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Quantitative Analysis of Expression of NeuroD, GAP43 and TrkB in Developing Mouse Olfactory Neuroepithelium

Abbreviated title: Quantative analysis of developing olfactory epithelium

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Abstract

Objective:Mammalian olfactory neuroepithelium(OE), harboring olfactory receptor neurons (ORNs) has an unusual ability of continuous neurogenesis throughout lifetime. This unique character has been providing an excellent model for neuronal differentiation. Recently, we have found dual phase expression of NeuroD, a member of the basic helix-loop-helix transcription factor family, in developing mouse OE, suggesting the multiple roles of NeuroD during the development of the mammalian ORNs.

Methods: To better understand the molecular mechanism of the development of ORNs, we performed quantitative analysis of expression of NeuroD, GAP43 and TrkB as well as BrdU-labeled cells in the developing mouse OE, from gestational day 10 to postnatal day 28.**Results:** During embryonic period, NeuroD expression is mostly confined in the basal compartment. During neonatal period, NeuroD expression is detected in two compartments; in the middle compartment and in the basal compartment. GAP43 expressing cells were located between these two NeuroD positive layers. TrkB expressing cells were located above the NeuroD positive layer in the middle compartment. As mice grew, the number of NeuroD

expressing cells and BrdU-labeled cells in the basal compartment significantly decreased, while NeuroD expressing cells in the middle compartment were gradually increased. The number of TrkB expressing cells dramatically increased. GAP43 expressing cells also gradually increased . However, the pr oportion of GAP43 cells relatively decreased as OE developed. **Conclusions:** NeuroD is a useful molecular marker to study the olfactory neurogenesis.

Introduction

The mammalian olfactory epithelium (OE), harboring olfactory recepotr neurons (ORN) has an unusual characteristic ability: neurogenesis in this tissue continuous throughout lifetime. The dying olfactory neurons are replaced by new ones, which display a topographical pattern of neuronal maturity. The globose basal cells(GBCs), which express GAP43, are located in the basal compartment of OE and continuously generate immature ORNs. Immature ORNs, which express TrkB (a preferred receptor for the neurotrophin BDNF), keep migrating up to the surface of the OE as they grow. The immature ORNs extend their axons to the olfactory bulb and finally become fully mature ORNs, which express OMP. The apical surface is covered by the supporting cells[Roskams, 1996 #32].

This regenerative ability of olfactory epithelium provides an unique model to study molecular mechanism of neurogenesis and neuronal differentiation[Nibu, 2002 #74]. NeuroD is a member of bHLH gene family, whose expression is detected late in neurogenesis, usually during and/or after the terminal mitosis of neuronal precursors. Because of the relatively l

ate expression,
NeuroD has been hypothesized to function largely in neuronal differentiation rather than determination[Lee, 1997 #45]. Recently, we have found dual phase expression of NeuroD in the developing mouse OE[Nibu, 2001 #75], suggesting the multiple roles of NeuroD during the development of the mammalian olfactory receptor neurons. To better understand the molecular
mechanism of the olfacotry neurogenesis,
we performed the quantitative analysis of expression of NeuroD, GAP43 and TrkB as well as BrdU-labeled
cells in the developing mouse OE.

Materials and Methods

Mice and BrdU injection, perfusion and fixation

Bulb/C mice were used at gestational period (G10) to postnatal ages ranging from 1 to 28days. To label dividing cells, mice were given single dose (50mg/kg body weight) of BrdU (5-bromo-2′-deoxyuridine, Sigma B 5002, St. Louis, MO) intraperitoneally (in case of embryos, intraperitoneal in mother) in a solution of 20g/ml PBS, pH 7.2. One hour later, mice were sacrificed by intraperitoneal (in case of embryo, intraperitoneal in mother) injection of a lethal dose of sodium pentobarbital (250g/kg body weight). Heads were removed, and fixed in 10% Formalin at room temperature for 7-14days. Then specimens were decalcified in decalcification buffer (0.27M EDTA, 1% Formalin). Dehydration was performed through graded alcohol and xylene series. Then specimens were embedded in paraffin. All experiments in this study were performed according to the “Principles of laboratory animal care” (NIH publication No. 86-23, revised 1985) and approved by the ethics committee of Kobe University.

Immunohistochemical Staining for NeuroD, GAP43 and TrkB

Five-micron-thick serial sections were cut from formalin-fixed paraffin-embedded specimens and mounted on silan coated slides (Dako Japan, Japan). Then the sections were deparaff

inized and rehydrated through xylene and alcohol series. Sections were placed in citrate-buffered solution (pH 6.0) and heated at 100℃ by microwave oven for 20 min. for antigen retrieval as previously described. Endogenous peroxidase was blocked with 3% hydrogen peroxide and nonspecific binding was blocked with 10% normal serum, respectively. Affinity-purified polyclonal anti-TrkB antibody (TrkB 794, Santa Cruz Biotec, Santa Cruz, CA), anti-NeuroD antibody (NeuroD G-20, Santa Cruz Biotec, Santa Cruz, CA), and anti-GAP43 antibody (NCL-GAP43, Novocastra, UK) were used at 1:100 dilution. Sections were incubated with primary antibody at room temperature overnight. Antibody binding was visualized with Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Diaminobenzidine (DAB) was used for coloration and nuclei were counterstained with Hematoxylin.

BrdU Immunohistochemistry

For BrdU (Bromodeoxyuridine) immunohistochemistry, sections were treated with 1N HCl for 30 min. at room temperature according to manufacturer’s protocol following deparaffinization and rehydration. Endogenous peroxidase was blocked with 3% hydrogen peroxide for 20 min. and nonspecific

binding was blocked with 10% normal horse serum for 20 min. The mouse monoclonal antibody against BrdU (M0744, Dako) was used at a dilution of 1:50. Slides were incubated with primary antibody at room temperature for 30 min. Antibody binding was visualized using Vectastain kit and DAB was used for coloration.

Quantitative analysis of the aged-dependent change in expression of NeuroD, GAP43, TrKB

To quantitativelty analyze the age-dependent change of expression of NeuroD, GAP43 and TrkB, we measured the number of cells expressing these markers in the OE of different developing periods. The results were compared with the rate of proliferation, measured as the number of BrdU-labeled basal cells. Three animals at each age were used for statistical analysis. Cells expressing markers on the bilateral olfactory epithelium overlying the nasal septum, were counted on coronal sections through the olfactory bulb and olfactory epithelium. Since turbinates were not fully developed during neonatal period, sections including the maximum olfactory epithelium area were used as each representative olfactory area. In the embryo at gestational age 10 and 12, entire olfactory epithelium in each section were counted. Counting was performed at 400times magnification in a NIKON

microscope. At least, three fields per each mouse were examined.

Results

Embryonic Period

During embryonic period, NeuroD expressing cells are mostly confined in the basal compartment of OE, as previously reported[Nibu, 2001 #75] (Fig. 1). BrdU-labeled cells and GAP43 expressing cells were observed throughout the developing ol factory epithelium(Fig.1). No immunoreactivity for TrkB was observed until gestational period 16. The number of BrdU-labeled cells dramatically decreased from E10 to E16 (Fig. 2).

Neonatal Peirod

During neonatal period, NeuroD expressing cells were detected in two compartments; in the middle compartment and in the basal compartment, as we previously reported[Nibu, 2001 #75]. GAP43 expressing cells were identified as the layer sandwiched between these two NeuroD positive layers(Fig.1). TrkB expressing cells were located above the NeuroD positive layer in the middle compartment. Only scattered BrdU-labeled cells were observed in the neonatal period. As mice grew, the number of NeuroD expressing cells in the basal compartment significantly decreased(Fig.2). On the other hand, the number of NeuroD expressing cells slightly increased

and this NeuroD positive layer in the middle compartment moved to relatively lower part of OE(Fig.3). While the number of GAP43 expressing cells also increased, the proportion of GAP43 expressing cells gradually decreased. The number and proportion of TrkB expressing cells dramatically increased (Fig.3).

Discussion

Traditionally, OE is divided into three major compartments: basal compartment, middle compartment and apical compartment. The basal compartment consists of basal cells, the middle compartment consists of ORNs expressing OMP[Margolis, 1972 #49], and the apical compartment consists of supporting cells. Recent studies have divided the first two compartments to subclasses. In the basal compartment, basal cells are divided to horizontal basal cells, and GBCs which express GAP43[Verhaagen, 1990 #43] and generate ORNs. In the middle compartment, ORNs are divided into immature ORNs expressing TrkB[Roskams, 1996 #32] and mature ORNs expressing both TrkB and OMP[Nibu, 1999 #77]. In this paper, we proposed additional subclasses to these two compartments, that is, "NeuroD expressing cells in the basal compartment" and " NeuroD expressing cells in the middle compartment ".

GAP43 expressing cells were observed between two disntinct NeuroD positive layers

According to the Calof's proposal[Calof, 1989 #61], so called "globose basal cells" consist of stem cells, transient amplifying cells (TAs) and immediate neuronal precursors (INP). Stem cells divide to d

aughter cells that can either differentiate to TAs or remain as stem cells. TAs are differentiating to INPs as they are dividing. During this differentiating process, TAs express basic Helix-Loop-Helix gene family genes, Mash1, Math3 and NeuroD in this order[Calof, 2002 #78]. As shown in our previous study, the NeuroD expressing cells in the basal compartment are partly overlapped with BrdU-labeled cells and partly located just above the BrdU-labeled cells[Nibu, 1999 #77]. In addition, in this paper, we show that these NeuroD expressing cells in the basal compartment were located just below the GAP43 expressing cells. Considering these findings, these NeuroD expressing cells in the basal compartment seems to be equivalent to INPs. On the other hand, NeuroD expressing cells in the middle compartment were located between GAP43 positive layer and TrkB positive layer as shown in this study, suggesting that these cells are at the development process of ORN lineage from GBCs to immature ORNs.

Dynamic changes in cell type composition of olfactory epithelium during development

Using these molecular markers, we have quantitatively shown dynamic changes in cell type composition of OE during development. For example, the number of BrdU-labeled cells significantly decreased. The number of NeuroD expressing cells in the basal compartment also significantly decreased, but relatively moderately in

comparison with BrdU-labeled cells. These observations suggest that most of NeuroD expressing cells at the basal compartment might be at the post mitotic period, rather than the at the end of mitotic period.

As for the GAP43 and TrkB, the proportion of GAP43 expressing cells gradually decreased, while the number of GAP43 expressing cells slightly increased. In contrast, the number and proportion of TrkB expressing cells significantly increased. These findings further support the idea that mature ORNs provides the negative feedback to the proliferation of GBCs, in order to regulate the olfacotry epithelial proliferation[Calof, 2002 #78].

Conclusion

We have quantitatively analyzed the development of OE, using molecular markers, including NeuroD, TrkB, BrdU, GAP43. Further studies using other molecular markers will provide more understanding of olfactory neurogenesis.

Acknowledgemnet

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Legends

Figure 1. NeuroD, GAP43 and TrkB expressing cells and BrdU-labeled cells in the mouse olfactory epithelium during development.

NeuroD1: NeuroD expressing cells in the basal compartment.
NeuroD2: NeuroD expressing cells in the middle compartment.
G:gestational period P:postnatal period

Figure 2. Quantitative analysis of NeuroD, GAP43 and TrkB expressing cells and BrdU-labeled cells in the mouse olfactory epithelium during development.

NeuroD1: NeuroD expressing cells in the basal compartment.
NeuroD2: NeuroD expressing cells in the middle compartment.
G:gestational period, P:postnatal period

Figure 3. Changes of the components in the olfactory epithelium during development.

In the embryonic period, GAP43 expressing cells, occupied the majority of olfactory epithelium. As mice grew, NeuroD expressing cells in the basal compartment significantly decreased and the center of the olfactory epithelum was filled with TrkB expressing immature and mature olfactory receptor neurons. The proportion of GAP43 expressing cells relatively decreased during neonatal period.
NeuroD1: NeuroD expressing cells in the basal compartment.
NeuroD2: NeuroD expressing cells in the middle compartment.

G:gestational period, P:postnatal period

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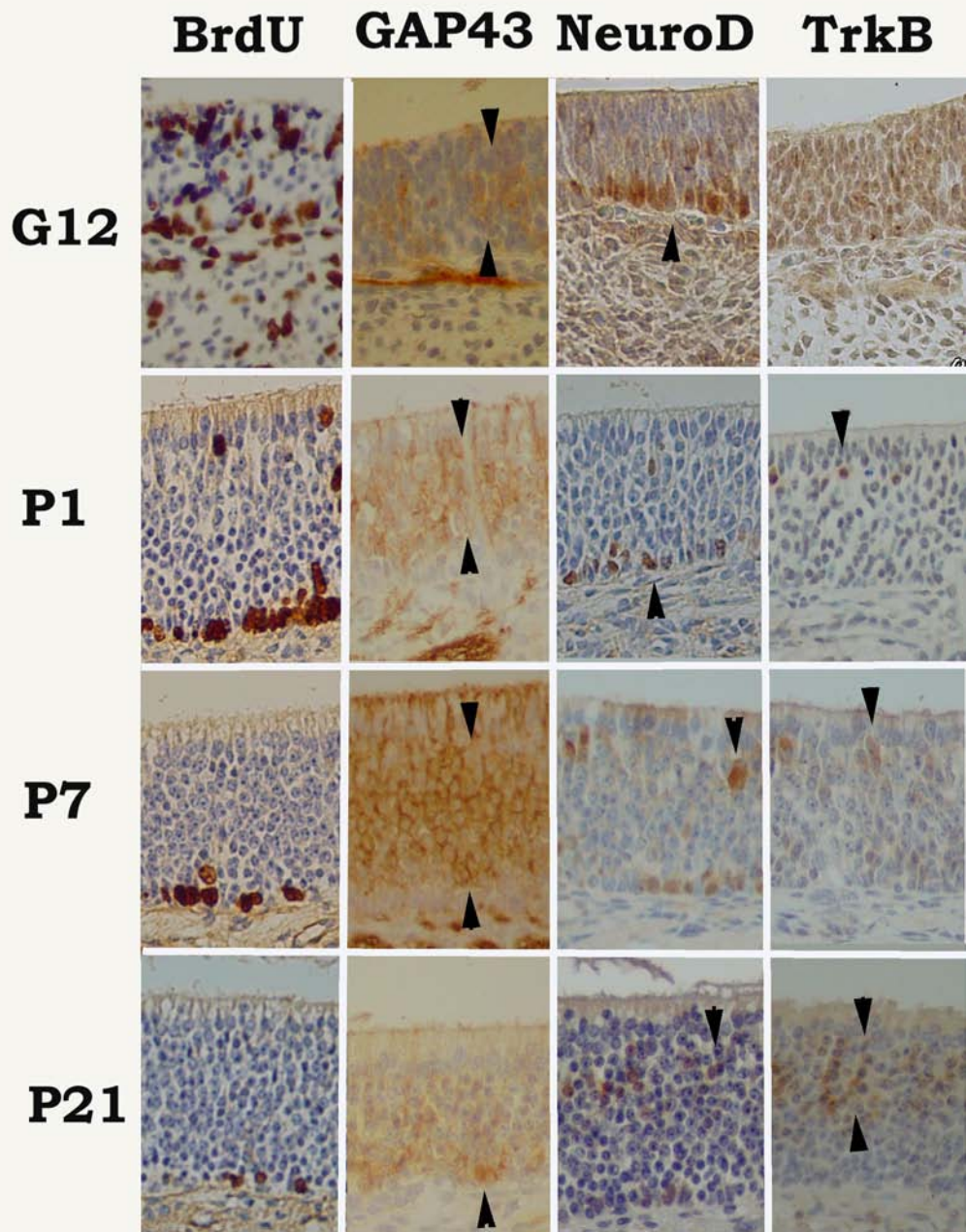
BrdU
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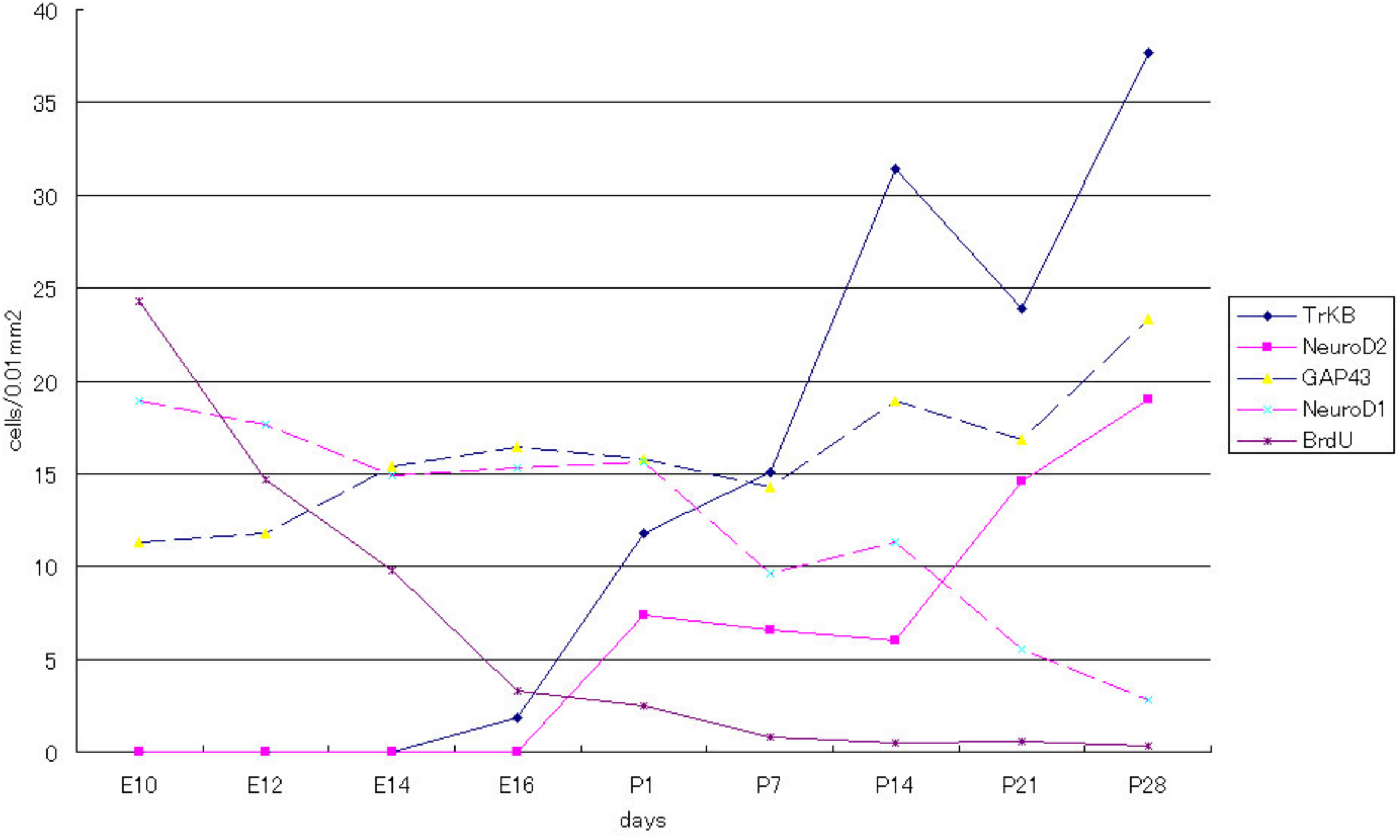
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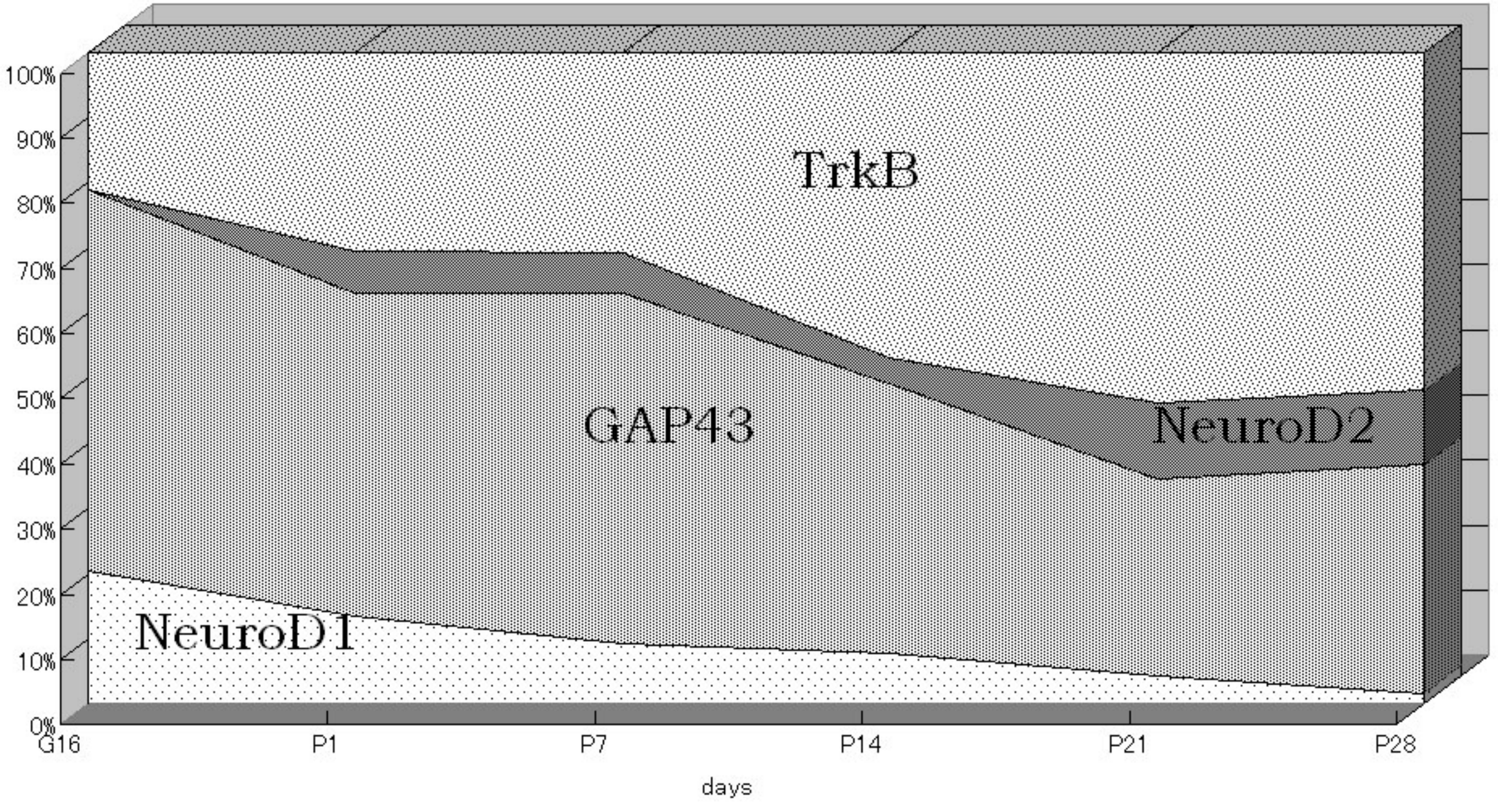
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