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Central catecholamine depletion inhibits peripheral lymphocyte proliferation and splenocyte cytokine production

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Wei Kou
aus Tanggu, Tianjin, China
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Medical Faculty of the University of Duisburg-Essen

Department of Medical Psychology

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Presented by
Wei Kou
From Tianjin, P.R. China
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Dekan: Univ.-Prof. Dr. med. H. Grosse-Wilde
1. Gutachter: Univ.-Prof. Dr. rer. biol. hum. Dipl.-Psych. M. Schedlowski
2. Gutachter: Prof. Dr. rer. nat. O.-E. Brodde

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1. Introduction

1.1 Overview

Traditionally, the nervous and immune systems have been regarded to function largely independently of each other. Evidence accumulated during the past two decades, however, has demonstrated the bidirectional communications between the central nervous system (CNS) and the immune system.

The first sustained program of research on brain-immune system interactions began in the 1920s and concerned the Pavlovian conditioning of 'immune' reactions. In the 1950s, there was a short-lived, but later received, interest in the immunologic effects of lesioning and electrical stimulation of the hypothelamus. At the same time, Fred Rasmussen, Jr., a virologist, and Norma Brill, a psychiatrist, initiated studies on the effects of stressful life experiences on susceptibility to experimentally induced infectious diseases (Ader et al., 2001).

Interest in this interdisciplinary research was rejuvenated when, in the 1970s, several independent lines of research converged to provide verifiable evidence of interactions between the brain and the immune system. John Hadden linked the immune system to the sympathetic nervous system (SNS) by documenting the existence of β-adrenergic receptors (ARs) on lymphocytes (Hadden et al., 1970); Robert Ader and Nicholas Cohen demonstrated behaviorally conditioned suppression of the immune system, providing a functional link between the brain and the immune system (Ader and Cohen, 1975); Roger Bartrop and his associates described immunologic changes associated with the bereavement that following the death of a spouse (Bartrop et al., 1977); Hugo Besedovsky began to piece together a neuroendocrine-immune system network with his studies of the effects of immune responses on neural and endocrine functions (Besedovsky et al., 1983); the sympathetic innervation of lymphoid tissues was documented by David and Suzanne Felten (Felten et al., 1987a; Felten and Olschowka, 1987) and connections to the thymus were documented by Karen Bulloch (Bulloch and Pomerantz, 1984); and Edwin Blalock and Eric Smith showed that lymphocytes, themselves, were capable of producing neuropeptides (Blalock and Smith, 1980). These seminal studies precipitated new research on the receptors for hormones and neurotransmitters that existed on lymphocytes, the

effects of SNS activity on immunity, conditioned immune responses, depression and immunity, the influence of stressful life experiences on immune function and resistance to disease, psychosocial factors in the progression of acquired immunodeficiency syndrome (AIDS), and immunologic effects on behavior (Ader et al., 2001). Thus, we have witnessed an explosive growth of a new research area that studies neuroimmune communication in the last two dacades.

1.2 Occurrence and regional distribution of catecholamines in the central nervous system

The CNS contains three types of catecholamines: norepinephrine (NE), dopamine (DA), and epinephrine (EPI) (Moore and Bloom, 1978). Biochemical measurement of transmitter levels in extracts of microdissected brain nuclei has helped clarify the distribution of them in the CNS. In table 1 the concentrations of NE, DA and EPI in several regions of rat brain are reported (Müller and Nisticò, 1989a).

The distinct neuronal groups in the brain stem are the major souce of catecholaminergic input to most parts of the brain. Histochemical techniques revealed three major catecholaminergic cell groups in the brain stem. These groups are distinguished by their transmitters: NE, DA and EPI (Role and Kelly, 1991).

Two principal nuclei in the brain stem contain noradrenergic neurons. These are (1) the locus ceruleus, located in the rostral pontine central gray region, and (2) the lateral tegmental neurons, which are more scattered in the medullary lateral pontine tegmentum. The neurons of the locus ceruleus have both descending and ascending axonal branches. The descending branches go to the spinal cord (predominantly to the ventral horn) and to the brain stem itself (primarily to sensory nuclei). Ascending projections terminate in the diencephalon (largely in the dorsal thalamus, with a smaller projection terminating in the hypothalamus), in the cerebellum, the basal forebrain (including the hippocampus), and the neocortex. The locus ceruleus receives only two major inputs. These come from two brain stem nuclei: the nucleus paragigantocellularis and the nucleus hypoglossi prepositus. Thus, the locus ceruleus receives restricted afferent input yet makes very broad efferent projections (Role and Kelly, 1991).

Table 1. Concentrations of norepinephrine, dopamine, and epinephrine in several nuclei and areas of the rat brain[a]

Site	Concentration (ng/mg protein)
Norepinephrine	
Nucleus interstitialis striae terminalis	80.2
Dorsomedial nucleus	55.1
Locus coeruleus	53.1
Paraventricular nucleus	52.9
Retrochiasmatic area	48.0
Periventricular nucleus	45.7
Nucleus tractus solitarii	37.4
Nucleus interstitialis striae medullaris	33.2
Medial forebrain bundle	29.8
Supraoptic nucleus	29.0
Dopamine	
Olfactory tubercle	104.5
Nucleus accumbens	87.2
Caudate-putamen	85.2
Median eminence	61.5
Ventral striatum	57.6
Anterior amygdala area	45.9
Globus pallidus	39.6
Nucleus tractus diadonalis	33.9
Area tegmentalis ventralis	22.4
Lateral septal nucleus	21.3
Epinephrine	
Periventricular nucleus	2.52
Dorsomedial nucleus	2.53
Nucleus tractus solitarii	2.19
Paraventricular nucleus	2.16
Supraoptic nucleus	2.14
Median eminence	1.83
Lateral mammillary nucleus	1.51
Arcuate nucleus	1.41
Midline thalamic nucleus	1.27

[a] From Müller and Nisticò (1989a)

The locus ceruleus only contains half of the total number of noradrenergic neurons in the brain stem. The rest are distributed diffusely throughout the ventral lateral tegmentum. An example of this group is the lateral tegmental neurons. Like the neurons of the locus ceruleus, the axons of the lateral tegmental neurons have extensive collaterals and dense terminal arborizations. The axons project to three major sites: (1) the spinal cord, (2) the brain stem and (3) the thalamus and cerebellar and cerebral cortices (Where the input is minor compared to that of the locus ceruleus). In general, neurons of the lateral tegmental region do not overlap their targets with those of the locus ceruleus. Thus, whereas the neurons of the locus ceruleus provide the principal noradrenergic input to the neocortex, the lateral tegmental neurons provide the major noradrenergic input to the brain stem and spinal cord. The physiological significance of these divergent noradrenergic projections is illustrated by the extensive behavioral effects produced by drugs that alter central NE action (Role and Kelly, 1991).

When neurons of the locus ceruleus are activated by novel sensory input, they respond as a group with an increased burst of activity. This coordinate response to a change in sensory input suggests that these neurons have a role in orienting and attending to sudden contrasting, or aversive sensory input. Neurons of the lateral tegmental noradrenergic system contribute to the integration of autonomic function in brain stem and spinal cord nuclei through projections to sympathetic preganglionic neurons in the intermediolateral cell column as well as to the nucleus of the solitary tract and the dorsal tegmental neurons leads to a profound decrease in mean arterial pressure, heart rate, and blood pressure (Role and Kelly, 1991).

There are about three to four times as many dopaminergic neurons as noradrenergic neurons in the brain. In contrast to the diffuse projections of the noradrenergic system, the dopaminergic system is highly organized topographically. On the basis of their efferent projections, dopaminergic cell groups have been broadly classified into two groups: (1) the mesostriatal system, and (2) the mesolimbic and mesocortical systems. The mesostriatal system projects from the substantia nigra and the ventral tegmentum to several striatal areas. This system plays an important role in the control of voluntary movement. Selective destruction or degeneration of the mesostriatal dopaminergic system results in the motor disorders of Parkinson's disease (PD). The mesolimbic and mesocortical systems project from the ventral tegmentum to limbic and cortical areas. The

function of these projections is not known, but they are thought to participate in cognition. The dopaminergic system is also considered the primary site of action of many stimulants (e.g. amphetamines) as well as antipsychotic drugs (Björklund and Lindvall, 1984).

Dopaminergic neurons are also found in other regions of the CNS including the tuberohypophyseal incertohypothalamic and medullary periventricular neurons, the dorsal and ventrolateral preoptic areas, the hypothalamus, the olfactory bulb and the retinal dopaminergic neurons (Björklund and Lindvall, 1984). The organization of dopaminergic systems in the rat brain is summarized in Table 2 (Müller and Nisticò, 1989a).

The use of an immunohistochemical method has made possible the demonstration in the mammalian CNS of neurons containing EPI (Hökfelt et al., 1984). In particular, by means of antibodies against phenylethanolamine-*N*-methyltransferase (PNMT), the enzyme converting NE into EPI, it has been clearly demonstrated that certain areas of the brain such as the olfactory bulb, olfactory tubercle, and the hypothalamus contain substantial amouts of the enzyme and therefore are capable of forming EPI *in vivo*. The EPI cell bodies have been found in the reticular formation of the medulla oblongata; they send descending axons to the sympathetic lateral column and ascending axons into the hypothalamus, the pericentricular gray, and the locus coeruleus. Furthermore, there is a dense EPI innervation of the nucleus dorsalis, nucleus vagi, and the nucleus tractus solitari (Hökfelt et al., 1984). The cell bodies of PNMT-immunoreactive neurons in the medulla oblongata are organized into two systems, a ventral C1 and a dorsal C2 group. The cells in the C1 group appear caudal to the area postrema, dorsolateral and/or ventromedial to the reticular nucleus (Müller and Nisticò, 1989a). Generally, PNMT-positive nerve endings have a more limited distribution than do those of the other two catecholamines although they extend from the forebrain to the spinal cord, mostly concentrated along the ventricular system. Telencephalic structures are poorly innervated, whereas a richer innervation exists in the diencephalon. In particular, a moderately dense network of PNMT-positive fibers is found in the periventricular area, including the suprachiasmatic part of the preoptic nucleus and the arcuate nucleus of the hypothalamus. A dense PNMT-reactive network is observed at the mid-hypothalamic level, in the paraventricular nucleus, and in the rostral part of the periventricular region (Müller and Nisticò, 1989a).

Table 2. Main dopaminergic pathways and systems in the rat brain[a]

System	Cells of origin	Projections
Mesostriatal	Substantia nigra (A9)	Caudate-putamen
	Ventral tegmental area (A10)	Ventral striatum (nucleus accumbens), olfactory tubercle, bed nucleus, striae terminalis
	Retrorubral nucleus (A8)	Globus pallidus
		Island of Galleja
		Subthalamic nucleus
Mesolimbocortical	Ventral tegmental area (A10)	Olfactory bulb
	Substantia nigra (A9)	Anterior septal nucleus
	Retrorubral nucleus (A8)	Piriform cortex
		Amygdala
		Ventral entorhinal cortex
		Suprarhinal cortex
		Pregenual anteromedial cortex
		Supragenual anteromedial cortex
		Perirhinal cortex and temporal association cortex
		Lateral habenular nucleus
		Locus coeruleus
Mesothalamic	Ventral tegmental area (A10)	Thalamus
Diencephalospinal	Dorsal and posterior hypothalamus, zona incerta, caudal thalamus (A11)	Spinal cord
Intertohypothalamic	Zona incerta, periventricular hypothalamic nuclei (A11, A13, A14)	Zona incerta, anterior medial preoptic and periventricular hypothalamus, septum
Tuberoinfundibular and tuberohypophyseal	Arcuate and periventricular hypothalamic nuclei (A12, A14)	Median eminence
		Pars intermedia and nervosa of pituitary
Periventricular	Mesencephalic periqueductal gray	Periqueductal gray
	Periventricular gray of caudal thalamus (A11)	Medial thalamus and hypothalamus
Periglomerular	Olfactory bulb (A16)	Dendritic processes into olfactory glomeruli
Retinal	Mainly in amacrine cells of inner nuclear layer of retina	Local dendritic projections

[a] From Müller and Nisticò (1989a)

1.3 Central catecholamines and immunity

It is well known that the CNS can influence the immune system via various pathways. This is primarily achieved by several neurotransmitters, neuropeptides, hormones and cytokines which interact with different immune effector cells and thereby ultimately regulate the homeostatic response of an individual to disease and other environmental stresses (Weigent and Blalock, 1987). Among these neural mediators of homeostasis, central catecholamines play a significant role, which has been proved by numerous clinical and experimental observations (Basu and Dasgupta, 2000; Kohm and Sanders, 2000).

1.3.1 Correlation between central catecholamines and immunity in pathophysiological conditions

Degeneration of nigrostriatal dopaminergic neurons is the cause of PD, resulting in hypodopaminergic activity of the CNS (Temlett, 1996). On the contrary, a hyperdopaminergic activity has been emphasized in schizophrenia (Birtwistle and Baldwin, 1998). Moreover, considerable evidence has also been accumulated, suggesting that both of these neurological disorders involving CNS catecholaminergic system are associated with significant alterations in immune response.

Evidence for the abnormalities in immune response in patients with PD appeared in the late 1970s, Hoffman *et al*. (1978) reported that the T-cell numbers and percentages and the total number of lymphocytes as well as their ability to respond to mitogenic stimulation were reduced in Guamanian patients with PD. Their later study showed that higher IgA and lower IgM levels in serum were also found in these patients (Hoffman et al., 1981). Following investigations demonstrated that patients with PD had a reduction in the proliferative response of peripheral lymphocytes to concanavalin A (Con A), phytohaemagglutinin (PHA) and pokeweed mitogen (PWM) and a decrease in antibody production (Marttila et al., 1985). Higher neutrophil count in peripheral blood, reduced killer cell activity and IL-2 production of peripheral lymphocytes after PHA stimulation were also reported (Bokor et al., 1992; Klüter et al., 1995). More recently, Bas *et al*. (2001) found that PD patients had a decrease in the number of $CD4^+$ T-helper (T_H) cells

and B cells, and a rise in activated, $CD4^+CD25^+$ T cells in their blood with the number of $CD8^+$ cytotoxic T lymphocytes (CTLs) remaining unchanged.

Although some results from schizophrenic patients are contradictory, majority of the evidence indicates an immune stimulation like an increase in the number of $CD4^+$ T_H cells (Henneberg et al., 1990; Muller et al., 1993) and increased plasma level of IL-6 (Maes et al., 1995). In some other studies, abnormal functions of T-cell-mediated immunity, as evidenced from deficient production of interleukin (IL)-2 and interferon (IFN)-γ and elevated level of soluble IL-2 receptor were also reported (Ganguli et al., 1995; Arolt et al., 1997).

Higher incidence of cancer in PD patients than age- and sex-matched normal controls was also reported (Iwasaki et al., 1990). On the contrary, patients with schizophrenia have a reduced incidence of malignancies, despite increased smoking and drinking habits in this population (Glubinat et al., 1992).

1.3.2 Pharmacological manipulation of central catecholamines and immunity

Removal of central catecholamines by treatment with neurotoxins, either 6-hydroxydopamine (6-OHDA) or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), has been widely used to assess immune function in absence of the central catecholamines.

The advantage of 6-OHDA is that it is highly selective for NE nerve fibers, and it does not cross the blood-brain barrier (BBB) when administered to adults (Kostrzewa and Jacobwitz, 1974). It has been widely used experimentally to deplete DA and NE either peripherally or centrally depending on the route of administration. A single intraperitoneal (i.p.) injection rapidly (<6h) destroys most NE nerve fibers in 6-OHDA-sensitive tissues; NE is depleted by 75-85% in spleen and lymph nodes for at least two weeks (de Champlain, 1971; Madden et al., 1994). It is taken up by the high affinity catecholamine uptake I system, and is blocked by uptake I blockers, such as the tricyclic antidepressant desipramine (de Champlain, 1971). On uptake, 6-OHDA rapidly destroys the integrity of the nerve terminal by a mechanism that is not well understood, resulting in the release of NE and other intracellular components from the nerve terminal. In adults, NE cell bodies are not destroyed, and reinnervation of the spleen begins as early as five days after 6-

OHDA treatment (Lorton et al., 1990). A gradual return of NE innervation occurs over the next several weeks, with complete recovery of the spleen observed sixty days after 6-OHDA treatment (Madden, 2001).

On the other hand, systemic administration of MPTP is neurotoxic to central DA neurons in several animal species (primates, mice) (Burns et al., 1983; Heikkila et al., 1984) and can cause Parkinsonism in humans (Langston et al., 1983). The mechanism of this action seems to be indirect. MPTP is oxidized in the brain to 1-methyl-4-phenyl-2,3-dehydropyridinium intermediate (MPDP) and this compound spontaneously oxidation to a pyridinium species, 1-methyl-4-phenylpyridine (MPP^+). MPTP oxidation is greatly decreased by inhibition of monoamine oxidase-B (MAO-B) (e.g., by deprenyl) (Langston and Irwin, 1986). In particular, MPTP selectively kills neurons in the zona compacta of the substantia nigra. All other areas of the brain appear to be spared, including the locus coeruleus and the dopaminergic ventral tegmental area. Thus, MPTP appears to be the first neurotoxin to affect a subset of catecholaminergic neurons selectively (Langston and Irwin, 1986).

Experimental data in the last two decades have confirmed that central catecholaminergic pathways modulate the humoral immune response in the periphery. Initially, Cross *et al*. (1986) reported that mice lesioned centrally with 6-OHDA displayed a profound inhibitory effect on the induction of both IgM and IgG primary antibody response to sheep red blood cells (SRBC) and immunological memory development, but was without effect on the secondary antibody response. More interestingly, these effects were abrogated by hypophysectomy three weeks before central 6-OHDA treatment, suggesting that central catecholamine depletion modulates immune function via the release of pituitary hormones (Cross et al., 1987). In addition, their further study extended these observations by showing that central 6-OHDA treatment inhibits the humoral antibody response to T-cell-dependent antigen trinitrophenyl-keyhole limpet hemocyanin (TNP-KLH), but does not affect the response to the T-cell-independent antigen trinitrophenyl-lipopolysaccharide (TNP-LPS) in mice (Cross and Roszman, 1988).

Furthermore, ablation of central dopaminergic structures with i.p. injection of MPTP reduces the number of IgM plaque-forming cells (PFC) of splenocytes to SRBC in mice (Bieganowska et al., 1993). Pre-treatment with i.p. injection of pargyline, a MAO-B

inhibitor, which prevents MPTP-induced striatal dopamine depletion, restores this humoral immune depression in these animals (Bieganowska et al., 1996). In the study of Filipov *et al.* (2002), selective central dopaminergic depletion in mice by intracerebroventricular (i.c.v.) injection of 6-OHDA and pretreatment of desipramine, which prevents uptake of 6-OHDA by noradrenergic terminals, resulted in decreases in both primary and secondary IgM anti-KLH titers in serum, which could not be restored by GBR-12935 dihydrochloride (GBR-12935), a selective DA uptake inhibitor. Primary, but not secondary IgG1 anti-KLH titers were also suppressed in these animals, and this suppression was not affected by co-administration of GBR-12935 as well. In contrast, the IgG2a anti-KLH titers were not influenced by the same treatment (Filipov et al., 2002). Similar results were also found in rats. Ten days after bilateral electrolytic destruction of brain areas containing DA cell bodies (nuclei A9 and A10) as well as terminal regions of the nigrostriatal and mesolimbic dopaminergic systems (nucleus caudatus and nucleus accumben), the number of splenocyte rosette-forming cell (RFC) to SRBC is decreased (Devoino et al., 1997). They also reported that the administration of SRBC to rats produced a marked rise in activity of central dopaminergic system at early stages of the immune response formation: the most pronounced elevation in the concentrations of DA and its metabolites was observed in the terminal regions of the nigrostriatal and mesolimbic dopaminergic systems (nuclei caudatus and accumbens), hypothalamus, hippocampus, amygdala within 20 minutes following antigen inoculation; by 60 minutes after immunization, DA metabolism had been retained at a high level in all brain regions examined; and the concentration of DA returned to control level in the amygdala and in the hypothalamus and had a tendency to reach control values in the rest of the structures 24 hours after antigen administration (Devoino et al., 1997). In addition, Qiu *et al.* (1996) reported that depletion of monoamine neurotransmitters in the brain 24 hours before immunization by i.c.v. injection of reserpine, which blocked the assimilation of the monoamine neurotransmitters by the vesicula of the nerve terminals and finally exhausted these neurotransmitters in the brain, led to a suppression of rat splenocyte anti-SRBC response five days after immunization, and during days 2-7 post-immunization in these rats, NE contents in the hypothalamus and hippocampus were increased and this paralleled to the peak period of antibody response. These results thus indicate a bi-directional signaling between the brain monoamines and the immune system as well.

However, the modulation of central catecholamine system on peripheral cellular immune functions is somewhat unclear. The initial *in vitro* experiments reported that central catecholamine depletion with 6-OHDA in mice resulted in an enhanced splenic suppressor T-cell activity, which, in turn, nonspecifically impaired humoral immune response only when this subpopulation of T cells was activated cocurrently by antigen (Cross et al., 1987). However, central 6-OHDA treatment does not affect the proliferative response of splenocytes to both the T-cell mitogen Con A and the B-cell mitogen LPS and the proliferative capacity of T cells stimulated with alloantigens in the mixed lymphocyte reaction in mice (Cross and Roszman, 1988). Neveu *et al*. (1992) also reported that four weeks after specific dopaminergic depletion in substantia nigra in mice, both Con A- and PHA-induced splenocyte proliferation were not changed. However, when tested six weeks after the depletion, splenocyte proliferative responsiveness to PHA is enhanced (Neveu et al., 1992). In contrast, later results from the same laboratory showed an impairment of splenocyte proliferation to both Con A and PHA while LPS-induced proliferation and NK cell activity remaining unchanged two weeks after specific dopaminergic depletion in the same brain area in mice (Deleplanque et al., 1992, 1994). When the same depletion was conducted in nucleus accumbens, no modification of splenocyte proliferative capacity to Con A, PHA and LPS, but a decrease in NK cell activity was observed in these animals (Deleplanque et al., 1992, 1994).

The association of MPTP-induced striatal DA depletion and immune function was also reported by Renoux *et al*. (1989). In their study, striatal DA level was depressed three days after the last of five daily i.p. injection of MPTP, the splenocyte proliferation in the presence of Con A was reduced when the PWM-induced proliferation was increased at the same time, and T-cell responses to alloantigens in the mixed lymphocyte culture (MLC) system were inhibited. These results were further confirmed by Bieganowska *et al*. (1993) who reported decreases in PHA-, Con A- and LPS-induced splenocyte proliferation seven days after i.p. injection of MPTP in mice which can be restored to control values by pretreatment with i.p. injection of pargyline. In addition, MPTP i.p. treatment in mice has no effects on both phagocytosis of yeast by peritoneal macrophages, and tumor necrosis factor (TNF)-α activity of peritoneal macrophages after LPS stimulation (Bieganowska et al., 1996).

Other experimental evidence also demonstrate that stimulation of D_1 dopaminergic receptors in different regions of the dopaminergic pathways of the brain is followed by either stimulation or inhibition of peripheral NK cell activity (Nistico et al., 1994).

On the other hand, central catecholamines have a consistent role on cellular immune functions when tested *in vivo*. For example, Basu *et al.* (1995) found MPTP-induced striatal dopaminergic depleted mice had an increased growth rate of transplanted malignant tumor and a decreased median survival time after the transplantation, tumor growth alone was also associated with a decrease in striatal DA level on 10^{th} day after tumor transplantation without MPTP injection, and depletion of central DA either experimentally by MPTP injection or during tumor growth was accompanied by significant immune depression like decreased Con A- or PHA-induced splenocyte proliferation, reduced CTL activity, splenic antibody-forming cell population, serum IgM, IgG levels and NK cell activity, suggesting central dopaminergic system influences tumor growth through the immune network of the host. In rats with a hyperactive dopaminergic system, reduced tumor growth, experimental metastasis formation and angiogenesis have also been reported (Teunis et al., 2002). A recent experiment by Alaniz and colleagues clearly illustrated the differences between *in vitro* and *in vivo* experiments: knock-out mice lacking dopamine beta-hydroxylase (DBH) ($dbh^{-/-}$ mice) (i.e. they cannot produce NE or EPI, but produce dopamine instead) had normal immunological status when housed in specific pathogen-free (SPF) conditions, and in the absence of an infecious challenge: the total number of leukocytes and the percentages of granulocytes, monocytes, and lymphocytes in the blood, and the numbers and subpopulations of cells in the thymus and spleen, were similar to age- and sex-matched controls; splenocyte and thymocyte proliferation in response to T-cell (Con A and anti-CD3) and B-cell (LPS) mitogens, splenocyte production of T-helper-1 (Th1) (IFN-γ) and T-helper-2 (Th2) (IL-4) cytokines, and the delayed-type hypersensitivity (DTH) response to TNP-KLH were similar in $dbh^{-/-}$ and control mice. All these indicate that $dbh^{-/-}$ mice do not have intrinstic developmental or functional immune defects (Alaniz et al., 1999). However, when $dbh^{-/-}$ mice were infected with the intracellular pathogens, Listeria monocytogenes (LM) or Mycobacterium tuberculosis (MT), or were immunized with a T-cell-dependent antigen, TNP-KLH, they were more susceptible to infection, had impaired T-cell function, and had impaired Th1-cell-dependent-IgG2a antibody production, indicating that physiological catecholamine production is not required for normal development of immune system, but plays an

important role in the modulation of T-cell-mediated immunity to infection and immunization (Alaniz et al., 1999). In addition, Filipov *et al*. (2002) reported that selective unilateral DA depletion by intratriatal injection of 6-OHDA in mice impaired subsequent *in vivo* immune responses to LM as well as to KLH, partial sparing of striatal DA levels by a co-administration of GBR-12935 eliminated the detrimental effect of 6-OHDA treatment on host resistance to LM, but not the impairment of antibody and DTH response to KLH. Mice administered with the combination of 6-OHDA and GBR12935 have approximately 35% more DA than the 6-OHDA along group, but about 60% less DA in the lesioned striatum than control animals, suggesting that antibody and DTH responses are more sensitive to the striatal DA depletion (Filipov et al., 2002).

The results concerning the association between central catecholamine depletion and peripheral lymphocyte distribution remain controversial. When MPTP-induced striatal DA depletion decreases T-cell percentage and increases B-cell percentage in the spleen (Renoux et al., 1989), MPTP i.p. treatment can not affect the T-cell subpopulations in the spleen but reduces the percentage of L3T4 T cells in peripheral blood in mice (Bieganowska et al., 1996). When 6-OHDA injected into substantia nigra bilaterally in rats fails to affect the percentage of activated $CD4^+CD25^+$ T cells in mesenteric lymph nodes, bilateral injection of MPP^+ into striatum causes an increase in it (Bas et al., 2001).

By the way, both Neveu *et al*. (1992) and Deleplanque *et al*. (1992, 1994) reported that central dopaminergic pathway was asymmetrically involved in modulation of immune response. They oberserved that after 6-OHDA-induced dopaminergic lesion of the substantia nigra in mice, the proliferative capacity of splenocyte was depressed only in right-lesioned group. Similarly, after lesioning of the nucleus accumbens, NK cell activity was depressed only in left-lesioned mice. Filipov *et al*. (2002) also found left-side DA depletion reduced the *in vivo* immune responses both to LM and to KLH in mice.

The time at which immune assays are performed after central catecholamine depletion also appears to be crucial to the results observed. Although no effects were reported on cellular immune variables two days after 6-OHDA injection into the cisterna magna (Cross and Roszman, 1988), mitogen-induced T-cell proliferation was proved to be suppressed two weeks after lesioning central dopaminergic structures in mice (Deleplanque et al., 1992). However, when immunological variables were analysed six

weeks after central dopaminergic lesioning, a significant enhancement in lymphocyte proliferation was observed (Neveu et al., 1992).

1.4 Aim of the study

It is well known that the central nervous system affects the immune response via various pathways. This is primarily achieved by several neurotransmitters, neuropeptides, hormones and cytokines. Among these neural mediators, central catecholamines play an important role, which has been proved by numerous clinical and experimental investigations. Patients with PD or schizophrenia, both of which are neurological disorders involving central catecholamine disfunction, are found to have significant abnormalitis in humoral and cellular immune responses. Numerous scientific investigations have also demonstrated that central catecholaminergic pathways play a pivotal role in modulating peripheral humoral and cellular immune functions both *in vitro* and *in vivo*. However, the modulation of central catecholamine depletion on peripheral cellular immune response remains somewhat unclear. To address this uncertainty, we designed a set of experiments to analyse the influence of central catecholamine depletion with the neurotoxin 6-OHDA on peripheral cellular immune functions.

2. Material and Methods

2.1 Animals

Naïve male Dark Agouti rats (Harlan Laboratories, Borchen, Germany), weighing between 220 and 250 g were used. All rats were allowed to habituate to the animal laboratory conditions for three weeks before starting the experiments. Animals were individually housed in standard plastic laboratory cages (40cm × 26cm × 15cm high) with a wire mesh lid. Cages were kept in an air-conditioned, sound-proof holding room at an ambient temperature of 24.0±0.5°C. The animals had access to standard lab chow and tap water *ad libitum*. A 12 hour light/dark cycle was maintained throught the experiment with lights off at 0700. All animal procedures were carried out as approved by the ethic committee of the Medical Faculty, University of Duisburg-Essen, Germany.

2.2 Experimental design

To centrally deplete catecholamines, experimental animals received bilateral injections of 6-OHDA into lateral ventricles. Control animals were injected only with the vehicle. Two, four, and seven days later, experimental and control animals were sacrificed by decapitation. Immediately afterwards, spleen and blood samples were obtained to measure the proliferative capacity of lymphocytes and cytokine production. Brain and spleen samples were collected and stored (-80°C) for later catecholamine determination.

2.3 Central catecholamine depletion

Central catecholamines were depleted by stereotaxically injecting 100 µg of freshly prepared 6-OHDA (Sigma, Taufkirchen, Germany) dissolved in a volume of 10 µl of ascorbate solution (2 mg/ml of ascorbic acid in sterile saline solution) as a vehicle into the lateral ventricles of the animals (5µl/ventricle). The stereotaxic coordinates used were ± 1.4 mm lateral, - 3.4 mm ventral, and -0.8 mm anterior with respect to bregma, according to a stereotaxic atlas (Paxinos and Watson, 1986).

2.4 Catecholamine determination

Levels of norepinephrine, dopamine, epinephrine and serotonin in the spleen and brain tissues (hypothalamus and cortex) were measured by high-performance liquid chromatography (HPLC) as described previously (van der Hoorn et al., 1989). Briefly, each sample was homogenized in 0.1-M perchloric acid containing 0.1-mM EDTA. Following centrifugation, 25 μl of the supernatant was used for catecholamine extraction by established methods (Smedes et al., 1982). Catecholamine concentrations were anlyzed by HPLC (Gynkotek, Germany) and expresessed as ng/mg wet tissue. Intra-assay variability was < 10%.

2.5 Splenocytes preparation

After sacrificing the rats, spleens were removed aseptically, then splenocytes were released from tissue by injecting cell culture medium (RPMI 1640 (PAA, Linz, Austria) + 7% fetal calf serum (FCS) (Sigma) inactivated + 1% penicillin (Sigma)) into spleens with a syringe. After washed with phosphate buffered saline (PBS) (ICN, USA), the erythocytes were removed by shaking with haemolysis buffer containing NH_4Cl and Tris (ICN, USA) for 10 minutes. Then the cells were washed with PBS twice again, resuspended with 5 ml of cell culture medium and counted under a microscope (Olympus, Japan).

2.6 Con A-induced splenocyte proliferation

Splenocytes were adjusted to 1.6×10^6 cells/ml in cell culture medium (RPMI 1640 + 7% FCS inactivated + 1% penicillin) and 100μl of cell suspension were added in triplicate to 96-well flat-bottom cell culture plates (NUNC, Denmark) cultured for 72 h in the presence of Con A (final concentrations: 0, 1.25 and 2.5 μg/ml) at 37°C in a 5% CO_2 incubator (Heraeus, Germany). Pre-test showed the optimal concentration of Con A was 2.5 μg/ml. After 48 h of mitogen stimulation the cells were pulsed with 20 μl/well [^{3}H]thymidine (0.5 μCi) and harvested 24 h later. Radioactivity was measured using a β-counter (WALLAC Oy, Finland).

2.7 Peripheral lymphocyte preparation

After decapitation, blood was put into tubes containing EDTA. Peripheral mononuclear cells were isolated and collected according to a standard Ficoll protocol. After washed with PBS (ICN, USA), the mononuclear cells were resuspended with 5 ml of cell culture medium (RPMI 1640 + 7% FCS inactivated + 1% penicillin) and transferred to a sterile petri-dish with 10 ml of cell culture medium inside for seperating monocytes from lymphocytes. After incubated at 37°C in a 5% CO_2 incubator (Heraeus, Germany) for 30 minutes, supernatants which containing lymphocytes were collected and washed with PBS (ICN, USA) again. Then the cells were resuspended with 1 ml of cell culture medium, counted under a microscope (Olympus, Japan).

2.8 Con A-induced peripheral lymphocyte proliferation

Peripheral lymphocytes were adjusted to 1.6 $\times 10^6$ cells/ml in cell culture medium (RPMI 1640 + 7% FCS inactivated + 1% penicillin) and 100μl of cell suspension were added in triplicate to 96-well flat-bottom cell culture plates (NUNC, Denmark) cultured for 72 h in the presence of Con A (final concentrations: 0, 1.25 and 2.5 μg/ml) at 37°C in a 5% CO_2 incubator (Heraeus, Germany). Pre-test showed the optimal concentration of Con A was 2.5 μg/ml. After 48 h of mitogen stimulation the cells were pulsed with 20 μl/well [^{3}H]thymidine (0.5 μCi) and harvested 24 hours later. Radioactivity was measured using a β-counter (WALLAC Oy, Finland).

2.9 ELISA detection for IL-2 and IFN-γ in splenocyte proliferation supernatant

2.9.1 ELISA detection for IL-2 in splenocyte proliferation supernatant

Splenocytes were adjusted to 1.6×10^6 cells/ml in cell culture medium (RPMI 1640 + 7% FCS inactivated + 1% penicillin), then cultured for 48 h in the presence of Con A (1.25 μg/ml) at 37°C in a 5% CO_2 incubator. Subsequently, plates were centrifuged and 50 μl of supernatant was removed for IL-2. IL-2 was assayed using commercial enzyme-linked immunosorbent assay (ELISA) kits for the detection of rat IL-2 (Biosource International, CA, USA).

Briefly, 12.5 μg of coating antibody were diluted in 10ml coating buffer A to a final concentration 1.25 μg/ml. Then 100 μl of diluted coating antibody was placed in 96 wells

U-bottomed flexible microtiter plates (Biosource International, CA, USA). The plates were covered and incubated overnight at 4°C. Afterward the coating antibody was aspirated from the wells and tapped on absorbent paper to remove excess liquid, 300 μl of blocking solution were then added to each well, covered and incubated at room temperature for two hours. Once again the blocking solution was aspirated from the wells and tapped on absorbent paper, and then the microplates were washed three times with wash solution.

Twenty-one ng of IL-2 standard were diluted in 2.1 ml standard diluent. Samples were diluted to 1:30, and then 100 μl of either standard or samples were added to the appropriate wells in duplicate. Plates were then incubated at room temperature for 90 minutes, after which the solution was aspirated from the wells and the plates were washed. Then 0.5 μg of detection antibody was added to each well, incubating the mixture at room temperature for 60 minutes.

Three μg of Streptavidin HRP conjugate were diluted in 10 ml standard diluent, and 100 μl were added to each well, incubating at room temperature for 45 minutes. Finally 100 μl of tetramethyl benzidine (TMB) were added to each well and incubated in the dark at room temperature for 30 minutes. The reaction was stopped by addition of 100 μl of stop solution.

Microplates were read at 450 nm (Mikrotek, Germany) within 30 minutes of adding the stop solution, and the average optical density was calculated at 450 nm for all standards, controls and samples. The concentration of each unknown sample was detected from the standard curve. The backgroud was subtracted from all groups.

2.9.2 ELISA detection for IFN-γ in splenocyte proliferation supernatant

Splenocytes were adjusted to 1.6×10^6 cells/ml in cell culture medium (RPMI 1640 + 7% FCS inactivated + 1% penicillin), then cultured for 48 h in the presence of Con A (1.25 μg/ml) at 37°C in a 5% CO_2 incubator. Subsequently, plates were centrifuged and 50 μl of supernatant was removed for IFN-γ. IFN-γ was assayed using commercial ELISA kits for the detection of rat IFN-γ (U-CyTech, Utrecht, The Netherlands).

Briefly, the coating antibody was diluted to 1:200 in PBS. Then 100 µl of diluted coating antibody was placed in 96 wells U-bottomed flexible microtiter plates (U-CyTech, Utrecht, The Netherlands). The plates were covered and incubated overnight at 4°C. Afterward the coating antibody was aspirated from the wells and tapped on absorbent paper to remove excess liquid, 300 µl of blocking solution were then added to each well, covered and incubated at 37ºC for one hour. Once again the blocking solution was aspirated from the wells and tapped on absorbent paper, and then the microplates were washed six times with wash solution.

Seven point five ng of IFN-γ standard were diluted in 500 µl distilled water. Samples were diluted to 1:30, and then 100 µl of either standard or samples were added to the appropriate wells in duplicate. Plates were then incubated at 37ºC for two hours, after which the solution was aspirated from the wells and the plates were washed. The detection antibody was diluted to 1:100 in PBS containing 0.5% bovine serum albumin (BSA) and 0.05% Tween-20. Then 100 µl of diluted detection antibody was added to each well, incubating the mixture at 37ºC for one hour.

The Streptavidin-HRP polymer was diluted to 1:100 in PBS containing 0.5% BSA and 0.05% Tween-20, and 100 µl were added to each well, incubating at 37ºC for one hour. Finally 100 µl of TMB were added to each well and incubated in the dark at room temperature for 15 minutes. The reaction was stopped by addition of 50 µl of stop solution.

Microplates were read at 450 nm (Mikrotek, Germany) within 30 minutes of adding the stop solution, and the average optical density was calculated at 450 nm for all standards, controls and samples. The concentration of each unknown sample was detected from the standard curve. The backgroud was subtracted from all groups.

2.10 RT-PCR analysis of IL-2 mRNA expression

Splenocytes were adjusted to 5×10^6 cells/ml in culture medium (RPMI 1640 + 7% FCS inactivated + 1% penicillin), one ml of the cell suspension were added in six-well tissue culture plates (Falcon) in the presence of Con A (1.25 µg/ml) and incubated for six hours, then harvested for reverse transcriptase-polymerase chain reaction (RT-PCR).

2.10.1 Total RNA isolation

Reagents: The denaturing solution was 4-M Guanidinium thiocyanate (GTC) (Sigma, USA), 25mM sodium citrate (Sigma, USA), pH 7, 0.5% sarcosyl (Sigma, USA), 0.1 M 2-mercaptoethanol (Sigma, USA). The stock solution was made as follows: 236.25 g guanidinium thiocyanate was dissolved with 500 ml DEPC, 4.18 ml 3-M NAOAc (Sigma, USA) and 3.75 ml β-mercaptoethanol (Sigma, USA).

5×10^6 splenocytes were denatured with 700 μl of 4-M GTC (Sigma, USA) after cultured for six hours in the presence of Con A (1.25 μg/ml). Sequentially, 50 μl of 2-M sodium acetate, 560 μl of Phenol (Sigma, USA), and 280 μl of phenol-chloroform-isoamyl alcohol mixture (Sigma, USA) were added to the cells, with thorough mixing by inversion after the addition of each reagent. The final suspension was cooled on ice for 20 minutes. Samples were centrifuged at 13,000 g for 20 minutes at 4°C. After centrifugation, RNA was present in the aqueous phase where DNA and proteins were present in the interphase and phenol phase. The aqueous phase was transferred to a fresh tube (Eppendorf, Germany), mixed with 500 μl of isopropanol (JT Baker, Netherland), and then placed at −20°C for at least two hours to precipitate RNA. Sedimentation at 13,000 g for 20 minutes was again performed and the resulting RNA pellet was dried for one hour, and then was dissolved in 300 μl 4-M GTC, and precipitated with 300 μl isopropanol at −20°C for at least two hours. After centrifugation at 13,000 g at 4°C for 20 minutes, the RNA pellet was resuspended in 75% ethanol, sedimented, air dried for 30 minutes, and dissolved in 10 μl DEPC at 59°C for 10 minutes. One μl of the RNA solution was removed and diluted with 99 μl DEPC. The RNA concentration was measured by SmartSpec™ 3000 (BIO-RAD, USA).

2.10.2 Reverse transcription

RNA was amplified by RT with an Oligo (dT-15) primer (Gibco/BRL, USA). One μg of total RNA sample and 2.5 μl of oligo-dT 15 were added to a sterile Rnase-free microcentrifuge tube (Eppendorf, Germany), filling the total volume with DEPC up to 12 μl. The tubes were heated to 68°C for 15 minutes to melt secondary structure within the

template, and then cooled immediately on ice to prevent secondary structure from reforming. Tubes were then spun briefly to collect the solution at the bottom of the tubes. The following components were then added to the tubes: 5 µl of M-MLV 5 × Reaction buffer (Promega, USA); 1.25 µl of 10 mM dATP, 1.25 µl of 10 mM dCTP, 1.25 µl of 10 mM dGTP, 1.25 µl of 10 mM dTTP (Promega, USA); 200 units of M-MLV Reverse transcriptase (Promega, USA). Finally, the nuclease-free water was added to make the final volume of 25 µl. The solution was mixed gently by flicking the tube and incubated for 90 minutes at 45°C, and then for 30 minutes at 52°C. The reation was halted by heating to 96°C for 15 minutes and this was followed by cooling on ice.

2.10.3 Amplification of specific complementary DNA (cDNA)

Specific cDNA products corresponding to mRNA for IL-2 and GAPDH were amplified using PCR. The following components were added to a microcentrifuge tube sitting on ice: five µl of 10 × PCR buffer (Gibco/BRL, USA), final concentration was 1 ×; four µl of 10 mM dNTP mixture (Promega, USA), final concentration was 0.2 mM each; 1.5 µl of 50 mM $MgCl_2$ (Gibco/BRL, USA), final concentration was 1.5 mM, one µl of 5' primer, one µl of 3' primer (MWG-Biotech, Germany), final concentration was 0.5 µM each; one µl of cDNA from RT reaction; 0.2 µl of Taq DNA polymerase (5 U/µl) (Gibco/BRL, USA). Autoclaved distilled water was added to bring the final volume to 50 µl. The concents of tube were mixed, and centrifuged briefly to collect the contents to the bottom.

Tubes were incubated in a Mastercycler 5330 system at 95°C for three minutes to completely denature the template. The program was set up as follows: (1). Denatured for 30 seconds at 94°C; (2). Annealed primers for 30 seconds at 61°C for GAPDH, 54°C for IL-2; (3). Extended the primers for 30 seconds at 72°C. The PCR technique employed 25 cycles of amplification for GAPDH and 30 cycles for IL-2. Incubation for an additional 10 minutes was carried out at 72°C and the reaction was maintained at 4°C. The amplified PCR products were identified by electrophoresis of 20 µl sample aliquots on 1.5% agarose gel (Gibco/BRL, USA), stained with 0.5 µg/ml of ethidium bromide (Roth, Germany). The products were visualised by UV transillumination and the gel was photographed. Specific products were identified by size in relation to a known 100 bp oligonucleotide DNA ladder (Gibco/BRL, USA) run with each gel. Imaging of the PCR products was performed using the fluorescence imaging analyzer (ITF, Germany). The image of

ethidium bromide stained DNA fragments were quantified using Aida 2 beta software. Relative fluorescent units (ruf) were used as an index of mRNA level. GAPDH served as an internal control. The data were expressed as amount of IL-2 produce over the amout of GAPDH produce (IL-2/GAPDH). Amplifying cDNA with PCR for GAPDH and IL-2, the following primers were used for gene amplification:

GAPDH (sense)

5' –ACC ACA GTC CAT GCC ATC AC-3'

GAPDH (antisense)

5' –TCC ACC ACC CTG TTG CTG TA-3'

IL-2 (sense)

5' –ATG TAC AGC ATG CAG CTC GCA TC-3'

IL-2 (antisense)

5' –TTA CTG AGT CAT TGT TGA GAT GAT GCT TTG-3'

2.11 Statistical analysis

One way analysis of variance (ANOVA) and Fisher post hoc test were used to examine statistical differences between the groups. Data are expressed as means ± SEM. $p < 0.05$ was considered statistically significant.

3. Results

3.1 Effects of i.c.v. 6-OHDA treatment on central and peripheral catecholamine concentrations

Six-OHDA, a neurotoxin which is highly selective for catecholamine nerve fibers, has been widely reported to deplete central catecholamines when administered centrally. In order to identify the severe, selective and systematic destruction of central catecholaminergic neurons by i.c.v. 6-OHDA treatment, two, four, and seven days after i.c.v. injection with 6-OHDA or vehicle, hypothalamus, cortex and spleen tissue samples were collected for catecholamine analysis by HPLC. These analyses demonstrated a pronounced reduction of NE ($p< 0.01$ on the second, fourth and seventh day after the treatment, respectively; Fig. 1a), EPI ($p< 0.05$ on the fourth and seventh day after the treatment, respectively; Fig. 1b) and DA ($p< 0.05$ on the seventh day after the treatment; Fig. 1c) levels in the hypothalamus of animals treated with i.c.v. 6-OHDA compared with controls. Similar results were found in the cortex, while the EPI concentrations were under the detectable limit, NE ($p< 0.01$ on the seventh day after the treatment; Fig. 2a) and DA ($p< 0.01$ on the second, fourth and seventh day after the treatment, respectively; Fig. 2b) levels significantly decreased in 6-OHDA injected animals, compared to the controls. One of the advantages of 6-OHDA is that it does not cross the BBB when administered to adults, so it has been used experimentally to deplete catecholamines either peripherally or centrally depending upon the route of administration. However, in the present study, we observed a significant but transient reduction in splenic concentrations of NE ($p< 0.05$; Fig. 3a) and EPI ($p< 0.05$; Fig. 3b) in 6-OHDA treated animals, compared to the controls on the fourth day after the injection. Although the DA level in the spleen of animals treated with i.c.v. 6-OHDA also decreased a lot compared with controls on the fourth day after the treatment, the statistical analysis revealed this decrease was not significant (Fig. 3c). Furthermore, in order to assess 6-OHDA's specificity for catecholamine depletion, serotonin levels were also assessed in hypothalamus, cortex and spleen by HPLC on the seventh day after the injection. These analyses revealed that there was no effect on serotonin levels in all these tissues by i.c.v. 6-OHDA treatment (Fig. 4).

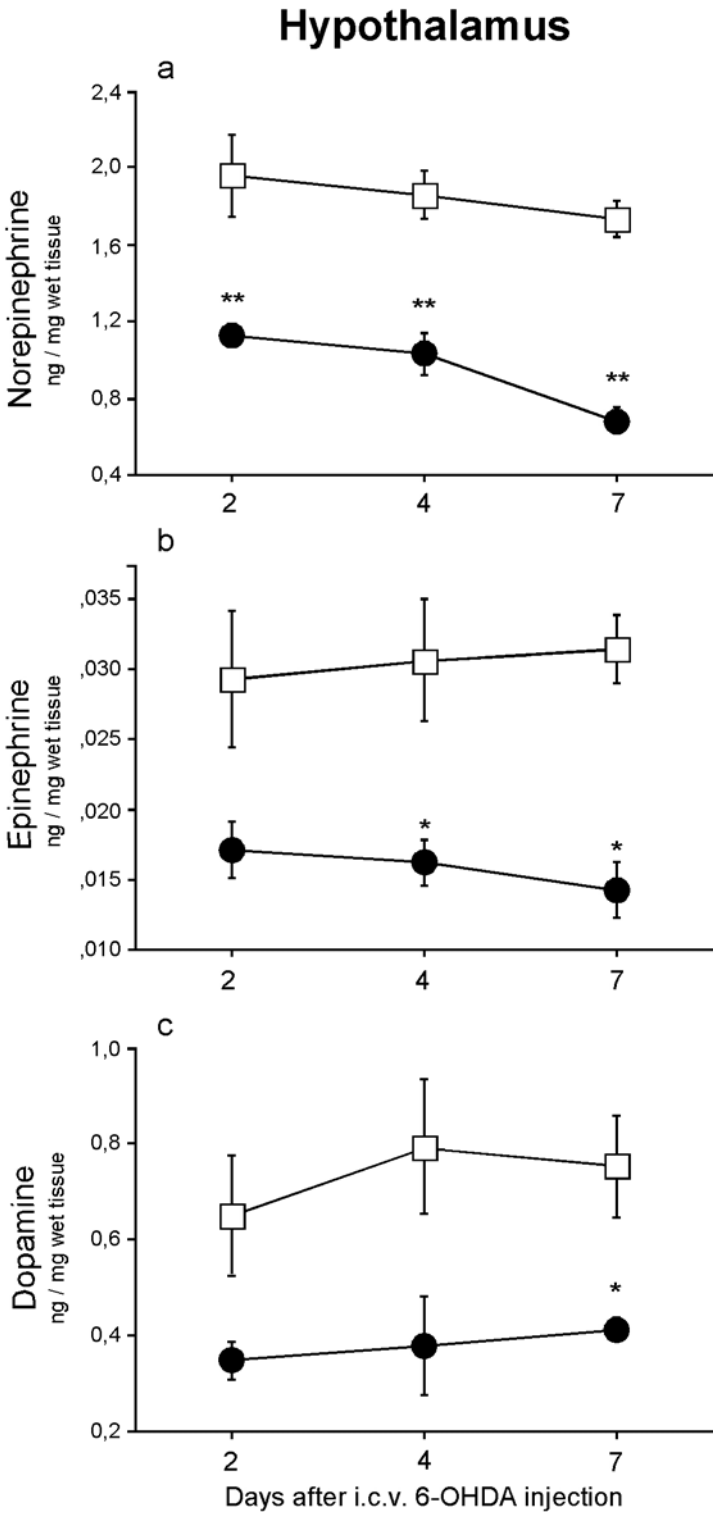

Figure 1. The effects of i.c.v. 6-OHDA treatment on catecholamine levels in hypothalamus. Dark Agouti rats received i.c.v. 6-OHDA (n=8, ●) or vehicle (n=8, □) injection and were killed 2, 4 and 7 days after injection and brain tissues were collected for measurement of norepinephrine, epinephrine and dopamine concentrations by HPLC. Results are expressed as mean ± SEM. *p<0.05**,** **p<0.01 compared to vehicle injection.

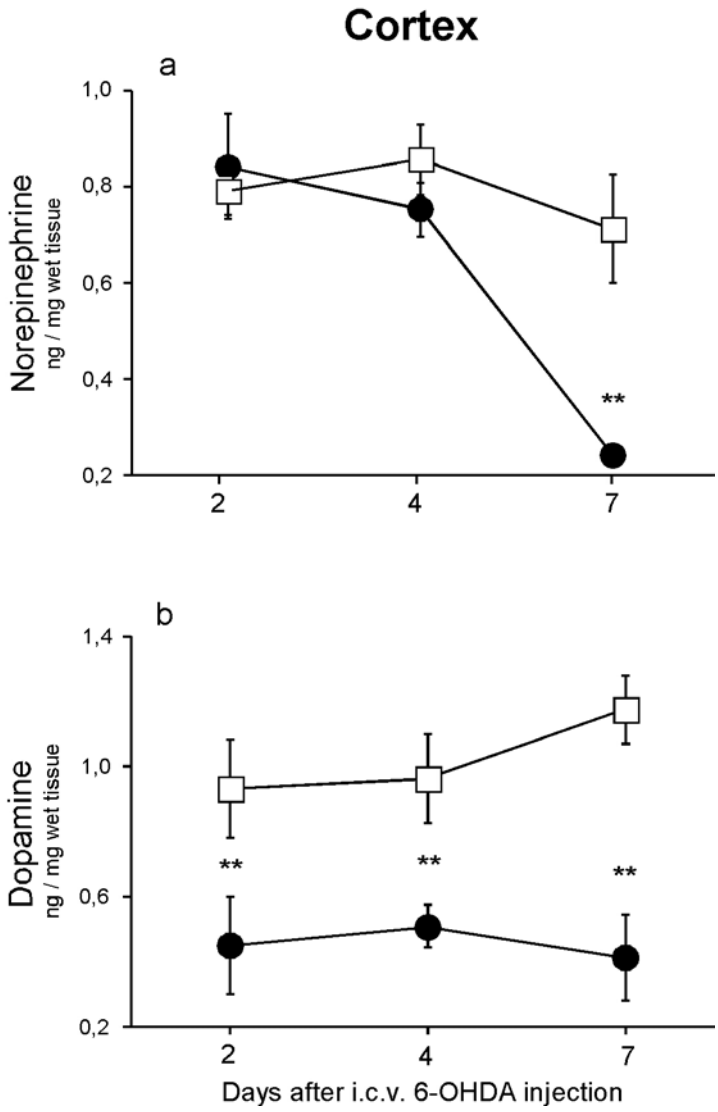

Figure 2. The effects of i.c.v. 6-OHDA treatment on catecholamine levels in cortex. Dark Agouti rats received i.c.v. 6-OHDA (n=8, ●) or vehicle (n=8, □) injection and were killed 2, 4 and 7 days after injection and brain tissues were collected for measurement of norepinephrine, epinephrine and dopamine concentrations by HPLC (epinephrine concentration was under the detectable limit). Results are expressed as mean ± SEM. ** $p<0.01$ compared to vehicle injection.

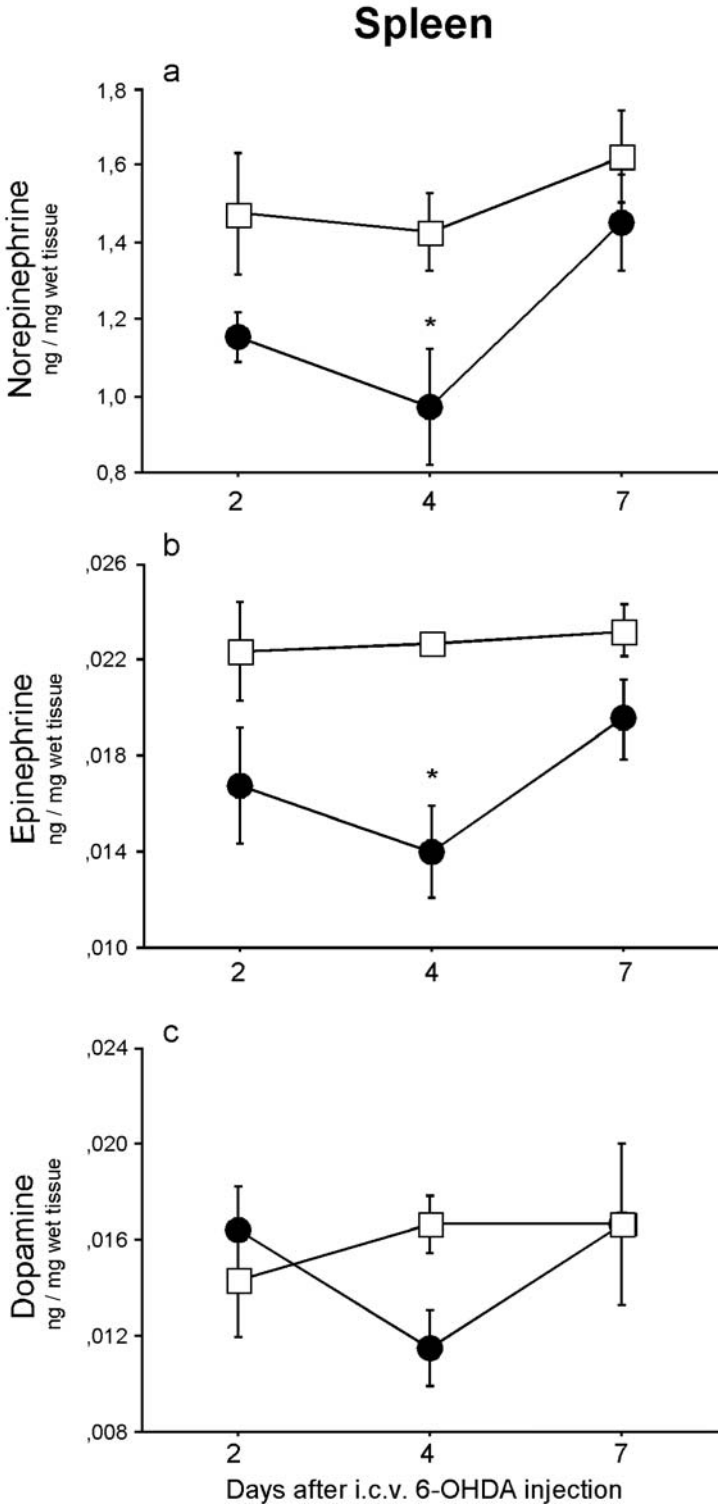

Figure 3. The effects of i.c.v. 6-OHDA treatment on catecholamine levels in spleen. Dark Agouti rats received i.c.v. 6-OHDA (n=8, ●) or vehicle (n=8, □) injection and were killed 2, 4 and 7 days after injection and spleen tissues were collected for measurement of norepinephrine, epinephrine and dopamine concentrations by HPLC. Results are expressed as mean ± SEM. *p<0.05 compared to vehicle injection.

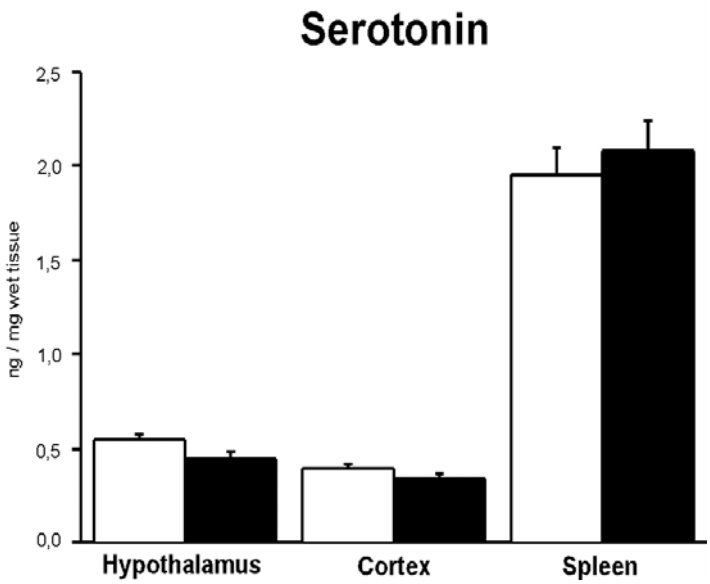

Figure 4. The effects of i.c.v. 6-OHDA treatment on serotonin levels in hypothalamus, cortex, and spleen. Dark Agouti rats received i.c.v. 6-OHDA (n=8, ■) or vehicle (n=8, □) injection and were killed 7 days after injection. Brain and spleen tissues were collected for measurement of serotonin concentration by HPLC. Results are expressed as mean ± SEM.

3.2 Effects of central catecholamine depletion on lymphocyte proliferation

In order to identify the influence of central catecholamine depletion by i.c.v. 6-OHDA injection on peripheral cellular immune response, four and seven days after the 6-OHDA treatment, the proliferative capacity of lymphocytes in the spleen and in peripheral blood was analysed. As shown in Figure 5, the proliferative response of splenocytes in the presence of Con A (final concentrations: 1.25 and 2.5 μg/ml), a T-cell mitogen, was not affected four days after the injection compared to the control group. However, a pronounced decrease in splenocyte proliferation in the presence of Con A (final concentrations: 1.25 and 2.5 μg/ml) seven days after the treatment compared with the controls was observed ($p< 0.05$ at both concentrations of Con A, respectively; Fig. 6). In the next step, we investigated whether this effect was limited to the spleen. Therefore, the proliferative capacity of peripheral blood lymphocytes at the same time point was tested. In parallel to the reduced splenocyte responsiveness, seven days after the treatment, we observed a significant reduction in the proliferative response of peripheral blood lymphocytes in the presence of Con A (final concentrations: 1.25 and 2.5 μg/ml) in animals with i.c.v. 6-OHDA injection as compared to the controls ($p< 0.05$ at both concentrations of Con A, respectively; Fig. 7).

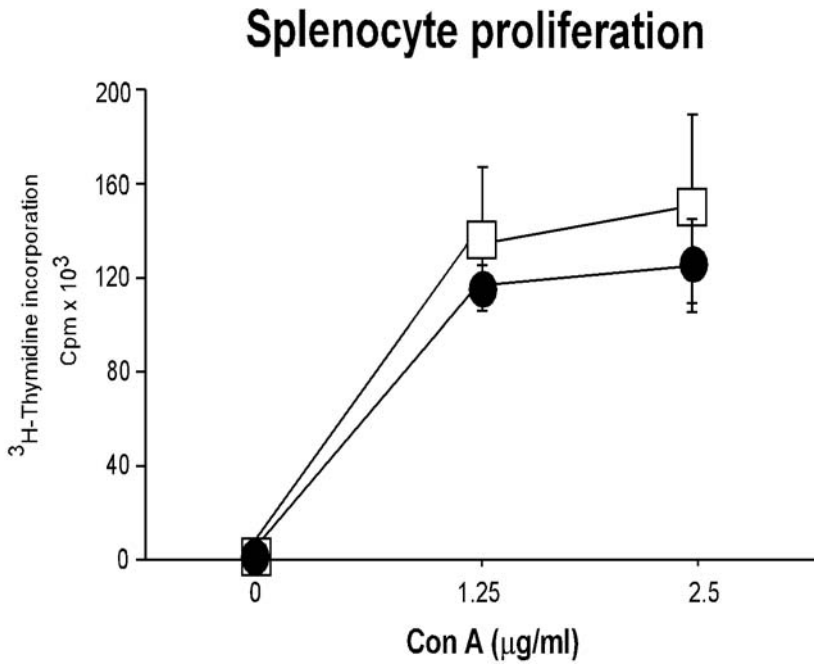

Figure 5. Con A (final concentrations: 0, 1.25 and 2.5μg/ml)-induced proliferation of splenocytes 4 days after i.c.v. treatment with 6-OHDA (n=8, ●) or vehicle (n=8, □). Results are expressed as mean ± SEM.

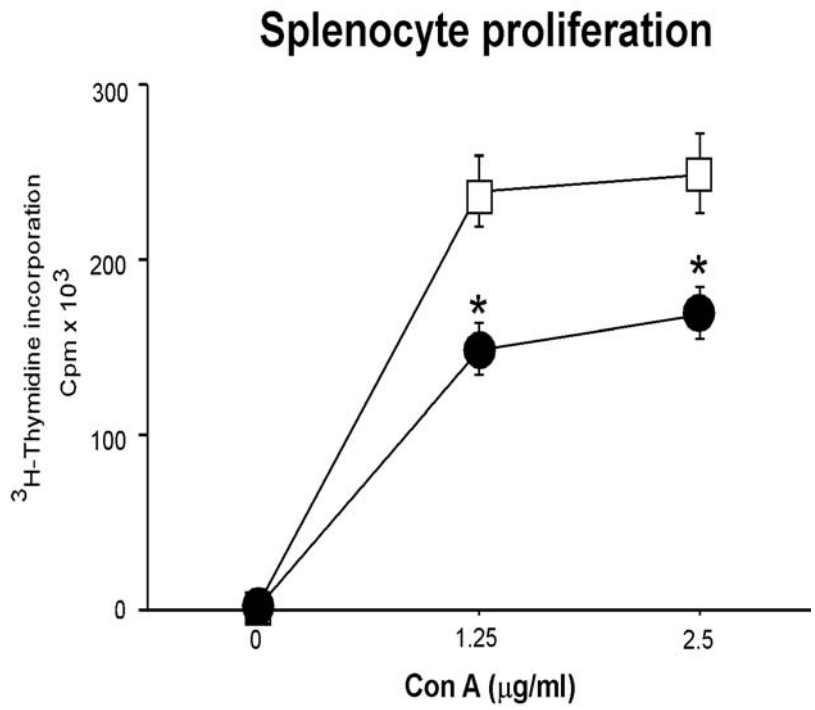

Figure 6. Con A (final concentrations: 0, 1.25 and 2.5μg/ml)-induced proliferation of splenocytes 7 days after i.c.v. treatment with 6-OHDA (n=8, ●) or vehicle (n=8, □). Results are expressed as mean ± SEM. *p<0.05 compared to vehicle injection.

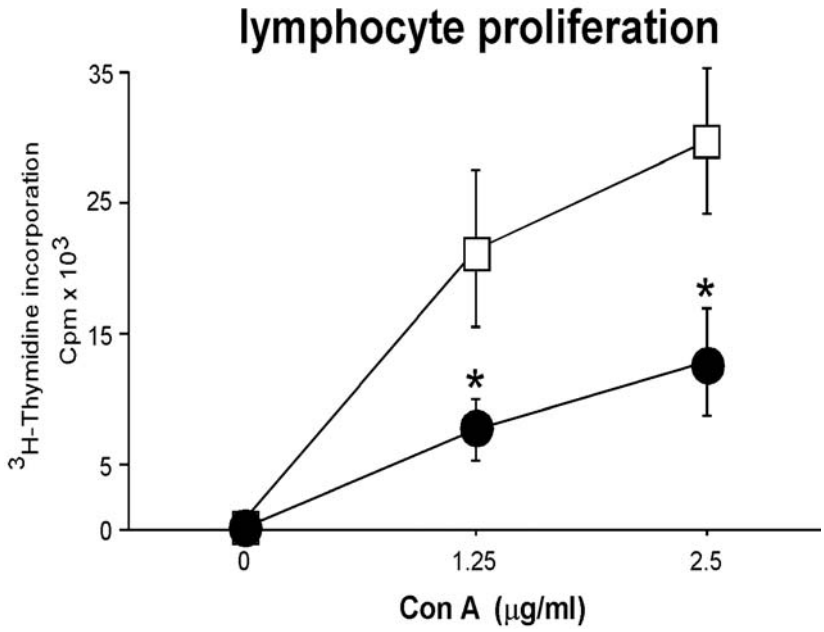

Figure 7. Con A (final concentrations: 0, 1.25 and 2.5μg/ml)-induced proliferation of peripheral blood lymphocytes 7 days after i.c.v. treatment with 6-OHDA (n=8, ●) or vehicle (n=8, □). Results are expressed as mean ± SEM. **p*<0.05 compared to vehicle injection.

3.3 Effects of central catecholamine depletion on splenocyte cytokine production

Since IL-2, which secreted by Th1 cells, have been proved to be of crucial importance for lymphocyte proliferation, we measured IL-2 concentration in the supernatant of Con A (final concentration 1.25 μg/ml) stimulated splenocytes seven days after central catecholamine depletion by i.c.v. 6-OHDA treatment to elucidate the possible cellular mechanisms of the reduced lymphocyte proliferation. As shown in Figure 8, experimental 6-OHDA treated animals had a significantly reduced splenic IL-2 production compared to the vehicle control group ($p< 0.05$).

IFN-γ, secreted by Th1 cells as well, have also been reported to play a role in determining the differentiation pathway of precursor T_H cells into effecotr Th1/Th2 cells and the antibody isotype produced by B cells. In order to further confirm the suppression of cytokine production from lymphocytes, especially from Th1 cells by central catecholamine depletion, IFN-γ was also analysed from the supernatants of the Con A (final concentration 1.25 μg/ml) stimulated splenocytes at the same time point. The experimental animals also showed a significant reduction in IFN-γ production compared to the controls ($p< 0.05$; Fig. 9).

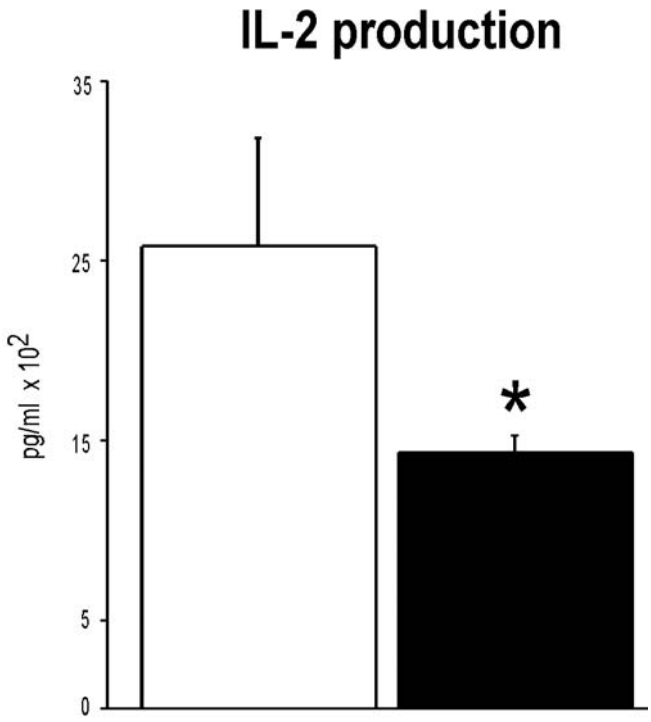

Figure 8. The effects of i.c.v. 6-OHDA treatment on splenocyte IL-2 production after Con A stimulation. 7 days after the i.c.v. injection of 6-OHDA (n=8, ■) or vehicle (n=8, □), animals were killed and splenocytes were collected and incubated for 48h in the presence of Con A (final concentration 1.25 μg/ml), and the supernatant was removed for IL-2 analysis using ELISA kits. Results are expressed as mean ± SEM. *$p<0.05$ compared to vehicle injection.

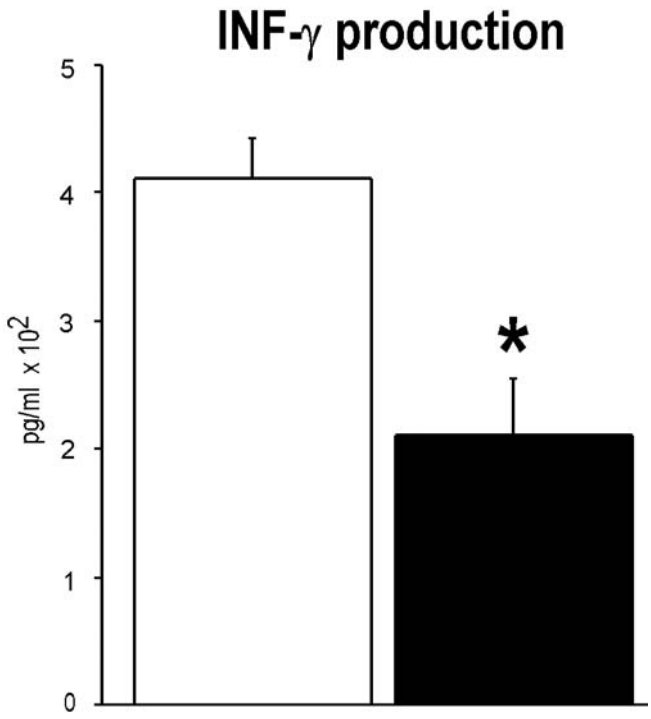

Figure 9. The effects of i.c.v. 6-OHDA treatment on splenocyte IFN-γ production after Con A stimulation. 7 days after the i.c.v. injection of 6-OHDA (n=8, ■) or vehicle (n=8, □), animals were killed and splenocytes were collected and incubated for 48h in the presence of Con A (final concentration 1.25 μg/ml), and the supernatant was removed for IFN-γ analysis using ELISA kits. Results are expressed as mean ± SEM. **p*<0.05 compared to vehicle injection.

3.4 Effects of central catecholamine depletion on splenocyte IL-2 mRNA expression

Having demonstrated that central catecholamine depletion by i.c.v. 6-OHDA treatment can decrease the proliferative capacity of lymphocytes as well as the splenocyte production of both IL-2 and IFN-γ in the presence of Con A, We were interested in determining at what level the reduction of splenocyte IL-2 production by the central 6-OHDA treatment occurred. In order to answer this question, the IL-2 mRNA expression in Con A (final concentration 1.25 μg/ml) stimulated splenocytes were analysed seven days after the treatment. Consistent with the observed effects of i.c.v. 6-OHDA injection on lymphocyte proliferation and splenocyte cytokine (IL-2 and IFN-γ) production, experimental 6-OHDA treated animals showed a pronounced suppression of IL-2 mRNA expression in the Con A (final concentration 1.25 μg/ml) stimulated splenocytes as compared to the vehicle injected controls ($p<0.05$; Fig. 10a).

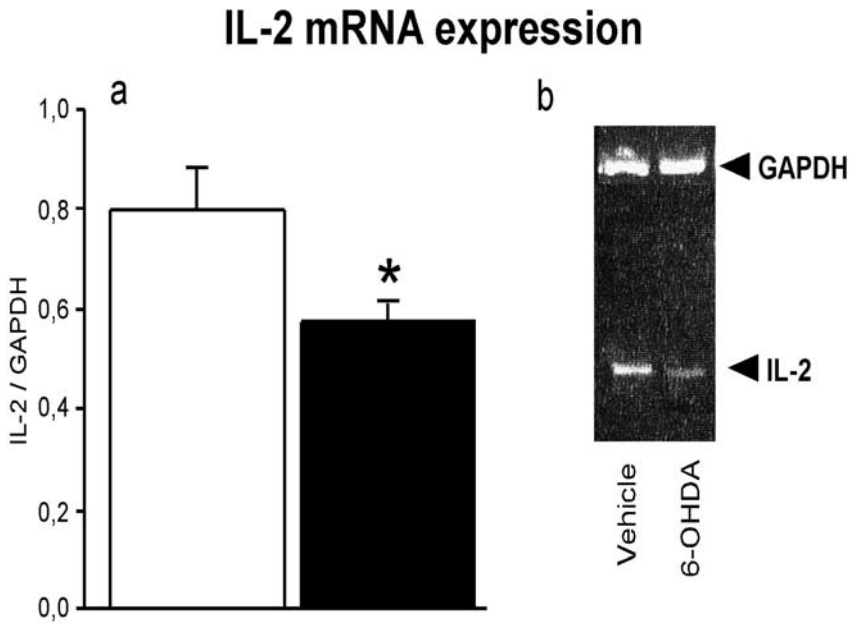

Figure 10. The effects of i.c.v. 6-OHDA treatment on splenocyte IL-2 mRNA expression (a and b) after Con A stimulation. 7 days after the i.c.v. injection of 6-OHDA (n=8, ■) or vehicle (n=8, □), animals were killed and splenocytes were collected and incubated for 6h in the presence of Con A (final concentration 1.25 μg/ml), and then the cells were collected for IL-2 mRNA expression detection using RT-PCR. Results are expressed as mean ± SEM. *$p<0.05$ compared to vehicle injection.

4. Discussion

The present investigation demonstrates that a selective and systematic depletion of central catecholamines by i.c.v. 6-OHDA injection influences peripheral cellular immune functions by decreasing lymphocyte proliferation in spleen and peripheral blood as well as splenocyte cytokine production at both protein (IL-2 and IFN-γ) and mRNA (IL-2) levels, thereby further contributing to our understanding of the role that central catecholamines play in regulating peripheral cellular immune response.

Intracerebroventricular injection of neurotoxin 6-OHDA causes severe, selective and systematic destruction of central catecholaminergic neurons. Currently, 6-OHDA, together with MPTP, are the two most widely used models of PD (Tolwani et al., 1999). A single central administration of 6-OHDA depletes the hypothalamus, midbrain and pons-medulla of NE and the striatum of DA in mice (Cross et al., 1986). In the present study, we also observed a severe, selective and systematic central catecholamine depletion in rats following i.c.v. 6-OHDA treatment. Two, four and seven days after the injection, NE, DA and EPI levels in the hypothalamus and in the cortex were significantly reduced in experimental animals than in the controls. Moreover, no effects of i.c.v. 6-OHDA injection on serotonin contents in the same tissues were found on the seventh day after the treatment, confirming the highly specificity of 6-OHDA for catecholaminergic nerve fibers.

Furthermore, although no effects of central catecholamine depletion with 6-OHDA on splenocyte and peripheral lymphocyte proliferation were found four days after the injection, the proliferative capacity of lymphocytes in spleen and peripheral blood to Con A, a T-cell mitogen, was significantly inhibited in experimental rats than in the controls seven days after the treatment. Since IL-2 have been shown to be of crucial importance for lymphocyte proliferation (Chouaib et al., 1985), we also measured the IL-2 production and mRNA expression of splenocytes in the presence of Con A seven days after i.c.v. 6-OHDA injection. Our data shows that splenic IL-2 production as well as mRNA expression is also significantly reduced by central catecholamine depletion. These observations are not only similar to those reported by Renoux *et al*. (1989) and Bieganowska *et al*. (1993) who found that splenocyte proliferation in the presence of Con A was inhibited by central catecholamine depletion, but also in agreement with those reported by Klüter *et al*. (1995) who found that IL-2 production of peripheral lymphocytes

after PHA stimulation was reduced in patients with PD. Moreover, our findings that central catecholamine depletion suppresses splenic IL-2 production not only at protein level, but also at mRNA level, and central catecholamine depletion suppresses splenocyte proliferation is associated with a marked synergistic decrease in IL-2 production, which indicating the inhibition of IL-2 production contributes to the suppressed splenocyte proliferation after the depletion, extend these previous observations.

Two $CD4^+$ T_H cell subsets have been identified and are characterized by the cytokines they secrete, Th1 cells secrete IFN-γ and IL-2, while Th2 cells secrete IL-4, IL-5, IL-6 and IL-10 (Mosmann et al., 1991). Th1 cells direct cellular immune response through the production of IL-2 to promote proliferation and expansion of $CD4^+$ T cells, and IFN-γ to play a role in determining the differentiation pathway of precursor T_H cells into effector Th1/Th2 cells and the antibody isotype produced by B cells. *In vitro* and *in vivo*, IFN-γ-producing Th1 cells induce B cells to produce IgG2a (Stevens et al., 1988; Finkelman et al., 1990). In the present study, we also found a significant reduction of splenocyte IFN-γ production in the presence of Con A seven days after i.c.v. 6-OHDA injection, which, together with the decreased splenocyte IL-2 production and mRNA expression, indicate Th1-cell activity is inhibited by central catecholamine depletion. Our results agree with those reported by Alaniz *et al*. (1999) who showed that splenocytes from $dbh^{-/-}$ mice produced less Th1 cytokines (IFN-γ and TNF-α) and more Th2 cytokine (IL-10) than splenocytes from controls in response to stimulation with MT culture filtrate proteins (CFP) and anti-CD3 four weeks after the infection by MT, and that after immunized with TNP-KLH, compared with $dbh^{+/-}$ controls, $dbh^{-/-}$ mice had lower titers of IFN-γ-dependent IgG2a anti-TNP antibody, but similar amouts of IgG1 and IgM anti-TNP antibody, which suggesting Th1 responses are impaired in $dbh^{-/-}$ mice in response both to immunization and to infection. Cross and Roszman (1988) also reported that central 6-OHDA treatment in mice inhibited the humoral antibody response to T-cell-dependent antigen TNP-KLH, but did not affect the response to the T-cell-independent antigen TNP-LPS. Taken together, it is likely that central catecholamine depletion inhibits peripheral cellular as well as humoral immune responses by reducing the activity of Th1 cells.

More interestingly, with no changes in splenic catecholamine concentrations were found two days after i.c.v. 6-OHDA injection, a transient but significant reduction in NE and EPI concentrations in the spleen was observed four days after the treatment in

experimental animals compared with the controls. Previous investigations have demonstrated that the spleen is innervated by NE-containing sympathetic nerve fibers (Felten et al., 1987a; Felten and Olschowka, 1987). However, 6-OHDA depletes NE by 75-85% in spleen and lymph nodes for at least two weeks when administered peripherally (Madden et al., 1994), and it does not cross the BBB when administered to adults (Kostrzewa & Jacobwitz, 1974). Therefore, this transient decrease of splenic NE and EPI concentrations is not due to the direct toxicity of 6-OHDA on sympathetic nerve fibers in the spleen, but a result of the modulation of central catecholamine depletion on peripheral sympathetic output.

At present, the precise mechanism of how central catecholamine depletion alters peripheral immunity is still unclear. However, the present data suggest that peripheral SNS plays a role in these effects.

The central catecholaminergic system seems to be involved in sensing and modulating peripheral immune functions. Significant alterations of catecholamine activity occur in specific brain regions during an immune response (Besedovsky et al., 1983; Qiu et al., 1996; Devoino et al., 1997), indicating that this central neurotransmission system is involved in the afferent arm of immune-to-brain communication. On the other hand, central noradrenergic networks can exert direct efferent immunomodulatory actions through fibres innervating immune organs (Bellinger et al., 1989; Felten, 1993). Specifically, it has been shown that the splenic sympathetic innervation is under the control of the ventromedial hypothalamic nucleus (Okamoto et al., 1996). Furthermore, these splenic efferent fibres originate from the sympathetic thoracolumbar column which is under the control of the A5 and A7 noradrenergic brainstem nuclei (Cano et al., 2001). As the neuronal activity (firing rates and patterns) of brain nuclei receiving projections from catecholaminergic structures changes after central catecholamine depletion (Ni et al., 2000, 2001), we hypothesize that centrally administrated 6-OHDA affects peripheral catecholamine output, with its immunomodulatory effects, by causing discrete alterations in central neural firing.

Evidence in the last decade has shown that NE-containing sympathetic nerve fibers penetrate primary and secondary lymphoid organs (Bulloch and Pomerantz, 1984; Felten et al., 1987a) and closely appose $CD4^+$ T_H cells residing within the periarteriolar lymphoid

sheath (Felten et al., 1987a; Felten and Olschowka, 1987), and the concentration of NE in these tissues is much higher than in blood (Felten et al., 1987b). In addition, hematopoietic cells express ARs on their surfaces (Bishopric et al., 1980), lymphocytes express both ARs and DA receptors (Sanders et al., 2001), NE regulates all aspects of immune function through α- and/or β-AR stimulation both *in vitro* and *in vivo* (Madden, 2001). More recently, we reported that β-AR agonist isoproterenol, but not α-AR agonists, could decrease splenocyte proliferation as well as IL-2 production at both protein and mRNA levels *in vitro*, which indicating NE modulates lymphocyte proliferation and cytokine production via β-ARs (Xie et al., 2002). In light of these findings, peripheral NE plays a physiological role in regulation of immune response. Depending on the fact that catecholamines and their metabolites are present in lymphocytes, Bergquist *et al*. (1994) proposed that catecholamines could regulate lymphocyte function via an autocrine or paracrine loop. Moreover, DA significantly modulates the proliferative capacity of murine and human lymphocytes *in vitro* and *in vivo* (Tsao et al., 1997; Saha et al., 2002). In the present investigation, we observed a reduction of peripheral lymphocyte proliferation and splenocyte cytokine production at both protein (IL-2 and IFN-γ) and mRNA (IL-2) levels as well as a transient but significant reduction in splenic NE and EPI contents following central catecholamine depletion by i.c.v. 6-OHDA injection. Token together, we suggest that peripheral SNS plays a role in the modulation of central catecholaminergic system on peripheral cellular immune functions.

Although no changes have been reported in cardiac concentrations of catecholamines two days after the central 6-OHDA treatment (Cross et al., 1986), this does not rule out the possibility that catecholaminergic output in other peripheral tissues changes after i.c.v. 6-OHDA injection. Indeed, we found a significant decrease in splenic catecholamine concentrations four days after the injection, but not two or seven days after the injection, supporting our hypothesis that central catecholamine depletion affects peripheral sympathetic output. In addition, Deng and colleaques (1983) reported that four weeks after i.c.v. 6-OHDA treatment, the contents of NE and EPI in the brain tissues as well as in the heart, in the aorta and in plasma were reduced in rats, suggesting that the overactivity of peripheral sympatho-adrenal system is dependent on the central catecholaminergic neurons. A decrease of endogenous DA in thymus and spleen one day after central DA depletion with MPTP in mice was observed as well (Tsao et al., 1997). Therefore, it is likely that central catecholamine depletion affects peripheral

catecholamine output from sympathetic nerve fibers and the changes of these neurotransmitters induce the observed immunosuppressive effects. Our hypothesis is also in line with Teunis *et al.* (2002) who suggest central dopaminergic activity directly and/or indirectly controls peripheral processes that are under control of peripheral DA as well. Since chemical sympathectomy by peripheral 6-OHDA administration has been well established (Madden, 2001), it might be of particular interest for us to test the effects of central catecholamine depletion on peripheral immune response in the absence of SNS tone to further confirm our hypothesis in further studies.

However, it is also important to realize that the effects of central catecholamine depletion are not only limited to SNS and the immune system, but also extended to other systems that could interact with both catecholaminergic and immune systems.

First of all, it is well known that several hormones exert their influence on immune system. This is primarily mediated through the hormonal receptors present on the surface or cytoplasm of different effector cells of the immune system (Bost, 1988; Weigent and Blalock, 1987). Central catecholamines are known to affect the activity of HPA axis, especially the release and synthesis of several anterior pituitary hormones including ACTH, GH, gonadotropin, prolactin and thyroid-stimulating hormone (TSH) (Müller & Nisticò, 1989b). Several reports which indicate a significant influence of these anterior pituitary hormones on the immune system are available now (Ader et al., 1990). It is also widely known that ACTH stimulates the synthesis and release of the glucocorticoids (e.g. cortisol and corticosterone) by the cortex of the adrenal glands, which in turn modulates immune functions. The facts that rats with a hyperreactive dopaminergic system, who share several characteristics with patients who suffer from schizophrenia, show a large and long-lasting endocrine response to stressors in terms of release of ACTH and corticosteroids (Rots et al., 1996), that hypophysectomy abrogates the immunomodulatory effects of intracisternal 6-OHDA injection in mice (Cross et al., 1987), and that basal serum corticosterone level is increased for at least 15 days after selective unilateral DA depletion in the striatum by 6-OHDA in mice (Filipov et al., 2002), suggest pituitary/adrenocortical hormones play a role in the immunomodulatory effects of central catecholamine system. Interestingly, MPTP-treated mice have increased basal serum corticosterone one week after MPTP injection, a period of time when MPTP-induced striatal DA depletion is maximal, but not earlier (Kaku et al., 1999). In addition, the

hypothalamus, which regulates pituitary hormone secretion and is affected in PD patients, also influences and is influenced by the striatum directly through hypothalamic-striatal projections (Sandyk et al., 1987). Moreover, a direct interaction between corticotropin releasing factor (CRF) and dopaminergic systems occurs in the striatum, and that interaction is altered in animals administered with 6-OHDA (Voilokova et al., 1999). Therefore, it is also possible that central catecholamines affect the peripheral immune functions through the activity of HPA axis. Assessment of HPA activity should be performed in our further investigation as well.

Furthermore, altered central catecholamine levels may also affect the synthesis and release of other neurochemicals which are immunomodulatory in nature. For example, enkephalins exert immunomodulating activity *in vitro* and *in vivo* and constitute important components of the neural-endocrine-immune network (Plotnikoff and Miller, 1983; Janković and Marić, 1990). Dopaminergic neurons that project to the striatum from the substantia nigra are thought to modulate methionine-enkephalin (Met-Enk) metabolism in the striatum. Gudehithlu *et al*. (1991) reported that the Met-Enk-like immunoreactivity and preproenkephalin mRNA content in the striatum increased due to central DA depletion after MPTP treatment in mice. Intracerebroventricular injections of rats with Met-Enk induce reduction in several immune variables like antibody production, development of DTH and number of $CD4^+$ T_H cells (Janković and Marić, 1990). Met-Enk can also stimulate HPA axis which, in turn, stimulates the cells of adrenal cortex to secrete corticosterone in circulation and thereby affecting the functional activities of circulating lymphocytes (Iyengar et al., 1987). Moreover, the level of somatostatin, known to have considerable influence on immune response, is also altered for a few days after MPTP treatment in mice (Stanisz et al., 1986; Asanuma et al., 1990). The role of these neurochemicals in the immunomodulatory effects of central catecholamine depletion also needs further investigations.

Although Bas *et al*. (2001) reported that PD patients had a decrease in the number of $CD4^+$ T_H cells and B cells, and a rise in activated, $CD4^+CD25^+$ T cells in their peripheral blood, the influence of central catecholamine depletion on lymphocyte distribution in primary and secondary lymphoid organs remains unclear (Bas et al., 2001; Bieganowska et al., 1996; Renoux et al., 1989). Since peripheral catecholamines can regulate lymphocyte trafficking as well as its entry, retention and release from lymphoid organs

(Madden, 2001), and central catecholamine depletion alters catecholamine output from sympathetic nerve fibres as reported by Deng *et al.* (1983) and in the present investigation, it might also be interesting for us to investigate the effects of central catecholamine depletion on lymphocyte circulation in peripheral blood and redistribution among the lymphoid organs in our future studies.

Data regarding the effects of central catecholamine depletion on peripheral immune response are still unclear (Cross et al., 1986; Renoux et al., 1989; Neveu et al., 1992; Deleplanque et al., 1992; Bieganowska et al., 1993; Deleplanque et al., 1994; Devoino et al., 1997). Observed discrepancies may be a result of differences in experimental design, specificity of the neurotoxin (6-OHDA vs. MPTP), central catecholamines depleted (NE and DA vs. DA along), species used (mice vs. rats), immunological variables measured (humoral vs. cellular), or immune compartments being investigated (spleen, peripheral blood or lymph nodes). In addition, the time point of the assay after central catecholamine depletion and the approach used to assess immunity (*in vitro* vs. *in vivo*) are likely to be critical (Alaniz et al., 1999; Filipov et al., 2002; Teunis et al., 2002). Therefore, it will be necessery to consider these methodological aspects carefully in designing future experiments.

Regardless of the mechanisms involved, the suppression of peripheral cellular immune functions including decreased lymphocyte proliferation in spleen and peripheral blood, reduced splenocyte cytokine production at both protein (IL-2 and IFN-γ) and mRNA (IL-2) levels after central catecholamine depletion by i.c.v. 6-OHDA treatment, which is crucial to interpretation of studies concerning neural-endocrine-immune communications, was found in the present study. Therefore, the present investigation makes further contribution to understanding the regulation of systematic depletion of central catecholamines on peripheral cellular immune functions in rats. Future work will involve determining the influence of central catecholamine depletion on peripheral cellular as well as humoral immune responses in the absence of SNS tone and the relationships among central catecholamine depletion, alterations of SNS, of HPA axis, and of other neurotransmitter systems in CNS, and peripheral immunological changes.

5. Abbreviations

ACTH	adrenocorticotropic hormone
AIDS	acquired immunodeficiency syndrome
ANOVA	analysis of variance
AR(s)	adrenergic receptor(s)
BBB	blood-brain barrier
BSA	bovine serum albumin
CFP	culture filtrate proteins
CNS	central nervous system
Con A	concanavalin A
CRF	corticotropin releasing factor
CTLs	cytotoxic T lymphocytes
DA	dopamine
DBH	dopamine beta-hydroxylase
DDC	diethyldithiocarbamate
DTH	delayed-type hypersensitivity
ELISA	enzyme-linked immunosorbent assay
EPI	epinephrine
FCS	fetal calf serum
GBR-12935	GBR-12935 dihydrochloride
GH	growth hormone
GTC	guanidinium thiocyanate
HPA	hypothalamic-pituitary-adrenocortical
HPLC	high-performance liquid chromatography
i.c.v.	intracerebroventricular
IFN	interferon
IL	interleukin
i.p.	intraperitoneal
KLH	keyhole limpet hemocyanin
LM	Listeria monocytogenes
LPS	lipopolysaccharide
MAO-B	monoamine oxidase-B
Met-Enk	methionine-enkephalin

MLC	mixed lymphocyte culture
MPDP	1-methyl-4-phenyl-2,3-dehydropyridine
MPP^+	1-methyl-4-phenylpyridine
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MT	Mycobacterium tuberculosis
NE	norepinephrine
NK	natural killer
PBS	phosphate buffered saline
PD	Parkinson's disease
PFC	plaque-forming cells
PHA	phytohaemagglutinin
PNMT	phenylethanolamine-*N*-methyltransferase
PWM	pokeweed mitogen
PCR	polymerase chain reaction
RFC	rosette-forming cell
RT	reverse transcription
6-OHDA	6-hydroxydopamine
SNS	sympathetic nervous system
SPF	specific pathogen-free
SRBC	sheep red blood cells
T_H	T-helper
Th1	T-helper-1
Th2	T-helper-2
TMB	tetramethylbenzidine
TNF	tumor necrosis factor
TNP	trinitrophenyl
TSH	thyroid-stimulating hormone

6. Summary

Evidence demonstrating that immune response is under the control of central nervous system has been accumulated during the past two decades. Central catecholamines have been proved to play a pivotal role in modulation of humoral and cellular immunity both *in vitro* and *in vivo*. However, the modulation of central catecholamine depletion on peripheral cellular immune response remains somewhat unclear. Therefore, we designed a set of experiments to analyse the influence of central catecholamine depletion by i.c.v. 6-OHDA injection on peripheral cellular immune functions. Two, four and seven days after the injection in rats, they were sacrificed and spleen and blood samples were collected to measure the lymphocyte proliferation, cytokine production and IL-2 mRNA expression. Brain and spleen samples were obtained for catecholamine determination as well. Our data demonstrate that two, four and seven days after i.c.v. 6-OHDA treatment, dopamine, norepinephrine and epinephrine levels in the hypothalamus and in the cortex are significantly reduced. Additionally, norepinephrine and epinephrine concentrations in the spleen are significantly decreased four days after the injection. In contrast, the serotonin levels in the brain and in the spleen are not changed seven days after the central 6-OHDA treatment. More importantly, Con A-induced lymphocyte proliferation and splenocyte cytokine production at both protein (IL-2 and IFN-γ) and mRNA (IL-2) levels are significantly reduced seven days after i.c.v. 6-OHDA injection. These results confirm the important role of central catecholamines in modulating peripheral cellular immune response. Although the precise mechanism of how central catecholamine depletion alters peripheral cellular immunity is still not clear, the fact that a significant reduction in splenic catecholamine contents is observed four days after i.c.v. 6-OHDA treatment suggests that peripheral sympathetic nervous system plays a role in these effects.

7. References

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9. Curriculum vitae

Personal Data:

Name:	Wei Kou
Sex:	Male
Date of birth:	March 12, 1973
Place of birth:	Tianjin, P. R. China
Marriage status:	Married
Nationality:	P. R. China
Permanent residence:	805, Xin Ji Shang Cun 162, Fangcun, Guangzhou 510080, China
Place of residence:	Essen, Germany
E-mail address:	freshman_k@yahoo.com.cn

Current Position:

Research Associate, Department of Medical Psychology, Universitätsklinikum Essen

Education:

1997-2000 Master of Science

Sun Yat-sen University of Medical Sciences, Guangzhou, P. R. China

1992-1997 Bachelor of Medicine

Tianjin Medical University, Tianjin, P. R. China

Professional Experiences:

Dec., 2001-Present:

Research Associate, Department of Medical Psychology, Universitätsklinikum Essen

- **In charge project:**
  - Immunomodulation of acupuncture in humans
- **Participated projects:**
  - The influence of central catecholamine depletion on cellular immune response in rats
  - The effects of certain brain area lesions on acquisition and expression of conditioned immune responses
  - Behavioral conditioning of IFN-β1b in humans

- Behavioral conditioning of immunity using staphylococcal enterotoxin B (SEB) as the unconditioned stimulus in rats

Jul., 2000-Dec., 2001:

Resident, Department of Internal Medicine, Sun Yat-sen memorial Hospital, Sun Yat-sen University of Medical Sciences, Guangzhou, P. R. China

Publication:

1. **Kou, W.**, Yin, S., Nie, D., et al. (2001). Changes in the activity of platelet L-arginine/nitric oxide pathway in hypercholesterolemia patients. Zhonghua Xue Ye Xue Za Zhi 22, 132-134.
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