Published in final edited form as:
In Vitro Cell Dev Biol Anim. 2010 September ; 46(8): 685–692. doi:10.1007/s11626-010-9324-0.

Coexpression of glutamate vesicular transporter (VGLUT1) and choline acetyltransferase (ChAT) proteins in fetal rat hippocampal neurons in culture

Neelima Bhargava, **Mainak Das**, **Darin Edwards**, **Maria Stancescu**, **Jung-Fong Kang**, and **James J Hickman***
NanoScience Technology Center, University of Central Florida, Orlando, FL 32826, USA

Abstract

A very small population of Choline acetyltransferase (ChAT) immunoreactive cells is observed in all layers of the adult hippocampus. This is the intrinsic source of the hippocampal cholinergic innervation, in addition to the well-established septo-hippocampal cholinergic projection. This study aimed at quantifying and identifying the origin of this small population of ChAT-immunoreactive cells in the hippocampus at early developmental stages, by culturing the fetal hippocampal neurons in serum-free culture and on a patternable, synthetic silane substrate N-1 [3-(trimethoxysilyl) propyl] diethylenetriamine (DETA). Using this method a large proportion of glutamatergic (glutamate vesicular transporter, VGLUT1-immunoreactive) neurons, a small fraction of GABAergic (GABA-immunoreactive) neurons and a large proportion of cholinergic (ChAT-immunoreactive) neurons were observed in culture. Interestingly, most of the glutamatergic neurons which expressed glutamate vesicular transporter (VGLUT1) also co-expressed choline acetyltransferase (ChAT) proteins. On the contrary, when the cultures were double stained with GABA and ChAT, colocalization was not observed. Neonatal and adult rat hippocampal neurons were also cultured to verify whether these more mature neurons also co-express VGLUT1 and ChAT proteins in culture. Colocalization of VGLUT1 and ChAT in these relatively more mature neurons was not observed. One possible explanation for this observation is that the neurons have the ability to synthesize multiple neurotransmitters at a very early stage of development and then with time follows a complex, combinatorial strategy of electrochemical coding to determine their final fate.

Keywords

Adult hippocampal neuron; Fetal; VGLUT1; ChAT; Serum-free culture

Introduction

In the adult mammalian hippocampus, Choline acetyltransferase (ChAT) immunoreactive cells are rare, but are observed in all layers of the hippocampus (Frotscher et al., 1986). This small number of ChAT-immunoreactive cells in the hippocampus are the intrinsic source of hippocampal cholinergic innervation, in addition to the well-established septo-hippocampal cholinergic projection (Baisden et al., 1984; Colom et al., 2005; De Lacalle et al., 1994; Ferencz et al., 2001; Frazier et al., 1996; Frotscher and Leranth, 1985; Frotscher et al., 1986; Gahwiler et al., 1987; Gilad et al., 1987; Ishimaru et al., 1995; Linke et al., 1994; Nilsson et al., 1992; Ransmayr et al., 1989; Ransmayr et al., 1992; Schwegler et al., 1996; Sotty et al.,

*Correspondence should be addressed to: NanoScience Technology Center, 12424 Research Parkway, Suite 400, University of Central Florida, Orlando, FL 32826, USA, Phone: 407-823-1925, Fax: 407-882-2819, jhickman@mail.ucf.edu.

2003; Sugaya et al., 1997; Vincent and McGeer, 1981; Williams et al., 1989). Interestingly, these endogenous cholinergic neurons, present in the hippocampus, have a functional role in the induction of long-term potentiation (LTP) at mossy fiber-CA3 synapses (Maeda et al., 1994). Furthermore, it has been observed that these small populations of cholinergic neurons in the rat hippocampus do not compensate for the loss of septo-hippocampal cholinergic fibers (Frotscher, 1988). However, the origin of these endogenous ChAT-immunoreactive cells in the hippocampus is not clear. In this study, the aim was to identify and quantify the origin of this rare population of ChAT-immunoreactive cells in the cultured hippocampal neurons that were obtained from fetal, neonatal and adult rats. A serum-free culture medium and patternable, synthetic silane substrate was utilized to grow the cells and perform the immunocytochemical studies. Our studies have shown that in the fetal rat, hippocampal neuron culture most of the glutamatergic neurons, which expressed glutamate vesicular transporter (VGLUT1), also co-expressed choline acetyltransferase (ChAT) proteins. On the other hand, fetal hippocampal neurons, which expressed GABA, did not express ChAT. In cultured neonatal and adult hippocampal neurons, co-localization of VGLUT1 and ChAT proteins was not observed. Based on these findings, we are proposing that at a very early developmental stage, neurons have the ability to synthesize multiple neurotransmitters. The neurons, at this early stage of development, then follow a complex combinatorial strategy of electrochemical coding in order to determine their final fate. However, they lose this ability to synthesize multiple neurotransmitters as they mature.

Methods

Surface modification of the coverslips

Glass coverslips (Thomas Scientific 6661F52, 22 × 22 mm^2 no. 1) were cleaned using an O_2 plasma cleaner (Harrick PDC-32G) for 20 min at 100 mTorr. The DETA (United Chemical Technologies Inc., Bristol, PA, T2910KG) films were formed by the reaction of the cleaned surface with a 0.1% (v/v) mixture of the organosilane in freshly distilled toluene (Fisher T2904), according to Ravenscroft et al. (1998) (Ravenscroft et al., 1998). The DETA (N-1[3-(trimethoxysilyl)propyl] diethylenetriamine)-coated coverslips were heated to just below the boiling point of toluene, rinsed with toluene, reheated to just below the boiling temperature, and then oven dried (Das et al., 2005; Das et al., 2006; Das et al., 2003; Das et al., 2007a).

Surface characterization of the coverslips after coating with DETA

Surfaces were characterized by contact angle measurements using an optical contact angle goniometer (KSV Instruments, Monroe, CT, Cam 200) and by X-ray Photoelectron Spectroscopy (XPS) (FISONS ESCALab 220i-XL). The XPS survey scans, as well as high-resolution N 1s and C 1s scans, using monochromatic Al Kα excitation, were obtained similar to the previously reported results (Das et al., 2007b; Das et al., 2007c; Hickman et al., 1994; Ravenscroft et al., 1998).

Fetal, Neonatal and Adult Rat Hippocampal Neuron isolation and Culture

The cell isolation procedures listed here are in agreement with and approved by the Animal Research Council of the University of Central Florida, which adheres to the IACUC policies.

Fetal hippocampal neuron culture

18 day-old fetuses were obtained from timed pregnant rats. The timed pregnant rats (fetal day 18) were euthanized using carbon dioxide. After C-sectioning, the fetuses were collected in ice cold Hibernate E/ B27/ Glutamax™/ Antibiotic-Antimycotic. The fetuses

were decapitated and the whole brain was transferred into fresh cold Hibernate E/ B27/ Glutamax™/ Antibiotic-Antimycotic. The hippocampi were isolated from the fetal brain and collected in a tube containing 1ml of Hibernate E/ B27/ Glutamax™/ Antibiotic-Antimycotic. The fetal hippocampal neurons were obtained by triturating the tissue using a Pasteur pipette. The 1 ml cell suspension was layered over a 4 ml step gradient (Optipep diluted 0.505: 0.495 (v/v) with Hibernate E/ GlutaMAX™ / Antibiotic-Antimycotic/ B27 and then made to 15%, 20%, 25% and 35% (v/v) in Hibernate E/ GlutaMAX™/ Antibiotic-Antimycotic/ B27) followed by centrifugation for 15 min, using 800g, at 4°C. This additional step helped to remove the debris that arose during dissection from the damaged cells. After centrifugation, one strong band of cells was obtained at the top. The pyramidal hippocampal neurons constituted this band with large somas. The cells were resuspended in culture medium (Neurobasal / B27 / Glutamax™ / Antibiotic-Antimycotic) and plated at a density of 75 cells/mm^2 onto a DETA silane coated substrate (Brewer, 1995; Brewer, 1997; Brewer, 1999; Brewer and Price, 1996; Brewer et al., 1993; Schaffner et al., 1995; Stenger et al., 1998; Stenger et al., 1993). Half of the medium was changed after every 3–4 days.

Neonatal hippocampal neuron culture

2 day-old neonatal rats were used for this culture. The neonatal rats were decapitated and the whole brain was transferred into fresh cold Hibernate A/ B27/ Glutamax™/ Antibiotic-Antimycotic. The hippocampi were isolated from the neonatal brain and collected in a tube containing 1 ml of Hibernate A/ B27/ Glutamax™/ Antibiotic-Antimycotic. The neonatal hippocampal neurons were obtained by digesting the hippocampal tissue for 15 minutes in Papain. The Papain solution was prepared by dissolving 1 mg of Papain in 1 ml of Hibernate A / B27/ Glutamax™/ Antibiotic-Antimycotic. The enzyme treatment was carried out in a shaking water bath at 37°C. After a 15 minute treatment, the enzyme solution was washed with fresh Hibernate A/ B27/ Glutamax™/ Antibiotic-Antimycotic and finally resuspended in 1 ml of fresh Hibernate A/ B27/ Glutamax™/ Antibiotic-Antimycotic. A Single cell suspension was obtained by triturating the tissue using a Pasteur pipette. The 1 ml cell suspension was layered over a 4 ml step gradient (Optipep diluted 0.505: 0.495 (v/v) with Hibernate A/ GlutaMAX™ / Antibiotic-Antimycotic/ B27 and then made to 15%, 20%, 25% and 35% (v/v) in Hibernate A/ GlutaMAX™/ Antibiotic-Antimycotic/ B27) followed by centrifugation for 15 min, using 800g, at 4°C. This additional step helped to remove the debris that arose during dissection from the damaged cells. After centrifugation, one strong band of cells was obtained at the top. The pyramidal hippocampal neurons constituted this band with large somas. The cells were resuspended in culture medium (Neurobasal / B27 / Glutamax™ / Antibiotic-Antimycotic) and plated at a density of 75 cells/mm^2 on DETA silane coated substrate (Brewer, 1995; Brewer, 1999; Brewer and Price, 1996; Brewer et al., 1993; Hickman et al., 1994; Schaffner et al., 1995). Half of the medium was changed after every 3–4 days.

Adult Rat Hippocampal Neuron isolation and Culture

4–6 month-old adult rats were used for the culture. The adult rats were decapitated and the whole brain was transferred into fresh cold Hibernate A/ B27/ Glutamax™/ Antibiotic-Antimycotic. The hippocampi were isolated from the adult brain and collected in tube containing 1 ml of Hibernate A/ B27/ Glutamax™/ Antibiotic-Antimycotic. The adult hippocampal neurons were obtained by digesting the hippocampal tissue for 15 minutes in Papain. Papain solution was prepared by dissolving 1 mg of Papain in 1 ml of Hibernate A / B27/ Glutamax™/ Antibiotic-Antimycotic. The enzyme treatment was carried out in a shaking water bath at 37°C. After 15 minutes treatment the enzyme solution was washed with fresh Hibernate A/ B27/ Glutamax™/ Antibiotic-Antimycotic and finally resuspended in 1 ml of fresh Hibernate A/ B27/ Glutamax™/ Antibiotic-Antimycotic. A single cell suspension was obtained by triturating the tissue using a Pasteur pipette. The 1 ml cell

suspension was layered over a 4 ml step gradient (Optipep diluted 0.505: 0.495 (v/v) with Hibernate A/ GlutaMAX™ / Antibiotic-Antimycotic/ B27 and then made to 15%, 20%, 25% and 35% (v/v) in Hibernate A/ GlutaMAX™/ Antibiotic-Antimycotic/ B27) followed by centrifugation for 15 min, using 800g, at 4°C. After centrifugation, three bands of cells were obtained at the top. The neurons constituted the second and the third band. The cells were resuspended in culture medium (Neurobasal A/ B27 / basic FGF (5ng/ml)/ Glutamax™ / Antibiotic-Antimycotic) and plated at a density of 150 cells/mm^2 on DETA silane coated substrate (Brewer, 1995; Brewer, 1997; Brewer, 1999; Brewer and Price, 1996; Brewer et al., 1993; Schaffner et al., 1995; Stenger et al., 1998; Stenger et al., 1993). Half of the medium was changed after every 3–4 days.

Immunocytochemistry

Four different antibodies were utilized, VGLUT1 (Guinea pig anti-vesicular glutamate transport polyclonal antibody, Cat # AB 5905, Chemicon, 1:100), ChAT (Rabbit anti-choline acetyl-transferase affinity purified polyclonal antibody, cat # AB 5042, Chemicon, 1:100), GAD65 (Anti-Glutamate Decarboxylase, 65 kDa Isoform, clone GAD-6, Cat # 351R, Chemicon, 1:100) and GABA (anti-mouse GABA, clone 5A9, cat # MAB 316, Chemicon, 1:100) for the immunostaining. In preparation for staining with anti-VGLUT1, anti-ChAT, anti-GAD65 and anti-GABA antibodies, 80 μl of paraformaldehyde was added (prepared in PBS) to 2 ml of medium for 5 minutes. This reaction was performed while keeping the 6-well plate on ice. After 5 minutes, the coverslips were rinsed free of medium with phosphate-buffered saline (PBS) and fixed for 20 min at room temperature with cold fixative (11.1 ml of Formalin+ 89.9 ml of PBS+ 200 μl of Glutaraldehyde+ 4g of Glucose). After 20 minutes, cells were permeabilized for 5 minutes with permeabilizing solution (50 mM Lysine+ 0.5% Triton X-100+ 100 ml of PBS). After rinsing with PBS, the nonspecific sites were blocked with 5% normal donkey serum and 0.5% Triton X-100 in PBS. The cells were blocked for 2 hours and then the cells were incubated with the primary antibodies for 12 h at 4°C. After 12 h, the cells were rinsed free of blocking solution with PBS and then further incubated with the secondary antibodies. After rinsing four times in PBS, the cover slips were mounted with Vectashield mounting medium (H1000, Vector Laboratories, Burlingame, CA) onto slides. The coverslips were observed and photographed using a confocal microscope. Controls without primary antibody were negative. In the case of hippocampal slice samples, the whole hippocampal slice was processed in the similar manner as above.

Results

This system uses a patternable (Ravenscroft et al., 1998; Stenger et al., 1998; Stenger et al., 1993), non-biological, cell growth promoting, organosilane substrate (DETA), coated on a glass surface, facilitating variable control and reproducibility of the growth surface (Das et al., 2003; Das et al., 2004; Das et al., 2007a; Ravenscroft et al., 1998; Schaffner et al., 1995). Quality of the surface modified coverslips used for the cell culture was evaluated using static contact angle measurements and X-ray photoelectron spectroscopy (XPS) analysis as previously described (Hickman et al., 1994). Stable contact angles (40.64° ± 2.9 / mean ± SD) throughout the study indicated high reproducibility and quality of the DETA coatings and were similar to previously published results. Based on the ratio of the N (401 and 399 eV) and the Si $2p_{3/2}$ peaks, XPS measurements indicated that a complete monolayer of DETA was formed on the coverslips.

In the first set of experiments, 20 day-old fetal hippocampal neuron cultures were stained separately with four different antibodies, glutamate vesicular transporter VGLUT1, GABA, GAD65 and choline acetyl transferase (ChAT). Based upon observation, 80% (n = 6, where n is the total number of coverslips used for evaluation) of the cells were of glutamatergic

(glutamate vesicular transporter, VGLUT1-immunoreactive) neurons (Figure 1A, B), a similar percentage of cells were cholinergic (ChAT-immunoreactive) neurons (Figure 1C, D) and a very small fraction (15–20%, n = 6) of cells were GABAergic (GABA-immunoreactive and GAD65-immunoreactive) neurons (Figure 1E, F, G and H) in the culture. Figure 1I–L indicates the double staining of the culture with anti-GABA and anti-VGLUT1 antibodies to view the network formation between inhibitory and excitatory hippocampal neurons in the culture.

The large proportion of ChAT-immunoreactive cells in the culture prompted a second set of experiments involving double staining of the cultures with VGLUT1 and ChAT antibodies. Interestingly, most of the glutamatergic neurons, which expressed glutamate vesicular transporter (VGLUT1), also co-expressed choline acetyltransferase (ChAT) proteins (Figure 2A–H). On the contrary, when the cultures were double stained with GABA and ChAT, co-localization was not observed (Figure 2I–L). The GABA positive fetal hippocampal neurons did not express ChAT. A similar colocalization of VGLUT1 and ChAT were obtained in fetal neurons cultured until day 5 (Data not shown) and day 13 (Figure 2H). In order to verify whether this VGLUT1+ ChAT colocalization was only seen in culture or whether it can also be observed *in vivo,* a whole fetal hippocampus slice was stained and it was found that indeed VGLUT1 and ChAT proteins colocalized *in vivo* at day 18 in the fetal hippocampus (Figure 2M–P). In order to determine whether cultured neonatal neurons have the ability to co-express VGLUT1 and ChAT, a third set of experiments was performed. In this experiment, 13 day-old, cultured neonatal hippocampal neurons were double stained with antibodies against VGLUT1 and ChAT proteins. In most of the neurons no co-expression of VGLUT1 and ChAT (Figure 3A–H) was observed. A very weak ChAT staining was observed in a very small population of neonatal neurons (Figure 3I–L).

Finally, to test whether adult hippocampal neurons have the ability to co-express VGLUT1 and ChAT in culture, 13 day-old cultured adult hippocampal neurons were double stained with antibodies against VGLUT1 and ChAT proteins. No ChAT immunostaining was observed (Figure 4A–L).

Discussion

Our studies indicated that during fetal development of the mammalian hippocampus, most of the hippocampal neurons are equipped with the cellular machinery to synthesize both glutamate and acetylcholine. But as the hippocampus matures, most of the neurons lose their ability to synthesize acetylcholine.

One of the possible explanations for this observation is that the neurons have the ability to express genes for synthesizing multiple transmitters at very early stages of development and then follow a combinatorial strategy of electrochemical coding to determine their final composition (Danik et al., 2005; Furshpan et al., 1986; Furshpan et al., 1976; Yang et al., 2002). During the last two decades, studies have indicated that single neurons appear to use multiple, colocalized transmitter signals (Bartfai et al., 1986; Hokfelt et al., 1980) and the final neurotransmitter phenotype of a neuron is decided either by the target organ, surrounding cells or by other unknown molecular switches (Asmus et al., 2000; Johnson, 1994; Landis, 2002). This combinatorial strategy at the synapse provides an individual neuron with extraordinary potential for combining signals not only at the single neuron level, but at the level of single synapse (Bartfai et al., 1986). Our studies indicate that fetal hippocampal neurons followed a similar combinatorial strategy during development, whereas at the fetal stage of development glutamatergic hippocampal neurons synthesize both glutamate and acetylcholine. Previously, it has been reported that there is a frequent co-expression of vesicular transporter 1 gene, as well as co-expression with genes for choline

acetyltransferase in different brain regions (Danik et al., 2005). However, whether these genes finally result in protein synthesis was not verified. In this study, the expression of the VGLUT1 and ChAT proteins was observed to be caused by these genes. Interestingly the neonatal and adult neurons appeared to have lost the ability to synthesize ChAT.

Conclusions

If our initial hypothesis is correct what remains to be answered is how this combinatorial strategy that is utilized by the VGLUT1+ ChAT immunopositive hippocampal neurons at very early phases of development leads to intrinsic cholinergic signaling within the hippocampus. In addition, do glial cells or projections into the hippocampus from other brain areas play any role in determining the neurotransmitter phenotypes in the hippocampal drug development? Further investigation needs to be done to understand the as yet unknown molecular switch(es) which reduce the number of ChAT immunoreactive cells in the mature hippocampus as this now could have important implications for regeneration of neurons after disease or injury.

Acknowledgments

This work was supported by the Department of Energy grant number DE-FG02-04ER-46171 and the National Institute of Health grant number 5RO1EB005459 for funding this project.

References

Asmus SE, Parsons S, Landis SC. Developmental changes in the transmitter properties of sympathetic neurons that innervate the periosteum. J Neurosci. 2000; 20:1495–504. [PubMed: 10662839]

Baisden RH, Woodruff ML, Hoover DB. Cholinergic and non-cholinergic septo-hippocampal projections: a double-label horseradish peroxidase-acetylcholinesterase study in the rabbit. Brain Res. 1984; 290:146–51. [PubMed: 6692131]

Bartfai T, Iverfeldt K, Brodin E, Ogren SO. Functional consequences of coexistence of classical and peptide neurotransmitters. Prog Brain Res. 1986; 68:321–30. [PubMed: 2882558]

Brewer GJ. Serum-free B27/neurobasal medium supports differentiated growth of neurons from the striatum, substantia nigra, septum, cerebral cortex, cerebellum, and dentate gyrus. J Neurosci Res. 1995; 42:674–83. [PubMed: 8600300]

Brewer GJ. Isolation and culture of adult rat hippocampal neurons. J Neurosci Methods. 1997; 71:143–55. [PubMed: 9128149]

Brewer GJ. Regeneration and proliferation of embryonic and adult rat hippocampal neurons in culture. Exp Neurol. 1999; 159:237–47. [PubMed: 10486191]

Brewer GJ, Price PJ. Viable cultured neurons in ambient carbon dioxide and hibernation storage for a month. Neuroreport. 1996; 7:1509–12. [PubMed: 8856709]

Brewer GJ, Torricelli JR, Evege EK, Price PJ. Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. J Neurosci Res. 1993; 35:567–76. [PubMed: 8377226]

Colom LV, Castaneda MT, Reyna T, Hernandez S, Garrido-Sanabria E. Characterization of medial septal glutamatergic neurons and their projection to the hippocampus. Synapse. 2005; 58:151–64. [PubMed: 16108008]

Danik M, Cassoly E, Manseau F, Sotty F, Mouginot D, Williams S. Frequent coexpression of the vesicular glutamate transporter 1 and 2 genes, as well as coexpression with genes for choline acetyltransferase or glutamic acid decarboxylase in neurons of rat brain. J Neurosci Res. 2005; 81:506–21. [PubMed: 15983996]

Das M, Bhargava N, Gregory C, Riedel L, Molnar P, Hickman JJ. Adult rat spinal cord culture on an organosilane surface in a novel serum-free medium. In Vitro Cell Dev Biol Anim. 2005; 41:343–8. [PubMed: 16448224]

Das M, Gregory CA, Molnar P, Riedel LM, Wilson K, Hickman JJ. A defined system to allow skeletal muscle differentiation and subsequent integration with silicon microstructures. Biomaterials. 2006; 27:4374–80. [PubMed: 16647113]

Das M, Molnar P, Devaraj H, Poeta M, Hickman JJ. Electrophysiological and morphological characterization of rat embryonic motoneurons in a defined system. Biotechnol Prog. 2003; 19:1756–61. [PubMed: 14656152]

Das M, Molnar P, Gregory C, Riedel L, Jamshidi A, Hickman JJ. Long-term culture of embryonic rat cardiomyocytes on an organosilane surface in a serum-free medium. Biomaterials. 2004; 25:5643–7. [PubMed: 15159080]

Das M, Patil S, Bhargava N, Kang JF, Riedel LM, Seal S, Hickman JJ. Auto-catalytic ceria nanoparticles offer neuroprotection to adult rat spinal cord neurons. Biomaterials. 2007a; 28:1918–1925. [PubMed: 17222903]

Das M, Rumsey JW, Gregory CA, Bhargava N, Kang JF, Molnar P, Riedel L, Guo X, Hickman JJ. Embryonic motoneuron-skeletal muscle co-culture in a defined system. Neuroscience. 2007b; 146:481–8. [PubMed: 17383103]

Das M, Wilson K, Molnar P, Hickman JJ. Differentiation of skeletal muscle and integration of myotubes with silicon microstructures using serum-free medium and a synthetic silane substrate. Nat Protoc. 2007c; 2:1795–801. [PubMed: 17641647]

De Lacalle S, Lim C, Sobreviela T, Mufson EJ, Hersh LB, Saper CB. Cholinergic innervation in the human hippocampal formation including the entorhinal cortex. J Comp Neurol. 1994; 345:321–44. [PubMed: 7929905]

Ferencz I, Leanza G, Nanobashvili A, Kokaia Z, Kokaia M, Lindvall O. Septal cholinergic neurons suppress seizure development in hippocampal kindling in rats: comparison with noradrenergic neurons. Neuroscience. 2001; 102:819–32. [PubMed: 11182245]

Frazier CJ, Rollins YD, Hall ME, Young DA, Rose GM. Cholinergic deafferentation enhances rat hippocampal pyramidal neuron responsiveness to locally applied nicotine. Brain Res. 1996; 727:217–20. [PubMed: 8842401]

Frotscher M. Cholinergic neurons in the rat hippocampus do not compensate for the loss of septohippocampal cholinergic fibers. Neurosci Lett. 1988; 87:18–22. [PubMed: 3380335]

Frotscher M, Leranth C. Cholinergic innervation of the rat hippocampus as revealed by choline acetyltransferase immunocytochemistry: a combined light and electron microscopic study. J Comp Neurol. 1985; 239:237–46. [PubMed: 4044938]

Frotscher M, Schlander M, Leranth C. Cholinergic neurons in the hippocampus. A combined light- and electron-microscopic immunocytochemical study in the rat. Cell Tissue Res. 1986; 246:293–301. [PubMed: 3779810]

Furshpan EJ, Landis SC, Matsumoto SG, Potter DD. Synaptic functions in rat sympathetic neurons in microcultures. I. Secretion of norepinephrine and acetylcholine. J Neurosci. 1986; 6:1061–79. [PubMed: 3701410]

Furshpan EJ, MacLeish PR, O'Lague PH, Potter DD. Chemical transmission between rat sympathetic neurons and cardiac myocytes developing in microcultures: evidence for cholinergic, adrenergic, and dual-function neurons. Proc Natl Acad Sci U S A. 1976; 73:4225–9. [PubMed: 186792]

Gahwiler BH, Enz A, Hefti F. Nerve growth factor promotes development of the rat septo-hippocampal cholinergic projection in vitro. Neurosci Lett. 1987; 75:6–10. [PubMed: 3574769]

Gilad GM, Rabey JM, Tizabi Y, Gilad VH. Age-dependent loss and compensatory changes of septohippocampal cholinergic neurons in two rat strains differing in longevity and response to stress. Brain Res. 1987; 436:311–22. [PubMed: 3435831]

Hickman JJ, Bhatia SK, Quong JN, Shoen P, Stenger DA, Pike CJ, Cotman CW. Rational Pattern Design for in-Vitro Cellular Networks Using Surface Photochemistry. Journal of Vacuum Science & Technology a-Vacuum Surfaces and Films. 1994; 12:607–616.

Hokfelt T, Rehfeld JF, Skirboll L, Ivemark B, Goldstein M, Markey K. Evidence for coexistence of dopamine and CCK in meso-limbic neurones. Nature. 1980; 285:476–8. [PubMed: 6105617]

Ishimaru H, Takahashi A, Ikarashi Y, Maruyama Y. Immunohistochemical and neurochemical studies of hippocampal cholinergic neurones after ischaemia. Neuroreport. 1995; 6:557–60. [PubMed: 7766863]

Johnson MD. Synaptic glutamate release by postnatal rat serotonergic neurons in microculture. Neuron. 1994; 12:433–42. [PubMed: 7906530]

Landis SC. Quick-change artist: from excitatory to inhibitory synapse in minutes. Nat Neurosci. 2002; 5:503–4. [PubMed: 12037514]

Linke R, Schwegler H, Boldyreva M. Cholinergic and GABAergic septo-hippocampal projection neurons in mice: a retrograde tracing study combined with double immunocytochemistry for choline acetyltransferase and parvalbumin. Brain Res. 1994; 653:73–80. [PubMed: 7982078]

Maeda T, Kaneko S, Satoh M. Roles of endogenous cholinergic neurons in the induction of long-term potentiation at hippocampal mossy fiber synapses. Neurosci Res. 1994; 20:71–8. [PubMed: 7984341]

Nilsson OG, Leanza G, Bjorklund A. Acetylcholine release in the hippocampus: regulation by monoaminergic afferents as assessed by in vivo microdialysis. Brain Res. 1992; 584:132–40. [PubMed: 1355389]

Ransmayr G, Cervera P, Hirsch E, Ruberg M, Hersh LB, Duyckaerts C, Hauw JJ, Delumeau C, Agid Y. Choline acetyltransferase-like immunoreactivity in the hippocampal formation of control subjects and patients with Alzheimer's disease. Neuroscience. 1989; 32:701–14. [PubMed: 2601840]

Ransmayr G, Cervera P, Hirsch EC, Berger W, Fischer W, Agid Y. Alzheimer's disease: is the decrease of the cholinergic innervation of the hippocampus related to intrinsic hippocampal pathology? Neuroscience. 1992; 47:843–51. [PubMed: 1374541]

Ravenscroft MS, Bateman KE, Shaffer KM, Schessler HM, Jung DR, Schneider TW, et al. Developmental neurobiology implications from fabrication and analysis of hippocampal neuronal networks on patterned silane- modified surfaces. Journal of the American Chemical Society. 1998; 120:12169–12177.

Schaffner AE, Barker JL, Stenger DA, Hickman JJ. Investigation of the factors necessary for growth of hippocampal neurons in a defined system. J Neurosci Methods. 1995; 62:111–9. [PubMed: 8750092]

Schwegler H, Boldyreva M, Linke R, Wu J, Zilles K, Crusio WE. Genetic variation in the morphology of the septo-hippocampal cholinergic and GABAergic systems in mice: II. Morpho-behavioral correlations. Hippocampus. 1996; 6:535–45. [PubMed: 8953306]

Sotty F, Danik M, Manseau F, Laplante F, Quirion R, Williams S. Distinct electrophysiological properties of glutamatergic, cholinergic and GABAergic rat septohippocampal neurons: novel implications for hippocampal rhythmicity. J Physiol. 2003; 551:927–43. [PubMed: 12865506]

Stenger DA, Hickman JJ, Bateman KE, Ravenscroft MS, Ma W, Pancrazio JJ, et al. Microlithographic determination of axonal/dendritic polarity in cultured hippocampal neurons. J Neurosci Methods. 1998; 82:167–73. [PubMed: 9700689]

Stenger DA, Pike CJ, Hickman JJ, Cotman CW. Surface determinants of neuronal survival and growth on self-assembled monolayers in culture. Brain Res. 1993; 630:136–47. [PubMed: 8118680]

Sugaya K, Clamp C, Bryan D, McKinney M. mRNA for the m4 muscarinic receptor subtype is expressed in adult rat brain cholinergic neurons. Brain Res Mol Brain Res. 1997; 50:305–13. [PubMed: 9406947]

Vincent SR, McGeer EG. A substance P projection to the hippocampus. Brain Res. 1981; 215:349–51. [PubMed: 6167321]

Williams LR, Jodelis KS, Donald MR. Axotomy-dependent stimulation of choline acetyltransferase activity by exogenous mouse nerve growth factor in adult rat basal forebrain. Brain Res. 1989; 498:243–56. [PubMed: 2790481]

Yang B, Slonimsky JD, Birren SJ. A rapid switch in sympathetic neurotransmitter release properties mediated by the p75 receptor. Nat Neurosci. 2002; 5:539–45. [PubMed: 11992117]

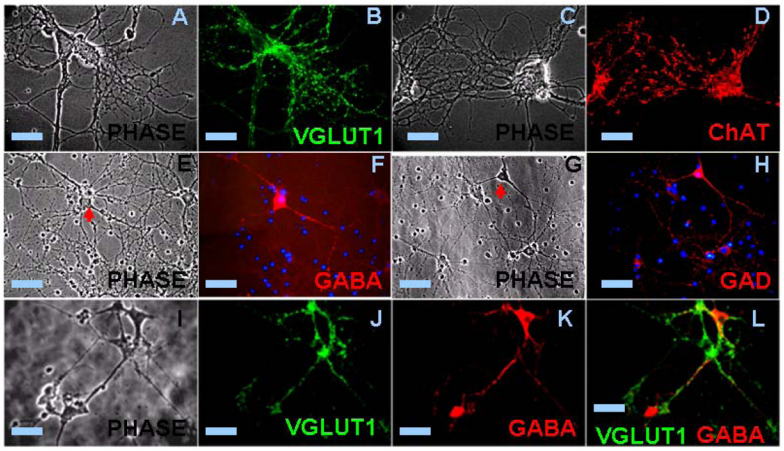

Figure 1. Single antibody immunostaining of fetal hippocampal neurons in culture

A. Phase picture of the hippocampal neuron which has been stained with VGLUT1 in B. Scale bar 30 μ. B. VGLUT1 stained hippocampal neuron shown in green. Scale bar 30 μ. C. Phase picture of a hippocampal neuron which has been stained with ChAT in D. Scale bar 30 μ. D. ChAT stained hippocampal neurons shown in red. Scale bar 30 μ. E. Phase picture of a GABA-immunoreactive hippocampal neuron marked with a red arrow in a field of other hippocampal neurons which are negative for GABA. Scale bar 60 μ. F. GABA-immunoreactive hippocampal neurons in culture are shown in red. DAPI indicated by a blue nucleus. Scale bar 60 μ. G. Phase picture of a GAD65-immunoreactive hippocampal neuron marked with a red arrow in a field of other cells which are negative for GAD65. Scale bar 60 μ. H. GAD65-immunoreactive hippocampal neurons in culture are shown in red. DAPI indicated by a blue nucleus. Scale bar 60 μ. I. Phase picture of GABA-immunoreactive and VGLUT1-immunoreactive hippocampal neurons forming a network in the culture. Scale bar 60 μ. J. VGLUT1-immunoreactive hippocampal neurons shown in green. Scale bar 60 μ. K. GABA-immunoreactive hippocampal neurons shown in red. Scale bar 60 μ. L. Immunostained picture of GABA-immunoreactive (Red) and VGLUT1-immunoreactive (Green) hippocampal neurons forming a network in the culture. Scale bar 60 μ.

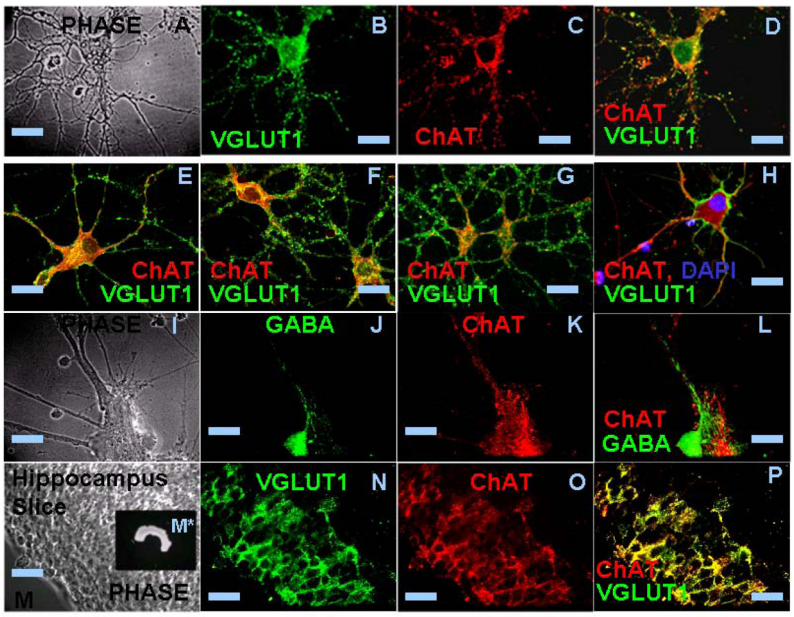

Figure 2. Double-staining of fetal hippocampal neurons in culture

A. Phase picture of a hippocampal neuron which had been double stained with VGLUT1 (Figure B), ChAT (Figure C) and co-localization of VGLUT1 and ChAT (Figure D). Scale bar 50 μ. B. VGLUT1-immunoreactive hippocampal neurons as shown in green. Scale bar 50 μ. C. Same neuron is also immunopositive for ChAT as shown in red. Scale bar 50 μ. D. Colocalization of VGLUT1 (green) and ChAT (red). Scale bar 50 μ. E, F and G. 20 day-old cultured fetal hippocampal neurons indicating the co-expression of ChAT (red) and VGLUT1 (green) proteins. Scale bar 50 μ. H. 13 day-old cultured fetal hippocampal neurons showing the co-expression of ChAT (red), VGLUT1 (green) proteins and DAPI (Blue). Scale bar 50 μ. I. Phase picture of a GABA-immunoreactive hippocampal neurons (Figure J) in close proximity of a ChAT positive neuron (Figure K). Scale bar 50 μ. J. GABA-immunoreactive hippocampal neurons shown in green. Scale bar 50 μ.

K. ChAT-immunoreactive hippocampal neurons shown in red. Scale bar 50 μ. L. No colocalization of ChAT was observed in GABA-immunoreactive hippocampal neurons on merging Figure J and Figure K. Scale bar 50 μ. M. Phase picture of a portion of the fetal hippocampus. Scale bar 100 μ. M*: Inset picture showed the intact hippocampus isolated from 18 day-old rat fetal brain which were used for culture after dissociation. N. VGLUT1-immunoreactive neurons in a hippocampal slice. Scale bar 100 μ. O. ChAT-immunoreactive neurons in a hippocampal slice. Scale bar 100 μ. P. Co-localization of the ChAT and VGLUT1 proteins in the hippocampal neurons in a slice preparation. Scale bar 100 μ.

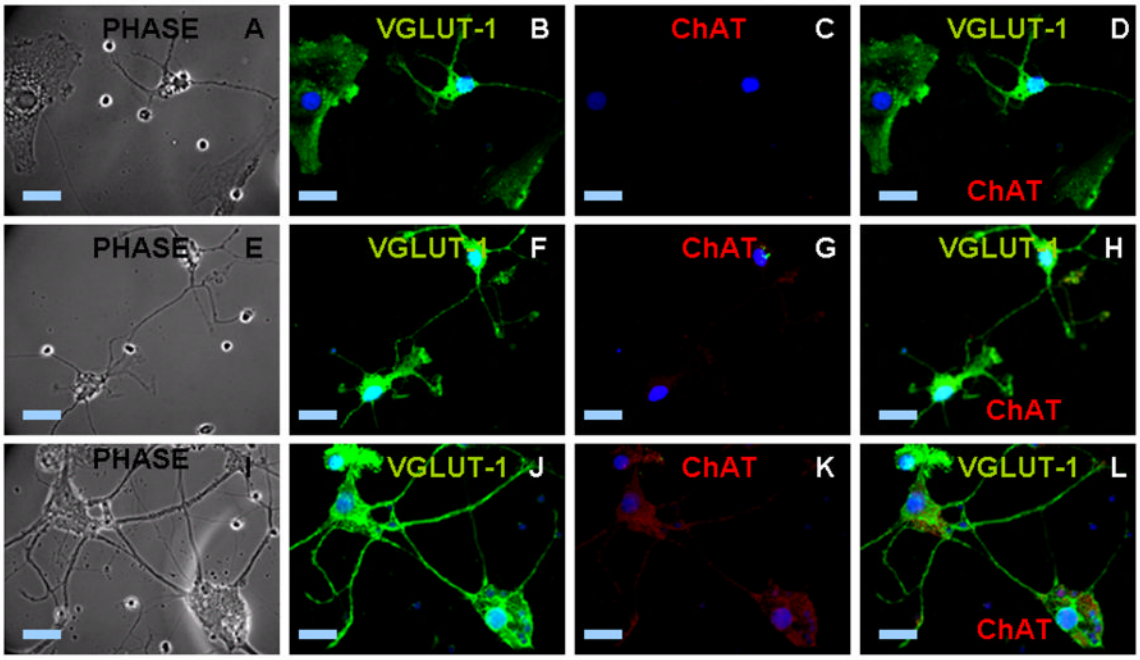

Figure 3. Immunostaining of neonatal hippocampal neurons in culture for 13 days: VGLUT1 (Green), ChAT (Red), DAPI (Blue)

A. Phase picture of a hippocampal neuron which has been double-stained with VGLUT1 (Figure B) and ChAT (Figure C). Scale bar 30 μ. B. A VGLUT1 stained hippocampal neuron shown in green. Scale bar 30 μ. C. No ChAT staining was observed in the hippocampal neuron stained with VGLUT1 in Figure B. Scale bar 30 μ. D. No colocalization of VGLUT1 and ChAT was observed after merging Figure B and Figure C. Scale bar 30 μ. E. Phase picture of the hippocampal neuron which was double-stained with VGLUT1 (Figure F) and ChAT (Figure G). Scale bar 30 μ. F. VGLUT1 stained hippocampal neuron shown in green. Scale bar 30 μ. G. No ChAT staining was observed in the hippocampal neuron stained with VGLUT1 in Figure F. Scale bar 30 μ. H. No colocalization of VGLUT1 and ChAT was observed after merging Figure F and Figure G. Scale bar 30 μ. I. Phase picture of a hippocampal neuron which has been double-stained with VGLUT1 and ChAT in I and J respectively. Scale bar 30 μ. J. A VGLUT1 stained hippocampal neuron shown in green. Scale bar 30 μ. K. Weakly ChAT stained hippocampal neuron shown in red. Scale bar 30 μ. L. Weak colocalization of VGLUT1 and ChAT was observed in a small population of 13 day-old, neonatal hippocampal neurons in culture. Scale bar 30 μ.

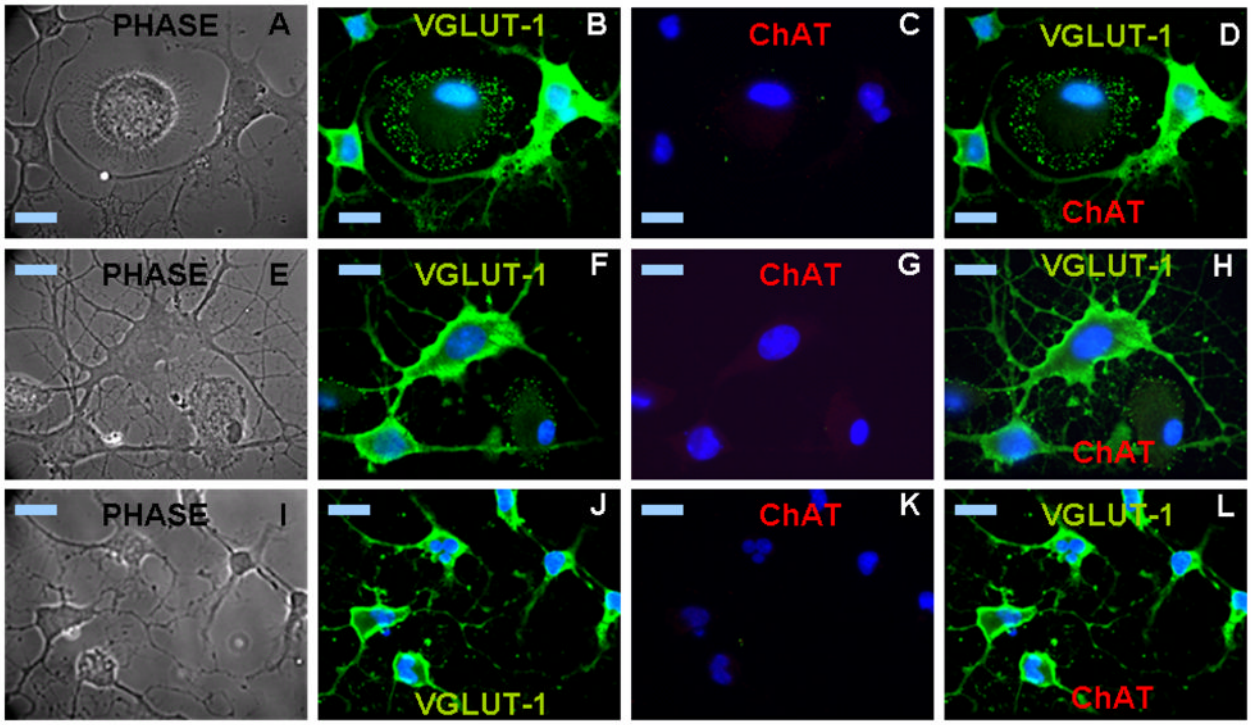

Figure 4. Immunostaining of adult hippocampal neurons in culture for 13 days: VGLUT1 (Green), ChAT (Red), DAPI (Blue)

A. Phase picture of a hippocampal neuron which has been double-stained with VGLUT1 (Figure B) and ChAT (Figure C). Scale bar 25 μ. B. A VGLUT1 stained hippocampal neuron shown in green. Scale bar 25 μ. C. No ChAT staining was observed in the hippocampal neuron stained with VGLUT1 in Figure B. Scale bar 25 μ. D. No colocalization of VGLUT1 and ChAT was observed after merging Figure B and Figure C. Scale bar 25 μ. E. Phase picture of the hippocampal neuron which was double-stained with VGLUT1 (Figure F) and ChAT (Figure G). Scale bar 25 μ. F. A VGLUT1 stained hippocampal neuron shown in green. Scale bar 25 μ. G. No ChAT staining was observed in the hippocampal neuron stained with VGLUT1 in Figure F. Scale bar 25 μ. H. No colocalization of VGLUT1 and ChAT was observed after merging Figure F and Figure G. Scale bar 25 μ. I. Phase picture of a hippocampal neuron which was double-stained with VGLUT1 (Figure J) and ChAT (Figure K). Scale bar 25 μ. J. A VGLUT1 stained hippocampal neuron shown in green. Scale bar 25 μ.

K. No ChAT staining was observed in the hippocampal neuron stained with VGLUT1 in Figure J. Scale bar 25 μ. L. No colocalization of VGLUT1 and ChAT was observed after merging Figure J and Figure K. Scale bar 25 μ.