Age- and region-dependent alterations in the GABAergic innervation in the brain of rats treated with amphetamine

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Abstract
Mechanisms underlying the pathogenesis of psychotic disorders were explored by monitoring the expression of GABAergic neurons in an animal model. Male rats of postnatal days 21 and 60 were intraperitoneally injected with amphetamine (Amph), 5 mg/kg, or saline three times daily for 6 d. After 1-d or 14-d withdrawal from Amph, they were challenged on day 8 (W1d) or on day 21 (W14d) with a single same dosage and then perfused. Immunostaining on the brain sections using an anti-glutamic acid decarboxylase (GAD67) antiserum revealed that the Amph treatment increased the densities of the GAD67-immunoreactive boutons by approx. 36 to 79% above controls in the layers of motor and somatosensory cortices of the W1d juvenile, whereas for those of W14d, the densities resembled controls. For the Amph-treated adults of both W1d and W14d, the GAD67 immunoreactivity increased 56–133% in these layers. In the striatum, the GAD67 densities responded to Amph in a similar manner to the neocortices. However, for the nucleus accumbens, the GAD67 terminals were up-regulated by 22–64% in all Amph-injected rats of both ages. In the hippocampal CA1–CA3 region of the Amph-administered juvenile, increases of 24–27% of GAD67 terminals occurred for W1d and W14d animals. By contrast, however, in the W1d Amph-injected adult, there were increases of 42–48% in CA1–CA3, at W14d the GAD67 boutons resembled controls or were reduced. An age-dependent correlation was implicated between behavioural and immunostaining observations. The data support the view that inhibitory regulation is involved in neuronal responses to chronic psychostimulant administration and reflect differential neuronal plasticity among the developing and adult brain regions.

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Key words: Glutamic acid decarboxylase-immunoreactivity, hippocampus, motor and somatosensory cortex, nucleus accumbens, synaptic plasticity.

Introduction
The abuse of the potent psychostimulant, amphetamine (Amph) and its derivatives produces adverse effects on the human central nervous system, leading to addiction and Amphetamine psychosis, which manifests paranoid schizophrenia-like symptoms and develops in long-term users of the drug (Ellison, 1994; Lortat-Jacob et al., 1997). Chronically Amph-treated animals display hyper-locomotion, stereotypy and behavioural sensitization, and therefore have been used as models for studying addiction and schizophrenia (Kuczenski et al., 1997; Seiden et al., 1993). Findings from animal models have suggested that the catecholamine, monoamine, glutamate and GABA systems are involved in mechanisms underlying the action of Amph on the central neurons (Bardo, 1998; Giorgetti et al., 2002). However, the mechanisms await further clarification, in order to identify pathogenic aspects of the psychotic disorders.

It has been proposed that Amph acts as a false substrate and binds to the dopamine, norepinephrine, and serotonin transporters (Hitri et al., 1994; White and Kalivas, 1998 for review). Thus, the drug and its derivatives, such as methamphetamine, could induce neuronal degeneration, including degeneration of dopaminergic terminals in the neostriatum and glutamatergic neurons in the somatosensory cortex of adult rats, but not of young rats (Pu and Vorhees,
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1993; Pu et al., 1996). Cell loss in the substantia nigra was likewise reported in the mouse treated with methamphetamine (Sonsalla et al., 1996). Moreover, we have found that acute Amph treatment leads to differential changes in the expression of the metabotropic glutamate receptor 5 in the layers of certain cortical regions, striatum and hippocampus of the young and adult rat brains (Yu et al., 2001).

Accumulated evidence has indicated that the GABA system plays an important role in the events of psychotic disorders. The efflux of striatal dopamine induced by an Amph derivative might be associated with the release of GABA in the substantia nigra (Yamamoto et al., 1995). Injection of the GABA_A agonist to the frontal cortex or striatum could hinder the Amph-elicited induction of behavioural sensitization or stereotypy (Karler et al., 1995, 1997). GABA_B receptors may also contribute to the expression of Amph-induced behavioural sensitization by participating in the altered neural transmission (Giorgetti et al., 2002; Zhang et al., 2000).

Furthermore, in the medial prefrontal cortex of adult gerbils following a single dose of methamphetamine (25 mg/kg), the GABAergic innervation increased 20% within 30 d (Dawirs et al., 1997). In another report on adult rats receiving four doses of methamphetamine, a decrease was seen in the density of presynaptic immunolabelling for GABA in the basal ganglia 1 wk later, and an increase after 4 wk (Burrows and Meshul, 1999). Nonetheless, the effect of Amph on the GABA system remains to be elucidated in key areas, such as distinct neocortical regions, nucleus accumbens and hippocampus of the brain, and the role age plays in the reaction of the GABA system to Amph is unclear.

Amph and related derivatives have been suggested to bring about synaptogenesis in the brain regions. Several studies using acute methamphetamine or chronic Amph treatment, have declared reversible or persistent changes in the number of dendritic spines, and dendritic length in the prefrontal cortex of adult gerbils or the nucleus accumbens of mature rats (Dawirs et al., 1993; Robinson and Kolb, 1997). In gerbils given a single dose of methamphetamine on postnatal day 14 resulted in higher total dendritic length and spine density of the pyramidal cell in the prefrontal cortex on postnatal day 90 (Blaesing et al., 2001). Thus, persistent alterations in the organization of the synaptic connection in the central nervous system have been proposed in interpreting the development of an addicted state (Nestler, 2001). However, relevant mechanisms for the reorganization require further exploration in order to understand more thoroughly the pathogenesis of the disease.

Thus, the purpose of our experiment was to primarily examine the responses of the GABAergic neurons in various brain regions of juvenile and adult rats to chronic treatment with Amph. Immunocytochemical staining was employed to localize the GABAergic boutons using an anti-glutamic acid decarboxylase (GAD_67) antiserum, which is the GABA-synthesizing enzyme. The densities of the boutons were determined in various brain regions. The behavioural responses of the animals were also investigated to Amph treatment.

Reactions of the neurons in distinct brain regions and the behaviour of the animals were compared between different drug-withdrawal periods and between developing and adult rats.

Methods

Drug administration

Male Wistar rats (postnatal days 21 and 60) were intraperitoneally injected with d-Amph (Sigma, St. Louis, MO, USA), 5 mg/kg, three times daily at 09:00, 13:00 and 17:00 hours for 6 d and then withdrawn from the injection. On day 8 (withdrawn 1 d, W1d) or day 21 (withdrawn 14 d, W14d) at 09:00 hours, they were challenged with a single dosage of Amph, and anaesthetized with 0.32 g/kg chloral hydrate 4 h later, followed by perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) or with Bouin’s fixative. The fixed brains were subjected to paraffin embedding. Before perfusion they were kept at 21 ± 1 °C with free access to food and water. The control animals received saline injections and saline challenge. The fact that the animals were perfused 4 h post-challenge injection allowed observation of the animals’ behaviour and matched injection intervals during the first 6 d. The animal experiments were performed in accordance with the guidelines of an Animal Research Review Committee of NTU. Histological observations were performed on coronal brain sections stained with Cresyl Violet.

Behavioural ranking

Approximately 30 min before the injection of Amph, the animals were placed in a 40 x 40 x 45 cm container. The behaviour of the animals was rated every 5 min beginning from 5 min before 09:00 hours until 150 min after the injection. The score of the behaviour was determined according to the following ranking (Ellinwood and Balster, 1974): 1, asleep; 2, alert with normal activity; 3, increased activity; 4, fast running movement; 5, slow repetitive searching activity; 6, fast repetitive exploring activity; 7, sniffing and rearing;
8, sniffing and gnawing; 9, dyskinesia and seizures. The most frequent rank of the behaviour was recorded within every 5-min interval. The determination of the scores within 5-min frames was based on the appearance of a certain score for more than 3 min and verified by different observers. The Kruskal–Wallis ANOVA was used to compare the behaviour scores between saline and Amph groups. The latencies to onset-of-peak scores were recorded after the Amph injections each day. One-way ANOVA analysis was performed to compare the latencies. Values less than 0.05 were considered as statistically significant.

**Immunocytochemistry**

Coronal paraffin brain sections (7 μm thick), were obtained and mounted on gelatin-coated slides. The brain sections were deparaffinized by xylene and treated with 0.1% H2O2 in methanol for 30 min. The samples were blocked with 5% normal serum for 30 min at room temperature and then incubated with a rabbit anti-GAD67 antibody (Chemicon, Temecula, CA, USA) diluted 1:2000 in PBS at 4 °C for 20 h. Thereafter, the sections were incubated with the biotinylated anti-rabbit antibody for 1 h at room temperature and with the avidin–biotin–peroxidase complex for 45 min (Vector, Burlingame, CA, USA). The immunoreaction products were visualized by incubating the samples with 0.05% 3-3′-diaminobenzidine tetra-chloride. Subsequently the samples were dehydrated and coverslipped with Permount.

To reduce variability among data, the procedures of immunocytochemistry were performed as regularly and consistently as possible. To quantify the GAD67 immunoreactive boutons, a computerized image analysis system (Image-Pro 3.0, Media Cybernetics, Silver Spring, MD, USA) was employed, including a Kodak digitized camera and Zeiss microscope. Coronal sections were analysed at the levels of nucleus accumbens, striatum, hippocampus and substantia nigra. Under a fixed level of illumination and a 40× objective, the sum of areas >0.05 μm², and optical densities ≥0.12, of the GAD- immunopositive profiles, i.e. the dark punctate structure, were measured in these brain sections. This measurement presumably selected the GAD-containing terminals (Keller and White, 1987). Six 40× fields in each of 10 sections, from every third of consecutive sections, were quantified for the motor cortex, somatosensory cortex, striatum, nucleus accumbens and hippocampus of each animal. There were six animals used for each control or drug-treated group. The density of the GAD67 boutons was determined by dividing the sum of the bouton areas by the area taken for quantification. Hippocampal CA regions were distinguished primarily according to the cytoarchitecture of the pyramidal cells on coronal brain sections. The CA2 pyramidal cell layer is relatively thicker than CA1 and CA3, and thus it appears to be a somewhat enlarged short segment connecting the pyramidal cell layers of CA1 and CA3. The data are presented as mean densities ± S.E. Effect of Amph on GAD-terminal densities was evaluated in relation to age and withdrawal period using three-way repeated-measure ANOVAs followed by post-hoc comparison. Values <0.05 were considered as statistically significant.

**Results**

**Behavioural observations**

The behaviour of saline-injected juvenile and adult rats resembled the behaviour of those untreated. They usually appeared alert and showed normal activities during the period of observation; however, some of them fell into sleep during the latter stage of the observation. Following the injection of Amph, the rats exhibited increased locomotion, hyperactivity and stereotypy (a score of approx. 7) (Figure 1). The maximal scores persisted for approx. 70–120 min, and then decreased. By 150 min post-injection, on days 1, 2 and 21, the behaviour of the animals reached the level of the saline controls, although on other days the behaviour scores were still higher than controls. The Kruskal–Wallis analysis revealed that the behaviour ranking of the Amph-injected animals displayed significant difference from that of saline-control animals (p <0.0001), for both age groups.

The sensitized behaviour of the Amph-treated rat was implicated by more rapid onset of stereotypy, and prolonged time of peak behaviour along with injection days. The latencies to reach peak behavioural scores were recorded following Amph injections. For juvenile rats, on day 1, the mean latency to reach the peak scores 6–7, was 36.9 ± 4.9 min post-first Amph injection (Figure 2). Beginning from day 2, the onset time of the peak following the daily first injection decreased along with injection days, from 21.7 ± 2.8 min of day 2 to 9.2 ± 2.0 min of day 8 (W1d) (p < 0.05). Nevertheless, on day 21, after the withdrawal period of 14 days (W1d), the latency was 18 ± 5.7 min, longer than that of day 8, although still shorter than day 1. Similarly to the juvenile, on day 1, it took the adult rats 35.8 ± 9.2 min post-injection to reach peak behavioural scores 6–8. For days 2–5, the latencies decreased to 15 ± 5 to 10.0 ± 0.0. On days 6 and 8 (W1d), it took the adult 12.5 ± 2.7 and 7.5 ± 2.7 min to approach the peak.
Similar to the value of day 8, the latency of day 21 (W14d) was $8.7 \pm 2.5 \text{ min.}$ The behavioural abnormality observed in the juvenile rats, appeared more variable than that of the adult. Within certain time-frames, different behavioural ranks appeared. During the manifestation of hyperactivity and stereotypy, behaviours such as jumping and backward walking were observed. The adults showed more uniform types of behaviour within the time-frames.

**The distribution of GAD$_{67}$ immunoreactivity**

In the brains of both age groups of rats, many dark GAD$_{67}$ (GAD) immunoreactive punctates were present adult, were determined for the first Amph injections of each day. The mean latencies, minutes $\pm$ s.e. ($n=7$), were plotted against the days of injections. For days 2–21, the latencies were shorter than that of day 1. On day 21, the latency of the juvenile is longer than that of adult ($p<0.05$).

![Figure 1](image1.png)

**Figure 1.** The behavioural responses of juvenile and adult rats during the course of amphetamine (Amph) treatment. The rats received three injections of 5 mg/kg Amph, daily for 6 d and then were challenged by a single injection of Amph on day 8 (withdrawn from the agent 1 d) or day 21 (withdrawn 14 d), as described in the Methods section. The control rats received saline injections, and a saline challenge. The behaviour of the rats was observed and rated from 1 to 9 according to the extent of their activity for 150 min post-injection. Data shown are scores of the first injections of each indicated day. The points are means $\pm$ s.d. ($n=7$). The behaviour scores of the Amph-injected animals display significant differences from that of the saline controls, for both age groups, by Kruskal–Wallis analysis ($p<0.0001$).

![Figure 2](image2.png)

**Figure 2.** Differential behavioural responses between juvenile and adult rats to the Amph treatment. The animals received three injections of 5 mg/kg Amph, or saline daily for 6 d and a single injection of Amph or saline on day 8 or day 21. The animal behaviour was rated from 1 to 9 following the injections for 150 min. After Amph injections, the latencies to onset of maximal scores, 6–7 for the juvenile and 6–8 for the
The GAD immunoreactivity was also seen in a number of neuronal somata. These terminals and somata were localized heterogeneously throughout the brain. In general, the Amph treatment generated increases in the levels of the GAD immunoreactivity in many regions of the brains. The density of the GAD terminals was defined by dividing the area sum of GAD-immunoreactive terminals by the area taken for quantification in each brain region. The values are listed in Tables 1–3. Although the individual terminal sizes and numbers of the terminals were not compared between the groups, in some Amph-treated samples, the sizes and numbers appeared to be increased. In addition, the optical densities of the GAD boutons remained quite similar among various experimental groups. As shown by Nissl stain, the layering of the cerebral cortex and the morphology of cells of Amph-treated rats did not seem to be significantly different from that of controls.

**Motor cortex**

In the motor cortex of frontal lobe and cingulate cortex, abundant GAD terminals were present in layers 2/3 and 5; some of the terminals were in close proximity to the cell bodies of pyramidal cells (Figure 3). The GAD-positive somata were scattered in the areas. For the Amph-treated juvenile rats, on day 8 (W1d) the densities of GAD terminals were $(1.02 \pm 0.39) \times 10^{-2}$ and $(1.09 \pm 0.08) \times 10^{-2}$ in layers 2/3 and 5 of the motor cortex, approx. 79 and 65% significantly higher than saline-injected control values ($p < 0.05$), whereas after 14-d withdrawal (at day 21), the levels of the GAD boutons were similar to controls. In W1d Amph-treated adult rats, the densities increased 92 and 88% above controls in layers 2/3 and 5 ($p < 0.01$). In

### Table 1. Repeated amphetamine (Amph) injections age-dependently up-regulate the densities of GAD boutons in the neocortex of the juvenile and adult rats [the values are mean (ratios ± s.e.) $\times 10^{-2}$]

<table>
<thead>
<tr>
<th></th>
<th>Juvenile 1-d withdrawal</th>
<th>Juvenile 14-d withdrawal</th>
<th>Adult 1-d withdrawal</th>
<th>Adult 14-d withdrawal</th>
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</thead>
<tbody>
<tr>
<td><strong>Motor cortex</strong></td>
<td></td>
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<tr>
<td>Layers 2/3</td>
<td>0.57 ± 0.09 (100%)</td>
<td>1.02 ± 0.39* (179%)</td>
<td>0.71 ± 0.08 (100%)</td>
<td>1.25 ± 0.14* (102%)</td>
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<td></td>
<td>(100%)</td>
<td>(102%)</td>
<td>(100%)</td>
<td>(192%)</td>
</tr>
<tr>
<td>Layers 5</td>
<td>0.66 ± 0.14 (100%)</td>
<td>1.09 ± 0.08* (165%)</td>
<td>0.65 ± 0.14 (100%)</td>
<td>1.39 ± 0.14* (113%)</td>
</tr>
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<td></td>
<td>(100%)</td>
<td>(102%)</td>
<td>(100%)</td>
<td>(188%)</td>
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<tr>
<td><strong>Somatosensory</strong></td>
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<tr>
<td>Layers 2/3</td>
<td>0.70 ± 0.02 (100%)</td>
<td>0.96 ± 0.05* (136%)</td>
<td>0.59 ± 0.06 (100%)</td>
<td>0.90 ± 0.06* (170%)</td>
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<td></td>
<td>(100%)</td>
<td>(102%)</td>
<td>(100%)</td>
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<tr>
<td>Layers 4</td>
<td>1.40 ± 0.10 (100%)</td>
<td>2.43 ± 0.18* (173%)</td>
<td>1.33 ± 0.16 (100%)</td>
<td>3.07 ± 0.31* (213%)</td>
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<td></td>
<td>(100%)</td>
<td>(113%)</td>
<td>(100%)</td>
<td>(100%)</td>
</tr>
<tr>
<td>Layers 5</td>
<td>0.64 ± 0.08 (100%)</td>
<td>0.96 ± 0.15* (150%)</td>
<td>0.69 ± 0.11 (100%)</td>
<td>1.29 ± 0.07* (189%)</td>
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<td></td>
<td>(100%)</td>
<td>(118%)</td>
<td>(100%)</td>
<td>(100%)</td>
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</table>

*p < 0.05–0.001, significantly different from saline.

Male rats of postnatal days 21 and 60 were injected intraperitoneally with 5 mg/kg of Amph or saline three times daily for 6 d and withdrawn from the drug for 1 d or 14 d. At day 8 (1-d withdrawal) or day 21 (14-d withdrawal), the Amph-injected animals received one challenge injection of Amph, and the saline-treated animals one challenge dose of saline, followed by perfusion and paraffin embedding of their brains. Coronal paraffin sections were immunostained with an anti-GAD$_{67}$ antiserum. The densities of the GAD$_{67}$-positive boutons in the layers were determined by dividing the area sum of the punctates by the area taken for quantification, as described in the Methods section. The values are mean (ratios ± s.e.) $\times 10^{-2}$. Percentages in parentheses are derived from dividing the densities by comparable saline values (100%). There are significant interactions between the effect of Amph and effect of different ages on the GAD-bouton densities in the motor layers and somatosensory layers respectively ($p < 0.001$), as analysed by the three-way ANOVA.
Table 2. Changes in the densities of GAD terminals in the striatum and nucleus accumbens in amphetamine (Amph)-treated juvenile and adult rats [the values are (mean ratios ± S.E.) x 10⁻²]

<table>
<thead>
<tr>
<th></th>
<th>Juvenile</th>
<th></th>
<th>Adult</th>
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<tr>
<td></td>
<td>1-d withdrawal</td>
<td>14-d withdrawal</td>
<td>1-d withdrawal</td>
<td>14-d withdrawal</td>
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<tr>
<td></td>
<td>Saline</td>
<td>Amph</td>
<td>Saline</td>
<td>Amph</td>
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<tr>
<td>Striatum</td>
<td></td>
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<tr>
<td></td>
<td>0.08 ± 0.01</td>
<td>0.14 ± 0.02*</td>
<td>0.13 ± 0.01</td>
<td>0.15 ± 0.01</td>
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<tr>
<td>(100%)</td>
<td>(175%)</td>
<td>(100%)</td>
<td>(115%)</td>
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<tr>
<td>N. accumbens</td>
<td></td>
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</tr>
<tr>
<td>Core</td>
<td>0.10 ± 0.03</td>
<td>0.15 ± 0.03*</td>
<td>0.11 ± 0.01</td>
<td>0.18 ± 0.03*</td>
</tr>
<tr>
<td>(100%)</td>
<td>(150%)</td>
<td>(100%)</td>
<td>(164%)</td>
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<tr>
<td>Shell</td>
<td>0.12 ± 0.06</td>
<td>0.16 ± 0.05*</td>
<td>0.15 ± 0.03</td>
<td>0.19 ± 0.04*</td>
</tr>
<tr>
<td>(100%)</td>
<td>(133%)</td>
<td>(100%)</td>
<td>(127%)</td>
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</table>

*p < 0.05, significantly different from saline.

The juvenile and adult rats were subjected to the repeated 5 mg/kg Amph, or saline injections and then sacrificed after a challenge injection at 1-d or 14-d withdrawal from the drug. The paraffin brain sections were immunostained with an anti-GAD₆₇ antiserum. The density of the GAD-positive boutons was quantified.

For the striatum, the effect of Amph on the GAD densities significantly interacts with the effect of different animal ages on the densities (p < 0.001). In the core and shell regions of the nucleus accumbens, the effect of different withdrawal periods on the GAD densities interacts with the effect of different ages on the densities (p < 0.01).

Table 3. Amphetamine (Amph)-induced alterations in the densities of GAD-axonal terminals in the pyramidal layers of hippocampal CA1–CA4 regions [the values are (mean ratios ± S.E.) x 10⁻²]

<table>
<thead>
<tr>
<th></th>
<th>Juvenile</th>
<th></th>
<th>Adult</th>
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<tbody>
<tr>
<td></td>
<td>1-d withdrawal</td>
<td>14-d withdrawal</td>
<td>1-d withdrawal</td>
<td>14-d withdrawal</td>
</tr>
<tr>
<td></td>
<td>Saline</td>
<td>Amph</td>
<td>Saline</td>
<td>Amph</td>
</tr>
<tr>
<td>Hippocampus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CA1</td>
<td>0.42 ± 0.06</td>
<td>0.52 ± 0.14*</td>
<td>0.31 ± 0.11</td>
<td>0.48 ± 0.17*</td>
</tr>
<tr>
<td>(100%)</td>
<td>(124%)</td>
<td></td>
<td>(100%)</td>
<td>(155%)</td>
</tr>
<tr>
<td>CA2</td>
<td>0.69 ± 0.12</td>
<td>0.93 ± 0.23*</td>
<td>0.62 ± 0.20</td>
<td>0.94 ± 0.27*</td>
</tr>
<tr>
<td>(100%)</td>
<td>(135%)</td>
<td></td>
<td>(100%)</td>
<td>(152%)</td>
</tr>
<tr>
<td>CA3</td>
<td>0.48 ± 0.14</td>
<td>0.61 ± 0.11*</td>
<td>0.52 ± 0.11</td>
<td>0.67 ± 0.18*</td>
</tr>
<tr>
<td>(100%)</td>
<td>(127%)</td>
<td></td>
<td>(100%)</td>
<td>(129%)</td>
</tr>
<tr>
<td>CA4</td>
<td>0.23 ± 0.04</td>
<td>0.26 ± 0.06</td>
<td>0.10 ± 0.02</td>
<td>0.17 ± 0.08*</td>
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<td>(100%)</td>
<td>(113%)</td>
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<td>(100%)</td>
<td>(170%)</td>
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</table>

*p < 0.05, significantly different from saline.

The juvenile and adult rats were subjected to the repeated Amph or saline injections and then sacrificed after a challenge injection at 1-d or 14-d withdrawal from the drug. The paraffin brain sections were immunostained with an anti-GAD₆₇ antiserum. The density of the GAD-positive punctates was quantified in the pyramidal cell layers of respective CA regions.

The values are (mean ratios ± S.E.) x 10⁻², derived from dividing the respective area sums of GAD punctates by the areas taken for quantification.

In CA1, CA2 and CA3, there are significant interactions among Amph-induced changes in the GAD densities, the effects of withdrawal times and animal ages (p < 0.01).
Figure 3. Micrographs illustrating that chronic amphetamine (Amph) treatment differentially up-regulates the expression of GAD67-immunoreactive boutons in layer 5 of the motor cortex of juvenile and adult rats. The animals were given the repeated Amph or saline injection, three times daily for 6 d, followed by 1-d or 14-d withdrawal from the drug. On either day 8 (W1d) or day 21 (W14d), after a challenge injection of Amph or saline, the animals were subjected to perfusion and paraffin embedding. The coronal brain sections were immunostained with an anti-GAD67 antiserum as described in the Methods section. Arrows indicate the GAD punctates, presumably the nerve terminals. Some punctates are seen abutting upon the pyramidal cell bodies. The GAD punctons appear increased in number and/or size in the Amph-treated juvenile and adult animals of W1d. At W14d, the level of GAD resembles saline in the juvenile, whereas it remains higher than saline in the adult. Arrowheads denote the GAD-immunoreactive cell bodies.
contrast to the juvenile, in the Amph-treated adult following 14-d withdrawal, the densities were still 56 and 95% higher than controls \((p < 0.05, p < 0.01)\). There was significant interaction between the effect of Amph and the effect of different ages on the GAD-bouton densities in the layers \((p < 0.001)\).

### Somatosensory cortex

For saline-injected animals, in the somatosensory cortex of the parietal lobe, the GAD-positive punctates and somata were distributed heterogeneously throughout the cortical layers. The GAD-immunoreactive punctates were relatively more densely distributed in layer 4 than other layers; some GAD terminals abutted the somata of a number of cells (Figure 4). In layer 5, the GAD punctates were also seen in the perimeter of pyramidal cell bodies, which were usually GAD-negative.

For the W1d Amph-treated juvenile rats, there were increases of approx. 36, 73 and 50% above control values of \((0.70 \pm 0.02) \times 10^{-2}\), \((1.40 \pm 0.10) \times 10^{-2}\) and \((0.64 \pm 0.08) \times 10^{-2}\) in the densities of the GAD terminals in layers 2/3, 4 and 5 \((p < 0.01–0.05)\), while at W14d, the values resembled controls. In the W1d Amph-treated adult rats the levels of the GAD terminals were elevated by approx. 70, 113 and 89%, compared to controls \((p < 0.01–0.001)\) (Figure 3). In contrast to the juvenile, in the W14d Amph-treated adult, the corresponding levels of GAD boutons were still 67, 133 and 91% greater than controls \((p < 0.01–0.001)\). For these layers, the effect of Amph on the GAD densities interacted with the effect of different ages \((p < 0.001)\).

### Striatum

The responsive pattern of striatal GAD terminals to Amph was similar to that of the neocortices (Figure 5). The GAD values were means of the medial and lateral striatal areas. For W1d Amph-injected juvenile rats, the density of GAD terminals was \((0.14 \pm 0.02) \times 10^{-2}\), approx. 75% higher than saline controls \((p < 0.01)\). On day 21 (W14d), the density was not significantly different from controls. Nevertheless, the densities of GAD terminals of Amph-treated adult rats were significantly raised 78 and 88% by Amph above controls at both W1d and W14d \((p < 0.01)\). Moreover, the effect of Amph on the GAD densities significantly interacted with the effect of different animal ages on the densities \((p < 0.001)\).

A number of Amph-injected adult rats were challenged on day 21 with saline. The GAD terminals in these animals appeared up-regulated compared to those of saline controls (saline-injected and saline-challenged) in the neocortical areas and striatum. Nonetheless, the data of this group were not quantified.

### Nucleus accumbens

The expression of GAD was up-regulated in both the core and shell of the nucleus accumbens of both ages, regardless of the different withdrawal periods (Figure 5). For W1d AMPH-administered juvenile rats, the densities of GAD terminals were raised by approx. 50 and 33% above the control values \((0.10 \pm 0.03) \times 10^{-2}\) and \((0.12 \pm 0.06) \times 10^{-2}\) in the core and shell \((p < 0.05)\). For W14d juveniles, increases of 64 and 27% still occurred in the levels of GAD terminals \((p < 0.01–0.05)\). For Amph-treated adults, the densities of the GAD terminals of W1d animals increased 31 and 25% in the core and shell from controls \((0.36 \pm 0.05) \times 10^{-2}\) and \((0.36 \pm 0.04) \times 10^{-2}\), and 33% in core and 25% in shell of W14d animals \((p < 0.05)\). In the core and shell regions, the effect of different withdrawal periods on the GAD densities interacted with the effect of different ages on the densities \((p < 0.01)\).

### Hippocampus

In the hippocampi of saline-injected animals, the GAD-positive punctates were present in the layers of both ages and abutted the perimeter of the pyramidal cell bodies (Figure 5). The GAD-immunoreactive somata were scattered among the different layers. The Amph administration elevated the levels of GAD immunoreactivity in almost all layers and regions of the hippocampus. After the Amph treatment, in the W1d juvenile, the densities of the GAD terminals increased approx. 24, 35 and 27% in the pyramidal cell layers of CA1, CA2 and CA3 from controls \((0.42 \pm 0.06\) to \(0.69 \pm 0.12\) \(\times 10^{-2}\) \((p < 0.05)\). The W14d Amph-injected juvenile still had increases of 55, 52 and 29% above controls in the CA1, CA2 and CA3 regions \((p < 0.05)\). Although the expression of GAD in the CA4 region of the W1d animal was similar to controls, the density of the GAD terminals of W14d animals became 70% higher than controls \((p < 0.05)\).

For the W1d Amph-treated adult animals, the levels of the GAD terminals showed increases of 48, 42 and 45% above the controls in the pyramidal cell layers of CA1, CA2 and CA3 (Figure 5) \((p < 0.05)\). Following 14-d withdrawal, the GAD density in the CA1 region was near control value, whereas reductions of 22 and 27% below control values occurred in CA2 and CA3 \((p < 0.05)\). In the CA4 region of the adult, the
Figure 4. Micrographs illustrating the increased levels of GAD$_{67}$-positive terminals induced by amphetamine (Amph) in layer 4 of the somatosensory cortex. The adult and juvenile rats were repeatedly injected with Amph or saline for 6 d, withdrawn from the drug, and subjected to perfusion after a challenge injection on day 8 (W1d) or day 21 (W14d). Paraffin brain sections were prepared and immunostained with an anti-GAD$_{67}$ antiserum. Arrows indicate the immunoreactive boutons and arrowheads the somata. Similar to the response pattern of motor cortex to Amph, the juvenile rat recovers a normal level of GAD boutons at W14d, but the adult has a persistent higher level.
expression of GAD did not evidently differ from the controls. In CA1, CA2 and CA3, there were significant interactions among Amph-induced changes in GAD densities, the effects of withdrawal times and animal ages \((p<0.01)\). GAD immunoreactivity was also seen in other brain areas of both ages, such as prefrontal cortex, amygdala, and substantia nigra. However, Amph did not induce apparent alterations in the expression of GAD in these areas.

**Discussion**

The present study compares the responses of the GABAergic innervation, by examining the...
GAD-containing axonal terminals in major brain areas, such as the neocortex, striatum, nucleus accumbens and hippocampus, to the chronic treatment of the psychomotor stimulant Amph. We have found that the alterations in the expression of the boutons are age- and region-specific. As revealed by monitoring the GAD terminals in W1d and W14d Amph-injected rats, although the juvenile animals show higher plasticity in the neocortex and striatum than adults, they did not exhibit the ability of recovery in the hippocampus and nucleus accumbens. However, for the adult, in the hippocampus of W14d animals, GAD returned towards the control level. The behaviour of the animals in response to Amph appears to age-dependently correlate with the immunocytochemical results.

Mechanisms underlying the behavioural abnormality produced by Amph treatment have been attributed to, at least in part, the agent-induced efflux of dopamine in the striatum, nucleus accumbens and prefrontal cortex (Whitton, 1997). The bell-shaped increases in the behavioural ranking along with post-injection time corresponds somewhat with the time-course of increases of extracellular dopamine in the striatum and nucleus accumbens of the rat following Amph injection, as demonstrated in another report (Kuczenski et al., 1997).

After 14-d withdrawal of Amph, in response to a challenge dose of Amph, the adult animals showed similar onset time of peak behaviour scores to that of 1-d withdrawal, whereas, in juvenile animals, the time to onset of peak rank at W14d was twice of that of W1d, although still shorter than day 1. This suggests partial recovery of the juvenile animals to Amph and more persistent alteration of the adult. Other studies disclosed that rats younger than 22 d treated with chronic Amph or methamphetamine administration, did not show behavioural sensitization to a challenge dose as did the treated older animals, following withdrawal from the drug (Fujisawa et al., 1987; Kolta et al., 1990; Laviola et al., 1994). It is possible that there is development-related difference in the neurochemical adaptation following the drug perturbation.

It has been shown that the maturation of the dopamine system, including dopamine transporter, presynaptic and post-synaptic dopamine receptors, would not be complete until around 1 month after birth (Kirksey and Slotkin, 1979; Shalaby and Spear, 1980). Thus, the differential behavioural changes seen between young and adult rats could probably be generated in part by the difference in the function of the dopamine system and/or other neurotransmitter systems. Furthermore, in our report, both age groups displayed longer duration of stereotypy in the latter injection days than in the initial days. Similarly, another study declared that under chronic low dose Amph injection, the initial increased locomotion was followed by increased duration of stereotypy in adult rats along with the days of injection (Segal and Kuczenski, 1987).

The distribution of the GAD boutons and terminals in the brain regions is quite extensive and generally corresponds to the results of previous studies on rats, and mice (Solberg et al., 1988; Warren et al., 1989). In general, the chronic Amphetamine treatment up-regulated the densities of GAD terminals in many areas of the brains of both ages at W1d. GABA-immunoreactivity up-regulation by acute or repeated methamphetamine administration was also shown earlier in the adult gerbil prefrontal cortex or in the basal ganglia of adult rats (Burrows and Meshul, 1999; Dawirs et al., 1997).

Amph is thought to act on the catecholamine and monoamine systems. The neocortex receives extensive innervations from dopamine, serotonin, and norepinephrine neurons in the brainstem. The striatum receives dopaminergic inputs from the substantia nigra and ventral segmental area and glutamatergic afferents from the neocortex. Dopaminergic and serotonergic fibres in the striatum and neocortex are known to be down-regulated by Amph (Axt and Molliver, 1991; Pu and Vorhees, 1993). When these fibres are affected by Amph, it is possible that the neural circuits undergo remodelling and the modified GABA synapses may possibly participate in reorganization of the inhibitory inputs to the post-synaptic neurons (Dawirs et al., 1993).

Layers 2/3, 4 and 5 in the neocortex, examined in our study, had predominant increases in the densities of GAD terminals. Neurons of these cortical layers are significantly involved in intrinsic and extrinsic cortical circuits. The Amph-induced abundant GABAergic innervation indicates a prominent modulation to the synaptic connectivity among the layers. From another viewpoint, Amph is a psychostimulant and the up-regulation of GABAergic innervation could be a compensatory response of the brain to the stimulation. An earlier study has noted that Amph could increase the release of GABA extracellularly in the striatum (Del Arco et al., 1998). In addition, using striatal slice and whole-cell patch recordings, Amph application depressed GABA-mediated IPSCs through a D2 dopamine receptor mechanism (Centonze et al., 2002).

The increased densities of GAD boutons induced by Amph might result from the presence of enlarged GAD terminals, the expression of a greater number of GAD terminals and/or sprouting of GABAergic neurons. The location of the cell bodies of the GABAergic
terminals is unknown. Nevertheless, these terminals may belong to intrinsic and/or extrinsic GABAergic neurons of each specific region. A number of GABAergic cell bodies are present in the areas investigated. Although the number of the cells and levels of GAD in the cell bodies were not quantified, the somatal content of GAD appeared higher than controls. To identify the source of these terminals may require double-labelling experiments using tracers and anti-GAD or GABA antibody. In addition, mapping the GAD-mRNA-containing neurons could likewise provide information about cell-body location and give further understanding on the mechanisms for up-regulated GAD-bouton densities.

It is interesting that after 14-d withdrawal from the drug the juvenile animal regained the normal densities of GAD terminals in the neocortex and striatum. By contrast, W14d adult animals had persistent higher densities of GAD terminals in the neocortex and striatum. In these areas, the young animals presumably have a better capacity to restore the regular expression of GAD than do the adults. We do not know whether the adult rats will recover following longer withdrawal period than 14 d. At 14-d withdrawal, the behavioural observation after a challenge injection of Amph indicates a partial recovery of the latency of peak behaviour scores towards the latency of day 1, while similar onset time to that of W1d was recorded for the adult. This behavioural discrepancy could be in accordance with the difference in the reversibility of the GAD expression between juvenile and adult.

Hippocampal areas receive monoaminergic afferents from the brainstem and GABAergic and cholinergic axons from the basal forebrain region. It has been proclaimed that acute Amph treatment elevates extracellular norepinephrine levels in the hippocampus (Kuczenski et al., 1995). The increases seen in the density of GAD terminals in the CA1–CA3 region may be due to the disturbance by Amph on the monoaminergic inputs. However, in contrast to the results of neocortex and striatum, at 14-d withdrawal, the young rat showed a consistent increase in GAD boutons, but the adult had regular GAD densities. These area- and age-dependent variations evidently denote the uniqueness of each specific brain region and its developmental characteristics. The neocortex, motor and somatosensory cortices, are intricately interconnected with the striatum; this may be related to similar response patterns of GAD in these regions. The hippocampus is an important part of the limbic system and is believed to be associated with memory.

We have found that GAD terminals in the nucleus accumbens were up-regulated by Amph, regardless of the withdrawal period and age. The nucleus accumbens receives afferent fibres from the ventral tegmental area, hippocampal formation and a number of basal forebrain areas, including the amygdaloid complex. The nucleus accumbens system has been one of the key targets in studying the neurobiological basis of behaviour sensitization, because it participates in mediating the psychomotor activating and rewarding effects of psychostimulant drugs. Amph could enhance the overflow of dopamine in the nucleus accumbens (Robinson and Becker, 1986); other neurotransmitter systems, including serotonin, glutamate, acetylcholine and GABA are involved in drug reward as well (Bardo, 1998). The persistent up-regulation of GAD boutons probably point to the critical role the nucleus accumbens plays in addiction.

The age- and region-specific changes in the level of GABAergic innervation found in our investigation attest that inhibitory regulation is involved in neuronal responses to chronic psychostimulant administration. Apparently the chronic Amph administration results in somewhat persistent synaptic changes in the brain areas, particularly of the adult animals, which could represent the differential plasticity among the brain regions and may account for, in part, the behavioural abnormality. Furthermore, the data may imply possible therapeutic strategy to Amph-induced psychotic disorders.

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Statement of Interest

None.

References

Effect of amphetamine on GAD boutons


