# Alternative splicing of AMPA receptor subunits: regulation in clonal cell lines

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**Abstract Alternative splicing of AMPA receptors was investigated in rat PC12 and human SH-SY5Y cells. PC12 cells predominantly expressed GluR-B flip mRNA before and after differentiation. In SH-SY5Y cells, each AMPA-receptor subunit showed a distinct splice variant expression profile throughout differentiation. GluR-B mRNA was comparable in expression levels for flip and flop splice variants. In the other AMPA-receptor subunit transcripts the flip form was the more prominent splice variant. After three days post induction a transient elevation of GluR-B and -D flop mRNA expression could be observed.**

*Key words:* AMPA; GluR-A to GluR-D; Flip; Flop; Alternative splicing

## 1. Introduction

AMPA-selective ionotropic glutamate receptors are composed of four closely related subunits termed GluR-A to -D or alternatively GluR-1 to -4 [1]. Heterogeneity within this receptor family is increased by alternative splicing of two mutually exclusive modules between transmembrane domains III and IV (TM III and TM IV) [2]. The two alternative versions of each subunit, designated flip and flop, constitute different functional characteristics of AMPA-gated channels and are expressed in a developmentally regulated and cell type-specific manner [3]. Changes in splice variant ratios have been suggested to play a role in certain pathophysiological phenomena. In rat hippocampus, a significant upregulation of GluR-A flip and GluR-B flip mRNA was observed after kindling [4]. A similar transient elevation of GluR-B flip was reported in hippocampus of rats with kainate-induced epilepsy [5].

To study this highly selective regulation of alternative exons in greater detail, we searched for clonal cell lines which express glutamate receptor mRNAs. The rat pheochromocytoma cell line PC12 and the human neuroblastoma line SH-SY5Y, cell lines of sympathoadrenal lineage, can be differentiated into a neuronal phenotype in culture [6,7] and express AMPA receptor subunit GluR-B. They were examined with respect to their potential to undergo changes in exon preference during the course of differentiation.

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*Abbreviations:* AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate; M-MLV RT, moloney murine leukemia virus reverse transcriptase.

## 2. Materials and methods

### 2.1. Cell culture

PC12 cells were cultured on collagen-coated plates in RPMI (Gibco) medium supplemented with 10% FCS (Gibco); SH-SY5Y cells were cultured in DMEM (Gibco) supplemented with 10% FCS. The cultures were grown at 37°C, in the presence of 5% $CO_2$ in a humidified atmosphere. Differentiation was induced by addition of nerve growth factor (NGF, 50 ng/ml) to the PC12 cell medium [6] and retinoic acid (RA, 10 μM) (Merck) to the SH-SY5Y cell medium [8]. Media including differentiating agents were changed every third day.

### 2.2. Preparation of RNA and polymerase chain reaction

Total RNA was extracted by the guanidinium isothiocyanate method [9] and reverse transcribed in a total volume of 20 μl with 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 10 mM DTT, 3 mM $MgCl_2$, 0.5 mM deoxynucleotides each, 1 μg RNA, 100 pmol of hexanucleotide primers, 10 U RNase inhibitor (BRL) 200U M-MLV RT (BRL) for one hour at 37°C [10]. Polymerase chain reaction (PCR) [11] was performed in 100 μl of 67 mM Tris-HCl (pH 8.8), 6.7 mM $MgCl_2$, 170 μg/ml bovine serum albumin, 16.6 mM $(NH_4)_2SO_4$, 5 mM tetra methyl ammonium chloride (TMACl), 10 μM deoxynucleotides each, 50 pmol of the appropriate primers and 2.5 U of Taq DNA polymerase (Perkin Elmer Cetus). PCR was carried out in a Thermocycler (Mod. 9600) for 38 cycles at 94 °C for 1 min, 60°C for 1 min, 72°C for 1 min. The following primer pairs were used for subunit specific amplification: 5′-GCGAATTCGCATTGCGACACCCAAGGGGTCCG-3′ encoding amino acids I A T P K G S A and 5′-GCGGTACCCTCCGCTGGCTCCTGCCCCACTGT-3′ complementary to amino acids S G A G A S G G of GluR-A; 5′-CCAAAGGCTACGGCATCGCCACAC-3′ encoding amino acids K G Y G I A T and 5′-AAAATTCTGGGAATTCTGCGAGG-3′ complementary to amino acids S Q N S Q N F of GluR-B; 5′-GCCTGCAGGTGTGGCAACCCCTAAAGGCTCAG- 3′ encoding amino acids V A T P K G S A and 5′-GCGGTACCGAGCAGGCTTAAAGTTTTGGGTGT-3′ complemetary to amino acids T Q N F K P A of GluR-C; 5′-GCGAATTCGTGTAGCAACGCCCAAGGGTTCCT-3′ encoding amino acids V A T P K G S and 5′-GCGGTACCCCCAGTGATGGATAACCTGGCTTT-3′ complementary to amino acids K A R L S I T G of GluR-D.

The primers for GluR-B recognize both human and rat sequences.

### 2.3. Analysis of PCR products

PCR products were separated on a 1.5% agarose gel. The gel was capillary blotted onto nitrocellulose membranes (Schleicher and Schuell Protran 0.45 μm) and hybridized to 5′-$^{32}$P-labelled splice variant specific oligonucleotides in 40% formamide, 6 × SSC, 5 × Denhardt's solution, 0.5% SDS, 0.1% sodium pyrophosphate and 500 μg/ml yeast tRNA at 42′C overnight. Filters were washed in 2 × SSC, 0.1% SDS at 42′C. Hybridization oligonucleotides were: 5′-CCTAGCGGTTTTGAAACTCAGTGA-3′ corresponding to amino acids L A V L K L S E of GluR-A flip; 5′-CCTGGCAGTGTTAAAACTGAACGA-3′ corresponding to amino acids L A V L K L N E of GluR-A flop; 5′-ACCCCAGTAAATCTTGCAGTATTG-3′ corresponding to amino acids T P N L A V L of GluR-B flip; 5′-AATGCGGTTAACCTCGCAGTACTA-3′ corresponding to amino acids N A V N L A V L of GluR-B flop; 5′-AACGCCTGTAAACCTTGCAGTATT-3′ corresponding to amino acids T P V N L A V L of GluR-C flip; 5′-AATGCTGTTAAC'CTGGCAGTATTA-3′ corresponding to amino acids A V N L A V L K of GluR-C flop; 5′-TCCTGTAAACCTTGCCGTTTTGAA-3′ corresponding to amino acids P V N L A V L K of GluR-D flip; 5′-TGCTGTTAACCTCGCAGTTTTAAA-3′ corresponding to amino acids A V N L A V L K of Glur-D flop.


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Quantitative analysis of PCR products was essentially as described previously [12]. Isolated PCR bands were digested with appropriate restriction enzymes and subsequently subcloned into M13mp18 RF DNA (predigested *Bam*HI/*Eco*RI for GluR-B and *Kpn*I/*Pst*I for GluR-C) or M13mp 19 RF DNA (predigested *Kpn*I/*Eco*RI for GluR-A and -D) [13]. Dual filter lifts from phage containing plates were hybridized with specific oligonucleotides. Hybridization and washing conditions were as specified above. Reliability of the assay was controlled by PCR amplification of premixed flip/flop ratios of plasmid-cloned GluR-B cDNAs (10 pg total) and subsequent subcloning and hybridization. The results are shown below (Table 1).

## 3. Results and discussion

Rat pheochromocytoma cells PC12 and human neuroblastoma cells SH-SY5Y were differentiated with NGF or retinoic acid (RA). In PC12 cells the induction of differentiation by NGF was slightly more pronounced, so this agent was subsequently used. For SH-SY5Y cells RA appeared to be the far more potent inducing agent based on morphological examination. Furthermore it has been described that RA-induced differentiation of SH-SY5Y cells gives rise to changes in the splicing pattern of the APP gene that are not observed when NGF is used [14]. During the first 3 days under differentiating conditions the cells started to extend short neurites. After 5 days of treatment with RA the cells presented a pronounced change in morphology characterized by an extensive network of neurite processes.

Total RNA was prepared from undifferentiated and from differentiating PC12 cells at various time points after NGF stimulus. RNA was reverse transcribed and subjected to PCR analysis with specific primers flanking the alternatively spliced region of GluR-B, the only AMPA receptor subunit RNA detected in PC12 cells [15] at any stage of differentiation (data not shown). A 300 bp PCR product containing a mixture of the mutually exclusive splice forms flip and flop of the GluR-B receptor encoding cDNA was obtained. Amplified products were subjected to gel electrophoresis and Southern analysis using splice variant specific probes. GluR-B flip was the dominant isoform throughout eight days in differentiating culture conditions. No changes in relative abundance could be detected at various stages of differentiation (Fig. 1a). PC12 cells are derived from a rat pheochromocytoma and display properties of adrenal chromaffin cells. Following treatment with NGF, these cells differentiate into a phenotype resembling sympathetic neurons [6]. AMPA receptor subunits have been detected in the rat adrenal gland by in situ hybridization [16]. Although distinct medullary sympathetic ganglion cells were found to express all four subunit mRNAs, GluR-B appears to be the prominent subunit of the adrenal medulla. In addition, mainly flip encoding AMPA receptor mRNAs were found in the adrenal gland, therefore the AMPA receptor expression pattern in PC12 cells most likely reflects that found in the originating tissue.

SH-SY5Y cells were analysed in a similar fashion. Total RNA was isolated from undifferentiated and differentiating cells up to ten days of induction. The results are shown in Fig. 1b. Changes in the ratio of GluR-B splice variant cDNAs could be detected at different time points of differentiation. In the presented experiment, both splice variants were of almost equal abundance in nondifferentiated cells. During the course of differentiation the ratio between alternative splice variants changed in favour of GluR-B flop mRNA, reaching peak levels 3 days after addition of RA. As differentiation proceeded, the proportion of mRNA encoding the flip isoform increased substantially and reached the levels of flop-encoding mRNA by day seven.

In order to assess the alternative exon selection of endogenous AMPA receptors in SH-SY5Y cells we applied a quantitative assay for all four AMPA receptor subunits [12]. In this assay, the relative contribution of each splice variant to the total amount of mRNA from a given subunit was measured irrespective of the absolute message levels. In brief, products from PCR reactions specific for human AMPA-receptor subunits GluR-A to -D harbouring the mutually exclusive exons flip and flop were subcloned into bacteriophage M13 RF DNA. The ratio between splice variants was determined by selective hybridization with isoform-specific oligonucleotides for each subunit. A subunit-specific pattern of splice variant expression could be observed (Fig. 2). The flop-encoding isoform constitutes about 30% of the total GluR-A message in undifferentiated cells. It decreases to 20% after 5 days of differentiation and returns to initial levels during further differentiation. Results obtained for GluR-B messages largely confirm the initial observation from Southern analysis. The flop-encoding isoform is more abundant in undifferentiated cells. In the early stages of differentiation (3 days) a slight increase of flop-encoding message (to more than 60% of total) can be observed, which declines below 40% of total GluR-B mRNA after 5 and 10 days post-induction. The flop-encoding message of GluR-C constitutes only a small percentage of the total in undifferentiated cells (7%) and decreases gradually below 2% during the course of the differentiation process. GluR-D flop mRNA is only a 2% portion before induction. Here, a dramatic transient elevation to almost 30% is observed at day 3 of differentiation which subsequently declines. At 10 days post-induction no flop-encoding message of the GluR-D subunit can be detected. The presented data were compiled from at least 3 independent experiments for each subunit and each timepoint. SH-SY5Y cells

Table 1
Hybridization selectivity was controlled for each subunit by growing and sequencing six hybridizing plaques on an automated sequencer A373 (Applied Biosystems).

| Ratio flip/flop | 100:1 | 50:1 | 10:1 | 1:1 | 1:10 | 1:50 | 1:100 |
|---|---|---|---|---|---|---|---|
| flip #: | 10000 | 10000 | 1480 | 9 | 270 | 100 | 14 |
| flop #: | 100 | 177 | 350 | 17 | 2000 | 2000 | 1000 |
| flip %: | 99 | 98 | 81 | 35 | 10 | 5 | 1.4 |
| flop %: | 1 | 2 | 19 | 65 | 90 | 95 | 98.6 |

Hybridization selectivity was controlled for each subunit by growing and sequencing six hybridizing plaques on an automated sequencer A373 (Applied Biosystems).

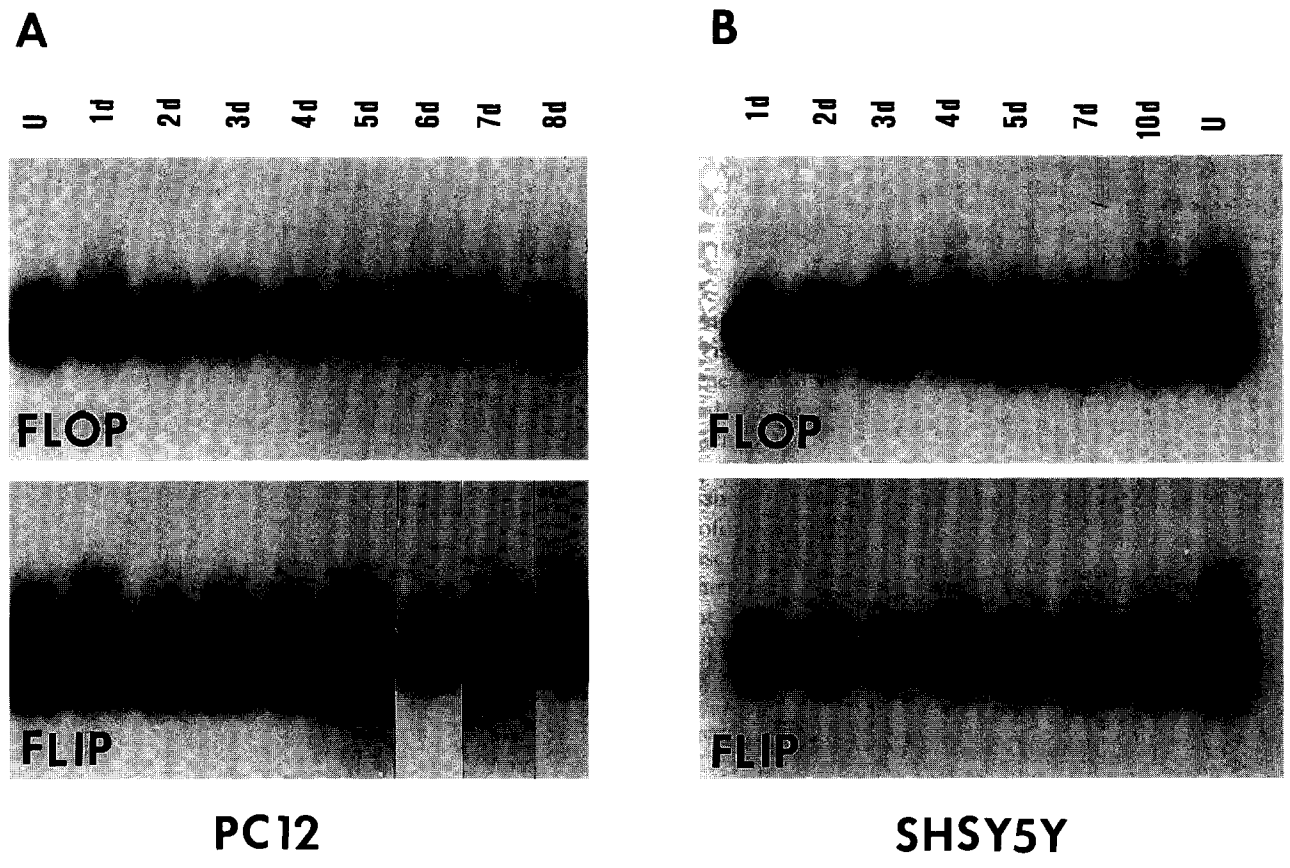


Fig. 1. Autoradiograph of a Southern blot showing the differential hybridization of flip and flop specific probes to PCR fragments of the GluR-B subtype. PC12 cells (a) and SH-SY5Y cells (b) were cultivated in the absence or presence of NGF (50ng/μl) and RA (10μM), respectively. U indicates untreated cells, 1d to 10d indicate timepoints of treatment in days. Total RNA was extracted, reverse transcribed and amplified with GluR-B specific primers. After transfer onto nitrocellulose membranes hybridization with specific oligonucleotide probes was performed. The size of the amplified fragment is 310 base pairs.

express all AMPA receptor mRNAs. Each subunit undergoes individual changes upon differentiation with RA with respect to the ratio of alternative transcripts. AMPA mediated changes in intracellular $Ca^{2+}$ as well as specific displacement of bound [$^3$H]glutamate by AMPA have been previously reported for SH-SY5Y cells [17]. Such findings, however, do not allow any conclusion with respect to the subunit and splice variant composition. Immunocytochemical studies on the sympathetic superior cervical ganglion revealed immunoreactivity for all AMPA receptor subunits in small intensely fluorescent (SIF) cells and for GluR-A, and -B/C in principle cells [18] being in good agreement with our findings on the neuroblastoma cell line. No information on the AMPA receptor splice preference in the originating neural-crest structure is available to date. Whether the difference of splice variant selection for individual AMPA receptor mRNAs in undifferentiated and differentiated SH-SY5Y cells may also be due to the involvement of specific regulatory factors needs to be determined.

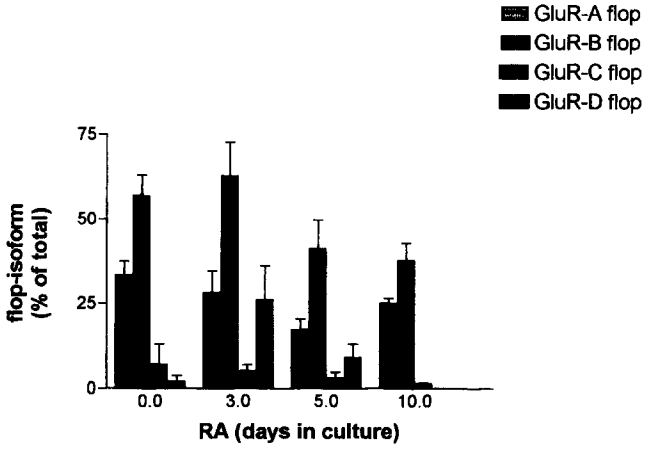


Fig. 2. Relative amounts of flop encoding mRNAs with respect to total message of AMPA receptor subunits. These were determined with isoform-specific oligonucleotides as described in section 2. The results were obtained from at least three independent experiments. Error bars represent S.E.M.

In summary, we demonstrated that rat pheochromocytoma and human neuroblastoma cells express transcripts of AMPA receptor subunits. For PC12 cells, the expression pattern and splice variant profile remains unchanged during differentiation. In SH-SY5Y cells the splice variant preference varies during differentiation in a distinct fashion for each individual subunit.

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