Chapter 3

The HSAS Assay: Optimizing tRNA Delivery to Mammalian Cells

3.1 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.

When developing a new translation system for unnatural amino acid incorporation, there are many variables to be considered. Importantly, one needs to deliver enough aminoacyl-tRNA to each cell in order to generate a detectable amount of protein. One of the greatest challenges in developing a mammalian cell expression system for unnatural amino acid incorporation arises from the fact that the aminoacyl-tRNA is a stoichiometric reagent. The amount of protein made containing the unnatural amino acid is limited by the amount of aminoacyl-tRNA that can be delivered to the cell. Because of the relatively small size of mammalian cells (10 - 30 μM in diameter) in comparison to *Xenopus* oocytes (diameter ~ 1 mm), much less aminoacyl-tRNA can be delivered to the former, hence less protein can be made.

Electroporation is a common method used to deliver many different types of macromolecules to cells, and is generally a favoured DNA transfection technique [16, 17]. It is operationally easy, reproducible, and works for many cell types including cultured neurons. Furthermore, cells that are either attached or in suspension can be transfected by electroporation. In essence, the application of an electric field to a population of cells results in reversible membrane breakdown, and the formation of large pores that allow the passage of macromolecules. The basic steps of electroporation include (i) electric field generation, (ii) polarization of the outer membrane, (iii) pore formation, (iv) transmembrane transport and (v) electropore resealing. The mechanism of pore formation and resealing is not known. Another advantage to this technique is that there are many parameters to control for optimization, including electric field strength, pulse duration and the number of pulses applied. A disadvantage is that there is no standard protocol, so optimization must be done for each individual system studied. Finally, electroporation has been used to successfully deliver aminoacyl-tRNA (rabbit liver tRNA) to CHO cells, although in this report the tRNA was found to not be functional within the cells [18]. Because of its generality, electroporation was tested as a method to deliver tRNA to adherent mammalian cells.

In order to minimize the amount of material required to transfect the cells, a "microporator" designed by Teruel and Meyer was used [19, 20]. This electroporation devise allows for the transfection of a small area of attatched cells (~ 1 cm^2), requiring small volumes of electroporation solution (1 - 10 μl). Teruel and Meyer have demonstrated successful transfection using both DNA and mRNA, with over 90% cell

survivability and over 50% transfection efficiency in rat basophilic leukemia cells, neocortical neuroblastoma cells and hippocampal neurons.

There are other variables that need to be considered. It has to be established that the mammalian cell translational machinery will recognize in vitro transcribed tRNA, while maintaining orthogonality to the endogenous synthetases. Also, one has to minimize hydrolysis of the aminoacyl-ester bond, which is labile at physiological pH, i.e. the required working pH when handling mammalian cells. Finally, it has to be determined if the tRNA THG73 - optimized for *Xenopus* oocyte nonsense suppression [21] - is functional in a mammalian expression system.

The HSAS assay described here was developed to reduce these variables, and to optimize tRNA delivery by electroporation. This assay involves the suppression of EGFP by a human serine amber suppressor tRNA (HSAS) first described by RajBhandary and coworkers [22] (Figure 3.1). They mutated the anticodon of the human serine tRNA to read the amber stop codon TAG. Because the tRNA anticodon is not a serine synthetase recognition element, this tRNA is still recognized and serylated in cultured mammalian cells. In our assay, HSAS was made by in vitro transcription. Likewise, the codon for Ser29 of EGFP was mutated to a TAG stop codon. It was hoped that when HSAS and Ser29TAG EGFP DNA were co-electroporated into mammalian cells, the HSAS would first be aminoacylated by the serine synthetase, and then used for nonsense suppression of EGFP. Electroporation of Ser29TAG mutant EGFP in the absence of HSAS tRNA would produce trunctated non-functional protein (Figure 3.2). The advantage of this assay is that it allowed us to test for tRNA delivery to cells and to see if in vitro transcribed tRNA is functional in mammalian cells, without worrying about

the stability of the aminoacyl-ester linkage of an aminoacyl-tRNA. It also allowed us to optimize tRNA delivery to mammalian cells, without consuming large quantities of aminoacyl-tRNA, which is chemically prepared and difficult to obtain in large amounts.

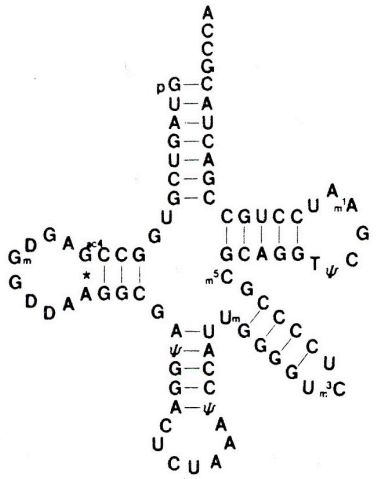

Figure 3.1. The human serine amber suppressor tRNA (HSAS) primary and secondary structure. In vitro transcribed tRNA does not contain the modified bases shown.

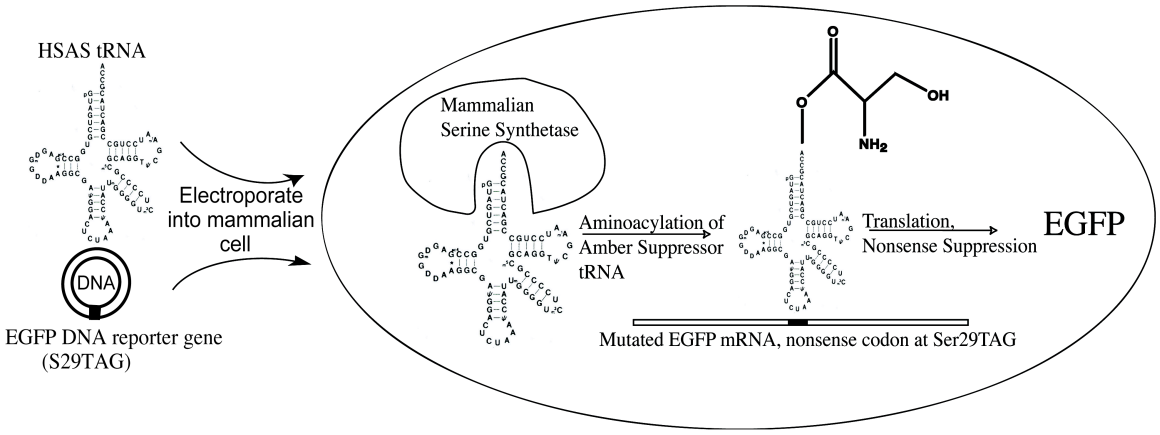

Figure 3.2. The HSAS suppression assay. Co-electroporation of HSAS and Ser29TAG EGFP DNA leads to serylation of HSAS, followed by EGFP expression by nonsense suppression.

3.2 Results

3.2.1 Electroporation of tRNA into adherent CHO-K1 and HEK cells: EGFP expression by nonsense suppression

The mutated EGFP construct Ser29TAG was combined with *in vitro* transcribed HSAS tRNA to a volume of 5 μl. A microelectroporator was used to transfect adherent mammalian cells with tRNA and DNA. The electroporator was designed to transfect a small section (~ 1 cm^2) of cells in a 35 mm dish, and therefore requires only small volumes of the transfection solution. Transfection was achieved by applying this solution to adherent CHO-K1 cells or HEK cells and applying four 120 V, 50 ms square wave pulses.

As shown in Figure 3.3, 2 hours after transfection there is high EGFP expression in CHO-K1 cells transfected with either wild-type EGFP or mutant Ser29TAG EGFP DNA and HSAS. When only the Ser29TAG mutant DNA is transfected without HSAS, no EGFP expression is observed in CHO-K1 cells. This demonstrates that both DNA and tRNA can be coelectroporated into cells with high efficiency; in vitro transcribed HSAS is aminoacylated by the endogenous CHO-K1 synthetase; and HSAS then functions as a suppressor tRNA in mammalian cells.

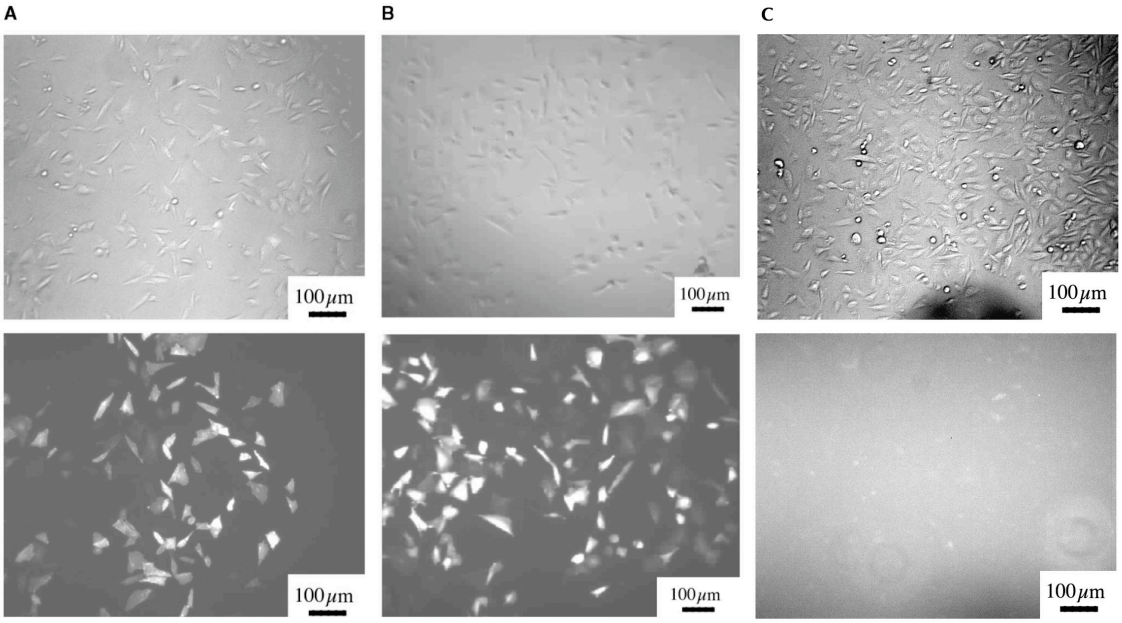

Figure 3.3. EGFP (pCS2gap EGFP) expression in CHO-K1 cells by nonsense suppression using HSAS tRNA. (A) CHO-K1 cells were electroporated with a 5 μl solution of HSAS (4 μg/μl) and Ser29TAG EGFP DNA (2.5 μg/μl). (B) CHO-K1 cells were electroporated with wt EGFP DNA (2.5 μg/μl). (C) CHO-K1 cells were electroporated with Ser29TAG EGFP DNA only (2.5 μg/μl). For all cases, four 120 V, 50 ms pulses were delivered to the cells. The bright-field (upper) and fluorescent (lower) images were taken 2 hr after transfection. The fluorescent image in (C) was taken with twice the exposure length as in (A) and (B).

In the case of HEK cells, lower expression was observed when compared to CHO-K1 cells. For this reason imaging of the cells was done 20 hours after transfection. Expression levels of the wild-type EGFP were similar to the HSAS suppressed EGFP, but there is more background signal evident in cells that were transfected with only the Ser29TAG DNA.

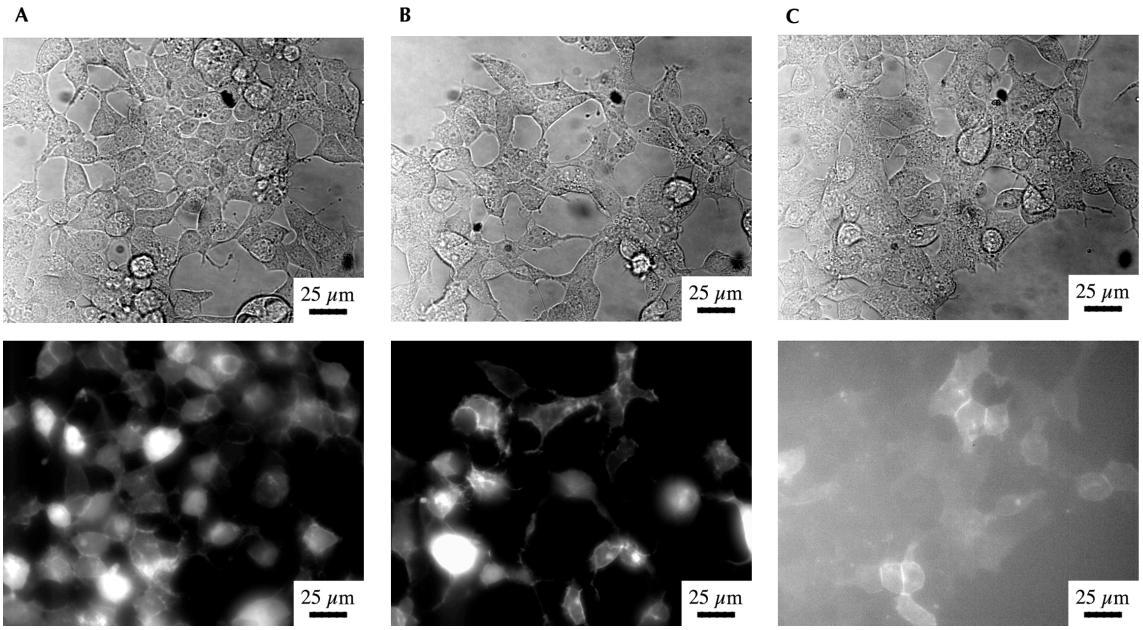

Figure 3.4. EGFP (pEGFP-F) expression in HEK cells by nonsense suppression using HSAS tRNA. (A) HEK cells were electroporated with wt EGFP DNA (2.5 μg/μl). (B) HEK cells were electroporated with a 5 μl solution of HSAS (4 μg/μl) and Ser29TAG EGFP DNA (2.5 μg/μl). (C) HEK cells were electroporated with Ser29TAG EGFP DNA only (2.5 μg/μl). For all cases, four 120 V, 50 ms pulses were delivered to the cells. The bright-field (upper) and fluorescent (lower) images were taken 20 hr after transfection. The fluorescent image in (C) was taken with four times the exposure length as in (A) and (B).

3.2.2 Nonsense suppression in hippocampal neurons

In order to determine the generality of this method, we tested the EGFP suppression assay in neurons. As with CHO and HEK cell transfection, HSAS tRNA and Ser29TAG EGFP DNA were coelectroporated into E18 rat hippocampal neurons (5 days in culture). As can be seen in Figure 3.5, 24 hours after transfection EGFP suppression by HSAS leads to comparable expression levels as electroporation of wild-type EGFP DNA. This demonstrates that electroporation also efficiently delivers tRNA and DNA to neurons and that the neurons also readily use in vitro transcribed tRNA for nonsense

suppression. As shown in Figure 3.5 (C), only low levels of fluorescence were detected when no tRNA is added, indicating that minimal read-through of the Ser29TAG construct occurred.

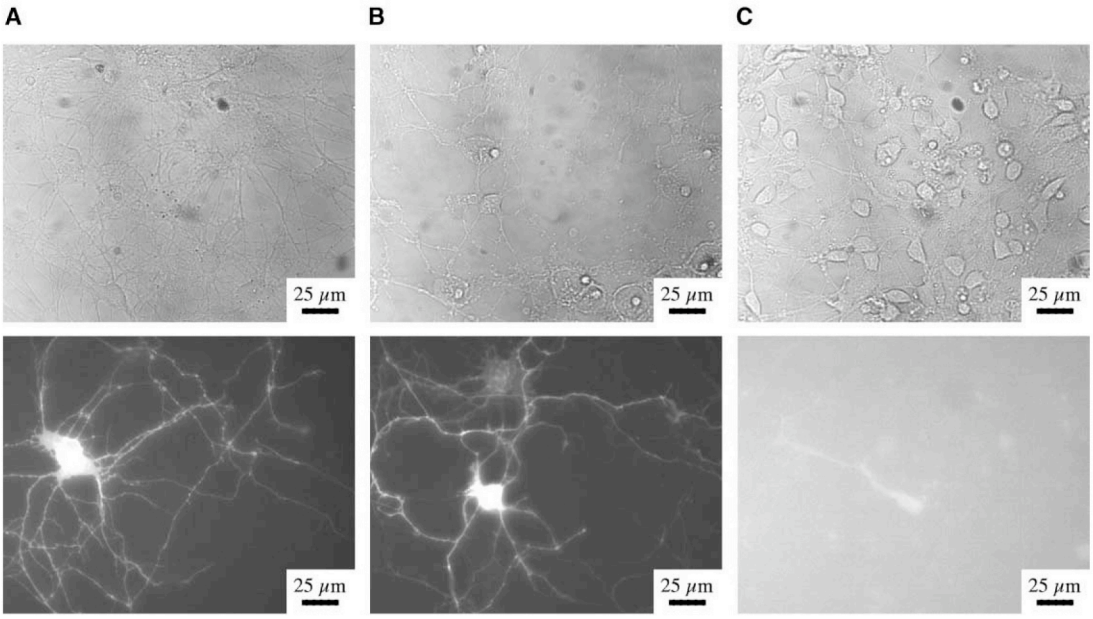

Figure 3.5. EGFP (pEGFP-F) expression in hippocampal neurons by nonsense suppression using HSAS tRNA. E18 rat hippocampal neurons (5 days in culture) were electroporated with a 5 μl solution of (A) HSAS (4 μg/μl) and Ser29TAG EGFP DNA (2.5 μg/μl); (B) wt EGFP DNA (2.5 μg/μl); (C) Ser29TAG EGFP DNA in the absence of HSAS tRNA. In all cases, four 160 V, 25 ms pulses were delivered to the cells. The bright-field (upper) and fluorescent (lower) images were taken 24 hr after transfection. The fluorescence image in (C) was taken with twice the exposure as (A) and (B).

3.3 Discussion and Future Directions

Presented is a general microelectroporation method to transfect mammalian cells with amber suppressor tRNA and DNA simultaneously. CHO-K1 cells, HEK cells and hippocampal neurons readily aminoacylate in vitro transcribed human amber suppressor tRNA (HSAS), and this tRNA is efficiently used by the translational machinery of these cells. The levels of HSAS suppressed EGFP expression all appear to be similar to the levels of wild-type EGFP expression. This suggests that enough HSAS tRNA is getting into the cells to not be a limiting factor for EGFP expression. However, it is important to note that HSAS suppression is most likely catalytic, in that each HSAS tRNA can be serylated and used for nonsense suppression multiple times.

Our results with the HSAS assay tRNA appear to contradict early reports by Deutscher and coworkers, who found that exogenous tRNA was not used by the translational machinery of mammalian cells [18]. They concluded that tRNA is "channeled" within the cell and that exogenous tRNA cannot enter the translational apparatus. Although their experimental design differed somewhat from ours (their tRNA was isolated from rabbit liver), the reason our results differ so significantly is unclear. What is clear from our results is that exogenous tRNA can easily enter into the protein synthesis pathway.

There are many advantages to the HSAS assay. One in particular is that it allowed for the optimization of tRNA delivery, without consuming large quantities of aminoacyl-tRNA, which is chemically prepared and difficult to obtain in large amounts. Another advantage of the HSAS assay is that it can be used to test other methods of

tRNA delivery to mammalian cells. In addition to electroporation, we investigated several other transfection techniques including the commercially available transfection reagents Effectene and Polyfect (Qiagen), GeneJammer (Stratagene), Lipofectamine (Invitrogen), as well as microinjection and biolistics. All of these approaches resulted in lower DNA delivery, with no convincing evidence of tRNA delivery (discussed in greater detail in Chapter 2).

In our hands, microelectroporation proved to be the most effective method for tRNA delivery to mammalian cells. First, it is highly general since many types of cells can be electroporated with equal efficiency and low cell mortality. Second, the method and instrumentation are very simple. The microelectroporator is easily built, is small and portable, and is easy to use [19]. Electroporation of adherent cells can be done on the benchtop or in a biological safety cabinet, and hundreds of cells can be transfected in a matter of seconds. Furthermore, protein expression is observable as soon as 2 hours after transfection.

The HSAS assay is now being used to test single cell electroporation [23-27], by Dr. Rigo Pantoja in the Lester lab. This method has the advantage of requiring significantly less material than the microelectroporator. Although the microelectroporator only uses 5 to 6 μl of transfection solution, the vast majority of this goes to waste since only a small percentage ($\leq$ 1%) of the material applied actually gets inside the cells. With single cell electroporation, a micropipette is filled with a small volume (hundreds of nanoliters) of transfection solution, and this can be used to transfect hundreds of cells in multiple dishes. This method is being developed and optimized by delivery of Ser29TAG EGFP mutant DNA and HSAS tRNA.

Another application of the HSAS assay that is currently in progress is the optimization of the nonsense suppression method using siRNA directed toward the eukaryotic release factor eRF1, by Joanne Xiu in the Dougherty lab. This is based on a previous report that demonstrated increased read-through of stop codons when siRNA designed to recognize eRF1 was delivered to HEK cells [28]. We believe that eRF1 may compete with our nonsense suppressor tRNAs and unnatural amino acid incorporation. Therefore if we can reduce the amount of eRF1 present in either *Xenopus* oocytes or mammalian cells, we may be able to increase the efficiency of unnatural amino acid incorporation. Miss Xiu is using the HSAS assay to look for enhanced suppression with the addition of siRNA in a variety of expression systems.

Another spin off of the HSAS assay involves mutation of the HSAS tRNA to remove the synthetase recognition elements, so that this tRNA can be used for unnatural amino acid incorporation. This approach is being pursued by Dr. Fraser Moss in the Lester lab. Because HSAS suppression is so efficient in all the cell lines tested, the translational machinery of the cell must efficiently recognize this tRNA even though it is generated by in vitro transcription and hence lacks all the post-transcriptional modifications that most tRNAs possess. If appropriately modified such that it is no longer serylated, but still recognized by the translational machinery, this may prove to be an effective tRNA for unnatural amino acid delivery in mammalian cells. The serine synthetase recognition elements have been identified for the human serine tRNA [29-36], and based on these a variety of modified HSAS tRNAs are being prepared and tested in vitro and in cell culture.

In conclusion, the HSAS assay has proved to be quite valuable in optimizing tRNA delivery to mammalian cells by electroporation. It has also become a routine assay for exploring other methods of tRNA delivery, as well as optimization of existing methods.

3.4 Experimental Methods and Materials

3.4.1 Materials

Synthetic DNA oligonucleotides were synthesized on an ABI 394 DNA Synthesizer on site. 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). Two membrane-localized GFP mammalian expression vectors were used, pCS2gapEGFP (Jack Horne, Caltech) and pEGFP-F (BD Biosciences Clontech, Palo Alto, CA). pEGFP-N1, a soluble EGFP construct was also purchased from BD Biosciences Clontech (Palo Alto, CA). Ham's F12 tissue culture media and DMEM were purchased from Irvine Scientific (Santa Ana, CA), and CO_2 independent and Neurobasal Media were purchased from GIBCO Introgen Corporation (Carlsbad, CA). The microelectroporator was built on site.

3.4.2 Mutagenesis and tRNA synthesis

The HSAS gene was constructed as follows: Two complementary synthetic oligonucleotides encoding for the T7 promoter, the HSAS gene, and the Fok I restriction site were annealed and ligated into the EcoR I and BamH I restriction sites of pUC19. After linearlization of the DNA with Fok I, in vitro transcription with the MegShortScript kit yields 74-mer tRNA (i.e. lacking the 3' terminal CA nucleotides). EGFP mutants (pCS2gapEGFP and pEGFP-F Ser29TAG) were made following the Quickchange mutagenesis protocol (Stratagene).

3.4.3 Tissue culture

CHO cells were grown in Ham's F12 media and HEK cells were grown in DMEM, enriched with glutamine, fetal bovine serum (FBS, 10 %), penecillin, and streptomycin at 37°C and 5% CO_2. 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.

Rat E18 hippocampal neurons were prepared as described previously [37]. Briefly, hippocampi were digested with 0.25% trypsin and then triturated. Cells plated in polylysine-coated 35 mm plastic dishes were maintained in Neurobasal medium supplemented with B27, 500 μM glutamax, and 5% horse serum (Invitrogen). Transfections were done after 5 days in culture.

3.4.4 Electroporation

The DNA or tRNA to be electorporated into either CHO-K1, HEK cells or neurons was precipitated alone or as coprecipitates in ethanol and ammonium acetate, and left at -20°C for at least 1 hour. 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. The cells were typically transfected with EGFP DNA at a concentration of 2.5 μg/μl, with or without HSAS tRNA at a concentration of 4 μg/μl. 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 and HEK cells this was typically four

120 V pulses of 50 ms duration, and for neurons, four 160 V pulses of 25 ms duration. The CO_2 independent media was immediately replaced with fresh Ham's F12 for CHO-K1 cells, DMEM for HEK cells or the original neurobasal media for neurons, and the cells were placed back into the 37°C incubator. Imaging of EGFP was done as soon as 2 hours after transfection.

3.4.5 Microscopy

CHO-K1 cells, HEK cells and neurons 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.

3.5 References

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