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## Hypercapnic ventilatory response in mice lacking the 65 kDa isoform of Glutamic Acid Decarboxylase (GAD65)

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### Abstract

**Background:** Recent reports have shown that there are developmental changes in the ventilatory response to hypercapnia in the rat. These are characterized by an initial large response to carbon dioxide immediately after birth followed by a decline with a trough at one week of age, followed by a return in sensitivity. A second abnormality is seen at postnatal day 5 (P5) rats in that they cannot maintain the increase in frequency for 5 min of hypercapnia. In mice lacking GAD65 the release of GABA during sustained synaptic activation is reduced. We hypothesized that this developmental pattern would be present in the mouse which is also less mature at birth and that GABA mediates this relative respiratory depression.

**Methods:** In awake C57BL/6J and GAD65<sup>-/-</sup> mice the ventilatory response to 5% carbon dioxide (CO<sub>2</sub>) was examined at P2, P4, P6, P7, P12.5, P14.5 and P21.5, using body plethysmography.

**Results:** Minute ventilation (VE) relative to baseline during hypercapnia from P2 through P7 was generally less than from P12.5 onwards, but there was no trough as in the rat. Breaking VE down into its two components showed that tidal volume remained elevated for the 5 min of exposure to 5% CO<sub>2</sub>. At P6, but not at other ages, respiratory frequency declined with time and at 5 min was less than at 2 and 3 min. GAD65<sup>-/-</sup> animals at P6 showed a sustained increase in respiratory rate for the five mins exposure to CO<sub>2</sub>.

**Conclusion:** These results show, that in contrast to the rat, mice do not show a decline in minute ventilatory response to CO<sub>2</sub> at one week of age. Similar to the rat at P5, mice at P6 are unable to sustain an increase in CO<sub>2</sub> induced respiratory frequency and GAD65 contributes to this fall off.

### Introduction

The postnatal period is characterized by relative respiratory instability. Three recent reports have shown that in the rat the ventilatory response to carbon dioxide (CO<sub>2</sub>) declines at P6–P7 compared to earlier and later postnatal ages. Stunden et al [1] found that the percent increase in

minute volume (V<sub>E</sub>) induced by 5% CO<sub>2</sub> declined from ~65% at P2 to ~10% at P8. Thereafter it gradually increased to ~70% at P18.5. These authors also determined the slope of minute ventilation at 1% CO<sub>2</sub> increases between 1 and 5% and found a fall from 240 ml/min/kg/% CO<sub>2</sub> at P1 to 27 ml/min/kg/% CO<sub>2</sub> at about P8,

with a subsequent increase to 67 ml/min/kg/% CO<sub>2</sub> at P21. The increases in V<sub>E</sub> were due to an increase in tidal volume with little change in respiratory frequency. Similarly Serra et al [2] showed that 7% CO<sub>2</sub> resulted in a 25% increase in V<sub>E</sub> in animals studied between P1 and P3. In these rats at P6–P7, however, hypercapnia failed to increase V<sub>E</sub>. In the same animals V<sub>E</sub> increased ~25% at P12–P13 and almost 100% at P18–P19. The components of minute ventilation, tidal volume and frequency were not separately reported. Abu-Shaweesh and co-authors [3] examined rats between P5 and P41–42. In 5% CO<sub>2</sub> V<sub>E</sub> increased ~40% at P5 then rose to a plateau of ~100% at P22–23. In the younger rats only tidal volume increased while in those at P22 – P23 both frequency and tidal volume increased. These authors showed a second altered response to CO<sub>2</sub> in the younger animals. At P5 rats differed from those at other ages in that they were unable to sustain their respiratory rate increased during a 5 min exposure. These later authors found that the GABAA receptor blocker bicuculline prevented the lengthening of expiratory time (T<sub>E</sub>) which characterized the decline in respiratory rate during hypercapnia. The actions of bicuculline are not confined to GABAA receptors as this agent also inhibits small-conductance calcium-activated potassium channels [4]. In addition when administered systemically bicuculline induces an increase in respiratory drive under basal conditions. Thus inspiratory time (TI) and T<sub>E</sub> are shortened [3] and peak phrenic nerve activity is increased about 3 fold [5]. Therefore it is not clear that the bicuculline effects are due to blocking the GABA inhibition which occurs in hypercapnia or to a generalized stimulation of respiratory activity.

GABA is synthesized by two GAD isoforms, GAD65 and GAD67. Their subcellular localization has suggested that each may have a distinct function. GAD65 is localized to axon terminals where it is bound to the synaptic vesicle membrane. GAD67 is distributed in the cytosol throughout the cell [6,7]. Electrophysiological studies (see Discussion for details) have shown that mice lacking GAD65 release GABA in a normal fashion under basal conditions. During sustained stimulation, however, the probability of release of the inhibitory neurotransmitter is significantly reduced [8].

The present studies were undertaken to determine if: 1) mice show a similar decline in CO<sub>2</sub> sensitivity in the first postnatal week as is seen in rats; and 2) mice lacking GAD65 are able to sustain an increase in respiratory rate during an acute exposure to elevated CO<sub>2</sub>.

## Materials and methods

### Animals

These experiments were performed in accord with The Guide for the Care and Use of Laboratory Animals (NIH).

The protocol was approved by the Oregon Health and Science University Institutional Animal Care and Use Committee. GAD65<sup>-/-</sup> breeding pairs were a kind gift from Professor Kunihiko Obata, National Institute for Physiological Sciences, Aichi, Japan. These animals are on a C57BL/6J background [9].

### Study design

The body plethysmograph method used to measure respiratory rate, tidal volume, TI and T<sub>E</sub> was the same as previously described [10]. The chamber was warmed to 32.5–33.5 °C for mice between P2 and P15 and to 30.5–31.5 °C for those at P21–P22. At an ambient temperature of 33 °C P6 mice maintain their body temperature [11]. Respiratory variables were recorded during a five minute baseline period in which warmed humidified 100% O<sub>2</sub> was passed through a cylinder which fit loosely over the head of the plethysmograph chamber. This was followed by a five minute period of 5% CO<sub>2</sub>/95% O<sub>2</sub> and then return to 100% O<sub>2</sub> for five min. Respiratory frequency and tidal volume were averaged for each minute of the fifteen minute protocol. IT and T<sub>E</sub> were obtained from thirty to fifty consecutive breaths in each minute.

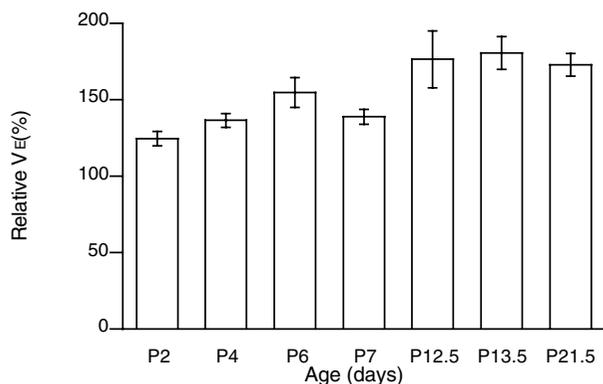
Previous developmental studies of ventilatory response to CO<sub>2</sub> in rats, as summarized in figure 10 of Stunden et al [1], show a decline from P1 reaching a trough at P6.5 to P8 with clear resumption of significant increases at P14. In order to evaluate this pattern in mice studies were carried out at P2, P4, P6, P7, P12.5, P14.5 and P21.5.

### Statistical analysis

Results are reported as mean and standard error. Mice were grouped at P12–P13, P14–P15 and P21–P22 and are designated as P12.5, P14.5 and P21.5. 13 to 16 mice were studied at P2, P4, P6, P7 and P13.5 days. 10 animals were studied at P12.5 and P21.5 days. One way repeated measures analysis of variance (ANOVA) followed by student-Newman-Keuls test was used to compare differences between baseline and each of the five minutes of hypercapnia; between the relative increase in minute ventilation in different age groups and between the changes in respiratory frequency at 2 min and 5 min in different age groups. Two way repeated measures ANOVA with time of exposure to CO<sub>2</sub> and mouse strain as the two factors was used to compare wild type to GAD65<sup>-/-</sup> animals. All tests were performed using Sigma Stat 2.03 Software (SPSS, Chicago, IL).

## Results

Exposure to 5% CO<sub>2</sub> increased minute ventilation in C57BL/6J (wild type) mice at all postnatal ages (figure 1). Minute ventilation increased above baseline by 0.5 min after the onset of hypercapnia (see for example Fig. 3). Therefore we chose the value at 2 min to determine the



**Figure 1**

Minute ventilation ( $V_E$ ) at 2 min of exposure to 5%  $\text{CO}_2$  relative to baseline (baseline = 100%). Values are mean  $\pm$  S.E.  $V_E$  at P2 significantly less than at P12.5 through P21.5 ( $p < 0.05$ )

changes in  $V_E$  relative to baseline.  $V_E$  at 2 min in P2 mice ( $124 \pm 5\%$  of control) was less than that seen at P12.5, P14.5 and P21.5 ( $176 \pm 7\%$ ,  $181 \pm 11\%$  and  $173 \pm 7\%$  respectively;  $P$  values of 0.008, 0.001 and 0.024 respectively). The relative increase at P4 ( $137 \pm 5\%$ ) was less than that at P14.5 ( $p = 0.046$ ). The increases in both respiratory frequency (Table 1) and tidal volume (Table 2) were less at P2 than at older postnatal ages. The smaller relative  $V_E$  at P7 ( $139 \pm 5\%$ ) compared to P6 ( $155 \pm 10\%$ ) and was not significant ( $p = 0.179$ ) (Fig. 1). The value at P7 was different from those at P12.5 and 14.5 ( $p = 0.033$  and 0.008) and approached significance compared to P21.5 ( $p = 0.06$ ). The number of animals in figure 1 is the same as that shown in Table 1 and gave a power of 0.90 with  $\alpha = 0.050$ .

In order to determine if the responses to hypercapnia for frequency and tidal volume were sustained the values at 5 min were compared to those at 2 min (Tables 1 and 2). At P6 respiratory frequency in the fifth min declined to a level significantly less than that at 2 or 3 min (Table 1, Fig. 2). At all other developmental stages the increase in respiratory frequency was sustained. The increase in tidal volume did not decline at any postnatal age (Table 2). The increase in respiratory frequency from baseline at 2 min was less at P7 ( $23 \pm 5$ ) than at P14.5 ( $53 \pm 9$ ) and P21.5 ( $67 \pm 6$ ) ( $p = 0.037$  and 0.002). The value at P7, however, was not different from that at P4 ( $44 \pm 3$ ) and P6 ( $49 \pm 8$ ) ( $p = 0.122$  and 0.124). The power was 0.927 in this analysis. There were no significant differences between age

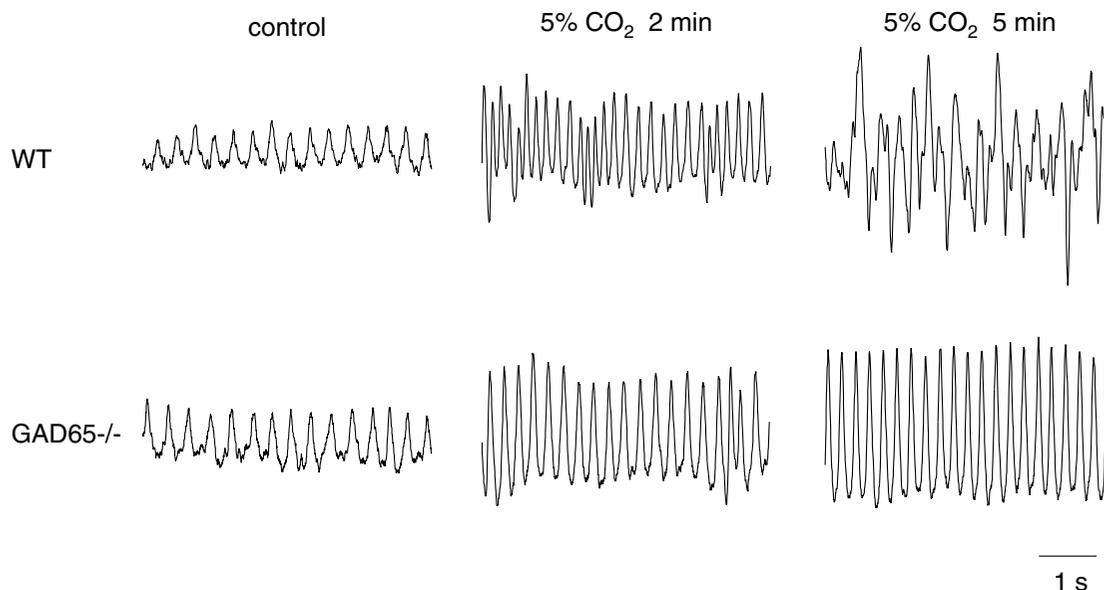
groups in the increases in frequency at 5 min compared to baseline ( $p = 0.117$ ).

In order to evaluate the contribution of GABA to the time dependent fall off in respiratory frequency during hypercapnia at P6, mice deficient in GAD65 were studied at this postnatal age. GAD65 $^{-/-}$  animals had baseline respiratory patterns similar to wild type: frequency ( $180 \pm 7$  vs.  $178 \pm 10$  bpm), tidal volume ( $15.0 \pm 1.3$  vs.  $14.2 \pm 1.0$   $\mu\text{l}$ ), inspiratory time ( $56 \pm 2$  vs.  $64 \pm 3$  msec); and expiratory time ( $297 \pm 14$  vs.  $280 \pm 15$  msec). Their increase in minute ventilation at 2 min in 5%  $\text{CO}_2$  ( $2.7 \pm 0.2$  to  $4.3 \pm 0.4$  ml/min) was of a similar magnitude as that in wild type ( $2.5 \pm 0.2$  to  $3.8 \pm 0.3$  ml/min). In contrast to WT animals GAD65 $^{-/-}$  mice had a sustained increase in frequency for the duration of the 5 min hypercapnic exposure (Figs. 2 and 3). In both the 4<sup>th</sup> and 5<sup>th</sup> min respiratory rate in GAD65 $^{-/-}$  animals exceeded that in WT (Fig. 3). Comparison of respiratory pattern in the 5<sup>th</sup> min showed that GAD65 $^{-/-}$  mice had a shorter inspiratory time ( $47 \pm 2$  vs.  $59 \pm 3$  msec) than WT ( $p = 0.024$ ) and a non-significantly different expiratory time ( $204 \pm 7$  vs.  $238 \pm 16$  msec ( $p = 0.162$ )).

## Discussion

There are two findings from this group of studies: 1) C57BL/6J mice show a developmental pattern in their ventilatory response to hypercapnia characterized by a smaller relative increases in minute volume from P2 to P7 compared to older ages. In contrast to the rat there is no trough at P6 and P7 compared to earlier ages. 2) At P6 this strain of mouse is unable to sustain the increase in respiratory frequency in hypercapnia, as seen in the rat at P5 3. GAD65 contributes to this as mice deficient in this enzyme do sustain their frequency.

In contrast to studies in rats,  $\text{CO}_2$  sensitivity in mice does not start from a high value immediately after birth and go through a trough at one week of age. Stunden et al 1 have combined data from five series of experiments in their figure 10. It is shown that in postnatal rats minute volume increases more than 60% above baseline at P1–P2, then declines to  $\sim 20\%$  at P6.5–P8, and returns to  $\sim 80\%$  at P18.5. For C57BL/6J mice there was no significant decline in relative  $V_E$  at P6 or P7. Relative minute ventilation from P12.5 onwards was above most values at earlier ages. Mice, however, do show one of the abnormal responses to hypercapnia seen in neonatal rats. At P6 there was, as in the rat [1], a decline in respiratory frequency such that at 5 min rate was less than that at 2 or 3 min. In mice lacking GAD65 this decline in frequency was not seen. In adult rats injections of a GABA synthesis inhibitor into the posterior hypothalamus augmented both the respiratory frequency and integrated diaphragmatic EMG activity responses to 5%  $\text{CO}_2$  [12].



**Figure 2**

Respiratory pattern during exposure to 5% CO<sub>2</sub> at P6. Upper traces: respiratory pattern in a WT mouse (inspiration is upwards) during control and at 2 and 5 min of exposure to CO<sub>2</sub>. Lower traces: pattern in a GAD65<sup>-/-</sup> mouse at same times. The larger variability in breath-to breath amplitude seen in the 5<sup>th</sup> min in WT was observed in 8 of 13 WT animals at P6. It was not seen in GAD65<sup>-/-</sup> mice.

**Table 1: Respiratory frequency response to hypercapnia**

Postnatal age (days)	N	Respiratory frequency (bpm)		
		Baseline	2 min	5 min
P2	16	144 ± 7	163 ± 6	164 ± 8
P4	15	203 ± 7	247 ± 7	246 ± 11
P6	13	178 ± 10	227 ± 15	202 ± 15 *
P7	16	194 ± 5	217 ± 7	235 ± 6
P12.5	10	195 ± 10	238 ± 10	232 ± 15
P14.5	16	194 ± 7	246 ± 9	247 ± 8
P21.5	10	176 ± 10	242 ± 11	243 ± 10

Values are mean ± S.E.; bpm, breaths per min; N, number of animals; \* significantly different from value at 2 min, p = 0.023

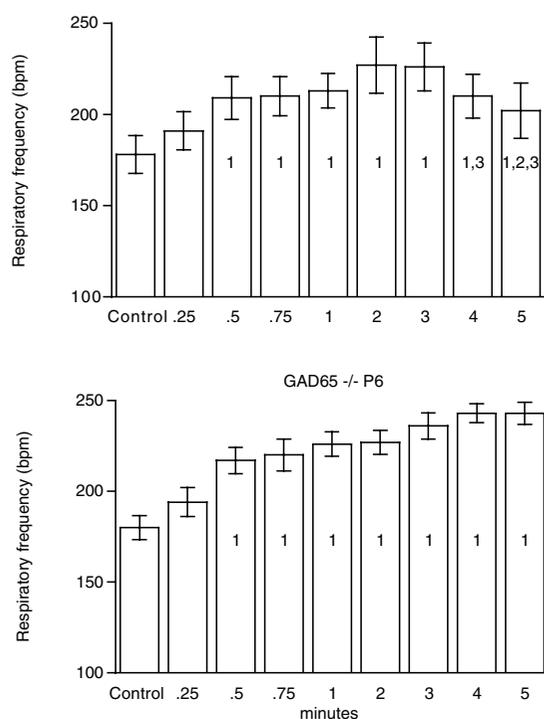
GABA is synthesized from glutamate by two GAD isoforms designated by their relative molecular sizes, GAD65 and GAD67. The two decarboxylases are encoded by separate genes [13]. A number of observations have suggested

that in the brain these two enzymes may serve separate functions. Immunohistochemistry has shown that GAD 67 is primarily localized to neuronal cell bodies while GAD65 has a relatively higher presence in axonal

**Table 2: Tidal volume response to hypercapnia**

Postnatal age (days)	N	Tidal volume ( $\mu$ l)	
		Baseline	2 min
P2	16	10.4 $\pm$ 0.2	11.3 $\pm$ 0.5
P4	15	12.0 $\pm$ 0.4	14.0 $\pm$ 0.6
P6	13	14.2 $\pm$ 1.0	16.9 $\pm$ 0.9
P7	16	14.7 $\pm$ 0.5	18.4 $\pm$ 0.8
P12.5	10	25.0 $\pm$ 2.5	39.3 $\pm$ 3.2
P14.5	16	45.0 $\pm$ 3.1	64.2 $\pm$ 5.8
P21.5	10	57.3 $\pm$ 6.2	69.3 $\pm$ 5.9

Values are mean  $\pm$  S.E.; N, number of animals

**Figure 3**

Respiratory frequency during 5 min exposure to 5% CO<sub>2</sub>. Upper panel wild type (WT) mice at P6, lower panel GAD65<sup>-/-</sup> animals at P6. Values are mean  $\pm$  S.E. 1 = significantly different from control, (P between 0.044 and < 0.001). 2 = significantly different from value at 2 and at 3 min ( $p = 0.023$  and  $0.025$ ) 3 = significantly different from corresponding min. in GAD65<sup>-/-</sup> ( $p = 0.049$  at 4 min and  $0.017$  at 5 min)

terminals [6,7]. This differential cellular localization is also supported by fractionation experiments which found that cytosol is relatively enriched in GAD67 while GAD65 is seen more prominently in synaptosomes [13]. GAD67,

however, is not excluded from nerve terminals. Studies utilizing GAD65<sup>-/-</sup> mice have shown that GAD67 is present in nerve terminals [14] where it co-localizes with the synaptic vesicle marker SV2 [15]. A significant amount of GAD in the brain exists as inactive apoenzyme, which is not associated with the necessary cofactor pyridoxal 5-phosphate [13,16]. GAD67 is present primarily as the active holoenzyme while GAD65 accounts for the majority of the apoenzyme. It has been suggested that this GAD65 inducibility may allow stimulated neurons to produce greater amounts of GABA as needed.

Electrophysiological studies directly demonstrate that GAD65 plays an important role in GABA release during neuronal stimulation. The basal frequency of spontaneous inhibitory postsynaptic currents (IPSCs) in both retinal ganglionic cells and CA1 hippocampal neurons was not different in GAD65<sup>-/-</sup> mice compared to WT [8]. When retinal cells were depolarized from -70 to 0 mV, however, IPSC frequency increased almost 7 fold in WT but did not change in cells from GAD65<sup>-/-</sup> mice. Similarly a number of indices showed that GABA release was increased by high frequency stimulation of CA1 neurons from WT animals but not in slices from GAD65<sup>-/-</sup>. It was these observations that prompted the present studies to determine if the absence of GAD65 prevented the depressive phase of carbon dioxide stimulation in young mice. To our knowledge GABA content in the brainstem has not been reported in GAD65<sup>-/-</sup> mice. In cerebral cortex, striatum, hippocampus and cerebellum no differences in GABA levels were found between GAD65<sup>-/-</sup> and WT adult mice [9,17]. The observation that baseline respiratory frequency and tidal volume are not affected by loss of GAD65 is consistent with the fact that resting GABA concentrations are normal in the medullary respiratory centers of GAD65 deficient mice.

In addition to being an essential component of the depressed CO<sub>2</sub> sensitivity at P6–P8, GABA may play a role in the absence of such depression at earlier postnatal ages.

In contrast to the hyperpolarizing effect seen in mature neurons, the GABAA-receptor mediated response is often depolarizing during early development [18]. GABA's actions are hyperpolarizing when the chloride equilibrium potential ( $E_{CL}$ ) is negative to the resting membrane potential and depolarizing when  $E_{CL}$  is positive.  $E_{CL}$  in turn is dictated by intracellular chloride concentrations. In rat hippocampal pyramidal neurons the primary chloride extruding transporter,  $K^+/Cl^-$  (KCC2) mRNA is hardly detectable at P0, appears at P5 and reaches near adult levels at P9 [19]. The change in membrane potential in response to the GABA agonist, muscimol, paralleled this developmental pattern for KCC2. At P0–P4 the agonist caused depolarization and at P13–P30 hyperpolarization was seen [19]. This maturation pattern has also been seen in respiratory related neurons. Ritter and Zhang [20] used the perforated patch approach to record from pre-Bötzing complex neurons in mice. From a resting membrane potential of -60 mV muscimol depolarized these neurons at P2 and caused hyperpolarization at P8. Thus GABA released during hypercapnia in the early postnatal period may be excitatory to respiratory neurons and inhibitory at later developmental stages. The return of  $CO_2$  sensitivity beyond P8 may be related to a decline in GAD enzymes. mRNA for both GAD65 and GAD67 in rat cervical spinal cord decline significantly between P7 and P14 [21].

### Conclusions

The present studies show that as in contrast to the rat, C57BL/6J mice do not have a fall in ventilatory response carbon dioxide to at one week of age, rather they increase their response from P12.5 onwards. In mice at P6 there is a failure to sustain the increase in respiratory frequency during a five min exposure to carbon dioxide. This may be due to the developmental switch in GABA<sub>A</sub> receptor mediated response from depolarizing to hyperpolarizing at a time when GAD enzymes are still elevated, as mice deficient in GAD65 did not display a time dependent fall in respiratory frequency during  $CO_2$  exposure.

### Abbreviations

ANOVA = analysis of variance

$CO_2$  = carbon dioxide

GABA =  $\gamma$ -aminobutyric acid

GAD = glutamic acid decarboxylase

kDa = kilodalton

P = postnatal

$T_E$  = expiratory time

$T_I$  = inspiratory time

$\mu$ l = microliter

$V_E$  = minute ventilation

VT = tidal volume

WT = wild type

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