Brief communication

Glucose intolerance induced by blockade of central FGF receptors is linked to an acute stress response

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ABSTRACT

Objective: Central administration of ligands for fibroblast growth factor receptors (FGFRs) such as fibroblast growth factor-19 (FGF19) and FGF21 exert glucose-lowering effects in rodent models of obesity and type 2 diabetes (T2D). Conversely, intracerebroventricular (icv) administration of the non-selective FGFR inhibitor (FGFRi) PD173074 causes glucose intolerance, implying a physiological role for neuronal FGFR signaling in glucose homeostasis. The current studies were undertaken to identify neuroendocrine mechanisms underlying the glucose intolerance induced by pharmacological blockade of central FGFRs.

Methods: Overnight fasted, lean, male, Long-Evans rats received icv injections of either PD173074 or vehicle (Veh) followed 30 min later by performance of a frequently sampled intravenous glucose tolerance test (FSIGT). Minimal model analysis of glucose and insulin data from the FSIGT was performed to estimate insulin-dependent and insulin-independent components of glucose disposal. Plasma levels of lactate, glucagon, corticosterone, non-esterified free fatty acids (NEFA) and catecholamines were measured before and after intravenous (iv) glucose injection.

Results: Within 20 min of icv PD173074 injection (prior to the FSIGT), plasma levels of lactate, norepinephrine and epinephrine increased markedly, and each returned to baseline rapidly (within 8 min) following the iv glucose bolus. In contrast, plasma glucagon levels were not altered by icv FGFRi at either time point. Consistent with a previous report, glucose tolerance was impaired following icv PD173074 compared to Veh injection and, based on minimal model analysis of FSIGT data, this effect was attributable to reductions of both insulin secretion and the basal insulin effect (BIE), consistent with the inhibitory effect of catecholamines on pancreatic β-cell secretion. By comparison, there were no changes in glucose effectiveness at zero insulin (GEZ) or the insulin sensitivity index (S). To determine if iv glucose (given during the FSIGT) contributed to the rapid resolution of the sympathoadrenal response induced by icv FGFRi, we performed an additional study comparing groups that received iv saline or iv glucose 30 min after icv FGFRi. Our finding that elevated plasma catecholamine levels returned rapidly to baseline irrespective of whether rats subsequently received an iv bolus of saline or glucose indicates that the rapid reversal of sympathoadrenal activation following icv FGFRi was unrelated to the subsequent glucose bolus.

Conclusions: The effect of acute inhibition of central FGFR signaling to impair glucose tolerance likely involves a stress response associated with pronounced, but transient, sympathoadrenal activation and an associated reduction of insulin secretion. Whether this effect is a true consequence of FGFR blockade or involves an off-target effect of the FGFR inhibitor requires additional study.

Keywords Central FGF receptors; FGF receptor inhibitor PD173074; Frequently sampled intravenous glucose tolerance test; Sympathoadrenal response; Minimal model; Glucose metabolism

1. INTRODUCTION

Growing evidence suggests that in rodent models of obesity and type 2 diabetes (T2D), glucose homeostasis is improved by activation of neuronal fibroblast growth factor receptors (FGFRs) [1–3]. Fibroblast growth factor-19 (FGF19) and its rodent homolog, FGF15 and FGF21 are members of the endocrine family of fibroblast growth factors (FGF) that elicit glucose-lowering effects following either central or systemic

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Abbreviations: AIRg, acute insulin response to glucose; AUC, area under the curve; bmax, basal insulin; BIE, basal insulin effect; CNS, central nervous system; DI, disposition index; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; FGF19, Fibroblast growth factor-19; FGFRi, FGFR inhibitor; FSIGT, frequently sampled intravenous glucose tolerance test; GE, glucose effectiveness; GEZ, glucose effectiveness at zero insulin; icv, intracerebroventricular; Kp, glucose disappearance constant; NEFA, non-esterified free fatty acids; SG, glucose effectiveness at basal insulin; S, insulin sensitivity

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administration [1–5]. The hypothesis that the central nervous system (CNS) is a target for the anti-diabetic action of systemic FGF19 administration is supported by evidence that its glucose-lowering effect is blunted by intracerebroventricular (icv) pre-treatment with the non-selective FGFR inhibitor (FGFRI) PD173074 [1]. Moreover, acute central FGFR blockade by icv PD173074 administration in normal, non-diabetic animals causes glucose intolerance [6], implying a physiological role for central FGFR signaling in the control of glucose homeostasis. To investigate the mechanism(s) whereby central FGFR antagonism impairs glucose tolerance, we injected PD173074, a highly selective inhibitor of FGFR1–3 [7,8] (with somewhat reduced affinity for FGFR4 [8,9]) into the third cerebral ventricle of normal male rats. We selected this inhibitor for our studies because of its wide use as a tool to investigate FGFR signaling in both in vitro and in vivo model systems [1,5–9], which include the previous demonstration that glucose intolerance is induced following its icv injection [6]. To quantify glucose tolerance and its determinants, we employed minimal model analysis of plasma glucose and insulin data obtained during a frequently sampled intravenous glucose tolerance test (FSIGT), a method that has been validated in humans [10], primates [11], dogs [12], and rodents [1,13,14]. This analysis provides estimates of insulin sensitivity (S) and glucose effectiveness at basal insulin (Sg, a measure of glucose disposal independent of glucose-induced insulin secretion), which can be subdivided into its two components: the basal insulin effect (BIE) and glucose effectiveness at zero insulin (GEZI) [15]. From the FSIGT we also calculated the acute insulin response to glucose (AIRg) as a measure of islet β-cell function in response to a glucose load. In addition, we measured plasma levels of lactate, glucagon, corticosterone, non-esterified free fatty acids (NEFA) and catecholamines both before and after intravenous (iv) glucose administration. Our findings show that the major determinant of glucose intolerance associated with acute central FGFR blockade is the inhibition of the acute phase insulin secretory response to glucose, which is potentially attributable to a marked, but transient sympathoadrenal activation.

2. METHODS

2.1. Animals
Adult, male, Long-Evans rats (Harlan Laboratories, Indianapolis, IN) were housed individually under specific pathogen-free conditions in a temperature-controlled room with a 12:12 h light:dark cycle and provided with ad-libitum access to water and standard laboratory chow (LabDiet, St. Louis, MO) unless otherwise stated. All procedures were performed in accordance with NIH guidelines for the care and use of animals and were approved by the Institutional Animal Care and Use Committee at the University of Washington.

2.2. Surgery
Cannulation of the 3rd ventricle (26-ga; Plastics One, Roanoke, VA) was performed under isoflurane anesthesia using stereotactic coordinates based on [16]; midline: –2.2 mm posterior to bregma; 7.7 mm below the skull surface. Catheterization of both the carotid artery and the internal jugular vein was performed during the same surgical session. Animals received buprenorphine hydrochloride (Reckitt Benckiser Pharmaceuticals Inc., Richmond, VA) at the completion of the surgery and were allowed to recover for at least 5–7 d while food intake and body weight were recorded.

2.3. Intracerebroventricular (icv) injections
The FGFRI PD173074 (Tocris Bioscience, Minneapolis, MN) was dissolved in dimethyl-sulfoxide (DMSO) at a concentration of 50 μg/μl. The dosage for icv administration (150 μg) was based on the findings of Ryan et al. [6], who showed that glucose intolerance is induced in normal rats following icv administration of this dose. Either PD173074 or DMSO vehicle (Veh) was administered into the 3rd ventricle in a final volume of 3 μl using a (33-ga) needle that extends 2 mm beyond the tip of the icv cannula over a period of 60 s. Correct icv cannula placement was confirmed by verifying that icv injection of 5-Thio-D-glucose (200 μg) provoked a doubling of blood glucose levels within 1 h post injection [17].

2.4. Frequently sampled intravenous glucose tolerance test (FSIGT)
The FSIGT was performed in body weight-matched, overnight fasted rats at least 5–7 d after cannulation of the 3rd ventricle. Blood sampling was performed via a surgically implanted carotid arterial catheter in unrestrained, conscious animals. Baseline fasting blood samples were drawn at t = 0 min. Blood samples were drawn at 10 and 0 min. Based on a previous protocol [13], a bolus of 50% dextrose (1 g/kg body weight) was injected iv over a period of 15 s at t = 0 min. Blood (100 μl) was sampled for measurement of glucose using a hand-held glucometer (Nova Max plus, Nova Diabetes Care, Billerica, MA) and subsequent assay of plasma insulin and lactate levels at time points 1, 2, 4, 8, 12, 16, 20, 30 and 60 min after commencing the glucose injection. Additional samples were obtained for blood glucose measurement at 3, 5, 6, 10, 14, 18, 25, 40 and 50 min using a hand-held glucometer.

2.5. Minimal model analysis and calculations
The plasma insulin and blood glucose profiles generated from the FSIGTs were analyzed using MinMod software to quantify Sg and S as previously described [13]. The AIRg was calculated based on insulin values between t = 0–4 min. BIE was calculated as the product of basal insulin (b, mU/l) and Sg. GEZI was calculated as Sg minus the BIE. Intravenous glucose tolerance was estimated as the glucose disappearance constant (Kg), calculated as the slope of the natural logarithm of blood glucose values from t = 4–25 min, expressed as percent change per min. The disposition index (DI), which is the product of Sg and AIRg, was calculated as a measure of β-cell function in response to a glucose load.

2.6. Plasma analysis
Arterial blood samples were collected into either EDTA-treated tubes (for measurement of insulin, lactate, glucagon, non-esterified free fatty acids (NEFA) and corticosterone) or EGTA/glutathione tubes (for measurement of catecholamines). Whole blood was centrifuged and plasma removed for subsequent measurement of plasma immunoreactive insulin, glucagon, and corticosterone levels by ELISA (Crystal Chem, Inc., IL; Mendocia, NC; and ALPCO, NH). Plasma lactate levels were determined using a GM90 glucose direct analyzer (Analox Instruments). Plasma NEFAs were quantified with an enzymatic colorimetric assay (Wako Diagnostics, VA). Plasma catecholamine levels were measured using a sensitive and specific radioenzymatic assay [19].

2.7. Statistical analysis
All results are expressed as mean ± SEM. Statistical analyses were performed using GraphPad Prism, version 5.04, 2010 (GraphPad Software, San Diego, CA). A two-sample unpaired Student’s t-test (normally distributed data) or a Mann–Whitney test (data not normally distributed) was used for two-group comparisons. In all instances, probability values of less than 0.05 were considered significant. To identify determinants of glucose tolerance (Kg) affected by icv administration of the FGFRI, we employed the Hayes PROCESS macro
for SPSS (http://www.processmacro.org/) to perform formal mediation analyses, an alternative to standard multiple regression for vetting potential causality [19]. In brief, mediation analysis yields an effect statistic and 95% confidence interval for each candidate mediator variable (M) considered for inclusion in the causal pathway through which a primary X variable (e.g., group membership) influences a Y variable of interest. When the 95% confidence interval for M excludes an effect value of zero, then the effect statistic is significant at \( p < 0.05 \), consistent with concept that M mediates a significant portion of the association of X with Y.

3. RESULTS

3.1. Effect of icv injection of the FGFRi PD173074 on glucose tolerance

Relative to icv vehicle (Veh), icv infusion of the FGFRi elevated basal blood glucose levels (t = 0 min; 171 ± 6 mg/dl for icv FGFRi vs. 124 ± 5 mg/dl for icv Veh, \( p < 0.0001 \); n = 16/group) and worsened glucose tolerance during the FSIGT (Figure 1A) as evidenced by a 24% reduction of Kg (Figure 1B, \( p < 0.0001 \)), consistent with the findings of Ryan et al. [6]. This effect was associated with a 38% decrease in the insulin response as estimated by the AIRg (Figure 1C and D, \( p < 0.001 \)), consistent with a role for reduced insulin secretion in the impairment of glucose tolerance induced by acute central FGFR blockade.

In agreement with previous evidence [1], plasma lactate levels increased rapidly, but modestly and transiently, following the iv glucose bolus; Figure 2C, \( p < 0.05 \)) and by evidence of behavioral agitation that was observed as early as 5 min post injection of PD173074, and which subsided within 30 min (data not shown).

3.2. Effect of icv FGFRi on the sympathoadrenal system

To explain the combination of glucose intolerance, reduced glucose-induced insulin secretion and elevated lactate levels, we considered the possibility that acute blockade of central FGFR signaling induces sympathoadrenal activation. To investigate this hypothesis, we measured plasma catecholamine levels both before and after the iv glucose bolus. We found that relative to icv Veh, plasma levels of norepinephrine and epinephrine increased by \( \sim 3 \)- and 8-fold, respectively, 20 min following icv PD173074 injection (10 min prior to iv glucose administration) (Figure 2A and B, \( p < 0.0001 \)) and rapidly declined thereafter, returning to baseline 8 min after iv glucose bolus (Figure 2A and B). This acute, transient sympathoadrenal response is highly suggestive of a stress response, a possibility strengthened both by the increase of plasma corticosterone levels (10 min prior to iv glucose bolus; Figure 2C, \( p < 0.05 \)) and by evidence of behavioral agitation that was observed as early as 5 min post injection of PD173074, and which subsided within 30 min (data not shown).

3.3. Role of iv glucose in resolution of sympathoadrenal activation

Because this evidence of sympathoadrenal activation subsided shortly following the iv glucose bolus, we considered the possibility that acute inhibition of CNS FGFR signaling activates neurocircuits that are also activated by neuroglucopenia [20], such that the effect is rapidly ameliorated by iv glucose administration. We tested this hypothesis in two ways. First, we found that plasma glucagon levels, which are known to increase in response to neuroglucopenia, did not increase following icv FGFRi injection relative to icv Veh (10 min prior to FSIGT; Figure 2D, \( p = ns \)), although the hormone levels did decrease following iv glucose (Figure 2D, \( p < 0.05 \)) as is widely reported [21]. These glucagon observations are therefore inconsistent with this hypothesis.

Secondly, to further refute the neuroglucopenia hypothesis, we measured the sympathoadrenal response induced by icv FGFRi in rats that subsequently received an iv bolus of saline, rather than glucose. Consistent with our earlier observations (Figure 2A and B), we again found that plasma norepinephrine and epinephrine levels were markedly elevated 20 min post icv FGFRi injection (10 min prior to iv bolus; Figure 2E and F) and that both hormone levels promptly returned to baseline (8 min post iv bolus) irrespective of whether iv glucose or iv saline was given (Figure 2E and F, \( p = ns \)). Collectively, these data suggest that the robust sympathoadrenal response to icv FGFRi is attributable to an acute stress distinct from that induced by neuroglucopenia.

3.4. Minimal model analysis of the effect of icv FGFRi on glucose tolerance

Icv FGFRi treatment had no significant effect on either SI or DI (Figure 3A and B, \( p = ns \)), although S0 was modestly reduced (by 23%; Figure 3C, \( p < 0.05 \)). Additional analysis of this effect revealed that the BIE was significantly reduced by icv FGFRi (Figure 3D, \( p < 0.01 \)), but GEZI was not (Figure 3E, \( p = ns \)). Our finding that acute blockade of central FGFRs impairs glucose tolerance via reductions of both insulin secretion and the BIE (and not GEZI) is consistent with the well-known effect of catecholamines to inhibit pancreatic β-cell insulin secretion [22].

3.5. Determinants of decreased glucose tolerance (Kg) with icv FGFRi

In a mediation model designed to jointly assess S0, AIRg and GEZI as potential mediators of the effect of the icv FGFRi on glucose tolerance (Kg), AIRg emerged as the sole significant potential mediator variable (effect statistic \(-0.0052 \pm 0.0024\%\text{min}, 95\%\text{ confidence interval} [-0.0120, -0.0018])\), while neither S0 nor GEZI were identified as potential mediator variables. This analysis also estimated that the effect of icv FGFRi on AIRg accounted for 35 ± 17% of the total effect of icv FGFRi administration on glucose tolerance, 95% confidence interval [0.13, 0.84]. Overall, this analysis is consistent with the interpretation that icv injection of the FGFRi inhibits acute phase insulin secretion, and that this effect was a major determinant of the resultant change of glucose tolerance (Kg).

4. DISCUSSION

Neuronal FGFR signaling is implicated in the regulation of both energy balance [6,23,24] and glucose homeostasis [1–3]. The current studies were undertaken to investigate the neuroendocrine and metabolic consequences of acute pharmacological blockade of central FGFR receptors induced by a single icv injection of the FGFRi PD173074. This compound has seen wide use in both in vivo and in vitro studies [1,5–9], and a recent study by Ryan et al. [6], showed that following icv administration, glucose tolerance was rapidly impaired in normal rats. This observation raises the important possibility that normal glucose tolerance depends on intact neuronal FGFR signaling. The strengths of
our current study are that 1) we employed the minimal model, which enables us to quantify insulin sensitivity and glucose effectiveness, while concurrently assessing insulin secretion and glucose tolerance, and 2) we measured a variety of neuroendocrine responses with the potential to impact glucose tolerance. This approach allows us to extend previous evidence that icv administration of PD173074 impairs glucose tolerance in rats [6] by demonstrating that this effect is associated with, and likely secondary to, a marked but transient sympathoadrenal activation suggestive of an acute stress response. Uncertainty as to whether the sympathoadrenal response we observed is a true consequence of acute FGFR blockade or instead involves an off-target effect of PD173074 in the brain is a key limitation of our study. Interestingly, Perry et al. [25], recently reported that icv administration of FGF19 ameliorates hyperglycemia in poorly

Figure 1: Effect of icv FGFR inhibitor on determinants of glucose tolerance in lean, fasted rats. icv FGFR inhibitor (FGFRi; PD173074; 150 μg) or vehicle DMSO (Veh) was administered via the 3rd cerebral ventricle in overnight fasted, lean rats (n = 16/group) and 30 min later (t = 0 min), animals underwent a frequently sampled intravenous glucose tolerance test (FSIGT). (A) Blood glucose levels (mg/dl), (B) glucose tolerance index, K_g (calculated as the slope of the natural logarithm of glucose from t = 4–25 min, expressed as percent change per min), (C) plasma insulin levels (ng/ml), (D) acute insulin response to glucose (AIR_g; mUL⁻¹min⁻¹), (E) plasma lactate levels (mmol/l), (F) the lactate area under the curve (AUC) for pre- (−10 to 0 min) and post (1−20 min) iv glucose bolus, (G) plasma NEFA levels (mmol/l), and (H) NEFA ΔAUC (−10 to 60 min). Data are presented as the mean ± SEM; *p < 0.05 vs. icv Veh; #p < 0.05 vs. icv Veh, pre iv bolus.
controlled diabetes by suppression of the stress (hypothalamic-pituitary-adrenal (HPA)) axis. This finding raises the possibility that FGFR signaling in the brain tonically inhibits HPA axis activity, in which case acute disruption of brain FGFR signaling might be expected to induce a stress response. It will therefore be of interest in future studies to determine whether the findings of Perry et al. [25] can be generalized to non-diabetic animals, and to test whether the effect of icv FGFRi treatment to impair glucose tolerance in rats is negated by adrenalectomy. Studies that investigate the neurocircuitry underlying HPA axis inhibition by FGF receptor activation will also be important; once the underlying mechanism is identified, it will be possible to determine if targeted disruption of the CNS FGF receptors involved replicates the stress response induced by icv injection of PD173074.

The combination of hormonal and metabolic responses observed in our studies is generally consistent with, and potentially explained by, the hypothesis that icv PD173074 injection induces robust sympathoadrenal activation. Among these responses is a marked and rapid elevation of plasma lactate levels, consistent with the known effect of catecholamines to stimulate glycogenolysis in both muscle and liver, with increased glycolysis leading to subsequent lactate release [26,27]. In addition, the increase of basal glucose levels may involve catecholamine-mediated stimulation of liver glycogenolysis via activation of adrenergic receptors, which, in turn, activates glycogen phosphorylase [28]. Although catecholamines are potent stimulators of adipocyte lipolysis, the degree of NEFA elevation was modest relative to the catecholamine response. One potential explanation for this
finding is that markedly elevated lactate levels suppressed catecholamine-induced release of NEFAs, consistent with previous in vitro and in vivo findings [29,30].

Although the above responses might have been expected to reduce whole-body glucose utilization, our findings point to reduced insulin secretion as the predominant mechanism underlying the observed impairment of glucose tolerance. Specifically, we found that central injection of the FGFR antagonist blunted both glucose-induced insulin secretion and, based on minimal modeling of FSIGT data, reduced glucose effectiveness at basal insulin ($S_G$). Post-analysis of $S_G$-derived parameters revealed that icv FGFRi suppressed the basal insulin effect, but not glucose effectiveness at zero insulin. Moreover, the mediation model designed to jointly assess $S_I$, AIRg, and GEZI as potential mediators of the effect of the icv FGFRi on glucose tolerance revealed inhibition of acute phase insulin secretion (AIRg) by the FGFRi to be the major determinant of the observed decrement of glucose tolerance ($K_g$). This collection of findings is compatible with the well-known effect of $\alpha_2$-adrenergic receptor activation by catecholamines to inhibit insulin secretion from $\beta$-cells [22].

In this context, it is somewhat surprising that insulin sensitivity was not reduced following icv FGFRi, given the known effect of catecholamines to decrease insulin sensitivity as previously reported using a hyperinsulinemic-euglycemic clamp in rats [31] (unlike the FSIGT, the clamp methodology does not allow for the concurrent measurement of insulin secretion). Several potential explanations for this outcome can be considered. First, it is possible that the observed activation of sympathoadrenal outflow was too short-lived to cause insulin resistance. Another possibility is that the transient increase of locomotor activity exhibited by rats following icv FGFRi injection may have offset the suppressive effect of catecholamines on SI, as exercise is known to increase insulin sensitivity [32]. A third possibility is that the acute reduction of insulin secretion following icv FGFRi induced compensatory mechanisms to increase SI, therefore offsetting the negative effect of catecholamines and resulting in no overall effect. Additional studies are needed to distinguish between these three possibilities.

Since the resolution of elevated circulating catecholamines induced by icv PD173074 injection occurred rapidly following an iv glucose bolus (during the FSIGT), we considered the possibility that FGFRi antagonism
activated neurocircuits that are also activated in response to neuroglucopenia [20]. Several observations argue against this hypothesis. First, we found that plasma glucagon levels were not elevated following icv FGFRi injection, and increased glucagon secretion is a reliable response to neuroglucopenia. To test this hypothesis more directly, we investigated whether iv glucose is required for the observed normalization of elevated plasma epinephrine and norepinephrine levels seen following icv PD173074 injection. Our finding that the sympathoadrenal response was normalized even when iv saline was given indicates that the stress induced by acute FGFR injection was not ameliorated by the subsequent glucose bolus and hence is unlikely to involve neuroglucopenia.

A key unanswered question related to our findings is whether the sympathoadrenal response induced by icv PD173074 is secondary to an acute blockade of one or more FGF receptor isoforms or instead is due to a non-specific or toxic effect of this particular FGFR antagonist. In theory, this question might best be answered by icv administration of the drug to mice in which CNS FGF receptors have been deleted. Should the effect of icv PD173074 to impair glucose tolerance and induce a sympathoadrenal response be blocked in mice with hypothalamic deletion of FGFR1, for example, we would conclude that effects of PD173074 reflect the true consequences of acute blockade of brain FGFR signaling. From a practical standpoint, however, such a study is problematic for at least two reasons. First, the drug antagonizes each of the four known FGF receptors isoforms [7–9], and information regarding the receptor isoform involved in the stress response is currently lacking. Indeed, it is possible that each of the four receptors must be blocked to elicit the observed stress response. Second, FGF receptor signaling is essential for brain development, and embryonic deletion of genes encoding these receptors has severe phenotypic consequences [33]. Finally, other investigators have noted that PD173074 has a “narrow therapeutic window” with high toxicity due either to its broad FGFR specificity or its non-specific off-target effects [34,35]. As a result, small molecule inhibitors and/or neutralizing monoclonal antibodies for FGFRs that have greater selectivity and lower toxicity may be needed to resolve this issue. In the meantime, however, a note of caution is warranted with respect to the selectivity and lower toxicity may be needed to resolve this issue. In the meantime, however, a note of caution is warranted with respect to the use of PD173074 for in vivo applications.

4.1. Conclusions

Collectively, our findings provide the first evidence that icv injection of an FGFR antagonist elicits a rapid but transient stress response that is characterized by sympathoadrenal activation, elevation of plasma lactate levels, and impairment of both glucose-induced insulin secretion and glucose tolerance. In addition, by using the minimal model to separate out the insulin-dependent from the insulin-independent components, we report that the injection of FGFRi reduced S, by decreasing the effect of basal insulin, while not altering S. Future studies are warranted both to investigate links between brain FGF receptors, glucose homeostasis and the sympathetic nervous system, and to determine the extent to which a non-specific, off-target mechanism might contribute to the stress response elicited by icv administration of PD173074.

CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists.

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