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Evaluation of role of septal nuclei in modulation of pain in selected pain models

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Abstract

The aim of the present work was to study the specific role of septal nuclei in modulation of pain. For this purpose bilateral septal lesion were done electrolytically in adult male wistar rats. The role of septal nuclei in modulation of pain was assessed by using two different pain tests such as the tail flick test and the formalin test.

The histological appearance showed that electrolytic lesion site involved all the parts of septal nuclei namely medial and lateral septal nuclei and anterior and posterior groups of neurons in septal region. No attempt was made to study the role of individual parts of septal nuclei in modulation of pain.

The pain threshold for thermal pain produced by radiant heat method in tail flick test was markedly low in the rats with bilateral lesions of septal nuclei, compared to the pain threshold for pain in the rats in control group. This difference in threshold was statistically significant ($p<0.001$).

Formalin test showed typical double peaked graph when pain score was plotted against time. Thus the biphasic response described for the formalin test was confirmed. There was no statistical significant difference in the threshold for pain produced by formalin in the control and operated groups.

The present work suggest that septal nuclei are involved in the processing of thermal pain sensation and its modulation, but probably not involved in the processing and modulation of chemically induced pain sensation.

Key words- Septal nuclei, thermal pain, modulation.

INTRODUCTION

The scientific study of pain has undergone rapid development in recent 20 years. Neural mechanisms that sub serve pain though much more complex are becoming clear.[1,2,3] Throughout the resurgence of attempts to understand pain and its mechanisms it has become increasingly apparent that pain is a multidimensional experience consisting of sensory cognitive evaluative and affective motivational components.[1-5] Appreciation of pain is considerably altered by various descending tracts originating in brain stem and various descending tracts originating in brainstem and various other parts of brain above the brainstem. [1-3] It is observed that very little work is carried out as regards elucidating the role of septal nuclei in modulating pain[5-7]. These nuclei are known to play role in hyper motility, spatial mapping, reproductory behavior and food intake. Septal nuclei are also one of the well established sites of self stimulation.[8] Considering these functions and taking into account their anatomical site and connections it was planned to manipulate septal nuclei and study the effect on pain rating in lab bred rats.

In our previous study we have suggested that the septum may be involved in modulation of pain and neurotransmitters like Acetylcholine, Noradrenaline and opioid peptides may have an individual or collective role to play in modulation of pain.[6-7] The present study was meant for exploring the role of septal nuclei in modulation of pain by using two different pain tests such as the tail flick test and the formalin test.

MATERIALS AND METHODS

1. Animals

40 adult male Wistar rats of 150 days old and weighing between 200gms and 220 gms were used for the experiments. The surgical animals were housed in separate polyvinyl cages under 12 hour light and dark regime [light on at 600 hours and off at 1800 hours] and were fed with standard diet and water ad libitum.

A prior permission was taken from the Institutional Ethics Committee of Rajarambapu College of Pharmacy, Kasegaon (RCPK) for use of rodents for the present research work. RCPK is registered under CPCSEA [Committee for the Purpose of Control and Supervision of Experiment on Animals]. The protocol of the present study was approved by the CPCSEA approved committee of RCPK.

2. Chemicals and Anaesthetics

Chemicals - 2.5% buffered formalin acetate was used to carry out formalin test. The chemicals used for perfusion of animal at the end of experimental session were formal saline and potassium ferrocyanide. The chemicals used for histological procedure were absolute alcohol and alcohol in various concentrations of chloroform and eosin.

Anaesthetics- Anaesthetics used for operative procedures was sodium pentobarbitone (LOBA chemicals, Mumbai) to maintain a steady level of anaesthesia while performing electrolytic lesions in the septal nuclei.

3. Instruments

a) Stereotaxic instrument (Inco, Ambala, India)- This was used for putting electrode in the septal nuclei for the purpose of sham operation and for making electrolytic lesions in the septal nuclei. It has two calibrated lateral bars. Calibrated aural bars are fixed at right angle to lateral bars through clamps provided. A rat head holder with longitudinal groove is present at the centre of the main frame. It can be moved in anterior-posterior direction. The head holder has a horizontal incisor bar which can be moved vertically to adjust its height. A nose bar helps to

keep the head pressed firmly in position. The electrode is fitted in electrode carrier which has arrangement for vertical, lateral and antero-posterior movement for exact placement of electrode.

b) Behavioral cage - The behavioral cage used for observation of animals for scoring pain behavior in formalin test was a wood box constructed specially with dimension 45x30x30 cms. It had transparent glass walls from all sides and transparent glass roof. It had a sliding glass door in front through which animals are introduced in to the cage.

c) Anagesiometer- Radiant heat type, mark I Analgesiometer (INCO, Ambala, India) was used for tail flick test. The instrument makes use of heated nichrome wire to provide radiant heat as a pain stimulus rat's tail. The description of the Analgesiometer is as follows- On top, are left hand side, there is the chrome plated water jacket in the centre of which is nichrome wire .On the sloping panel are: 1) Pilot lamp –TO see weather current is flowing through the nichrome wire. 2) On –off switch. 3) Ampere meter – To adjust and keep constant amount of current flowing through the nichrome wire.

Operation of the instrument- One end of water jacket is attached to a water tap by suitable rubber tubing. To another end of water jacket is attached another rubber tubing to allow the water to run waste. During experiments free circulation of water is maintained through the water jacket to provide a constant ambient temperature around the stimulus area. Instrument is connected to 250 volt A.C supply. When switch on, the pilot lamps light up. The knob on the ampere meter is adjusted so that desired fixed amount of current flows through the nichrome wire. Before doing the test procedure, it is seen that every time same amount of current flows through the nichrome wire. Experiments are generally carried out with current of the amplitude between 3 and 4 amperes flowing through the nichrome wire. During the test, the rat is placed in the rat carrier with its tail protruding through the specially cut slit in the shutter of the carrier. The rat carrier is held with the right hand on the top of the instrument. The tail passes through the water jacket at right angle to heated wire without maki8ng any contact with the wire. The heat radiated from nichrome wire due to current flowing through it thus gets projected to the segment of the tail.

4. Experimental protocol

A] Operative procedure (Stereotaxic procedure) [6-7]

Each rat was anaesthetized with pentobarbital sodium dissolved in sterile distilled water. The anesthetic solution was injected intraperitoneally in the dose of 35 mg/kg of body weight. It took, on an average 10 minutes for rat to come under anesthesia. When required, intermittent ether inhalation was use to maintain the level of anaesthesia.Then the scalp of animal was shaved, the animal was kept in the stereotaxic frame and head was fixed by two calibrated aural bar was kept same and this confirmed that head is exactly in the center of 2 bars. The incision bar was kept at zero position i.e. 5mm above the interaural line and the upper jaw of the animal was fixed in this incisor bar with the help of upper incisor teeth. Midline skin incision was taken on the skull and fascia and muscle were retracted after retracting the skin. The clean bone surface with its sutural landmarks was exposed after proper hemostasis. The bregma was located. After adjusting the stereotaxiccoardinates for septal nuclei (described below), the points were marked on the skull on either sides of midline with the help of sharp stainless steel pointer of the stereotaxic apparatus. The skull bone overlying these points was bored with the help of special bone drilling screw, so as to make a hole to pass the electrode. The stereotaxic co-ordinate was measured with reference to bregma. The coordinates selected for septal nuclei were from stereotaxic atlas for rats brain and were as follows, 1] 1 mm anterior to bregma. 2] 0.5 mm on either side of sagital sutures line. 3] 5.5 mm deep depth below the surface of skull bone. Unipolar electrode (gauge 28) varnished except at the tip was placed stereotaxically in the septal nuclei first on one side and then on the other side. Another electrode was fixed to the ear of the animal. From dry cell battery source anodal DC current adjusted through multimiter to 1.5 milliamperes was passed for 20 seconds. This was done on either side to make the anodal lesions bilaterally in the septal nucei. In case of sham operated group (sham group) the whole procedure remained the same except that no current was passed through the electrode. After the procedure was over, electrode was taken out, the skin was sutured and the site was cleaned with spririt. Nebasulf powder was applied. Injection benezathine penicillin 10,000 I U was given intramuscularly and animal was transferred to its cage. The animals were observed for 7 days post-operatively when the aggressive behavior or such other affective responses disappeared. Septally lesioned 20 animals were divided in to two groups consisting of 10 animals each. One septally lesioned group and one sham operated group and sham operated group underwent tail flick test.

B] The formalin test [10,11,12]

The details regarding formalin test are given below: The formalin testing was performed as described for rats. Each rat was habituated to behavioral cage for 1 hour in a day of the 3 days prior to testing to minimize stress. This is necessary because novel environments have been shown to reduce pain during formalin test. On the day of testing, 1.25 ul of 2.5% buffered formalin acetate was injected subcutaneously with the help tuberculin syringe, in to the planter surface of right hind paw. The rat was then observed in behavioral cage for one hour following injection and pain score was determined. The rat's tendency to favor the injected paw was rated using the pain rating scale described by Cohen et al [10] as summarized in table 1.

Table 1- The pain rating scale described by Cohen et al.[10]

Score	Response
0	The injected paw is placed normally on the floor.
1	The injected paw is favored but still in contact with floor.
2	The injected paw is elevated and not in contact with the floor.
3	The injected paw is licked or chewed.

C] Tail flick test [6,13]

The details regarding the tail flick test are as follows: The animals were put in a rat carrier with its tail protruding out through the slit in the shutter. After an 15 minutes adaption period, the 1 cm segment of the tail, 5 cms away from the tip of tail was kept in the slit of water jacket above the heating wire of Analgesiometer. The A.C current of 4 ampere was switched on through the heating wire and simultaneously the stop watch was started.Radient heat was thus focused on the tail of the rat. The latency between the onset of heat stimulus and abrupt flicking movement of tail was measured with the help of stopwatch. The radiant heat threshold depends only on the temperature attained in the tissue and very little on the rate of rise of temperature or the size of body area simulated. All the animals were tested for trials separated by an interval of 15 seconds. A cut off line of 45 seconds was fixed to avoid damage to the tail. The sham group and operated group were tested under the same environmental conditions.

D] Histology [6-7]

At the end of the experiments, each animal was anaesthetized with pentobarbitone given intraperitoneally in the dose of 35 mg/kg of body weight. Thorax was opened. The animal was per fused with 5% potassium ferrocyanide solution prepared in 10 % formal saline for Prussian-blue reaction to occur. The tip of the needle attached to the perfusion set of formal saline bottle was put in the cavity of left ventricle after piercing its wall and formal saline drip was started. The tip of the needle of another perfusion set was put in the cavity of right ventricle and blood was allowed to drain out of the body till the clear formal saline fluid started coming out. This ensured that potassium ferrocyanide in formal saline injected through the left ventricle could reach to every part of the body including the brain. After this the head of the animal was removed: skin was reflected and skull was opened. The brain was taken out and treated with 10% HCL solution for Prussian-blue reaction to occur. After the fixation the sections were stained with eosin. After drying sections were mounted in glycerine and observed under light microscope.

5. Statistical analysis

The mean of formalin scores (pain response) and tail flick latencies in sham operated and septally lesionsed rats were calculated and students 't' test was applied to assess the level of significance in observed differences.

RESULTS

1. Formalin test

For formalin test 20 animals were used in all. These are divided into two groups. One group of 10 animals was treated as control group and underwent the sham operative procedure. Second group of 10 animals was treated as operated group and underwent the procedure of bilateral electrolytic lesioning of the septal nuclei. The pain score was recorded in both the groups by Cohen's method after injecting the buffered formalin acetate in the right hind paw. The score was recorded in blocks of 5 mins for the total duration of 1 hour. Thus there were 12 such blocks in which recording was done. Time of injection was taken as zero time. In each group the mean of the reading obtained in all 10 animals was calculated separately for each block. As seen in figure 1 the score recorded in each block of 5 minutes in each sham operated animal has been depicted along with the septally lesioned animals. Formalin test showed typical double peaked graph when pain score was plotted against time. Thus the biphasic response described for the formalin test was confirmed. There is no statistically significant difference in the threshold for chemical pain produced by formalin between the control group and operated. Figure 1 show curves obtained for control group as well as operated group. The curves show two distinct peaks of pain score one at the end of 5 minutes and another at the end of 40 minutes. The first peak is supposed to be due to acute chemical pain and second due to inflammation induced by formalin. In the present study there was marked but localized visible swelling of the injected paw starting 10 minutes after the zero time, redness was also seen. The swelling and redness became maximum 30 to 40 minutes after the injection. The second peak of the pain score occurred about the same time.

The mean of Pain responses along with area under curve in formalin induced pain test is shown in figure 2. The area under curve was calculated in both the groups such as control and operated. Area under curve for control group was calculated to be 157 cm^2. The area under curve for lesioned group was 149 cm^2.

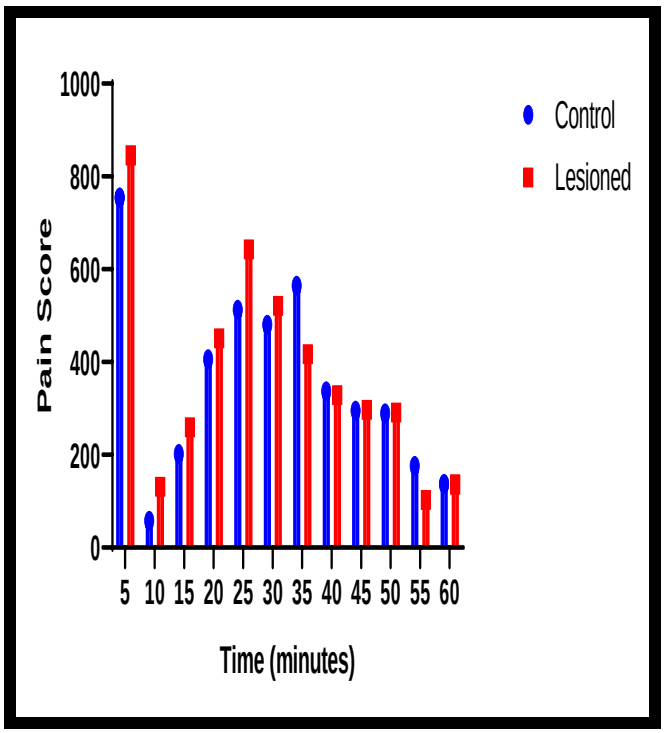

Figure 1 Pain response in formalin induced pain

The graph shows twelve time blocks (each of 5 minutes) in which score was recorded, where n=10 for each group. The results are shown as mean ± standard error of mean.

2. Tail flick test

For tail flick test, 20 rats were used in all. These were divided into two groups. Each consisting of 10 animals each where first group served as control and the second was treated as operated group and underwent the procedure of bilateral electrolytic lesioning of the septal nuclei. Tail flick latency was measured in both the groups under similar experimental situations.

Figure no. 3 shows the bar diagram for the tail flick test .The height of the bar shows the mean of the tail flick latencies obtained for 10 animals in that group . The bar diagram stresses the significant difference in the radiant heat pain threshold between operated group and the control group, in a simpler way. In both sham operated and lesioned group mean of the 10 mean readings obtained for 10 animals was calculated. Student's t test was applied. As the value of P is less than 0.001, the results are statistically significant.

The pain threshold for thermal pain which was produced by radiant heat method used in tail flick test was marked low in the rats with bilateral lesions of septal nuclei compared to the pain in the rats in control group.

Thus, there is no significant difference in the chemical pain threshold between the control group and operated group as evident from the results of formalin test. However there is significant difference in the radiant heat pain threshold between the control group and operated group as evident from the results of tail flick test. This clearly indicates the relationship between septal nuclei and threshold for acute thermal pain. This is discussed further under discussion.

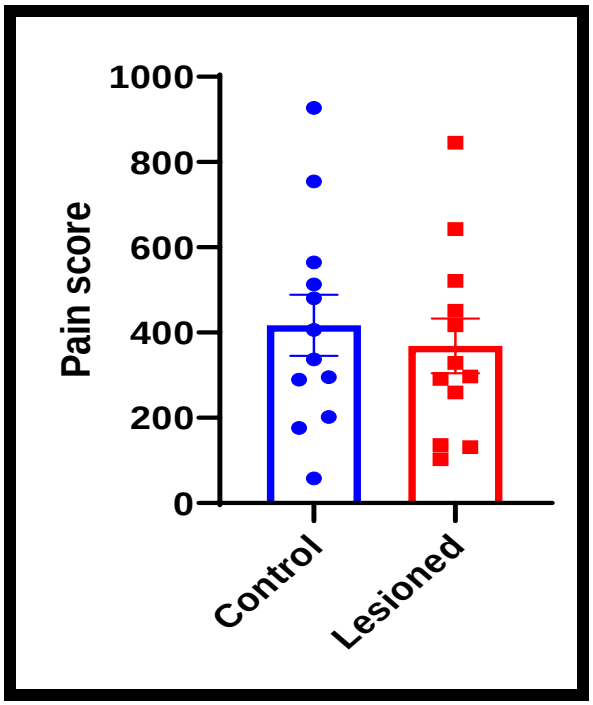

Figure 2 Mean of Pain responses in formalin induced pain.

The results are shown as mean ± standard error of mean, where n=10 for each group. The difference of mean of the pain score of the lesioned animals compared with the control.

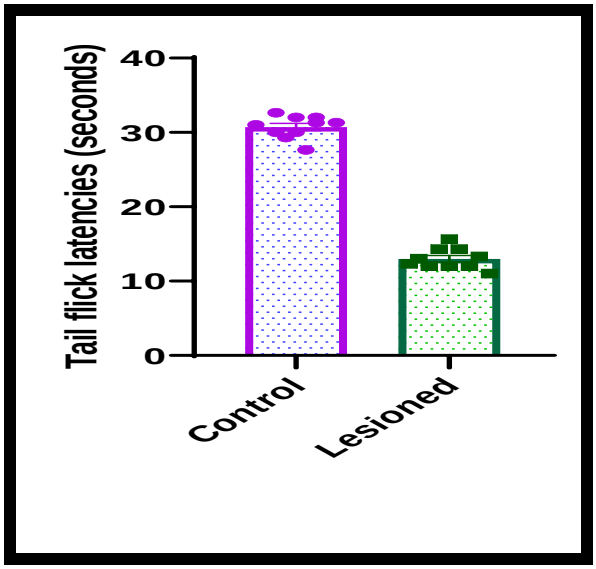

Figure 3 Pain responses in Tail flick test

3. **Histological Studies-** The histological appearance showed that electrolytic lesion site involved all the parts of septal nuclei namely medial and lateral septal nuclei and anterior and posterior groups of neuron in septal region. Figure 4 Stained section of rat's brain with typical bilateral lesions of septal nuclei. Stained sections of rat's brain with typical bilateral lesions of septal nuclei are shown in Figure 4. No attempt was made to study the role of individual parts of septal nuclei in modulation of pain.

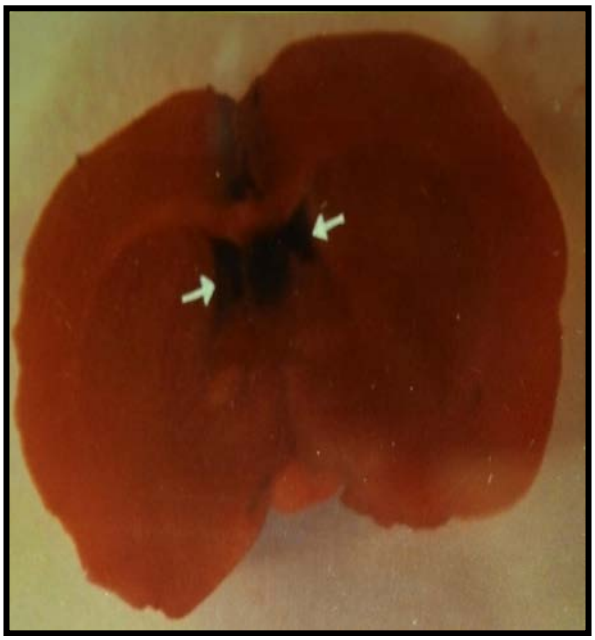

Figure 4 Stained section of rats brain with typical bilateral lesions of septal nuclei, shown with arrows

DISCUSSION

In the present study two tests were used to study the modulation of pain threshold by septal nuclei. Septal nuclei were lesioned electrolytically in wistar male rats. The type of pain test used to determine the antinosiceptive activity of any area in the central nervous system is of

outmost importance.[1-4] This is because different pain tests yield different results even when the same area in the brain is manipulated pharmacologically or by electrical stimulation or by electrolytic lesions or when the same stress is used to evaluate stress induced analgesia.[12-14] For example procedures like electrical stimulation or lesions of rostral brain regions influence formalin pain but have no effect the foot flick latencies of foot flick test.[12-13] On the other hand lesions of periaqueductal gray attenuate morphine analgesia in the tail flick test but not in the formalin test.[12]

The results of the present study are discussed under two headings-

1. The pain test used type of pain it produces and probable neural substrate affected by that test.
2. Connections of septal nuclei relevant to this study and the possible mechanism by which septal nuclei influence the pain sensation in tail flick test.

1. The pain test used type of pain it produces and probable neural substrate affected by that test- The pain test was used in the present study, namely Formalin test and Tail flick test.

A. Formalin Test

Formalin test represents a model of continuous moderate pain that seems to be a valid technique for evaluating analgesia in rodents.[11,15,16] The formalin test permits the freedom of movement to the animal and elicits complex integrated behavior in response to pain produced by a chemical stimulus associated with inflammation.[11,15,16] The physiological mechanisms carrying the phasic pain sensation produced by brief exposure to a noxious stimulus as elicited by radiant heat in the tail flick may be different from those involved in the tonic pain of formalin test. Thus, continuous or tonic pain may be sub served by different mediating and modulating substrate of central nervous system than those involved in the transient or phasic pain. In addition, animal models of nociception which produces moderate, inescapable and prolonged (tonic) pain, such as the formalin test are presumed to have a greater affective component than tests which measure reflexive responses to threshold level, escapable, brief pain as the tail flick test.[11]

Thus for evaluation of the role of septal nuclei (in modulation of pain), which form integral part of the limbic system and are presumed to have role in various behavioral response, formalin test was considered equally appropriate as the tail flick test.[6,8,9] The assays of tail flick test and formalin test was considered equally appropriate as the tail flick test. Tail flick test involves brief rapidly rising pain whereas the time course of the pain produced by formalin injection is biphasic.

Initially pain subsides in 5 to 10 minutes but the reappears 10 to 15 minutes later and persists for 1 to 2 hours. Moreover there is an excellent temporal relationship of biphasic response in formalin test with electrophysiological findings. In a report, Dickenson and Sullivian [17] demonstrated a biphasic firing pattern of lumbar dorsal horn neurons from the receptive field of ipsilateral hind paw following injection of formalin. The researchers observed that an initial high frequency increase in firing was followed, after a quiescent period by a more persistent lower frequency discharge. The transient early phase of nociceptive response in this test has been attributed to direct effects on sensory receptors and the late phase to additional factor of ensuring inflammatory response.[10-11]

The time course of formalin induced spontaneous nociceptive behavior from the present study correlates well with those of other researchers who used a behavioral rating scale.[11,16,17] The biphasic pattern of pain is clearly seen in the curves displayed in fig 1.

However, there is no significant difference in the pain threshold for chemical pain, between the control group and operated group. This indicates that septal nuclei are probably not involved in the modulation of chronic, inescapable chemical pain as produced by formalin.

B. Tail Flick Test

The test tail flick is very commonly used in studies of analgesic mechanisms in rats. The tail flick response is spinally mediated reflex, and its latency represents a spinal thermal pain threshold.[13-14] The spinal organization of the tail flick reflex was initially established in studies showing that spinalized rats retained the ability to demonstrate the reflex. [13-14] The test has been used extensively as an index of nociceptive transmission at spinal levels and as an assay for studying the influence of antinociceptive treatment on the segmental processing of nociceptive information.

As already observed in the results there is significant difference between the thermal pain thresholds of control and operated group. With lesions of bilateral septal nuclei animal develops very low threshold for the thermal pain as compared to the control group animals. This clearly indicates that septal nuclei are involved in the processing and modulation of acute thermal pain as used in the tail flick test.

The results are shown as mean ± standard error of mean, where n=10 for each group. The difference of mean tail flick latencies was stastically significant ($p<0.001$) of the lesioned animals as compared with the control.

2. Connections of septal nuclei relevant to this study and possible mechanisms by which they influence the pain sensation in tail flick test.

The septal neurons are sensitive to somatic stimulation is known for a long time. These authors observed that somatosensory stimulation could elicit bursting activity in the septum. [18-20] Further the septal neurons are sensitive to somatic stimulations also. Dutar et.al[21] noted that majority of identified septohippocampal neurons in rats were activated by noxious stimuli. Stimulation of septal region can modify the consequences of painful stimulation. For example septal intracranial stimulation (SICS) can impair conditioned emotional response learning and can attenuate acquisition of a passive avoidance response.[23]

The present study has shown that there is decreased threshold of acute short term pain as in tail flick test but no alteration in the threshold for the chronic prolonged pain as in formalin test. As already discussed, acute and chronic pain are sub served and modulated by different neuronal

substrates. It seems that septal nuclei somehow maintain the inhibitory influence on the transmission of acute pain in the ascending fast pain transmitting neuronal pathways. Therefore the latency for the reflex response evoked by acute pain i.e. tail flick latency in tail flick test is decreased after bilateral lesions of septal nuclei. This is probably because tonic inhibitory influence by septal nuclei no more exists and this causes facilitation of impulse transmission in fast pain transmission pathways.

Exact mechanism and nature of the inhibitory control of septal nuclei on transmission of acute pain is however not known. No direct connections of septal nuclei with spinal cord dorsal horn neurons have been described.[22.23,24,25,26]

The efferent, afferent and intrinsic connections of the septal nuclei have been analyzed in a number of studies in the rat, autoradiographic method in a study undertaken by Swanson and Cowan[8].

The important connections with the areas concerned with the pain transmission and /or modulation are briefly as follows: The lateral septal nucleus receives major input from hippocampal formation and projects to medial septal diagonal band complex.[2,8] The ventral part of the lateral septal nucleus also sends fibers through medial forebrain bundle to the various nuclei of hypothalamus, mammilary body and to the ventral tegmental area. The medial septal nucleus / diagonal band complex projects back to the hippocampal formation by way of fornix and fimbria. The medial septal nucleus also sends fibers to the ventral tegmental area in the midbrain and raphe nuclei. Ascending inputs to the septal nuclei arise in several hypothalamic nuclei and in the brainstem aminergic cell groups.[27]

Thus the septal nuclei are closely connected by afferent and efferent connections with the most important components of descending analgesia system, i.e. with the ventral tegmental area in the midbrain and raphe nuclei. So it can be hypothesized that probably through these areas septal nuclei maintain an inhibitory control probably at the level of dorsal horn neurons on the transmission of acute thermal pain used in the tail flick test. Our experimental findings described below strongly support this hypothesis.

The affects of stimulation of lateral and medial septum with single pulses of current were assessed on the neural activity evoked by a noxious tail shock as well as spontaneous activity in 72 cells located in periventricular nuclei, periaqueductal gray (PAG), dorsal raphe and nucleus raphe magnus of anaesthetized rat.[28] Pronounced inhibitory effects of evoked activity were found primarily in dorsal rap he and periaqueductal gray. Inhibitory effect on dorsal horn neurons responsible to noxious stimuli have been reported by stimulation of septal areas in the cat.[24]

Future study involving simultaneous electrical potentials recording from the septal nuclei, ventral tegmental area and raphe nuclei when thermal pain stimulus is being given need to be done for conforming undoubtedly these as neural substrate through which septal nuclei might modulate the pain. Also to know exactly which part of the septal nuclei are involved in modulation of pain, different portions of nuclei like only medical or only lateral groups can be lesioned in different groups of experimental animals and then each group should be tested for pain threshold.

To conclude the present work suggest that septal nuclei are involved in the processing of thermal pain sensation and its modulation, but probably not involved in the processing and modulation of chemically induced pain sensation. Thus septal area may become a future target of study in neurophysiology of pain for exploring their analgesic mechanisms and their manipulation by pharmacological and other means for the reduction of pain in humans.

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