Review

DYNAMICS OF CORTICOSTEROID RECEPTORS: LESSONS FROM LIVE CELL IMAGING

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Abstract: Adrenal corticosteroids (cortisol in humans or corticosterone in rodents) exert numerous effects in the central nervous system that regulate the stress response, mood, learning and memory, and various neuroendocrine functions. Corticosterone (CORT) actions in the brain are mediated by two corticosteroid receptors, glucocorticoid receptor (GR) and mineralocorticoid receptor (MR), and they show a high degree of colocalization in the hippocamal region. These receptors are predominantly resided in the cytoplasm without ligand and translocated into the nucleus upon ligand binding to act as transcriptional factors. Thus their subcellualr localizations are important component of their biological activity. Given the differential action of MR and GR in the central nervous system, it is important to elucidate how the trafficking of these receptors between cytoplasm and nucleus and their interaction are regulated by ligand or other molecules to exert transcriptional activity. In this review, we focus on the nucleocytoplasmic and subnuclear trafficking of GR and MR in neural cells and non-neural cells, and discuss various factors affecting the dynamics of these receptors.

Key words : glucocorticoid receptor, mineralocorticoid receptor, hippocampus, GFP, importins, real-time imaging

INTRODUCTION

GR and MR show a high degree of colocalization in the hippocampus[1]. Since MR has about 10-fold higher affinity for CORT than does GR, hippocampal MR responds strongly to CORT[2]. Thus, in the hippocampus, one compound, CORT serves to regulate two signaling pathways via MR and GR[3]. The progressive activation of MR at a low CORT concentration and additional activation of GR when CORT levels increase can cause extreme alterations of neuronal integrity for responding to stress conditions[4] and of neuronal excitability[5] associated with changes in neuroendocrine regulation and behavior.

These corticosteroid receptors are localized predominantly in the cytoplasm in the absence of ligand associated with various chaperone proteins such as heat shock protein 90 (hsp90). After binding with hormone, the hormone-receptor complex becomes activated leading to dynamic conformational changes of protein complex, and translocates into the nucleus. For inducing transactivation, the hormone-receptor complex binds to glucocorticoid responsive elements (GRE) in the promoter regions in a homodimer or a heterodimer form. Thus, the elucidation of mechanisms for subcellular and subnuclear trafficking of these receptors is a

remarkably important issue.

Nucleocytoplasmic Trafficking of GR and MR

Differential responses to the common natural ligand, corticosterone.

Adrenal corticosteroids regulate their own secretion via a negative feedback system at the level of the hypothalamus and pituitary that is mediated by GR. In addition to these regulatory systems, recent studies have indicated that the tonic inhibitory action of corticosteroid on hypothalamus-pituitary-adrenal (HPA) activity is exerted via MR in the suprahypothalamic structures including the hippocampus. The limbic structure controls HPA activity via the inhibitory GABAergic system[6]. At the lower level of CORT during the circadian trough, MR is predominantly occupied and operated in a pro-active fashion in the maintenance of homeostasis, while at the higher level of CORT observed at the circadian peak or after stress, GR is mainly activated. Thus, the cytoplasm- to-nuclear translocation of these two receptors in response to CORT in single cells is intriguing.

We investigated the trafficking manners of GR and MR in response to the common natural ligand, CORT, in single living cells cotransfected with GR and MR using dual-color labeling with two different GFP spectral variants, CFP (cyan fluorescent protein) and YFP(yellow fluorescent protein). The double labeling strategies with CFP and YFP have allowed time-lapse imaging of two different receptors in single living cells simultaneously 7). In the absence of CORT, CFP-GR was predominantly localized in the cytoplasmic regions, whereas YFP-MR was distributed in both the cytoplasm and nucleus. The trafficking manners of these fusion proteins in the cotransfected cells were basically the same as those in the singly transfected cells.

In COS-1 cells, YFP-MR was accumulated in the nucleus faster than CFP-GR in the presence of 10^{-9} M CORT (Fig.1), a concentration between the Kd values of MR and GR. In

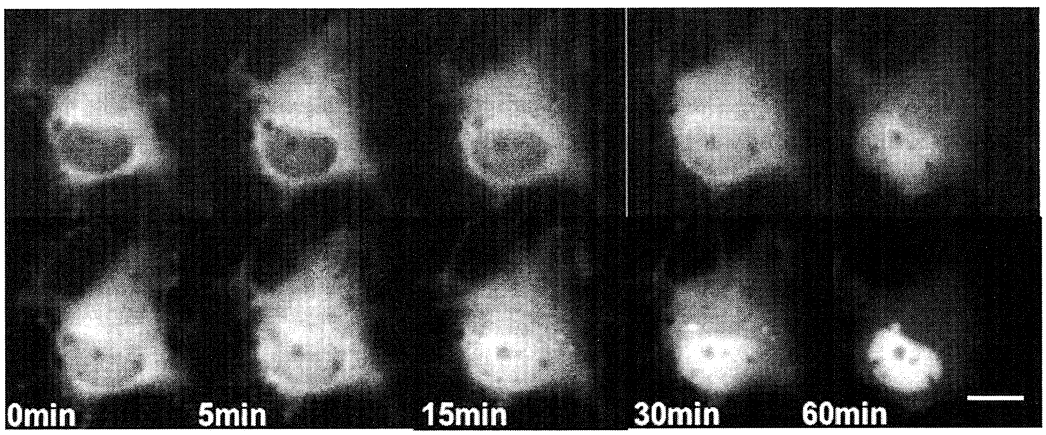

Fig. 1. Dual-color imaging of GR and MR with GFP color variants in a single COS-1 cell
COS-1 cells co-transfected with CFP-GR and YFP-MR were cultured in the absence of serum and steroids for 24h before observation. Upper images were representative time-lapse images of CFP-GR, and bottoms were those of YFP-MR. Note that YFP-MR was accumulated in the nuclear region faster than CFP-GR after treatment with 10^{-9} M CORT.
Bar = 10 μm.

contrast, no significant difference was observed in the accumulation rate in the presence of 10^{-6}M CORT, a concentration much higher than the Kd values of both receptors. Since COS-1 cells have no endogenous MR or GR, the difference in trafficking kinetics detected in the presence of 10^{-9} M CORT is considered to directly reflect the difference in affinities for CORT between MR and GR; more specifically, MR has about 10-fold higher affinity for CORT than that of GR. The findings suggest that both MR and GR are saturated in cells treated with 10^{-6}M CORT, causing the lack of difference in trafficking kinetics. These results are in agreement with the present understanding that MR plays major roles at physiological concentrations of CORT, while GR is mainly effective at high concentrations of CORT[8].

Contrary to COS-1 cells, hippocampal neurons did not show any obvious difference in the nuclear accumulation rates of MR and GR in the presence of either 10^{-9}M or 10^{-6}M CORT. Since hippocampal neurons express endogenous MR and GR, these endogenous receptors may affect the trafficking of YFP-MR and CFP-GR. Another possible explanation is that hippocampal neurons may have an unique nuclear transporting system for accumulating MR and GR in the nucleus together, which is different from that in COS-1 cells, expressing no endogenous receptors. Recent study showed that vesicles containing NMDA receptor 2B are transported along microtubules by KIF (kinesin superfamily) 17, a neuron-specific molecular motor[9]. Although our previous data indicated that microtubules are not essentially involved in the nuclear import of GFP-MR or GFP-GR, the results of Setou and coworkers suggest that some receptors expressed in neuronal cells are transported by a neuron-specific molecular motor[9]. These results lead us to speculate that MR and GR could be translocated into the nucleus at mostly the same speed using specific motor molecules in cultured hippocampal neurons.

Role of carrier proteins

It has been considered that the cytoplasmic/nuclear distribution of steroid hormone receptors is primarily regulated by conditional interaction of nuclear localization signals (NLS) with the import/export apparatus in the nuclear pores[10]. In the absence of hormone, steroid hormone receptors are associated with a complex set of chaperones in a large complex, and the interaction of the cognate ligand with these receptors induces a conformational change resulting in dissociation of the complex and loss of many associated factors. This reconstruction is thought in some cases to expose previously masked NLS, and the receptors are then recognized by the transport machinery, such as importin family members[11]. Because macromolecules greater than about 40kDa, including corticosteroid receptors, are transported through gated channels of the nuclear pore complex (NPC) by active mechanisms, whereas molecules less than 20–40 kDa can passively diffuse through NPC[12]. In the classical nuclear import pathway, importin α recognizes and binds to the NLS on the cargo protein, and also binds to importin β, which then docks the NPC and mediates translocation from the cytoplasm to the nucleus[13]. We showed that corticosteroid receptors were translocated from the cytoplasm to the nucleus in association with importin α after ligand binding in single living COS-1 cells coexpressing fusion proteins with GFP color variants, which means importin a was also translocated from the cytoplasm to the nucleus in mostly the same time course as that of corticosteroid receptors[14]. In contrast, the distribution

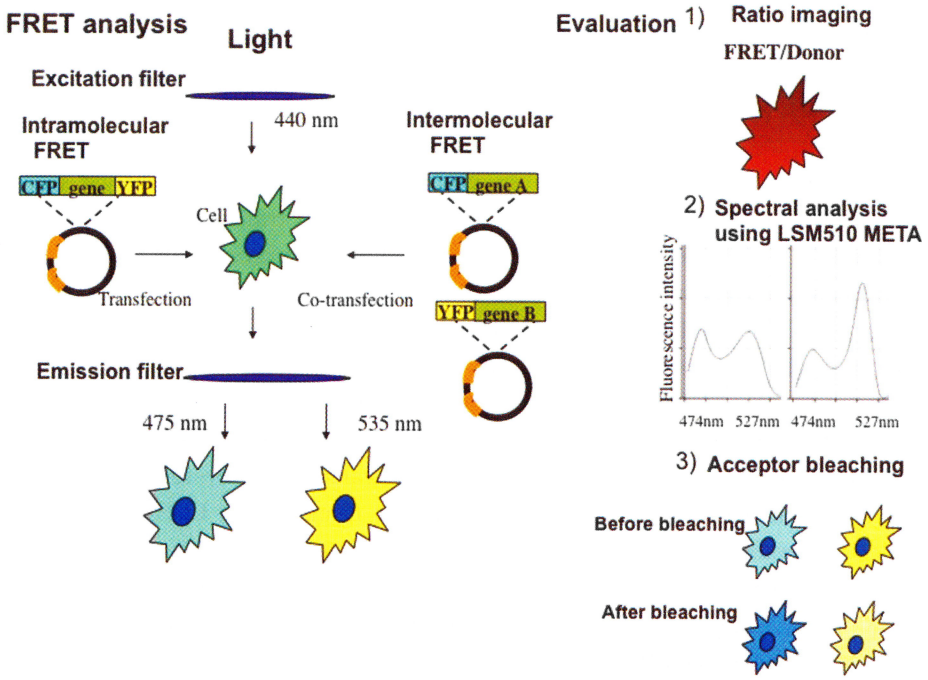
FRET analysis
Light
Excitation filter
440 nm
Intramolecular FRET
CFP gene YFP
Cell
Transfection
Intermolecular FRET
CFP gene A
YFP gene B
Co-transfection
Emission filter
475 nm
535 nm
Evaluation 1) Ratio imaging
FRET/Donor
2) Spectral analysis using LSM510 META
Fluorescence intensity
474nm 527nm 474nm 527nm
3) Acceptor bleaching
Before bleaching
After bleaching

Fig. 2.

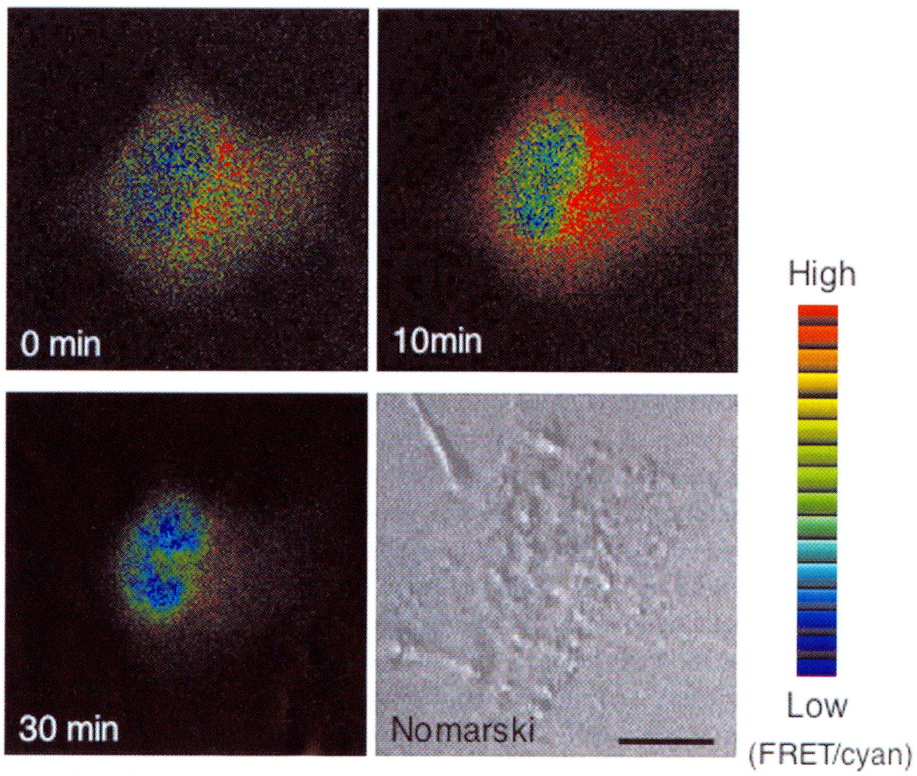
0 min
10min
30 min
Nomarski
High
Low
(FRET/cyan)

Fig. 3.

of importin β was predominantly around perinuclear sites and little changed after ligand binding. Furthermore, analysis using fluorescence resonance energy transfer (FRET) (Fig. 2) proved that GR directly interacted with importin α in the whole area of the cytoplasm upon ligand treatment and detached importin α shortly after nuclear import (Fig. 3). However direct interaction between GR and importin β was not detected. The study of a mutant in NLS of corticosteroid receptors support these data[14].

Subnuclear Trafficking of GR and MR

Nuclear profile after translocated in the nucleus

After GR and MR enter the nucleus affected by various kinds of factors, what is happening in the nucleus? In live cell studies using GFP-GR and GFP-MR, the GFP fluorescence appears to be accumulated in certain specific nuclear regions, and is distributed in heterogeneous dot-like distributions in the nucleus[15,16]. Fejes-Toth[17] reported that agonist-activated GFP-MR accumulates in discrete clusters in the nucleus, and that this phenomenon occurs only with transcriptionally active MR. In contrast, van Steensel and colleagues[18] demonstrated the spatial distribution of MR and GR in clusters in specific nuclear domains using an immunofluorescence technique with confocal microscopy. They indicated that there is no correlation between the clusters of receptor and the distribution of newly synthesized pre-mRNA, suggesting that the clusters of receptor are not directly involved in active transcription.

Fig. 2. Procedure and evaluation of fluorescence resonance energy transfer (FRET) experiment using fusion proteins with cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP)
In our experiment, FRET is evaluated with three ways: 1) Ratio imaging (FRET image was divided by Donor image). Ratio images were pseudocolored where the red range indicated a high ratio and the blue range indicated a low ratio. 2) For detecting an emission spectral changes in FRET imaging, Emission Fingerprinting method using confocal laser-scanning microscope LSM 510 META (Zeiss) was employed. First, spectral signatures of the fluorescence within the specimen were captured by means of lambda stack acquisition with excitation at 458 nm and detection at 10 nm-intervals from 458 through 596 nm using an HFT 458/543 dichroic mirror. Several regions of interest (ROIs) with a diameter of 2 mm were then randomly selected for obtaining emission spectral patterns, and the mean ratio of fluorescence intensity of 527 nm and 474 nm was calculated from selected ROIs at each time point after ligand addition. 3) For acceptor photobleaching, we used the confocal laser-scanning microscope. Energy transfer was detected as an increase in donor fluorescence (CFP) after photobleaching of the acceptor molecules (YFP). The acceptor was photobleached by using a 514 nm laser for 1 min at maximum power (25mW).

Fig. 3. Ratio images of the cell co-expressing CFP-GR and YFP-importin α detected by FRET
COS-1 cells were co-expressed with CFP-GR and YFP-importin α and cultured in the absence of serum and steroids for at least 15h before observation. Fluorescent images of CFP-GR and YFP-importin α were captured using a filter set of CFP (440AF21 excitation, 480AF30 emission, and 455DRLP dichroic mirror) and YFP (500AF25 excitation, 545AF35 emission, and 525DRLB dichroic mirror), respectively. FRET image was detected using a filter set with 440AF21 excitation and 535AF26 emission, and 455DRPL dichroic mirror at 0, 10, and 30 min after treatment with 10^{-6}M CORT. Filter sets were purchased from Omega Optical Inc. The ratio of the FRET image was divided by donor image to obtain the ratio images using MetaMorph software (Universal Image Corp.). The ratio images were pseudo colored. The red range showed high ratio and blue range showed low ratio. High ratio was observed in the cytoplasm, indicating an interaction of CFP-GR and YFP-importin α, whereas low ratio was observed in the nucleus, indicating a dissociation of these two molecules. bar : 10 μm.

Recent studies showed that various nuclear proteins such as transcription factors, splicing factors and chromatin remodeling factors, continuously and rapidly associate and dissociate with nuclear compartments such as regulatory sites in living cells[19]. These studies investigated the nuclear dynamics of GFP-labeled proteins in living cells using FRAP (fluorescence recovery after photobleaching) and FLIP (fluorescence loss in photobleaching) to ask the question how fast these nuclear proteins move within the nucleus. McNally and colleagues elegantly visualized the direct interaction of GR with hormone response elements in living cells by applying a large tandem array of a mouse mammary tumor virus/Harvey viral ras reporter[19]. Using FRAP techniques, they proposed a dynamic "hit-and-run" model in which the receptor undergoes continuous exchange between chromatin regulatory elements and the nucleocytoplasmic compartment during a constant existence of ligand. In the case of cultured hippocampal and cortical neurons, FRAP study showed mostly the same rapid movement of GR and MR as detected in non-neural cells (Nishi unpublished data). The techniques of FRAP and FLIP showed the possibilities that the nuclear proteins are freely diffusing or constrained by structure, perhaps actively recruited from one place to the next.

Interaction of corticosteroid receptors in the nucleus

Heterodimerization between transcription factors is not uncommon and seems to increase level of functional diversity[20]. Likewise, the formation of heterodimers between members of the nuclear receptor superfamily is a common property. Interactions have been reported between the retinoic X receptor and the retinoic acid receptor, and between the vitamin D receptor and the thyroid receptor[21]. The same could be true for the case of GR and MR. Previous molecular biological studies have indicated that in cells expressing only one of the receptors, transcriptional regulation from hormone response element (HRE), many of which are imperfect inverted hexanucleotide repeats, is mediated by receptor homodimers[22]. However, physiological studies in various systems suggest that GR and MR also functionally interact with each other[5]. Biogenetic evidence demonstrated that GR and perhaps MR form homodimers through a dimer interface within their zinc finger regions (ZFRs), and these receptors share complete sequence identity in this ZFR dimer interface, suggesting that this region might mediate heterodimerization as well[23]. To visualize such an interaction in spatio-temporal specific manner in living cells, we performed a FRET analysis coupled with a new technique, spectral imaging fluorescence microscopy[24, 25] to compensate for varying levels of protein expression (Fig. 2). This technique allowed us to detect spectral changes in fluorescence in living cells and to address several argued points of intermolecular FRET[26]. We calculated mean ratio of fluorescence intensity at acceptor and donor emission maximum wave lengths, 527 nm and 474 nm, respectively. By using these methods, we observed that CFP-GR and YFP-MR directly interact each other in the nucleus, but not in the cytoplasm, after treatment with CORT, both in COS-1 cells and cultured hippocampal neurons. These results suggest that heterodimer formation depends on the content of GR and MR in the nucleus. Then we investigated whether GR and MR heterodimer formation is affected by concentrations of CORT, because there is a possibility that GR and MR exert various functions reflecting the differences in affinity for the common ligand, CORT. Particularly, in structures such as the hippocampus where both GR and MR are co-expressed in the same

cells[18], heterodimerization of these receptors may have a decisive influence on the regulation of corticosteroid-responsive genes in the brain. We employed two different concentrations, 10^{-6}M and 10^{-9}M , and found that content of heterodimer of CFP-GR and YFP-MR detected at 10^{-6}M was higher than that at 10^{-9}M. These results suggest that MR, with 10-fold higher affinity than GR, may form predominantly homodimers at a lower concentration, whereas at a higher concentration mimicking stressful conditions, occupancy of GR increases the probability of heterodimerization. Our findings could be consistent with the previous demonstrations that MR is dominantly activated at lower concentrations of CORT to explore tonic influences, while the additional occupancy of GR with higher levels of CORT mediates the feedback actions to restore disturbances of homeostasis[9]. The physiological significance of the formation of GR-MR heterodimers has been proposed from the co-localization of these receptors in a variety of tissues and cells[27]. Hence, having two types of receptors may allow a more flexible response to widely varying corticosteroid concentrations that may be present under physiological and pathological conditions[28,29]. The availability of a variety of corticosteroid receptor dimers gives the potential to provide a more finely orchestrated regulation of corticosteroid-responsive genes than the previous model of corticosteroids action based on homodimerization (Fig. 4). But real functional role of heterodimerization in

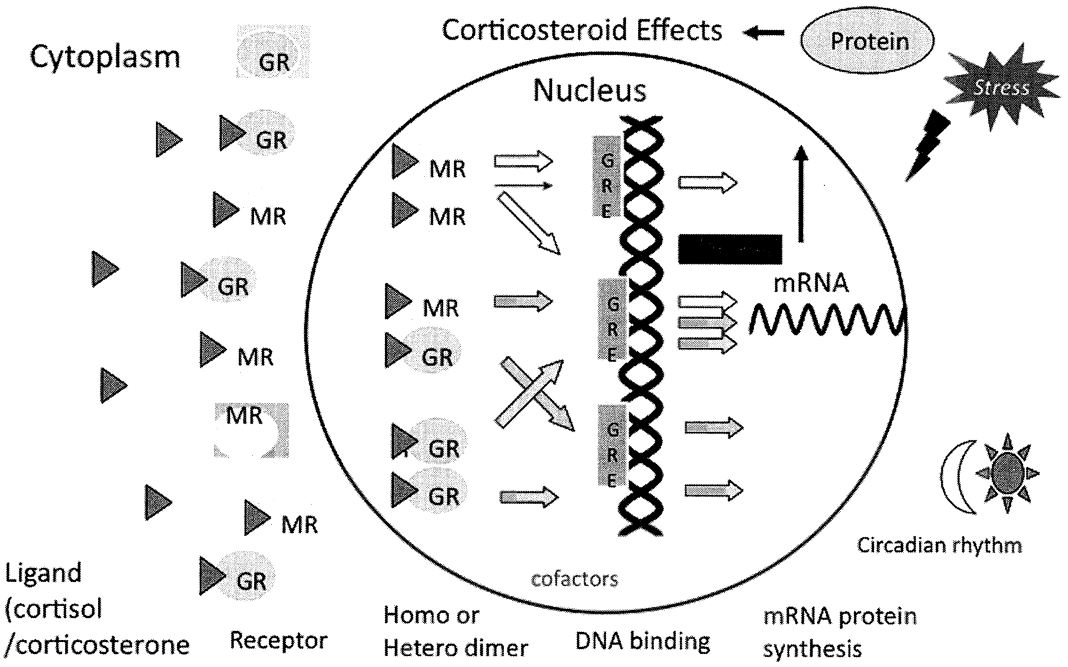

Fig. 4. A schematic model for dimer formation of corticosteroid receptor
A variety of corticosteroid receptor dimers including homodimers and heterodimers may give the potential to provide a more finely tuned regulation of corticosteroid-regulated gene for responding to fluctuations in plasma cortisol/corticosterone level affected by stress responses, circadian rhythm, and so on.

vivo remains controversial. Furthermore, the recruitment of transcriptional cofactors is affected by dimerization manner, homodimer or heterodimer, which leads to differential regulation of transcription activities of GR and MR. These findings indicate that heterodimerization of GR and MR may provide a diversity for regulating gene expression in response to various cellular environments such as fluctuating CORT concentrations induced by stress and/or circadian rhythm.

CONCLUSION

These studies of receptor trafficking in living cells reveal a dynamic alteration in the subcellular localization of receptors in response to various extracellular and intracellular environments. Although there are still problems in tagging proteins with GFP and overexpressing the receptors, this approach makes it possible to observe varieties of events in living cells, which have never been detected in fixed cells. Finally, it is particularly important to elucidate whether the functional significance of the observations in living neurons should be true as whole brain levels. This could come true with recent progress in multidisciplinary studies of life science areas including varieties of combinations of genetically engineered animals with sophisticated optical instruments in the near future.

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