying classes of analgesic drugs.
The pain signaling process is a complicated one involving a large number of neurotransmitters, molecules, and chemicals in the body. Nitric oxide (NO), a free radical diffusible gas molecule that is synthesized on demand (in response to inflammatory stimulation) with a half-life of few seconds, is one of the most important neurotransmitters involved in neuropathic pain in the spinal cord. It also plays an important role in the guidance and modulation of signals related to pain, neuronal protection, neurotoxicity, neuroplasticity and persistent inflammation\textsuperscript{16}. Nitric oxide synthase (NOS) produces NO by oxidation of L-arginine to L-citrulline. The essential reductive cofactor NADPH or the NOS enzyme involved in NO synthesis can be used as a marker of NO presence\textsuperscript{17}. Many researchers have reported that inhibition or knockout of NOS results in pain persistence\textsuperscript{18}.

The expression of the proto-oncogene protein c-Fos, encoded by a member of the immediate-early gene family, is an important neurophysiological response to a variety of painful stimuli. c-Fos is rapidly induced to control the expression of other genes whose products are involved in long-term changes in neuronal excitability, and can be detected using immunohistochemistry\textsuperscript{19}. During nociceptive transmission, Fos-like immunoreactivity is reported through the spinal dorsal horn up to the cerebral cortex, indicating the neural cascade of signal transduction. Therefore, mapping c-Fos expression in the areas is a valid and powerful tool to trace populations of nociceptive neurons responding to pain\textsuperscript{20,21}.

Given the promising results reported in clinical trials on the anti-inflammatory and pain-reducing effects of B-complex vitamin supplements, in this study we utilized the formalin test in rats to evaluate a pain model that is closest to clinical pain. We aimed to determine whether the Neurobion B-complex modified expressed pain behavior, and if so, delineate in which phase (acute and/or chronic) any effect was seen. Furthermore, we chose to delineate any such effects on both nitric oxide-producing neurons and c-Fos expression in the dorsal horn of the lumbar spinal cord and supra-spinal centers that are involved in pain processing.

\textbf{Materials And Methods}

\textbf{Animals and Treatment}

Male Wistar rats (\textit{Rattus norvegicus}) with an average weight of 195 ± 15 g were the model animal used in this study. All animals were kept single-housed, with free access to food (standard chow diets, without any dietary deficiency in B vitamins) and water, and maintained at a constant temperature of 22 ± 2°C in a laboratory with a 12 h light/dark cycle. The animal research fully complied with the principles and protocols of working with animals and experiments, guidelines for animal care established by the International Association for the Study of Pain (IASP), and consistent with NIH and our institute's Animal Ethics Committee guidelines for studies in animals.

The rats were divided into three groups and each group contained eight rats (\textit{n} = 8); the number of rats was chosen based on previously published studies\textsuperscript{22-25}. For behavioral observation and histochemical evaluation, six and four rats were chosen respectively, and randomly from each group. The first
experimental group received injections of 50 µl of formalin subcutaneously on the plantar surface of the right hind paw using needle number 30, with concentrations of 5% as used previously \(^{26,27}\). Neurobion is usually administered by either oral administration or intramuscular injection (higher bioavailability) for 7 to 10 days continuously to treat acute or chronic pain clinically \(^{28}\). We chose a 10-day intraperitoneal (IP) Neurobion administration using a higher dose based on the clinical literature \(^{29,30}\). The second group was therefore predosed with NEUROBION® (injected at 1 ml/kg of body weight [1 ml containing 33.3 mg B1, 33.3 mg B6, and 0.333 mg B12]. The injections were continued for 10 days, and were then injected with formalin (TG) on the last day (an hour after the last Neurobion injection) using the same protocol as group 1. To evaluate the effect of pain or stress following the intraperitoneal (IP) injection alone, we added a third (Vehicle) group, which like group 2 was acclimatized and handled for 10 days, but received saline intraperitoneally every day and one hour before the formalin test for the last time.

**Pain measurement evaluation (pain score)**

Formalin causes inflammation by injection into the plantar area of the rat's hind paw allowing us to evaluate how the animal responds to pain in both acute and chronic phases. Researchers suggest that formalin testing, compared to thermal and mechanical pain models, is one of the most reliable models for assessing clinical pain \(^{31}\), and it allows us to determine the acute and chronic phases in which Neurobion exerts its effect. It has been previously reported that a biphasic nociceptive behavior could be clearly observed after formalin injection \(^{32}\); the first phase is known as the acute phase, which includes the first five minutes after injection. This phase is mainly caused by pain afferent fibers, mostly the C fibers through direct chemical stimulation. However, it is also accompanied by direct and strong stimulation of myelinated and non-myelinated fibers that represent neurogenic pain. The second phase starts from around minute 10 to 35. This phase is a clear sign of inflammatory response to tissue damage during the initial phase resulting in inflammation-driven pain. It can be characterized by the activity of non-myelinated fibers and hyperalgesia, which is caused by functional changes in the dorsal horn of the spinal cord as a result of central sensitivity \(^{33}\). Interphase (the time between 5–10 minutes) separates these two phases and is thought to result from an active inhibitory process. Pain reduces after the second phase and during the recovery phase, from around minute 35 to 60.

Formalin was injected, and pain-related behavior (pain score) was accurately monitored for an hour and recorded every 15 seconds, continuously. The score was given to the rat's behavior according to the following quantification of pain-related behavior \(^{34}\): Score zero was used when no pain-related behavior was observed, and the rat did not pay attention to the injected foot; Score 1 was used when the rat tried to walk or rest while putting most of his body weight on the non-injected leg; Score 2 was used when the rat lifted the injected leg; Score 3 was used when the rat was shaking or licking the injected foot. For pain intensity, the flinching and licking time and number were carefully observed for an hour and recorded every five minutes.

**Spinal cord and brain tissue collection**
For histochemical evaluation, four rats were randomly chosen from each group following behavioral observation. The spinal cord and brain tissue were fixed exactly two hours after formalin injection (an hour after the end of the one-hour behavioral testing process). First, rats were deeply anesthetized by intravascular injection of Urethane (1.5 gr/kg). Subsequently, to perfuse and fix the brain and spinal cord tissue, the chest was opened and the pericardium and thymus were separated from the heart. Sodium nitrite (0.5 mL, 1%) and heparin (5000 IU) were injected into the left intraventricular space for opening the vessels and preventing clot formation, respectively. Following insertion of a cannula in ascending aorta, saline solution (500 mL) was circulated and the right atrium was cut with scissors subsequent to complete exsanguination. Fixative (containing 250 mL phosphate-buffered saline (PBS) (0.2 M, pH = 7.4), 50 mL picric acid (1.33%) and 100 mL paraformaldehyde (20%) in 100 mL deionized water; 500 mL, 4°C) was circulated for 3 h. The brain and spinal cord were carefully removed and stored in the same fixative for 24 h at 4°C. Subsequently, the brain and spinal cord were immersed in 20% sucrose and PBS at 4°C, followed by washing three times with PBS and freezing using liquid nitrogen. A Cryostat freezing microtome device (Leica, Germany) was used to cut the frozen tissues to thin sections of 40–45 µm, and the lumbar spinal cord and brain sections were kept in PBS at 4°C.

Histochemical and Immunohistochemical analysis:

For NADPH-diaphorase (NADPH-d) histochemistry, the lumbar spinal cord and brain stem sections were kept at room temperature and rinsed three times with PBS containing Triton X-100 (0.1%), then incubated in the PBS (0.1M) containing Triton X-100 (0.3%), β-NADPH (0.1 mg/ml) and nitro blue tetrazolium (NBT) (0.1mg/mL) with light protection at 37°C for 1h, till a purple color was observed. Once the reaction had completed, the sections were taken out and washed three times with PBS and deionized water, and mounted on glass slides, air-dried and cover-slipped with Entellan rapid mounting (Merck, Germany).

Alternate sections from the lumbar spinal cord and brain stem were used for immunohistochemical evaluation of c-Fos. The sections were rinsed three times (5 min) in PBST followed by immersing into hydrogen peroxide solution (0.3%) for 30 min at room temperature and were washed two times again with PBST (5min). After incubation in PBS solution containing 9% Bovine Serum Albumin (BSA) for 60 minutes at room temperature and washed again with PBST (5min), the samples were incubated in PBST, BSA, Azide (3 mg), and primary antibody *Anti-c-Fos rabbit Ab* (Calbiochem, USA; RRID:AB_213451) diluted 1:10000 at 4°C for 72 h. Sections were washed three times in PBST and incubated in a solution of BSA (3%), 3 mg of Azide, and biotinylated secondary antibody (Anti Rabbit IgG (whole molecule) (Sigma, USA; RRID:AB_916366), with the concentration of 1:1000 at 4°C for 24 hr. After 3 washes with PBST the sections were placed in biotin peroxidase complex (ABC-vector) in PBST solution for 90 min. Following 3 further washes in PBS and tris-HCl buffer (0.05 M, PH 7.4), the sections were placed for 10 min in Tris-HCl buffer containing Diaminobenzamide tetrahydrochloride (5 mg), ammonium nickel sulfate (50 mg), and hydrogen peroxide (0.5 µl). The reaction was stopped by washing the sections in Tris-HCl buffer. Sections were then mounted onto glass slides, air-dried and cover-slipped. The c-Fos positive neurons turn dark blue with an ovaloid shape. An Olympus microscope was used to capture photos, and a camera lucida microscope was used for counting the neurons from both sides; at least 20 sections were taken from the
lumbar region (L4 and L5) of each rat. We used the hypothalamic region as a control for c-Fos immunoreactivity after formalin paw injection.

**Statistical analysis**

All data are expressed as the mean ± SEM. Visualizations of our sample group data were plotted using Raincloud plots \(^{35}\) and analysis was performed using JASP V0.16.3 \(^{36}\). We used the Shapiro-Wilk test to check the normality of our data distribution, and Levene's test to check the homogeneity of variance between groups. For the behavioural results, where Levene's test of homogeneity passed, we use a two-way ANOVA with tukey post-hoc correction. For groups that exhibited significant inhomogeneity (p < 0.001) we reverted to individual Welch's tests (for non-equal variances) and Mann-Whitney U tests (for non-normal distributions). p values less than 0.05 indicated statistical significance. Where available we also calculated Cohen's d to estimate the effect size, taking d > 0.2 = small effect, d > 0.6 = medium effect and d > 0.8 = large effect.

**Results**

The results of this research are separated into two parts; behavioral observation and histological investigation. For the behavioral observation, the formalin test was used for pain induction, and quantitative measurement of the number and duration of licking and flinching (along with a resultant pain score) compared between the control and the pretreatment group. For histological investigation, exemplar rats were chosen randomly after behavioral observations. NADPH-d and c-Fos reactivity were used to investigate changes in the pain pathways, from the lumbar spinal cord to supra-spinal structures which are involved in pain processing.

**Behavioral observations**

The biphasic nociceptive behavior could be clearly observed after formalin injection in our study. The first phase (phase I) is known as the acute phase, which includes the first five minutes after injection. The second phase (phase II) starts shortly after the phase I and lasts longer, around min 10 to 35. Interphase (the time between 5–10 minutes) separates these two phases. Pain reduces during the recovery phase, which lasts from around minute 35 to 60 (Fig. 1).

**Behavioral observation and quantification**

Behavioral evaluations were performed for the control group (CG) and the Neurobion pretreatment group (TG) across the four time periods illustrated in Fig. 1. A two-way ANOVA showed that there were significant and large difference between CG and TG \((F(1, 32) = 33.22, p < 0.001; \text{effect size } \omega = 0.19)\), a significant and large difference across time period \((F(3, 32) = 26.58, p < 0.001; \text{effect size } \omega = 0.46)\), and a significant and medium-sized interaction \((F(3, 32) = 4.72, p = 0.006; \text{effect size } \omega = 0.07)\). Simple main effects analysis showed that there were significant differences between CG and TG for phase 1 \((p = 0.025)\), interphase \((p < 0.001)\) and phase 2 \((p < 0.001)\); but no significant difference for recovery \((p = \text{not shown})\).
Post-hoc corrected multiple-group comparisons confirmed highly significant differences between CG and TG for interphase and phase 2 only (Fig. 2a; \( p < 0.001 \) & \( p = 0.005 \) respectively).

Decreases in the respective behavioral response composites were seen for all phases of both licking time and licking number; these reached significance in the interphase (\textit{licking time}: \( p = 0.046 \) Student’s T; Cohen’s \( d = 0.85 \)) and the second phase (\textit{licking time}: \( p = 0.014 \) Student’s T, Cohen’s \( d = 1.72 \pm 0.76 \); \textit{licking number}: \( p = 0.04 \) Student’s T, Cohen’s \( d = 1.36 \pm 0.7 \)) for TG compared to CG (Fig. 2b and c). Figure 2d and E plots flinching time and flinching number, which clearly show significant reductions in TG in both the interphase (\textit{flinching time}: \( p = 0.003 \) Welch’s T, Cohen’s \( d = 2.94 \pm 1.03 \); \textit{flinching number}: \( p = 0.004 \) Welch’s T, Cohen’s \( d = 2.57 \pm 0.94 \)) and the second phase (\textit{flinching time}: \( p = 4.3 \times 10^{-4} \) Student’s T, Cohen’s \( d = 2.97 \pm 1.03 \); \textit{flinching number}: \( p = 0.026 \) Welch’s T, Cohen’s \( d = 1.57 \pm 0.73 \)).

To summarize the behavioral assessment, Neurobion resulted in significant reductions of pain in the second phase and interphase but not in the first phase and recovery time, which indicates that the greatest effect of Neurobion is in the chronic phase of the pain response.

In order to monitor the effect of the needle pain caused by the IP injection of Neurobion, a Vehicle group (VG) received the saline followed by a 5% formalin injection (after 1 h). The result obtained from this group was the same as the CG (Fig. 3).

Histochemical analysis

For histochemical and immunohistochemical evaluation, we utilized at least 20 sections from the spinal cord (the focus was on L4 and L5 of the lumbar region) and 5 sections from the supra-spinal areas from each rats.

To assess the NADPH-d positive neurons in this study, we investigated the dorsal horn region that contains laminae I to IV, and also areas around the central canal, including lamina X. Laminae I - II as superficial laminae of the dorsal horn and laminae III - IV as deep laminae were examined separately. The mentioned above classification is shown schematically in the schematic diagram (Fig. 4).

**Number of NADPH-d positive neurons in the dorsal horn**

In the superficial laminae of the dorsal horn, the small axons had a spindle-shaped cell body and appendages that projected a short distance around neurons. In the deep laminae of the dorsal horn, larger neurons with a multipolar cell body were seen, with their appendages branching out in all directions. Extensions of some of these neurons also extended to the superficial laminae. Following the formalin injections, the number of NADPH-d positive neurons in the superficial and deep laminae of the dorsal horn was investigated in CG and compared with TG in the injected side and the opposite side of injection. After the formalin injection, the number of NADPH-d positive neurons of the TG group was significantly lower than CG in the dorsal horn laminae of the spinal cord, especially in the superficial laminae (I – II) on both sides (Fig. 4. \textit{a and b} and Fig. 5).
In the superficial lamina (I and II) there was a 25% decrease (SD = 0.4) in NADPH-d positive neurons on the injection side (Laminae I + II IPSI p < 0.001 Student’s T; effect size 1.05 ± 0.3) and a 15% (SD = 0.6) decrease on the opposite side of the injection (CONTRA I + II p < 0.001 Mann Whitney; effect size 0.30 ± 0.7), and in the deep laminae (III and IV) 34% decrease (SD = 0.4) was observed in the number of neurons on the injection side (Laminae III + IV IPSI p = 0.013 Mann Whitney; effect size 0.37 ± 0.1), but not in the number of neurons on the contralateral side (CONTRA III + IV p = 0.38 Mann Whitney) (Fig. 5a-d).

The number of NADPH-d positive neurons in lamina X

The NADPH-d positive neurons in lamina X contain bigger cell bodies and indicate a variety of morphologies. It was observed that some of the neuronal appendages extended into the dorsal horn (Fig. 6).

Around the central canal (lamina X), the number of positive NADPH-d neurons in the upper-quarter of the injected area (synapsing on the same side as the dorsal horn) demonstrated a significant (p = 0.016 Mann Whitney) reduction in the number of positive NADPH-d neurons in TG compared to CG (Fig. 7).

The supra-spinal areas focused on for histochemical evaluation in this study were the PAG, parabrachial nucleus, and laterodorsal tegmental nucleus (LDTg). However, given the high density of neurons in these areas in multiple layers, it was impractical to count them accurately with a camera lucida, and visual observation showed no change on the injected side compared with the noninjected side and also for TG compared with CG (Fig. 8a-c).

Immunohistochemistry reaction of c-Fos protein

The immunohistochemical reaction of c-Fos protein in the lumbar area of the dorsal spinal cord and supra-spinal centers was also investigated in response to the inflammatory pain induced by formalin. The results indicated that positive c-Fos neurons in the dorsal spinal cord of both CG and TG were observed just in the injected side (Fig. 9a,b). There was a highly significant reduction in the number of positive c-Fos neurons in lamina (I – II) for TG compared to CG (p = 5.03×10⁻²⁰ Student’s T; effect size 0.78 ± 0.16).

Discussion

In this study, the effect of chronic pretreatment of Neurobion on an inflammatory pain test was investigated using both behavioral and morphological methods. To our knowledge, this is the first study comparing B vitamin treatments that utilizes NADPH-d and c-Fos to evaluate the potential role of NO in the pain pathway in the dorsal horn of the spinal cord and supra-spinal centers.

We found that Neurobion pretreatment resulted in a clear and significant reductions of behavioral responses to pain in the chronic phase. This result is in accordance with studies, which reported that chronic pretreatment of B-complex could decrease the pain response behavior i.e. the licking time and flinching scores in response to pain induced by the formalin injection model.
Our results indicated that formalin injection in rats right paw could increase the number of the NADPH-d positive neurons on both sides of the dorsal horn at the lumbar level of the spinal cord as well as in the X lamina. More interestingly, after formalin injection in the Neurobion pretreated group, the number of NADPH-d positive neurons on both sides of the surface lamina in the dorsal horns showed a significant decrease compared to the control group. The same pattern was observed in the area around the central canal.

Studies have shown that neurons containing NOS in these laminae are actually spinothalamic projection neurons involved in spinal cord pain processing, and bilaterally activation of these neurons in the spinal cord has been reported following inflammation caused by formalin or carrageen injection in a rat’s paw.

Considering the reduction of pain behavior alongside the reduction of the number of NADPH-d positive neurons in the superficial laminae of the dorsal horn and lamina X, it is reasonable to assume reduced pain is associated with changes in nitric oxide mediated neural transmission. Supporting this view following formalin injection in the hind paw of the rat, the number of neurons that express nNOS in the lumbar of the spinal cord increases, along with blocking NO synthesis(L-NAME) reduced the effect of B vitamins, which suggests that B vitamins exert some of their analgesic effects by releasing NO.

It has been also shown that stimulation of primary sensory neurons by a variety of painful stimulations causes rapid observation of the expression of the c-Fos protein in the nucleus of post-synaptic neurons in the superficial layers of the dorsal horn of the spinal cord unilaterally, which is used as an indirect marker of neuronal activity in determining pain pathways.

The expression of c-Fos pattern in visceral stimulations is different from its pattern in somatic stimulation which expresses bilaterally. Expression of c-Fos after stimulation of pain receptor neurons in the spinal cord can be stopped by analgesics and pain killers such as morphine, Indomethacin and ketoprofen before carrageenan test. Each of these demonstrations confirms the relation between c-Fos expression and pain. In our study, we also observed a significant decrease in the number of c-Fos positive neurons in the posterior spinal cord lamina in the Neurobion pretreatment group compared to the control group. However, studies have shown that c-Fos is still expressed after anesthesia which reduces pain or pain responses. It has been reported that after pain relief, c-Fos expression is still high, which indicates that c-Fos expressing cells are still sensitive, and there are theories that c-Fos expression represents central sensitivity.

It has been previously suggested that NO-producing cells may have an intrinsic property that creates a lower threshold for the expression of c-Fos than other neurons. As well, it is possible that NO released from the cellular body or axon-dendrites of these neurons induces the c-Fos expression by affecting nearby neurons. However, Todd et al. have reported that some inhibitory neurons in the spinal cord cause c-Fos expression after formalin injection. It seems that licking, as a non-painful stimulation, affects the threshold of nearby neurons by stimulating primary sensory neurons, therefore, increasing the
expression of c-Fos in painful situations. The c-Fos positive cells may contain some interneurons containing inhibitory neurotransmitters such as GABA, glycine, enkephalin, or dynorphin. It is probably possible that licking plays an important role in modulating the expression of c-Fos in the dorsal horn of the spinal cord, especially in the superficial lamina on the same side.

There is also evidence suggesting the role of c-Fos neurons in modulating hyperalgesia. Hunter et al. reported the importance of c-Fos in the formalin test, which is necessary to reduce pain (possibly by the mechanism of dynorphin)\(^4^6\). During hyperalgesia, the adjustment or compensation mechanism is upregulated. Therefore, the nervous system tries to weaken hyperalgesia with the Fos mechanism. This explains why L-NAME reduces c-Fos because it reduces progression by the NMDA-NOS knockout mechanism and suggests that NO may be involved in understanding chronic pain by strengthening the activity of spinal cord pain receptor neurons by the NMDA receptor-dependent mechanism. It is also suggested that NO be involved in the production of Fos protein. Therefore, the use of NO-blocking agents may be a way to inhibit the progression of hyperalgesia after inflammation and perhaps even after peripheral nerve injury.

Combining previous works and the results of our study, the expression of c-Fos due to the increase of pain indices in the dorsal spinal cord and their reduction after the pretreatment with Neurobion could indicate the role of c-FOS in the induction of pain. In this model of inflammatory pain, at least part of the analgesic effects of Neurobion is probably due to a reduction in the number of active neurons that receive or transmit pain which uses nitric oxide as a pain neurotransmitter.

Some studies also have suggested that the analgesic effect of some painkillers is due to the activation of descending inhibitory pathways. For instance, in a study that used the formalin test and then evaluated the effect of intracerebroventricular injection of enkephalin, the results demonstrated that pain score decreased, and c-Fos expression in laminae related to pain processing in the spinal cord was inhibited\(^2^1\). This experiment is strong evidence for the theory that supra-spinal opioids reduce pain by activating descending inhibitory control. In particular, the activation of PAG neurons stimulates neurons in the raphe nuclei, which synapse with the sensory neurons in the spinal cord and inhibit them. This study suggests that the descending inhibitory mechanism (which inhibits the firing of the neurons responding to pain), that acts at the spinal cord level is essential for pain-reducing behavior in the formalin test. Bilateral injury on the posterior surface of the pectoral spinal cord prevented pain relief and reduces the c-Fos expression of intracerebroventricular injection of enkephalin.

Although we were not able to show the effect of pain and its reduction by Neurobion in the supra-spinal region using histochemical and immunohistochemical reactions, as licking is a supra-spinal behavior, it can be concluded that Neurobion can play a part of its analgesic role by affecting these areas. On the other hand, there are positive NADPH-d, not only in areas of the brain that are associated with the pain pathway, such as the hypothalamus, gray matter around the central aqueduct, reticular nucleus, Raphe Magnus, and Locus coerules\(4^7\), but also in other areas that are not involved in pain, such as tegmental nuclei, superior and inferior colliculus, pedunculopontine, hippocampal gyrus, supraoptic nucleus and
striatum. This indicates a clear role of nitric oxide in the transmission and processing of other information in addition to pain across differing brain areas. Therefore, due to the different roles of supra-spinal regions, it prevents observation and measurement of the pain reduction effect of Neurobion without the investigation of other factors.

Conclusion

Our results suggest that B-complex pretreatment can reduce the chronic phase of pain. Downregulation of c-Fos and NADPH-d in dorsal spinal neurons in the vitamin-treated group suggested that the antinociceptive effect induced by Neurobion could be due to a suppression of nociceptive transition at the spinal level, especially at the afferent region in the lumbar section of dorsal spinal horn. At least a part of the analgesic effects of Neurobion could be the result of decrement in the number of the active neurons after pain inducement, in the site of afferent fibers entering the spinal cord. Those active neurons use NO at least as one of their pain neurotransmitters and reductions in their activity correlates with a decrease the behavioral pain measure.

Declarations

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Author Contributions

Shahab A. Zarei, Conceptualization, Experimental section, Data collection and analysis, Investigation, Methodology, Writing original draft, review and editing, and incorporated feedback from all authors; Mina Shahriari-Khalaji: Writing original draft, review and editing, Visualization, Design of graphical abstract; Ian Max Andolina: Supervision, Formal analysis, Data analysis and curation, wrote parts of the manuscript and provided feedback on manuscript drafts, review and editing, Visualization; Gila Behzadi: Supervision, Conceptualization, Material preparation, Curation, Investigation, Methodology, Data monitoring, Original draft, Provided feedback on manuscript drafts; All authors read and approved the final manuscript.

Declaration of Conflicting Interests

The authors declare that there is no conflict of interest.

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**Figures**
**Figure 1**

The separate phases of the pain response in formalin test. First phase, interphase, second phase and recovery time in the pain score of the control group (n=6)
Figure 2

Distribution plot comparison of pain behavior evaluation between Neurobion pretreatment (TG) and its control (CG) in the formalin test. Significant decreases were most clearly seen in the composite pain score (a) and behavioral responses (b-d) for interphase and Phase 2 periods (n=6, *P<0.05, **P<0.01, and ***P<0.001)
Figure 3

Comparison of pain score between Vehicle group (VG) and control group (CG) in the formalin test. The mean pain score has not any significant difference (n=6)
Figure 4

Schematic diagram (upper) and Photomicrograph of NADPH-d positive neuron (lower) in lumbar spinal cord. The Schematic diagram shows white and grey matter of the spinal cord and its dorsal and central horn laminae. Roman numerals in the schematic figure indicate the corresponding laminae. M, shows the medial side and L shows the lateral side of the dorsal horn. The Photomicrograph of NADPH-d positive neuron shows the dorsal horn laminae of the CG (a) and TG (b) groups. (Scale bar = 100μm)
Figure 5

The NADPH-d positive neurons after formalin injection in superficial and deep laminae of the dorsal horn in CG and TG. The population plots show the number of NADPH-D positive neurons in the lamina of the dorsal horn ipsilateral to (a and b) and contralateral to (c and d) the injected side. (*P<0.05 and ***P<0.001)
Figure 6

NADPH-d positive neurons in dorsal horn and X lamina. (a) and (b) represent spinal cord sections and activated neurons in the dorsal horn of the injection site in the control group. (c) NADPH-d positive neurons whose axons extend from the central canal (lamina X) to the dorsal horn. (d) neurons at a higher magnification. The white arrow in (a) indicates the knife cut determining the side opposite the injection. (Scale bar = 200 μm) (C.C: Central Canal)
Figure 7

Photomicrograph of lumbar spinal cord sections of positive NADPH-d neurons around the central canal, in CG (a) and TG (b). The raincloud plot (c) shows the positive NADPH-d neurons on the injected side of the central canal in CG and TG groups after formalin injection. (Scale bar=100μm) (*p<0.05)
Figure 8

NADPH-d positive neurons in the supra-spinal areas. (a) The positive NADPH-d neurons (in purple) in LDTg (a) and dorsal area of the PAG in CG(b) and TG (c). There was no significant change on either side of the injection and in both control and Neurobion pretreatment groups. (Scale bar=500 μm). LDTg: laterodorsal tegmental nucleus, mlf: medial longitudinal fasciculus, 4V: 4th ventricle, and PAG: Periaqueductal gray

Figure 9

Immunohistochemical reaction of c-Fos protein in the superficial lamina (I – II) in CG and TG groups. Microscopic images indicate the dorsal spinal cord in the injected side of CG (a) and TG (b) and the mean difference in their neurons number has shown on the chart (c). The black arrows indicate c-Fos positive neurons. (***P<0.001)
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