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# Interneurons secrete prosaposin, a neurotrophic factor, to attenuate kainic acid-induced neurotoxicity

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ABSTRACT

Prosaposin (PS) is a secretory neurotrophic factor, as well as a regulator of lysosomal enzymes. We previously reported the up-regulation of PS and the possibility of its axonal transport by GABAergic interneurons after exocitotoxicity induced by kainic acid (KA), a glutamate analog. In the present study, we performed double immunostaining with PS and three calcium binding protein markers: parvalbumin (PV), calbindin, and calretinin, for the subpopulation of GABAergic interneurons, and clarified that the increased PS around the hippocampal pyramidal neurons after KA injection existed mainly in the axons of PV positive interneurons. Electron microscopy revealed PS containing vesicles in the PV positive axon. Double immunostaining with PS and secretogranin or synapsin suggested that PS is secreted with secretogranin from synapses. Based on the results from in situ hybridization with two alternative splicing forms of PS mRNA, the increase of PS in the interneurons was due to the increase of PS + 0 (mRNA without 9-base insertion) as in the choroid plexus, but not PS + 9 (mRNA with 9-base insertion). These results were similar to those from the choroid plexus, which secretes an intact form PS + 0 to the cerebrospinal fluid. Neurons, especially PV positive GABAergic interneurons, produce and secrete the intact form of PS around hippocampal pyramidal neurons to protect them against KA neurotoxicity.



## 1. Introduction

Secretory neurotrophic factors including brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin 3 or 4/5 (NT-3 or NT-4/5) promote neuronal survival. Unlike other growth factors, which are secreted via a constitutive pathway, BDNF is sorted into a regulated pathway with sortilin in response to neuronal activity (Lou et al., 2005; Evans et al., 2011).

Prosaposin (PS), a neurotrophic factor (Sano et al., 1994; Kotani et al., 1996a, 1996b; Tsuboi et al., 1998; Gao et al., 2013a, 2016), is also secreted by a mechanism for regulated secretion with sortilin (Yuan and Morales, 2011). Previously, we have reported the possible PS secretion from the GABAergic interneurons via tau-positive axons around the hippocampal pyramidal neurons after kainic acid injection (Nabeka et al., 2014). PS is originally reported to be the precursor protein of four small lysosomal glycoproteins, saposins A, B, C, and D (Sano et al., 1988; O'Brien et al., 1988; O'Brien and Kishimoto, 1991; Kishimoto et al., 1992). Both saposins and PS are widely expressed in various tissues, although the brain, skeletal muscle and heart cells predominantly contain unprocessed PS rather than saposins (Sano et al., 1989, 1992; Kondoh et al., 1991, 1993; Hosoda et al., 2007; Terashita et al., 2007). In addition, unprocessed PS is found in various secretory fluids, such as seminal plasma, bile, pancreatic juice, human breast milk and cerebrospinal fluid (Hineno et al., 1991; Hiraiwa et al., 1992), and PS mRNA is strongly expressed in the choroid plexus (Saito et al., 2014).

Kainic acid (KA), a glutamate analogue, is a powerful neurotoxic agent (Olney and de Gubareff, 1978) that stimulates excitatory neurotransmitter release (Ferkany et al., 1982). Systemic KA injection induces neuronal degeneration in certain neuronal areas, includ-

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ing the hippocampus (Schwob et al., 1980; Nadler and Cuthbertson, 1980; Nadler et al., 1981; Heggli et al., 1981; Lothman and Collins, 1981; Nabeka et al., 2015). Hippocampal CA3 neurons are selectively vulnerable to KA, due to high levels of KA receptors in this region (Malva et al., 1998). KA can bind to the AMPA/KA receptors, and activation of its receptor has been shown to elicit a number of cellular events, including the increase in intracellular Ca2+, production of reactive oxygen species, and other biochemical events leading to neuronal cell death (see the review of Wang et al., 2005). This nature of neuronal degeneration caused by systemic KA injection resembles some forms of ischemia (Coyle, 1987) or epilepsy (Lévesque and Avoli, 2013). In recent years, neurodegeneration caused by systemic KA injection has been used to investigate mechanisms of the excitotoxic events (Sun and Chen, 1998; Dawson et al., 1995), and we also used KA to define the mechanisms of neurodegeneration and neuroprotection with PS (Nabeka et al., 2014, 2015).

Although the PS receptors have been defined as GPR37 and GPR37L1, orphan G protein- coupled receptors (Meyer et al., 2013), the movement of intrinsic PS in injured, as well as normal, nervous tissue remains unclear. We have shown that intrinsic PS and its mRNA increase in the facial nerve nucleus after nerve transection (Unuma et al., 2005; Chen et al., 2008) and decrease in the brain of mdx mice (Gao et al., 2013b), indicating its pivotal role in the survival of neurons and muscles.

In the previous study, we have shown the increase of PS immunoreactivity and its mRNA expression in the hippocampal and cortical neurons on day 3 after KA injection, and high PS levels were maintained even after 3 weeks. The increase in PS, but not saposins, suggested that the increase in PS-like immunoreactivity after KA injection was not due to an increase in PS for lysosomal enzymes after neuronal damage, but rather in PS for a neurotrophic factor to improve neuronal survival (Nabeka et al., 2014, 2015). The same study indicated that inhibitory interneurons as well as stimulated hippocampal pyramidal and cortical neurons synthesize PS for neuronal survival, and the choroid plexus is highly activated to synthesize PS, which may prevent neurons from excitotoxic neuronal damage (Nabeka et al., 2014).

In the present study, we aimed to clarify how PS act as a neurotrophic factor against exocitotoxic stimulation after systemic KA injection, for example which type of interneurons synthesize and transport PS, and whether up-regulated PS is secretion type or intracellular type.

## 2. Materials And Methods

### 2.1. Animals

Ten-week-old male Wistar rats (320–350 g, total number = 28) were used in this study. All animals were provided by CLEA-Japan (Kyoto) and housed at a constant temperature (22 °C) under a 12:12-h light: dark cycle and given food and water *ad libitum*. This study was carried out in strict accordance with the recommendations of the Guidelines of the Animal Care Committee of Ehime University. The protocol was approved by the Animal Care Committee of Ehime University (Permit Number: 05A261) All animal experimentation have been conducted in accordance with the Society's Policies on the Use of Animals and Humans in Research. All surgery was performed under chloral hydrate (10 mg/kg) anesthesia, and all efforts were made to minimize suffering.

### 2.2. Antibodies

Anti-PS IgG (0.1 μg/mL) was prepared by Medical and Biological Laboratories (Nakaku, Nagoya, Japan) (Shimokawa et al., 2013). From the amino acid sequence of rat PS (M19936; Collard et al., 1988), a synthetic oligopeptide corresponding to the proteolytic portion of PS (409- PKEPAPPKQPEEPKQSALRAHVPPQK-434), which did not encode saposins, was used to generate a rabbit polyclonal antibody against rat PS. So, this anti-PS IgG reacts with PS but not any of four saposins (Nabeka et al., 2014).

To determine which type of interneurons synthesized and secreted PS, we used immunofluorescence method with the combination of rabbit anti-PS IgG and mouse anti-GAD67 (gulitamic acid decarboxylase 67, 1:500, Millipore, Temecula, CA, USA), mouse anti-PV (parvalbumin, 1:500, Sigma, St. louis, MO, USA), mouse anti-CB, or mouse anti-CR (calbindin, 1:500, calretinin, 1:500, SWANT, Bellinzona, Switzerland) as the primary antibodies (Figs. 1–6). As the second antibodies, we used Alexa Fluor 546 goat anti-rabbit IgG (H + L) (1:1000; Invitrogen, CA, USA) or Alexa

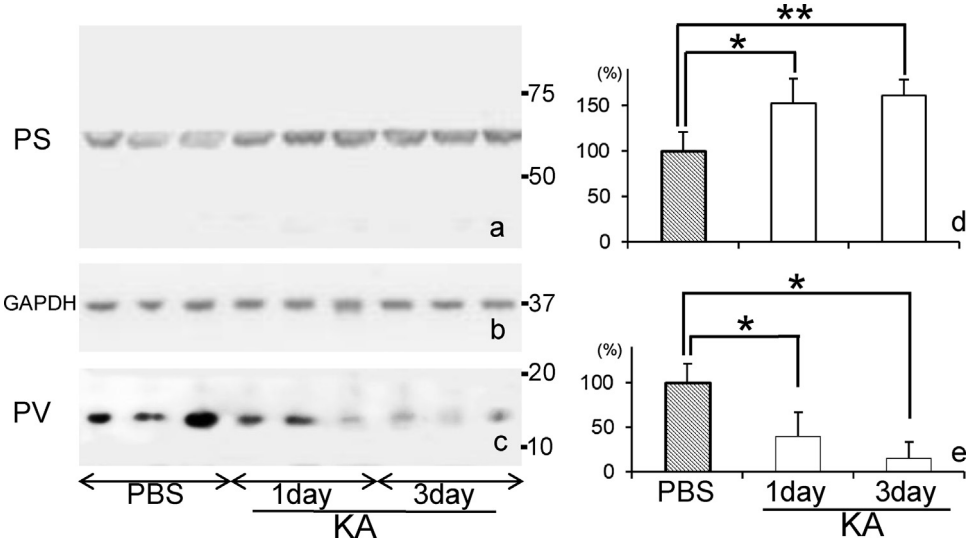


**Fig. 1.** a-d: Crude hippocampal extracts from normal control and from animals 1or 3 days after kainic acid (KA) injection were examined using anti-PS, GAPDH, PV antibodies. When stained with anti-PS (a), the single band observed at ~ 69 kDa, which likely corresponded to PS, significantly increased in intensity after KA treatment (d). When stained with anti-PV (c), the single band observed at ~ 12 kDa, which likely corresponded to PV, significantly decreased in intensity after KA treatment (e). The intensities of the protein bands were quantified using the NIH Image software (b, d), and PS and PV protein levels were expressed as % of vehicle (mean ± SD) normalized relative to GAPDH protein level. (*P < 0.05, **P < 0.01).