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Neuronal Ablation of p-Akt at Ser473 Leads to Altered 5-HT_{1A/2A} Receptor Function

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Abstract

The serotonergic system regulates a wide range of behavior, including mood and impulsivity, and its dysregulation has been associated with mood disorders, autism spectrum disorder, and addiction. Diabetes is a risk factor for these conditions. Insulin resistance in the brain is specifically associated with susceptibility to psychostimulant abuse. Here, we examined whether phosphorylation of Akt, a key regulator of the insulin signaling pathway, controls serotonin (5-HT) signaling. To explore how impairment in Akt function regulates 5-HT homeostasis, we used a brain-specific rictor knockout (KO) mouse model of impaired neuronal phosphorylation of Akt at Ser473. Cortical 5-HT_{1A} and 5-HT_{2A} receptor binding was significantly elevated in rictor KO mice. Concomitant with this elevated receptor expression, the 5-HT_{1A} receptor agonist 8-Hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) led to an increased hypothermic response in rictor KO mice. The increased cortical 5-HT_{1A} receptor density was associated with higher 5-HT_{1A} receptor levels on the cortical cell surface. In contrast, rictor KO mice displayed

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significantly reduced head-twitch response (HTR) to the 5-HT_{2A/C} agonist 2,5-dimethoxy-4-iodoamphetamine (DOI), with evidence of impaired 5-HT_{2A/C} receptor signaling. *In vitro*, pharmacological inhibition of Akt significantly increased 5-HT_{1A} receptor expression and attenuated DOI-induced 5-HT_{2A} receptor signaling, thereby lending credence to the observed *in vivo* cross-talk between neuronal Akt signaling and 5-HT receptor regulation. These data reveal that defective central Akt function alters 5-HT signaling as well as 5-HT-associated behaviors, demonstrating a novel role for Akt in maintaining neuronal 5-HT receptor function.

Keywords

serotonin; Akt; cortex; 5HT_{1A} receptor; 5HT_{2A} receptor

1. INTRODUCTION

In the brain, insulin acts through its receptor to stimulate phosphatidylinositol 3-kinase, and downstream protein kinase B (PKB)/Akt isoforms 1 and 2. Akt, a metabolic kinase, is involved in neuronal development, synaptic transmission, and monoamine transporter trafficking and function (Garcia et al., 2005; Robertson et al., 2010; Speed et al., 2011). The mammalian target of rapamycin (mTOR) complex 2 (mTORC2), that comprises the protein rictor, is a protein kinase regulating cell growth and metabolism (Sarbasov et al., 2005) and Akt activity. Akt function is regulated by phosphorylation at 2 key residues: Ser473 by mTORC2 and Thr308 by phosphoinositide-dependant kinase 1. Akt dysregulation has been linked to numerous metabolic diseases including diabetes and obesity, and mental disorders such as schizophrenia (Nicodemus et al., 2010; Thiselton et al., 2008) and drug abuse (Ikeda et al., 2006). As aberrant Akt function has been progressively linked to a growing number of brain disease states, the mechanism of how it contributes to their pathophysiology is under intense study. Recently, it has been proposed that Akt may represent a molecular link between diseases driven by insulin resistance (e.g. diabetes) and disorders associated with central monoaminergic disturbances, including depression, schizophrenia and drug abuse (Daws et al., 2011; Robertson et al., 2010).

The serotonin (5-HT) system mediates a variety of brain functions, including temperature regulation, appetite, mood, libido, and social behaviors (Berger et al., 2009). Perturbations in the serotonergic system are involved in a number of psychiatric disorders, including panic disorder (Nash et al., 2008), anxiety, aggression (Gingrich and Hen, 2001) and most recently, autism spectrum disorder (Veenstra-VanderWeele et al., 2012). The emergence of selective serotonin reuptake inhibitors (SSRIs) as the most prescribed medications in the U.S. has centered attention on the role of the 5-HT system in depression and anxiety. Genetic evidence also points to the involvement of the serotonin system in susceptibility to stress and depression (Jans et al., 2007). More than a quarter of the population with diabetes suffers from depression (Lustman and Clouse, 2005). This common comorbidity could reflect an important relationship between dysfunction in the insulin-signaling pathway and altered 5-HT neurotransmission.

We have previously shown that at the neuronal level, insulin signaling regulates monoamine transporter trafficking and that changes in insulin status profoundly affect monoamine homeostasis (Robertson et al., 2010; Speed et al., 2011; Williams et al., 2007). For example, dysregulated brain insulin signaling alters dopamine and norepinephrine homeostasis (Robertson et al., 2010; Speed et al., 2011; Williams et al., 2007) and related behaviors. With respect to its effect on 5-HT regulation, animal models of both diet-induced obesity (DIO) and type 2 diabetes have been shown to increase 5-HT_{2A} receptor density (Park et al., 1999; Sumiyoshi et al., 1997), whereas animal models of food restriction and experimentally-induced type 1 diabetes result in decreased *in vivo* response to 5-HT_{1A} and 5-HT_{2A} receptor agonists. Thus, the relationship between central insulin signaling and 5-HT neurotransmission is complex. Here, we focused on determining whether genetic impairment of neuronal insulin signaling (i.e. Akt function) affects 5-HT homeostasis, 5-HT receptor signaling, and *in vivo* response to 5-HT receptor stimulation.

2. MATERIAL AND METHODS

2.1. Generation of mice

All neuronal rictor KO mice were generated and fully backcrossed to the C57BL/6 background as previously described (Siuta et al., 2010). Breeding pairs consisted of animals homozygous for knock-in of loxP sites flanking exon 3 of the rictor gene (Rictor flox/flox) and heterozygous for Nestin-Cre (Nes +/-). Experimental mice (rictor KO) lacked expression of functional rictor due to excision of exon 3 in all neurons (Nes +/-). Control animals (FLOX) contained the loxP sites but did not have Cre (Rictor flox/flox, Nes -/-).

2.2. Drugs

DOI ((±)-2,5-dimethoxy-4-iodoamphet-amine hydrochloride), 8-hydroxy-2-(di-*n*-propyl-amino)tetralin (8-OH-DPAT), WAY100635, SB269970, WAY100635, fluoxetine, methysergide, ritanserin, and spiperone were purchased from Sigma (St. Louis, MO, USA). The 5-HT_{2A} antagonist MDL 100907 was a gift from Merrell Dow Pharmaceuticals (Kansas City, MO, USA). [³H] citalopram, [³H]-ketanserin, and [³H]-8-OH-DPAT [8-hydroxy-2-(di-*n*-propylamino)-tetralin] were purchased from PerkinElmer Inc. (Boston, MA, USA). [³H]-mesulergine was purchased from G.E.Healthcare, Inc. (Piscataway, NJ, USA).

2.3. Brain tissue extraction for serotonin content

Tissue extraction and analysis was done by a specific HPLC assay (Vanderbilt Neurochemistry Core) as described previously (Siuta et al., 2010).

2.4. Serotonin receptor and transporter binding

Binding assays for serotonin receptors and serotonin transporter were performed with modification as previously reported (Canal et al., 2010). **SERT binding:** Homogenized cortical and midbrain membranes were incubated with [³H]citalopram [3.8 nM] in the presence or absence of fluoxetine [10 μM] to measure non-specific and total binding, respectively. **5-HT_{1A} receptor binding:** Homogenized cortical membranes were incubated with [³H]8-OH-DPAT [10 nM] in the presence or absence of WAY100635 [10 μM] to measure non-specific and total binding, respectively. Reaction buffer included the 5-HT₇

receptor antagonist SB269970 [100 nM]. **5-HT_{2A} receptor binding:** Homogenized cortical membranes were incubated with [³H]ketanserin [12 nM]. Non-specific binding was determined using excess methysergide [100 μM]. **5-HT_{2C} receptor binding:** Homogenized cortical membranes were incubated with [³H]-mesulergine [10 nM] in the presence or absence of ritanserin [8 μM] to measure non-specific and total binding, respectively. The reaction buffer included the 5-HT_{1A/2A} receptor antagonist spiperone [100 nM] and the 5-HT₇ receptor antagonist SB269970 [100 nM]. Single point saturating concentration was chosen based on pilot experiments of complete saturation binding curves in FLOX and rictor KO mice for [³H] citalopram, [³H]ketanserin, [³H]-8-OH-DPAT and [³H]mesulergine binding. For all experiments, specific binding was calculated by subtracting non-specific binding from total binding and expressed as bound ligand (fmol) per mg protein. All experiments were performed in duplicate.

2.5. DOI-induced head twitch response (HTR)

This was conducted as previously described (Veenstra-VanderWeele et al., 2012). Mice received an intraperitoneal (IP) injection of 1.0 mg/kg of 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI, Sigma), a 5HT_{2A/2C} agonist, 30 minutes before testing. After the injection, each mouse was placed in a large glass beaker containing bedding after the injection. A HTR was defined as a clear, rapid, left to right or right to left tic movement of the head of the mouse following DOI treatment. Head twitches were recorded over three 5-minute periods in a quiet room by 3 trained observers blind to the genotype. The mean of the three observers was taken as the HTR. For the studies done with the 5-HT_{2A} antagonist, more than one week later, the same mice were injected with 0.25 mg/kg M-100907 17 minutes prior to the DOI injection. At least one week later, mice were also tested for HTR in response to saline solution alone.

2.6. 8-OH-DPAT-induced hypothermia

Body temperature of the mice was measured using implantable programmable temperature transponders (IPTT-300, Bio Medic Data Systems, Inc., Seaford, DE) and probe (DAS-7007 transponder reader system, BMDS). The implantable electronic ID transponders were inserted by means of a needle assembly subcutaneously under brief isoflurane anesthesia at least 24-48 hours prior to conducting the experiment. On the day of the experiment, mice were injected subcutaneously with 2 mg/kg 8-OH-DPAT, as described previously (Veenstra-VanderWeele et al., 2012). Control animals were treated with 0.9% saline in equivalent volumes. The ambient room temperature was recorded for every experiment and ranged between 19.5 –21.5°C. Body temperature measurements were taken every 10 minutes for an hour post-injection, as well as 20 minutes prior to injection. Mice were also monitored at 2 and 4 hours post-injection to ensure a stable return to baseline body temperature.

2.7. Creation of cell lines stably expressing 5-HT_{1A} and 5-HT_{2A} receptors

Cloned human 5-HT_{1A} and 5-HT_{2A} receptors were stably expressed in HEK293 cells and grown under constant selective pressure using 100 μg/mL G418 as previously described (Chang et al., 2009; Cummings et al., 2010).

2.8. Brain slice preparation and biotinylation

This procedure was done as previously described (Siuta et al., 2010) with few modifications. Brain slices were prepared from 6- to 10-week-old mice. The brain was chilled in oxygenated $\approx 4^{\circ}\text{C}$ sucrose solution, and while in sucrose solution, 300 μm coronal slices were made using a vibratome (Leica VT1000S). Slices were collected in oxygenated artificial cerebral spinal fluid (ACSF), washed twice with oxygenated 4°C ACSF, and then incubated with 4°C ACSF solution containing 1mg/mL of EZ-Link Sulfo-NHS-SSBiotin (Pierce Chemical; Rockford, IL) for 45min. The reaction was quenched with oxygenated 4°C ACSF containing glycine. Slices were frozen on dry ice and the cortex and hippocampus cut out. These specific brain regions were lysed in 1% Triton buffer and centrifuged at 17,000xg for 30min at 4°C . Biotinylated proteins were then isolated using Streptavidin Agarose Resin (Thermo Scientific) overnight at 4°C with agitation. Biotinylated proteins were eluted in 50 μL of 2X SDS-PAGE sample loading buffer at 95°C and then cooled to room temperature. Total slice lysates and the biotinylated (slice surface) fraction underwent immunodetection for 5-HT_{1A} receptors.

2.9. Immunoblot analysis

Mice were rapidly decapitated for brain removal. Brains were chilled on ice and dissected under a microscope. Tissue was homogenized and lysed, and immunoblotting done as previously described (Siuta et al., 2010). Immunoblotting of protein in the HEK-293 cell lines was done as previously described (Garcia et al., 2005). All Akt antibodies were obtained from Cell Signaling. Other antibodies included actin (Sigma), insulin receptor β (Santa Cruz), ERK1/2 (Promega), GSK-3 β (Santa Cruz), NaK-ATPase (DBH), and TH (Millipore). Secondary antibodies were obtained from Santa Cruz Biotechnology.

2.10. Statistical analysis

All data are expressed as the mean $\pm$ S.E.M. Statistical significance between groups was determined using *t*-tests or one and two-way ANOVAs followed by post hoc tests when the main effect or interaction was significant at $p < 0.05$. Statistical analyses were conducted using software from Graph-Pad Prism5®.

3. RESULTS

3.1. Rictor deletion does not alter brain 5-HT content

In order to explore how central Akt might regulate 5-HT receptor function and its related behaviors, we used our mouse model of defective neuronal Akt signaling in which Akt phosphorylation at Ser473 is ablated by Cre-LoxP-mediated and therefore, neuronal-specific deletion of the gene encoding the mTORC2 regulatory subunit, rictor (Siuta et al., 2010). Neuronal rictor deletion abolishes Akt Ser473, but not Thr308, phosphorylation within the cortex and substantia nigra/ventral tegmental area in these null (rictor KO) mice (Siuta et al., 2010). We have previously shown that in these rictor KO mice, brain norepinephrine and dopamine tissue content and homeostasis are altered (Siuta et al., 2010). We evaluated brain 5-HT content to determine whether Akt dysfunction leads to imbalance in 5-HT homeostasis. We found no significant changes in 5-HT content in the brain regions

examined, including cortex, in the rictor KO mice compared to FLOX mice (Fig. 1). We also examined the turnover rate of 5-HT (5-HIAA/5-HT) and found no significant difference between rictor KO and FLOX mice (data not shown). To determine whether altered central insulin (Akt) signaling might influence peripheral insulin levels, we measured blood insulin levels in both phenotypes. There was no difference in insulin levels between FLOX and rictor KO mice (FLOX: 1.15 ± 0.23 ng/ml vs. KO: $1.07 \pm .12$ ng/ml; $n=8-11$, $p>0.05$ by Student's *t*-test).

3.2. [³H]Citalopram binding shows no difference in FLOX vs. rictor KO mice in either cortex or midbrain

We previously observed changes in cortical norepinephrine transporter membrane expression in our rictor KO mice (Siuta et al., 2010). Here, we sought to evaluate the serotonin transporter (SERT) levels in the rictor KO mice in cortex as well as midbrain by radioligand binding. We first verified that phosphorylation of Akt at Ser473 (but not Thr308) is indeed absent in the midbrain of rictor KO mice (Fig. 2, inset), as has been shown for the cortex (Siuta et al., 2010). With respect to SERT levels, we found no significant difference in [³H]citalopram binding between the FLOX and rictor KO mice, in either cortex or midbrain (Fig. 2). Thus, the impaired insulin signaling of phosphorylation of Akt-473 does not alter central SERT expression.

3.3. 5-HT_{1A} and 5-HT_{2A} receptor binding is increased in rictor KO mice

We next examined radioligand binding to 5-HT_{1A}, 5-HT_{2A}, and 5-HT_{2C} receptors. We found a significant increase in radiolabel binding to 5-HT_{1A} and 5-HT_{2A} receptors in the cortex of KO mice compared to the FLOX mice (Fig. 3A,B), and no significant increase for the 5-HT_{2C} receptor binding (data not shown). Elevated expression of these 5-HT receptor subtypes was specific, since expression of the unrelated insulin receptor β (IR β), as measured by immunoblotting, was unaltered in KO mice compared to FLOX mice (expressed as a % of control: FLOX: 100.2 ± 9.6 % vs. KO: 107.8 ± 18.6 %; $n=5-7$; $p>0.05$ by Student's *t* test).

3.4. Neuronal rictor KO causes increased 5-HT_{1A} receptor membrane expression

In figure 3, we estimated levels of 5-HT_{1A} receptor by [³H]8-OH-DPAT binding. However, [³H]-8-OHDPAT is not without limitations, since it binds with high affinity only to those 5-HT_{1A} receptors which are G protein-coupled (Emerit et al., 1990; Hall et al., 1985; Mongeau et al., 1992; Nenonene et al., 1994). Moreover, the ligand binding does not completely discriminate between surface and intracellular receptors. Thus, we examined whether the increased 5-HT_{1A} receptor binding observed in KO mice is a consequence of enhanced receptor at the plasma membrane. In brain, protein cell surface redistribution can be assessed by brain slice biotinylation (Matthies et al., 2010; Robertson et al., 2010; Siuta et al., 2010). Using this biotinylation assay for the first time to quantitate 5-HT_{1A} receptor levels, we observed a significant increase of the amount of surface 5-HT_{1A} receptor levels in KO mice in cortical slices (surface) relative to controls (Fig. 4). This increase was not associated with an increase in total 5-HT_{1A} receptor levels. Thus, the increase in functional receptor binding observed in figure 3A is due, largely, to increased receptor expression at the cortical cell

surface. Interestingly, there was no significant increase in surface 5-HT_{1A} receptor expression in hippocampal slices of the rictor KO mice (Fig. 4, inset: % of control FLOX: $100 \pm 24\%$ vs. KO: $71 \pm 19\%$; $n=4$; $p = 0.3$ by Student's t-test). These results underscore a more prominent role for Akt phosphorylation at Ser473 at cortical serotonergic pathways.

3.5. Rictor KO mice display hypersensitivity to the 5-HT_{1A/7} receptor agonist, 8-OH-DPAT

In an effort to explore the *in vivo* consequences of this increase in cortical 5-HT_{1A} plasma membrane receptor, we conducted a hypothermia test with the 5-HT_{1A/7} receptor agonist, 8-OH-DPAT. In mice, 8-OH-DPAT-induced hypothermia is thought to be mediated by presynaptic 5-HT_{1A} autoreceptors (Bill et al., 1991; Rusyniak et al., 2007). The dose of 2.0 mg/kg of 8-OH-DPAT was selected based on published data where the administered doses ranged from 0.1-0.25 mg/kg (Heisler et al., 1998; Veenstra-VanderWeele et al., 2012; Wozniak et al., 1991) to 2-5 mg/kg (Abbas et al., 2009; El Yacoubi et al., 2003). As seen in figure 5, we found that in the rictor KO mice, 8-OH-DPAT induced a significant increase in the hypothermic response compared to FLOX mice. Thus, this pronounced increase in presynaptic 5-HT_{1A} receptor signaling/function is likely a consequence of increased 5-HT_{1A} receptor expression (Fig. 3).

3.6. DOI-induced head twitch response is attenuated in KO mice

To explore the *in vivo* consequence of the increase in 5-HT_{2A} binding, we evaluated head twitch response (HTR) induced by the hallucinogen 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI), a 5HT_{2A/2C} agonist. The dose of 1.0 mg/kg of DOI was selected based on a literature review where the doses ranged from 1 mg/kg (Veenstra-VanderWeele et al., 2012) to 5-10 mg/kg (Abbas et al., 2009; Darmani et al., 1991), with 1 mg/kg being a standard dose (Canal et al., 2010). Despite the increase in 5-HT_{2A} binding (Fig. 3B), the KO animals showed significantly decreased head twitches in response to DOI compared to the FLOX mice (Fig. 6). No differences between genotypes were seen after treatment with saline alone or after pre-treatment with the 5-HT_{2A} antagonist M-100907 followed by DOI (Fig. 6).

3.7. Cortical 5-HT_{2A} receptor signaling is impaired in KO mice

We observed an increase in total cortical 5-HT_{2A} receptors in rictor KO mice associated with a decrease in DOI-induced HTR. Therefore, it is possible that in rictor KO mice, 5-HT_{2A} receptor signaling is impaired. As shown by others, 5-HT_{2A} receptor activation increases phosphorylation of ERK1/2 (Chang et al., 2009; Schmid et al., 2008; Watts, 1996) as well as of GSK-3 (Abbas et al., 2009; Beaulieu et al., 2009), although the latter has been somewhat controversial (Li et al., 2004). The GSK-3 β phosphorylation is through activation of Akt. To test ERK1/2 and GSK-3 phosphorylation, the agonist DOI (5 mg/kg, IP) was administered *in vivo* (Abbas et al., 2009) and cortical tissue was collected for quantification of phosphorylation levels of ERK1/2 and GSK-3 β . As expected in FLOX mice, DOI (gray bars) significantly increased p-ERK1/2 (Fig. 7A) and p-GSK-3 β (Fig. 7B) with respect to vehicle (white bars). In contrast, there was no DOI-induced increase of cortical p-ERK1/2 (Fig. 7A) or p-GSK-3 β (Fig. 7B) in rictor KO mice. Despite the basal levels of p-GSK-3 β being comparable in FLOX vs. rictor KO mice, DOI decreased p-GSK-3 β significantly to a

level below vehicle control (Fig. 7B) in the mutant mice. These data demonstrate that the 5-HT_{2A} receptor has an aberrant signaling response to agonist stimulation in the rictor KO animals.

3.8. Inhibition of Akt in vitro attenuates 5HT_{2A} receptor-mediated ERK1/2 phosphorylation

To define whether the decrease in Akt function alone in KO mice is responsible for the impaired agonist-mediated 5-HT_{2A} receptor signaling (Fig. 7), we inhibited Akt pharmacologically in HEK-293 cells stably expressing the 5HT_{2A} receptor. This was achieved with an allosteric Akt inhibitor specific for isoforms 1 and 2 (Akt1/2 inhibitor), which blocks phosphorylation of Akt (Lindsley et al., 2005; Speed et al., 2010). In figure 8A, we show that in cells treated with vehicle, DOI (5 min) induced an increase in p-ERK1/2. This increase was also associated with an increase in Akt phosphorylation (Fig. 8A). In contrast, in cells treated with the Akt1/2 inhibitor, there was a significant decrease in this DOI-mediated effect. The Akt inhibitor was effective in ablating the p-ERK1/2, pAkt-473 and pAkt-308 response, but did not alter the total levels of either ERK1/2 or Akt (Fig. 8A). Quantitation of Akt inhibition on p-ERK1/2 levels is shown in Fig. 8B. (Inset: The cells display a typical response to 5-HT, substantiating the viability of the cell line for 5-HT receptor signaling). These data suggest that Akt is directly influencing 5-HT_{2A} receptor signaling.

3.9. Inhibition of Akt in vitro increases 5HT_{1A} receptor expression

We next wanted to understand whether pharmacological inhibition of Akt activity alters 5HT_{1A} receptor expression. HEK-293 cells stably expressing 5HT_{1A} receptors were incubated (16 hr) with the Akt1/2 inhibitor (Lindsley et al., 2005) since 1 hr incubation was ineffective in causing changes in 5HT_{1A} receptor expression (data not shown). Treatment with the Akt1/2 inhibitor (5 μ M) caused a significant increase in the amount of detectable 5HT_{1A} receptor in the immunoblots (Fig. 9A,B). These data are consistent with the increased receptor plasma membrane expression observed in the rictor KO cortices (Fig. 4).

4. DISCUSSION

The global obesity epidemic correlates with a staggering increase in diabetes. In 2000, the WHO estimated that 2.2% of the total global population was diabetic and predicted a two-fold increase to 4.4% by 2030 (Wild et al., 2004). Notably, a key aspect of type 2 diabetes is its comorbidity with neuropsychiatric disorders including depression, schizophrenia, anxiety, and drug addiction (Niswender et al., 2011), as well as gestational diabetes contributing to offspring risk of neurodevelopmental disorders including autism and attention deficit hyperactivity disorder (Krakowiak et al., 2012; Lyall et al., 2012; Nomura et al., 2012). These brain disorders have been linked, at least in part, with dysregulated 5-HT neurocircuitry (Daws et al., 2011). The association between mental and metabolic disorders (e.g. diabetes) has been well established, but the underlying mechanism(s) for this link, which probably include genetic, environmental as well as behavioral factors, remain under investigation. Recently, we have shown in rodents that an obesogenic diet that promotes diabetes reduces central insulin signaling and impairs brain Akt function (Speed et al., 2011). Thus, considering the role played by Akt in diabetes and obesity as well as the role of

5-HT in neuropsychiatric disorders, we sought to investigate a possible role of Akt in controlling neuronal 5-HT signaling and homeostasis.

To probe the specific impact of Akt signaling dysfunction on the 5-HT system, we used neuronal deletion of the rictor protein, thereby ablating Akt phosphorylation at Ser473. The resulting rictor KO mice do not show any changes in the levels of 5-HT in any of the brain regions examined (Fig. 1). Furthermore, there were no significant changes in the turnover rate of brain 5-HT. Since promoter gene polymorphisms in the SERT have been described to be associated with type 2 diabetes, eating disorders, and obesity (Lan et al., 2009; Toperoff et al., 2012; Zhao et al., 2012), we examined the levels of SERT in our FLOX and rictor KO mice. They were not significantly different between the genotypes in either the midbrain or the cortex (Fig. 3).

In contrast to normal 5-HT levels and SERT binding, we show that 5-HT_{1A} and 5-HT_{2A} receptor binding are significantly elevated in the rictor KO mice (Fig. 3). The changes in cortical receptor expression are specific since the levels of 5-HT_{2C} receptor and IR β (data not shown) were unchanged. Experimentally-induced diabetes, which induces impairments in Akt function in brain (Robertson et al., 2010), has been shown to increase cortical 5-HT_{2A} receptor density (Sumiyoshi et al., 1997). Moreover, diet-induced obesity has been linked to an increase in 5-HT_{1A} and 5-HT_{2A} receptor density in rats (Park et al., 1999). There is also evidence that diabetes leads to increased cortical 5-HT_{1A} receptor levels in humans (Price et al., 2002). The mechanism for this observed increased receptor density is not readily apparent. In theory, exposure to decreased levels of cortical dopamine in these rictor KO mice (Siuta et al., 2010) could alter receptor expression, since dopamine has been shown to be a ligand for 5-HT_{1A} and 5-HT_{2A} receptors (Bhattacharyya et al., 2006; Oz et al., 2003), and causes internalization and recycling of the 5-HT_{2A} receptor (Bhattacharyya et al., 2006). Taken together, our data suggest that neuronal Akt-473 phosphorylation can influence cortical 5-HT receptor expression.

It is noteworthy that SERT levels remain unaltered in the face of these changes in 5-HT receptor levels, a finding which does not preclude, however, the possibility of SERT/5-HT interactions. Studies of transporter/receptor interactions have increased considerably over the last decade (such as D1 dopamine and NMDA receptors mediating GABA transporter function in rat striatum (Schoffelmeer et al., 2000), as well as glycine transporter activity in rat hippocampus, and DAT and D2 dopamine receptors (Bertolino et al., 2009) or cannabinoid receptors (Schulze et al., 2012), as well as NET with the neurokinin-1 receptor (Arapulisy et al., 2013)). Yet, those of specifically SERT/5-HT receptors are emerging slowly. Functional interactions between SERT and 5-HT receptors have been demonstrated in pulmonary arteries (Morecroft et al., 2005). Indirect interactions between the two have been studied in SERT-deficient mice, where the consequences of 5-HT receptor activation stem from increased availability of 5-HT in the SERT KO background (Fox et al., 2010). Centrally, SERT interactions with other G protein-coupled receptors, such as the A3 adenosine receptors in mouse midbrain serotonergic neurons and heterologous expression systems (Zhu et al., 2011), where SERT physically complexes with and functionally regulates the receptor. In humans, the interaction of SERT and striatal D2/D3 dopamine receptors has recently been hypothesized to contribute to the mechanism of harm avoidance in

eating disorders (Bailer et al., 2013). In the future, therefore, it will be interesting to see whether central, direct SERT and 5-HT receptors interact not only physiologically, but also pathophysiologically as in models of diabetes or insulin signaling impairment.

The genetic neuronal deletion of RICTOR also leads to profound increase in cortical 5-HT_{1A} receptor ligand binding. This increase appears to not be due to augmented synthesis of the receptor since total receptor expression of cortical 5-HT_{1A} receptor is unaltered in KO mice (Fig. 4). The cortical slice biotinylation experiments reveal more receptor at the cell surface available for ligand binding, an effect that could be due to altered trafficking dynamics such as a slower rate of internalization of the 5-HT_{1A} receptor. The increased cell surface receptor expression could explain the increased cortical radioligand binding observed in rictor KO mice. This is because [³H]-8-OH-DPAT binds with high affinity only to those 5-HT_{1A} receptors which are G protein-coupled (Emerit et al., 1990; Hall et al., 1985; Mongeau et al., 1992; Nenonen et al., 1994). We find that ablation of Akt-473 phosphorylation alters the cortical distribution of the 5-HT_{1A} receptor in the rictor KO mice, but not that in the hippocampus (Fig. 4). The reason for this increase in cell surface 5-HT_{1A} receptor in the cortex is not readily obvious. Since central serotonergic imbalance or drugs that function centrally on serotonin levels such as SSRIs have been linked to hyperglycemia (Nonogaki et al., 1998; Yamada et al., 1999), insulin resistance (Chen et al., 2012) and diabetes (Lam and Heisler, 2007), our observed 5-HT_{1A} receptor increase might represent a compensatory strategy to overcome the manifestations of the disease. In support of this hypothesis is the finding that SERT KO mice exhibit an attenuated insulin-induced increase of pAKT (Chen et al., 2012). Furthermore, a recent study showed that insulin-resistance rendered normal serotonin-dependent phosphorylation of Akt in the hypothalamus nonfunctional (Papazoglou et al., 2012). Thus, our data underscores the importance of an intact central insulin-serotonergic signaling axis under physiologic conditions.

The 5-HT_{1A} receptor has important roles in behavior and physiology, including thermoregulation (Bert et al., 2006; Ramboz et al., 1998; Richardson-Jones et al., 2010; Richardson-Jones et al., 2011). We show that the increase in 5-HT_{1A} surface expression is associated with a greater hypothermic response to 8-OH-DPAT in rictor KO mice (Fig. 5). These data are consistent with a transgenic mouse model of 5-HT_{1A} receptor overexpression in the cortex that shows enhanced 8-OH-DPAT-induced hypothermia (Bert et al., 2006). Thus, our data suggests that dysregulated Akt phosphorylation disrupts cortical 5-HT receptor expression and function with significant physiological consequences.

Although expression of the 5-HT_{2A} receptor is significantly increased in rictor KO mice, it is functionally impaired. Rictor KO mice fail to show the expected DOI-induced kinase activation (Schmid et al., 2008) after *in vivo* administration of DOI (Fig. 7), as evidenced by the lack of ERK1/2 as well as GSK-3 β phosphorylation. Unfortunately, slice biotinylation studies of 5-HT_{2A} receptor expression on the cell membrane are difficult due to the dearth of reliable antibodies. 5-HT_{2A} receptors, mainly found in cortical layers II, III, V and VI, are the main site of action of LSD-like hallucinogens (Nichols, 2004). The functional consequence of this lack of signaling is the blunted HTR in the rictor KO mice (Fig. 6). These findings are in line with previous work in which experimentally-induced diabetes significantly reduced the DOI-mediated HTR (Li and France, 2008; Miyata et al., 2004), an

alteration which could be restored by insulin replacement (Li and France, 2008). Together, these data suggest that the diminished behavioral response in the rictor KO mice is mediated by impaired 5-HT_{2A} receptor signaling.

In order to mechanistically probe whether ablation of Akt phosphorylation at Ser473 itself in the rictor KO mice contributes to altered 5-HT receptor signaling, we assessed the consequences of Akt inhibition in a heterologous expression system. Here, we show that pretreatment with an Akt1/2 inhibitor significantly diminished DOI-induced 5-HT_{2A} receptor-mediated ERK1/2 activation (Fig. 8). In mouse cortex, previous intriguing studies identify β -arrestin, Src and Akt as critical for the HTR to the agonist 5-HT (Gonzalez-Maesos et al., 2007; Schmid and Bohn, 2010) but not N-methyltryptamines or, presumably, DOI (Schmid et al., 2008). To our knowledge, our data are the first to demonstrate dependence of 5-HT_{2A} receptor signaling on Akt phosphorylation, specifically at Ser473. While 5-HT-mediated 5-HT_{2A} receptor signaling and behavior (HTR) are β -arrestin- and Akt-dependent, DOI-mediated 5-HT_{2A} receptor signaling (Schmid et al., 2008) and behavior (Schmid and Bohn, 2010) are not. Thus, it is conceivable that in our studies, DOI could also partially be acting *via* the β -arrestin/Akt-dependent pathway, thereby explaining our observations of significant (albeit not complete) reduction of the HTR in rictor KO mice in response to DOI. We could hypothesize, then, that rictor KO mice are not able to signal (Fig. 7) or elicit HTR (Fig. 6) in response to 5-HT_{2A} receptor activation because Akt is not functioning. Furthermore, if β -arrestin is also not functioning (by virtue of not forming the complex with Akt and Src (Schmid and Bohn, 2010)), this mechanism might explain why we observe more cortical 5-HT_{2A} receptor on the cell surface, since β -arrestin has been reported to mediate many 5-HT induced downstream events at 5-HT_{2A} receptors, including internalization (Abbas and Roth, 2008).

We also demonstrated that inhibition of Akt function stimulates an increase in 5-HT_{1A} receptor density (Fig. 9). This increase is comparable to that observed in the cortexes of the rictor KO mice. No previous studies have demonstrated an effect of Akt on 5-HT_{1A} expression or trafficking.

CONCLUSIONS

Our data pose that inhibition of phosphorylation of Akt is, at least in part, responsible for the upregulation and potential uncoupling of two 5-HT receptor subtypes, both *in vivo* and *in vitro*. Together, they suggest that the cross-talk between these two signaling pathways (insulin and 5-HT receptors) could contribute to the comorbidity of diseases driven by insulin resistance (e.g. diabetes) and disorders associated to central serotonergic disturbances, including depression and drug abuse.

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Abbreviations

5-HT	serotonin
Akt	protein kinase B
DOI	(±)-2,5-dimethoxy-4-iodoamphetamine hydrochloride
ERK1/2	extracellular signal-regulated protein kinases 1 and 2
GSK-3β	glycogen synthase kinase-3beta
KO	knock out
8-OH-DPAT	[8-hydroxy-2-(di- <i>n</i> -propylamino)tetralin]
HTR	head-twitch response
SERT	serotonin transporter

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HIGHLIGHTS

Neuronal insulin regulates central monoamine homeostasis via down-stream kinase Akt.

Neuronal ablation of p-Akt at Ser473 increases cortical 5-HT_{1A} and -_{2A} receptors.

Elimination of p-Akt at Ser473 changes 5-HT receptor subtype signaling and behaviors.

We identified a novel link between Akt dysfunction (diabetes) and mental illness.

Figure 1.

5-HT content in brain. Tissue content of 5-HT in different brain regions. HPLC results are the mean $\pm$ SEM ng/mg of protein, $n=4-15$; $p>0.05$ by Student's t test comparing FLOX against KO mice.

Figure 2.

Neuronal rictor deletion does not alter SERT expression in cortex and midbrain.

Homogenized membranes were incubated with [³H]Citalopram [3.8 nM] for 45 minutes at room temperature. Non-specific binding was determined using unlabeled Fluoxetine [10 μM]. Data are expressed as mean ± SEM, n=3-10; p > 0.05 by Student's *t* test. Inset: Neuronal rictor deletion in midbrain ablates pAkt-473, but not pAkt-308 or Akt protein, as demonstrated by immunoblotting. pAkt-473 values in both FLOX and rictor KO midbrain were quantified and normalized to Akt. n=5/genotype, *p < 0.05 by Student's *t* test.

Figure 3.

Cortical receptor binding of 5-HT_{1A} and 5-HT_{2A} receptors is significantly increased in KO mice. A. Homogenized membranes were incubated with [³H]mesulergine [10 nM] in the presence or absence of ritanserin [8 mM] to measure non-specific and total binding, respectively. Specific binding was calculated by subtracting non-specific binding from total binding for 5-HT_{1A} receptors and corrected for bound protein (fmol/mg). Reaction buffer included the 5-HT_{1A/2A} receptor antagonist spiperone [100 nM] and the 5-HT_{7R} antagonist SB269970 [100 nM]. B. Homogenized membranes were incubated with [³H]8-OH-DPAT [10 nM] in the presence or absence of WAY100635 [10 μM] to measure non-specific and total binding for 5-HT_{2A} receptor, respectively. Reaction buffer included the 5-HT₇ receptor antagonist, SB269970 [100 nM]. Data are expressed as mean ± SEM, n=4-9/genotype, *p<0.05 by Student's *t* test.

Figure 4.

Neuronal rictor deletion results in increased cortical 5-HT_{1A} receptor surface density. Levels of surface 5-HT_{1A} receptor are obtained from the biotinylation fraction of cortical slices (surface). The optical density is shown for both FLOX (white bar) and rictor KO mice (black bar), where the surface optical density is normalized to its respective total. Representative immunoblots are shown, including a blot for Na⁺/K⁺ ATPase to serve as a plasma membrane/loading control, and tyrosine hydroxylase (TH) which is absent in the biotinylated fractions since it is a cytosolic protein. n=13/genotype, mean ± S.E.M., *p < 0.05 by Student's *t* test. Inset: Hippocampal slices show no difference in surface 5-HT_{1A} receptor expression between FLOX and rictor KO mice. n=6/genotype, p > 0.05 by Student's *t* test.

Figure 5.

Hypersensitivity to 8-OH-DPAT-induced hypothermia in rictor KO mice. Body temperature of the mice was measured using implantable programmable temperature transponders and scanning them with temperature probe. Mice received a s.c. injection of 2.0 mg/kg of the 5-HT_{1A/7} receptor agonist 8-OH-DPAT, which mediates a hypothermic response. Body temperature measurements were taken every 10 minutes for an hour post-injection, as well as 20 minutes prior to injection. Data are expressed as mean $\pm$ SEM, n=7/genotype; *= p <0.05 by two-way ANOVA, followed by Bonferroni post-hoc test.

Figure 6.

DOI-induced head twitch response is attenuated in KO mice. Mice received an i.p. injection of 1.0 mg/kg of DOI (black bars) or saline (white bars) 30 minutes before testing. Head twitches were recorded over three 5-minute periods, which were averaged together. For the studies done with the 5-HT_{2A} receptor antagonist (gray bars), the same mice were injected with 0.25 mg/kg M-100907 17 minutes prior to the DOI injection, and the same procedure for counting head twitches was used. Data are expressed as mean $\pm$ SEM, n=5/genotype; *= p <0.05 by two-way ANOVA, followed by Bonferroni post-hoc test.

Figure 7.

Cortical 5-HT_{2A} receptor-mediated ERK1/2 and GSK-3β activation is impaired in rictor KO mice. Mice received an i.p. injection of 5.0 mg/kg of 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI, gray bars), a 5HT_{2A/2C} agonist, or vehicle (white bars) for 15 minutes. Cortical microdissection was performed rapidly thereafter. Representative immunoblots for ERK1/2 (A) and GSK-3β (B) are shown. Quantification was done by normalizing the levels of kinase phosphorylation to the total kinase. Basal active and total levels of ERK1/2 and GSK-3β were the same in FLOX and KO mice. Data are expressed as mean ± SEM, n=6/genotype; *= $p < 0.05$ by one-way ANOVA followed by Dunnett's post-hoc test in FLOX mice; †= $p < 0.05$ by one-way ANOVA followed by Dunnett's post-hoc test in KO mice.

Figure 8.

Akt1/2 inhibitor-mediated attenuation of DOI-induced ERK1/2 phosphorylation in HEK-293 cells expressing the 5-HT_{2A} receptor. **A.** Representative immunoblots of p-ERK1/2, ERK1/2, pAkt-473, pAkt-308 and Akt in HEK-293 cells expressing 5-HT_{2A} receptors. Cells were preincubated with an Akt1/2 inhibitor (5 μ M) or vehicle for 60 minutes. DOI (10 μ M)-induced ERK1/2 activation for 5 minutes was measured. **B.** Quantitation of the immunoblots, expressed as percent of control (vehicle) stimulation at 5 minutes. Data are expressed as mean $\pm$ SEM, n=4, *p<0.05 by Student's *t* test. Inset: 5-HT-mediated pERK1/2 activation (10 μ M, 5 min) in HEK-293 cells expressing the 5-HT_{2A} receptor.

Figure 9. Akt1/2 inhibitor-mediated increase in 5-HT_{1A} receptor expression. HEK-293 cells expressing 5-HT_{1A} receptors were pretreated for 16 hours with the Akt1/2 inhibitor (5 μ M) or vehicle. A. Representative immunoblots of 5-HT_{1A} receptor and actin. B. Quantitation of the immunoblots, expressed as % of control. Data are expressed as mean $\pm$ SEM, n=4, *p<0.05 by Student's *t* test.