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Insulin Regulates Astrocyte Gliotransmission and Modulates Behavior

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Abstract

Complications of diabetes affect tissues throughout body, including central nervous system. Epidemiological studies show that diabetic patients have increased risk of depression, anxiety, age-related cognitive decline and Alzheimer’s disease. Mice lacking insulin receptor in brain or on hypothalamic neurons display an array of metabolic abnormalities, however, the role of insulin action on astrocytes and neurobehaviors remains less well-studied. Here, we demonstrate that astrocytes are a direct insulin target in the brain and that knockout of IR on astrocytes causes increased anxiety and depressive-like behaviors in mice. This can be reproduced in part by deletion of IR on astrocytes in the nucleus accumbens. At a molecular level, loss of insulin signaling in astrocytes impaired tyrosine phosphorylation of Munc18c. This led to decreased exocytosis of ATP from astrocytes, resulting in decreased purinergic signaling on dopaminergic neurons. These reductions contributed to decreased dopamine release from brain slices. Central administration of ATP analogues could reverse depressive-like behaviors in mice with astrocyte IR knockout. Thus, astrocytic insulin signaling plays an important role in dopaminergic signaling, providing a potential mechanism by which astrocytic insulin action may contribute to increased rates of depression in people with diabetes, obesity and other insulin resistant states.
Introduction

Over the past decade it has become clear that the brain is an insulin sensitive organ. Insulin receptors are widely distributed in the brain (1). Intracerebroventricular insulin infusion reduces food intake and body weight from rodents to primates (2, 3). Intranasal insulin administration in healthy men has also been shown to decrease appetite and reduced body fat (4, 5). Conversely, IR deletion in the whole brain (NIRKO) leads to an array of metabolic abnormalities in mice, including increased food intake, obesity, altered response to hypoglycemia, and hypothalamic hypogonadism (6, 7). In addition, insulin action in the brain, especially in the hypothalamus, has also been shown to mediate systemic energy homeostasis through regulating peripheral metabolic tissue activities, including suppressing hepatic glucose output (8) and lipolysis in white adipose tissue (9), and increasing thermogenesis in brown adipose tissue (10).

Although less well-studied, insulin action on brain may also modulate neurobehaviors including cognition and mood. Mice with knockout of insulin receptor in whole brain have been shown to have increased signs of depression and anxiety, especially at older ages (11). Studies also suggest that intranasal insulin administration can improve memory and mood in healthy men and women (4, 12). Conversely, individuals with diabetes and obesity have been shown to have higher rates of depression, anxiety, cognitive decline and dementia (13-15). Many of these disorders are associated with impaired insulin signaling in the brain, even in non-diabetic individuals (16-18). While most of these effects are thought to occur through insulin action on neurons (19-21), recently it has been shown that insulin may also play a role on astrocytes and glial cells, affecting metabolism in both the cells themselves (22, 23) and the body as a whole (24).

Astrocytes, along with microglia and oligodendrocytes, are important for homeostasis of the brain microenvironment (25, 26). Astrocytes are responsible for the
uptake of glucose and other nutrients, which will ultimately be used by energy consuming neurons, and are part of the blood brain barrier. Astrocytes are also responsible for uptake and degradation of excessive neurotransmitters and neuromodulators, an activity that is essential for the spatial and temporal control of neuronal activation and for avoidance of excitotoxicity. Recent studies have demonstrated that astrocytes respond to multiple stimuli to secrete neurotransmitters, like glutamate, D-serine and ATP, in an intracellular Ca$^{2+}$-dependent manner, thereby actively modulating synaptic strength and neuronal activity (27-29).

In the present study, we have investigated the role of insulin receptor in the astrocytes on both whole-body energy homeostasis and neurobehaviors. We find that loss of insulin receptor in the astrocytes leads to moderate glucose intolerance in young males. More importantly, mice lacking IR in astrocytes display increased depressive-like behavior, which is accompanied by impaired dopamine release from brain slices, particularly in the nucleus accumbens. Mechanistically, astrocytes respond to hormonal stimulation, i.e. insulin, to induce tyrosine phosphorylation of Munc18c, which activates exocytosis of ATP from astrocytes. This in turn modulates dopaminergic neuronal activity and dopamine release, which may contribute to the mood modulation in mice. Thus, insulin signaling plays an important role for astrocyte-derived purinergic signaling on dopaminergic system and behavior modulation.
Results

Targeting reduction of insulin receptors in astrocytes of intact mice.

To determine the role of insulin receptor signaling in astrocytes on metabolic and neurobehavioral control, we generated mice in which the IR gene in astrocytes was inactivated by crossing mice expressing GFAP-promoter-driven Cre with homozygous IR-flox (IR^{fl}) mice to create GIRKO mice (Supplemental Figure 1A). The efficiency and specificity of GFAP-Cre-mediated recombination was confirmed using mTmG reporter mice, which showed recombined GFP^+ cells in the brain, i.e. cells that had unique floxed allele recombination (30), also expressed the astrocyte-specific marker S100β (Supplemental Figure 1, B and C). Furthermore, when a GFAP-GFP reporter transgene was introduced into GIRKO and control IR^{fl} mice (Supplemental Figure 1D) and the GFP^+ cells isolated from the brain by digestion and FACS sorting, there was a ~75% reduction of IR mRNA in GFP^+ cells from GIRKO mice compared to those from IR^{fl} mice (Figure 1A). At the tissue level, both mRNA and protein levels of IR were also reduced by 40% in dissected nucleus accumbens (Supplemental Figure 1, E-G), consistent with the relative abundance of astrocytes in this region. By contrast, there was no change in the expression of the insulin-like growth factor-1 receptor (IGF1R), and GIRKO mice showed similar percentage of GFP^+ cells over the total live cells as in the control (IR^{fl}/GFP: 7.538 ± 0.595% vs GIRKO/GFP: 7.050 ± 1.104%, Figure 1B), indicating no toxicity by deletion of IR in astrocytes.

Metabolic assessment of both male and female GIRKO mice at 3 months of age revealed normal body weight, body composition, fed blood glucose, plasma insulin levels, oxygen consumption rate (VO_{2}), and respiratory exchange ratio (RER) (Supplemental Figures 2 and 3). Three-month-old male GIRKO mice also responded normally to exogenous insulin with a fall in blood glucose (Supplemental Figure 2F), but, consistent with a previous study (24), had slightly impaired i.p. glucose tolerance (Figure
At one year of age, both male and female mice showed normal body weight, fasting glucose level, glucose tolerance and insulin tolerance at 1 year of age (Supplemental Figure 4).

*Mice with astrocytic IR deletion exhibit anxiety and depressive-like behaviors.*

We have previously shown that insulin receptor deletion in whole-brain of mice leads to an age-dependent increase in anxiety and depressive-like behaviors (11). This phenotype was even stronger in mice with IR knockout in astrocytes only and was marked by multiple behavioral abnormalities. Thus, female GIRKO mice showed anxiety-like behaviors as early as 4 months-of-age with a 60% decrease in the number of entries into the central zone in the open field test (Figure 1, D and E). This persisted at 1 year of age (Figure 1E) and was not due to defects in locomotion. Thus young GIRKO mice travelled similar distances and at similar speeds as control IR$^{ff}$ littermates in the open field (Supplemental Figure 5, A and B), while 1-year-old GIRKO mice travelled less distances, but same speeds as IR$^{ff}$ littermates (Supplemental Figure 5, C and D).

Likewise, in the novelty-suppressed feeding test, overnight-fasted female GIRKO mice showed a 90% increase in the latency to feed compared to IR$^{ff}$ controls (Figure 1F) consistent with increased anxiety. This occurred despite having similar food intake as controls during the 1 hour after the test (Supplemental Figure 5E) indicating no alterations in hunger.

Another common indicator for altered mood and depression in rodents is anhedonia, which can be assessed using the sucrose preference test. Again, compared to IR$^{ff}$ littermates, which showed a strong preference for sucrose solution over water, female GIRKO mice displayed a significantly decreased preference for sucrose (Figure 1G). Likewise, in the forced swimming test, female GIRKO mice exhibited an ~2-fold increase in time of immobility at both 4-months and 1-year of age (Figure 1, H and I),
indicating increased depressive-like behavior. Importantly, this behavior was completely rescued by pretreatment with the antidepressant imipramine 1 h prior to the test (Figure 1H), indicating that the difference in swimming was not due to defects in motor function in these mice. These differences in anxiety and depression were not driven by differences in the stress response, as indicated by similar increases in serum corticosterone levels in GIRKO mice and IR<sup>ff</sup> littersmates subjected to restraint stress (Figure 1J). Thus, loss of IR in astrocytes resulted in increased anxiety and depressive-like behaviors in both young and aged mice. Consistent with other models showing sexual dimorphism in rodent models of anxiety and depression (31, 32), these behavioral abnormalities were strongest in female GIRKO mice. Four-month-old male GIRKO mice showed mild, non-significant changes in the open field, novelty-suppressed feeding and sucrose preference tests (Supplemental Figure 6, A-D and F), but did exhibit significantly increased immobility time in the forced swimming test (Supplemental Figure 6, E).

*Lack of IR in astrocytes impairs dopamine release in the brain.*

Dopamine signaling has been linked to depression, and many antidepressants target dopamine pathways in the brain (33, 34). Indeed, we previously showed that dopamine signaling is defective in mice with a whole-brain IR knockout (NIRKO mice) (11). The nucleus accumbens (NAc), medial prefrontal cortex (mPFC) and dorsal caudate putamen (CPu) are three major sites that receive dopaminergic projections from ventral tegmental area (VTA) and substantia nigra (SN) in the midbrain and are involved in mood control, reward and movement (reviewed in ref 35). To analyze dopamine signaling, we used carbon fiber amperometry to measure electrically evoked dopamine release in these regions from ex vivo cultured brain slices (36). We found that dopamine release in the NAc, CPu and mPFC was decreased by about 50% in GIRKO mice
compared to IR^{f/f} littermates (Figure 2, A and B). This was due primarily to a decrease in event amplitude (Figure 2C), with no change in clearance of the released dopamine, as estimated by rate of signal decay (Figure 2D). This also occurred with no change in total dopamine content in the brain (Figure 2E) nor change in the expression of tyrosine hydroxylase (TH), the rate-limiting enzyme for dopamine synthesis, in the nucleus accumbens, dorsal caudate putamen, medial prefrontal cortex and VTA/SN of GIRKO mice (Figure 2, F and G, and Supplemental Figure 7). In addition, the overall distribution of dopaminergic fibers and astrocytes in the nucleus accumbens of GIRKO mice was similar to IR^{f/f} littermates (Figure 2, H-J, and Supplemental Figure 8). mRNA expression of exocytotic vesicular monoamine transporters (VMAT1 and VMAT2), and proteins involved in dopamine uptake (DAT, Drds) and degradation (MaoA and MaoB) in the NAc, CPu and mPFC were also comparable between GIRKO and IR^{f/f} littermates (Figure 2G, and Supplemental Figure 7). While dopamine signaling in the brain, particularly in the nigrostriatal pathway, is important for normal motor behaviors (37), both 6-month-old and 18-month-old female GIRKO mice exhibited similar stride length, grip strength and total work on treadmill as control littermates (Supplemental Figure 9).

Impaired dopaminergic signaling in the nucleus accumbens contributes to the depressive-like behavior in mice with astrocytic insulin signaling deficiency.

To confirm that the effect of IR loss on mood behavior and dopamine signaling was on astrocyte function and not astrocyte development, we created mice with an inducible astrocyte-specific IR knockout (iGIRKO) by crossing homozygous IR-flox (IR^{f/f}) mice with mice carrying GFAP-CreERT2 transgene (Figure 3A). Cre-mediated recombination in astrocytes was then induced by a series of five tamoxifen injections at 8 weeks of age, and recombination was confirmed six weeks post-tamoxifen injections using mTmG reporter mice (Figure 3B and Supplemental Figure 10). IR^{f/f} littermates
given the same tamoxifen injection regimen were used as controls. Consistent with the
conservative knockout model above, female iGIRKO mice showed multiple signs of
anxiety and depression, including a 33% reduction in central zone entries in the open
field test (Figure 3C, $P < 0.05$), a significantly decreased preference for sucrose over
water in the sucrose preference test (Figure 3D, $P < 0.05$) and a 42% increase in time of
immobility in the forced swimming test compared with IR$^{f/f}$ littermates (Figure 3E, $P <$
0.05). Importantly, the increased immobility during forced swimming indicative of
depressive-like behavior in iGIRKO mice was rescued by pre-treatment with the specific
and potent dopamine D2/D3 receptor agonist pramipexole (Figure 3E) (38, 39), but not
by the specific serotonin 5-HT$_{1A}$ receptor agonist 8-OH-DPAT (Figure 3F and
Supplemental Figure 11) (40), indicating the importance of defective dopamine release
in the depressive-like behavior in this model.

Over 95% of the neurons in the nucleus accumbens express dopamine receptors
(41). To further assess the downstream effect of impaired dopamine release, we
assessed neuronal activation in the NAc in iGIRKO mice by c-fos immunostaining
following the forced swimming test. In the control IR$^{f/f}$ littermates, the percentage of c-
fos$^+$ neurons (i.e., c-fos$^+$ neurons/total mature NeuN$^+$ neurons) increased by over 6-fold
following forced swimming. This increase in c-fos$^+$ neurons was significantly blunted in
iGIRKO mice, which showed only ~2-fold induction following forced swimming (Figure 3,
G and H), consistent with impaired dopamine input in the iGIRKO mice.

To determine if insulin signaling in astrocytes in either the nucleus accumbens or
medial prefrontal cortex was important in the observed behavioral changes, we also
induced site-specific deletion of insulin receptors in astrocytes by stereotaxic injections
of AAV encoding Cre or GFP under control of the GFAP promoter into NAc or mPFC
(Figure 3I) of IR$^{f/f}$ mice. Interestingly, deletion of astrocytic IR in NAc, but not deletion in
mPFC, caused a 30% increase in time of immobility in forced swimming test, indicating
increased depressive behavior (Figure 3, J and K). However, the astrocytic insulin signaling in NAc appears dispensable for anxiety-like behavior, since the IR^{fl} mice injected with AAV-GFAP-Cre showed normal center zone entries and locomotive activity in the open field test as control groups receiving AAV-GFAP-GFP (Supplemental Figure 12). Thus, loss of insulin signaling in astrocytes, particularly in the nucleus accumbens, results in increased depressive-like behavior in mice, which is associated with decreased dopamine release and rescued by a dopamine receptor agonist.

**Insulin signaling regulates ATP release in astrocytes to modulate dopaminergic neuronal activity.**

To define the molecular mechanisms by which insulin signaling in astrocytes could affect dopamine release from neurons, we developed a primary cell model using astrocytes isolated from newborn IR^{fl} pups which could be infected with an adenovirus encoding Cre:GFP fusion protein to induce gene deletion or adenovirus-GFP as a control. In both cases, the astrocytic nature of these cells was confirmed by high expression of GFAP with no expression of the neuronal marker NeuN (Supplemental Figure 13A). As expected, the IRKO astrocytes showed almost complete loss of IR at both mRNA and protein levels (Figure 4A and Supplemental Figure 13B). This was associated with a small and insignificant increase in the expression of the related IGF-1 receptor (Figure 4A and Supplemental Figure 13B). This was paralleled by a dramatic reduction in insulin-stimulated phosphorylation of IRS-1, Akt, ERK1/2 and GSK3 (Figure 4B). Loss of IR in the primary astrocytes also resulted in a 25-50% decrease in expression in several proteins important in astrocyte function, including GFAP, aquaporin 4 (AQP4), glutamate transporter 1 (GLT-1), aldolase c (Aldoc) and glutamine synthetase (GS), while ApoE expression was increased by ~40% in the IRKO cells (Figure 4C). Expression of other astrocyte-specific proteins like glutamate aspartate
transporter (GLAST), monocarboxylate transporters (MCT-1 and MCT-4) and connexin 43 (Cx43) was not changed upon IR deletion (Figure 4C). Astrocytes can modulate neuronal activity through multiple mechanisms. Recent studies have shown that astrocytes can secrete a series of neurotransmitters, including glutamate, D-serine and ATP, which are able modulate activity of nearby neurons (28, 42, 43). Indeed, ATP released from astrocytes has been suggested to have anxiolytic and antidepressant effects (44). Interestingly, in control astrocytes, insulin stimulation triggered a 2-fold increase in ATP release from the culture astrocytes, and this response was completely lost in IRKO astrocytes (Figure 4D). This occurred with no change in total cellular ATP content (Figure 4E).

To assess the potential importance of ATP release by astrocytes on purinergic receptor signaling and dopamine release by neurons in the nucleus accumbens, we subjected brain slices containing nucleus accumbens to ex vivo electrical stimulation in the presence or absence of two different purinergic ligands: ATP-γ-S, a nonhydrolyzable ATP analog, and the purinergic 2 (P2) receptor agonist 2-Me-S-ATP. Both treatments, and especially 2-Me-SATP, markedly and significantly potentiated dopamine release from NAc (Figure 4, F and G), indicating that ATP acting through the P2Y receptor is able to positively modulate dopamine release in NAc. To confirm the significance of astrocyte-derived purinergic signaling on dopamine release and depressive-like behavior in vivo, both IR\textsuperscript{f/f} and iGIRKO mice were infused with 20 pmol 2-Me-SATP intracerebroventricularly and then subjected to the forced swimming test. Again, consistent with the concept that impaired ATP release by astrocytes in iGIRKO mice was responsible for the depressive-like behaviors, 2-Me-SATP infusion decreased the time of immobility in iGIRKO mice by 50%, while the same procedure in IR\textsuperscript{f/f} littermates had no significant effect (Figure 4H and Supplemental Figure 14).
**Insulin signaling regulates exocytosis via tyrosine phosphorylation of Munc18c in astrocytes.**

Astrocytes and neurons are enriched with two distinct sets of SNARE proteins, which provide a foundation for differential regulation of exocytosis in these two cell types (45). Astrocytes highly express syntaxin-4, SNAP23, Munc18c and VAMP3, and astrocyte-derived ATP has been shown to be dependent on SNARE complex-dependent exocytosis (44). Compared to control cells, IRKO astrocytes showed similar protein and mRNA levels of all of the astrocyte-enriched SNARE proteins, including syntaxin-4, SNAP23, VAMP3 and Munc18c, as well as the vesicular ATP transporter (VNUT) (Figure 5A). In control astrocytes, assembly of the SNARE core complex, as assessed by co-immunoprecipitation of syntaxin-4 and VAMP3 was induced by over 2-fold following insulin stimulation. By contrast, this induction was severely blunted in IRKO astrocytes (Figure 5B), despite comparable levels of intracellular Ca\(^{2+}\) (Supplemental Figure 15A) and similar expression of the three major inositol 1,4,5-trisphosphate receptors, ITPR1,2,3 (Supplemental Figure 15B).

Given the role of insulin to stimulate ATP release from astrocytes, we hypothesized that the reduced ATP release from IRKO astrocytes that resulted in decreased dopaminergic neuron activation and decreased dopamine release was secondary to a loss of insulin regulation of a component of the SNARE complex. Munc18c has been shown to bind to and have an inhibitory role on syntaxin-4 (46, 47). Munc18c can be relieved of this inhibitory effect by phosphorylation at tyrosine 521, which uncovers the docking sites on syntaxin-4 for VAMP3-containing vesicles (48, 49). Consistent with the hypothesis that insulin might regulate this process, in primary control astrocytes, insulin stimulated the tyrosine phosphorylation of transfected Flag-tagged Munc18c by 2-fold, and this tyrosine phosphorylation was completely lost in IRKO astrocytes (Figure 5C). Thus, insulin stimulation of IR in astrocytes results in tyrosine
phosphorylation of Munc18c. This blocks the inhibitory role of Munc18c on SNARE complex formation, allowing a stimulation of ATP release. ATP then binds to purinergic receptors on neurons, enhancing dopaminergic neuronal activity, contributing to a reduction in depression-related behaviors. In the absence of the insulin receptor, this chain of events is lost, leading to alterations in mood and behavior (Figure 6).
Discussion

While the brain has been classically regarded as an insulin-insensitive tissue, many recent studies have shown that this is not the case. Indeed, insulin action on the brain has been shown to regulate feeding, hepatic glucose output, body temperature and energy homeostasis, as well as other metabolic and endocrine functions (reviewed in ref 1). These defects have been thought to be mediated predominantly by insulin action on neurons, although we and others have shown that insulin acting on glial cells can regulate cholesterol synthesis (22) and alter some aspects of whole-body metabolic control (24). In the present study, using two genetically-engineered mouse models, as well as primary cells, we demonstrate that insulin signaling in astrocytes plays a crucial role in potentiating release of dopamine and that loss of this function has important effects on regulation of mood and behavior.

Diabetes is associated with a number of CNS abnormalities including increased risks of cognitive impairment, Alzheimer’s disease, anxiety and depression (50, 51). Impaired signaling downstream of the insulin receptor has been demonstrated in brains from patients with Alzheimer’s patients even in the absence of diabetes (17). Mice with knockout of insulin receptor in all cells of the brain have been shown to display anxiety and depressive-like behaviors as they age (11). Likewise, mice with insulin resistance secondary to diet-induced obesity (52) or genetic defects (53) display anxiety and depressive-like behaviors. Conversely, systemic insulin delivery has been shown to have an antidepressant effect in diabetic mice (54), and some studies have suggested that insulin can slow the rate of cognitive decline in patients with Alzheimer’s disease (55) and even have beneficial effects on mood and cognition in healthy subjects (12). The present study demonstrates that insulin signaling in astrocytes is critical for the mood control. Indeed, loss of insulin receptors on astrocytes in the nucleus accumbens is
sufficient to induce depressive-like behavior in mice, demonstrating the importance of
astrocytic insulin signaling in behavioral control.

Insulin receptors are expressed on both neurons and glial cells. The action of
insulin in neurons in the arcuate nucleus (ARC) of the hypothalamus has been shown to
inhibit orexigenic AgRP/NPY neurons, while activating anorexigenic POMC neurons (20,
56). As a result, modulation of these neuronal populations by insulin suppresses food
intake. In addition, insulin action on AgRP neurons has been shown to be critical for
suppression of hepatic glucose production (19), while its action on POMC neurons
controls adipose tissue lipolysis (21). Hypothalamic insulin receptors have also been
shown to be important for the regulation of thermogenesis and reproductive fertility (6,
57). The effects of insulin signaling, however, are not limited to hypothalamic neurons,
since insulin has also been shown to act on cholinergic interneurons in striatum to
potentiate the reward response (58).

The present study demonstrates that besides neurons, astrocytes are an
important insulin-responsive element in the brain. Thus, insulin acutely stimulated
astrocyte exocytosis by phosphorylation of Munc18c on tyrosine residues, alleviating its
inhibitory effect on syntaxin-4. This results in increased release of ATP and possibly
other gliotransmitters and metabolites from astrocytes, which can modulate the activity
of dopaminergic neurons and potentially other neurons (i.e. serotonergic neurons). In
this regard, the regulation of syntaxin-4/Munc18c complex in astrocytes is similar to that
in peripheral insulin sensitive tissues, such as adipose tissue and muscle, where
Munc18c has been shown to be involved in insulin-stimulated translocation of
intracellular GLUT4-containing vesicles to the plasma membrane (48, 59, 60). This
enrichment of syntaxin-4/Munc18c in astrocytes, but not neurons, provides a unique
mechanism by which insulin can specifically target astrocytes in the central nervous
system.
This acute action of insulin on astrocytes is in addition to more chronic effects of insulin on cellular metabolism. For example, we and others have shown that insulin regulates cholesterol synthesis in astroglial cells (22), and this astrocyte-derived cholesterol is released from astrocytes complexed to ApoE-containing lipoproteins which can then be taken up by neurons where the cholesterol participates in membrane homeostasis and synaptic remodeling (61). Somewhat paradoxically, we find that ApoE mRNA is increased in the astrocytes lacking IR, suggesting that at the level of gene expression, insulin signaling suppresses ApoE expression in the astrocytes. Since ApoEε is the most significant risk allele for Alzheimer’s disease (62), the impact of insulin regulation on ApoE in Alzheimer’s disease requires further investigations. Others have found that loss of insulin receptors in astrocytes can also result in decreased expression of GLUT1 and increased expression of carnitine palmitoyltransferase 1C (CPT1C), shifting the fuel preference of the astrocytes from glucose to lipids and changing mitochondria oxidation (24). Thus, insulin can regulate multiple aspects of astrocyte function, which can modulate neuronal plasticity and activity in the brain.

While our study focuses on the role of insulin in astrocytes, astroglial dysfunction can occur in individuals with mental illness, even in the absence of diabetes. Histopathological studies have shown that glial cell density is reduced in the prefrontal cortex, hippocampus and amygdala of patients with major depressive disorders and that this is associated with alterations in the expression of astrocyte-specific markers (reviewed in ref 63, 64). Treatment with antidepressants might also indirectly alter insulin receptor expression and signaling through effects on structural proteins, receptor expression and activation of intracellular pathways in astrocytes (reviewed in ref 65).

The present study demonstrates that insulin-dependent release of ATP from astrocytes is critical for normal mood control. This is supported by other studies showing that ATP
levels are decreased in many brain regions in rodent models of depression (44) and that a P2Y1 receptor agonist can cause anxiolytic effects in the rat (66).

Our data indicate that the dopamine system may be an important mechanistic link between astrocytic insulin action and mood regulation, since selective dopamine D2/D3 receptor agonist, but not serotonin 5-HT$_{1A}$ receptor agonist, acutely reversed the depressive-like behavior in mice lacking IR in astrocytes. However, this does not rule out the possibility that astrocytic insulin signaling-mediated ATP release may have effects on other neural systems, including the serotonin and cannabinoid systems (67, 68), to modulate mood behaviors in mice. For example, impaired serotonin signaling may play a role in the depressive-like behavior in these mice by modulating dopaminergic neuronal activity through other serotonin receptor subtypes like 5-HT$_{2C}$ (69). In addition to dopaminergic neurons, modulation of neuronal activity by astrocyte-derived “gliotransmitters” has also been reported in hippocampal pyramidal neurons through effects of glutamate (42) and in hypothalamic AgRP neurons through the action of AMP (70, 71). Whether insulin signaling-dependent gliotransmission from astrocytes plays a significant role in other neural circuits awaits further investigations.

In summary, we have demonstrated that astrocytes are an important target for insulin signaling in control of mood and behavior. Astrocytic insulin signaling regulates Munc18c phosphorylation and syntaxin-4-dependent ATP exocytosis, which in turn modulates presynaptic dopaminergic neuronal activity and dopamine release. Thus, astrocytic IR deficient mice exhibit impaired dopamine release in brain slices and increased depressive-like behaviors. Developing new agents to target this aspect of astrocyte biology could provide a novel approach to the treatment of mood and behavioral disorders in both diabetic and non-diabetic individuals.
Methods

Animal models. Mice were housed in standard cages with a 12-h light/dark cycle and fed a 22% fat chow diet (Mouse Diet 9F, LabDiet). Both male and female mice were used as indicated. All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) at Joslin Diabetes Center and Tufts University and were in accordance with NIH guidelines. Additional information about animal studies is described in Supplemental Experimental Procedures.

Behavioral assessment. Male and female (4-month-old and 1-year-old) mice were used for behavioral tests. Each mouse was exposed to each specific behavioral test only once in its lifetime unless otherwise indicated. Only one behavioral test was performed per week to minimize the stress. All mice were transferred to the behavioral testing room the day before the test for acclimation.

In the open field test, each mouse was placed in an open box 57 X 37 X 31 cm. During the 5 min session, the movement of the mouse was recorded using a HD webcam and analyzed by Anymaze software (Stoelting Co.). Central zone entries, total distance travelled and maximum speed were measured.

For the novelty suppressed feeding test, mice were fasted overnight (16 h). Each mouse was placed in a well-illuminated open field box (57 X 37 X 31 cm) with a white 15 cm disc containing a food pellet in the center of the box. The movement of the mouse was recorded for maximum 15 min and analyzed by Anymaze software. The latency to feeding and food intake by each mouse in a housing cage in the hour immediately following testing were measured.

In the forced swimming test, each mouse was placed in a vertical plexiglass cylinder (40 cm height; 18 cm diameter) containing 15 cm deep of water (23-25°C). The mouse was allowed to swim inside the cylinder and videotaped for 6 min. Videos were
analyzed by Anymaze software. During the testing period, the total time of immobility was assessed as a measure of “despair” or depression. In the same studies, the antidepressant imipramine (16 mg/kg, Sigma), dopamine receptor D2/D3 agonist pramipexole (0.3 mg/kg, Sigma) or serotonin 5-HT₁A receptor agonist 8-OH-DPAT (0.3 mg/kg, Tocris) was i.p. injected into subgroup of GIRKO mice and IR<sup>fl</sup> littermates 1 h prior to the test.

In the sucrose preference test, mice were single housed and habituated for 24 h in the behavioral testing room with both bottles containing water. After habituation, mouse was given free access to two water bottles: one containing 1% sucrose solution and the other plain water. Daily water and sucrose intake was measured for the next 4 days. The placement of water and sucrose bottles was switched each day to avoid memory-driven behavior. Sucrose preference was presented as the percentage of the volume of sucrose intake over the volume of total fluid intake.

**Brain slice preparation.** 11- to 12-week-old female mice fed normal chow were euthanized using a ketamine-xylazine cocktail. The brain was placed in ice-cold oxygenated sucrose bicarbonate solution (210 mM sucrose, 10 mM glucose, 3.5 mM KCl, 1 mM CaCl<sub>2</sub>, 4 mM MgCl<sub>2</sub>, 1.25 mM NaH<sub>2</sub>PO<sub>4</sub>). The two halves of the neocortex were promptly glued to a metallic base fitting a Leica VT1000S Vibratome (Leica Microsystems) and cut in 300 μm coronal brain slices, which were transferred to a container filled with oxygenated aCSF (124 mM NaCl, 2 mM KCl, 1.25 mM KH<sub>2</sub>PO<sub>4</sub>, 2 mM MgSO<sub>4</sub>, 25 mM NaHCO<sub>3</sub>, 2 mM CaCl<sub>2</sub> and 11 mM glucose) at room temperature. Brain slices were allowed to recover for an hour after dissection. Slices with the three sites that contain the bulk of terminals projecting from the dopaminergic midbrain (prefrontal cortex, nucleus accumbens or dorsal caudate putamen) were used for testing.
**Dopamine release measured by carbon fiber amperometry.** Amperometric electrodes were 5 µm carbon fibers (Amoco). The electrodes were back filled with 3 M KCl and beveled at the tip. Electrode response was tested by cyclic voltammetry. A positive 700 mV voltage (vs. a Ag-AgCl ground) was applied to the carbon fiber electrode using a 200B amplifier (Axon instruments). The amperometric electrode was placed in the prefrontal cortex, dorsal striatum or nucleus accumbens. A bipolar stimulating electrode (PlasticsOne, Inc.) was placed 100-200 µm away from the carbon fiber electrode. A current stimulus of +500 µA was applied 5 times per site every 5 min for 2 msec. The response of the amperometric electrode (increase above baseline) was recorded using Axograph. The output was digitized at 50 kHz, low-pass filtered at 1 kHz, and analyzed using Axograph. The number of molecules oxidized was determined by the relation $N = Q/nF$ where $Q$ is the charge of the spike, $n$ is the number of electrons transferred (two for catecholamines), $n$ is the number of moles, and $F$ is Faraday’s constant (96485 coulombs per unit charge).

To test the role of purinergic signaling on dopamine release, two slices that contained nucleus accumbens were used. Electrically evoked dopamine release from one slice was measured in oxygenated aCSF incubated with either 150 µM ATP-γ-S for 10 min or 10 µM 2-Me-S-ATP for 40 min, and measured again. The other slice was subjected to the same procedure with oxygenated aCSF for the same time period as experimental slice.

**Dopamine content measurement by HPLC.** Whole brains from 3-month-old GIRKO mice and IR$^{ff}$ littermates were rinsed in aCSF solution to remove blood and homogenized in 1 ml 0.3 M perchloric acid. Lysates were repeatedly sonicated (1 min) and frozen on dry ice (10 min) 3 times. The samples were spun for 25 min at 1,200 rpm, the supernatant collected, and total volume measured. 20 µl supernatant from each
sample was injected into HPLC system coupled to an electrochemical detector (Antec INTRO) with the GBC LC1120 HPLC pump pressure of 1,200 psi. The total amounts of dopamine and other neurotransmitters were measured. The pellets were solubilized in 1X RIPA buffer with protease inhibitor cocktail (CalBiochem) by sonication. Protein concentrations, measured using the BCA protein assay, were used for normalization.

**Stereotaxic injections.** IR^{lof} mice were anesthetized with a ketamine (100 mg/ml) and xylazine (20 mg/ml) mix diluted in saline and placed into a stereotaxic station (Kopf Instruments). The skull was exposed via a small incision, and a small hole was drilled to the desired coordinates for viral injection. A 33-gauge needle (Hamilton) was inserted bilaterally into the nucleus accumbens (from bregma, AP: +1.2 mm, ML: ± 2.5 mm, DV: -4.8 mm, angle: ± 18%) and medial prefrontal cortex (from bregma, AP: +2.0 mm, ML: ± 0.4 mm, DV: -2.5 mm). 1 μl (2 X 10^8 viral genome particles) AAV-DJ/8-GFAP-Cre:GFP or GFP only viruses were injected bilaterally into NAc or mPFC using a 2.5 μl Hamilton microsyringe. The needle was withdrawn 5 min after the injection.

**Intracerebroventricular (i.c.v.) injection.** Both 13-week-old IR^{lof} and iGIRKO female mice were anesthetized with ketamine (100 mg/ml) and xylazine (20 mg/ml) mix diluted in saline and placed into a stereotaxic station (Kopf Instruments). A stainless steel guide cannula (Plastic One, Torrington, CT) with 2 mm guide length was implanted into the lateral cerebral ventricle. The stereotaxic coordinates were -0.5 mm posterior and +1.0 mm lateral from bregma. Correct placement of the cannula was verified by i.c.v. injection of angiotensin II (1 μg in 1 μl 0.9% saline, Sigma) three days after the cannula implantation. Mice failed to consume water within 30 min after the injections were removed from the following study. One week after the implantation, mice were injected with 2 μl saline or 2-Me-SATP (10 μM) i.c.v. in a randomized fashion 1 h prior to the
forced swimming test. One group of mice were administrated saline followed by forced
swimming test at week 1 and then administrated 2-Me-SATP followed by forced
swimming test at week 2. In the second group of mice the order of tests was reversed.

**Primary astrocyte culture.** Primary cultures of cortical astrocytes were prepared from
IR^{ff} newborn pups. Both cortices were surgically dissected in ice-cold Hybernate A
media (Invitrogen). After the removal of meninges, the cortical tissues were dissociated
by treatment for 30 min at 37°C in Hybernate A supplemented with 4 mg/mL papain
(Sigma) and 0.0025 mg/mL DNase I (Sigma), subjected to gentle trituration in DMEM
plus 10% FBS, and plated on 75 cm^{2} cell culture flasks. Following incubation overnight in
a humidified incubator at 37°C and 5% CO_{2}, the flasks were vigorously shaken to detach
neurons and microglia. Culture medium (DMEM supplemented with 10% FBS, 100
units/mL penicillin, and 100 µg/mL streptomycin) was replaced every 3 d thereafter until
the cell monolayer reached confluence.

**ATP release assay.** Cells cultured in 12-well plates were washed twice with 1X HBSS
and serum starved in HBSS for an additional 4 h. After the starvation period, the media
was replaced with 1 X HBSS + 100 µM ARL67156 (an ecto ATPase inhibitor) with or
without 100 nM insulin. After 30 min incubation, the supernatant was collected, and ATP
quantitated using a luciferase-based ATP determination kit (ThermoFisher Scientific).
Cells were lysed with RIPA lysis buffer complemented with 50 mM KF, 50 mM β-
glycerolphosphate, 2 mM EGTA (pH 8), 1 mM Na_{3}VO_{4} and 1 X protease inhibitor
cocktail (Calbiochem). Protein concentrations were determined using the Pierce 660
nm Protein Assay Reagent (Bio-Rad) and used for normalization.

To quantify the total ATP content in the cells, 50 µl of cell lysates were mixed
with 50 µl 2 M perchloric acid by vortexing and incubated on ice for 5 min. After
centrifugation, the supernatant was mixed with 50 µl 3 M KOH, vortexed and centrifuged to precipitate the remaining perchloric acid. The resultant supernatant was neutralized using HCl and subjected to ATP assay as described above.

**Munc18c tyrosine phosphorylation.** Both control and IRKO astrocytes were infected with adenovirus encoding Flag-tagged Munc18c (1 X 10⁹ genomic copies/ml) for 24 h. The cells were cultured for an additional 5 days before the experiment. Cells were serum starved in DMEM + 0.1% BSA for 5 h before 10 nM insulin stimulation for 15 min. After stimulation, cells were washed immediately with ice-cold PBS once and lysed in lysis buffer [20 mM Hepes (pH 7.4), 150 mM NaCl, 50 mM KF, 50 mM β-glycerolphosphate, 2 mM EGTA (pH 8), 1 mM Na₃VO₄, 1% Triton X-100, 10% glycerol and 1 × protease inhibitor cocktail (Calbiochem)]. Protein concentrations were determined using the Pierce 660 nm Protein Assay Reagent (Bio-Rad). To immunoprecipitate total phosphotyrosine containing proteins, 600 µg protein lysates were incubated with 1 µg anti-phosphotyrosine antibody (Santa Cruz) in a total volume of 800 µl overnight at 4°C with end-to-end rotation, followed by a 1-hour incubation with 20 µl protein A/G-conjugated magnetic beads at 4°C with end-to-end rotation. The immunocomplexes were pulled down with magnetic rack and washed sequentially: 1 time with lysis buffer, two times with lysis buffer + 500 mM NaCl, and two times with lysis buffer. Bound proteins were eluted by incubation for 5 min at 100°C in 1X SDS loading buffer. The bound proteins along with 10 µg total cell lysates from each sample were resolved using SDS-PAGE, transferred to PVDF membranes. The level of Munc18c tyrosine phosphorylation was assessed by immunoblotting using anti-Flag antibodies (Sigma).
**Statistical analysis.** All the data are presented as mean ± SEM. Two groups were compared using unpaired two-tailed Student’s t-test. 2-way ANOVA was performed to detect the interactions between genotype and treatment (i.e. insulin, antidepressants, receptor agonists, restraint), and Tukey’s *post hoc* analysis was performed when appropriate. Repeated 2-way ANOVA was performed to detect the significant interaction between treatment and time followed by Sidak’s multiple comparisons. A *P* value less than 0.05 was considered significant.

**Study approval.** All animal studies were approved by the IACUC of the Joslin Diabetes Center, Boston, MA 02215 and Tufts University School of Medicine, Boston, MA 02111, and were in accordance with NIH guidelines.
Author contributions

W.C. designed the study, researched data, and wrote the manuscript. C.X., M.S., M.K., A.S., H.A.F., M.L., R.Y., A.K. and E.N.P. researched data and helped design experiments. C.R.K. designed the study, supervised all work and helped write the manuscript.

Acknowledgements

This work was supported by NIH grants R01 DK031036 and R01 DK033201 (to C.R.K.) and R01 DK065872 (to E.N.P.). The work was also supported by the Boston University/Joslin Diabetes Center Pilot and Feasibility Award (E.N.P.), and the Tufts Center for Neuroscience Research P30 NS047243 (Jackson). M.S. was supported by MSD Life Science Foundation and Takeda Science Foundation. H.A.F. was supported by NIH grant 5 K08 DK097293. A.K. was supported by the Deutsche Forschungsgemeinschaft (DFG) grant project KL 2399/4-1 and the Federal Ministry of Education and Research (German Center for Diabetes Research, Grant No. 01GI092). The Joslin Diabetes Center DRC Advanced Microscopy Core, Flow Cytometry Core and Animal Physiology Core (P30 DK36836) also provided important help. The authors declare no conflict of interest.

Conflict of interest statement

The authors have declared that no conflict of interest exists.
References


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Figure 1

A. Relative expression (a.u.)

B. Percentage of GFP+ cells (Average ± SEM)

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<tr>
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<th>IR/+/GFP</th>
<th>GIRKO/GFP</th>
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<td>IR/+/GFP</td>
<td>7.538% ± 0.595%</td>
<td>7.050% ± 1.104%</td>
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C. Blood glucose (mg/dl)

D. Percentage of GFP+ cells (Average ± SEM)

E. Center zone entries

F. Latency to feed (sec)

G. Sucrose preference (%)

H. Time of immobility (sec)

I. Time of immobility (sec)

J. Serum corticosterone (ng/ml)
Figure 1. Loss of IR in astrocytes increases anxiety and depressive-like behavior in mice. (A) mRNA levels of IR and IGF1R from FACS-sorted GFP+-astrocytes from GIRKO/GFP and IRff/GFP mice normalized to TBP. **, $P < 0.01$, two-tailed Student's t-test, $n=4$ for IRff/GFP, $n=3$ for GIRKO/GFP. (B) Representative FACS profiles of dissociated cells from brains of control GFAP-GFP reporter (IRff/GFP) and GIRKO GFAP-GFP reporter (GIRKO/GFP) mice. x-axis: GFP fluorescence; y-axis: side-scattered light (SSC). Dead cells were excluded by propidium iodide staining. $n=4$ for IRff/GFP; $n=3$ for GIRKO/GFP. (C) Glucose tolerance tests on overnight-fasted 3-month-old male IRff and GIRKO mice following i.p. glucose (2 g/kg). Right: Area under the curve of GTT. *, $P < 0.05$, two-tailed Student's t-test, $n=7$ for IRff, $n=6$ for GIRKO. (D) Open field test representative tracing of movement by IRff and GIRKO mice. (E) Number of center zone entries of 4-month-old and 1-year-old female IRff and GIRKO mice in the open field test. *, $P < 0.05$, ***, $P < 0.001$, two-tailed Student's t-test, 4-month-old: $n=13$ for IRff, $n=11$ for GIRKO, 1-year-old: $n=6$ for IRff and GIRKO. (F) Latency to feeding of 4-month-old female mice in novelty suppressed feeding test. *, $P < 0.05$, two-tailed Student's t-test, $n=12$ for IRff, $n=12$ for GIRKO. (G) Sucrose preference of 4-month-old female IRff and GIRKO mice. *, $P < 0.05$, two-tailed Student's t-test, $n=9$ for IRff, $n=19$ for GIRKO. (H) Time of immobility of 4-month-old female IRff and GIRKO mice in forced swimming test following saline or 16 mg/kg imipramine i.p. injection 1 h prior to the test. *, $P < 0.05$, two-way ANOVA followed by Tukey's multiple comparisons, $n=11$ for IRff groups, $n=10$ for GIRKO with saline, $n=12$ for GIRKO with imipramine treatment. (I) Time of immobility of 1-year-old female IRff and GIRKO mice in forced swimming test. **, $P < 0.01$, two-tailed Student's t-test, $n=6$. (J) Serum corticosterone of 6-month-old female IRff and GIRKO mice before and after 5 min of restraint. ***, $P < 0.001$, **, $P < 0.01$, two-tailed Student's t-test, $n=6$. All data are mean ± SEM.
**Figure 2**

A. 

B. 

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J.
Figure 2. Loss of IR in astrocytes leads to decreased dopamine release. (A) Representative tracing of electrically evoked dopamine release from nucleus accumbens in 3-month-old female IR<sup>f/f</sup> and GIRKO mice using carbon fiber amperometry. (B-D) Total number of dopamine molecules released (B), average peak amplitude of dopamine release (C) and average peak width (D) from dorsal caudate putamen (CPu), nucleus accumbens (NAc) and medial prefrontal cortex (mPFC) of 3-month-old female IR<sup>f/f</sup> and GIRKO mice. ***, P < 0.001, **, P < 0.01, *, P < 0.05, two-tailed Student’s t-test, n=42 measurements from 19 IR<sup>f/f</sup> mice, n=43 measurements from 19 GIRKO mice. (E) Total dopamine content in the brains of 3-month-old female IR<sup>f/f</sup> and GIRKO mice. n=5, two-tailed Student’s t-test. (F) Relative expression of tyrosine hydroxylase (TH) in the substantia nigra/ventral temgmental area of IR<sup>f/f</sup> and GIRKO mice. TBP was used as housekeeping gene. n=6. (G) Relative expression of genes involved in dopamine signaling pathways in the nucleus accumbens of 3-month-old female IR<sup>f/f</sup> and GIRKO mice. TBP was used as housekeeping gene. n=6. (H) Representative images of TH/GLAST/NeuN co-immunostaining in the nucleus accumbens of 4-month-old female IR<sup>f/f</sup> and GIRKO brain sections. Scale bar: 20 µm. (I) Mean TH intensity in the nucleus accumbens of IR<sup>f/f</sup> and GIRKO brain sections. n=8 random fields in nucleus accumbens from four mice. (J) Mean GLAST intensity in the nucleus accumbens of IR<sup>f/f</sup> and GIRKO brain sections. n=8 random fields in nucleus accumbens from four mice. All data are mean ± SEM.
**Figure 3**

A. **iGIRKO**

B. **GFAP-CreERT2 X mTrmG with i.p. Tamoxifen**

C. **Center zone entries**

D. **Sucrose preference (%)**

E. **Time of immobility (sec)**

F. **Time of immobility (sec)**

G. **CTR**

H. **% (c-fos NeuN* / total NeuN**)

I. **AAV-GFAP-Cre at NAc**

J. **Time of immobility (sec)**

K. **Time of immobility (sec)**
Figure 3. Deletion of IR in astrocytes in adult mice leads to depressive-like behavior.

(A) Strategy to generate inducible astrocyte-specific IR knockout mice by crossing IR^{fl/fl} mice with mice transgenic for human GFAP-promoter-driven CreERT2. To induce recombination, 8-week-old mice were injected with tamoxifen (100 mg/kg, i.p.) for 5 consecutive days. All experiments were performed 6 weeks later. Control IR^{fl/fl} littermates were subjected to the same tamoxifen injections. (B) Left, GFP immunostaining of brain sections containing striatum from GFAP-CreERT2 X mTmG mouse. Scale bar: 1 mm. Right, Co-immunostaining of GFP and the astrocyte-specific marker S100β in nucleus accumbens. Scale bar: 20 μm. (C) Number of center zone entries of 4-month-old female IR^{fl/fl} and iGIRKO mice in the open field test. *, P < 0.05, two-tailed Student’s t-test, n=13 for IR^{fl/fl}, n=11 for iGIRKO. (D) Sucrose preference of 4-month-old female IR^{fl/fl} and iGIRKO mice. *, P < 0.05, two-tailed Student’s t-test, n=7 for IR^{fl/fl}, n=11 for iGIRKO. (E) Immobility time of 4-month-old female IR^{fl/fl} and iGIRKO mice in forced swimming test following saline or 0.3 mg/kg pramipexole injection (i.p.) 1 h prior to the test. *, P < 0.05, ****, P < 0.0001, two-way ANOVA followed by Tukey’s multiple comparisons, n=10 for saline, n=9 for IR^{fl/fl} with pramipexole, n=10 for iGIRKO with pramipexole. (F) Immobility time of 4-month-old female IR^{fl/fl} and iGIRKO mice in forced swimming test following saline or 0.3 mg/kg 8-OH-DPAT injection (i.p.) 1 h prior to the test. *, P < 0.05, repeated two-way ANOVA followed by Sidak’s multiple comparisons, n=10 for IR^{fl/fl}, n=9 for iGIRKO. (G) Immunostaining of c-fos (red) and neuron marker, NeuN (green), in the NAc of IR^{fl/fl} and iGIRKO mice under basal conditions (CTR) or 1 h post-forced swimming test (FST). (H) Percentage of c-fos+/NeuN+ neurons over total neurons (NeuN+) in the NAc of IR^{fl/fl} and iGIRKO mice under basal conditions or 1 h post FST. *, P < 0.05, **, P < 0.01, two-way ANOVA followed by Tukey’s multiple comparisons, n=4 for CTR, n=7 for FST. (I) Left, Images of GFP and GFAP co-immunostaining on a brain section from an mTmG mouse injected with AAV-GFAP-Cre in the nucleus accumbens (top) and medial prefrontal cortex (bottom). Scale bar: 1 mm. Right, co-localization of GFP and GFAP in an astrocyte. Scale bar: 20 μm. (J) Immobility time in the forced swimming test of 4-month-old female IR^{fl/fl} mice injected with AAV-GFAP-Cre or AAV-GFAP-GFP in NAc. *, P < 0.05, two-tailed Student’s t-test, n=12 for GFP injected mice, n=10 for Cre injected mice. (K) Immobility time in the forced swimming test of 4-month-old female IR^{fl/fl} mice injected with AAV-GFAP-Cre or AAV-GFAP-GFP in mPFC. n=9 for GFP injected mice, n=10 for Cre injected mice. All data are mean ± SEM.
**Figure 4**

A) Relative expression (a.u.)

B) Heatmaps showing protein expression for CTR and IRKO:
- p-IR/IGF1R
- IRβ
- p-IRS-1 (Y612)
- IRS-1
- p-Akt (S473)
- Akt
- p-GSK3α/β
- GSK3β
- p-ERK1/2
- ERK1/2
- β-Actin

C) Relative expression (a.u.)

D) ATP release/mg protein:

E) nmol ATP/mg protein:

F) G) H) Graphs showing dopamine release and time of immobility.
Figure 4. IR in astrocytes regulates ATP exocytosis to modulate dopamine release. (A) mRNA expression of IR and IGF1R in primary IR<sup>f/f</sup> astrocytes infected with adenovirus encoding Cre or GFP. TBP was used as a housekeeping gene. ***, P < 0.001, two-tailed Student’s t-test, n = 6. (B) Immunoblotting of phosphorylation of insulin signaling molecules in CTR and IRKO astrocytes following indicated concentrations of insulin stimulation for 10 min. (C) Relative expression of astrocyte-specific markers in CTR and IRKO astrocytes. TBP was used as housekeeping gene. ***, P < 0.001, **, P < 0.01, *, P < 0.05, two-tailed Student’s t-test, n = 6. (D) ATP release from CTR and IRKO astrocytes in the presence or absence of 100 nM insulin stimulation for 30 min. *, P < 0.05, two-tailed Student’s t-test, n = 6. (E) Total ATP content in both CTR and IRKO astrocytes. n = 6. (F) Peak amplitude of electrically evoked dopamine release from nucleus accumbens before and after infusion of aCSF or ATP-γ-S. **, P < 0.01 vs basal, repeated two-way ANOVA followed by Sidak’s multiple comparisons, n = 5. (G) Peak amplitude of electrically evoked dopamine release from nucleus accumbens before and after infusion of aCSF or 2-Me-S-ATP. ***, P < 0.001 vs basal, repeated two-way ANOVA followed by Sidak’s multiple comparisons, n = 5. (H) Time of immobility of 4-month-old female IR<sup>f/f</sup> CTR and iGIRKO mice in forced swimming test with i.c.v. infusion of saline or 20 pmol 2-Me-SATP 1 h prior to the test. *, P < 0.05, **, P < 0.01, two-way ANOVA followed by Sidak’s multiple comparisons, n = 9 for CTR groups, n = 10 for iGIRKO groups. All data are mean ± SEM.
Figure 5. IR in astrocytes regulates exocytosis through Munc18c tyrosine phosphorylation. (A) Top: Immunoblotting of astrocyte-enriched SNARE complex proteins in CTR and IRKO astrocytes. Bottom: Relative mRNA expression of astrocyte-enriched SNARE complex proteins and vesicular ATP transporter VNUT in CTR and IRKO astrocytes. TBP was used as housekeeping gene. n=6. (B) Top: Co-immunoprecipitation showing syntaxin-4 (Sxt4):VAMP3 interaction in CTR and IRKO astrocytes under basal state and after insulin (100 nM, 30 min) stimulation. Bottom: Densitometry analysis showing impaired Sxt4:VAMP3 interaction in IRKO astrocytes following insulin stimulation. **, P < 0.01, two-way ANOVA followed by Tukey’s multiple comparisons, n=6. (C) Top: Tyrosine phosphorylation of Flag-Munc18c in CTR and IRKO astrocytes under basal state and after insulin (100 nM, 30 min) stimulation. Bottom: Densitometry analysis showing loss of insulin-stimulated Munc18c tyrosine phosphorylation in IRKO astrocytes. *, P < 0.05, two-way ANOVA followed by Tukey’s multiple comparisons, n=3. All data are mean ± SEM.
Figure 6. Proposed model of astrocytic insulin signaling on the regulation of dopaminergic neuronal activity and behavior. *Left:* Insulin activates IR on astrocytes to induce syntaxin-4 (Stx4):Munc18c SNARE complex-dependent ATP exocytosis, which contributes to the modulation of dopaminergic neuronal activity and mood behavior. *Right:* Loss of IR in astrocytes results in dysregulation of the proposed signaling pathway, leading to depressive-like behavior.