Chapter 4

Natural and Unnatural Amino Acid Incorporation into Ion Channels Expressed in Mammalian Cells by Nonsense Suppression

4.1 Introduction

Unnatural amino acid incorporation into proteins by nonsense suppression has proven to be a valuable tool for structure-function studies [1-5]. Using the *in vivo* nonsense suppression methodology [6], information on ligand binding and ion channel gating mechanisms has been obtained on a variety of ion channels including the nicotinic ACh receptor (nAChR) [7-12], 5-$HT3_A$ receptor [13], and the Shaker [14] and Kir2.1 [15] potassium channels. To date, such studies have been limited to the *Xenopus* oocyte heterologous expression system. There would be clear benefits to expanding the technology to a mammalian cell expression system. This would provide a more relevant environment for many proteins of mammalian origin and would allow for studies of cell-specific signal transduction pathways.

With the electroporation protocol in hand (developed using the HSAS assay, Chapter 3), we were ready to apply the methodology toward the delivery of aminoacyl-tRNA to mammalian cells. Our initial assay involved the suppression of EGFP using THG73-aa. THG73 tRNA has proven to be effective for unnatural amino acid incorporation in the *Xenopus* oocyte expression system. We co-electroporated a variety of EGFP TAG mutants, with a variety of amino acids ligated to THG73, but never observed EGFP expression by nonsense suppression (details in Chapter 2). Although discouraging, we felt that this was most likely because the suppressed EGFP was below our detection limit. What we needed, therefore was a more sensitive assay.

Electrophysiology is one of the most sensitive assays available. Single ion channels can be detected within a cell because ion channels conduct electrical current, a

very sensitive measurement. Because it was ultimately of interest to incorporate unnatural amino acids into ion channels using the mammalian cells expression system, we decided to abandon the EGFP assay and to move on to ion channel expression instead. As described in the following sections, we first demonstrate natural and unnatural amino acid incorporation by nonsense suppression into the nicotinic acetylcholine receptor (nAChR), an ion channel that our lab is very familiar with. We then try to apply the methodology to less familiar systems, including the neuronal α4β2 AChR, and the neuronal NMDA (N-methyl D-aspartate) receptor.

4.2 Results and Discussion

4.2.1. nAChR expression by HSAS suppression

The nicotinic acetylcholine receptor (nAChR) is the most well-studied neuronal receptor [16]. The nAChR belongs to a superfamily of ion channels that includes the 5-HT3, GABA and glycine receptors, and functions both at the neuromuscular junction and within the CNS. The muscle-type nAChR is a pentameric channel composed of two α subunits, and one of each β, δ, and γ. Our lab has studied the ligand-binding domain of the muscle-type nAChR extensively, using unnatural amino acid incorporation. These experiments thus far have been limited to the *Xenopus* oocyte expression system [6], so we were interested in extending the methodology to a mammalian cell system. This will allow us to perform studies of cell-specific signal transduction pathways, including those that involve the muscle-type nAChR.

In initial experiments, we wanted to establish that ion channels are efficiently expressed in mammalian cells by nonsense suppression. To do this, we turned to the HSAS assay. We relied on the mutation of a Leu residue in the M2 pore lining region, termed Leu9' [17], that is conserved in all known nAChR subunits. Earlier studies in *Xenopus* oocytes showed that the Leu9'Ser mutation of the β subunit leads to a ~ 40-fold decrease in the EC_{50} when compared to the wild-type channel [10, 18]. Therefore, suppression of the β9'TAG by HSAS should lead to expression of channels that display a substantial shift in the dose-response relation, the characteristic βLeu9'Ser phenotype.

4.2.1.1 Results

Transfection of CHO-K1 cells was achieved by electroporation of a 5 μl solution containing HSAS, mutant β subunit mRNA (Leu9'TAG), and mRNA for the remaining wild-type subunits (α, δ, and γ). Also included was a reporter EGFP plasmid. Expression of the nAChR was determined from whole-cell recordings of ACh-induced currents in EGFP-expressing CHO-K1 cells.

As shown in Figure 4.1, 24 hours after transfection the cells exhibit a strong ACh response that is not observed in non-transfected cells. All GFP-expressing cells exhibit an ACh response. Both the receptors generated from HSAS-suppressed β9'TAG mutant mRNA and from the βLeu9'Ser conventional mutant showed substantial decreases in their EC_{50} values, relative to wild-type. Interestingly, the shift seen in CHO cells (~ 10 fold) is smaller than that seen in *Xenopus* oocytes, perhaps due to differential processing in the two different cell types. For the present purposes, however, the key is that the shift seen is the same whether the Leu9'Ser mutant is made by conventional mutagenesis or

nonsense suppression. This demonstrates that HSAS did indeed deliver serine during translation of the βLeu9'TAG subunit.

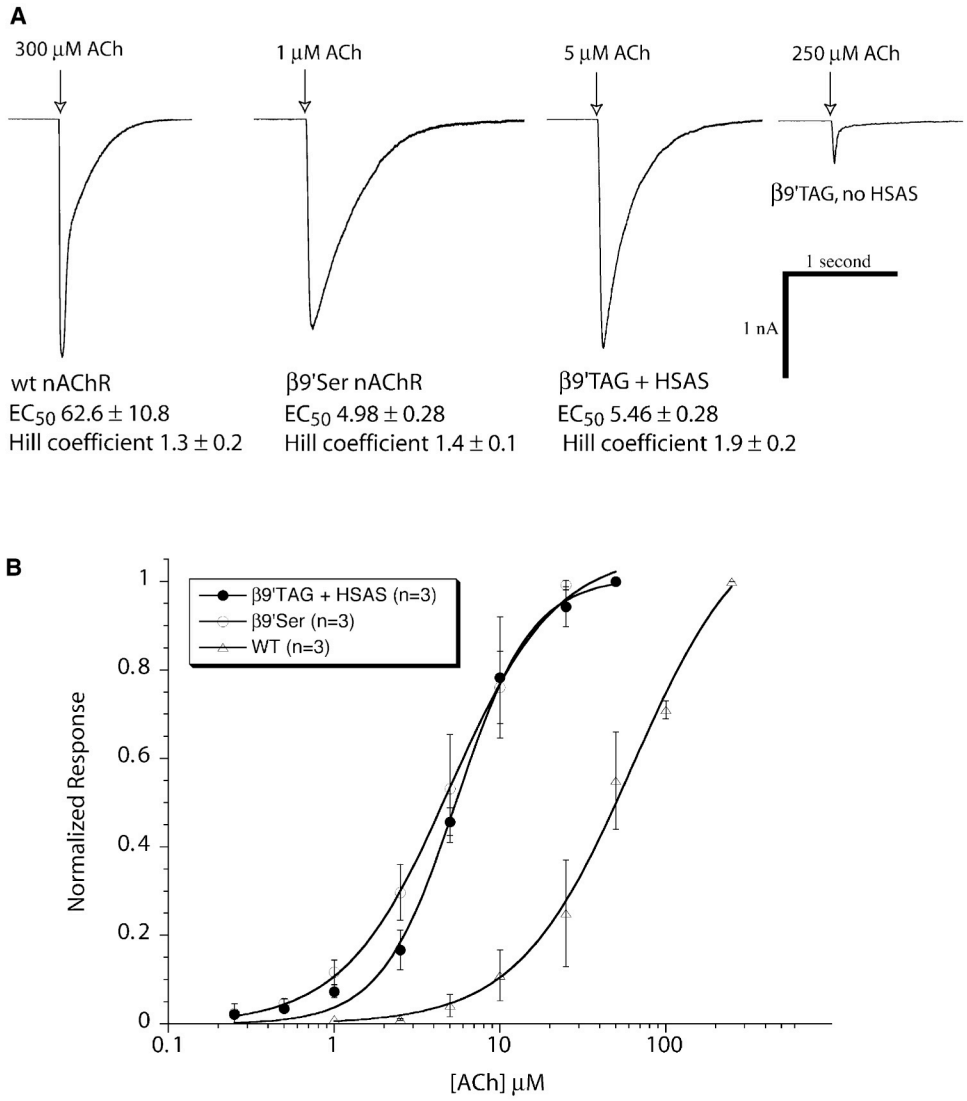


Figure 4.1. nAChR expression in CHO-K1 cells by nonsense suppression using HSAS tRNA. (A) CHO-K1 cells were electroporated with a 5 μl solution containing α, β, δ, and γ, nAChR subunit mRNA, and reporter plasmid. Arrows indicate 25 ms pulses of ACh application. The first trace shows a typical response from cells transfected with the wild-type nAChR subunits (2 μg/μl α, 0.5 μg/μl each β, δ, and γ). The second trace is a response of cells transfected with the βLeu9'Ser mutant (0.15 μg/μl) and the remaining wild-type subunits (2 μg/μl α, 0.5 μg/μl each δ, and γ). The third trace is a response from cells transfected with mutant βLeu9'TAG mRNA (0.15 μg/μl), the remaining wild-type subunits (1 μg/μl α, 0.5 μg/μl each δ, and γ), and HSAS tRNA (2 μg/μl). The fourth trace shows a response from cells transfected with mutant βLeu9'TAG mRNA and other subunits, in the absense of HSAS tRNA (read-through). The reporter plasmid EGFP-N1 (0.5 μg/μl) was included in all cases, and recordings were done from EGFP-expressing cells. The corresponding EC_{50} values are also shown. (B) ACh dose-response curves for wt nAChR (triangle), βLeu9'Ser nAChR (open circle), and HSAS-suppressed nAChR (closed circle).

4.2.1.2 Discussion

We demonstrate here that CHO-K1 cells readily aminoacylate exogenous HSAS tRNA with serine, and use it for expression of the nAChR by nonsense suppression. This is clearly demonstrated by HSAS nonsense suppression of β Leu9'TAG of the muscle type nAChR, which shows the same characteristic ACh EC_{50} shift as the conventional β Leu9'Ser mutant. Importantly, the magnitude of the ACh response for the HSAS-suppressed channels is comparable to those for the wild-type and βLeu9'Ser nAChR systems. This establishes that ion channel expression is not tRNA limited, validating that electroporation leads to highly efficient delivery of tRNA to the cells.

4.2.2 nAChR expression by unnatural amino acid incorporation

With the basic protocol being established using the HSAS suppressor, we turned our attention to unnatural amino acid incorporation. Because we are familiar with incorporating unnatural amino acids into the muscle-type nAChR in the *Xenopus* oocyte expression system, we turned to this receptor to optimize aminoacyl-tRNA delivery to mammalian cells. Using our previous experience, we designed an experiment that would produce a distinct phenotype upon unnatural amino acid incorporation. Our studies of the agonist-binding site of the nAChR established a critical role for Trp α149 in agonist binding [12]. Specifically, incorporation of a fluoronated Trp series at α Trp149 - and only α Trp149 - leads to a right shift of the ACh EC_{50} with increasing Trp fluorination. In fact, when the EC_{50} ($\log[EC_{50}/EC_{50}wt]$) is plotted against the calculated gas-phase cation-π binding energy for each fluorinated Trp at α149, there is a linear relationship (Figure 4.2). We attribute this to a decrease in the cation-π binding ability of the fluoro-

Trp derivatives. We therefore set out to reproduce these results in a mammalian cell expression system.

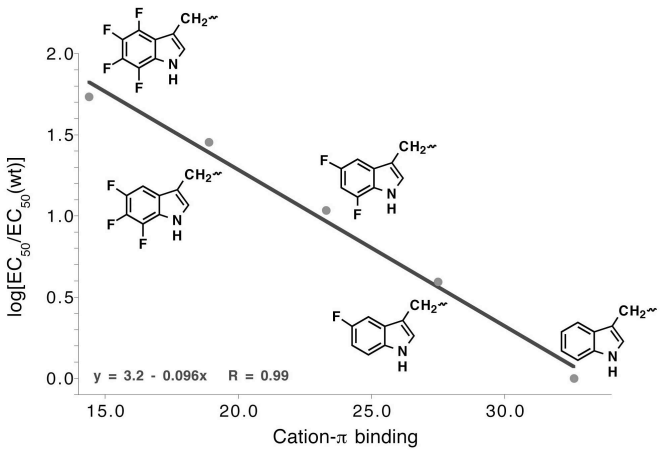


Figure 4.2. The αTrp149 of the muscle-type nAChR interacts with acetylcholine via a cation-π type interaction. This plot demonstrates a linear correlation between *ab initio* calculations of the cation-π binding energy of the fluorinated Trp residues shown, and the in vivo measurements of the EC_{50}, measured for acetylcholine.

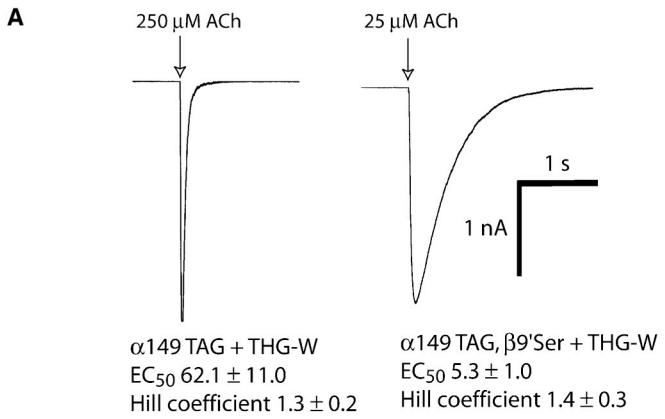
4.2.2.1 Results

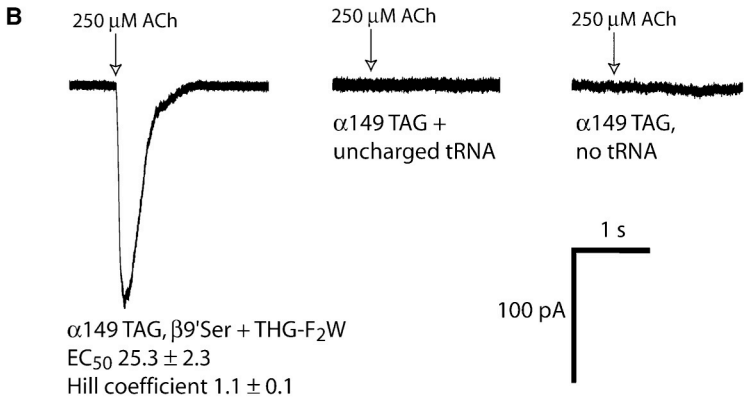
Using the electroporation protocol, we delivered mutant α subunit mRNA (149TAG), mRNA for the remaining subunits (βLeu9'Ser mutant, δ, and γ), and a reporter EGFP plasmid. Also included was the tRNA THG73 that has proven to be effective for studies in *Xenopus* oocytes, chemically aminoacylated with either Trp (wild-type) or 5,7-difluorotryptophan (THG-F_2Trp).

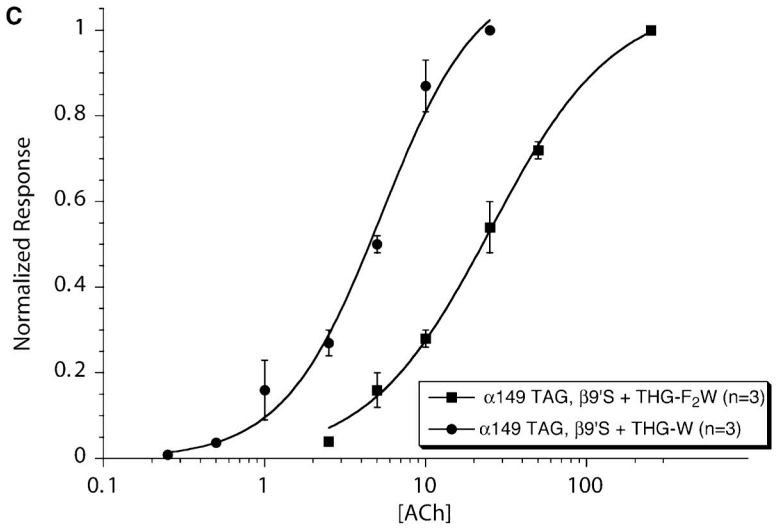
In measurements 24 hours after transfection, currents from 100 pA to 2 nA are seen in response to saturating ACh concentrations. As shown in Figure 4.3, when THG73 is used to deliver Trp, a wild-type channel is produced. Most importantly, THG73 aminoacylated with F_2Trp leads to a characteristic shift in the dose-response curve to higher EC_{50}. The results agree well with analogous experiments performed in

Xenopus oocytes, and they convincingly demonstrate successful incorporation of an unnatural amino acid. Control experiments using THG73 that has not been aminoacylated gave no response.

Figure 4.3 (*next page*). Incorporation of natural and unnatural amino acids into the nAChR expressed in CHO-K1 cells by nonsense suppression using chemically aminoacylated THG73 tRNA. CHO-K1 cells were elctroporated with a 5 μl solution containing α149TAG, β or βLeu9'Ser, δ, and γ nAChR subunit mRNA, THG-aa tRNA, and reporter plasmid. Arrows indicate 25 ms pulses of ACh application. (A) The first trace shows wild-type recovery of the nAChR by suppression of α149TAG mRNA with THG73 tRNA aminoacylated with Trp (THG-W) (2μg/μl α149TAG, 0.5 μl each β, δ, and γ, 4 μg/μl THG-W). The second trace shows wild-type recovery of the βLeu9'Ser mutant nAChR channel by suppression of α149TAG mRNA with THG-W (2μg/μl α149TAG, 0.5 μl each βLeu9'Ser, δ, and γ, 4 μg/μl THG-W). (B) The first trace shows ACh response from a cell expressing the unnatural amino acid 5,7-difluorotryptophan (THG-F_2W) at α149 of the nAChR (2μg/μl α149TAG, 0.5 μl each βLeu9'Ser, δ, and γ, 3 μg/μl THG-F_2W). The second and third traces show that there is no ACh response from cells transfected with mRNA only, with or without uncharged tRNA. (C) ACh dose response curves for THG-W suppressed α149TAG/βLeu9'Ser nAChR (closed circle) and THG-F_2W suppressed α149TAG/βLeu9'Ser nAChR (closed square).


A
250 μM ACh
25 μM ACh
1 s
1 nA
α149 TAG + THG-W
EC50 62.1 ± 11.0
Hill coefficient 1.3 ± 0.2
α149 TAG, β9'Ser + THG-W
EC50 5.3 ± 1.0
Hill coefficient 1.4 ± 0.3


B
250 μM ACh
250 μM ACh
250 μM ACh
α149 TAG +
uncharged tRNA
α149 TAG,
no tRNA
1 s
100 pA
α149 TAG, β9'Ser + THG-F2W
EC50 25.3 ± 2.3
Hill coefficient 1.1 ± 0.1


C
Normalized Response
1
0.8
0.6
0.4
0.2
0
0.1
1
10
100
[ACh]
α149 TAG, β9'S + THG-F2W (n=3)
α149 TAG, β9'S + THG-W (n=3)

4.2.2.2 Discussion

We show that microelectroporation can deliver chemically aminoacylated tRNA to mammalian cells. This is the first example of site-specific incorporation of an unnatural amino acid into a protein expressed in a mammalian cell using chemically aminoacylated-tRNA. This extension of the nonsense suppression methodology for unnatural amino acid incorporation should greatly expand the utility of the method for studying mammalian proteins in a more physiologically relevant system.

Recently, RajBhandary and coworkers have shown the delivery of aminoacyl-tRNA obtained intact from *E. coli* to COS-1 cells using the transfection reagent Effectene (Qiagen) [19]. We saw no success with this method, as described in Chapter 2. Most likely, the difference between the two studies is the nature of the assays employed. In their studies, Kohrer et al. harvested transfected COS-1 cells and then employed the highly sensitive biochemical CAT assay because protein expression was too low to be observed on a single-cell level. In the present work, we observe much higher levels of protein expression, and single cells can be assayed. While there are other important differences between the two studies, our work thus far indicates that for studies at the single-cell level, electroporation is a more promising transfection method.

A recent report from Yokoyama and coworkers showed site-specific incorporation of unnatural amino acids in proteins in mammalian cells [20]. They expressed in CHO-Y cells a mutant E. coli tyrosine synthetase that aminoacylates B. Stearothermophilus amber suppressor tRNA with 3-iodo-L-tyrosine. This is significant work toward engineering cells with novel amino acids, but is complicated by the requirement that each new amino acid has a specific engineered synthetase and tRNA.

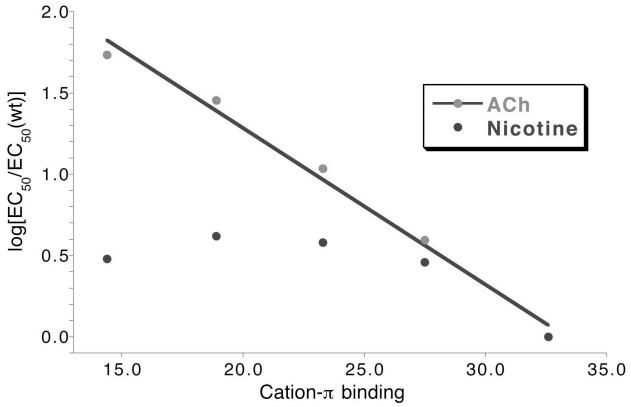
For our purposes, chemical aminoacylation of tRNA has the distinct advantage of not being amino acid specific and no protein engineering is required, and therefore it is a more general technique.

Finally, Vogel and coworkers independently demonstrated nonsense suppression of EGFP with aminoacyl-tRNA [21]. They microinjected CHO cells with in vitro transcribed *E. coli* amber suppressor tRNA that was chemically aminoacylated with wild-type leucine, along with the Leu64TAG mutant EGFP mRNA reporter gene, leading to the recovery of wild-type EGFP expression. This is promising work because like THG73, this tRNA was shown to be orthogonal, such that delivery of nonaminoacyl tRNA did not lead to EGFP expression. For many systems, microinjection may represent a viable approach. However, in our hands electroporation is far less tedious, since hundreds of cells can be transfected in a matter of seconds. The present method also appears to be more general because we were able to transfect different types of adherent cells with equal efficiency and with less cell mortality than single-cell gene transfer methods such as microinjection.

4.2.3 Attempts at neuronal α4β2 expression by nonsense suppression

We are particularly interested in studying the biophysical properties of nicotine binding to neuronal receptors. It is of particular interest that, from our studies, the step-wise increase in the EC_{50} observed for ACh binding with increasing fluorination at αTrp149 of the muscle-type nAChR is not observed for nicotine [13] (Figure 4.4). In fact, no aromatic residue located within the binding cleft of the muscle type nAChR has

been shown to interact with nicotine via a cation-π type interaction [22]. However, this may not be surprising since nicotine is a poor agonist for this receptor. The majority of high affinity nACh receptors in the brain are the α4β2 receptor. Nicotine binding has been accounted for by the neuronal nAChR receptors α4β2 and α7, and these receptors show a higher affinity to nicotine than they do to ACh [16]. Furthermore, studies suggest that the behavioral effects of nicotine in animals is predominantly mediated by the α4β2 receptor (reviewed in [23]).



Figue 4.4. The anomalous behavior of nicotine binding at the muscle-type nAChR. This plot demonstrates that nicotine does not follow the same trend as acetylcholine when the fluorinated Trp residues are incorporated at αTrp149, and thus does not interact at this site by a cation-π type interaction.

We are interested in incorporating the fluoro-tryptophan series at key aromatic residues within the neuronal nAChRs to test if nicotine is binding via a cation-π interaction. The homologous tryptophan to α149 of the muscle type receptor is Trp182 in α4, so we are particularly interested in incorporating the fluorinated Trp series at this position of the α4β2 receptor. Unfortunately, attempts thus far have proved to be disappointing, as these receptors were resistant to unnatural amino acid incorporation in

the *Xenopus* oocyte expression system. Dr. Gabriel Brandt went to heroic efforts to increase the surface expression of both α4β2 and α7 in *Xenopus* oocytes [22]. These included optimizing the expression vector, overexpressing elongation factors, trying to reduce clatherin-mediated endocytosis by co-expression of a dominant-negative dynamin, overexpression of ER-chaperones (BiP), insertion of forward-trafficking signals for greater surface expression, and multiple injections into oocytes. Of all of these, only multiple injections had any measurable effect, but the whole-cell currents were still too small to generate reliable dose-response relations. Given that the neuronal nAChRs we are studying are mammalian in origin, we speculated that the *Xenopus* oocyte expression system may not be appropriate for these receptors. This in turn may have lead to the poor expression observed. With a mammalian expression system now in hand, we set out to express α4β2 receptors in mammalian cells by nonsense suppression.

4.2.3.1 Results

The mRNA for the human α4 and β2 subunits was made by in vitro transcription, from the Not I linearized pAMV constructs (optimized for *Xenopus* oocyte expression). In initial experiments, CHO-K1 cells were transfected with mRNA, along with the reporter plasmid EGFP-N1, by electroporation. ACh and nicotine responses were measured from EGFP expressing cells by whole-cell patch clamping. The responses from transfected CHO-K1 cells were small, at best, on the order of 10s of pA. There was also a great deal of inconsistency in expression levels from day to day, using the same mRNA stocks and same transfection protocol. On many days the cells would fail to express α4β2 all together.

Given the inconsistency of mRNA expression, the α4 and β2 subunits were subcloned into the mammalian expression vector pCI-neo at the Mlu I and Sal I sites. Using electroporation, CHO-K1 cells were transfected with these constructs. Whole-cell currents measured in response to ACh and nicotine were significantly larger in comparison to the mRNA transfections, on the order of ~ 100 pA to 1 nA (Figure 4.5).

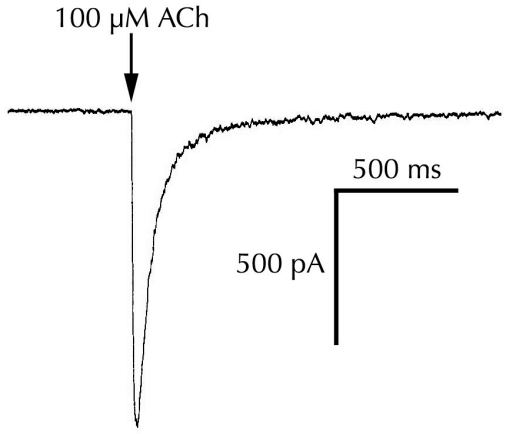


Figure 4.5. A typical whole-cell patch clamp response from a CHO-K1 cell transfected with a 5 μl solution containing the α4 and β2 subunits in pCI-neo, (2 μg/μl each), along with an EGFP-N1 reporter plasmid (0.15 μg/μl). The arrow indicates a 25 ms pulse of a 100 μM solution of ACh.

We next set out to express the α4β2 receptor by nonsense suppression in CHO-K1 cells using THG73 tRNA. The homologous tryptophan to α149 of the muscle type receptor is Trp182 in α4, so we were interested in incorporating the fluorinated Trp series at this site. Unfortunately, when cells were transfected with the αTrp182TAG mutant DNA (or mRNA) along with β2 and THG-W or THG-F_2W tRNA, no expression was observed. Transfection was done using a transfection solution at a variety of concentrations, up to concentrations as high as 7 μg/μl αTrp182TAG DNA (or mRNA)

and 7 μg/μl of THG-aa (along with 1 μg/μl of the β4 subunit and 0.5 μg/μl of the EGFP reporter plasmid). Under no circumstances was any sign of suppression observed.

4.2.3.2 Discussion

It is not readily apparent why expression of the α4β2 receptor by nonsense suppression failed in both the *Xenopus* oocyte and mammalian cell expression systems. The HSAS suppression experiments described in Chapter 3 and in section 4.2.1 demonstrate that the electroporation protocol delivers sufficient tRNA to cells such that suppression is not tRNA limited. Furthermore, the successful incorporation of natural and unnatural amino acids into the muscle-type nAChR using THG73-aa demonstrates that this tRNA is fully functional in mammalian cells as a suppressor tRNA. It is therefore curious that the wild-type α4β2 receptor expresses so well in mammalian cells (when DNA is used in transfection), while expression by nonsense suppression cannot be achieved. It may that upon translation, the α4β2 receptor is processed differently than the muscle-type receptor, and that this processing is not compatible with nonsense suppression. Or perhaps the mutant α4 Trp182TAG mRNA is more susceptible to nonsense-mediated decay (NMD), a process by which transcripts containing premature termination codons are degraded within a cell [24, 25]. NMD seems unlikely, however, since we don't believe it has been a problem for any other systems we have studied. Also, NMD has more to do with the relative position of premature termination codons relative to introns, and our mRNA lacks introns.

It seems that any future studies of the α4β2 receptor using unnatural amino acids will have to look beyond what we now know as the nonsense suppression methodology.

I suspect that differential processing of the neuronal α4β2 receptor, compared to other proteins that we have studied, is possibly incompatible with our current methodology. Perhaps if expressed in neurons, this ion channel would be processed and delivered to the surface more efficiently, and this could be one way to address this problem. Having a better understanding of how these ion channels are processed during translation will help us to optimize the method.

4.2.4 NMDA receptor expression by nonsense suppression

The NMDA receptor belongs to the family of glutamate ion channels, found primarily in the central nervous system, which constitutes the vast majority of excitatory neurotransmission in the brain. This receptor is a coincidence detector, important for long-term potentiation (LTP) in the hippocampas. Channel opening requires both agonist-binding as well as depolarization to relieve Mg^{2+} block of the pore. This receptor has nonspecific cation conductance, with high calcium permeability ($P_{Ca}/P_{K,Na,Cs} = 3.1$ - 11). Ca^{2+} entry into the cell combined with simultaneous firing of the pre and post synapse strengthens synaptic connections and induces LTP. Therefore, its role in neuronal development and synaptic plasticity is central to the mechanisms of learning and memory (reviewed in [26, 27]).

The NMDA receptor subunit stoichiometry is believed to be tetrameric, consisting of two NR1 subunits and two NR2 subunits. The subunits resemble those of potassium channels with inverted topology, with three transmembrane domains and a re-entrant loop

(M2) that lines the pore. Activation of the NMDA receptor is achieved by the binding of glutamate to NR2 and the essential co-agonist glycine to NR1 [26, 27].

There is now enough known about the NMDA receptor at a molecular level to design experiments using unnatural amino acids. For example, the crystal structure of the extracellular glycine-binding domain has recently been solved [28], and it provides a good framework to develop biophysical experiments aimed at understanding glycine modulation and channel gating. Also, residues implicated in channel modulation by phosphorylation, nitrosylation, glycosylation, reduction and oxidation, as well as modulation by pH and zinc, have been identified [26, 27]. We are therefore very interested in using unnatural amino acid incorporation to better understand the functioning of the NMDA receptor.

Because the NMDA receptor has never been studied using unnatural amino acids, we wanted to first design a relatively straightforward "proof of principle" experiment, with interpretable results. As discussed above, Mg^{2+} blocks the channel of the NMDA receptor by binding to residues in the M2 pore-lining region of the receptor. It has been shown that for receptors composed of NR1-1a and NR2b, that when Trp607 of the NR2b subunit was mutated to the nonaromatic residues Leu, Asn or Ala, the Mg^{2+} IC_{50} increased by an order of magnitude from 20 μM to 200 - 300 μM [29]. Mutation of Trp607 to the aromatic residues Phe and Tyr had minimal effect on the Mg^{2+} IC_{50}. From these data it was suggested that Mg^{2+} binds at Trp607 via a cation-π type interaction. This effect was not seen at any other Trp residue in the NR2b subunit (Trp610), in the homologous NR2a Trp residues (Trp606 and Trp609), or in the homologous NR1-1a Trp residues (Trp608, Trp611). We are therefore interested in incorporating the fluorinated

Trp series (that was used to identify a cation-π interaction between ACh and the nAChR) to identify a cation-π interaction between Mg^{2+} and Trp607 of NR2b. Since we already have all the unnatural amino acids necessary, no synthesis is required. Furthermore, these residues have already been shown to be functional in a variety of expression systems. Finally, if Mg^{2+} binding does occur via a cation-π type interaction, then the receptor has a measurable phenotype - the Mg^{2+} IC_{50}.

4.2.4.1 Results

The NMDA constructs (rat) NR1-1a (pCI-neo), NR2a (pCI-neo) and NR2b (pcDNA1) were obtained from the labs of Stephen Traynelis. NR1-1a and NR2a or NR2b were co-electroporated into CHO-K1 cells, along with an EGFP-N1 reporter plasmid. It can be seen in Figure 4.6 that the NR1-1a and NR2a constructs express well in CHO-K1 cells. Responses on the order of ~ 1 nA or greater were typical. Expression of the NR2b construct was less efficient, however, and responses usually were on the order of 10s to 100s of pA.

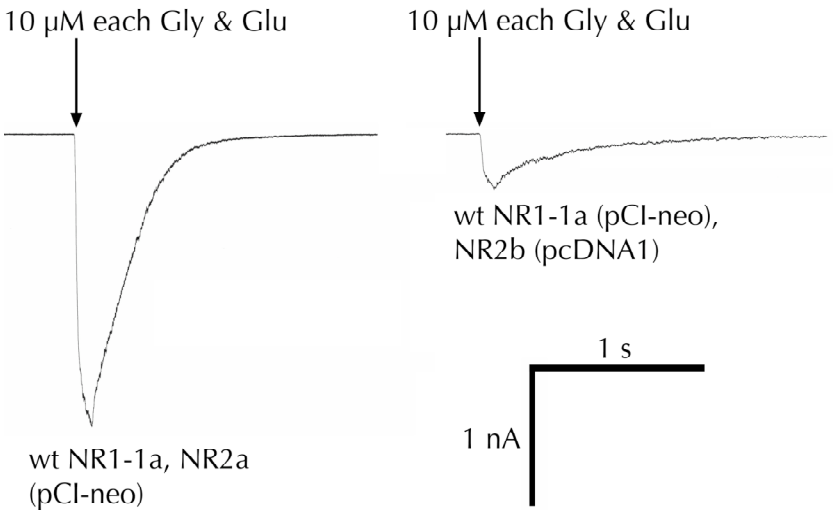


Figure 4.6. Expression of wt NMDA receptors in CHO-K1 cells. CHO-K1 cells were transfected with a 5 μl solution containing the NR1-1a and NR2a (in pCI-neo) or NR2b (in pcDNA1) subunits (2 μg/μl each). The first trace shows a whole-cell response from a cell expressing the NR1-1a and NR2a subunits. The second trace is a response of a cell expressing the NR1-1a and NR2b subunits. The arrows indicate a 50 ms pulse of 10 μM each glutamate and glycine.

Initial experiments in studying Mg^{2+} block of the NMDA receptor were done using the same fast perfusion system as done for all the electrophysiology experiments, with quick pulses of drug application (25 - 50 ms). Since the IC_{50} for Mg^{2+} is known to be ~ 20 μM, a 300 μM Mg^{2+} solution along with 10 μM of each glutamate and glycine was applied to CHO-K1 cells expressing the NMDA receptor. It was expected that there would be no response due to total channel block. Surprisingly, there was minimal difference between the responses with or without Mg^{2+} (Figure 4.7).

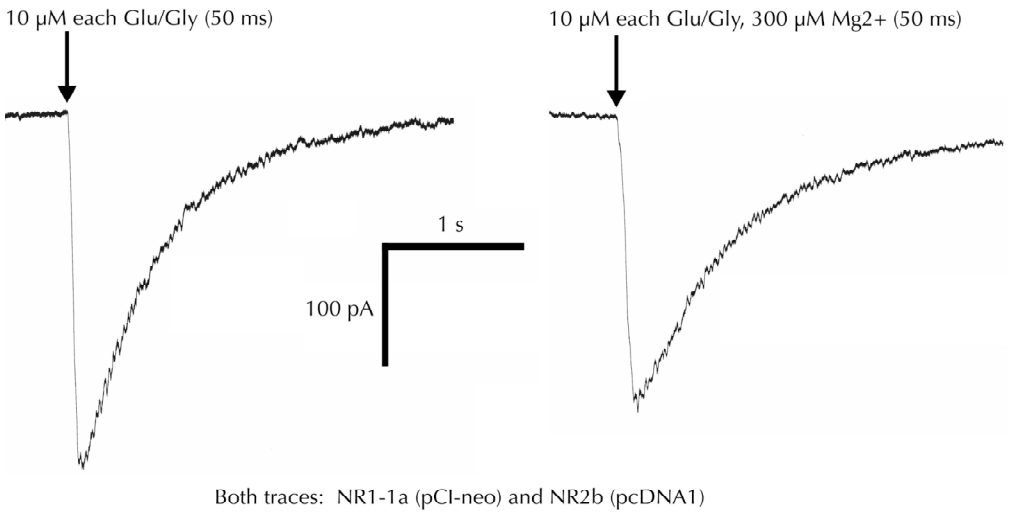


Figure 4.7. Expression and Mg^{2+} block (50 ms application) of wt NMDA receptors. CHO-K1 cells were transfected with a 5 μl solution containing the NR1-1a (in pCI-neo) and NR2b (in pcDNA1) subunits (2 μg/μl each). The first trace shows a whole-cell response upon a 50 ms pulse of 10 μM each glutamate and glycine. The second trace is a response upon a 50 ms pulse of 10 μM each glutamate and glycine and 300 μM Mg^{2+}.

It was speculated that the Mg^{2+} was having no effect because the duration of drug application was too short. That is, the rate of agonist binding and channel opening may be faster than the kinetics of Mg^{2+} block. To assess this, the drugs were applied for longer durations. As expected, with a 1 second drug application, channel block was observed with a 10 μM Mg^{2+} application, and almost total channel block was observed with 300 μM Mg^{2+} (Figure 4.8). This demonstrates that longer applications of Mg^{2+} are required to observe channel block.

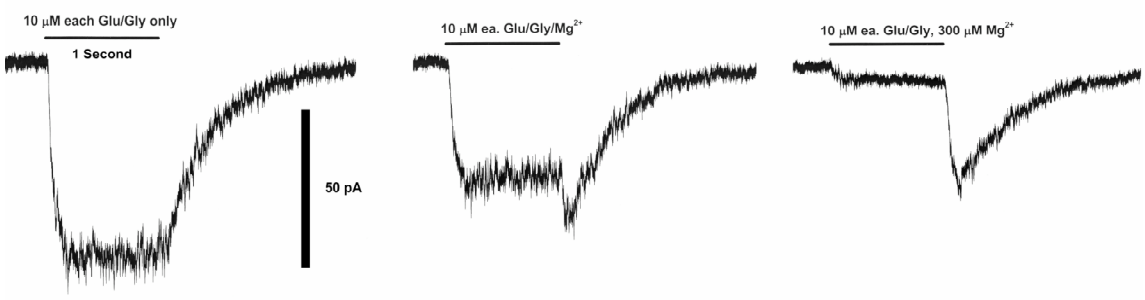


Figure 4.8. Expression and Mg^{2+} block (1 s application) of wt NMDA receptors. CHO-K1 cells were transfected with a 5 μl solution containing the NR1-1a (in pCI-neo) and NR2b (in pcDNA1) subunits (2 μg/μl each). The first trace shows a whole-cell response upon a 1 s pulse of 10 μM each glutamate and glycine. The second trace is a response upon a 1 s pulse of 10 μM each glutamate, glycine and Mg^{2+}. The third trace is a response upon a 1 s pulse of 10 μM each glutamate, glycine and 300 μM Mg^{2+}.

An attempt to make a variety of NMDAR mutants was pursued in order to study the role of pore-lining tryptophan residues in Mg^{2+} channel block (both by Quickchange and PCR mutagenesis). These included the NR2b Trp607TAG and Trp610TAG mutants, the NR2a Trp606TAG and Trp609TAG mutants and the NR1-1a Trp608TAG and Trp611TAG mutants. Of these, the only two that were successfully obtained (by PCR mutagenesis) were the NR2b Trp607TAG and NR2a Trp609TAG. With these in hand, CHO-K1 cells were transfected with NR1-1a, one of the mutant NR2 constructs, along with THG-W tRNA (to look for wild-type recovery of both NMDA receptors). Unfortunately, no expression by nonsense suppression was observed with the NR2b Trp607TAG mutant. However, it can be seen in Figure 4.9 that wild-type recovery by nonsense suppression of the NR2a subunit was successful. Responses on the order of 50 pA were observed with a 50 ms pulse of 10 μM each glutamate and glycine. There was minimal read-through of the mutant NR2a Trp606TAG in the absence of aminoacyl-tRNA. This demonstrates that the NMDA receptor can be expressed by nonsense suppression.

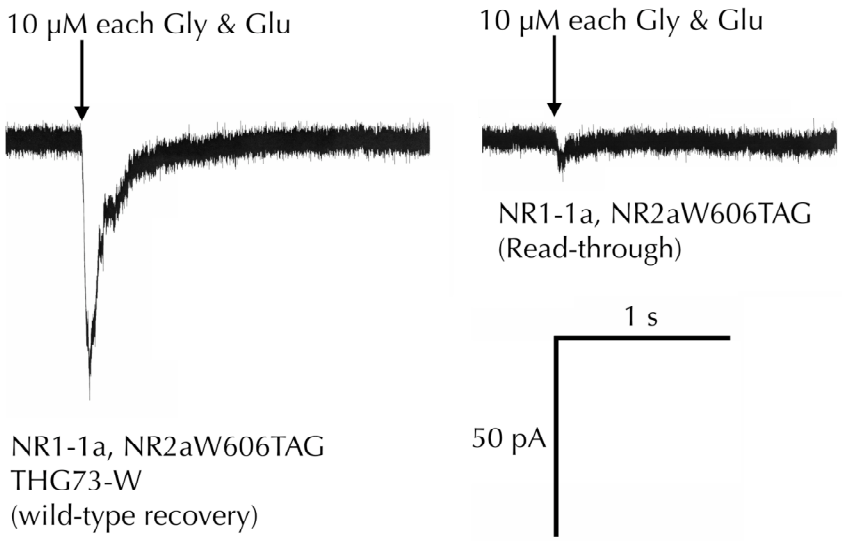


Figure 4.9. Expression of wt NMDA receptors in CHO-K1 cells by nonsense suppression. The first trace shows whole-cell response from CHO-K1 cells that were transfected with a 5 μl solution containing the NR1-1a (1 μg/μl) and NR2a Trp606TAG (4 μg/μl each) (both in pCI-neo), and THG-W (5 μg/μl). The second trace is a response of a cell expressing the NR1-1a and mutant NR2b Trp606TAG subunits, with 74mer THG73 tRNA (5 μg/μl). The arrows indicate a 50 ms pulse of 10 μM each glutamate and glycine.

4.2.4.2 Discussion

The above results demonstrate that the NMDA receptor expresses well in mammalian cells, using the electroporation transfection protocol. The wild-type receptor containing the NR2a subunit consistently had maximal responses on the order of nAs. However, NR2b expressing receptors had much smaller signals. This difference is most likely due to the fact that the NR2a was in pCI-neo, a high expression vector, and NR2b construct was in the poorly expressing vector pcDNA1.

Attempts were made to subclone the gene encoding for the NR2b subunit into pCI-neo in order to increase its expression levels in CHO-K1 cells. However, the

pcDNA1 construct was extremely resistant to sequencing, as well as to mutagenesis by Quickchange or PCR. I was unfortunately unable to determine the sequence flanking the NR2b coding region, making it impossible to do the necessary molecular biology. The best course of action to take for future studies would be to obtain an NR2b construct that is already in a mammalian expression vector from another lab.

An important result from these experiments is that the NMDA receptor can be expressed by nonsense suppression. This was shown by wild-type recovery of receptors containing the NR1-1a and NR2a subunits, using THG-W. Nonsense suppression of the NR2a Trp606TAG mutant DNA gave maximal responses on the order of 50 pA, while cells transfected with this mutant in the absence of aminoacyl-tRNA had minimal read-through.

Unfortunately, no wild-type recovery was observed for the NR2b Trp607TAG subunit, which is the residue implicated in making a cation-π interaction with Mg^{2+}, and therefore was the site at which we had hoped to incorporate the fluoro-tryptophan series. We suspect that nonsense suppression did not work for this construct was because it is in a poorly expressing vector (pcDNA1). Judging from the nAChR nonsense suppression experiments (4.2.2), and the NR2a suppression shown above, it seems that the wild-type receptor needs to generate whole-cell responses on the order of ~ 1 nA or greater in order to observe expression by nonsense suppression.

Another important result from these experiments is that nonsense suppression was achieved by electroporation of DNA that encodes for the NMDA subunits, rather than mRNA as has been traditionally done. This further simplifies the mammalian cell

nonsense suppression method, since mRNA synthesis can be of inconsistent quality, and since mRNA itself is inherently less stable and more difficult to work with than DNA.

Finally, these results demonstrate that NMDA expression is compatible with nonsense suppression. This is very exciting, because one can imagine many experiments using unnatural amino acids for this receptor. For example, zinc and redox modulation can be studied using caged-cysteine; phosphorylation can be studied using the phospho-amino acid analogues that are currently in progress (Dr. Gabriel Brandt and E. James Petersson); glycine modulation can be studied using the crystal structure as a model; and channel gating can be studied using hydroxy acids and the backbone cleaving residues 5-(*o*-nitrobenzyl)selenyl-2-hydroxypentanoic acid (NbSeOH) [30] and nitrophenylglycine [14]. This will be an exciting area for future studies.

4.3 Conclusions

Presented is a general method to deliver aminoacyl-tRNA, mRNA and DNA simultaneously to mammalian cells by electroporation. Both natural and unnatural amino acids chemically appended to a suppressor tRNA are site-specifically incorporated into the nicotinic acetylcholine receptor. These are very exciting results, as they demonstrate that the nonsense suppression method of unnatural amino acid incorporation can be done in a mammalian cell expression system. Furthermore, we show the first demonstration of NMDA expression by nonsense suppression. This demonstrates that the methodology can be applied to the family of glutamate receptors, an entirely new class of neuronal ion channels in our hands.

In conclusion, we describe the first general method for unnatural amino acid incorporation in mammalian cells. By not being limited to the *Xenopus* oocyte or in vitro expression systems, this will greatly expand the use of unnatural amino acids to studying protein structure-function relationships in cell-specific signaling cascades. We therefore feel that this method will advance our own studies on neuronal ion channels, as well as making the use of unnatural amino acids more attainable to a broader cross-section of researchers.

4.4 Experimental Methods and Materials

4.4.1 Materials

Restriction enzymes and T4 RNA ligase were purchased from New England Biolabs (Beverly, MA). The mMessage mMachine and MegaShortScript in vitro transcription kits were purchased from Ambion (Austin, TX). Maxiprep kits used for plasmid isolation were purchased from Qiagen (Valencia, CA). pEGFP-N1, a soluble EGFP construct was purchased from BD Biosciences Clontech (Palo Alto, CA). The NR1-1a (pCI-neo), NR2a (pCI-neo) and NR2b (pcDNA1) were obtained from Prof. Stephen Traynelis (Emory, GA). Ham's F12 tissue culture media was purchased from Irvine Scientific (Santa Ana, CA), and CO_2 independent was purchased from GIBCO Introgen Corporation (Carlsbad, CA). The microelectroporator was built on site.

4.4.2 Mutagenesis, mRNA and tRNA synthesis

THG73 tRNA, THG-W and THG-F2W have been described elsewhere. Briefly, linearization of pUC19 containing the THG73 gene with Fok I yeilds 74-mer tRNA upon in vitro transcription with the MegaShortScript kit. THG73 74-mer was then ligated to dCA-W or dCA-F2W with T4 RNA ligase to generate THG73-aa.

Mutagenesis of the NMDA subunits was done using the Quickchange mutagenesis protocol (Stratagene), or using standard PCR mutagenesis. The wild-type neuronal nAChR subunits α4, α4 182TAG and wild-type β2 were removed from the pAMV expression vector by PCR, while introducing an Mlu I mutation at the 5'terminus

at the same time. This was trimmed with Mlu I and Sal I, and subcloned into pCI-neo at the corresponding sites.

The mRNA that codes for the muscle-type nAChR (α, β, δ and γ) or the neuronal α4 and β2 AChR subunits was obtained by linearization of the expression vector (pAMV) with Not I, followed by in vitro transcription using the mMessage mMachine kit.

4.4.3 Tissue culture

CHO cells were grown in Ham's F12 media and HEK cells were grown in DMEM, at 37°C and 5% CO_2, enriched with glutamine, fetal bovine serum (FBS, 10 %), penecillin, and streptomycin. 1 to 2 days prior to electroporation, the cells were passaged onto 35 mm tissue culture dishes such that confluency was typically 50% or less at the time of transfection.

4.4.4 Electoporation

The DNA, mRNA or tRNA to be electroporated into CHO-K1 was precipitated alone or as co-precipitates in ethanol and ammonium acetate, and left at -20°C for at least 1 hour. For THG73-aa, the amino acids have an o-nitroveratryloxycarbonyl (NVOC) protecting group at the N-terminus and were photo-deprotected immediately prior to electroporation. This consisted of irradiating a 15 μl solution of THG73-aa (1 μg/μl) in 1 mM NaOAc (pH 4.5) for 6 minutes, using a 1000 W Hg/Xe arc lamp (Oriel, Stratford, CT) operating at 400 W, equipped with WG355 and UG11 filters (Schott, Duryea, PA). Reporter EGFP DNA and the AChR subunit mRNA or NMDA subunit DNA was then

combined with this and precipitated with ammonium acetate and ethanol. This was then microcentrifuged at 15,000 rpm, 4°C for 15 minutes, vacuum dried for 5 minutes, and resuspended in CO_2 independent medium to the desired final concentration. Immediately prior to electroporation, the cell tissue culture media was swapped to CO_2 independent media (with no glutamine, FBS or antibiotics). Approximately 5 μl of the electroporation solution was applied to the cells, followed by application of electrical pulses. For CHO-K1 cells this was typically four 120 V pulses of 50 ms duration. The CO_2 independent media was immediately replaced with fresh Ham's F12, and the cells were placed back into the 37°C incubator. Electrophysiological recordings were done 24 hours after transfection.

4.4.5 Microscopy

CHO-K1 cells were visualized with an inverted microscope (Olympus IMT2), a 250 W Hg/Xe lamp operating at 150 W, a GFP filter set (Chroma, model 41017) with an excitation band pass of 450 to 490 nm and an emission band pass of 500 to 550 nm, 10x/0.25NA or 40x/1.3NA lens, and a Photometrix Quantix CCD camera running Axon Imaging Workbench 4.0.

4.4.6 Electrophysiology

Whole-cell recordings were performed on EGFP-expressing (reporter gene) cells. The cells were visualized using an inverted microscope as described above. Patch electrodes (borosilicate, 4-6 MΩ) were filled with a pipette solution containing 88 mM KH_2PO_4, 4.5 mM $MgCl_2$, 0.9 mM EGTA, 9 mM HEPES, 0.4 mM $CaCl_2$, 14 mM creatine

phosphate, 4 mM Mg-ATP, 0.3 mM GTP (Tris salt), adjusted to pH 7.4 with KOH. The recording solution contained 150 mM NaCl, 4 mM KCl, 2mM $CaCl_2$, 2mM $MgCl_2$, 2 mM glucose, 10 mM HEPES, adjusted to pH 7.4 with NaOH. When recording from ACh receptors, 1 μM atropine was added (metabotropic AChR blocker). When recording from NMDA receptors, the Mg^{2+} was left out, and added back at the desired concentration. Standard whole-cell recordings were done using an Axopatch 1-D amplifier, low-pass filter at 2-5 kHz and digitized online at 20 kHz (pClamp 8, Foster City, CA). The membrane potential was held at -60 mV.

Acetylcholine (ACh), glutamate (Glu), glycine (Gly) or Mg^{2+} was delivered using a two-barrel glass theta tube (outer diameter ~ 200 μm, pulled from 1.5 mm diameter theta borosilicate tubing) connected to a peizo-electric translator (Burleigh LSS-3100, Fisher, NY). Each barrel of the theta tube was fed from a 12-way manifold. This allowed up to 12 different solutions to be fed in either the control or agonist barrel. Agonists were applied for 25 ms for AChR, and 50 ms or 1 s for the NMDAR, which weas triggered by pClamp 8 software. The voltage input to the high-voltage amplifier (Burleigh PZ-150M, Fishers, NY) used to drive the piezo translator was filtered at 150 Hz by an 8-pole Besel filter (Frequency Devices, Haverhill, MA), to reduce oscillations from rapid pipette movement. Solution exchange rates measured from open tip junction potential changes upon application with 10% recording solution were typically ~ 300 μs (10% - 90% peak time).

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