Improved antioxidant defense in the ventral tegmental area increases pain tolerance in male rats

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Summary

The ventral tegmental area (VTA) is a brain region recently shown to interfere with pain perception. According to previous studies, improvement of antioxidant defense mechanisms reduces pain. The aim of this study was to show that lack of social interaction influences oxidative stress in the VTA and that this results in increased pain tolerance.

In this study, 21 male Sprague-Dawley rats were randomly divided into 2 groups: paired and socially isolated (SI). After one week of acclimatization, the SI rats were isolated for 14 days and the other rats were paired for the same period. On day 15, pain tolerance was assessed through the tail flick test, and two days later the rats were investigated for anxiety in an elevated plus maze (EPM). Indices of oxidative stress (levels of malondialdehyde, glutathione, nitrite/nitrate and catalase activity in the VTA) were then studied in the animals.

Malondialdehyde in the VTA was found to be reduced in the SI rats compared with the paired rats. Furthermore, catalase activity, glutathione and nitrite/nitrate levels in the VTA were increased in SI compared with paired rats. Pain tolerance on the tail flick test was increased in SI rats compared with paired rats, while anxiety, as assessed by EPM, was reduced in the SI rats compared with the paired rats.

Improvement of antioxidant defense and reduction of oxidative stress in the VTA increases pain tolerance and reduces anxiety. In this sense, environmental conditions play an important role in pain control.

KEY WORDS: catalase activity and nitrite/nitrate, glutathione, malondialdehyde, pain and VTA.

Introduction

Pain is an undesirable phenomenon whose nature, in some conditions, is unclear. Furthermore, poor localization of pain makes its alleviation difficult in some conditions. Thus, distinguishing brain regions that can interfere with the experience of pain may be a significant help in shedding light on this experience. The ventral tegmental area (VTA) is a brain region recently shown to interfere with pain perception (Li et al., 2016). Negative or positive reinforcement of pain in response to certain stimuli is perhaps the most useful mechanism for distinguishing different experiences of pain phenomena, because it makes it easier to identify factors that worsen or alleviate pain. The VTA has recently been investigated for this purpose, and one study in particular has shown that the VTA can balance pain in a negative-reinforcing manner. This means that negative reinforcement lessens pain perception (Navratilova et al., 2012). Oxidative stress is a factor known to influence brain function. Pain perception can also be alleviated by improving antioxidant defense mechanisms (Adebayo et al., 2015). In the present study, these mechanisms were evaluated in the VTA.

Emotional state can also modulate pain perception (Lumley et al., 2011), but the exact molecular mechanism involved in this has not been well elucidated. The VTA, as part of the limbic system (Ikemoto, 2007), may possibly alter emotional state, and in turn, affect pain perception.

The aim of this study is to provide new evidence that lack of social interaction increases pain tolerance. A further aim is to show that this increase in pain tolerance is associated with improvement of antioxidant defense mechanisms in the VTA.

Materials and methods

Animal care

Rats (8 to 10 weeks old) were housed individually in small standard polycarbonate cages (27x15x21) or together in large (42x15x21) polycarbonate cages with ad libitum access to food and water. The cages were kept in a temperature-controlled (22 ± 1°C) and humidity-controlled (40-70%) vivarium with a 12 h/12 h light/dark cycle (lights on at 7:00 a.m.). All behavioral testing took place during the lights-on phase of the cycle (specifically, from 11:00 a.m. to 3:00 p.m.) in the experimental room. All rats were habituated to the Tehran University of Medical Science vivarium for at least 7 days before the start of the experiments. In this study, 21 male Sprague-Dawley rats weighing about 200-250 grams were used. Seven rats were kept in isolation. To induce the paired state the other 14 rats were housed in pairs.

Ethical approval

All the experiments were performed in accordance with the guidelines for the care and use of laboratory animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996). The experimental
protocol was approved by the institutional animal care and use committee of Tehran University of Medical Sciences (approval reference code: 91-01-158-18022, Tehran, Iran). Every effort was made to minimize the number of rats used in this study and their suffering. Anesthesia and sedation before euthanizing animals for obtaining specimens were properly conducted through a skilled procedure performed using well-maintained equipment that ensured rapid death.

**Experimental procedure and tissue preparation**

After 14 days, the rats were assessed for pain tolerance and anxiety. They were then sedated with xylazine (10 mg/kg) and anesthetized and euthanized with ketamine (100 mg/kg). The VTA was removed and immediately frozen in liquid nitrogen. For the preparation of homogenized tissue, the VTA was homogenized with buffer phosphate after weighing with a digital balance (1/10).

**Malondialdehyde assessment**

The extent of lipid peroxidation was measured as formation of thiobarbituric acid (TBA) reactive substances, according to the method of Esterbauer and Cheeseman (1990). A sample containing 100 microliters of homogenized VTA was mixed with 1 ml TCA (20%) (Sigma-Aldrich Co.) and 2 ml TBA (1%) (Sigma-Aldrich Co.) and heated for 1 h at 100°C. It developed a purple color after boiling. After immediate cooling, the precipitate was removed by centrifugation.

The final product was assessed with a spectrophotometer at a wavelength of 532 nm. The final malondialdehyde (MDA) concentration was obtained with application of an equation calculated using Excel software (y = 0.0918x + 0.0452). For obtaining this equation the wavelengths of maximum absorptions of sulfanilamide and phosphoric acid utilized in spectrophotometric analysis were used. The final concentration was expressed as µmol/g of tissue (Esterbauer and Cheeseman 1990).

**Glutathione assessment**

The spectrophotometric reader assay method for glutathione (GSH) involves oxidation of GSH by the sulfhydryl reagent 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB) to form the derivative 5′-thio-2-nitrobenzoic acid (Ellman reaction). To perform this investigation a mixture of Tris buffer (Sigma-Aldrich Co.), DTNB (Sigma-Aldrich Co.) and methanol (Sigma-Aldrich Co.) was utilized. Then, homogenized VTA tissue (100 microliters) was mixed with the above solution. The mixture was then incubated at room temperature for 15 minutes and developed a yellow color.

The final product was assessed with a spectrophotometer at a wavelength of 412 nm. The final GSH concentration was obtained with the equation calculated using Excel software (y = 0.0918x + 0.0452). For obtaining this equation the wavelengths of maximum absorptions of above mentioned utilized substances (DTNB, Tris buffer and methanol) in spectrophotometric analysis were used. The final concentration was expressed as µmol/g of tissue (Roma et al., 2012).

**Nitrite/nitrate assessment**

The Griess Reagent System is based on the chemical diazotization reaction that was originally described by Griess in 1879. Basically, to perform this examination n-1 (naphthyl) ethylenediamine (NEDD) (Sigma-Aldrich Co., Taufkirchen), sulfanilamide (Sigma-Aldrich Co.) and phosphoric acid (Sigma-Aldrich Co.) were combined. The resulting solution was then mixed with 100 microliters of homogenized VTA tissue. The mixture was then incubated at room temperature for 15 minutes and developed a pink color. The final product was assessed with a spectrophotometer at a wavelength of 540 nm. The final nitrite/nitrate content (an indicator of nitric oxide activity) was obtained with the equation calculated using Excel software (y = 0.02775x + 0.05114). To obtain this equation the wavelengths of maximum absorptions of the above-mentioned substances (NEDD, sulfanilamide and phosphoric acid) utilized in spectrophotometric analysis were used. The final concentration was expressed as µmol/g of tissue (Miranda et al., 2001).

**Catalase activity**

The assessment of catalase activity was assessed by measurement of the breakdown of H₂O₂. Briefly, the reaction mixture was composed of 50 mM phosphate buffer (pH 7.0), 10 mM H₂O₂ and VTA homogenate. The reduction rate of H₂O₂ was followed at 240 nm for 30 seconds at room temperature. The enzymatic activity was expressed as units of enzymatic activity per milligram of protein (Genet et al., 2002).

**Tail flick test**

The tail flick test is a nociceptive assay involving measurement of the latency of the avoidance response to a thermal stimulus in rodents. Basically, a thermal stimulus is applied to the tail; when the animal feels discomfort, it reacts with a sudden tail movement. The tail flick reaction time is measured and used as an index of the animal’s pain sensitivity (South et al., 2009).

**Elevated plus maze**

The elevated plus maze (EPM) was used for assessing anxiety levels. The test involves the use of a plus-shaped apparatus with two open and two closed arms, each with an open roof. The arms were elevated 67 cm from the floor. Each animal was placed in the center of the apparatus and then allowed to move freely in the four arms. This model is based on rodents’ aversion to open spaces and tendency to prefer closed spaces. The number of entries into the closed arms was recorded. Reduced anxiety levels were indicated by fewer entries into the closed arms (Hill et al., 2015).

**Statistical analysis**

Data were analyzed using SPSS version 22 and GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). A two-tailed independent samples t-test was performed for all experiments. Data are as represented as mean values ± SEM; p<0.05 was considered statistically significant.
Results

**MDA in the VTA**

After 14 days, the level of MDA recorded in the socially isolated (SI) rats was lower than that recorded for the rats in the paired state ($t_{5,179} = 1.488 \pm 0.04329$ vs $0.8936 \pm 0.1127$, $p<0.0002$) (Fig. 1).

**GSH in the VTA**

After 14 days, the SI rats showed increased GSH versus the level recorded for the animals in the paired state ($t_{19.63} = 1.420 \pm 0.0075$ vs $1.919 \pm 0.02424$, $p<0.0001$) (Fig. 2).

**Nitrite/Nitrate in the VTA**

Nitrite/nitrate was increased in the SI rats after 14 days compared with the level recorded for the paired state ($t_{22.11} = 10.02 \pm 0.08333$ vs $15.53 \pm 0.2348$, $p<0.0001$) (Fig. 3).

**Catalase activity in the VTA**

After 14 days, catalase activity was increased in the SI rats compared with the value recorded in the paired rats ($t_{15.296} = 2.717 \pm 0.6872$ vs $6.125 \pm 0.2266$, $p<0.0002$) (Fig. 4).

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Figures:

- **Figure 1** - Concentration of MDA in the VTA (n=7 measurements). MDA concentration was assessed by spectrophotometer and the final concentration was corrected with a standard curve. Data are represented as mean ± SEM; *significant difference at $p<0.05$. Pair=paired; SI =socially isolated.

- **Figure 2** - Concentration of GSH in the VTA (n=7 measurements). GSH concentration was assessed by spectrophotometer and the final concentration was corrected with a standard curve. Data are represented as mean ± SEM; *significant difference at $p<0.05$. Pair=paired; SI =socially isolated.

- **Figure 3** - Concentration of nitrite/nitrate in the VTA (n=7 measurements). Nitrite/nitrate concentration was assessed by spectrophotometer and the final concentration was corrected with a standard curve. Data are represented as mean ± SEM; *significant difference at $p<0.05$. Pair=paired; SI =socially isolated.

- **Figure 4** - Catalase activity in the VTA (n=7 measurements). Catalase activity was assessed by spectrophotometer and the final concentration was obtained by means of a formula. Data are represented as mean ± SEM; *significant difference at $p<0.05$. Pair=paired; SI =socially isolated.
Pain tolerance as assessed with the tail flick test

The SI rats showed increased pain tolerance the end of 14 days when compared with the animals in the paired state. In the tail flick test increased pain tolerance is manifested as an increased latency to tail withdrawal ($t_{3.401} = 8.525 \pm 0.5388$ vs $15.08 \pm 1.849$, $p<0.0043$) (Fig. 5).

![Tail Flick Test Graph]

Figure 5 - Pain tolerance in the tail flick test (n=7 measurements). Pain tolerance was characterized by the latency to tail withdrawal. Data are represented as mean ± SEM; *significant difference at $p<0.05$. Pair=paired; SI =socially isolated.

Anxiety as assessed in the EPM

After 14 days, anxiety in the SI rats was reduced compared with that found in the paired rats. In the EPM, reduced anxiety is shown by an increase in the number of entries into closed arms of the maze ($t_{3.871} = 10.00 \pm 1.633$ vs $2.000 \pm 0.4082$, $p<0.0047$) (Fig. 6).

![Elevated Pluse Maze Graph]

Figure 6 - Anxiety in the EPM (n=7 measurements). Anxiety was measured by a number of entries into closed arms. Data are represented as mean ± SEM; *significant difference at $p<0.05$. Pair=paired; SI =socially isolated.

Discussion

In this study, it was seen that improvement in antioxidant defense mechanisms at the level of the VTA can increase resilience to pain, and also that environmental conditions such as the paired state (socialization) can influence pain tolerance by regulating oxidative stress. Additionally, emotional state affects pain perception.

There exist various experiments through which pain can be evaluated in animal models. It is necessary to be aware that the pain assessed in the tail flick test is pain caused by phasic nociceptive stimulation (Miranda et al., 2011). This is important to mention as other experiments involve the use of tonic stimulation.

In this study, the paired state was compared with social isolation in order to show that environment can influence pain perception. The reward circuit is an interesting pathway that is very useful to consider in pain studies, because, through it, pain can be controlled by positive and negative reinforcement. The VTA is part of the reward circuit. Indeed, the reward circuit consists of the hippocampus, the nucleus accumbens and the VTA. If VTA function can be controlled in a reward-based way, this means that, by strengthening or weakening stimuli, it will be possible to control pain as desired. The main evidence supporting involvement of the reward circuit in pain control is the finding of pain reduction mediated by a negative reinforcement mechanism (Navratilova et al., 2012). There also exists evidence to support the idea that dopaminergic neurons in the VTA are modulated by hippocampal neurogenesis (Luo and Huang, 2016).

Changes in hippocampal neurogenesis and the resultant behavioral changes may be taken as further evidence that the reward center is influenced by environment (Valero et al., 2011). This evidence is in agreement with the current study which supports the idea that environment may cause or diminish a behavior that may be desirable or undesirable.

The striking feature of VTA involvement in the regulation of reward-dependent behavior is that dopamine release is necessary for both negative and positive reinforcement (Taylor et al., 2016). In this regard, the antioxidant defense mechanism probably acts by increasing dopamine storage in VTA neurons. As 60% of VTA neurons are dopaminergic, the neurotransmitter most likely to be involved in reinforcing behaviors is dopamine (Oliva and Wanat, 2016).

In the present study, anxiety was assessed to investigate whether or not pain sensitivity is associated with changes in the emotional state of rats. This is important because emotional control is an effective means of controlling pain. As we showed, reduced anxiety was associated with increased pain tolerance. There are two possible mechanisms through which the VTA may reduce anxiety, which are worth mentioning here: 1) the VTA can, itself, through muscarinic receptors, change emotional state (Small et al., 2016); 2) the VTA can modify emotional state through other parts of the reward circuit (Marusak et al., 2017). In previous studies, it is well established that the emotional state of an individual can influence pain perception (Lumley et al., 2011). In this study, reduction of anxiety was associated with a better outcome in terms of pain perception.

Overall, in previous studies, improved antioxidant defense was associated with better brain function (Shirley...
et al., 2014). However, in the present study, the interesting finding is that improvements in the antioxidant defense mechanisms (increases in glutathione, nitrite/nitrate, and catalase activity) were associated with a reduction of MDA in the VTA. So, the possible mechanism underlying the observed increase in pain tolerance may be related to the reduction of oxidative stress in the VTA. Pain is an undesirable and unpleasant phenomenon that, in many situations, is difficult to control. As we know, in many conditions characterized by pain, such as migraine, patients look for a silent environment. In this study, too, we saw that isolation alleviates pain. Inducing negative reinforcement of pain is an effective mechanism that effectively reduces pain. It is concluded that enhancing antioxidant defense mechanisms in the VTA, through social isolation, may activate this negative reinforcement mechanism for reduction of pain.

In conclusion, in this study, it was shown that during social isolation oxidative stress was reduced and, in turn, the antioxidant defense mechanisms were improved in the VTA. Changes in oxidative stress status in the VTA increased pain tolerance and reduced anxiety.

References


