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Viral delivery of shRNA to amygdala neurons leads to neurotoxicity and deficits in Pavlovian fear conditioning

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Abstract

The use of viral vector technology to deliver short hairpin RNAs (shRNAs) to cells of the nervous system of many model organisms has been widely utilized by neuroscientists to study the influence of genes on behavior. However, there have been numerous reports that delivering shRNAs to the nervous system can lead to neurotoxicity. Here we report the results of a series of experiments where adeno-associated viruses (AAV), that were engineered to express shRNAs designed to target known plasticity associated genes (i.e. Arc, Egr1 and GluN2A) or control shRNAs that were designed not to target any rat gene product for depletion, were delivered to the rat basal and lateral nuclei of the amygdala (BLA), and auditory Pavlovian fear conditioning was examined. In our first set of experiments we found that animals that received AAV (3.16E13 – 1E13 GC/mL; 1ul/side), designed to knockdown Arc (shArc), or control shRNAs targeting either luciferase (shLuc), or nothing (shCntrl), exhibited impaired fear conditioning compared to animals that received viruses that did not express shRNAs. Notably, animals that received shArc did not exhibit differences in fear conditioning compared to animals that received control shRNAs despite gene knockdown of Arc. Viruses designed to harbor shRNAs did not induce obvious morphological changes to the cells/tissue of the BLA at any dose of virus tested, but at the highest dose of shRNA virus examined (3.16E13 GC/mL; 1ul/side), a significant increase in microglia activation occurred as measured by an increase in IBA1 immunoreactivity. In our final set of experiments we infused viruses into the BLA at a titer of (1.60E+12 GC/mL; 1ul/side), designed to express shRNAs designed to target Egr1 (shEgr1), GluN2A (shGluN2A), shArc, shLuc, shCntrl, or a virus which did not express an shRNA, and found that all groups exhibited impaired fear conditioning compared to the group which received a virus that did not express an shRNA. The shEgr1 and shGluN2A groups exhibited gene knockdown of Egr1 and GluN2A compared to the other groups examined respectively, but Arc was not knocked down in the shArc group under these conditions. Differences in fear conditioning among the shLuc, shCntrl, shArc and shEgr1 groups were not detected under these circumstances, however the shGluN2A group exhibited significantly impaired fear conditioning compared to most of the groups, indicating that gene

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specific deficits in fear conditioning could be observed utilizing viral mediated delivery of shRNA. Collectively, these data indicate that viral mediated shRNA expression was toxic to neurons *in vivo*, under all viral titers examined and this toxicity in some cases may be masking gene specific changes in learning. Therefore, the use of this technology in behavioral neuroscience warrants a heightened level of careful consideration and study design and potential methods to alleviate shRNA induced toxicity are discussed.

Introduction

The ability to genetically modify organisms provides the means to determine how individual genes contribute to the functioning of the nervous system. Currently, mouse and rat gene “knockouts/ins” and transgenics are the main systems used to investigate the role and function of individual mammalian genes in behavioral neuroscience (Capeocchi, 2005). One technology to study gene function that offers alternatives to the above mentioned technology is to use viruses to deliver transgenes of interest to specific cells or regions of the nervous system. If the virus is designed to contain a gene that codes for a short hairpin RNA (shRNA), specific gene products (i.e. mRNAs) can be targeted for degradation via the RNA interference (RNAi) pathway (Hommel, Sears, Georgescu, Simmons, and DiLeone, 2003). This technology can essentially be used to knockout genes in particular tissues/cells quickly, before or after behavioral training and relatively easily.

Intriguingly, there are numerous reports that *in vivo* use of shRNAs can be problematic. For example, there have been studies that report that viral delivery of shRNAs to the mouse and rat brain is associated with neural toxicity. For example, AAV mediated delivery of shRNA to the striatum of mice has been reported to induce neural toxicity, neuronal cell loss, increased inflammation as measured by an increase in microglia activation, motor disturbances and early demise (Martin, Wolken, Brown, Dauer, Ehrlich, and Gonzalez-Alegre, 2011; McBride, Boudreau, Harper, Staber, Monteys, Martins, Gilmore, Burstein, Peluso, Polisky, Carter, and Davidson, 2008). These findings were caused by the expression of all shRNAs examined, even shRNAs that were not designed to target any gene product for depletion. Similar neurotoxicity has been reported to be induced by viral delivery of shRNA to the rat substantia nigra (Khodr, Sapru, Pedapati, Han, West, Kells, Bankiewicz, and Bohn, 2011; Ulusoy, Sahin, Bjorklund, Aebischer, and Kirik, 2009), cerebellum (Boudreau, Martins, and Davidson, 2009), and red nucleus (Ehlert, Eggers, Niclou, and Verhaagen, 2010). However, despite these previous findings that viral mediated delivery of shRNA may cause neural toxicity when administered to the central nervous system of mice and rats, it remains a common tool to examine the role of genes in behavior.

In this series of experiments, we were specifically interested in determining if robust learning and memory deficits could be created when viral mediated delivery of shRNA technology was used to target the mRNAs for the Activity regulated cytoskeletal (Arc) gene, Early Growth Response 1 (Egr1) gene, and the GluN2A gene - a subunit of the *N*-Methyl-D-aspartate receptor (NMDAR). Because these genes have previously been shown to be critical for amygdala dependent Pavlovian fear conditioning (Jones, Errington, French, Fine, Bliss, Garel, Charnay, Bozon, Laroche, and Davis, 2001; Maddox, Monsey, and Schafe,

2011; Plath, Ohana, Dammermann, Errington, Schmitz, Gross, Mao, Engelsberg, Mahlke, Welzl, Kobalz, Stawrakakis, Fernandez, Waltereit, Bick-Sander, Therstappen, Cooke, Blanquet, Wurst, Salmen, Bosl, Lipp, Grant, Bliss, Wolfer, and Kuhl, 2006; Ploski, Pierre, Smucny, Park, Monsey, Overeem, and Schafe, 2008; Walker and Davis, 2008), we reasoned that if this technology was effective, it should be able to knockdown these genes within the amygdala and impair Pavlovian fear conditioning. Therefore, we designed adeno-associated viruses that harbored short-hairpin RNAs (shRNA) designed to target the mRNAs for each of these genes. These viruses, along with control viruses which harbor shRNA genes that are not designed to target any rat mRNA for degradation, were bilaterally infused into the rat basal and lateral amygdala and subsequently these animals were auditory fear conditioned and fear memory was assessed. We found that the viruses designed to express control shRNAs and shRNAs designed to target known plasticity associated genes (i.e. Arc, Egr1 and GluN2A) were toxic and auditory Pavlovian fear conditioning was significantly impaired. Our results indicate that the impairments in Pavlovian fear conditioning were due to the viruses harboring shRNA genes and were not due to surgery, the virus itself or viral mediated GFP expression and these results were dose dependent. Unfortunately, even when significantly lower doses of shRNA harboring viruses were infused into the amygdala, impairments in fear conditioning were observed, which likely prevented the detection of gene specific deficits in fear learning. Here we report our findings and discuss the implications of these results.

Results

Viral delivery of shRNA to the rat amygdala results in relatively weak fear conditioning

In our first set of experiments we aimed to knockdown Arc gene expression within neurons of the rat basal and lateral amygdala (BLA) utilizing viral mediated delivery of shRNA, and then we wanted to determine how viral mediated knockdown of Arc influenced learning and memory utilizing Pavlovian auditory fear conditioning. We developed AAV2 viral genome vectors that contain a gene expression cassette designed to express GFP from a CMV promoter, and an shRNA expression cassette designed to express the shRNA from an H1 RNA polymerase III promoter. To target Arc for depletion, we utilized an shRNA sequence (shARC), that has previously been shown to be effective at lowering Arc levels within neurons (Rial Verde, Lee-Osbourne, Worley, Malinow, and Cline, 2006; Waung, Pfeiffer, Nosyreva, Ronesi, and Huber, 2008). As a control, we developed a similar viral vector that contained an shRNA sequence (shLuc), that was designed to target and deplete luciferase gene products and this shRNA has been widely utilized within many *in vitro* studies as a control, since it is not designed to target any rat gene product for depletion (Hung, Sung, Brito, and Sheng, 2010).

To measure the effectiveness of the shArc viral plasmid *in vitro*, the AAV-shArc and AAV-shLuc plasmids were co-transfected into 293FT cells with a plasmid that was designed to express Arc protein fused to red fluorescent protein (RFP). Ninety six hours post transfection, the levels of RFP and GFP were examined via fluorescence microscopy. Each well contained similar levels of GFP indicating that the transfection efficiency for each plasmid was similar among samples. However, the levels of Arc-RFP were lower in the cells

that received shArc compared to the cells that received shLuc, indicating that the shArc was effective at depleting Arc-RFP levels, as intended. To quantitatively assess shArc effectiveness, cells were treated in a similar manner as above, but they were harvested at forty eight hours post transfection, and Arc mRNA levels were assessed by qRT-PCR. Arc mRNA levels were found to be significantly lower in cells that received shArc versus cells that received shLuc [$t_{(4)} = 4.3812$, $p = 0.0006$] (Figure 1C).

Next, we created adeno-associated viruses with the viral shArc and shLuc plasmids, pseudotyped as AAV2/DJ8 and infused these viruses bilaterally into the rat BLA at a titer of 3.16×10^{13} GC/mL DNase resistant viral particles. Twenty one days later, the animals were sacrificed, and the brains were removed and processed so the viral transduced areas of the BLA could be laser microdissected (LMD) and Arc mRNA levels could be subsequently examined via qRT-PCR. The LMD/qRT-PCR approach was used to examine gene expression levels, because it provides the most accurate means possible to assess gene expression changes within such a relatively small region of the brain and provides the means to specifically dissect areas of tissue that are transduced by virus. More traditional approaches, such as using punch tools to dissect the amygdala, would inevitably dissect out tissue that was not entirely of BLA origin and areas of tissue that were not transduced by virus. The qRT-PCR results revealed a significant decrease in Arc mRNA in animals that received an infusion of shArc compared to animals infused with a virus expressing shLuc [$t_{(9)} = -5.07$, $p = 0.0007$; (shArc, $n = 6$; shLuc, $n = 5$)] (Figure 1F). Collectively, these data indicate that these shRNA vectors are effective at depleting Arc mRNA levels *in vivo* and *in vitro*.

To examine how viral mediated delivery of shRNA may influence learning in a Pavlovian auditory fear conditioning paradigm, shArc and shLuc viruses were infused bilaterally into the BLA at a titer of 3.16×10^{13} GC/mL. Twenty one days later, the animals were auditory fear conditioned to a 30 sec, 5 kHz, 75 dB tone that co-terminated with a 1.5 mA foot shock. Three hours following fear conditioning training, retention of short term memory (STM) was assessed by exposing the rats to three 5 kHz, 75 dB tones within a novel context. The tone induced freezing behavior was determined for each rat and an ANOVA revealed there was not a significant difference in freezing levels between the groups [$F_{(1,17)} 0.3560$, $p = 0.5585$] (Figure 1G). Long-term memory (LTM) was assessed 24 hours post-training, in a similar manner as to STM, and freezing levels did not differ between the groups [$F_{(1,17)} = 0.020$, $p = 0.8883$] (Figure 1H). Despite the fact that freezing levels did not differ between the shArc and shLuc groups across STM and LTM measures within these experiments, the most striking observation was the extremely low levels of freezing observed in these experiments.

Viral mediated shRNA expression within the BLA interferes with fear conditioning and leads to dysregulation of gene expression

In our first series of experiments, we observed very low levels of cue induced freezing in animals that received bilateral BLA infusions of viruses designed to deliver RNAi. We reasoned that the low freezing levels may have been caused by the viruses that were designed to express the shRNA genes. To test this hypothesis, we developed a series of

AAV2 viral genome plasmids that differed in their ability to express shRNA. Viral plasmids were developed that did not contain an shRNA expression cassette (GFP only), or that contained an shArc and shLuc expression cassette that contained a mutated/truncated H1 promoter (shArc(H1⁻), shLuc(H1⁻)). This particular H1 promoter mutation has previously been shown to virtually abolish transcription from this promoter (Myslinski, Ame, Krol, and Carbon, 2001). Viral plasmids were also constructed to contain 4 shRNA expression cassettes in tandem (shArc(4) and shLuc(4)).

To confirm that the shArc(4) viral plasmids indeed expressed more shRNA, shArc and shArc(4) plasmids were transfected into 293FT cells and forty eight hours later, small RNAs were harvested from these cells and qRT-PCR was performed to specifically measure shARC levels, utilizing PCR primers specific for the shARC short hairpin RNAs these constructs were designed to express. Quantitative RT-PCR revealed that cells which received shArc(4) plasmids expressed higher levels of shRNAs, as compared to cells that received shArc plasmids which contain only one shArc expression cassette [$t_{(4)} = 3.98$, $p = 0.00164$] (Figure 2B). Next, each of the viral plasmids, (GFP, shArc(H1⁻), shLuc(H1⁻), shArc, shLuc, shArc(4) and shLuc(4)), were co-transfected into 293FT cells with the Arc-RFP plasmid. Ninety six hours post transfection, the levels of RFP and GFP were examined via fluorescence microscopy. Each well contained similar levels of GFP, indicating that the transfection efficiency for each plasmid was similar among samples (Figure 2Cii, iv, vi, viii, x, xii, xiv). However, the levels of Arc-RFP were lower in the cells that received shArc and shArc(4), compared to all other groups, indicating that the shArc and shArc(4) plasmids were effective at depleting Arc-RFP levels and the H1 promoter mutant plasmids were indeed impaired in their ability to knockdown Arc, as expected. To quantitatively assess the effectiveness of these plasmids for knocking down Arc, cells were treated in a similar manner as above, but they were harvested at forty eight hours post transfection, and Arc mRNA levels were assessed by qRT-PCR. An ANOVA revealed an overall significant effect [$F_{(3,8)} = 73.492$, $p < 0.0001$], where Arc mRNA was significantly reduced in cells transfected with shArc and shArc(4), compared to cells transfected with shLuc and shLuc(4) ($p < 0.0001$) (Figure 2D), and Arc mRNA was significantly lower in the shArc group ($p = 0.002$), as compared to the shLuc and promoter mutants groups (shArc(H1⁻), shLuc(H1⁻)) [$F_{(3,8)} = 20.525$, $p = 0.0004$] (Figure 2E).

Next, adeno-associated viruses pseudotyped as AAV2/DJ8 were produced using these 7 different viral genome plasmids and these viruses were bilaterally infused into the BLA at a titer of $1.0E+13$ GC/mL – a half log lower titer of virus than what was used for our first experiment. Twenty one days later, the animals were sacrificed, the brains were removed, and processed via LMD/qRT-PCR to examine Arc mRNA levels within the BLA (Figure 2F). There was an overall significant effect across groups [$F_{(6,32)} = 4.385$, $p = 0.0024$; GFP $n = 5$, shLuc(H1⁻) $n = 5$, shArc(H1⁻) $n = 5$, shLuc $n = 6$, shArc $n = 6$, shLuc(4) $n = 6$, shArc(4) $n = 6$]. Post hoc analysis revealed that the shArc and shArc(4) groups did not differ significantly ($p = 0.4833$), but both of these groups exhibited significantly lower Arc mRNA levels compared to the shLuc and shLuc(4) groups ($p < 0.0056$). The GFP, shArc(H1⁻) and shLuc(H1⁻) groups did not differ significantly ($p > 0.2383$). Strikingly, the GFP and shArc(H1⁻) groups exhibited significantly lower levels of Arc mRNA compared to the

shLuc and shLuc(4) groups, ($p < 0.0413$), indicating that shLuc expression was dysregulating Arc levels, leading to higher than normal levels of Arc mRNA.

These 7 viruses were infused into the BLA as described above and twenty one days later, the animals were auditory fear conditioned to a 30 sec, 5 kHz, 75 dB tone that co-terminated with a 1.5 mA foot shock. Three and twenty four hours following fear conditioning training, STM and LTM were assessed as described above, respectively. A significant overall effect was observed across groups in STM [$F_{(6,37)} = 7.841$, $p < 0.0001$; (GFP $n = 6$, shLuc(H1) $n = 6$, shArc(H1) $n = 7$, shLuc $n = 6$, shArc $n = 7$, shLuc(4) $n = 6$, shArc(4) $n = 6$)] (Figure 2G) and LTM [$F_{(6,37)} = 7.611$, $p < 0.0001$] (Figure 2H). For STM and LTM, animals that received viruses designed to express shRNAs (shArc, shArc(4), shLuc, and shLuc(4)) did not exhibit significantly different freezing levels, indicating that abnormalities in fear learning due to Arc gene knockdown could not be detected ($p > 0.1829$), but these groups exhibited significantly lower freezing levels as compared to the shArc(H1), shLuc(H1) and GFP groups ($p < 0.0034$). During STM, the shLuc(H1) group displayed significantly lower freezing to the tone, compared to the GFP and shArc(H1) groups ($p < 0.0463$), while, during LTM, the three groups did not differ among each other ($p > 0.5524$). Collectively, these experiments indicate that viral mediated shLuc expression interferes with fear conditioning and shLuc expression can lead to a dysregulation of Arc gene expression.

Viral mediated delivery of shRNA to the BLA, dose dependently induces neurotoxicity and deficits in fear conditioning

In this next experiment, we aimed to determine if viral mediated delivery of shRNA to BLA neurons dose dependently interferes with fear conditioning. Animals were bilaterally infused with differing doses of the shLuc virus, which spanned an order of magnitude (Low, $3.16E + 12$ GC/mL, Medium $1.0E + 13$ GC/mL, High $3.16E + 13$ GC/mL) or GFP virus (High $3.16E + 13$ GC/mL). Twenty one days post infusion, the animals were trained and tested in Pavlovian fear conditioning for STM and LTM as described above. An ANOVA revealed an overall significant effect for STM [$F_{(3,29)} = 3.981$, $p = 0.0172$; (GFP $n = 10$, shLuc-High $n = 6$, shLuc-Med $n = 8$, shLuc-Low $n = 9$)] (Figure 3B) and LTM [$F_{(3,29)} = 5.846$, $p = 0.0030$] (Figure 3C). Post-hoc analysis revealed that freezing levels between the Low, Medium and High dose groups, were significantly different compared to the GFP only group during the STM test ($p < 0.002$). Animals in the Medium and High dose groups froze significantly less, as compared to the GFP only group during LTM ($p < 0.0012$). Animals in the Low and Medium dose groups froze significantly more than the animals in the High groups during both the STM and LTM tests ($p < 0.0325$). Collectively, these data indicate viral mediated overexpression of shLuc, within neurons of the BLA, dose dependently interferes with fear conditioning. Notably, even the lowest dose of virus interfered with fear conditioning and this dose was 10 fold lower than the high dose.

Next, we examined coronal tissue slices within the area of viral infusion/transduction from animals that received the shLuc (Low, Med, High) and GFP only (High) viruses. There was no evidence that the infusion of viruses through the stereotactically placed infusion needle or the virus itself created gross morphological damage or abnormalities within the BLA. In most cases, detecting where the infusion needle itself was placed was impossible and this is

likely due to the fact that the needle itself is very small and it was only inserted into the BLA for a very short time. Because there was no evidence of gross abnormalities, we reasoned that there might be other anomalies that could be detected using immunohistochemistry (IHC). We performed IHC for the microglia protein IBA1. An increase in IBA1 signal is an indication of microglia activation, which is routinely used as a marker to infer an ongoing inflammatory response. Coronal tissue slices containing the BLA transduced with either of the shLuc (Low, Med, High) and GFP only (High) groups were subjected to IBA1 IHC. The shLuc-High dose group exhibited an increase in the amount of IBA1 signal that was noticeably different compared to the shLuc-Medium, shLuc-Low and GFP only groups (Figure Ei–iv). Quantification of IHC for IBA1 revealed a significant difference of IBA1 staining [$F_{(3,20)} = 195.665$, $p < 0.0001$, (GFP $n = 6$, shLuc-High $n = 6$, shLuc-Med $n = 6$, shLuc-Low $n = 6$)], where the shLuc-High group exhibited significantly more IBA1 signal compared to the other groups ($p < 0.0001$). IBA1 staining for the shLuc-Low and shLuc-Medium groups was not different compared to the GFP only groups, despite the fact that the shLuc-Low and shLuc-Medium groups exhibited impairments in Pavlovian fear conditioning. These findings indicate that expression of shLuc can cause cellular dysfunction that is not necessarily associated with an inflammatory response.

AAV naturally transduces neurons preferentially *in vivo*. To confirm that our viruses are predominantly transducing neurons *in vivo*, we performed IHC on BLA containing tissue that was transduced with the GFP only virus at the High titer, with the neuronal specific marker, NeuN (Figure Bi–iii). Quantification of the IHC revealed that 93.3% of the GFP expressing cells were also positive for NeuN expression (Figure 4a), indicating that, as expected, these viruses were predominantly transducing neurons. Collectively, because these viruses are predominately targeting neurons, and viruses that harbor shLuc, impair fear conditioning, these findings support the notion that viral mediated shLuc expression is causing damage to neurons and therefore it would be accurate to state that neuronal shLuc expression is causing neural toxicity. However there is no indication that shLuc expression is causing cell death, given that the gross tissue morphology appears normal.

The experiments described thus far all utilized shLuc as a control. This control shRNA is designed to express an shRNA that is designed to not target any mRNA for degradation. However, it remains possible that there is something about the shLuc shRNA that is inherently toxic. Therefore, in our next experiment we examined another control shRNA that is not designed to target any mRNA for degradation and it is referred to here as shCntrl. In this experiment we bilaterally infused, into the BLA, the GFP only virus or the shCntrl virus at a titer of 3.16×10^{13} GC/mL. We also included a third group of animals in this experiment that were subjected to stereotaxic surgery and had the infusion needle lowered into the BLA, but nothing was infused. This group is referred to as the Sham group and it serves as a control for how the GFP only virus may influence Pavlovian fear conditioning. Twenty one days post infusion, the animals were fear conditioned and examined for cue induced freezing behavior during STM and LTM tests as described above. An ANOVA revealed an overall effect for STM [$F_{(2,20)} = 15.172$, $p < 0.0001$; (Sham $n = 6$, GFP $n = 9$, shCntrl $n = 8$)], where the shCntrl group was freezing significantly less than the GFP only and Sham groups ($p < 0.0001$) (Figure 5C). An overall effect was observed for LTM [$F_{(2,20)}$

=19.388, $p < 0.0001$], where the shCntrl group was freezing significantly less than the GFP only and Sham groups ($p < 0.0001$) (Figure 5D). Freezing levels did not differ between the Sham and GFP only groups during STM or LTM ($p > 0.1071$), indicating that the presence of the virus and the fluorescent protein expressed in BLA neurons does not influence Pavlovian fear conditioning.

Next, animals were bilaterally infused with the GFP only virus or the shCntrl virus at a titer of 3.16×10^{13} GC/mL. In addition, a group of animals that received a sham surgery were also included. Twenty one days post infusion, the animals were sacrificed and IHC for IBA1 was performed on coronal tissue slices that contained transduced BLA. BLA transduced with the shCntrl virus exhibited obvious IBA1 staining in comparison to the Sham and GFP groups, indicative that the shCntrl virus is neurotoxic and leads to microglia activation (Figure 5B). Collectively, these data indicate that shRNA overexpression induces neurotoxicity and leads to deficits in Pavlovian fear conditioning.

Virally mediated shRNA induced depletion of GluN2A within BLA neurons impairs Pavlovian fear conditioning, as compared to the GFP only, shLuc, shArc and shEgr1 groups

In the experiments depicted thus far, a significant difference in learning between the shLuc and shArc groups was not observed, and this is likely due to the fact that the toxicity induced by shRNA overexpression is masking the influence of gene specific knockdown on Pavlovian fear conditioning. Therefore, in our last set of experiments, we examined whether lowering the dose of virus further might result in decreased shRNA induced toxicity, so gene specific deficits in Pavlovian fear conditioning could be detected. We also examined how gene knockdown of Egr1 and GluN2A within BLA neurons might influence Pavlovian fear conditioning.

Viral plasmids were created to express shRNAs designed to target Egr1 and GluN2A. The shEgr1 was designed based on a previously reported algorithm (Reynolds, Leake, Boese, Scaringe, Marshall, and Khvorova, 2004). To target GluN2A for depletion, we utilized an shRNA sequence (shGluN2A), that has previously been shown to be effective at lowering GluN2A levels within neurons (Foster, McLaughlin, Edbauer, Phillips, Bolton, Constantine-Paton, and Sheng, 2010; Kim, Dunah, Wang, and Sheng, 2005). To measure the effectiveness of the shEgr1 viral plasmid *in vitro*, the AAV-shEgr1 and a control shRNA plasmid (AAV-shScram, previously described (Hommel et al., 2003)), were co-transfected into 293FT cells with a plasmid that was designed to express Egr1 protein fused to RFP. Ninety six hours post transfection, the levels of RFP and GFP were examined via fluorescence microscopy. Each well contained similar levels of GFP, indicating that the transfection efficiency for each plasmid was similar among the samples (Figure 6Bii, iv). However, the levels of Egr1-RFP were lower in the cells that received shEgr1, compared to the cells that received shScram (Figure 6Bi, iii,) indicating that the shEgr1 was effective at depleting Egr1-RFP levels, as intended. To quantitatively assess shEgr1 effectiveness, cells were treated in a similar manner as above, but they were harvested at forty eight hours post transfection, and Egr1 mRNA levels were assessed by qRT-PCR. Egr1 mRNA levels were found to be lower in cells that received shEgr1, versus cells that received shScram (Figure

6C). To measure the effectiveness of the shGluN2A viral plasmid *in vitro*, the AAV-shGluN2A and shLuc were co-transfected into 293FT cells with a plasmid that was designed to express GluN2A protein fused to a GFP. Forty eight hours post transfection, and GluN2A mRNA levels were assessed by qRT-PCR. GluN2A mRNA levels were found to be lower in cells that received shGluN2A versus cells that received shLuc [$t_{(4)} =$, $p = 0.0107$] (Figure 6D).

Next, we created adeno-associated viruses with the viral shEgr1 and shGluN2A plasmids, pseudotyped as AAV2/DJ8, and infused these viruses, along with GFP only, shLuc, shCntrl, and shArc viruses bilaterally into the BLA at a titer of 1.60×10^{12} GC/mL. This dose of virus is ~20 times lower than the High dose of virus used in previous experiments. Twenty one days post infusion, the animals were sacrificed, and the brains were removed and processed via LMD/qRT-PCR to examine Arc, Egr1 and GluN2A mRNA levels within the BLA (GFP $n = 6$, shLuc $n = 6$, shCntrl $n = 6$, shArc $n = 6$, shEgr1 $n = 8$, shGluN2A $n = 6$). Surprisingly, there was no significant difference in Arc mRNA levels among the groups [$F_{(5,31)} = 1.317$, $p = 0.2828$] (Figure 6E). There was, however, a significant difference among the groups for Egr1 mRNA levels [$F_{(5,31)} = 3.282$, $p = 0.0171$] (Figure 6F). A post hoc analysis revealed Egr1 mRNA levels were significantly lower in the shEgr1 group compared to the GFP, shLuc, shArc, and shGluN2A groups ($p < 0.0087$), however, Egr1 mRNA levels in the shEgr1 group were not significantly lowered, as compared to the shCntrl group, however, there was a trend ($p = 0.0511$). When we examined GluN2A mRNA levels, we found a significant difference among the groups [$F_{(5,31)} = 6.650$, $p = 0.0003$] (Figure 6G). A post hoc analysis revealed that GluN2A mRNA levels were significantly different among the shGluN2A group as compared all other groups ($p < 0.0029$).

Next, these 6 viruses were infused into the BLA and twenty one days post infusion, animals were auditory fear conditioned, and cue induced freezing was examined during STM and LTM tests, as described above. A significant difference was not observed among the groups for STM [$F_{(5,34)} = 2.162$, $p = 0.0816$; (GFP $n = 6$, shLuc $n = 7$, shCntrl $n = 7$, shArc $n = 7$, Egr1 $n = 7$, shGluN2A $n = 6$)] (Figure 6H). However, for LTM, there was a significant difference among the groups [$F_{(5,34)} = 2.573$, $p = 0.0445$] (Figure 6I). For LTM, a post hoc analysis revealed that each group that received virus designed to express shRNA exhibited significantly lower freezing levels as compared to the group that received virus that was not designed to express shRNA, (GFP only) ($p < 0.0021$). The shArc, shEgr1, shLuc and shCntrl groups did not differ significantly indicating that gene specific influences on learning and memory could not be detected, under these circumstances for Egr1 or Arc ($p > 0.4235$). The shGluN2A group froze significantly less than the GFP only, shLuc, shArc, and shEgr1 groups ($p < 0.0195$). The shCntrl and shGluN2A group did not significantly differ from each other, however there was a trend ($p = 0.0890$). Collectively, these data indicate that it is possible to detect learning deficits induced by targeting specific mRNAs utilizing viral mediated shRNA delivery, but this may be restricted to situations when highly potent shRNAs are utilized.

To determine if there was an observable difference in the intensity of GFP reporter signal and number of neurons fluorescing across the different doses of AAV virus used in these experiments, we imaged brain tissue sections that were transduced with shLuc virus at

various titers. We infused shLuc virus bilaterally into the BLA at 4 different titers (High 3.16×10^{13} GC/mL, Medium 1.0×10^{13} GC/mL, Low 3.16×10^{12} GC/mL, and Extra Low (1.6×10^{12} GC/mL). Twenty one days post infusion, the animals were perfused and the brains were sectioned within the coronal plane. In addition, tissue sections that contained no virus (naïve) were used as a comparison. Imaging of GFP fluorescence in the BLA was performed at 4 different exposure times, in which each exposure time is optimal for one of the specific viral titers (Figure 7A). These images were used to evaluate cellular detail by magnifying the existing image (Figure 7B). There was an observable decrease in amount of cells fluorescing and overall GFP fluorescence as the dose of virus is decreased. These data demonstrate that lowering the titer of AAV virus infused will result in less neurons transduced and an overall decrease in GFP, thus, indicating that this technology may be less effective at lower titers.

Discussion

Here we report that viral mediated delivery of shRNA to rat neurons *in vivo* is toxic and impairs auditory Pavlovian fear conditioning. These findings were not due to surgery, the virus or GFP expression, but rather they were due to shRNA expression. ShRNAs delivered to BLA neurons, using varying doses of virus, induced highly reproducible deficits in Pavlovian fear conditioning, even at the lowest dose tested, which was ~20 times lower than the highest dose we examined. These findings were surprising, considering the widespread *in vivo* use of viral mediated delivery of shRNA in the behavioral neuroscience field. However, despite its consistent use, it is well established that viral mediated delivery of shRNA can be toxic to cells (Grimm, 2011), including neurons (Boudreau et al., 2009; Ehlert et al., 2010; Khodr et al., 2011; Martin et al., 2011; McBride et al., 2008; Ulusoy et al., 2009). This is the first report that a common learning and memory paradigm was adversely influenced by viral mediated delivery of shRNA.

Gene knockdown approaches that utilize viral mediated delivery of shRNA are attractive because they can be implemented quickly and delivered in a spatially and temporally restricted manner to the CNS of numerous model organisms. However, unlike conventional mouse and rat knockouts that are designed to have every cell or cell type genetically manipulated within the organism, viral mediated delivery of shRNA does not necessarily target every cell or neuron within a brain region. This is because the number of cells the virus transduces is dependent on how efficiently the virus transduces the cell type/brain region, and the concentration and volume of the virus administered. In practice, not every cell is transduced by virus and some cells are transduced by multiple viruses, thus, delivering many copies of the viral transgene to the cell, and as the dose of virus is lowered, fewer cells are transduced. It would be ideal to be able to target every neuron within a specific brain region since the odds of targeting neurons relevant to the behavior would increase as the number of genetically modified neurons increased within the targeted brain region. This is because not every neuron within a brain region would likely contribute to a specific behavior. As a greater number of neurons within a brain region are targeted and genetically manipulated, a greater augmentation of behavior would be expected, and this could lead to more robust and reproducible findings. Unfortunately, our findings and findings from others indicate that viral mediated delivery of shRNA can be toxic to neurons,

which of course can limit one's ability to use this technology in a manner that would allow the targeting of every neuron within the target region. For example, McBride and colleagues previously raised the concern that using doses of virus that transduced most of the neurons within a brain region resulted in shRNA induced toxicity, and doses of virus that did not exhibit shRNA induced toxicity transduced significantly less neurons within the target region, therefore limiting its utility (McBride et al., 2008). However, this prior study solely utilized microglia activation as a marker of toxicity. We found that there was likely neuronal toxicity induced at viral doses that did not necessarily lead to microglia activation, since deficits in Pavlovian conditioning were detected when these lower doses were used. Therefore, lowering the dose of virus may not only lead to transducing less cells, but it also may not completely alleviate the shRNA induced toxicity. But our data clearly indicate that the amount of virus infused into the CNS needs to be chosen wisely, because infusing too much of a virus that harbors an shRNA expression cassette can cause toxicity and too little may not be effective at knocking down the gene of interest or producing a gene specific behavioral impairment.

All shRNAs are not equally effective at depleting the gene products that they are intended to. Because of this, we specifically chose to work with shArc and shGluN2A, because these shRNAs have been previously reported to very effective at knocking down these gene products in neurons. Across experiments that utilized shLuc and shArc, we found Arc knockdown was dependent on the dose of virus used. For example, the greatest difference in Arc levels between the shLuc and shArc groups occurred when the High dose was utilized. We observed a moderate degree of Arc knockdown at the Medium dose. At the lowest dose of virus, we did not detect a significant difference in Arc levels between the shLuc and shArc groups. However, the fact that shLuc expression alone led to increases in levels of Arc as compared to the GFP only group, complicates these findings. Short-hairpins designed to target Egr1 and GluN2A were effective at knocking down these gene products at the lowest dose of virus utilized. Despite gene knockdown across numerous experiments that utilized differing doses of virus, we were never able to detect a difference in fear conditioning among the shLuc, shCntrl, shArc and shEgr1 groups. We suspect that this might be due the fact that shRNA induced toxicity may be masking a subtle impairment in fear conditioning that might be due to gene specific knockdown. For example, mouse knockouts of both Arc and Egr1 exhibit deficits in Pavlovian fear conditioning, but in both of these cases, these deficits are small (Jones et al., 2001; Plath et al., 2006). We found that knockdown of GluN2A exhibited significantly lower freezing levels during the LTM test, as compared to all the groups examined, except shCntrl (trend $p = 0.0890$), indicating that, despite shRNA induced toxicity, gene specific deficits in fear conditioning could be observed utilizing viral mediated delivery of shRNA.

Considering that shRNA overexpression can induce neuronal toxicity, potential "gene specific" findings obtained using this technology might not be physiologically relevant, since they may be a byproduct of, or in response to, ongoing neuronal dysfunction. Also, shRNA induced neuronal toxicity is not isolated to the viral delivery approach. A recent study reported that shRNA delivered to cortical neurons via *in vivo* electroporation resulted in a non-specific deficit in neural migration (Baek, Kerjan, Bielas, Lee, Fenstermaker,

Novarino, and Gleeson, 2014). Collectively, these results also raise concerns regarding the use of shRNA in primary neurons grown in culture or in organotypic slice cultures. ShRNA is frequently delivered to primary neurons grown in culture or in organotypic slice cultures using a variety of approaches, (i.e. lipofection, viral transduction, gene guns, and electroporation) and, therefore, observations made from such studies need to be carefully interpreted with proper controls, especially since it is difficult to accurately titrate the amount of shRNA that is delivered to each neuron.

It is currently believed that shRNA induced cellular toxicity is due at least in part, to the shRNAs interfering with the endogenous cellular microRNA (miRNA) pathways (Baek et al., 2014; Grimm, 2011; Grimm, Streetz, Jopling, Storm, Pandey, Davis, Marion, Salazar, and Kay, 2006; Grimm, Wang, Lee, Schurmann, Gu, Borner, Storm, and Kay, 2010). For example when highly potent AAVs harboring shRNAs were intravenously administered to mice, they were found to cause hepatocellular toxicity and in some cases, caused death. AAV mediated shRNA over-expression was found to interfere with the processing of endogenous miRNAs, leading to lower levels of mature miRNAs, and this was believed to occur in part due to a saturation of exportin-5 mediated nuclear export of the shRNA/miRNAs (Grimm et al., 2006). More recently similar findings that endogenous miRNA levels are dysregulated by AAV mediated shRNA overexpression within the rat brain were found, and the severity of the miRNA dysregulation increased over time (van Gestel, van Erp, Sanders, Brans, Luijendijk, Merkestein, Pasterkamp, and Adan, 2014). Notably the use of siRNAs, which are frequently used in cell culture systems, do not interfere with miRNA export, since they begin their cellular processing at a later stage of this pathway; however, off target effects can still be a problem with siRNAs (Fedorov, Anderson, Birmingham, Reynolds, Karpilow, Robinson, Leake, Marshall, and Khvorova, 2006).

Despite the fact that the *in vivo* use of shRNAs can be toxic, there may be possible ways to mitigate these issues and successfully use this technology *in vivo*. For example embedding the sense and antisense portions of the shRNA into a miRNA scaffold creating an artificial miRNA can alleviate some of the toxicity associated with conventional shRNAs (McBride et al., 2008). Additionally reducing the overall expression of shRNAs within the cell could significantly reduce the shRNA induced toxicity, by preventing the oversaturation of the endogenous miRNA pathways. This could be achieved using weaker promoters to drive shRNA expression by opting to use RNA polymerase II based promoters instead of the strong U6 and H1 RNA polymerase III promoters that are typically used or by mutating the existing U6 and H1 promoters to reduce their ability to drive transcription (Giering, Grimm, Storm, and Kay, 2008). Alternatively administering less viral particles or opting to use a viral serotype with reduced cellular transduction ability, could reduce the shRNA induced toxicity; however, as stated above, these two methods would sacrifice the number of cells likely transduced *in vivo*. Lastly performing behavioral analysis at early time points following viral infusion could limit the adverse influence of shRNA induced toxicity, since miRNA dysregulation appears to get worse with increasing duration of shRNA overexpression (van Gestel et al., 2014). Since there can be large differences in viral transduction efficiency that exist across types of viruses and serotypes of viruses (Holehonnur, Luong, Chaturvedi, Ho, Lella, Hosek, and Ploski, 2014), differences in

potency of the shRNA or artificial miRNA used (Knott, Maceli, Erard, Chang, Marran, Zhou, Gordon, El Demerdash, Wagenblast, Kim, Fellmann, and Hannon, 2014), and differences in the half-life of protein products that are intended to be depleted, the exact experimental conditions for viral based shRNA delivery that will achieve the best results likely need to be empirically determined for individual cases.

In conclusion, we believe that the use of shRNAs in behavioral neuroscience warrants careful consideration and careful study design. Unfortunately there are a large number of variables that will influence the success of these types of experiments since ectopic expression of shRNAs can induce neurotoxicity.

Materials and Methods

Subjects

3–4 month male Sprague Dawley rats (Harlan) were individually housed in polycarbonate cages on a 12 hour light/dark cycle. Food and water were provided *ad libitum*. Animal use procedures were in accordance with the National Institutes of Health Guide for the Care and Use of laboratory animals and were approved by the University of Texas at Dallas Animal Care and Use Committee.

Plasmid Constructs

Plasmids used within this study have been developed using standard recombinant cloning procedures. Short hairpin RNAs (shRNAs) previously described for targeting Arc (Rial Verde et al., 2006) were cloned into pSuper (Oligoengine). Specifically, the following oligos (shARC-TOP
GATCcccGCTGATGGCTACGACTACAttcaagagaTG TAGTCGTAGCCATCAGCttttt, shARC-BOT
AGCTaaaaaGCTGATGGCTACGACTACAtctcttgaaTG TAGTCGTAGCCATCAGCggg) were annealed and ligated to the BglII and HindIII restriction enzyme sites of the pSUPER vector (Oligoengine) to create the pSuper-shARC plasmid. The plasmid (pSuper) containing the shRNA designed to target Luciferase (shLuc) and GluN2A (shGluN2A), were kindly provided by Dr. Morgan Sheng (Foster et al., 2010; Hung et al., 2010). To create the pAAV-GFP-shArc(1) and pAAV-GFP-shLuc(1) plasmids, the shArc and shLuc expression cassettes, which include the 226 base pair H1 RNA polymerase III promoter, were PCR amplified using the following DNA primers, (Super FP Acc65I
aaaggtaccGAATTCTGAACGCTGACGTCATC, Super RP AscI
CAAGGCGCGCCTACCGGGCCCCCCTCG), and these DNA products were ligated to the Acc65I and AscI restriction enzymes sites of the pNEB193 plasmid (New England Biolabs), creating pNEB193-shArc1 and pNEB193-shLuc, respectively. Subsequently, the entire shRNA expression cassette was PCR amplified from pNEB193 using the following DNA primers, (pNeb193 FP GGCGAAAGGGGATGTGCTG, pNeb193 RP BlnI
cacGctcagcCACCTCTGACTTGAGCGTCTG), and these PCR products were ligated to the Acc65I and BlnI sites of the AAV2 viral genome plasmid, pAAV-GFP-shRNA, (Hommel et al., 2003) to create pAAV-GFP-shArc(1) and pAAV-GFP-shLuc(1) plasmids, respectively. The pAAV-GFP-shArc(4) and pAAV-GFP-shLuc(4) plasmids that contain 4 shRNA

expression cassettes in tandem were created in a similar manner as to pAAV-GFP-shArc(1) and pAAV-GFP-shLuc(1) plasmids but the 2nd, 3rd and 4th shRNA expression cassettes were sequentially cloned into the pNEB193-shArc1 and pNEB193-shLuc plasmids via the AscI/BamHI, BamHI/SphI, and the SphI/AflIII restriction enzymes sites, respectively, using the following DNA primers, (Super FP AscI
 aaaGGCGCGCCGAATTCGAACGCTGACGTCATC, Super RP
 BamHICAAGGATCCTACCGGGCCCCCCTCG; Super FP BamHI
 CAAGGATCCGAATTCGAACGCTGACGTCATC, Super RP SphI
 CAAGCATGCTACCGGGCCCCCCTCG; Super FP SphI
 CAAGCATGCGAATTCGAACGCTGACGTCATC, Super RP AflIII
 CAAACATGTTACCGGGCCCCCCTCG). Subsequently, the entire region containing the 4 shRNA expression cassettes was PCR amplified from pNEB193 using the following DNA primers, (pNeb193 FP GGCGAAAGGGGATGTGCTG, pNeb193 RP BlnI
 cacGctcagcCACCTCTGACTTGAGCGTCG), and this PCR product was ligated to the Acc65I and BlnI sites of the pAAV-GFP-shRNA vector to create pAAV-GFP-shArc(4) and pAAV-GFP-shLuc(4) plasmids, respectively. The pAAV-GFP vector was created by digesting the pAAV-GFP-shRNA plasmid with AscI and XbaI and re-ligating it to remove the existing U6 promoter/shRNA Expression cassette to create a GFP transgene only pAAV vector. To create AAV vectors that contain H1 promoter mutants, shArc and shLuc expression cassettes were PCR amplified from pSuper-shArc and pSuper-shLuc, respectively, using the following primers, (Super FP AscI mutant
 AAAGGCGCGCCAATTTGCATGTCGCTATGTGTTCgtttAAATCACCATAAACGT
 G, Super RP XbaI CAATCTAGATACCGGGCCCCCCTCG), and these DNA products were ligated to the AscI and XbaI restriction enzymes sites of the pAAV-GFP-shRNA vector. These ~100 bps H1 promoter mutants contain a truncated promoter with a TGGG > GTTT mutation occurring at position -72. This mutation is indicated in the forward primer with lower case letters. pAAV-GFP-shGluN2A was created by PCR amplifying the shGluN2A expression cassette from pSuper-shGluN2A using the following DNA primers, (Super FP Acc65I aaaggtaccGAATTCGAACGCTGACGTCATC, Super RP AscI
 CAAGGCGCGCCTACCGGGCCCCCCTCG), and ligating the DNA product to the Acc65I and AscI sites of pAAV-GFP-shRNA vector (Hommel et al., 2003). To create pAAV-GFP-shControl, the pAAV-GFP-shArc (Asc-XbaI) plasmid was first digested with BglII and HindIII and religated to destroy the intervening XhoI sites and the BglII sites. Then this new plasmid was digested with AscI and XbaI and the empty shRNA expression cassette from pSuper was PCR amplified using the following DNA primers, (Super FP AscI
 aaaGGCGCGCCGAATTCGAACGCTGACGTCATC, Super RP XbaI
 CAATCTAGATACCGGGCCCCCCTCG), and this DNA product was ligated to the AscI and XbaI sites to create pAAV-GFP-(H1)shRNA (empty) plasmid. Subsequently, the control shRNA oligonucleotides (Top:
 GATCCCCGCGCGCTTTGTAGGATTCGTTCAAGAGACGAATCCTACAAAGCGCGC
 TTTTAA, Bottom:
 TCGATAAAAAGCGCGCTTTGTAGGATTCGTTCTTGAACGAATCCTACAAAGCG
 CGCGGG) were annealed and ligated to the BglII and XhoI sites of pAAV-GFP-H1shRNA (empty) plasmid to create pAAV-GFP-shControl. To create pAAV-GFP-shEgr1, the following oligonucleotides (Top:

tttGATGAGTTGGGACTGGTAGGTGTTCTTCCTGTCAAACACCTACCAGTCCCAAC TCATaTTTTT, Bottom:
 ctagAAAAAATGAGTTGGGACTGGTAGGTGTTTGACAGGAAGAACACCTACCAGT CCCAACTCATC), were annealed and ligated to the SapI and XbaI sites of the pAAV-GFP-shRNA vector, (Hommel et al., 2003). The AAV genome plasmids created in this study all consisted of a viral genome that was ~3–3.5 kb, except for the shArc4/Luc4 viral genomes which were ~4 kb in length. To create plasmids that were designed to express Arc and Egr1 coding regions fused with red fluorescent protein (RFP) at the c-terminus, the Arc and Egr1 rat coding regions were PCR amplified from rat brain cDNA using the following primers (Arc: FP Xho AAACCTCGAGcagatggagctggaccatagac, RP BamHI TTTTGGATCCCGTTCAGGCTGGGTCCTGTAC; Egr1: FP X1 cCTCGAGatggacaactacccaaactg, RP R1 CAGAATTCGGCAAATTTCAATTGTCC) and these DNA products were ligated into the XhoI and BamHI sites or XhoI and EcoRI sites of pdsRedN-1 plasmid (Clontech), to create pdsRedN-1-Arc and pdsRedN-1-Egr1, respectively. The pCI-GFP-GluN2A plasmid designed to express GluN2A as a GFP fusion protein was generously provided by Andreas Barria (Barria and Malinow, 2002; 2005). All constructs were confirmed through sequencing. Plasmids and plasmid sequences will be provided upon request.

In vitro Knockdown Experiments

In vitro knockdown experiments were performed as previously described [13]. For *in vitro* knockdown of Arc-RFP, Egr1-RFP and GFP-GluN2A, 293FT cells (Invitrogen, Cat# R700-07) were grown in a 24 well plate to 30–50% confluency. Cells were then co-transfected with a pdsRedN-1-Arc, pdsRedN-1-Egr1 or pCI-GFP-GluN2A, and the appropriate shRNA expression cassette containing plasmids in a 1:1 ratio using lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. RFP or GFP intensity was observed 96 hours post transfection to visually/qualitatively observe knockdown of the Arc-RFP or Egr1-RFP fusion proteins using an Olympus IX51 inverted fluorescent microscope under RFP and GFP filters and the images were acquired using an Olympus DP72 Digital Camera and Cellsens software (Olympus). To quantitatively measure shRNA mediated gene knockdown of Arc, Egr1, or GluN2A, cells were treated as above, but the cells were harvested forty eight hours post transfection and the RNA was extracted with Trizol reagent (Invitrogen) following the manufacturer's instructions. RNA was precipitated with PelletPaint (Novagen) and converted to cDNA with Superscript Reverse Transcriptase II (Invitrogen) following the manufacturer's instruction, respectively, and cDNA levels were measured with qRT-PCR in a standard 20 µL Taqman PCR assay (Applied Biosystems) using a CFX96 Real-time PCR system (BioRad). One µl of a 20X Taqman custom RFP Primer/Probe to measure RFP levels was used (RFP FP: AGCGCGTGATGAACTTCGA, RFP RP: GCCGATGAACTTCACCTTGATAGAT, RFP Probe: 6FAM-ACCCAGGACTCCTCC) (as previously described (Althage, Ford, Wang, Tso, Polonsky, and Wice, 2008)). One µl of a 20X Taqman GluN2A Primer/Probe was used to measure GluN2A levels (Life Technologies, Cat#Rn00561341_m1) Complementary DNA input was normalized to GAPDH using GAPDH specific primers/Vic labeled probe (ID# Hs02758991_g1, Invitrogen). Quantitative RT-PCR was performed using the Ct method as described previously (Ploski, Monsey, Nguyen, DiLeone, and Schafe, 2011;

Ploski, Newton, and Duman, 2006; Ploski, Park, Ping, Monsey, and Schafe, 2010) and these data are represented as the average threshold cycle (Ct) difference values for each group after normalization to GAPDH, with the error bars representing the standard error of the mean for each group, (average fold change = $2^{(\text{average Ct difference value})}$). Wells were seeded in triplicate and qRT-PCR data was compared via 2 tailed t-test assuming equal variances or One-Way ANOVA, where appropriate.

Viral Production, Purification, and Titering

Viral production, purification, and titering were performed as previously described (Holehonnur et al., 2014). Briefly, AAV2 genome plasmids were pseudotyped as AAV2/DJ8. Pseudotyped viruses were produced using a triple-transfection, helper-free method using 293FT cells, and the resultant viruses were purified on an iodixanol step gradient and further concentrated using Amicon Ultra-15 centrifugal filter units (Millipore). Purified AAV viruses were titered using a quantitative-PCR based titering method with the following DNA primers (GFP Primer/Probe (ID# Mr04329676_mr, Invitrogen)), to calculate DNase resistant viral particles. The titers were reported as genome copies GC/ml.

Viral infusions to the Basolateral complex (BLA)

Viral infusions targeting the BLA were performed as previously described (Holehonnur et al., 2014). Briefly, rats were rendered unconscious with an intra-peritoneal injection of ketamine (100mg/kg) and xylazine (10mg/kg) prior to stereotaxic surgery. Thirty-three gauge custom infusion cannulas (C315G, PlasticsOne) were inserted into polyethylene tubing (I.D. 0.0150 in, O.D. 0.043 in, wall thickness 0.0140 in)(A-M systems, Inc.) that were ~50 cm in length. These tubes were first backfilled with 1 x phosphate buffered saline, pH 7.4 (PBS), followed by sesame oil so that the ~5 cm closest to the infusion needle contained PBS and the rest of the infusion line was filled with sesame oil. These lines were then connected to 2 µl, 23-gauge (88500) stainless steel Hamilton syringes (Hamilton Company). Rats were bilaterally infused into the BLA [AP -2.9, ML ±4.7, DV -8.6], with 1µL/side at a rate of 0.07 µl/min for 15 minutes. The titers used were as follows: high (3.16 E + 13 GC/mL), medium (1.0 E + 13 GC/mL), low (3.16 E + 12 GC/mL), and extra-low (1.6E + 12 GC/mL). Behavioral and molecular experiments were performed 21 days post viral infusion.

Fear Conditioning

Fear conditioning training and testing was essentially completed as previously performed (Banerjee, Engineer, Sauls, Morales, Kilgard, and Ploski, 2014; Ploski et al., 2011). Twenty one days post-viral infusion the rats were fear conditioned. *Training* consisted of a 2 min acclimation period, followed by exposure to a single tone (30 sec, 5 kHz, 75 dB), which co-terminated with a 1.5 sec, 1.5 mA foot shock. Animals remained in the training chamber for an additional minute following the delivery of the foot shock and subsequently the animals were placed back into their home cages. Short-term memory (STM) was examined 3 hours after training by exposing the rats to 3 tones (2 min intertrial interval; 30 sec, 5 kHz, 75 dB) in an altered context (a modified chamber and the absence of light, with distinct olfactory and tactile cues). Long-term memory, (LTM) was examined 24 hours post-training in a

similar context as the STM test. Freezing behavior of each animal was measured during the exposure to each of the three tones presenting during STM an LTM testing. A Coulbourn Instruments fear conditioning system with computer controlled shockers, USB cameras for video monitoring/video capture and FreezeFrame Software (Actimetrics) for unbiased behavioral analysis was used to auditory fear condition rats and to test for conditioned fear responses. 154 animals were used for pavlovian fear conditioning experiments. Data was analyzed using a One-Way ANOVA for repeated measures or 2 tailed t-test assuming equal variances where appropriate. The individual that scored the freezing behavior and analyzed the data was blind to the experimental conditions. Rats were perfused the day following LTM, their brains were sectioned, and evaluated for placement analysis. Animals with poor viral placement within the BLA were excluded from the experiment.

Measurement of in vivo knockdown via qRT-PCR

The effectiveness of viral delivered shRNAs to knockdown gene expression was examined by qRT-PCR. Animals were sacrificed 21 days post infusion. Rats were anesthetized lightly with CO₂ and quickly decapitated. Brains were immediately removed and placed on dry ice and stored at -80 C°. Ten micron coronal sections containing viral transduction within the amygdala were obtained and placed on MMI Laser Microdissection (LMD) slides (product #50102) and were dissected via laser microdissection on a SmartCut Laser Microdissection System configured on an Olympus CKX41 inverted microscope. Section preparation, dehydration and laser microdissection were performed as previously described (Partin, Hosek, Luong, Lella, Sharma, and Ploski, 2013). Viral transduced BLA tissue was microdissected from approximately 10 sections and these microdissected tissue pieces were collected in 25 µL of cell lysis buffer (RNAqueous-Micro Kit; Ambion) and the RNA was purified according to the manufacturer's instructions. The RNA was precipitated using Pellet Paint NF (Novagen) and converted to cDNA with Superscript Reverse Transcriptase II (Invitrogen) following the manufacturer's instructions. Quantitative RT-PCR was performed in a similar manner as for the *in vitro* knockdown experiments, using RNA prepared from at least 5 individual amygdala, per group (i.e. n > 5). ANOVA or 2 tailed t-test assuming equal variances, where appropriate, were used for statistical analysis.

Measurement of shArc levels

293FT cells were seeded in a 12 well plate. Twenty four hours later the cells were co-transfected in triplicate with 0.08 µgs of shArc or shArc(4) plasmids and 1.5 µgs of pNEB193 plasmid following the standard lipofectamine 2000 protocol (Invitrogen). Forty eight hours post transfection, the cells were harvested and the RNA was purified using the mirVana miRNA Isolation Kit, (Ambion, Cat #AM1560) following the manufacturer's instructions. Specific qRT-PCR primers and reverse transcription primers were designed against the antisense portion of the Arc shRNA (5' TGTAGTCGTAGCCATCAGC 3') utilizing the Custom TaqMan Small RNA Assay Design Tool (Invitrogen; Batch ID: w1211234309000; Assay ID# CS0IWAH). The purified RNA samples were reverse transcribed with the custom stem loop primer provided in the Custom TaqMan Small RNA Assay, using the TaqMan MicroRNA Reverse Transcription Kit, (Ambion, cat # 4366596), and these samples were subjected to Taqman qRT-PCR. RNA from the same samples was also reverse transcribed with the stem loop primer for the U6 snRNA (Invitrogen, TaqMan

MicroRNA Assay; U6 snRNA Assay, ID# 001973) using the TaqMan MicroRNA Reverse Transcription Kit and these samples were subjected to Taqman qRT-PCR. Quantitative RT-PCR was performed using the Ct method as described previously (Ploski et al., 2011; Ploski et al., 2006; Ploski et al., 2010) and these data are represented as the average threshold cycle (Ct) difference values for each group after normalization to U6 snRNA levels, with the error bars representing the standard error of the mean for each group, (average fold change = $2^{(\text{average Ct difference value})}$).

Immunohistochemistry and Quantification

Immunohistochemistry (IHC) for IBA1 was carried out to detect microglia activation. Animals were perfused through the heart with 4% paraformaldehyde in PBS and cryoprotected with a 30% sucrose solution in PBS. Tissue was sectioned coronally using a cryostat sectioner. Slices containing amygdala that were transduced with virus were collected in PBS with 0.1% sodium azide. Free-floating sections were washed in PBS and quenched in a solution containing 3% H₂O₂ in H₂O for 5 min. Following quenching, the tissue was rinsed in PBS once, and then subsequently placed in blocking solution (1.5% normal goat serum and 0.3% Triton X-100 in PBS-A (NaCl 150mM, NaOH 96mM, NaH₂PO₄ 106mM)) for one hour, followed by an overnight incubation in blocking solution containing anti-IBA1 antibody (anti-IBA1 rabbit polyclonal; 1:1000; Wako 019-19741). Tissue was washed in PBS-B (NaCl, 150mM, NaOH 10mM, NaH₂PO₄ 11mM) 3 times for 10 min each, following by processing using VectaStain ABC kit (Vector Laboratories PK-6801) following the manufacturer's instructions. The tissue was developed in DAB peroxidase substrate and H₂O₂ (Sigma) for 7 min. The tissue was then mounted on Fisher Super Frost glass slides with DPX mountant media (Sigma). The IBA1 signal was quantified by calculating the integrated density of the IBA1 signal using ImageJ software from no less than 6 amygdala per group.

Immunohistochemistry for NeuN was performed to determine if virus was localized to neurons. Animals received viral infusions to the BLA and were perfused twenty one days post infusion with 4% PFA in PBS. Free floating sections were washed 3 times for 10 min in PBS at RT. Tissue was then transferred to blocking solution (5% normal goat serum and 0.3% triton-100 in PBS) for 30 min. Sections were then placed in blocking solution with a NeuN Antibody (anti-NeuN mouse monoclonal; 1:500; Millipore MAB377) and incubated overnight at 4°C. Sections were washed 3 times for 10 min at RT before being transferred to blocking solution containing secondary antibody (anti-mouse Texas Red-X conjugate; 1:1000; Invitrogen T6390) for two hours. Sections were then washed 3 times for 10 min and then mounted on VectaShield mounting medium(H-1000). Localization of virus to neurons was quantified using Adobe Photoshop across 3 40uM slices that were a considerable distance apart from each other.

Statistical Analysis

Data presented in graphs is displayed as the mean $\pm$ standard error of the mean (SEM). A One-Way ANOVA or two-tailed t-test assuming equal variances was used to compare group means for qRT-PCR analysis. A One-Way ANOVA for repeated measures was used for

STM and LTM fear conditioning experiments. All post hoc analysis was done using Fischer's LSD.

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Highlights

- Viral delivery of shRNA to amygdala rat neurons *in vivo* impaired fear conditioning
- These findings were independent of the shRNA sequence used
- The use of shRNAs in behavioral neuroscience warrants careful study design
- Potential methods to alleviate shRNA induced toxicity are discussed

Figure 1. Viral delivery of RNAi to the rat amygdala results in relatively weak fear conditioning (A) AAV genome maps for AAV-shLuc and AAV-shArc. ITR = inverted terminal repeats (B) 293FT cells were co-transfected with plasmids designed to express Arc-RFP and AAV-shLuc (i, ii) or Arc-RFP and AAV-shArc (iii, iv) and were imaged for GFP(ii, iv) and Arc-RFP(i, iii) 96 hrs post-transfection. AAV-shArc causes an observable decrease in Arc RFP signal compared to AAV-shLuc (i vs. iii). (C) 293FT cells were treated in a similar manner as in B, but they were harvested 48 hrs post-transfection and used to assess Arc mRNA levels via qRT-PCR. Cells transfected with AAV-shARC exhibited a decrease in Arc mRNA levels compared to cells that received AAV-shLuc ($p = 0.0006$). (D) Images of the BLA that received AAV2/DJ8 virus designed to express GFP; brightfield image (i), GFP fluorescence (ii). (E) Similar images as described in D, at different stages of laser microdissection of viral transduced BLA tissue. Brightfield and GFP images before LMD (i, ii) and after LMD (iii, iv). (F) AAV-shLuc and AAV-shArc viruses were infused into the BLA and 21 days post infusion the brains were extracted and processed via LMD/qRT-PCR to examine Arc mRNA levels (shArc, $n = 6$; shLuc, $n = 5$). Quantitative RT-PCR revealed a significant decrease of Arc mRNA in tissue transduced with the shArc virus compared to tissue transduced with shLuc virus ($p = 0.0007$). (G–H) Animals infused with either shLuc virus or shArc virus (shLuc $n = 9$, shArc $n = 10$) did not exhibit significant differences in freezing during STM (G) and LTM (H). Error bars represent standard error of the mean (SEM) (***) = $p < 0.001$).

Figure 2. Viral mediated shRNA expression within the BLA interferes with fear conditioning and leads to dysregulation of gene expression

(A) AAV genome maps depicting viral genomes for viruses designed to: not express an shRNA (GFP only), contain shLuc or shArc with H1 promoter mutations (shArc(H1⁻), shLuc(H1⁻)), contain one copy of shLuc or shArc, or contain 4 copies of shLuc and shArc (shLuc(4), shArc(4)). (B) 293FT cells were transfected with plasmids designed to express AAV-shArc or AAV-shArc(4) and were harvested 48 hrs post-transfection to examine shArc RNA levels via qRT-PCR. AAV-shArc displayed less shRNA expression compared to shArc(4) ($p = 0.00164$). (C) 293FT cells were co-transfected with a plasmid designed to express Arc-RFP and the AAV viral plasmids depicted in A and these were imaged for GFP(ii, iv, vi, viii, x, xii, xiv) and Arc-RFP(i, iii, v, vii, ix, xi, xiii) 96 hrs post-transfection. An observable decrease in Arc-RFP signal is only observed in cells transfected with either shArc or shArc(4), as compared to all other groups. (D) 293FT cells were transfected with a plasmid designed to express Arc-RFP and either shArc, shArc(4), shLuc or shLuc(4) plasmids. Forty eight hrs post-transfection, the cells were harvested and Arc mRNA levels were assessed via qRT-PCR. ShArc and shArc(4) exhibited lower Arc mRNA levels compared to the shLuc and shLuc(4) groups ($p < 0.0001$). (E) A similar experiment was performed as described in D to compare Arc mRNA levels among the shArc, shLuc, shArc(H1⁻) and shLuc(H1⁻) groups. shArc displayed significantly lower levels of Arc

mRNA compared to all other groups ($p < 0.002$). **(F)** Viruses for the above mentioned plasmids were generated and infused bilaterally into the BLA. Twenty one days post infusion, the brains were extracted and processed via LMD/qRT-PCR to examine Arc mRNA levels. (GFP $n = 5$, shLuc(H1) $n = 5$, shArc(H1) $n = 5$, shLuc $n = 6$, shArc $n = 6$, shLuc(4) $n = 6$, shArc(4) $n = 6$). A post hoc analysis revealed that the shArc and shArc(4) groups had lower levels of Arc mRNA, compared to the shLuc and shLuc(4) groups ($p < 0.0056$). The GFP, shArc(H1) and shLuc(H1) groups did not differ significantly from each other ($p > 0.2383$). The GFP and shArc(H1) groups exhibited significantly lower levels of Arc mRNA compared to the shLuc and shLuc(4) groups, ($p < 0.0413$). *, ** indicates significance compared to shLuc group. **(G)** These viruses were again infused bilaterally into the BLA and animals were tested in Pavlovian fear containing 21 days post infusion (GFP $n = 6$, shLuc(H1) $n = 6$, shArc(H1) $n = 7$, shLuc $n = 6$, shArc $n = 7$, shLuc(4) $n = 6$, shArc(4) $n = 6$). Animals that received viral infusions of virus designed to express an shRNA (shLuc, shArc, shLuc(4) and shARC(4)) exhibited lower freezing levels during STM **(G)** and LTM testing **(H)** as compared to animals that received infusion of viruses not designed to express shRNAs (GFP only, shLuc(H1), shArc(H1)) ($p < 0.0031$). Error bars represent standard error of the mean (SEM) (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; † $p < 0.0001$).

Figure 3. Viral mediated expression of shLuc within the BLA dose dependently interferes with Pavlovian fear conditioning and increases microglia activation

(A) AAV-shLuc virus was infused into the BLA at 3 different titers (High 3.16×10^{13} GC/mL; Med 1.0×10^{13} GC/mL; Low 3.16×10^{12} GC/mL) along with a group that was infused with a GFP only virus (High 3.16×10^{13} GC/mL) (GFP $n = 10$, shLuc-High $n = 6$, shLuc-Med $n = 8$, shLuc-Low $n = 9$). Twenty one days post infusion, animals were tested in Pavlovian fear conditioning. (B) During STM, lower freezing levels were observed in the High, Med and Low groups, as compared to the GFP group ($p < 0.002$). (C) During LTM, the High and Medium groups displayed lower freezing compared to the GFP group during LTM ($p < 0.0012$). (D) Animals were infused with the different doses of shLuc expressing virus and GFP only virus as described in A, and twenty one days later, the transduced tissue was subjected to IHC for IBA1 immunoreactivity to assess microglia activation (GFP $n = 6$, shLuc-High $n = 6$, shLuc-Med $n = 6$, shLuc-Low $n = 6$). Image analysis revealed tissue transduced with the high dose of shLuc virus exhibited increased IBA1 immunoreactivity as compared to all other groups ($p < 0.0001$). (E i–iv) Representative images for IBA1 IHC and GFP (E v–viii) for the experiment described in D. The shLuc High dose exhibits an obvious increase in IBA1 staining compared to the other doses and GFP only virus. Corresponding GFP reporter, exhibits dose dependent GFP fluorescence across the different doses of virus.

Error bars represent standard error of the mean (SEM) (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; † $p < 0.0001$).

Figure 4. AAV2/DJ8 viruses predominantly transduce neurons when infused into the BLA

In this experiment, animals were infused with the GFP only virus into the BLA at a titer of 3.16×10^{13} GC/mL. Twenty one days post infusion, animals were sacrificed and tissue sections containing transduced amygdala were examined for NeuN IHC. (A i) Image depicting GFP expressing, virally transduced cells (A ii). Same field as depicted in (A i), but depicting NeuN IHC using a TxRed secondary antibody. (A iii) Depicts the merge of the AAV-GFP image and the NeuN-TxRed image. Arrows point to a subset of NeuN positive neurons that are also AAV-GFP positive. (B) Quantification revealed that 93.3% NeuN positive neurons were also AAV-GFP positive neurons ($n = 3$).

Figure 5. Viral mediated expression of shCntrl in BLA neurons induces deficits in fear conditioning and increases in microglia activation compared to GFP only, and Sham control groups

(A) An adeno-associated virus was designed to express a control shRNA (shCntrl) that was designed not to target any rat mRNA for degradation. This virus and the GFP only virus were bilaterally infused into the rat BLA at a titer of 3.16×10^{13} GC/mL. A third group of animals was included that underwent surgery and the infusion needle was lowered into the BLA, but nothing was infused into the BLA (Sham). (B i–iii) Twenty one days post-surgery/infusion, the animals were perfused and BLA transduced tissue was examined for IBA1 IHC. An obvious increase in IBA1 immunoreactivity is apparent in the group that received the virus designed to express the shCntrl, but not in the GFP only or Sham groups. (B iv–vi) GFP reporter expression was similar between the GFP and shCntrl group, as expected. (C, D) Animals received either infusions of shCntrl, GFP only virus, or a sham surgery and were fear conditioned twenty one days post surgery/infusion (Sham $n = 6$, GFP $n = 9$, shCntrl $n = 8$). Animals that received infusion of shCntrl virus exhibited significantly lower freezing levels during STM (C) and LTM (D), as compared to the GFP and Sham groups ($p < 0.0001$). Error bars represent standard error of the mean (SEM) ($\dagger p < 0.0001$).

Figure 6. Virally mediated RNAi induced depletion of GluN2A within BLA neurons impairs Pavlovian fear conditioning, as compared to the GFP only, shLuc, shArc and shEgr1 groups (A) AAV genome maps depicting viral genomes for viruses designed to express GFP only, or in addition, one of the following shRNAs expression cassettes: shLuc, shCntrl, shArc, shEgr1, shGluN2A. (B i–iv) 293FT cells were co-transfected with plasmids designed to express Egr1-RFP and shEgr1 (i, ii) or Egr1-RFP and shScram (iii, iv) and were imaged for GFP(ii, iv) and Egr1-RFP(i, iii) 96 hrs post-transfection. AAV-shEgr1 causes an observable decrease in Egr1-RFP signal compared to AAV-shScram (i vs. iii). (C) 293FT cells were treated in a similar manner as in B, but they were harvested 48 hrs post-transfection and used to assess Egr1 mRNA levels via qRT-PCR. Cells transfected with AAV-shEgr1 exhibited a decrease in Egr1 mRNA levels compared to cells that received AAV-shScram. (D) 293FT cells were co-transfected with plasmids designed to express GluN2A and shGluN2A or GluN2A and shLuc. Forty eight hours later, cells were harvested and GluN2A mRNA levels were measured by qRT-PCR. GluN2A mRNA levels were significantly lower in cells transfected with AAV-shGluN2A as compared to cells transfected with AAV-shLuc

($p = 0.0107$). **(E–G)** Viruses from the above mentioned AAV plasmids were generated and infused bilaterally into the BLA at a titer of 1.60×10^{12} GC/mL. Twenty one days post infusion, the brains were extracted and processed via LMD/qRT-PCR to examine Arc, Egr1 and GluN2A mRNA levels. (GFP $n = 6$, shLuc $n = 6$, shCntrl $n = 6$, shArc $n = 6$, shEgr1 $n = 8$, shGluN2A $n = 6$). **(E)** Arc mRNA levels were relatively the same across all groups and were not significant across groups. **(F)** Egr1 mRNA levels were significantly lower in the shEgr1 group, compared to the GFP, shLuc, shArc, and shGluN2A groups ($p < 0.0087$), however, Egr1 levels were not significantly different from levels in the shCntrl group, there was a trend ($p = 0.0511$). **(G)** GluN2A mRNA levels were significantly lower compared to all other shRNA groups ($p < 0.0029$). Interestingly, GluN2A mRNA levels were significantly higher in the shArc group, compared to the shCntrl and shEgr1 group ($p < 0.0329$). **(H–I)** These viruses were infused into the BLA as described above and animals were fear conditioned twenty one days post infusion (GFP $n = 6$, shLuc $n = 7$, shCntrl $n = 7$, shArc $n = 7$, Egr1 $n = 7$, shGluN2A $n = 6$). **(H)** For STM, an ANOVA indicated there was not a significant difference in freezing levels between the groups. **(I)** For LTM, freezing levels were significantly lower in all shRNA groups compared to the GFP group ($p < 0.0021$). The shArc, shEgr1, shLuc and shCntrl groups did not differ significantly ($p > 0.4235$). The shGluN2A group froze significantly less than the GFP only, shLuc, shArc, and shEgr1 groups ($p < 0.0195$). The shCntrl and shGluN2A group did not significantly differ from each other, however there was a trend ($p = 0.0890$). Error bars represent standard error of the mean (SEM) (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; † $p < 0.0001$).

Figure 7. Lowering the titer of AAV virus infused, will result in less neurons transduced and an overall decrease in GFP expression

We imaged tissue sections containing transduced BLA that were infused with virus at either of the 4 titers used in these experiments (High 3.16×10^{13} GC/mL, Medium 1.0×10^{13} GC/mL, Low 3.16×10^{12} GC/mL, and Extra Low 1.6×10^{12} GC/mL). Each group was imaged at 4 different exposure times; each exposure time is optimal for one of the 4 titers. Tissue sections were imaged at (A) 20x, in addition, we include a (B) magnified view of this image to visualize cellular detail of the transduced neurons.