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CHOLINERGIC REGULATION OF STRIATAL NOVA mRNAs

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Abstract—Alternative splicing is an important mechanism for expanding proteome diversity from a limited number of genes, especially in higher vertebrates. Brain-specific splicing factors play an important role in establishing specific patterns of alternative splicing in the brain and thereby contribute to its complex architecture and function. Nova proteins are splicing factors that are expressed specifically in the central nervous system, where they regulate a large number of pre-mRNAs encoding synaptic proteins that are important for the balance of neuronal excitation and inhibition. Since this balance is interrupted in epileptic seizures, we explored whether LiCl/pilocarpine- or kainate-induced epileptic seizures would induce changes in the levels of Nova mRNAs in the rat brain. We found that the muscarinic agonist, pilocarpine, but not the glutamatergic agonist, kainate, induced a significant downregulation of Nova2 mRNA and upregulation of all three Nova1 mRNA isoforms in the striatum. Treatment with the muscarinic antagonist, scopolamine, at the onset of pilocarpine-induced seizures inhibited the seizures and the changes in Nova mRNA levels. Therefore it seems likely that pilocarpine stimulation of muscarinic acetylcholine receptors was a prerequisite for the observed changes, while the contribution of other striatal neurotransmitter systems activated by seizures could not be excluded. We propose that the LiCl/pilocarpine seizure model could serve as a valuable tool for studying mechanisms of Nova-regulated alternative splicing in rat striatum. **© 2010 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: Nova, alternative splicing, pilocarpine, kainate, seizures, striatum.

Nova proteins are evolutionary conserved neuron-specific splicing factors that are expressed specifically in the central nervous system (Buckanovich et al., 1993, 1996; Yang et al., 1998; Jelen et al., 2007). Two members of the Nova family have been cloned, termed Nova1 and Nova2. Studies showed that Nova proteins regulate alternative splicing and alternative polyadenylation of a large number of genes that function primarily at synapses (Jensen et al., 2000; Ule et al., 2003, 2005, 2006; Licatalosi et al., 2008). Nova1 and Nova2 have distinct and largely reciprocal patterns of expression in the mouse brain (Buckanovich et al., 1993, 1996; Yang et al., 1998). Nova2 shows diffuse and regionally variable expression in the brain, with the highest levels found in the cortex and thalamus. On the other hand, Nova1 has been found to be largely absent from these two brain structures and is expressed primarily in the midbrain and brain stem.

Three mRNA isoforms of Nova1 have been described, which differ in the presence of the internal cassette exon E4 and/or the 3′ terminal alternative exon. Isoform Nova1S lacks the E4 exon, while Nova1L contains it. Nova1T is supposedly a non-functional isoform and it harbours an alternative 3′ terminal exon. It has also been reported that Nova2 and Nova1 can regulate alternative splicing of Nova1 pre-mRNA (Dredge et al., 2005). While the alternative exon E4 codes for a protein domain that can be phosphorylated *in vivo*, the possible physiological role of its inclusion is not yet known.

It has been suggested that by regulating RNAs encoding synaptic proteins, Nova participates in the balance of neuronal excitation and inhibition and is necessary for proper synaptic development and function (Ule et al., 2005; Ruggiu et al., 2009). In the paraneoplastic neurologic disease POMA (paraneoplastic opsoclonus-myoclonus ataxia), antibody targeting of Nova leads to a failure of brain stem and spinal motor inhibition (Buckanovich et al., 1993). Similarly, Nova knockout mice appear normal at birth, but die soon after due to the failure of motor inhibition (Jensen et al., 2000). Finally, a study showed that Nova2 is necessary for the induction of slow inhibitory postsynaptic currents in hippocampal neurons (Huang et al., 2005). This finding suggested the interesting possibility that one of the physiological roles of Nova is to enable neurons to adapt their synaptic inhibition in response to neuronal activity. We thus speculated that epileptic seizures that perturb the balance between synaptic excitation and inhibition (Scharfman, 2007) could serve as a model to study the regulation of Nova expression in the brain. Pilocarpine or kainate can induce acute epileptic seizures and excitotoxic injury in rats followed by the development of spontaneous recurrent seizures reminiscent of temporal lobe epilepsy in humans (Leite et al., 2002). Although pilocarpine and kainate both cause similar epileptic seizures and pathological changes in the brain, they differ in their initial mode of action. Pilocarpine is a selective agonist of muscarinic acetylcholine receptors (mAChR) that provokes seizures indirectly by overstimulation of cortical glutamatergic neurons (Turski et al., 1983). Once triggered by the excessive cholinergic activity, seizures are propagated by the sustained cortical glutamatergic overactivity. In this model, LiCl pre-treatment is given in order to decrease the threshold for the onset of seizures triggered by pilocarpine. In

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Abbreviations: ACh, acetylcholine; GluR, glutamate receptor; mAChR, muscarinic acetylcholine receptor; POMA, paraneoplastic opsoclonus-myoclonus ataxia; ROD, relative optical density; ROI, regions of interest; SCO, scopolamine hydrobromide.

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contrast, kainate is a subtype selective agonist of glutamate receptors (GluR), and thus overstimulates the cortex directly by mimicking cortical glutamatergic overactivity (Ben-Ari and Cossart, 2000).

In order to shed new light on the function of Nova in the brain, we investigated whether epileptic seizures induced with LiCl/pilocarpine or kainate would affect the levels of Nova mRNAs in the rat brain. In this study, we report that seizures induced with LiCl/pilocarpine, but not by kainate, induced significant changes in the levels of Nova1 and Nova2 mRNAs in rat striatum that could be inhibited by the muscarinic antagonist scopolamine, implying a cholinergic regulation of striatal Nova expression.

EXPERIMENTAL PROCEDURES

Animals

We used 63 adult male Wistar rats (220–300 g). They were housed in groups of four in polycarbonate cages under standard housing conditions (50% humidity) and were maintained on a 12 h light–dark cycle (light on: 07.00 h–19.00 h) in a temperature-controlled colony room at 22–24 °C with free access to rodent pellets and tap water. They were sacrificed by decapitation under CO_2 anaesthesia. We made all efforts to minimize the number of animals used and their suffering. Rats were handled according to the European Communities Council Directive of 24th of November 1986 (86/609/EEC) and National Veterinary Institute Guide for the Care and Use of Laboratory Animals.

Drugs

The following drugs were used: lithium chloride (LiCl), pilocarpine hydrochloride (PI), scopolamine hydrobromide (SCO) and kainic acid (KA), all purchased from Sigma, St. Louis, MO, USA. All drugs were dissolved in 0.9% saline (SAL) and were administered i.p. (LiCl, SCO) or s.c. (PI, KA) in the dorsal neck region in a volume of 1 ml/kg. Drug solutions were freshly prepared no more than 30 min before the injections.

Treatment protocols

We performed three experiments in two animal models of epilepsy. The first two experiments consisted of a time-course study in which groups of four animals were sacrificed at different time points (30 min, 4 h, 8 h, and 12 h) after the beginning of grade 4-5 seizures according to the Racine scale (Racine, 1972) induced by KA or LiCl/PI. In KA experiment, animals were treated with 15 mg/kg of kainic acid. KA-induced grade 4 seizures (rearing with forelimb clonus) started in about 60 min. A control group of four animals received an injection of saline and were killed after 9 h. In the LiCl/PI experiment, animals were pretreated with LiCl (3 mEq/kg) 24 h before the injection of PI (30 mg/kg). Grade 4 seizures started about 20 min after the injection of PI. Control animals in this experiment were pretreated with LiCl (3 mEq/kg) 24 h before the injection of SAL and were sacrificed 8 and 20 min after the injection of SAL. After injection of the seizure-inducing agent (or control injection of SAL) the animals were housed in individual cages.

In the third experiment, animals were divided into seven groups. The first group ($n=4$) received SAL injection (1 ml/kg) and served as the control group. The second group ($n=4$) received LiCl (3 mEq/kg) 24 h before SAL (1 ml/kg) and also served as a control group. The third group ($n=3$) was pretreated with LiCl (3 mEq/kg) 24 h before the injection of PI (30 mg/kg). The animals from this group developed epileptic seizures of magnitude 4-5 after approximately 20 min and were sacrificed 12 h after the beginning of grade 4 seizures. Animals from control groups were killed 12 h and 20 min after the injection of SAL. The fourth group ($n=3$) was pretreated with LiCl (3 mEq/kg) 24 h before the injection of PI (30 mg/kg). When animals from this group started to display first signs of grade 4 seizures, they received an injection of SCO (2 mg/kg). Group five ($n=3$) received the same treatment as group four, except for receiving a lower dose of SCO (1 mg/kg). Animals from these two groups were observed for an additional 1 h period. Group six ($n=3$) received SCO (2 mg/kg) 24 h after the pre-treatment with LiCl (3 mEq/kg). Animals from groups four, five and six were killed 12 h after the injection of SCO. The seventh group ($n=4$) received the injection of KA (15 mg/kg) and developed grade 4 seizures which started in about 60 min. During the remaining experimental period, all animals were housed individually, but were not behaviourally observed. Animals from the seventh group were killed 12 h after the beginning of seizures.

Preparation of brain cryo-sections

Animals in all experimental groups were sacrificed by decapitation under CO_2 anaesthesia. Brains were rapidly removed and quickly frozen on dry-ice powder, wrapped in Parafilm to prevent desiccation, and stored at −80 °C. Before cutting, the brains were allowed to equilibrate to the temperature of the cryostat chamber (−20 °C). Coronal sections (10 μm) were cut at three evenly spaced rostro–caudal levels through the striatum (rostrally to the anterior commissure), rostral and dorsal part of hippocampus, respectively. Each section was thaw-mounted onto an RNA-se free glass slide coated with 0.01% solution of (poly)L-lysine in dimethylpyrocarbonat. The sections were vacuum-packed together with a small amount of silica gel and stored at −80 °C until further processing for *in situ* hybridization or immunohistochemistry.

Immunohistochemistry

Immunohistochemistry (IHC) was performed using avidin–biotin peroxidase (ABC) substrate. Briefly, sections were fixed in 100% methanol for 5 min and then in 1% H_2O_2 made in 100% methanol for 15 min and rinsed with potassium phosphate buffered saline (KPBS) (50 mM; pH 7.2). Sections were then incubated in 10 mM citrate buffer (pH=6, temp. 100 °C) for 10 min and rinsed with KPBS. They were covered for 30 min by KPBS with 4.0% Normal Horse/Donkey Serum (NS) and 1.0% Bovine Serum Albumin (BSA) followed by an incubation with Nova1 antibody (Abnova, polyclonal antibody, H00004857-A01) made in mouse diluted 1:1000 in KPBS with 1.0% NS and 1.0% BSA or Nova2 (Santa Cruz, C-16; sc-10546) made in goat diluted 1:1000 in KPBS with 1.0% NS and 1.0% BSA for 2.5 days at 4 °C. After rinses in KPBS, biotinylated secondary antibodies (anti-mouse made in horse or anti-goat made in donkey; Vector Laboratories, Inc. Burlingame, CA, USA) were applied at 1:1000 for 1 h. Rinses in KPBS and ABC incubation followed for 1 h (ABC elite standard kit, Vector, Laboratories, Burlingame, CA, USA). The immunoreactive product was visualized by 3,3′-diaminobenzidine (DAB, Aldrich Chemicals, Milwaukee, WI, USA). Processed sections were coverslipped with DePeX (SERVA Electrophoresis GmbH, Heidelberg, Germany) and analyzed under light microscope (Olympus IX81, Olympus Optical Co., Tokyo, Japan) with an attached digital camera (Olympus DP71).

Oligonucleotide probes

For *in situ* hybridization we used oligodeoxyribonucleotide (antisense) probes (Operon, Ebersberg, Germany) that were 45 bases long. Sequences of the probes were (5′ → 3′):

Nova1 (XM_001075031): CCAGCAGCATAACTAGGTATGAGGACCTTTAGAAAGTACTGGCCG,

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