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Non-vagal apnea evoked by intra-common carotid artery injection of N-methyl-D-aspartic acid (NMDA) in anesthetized rats

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Short communication

Abstract. Respiratory effects of an intra-common carotid artery injection of N-methyl-D-aspartic acid (NMDA) were investigated in anesthetized spontaneously breathing rats, using three experimental paradigms: (1) midcervical vagotomy followed by supranodosal vagotomy, (2) midcervical vagotomy followed by section of the carotid sinus nerves (CSNs), and (3) midcervical vagotomy followed by pharmacological blockade of NMDA receptors. The intra-common carotid artery injection of NMDA (4 mg/kg) induced transient expiratory apnea followed by a brief and variably occurring period of breathing at reduced tidal volume. There were no consistent changes in respiratory rate in rats subjected to midcervical vagotomy alone. Supranodose vagotomy exerted no effect on NMDA-induced respiratory arrest, whereas CSNs' section or blockade of NMDA receptors with AP-7 abolished the apnea. These results indicate that the apnea induced by intra-arterial NMDA challenge is due to activation of peripheral NMDA receptors and is mediated *via* carotid body afferents.

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Glutamate is the primary excitatory neurotransmitter of the visceral afferent fibers (Talman et al. 1980, Sykes et al. 1997). The nucleus tractus solitarii that is the target of afferent fibers from peripheral chemoreceptors and cardiopulmonary receptors shows substantial glutamate concentration and the presence of N-methyl-D-aspartic acid (NMDA) glutamatergic receptors (Aicher et al. 1999, Yamazaki et al. 2000). NMDA receptors mediate many reflexes elicited by stimulation of pulmonary C-fiber afferents (Vardhan et al. 1993a), baroreceptors (Canesin et al. 2000), Breuer-Hering volume endings (Bonham et al. 1993) and carotid chemoreceptor pathways (Vardhan et al. 1993b).

The effects of peripherally administered NMDA on respiratory control mechanisms have not been investigated at length. Our recent studies have shown that intravenous (i.v.) or intra-atrial NMDA challenge caused depression of respiratory motor output due to the decline in both tidal volume and respiratory rate. These responses resulted from the excitation of peripheral NMDA receptors and were mediated by the afferent pathway from the carotid bodies, i.e. beyond the vagal pathway (Kaczyńska and Szereda-Przestaszewska 2004, 2005).

The present study tested the hypothesis that NMDA administered to the bloodstream perfusing the carotid bodies will result in a different pattern of respiratory response than that evoked by the aforementioned intravenous (pulmonary circulation) challenge. To verify whether post-NMDA apnea is caused by excitation of NMDA receptors on carotid body afferents, we eliminated the vagal afferent input to the medulla at infra- and supranodose levels and examined the effect of NMDA receptor blockade on the respiratory response.

Adult male Wistar rats weighing 250–300 g were anesthetized with an intraperitoneal injection of 600 mg/kg of urethane (Sigma) and 120 mg/kg of alpha-chloralose (Fluka AG). The animals were placed in the supine position and breathed with room air spontaneously. The trachea was exposed in the neck, sectioned below the larynx, and cannulated. The right common carotid artery (c.c.a.) was tied off and the catheter for NMDA injections was inserted cephalad. The right femoral artery was catheterized for blood pressure recording, and another catheter was inserted into the left femoral vein for administration of additional urethane doses at re-emergence of nociperception. Rectal temperature was maintained at 38°C with a heating pad. The midcervical and supranodose segments of the vagi were isolated and prepared for bilateral vagotomy prior to measuring respiratory variables. The carotid sinus nerves (CSNs) were isolated under an operating microscope and prepared for section at their junctions with glossopharyngeal nerves.

First measurements were performed about 30 min after the bilateral midcervical vagotomy and were immediately followed by NMDA challenge. The next surgical intervention (bilateral supranodose vagotomy or CSNs' cut) or AP-7 blockade was applied immediately after the accomplishment of the initial measurements. The second (final) NMDA challenge was given 30–45 min later, i.e. after stabilization of the studied parameters.

The respiratory effects of NMDA challenge were tested in three groups of midcervically vagotomized rats using single NMDA boluses in the following experimental conditions: before and after (i) bilateral supranodose vagotomy (n=10), (ii) bilateral transsection of the CSNs (n=6), (iii) pharmacological blockade of NMDA receptors (n=6).

N-metyl-D-aspartic acid (Sigma) was injected into the right c.c.a. at a dose of 4 mg/kg. The dose was derived from the dose-response relationship reported earlier (Kaczyńska and Szereda-Przestaszewska 2004). The specific NMDA receptor antagonist D,L-2-amino-7-phosphonoheptanoic acid (AP-7; Sigma) was injected i.v. at a dose of 200 μg/kg (Sitniewska et al. 2003). Each drug bolus was immediately flushed with 0.2 ml of physiological saline.

All animal use procedures were in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the local ethics committee.

Tidal volume (V_T), minute ventilation (V_E), respiratory rate (f), expiratory time (T_E), mean arterial pressure (MAP), and costal diaphragm electromyogram were acquired, recorded and calculated as described earlier (Kaczyńska and Szereda-Przestaszewska 2004, 2005). The ventilatory response parameters were assessed just before NMDA challenge, immediately after the post-challenge apnea (over the first five post-apnoeic breaths), and 30 s and 60 s after the challenge. T_E prolongation was measured as the ratio of maximal T_E during post-NMDA apnea to the respective control T_E value ($T_{E\ NMDA}/T_{E\ control}$). The duration of the apnoeic period in diaphragm activity or V_T was used as an index of respiratory inhibition.

V_T, V_E, f and T_E data were first analyzed by two-way ANOVA with repeated measures on post-NMDA challenge time (pre-challenge, early post-apneic phase, and 30 s and 60 s after the challenge) and on innervation status (vagotomy combined with supranodose section, or with CNSs' section, or with AP-7 treatment). Differences in the ventilatory parameters between various time points and innervation states, and T_E prolongation were evaluated by Student's *t*-test for paired data when appropriate. All statistical analyses were performed using Statistica for Windows 5.1 software (StatSoft, Tulsa, OK, USA). In all cases, a $P \leq 0.05$ was considered significant. The results shown are means ± 1 SE.

In rats subjected to midcervical vagotomy alone, injection of physiological saline (0.2 ml) caused no respiratory effect (not shown), while intra-c.c.a. NMDA injection produced significant respiratory effects. The effects were invariably comprised of apnea followed by breathing at a steady rate and wavering changes in V_T that showed a tendency for post-apnoeic depression.

In midcervically vagotomised rats, two-way ANOVA showed a significant effect of the NMDA challenge on each respiratory parameter studied ($P \leq 0.013$), a significant effect of supranodose vagotomy on V_E ($F_{1,9}$=5.60, P=0.042) and a tendency for an effect on V_T ($F_{1,9}$=4.45, P=0.064), a significant midcervical vagotomy × supranodose vagotomy interaction effect on V_E ($F_{3,27}$=4.30, P=0.013), and a borderline-significant effect of the interaction on V_T ($F_{3,27}$ =2.90, P=0.053). The supranodose vagotomy did not significantly affect the duration of post-challenge apnea (3.12 ± 0.81 s and 1.85 ± 0.4 s, pre- and post-supranodose nerve section, respectively; t_9=1.26, P=0.24) or T_E prolongation (t_9=1.35, P=0.21; Fig. 1D). *Post-hoc* analysis showed that supranodose vagotomy significantly increased V*T* and V*E* shortly after the post-NMDA-induced apnea (immediately after and at 30 s; Fig. 1A).

To elucidate if the CNSs contribute to the response to the NMDA challenge, we tested the effect of CNSs' section on the respiratory effects. Two-way ANOVA showed no significant effect of the deafferentation on the respiratory parameters studied ($P \geq 0.17$), significant effects of the NMDA challenge on VT ($F_{3,15}$=5.64, P=0.0086) and MAP ($F_{3,15}$=4.89, P=0.014), and a significant midcervical vagotomy × CSNs' section interaction effects on V_T ($F_{3,15}$=4.19, P=0.024); the interaction effect evidenced a CSNs' cut-related change in the

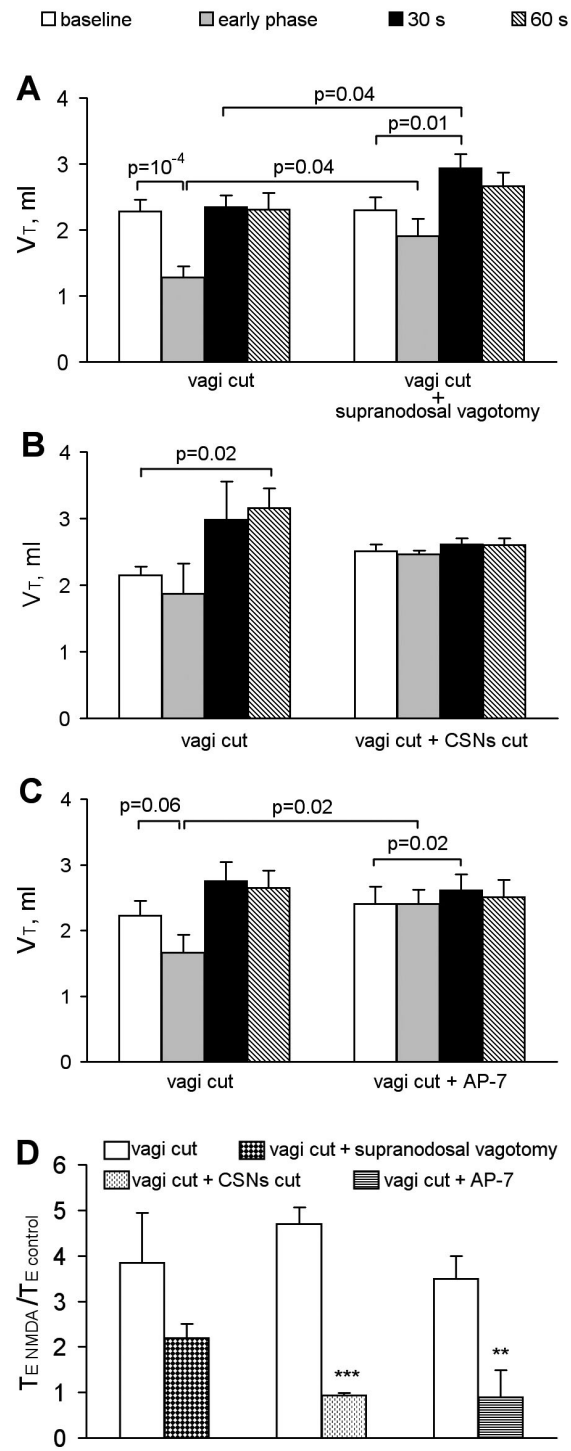

Fig. 1. Mean changes in tidal volume (V_T) evoked by an intra-common carotid artery administration of NMDA in midcervically vagotomised rats before and after supranodose vagotomy (A) (n=10), before and after CSNs section (B) (n=6), and before and after NMDA receptors' blockade with AP-7 (C) (n=6). Panel (D) shows the respective treatment effects on T_E prolongation; $^{**}P<0.01$, $^{***}P<0.001$ *versus* the respective baseline value, Student *t*-test for paired data.

Table I

Changes in MAP (mmHg) and respiratory rate after intracarotid NMDA challenge

Respiratory parameter	Innervation status	Baseline	Immediate post-apneic phase	30 s post-apnea	60 s post-apnea
Mean arterial blood pressure	Vagi cut	75.4 ± 3.5	100.0 ± 9.3*	87.0 ± 8.8	85.2 ± 7.3
	Vagi cut + supranodose vagotomy	72.9 ± 5.0	103.5 ± 9.3**	76.4 ± 6.3	81.8 ± 6.1
	Vagi cut	86.2 ± 7.1	111.2 ± 13.4	80.0 ± 8.4	90.0 ± 5.5
	Vagi cut + CSNs' cut	93.6 ± 6.4	108.2 ± 5.8**	107.5 ± 3.8	108.6 ± 4.7**
	Vagi cut	81.3 ± 8.5	102.8 ± 5.9	84.8 ± 7.7	89.7 ± 8.0
	Vagi cut + AP-7	80.2 ±8.3	95.8 ± 8.4	90.3 ± 9.7	91.3 ± 9.4
Respiratory rate	Vagi cut	58.1 ± 1.9	59.2 ± 4.2	49.9 ±4.3	51.9 ±3.7
	Vagi cut + supranodose vagotomy	54.0 ±2.6	61.2 ±4.5	53.9 ±3.2	52.8 ±3.2
	Vagi cut	61.5 ±2.8	60.6 ±8.5	53.2 ±4.7	47.2 ±4.6
	Vagi cut + CSNs' cut	52.6 ±4.3	55.5 ±3.7	54.0 ±4.1	52.6 ±4.4
	Vagi cut	59.2 ±3.0	62.8 ±4.0	54.8 ±2.0	55.0 ±1.5
	Vagi cut + AP-7	60.3 ±3.1	65.2 ±2.4	65.0 ±1.7###	61.8 ±2.1

All values are means ± SEM. *P<0.05, **P<0.01 vs. the respective baseline value, ###P<0.001 vs. the respective pre-AP-7 blockade value; two-way ANOVA followed by t-test for paired data.

respiratory response pattern. *Post-hoc* analysis revealed that the CSNs' section abolished a delayed (at 60 s) post-NMDA increase in V_T in this rat group (Fig. 1B), but elevated post-NMDA MAP. NMDA-induced apnea of mean duration of 3.2 ± 0.3 s and T_E prolongation were abolished by the CSNs' section (Student t_5=9.77; Fig. 1D).

To verify that the NMDA-induced effects are specific for peripheral NMDA receptors' excitation, we used the highly selective NMDA antagonist AP-7 that does not show propensity for blood-brain barrier crossing (Lodge and Danysz 2002). Two-way ANOVA showed significant effects of the NMDA challenge and of the NMDA receptors' blockade with AP-7 × NMDA challenge interaction on V_T ($F_{3,15}$=12.84, P=0.0002 and $F_{3,15}$=5.60, P=0.0088, respectively; Fig. 1C) and a significant effect of the NMDA receptors' blockade on respiratory rate ($F_{1,5}$=12.59, P=0.016). NMDA given 2 min after AP-7 evoked no respiratory effects; the pre-blockade NMDA-induced apnea (mean duration: 2.41 ± 0.4 s) and TE prolongation (Student t_9=5.57, P=0.0026) and the tendency (P=0.06) for post-apneic decline in V_T were abolished by the administration of the antagonist (Fig. 1D).

NMDA challenge induced an increase in mean arterial pressure immediately in post-apneic phase in all neural states (Table I), which is consistent with our previous results (Kaczyńska and Szereda-Przestaszewska 2004, 2005).

This study showed that the principal effect of the intra-c.c.a. NMDA challenge was a prompt apnea followed by a short period of breathing at reduced V_T and usually a subsequent spurt of breathing that consisted of augmented tidal volume and occasional acceleration of respiration. Importantly, all three tested experimental manipulations (supranodosal vagotomy, CSNs' section and peripheral NMDA receptors' blockade) showed a significant interaction effect with the challenge on V_T.

Microinjections of NMDA to ventrolateral nucleus tractus solitarii induce apnea (Berger et al. 1995, Bonham et al. 1993). When applied to the chemoreceptor projection site (commissural nucleus) in the rat, NMDA causes an increase in minute ventilation (Vardhan et al. 1993b). The brief apnea revealed in our study is consistent with the respiratory effects of centrally applied NMDA. The initial suppression of central inspiratory activity induced by intra-c.c.a. NMDA

challenge may depend on several factors. Since NMDA shows low blood-brain barrier penetrability (Engström and Woodbury 1988), the respiratory changes observed in our study likely involve peripheral effects. This is supported by the abolition of the apnea in rats pretreated with AP-7; this selective NMDA antagonist is active in the peripheral nervous system (Lodge and Danysz 2002).

Apnea is assumed to arise from stimulation of vagal sensory receptors in the lungs. Our rats were lacking lung vagal afferentation, therefore the breathing that followed the NMDA-induced apnea did not present the pattern typical for C-fiber activation (Lee and Pisarri 2001). The apnea provoked by NMDA was not significantly reduced after removal of the input from nodose ganglia, which indicates that it was not merely confined to the vagal reflex. Hence, the NMDA receptors located on the vagal afferents and the nodose ganglia (Erdö 1991, Shigemoto et al. 1992), which are within the reach of the blood supply of the carotid bodies, do not seem to interfere with the respiratory response.

We tried to determine whether the NMDA-induced respiratory response occurring after disconnecting the vagal sensory afferents relies on the chemoafferent nerves. The deafferentation abolished the arrest of breathing, which speaks for this hypothesis. This is the first report on the effect of intra-c.c.a. NMDA administration to midcervically vagotomised chemosensory-deprived rats.

The respiratory response to stimulation of peripheral chemoreceptors is characterized by an increase of the tidal volume and respiratory rate. The response to NMDA injections that we observed was somewhat different, because it included a short apnea followed by a drop and then an increase of V_T, and there were no consistent changes in the respiratory rate. The cessation of breathing induced by the peripheral NMDA application is consistent with the inhibition of neuronal activity *via* the central receptors. Nevertheless, our results imply that intra-c.c.a. NMDA has a stimulatory effect on carotid chemoreceptors, and cutting the carotid sinus nerves, thereby denervating the carotid bodies, precludes this respiratory response. The same pathway was shown to mediate the respiratory response to NMDA administered to the right atrium (Kaczyńska and Szereda-Przestaszewska 2005); however, that response involved depression of both tidal and timing components of the breathing pattern.

There is no evidence in the literature for a role of peripheral NMDA receptors in chemoreceptive functions. Albeit both glutamate and aspartate were immunocytochemically identified in the carotid nerve afferent neurons within the petrosal ganglia (Okada and Miura 1992), the carotid body has not been shown to contain NMDA receptors. There are numerous studies showing that hypoxic activation of carotid chemoreceptors causes glutamate release from the chemosensitive neurons of the NTS (e.g. Houseley and Sinclair 1988, Richter et al. 1999), and the activation is mediated by NMDA glutamate receptors in the rat, cat and dog (Ang et al. 1992, Gozal et al. 1999, Richter et al. 1999). However, we cannot explain the mechanism of carotid body or sinus nerve excitation by NMDA based on the available reports. Further experiments are required to demonstrate the presence of NMDA receptors in the carotid chemoreceptor structures.

In summary, this study has shown that: (i) intra-c.c.a. NMDA challenge consistently produces apnea in the rat, which is in contrast with the effects of i.v. NMDA challenge (Kaczyńska and Szereda-Przestaszewska 2004); (ii) the apneic spells are executed *via* activation of NMDA receptors outside the vagal afferentation to the brain medulla; (iii) CSNs' section abolishes NMDA-induced apnea, which implies that carotid chemoreceptors most probably constitute a crucial neural pathway to the medullary respiratory network.

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