Department of Basic Medical Sciences

Division of Neuronal Network
神経ネットワーク分野

Professor	Toshiya Manabe, M.D., Ph.D.	教　授	真　鍋　俊　也
Assistant Professor	Hiroyuki Katagiri, Ph.D.	助　教	片　桐　大　之
Assistant Professor	Shizuka Kobayashi, Ph.D.	助　教	小　林　静　香
Assistant Professor	Takahiko Chimura, Ph.D.	助　教	千　村　崇　彦

Our major research interest is the molecular mechanisms of higher brain functions in mammals such as emotion, and learning and memory. We are especially focusing on the roles of functional molecules localized in synapses, for instance, neurotransmitter receptors, signal transduction molecules and adhesion molecules, in neuronal information processing. We are examining receptor functions, synaptic transmission and plasticity, and their roles in the whole animal with electrophysiological, biochemical, molecular genetic and behavioral approaches. We are also trying to elucidate fundamental aspects of psychiatrical and neurological disorders using model animals.

1. The adhesion molecule cadherin 11 is essential for acquisition of normal hearing ability through middle ear development in the mouse

Yuji Kiyama, Yayoi S. Kikkawa[1], Tatsuya Yamasoba[1] and Toshiya Manabe: [1]Department of Otorhinolaryngology, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan

Cadherin 11 (Cdh11), a member of the cadherin adhesion molecule family, is expressed in various regions of the brain as well as the head and ear. To gain further insights into the roles of Cdh11 in the development of the ear, we performed behavioral tests using Cdh11 knockout (KO) mice. KO mice showed reduced acoustic startle responses and increased thresholds for auditory brainstem responses, indicating moderate hearing loss. The auditory bulla volume and ratio of air-filled to non-air-filled space in the middle ear cavity were reduced in KO mice, potentially causing conductive hearing loss. Furthermore, residual mesenchymal and inflammatory cells were observed in the middle ear cavity of KO mice. Cdh11 was expressed in developing mesenchymal cells just before the start of cavitation, indicating that Cdh11 may be directly involved in middle ear cavitation. Since the auditory bulla is derived from the neural crest, the regulation of neural crest-derived cells by Cdh11 may be responsible for structural development. This mutant mouse may be a promising animal model for elucidating the causes of conductive hearing loss and otitis media.

2. AMPA receptor activation-independent antidepressant actions of ketamine metabolite

Shizuka Kobayashi, Kenji Hashimoto[2] and Toshiya Manabe: [2]Division of Clinical Neuroscience, Chiba University Center for Forensic Mental Health, 1-8-1 Inohana, Chiba 260-8670, Japan

Ketamine, an N-methyl-D-aspartate receptor antagonist, exerts robust antidepressant effects in patients with treatment-resistant depression. The precise mechanisms underlying ketamine's antidepressant actions remain unclear, although previ-

ous research suggests that α-amino-3-hydroxy-5-methyl - 4 - isoxazole propionic acid receptor (AMPAR) activation plays a role. We investigated whether (S)-norketamine and (R)-norketamine, the two main metabolites of (R,S)-ketamine, also played a significant role in ketamine's antidepressant effects and whether the effects were mediated by AMPARs. Cellular mechanisms of antidepressant action of norketamine enantiomers were examined in mice. (S)-Norketamine had more potent antidepressant effects than (R)-norketamine in inflammation and chronic social defeat stress models. Furthermore, (S)-norketamine induced more beneficial effects on decreased dendritic spine density and synaptogenesis in the prefrontal cortex and hippocampus compared with (R)-norketamine. Unexpectedly, AMPAR antagonists did not block the antidepressant effects of (S)-norketamine. The electrophysiological data showed that, although (S)-norketamine inhibited N-methyl-D-aspartate receptor-mediated synaptic currents, (S)-norketamine did not enhance AMPAR-mediated neurotransmission in hippocampal neurons. Furthermore, (S)-norketamine improved reductions in brain-derived neurotrophic factor-tropomyosin receptor kinase B signaling in the prefrontal cortex of mice susceptible to chronic social defeat stress, whereas the tropomyosin receptor kinase B antagonist and a mechanistic target of rapamycin inhibitor blocked the antidepressant effects of (S)-norketamine. In contrast to (S)-ketamine, (S)-norketamine did not cause behavioral abnormalities, such as prepulse inhibition deficits, reward effects, loss of parvalbumin immunoreactivity in the medial prefrontal cortex, or baseline γ-band oscillation increase. Our data identified a novel AMPAR activation-independent mechanism underlying the antidepressant effects of (S)-norketamine. (S)-Norketamine and its prodrugs could be novel antidepressants without the detrimental side effects of (S)-ketamine.

3. Combating herpesvirus encephalitis by potentiating a TLR3-mTOR axis

Takahiko Chimura, Ryota Sato[3], Yasushi Kawaguchi[4], Kensuke Miyake[3] and Toshiya Manabe: [3]Division of Innate Immunity, Department of Microbiology and Immunology. [4]Division of Molecular Virology, Department of Microbiology and Immunology.

TLR3 is a sensor of double-stranded RNA that is indispensable for defense against infection with herpes simplex virus type 1 (HSV-1) in the brain. We found here that TLR3 was required for innate immune responses to HSV-1 in neurons and astrocytes. During infection with HSV-1, TLR3 recruited the metabolic checkpoint kinase complex mTORC2, which led to the induction of chemokines and trafficking of TLR3 to the cell periphery. Such trafficking enabled the activation of molecules (including mTORC1) required for the induction of type I interferons. Intracranial infection of mice with HSV-1 was exacerbated by impairment of TLR3 responses with an inhibitor of mTOR and was significantly 'rescued' by potentiation of TLR3 responses with an agonistic antibody to TLR3. These results suggest that the TLR3-mTORC2 axis might be a therapeutic target through which to combat herpes simplex encephalitis.

Publications

Kiyama, Y., Kikkawa, Y.S., Kinoshita, M., Matsumoto, Y., Kondo, K., Fujimoto, C., Iwasaki, S., Yamasoba, T. and Manabe, T. (2018). The adhesion molecule cadherin 11 is essential for acquiring normal hearing ability through middle ear development in the mouse. *Lab. Invest.* 98: 1364-1374.

Yang, C., Kobayashi, S., Nakao, K., Han, M., Qu, Y., Ren, Q., Zhang, J.-c., Ma, M., Dong, C., Toki, H., Yamaguchi, J.-i., Chaki, S., Shirayama, Y., Nakazawa, K., Manabe, T. and Hashimoto, K. (2018). AMPAR activation-independent antidepressant actions of ketamine metabolite. *Biol. Psychiat.* 84: 591-600.

Sato R., Kato A., Chimura T., Saitoh S.-I., Shibata T., Murakami Y., Fukui R., Liu K., Zhang Y., Arii J., Sun-Wada G.-H., Wada Y., Ikenoue T., Barber G.N., Manabe T., Kawaguchi Y. and Miyake K. (2018). Combating herpesvirus encephalitis by potentiating a TLR3-mTOR axis. *Nat. Immunol.* 19: 1071-1082.

Department of Basic Medical Sciences

Division of Cell Signaling and Molecular Medicine
分子シグナル制御分野

Professor	Mutsuhiro Takekawa, M.D., Ph.D.	教　授	博士(医学)	武　川　睦　寛
Assistant Professor	Yuji Kubota, Ph.D.	助　教	博士(理学)	久保田　裕　二
Assistant Professor	Takanori Nakamura, Ph.D.	助　教	博士(理学)	中　村　貴　紀

The aims of the ongoing research projects in our laboratory are to elucidate the regulatory mechanisms of intracellular signal transduction systems responsible for cell-fate decisions, such as MAP kinase cascades and Stress granules. Perturbation of these signaling systems is involved in a variety of life-threatening diseases, including cancer, autoimmune diseases, neurodegenerative disorders and type 2 diabetes. Our laboratory also aims to develop new diagnostic or therapeutic tools for currently intractable disorders in which these pathways are involved.

1. Identification of novel substrates of human mitogen-activated protein kinases.

Seina Oe, Hisashi Mori-izumi, Natusno Suzuki, Sho Tanaka, Takanaori Nakamura, Yuji Kubota, and Mutsuhiro Takekawa

Sequential activation of protein kinases within MAPK cascades is an evolutionary-conserved mechanism of intracellular signaling among eukaryotes. In mammals, at least three distinct subfamilies of MAPKs are present, namely, ERK, JNK, and p38. While the classical ERK MAPK is mainly activated by growth factors and mitogens, two relatively newly identified MAPKs, p38 and JNK, are preferentially activated by various environmental stresses (e.g., ultraviolet-light and γ irradiation, oxidative stress, DNA-damaging reagents, osmotic stress, and pro-inflammatory cytokines). Therefore, p38 and JNK MAPKs are collectively called stress-activated protein kinases (SAPKs). Each of these MAPK cascades can regulate several different and sometimes overlapping biological functions. In general, the ERK pathway mediates growth-promoting and anti-apoptotic signaling, while the p38 and JNK pathways play crucial roles in cellular stress responses such as growth arrest and apoptosis. In addition, the p38 and JNK pathways are involved in inflammatory responses. Perturbation of these crucial signal transduction pathways is involved in the pathophysiology of various life-threatening diseases, including cancer, autoimmune diseases, and neurodegenerative disorders.

Since these MAPKs exert their biological effects through the phosphorylation of their substrate proteins, the identification of which is a prerequisite for the understanding of regulatory mechanisms of critical biological phenomena. By developing a novel screening strategy, we have isolated several new MAPK substrate proteins from human cDNA libraries. These substrates include regulatory molecules for the expression of growth-promoting genes and for centrosome duplication, and several Ser/Thr protein kinases that regulate inflammation and cell death. We confirmed that these molecules were indeed directly phosphorylated by one (or more) of the human MAPKs *in vitro* as well as *in vivo* in response to mitogenic and/or stress stimuli. Thus, these molecules are bona fide substrates of MAPKs. The biological functions of these novel substrate proteins are currently under investigation in our laboratory.

2. Identification of novel genes whose expression is controlled by MAPK signaling pathways.

Yuji Kubota, Noriko Nishizumi-Tokai, Yusuke Takagi, Shiho Hirose, Yukari Shiozaki, and Mutsuhiro Takekawa

The initial cellular response to various environmental cues, such as growth factors, environmental stresses, and cytokines, is the transcriptional regulation of a set of genes that control a wide variety of biological functions. It is well known that MAPK signaling pathways play crucial roles in this process. Previous studies have shown that MAPKs directly phosphorylate and activate a bunch of transcription factors and regulators. For instance, the transcription factor ELK-1, which is a member of the ternary complex factor (TCF) subfamily, is a substrate of ERK. TCFs interact with a second transcription factor, serum response factor (SRF), and these two transcription factors jointly bind and activate serum response elements (SREs) in the promoters of immediately early genes (IEGs). Moreover, upon stress stimulation, p38 and JNK MAPKs directly phosphorylate activating transcription factor 2 (ATF2). ATF2 binds either to CRE response elements as a homodimer, or to both AP-1 and CRE sequences as a heterodimer, in which ATF2 forms a complex with other members of the ATF family or with Jun/Fos family members, thereby inducing target gene expression.

We have previously shown that TGFβ-induced p 38 activation elicits the transcriptional activation of a tumor angiogenesis inhibitor TSP1 and that the perturbation of this signaling is involved in the development of human pancreatic cancer. In this year, we comprehensively searched for human genes whose expression is transcriptionally regulated by the MAPK pathways, and have succeeded in identifying dozens of such genes. Interestingly, these transcripts include not only protein-coding mRNAs but also various non-coding, functional RNAs. The roles of these MAPK-dependent transcripts in the regulation of cell fate decisions are currently under investigation in our laboratory.

3. Development of a novel method for the detection of O-GlcNAc-modified proteins by lectin affinity gel electrophoresis.

Yuji Kubota, Ko Fujioka, and Mutsuhiro Takekawa

O-linked β-N-acetyl-glucosamine modification (O-GlcNAcylation) is a protein post-translational modifications (PTMs) that regulate various properties of proteins such as their stability, subcellular localization, and catalytic activity. O-GlcNAcylation is the result of a reaction in which a single-sugar, N-acetylglucosamine (GlcNAc), is conjugated to the hydroxyl moiety of a serine or a threonine residue of cytoplasmic and nuclear proteins, thereby dramatically altering the molecular functions of these proteins. O-GlcNAcylation is dynamically and reversibly regulated by only two enzymes: O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA). OGT catalyzes the addition of a GlcNAc moiety from a uridine diphosphate (UDP)-GlcNAc to target proteins, while OGA removes O-GlcNAc from the modified proteins. Since both O-GlcNAcylation and phosphorylation occur at serine and threonine residues, these two types of PTMs are mutually exclusive on the same target site. Indeed, various cellular proteins have been demonstrated to show dynamic crosstalk between O-GlcNAcylation and phosphorylation. Despite recent advances in understanding the importance of O-GlcNAcylation in diverse physiological processes, stoichiometric analysis of only a few O-GlcNAc proteins has been reported. The main reasons for such few reports are the high cost and the time-consuming processes required by conventional methods for quantification of protein O-GlcNAcylation. In addition, the O-GlcNAc moiety tends to be released from the modified peptide in collision-induced dissociation (CID) in mass-spectrometric analysis, which limits evaluation of the O-GlcNAcylation level of a specific protein by using this method. Hence, the development of novel methods for quantitative analysis of protein O-GlcNAcylation remains an important challenge.

To overcome this drawback, we established a novel technique for the separation of cellular O-GlcNAcylated proteins by incorporating wheat germ agglutinin (WGA), a lectin from *Triticum vulgaris,* into an electrophoretic gel. WGA has multiple GlcNAc-binding sites, and the dimerized form of this protein is highly resistant to protein denaturing conditions including acidic environments, chaotropic agents, and high temperature. This year, we developed a new electrophoretic method, termed WGA-SDS-PAGE, that separates O-GlcNAcylated and unmodified proteins, thereby allowing the detection and quantification of O-GlcNAcylated proteins. Electrophoresis of cell lysates through a gel containing copolymerized WGA selectively induced retardation of the mobility of O-GlcNAcylated proteins, thereby allowing the simultaneous visualization of both the O-GlcNAcylated and the unmodified forms of proteins. Using this novel method, we successfully detected and quantified the O-GlcNAcylation status of several proteins that regulate critical cellular functions such as signal transduction pathways and nuclear-cytoplasmic transport of mRNA and proteins. Therefore, this method enables the quantitative detection of O-GlcNAcylated proteins and is thus useful for the analysis of biological phenomena in which protein O-GlcNAcyla-

tion is involved.

4. Role of stress granule assembly in cellular stress response

Natsuha Hashimoto, Daisuke Yoshioka, Mari Taguchi, Daichi Fujikawa, Takanaori Nakamura, and Mutsuhiro Takekawa

When confronted with environmental stresses, cells either activate defense mechanisms to survive or initiate apoptosis, depending on the level and type of stress. One of the major cellular defense mechanisms is the assembly of stress granules (SGs). SGs are cytoplasmic ribonucleoprotein foci that appear when eukaryotic cells are exposed to specific types of stress such as ER stress, heat shock, hypoxia, arsenite or viral infection. The core components of SGs are large aggregates of stalled translation pre-initiation complexes that contain mRNA, 40S ribosomal subunits, translation initiation factors and several RNA-binding proteins (RBPs). In general, the assembly of SGs is triggered by stress-induced phosphorylation of eIF2α, and requires self-aggregation of certain RBPs such as TIA 1 or G3BP. In cells under various stresses, eIF2α is phosphorylated by a number of different stress-sensing kinases including PKR. Phosphorylation of eIF2α suppresses productive translation initiation by preventing formation of the eIF2-GTP-Met-tRNAi complex. Under the stress conditions, specific RBPs such as G3BP, instead of the ternary complex, interact with an mRNA in the 43S complex, leading to the assembly of a translationally stalled 48S complex. Self-oligomerization of RBPs then promotes the aggregation of these 48S complexes at discrete cytoplasmic foci termed SGs. Therefore, both RNA binding and self-oligomerization of these RBPs are crucial for the assembly of SGs.

SGs serve as sites for mRNA storage and triage. While housekeeping mRNAs are recruited to and sequestered into SGs during stress, certain mRNAs encoding proteins involved in stress tolerance are excluded from SGs. Therefore, by assembling SGs, cells temporarily reduce the synthesis of housekeeping proteins to prevent accumulation of misfolded proteins, and optimize translation of stress-responsive anti-apoptotic mRNAs. Besides mRNA sorting and translational suppression, SGs sequester several apoptosis regulatory factors into granules and thereby inhibit stress-induced cell death. We have previously reported that when cells are exposed to a SG-inducing stress, the signaling adaptor protein RACK1 is sequestered into SGs, and this sequestration inhibits the SAPK pathways and subsequent apoptosis. Thus, formation of SGs serves as a cellular adaptive defense mechanism and protects cells from apoptosis under adverse conditions, by regulating mRNA translation as well as by sequestering signaling molecules. This year, by developing a strategy to identify the molecules that reside in SGs, we discovered novel SG-components including nucleotide-binding proteins, cytoskeletal proteins, and signaling molecules (e.g., kinases, scaffold proteins, and transcriptional regulators). By analyzing some of these SG-components, we elucidated the molecular mechanism as to how SG assembly suppresses stress-induced apoptosis.

5. Elucidation of the physiological functions of MCRIP1, an ERK substrate, during epithelial-to-mesenchymal transition and embryonic development.

Takanori Nakamura, Hisashi Mori-izumi, and Mutsuhiro Takekawa

We have recently identified a novel ERK substrate, designated MCRIP1, which mediates functional crosstalk between ERK signaling and CtBP-mediated gene silencing. MCRIP1 is a previously uncharacterized protein, but is highly conserved in all vertebrates. CtBP is recruited to the promoter elements by interacting with the DNA-binding transcriptional repressor ZEB1. We found that MCRIP1 binds to CtBP, thereby competitively inhibiting CtBP-ZEB1 interaction. However, when phosphorylated by ERK, MCRIP1 dissociates from CtBP, allowing CtBP to interact with ZEB1. In this manner, the CtBP complex is then recruited to, and silences the E-cadherin promoter by inducing histone modifications. Expression of a constitutively-CtBP-binding MCRIP1 mutant profoundly inhibited ERK-induced EMT. These results delineate a molecular mechanism by which ERK signaling induces epigenetic silencing of tumor suppressive genes. This year, to more comprehensively identify the genes whose expression is regulated under the control of MCRIP1, we performed transcriptome analyses of cells that stable express either wild-type or a phosphorylation-defective mutant of MCRIP1. Furthermore, by establishing gene-engineered mice, we investigated the physiological function of MCRIP1 in the epigenetic regulation of gene expression during embryonic development.

6. Regulation of the MTK1 MAPKKK by a family of GADD45 proteins

Saeko Kawataki, Mamiko Komori, Yukari Shiozaki, Takanori Nakamura, Hisashi Mori-izumi, and Mutsuhiro Takekawa

The MTK1 MAPKKK is a selective mediator for the stress-responsive p38 and JNK signaling. Although its C-terminal kinase domain is homologous to other stress-activated MAPKKKs, its N-terminal

regulatory domain has only limited similarity, suggesting that the regulatory mechanism of MTK1 is unique. Indeed, we have previously identified three GADD45 family proteins (GADD45α/β/γ) as activators of MTK1. All GADD45 family genes are induced by various stress stimuli such as UV irradiation and DNA-damaging reagents, although the optimal stress stimuli for each gene are different. Enforced expression of any of these GADD45 proteins in cells leads to the activation of MTK1 and its downstream p38 and JNK MAPKs. GADD45-mediated activation of SAPK pathways is important particularly in the late phase of cellular stress responses, because it requires transcriptional induction and protein synthesis of GADD45 prior to activation of MTK1. Thus, GADD45-mediated MTK1 activation provokes prolonged activation of p38 and JNK signaling, which is, in some cases, critical for induction of apoptotic cell death. Indeed, overexpression of each individual GADD45 protein in cells induces prolonged p38/JNK activity and subsequent apoptotic cell death. Thus, the stress-inducible GADD45 proteins are critical signal transducers that convert various extracellular stress stimuli into intracellular SAPK signaling through activation of MTK1.

Previous studies have demonstrated that GADD 45-MTK1 signaling is involved in various biological processes during embryogenesis. Mice deficient in MTK1 develop highly penetrant neural tube defects and exencephaly, which are associated with abnormal apoptosis in neuroepithelia. Embryos homozygous for a kinase-dead MTK1 mutation exhibit a dramatic loss of phosphorylation of MKK3/6 in various tissues and display neural tube defects, skeletal malformations and dysregulated placental development. Moreover, during male genital differentiation, GADD45γ-mediated MTK1 activation leads to the expression of the Y-chromosome-linked gene *SRY*, a key molecule for initiation of the male differentiation program. Accordingly, both mice deficient in GADD45γ and those lacking MTK1 show reduced expression of male-specific gonadal markers and complete male-to-female sex reversal. While GADD45-MTK1 signaling plays pleiotropic roles during embryogenesis, it also functions in adult tissues. We have previously demonstrated that activation of the GADD45-MTK1 signaling system contributes to stress-induced apoptosis, TGFβ-mediated TSP1 expression, and IFN-γ production from Th1 cells. This year, we further examined physiological function of MTK1 in various biological processes and uncovered the new roles of MTK1 in cellular stress responses, immune reactions, and cell cycle control. Thus, MTK1-inducing signaling regulates not only embryonic development but also various cellular functions in postnatal tissues.

Publications

Kubota Y, Fujioka K and Takekawa M. Wheat germ agglutinin (WGA)-SDS-PAGE: A novel method for the detection of O-GlcNAc-modified proteins by lectin affinity gel electrophoresis. Bio-protocol 8: doi: 10.21769/BioProtoc.3098 (2018)

Ichimanda M, Hijiya N, Tsukamoto Y, Uchida T, Nakada, C, Akagi T, Etoh T, Iha H, Inomata M, Takekawa M and Moriyama M. Downregulation of DUSP4 enhances cell proliferation and invasiveness in colorectal carcinomas. Cancer Science. 109, 250-258, (2018)

久保田裕二，藤岡興，武川睦寛 O-GlcNA c化蛋白質の検出と定量的解析を可能にするレクチン親和性ゲル電気泳動法の開発と応用．生物物理化学 電気泳動．in press.