BEYOND DRUG SCREENS: A GENETIC APPROACH TO C. ELEGANS CHOLINERGIC SIGNALING

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cholinergic, neurobiology, neuromuscular junction
ABSTRACT

The identification of genes based on mutations that alter responsiveness to the paralyzing effects of the cholinergic agonist levamisole and the acetylcholinesterase inhibitor aldicarb has greatly enhanced our understanding of the mechanisms underlying neurotransmitter release and cholinergic signaling in *C. elegans*. However, mutations that only subtly affect drug sensitivity may still produce significant effects on synaptic function and be difficult to isolate in these kinds of screens. Alternative strategies for probing the genetic pathways that regulate neurotransmission may be valuable in identifying these classes of genes. Recent studies of cholinergic signaling in mammals have explored potential roles for nicotinic receptors in the mammalian brain by utilizing nAChR knockin mice that are hypersensitive to nicotine. We have generated strains expressing levamisole receptor subunits carrying a similar gain-of-function mutation. These strains are hypersensitive to levamisole and aldicarb, are hypercontracted, display exaggerated body bends, have kinked noses, and show reduced movement compared to wild type worms. We have used these animals to explore the downstream effects of hyper-activation and have found a dramatic effect in the localization of the nAChR *acr-16*. To identify new genes that may be involved in levamisole receptor signaling we have conducted a forward genetic screen for suppressors of the movement phenotype. We have isolated 15 suppressors, many of which retain levamisole sensitivity and thus would not have been isolated from traditional levamisole screens. We believe that this strategy will help us to find novel factors that play important roles in cholinergic function.
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INTRODUCTION

To understand nervous system function it is essential to know how neurons communicate. Most neuronal communication occurs at specialized points of cell-cell contact called synapses. A chemical messenger, or neurotransmitter, is released at synapses from the presynaptic cell and activates receptors located on the postsynaptic cell. This process is a universally conserved feature of nervous systems, and deficits in synaptic transmission underlie many neurological disorders. By elucidating mechanisms for the regulation of neurotransmission and, in particular, postsynaptic receptors, we hope to understand neural function better. With a better understanding of these mechanisms, we hope be able to identify factors important in neuronal plasticity, an essential part of learning and memory, and to find potential targets for therapies against diseases that affect these synapses.

THE C. ELEGANS NEUROMUSCULAR JUNCTION

For our work, we have chosen to study the neuromuscular junction (NMJ) of the nematode Caenorhabditis elegans. C. elegans offers an incredibly useful system for studying the basic properties of the nervous system. On the molecular level, many aspects of the C. elegans nervous system are conserved in the mammalian nervous system. Mutations in genes that are important for nervous system function are often lethal in mammals and are thus difficult to study. In contrast, homologous mutations in C. elegans are well tolerated. C. elegans are also hermaphroditic, meaning that they do not need to mate in order to reproduce. In the lab, we can culture these animals directly on their bacterial food source, allowing survival despite severe movement defects. Taken together, these traits allow us to study deficient nervous systems in intact animals. C. elegans is also amenable to transgenic approaches, allowing us to express wild type and mutated genes of interest under cell-specific promoters. The animals are transparent, allowing us to use photomicroscopy to study expression patterns and subcellular localization of proteins in live, intact animals using florescent reporter molecules such as green florescent protein (GFP).
Figure 1. Model of the C. elegans NMJ. Previously characterized genes in C. elegans are labeled. Levamisole is an agonist of the levamisole receptor (LevR). Aldicarb inhibits acetylcholinesterase (AChE) causing acetylcholine (ACh) to build up in the synapse. When the synapse is exposed to these drugs, many channels are opened and the postsynaptic cell becomes hyperactivated, resulting in muscle contraction and paralysis. Adapted from (Rand, 2007).

The basic properties of the C. elegans NMJ have been well characterized. This allows a great potential for genetic analysis of the regulation of this system. We hope to identify genes that affect the trafficking, localization, and modulation of post-synaptic receptors that have conserved function in the mammalian nervous system. Acetylcholine (ACh) plays highly conserved roles in neurotransmission. ACh is the neurotransmitter involved in cholinergic signaling, which is essential to reward systems, nicotine addiction, and at the NMJ in mammals. It is the primary excitatory neurotransmitter in C. elegans, and is released at the excitatory NMJ, as well in many connections in the nervous system. Figure 1 shows a model of the C. elegans NMJ. Synaptic vesicles loaded with ACh fuse with the presynaptic cell membrane and release ACh into the synaptic cleft. ACh binds to the ligand-gated ACh channels (AChR), resulting in the opening of the channels. This allows influx of Na+ and Ca2+ ions, depolarizing the post-synaptic cell. The ACh in the synapse is inactivated when ACh esterase (AChE) removes the acetyl group from the choline, which is then recycled into the presynaptic cell.
AChRs are pentameric receptors that are homo- or heteromeric. At least 27 different AChR subunits are transcribed from the C. elegans genome (Rand, 2007). This array of receptors allows for enormous variety in the receptor population. Presumably, this diversity plays an important role in mediating fast synaptic transmission in both C. elegans and human nervous systems. There are two classes of cholinergic receptors at the C. elegans NMJ: the heteromeric levamisole receptor (LevR), and the homomeric ACR-16. The LevR resembles heteromeric mammalian brain nAChRs, and ACR-16 resembles the mouse alpha 7 homomeric receptor (Rand, 2007).

Many of the genes involved in cholinergic signaling at the NMJ have been previously characterized using pharmacological screens (Rand, 2007). AChE inhibitors such as aldicarb prevent the inactivation and removal of ACh from the synapse. In addition, levamisole acts as an agonist of the LevR. When exposed to these drugs, the postsynaptic muscle cells in C. elegans are hyperactivated. The muscles become hypercontracted, resulting in paralysis. Knockout of genes required for cholinergic signaling confer resistance to these drugs. Forward genetic screens for levamisole resistance have defined the subunits of the LevR, as well as genes involved in folding and trafficking of the receptor (Lewis et al, 1980). Many of these mutations result in a complete loss of functional LevRs at the surface. The screens, however, have not found mutations in genes where the receptors would still be found at the surface of the cell, such as in genes that are specifically involved in the localization of the LevR. As a result, an alternative strategy for identifying these genes will be an important tool for gaining a complete understanding of the mechanisms that regulate cholinergic signaling.

**A Gain-of-function LevR**

All AChR subunits have a conserved structure (Figure 2). Each receptor subunit consists of four transmembrane domains. The M2 transmembrane domain is a highly conserved region in these subunits and is thought to play an essential role in ion specificity and gating. As in mammalian nAChRs, the LevR is composed of five different subunits. The LevR is made up of three α subunits (LEV-8, UNC-38 and UNC-63) and two non-α subunits (LEV-1 and UNC-29).
**Figure 2. Heteromeric Acetylcholine Receptors and the LevR(L/S) Mutation.**

[A] Structure of heteromeric AChRs. Five distinct subunits combine to make the ionotropic receptor. The M2 transmembrane domain lines the inside of the pore, and participates in the mediation of channel gating.  

[B] The membrane topology of an AChR receptor subunit. The site of the L/S mutation is indicated.  

[C] Alignment of AChR subunits in the M2 transmembrane domain. The site of an engineered serine mutation is indicated by the blue box in the subunits labeled with a red box. UNC-29, UNC-38, LEV-1, LEV-8, and UNC-63 are combined to make the LevR, a heteromeric acetylcholine receptor. This region is highly conserved in ACR-16, the homomeric receptor found at the NMJ, and the mouse acetylcholine receptor subunits alpha 7 and beta 4. Crystal structure taken from (Sixma, 2007).
In the mouse model, generation of transgenic mice with a point mutation in acetylcholine receptors has helped to further understanding of the role these receptors play in the nervous system (Labarca, 2001). A substitution with a polar amino acid near the gate in the M2 transmembrane region of the pore causes the receptor to stay open for much longer than in the wild type. This M2 transmembrane region is conserved across the LevR subunits (UNC-29, UNC-38, LEV-1, LEV-8, and UNC-63), ACR-16, and mouse ALPHA7 and BETA4 receptor subunits (Figure 2 C). Previously, our lab introduced this mutation into the UNC-29, UNC-38, and LEV-1 subunits of the LevR to generate a gain-of-function LevR (LevR(L/S)). UNC-29 and LEV-1 are specifically found in muscle (Rand, 2007), and all three of these subunits have shorter, more easily managed coding sequences.

Coexpression of these mutated subunits on a single transgene caused a dramatic movement phenotype (Figure 3). These animals are hypercontracted and have exaggerated body bends and a hooked head. In addition, worms expressing the LevR(L/S) transgene were hypersensitive to levamisole and aldicarb. In C. elegans, it is possible to dissect live animals and directly monitor cellular excitability using patch clamp electrophysiology in body wall muscles. Animals are dissected so that their muscle cells are exposed, and electrodes are placed in the muscle cells of the animals to allow voltage-clamp and current-clamp recordings. We have previously used this technique to test if these phenotypes were the result of changes in receptor function. We found that current responses from levamisole exposure were markedly slower in animals expressing LevR(L/S). These results suggest that the LevR(L/S) mutation causes a dramatic change in receptor function underlying the movement and hypersensitivity phenotypes we observed.

**The Effect of LevR(L/S) on Motor Neurons and Muscles**

Modulation of the signal from the presynaptic cell to the postsynaptic cell is an essential part of neural function, however, very little is known about the mechanisms that modify activity at the synapse. The LevR(L/S) mutation may offer a way to probe how perturbations in neuronal activity can effect protein localization and connectivity in the motor circuit.
**Figure 3. LevR(L/S) Induces a Change in LevR Function.** [A] Phenotype of LevR(L/S) worms. Introduction of LevR(L/S) creates a dramatic movement phenotype, which is suppressed by several mutations acquired from a genetic screen. [B] Worms expressing the LevR(L/S) are hypersensitive to the paralytic effects of aldicarb and levamisole. Error bars depict standard error of the mean. [C] Patch-clamp recordings from wild type and LevR(L/S) worms with application of levamisole. The black bar indicates a brief exposure of the muscle cell to levamisole. In the gain-of-function, there is a very slow return to baseline, indicative of the channels staying open for longer and increased ion flow.

In addition to agonistic, cholinergic neurons, *C. elegans* muscle cells are innervated by the motor neurons that release γ-aminobutyric acid (GABA), an inhibitor neurotransmitter at these synapses (Jorgensen, 2005). When GABA is released, it opens chloride channels and hyperpolarizes the muscle. The GABAergic motor neurons are also innervated by the cholinergic motor neurons (Figure 4). One possible way in which the *C. elegans* locomotory circuit may be adapting to the presence of a gain-of-function ACh receptor is to increase GABAergic signaling in a compensatory fashion.
Figure 4. Diagram of the Motor Circuit. Note that muscle cells are innervated by both excitatory cholinergic and inhibitory GABAergic motor neurons, and that the GABAergic motor neurons are innervated by the cholinergic neurons. When the cholinergic motor neurons release ACh, the muscle they innervate contracts, but the GABAergic neuron it innervated causes the muscle on the other side to relax. This in turn causes the characteristic sinusoidal motion of nematodes. Figure taken from (Jorgensen, 2005).

One way to assay GABAergic and cholinergic function is to examine the distribution of tagged release proteins expressed in the presynaptic cell. RAB-3 is a G-protein that associates with the docking sites in neurons (Gracheva, Hadwiger, Nonet, & Richmond, 2008). In this project, we looked for changes in presynaptic release sites by examining the subcellular localization of a RAB-3 that is tagged with a red fluorescent reporter protein, mCherry. In addition to effects on GABA and ACh release, we expected to see changes in levels and localization of the receptor populations on the muscle. We explored these possibilities using fluorescent microscopy to examine the distribution of receptor subunits tagged with fluorescent reporter molecules.

Behavioral Impacts of LevR(L/S)

Cholinergic signaling mediates much more than merely muscular activity. In C. elegans, cholinergic signaling is an important mediator of egg laying (Rand, 2007). However, knowledge of the full role of ACh in this process is complex. ACh deficient animals are hyperactive egg-layers; however, levamisole has also been shown to induce egg laying. Therefore, egg-laying behaviors may be strongly affected by the LevR(L/S) mutation, and may be a sensitive way of monitoring LevR activity. We tested this hypothesis by recording quantitative differences in egg laying behaviors.
A Suppressor Screen of LevR(L/S)

The marked phenotype caused by the LevR(L/S) mutation in combination with the ease of genetics in *C. elegans* allows us to probe gene mutations that modify LevR signaling. Mutations in genes that suppress the phenotype of LevR(L/S) are likely to be involved in LevR signaling, and if these mutations do not confer levamisole resistance it is likely that they would not have been discovered using traditional pharmacological screens.

Proteins can be easily removed in *C. elegans* via crossing deletion mutations into the LevR(L/S) background or knocking down with RNA interference (RNAi). Previous studies have identified genes that are associated with the LevR using Tandem Affinity Purification of LevR complexes in combination with mass spectrometry to identify compounds associated with LevRs precipitated with antibodies (Gottschalk et al., 2005). Because these proteins associate with the LevR, they may play an important role in its function. Several of the genes (*nra-2, nra-4*) have been since implicated in homomeric receptor subunit composition at the *C. elegans* NMJ, but many of the isolated genes’ roles at the NMJ remain unclear (Banerjee et al. 2005, Almedon et al. 2009). The LevR(L/S) mutation may provide a tool for further exploration into candidate genes involved in LevR signaling.

We previously used suppression of the movement phenotype caused by LevR(L/S) to isolate suppressors from a forward genetic screen. Alleles isolated from the screen have been given codes in the form *ufXX*. As part of this project, we performed complementation tests to determine if the recessive mutations isolated were in the same gene. Identified complementation groups are groups of mutations that fall in the same gene, and have been assigned the gene name Sal, for Suppressor of Activated LevR. For more information on nomenclature, please see Appendix B. One of the suppressors, *sal-2(uf33)*, is a dominant suppressor of the gain-of-function movement phenotype. Worms that have *sal-2(uf33)* have no other obvious movement phenotype. Like LevR(L/S) alone, LevR(L/S);*sal-2(uf33)* animals are hypersensitive to levamisole and aldicarb (Figure 5). Because the strain retains this sensitivity, we know that functional receptors are making their way to the surface. Therefore, we hypothesize that the action the gene product identified by *sal-2(uf33)* is in the localization or modulation of LevRs. A major focus of this MQP was
mapping the general location of the gene affected by *sal-2(uf33)*, as well as characterizing other suppressors from the screen.

**Figure 5. Levamisole and Aldicarb Sensitivity of *sal-2(uf33)*.** *sal-2(uf33)* worms retain hypersensitivity to levamisole and aldicarb. Note that *unc-29(x29)* animals, which lack the LevR, are completely resistant to levamisole. Error bars depict standard error of the mean.

**Single Nucleotide Polymorphism Mapping**

The potential for high throughput mapping of genes is one of the major advantages of *C. elegans*. Advances in sequencing have allowed us to obtain the precise sequence of the entire genome of the wild type and many related *C. elegans* strains. The N2 strain, which is the standard wild type strain, was isolated in Bristol, England (Davis *et al.*, 2005). Another closely related strain, CB4856, was isolated from Hawaii. These strains have many single base pair differences between their genomes, called single nucleotide polymorphisms (SNPs). Many of these changes fall within recognition sequences for restriction enzymes,
with a cut site only found in one genotype. This allows for the genotype at these SNPs to be easily determined: the area of DNA surrounding the SNP can be amplified using PCR and cut with the specific enzyme. This provides us with an array of easily tracked genetic markers for mapping mutations. Davis et al (2005) have developed a method for rapidly screening the genome using DraI SNPs evenly placed along chromosomes.
RESULTS

In order to characterize the LevR(L/S) phenotype further, we created transgenic animals expressing GFP fused to proteins that function at the synapse to observe synapse morphology and localization defects. We also performed an analysis of the egg laying behavior in the animals. To determine whether specific genes were required for normal function of the LevR, we crossed deletions in candidate suppressors into the LevR(L/S) background and used RNAi to knock down the expression of candidate suppressing genes. To use an unbiased approach for identifying genes that modify LevR function, we conducted a forward genetic screen prior to this project for mutations that suppress the movement phenotype caused by the LevR(L/S) mutation. We characterized these candidate suppressors using body bend analysis, levamisole resistance, and SNP mapping.

LEV(R(L/S)) INDUCES A MOLECULAR HOMEOSTATIC RESPONSE

In order to examine adaptive homeostatic changes at the NMJ in response to LevR(L/S) expression, we expressed fluorescently labeled markers in the LevR(L/S) background. We chose both of the other known receptors at the C. elegans NMJ, ACR-16 and the GABA receptor, as well as vesicle markers in the different classes of motor neuron to look for changes in presynaptic morphology. We also mis-expressed a subtype of ionotropic glutamate receptor (GluR) in body wall muscle, which normally does not express GluRs, as a negative control. In C. elegans, the muscles extend cellular projections to the central nerve cord called muscle arms. At the nerve cord, these muscle arms form synaptic contacts with motor neuron processes. Receptors and vesicle markers cluster at synapses, and thus puncta observed in animals expressing these markers represent synapses.

We observed no significant change in the localization of any of these markers, excepting ACR-16. In LevR(L/S) animals, we observed that ACR-16 was dramatically absent from the nerve cord, and was instead found retained in the muscle cell bodies (Figure 6). A suppressor we isolated in the forward genetic screen, sal-2(uf33), rescued this mislocalization. We also expressed a membrane-bound yellow fluorescent protein in
muscle cells that allowed us to observe gross muscle arm structure. Muscle arms in LevR(L/S) animals appeared to be thinner. These results suggest the cells may be compensating for the LevR(L/S) by mislocalizing or retaining the ACR-16 receptor and by a change in muscle arm morphology.

**Figure 6. LevR(L/S) induced a rescuable mislocalization of ACR-16 without affecting other markers.** [A] Cartoon of the ventral view of *C. elegans*. Images were taken in the boxed area. [B] When LevR(L/S) was co-expressed with a GFP fused ACR-16, the ACR-16 was largely absent from the nerve cord. It was instead aggregated in the muscle cell bodies, indicated by white triangles. (Top two panels). This mislocalization was rescuable by sal-2(uf33) (Third panel). Co-expression with other markers revealed no significant difference in the localization of presynaptic components or other receptors expressed in muscle cells. [C] Quantification of number of puncta in a fixed area of the ventral nerve cord. For ACR-16, statistical significance was calculated using ANOVA and Tukey HSD. *=p<0.001. All other samples were compared using the Independent Samples T-test, and were found to not be significantly different. Error bars depict standard error of the mean.
LEVR(L/S) INDUCES CONSTITUTIVE EGG LAYING

Cholinergic signaling plays an important role in the egg-laying behaviors of *C. elegans* (Rand, 2007), so we hypothesized that a hyper-activated LevR would induce constitutive egg laying. To test this we monitored the number of eggs retained in the uterus after 24 hours, as well as the number of eggs laid every 12 hours following L4 stage (Figure 7). Twenty-four hours after L4 stage, worms that express LevR(L/S) had very few eggs in their uteri as compared to wild type. The eggs laid by these animals were much earlier staged, usually between the 4 and 12 egg stage as opposed to the >40 cell stage of eggs laid by wild type animals. Although eggs were laid earlier by worms expressing LevR(L/S) than by wild type animals, the animals had no overall change in brood size. Taken together, these results suggest that LevR(L/S) induces constitutive egg laying. In addition, the suppressor of the LevR(L/S) movement phenotype sal-2(uf33) only partially suppresses this phenotype, and thus quantification of egg laying may represent a more subtle way of looking at cholinergic signaling than the movement phenotype or indicating a distinct, cell-specific, role of sal-2(uf33) in the egg laying circuitry.

RESULTS OF A CANDIDATE SUPPRESSOR SCREEN

As previously mentioned, suppressors of the LevR(L/S) movement phenotype may be involved in the regulation or localization of the receptor. To look for genes of interest and to demonstrate the efficacy of a forward suppressor screen for novel mutations, we used a candidate gene approach to find gene knockouts that suppress the movement phenotype. We chose to examine the effect of knocking down genes representing distinct functional classes: *acr-16*, the homomeric nAChR found at the *C. elegans* NMJ, *itsn-1*, which has been implicated in cholinergic function (Wang *et al.*, 2008), and *tam-1*, a gene require for expression of repetitive DNA sequences as in the multi-copy transgene carrying the LevR(L/S) mutation (Hsieh *et al.*, 1999). We also chose genes that are known levamisole resistance genes or are identified as interacting with LevRs (Gottschalk *et al.*, 2005). Using RNAi or by crossing in deletions in the genes, we determined whether each of these genes were required for the LevR(L/S) movement phenotype. Results are shown in Table 1.
Figure 7. LevR(L/S) Induced Constitutive Egg Laying. [A] Eggs laid by LevR(L/S) animals were at an earlier stage than those laid by wild-type animals. Later stage eggs had more cells, and there were fewer eggs retained in the vulva. Image taken by DIC. [B] Quantification of number of eggs retained in the uterus after 24 hours. LevR(L/S) animals had a marked decrease in the number of eggs retained. Animals with the sal-2(uf33) mutation partially suppressed this phenotype. Statistical significance was calculated using ANOVA and Tukey HSD. [C] Time course of eggs laid by staged animals. LevR(L/S) animals laid more eggs earlier than wild-type animals as indicated by the change in inflection point of the curve. Error bars depict standard error of the mean.
**Table 1. Candidate Suppressor Screen of the LevR(L/S) Movement Phenotype.** Genes were chosen because they were known levamisole resistance genes, they are associated with the LevR (Gottschalk *et al*, 2005), and from other evidence that they may be involved in LevR localization.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
<th>Knockdown Type</th>
<th>Suppression</th>
</tr>
</thead>
<tbody>
<tr>
<td>lev-10</td>
<td>CUB domain protein, involved in LevR localization</td>
<td>Deletion</td>
<td>-</td>
</tr>
<tr>
<td>ric-3</td>
<td>LevR chaperone</td>
<td>RNAi</td>
<td>+</td>
</tr>
<tr>
<td>unc-50</td>
<td>LevR trafficking</td>
<td>RNAi</td>
<td>+</td>
</tr>
<tr>
<td>unc-63</td>
<td>LevR subunit</td>
<td>Deletion</td>
<td>+</td>
</tr>
<tr>
<td>unc-74</td>
<td>Required for Surface Expression</td>
<td>Deletion</td>
<td>+</td>
</tr>
<tr>
<td>kin-20</td>
<td>Casein kinase I, Drosophila <em>double-time</em> homolog (Banerjee <em>et al</em>, 2005)</td>
<td>RNAi</td>
<td>+</td>
</tr>
<tr>
<td>nra-1</td>
<td>Copine</td>
<td>RNAi, Deletion</td>
<td>-</td>
</tr>
<tr>
<td>nra-2</td>
<td>Nicalin homologue, ER transmembrane involved in receptor composition (Almedom <em>et al</em>, 2009)</td>
<td>RNAi</td>
<td>-</td>
</tr>
<tr>
<td>nra-3</td>
<td>PHD (Zn-)finger</td>
<td>RNAi</td>
<td>-</td>
</tr>
<tr>
<td>pgp-12</td>
<td>P-glycoprotein</td>
<td>RNAi</td>
<td>-</td>
</tr>
<tr>
<td>soc-1</td>
<td>Pleckstrin homology domain</td>
<td>RNAi</td>
<td>-</td>
</tr>
<tr>
<td>tax-6</td>
<td>Calcinerurin A subunit</td>
<td>RNAi</td>
<td>-</td>
</tr>
<tr>
<td>acr-16</td>
<td>nAChR subunit</td>
<td>Deletion</td>
<td>-</td>
</tr>
<tr>
<td>itsn-1</td>
<td>Intersectin</td>
<td>Deletion</td>
<td>-</td>
</tr>
<tr>
<td>tam-1</td>
<td>Regulates transgene expression</td>
<td>Deletion</td>
<td>+</td>
</tr>
</tbody>
</table>

Deletions of ric-3, unc-50, unc-63, and unc-74 all eliminate surface expression of the LevR and suppressed the LevR(L/S) movement phenotype as expected. *lev-10* is a CUB-domain protein thought to be required for LevR localization (Gally *et al* 2004). Interestingly, deletion of *lev-10* did not suppress LevR(L/S). We hypothesized that *lev-10* is not sufficiently involved in the localization of the LevR for the deletion to affect LevR signaling. To test this, we created a strain carrying deletion alleles of both *acr-16* and *lev-10*. With no ACR-16 receptors at the neuromuscular junction, if *lev-10* had a strong effect on the localization of LevR(L/S) we would expect this strain to be paralyzed or severely uncoordinated, However, these animals displayed no visible phenotype (data not shown).
Of the genes isolated by Gottschalk et al, only *kin-20* substantially suppressed the LevR(L/S) phenotype. *kin-20* is a *Drosophila double-time* homolog, and has been implicated as a major player in developmental timing. *acr-16* did not suppress the LevR(L/S) phenotype. *itsn-1* plays a role in cholinergic vesicle release, and did not suppress the LevR(L/S) phenotype. This suggests that suppressors of the phenotype are likely to act post-synaptically. Deletion of *tam-1* suppressed the LevR(L/S) phenotype, presumably by knocking down expression of the LevR(L/S) transgene. We would therefore expect to pull alleles of *tam-1* out of a forward genetic screen for suppressors. Taken together, these results support the view that a forward genetic screen for suppressors may be a useful tool for identifying novel genes involved in cholinergic signaling.

**Analysis of Suppressors of LevR(L/S) Found in a Forward Genetic Screen**

To find novel genes involved in LevR signaling, we conducted a forward mutagenesis screen for suppressors of the LevR(L/S) movement phenotype (Table 2). We performed complementation tests on the alleles, and determined that they represent at least seven different genes, including *tam-1* (Table 3).

We hypothesized that mutations that suppress the LevR(L/S) phenotype and confer levamisole resistance are likely to represent mutations in genes already characterized from levamisole resistance screens. Of interest to us are novel genes that have more subtle effects on LevR signaling and are therefore not likely to have been previously described. We therefore predicted that mutant stains of interest would be sensitive to the paralytic effects of levamisole. To focus our characterization on these likely novel candidates, we examined the movement speed and sensitivity to the paralytic effects of levamisole (Figure 8). Many of the strongest suppressors of the LevR movement phenotype remained highly sensitive to levamisole, and thus are target mutations of interest.
**Table 2. Summary of Alleles Isolated from Suppressor Screen.** Alleles that have been found to fail-to-complement the known gene *tam-1* are marked. Complementation groups for which the gene is not yet known have been temporarily assigned the gene name *sal*, for suppressor of activated LevR. Alleles with no entry in the gene column are not yet mapped or are not yet known to fall in a complementation group.

<table>
<thead>
<tr>
<th>Allele</th>
<th>Gene</th>
<th>Dominance</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>uf14</td>
<td>sal-1</td>
<td>Recessive</td>
<td>Runs into body on direction changes</td>
</tr>
<tr>
<td>uf33</td>
<td>sal-2</td>
<td>Dominant</td>
<td>Wild type</td>
</tr>
<tr>
<td>uf40</td>
<td>sal-3</td>
<td>Recessive</td>
<td>Very deep body bends, few reversals</td>
</tr>
<tr>
<td>uf42</td>
<td>sal-3</td>
<td>Recessive</td>
<td>Long, uncoordinated reversals</td>
</tr>
<tr>
<td>uf44</td>
<td>sal-4</td>
<td>Dominant</td>
<td>Few reversals</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Stuttering movement, faster but visibly uncoordinated</td>
</tr>
<tr>
<td>uf50</td>
<td>sal-5</td>
<td>Recessive</td>
<td>Very short reversals, backwards movement much less fluid</td>
</tr>
<tr>
<td>uf55</td>
<td>sal-6</td>
<td>Recessive</td>
<td>Very short reversals, backwards movement much less fluid</td>
</tr>
<tr>
<td>uf13</td>
<td>tam-1</td>
<td>Recessive</td>
<td>Wild type</td>
</tr>
<tr>
<td>uf31</td>
<td>tam-1</td>
<td>Recessive</td>
<td>Wild type</td>
</tr>
<tr>
<td>uf32</td>
<td>tam-1</td>
<td>Recessive</td>
<td>Wild type</td>
</tr>
<tr>
<td>uf45</td>
<td>tam-1</td>
<td>Recessive</td>
<td>Wild type</td>
</tr>
<tr>
<td>uf49</td>
<td>tam-1</td>
<td>Recessive</td>
<td>Wild type</td>
</tr>
<tr>
<td>uf47</td>
<td></td>
<td>Dominant</td>
<td>Wild type</td>
</tr>
<tr>
<td>uf48</td>
<td></td>
<td>Dominant</td>
<td>Some short, jerky reversals</td>
</tr>
<tr>
<td>uf52</td>
<td></td>
<td>Dominant</td>
<td>Head lifts off agar</td>
</tr>
<tr>
<td>uf53</td>
<td></td>
<td>Recessive</td>
<td>Jerky movement and shallow body bends. Head lifts off agar</td>
</tr>
<tr>
<td>uf54</td>
<td></td>
<td>Recessive</td>
<td>Uncoordinated</td>
</tr>
<tr>
<td>uf56</td>
<td></td>
<td>Recessive</td>
<td>Short reversals stuttered, long reversals very long</td>
</tr>
<tr>
<td>uf57</td>
<td></td>
<td>Recessive</td>
<td>Very uncoordinated</td>
</tr>
<tr>
<td>uf58</td>
<td></td>
<td>Recessive</td>
<td>Long, uncoordinated reversals. Worms uncoordinated and small.</td>
</tr>
<tr>
<td>uf59</td>
<td></td>
<td>Dominant</td>
<td>Hyper foraging, short distances before stop, stuttering movement</td>
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**Table 3. Complementation Summary of Suppressors.** Complementation tests that have resulted in complementation (different genes) are highlighted in blue, failed to complement (same gene) are highlighted in red. White tests remain to be completed, and grey indicates a redundant test.

<table>
<thead>
<tr>
<th>tam-1</th>
<th>uf13</th>
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<th>uf31</th>
<th>uf32</th>
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<td>uf13Xtam-1</td>
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</tbody>
</table>

![Redundant](#) ![Fail to complement](#) ![Complement](#)
Suppressors that are most likely to be not previously characterized are those that restore wild type movement, while still being hypersensitive to levamisole. We measured movement speed by counting the number of body bends made by an animal per minute, and examined levamisole sensitivity by observing the percentage of animals paralyzed after one hour on plates containing 200 μM levamisole. LevR deletion animals contain the \textit{unc-63(ok789)} knockout allele, have no functional LevRs, and thus are completely resistant to levamisole. The grey dotted line shows the average levamisole sensitivity of N2 animals, and the black dotted line shows the average movement speed of LevR(L/S) animals. For example, sal-2(uf33) fully rescues the movement phenotype of LevR(L/S), while retaining levamisole sensitivity. Error bars depict standard error of the mean.

\textbf{Figure 8. Body Bend and Levamisole Resistance Analysis of Suppressors.} Suppressors that are most likely to be not previously characterized are those that restore wild type movement, while still being hypersensitive to levamisole. We measured movement speed by counting the number of body bends made by an animal per minute, and examined levamisole sensitivity by observing the percentage of animals paralyzed after one hour on plates containing 200 μM levamisole. LevR deletion animals contain the \textit{unc-63(ok789)} knockout allele, have no functional LevRs, and thus are completely resistant to levamisole. The grey dotted line shows the average levamisole sensitivity of N2 animals, and the black dotted line shows the average movement speed of LevR(L/S) animals. For example, sal-2(uf33) fully rescues the movement phenotype of LevR(L/S), while retaining levamisole sensitivity. Error bars depict standard error of the mean.
To identify the genes mutated by the suppressors of LevR(L/S) we isolated, we used a SNP mapping. We obtained several dominant suppressors of the LevR(L/S) movement phenotype from our screen that may represent gain-of-function alleles, dominant negative alleles, or genes that are haploinsufficient for enabling the LevR(L/S) phenotype. For dominant suppressors, it is sufficient to backcross the suppressor into the Hawaiian background when the LevR(L/S) mutation is kept present. We have performed extensive mapping analysis of \textit{sal-2(uf33)}. \textit{sal-2(uf33)} is of particular interest because it restores wild-type movement, while conferring no resistance to levamisole. The most likely position of \textit{sal-2(uf33)} is on the left side of chromosome II, as that is where N2 markers are most conserved (Figure 9).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure9.png}
\caption{SNP Markers in \textit{sal-2(uf33)} Backcrossed Strains.} The area boxed in grey is the area close to the LevR(L/S) transgene. Because the markers are most constantly N2 type on the left side of two, it is likely that \textit{sal-2} is somewhere on the left of two. Markers marked null have not enough data to determine whether any markers are Hawaiian. Isolates 5-n are a 3x backcross to LevR(L/S);CB4856, isolates 1-n are a 9 X backcross. PCR was only performed on the 9x backcrosses on chromosomes I and II.
\end{figure}
DISCUSSION

Patients with slow-channel congenital myasthenic syndrome (SCCMS) have a mutation in AChRs in their skeletal muscles similar to the one found in LevR(L/S), causing a dramatic change in channel kinetics. Over time, hyperactivation of the nAChRs induces homeostatic pathways that lead to endplate myopathy, muscle weakness, and loss of muscle control (Engel et al, 2010). A homologous mutation in brain nAChRs has been shown to cause autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) and benign familial neonatal convulsions (Barrantes et al, 2000). The distinct mislocalization phenotype of ACR-16 and apparent effect on the morphology of muscle arms in LevR(L/S) animals may be the result of activation of homologous pathways in the worm. Our LevR(L/S) may provide a model system for these diseases, and the suppressors of LevR(L/S) may identify targets for therapies for SCCMS and ADNFLE patients. In addition to the use of this as a model system, this phenotype may indicate that there is an important balancing act between cholinergic signaling by the LevR and the ACR-16 receptor. The homeostatic mechanisms that maintain this balance may play important roles in maintenance of excitability and in plasticity.

An obvious potential homeostatic response to increased LevR activity would be a change in localization of the LevR itself. However, attempts to visualize the LevR by addition of a GFP tagged LevR subunit, UNC-29, reduced the LevR(L/S) movement phenotype. We hypothesize that this result occurred because the GFP tagged UNC-29 was competing with the gain-of-function UNC-29 in the LevR(L/S) transgenic array (work done prior to MQP). Possible ways to view localization of the receptor would be to tag a subunit not in the LevR(L/S) mutation and express it only in muscle or to create antibodies against the LevR. We generated a cloning strategy to tag the LevR subunit UNC-63 with GFP that can be seen in Appendix A.

The constitutive egg laying phenotype caused by the LevR(L/S) mutation represents a dramatic effect of increased cholinergic signaling. In addition, sal-2(uf33) only partially rescues the egg laying phenotype, while fully rescuing the movement phenotype. This result suggests that cholinergic signaling via the LevR may still be increased in
LevR(L/S);sal-2(uf33) and that the movement rescue by sal-2(uf33) is caused by some subtle effect on LevR signaling, or that sal-2(uf33) acts differently in the vulval musculature than it does in body-wall muscle. This also supports the differential role of ACh in muscles as promoting egg laying, while inhibiting egg laying in the nervous system. Egg laying is known to be suppressed only when cholinergic signaling is present, but the drug levamisole also causes the release of eggs (Rand, 2007). The partial rescue by sal-2(uf33) demonstrated that this technique may serve as a useful assay for uncovering more subtle effects on LevR signaling.

Of the candidate suppressors chosen, only strong levamisole resistance genes, kin-20, and tam-1 knockouts suppressed the LevR(L/S) phenotype. The strong levamisole resistance genes were expected to suppress LevR(L/S) as the receptor is eliminated from the cell surface, and tam-1 knockout is known to silence repeated sequences such as the multi-copy array containing our mutated subunit. kin-20 was the only other candidate that suppressed the LevR(L/S) phenotype by RNAi; however, kin-20 in heavily involved in development and animals with the knockout of kin-20 are very sick and uncoordinated. That none of the knockouts of known genes that associate with the LevR that do not affect levamisole sensitivity suppressed the LevR(L/S) and that many of the suppressors isolated from the forward genetic screen do suggest that these mutations may be in genes that have not yet been implicated in cholinergic signaling. We may expect that a forward genetic screen will reveal novel genes involved in modulation and localization of LevR.

We believe that the LevR(L/S) mutation provides a useful approach to exploring cholinergic function. We have shown that the LevR(L/S) mutation induces a dramatic movement and egg laying phenotype, as well as a potential homeostatic response. We have obtained many suppressors from a forward genetic screen that would have been difficult to identify using pharmacological screen. We have begun to identify these genes using SNP mapping, and are confident that identification of these genes will lead to new insights in how receptors are managed in synaptic communication.
COMPLEMENTATION TESTS

To determine whether mutations isolated from the forward genetic screen fell on the same gene, we performed complementation tests (Figure 10). Males of one recessive suppressor were crossed with hermaphrodites of a different recessive suppressor strain. If all of the cross progeny moved normally, they each received a mutant allele of the gene that suppressed LevR(L/S), and thus both mutations were in the same gene. Males are denoted by a ♂, hermaphrodites by ♀.

\[
♀\text{LevR}(L/S); \text{suppressorA} \times \text{LevR}(L/S); \text{suppressorB}♀
\]

- **Same gene**: Wild type movement
- **Different genes**: Uncoordinated

**Figure 10. Diagram of Complementation Test.** If suppressor A and suppressor B are in the same gene, then all cross progeny will carry two mutant alleles of the gene and will continue to suppress LevR(L/S). However, if the genes are different, then they will be heterozygous for both recessive mutations, and the worms will be uncoordinated as LevR(L/S) animals are.

CROSS STRATEGIES

In order to explore aspects of cholinergic signaling, we took advantage of the genetic flexibility in *C. elegans* to perform crosses. Males were generated by heat shocking L4 stage animals for two six-hour periods separated by a 15-minute room temperature recovery period. Worms carrying *lin-15(n765ts)*, a temperature sensitive marker we used for transgenics, were similarly generated, except they were allowed to recover at 15 degrees and were returned to 15 degrees immediately following heat shock. Some of the stains included here were generated, but have not yet be used to generate data. There were four general strategies used to create strains, shown in the figures below.
ufls23 is a transgenic array where a GFP fused channelrhodopsin-2 (ChR2) is expressed under a cholinergic-motor neuron specific promoter, \textit{pacr-2}. ChR2 is a light-gated ion channel involved in photosensitivity in algae (Nagel \textit{et al}, 2003). We can express ChR2 cell specifically using the transgenic power of \textit{C. elegans}. ChR2 changes conformation under exposure to blue light, allowing allow ion flow and depolarizing the neurons expressing it. We can use this as a switch to turn on specific neurons, allowing us to use electrophysiology to directly see synaptic responses.

\[
\text{\textit{LevR(L/S); uf33 \times LevR(L/S); ufls23(pacr-2 :: ChR2 :: GFP)}}
\]

\[
\downarrow \text{Pick non unc hermaphrodites}
\]

\[
\text{LevR(L/S); uf33/+; ufls23/+}
\]

\[
\downarrow \text{Pick 26 non unc, GFP positive hermaphrodites}
\]

\[
\text{From the next generation screen plates for homzygous non unc, GFP positive}
\]

\[
\text{LevR(L/S); uf33; ufls23}
\]

\textbf{Figure 11. Cross Strategy for Generation of \textit{LevR(L/S);sal-2(uf33);ufls23(pacr-2::ChR2::GFP)}}. ufls23(pacr-2::ChR2::GFP) is the acr-2 promoter driving expression of a GFP fused channelrhodopsin, allowing us to use light to activate the motor neurons that innervate muscles.
\( \text{Fig. 12. Cross Strategy for Generation of } \text{levR}(L/S)\text{; sal-2(uf33); acr-16(ok789)}. \) In the absence of acr-16, we can use ChR2 to specifically drive the response of the levR at synapses.

\( \text{Fig. 13. Cross Strategy for Marking } \text{levR}(L/S) \text{ with a Fluorescent Tag}. \)
\( \varnothing N2 \times \text{mut}\varnothing \)

\[
\downarrow
\]

\( \varnothing \text{mut}/+ \times \text{LevR(L/S)}\varnothing \)

\[
\downarrow
\]

\( \text{mut}/+; \text{LevR(L/S)}/+ \)

\[
\downarrow \text{Confirm visually or by PCR}
\]

mut; LevR(L/S)/+

\[
\downarrow \text{Confirm visually or by PCR}
\]

mut; LevR(L/S)

**Figure 14. Cross Strategy for Making Double Mutants with LevR(L/S).**

**Single Nucleotide Polymorphism (SNP) Mapping**

To determine where the suppressors isolated from of the forward genetic screen were located in the *C. elegans* genome, we used a modification of the protocol developed by Davis *et al* for mapping using single nucleotide polymorphisms, or SNPs, between the Bristol strain (N2) and the Hawaiian strain (CB4856). These polymorphisms can introduce or eliminate a restriction enzyme recognition site, and in combination with PCR can be used to determine the location of genes quickly.

Prior to this MQP, we developed a variant of the technique developed by Davis *et al* to begin to map dominant mutations (Figure 15). The mutations acquired from the genetic screen were selected for their lack of phenotype other than suppressing LevR(L/S). As a result, the suppressors had to be mapped in the presence of the LevR(L/S) transgene. To overcome this obstacle, we backcrossed two integrants of the LevR(L/S) transgene to the Hawaiian strain: one integrated on LGII and one integrated LGV. We compared the backcrossed lines to N2, and showed that the LGII variant displayed Hawaiian markers on all other chromosomes. The LGII variant shows Hawaiian SNP markers at -18, -14, and +22 CM, whereas the LGV variant shows Hawaiian SNP markers all across LGII (Figure 16). SNP
mapping could then be performed by selecting for suppression of the LevR(L/S). Dominant mutations can be serially backcrossed to these strains, allowing fine breakpoints to be determined by crossing away N2 markers.

The genome of the Hawaiian strain CB4856 has various single base pair differences to the parent strain of all of our mutants, N2. These changes are called SNP markers.

When crossed with a dominant suppressor in the LevR(L/S) background, all normally moving males carry the mutation.

When crossed again, crossing over between chromosomes can occur and N2 markers can be lost.

When continuously repeated, N2 markers will eventually be lost everywhere but very close to the mutation. Across many different lines, N2 markers will likely be conserved only near the mutation.

Using PCR and restriction digest, the location of areas conserved for N2 can be determined.

**Figure 15. SNP Mapping Strategy for Dominant Suppressors.** The N2 (blue) and Hawaiian (red) regions of the chromosomes are distinguished by SNPs, which can be identified by PCR and restriction digests. A mutation shown by the black bar is crossed into the Hawaiian background. When cross progeny have wild type movement, they are heterozygous for the dominant suppressor. Males are continuously crossed back into the Hawaiian background. When crossing over occurs, N2 markers are lost at positions far from the mutation. When the mutation is re-isolated, the region very close to the mutation will always show N2 SNPs. Further away from the mutation, however, recombination will occur and Hawaiian SNPs will be observable.
**Figure 16. Genotypes on LGII of LevR(L/S) Strains Used for Mapping.** Genetic locations in cM are surrounded by brackets. Each bracket shows the N2 marker, followed by the marker carried by the LevR(L/S) backcrossed line, followed by the Hawaiian marker. Asterisks show markers that present the Hawaiian SNP. [A] The integrant for which the screen was performed; the LevR(L/S) transgene is integrated on LGII. The Hawaiian backcross presents Hawaiian markers at all locations other than LGII (not shown). [B] Another LevR(L/S) integrant, integrated on LGV, was also backcrossed and presents Hawaiian markers all across LGII, allowing us to locate suppressors anywhere in the genome.

**Cloning of pmyo-3::UNC-63::GFP**

An important part of characterizing the function of the LevR is viewing expression patterns. However, when a GFP tagged UNC-29 subunit was co-expressed with the gain-of-function LevR(L/S) transgene, there was a marked reduction in the LevR(L/S) movement phenotype, presumably due to competition between the gain-of-function and GFP tagged
subunits. In order to overcome this obstacle, we wanted to express GFP in a subunit not expressed in the LevR(L/S) transgene, namely, UNC-63. Previous work has shown that GFP tagged receptor subunits are primarily functional when inserted into the cytosolic loop of the subunit. The strategy developed can be found in Appendix A. Our initial attempts to make the clone were unsuccessful.

**Body Bend Analysis**

In order to measure the suppression of the LevR(L/S) phenotype quantitatively, we used analysis of the rate of body bends in the animals. We staged worms at L4 stage. Twenty-four hours later, the worms were videotaped on plates without food. The number of body bends over the course of five minutes was counted.

**Levamisole Sensitivity**

To measure the function of the LevR, we used assays to measure levamisole sensitivity. Worms were assayed twenty-four hours after L4 stage. Worms were placed on NGM plates containing levamisole. For time course assays, 100 μM levamisole was used and paralyzed worms were removed and counted every fifteen minutes. For rough levamisole assays, worms were placed on 200 μM levamisole for one hour.

**Egg Laying Assays**

Egg laying is one function that is dependent on cholinergic processes (Rand, 2007). Treatment with levamisole can induce constitutive egg laying. To determine the egg laying phenotypes of LevR(L/S), worms were dissolved in bleach twenty-four hours following L4, and the eggs in the vulva were counted. In addition, worms were staged at L4 and every 12 hours picked to new plates. The numbers of eggs laid by each worm during each 12-hour interval were then counted.
Although not conducted as part of this project, the resulting strains from the genetic screen for suppressors of LevR(L/S) play an important role in this project. As a pilot screen, LevR(L/S) worms were mutagenized using ethyl-methanesulfate according to the protocol by Koelle (1994). F2s from the mutagenesis were visually scanned for improved movement. Following this screen an additional, more directed screen was performed. Plates were marked with 1 mm markers, and were referred to as race plates. F2s from mutagenesis were pelleted in M9 solution when the worms were primarily young adults. Pelleted worms were dropped along the starting line in 2 μL drops, each drop containing ~50 worms (Figure 17). The worms would then tax towards the drop of food on the finish line. In general, wild type animals would arrive at the food after 30 minutes, and LevR(L/S) animals would arrive after 2 hours. Every 15 minutes, the mutagenized worms that made it to the food were picked clonally to new plates, and in the next generation, the worms were screened for improved movement.

**Figure 17. Race Plate Diagram.** Pelleted adult animals were dropped along the left, and a drop of food was placed on the right.


APPENDIX A. PMYO-3::UNC-63::GFP CLONING STRATEGY

pJRC2 Pmyo-3::UNC-63 cDNA

PCR

OMF457

GFP Insertion Site

OMF458

Unc-63 cDNA

Digest with Nhel and Ncol (2+BSA)

Ligate

Nhel+BamHI (2+BSA)
**pJRC3 GFP::UNC-63 3’**

**PCR**

OMF459
CAGTACGAGGAGAATCATATGAGGGACAGAA

OMF460
GATCCATGAGGAGAATCATATGAGGGAGGTTAT

Serial digest with Nhel (2+BSA)
and AccI (F)
Ligate

Confirm by PCR OMF355 and
OMF462
**APPENDIX B. C. ELEGANS NOMENCLATURE AND TRANSGENICS**

*C. elegans* is a useful genetic organism, so it is important to discuss the nomenclature of the field of *C. elegans* research and to clarify how transgenic lines are created.

*C. elegans* strains are each given a strain name. The most common of these strains is the N2 strain, isolated in Bristol, England. This is a wild type strain, and variants of this strain are used in many fields of research. Each gene in the *C. elegans* genome is given a three-letter code, followed by a number. The three-letter code refers to the type of the gene, and the number is specific to that gene. For example, *acr-16* is an Acetyl Choline Receptor subunit, coded 16. When referring to the gene, it is shown in lower case italics, and when referring to the protein product of the gene it is shown in all upper case. Alleles of a gene are shown following the gene name in parentheses. For example, *acr-16(ok789)* is a deletion allele of *acr-16*. When a strain caries more than one mutation, it is separated by a semicolon (e.g. *acr-16(ok789);unc-29(x29)*).

*C. elegans* are also extremely useful in the creation of transgenics. When plasmid DNA is injected into the gonad of a young adult animal, the developing ova create a chromosome-like structure from the inserted DNA called an extrachromosomal array. This array is randomly lost in some cells of each animal, as well as in some progeny of the animal. In order to create a stability transmitting transgenic line, X-rays are used to introduce double stranded breaks in the chromosomal DNA. In a small percentage of animals, DNA repair mechanisms will randomly incorporate the extrachromosomal array into the chromosomal DNA, and the array becomes integrated. These animals are selected based on the stable transmission to all progeny.