

Developmental Variation in Nitrous Oxide–induced c-Fos Expression in Fischer Rat Spinal Cord

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RECENT studies have further clarified the mechanism for the analgesic–antinociceptive action of nitrous oxide (N₂O).¹ N₂O induces opioid peptide release in the mid brain, which activates descending noradrenergic inhibitory neurons and modulates pain and nociceptive processing in the spinal cord. Activation of descending noradrenergic inhibitory neurons by N₂O can be assessed by the induction of c-Fos expression (a protein product of the immediate early gene, *c-fos*, which is commonly used as an immunohistochemical marker of neuronal activation²) in the spinal cord in adult rats.³ Because the descending noradrenergic inhibitory neurons are not yet functional at birth in rats,^{4,5} we posit that N₂O is not an effective analgesic–antinociceptive agent in newborns. In support of this, we have shown that N₂O lacks antinociceptive effect in the tail flick test (thermal stimulation on the tail) in newborn rats.⁶ In this report, we show that N₂O administration does not induce c-Fos expression in the spinal cord of newborn rats, possibly due to a lack of functional descending noradrenergic inhibitory neurons.

Materials and Methods

Animal and Gas Exposure

The study protocol of animal experiments was approved by the Home Office of the United Kingdom (London, United Kingdom), and all efforts were made to minimize animal suffering and reduce the number of animals used. Fischer rats⁷ of different ages, including adults, were obtained from the breeder (B&K Universal, Grimston Aldbrough Hull, United Kingdom). One- and 2-week-old pups were received from the breeder with their mothers and were kept together until the experiment was initiated. Those pups were randomly divided

into two groups without consideration of sex (table 1). (In the previous study, we found no sex difference in response to noxious thermal stimulation or in response to N₂O at these age groups.⁶) Three- and 4-week-old rats were received without mothers, and only male animals were used for the experiment, as for adults. To estimate more accurately the age of each animal, a growth curve of the body weight was created from 12 pups (7 males and 5 females that were obtained from the timed-pregnant rats that delivered at our facility), and the curve fit was calculated as follows:

$$y = 4.8168 + 0.7439x + 1.9774e^{-2}x^2 + 6.5455e^{-4}x^3 \quad (R^2 = 0.999)$$

where *y* = body weight, and *x* = age by day. The day of birth was defined as 0 days old. The age of each animal was then estimated from the inverted formula (table 1):

$$y = -4.4548 + 1.1298x - 1.3568e^{-2}x^2 + 6.7857e^{-5}x^3,$$

where *y* = age by day, and *x* = body weight. The animals were exposed to either air (control group) or 75% N₂O–25% O₂ (N₂O group) for 90 min in an acrylic chamber as described in our previous study.³

Spinal Cord Preparation and Immunohistochemical Analysis of c-Fos

The spinal cord collection and its cryosection and immunohistochemical analysis are described in detail in our previous study.³ In brief, animals were injected intraperitoneally with sodium pentobarbital (100 mg/kg) immediately after gas exposure, and the spinal cord was collected after paraformaldehyde perfusion. The spinal cord was cryosectioned at 30 μm, and sections were collected in 0.1-M phosphate-buffered saline as free-floating sections. Sections were incubated with anti-c-Fos antibody (1:10000, catalog No. sc-52-G; Santa Cruz Biotechnology, Santa Cruz, CA), and the immunohistochemical reaction was visualized using enhanced diaminobenzidine (DAB) reaction (DAB kit; Vector Laboratories, Burlingame, CA). Slides were examined for c-Fos-positive cells, which were identified by dense black nuclear staining. For adult animals, the number of c-Fos-positive cells was counted for each area of the spinal cord, *i.e.*, laminae I–II (superficial area), laminae III–IV (nucleus proprius area), laminae V–VI (neck area), and laminae VII–X (ventral area), according to the method described by Presley *et al.*⁸ For young animals, such a scheme

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Table 1. Summary Data from Experimental Groups at Different Developmental Stages

Age (weeks)	Group	No. of Animals Examined	Body Weight (g, Mean \pm SD)	Estimated Age (days, Mean \pm SD)	Sex Ratio (M/F)
1	Control	4	12.7 \pm 0.7	7.9 \pm 0.6	2/2
	N ₂ O	4	12.9 \pm 0.9	7.9 \pm 0.7	3/1
2	Control	4	26.2 \pm 1.3	17.0 \pm 0.8	2/2
	N ₂ O	4	26.0 \pm 0.9	16.9 \pm 0.5	2/2
3	Control	4	34.4 \pm 5.8	21.0 \pm 2.4	4/0
	N ₂ O	4	35.1 \pm 5.7	21.3 \pm 2.4	4/0
4	Control	4	71.3 \pm 6.5	31.7 \pm 1.5	4/0
	N ₂ O	4	76.4 \pm 4.9	33.0 \pm 1.2	4/0

N₂O = nitrous oxide.

could not be used because of immature development of the spinal cord. Therefore, a simplified scheme developed by Yi and Barr⁹ was used to count the number of c-Fos-positive cells in four different areas of the spinal cord, *i.e.*, A-B, C, D, and E. At least four animals were examined for each group, and the number of c-Fos-positive cells in each area and group was calculated as mean \pm SD. An investigator who conducted the counting of c-Fos-positive cells was blinded for experimental groups.

Statistical Analysis

The Mann-Whitney U test was performed on the data for the numbers of c-Fos-positive cells between control and N₂O groups at each developmental stage. A *P* value less than 0.05 was considered to be statistically significant. No attempt was made to compare among different levels of the spinal cord and different age groups.

Results

In adult rats, exposure to 75% N₂O increased the number of c-Fos-positive cells at all levels of the spinal cord examined, *i.e.*, cervical, thoracic, and lumbar levels, and within all laminae except for laminae I and II (table 2). N₂O-induced c-Fos expression was specifically localized within laminae III and VI. In younger rats, two levels of the spinal cord, cervical and lumbar, were examined, which showed similar results (table 3). (Those at the thoracic and sacral levels were too small to be processed.) In 1-week-old animals, there were no differences in the number of c-Fos-positive cells between control and N₂O groups, *i.e.*, N₂O did not induce c-Fos expression. In 2-, 3-, and 4-week-old animals, the number of c-Fos-positive cells was increased in all areas.

Discussion

Descending inhibitory neurons are essential components of the "endogenous pain-suppression system."¹⁰ Recent studies have indicated that activation of descending noradrenergic inhibitory neurons has a pivotal role in the analgesic-antinociceptive effect of N₂O.¹ During de-

velopment, descending inhibitory neurons extend their axons *caudad* from the brain stem, reaching the spinal cord during the fetal period.¹¹ Evidence has shown that these neurons are not functionally mature until a few weeks after birth in rats. For example, immunohistochemical studies have shown that it takes more than 2 weeks after birth for noradrenergic neurons to establish their adult distribution pattern in the dorsal horn of the spinal cord.¹²⁻¹⁴ Electrophysiologic studies have also indicated that descending inhibitory neurons become functionally mature approximately 3 weeks after birth.^{4,5} In the current study, we have demonstrated that N₂O does not induce c-Fos expression in the spinal cord in rats until 2 weeks after birth. This result is consistent with our hypothesis that N₂O does not induce c-Fos expression in the spinal cord of newborn rats because of a lack of functionally mature descending noradrenergic inhibitory neurons. It is also in accordance with our previous study indicating that N₂O does not induce an antinociceptive response to the tail-flick test in newborn rats.⁴

Table 2. Number of c-Fos-positive Cells at Different Laminae of the Spinal Cord at Different Levels in Adult Animals

	Control	75% N ₂ O
Body weight (g)	233.0 \pm 26.2	235.8 \pm 29.4
Cervical		
Laminae I-II	5.5 \pm 3.5	7.1 \pm 1.7
Laminae III-IV	6.1 \pm 0.9	42.8 \pm 2.7*
Laminae V-VI	1.8 \pm 1.0	24.7 \pm 8.7*
Laminae VII-X	3.3 \pm 1.7	15.3 \pm 3.1*
Total	16.8 \pm 4.4	89.9 \pm 10.1*
Thoracic		
Laminae I-II	2.6 \pm 1.2	4.3 \pm 2.2
Laminae III-IV	5.8 \pm 2.1	29.4 \pm 8.7*
Laminae V-VI	2.6 \pm 1.1	13.9 \pm 6.1*
Laminae VII-X	1.7 \pm 1.1	6.8 \pm 2.2*
Total	12.8 \pm 2.5	54.4 \pm 15.8*
Lumbar		
Laminae I-II	9.8 \pm 5.7	12.3 \pm 4.3
Laminae III-IV	18.3 \pm 7.6	64.8 \pm 8.9*
Laminae V-VI	5.3 \pm 1.4	27.0 \pm 6.2*
Laminae VII-X	3.8 \pm 1.4	15.6 \pm 2.6*
Total	37.0 \pm 12.8	120.0 \pm 4.2*

Values are mean \pm SD; n = 4.

* *P* < 0.05 versus control.

Table 3. Number of c-Fos-positive Cells at Different Areas of the Spinal Cord at the Cervical and Lumbar Levels in Newborn Rats of Different Ages

Age (weeks)	Area	Cervical Level		Lumbar Level	
		Control	N ₂ O	Control	N ₂ O
1	A-B	11.0 ± 3.2	9.8 ± 2.5	4.3 ± 1.7	3.9 ± 3.3
	C	12.2 ± 9.2	7.0 ± 2.5	9.3 ± 3.3	7.1 ± 4.6
	D	5.2 ± 3.2	3.8 ± 1.2	1.6 ± 0.9	3.3 ± 2.4
	E	6.2 ± 2.7	4.8 ± 2.1	1.8 ± 1.0	1.6 ± 1.3
	Total	34.5 ± 11.8	25.4 ± 2.8	16.9 ± 1.9	15.8 ± 8.1
2	A-B	9.8 ± 3.7	33.9 ± 6.1*	10.4 ± 5.5	22.8 ± 13.3*
	C	24.4 ± 9.4	42.3 ± 5.5*	5.7 ± 3.9	30.7 ± 13.8*
	D	7.8 ± 3.2	23.0 ± 5.7*	7.9 ± 5.3	16.3 ± 10.6
	E	3.4 ± 1.8	12.1 ± 3.1*	1.1 ± 0.5	6.8 ± 4.9*
	Total	45.3 ± 9.1	111.3 ± 9.6*	25.1 ± 14.7	76.5 ± 41.6*
3	A-B	15.2 ± 7.7	40.2 ± 7.8*	9.2 ± 5.6	31.6 ± 11.9*
	C	9.3 ± 6.7	29.9 ± 7.6*	5.8 ± 3.4	35.7 ± 19.9*
	D	3.2 ± 1.7	28.7 ± 12.2*	1.9 ± 0.6	21.3 ± 14.2*
	E	1.7 ± 1.2	13.4 ± 2.6*	0.8 ± 0.8	8.5 ± 5.9*
	Total	29.3 ± 13.0	112.2 ± 25.7*	17.7 ± 9.1	97.1 ± 45.0*
4	A-B	12.9 ± 2.3	21.1 ± 4.4*	26.0 ± 11.9	39.2 ± 5.8*
	C	12.5 ± 5.0	45.0 ± 7.0*	12.4 ± 5.2	23.5 ± 5.4*
	D	3.6 ± 2.0	26.9 ± 3.1*	2.6 ± 1.5	12.8 ± 2.7*
	E	3.4 ± 4.4	11.4 ± 4.6*	1.8 ± 1.0	7.2 ± 1.9*
	Total	32.4 ± 8.2	104.4 ± 6.6*	42.8 ± 18.6	82.6 ± 8.9*

Values are mean ± SD; n = 4.

* $P < 0.05$ versus control.

N₂O = nitrous oxide.

The quantitative difference in c-Fos expression between adult and young animals may be confounded by the different laminar schemes used. In the laminar scheme that we have adopted for young animals,⁹ A-B includes adult laminae that both express (lamina III) and do not express (laminae I and II) c-Fos with N₂O exposure. In addition, there seems to be a lack of congruity with N₂O exposure between our current findings (in which c-Fos expression appears at 2 weeks) and our earlier report (in which antinociceptive effect on tail-flick test only appears at 4 weeks⁶). However, these can be reconciled by the fact that although activation (and hence c-Fos expression) of γ -aminobutyric acid-mediated (GABAergic) interneurons is required,³ it is not sufficient to produce antinociceptive effect with N₂O exposure. Therefore, other downstream consequences of GABA release may still be immature and nonfunctional, including signaling through the GABA_A receptor.¹⁵ Furthermore, the sacral elements are involved in the tail-flick latency paradigm, and this region of the spinal cord was not examined in this study for technical reasons.

In summary, we have demonstrated that N₂O administration induces c-Fos expression in the spinal cord of the adult rat; this expression is deficient in newborn rats and appears only 2 weeks after birth. These findings are consistent with our hypothesis that a lack of functional descending noradrenergic inhibitory neurons precludes

both the induction of c-Fos expression and the antinociceptive effect during N₂O exposure in newborn rats.

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