# Insulin modulates emotional behavior through a serotonin-dependent mechanism

## Running title:

Insulin modulates the 5-HT system to control mood

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## Abstract

Type-2 Diabetes (T2D) is characterized by insulin resistance and accompanied by psychiatric comorbidities including major depressive disorders (MDD). Patients with T2D are twice more likely to suffer from MDD and clinical studies have shown that insulin resistance is positively correlated with the severity of depressive symptoms. However, the potential contribution of central insulin signaling in MDD in patients with T2D remains elusive. Here we hypothesized that insulin modulates the serotonergic (5-HT) system to control emotional behavior and that insulin resistance in 5-HT neurons contributes to the development of mood disorders in T2D. Our results show that insulin directly modulates the activity of dorsal raphe (DR) 5-HT neurons to dampen 5-HT neurotransmission through a 5-HT$_{1A}$ receptor-mediated inhibitory feedback. In addition, insulin-induced 5-HT neuromodulation is necessary to promote anxiolytic-like effect in response to intranasal insulin delivery. Interestingly, such an anxiolytic effect of intranasal insulin as well as the response of DR 5-HT neurons to insulin are both blunted in high fat diet-fed T2D animals. Altogether, these findings point to a novel mechanism by which insulin directly modulates the activity of DR 5-HT neurons to dampen 5-HT neurotransmission and control emotional behaviors, and emphasize the idea that impaired insulin-sensitivity in these neurons is critical for the development of T2D-associated mood disorders.

**Introduction**

Type 2 diabetes (T2D) is among the most prevalent chronic disease worldwide, and it is strongly associated with comorbidities including Major Depressive Disorder (MDD). Recent epidemiological studies indicate that the prevalence of MDD is at least twice as high among patients suffering from T2D (1). Interestingly, clinical evidence suggests that impairment of insulin signaling, resulting from insulin resistance, might be one of the underlying mechanisms for the development of MDD in patients with T2D. (2–4). Preclinical studies support this hypothesis since it has been shown that insulin resistant animal models of T2D exhibit both depressive- and anxiety-like behaviors (5–9). Nevertheless, it remains unclear whether peripheral or brain insulin resistance plays a causal role in T2D-associated mood disorders.

The impact of impaired brain insulin signaling on mood has been investigated using transgenic animal models presenting a deletion of the insulin receptor (IR) in selective brain cells or structures (10). For instance, Neuronal Insulin Receptor Knock-Out (NIRKO) mice, which display neuronal IR deletion, exhibit spontaneous depressive- and anxiety-like behaviors (11). These findings support the hypothesis that brain IR signaling is crucial for the regulation of emotional behavior. The dopaminergic system is known to modulate mood (12) and is a target of the action of insulin (13,14). However, the effect of insulin on the dopaminergic system does not seem to impact anxiety- or depressive-like behaviors (15). As such, the identity of the neuronal networks underlying the effects of insulin on mood remains to be determined.

The serotonergic (5-HT) neurotransmission is a well-established monoaminergic system involved in the etiology of psychiatric disorders and more specifically in the physiopathology of MDD and anxiety disorders (16). This system is also the main target of antidepressant drugs such as selective serotonin reuptake inhibitors (SSRIs), which represent the most prescribed class of antidepressants. Previous studies from our group have demonstrated that 5-HT neurotransmission is impaired in animal models of T2D-associated mood disorders (6,7,10).

Thus, we hypothesized that insulin directly modulates the 5-HT system to control emotional behavior.

Here, we used multiple approaches to assess the role of insulin in the modulation of 5-HT neurons and associated emotional behaviors. Our data reveal that insulin directly controls the activity of 5-HT neurons in the dorsal raphe (DR). In addition, we provide evidence that the anxiolytic-like effect of insulin requires modulation of the 5-HT system. Finally, we show that DR 5-HT neurons do not respond anymore to insulin in a model of T2D-associated mood disorders. Altogether, these findings identify a new mechanism by which insulin directly modulates the activity of DR 5-HT neurons to control emotional behaviors.

## Materials and Methods

### *Animals.*

Adult male mice (8-12 weeks) from different strains were collectively housed in conventional housing cages at NutriNeuro' or CRCA's animal facilities. Animals were maintained on a 12 hours light-dark cycle with *ad libitum* access to food and water. All experimental procedures were conducted in accordance with the European directive 2010/63/UE and approved by the French Ministry of Research and local ethic committees (APAFIS#: 16853; 12898; 22415; 8928). C57BL/6J were purchased from Janvier laboratory (Le Genest-Saint-Isle, France). Pet1-cre-mCherry mice were obtained by crossing Pet1-cre mice (gift from Dr. P Gaspar, (17)) with B6.Cg-Gt(ROSA)26Sortm9(CAG-tdTomato)Hze/J mice (Jackson Laboratory, Bar Harbor, Maine, USA). SeIRKO (**Se**rotonin **I**nsulin **R**eceptor **K**nock-**O**ut;, $cre^+::IRflox^{+/+}::mCherry^{+/-}$) mice were obtained by crossing $IR^{lox/lox}$ ($cre^-::flox^{+/+}$) mice (Jackson Laboratory) and Pet1-cre-mCherry ($cre^+::mCherry^{+/-}$) to specifically study insulin default of action only in 5-HT neurons. Littermates with $cre^+::flox^{-/-}::mCherry^{+/-}$ genotype were used as controls for the SeIRKO mice. To model T2D-associated emotional disorders in rodents, a sub-group of mice was fed a high fat diet (HFD; #D12451, Research Diet, New Brunswick, NJ, USA) or a standard diet (STD; #A04, Research Diet) for 16 weeks as previously described (6,7).

### *Drugs and treatments.*

*Serotonergic modulators:* To deplete 5-HT levels, mice were injected i.p. with p-chlorophenylalanine (pCPA, 150 mg/kg; Sigma, St-Quentin-Fallaviers, France) twice per day (9.00 AM and 6.00 PM) for three consecutive days prior to experiments. The 5-HT$_{1A}$ antagonist WAY 100635 (0.5-1 mg/kg; Tocris, Bristol, UK) was injected i.p. one hour before behavioral tests.

*Intranasal delivery procedure:* Mice were handled and then habituated to intranasal delivery injections two weeks prior to testing. The day of the experiment, mice were fasted for 18 hours before intranasal delivery of Human recombinant insulin (7 µg/µL insulin, diluted in NaCl 0.9%; Sigma) or vehicle (NaCl 0.9%) into mouse nares (5 µL/nare) to reach a final dose of 2IU insulin (18). Mice were replaced into their home cages and tested 60 minutes after IND injections. The dose of insulin was established based on a modified protocol (18). Compounds (incl. insulin) injected through the intranasal route reach the brain either by crossing the epithelium of the nasal cavity or by axonal transport along the trigeminal nerve (19). This last mechanism is the most relevant as the trigeminal nerve innervates the brain stem where the DR is located. Thus, intranasal injection of insulin lead to a rapid increase of insulin concentration in the brain stem with a $t_{max}$ of 15 min (20).

*Intra-raphe insulin injection:* Mice were anesthetized with chloral hydrate (400 mg/kg, i.p., with additional doses when necessary) and placed in a stereotaxic apparatus. A cannula was implanted 0.5 mm posterior to the interaural line on the midline and lowered into the DR, attained at 2.5 mm depth from the brain surface. Two days after half of the mice received 0.5 µL of artificial cerebro-spinal fluid (aCSF) (147 mM NaCl, 2.7 mM KCl, 1 mM MgCl2, 1.2 mM CaCl2, adjusted to pH 7.4 with sodium phosphate buffer 2 mM). The other half received 100 µIU of insulin in 0.5 µL aCSF. The injection flow was set to 0.1 µU/min and behavioral tests were performed 10 min after the end of the injection.

*Antidepressant add-on treatment experiment:* One hour prior to be subjected to the tail suspension test, mice were injected with an appropriate combination intranasal (insulin 2 IU or vehicle NaCl 0.9%) and intraperitoneal treatment (fluoxetine-hydrochloride 18 mg/kg; Sigma; or vehicle 0.9%).

*Behavioral tests.*

Animals were tested in different anxiety-like and depressive-like behavioral paradigms as previously described (Supplementary Table 1; (6,7)). Tests were spaced by three days of recovery time and increasing in term of stress-related paradigm, starting with the less stressful test. Mouse behavior was tracked and analyzed with *Smart* (Panlab, Barcelona, Spain) or *Bioseb* (Bioseb, Vitrolles, France) software. All behavioral experiments were subject-randomized and blind analyzed.

*FISH for IR combined with IHC for TPH2*

Digoxigenin-labeled riboprobe against mouse Insr (IR-DIG, Allen Brain Atlas, #RP_050503_02_H08) was prepared as previously described (4) (Supplementary Table 2).

*Tissue sampling and high-pressure liquid chromatography coupled to electrochemical detection (HPLC-ECD).*

Tissue sampling and conditioning for HPLC-ECD have been performed as previously described (21,22) (Supplementary Table 2).

*In vivo single-unit recordings of 5-HT neurons in the DR.*

Mice were anesthetized with chloral hydrate (400 mg/kg, i.p., with additional doses when necessary) and extracellular recordings of DR 5-HT neurons were performed using single glass micropipettes (Stoelting Europe, Dublin, Ireland) pulled on a pipette puller (Narishige, Tokyo, Japan) and preloaded with a 2 M NaCl solution. Micropipettes were positioned 0.2–0.5 mm posterior to the interaural line on the midline and lowered into the DR, attained at a depth between 2.5 and 3.5 mm from the brain surface. Presumed 5-HT neurons were identified using

the following criteria: a slow (0.5–2.5 Hz) and regular firing rate and a long-duration positive action potential (please see references (7,23,24) for additional information regarding presumed DR 5-HT neurons recordings and their pharmacological characterization). In each mouse, presumed 5-HT neurons were identified based on these criteria and their spontaneous firing rate was recorded for 2 minutes. Insulin was then injected i.p. at doses of 0.75 and 1.5 IU/kg either alone or in combination with WAY100635 as described in figure legend.

*Intracerebral microdialysis in the ventral Hippocampus or Dorsal Raphe of awake freely moving mice.*

Mice were implanted with microdialysis probes in the ventral hippocampus (vHP) or the DR. Stereotaxic coordinates from bregma were as follows (in mm): vHP: antero-posterior (AP): -2.5; lateral (L): ±2.7; and dorso-ventral (DV): -3.0; DR: AP: -4.5; L: 0; and DV: -3.5. Twenty-four hours later, probes were connected to a microinjection pump for a continuous perfusion (flow rate: 1.5 µL/min for the vHP and 0.5 µL/min for the DR) of artificial cerebrospinal fluid (147 mM NaCl, 2.7 mM KCl, 1 mM MgCl2, and 1.2 mM CaCl2, adjusted to pH 7.4 with 2 mM sodium phosphate buffer) containing the 5-HT reuptake inhibitor escitalopram (1 µM), as previously described (25). Samples were collected every 15 min and 30 min in the vHP and DR, respectively. The four initial fractions were used to determine the basal extracellular levels of serotonin ([5-HT]ext) before injecting insulin in mice pre-treated with saline or WAY100635 (0.5 mg/kg; i.p.). The amount of 5-HT in dialysate samples post-insulin injection was expressed as percentage of the basal value. Samples were analyzed using high-performance liquid chromatography system equipped with a 2.6 µm C18 reverse-phase analytical column (50 x 3.0 mm; Accucore, Thermo Fisher Scientific, Waltham, MA, USA) coupled with electrochemical detection (Dionex Ultimate 3000, Thermo Fisher Scientific).

*Brain slice patch clamp recordings of DR 5-HT neurons*

*Ex-vivo* patch-clamp recordings were performed on brain slices from Pet1-cre-mCherry mice, as previously described (7). Briefly, mice were intracardially perfused during euthanasia (exagon/lidocaine: 300/30 mg/kg, i.p) with ice-cold NMDG solution containing the following (in mM): 1.25 $NaH_2PO_4$, 2.5 KCl, 7 $MgCl_2$, 20 HEPES, 0.5 $CaCl_2$, 28 $NaHCO_3$, 8 D-glucose, 5 L(+)-ascorbate, 3 Na-pyruvate, 2 thiourea, 93 NMDG, and 93 HCl 37%; pH: 7.3–7.4; osmolarity: 305–310 mOsM. Brains were quickly removed and 250 μm slices containing the DR were cut with a vibroslice (Leica VT1000S, Wetzlar, Germany) and transferred at room temperature into aCSF solution (containing the following in mM: 124 NaCl, 2.5 KCl, 1.25 $NaH_2PO_4$, 2 $MgCl_2$, 2.5 $CaCl_2$, 2.5 D-glucose, and 25 $NaHCO_3$; pH: 7.3–7.4; osmolarity: 305–310 mOsM) for at least 1 hour. For whole cell recording, borosillicate pipette (4-6 MΩ; 1.5 mm OD, Sutter Instrument) were filled with an intracellular K-gluconate solution (containing the following (in mM): 128 K-gluconate, 20 NaCl, 1 $MgCl_2$, 1 EGTA, 0.3 $CaCl_2$, 2 Na2-ATP, 0.3 Na-GTP, 0.2 cAMP, and 10 HEPES; pH: 7.3–7.4; osmolarity: 280–290 mOsM), cesium chloride (containing the following (in mM):150 CsCl, 2 MgCl2, 1 EGTA, 2 Na2-ATP, 0.2 Na-GTP, 0.2 cAMP, 10 HEPES) or filled with filtered aCSF for cell-attached recording. Recordings were made using a Multiclamp 700B amplifier, digitized using the Digidata 1440A interface and acquired at 2 kHz using pClamp 10.5 software (Axon Instruments, Molecular Devices, San José, CA, USA). Pipette and cell capacitances were fully compensated and junction potential was corrected off-line.

Firing frequency of DR 5-HT neurons was recorded in cell-attached mode. Because DR 5-HT neurons are silent *ex vivo*, phenylephrine (PHE, 5 μM; Tocris) was perfused to stimulate basal action potential activity for 5 minutes before application of insulin (300 nM) and recorded for 10 additional minutes. Change in firing rate frequency was analyzed during the last minute of insulin application. Spontaneous excitatory (sEPSC) or inhibitory (sIPSC) postsynaptic

currents were recorded in whole-cell Vclamp mode on cells held at -60 mV. Gabazine (5 µM; Tocris) was added into the bath to record sEPSC while D-AP5 (10 µM; Tocris) and CNQX (10 µM; Tocris) were added to monitor sIPSC. Spontaneous EPSC and IPSC frequency was recorded before and after 10 minutes insulin (300 nM; Sigma) perfusion period and analyzed during the last minute of each period.

*Statistical analysis*

Sample size has been estimated based on previous work from our group or others. No randomization was used. Investigator was not blinded to the group allocation for most experiment. Statistical analysis was performed using *Prism GraphPad 9* (San Diego, CA, USA). Data are expressed as mean ± SEM and individual values are plotted on graph when possible. After normal Gaussian distribution was assessed using Shapiro-Wilk test, appropriate parametric (unpaired or paired Student's t-test) or non-parametric (Mann-Whitney or Wilcoxon matched-pairs signed rank test) tests were chosen to compare two population samples. One-way ANOVA was used when comparing one variable of several population samples and two-way ANOVA was used when analysis accounted for two distinct variables. Post-hoc analyses are mentioned in each figure legend. Outliers were identified using *Prism GraphPad 9* and discarded from the analyses. Statistics are detailed in supplementary table 3 (Supplementary Table 3).

## Results

***Insulin increases firing activity of DR 5-HT neurons.*** We first verified that the IR is expressed at the mRNA and protein level in the DR (Supplementary Fig. S1A, B). To determine whether the IR is expressed in DR 5-HT neurons, we used *in situ* fluorescence hybridization targeting IR mRNA combined with an immunodetection against the rate limiting 5-HT synthesis enzyme Tryptophan Hydroxylase 2 (TPH2), which is selectively expressed in 5-HT neurons. This analysis shows that the IR is expressed in DR 5-HT neurons (Fig. 1A). The functional activity of the IR expressed onto DR 5-HT neurons was assessed using cell-attached patch-clamp recording on Pet1-cre-mCherry mice (Fig. 1B). Insulin (300 nM) increases action potentials frequency in 80 % of DR 5-HT neurons by $64 \pm 21$ % (Fig. 1C: 10/12 neurons tested). The selective IR inhibitor S961 prevents the excitatory effect of insulin (Fig. 1D). In the DR, the IR is expressed in both 5-HT and non-5-HT neurons (Fig. 1A). Thus, we assessed whether the action of insulin onto 5-HT neurons is direct rather than mediated by pre-synaptic inputs. We found that insulin fails to modulate the frequency of both spontaneous excitatory (EPSC) and inhibitory (IPSC) postsynaptic currents (Fig. 1E-F). To further confirm that insulin directly activates DR 5-HT neurons, we evaluated the response of DR 5-HT neurons to insulin in mice lacking the IR selectively in 5-HT neurons (SeIRKO mice; supplementary figure S1D, E; Fig. 1G). Similarly, to what we have observed before, insulin increases action potentials frequency of DR 5-HT neurons in control animals but fails to activate 5-HT neurons in SeIRKO mice (Fig. 1H), leading to conclude that insulin directly modulates DR 5-HT neurons through an IR-dependent mechanism.

***Insulin inhibits the serotonergic system in vivo through a 5-HT$_{1A}$ receptor-dependent mechanism.*** Extracellular recordings of DR 5-HT neurons were then performed to study the effects of insulin on this neuronal population *in vivo*. While we were expecting an increase in

the activity of presumed DR 5-HT neurons, we found that the intraperitoneal administration of 0.75 or 1.5 UI insulin produces, on average, a reduction of firing by $30 \pm 3\%$ and $20 \pm 4\%$, respectively, relative to baseline (Figs. 2A-C). Injecting glucose i.p. does not reverse insulin-induced decreased presumed DR 5-HT neuronal activity suggesting that insulin, rather change in blood glucose level, impacts DR 5-HT neurons activity (supplementary figure S2). This non-intuitive, inhibitory response of presumed DR 5-HT neurons *in vivo* could be consequent to the recruitment of autoinhibitory mechanisms inherent to the raise in DR 5-HT neurons excitability induced by insulin, as observed *in vitro*. Interestingly, the ability of insulin to inhibit DR 5-HT neuronal activity is completely prevented by the selective 5-HT$_{1A}$ receptor antagonist WAY100635. Indeed, the firing rate of presumed DR 5-HT neurons in mice receiving the combination of insulin and WAY100635 is significantly higher compared to the group injected with insulin alone at both doses (Figs. 2A-C). This finding therefore suggests that, *in vivo*, insulin inhibits DR 5-HT neurons through a 5-HT$_{1A}$-dependent mechanism. In support of this hypothesis, assessment of DR extracellular 5-HT levels ([5-HT]ext) by intra-DR microdialysis shows that i.p. administration of insulin increases [5-HT]ext in presence of WAY100635 whereas [5-HT]ext level are not altered in absence of this pharmacological compound (Figs. 2D,E).

In addition, based on the importance of 5-HT projections in the ventral-hippocampus (vHP) in the control of mood, we then conducted intra-vHP microdialysis to determine to what extent insulin influences [5-HT]ext at the nerve terminals. Acute administration of insulin decreases [5-HT]ext in the hippocampus relative to baseline, whereas it fails to do so when combined with WAY100635 (Figs. 2F). Analysis of the area under the curves indicates that differences between the two groups are statistically significant (Figs. 2G), demonstrating that the inhibitory effect of insulin on vHP [5-HT]ext is blunted when the inhibitory feedback exerted by the 5-HT$_{1A}$ autoreceptor on 5-HT release is prevented.

***Anxiolytic-like effect of brain insulin requires 5-HT neurotransmission.*** Clinical and preclinical studies have suggested that insulin modulates emotional behaviors by acting onto the brain (26,27). In the present study, we determined whether the action of insulin on mood requires the 5-HT system. Intranasal insulin delivery has been widely used to assess brain insulin effect in both rodents and humans in order to avoid symptomatic insulin-induced hypoglycemia bias (28). Accordingly, our results showed that intranasal insulin delivery does not modify peripheral blood glucose (supplementary figure S3A). However, it significantly decreases tissue 5-HT content in both the amygdala and nucleus accumbens (NAc), with a non-significant trend in the vHP (supplementary figure S3B). Remarkably, overall, no modifications in brain noradrenaline (NA) and dopamine (DA) contents were detected, thereby highlighting the specificity of insulin action on 5-HT neurons (supplementary figure S3C, D).

In the forced swim test (FST), intranasal insulin does not affect the time of immobility compared to intranasal vehicle treatment (Fig. 3A). Intranasal insulin does not modify latency to feed in the novelty suppressed feeding (NSF) test (Fig. 3B) or the time spent in the light compartment of the Light-dark box test (Fig. 3C). However, intranasal insulin increases the time spent in the open arms in the elevated plus maze (EPM) (Fig. 3D). Consistent with the latter, intranasal insulin also elicits an anxiolytic-like effect since it increases the time spent in the center of the open field (OF) without any changes in the locomotor activity (Fig. 3E). Interestingly, injection of insulin directly in the DR produces a similar increase in the time spent in the open arm in the EPM (Fig. 3F). To confirm the contribution of the 5-HT system in the anxiolytic effects of insulin, mice were pre-treated with the TpH2 inhibitor pCPA. As expected, pCPA treatment significantly decreases tissue 5-HT levels in all brain areas analyzed, without overall change of tissue DA or NA contents (supplementary figure S4). At the behavioral level, pCPA treatment prevents the anxiolytic-like response of intranasal insulin assessed in the EPM (Fig. 3G). Importantly, the anxiolytic-like effect of intranasal insulin in the EPM is also blunted

in SeIRKO animals as compared to their wild-type littermates (Fig. 3H). Finally, since we found that the 5-HT$_{1A}$ autoreceptor is involved in insulin-induced inhibition of the 5-HT tone *in vivo*, we sought to determine to what extent the pharmacological inactivation of this receptor influences insulin behavioral response. Our results show that the 5-HT$_{1A}$ receptor antagonist WAY100635 blocks the anxiolytic-like effect of intranasal insulin in the EPM (Fig. 3I).

***Potentiated antidepressant response to fluoxetine in combination with intranasal insulin delivery.*** Since insulin signaling pathway has been shown to cross-talk with 5-HT signaling (29), we investigated whether intranasal insulin potentiates the response of acute fluoxetine administration. Consistent with our previous experiments assessing despair behavior in the FST, intranasal insulin treatment does not modify immobility time in the tail suspension test (TST) (supplementary figure S5A) whereas fluoxetine significantly decreases the immobility time during TST (supplementary figure S5A). Interestingly, the combination of intranasal insulin and fluoxetine further reduces the immobility time compared to fluoxetine treatment alone (supplementary figure S5A). Acutely, fluoxetine induces anxiogenic effects (30). Here we show that intranasal insulin does not reverse or potentiate fluoxetine-induced anxiogenic-like response in the EPM (supplementary figure S5B). These data suggest that brain insulin signaling improves the antidepressant-like response of fluoxetine but does not impact its anxiogenic property.

***DR 5-HT neurons harbor insulin-resistance in a model of T2D.*** Mice fed a HFD for 16 weeks develop hallmarks of T2D including basal hyperglycemia, basal hyperinsulinemia, glucose intolerance and insulin resistance (supplementary figure S6). We previously showed that HFD fed mice display T2D-associated anxiety- and depressive-like behaviors as well as alterations

in the activity of DR 5-HT neurons (6,7). In the present study, we aimed at determining whether such alterations in 5-HT tone are associated with impaired response to insulin in DR 5-HT neurons. We found that insulin (300 nM) fails to increase action potentials frequency of DR 5-HT neurons in Pet1-cre-mCherry mice fed an HFD for 16 weeks (Fig. 4A). Interestingly, the anxiolytic-like response to intranasal insulin is blunted in both the EPM (Fig. 4B) and the OF (Fig. 4C), raising the possibility that DR 5-HT neurons are insulin-resistant in a model of HFD-induced T2D.

**Discussion**

Our study demonstrates that insulin inhibits the 5-HT system to elicits anxiolytic-like responses in mice. Such effect is mediated by direct action of insulin onto DR 5-HT neurons through the enhancement of inhibitory somatodendritic 5-HT$_{1A}$R. We also show that, in HFD-fed T2D mice, insulin fails to modulate the electrical activity of DR 5-HT neurons and to promote anxiolytic-like effects. This study provides novel mechanistic insights into how insulin modulates the serotonergic system to influence emotional behavior.

Previous studies have reported inconsistent findings on the neuronal response to insulin including increase (31) or decrease (32) in 5-HT neurotransmission. Using complementary approaches consisting in *ex vivo* patch-clamp electrophysiology, *in vivo* electrophysiology and intracerebral microdialysis, our data suggest that the action of insulin on the 5-HT system is more complex than originally thought. Indeed, using patch-clamp, we show that insulin increases the electrical activity of DR 5-HT neurons. This effect of insulin on DR 5-HT neurons is direct since insulin does not modify the frequency of IPSC nor EPSC thereby excluding a modulation from GABAergic or glutamatergic inputs. In addition, insulin fails to increase DR 5-HT neurons firing frequency in SeIRKO mice, which lack the IR selectively in 5-HT neurons. While these *ex vivo* experiments suggest that insulin activates the 5-HT system, *in vivo* studies show, on the contrary, that its systemic administration elicits inhibitory responses. Indeed, *in vivo*, insulin decreases both the firing rate frequency of DR 5-HT neurons and the tissue or extracellular 5-HT levels ([5-HT]ext) in different brain areas including the hippocampus. The latter data echo previous findings showing that peripheral insulin administration decreases the firing rate of 5-HT neurons in both the Raphe Pallidus and the Raphe Obscurus (33). Going one step further, we demonstrate the involvement of a 5HT$_{1A}$ receptor (5-HT$_{1A}$R)-dependent mechanism in the inhibitory effects of insulin *in vivo*. Studies have repeatedly shown that

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activation of $5HT_{1A}$ autoreceptors located at the somatodentric level of DR 5-HT neurons may silence these neurons and decrease [5-HT]ext in both brainstem (i.e., the DR) and forebrain projections (34,35). Thus, we hypothesized and demonstrate that insulin first activates DR 5-HT neurons and consequent accumulation of endogenous [5-HT]ext in the DR ultimately inhibits DR 5-HT neurons through $5-HT_{1A}R$ activation (Fig. 5A). Accordingly, our findings show that the $5-HT_{1A}R$ antagonist WAY100635 prevents the inhibition of firing rate frequency of DR 5-HT neurons. At the neurochemical level, it also appear that insulin increases [5-HT]ext in both the DR and the vHP specifically in presence of WAY100635 compared to control. Thus, DR 5-HT neuronal activity is decreased in biological settings where local inhibitory feedback is preserved whereas the excitatory effects of insulin is revealed when blocking $5-HT_{1A}$ autoreceptors *in vivo*. Nevertheless, it is important to note that there is evidence for post-synaptic $5-HT_{1A}$ receptors control of DR 5-HT neurons (i.e., in the amygdala, hippocampus or median prefrontal cortex (mPFC)) (36,37). In particular, data suggest that post-synaptic $5-HT_{1A}$ are found on glutamatergic pyramidal neurons in the mPFC and given the fact that $5-HT_{1A}$ receptors are usually inhibitory, it has been speculated that the excitation of mPFC by selective agonists involves inhibitory interneurons (38). In such cases, one may assume that $5-HT_{1A}$ receptor agonist-induced excitation of mPFC neurons would produce excitatory amino acid release in the DR, which would activate inhibitory interneurons leading to inhibition of 5-HT neurons. Thus, such model of regulation would support the possibility that the excitatory effects of insulin in presence of WAY100635 on DR 5-HT neuronal activity and extracellular 5-HT levels (in the DR and hippocampus) involves, at least in part, a disinhibition of this long neuronal feedback loop. From these data, one could wonder why such $5-HT_{1A}$ receptors-dependent mechanism is not observed during patch-clamp recordings *ex vivo*. Studies have suggested that in *ex-vivo* patch-clamp conditions the $5HT_{1A}R$-inhibition tone cannot be assessed due to the depletion of endogenous 5-HT synthesis and release (39–41).

The effect of intranasal insulin on anxiety has been previously investigated (27). In agreement with the literature we show that insulin exerts anxiolytic effects. Yet, neuronal circuits underpinning such behavioral outcome have not been unraveled. Even if we cannot rule out the involvement of extra-DR neurons, the anxiolytic-like effect of intra-DR administration of insulin supports a role of the serotonergic system in the central effect of brain insulin. Going one step further, using pharmacological and genetic-based approaches, we confirm that anxiolysis induced by intranasal insulin relies, at least in part, on the serotonergic system. Indeed, both 5-HT depletion and selective deletion of the IR in 5-HT neurons result in a lack of anxiolytic-like effect of intranasal insulin. Interestingly, we found that intranasal insulin significantly reduces 5-HT level in the vHP, the amygdala and the NAc. Importantly, decrease in 5-HT levels or lesion of serotonergic projections to the amygdala have been shown to mitigate anxiety-like behavior (42,43). Thus, such a drop in 5-HT in response to intranasal insulin is consistent with the associated anxiolytic-like effect. In addition, we also show that blocking decreased 5-HT level using the $5HT_{1A}R$ antagonist WAY100635 prevents the anxiolytic effect of intranasal insulin administration. It is noteworthy that we cannot generalize the effect of insulin to all the behavioral tasks since our results clearly show that it produces anxiolytic effects in the EPM and in the OF while it fails to do so in the light-dark test. These paradigms rely on competition between spontaneous exploratory behavior and the innate avoidance of open or illuminated areas respectively. Since evidence suggests the existence of a high level of specialization at the level of microcircuits and cell populations involved, it is tempting to speculate that there is an overlap of the neuronal networks recruited during these tasks (44). Nevertheless, the testing conditions are somewhat different with mice subjected to a dim lightening in the EPM/OF and a choice between a brightly-lit and dark compartment in the light-dark. A recent study demonstrates that light-dark preference behavior engages complex regulation due to the emotional valence of light (45). Thus, even if the tests probe the

same anxious behavior, the variations in experimental conditions strongly suggest that insulin could trigger a circuit specifically recruited during the EPM and the OF.

Our data show that intranasal injection of insulin promotes anxiolytic-like effects. Interestingly, we did not find any antidepressant-like effects in response to acute intranasal insulin administration in the FST or in the TST. According to the *Research domain criteria* (RDoC) approach (46), we can assume that the acute effect of insulin might be involved in the anxiety dimension of MDD, a core symptom of the disease. However, in animal models of T2D induced by a HFD, both anxiety- and depressive-like symptoms have been reported (5–7,47). In these models, insulin resistance has been found in several discrete brain areas including the hippocampus, the ventral tegmental area and or the striatum (48,49). Here, we provide the first evidence that such resistance also targets DR 5-HT neurons after HFD feeding and that such impairment likely explains our previous observation that 5-HT neurons display a lower electrical activity in response to a HFD (7). Thus, we could hypothesize that insulin resistance at the level of 5-HT neurons takes a major part in the development of anxiety-like symptoms whereas decreased insulin sensitivity in other neuronal populations would rather promote depressive-like symptoms.

Clinical data show that antidepressant response efficacy is not optimal in patient with T2D as only one third present an adequate treatment response following the first antidepressant trial (50). Add-on treatment strategy represents a relevant approach to increase antidepressant response efficiency (51). Interestingly, the insulin-sensitizer agent metformin has been shown to potentiate the antidepressant effect of fluoxetine (52). Thus, in a translational perspective, we investigated whether acute intranasal insulin could also potentiate fluoxetine antidepressant efficacy. In STD-fed animals, we observed that intranasal insulin indeed increases the antidepressant-like response of fluoxetine. However, the anxiogenic response of fluoxetine was

not reversed by insulin. Although the reason of this lack of potentiating effect in the EPM remains unknow, it is possible that 1/ the fluoxetine effect is too strong to be reversed by insulin; 2/ the neuronal networks involved the anxiogenic effect of fluoxetine are different from, those mobilized by insulin. Clinical investigations are needed to support our data suggesting that enhancing insulin signaling represents an original therapeutic approach for patients with MDD presenting, or not, comorbid metabolic disorders associated to T2D.

## Authors contribution

HM, SB designed and performed experiments. MM, MC, MDM, VS, SJ, JB, MS, SC performed experiments. FC, AK, DC, PDD, LP, SL edited the manuscript. BG and XF designed the project and wrote the manuscript.

## Conflict of interest

The authors declare no competing interests

**Supplementary information is available at MP's website**

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**Figure 1: Insulin increases DR 5-HT neurons firing frequency by direct activation of insulin receptor expressed onto that neuronal population. A**. Representative confocal image of TPH2-positive 5-HT neurons (red) and IR mRNA punctae (green) using fluorescence *in situ* hybridization. *Right panel*: magnification of the white dotted square area from the left pannel. Scale: 10µm. White arrows show IR mRNA in 5-HT neurons. **B**. Pet1-cre-mCherry mice were used for patch-clamp experiments to specifically visualize fluorescent mCherry-DR 5-HT neurons. Scale: 10µm. **C, D**. Quantification of firing rate and representative traces of cell-attached mode recording before and after insulin (300 nM) perfusion and of DR 5-HT neurons under control condition (**C**) or during bath application of the IR inhibitor S961 (100 nM, **D**). **E-F**. Frequency of spontaneous excitatory postsynatpic currents (EPSC, **E**) or inhibitory postsynaptic currents (IPSC, **F**) onto DR 5-HT neurons before and after insulin (300 nM) perfusion. **G**. SeIRKO mice were obtained by crossing Pet1-cre-mCherry mice with IR$^{lox/lox}$ mice allowing selective deletion of IR only in 5-HT neurons. **H**. Quantification of firing rate and representative traces of cell-attached mode recording before and after insulin (300 nM) perfusion of DR 5-HT neurons in control (*Left panel*) and in SeIRKO (*Right panel*) mice. The data are represented as mean ± SEM. Statistics: Student's paired t-test: *p<0.05; **p<0.01.

**Figure 2: Insulin decreases DR 5-HT neurons *in vivo* through a 5-HT$_{1A}$ receptor-dependent mechanism. A-C**. **A.** *In vivo* extracellular recordings of presumed DR 5-HT neurons. Data are mean ± SEM of DR 5-HT firing rate (% of baseline) in response to the intraperitoneal (i.p.) administration of insulin alone (0.75 IU, n=5 and 1.5 IU, n=14) or in combination with the 5-HT$_{1A}$ receptor antagonist (WAY100635: 0.5 mg/kg, n=6). **B**. Integrated firing histograms showing the effects of cumulative doses of insulin. **C.** Representative recordings of a single presumed 5-HT neuron in response to insulin (0.75 U/kg) alone (**C**-*left panel*) or in presence of the 5-HT1A antagonist WAY100635 (**C**-*right panel*). **D-G**. *In vivo* intracerebral microdialysis in the DR (**D-E**) or the ventral hippocampus (**F-G**). (**D, F**) Time course of the effect of cumulative doses of insulin alone (n=5) or in combination with WAY100635 (0.5 mg/kg; i.p, n=5) given at T0 on 5-HT release in the DR (**D**) or ventral hippocampus (**F**). (**E, G**) Data are mean ± SEM of Area Under the Curve reflecting extracellular 5-HT concentrations ([5-HT]ext) over the 0-150 min post-treatment injections in the DR (**E**) or the ventral hippocampus (**G**). Statistics: Student's unpaired t-test: *p<0.05; **p<0.01; ***p<0.001. One sample t-test compared to hypothetical value (0): #p<0.05; ##p<0.01

**Figure 3: The anxiolytic like effect of intranasal insulin delivery relies on the central serotonergic system. A-E**. Immobility time in the FST (**A**), latency to feed in the NSF (**B**), time spent in light box of the L/D (**C**), time spent in the open arms of the EPM (**D**) and time in the center (left panel, **E**) and locomotor activity in the OF (right panel, **E**) in response to intranasal 2IU or vehicle delivery in naïve mice. **F**. Time spent in the open arms of the EPM in response to intra-DR injection of insulin (100μIU) or vehicle in naïve mice. **G-I**. Time spent in the open arms of the EPM in naïve mice pre-treated with pCPA (300 mg/kg/day for three consecutive days; **G**), in control and SeIRKO animals (**H**) or naïve mice pre-treated with WAY100635 (1 mg/kg; **I**) in response to intranasal insulin 2 IU or vehicle delivery. Data are represented as mean ± SEM. Statistics: Student's unpaired t-test: *p<0.05; **p<0.01.

**Figure 4: HFD feeding impairs the insulin response of the 5-HT system. A**. Pet1-cre-mCherry mice were fed a HFD for 16 weeks prior to *ex-vivo* electrophysiology experiments. Quantification of firing rate and representative trace of cell-attached mode recording before and after insulin (300 nM) perfusion of DR 5-HT neurons. **B-C**. Time spent in the open arms of the EPM (**B**), time spent in the center of the OF (**C**, *left panel*) and locomotor activity in the OF (**C**, *right panel*) of HFD-fed mice in response to intranasal insulin 2 IU or vehicle delivery. Data are represented as mean ± SEM. Statistics: Student's unpaired t-test.

**Figure 5: Schematic representation of the putative action of insulin onto DR 5-HT neurons and related control of emotional behaviors. A. At resting state, the 5-HT$_{1A}$ autoreceptors would exert limited effects on DR 5-HT neurons activity as previously reported (1)**. Acute insulin binding to IR onto DR 5-HT neurons increases action potential firing activity and presumably extracellular 5-HT release in both projection areas and the DR through collaterals in a shorter timescale (**2**). Such an accumulation of endogenous extracellular 5-HT levels around 5-HT cell bodies would, in turn, activate the autoinhibitory 5-HT$_{1A}$R resulting in a decrease in action potential firing activity and endogenous extracellular 5-HT release in an expanded timescale (**3**). Reduced 5-HT levels, in regions such as the amygdala, might be responsible for the anxiolysis induced by intranasal insulin administration. **B-C**. Selective deletion of the IR in 5-HT neurons (**B**) or pre-administration of a 5-HT$_{1A}$R antagonist (**C**) block

the anxiolytic-like effect of intranasal insulin assessed in the EPM. Altogether, this study reveals that the anxiolytic-like effect of insulin relies on the 5-HT system.
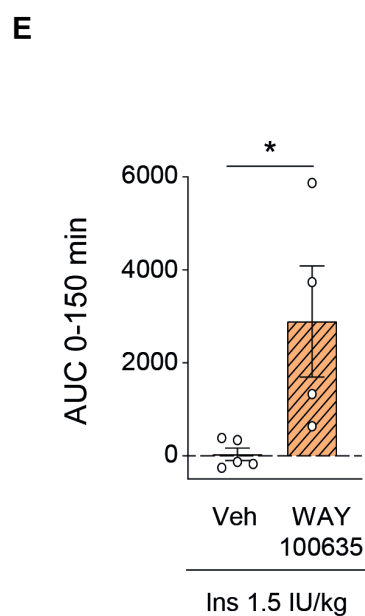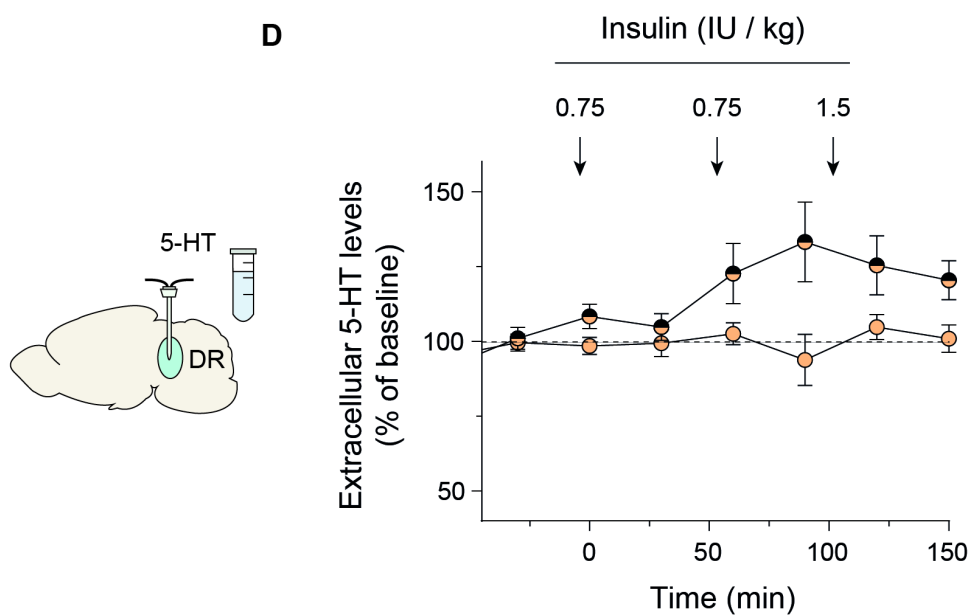
**A** TPH2/IR

**B** Pet1-cre-mcherry

AP: -4.6 mm

**C**

Firing frequency (Hz)

Ins  −  +

aCSF
aCSF + insulin

5 s
100 pA

**D**

Firing frequency (Hz)

Ins    −    +
S961  +    +

S961
S961+ insulin

40 pA  5 s

**E** sEPSC

Frequency (Hz)

Ins  −  +

aCSF
aCSF + insulin

1 s
100 pA

**F** sIPSC

Frequency (Hz)

Ins  −  +

aCSF
aCSF + insulin

0,1 s
20 pA

**G**

Pet1-cre-mCherry

X ⟶

**SeIRKO**

Pet1-cre-mCherry::IRlox/lox

| Pet1 | Cre | mCherry |

| LoxP | | LoxP |
| LoxP | | LoxP |

IRlox/lox

**H** Control          SeIRKO

Firing frequency (Hz)

Ins  −  +

Firing frequency (Hz)

Ins  −  +

aCSF
aCSF + insulin

aCSF
aCSF + insulin

5 s
20 pA

A

B

C

Insulin                    Control

Insulin                    WAY100635

1 min

1 min

D

Insulin (IU / kg)

0.75    0.75    1.5

E

F

Insulin (IU / kg)

0.75    0.75    1.5

G

**C57BL/6J**

Intranasal Insulin 2 IU

**A** Immobility time (sec) — Veh, Ins

**B** Latency to feed (sec) — Veh, Ins

**C** Time in light box (sec) — Veh, Ins

**D** Time in open arms (sec) — Veh, Ins *

**E** Time in center (sec) — Veh, Ins ** ; Total distance travelled (cm) — Veh, Ins

**F** Intra-DR insulin injection (100 µIU) — Time in open arms (sec) — Veh, Ins *

**G** C57BL/6J + *pCPA* — Intranasal Insulin 2 IU — Time in open arms (sec) — Veh, Ins
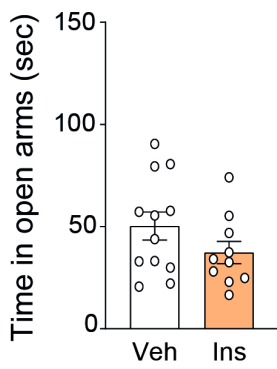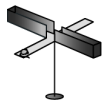
**H** SeIRKO — Intranasal Insulin 2 IU

Control — Time in open arms (sec) — Veh, Ins *

SeIRKO — Time in open arms (sec) — Veh, Ins

**I** C57BL/6J + *WAY100635* — Intranasal Insulin 2 IU — Time in open arms (sec) — Veh, Ins

A

Insulin | IR | 5-HT1AR | 5-HT

C57BL/6J

Intranasal Insulin 2 IU

DR 5-HT neuron

Projection areas

Anxiolysis

B

SeIRKO

Intranasal Insulin 2 IU

DR 5-HT neuron

C

C57BL/6J

+ *WAY100635*

Intranasal Insulin 2 IU

DR 5-HT neuron

WAY100635

No effect