原代培养的乙酰胆碱能神经元的表型表达与在体不同

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摘要 为了进一步了解原代培养神经元技术是否可以用于建立可靠的乙酰胆碱能神经元体外培养细胞模型, 该实验检测了分别培养自E18胎鼠基底前脑(basal forebrain, BF)和海马(hippocampus, HIP)原代培养神经元中的乙酰胆碱能神经元的标志物, 同时比较了离体培养细胞与在体在相同脑区中乙酰胆碱能标志物表达的差异。使用免疫标记荧光方法, 检测了来自E18胎鼠的基底前脑脑区和海马脑区的离体原代培养神经元中在DIV 3和DIV 21时间点上表达ChAT和p75NTR(两种常用的胆碱能神经元标记物)的神经元的数量, 分析其占总神经元数量的比例, 并与E18胎鼠和成年小鼠相同脑区的在体组织切片中的结果相比较。结果显示, ChAT和p75NTR均在来自基底前脑和海马的培养神经元的DIV 3和DIV 21中高比例表达。然而, 虽然在E18胎鼠和成年小鼠的基底前脑的组织切片中有ChAT和p75NTR的表达, 但是在同时期的海马组织切片中并无ChAT的表达, 并且来自基底前脑和海马脑区的培养神经元中表达乙酰胆碱能神经元标志物的神经元数量占总神经元数量比例与在体并不一致。这些结果显示, 乙酰胆碱能标志物在离体原代培养和在体中的表达状况可能存在不同。根据实验结果推测, 在体外应用原代培养方法培养乙酰胆碱能标志物免疫阳性神经元可能并不是乙酰胆碱能神经元。除了通过免疫组织化学方法, 还需要更多的技术和方法来鉴定培养细胞中的乙酰胆碱能神经元。

关键词 乙酰胆碱能神经元; 基底前脑; 海马; ChAT; p75NTR; 神经元原代培养

Differential Cholinergic Phenotypes in Primary Culture Neurons and Brain *In Vivo*

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Abstract To further understand whether primary cultured cholinergic neurons can be used as a viable cell model for cholinergic neurons *in vitro*, we compared differences in proportion of neurons expressed cholinergic biomarkers and total neurons in cultured neurons cultivated from E18 basal forebrain (BF) and E18 hippocampus (HIP), and the differences in expression ratio of cholinergic markers between primary culture neurons and brain slices in the same brain regions. Immunofluorescence was used to detect the proportion of ChAT and p75NTR (two commonly used cholinergic neuron markers) expression in DIV 3 and DIV 21 of cultured neurons of basal forebrain and hippocampus from E18 fetus, and also in E18 fetus and adult mice in brain slice. Both ChAT and p75NTR showed high expression ratio in cultured neurons from BF and HIP in DIV 3 and DIV 21. However, there was no expression of ChAT in hippocampal slices of E18 fetus and adult mice. Therefore, the ratio of neurons expressed cholinergic

收稿日期: 2018-04-09 接受日期: 2019-05-23

国家自然科学基金(批准号: 31471027)资助的课题

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Received: April 9, 2018 Accepted: May 23, 2019

This work was supported by the National Natural Science Foundation of China (Grant No.31471027)

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网络出版时间: 2019-09-12 12:14:16 URL: http://kns.cnki.net/kcms/detail/31.2035.Q.20190912.1214.006.html

markers in cultured neurons from basal forebrain and hippocampus is inconsistent with *in vivo*, suggesting that it is temporarily not feasible to culture primary cholinergic neurons *in vitro* by using primary neuronal culture methods. Meanwhile, in addition to immunostaining more techniques and methods are needed to identify cholinergic neurons in cultured cells.

Keywords cholinergic neuron; basal forebrain; hippocampus; ChAT; p75NTR; primary neuronal culture

The cholinergic system of neurons plays a key role in neural modulation of central neural system. Many researches in recent years have focused on cholinergic neurons associated with aging and neurodegenerative diseases. In early on se1 Alzheimer's patients, the drop of basal forebrain cholinergic neurons (BFCNs) has been deemed to pathological hallmark of neurodegeneration[1-2]. The most important physiological function of cholinergic neurons is releasing acetylcholine (ACh). Releasing ACh is also the vital definition of cholinergic neurons. As long axonal projection neurons, basal forebrain cholinergic neurons innervate into hippocampus, neocortex and olfactory cortex, regulating neural circuits in targeting location areas via releasing ACh[3-4].

Cholinergic neurons are able to be stained by several cholinergic specific biomarkers. Two typical cholinergic specific biomarkers; choline acetyltransferase (ChAT) participates in synthesizing Ach and the low-affinity receptor for neurotrophins (p75 neurotrophin receptor, p75NTR) modulates cholinergic phenotype via binding nerve growth factor (NGF)[5-7]. These specific cholinergic neuron markers contribute to not only recognizing cholinergic neurons, but also researching on pathological morphology and function of cholinergic neurons *in vivo*. One popular method of detecting cholinergic neurons *in situ* is immunohistochemistry. For example, Wu[8] established the colocalization of FADD immunohistochemistry with ChAT positive neurons in AD and more recently. Lazao[9] showed that the slow reduction of cholinergic cells labeled by ChAT and p75NTR after axotomy without being accompanied by death of cholinergic neurons.

However, although the cellular and molecular of neurodegenerative cholinergic neurons was investigated during the past two decades, little attention has been paid to the research on primary cultured cholinergic neurons. In this paper we present that cultivating primary cultured cholinergic neurons in different culture DIV and compare difference of cholinergic phenotypes *in vivo*. Our results acknowledged that the expression of typical cholinergic markers, ChAT and p75TNR, *in vivo*, was different from those in primary neuron culture *in vitro*. Besides, whether these exemplary markers of cholinergic neurons were able to determine the positive cultured cholinergic neurons by immunocytochemistry are questionable.

1 Methods

1.1 Animals

SD (Sprague Dawley) rats and 8 weeks aged WT C57BL6 mice provided by the Shanghai SLAC Animal Co Ltd. The animals were housed in a regulated environment (22±1) °C with a 12 h light–dark cycle. Food and water were available ad libitum. All experiments were carried out in accordance with the local animal protection law, and approved by the Experimental Animal Ethics Committee of Fudan University.

1.2 Neurons primary culture

Primary hippocampus and basal forebrain neurons were prepared from embryonic day 18-19 SD rats. The tissue was bathed in cold HBSS before dissection. Then the dissected tissue was digested with 0.05% trypsin-EDTA for 20 min at 37 °C, after that, terminating enzyme reaction by plating media (80% DMEM, 10% F12, 10% FBS) with 10% DF12. And then rinsing twice, cells were counted and plated onto glass coverslips precoated with 0.1 mg/mL poly-D-lysine. After culturing for 1 day, half of the plating media were replaced into neuronal culture media NB27 (98% Neurobasal Media GlutaMax & 2% B27) and fed twice

weekly. All cells were culture in 5% CO_2 at 37 °C.

1.3 Fixed tissue preparation, brain slices and cells immunostaining

1.3.1 Tissue preparation Fetal rats and adult mice were deeply anesthetized with isoflurane and transcranial heart perfused with 0.01 mol/L sodium phosphate buffer (PBS) following by 4% paraformaldehyde (PFA) in 0.1 mol/L sodium phosphate buffer. Mice brains were post fixed in 4% PFA for one-night at 4 °C, dehydrated with 15% (×1) and 30% (×2) sucrose. The whole brain frozen in tissue freezing medium (Leica) at –80 °C, and then sliced into 20 or 40 μm sections, adhered to SuperFrost Plus slides (ThermoFisher).

1.3.2 Cultured cells Culture media were decanted and the cell glass coverslips were washed immediately by 0.01 mol/L PBS twice. Cultured cells were post fixed in 4% PFA for 25 min in room temperature. Slides and cultured coverslips were washed by 0.01 mol/L PBS.

1.3.3 Immunostaining Both brain tissue and cultured cells were blocked out by 10% normal donkey serum for 90 min and then incubated first antibodies (Anti-Choline Acetyltransferase antibody AB144P Goat 1:500; Anti-p75NGF Receptor antibody ab8874 Rabbit 1:300; Anti-NeuN Mouse Millipore MAB377 1:500) for one-night at 4 °C. Tissue slices or cultured coverslips were washed by 0.01 mol/L PBS and incubated second antibodies (Alexa Fluor 488 anti-Rabbit second antibody; Alexa Fluor 488 anti-mouse second antibody; Alexa Fluor 555 anti-mouse second antibody; Alexa Fluor 555 anti-Goat second antibody; Alexa Fluor 647 anti-Goat second antibody; Alexa Fluor 647 anti-Mouse second antibody) 1:500 protected from light for 2 h at room temperature. Following second antibodies incubation, brain slices and glass coverslips washed triple in 0.01 mol/L PBS. Sections and glass coverslips were covered with Prolong anti-fade reagent.

1.4 Imaging

Confocal images were acquired with NIKON air scanning confocal microscope through a 25× water-immersion objective and a 60× water-immersion objective.

1.5 Data Analysis

Confocal images were analyzed by NIKON. Cell counting was collected by Photoshop and ImageJ. All statistics were analyzed by GraphPad Prism 7. Data are presented by mean±standard error of the mean (S.E.M.). The results of comparisons among groups were statistically evaluated using unpaired Student's *t*-test. Statistical significance levels were set at $P<0.05$, and no significant differences ($P>0.05$) were abbreviated by ns.

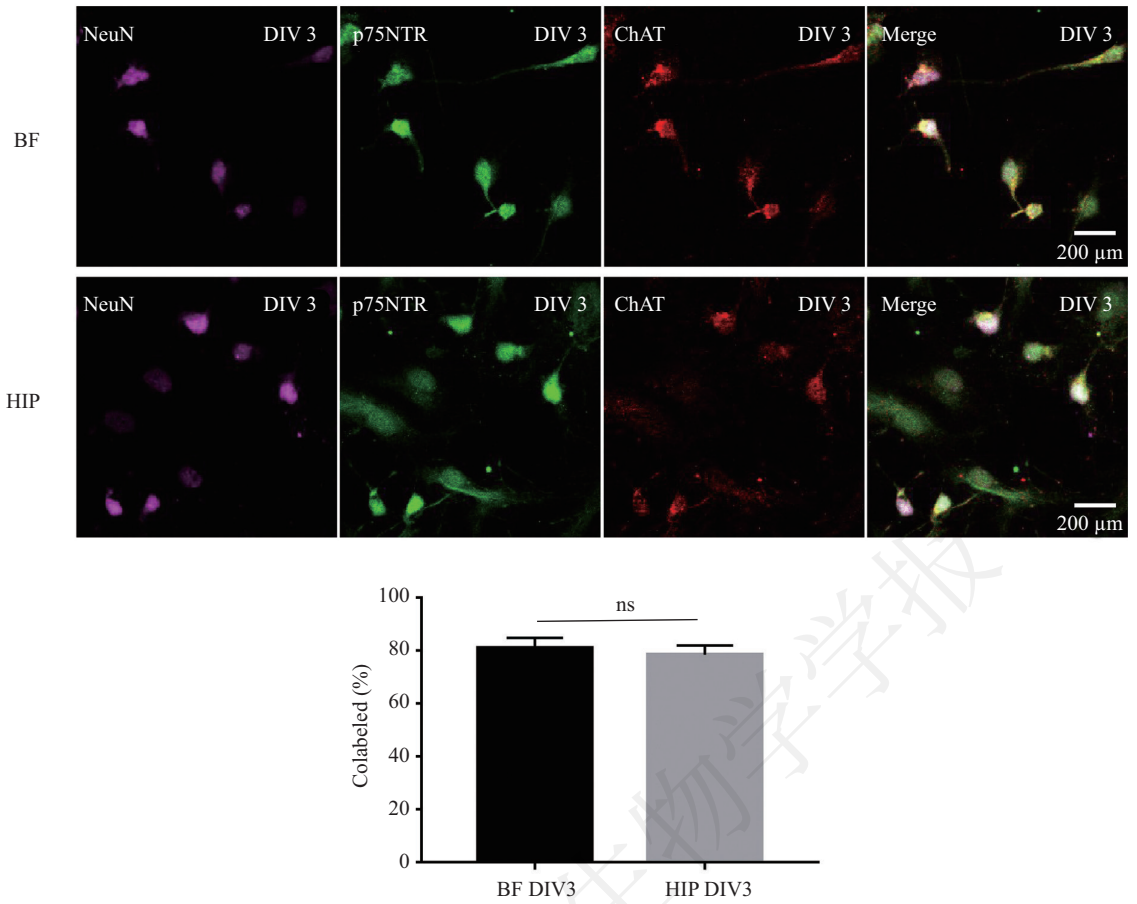
2 Results

2.1 Cholinergic markers expressed both in BF and HIP primary culture neurons in DIV 3

As discussed previously, basal forebrain cholinergic neurons can be well labeled on the basal forebrain tissue by specific markers, ChAT and p75NTR. Here, we applied immunostaining for detecting primary cultured neurons that express positive labeled signals of ChAT and p75NTR. Basal forebrain and Hippocampus tissues were separated from E18 fetus and digested by Trypsin-EDTA. Then, we detected expression of p75NTR and ChAT at cultured day 3 (DIV 3) of cholinergic primary cultures. As shown in Fig.1, there were strong p75NTR, ChAT and NeuN staining signals triple-colocalized in DIV 3. Based on the approach we used, high colocalization ratio of p75NTR and ChAT with NeuN in DIV 3 cultured neurons from BF (81.0%±3.9%, n=329 cells, N=4) and HIP (78.5%±3.5%, n=256 cells, N=4). These results demonstrated that ChAT and p75NTR began to express tremendously at DIV 3 in primary neuronal culture, which were defined by cholinergic immunostaining positive signals.

2.2 Cholinergic markers expressed both in BF and HIP primary culture neurons in DIV 21

Previous results showed the high expression level of cholinergic phenotypes in DIV 3 both in BF and HIP cholinergic cultured neurons. Next, we investigated mature primary cultured cholinergic neurons in DIV 21, which were separated from BF and HIP in E18 fetus. As shown in Fig.2, high colocalization ratio



免疫荧光结果显示p75NTR、ChAT和NeuN在离体培养神经元DIV 3时高比例共标, ns: 没有显著性差异。

Immunostaining of p75NTR, ChAT and NeuN showed strong colocalization in culture for 3 d from BF and HIP, ns: no significance.

图1 DIV 3 原代培养神经元中p75NTR和ChAT的表达情况

Fig.1 Expression of p75NTR and ChAT in primary cell culture in DIV 3

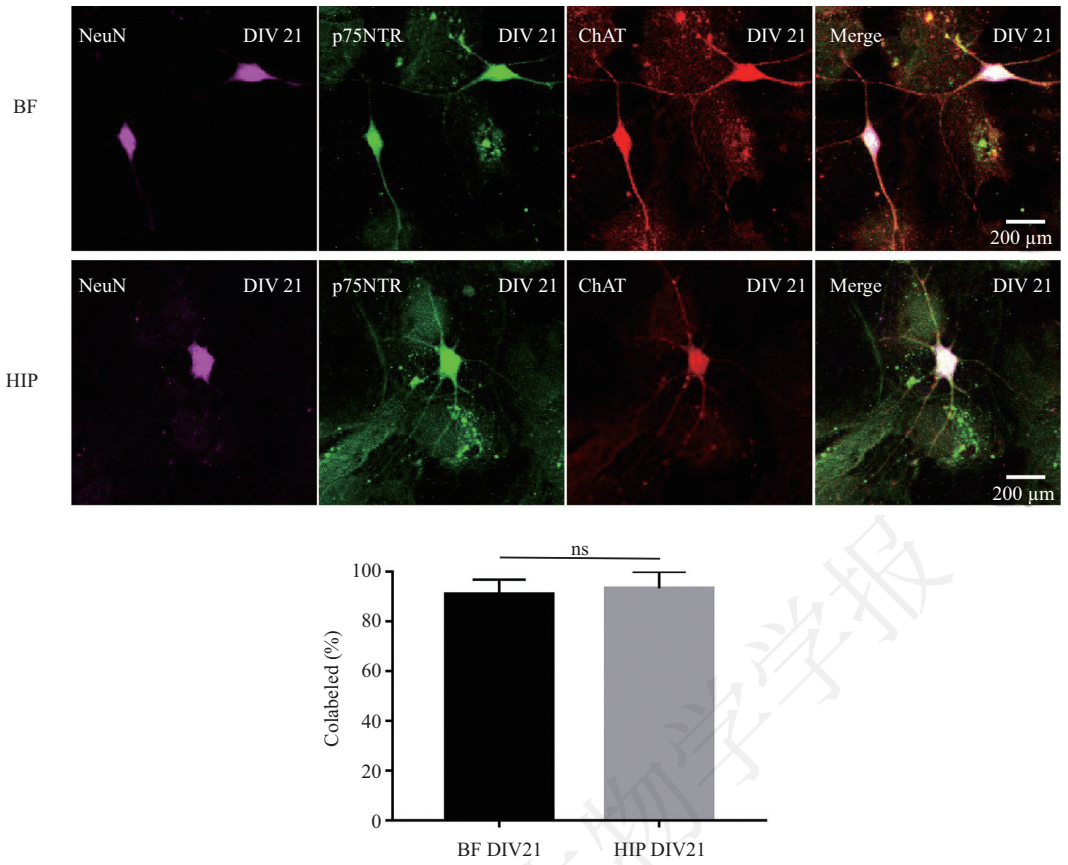
of p75NTR and ChAT with NeuN in DIV 21 cultured neurons from BF (90.9%±5.9%, n=198 cells, N=4) and HIP (93.3%±6.7%, n=170 cells, N=3) was also presented in mature primary cultured neurons. Hence, it was revealed that ChAT and p75NTR still highly expressed in mature primary cultured neurons.

2.3 Expression of cholinergic markers in basal forebrain areas but not in hippocampus in E18 fetus

Immunocytochemistry results of BF and HIP cultured neurons showed that p75NTR and ChAT expressed broadly in cultivated neurons in DIV 3. Then, we researched on cholinergic phenotypes in hippocampus and basal forebrain tissues in E18 fetus. According to Fig.3, there was no expression of ChAT in hippocampus but relatively high ratio of positive cholinergic neurons counting has been showed by ChAT specifically in basal forebrain in E18 fetus (Fig.3, 30.5%±1.9%, N=3). This result suggested that there were no ChAT positive neurons in E18 hippocampus and immunostaining signals of ChAT in basal forebrain areas and hippocampus formations in E18 fetus tissues were significantly distinctive.

2.4 Expression of cholinergic markers in basal forebrain areas but not in hippocampus in adult mice

Previous results suggested that cultured neurons (DIV 3 and DIV 21) from basal forebrain and hippocampus areas in E18 fetus both showed cholinergic-immunoreactive neurons, but there were no cholinergic neurons in E18 hippocampus *in vivo*. Finally, we determined whether ChAT positive neurons existed in hippocampus in adult animals. The results showed that whole hippocampus was not discovered any ChAT-



免疫荧光结果显示p75NTR、ChAT和NeuN在离体培养神经元DIV 21时高比例共标, ns: 没有显著性差异。

Immunostaining of p75NTR, ChAT and NeuN showed strong colocalization in culture for 21 d from BF and HIP, ns: no significance.

图2 DIV 21原代培养神经元中p75NTR和ChAT的表达情况

Fig.2 Expression of p75NTR and ChAT in primary cell culture in DIV 21

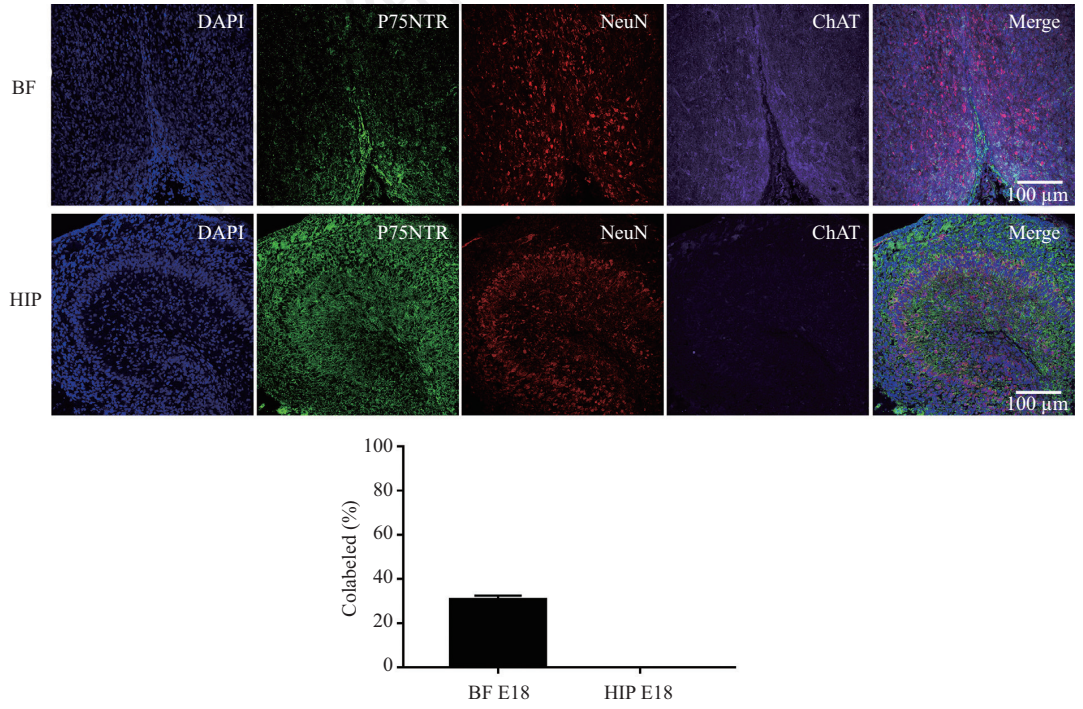


图3 E18胎鼠中ChAT在海马脑区中不表达

Fig.3 Differential expression of ChAT between hippocampus and basal forebrain in E18 fetus

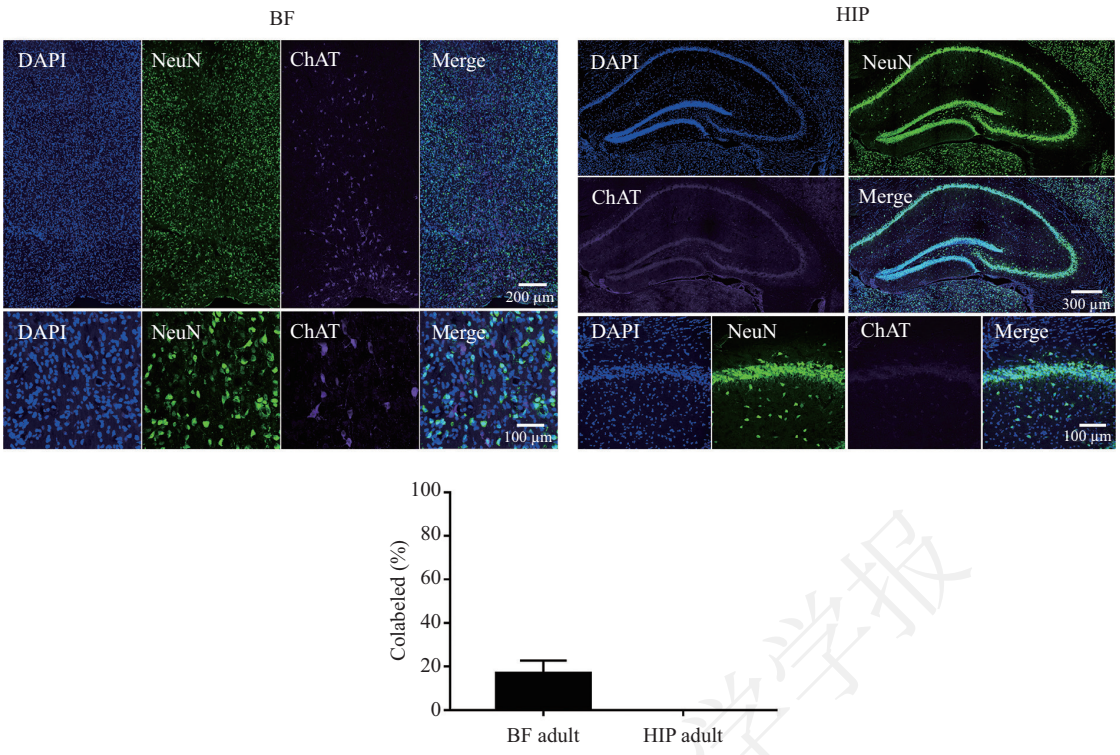


图4 成年小鼠海马组织中没有ChAT表达

Fig.4 No expression of ChAT in hippocampus in adult mice.

immunoreative neuronal cell bodies (Fig.4). In the same animal, the proportion of numbers of ChAT-immunoreative neurons and total neurons in MS/DB were 16.9%±6.0% (Fig.4, *N*=3), and these ChAT positive neurons projected axons into pyramidal layer of hippocampus, which converged BF axonal terminals and branches. This result indicated that proportion of mature cholinergic neurons numbers were not existed in hippocampus.

3 Discussion

Cultured neurons dissociated from basal forebrain and hippocampus in E18 fetus both showed that ChAT and p75NTR-immunoreactive neurons existed in DIV 3 and DIV 21 (Fig.1 and Fig.2). However, we did not observe any cholinergic positive signals in hippocampus in E18 fetus and adult mice *in vivo* (Fig.3 and Fig.4). This study offered the evidence that cholinergic phenotypes in primary cultured neurons by detecting these cholinergic specific biomarkers were not in agreement with which in animals *in vivo*.

Some researchers believe that the hippocampus has intrinsic cholinergic neurons[10-11], but this ChAT positive neurons emerge in BAC-mice, which genes combination might disturb normal cholinergic related genes transcription[11-13]. To date, attempts to confirm that these neurons co-express the endogenous cholinergic marker ChAT, or release ACh, have been unsuccessful. Based on previous evidence and current data, we conjecture that these hippocampal intrinsic cholinergic neurons expressed fluorescent proteins, which under the control of the ChAT promoter, might express ChAT transiently during cholinergic development *in vivo*. But the probability of inaccuracy of transgenic methods should not be ignored as well.

Because of totally different development circumstances, culture neurons are hardly to mimic cell growth process in animal body completely. The study showed that microglia can regulate differentiation of embryonic cholinergic neurons[14]. Especially for the neurons with releasing particular neurotransmitter, primary culture shows inescapable defects. Basal forebrain area has been regard as collective cholinergic neurons in brain areas. These projection axonal cholin-

ergic neurons innervate their axon fibers into various brain sub-regions, including hippocampus. In addition, BFCNs cooperate with other types of neurons in basal forebrain areas send the admixture projection into hippocampus in order to modulating cognitive activities in hippocampus[15-17]. Hence, cultured cholinergic neurons can help researchers to build up disease models *in vitro*, such as Alzheimer's disease, which correspond with degeneration of BFCNs. Our results doubted that ChAT positive neurons could not represent cultured cholinergic positive neurons absolutely. Another indication for us was to consider the feasibility of dedifferentiation and regeneration in cultured neurons. It is not difficult to assume that conclusion the regrowth of neurite is the course of neuronal regeneration in culture.

Some experimental methods might help us to obtain pure and stable BFCNs for culture *in vitro*. After digesting brain tissue from E18 fetus, ChAT immunoreative neurons selected by flow cytometry. But the neuron regeneration *in vitro* could not be avoided even though we select at first. The other solution is to culture adult cholinergic neurons for reducing possibility of dedifferentiation and regeneration[18]. The problem of livability of adult cultured neurons *in vitro* can be fixed via flavoring NGF/BDNF and BMP9[19-22] for sake of maturing and maintaining typical phenotype of cholinergic neurons. Consequently, primary cultivation of cholinergic neurons is needed further investigation for classifications and identifications in variable approaches.

4 Conclusion

Our results acknowledge that the expression of typical cholinergic markers detected by immunofluorescence staining, *in vivo* is different from those in primary neuron culture *in vitro*. Besides, exemplary markers of cholinergic neurons, ChAT and P75NTR, are able to determine the positive cultured cholinergic neurons by immunocytochemistry. What were questionable. Further investigation needs to study on identifying functional cholinergic neurons *in vitro* applying valid methods.

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