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**A common genetic mechanism underlying susceptibility to posttraumatic stress disorder**

He Z *et al*. Aberrant neuronal gene expression and PTSD

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**Abstract**

We hypothesize that susceptibility to post-traumatic stress disorder (PTSD) may be determined in part by aberrant microtubule-associated protein tau expression in neurons of critical brain structures. The following lines of evidence support this hypothesis. First, epidemiologic data suggest the involvement of genetic factors in the susceptibility to PTSD. Second, the common features of both abnormal tau expression and PTSD include amygdalar and hippocampal atrophy, upregulation of norepinephrine biosynthetic capacity in the survivinglocus coeruleus neurons and dysfunction of *N*-methyl-*D*-aspartate-receptors. Finally, our experiments using rTg4510 mice, a model that over-expresses human mutant tau and develops age-dependent tauopathy, demonstrate that these animals display circling behavior thought to be related to states of anxiety. To detect the potential molecular mechanisms underlying PTSD episodes, laser-assisted/capture microdissection can be used with microarray analysis as an alternative approach to identify changes in gene expression in excitatory and/or inhibitory neurons in critical brain structures (*i.e.*, hippocampus and amygdala) in response to the onset of PTSD.

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**Key words:** Amygdalar damage; Anxiety behavior; Microarrays; Microdissection; Microtubule-associated protein tau; Post-traumatic stress disorder; RNA quality

**Core tip:** We propose that susceptibility to post-traumatic stress disorder (PTSD) may be determined, in part, by aberrant microtubule-associated protein tau expression in neurons of critical brain structures. We review several lines of evidence to support this novel hypothesis. In addition, we review types of PTSD, namely non-classical PTSD, induced by various medical conditions and address this issue of why non-classical PTSD can be reliably elicited. To verify our hypothesis, we propose to use animal models of PTSD combined with laser-assisted/capture microdissection and microarray analysis to examine gene expression changes in selected cellular elements in response to the occurrence of PTSD.

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**INTRODUCTION**

***Defining posttraumatic stress disorder***

Posttraumatic stress disorder (PTSD) is an anxiety disorder which can develop following exposure to a traumatic event such as combat, natural disasters, domestic violence, or other catastrophes. Epidemiological studies demonstrate that 4%-23% of those experiencing a traumatic event develop PTSD[1-4]. The lifetime prevalence of PTSD among US citizens is approximately 8%[5,6]. Functionally, abnormalities in amygdala, prefrontal cortex, and hippocampus as well as abnormalities in neuroendocrinologicol characteristics may be associated with PTSD[7].

The development of PTSD requires exposure to a traumatic event which is then followed by the altered regulation of the neural circuits that govern what is often termed the “fight-or-flight” response. As reviewed by Sherin and Nemeroff[8],this dysregulation likely involves norepinephrine, γ-aminobutyric acid (GABA), serotonin, and neuropeptide Y and includes the hippocampus, amygdala and the prefrontal cortex. Still, the majority of those exposed to a traumatic event do not develop PTSD and thus, risk factors have been identified which indicate increased vulnerability. These risk factors include smaller hippocampal volume, below normal executive function abilities, poorer attention, older age at time of traumatic event, female gender, and co-morbid disease[3,4,9]. Further, gene-environment interactions as well as epigenetic influences are likely to be important factors in the consideration of PTSD risk[10,11]. Still, the molecular and pathogenic bases underlying vulnerability to PTSD are largely unknown.

***Non-classical PTSD***

PTSD-like symptoms can arise after serious health events that may be associated with brain damage/neural loss. We call this medically induced condition “non-classical PTSD” to discriminate it from the classical PTSD qualified for standards of the fifth edition of the American Psychiatric Association's Diagnostic and Statistical Manual of Mental Disorders (DSM-5). Such medical events include stroke[12,13],brain trauma[14],acute coronary syndromes (ACS) , [7] a brief treatment in an intensive care unit[15] and even hematopoietic stem-cell transplantation therapy for cancer[16,17].The diagnostic criteria for non-classical PTSD are similar to those for classical PTSD and include symptoms such as re-experiencing, cognitive or behavioral avoidance of reminders of the event, and physiological hyperarousal following occurrence of the above mentioned critical medical events. Per the DSM-5, non-classical PTSD should not be included in the PTSD category since one of its exclusive criteria is that the medical condition(s) and not a traumatic/catastrophic event(s) is the eliciting cause. Interestingly, the prevalence of non-classical PTSD is similar to that of classical PTSD. For example, the overall prevalence of ACS-induced PTSD is 12% and individual study prevalence estimates range 0%-32%[7]; and incidence of stroke-elicited non-classical PTSD is 10%-31%[13,18]. Further, non-classical PTSD appears to be unrelated to neurologic impairment[18].In addition, the biological basis underlying the occurrence of non-classical PTSD remains unclear, even when substantial brain damage is involved. One approach is to examine whether non-classical PTSD shares, at least partially, a common biological basis/pathogenic pathway with that described for classical PTSD. Modeling non-classical PTSD should be feasible and/or reproducible in experimental animals because the key PTSD brain structures, such as the amygdala and/or the hippocampus, could be directly targeted.

**HYPOTHESIS**

Here, we hypothesize that susceptibility to PTSD may be determined in part, by aberrant tau expression in the amygdala and hippocampus. This abnormal expression is thought to then interfere with the normal cognitive processes in response to traumatic events, thus conferring vulnerability to PTSD development.

**GENETIC FACTORS AND PTSD**

As defined by the DSM-5, PTSD symptoms include four main types: re-experiencing the traumatic event, avoiding reminders of the trauma, negative cognitions and mood, and increased anxiety/emotional arousal. Clinical reports clearly support a role for genetic factors in the development of PTSD[19].Quantitative genetic analyses of monozygotic and dizygotic male twin pairs reveal that genetic factors account for 13%-30% of the variance in liability for symptoms in the ”re-experiencing“ cluster, 30%-34% for symptoms in the ”avoidance cluster“ and 28%-32% for symptoms in the ”arousal cluster” [20].Hyperresponsivity in the dorsal anterior cingulate is proposed as a familial risk factor for the development of PTSD following psychological trauma[21]. A report on 200 members of 12 multigenerational families that experienced an earthquake demonstrated the likelihood of inherited vulnerability to symptoms of PTSD[22].The specific genes that may cause increased PTSD susceptibility have not been identified. However, in the first genome-wide association study of PTSD, several single-nucleotide polymorphisms (SNPs) were associated with PTSD[23]. It has been hypothesized that strong memory of a traumatic event could contribute to PTSD development and symptoms, and a genetic inclination for strong memories might confer an increased risk. In support of this, a specific SNP within the gene that encodes protein kinase C alpha, a memory-relevant gene, may be linked to increased PTSD risk[24]. Nevertheless, clinical association studies have not established a causative relationship between any specific gene and PTSD.

**PTSD AND ALZHEIMER’S DISEASE**

At least four million Americans suffer from Alzheimer’s disease (AD) and associated disorders in which tau pathology is one of the hallmarks. While there are no reports directly linking PTSD and AD (or mild cognitive impairment), common features of the disorders include amygdalar and hippocampal atrophy[25-29],upregulation of norepinephrine biosynthetic capacity in survivinglocus coeruleus neurons[30],and NMDA-receptor activation dysfunction[31]. A review of imaging studies (single photon emission tomography; positron emission tomography; magnetic resonance imaging; and functional magnetic resonance imaging) described morphological similarities between AD and PTSD in the medial temporal lobe, hippocampus, and cingulate cortex[32]. In addition, there is increasing evidence to suggest that amygdalar degeneration is associated with emotional disorders, including AD and PTSD, and that unilateral amygdalar atrophy can manifest in tauopathies[33].

Anatomical connections may provide an explanation of the aforementioned similarities between PTSD and AD: noradrenergic projections to the amygdalar complex and hippocampus originate in the locus coeruleus[34]. In response to stressful stimuli, the hypothalamic-pituitary-adrenocortical (HPA) axis acts with a surge in adrenocorticotropic hormone and glucocorticoid release which initiates a response in central nervous system circuitry[35]. Locus coeruleus norepinephrine projections are some of the pivotal structures bridging the central stress response pathways to HPA activity[36,37]. The locus ceruleus, *via* release of norepinephrine, can modulate cellular excitability and synaptic efficacy and, thus, influence behavioral performance[38]. Nevertheless, there is little information concerning how this anatomical link may contribute to vulnerability to PTSD in AD or AD-susceptible populations.

**ANIMAL MODELS OF PTSD**

Several paradigms for inducing PTSD in animal models have been accepted. Generally, they include the use of brief stressors which result in biological and behavioral outcomes that simulate PTSD symptoms. As reviewed by Pitman *et al*[39],models with both face and construct validity include predator exposure, serial exposure to multiple stressors, and footshock with additional stressors. Nevertheless, the complexity and variability of human PTSD symptoms make it difficult to establish animal models that precisely mimic human PTSD.

**RTG4510 TRANSGENIC MOUSE MODEL AND PTSD**

The rTg4510 transgenic mouse was created as a model of inducible tauopathy[40].With age, rTg4510 mice develop neurofibrillary tangles (NFTs) and neuronal and memory loss. The tau transgene is driven by a tetracycline-operon-responsive element. Tet transactivator binds the tetracycline operator sequences within the cytomegalovirus promoter and drives the expression of the human tau transgene (human 4-repeat tau containing the P301L mutation). A 15-fold over-expression of tau in the forebrain (hippocampus and cortex) can occur and can be repressed with doxycycline in this model. This model has been widely investigated with reports of decreased amygdala and hippocampal activity[41],loss of synapses[42],and poor spatial learning and memory, particularly in females[43]. Importantly, the cognitive dysfunction in older rTg4510 mice can be reversed by repressing tau expression, despite the pre-existence of brain atrophy, neuronal loss, and the continued accumulation of the 64 kDa insoluble tau species and NFTs[40].

Our hypothesis that tau expression may be linked to PTSD risk is based on our recent report describing injection of 2 μL of 1% fluorogold, a “harmless” fluorescent tracer, into the right amygdala elicited circling behavior thought to be related to an anxiety-like state[44].This circling behavior was transient in control mice, but persisted for 14 days in rTg4510 mice. The post-injection clinical signs observed in the rTg4510 mice were of the type thought to be relevant to those appropriate for an animal model of PTSD[45]. Specifically, the fluorogold injections elicited: seizure-like attacks which were characterized by high-amplitude motor spasms of the extremities and trunk while the animal was lying on its back; rolling along the longitudinal body axis and/or turning over spontaneously; persistent circling behavior, in the presence or absence of stimuli, that occurred primarily during the light period when mice would normally be sleeping; and hyperexcitablity (the circling behavior often occurred following minimal stimulation, such as a gentle push)[44].

**MECHANISMS UNDERLYING THE VULNERABILITY OF THE RTG4510 MOUSE MODEL TO PTSD**

***Reduced volume in key brain structures***

Reduced hippocampal and anterior cingulate volumesappear to be a characteristic of PTSD[46-50] as well as dysfunction in the medial prefrontal cortex, amygdala, and hippocampus[51]. rTg4510 mice develop NFTs and neuronal and memory loss in an age-dependent fashion[40].Very little tau pathology exists at 1 month of age, but hippocampal and cortical pre-tangle structures are detectable by 2.5 mo and argyrophilic tangles develop by 4-5.5 mo. By 5.5 mo of age, brain weight is significantly less and the total number of CA1 hippocampal neurons is decreased by about 60%[40].Reduced hippocampal volume suggests a reduced capacity to handle stress. Neuronal loss may involve those cells that are critical to maintain the balance of corticosteroid receptors/responses in these regions, which together with other modulators control the final output of the stress response[52].In rTg4510 mice treated with fluorogold, the abnormal behavior was observed at 2.5 mo of age, when no significant brain weight or neuron loss would be detectable[44].Animals at this age, however, begin to show pre-tangles, an early sign of neuronal tau pathology, implying a reduced functional neuron capacity[40,53].

Interestingly, mortality occurred exclusively in the rTg4510 mice following the fluorogold injection and this did not correlate with either age or severity of tauopathy[44]: seventeen to twenty-five percent of the fluorogold injected rTg4510 mice died while all fluorogold injected wild type mice survived. Fourteen days after the fluorogold injection, in both rTG4510 and control mice, the fluorogold was well distributed and easily detected on the side of injection throughout the hippocampus and parietal cortex[44],both pivotal structures involved in the development of PTSD. Fluorogold deposition in the amygdala, hippocampus and primary and secondary motor cortices (which occurred *via* axonal transport whereas in the amygdala it occurred *via* direct injection) may have served as an enduring “traumatic event” which resulted in the abnormal behavior in the rTg4510 mice. Describing fluorogold injection as a “traumatic event” may be valid even though the amygdala did not exhibit significant caspase-3 immunoreactivity or terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay-measured neuronal death[44].Because the distribution of fluorogold *via* axonal transport appeared similar in control and rTg4510 mice[44] (our unpublished data), the behavioral abnormalities expressed by the rTg4510 mice are, at least partially, attributable to a vulnerability associated with overexpression of human mutant tau.

***Over-activation of excitatory pathways***

**Increased excitatory activity:** The only FDA-approved treatments for AD involve pharmacological manipulation of the glutamatergic NMDA receptor pathway. One of those treatments is memantine, an adamantane derivative and NMDA receptor antagonist. NMDA receptors in the amygdala are thought to participate in the modulatory effect of glucocorticoids on the extinction of fear memories. [54] NMDA receptors are also involved in stress-induced anxiety: administration of the NMDA receptor antagonist, MK-801, before exposure to a predator prevented the increase in anxiety-like behaviors typically exhibited after that stress[55]. That same administration (*i.e.*, MK-801) increased the number of approaches to the predator[55]. Hippocampal-associated memory impairments after stress are likely influenced by stress-induced elevations in corticosteroid levels which modulate fast excitatory amino acid-mediated synaptic transmission and synaptic plasticity[56]. Relevant to our hypothesis here, the excitotoxin quinolinic acid can induce tau phosphorylation *via* NMDA receptor activation[57]. Tau-tubulin kinase-1 (TTBK1)levels were reported to be up-regulated in human AD brains compared with age-matched controls. Additionally, in TTBK1 transgenic mice, up-regulation of TTBK1 was associated with the aggregation of phosphorylated neurofilaments in brain and reduced expression of NMDA receptor types 2B and D[58],suggesting aberrant activities of NMDA receptors in these animals. Nevertheless, mechanisms underlying how over-expression of human mutant tau protein elicits over-activity of NMDA receptors or NMDA pathways remain unclear.

**Reduced “inhibitory” activity associated with NMDA receptor activation**: Neuronal activity often involves the NMDA receptor in the transfer of electrical signals and it is thought that the NMDA receptor ion channel must be open for it to be functional. Inhibition of NMDA receptors *via* NMDA antagonist treatment can induce an anesthetic state characterized by [catalepsy](http://en.wikipedia.org/wiki/Catalepsy), [amnesia](http://en.wikipedia.org/wiki/Amnesia), and [analgesia](http://en.wikipedia.org/wiki/Analgesia)[59]. On the other hand, NMDA receptors can be modulated by various endogenous and exogenous molecules. Local ions, such as Mg2+ and Zn2+, can block the NMDA receptor ion channel[60,61] and external and/or internal cellular Ca2+, Na+ and K+ concentrations can modulate NMDA receptor activity[62-64]. Perhaps importantly, extracellular proton concentrations regulate NMDA channels[65]: the responses of NMDA receptors to glutamate can be down-regulated by increasing extracellular H+ ions[60],whereas under alkaline conditions, NMDA-evoked cytosolic calcium influxes can be increased[66].Synaptically-evoked H+ shifts modulate NMDA receptor activity[67].In addition, polyamines can modulate NMDA-induced depolarization[68]. Mechanistically, this modulation may depend upon on polyamine interactions with a proton-sensitive location on the extracellular N-terminal of the NR1 subunit[69].In addition, polyamines can function as allosteric modulators of NMDA receptors *via* N-terminal interactions on NR2 subunits[70,71].

The vulnerability of rTg4510 mice to fluorogold treatment may be attributable to aberrant NMDA receptor function. Fluorogold may act on NMDA receptors in the transgenic mice differently than those in the normal/wild type controls. The active constituent of fluorogold is the weak base hydroxystilbamidine. Accordingly, fluorogold may affect NMDA receptor function by increasing the extracellular pH at the injection site. It has also been suggested that fluorogold crosses cell membranes in its uncharged form and then is trapped intracellularly in acidic cellular compartments due to a favorable pH gradient[72].This action may then regulate NMDA receptor activity by changing intracellular pH. Second, because hydroxystilbamidine, as an aromatic diamidine, can inhibit the cellular uptake of polyamines[73,74],fluorogold may alter polyamine metabolism, thereby indirectly affecting NMDA receptor activity. Finally, fluorogold may cause imbalances in neurotransmitter concentrations at the injection site: micromolar concentrations of fluorogold inhibit dopamine release and fluorogold abolishes the dopamine release evoked by glutamate or Ca2+[75].

**FUTURE STUDIES**

Two specific questions will be addressed in future studies. One aim of investigation will involve an examination of the hypothesis that expression of human mutant tau in amygdalar and hippocampal neurons enhances susceptibility to development of PTSD and that inhibition of mutant tau expression will decrease this vulnerability. Young female rTg4510 and wild-type mice with/without doxycycline treatment could be subjected to PTSD modeling (such as electric foot-shock), followed by the measurement of anxiety-relevant behaviors, such as elevated plus maze behavior. If pathophysiological changes occur in the amygdala and hippocampus as a result of specific traumatic events (*e.g.*, foot shock), this may trigger the cascade needed to model PTSD. rTg4510 mice would be expected to exhibit increased vulnerability to foot shock which would be reflected in increased anxiety-like behavior as a result. It is highly likely that rTg4510 mice are also susceptible to other types of traumatic events due to their tau pathology burden and/or aberrant gene expression in neurons in the amygdala and hippocampus. Accordingly, lifelong suppression of tau gene expression by treatment with doxycycline may reverse this vulnerability in rTg4510 mice.

Another aim of investigation will test the hypothesis that intra-amygdala injection of fluorogold as a traumatic stimulus can produce animals with reliable and reproducible behavioral profiles reminiscent of PTSD. In addition, the fluorogold model could be used to optimize manipulations to decipher the molecular mechanisms underlying the susceptibility of the rTg4510 mouse to stress-induced abnormalities. Here, rTg4510 and wildtype mice would be unilaterally injected with fluorogold or vehicle into the amygdala and then subjected to footshock or sham-treatment. Subsequently, all mice would be assessed for anxiety-relevant behaviors. Mice would be sacrificed at various times following anxiety measures and brains harvested for evaluation. Neurons in the contralateral (*i.e.*, intact side) amygdala and hippocampus would be collected *via* laser-capture microdissection (LCM) or laser-assisted microdissection (LAM). A T7 method (the Eberwine T7 protocol) that linearly increases mRNA copies could be used for mRNA signal amplification and RNA quantity and quality could be determined using microfluidic technology (*e.g.*, Bioanalyzer, Agilent Technologies, Palo Alto, CA, United States). Gene expression could then be profiled using genome-wide/pathway microarrays. Validation of microarray outcomes would be performed at the transcriptional and translational levels.

The molecular mechanisms underlying the pathology of PTSD are poorly understood. A traumatic event directly targeting the amygdala unilaterally may result in an animal model characterized by reliable and reproducible behavioral characteristics that are relevant to the study of PTSD. The contralateral (untreated) amygdala would remain “intact” and thus serve as a within-subject control, facilitating analyses of potential molecular mechanisms. Utilization of LCM/LAM to collect the targeted tissue for subsequent microarray analyses will allow for the evaluation of cell-specific gene expression. Validation of the information using independent molecular biological techniques will be important and may lead to the identification of new research and therapeutic and preventive strategies with direct relevance for PTSD.

Interestingly, there have been multiple studies using LCM/LAM and microarray analyses to define the genetics associated with the functional responses in the decisive components (neurons) within the amygdalar complex[76-78].In addition, integrating LCM/LAM techniques with RNA amplification (PCR/quantitative PCR) has also been described in efforts to define changes in the targeted amygdalar gene(s) that may be responsible for the control of emotion or memory[79-83].However, it appears that monitoring RNA quality before microarray/RNA amplification of microdissected neurons has not been properly addressed: the RNA quality in the cited references was either indirectly examined or was not verified at all; it is arguable, though, that the reproducibility of microarray data and/or the detectability of targeted genes provide evidence for a certain degree of reliability.

In our laboratory, neurons in the mouse spinal cord (NeuN-positive profiles) or the rat hippocampus (methyl green stained cells in the CA1 layer and dentate gyrus granule cell layer) were harvested either singly in the case of NeuN-stained motor neurons, or in groups in the case of methyl green-stained hippocampal cells (Figure 1) using a laser microdissection system (Version 4.0, Leica, Bannockburn, IL, United States) under a × 40 objective (final magnification × 400) for single cells or a × 10 objective (final magnification × 100) for groups of cells. A total of 37 tubes of microdissected cells from the hippocampus and dentate gyrus were collected. Each of these tubes contained variable numbers of cells, up to several thousand cells per tube. RNA extracts were then analyzed with a Pico Chip (Agilent Technologies, Palo Alto, CA, United States). A few of these samples exhibited noticeable degradation (*e.g.*, Figure 2 lane 7) although multiple, constrained, standard operating procedures were followed to ensure RNA quality used in profiling the 18S and 28S rRNAs with the Model 2001 Bioanalyzer (Agilent Technologies, Palo Alto, CA, United States). The amount of RNA in these tubes ranged from 193 pg/μL to 9475 pg/μL, RNA samples provided reasonable microarray data (Figure 3), while the RNA samples that did not qualify with the 18S and/or 28S rRNAs profiles were not analyzed. On the other hand, from approximately 1000 motor neurons that were individually harvested *via* microdissection from the anterior horn of the mouse spinal cord, the yield of RNA was 116 ± 68 pg/μL[84], which is beyond the recommended capability of the Bioanalyzer (limit of 200 pg/μL: below this value, the Bioanalyzer may not display the electrophoretic profiles including the 18S and/or 28S rRNAs). Thus, the RNA quality cannot be determined using the criteria used previously for the 18S and 28S rRNAs. Forty-nine out of 50 sets of the 1000-neuron RNA samples were amplifiable using a T7 amplification method and the electropherograms (Figure 4) indicated that the aRNA sizes extended to well beyond 6000 nucleotides, providing an alternative measure: the number of nucleotides might be used as a type of criteria for addressing the quality of the aRNA. Actually, the aRNAs yielded reproducible microarrays with correlation coefficients of > 0.9 (Figure 5) between 2 microarrays that were randomly chosen. Practically, a subset of neurons that express the targeted proteins - such as excitatory glutamatergic neurons with CamKIIα as a marker or inhibitory GABAergic neurons with GAD67 as a marker[85] - can be selectively collected using an optimized immunohistochemical labeling technique followed by the LAM/LCM procedures. Presumably, the altered gene expressions in the excitatory and/or inhibitory neurons may indicate the signaling pathways accountable for the vulnerability to and onset of PTSD.

**CONCLUSION**

Susceptibility to PTSD may be related, in part, to aberrant tau expression in neurons of critical brain structures. This abnormal expression is postulated to interfere with the function of those central nervous system circuits that normally respond to traumatic stress, thus conferring vulnerability to PTSD development. Verification of the vulnerability of the brain to develop PTSD due to an overabundance of tau expression may require a model that does not employ direct intra-brain/amygdalar damage. On the other hand, modeling PTSD might be more feasible using this approach because the key PTSD brain structures, the amygdala and the hippocampus, could be directly targeted. Defining the molecular mechanism(s) underlying the expression of PTSD will be challenging. The integration of the LAM/LCM technique with gene expression analyses in neurons of brain structures critical to the development of PTSD seems a useful approach, provided that the quality of the RNA obtained using LAM/LCM can be demonstrated.

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**Figure 1 Examples of tissues following microdissection using a Leica DMLA laser-assisted microdissection system.** A: A scanned image of a foil slide with multiple coronal sections of mouse spinal cord mounted. These sections are 16 μm thick; B: A high-power (× 40 objective, × 400 final magnification) image of the ventral gray region of mouse spinal cord that was immuno-stained for the NeuN neuronal marker. The yellow-brown profiles are motor neurons; C: The same view as in panel B, but after laser microdissection and collection of four motor neurons; C: A low-power (× 5 objective, × 50 final magnification) image of the spinal cord section (shown upside down), with the red square marking the region shown in panels B and C; D: A low-power view (× 10 objective, × 100 final magnification) of methyl green-stained rat dentate gyrus. The section is 10 μm thick; E: An image of the same section shown in panel D, but after microdissection and collection of the neurons in the granule cell layer.



**Figure 2 Ensuring quality of the microdissected neural RNA samples.** For the 28S and 18S rRNA species/bands, a Bioanalyzer Model 2001 was used to examine the quality of the RNA sample derived from the neurons collectively harvested from the hippocampus (Figure 1D and E). Lane L: The RNA ladder; lanes 1-4: Non-sample controls; lanes 5-11: Electrophoretic profiles of RNA from multiple tubes of cells collected *via* laser-assisted microdissection; lane 7: The sample to be degraded and was excluded from further evaluation.



**Figure 3 Example of an Agilent Rat Oligo microarray hybridized with probes from microdissected neurons.** The upper, right-hand panel is an enlarged view of a portion of the microarray. The two probes used were made from RNA amplified through one stage of the T7 method, the Cy3-labeled probe was synthesized from total RNA extracted from several thousand CA1 pyramidal neurons and the Cy5-labeled probe was synthesized from several thousand dentate gyrus granule cells, after laser-assisted microdissection. The two probes are shown overlaid; the predominance of yellow spots indicates that most of the genes in the two samples were at or near equivalent levels. Only a few spots are saturated (white). Shown in the lower panel, the genes of interest can be identified on the scatterplot (CA1 neurons *vs* dentate granular neurons). An example of one gene of interest is the highlighted black spot, which represents caspase-3, a key apoptotic mediator.



**Figure 4 Bioanalyzer profiles of aRNA products from microdissected mouse spinal cord motor neurons using the Arcturus PicoAmp kit.** A and B: These are the standard views obtained from the Bioanalyzer of the Pico Chip and Nano Chip data, providing electrophoretic profiles in time (s). The ladder is shown in the uppermost profiles. Note that the quantity of ladder used in the Pico Chip (A, upper panel) was 1000 pg, but the quantity of ladder used in the Nano Chip (B, upper panel) was 150 ng (150000 pg). Thus, the scales for the Pico Chip (A) and Nano Chip (B) profiles are approximately 150-fold different. The samples shown (S1, S2, S8, and S11) are aRNA products obtained after the first round of PicoAmp amplification (A) and the second round of amplification (B). The black arrowheads in the Pico Chip data for S8 and S11 show visible points of maximal migration for RNA in these two samples; C: RNA concentrations before and after one- and two-rounds of amplifications in 4 representative samples of S1, 2, 8, and 11.



**Figure 5 Examples of microarray data obtained from microdissected neurons. Two microarray experiments using spinal cord motor neurons from four mice were performed.** Scatterplots of processed data generated using the program GeneSpring are shown. In each microarray experiment, a Cy3-labeled probe made from approximately 1000 motor neurons from a P301L transgenic mouse was co-hybridized with a Cy5-labeled probe made from approximately 1000 motor neurons from a non-transgenic littermate. The results are shown in the top two scatterplots. The colors of the plotted data are derived from the scale shown at right, indicating the expression fold change. To further examine the quality of the data, a derivative plot was made (bottom). The data from two non-transgenic littermates from the two microarray experiments were plotted against each other. The correlation coefficient of these two biological replicates was *r* = 0.9 (bottom panel).