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Disruption of subcellular *Arc/Arg 3.1* mRNA expression in striatal efferent neurons following partial monoamine loss induced by methamphetamine

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Abstract

The immediate-early gene *Arc* (activity-regulated cytoskeleton-associated protein) is provocative in the context of neuroplasticity because of its experience-dependent regulation and mRNA transport to and translation at activated synapses. Normal rats have more *preproenkephalin*-negative (*ppe*-neg; presumed striatonigral) neurons with cytoplasmic *Arc* mRNA than *ppe*-positive (*ppe*-pos; striatopallidal) neurons, despite equivalent numbers of these neurons showing novelty-induced transcriptional activation of *Arc*. Furthermore, rats with partial monoamine loss induced by methamphetamine (METH) show impaired *Arc* mRNA expression in both *ppe*-neg and *ppe*-pos neurons relative to normal animals following response-reversal learning. In this study, *Arc* expression induced by exposure to a novel environment was used to assess transcriptional activation and cytoplasmic localization of *Arc* mRNA in striatal efferent neuron subpopulations subsequent to METH-induced neurotoxicity. Partial monoamine depletion significantly altered *Arc* expression. Specifically, basal *Arc* expression was elevated, but novelty-induced transcriptional activation was abolished. Without novelty-induced *Arc* transcription, METH-pretreated rats also had fewer neurons with cytoplasmic *Arc* mRNA expression, with the effect being greater for *ppe*-neg neurons. Thus, METH-induced neurotoxicity substantially alters striatal efferent neuron function at the level of *Arc* transcription, suggesting a long-term shift in basal ganglia neuroplasticity processes subsequent to METH-induced neurotoxicity. Such changes potentially underlie striatally-based learning deficits associated with METH-induced neurotoxicity.

Keywords

methamphetamine; dopamine; *Arc/Arg 3.1*; striatum; immediate-early gene

Introduction

Methamphetamine (METH) is a highly addictive psychostimulant that can induce substantial forebrain dopamine (DA) and serotonin depletions when administered at high doses (Wagner *et al.* 1980, Woolverton *et al.* 1989). While METH-induced monoamine depletions are significantly less than those observed in Parkinson's disease, these partial depletions lead to impairments in basal ganglia-mediated behavioral and cognitive abilities in both rodents and humans (Chapman *et al.* 2001, Kalechstein *et al.* 2003, Volkow *et al.*

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2001, Johanson *et al.* 2006). Furthermore, recent data show that individuals with a history of methamphetamine or other amphetamine abuse are more likely to develop Parkinsonism (Callaghan *et al.* 2012), suggesting that METH exposure may be associated with a significant preclinical period of partial striatal DA loss and associated consequences.

The bases for the cognitive and behavioral sequelae of METH-induced partial DA loss are unknown. Approximately 95% of neurons in striatum are medium spiny neurons, and these efferent neurons can be divided into two equal subpopulations: striatonigral “direct” pathway neurons and striatopallidal “indirect” pathway neurons (Wichmann & DeLong 1996, Obeso *et al.* 1997, Grillner *et al.* 2005). These efferent neurons can be phenotypically differentiated at the cell body level by their selective expression of neuropeptides: striatopallidal neurons express *preproenkephalin* (*ppe-pos*), whereas striatonigral neurons do not (*ppe-neg* (Gerfen & Young 1988)). Striatonigral neurons predominately express D1-DA receptors while striatopallidal neurons predominately express D2-DA receptors. Prior work suggests that METH-induced functional impairments are substantially more pronounced in striatonigral neurons (Daberkow *et al.* 2008, Chapman *et al.* 2001, Johnson-Davis *et al.* 2002), consistent with findings reported for rats with partial DA loss induced by 6-hydroxydopamine (Nisenbaum *et al.* 1996). Such changes may be due, in part, to impairments in phasic DA signaling associated with the METH-induced neurotoxicity (Howard *et al.* 2011), as the D1-DA receptor may be particularly sensitive to such changes (Dreyer *et al.* 2010, Floresco *et al.* 2003).

The immediate-early gene *Arc/Arg3.1* (activity-regulated cytoskeleton-associated protein) is a critical mediator of normal neuroplasticity processes and is induced by N-methyl-D-aspartate (NMDA)-receptor activation before being trafficked to activated synapses where it is locally translated (Bramham *et al.* 2008, Lyford *et al.* 1995). *Arc* mRNA expression is induced in an experience-dependent manner (Guzowski *et al.* 1999, Daberkow *et al.* 2007) within GABAergic striatal neurons (Vazdarjanova *et al.* 2006). *Arc* protein itself is critical to modulating excitatory glutamatergic inputs via endocytosis of AMPA-type glutamate receptors (Chowdhury *et al.* 2006, Rial Verde *et al.* 2006) and to maintaining synaptic plasticity via modulation of long-term potentiation (LTP) and long-term depression (LTD) (Bramham *et al.* 2008, Bloomer *et al.* 2008). Blocking *Arc* translation via antisense oligonucleotide infusions impairs consolidation of learning (Guzowski *et al.* 2000), including basal ganglia-mediated learning (Hearing *et al.* 2011, Pastuzyn *et al.* 2012). Thus, *Arc* is critical for consolidation of learning, including those processes mediated by dorsal striatum.

We have previously reported that despite equivalent numbers of neurons showing transcriptional activation of the *Arc* gene, there are more striatonigral neurons with *Arc* mRNA in the cytoplasm than striatopallidal neurons in normal animals following exploration of a novel environment (Daberkow *et al.* 2007)—the prototypical behavioral paradigm used to assess *Arc* expression (Vazdarjanova *et al.* 2006, Guzowski *et al.* 1999, Chawla *et al.* 2005). Furthermore, we have previously reported that rats with prior exposure to a neurotoxic regimen of METH show decreased numbers of striatal neurons with cytoplasmic *Arc* mRNA expression after engaging in a striatally-mediated, response-reversal learning task, with the greatest impairment being in the numbers of striatonigral neurons (Daberkow *et al.* 2008). Also, the correlation between *Arc* mRNA expression in dorsomedial (DM) striatum and trials to criterion on the response-reversal learning task observed in normal animals (Daberkow *et al.* 2008, Daberkow *et al.* 2007) is lost in METH-pretreated animals (Daberkow *et al.* 2008). Furthermore, reversal learning in METH-pretreated rats is no longer sensitive to antisense oligonucleotide-mediated disruption of *Arc* translation in DM striatum (Pastuzyn *et al.* 2012). However, the paradigms examined to date do not allow us to discern whether the decreased numbers of striatal neurons with *Arc*

mRNA expression in the cytoplasm in METH-pretreated rats arises due to deficits in transcriptional activation of the *Arc* gene or deficits in the trafficking of *Arc* mRNA into the cytoplasm. Thus, the goal of the present studies was to determine whether METH-induced neurotoxicity is associated with acute disruption of *Arc* transcriptional activation vs. longer-duration processes regulating *Arc* trafficking/cytoplasmic stability. Furthermore, an additional goal of the present work was to examine further whether there are phenotypic differences in the acute regulation of *Arc* mRNA transcriptional activation and cytoplasmic expression following METH-induced neurotoxicity so as to better define how partial DA depletion impacts upon striatal efferent neuron function. Clarifying the impact of METH-induced neurotoxicity on the transcriptional activation and cytoplasmic regulation of *Arc* mRNA in striatum is critical so as to better inform treatment strategies to ameliorate cognitive and behavioral impairments in human METH abusers (Volkow et al. 2001, Kalechstein et al. 2003, Johanson et al. 2006).

Methods

Animals

Male Sprague-Dawley rats (Charles River Laboratories, Raleigh, NC; 275-300g) were singly housed in tub cages in a room controlled for temperature and lighting (12:12 hr). All animal care and experimental procedures conformed to the *Guide for the Care and Use of Laboratory Animals* (8th Ed.), followed the ARRIVE guidelines (Kilkenny et al. 2010) and were approved by the Institutional Animal Care and Use Committee at the University of Utah.

Methamphetamine treatment

Rats were treated with ($\pm$)-methamphetamine hydrochloride (NIDA, Research Triangle Park, NC) as previously described (Son et al. 2011, Daberkow et al. 2008). This specific regimen was chosen to model METH-induced damage to striatal monoamine systems. While METH self-administration models may more closely mimic human exposure by self-administration, such contingent models typically do not recapitulate long-lasting striatal DA toxicity (Brennan et al. 2010, Schwendt et al. 2009, McFadden et al. 2012, Reichel et al. 2012)), perhaps due to the duration of the exposure, as one study (Krasnova et al. 2010)), with much longer daily access (15 hr/day for 8 days) did report DA neuron toxicity. Thus, the single-day, non-contingent binge regimen implemented in the present study serves as a more rapid and reproducible model with face validity to the striatal DA depletions present in individuals with a history of METH exposure and who may, in fact, be in a preclinical Parkinsonian state. On the METH treatment day, rats were housed 5-6 animals per cage. Rats received four injections of either ($\pm$)-METH (10 mg/kg free base, s.c.) or 0.9% saline (1 mL/kg, s.c.) at 2-hr intervals, with rectal temperatures recorded hourly (Figure 1A). If core temperature exceeded 41°C, the rat was removed and placed in a separate cage over ice to decrease hyperthermia. Twelve hours after the final injection, rats were returned to home cages and given free access to food and water until behavioral manipulations three weeks later.

Behavioral activation via novel environment exploration

Rats were divided into three experimental groups (“5-min”, “30-min”, caged controls (CC)), each consisting of 6-8 saline- or 10-11 METH-pretreated rats. After initially observing elevated basal *Arc* expression in CC METH rats, an additional 7 rats were treated with METH as described above and sacrificed as CC. Thus, the METH CC group consisted of 18 animals. Rats were removed from their home cage after having been isolated for 24 hours. CC rats were sacrificed immediately upon removal from the home cage. Rats in the “5-min” group were exposed to a novel environment (50 × 40 × 40 cm tall Rubbermaid tub with

pictures on two walls, as previously described (Daberkow et al. 2007, Guzowski et al. 1999)) for 5 min, then immediately sacrificed. Rats in the “30-min” group were exposed for 5 min to the novel environment, returned to the home cage for 25 min, and then sacrificed. Animals were sacrificed by CO₂ exposure, decapitated, and brains immediately removed and flash-frozen in 2-methylbutane (Mallinckrodt Baker, Phillipsburg, NJ) chilled on dry ice.

Tissue preparation

Twelve- μ m cryosections of striatum (Cambridge Instruments, Bayreuth, Germany; Bregma: +1.60 to –0.8 mm (Paxinos and Watson, 1998)) were thaw-mounted onto SuperFrost Plus slides (VWR, Batavia, IL), then postfixed as previously described (Ganguly & Keefe 2001).

DAT Autoradiography

METH-induced DA depletions in dorsal striatum were analyzed using [¹²⁵I]RTI-55 binding to the dopamine transporter (DAT), as described previously (Boja *et al.* 1992, Pastuzyn *et al.* 2012). DAT binding levels were normalized to percent of saline controls for DM and dorsolateral (DL) striatum (Figure 1B).

Fluorescent in situ hybridization for *Arc*/*ppe* mRNAs

Expression of *Arc* mRNA in striatal efferent neurons was determined by double-label fluorescence *in situ* hybridization histochemistry (FISH) for *Arc* and *ppe* mRNAs, as previously described (Daberkow *et al.* 2008, Daberkow *et al.* 2007). Full-length ribonucleotide probes complementary to mRNAs for *Arc* (Lyford *et al.*, 1995) and *ppe* (Yoshikawa *et al.*, 1984) were synthesized from cDNAs using digoxigenin-UTP (DIG-UTP) and fluorescein-UTP (FITC-UTP) with T7 and SP6 RNA polymerases and DIG- and FITC-UTP RNA-labeling kits, respectively (Roche Applied Science, Indianapolis, IN).. Ribonucleotide probes were hybridized and detected as previously described (Daberkow *et al.* 2008, Daberkow *et al.* 2007).

Image acquisition

DAT film autoradiograms were digitized, and four sections per animal in rostral (+1.6 mm from bregma), middle (+0.5 mm from bregma) and caudal (–0.8 mm from bregma) dorsal striatum analyzed with *ImageJ* (Pastuzyn *et al.* 2012).

For FISH, a 0.18 mm² montage from DM striatum was captured with an FV1000 confocal laser-scanning microscope (Olympus) with motorized stage (Prior Scientific) using a 60x, 1.45 NA oil-immersion lens (plan APO) and 488-nm Ar and 543-nm and 633-nm HeNe lasers. Areas of analysis were z-sectioned in 1- μ m-thick optical sections (10 z-sections per image) (Daberkow *et al.* 2008, Daberkow *et al.* 2007). The numbers of *ppe*-pos and *ppe*-neg neurons with *Arc* mRNA staining in the nucleus and in the cytoplasm were determined, as previously described (Daberkow *et al.* 2007, Guzowski *et al.* 1999, Daberkow *et al.* 2008).

Statistical analysis

Analysis of DAT binding was accomplished by two-sample *t*-tests. METH and saline effects on body temperature were analyzed by MANOVA. Expression of *Arc* mRNA in each subcellular compartment was first compared across saline- and METH-pretreated rats using MANOVA (treatment $\times$ time $\times$ phenotype), although for clarity, *Arc* expression data are shown in separate, side-by-side graphs for saline- and METH-pretreated rats (Figs. 3, 4). *Post-hoc* analyses of significant interactions were achieved via Tukey-Kramer test for between-subjects factors (treatment, time) or *post-hoc* *t*-tests for the within-subjects factor (phenotype). For all analyses, *p*-values <0.05 were significant.

Results

METH-induced partial dopamine depletions

Treatment with METH resulted in hyperthermia necessary to produce long-term neurotoxicity (Ali *et al.* 1994). METH-treated rats reached maximum temperatures of 39.7°C ($\pm 0.8^\circ\text{C}$; Figure 1A), whereas saline-treated rats achieved maximum temperatures of 36.8°C ($\pm 0.5^\circ\text{C}$). There were significant main effects of treatment ($F_{(1,54)}=192.9$, $p<0.0001$) and time ($F_{(3,54)}=102.1$, $p<0.0001$) and a significant time $\times$ treatment interaction ($F_{(3,54)}=113.0$, $p<0.0001$). At all time points after baseline, core temperatures in METH-treated rats were significantly elevated over saline (baseline, $p=0.3$; temp 1-temp 7, $p<0.0001$). Three-weeks later, DAT binding in DM striatum was $57.2\pm 4.2\%$ of control (mean $\pm$ SEM; $n=40$ METH, $n=23$ saline; $t_{(1,26)}=-9.51$, $p<0.0001$), and that in DL striatum was $65.5\pm 3.7\%$ of control ($t_{(1,26)}=-10.14$, $p<0.0001$; Figure 1B).

METH-induced neurotoxicity is associated with impaired transcriptional activation of Arc mRNA

Using catFISH (Guzowski *et al.* 1999, Daberkow *et al.* 2008, Daberkow *et al.* 2007), we determined the subcellular localization of *Arc* mRNA in striatal efferent neurons following spatial exploration of a novel environment (Figure 2A-F). As previously reported in normal animals (Daberkow *et al.* 2007, Guzowski *et al.* 1999), exposure to the novel environment increased nuclear *Arc* mRNA expression within 5 min (Figure 2 and 3A). MANOVA revealed significant main effects of time ($F_{(2,54)}=6.0$, $p<0.005$) and treatment ($F_{(1,54)}=5.96$, $p<0.02$) and a significant time $\times$ treatment interaction ($F_{(2,54)}=4.99$, $p<0.02$). There was no main effect of phenotype ($F_{(1,54)}=2.72$, $p=0.1$) and no phenotype $\times$ time ($F_{(2,54)}=0.7$, $p=0.5$), phenotype $\times$ treatment ($F_{(1,54)}=0.5$, $p=0.5$) or phenotype $\times$ time $\times$ treatment ($F_{(2,54)}=0.6$, $p=0.6$) interactions. *Post-hoc* analysis of the time $\times$ treatment interaction revealed that in saline-pretreated rats the number of cells with nuclear *Arc* mRNA expression was significantly greater in the 5-min group than in the CC ($p<0.01$) or 30-min ($p<0.001$) groups. There were no such effects in METH-pretreated rats, as the numbers of cells with intranuclear foci of *Arc* mRNA in the METH-pretreated groups were not different from each other ($p>0.1$). However, overall (main effect of treatment) there were more cells with nuclear *Arc* mRNA in the METH-vs. saline-pretreated rats ($p<0.02$; Figure 3A vs. 3B). Thus, rats with METH-induced neurotoxicity have higher basal *Arc* transcription, but fail to induce *Arc* transcription in response to behavioral activation.

METH-induced neurotoxicity is associated with impaired cytoplasmic Arc mRNA expression

As previously reported (Daberkow *et al.* 2007), saline-pretreated rats in the 30-min group had a significant increase in the number of striatal efferent neurons with *Arc* mRNA in the cytoplasm (Figure 4, 5). Furthermore, as we have previously reported (Daberkow *et al.*, 2007, 2008), normal animals had more striatonigral (*ppe*-neg) than striatopallidal (*ppe*-pos) neurons with cytoplasmic *Arc* mRNA expression (Fig. 4, 5A). Specifically, MANOVA revealed a significant main effect for time ($F_{(2,54)}=16.9$, $p<0.0001$) and significant time $\times$ treatment ($F_{(2,54)}=3.3$, $p<0.05$) and phenotype $\times$ time $\times$ treatment ($F_{(2,54)}=3.8$, $p<0.03$) interactions. There was no significant main effect of treatment ($F_{(1,54)}=0.7$, $p=0.4$) or phenotype ($F_{(1,54)}=3.1$, $p=0.08$) and no significant phenotype $\times$ time ($F_{(2,54)}=0.6$, $p=0.5$) or phenotype $\times$ treatment ($F_{(1,54)}=0.8$, $p=0.4$) interactions. *Post-hoc* analysis of the time $\times$ treatment interaction revealed greater numbers of neurons with cytoplasmic *Arc* mRNA expression in saline-pretreated, 30-min rats relative to saline-pretreated, CC ($p=0.002$) and 5-min ($p<0.001$) groups, consistent with previous reports regarding the time course of normal *Arc* trafficking (Guzowski *et al.* 1999, Daberkow *et al.* 2007). Conversely, there were no significant differences between the numbers of cells with cytoplasmic *Arc* mRNA

expression in the METH-pretreated, 30-min group relative to the METH-pretreated, CC ($p=0.6$) and 5-min ($p=0.1$) groups, again indicating a general overall lack of *Arc* mRNA induction associated with spatial exploration in METH-pretreated rats.

To examine the phenotypic differences, *post-hoc* t-tests were performed on the numbers of *ppe*-neg and *ppe*-pos neurons with cytoplasmic *Arc* mRNA in saline- and METH-pretreated rats. Paired t-tests confirmed (Daberkow et al., 2007) greater numbers of both *ppe*-neg ($t=3.9$, $p=0.003$) and *ppe*-pos ($t=3.9$, $p=0.001$) neurons with cytoplasmic *Arc* in the saline-pretreated, 30-min group relative to saline-pretreated, CC group (Figure 4A) and that there were more *ppe*-neg than *ppe*-pos neurons with cytoplasmic *Arc* mRNA expression in the saline-pretreated, 30-min group (Figure 4A, 5A; $t=2.5$, $p=0.02$). In METH-pretreated, CC rats, there was no significant difference from saline-pretreated, CC rats in the numbers of *ppe*-neg neurons with cytoplasmic *Arc* mRNA (Figure 4; $t=-0.28$, $p>0.1$). Additionally, in METH-pretreated rats there was no significant increase in numbers of *ppe*-neg neurons with cytoplasmic *Arc* at 30-min (Figure 4B; $t=0.44$, $p=0.31$) relative to METH-pretreated, CC group, whereas there was a significant increase in the numbers of *ppe*-pos neurons (Figure 4B; $t=2.2$, $p=0.02$). Thus, in the METH-pretreated, 30-min group there was no longer a phenotypic difference between the numbers of *ppe*-neg and *ppe*-pos neurons with cytoplasmic *Arc* (Figure 4B, 5A; $t=-0.95$, $p=0.82$), but there were significantly fewer *ppe*-neg neurons with cytoplasmic *Arc* mRNA expression relative to the saline-pretreated, 30-min group (Figure 4B, 5A; $t=3.0$, $p=0.004$). Interestingly, there were more *ppe*-pos neurons with cytoplasmic *Arc* mRNA expression in the METH-pretreated, CC group than in the saline-pretreated, CC group (Figure 4; $t=-2.4$, $p=0.01$), and also slightly more *ppe*-pos neurons with cytoplasmic *Arc* mRNA in METH-pretreated, 30-min relative to METH-pretreated, CC rats (Figure 4B; $t=1.9$, $p=0.04$). However, there was no difference between the saline- and METH-pretreated 30-min groups in the numbers of *ppe*-pos neurons with cytoplasmic *Arc* mRNA expression (Figure 4, 5A; $t=0.18$, $p=0.43$).

Upon observing impaired cytoplasmic *Arc* expression in *ppe*-neg neurons in the METH-pretreated, 30-min group, we assessed whether this impairment was uniformly distributed across METH-pretreated rats or whether this difference correlated to the DA depletion in each animal. Using only cell counts from the METH-pretreated, 30-min group, we correlated the numbers of striatal efferent neurons with *Arc* mRNA in the cytoplasm with the percent DA depletion as assessed by DAT autoradiography (Figure 5B, C). The numbers of *ppe*-pos neurons with cytoplasmic *Arc* mRNA were not significantly correlated with DA loss (Figure 5B; $R^2=0.015$, $p=0.7$). Conversely, the numbers of *ppe*-neg neurons with cytoplasmic *Arc* mRNA expression was significantly inversely correlated to the DA depletion (Figure 5C; $R^2=0.518$, $p=0.019$), indicating that greater DA loss is associated with fewer *ppe*-neg neurons with cytoplasmic *Arc* mRNA expression. In the METH-pretreated, 5-min group, there was no significant correlation between cells with nuclear *Arc* and DA depletion (*ppe*-pos $R^2=0.005$, $p>0.5$; *ppe*-neg $R^2=0.05$, $p>0.05$).

Discussion

Herein, we report novel findings that basal *Arc* transcription in striatal efferent neurons is enhanced, but *Arc* mRNA transcriptional activation and cytoplasmic localization in response to behavioral activation is severely impaired, in rats with METH-induced neurotoxicity. Specifically, behavioral activation associated with spatial exploration of a novel environment – the classic paradigm used to examine *Arc* mRNA induction and trafficking (Guzowski et al. 1999, Daberkow et al. 2007, Vazdarjanova et al. 2006, Chawla et al. 2005) – resulted in no concomitant increase in cells with nuclear *Arc* expression above the elevated CC levels in METH-pretreated groups. There was also no significant overall increase in total number of neurons with cytoplasmic *Arc* mRNA in the METH-pretreated,

30-min group, particularly with respect to *ppe*-neg neurons, similar to the suppressed cytoplasmic expression previously reported following a striatally-mediated response-reversal task (Daberkow et al. 2008). Interestingly, the degree of DA depletion was significantly negatively correlated with the numbers of *ppe*-neg cells with cytoplasmic *Arc*, suggesting that the loss of this expression may arise as a consequence of disrupted DA signaling in striatum. Taken together, these data reveal a profound dysregulation of *Arc* transcriptional activation and subcellular trafficking. As *Arc* mRNA, and the resulting *Arc* protein, is critical to normal neuroplasticity processes (Chowdhury et al. 2006), such disruption may contribute to disrupted *Arc*-mediated memory processes of the basal ganglia subsequent to METH pretreatment (Daberkow et al. 2008, Pastuzyn et al. 2012).

We presently demonstrate an intriguing increase in basal (CC) *Arc* mRNA transcription following METH-induced neurotoxicity relative to saline-pretreated controls. Persistent changes in basal gene expression are not uncommon after repeated exposure to psychostimulants (*c.f.* (Unal et al. 2009), including reports from our lab showing long-term decreases in *preprotachykinin* mRNA expression after METH-induced neurotoxicity (Chapman et al. 2001, Johnson-Davis et al. 2002). However, to our knowledge, there are no prior reports of long-term changes in basal immediate early gene expression in striatal neurons as a consequence of METH-induced neurotoxicity. Given that prior work suggested a relatively preferential impact of METH-induced neurotoxicity on striatonigral neuron function (Johnson-Davis et al. 2002, Chapman et al. 2001, Daberkow et al. 2008), the equivalent increases in basal *Arc* transcription observed in *ppe*-pos and *ppe*-neg neurons were surprising and suggest global alterations in striatal efferent neuron function following METH-induced neurotoxicity. This up-regulation may reflect alterations in signals regulating *Arc* transcription, including brain-derived neurotrophic factor or glutamate. While both of these molecules are elevated 24-72 hrs after exposure of animals to a neurotoxic regimen of METH (Mark et al. 2007, Thomas et al. 2004), whether they remain elevated in striatum weeks later is unknown. Clearly, further studies are needed to examine the mechanisms underlying long-term dysregulation of basal *Arc* transcription and also the extent to which the expression of other plasticity-related genes is similarly altered.

The present studies also reveal a profound impairment of acute, novelty-induced transcription of *Arc* in rats with METH-induced neurotoxicity. The basis for this observation is presently unknown, but changes in phasic DA release seem a likely contributing factor. Partial monoamine loss is associated with impaired phasic-like, but not tonic-like, DA release (Bergstrom & Garris 2003, Howard et al. 2011). Modeling data suggest that D1-DA receptors should be particularly sensitive to changes in phasic DA amplitude (Dreyer et al. 2010). Consistent with this model, phasic-like stimulation of DA transmission increases gene expression in and electrophysiological activity of striatonigral, but not striatopallidal, neurons (Gonon 1997, Onn et al. 2000, Chergui et al. 1997). Finally, we presently report that in METH-pretreated rats, the impairment in numbers of *ppe*-neg neurons with cytoplasmic *Arc* mRNA correlates with the extent of DA loss, which is further known to correlate with the degree of impairment of phasic-like DA neurotransmission (Bergstrom & Garris 2003, Howard et al. 2011). Thus, impaired phasic DA neurotransmission may ultimately contribute to the observed deficits in *Arc* expression subsequent to METH-induced neurotoxicity.

Decreased phasic DA neurotransmission and consequent decreases in D1-DA receptor activation may also impair *Arc* transcription by altering upstream signaling cascades. Activation of D1-DA receptors enhances NMDA receptor-mediated currents in striatonigral neurons (Jocoy et al. 2011, Cepeda et al. 1993, Andre et al. 2010) and increases activation of ERK1/2 (Valjent et al. 2005, Pascoli et al. 2011). *Arc* transcription is regulated by NMDA receptor and ERK1/2 activation (Korb & Finkbeiner 2011). Thus, decreased D1-DA

receptor activation due to attenuated phasic DA transmission likely results in decreased NMDA receptor and ERK1/2 activation and, thus, decreased activity-dependent *Arc* induction in *ppe*-neg neurons.

While the above-delineated effects can explain the basis for the impairment of acute, novelty-induced *Arc* transcription in *ppe*-neg neurons, the basis for the impaired transcriptional activation in *ppe*-pos neurons is less apparent. One possibility is that it also arises as a consequence of impaired phasic DA signaling and decreased D1-DA receptor activation in striatum. Disruption of striatal D1-DA receptor activation decreases cortical excitability (Yano *et al.* 2006, Gross & Marshall 2009, Steiner & Kitai 2000). Additionally, cortical immediate early gene induction by DA receptor agonists is blunted in rats with METH-induced neurotoxicity (Belcher *et al.* 2009). Furthermore, novelty-induced *c-fos* mRNA expression in striatopallidal neurons is dependent on corticostriatal transmission (Ferguson & Robinson 2004), sensitive to decreases in D1-DA receptor activation (Ferguson *et al.* 2003), and sensitive to blockade of NMDA receptors or ERK1/2 signaling (Ferguson *et al.* 2003, Ferguson & Robinson 2004). These data thus suggest a model wherein loss of D1-DA receptor activation in striatum due to disrupted phasic signaling secondary to METH-induced monoamine toxicity may lead to decreased cortical excitability and, consequently, decreased gene expression in *ppe*-pos neurons.

An alternative explanation for the overall loss of novelty-induced *Arc* transcription is that chronic elevations in basal *Arc* mRNA expression suppress further transcriptional activation. Massed exposures to a spatial environment in a single day impair *Arc* transcription (Guzowski *et al.* 2006) and AMPA receptor activation can inhibit *Arc* transcription (Rao *et al.* 2006). While there are DNA regulatory elements that repress *Arc* transcription (Pintchovski *et al.* 2009), what binds to those regulatory sites and the situations under which they are engaged are currently undefined. Therefore, the extent to which transcriptional repression of the *Arc* gene vs. impaired activation of postsynaptic receptors (NMDA, D1) or intracellular signaling cascades contributes to the loss of evoked *Arc* expression in rats with METH-induced neurotoxicity remains to be determined.

The present results confirm prior observations (Daberkow *et al.* 2008, Daberkow *et al.* 2007) that normal, saline-pretreated animals have more *ppe*-neg neurons than *ppe*-pos neurons with *Arc* mRNA in the cytoplasm. At present, the basis for this phenotypic difference in normal animals is unknown, but it may be related to differences in *Arc* trafficking or cytoplasmic mRNA stability, given that *Arc* mRNA is a substrate for translation-dependent decay (Giorgi *et al.* 2007). The lack of transcriptional activation of *Arc* in rats with METH-induced neurotoxicity makes it somewhat difficult to discern whether DA contributes to this phenotypic difference; however, the present results suggest that the trafficking/stability of *Arc* mRNA in *ppe*-neg neurons in normal animals is dependent on DA signaling. First, despite the lack of novelty-induced transcriptional activation, METH-pretreated, CC rats have more *ppe*-pos neurons with cytoplasmic *Arc* than do saline-pretreated, CC rats, suggesting that METH-pretreated rats still traffic basally transcribed *Arc* mRNA into the cytoplasm in *ppe*-pos neurons. Conversely, despite having high basal numbers of *ppe*-neg neurons with transcriptional activation of *Arc*, METH-pretreated, 30-min rats do not have greater numbers of *ppe*-neg neurons with cytoplasmic *Arc* expression compared to saline-pretreated, 30-min rats. Thus, the elevated basal transcription of *Arc* mRNA in *ppe*-neg neurons of METH-pretreated rats does not translate into greater numbers of neurons with cytoplasmic *Arc*, suggesting some disruption of *Arc* trafficking/stability in *ppe*-neg neurons. Second, whereas there is a slight, but significant, increase in the numbers of *ppe*-pos neurons with cytoplasmic *Arc* mRNA in the METH-pretreated, 30-min group relative to the numbers in the METH-pretreated, CC group, there is no such increase in the numbers of *ppe*-neg neurons. Taken together, these observations suggest that METH-induced

neurotoxicity is associated with impaired acute transcriptional activation of *Arc* in both subtypes of striatal efferent neurons, as well as impaired trafficking/stability specifically in *ppe*-neg neurons.

We presently report that a neurotoxic regimen of METH has significant, long-term impact on the regulation of transcriptional activation and subcellular expression of *Arc* mRNA. This METH-induced effect on *Arc* mRNA transcription and expression may thus impair Arc protein translation, with Arc protein synthesized within 60 min of neuronal stimulation (Baez *et al.* 2011, Vazdarjanova *et al.* 2006, Lyford *et al.* 1995). Importantly, this disrupted activity-dependent striatal *Arc* expression, and hypothesized impact on protein synthesis, may underlie previously reported changes in basal ganglia-mediated learning and memory processes in METH-pretreated rats, including response-reversal (Daberkow *et al.* 2008, Pastuzyn *et al.* 2012), sequential motor (Chapman *et al.* 2001), and stimulus-response vs. action-outcome (Son *et al.* 2011) learning. Our present results therefore suggest *Arc* mRNA or the factors regulating it as potential novel therapeutic targets in the treatment of METH-induced cognitive impairments (Kalechstein *et al.* 2003, Volkow *et al.* 2001, Johanson *et al.* 2006), as Arc is a critical mediator underlying normal neuroplasticity processes (Chowdhury *et al.* 2006, Rial Verde *et al.* 2006). Future work will thus need to examine whether therapeutically restoring partial DA loss will resolve the molecular and cellular changes that occur, as well as improve cognitive functionality compromised with METH addiction and abuse.

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Abbreviations

Arc/Arg3.1	activity-regulated cytoskeleton-associated protein
METH	(±)-methamphetamine hydrochloride
DA	dopamine
ppe	preproenkephalin
LTP	long-term potentiation
LTD	long-term depression
CC	cage control rats
DIG-UTP	digoxigenin-UTP
FITC-UTP	fluorescein-UTP
DAT	dopamine transporter
DM	dorsomedial
DL	dorsolateral
BDNF	brain-derived neurotrophic factor
ERK1/2	extracellular signal-regulated kinase

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Figure 1. Methamphetamine pretreatment and subsequent DA depletions

A) A neurotoxic regimen of methamphetamine (METH; 4×10 mg/kg) leads to a significant elevation of core body temperature relative to saline-treated animals (treatment effect $F_{(1,54)}=192.9$, $p<0.0001$). B) METH-pretreated rats ($n = 40$) show significant DA depletions in both dorsomedial (DM) and dorsolateral (DL) striatum as measured by [125 I]RTI-55 dopamine transporter autoradiography three weeks later (mean gray values (arbitrary units) $\pm$ SEM expressed as % of saline control ($n = 23$)). * Significantly different from saline control, $p \leq 0.05$.

Figure 2. Representative photomicrographs of *Arc* mRNA expression in rat dorsomedial striatum

Representative *in situ* hybridization histochemical images from rats pretreated with saline ($4 \times 1\text{mL/kg}$; A-C) or a neurotoxic regimen of ($\pm$)-methamphetamine (METH; $4 \times 10\text{mg/kg}$, s.c. at 2-hr intervals) and then either sacrificed immediately upon removal from the home cage (“caged control”; A, D), allowed to explore a novel environment for 5 min and then sacrificed (5-min; B, E), or allowed to explore the novel environment for 5 min before being returned to the home cage for 25 min before being sacrificed (30-min; C, F). Green is Sytox nuclear counter stain, blue is *preproenkephalin* mRNA and red is *Arc* (activity-regulated, cytoskeleton-associated) mRNA expression. Scale bar = $10\text{ }\mu\text{m}$.

Figure 3. Nuclear *Arc* mRNA expression in striatal efferent neurons

Data are mean ($\pm$ SEM; saline $n = 6-8$; METH $n = 10-18$ per time point) numbers of labeled neurons per 0.5 mm^2 determined from double fluorescent *in situ* hybridization staining for *preproenkephalin* and *Arc* (activity regulated, cytoskeleton-associated) mRNAs in the dorsomedial striata of rats treated approximately three weeks prior to sacrifice with A) saline ($4 \times 1 \text{ mL/kg}$) or B) a neurotoxic regimen of ($\pm$)-methamphetamine (METH; $4 \times 10 \text{ mg/kg}$, s.c. at 2-hr intervals). Rats were either sacrificed immediately upon removal from the home cage (“caged control”; CC), allowed to explore a novel environment for 5 min and then sacrificed (5-min), or allowed to explore the novel environment for 5 min before being returned to the home cage for 25 min before being sacrificed (30-min). There is a significant main effect of METH-pretreatment relative to saline-pretreatment ($p < 0.02$). * Significant main effect of time (5-min point significantly different from both the CC and 30-min groups).

Figure 4. Cytoplasmic *Arc* mRNA expression in striatal efferent neurons

Data are mean ($\pm$ SEM; saline $n = 6-8$; METH $n = 10-18$ per time point) numbers of labeled neurons per 0.5 mm^2 determined from double fluorescent *in situ* hybridization staining for *preproenkephalin* and *Arc* (activity regulated, cytoskeleton-associated) mRNAs in the dorsomedial striata of rats treated approximately three weeks prior to sacrifice with A) saline ($4 \times 1 \text{ mL/kg}$) or B) a neurotoxic regimen of ($\pm$)-methamphetamine (METH; $4 \times 10 \text{ mg/kg}$, s.c. at 2-hr intervals). Rats were either sacrificed immediately upon removal from the home cage (“caged control”; CC), allowed to explore a novel environment for 5 min and then sacrificed (5-min), or allowed to explore the novel environment for 5 min before being returned to the home cage for 25 min before being sacrificed (30-min). * Significantly different from all other saline-pretreated groups, $p < 0.05$. # Significantly greater than number of *ppe*-pos neurons in same group. ^ Significantly greater than the number of *ppe*-pos neurons with *Arc* mRNA in the cytoplasm in the saline-pretreated, CC group. § Significantly greater than the number of *ppe*-pos with *Arc* mRNA expression in the cytoplasm in the METH-pretreated, CC group. ¶ Significantly less than the number of *ppe*-neg neurons with *Arc* mRNA in the cytoplasm in the saline-pretreated, 30-min group.

Figure 5. Cytoplasmic *Arc* mRNA expression in striatal efferent neurons of rats sacrificed 30 min after exposure to a novel environment and relation to degree of methamphetamine-induced dopamine depletion

Partial monoamine depletion significantly only affects the numbers of *ppe-neg* neurons with cytoplasmic *Arc* mRNA expression. A) Mean ($\pm$ SEM; saline $n = 8$; METH $n = 11$) numbers of labeled neurons per 0.5 mm² determined from double fluorescent *in situ* hybridization staining for *preproenkephalin* and *Arc* (activity regulated, cytoskeleton-associated) mRNAs in the dorsomedial striata of rats treated approximately three weeks prior to sacrifice with saline (4 $\times$ 1 mL/kg) or a neurotoxic regimen of ($\pm$)-methamphetamine (METH; 4 $\times$ 10 mg/kg, s.c. at 2-hr intervals). These data are the same “30-min” data presented in Figure 4, but are re-graphed to highlight the differences between the striatal efferent neuron subtypes at this time point. * Significantly different from number of *ppe-neg* neurons in saline-pretreated rats. # Significantly different from number of *ppe-pos* neurons in saline-pretreated rats. B) Correlation between the numbers of *ppe-pos* neurons with *Arc* mRNA in the cytoplasm and the percent DA loss, as assessed by [¹²⁵I]RTI-55 autoradiography for dopamine transporter binding, induced by the neurotoxic regimen of METH (4 $\times$ 10 mg/kg, s.c. at 2-hr intervals) three weeks prior to sacrifice. C) Correlation between the numbers of *ppe-neg* neurons with *Arc* mRNA in the cytoplasm and the percent DA loss, as assessed by [¹²⁵I]RTI-55 autoradiography for dopamine transporter binding, induced by the neurotoxic regimen of METH (4 $\times$ 10 mg/kg, s.c. at 2-hr intervals) three weeks prior to sacrifice. * Significant correlation, $p=0.019$.