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Activity-dependent regulation of alternative splicing patterns in the rat brain

Rosette Daoud, Maria da Penha Berzaghi, Frank Siedler,[1] Mark Hübener and Stefan Stamm
Max-Planck Institute of Neurobiology and [1]Max-Planck Institute of Biochemistry, Am Klopferspitz 18a, D-82152 Martinsried, Germany

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Abstract

Alternative splicing plays an important role in the expression of genetic information. Among the best understood alternative splicing factors are transformer and transformer-2, which regulate sexual differentiation in *Drosophila*. Like the *Drosophila* genes, the recently identified mammalian homologues are subject to alternative splicing. Using an antibody directed against the major human transformer-2 beta isoform, we show that it has a widespread expression in the rat brain. Pilocarpine-induced neuronal activity changes the alternative splicing pattern of the human transformer-2-beta gene in the brain. After neuronal stimulation, a variant bearing high similarity to a male-specific *Drosophila* tra-2^{179} isoform is switched off in the hippocampus and is detectable in the cortex. In addition, the ratio of another short RNA isoform (htra2-beta2) to htra2-beta1 is changed. Htra2-beta2 is not translated into protein, and probably helps to regulate the relative amounts of htra2-beta1 to beta3. We also observe activity-dependent changes in alternative splicing of the clathrin light chain B, c-*src* and NMDAR1 genes, indicating that the coordinated change of alternative splicing patterns might contribute to molecular plasticity in the brain.

Introduction

Neurons are capable of responding to external stimuli by changing their protein composition. This can be achieved either by a change in promoter activity or by post-transcriptional events, such as differential mRNA stability or alternative splicing, which is partly made possible by a neuron-specific set of RNA binding proteins, e.g. ELAV (King *et al*., 1994) or ELAV-related proteins (Okano & Darnell, 1997) and the neuron-specific Sm protein N (Schmauss *et al*., 1989). This ability to respond to external stimuli by changing the molecular composition is the basis for neuronal plasticity and thus for complex physiological phenomena, such as learning and memory (Bailey *et al*., 1996).

Alternative splicing is an important mechanism for the creation of protein isoforms from a single gene. While the biological significance of these isoforms is often unclear, it is nevertheless well understood in some instances. In many cases, stop codons are introduced by alternative splicing, which changes the carboxy terminal of proteins. This has various physiological consequences; it can create soluble instead of membrane-bound receptors (Baumbach *et al*., 1989; Eipper *et al*., 1992; Toksoz *et al*., 1992; Zhang *et al*., 1994; Hughes & Crispe, 1995; Tabiti *et al*., 1996), change the affinity towards ligands (Sugimoto *et al*., 1993; Xing *et al*., 1994; Suzuki *et al*., 1995), truncate proteins to produce inactive variants (Swaroop *et al*., 1992; van der Logt *et al*., 1992; Duncan *et al*., 1995; Sharma *et al*., 1995; Eissa *et al*., 1996) and change endocytotic pathways (Wang & Ross, 1995). In addition, inclusion or skipping of alternative exons can add or delete protein modules that change the affinity towards ligands (Chiquet *et al*., 1991; Danoff *et al*., 1991; Giros *et al*., 1991; Flier, 1992; Miki *et al*., 1992; Ogura *et al*., 1993; Ohsako *et al*., 1993; Guiramand *et al*., 1995; Suzuki *et al*., 1995; Yan *et al*., 1995; Strohmaier *et al*., 1996), modulate enzymatic activity (O'Malley *et al*., 1995), create different hormones (Amara *et al*., 1982; Courty *et al*., 1995) and change properties of ion channels (Sommer *et al*., 1990; Kuhse *et al*., 1991). Finally, numerous transcription factors are subject to alternative splicing, which contributes to control of gene expression (reviewed in Lopez, 1995).

Mechanistically, pre-mRNA splicing is catalysed by numerous proteins in a macromolecular complex, the spliceosome (Krämer, 1996). Major components of the spliceosome are the small nuclear ribonucleoproteins (or snRNPs) (Will *et al*., 1993), proteins associated with hnRNA (hnRNPs) (Weighardt *et al*., 1996) and a large family of serine/arginine rich proteins (Fu, 1995; Manley & Tacke, 1996). Based on their reactivity with an antibody and biochemical characteristics, the latter group is subdivided into SR-proteins and SR-related proteins. SR and SR-like proteins are characterized by a modular composition, consisting of one or more RNA recognition motifs, and a domain rich in serine and arginine residues (the SR domain), in which the serine residues are phosphorylated. SR proteins can complement splicing-deficient nuclear extracts, and are expressed with different concentrations in all tissues (Zahler *et al*., 1993), indicating that they are essential splicing factors. Using their SR domain and RNA recognition motif, they can bind to other SR proteins and RNA, forming networks of SR-domain-mediated protein–protein interactions (Wu & Maniatis, 1993; Manley & Tacke, 1996) that identify splice sites on the pre-mRNA. In addition, SR and SR-like proteins can bind to exon-enhancer motifs, often purine rich sequences, that can stimulate usage of suboptimal splice sites (Watakabe *et al*., 1993; Manley & Tacke, 1996). The interaction with exon enhancers results in a concentration-dependent influence of SR-proteins on alternative splicing decisions, both *in vivo* (Cáceres *et al*., 1994; Screaton *et al*., 1995; Wang & Manley, 1995) and *in vitro* (Ge & Manley, 1990; Mayeda & Krainer, 1992).

Correspondence: Dr Stefan Stamm, as above.
E-mail: stamm@pop1.biochem.mpg.de

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In mammalian systems, the physiological targets of various SR-proteins have not been determined. In contrast, genetic analysis in *Drosophila* has revealed a direct influence of the SR-like proteins, transformer and transformer-2 on exon 4 of the double-sex pre-mRNA, on an alternative fruitless 5′ splice sites (Heinrichs *et al*., 1998), as well as on exuperantia transcript (Hazelrigg & Tu, 1994) and alternative testis transcript (Madigan *et al*., 1996). Recently, two mammalian homologues of transformer-2 have been isolated. The human alpha gene was identified as an expressed sequence tag, and shown to complement transformer-2 deficiency in *Drosophila* (Dauwalder *et al*., 1996).

We have cloned another human homologue of transformer-2 in a yeast two-hybrid screen aimed at identifying new components of the neuronal spliceosome (Beil *et al*., 1997). This beta gene has also been isolated in rat (Matsuo *et al*., 1995) and mouse (Segade *et al*., 1996), in a differential display and subtractive library screen performed to identify genes involved in ischaemia and in silica induction, respectively. The characterization of the beta gene in the rat, mouse and human demonstrated that the expression of this gene is influenced by various physiological stimuli: in the brain, ischaemia and re-oxygenation dramatically increase the message level (Matsuo *et al*., 1995); in macrophages, silica particles increase levels twofold (Segade *et al*., 1996) and stimulation of human cultured T-cells changes the expression level of its alternatively spliced variants (Beil *et al*., 1997).

We have determined the gene structure of the htra2-beta gene and found that at least four alternatively spliced mRNAs are created, that can encode three protein isoforms (Nayler *et al*., 1998) (Fig. 1). They are structurally related to isoforms found in *Drosophila melanogaster* (Mattox *et al*., 1990) and *virilis* (Chandler *et al*., 1997) where they probably autoregulate their expression levels.

As transformer-2 regulates sex-specific expression of the neuronal gene fruitless in *Drosophila* (Heinrichs *et al*., 1998), we wished to determine whether the alternative splicing patterns of its mammalian homologue are subject to regulation in the brain as well. We used a model in which neuronal activity is changed by administration of pilocarpine, a cholinergic agonist that crosses the blood–brain barrier. On the basis of electroencephalographic monitoring, behavioural analysis and morphological sequelae, pilocarpine-mediated progressive increases in neuronal activity have been used in an animal model resembling some aspects of human temporal lobe epilepsy (Turski *et al*., 1983; Turski *et al*., 1984). In this report, we demonstrate that pilocarpine administration changes alternative splicing patterns in the tra2-beta gene (Fig. 1). As the alternative splicing of other genes, such as clathrin light chain B (Stamm *et al*., 1992), NMDAR1 (Zimmer *et al*., 1995) and c-*src* (Black, 1991), changes in response to neuronal activation, this could be part of a cascade of alternative splicing decisions ultimately modifying the protein isoform composition in neurons.

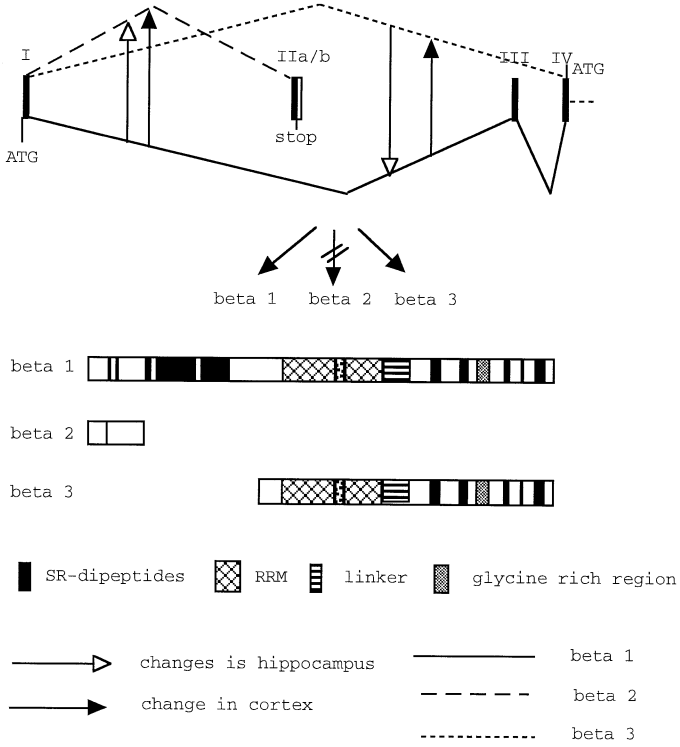


FIG. 1. Gene structure and change of alternative splicing events of the tra2-beta gene after pilocarpine treatment. The 5′ end of the gene is schematically shown. (*top*) The predominant splicing event generates htra2-beta1 (thick line). The joining of exon I to II leads to a truncated form, beta2 (dashed line). The joining of exon 1 to exon 4 generates the beta3 form (dotted line). Due to a frameshift caused by the skipping of exon III, the beta3 isoform lacks the first SR-rich domain of beta1, and begins with the start codon indicated in exon 4. The effects of pilocarpine stimulation after ≈ 6 h are indicated by open arrows for hippocampus and closed arrows for cortex. The direction of the arrow indicates the change of isoforms observed. The translational products and their domain structures are shown below. The SR-dipeptides are indicated by black lines and the RNA recognition motif is shown as a striped box. The beta2 isoform is not translated into protein.

Materials and methods

Synthesis of tra2-beta2 peptide

Preloaded p-benzyloxybenzyl alcohol resin [Fmoc-Lys (Boc)-WANG resin, 0.41 mmol/g] was obtained from Calbiochem-Novabiochem (Bad Soden, Germany), the protected amino acid derivatives and the coupling reagent HBTU were from Alexis (Grünberg, Germany) and all other reagents and solvents from Fluka (Neu Ulm, Germany). The peptide of the sequence MSDSGEQNYGERVNVEEGKCGSRHLTS-FINEYLKLRNK, corresponding to htra2-beta2, was synthesized automatically on an Applied Biosystems Peptide Synthesizer, Model 431 A, using the Fmoc/tert-butyl strategy. For the side chain protection, trityl (Trt) was used for glutamine, asparagine, histidine, and cysteine), t-butyloxycarbonyl (Boc) for lysine and 2,2,5,7,8-pentamethylchromansulphonyl (Pmc) for arginine. Couplings were performed following the HBTU/HOBt [2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate/N-hydroxybenzotriazol] procedure with prolonged coupling times in comparison with Applied Biosystems 'FastMoc' modules, whereas deprotection of the growing chain was performed with piperidine in the usual manner. For the final cleavage from the resin, a cocktail of trifluoroacetic acid/H_2O/tri-isopropylsilane (95 : 2.5 : 2.5; v : v) was used for 5 h. The crude peptide was purified to homogeneity by reversed-phase HPLC on a 20 × 250-mm column (100–5 C18-PPN; Macherey & Nagel, Düren, Germany) using the standard acetonitrile/trifluoroacetic acid system with gradient elution (from 24% to 33% acetonitrile in 15 min with a flow rate of 12.5 mL/min). The final product was characterized by quantitative amino acid analysis and electrospray mass spectrometry. The peptide was coupled to keyhole limpet haemocyanin, and New Zealand rabbits were used in a standard immunization protocol to create the htra2-beta serum.

Dephosphorylation

Dephosphorylation of nuclear extract was performed for 45 min at 37 °C using 1 U of calf intestine phosphatase (Boehringer Mannheim, Germany) in (in mM) Tris-HCl, 25; $MgCl_2$, 1; $ZnCl_2$,0.1.

Cloning

Enhanced green fluorescent protein (EGFP)-tra2 was cloned by amplifying htra2-beta1 using 26-EcoRI (ttgaattcGGAGTCATG-AGCGACAGCGGCGAGCAG) and 26-BamHI (ttggatccCAAAA-CATTTGAGTGAAATTGG) (Beil *et al*., 1997), and subcloning the EcoRI-BamHI fragment into pEGFP-C2 (Clontech, Palo Alto, USA). Glutathione S-transferase (GST)-tra2 and Flag-beta1 was constructed by subcloning the same fragment into a modified GEX-vector (Pharmacia, Freiburg, Germany) or a modified pCR3.1 (Invitrogen, Carlsbad, USA) vector. Flag-beta3 was constructed by subcloning an amplicon generated with tradelSR1 (gggaattcaccatggattatcgtagacggca-cagc) and 26-BamHI into the same modified pCR3.1 vector.

Haemagglutinine (HA)-beta2 was amplified with the primers 26-HA (ttaagcttggatccgccaccatgtacccatacgatgttccagattacgctATGAG-CGACAGCGGCGAGCAGAAC) and 26–17 (ccggtaccCATTTGA-GTGAAATTGGGCCTCCTTTGG) (Beil *et al*., 1997) and subcloned into pCR3.1 (Invitrogen).

Transfection

A293 cells (ATCC, Rockville, USA) were grown in Dulbecco's modified Eagle's medium (Gibco BRL, Germany) supplemented with 10% foetal calf serum and transfected with 10 μg of the appropriate plasmid DNA using the calcium phosphate method. Protein was prepared after one day of transfection and detected on 15% sodium dodecyl sulphate (SDS) polyacrylamide gels.

Western blot

Protein for immunoblotting was prepared from different tissues by homogenizing 0.25 g of tissue in 1 mL of sample buffer (60 mM tris/HCL, pH 6.8; 2% SDS, 0.1 M dithiothreitol), followed by boiling and centrifugation. Protein was quantified using the Bio-Rad Bradford assay. Each 30 μg of protein was subjected to SDS/polyacrylamide gel electrophoresis (12%) as described (Laemmli, 1970), transferred onto ECL-menbranes (Amersham, Braunschweig, Germany), incubated with rabbit htra2 antibody diluted 1 : 2000 in 1 × NET/2.5% gelatine [10 × NET (in M): NaCl, 1.5; EDTA, 0.05; Tris, 0.5, pH 7.5, with 0.5% triton X-100] and detected with an anti-rabbit antibody coupled to horseradish peroxidase (Amersham) (1 : 3000). Other antibodies used were anti-actin (Amersham), 1 : 1000, anti-SF2/ASF (AK96, Oncogene Science, Cambridge, USA) 1 : 1000, antihistone (Fitzgerald, Concord, USA), 1 : 1000, anti-HA (Berkley antibodies, USA), 1 : 500 and anti-Flag (Sigma) 1 : 2000.

Treatment of animals

All the animal experiments reported in this study were conducted in accordance with the statement regarding the care and use of animals, in the Handbook for the Use of Animals in Neuroscience Research (1991) of the Society for Neuroscience. Adult male Wistar rats were used. Pilocarpine was administered intraperitoneally at 300 mg/kg. Methylscopolamine (1 mg/kg) was administered subcutaneously 30 min prior to pilocarpine. Both pilocarpine and methylscopolamine were freshly dissolved in saline before use. Ninety minutes after pilocarpine administration, the animals received diazepam (7.5 mg/kg, i.p.) to suppress excessive neuronal activity (Berzaghi *et al*., 1993). Control animals received an equal volume of saline.

Preparation of total RNA and RT-PCR

Total RNA was extracted from the hippocampus and cortex of rats as described by the guadinium thiocyanate method (Chomcyznski & Sacchi, 1987). For RT-PCR, cDNA was made from 1 μg of total RNA using H^- MMLV reverse transcriptase (Gibco, Karlsruhe, Germany), 5 mM random primers (Promega, Madison, USA), 0.1 mM desoxy nucleotide triphosphates (dNTPs), 10 units of RNasin and 10 mM dithiothreitol. The reactions were performed for 60 min at 42 °C. PCR was performed using the primers shown in Table 1.

TABLE 1. The primers used for PCR

LCB/EN	
sense	ACCGAACAGGAGTGGCGGGAG
antisense	GGGGTCTCCTCCTTGGATTCT
NMDA/exon 21	
sense	ATGCCCGTAGGAAGCAGATGC
antisense	CGTCGCGGCAGCACTGTGTC
GABA2 exon 24	
sense	TTGGATCGTTGCTGATCTGGGACG
antisense	GTATGGTACCCTGCACTATTTTGTG
traex1f	GGAGTCATGAGCGACAGCGGCGAG
traex4rev	CCGGTTCCCAACATGACGCCTTCG
c-*src*	
sense	AGGCTTCAACTCCTCGGACA
antisense	ATCTTGCCAAAGTACCACTCCT

The PCR conditions used were denaturation at 94 °C for 2 min, followed by 40 (light chain B) or 30 cycles of denaturation (94 °C for 30 s), annealing (55 °C for 30 s) and elongation (72 °C for 30 s). The final elongation was performed at 72 °C for 10 min. PCR products for c-*src* were analysed on 15% native polyacrylamid gels. All other products were resolved on 2% agarose and quantified with the enhanced analysis system of Herolab (Wiesloch, Germany).

RNase protection assays

RNase protection assays were performed as previously described (Beil *et al*., 1997) using an Ambion Hybspeed RPA kit (Ambion, USA). For each experiment, 10 μg of total RNA was used with 10^4 c.p.m. of ^{32}P radio-labelled htra2-beta2. The RNA fragments were analysed on 5% native polyacrylamide gels. Dried gels were exposed overnight on Fuji X BAS phosphoimager plates. Band intensities were quantified using BAS software (Raytest, Straubenhardt, Germany).

Immunohistochemistry

For immunohistochemistry, rats were deeply anaesthetized with ether and killed by decapitation. The brains were immediately removed and frozen on dry ice. Coronal cryostat sections were cut at 20 μm, thawed onto gelatinized slides, and stored at –20 °C. The sections were fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) for 30 min and washed three times in PBS. They were preincubated for 1 h in 3% normal goat serum with 0.5% TritonX in PBS at room temperature followed by overnight incubation at 4 °C with the htra2 antibody (1 : 500) in PBS containing 0.3% normal goat serum and 0.5% TritonX. After three washes in PBS, the sections were incubated with the secondary Cy3-fluorochrome-conjugated goat anti-rabbit antibody (Jackson ImmunoResearch Laboratories, Inc., Westgroove, USA) at a dilution of 1 : 200 in PBS for 2 h. Next, the sections were counterstained with 0.5 μg/mL DAPI (4′,6-diamidino-2-phenylindol; Sigma) in PBS for 10 min, washed again three times with PBS, and coverslipped with Gel-Mount (Biomeda Corporation, Forster City, USA). To test the specificity of the htra2 antibody for immunohistochemical staining, we incubated the undiluted rabbit serum with the peptide (2.4 mg/mL) used for immunization for 1 h at room temperature. Staining with the preabsorbed antibody was then carried out as described above.

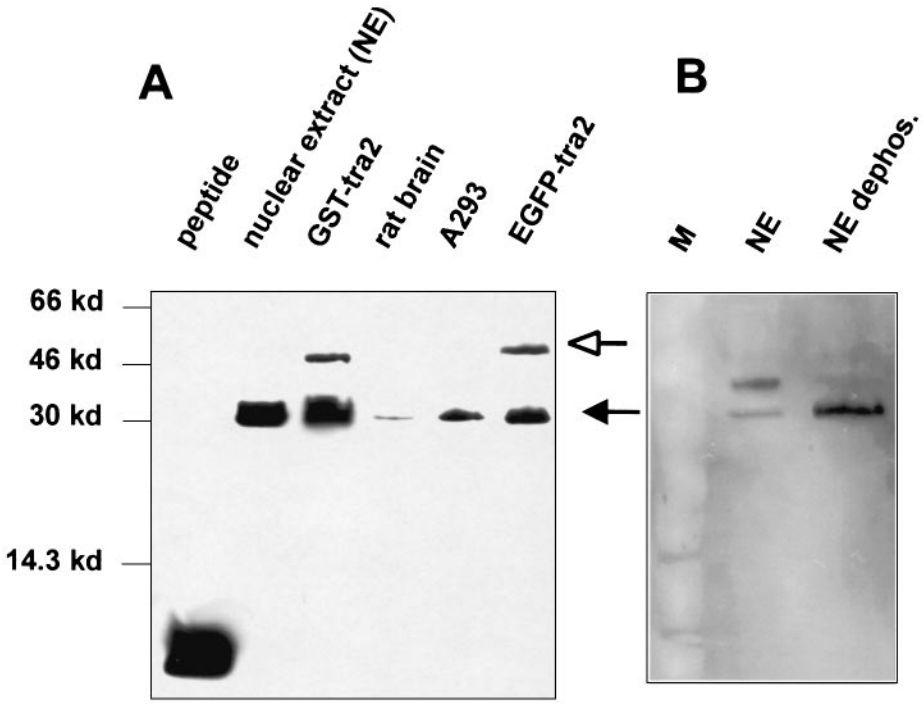


FIG. 2. Properties of the htra2-beta1 antiserum. (A) Approximately 30 μg of the proteins indicated were separated on 15% polyacrylamide gels and analysed in Western blots with anti-tra antiserum. Peptide: peptide htra2-beta2 used to raise the antibody, this peptide is identical to the predicted open reading frame of htra2-beta2. Nuclear extract (NE): nuclear extract from HeLa cells. GST-tra2: recombinant GST-htra2-beta1 from *E. coli*. Rat brain: total lysates from rat brain. A293: total lysate from A293 cells. EGFP-tra: total lysates from cells transfected with EGFP-tra2 expression plasmid. The position of endogenous htra2-beta1 is indicated with a closed arrow, the position of the fusion proteins is indicated with an open arrow. The GST-fusion protein is degraded in *E. coli* and gives rise to a degradation product of ≈ 34 kDa that comigrates with endogenous htra2-beta1. (B) 5 μg of HeLa nuclear extract (NE) was dephosphorylated (NE dephos.) and separated on a 5% polyacrylamide gel. Protein was detected with the anti-tra2 antiserum. The upper band disappears after dephosphorylation.

Results

Htra2-beta1 and beta2 protein levels in rat brain

We recently identified htra2-beta1 as an interactor of splicing components (Beil *et al*., 1997). At that time, our analysis was limited to RNA levels measured by semiquantitative RT-PCR and RNAse protection analysis. In mouse, it was shown that several transcripts are created due to alternative polyadenylation that changes mRNA stability (Segade *et al*., 1996), which would ultimately influence protein expression. We therefore generated a rabbit antiserum to study the protein levels.

Characterization of an antiserum against htra2-beta1

In order to create a specific antiserum that does not crossreact with other members of the large family of RNA binding proteins (Birney *et al*., 1993) or with the SR-family of proteins (Fu 1995; Valcárcel & Green, 1996), we synthesized a peptide corresponding to htra2-beta2, an alternative splice variant of beta1 (Beil *et al*., 1997). It contained the common N-terminus of htra2-beta1 and beta2, which has only weak similarities to htra2-alpha and other SR-proteins and the unique C-terminus of htra2-beta2. Database searches revealed no high homologies of this peptide to other known proteins. The peptide was coupled to keyhole limpet haemocyanin and injected into rabbits. The specificity of the antibody was tested against the peptide, HeLa nuclear extract, recombinant htra2-beta1, total rat brain lysates, A293 lysates, and lysates of cells transfected with pEGFPtra-2 (Fig. 2A). A band of 34 kDa corresponding to the predicted size of htra2-beta1 was observed in all lanes. In addition, GST-tra2 and EGFP-tra2 were detected with the predicted sizes of 61 and 62 kDa, respectively. In order to separate htra2-beta1 and beta2 on one gel, we used a 15% polyacrylamide gel. Using a lower acrylamide concentration, an additional band of lower intensity running slightly above the predicted 34-kDa band was detected. As this band disappears after dephosphorylation, it probably represents a different phosphorylation status (Fig. 2B). In addition, in total brain lysates, the antibody weakly recognizes a band of 110 kDa, which is more strongly expressed in testis (Nayler *et al*., 1998).

We were unable to detect a band corresponding to the translational product of beta2 (4.4 kDa). We therefore tested whether this variant can be expressed as a protein in cells. A293 cells were transfected with an HA-tagged expression plasmid pCR-HA-beta2 and probed with the htra2-beta1 antibody. The antibody detected htra2-beta1 as well as the beta2 peptide used to raise it, but it failed to recognize htra2-beta2 in transfected cells (Fig. 3A). The signal was abolished after preabsorbtion of the antiserum with tra2-peptide, indicating the specificity of the antiserum (Fig. 3B). To further test the protein expression of htra2-beta2 and -beta3 we expressed them as HA- or Flag-tagged cDNAs in A293 cells (Fig. 3C,D). While we can detect htra2-beta1 (36 kDa) and -beta3 (24 kDa) with antibodies against their tags, we cannot detect the htra2-beta2 isoform (5.6 kDa).

In summary, we conclude that the serum is specific for htra2-beta1. Furthermore, htra2-beta3 can be translated into protein. In contrast, htra2-beta2 is not translated. As the stop codon found in htra2-beta2 is located 195 nt upstream, the 3′ most exon–exon junction, the termination-codon position rule (Nagy & Maquat, 1998) predicts that this is probably due to nonsense-mediated RNA decay (Ruiz-Echevarria *et al*., 1996).

Level of htra2-beta1 in brain development

SR-proteins have been shown to change splice site selection in a concentration-dependent manner, both *in vivo* (Cáceres *et al*., 1994;

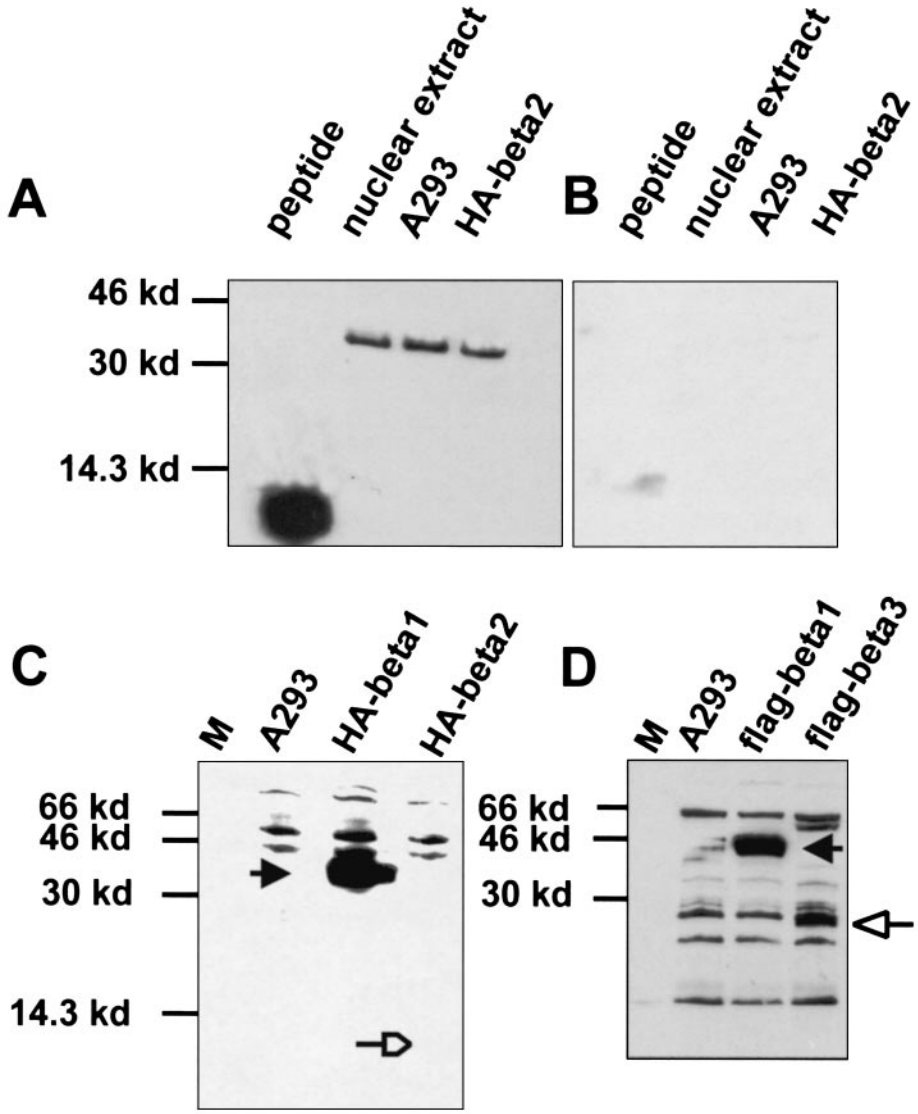


FIG. 3. Htra2-beta2 is not translated into protein. (A) Protein extracts and cell lysates, from the transfection experiments indicated, were tested with anti-tra2 antiserum in Western blot. Peptide: peptide htra2-beta2 used to raise the antibody; this peptide is identical to the predicted open reading frame of htra2-beta2. Nuclear extract: nuclear extract from HeLa cells. HA-beta2: expression plasmid of beta-2. (B) An identical filter was probed with anti-tra antiserum preabsorbed with peptide htra2-beta2. (C) Transfection of HA-tagged htra2-beta1 (HA-beta1) and HA-tagged htra2-beta2 (HA-beta2) in A293 cells. A293: untransfected cells. The closed arrow indicates the position of the expected HA-beta1 protein (36 kDa) and the open arrow the expected position of HA-beta2 (5.6 kDa). (D) Transfection of flag-tagged htra2-beta1 (flag-beta1) and flag-tagged htra2-beta3 (flag-beta3) in A293 cells. A293: untransfected cells. The closed arrow indicates the position of the expected flag-beta1 protein (36 kDa) and the open arrow the expected position of flag-beta3 (24 kDa).

Screaton *et al*., 1995; Wang & Manley, 1995) and *in vitro* (Ge & Manley, 1990; Mayeda & Krainer, 1992). As there are numerous examples of changes in alternative splice site selection during development in the brain (Stamm *et al*., 1994), we examined the levels of htra2-beta1 protein in the developing rat brain. Using constant amounts of total protein lysate, we observed a steady decline in the concentration of the htra2-beta1 protein between embryonic day 14 and adulthood (Fig. 4A). Equal loading of protein was confirmed by probing against actin (Fig. 4B). Using an antibody against SF2/ASF, a member of the SR-protein family, we observed a similar decline of the signal during development (Fig. 4C). In order to test if this decline reflects a change in the ratio of nuclear proteins towards total protein in the brain lysates, we used an anti-histone antibody and observed a similar decline of signal. Thus, on average, the amount of htra2-beta1 and SF2/ASF remains constant in the nuclei during brain development. However, reflecting the decrease of nuclei during development (Williams & Herrup, 1988), the fraction of nuclear protein present in total lysate seems to decrease as brain development continues, and this results in a decline of the htra2-beta1 and SF2/ASF signal in total lysates.

Level of htra2-beta1 in brain regions

Next, we examined the amount of htra2-beta1 immunoreactivity in lysates prepared from different regions of the brain (Fig. 5). There are slight differences between regions that correspond to changes in intensities of the histone signal, and they can therefore be explained by different amounts of cell nuclei in various tissues. Again, we did not detect protein corresponding to htra2-beta2 in any region of the brain. Thus, on average, the protein levels of htra2-beta1 remain constant during CNS development and are similar in different regions of the adult brain.

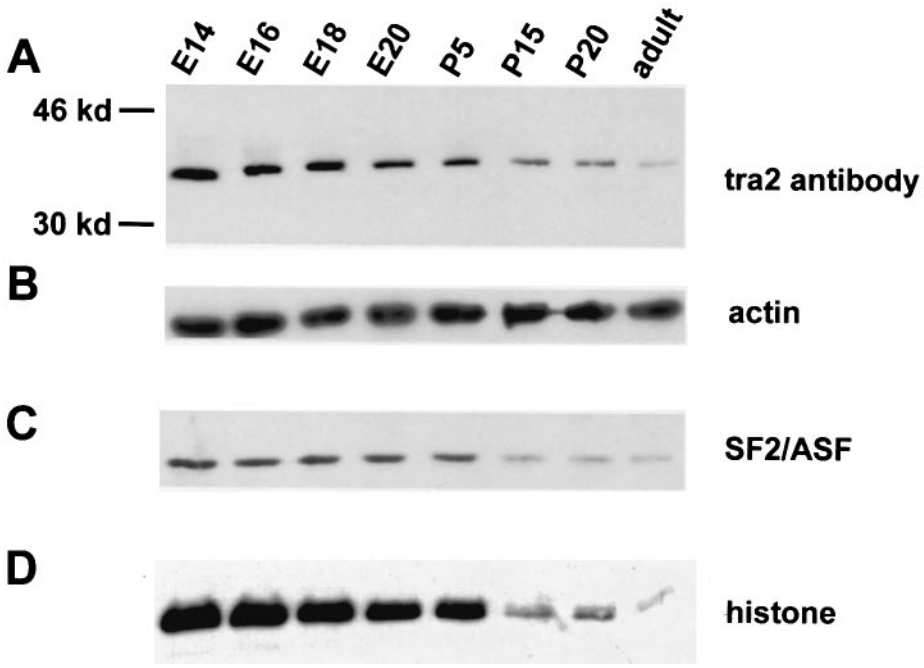


FIG. 4. Expression of htra2-beta1 in brain development. Each 30 μg of total rat protein was subjected to Western blot analysis with the antibodies indicated. Protein samples were from whole brain at embryonic stages E14, E16, E18 and E20, postnatal stages P5, P15 and P20, and adult rat brain. (A) Htra2-beta1 immunoreactivity, detected with the anti-tra2 antibody. Antibody was removed and the filter was probed with anti-actin (B), anti-SF2/ASF (C) and anti-histone (D).

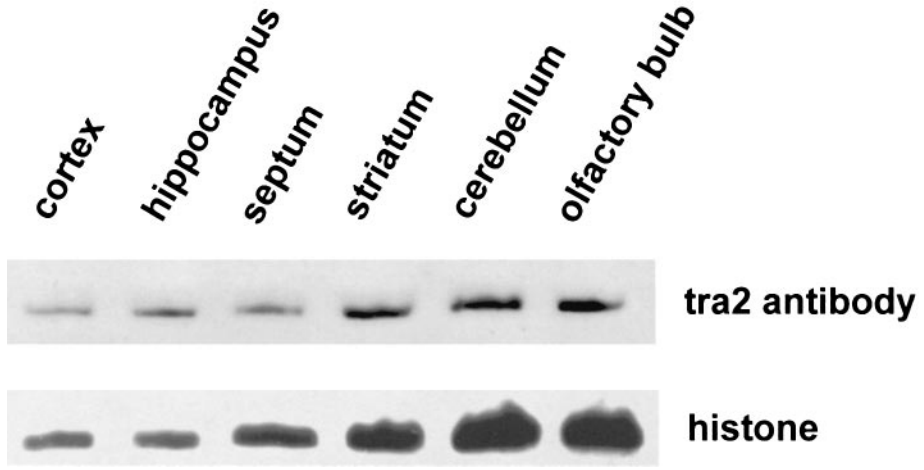


FIG. 5. Expression of htra2-beta1 in various adult brain regions. Each 30 μg of total rat protein, from the regions indicated, were subjected to Western blot analysis. (*Top*) tra-2 immunoreactivity and (*bottom*) the same blot was reprobed with anti-histone.

Immunohistochemistry

The protein analysis described so far was limited to total tissue. In order to obtain information on protein expression at the cellular and subcellular levels, we performed immunohistochemistry with the htra-2 antibody (Fig. 6). In agreement with the Western blot analysis shown in Fig. 5, we found that htra-2-positive cells were present in all parts of the adult rat brain. Figure 6 shows the staining pattern in the CA1 region of the hippocampus. Overall, we observed a close match between the distribution of htra-2-labelled and DAPI-counterstained nuclei, indicating that the majority of cells are htra-2-positive (Fig. 6A and B). We did not detect any obvious changes in the staining intensity in the hippocampus of pilocarpine treated animals (compare Fig. 6A and C). This is in agreement with our PCR (Fig. 7), RNAse protection (Fig. 8) and Western blot analysis (Fig. 9), indicating that there is no major change in htra2-beta1 concentration after neuronal stimulation.

At higher magnification it becomes evident that most cells in the pyramidal cell layer are htra-2-positive (Fig. 6E and F). However, a small number of DAPI-labelled nuclei outside the pyramidal cell layer were not labelled with the htra-2 antibody (arrowheads in Fig. 6E and F). The location, as well as the size and shape of these nuclei, suggests that most of these htra-2-negative cells are glial cells. Thus, despite its widespread expression in the brain, htra-2 seems to be regulated in a cell-type-specific manner. Figure 6 (E and F) also confirms that the staining was always confined to the nuclei. Moreover, confocal microscopy revealed a speckled staining pattern in the nuclei (Fig. 6I), a pattern which has previously been shown for htra2-beta1 (Beil *et al*., 1997).

Change of htra2-beta isoforms in response to pilocarpine administration

Due to alternative usage of exons I–IV, the htra2-beta gene can create at least four RNA isoforms (beta1–4) that can be conceptually translated into three proteins. A schematic diagram of the 5′ gene structure is shown in Fig. 7A. In all species examined, the transformer-2 protein is composed of three major protein motifs: an N- and C-terminal SR domain and an RNA recognition motif (Fig. 1). In *Drosophila*, alternative usage of intron M1 creates a splice variant lacking the first SR domain, which exists only in cells of the male germline. In the rat and human, a structural related protein lacking the N-terminal SR-domain is created in the tra2-beta gene by skipping exons 2 and 3. This variant, termed beta3, is predominantly expressed in adult brain, liver and testis (Nayler *et al*., 1998).

Beta3-isoform

In order to test the effects of neuronal activity on this isoform, rats were treated with pilocarpine and sacrificed after 1, 3, 6, 12, 24 and 48 h. Following extraction from hippocampus and cortex, RNA from several time points after pilocarpine induction was reverse-transcribed and amplified using exon 1- and 4-specific primers. In untreated hippocampus we found roughly equal amounts of beta1 and beta3 mRNA. One hour after pilocarpine stimulation, the beta3 isoform can no longer be detected, whereas it reappears 24 h after stimulation (Fig. 7B).

In contrast, the beta3 isoform cannot be detected in the cortex. However, after 6 and 12 h of stimulation, this isoform appears in the cortex and later disappears again (Fig. 7C).

These findings show that pilocarpine treatment influences the generation of the beta3 isoform in the brain. The isoform is switched off in the hippocampus and, later in time, turned on in the cortex. The time course of switching between the beta1 and beta3 isoform in the cortex is slightly slower than in the hippocampus. This might reflect the fact that, characteristically, the pilocarpine-induced activity changes originate in the hippocampus and progressively build up into an electrographic seizure that is registered initially in hippocampus while cortical recordings display minor changes. Later, the electrographic seizures become highly synchronized in the hippocampus and cortex (Turski *et al*., 1983, 1984).

Beta1/beta2 isoform ratio

Inspection of the alternatively spliced exon 2 (Beil *et al*., 1997) reveals the existence of a palindrome flanked by two repeats. This structure is reminiscent of a sequence found in the male-specific intron M1 of the *Drosophila* transformer-2 gene and could be a target for regulatory proteins (Beil *et al*., 1997). In fact, it has been shown that *Drosophila* tra-2 protein binds to this region of the *Drosophila* M1 intron (Amrein *et al*., 1994), indicating that this region is involved in autoregulation. To this end, we could previously show that the ratio of htra2-beta1 to htra2-beta2 changes in response to stimulation in T-cells and primary spleen cultures (Beil *et al*., 1997). These observations prompted us to examine whether neuronal activity would also influence the ratio of htra2-beta1 to htra2-beta2.

In order to measure the ratio of htra2-beta1 to -beta2, we employed an RNA protection assay using a probe complementary to rat tra2-beta2. This probe protects a 114 nt fragment corresponding to htra2-beta2 and a 42 nt fragment corresponding to htra2-beta1 or beta3 (Fig. 8A and B). Each 10 μg of total RNA was tested in a RNAse protection assay. As can be seen in Fig. 8(C and D), neuronal stimulation decreases the ratio of the 114–42-nt protected fragment in the hippocampus. As the beta3 isoform is only significantly expressed in untreated hippocampus (Fig. 7B), the change in this ratio reflects, approximately, the change from beta1 to beta2 isoforms.

The maximum decrease occurs after 3 h, when the ratio drops by about two-thirds. After 3 h, the ratio begins to go back to normal levels, which are reached after 24 h.

A similar effect was observed in the cortex. Here, the ratio of the beta1 to beta2 isoforms also drops by two-thirds after pilocarpine treatment. However, the time course is slightly slower than in the hippocampus. The maximum decrease is found after 6–12 h and normal levels are reached after ≈ 48 h. In addition, the basal ratio of tra2-beta1 to beta2 is higher in the cortex than in the hippocampus.

We conclude that pilocarpine-induced activity changes the ratio of the beta1 and beta2 isoforms as well. As with the beta3 isoform, the time course of changes in the cortex is slightly slower than in the hippocampus, which again might reflect the time necessary for the activity to move from the hippocampus to cortex.

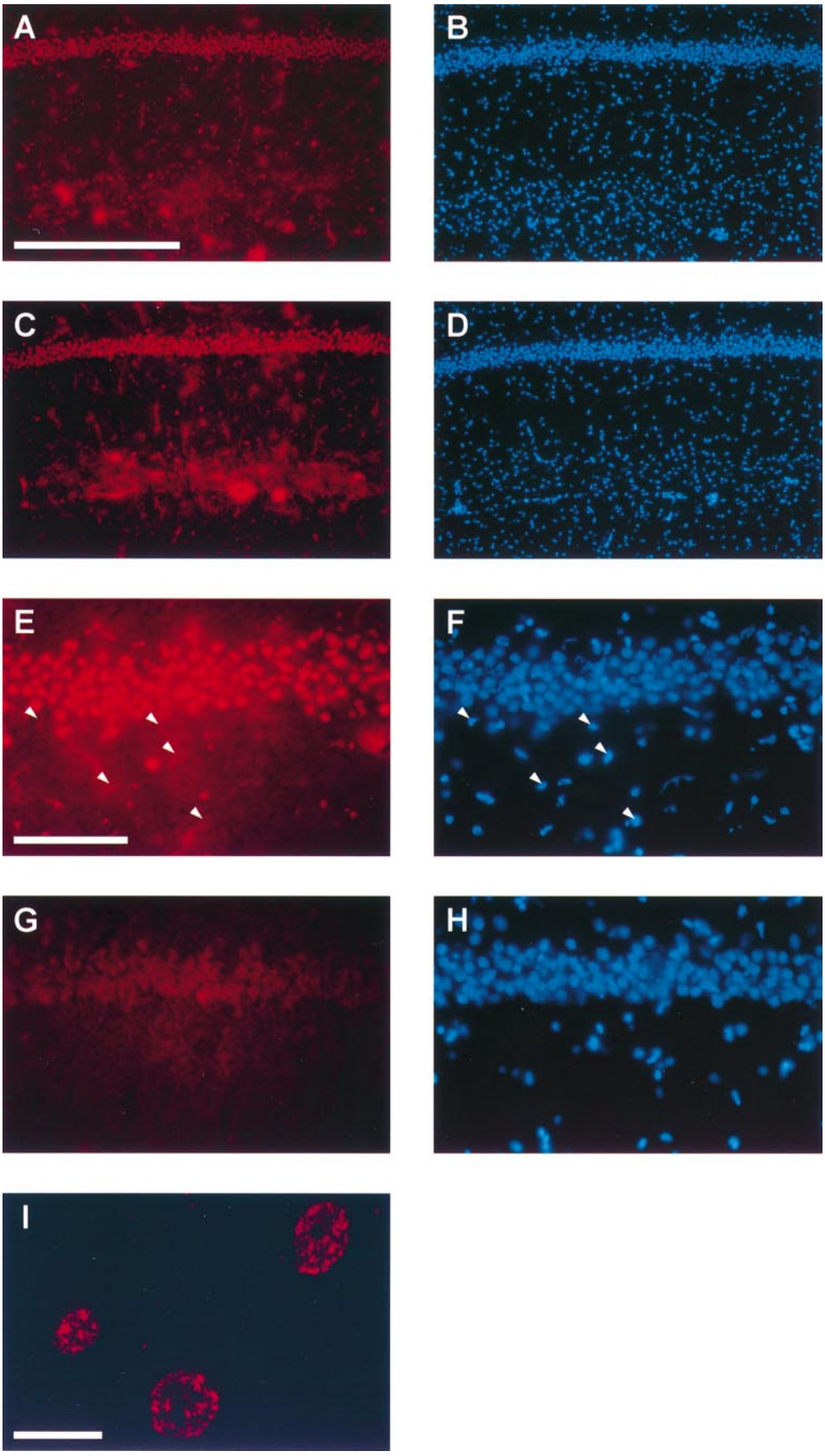
Protein level of htra2-beta1 in stimulated brain

The experiments shown in Figs 7 and 8 were designed to determine the relative ratios of the RNA of htra2-isoforms. Since it is not clear which messages would stay constant during pilocarpine treatment, we used the same amount of total RNA in each protection. Based on the total RNA, there might be an increase (in hippocampus only) of total htra2-beta mRNA levels between 1 and 6 h of pilocarpine treatment. However, we did not observe a dramatic change in the amount of htra2-beta1 mRNA like that detected in ischaemia (Matsuo *et al*., 1995) and upon macrophage induction (Segade *et al*., 1996). In order to determine whether there is an effect on htra2-beta1 protein, we tested its protein expression levels in Western blots using total lysates from different time points after induction and our htra2-beta1 antibody, which recognizes beta1 and 2, but not beta3. As shown in Fig. 9, we did not observe a change in htra2-beta1 protein in hippocampus (Fig. 9A) or cortex (Fig. 9C). In addition, we did not observe expression of beta2 isoform at any time point (Fig. 9C) in cortex or hippocampus (data not shown).

Effect of neuronal activity on other alternatively spliced genes

These experiments demonstrate that pilocarpine treatment can change the alternative splicing patterns of the htra2-beta gene. Genetic analysis in *Drosophila* has revealed a direct influence of transformer-2 on exon 4 of the double-sex pre-mRNA, on an alternative 5′ splice site in fruitless (Heinrichs *et al*., 1998), as

FIG. 6. Immunohistochemical localization of the tra2-beta1 protein in the hippocampus. Panels on the left show staining with the htra-2 antibody; those on the right show the same fields of view with a DAPI-counterstain. (A and B) Staining with the htra-2 antibody in the CA1 region of the hippocampus. The pyramidal cell layer contains many densely packed stained nuclei, while there are only a few scattered nuclei in other layers of the hippocampus. (C and D) Increased neuronal activity after pilocarpine treatment (3 h) does not change the staining intensity. (E and F) High power view of htra-2 staining in the hippocampal CA1 region, showing that the staining is strictly confined to the nuclei. While most nuclei are htra-2-positive, some are clearly devoid of staining (arrowheads). (G and H) Negative control: no stained nuclei are found after preabsorption of the antibody with the peptide used to generate the antibody. (I) Confocal microscopy reveals the speckled distribution of the antigen in the nuclei. Scale bars: 500 μm (A–D), 100 μm (E–H) and 10 μm (I).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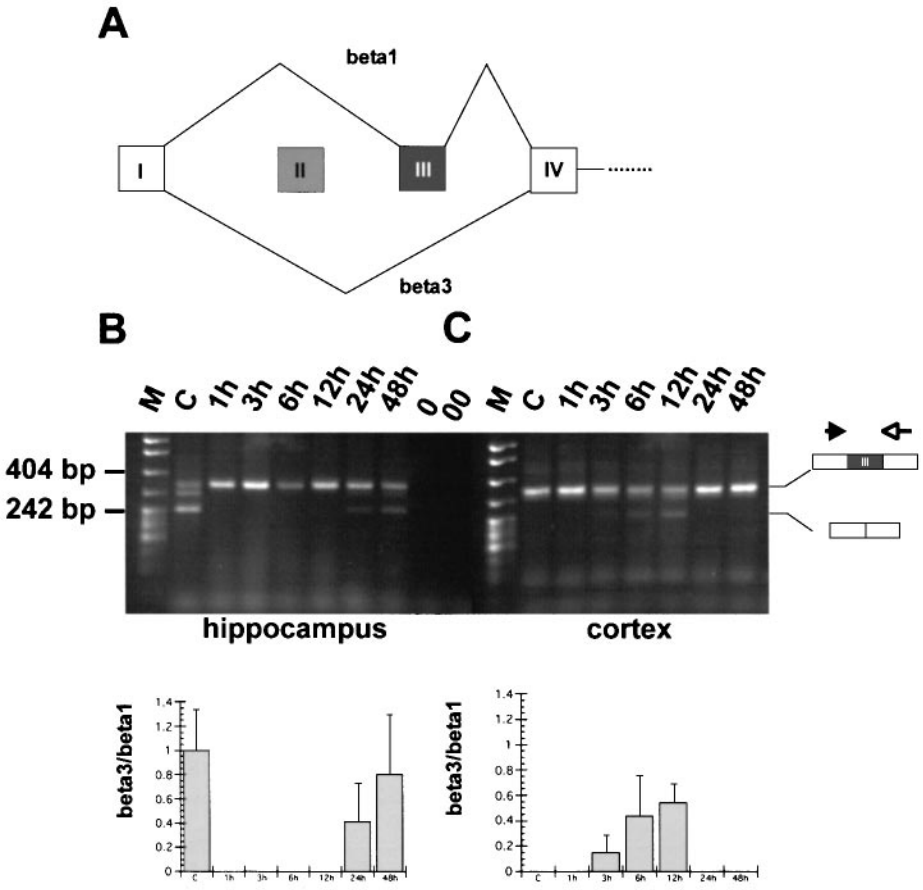

FIG. 7. Change of htra2-beta1 to -beta3 ratio after stimulation with pilocarpine (A). Schematic diagram of the 5′ end of the htra2-beta gene. Location of the primers and splicing patterns generating the beta1 and beta3 isoforms are indicated. (B) RT-PCR using 1 μg total RNA from normal (C) and stimulated (1–48 h) hippocampus (*left*) and cortex (*right*). The structure of the PCR products is schematically indicated on the right. The band running between the 404 and 242-nt bands is probably a heterodimer (Zorn & Krieg, 1991) of those two bands. 0: PCR with RNA. 00: PCR without template. The diagram below shows a statistical evaluation of three independent experiments, error bars are standard deviations. (C) The same experiment was performed for cortex.

well as on exuperantia (Hazelrigg & Tu, 1994) and on alternative testis transcript (Madigan *et al*., 1996). However, there are no mammalian homologues for these target genes and, owing to the lack of genetic systems, target RNAs for various splice factors have not been determined in mammals (Manley & Tacke, 1996).

We therefore tested the influence of pilocarpine induction on several alternatively spliced cassette exons, where the ratio of exon inclusion to exclusion can be determined by RT-PCR. First, we tested the clathrin light chain B gene expressing a neuron-specific exon EN (Stamm *et al*., 1992). Exon EN is surrounded by weak splice sites and its expression is enhanced by the SR-protein SF2/ASF *in vivo* (Cáceres *et al*., 1994). As shown in Fig. 10A–D, pilocarpine treatment leads to an increase of exon-EN-skipping both in hippocampus and cortex. Neuron-specific exons have been analysed in cerebellar neurons by quantitative single cell PCR, and it was found that the alternative exon of the $GABA_A(\gamma 2)$-receptor and NMDAR1 exon 21 show expression profiles similar to those of clathrin light chain B (Wang & Grabowski, 1996). We therefore analysed these exons by RT-PCR, using RNAs from stimulated rat hippocampus and cortex. As shown in Fig. 10E–H, we did not detect a change in the alternative splicing pattern of the $GABA_A(\gamma 2)$-receptor. However, we found a small change in the expression of NMDAR1 exon 21 (Fig. 10K–L) in cortex. Finally, we tested the influence of neuronal activity on the neuron-specific exon NI of c-*src* (Levy *et al*., 1987; Pyper & Bolen, 1989; Black, 1992) and, as with clathrin light chain B, we found repression of the neuron-specific form after stimulation, both in hippocampus (Fig. 10M and N) and cortex (Fig. 10O and P).

These experiments indicate that pilocarpine-induced neuronal activity changes the alternative splicing pattern of some, but not all, alternatively spliced messages.

Discussion

Our results provide evidence that alternative splicing patterns are influenced by pilocarpine-induced changes of neuronal activity in the rat brain. Pilocarpine is a muscarinic cholinergic agonist whose systemic application leads to electrographic seizures, brought about by the stimulation of cholinergic neurons (Turski *et al*., 1983, 1984). As pre-mRNA splicing takes place in a multienzyme complex, the spliceosome, we investigated the effects of neuronal stimulation on a component of this complex, htra2-beta. First, we raised an antibody against htra2-beta1 and used it to test the protein levels of htra2-beta1 in developing and adult brain. We found that, on average, the amount of htra2-beta1 stays constant during development. Often, alternative exons are surrounded by weak splice sites (Stamm *et al*., 1994), whose usage can be stimulated by the presence of exon-enhancer motifs that are recognized by members of the SR or SR-like family of proteins (Fu, 1995; Valcárcel & Green, 1996). It has been demonstrated both *in vivo* and *in vitro* that alternative splicing is influenced by the concentration of SR-proteins or other RNA

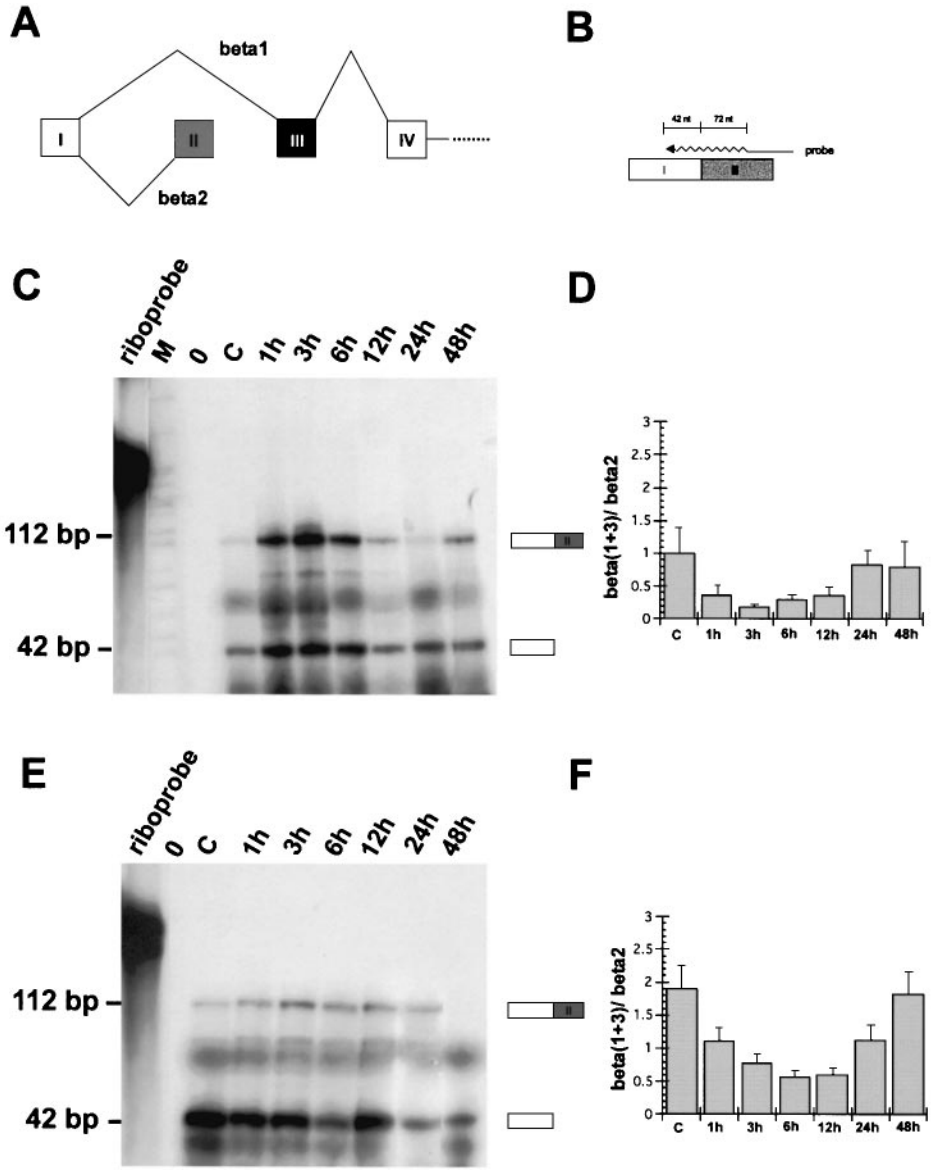

FIG. 8. Change of htra2-beta1 to -beta2 RNA ratio after stimulation with pilocarpine. (A) Schematic diagram of the 5′ end of the htra2-beta gene. (B) Schematic diagram of RNAse protection assay used. The alternative exon II is indicated by dark shading. The protected fragments are 42-nt nucleotides for htra2-beta1 and 114 nt for htra2-beta2. Exon I is used by the variants beta1 and beta3, and exon I and exon II are joined in the beta2 and beta4 RNA isoform. (C) Analysis of exon I to exon (I + II) ratio in hippocampus with RNAse protection assay. The structure of the protected fragment is indicated on the right. Riboprobe: untreated riboprobe. M: end-labelled MspI pBR 322 marker. 0: riboprobe without RNA. C: untreated hippocampal RNA. 1–48 h: time after pilocarpine induction when the hippocampi were isolated. (D) Statistical representation of four independent experiments from hippocampus. The ratio of exon I to exon II was set to one in control hippocampus. Standard deviations are indicated. (E) Analysis of exon I to exon (I + II) ratio in cortex with RNAse protection assay. The details are similar to those in (C). (F) Statistical representation of four independent experiments from cortex. The ratio of exon I to exon II in control hippocampus was set to one and used to normalize the ratio in cortex. Standard deviations are indicated.

binding factors, such as hnRNPs (Ge & Manley, 1990; Mayeda & Krainer, 1992; Cáceres *et al*., 1994; Mayeda *et al*., 1994; Wang & Manley, 1995). *In vitro* experiments show that htra2-alpha1 and beta1 can act as exon enhancers, binding to the purine-rich enhancer sequence A3 (Tacke *et al*., 1998). However, since the htra2-beta1 concentration remains constant, we conclude that it does not act as a global alternative exon enhancer during brain development.

We next investigated tra2-beta1 levels in adult rat brain and found that they are expressed roughly at the same concentrations in different brain regions. However, using immunohistochemistry we could show that not all nuclei contain the htra2-beta1 protein. Thus, while the protein is present in all regions of the adult brain, its expression at the cellular level seems to be regulated in a highly specific manner.

Using the muscarinic cholinergic agonist pilocarpine, we induced seizures in rats, and determined the effects on the splice variants of htra2-beta. A schematic diagram of the human htra2-beta gene and its splicing variants is given in Fig. 1. Sequencing of various rtra2-beta isoforms generated by RT-PCR from rat tissues revealed that the exon–intron structure is completely conserved between human and rat (data not shown).

In hippocampus, pilocarpine induction represses the beta3 isoform and simultaneously increases the amount of the beta2 isoform. We have no indication that the promotor activity of the htra2-beta gene or its mRNA stability is severely affected by pilocarpine treatment. Therefore, we assume that this splicing regulation will allow the htra2-beta1 isoform to stay constant by replacing the beta3 with the

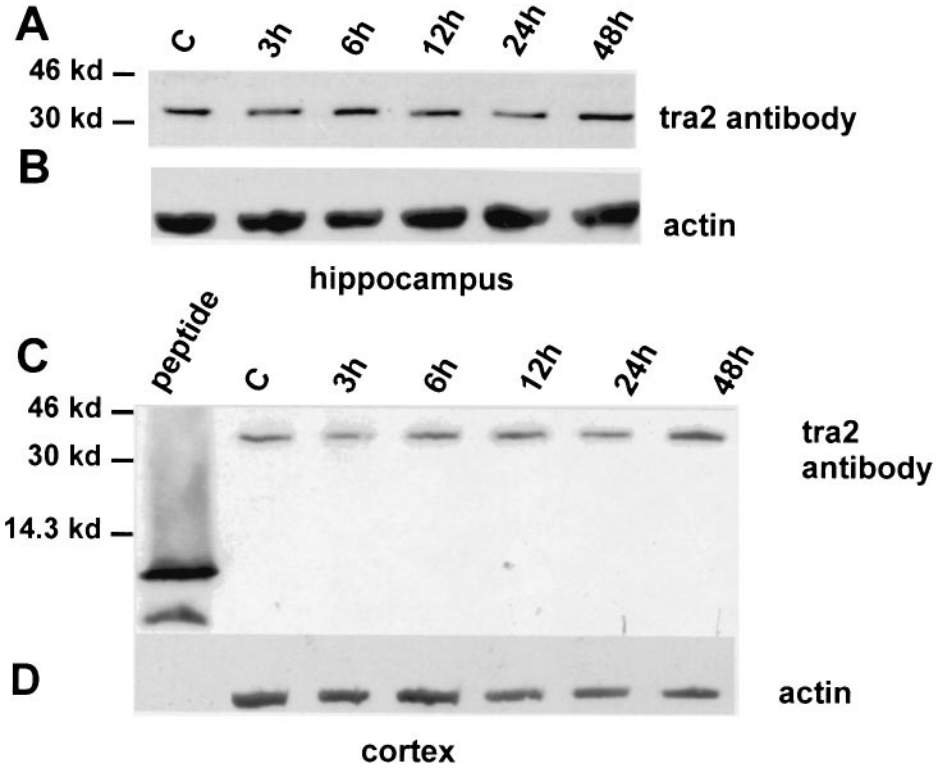

FIG. 9. Expression level of htra2-beta1 protein in stimulated hippocampus and cortex. Lysates from brains at the time indicated after pilocarpine injection were tested with anti-tra2 antiserum and anti-actin in Western blot. C: untreated control. (A) The tra-2-beta1 immunoreactivity in stimulated hippocampus, (B) the same filter reprobed with anti-actin and (C) tra-2-beta1 immunoreactivity in stimulated cortex, tra-beta2 peptide was loaded to demonstrate that no beta2 expression can be detected in stimulated cortex. (D) The same filter reprobed with antiactin.

beta2 isoform from a possibly constant pre-messenger tra2-beta RNA pool. Thus, the beta2 isoform could form an RNA 'reservoir' helping to regulate the relative amounts of beta1 to beta3.

In the cortex, pilocarpine-induced stimulation shifts the splicing pattern towards the beta2 isoform as well. In addition, there is a small amount of beta3 isoform detectable after 6 and 12 h of stimulation. Taken together, pilocarpine induction changes the expression of the beta3 protein, which disappears in the hippocampus and appears in the cortex. Since the beta2 variant is not translated into protein, this means that, at the protein level, the beta3 isoform expression changes while the full length beta1 isoform protein levels remain constant. The changes of alternative splicing are reversible, because the ratios of splice variants approach normal levels after 48 h, indicating that the changes we see are probably caused by an increase in neuronal activity and not by cell death or damage, which is also suppressed by the diazepam administered 90 min after pilocarpine.

In *Drosophila*, a tra-2 protein, $tra2^{179}$, with a domain structure similar to tra2-beta3, has been described as a male germline specific factor (Mattox *et al*., 1990). Complementation experiments in *Drosophila* (Amrein *et al*., 1994) and binding studies of the htra2 beta variants (Nayler *et al*., 1998) did not reveal differences between the isoforms containing/lacking the first SR-domain.

Therefore, it has been proposed that the $tra2^{179}$ isoform is involved in the 'fine tuning' of splicing decisions (Mattox *et al*., 1990). Using a minigene of clathrin light chain B bearing a splice site mutation in the alternatively spliced exon EN that causes neuronal exon usage in fibroblasts, we observed exon repression by htra2-beta1 but not htra2-beta3 *in vivo* (Stamm *et al*., 1999), indicating a difference between the two isoforms.

Therefore, it is likely that changes in the alternative splicing pattern of the htra2-beta gene will affect other alternatively spliced genes as well. In fact, we found a strong effect on the alternative splicing of the clathrin light chain B gene; pilocarpine-induced neuronal activity increases skipping of the neuron-specific exon EN (Fig. 10A–D). This exon encodes a calmoduline binding site that probably results in an enhanced calcium sensitivity of neuronal clathrin-coated vesicles (Jackson, 1992). A decrease of the neuronal relative to the non-neuronal form would therefore result in an endocytotic machinery which is less sensitive to calcium after pilocarpine stimulation. Changes of clathrin light chains after stimulation have also been demonstrated in *Aplysia*. Here, serotonine-induced increases in the amount of clathrin light chains in sensory neurons have been linked to long-term facilitation (Hu *et al*., 1993). It is conceivable that a change in alternative isoforms of clathrin light chains might also accompany synaptic plasticity in rat brain.

A similar effect of neuronal stimulation was observed on the exon NI of c-*src*. Again, stimulation represses usage of the neuron-specifc exon NI (Fig. 10M–P). As NI is located in a conserved SH3 domain (Levy *et al*., 1987; Martinez *et al*., 1987), this change might also modulate receptor–ligand interaction in response to neuronal activity.

Not every alternative exon is influenced by pilocarpine. For example, we detected a modest response of NMDAR1 receptor exon 21 after pilocarpine treatment only in cortex. A similar observation was made by Vezzani *et al*. (1995); prolonged kindling of rats was shown to change this splice variant after 1 week. In contrast, we did not observe a significant change of splice variants of the $GABA_A(\gamma 2)$-receptor. These experiments show a high degree of specificity in the regulation of alternative splicing in the CNS; some, but not all

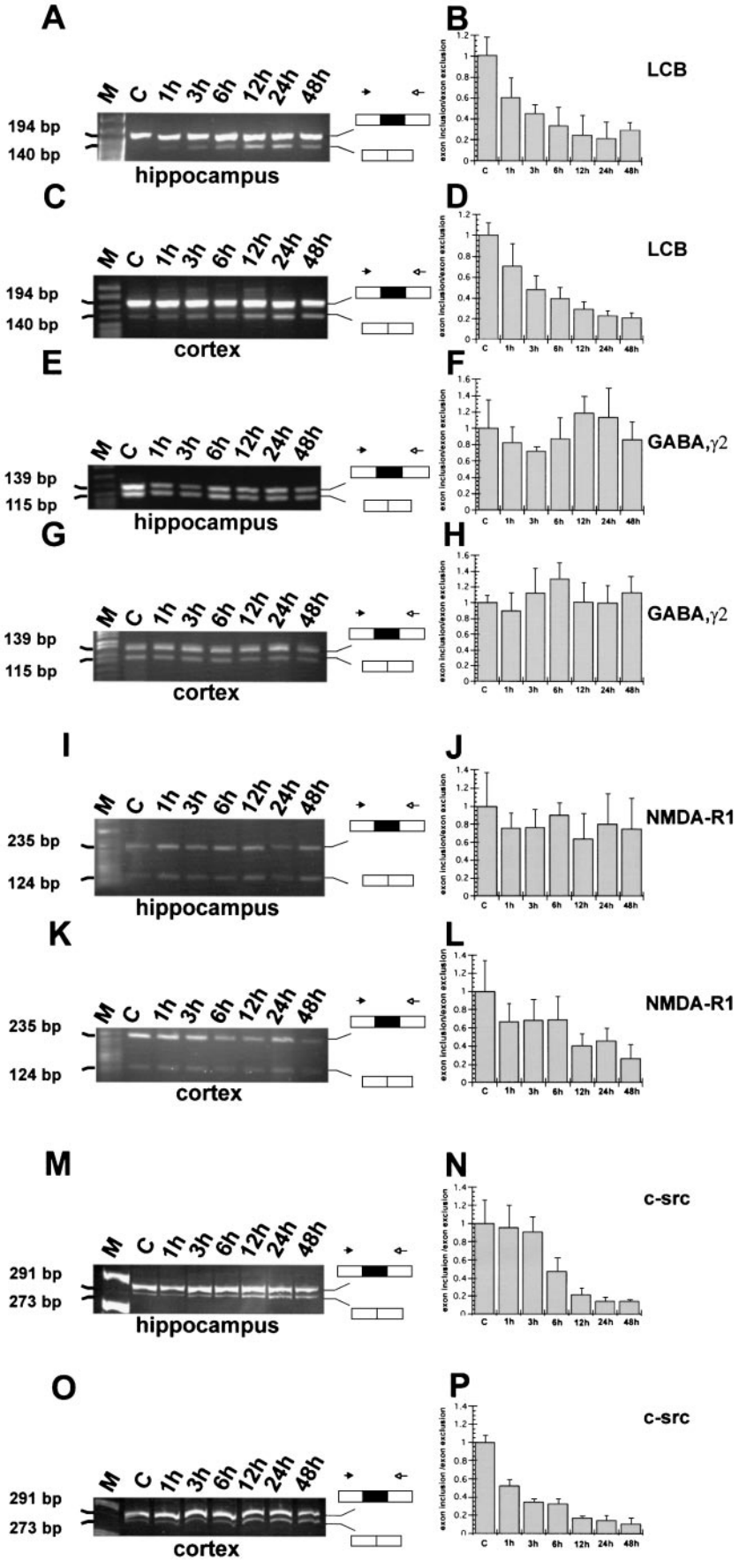

FIG. 10. The effect of pilocarpine on other alternatively spliced genes. RT-PCR analysis of RNA from pilocarpine-treated hippocampus and cortex. Removal of tissue after pilocarpine injection was at the time indicated. C: untreated control. A statistical evaluation for three experiments is given on the right. Standard deviations are indicated. Location of primers and cDNA structure are schematically indicated for each gene. For each reaction, the ratio of exon inclusion to exon exclusion was arbitrarily set to one in the control tissue. (A) RT-PCR for clathrin light chain B in hippocampus, alternative exon EN. (B) Statistical evaluation for (A). (C) RT-PCR for clathrin light chain B in cortex. (D) Statistical evaluation for (C). (E) RT-PCR for $GABA_A(\gamma 2)$-receptor, in hippocampus, alternative exon 24. (F) Statistical evaluation for (E). (G) RT-PCR for $GABA_A(\gamma 2)$-receptor in cortex, alternative exon 24. (H) Statistical evaluation for (G). (I) RT-PCR for NMDARI in hippocampus, alternative exon 21. (J) Statistical evaluation for (I). (K) RT-PCR for NMDARI in cortex, alternative exon 21. (L) Statistical evaluation for (K). (M) RT-PCR for c-*src* in hippocampus, alternative exon NI. (N) Statistical evaluation for (M). (O) RT-PCR for c-*src* in cortex, alternative exon NI. (P) Statistical evaluation for (O).

alternative splicing patterns are changed in the brain after pilocarpine treatment.

In mammalian systems, specific targets of splice factors are not known. In contrast, in *Drosophila*, genetic analysis has enabled identification of target genes of splice factors that often play an important role in development, e.g. in suppression of the white apricot phenotype or in sex determination. Sexual differentiation in *Drosophila* is controlled by a cascade of alternative splicing decisions, in which the two SR-related proteins tra and tra-2 play a crucial role (Baker, 1989). Sex determination in mammals and flies is completely different, but the biochemical characteristics of a major component, tra-2, have been conserved (Dauwalder *et al*., 1996). It has therefore been proposed that a 'regulatory module' consisting of several components of the *Drosophila* sex-determination pathway has been conserved and is used for other purposes in humans (Dauwalder *et al*., 1996). Our finding, that the alternative splicing of the tra2-beta gene is changed after pilocarpine induction, and the observation of other changes in alternative splicing support this view. From the experiments presented here we cannot conclude that changes in alternative splicing, e.g. clathrin light chain B, are influenced directly by the change in htra2-beta isoforms. It is possible that parallel pathways exist that eventually affect alternative splicing. However, in mammals, htra2-beta1 probably has specific target genes, as is the case in *Drosophila*. Therefore, a change in alternative splicing patterns of htra2 will probably coordinate changes in alternative splicing for a subset of genes in response to neuronal stimulation.

Neurons are different from other cell types in that they cannot divide. Therefore, for these cells it will be more difficult to change the DNA methylation pattern or the histone acetylation pattern in response to external stimuli. As these mechanisms regulate transcription by influencing binding of transcription factors (Tate & Bird, 1993; Siegfried & Cedar, 1997) and chromatin structure (Jeppesen, 1997), respectively, alternative splicing might be an attractive alternative for the regulation of gene activity, e.g. by producing nonfunctional protein isoforms. In fact, it has been estimated that $\approx$ 5% of on/off regulation in *Drosophila* is caused by alternative splicing (Bingham *et al*., 1988). In conclusion, a change of alternative splicing in response to neuronal activity might be an important mechanism to convey molecular plasticity in the brain.

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Abbreviations

DAPI, 4′,6-diamidino-2-phenylindol; EGFP, enhanced green fluorescent protein; ELAV, embryonic lethal abnormal vision; GST, glutathione S-transferase; HA, haemagglutinine; hnRNP, protein associated with hnRNA; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulphate; SR, serine arginine rich (domain).

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