

Bren Professor of Biology: Henry A. Lester

George Grant Hoag Professor Chemistry: Dennis Dougherty

Visiting Associate: Johannes Schwarz

Members of the Professional Staff: Bruce N. Cohen, Cesar G. Labarca

Postdoctoral Fellows: Daniel J. Clayton, Chi-Sung Chiu, David S. Dahan, Mohammed Dibas, Carlos Ivan Fonck, Joanna Louisa Jankowsky, Abraham Kovoov, Ping Li, John F. Leite, Sacha Malin, Fraser John Moss, Raad Nashmi, Irina Sokolova, Andrew R. Tapper

Associate Biologist: Purnima G. Deshpande

Graduate Students: Darren Lee Beene¹, Gabriel Brandt¹, Amanda Leigh Cashin¹, Donald E. Elmore, Jr.¹, Lori WaiHang Lee¹, Sarah L. Monahan¹, Tingwei Mu¹, Julien Muffat, E. James Petersson¹, Nivalda Rodrigues-Pinguet², Julian Revie¹, George G. Shapovalov³, Eric Slimko, Amber Southwell, Steven A. Spronk¹, Michael Torrice¹, Niki Zacharias¹

Research and Laboratory Staff: Sami Barghshoon, Pamela Y.C. Fong, Kathleen Hamilton, Kyra Kostenko, Steven Kwoh, Rain K. Lynham, Sheri McKinney, Carrie Shilyansky, Michael P. Walsh

Volunteers: Qi Huang

¹*Division of Chemistry and Chemical Engineering, California Institute of Technology*

²*Special Graduate Student, Division of Biology, California Institute of Technology*

³*Division of Physics, Mathematics, and Astronomy, California Institute of Technology*

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Summary: We continue our work on ion channels, receptors, and transporters. We have continued to analyze several strains of knock-in mice generated in our laboratory for two ligand-gated channels, the nicotinic $\alpha 4$ receptor and the serotonin 5-HT₃ receptor. The nicotinic receptor work is enhanced by a promising new strain, Leu9'Ala. This work has generated interesting insights into nicotine addiction, neurodegenerative disease, and epilepsy. The 5-HT₃ receptor studies have generated insights into murine urologic syndrome.

Our work on selective silencing of mammalian neurons has generated a promising set of techniques and reagents based on ligand-activated chloride channels. We are now generating "proof of concept" transgenic mouse strains.

We continue our joint work with the Dougherty group, in Caltech's Chemistry Division, on aspects of ion channel structure-function. We have brought novel techniques to these studies, including mass spectrometry and fluorescence. We work on unnatural amino-acid

mutagenesis, and a newly acquired instrument, the OpusXpress, speeds data collection. We have now extended unnatural amino-acid incorporation to mammalian cells.

We collaborate with both Dougherty and Doug Rees, also in the Chemistry Division, on bacterial ion channels of known atomic-scale structure. This year, we helped to accomplish total synthesis and reconstitution of a functional multipass ion channel. We also gathered data at bandwidths an order of magnitude greater than usual; but gating transitions are still too fast to measure.

Our work continues on quantitative aspects of transporter function, primarily measured with fluorescence and with knock-in mice. As an interesting side benefit of the GABA transporter knock-in mouse, we have generated and analyzed a knockout mouse for the same molecule.

The late Norman Davidson led a subgroup working on aspects of synaptic plasticity, particularly those that depend on A kinase stimulation. Members of this subgroup are now analyzing their data and preparing papers for publication.

Our group's home page has additional up-to-date information, images, and notices of positions. It's at <http://www.its.caltech.edu/~lester>.

1. **Spatial-temporal separation of nicotine-induced seizures in knock-in mice with hypersensitive nicotinic receptors**

Carlos Fonck, Bruce N. Cohen, Purnima Deshpande, Cesar Labarca

We studied nicotine-induced seizures in mice with hypersensitive nicotinic acetylcholine receptors (nAChR). These mice contain the Leu9'Ala mutation in the M2 region of the nAChR $\alpha 4$ subunit which increases receptor sensitivity to agonists such as acetylcholine and nicotine. Seizure studies on $\alpha 4$ mutated mice may be relevant to epilepsy research because all known mutations linked to autosomal dominant nocturnal frontal lobe epilepsy occur in the M2 region of the $\alpha 4$ or $\beta 2$ subunits of nAChR. L9'A homozygous (hom) and heterozygous (het) mice, and their wild-type (WT) littermates received a single subcutaneous nicotine injection and the latency and intensity of seizures were recorded. 1 mg/kg nicotine caused rapid onset (20 sec) seizures in hom and het, but had no visible effect on WT mice. In WT mice, 10 mg/kg nicotine was necessary to elicit a seizure, which started 2-3 min following injection. Hom and het seizures (1 mg/kg) were clonic with rapid and repetitive movement of the extremities, whereas, WT seizures (10 mg/kg) were tonic-clonic and more violent. EEGs obtained from screw electrodes placed above the primary motor cortex and the visual cortex showed spike and wave activity in WT during seizures, but there were no EEG changes during hom and het seizures. A 10 mg/kg nicotine injection in hom resulted in two successive seizures: the first seizure started 20-30 sec following injection, was clonic and showed no EEG changes (similar to the 1 mg/kg seizures in mutant mice described above). The second seizure began 2-3 min after injection, was tonic-clonic and had

spike and wave shaped traces on the EEG (similar to the 10 mg/kg seizures in WT mice described above). In conclusion, seizures mediated by the mutated nAChR are initiated faster than those caused by WT receptors and, as assessed by EEG recordings, may involve the activation of a separate neuronal circuit. We are currently using various experimental approaches, such as c-fos expression, fMRI and multielectrode recordings to localize the mutant-like seizures in L9'A mice.

2. **Knock-in mice carrying hypersensitive $\alpha 4$ nicotinic receptors: Nicotine and morphine nociception responses**

*Carlos Fonck, Purnima Deshpande, Cesar Labarca, Raad Nashmi, M. Imad Damaj**

Neuronal nicotinic receptors (nAChR) are involved in a number of rodent behavioral responses including sedation, decreased nociception, hypothermia and seizures. It is not known what roles the various individual nicotinic receptor types play in the different behavioral responses. We created gain-of-function mice carrying hypersensitive $\alpha 4$ nAChR by introducing a Leu9'Ser mutation in the M2 region (Labarca *et al.*, 2001). In terms of agonist sensitivity, abundance and widespread brain distribution, the most important nicotinic receptor appears to be the one formed by $\alpha 4$ coassembled with $\beta 2$ subunits. We examined the role of $\alpha 4$ nAChR in acute nociceptive responses by testing mice heterozygous for the L9'A mutation (hets) and their wild-type (WT) littermates in the hot plate and the tail flick apparatus (TF), following a single injection of either nicotine or morphine. It is thought that nicotine and morphine cause analgesia, through the activation of nicotinic or μ -opioid receptors, respectively, present in descending pain-modulating pathways. In the hot plate assay, nicotine increased the latency of the pain avoidance response in hets at 0.05 to 0.5 mg/kg, and in WT at 0.5 to 2 mg/kg. Hets displayed a 5.3-fold lower ED50 than WT. The specific nicotinic-binding site blocker mecamylamine (1 mg/kg) almost completely abolished nicotine effects in both WT and het. In the TF assay, hets showed no increase in response times at informative nicotine levels. Morphine (1 to 16 mg/kg), unlike nicotine, caused equal analgesia in het and WT, both in the hot plate and the TF. These data support (1) the importance of the $\alpha 4$ subunit in mediating nicotine analgesia in the supraspinal responses measured by the hot plate, (2) the minimal $\alpha 4$ modulation of the primarily spinal reflex-dominated pathway assessed by TF and (3) the independent modulation of acute nociceptive responses by $\alpha 4$ nAChR and morphine-sensitive receptors.

**Department of Pharmacology and Toxicology, Medical College of Virginia Campus/VCU, Richmond VA 23298*

3. **Alpha4-containing neuronal nicotinic receptors modulate appetitive learning**

Cesar Labarca, Seth A. Balogh, B.J. Bowers*, S.F. Logue*, J. Ernisse*, Jeanne M. Wehner**

The present study characterized the role of $\alpha 4$ -containing neuronal nicotinic receptors (nAChRs) in learning and memory using a four-stage appetitive signaled-nosepoke task (Logue *et al.*, 1998) in 13 inbred mouse strains and in a gain-of-function $\alpha 4$ nicotinic receptor mutant (Labarca *et al.*, 2001). In inbred mouse strains, a naturally occurring polymorphism in the $\alpha 4$ nAChR subunit gene encodes either an alanine or threonine (A/T) at position 529 (Stitzel *et al.*, 2000). This A/T polymorphism is associated with differential receptor function and behavioral sensitivity to nicotine and ethanol in both inbred and recombinant inbred mouse strains. The first three phases of the nosepoke task consisted of training to associate an auditory cue with reinforcer availability. The last phase required that each mouse nosepoke only when the cue was presented. Inbred mouse strains with the 529alanine form of the polymorphism required a significantly greater number of days to learn to associate the auditory cue with the reward than those containing the 529threonine residue. The $\alpha 4$ Leu9'Ser mice are hypersensitive to acetylcholine and nicotine and have several behavioral alterations. The $\alpha 4$ Leu9'Ser mice showed enhanced associative learning in the signaled nosepoke task, relative to their wild-type littermates. These data suggest that nAChRs that contain the $\alpha 4$ subunit modulate appetitively-motivated associative learning.

**Institute for Behavioral Genetics, University of Colorado, Boulder, CO*

4. **Nicotinic acetylcholine receptors modulate the effects of ethanol and nicotine on acoustic startle**

Cesar Labarca, Jeremy C. Owens¹, Seth A. Balogh¹, Tristan D. McClure-Begley¹, Marina R. Picciotto², Jeanne M. Wehner¹, Allan C. Collins¹

Recent evidence suggests that common genes influence sensitivity to both alcohol and tobacco in humans. The studies described here tested the hypothesis that $\alpha 4\beta 2$ -containing (abbreviated $\alpha 4\beta 2^*$) nAChRs are one site of overlap. This postulate was suggested by the results of a genetic mapping analysis that used recombinant inbred strains derived from Long Sleep (LS) and Short Sleep (SS) mice. An association between ethanol effects on acoustic startle and a naturally occurring polymorphism in the $\alpha 4$ subunit of the nAChR was found in the RI strains. This agrees with our previous finding that variability in nicotine effects on acoustic startle response and the $\alpha 4$ polymorphism are significantly associated in these RI strains. We tested this hypothesis further using two mouse lines carrying targeted mutations, the $\alpha 4$ Leu9'Ser "gain-of-function" mutant and $\beta 2$ subunit null mutant mice, which do not express $\alpha 4\beta 2$ -type nAChRs. The $\alpha 4$ mutants were more sensitive to the

effects of both drugs on acoustic startle, whereas, the $\beta 2$ null mutants were less sensitive to both drugs relative to wild-type controls. These results support the postulate that $\alpha 4\beta 2^*$ nAChRs regulate the effects of both nicotine and ethanol on acoustic startle.

¹*Institute for Behavioral Genetics, University of Colorado, Boulder, CO 80309*

²*Department of Psychiatry, Yale University, New Haven, CT 06508*

5. Mice expressing a mutant form of the $\alpha 4$ nicotinic receptor subunit show altered GABAergic function as measured by nicotinic acetylcholine receptor-stimulated [³H]-GABA release

Cesar Labarca, Michael J. Marks, Tristan D. McClure-Begley*, S.R. Grady*, Jeremy C. Owens*, Seth A. Balogh*, Jeanne M. Wehner*, Allan C. Collins**

Many behavioral effects of nicotine appear to be modulated by $\alpha 4$ -containing nicotinic receptors (nAChRs). Leu9'Ser alleles with wild-type (WT) expression levels die neonatally, but heterozygotes with intact neo selection cassette in a nearby intron have decreased expression throughout the brain (Fonck *et al.*, 2003), are viable, and were studied in these experiments. L9'S mice differ from WT in sensitivity to several behavioral effects of nicotine. For example, L9'S mice are more sensitive than WT to nicotine-induced seizures (Fonck *et al.*, 2003). Many $\alpha 4$ -containing nAChRs are expressed in GABAergic neurons. Therefore, we evaluated the effects of the L9'S mutation on nAChR function by measuring nicotinic agonist-stimulated evoked [³H]-GABA release from synaptosomes prepared from several brain regions known to express $\alpha 4$ subunit-containing presynaptic receptors. Concentration-effect curves were constructed by stimulating [³H]GABA release using acetylcholine concentrations that ranged over four log units. Acetylcholine produced a concentration-dependent release of [³H]-GABA in both the WT and L9'S mice. The release profile was best fit to a two-site model in wild-type mice, but a one-site model was the best fit for the L9'S data. As expected, L9'S mice displayed an overall decrease in release; this was mostly due to a decreased low-affinity release process (stimulated by high agonist concentrations). This shift in receptor mediated GABA release is evidence for an increase in the fraction of high-affinity $\alpha 4$ containing receptors in the L9'S mice.

**Institute for Behavioral Genetics, University of Colorado, Boulder, CO 80309*

6. Hypersensitivity to peripheral thermal nociception, decreased startle reactivity, and modulation of sensorimotor gating in knock-in mice carrying hypersensitive 5-HT3 receptors

Andrew R. Tapper, Amber L. Southwell, Carrie Shilyansky, Hong Dang, Bruce N. Cohen

5-hydroxytryptamine type 3 receptors (5-HT3Rs) are the only serotonin receptors belonging to the nicotinic acetylcholine superfamily of excitatory ligand-gated ion channels. To date, 5-HT3A and 5HT3B subunits have been cloned. 5HT3A subunits can self-assemble in heterologous expression systems forming homopentameric channels while 5HT3B subunits must coassemble with 5-HT3A subunits to form functional receptors. 5-HT3 receptors have been implicated in nociception, emesis, anxiety, and alcohol abuse. In addition, 5-HT3 receptors may play a role in certain neuropsychiatric disorders such as schizophrenia and bipolar affective disorder. To gain further insight into the physiological role of 5HT3Rs we have generated a hypersensitive 5-HT3R knock-in mouse line by introducing a point mutation, V13'S, in the pore-forming M2 region of the 5-HT3A subunit via homologous recombination. When expressed in *Xenopus* oocytes, V13'S 5-HT3A subunits form receptors ~70 fold more sensitive to serotonin and become constitutively active when combined with 5-HT3B subunits. Homozygous animals exhibit reduced expression of 5-HT3A mRNA in brain and SCG as evidenced by RT-PCR. However, 5-HT induced whole-cell currents from primary cultured SCG neurons, while small, are maximally activated by 0.1 μ M serotonin, indicating that hypersensitive 5-HT3 receptors are expressed on the cell surface. Homozygous mutant mice are hypersensitive to peripheral thermal nociception compared to wild-type controls, as measured by latency to respond in the hot plate assay. However, mutant mice do not significantly differ from wild-type mice in the tail flick assay, a measure of spinal nociception. To investigate the role of 5HT3Rs in anxiety and neuropsychiatric disorders, we tested these mice with the acoustic startle response (ASR) and prepulse inhibition (PPI) assays. Male homozygous mutant animals have a lower baseline ASR and a decrease in PPI compared to wild-type mice. Interestingly, female homozygous mutants show no significant difference from wild-type in ASR or PPI. Our data indicate that 5-HT3 receptors play a role in peripheral thermal nociception and, in males, can modulate startle reactivity, as well as sensorimotor gating.

7. Design and characterization of ADNFLE mutant nAChR knock-in mice

Andrew R. Tapper, Carlos Fonck, Purnima Deshpande, Cesar Labarca, Bruce N. Cohen

Autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) is an idiopathic epileptic disorder characterized by nocturnal seizures localized within the frontal lobe arising during stage 2 sleep. Mutations within the putative pore-lining M2 helix of ionotropic neuronal nicotinic acetylcholine receptor $\alpha 4$ (CHRNA4) and $\beta 2$

(CHRNAB2) subunits have been linked to ADNFLE. When expressed in heterologous expression systems, ADNFLE-associated mutant receptors have altered channel properties compared to wild-type, suggesting that malfunctioning nicotinic receptors are responsible for the disease phenotype. Despite the molecular identification and characterization of nAChR mutations that may underlie some cases of ADNFLE, many questions remain regarding the pathophysiology of the disease.

To test the hypothesis that a knock-in mouse carrying a human mutation for ADNFLE will display seizures like the human disease, thus providing an ADNFLE animal model, we have generated a knock-in mouse line by introducing an ADNFLE-linked point mutation, Ser10^{Leu}, into the M2 transmembrane region (exon 5) of the $\alpha 4$ nicotinic acetylcholine receptor gene using homologous recombination. Knock-in mice heterozygous for this mutation are viable and fertile. We are monitoring these animals for spontaneous seizures using chronic video and EEG analysis. Characterization of mutant mice on a behavioral, neuronal, cellular and molecular level should provide valuable insights into the pathogenic mechanism and pathophysiology of ADNFLE, as well as the role of $\alpha 4\beta 2$ acetylcholine receptors in the forebrain.

8. Five ADNFLE mutations reduce Ca^{2+} dependence of the $\alpha 4\beta 2$ acetylcholine response

Nivalda Rodrigues-Pinguet, Li Jia², Maureen Li¹, Antonio Figl¹, Alwin Klaassen³, Anthony Truong¹, Bruce N. Cohen

Five nicotinic acetylcholine receptor (nAChR) mutations are currently linked to autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE). The similarity of their clinical symptoms suggests that a common functional anomaly of the mutations underlies ADNFLE seizures. To identify this anomaly, we constructed rat orthologs (S252F, +L264, S256L, V262L, V262M) of the human ADNFLE mutations, expressed them in *Xenopus* oocytes with the appropriate wild-type (WT) subunit ($\alpha 4$ or $\beta 2$), and studied the Ca^{2+} dependence of their ACh responses. All the mutations significantly reduced 2 mM Ca^{2+} -induced increases in the 30 μM ACh response. Consistent with a dominant mode of inheritance, this reduction persisted in oocytes injected with a 1:1 mixture of mutant and WT cRNA. BAPTA injections showed that the reduction was not due to a decrease in the secondary activation of Ca^{2+} -activated Cl^- currents. The S256L mutation also abolished 2 mM Ba^{2+} potentiation of the ACh response. The S256L, V262L, and V262M mutations had complex effects on the ACh concentration-response relation but all three mutations shifted the concentration-response relation to the left at $[\text{ACh}] \geq 30 \mu\text{M}$. Co-expression of the V262M mutation with a mutation (E180Q) that abolished Ca^{2+} potentiation resulted in 2 mM Ca^{2+} block, rather than potentiation, of the 30 μM ACh response, suggesting that the ADNFLE mutations reduce Ca^{2+} potentiation by enhancing Ca^{2+} block of the

$\alpha 4\beta 2$ nAChR. Ca^{2+} modulation may prevent presynaptic $\alpha 4\beta 2$ nAChRs from over-stimulating glutamate release at central excitatory synapses during bouts of synchronous, repetitive activity. Reducing the Ca^{2+} dependence of the ACh response could trigger seizures by increasing $\alpha 4\beta 2$ -mediated glutamate release during such bouts.

¹Division of Biomedical Sciences, ²Computer Science Department, University of California, Riverside, CA 92521-0121

³Psychiatry and Biobehavioral Sciences, University of California, Los Angeles, CA 90095-1759

9. Selective silencing of mammalian neurons: Optimizing the strategies using chloride channels

Eric M. Slimko, Ping Li

Glutamate-gated (GluCl) chloride channels from invertebrates can be activated by ivermectin (IVM) to produce electrical silencing in mammalian neurons. To improve this GluCl/IVM strategy, we sought to mutate the *C. elegans* GluCl channels so that they become insensitive to glutamate but retain their sensitivity to IVM. Based on structure-function studies of nAChR superfamily members, we tested in oocytes 19-point mutants at 16 residues in the β subunit likely to be involved in the response to glutamate. Y182F reduces the glutamate response by greater than 6 fold, with little change to IVM responses, when coexpressed with WT GluCl α . For GluCl $\alpha\beta$ (Y182F), the EC_{50} and Hill coefficient for glutamate is similar to those of WT, indicating that the mutant decreases the efficacy of glutamate, but not the potency. Also, fluorescent proteins (EGFP, EYFP, ECFP; XFP) were inserted into the M3-M4 loop of the GluCl α , β and β (Y182F). We found no significant functional difference between these XFP-tagged receptors and WT receptors.

Also, organisms use synonymous codons in a highly non-random fashion. These codon usage biases sometimes frustrate attempts to express high levels of exogenous genes in hosts of widely divergent species. The *C. elegans* GluCl $\alpha 1$ and GluCl β genes form a functional glutamate and ivermectin-gated chloride channel when expressed in *Xenopus* oocytes, but expression is weak in mammalian cells. We have constructed synthetic genes that retain the amino acid sequence of the wild-type GluCl channel proteins, but use codons that are optimal for mammalian cell expression. We have tagged the native and codon-optimized GluCl cDNAs with enhanced yellow fluorescent protein (EYFP, GluCl $\alpha 1$ subunit) and enhanced cyan fluorescent protein (ECFP, GluCl β subunit), expressed the channels in E18 rat hippocampal neurons, and measured the relative expression levels of the two genes with fluorescence microscopy, as well as with electrophysiology.

Codon optimization provides a six- to nine-fold increase in expression, allowing the conclusions that the ivermectin-gated channel has an EC_{50} of 1.2 nM and a Hill coefficient of 1.9. We also confirm that the Y182F

mutation in the codon-optimized β subunit results in a heteromeric channel that retains the response to ivermectin while reducing the response to 100 μM glutamate by seven-fold. The engineered GluCl channel is the first codon-optimized membrane protein expressed in mammalian cells. The modified GluCl channel, without glutamate sensitivity, with a fluorescent tag, and with optimized codons, is now being used to construct transgenic mice with cell-specific expression.

10. **Localization, trafficking and resonance energy transfer in $\alpha 4\beta 2$ nicotinic receptor-fluorescent protein chimeras**

Raad Nashmi, Mary E. Dickinson, Sheri McKinney, Mark Jareb, Cesar Labarca, Scott E. Fraser

Although the mechanisms of nicotine addiction remain unclear, altered trafficking of neuronal nicotinic receptors (nAChR) may be one contributing mechanism that modulates neuronal excitability. To study such mechanisms, we made fluorescently-tagged neuronal nicotinic receptor (nAChR) subunits. Yellow fluorescent protein (YFP) was inserted at the N-terminus or the M3-M4 intracellular loop of $\alpha 4$, and cyan FP (CFP) at the C-terminus or the M3-M4 loop of $\beta 2$. We expressed labeled $\alpha 4\beta 2$ nAChRs in HEK293T cells and cultured mesencephalic neurons, and compared their functional properties with those of unlabeled wild-type (WT) $\alpha 4\beta 2$ receptors. Nearly normal ACh sensitivity and calcium permeability was noted for receptors with YFP and CFP in $\alpha 4$ and/or $\beta 2$ M3-M4 intracellular loops; these constructs were studied further. In contrast, inserting YFP in the $\alpha 4$ N-terminus or CFP in the $\beta 2$ C-terminus dramatically inhibited nAChR function.

The somatic and dendritic distribution of fluorescently-tagged $\alpha 4$ and $\beta 2$ subunits was similar to that of endogenous $\alpha 4$ -containing receptors. Co-expressing the $\alpha 4$ -YFP and $\beta 2$ -CFP subunits resulted in fluorescence resonance energy transfer (FRET) between the subunits. In midbrain neurons, dendritic $\alpha 4\beta 2$ nAChRs displayed greater FRET than receptors inside the soma; and in HEK293T cells, a similar increase occurred for receptors that were translocated to the surface upon PKC stimulation. The maximal FRET efficiency between the $\alpha 4$ and $\beta 2$ subunits was $48 \pm 3\%$ (mean \pm SEM), suggesting a distance of 50 angstroms between the $\alpha 4$ and $\beta 2$ M3-M4 intracellular loops, in rough agreement with higher-resolution structural studies. Furthermore, $\alpha 4\beta 2$ nAChRs in neurons that were incubated with 1 μM nicotine for 24 hr displayed greater FRET than those in untreated neurons. Thus, fluorescently-tagged $\alpha 4$ and $\beta 2$ nicotinic subunits provide information about $\alpha 4\beta 2$ nAChR trafficking, assembly, and localization. In future experiments, we plan to examine the time-course of altered nicotinic receptor function and $\alpha 4$ and $\beta 2$ subunit expression with chronic nicotine exposure in cultured neurons.

11. **Conformational state-dependent hydrophobic photolabeling of the nicotinic acetylcholine receptor using electrophysiology-coordinated photochemistry and mass spectrometry**

John F. Leite, Mona Shahghol¹, Dennis A. Dougherty¹, Michael P. Blanton²

We characterized the differential accessibility of the nicotinic acetylcholine receptor $\alpha 1$ subunit (nAChR $\alpha 1$) in the open, closed and desensitized states, using electrophysiology-coordinated photolabeling by several lipophilic probes followed by mass spectrometric analysis. Voltage-clamped *Xenopus* oocytes expressing receptors were preincubated with one of the lipophilic probes and were continually exposed to acetylcholine; UV irradiation was applied during 500 msec pulses to +40 or to -140 mV (which produced closed or $\sim 50\%$ open receptors, respectively). In the open state, there was specific probe incorporation within the N-terminal domain at residues that align with the $\beta 8$ - $\beta 9$ loop of the acetylcholine-binding protein. In the closed state, probe incorporation was identified at several sites of the N-terminal domain within the conserved cysteine loop (residues 128-142), the cytoplasmic loop (M3-M4), and M4. The labeling pattern in the M4 region is consistent with previous results, further defining the lipid-exposed face of this transmembrane α -helix. These results show regions within the N-terminal domain that are involved in gating-dependent conformational shifts, confirm that the cysteine loop resides at or near the protein-membrane interface, and show that segments of the M3-M4 loop are near to the lipid bilayer.

¹*Division of Chemistry and Chemical Engineering, California Institute of Technology*

²*Texas Tech University Health Sciences Center, Departments of Pharmacology and Anesthesiology, Lubbock, TX 79430*

12. **Investigation of apparent mass deviations in electrospray ionization tandem mass spectrometry of a benzophenone-labeled peptide**

John F. Leite, Mona Shahgholi^{}, Dennis A. Dougherty^{*}*

In the study summarized above, using benzophenone-based topological probes to study conformational-dependent changes in mouse muscle nicotinic acetylcholine receptor (nAChR) topology, ESI-MS-MS analysis led to a consistent -2.0 Da mass deviation from expected values. In the present study we photolabeled a synthetic peptide corresponding to nAChR $\alpha 1$ subunit residues 130-139. MS-MS analysis of this peptide confirmed the previously observed mass deviation, associated only with fragment ions that contain the incorporated benzophenone moiety. Analysis of peak profiles for the photolabeled ions does not indicate the typical "peak fronting" that produces a mass shift when labile ions are prematurely ejected from the ion trap. Rather, H/D exchange experiments support the hypothesis that a chemical rearrangement involving phenyl migration

and ketone formation has formed an unexpected oxidized peptide, with molecular mass 2 Da less than that expected, that is isolated for collision induced dissociation in the ion trap together with the predicted precursor due to the broad ion isolation window specified.

**Division of Chemistry and Chemical Engineering,
California Institute of Technology*

13. A fluorophore attached to nicotinic acetylcholine receptor β M2-domain detects high-affinity-binding and desensitization

David S. Dahan¹, Vincent A. Auyeung², Dennis A. Dougherty¹

To study conformational transitions at the muscle nicotinic acetylcholine (ACh) receptor (nAChR), a rhodamine fluorophore was tethered to a cysteine side chain introduced at the β 19' position in the M2 region of the nAChR expressed in *Xenopus* oocytes. This procedure led to only minor changes in receptor function. Fluorescence increases ($\Delta F/F$) of ~10% occurred during agonist activation. The dose-response relations for ΔF agreed well with those for epibatidine-induced currents, but were shifted ~100-fold to the left of those for ACh-induced currents. Because (i) epibatidine binds more tightly to the γ -binding site than to the $\alpha\delta$ site and (ii) ACh binds with reverse site selectivity, these data suggest that ΔF monitors an event linked to binding at the $\alpha\delta$ subunit interface. The data do not yet allow us to determine whether the earliest detectable fluorescent state is activation or a rapid phase of desensitization. At low [ACh] ($\leq 10 \mu\text{M}$), a phase of ΔF occurs with the same time constant as desensitization, presumably monitoring an increased population of agonist-bound receptors. Following agonist washout, ΔF returns to baseline after a noticeable lag compared with the delay of agonist-induced current, but several-fold more rapidly than the agonist-induced current recovers from desensitization, showing that one or more desensitized states have fluorescence like that of the resting channel. That conformational transitions at the $\alpha\delta$ -binding site are not tightly coupled to channel activation, suggests that sequential rather than fully concerted transitions occur during receptor gating. Thus, time-resolved fluorescence changes provide a powerful probe of nAChR conformational changes.

*¹Division of Chemistry and Chemical Engineering,
California Institute of Technology*

²Caltech Undergraduate Student

14. The role of tyrosine residues at the mouse-5HT_{3A} receptor ligand-binding site investigated by unnatural amino-acid mutagenesis

Darren L. Beene¹, K.L. Price², S.C.R. Lummis²

The 5-HT₃ receptor is a member of the Cys-loop family of ligand-gated ion channels and shares a high degree of homology with nicotinic acetylcholine, GABA_A, glycine, and GluCl receptors. Previous data show that the amino acids involved in ligand binding comprise six non-

contiguous loops (A-F). Recently, tyrosine residues from binding loops C (Y234) and E (Y141, Y143 and Y153) were shown to be important for ligand binding and/or receptor gating transitions in the 5-HT₃ receptor (5HT₃R). To further characterize the role of these residues, we have used the *in vivo* nonsense suppression method to incorporate unnatural amino acids site-specifically into 5-HT₃Rs expressed in *Xenopus* oocytes. The results indicate that the -OH groups of Y143 and Y153 are critical for binding and/or function, whilst that of Y141 is not, as aromatic substitutions are well tolerated. At Y234, substitution with Phe leads to a ten-fold increase in the EC₅₀ for 5-HT; whereas, incorporation of unnatural Phe derivatives with substituents (-F, -Br, and -CH₃) at the 4 position of the aromatic ring yield wild-type EC₅₀s, suggesting that steric bulk here enhances agonist binding. These data provide support for a homology model of the 5-HT₃R extracellular domain.

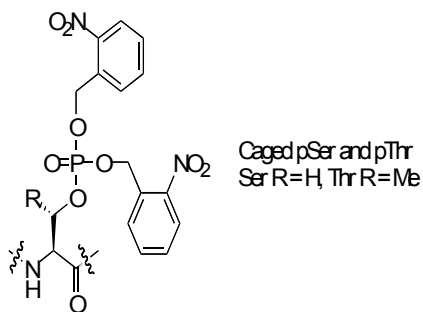
*¹Division of Chemistry and Chemical Engineering,
California Institute of Technology*

*²Department of Biochemistry, Tennis Court Road,
Cambridge, CB2 1GA*

15. Temporal control of protein phosphorylation effects with caged phosphoamino acids

E. James Petersson, Gabriel S. Brandt*, Fraser Moss, Dennis A. Dougherty**

Phosphorylation of serine, threonine, and tyrosine residues is a ubiquitous and dynamic posttranslational protein modification that results in a wide array of alterations in protein function. In the case of ion channel proteins, these effects can result in changes in the electrophysiological properties of the channel or in the channel's trafficking. Traditionally, changes in phosphorylation have been controlled by the upregulation of kinases or the downregulation of phosphatases.



16. RGS9 via its DEP domain targets to D2 dopamine receptors and mice lacking RGS9 develop dyskinesia associated with dopamine pathways

Abraham Kooor¹, Sami Barghshoon, Jason C.K. Chen², Mel I. Simon, Sigrid Schwarz³, Johannes Schwarz⁴

We discovered that RGS9-2, a member of the RGS family of G α GTPase accelerating proteins, associates with the D2-dopamine receptor (D2-DR), and preferentially accelerates the termination of D2-DR signals. We established that the DEP domain of RGS9 was both necessary and sufficient for association with D2-DR. DEP domains present in other proteins, such as disheveled, associate with G protein-coupled receptors (GPCRs) in addition to D2-DR, suggesting that the DEP domain is a GPCR targeting domain. RGS9-2 is expressed specifically in the striatum, the brain region involved in the development of neuroleptic-induced tardive dyskinesia and levodopa-induced dyskinesia. We produced similar disorders in RGS9 knockout mice when inhibition of dopaminergic transmission was followed by activation of D2 dopamine receptors (D2-DR). In addition we showed that in wild-type striatal neurons RGS9-2 and D2-DR had an identical cellular distribution pattern and D2-DR abnormally inhibited glutamate-elicited currents in striatal neurons from RGS9 knockout mice. These data support a role for RGS9-2 in suppressing the side effects associated with the treatment of psychoses and Parkinson's disease.

¹UC, San Francisco

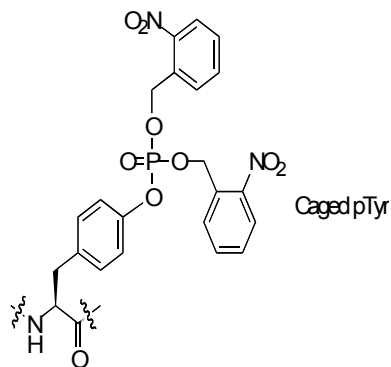
²University of Utah

³NeuroProgen GmbH Leipzig, Germany

⁴Department of Neurology, University of Leipzig, Germany

However, even in a perfectly designed experiment, these experiments are limited by the rates of the ion channel's interaction with the regulatory proteins. This prevents any precise kinetic analysis of the downstream effects of phosphorylation. We are in the process of site-specifically introducing caged, chemically-phosphorylated amino acids that can be photolyzed to reveal an authentically phosphorylated amino acid. This permits one to establish a "t=0" point for any phosphorylation experiment with \square s time resolution.

**Division of Chemistry and Chemical Engineering, California Institute of Technology*



17. RGS9 modulates dopamine signaling in the basal ganglia

J. Schwarz, A. Kooor, S.C. Schwarz, M.I. Simon, C.K. Chen, Z. Rahman^{1,2,3}, S.J. Gold^{1,3}, V. Zachariou¹, M.N. Wein³, K.H. Choi¹, R. DiLeone¹, D. Selley⁴, L. Sim-Selley⁴, M. Barrot¹, R.R. Luedtke⁵, D. Self¹, R.L. Neve⁶, E.J. Nestler^{1,3}

Regulators of G-protein signaling (RGS) proteins modulate the function of heterotrimeric G proteins in part by serving as GTPase activating proteins for G α subunits. We examined a role for RGS9-2, an RGS subtype highly enriched in striatum, in modulating dopamine D2 receptor function. Viral-mediated overexpression of RGS9-2 in rat nucleus accumbens (ventral striatum) reduced locomotor responses to cocaine (an indirect dopamine agonist) and to D2 but not to D1 receptor agonists. Conversely, mice with a null mutation in the RGS9 gene showed heightened locomotor and rewarding responses to cocaine and related psychostimulants. *In vitro* expression of RGS9-2 in *Xenopus* oocytes substantially accelerated the off-kinetics of D2 receptor-induced GIRK currents, consistent with the *in vivo* data of a net inhibitory influence of RGS9-2 on D2 receptor function. Finally, chronic exposure to cocaine increased levels of RGS9-2 in nucleus accumbens. Together, these data demonstrate a functional interaction between RGS9-2 and D2 receptor signaling and the behavioral actions of psychostimulants, and suggest that increased levels of RGS9-2 induced by psychostimulant exposure represents a compensatory adaptation that diminishes drug responsiveness.

¹Department of Psychiatry and Center for Basic Neuroscience, The University of Texas Southwestern Medical Center, Dallas, TX

²Department of Molecular, Cellular and Developmental Biology, Yale University, New Haven, CT

³Laboratory of Molecular Psychiatry, Yale University, New Haven, CT

⁴Department of Pharmacology, Virginia Commonwealth University, Richmond, VA

⁵Department of Pharmacology and Neuroscience, University of North Texas Health Science Center, Fort Worth, TX

⁶Department of Genetics, Harvard Medical School, Belmont, MA

⁷Department of Genetics, Harvard Medical School, Belmont, MA

18. Knock-in mice with hypersensitive nicotinic $\alpha 4$ receptors show selective excitotoxic cell death of midbrain dopaminergic neurons

Johannes Schwarz*, Johannes Wieacker*, Sabine Orbl*, Cesar Labarca, Carlos Fonck

We have previously shown that heterozygous neo-deleted or homozygous neo-intact knock-in mice with Leu⁹Ser hypersensitive nicotinic $\alpha 4$ receptors die shortly after birth and have a severe reduction of midbrain dopaminergic neurons. However, heterozygous neo-intact mice that express only 20–30% of mutant receptors are viable and do not exhibit any gross abnormal phenotype.

Recent analyses of these heterozygous neo-intact mice show that locomotor responses to amphetamine are reduced by ~50% compared to wild-type littermates with only subtle changes in baseline behavior. Histology revealed no major cell loss except for substantia nigra pars compacta. Cell counts of tyrosine hydroxylase-immunoreactive neurons revealed a 39.1% reduction in heterozygous animals compared to wild-type littermates in young adult animals (four months). There was an age-dependent reduction of tyrosine-hydroxylase positive neurons in both wild-type and heterozygous mice (age eight months), independent of the genotype. As a control, we counted tyrosine hydroxylase-positive neurons in locus coeruleus, revealing no difference between mutant and wild-type animals. Electrophysiological recordings from dopaminergic neurons in acute midbrain slices showed an increased sensitivity of mutant dopaminergic neurons following applications of nicotine (100 μ M). Spontaneous action potentials increased by 1.02 ± 0.25 Hz in mutant but did not change in wild-type neurons (0.05 ± 0.33 Hz). There was a shift in membrane potential (peak of afterhyperpolarization) by 1.83 ± 0.38 mV in mutant but only 0.71 ± 0.6 mV in wild-type neurons. Thus, in heterozygous neo-intact L⁹S nicotinic $\alpha 4$ receptor knock-in mice with limited expression of mutant receptors, there is loss of dopaminergic neurons most likely due to cholinergic excitotoxicity.

*Department of Neurology, University of Leipzig, Leipzig, Germany

19. GABA transporter (GAT1)-deficient mice display ataxia, tremor, reduced locomotor activity, increased body temperature fluctuation, and increased GABA receptor-mediated tonic conductance in cerebellar granule and Purkinje cells

Chi-Sung Chiu, Stephen Brickley¹, Kimmo Jensen^{2,3}, Amber Southwell, Sheri McKinney, Stuart Cull-Candy¹, Istvan Mody²

We created GABA transporter subtype I-deficient mice, mGAT1 knockout (KO), by gene targeting. The mGAT1-deficient mice reproduce and have normal muscle strength and life span, but reduced body weight (female -10%; male -20%), and motor disorders including gait abnormality, reduced time on rotarod, constant tremor at 25-32 Hz, and reduced locomotor activity in the home cage. In open-field tests, mGAT1-deficient mice display delayed exploratory activity, reduced rearing, and reduced visits to the central area, although without change in total distance traveled. Furthermore, the mGAT1-deficient mice show no difference in acoustic startle response, but a deficiency in prepulse inhibition. The open-field and prepulse inhibition results suggest that the mGAT1-deficient mice show mild anxiety or episodic nervous behavior. The knockouts also displayed higher body temperature fluctuations in 0.2-0.9 h⁻¹ frequency. These behaviors are partially phenocopy effects of tiagabine, a GAT1-specific inhibitor, suggesting that they arise directly from GAT1 deficiency. Compromised levels of GABA uptake resulted in an increased GABA_A receptor-mediated tonic conductance in cerebellar granule and Purkinje cells and prolonged decay of spontaneous IPSCs. The behavioral defects associated with excessive extracellular GABA in this animal illustrate the importance of GABA transporters in the regulation of correct neural function. Immunocytochemistry shows no detectable loss of GABAergic interneurons, no change in the number of GABA_A-, GABA_B-, or GAD65-immunoreactive structures, and no change in GAT3 expression pattern in hippocampus and cerebellum, suggesting little or no compensatory expression of other proteins related to GABA transmission.

¹Department of Pharmacology, University College London, WC1E 6BT, London

²Departments of Neurology and Physiology, UCLA School of Medicine, Los Angeles, CA 90095-1769

³University of Aarhus, Denmark

20. GABA transporter-1 (GAT1) deficient mice: Differential tonic activation of GABA_A versus GABA_B receptors in the hippocampus

Chi-Sung Chiu, Irina Sokolova, Kimmo Jensen*, Istvan Mody*

Following its release from interneurons in the central nervous system (CNS), the major inhibitory neurotransmitter GABA is taken up by GABA transporters (GATs). The predominant neuronal GABA transporter GAT1 is localized in GABAergic axons and nerve terminals, where it is thought to influence GABAergic

synaptic transmission, but the details of this regulation are unclear. To address this issue, we have generated a strain of GAT1-deficient mice. We observed a large increase in a tonic postsynaptic hippocampal GABA_A receptor-mediated conductance. There was little or no change in the waveform or amplitude of spontaneous IPSCs or miniature IPSCs. In contrast, the frequency of quantal GABA release was one-third of WT, although the densities of GABA_A-receptors, GABA_B-receptors, GAD65 and VGAT1 were unaltered. The GAT1-deficient mice lacked a presynaptic GABA_B-receptor tone, present in WT mice, which reduces the frequency of spontaneous IPSCs. We conclude that GAT1 deficiency leads to enhanced extracellular GABA levels resulting in an overactivation of GABA_A-receptors responsible for a postsynaptic tonic conductance. Chronically elevated GABA levels also downregulate phasic GABA release and reduce presynaptic signaling via GABA_B receptors, thus causing an enhanced tonic and a diminished phasic inhibition.

**Departments of Neurology and Physiology, UCLA School of Medicine, Los Angeles, CA 90095*

21. Resting tremor of GABA transporter type I (GAT1)-deficient mice is modulated by benzodiazepine treatment

Amber L Southwell, Chi-Sung Chiu

Tremor, or the involuntary contraction of opposing muscles in a rhythmic or oscillatory way, is the most common movement disorder in humans. To investigate the role of GABA in tremorgenesis, our laboratory has studied a GAT1-deficient mouse. This mouse exhibits several abnormal phenotypic characteristics, including a 25-32 Hz resting tremor. The anticonvulsant Tiagabine, a specific inhibitor of GAT1, induces tremor in human patients, indicating that the tremor of the GAT1-deficient mouse is the result of increased extracellular GABA concentrations rather than developmental alterations. Power spectrum analysis of mechanical transducer data gathered from wild-type mice, GAT1-deficient heterozygous and GAT1-deficient homozygous mutants show a peak at 25-32 Hz that occurs only in the homozygous mutant representing the pathological tremor. Flunitrazepam treatment (10 and 15 mg/kg IP) dose dependently increases the amplitude and decreases the frequency of the pathological tremor peak, while having very little effect on the spectrum of wild-type mice. This indicates that the tremor of the GAT1-deficient mouse results from over stimulation of benzodiazepine-sensitive GABA_A receptors

22. Activity of *E. coli* mechanosensitive channels recorded at bandwidths of up to 300 kHz

George Shapovalov

We have modified a patch-clamp setup to record and analyze at low noise and high temporal resolution recordings from bacterial ion channels. We modified the headstage to house a specially created Teflon pipette holder following Parzefall, Wilhelm *et al.* (1998) and used quartz pipettes in order to decrease stray capacitance. The

chamber was shielded by placing it inside a specially prepared aluminum shed that formed a Faraday cage. An inner layer of shielding was created from aluminum foil near chamber and pipette assembly. The Axoclamp 200B amplifier was modified to bypass the internal 10 kHz filter, allowing faster than 100 kHz signals to be recorded. We used an external 8-pole Bessel unit to provide anti-alias filtering. The signal was digitized at 1 MHz with a modified Digidata 1322A by Axon.

Bacterial MscL and MscS channels have an unusually large conductance, which renders them suitable for achieving higher signal-to-noise ratio. For example, the MscL channel produces single channel currents of 300 pA at 100 mV applied across the membrane. Combination of these properties of the channels and modifications that we did to instrumentation allowed us to achieve an acquisition bandwidth of 300 kHz with a corresponding temporal resolution of 2-3 μ s. We registered closed channel rms. noise of 15 pA. At such resolution *E. coli* MscL channels demonstrated rich gating kinetics, visiting many subconductive states. The *E. coli* MscS channel has smaller total conductance of 1 nS, however, only one conductive state has been reported. This renders the MscS channel a more favorable candidate for studying properties of state transitions.

Elementary transition events were collected via the template search routine provided by Clampfit 9. Selected fragments were further aligned with a sub-microsecond resolution to minimize deviation of central parts of individual fragments from average. Mean 10-90 rise time of observed transitions corresponds to the resolution limit set by the instrumentation (2-3 μ s).

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23. Synthesis and reconstitution of functional mechanosensitive channels

Daniel Clayton, George Shapovalov, Joshua Maurer¹, Dennis A. Dougherty¹, Gerd Kochendoerfer²

Our objective was to achieve robust synthetic access to functional MscL protein. We have succeeded in producing multi-mg quantities of biotin-labeled full-length MscL from both *M. tuberculosis* and *E. coli*. Biotin was incorporated as an affinity tag for Alexafluor-488-labeled streptavidin to facilitate analysis of the reconstitution of MscL into lipid vesicles. We then proceeded to incorporate both polypeptides into multilamellar vesicles. Bright-field and fluorescence images of *E. coli*-MscL incorporated into azolectin vesicles showed intense fluorescence in the lipid bilayer regions of the vesicles only after MscL reconstitution, indicating preferential incorporation of the synthetic channel protein into the lipid bilayer. Single- and multi-channel conductance recordings were then obtained on inside-out patches from vesicle preparations similar to those used in fluorescence experiments. Synthetic MscL exhibited comparable

activity to recombinant wild-type protein, as indicated by a single-channel conductance of ~2.5 nS, and similar gating characteristics under suction [average midpoints for the probability of opening curves are -1.0 (SD 0.16, n=6) for synthetic and -1.3 PSI (SD 0.25, n=10) for recombinant *E. coli*-MscL]. This reconstitution procedure was successfully applied to full-length Tb-MscL protein, with comparable results for reconstitution, and wild-type channel behavior was observed.

¹*Division of Chemistry and Chemical Engineering, California Institute of Technology*

²*Gryphon Therapeutics, San Francisco, CA*

24. Gain-of-function mutation of 5-HT₃ receptor leads to obstructive uropathy in mice: *In vivo* and *in-vitro* pharmacology

Annindya Bhattacharya¹, Quan-Ming Zhu¹, Hong Dang, Gary Cain¹, Nora Rosengurt², Debra A. Cockayne¹, Anthony P.D.W. Ford¹

Gene knock-in mice carrying a gain-of-function (V to S) mutation in the 13' position of the α subunit of the 5-HT₃ receptor (5-HT₃^{*}) exhibit premature death due to obstructive uropathy. To characterize this urological pathology, we measured bladder activity in homozygous 5-HT₃^{*} mice and investigated responses to pharmacological manipulation. Histopathology of lower urinary tract tissues from 5-HT₃^{*} mice revealed mucosal and smooth muscle hyperplasia of urinary bladder and prostate with secondary inflammation and bacterial infection. Cystometric evaluation of 14 week-old 5-HT₃^{*} mice revealed highly distended bladders with impaired voiding. In contrast to the micturition contractions seen in wild-type controls, 5-HT₃^{*} mice exhibited urinary dribbling and frequent non-micturition bladder contractions (NMBC). NMBC in 5-HT₃^{*} mice were attenuated by prazosin (0.3 mg/kg, sc), and bladder contractions appeared to be less responsive to carbachol (ivc) with contractions seen only at 10-4 M, compared to the potent contractions seen in wild-type controls at 10-6 – 10-4 M. *In vitro* analysis on isolated bladder strips from these animals demonstrated that carbachol (10-5 M) and KCl (67 mM) produced significantly reduced contractions. In contrast, isolated bladder strips from naïve eight week-old 5-HT₃^{*} mice contracted to carbachol and KCl with same maximal response as that of wild-type controls, but failed to exhibit bladder contractions to ATP (10-4 M) and neurogenic electrical field stimulation. Therefore, 5-HT₃^{*} mice exhibit histopathological and cystometric changes indicative of bladder outlet obstruction. Compared to wild-type controls these mice show bladder and prostate hyperplasia, bladder distension, urine dribbling and frequent NMBCs. Bladder tissue from 5-HT₃^{*} mice fail to contract to nerve stimulation and ATP, and show a progressive loss of carbachol and KCl-induced maximal contraction. This is the first study showing that disturbances of 5-HT₃ receptor function lead to genitourinary disease.

¹*Genitourinary Therapy Area, Roche Palo Alto, Palo Alto, CA 94304*

²*Department of Pathology and Laboratory Medicine, UCLA, Los Angeles, CA 90095*

25. Site-specific incorporation of unnatural amino acids into receptors expressed in mammalian cells

Sarah L. Monahan^{}, Dennis A. Dougherty^{*}*

We describe an approach to achieve unnatural amino acid incorporation into channels and receptors expressed in mammalian cells. We show that microelectroporation provides a general method to deliver DNA, mRNA, and tRNA simultaneously. In both CHO cells and cultured neurons, microelectroporation efficiently delivers an *in vitro* transcribed, serine amber suppressor tRNA, leading to nonsense suppression in a mutant EGFP gene. In CHO cells both natural and unnatural amino acids chemically appended to a suppressor tRNA are site-specifically incorporated into the nicotinic acetylcholine receptor (nAChR). Electrophysiology confirms the expected functional consequences of the unnatural residue. The microelectroporation strategy described here is more general, less tedious, and less damaging to mammalian neuronal and non-neuronal cells than previous approaches to nonsense suppression in small cells, and provides the first example of unnatural amino acid incorporation in mammalian cells using chemically aminoacylated tRNA.

^{*}*Division of Chemistry and Chemical Engineering, California Institute of Technology*

26. Different binding orientations for the same agonist at homologous receptors: A lock and key or a simple wedge?

Tingwei Mu^{}, Dennis A. Dougherty^{*}*

We studied two homologous binding sites for the neurotransmitter serotonin (5-HT) the 5-HT₃ receptor and the MOD-1 receptor of *C. elegans*. Key aromatic residues of the agonist-binding site are conserved in the two. We saw MOD-1 as presenting an opportunity to quantify a cation- π interaction between serotonin and a Tyr, allowing a direct comparison with the serotonin•••Trp interaction in 5-HT₃. Therefore we used the *in vivo* nonsense suppression methodology for unnatural amino acid incorporation to substitute Tyr 180 of MOD-1 with several Tyr analogues, including fluorinated residues of the sort that were so informative in studying the tryptophan interaction. These studies led to no clear conclusions. However, another canonical aromatic residue of the agonist-binding site is Tyr 198 of the nAChR, aligning with Tyr 234 of 5-HT₃ and Tyr 192 of AChBP. This Tyr is conserved in essentially all members of the family, but it is Trp 226 in MOD-1. Given our earlier successes with studies of fluorinated Trp derivatives, we applied the same protocol to Trp 226 of MOD-1. The same trend seen for Trp 183 of 5-HT₃ is seen for Trp 226 of MOD-1. Both serotonin lines have steeper slopes than the analogous ACh line (as expected based on electrostatic arguments), and the two serotonin lines have identical slopes. Such agreement means that the primary ammonium of serotonin makes a strong cation- π interaction with a Trp in both systems, but

the two homologous receptors use a different tryptophan to make the cation- π interaction to serotonin. Our results show that, instead of settling for the tyrosine, the agonist reorients in the binding site to contact a nearby tryptophan and thus, maximize the cation- π interaction.

**Division of Chemistry and Chemical Engineering, California Institute of Technology*

27. **Modulation of postsynaptic proteins is essential for forskolin-induced potentiation of synaptic transmission**

Irina V. Sokolova, Norman Davidson

Activation of protein kinase A (PKA) by forskolin induces enhancement of glutamatergic synaptic transmission in the CA1 area of the hippocampus. Classical quantal analysis of synaptic transmission suggests that PKA-induced potentiation arise via increased presynaptic release probability. To investigate whether postsynaptic events also contribute to the forskolin-induced potentiation of excitatory synapses in culture, we studied dissociated hippocampal cultured neurons using either perforated patch or whole-cell configuration (PPC or WCC, correspondingly). Paired recordings using PPC revealed that 15 min forskolin perfusion leads to the short-term potentiation (STP) of evoked EPSCs (eEPSCs) in all six cell pairs tested [$234 \pm 10\%$ (SE)]. STP was followed by the long-term potentiation (LTP) of eEPSCs in five of six cell pairs tested ($143 \pm 7\%$ (SE)). LTP lasted for ≥ 80 min after forskolin washout. When a postsynaptic neuron was voltage-clamped by WCC, however, forskolin-induced STP was significantly attenuated ($152 \pm 8\%$ (SE), $p < 0.01$) and LTP was disrupted in all six-cell pairs tested. In experiments using PPC, 15 min forskolin perfusion also induced an increase of mEPSC frequencies. The frequency of mEPSCs was maximal after 15 min of forskolin perfusion ($445 \pm 106\%$ (SD) of baseline level, five cells) and gradually declined reaching the baseline level 45 min after the start of forskolin washout (three cells). The time-course of mEPSC frequencies in WCC experiments resembled that in the PPC experiments; however, in WCC the maximal upregulation of mEPSC frequency 15 min after the start of forskolin perfusion was significantly attenuated ($275 \pm 18\%$ (SD) of baseline, three cells, $p < 0.05$). Overall, these results demonstrate that modulation of postsynaptic proteins by PKA, and diffusible postsynaptic factors, are essential for the forskolin-induced potentiation of synaptic transmission.

28. **Requirement of a critical period of GABAergic receptor blockade for induction of a cAMP-mediated long-term depression at CA3-CA1 synapses**

Tzu-ping Yu, Norman Davidson

Previous reports show that bath application of the adenosine 3':5'-cyclic monophosphate (cAMP) analog, Sp-cAMPS, induces a protein kinase A (PKA)-dependent and protein synthesis-dependent long-term potentiation (LTP) at hippocampal CA3-CA1 synapses. Recently we reported

a novel form of long-term depression (LTD) induced by concurrent application of Sp-cAMPS and picrotoxin, a γ -aminobutyric acid type A (GABA_A) receptor antagonist. In the present study, we further investigate the mechanisms underlying such cAMP-mediated LTD. Synaptically connected CA3 and CA1 cells of hippocampal slice cultures were impaled by sharp electrodes. Excitatory postsynaptic potentials recorded from a CA1 pyramidal cell were evoked by single action potentials in a CA3 cell. Picrotoxin was applied to slices at various time points after Sp-cAMPS was perfused. We found that Sp-cAMPS-induced potentiation could be converted to depression when picrotoxin was applied within 30 min after perfusion of Sp-cAMPS. Picrotoxin applied 1 h after perfusion of Sp-cAMPS had no effect on Sp-cAMPS-induced synaptic potentiation. Once LTP was induced by Sp-cAMPS and expressed for 1 h, the subsequent application of Sp-cAMPS and picrotoxin produced no new changes in synaptic strength. Also, once LTD was induced and expressed for 1 h, subsequent Sp-cAMPS produced no new changes in synaptic strength. These findings suggest that a synapse is committed irreversibly to cAMP-mediated LTP or LTD during a critical period, and that later signals cannot interconvert these two fates.

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