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| 著者 | Tanii Hideji, Higashi T., Saijoh Kiyofumi |
|---|---|
| journal or publication title | Food and Chemical Toxicology |
| volume | 48 |
| number | 2 |
| page range | 750-754 |
| year | 2010-02-01 |
| URL | http://hdl.handle.net/2297/20342 |

doi: 10.1016/j.fct.2009.12.010

**Preconditioning with subneurotoxic allyl nitrile: protection against allyl nitrile neurotoxicity**

H. Tanii, T. Higashi, K. Saijoh

Department of Hygiene, Graduate School of Medical Science, Kanazawa University, 13-1 Takara-machi, Kanazawa 920-8640, Japan

**Corresponding author:** Hideji Tanii

Department of Hygiene, Graduate School of Medical Science, Kanazawa University, 13-1 Takara-machi, Kanazawa 920-8640, Japan

Fax: +81 76 234 4232; Tel: +81 76 265 2211; E-mail: taniih@med.kanazawa-u.ac.jp

**Running title**: Preconditioning with allyl nitrile and its neurotoxicity

**Keywords:** Allyl nitrile, Glutathione S-transferase, Quinone reductase, Glutathione, Behavioral abnormalities, γ-Aminobutyric acid

**Abbreviations**: GABA, γ-Aminobutyric acid; GSH, glutathione; GST, glutathione S-transferase; QR, quinine reductase

**ABSTRACT**

High-dose cruciferous allyl nitrile can induce behavioral abnormalities in rodents, while repeated exposure to allyl nitrile at subneurotoxic levels can increase phase 2 detoxification enzymes in many tissues, although the brain has not been investigated yet. In the present study, we examined the effect of five days repeated exposure to subneurotoxic allyl nitrile (0—400 μmol/kg/day) on the brain. Elevated glutathione S-transferase activity was recorded in the striatum, hippocampus, medulla oblongata plus pons, and cortex. Enhancement of quinone reductase activity was observed in the medulla oblongata plus pons, hippocampus, and cortex. In the medulla oblongata plus pons, elevated glutathione levels were recorded. Following repeated subneurotoxic allyl nitrile exposure (0—400 μmol/kg/day), mice were administered a high-dose allyl nitrile (1.2 mmol/kg) which alone led to appearance of behavioral abnormalities. Compared with the 0 μmol/kg/day group, animals in the 200 and 400 μmol/kg/day pre-treatment groups exhibited decreased behavioral abnormalities and elevated GABA-positive cell counts in the substantia nigra pars reticulata and the interpeduncular nucleus. These data suggest that repeated exposure to subneurotoxic levels of allyl nitrile can induce phase 2 enzymes in the brain, which together with induction in other tissues, may contribute to protection against allyl nitrile neurotoxicity.

## 1. Introduction

Nitriles are widely used in the manufacture of plastics, solvents, and synthetic industries, and are common in cruciferous vegetables. There is increasing evidence suggesting that some nitriles, including allyl nitrile, crotononitrile, and 2-pentenenitrile, are neurotoxic (Tanii et al., 1989a, 1989b; Llorens et al., 1993; Balbuena and Llorens, 2001, 2003). Allyl nitrile may be particularly relevant to human health as it is present in cruciferous vegetables, suggesting the potential for repeated exposure (West et al., 1977; Fahey et al., 2001; Tolonen et al., 2002; Tanii et al., 2004).

In rodents, exposure to a high-dose of allyl nitrile induces behavioral abnormalities including alterations in reflex behavior, increased locomotor activity, circling, head twitching, and occasional backward pedaling (Tanii et al., 1989a; Zang et al., 1999). Allyl nitrile can also induce apoptotic changes habenular and raphe nuclei neurons (Zang et al., 1999), and can alter the γ-Aminobutyric acid (GABA) system through the medial habenula-interpeduncular nucleus-ascending raphe nuclei relay and through the substantia nigra (Tanii et al., 2000). Furthermore, allyl nitrile causes vestibular and auditory hair cell degeneration, corneal opacity, and gliosis in the retina and olfactory bulbs (Balbuena and Llorens, 2001). Nevertheless, the majority of these data are from rodents exposed to relatively high doses.

Recently, repeated exposure to allyl nitrile at subneurotoxic levels was shown to increase phase 2 antioxidant and detoxification enzymes in mice (Tanii et al., 2005, 2008), with increased activity of thioredoxin reductase in the rectum, kidneys, and liver, increased activity of glutathione peroxidase in the small intestine and kidneys, increased activity of glutathione S-transferase (GST) in the stomach, rectum, kidneys, and lungs, increased activity of quinone reductase (QR) in the stomach, small intestine, urinary bladder, kidneys, and lungs, and increased levels of glutathione (GSH) in the stomach, rectum, and urinary bladder. These inductive effects may be related to epidemiological studies showing an inverse association between the consumption of cruciferous vegetables and the risk of various cancers (Graham et al., 1978; Haenszel et al., 1980; Verhoeven et al., 1996). However, it is unknown whether repeated subneurotoxic exposure to allyl nitrile can also increase phase 2 enzymes in the brain.

In the present study, we examined the effect of five days repeated subneurotoxic allyl nitrile exposure (less than or equal to 400 μmol/kg/day) on the mouse brain by measuring the activities of GST and QR, and the level of GSH, in various brain regions. Furthermore, we examined whether repeated exposure to subneurotoxic allyl nitrile protects the brain against the neurotoxic effects of high-dose exposure.

## 2. Materials and methods

*2.1. Materials*

2,6-dichloroindophenol, nicotinamide adenine dinucleotide phosphate (reduced) and dicumarol were purchased from Sigma-Aldrich Ltd. (St. Louis, MO, USA), allyl nitrile (3-butenenitrile, CAS No. 109-75-1, purity >98%) from Tokyo Kasei Kogyo Co. (Tokyo, Japan), rabbit anti-GABA polyclonal antibody (AB131) from Chemicon (Temecula, CA, USA), Vectastain ABC kit (peroxidase rabbit IgG) from Vector Laboratories (Burlingame, CA, USA), and sodium pentobarbital from Dainihon Seiyaku Co. (Tokyo, Japan). All other chemicals were purchased from Nacalai Tesque (Kyoto, Japan).

*2.2.Animals and treatments*

All animal experiments were conducted according to the Guidelines of the Committee on Animal Experimentation of Kanazawa University. Male ddY mice weighing 26—30 g were obtained from Japan SLC Co. (Shizuoka, Japan), and were maintained at 22±2°C under a 12:12 h light/dark cycle with free access to tap water and laboratory food (CRF-1; Charles River Japan, Inc., Yokohama, Japan).

Groups of five to six animals were administered subneurotoxic doses of allyl nitrile (100, 200, or 400 μmol/kg) or vehicle-distilled water (control; 4 mL/kg) daily for

five days by gastric intubation, based on our previous findings (Tanii et al., 2008). For enzyme and GSH analyses, the animals were sacrificed on the sixth day. For behavioral assessment and immunohistochemistry, the animals were given a high-dose of allyl nitrile (1.2 mmol/kg) by gastric intubation on the sixth day, which we have previously shown to be neurotoxic (Zang et al., 1999).

### *2.3. Tissue preparation for biochemical assays*

Mice were anesthetized with 100 mg/kg sodium pentobarbital and were perfused transcardially with 1.15% KCl. The striatum, medulla oblongata plus pons, hippocampus, and cortex were immediately removed and stored at -80°C until analysis. Supernatant for enzyme analyses was prepared as previously described (Tanii et al., 2005). Protein concentrations were measured according to the method of Bradford (1976) using bovine serum albumin as the standard.

### *2.4. Enzyme and GSH analyses*

GST activity was measured according to the spectrophotometric method of Habig et al. (1974) using 1-chloro-2,4-dinitrobenzene as the substrate. QR activity was measured according to the spectrophotometric method of Ernster (1967), and GSH concentrations were measured according to the method of Jaeger et al. (1974).

### *2.5. Behavioral assessment*

Changes in the spontaneous and reflex behaviors of the mice were measured as previously described (Zang et al., 1999). In brief, observation was performed between 09.00 and 13.00 h by one of the authors unaware of the treatment groups over a period of 22 days. Animals were placed on a table top, and their behavior was observed for five minutes. The four dyskinetic behaviors of circling, retropulsion, head twitch, and alterations in tail hanging were assessed using a dyskinetic scale: 0, absence of behavioral abnormalities; 1, intermediate responses; and 2, presence of severe abnormalities. Circling was defined as stereotyped circling. Retropulsion consisted of displacement of the animal toward his back. Head twitch consisted mostly of backward movement. When normal mice were hung by the tail and slowly lowered to the table surface, they landed on their forelimbs with the head turned dorsal, while by contrast, mice with dyskinetic syndrome bent ventrally, resulting in occipital landing.

*2.6. GABA immunohistochemistry*

Immunohistochemical staining for GABA was performed on brains from non treated mice or animals killed at two days after high-dose allyl nitrile, as previously described (Tanii et al., 2000). In brief, the animals were anesthetized with 100 mg/kg sodium pentobarbital, and perfused transcardially with 0.01 M phosphate buffer containing 0.9% sodium chloride (pH 7.4) followed by cold fixative containing 4%

paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were removed and postfixed in 4% paraformaldehyde in 0.1 M phosphate buffer overnight at 4°C , and then placed in 30% sucrose in 0.1 M phosphate buffer (pH 7.4) at 4°C for a minimum of 2 days. The brains were rapidly frozen and sectioned coronally in a cryostat at 20 μm. After washing and blocking, tissue sections were incubated with primary antibody (rabbit anti-GABA, 1:2000) for four days at 4°C. Sections were then incubated in Tris-HCl buffered saline (pH 7.4) containing 0.04% diaminobenzidine, 0.08% nickel ammonium sulfate, and 0.003% $H_2O_2$ for 10 min for visualization. The number of immunopositive neurons per brain structure is presented as the mean±SD of three to four animals.

*2.7. Statistics*

Statistical analyses were performed by analysis of variance for factorial (enzyme and GSH analyses, and immunohistochemical studies) or by repeated measures (behavioral assessment), followed by Fisher's least significant difference test for multiple comparisons. The level of significance was set at $P<0.05$.

## 3. Results

*3.1.Induction of phase 2 detoxification enzymes in the brain*

Consistent with our previous report (Tanii et al., 2004), repeated exposure to subneurotoxic allyl nitrile (0—400 μmol/kg/day) did not induce any behavioral abnormalities in experimental mice, while there were significant changes in the levels of detoxification enzymes and GSH in the brain. There was no significant difference in GST activity in the hypothalamus of mice receiving allyl nitrile at any concentration ( $F(3,16)=1.682$, $P=0.2108$ ). By contrast, elevated GST activity was observed in the striatum ( $F(3,16)=5.030$, $P=0.0120$ ) and hippocampus ( $F(3,16)=6.825$, $P=0.0036$ ) at 100, 200, and 400 μmol/kg/day, in the medulla oblongata plus pons ( $F(3,16)=3.874$, $P=0.0294$ ) at 400 μmol/kg/day, and in the cortex ( $F(3,16)=5.055$, $P=0.0119$ ) at 200 and 400 μmol/kg/day (Table 1). There was no change in QR activity in the striatum ($F(3,16)=0.891$, $P=0.4672$ ) or the hypothalamus ($F(3,16)=1.231$, $P=0.3311$ ) at any concentration. By contrast, increased QR activity was observed in the medulla oblongata plus pons ( $F(3,16)=3.496$, $P=0.0402$ ) at 200 and 400 μmol/kg/day, in the hippocampus ( $F(3,16)=11.525$, $P=0.0003$ ) at 100, 200, and 400 μmol/kg/day, and in the cortex ( $F(3,16)=4.209$, $P=0.0225$ ) at 400 μmol/kg/day (Table 2). The changes in GSH levels can be seen in Table 3. In the medulla oblongata plus pons ( $F(3,12)=7.461$, $P=0.0044$ ), elevated GSH levels were seen at 100, 200, and 400 μmol/kg/day, while in

the striatum, hypothalamus, hippocampus or cortex, no differences were detected at any concentration.

<Insert tables 1, 2 and 3 here>

*3.2.Protection against behavioral abnormalities*

Animals (n=5/group) were administered a high-dose of allyl nitrile (1.2 mmol/kg) following repeated exposure to subneurotoxic allyl nitrile (0—400 μmol/kg/day). Two out of five animals died at one day post-dosing in the 0 and 100 μmol/kg/day repeated exposure groups, while there were no deaths in the of 200 and 400 μmol/kg/day groups. All survivors began to exhibit behavioral abnormalities (Fig. 1). Analysis of variance for repeated measures showed a significant effect of treatment ($F(3,12)=3.544$, $P<0.05$), time ($F(4,48)=15.472$, $P<0.0001$), but not the interaction between treatment and time ($F(12,48)=1.226$, $P<0.2938$). Control animals repeatedly exposed to 0 μmol/kg/day allyl nitrile showed high behavioral abnormality rating scores throughout the 22 day observation period. For the 100 μmol/kg/day group, there were no significant differences in scores compared with the control throughout the observation period. The 200 μmol/kg/day group showed the low mean rating scores with significant differences compared with the control group at 5, 15, and 22 days post-dosing ($P<0.05$), while the mean rating scores in the 400 μmol/kg/day were significantly low compared with the

control group at 2, 5, 10, 15, and 22 days post-dosing ($P<0.05$).

<Insert figure 1 here>

*3.3.GABA immunolabeling in the brain*

In the present study, mice (0 μmol/kg/day) administered high-dose allyl nitrile exhibited decreased GABA immunolabeling of various brain structures at two days post-dosing, this was previously shown to be associated with the neurotoxic effects of allyl nitrile (Tanii et al., 2000). By contrast, repeated exposure to allyl nitrile (100—400 μmol/kg/day) followed by high-dose exposure (1.2 mmol/kg) significantly changed GABA immunolabeling in the mouse brain (Fig. 2). For instance, in the substantia nigra pars reticulata ( $F(4,15)=5.922$, $P=0.0046$ ), the GABA-positive cell count was decreased in the 0 μmol/kg/day group, and comparable to the Non group in the 100, 200, and 400 μmol/kg/day groups (Fig.2A-C,G). Similarly, in the interpeduncular nucleus ( $F(4,15)=19.769$, $P<0.0001$), the GABA-positive cell count was decreased in the 0 μmol/kg/day group, and was increased in the 100, 200, and 400 μmol/kg/day groups, compared with those in the Non and 0 μmol/kg/day groups (Fig.2D-F,G).

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## 4. Discussion

We demonstrated that the activities of GST and QR were elevated in the medulla oblongata plus pons, hippocampus, and cortex, while GST activity alone was enhanced in the striatum, following repeated exposure to subneurotoxic levels of allyl nitrile in the mouse. The observed changes did not always show dose-response relationship. Further works will be needed to examine what level of allyl nitrile shows dose-response relationship. Although it is unclear why the activities of GST and QR were not always both increased in the same brain areas, a similar phenomenon was described in tissues other than the brain (Tanii et al., 2005). In the present study, GSH was only elevated in the medulla oblongata plus pons, which overall was the most responsive area to allyl nitrile stimulation. Administration of neurotoxic levels of allyl nitrile was previously shown to activate the 5-hydroxytrypaminergic system in all brain areas examined (Tanii et al., 1993). The 5-hydroxytrypaminergic system originating in the raphe nuclei, and which is part of the medulla oblongata plus pons, exerts a tonic modulatory influence on widespread targets including the basal ganglia-motor systems and limbic structures. Further studies are needed to clarify why the medulla oblongata plus pons is the most responsive area to allyl nitrile.

Allyl nitrile neurotoxicity is characterized by dose-dependent behavioral

abnormalities (Tanii et al., 1989a; Llorens et al., 1993; Zang et al., 1999; Balbuena and Llorens, 2001). To assess behavioral abnormalities, we employed the original method by Llorens et al. (1993) which was specifically designed to assess the behavioral effects consequent to the vestibular toxicity of iminodipropionitrile and similar nitriles. Thus, the present rating scores mostly reflect vestibular dysfunction. In the present study, repeated pre-exposure to allyl nitrile at 200 and 400 μmol/kg/day suppressed the behavioral abnormalities produced by a later neurotoxic high-dose allyl nitrile, suggesting a preconditioning effect of repeated exposure in the body. These data are further supported by the reduced mortality rate observed after a high-dose allyl nitrile in the 200 and 400 μmol/kg/day groups. The issue of the role of allyl nitrile metabolism on allyl nitrile toxicity has been treated previously. A role for hepatic bioactivation for acute toxicity was first indicated ( Tanii and Hashimoto, 1984), and the involvement of the enzymes involved in ethanol metabolism was subsequently supported ( Tanii and Hashimoto, 1986 ). Recently, the ethanol-inducible isoform of P450 ( CYP2E1 ) has been demonstrated to bioactivate allyl nitrile for acute lethality but not for behavioral toxicity ( Boadas-Vaello et al., 2009 ). Thus, the change in allyl nitrile toxicity following previous exposure to low dose allyl nitrile may depend on changes in phase 2 activities, but also on changes in phase 1 activities. Phase 2 detoxification enzymes, including

GST and QR, promote the conjugation of phase 1 products with endogeneous ligands such as GSH and glucuronic acid, usually resulting in an increase in water-soluble products. Elevated levels of GST, QR, and GSH in the brain may be involved in a preconditioning effect of repeated exposure in the body. Additionally, reactive oxygen species seems to be involved in the pathogenic processes of many diseases, however, to our knowledge, there is no report indicating involvement of reactive oxygen species in the allyl nitrile-induced neurotoxicity. Hence, for exogeneous chemicals including neurotoxicants, repeated consumption of cruciferous vegetables can be beneficial to human health.

The behavioral abnormalities observed following high-dose allyl nitrile were previously demonstrated to be associated with decreased GABA immunolabeling in various brain structures including the substantia nigra pars reticulata and the interpeduncular nucleus (Tanii et al., 2000). In the present study, repeated exposure to allyl nitrile (100, 200, or 400 μmol/kg/day) protected against the decrease in GABA immunolabeling observed following high-dose allyl nitrile alone; however, as described above, there was no effect of repeated exposure to 100 μmol/kg/day allyl nitrile on behavioral abnormalities. Although the mechanism underlying allyl nitrile-induced neurotoxicity is largely unknown, these data suggest that the GABAergic system may

play an important role. However, there are many studies providing evidence that the effects of allyl nitrile on behavior are due to loss of vestibular function ( Llorens et al., 1993; Balbuena and Llorens, 2001; Boadas-Vaello et al., 2005, 2009 ).

In conclusion, repeated exposure to allyl nitrile resulted in induction of phase 2 detoxification enzymes and GSH in the brain, which correlated with suppressed behavioral abnormalities and a recovery in GABA immunolabeling following a later high-dose of allyl nitrile compared with those observed in animals receiving a high-dose only.

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**Figure legends**

Fig. 1. Behavioral abnormalities induced by high-dose allyl nitrile. Mice were administered oral allyl nitrile at 0, 100, 200, or 400 μmol/kg for five days, followed by high-dose allyl nitrile (1.2 mmol/kg) on the sixth day, and were then assessed for behavioral abnormalities over a period of 22 days. Behavioral abnormalities including circling, retropulsion, head twitch, and alteration in tail hanging were scored, and a total score was calculated based on a summation of each test score, with a maximum possible score of eight. Values represent mean±SD of three to five animals. Values with different superscript letters within days after dosing are significantly different ( $P<0.05$ ).

Fig. 2. GABA immunolabeling in the brain of allyl nitrile-treated mice. Mice were

administered oral allyl nitrile at 0, 100, 200, or 400 μmol/kg for five days, followed by high-dose allyl nitrile (1.2 mmol/kg) on the sixth day, and were then assessed for GABA immunohistochemistry two days later. Non treated animals ( Non ) were also assessed for GABA immunohistochemistry. Substantia nigra pars reticulata (A, B, C) and interpeduncular nucleus (D, E, F) of mice repeatedly exposed to none (A, D), 0 (B, E) or 400 (C, F) μmol allyl nitrile/kg for five days. Scale bar: 100 μm. GABA-positive cell counts were performed quantitatively (G). Values represent mean±SD of three to four animals.Values with different superscript letters within SNR or IP are significantly different ($P<0.05$). SNR, substantia nigra pars reticulata; IP, interpeduncular nucleus.

Table 1. Glutathione S-transferase (GST) activity in discrete brain regions of mice administered allyl nitrile for five days.

| | GST activity (μmol of 1-chloro-2,4-dinitrobenzene conjugated/min/mg of protein) | | | | |
|---|---|---|---|---|---|
| Dose level (μmol/kg/day) | Striatum | Hypothalamus | Medulla oblongata plus pons | Hippocampus | Cortex |
| 0 (control) | 0.194±0.017[a] | 0.274±0.042 | 0.155±0.022[a] | 0.189±0.043[a] | 0.174±0.027[a,c] |
| 100 | 0.270±0.043[b] | 0.284±0.035 | 0.170±0.019[a] | 0.250±0.029[b] | 0.192±0.012[a,c] |
| 200 | 0.239±0.044[b] | 0.307±0.049 | 0.172±0.030[a] | 0.267±0.035[b] | 0.208±0.027[b,c] |
| 400 | 0.236±0.024[b] | 0.255±0.014 | 0.204±0.024[b] | 0.279±0.027[b] | 0.224±0.013[b,d] |

Data are expressed as means±SD of five animals. Values with different superscript letters within tissue are significantly different ($P<0.05$).

Table 2. Quinone reductase (QR) activity in discrete brain regions of mice administered allyl nitrile for five days.

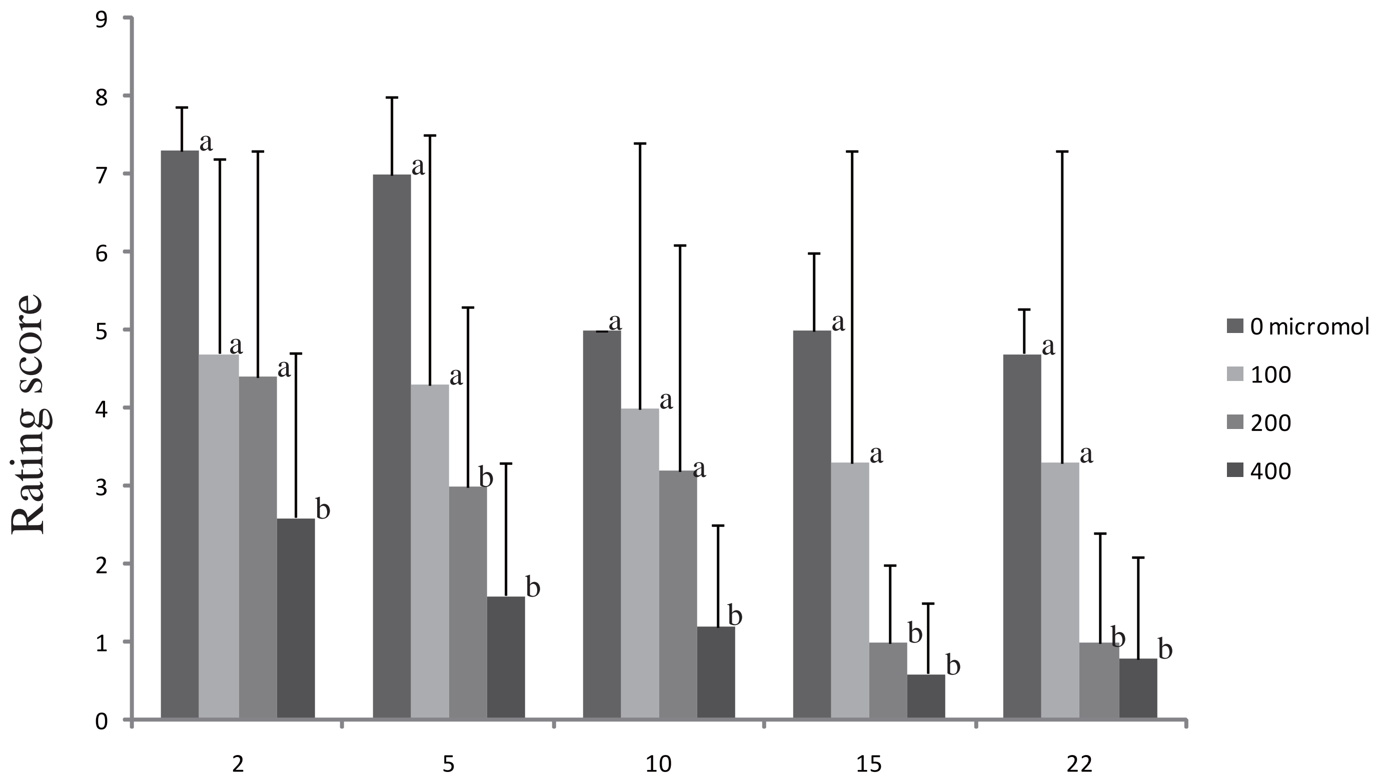
| | QR activity (µmol of 2,6-dichloroindophenol reduced/min/mg of protein) | | | | |
|---|---|---|---|---|---|
| Dose level (µmol/kg/day) | Striatum | Hypothalamus | Medulla oblongata plus pons | Hippocampus | Cortex |
| 0 (control) | 0.045±0.013 | 0.059±0.0162 | 0.044±0.011[a] | 0.046±0.008[a] | 0.042±0.018[a] |
| 100 | 0.047±0.013 | 0.055±0.013 | 0.045±0.004[a] | 0.074±0.009[b] | 0.043±0.004[a] |
| 200 | 0.041±0.002 | 0.058±0.007 | 0.057±0.005[b] | 0.071±0.002[b] | 0.049±0.005[a] |
| 400 | 0.038±0.006 | 0.047±0.001 | 0.056±0.008[b] | 0.069±0.012[b] | 0.064±0.009[b] |

Data are expressed as means±SD of five animals. Values with different superscript letters within tissue are significantly different ($P<0.05$).

Table 3. Glutathione (GSH) levels in discrete brain regions of mice administered allyl nitrile for five days.

| | GSH level (µmol /g of tissue) | | | | |
|---|---|---|---|---|---|
| Dose level (µmol/kg/day) | Striatum | Hypothalamus | Medulla oblongata plus pons | Hippocampus | Cortex |
| 0 (control) | 3.087±0.337 | 3.763±0.782 | 1.900±0.055[a] | 3.253±0.438 | 4.035±0.402 |
| 100 | 3.613±0.502 | 3.421±0.444 | 2.041±0.076[b] | 3.345±0.845 | 3.481±0.855 |
| 200 | 3.743±0.734 | 4.256±0.284 | 2.025±0.078[b] | 3.573±0.500 | 3.344±0.641 |
| 400 | 3.575±0.419 | 3.551±0.214 | 2.113±0.040[b] | 3.115±0.109 | 3.129±0.250 |

Data are expressed as means±SD of four animals. Values with different superscript letters within tissue are significantly different ($P<0.05$).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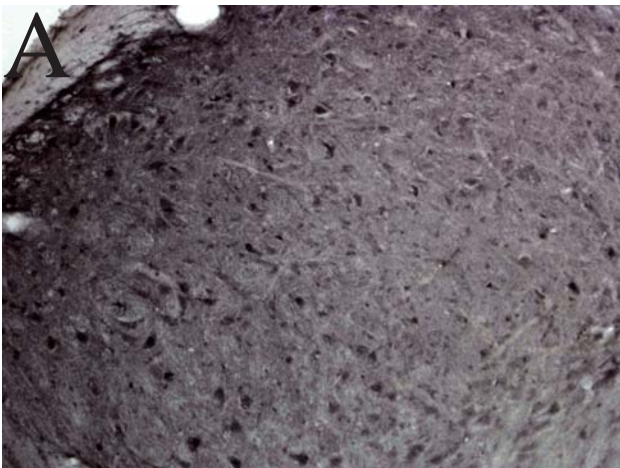
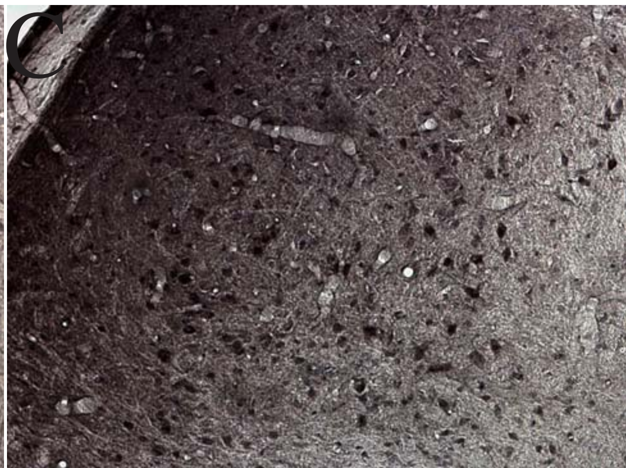
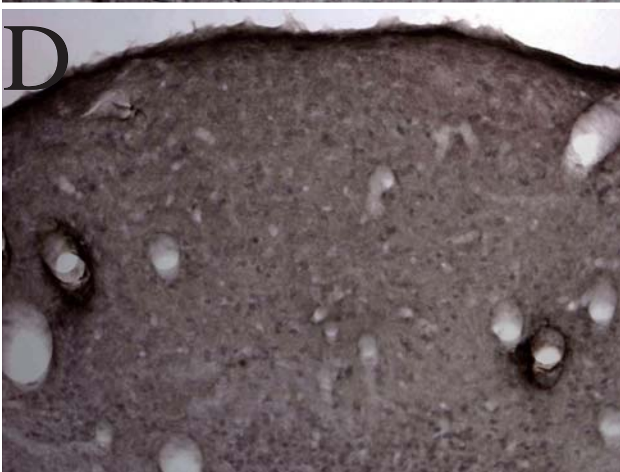
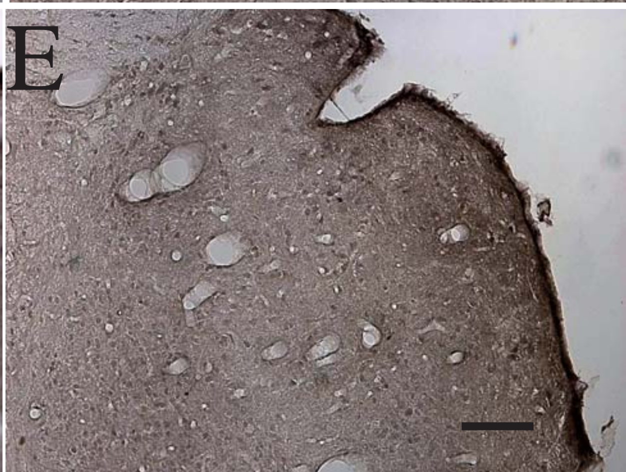
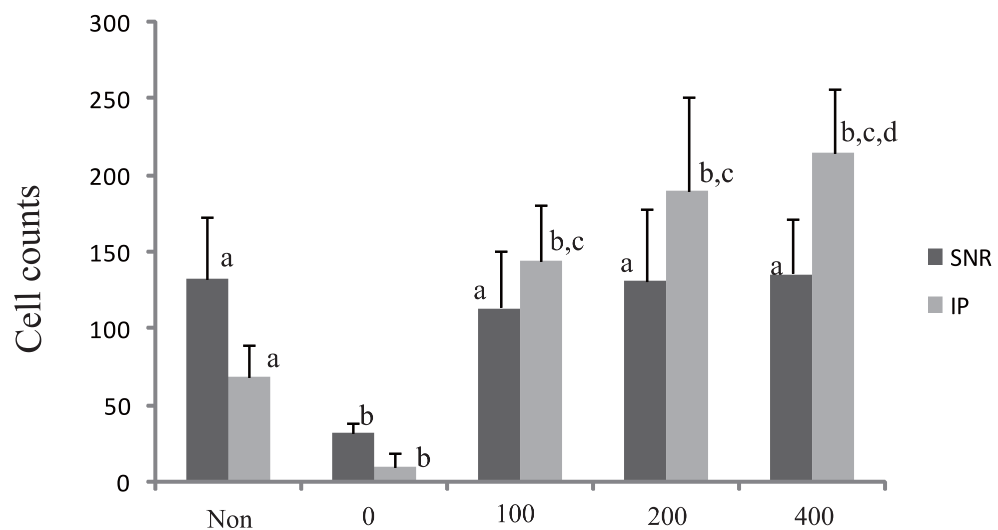

A

B

C

D

E

F


G
Cell counts
300
250
200
150
100
50
0
a
a
b
b
a
b,c
a
b,c
a
b,c,d
Non
0
100
200
400
SNR
IP
Treatment with allyl nitrile (µmol/kg/day)