CHAPTER IV

RESULTS

Effect of dural stimulation on trigeminal nociception in normal rats

Dural inflammation was induced by topical application of inflammatory soup (IS) containing a mixture of histamine, serotonin, bradykinin, each at 1 mM, and 0.1 mM prostaglandin E_2, pH 5.5 or low-pH phosphate-buffered artificial cerebrospinal fluid (low-pH CSF, pH 4.7) on exposed dura of right hemisphere for 30 minutes. Fos expression was detected by immunohistochemistry (IHC) at 30 minutes and 2 hours after the beginning of dural stimulation. Dural stimulation by IS can activate trigeminal nociception indicated by the increase in the number of Fos-immunoreactive (-ir) cells in Lamina I and II of trigeminal nucleus caudalis (TNC) and C1, C2 of spinal cord as compared to control group. In contrast, application of low-pH CSF cannot activate trigeminal nociception because it did not produce a significant change in Fos expression as compared to control.

A. Distribution and pattern staining

Chemical stimulation applied to the dural surface underlying parietal bone produced a widespread distribution of Fos-ir cells in the upper cervical and medullary dorsal horn, which was mostly ipsilateral to the stimulus. Labeling dorsal horn extended over a long distance, from C2 to the trigeminal nucleus caudalis. The labeling was most dense in lamina I, II of the ventrolateral part and in lamina I of the dorsomedial part of the dorsal horn (figure 9).

Fos staining was restricted to the nucleus (figure 10). In naïve rats, there was no expression of Fos (data not shown). Our results from the immunohistochemical study demonstrated that the Fos expression could not be detected in any rats at 30 minutes after stimulation. Thus, only Fos expression at 2 hours after dural stimulation was reported in this study.

Figure 9 Distribution of Fos-ir cells in dorsal horn of TNC. The labeling was most dense in lamina I, II of the ventrolateral part and in lamina I of the dorsomedial part of the dorsal horn. (10x objective lens).

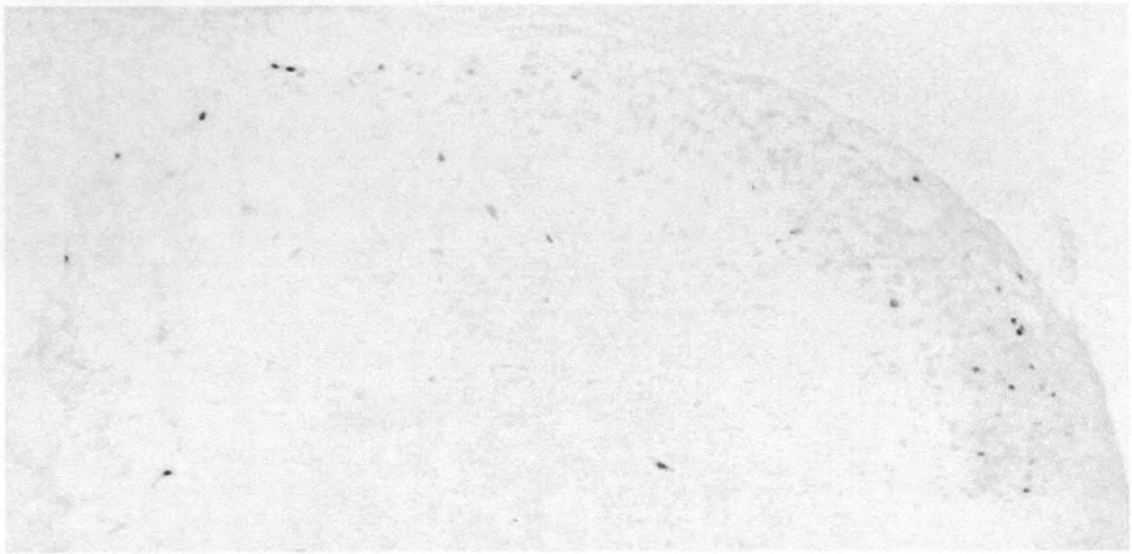
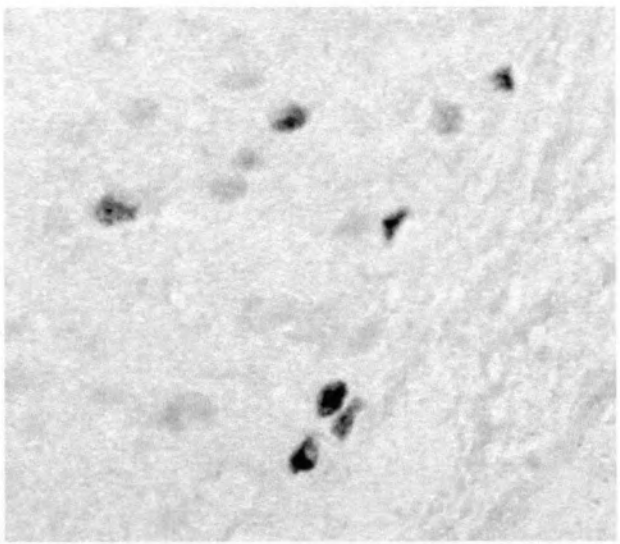


Figure 10 Cellular pattern staining of Fos. Fos staining was restricted to the nucleus. (40x objective lens).



B. Quantitative analysis

The results described in the text are the data of ipsilateral side to dural stimulation. The data of contralateral side are shown in table and graph. Quantitative analysis (Table 1 and Figure 11) showed that dural stimulation by IS significantly increased the number of Fos-ir cells above control value in the dorsal horn ($P = .000$, ANOVA; LSD post hoc comparison was used for all ANOVA). The magnitude of increase was 2-fold. There were 15 ± 3 Fos-ir neurons per slide in rats receiving dural stimulation by IS vs. 7 ± 1 in controls. Significant increase in Fos-ir cells was also present when compared to non-stimulation side ($P = .001$). The magnitude of increase was 2.5-fold.

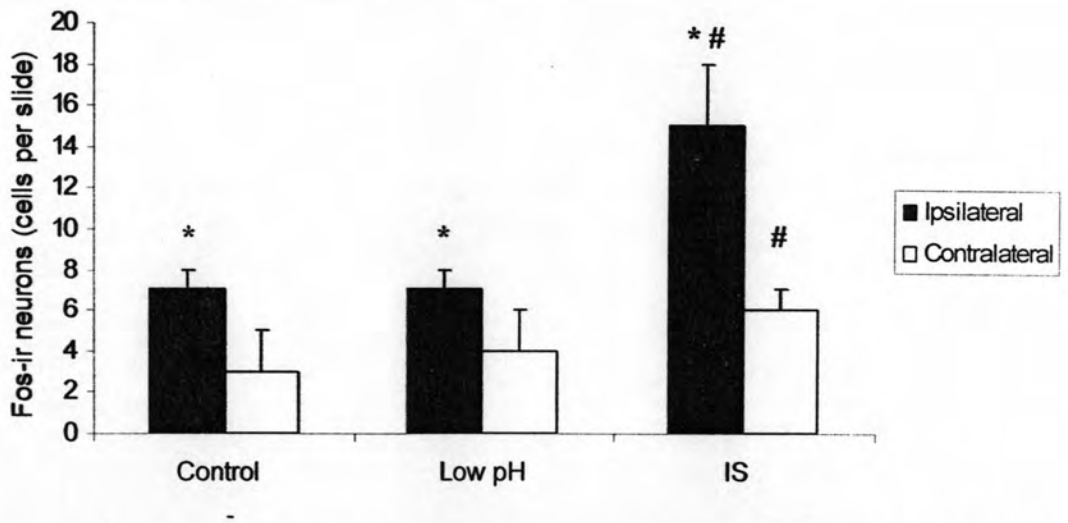
Application of low-pH CSF caused an increase in the number of Fos-ir cells as compared to non-stimulation side. However, the number of Fos-ir cells in this group was not significantly different form control group ($P = .999$). The average numbers of Fos-ir neurons were 7 ± 1 and 7 ± 1 cells per slide for rats receiving the dura stimulation by low-pH CSF and control, respectively.

Table 1 Number of Fos-ir cells in TNC of normal rats with dural stimulation by IS or low-pH CSF compared to control

		Number of Fos-ir cells (cells per slide)			
Group	n	Ipsilateral	*P*	Contralateral	*P*
Control	5	$7 \pm 1^{*}$		3 ± 2	
Low pH	4	$7 \pm 1^{*}$	.999	4 ± 2	.634
IS	5	$15 \pm 3^{*\#}$	.000	$6 \pm 1^{\#}$	.017

Note: * significantly different compared to correspondent contralateral side ($P < .05$)
significantly different compared to control group ($P < .05$)

Figure 11 Bar graph showing the number of Fos-ir cells in TNC of normal rats with dural stimulation by IS or low-pH CSF compared to control



Note: * significantly different compared to correspondent contralateral side ($P < .05$)
significantly different compared to control group ($P < .05$)

Effect of dural stimulation on NR1 receptor expression in normal rats

Topical application of IS on exposed dura of right hemisphere for 30 minutes were performed to induce dural inflammation. NMDA receptor NR1 subunit expression was detected using IHC at 30 minutes and 2 hours after the beginning of dural stimulation. The results from NR1 IHC demonstrated that stimulation of dura by IS had no effect on NR1 expression in TNC. The number of NR1-ir cells in TNC of the dural stimulation group was not different from those obtained from control group.

A. Distribution and pattern staining

In normal rats, NR1 receptor was abundantly expressed throughout the spinal and medullary dorsal horn. Densely NR1 staining was found in the spinal lamina I, II (figure 12). Most of labeled-cells in this lamina were small to medium cells. There was no difference in NR1 distribution between control rats and rats with dural stimulation.

NR1 staining is found in the cytoplasm of the cell (figure 13). NR1 expression was detected by IHC at 30 minutes and 2 hours and also detected in naïve rats (data not shown).

Figure 12 Distribution of NR1-ir cells in dorsal horn of TNC. Densely NR1 staining was found in the spinal lamina I, II. (8x magnification in Image Scope Program).

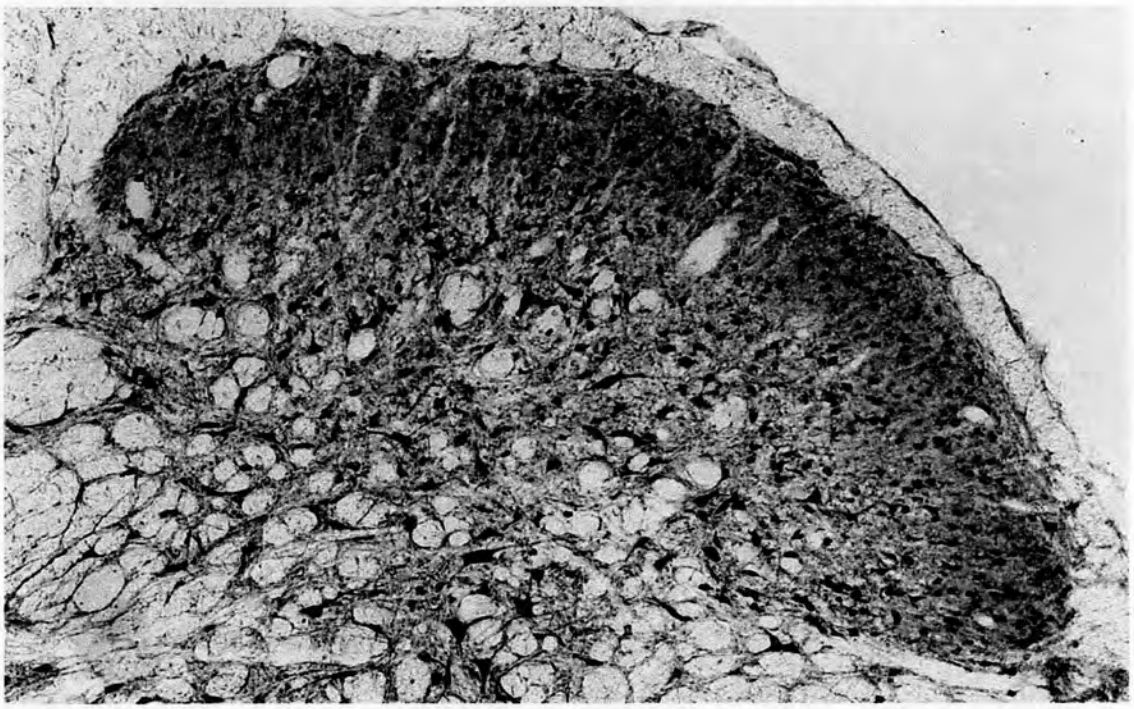
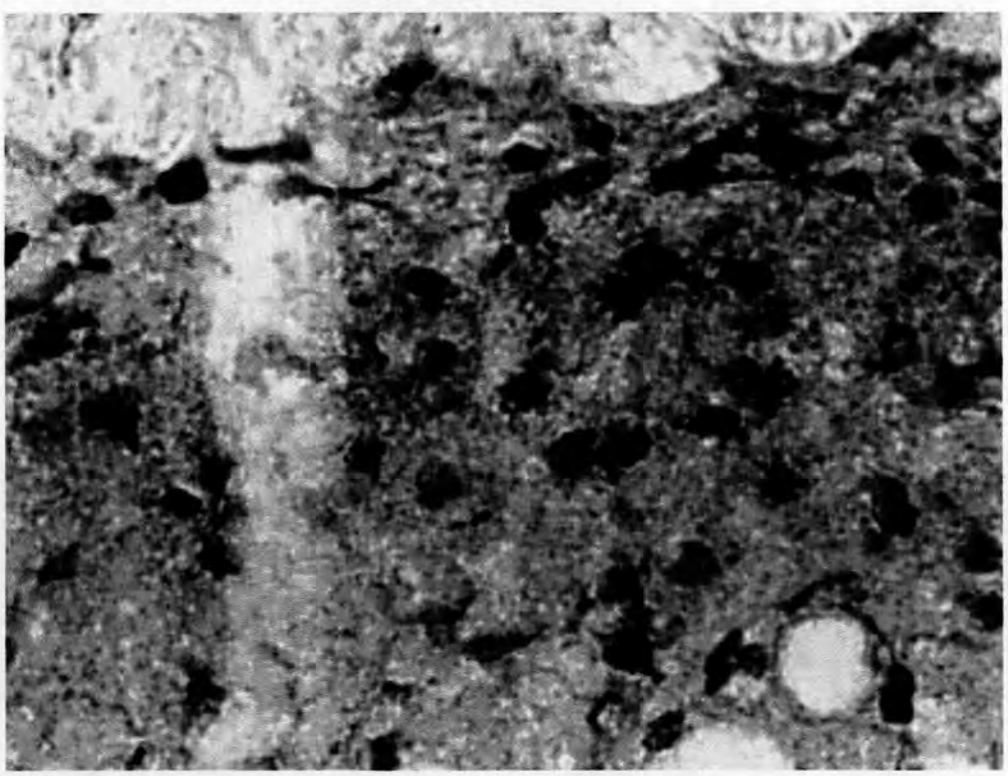


Figure 13 Cellular pattern staining of NR1. NR1 staining is found in the cytoplasm of the cell. (20x magnification in Image Scope Program).



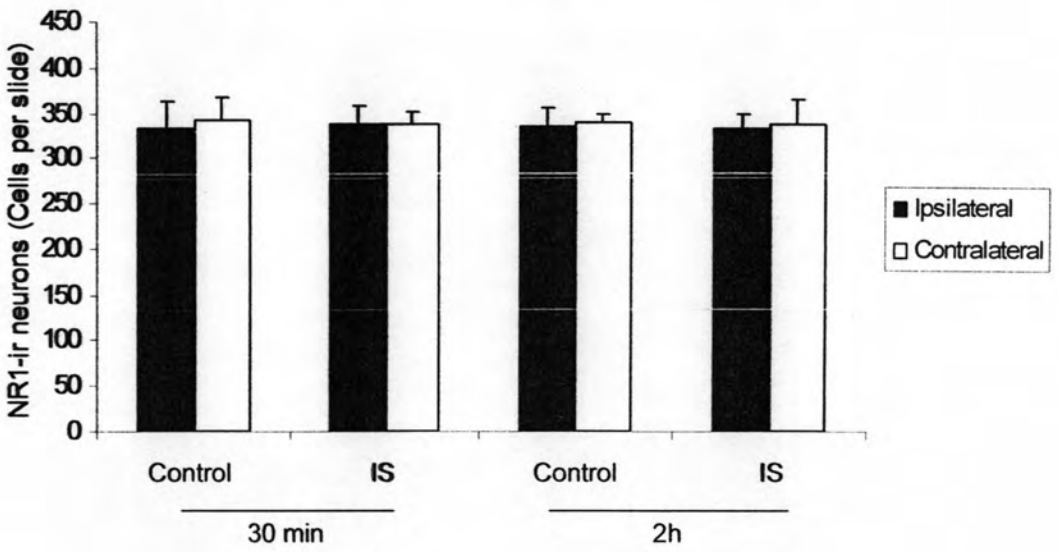
B. Quantitative analysis

Quantitative analysis (table 2 and figure 14) showed that dural stimulation by IS had no effect on the expression of NR1 as compared to the control. There were 337 ± 21 and 332 ± 16 NR1-ir neurons per slide in rats receiving dural stimulation by IS vs. 334 ± 30 and 334 ± 21 in controls at 30 minutes (P = .997) and 2 hours (P = .999), respectively. There was no significant difference in NR1 expression between ipsilateral to the stimulation and contralateral side. The number of NR1-ir neurons were 337 ± 21 and 332 ± 16 neurons per slide on ipsilateral side vs. 338 ± 14 and 337 ± 28 on contralateral side at 30 minutes (P = .977) and 2 hours (P = .639), respectively.

Table 2 Number of NR1-ir cells in TNC of normal rats with dural stimulation by IS compared to controls at 30 minutes and 2 hours

			Number of NR1-ir cells (cells per slide)			
Time	Group	n	Ipsilateral	*P*	Contralateral	*P*
30 min	Control	5	334 ± 30		343 ± 25	
	IS	5	337 ± 21	.997	338 ± 14	.989
2 h	Control	5	334 ± 21		340 ± 9	
	IS	5	332 ± 16	.999	337 ± 28	.992

Figure 14 Bar graph showing the number of NR1-ir cells in TNC of normal rats with dural stimulation by IS compared to controls at 30 minutes and 2 hours



Effect of dural stimulation on NR1 receptor phosphorylation in normal rats

The phosphorylation at serine-896 of NMDA receptor NR1 subunit was detected using IHC at 30 minutes and 2 hours after the beginning of dural stimulation. Dural stimulation by IS can induce NR1 receptor phosphorylation in Lamina I and II of TNC and C1, C2 of spinal cord as compared to control. In contrast, application of low-pH CSF cannot induce NR1 receptor phosphorylation because it did not produce a significant change in NR1 phosphorylation as compared to control.

A. Distribution and pattern staining

Like Fos expression, chemical stimulation applied to the dural surface underlying parietal bone produced a widespread distribution of serine-896 phosphorylated NMDA receptor NR1 subunit (pNR1)-ir cells in the upper cervical and medullary dorsal horn, which was mostly ipsilateral to the stimulus. Labeling dorsal horn extended over a long distance, from C2 to the TNC. Pattern of distribution of pNR1-ir cells on dorsal horn was as same as Fos-ir cells. The labeling was most dense in lamina I, II of the ventrolateral part and in lamina I of the dorsomedial part of the dorsal horn (figure 15).

The staining pattern of pNR1 seems to be like Fos staining pattern, which was nuclear staining, when observed by light microscope. However, pNR1 staining pattern is predominantly perinuclear (endoplasmic reticulum region; figure 16). There was very little pNR1 expression in naïve rats (data not shown). Unlike Fos expression, pNR1-ir cells were detected by IHC at 30 minutes and 2 hours.

Figure 15 Distribution of pNR1-ir cells in dorsal horn of TNC. The labeling was most dense in lamina I, II of the ventrolateral part and in lamina I of the dorsomedial part of the dorsal horn. (10x objective lens).

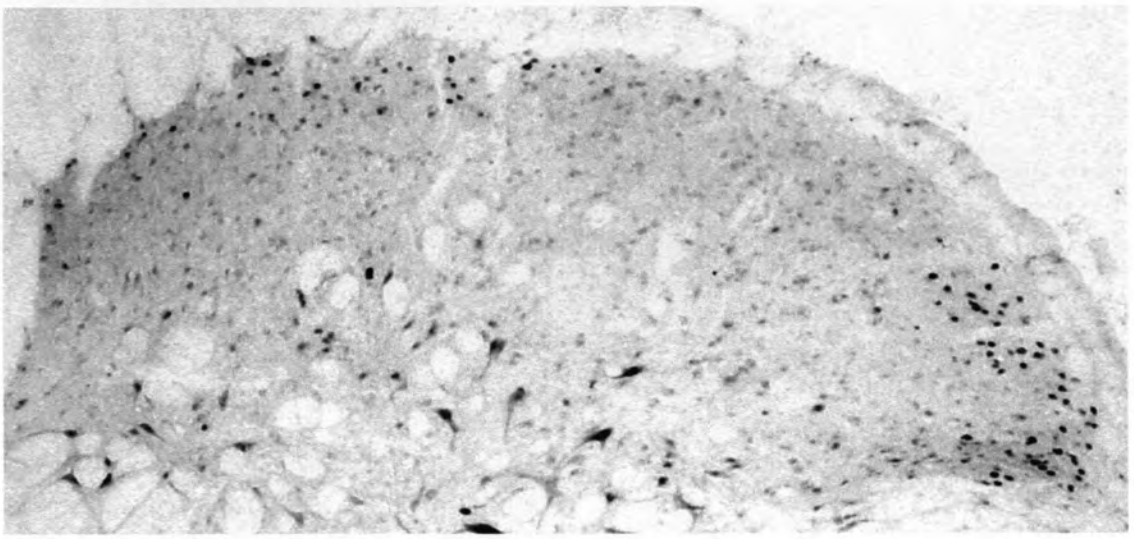
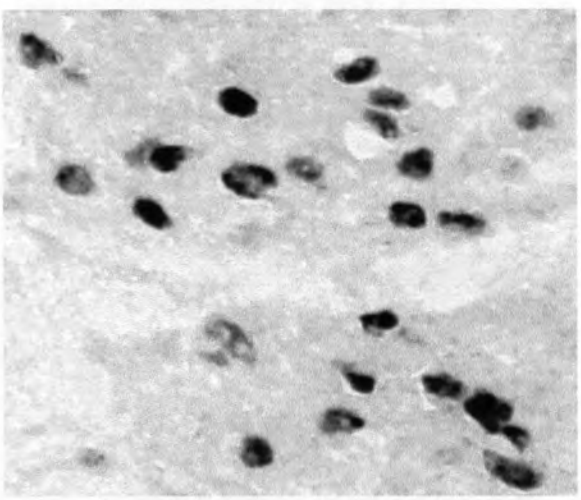


Figure 16 Cellular pattern staining of pNR1. pNR1 staining pattern is predominantly perinuclear (endoplasmic reticulum region). (40x objective lens)



B. Quantitative analysis

Quantitative analysis (table 3 and figure 17) showed that dural stimulation by IS significantly increased the number of pNR1-ir cells above control value in the dorsal horn. The magnitude of increase was approximately 1.5-fold. There were 31 ± 5 and 29 ± 4 pNR1-ir neurons per slide in rats receiving dural stimulation by IS vs. 22 ± 3 and 18 ± 3 in controls at 30 minutes (P = .004), and 2 hours (P = .002), respectively. Significant increase in pNR1-ir cells was also present when compared to non-stimulation side. The magnitude of increase was 2.5-fold. There were 31 ± 5 and 29 ± 4 pNR1-ir neurons per slide on ipsilateral side vs. 12 ± 2 and 12 ± 5 on contralateral side at 30 minutes (P = .001) and 2 hours (P = .000), respectively. There was no significant difference between the number of pNR1-ir neurons at 30 minutes and 2 hours (P = .056).

Low-pH CSF application increased in the number of pNR1-ir cells compared to non-stimulation side. However, the number of pNR1-ir cells was not significantly different form control group. There were 17 ± 2 Fos-ir neurons per slide in rats receiving dural stimulation by low-pH CSF vs. 18 ± 3 in controls at 2 hours (P = .998).

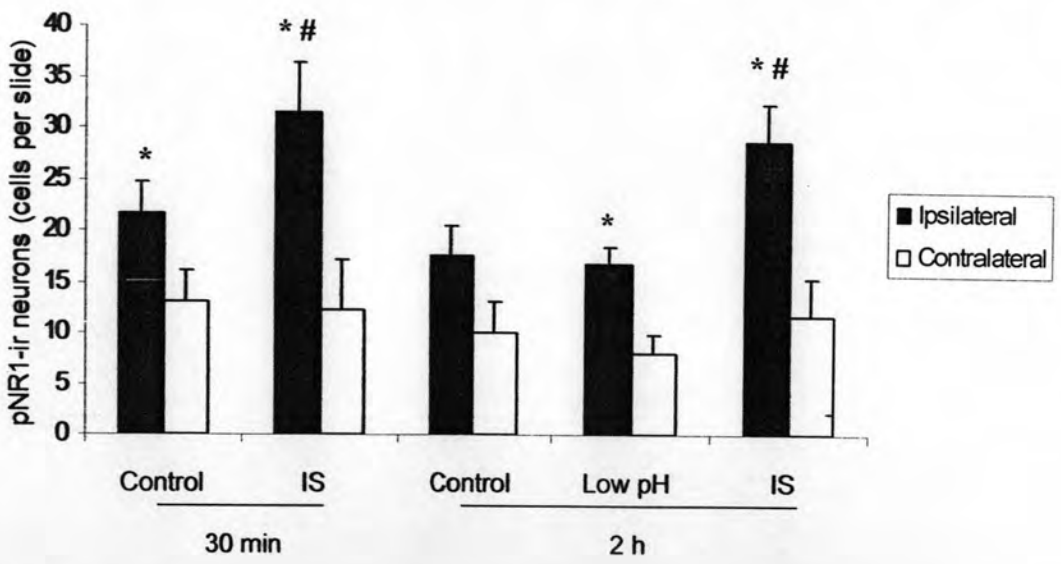
The number of pNR1-ir cells and NR-ir cells of the same rat were used to calculate the percentage of phosphorylation (figure 18). The number of pNR-ir cells and percentage of phosphorylation were in accordance because the number of NR1-ir cells was not significant different in all groups.

Table 3 Number of pNR1-ir cells in TNC of normal rats with dural stimulation by IS or low-pH CSF compared to controls at 30 minutes and 2 hours

			Number of pNR1-ir cells (cells per slide)			
Time	Group	n	Ipsilateral	*P*	Contralateral	*P*
30 min	Control	5	$22 \pm 3^{*}$		13 ± 7	
	IS	5	$31 \pm 5^{*\#}$	.004	12 ± 2	1.000
2h	Control	5	18 ± 3		10 ± 3	
	Low pH	4	$17 \pm 2^{*}$	.998	8 ± 2	.967
	IS	5	$29 \pm 4^{*\#}$	.002	12 ± 5	.982

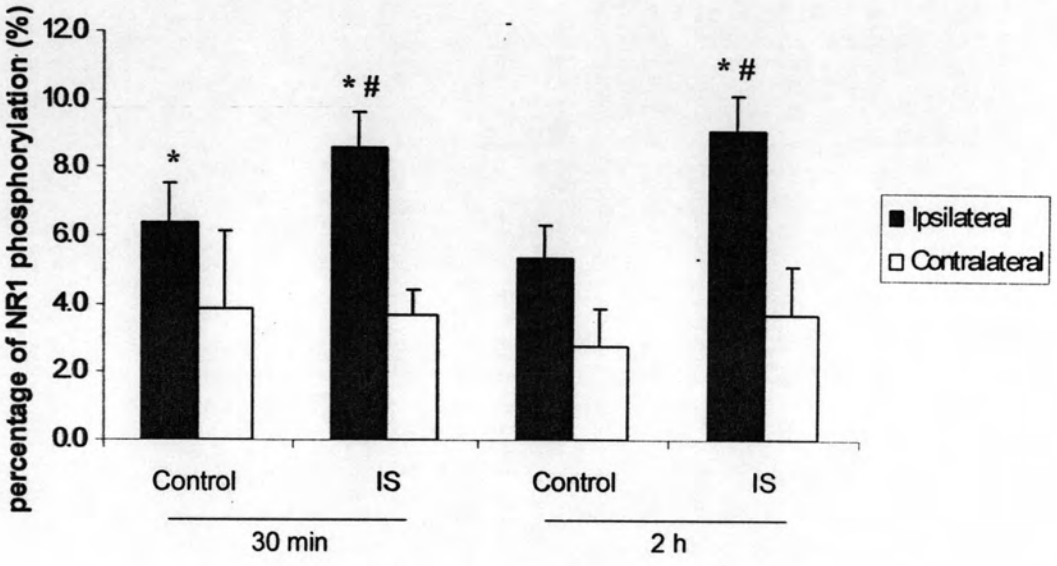
Note: * significantly different compared to correspondent contralateral side ($P < .05$)
significantly different compared to correspondent control group ($P < .05$)

Figure 17 Bar graph showing the number of pNR1-ir cells in TNC of normal rats with dural stimulation by IS or low-pH CSF compared to controls at 30 minutes and 2 hours



Note: * significantly different compared to correspondent contralateral side ($P < .05$)
significantly different compared to correspondent control group ($P < .05$)

Figure 18 Bar graph showing the percentage of NR1 phosphorylation in TNC of normal rats with dural stimulation by IS or low-pH CSF compared to controls at 30 minutes and 2 hours



Note: * significantly different compared to correspondent contralateral side ($P < .05$)
significantly different compared to correspondent control group ($P < .05$)

Effect of serotonin depletion on trigeminal nociception induced by dural stimulation

In serotonin-depleted rats, para-chlorophenylalanine (PCPA) was intraperitoneally administered (100 mg/kg body weight) three days before the operation. In the day of experiment, dural inflammation was induced by topical application of IS or low-pH CSF on exposed dura of right hemisphere for 30 minutes. Fos expression was studied using immunohistochemical technique. The results are described as following and also shown in table 4 and figure 19. The results described in the text and in table are the data of ipsilateral side to dural stimulation. The data of contralateral side are shown in graph.

A. Comparison between serotonin-depleted groups

In low serotonin condition, stimulation of dura by IS could activate trigeminal nociception as they did in normal serotonin condition. Dural stimulation by IS significantly increased the number of Fos-ir cells from the serotonin depleted rat without stimulation ($P = .000$). The magnitude of increase was approximately 2.5-fold. There were 39 ± 11 Fos-ir neurons per slide in rats receiving dural stimulation by IS vs. 15 ± 3 neurons per slide in controls. Significant increase in the number of Fos-ir cells was also present when compared to non-stimulation side ($P = .007$). The magnitude of increase was approximately 2-fold.

Low-pH CSF application caused an increase in the number of Fos-ir cells compared to non-stimulation side. However, the number of Fos-ir cells is not significantly different form control group ($P = .684$). There were 16 ± 2 Fos-ir neurons per slide in rats receiving dural stimulation by low-pH CSF vs. 15 ± 3 neurons per slide in controls.

B. Comparison between serotonin-depleted and normal groups

Our study showed that serotonin depletion potentiated trigeminal nociception induced by dural stimulation. The number of Fos-ir cells in the rat with PCPA pretreatment was higher than those obtained from the rat without PCPA pretreatment in every experiment. Without dural stimulation, the number of Fos-ir cells significantly increased from 7 ± 1 in normal rats to 15 ± 3 cells per slide in serotonin depleted rats

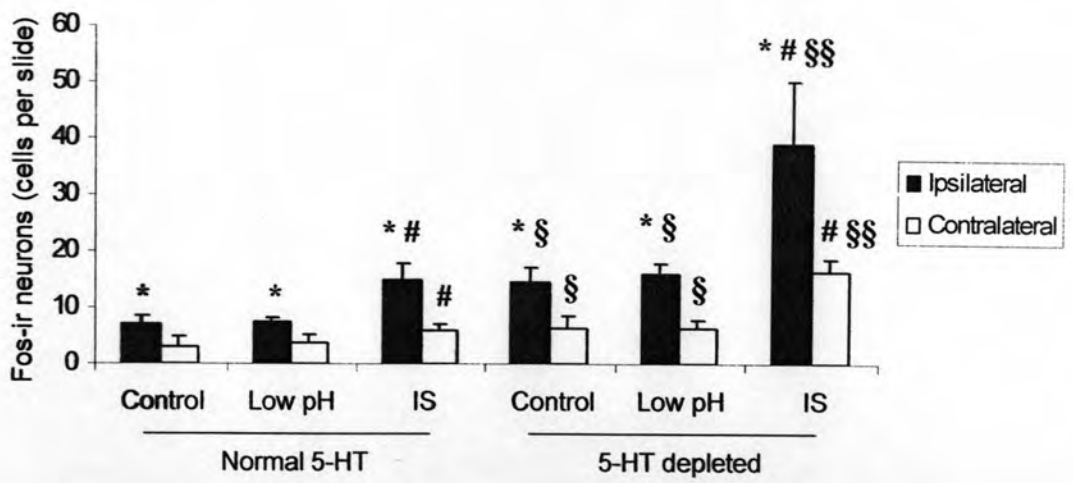
(P = .031). With dural stimulation, the number of Fos-ir cells in rats with serotonin depletion were 16 ± 2 and 39 ± 11 cells per slide for the low-pH CSF and IS stimulation, respectively, which was significantly higher than those from normal rats with dural stimulation (7 ± 1, and 15 ± 3 cells per slide for the low-pH CSF (P = .023) and IS stimulation (P = .000 respectively). It was noted from this result that serotonin depletion strongly enhanced trigeminal nociception in IS group. The magnitudes of increase were 114 %, 129 %, and 160 % in control, low pH, and IS groups, respectively.

Table 4 Number of Fos-ir cells in TNC with and without dural stimulation of 5-HT depleted rats compared to normal 5-HT rats

	Number of Fos-ir cells (cells per slide)				
Group	Control	Low pH	IS	*P* (low pH vs. control)	*P* (IS vs. control)
Normal 5-HT	7 ± 1	7 ± 1	15 ± 3$^{\#}$	.988	.023
5-HT depleted	15 ± 3§	16 ± 2§	39 ± 11$^{\# \S}$	.684	.000
P (compared to normal)	.031	.023	.000		

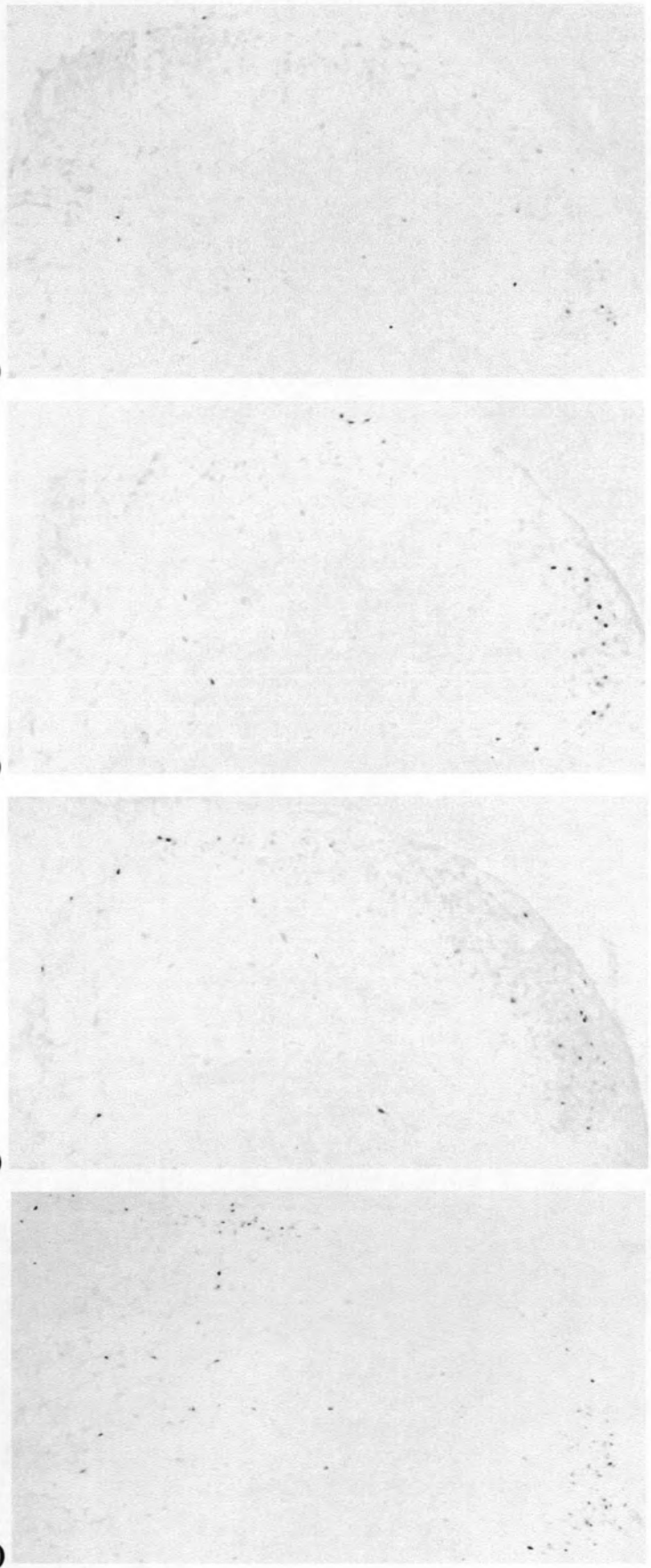
Note # significantly different compared to correspondent control group ($P < .05$)
§ significantly different compared to correspondent normal 5-HT group ($P < .05$)

Figure 19 Bar graph showing the number of Fos-ir cells in TNC with and without dural stimulation of 5-HT depleted rats compared to normal rats



Note * significantly different compared to correspondent contralateral side ($P < .05$)
significantly different compared to correspondent control group ($P < .05$)
§ and §§ significantly different compared to correspondent normal 5-HT group ($P < .05$ and $P < .001$, respectively)

Figure 20 Fos-ir cells in dorsal horn of TNC of A) normal rat; B) normal rat with IS; C) serotonin-depleted rat; D) serotonin-depleted rat with IS. (10x objective lens).



Effect of serotonin depletion on NR1 receptor expression

The results showed that low serotonin condition did not alter the baseline of NR1 expression. Number of NR1-ir cells in serotonin-depleted and normal rats of control groups is not significant different at 30 minutes (P = .594) and 2 hours (P = .500). There were 342 ± 21 and 344 ± 6 NR1-ir neurons per slide in serotonin-depleted rats vs. 334 ± 30 and 334 ± 21 neurons per slide in normal serotonin rats at 30 minutes and 2 hours, respectively.

Similar to the effect of dural stimulation on NR1 expression in normal rats, the meningeal inflammation did not alter the expression of NR1 in serotonin depleted rats. In serotonin depleted groups, the number of NR1-ir cells in rats with dural inflammation and control rats is not significant different at 30 minutes (P = .883) and 2 hours (P = .626). There were 339 ± 8 and 351 ± 13 NR1-ir neurons per slide in IS rats vs. 342 ± 21 and 344 ± 6 neurons per slide in control rats at 30 minutes and 2 hours, respectively. In brief, serotonin depletion or/and dural inflammation did not alter the expression of NR1 receptor (table 5 and figure 21).

Table 5 Number of NR1-ir cells in TNC with and without dural stimulation of 5-HT depleted rats compared to normal rats at 30 minutes and 2 hours

		Number of NR1-ir cells (cells per slide)		
Time	Group	Control	IS	*P*
30 min	Normal 5-HT	334 ± 30	337 ± 21	.809
	5-HT depleted	342 ± 21	339 ± 8	.883
	P (compared to normal)	.594	.900	
2 h	Normal 5-HT	334 ± 21	332 ± 16	.858
	5-HT depleted	344 ± 6	351 ± 13	.626
	P (compared to normal)	.500	.153	

Figure 21 Bar graph showing the number of NR1-ir cells in TNC with and without dural stimulation of 5-HT depleted rats compared to normal rats at 2 hours

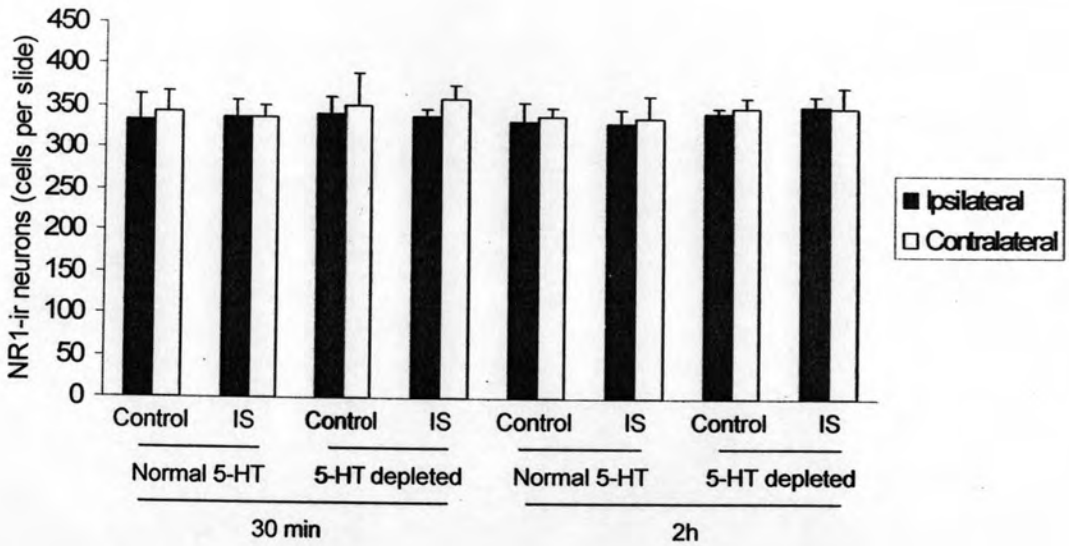
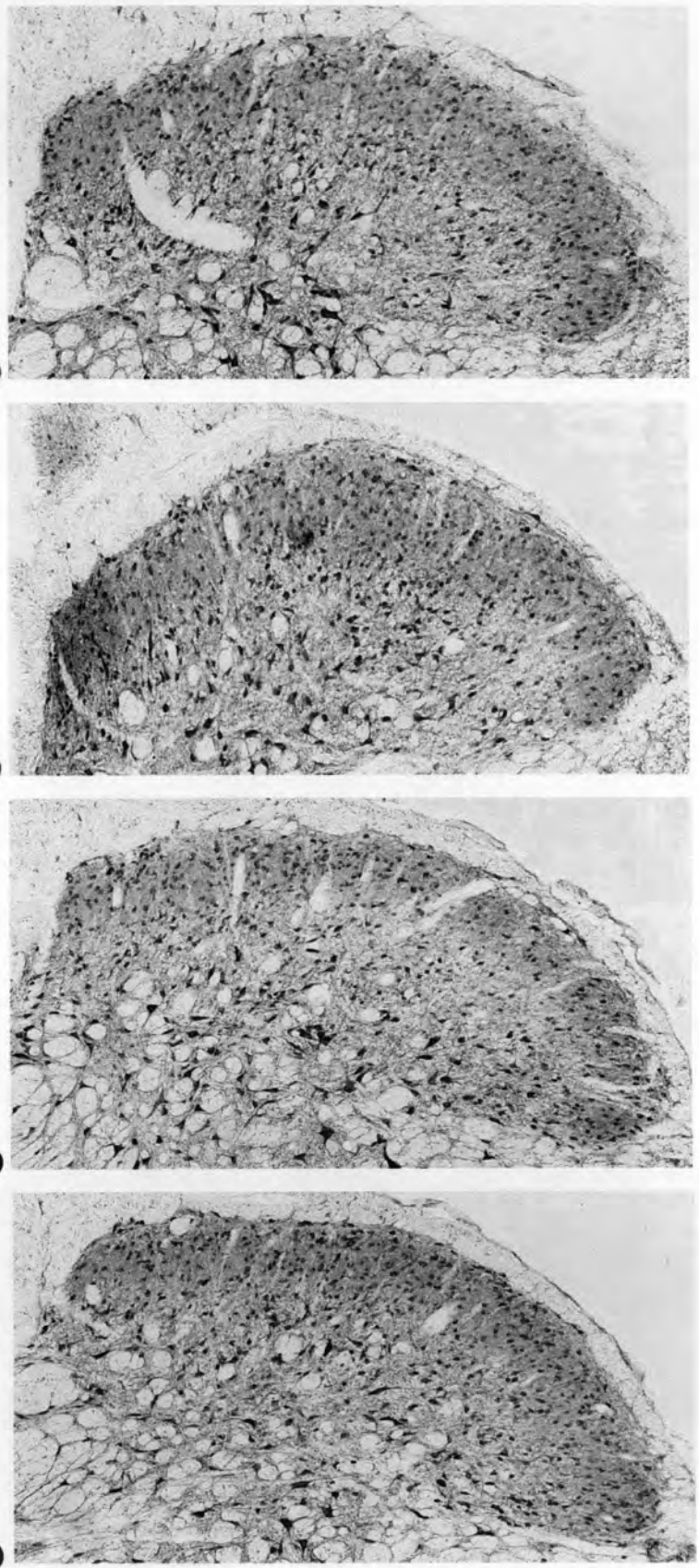


Figure 22 NR1-ir cells in dorsal horn of TNC of A) normal rat; B) normal rat with IS; C) serotonin-depleted rat; D) serotonin-depleted rat with IS. (8x magnification in Image Scope program)



Effect of serotonin depletion on NR1 receptor phosphorylation induced by dural stimulation

The results are shown in table 6 and figure 23, 24, 25 and also described in the following text.

A. Comparison between serotonin-depleted groups

In a low serotonin condition, stimulation of dura by low-pH CSF and IS had the same effect of NR1 phosphorylation as in normal serotonin condition. Dural stimulation by IS significantly increased the number of pNR1-ir cells as compared with the control value in the dorsal horn at 30 minutes ($P = .001$) and 2 hours ($P = .000$). There were 47 ± 5 and 53 ± 9 pNR1-ir neurons per slide in rats receiving dural stimulation by IS vs. 33 ± 3 and 25 ± 3 neurons per slide in controls at 30 minutes and 2 hours, respectively. Significant increase in pNR1-ir cells was also present when compared to the non-stimulation side ($P = .010$ and $P = .010$ at 30 minutes and 2 hours, respectively).

The application of low-pH CSF caused an increase in the number of pNR1-ir cells as compared to the non-stimulation side ($P = .003$). However, when compared to the control group, no significant difference was observed ($P = .775$). There were 26 ± 2 pNR1-ir neurons per slide in rats receiving dural stimulation by low-pH CSF vs. 25 ± 3 neurons per slide in controls.

B. Comparison between serotonin-depleted and normal groups

Our study showed that serotonin depletion potentiated NR1 receptor phosphorylation. Pretreatment with PCPA caused an increased in the number of pNR1-ir cells in TNC in all experimental groups. When compared to the rat with normal serotonin level, serotonin depletion significantly increased the number of pNR1-ir cells in control group ($P = .048$), in low pH stimulation group ($P = .017$), and in IS stimulation group ($P = .000$). In serotonin depleted rats, the number of pNR1-ir neurons was 25 ± 3, 26 ± 2, and 53 ± 9 neurons per slide for the control, low-pH CSF stimulation, and IS stimulation groups, respectively, while in normal serotonin rats, the number of pNR1-ir neurons was 18 ± 3, 17 ± 2, and 29 ± 4 neurons per slide for the control, low-pH CSF stimulation, and IS stimulation groups, respectively. It was noted

from this result that serotonin depletion strongly enhanced NR1 receptor phosphorylation in IS group. The magnitudes of increase were 39 %, 53 %, and 83 % in control, low pH, and IS groups, respectively.

The number of pNR1-ir cells and NR-ir cells of the same rat were used to calculate the percentage of NR1 phosphorylation. Percentage of NR1 phosphorylation and the number of pNR-ir cells were in accordance because the number of NR1-ir cells was not significant different in all groups.

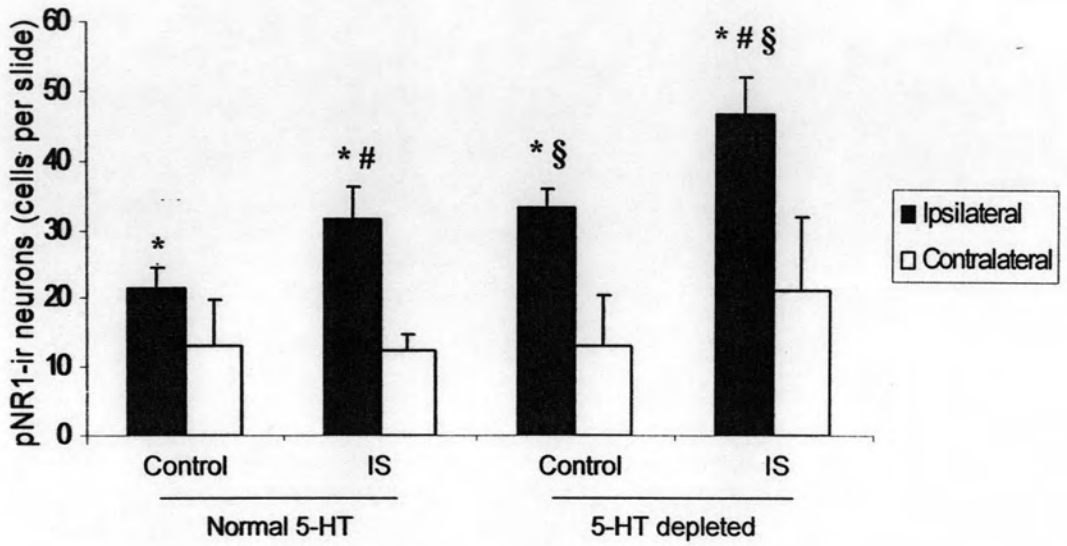
Table 6 Number of pNR1-immunoreactive cells in TNC with and without dural stimulation of 5-HT depleted rats compared to normal rats at 30 minutes and 2 hours

		Number of pNR1-ir cells (cells per slide)				
Time	Group	Control	Low pH	IS	*P* (low pH vs. control)	*P* (IS vs. control)
30 min	Normal 5-HT	22 ± 3	-	31 ± 5$^{\#}$	-	.003
	5-HT depleted	33 ± 3§	-	47 ± 5$^{\# \S}$	-	.001
	P (compared to normal)	.001	-	.000		
2 h	Normal 5-HT	18 ± 3	17 ± 2	29 ± 4$^{\#}$	.830	.003
	5-HT depleted	25 ± 3§	26 ± 2§	53 ± 9$^{\# \S}$	.775	.000
	P (compared to normal)	.048	.017	.000		

Note # significantly different compared to correspondent control group ($P < .05$)

§ significantly different compared to correspondent normal 5-HT group ($P < .05$)

Figure 23 Bar graph showing the number of pNR1-immunoreactive cells in TNC with and without dural stimulation of 5-HT depleted rats compared to normal rats at 30 minutes

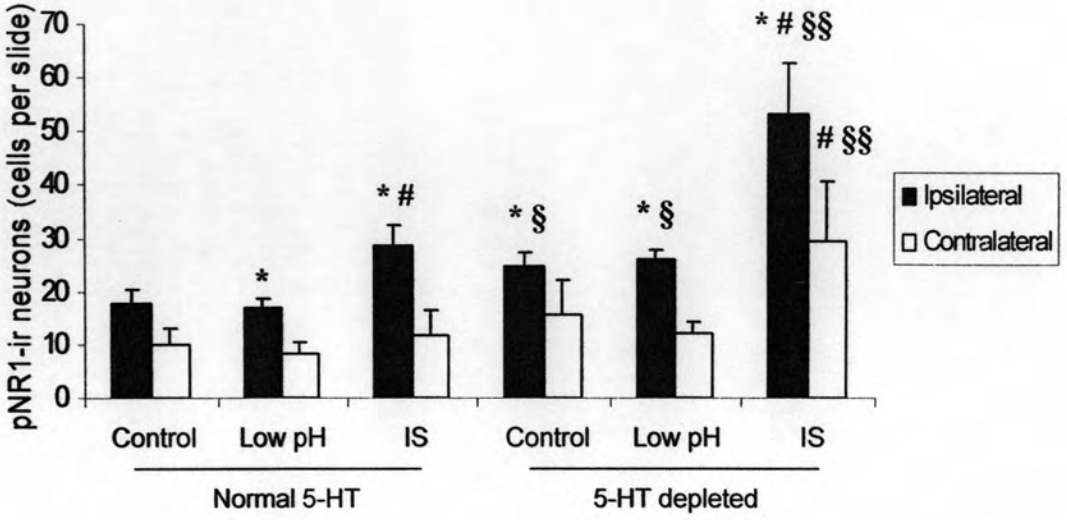


Note * significantly different compared to correspondent contralateral side ($P < .05$)

significantly different compared to correspondent control group ($P < .05$)

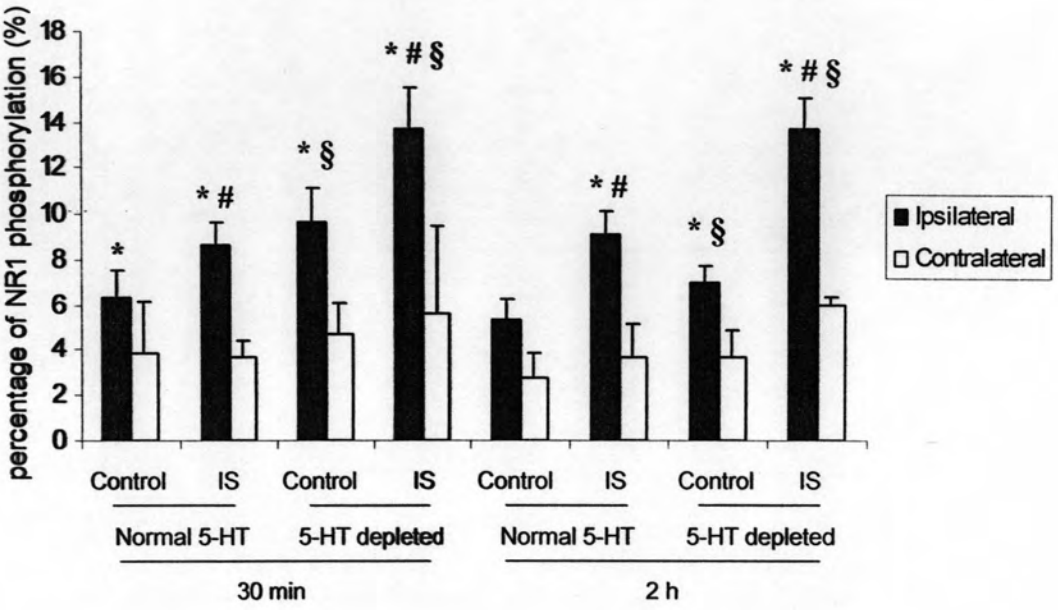
§ significantly different compared to correspondent normal 5-HT group ($P < .05$)

Figure 24 Bar graph showing the number of pNR1-immunoreactive cells in TNC with and without dural stimulation of 5-HT depleted rats compared to normal rats at 2 hours



Note * significantly different compared to correspondent contralateral side ($P < .05$)
significantly different compared to correspondent control group ($P < .05$)
§ and §§ significantly different compared to correspondent normal 5-HT group ($P < .05$ and $P < .001$, respectively)

Figure 25 Bar graph showing the percentage of NR1 phosphorylation in TNC with and without dural stimulation of 5-HT depleted rats compared to normal rats at 30 minutes and 2 hours

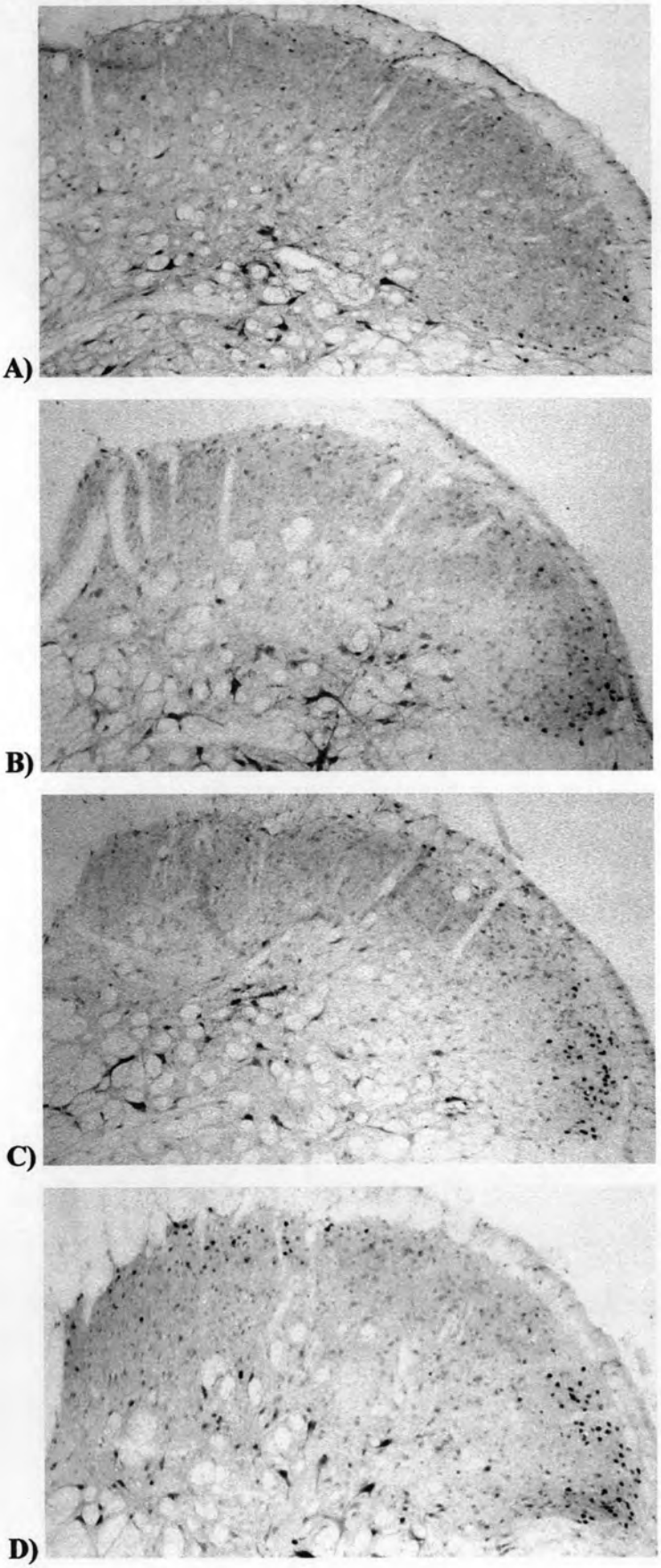


Note: * significantly different compared to correspondent contralateral side ($P < .05$)

significantly different compared to correspondent control group ($P < .05$)

§ significantly different compared to correspondent normal 5-HT group ($P < .05$)

Figure 26 pNR1-ir cells in dorsal horn of TNC of A) normal rat; B) normal rat with IS; C) serotonin-depleted rat; D) serotonin-depleted rat with IS. (10x objective lens).



Relationship between NR1 receptor phosphorylation and trigeminal nociception

To show the relationship between phosphorylation of NR1 receptor and trigeminal nociception, linear regression was used to predict this relationship. Number of pNR1- and Fos-ir cells in TNC of the same rat in both control and inflammation groups at time point of 2 hours was used in the calculation and data from normal and serotonin-depleted groups were separately calculated. The result showed that phosphorylation of NR1 receptor in TNC was highly related to trigeminal nociception (figure 27). In normal rats, there was a strong positive correlation between the number of pNR1-ir cells and the number of Fos-ir cells ($r^2 = 0.957$, $P < .001$). Besides, the correlation could be presented by the linear regression of $y = 0.520x$. In serotonin-depleted rats, there also was a strong positive correlation between the number of pNR1-ir cells and the number of Fos-ir cells ($r^2 = 0.941$, $P < .001$). Besides, the correlation could be presented by the linear regression of $y = 0.706x$. It was noted that the slope of the serotonin-depleted group was higher than the slope of normal group.

Figure 27 Correlation between number of pNR1- and Fos-ir cells in normal serotonin and serotonin-depleted group