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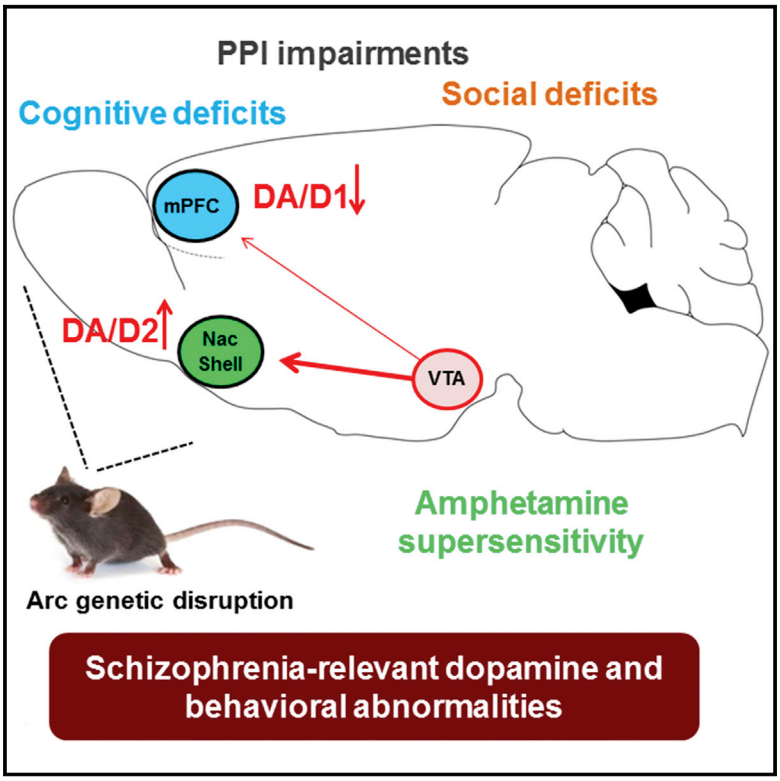
Genetic Disruption of Arc/Arg3.1 in Mice Causes Alterations in Dopamine and Neurobehavioral Phenotypes Related to Schizophrenia

Graphical Abstract



Highlights

- Arc genetic disruption recapitulates schizophrenia-relevant behavioral abnormalities
- Arc disruption leads to hypoactive PFC dopamine (DA) and hyperactive striatal DA system
- Hypo-PFC and hyper-striatal DA mediates cognitive and motor changes, respectively
- Arc is a convergence point for genes implicated in schizophrenia risk

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In Brief

Managò et al. find that, consistent with schizophrenia-related phenotypes, disruption of Arc in mice produces deficits in sensorimotor gating, cognitive functions, social behaviors, and amphetamine-induced psychomotor responses. Furthermore, genetic disruption of Arc leads to concomitant hypoactive mesocortical and hyperactive mesostriatal dopamine pathways.

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Genetic Disruption of Arc/Arg3.1 in Mice Causes Alterations in Dopamine and Neurobehavioral Phenotypes Related to Schizophrenia

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SUMMARY

Human genetic studies have recently suggested that the postsynaptic activity-regulated cytoskeleton-associated protein (Arc) complex is a convergence signal for several genes implicated in schizophrenia. However, the functional significance of Arc in schizophrenia-related neurobehavioral phenotypes and brain circuits is unclear. Here, we find that, consistent with schizophrenia-related phenotypes, disruption of Arc in mice produces deficits in sensorimotor gating, cognitive functions, social behaviors, and amphetamine-induced psychomotor responses. Furthermore, genetic disruption of Arc leads to concomitant hypoactive mesocortical and hyperactive mesostriatal dopamine pathways. Application of a D1 agonist to the prefrontal cortex or a D2 antagonist in the ventral striatum rescues Arc-dependent cognitive or psychomotor abnormalities, respectively. Our findings demonstrate a role for Arc in the regulation of dopaminergic neurotransmission and related behaviors. The results also provide initial biological support implicating Arc in dopaminergic and behavioral abnormalities related to schizophrenia.

INTRODUCTION

Schizophrenia is a devastating disorder strongly related to genetic factors (Sullivan et al., 2003). This debilitating disorder is defined by behavioral symptoms that encompass a large spectrum of abnormalities, including cognitive and sensorimotor deficits, social withdrawal, and hallucinations. Although the neurobiological mechanisms underlying these behavioral abnormalities remain unclear, the pathophysiology of schizophrenia, its treatments, and aspects of its associated behavioral deficits have been consistently linked with a dysregulation of dopaminergic, glutamatergic, and GABAergic neural transmission, especially in brain areas such as the prefrontal cortex (PFC) and striatum (Howes and Kapur, 2009; Simpson et al., 2010; Winterer and Weinberger, 2004).

Recent large-scale genetic studies of schizophrenia have noted that many relevant variants involve genes encoding proteins interacting with the activity-regulated cytoskeletal-associated (Arc) postsynaptic signaling complexes (Fromer et al., 2014; Purcell et al., 2014; Huentelman et al., 2015). Arc chromosomal microdeletion and intragenic SNPs have also been found in association with neurodevelopmental disorders such as schizophrenia (Hu et al., 2015; Huentelman et al., 2015). In addition, reduced expression of Arc mRNA has been detected in the PFC of individuals with schizophrenia (Guillozet-Bongaarts et al., 2014). Despite this consistent evidence pointing to convergence on Arc, the neurobiological implication of Arc in schizophrenia-related behavioral phenotypes and brain systems remains unclear.

Arc is a neural activity-regulated immediate early gene that is expressed selectively in Ca^{2+}/calmodulin-dependent kinase II alpha (CaMKIIa)-expressing neurons in the neocortex, hippocampus, and striatum (Miyashita et al., 2008; Vazdarjanova et al., 2006). Extensive studies have shown that the Arc protein is functionally involved in long-lasting forms of synaptic plasticity (Plath et al., 2006; Shepherd et al., 2006; Rial Verde et al., 2006; Waung et al., 2008; Park et al., 2008; Jakkamsetti et al., 2013). In particular, Arc has been reported to regulate synaptic activities and neuronal firing patterns through AMPA and NMDA-type glutamate receptors (Chowdhury et al., 2006; Zhang et al., 2015). Furthermore, genetic disruptions of the Arc gene have been shown to impair experience-dependent cortical circuit functions (McCurry et al., 2010; Wang et al., 2006) and consolidation of memories (Plath et al., 2006; Ren et al., 2014; Cao

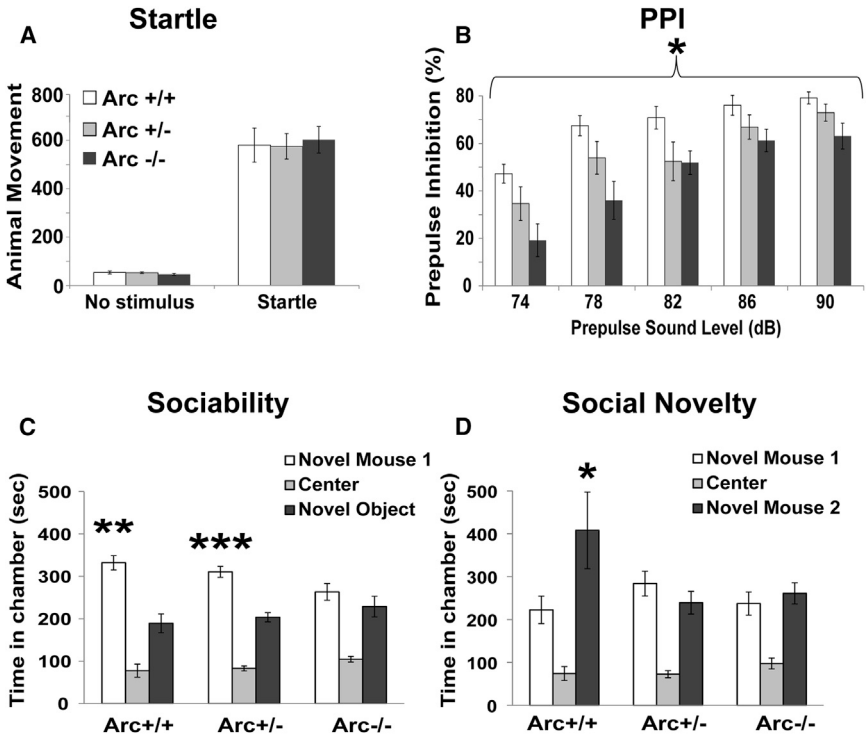

Figure 1. Impaired Sensorimotor Gating and Social Abilities in Arc Knockout Mice

(A) Animal movements (a.u.) displayed by $Arc^{+/+}$, $Arc^{+/-}$, and $Arc^{-/-}$ littermates following the presentation of no stimulus or a 120 dB acoustic stimulus (Startle).

(B) Percent PPI of the acoustic startle response displayed by the same mice after the presentation of 74, 78, 82, 86, and 90 dB prepulse stimuli. n = 20–26/group. *p < 0.05, $Arc^{-/-}$ versus $Arc^{+/+}$.

(C and D) Time spent in each compartment of the three-chambered arena during (C) the sociability and (D) the social novelty tests displayed by $Arc^{+/+}$, $Arc^{+/-}$, and $Arc^{-/-}$ littermates. n = 9–17/group. ***p < 0.0005, **p < 0.005, and *p < 0.05 versus the novel object or the novel mouse within the same genotype. Values represent mean ± SEM throughout all figures.

RESULTS

Arc Genetic Disruption Impaired Prepulse Inhibition Abilities, but Not Startle Responses

Prepulse inhibition (PPI) is a sensorimotor gating measure that is attenuated in patients with schizophrenia (Swerdlow et al., 2006), is highly conserved across mammalian species, and can be studied experimentally in rodents (Papaleo et al., 2012).

Reduction (+/−) or absence (−/−) of Arc did not affect general health or physical abilities (Table S1; Supplemental Results). Arc genotype did not affect acoustic startle reactivity or basal activity ($F_{2,64}$ = 0.03, p = 0.97; Figure 1A). Instead, PPI showed a genotype effect ($F_{2,64}$ = 4.46, p < 0.02; Figure 1B), with $Arc^{-/-}$ mice displaying disrupted PPI (p = 0.008) and $Arc^{+/-}$ mice a tendency (p = 0.08) compared to $Arc^{+/+}$ littermates. These results indicate that genetic modifications reducing Arc decrease sensorimotor gating abilities, consistent with PPI deficits in schizophrenia.

Arc Genetic Disruption Impaired Social Abilities

Disrupted social behaviors are characteristic features in schizophrenia, embedded in so-called negative symptoms (Millan et al., 2014). We thus assessed $Arc^{+/+}$, $Arc^{+/-}$, and $Arc^{-/-}$ littermates in a well-validated social approach task (Papaleo et al., 2011).

Sociability, defined as spending more time in the chamber with a novel mouse than in the chamber with a novel object, was evident in both $Arc^{+/+}$ ($F_{1,8}$ = 15.58, p < 0.005) and $Arc^{+/-}$ ($F_{1,16}$ = 22.31, p < 0.0005; Figure 1C) mice. In contrast, $Arc^{-/-}$ mice presented sociability deficits as they spent the same amount of time with the novel mouse and the novel object ($F_{1,5}$ = 0.63, p = 0.46; Figure 1C). Thus Arc deletion completely abolished social preference versus a conspecific compared to an inanimate object.

Deficits in social novelty recognition were also detected in Arc mutant mice. $Arc^{+/+}$ mice spent more time in the chamber containing the newly introduced mouse (novel mouse 2) than in the chamber containing the now familiar mouse ($F_{1,8}$ = 7.65, p < 0.05; Figure 1D). In contrast, both $Arc^{+/-}$ and $Arc^{-/-}$ mice

Using genetically modified mice with targeted deletion of the Arc gene, we aimed to investigate the effects of Arc genetic disruptions on behavioral functions that have been studied in rodents as relevant to schizophrenia. We did not attempt to find a mouse model of an entire psychiatric disorder but rather focused on disease-related functional dimensions of behavior. Particularly, because one of the long-standing pathophysiological hypotheses of schizohprenia involves a dysregulated dopaminergic system (Weinstein et al., 2016), we investigated the effects of Arc genetic disruption on different aspects of dopaminergic system functions. We found that Arc gene disruption leads to behavioral abnormalities comprising a broad range of cognitive, negative and positive schizophrenia-like symptoms. In addition, using a combination of ex vivo molecular assessments, in vivo microdialysis, and electrical stimulation coupled with in vivo two-photon imaging, we show that Arc genetic disruption leads to divergent alterations between the PFC and striatal dopaminergic system that capture aspects of schizophrenia-related neuropathophysiology. Specifically, reduced Arc gene expression resulted in a hypo-dopaminergic response in the PFC and increased dopamine/D2 signaling in the striatum. These data provide initial biological support that abnormal Arc function may cause dysfunctions in the dopaminergic system and behavioral abnormalities related to schizophrenia.

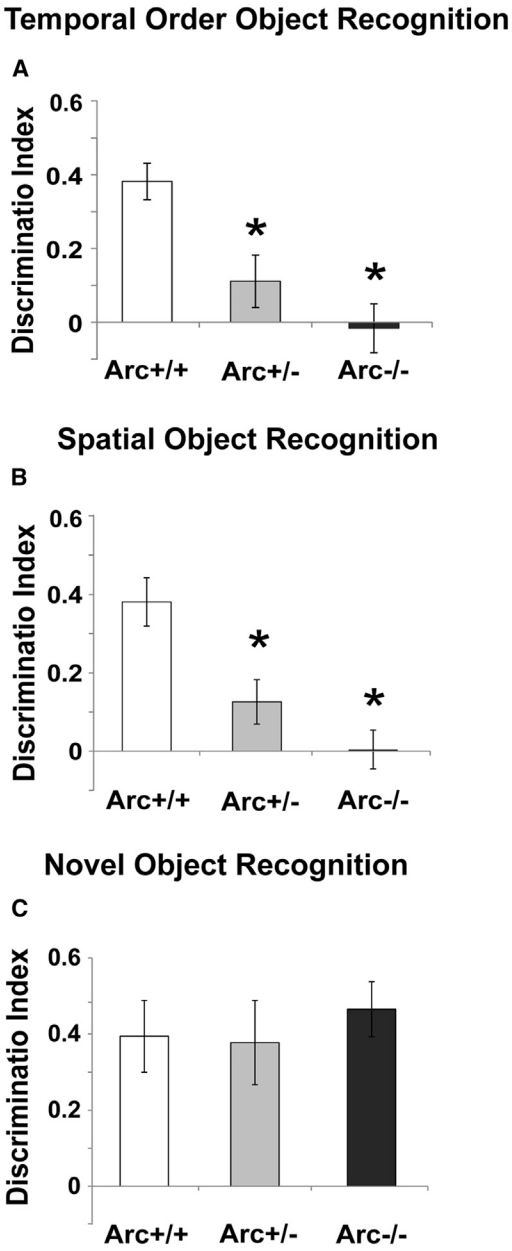

Figure 2. Impaired Temporal Order and Spatial Object Recognition Memory in Arc Knockout Mice

Discrimination ratio displayed by $Arc^{+/+}$, $Arc^{+/-}$, and $Arc^{-/-}$ littermates (A) during the 5-min temporal order object recognition test (n = 18–20/group), (B) during the 5-min spatial object recognition test (n = 17–22/group), and (C) during the 5-min novel object recognition test (n = 6–12/group). *p < 0.01 versus $Arc^{+/+}$ mice.

spent the same amount of time between the newly introduced and familiar mice ($F_{1,16}$ = 0.67, p = 0.43 and $F_{1,5}$ = 0.22, p = 0.66 for $Arc^{+/-}$ and $Arc^{-/-}$ mice, respectively; Figure 1D). For both tests, entries into the left and right side chambers did not differ within each genotype or across genotypes (Figure S1). No innate side preference was observed across genotypes before the start of the testing. These data indicate that genetic mutations reducing Arc disrupt social discrimination abilities, consistent with similar deficits in schizophrenia.

Arc Genetic Disruption Produced Schizophrenia-Relevant Cognitive Impairments

Cognitive deficits have been suggested as core symptoms of schizophrenia. We tested Arc genetically modified mice in a temporal order object recognition task that measures recency discrimination, a cognitive ability disrupted in patients with schizophrenia (Rizzo et al., 1996; Schwartz et al., 1991).

An Arc genotype effect was evident during the test phase ($F_{2,54}$ = 9.86; p < 0.0002; Figure 2A). In particular, the performance of $Arc^{+/-}$ and $Arc^{-/-}$ mice was significantly worse than that of $Arc^{+/+}$ mice (p < 0.005). While $Arc^{+/+}$ mice were spending more time exploring the object presented least recently, $Arc^{+/-}$ and $Arc^{-/-}$ mice failed to show any preference for the less recent object. Thus, Arc genetic reduction impaired cognitive functions dependent on the medial prefrontal cortex (mPFC), hippocampus, and perirhinal cortex (PRH; Barker and Warburton, 2011).

To better dissect the neuroanatomical components of Arc-dependent cognitive deficits, we tested two other cohorts of naive mutants in a spatial object recognition task, exclusively dependent on hippocampal functions, and in a novel object recognition task that depends on the PRH, but not on the mPFC or the hippocampus (Barker et al., 2007; Barker and Warburton, 2011). In the spatial object recognition task, there was an Arc effect ($F_{2,54}$ = 10.66, p < 0.002; Figure 2B). In contrast to $Arc^{+/+}$ mice, $Arc^{-/-}$ and $Arc^{+/-}$ mice were not able to recognize which object was displaced (p < 0.003). These results indicate that Arc is fundamental for spatial memory functions.

The novel object recognition task revealed no Arc effects ($F_{2,24}$ = 0.16; p = 0.85; Figure 2C). As well as $Arc^{+/+}$ mice, $Arc^{+/-}$ and $Arc^{-/-}$ mice spent more time exploring the novel object compared to the familiar one. This indicates unimpaired novel-familiar object discrimination abilities and unaltered PRH functioning. In all three experiments, $Arc^{+/+}$, $Arc^{+/-}$, and $Arc^{-/-}$ mice spent equal amount of time exploring the objects in every phase of the task (Figure S2). Thus, Arc genetic reduction did not alter motivation, curiosity, motor, olfactory, tactile, or visual functions that might affect object recognition.

Overall, these results indicate that the Arc-dependent cognitive deficits might derive from a dysfunctional mPFC and/or hippocampus, two brain regions implicated in the cognitive deficits found in schizophrenia (Dreher et al., 2012; Rasetti et al., 2014).

Arc Genetic Disruption Produced Amphetamine Supersensitivity

Amphetamine exacerbates psychotic experiences in patients with schizophrenia and can be psychogenic in normal human subjects (Laruelle et al., 1999). We thus tested $Arc^{+/+}$, $Arc^{+/-}$, and $Arc^{-/-}$ littermates in an open field arena in basal conditions and following amphetamine injection.

A genotype × time interaction effect was detected in the basal locomotor activity during the first day ($F_{22,572}$ = 1.98, p < 0.005). All three genotypes decreased the distance traveled in the open field arena over time. However, $Arc^{-/-}$ mice were slightly more active than $Arc^{+/-}$ and $Arc^{+/+}$ mice during the first 5 min (p < 0.05; Figure 3A).

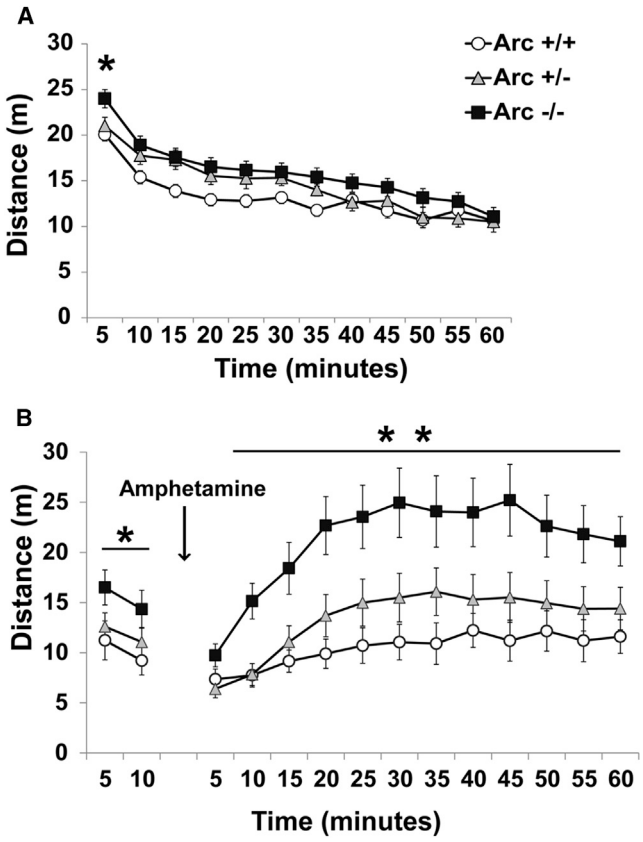

Figure 3. Amphetamine Supersensitivity in Arc Knockout Mice

(A) Ambulatory distance displayed by $Arc^{+/+}$, $Arc^{+/-}$, and Arc $^{-/-}$ littermates during the first exposure to the empty open field arena (n = 14–24/group). *p < 0.05 versus $Arc^{+/+}$ within the same time point.

(B) Ambulatory distance displayed by $Arc^{+/+}$, $Arc^{+/-}$, and Arc $^{-/-}$ mice during the 10 min before and 60 min after the amphetamine injection (1.5 mg/kg, i.p.) (n = 13–20/group).

*p < 0.01 and **p < 0.001 versus $Arc^{+/+}$ and $Arc^{+/-}$.

Two days later, a genotype effect was present again during the first 10 min in the open field ($F_{2,42} = 4.97$, $p \leq 0.01$), with $Arc^{-/-}$ mice being more active than $Arc^{+/+}$ and $Arc^{+/-}$ mice ($p < 0.008$; Figure 3B). Afterward, mice were injected with amphetamine (1.5 mg/kg), revealing a genotype × time interaction effect ($F_{22,462} = 1.92$, $p < 0.008$). Locomotor activity responses to amphetamine were higher in $Arc^{-/-}$ mice than in $Arc^{+/-}$ and $Arc^{+/+}$ mice ($p < 0.005$; Figure 3B). Thus, genetic mutations disrupting Arc produced a hyperactive phenotype and amphetamine supersensitivity consistent with rodents' correlates of schizophrenia-like symptoms. Importantly, these results may suggest a previously unexpected effect of Arc in the modulation of the dopaminergic system.

Arc Genetic Disruption Decreased Dopamine Turnover in the PFC and Increased Post-synaptic D2 Signaling in the Striatum

To investigate how Arc genetic mutations might affect the dopaminergic system, we first analyzed Arc mutants for dopamine content in PFC and striatum, two major areas involved in the dopamine hypothesis of schizophrenia (Koch et al., 2014; Simpson et al., 2010; Weinstein et al., 2016; Winterer and Weinberger, 2004). In the PFC, an Arc effect was present on dopamine tissue content ($F_{2,71} = 6.72$, $p = 0.002$; Figure 4A), but not on noradrenaline (Figure S3). $Arc^{-/-}$ mice had increased dopamine levels ($p = 0.003$) and a decrease in both the HVA:dopamine and DOPAC:dopamine ratios ($p < 0.05$; Figures 4B and 4C), suggesting a decreased dopamine turnover. In agreement, measurements of tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis, revealed that Arc knockout mice had reduced pTH(Ser40) levels ($F_{2,15} = 9.58$, $p < 0.003$; Figure 4D), a marker of TH enzymatic activity (Anzalone et al., 2012), and normal TH total protein levels ($p = 0.7$; Figure 4E). In contrast, in the striatum there was no Arc-dependent effect on the dopamine content ($F_{2,47} = 0.46$, $p = 0.63$; Figure S3) or on HVA and DOPAC ratios (Figure S3). These results indicate that Arc genetic disruption affects the maintenance of dopaminergic transmission in the PFC.

Alterations of the dopamine system in the PFC can change D2 receptor transmission in the striatum (Clarke et al., 2014; Pycock et al., 1980), and dopamine/D2 pathways in the PFC-striatal loop have been implicated in the manifestations of schizophrenia (Simpson et al., 2010; Winterer and Weinberger, 2004). Thus, we next analyzed total and surface protein levels of the D2 receptors in Arc mutants. Genetic reduction of Arc (both +/− and −/−) increased surface D2 in the striatum ($F_{2,6} = 228.41$, $p < 0.0001$, Figure 4F), but not in the PFC ($F_{2,6} = 1.98$, $p = 0.22$; Figure S4). In contrast, no Arc effect was evident in the total levels of D2 in the striatum ($F_{2,6} = 0.16$, $p = 0.85$; Figure 4G) and PFC ($F_{2,6} = 0.59$, $p = 0.58$; Figure S4). Several studies indicate that increased D2 transmission in the striatum is an important component in the pathophysiology of schizophrenia (Abi-Dargham et al., 2000; Laruelle, 1998; Simpson et al., 2010; Winterer and Weinberger, 2004; Kellendonk et al., 2006). In particular, D2 density and surface availability is increased in drug-naive or drug-free patients with schizophrenia (Abi-Dargham et al., 2000; Laruelle, 1998).

To further examine striatal D2 signaling in Arc knockout mice, we analyzed total and phosphorylated levels of different dopamine/D2-related intracellular regulatory proteins in baseline conditions and following the amphetamine challenge used for behavioral experiments. The Ser40 pTH levels have been directly correlated with the activity of pre-synaptic D2 (Anzalone et al., 2012). Consistent with this, we found that amphetamine reduced striatal pTH levels ($F_{1,30} = 5.83$, $p < 0.02$), but in an Arc-independent manner ($F_{2,30} = 0.31$, $p = 0.74$; Figure 4I). The total levels of TH were unassociated with Arc ($F_{2,34} = 0.74$, $p = 0.49$), as well as with amphetamine treatment ($F_{1,34} = 0.13$, $p = 0.72$, Figure 4H). Thus, our data suggest that Arc might not alter presynaptic D2 pathways in the striatum.

Reduced expression of Akt kinase has been reported in patients with schizophrenia (Emamian et al., 2004; Zhao et al., 2006). We found a similar reduction of total Akt under basal condition in both $Arc^{+/-}$ and Arc $^{-/-}$ mice ($F_{2,32} = 8.97$ $p < 0.001$; Figure 4J). The thr308 pAkt levels have been directly correlated with the activity of postsynaptic D2 (Beaulieu et al., 2007). We found that at the basal level, $Arc^{-/-}$ mice had reduced pAkt compared to $Arc^{+/+}$ mice ($F_{2,29} = 4.77$, $p < 0.01$; Figure 4K). Following amphetamine treatment, Arc knockout mice did not show further

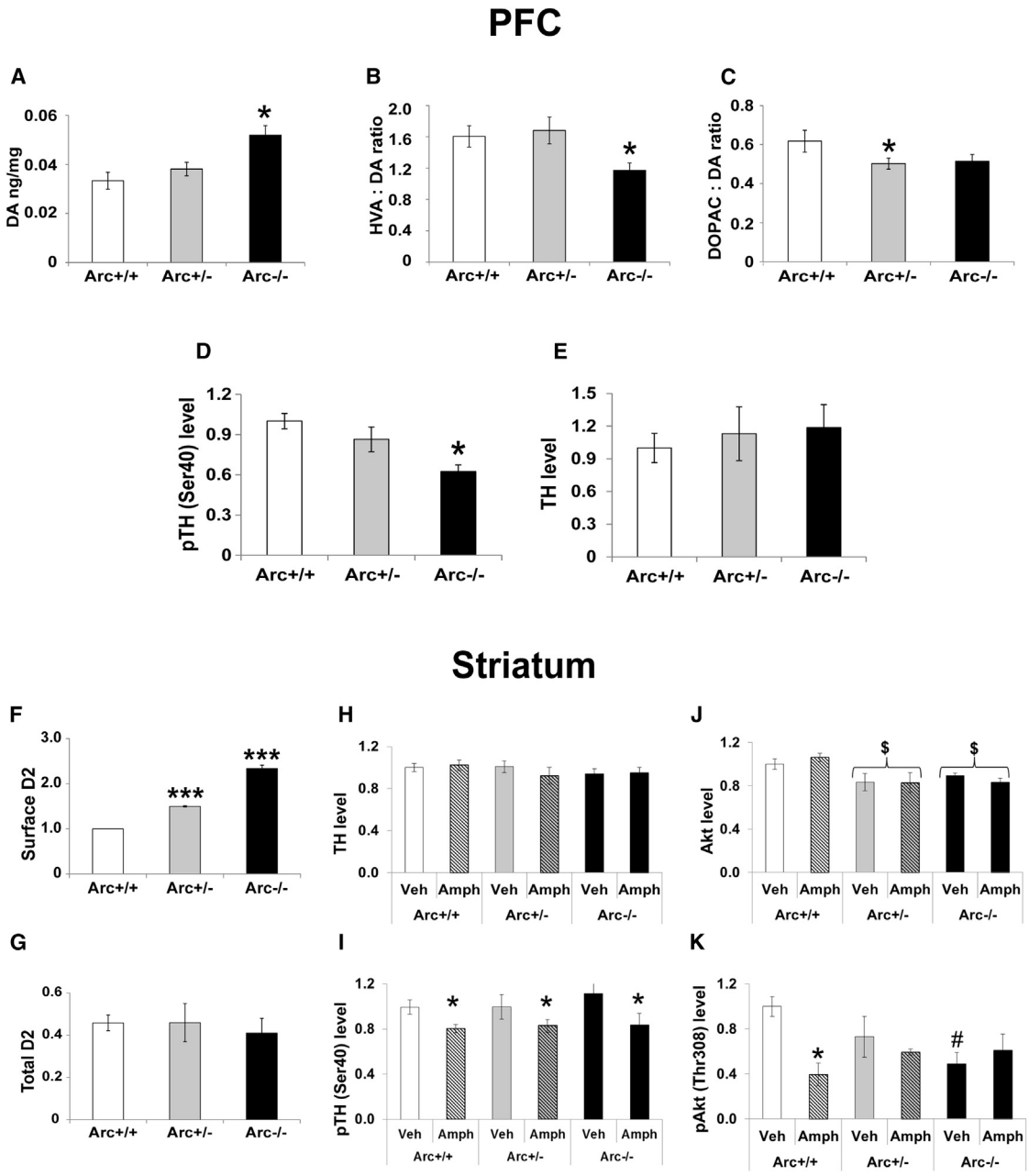

Figure 4. PFC Decreased Dopamine Turnover and Striatal Increased Post-synaptic D2 Signaling in Arc Knockout Mice

(A–E) Total dopamine (DA) content expressed as nanograms per milligram of tissue (A), HVA:DA (B) and DOPAC:DA (C) metabolite ratios, levels of TH phosphorylated at the Ser40 site (pTH Ser40) (D), and TH in dissected PFC (E) of $Arc^{+/+}$, $Arc^{+/-}$, and $Arc^{-/-}$ littermates (n = 14–20/group for HPLC, n = 5–7/group for western blots). *p < 0.05 versus $Arc^{+/+}$ mice.

(F–K) Densitometric analyses of D2 receptors (F and G) and relative levels of total TH (H), pTH (Ser40) (I), total Akt (J), and pAKT (Thr-308) (K) in dissected striatum of $Arc^{+/+}$, $Arc^{+/-}$, and $Arc^{-/-}$ littermates. For the D2 analysis, striatal samples were loaded 1:20 compared to PFC samples, and for each brain area, the input was 1:10 compared to the pull down. Samples without Streptavidin beads and Synaptophysyn proteins were used as a pulldown control. Data are normalized to transferrin receptor proteins. For total TH and Akt levels, actin was used as loading controls, while for measurement of phospho-protein levels, total protein were used as a loading control. *p < 0.05 and ***p < 0.0005 versus $Arc^{+/+}$. #p < 0.05 versus $Arc^{+/+}$ within the same treatment, $^{\$}$p < 0.05 versus $Arc^{+/+}$. Representative western blots are shown in Figure S7.

reduction in pAkt, in contrast to wild-type mice. Taken together, the D2 expression and Akt data (Figures 4F–4K) suggest that Arc disruption increased postsynaptic dopamine/D2 signaling in the striatum. Notably, mice with selective overexpression of post-synaptic D2 in the striatum show molecular and behavioral alterations (Kellendonk et al., 2006) overlapping with Arc knockout

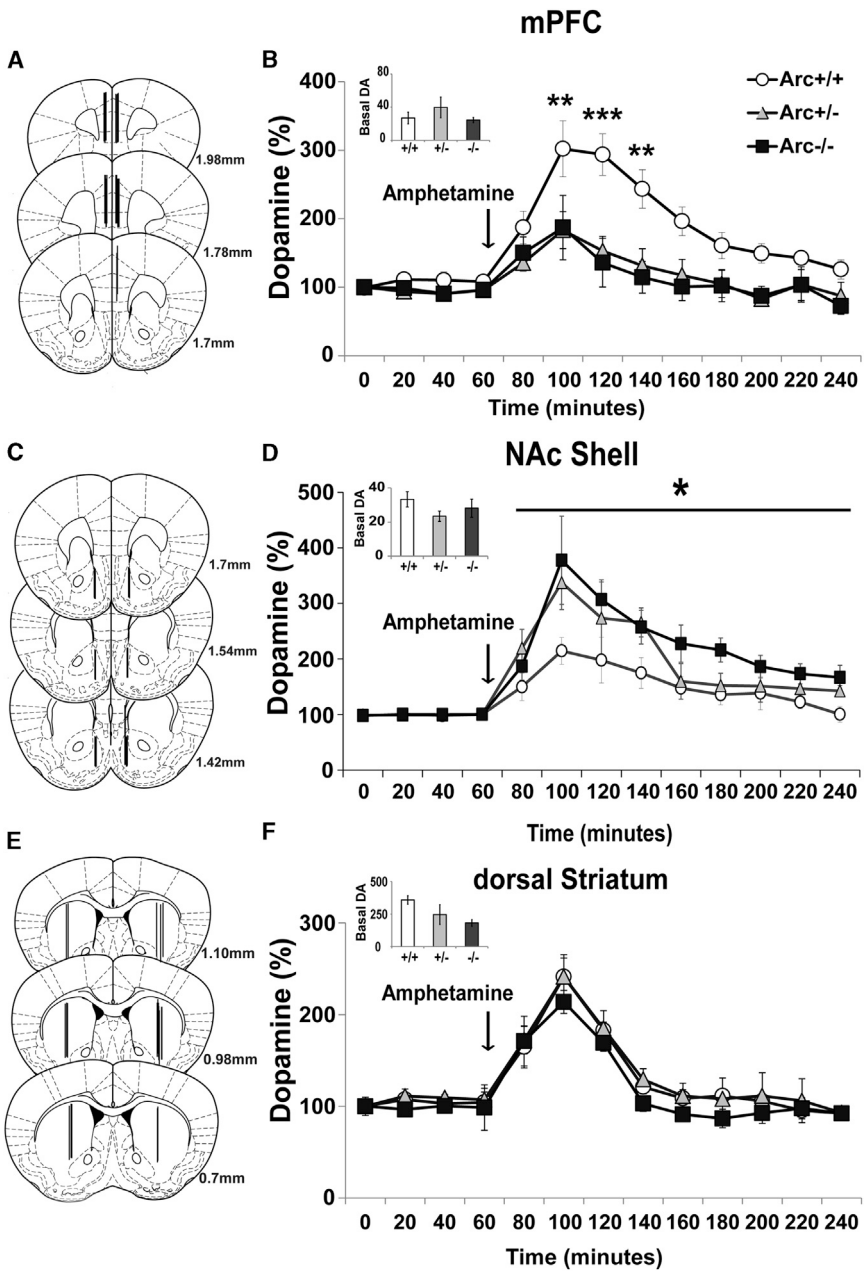

Figure 5. Opposite Regulation of Amphetamine-Evoked Dopamine Release in the mPFC and Nucleus Accumbens Shell in Freely Moving Arc Knockout Mice

(A, C, and E) Coronal sections showing the placements in mPFC (A), ventral striatum nucleus accumbens (NAc) shell portion (C), and dorsal striatum (E) of the in vivo microdialysis probes.

(B, D, and F) Effect of amphetamine (1.5 mg/kg i.p.) on the dopamine (DA) dialysate levels in mPFC (B), nucleus accumbens shell (D), and dorsal striatum (F) in freely moving $Arc^{+/+}$, $Arc^{+/-}$, and $Arc^{-/-}$ littermates.

Data are expressed as the percentage of change in dopamine extracellular levels from the basal values. Inset quadrants show basal values of extracellular dopamine expressed as fmol/20 μl sample. n = 6–8/group. ***p < 0.0005 and **p < 0.005 versus $Arc^{+/-}$ and $Arc^{-/-}$ within the same time point.

mice, further supporting Arc as a converging point of schizophrenia genetics.

Arc Genetic Disruption Dampened Amphetamine-Induced Dopamine Release in the mPFC but Intensified It in the Nucleus Accumbens Shell

To directly and more precisely characterize the active processes of dopamine release, we measured its dynamics in the extrasynaptic space by in vivo microdialysis in freely moving $Arc^{+/+}$, $Arc^{+/-}$, and $Arc^{-/-}$ littermates.

Notably, the same amphetamine challenge to which Arc knockout mice were supersensitive revealed a genotype effect on dopamine release in the mPFC ($F_{2,20}$ = 6.1, p < 0.009; Figures 5A and 5B), in the ventral striatum at the level of nucleus accumbens shell ($F_{2,11}$ = 4.31, p < 0.05; Figures 5C and 5D), but not in the dorsal striatum ($F_{2,17}$ = 0.87, p = 0.44; Figures 5E and 5F). In particular, compared to wild-type mice, amphetamine-dependent dopamine release in Arc knockout mice was reduced in the mPFC (p < 0.02; Figure 5B) but increased in the nucleus accumbens shell (p < 0.05; Figure 5D). We also replicated in these same mice the Arc-dependent locomotor supersensitivity to amphetamine (Figure S5). Consistently, previous studies indicated that pharmacological inhibition of PFC dopaminergic input leads to supersensitivity in amphetamine-induced locomotion (Tzschentke, 2001), and amphetamine-induced locomotion is preferentially related to an increase in dopamine neurotransmission in the nucleus accumbens shell more than to dorsal striatum (Ikemoto, 2002; Heidbreder and Feldon, 1998). Together with the ex vivo measurements (Figure 4), these findings indicate that Arc genetic disruption results in a hypoactive PFC and a hyperactive striatal dopaminergic system. Importantly, these data parallel similar findings in patients with schizophrenia (Slifstein et al., 2015; Howes and Kapur, 2009).

Arc Genetic Disruption Reduced VTA-Induced Cortical Activity in the PFC

We next examined whether Arc PFC deficit may be associated with changes in frontal cortical activity. Electrophysiological studies have shown that dopaminergic input plays an important

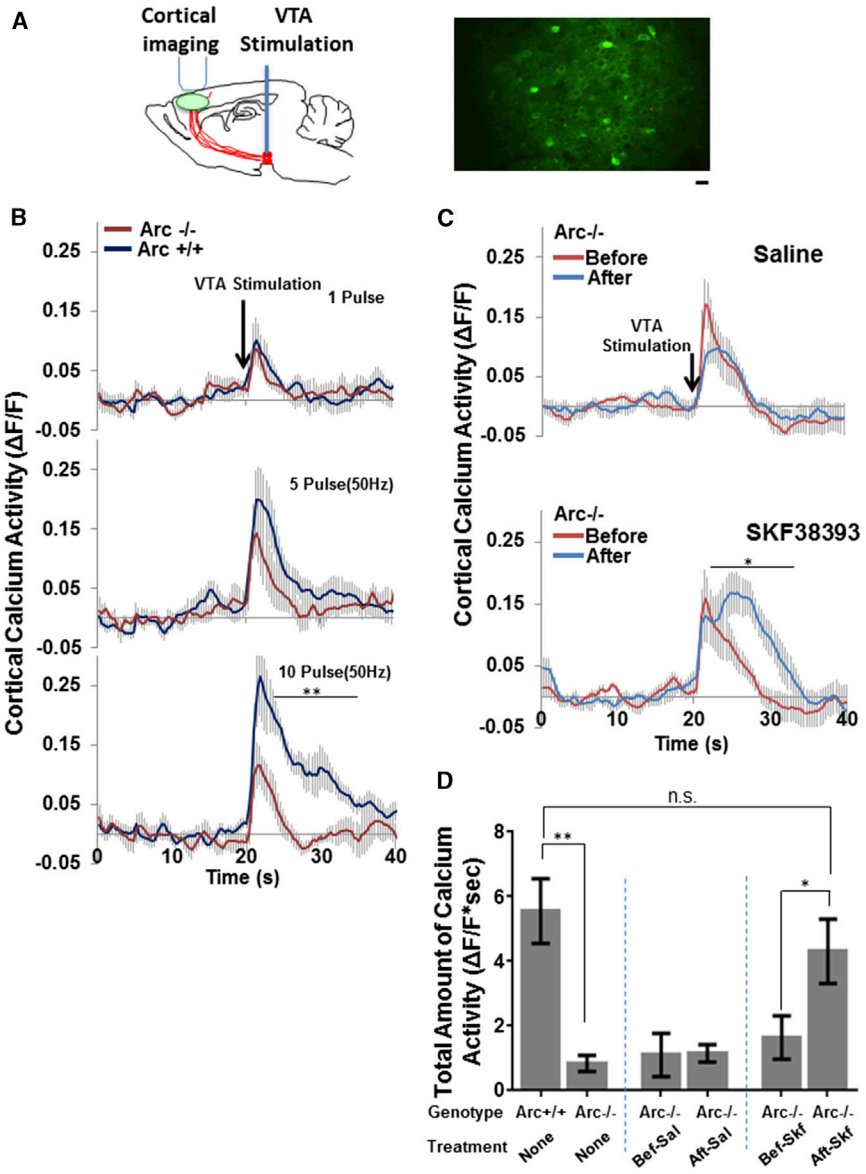

Figure 6. VTA-Induced PFC Activation Is Reduced in Arc Knockout Mice, and This Reduction Is Recovered by Pharmacological Activation of D1 Receptors

(A) Diagram showing the experimental setup for imaging VTA-stimulation evoked frontal cortical response (left). An AAV virus encoding a synapsin-promoter-driven calcium reporter GCaMP6 was locally injected into the PFC. GCaMP6 fluorescence was imaged in the superficial layers of the PFC by two-photon microscopy (right). Scale bar, 15 μm.

(B) Time courses of cortical calcium signals in response to VTA stimulation in $Arc^{+/+}$ and $Arc^{-/-}$ mice. One, five, or ten pulses of electrical stimuli (50 Hz) were delivered at 20 s after the start of imaging. The cortical calcium activity at each time point is represented by the change in image fluorescence relative to the baseline image fluorescence (ΔF/F). n = 6/group, **p < 0.001 versus $Arc^{+/+}$.

(C) Time courses of VTA-stimulation evoked cortical responses in $Arc^{-/-}$ mice before and after saline or D1 agonist SKF38393 injection (10 mg/kg, i.p.). Ten pulses of electrical stimuli (50 Hz) were delivered at 20 s after the start of imaging in each session. The frontal cortical activation was significantly increased after SKF38393 injection (*p < 0.02). n = 6/group.

(D) Total amount of calcium activity in the PFC following ten-pulse (50-Hz) VTA stimulation (sum of 20–35 s) showing significant reduction in $Arc^{-/-}$ compared to $Arc^{+/+}$ mice (**p < 0.006) and significant increase in $Arc^{-/-}$ mice after SKF compared to before SKF (*p < 0.02). No significant difference between $Arc^{+/+}$ and Skf-injected $Arc^{-/-}$ animals (p = 0.40). n = 6/group.

role in regulating the activity of frontal cortical neurons (Robbins and Arnsten, 2009). For example, burst electrical stimulation of dopamine neurons in midbrain ventral tegmental area (VTA) evokes sustained neural activity in the frontal cortex (Lavin et al., 2005; Mastwal et al., 2014).

To efficiently sample the activity of neurons in the PFC and compare cortical activity between Arc wild-type and knockout mice, we adopted an in vivo optical imaging approach. We expressed the genetically encoded calcium sensor GCaMP6 in frontal cortical neurons through a locally injected adeno-associated virus (AAV) vector, and applied in vivo two-photon microscopy to image cortical calcium responses to VTA electrical stimulation (Figure 6A). While calcium activity cannot be equated with dopamine activation, we conducted experiments to test whether reduced dopamine release in the PFC of Arc knockout mice would lead to reduction in mesofrontal circuit activation.

In wild-type mice, we observed a robust activation of the PFC, as represented by the increase of calcium activity, in response to burst stimulation of VTA (Figure 6B). However, in Arc knockout animals, the activation of PFC in response to VTA stimulation was reduced compared to $Arc^{+/+}$ animals (Figure 6B). This difference in activation becomes more prominent with increasing strength of VTA stimulation ($F_{1,10}$ = 20.94, p = 0.001). Thus, our results show that the reduction of evoked cortical dopamine levels in Arc knockout mice is associated with reduced cortical response to VTA stimulation.

We next tested whether the impaired prefrontal response to VTA stimulation in Arc knock out mice could be rescued by pharmacologically enhancing dopaminergic signaling. Dopamine D1 receptors have been suggested to mediate the sustained neural activity of the frontal cortex in response to VTA activation (Lewis and O'Donnell, 2000). We confirmed that VTA evoked calcium responses in the PFC of wild-type mice were reduced by dopamine/D1 antagonist (Figure S6). Moreover, we administered D1 agonist SKF38393 (10 mg/kg, intraperitoneally [i.p.]) to Arc knockout mice and found that VTA-evoked frontal cortical

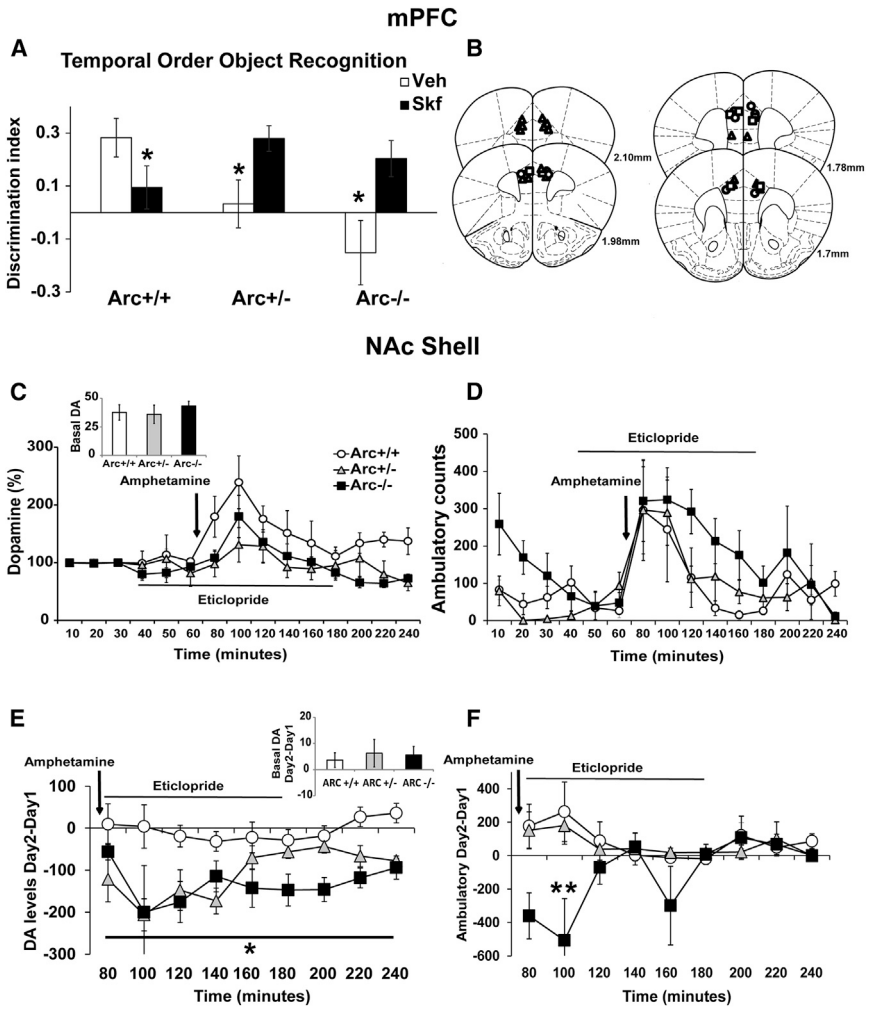

Figure 7. Pharmacological D1 Stimulation in mPFC and D2 Inhibition in the Nucleus Accumbens Shell Rescued Arc-Dependent Cognitive and Dopamine/Locomotor Deficits, Respectively

(A) Discrimination ratio displayed by $Arc^{+/+}$, $Arc^{+/-}$, and Ar $^{-/-}$ littermates treated 15 min before the retrieval phase of the temporal order object recognition task with vehicle or the dopamine D1 agonist SKF38393 (0.06 μg in 0.3μl), bilaterally microinjected in the mPFC (n = 5–15/group). *Groups showing no discrimination.

(B) Coronal sections showing the injection sites in mPFC for $Arc^{+/+}$ (circles), $Arc^{+/-}$ (triangles), and $Arc^{-/-}$ (squares).

(C) Dopamine extracellular levels measured by in vivo microdialysis in the nucleus accumbens shell of freely moving $Arc^{+/+}$, $Arc^{+/-}$, and Arc $^{-/-}$ littermates in basal conditions, following the infusion of the D2-like antagonist eticlopride (1 μM) with reverse dialysis (30 min alone, and 2 hr after the i.p. injection of amphetamine 1.5 mg/kg) and during the 1-hr washout period. Data are expressed as the percentage of change in dopamine extracellular levels from the basal values. Basal values of extracellular dopamine (expressed as fmol/20-μl sample) are shown in the inset quadrants. n = 5–6/group.

(D) Ambulatory distance displayed by these same $Arc^{+/+}$, $Arc^{+/-}$, and $Arc^{-/-}$ mice while going through the different phases of the in vivo microdialysis experiment.

(E and F) Differences in dopamine levels (E), ambulatory activity (in beam crossing) (F), and basal dopamine levels (inset) between the day of eticlopride infusion and the day without eticlopride in $Arc^{+/+}$, $Arc^{+/-}$, and Arc $^{-/-}$ mice. **p < 0.005 and *p < 0.05 versus $Arc^{+/+}$.

activation was significantly increased in contrast to saline control ($p < 0.027$, $F_{1,5} = 9.63$; Figure 6C). The total amount of calcium activity in the frontal cortex following ten-pulse (50 Hz) VTA stimulation was not different between $Arc^{+/+}$ and SKF38393-injected $Arc^{-/-}$ animals (p = 0.397; Figure 6D), indicating that the deficit in $Arc^{-/-}$ mice is rescued by D1 agonist treatment. Taken together, these data support a deficiency of dopaminergic modulation in the PFC of Arc knockout mice and suggest that enhancing D1 signaling can rescue this circuit deficit.

D1 Stimulation in mPFC and D2 Inhibition in the Ventral Striatum Reverses Cognitive and Psychomotor Deficits, Respectively, in Arc Knockout Mice

The suggested dopaminergic imbalance in schizophrenia seems to involve a hypostimulation of D1 receptors in the PFC, as a possible mediator of negative symptoms and cognitive impairment, and a hyperstimulation of D2 receptors in striatal regions, as a possible mediator of positive symptoms (Slifstein et al., 2015; Tzschentke, 2001; Winterer and Weinberger, 2004). Thus, guided by our first findings (Figures 3, 4, 5, and 6), we directly tested this hypothesis in Arc knockout mice.

To rescue putatively PFC-dependent cognitive impairment in Arc mutant mice, we applied dopamine D1 agonist SKF38393 in the mPFC during the temporal order object recognition task. A genotype-by-treatment interaction effect was evident ($F_{2,47} = 3.87$, p = 0.02). In agreement with the in vivo two-photon optical imaging data (Figure 6), pre-test microinjection of the dopamine D1 agonist in the mPFC rescued the cognitive deficits found in $Arc^{+/-}$ mice (t = 5.36; df = 6; p = 0.002; Figure 7A) and partially in $Arc^{-/-}$ mice (t = 2.57; df = 4; p = 0.08; Figure 7A). In contrast, SKF38393 in the mPFC disrupted temporal order object recognition in wild-type mice (t = 1.08; df = 6; p = 0.32; Figure 7A). In mPFC vehicle-treated mice, we replicated the inabilities of $Arc^{+/-}$ mice (t = 0.35; df = 14; p = 0.73) and $Arc^{-/-}$ (t = −1.14; df = 5; p = 0.31) to distinguish between the object presented less recently and the one presented more recently (Figure 7A). These data are consistent with the inverted U-shaped relationship between cortical dopamine and cognitive performance (Papaleo et al., 2014; Vijayraghavan et al., 2007) and suggest that Arc genetic disruption generated dopamine/D1-mediated PFC deficiency and related cognitive deficits.

To rescue the psychomotor hyperactivity in Arc mutant mice, we applied D2-like antagonist eticlopride in the nucleus accumbens shell. The local infusion of eticlopride abolished the amphetamine-supersensitivity of Arc knockout mice, in terms of both dopamine release ($F_{2,55}$ = 1.70, p = 0.23, Figure 7C) and locomotor responses ($F_{2,55}$ = 1.52, p = 0.26, Figure 7D).

These data demonstrate that Arc knockout mice are more sensitive to a D2 antagonist. Compared to the phenotypes of these mice in the absence of eticlopride treatment, the D2 antagonism did not cause any significant change in $^{+/+}$ mice, but consistently reduced dopamine release ($F_{2,55}$ = 8.17, p = 0.007; Figure 7E) and corresponding locomotor activation ($F_{2,55}$ = 5.56, p = 0.02; Figure 7F) in Arc$^{+/-}$ and Arc $^{-/-}$ mice. Previous studies have indicated that elevated dopamine transmission in nucleus accumbens leads to hyperlocomotion (Ikemoto, 2002) and D2 antagonism in nucleus accumbens reduces psychostimulant-induced locomotion in a dose-dependent manner (Baker et al., 1996; Chausmer and Ettenberg, 1999; van den Boss et al., 1988). The stronger effect of eticlopride on amphetamine-induced locomotor activity in Arc mutant mice compared to Arc$^{+/+}$ mice is consistent with the elevated surface expression of D2 receptors in Arc mutant mice (Figure 4F). Taken together, these data suggest that the supersensitivity to amphetamine found in Arc knockout mice is mediated by altered D2 signaling in the striatum.

DISCUSSION

The data reported here show that genetic mutations resulting in loss or reduction in Arc function cause behavioral features spanning multiple domains of rodent correlates of cognitive, negative, and positive schizophrenia-like symptoms. Furthermore, our data implicate a previously unexplored role of Arc as a modulator of the brain dopaminergic system. The observed changes were consistent with a notion of schizophrenia pathophysiology that includes a hypodopamine/D1 functioning in the PFC but a hyperdopamine/D2 system in striatal regions. These findings provide initial biological evidence that genetic variations resulting in impaired Arc function contribute to pathophysiologic correlates of schizophrenia.

Arc knockout mice recapitulated many behavioral abnormalities considered rodent correlates of schizophrenia-like symptoms. In particular, Arc knockout deficits in PPI are analogous to PPI deficits found in patients with schizophrenia (Swerdlow et al., 2008). Similarly, the deficits in Arc knockout mice in sociability and social cognition are potentially mouse correlates of low social reciprocity and deficits in social cognition found in schizophrenia (Millan et al., 2014). Furthermore, Arc knockouts impairments in the temporal order and spatial object recognition tasks, with a preserved ability to recall and recognize objects in the novel object recognition task, is reminiscent to cognitive deficits found in schizophrenia. Indeed, patients with schizophrenia show analogous impairments in temporal and spatial memory tasks, while they are able to recall and recognize target items (Dreher et al., 2001; Schwartz et al., 1991; Rizzo et al., 1996; Folley et al., 2010). Finally, Arc knockout mice's slight hyperactivity in a novel environment and amphetamine supersensitivity might parallel psychotic agitation and amphetamine's ability to be psychogenic in healthy subjects and to exacerbates psychotic experiences in patients with schizophrenia (van den Buuse, 2010).

Earlier studies of Arc knockout mice have emphasized the deficits in long-term memory tasks such as the Morris water maze, auditory fear conditioning, and taste aversion, whereas the general performance and short-term memory of the knockout mice in these tasks were mostly normal (Plath et al., 2006). In this study, all the behavioral tasks were performed without prior training and do not require formation of long-term episodic memory. The deficits in these tasks therefore may reflect a pre-existing deficit in the steady-state function of the underlying brain circuits rather than the consolidation of new memories. Notably, the behavioral tasks reported in earlier studies mainly rely on the visual cortex, auditory cortex, taste cortex, hippocampus, or amygdala (Plath et al., 2006). By contrast, the tasks used in the current study involve PFC and also the dopaminergic system (Barker et al., 2007; Hanks et al., 2013; Lacroix et al., 2000; Tzschentke, 2001). These findings raise the possibility that the different susceptibility of behavioral tasks to Arc genetic disruption may reflect differences in the underlying brain circuits. Furthermore, in the cortex and hippocampus, Arc is only expressed in the CamKII-positive excitatory neurons (Ren et al., 2014; Vazdarjanova et al., 2006). Thus, Arc genetic disruption will not directly affect interneuron function in a cell-autonomous manner. However, as Arc genetic disruption leads to changes of both glutamate receptors in excitatory neurons and dopamine release, cortical inhibitory neuron activities will be affected through network interactions with dopamine and glutamatergic neurons. Some of the behaviors we assessed may indeed reflect this network level imbalance, as what has been proposed for the pathophysiology of schizophrenia.

More surprisingly, following Arc disruption, we identified contrasting deficits in the mesocortical and mesostriatal dopaminergic pathways. The Arc-dependent effects found in the PFC and in the ventral striatum, but not in the dorsal striatum, might be related to the fact that the source of dopamine in both the PFC and nucleus accumbens is the VTA, while in the dorsal striatum, it is the substantia nigra (Beckstead et al., 1979). The opposite effects of Arc genetics in amphetamine-induced dopamine release in PFC versus nucleus accumbens might originate from a circuit mechanism. Previous pharmacological studies have suggested that dopamine transmission in the PFC and nucleus accumbens are inversely coupled (Scornaiencki et al., 2009; Simpson et al., 2010; Tzschentke, 2001) and direct anatomical connections with opposite feedbacks exist among the PFC, VTA, and nucleus accumbens to mediate these effects (Carr and Sesack, 2000; Ferenczi et al., 2016). Remarkably, the Arc-dependent dopaminergic alterations resemble abnormalities considered key features in patients with schizophrenia (i.e., hypoactive D1 mesocortical and hyperactive D2 mesostriatal pathways) (Simpson et al., 2010; Slifstein et al., 2015; Winterer and Weinberger, 2004). Our findings suggest that Arc function is important for establishing the proper activity balance between mesocortical and mesostriatal dopaminergic circuits. Moreover, our studies further suggest that these circuit deficits can arise together from the same genetic background, which has been difficult to determine in humans due to different dopamine molecular imaging methods in striatal and cortical regions and genetic diversity (Weinstein et al., 2016).

Previous studies have reported that PFC dopaminergic inputs show protracted postnatal maturation through adolescence and are susceptible to activity-dependent modification during this period (Kalsbeek et al., 1988; Lewis and O'Donnell, 2000; Mastwal et al., 2014). Recurrent network activity in frontal-striatal loops can also affect striatal circuit maturation (Kozorovitskiy et al., 2012). As a neural activity-induced plasticity-related protein abundantly expressed in cortical excitatory and striatal GABAergic projection neurons (but not detected in midbrain dopamine neurons) (Shepherd and Bear, 2011), Arc may regulate activity-dependent maturation of the VTA-PFC-striatal circuits during postnatal development. Considering the well-known role of Arc in modulating glutamate receptors (Jakkamsetti et al., 2013; Ren et al., 2014; Shepherd and Bear, 2011), our findings raise the possibility that Arc-dependent changes in glutamatergic signaling might be the effector of the dopamine system changes. Since alterations in mesocortical and mesostriatal dopaminergic pathways are often inversely coupled to each other (Clarke et al., 2014; Simpson et al., 2010; Tzschentke, 2001; Saunders et al., 1998), future studies are needed to differentiate the cell-autonomous and network effects of Arc genetic disruption.

In conclusion, Arc knockout mice recapitulate important features of rodent correlates of schizophrenia-related functional dimensions. Moreover, we describe a previously unexplored role of Arc in the modulation of dopaminergic neurotransmission, again consistent with schizophrenia-relevant endophenotypes. Together with the already well-known role of Arc in the modulation of the glutamatergic signaling (Ren et al., 2014; Jakkamsetti et al., 2013; Shepherd and Bear, 2011), the present data strongly implicate Arc as a key regulator of biological features associated with schizophrenia that involve PFC-striatal dopaminergic circuitry. Indeed, Arc may provide not only a converging point for genes encoding for glutamatergic post-synaptic proteins (Fromer et al., 2014) but also a crucial link between the dopaminergic and glutamatergic fronto-striatal systems.

EXPERIMENTAL PROCEDURES

Subjects

All procedures were approved by the Italian Ministry of Health (permit n. 230/2009-B) and local animal use committee and were in accordance with the *Guide for the Care and Use of Laboratory Animals* of the NIH and the European Community directives. Arc knockout mice (Wang et al., 2006) of both sexes (3–7 months old) were used. Because no sex-dependent differences were found in any parameter measured, male and female mice were pooled together. Arc null mutant (−/−) mice and their heterozygous (+/−) and wild-type (+/+) littermates were bred in-house by +/− mating. Mice were housed two to four per cage, in a climate-controlled animal facility (21 ± 2°C), and maintained on a 12-hr light/dark cycle (7 a.m. to 7 p.m.) with ad libitum access to food and water. Testing was conducted during the light phase. Different cohorts of mice were used for each task. Experimenters were blind to the genotype during testing.

Behavioral Experiments

Startle, PPI, and general health parameters were measured as previously described (Papaleo et al., 2008, 2012).

Locomotor activity, temporal order, spatial and novel object recognition tasks were performed as previously described (Huang et al., 2014; Papaleo et al., 2008; Barker et al., 2007).

Sociability and preference for social novelty task as previously described (Papaleo et al., 2011).

TOR with Skf Treatment

Mice were bilaterally implanted with 7-mm-long stainless steel guide cannulae (Unimed) under an isoflurane/oxygen mixture anesthesia. The mPFC stereotax coordinates used were antero posterior (AP) +1.95 mm, lateral (L) ±0.5 mm, and dorso ventral (DV) −1.5 mm, relative to the bregma (Franklin and Paxinos, 1997). Guide cannulae were secured in place with dental cement. Mice were then allowed to recover for 7 days after surgery. The Skf38393 D1 agonist (Sigma-Aldrich) was dissolved in 0.9% saline solution and then injected in a dose of 0.06 μg/0.3 μl/side. An injection needle was inserted into the guide cannula protruding 1 mm below (final DV, −2.5 mm), which was connected by plastic tubing to a 10-μl Hamilton syringe; the process took 1 min, with an additional 1 min to allow diffusion. The drug injection was performed 15 min before the temporal order object recognition task (TOR) testing phase. At the completion of the experiments, mice were sacrificed, and the brains were removed and fixed in a 4% paraformaldehyde solution. Cannula placements were determined by examining serial 80-μm coronal sections stained with cresyl violet.

HPLC

For dopamine determination by HPLC, PFC and striatum were rapidly dissected from naive $Arc^{-/-}$, $Arc^{+/-}$, and $Arc^{+/+}$ littermates, immediately frozen in dry ice, and then stored at −80°C. Samples were then lysed in 0.1 M perchloric acid, then sonicated and spun in a microcentrifuge at 10,000 × *g* for 10 min. The supernatant was transferred in ultra-free microcentrifuge tubes (Millipore, catalog UFC30GV0S) and spun for 2 min. Samples (11 μl) were injected into the HPLC and measurements of dopamine and metabolites were made with an electrochemical detection system (ALEXYS LC-EC; Antec Leyden BV) equipped with a reverse-phase column (3-μm particles, ALB-215 C18, 1 × 150 mm; Antec Leyden BV) at a flow rate of 200 μl/min and electrochemically detected by a 0.7-mm glass carbon electrode (VT-03; Antec Leyden BV). The mobile phase contained 50 mM H_3PO_4, 50 mM citric acid, 8 mM KCl, 0.1 mM EDTA, 400 mg/l octanesulfonic acid sodium salt, and 3% (v/v) methanol (pH 75).

In Vivo Microdialysis

Vertical concentric dialysis probes, with a dialyzing portion of 2 mm for dorsal striatum and mPFC and 1 mm for nucleus accumbens shell, were prepared using AN69 fibers (Hospal Dasco), as previously described (De Luca et al., 2014). More details can be found in Supplemental Experimental Procedures.

Electrical Stimulation Coupled with In Vivo Two-Photon Imaging

Viral Injection

Using a glass micropipette connected to a 10-μl Hamilton syringe and a syringe pump, AAV1.Syn.Flex.GCaMP6s.WPRE.SV40 (1 μl) was injected into a single site in the PFC (from bregma: AP +2.0, medio lateral [ML] +0.5, and DV −0.3 mm) of $Arc^{+/+}$ and Arc $^{-/-}$ littermates. Animals were allowed to recover for ~1–2 weeks.

Electrode Implantation and Cranial Window

A bipolar stimulation electrode was lowered into the VTA (from bregma: AP −3.2 mm, ML 0.5 mm, and DV 4.5 mm) and glued in place in animals anesthetized with isoflurane (~1.5%). A cranial window was opened above the AAV-GCaMP6 injected region in the frontal cortex (from bregma: AP 1.0–3.0 mm, ML 0.3–1.3 mm, covering the M2/FrA region). The cranial window was filled with silicon gel, covered with a glass coverslip, and sealed with dental cement. A head plate was glued on the skull for fixation during imaging. The animals were then taken off the anesthesia and allowed to recover for ~1 hr before imaging.

VTA Stimulation and GCaMP6 Imaging

VTA was stimulated with electrical pulses (0.7 mA, 0.2 ms per pulse, and one, five, or ten pulses at 50 Hz per stimulus train). A two-photon microscope (FV1000, Olympus) was used to image the brain under the cranial window (excitation laser: 900 nm) using a 20× water-immersion lens (numerical aperture [NA] 0.95). Time series images lasting ~40 s (115 frames at 0.351 s/frame) were taken for each stimulus train, with the VTA stimulus delivered at 20 s after the start of imaging. For saline, SKF38393 (10 mg/kg, i.p.) and SCH23390 (1 mg/kg, i.p.) injection experiments, GCaMP6 response to the ten-pulse (50 Hz) VTA stimulation was imaged before and 30 min after the injection.

Image Analysis

Images were analyzed using NIH ImageJ. The mean pixel intensity in each image frame of the time series was calculated as Ft. Baseline fluorescence (F0) was defined as the average of the fluorescent signals (Ft) in the first 15 s of the time series. Changes in calcium signals (ΔF/F) are calculated as (Ft − F0)/F0.

Slice Surface Biotinylation, Antibodies, and Western Blot Analyses

Details are reported in the Supplemental Experimental Procedures.

Statistical Analysis

Results are expressed as mean ± SEM throughout. One- or two-way ANOVAs were applied to compare the group means. Newman-Keul's post hoc test was used for making comparisons between groups when the overall ANOVA showed statistical significant differences for the main factors. The accepted value for significance was $p \leq 0.05$. Statistical analyses were performed using Statistica 11 (StatSoft).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Results, Supplemental Experimental Procedures, seven figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.07.044.

AUTHOR CONTRIBUTIONS

Conceptualization, F.M., D.R.W., K.H.W., and F.P.; Methodology, F.M., M.M., S.M., K.H.W., and F.P.; Investigation, F.M., M.M., S.M., D.S., M.E., R.M., M.D.L., and F.P.; Resource, K.H.W. and F.P.; Writing, F.M., D.R.W., K.H.W., and F.P.; Visualization and Analysis, F.M., S.M., and F.P.; Supervision, K.H.W. and F.P.; Funding Acquisition, D.R.W., K.H.W., and F.P.

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