Central noradrenergic activity affects analgesic effect of Neuropeptide S
（中枢神経系のノルアドレナリン作動性活性は、ニューロペプチド S の鎮痛効果に影響する）

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Abstract

**Background:** Neuropeptide S (NPS) is an endogenous neuropeptide controlling anxiolysis, wakefulness, and analgesia. NPS containing neurons exist near to the locus coeruleus (LC) involved in the descending anti-nociceptive system. NPS interacts with central noradrenergic neurons; thus brain noradrenergic signaling may be involved in NPS-induced analgesia. We tested NPS analgesia in noradrenergic neuron-lesioned rats using a selective LC noradrenergic neurotoxin, N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4).

**Methods:** A total 66 male Sprague–Dawley rats weighing 350–450 g were used. Analgesic effects of NPS were evaluated using hot-plate and tail-flick test with or without DSP-4. The animal allocated into 3 groups; hot-plate with NPS alone icv (0.0, 1.0, 3.3, and 10.0 nmol), tail-flick NPS alone icv (0.0 and 10.0 nmol), and hot-plate with NPS and DSP-4 (0 or 50 mg/kg ip). In hot-plate with NPS and DSP-4 group, noradrenaline content in the cerebral cortex, pons, hypothalamus, were measured.

**Results:** NPS 10 nmol icv prolonged hot plate (%MPE) but not tail flick latency at 30 and 40minutes after administration. DSP-4 50 mg/kg decreased noradrenaline content in the all 3 regions. The NA depletion inhibited NPS analgesic effect in the hot plate test but not tail flick test. There was a significant correlation between hot plate latency (percentage of maximum possible effect: %MPE) with NPS 10 nmol and NA content in

the cerebral cortex ($p = 0.017$, $r^2 = 0.346$) which noradrenergic innervation arisen mainly from the LC. No other regions had the correlation.

**Conclusions:** NPS analgesia interacts with LC noradrenergic neuronal activity.

## Introduction

Neuropeptide S (NPS) is an endogenous neuropeptide that has various biological properties [1] that include anxiolytic-like effect [2-6], improving N-methyl-D-aspartate (NMDA)-induced memory impairment [7], promoting hyper-locomotion [4, 8], anti-aggressiveness [9, 10], promoting wakefulness [4, 11-13], promoting anesthesia emergence [14], and reduction of anesthesia depth [15]. Moreover supraspinal administration of NPS has been demonstrated to elicit antinociceptive effects in rodents [8, 16-20]. NPS receptor are widely distributed across brain regions especially in brain regions involved in processing of emotion, olfaction, sleep/wake and arousal modulation and learning and memory [21]. Most of the NPS containing neurons are located in the principle sensory trigeminal nucleus, the lateral parabrachial nucleus, and the peri-locus coeruleus (LC) area [22] where noradrenergic neuron arising from. Noradrenergic activity interacts with NPS. For example, noradrenergic signaling enhances memory consolidation by NPS [23]. Numerous studies indicated that brain noradrenergic signaling

is important for nociceptive transmission [24]. The LC noradrenergic neurons plays crucial role in the descending anti-nociceptive system [25]. This anatomical distribution suggests that NPS could interact with LC noradrenergic neuron and NPS could provide analgesic effect through the LC noradrenergic neuronal activity. We tested the NPS analgesia in selective noradrenergic neuron lesioned model by a selective neurotoxin, N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4).

## Materials and methods

With the approval of the institutional committee on animal research of Hirosaki University Graduate School of Medicine, a total 66 male Sprague–Dawley rats weighing 350–450 g were used. All rats were housed for at least 1 week before surgery and were maintained in a 12 h light/dark cycle environment (lights on 20:00–08:00 h) at a temperature of 24±0.5 ºC and 40% humidity. The animals were provided free access to food and water. The NPS was synthesized according to published methods; details of which have been described previously [26].

Surgery

All surgery was performed under ketamine-xylazine (35 and 5 mg/kg intraperitoneal injection) anesthesia as previously described [27]. The rats were surgically implanted with a lateral intracerebroventricular (icv) cannula. All rats were allowed to spend 10days as a recovery period. They were housed in the same condition as pre-surgery.

Experimental Protocol:

We used two analgesiometric assays: the hotplate and tail flick test. In the hotplate test, two subgroups were performed. One tested NPS alone effect and another tested NPS and DSP-4, a selective toxin of noradrenergic neuron [28]. In tail flick test, only NPS alone effect was evaluated.

Hotplate test

In the NPS alone study, each rat was placed individually on a hot-plate maintained at 50 °C (MK-350D™; Muromachi Kikai Co, Ltd., Tokyo) and the reaction time was measured starting from time at the rat was placed on the plate until the rat either

demonstrated hind paw licking or jumping. The cut-off latency used was 60 s to prevent tissue damage. Two successive values were measured as pre-injection values; then, five successive values were measured every 10 min in each animal after receiving an injection of icv with one of four doses of the NPS: 0.0 nmol, 1.0 nmol, 3.3 nmol, 10.0 nmol (n = 10 each). The injection volume was 4 μl. The injections took place between 1830 and 1930.

In the NPS combined with DSP-4 study, Other 16 rats were used. At first, time course of hotplate latency with NPS 10.0 nmol icv without DSP-4 (control values) were obtained in the same manner as the NPS alone study group. Then, they received DSP-4 ip: 0 mg/kg (physiological saline) or 50 mg/kg (n = 8 each). Ten days after the DSP-4 administration, hot-plate latency with NPS 10 nmol icv was measured again in the same manner as the control. After completion of the behavioral tests, each rat was decapitated, the brain quickly removed. The cerebral cortex, the pons, and the hypothalamus were dissected from their surrounding structures on ice-cold glass dish and weighed. Each brain tissue was immediately sonicated in 2ml of physiological saline (NaCl 155 mM). The supernate was collected from centrifugation of the sonicated material at 15,000×g for 30 min and stored at −70 ◦C until catecholamine extraction was performed. Noradrenaline contents were determined directly by high-performance liquid

chromatography with electrochemical detection (ESA Coulochem Model 5100A™, Tokyo, Japan).

Tail flick test

Additional 10 rats were used. Each rat was placed individually on a tail-flick apparatus (MK-330B™; Muromachi Kikai Co, Ltd., Tokyo) to apply radiant heat to the tail and the reaction time from the onset of the heat to the withdrawal of the tail was measured. The intensity of the radiant heat was adjusted so that baseline latencies would fall between 2 and 6 s. To avoid tissue damage, the heat stimulus was discontinued after 10 s (cut-off latency). Two successive values were measured as pre-injection values; then, five successive values were measured every 10 min in each animal after receiving an injection of NPS 0.0 nmol or 10.0 nmol icv (n=5 each). The injections was done as same time as the hotplate test.

All values were expressed as mean ± SD. The percentage of maximum possible effect (%MPE) of reaction time for the hot-plate and tail flick test was calculated for each rat and time point according to the following formula: %MPE = (post-treatment latency-pretreatment latency)/ (cut-off latency-pretreatment latency) ×100 [29]. Statistical analyses of hotplate and tail flick data in time-matched across all doses were performed

with two-way analysis of variance for repeated measures (ANOVA) followed by Tukey's multiple comparisons test in NPS alone group, and were performed with two-way analysis of variance for repeated measures (ANOVA) followed by Bonferroni's multiple comparisons test in NPS combined with DSP-4 group. Hotplate latencies in pre NPS administration with or without DSP-4 were analyzed by student paired t-test. Noradrenaline contents in the brain regions were analyzed by student unpaired t-test. All statistical analyses were performed using GraphPad Prism version 6.01 for Windows (GraphPad Software, La Jolla, CA, USA). A significance level of $p<0.05$ was accepted.

## Results

Figure 1A showed that NPS 10 nmol prolonged hot plate latency (%MPE) in the NPS alone study at 30 minutes after NPS icv [10 nmol vs 1.0 nmol; $q (3, 36)= 4.066$, $p = 0.023$: 10 nmol vs 3.3 nmol; $q (3, 36)= 3.967$, $P = 0.028$] and at 40 minutes after NPS icv [10 nmol vs 0.0 nmol; $q (3, 36)= 5.284$, $p = 0.002$: 10 nmol vs 1.0 nmol; $q (3, 36)= 3.672$, $p = 0.049$: 10 nmol vs 3.3 nmol; $q (3, 18)= 3.883$, $p = 0.033$]. On the other hand, NPS 10 nmol did not prolonged tail flick latency (figure 1B). In the NPS combined DSP-4 study,

hot plate latency of pre-injection NPS without DSP-4 50 mg/kg (physiological saline administration) was 23.9 ± 6.9 seconds and with DSP-4 50 mg/kg was 16.2 ± 4.2 seconds (figure 2A). There was significant difference between them (p=0.046). DSP-4 50 mg/kg inhibited NPS-induced prolongation of hot plate latency at after 30 minutes [ANOVA treatment effect; F (1, 14) = 5.658, p < 0.0001], 40 minutes [ANOVA treatment effect; F (1, 14) = 3.920, p = 0.001], 50 minutes [ANOVA treatment effect; F (1, 14) = 3.743, p = 0.002] of NPS 10 nmol injection (figure 2B). DSP-4 50 mg/kg decreased noradrenaline content (pg/mg wet tissue) in the cerebral cortex (15.4 ± 8.2 in control vs 6.0 ± 5.3 in DSP-4 50 mg/kg: p=0.017), in the pons (49.2 ± 32.1 in control vs 21.8 ± 14.6 in DSP-4 50 mg/kg: p=0.045), and in the hypothalamus (218.1 ± 130.0 in control vs 91.0 ± 56.9 in DSP-4 50 mg/kg: p=0.024) (figure 3). Statistically significant positive correlation was only observed between hot plate latency with 10 nmol NPS icv and NA content of the cerebral cortex (p = 0.017, $r^2$ = 0.346) but 2 other regions tested had no correlation (figure 4).

## Discussion

The major findings in this study are activation of central NPS tone exerted analgesia and depletion central noradrenergic neuron by DSP-4 inhibits the NPS action. DSP-4 is a selective neurotoxin for the locus coeruleus noradrenergic system in the rodent and bird brain [30]. Previous study reported that elimination half-life of both agents (intraperitoneal injection) in rats were 1.26 hour (ketamine 125mg/kg) and 1.30 hour (Xylazine 10mg/kg) [31]. Therefore, these drugs unlikely affect the behavioral results after the 10days recovery period.

Brain noradrenergic neurons are consisted with two groups, dorsal and ventral noradrenergic bundles [32]. The dorsal region is LC noradrenergic neuron. The LC noradrenergic neuron is involved in the descending anti-nociceptive system but ventral noradrenergic bundle is not involved in it. The noradrenergic neuron in the cerebral cortex is mainly innervated from LC noradrenergic neuron [32]. On the other hand, the pons is innervated from dorsal and ventral noradrenergic bundle and the hypothalamus is mainly innervated from the ventral bundle. Therefore, changes in NA content in the cerebral cortex closely reflect LC noradrenergic neuronal activity; whereas, other regions would not. In this study, DSP-4 depleted NA in the cerebral cortex as previous study [30]. Moreover, NA content and hotplate latency was correlated only in the cerebral cortex but no other regions studied. These results suggest the NPS analgesia affected

by noradrenergic neuronal activity originated from the LC. This could be one reason why hotplate latency was correlated only with NA content in the cerebral cortex but other 2 regions. The NPS dose used in this study was based on our previous study that elucidated effect of NPS on the anesthetic time [14]. In this study, intra-cerebroventricular NPS 0.3 to 1.0 nmol significantly reduced thiopental anesthesia time but 10 nmol had no effect on the anesthesia time; thus, NPS may not have simple dose dependent effect on a biological action. We added 3.3 nmol in this study to clarify if NPS had simple dose dependent effect on hot plate latency. We found that only the highest dose, 10 nmol, increased hotplate latency in this study. This result confirmed that effect of NPS on various biological actions including analgesia is not in simple dose dependent manner.

Exogenous NPS prolonged hotplate latency; while, it failed to affect tail flick latency. These results were consisted with our previous data that DSP-4 inhibited prolongation hotplate latency but not tail flick latency [33]. As response to hot-plate and tail-flick implies supra-spinal process and spinal reflex, respectively [34]; thus, NPS analgesia could act on a supra-spinal process.

The role of serotonergic neuron in NPS-induced analgesia would also be considered because serotonergic neuron is one of main component of the descending anti-nociceptive system. We did not measure serotonin in this study. Role of serotonin

in pain process is not simple. Various serotonin receptors play inhibitory and facilitatory modulations of chronic neuropathic pain symptoms [35]. For example, rats in the formalin test, low doses of 5-HT injected via the intrathecal route are known to exert anti-nociceptive effects whereas larger doses induce a pro-nociceptive effect [35].

## Conclusion

In conclusion, NPS produced its analgesic effect, at least in part, by activation of central noradrenergic neuronal activity.

## Conflicts of interest

The authors declare no conflicts of interest.

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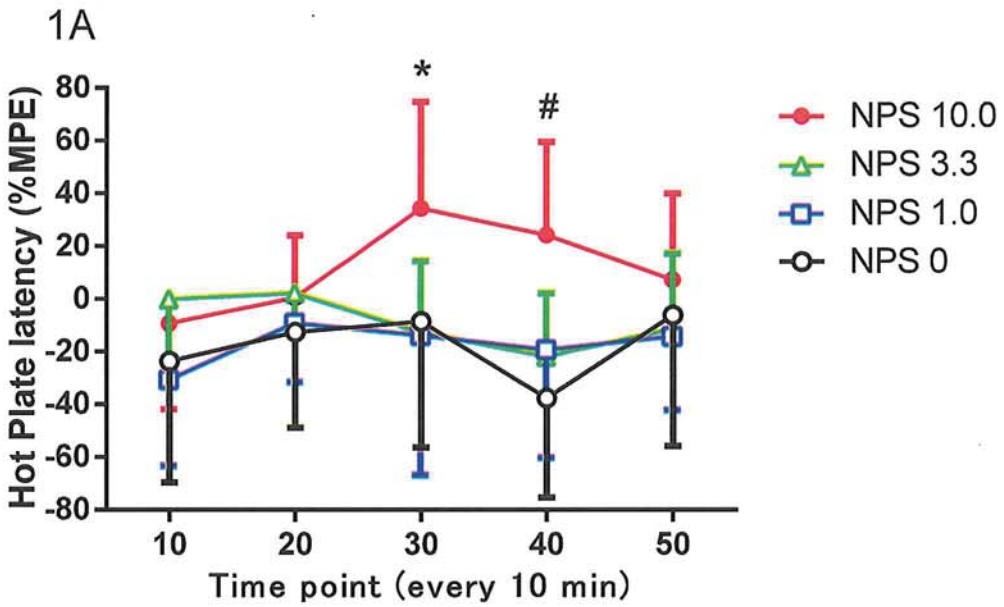
Figure legends




* P<0.05 vs NPS 1.0, 3.3 nmol

#P<0.05 vs NPS 0.0, 1.0, 3.3 nmol

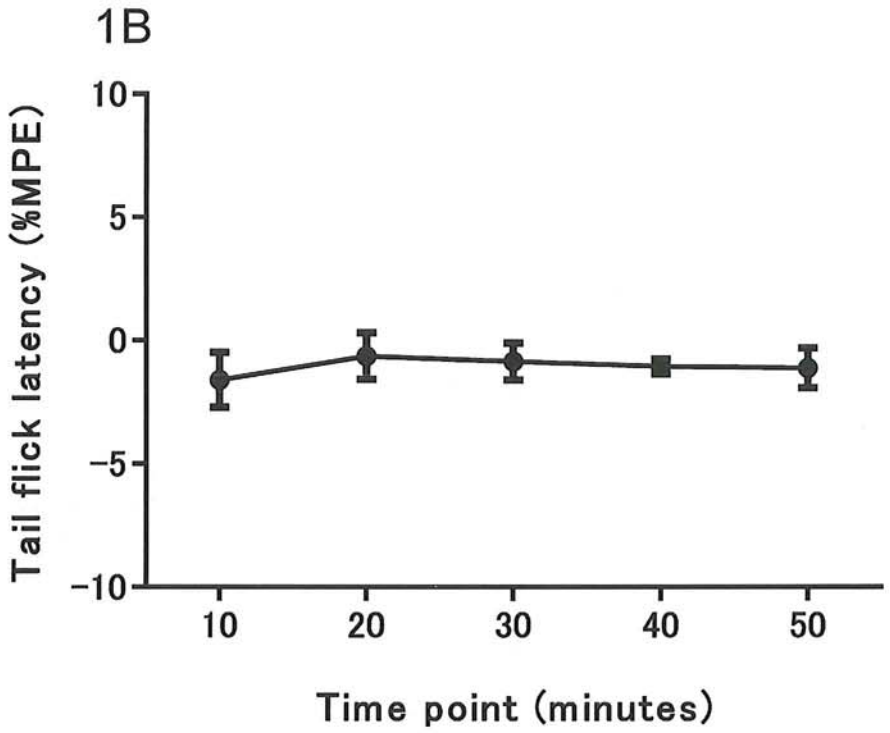


Figure 1

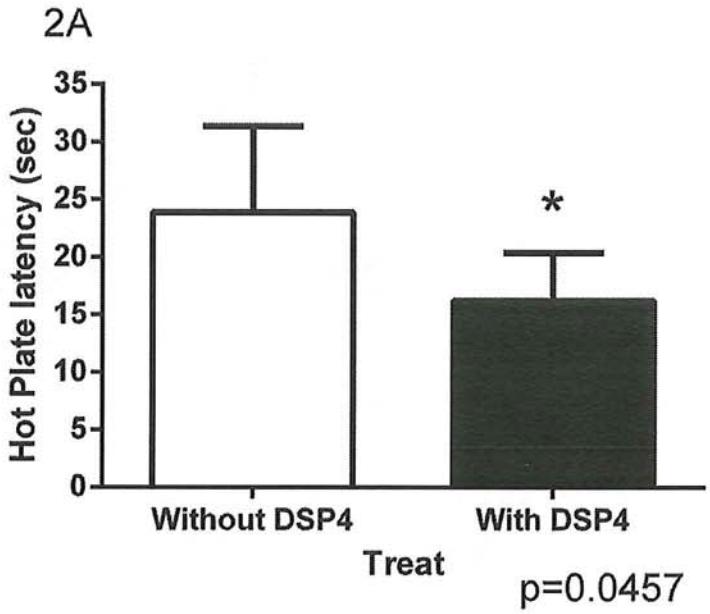
Effects of NPS icv in the hot plate (panel A) and tail flick (panel B) tests.

Panel A

Closed red circle indicates changes in hotplate latency by NPS 10 nmol icv. Open green triangle indicates changes in hotplate latency by NPS 3.3 nmol icv. Open blue square indicates changes in hotplate latency by NPS 1.0 nmol icv. Open black circle indicates changes in hotplate latency by NPS 0 nmol icv. NPS 10 nmol prolonged hot plate latency at 30 minutes after NPS icv compared to 1.0, 3.3 nmol. NPS 10 nmol also prolonged hot plate latency at 40 minutes after NPS icv compared to 0, 1.0, 3.3 nmol.

Panel B

NPS 10 nmol icv did not affect tail flick latency.



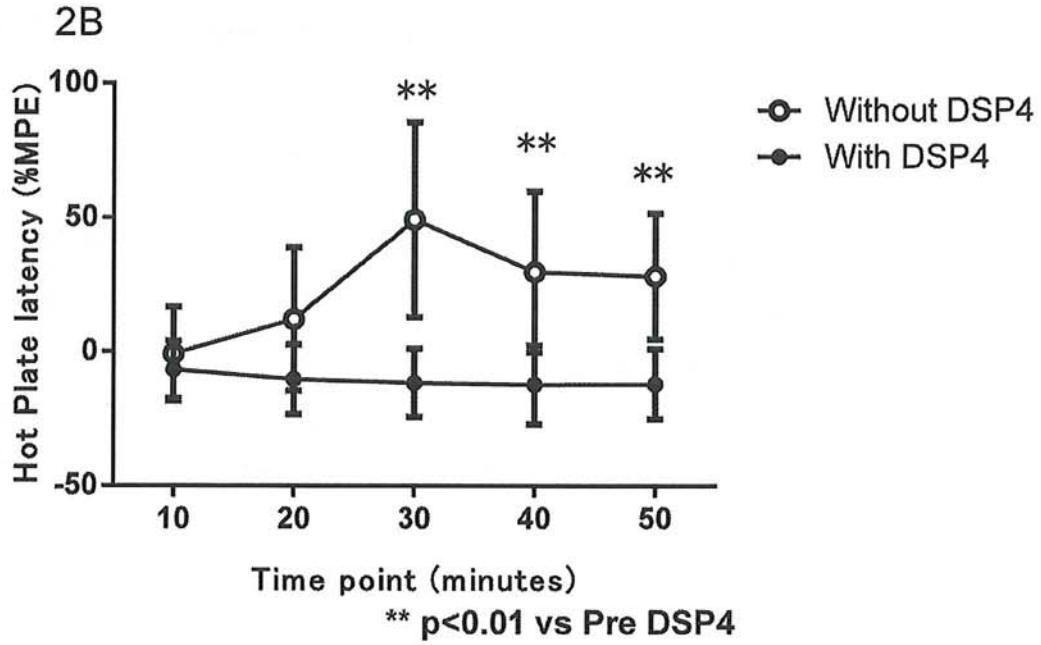




Figure 2

Changes in hot plate latency in NPS combined DSP-4 study.

Panel A

Hot plate latency of pre-injection NPS with or without DSP-4

Open rectangle indicates the latency without DSP-4 50 mg/kg (23.9 ± 6.9 seconds).

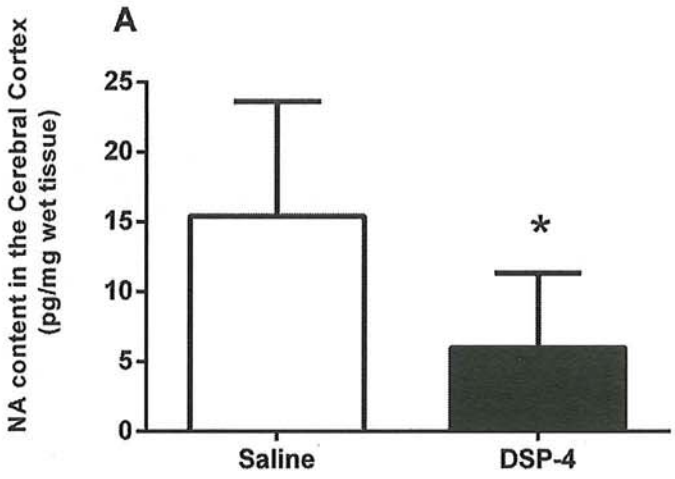
Closed rectangle indicates the latency with DSP-4 50 mg/kg (16.2 ± 4.2 seconds).

There was significant difference between them ($p=0.046$)

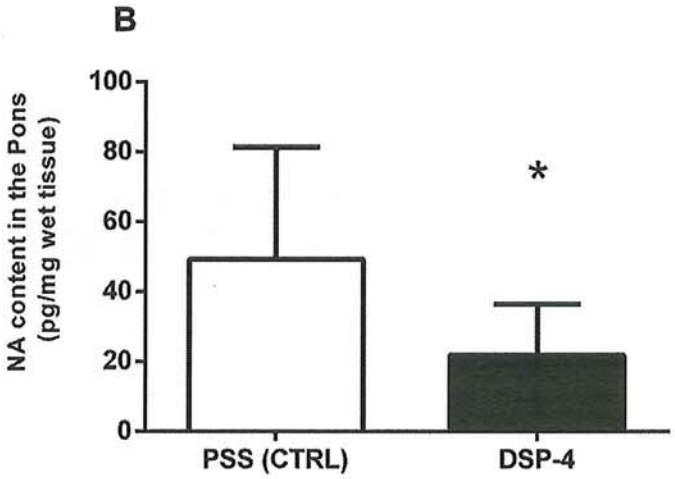
Panel B

Time course of hotplate latency by NPS 10 nmol icv with or without DSP-4

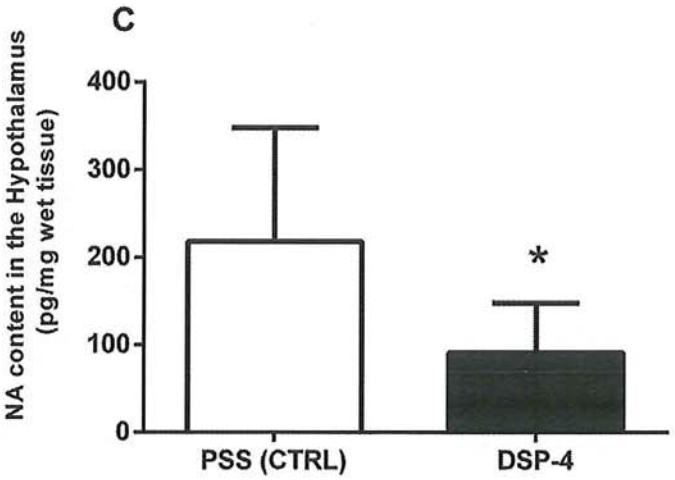
Open circle indicates changes in hotplate latency by NPS 10 nmol icv without DSP-4 50 mg/kg. Close circle indicates changes in hotplate latency by NPS 10 nmol icv with DSP-4 50 mg/kg. DSP-4 50 mg/kg inhibited NPS-induced prolongation of hot plate latency at after 30, 40, 50 minutes of NPS 10 nmol injection.




*p=0.017 vs Control




*p=0.045 vs Control

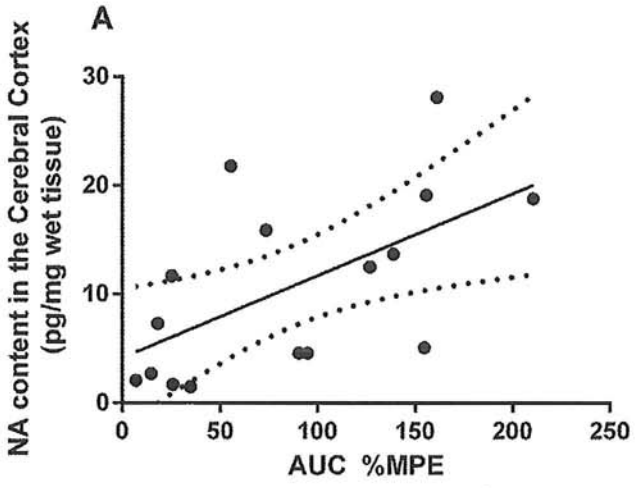



*p=0.024 vs Control

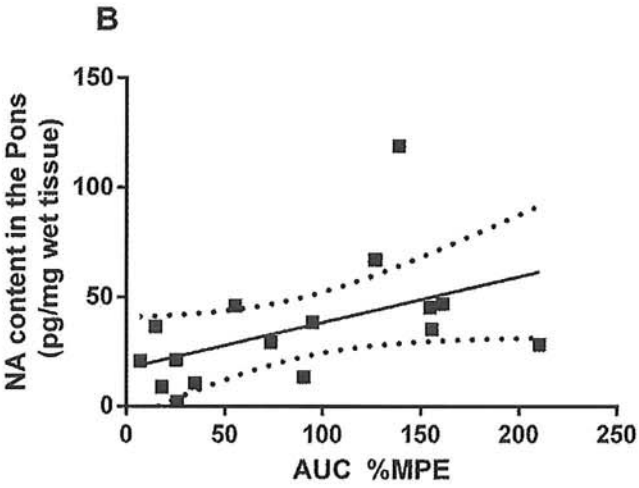
Figure 3

Effect of DSP-4 50 mg/kg administration on noradrenaline contents in brain regions.

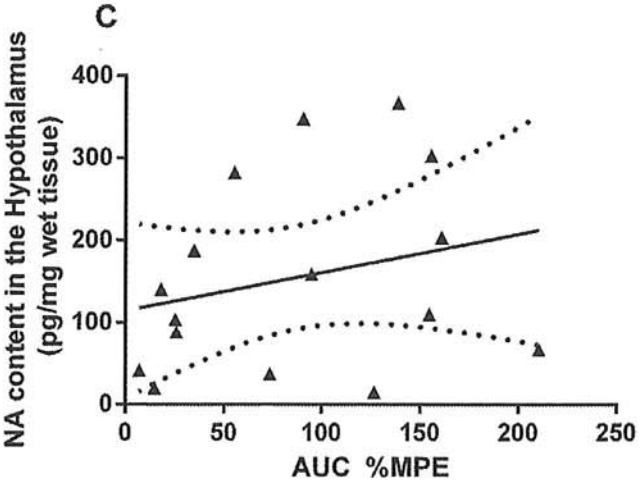
DSP-4 50 mg/kg decreased noradrenaline content (pg/mg wet tissue) in the cerebral cortex (15.4 ± 8.2 in control vs 6.0 ± 5.3 in DSP-4 50 mg/kg: p=0.017: Panel A), in the pons (49.2 ± 32.1 in control vs 21.8 ± 14.6 in DSP-4 50 mg/kg: p=0.045: Panel B), hypothalamus (218.1 ± 130.0 in control vs 91.0 ± 56.9 in DSP-4 50 mg/kg: p=0.024: Panel C).




$r^2$=0.346
p=0.017




$r^2$=0.232
p=0.058




$r^2$=0.066
p=0.339

Figure 4

Correlation between Noradrenaline content and hot plate latency with NPS icv.

Statistically significant positive correlation was only observed between hot plate latency with 10 nmol NPS icv and NA content of the cerebral cortex ($p = 0.017$, $r^2 = 0.346$: Panel A) but no other regions tested had the correlation (Panel B, C).