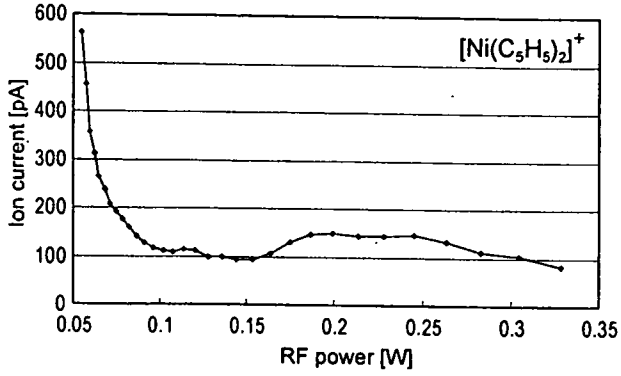

Figure 1. Nickelocene ion current as a function of the RF power.

peak current of the nickelocene ions with the RF power. As shown, an RF power of 54.7 mW is sufficient for the ion to be detected. This shows that the plasma is also generated at this RF power. Moreover, the emission of light of the ECR plasma has been visually checked in this state. Subsequently, when the RF power was adjusted to 50 mW, the plasma suddenly disappeared and nickelocene ions were not detected. Although the values of the RF power for the generation and disappearance of the plasma differed from the abovementioned value, similar tendencies were observed for other organometallic compounds (ferrocene and osmoceme) and their ions were detected. This value of RF power corresponded to that at the exit of a high-frequency amplifier (travelling wave tube amplifier: TWTA) and differed from the value at which the plasma was actually absorbed, which was lower. The electron temperature in the ECR plasma had a certain distribution that could be determined by careful measurements with a Langmuir probe and/or laser Thomson scattering measurement.[4]

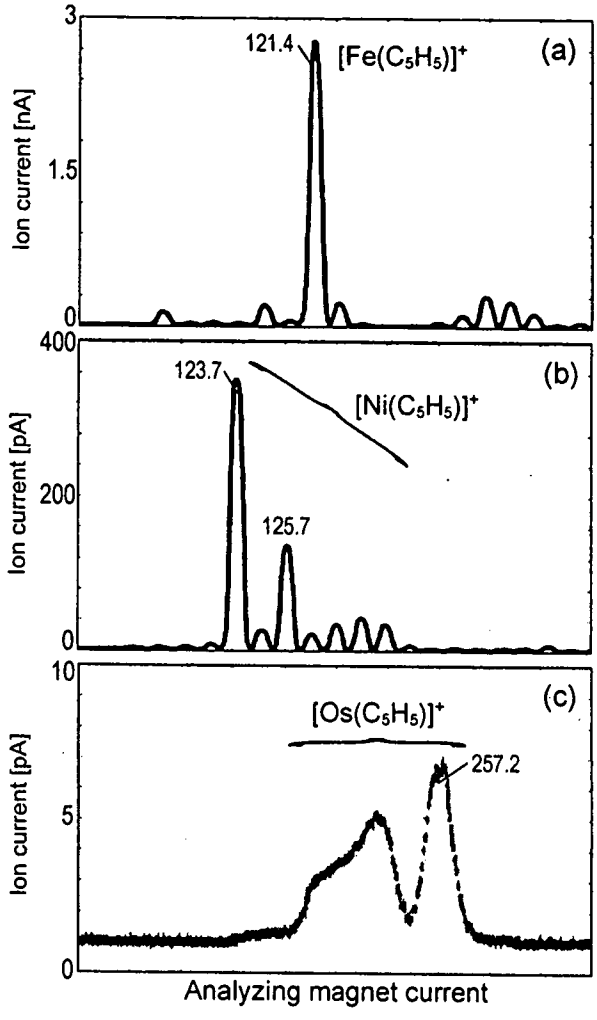

Figure 2. Spectrum of the fragment ions of the organometallic compounds.

Fragmentation and ionization control by varying RF power

Ferrocene, nickelocene and osmocene, which are organometallic compounds, were used for the ionization of a molecule and a fragment. These compounds have a structure wherein one metal element is sandwiched by two pentagons. These compounds have high vapor pressure at room temperature and, since these samples can be used in the form of gases, they are suitable for introducing a sample into the ECR ion source. The ECRIS mass spectrometer[2] was used for performing the analyses. The specifications of the ECR ion source can be obtained from Reference 2. Figures 2(a), (b) and (c) show the spectra of the fragment ions in which one pentagon is separated in each organometallic compound. This shows that ions can be stably generated from such chemically unstable fragments by using an ECR plasma. Figures 3(a), (b) and (c) show the variations in the maximum peak current corresponding to each spectrum of Figure 2 with the RF power. Since the RF power controls the electron temperature in the ECR plasma, the horizontal axis in Figure 3 can also be considered to represent the electron temperature. Since the electron temperature in the ECR plasma was controlled by varying the RF power, the generated quantity of the fragment ions was controlled.

Rough estimation of electron temperature

Here, we roughly estimate the mean electron temperature in the ECR plasma. In an ECR ion source, the electron confinement time (τ_e) usually increases with the electron temperature (T_e). For example, in the case of a minimum-B-type ECR ion source[1] that generates highly charged ions, τ_e is of the order of milliseconds. The transfer time (τ_t) for which energy is transferred from the RF power to a resonating electron is of the order of nanoseconds.[1] The electron traces a spiral orbit along magnetic lines of force and precesses

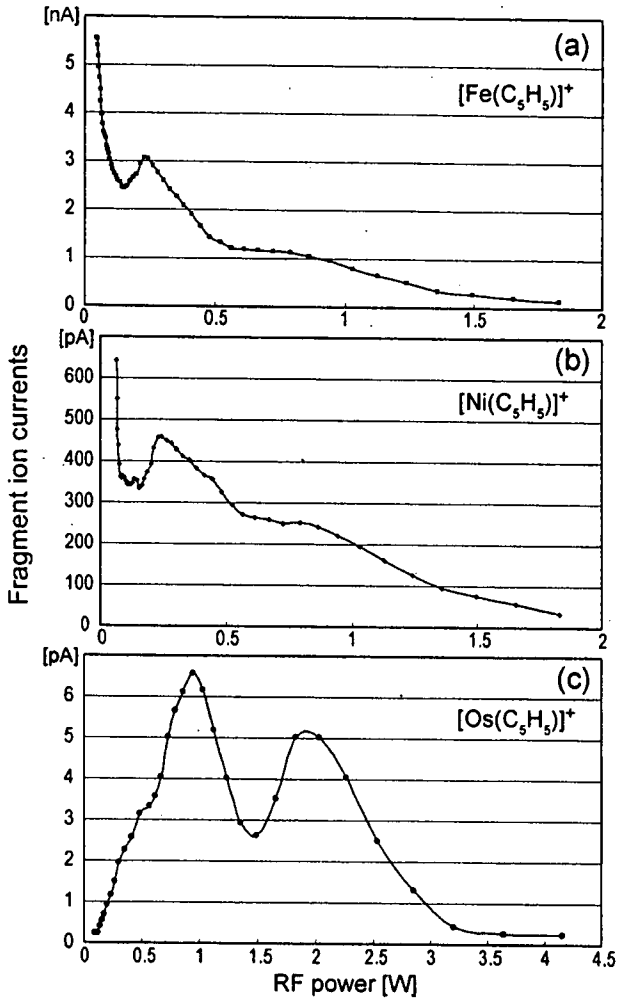

Figure 3. Fragment ion current of the organometallic compounds as a function of the RF power.

between the mirror magnetic fields. While the electron is confined by the mirror magnetic field, it passes along a resonating point repeatedly and energy is supplied to it from the RF power. On the other hand, τ_e becomes extremely small when T_e is very low. Here, when τ_e is very small (of the order of microseconds) and energy is supplied 1000 times (because τ_e and τ_t are of the order of microseconds and nanoseconds, respectively) at the resonating point during τ_e, the mean electron temperature $<T_e>$ is given as follows:

$$<T_e> \cong 1.602\times10^{19}\times10^{3}\times(C_a P_f \tau_t)(V_p n_e)^{-1}\ [\mathrm{eV}] \qquad (1)$$

where C_a denotes the absorptivity coefficient of RF power; P_f [W], the input RF power; V_p [cm^3], the plasma volume; and n_e [cm^{-3}], the electron density. Here, by using typical values—$C_a=0.7$,[5] $\tau_t=10^{-9}$ s^{-1} and $V_p=200$ cm^3—for an ECR ion source, we obtain the expression

$$<T_e> \cong 5.6\times10^{10}\times(P_f n_e^{-1}) \qquad (2)$$

From Golovanivsky's diagram,[1] which is obtained by employing the step-by-step ionization criterion, the value of $n_e\tau_i$ optimized for the value of T_e that is required for (highly charged) ionization can be approximately estimated (τ_i is the ion confinement time). Here, we assume $n_e\tau_i=10^6$ cm^{-3}·sec based on the extrapolation of the diagram since the ion in the plasma has mainly 1+; therefore, considering $\tau_i \cong \tau_e$, Equation (2) becomes

$$<T_e> \cong 5.6\times10^{4}\times P_f\tau_e \qquad (3)$$

$<T_e>$ caused by the RF power is shown in Figure 4 for a specific value of τ_e by using Equation (3). For example, when τ_e is 0.01 ms for an RF power of 55 mW, low-temperature electrons (super cold electrons) with a $<T_e>$ value of 31 meV will exist with a density of 10^{11} cm^{-3}. It is considered that the molecule ions and fragment ions (or fragmentation) are accomplished by such super cold electrons.

At electron temperatures of 1 eV or less, an electron–molecule collision involves a dominant momentum transfer process. However, the mechanism of fragmentation and fragment ionization in the ECR plasma is not well understood. It is expected that the mechanism will be clarified by future researches.

With regard to the plasma of the minimum-B-type ECR ion source, the plasma domain has not been researched extensively. Therefore, the above-mentioned estimates are rough approximations.

Summary

Organometallic compound molecules were soft-ionised by "super cold electrons" generated from an ECR plasma. Moreover, it was observed that the produced quantity of the fragment ions can be controlled by varying the RF power. Since the ionization of molecules and fragments by super cold electrons does not require a matrix, this ionization technique may potentially be used for the ionization of various chemical substances. Further, the dynamic control of the

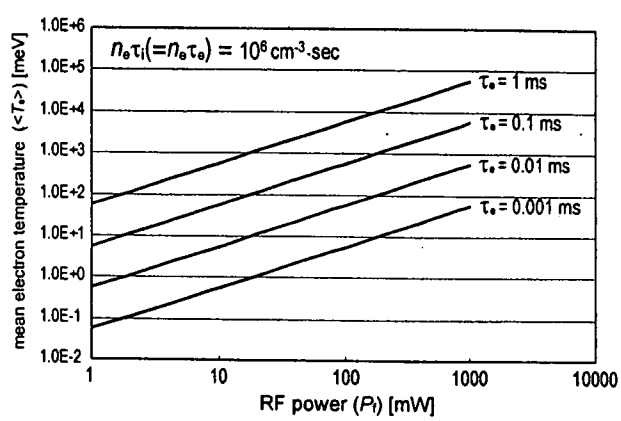

Figure 4. $<T_e>$ in the ECR plasma as a function of the RF power for τ_e = 1, 0.1, 0.01 and 0.001 ms.

fragmentation may potentially be used as a new method for the structural analysis (for example, from information on the strength of a chemical bond) of a chemical substance.

Acknowledgements

We are grateful to Mr M. Hemmi for assisting us with the microwave technique, electronics and ion current measurement. This study was supported by a Grant-in-Aid from the President's Discretionary Fund, RIKEN and a Grant-in-Aid for Scientific Research (KAKENHI: 19540515).

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Received: 28 September 2007
Revised: 29 October 2007
Accepted: 21 November 2007
Publication: 10 December 2007

DOI: 10.1007/s10967-007-0541-2 Journal of Radioanalytical and Nuclear Chemistry, Vol. 272, No.2 (2007) 429–431

Change of concentrations of trace elements and protein contents in the liver of zinc deficient mice

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(Received June 30, 2006)

The concentrations of essential trace elements and proteins in cytosolic fraction of hepatic cells of mice fed with Zn-deficient diet (Zn-def. mice) and control were determined by ICP-MS and BCA protein assay method, respectively, after division into forty fractions by gel filtration chromatography. The concentrations of zinc and proteins decreased in the 14–17th fractions of Zn-def. mice, whereas cobalt concentrations increased in the 14, 17, and 18th fractions. However, no significant differences were found on the gel after SDS-PAGE for the 12–21st fractions, although the BCA protein assay date showed the decrease of protein amounts in 13–15th fractions of Zn-def. mice.

Introduction

Zinc is one of the most important essential trace elements in living organisms, because of a variety of biochemical and physiological action. The zinc content in adult human is about 2.3 g.[1] This element is contained in detectable concentrations in skin, bone, liver, pancreas, kidney, and blood. It influences the activity of more than 300 enzymes that participate in a wide variety of metabolic processes, such as the metabolism and the synthesis of proteins and nucleic acid. A deficiency of zinc leads to various symptoms, such as growth retardation, hypogonadism, immunodeficient, alopecia, osteogenetic inhibition and so on.[2–4]

Previously, concentrations of zinc and other trace elements in various organs and tissues of Zn-def. mice were determined by means of instrumental neutron activation analysis (INAA).[5–7] The zinc concentrations in bone and pancreas of Zn-def. mice were significantly lower than those of control ones, although no differences were found in the other organs and tissues, such as liver, kidney and brain. On the other hand, concentrations of Co increased in all the organs and tissues of Zn-def. mice. Two works were carried out, in order to investigate the behavior of the metallic elements which bound proteins. One is the determination of the concentrations of trace elements and proteins after gel filtration chromatography for cytosolic fraction of hepatocytes. Another one is the investigation of affinities between metals and proteins which are contained in these fractions.

Experimental

Eighty 7-week old male mice of ICR strain were purchased from Clea Japan, Inc. (JCL), Tokyo, Japan, and fed with ordinary commercial diet and tap water. After a week, they were divided into two groups. One group was fed with Zn-deficient diet (<1 μg of Zn/g diet) and ultra pure water ad libitum (Zn-def. mice), and the other group fed with control diet (30 μg of Zn/g diet) and the same water as the former (control mice) ad libitum. The control diet consisted of raw materials for Zn-deficient diet and Zn as zinc carbonate, basic. Concentrations of minerals except for Zn in control diet were equal to those in Zn-deficient diet. After three weeks, their livers were removed and weighed, immediately. Then, every eight livers of each group was together homogenized and separated into two subcellular fractions, such as supernatant (cytosolic fraction) and the other fractions by ultracentrifugation. The cytosolic fraction, of 300 μl was loaded and was further divided into forty fractions (2 ml per a fraction) by gel filtration chromatography (Sephadex G-100 column, 1.5×70 cm^2) using Tris-HCl buffer (pH 7.4) as the eluent, at a flow rate of 10 ml/h, at 4 °C. Then, the concentration of the metallic elements and proteins in each fraction were determined by inductively coupled plasma mass spectrometry (ICP-MS) and BCA protein assay method, respectively. According to the results of ICP-MS, the 12–19th fractions in which the concentrations of zinc decreased were used for the following experiments. Proteins in these fractions were further separated with sodium dodecyl sulfate poly-achrylamide gel electrophoresis (SDS-PAGE).

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Then, the gel was stained with silver stain. The affinities between trace elements and metalloproteins existing in the 14–16th fractions were examined by the multitracer technique.[8] Multitracer solution prepared from silver target was added to the fractions and incubated for 1 hour at 4 °C. Then, each solution was poured into several centrifugal filter tubes, such as Micron YM-3, -10, -30, and -50 (Millipore Co.), and centrifuged at 2000 *g* for 30 minutes in order to estimate the percentage of RI tracer bound to materials whose molecular weight are over 3, 10, 30, and 50 kDa, respectively. Radioactivities in those samples were measured with HPGe detectors. In each examination process, such as gel filtration chromatography, SDS-PAGE, and the multitracer experiment, the amounts of proteins in the samples for two groups were adjusted to be equal to each other.

The experiments were performed under the Guidelines for Animal Welfare and Experimentation of Faculty of Science, Shizuoka University.

Results and discussion

Figure 1 shows the concentrations of zinc and cobalt, determined by ICP-MS, in the 11–40th fractions of forty fractions divided by gel filtration chromatography for cytosolic fraction of hepatic cell. The concentrations of proteins in the same fractions were also shown in Fig. 2. The zinc concentrations in the 13–18, 21, and 22nd fractions of Zn-def. mice were much lower, and the cobalt concentrations in 14, 17, 18, 21, and 22nd fractions were higher than control ones as shown in Fig. 1. On the other hand, as shown in Fig. 2, the concentrations of proteins in 13–15th fractions of Zn-def. mice were low. These results indicate that some specific proteins in this region, especially in the 13–15th fractions, were related to a decrease in zinc and an increase in cobalt in Zn-def. mice. It is thought that the variations in the concentrations of zinc and cobalt were caused by changes to alternative states of proteins, such as liberation of metals from proteins, replacement of zinc in some specific proteins by cobalt and so on. Then, SDS-PAGE was performed in order to examine whether disappearance or induction of proteins occurred or not.

Typical results of the SDS-PAGE for the 14–17th fractions are shown in Fig. 3. No significant differences between Zn-def. and control mice were found on the positions and the number of the protein bands of the lanes on gel and also for the other fractions (12–21st).

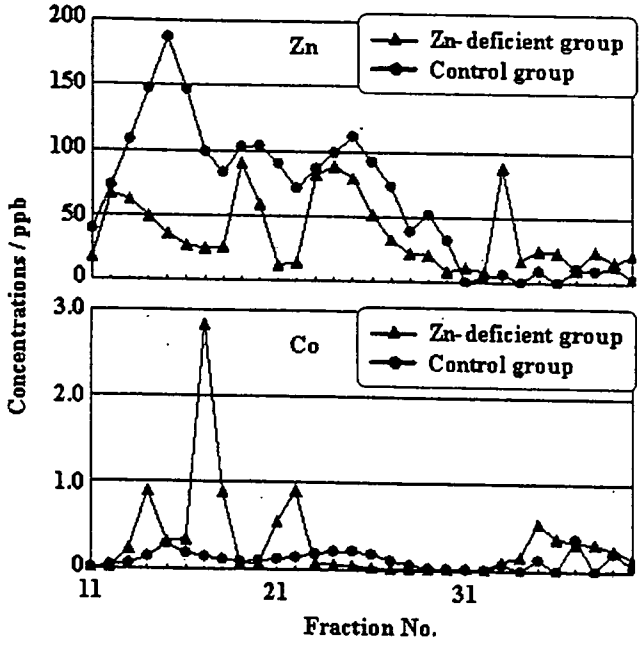

Fig. 1. Concentrations of zinc and cobalt after gel filtration chromatography for cytosolic fraction of hepatocytes in the 11–40th fractions

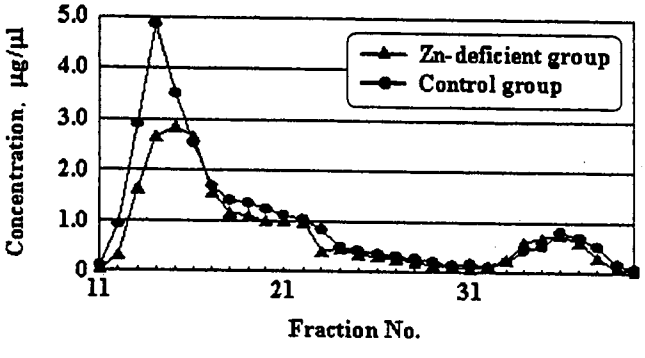

Fig. 2. Concentrations of proteins in the 11–40th fractions

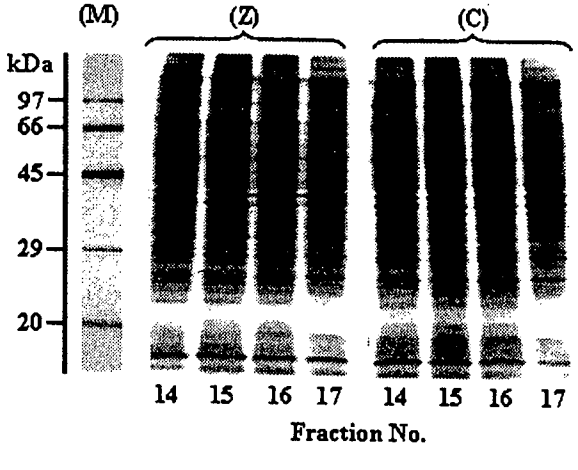

Fig. 3. Typical SDS-PAGE after silver staining for the 14–17th fractions; (M) protein marker, (Z) zinc-deficient mice, (C) control mice

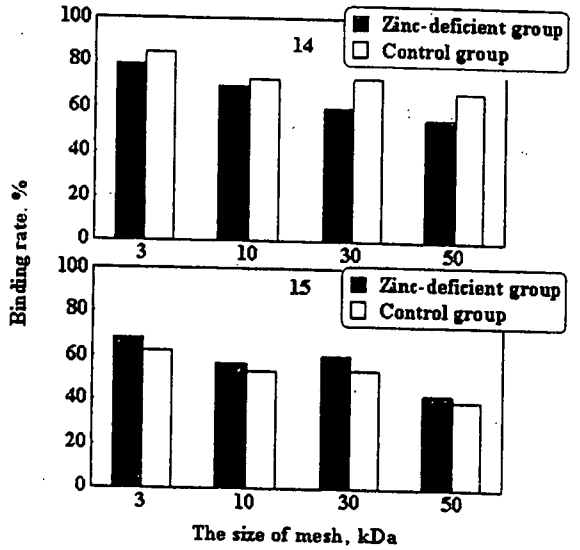

Fig. 4. Affinities between proteins and ^{65}Zn in the 14th and 15th fractions

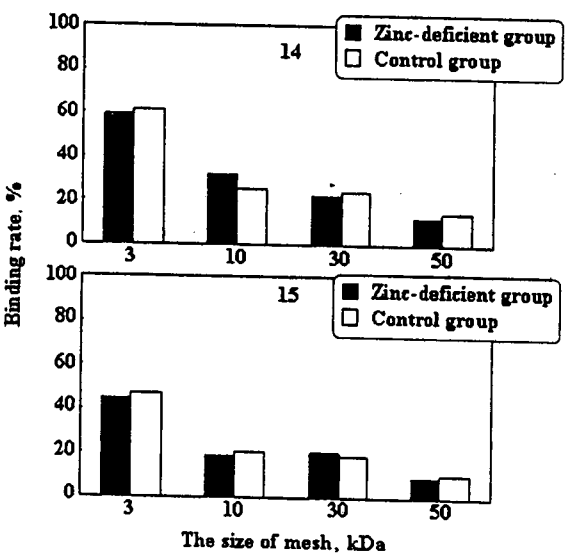

Fig. 5. Affinities between proteins and ^{56}Co in the 14th and 15th fractions

It might indicate that the possibilities of disappearance or a new appearance of proteins in the region of 17–116 kDa in these fractions are low. However, total amount of proteins in this region for the zinc deficient mice decreased as mentioned above.

The affinities between proteins and the radionuclides of ^{65}Zn, and ^{56}Co in the 14th and 15th fractions are shown in Figs 4 and 5, respectively. The affinities between ^{65}Zn and proteins over 30 kDa in the 14th fraction of Zn-def mice decreased, whereas the affinities to proteins over 10 kDa did not distinctly decrease. This result suggests that the zinc-binding proteins whose size is in a range of 10–30 kDa decreased or other metals replaced instead of zinc by zinc deficiency. On the other hand, as shown in Fig. 5, these were no differences between two groups on the affinities between ^{56}Co and proteins in the 14th and 15th fractions. Furthermore, the results in Fig. 5 also indicate that the size of cobalt affinitic proteins is almost in a range of 3–10 kDa. The direct evidence for the replacement of zinc with cobalt was not obtained by this carrier-free RI tracer examination for binding affinities. However, if it is considered that both elements of zinc and cobalt may be antagonistic[5–7] and also content of cobalt in organs is distinctly less than that of zinc, the enhancement of cobalt concentration in Zn-def. mice may be explained by substitution of zinc in some proteins with cobalt.

Conclusions

The concentrations of many essential trace elements and proteins of cytosolic fraction divided into forty fractions in Zn-def. mice were determined. The concentrations of zinc, cobalt, and proteins of Zn-def. mice were remarkably different from those of the control one. It indicates that zinc deficiency state leads to the change of the metalloproteins. The result of the multitracer experiments, affinities between ^{65}Zn and proteins in the 14th fraction, indicated that the zinc-binding proteins in a range of 10–30 kDa decreased or zinc was replaced by the other metal elements, whereas the results of SDS-PAGE showed that there were neither disappearance of metalloproteins nor induction of other proteins.

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Trace Nutrients Research 24 : 56 – 58 (2007)

牡蠣抽出物の胃液分泌能

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Effects of the Oyster Extract on the Gastric Secretion in Rats

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Summary

Digestion is the process in which the bulk of ingested nutrients are broken down to oligomers or monomers in the mouth, stomach and intestine before they are absorbed and made available to all the cells of the body. Gastric juice is characterized by the presence of HCl, pepsin, mucus and an intrinsic factor. It is well established that the secretion of gastric juice is promoted by a type of amino acid and peptide. Oyster is a shellfish classified as Pelecypoda and is a nutritious food containing a great deal of zinc and taurine. Taurine has been reported to reduce myocardial damage and to have a beneficial effect on blood glucose and lipid levels. Thus, we investigated the effect of oyster extract on the gastric secretion in rats. Pylorus-ligated rats were sacrificed 6 h after the administration of oyster extract or taurine, and the gastric juice was collected and analyzed for volume, pH, acidity and pepsin activity. The results indicate that oyster extract may decrease pepsin activity and pepsin output; however, oyster extract significantly raised the acidity. Furthermore, it is suggested that the composition of oyster extract, excluding the taurine, may promote the secretion of HCl and organic acid because oyster extract more significantly raised acidity than taurine.

消化とは，摂取した食物を消化管内で吸収できる小分子の物質に変える働きであり，消化において，消化管の運動，消化液の生成，分泌およびその作用，腸粘膜における物質輸送は重要な要素である。

胃における消化は，食道から送り込まれた食塊を体温にまで温め，胃液含有物質により均質な液状に近い消化粥に変化させ，少量ずつ規則的に十二指腸へ送りこむことにより行われる[1)]。胃液は，胃の外分泌腺から分泌される種々の有機物質と無機電解質を含む液体であり，生理的に重要なのは，酸，ペプシン，粘液および内因子である[2)]。胃液の分泌は，ある種のアミノ酸およびペプチドにより促進されることが知られており[3)]，摂取する食物の含有成分が胃液分泌物やその量に影響をおよぼすことが推測される。

牡蠣は軟体動物斧足類に属する貝であり，必須微量元素のひとつである亜鉛などのミネラルを多く含む栄養食品として知られている。また，牡蠣は，心筋保護作用，血糖および血中コレステロール低下作用などを有する含硫アミノ酸であるタウリンを多く含んでいる[4,5)]。

そこで，我々は，牡蠣抽出物およびタウリンが胃液の分泌に及ぼす影響を検討するため，ラットを用いて胃液分泌実験を行った。

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方　法

動物は，8週齢Wistar系雄性ラットを用いた。ラットの体重は209 ± 1.0 gであり，各群5匹とした。絶食後，エーテル麻酔下で開腹して幽門部を固く縛り，直ちに閉腹した[6]。その後，生理食塩水，牡蠣抽出物（日本クリニック株式会社製），タウリンをラットにゾンデにより経口投与した。牡蠣抽出物中のタウリン含有量は，5.4 g/100 gであり，投与溶液は，牡蠣抽出物138 mgを蒸留水で0.5 mLに調整したものとした。タウリンは，牡蠣抽出物溶液中タウリン含有量に相当する量を蒸留水で0.5 mLに調整した。試料投与6時間後にラットを解剖して胃を摘出し，内容物を採取して胃液量，ペプシン活性，ペプシン分泌量，酸度およびpHを測定した。ペプシン活性の測定にはAnson-Mirsky変法，胃液酸度の測定には滴定酸度測定法を用いた[7]。

結果および考察

生理食塩水，タウリンまたは牡蠣抽出物溶液を投与した場合におけるペプシン活性，ペプシン分泌量，胃液分泌量，酸度，pHをTable 1に示した。

タウリンを投与した群は，ペプシン活性がコントロール群に比べ有意に低値を示した。ペプシン分泌量，胃液分泌量およびpHではコントロール群に比べ低い傾向にあったが，有意な差は認められなかった。タウリン投与群の酸度は有意な差は認められなかったが，コントロール群に比べ高い傾向が認められた。これらのことから，タウリンは，ペプシン活性を低下させ，分泌量を低下させる可能性が示唆された。

牡蠣抽出物を投与した群におけるペプシン活性およびペプシン分泌量は，コントロール群およびタウリン投与群と比較し有意に低値を示した。胃液分泌量およびpHは，牡蠣抽出物投与群がコントロール群およびタウリン投与群に比べ低値であったが，有意な差は認められなかった。一方，牡蠣抽出物を投与した群における酸度は，コントロール群およびタウリン投与群と比較し有意に高値を示した。これらのことから，牡蠣抽出物は，タウリンに比べペプシン活性およびペプシン分泌量を低下させるが，酸度を上昇させることが示唆された。

本実験の結果から，タウリンは，ペプシン活性およびペプシン分泌量を低下させる傾向が認められた。一方，牡蠣抽出物は，ペプシン活性およびペプシン分泌量を抑制するが，胃液酸度を上昇させる可能性が示唆された。

胃酸は，胃液に含まれるタンパク質分解酵素であるペプシンの活性や作用を促進することにより間接的に消化に影響を及ぼす。牡蠣抽出物は，酸度を上昇させるにもかかわらずペプシンの活性および分泌量を低下させているが，消化におけるペプシンの作用が膵液の消化酵素により完全に代用されることから[2]，牡蠣抽出物投与によるペプシン活性および分泌量の低下は，消化に顕著な影響を及ぼさないことが推測された。本郷らは，胃酸分泌は，むしろ，膵外分泌機能を高める上で重要であると報告している[2]。胃酸は，十二指腸に到達すると粘膜の内分泌腺細胞を刺激し，セクレチンやコレシストキニンを分泌させる[2, 8]。セクレチンは，膵臓の導管細胞に作用して，Na^+，HCO_3^-の分泌を促進し，膵臓から分泌される消化酵素の活性を高める[2]。また，コレシストキニンは，セクレチンの作用を増強する[8]。本実験では，牡蠣抽出物投与時における膵液分泌の変化について検討は行っていないが，牡蠣抽出物が，上記の機構により膵液に含有される酵素活性を高める可能性が推測された。

また，牡蠣抽出物の酸度上昇作用は，タウリンに比べ顕著であったことから，牡蠣抽出物に含有されるタウリン以外

Table 1 Pepsin activity, pepsin output, volume, acidity and pH in the control, oyster extract- and taurine-administered rats

	Pepsin activity (μg/mL/min)	Pepsin output (trypsine μg)	Volume (mL)	Acidity (mEq/L)	pH
Control	1085 ± 149[a]	4079 ± 1531[a]	3.4 ± 0.9	38.4 ± 15.3[b]	4.2 ± 0.8
Oyster Extract	311 ± 65[c]	992 ± 290[b]	3.1 ± 0.3	119.4 ± 6.5[a]	3.6 ± 0.1
Taurine	725 ± 48[b]	2233 ± 570[a]	3.2 ± 0.9	49.0 ± 12.5[b]	3.8 ± 0.5

Values are means ± SE (n = 5). [a,b,c] Values with different superscript letters are significantly different ($p < 0.05$).

のアミノ酸またはペプチド等の影響である可能性が示唆された。Cieszkowskiらは，ポリペプチドおよび必須アミノ酸は胃酸分泌刺激物質であると報告している[9]。また，非必須アミノ酸であるアラニンは酸分泌を増加させるという報告もある[10]。本実験に用いた牡蠣抽出物には，必須アミノ酸，および比較的多くのアラニンが含まれることから，これらのアミノ酸の影響により，牡蠣抽出物投与時の酸度が上昇したと考えられた。

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J Neural Transm (2007) 114: 555–561
DOI 10.1007/s00702-006-0608-1
Printed in the Netherlands

Journal of Neural Transmission

Mental fatigue-induced decrease in levels of several plasma amino acids

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Received: August 8, 2005 / Accepted: November 5, 2006 / Published online: December 11, 2006

Summary To investigate the relation between plasma amino acid levels and mental fatigue, we measured the plasma concentrations of 20 amino acids in 9 healthy volunteers before and after a fatigue-inducing mental task session for 8 hr. As fatigue-inducing mental tasks, the subjects performed an advanced trail making test, a Japanese KANA pick up test, and a mirror drawing test. As a control, 8-hr relaxation session was performed in the same subjects at an interval of 4 weeks. Immediately after the fatigue session, the plasma levels of branched-chain amino acids, tyrosine, cysteine, methionine, lysine, and arginine were below those after a relaxation session. The values for other blood parameters including total protein, albumin, glucose, and total cholesterol did not show any differences between the 2 sessions. These results indicate that mental fatigue may be characterized by a decrease in the plasma level of these amino acids.

Keywords: Mental fatigue, amino acids

Introduction

Fatigue is an everyday experience. However, in the case of chronic or accumulated fatigue, they affect the person's performance. In addition, long-term accumulated fatigue can lead to *Karoshi* (death as a result of overwork). For clinical use, mental fatigue is defined as difficulty in the initiation of, or the ability to, sustain voluntary activities (Chaudhuri and Behan, 2004). Mental fatigue, in contrast with neuromuscular or peripheral fatigue, represents a failure to complete mental tasks that require self-motivation and internal cues, in the absence of demonstrable cognitive failure or motor weakness (Chaudhuri and Behan, 2000). Based on this definition, Chaudhuri and Behan (2004) proposed a conceptual model for central fatigue. The work output of voluntary activity depends on the applied voluntary effort, which is controlled by motivational input and perceived effort via feedback from motor, sensory, and cognitive systems. Hence, any dissociation between the level of internal input (motivational and limbic) and that of the perceived effort from applied voluntary effort results in the sense of fatigue.

Physical fatigue can be derived from the action of the muscles, and is also known as peripheral fatigue. There are a large number of reports about the biochemical mechanisms of peripheral fatigue, e.g., depletion of glycogen and phosphocreatine, which are physical energy sources; a decrease in the resting membrane potential or dysfunction of the calcium pump in the sarcoplasmic reticulum in the skeletal muscles; and failure of neuromuscular transmission (For a review, see Fitts, 1994).

Several animal studies have shown that sustained exercise causes an increase in 5-HT turnover in some brain regions (Blomstrand et al., 1989; Chaouloff et al., 1989). Administration of a 5-HT agonist, *m*-chlorophenyl piperazine, to rats impaired running performance in a dose-dependent manner (Bailey et al., 1992) and that of a

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5-HT antagonist, LY 53857, improved running performance (Bailey et al., 1993). These results suggest that this increased brain 5-HT level induced by sustained exercise is associated with physical fatigue. After exhaustive exercise, serum branched-chain amino acids (BCAA) levels were decreased by 22% from those before the exercise, whereas the level of free tryptophan in the serum increased by 74% (Lehmann et al., 1995). It has also been reported that plasma BCAA levels were decreased by 19%, whereas the plasma level of free tryptophan was increased by 15–17% after sustained exercise (Blomstrand et al., 1991). It is known that blood BCAA and free tryptophan compete for being transported into the brain through the blood-brain barrier, since they are carried by the same transport system (Pardridge, 1977). Thus, an increase in the ratio of plasma free tryptophan to BCAA accelerates the transport of free tryptophan into the brain; and thus, because tryptophan is a precursor of 5-HT, an increase in the 5-HT content in the brain may be expected. Therefore, plasma BCAA and free tryptophan levels are considered to be associated with physical fatigue.

Although the roles of amino acids in physical fatigue have to some extent been clarified, little is known about those in mental fatigue. From our preliminary experiments, it was not enough to evaluate the changes in plasma amino acid levels associated with fatigue by performing mental tasks for 4 hr. Therefore, in order to clarify the relationship between amino acids and mental fatigue, we evaluated changes in the level of all 20 plasma amino acids in healthy volunteers subjected to fatigue-inducing mental tasks for 8 hr.

Materials and methods

The study group consisted of 9 healthy volunteers [27.4 ± 5.2 years of age (mean ± SD), 4 females and 5 males]. The participants were recruited at Osaka City University. Current smokers, subjects having history of medical illness, taking chronic medication or supplemental vitamins, body weight less than 40 kg, having performed blood donation within one month before the study, or blood hemoglobin level less than 12.0 g/dl were excluded from the study. Good health was also assessed by physical examination, blood pressure, heart rate, blood chemistry panel (glucose, creatinine, uremic nitrogen, sodium, potassium, chloride, uric acid, and creatine phosphokinase), lipid profile (total cholesterol and triglyceride), complete blood count, and urinalysis. Furthermore, subjects who manifested psychiatric morbidity (e.g., depression) were excluded from the study. Psychiatric diagnosis was made by a psychiatrist (O. K.). The protocol was approved by the Ethics Committee of Kansai University of Welfare Sciences and all the subjects gave their written informed consent for the study.

All subjects underwent both fatigue and relaxation sessions. Four weeks after the relaxation session, the fatigue session was performed. Considering menstrual cycle for the female subjects, we conducted the fatigue session 4 weeks after the relaxation session. The day before either type of session, the subjects were instructed to avoid intensive physical and mental activities, to finish dinner by 10:00 p.m., and to fast overnight. At 9:00 a.m. on the following morning, the subjects recorded their subjective sensation of fatigue on a visual analogue scale (VAS) from 0 (no fatigue) to 100 (full exhaustion), and blood samples were collected. Thereafter, the subjects had breakfast (carbohydrate, 73.6 g; protein, 26.9 g; lipid, 32.3 g; total calories, 707 kcal) between 9:45 and 10:15 a.m. After breakfast either a relaxation or fatigue session was undertaken. As fatigue-inducing mental tasks, the subjects performed an advanced trail making test (Kajimoto et al., 2007) for 45 min, a Japanese KANA pick up test (Yamamoto, 1992) for 30 min, and a mirror drawing test (Yoshiuchi et al., 1997) for 45 min. These series of tasks began at 10:15 a.m., and 4 sessions of each series of tasks were performed, thus resulting in 8 hr of mental fatigue-inducing activities. After the end of the second series of tasks, the subjects had lunch (carbohydrate, 124.2 g; protein, 26.4 g; lipid, 16.3 g; total calories, 844 kcal) between 3:15 and 3:45 p.m. The time-interval between the first and second series of tasks and that between the third and fourth series were 30 min. After the end of the fourth series of the task, the subjects recorded again their subjective sensation of fatigue on the VAS, and blood samples were collected between 8:15 and 8:45 p.m. Then, they had dinner (carbohydrate, 126.3 g; protein, 23.4 g; lipid, 25.0 g; total calories, 882 kcal) between 8:45 and 9:15 p.m., and stayed at a hotel until the next morning. On the next morning at 7:30 a.m., the subjects recorded their subjective sensation of fatigue on the VAS, and blood samples were again collected. As for the relaxation sessions, the subjects read books, watched movies or chattered in the same time frame as the sessions for the mental tasks.

For the advanced trail-making test, the subjects performed visual search trials. In the test, circles numbered from 1 to 25 were first randomly located on the display of a personal computer. The subjects were required to touch these circles in sequence, starting with circle number 1. When the subjects touched the first target circle by using the computer mouse, it disappeared and circle number 26 appeared in a different position on the screen. The positions of the other circles remained the same. The subjects were required to memorize the positions of the other circles while searching for the target circle. This test is an advanced version of the conventional trail-making test (Reitan, 1955). They performed this test as quickly and as correctly as possible.

In the Japanese KANA pick up test, the subjects identified vowels in a novel as many and as correctly as possible. Every 8 min after the start of the test, the subjects were asked questions about the contents of the novel for 2 min.

In the mirror drawing test, the subjects traced a character on a hand glass mirror, which reversed the image. They performed this test as quickly and as correctly as possible.

Blood samples for the analysis of amino acids were collected from a brachial vein into a heparin-containing tube and kept on ice until centrifuged at 1700 *g* for 10 min at 4°C. The plasma sample was deproteinized with 5% trichloroacetic acid for 30 min on ice and centrifuged at 7500 *g* for 10 min at 4°C, after which the supernatant was stored at −80°C until analyzed. The concentration of amino acids in the supernatant was measured by using HPLC. Blood samples for the analysis of plasma glucose were collected in fluorosodium-containing tubes, and centrifuged at 1700 *g* for 10 min at 4°C. The supernatant of the plasma was stored at 4°C until analyzed. In the case of analysis of serum total protein, albumin, total cholesterol, high density lipoprotein cholesterol, triacylglycerol, creatine phosphokinase, and cortisol, the blood was allowed to coagulate at room temperature, and then the serum supernatants were obtained by centrifugation as described above and stored at −80°C until analyzed. All these assays were performed by the Special Reference Laboratories (SRL, Tokyo, Japan).

The values presented were shown as the mean ± SD unless otherwise stated. In order to exclude the influences of the confounding variables (such as diet, circadian rhythm, and gender) on the levels of plasma amino acids, we conducted the fatigue and relaxation sessions in the same time schedule and dietary contents, and compared just intra-individual differences across the sessions. Furthermore, in order to control for intra-individual variability of baseline values over the 4-week period that transpired between the 2

types of sessions, we determined baseline-adjusted net changes, which were the values obtained by subtracting those before the session from those after the session, as data for each time point (Ellenbogen et al., 1996). Comparisons between the 2 groups were performed by using 2-way analysis of variance (ANOVA) for repeated measures. When statistically significant effects were found, inter-group differences between the 2 groups were compared by using the paired *t*-test with Bonferroni correction. Statistical analyses were performed by using a Statview 5.0 software package (SAS Institute Inc., Cary, NC).

Results

Baseline characteristics of the various parameters before relaxation and fatigue sessions are summarized in Tables 1 and 2. Plasma cysteine and histidine levels before the fatigue session were significantly higher than those before the relaxation session. In the other parameters,

Table 1. *Various parameter levels before and after relaxation or fatigue session and the next morning*

	Before		8 hr		Next morning	
	Relaxation	Fatigue	Relaxation	Fatigue	Relaxation	Fatigue
VAS	36.8 ± 15.4	27.5 ± 17.5	42.4 ± 19.1	74.9 ± 16.2*	28.2 ± 13.0	44.2 ± 27.9
Total protein, g/l	80.6 ± 4.4	77.1 ± 4.0	76.7 ± 3.8	76.6 ± 4.2	76.8 ± 3.2	72.3 ± 2.9
Albumin, g/l	51.7 ± 3.0	49.3 ± 3.6	49.1 ± 1.9	48.6 ± 2.9	49.3 ± 3.0	45.4 ± 2.4
Plasma glucose, mmol/l	5.40 ± 0.39	5.21 ± 0.28	5.08 ± 0.54	4.96 ± 0.53	5.60 ± 0.25	5.43 ± 0.39
Total cholesterol, mmol/l	5.03 ± 0.69	5.05 ± 0.67	4.74 ± 0.63	4.98 ± 0.62	4.78 ± 0.58	4.69 ± 0.62
HDL cholesterol, mmol/l	1.77 ± 0.35	1.70 ± 0.44	1.66 ± 0.31	1.68 ± 0.33	1.65 ± 0.34	1.57 ± 0.32
Triacylglycerol, mmol/l	0.74 ± 0.47	0.87 ± 0.30	0.88 ± 0.63	0.90 ± 0.41	1.03 ± 0.57	1.05 ± 0.54
Creatine phosphokinase, IU/l	98.2 ± 38.6	109.8 ± 79.4	92.4 ± 37.6	97.7 ± 61.7	114.8 ± 72.9	99.9 ± 60.4
Cortisol, nmol/l	346.7 ± 119.5	308.1 ± 105.9	192.8 ± 150.3	136.4 ± 37.7	436.8 ± 146.6	413.9 ± 101.3

VAS Visual analogue scale; *HDL* high density lipoprotein.
Data are presented as the mean ± SD.
* $P < 0.05$, significantly different from the corresponding values of the relaxation session (2-way ANOVA for repeated measures, followed by paired *t*-test with Bonferroni correction).

Table 2. *Plasma.amino acid levels before and after relaxation or fatigue session and the next morning*

	Before		8 hr		Next morning	
	Relaxation	Fatigue	Relaxation	Fatigue	Relaxation	Fatigue
Valine, μmol/l	213.1 ± 47.9	225.2 ± 44.5	275.8 ± 45.8	242.6 ± 36.0	237.6 ± 34.4	233.5 ± 26.9
Leucine, μmol/l	106.4 ± 25.5	119.3 ± 25.9	154.3 ± 35.1	116.0 ± 21.8[a]	126.6 ± 21.7	121.7 ± 17.6
Isoleucine, μmol/l	59.0 ± 14.8	63.2 ± 15.2	95.9 ± 21.8	72.6 ± 15.5[c]	78.6 ± 14.2	73.9 ± 11.2
BCAA, μmol/l	378.4 ± 86.5	407.7 ± 82.0	525.9 ± 101.0	431.2 ± 69.9[c]	442.8 ± 67.3	429.1 ± 52.1
Tyrosine, μmol/l	53.5 ± 7.5	57.7 ± 7.5	69.7 ± 9.3	55.3 ± 5.8[a]	66.6 ± 11.4	67.6 ± 9.0
Phenylalanine, μmol/l	53.8 ± 5.3	56.2 ± 6.6	68.2 ± 8.1	60.7 ± 3.8	59.5 ± 4.8	55.6 ± 5.2
Tryptophan, μmol/l	60.6 ± 12.5	59.8 ± 9.6	70.0 ± 14.2	54.9 ± 9.4	64.1 ± 14.5	57.5 ± 9.8
Cysteine, μmol/l	30.6 ± 3.8	40.5 ± 4.3[a]	33.7 ± 4.6	36.7 ± 4.2[c]	38.1 ± 4.0	38.5 ± 4.8
Methionine, μmol/l	24.0 ± 3.1	28.0 ± 4.7	38.6 ± 9.0	26.2 ± 5.7[b]	30.1 ± 5.3	28.6 ± 3.1
Lysine, μmol/l	167.5 ± 26.0	189.3 ± 31.3	231.7 ± 48.9	192.1 ± 34.9	194.5 ± 33.3	174.0 ± 18.2
Arginine, μmol/l	69.5 ± 16.6	76.5 ± 14.6	107.7 ± 23.2	84.1 ± 17.4	86.0 ± 12.9	81.9 ± 14.3
Histidine, μmol/l	71.2 ± 8.7	79.0 ± 9.8[a]	80.1 ± 11.4	79.6 ± 9.4	76.3 ± 8.7	74.2 ± 7.6
Serine, μmol/l	117.1 ± 24.7	112.7 ± 15.5	127.1 ± 30.3	113.6 ± 25.9	124.7 ± 30.2	117.7 ± 22.6
Threonine, μmol/l	127.5 ± 17.6	130.0 ± 24.5	144.7 ± 24.9	124.0 ± 26.0	146.8 ± 29.1	133.9 ± 20.1
Asparagine, μmol/l	43.1 ± 5.2	47.0 ± 4.2	54.2 ± 6.8	52.8 ± 9.2	49.4 ± 7.2	49.2 ± 6.5
Aspartate, μmol/l	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Glutamine, μmol/l	567.9 ± 63.3	536.9 ± 76.1	631.4 ± 83.1	557.5 ± 88.4	607.5 ± 79.5	526.4 ± 60.8
Glutamate, μmol/l	30.0 ± 12.0	33.3 ± 10.7	29.6 ± 13.8	30.6 ± 12.9	33.3 ± 12.2	38.2 ± 13.8
Glycine, μmol/l	222.7 ± 67.7	229.5 ± 56.3	255.0 ± 70.1	245.2 ± 78.9	247.4 ± 69.8	248.8 ± 67.1
Alanine, μmol/l	299.1 ± 53.1	302.2 ± 58.8	375.1 ± 43.9	335.8 ± 46.4	332.5 ± 47.0	342.7 ± 70.2
Proline, μmol/l	139.8 ± 35.9	124.7 ± 29.4	172.1 ± 27.4	147.9 ± 26.9	153.9 ± 13.9	144.4 ± 23.2

BCAA Branched-chain amino acids (total for valine, leucine, and isoleucine); *N.D.* not detected.
Data are presented as the mean ± SD.
[a] $P < 0.05$, [b] $P < 0.01$ significantly different from the corresponding values of the relaxation session (2-way ANOVA for repeated measures, followed by paired *t*-test with Bonferroni correction).
[c] $P < 0.1$, different from the corresponding values of the relaxation session (2-way ANOVA for repeated measures, followed by paired *t*-test with Bonferroni correction).

including the VAS value for fatigue, no statistical differences were observed between the 2 sessions. We analyzed baseline-adjusted net changes in order to evaluate the effects of mental fatigue on all parameters and in order to control for intra-individual variability of baseline values over 4 weeks; and these changes after 8 hr of relaxation or mental fatigue and those assessed the next morning are shown in Tables 3 and 4. Two-way ANOVA for repeated measures of the VAS value for fatigue revealed that the baseline-adjusted net change in the value after the fatigue session was significantly higher relative to that after the relaxation session ($F = 17.11$; $df = 1, 16$; $P < 0.001$). The

Table 3. *Baseline-adjusted net changes in various parameters after relaxation or fatigue session and the next morning*

	8 hr		Next morning	
	Relaxation	Fatigue	Relaxation	Fatigue
VAS	5.6 ± 24.7	47.4 ± 21.3[a]	−8.6 ± 16.0	16.7 ± 20.0
Total protein, g/l	−3.9 ± 3.4	−0.6 ± 5.3	−3.8 ± 2.6	−4.8 ± 4.5
Albumin, g/l	−2.6 ± 1.9	−0.8 ± 3.5	−2.3 ± 1.1	−4.4 ± 2.8
Plasma glucose, mmol/l	−0.32 ± 0.30	−0.25 ± 0.43	0.20 ± 0.35	0.22 ± 0.27
Total cholesterol, mmol/l	−0.29 ± 0.17	−0.07 ± 0.39	−0.25 ± 0.13	−0.36 ± 0.39
HDL cholesterol, mmol/l	−0.11 ± 0.06	−0.02 ± 0.18	−0.12 ± 0.09	−0.16 ± 0.15
Triacylglycerol, mmol/l	0.14 ± 0.20	0.03 ± 0.16	0.29 ± 0.29	0.18 ± 0.39
Creatine phosphokinase, IU/l	−5.8 ± 12.1	−12.1 ± 26.5	16.6 ± 78.1	−9.9 ± 29.8
Cortisol, nmol/l	−153.9 ± 135.0	−171.7 ± 101.4	90.1 ± 129.9	105.8 ± 135.5

VAS Visual analogue scale; *HDL* high density lipoprotein.
Data are presented as the mean ± SD.
[a] $P < 0.01$, significantly different from the corresponding values of the relaxation session (2-way ANOVA for repeated measures, followed by paired *t*-test with Bonferroni correction).

Table 4. *Baseline-adjusted net changes in plasma amino acid levels after relaxation or fatigue session and the next morning*

	8 hr		Next morning	
	Relaxation	Fatigue	Relaxation	Fatigue
Valine, mmol/l (%)	62.7 ± 35.9 (32.5)	17.4 ± 37.1 (10.4)[a]	24.5 ± 35.1 (14.5)	8.3 ± 31.1 (6.2)
Leucine, mmol/l (%)	48.0 ± 29.2 (48.0)	−3.3 ± 24.1 (−0.5)[a]	20.2 ± 24.2 (22.8)	2.4 ± 16.1 (4.4)
Isoleucine, mmol/l (%)	36.9 ± 20.2 (67.6)	9.4 ± 17.9 (19.6)[b]	19.6 ± 16.7 (38.7)	10.7 ± 6.4 (20.1)
BCAA, mmol/l (%)	147.6 ± 81.9 (41.8)	23.5 ± 75.9 (8.4)[a]	64.4 ± 74.3 (20.4)	21.5 ± 51.4 (7.7)
Tyrosine, mmol/l (%)	16.2 ± 14.6 (33.9)	−2.4 ± 10.5 (−2.4)[b]	13.1 ± 13.2 (26.3)	9.9 ± 6.9 (17.7)
Phenylalanine, mmol/l (%)	14.4 ± 8.1 (27.4)	4.5 ± 8.2 (9.5)	5.6 ± 6.0 (11.2)	−0.6 ± 7.3 (0)
Tryptophan, mmol/l (%)	9.5 ± 20.7 (20.5)	−4.9 ± 5.2 (−7.8)	3.5 ± 19.2 (9.6)	−2.3 ± 5.7 (−3.5)
Cysteine, mmol/l (%)	3.0 ± 3.7 (10.4)	−3.8 ± 3.2 (−9.1)[a]	7.5 ± 3.2 (25.3)	−2.0 ± 1.9 (−5.0)[c]
Methionine, mmol/l (%)	14.6 ± 11.0 (65.4)	−1.8 ± 6.9 (−4.5)[a]	6.1 ± 7.4 (28.5)	0.6 ± 3.6 (3.7)
Lysine, mmol/l (%)	64.1 ± 36.5 (38.1)	2.8 ± 35.5 (2.7)[b]	27.0 ± 29.1 (16.9)	−15.3 ± 20.6 (−6.9)[b]
Arginine, mmol/l (%)	38.2 ± 19.2 (59.3)	7.6 ± 14.5 (11.3)[b]	16.5 ± 13.2 (27.8)	5.4 ± 4.9 (7.7)
Histidine, mmol/l (%)	9.0 ± 6.1 (12.5)	0.6 ± 7.9 (1.3)	5.2 ± 6.7 (7.7)	−4.8 ± 6.6 (−5.5)
Serine, mmol/l (%)	10.0 ± 45.4 (14.3)	0.9 ± 13.6 (0)	7.6 ± 42.7 (11.4)	4.9 ± 9.9 (3.8)
Threonine, mmol/l (%)	17.2 ± 22.8 (14.4)	−6.0 ± 22.3 (−3.8)	19.2 ± 35.5 (17.5)	3.9 ± 18.4 (4.5)
Asparagine, mmol/l (%)	11.1 ± 11.0 (28.7)	5.8 ± 8.6 (12.5)	6.3 ± 10.6 (17.0)	2.2 ± 5.7 (4.8)
Aspartate, mmol/l (%)	N.D.	N.D.	N.D.	N.D.
Glutamine, mmol/l (%)	63.5 ± 35.5 (11.1)	20.6 ± 55.9 (4.2)	39.5 ± 42.7 (6.9)	−10.5 ± 33.1 (−1.5)
Glutamate, mmol/l (%)	−0.4 ± 5.5 (−2.8)	−2.7 ± 9.8 (−7.7)	3.4 ± 8.6 (16.6)	4.9 ± 9.7 (16.1)
Glycine, mmol/l (%)	32.3 ± 117.0 (25.6)	15.7 ± 31.8 (5.9)	24.7 ± 119.0 (22.2)	19.3 ± 18.1 (8.1)
Alanine, mmol/l (%)	75.9 ± 51.1 (27.6)	33.6 ± 48.1 (13.2)	33.4 ± 63.9 (13.5)	40.5 ± 37.7 (13.6)
Proline, mmol/l (%)	32.3 ± 29.3 (27.0)	23.2 ± 18.5 (20.9)	14.0 ± 34.5 (15.1)	19.7 ± 16.5 (18.2)

BCAA Branched-chain amino acids; *N.D.* not detected.
Data are presented as the mean ± SD.
The percent change in mean plasma level of amino acids after the 8-hr session or the next morning relative to that of the baseline level is given in parentheses.
[a] $P < 0.05$, [c] $P < 0.01$, significantly different from the corresponding values of the relaxation session (2-way ANOVA for repeated measures, followed by paired *t*-test with Bonferroni correction). [b] $P < 0.1$, different from the corresponding values of the relaxation session (2-way ANOVA for repeated measures, followed by paired *t*-test with Bonferroni correction).

VAS value for the next morning after the fatigue session tended to show recovery. There were no significant differences in baseline-adjusted net changes in plasma glucose, serum total protein, albumin, total cholesterol, high density lipoprotein, cholesterol, triacylglycerol, creatine phosphokinase, and cortisol levels between the 2 groups just after the sessions or the next morning. ANOVA analyses also revealed main effects of the session on plasma valine ($F=4.51$; $df=1$, 16; $P=0.049$), leucine ($F=12.94$; $df=1$, 16; $P=0.002$), isoleucine ($F=9.24$; $df=1$, 16; $P=0.008$), BCAA ($F=8.39$; $df=1$, 16; $P=0.011$), tyrosine ($F=5.68$; $df=1$, 16; $P=0.030$), cysteine ($F=38.98$; $df=1$, 16; $P<0.001$), methionine ($F=11.67$; $df=1$, 16; $P=0.004$), lysine ($F=15.53$; $df=1$, 16; $P=0.001$), and arginine ($F=15.39$; $df=1$, 16; $P=0.001$) levels. Baseline-adjusted net changes in plasma valine, leucine, BCAA, cysteine, and methionine levels after the fatigue session were significantly lower than those after the relaxation session. In addition, baseline-adjusted net changes in plasma isoleucine, tyrosine, lysine, and arginine levels after the fatigue session had a trend toward lower than those after the relaxation session. Baseline-adjusted net change in plasma cysteine level for the next morning following the fatigue session was significantly lower, and that in plasma arginine level had a trend toward lower than those following the relaxation session.

Discussion

Although there are a number of reports in the literature about the relationship between physical fatigue and plasma levels of amino acids (Blomstrand et al., 1991; Blomstrand, 2001; Lehmann et al., 1995), the influence of mental fatigue on these levels remains unknown. Plasma amino acid levels are thought to be influenced by brain activities; e.g., a molecular imaging study showed that the plasma level of tyrosine, which is a precursor amino acid of dopamine, was correlated with the extent of neurotransmission (Leyton et al., 2004). Therefore, in order to investigate the relationship between amino acid metabolism and mental fatigue, we measured the plasma levels of all 20 amino acids before and after relaxation and fatigue sessions. This is the first study to show that the concentrations of 8 plasma amino acids were significantly or trend toward changed by mental fatigue.

The baseline-adjusted net change in the VAS value after the fatigue session was approximately 8-times higher relative to that after the relaxation session, indicating that the subjects had a fatigue sensation by performing the series of mental tasks during the fatigue session. There was a high degree of variability in the VAS values given by the individuals during the relaxation and fatigue sessions. Since subjective fatigue sensation is influenced not only by individual's fatigue level, but also by many factors (e.g., motivation and sensitivity for fatigue), such a high degree of variability in the subjective fatigue score might happen. There were no significant differences in baseline-adjusted net changes in creatine phosphokinase level between fatigue and relaxation sessions. These results suggest that the subjects performed continual mental tasks without marked physical load. As for stress load, we have no available data about saliva cortisol level, self report-scale of stress, skin conductance, or heart rate variability. Therefore, results of our study seem to reflect mental fatigue and/or stress associated with performing the tasks.

The baseline-adjusted net changes in the plasma levels of valine, leucine, cysteine, and methionine levels after the fatigue session were significantly lower and those in the plasma isoleucine, tyrosine, lysine, and arginine had a trend toward lower than those after the relaxation session. Blood levels of other parameters including total protein, albumin, glucose, and total cholesterol did not show any differences between the 2 sessions. These results indicate that consumption of the above 8 amino acids was accelerated when the subjects performed the mental tasks. In contrast, the plasma levels of glycine, proline, asparagine, aspartate, and glutamate, all of which show low potential extraction rates through the blood–brain barrier (Oldendorf, 1971), were not affected by the mental task. These results suggest that the consumption of the 8 amino acids was caused by the brain activities.

Besides being used for protein synthesis, amino acids are also involved in various metabolic activities in the brain: e.g., BCAA are transaminated to α-ketoglutarate for the synthesis of glutamate (Yudkoff, 1997); tyrosine is utilized for the synthesis of dopamine (Wurtman et al., 1974); cysteine is used for the synthesis of glutathione and taurine (Kranich et al., 1998; Parcell, 2002); and arginine is required for nitric oxide production and for creatine synthesis (Bredt and Snyder, 1994; Wyss and Kaddurah, 2000). It has been reported that in animals subjected to stress for several hours the release and synthesis of glutamate (Gilad et al., 1990; Moghaddam, 1993) and dopamine (Imperato et al., 1991; Nakahara and Nakamura, 1999) were enhanced in their brain. These findings suggest that mental load accelerates the consumption of neurotransmitters. Accordingly, utilization of tyrosine may be increased for the synthesis of catecholamines; and therefore the supply of tyrosine through phenylalanine by liver phenylalanine hydroxylase may proceed. Although we did not measure plasma free-

tryptophan level, change of plasma tryptophan level after the fatigue session had a trend toward lower than that after the relaxation session ($P=0.056$, paired t-test without Bonfferoni correction). Increase in the utilization of tryptophan for the synthesis of serotonin may also happen. In addition, prolonged psychological stresses induced an increase in the level of thiobarbituric acid reactive substance, an index of lipid preoxidation activity, in the brain (Matsumoto et al., 1999), thus suggesting that oxidative stress is brought about by mental load. Thus, utilization of cysteine and arginine may be increased for the synthesis of antioxidants. Taken together, a possible interpretation of these results might be that mental fatigue could facilitate utilization of amino acids for the synthesis of neurotransmitters or antioxidants in the central nervous system.

On the morning after the fatigue session, baseline-adjusted net changes in plasma cysteine and lysine levels remained lower as compared with those for the relaxation session, thus suggesting prolonged effects of mental fatigue on the plasma levels of these particular amino acids. We speculate that the consumption of the amino acids that are precursors of antioxidants, was accelerated during the recovery phase from mental fatigue and that antioxidants are essential for the recovery from mental fatigue.

There are some limitations to our study, such as the small number of subjects and study design. To generalize our results, the study with a larger number of subjects is essential. However, not only decreased levels of plasma amino acids (valine, leucine, isoleucine, tyrosine, cysteine, methionine, lysine, and arginine) were shown just after the mental tasks, but also those of plasma cysteine and lysine were shown in the next morning relative to the relaxation tasks. We believe the validity of our results to some extent.

The plasma levels of these amino acids levels might thus be good biomarkers of mental fatigue or at least mental task-induced fatigue. Supplementation of the diet with the above amino acids may be useful for lowering mental fatigue and for facilitating recovery from it. We now plan to examine the uptake of radiolabeled amino acids into the brain by using positron emission tomography (PET) as well as the effects of amino acid administration on mental fatigue and the fatigue sensation.

Acknowledgements

This work was supported in part by Special Coordination Funds for Promoting Science and Technology, and the 21st Century COE Program "Base to Overcome Fatigue", from the Ministry of Education, Culture, Sports, Science and Technology, the Japanese Government. We thank Dr. Larry D. Frye for editorial help with the manuscript.

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European Journal of Neuroscience, Vol. 25, pp. 3489–3498, 2007 doi:10.1111/j.1460-9568.2007.05617.x

Multi-directional differentiation of doublecortin- and NG2-immunopositive progenitor cells in the adult rat neocortex *in vivo*

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Keywords: BrdU, cerebral cortex, gliogenesis, neural progenitor cells, neurogenesis

Abstract

In the adult mammalian brain, multipotent stem or progenitor cells involved in reproduction of neurons and glial cells have been well investigated only in very restricted regions; the subventricular zone of the lateral ventricle and the dentate gyrus in the hippocampal formation. In the neocortex, a series of *in vitro* studies has suggested the possible existence of neural progenitor cells possessing neurogenic and/or gliogenic potential in adult mammals. However, the cellular properties of the cortical progenitor cells *in vivo* have not been fully elucidated. Using 5′-bromodeoxyuridine labeling and immunohistochemical analysis of cell differentiation markers, we found that a subpopulation of NG2-immunopositive cells co-expressing doublecortin (DCX), an immature neuron marker, ubiquitously reside in the adult rat neocortex. Furthermore, these cells are the major population of proliferating cells in the region. The DCX(+)/NG2(+) cells reproduced the same daughter cells, or differentiated into DCX(+)/NG2(−) (approximately 1%) or DCX(−)/NG2(+) (approximately 10%) cells within 2 weeks after cell division. The DCX(+)/NG2(−) cells were also immunopositive for TUC-4, a neuronal linage marker, suggesting that these cells were committed to neuronal cell differentiation, whereas the DCX(−)/NG2(+) cells showed faint immunoreactivity for glutathione S-transferase (GST)-pi, an oligodendrocyte lineage marker, in the cytoplasm, suggesting glial cell lineage, and thereafter the cells differentiated into NG2(−)/GST-pi(+) mature oligodendrocytes after a further 2 weeks. These findings indicate that DCX(+)/NG2(+) cells ubiquitously exist as 'multipotent progenitor cells' in the neocortex of adult rats.

Introduction

In adult mammals, continuous reproduction of neurons is known in two restricted regions: the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone of the hippocampal dentate gyrus (Cameron *et al.*, 1993; Lois & Alvarez-Buylla, 1993). These neurogenic regions contain neural progenitor cells that possess proliferative potential including self-reproduction, and can generate a wide variety of neural cells including neurons, astrocytes and oligodendrocytes, *in vitro* (Morshead *et al.*, 1994; Gage *et al.*, 1995; Gritti *et al.*, 1999). The neural progenitor cells in the neurogenic regions *in vivo* include glial fibrillary acidic protein-immunopositive [GFAP(+)] cells and the polysialyated form of neural cell adhesion molecule-immunopositive [PSA-NCAM(+)] cells, which are derived from GFAP(+) cells (Doetsch *et al.*, 1999, 2002; Seri *et al.*, 2001). Some neural progenitor cells in the SVZ have also been reported to express NG2 chondroitin sulfate proteoglycan (Belachew *et al.*, 2003).

Observations that cells isolated from the adult rat cerebral cortex self-renewed and generated both neurons and glial cells under cultured conditions have implied that the cerebral cortex also includes neural progenitor cells (Palmer *et al.*, 1999). However, very little is known about the cellular properties of cortical progenitor cells *in vivo*. Recently, NG2(+) cells isolated from the cerebral gray matter of early postnatal mice were shown to form neurospheres and gave rise to neurons, as well as astrocytes and oligodendrocytes *in vitro* (Belachew *et al.*, 2003). Moreover, NG2(+) cells were found to be the major proliferating cell population in the cerebral cortex of adult rats *in vivo* (Dawson *et al.*, 2003; Tamura *et al.*, 2004). These reports indicate that NG2(+) cells may be a candidate for neural progenitor cells in the adult cortex. However, it remains unclear whether all NG2(+) cells are cortical progenitor cells and whether the cells have multipotent activities *in vivo*. Indeed, it has been reported that only a small fraction of cortical NG2(+) cells isolated from early postnatal mice

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Received 10 August 2006, revised 20 April 2007, accepted 7 May 2007

are immunopositive for Tuj1 and HuC/D, markers for immature neurons (Chittajallu *et al.*, 2004). Moreover, different subtypes of NG2(+) cells have been identified in the gray matter of adult rodent brain on the basis of morphological criteria (Nishiyama *et al.*, 1999) and physiological properties (Chittajallu *et al.*, 2004). These observations suggest that NG2(+) cells are composed of heterogeneous cellular populations.

In the present study, we identified progenitor cells that express doublecortin (DCX), an immature neuron marker, among NG2(+) cells in the neocortex of adult rats *in vivo*. Furthermore, we examined whether these cells have multipotent activity by tracing their differentiation.

Materials and methods

Animals and BrdU injections

Adult male Wistar rats (SLC, Hamamatsu, Japan; 250–350 g body weight) were used. All experimental protocols were approved by the Ethics Committee on Animal Care and Use, Kansai Medical University, and were performed in accordance with the Principles of Laboratory Animal Care (NIH publication no. 85-23, revised 1985). For labeling of proliferating cells, adult rats ($n = 15$) were intraperitoneally injected with 5-bromodeoxyuridine (BrdU) at 50 mg/kg body weight. Each animal underwent a single injection of BrdU.

Immunohistochemistry

Animals were deeply anesthetized with diethyl ether and perfused transcardially with 4% formaldehyde buffered with 0.1 M phosphate-buffered saline (PBS; pH 7.4) at 2 h ($n = 5$), 14 days ($n = 5$) and 28 days ($n = 5$) after injection of BrdU (Sigma, St Louis, MO, USA). Brains were removed, postfixed overnight at 4 °C in 4% formaldehyde buffered with 0.1 M PBS and then immersed in 20% (w/v) sucrose solution. Coronal brain sections (30 μm thickness) were prepared using a cryostat and collected as free-floating sections. For detection of BrdU incorporation, brain sections were preincubated in 50% formamide/2 × standard sodium citrate (SSC) for 2 h at 65 °C, incubated in 2 N HCl for 30 min at 37 °C, rinsed in 0.1 M boric acid (pH 8.5) for 10 min at 25 °C, and then washed with 0.3% Triton X-100 in PBS (PBST). For multiplex-immunostaining, coronal sections were incubated with several primary antibodies for 12–36 h at 4 °C. The primary antibodies used in this study were: monoclonal rat anti-BrdU IgG (1 : 10, Oxford Biotechnology, Oxford, UK); polyclonal rabbit anti-Ki67 IgG (1 : 1000, NovoCastra, Newcastle, UK); polyclonal goat anti-DCX (C-18) IgG (1 : 100, Santa Cruz Biotechnology, Santa Cruz, USA); monoclonal mouse anti-PSA-NCAM IgM (1 : 200, Chemicon, Temecula, CA, USA); polyclonal rabbit anti-TUC-4 protein IgG (1 : 1000, Chemicon); monoclonal mouse anti-neuronal nuclei (NeuN) IgG (1 : 200, Chemicon); monoclonal mouse anti-NG2 IgG (1 : 200, Chemicon); polyclonal rabbit anti-NG2 IgG (1 : 200, Chemicon); polyclonal rabbit anti-glutathione S-transferase (GST)-pi IgG (1 : 500, Medical & Biological Laboratories, Nagoya, Japan); monoclonal mouse anti-oligodendrocytes (clone RIP) IgG (1 : 20 000, Chemicon); and polyclonal rabbit anti-GFAP IgG (1 : 100, Sigma). After washing for 30 min (three washes of 10 min each) with PBST, brain sections were incubated in the appropriate secondary antibodies conjugated with either Cy2, Cy3 or Cy5 (1 : 200, Jackson ImmunoResearch, West Grove, PA, USA) for 4 h at 4 °C and washed with PBST for 30 min. Some of the staining sections were mounted with solution containing TO-PRO3 (1 : 1000, Molecular Probes, Eugene, OR, USA) and then examined using a confocal laser microscope (LSM510META Ver. 3.2; Carl Zeiss).

Cell counting procedure

Coronal brain sections (30 μm thickness) were randomly selected from 2.30 mm to 3.80 mm posterior to the bregma from each animal. Confocal images were captured at 1-μm intervals along the Z-axis (depth) from each section and were reconstructed into three-dimensional images. The percentages of cells expressing each cellular marker among all BrdU-labeled cells were evaluated in the neocortical parenchyma, including the motor cortex, somatosensory cortex and auditory cortex of both hemispheres of three coronal sections from each animal. The proportion of DCX(+) cells in the neocortical NG2(+) cells was estimated in 12 square areas (150 × 150 μm), which were randomly selected in the neocortical areas described above from each hemisphere. Vascular cells, including endothelial and perivascular cells, which were defined as cells having crescent or narrow nuclei located along the wall of the blood vessels, were omitted from analysis. Data from each animal were averaged.

Results

NG2-immunopositive [NG2(+)] cells were abundantly observed in all cortical layers in the adult rat neocortex (Fig. 1A). Some were closely attached to the somata of the NeuN(+) neurons (perineuronal territory, Kataoka *et al.*, 2006; arrowhead in Fig. 1B). We investigated whether neocortical cells express immature neuron markers by immunofluorescent staining for neuronal-lineage markers (PSA-NCAM and DCX). The studies revealed that many DCX(+) cells existed throughout the neocortex (Fig. 1C), although none of the cortical cells was immunopositive for PSA-NCAM within the neocortex (data not shown). The majority of DCX(+) cortical cells were found in the perineuronal territory of NeuN(+) neurons, and the multiple processes of these cells appeared to wrap around the cell bodies of the neurons (Fig. 1D), as did some of the NG2(+) cells (arrowhead in Fig. 1B). In order to investigate the coexistence of DCX and NG2 in these cells, we then performed triple immunofluorescent staining of DCX, NG2 and TO-PRO3, a DNA/RNA marker contained abundantly in neuronal somata. The study clearly revealed that such NG2(+) cells in the perineuronal territory had positive immunoreactivity for DCX (arrowheads in Fig. 2A–D), and that DCX(–)/NG2(+) cells were also observed in the cortex (arrows in Fig. 2A–D) but not in the perineuronal territory. As shown in Fig. 2G, cell pairs consisting of a DCX(+)/NG2(+) cell (arrowhead) and a DCX(–)/NG2(+) cell (arrow) were observed, suggesting that these distinctive cells originated from identical cells. Cell counting studies showed that 63 ± 3% (mean ± SD, $n = 2938$ cells from five animals) of neocortical NG2(+) cells were immunopositive for DCX. The cell densities of DCX(+)/NG2(+) cells were 70 ± 7 cells/mm^2 (mean ± SD, $n = 5$ animals) in the motor cortex (M1 and M2 defined by Paxinos & Watson, 1998), 75 ± 7 cells/mm^2 in the somatosensory cortex (S1BF and S1Tr) and 68 ± 5 cells/mm^2 in the auditory cortex (Au1, Au and AuD). These results indicate that two subtypes of NG2(+) cells exist in the neocortex, based on the presence or absence of DCX co-expression. DCX(+)/NG2(–) cells were also very occasionally observed in the adult rat neocortex (asterisk in Fig. 2H–L).

It has been reported that immature neuron markers such as PSA-NCAM and DCX are expressed only in cells observed in the entorhinal and piriform cortex (Seki & Arai, 1991; Nacher *et al.*, 2001), and that the majority of DCX-expressing cells are

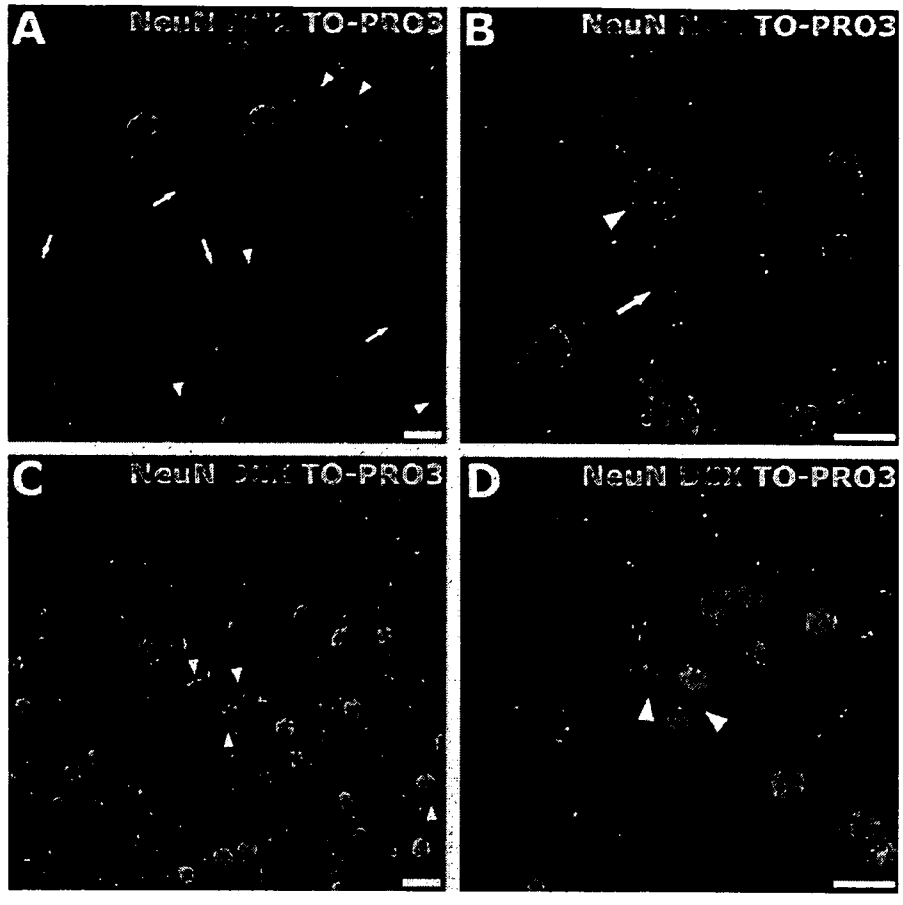

FIG. 1. The existence of NG2- or doublecortin (DCX)-positive cells in the neocortex of adult rats. (A) NG2(+) cells (red) were abundantly present in the neocortex. (B) An NG2(+) cell (arrowhead) close to NeuN(+) neuronal soma (green) and NG2(+) cell (arrow) out of the perineuronal territory. (C and D) DCX(+) cells (red) located in the perineuronal territory of NeuN(+) neurons (green). All cell nuclei and neuronal somata were stained with TO-PRO3. Scale bars: 20 μm.

immunopositive for PSA-NCAM in these areas of adult rats (Nacher *et al.*, 2001). In the present study, we found DCX(+) cells throughout all layers of the neocortex including the motor cortex, somatosensory cortex and auditory cortex, in addition to the entorhinal cortex and piriform cortex. Triple staining revealed that the cortical DCX(+)/NG2(+) cells were not immunopositive for PSA-NCAM; Fig. 3 shows that PSA-NCAM was observed only in DCX(+)/NG2(–) cells (arrows), but not in DCX(+)/NG2(+) cells (arrowheads) in the piriform cortex. These observations indicate that DCX(+)/NG2(+) cells ubiquitously observed in the adult cortex and DCX(+)/PSA-NCAM(+) cells in the piriform cortex are distinct cell populations.

NG2(+) cells have been reported to be the major population of proliferating cells in the cerebral cortex of adult rats, based on analysis of BrdU-incorporated cell nuclei 2 h after BrdU injection (Dawson *et al.*, 2003; Dayer *et al.*, 2005; Kataoka *et al.*, 2006). In the present study, we determined whether cortical proliferating cells are DCX(+)/NG2(+) cells or DCX(–)/NG2(+) cells by triple staining for NG2, DCX and BrdU using the same BrdU labeling method (Table 1). Almost all the BrdU-incorporated cells (94.2 ± 1.8%; $n = 5$ animals) were DCX(+)/NG2(+) cells (Fig. 4A–D), while none of the DCX(–)/NG2(+) cells or DCX(+)/NG2(–) cells showed BrdU incorporation at 2 h after BrdU injection (Table 1). We also confirmed that almost all the Ki67-positive cells were DCX(+)/NG2(+) cells (data not shown), indicating that these cells are the major cell population entering the cell cycle. The remaining proliferating cells were microglia showing immunoreactivity for OX-42 (data not shown).

We investigated cellular differentiation after proliferation of DCX(+)/NG2(+) cells in the adult neocortex by multiple immunodetection of cell differentiation markers and BrdU at various time points after BrdU injection. At 14 days after BrdU injection, approximately 80% (79.5 ± 3.8%), 10% (9.5 ± 3.4%) and 1% (0.61 ± 0.44%) of BrdU-labeled cells (1168 cells from five animals) were DCX(+)/NG2(+) (small arrow in Fig. 4E), DCX(–)/NG2(+) (big arrow in Fig. 4H) and DCX(+)/NG2(–) cells (arrowheads in Fig. 4E and I), respectively (Table 1). These results indicate that DCX(+)/NG2(+) cells may produce both DCX(+)/NG2(–) and DCX(–)/NG2(+) cells in addition to the reproduction of DCX(+)/NG2(+) cells. We performed other multiple immunohistochemical staining analyses of these cells to determine whether the cells were committed to the neuronal or glial cell lineage. TUC-4, a neuronal lineage marker (Seki, 2002), was found in all DCX(+)/NG2(–) cells ($n = 11$ cells at 14–28 days after BrdU injection; arrowhead in Fig. 5), but not in DCX(+)/NG2(+) or DCX(–)/NG2(+) cells, suggesting that the DCX(+)/NG2(–) cells were committed to the neuronal cell lineage after generation of the cells from DCX(+)/NG2(+) cells. We were unable to detect mature neuronal markers, including NeuN, γ-aminobutyric acid (GABA) or GAD-67, in BrdU-incorporated cells at 14 and 28 days after BrdU injection.

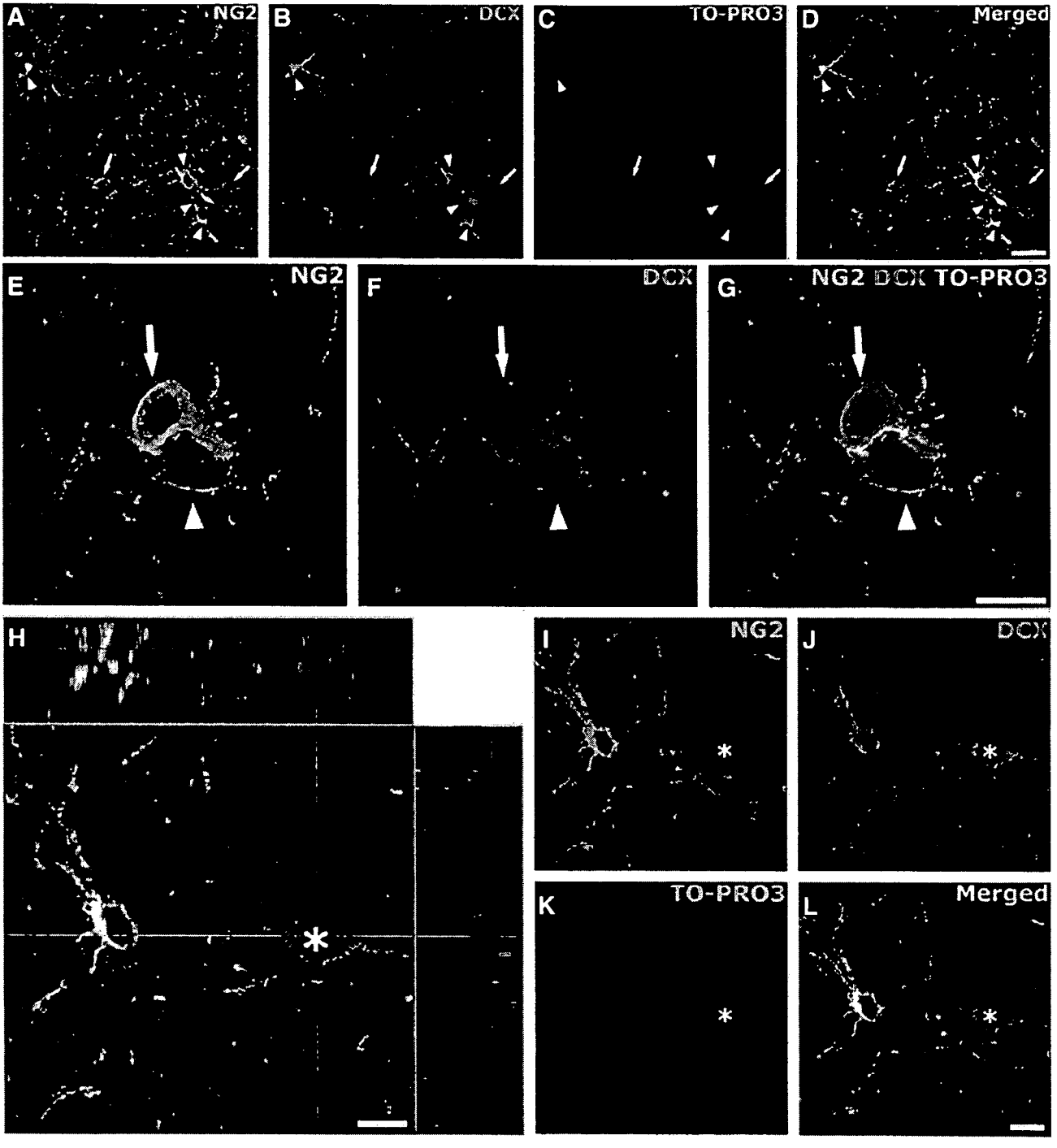

FIG. 2. Two subtypes of NG2(+) cells were distinguished with triple staining for NG2 (green), doublecortin (DCX; red) and TO-PRO3 (blue) in the neocortex. Arrowheads, DCX(+)/NG2(+) cells (yellow in D and G); arrows, DCX(–)/NG2(+) cells; asterisks, an NG2(–)/DCX(+) cell. Scale bars: 20 μm (A–D); 10 μm (E–L).

In order to examine differentiation of DCX(+)/NG2(+) cells into the glial lineage, we performed triple immunohistochemistry for BrdU, NG2 and GST-pi, an oligodendrocyte lineage marker, or for BrdU, DCX and GST-pi. At 14 days after BrdU injection, a portion of BrdU(+)/NG2(+) cells started to show faint immunoreactivity for GST-pi in the cytoplasm (Fig. 6A–D). GST-pi immunoreactivity was detected in all DCX(–)/NG2(+) cells found in this study, but not in BrdU(+)/NG2(–) and BrdU(+)/DCX(+) cells. These findings suggested that only DCX(–)/NG2(+) cells were committed to the oligodendrocyte lineage at that time point. Furthermore, among the BrdU-labeled cells at Day 28, potent GST-pi immunoreactivity was observed only in DCX(–)/NG2(–) cells (Fig. 6E–H), and was faint in DCX(–)/NG2(+) cells, but not at all present in any DCX(+) cells (Table 1). Immunohistochemical staining for RIP (Friedman *et al.*,