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PICK1 facilitates lasting reduction in GluA2 concentration in the hippocampus during chronic epilepsy

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ABSTRACT

Overstimulation of glutamate receptors resulting in excessive intracellular calcium concentrations is a major cause of neuronal cell death in epilepsy. The main source of increased calcium concentration during this excitotoxicity is an influx through NMDA subtype of glutamate receptors. The GluR2 (GluA2) hypothesis states that following a neurological insult such as an epileptic seizure, the AMPA receptor subunit GluR2 protein is downregulated. This increases the likelihood of the formation of GluR2-lacking, calcium-permeable AMPA receptor which might further enhance the toxicity of the neurotransmitter, glutamate. The cytosolic protein, PICK1, facilitates the removal of GluA2 subunits from the synaptic plasma membrane. High calcium concentrations may cause PICK1 to bind to the GluA2 subunit of calcium-impermeable AMPARs, leading to an increased internalization of these receptor subunits and a relative increase of GluA2-lacking, calcium-permeable AMPARs. This further escalates the cytosolic calcium concentration. In order to test this hypothesis, we have used kainic acid to induce epilepsy in rats. Using semi-quantitative western blotting combined with univariate and multivariate statistical analyses, we found that both GluA2 and PICK1 were down-regulated in kainate-treated rats for as long as eight weeks after induction of epilepsy. An interesting finding was that statistical analysis indicates that the functional role of PICK1 in our material is to increase GluA2 concentrations in the cells. The observed reduction in PICK1 concentration may thus be an independent contributor to the observed GluA2 reduction. This reduction may possibly be an adaptive mechanism, serving to prevent further loss of GluA2 from the synapses.

1. Introduction

Epilepsy is a common chronic neurological disorder with a prevalence of 1–2% (Kobau et al., 2008). The disease is characterized by transient and recurrent seizures. The underlying cause of the seizures is abnormal neuronal activity in the brain, either excessive or synchronous (Fisher et al., 2005). Out of the world's population of epilepsy patients, about 80% of them live in developing countries (Meyer et al., 2010). Possible causes of the dysfunction might be genetic abnormalities, brain damage at birth, infections, stroke and tumours. In medial temporal lobe epilepsy (MTLE), the abnormal neuronal activity commences from temporal lobe structures like the hippocampus. There is a genetic predisposition to the occurrence of MTLE (Engel, 1996b). MTLE is the commonest form of epilepsy (Yang et al., 2006). It is markedly associated with neuronal cell loss and gliosis in the hippocampus. According to Engel (1996b), these seizures often recur throughout life. Among MTLE patients, about 40% of the cases become medically intractable (de Lanerolle and Lee, 2005). People with medically intractable epilepsy have a high mortality rate (Mohanraj et al., 2006). Medical treatment of MTLE is often difficult but surgical treatment has been effective in curing about 70–80% of such cases (Engel, 1996a).

One reason for the high portion of patients that are unable to control their epilepsy through medication might be that we still know too little about disease-related changes in surviving neurons in the chronic phase of epilepsy. Do these neurons undergo molecular changes that contribute to maintain the disease? Or, conversely, do the neurons adapt to the disease, with plastic changes that, to some degree, protect against further propagation of pathology? It is necessary to answer these questions on cellular and molecular levels in order to design successful therapeutic interventions in the future.

Glutamate is the most common excitatory neurotransmitter in the human brain. It is present in a majority of the excitatory synapses. According to Danbolt (2001), "glutamate uptake appears to be modulated on virtually all possible levels, i.e. DNA transcription, mRNA

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splicing and degradation, protein synthesis and targeting, and actual amino acid transport activity and associated ion channel activities". In neurons, glutamate is present throughout the cytosol but is concentrated and stored in presynaptic vesicles (Oltedal et al., 2008). When an action potential reaches the nerve terminal, these vesicles are fused to the synaptic membrane to release glutamate into the synaptic cleft by exocytosis. The transmitter then diffuses to postsynaptic receptors.

Complex molecular mechanisms contribute to the regulation and modification of the physiological effect of glutamate activation of synaptic receptors (Auger and Attwell, 2000; Danbolt, 2001; Smith, 2000). In spite of these complex regulatory mechanisms, glutamate has still been found to be toxic to neurons and contribute to cell damage in a variety of central nervous system (CNS) diseases, especially when neurons are subjected to ischemia and glucose deprivation (Lee et al., 2000). In many CNS diseases, increased glutamate stimulation might reach pathological levels due to malfunctioning regulatory mechanisms, resulting in cell damage or death by excitotoxicity.

During seizures, there is an increase in extracellular glutamate concentrations (Delorenzo et al., 2005). This leads to overstimulation of postsynaptic glutamate receptors. Excitotoxicity occurs when glutamate receptor stimulation becomes excessive. According to Olney and de Gubareff (1978), excitotoxicity refers to neuronal death that is instigated by excitatory amino acid neurotransmitters such as glutamate. This phenomenon occurs because excessive neuronal excitation leads to pathological energy deficits in the affected neurons in diseases such as epilepsy. In some cases, excitotoxicity may stem from substances produced within the body (endogenous excitotoxins), or from substances that are ingested into the body (exogenous excitotoxins). Excitotoxicity is thought to contribute to neurodegeneration in a wide range of neurological insults such as ischemia, trauma, hypoglycaemia and epileptic seizures. It is, also, suspected that excessive glutamate receptor activation participates in the pathological processes of chronic neurodegenerative disorders such as Alzheimer's disease, Huntington's chorea, AIDS encephalopathy and amyotrophic lateral sclerosis (Kim et al., 2002).

The main route through which glutamate induces cell death is by increasing intracellular [Ca^{2+}] through various ways: activation of Ca^{2+}-permeable NMDA receptors, activation of Ca^{2+}-permeable AMPA receptors, opening of voltage-gated Ca^{2+}-channels following membrane depolarization induced by activation of AMPA receptors, and via activation of metabotropic glutamate receptors linked to phosphoinositide hydrolysis, which releases Ca^{2+} from intracellular stores (Pellegrini-Giampietro et al., 1997). Increased intracellular [Ca^{2+}] leads to increased generation of free radicals and activation of proteases, phospholipases and endonucleases. Furthermore, transcriptional activation of specific cell-death program is activated. Through these processes, cell structures are damaged and if cell damage is sufficient, the cell might die.

Calcium influx into the cell is mainly related to NMDA receptors (Pellegrini-Giampietro et al., 1997). However, GluA2-lacking AMPA receptors are, also, involved in the calcium influx. In normal neurons, more than 95% of AMPA receptors contain the subunit GluA2 hence most AMPA receptors are calcium impermeable. However, according to the GluA2 hypothesis (Pellegrini-Giampietro et al., 1997), these changes after a neurological disease. It states that following a neurological insult, the AMPA-receptor subunit GluA2 protein is down-regulated. This increases the likelihood of the formation of GluA2-lacking, calcium-permeable AMPA receptors which might enhance the toxicity of endogenous and exogenous glutamate.

Protein interacting with C kinase 1, PICK1, is involved in both the endocytosis and exocytosis of GluA2-containing AMPARs (Hanley, 2008; Lu et al., 2014). PICK1 is expressed in many tissues with the highest expression found in the brain. In neurons, PICK1 has been found in synapses and at the perinuclear region (Haglerod et al., 2009; Xu and Xia, 2007).

While most studies on GluA2 changes in epilepsy focused on short-term changes and last only for days or up to two weeks (Borbely et al., 2009; Porter et al., 2006; Sommer et al., 2001), our study focused on changes evident after eight weeks, which may be more relevant for detecting lasting changes during epilepsy. This study examined a kainate model of MTLE with a focus on possible changes that may occur in the concentration of the AMPA receptor subunit GluA2 during chronic epilepsy. Our hypothesis was that PICK1 contributes to a reduction in GluA2 in the hippocampus during epilepsy.

2. Materials and methods

2.1. Animals

Protein homogenates were obtained from adult male rats (Wistar, weighing approximately 250 g, from Møllegaard Breeding Centre, Copenhagen, Denmark). For the 8 weeks experiments, a total of 19 rats were used (8 in the epilepsy group, and 11 controls), while in the 2 weeks experiments, a total of 13 rats were used (6 in the epilepsy group, and 7 controls). The procedure by which the animals were treated was in accordance with the European Convention (ETS 123 of 1986), and the Norwegian National Animal Research Authority approved all protocols.

2.2. Inducing epilepsy in animals

Epilepsy was induced in animals by intraperitoneal injection of kainate, 10 mg/kg in buffered saline in a volume of 10 ml/kg. Control rats received only saline.

To ensure that the kainate-injected rats underwent status epilepticus, the rats were placed in separate cages after injection and monitored for at least four hours using a DVD recorder device with a CCTV camera. The seizure activity was assessed using the five-stage Racine model (Racine, 1972). Status epilepticus was defined as "continuous limbic seizures scored as class 4 or 5 on the Racine model and lasting for more than 90 min" (Hammer et al., 2008). Of the kainate injected rats, only those that underwent status epilepticus were used in the study.

2.3. Tissue preparation

Two weeks following the injections, 6 rats that developed epilepsy and 7 controls were sacrificed by decapitation. This corresponds to the latent phase of epilepsy. Eight weeks following the injections, eight remaining rats with epilepsy (chronic epilepsy group) and 11 control rats were sacrificed. The epileptic rats had been video-monitored 8–12 h/day and had all exhibited two or more spontaneous seizures. The brains were immediately removed from the skull and the left hippocampi were isolated and snap-frozen en bloc in liquid nitrogen, and the tissue was stored at −80 °C. They were later sonicated on ice in homogenisation buffer [1% SDS, 10 mM sodium phosphate buffer, pH 7.4, 150 mM NaCl, 1 mM phenylmethylsulphonyl fluoride (PMSF), 10 mM ethylenediamine tetraacetic acid (EDTA)] in order to disrupt cell and organelle membranes. There was no further fractionation.

Protein concentrations were determined with a Bicinchoninic Acid (BCA) protein assay kit and measured on a microplate reader at 570 nM (Tecan Sunrise). Homogenized rat hippocampus was used to make a standard curve, which was used to standardize the measures of the quantified proteins. Thus, the observed number from quantifying the signals, is equivalent to the number of micrograms of protein from the homogenized hippocampus standard protein that will give an equally strong signal.

For GluA2- and β-tubulin immunoblots, 10 μg of protein per well was used, while 5 μg was used for synaptophysin and PICK1 immunoblots. After separation of the proteins by SDS-PAGE gel electrophoresis, the proteins were transferred to a PVDF membrane and then blocked with TBS-T (20 mM Tris pH 7.6, 137 mM NaCl, 0.05% Tween 20) with 5% milk powder solution before washing and subsequent overnight incubation at room temperature with TBS-T containing the