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# IDENTIFICATION OF ADRENOCEPTOR SUBTYPE-MEDIATED CHANGES IN THE DENSITY OF SYNAPSES IN THE RAT VISUAL CORTEX

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**Abstract—Both serotonin and noradrenaline affect synapse formation and maintenance in the CNS. Although we previously demonstrated that serotonin regulates synaptic density via activation of serotonin$_{2A}$ receptor, it was still unclear which receptor subtype mediates the function of noradrenaline. In the present study we tried to identify the noradrenaline receptor (adrenoceptor) subtype, which could regulate the density of synapses in the rat visual cortex. Selective antagonists and/or agonists of adrenoceptor subtypes were administered to six weeks old rats. Changes in the density of axodendritic synapses were quantitatively examined in lamina I, where noradrenaline rather than serotonin is known to regulate the density of synapses. The α1 adrenoceptor antagonists (prazosin and 2-{[b-(4-hydroxyphenyl)ethyl]aminomethyl}-1-tetralone) decreased the number of synapses in a dose-dependent manner. In contrast, administrations of the α1-agonist (methoxamine) increased the density of synapses. The β1 adrenoceptor antagonist (atenolol) had no effect on the density of synapses. The α2-antagonist (rauwolscine) increased synaptic density, whereas the β2-antagonist (ICI-118,551) decreased synaptic density. Simultaneous treatments with the α1-antagonist and α1-agonist caused the α1-agonist to competitively block the effect of the α1-antagonist and recover the density of synapses to the control values. In addition, the α1-antagonist/agonist appeared to show a reverse effect on the changes in synaptic density following α2- or β2-antagonist treatment by acting via the α1 receptor. Moreover, decreased synaptic density when a selective noradrenergic neurotoxin (*N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine) was counterbalanced by the α1-agonist. These data suggest that noradrenaline regulates the density of synapses in the rat visual cortex primarily via the α1 receptor subtype. Both serotonin$_{2A}$ and α1 receptors are known to couple with phospholipase C, which has been shown to increase intracellular calcium. It may help us to understand the underlying mechanisms for synaptic plasticity in the CNS. © 2005 Published by Elsevier Ltd on behalf of IBRO.**

**Key words: plasticity, synaptic maintenance, noradrenaline, quantitative study, α1 adrenoceptor, electron microscope.**

It was previously indicated that serotonin (5-HT), noradrenaline (NA), dopamine (DA) and acetylcholine play a trophic-like role in facilitating the formation and maintenance of synapses in the CNS (Okado et al., 1993; Chen et al., 1994; Matsukawa et al., 1997, 2003; Imai et al., 2004). In the rat visual cortex, NA- and/or 5-HT-mediated changes in the density of synapses are most prominent between one and two weeks after birth, and this has an effect on synapse formation (Matsukawa et al., 2003). In this study, following the depletion of NA and/or 5-HT, the magnitude of synaptic density reduction was quite similar and dramatically regulated (29–56%) in all the laminae examined. However, in adult animals, it was less prominent in facilitating synaptic formation, but had a maintenance effect on synaptic density in some selected laminae. It was also revealed that the density of synapses was regulated by NA in lamina I, by 5-HT in lamina II, and by both NA and 5-HT in lamina IV at 6–7 weeks of age.

It is known that the function of 5-HT in facilitating synapse formation in embryonic chicken spinal cords is mediated via the 5-HT$_{2A}$ receptor subtype (Niitsu et al., 1995). The density of 5-HT$_{2A}$ receptors in the rat cerebral cortex increases after birth, and reaches a peak value by postnatal day 12. Subsequently it decreases to adult values by one month after birth (Roth et al., 1991). In addition, increased expression of the 5-HT$_{2A}$ receptor and increased 5-HT-mediated synaptic plasticity (relative to the adult value) occur concurrently between one and two weeks after birth in the rat cerebral cortex.

The β1-adrenoceptor was previously shown to be involved in regulation of ocular dominance plasticity in the kitten visual cortex (Kasamatsu, 1991). If a similar receptor mechanism is involved in NA-mediated changes in the density of synapses, the β1-adrenoceptor should be upregulated between one and two weeks after birth. However, the β1-adrenoceptor actually reaches a peak value between postnatal days 20 and 40 (Pittman et al., 1980). In contrast, the spatiotemporal changes in NA-mediated density of synapses and the rapid increase in α1-adrenoceptor, which occurs between one and two weeks after birth, are correlated in the rat visual cortex (Zilles et al., 1991). These developmental changes in NA receptor subtypes suggest that facilitation of synapse formation and mainte-

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*Abbreviations:* AC, adenylyl cyclase; $[Ca^{2+}]_i$, intracellular calcium; DAG, diacylglycerol; DSP-4, *N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine; HEAT, 2-{[b-(4-hydroxyphenyl)ethyl]aminomethyl}-1-tetralone; $IP_3$, inositol triphosphate; NA, noradrenaline; NAD, number of axodendritic synaptic profiles in a unit volume; NAD(A), number of axodendritic synaptic profiles in a unit area; NMDA, *N*-methyl-D-aspartic acid; PLC, phospholipase C; 5-HT, 5-hydroxytryptamine.

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nance is, in fact, mediated by the α1-adrenoceptor not the β1-adrenoceptor subtypes. To clarify this hypothesis, the present study attempted to determine the NA receptor subtypes that mediate the function of NA in the formation and maintenance of synapses in the rat visual cortex. To identify the selective adrenoceptor subtypes that mediate the synaptic plasticity of NA, we administered selective antagonists and/or agonists to six weeks old rats. The density of synapses in the lamina I, where synaptic density is regulated by NA not 5-HT, was quantitatively examined using electron microscopy. Since we have been assessed the synaptic density following one-week administrations of drugs in our previous studies, every antagonist and/or agonist used in this study was administered for one week.

## EXPERIMENTAL PROCEDURES

### Animals

Six-week-old male Wistar rats were used in all experiments. Four control and four experimental animals were used for each dose of each drug treatment. They were housed in groups of two animals per cage at 24 °C (room temperature) with a 12-h light/dark cycle, and given *ad libitum* access to food and water. Efforts were made to minimize animal suffering and to reduce the number of animals used. All animal experimental procedures used in this study followed the Guide for the Care and Use of Laboratory Animals described by the National Institutes of Health (USA) and were approved by the Animal Experimentation Committee of the University of Tsukuba.

### Pharmacological procedures

Specific NA receptor antagonists and/or agonists (Table 1), and a selective noradrenergic neurotoxin were used in the present study. 2-{[b-(4-Hydroxyphenyl)ethyl]aminomethyl}-1-tetralone (HEAT) hydrochloride was purchased from Tocris Cookson (Bristol, UK). All other drugs were purchased from Research Biochemicals International (Natick, MA, USA). Agonists and antagonists were dissolved in saline, and injected in the IP space once daily for one week. The same volume of vehicle (saline) was similarly injected into the control animals. Twenty-five mg/kg weight of a selective neurotoxin, *N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine (DSP-4), was intraperitoneally injected twice on the first day of the experiment, and three doses of methoxamine (α1-agonist) were injected once daily for one week in animals administered DSP-4.

### Fixation

One week after beginning treatment, animals were deeply anesthetized with an overdose of pentobarbiturate (Nembutal; Abbott Laboratories, North Chicago, IL, USA), and perfused with a mixed solution of glutaraldehyde (2.5%) and paraformaldehyde (2%) in a 0.1 M phosphate buffer (pH 7.4). The brain was then removed and immersed in freshly prepared fixative for 12 h at 4 °C. The right hemisphere was used for counting synaptic profiles, and the left was used for measuring the size and weight of the cerebrum. The rostrocaudal length and mediolateral width of the cerebrum were measured using a binocular microscope (Fig. 1a).

**Table 1.** The antagonists and agonists used in this study

| Selective drugs | α Receptor | | β Receptor | |
|---|---|---|---|---|
| | α1 Receptor | α2 Receptor | β1 Receptor | β2 Receptor |
| Antagonist | Prazosin<br>HEAT | Rauwolscine | Alprenolol<br>Atenolol | Alprenolol<br>ICI-118,551 |
| Agonist | Methoxamine | | | |

### Electron microscopy

Four rats were used for quantitative electron microscopic analyses following each dose of pharmacological treatment and saline (control). The procedures used in this study were almost the same as in our previous studies (Matsukawa et al., 1997, 2003; Imai et al., 2004). Coronal sections (1 mm thick) were cut with a razor blade from the central part of V1 M region in the visual cortex (Fig. 1a and b) (Paxinos and Watson, 1998). Specimens were postfixed with 2% osmium tetroxide, dehydrated with ethanol, and embedded in Epon. Semithin (4 μm thick) plastic sections were cut perpendicular to the surface of the cortex and stained with Toluidine Blue (Fig. 1c). The thickness of the visual cortex was measured in these sections under a light microscope. Following light microscopic observations, the sections were re-embedded in Epon. Based on the interference colors, 75 nm thick ultrathin sections were cut, picked up on #100 mesh grids then stained with lead citrate and uranyl acetate. Photomicrographs were taken using 24×36 mm film (Minicopy; Fuji Film, Tokyo, Japan) and an electron microscope (H-600; Hitachi, Tokyo, Japan).

Selection of specific lamina and developmental stage is important for identifying NA receptor subtypes. The results of a previous study (Matsukawa et al., 2003) revealed interactions between 5-HT and NA in facilitating synapse formation and maintenance in all layers examined between one and two weeks after birth. An appropriate region for the present study was the lamina(e) where the density of synapses is modulated only by NA and β adrenoceptors are not yet fully developed at five weeks after birth (Pittman et al., 1980). For this reason, changes in the density of synapses in lamina I were examined between 6 and 7 weeks after birth following drug administration.

Because neuronal soma profiles were infrequently located in lamina I, changes in the density of axodendritic synapses were analyzed in the present study. Two non-sequential sections from each rat were re-embedded for the quantitative analyses using electron microscopy. The electron micrographs for counting the synapses were taken at a primary magnification of ×4600; each photograph covered an area of 41 $\mu m^2$ at this magnification. More than 13 photographs were randomly taken from the ultrathin sections cut from the re-embedded semithin sections. In total, more than 120 photographs were taken from four animals for each dose of given drug and saline-treated control.

The number of synaptic profiles was counted at a final magnification of ×20,400 (Fig. 1d). Synaptic profiles were identified as structures with an aggregation of synaptic vesicles in the terminal bouton and pre- and post-synaptic membrane thickenings. Single terminal boutons with multiple synaptic vesicle aggregation sites and membrane thickenings were counted as single synaptic profiles. For all quantitative analyses the final magnification was determined by photographs of a test specimen (carbon-grating replica) taken and enlarged in the same way as those for the synaptic profiles.

The profile counting method rather than the physical disector method was adopted for estimations of synaptic density. Although the physical disector method is strongly recommended for estimating the absolute number of synapses (Coggeshall, 1992), the profile counting method can be used to compare experimental and control values (Guillery and Herrup, 1997; DeFelipe et al., 1999). Furthermore, we previously demonstrated that both methods showed almost the same value for the number of synapses in the rat visual cortex at 6–7 weeks of age (Matsukawa et al., 2003).

The lengths of the membrane thickenings of the synaptic profiles were measured using a tracing tablet (Image Measuring System; Fine Tech., Tokyo, Japan) and personal computer. In