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VISUALIZATION OF GLUCOCORTICOID RECEPTOR IN THE BRAIN OF GREEN FLUORESCENT PROTEIN–GLUCOCORTICOID RECEPTOR KNOCKIN MICE

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Abstract—Glucocorticoids exert various neuroendocrinological effects, including stress response, in the central nervous system via glucocorticoid receptor (GR). GRs are transported from the cytoplasm to the nucleus upon ligand binding, and then exert the transcriptional activity. Although it is important for unraveling the actual property of the GR *in vivo*, subcellular dynamics of the GR are still unclear within the brain tissue in which the neuronal circuitry is maintained. To address this issue, we generated green fluorescent protein (GFP)–GR knockin mice, whose GR has been replaced by a GFP–GR fusion protein that is functionally indistinguishable from endogenous GR. In fixed brain sections of the GFP–GR knockin mice, the distribution of the green fluorescence was similar to that of GR immunoreactivity. By subtracting autofluorescence using fluorescent emission fingerprinting method with confocal laser scanning microscope, nuclear localization of GFP–GR was identifiable in the hippocampal CA3 subregion, where subcellular localization of the GR has been unsolved compared with other areas. To examine the subcellular trafficking of GFP–GR *in vivo*, we performed adrenalectomy on the GFP–GR knockin mice. GFP–GR was translocated from the nucleus to the cytoplasm and neurites two days after adrenalectomy. Furthermore, laser scanning cytometry by which fluorescence intensity *in situ* can be quantitatively measured revealed the entire GFP–GR expression level was increased. We then examined the dynamic changes in the subcellular localization of GFP–GR in living hippocampal neurons both in dissociated culture and in tissue slices. GFP–GR was localized in not only the perikarya but also neurites in the absence of ligand, and nuclear translocation following ligand treatment was observed. This is the first report visualizing subcellular trafficking of the GR in the mouse brain in more physiological condition. The present results propose new avenues for the research of the GR dynamics both *in vitro* and *in vivo*.

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Abbreviations: ACSF, artificial cerebrospinal fluid; ADX, adrenalectomy; ADXed, adrenalectomized; GFP, green fluorescent protein; GR, glucocorticoid receptor; NSE, neuron specific enolase; P, postnatal day; PBS, phosphate-buffered saline; PVN, paraventricular hypothalamic nucleus; SAB, streptavidin–biotin.

Key words: living cell imaging, fluorescence quantification, fluorescent emission fingerprinting, hippocampal slice, stress response.

Glucocorticoids diffuse through the cell membrane and bind to intracellular glucocorticoid receptor (GR). These receptors, which belong to the nuclear receptor superfamily, act as ligand-dependent transcription factors (Parker, 1993; Kawata, 1995), and are intimately involved in the alteration of neural functions, such as stress responses, learning and memory, and post-ischemic changes (Sapolsky and Pulsinelli, 1985; Kawata, 1995; McEwen and Sapolsky, 1995; De Kloet et al., 1998; Krugers et al., 1999). Previously, the distribution of GR in the brain has been investigated mainly by using receptor autoradiography, immunohistochemistry, and *in situ* hybridization (Ahima and Harlan, 1990; Cintra et al., 1994; Morimoto et al., 1996). GR positive cells are detected extensively throughout the brain, but found densely in the olfactory bulb, cerebral cortex, hypothalamus, and hippocampus. It is widely accepted that GRs are present in cytoplasm and nucleus (Akner et al., 1995), and following ligand binding, they accumulate in the nucleus where they activate or repress gene transcription (Parker, 1993; Kawata, 1995).

Recent technology has made it possible to visualize a molecule in a less invasive way using a chimera of green fluorescent protein (GFP) and target molecule permitting the examination of real-time changes in expression of the molecule *in vitro* and *in vivo* (Hadjantonakis and Nagy, 2001). By transfection of GFP-tagged GR molecules into non-neuronal (Htun et al., 1996) and neuronal cells (Nishi et al., 1999, 2004), dynamic translocation of GR from cytoplasm into nucleus in response to glucocorticoid was demonstrated *in vitro* as an alteration in the pattern of green fluorescence regardless of the cell type. Recently, GFP–GR knockin mice were generated (Brewer et al., 2002), in which a GFP–GR fusion gene was knocked into the GR locus. The expression and function of GFP–GR were indistinguishable from endogenous GR *in vitro* and *in vivo*.

In the present study, we focused on the brain of the GFP–GR knockin mouse to investigate the properties of GFP–GR in brain tissue. The distribution of the green fluorescence of the GFP–GR was compared with GR immunoreactivity in the brain regions where endogenous GR was abundantly found in wild-type mice. In combination with fluorescent emission fingerprinting, the GFP–GR signal could be distinguished from strong autofluorescence,

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thus subcellular distribution of the GR in the pyramidal neuron in hippocampal CA3 was visualized. To examine subcellular trafficking of the GFP–GR fusion protein, as observed in endogenous GR, adrenalectomy (ADX) was performed on the GFP–GR mice and the change in the fluorescence intensity in the brain section was investigated in combination with laser scanning cytometry. GFP fluorescence significantly decreased in the nuclei, whereas increased at the whole cell level two days after ADX. In addition, a change in fluorescence intensity *in situ* was examined quantitatively. Dynamic translocation of GFP–GR from cytoplasm to nucleus in response to ligand was also visualized in living hippocampal neurons within dissociated culture and the hippocampal tissue slices. Especially, in dissociated culture, faint GFP–GR signals in dendrites were found before ligand treatment. These observations suggest that the GFP–GR knockin mice may provide us with a promising *in vivo* method for analyzing the role of GR in the brain.

EXPERIMENTAL PROCEDURES

Generation of the GFP–GR knockin mice

The detailed procedure for the generation of the GFP–GR knockin mouse has been described elsewhere (Brewer et al., 2002). Briefly, a targeting vector was designed in which GFP was fused to the initiator methionine in exon 2 of the GR gene, downstream of the exon 2 splice acceptor site, and a phosphoglycerate kinase neomycin resistance (PGKneo) cassette containing flanking loxP sites was subcloned into intron 2. After homologous recombination in embryonic stem cells and subsequent germline transmission, the PGKneo gene was removed by mating to EIIA-Cre transgenic mice, thus maintaining all endogenous regulatory sequences in the GR gene. Normal function of GFP–GR fusion protein as endogenous GR was confirmed *in vitro* and *in vivo* (Brewer et al., 2002).

Tissue preparation and immunohistochemistry

All surgical and experimental procedures were permitted by the Committee for Animal Research, Kyoto Prefectural University of Medicine and carried out in accordance with the guidelines of the National Institutes of Health on animal care. We tried to minimize the number of animals used and their suffering. The animals were maintained on a 12-h light/dark schedule with access to food and water *ad libitum*. Immunohistochemistry of the brain sections of wild-type mouse was performed based on a previously described method (Morimoto et al., 1996). Briefly, wild-type male C57BL/6 mice aged 6 weeks were deeply anesthetized with 50 mg/kg sodium pentobarbital and perfused transcardially with saline, followed by 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.4). After post-fixation and cryoprotection, the brains were sectioned in the frontal plane at 20 μm using a cryostat (CM3050S, Leica, Nussloch, Germany). After blocking with 1% bovine serum albumin, the sections were incubated with rabbit polyclonal antibody against rat GR (Morimoto et al., 1996) diluted 1:8000 with 0.3% Triton X-100 in PBS for 3 days at 4 °C. Subsequently, staining was performed by the streptavidin–biotin (SAB) method (Histofine Simple Stain PO, Nichirei, Tokyo, Japan).

ADX

Bilateral ADX was performed under the anesthesia with 50 mg/kg sodium pentobarbital on male wild type and GFP–GR knockin mice aged 6 weeks. After the ADX, the animals were maintained in their cage and their drinking water was replaced with saline. Two days after the operation, the animals were anesthetized again and killed. Plasma corticosterone concentrations were examined to verify that the adrenal glands were totally removed.

Immunofluorescence histochemistry

Male GFP–GR homozygous mice aged 6 weeks were fixed by transcardial perfusion and 20 μm thick sections were cut as described above. The frozen sections were mounted on Silane-coated slide glass and coverslipped with Gelvatol (20% polyvinyl alcohol and glycerol mixture). They were viewed using a confocal laser microscope (LSM 510 META, Zeiss, Jena, Germany). For immunofluorescent staining, the mounted sections were incubated with the rabbit polyclonal antibody against rat GR diluted 1:2000 or neuron specific enolase (NSE, Polyscience, Niles, IL, USA) diluted 1:4000 for 3 days at 4 °C after blocking with bovine serum albumin in humidified chamber. After rinsing the first antibody from the sections, they were incubated with goat anti-rabbit IgG labeled with Alexa 546 (Molecular Probes, Eugene, OR, USA) diluted 1:1000 for 2 h at room temperature and subsequently coverslipped with Gelvatol.

Fluorescent emission fingerprinting

To distinguish GFP–GR fluorescence and background autofluorescence in GFP–GR knockin mice, the emission fingerprinting method of LSM 510 META was employed. A lambda stack acquisition was performed with excitation at 488 nm and detection at 10 nm intervals from 490 nm to 575 nm using a HFT488 dichroic mirror. For spectral reference, regions of interest were selected: autofluorescence was observed in a wild-type mouse brain section; and GFP fluorescence in a neuronal nucleus of the hippocampal CA1 subregion of a GFP–GR knockin mouse brain section, where GFP–GR densely localized. These spectra were then extracted from obtained images of brain sections of GFP–GR knockin mice by linear unmixing and colored green as GFP fluorescence and red as background autofluorescence.

Fluorescence quantification

We compared regional differences of GFP–GR fluorescence intensity in brain sections between adrenalectomized (ADXed) and sham-operated mice by using laser scanning cytometer (iCys, Olympus, Tokyo, Japan). Because this system measures all fluorescent signals within a 20 μm thickness in a single scan, the fixed brain sections 20 μm in thickness were used. Brain sections from ADXed and sham-operated mice were mounted on a slide glass and examined with an air-cooled 5mW 488 nm argon-ion laser using a ×40 objective. Before the evaluation, the target brain regions were determined by the observation with transmitted light. GFP signals were detected through a 515–545 nm green filter. Data analysis was performed with iCys acquisition software (Olympus, Tokyo, Japan). For each region, the difference between the mean fluorescence intensity of ADXed ($n=5$) and sham-operated group ($n=5$) was determined by Student's *t*-test.

Hippocampal neuronal culture

Dissociated hippocampal primary neuronal cultures were prepared from postnatal day 4 (P4) GFP–GR knockin mice according to a previously reported method (Nishi et al., 1999). Briefly, the mice brains were transferred to ice-cold dissecting solution (0.8% NaCl, 0.04% KCl, 0.006% $Na_2HPO_4.12H_2O$, 0.003% KH_2PO_4, 0.5% glucose, 0.00012% Phenol Red, 0.0125% penicillin G, and 0.02% streptomycin). The isolated hippocampi were mechanically dissociated by triturating through a fire-polished glass pipette. The dissociated cells were plated on a 35 mm glass-bottomed dish