Golgi cells and Lugaro cells. These two types have been characterized so far, but we have observed another type of interneurons; a globular-like neuron (GloLN), which has a small globular soma with several processes extending along the PC layer. Here, in order to identify interneurons underneath the PC layer by fluorescence microscopy we made cerebellar slices from a glutamic acid decarboxylase (GAD) 67/green fluorescent protein (GFP) knock-in mouse expresses GFP in GABAergic neurons. To establish electrophysiological criteria for the types of interneurons, we applied patch-clamp techniques and studied their electrophysiological properties, combined with pharmacological assays. We found that Golgi cells fired spontaneously at rest, but neither Lugaro cells nor GloLNs caused spontaneous action potential. Serotonin facilitated spontaneous firing in not only Lugaro cells but also Golgi cells and GloLNs. The frequency of spontaneous IPSCs (sIPSCs) recorded in GloLNs was much higher (~40 Hz) than those in Golgi (~2 Hz) and Lugaro cells (~20 Hz). Serotonin facilitated sIPSCs in Golgi cells but not in Lugaro cells or GloLNs. Membrane properties of GloLNs were different from Golgi and Lugaro cells, but similar to basket cells except for the membrane potential. Double-patch recordings demonstrated that sIPSCs in GloLNs and action potentials of PCs located close to the GloLNs were coincidentally detected, meaning that GloLNs could receive PC axon collateral inputs. These results suggest that GloLNs we observed here are likely to integrate signals from PCs.

3P-201 薬剤投与マウスの歩行指標の開発

New index for a behavioral differentiation across psychomotor stimulants

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When mice are placed at an open-field, they explore mainly the peripheral zone of the open-field, which are called thigmotaxis. The thigmotaxis have been used as an index of anxiety in anxiogenic drug applied BALB/C mice. However, mice of another strains (C57BL/6 and DBA/2) walk mainly in center zone of the open-field after applying drug. That indicates the thigmotaxis cannot be adapted for the index for the anxiety in all the species. We have investigated the locomotor activity of cocaine-applied mice in an open-field (the circular field 60cm in diameter, with the wall 40cm high) test through the image analysis. Comparing the index of statistical physics concerning about the random walk behavior, we have investigated the characteristics of locomotor activity. We found plots of log(duration time of walking) vs log(numbers of walking event) are essentially linear with slopes which appear to depend on doses of applied cocaine. Then, we develop new index depending on obtained slopes. Our index can integrate locomotor behavior of all three strains (C57BL/6, DBA/2, BALB/c) without any contradiction.

3P-202 ヨーロッパモノアラガイの中枢神経系で見られる陰影反射の神経基盤

The neuronal substrates of predator induced stress on the shadow response in *Lymnaea*

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In the pond snail *Lymnaea* a tactile or shadow stimulus induces an avoidance behavior, that is part of the whole body withdrawal (WBW) response. We tested at both the behavioral and electrophysiologic level whether WBW was altered in the presence of an ecologically relevant stress. Snails in the predator-exposed group had a significantly more pronounced and longer lasting WBW to the shadow stimulus compared to a control. Since RPeD11 is assumed to be the key interneuron controlling WBW, we focused on the activity of RPeD11. Presenting a shadow stimulus to the pneumostome area evoked an EPSP in RPeD11 in semi-intact preparations (without ocular photoreceptors). This EPSP is monosynaptic and is mediated by dermal photoreceptors in the pneumostome area. That is, the EPSP is present in hi Mg^{2+} /hi Ca^{2+} saline while it disappears in Ca^{2+} free saline. The amplitude and duration of the EPSP are significantly enhanced in the presence of the predator compared to naive preparations. These data strongly suggest that augmentation of sensory input to RPeD11 plays a key role in the enhanced WBW when predator is present.

3P-203 軟体動物腹足類における神経型 NO 合成酵素の同定

Gene Expression of Neuronal Nitric Oxide Synthase in the mollusk, *Lymnaea stagnalis*

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Nitric oxide is well known gaseous signaling molecule which works in numerous biological mechanisms, including retrograde transmission in the central nervous system (CNS). Generally, NOS proteins were classified into three groups, and each referred as inducible NOS (iNOS), endothelial NOS (eNOS) and

neuronal NOS (nNOS). The nNOS protein is different from other NOS proteins in possessing PSD/Discs-large/ZO-1 homologous (PDZ) domain at its N-terminus. The PDZ domain of nNOS is known to heterodimerize with other PDZ domain proteins in discrete subcellular regions, which is essential for anchoring nNOS at the synaptic sites. As well as in mammalian brain system, the regulatory mechanisms of NO have been investigated in the invertebrate CNS. In the pond snail *Lymnaea stagnalis*, previous reports showed the regulatory role of NO in feeding behavior and memory formation. More, histological analyses showed that the NADPH-diaphorase positive signal in the neuropil, indicating the subcellular localization of NOS proteins in the *Lymnaea* CNS. These findings were consistent with the idea that *Lymnaea* NOS proteins should be expressed as neuronal NOS, however, none of NOS related gene encoding the PDZ domain has been isolated. To start our study for analyzing the functional NOS involved in the synaptic regulatory mechanism, we first identified neuronal NOS gene with the PDZ domain. The deduced amino acid sequence (1645 AA) was highly identical with other species nNOS, and it was named as *Lymnaea* neuronal NOS (Lym nNOS). We also examined in situ hybridization for Lym nNOS mRNA and NADPH-d staining to examine the function of nNOS in the CNS.

3P-204 老化マウスの瞬目反射条件付け学習におけるニコチン性アセチルコリン受容体の関与

Contribution of nicotinic acetylcholine receptor to the classical eyeblink conditioning in aged mice

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Brain $\alpha 7$ nicotinic receptor ($\alpha 7$ nAChR) has become a therapeutic target for Alzheimer's disease (AD) based on their memory-enhancing and neuroprotective actions. In this study, we aim at revealing contribution of $\alpha 7$ nAChR on aging-related learning deficits using delay version of eyeblink classical conditioning. For conditioning in mice, 85 dB, 1 kHz tone was used as conditioned stimulus (CS) and 100 Hz electric stimulus to eyelid was as unconditioned stimulus (US). Conditioned response (CR) was evaluated by measuring myogenic potentials from eyelid. Interval from the CS onset to the US onset was fixed at 250 ms and the two stimuli terminated simultaneously. After seven days of conditioning sessions, 12-month-old (aged) mice showed a marked impairment in the percentage of conditioned responses (CR) compared with 8-week-old (young) mice. Treatment of selective $\alpha 7$ nAChR agonist, PNU282987, ameliorated the impairment of learning in aged mice. Central acetylcholinesterase inhibitor, Galanthamine, was more effective in the ameliorating learning deficit than PNU282987. It suggests that there may be another mechanism which contributes to the acquisition of delay CR without $\alpha 7$ nAChR signaling. The level of $\alpha 7$ nAChR expression was decreased in aged mice in the prefrontal cortex, hippocampus and cerebellum. Interestingly, it is known that cerebellum is essential to delay CR acquisition. These results suggest that impairment of acquisition of delay conditioning observed in aged mice caused by hypoactivity of $\alpha 7$ nAChR function.

3P-205 大腸菌発現系を用いた *salinarum* ハロロドプシンの機能的再構成

Functional Reconstitution of *salinarum* Halorhodopsin Expressed in *Escherichia coli*

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Halorhodopsin (HR) is a light-driven inward chloride pump expressed in the archaeal membranes. The representative HRs are from *Halobacterium salinarum* (HsHR) and *Natronomonas pharaonis* (NpHR). Despite of high sequence similarities, their characteristic differences were reported in chloride-dependent spectral shifts, other transportable anions, schemes and the chloride-concentration dependencies of the photocycles, and so on. In addition, the functional expression in *E. coli* membrane was reported for NpHR but not for HsHR. In this work, to facilitate the comparative studies between NpHR and HsHR, we established the procedures to prepare photoactive HsHR from *E. coli* cells and reconstitute purified HsHR functionally.

Expressed HsHR had no color in the *E. coli* membrane, but turned purple like in archaeal membrane after solubilization in the presence of all-*trans* retinal. The colored HsHR was highly purified and reconstituted in alectin liposomes. Light-induced outward directed transport of chloride by HR was evaluated by measuring passive proton efflux in the presence of uncoupler CCCP. By using this reconstitution system, the chloride-concentration dependence of the pumping activity was examined. From the relations between the pumping activity and the corresponding photocycle, we will discuss the chloride pumping mechanism of HsHR and the differences from NpHR.

3P-206 共鳴ラマン分光法によるファラオニスフォロドプシンN中間体の検出

Photointermediates of *Natronomonas pharaonis* phoborhodopsin revealed by resonance Raman spectroscopy

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pharaonis phoborhodopsin (ppR, also called *pharaonis* sensory rhodopsin II; pSRII) is a retinal protein in *Natronomonas pharaonis* and acts as a receptor of negative phototaxis. ppR has been extensively studied, because this protein is stable and can be expressed in *E. coli* membrane in a large amount. The X-ray structure of ppR has been reported, and the structure is similar to that of bacteriorhodopsin (bR), which is a light-driven proton pump. Furthermore, the photocycle of ppR is also similar to that of bR, where photoexcitation of the dark state bR produces a series of intermediates denoted as K, L, M, N, and O intermediates. A notable difference between the photocycle of bR and ppR is a long lifetime of M and O intermediates of ppR. Moreover the existence of N intermediate has not been clearly identified for ppR. In fact, transient absorption spectroscopy suggested the presence of the N intermediate [1], whereas FTIR spectroscopy failed to observe the N intermediate under alkaline pH, where N-intermediate of bR is visible [2]. More recently Kamo and co-workers suggested the presence of N intermediate at acidic pH [3]. In this study, in order to clarify the controversy regarding the presence or absence of N intermediate in ppR, we have carried out resonance Raman investigations of ppR and present the evidence indicating the presence of the N intermediate at low pH.

[1] Chizhov et al., *Biophys. J.* 75, 999-1009 (1998)

[2] Furutani et al., *Biophys. J.* 83, 3482-3489 (2002)

[3] Abe et al. *Seibutsu butsuri Supplement 1* 47, S260 (2007)

3P-207 ファラオニスフォボロドプシンの光反応サイクルに対する pH の影響

pH effect on the photoreaction cycle of *pharaonis* phoborhodopsin

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Pharaonis phoborhodopsin (ppR, also called *Natronomonas pharaonis* sensory rhodopsin II, or NpSRII), is a photoreceptor of *Natronomonas* (*Natronobacterium*) *pharaonis*, responsible for the photophobic response of this bacterium. Light irradiation of ppR causes cyclic and linear photoreactions, photocycle, during which the light energy is used to generate the photophobic response. The photocycle intermediates in ppR are denoted as K, L, M, and O analogous to that of bacteriorhodopsin, the most intensively studied retinal protein. Acidification of ppR causes a red shift in the absorption maximum probably due to the protonation of D75 which is a counter ion of the protonated Schiff base. In the present work, the low temperature photoreactions of ppR at two different pH conditions, pH=5 (neutral-ppR) and pH=2 (acid-ppR), were studied. Formations of the K-, KL- and M-like intermediates were observed in neutral-ppR. On the contrary, even the formation of K-like intermediate was also observed in acid-ppR, formations of KL- and M-like intermediates were not detected in acid-ppR. An O-like intermediate, moreover, was obtained by warming the K-like intermediates of acid-ppR. Thus, it can be concluded that the protonated states of the counter ion have a significant effect on the photoreaction pathway of this protein.

3P-208 *Halobacterium salinarum* 由来のフォボロドプシン (センサリーロドプシン II) の光化学反応サイクルとプロトン移動

The photochemical reaction cycle and photo-induced proton transfer of phoborhodopsin from *Halobacterium salinarum* (sensory rhodopsin II)

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Phoborhodopsin (pR or sensory rhodopsin II, SRII) is a negative phototaxis receptor of halobacteria. The photo-induced isomerization of all-*trans* retinal triggers the photochemical reaction cycle (called photocycle) accompanied by the proton uptake and release. The existence of pR (SRII) has been first reported in *Halobacterium salinarum* (spR or HsSRII) [1] and later in *Natronomonas pharaonis* (ppR or NpSRII). spR has not been studied well in contrast with ppR due to low content in the membrane, and instability against detergent and under the low salt condition. However, the expression system of spR in *E. coli* was reported recently [2], which enabled us to obtain many proteins more easily. In this study, we obtained spR using this expression system, and examined the photocycle and the accompanying proton uptake/release using the transparent SnO₂ electrode. For all measurements, we used phospholipid-reconstituted spR which was stable under wide pH and temperature ranges. We found M- and O-intermediates in the photocycle, but another intermediate ($\lambda_{\max}$ is close to that of

spR) was necessary at the end of photocycle for the fitting of the kinetic data. In addition, the photocycle and proton movement showed different schemes depending on the medium pH, suggesting the association/dissociation of a certain amino acid residue changes the scheme. Details of will be presented.

[1] Takahashi et al. (1985) *FEMS Microbiol. Lett.* 28, 161-164. [2] Mironova et al. (2005) *FEBS Lett.* 579, 3147-3151.

3P-209 ファラオニスハロロドプシンの Cl⁻輸送機構における Asp252 の役割

Role of Asp252 in chloride transport by *pharaonis* halorhodopsin

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Halorhodopsin (HR) is a light-driven Cl⁻ pump. Like bacteriorhodopsin (BR), HR has all-*trans* retinal bound through a protonated Schiff base (PSB) to a conserved lysine residue. From the analogy to BR, the PSB region is considered to play a crucial role for the Cl⁻ pumping function. At present study, we used HR from *Natronomonas pharaonis* and focused on D252, a negative counterpart of the PSB. The Cl⁻ titration to the unbound wild-type NpHR caused spectral blue-shift. It is known that this Cl⁻ dependency is almost parallel with the dependencies of both the amplitude of the flash-induced absorbance change and the Cl⁻ transport activity. The Cl⁻ titration to the D252N mutant caused spectral red-shift contrary to the wild-type. Although the directions are opposite, the spectral shifts of both wild-type NpHR and the D252N mutant occurred in the similar Cl⁻ concentration range and almost completed up to 100 mM Cl⁻. Thus, we may conclude that Cl⁻ can bind to D252N by the affinity similar to the wild-type NpHR (K_d = 3-4 mM). Under this low Cl⁻ concentration range (< 100 mM), however, D252N showed very small Cl⁻ pumping activity with a photocycle being totally different from that of wild-type. In the D252N photocycle, only the K-like intermediate was observed. With increasing Cl⁻ concentration beyond 100 mM, L intermediate became detectable, and at the same time, the pumping activity was observed. For the Cl⁻ pumping function, thus, the negative charge of D252 should be important but not essential. The D252N mutation might perturb the hydrogen-bonding network around the PSB, which might inhibit the inherent transition of K to L intermediate. The Cl⁻ bound to a second binding site might restore the appropriate hydrogen-bonding network which leads to the Cl⁻ pumping.

3P-210 FMN 結合型蛍光タンパク質 iLOV の光反応

Photoreaction of iLOV, an FMN-binding fluorescence protein.

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LOV domain, the chromophoric domain in phototropins, is composed of about 100 amino acids and flavin mononucleotide (FMN) as a chromophore. Upon illumination of LOV domain, FMN forms a covalent adduct with a conserved cysteine through an excited triplet state, which initiates conformational changes of protein for signal transduction. When the cysteine is mutated into other residues such as alanine (C/A mutant), however, normal photocycle does not occur and the excited FMN chromophore is reverted to the original state by radiative or non-radiative relaxation. Recently, effective fluorescent proteins were screened using the LOV2 domain's C/A mutation of Arabidopsis phot2 as a template, and one variant, designated iLOV, showed high fluorescence that is promising as a tool for cell imaging [1]. iLOV has multiple mutation sites in addition to the C/A mutation, whereas it is unclear why the protein becomes highly fluorescent.

Here we studied photochemical properties of iLOV by means of various spectroscopic techniques. Protein structure in the excited triplet state can be measured by FTIR spectroscopy at 77 K [2], and we found that the spectra of iLOV are similar to those of the C/A mutant and WT of neochrome1-LOV2. We also compare the photoreduction of FMN between iLOV and the C/A mutant, and the corresponding FTIR spectra will be presented. Molecular mechanism of highly fluorescent nature in iLOV will be discussed based on these spectral observations.

3P-211 (6-4) 光回復酵素による DNA 修復の赤外分光観測

Direct observation of enzymatic (6-4) photoproduct conversion by FTIR spectroscopy

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Photolyases (PHRs) are DNA repair proteins that specifically revert UV-induced photoproducts, cyclobutane pyrimidine dimers (CPD) and (6-4) photoproducts into normal bases to maintain genetic integrity. The (6-4) PHR must catalyze not only covalent bond cleavage, but also a hydroxyl or an amino group transfer. Thus, a more complex mechanism has been postulated for (6-4) PHRs than for