１．研究課題名：

成熟脳組織の試験管内における代謝活動維持に関する基礎的条件の研究

２．研究機関：

秋田大学

３．研究者名と所属　：

山田勝也（秋田大学医学部生理学第一講座）

4，研究協力者名と所属：

山本亘彦（京都府立医科大学第二生理学講座）
外山敬介（京都府立医科大学第二生理学講座）
小川哲朗（秋田大学医学部生理学第一講座）

５．研究期間：1996 年－1999 年

６．要約

試験管内でスライス状の大脳皮質視覚野と視覚系の中継核である視床外側膝状体、または他の視覚野とを共培養した。皮質各層において細胞の電気生理学的膜特性ならびに神経伝達の試験管内での発達について調べたところ、膜特性は正常皮質における生後発達と類似した成長を示した。特に受動的な膜特性の変化の大きさは浅層の方が深層より大きく、また深層の方が成熟の時間経過が先行して進んでいることが示唆された。また、層によって膜特性に違いがみられ、これは層特異的な発達のメカニズムが培養下でも作用することが示唆された。

一方、神経伝達も正常に類似した発達を示したが、特に興奮性伝達における各受容体の寄与が培養日数の経過により層特異的にしかも正常に変化したことは、脳における神経伝達の発達において少なくとも興奮性アミノ酸受容体の発現にはパターン化された感覚入力が必要ないことを示唆している。また以上の結果により、幼弱期に取り出した脳組織は通常の中枢神経系の培養条件下で少なくとも神経細胞に関してはかなりの機能的成熟が可能であり、またその活動を数ヶ月以上の長期にわたり維持できることが明らかとなった。

幼弱期の組織と異なり、完全に成熟した組織を試験管内に取り出す場合には、通常の培養条件下ではその維持は困難であった。しかし、組織の代謝環境を整えることで長期にわたり維持できる可能性が示唆された。

７．研究目的

脳内の異なる部位から脳組織を別々にスライス状に切り出し、試験管内で互いに少

し離して静置すると、それらの組織間に正常に類似した神経結合が形成されることが我々を含むいくつかのグループの研究によって次第に明らかにされつつある（参考１-4)。この方法（スライス共培養）は多くの応用可能性をもつ反面、胎児や生後直後の幼弱な脳組織を用いる必要があるという大きな欠点がある。実際、成熟した脳組織を試験管に取り出すと通常一日程度で神経細胞がほとんど死滅するが、その原因の一つとして脳組織の代謝環境が幼弱期と成熟期で大きく異なることが関係するのではないかと考えられる（山田他 1997)。しかし、逆に成熟脳組織を試験管内で生存させ、さらに正常な神経活動を維持する為に必要な、グルコースや酸素供給など各種基礎的代謝条件をスライス共培養を用いて検討すれば、神経活動を支える二次信号に関して細胞レベルで研究できる有力なモデルとなる可能性がある。スライス培養の研究はこれまで大半が組織学的な研究に留まっており、周辺の組織から切り離され血流供給もない状態の大脳皮質で、はたして神経活動がどの程度発達し、また維持されるかについてもほとんど詳細な検討はなされていない。そこで本研究では幼弱期に取り出された大脳皮質視覚野における神経細胞の電気生理学的膜特性ならびに神経伝達が視覚入力のない人工的環境下でどのように、またどこまで発達可能か、についてまず検討した。また、成熟大脳皮質の培養に対する酸素分圧の影響や、組織内の透過性、代謝産物の除去に大きく関わる培養液の潅流条件などについてさまざまな検討もおこなった。

８．材料と方法

胎生 15-17 日令のラット胎児から取り出した外側膝状体ならびに生後 2-3 日令の大脳皮質視覚野、または視覚野同士を互いに 0.5mm 離してフィルター膜上に静置した。各種ホルモンを添加した D-MEM/Ham F-12 培養液を用い、5% 炭酸ガス培養器にて 37℃で最大数ヶ月に渡り培養した。

電気生理学的実験には、約 0.5ml/min で Krebs-Ringer solution (in mM; 127 NaCl, 2 KCl, 4 CaCl2, 4 MgSO4, 1.24 KH2PO4, 26 NaH CO3, 10 dextrose, pH 7.4) を 33℃で潅流した。外側膝状体やペアとなる視覚野を bipolar の刺激電極で電気刺激し、皮質各層で細胞外ならびに細胞内記録をおこなった。細胞はパートナーとなる神経核から単シナプス性伝達を受けていることを確認したもののみを用いた。実験後、Nissl 染色により組織学的検索を行った。

成熟組織の培養は上記に類似するが、拡散を促進するために大脳皮質を極めて薄く（100-150μ程度）切り出した上、培養液の交換頻度を高めて培養した。実際には培養液の交換頻度を数時間おきにする、あるいは連続的に培養液を潅流するという方法を用いた。

９．結果

神経核の間には約 1 週間で神経結合が形成された。培養開始後、7-11days in vitro (DIV), 12-15DIV, 16-52 DIV に分けて、皮質各層において細胞内記録を行い、細胞の電気生理学的膜特性ならびに神経伝達の試験管内での発達について調べた。その結果、膜特性は正常皮質における生後発達と類似した成長を示し、培養開始 7 日以降でみると特に受動的な膜特性の変化の大きさは浅層の方が深層より大きく、また深層の方が成熟の時間経過が先行して進んでいることが示唆された。また、各層によって膜特性に違いがみられ、これは層特異的な発達のメカニズムが培養下でも作用することを示唆している (Fig. 3; Yamada et al., 1999)。

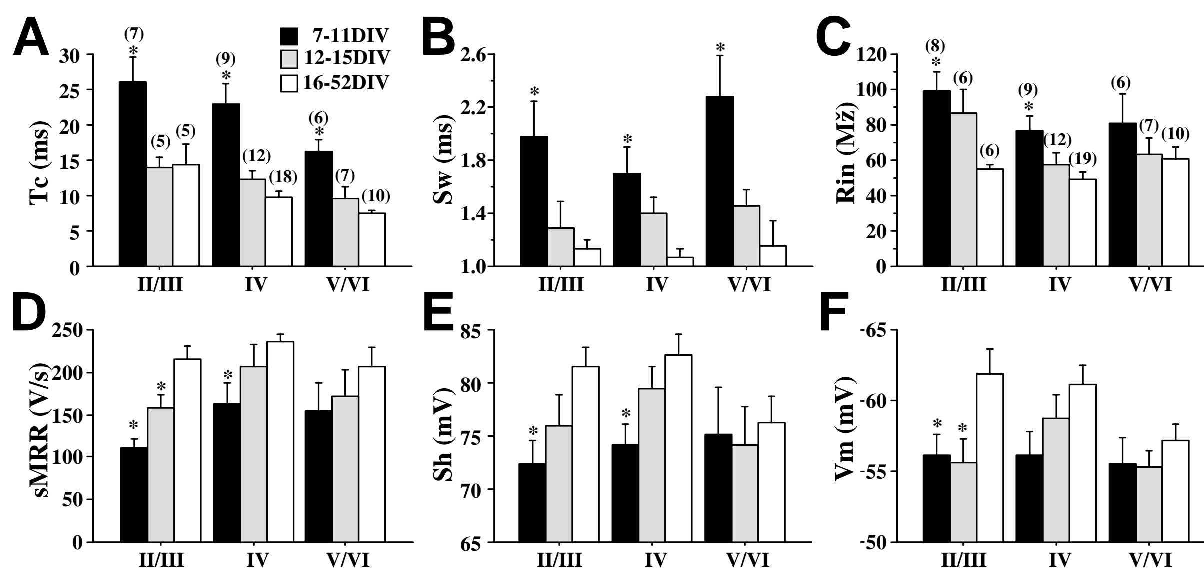

Fig. 3 皮質視覚野の各層における神経細胞の膜特性の試験管内における発達

A, 膜の時定数の変化。DIV は培養開始後の日数を示す。B, 活動電位の半値幅の変化。C, 膜抵抗の変化。D, 活動電位の最大初期傾斜の変化。E, 活動電位の大きさの変化。F, 静止膜電位の変化。

一方、神経伝達も正常に類似した発達を示したが、特に興奮性伝達における各受容体の寄与が培養日数の経過により層特異的にしかも正常に変化したことは、脳における神経伝達の発達において少なくとも興奮性アミノ酸受容体の発現にはパターン化された感覚入力が必要ないことを示唆している (Fig. 4; Yamada et al., in press)。

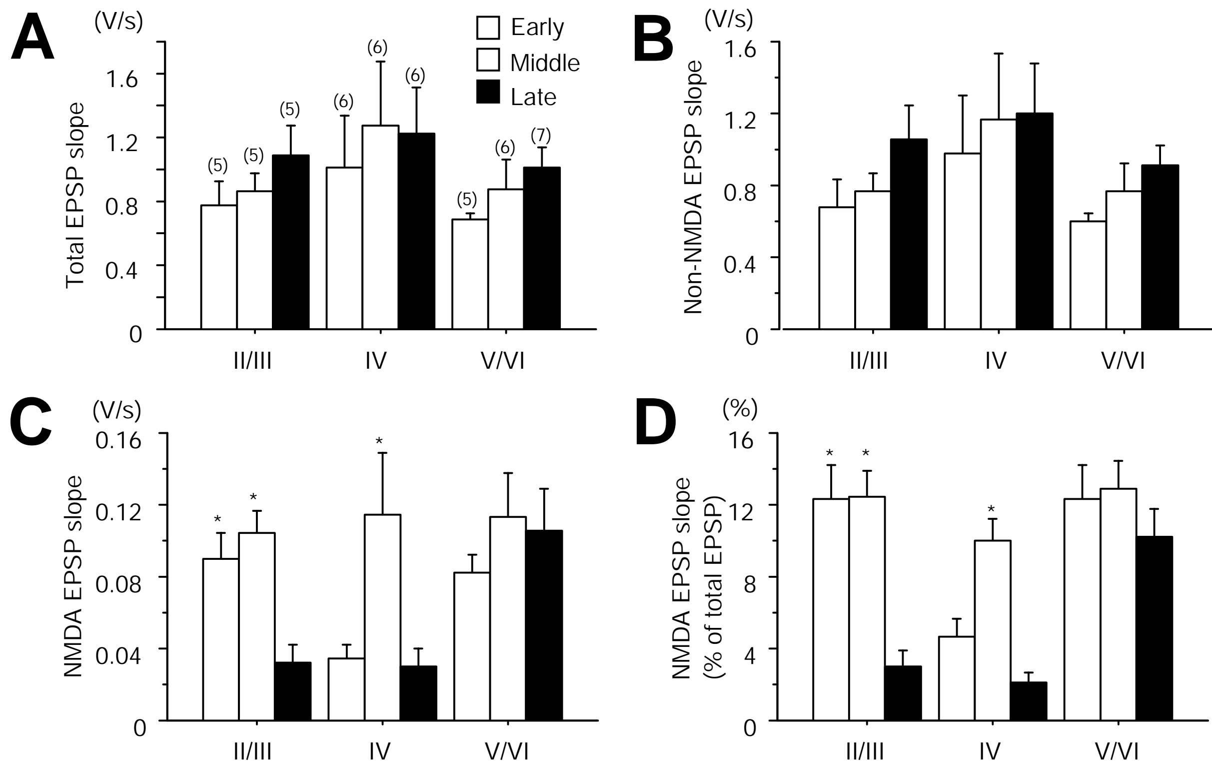

Fig. 4 生体外に取り出した視覚皮質と視床外側膝状体およびもう一つの視覚皮質との間に形成された層特異的神経結合の試験管内における電気生理学的発達

A, 興奮性神経伝達(EPSP)の皮質各層における発達。Early, Middle, Late はそれぞれ培養開始後の日数の経過を示す。B, 興奮性アミノ酸の受容体の内、non-NMDA 受容体を介する EPSP の発達。層による顕著な違いは見られない。C, 同じく NMDA 受容体を介する発達。層特異的な発達の時間経過を示す。D, 全体の EPSP に対する NMDA-EPSP の寄与の変化。

また以上の結果により、幼弱期に取り出した脳組織は通常の中枢神経系の培養条件下で少なくとも神経細胞に関してはかなりの機能的成熟が可能であり、またその活動を数ヶ月以上の長期にわたり維持できることが明らかとなった。

一方、完全に成熟した組織を試験管内に取り出す場合には、通常の培養条件下ではその維持は困難であった。組織の代謝環境として、異なる酸素分圧下で培養を行ったが、改善は認められなかった。しかし、組織を 150 ミクロン程度に薄く薄切した上で培養すると 1 週間にわたり神経繊維の伸長を認めた。また各種アミノ酸等を含まないリンガー液でも、潅流速度を速めることで通常数時間から長くて 10 数時間しか維持できない神経活動が、3 倍以上の期間維持された。これらの事実は成熟脳組織を長期にわたり維持する上で、組織の代謝環境を整えることの重要性を示唆している (Yamada and Ogawa, 1997)。

10. 考察

今回の研究により、生体外に取りだした幼弱な脳組織中の神経細胞は、試験管内でも正常に類似した電気生理学的発達を示し、更に組織同士の間に形成される神経結合は単に組織学的に層特異的に正常に発達するだけでなく、その神経伝達の受容体の発現に関しても正常に類似した時間経過をたどることが少なくとも興奮性の神経伝達については明らかとなった。このことは、通常の培養環境で神経細胞の基礎的代謝活動を原理的には支えることができることを示唆しており、更に別の実験により実際成熟脳組織を体外に取り出した場合にもその代謝環境を改善することで長期に渡り維持できる可能性が示唆された。

従来、脳組織が培養可能な時期はラット視覚野では、胎生期から生後 11 日目ごろまでと言われ、この中でも生後日数がすすめば培養の成功率は急激に低下する(7)。実際、通常の培養法である静置培養法をそのまま成熟脳に適用すると、短時間の内に組織に浮腫を生じ、電気生理学的応答が消失した。形態を見ると、神経細胞に著しい委縮がおきており、その様子は典型的な虚血性脳疾患の様相に類似していた。

なぜこうした変化がおこるのか、またどうしたら防げるのかは関心の持たれるところである。周知のように成熟脳ではアストロサイトが発達しており、毛細血管の周辺などを隈なく覆っている(8)。幼弱な組織にくらべ細胞間隙も小さい。従って、静的な物理拡散に頼る培養法では、酸素や栄養が十分速いスピードで組織内の細胞に到達せず、また細胞活動の結果生じる炭酸ガス、あるいは興奮性アミノ酸や活性酸素などの代謝産物の除去にも支障を来たすのかもしれない。

実際、今回拡散を促進するために大脳皮質を極めて薄く切り出した上、培養液の交換頻度を高めて培養した。その結果、厚い組織では神経繊維が死滅し消失するのに、薄い組織内では１週間に渡ってその形態を保つことが観察されたことはこうした脳神経活動によって生じる代謝産物の供給ならびにその除去の重要性を示唆している。いずれにしても試験管内に取り出した脳組織の代謝について考察することは、実際の脳の神経活動と二次信号との関係を調べる上でもきわめて有益な情報を与えてくれるものと考えられる。

11．今後の展開

成熟脳組織の培養は、培養関係者の長年の夢の一つであり、それが可能になることにより脳科学に与える影響は計り知れない。今回はその基礎的な条件の検討として、少なくとも神経細胞機能の発達は通常の培養環境でかなりの程度まで達成されることが明らかとなったが、一方３次元的な神経回路の構築という脳のもっとも重要な特徴を達成することは、脳を薄く切って代謝を改善する方法では根本的に解決されない。

今後、安定に長期に渡り成熟神経細胞を維持する上での条件を更に探ると共に、この問題をどのように解決していくかが鍵を握るものと考えられる。その意味で、生体脳に張り巡らされた血管の存在がクローズアップされてくるのであって、生体での研究と試験管内での研究の両面からのアプローチが今後ますます重要になるのではないかと予想される。

12．参考文献

[1] Nobuhiko Yamamoto, Tohru Kurotani and Keisuke Toyama (1989) Neural connections between the lateral geniculate nucleus and visual cortex in vitro, Science, 245, 192-194.

[2] Nobuhiko Yamamoto and Katsuya Yamada (1991) Formation of Specific Neuronal Connections by Using Organotypic Slice Culture. Cell Technology 10, (3), 209-214.

[3] Keisuke Toyama, Yukio Komatsu, Nobuhiko Yamamoto, Tohru Kurotani and Katsuya Yamada (1991) In vitro approach to visual cortical development and plasticity. Neuroscience Research 12, 57-71.

[4] Nobuhiko Yamamoto, Katsuya Yamada, Tohru Kurotani and Keisuke Toyama (1992) Laminar Specificity of Extrinsic Cortical Connections Studied in Coculture Preparations. Neuron, 9, 1-20.

13.　研究業績：

13-1. 原著論文

[1] K. Yamada, N. Yamamoto, and K. Toyama: Development of membrane properties of rat neocortical neurons studied in organotypic cocultures. Neurosci. Lett., 275, 65-68 (1999).

[2] K. Yamada, N. Yamamoto, and K. Toyama: Development of NMDA and non-NMDA receptor-mediated excitatory synaptic transmission in geniculocortical and corticocortical connections studied by organotypic coculture preparations. *Eur. J. Neurosci.* (in press).

13-2.総説など：

[1] 山田勝也「試験管内で成長する大脳皮質神経回路」ミクロの世界から認識まで, p.3-15、本道医学振興会 (1996).

[2] 山田勝也、小川哲朗「脳切片の培養法」Clinical Neuroscience, 15, (7), 714-715

(1997).

13-3.国際学会発表：なし

13-4.国内学会発表：

[1] 山田勝也、小川哲朗：成熟大脳皮質組織培養における酸素分圧の影響、第 19 回日本神経科学大会、平成 8 年 11 月、神戸。

[2] 山田勝也、小番篤、小川哲朗：成熟大脳皮質組織培養における酸素分圧の影響、第 29 回東北生理談話会、平成 8 年、岩手。

[3] 山田勝也、太田英樹、石井治佳、小川哲朗：成熟大脳皮質の静置培養と潅流培養、第 20 回日本神経科学大会、平成 9 年 7 月 16 日、仙台。

14. Basis for maintenance of physiological metabolisms of adult brain tissue in vitro

15. Akita University School of Medicine

16. Katsuya Yamada, Department of Physiology, Akita University School of Medicine

17.

Nobuhiko Yamamoto (Department of Physiology, Kyoto Prefectural University of Medicine)

Keisuke Toyama (Department of Physiology, Kyoto Prefectural University of Medicine)

Tetsuro Ogawa (Department of Physiology, Akita University School of Medicine)

18. 1996-1999

19. Abstract

Development of membrane properties and excitatory postsynaptic transmissin of layer IV neocortical neurons - which receive thalamic inputs - and other layer neurons - which receive cortical inputs - were studied in organotypic cocultures of a rat visual cortical slice with either a block of the lateral geniculate nucleus or another cortical slice. The results suggest that the membrane properties of cortical neurons develop primarily based on laminar specificity. On the other hand, the non-NMDA component of excitatory amino acid-mediated synaptic transmission tended to increase in both the geniculocortical and corticocortical connections with progression of the culture stage. Whereas the NMDA component

exhibited distinct developmental changes. Our results suggest that glutamatergic transmission in the visual cortex develops differently in the geniculocortical and corticocortical connections without activity of patterned afferent inputs. In addition, these results suggest that immature brain tissue can not only be maintained for a long period, but also functionally mature in vitro. On the contrary, adult brain tissue could only survive for a very limited period. The fact that the culture period was prolonged when medium exchange rate around tissue was increased, suggest importance of metabolic factors that linked neuronal activity of adult tissue.

ELSEVIER

NSL 16008

Neuroscience Letters 273 (1999) 1–4

Neuroscience Letters

www.elsevier.com/locate/neulet

Development of membrane properties of rat neocortical neurons studied in organotypic cocultures

Katsuya Yamada[a,*], Nobuhiko Yamamoto[b], Keisuke Toyama[c]

[a]*Department of Physiology, Akita University School of Medicine, Akita 010–8543, Japan*
[b]*Division of Biophysical Engineering, Graduate School of Engineering Science, Osaka University, Toyonaka, Osaka 560–8531, Japan*
[c]*Department of Physiology, Kyoto Prefectural University of Medicine, Kyoto 602–8566, Japan*

Received 31 August 1999; accepted 13 September 1999

Abstract

Development of membrane properties of layer IV neocortical neurons, which receive thalamic inputs, and other layer neurons, which receive cortical inputs, were studied in organotypic cocultures of a rat visual cortical slice with either a block of the lateral geniculate nucleus or another cortical slice. In all types of cells, the membrane time constant, input resistance and spike width decreased markedly with days in culture whereas maximum rate of rise of the spike increased. Except for layer V/VI neurons, spike height and resting potentials also tended to increase during development. These changes were more prominent in superficial neurons regardless of afferent connectivity. The results suggest that the membrane properties of cortical neurons develop primarily based on laminar specificity. © 1999 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Visual cortex; Lateral geniculate nucleus; Organotypic culture; Membrane property; Cortical development

During development, neocortical neurons differentiate into various neuronal types. The membrane properties, which affect neuronal firing and transmission, are also thought to develop and become unique to each neuronal type. In general, the neurons tend to have larger and narrower action potentials, smaller input resistance and membrane time constant with maturation. However, it has not been fully understood how these properties develop in specific neuronal types in the neocortex. In particular, to what extent they are determined by laminar locations and afferent inputs remains unknown. To address these issues, organotypic coculture preparations [17] would provide a useful model, since cortical cytoarchitecture and extrinsic neuronal connections are formed with the normal laminar specificity in these preparations [4,5,11,12,17,18]. In addition, cellular morphology and afferent innervation develop with a time course which is similar to that found in vivo [1,5,15,18]. In the present study, we examined the development of the membrane properties of neocortical neurons by using cocultures of a visual cortical slice (VC) with either a chunk of the lateral geniculate nucleus (LGN) or another cortical slice. Measurements were focused on layer IV neurons and layers II/III and V/VI neurons which receive thalamic and cortical inputs, respectively.

Cocultures were prepared as described previously [18]. In brief, a block of LGN obtained from embryonic 15- to 17-day-old fetus and a VC coronal slice from a 2- to 3-day-old rat, or, a pair of VC slices were plated side by side on a filter membrane. They were maintained in a hormone-supplemented DMEM/F-12 medium in 95% air and 5% CO_2 at 37°C. For electrophysiological experiments, cocultures were continuously perfused (flow rate, 0.4–0.6 ml/min) in an interface-type chamber with Krebs–Ringer solution (in mM; 127 NaCl, 2 KCl, 4 $CaCl_2$, 4 $MgSO_4$, 1.24 KH_2PO_4, 26 $NaHCO_3$, 10 dextrose; pH 7.4) saturated with 5% CO_2 in air (33 ± 1°C). To test the synaptic connectivity of impaled neurons, electrical stimulation (0.06–0.4 mA, 100 μs) was applied through a pair of platinum-iridium electrodes placed in the LGN or the partner VC. Glass microelectrodes filled with 2 M K-methylsulphate (electrical resistance, 80–200 MΩ) were used for the recordings. Input resistance (R_{in}) and membrane time constant (T_c) was determined by injecting a small (< 10 mV) hyperpolarizing current pulse (duration, 200–500 ms) through the microelectrode [3]. To examine the active properties, action potentials were produced by injecting a depolarizing current pulse through the electrode.

* Corresponding author. Tel.: + 81-18-884-6070; fax: + 81-18-836-2604.
E-mail address: kyamada@med.akita-u.ac.jp (K. Yamada)

0304-3940/99/$ - see front matter © 1999 Elsevier Science Ireland Ltd. All rights reserved.
PII: S0304-3940(99)00745-4

Signals were amplified, digitized and stored on disk. After the recording session cocultures were fixed with 4% paraformaldehyde and stained with cresyl violet to determine the laminar locations of recorded neurons.

The membrane properties of layer IV neurons which receive thalamic inputs were examined by intracellular recording (resting potential deeper than − 50 mV; Table 1) in cocultures of the VC with the LGN. The activation of the impaled neurons by the LGN was determined to be monosynaptic if both of the following criteria [13] were satisfied: (i) the initial slopes of the excitatory postsynaptic potentials (EPSPs) were smoothly graded with the same onset latency when electrical stimulation was applied to the LGN with varying strength; (ii) the EPSPs followed high frequency stimulus of 20 Hz. On the other hand, layer II/III or V/VI neurons with cortical inputs were examined in cocultures of two VC slices. Whether these neurons receive monosynaptic inputs from the partner VC was also tested using the criteria as described above. In both cocultures, monosynaptic EPSPs were not detected before 7 days in vitro (DIV). Therefore, the membrane properties were examined after 7–52 DIV when the developmental change seemed to cease. Most of these neurons were identified as regular spiking neurons by their firing properties [10,16].

Passive membrane properties were determined from a voltage deflection in response to a small hyperpolarizing current and active properties were determined from the shape of the action potentials. A common feature in all three types of VC neurons was that T_c, spike width at half amplitude (Sw) and R_{in} markedly decreased with the progression of the culture stage, whereas maximum rate of rise of the spike (sMRR) increased (Fig. 1A,B). In addition, spike height (Sh) tended to increase and resting potentials (V_m) shifted towards the hyperpolarized direction, although these two parameters did not appear to alter in layer V/VI neurons during the culture period. To quantify the developmental changes in each neuronal type, the culture period was divided into three different stages, early (7–11 DIV), middle (12–15 DIV) and late (16–52 DIV), according to a previous study in acute cortical slices [7]. These parameters were compared among the three groups.

Table 1
Cultures used in the present experiments[a]

Coculture	Layer	*N*	*M*
VC–VC	II/III	19	20
	V/VI	23	23
LGN–VC	IV	35	40
	Total	77	83

[a] VC–VC, visual cortex-visual cortex coculture; LGN–VC, lateral geniculate nucleus-visual cortex coculture. *N* represents number of cultures. *M* represents the numbers of identified visual cortical neurons which responded with monosynaptic EPSPs or EPSPs followed by IPSPs to electrical stimulation of the partner explant.

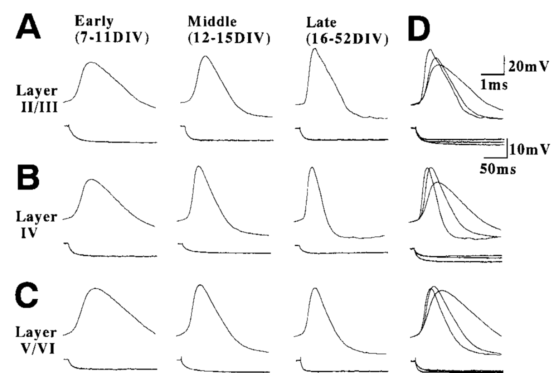

Fig. 1. Representative spikes and passive hyperpolarizing responses of the VC neurons. (A) Upper traces are representative spikes elicited by a depolarizing current pulse injection in layer II/III neurons in the VC of VC–VC at each culture stage. Lower traces, voltage deflections elicited by a small hyperpolarizing current pulse injection (0.1 nA, $>$ 200 ms) in the same cell as in the upper traces. (B,C) Similar to (A), but pulses were applied to layer IV neurons in the VC of LGN–VC (B) and to layer V/VI neurons in the VC of VC–VC (C). DIV, days in vitro. (D) The records were constructed by superimposing the three traces in (A–C). The same scales were applied in (A–D).

During the late stage the average values of T_c and Sw were 43–63% of those during the early stage for all neuronal types. The significance of the differences were tested by ANOVA and Dunnett's test with $P < 0.05$ (Fig. 2A,B). On the other hand, the changes in R_{in}, sMRR, Sh and V_m were not identical for all neuronal types (Fig. 2C–F). The most remarkable change in development was observed in layer II/III neurons that were monosynaptically activated by the partner VC stimulation. R_{in} (mean ± SD, 54.8 ± 6.4 MΩ) was almost half of that at the early stage (99.3 ± 31.0 MΩ). sMRR during the late stage (214.4 ± 35.9 V/s) was almost double of that in the early stage (111.3 ± 25.4 V/s). Sh increased by about 10 mV and V_m shifted slightly but significantly in the hyperpolarized direction by several millivolts. Layer IV neurons receiving LGN input showed similar trends during development, although the difference was not statistically significant for V_m. By contrast, layer V/VI neurons innervated by cortical afferents did not exhibit prominent changes during the culture period. Sh and V_m remained small even during the late stage (Fig. 2E,F). R_{in} and sMRR showed developmental changes which were similar to those found in other layers but the differences were not significant (Fig. 2C,D).

A difference between neuronal types was found in the mature stage as well as in the developmental process. In fact, average T_c of layer II/III neurons at the late stage (14.4 ± 6.4 ms) was two times larger than that of layer V/VI neurons (7.4 ± 1.3 ms), and the values differed significantly between layers ($P < 0.05$, ANOVA; Fig. 2A). Sh during the late stage also differed significantly between layers ($P < 0.05$, ANOVA; Fig. 2E).

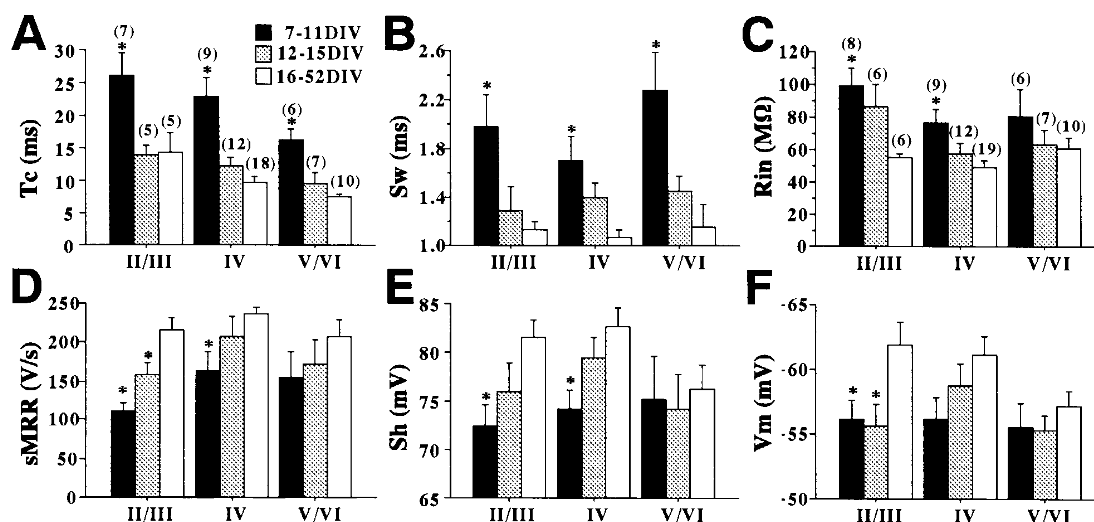

Fig. 2. In vitro changes in the membrane properties of the VC neurons. (A–C) Change in the time constant (T_c), the spike width at half amplitude (Sw) and the input resistance (R_{in}). (D–F) Change in the maximum rate of rise of the spike (sMRR), the spike height (Sh) and the resting membrane potentials (V_m) with culture stages. DIV, days in vitro. Numbers in parenthesis represent the number of recorded neurons; those in (A) are common to (B,D), and those in (C) are common to (E,F). The asterisk indicates that the value differs significantly from that for the late stage ($P < 0.05$, ANOVA and Dunnett's test).

The present study demonstrated that electrical membrane properties of cortical neurons developed more prominently in the upper layers than the deep layers irrespective of afferent types. To date only a few acute slice studies have reported the development of membrane properties for layer II/III [7] and V neurons [9], although neuronal connection patterns were not identified in these studies. The time course of the developmental changes in Sw and R_{in} of the layer II/III neurons [7] was similar to those obtained in this study. Changes in the six membrane properties of the layer V neurons [9] tended to be similar to that obtained in this study. In addition, the values of R_{in} and V_m during the late stage of the coculture are also comparable with those reported for mature cortical neurons in the acute slice [6,14]. Therefore, it is likely that the developmental changes in this culture system reflect those in the normal cortex to a large extent.

The developmental difference between cortical cell types observed in this study may be due to the order of birth of cortical neurons. This is because deep layer neurons are born earlier and upper layer neurons appear later [2,18]. However, this does not seem to be the sole mechanism. The fact that Sh and V_m did not change in layer V/VI neurons, while other parameters such as T_c and Sw developed in the same fashion as those in other layers, suggests the existence of a lamina-specific developmental program [8] which regulates electrical properties. Supporting this view is the fact that the values representing the characteristics during the late stage were not the same for all neuronal types tested. We cannot exclude the possibility that afferent connectivity determines the properties, but it is unlikely that this is a major factor [1], since the developmental changes between layer II/III and IV neurons were much smaller than those between layer II/III and V/VI neurons with the same afferent innervation.

We wish to thank Drs. Y. Komatsu, T. Kurotani, Y. Kawaguchi and J. Kershaw for helpful suggestions, T. Ogawa and N. Inagaki for their encouragement and N. Horimoto, S. Sato and E. Akase for data analysis. This work was supported by Grants-in-Aid for Scientific Research Projects 01770085 and 02220217 from the Ministry of Education, Science and Culture of Japan.

[1] Annis, C.M., Robertson, R.T. and O'Dowd, D.K., Aspects of early postnatal development of cortical neurons that proceed independently of normally present extrinsic influences. J. Neurobiol., 24 (1993) 1460–1480.
[2] Berry, M. and Rogers, A.W., The migration of neuroblasts in the developing cerebral cortex. J. Anat., 99 (1965) 691–709.
[3] Bindman, L.J., Meyer, T. and Prince, C.A., Comparison of the electrical properties of neocortical neurons in slices in vitro and in the anaesthetized rat. Exp. Brain Res., 69 (1988) 489–496.
[4] Bolz, J., Novak, N., Götz, M. and Bonhoeffer, T., Formation of target-specific neuronal projections in organotypic slice cultures from rat visual cortex. Nature, 346 (1990) 359–362.
[5] Götz, M. and Bolz, J., Formation and preservation of cortical layers in slice cultures. J. Neurobiol., 23 (1992) 783–802.
[6] Komatsu, Y., Age-dependent long-term potentiation of inhibitory synaptic transmission in rat visual cortex. J. Neurosci., 14 (1994) 6488–6499.
[7] Luhmann, H.J. and Prince, D.A., Postnatal maturation of the GABAergic system in rat neocortex. J. Neurophysiol., 65 (1991) 247–263.

[8] McConnell, S.K., The determination of neuronal fate in the cerebral cortex. Trends Neurosci., 12 (1989) 342–349.

[9] McCormick, D.A. and Prince, D.A., Post-natal development of electrophysiological properties of rat cerebral cortical pyramidal neurones. J. Physiol., 393 (1987) 743–762.

[10] McCormick, D.A., Connors, B.W., Lighthall, J.W. and Prince, D.A., Comparative electrophysiology of pyramidal and sparsely spiny stellate neurons of the neocortex. J. Neurophysiol., 54 (1985) 782–806.

[11] Molnár, Z. and Blakemore, C., Lack of regional specificity for connections formed between thalamus and cortex in coculture. Nature, 351 (1991) 475–477.

[12] Plenz, D. and Aertsen, A., Neural dynamics in cortex-striatum co-cultures-I. Anatomy and electrophysiology of neuronal cell types. Neuroscience, 70 (1996) 861–891.

[13] Sah, P. and Nicoll, R.A., Mechanisms underlying potentiation of synaptic transmission in rat anterior cingulate cortex in vitro. J. Physiol., 433 (1991) 615–630.

[14] van Brederode, J.F.M. and Snyder, G.L., A comparison of the electrophysiological properties of morphologically identified cells in layers 5B and 6 of the rat neocortex. Neuroscience, 50 (1992) 315–337.

[15] Wolburg, H. and Bolz, J., Ultrastructural organization of slice cultures from rat visual cortex. J. Neurocytol., 20 (1991) 552–563.

[16] Wolfson, B., Gutnick, M.J. and Baldino Jr, F., Electrophysiological characteristics of neurons in neocortical explant cultures. Exp. Brain Res., 76 (1989) 122–130.

[17] Yamamoto, N., Kurotani, T. and Toyama, K., Neural connections between the lateral geniculate nucleus and visual cortex in vitro. Science, 245 (1989) 192–194.

[18] Yamamoto, N., Yamada, K., Kurotani, T. and Toyama, K., Laminar specificity of extrinsic cortical connections studied in coculture preparations. Neuron, 9 (1992) 217–228.

Development of NMDA and non-NMDA receptor-mediated excitatory synaptic transmission in geniculocortical and corticocortical connections studied by organotypic coculture preparations

Katsuya Yamada (a), Nobuhiko Yamamoto (b), and Keisuke Toyama (c).

(a) Department of Physiology, Akita University School of Medicine. 1-1-1 Hondo, Akita 010-8543, Japan.
(b) Department of Biophysical Engineering, Faculty of Engineering Science, Osaka University, Toyonaka, Osaka 560-8531, Japan.
(c) Department of Physiology, Kyoto Prefectural University of Medicine, Kamigyo-ku, Kyoto 602-8566, Japan.

和文要約

生体外に取り出した視覚皮質と視床外側膝状体およびもう一つの視覚皮質との間に形成された層特異的神経結合の試験管内における電気生理学的発達について調べた。その結果、興奮性アミノ酸受容体の内、non-NMDA受容体を介するEPSPの発達には層による顕著な違いは見られなかった。しかし、NMDA受容体を介する発達は神経結合特異的な発達の時間経過を示し、更に層特異性も示した。興奮性伝達における各受容体の寄与が培養日数の経過により正常に類似した形で変化したことは、脳における神経伝達の発達において少なくとも興奮性アミノ酸受容体の発現にはパターン化された感覚入力が必要ないことを示唆している。また以上の結果より、幼弱期に取り出した脳組織は通常の中枢神経系の培養条件下で少なくとも神経細胞に関してはかなりの機能的成熟が可能であり、またその活動を数ヶ月以上の長期にわたり維持できることも明らかとなった。

Abstract

Development of *N*-methyl-D-aspartate (NMDA) and non-NMDA receptor-mediated excitatory synaptic transmission was studied in the visual cortex by using organotypic slice cocultures. A slice of visual cortex (VC) dissected from newborn rats was cocultured with either a chunk of embryonic lateral geniculate nucleus (LGN) or another VC. During 7-38 days *in vitro* (DIV), geniculocortical monosynaptic excitatory postsynaptic potentials (EPSPs) were recorded from layer IV neurons in response to stimulation of the LGN in cocultures of the VC with the LGN. Similarly, corticocortical monosynaptic EPSPs were recorded from layers II/III and V/VI neurons in cocultures of two VCs when stimulating the partner VC. The initial slopes of the non-NMDA and NMDA receptor-mediated components of the EPSPs, which were dissociated pharmacologically, were assessed and compared among three different culture stages, early (7-11 DIV), middle (12-15DIV) and late (17-38 DIV). With progression of the culture stage, the non-NMDA component tended to increase in both the geniculocortical and corticocortical connections. In contrast, the NMDA component exhibited distinct developmental changes. The NMDA component in layer IV neurons, which receive geniculate inputs, showed a transient increase in the middle stage. In the corticocortical connection, the magnitude of the NMDA component was large in the early stage and maintained through all culture stages in layer V/VI cells, whereas in layer II/III cells it

decreased sharply by the late stage. **Our results suggest that glutamatergic transmission in the visual cortex develops differently in the geniculocortical and corticocortical connections.**

Introduction

Excitatory synaptic transmission in mammalian neocortical circuits is mediated primarily by *N*-methyl-D-aspartate (NMDA) and non-NMDA subtypes of glutamate receptors (Watkins, 1989). In the visual cortex, developmental changes or modification of these synaptic transmissions are thought to be a basis for plasticity of cortical circuitry and the resulting visual responses (Hebb, 1949; Shatz, 1990). A possible mechanism in this modification would be that non-NMDA receptor-mediated synaptic transmission is regulated by NMDA receptor-dependent processes (Brown et al., 1990; Madison et al., 1991; Bliss & Collingridge, 1993; Klintsova & Greenough, 1999). From this point of view, it is of interest to study how non-NMDA and NMDA receptors are expressed during development at specific synapses in the visual cortex since this may be related to the modifiability of each synapse during the formation of neocortical circuitry.
A number of anatomical binding and immunohistological assays (Bode-Greuel & Singer, 1989; Kumar et al., 1994; Catalano et al., 1997), *in vivo* visual responses (Hagihara et al., 1988; Fox et al., 1989)**,** and *in vitro* slice studies (Jones & Baughman, 1988; Shirokawa et al, 1989; Kato et al., 1991; Carmignoto & Vicini, 1992; Iwakiri & Komatsu, 1993) have suggested that NMDA receptors are heterogeneously expressed in the visual cortex. However, the results from these studies do not always seem consistent with each other (Kaczmarek et al., 1997). In fact, different contributions of the NMDA receptors to excitatory transmission during development have been reported for each layer of the visual cortex from studies on the amount of the NMDA receptor expression, visual responsiveness which is sensitive to an NMDA receptor antagonist, and studies of NMDA receptor-mediated EPSPs in acute slices. These discrepancies might arise partly because of the difficulty in isolating and identifying synaptic connections in the visual cortex. Moreover, in most electrophysiological studies, the NMDA receptor contribution was investigated only in a particular layer at a particular developmental stage and no comparison was made among each synaptic pathway in the visual cortex.

Our aim in the present study is to elucidate systematically how non-NMDA and NMDA synaptic transmissions develop in divergent cortical circuits. For this purpose, we used organotypic cocultures (Gähwiler, 1988) comprised of a slice of visual cortex (VC) with either a chunk of lateral geniculate nucleus (LGN) or another VC**,** and compared EPSPs that were mediated by NMDA receptors (NMDA EPSPs) with those mediated by non-NMDA receptors (non-NMDA EPSPs) in identified geniculocortical and corticocortical connections. We and other groups have previously demonstrated that these two types of afferent connections are established *in vitro* with essentially the same laminar specificity as found *in vivo* (Yamamoto et al., 1989, 1992; Molnár & Blakemore, 1991; Bolz et al., 1992). Thus, these preparations may provide a suitable model to study the development of each receptor subtype in geniculocortical synapses with minimum contamination by other corticocortical synaptic connections, and *vice versa*.

Materials and methods

Culture

Cocultures of the VC with the LGN or with another VC were prepared as described

previously (Yamamoto, et al., 1992). In brief, blocks of LGN were dissected from embryonic day 15-17 fetuses (Sprague-Dawley), and VC slices (300-400 μm thickness) were coronally sectioned from the occipital cortices of newborn rats (postnatal day 2-3). A block of LGN and a slice of VC, or a pair of VC slices were plated side by side on a collagen-coated membrane (Transwell-COL, Coster, Cambridge, MA, USA), separated by a distance of about 0.5 mm. They were maintained in a hormone-supplemented DMEM/F-12 medium (Life Technologies, Grand Island, NY, USA) in 95% air and 5% CO_2 at 37°C. In this way, the cultures were maintained for up to several weeks to months (Yamamoto, et al., 1989).

Recording solution and chamber

For electrophysiological recordings of evoked potentials, the cultures were transferred to an interface-type recording chamber and continuously perfused at a flow rate of 0.4-0.6 ml/min with Krebs-Ringer solution (in mM; 127 NaCl, 2 KCl, 4 $CaCl_2$, 4 $MgSO_4$, 1.24 KH_2PO_4, 26 $NaHCO_3$, 10 glucose; pH 7.4) saturated with 5% CO_2 in air. Glycine (1 μM) was routinely added to the solution to fully activate the glycine site of NMDA receptor (Johnson & Ascher, 1987). High concentrations of calcium and magnesium ions (4 mM) were used to reduce excessive polysynaptic activities (Streit et al., 1989). The temperature of the bathing solution in the recording chamber was maintained at 33°C. Cultures were allowed to equilibrate in the recording solution for at least 1h before each recording session. To record spontaneous activity, cocultures were perfused in a manner similar to that described above, but with the DMEM/F12 culture medium containing the hormone-supplement saturated with 5% CO_2 in air.

Stimulation and recordings

As illustrated in Fig.1, stimulating electrodes (s), consisting of three to four platinum-iridium wires (diameter, 30 μm; interpolar distance, 250 μm), were placed in the LGN or the partner VC. Constant current pulses with intensities of 0.06-0.4 mA were applied for a duration of 100 μs.

Glass microelectrodes filled with 2M K-methylsulphate (electrical resistance, 80-200 MΩ) were used for the intracellular recordings. A bridge circuit was used to record membrane potentials while currents were injected through the recording electrode (Axoprobe-1A, Axon Instruments, Foster City, CA, USA). **Glass microelectrodes filled with 0.9 % NaCl and pontamine sky blue (resistance, 5-10 MΩ) were used to record the spontaneous activity in the cortical explant. The spontaneous unit activity was usually recorded for more than 10 min.** Signals were digitized and stored on an IBM PC-AT compatible computer and videotapes.

Input resistance was determined by injecting a small hyperpolarizing current pulse (0.05-0.1 nA, 200-500 ms) to the neuron and measurements were taken at the plateau of the voltage response. Spike height was determined by injecting a depolarizing (just above threshold) current pulse. The resting membrane potential was determined by the difference between the potentials before and after withdrawal of the electrode from the recorded neuron.

With the assistance of extracellular field potentials or current source density (Mitzdorf & Singer, 1978) profiles elicited by electrical stimulation of the partner explant, the approximate laminar location for the recording of monosynaptic EPSPs was predetermined. In the LGN-VC cocultures, this region, which elicited field potentials with the shortest onset latency, was in the middle cortical layer (300-500 μm from the pial surface) of the VC. In the VC-VC cocultures, the region was in the upper and lower layers (150-300 μm and 500-800 μm from the pia, respectively; Yamamoto, et al., 1992).

Quantification of non-NMDA and NMDA EPSPs

To evaluate non-NMDA and NMDA receptor-mediated synaptic components, EPSPs were quantified by measuring the initial slope, as determined from the line connecting the 10 and 90 % values of the peak amplitude. The magnitude of these EPSP slopes was compared among sampled neurons at a restricted membrane potential because of the well-known voltage-dependency of NMDA EPSPs (Nowak, et al., 1984; Hestrin et al., 1990). In practice, a number of small steps (0.05-1 nA) of both depolarizing and hyperpolarizing current pulses (200-500 ms) were applied to every neuron tested, and EPSPs were evoked at the plateau of the voltage deflection. Actual membrane potentials were determined with reference to the resting membrane potentials after the experiment. From the set of EPSPs, an EPSP which was elicited at a membrane potential at -54 to -58mV (–55.9±1.4mV on average, n=51) was selected and compared before and after pharmacological manipulations. Around this voltage, the initial slope of non-NMDA EPSPs did not change noticeably and the change in the slope of NMDA EPSPs was monotonic against membrane potential.
Electrical stimulation, with an intensity of 1.4±0.2 times the threshold current (T) necessary to evoke a detectable EPSP, was applied to all neurons tested since the stimulus strength can also affect the magnitude of EPSPs. Under these conditions, both non-NMDA and NMDA components were discerned without interference of orthodromic spikes and robust polysynaptic activities.

The duration of the NMDA EPSPs was also measured. The duration was determined as the width at half amplitude of the EPSP peak.

Pharmacological experiments

DL-2-amino-5-phosphonovalerate (APV) (Sigma, St. Louis, MO, USA) and 6,7-dinitro-quinoxaline-2,3-dione (DNQX) (Tocris Neuramin, Essex, England) were used to block NMDA and non-NMDA synaptic components, respectively. DNQX was used at 20 μM, since large doses of DNQX are known to suppress both non-NMDA and NMDA components. Both drugs were applied to the bath and their effects reached a saturated state by about 20 min after application. Therefore, we estimated the effect of the drugs at least 25-30 min after application.

Histology

Nissl staining was carried out to determine laminar locations of recorded neurons. After the recording session, cultures were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, then cut into 30 μm-thick sections, and stained by a standard cresyl violet procedure.

Statistical analysis

Values were expressed as mean ±S.D., unless otherwise noted. All statistical analyses were performed using commercially available software (JMP ver.3 and Stat View ver.4.5, SAS Institute, Cary, NC, USA).

Results

General features

A bundle of afferent and efferent fibers connected the cocultured explants by about a week *in vitro* (Fig. 1). The laminar organization of the VC in the cultured condition was similar to that observed in normal visual cortex as has been described previously (Yamamoto, et al., 1989, 1992, 1997).

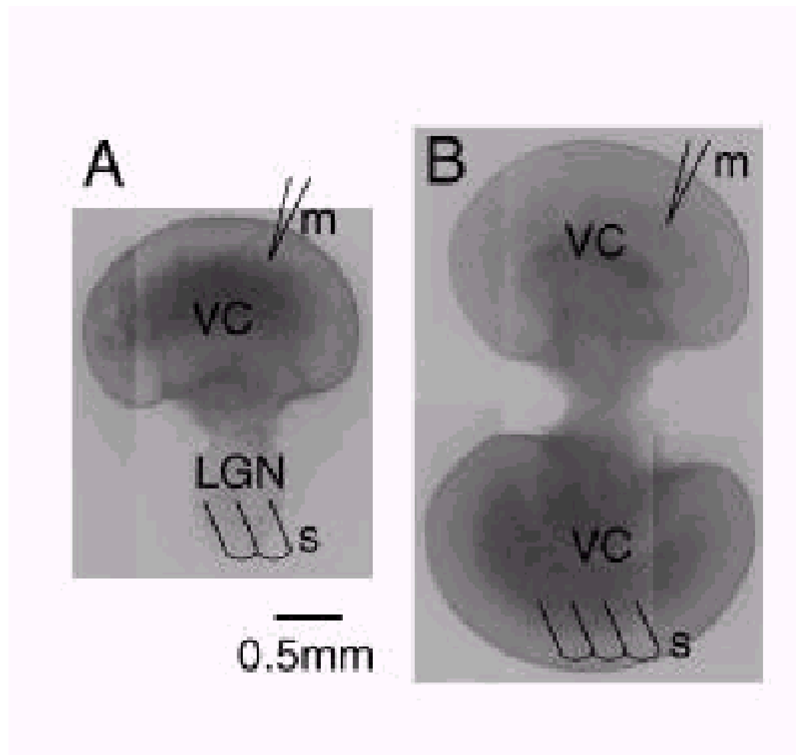

Fig.1 Experimental arrangements in coculture preparations. (A) Lateral geniculate nucleus (LGN)-visual cortex (VC) coculture preparation photographed under phase-contrast microscopy, and arrangements of stimulating (s) and recording (m) electrodes. (B) Similar to A but for VC-VC coculture. Calibration is common to A and B.

In the present study, we analyzed neurons with stable (> 1hr) resting membrane potentials deeper than -50 mV. These neurons showed spike firing properties similar to those in regular spiking neurons (McCormick et al., 1985; Wolfson et al., 1989). We found that electrical stimulation of the partner explant rarely evoked postsynaptic potentials (PSPs) in the VC neurons before 7 days in vitro (7 DIV). Therefore, electrophysiological experiments were conducted between 7 and 38 DIV, and we compared the results among early (7-11 DIV), middle (12-15 DIV) and late (17-38 DIV) culture stages (Table 1) according to a previous study in acute cortical slices (Luhmann & Prince, 1991). Thus, 18 layer IV neurons in 18 LGN-VC cocultures and 33 layers II/III and V/VI neurons in 33 VC-VC cocultures were recorded successfully. **Spontaneous activity was not observed in Krebs-Ringer solution where evoked potentials were recorded, but it was found when the recordings were made in the culture medium. Cortical neurons sampled from all layers (9-21 DIV) exhibited spontaneous firing with a frequency range of 0.08-8.3 Hz (mean, 1.8 ± 2.1, n=17). Spontaneous activity was also observed in LGN neurons (0.05-5.4 Hz; mean, 1.7 ± 1.6, n=26).**

Table 1. Cultures used in the present study and membrane properties of the monosynaptic neurons recorded in the VC.

coculture	layer	C	N	culture stage	Rin (MΩ)	Sh (mV)	Vm (mV)
VC-VC	II/III	5	5	E (7-11 DIV)	72.0±27.4	75.0±7.2	-58.8±2.8
		5	5	M (12-15 DIV)	89.0±34.9	77.6±6.8	-56.4±3.8
		5	5	L (17-37 DIV)	55.6±6.8	81.2±5.0	-62.2±4.9
LGN-VC	IV	6	6	E (9-11 DIV)	75.2±13.6	73.5±6.2	-56.3±5.9

		6	6	M (13-15 DIV)	61.3±28.2	82.3±8.7	-58.8±6.9
		6	6	L (18-26 DIV)	48.0±23.0	80.0±10.2	-59.7±4.9
VC-VC	V/VI	5	5	E (8-10 DIV)	90.4±40.5	70.0±12.3	-54.0±5.4
		6	6	M (12-15 DIV)	55.8±13.9	70.8±10.0	-53.3±2.8
		7	7	L (18-38 DIV)	65.6±21.3	74.1±8.3	-57.6±4.7
	Total	51	51	(7-38 DIV)	67.4±26.6	76.0±8.9	-57.4±5.2

VC-VC, visual cortex-visual cortex coculture; LGN-VC, lateral geniculate nucleus-visual cortex coculture. C and N represent the number of cocultures used and VC cells which responded with monosynaptic EPSPs for electrical stimulation of the partner explant, respectively. E, M, L and DIV represent the early, middle, late culture stage and days **in vitro*****. Rin represents membrane input resistance which was determined by injecting a small hyperpolarizing (0.05-0.1 nA, 200-500ms) current pulse. Sh, spike height determined by injecting depolarizing (just above threshold) current pulse. Vm, resting membrane potential. Data are expressed by mean ± S.D. The number in the parenthesis represents minimum and maximum days*** **in vitro** ***of the culture.***

Monosynaptic responses in geniculocortical and corticocortical connections in coculture

To evaluate synaptic transmission in the visual cortex, monosynaptic EPSPs elicited in the cocultured VC by stimulating the partner explant were analyzed. EPSPs obtained from the presumably monosynaptic regions (see Materials and Methods) had a wide range of onset latency (4.1-9.6 and 4.0-11.2 ms for LGN-VC and VC-VC, respectively), which was probably due to the slow conduction velocity of afferent fibers and variability of the distance between the recording and stimulation electrodes in each preparation (Fig.1; Yamamoto, et al., 1989). Moreover, some of the EPSPs could be polysynaptic. Therefore, we adopted a criterion for identification of monosynaptic EPSPs based on constant latency at different stimulus intensities and during high-frequency stimulation (Sah & Nicoll, 1991). Figs.2A-B show representative monosynaptic EPSPs elicited in a layer IV neuron by LGN stimulation of an LGN-VC coculture (21 DIV). As the stimulus intensity was increased, the initial slope of the EPSP gradually increased without changing its onset latency. For higher frequency stimuli (20-50 Hz), the onset latency was constant, although the initial slope decreased. On the other hand, we considered EPSPs polysynaptic when the onset latency increased with the reduction of stimulus intensity, or it increased during high-frequency stimulation (Figs.2C-D). For all stages of LGN-VC cocultures, EPSPs that met these criteria were obtained only in layer IV of the VC, although we did not try to impale deep layer VI neurons. Similarly in VC-VC cocultures, monosynaptic EPSPs were easily found by stimulation of the partner VC in layers II/III and V/VI, but not in layer IV of the VC. These results indicate that electrophysiologically identified monosynaptic geniculocortical and corticocortical synapses are formed and function at early stages in coculture and that they can be maintained for at least several weeks.

Inhibitory postsynaptic potentials (IPSPs) usually followed EPSPs. These IPSPs were easily discriminated from EPSPs by current pulse injections (see below). EPSPs analyzed in the present study were confined to those whose initial component was not influenced by the IPSPs and met all the above criteria for being monosynaptic.

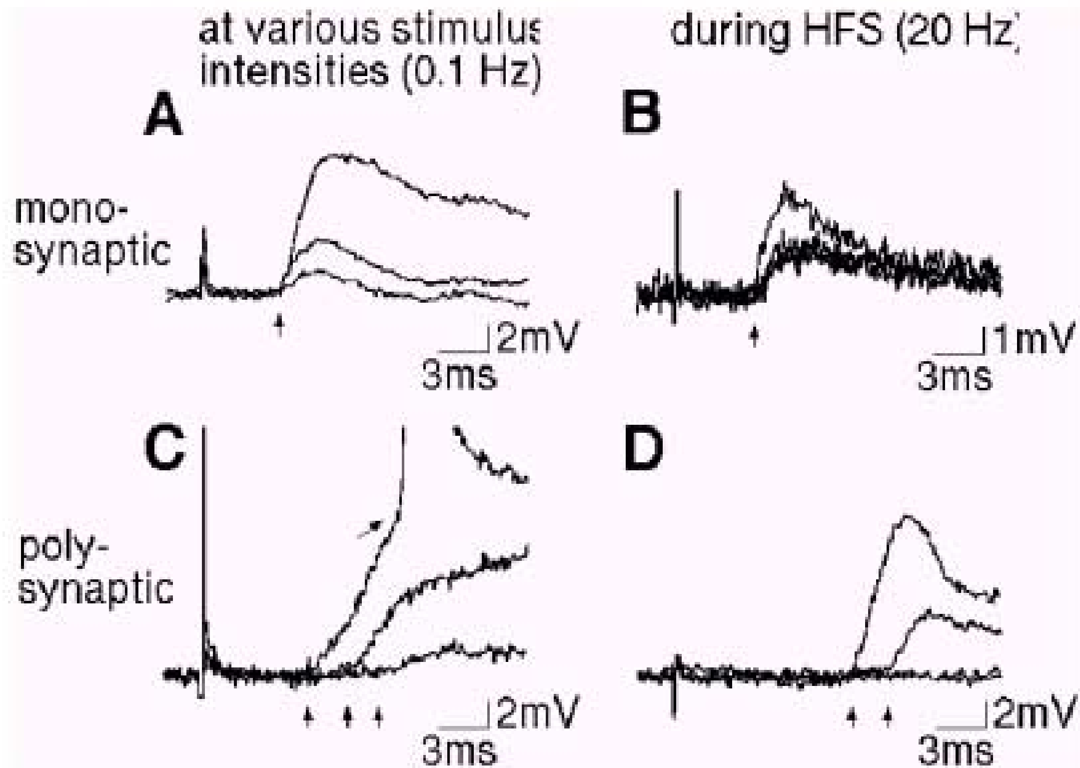

Fig.2 Identification of mono and polysynaptic excitatory postsynaptic potentials (EPSPs). (A, B) Superimposed traces of monosynaptic EPSPs evoked in a layer IV neuron of a VC slice of LGN-VC coculture (21 DIV). (A) Those with the LGN stimulation at intensities of 1.1, 1.4 and 1.8 times the threshold stimulus current (T; it was 110 µA in this example) needed for evoking postsynaptic potentials. Upward arrow indicates the onset of EPSPs. (B) Those during high frequency stimulation (HFS, 20 Hz, 1.4 T) of the LGN. The records were constructed by superimposing four representative traces (1st, 7th, 13th and19th) of EPSPs during the HFS. (C, D) Similar to A, B, but for polysynaptic EPSPs evoked in upper layer II/III neurons of VC explants by the LGN stimulation (18 DIV). (C) Those with stimuli at 1.1, 1.4 and 2.1 T (T, 70 µA). Arrows indicate variable onset latencies. An oblique arrow indicates an orthodromic spike potential. (D) Those during the HFS (1.4 T; T, 140 µA) in another neuron.

Development of non-NMDA and NMDA receptor-mediated synaptic components

Development of non-NMDA and NMDA receptor-mediated components of synaptic transmission in the visual cortex were studied in the cocultured VC by stimulating the partner explant. The monosynaptic EPSPs elicited in layer IV neurons by LGN stimulation were comprised of non-NMDA and NMDA components. This was verified pharmacologically. Fig. 3A depicts representative PSPs recorded from a layer IV neuron by stimulating the LGN in an LGN-VC coculture (15 DIV). Bath application of 20 µM DNQX, a non-NMDA receptor antagonist, clearly reduced the initial slope of the EPSP but its onset latency remained unchanged (Fig. 3B). This indicates that the fast component of the EPSPs were largely comprised of the non-NMDA receptor-mediated component. The resultant EPSP with a slow rising slope was larger at more depolarized potentials, suggesting that it was mediated by NMDA receptors. Indeed, addition of 50 µM of NMDA receptor antagonist APV completely blocked the EPSP (Fig.3C). In addition, it was noted that the IPSP following the EPSP was also completely abolished in the presence of DNQX, indicating that it was polysynaptic in origin. The monosynaptic EPSPs elicited in layers II/III and V/VI neurons of the VC by stimulation of another VC were also comprised of non-NMDA and NMDA components, and exhibited essentially similar pharmacological characteristics.

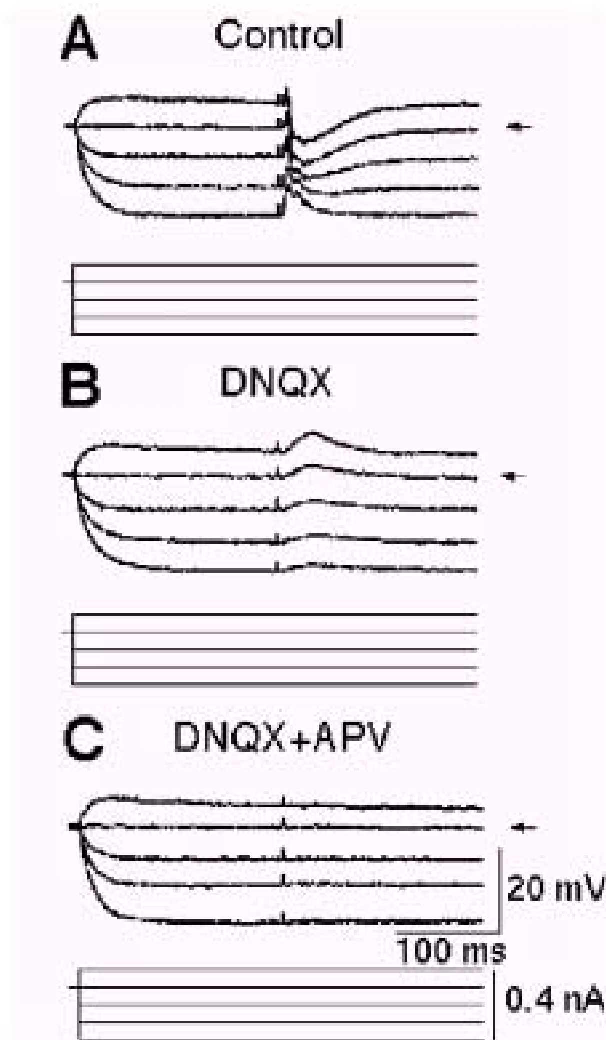

Fig.3 Effects of ionotropic glutamate receptor antagonists on postsynaptic potentials. (A) Upper traces represent postsynaptic potentials evoked in a layer IV neuron of LGN-VC coculture (15 DIV) by electrical stimulation of the LGN in control solution. Membrane potential was depolarized or hyperpolarized by small steps (0.1 nA) of current pulse injection with duration of > 200 ms (lower traces). (B) The same neuron as in A, but in solution containing the non-NMDA receptor antagonist 6,7-dinitro-quinoxaline-2,3-dione (DNQX; 20 µM). (C) Similar to B, but in the presence of DNQX (20 µM) and the NMDA receptor antagonist DL-2-amino-5-phosphonovalerate (APV; 50 µM). Arrowheads indicate –56 mV.

By these pharmacological methods, the non-NMDA and NMDA components in geniculocortical and corticocortical transmission were identified and compared quantitatively at three culture stages. For this purpose, the initial slope of each component of the monosynaptic EPSPs elicited by intermediate stimulus strengths (1.4 T) was measured before and after application of DNQX at the same membrane potential (-56 mV, see Materials and Methods).

Fig. 4 depicts representative geniculocortical and corticocortical EPSPs. A common feature in these synaptic connections was that the total and non-NMDA EPSPs were much larger than NMDA EPSPs and tended to increase with the progression of the culture stage (Fig. 4). This was confirmed quantitatively. The initial slope of the total

EPSPs (Fig. 5A) was about 10 times larger than the NMDA EPSPs (Fig. 5C). The total EPSPs in the late stage were roughly 1.3-fold larger (1.11±0.48 V/s) than those in the early stage (0.83±0.52 V/s), but the difference between these two stages was not statistically significant for each transmission (Fig. 5A). A similar developmental change was also found in the initial slope of the non-NMDA EPSPs: it increased slightly on average, but the difference between the early and late stages was not significant (Fig. 5B).

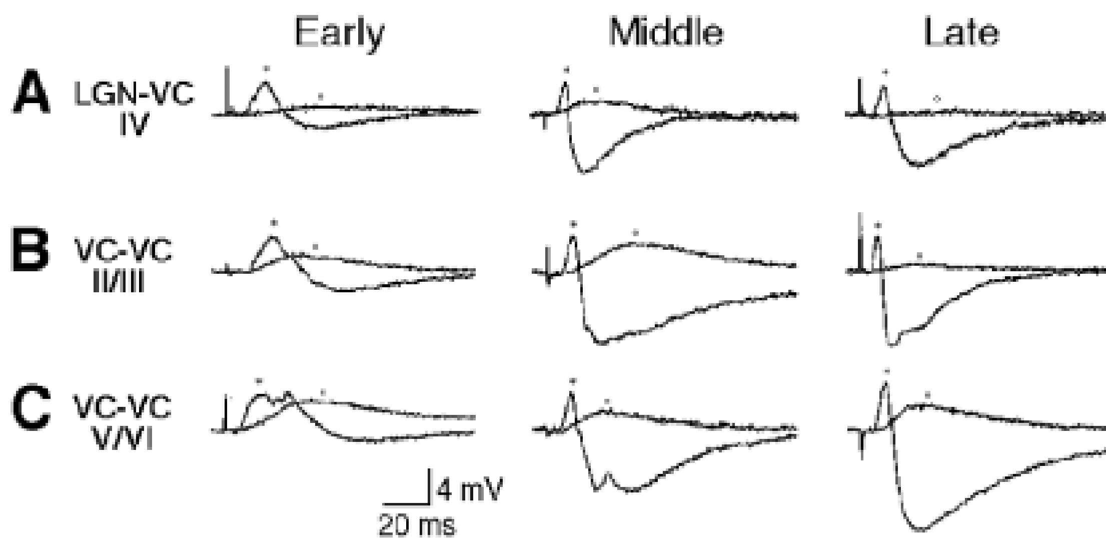

Fig.4 EPSPs and NMDA receptor-mediated components in three synaptic connections during different developmental stages. (A) Superimposed postsynaptic potentials evoked in layer IV neurons during the early, middle and late stage of LGN-VC cocultures elicited by LGN stimulation in control (asterisk) and in the presence of 20 µM DNQX (open circle). (B) Similar to A but those evoked in layer II/III neurons of VC-VC cocultures by stimulation of the partner VC slice. (C) Similar to B but those evoked in layer V/VI neurons. All traces were recorded at the same membrane potential from –54 to -58 mV. Calibrations were common to all traces.

By contrast, the NMDA EPSPs changed remarkably (Figs. 4 and 5C). In geniculocortical transmission, the initial slope of the NMDA EPSPs was about 0.035 V/s (membrane potential –56 mV) during the early stage, was three times larger at the middle stage and then was much reduced during the late stage. The differences between the middle and the other stages were statistically significant ($p<0.05$, ANOVA and Bonferroni/Dunn test, n=6, 6 and 6 for the early, middle and late stages, respectively. Fig. 5C). Since absolute values of the EPSP slope might be affected by the intensity of stimulation, we also compared the ratio of the NMDA EPSPs to the total EPSPs for each culture stage (Fig. 5D). The development of the ratio was essentially the same as that seen in the absolute value, except that the value appeared slightly larger during the early stage and smaller at the late stage.

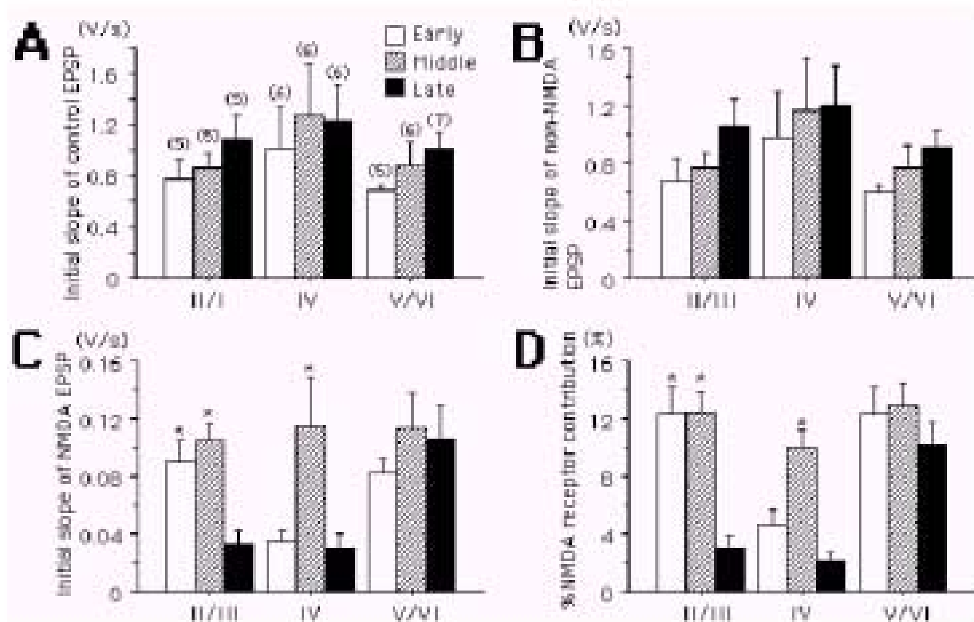

Fig.5 Quantitative analysis of developmental changes in the non-NMDA and NMDA receptor-mediated EPSPs. (A) Initial slopes of EPSPs recorded from layers II/III and V/VI neurons in VC-VC and from layer IV neurons in LGN-VC in control solution during the early (7-11 DIV; empty), middle (12-15 DIV; hatched) and late (17-38 DIV; black) stages. (B) Similar to A, but initial slopes of non-NMDA EPSPs, which were calculated by subtracting slopes in the presence of 20 µM DNQX from those in control solution. (C) Initial slopes of NMDA EPSPs measured in the presence of 20 µM DNQX. (D) Similar to C, but ratio of NMDA EPSPs to total EPSPs. Values were obtained at the same membrane potential from –54 to -58 mV, and expressed as mean±S.E.M. Numbers in parenthesis indicate number of recorded neurons, and are common to A-D. An asterisk indicates that the value differs significantly (p<0.05, ANOVA; Bonferroni/Dunn test) from that at the late stage for layer II/III neurons, those at the early and late stage for layer IV neurons.

In corticocortical transmission, the development of NMDA EPSPs was distinct from that in geniculocortical transmission (Fig. 4B-C). A marked difference in time-course was found even between the upper and deep layers in corticocortical transmission. For layer II/III neurons that received corticocortical inputs, the initial slope of the NMDA EPSPs and their ratio to total EPSPs were 0.09 V/s and 12% during the early stage (Fig. 5C-D). These values were significantly larger than those at the early stage of layer IV neurons that received geniculocortical inputs (p<0.005, ANOVA and Bonferroni/Dunn). They remained at a high level during the middle stage, but reduced significantly by the late stage (p<0.01, ANOVA and Bonferroni/Dunn, n=5, 5 and 5 for the early, middle and late stages, respectively). Development of the NMDA component recorded for layer V/VI neurons in corticocortical transmission was quite different from that for layer II/III neurons: the NMDA component did not reduce significantly even at the late stage (Fig. 4C). The absolute value and ratio of the initial slope of the NMDA EPSPs during the late stage of layer V/VI neurons were 0.10 V/s and 10%, respectively. These values were significantly larger than those at the same stage of layer II/III neurons in VC-VC cocultures and layer IV neurons of the LGN-VC coculture (p<0.01, ANOVA and Bonferroni/Dunn; n=5, 6 and 7 for the layer II/III, IV and V/VI neurons, respectively. Fig. 5C-D).

Developmental changes in the duration of the NMDA EPSPs

It has been reported that the duration of the NMDA receptor-mediated excitatory postsynaptic currents (EPSCs) evoked in layer IV neurons changes with development (Carmignoto &

Vicini, 1992). To examine whether similar changes occur in cocultured VC slices, the duration of the NMDA EPSPs at their half amplitude was measured (Fig. 6). In geniculocortical transmission recorded in layer IV neurons, this value significantly decreased with progression of the culture stage. The mean value during the late stage was about one-third of that during the early stage. A similar tendency was observed in corticocortical transmission, although the decrease was slightly less than that in geniculocortical transmission and insignificant in layer II/III neurons.

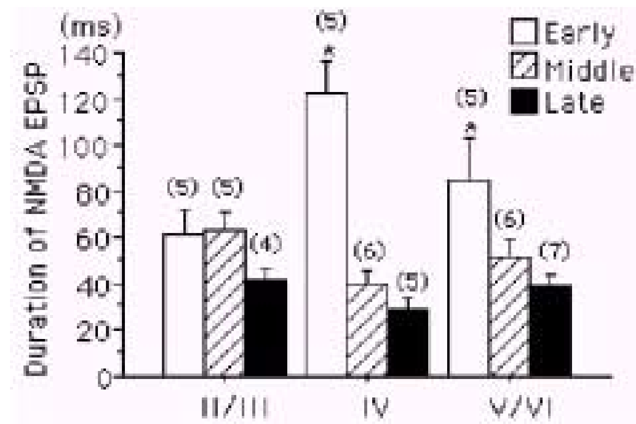

Fig.6 Developmental changes of the duration of NMDA EPSPs. The duration (mean±S.E.M) was determined as the width at half amplitude of the EPSP peak measured at the same membrane potential from -54 to -58 mV in the presence of 20 µM DNQX during the early (empty), middle (hatched) and late (black) stages. An asterisk indicates that the value differs significantly ($p<0.05$, ANOVA; Bonferroni/Dunn test) from that at the middle and late stage for layer IV neurons, at the late stage for layer V/VI neurons. Numbers in parenthesis represent number of recorded neurons. EPSPs that were too small for reliable measurement of the duration at -56 mV were excluded from the analysis.

Discussion

Our results show the developmental sequence of non-NMDA and NMDA receptor-mediated synaptic response in different cortical circuits *in vitro*. At all culture stages the amplitude of the non-NMDA EPSP, estimated by its initial slope, was about 10 times larger than that of the NMDA EPSP at the same membrane potential (-56 mV). During culture development, the NMDA EPSP showed remarkable changes, while the total and non-NMDA EPSPs only increased slightly. In addition, the developmental time-course of the change in the NMDA EPSPs was quite different, not only between geniculocortical and corticocortical connectivity, but also between upper and deep layer neurons that received direct cortical inputs.

Specificity of afferent connectivity in cocultures

Although specific neural pathways are stimulated far more selectively in the cocultures than in the acute slice preparation of the visual cortex, responses evoked in this study could still be contaminated by some unintended component (Bolz et al., 1992). Indeed, in LGN-VC cocultures it is possible that stimulation of the LGN could antidromically activate layer VI neurons projecting to the LGN (Yamamoto, et al., 1992). It has been reported that in normal visual cortex layer VI neurons send their axon collaterals to layer IV (Burkhalter, 1989). However, we have previously shown that in our coculture preparation the contribution of layer VI neurons to responses of layer IV neurons was small. This was because almost no

response was evoked in layer IV neurons by layer VI stimulation after degeneration of LGN axons was produced by severing axon bundles between the VC and the LGN (Yamamoto, et al., 1989). Therefore, it is likely that under our experimental conditions, EPSPs recorded from layer IV neurons are produced primarily by stimulation of LGN neurons.
Our previous electrophysiological and morphological studies of VC-VC cocultures showed that the neurons in the partner VC slice projected to the VC slice with a tendency to avoid layer IV (Yamamoto et al., 1992), as is seen in normal visual cortex (Miller & Vogt, 1984). These corticocortical synaptic connections form symmetrically between the two VC slices. Thus, in VC-VC cocultures electrical stimulation of the partner VC may activate two sets of axons: those that originate in the neurons of the partner VC and those that arise from neurons located in the recorded VC. In the latter case, if the neurons have sent long-distance projections across the white matter, EPSPs may be produced in layer II/III and V/VI neurons through antidromic activation of the axon collaterals of the neurons. However, it should be noted that within the range of our stimulation strength, antidromic spikes were rarely recorded in the VC. Thus, it is more likely that the VC neurons were orthodromically activated in our recordings.

Development of total and non-NMDA EPSPs

In the cocultured cortex, the initial slope of the total EPSP increased slightly (1.3 fold on average) between the early and late stages of both geniculocortical and corticocortical connections, although differences in the change between culture stages were not statistically significant in each transmission. In accordance with this result, Iwakiri and Komatsu (1993) have reported a developmental increase in EPSPs in layer III and IV neurons in cat visual cortex slices. In that study, monosynaptic EPSP slope evoked by the white matter stimulation was about 3.5 times larger for adult cats (7 V/s) than for kittens (2 V/s; 1 week-old). Similar changes also seemed to occur in rat cortex (Kato, et al., 1991; Burgard & Hablitz, 1993), although detailed values were not explicitly indicated in those studies. In addition, it is noted that, when using a comparable stimulus strength, the absolute values of the EPSP amplitude and slope observed in the cocultures were in a range similar to that seen in the adult rat VC (approximately 4-10 mV and 1-2V/s, respectively; Sah & Nicoll, 1991). Therefore, it is more likely that changes in the total EPSP of the cocultured VC neurons exhibited a similar tendency to that observed in normal cortex, but the change was smaller in the coculture than that normally observed *in vivo*.
The initial slope of non-NMDA EPSPs also tended to increase slightly with the culture stage regardless of connectivity. Similar development of the non-NMDA component has been suggested in layer III and IV neurons of cat visual cortex slices (Iwakiri & Komatsu, 1993). This electrophysiological finding also appears to be consistent with data from an autoradiographic ligand binding study to alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors in the rat visual cortex (Kumar et al., 1994). **Taken together, the total and non-NMDA EPSPs in cultured cortical neurons are likely to develop in a fashion similar to those *in vivo*, but those in the coculture might not develop as fully as those *in vivo*.**

Development of NMDA EPSPs in geniculocortical transmission

For layer IV neurons of the VC that received geniculocortical inputs, the NMDA EPSPs were relatively small during the early stage, peaked at the middle stage, and disappeared at the late stage. Evidence in accordance with these results has been reported in *in vivo* visual responses (Hagihara et al, 1988; Fox et al., 1989) and in acute slices (Kato et al., 1991; Iwakiri & Komatsu, 1993), although synaptic connectivity was not identified in those studies. **In**

particular, a previous study of rat VC slices has demonstrated that NMDA EPSP amplitudes in layer II-IV neurons at two weeks of age (about 3.2 mV) were significantly greater than those in the adult (2.0 mV; stimulus strength, just subthreshold levels for spike elicitation; Kato et al., 1991). A comparable change in the amplitude of the NMDA EPSP was obtained in the cocultures, where the amplitudes at the middle and late stages were 2.55±1.26 and 0.74±0.13 mV, respectively. Moreover, in neurons from layers III and IV of cat visual cortex slices, typical NMDA EPSP slopes (0.43 V/s) obtained from 5-week-old cats using a weak stimulus were about three times larger than those in both 1-week-old and adult cats (0.15 V/s) (Iwakiri & Komatsu, 1993). This was compatible with the increase from the early (0.035 V/s) to the middle stage (0.115 V/s) observed in the coculture.

In accordance with our present results, APV-sensitive [^{3}H] glutamate binding (Bode-Greuel & Singer, 1989) and immunoreactivity to NMDAR1 (Catalano et al., 1997) in cat visual cortex have suggested that the NMDA receptor decreases by the mature stage. In contrast, a [^{3}H] MK-801 binding study in rat has shown a monotonic increase of NMDA receptors in layer IV during postnatal life (Kumar et al., 1994). However, it is noted that thalamocortical synapses comprise only about 10-15% of excitatory synapses in layer IV neurons. Most of the remaining synapses originate from spiny stellate neurons in layer IV (White & Rock, 1980). In fact, the latter synapses are known to have substantial NMDA components in mouse barrel cortex (Fleidervish et al., 1998). Thus, it is possible that, as a whole, the NMDA synapses in layer IV neurons increased monotonically, even though NMDA components of geniculocortical synapses peaked during development. This idea is further supported by a recent result from the somatosensory barrel cortex of the rat (Crair & Malenka, 1995).

Another feature in the coculture development was that the duration of the NMDA EPSP of the geniculocortical transmission was decreased with culture stage (122 ms and 29 ms on average during the early and late stages, respectively). A similar change in the duration of the NMDA component has been reported for layer IV neurons in acute cortical slices of the rat (Carmignoto & Vicini, 1992). In that study, NMDA EPSCs were described by a double exponential function with a fast and slow component. The relative contribution of the slow component (time constants, 200-300 ms) to the EPSCs decreased from 92 % at day 12 to less than 20% in the adult. A decrease in the EPSP duration has also been reported in layer II/III and IV neurons of the cat visual cortex, where the duration of the NMDA EPSP was about 70 ms for one week-old animals and 25 ms for adult animals (Iwakiri & Komatsu, 1993). Thus, in consideration of these facts, our results not only confirm previous findings, but also clarify the development of the NMDA component in the geniculocortical connection.

Development of NMDA EPSPs in corticocortical transmission

The NMDA components in layer II/III and V/VI VC neurons that received corticocortical inputs, displayed very different time-courses during development: the NMDA component in layer II/III neurons sharply decreased after the middle stage, while that in layer V/VI neurons remained unchanged throughout all culture stages (up to 38 DIV). To our knowledge, there have not been any studies that systematically investigate the postnatal development of the NMDA and non-NMDA receptor-mediated monosynaptic EPSPs in corticocortical transmissions within the visual cortex. Previous in vivo *visual response (Fox et al., 1992) and binding assays (Bode-Greuel & Singer, 1989; Kumar et al., 1994; Catalano et al., 1997) have reported that large amounts of NMDA receptors exist in layer II/III even in the adult. The discrepancy between these and our results might be explained if high levels of NMDA receptors are preserved in synapses other than those examined in the present study, such as synapses formed on layer II/III neurons by axons that arise from layer IV neurons (Thomson,*

1986; Sutor, 1989). Indeed, EPSPs consisting solely of non-NMDA receptors have been reported in layer II/III neurons in adult slices using white matter stimulation (Shirokawa et al, 1989).
In layer V/VI, visual response (Fox et al., 1992) and immunoreactivity to NMDAR1 (Catalano et al., 1997) in cat visual cortex have shown that the NMDA component declines during the early postnatal period. However, a substantial NMDA component has been observed in acute slice studies of the adult rat: in the anterior cingulate cortex with callosal stimulation (Sah & Nicoll, 1991), and in the visual and prefrontal cortices using layer II/III stimulation (Jones & Baughman, 1988; Hirsch & Crepel, 1990). Thus, it is possible that NMDA EPSPs are preserved in deep layer visual cortex neurons that receive corticocortical inputs, but not in neurons that receive some intracortical and/or geniculocortical inputs. However, the existence of some species specific differences cannot be excluded either.

Possible mechanisms for developmental regulation of glutamate receptor expression
One interesting feature of this study is that the glutamate receptors develop in cortical circuits in the absence of patterned afferent activity. Since the absence of activity might cause a delay in the development of NMDA receptors, large NMDA receptor currents with slow kinetics would be expected in our culture conditions regardless of layer (Quinlan, et al., 1999). The fact that the development of NMDA components appears to mimic that *in vivo*, especially in geniculocortical transmission, might indicate that the expression is not governed by visual inputs. **It is possible that spontaneous activity is also required for the expression of these specific receptors (Lissin, et al., 1998). This view is consistent with the finding that the shorting of NMDA EPSPs is prevented by tetrodotoxin (Camignoto and Vici, 1992). The spontaneous spike activity, which was observed in the present culture conditions, might contribute to the normal development of this receptor expression. However, this does not seem to be the sole mechanism because the NMDA component developed differently in each connection although the spontaneous activity was observed for neurons in all layers. A plausible mechanism for this may be that the development of glutamatergic synaptic transmission is preprogrammed in neurons in each layer of the visual cortex (Yamada, et al., 1999), or is controlled by some molecular interactions between afferent fibers and postsynaptic cells. Further study is necessary to determine the factors that predominate in the regulation of glutamate receptor subtype expression for each synaptic pathway.**

Acknowledgements

We are grateful to Drs. Y. Komatsu, T. Kurotani for many helpful suggestions, M. Crair, T. Ogawa, N. Inagaki, Y. Kawaguchi, J. Kershaw for their encouragement and valuable comments on the manuscript, and N. Horimoto, S. Sato and E. Akase for data analysis. This work was supported by Grants-in-Aid for Scientific Research Projects 01770085 and 02220217 from the Ministry of Education, Science and Culture of Japan.

Abbreviations

AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APV	DL-2-amino-5-phosphonovalerate
DIV	days *in vitro*
DNQX	6,7-dinitro-quinoxaline-2,3-dione
EAA	excitatory amino acid

EPSC	excitatory postsynaptic current
EPSP	excitatory postsynaptic potential
IPSP	inhibitory postsynaptic potential
LGN	lateral geniculate nucleus
NMDA	*N*-methyl-D-aspartate
NMDA EPSP	NMDA receptor-mediated EPSP
PSP	postsynaptic potential
T	the threshold current to evoke a detectable EPSP
VC	visual cortex

References

Bliss, T. V. P. & Collingridge, G. L. (1993) A synaptic model of memory: long-term potentiation in the hippocampus. *Nature*, **361**, 31-39.

Bode-Greuel, K. M. & Singer, W. (1989) The development of *N*-methyl-D-aspartate receptors in cat visual cortex. *Dev.Brain Res.*, **46**, 197-204.

Bolz, J., Novak, N. & Staiger, V. (1992) Formation of specific afferent connections in organotypic slice cultures from rat visual cortex cocultured with lateral geniculate nucleus. *J. Neurosci.*, **12**, 3054-3070.

Brown, T. H., Kairiss, E. W. & Keenan, C. L. (1990) Hebbian synapses: biophysical mechanisms and algorithms. *Annu. Rev. Neurosci.*, **13**, 475-511.

Burkhalter, A. (1989) Intrinsic connections of rat primary visual cortex: laminar organization of axonal projections. *J. Comp. Neurol.*, **279**, 171-186.

Carmignoto, G. & Vicini, S. (1992) Activity-dependent decrease in NMDA receptor responses during development of the visual cortex. *Science*, **258**, 1007-1011.

Catalano, S. M., Chang, C. K. & Shatz, C. J. (1997) Activity-dependent regulation of NMDAR1 immunoreactivity in the developing visual cortex. *J. Neurosci.*, **17**, 8376-8390.

Crair, M. C. & Malenka, R. C. (1995) A critical period for long-term potentiation at thalamocortical synapses. *Nature*, **375**, 325-328.

Fleidervish, I. A., Binshtok, A. M. & Gutnick, M. J. (1998) Functionally distinct NMDA receptors mediate horizontal connectivity within layer 4 of mouse barrel cortex. *Neuron*, **21**, 1055-1065.

Fox, K., Sato, H. & Daw, N. (1989) The location and function of NMDA receptors in cat and kitten visual cortex. *J. Neurosci.*, **9**, 2443-2454.

Fox, K., Daw, N., Sato, H. & Czepita, D. (1992) The effect of visual experience on development of NMDA receptor synaptic transmission in kitten visual cortex. *J. Neurosci.*, **12**, 2672-2684.

Gähwiler, B. H. (1988) Organotypic cultures of neural tissue. *Trends. Neurosci.,* **11,** 484-489.

Gustafsson, B. & Wigström, H. (1988) Physiological mechanisms underlying long-term potentiation. *Trends. Neurosci.*, **11**, 156-162.

Hagihara, K., Tsumoto, T., Sato, H. & Hata, Y. (1988) Actions of excitatory amino acid antagonists on geniculo-cortical transmission in the cat's visual cortex. *Exp. Brain Res.*, **69**, 407-416.

Hebb, D. O. (1949) The Organization of Behaviour. Wiley, New York.

Hestrin, S., Nicoll, R. A., Perkel, D. J. & Sah, P. (1990) Analysis of excitatory synaptic action in pyramidal cells using whole-cell recording from rat hippocampal slices. *J. Physiol.,* **422,** 203-225.

Hirsch, J. C. & Crepel, F. (1990) Use-dependent changes in synaptic efficacy in rat prefrontal

neurons *in vitro*. *J. Physiol.*, **427**, 31-49.
Iwakiri, M. & Komatsu, Y. (1993) Postnatal development of NMDA receptor-mediated synaptic transmission in cat visual cortex, *Dev. Brain Res.*, **74**, 89-97.
Johnson, J. W. & Ascher, P. (1987) Glycine potentiates the NMDA response in cultured mouse brain neurons. *Nature*, **325**, 529-531.
Jones, K. A. & Baughman, R. W. (1988) NMDA- and non-NMDA-receptor components of excitatory synaptic potentials recorded from cells in layer V of rat visual cortex. *J. Neurosci.*, **8**, 3522-3534.
Kaczmarek, L., Kossut, M. & Skangiel-Kramska, J. (1997) Glutamate receptors in cortical plasticity: molecular and cellular biology. *Physiol. Rev.*, **77**, 217-255.
Kato, N., Artola, A. & Singer, W. (1991) Developmental changes in the susceptibility to long-term potentiation of neurones in rat visual cortex slices. *Dev. Brain Res.*, **60**, 43-50.
Klintsova, A. Y. & Greenough, W. T. (1999) Synaptic plasticity in cortical systems. *Curr. Opin. Neurobiol.,* **9**, 203-208.
Kumar, A., Schliebs, R. & Bigl, V. (1994) Postnatal development of NMDA, AMPA and kainate receptors in individual layers of rat visual cortex and the effect of monocular deprivation. *Int. J. Devl. Neurosci.*, **12**, 31-41.
Lissin, D. V., Gomperts, S. N., Carroll, R. C., Christine, C. W., Kalman, D., Kitamura, M., Hardy, S., Nicoll, R. A., Malenka, R. C. & von Zastrow, M. (1998) Activity differentially regulates the surface expression of synaptic AMPA and NMDA glutamate receptors. *Proc. Natl. Acad. Sci. USA,* **95,** 7097-7102.
Luhmann, H. J. & Prince, D. A. (1991) Postnatal maturation of the GABAergic system in rat neocortex. *J. Neurophysiol.*, **65**, 247-263.
Madison, D. V., Malenka, R. C. & Nicoll, R. A. (1991) Mechanisms underlying long-term potentiation of synaptic transmission. *Annu. Rev. Neurosci.*, **14**, 379-397.
McCormick, D. A., Connors, B. W., Lighthall, J. W. & Prince, D. A. (1985) Comparative electrophysiology of pyramidal and sparsely spiny stellate neurons of the neocortex. *J. Neurophysiol.*, **54**, 782-806.
Miller, M. W. & Vogt, B. A. (1984) Direct connections of rat visual cortex with sensory, motor, and association cortices. *J. Comp. Neurol.*, **226**, 184-202.
Mitzdorf, U. & Singer, W. (1978) Prominent excitatory pathways in the cat visual cortex (A17 and A18): a current source-density analysis of electrically evoked potentials. *Exp. Brain Res.*, **33**, 371-394.
Molnár, Z. & Blakemore, C. (1991) Lack of regional specificity for connections formed between thalamus and cortex in coculture. *Nature*, **351**, 475-477.
Nowak, L., Bregestovski, P., Ascher, P., Herbet, A., & Prochiantz, A. (1984) Magnesium gates glutamate-activated channels in mouse central neurones. *Nature*, **307,** 462-465.
Quinlan, E. M., Philpot, B. D., Huganir, R. L. & Bear. M. F. (1999) Rapid, experience-dependent expression of synaptic NMDA receptors in visual cortex *in vivo*. *Nature Neurosci.*, **2**, 352-357.
Sah, P. & Nicoll, R. A. (1991) Mechanisms underlying potentiation of synaptic transmission in rat anterior cingulate cortex *in vitro*. *J. Physiol.*, **433**, 615-630.
Shatz, C. J. (1990) Impulse activity and the patterning of connections during CNS development. *Neuron*, **5**, 745-756.
Shirokawa, T., Nishigori, A., Kimura, F. & Tsumoto, T. (1989) Actions of excitatory amino acid antagonists on synaptic potentials of layer II/III neurons of the cat's visual cortex. *Exp. Brain Res.*, **78**, 489-500.
Streit, P., Thompson, S. M. & Gähwiler, B. H. (1989) Anatomical and physiological properties of GABAergic neurotransmission in organotypic slice cultures of rat

hippocampus. *Eur. J. Neurosci.*, **1**, 603-615.
Sutor, B. & Hablitz, J. J. (1989) EPSPs in rat neocortical neurons in vitro II. Involvement of *N*-methyl-D-aspartate receptors in the generation of EPSPs. *J. Neurophysiol.*, **61**, 621-634.
Thomson, A. (1986) A magnesium-sensitive post-synaptic potential in rat cerebral cortex resembles neuronal responses to N-methylaspartate. *J. Physiol.*, **370**, 531-549.
Watkins, J. C. (1989) The NMDA receptor concept: origins and development. In Watkins, J. C. & Collingridge, G. L. (eds), The NMDA Receptor. Oxford University Press, Oxford, pp. 1-17.
White, E. L. & Rock, M. P. (1980) Three-dimentional aspects and synaptic relationships of a Golgi-impregnated spiny stellate cell reconstructed from serial thin sections. *J. Neurocytol.*, **9**, 615-636.
Wolfson, B., Gutnick, M. J. & Baldino, Jr. F. (1989) Electrophysiological characteristics of neurons in neocortical explant cultures. *Exp. Brain Res.*, **76**, 122-130.
Yamada, K., Yamamoto, N. & Toyama, K. (1999) Development of membrane properties of rat neocortical neurons studied in organotypic cocultures. *Neurosci. Lett.*, **273,** 1-4.
Yamamoto, N., Kurotani, T. & Toyama, K. (1989) Neural connections between the lateral geniculate nucleus and visual cortex *in vitro*. *Science*, **245**, 192-194.
Yamamoto, N., Yamada, K., Kurotani, T. & Toyama, K. (1992) Laminar specificity of extrinsic cortical connections studied in coculture preparations. *Neuron*, **9**, 217-228.
Yamamoto, N., Higashi, S. & Toyama, K. (1997) Stop and branch behaviors of geniculocortical axons: a time-lapse study in organotypic cocultures. *J. Neurosci.*, **17**, 3653-3663.