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# CELLULAR LOCALIZATION OF METABOTROPIC GLUTAMATE RECEPTORS IN CORTICAL TUBERS AND SUBEPENDYMAL GIANT CELL TUMORS OF TUBEROUS SCLEROSIS COMPLEX

**K. BOER,[a] D. TROOST,[a] W. TIMMERMANS,[a] J. A. GORTER,[f] W. G. M. SPLIET,[b] M. NELLIST,[d] F. JANSEN[c] AND E. ARONICA[a,e]***

[a]*Department of (Neuro)Pathology, Academic Medical Center, University of Amsterdam, The Netherlands*

[b]*Department of Pathology, University Medical Center Utrecht, The Netherlands*

[c]*Department of Child Neurology/Rudolf Magnus Institute for Neurosciences, University Medical Center Utrecht, The Netherlands*

[d]*Department of Clinical Genetics, Erasmus Medical Center, The Netherlands*

[e]*Stichting Epilepsie Instellingen Nederland, Heemstede, The Netherlands*

[f]*Swammerdam Institute for Life Sciences, University of Amsterdam, The Netherlands*

**Abstract—Tuberous sclerosis complex (TSC) is an autosomal dominant disorder associated with cortical malformations (cortical tubers) and the development of glial tumors (subependymal giant-cell tumors, SGCTs). Expression of metabotropic glutamate receptor (mGluR) subtypes is developmentally regulated and several studies suggest an involvement of mGluR-mediated glutamate signaling in the regulation of proliferation and survival of neural stem-progenitor cells, as well as in the control of tumor growth. In the present study, we have investigated the expression and cell-specific distribution of group I (mGluR1, mGluR5), group II (mGluR2/3) and group III (mGluR4 and mGluR8) mGluR subtypes in human TSC specimens of both cortical tubers and SGCTs, using immunocytochemistry.**

**Strong group I mGluR immunoreactivity (IR) was observed in the large majority of TSC specimens in dysplastic neurons and in giant cells within cortical tubers, as well as in tumor cells within SGCTs. In particular mGluR5 appeared to be most frequently expressed, whereas mGluR1$\alpha$ was detected in a subpopulation of neurons and giant cells. Cells expressing mGluR1$\alpha$ and mGluR5, demonstrate IR for phospho-S6 ribosomal protein (PS6), which is a marker of the mammalian target of rapamycin (mTOR) pathway activation. Group II and particularly group III mGluR IR was less frequently observed than group I mGluRs in dysplastic neurons and giant cells of tubers and tumor cells of SGCTs. Reactive astrocytes were mainly stained with mGluR5 and mGluR2/3.**

**These findings expand our knowledge concerning the cellular phenotype in cortical tubers and in SGCTs and highlight the role of group I mGluRs as important mediators of glutamate signaling in TSC brain lesions. Individual mGluR subtypes may represent potential pharmacological targets for the treatment of the neurological manifestations associated with TSC brain lesions.** 

**Key words: metabotropic glutamate receptors, astrocytes, dysplastic neurons, giant cells, epilepsy.**

Tuberous sclerosis complex (TSC) is an autosomal dominant, multisystem disorder caused by a mutation of one of the tumor suppression genes, *TSC1* or *TSC2* (European Chromosome 16 Tuberous Sclerosis Consortium, 1993; van Slegtenhorst et al., 1997). CNS involvement is common in TSC and includes three major lesions: cortical tubers, subependymal nodules and subependymal giant cell tumors (SGCTs) (Mizuguchi and Takashima, 2001; DiMario, 2004). The neurological manifestations of TSC are the most disabling and include: developmental delay, neurobehavioral dysfunctions (such as autism) and severe epilepsy (Curatolo et al., 2002; Bolton, 2004). Epilepsy occurs in up to 80% of TSC patients and is often poorly controlled by anti-epileptic drugs (AEDs). In selected patients resection of the epileptogenic zone, which is mainly represented by cortical tubers, is performed (Weiner et al., 2004; Jansen et al., 2007). The cellular mechanism(s) underlying the epileptogenicity of cortical tubers remain largely unknown. Several mechanisms are possibly involved. Recent studies support the role of developmental alterations of the balance between excitation and inhibition in the pathogenesis of focal discharges in cortical tubers (for review see Holmes and Stafstrom, 2007). Selective alterations in ionotropic glutamate and GABA receptor subunit mRNA expression have been observed in the different cellular components of cortical tubers (White et al., 2001). Abnormal glutamate homeostasis, altered synaptic excitation and increased AMPA-receptor-mediated currents, which would favor seizure generation, have been described in mouse models of TSC (Tavazoie et al., 2005; Wang et al., 2007; Zeng et al., 2007).

Metabotropic glutamate receptors (mGluRs) are a family of eight G-protein-linked receptors that regulate a variety of intracellular signaling systems (Pin and Acher, 2002). They have been subdivided into three main groups on the basis of their sequence homology, second messen-

*Correspondence to: E. Aronica, Department of (Neuro)Pathology, Academic Medical Center, University of Amsterdam, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands. Tel: +31-20-5662943; fax: +31-20-5669522.
E-mail address: e.aronica@amc.uva.nl (E. Aronica).

*Abbreviations:* Abs, antibodies; FRAXS, fragile X syndrome; GFAP, glial fibrillary acidic protein; GW, gestational week; IR, immunoreactivity; MAP2, microtubule-associated protein; mGluR, metabotropic glutamate receptor; mTOR, mammalian target of rapamycin; NeuN, neuronal nuclear protein; NF, neurofilament; PS6, phospho-S6 ribosomal protein; SGCT, subependymal giant cell tumor; SVZ, subventricular zone; TSC, tuberous sclerosis complex; VZ, ventricular zone.

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ger systems and pharmacology. Group I includes mGluR1 and mGluR5, which are coupled to phosphoinositide hydrolysis and generally mediate postsynaptic excitatory effects in neurons. Group II (mGluR2 and mGluR3) as well as group III (mGluR4, -6, -7, -8) mGluRs are negatively coupled to adenylyl cyclase and generally mediate presynaptic inhibitory effects on synaptic transmission and neurotransmitter release (Conn, 2003). Both group I (mGluR5) and II (mGluR3) subtypes are expressed in human astrocytes and these glial mGluR subtypes may play a role in regulating the extracellular levels of glutamate (Aronica et al., 2003a). Several studies suggest that mGluR subtypes are significant molecular targets for treatment of epilepsy (Conn, 2003; Meldrum and Rogawski, 2007). In particular activation of group II and III mGluRs have been shown to have anticonvulsant effects, as opposed to the convulsant action of group I mGluRs reported in a variety of experimental models (Meldrum and Rogawski, 2007).

Group I mGlu receptors have been shown to regulate proliferation, differentiation, and survival of neural stem/progenitor cells, suggesting a role for these receptors in brain development and developmental disorders (for review see Catania et al., 2007). Recently, activation of the mammalian target of rapamycin (mTOR) pathway has been reported after stimulation of group I mGluRs, suggesting a link between mGluR activation and the major pathway disrupted in TSC (Hou and Klann, 2004; Banko et al., 2006; Page et al., 2006).

In the present study, immunocytochemistry with antibodies (Abs) specific for mGluR1, mGluR2/3, mGluR4, mGluR5 and mGluR8 was performed in surgical specimens of cortical tubers and SGCTs from TSC patients. Our major aim was to contribute to the definition of the cellular distribution of mGluRs within the two major TSC brain lesions and give better insights into the mechanisms underlying the heterogeneous neurological manifestations observed in TSC patients.

## EXPERIMENTAL PROCEDURES

### Subjects

We examined a total of 13 surgical specimens, eight cortical tubers (male/female: three/five; mean age at surgery: 13.8 years, range: 1–35) and five SGCTs (male/female: three/two; mean age at surgery: 12 years, range: 1–23) from patients undergoing epilepsy surgery or surgery for obstructive hydrocephalus and one autopsy cortical tuber specimen (age 32 years; male). Patient data were obtained from the databases of the departments of Neuropathology of the Academic Medical Center (University of Amsterdam; UVA) in Amsterdam, The Netherlands, the University Medical Center in Utrecht (UMCU), The Netherlands and the Free University Medical Center (VUMC) in Amsterdam, The Netherlands. Informed consent was obtained for the use of brain tissue and for access to medical records for research purposes. Tissue was obtained and used in a manner compliant with the Declaration of Helsinki.

All patients fulfilled the diagnostic criteria for TSC (Gomez et al., 1999). For the SGCTs we used the revised WHO classification of tumors of the CNS (Louis et al., 2007). *TSC1* mutations were detected in five patients (four cortical tubers and one SGCT) and *TSC2* mutations in nine patients (five cortical tubers and four SGCTs). The patients with cortical tubers who underwent epilepsy surgery had, predominantly, medically intractable complex partial seizures. In five patients a significant amount of perituberal tissue (normal-appearing cortex/white matter adjacent to the lesion) was resected as well. In addition, normal-appearing control cortex/white matter was obtained at autopsy from one TSC patient. This material represents good control tissue, since it is exposed to the same seizure activity, drugs and fixation protocol, and age and gender are the same. Normal-appearing control cortex/white matter from the temporal region was obtained at autopsy from five young adult control patients (male/female: three/two; mean age 29; range 14–35), without a history of seizures or other neurological diseases. All autopsies were performed within 12 h of death. We also included control material from 9 and 10 gestational weeks (GW) human brain.

### Tissue preparation

Tissue was fixed in 10% buffered formalin and embedded in paraffin. Paraffin-embedded tissue was sectioned at 6 $\mu$m, mounted on organosilane-coated slides (Sigma, St. Louis, MO, USA) and used for immunocytochemical staining as described below.

### Antibody characterization

Abs specific for glial fibrillary acidic protein (GFAP; polyclonal rabbit, DAKO, Glostrup, Denmark; 1:4000; monoclonal mouse; DAKO; 1:50), vimentin (mouse clone V9; DAKO; 1:1000), neuronal nuclear protein (NeuN; mouse clone MAB377; Chemicon, Temecula, CA, USA; 1:2000), synaptophysin (mouse clone Sy38; DAKO; 1:200), neurofilament (NF, SMI311; Sternberger Monoclonals, Lutherville, MD, USA; 1:1000), microtubule-associated protein (MAP2; mouse clone HM2; Sigma 1:100), anti-human leukocyte antigen (HLA)-DP, DQ, DR (mouse clone CR3/43; DAKO; 1:100), CD68 (mouse monoclonal, clone PG-M1; DAKO; 1:200) and phospho-S6 ribosomal protein (Ser235/236; PS6, rabbit polyclonal, Cell Signaling Technology, Beverly, MA, USA; 1:50) were used in the routine immunocytochemical analysis of TSC specimens to document the presence of a heterogeneous population of cells and the activation of the mTOR pathway (Baybis et al., 2004).

For the detection of group I mGluRs, we used Abs specific for the mGluR subtypes 1$\alpha$ (polyclonal rabbit, Chemicon; 1:100) and 5 (polyclonal rabbit, Upstate Biotechnology, Lake Placid, NY, USA; 1:100). For the detection of group II mGluRs we used the mGluR2/3 antibody (polyclonal rabbit, Chemicon; 1:50); it recognizes both mGluR2 and mGluR3. For the detection of group III mGluRs, we used Abs specific for the mGluR4 (polyclonal rabbit, Upstate Biotechnology, 1:70) (Bradley et al., 1996; Tang et al., 2001) and mGluR8 (polyclonal guinea pig, 1:50; a generous gift from Dr. R. Shigemoto (Shigemoto et al., 1997)). Characterization of these Abs in human brain tissue has been documented previously (Aronica et al., 2001a,c, 2003b; Geurts et al., 2003, 2005).

Since the availability of frozen material from cortical tubers and SGCTs and age-matched controls is very limited, immunoblot with complete statistical comparison between controls and TSC lesions could not be performed.

### Immunocytochemistry

Immunocytochemistry was carried out as previously described (Aronica et al., 2001b, 2003b). Single-label immunocytochemistry was performed with the Powervision peroxidase system (Immunologic, Duiven, The Netherlands) for the monoclonal mouse and polyclonal rabbit Abs and with the biotin-labeled goat–anti-guinea-pig antibody (Chemicon, 1:100) for the mGluR8 antibody. 3,3-Diaminobenzidine was used as chromogen. Sections were counterstained with hematoxylin.

For double-labeling studies, sections, after incubation with the primary Abs (mGluR1$\alpha$, mGluR2/3, mGluR5, mGluR4 or mGluR8