Research report

The formation of glutamatergic synapses in cultured central neurons: selective increase in miniature synaptic currents

K. Gottmann *, F.W. Pfrieger, H.D. Lux

Max-Planck-Institute for Psychiatry, Department of Neurophysiology, Am Klopferspitz 18A, D-82152 Planegg-Martinsried, Germany

Accepted 10 March 1994

Abstract

The formation of synapses between cultured rat thalamic neurons was studied with electrophysiological and immunocytochemical methods. Thalamic neurons in culture form predominantly glutamatergic synapses. Already after 3 days in vitro glutamatergic miniature EPSCs occurred spontaneously and their frequency was strongly increased after K^+ depolarization, while GABAergic mIPSCs were found after K^+ depolarization at lower frequency. This demonstrates that both, excitatory glutamatergic and inhibitory GABAergic synapses were functional in close succession to initial neurite outgrowth. Synapses formed independent of spontaneous electrical activity, which was absent during the first week in culture. Spontaneous action potentials appeared during the second week and chronic action potential blockade by addition of tetrodotoxin reduced neuronal survival and the number of glutamatergic synapses per neuron. During in vitro differentiation the number of synapsin I immunoreactive presynaptic terminals and the frequency of spontaneous glutamatergic miniature EPSCs increased closely correlated, while the frequency of GABAergic mIPSCs after K^+ depolarization did not increase. Thus, the continuous formation of presynaptic terminals, including possible maturation of transmitter release, appeared to underlie the increase in mEPSC frequency. Analysis of miniature EPSC amplitudes at different stages in vitro revealed an increase in amplitudes, suggesting synaptic differentiation after initial establishment of functional transmission in glutamatergic synapses. This process was synapse specific as amplitudes of GABAergic mIPSCs were invariant.

Key words: Synaptogenesis; Central neuron; Glutamatergic synapse; GABAergic synapse; Synapsin I; Miniature synaptic current; Tetrodotoxin

1. Introduction

Synaptogenesis constitutes the crucial event in the development of functional neuronal networks. Previous studies on neuromuscular cocultures demonstrated that after physical contact between a neuronal growth cone and a muscle target cell neuromuscular junctions differentiate functionally as indicated by an increase in the frequency and the amplitudes of miniature synaptic currents [9,22,48]. Junctional differentiation includes presynaptic maturation of acetylcholine (ACh) release [23,29] and postsynaptic accumulation of ACh receptors [1,13,22,41]. Moreover, developmental changes in

single channel properties of ACh receptors occur [11,42].

In the brain, glutamatergic synapses are the main source of excitatory input to neurons. Postsynaptic receptors and plasticity phenomena in mature glutamatergic synapses are well characterized, the processes leading to synapse formation, however, are poorly understood. In mature synapses glutamate receptors have been demonstrated to be accumulated in the subsynaptic membrane [16,21,36,39,45]. This suggests that synaptic differentiation processes, like postsynaptic receptor accumulation, play major roles in the formation of glutamatergic synapses.

In cultured central neurons the formation of synapses has been studied ultrastructurally [2] and by immunocytochemical staining of presynaptic terminals [12,47]. These studies demonstrated a developmental increase in the number of presynaptic terminals, how-

* Corresponding author. Present address: Ruhr-Universität Bochum, Fakultät für Biologie, Lehrstuhl für Zellphysiologie, Gebäude ND 4, D-44780 Bochum, Germany.
Fax: (49) (234) 709-4129.

ever, this was not compared with electrophysiological recordings of synaptic activity. Studies correlating morphological and electrophysiological data focussed on the development of inhibitory, GABAergic synapses [8,24]. We have now investigated the formation of excitatory, glutamatergic synapses in cultured thalamic neurons, which are synaptically connected preferentially by glutamatergic synapses, while inhibitory, GABAergic synapses connect only a low percentage of cell pairs [38]. Monitoring of synaptogenesis by counting synapsin I immunoreactive terminals and by recording postsynaptic currents indicated that functional synapses formed in close succession to neurite outgrowth. A prominent increase in the number of presynaptic terminals during further differentiation was reflected by an increased frequency of miniature EPSCs. The amplitudes of miniature EPSCs, but not that of miniature IPSCs, also increased, suggesting synaptic differentiation after initial establishment of functional transmission selectively in glutamatergic synapses.

2. Materials and methods

2.1. Cell culture

After 17 days of gestation wistar rats were anesthetized and decapitated. Whole thalami including the epithalamus, dorsal thalamus and ventral thalamus were dissected out of embryos and triturated with fire-polished Pasteur pipettes. No enzymatic treatment was used. Dissociated cells were plated at a density of $150\text{--}200 \cdot 10^3/\text{cm}^2$ in polyornithine/laminin-coated culture dishes. Cultures were incubated at 37°C in 5% CO_2 atmosphere. The culture medium consisted of Eagle's basal medium with addition of horse serum (10%), L-glutamine, glucose and insulin. After 4–5 days in vitro (DIV) cytosine- β -D-arabinofuranoside hydrochloride ($10 \mu\text{M}$) was added for 2 days to minimize proliferation of non-neuronal cells.

2.2. Synapsin I immunostaining

Immunofluorescence staining of presynaptic terminals was performed according to Fletcher et al. [12]. Cells were fixed with warm 4% paraformaldehyde in PBS containing 0.12 M sucrose for 20 min, permeabilized in 0.3% Triton X-100 for 5 min and rinsed in PBS. Cells were incubated first with fresh 10% BSA for 1 h at 37°C , then with primary antibody (rabbit polyclonal anti-synapsin I diluted 1:100 in 1% BSA/PBS) overnight at 4°C . After primary antibody exposure, cells were rinsed in PBS and incubated with biotinylated goat anti-rabbit IgG (1:800, Sigma) for 1 h at 37°C . After washing again with PBS cells were incubated with fluorescein-conjugated avidin (1:1000, Sigma) for 1 h at 37°C . After rinsing with PBS cells were mounted in PBS:glycerol (1:1), containing 0.05% *p*-phenylenediamine. Representative areas of each culture dish were photographed using Kodak TX 400 film and the number of stained terminals per neuron was determined.

2.3. Electrophysiological recordings and data analysis

Membrane potential and spontaneous synaptic currents were recorded in the whole cell configuration [15] using an EPC9 patch

clamp amplifier (HEKA). Patch electrodes ($5\text{--}10 \text{ M}\Omega$) were fabricated from borosilicate glass and filled with a solution containing a physiologically low chloride concentration (100 mM potassium gluconate, 10 mM KCl, 0.25 mM CaCl_2 , 10 mM EGTA, 20 mM Hepes, pH = 7.3). The extracellular solution contained 130 mM NaCl, 5 mM KCl, 5 mM CaCl_2 , 1 mM MgCl_2 , 20 mM Hepes, pH = 7.3. All experiments were performed at room temperature. To isolate miniature synaptic currents action potentials were blocked by $1 \mu\text{M}$ TTX. High potassium solution and drugs were applied to the bath solution.

For analog–digital conversion and stimulation EPC9 software was used. To analyse spontaneous synaptic activity routinely 20–40 samples of 1 s duration were recorded from each cell. Comparison to longer duration records in some cells showed that this protocol was sufficient to obtain a typical sample of synaptic currents at all stages of development. EPSCs and IPSCs were analyzed quantitatively using a PSC detection and analysis program [38] based on AUTESP software. Critical detection parameters were the time-to-peak and the amplitude. The threshold for PSC detection was chosen according to the rms noise level (3-fold SD value). To obtain frequency-amplitude histograms data from individual cells were pooled for each stage. For statistical comparison of amplitude distributions of miniature postsynaptic currents the Kolmogorov–Smirnov test was used. Miniature postsynaptic currents $> 5 \text{ pA}$ were grouped into eight amplitude classes with a bin width of 10 pA.

2.4. Drugs

6,7-Dinitroquinoxaline-2,3-dione (DNQX), ($\pm$)-2-amino-5-phosphonopentanoic acid (AP-5), kynurenic acid, (–)-bicuculline methochloride and TTX were purchased from Sigma.

3. Results

3.1. Morphological differentiation of thalamic neurons in vitro

Dissociated thalamic neurons from embryonic rats formed neuritic processes within hours after plating on polyornithine/laminin-coated culture dishes. Already after 2 DIV neuronal growth cones established contacts to neighbouring cells (Fig. 1A) and during the first week in culture the density of neurites strongly increased (Fig. 1B). Concomitantly to neurite outgrowth proliferation of non-neuronal cells led to the formation of a confluent cell monolayer after 4–5 DIV. Further proliferation was minimized by transient addition of cytosine- β -D-arabinofuranoside (AraC). Throughout the culture period the mean density of neurons, as characterized by phase bright cell bodies and the presence of neurites, decreased from $160.9 \pm 55.7 \cdot 10^3/\text{cm}^2$ ($n = 11$) after 2–4 DIV to $16.5 \pm 8.9 \cdot 10^3/\text{cm}^2$ ($n = 13$) after 9–11 DIV and finally to $9.7 \pm 5.3 \cdot 10^3/\text{cm}^2$ ($n = 14$) after 15–17 DIV. The surviving neurons continued to differentiate as indicated by the further outgrowth of neurites and the pronounced increase in soma size (Fig. 1A–C).

Fig. 1. Morphological differentiation and development of synapsin I immunoreactive presynaptic terminals in cultured thalamic neurons. Morphological appearance of neurons (A–C) and synapsin I immunoreactivity (D–F) at different stages of in vitro differentiation (A,D: 2–4 DIV; B,E: 9–11 DIV, C,F: 15–17 DIV). Scale bars represent 20 μm in C and 10 μm in F. G–I: quantitative determination of synapsin I immunoreactive terminals at different stages (I: 2–4 DIV, II: 9–11 DIV, III: 15–17 DIV). The number of synapsin I immunoreactive terminals per neuron increased throughout the culture period (G). I: the fraction of somatic terminals (H) decreased due to the increasing arborization of neurons. Error bars represent standard deviations.

3.2. Formation of synapsin I immunoreactive presynaptic terminals

Synapsins are accumulated at presynaptic terminals, associated with synaptic vesicles and involved in the regulation of transmitter release [46]. Immunohistochemical staining of synapsin I results in brightly fluorescent puncta labelling presynaptic terminals [12,47].

To study synapse formation in thalamic neurons, cultures were stained with polyclonal synapsin I antibodies at three stages (after 2–4, 9–11 and 15–17 DIV; Fig. 1D–F) and the number of synapsin I immunoreactive puncta per neuron was determined. This parameter increased from 3.2 ± 3.6 at 2–4 DIV ($n = 8$) to 54.5 ± 37.8 at 9–11 DIV ($n = 13$) and finally to 206.0 ± 80.8 ($n = 7$) after 15–17 DIV (Fig. 1G). The number of

Fig. 2. Development of spontaneous action potentials (APs) and spontaneous postsynaptic currents. A: AP frequency at different stages (I: 2–4 DIV, II: 9–11 DIV, III: 15–17 DIV). Spontaneous APs were absent during the first week in culture. B: spontaneous APs after 15–17 DIV were triggered by EPSPs (arrow). Scale bars represent 10 mV and 100 ms (single spike) or 500 ms (spike burst). C–E: typical spontaneous postsynaptic currents at different stages (holding potentials: –60 and –40 mV). The inset in C shows a postsynaptic event at 10-fold magnification and the arrow in E marks an EPSC burst evoked by presynaptic APs. F: percentages of neurons showing a particular type of synaptic activity, as illustrated in C (empty bars, type A), D (hatched bars, type B) and E (filled bars, type C), at three different stages in culture (I: 2–4 DIV, II: 9–11 DIV, III: 15–17 DIV). Type A: 5 or less EPSCs at –60 mV and no IPSCs were observed within 20 s. Type B: more than 5 EPSCs at –60 mV and no IPSCs. Type C: more than 5 EPSCs at –60 mV and IPSCs at least at –40 mV.

somatic synapsin I immunoreactive terminals increased from 0.4 ± 0.3 at 2–4 DIV to 3.5 ± 2.1 at 9–11 DIV and finally to 10.4 ± 4.1 at 15–17 DIV (Fig. 1H). We did not observe subcellular selectivity of terminal formation, except that due to the increasing neuritic arborization the proportion of somatic synapses decreased during *in vitro* differentiation from 23% to 5% (Fig. 1I). Thus, synapsin I immunostaining showed that presynaptic terminals formed throughout the whole culture period with the first terminals appearing after a few days.

3.3. Formation of functional synaptic contacts

After 2 weeks in culture spontaneous EPSCs and IPSCs mediated by glutamate and by GABA_A receptors, respectively are frequently observed in cultured thalamic neurons [38] demonstrating *in vitro* formation of functional synapses. To study their development we monitored spontaneous synaptic activity at different stages of *in vitro* differentiation with whole cell patch-clamp recording. Fig. 2C–E illustrates typical recordings of spontaneous postsynaptic currents at -60 mV (EPSCs) and at -40 mV (IPSCs) holding potential after 2–4, 9–11 and 15–17 DIV. As a physiological intracellular Cl[−] concentration was used in the patch pipette (10 mM Cl[−]), GABAergic IPSCs, mediated by Cl[−] channels, were outwardly, whereas glutamatergic EPSCs, mediated by cationic conductances, were inwardly directed.

During the culture period the mean frequency of EPSCs increased 35-fold from 0.2 ± 0.7 events/s at 2–4 DIV ($n = 20$) to 7.3 ± 8.0 events/s at 9–11 DIV ($n = 20$). After 15–17 DIV ($n = 18$) an only 4-fold further increase to 29.7 ± 17.8 events/s was observed (Fig. 3B). To further analyse the release mechanisms underlying EPSCs, miniature EPSCs (mEPSCs), reflecting action potential independent, spontaneous vesicle fusion, were isolated with TTX ($1 \mu\text{M}$) and the presence of spontaneous action potentials (APs) was investigated with current-clamp recording. The mean frequency of mEPSCs increased from 0.15 ± 0.3 events/s ($n = 13$) at 2–4 DIV to 5.2 ± 3.7 events/s ($n = 10$) at 9–11 DIV and finally reached 17.1 ± 10.4 events/s ($n = 9$) at 15–17 DIV (Fig. 3B). TTX-sensitive EPSCs with large amplitudes (> 50 pA) ranging up to 250 pA (Fig. 3A) were absent during the first week and appeared after 9–11 DIV. Thus, spontaneous excitatory synaptic activity up to 11 DIV consisted almost exclusively of mEPSCs. The increase in mEPSC frequency was closely correlated to the increase in the number of synapsin I immunoreactive terminals (30-, respectively 20-fold between 2–4 and 9–11 DIV and 3–4-fold between 9–11 and 15–17 DIV), suggesting that the former reflected the formation of presynaptic terminals. EPSCs evoked by spontaneous action poten-

Fig. 3. Frequency of spontaneous EPSCs and mEPSCs at different stages of *in vitro* differentiation. A: current trace containing large amplitude EPSCs evoked by presynaptic APs and miniature EPSCs (15–17 DIV). Holding potential: -60 mV. B: the frequency of EPSCs (empty bars) and mEPSCs (isolated by addition of $1 \mu\text{M}$ TTX during recording, hatched bars) increased throughout the culture period. The increase in mEPSC frequency was closely correlated to the increase in the number of synapsin I immunoreactive terminals. Error bars represent standard deviations.

tials appeared delayed during the second week in culture.

Spontaneous APs emerged after 9–11 DIV, but at a low frequency ($0.03 \pm 0.09 \text{ s}^{-1}$; $n = 14$), although thalamic neurons were excitable by current injection at the first days in culture. During further differentiation AP frequency increased to $0.76 \pm 0.47 \text{ s}^{-1}$ after 15–17 DIV ($n = 30$) (Fig. 2A). In more than 80% of cells all observed APs were triggered by excitatory synaptic input (Fig. 2B) as they were blocked by addition of the glutamate receptor antagonist kynurenic acid (1 mM) or the Ca²⁺ channel blocker Cd²⁺ ($50 \mu\text{M}$) ($n = 13$). The delayed occurrence of spontaneous APs presumably reflects strengthening of excitatory synaptic transmission due to the increasing number of presynaptic terminals. The mean resting membrane potential of thalamic neurons was -49 ± 5 mV ($n = 53$) and did not change significantly throughout the culture period.

IPSCs were absent during the first week in culture (Fig. 2C,D) and appeared after 9–11 DIV. After 15–17 DIV IPSCs occurred with a mean frequency of 1.35 ± 1.94 events/s with amplitudes ranging up to 250 pA (mean 36.1 ± 44.9 pA). They were abolished by the

Fig. 4. Effects of chronic action potential blockade. Neither EPSC frequency (A), number of synapsin I immunoreactive terminals (B) nor neuronal survival (C) were affected by chronic TTX treatment up to 9–11 DIV. After 15–17 DIV, however, the number of functional glutamatergic terminals per neuron and neuronal survival were significantly (*) reduced. Empty bars: control cultures. Hatched bars: cultures chronically treated with 1 μ M TTX. Error bars represent standard deviations.

addition of TTX, indicating that they were evoked by presynaptic action potentials. Thus, the delayed occurrence of IPSCs reflected the delayed appearance of action potentials. Miniature IPSCs were not resolved at -40 mV holding potential (but see K^+ stimulation experiments below).

EPSCs and IPSCs were characterized pharmacologically. EPSCs at 9–11 and 15–17 DIV were strongly reduced in amplitude by 1 mM kynurenic acid ($n = 4$) or 20 μ M DNQX ($n = 5$), whereas 50 μ M ($\pm$)-AP-5 ($n = 5$) in the presence of 1 mM Mg^{2+} had no effect. Thus EPSCs in cultured thalamic neurons were mediated mainly by non-NMDA glutamate receptors. IPSCs were blocked by the GABAA antagonist bicuculline methchloride (50 μ M, $n = 3$). Spontaneous postsynap-

tic currents after 2–4 DIV were not characterized pharmacologically, since frequencies were too low.

3.4. Effects of chronic action potential blockade

In order to investigate whether APs evoked by excitatory synaptic input are involved in synapse formation and maintenance, we chronically treated cultured neurons with TTX (1 μ M), which was added repeatedly to the culture medium to compensate for breakdown during the application period. For recording of spontaneous EPSCs TTX was omitted from the bath solution. Up to 11 DIV chronic application of TTX did neither affect the mean EPSC frequency (5.9 ± 7.5 ev/s; $n = 11$; control values see above), the mean number of

Fig. 5. Amplitude distributions of EPSCs at 15–17 DIV. A: control conditions (10551 events). B: chronic TTX treatment (1482 events). Holding potential: -60 mV.

synapsin I immunoreactive terminals per neuron (24.4 ± 15.5 compared to 33.5 ± 20.3 in controls at 8–10 DIV; $n = 4,7$) nor the mean cell density ($15.1 \pm 7.4 \cdot 10^3/\text{cm}^2$; $n = 7$) significantly (Fig. 4). Thus, during the first week excitatory synapses formed in the absence of spontaneous APs.

In contrast, chronic TTX treatment during the second week strongly influenced the synaptic network. The mean EPSC frequency was significantly (t -test, $P < 0.01$) reduced to 18% of sister control cultures after 15–17 DIV (5.4 ± 4.8 ev/s; $n = 14$, control values see above). Also the mean number of synapsin I immunoreactive terminals per postsynaptic neuron was significantly (t -test, $P = 0.01$) reduced to 42% of controls (72.4 ± 23.3 compared to 172.0 ± 63.7 at 15 DIV; $n = 5,5$) and the mean cell density ($3.3 \pm 1.6 \cdot 10^3/\text{cm}^2$, $n = 6$) was significantly (t -test, $P = 0.01$) reduced to 34% of control values (Fig. 4). The slightly stronger reduction in EPSC frequency as compared to the number of presynaptic terminals appears to be due to reduction of the frequency of EPSCs evoked by APs in addition to reduction of mEPSC frequency. The amplitude distribution of EPSCs was not affected with few large amplitude EPSCs (> 50 pA) evoked by spontaneous presynaptic action potentials still present (Fig. 5). Thus, during the second week in culture the number of functional, excitatory synaptic contacts and the survival of neurons depended on spontaneous APs.

3.5. Miniature synaptic currents evoked by K^+ stimulation

After 2–4 DIV few mEPSCs were already observed, however, their frequency was too low to allow for quantitative analysis. In order to increase the frequency of mEPSCs, presynaptic terminals were depolarized by increasing the extracellular K^+ concentration to 32 mM. Bath application of K^+ after 3 DIV increased the mean EPSC frequency at -60 mV holding potential more than 100-fold from 0.05 ± 0.07 events/s ($n = 21$) to 6.1 ± 5.6 events/s ($n = 7$) 5 to 25 s after application (Fig. 6A,C). After 40–60 s the mean EPSC frequency was still 46-fold increased (2.3 ± 2.1 events/s) and during the following 20–30 min in elevated K^+ this frequency was maintained. K^+ evoked EPSCs were blocked by $20 \mu\text{M}$ DNQX ($n = 5$), whereas $50 \mu\text{M}$ ($\pm$)-AP-5 ($n = 3$) had no effect in the presence of 1 mM Mg^{2+} , demonstrating that they were mediated by non-NMDA receptors. Amplitudes ranged up to 25 pA with the smallest amplitudes buried in the recording noise (see below). K^+ depolarization increased the frequency of miniature EPSCs as coapplication of TTX ($1 \mu\text{M}$; $n = 5$) did neither affect the increase in EPSC frequency (6.9 ± 3.8 ev/s) (Fig. 6C) nor the amplitude distribution of EPSCs. The amplitude distribution of mEPSCs recorded within 5–25 s after application ($n =$

Fig. 6. Miniature postsynaptic currents evoked by K^+ stimulation. Frequency and time course of mEPSCs (A, holding potential: -60 mV) and mIPSCs (B, holding potential: -10 mV) after K^+ depolarization at 3 DIV. C: the K^+ induced increase in mEPSC frequency (empty bars) was not affected by coapplication of $1 \mu\text{M}$ TTX (hatched bars). Coapplication of $100 \mu\text{M}$ Cd^{2+} (filled bar) significantly (*) reduced the increase in mEPSC frequency, demonstrating its dependence on Ca^{2+} influx. The time intervals after K^+ depolarization, used to determine mEPSC frequency, are indicated below bars. Error bars represent standard deviations.

5) did not differ significantly from those recorded more than 40 s after application ($n = 13$, Fig. 8A), suggesting that amplitudes were not affected by depletion phenomena.

To investigate, whether the K^+ -evoked increase in mEPSC frequency was due to a depolarization-induced increase in intracellular Ca^{2+} , as described previously [10,38,50], Cd^{2+} ($50 \mu\text{M}$) was simultaneously applied to block voltage dependent Ca^{2+} channels. In the presence of Cd^{2+} the increase in EPSC frequency (1.3 ± 1.4 ev/s, $n = 8$) was reduced significantly (t -test, $P < 0.05$) to 20% of controls (Fig. 6C), demonstrating the ability of early excitatory synapses to respond to

increased intracellular Ca^{2+} with increased transmitter release.

Additionally after 3 DIV mIPSCs were observed at -10 mV holding potential after K^+ depolarization ($n = 8$, Fig. 6B) with a mean frequency of 1.1 ± 0.9 events/s and amplitudes ranging up to 20 pA. They were mediated by GABA_A receptors as they were blocked by 50 μM bicuculline methchloride ($n = 3$). Thus, excitatory as well as inhibitory synapses were functional rapidly after establishment of intercellular contacts. The frequency of mIPSCs evoked by K^+ depolarization did not increase during the culture period (0.7 ± 0.7 events/s at 15 DIV), suggesting that mainly glutamatergic synapses formed in cultured thalamic neurons.

3.6. A developmental increase in amplitudes of mEPSCs indicated synaptic differentiation

Miniature postsynaptic currents represent the postsynaptic responses to single vesicles released spontaneously from presynaptic terminals [40]. Their amplitudes and time courses reflect the transmitter content of vesicles and the number and properties of postsynaptic receptors. Comparison of the amplitudes and time courses of miniature currents at different stages should thus allow to detect differentiation processes occurring in developing synapses.

Fig. 7 illustrates typical spontaneous mEPSCs recorded after 2–4, 9–11 and 15–17 DIV. During the first week in culture the amplitudes of mEPSCs tended to increase, with the maximum values increasing from about 25 pA to about 50 pA. Since the frequency of spontaneous mEPSCs at early stages was low, the amplitudes of mEPSCs evoked by K^+ depolarization were quantitatively compared. Amplitudes of mEPSCs evoked by K^+ stimulation clearly increased between 3 and 5 DIV. The distribution at 3 DIV ($n = 13$, > 40 s K^+ depolarization, Fig. 8A) was significantly ($P < 0.01$) different from that at 5 DIV ($n = 6$, > 40 s K^+ depolarization, Fig. 8B,E) as shown by the Kolmogorov–Smirnov test. After 9–11 DIV ($n = 10$) and 15–17 DIV ($n = 9$) no further increase in spontaneous mEPSC amplitudes was observed (Fig. 8C,D). Thus, an increase in mEPSC amplitudes during the first week in culture indicated synaptic differentiation.

To analyse the time course of mEPSCs, the time to peak (10–90%; TTP) and the time constant of decay of individual events were determined at each stage. After 2–4 DIV the mean TTP was 1.1 ± 0.4 ms ranging from 0.5 up to 2.5 ms and the mean time constant of the monoexponential decay was 3.5 ± 1.3 ms ranging from 1 ms up to 6 ms. Neither TTP nor time constant of decay changed significantly during the culture period, however, developmental alterations might have been obscured by the large variability of mEPSC kinetics.

Fig. 7. Spontaneous mEPSCs at different stages in culture. A: 2–4 DIV. B: 9–11 DIV. C: 15–17 DIV. Amplitudes of mEPSCs increased during in vitro differentiation, while the time course of mEPSCs remained unchanged. Holding potential: -60 mV.

Time courses of individual mEPSCs varied presumably due to electrotonic distortion. However, the time constants of decay and the amplitudes of mEPSCs were not clearly correlated suggesting, that factors other than electrotonic attenuation also influenced the variability of mEPSC amplitudes [3,18].

Increases in amplitudes of miniature synaptic currents occurred synapse specific, as, in contrast to mEPSCs, mIPSC amplitudes did not increase throughout the culture period. Amplitude distributions of mIPSCs recorded at -10 mV holding potential at 3 DIV ($n = 8$), 5 DIV ($n = 5$) and after 15 DIV ($n = 9$) were not significantly different (Fig. 8F).

4. Discussion

To study synaptogenesis in central neurons, we established cultures of embryonic rat thalamic neurons, which permitted the formation of a functional neuronal

Fig. 8. Amplitude distributions of miniature postsynaptic currents at different stages in culture. Amplitude distributions of mEPSCs (holding potential: -60 mV) at 3 DIV after K^+ depolarization (A, 1208 events), at 5 DIV after K^+ depolarization (B, 1048 events), at 9–11 DIV (C, 1637 events) and at 15–17 DIV (D, 5742 events). E: cumulative plots of mEPSC amplitudes demonstrated an increase in amplitudes between 3 DIV and later stages. F: cumulative plots of mIPSC amplitudes (holding potential: -10 mV) after K^+ depolarization at 3 (358 events), 5 (240 events) and 15 (533 events) DIV. mIPSC amplitudes did not increase significantly.

network. Thalamic neurons were removed from embryos after 17 days of gestation as at this embryonic stage genesis of the majority of thalamic neurons is completed [32] and thalamocortical fibres are just invading the immature cortex [30]. Thus, it is likely, that postmitotic thalamic neurons were cultured prior to or during the embryonic stage, at which synaptogenesis occurs *in vivo*. *In vitro* differentiation led to the formation of functional, mainly glutamatergic synapses. Excitatory, glutamatergic and inhibitory, GABAergic synapses were distinguished by the reversal potential of postsynaptic currents. In the presence of Mg^{2+} EPSCs were mediated by non-NMDA glutamate receptors and IPSCs by GABA_A receptors. As shown previously by application of selective agonists cultured thalamic neurons expressed NMDA and non-NMDA glutamate as well as GABA_A receptors [25].

4.1. Synapse formation *in vitro*

To monitor synapse formation presynaptic terminals were identified by immunostaining for synapsin I. As synapsin I is thought to mediate clustering of synaptic vesicles at release sites [37] and weak immunostaining is associated even with small clusters of a few vesicles [12], it appears a reliable marker of accumulation of synaptic vesicles in forming presynaptic terminals. The first synapsin I immunoreactive terminals were found after 2–4 DIV shortly after initial neurite outgrowth. The number of immunoreactive terminals per thalamic neuron increased throughout the whole culture period

and was paralleled by an increasing neuritic arborization. Qualitatively similar results have been obtained in cultured striatal [8,47] and hippocampal neurons [12]. However, presynaptic synapsin I accumulation has previously not been correlated quantitatively to electrophysiological recordings of postsynaptic currents and synapsin I immunostaining does not distinguish excitatory and inhibitory synapses [7].

Recording of spontaneous postsynaptic currents revealed characteristic types of synaptic activity accompanying each stage of *in vitro* development (Fig. 2F). Within a few days after initial neurite outgrowth, EPSCs occurred at a low frequency. As shown by TTX addition these events were miniature EPSCs due to probabilistic release of single synaptic vesicles [40]. K^+ stimulation of early thalamic cultures strongly increased the frequency of mEPSCs and revealed mIPSCs. Thus, both, glutamatergic and GABAergic synapses were functional rapidly after initial neurite outgrowth, without any delay in the formation of inhibitory synapses.

During the first week in culture mEPSC frequency strongly increased. This increase was closely correlated to the increase in synapsin I immunoreactive terminals, suggesting that the former reflected the formation of new synapses. Functional presynaptic maturation following terminal formation might also contribute to the enhancement in mEPSC frequency.

Additionally to mEPSCs spike-evoked, large EPSCs and spike-evoked IPSCs appeared during the second week due to the delayed occurrence of spontaneous

APs, which most likely reflected strengthening of synaptic transmission with increasing numbers of presynaptic terminals. Developmental increases in spontaneous excitatory postsynaptic responses have also been reported in other central neurons in culture [8,20,35,44].

4.2. *Electrical activity and trophic interactions*

To study whether electrical activity of cultured neurons influences *in vitro* synaptogenesis, spontaneous APs were chronically blocked. Prior to the developmental appearance of spontaneous APs (up to 11 DIV) tetrodotoxin did not affect the formation of excitatory, glutamatergic synapses corroborating that synapses form independently of electrical activity [17]. During a later phase of *in vitro* differentiation, however, blockade of spontaneous APs reduced the number of excitatory synapses per postsynaptic neuron, suggesting that electrical activity stabilized excitatory synapses. In line with our results long-term electrical stimulation has been shown to increase the number of synaptic inputs on cultured spinal neurons [34].

Chronic AP blockade additionally led to a decrease in neuronal cell density, suggesting an effect on trophic interactions. An increased cell death in the presence of TTX was also observed in other cultured central neurons [4,26,27] and at the ultrastructural level disorganized synaptic terminals have been described after treatment with TTX in cultured hippocampal neurons [31]. As the amplitude distribution of spontaneous EPSCs was unaltered by TTX treatment, the synapses formed by surviving cells seemed unaffected. However, since amplitude distributions of spontaneous EPSCs were dominated by miniature EPSCs, effects of chronic TTX treatment on the amplitudes of EPSCs evoked by APs cannot be excluded. In spinal cord cultures TTX blocked release of neurotrophic factors [5] and the expression of neurotrophic factor genes was reduced by TTX in hippocampal neurons [28,49]. In summary, our results support the hypothesis, that trophic interactions play a role in electrical activity-dependent synaptic stabilization.

4.3. *Synaptic differentiation as suggested by an increase in glutamatergic miniature synaptic currents*

Miniature synaptic currents reflect the number and properties of postsynaptic receptors and the transmitter content of presynaptic vesicles at individual synapses and thus allow to study synaptic differentiation processes. We observed a marked increase in miniature EPSC amplitudes during *in vitro* differentiation of thalamic neurons (Fig. 8), suggesting synaptic differentiation processes, which occur after the initial establishment of a functional synapse.

Postsynaptic differentiation by receptor accumulation seems most likely to underlie this increase in mEPSC amplitudes. The accumulation of postsynaptic receptors is well known from studies of ACh receptor clustering at developing neuromuscular junctions, which is associated with increased miniature synaptic currents [9,22,48]. Receptor clustering has also been suggested to occur at developing cholinergic synapses in sympathetic neurons [14,19,33]. Furthermore, in mature glutamatergic synapses non-NMDA glutamate receptors have been shown to be accumulated at postsynaptic sites [16,21,36,39,45]. A recent immunocytochemical investigation of the subcellular distribution of AMPA receptors in cultured hippocampal neurons demonstrated a delay of several days in the appearance of GluR1-enriched spines compared to the occurrence of synapsin I immunoreactive presynaptic terminals [6]. This study thus strongly corroborates the interpretation that the observed increase in mEPSC amplitudes reflects accumulation of receptors at postsynaptic sites.

However, a contribution of presynaptic differentiation processes cannot be excluded: the transmitter content of synaptic vesicles could increase and thus account for the observed increase in mEPSC amplitudes. Additionally, changes in the geometry of the synaptic cleft might lead to an increased transmitter concentration. Theoretically, the delayed formation of somatic synapses could also lead to an increase in mEPSC amplitudes due to reduced electrotonic attenuation. However, this explanation was excluded by synapsin I immunostaining demonstrating a decrease in the proportion of somatic synapses during *in vitro* differentiation.

Contrary to mEPSCs GABAergic mIPSC amplitudes were invariant throughout the culture period. In agreement to our results no developmental change in mIPSC amplitudes was observed in cultured superior colliculus neurons [24]. Thus, in GABAergic synapses synaptic differentiation following initial establishment of functional transmission seems to play a minor role. Consistent with this notion, GABA_A receptors are not confined to postsynaptic sites [43] and no hot spots of GABA sensitivity were found in cultured central neurons [39].

In summary, our results suggest that the formation of glutamatergic synapses includes synaptic differentiation processes occurring after the establishment of functional synaptic transmission. The determination of the relative contributions of pre- and postsynaptic factors, however, will require detailed analysis of the development of individual synaptic sites.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft SFB 220 (A1). We would like

to thank Silke Engers and Helma Tyrlas for excellent technical assistance and, in particular Nicole Brusi for performing synapsin I immunostaining of cultured neurons. We further wish to thank Dr. Horstmar Holländer for providing polyclonal synapsin I-antibodies.

References

- [1] Anderson, M.J., Cohen, M.W. and Zorychta, E., Effects of innervation on the distribution of acetylcholine receptors on cultured muscle cells, *J. Physiol.*, 268 (1977) 731–756.
- [2] Bartlett, W.P. and Banker, G.A., An electron microscopic study of the development of axons and dendrites by hippocampal neurons in culture, II. Synaptic relationships, *J. Neurosci.*, 4 (1984) 1954–1965.
- [3] Bekkers, J.M., Richerson, G.B., Stevens, C.F., Origin of variability in quantal size in cultured hippocampal neurons and hippocampal slices, *Proc. Natl. Acad. Sci. USA*, 87 (1990) 5359–5362.
- [4] Bergey, G.K., Fitzgerald, S.C., Schrier, B.K. and Nelson, P.G., Neuronal maturation in mammalian cell culture is dependent on spontaneous electrical activity, *Brain Res.*, 207 (1981) 49–58.
- [5] Brenneman, D.E., Fitzgerald, S. and Nelson, P.G., Interaction between trophic action and electrical activity in spinal cord cultures, *Dev. Brain Res.*, 15 (1984) 211–217.
- [6] Craig, A.M., Blackstone, C.D., Huganir, R.L. and Banker, G., The distribution of glutamate receptors in cultured rat hippocampal neurons: postsynaptic clustering of AMPA-selective subunits, *Neuron*, 10 (1993) 1055–1068.
- [7] DeCamilli, P., Harris, S.M., Huttner, W.B. and Greengard, P., Synapsin I (protein I), a nerve terminal-specific phosphoprotein. II. Its specific association with synaptic vesicles demonstrated by immunocytochemistry in agarose-embedded synaptosomes, *J. Cell Biol.*, 96 (1983) 1355–1373.
- [8] Dubinsky, J.M., Development of inhibitory synapses among striatal neurons *in vitro*, *J. Neurosci.*, 9 (1989) 3955–3965.
- [9] Evers, J., Laser, M., Sun, Y., Xie, Z. and Poo, M., Studies of nerve-muscle interactions in *Xenopus* cell culture: analysis of early synaptic currents, *J. Neurosci.*, 9 (1989) 1523–1539.
- [10] Finch, D.M., Fisher, R.S. and Jackson, M.B., Miniature excitatory synaptic currents in cultured hippocampal neurons, *Brain Res.*, 518 (1990) 257–268.
- [11] Fischbach, G.D. and Schuetze, S.M., A post-natal decrease in acetylcholine channel open time at rat end-plates, *J. Physiol.*, 303 (1980) 125–137.
- [12] Fletcher, T.L., Cameron, P., De Camilli, P. and Banker, G., The distribution of synapsin I and synaptophysin in hippocampal neurons developing in culture, *J. Neurosci.*, 11 (1991) 1617–1626.
- [13] Frank, E. and Fischbach, G.D., Early events in neuromuscular junction formation *in vitro*, *J. Cell Biol.*, 83 (1979) 143–158.
- [14] Gardette, R., Listerud, M.D., Brussaard, A.B. and Role, L.W., Developmental changes in transmitter sensitivity and synaptic transmission in embryonic chicken sympathetic neurons innervated *in vitro*, *Dev. Biol.*, 147 (1991) 83–95.
- [15] Hamill, O.P., Marty, A., Neher, E., Sakmann, B. and Sigworth, F.J., Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches, *Pflügers Arch.*, 391 (1981) 85–100.
- [16] Hampson, D.R., Huang, X.P., Oberdorfer, M.D., Goh, J.W., Auyeung, A. and Wenthold, R.J., Localization of AMPA receptors in the hippocampus and cerebellum of the rat using an anti-receptor monoclonal antibody, *Neuroscience*, 50 (1992) 11–22.
- [17] Harris, W.A., Neural activity and development, *Annu. Rev. Physiol.*, 43 (1981) 689–710.
- [18] Hestrin, S., Activation and desensitization of glutamate-activated channels mediating fast excitatory synaptic currents in the visual cortex, *Neuron*, 9 (1992) 991–999.
- [19] Hume, R.I. and Honig, M.G., Physiological properties of newly formed synapses between sympathetic preganglionic neurons and sympathetic ganglion neurons, *J. Neurobiol.*, 22 (1991) 249–262.
- [20] Jackson, M.B., Lecar, H., Brenneman, D.E., Fitzgerald, S. and Nelson, P.G., Electrical development in spinal cord cell culture, *J. Neurosci.*, 2 (1982) 1052–1061.
- [21] Jones, K.A. and Baughman, R.W., Both NMDA and non-NMDA subtypes of glutamate receptors are concentrated at synapses on cerebral cortical neurons in culture, *Neuron*, 7 (1991) 593–603.
- [22] Kidokoro, Y., Anderson, M.J. and Gruener, R., Changes in synaptic potential properties during acetylcholine receptor accumulation and neurospecific interactions in *Xenopus* nerve-muscle cell culture, *Dev. Biol.*, 78 (1980) 464–483.
- [23] Kidokoro, Y., Two types of miniature endplate potentials in *Xenopus* nerve-muscle cultures, *Neurosci. Res.*, 1 (1984) 157–170.
- [24] Kraszewski, K. and Grantyn, R., Development of GABAergic connections *in vitro*: increasing efficacy of synaptic transmission is not accompanied by changes in miniature currents, *J. Neurobiol.*, 23 (1992) 766–781.
- [25] LeBmann, V., Gottmann, K. and Lux, H.D., Evans blue reduces macroscopic desensitization of non-NMDA receptor mediated currents and prolongs excitatory postsynaptic currents in cultured rat thalamic neurons, *Neurosci. Lett.*, 146 (1992) 13–16.
- [26] Ling, D.S.F., Petroski, R.E. and Geller, H.M., Both survival and development of spontaneously active rat hypothalamic neurons in dissociated culture are dependent on membrane depolarization, *Dev. Brain Res.*, 59 (1991) 99–103.
- [27] Lipton, S.A., Blockade of electrical activity promotes the death of mammalian retinal ganglion cells in culture, *Proc. Natl. Acad. Sci. USA*, 83 (1986) 9774–9778.
- [28] Lu, B., Yokoyama, M., Dreyfus, C.F. and Black, I.B., Depolarizing stimuli regulate nerve growth factor gene expression in cultured hippocampal neurons, *Proc. Natl. Acad. Sci. USA*, 88 (1991) 6289–6292.
- [29] Lu, B., Greengard, P. and Poo, M., Exogenous synapsin I promotes functional maturation of developing neuromuscular synapses, *Neuron*, 8 (1992) 521–529.
- [30] Lund, R.D. and Mustari, M.J., Development of the geniculocortical pathway in rats, *J. Comp. Neurol.*, 173 (1977) 289–306.
- [31] Mattson, M.P., Lee, R.E., Adams, M.E., Guthrie, P.B. and Kater, S.B., Interactions between entorhinal axons and target hippocampal neurons: a role for glutamate in the development of hippocampal circuitry, *Neuron*, 1 (1988) 865–876.
- [32] McAllister, J.P. and Das, G.D., Neurogenesis in the epithalamus, dorsal thalamus and ventral thalamus of the rat: an autoradiographic and cytological study, *J. Comp. Neurol.*, 172 (1977) 647–686.
- [33] Moss, B.L. and Role, L.W., Enhanced ACh sensitivity is accompanied by changes in ACh receptor channel properties and segregation of ACh receptor subtypes on sympathetic neurons during innervation *in vivo*, *J. Neurosci.*, 13 (1993) 13–28.
- [34] Nelson, P.G., Yu, C., Fields, R.D. and Neale, E.A., Synaptic connections *in vitro*: modulation of number and efficacy by electrical activity, *Science*, 244 (1989) 585–587.
- [35] O'Brien, R.J. and Fischbach, G.D., Excitatory synaptic transmission between interneurons and motoneurons in chick spinal cord cell cultures, *J. Neurosci.*, 6 (1986a) 3284–3289.
- [36] O'Brien, R.J. and Fischbach, G.D., Modulation of embryonic chick motoneuron glutamate sensitivity by interneurons and agonists, *J. Neurosci.*, 6 (1986) 3290–3296.

- [37] Petrucci, T.C. and Morrow, J.S., Synapsin I: an actin-bundling protein under phosphorylation control, *J. Cell Biol.*, 105 (1987) 1355–1363.
- [38] Pfrieger, F.W., Veselovsky, N.S., Gottmann, K. and Lux, H.D., Pharmacological characterization of calcium currents and synaptic transmission between thalamic neurons *in vitro*, *J. Neurosci.*, 12 (1992) 4347–4357.
- [39] Ransom, B.R., Bullock, P.N. and Nelson, P.G., Mouse spinal cord in cell culture. III. Neuronal chemosensitivity and its relationship to synaptic activity, *J. Neurophysiol.*, 40 (1977) 1163–1177.
- [40] Redman, S., Quantal analysis of synaptic potentials in neurons of the central nervous system, *Physiol. Rev.*, 70 (1990) 165–198.
- [41] Role, L.W., Matossian, V.R., O'Brien, R.J. and Fischbach, G.D., On the mechanism of acetylcholine receptor accumulation at newly formed synapses on chick myotubes, *J. Neurosci.*, 5 (1985) 2197–2204.
- [42] Sakmann, B. and Brenner, H.R., Change in synaptic channel gating during neuromuscular development, *Nature*, 276 (1978) 401–402.
- [43] Somogyi, P., Takagi, H., Richards, J.G. and Mohler, H., Subcellular localization of benzodiazepine/GABA_A receptors in the cerebellum of rat, cat and monkey using monoclonal antibodies, *J. Neurosci.*, 9 (1989) 2197–2209.
- [44] Swandulla, D. and Misgeld, U., Development and properties of synaptic mechanisms in a network of rat hypothalamic neurons grown in culture, *J. Neurophysiol.*, 64 (1990) 715–726.
- [45] Trussel, L.O., Thio, L.L., Zorumski, C.F. and Fischbach, G.D., Rapid desensitization of glutamate receptors in vertebrate central neurons, *Proc. Natl. Acad. Sci. USA*, 85 (1988) 4562–4566.
- [46] Valtorta, F., Benfenati, F. and Greengard, P., Structure and function of the synapsins, *J. Biol. Chem.*, 267 (1992) 7195–7198.
- [47] Weiss, S., Pin, J., Sebben, M., Kemp, D.E., Sladeczek, F., Gabrion, J. and Bockaert, J., Synaptogenesis of cultured striatal neurons in serum-free medium: a morphological and biochemical study, *Proc. Natl. Acad. Sci. USA*, 83 (1986) 2238–2242.
- [48] Xie, Z. and Poo, M., Initial events in the formation of neuromuscular synapse: rapid induction of acetylcholine release from embryonic neuron, *Proc. Natl. Acad. Sci. USA*, 83 (1986) 7069–7073.
- [49] Zafra, F., Castren, E., Thoenen, H. and Lindholm, D., Interplay between glutamate and γ -aminobutyric acid transmitter systems in the physiological regulation of brain-derived neurotrophic factor and nerve growth factor synthesis in hippocampal neurons, *Proc. Natl. Acad. Sci. USA*, 88 (1991) 10037–10041.
- [50] Zucker, R.S. and Haydon, P.G., Membrane potential has no direct role in evoking neurotransmitter release, *Nature*, 335 (1988) 360–362.