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Effects of Arginine Vasopressin on Islet Cells in Pancreatic Tissue Slices: Glucose-Dependent Modulation of Alpha and Beta Cell Activity

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eLife Assessment

This study provides a **useful** analysis of the effects of arginine vasopressin (AVP) on islet cells in pancreatic tissue slices, using sophisticated spatiotemporal calcium recordings to show that AVP affects α and β cells differently depending on glucose concentration. The calcium imaging, analytical approaches, and V1b receptor-targeted peptide ligands are strengths of the work. However, the reviewers were concerned that the proposed mechanistic model is not sufficiently supported by the data. Characterisation of β-cell responses remains **incomplete**, and potential off-target effects and limited receptor specificity raise alternative explanations, including indirect effects mediated through α cells. The study would have been strengthened by signalling pathway analyses, genetic validation (e.g. β-cell-specific V1bR deletion), or selective V1b receptor silencing. The RNAscope data included in the revision indicate broader expression patterns but do not clearly establish receptor localisation within specific endocrine populations.

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Abstract

Arginine vasopressin (AVP) is well known for regulating fluid volume, osmotic balance, and vascular tone. Its role in the regulation of pancreatic α and β cell function has been reported, yet its effects are not fully understood, particularly regarding its interaction with plasma glucose levels. The osmotic and volume challenges posed by hyper- and hypoglycaemia in diabetes can be a significant complication of effective hormonal regulation of metabolism. In this study, we primarily investigated the effects of AVP and synthetic peptide receptor agonists and antagonists on α and β cells in pancreatic tissue slices using live confocal Ca^{2+} imaging. Our findings demonstrate that AVP exerts glucose-dependent effects on both cell types. At low glucose concentrations, AVP, in combination with physiologically or pharmacologically increased cAMP levels, selectively activated α cells without significantly affecting β cells. In contrast, at higher glucose concentrations and pharmacologically elevated cAMP levels, physiological levels of AVP enhanced β cell activity, leading to increased Ca^{2+} oscillations and insulin release. In both cell types, AVP displayed a bell-shaped concentration dependence, with lower AVP concentrations stimulating hormone release and higher concentrations leading to diminished responses,

consistent with inositol trisphosphate receptor (IP_3R) activation and inactivation properties. Furthermore, our results indicate that AVP acts primarily through V_{1b} receptors in β cells, with no involvement of V_{1a}, V_2 or oxytocin receptors. These findings provide new insights into the modulation of glucose-dependent release of pancreatic hormones by AVP in the context of changed blood osmolality due to hyper- or hypoglycemia in diabetes. Importantly, our results emphasize the potential of targeting AVP signaling pathways as a therapeutic approach in diabetes research, aiming to improve hormone regulation and nutrient homeostasis.

Highlights

- Highly spatio-temporally resolved imaging of islet Ca^{2+} oscillations on pancreatic tissue slices provides an in situ-like model for physiological and pharmacological approaches.
- Physiological glucose stimulation triggers non-linear β cell collective responses that must be taken into account when interpreting single concentration pharmacological experiments.
- In a high cAMP context, AVP acts through V_{1b} receptors on islet α and β cells, exhibiting a bell-shaped dependence driven by the activation-inactivation properties of IP_3 receptors.
- AVP modulates glucose-dependent effects on α and β cells in a physiological concentration range, in the presence of altered blood osmolality or volume due to hyperglycemia, or to the direct effects of hypoglycemia in diabetes.

Introduction

The most extensively studied physiological roles of arginine vasopressin (AVP; a.k.a. ADH) include the regulation of body fluid volume by promoting water reabsorption in the kidneys, maintenance of osmotic and electrolyte balance, and contribution to cardiovascular homeostasis through regulation of vascular tone and arterial pressure (1, 2). Other actions of AVP, particularly its connection to aspects of metabolic syndrome, are less frequently highlighted (3, 4). AVP has been described to elevate blood glucose levels through two primary mechanisms: enhancing glycogenolysis in the liver and promoting glucagon release from pancreatic islets (5, 6). However, the direct effects of AVP agonists and antagonists on pancreatic β and α cells remain contentious, with ongoing debates in the literature (7–10). This uncertainty is partly because systemic AVP responses can originate from extra-islet targets, making it necessary to examine AVP actions directly within intact pancreatic tissue.

The physiology of pancreatic β cells, which secrete insulin, and α cells, which secrete glucagon, are intricate and remains incompletely understood. The principal hormones released by these cell types are essential for metabolic homeostasis, responding dynamically to nutrient availability and metabolic intermediates present in the plasma (4, 11). These responses to nutrients are further modulated by neurohormonal signals, including AVP. AVP is a nonapeptide synthesized mainly in magnocellular AVP neurons in the supraoptic and paraventricular nuclei of the hypothalamus (12). Its release from the posterior pituitary into the systemic circulation is mainly stimulated by increased plasma osmolality, substantial blood-volume depletion, and diverse neuronal, endocrine or metabolic inputs to the hypothalamus (13). The resulting physiologically active plasma AVP concentration in rodents and humans has been reported to be in the low to mid-picomolar range (14–16). Some studies suggest that circulating AVP and the AVP produced in the pancreas could affect insulin and glucagon secretion via paracrine signaling (17).

One significant factor contributing to the controversies regarding the direct effects of AVP is the glucose concentration used in experimental settings. Insulin and glucagon secretions are reciprocally correlated with plasma glucose concentrations (18). While glucose alone stimulates oscillations in intracellular Ca^{2+} concentration in pancreatic β cells and causes insulin secretion (19), AVP has been reported to act as a positive modulator of glucose-stimulated insulin release (20). Notably, when AVP is administered intraperitoneally together with glucose in fasted mice, there is no significant increase in overall insulin secretory activity compared to glucose alone; however, it results in a marked reduction in blood glucose levels (8). Independently of glucose levels, several in vivo studies across species utilizing intravenous or intramuscular AVP

administration have reported increased pancreatic hormone release (6, 13, 20–22). Conversely, other studies have reported no or selective effects on glucagon (23, 24) and insulin release (6, 25), and in vitro experiments in rats have also described AVP-dependent inhibition of insulin release (25). In addition to its effects on insulin secretion, AVP has been reported to enhance proliferation of rodent and human β cells and to protect against cytokine-induced β cells apoptosis (8).

In an organism, AVP exerts its diverse physiological actions by binding to four transmembrane G-protein-coupled receptors (GPCRs): V_{1a}, V_{1b}, V_2, as well as oxytocin receptors (26). These receptors differ in their tissue expression, signaling mechanisms, and physiological functions. V_{1a} receptors are primarily expressed in vascular smooth muscle, heart, liver, and central nervous system, whereas V_{1b} receptors are found in the anterior pituitary gland and pancreas. Activation of V_{1a} receptors is associated with vascular contraction, cardiac function (13) and modulation of glucose and lipid metabolism, whereas activation of V_{1b} receptors is involved in the release of adrenocorticotropic hormone (ACTH), glucagon and insulin. Both receptor types couple to the $G_{q/11}$ proteins and activate the phospholipase C (PLC) pathway, leading to increased intracellular Ca^{2+} levels. In contrast, V_2 receptors preferentially couple to the G_s proteins and activate the adenylate cyclase pathway, increasing cyclic AMP (cAMP). This signaling cascade is crucial for water reabsorption in the kidneys, where V_2 receptors are primarily expressed in the renal collecting ducts and vascular endothelial cells, facilitating the insertion of vesicles containing aquaporin channels into the cell membrane, thereby enhancing water permeability (27).

For pancreatic islets specifically, available transcriptomic datasets indicate that V_{1b} receptor expression is strongly enriched in α cells, whereas V_2 receptor expression is absent or very low in endocrine islet cells, and β cell V_{1b} receptor expression appears low and remains difficult to resolve conclusively (28). The binding of AVP to either V_{1a} or V_{1b} receptor and activation of PLC leads to intracellular Ca^{2+} release due to inositol trisphosphate (IP_3) production. Such IP_3-mediated Ca^{2+} release has been shown to stimulate the secretion of glucagon (13) or insulin in primary (20) and clonal β cell preparations (29). Pharmacological studies have also supported the role of $G_{q/11}$ coupled receptors, as specific V_{1a} or V_{1b} receptor antagonists abolished, and an oxytocin receptor antagonist partially reduced an insulinotropic action of AVP in rodent BRIN BD11 β cells (8). Nevertheless, recent transcriptomic studies support α cells as the dominant V_{1b} receptor-expressing endocrine population, and therefore β cell responses to AVP may reflect a combination of low-level direct receptor-depending effects, indirect intra-islet signaling, and state-dependent modulation of the β cell collective. The potential involvement of V_{1a} receptors in α cells has also not been completely ruled out (8). Studies using genetically modified rodents have provided further evidence for the role of AVP and $V1_b$ receptors in islet function. Mice deficient in V_{1b} receptors could not release insulin after AVP stimulation (7, 30), with no difference in fasting blood glucose levels (7). Similarly, glucagon levels were significantly reduced in $V_{1b}^{-/-}$ mice (9). On the other hand, Brattleboro rats, which carry a naturally occurring mutation that abolishes AVP production, have been reported to have improved glucose tolerance (31).

It is important to emphasize that AVP does not act primarily as an initiator of hormone release, but rather has a permissive and potentiating role (32). For instance, AVP facilitates corticotropin-releasing hormone (CRH) (33) or histamine-dependent (34) release of adrenocorticotropic hormone (ACTH) from the anterior pituitary, both of which involve G_s/cAMP-dependent-signaling. β and α cells also utilize signaling pathways involving G_s-coupled protein receptors, where the permissive and potentiating roles of AVP may be significant (35, 36). In islets, this is particularly relevant because cAMP-elevating conditions may amplify the secretory consequences of Gq-dependent Ca^{2+} signals, although this does not necessarily mean that the upstream AVP-evoked Ca^{2+} response itself is cAMP-dependent. Thus, experiments performed in cAMP-elevated conditions should be interpreted as testing AVP action in a permissive signaling background, rather than as proof of a direct cAMP requirement for AVP receptor activation.

Finally, because the downstream readout of V_{1b} receptor activation involves IP_3 receptors with well-described activation and inactivation properties (37), such an arrangement could accommodate the broad spectrum of previously observed effects of AVP on the function of α and β cells. However, the relative contributions of direct receptor activation, intra-islet paracrine

interactions, and the current dynamic state of the β cell population remain unresolved. On that note, the present study reassessed the effects of AVP and the selection of specific novel AVP receptor agonists and antagonists on pancreatic β and α cells using fresh pancreatic tissue slices combined with live confocal imaging of intracellular Ca^{2+} oscillations. Pancreatic slices provide a complementary preparation that preserves local tissue architecture and intercellular interactions, while also carrying limitations shared with other ex vivo preparations, including the absence of intact circulation and innervation. In this preparation, the majority of AVP stimulatory activity occurs within the known physiological AVP range (1), a range that correlates with the correction of plasma osmolarity through water retention in the distal nephron or oral water intake, and with physiological scenarios related to blood volume depletion.

Material and methods

Ethics Statement

The study strictly adhered to all national and European guidelines regarding the care and treatment of laboratory animals. Every effort was made to minimize animal suffering and improve animal welfare. The experimental protocol received approval from the Administration of the Republic of Slovenia for Food Safety, Veterinary and Plant Protection (grant No.: U34401-12/2018/2) and The Ministry of Education, Science and Research, the Republic of Austria (GZ 2022-0.325.009).

Animals, Tissue Slice Preparation and Indicator Loading

Experiments were conducted 8–25-week-old C57BL/6J mice of both sexes, which were housed in individually ventilated cages (Allentown LLC, USA) at a room temperature 22-24 °C and 45-55% relative humidity with a 12-hour light/dark cycle. The mice had *ad libitum* access to water and a standard chow (Ssniff, Soest, Germany). Acute pancreatic tissue slices were prepared as previously described (38). Briefly, after CO_2 anesthesia, cervical dislocation and opening of the abdominal cavity, the common bile duct was clamped distally at the major duodenal papilla 1.9% low melting point agarose (Lonza, Basel, Switzerland) was injected proximally. Agarose was dissolved in an extracellular solution (ECS) containing (in mM) 125 NaCl, 26 $NaHCO_3$, 6 glucose, 6 lactic acid, 3 myo-inositol, 2.5 KCl, 2 Na-pyruvate, 2 $CaCl_2$, 1.25 NaH_2PO_4, 1 $MgCl_2$, 0.5 ascorbic acid and kept in a prewarmed water bath at 40 °C. Following the agarose injection, the pancreatic tissue was immediately cooled with ice-cold ECS, extracted from the abdominal cavity, and placed into a large Petri dish containing ice-cold ECS. Next, 3-5 mm^3 large tissue cubes were cut from the pancreas, cleared from the connective tissue, and embedded into agarose. The embedded tissue blocks were sectioned into 140 µm thick slices using a vibratome (VT1000S, Leica Microsystems, Wetzlar, Germany). The tissue slices were stored at room temperature in HEPES-buffered saline containing 6 mM glucose (HBS, consisting of (in mM) 150 NaCl, 10 HEPES, 6 glucose, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$; titrated to pH=7.4 with 1 M NaOH). For Ca^{2+} indicator loading, the slices were incubated for 50 minutes in 3.33 ml of HEPES-buffered saline containing 6 mM glucose, 3.75 µl dimethylsulfoxide, 1.25 µl Pluronic F-127, and 6 µg Calbryte 520 AM (AAT Bioquest, Pleasanton, CA, USA). Unless specified otherwise, all chemicals were obtained from Sigma Aldrich (St. Louis, MO, USA).

Immunofluorescence and RNAscope

Fresh-frozen samples were sectioned at 20 µm using a cryostat (Leica CM1950). Tissue fixation was performed in cold 4% paraformaldehyde (PFA) for 1 h. Sections were blocked with 5% BSA and 0.3% Triton X-100 in PBS for 1 h at room temperature (RT). Incubation with the somatostatin primary antibody (Sigma-Aldrich, SAB4502861) was carried out overnight at 4 °C. The secondary antibody (Invitrogen, A-21245) was incubated for 2 h at RT. To assess tissue morphology, DAPI staining was performed at a concentration of 1 µg/mL.

RNAscope was performed using the RNAscope™ Multiplex Fluorescent Reagent Kit v2 (ACD Bio-Techne, 323136) according to the manufacturer's recommended conditions and supplied reagents, with minor modifications (39). Fresh-frozen samples were sectioned at 20 µm and dried inside a

cryostat (Leica CM1950) for 2 h at −26 °C. When not used immediately, slides were stored at −80 °C according to the manufacturer's instructions. Protease IV digestion was prolonged by an additional 10 min, and the number of wash steps was increased to three washes of 5 min each. The RNAscope™ probes used were Probe Mm-Avpr1b C1 (ACD Bio-Techne, 480141) and Probe Mm-Gcg C2 (ACD Bio-Techne, 400601-C2). For probe detection, TSA Vivid dyes 520 (ACD Bio-Techne, 323271) and 570 (ACD Bio-Techne, 323272) were used at a 1:1500 dilution, respectively. Probe visualization was acquired using a confocal microscope (Olympus IXplore SpinSR) equipped with a Hamamatsu ORCA-Fusion 2 camera. Overview images were acquired using a 20× dry objective, and islet images were acquired using a 60× silicone oil immersion objective (NA 1.3).

The cell segmentation of islet images was based on the fluorescent DAPI signal. Briefly, the 2D Gaussian filter of a kernel size of 20 was applied over the DAPI channel and the background signal was removed using Otsu-thresholding (40). Next, the local maxima was found defining the nuclei center. As a last step, watershed (41) segmentation was used with a maximum cell radius of 150 pixels (~15 µm) using the detected peaks. RNAscope signal was counted by applying a maximum entropy thresholding on the respective fluorescence data. Next, we detected dots by using a Laplacian of Gaussian (42) approach with a minimum sigma of 0.75 and a maximum sigma of 2. Detected dots were mapped back to the DAPI segmentation data. Each cell with more than 2 dots was counted as positive.

Compound Synthesis, Purification, and Quality Control

VP analogs [(D-Leu)^2,Ile^3,Thr^4]-VP, d[Cha^4,Dab^8]-VP, and d[(CH$_2$)$_5$^{1},Tyr(Me)^2,Dab^5,Tyr^9]-VP were prepared following established procedures *via* Fmoc-SPPS (9-fluorenylmethyloxycarbonyl solid-phase peptide synthesis) and standard purification and quality control methods (43) (Fig. 5, Suppl. Fig. 1, Suppl. Table 1). Shortly, peptides were obtained through manual synthesis on a Rink amide aminomethyl resin followed by TFA cleavage, oxidative folding in a 0.1 M ammonium bicarbonate buffer (pH 8.2, 24 h), and purification *via* reversed phase-high performance liquid chromatography (RP-HPLC) on a Waters Auto Purification HPLC-UV system (Kromasil Classic C$_{18}$ 21.2 x 250 mm, 300 Å, 10 µm) at a solvent flow of 20 mL/min and a linear gradient of 5-45% solvent B in solvent A (A: ddH$_2$O + 0.1 % TFA, B: ACN + 0.08 % TFA) in 50 min. Reactions were monitored by analytic RP-HPLC-mass spectrometry (RP-HPLC-MS) on a Thermo Scientific Dionex Ultimate 3000 system equipped with a Waters XSelect CSH UPLC C$_{18}$ XP column (3.0 x 75 mm, 130 Å, 2.5 µm), UV detection (measurement at 214 nm and 280 nm), and Thermo Scientific MSQ Plus ESI-MS unit (positive ionization mode). Compound purity was determined through analytical RP-HPLC peak integration at 214 nm, and compound identity was verified through direct injection MS (Supplementary Information). A Thermo Fisher Scientific Vanquish Horizon UHPLC system using a designated column (Kromasil Classic C$_{18}$ 2.1 x 100 mm, 100 Å, 5 µm) was used to determine the product concentration based on peptide absorption at 214 nm, and a comparison of absorption areas to peptide standards with known concentration (44).

Pharmacological Characterization

HEK293 cells stably expressing the EGFP-tagged receptor of interest (human oxytocin, V$_{1a}$, or V$_{1b}$ receptor) were cultured. Radioligand displacement was performed in duplicates of cell membranes (6–20 µg) incubated with the radioactive agonist ^{3}H-OT or ^{3}H-VP and the competing peptide according to previously published protocols (45–47). The used radioligand concentrations refer to the K$_d$ of each receptor subtype which was obtained from saturation binding experiments (0.65 nM for OTR, 0.21 nM for V$_{1a}$R and 0.14 nM for V$_{1b}$R). Nonspecific binding was determined by addition of 10 µM OT or VP. After one hour, the reaction was stopped by filtering through glass fiber filters, which were subsequently used to determine the retained radioactivity measured by liquid scintillation.

Activation of G$_{q/11}$-signalling was measured by IP$_1$ quantification using the homogeneous time-resolved fluorescence IP-One assay kit (Revvity) according to the manufacturer's recommendations. Briefly, stable cell lines were seeded at a density of 10,000 cells per well and incubated for 2 days. At the time of the assay, the cells were equilibrated in the provided

stimulation buffer for 15 min prior to the addition of the peptide ligands. After 1 h at 37°C, the stimulation was stopped by adding labeled immunoreagents. The mixtures were then incubated for a further hour at room temperature before the fluorescence ratio 665/620 nm was measured on a FlexStation 3 plate reader (Molecular Devices, USA) at an excitation wavelength of 340 nm.

Data analysis of the pharmacological characterization was performed with GraphPad Prism (GraphPad Software, USA) (Fig. 5, Table 1). The potency (EC_{50}) and maximum efficacy (E_{max}) values were generated by fitting the obtained data to three-parameter nonlinear regression curves with a bottom constrained to zero. Graphs were normalized to the highest response by the control, i.e. oxytocin or vasopressin. For radioligand displacement IC_{50} values were calculated by a three-parameter logistic Hill equation and then further used to approximate $K_i$values according to Cheng and Prusoff (48). The normalization refers to the specific binding of the radioligand in the absence of peptides as a maximum with an average of 4.2 pmol/mg for OTR, 2.6 pmol/mg for $V_{1a}R$ and 2.9 pmol/mg for $V_{1b}R$. All data regarding pharmacological characterization were presented as mean ± SD of at least three independent experiments unless otherwise stated.

Calcium Imaging

Imaging was performed with upright confocal microscope Leica TCS SP5 AOBS Tandem II (20x HCX APO L water immersion objective, NA 1.0) and inverted confocal microscope Leica TCS SP5 DMI6000 CS (20x HC PL APO water/oil immersion objective, NA 0.7). The Ca^{2+} indicator Calbryte 520 was excited using a 488 nm argon laser, and the emitted fluorescence was detected by Leica HyD hybrid detectors in photon-counting mode (Leica Microsystems, Wetzlar, Germany) in the range of 500-700 nm, as previously described (38). The laser power was adjusted to maintain a satisfactory balance between photobleaching and signal-to-noise ratio. The optical imaging thickness was set to nearly 5 µm to prevent recording from multiple cell layers. Time series were acquired with a frequency of 20 Hz and a resolution of 256 x 256 pixels and pixels size of approximately 1 mm^2.

Stimulation Protocol

Before imaging, the stained slices were kept in a substimulatory glucose concentration (HEPES with 6 mM glucose) at room temperature. For Ca^{2+} imaging slices were transferred into the recording chamber on the microscope stage, perfused continuously with carbonated ECS containing 6 mM glucose at 37 °C. After recording basal Ca^{2+} signals in 6 mM glucose, the perfusion was exchanged with stimulatory ECS containing 8 (or 9 mM as indicated) glucose for 15-20 minutes. When a stable second phase plateau response with fast Ca^{2+} oscillations was achieved (49), AVP or AVP receptor agonist/antagonist was added to the perfusion for 10-15 minutes. Following AVP/agonist/antagonist testing, the perfusion was again changed to ECS with either 8 or 9 mM glucose for wash-out period of about 15-20 minutes and then to the substimulatory ECS with 6 mM glucose until the cessation of fast Ca^{2+} oscillations. For AVP concentration-dependent responses, islets were stimulated with 8 mM glucose and 500 nM forskolin until the plateau phase has been achieved. A version of this experiment has been performed with 2.5 nM epinephrine. In both cases, a series of AVP concentrations were applied, each of which was static incubated in the recording chamber for 15 minutes before the next concentration was applied.

Hormone measurements

For acute insulin release in response to glucose, mouse pancreatic tissue slices were preincubated (1 h) in ESC buffer containing 6 mM glucose at 37°C. The sample for the insulin release from the slices was then collected in ESC with 6 mM glucose for 5 min (basal), after which the solution was replaced with stimulatory glucose in ESC 8 mM glucose or ESC 8 mM glucose plus 10 nM AVP and incubated for 15 min. Each solution replacement step involved washing the dish containing the slices at least 3 times to ensure no remaining insulin being present. Insulin concentration was determined using the Insulin Ultra-Sensitive assay kit from Cisbio (Bagnols-sur-Ceze, France).

	K_i ± SD (nM)			EC_{50} ± SD (nM)	E_{max} ± SD (%)	pA_2
	OTR	$V_{1a}R$	$V_{1b}R$	$V_{1b}R$	$V_{1b}R$	$V_{1a}R$
OT	0.5 ± 0.2	18 ± 5	588 ± 19	n.d.	n.d.	-
AVP	8 ± 5	0.2 ± 0.1	0.3 ± 0.1	15 ± 2	97 ± 4	-
[(D-Leu)^2,Ile^3,Thr^4]-VP	209 ± 36	5 ± 1	70 ± 7	361 ± 176	79 ± 8	n.d.
d[Cha^4,Dab^8]-VP	54 ± 19	23 ± 5	0.2 ± 0.02	130 ± 52	104 ± 13	n.d.
d[$(CH_2)_5^1$,Tyr(Me)^2,Dab^5,Tyr^9]-VP	2300 ± 190	66 ± 15	>10,000	-	-	5.27

Cha = cyclohexylalanine; $(CH_2)_5$ = β-mercapto-β,β-cyclopentamethylenepropionic acid; d = deaminated N-terminus; Dab = 2,4-diaminobutyric acid.

Table 1. Affinity, potency and efficacy of [(D-Leu)^2,Ile^3,Thr^4]-AVP, d[Cha^4,Dab^8]-AVP, and d[$(CH)^1$,Tyr(Me)^2,Dab^5,Tyr^9]-AVP.

Affinity (K) and potency (EC) data are indicated as mean ± SD (nM, n = 3); K_i values were calculated from IC_{50} values according to Cheng and Prusoff, assuming K_d values of 0.65 nM for OTR, 0.21 nM for $V_{1a}R$ and 0.14 nM for $V_{1b}R$. Efficacy (E_{max}) data is normalized to the maximum IP_1 formation by the control. #controls were OT at the OTR or AVP at the $V_{1a}R$ and $V_{1b}R$; n.d., not determined.

Dynamic insulin secretion from pancreatic tissue slices was assessed using an in-house built perifusion system with automated tray handling (Lambda Omnicoll, Lambda Laboratory Instruments). Two perifusion columns were connected to the system, each consisting of three chambers positioned vertically. Three pancreatic tissue slices were placed on perforated slice supports (Biorep), one slice per chamber. To equilibrate the slices to 37 °C and wash out accumulated enzymes and hormones, a 60-min pre-perifusion step was performed using a BSA-HEPES–based solution containing 6 mM glucose and soybean trypsin inhibitor (STI) at a flow rate of 140 µL min^{-1}. Subsequently, the experimental perifusion protocol was applied at the same flow rate. Samples were collected at 5-min intervals into 1.5 mL tubes and immediately placed on ice. All samples were stored at −80 °C until insulin or glucagon concentrations were determined using the corresponding HTRF assay kits (Revvity, 62IN1PEG and 62CGLPEG, respectively).

The stimulation solution contained (in mM): NaCl 125; KCl 2.5; HEPES 10; $NaHCO_3$ 10; Na_2HPO_4 1.25; sodium pyruvate 2; ascorbic acid 0.25; myo-inositol 3; lactate 6; $MgCl_2$ 1; $CaCl_2$ 2; forskolin 0.0005; BSA 0.1% (w/v); and soybean trypsin inhibitor 0.025% (w/v). Glucose (6 or 8 mM) and AVP (1 pM, 100 pM, or 10 nM) were added according to the experimental protocol. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated.

Insulin and glucagon concentrations measured in perifusate samples were normalized to the total hormone content of the tissue slices from the corresponding perifusion column. At the end of each experiment, the three pancreatic tissue slices from each perifusion column were collected and pooled for lysis in acid ethanol to extract intracellular hormone content. Insulin and glucagon concentrations in the lysates were determined using the same assay as for perifusate samples. Hormone secretion during each stimulation period was calculated as cumulative hormone release by summing hormone concentrations measured in all collected fractions during the respective stimulation period and normalizing the obtained value to the total hormone content of the corresponding lysate. To reduce heteroscedasticity, normalized hormone secretion values were log-transformed before statistical analysis and visualization. Outliers were identified after log transformation using a predefined 1.5×IQR rule within each experimental condition and excluded before statistical testing. Normalized and log transformed values from individual experiments were compared across replicates and presented as median ± IQR.

Data Analysis

The analysis of Ca^{2+} events has been performed as previously described (49). Briefly, the movies were processed using a custom Python script to detect ROIs corresponding to individual cells automatically. Cells have been identified based on their typical activity pattern and intraislet localization as described before (50, 51). β cells have been inactive at non-stimulatory glucose concentration (6 mM) and activated in a biphasic manner when stimulated with either 8 or 9 mM glucose. In contrast, α cells were still active at 6 mM glucose, progressively inhibited in 8 mM glucose, and further stimulated during epinephrine stimulation. Smooth muscle cells were localized around vascular structures in the slices and were used as a readout of V_{1a} receptor activity. All other cells outside the histologically identifiable islet were discarded from further analysis. In the next step, Ca^{2+} events from each ROI were automatically distilled and annotated (49).

For this study, altogether 172 pancreatic tissue slices have been imaged altogether. In addition to the frequency of events per roi per minute (epmpr), for each detected Ca^{2+} event, we measured the height, duration at half of the amplitude (halfwidth) and area under the curve (AUC) (Fig. 2E). Halfwidths between 0.01 to 100 seconds were collected and were pooled together for the global analysis. For the analysis within individual islets, the parameters from each ROI were checked for normality and then parametric analysis (ANOVA/Tukey HSD post hoc) or non-parametric analysis (Kruskal-Wallis/Dunńs test post hoc) has been performed. A similar strategy has been used when we compared several islets exposed to the same treatment. The rois with less than 5 events in the whole length of recording were eliminated from the analysis. The p-value below 0.05 has been taken as statistically significant.

Results

V_{1b} receptor transcript expression is enriched in α cells but not restricted to α cells

Previous transcriptomic analyses of purified pancreatic endocrine cells identified V_{1b} receptor expression as strongly enriched in α cells, whereas expression in β cells was reported to be low or absent (28). These data provided an important reference point for interpreting AVP effects in the islet, but they do not fully resolve receptor distribution within the preserved tissue context. We therefore performed RNAscope in situ hybridization in mouse pancreatic sections to assess the localization of V_{1b} receptor transcripts directly in intact pancreatic tissue (Fig. 1).

Consistent with previous transcriptomic data, V_{1b} receptor transcripts were readily detected in glucagon transcript-positive α cells. However, the signal was not restricted exclusively to glucagon-positive cells, indicating a broader intra-islet expression pattern than would be expected from a strictly α cell-specific marker (Fig. 1A). Quantification confirmed that 67.9 ± 7.5% of α cells were V_{1b} receptor-positive, confirming substantial V_{1b} receptor expression in α cells (Fig. 1B). At the same time, comparison of the V_{1b}-positive and glucagon-positive populations indicated that only approximately one third of the V_{1b} receptor-positive signal/cells colocalized with α cells. Thus, V_{1b} receptor expression in the islet is clearly not restricted to α cells.

Together, these data place the functional AVP experiments in the context of previous transcriptomic studies. They confirm that α cells represent an important V_{1b} receptor-positive endocrine population, but also indicate that V_{1b} receptor transcript expression is broader than expected from an exclusively α cell-restricted model. This supports a more cautious interpretation of AVP effects in intact tissue, where β cell modulation may arise from a combination of direct V_{1b} receptor-dependent effects in non-α cells, intra-islet paracrine interactions, and state-dependent collective β-cell dynamics.

The glucose-dependence of AVP modulation of α and β cell activity

Having established that V_{1b} receptor transcript expression in intact pancreatic islets is not restricted to α cells, we next examined how AVP modulates α and β cell Ca^{2+} activity under different glucose conditions. Since previous work has shown that both AVP responsiveness and islet hormone secretion depend strongly on the metabolic state of the islet, we first tested AVP effects at different glucose concentrations using live Ca^{2+} imaging in acute pancreatic tissue slices.

We have fully reproduced the previously reported glucose-dependent effects of AVP on both α and β cells (Fig. 2). It is crucial to emphasize that at the substimulatory glucose, neither forskolin, an activator of cAMP production alone nor in combination with AVP could significantly activate β cells (Fig. 2BF). On the other hand, forskolin alone or combined with AVP, activated α cells (Fig. 2DG). However, subsequent increase to stimulatory glucose in the same islet activated β cells (Fig. 2B) and progressively reduced the activity of α cells (Fig. 2D). The activation of α cells with AVP did not have a significant effect on β cells (Fig. 2BD).

As previously observed, the application of forskolin to islets after stimulation with physiological levels of glucose (8 or 9 mM), significantly increased the activity of β cells (Fig. 3B). Forskolin, even at low concentration used here (500 nM), increased the frequency of Ca^{2+} oscillations in all islets tested at 8 mM glucose (Fig. 3BE). Application of 10 nM AVP further increased the oscillation frequency, which was often associated with a visible decrease in the halfwidth of the events (Fig. 3B) and in the overall AUC, a parameter that reflects both changes in oscillation frequency and duration (Fig. 3H), although this was not significantly different after pooling the data (Fig. 3GH). It is worth noting that the AUC values varied significantly between different islets, ranging from an increase in overall activity after AVP application to no change or even a decrease in AUC.

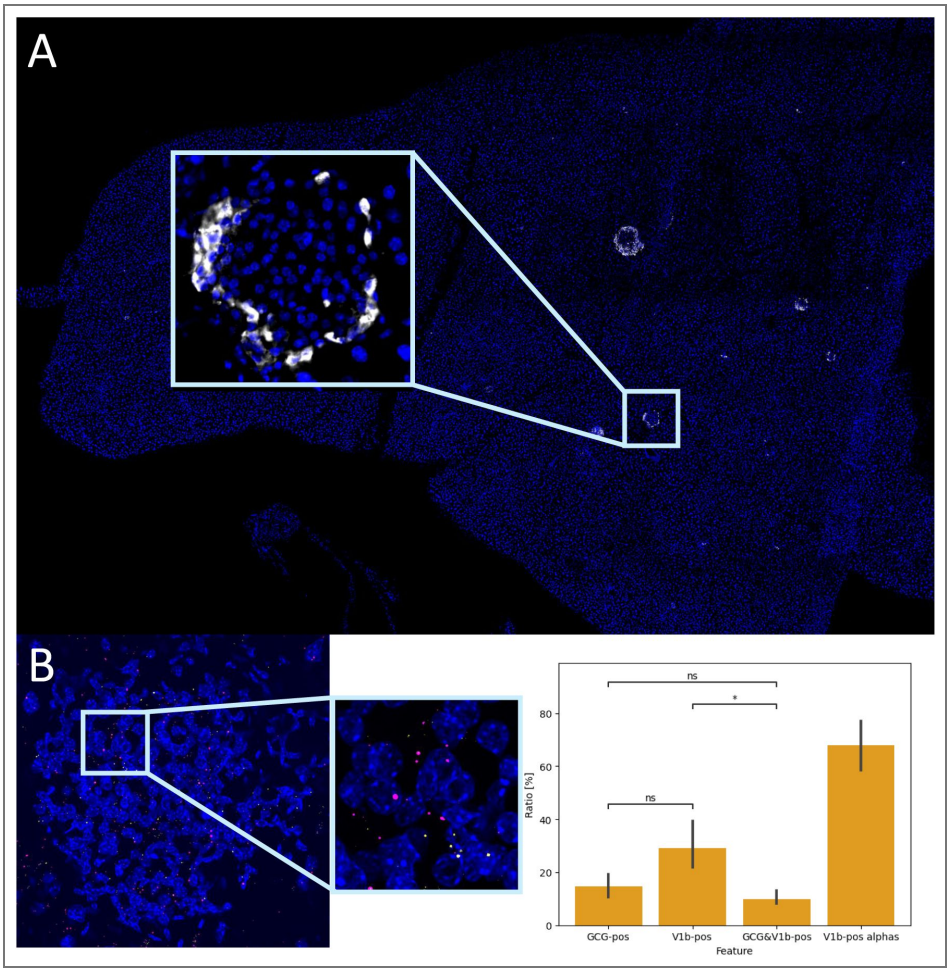


Figure 1. V_{1b} receptor transcript expression in pancreatic islets is enriched in α cells but not restricted to α cells.

(A) Mouse pancreatic section showing immunostaining for somatostatin and DAPI, with the islet region shown at higher magnification. (B) RNAscope in situ hybridization of the same islet region. Magenta puncta indicate V_{1b} receptor / Avpr1b transcripts and yellow puncta indicate Gcg transcripts. The magnified inset shows V_{1b} receptor transcript signal in glucagon-positive α cells as well as in other glucagon-negative islet cells. C) Quantification of RNAscope signals showing the relative abundance of glucagon-positive cells, V_{1b} receptor-positive cells, double-positive glucagon/V_{1b} receptor transcript-expressing cells, and the fraction of α cells positive for V_{1b} receptor transcripts. In this dataset, 67.9 ± 7.5% of α cells were V_{1b} receptor-positive, whereas only approximately one third of V_{1b} receptor-positive cells/signals colocalized with glucagon-positive α cells.

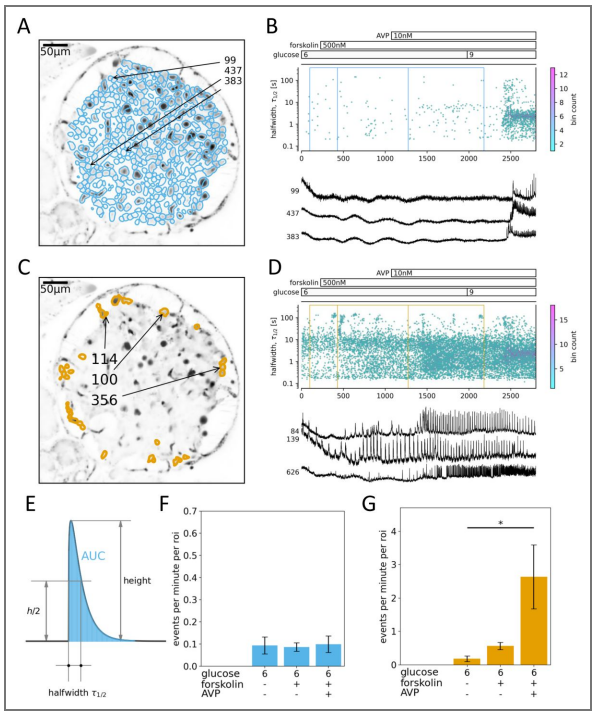


Figure 2. The effect of AVP at the substimulatory glucose on β (A, B, F) and α (C, D, G) cells.

(A, C) Regions of interest (ROIs) were obtained with our analytical pipeline. Indicated are the ROIs whose filtered traces correlate best with the average trace for the whole islet. (B, D) (top panel) Time distribution of all measured eventś halfwidths at their peak times. Stimulation protocol is indicated above. The color bar indicates the bin count in each time/halfwidth point. The dashed-line rectangles indicate the 3 time periods where events per minute per ROI parameter was sampled for panels F and G. (bottom panel) Representative traces from ROIs indicated in A and C. (E) A scheme showing how the height, duration at half amplitude (halfwidth) and area under the curve (AUC) were measured for each Ca^{2+} event detected. (F, G) Events per minute per ROI were normally distributed, we used one-way ANOVA and Tukey post-hoc test. The data are presented as mean +/− SD. The significant differences in AVP treatment at the substimulatory glucose were found in α cells only (*$p < 0.05$).

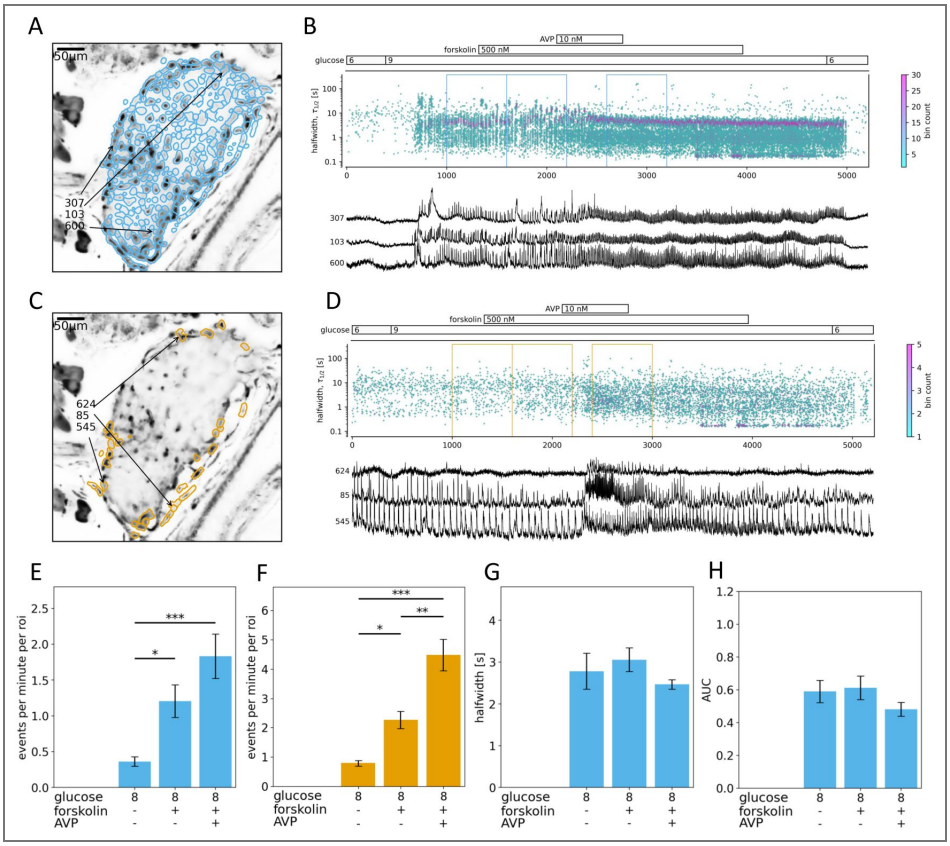


Figure 3. The effect of AVP in the physiological stimulatory glucose range on β (A, B, E, G, H) and α (C, D, F) cells.

(A, C) Indicated are the ROIs whose filtered traces correlate best with the average trace for the whole islet. (B, D) (top panel) Time distribution of all measured eventś halfwidths at their peak times. Stimulation protocol is indicated above. The color bar indicates the bin count in each time/halfwidth point. The dashed-line rectangles indicate the 3 time periods where events per minute per ROI, halfwidth and AUC parameters were sampled for panels E-H. (bottom panel) Representative traces from ROIs indicated in A and C. (E, F) Forskolin significantly increased the number of Ca^{2+} oscillations per minute per ROI in β cells that were further stimulated by addition of AVP. A similar effect was observed also in α cells. (G, H) A non-significant but visible decrease in the events' halfwidth and AUC values due to forskolin and AVP administration in β cells were noted. The parameters were distributed normally, and we used one-way ANOVA and Tukey post-hoc test. The data are presented as mean +/− SD (*p < 0.05, **p < 0.01, **p < 0.001).

Using low levels of forskolin in the same islet increased the activity of α cells despite the presence of high glucose concentration and glucose-dependent Ca^{2+} oscillations in β cells, both of which should inhibit the activity of α cells (Fig. 3DF). This increased activity was further enhanced by adding 10 nM AVP (Fig. 3DF). Again, the intense stimulation of α cells had no detectable effect on AVP modulation of β cell activity. The control experiment without AVP application did not produce significant changes in oscillation frequency in a comparable recording time (Suppl. Fig. 3). In this study we did not observe any sex differences in AVP responses.

The effect of physiological epinephrine concentration on AVP modulation of α and β cells

To further test whether AVP-dependent modulation of islet cell activity could be explained by a cAMP-like stimulatory mechanism, we used physiological epinephrine concentrations. We have previously shown that epinephrine suppresses Ca^{2+} activity in β cells at physiological glucose concentrations through a G_i-protein-coupled pathway, while simultaneously stimulating α-cell activity through G_s-protein-coupled signaling (51).

In the stimulatory 9 mM glucose, the administration of epinephrine rapidly reduced the frequency of β cell oscillations to a non-stimulatory activity level (Fig. 4). Subsequent perfusion with progressively increasing AVP concentration did not restore β cell activity beyond this level, indicating that AVP does not counteract epinephrine-induced inhibition in a manner consistent with activation of G_s-protein-coupled V_2 receptors (Fig. 4E).

In contrast, α cells were activated by AVP and responded further to AVP in a frequency-coded epinephrine concentration-dependent manner (Fig. 4DF). This supports the view that AVP acts as a potent modulator of α cell activity under physiological adrenergic conditions. Importantly, these experiments were performed without forskolin, demonstrating that forskolin is not required for AVP-dependent activation of α cells or for detectable modulation of β cell activity. Rather, forskolin was used in subsequent experiments to provide a common cAMP-permissive background in which AVP effects on α and β cell activity could be compared under more standardized conditions.

AVP enhances the activity of α and β cell within its physiological osmoregulatory range

To further investigate the sources of variability in the observed AVP action in the presence of forskolin and 8 mM glucose, we performed a series of experiments in which slices were exposed to an AVP concentration ramp. AVP administration reproducibly induced concentration-dependent activation of both α and β cells (Fig. 5). The most prominent stimulatory effect of AVP on both cell types was observed in its physiological osmoregulatory range between 10 and 100 pM (Fig. 5BFG).

In β cells, lower concentrations of AVP resulted in an increased frequency of Ca^{2+} oscillations, but this was reduced at higher AVP concentrations (Fig. 5G). In contrast, the halfwidth of oscillations decreased progressively and significantly with increasing AVP concentration (Fig. 5I). The overall effect of AVP produced a bell-shaped relationship for both the frequency parameter, measured as events per minute per region of interest (epmpr) (Fig. 5F), and normalized AUC values (Fig. 5G).

Such bell-shaped relationships are consistent with the known activation and inactivation properties of IP_3 receptors (37), which represent a major downstream component of V_{1b}/G_q/PLC-dependent Ca^{2+} signaling. IP_3 receptors are activated with lower IP_3 and Ca^{2+} levels, but become progressively inactivated at higher concentrations. Thus, unphysiologically high AVP concentrations may strongly stimulate of G_q protein-coupled signaling and thereby contribute to IP_3 receptor inactivation, reducing the dynamic range of the AVP response and potentially resulting in an inhibitory effect, with AUC values falling below those of 8 mM glucose stimulation alone (Fig. 5G). This inhibitory effect was also reflected in the reduced insulin release in static

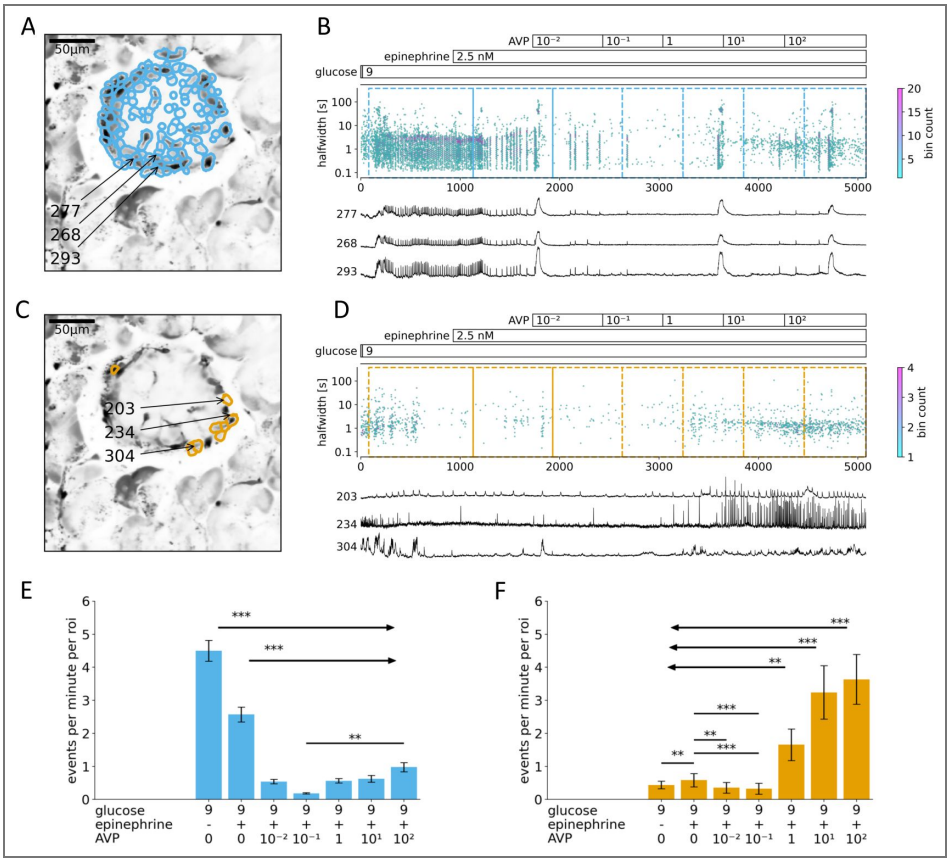


Figure 4. The effect of physiological epinephrine concentration β (A, B, E) and α (C, D, F) cells.

(A, C) Indicated are the ROIs whose filtered traces correlate best with the average trace for the whole islet. (B, D) (top panel) Time distribution of all measured eventś halfwidths at their peak times. Stimulation protocol is indicated above. The color bar indicates the bin count in each time/halfwidth point. The dashed-line rectangles indicate the 7 time periods where events per minute parameter was sampled for panels E and F. (bottom panel) Representative traces from ROIs indicated in A and C. (E, F) The analysis of events per minute per ROIs shows that inhibition by epinephrine of glucose-dependent activation is reversed with increasing vasopressin concentration (0.01, 0.1, 1, 10 and 100 nM) in α cells but not in β cells. The parameters were distributed normally, and we used one-way ANOVA and Tukey post-hoc test. The data are presented as mean +/− SD (*p < 0.05, **p < 0.01, **p < 0.001).

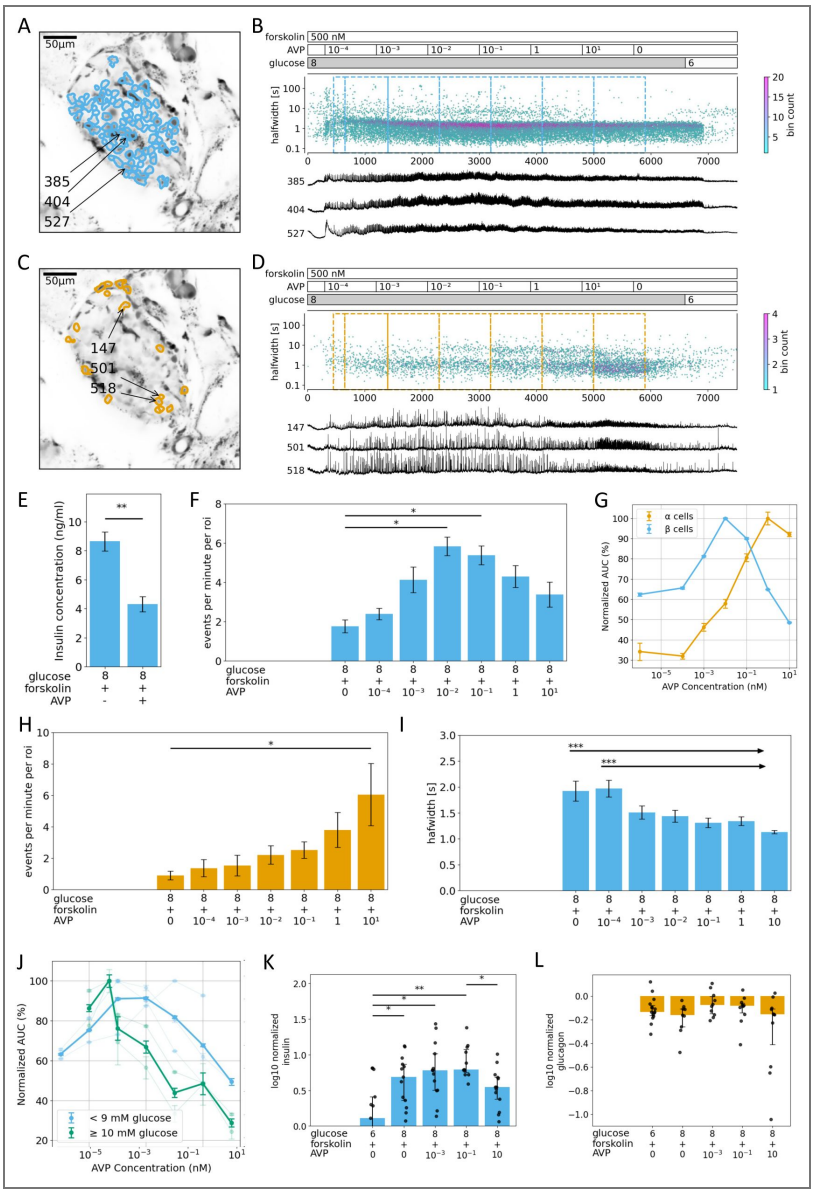


Figure 5. AVP modulates the activity of β (A, B, E-G, I, J, K) and α (C, D, G, H, L) cells in a concentration-dependent manner.

(A, C) Indicated are the ROIs whose filtered traces correlate best with the average trace for the whole islet. (B, D) (top panel) Time distribution of all measured eventś halfwidths at their peak times. Stimulation protocol is indicated above. The color bar indicates the bin count in each time/halfwidth point. The dashed-line rectangles indicate the 7 time periods where events per minute per ROI and halfwidth parameters were sampled for panels F, H and J. (bottom panel) Representative traces from ROIs indicated in A and C. (E) Supraphysiological concentrations of AVP (above 10 nM) reduced insulin release in static incubation conditions from β cells below that measured at 8 mM glucose and forskolin alone. (F, H) Events per minute per ROI in β cells was gradually increased from 0.0001 nM to the physiological osmoregulatory range of AVP, between 0.01 and 0.1 nM, and then gradually decreased with further increases in AVP concentration. In the same islets the number of events per minute increased gradually in α cells also at higher AVP. (G) Pooling several islets revealed that bell-shaped distribution of the AUC could be found in both β and α cells, although the latter being shifted towards higher AVP concentration. (I) The halfwidth of events in β cells progressively declined with increased AVP concentration. (J) The AVP-dependent activation/inactivation curve for β cells was left-shifted at supraphysiological glucose concentration. All the parameters were distributed normally, and we used one-way ANOVA and Tukey post-hoc test. The data are presented as mean +/− SD (*p < 0.05, **p < 0.01, **p < 0.001). (K, L) Concentration-dependent effects of AVP (in nM) on hormone secretion at 8 mM glucose and forskolin. (K) Insulin secretion exhibited a bell-shaped response, with maximal stimulation at low AVP concentrations and reduced secretion at 10 nM AVP. (L) Glucagon secretion was not significantly affected. Bars indicate median ± IQR; dots denote individual experiments. *P < 0.05, **P < 0.01.

incubation insulin release assay (Fig. 5E) as well as in dynamic insulin secretion (Fig. 5K). In the latter, insulin secretion showed a bell-shaped AVP concentration dependence, with increased release at low AVP concentrations and reduced release at 10 nM AVP.

A similar paradoxical inhibitory effect on β cells has previously been documented for muscarinic receptor signaling, which also involves the activation of Gq/PLC/IP_3 pathway (52). Additionally, at supraphysiological glucose concentration, the AVP-dependent activation/inactivation curve for β cells was shifted to the left (Fig. 5J), allowing for inhibitory modulation by AVP at lower concentrations and suggesting that glucose-dependent excitability state of β cells strongly shapes their sensitivity to AVP.

Notably, peak AVP activation in α cells occurred at an approximately one order of magnitude higher AVP concentration than in β cells (Fig. 5G), with limited evidence of inactivation at physiological AVP levels (Fig. 5GH), supporting previously published findings (13). Although AVP induced concentration-dependent changes in α cell Ca^{2+} activity, these effects were not reflected in dynamic glucagon secretion measurements. (Fig. 5L).

V_{1b} receptor-selective agonists reproduce key AVP effects on α- and β-cell Ca^{2+} activity

One of the primary objectives of this study was to evaluate the exclusive role of V_{1b} receptors without confounding effects of the activation of V_{1a}, V_2 and oxytocin receptors in α and β cells using fresh pancreatic tissue slices. To accomplish this, we employed AVP, alongside a set of newly synthesized specific peptides (Fig. 6A): d[Cha^4,Dab^8]-VP, a specific V_{1b} receptor agonist; [(D-Leu)^2,Ile^3,Thr^4]-VP, a mixed V_{1b} receptor agonist and V_{1a} and oxytocin receptor antagonist; and d[$(CH_2)_5{}^1$,$Tyr(Me)^2$,Dab^5,Tyr^9]-VP, specific V_{1a} receptor antagonist. Further, we used a commercially available compound tolvaptan to further substantiate the absence of V_2 receptors in β cells – evidenced by our inability to reverse epinephrine inhibition with AVP (Fig. 4).

The three peptide ligands were rationally designed based on available earlier structure-activity relationship data from our own previous work (47) and others (53–55), incorporating specific modifications to optimize receptor selectivity and activation. The functional properties of the three peptide ligands were assessed using a panel of cell-based *in vitro* assays expressing human oxytocin, V_{1a}, and V_{1b} receptors. Receptor-subtype selectivity was assessed using radioligand binding studies (Fig. 6B) measuring the displacement of tritiated OT/AVP with increasing concentrations of peptides. This demonstrated that d[Cha^4,Dab^8]-VP had the highest affinity for V_{1b} receptor (200 pM) and a selectivity gain over oxytocin (~270-fold) and V_{1a} receptor (~115-fold) (Table 1). On the other hand, [(D-Leu)^2,Ile^3,Thr^4]-VP had comparable affinity for all three receptors, ranging between 5 nM and 209 nM (Table 1), and lastly d[$(CH_2)_5{}^1$,$Tyr(Me)^2$,Dab^5,Tyr^9]-VP exhibited only affinity for V_{1a} receptor (66 nM), but did not bind to oxytocin (>2.3 μM) or V_{1b} receptors (>10 μM) (Table 1).

To determine their activation profile, intracellular signaling was evaluated using IP_1 second messenger quantification in two steps: a first single-concentration screen allowed to distinguish agonist/antagonist properties of the peptide ligands (Fig. 6C) and a comprehensive concentration-response determination yielded reliable values for potency (EC_{50}), efficacy (E_{max}) and inhibitory potency (pA_2) (Fig. 5DE). Peptide d[Cha^4,Dab^8]-VP is a moderate potent full agonist at V_{1b} receptor (EC_{50} = 130 nM; E_{max} = 104%) (Table 1), with no significant activation of V_{1a} or oxytocin receptors (Fig. 5C). Its potency was slightly reduced as compared to endogenous AVP (15 nM). Peptide [(D-Leu)^2,Ile^3,Thr^4]-VP functioned as a moderate/weak partial agonist at V_{1b} receptor (EC_{50} = 361 nM; E_{max} = 79%) (Table 1), while displaying antagonist properties at oxytocin and V_{1a} receptors (Fig. 6C). Peptide d[$(CH_2)_5{}^1$,$Tyr(Me)^2$,Dab^5,Tyr^9]-VP demonstrated selective antagonism for V_{1a} receptor, effectively inhibiting endogenous ligand-induced signaling in a competitive manner (as exemplified by Schild regression analysis, Fig. 6D). Its inhibitory potency (pA2) was determined to 5.27 (Table 1), corresponding to 5.37 μM. These findings demonstrate that the newly designed peptide ligands provide valuable tools for probing the role of V_{1b} receptors in in α and β cells.

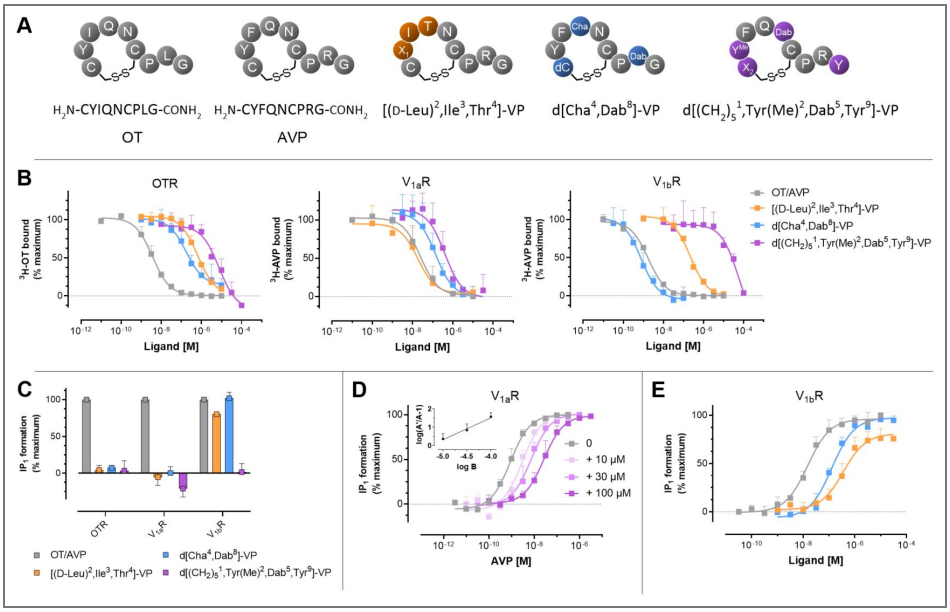


Figure 6. Schematic structures and pharmacology of peptide probes [($_D$-Leu)^2,Ile^3,Thr^4]-VP, d[Cha^4,Dab^8]-VP, and d[(CH$_2$)$_5^1$,Tyr(Me)^2,Dab^5,Tyr^9]-VP.

(A) Schematic structures and amino acid compositions of oxytocin (OT), vasopressin (AVP), and their derivatives. (B) Radioligand displacement assay of [($_D$-Leu)^2,Ile^3,Thr^4]-VP, d[Cha^4,Dab^8]-VP, and d[(CH$_2$)$_5^1$,Tyr(Me)^2,Dab^5,Tyr^9]-VP. Concentration-dependent displacement of ^{3}H-OT (0.65 nM) or ^{3}H-AVP (0.21 nM for V_{1a}R, 0.14 nM for V_{1b}R) in HEK293 cell membranes expressing the human OTR, V_{1a}R or V_{1b}R by either test ligand or the respective control. Data is presented as specific binding by subtracting nonspecific from total binding normalized to the maximum binding of the radioligand in the absence of the peptides (n = 3). (C) Ligand induced formation of intracellular IP_1 by 10 µM OT/AVP, [($_D$-Leu)^2,Ile^3,Thr^4]-VP, d[Cha^4,Dab^8]-VP, and d[(CH$_2$)$_5^1$,Tyr(Me)^2,Dab^5,Tyr^9]-VP demonstrating agonistic properties of [($_D$-Leu)^2,Ile^3,Thr^4]-VP and d[Cha^4,Dab^8]-VP at V_{1b}R, whereas in the sense of an antagonism no increased IP_1 accumulation was observed at OTR and V_{1a}R (n = 2). Activation data was normalized to maximum response generated by the control (OT for OTR, AVP for V_{1a}R and V_{1b}R). All data points are shown as mean ± SD. (D) Antagonistic properties of d[(CH$_2$)$_5^1$,Tyr(Me)^2,Dab^5,Tyr^9]-VP at V_{1a}R were assessed through concentration-dependent displacement of AVP according to the Schild method (n=3). (E) Concentration-response curves of AVP, [($_D$-Leu)^2,Ile^3,Thr^4]-VP, and d[Cha^4,Dab^8]-VP were obtained for the V_{1b}R in order to assess their potency (n = 3). Affinity constants (K_i), potency (EC50) and maximum efficacy (E_{max}) values for [($_D$-Leu)^2,Ile^3,Thr^4]-VP, d[Cha^4,Dab^8]-VP, d[(CH$_2$)$_5^1$,Tyr(Me)^2,Dab^5,Tyr^9]-VP, and the respective controls are listed in Table 1. Abbreviations: Cha = cyclohexylalanine; Dab = 2,4-diaminobutyric acid; d = desamino; X_1 = $_D$-Leu; X_2 = d(CH$_2$)$_5$ (β-mercapto-β,β-cyclopentamethylenepropionic acid); Y^{Me} and Tyr(Me)= O-methylated tyrosine.

Applied in pancreatic tissue slices, [(D-Leu)^2,Ile^3,Thr^4]-VP produced effects similar to AVP, characterized by an increase in the frequency and a decrease in the halfwidth of the Ca^{2+} oscillations (Fig. 7). However, paired before-after analysis revealed heterogeneous β cell responses, with some islets showing no change or even a reduction in Ca^{2+} oscillation frequency after ligand application. Consequently, the pooled AUC data did not show a significant difference (Fig. 7E). In contrast, α cells were activated in a frequency-coded manner (Fig. 7DF).

Insulin release from islets within pancreas slices did not show statistically significant differences following treatment with [(D-Leu)^2,Ile^3,Thr^4]-VP in 8 mM glucose and substimulatory concentration of forskolin (Fig. 7G). Given that [(D-Leu)^2,Ile^3,Thr^4]-VP also antagonized V_{1a} and oxytocin receptors, it is plausible that the stimulatory effect through V_{1b} receptors was attenuated. Analysis of the effects of d[Cha^4,Dab^8]-VP or AVP under similar experimental conditions, revealed that both produced comparably heterogenous responses. Paired analysis indicated a non-significant change in AUC with both V_{1b} receptor-selective agonists (Fig. 7EH) as well with AVP (Fig. 7I).

Subsequently, we tested the involvement of V_{1a} receptors using d[$(CH_2)_5^1$,Tyr(Me)^2,Dab^5,Tyr^9]-VP, a V_{1a} receptor antagonist. This antagonist did not inhibit AVP-stimulated Ca^{2+} activity in either α and β cells (Fig. 8A-F). To validate its V_{1a} antagonistic action in the same tissue preparation, we assessed Ca^{2+} oscillations in smooth muscle cells of blood vessels adjacent to the islets (Fig. 8G). AVP increased the frequency of Ca^{2+} oscillations in vascular smooth muscle cells, and this activity was specifically inhibited by d[$(CH_2)_5^1$,Tyr(Me)^2,Dab^5,Tyr^9]-VP and restored when antagonist washout, consistent with AVP action through V_{1a} receptor in these cells. In contrast, application of V_2 receptor antagonist tolvaptan did not have any significant effect on α cells, β cells, or any other cell types in the slice, and did not reverse any of the AVP effects (Suppl. Fig. 2).

State-dependent pharmacological modulation of β cell activity by AVP and V_{1b} receptor-selective agonists

Fig. 9 summarizes the analysis of the pharmacological modulation of AVP receptor-dependent responses in β cells within pancreatic tissue slices. During the AVP concentration ramp, both the amplitude and direction of changes in the rate of Ca^{2+} oscillations varied substantially across islets (Fig. 9A). The initial activation at lower pM AVP concentrations was followed by a relatively stable response near the peak of a bell-shaped concentration-dependence curve, which then transitioned towards inhibition at concentrations approaching or exceeding nM range.

The distribution of the rate of changes at different AVP concentrations resembled that observed during static incubation experiment with AVP or V_{1b} receptor-selective agonists (Fig. 9B), showing a range of responses: activation (46%), no effect (27%), and inhibition (17%) after applying AVP, [(D-Leu)^2,Ile^3,Thr^4]-VP (Fig. 8C) or d[Cha^4,Dab^8]-VP (Fig. 8D). In contrast, control without agonists (Fig 8E) or the use of antagonists for non-expressing AVP receptors following AVP stimulation, did not significantly affect the relative rate change (Fig. 8FG). One interpretation of the observed variability with V_{1b} receptor activation is that different islets, during their plateau phase stimulated by 8 mM glucose and forskolin, may achieve varying levels of IP_3 receptor activation (IP_3 receptor activation equivalent), before being further influenced by AVP or specific V_{1b} receptor agonists (Fig. 8H). Overall, the effect of V_{1b} receptor stimulation depends on the current functional status of an islet. Resting β cells permit activation and increase insulin secretion, whereas in highly activated β cells, high levels of AVP and V_{1b} receptor agonists tend to promote inactivation and interfere with insulin release (Fig. 5K).

Discussion

The use of freshly prepared pancreatic tissue slices was important for resolving the context-dependent role that AVP receptor signaling plays in the physiology of pancreatic β and α cells. This preparation represents acutely isolated, viable pancreatic tissue from healthy mice that can be imaged in real time using high-resolution confocal microscopy shortly after extraction. Tissue manipulation during slicing and vital Ca^{2+} indicator loading is kept to a minimum. Pancreatic

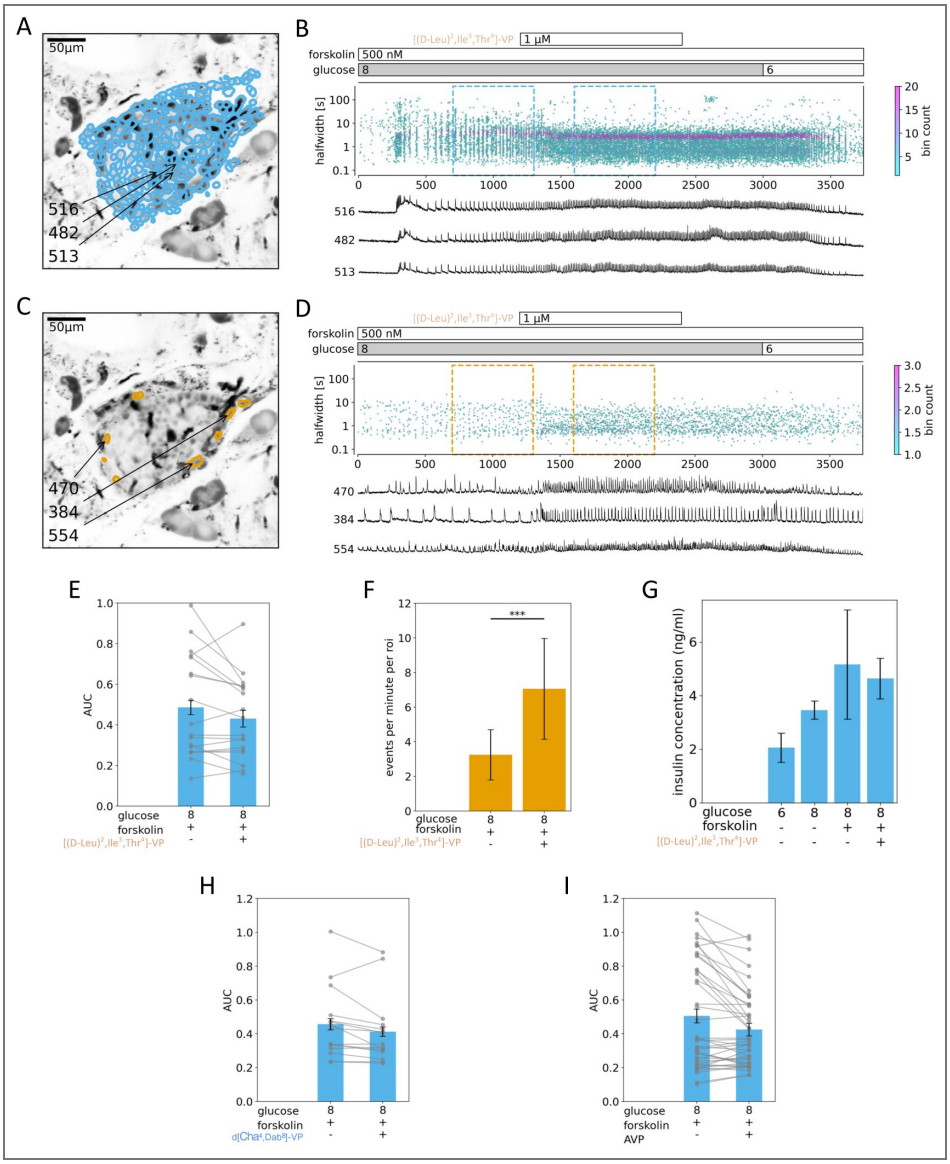


Figure 7. A specific V_{1b} receptor agonist and V_{1a} and oxytocin receptor antagonist, has a similar effect on β (A, B, E) and α (C, D, F) cells as AVP.

(A, C) Indicated are the ROIs whose filtered traces correlate best with the average trace for the whole islet. (B, D) (top panel) Time distribution of all measured eventś halfwidths at their peak times. Stimulation protocol is indicated above. The color bar indicates the bin count in each time/halfwidth point. The dashed-line rectangles indicate the 2 time periods where events per minute per ROI and AUC parameters were sampled for panels E and F. (bottom panel) Representative traces from ROIs indicated in A and C. (E) There were no significant differences in the pooled AUC data for β cells treated with the [(D-Leu)^2,Ile^3,Thr^4]-VP and forskolin or with forskolin only. Direct comparison of the AUC values before and after the ligand application shows a large heterogeneity of responses. (F) A significant increase in the events per minute per ROIs due to [(D-Leu)^2,Ile^3,Thr^4]-VP administration in α cells was noted. (G) There was no significant effect on insulin release from slices after [(D-Leu)^2,Ile^3,Thr^4]-VP exposure in comparison to stimulatory forskolin only. (H, I) The d[Cha^4, Dab^8]-VP (a specific V_{1b} receptor agonist) and AVP produced similarly heterogeneous responses in β cells with non-significant changes in the AUC compared to forskolin only. All the parameters were distributed normally, and we used one-way ANOVA and Tukey post-hoc test. The data are presented as mean +/− SD (*p < 0.05, **p < 0.01, **p < 0.001).

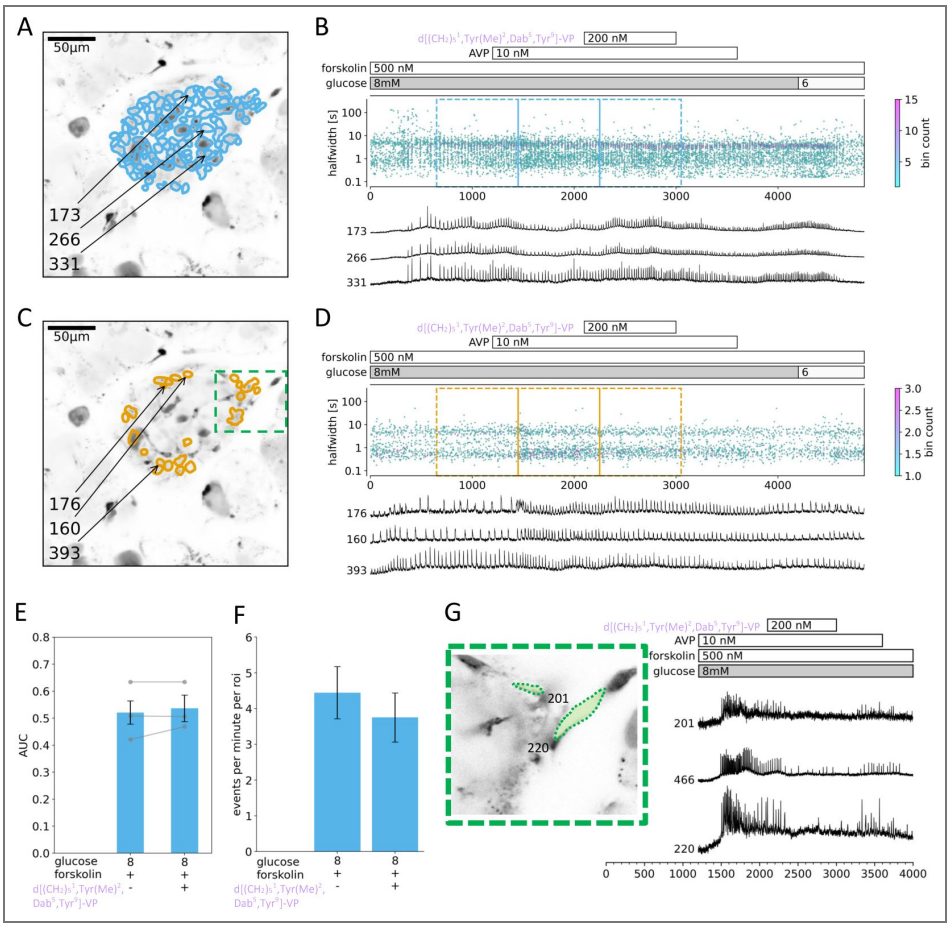


Figure 8. A specific V_{1a} receptor antagonist has no significant effect on β (A, B, E, F) and α (C, D) cells, but inhibits activity in smooth muscle cells (G).

(A, C) Indicated are the ROIs whose filtered traces correlate best with the average trace for the whole islet. Inset labelled with green dashed line is expanded in panel G. (B, D) (top panel) Time distribution of all measured eventś halfwidths at their peak times. Stimulation protocol is indicated above. The color bar indicates the bin count in each time/halfwidth point. The dashed-line rectangles indicate the 3 time periods where events per minute per ROI and AUC parameters were sampled for panels E and F. (bottom panel) Representative traces from ROIs indicated in A and C. (E) There were no significant differences in the pooled AUC data for β cells treated with the d[$(CH_2)_5^1$, Tyr(Me)^2, Dab^5, Tyr^9]-VP and forskolin or with forskolin only. Direct comparison of the AUC values before and after the antagonist application shows no difference in responses. (F) No significant differences in the events per minute per ROIs due to d[$(CH_2)_5^1$, Tyr(Me)^2, Dab^5, Tyr^9]-VP administration in α cells were noted. (G) (left) Inset from C, showing location of two smooth muscle cells lining the blood vessel adjacent to the islet (right) Representative traces from ROIs on smooth muscle cells exposed to stimulatory glucose, forskolin, AVP, and d[$(CH_2)_5^1$, Tyr(Me)^2, Dab^5, Tyr^9]-VP. AVP administration increased the frequency of Ca^{2+} oscillations in smooth muscle cells, but V_{1a} receptor antagonist fully, but reversibly inhibited this activity. All the parameters were distributed normally, and we used one-way ANOVA and Tukey post-hoc test. The data are presented as mean +/− SD.

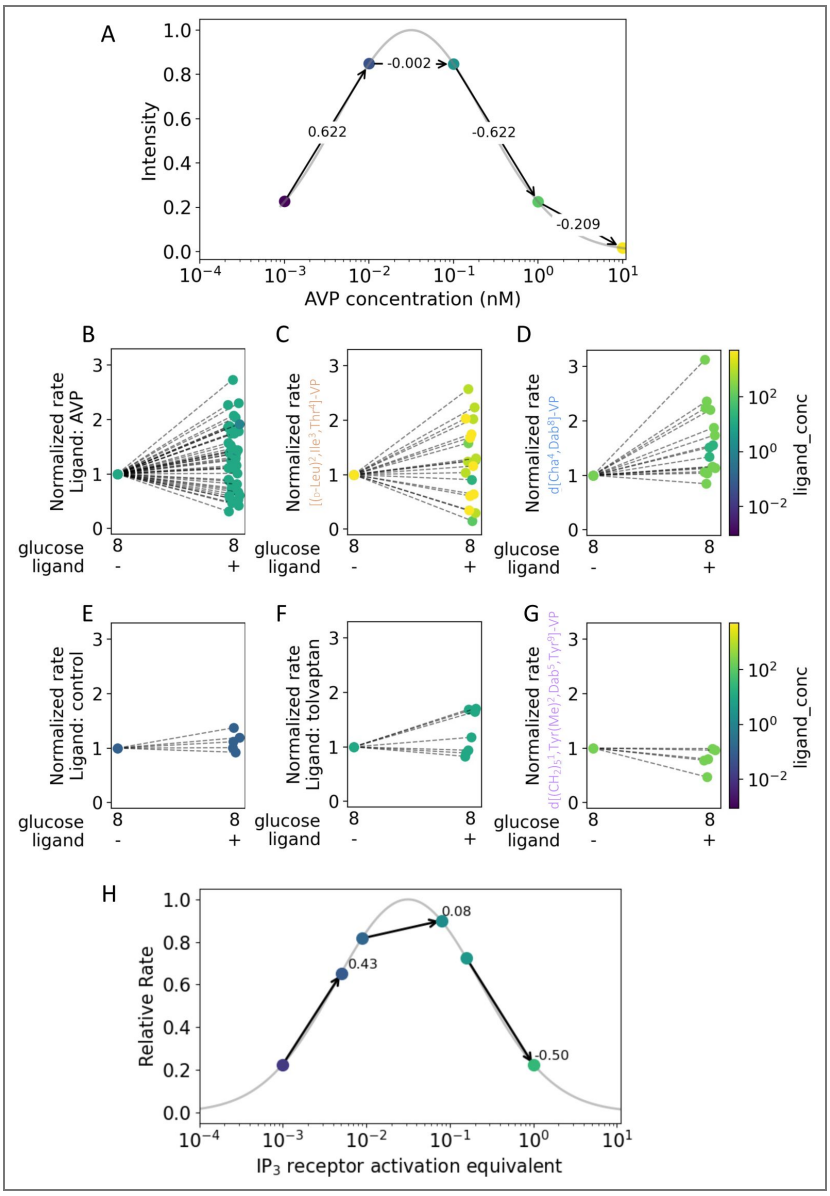


Figure 9. Overview of the analysis of pharmacological modulation of V_{1b} receptors in β cells in pancreatic tissue slices.

(A) The relative rates of Ca^{2+} oscillations obtained during the AVP concentration ramp experiment. The bell-shaped dependence curve rises at the lower pM range of AVP, followed by a relatively stable response around the peak of the curve, and then falls at higher concentrations (nM range) of AVP. (B, C, D) Normalized rates to the basal stimulatory conditions (8 mM glucose) in the context of single concentration static incubation with AVP, $[(D\text{-}Leu)^2$, Ile^3, $Thr^4]$-VP and $d[Cha^4,Dab^8]$-VP are widely scattered. (E, F, G) Normalized rates to the basal stimulatory conditions (8 mM glucose) in the context of single concentration static incubation without AVP receptor agonists (control), with tolvaptan (V_2 antagonist) and $d[(CH_2)_5{}^1,Tyr(Me)^2,Dab^5,Tyr^9]$-VP ($V_{1a}$ antagonist) are only slightly scattered. (H) Different islets during their plateau phase stimulated by 8 mM glucose and forskolin can achieve different levels of IP_3 receptor activation equivalent, contributing to the heterogeneity of further IP_3R stimulation with AVP or specific V_{1b} agonists.

slices therefore provide a valuable ex vivo preparation that preserves many properties of intact cells, including local tissue architecture and intercellular communication between homotypic and heterotypic cell types. These interactions are disrupted in research models involving isolated islets, dispersed islet cells (56) and cell lines, and the slice preparation also avoids the enzymatic digestion step used for islet isolation (38, 57). At the same time, pancreatic slices remain an ex vivo model with limitations, including the absence of intact vascular perfusion and innervation.

Existing evidence regarding the role of AVP for the activity of pancreatic β and α cells remains controversial. Some studies suggest that AVP plays a significant role in insulin and glucagon release, whereas others report minimal or selective effects (7, 13). There has generally been stronger functional evidence for involvement of V_{1b} receptors than direct evidence for their mRNA or protein abundance in β cells. Previous single-cell transcriptomic analysis (58) or protein expression analysis (8) reported absent or very low AVP receptor expression in β cells, whereas overexpression of V_{1b} receptors in β cells improved the function of transplanted pseudoislets in vitro (59). Our new RNAscope analysis adds spatial information to this discussion (Fig. 1). It confirms that V_{1b} receptor transcripts are present in glucagon-positive α cells, but also shows that V_{1b} receptor transcript signal in the islet is not restricted to α cells. In our dataset, 67.9 ± 7.5% of α cells were V_{1b} receptor-positive, whereas only approximately one third of the total V_{1b} receptor-positive signal/cells colocalized with glucagon-positive α cells. Thus, the data support α cells as an important V_{1b} receptor-positive endocrine population, but argue against a strictly α-cell-restricted model of V_{1b} receptor expression in intact pancreatic tissue (28). Future approaches combining spatial expression analysis, cell-specific genetic models, and physiological recordings in intact tissue will be needed to determine how AVP responses arise from direct receptor expression, α-to-β-cell interactions (60), and the dynamic state of the β-cell collective.

Our research aimed to reassess the role of AVP in the function of both α and β cells in fresh pancreas tissue slices, a preparation with in vivo-like sensitivity to physiological and pharmacological stimuli while enabling high spatial and temporal resolution measurements (38, 49, 51, 61). We focused on signaling through G_q-coupled AVP receptor, particularly V_{1b} receptors, and downstream Ca^{2+} signaling, involving IP_3 receptors. The bell-shaped response curve observed with AVP receptor agonists, where lower concentrations stimulate and higher concentrations inhibit cell activation and insulin release, is consistent with the biphasic activation and inactivation properties of the IP_3 receptors (37, 62, 63). The single-concentration agonist experiments with 15-minute incubations allowed us to reduce the possibility that the observed responses during the prolonged AVP ramp were explained solely by time-dependent receptor internalization or desensitization. The bell-shaped relationship has important implications for understanding how β and α cells respond to fluctuating AVP levels of AVP under varying plasma glucose levels, plasma osmolality and hormonal context. Importantly, it underscores the necessity of controlling AVP to avoid dysfunctional endocrine responses, or improve it after transplantation (59). This interpretation is also consistent with the emerging clinical view that AVP, as a hydration-sensitive hormone, links osmoregulatory state to metabolic outcomes, including glucose homeostasis, insulin sensitivity, lipid metabolism, and energy balance(64).

Hormones acting through IP_3 as a Ca^{2+}-releasing messenger can induce intracellular Ca^{2+} oscillations that are shaped by Ca^{2+} feedback on the IP_3 receptor (65). We combined high-resolution measurements of cytosolic Ca^{2+} oscillations and a high-throughput analysis of newly synthesized V_{1b} receptor-selective ligands, including ligands that simultaneously antagonized V_{1a} and oxytocin receptors.Our findings support a model in which AVP modulates the activity of β and α cells in a non-linear, state-dependent manner. The observed effects across the physiological AVP concentration range, with IP_3 receptor-dependent signaling as a functional downstream readout, followed a bell-shaped activation/inactivation profile. This non-linear dependence may help explain why previous studies reached conflicting conclusions, particularly when experiments were performed under different glucose, cAMP, or hormonal conditions, or were interpreted without considering the activation/inactivation profile previously observed in perfused rat pancreas (18) and insulinoma cells (29).

By measuring the AVP-dependent activity of both cell types simultaneously, our results do not support a simple model in which activation of V_{1b} receptors in α cells is sufficient to indirectly activate β cells in a coordinated paracrine fashion. Rather, β and α cell responses were temporally and functionally distinct, suggesting that β cell modulation cannot be explained solely as a secondary consequence of α cell activation. Nevertheless, paracrine interactions within the intact islet cannot be excluded and may contribute to the overall response under specific conditions, for example possible direct AVP activation of δ cells that would in turn inhibit insulin release from β cell. However, a qualitatively similar bell-shaped dependence has also been described for β cell responses to acetylcholine stimulation, although interpreted differently (66). Together, these findings support the interpretation that AVP acts less as a simple on/off stimulus and more as a context-dependent perturbation of the islet Ca^{2+} signaling network.

Glucose-Dependent Modulation by AVP

Our results reproduced the glucose-dependent effects of AVP on both α and β cells, corroborating previous studies. One important differences from most previous work is that AVP reached its peak activity within its physiological osmoregulatory range of 10-100 pM (1), rather than in the nM range. We showed that AVP-dependent augmentation of β cell activity required stimulatory glucose concentrations. In contrast, AVP was able to activate α cells even at glucose-concentrations that are typically inhibitory for these cells, particularly when cAMP levels were elevated either directly by forskolin-mediated stimulation of adenylyl cyclase or physiologically by epinephrine acting through G_s-protein-coupled β-adrenergic receptors. Furthermore, simultaneous α and β cell activity, enabled by forskolin, revealed no major α-β cell-cell interaction at the time scales analyzed in this study. β cell were more sensitive to AVP, with peak activity between 10 and 100 pM, whereas α cell activity peaked at approximately 1 nM AVP. It remains to be investigated whether this apparent lack of α-β cell communication reflects the fact that AVP primarily drives Ca^{2+} release through IP_3 receptors, rather than through plasma membrane-related Ca^{2+} fluxes.

Even moderately supraphysiological glucose concentrations shifted the AVP concentration dependence of β cells to the left, such that physiologically relevant concentration of AVP were more likely to exert inhibitory effect on the activity of these cells. This suggests that, during hyperglycemia and increased plasma osmolality the rise in AVP concentration with concomitant elevation of cytosolic cAMP concentration through GLP-1 or other G_s-protein-coupled receptor stimulation, could diminish the capacity of β cells to respond adequately, or even suppress glucose-dependent insulin release (64). Similarly, a glucose-dependent modulation of AVP sensitivity could affect the activation capacity of α cells in hypoglycemic conditions and contribute to the inability of α cells to counteract hypoglycemia and contribute to impaired counterregulatory responses. The rightward shift in AVP dependence in α cells compared with β cells may reflect a generally lower activation state under glucose concentrations that are suboptimal for α cell activity, and this sensitivity may shift under more favorable glucose conditions. Further experiments will be required to clarify this issue.

It is important to note that although AVP can potentiate α cell activity, this potentiation has been reported to be insufficient in T1D patients to support an adequate counterregulatory response during hypoglycemia, a context associated with high systemic AVP concentrations (13). Overactivation of α cells by AVP in the presence of high cAMP levels, for example during concomitant stress-related adrenergic stimulation, could potentially desensitize or impair the ability of α cells to respond appropriately to dangerously low glucose concentrations. Thus, under certain pathological or extreme physiological conditions, AVP together with elevated cAMP may disrupt normal glucagon release and compromise the capacity to counteract hypoglycemia.

Permissive Role of AVP in cAMP-Dependent Signaling

A critical aspect of AVP's role in β and α cells is its dependence on the prevailing cAMP signaling environment. Our study provides evidence that AVP effects were strongly shaped by intracellular cAMP environment, with forskolin enabling the simultaneous assessment of AVP responses in both cell types, despite their differential sensitivity to glucose. This suggests that AVP does not act

primarily as a universal initiator of β and α cell activity, but rather enhances or reshapes the response of these cells to other stimuli when cAMP levels are elevated. The permissive role of AVP, particularly in the context of cAMP-dependent signaling, aligns with the broader understanding of its function in other endocrine systems, such as CRH- or histamine-dependent potentiation of ACTH release in the pituitary (32, 34).

The observation that epinephrine, which reduces cAMP levels and suppresses Ca^{2+} activity in β cells, did not allow AVP to restore β cell activity beyond the resting level further supports the interpretation that AVP action is closely linked to the cAMP signaling axis. This cell-type-specific interaction may help explain the variability in AVP effects observed across different studies and species, where differences in glucose concentration, adrenergic tone, incretin signaling, or experimental cAMP background could strongly modulate the apparent AVP response. Consistent with this permissive framework, antagonism of GLP-1 receptors prevented AVP-dependent action in β cells (58).

V_{1b} Receptor-Specific Modulation

The heterogeneous responses observed across different islets, where some showed increased activity while others showed no detectable change or inhibition, could intuitively be attributed to variability in V_{1b} receptor expression or signaling capacity among β cells. Such an explanation would be consistent with differences in receptor density, coupling efficiency to G_q proteins, or downstream signaling components such as PLC or IP_3 receptors. However, our data suggest that receptor-level variability alone is unlikely to fully explain the observed response spectrum, and that the current functional state of the islet collective must also be considered. The islet behaves as a non-linear dynamic system in which the same molecular perturbation can produce different functional outcomes depending on the current state of the β cell collective. In such systems, cells or cell populations do not occupy a single deterministic activity state, but rather move within a landscape of possible states, with perturbations shifting the probability distribution of transitions between them (67). This concept is well established in dynamical systems approaches to biological cell-state transitions, where attractor landscapes, noise, and signaling inputs determine the probability of moving between alternative functional states rather than enforcing a single fixed output.

In this framework, AVP and V_{1b} receptor-selective agonists may reshape the probability landscape of β cell activity. Depending on the initial metabolic, electrical, and Ca^{2+}-handling state of the islet, the same stimulus may increase oscillation frequency, produce little detectable effect, or shift the system toward reduced activity or functional inactivation. This interpretation is also consistent with studies of pancreatic islet dynamics showing that β cell Ca^{2+} activity emerges from coupled electrical, metabolic, and network interactions rather than from the properties of individual cells alone (68). Thus, molecular variability in V_{1b} receptor expression or signaling capacity may contribute to the heterogeneous responses, but it is unlikely to determine them without considering the collective dynamic state of the islet.

The study specifically targeted the V_{1b} receptor to distinguish its role from other AVP receptor subtypes that have been previously described to contribute to islet cell responses, including V_{1a}, V_2 and oxytocin receptors. The use of V_{1b} receptor-selective agonists together with V_{1a} and oxytocin receptor antagonists allowed a more precise characterization of AVP modulation of β and α cell function. The findings support V_{1b} receptor-dependent signaling as a major mediator of AVP effects in pancreatic tissue slices, with no detectable contribution from V_{1a}, V_2 or oxytocin receptors under the conditions tested. In addition, previous functional studies using selective V_{1b} receptor agonists, such as d[Cha4]AVP, revealed nanomolar affinity for V_{1b} receptors across species and concentration-dependent activation of natural biological models expressing these receptors (69).

Physiological Implications and Broader Context

Maintaining AVP levels within a physiological range is particularly challenging in diabetes mellitus. Hyperglycemia can increase plasma osmolality and promote water loss, whereas hypoglycemia can also directly stimulate AVP release (13). In these scenarios, AVP signaling becomes physiologically relevant not only as an osmoregulatory response, but also as a potential modulator of α and β cell activity(64). Elevated AVP may therefore contribute to endocrine dysregulation when the islet is already exposed to altered glucose, cAMP, and stress-related signaling conditions.

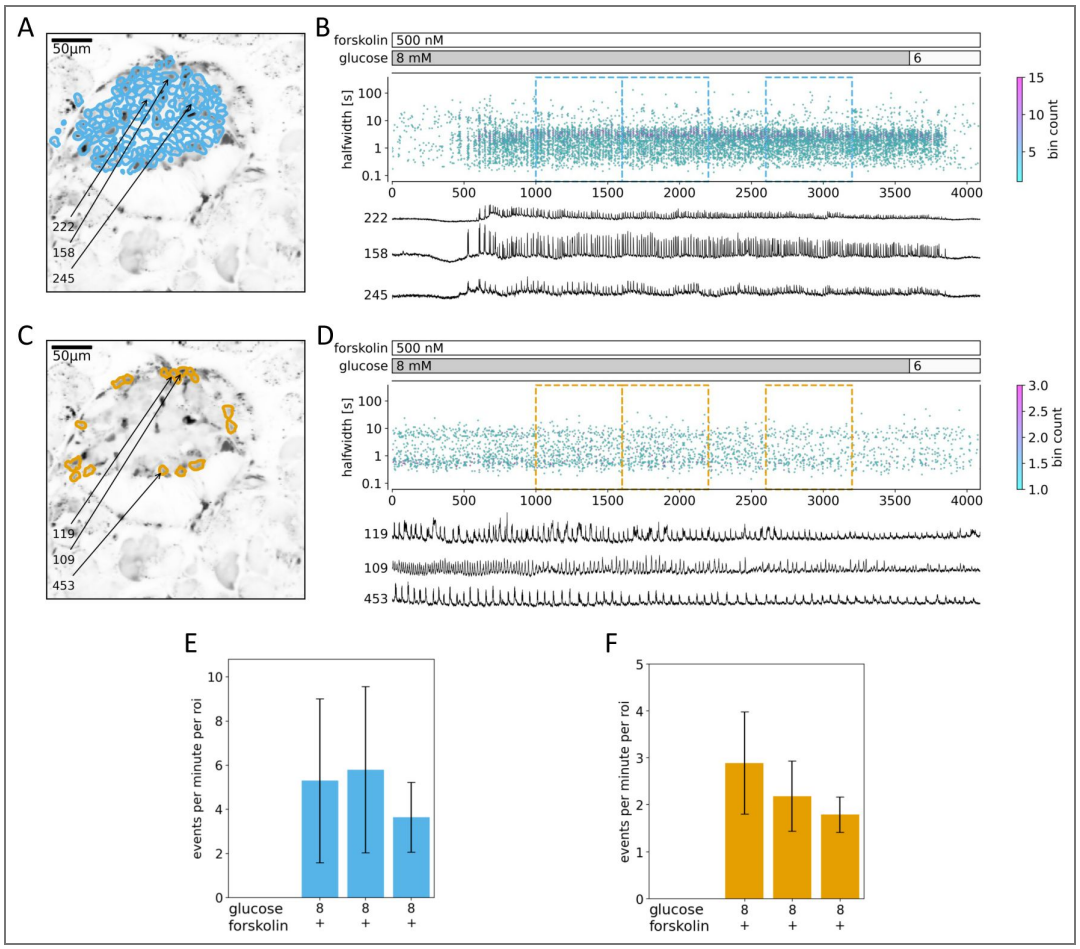
The complex interplay between AVP, glucose concentration, and the endocrine state of the islet also highlights a broader conceptual issue. AVP does not appear to impose a single deterministic response on β cells. Rather, as summarized in the graphical abstract, the same AVP stimulus may shift an islet between different activity states depending on its initial position within a non-linear functional landscape. At 8 mM glucose, a weakly active β cell collective may remain responsive and be further activated by AVP, whereas a highly active collective may be pushed toward a damped or trapped state. This state dependence provides a framework for understanding why AVP can stimulate, fail to affect, or inhibit β cell activity under apparently similar experimental conditions. In a diabetes, where AVP levels may be elevated because of chronic hyperosmolarity, dehydration or hypoglycemia, the probability of tipping the islet towards an inhibitory or desynchronized state may increase. This could impair insulin secretion and, depending on the glucose and cAMP context, may also disturb the glucagon response during hypoglycemia. Thus, AVP signaling should be considered not simply as an endocrine stimulus, but as a context-dependent perturbation that reshapes the probability distribution of accessible islet activity states.

Given these complexities, therapeutic strategies targeting AVP signaling in diabetes would require a nuanced approach. Modulation of AVP or its receptor pathways, particularly V_{1b} receptor-dependent signaling, could in principle fine-tune pancreatic islet responses under selected conditions. However, inappropriate activation or desensitization of α and β cells by fluctuating or persistently elevated AVP may also worsen glycemic instability, especially in patients experiencing frequent transitions between hyperglycemia and hypoglycemia.

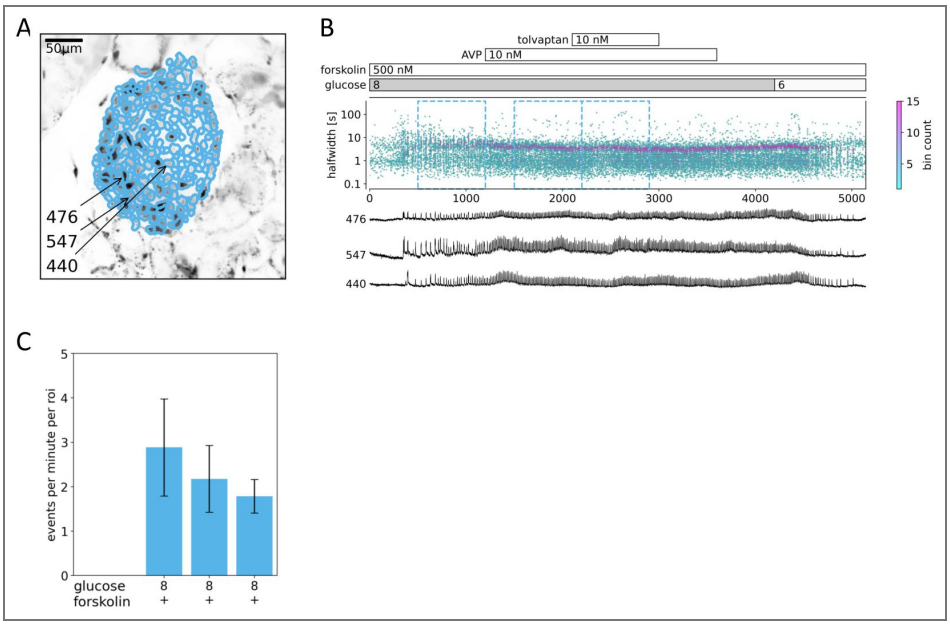
In summary, AVP plays an important role in modulating pancreatic endocrine function, but its effects depend strongly on glucose concentration, cAMP signaling, and the current dynamic state of the islet. The difficulty of maintaining AVP within a physiological range during hyperosmolality, dehydration, or hypoglycemia underscores the need to consider AVP signaling in the broader neurohormonal regulation of diabetes, particularly in relation to insulin secretion, glucagon counterregulation, and prevention of hypoglycemic episodes.

This study has several limitations. Although pancreatic slices preserve local tissue architecture and enable high-resolution analysis of α and β cell Ca^{2+} dynamics, they do not fully reproduce the intact in vivo environment, including vascular perfusion, innervation, and systemic hormonal regulation. Ca^{2+} imaging is a surrogate readout for hormone secretion; although we expanded insulin and glucagon secretion measurements, the used stimulation protocols could not establish a strict quantitative relationship between Ca^{2+} dynamics and secretory output. In addition, most experiments were performed under cAMP-permissive conditions, which increases sensitivity for detecting AVP-dependent modulation but complicates the separation of direct beta cell effects from intra-islet interactions. Finally, we were not yet able to sufficiently standardize or dynamically monitor the metabolic state of individual islets, which remains an important source of variability and a key objective for future studies.

Supplementary figures



Supplementary Figure 1. The effect of physiological levels of glucose and forskolin on β (A, B, E, G, H) and α (C, D, F) cells. (A, C) Indicated are the ROIs whose filtered traces correlate best with the average trace for the whole islet. (B, D) (top panel) Time distribution of all measured eventś halfwidths at their peak times. Stimulation protocol is indicated above. The color bar indicates the bin count in each time/halfwidth point. The dashed-line rectangles indicate the 3 time periods where events per minute per ROI parameter was sampled for panels E and F. (bottom panel) Representative traces from ROIs indicated in A and C. (E, F) Prolonged exposure to physiological glucose stimulation and low forskolin concentration resulted in a stable Ca^{2+} oscillations over almost an hour in β and α cells. The parameters were distributed normally, and we used one-way ANOVA and Tukey post-hoc test. The data are presented as mean +/− SD.

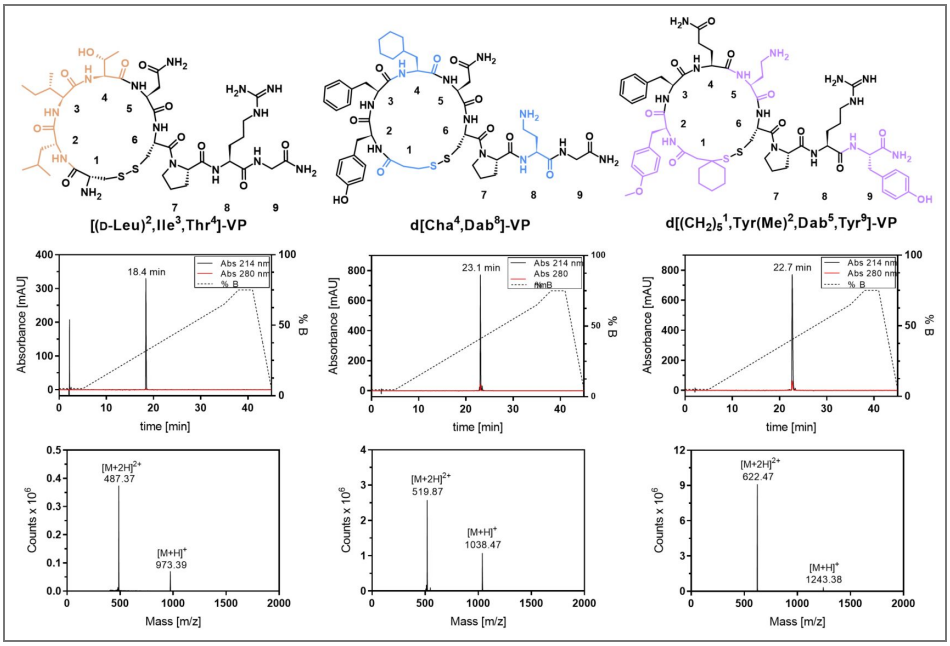


Supplementary Figure 2. Tolvaptan, a V_2 receptor antagonist, has no significant effect on β cells.

(A) Indicated are the ROIs whose filtered traces correlate best with the average trace for the whole islet. (B) (top panel) Time distribution of all measured eventś halfwidths at their peak times. Stimulation protocol is indicated above. The color bar indicates the bin count in each time/halfwidth point. The dashed-line rectangles indicate the 3 time periods where events per minute per ROI parameter was sampled for the panel. (bottom panel) Representative traces from ROIs indicated in A (C) Tolvaptan had no significant effects on the number of events per minute per ROI. The parameters were distributed normally, and we used one-way ANOVA and Tukey post-hoc test. The data are presented as mean +/− SD.

Supplementary Figure 3. Overview of molecular structures, chromatograms, and mass spectra of synthesized compounds.

Amino acid residues that differ from native vasopressin were marked in different colors. Analytic RP-HPLC chromatograms were measured for product purity determination at 214 nm. Solvent A (ddH_2O + 0.1% TFA) and B (ACN + 0.08% TFA) were used as eluents at 1 mL/min flow rate and a linear gradient of 5-65% B in 30 min on a Thermo Fisher Scientific Vanquish Horizon UHPLC system using a Kromasil Classic C_{18} column (4.6 x 150 mm, 300 Å, 5 µm). Mass spectra were measured for product identity verification through direct injection on a Thermo Scientific Dionex Ultimate 3000 system equipped with a Thermo Scientific MSQ Plus ESI-MS unit (positive ionization mode).



Supplementary Table 1. Name, sequence, purity, and mass identity of synthesized vasopressin analogs and vasopressin.

Amino acid residues that differ from native vasopressin were marked in different colors.

Name	Sequence	Purity	m/z (calc.)	m/z (obs.)
Vasopressin (AVP)	CYFQNCPRG	–	–	–
[(D-Leu)^2,Ile^3,Thr^4]-VP	CIITNCPRG*	>99%	973.47 $[M+H]^+$	973.39 $[M+H]^+$
d[Cha^4,Dab^8]-VP	dCYF(Cha)NCP(Dab)G*	92.3%	1038.45 $[M+H]^+$	1038.47 $[M+H]^+$
d[$(CH_2)_5^1$,Tyr(Me)^2,Dab^5,Tyr^9]-VP	d$(CH_2)_5$Y^{Me}FQ(Dab)CPRY*	98.3%	1243.58 $[M+H]^+$	1243.38 $[M+H]^+$

Cha = cyclohexylalanine; $(CH_2)_5$ = β-mercapto-β,β-cyclopentamethylenepropionic acid; d = deaminated N-terminus; Dab = 2,4-diaminobutyric acid ; * = C-terminal amide.

Data availability

Raw data supporting the peptide synthesis & analysis, as well as pharmacological and physiological measurements of this study are available from the corresponding authors upon reasonable request.

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Additional information

Author Contributions

K., N. M., L. K. B., E. P. L, J. P., S. P. performed pancreatic tissue slice imaging experiments. N. M. and J. P. performed hormone release assays, S. P. performed RNAscope experiments. M. S. R and J. P. analyzed the data and created the figures. M. S. R., J. K., L. K. B., C. W. G, drafted the paper and all authors edited the paper. C. W. G,, M. P., M. M., and X. K. provided resources. M. S. R., A. S., D. K., C. W. G., and M. M. acquired funding.

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Peer reviews

Reviewer #1 (Public review):

Summary:

The paper investigates how AVP modulates pancreatic alpha and beta cell activity using acute mouse pancreatic tissue slices, calcium imaging, hormone secretion assays, RNAscope, and newly synthesized receptor-selective ligands. The Authors report that AVP regulates islet cell activity in a glucose- and state-dependent manner, with maximal effects occurring within physiological AVP concentrations and a bell-shaped concentration-response profile. They conclude that V1b receptors are the principal mediators of these effects and propose that IP3 receptor-dependent signaling underlies the observed nonlinear responses.

Strengths:

The use of fresh pancreatic tissue slices preserves islet architecture and cell-cell interactions, providing a physiologically relevant experimental model compared with isolated islets or immortalized cell lines.

The combination of live calcium imaging, hormone secretion measurements, RNAscope, and pharmacological characterization of newly synthesized receptor-selective ligands represents a technically comprehensive experimental approach that addresses AVP signaling from multiple complementary perspectives.

Weaknesses:

(1) The central mechanistic model of the manuscript is not supported by the experimental data. Although the Authors repeatedly attribute the observed bell-shaped responses to IP3 Receptor activation and inactivation, no direct mechanistic evidence is provided to implicate IP3 receptors. Experiments assessing IP3 receptor function using genetic manipulation and direct measurements of IP3 signaling are necessary before such mechanistic conclusions can be drawn.

(2) The Authors should directly demonstrate V1b receptor expression in β cells using complementary approaches, since the RNAscope data indicate broader expression but do not convincingly establish receptor localization within specific endocrine populations.

(3) In my opinion, the central conclusion that V1b receptors are the predominant mediators of the observed effects is insufficiently supported because definitive loss-of-function experiments are lacking. Genetic deletion or selective silencing of V1b receptors should be provided to validate the proposed mechanism.

(4) The heterogeneous responses observed among islets substantially weaken the proposed mechanistic model. Data should be provided to identify the determinants responsible for activation, absence of response, or inhibition in individual islets.

(5) Please explain why the marked changes in alpha-cell calcium activity were not accompanied by corresponding alterations in glucagon secretion. This apparent discrepancy requires additional experimental evidence.

(6) The Authors need to provide stronger evidence linking the observed calcium dynamics with insulin secretion, since calcium measurements alone cannot establish the proposed functional consequences.

(7) Proper assays should be provided to assess whether the newly synthesized ligands exhibit comparable selectivity and efficacy at murine receptors rather than relying primarily on pharmacological characterization performed using human receptor-expressing cell lines.

(8) The proposed absence of V1a receptor involvement is based primarily on pharmacological inhibition. Independent experimental approaches should be provided to exclude a contribution of this receptor subtype.

(9) They must provide additional quantitative analyses demonstrating that the reported bell-shaped concentration-response relationship is robust across individual experiments rather than reflecting substantial biological variability.

(10) The Authors should include experiments evaluating endogenous AVP signaling under more physiological conditions instead of relying predominantly on exogenous agonist administration.

(11) I believe the role of forskolin deserves further clarification because many conclusions were obtained under cAMP-permissive conditions that may substantially influence AVP responses. Additional experiments without pharmacological cAMP stimulation should be presented.

(12) Please clarify how beta cells and alpha cells were identified exclusively from functional activity patterns during calcium imaging and provide independent validation of cell identity within the analyzed recordings.

(13) In my opinion, the manuscript relies heavily on changes in intracellular calcium activity as a surrogate for endocrine function, whereas the secretion data do not consistently support the proposed functional conclusions. Additional evidence is needed to establish a direct relationship between the observed calcium dynamics and hormone release.

(14) The Authors should better reconcile their findings with previous reports showing minimal or absent AVP receptor expression in β cells and explain how the current data resolve these discrepancies rather than adding another possible interpretation.

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Reviewer #2 (Public review):

Summary:

In this paper Drs. Kercmar, Murko and Bombek make a series of observations related to the role of AVP in pancreatic islets. They use the pancreatic slice preparation that their group is well known for. The observations on the slide physiology are technically impressive. However, I am not convinced by the conclusions of this manuscript for a number of reasons. At the core of my concern is perhaps that this manuscript appears to be motivated to resolve 'controversies' surrounding the actions of AVP on insulin and glucagon secretion. This manuscript adds more observations, but these do not move the field forward in improving or solidifying our mechanistic understanding of AVP actions on islets. A major claim in this manuscript is the beta cell expression of the V1b Receptor for AVP, but the evidence

presented in this paper fall short of supporting this claim. Observations on the activation of calcium in alpha cells via V1b receptor align with prior observations to this effect and can explain the effects of beta cell calcium and insulin secretion better than an explanation where beta cells express functional V1BR, for which direct evidence is lacking.

I have focused my main concerns below. I hope the authors will consider these suggestions carefully - please be assured that they were made with the intent to support the authors and increase the impact of this work.

Strengths:

The main strength of this paper is the technical sophistication of the approach and the analysis and representation of the calcium traces from alpha and beta cells.

Weaknesses:

(1) There are excellent data that indicate that the actions of AVP are mediated via V1bR on alpha cells and that V1bR is 1) not expressed by beta cells and 2) does not activate beta cell calcium at all at 10 nM - which is the same concentration used in this paper (Figure 4G) for peak alpha cell Ca2+ activation (see *https://doi.org/10.1016/j.cmet.2017.03.017;* cited as ref 30 in the current manuscript). Any published stimulatory actions of AVP on insulin secretion can be explained by the potentiating effects of glucagon, released in response to AVP stimulation of alpha cells.

(2) The RNAscope data offered in the revision as a second line of evidence for the expression of the V1bR in beta cells do not convince. I applaud the authors for trying as these are hard experiments to do well, as evidenced from the Gcg RNAscope signal that is not at all concentrated in the islet periphery, and in fact both color puncta occur outside of the islet at similar density. Absent a convincing concentration of Gcg signal (which is a very abundant transcript in alpha cells), it is hard to depend on these results. They certainly do not substitute experiments to determine cell autonomous activation of isolated beta cells by AVP. Claim of beta cell expression of V1br, require a more direct demonstration by staining (if appropriate antibodies exist), by beta cell-specific deletion of V1br, or by documenting the direct calcium activation in isolated beta cells in the absence of alpha cells. This should include a demonstration of Gaq-dependence in isolated beta cells.

(3) We know from bulk RNAseq data on purified alpha, beta, and delta cells from both the Huising and Gribble groups that there is no expression of V2a. I will point you to the data from the Huising lab website published almost a decade ago (*http://dx.doi.org/10.1016/j.molmet.2016.04.007*) - which is publicly available and can be used to generate figures (*https://huisinglab.com/data-ghrelin-ucsc/index.html*). They indicate the absence of expression of not only AVP2 receptors anywhere in the islet - but the lack of expression of V1bra, V1brb, and Oxtr in beta cells. These AVP/OXT receptor expression data are largely and helpfully confirmed by the efforts in this paper that involved the generation of the V1aR agonist and V2R antagonist.

(4) Importantly, the lack of V1br from beta cells does not invalidate observations that AVP affects calcium in beta cells, but it does indicate that these effects are mediated 1) indirectly, downstream of alpha cell V1br or 2) via an unknown off-target mechanism (less likely). The different peak efficacies in Figure 4G would also suggest they are not mediated by the same receptor. The recent work by Huixia Ren and colleagues (PMID: 41916313) that demonstrates that glucagon accelerates the frequency of beta cell calcium is in line with such a scenario.

(5) The use of forskolin across almost all traces complicates the interpretation of the results. The design does not account for the elevation of cAMP in alpha cells and subsequent release of glucagon - particularly upon co-stimulation with AVP which permits glucagon release by activating a calcium response in alpha cells. This glucagon then could activate beta cells. If

resolving the mechanism of action is the goal, often less is more. The activation of Gaq-mediated calcium is not cAMP dependent (although the downstream hormone secretion clearly often is). As was shown, AVP does not activate calcium in beta cells in the absence of cAMP. The experiments should have been completed in the absence of cAMP/forskolin, which would likely have had different outcomes on the beta cell responses and to the hormone secretion.

(6) It is motivated by a desire to 'study the AVP dependence of both alpha and beta cells at the same time'. As best as I can determine, the design choice to conduct most studies under sustained forskolin stimulation is related to the permissive actions of AVP on hormone secretion in response to cAMP-generating stimuli. The permissive actions by AVP that are cited are on hormone secretion - which in many cell types requires activation of both calcium and cAMP signaling. Whether the activation of V1br and subsequent calcium responsive is permitted by cAMP is unclear. I believe the argument the authors are making here is that the activation of beta cell calcium by AVP is permitted by forskolin. i.e. the cAMP stimulated by it in beta cells.

(7) Figure 9 suggests a pharmacological activation of beta cell V1bR in the low pM range. How do the authors reconcile this compare with the apparent absence of an effect of AVP stimulation at low pM to low nM doses in beta cells (Figure 5A). I note that there are changes over time with sustained beta cell stimulation with 8 mM glucose, but these changes are relatively subtle, gradual and quite likely represent the progression of calcium behaviors that would have occurred under sustained glucose irrespective of these very low AVP concentrations. I will note that the Kd of the V1bR for AVP is around 1 nM, with tracer displacement starting around 100 pM according to the data in figure 6B, which is hard to reconcile with changes in beta cell calcium by AVP doses that start 10-100-fold lower than this dose at 1 and 10 pM (Figure 9).

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Reviewer #3 (Public review):

Summary:

This work aims to better understand the role of arginine vasopressin (AVP) in the control of islet hormone secretion. This builds on previous literature in this area reporting on the actions of AVP to stimulate islet hormones. The gap in literature being addressed by these studies is primarily focused on the glucose-dependency of AVP on both insulin and glucagon secretion. A secondary objective is to explore the role of individual receptors with the use of newly generated peptides and existing tools. The methods include the use of Ca2+ imaging in pancreas slices from mice, with additional outcomes including insulin secretion in some areas. The conclusions presented are that AVP acts through V1b receptors in both alpha- and beta-cells, that this activity occurs in the high cAMP environment, and is glucose dependent.

Strengths:

The area of research is emerging with plenty of room for new contributions. The concept of AVP stimulating islet hormone secretion is important and deserving of further insight. The use of pancreas tissue to image primary cells makes the experiments physiologically relevant. The advancement of novel tools in this area should be helpful to other groups investigating the actions of AVP.

Comments on revised version:

Overall, the authors have modified their conclusions to more accurately capture the results of this manuscript. They also add the significant limitations outlined in the review process to

the Discussion. With the addition of new data, however, a few concerns have emerged.

(1) The rational for showing somatostatin staining in Figure 1 a is unclear. It also does not appear to be the same region of interest as in panel B.

(2) It is difficult to assess the success of the RNAscope with the representative image used in Figure 1b. It is surprising how low the Gcg signal is in this image, suggesting some optimization is required. Additionally, how many mice were used (biological replicates) and how many V1b receptor+ and Gcg+ cells per mouse were quantified for the RNAscope images? This must be indicated in the methods section and should be sufficiently powered to make a conclusion.

(3) While the addition of insulin and glucagon secretions with AVP ramp provide a functional output to the calcium imaging, it is unclear why the measurements are sometimes log10 transformed (Figure 5 K and L) but not always (Figure 5E). It is difficult to interpret negative glucagon values. What is the functional output of the dose-dependent calcium response to AVP in alpha cells if it is not glucagon?

(4) Finally, the highlights section has not been refined to the revised interpretations of the manuscript.

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Author response:

The following is the authors' response to the original reviews.

> ***eLife Assessment***
>
> *This study presents a useful finding on the effects of arginine vasopressin (AVP) on islet cells in pancreatic tissue slices, using technically sophisticated spatio-temporal calcium recordings to confirm that AVP influences α and β cells differently depending on glucose concentrations. While the study's methods - particularly the calcium imaging techniques and peptide ligand design targeting V1b receptors - are strong, the reviewers were concerned about several aspects of the experimental design. However, the results on βcell responses are incomplete and insufficient to support the manuscript's claims, especially due to the high variability of islet responses and lack of mechanistic and functional (hormone release) data. There are also concerns about the possibility of off-target effects and incomplete receptor specificity, noting that the study would have been significantly strengthened by inclusion of signaling pathway interrogation, hormone output assays, genetic validation (e.g., β cell-specific deletion of V1br), and receptor localization, although the work will still be of interest to researchers studying islet physiology in the context of health and diabetes.*

We sincerely thank the reviewers and editors for their thorough evaluation of our manuscript and their recognition of its technical strengths, including the advanced spatio-temporal calcium imaging and the rational design of selective V1b receptor ligands. We appreciate their acknowledgement of the study's relevance for understanding AVP effects in a physiologically intact islet context and their positive assessment of our methodological rigour and innovation. The reviewers' constructive feedback has helped us clarify the boundaries and intent of our study, which focuses on the glucose- and context-dependent modulation of α- and β-cell activity, rather than exhaustive molecular dissection.

While the reviewers rightly emphasize the importance of receptor specificity and downstream signaling validation, we respectfully suggest that some of their concerns may reflect a lingering bias toward reductionist frameworks. Our interpretation is rooted in the emerging understanding that β-cell behaviour is largely defined by dynamic intercellular

interactions within the islet collective, rather than by static gene expression or receptor localization alone (Jin et al., 2025; Korošak et al., 2021; Rutter et al., 2024). Recent studies have demonstrated that roles such as “leader” or “hub” β cells are transient and emergent, governed more by timing, environment, and local network structure than by fixed molecular identity (Postic et al., 2023; Gosak et al., 2018).

This has profound implications for how we interpret cell responsiveness to agents like AVP: what appears as biological variability may in fact reflect context-sensitive transitions within a non-linear, self-organizing system (Stožer et al., 2021). Hence, we chose to focus on functional collective dynamics using intact pancreatic slices, rather than isolated cell models which fail to preserve the essential network architecture of islets. Although the addition of genetic models or isolated receptor measurements would strengthen receptor-specific conclusions, we argue that such approaches alone cannot resolve the physiological complexity of a system where function arises from cell–cell communication and spatiotemporal context.

Indeed, the lack of direct correlation between receptor transcript abundance and functional outcomes has been noted in prior studies, reinforcing the view that function cannot be strictly predicted by molecular presence (Rutter et al., 2024). As articulated in our manuscript, the islet behaves as a sensory collective (Fancher & Mugler, 2017), where emergent patterns— not static cell identity—determine behaviour. This perspective aligns with broader shifts in biology away from strict genetic determinism toward causal emergence and collective agency (Ball, 2023; Levin, 2021).

We therefore believe our study contributes not only new pharmacological insights but also a conceptual reframing of how AVP responses should be interpreted in a complex organ like the pancreas. We have added new data addressing reviewer suggestions—such as glucagon secretion assays, clarifications on the role of forskolin, and an analysis of event timing—that further support our conclusions. We also expanded the discussion on how islet variability is functionally meaningful, not just noise, and explained why β-cell responses to AVP must be interpreted within this probabilistic framework.

We agree that future work should include receptor-specific knockouts and more direct signaling pathway assays, but these would need to be designed with careful consideration of the islet’s dynamic topology and the emergent nature of β-cell roles. In this light, we see our study not as the final word, but as a necessary systems-level foundation for more targeted interventions. We thank the reviewers again for their careful critiques and hope that our response clarifies both the rationale and scope of our work. Our revisions aim to enhance the paper’s clarity while maintaining its commitment to an integrative, physiology-rooted approach.

We thank the reviewers and editors for their thoughtful and constructive assessment of our work. We are especially grateful for their recognition of the study’s technical strengths, including the use of spatio-temporal calcium imaging in intact pancreatic tissue and the strategic development of receptor-selective peptide ligands. We also appreciate their acknowledgement that our study contributes to the understanding of glucose-dependent AVP effects in islet physiology. The reviewers’ concerns regarding variability, receptor specificity, and functional validation helped us further clarify the scope and context of our study.

We respectfully submit that some reservations stem from a reductionist framing that may not fully account for the collective behaviour of islets. As we and others have shown, β-cell function arises from emergent, self-organizing network dynamics, not just from static gene expression or receptor abundance (Jin et al., 2025; Korošak et al., 2021; Postic et al., 2023). In this view, pharmacological heterogeneity across islets is not simply noise or experimental inconsistency, but a signature of dynamic attractor states within the islet network (Stožer et al., 2021). Because an islet functions as a coupled system, most response variability originates

from its emergent collective behavior, which eclipses variability in receptor expression or metabolic state.

For this reason, even single-islet receptor quantification or ATP measurements would provide limited explanatory power: it is the state of the network—not absolute receptor levels—that determines whether a perturbation elicits activation or inhibition. As we illustrate in our graphical abstract, a single islet tested repeatedly under identical glucose conditions can yield divergent responses, simply because it occupies different dynamic states. These findings are in line with systems biology and network science approaches, which have revealed that cell function, especially in the β-cell collective, cannot be fully understood through reductionist parameters alone (Gosak et al., 2018; Ball, 2023).

We have included glucagon secretion assays and new analyses to address key reviewer suggestions. Still, we chose not to pursue extensive knockouts or cAMP imaging, as these would require a different experimental scope and could risk disrupting the very dynamics we aim to understand. Likewise, while direct measurements of V1bR or IP3R expression would add molecular detail, they are not definitive without network context. The bell-shaped AVP dose-response curve and its explanation through IP3R inactivation are supported by prior studies; we invoke this mechanism not speculatively, but because it provides the most parsimonious explanation for the glucose-dependent shift in β-cell responsiveness.

We also clarify that our study does not aim to resolve every mechanistic detail, but rather to offer a systems-level insight into how AVP modulates islet dynamics across varying glucose and cAMP contexts. The implications extend beyond AVP pharmacology, suggesting that perturbations to β-cell function must be understood within a probabilistic, state-dependent framework (Fancher & Mugler, 2017). This resonates with emerging concepts in cell physiology that emphasize causal emergence and local agency over static molecular determinism (Levin, 2021; Rutter et al., 2024).

In summary, we see our work as part of a necessary shift in perspective—from linear receptor-function models to context-sensitive dynamic systems. We are grateful for the opportunity to revise our manuscript in response to insightful feedback and hope our clarifications and new data will strengthen its impact for the islet research community.

> ***Public Reviews:***
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> ***Reviewer #1 (Public review):***
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> *Summary:*
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> *The authors confirmed earlier findings that AVP influences α and β cells differently, depending on glucose concentrations. At substimulatory glucose levels, AVP combined with forskolin - an activator of cAMP -did not significantly stimulate β cells, although it did activate α cells. Once glucose was raised to stimulatory levels, β cells became active, and α cell activity declined, indicating glucose's suppressive effect on α cells and permissive effect on β cells. Under physiological glucose levels (8-9 mM), forskolin enhanced β-cell calcium oscillations, and AVP further modulated this activity. However, AVP's effect on β cells was variable across islets and did not significantly alter AUC measurements (a combined indicator of oscillation frequency and duration). In α cells, forskolin and AVP led to increased activity even at high glucose levels, suggesting that α cells remain responsive despite expected suppression by insulin and glucose.*
>
> *Experiments with physiological concentrations of epinephrine suggest that AVP does not operate via Gs-coupled V2 receptors in β cells, as AVP could not counteract epinephrine's inhibitory effects. Instead, epinephrine reduced β cell activity while increasing α cell activity through different G-proteincoupled mechanisms. These results emphasize that*

> *AVP can potentiate acell activation and has a nuanced, context-dependent effect on β cells.*
>
> *The most robust activation of both α and β cells by AVP occurred within its physiological osmo-regulatory range (~10-100 pM), confirming that AVP exerts bell-shaped concentration-dependent effects on β cells. At low concentrations, AVP increased β cell calcium oscillation frequency and reduced "halfwidths"; high concentrations eventually suppressed β cell activity, mimicking the muscarinic signaling. In α cells, higher AVP concentrations were required for peak activation, which was not blunted by receptor inactivation within physiological ranges.*
>
> *Attempting to further dissect the role of specific AVP receptors, the authors designed and tested peptide ligands selective for V1b receptors. These included a selective V1b agonist; a V1b agonist with antagonist properties at V1a and oxytocin receptors; and a selective V1a antagonist. In pancreatic slices, these peptides seem to replicate AVP's effects on Ca^{2+} signaling, although responses were highly variable, with some islets showing increased activity and others no change or suppression. The variability was partly attributed to islet-specific baseline activity, and the authors conclude that AVP and V1b receptor agonists can modulate β cell activity in a statedependent manner, stimulating insulin secretion in quiescent cells and inhibiting it in already active cells.*

We applaud the reviewer to capture the essence of work in their introduction.

> *Strengths:*
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> *Overall, the study is technically advanced and provides useful pharmacological tools. However, the conclusions are limited by a lack of direct mechanistic and functional data. Addressing these gaps through a combination of signaling pathway interrogation, functional hormone output, genetic validation, and receptor localization would strengthen the conclusions and reduce the current (interpretive) ambiguity.*

Thank you!

> *Weaknesses:*
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> *(1) The study is entirely based on pharmacological tools. Without genetic models, off-target effects or incomplete specificity of the peptides cannot be fully ruled out.*

We partially agree with this comment and acknowledge that genetic models would provide a valuable complementary approach to address possible off-target effects or incomplete peptide specificity. However, genetic models also have important limitations, particularly when the aim is to resolve subtle, population-level physiological differences in beta cell activity. We therefore used pharmacological tools at different concentrations to test whether the observed effects were concentration-dependent and consistent with the expected receptor-mediated actions. An advantage of the pancreatic slice preparation is that it preserves much of the native tissue environment and allows pharmacological manipulation within concentration ranges closer to *in vivo* efficacy, thereby reducing the likelihood of nonspecific effects. To compensate for the lack of genetic models, we now emphasize the collective activity analysis as an additional strength of the study and have clarified this limitation in the revised manuscript.

> *(2) Despite multiple claims about β cell activation or inhibition, the functional output - insulin secretion - is weakly assessed, and only in limited conditions. This aspect makes it very hard to correlate calcium dynamics with physiological outcomes.*

We agree that the functional output needed stronger support and have therefore expanded the hormone secretion experiments. While the effects of AVP and its analogues were tested

during a stable plateau phase in the Ca^{2+} imaging experiments, this phase provides only a narrow dynamic range for insulin release measurements in mouse slices. We therefore added a sequence of stimulations on the same slices, using 8 mM glucose and 500 nM forskolin, with glucose lowered to a non-stimulatory range between different AVP concentrations. These new experiments better define how AVP-dependent changes in Ca^{2+} dynamics translate into insulin secretion under conditions with a broader secretory dynamic range. The new insulin and glucagon secretion data have now been added to the manuscript as Figure 5, and the text has been revised accordingly.

> *(3) Insulin and glucagon secretion assays should be provided; the authors should measure hormone release in parallel with Ca2+ imaging, using perifusion assays, especially during AVP ramp and peptide ligand applications.*

We added insulin and glucagon secretion assays for AVP ramp to Figure 5.

> *Additionally, there is no standardization of the metabolic state of islets. The authors should consider measuring islet NAD(P)H autofluorescence or mitochondrial potential (e.g., using TMRE) to control for metabolic variability that may affect responsiveness.*

We agree that standardization of the metabolic state of the islets would further strengthen the interpretation of the responsiveness data. We attempted to address this experimentally, but the results were inconclusive and therefore not included in the manuscript. Based on our previous unpublished observations, NAD(P)H levels appear to be significantly higher and less variable in islets within tissue slices than in isolated islets, suggesting that the slice preparation may better preserve the native metabolic state. However, we acknowledge that this remains an important limitation and we now indicate that in the manuscript. Additional experiments will be required to establish a robust and standardized approach, for example by combining NAD(P)H autofluorescence and/or mitochondrial potential measurements with Ca^{2+} imaging.

> *(4) There is a high degree of variability in response to AVP and V1b agonists across islets (activation, no effect, inhibition). Surprisingly, the authors do not fully explore the cause of this heterogeneity (whether it is due to receptor expression differences, metabolic state, experimental variability, or other conditions).*

This is a well-taken point and has indeed been one of the major bottlenecks in interpreting the results of this study. We agree that the variability in responses to AVP and V1b agonists may reflect several factors, including receptor expression, metabolic state, experimental conditions, and differences in the functional state of individual islets. However, our data also suggest that the beta cell population within an islet should be considered as a dynamic, non-linear system, in which even small differences in initial conditions or collective state can result in qualitatively different outcomes, including activation, no apparent effect, or inhibition. In this framework, the response to AVP is not determined by receptor expression alone, but by the current physiological context of the islet network. This is also why we believe that pharmacological tests are most informative when interpreted within a defined functional state rather than as isolated receptor-specific readouts. As indicated in the graphical abstract, apparently similar islets may occupy different dynamic states and therefore respond differently to the same Gq/PLC/IP3R stimulus. We have now expanded the discussion to make this interpretation more explicit and to acknowledge that receptor expression, metabolic variability, and experimental factors remain possible contributors that will require further targeted studies.

The following text has been added to expand the discussion:

“The heterogeneous responses observed across different islets, where some showed increased activity while others showed no detectable change or inhibition, could intuitively

be attributed to variability in V1b receptor expression or signaling capacity among β cells. Such an explanation would be consistent with differences in receptor density, coupling efficiency to Gq proteins, or downstream signaling components such as PLC or IP_3 receptors. However, our data suggest that receptor-level variability alone is unlikely to fully explain the observed response spectrum, and that the current functional state of the islet collective must also be considered. The islet behaves as a non-linear dynamic system in which the same molecular perturbation can produce different functional outcomes depending on the current state of the β-cell collective. In such systems, cells or cell populations do not occupy a single deterministic activity state, but rather move within a landscape of possible states, with perturbations shifting the probability distribution of transitions between them. This concept is well established in dynamical systems approaches to biological cell-state transitions, where attractor landscapes, noise, and signaling inputs determine the probability of moving between alternative functional states rather than enforcing a single fixed output.

In this framework, AVP and V1b receptor-selective agonists may reshape the probability landscape of β-cell activity. Depending on the initial metabolic, electrical, and Ca^{2+}-handling state of the islet, the same stimulus may increase oscillation frequency, produce little detectable effect, or shift the system toward reduced activity or functional inactivation. This interpretation is also consistent with studies of pancreatic islet dynamics showing that βcell Ca^{2+} activity emerges from coupled electrical, metabolic, and network interactions rather than from the properties of individual cells alone. Thus, molecular variability in V1b receptor expression or signaling capacity may contribute to the heterogeneous responses, but it is unlikely to determine them without considering the collective dynamic state of the islet."

> *(5) There is no validation of V1b receptor expression at the protein or mRNA level in α or β cells using in situ hybridization, immunohistochemistry, or spatial transcriptomics.*

We agree with the reviewer that spatial validation of V1b receptor expression is important for interpreting the cellular targets of AVP signaling in the islet. We have therefore added RNAscope in situ hybridization data to the revised manuscript to assess V1b receptor mRNA expression within the pancreas and islet. These new data show a broader expression pattern of V1b receptor transcripts within the islet than originally assumed, suggesting that AVP signaling may not be restricted to a single endocrine cell population. At the same time, the RNAscope analysis confirms previous reports of higher AVP receptor expression in glucagon-positive alpha cells. We have added these results to Figure 1 and revised the corresponding Results and Discussion sections to clarify that the observed functional responses may reflect both direct effects on beta cells and indirect intra-islet effects mediated through alpha-cell signaling.

> *(6) AVP effects are described in terms of permissive or antagonistic effects on cAMP (especially in relation to epinephrine), but direct measurements of cAMP in α and β cells are not shown, weakening these conclusions. The authors should use Epac-based cAMP FRET sensors in α and β cells to monitor the interaction between AVP, forskolin, and epinephrine more conclusively.*

We agree that direct measurements of cAMP dynamics in alpha and beta cells would provide a more conclusive assessment of the interaction between AVP, forskolin, and epinephrine signaling. We attempted to address this experimentally; however, within the time domain of the Ca^{2+} oscillations analyzed here, the temporal resolution and robustness of currently available cAMP readouts were not sufficient to resolve these interactions reliably. Even at slower time scales, cAMP sensor signals can be difficult to interpret quantitatively and may be overinterpreted if not tightly linked to the functional readout. We have therefore moderated the wording of the manuscript and now describe the proposed permissive or antagonistic interaction between AVP/V1b and cAMP-dependent signaling as an interpretation supported by the pharmacological Ca^{2+} response patterns, rather than as a directly demonstrated cAMP mechanism. We now explicitly acknowledge in the limitations

that most experiments were performed under cAMP-permissive conditions, which increases sensitivity for detecting AVP-dependent modulation but complicates the separation of direct beta cell effects from intra-islet interactions. Future studies using optimized cell-type-specific Epac-based sensors will be required to resolve this interaction.

> *(7) Single-islet transcriptomics or proteomics (also to clarify variability) should be provided to analyze receptor expression variability across islets to correlate with response phenotypes (activation vs inhibition). Alternatively, the authors could perform calcium imaging with simultaneous insulin granule tracking or ATP levels to assess islet functional states.*

We agree that single-islet transcriptomics, proteomics, or simultaneous metabolic readouts could provide useful complementary information, particularly for describing molecular variability across islets. However, we do not think that differences in receptor expression or ATP levels alone are sufficient to explain the diversity of response phenotypes observed here. Our interpretation is that the beta cell population behaves as a collective dynamic system, in which the same input can lead to different outcomes depending on the current state of the network and its local physiological context. In such a system, AVP/V1b signaling does not necessarily impose a single deterministic response, but changes the probability distribution of accessible states, including activation, inhibition, or no detectable response. Theoretically and partially confirmed by the preliminary data, even the same islet exposed repeatedly under apparently identical conditions could be expected to display different responses if it occupies a different position within this dynamic state space at the time of stimulation. This concept is summarized in the graphical abstract and is central to our interpretation of the pharmacological data. We have therefore clarified in the Discussion that receptor expression, ATP levels, and other molecular parameters may modulate the response landscape, but are unlikely to fully define the observed functional phenotype without considering the collective dynamics of the islet.

Added to Discussion section: “In this framework, AVP and V1b receptorselective agonists may reshape the probability landscape of β-cell activity. Depending on the initial metabolic, electrical, and Ca^{2+}-handling state of the islet, the same stimulus may increase oscillation frequency, produce little detectable effect, or shift the system toward reduced activity or functional inactivation. This interpretation is also consistent with studies of pancreatic islet dynamics showing that β cell Ca^{2+} activity emerges from coupled electrical, metabolic, and network interactions rather than from the properties of individual cells alone (63). Thus, molecular variability in V1b receptor expression or signaling capacity may contribute to the heterogeneous responses, but it is unlikely to determine them without considering the collective dynamic state of the islet.”

> *(8) While the study implies AVP acts through V1b receptors on β cells, the signaling downstream (e.g., PLC activation, IP3R isoforms involved) is simply inferred but not directly shown.*

We agree that downstream signaling was not directly resolved at the level of PLC activation or specific IP3R isoforms. However, we did not infer Gq/PLC/IP3R involvement solely from AVP pharmacology, but used ACh as an independent Gq-coupled receptor reference stimulus in the same pancreatic slice preparation. With ACh concentration ramps, we could reproduce both activation and inactivation patterns observed with AVP/V1b stimulation, supporting the interpretation that these responses arise from modulation of the Gq-dependent Ca^{2+} signaling axis.

In addition, in prelilminary expriments we could observe that inhibition of Gq activity with YM254890, as well as interference with IP3R-dependent signaling using Xestospongin C, diminished the response, although not completely. This incomplete suppression is important, because it suggests that beta cell Ca^{2+} homeostasis and collective islet activity are not

controlled by a single linear pathway, but by partially redundant and context-dependent mechanisms. We have therefore revised the manuscript to state more cautiously that our data support the involvement of Gq/PLC/IP3R-dependent signaling, while acknowledging that direct measurements of PLC activity and IP3R isoform-specific contributions remain outside the scope of the present study.

> *(9) The interpretation that IP3R inactivation (mentioned in the title!) underlies the bell-shaped AVP effect is just hypothetical, without direct measurements. Assays in β (and/or α)-cell-specific V1b KO mice and IP3R KO mice must be provided to support these speculations.*

We agree that the involvement of IP3R-dependent signaling should be stated with appropriate caution. However, the concept of IP3R inactivation as a mechanism contributing to bell-shaped Gq-dependent Ca^{2+} responses is not purely hypothetical, since IP3R inactivation has been directly demonstrated in previous studies and provides a parsimonious explanation for the shift from activation to suppression at higher AVP concentrations. In the present study, this interpretation is further supported by the glucose dependence of the AVP concentration-response relationship, where different stimulatory glucose conditions shift the apparent efficacy peak.

We also agree that cell-specific V1b receptor and IP3R knockout experiments would be valuable future approaches. In this respect, we have obtained preliminary results from a small sample of IP3R triple-knockout mice, which cannot yet be fully included because they are part of an ongoing collaboration. In these experiments, supraphysiological AVP concentrations did not produce the IP3R-like beta-cell response pattern observed in controls, namely reduced halfwidth and increased frequency, whereas alpha cell stimulation was preserved similarly to WT slices.

At the same time, we believe that definitive knockout experiments must be carefully designed, because the beta cell population behaves as a dynamic collective system in which the response to AVP depends on the current functional state of the islet, glucose context, and intercellular coupling. We therefore now present IP3R inactivation as a strongly supported mechanistic interpretation rather than as a directly proven mechanism in this study, and we explicitly acknowledge that cell-specific V1b and IP3R genetic models will be required to fully resolve this pathway.

> ***Reviewer #2 (Public review):***
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> *Summary:*
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> *In this paper, Drs. Kercmar, Murko, and Bombek make a series of observations related to the role of AVP in pancreatic islets. They use the pancreatic slice preparation that their group is well known for. The observations on the slide physiology are technically impressive. However, I am not convinced by the conclusions of this manuscript for a number of reasons. At the core of my concern is perhaps that this manuscript appears to be motivated to resolve 'controversies' surrounding the actions of AVP on insulin and glucagon secretion. This manuscript adds more observations, but these do not move the field forward in improving or solidifying our mechanistic understanding of AVP actions on islets. A major claim in this manuscript is the beta cell expression of the V1b Receptor for AVP, but the evidence presented in this paper falls short of supporting this claim.*
>
> *Observations on the activation of calcium in alpha cells via V1b receptor align with prior observations of this effect.*
>
> *I have focused my main concerns below. I hope the authors will consider these suggestions carefully - please be assured that they were made with the intent to support the authors and increase the impact of this work.*

We thank the reviewer for their detailed input and support to increase the impact of our work and our understanding of important cellular processes overall. We have considered their suggestions carefully to further expand the strenghts of our approach and analysis.

> *Strengths:*
>
> *The main strength of this paper is the technical sophistication of the approach and the analysis and representation of the calcium traces from alpha and beta cells.*

Thank you!

> *Weaknesses:*
>
> *(1) The introduction is long and summarizes a substantive body of literature on AVP actions on insulin secretion in vivo. There are a number of possible explanations for these observations that do not directly target islet cells. If the goal is to resolve the mechanistic basis of AVP action on alpha and beta cells, the more limited number of papers that describe direct islet effects is more helpful. There are excellent data that indicate that the actions of AVP are mediated via V1bR on alpha cells and that V1bR is a) not expressed by beta cells and b) does not activate beta cell calcium at all at 10 nM - which is the same concentration used in this paper (Figure 4G) for peak alpha cell Ca2+ activation (see https://doi.org/10.1016/j.cmet.2017.03.017; cited as ref 30 in the current manuscript).*

We thank the reviewer for this important comment and agree that the literature on AVP actions *in vivo* is complex, with several possible sites of action outside the islet. We have therefore revised the Introduction to make the rationale more focused and to better separate systemic effects of AVP from studies addressing direct actions on pancreatic islet cells. At the same time, we chose not to restrict the Introduction only to the alpha cell V1bR literature, because one of the aims of the manuscript is precisely to address why AVP effects on insulin secretion have remained difficult to interpret across experimental contexts.

Our results fully confirm a central aspect of the study cited by the reviewer, namely that V1bR activation robustly stimulates alpha cell Ca^{2+} activity under non-stimulatory glucose conditions, and that 10 nM AVP does not produce a uniform activation of beta cell Ca^{2+}

activity. In fact, in a substantial fraction of beta cell populations, 10 nM AVP failed to activate oscillations, consistent with the view that alpha cells are the more sensitive and more direct cellular target of AVP/V1bR signaling. However, we do not think that the available transcriptomic evidence is sufficient to categorically exclude V1bR expression or functional relevance in beta cells. Re-analysis of the published dataset, together with more recent datasets and our newly added RNAscope data, supports a higher relative expression of V1bR transcripts in alpha than in beta cells, but does not justify treating beta (or non-alpha) cell expression as absent.

We have therefore revised the manuscript to avoid overstating beta cell V1bR expression as a major isolated claim. Instead, we now present the data as evidence that AVP/V1bR signaling acts most prominently through alpha cells, while beta cell responses emerge in a concentration-, glucose-, and statedependent manner within the intact islet. This interpretation is consistent with the reviewer's concern that 10 nM AVP preferentially activates alpha cells, but it also accommodates our observation that beta cell collective activity can be modulated under defined pharmacological and metabolic conditions. We believe that this is an important distinction, because the absence of a uniform beta cell Ca^{2+} activation at one AVP concentration does not exclude beta cell modulation by AVP/V1bR signaling within the intact islet network. The Introduction and Discussion have been revised accordingly to clarify that our study does not simply challenge the alpha cell V1bR model, but expands it by examining how AVP-dependent alpha cell activation, possibly lower beta-cell receptor expression, and collective beta cell dynamics interact in the native pancreatic slice preparation.

> *(2) We know from bulk RNAseq data on purified alpha, beta, and delta cells from both the Huising and Gribble groups that there is no expression of V2a. I will point you to the data from the Huising lab website published almost a decade ago (http://dx.doi.org/10.1016/j.molmet.2016.04.007) - which is publicly available and can be used to generate figures (https://huisinglab.com/dataghrelin-ucsc/index.html). They indicate the absence of expression of not only AVP2 receptors anywhere in the islet, but also the lack of expression of V1bra, V1brb, and Oxtr in beta cells. Instead of the detailed list of expression of these 4 receptors elsewhere in the body, it would be more directly relevant to set up their pancreatic slice experiments to summarize the known expression in pancreatic islets that is publicly available. It would also have helped ground the efforts that involved the generation of the V1aR agonist and V2R antagonist, which confirm these known AVP/OXT receptor expression patterns.*

We thank the reviewer for pointing us more directly to the publicly available islet expression datasets. We agree that the expression of AVP/OXT receptors in purified alpha, beta, and delta cells provides an important reference frame for interpreting our pharmacological data, and we have revised the manuscript to summarize these islet-specific datasets more directly rather than emphasizing receptor expression in other organs. These data support the absence or very low expression of V2 receptors in islet endocrine cells and confirm that V1b receptor expression is substantially enriched in alpha cells compared with beta cells.

At the same time, as outlined in our response above, we do not think that the currently available transcriptomic datasets are sufficient to categorically exclude low-level V1bR transcript expression or functional relevance in beta cells within the intact islet. For this reason, we added independent RNAscope validation to assess V1bR transcripts in the pancreatic slice preparation. These data confirm stronger V1bR expression in glucagon-positive alpha cells, while also showing a broader expression pattern within the islet and pancreas.

We have also revised the rationale for the pharmacological experiments using V1aR- and V2R-directed tools. We now present these experiments not as evidence for unexpected receptor expression, but as functional controls that are consistent with the known AVP/OXT

receptor expression patterns in pancreatic islets. This better aligns the manuscript with the existing transcriptomic literature while preserving the main physiological question of the study: how AVP/V1bR-dependent signaling reshapes alpha-cell activity and beta-cell collective dynamics in intact pancreatic tissue.

> *(3) Importantly, the lack of V1br from beta cells does not invalidate observations that AVP affects calcium in beta cells, but it does indicate that these effects are mediated a) indirectly, downstream of alpha cell V1br or b) via an unknown off-target mechanism (less likely). The different peak efficacies in Figure 4G would also suggest that they are not mediated by the same receptor.*

We agree with the reviewer that the absence or very low abundance of V1bR transcripts in beta cells in published transcriptomic datasets would not invalidate the observation that AVP modulates beta-cell Ca^{2+} activity. It does, however, raise the important question of whether this modulation is mediated indirectly through alpha-cell V1bR activation, through V1bR expression in beta cells that is difficult to resolve transcriptomically, or through another mechanism. To address this more directly, we have now added RNAscope data, which confirm relatively stronger V1bR transcript enrichment in glucagon-positive alpha cells, but also show a broader V1bR transcript signal within the islet and pancreas. Thus, while our data support alpha cells as the dominant V1bR-positive endocrine population, they do not support a strict absence of V1bR-associated signaling capacity in the beta cell compartment.

We also agree that different peak efficacies in alpha and beta cells could be interpreted as evidence for distinct receptors or indirect mechanisms. However, we favor a different interpretation: the apparent efficacy of AVP depends strongly on the physiological state in which the cells are tested. This is particularly evident in beta cells, where the AVP efficacy peak shifts with glucose concentration, suggesting that the beta-cell response is shaped by the metabolic and Ca^{2+}-handling context rather than by receptor occupancy alone. In this framework, the same V1bR/Gq-dependent input can generate different downstream Ca^{2+} outcomes in alpha and beta cells because the two cell types operate in different dynamic regimes.

We have therefore revised the manuscript to acknowledge this dilemma more explicitly. We now state that beta cell effects of AVP could include indirect alpha cell-dependent components, but given the magnitude and statedependence of the beta cell Ca^{2+} response it is unlikely to be driven by alpha cell activation. Instead, our preferred interpretation is that AVP/V1bR signaling acts within the intact islet as a context-dependent perturbation of the collective beta cell Ca^{2+} system, with IP3R-dependent mechanisms being modulated by glucose-dependent changes in beta cell excitability and intracellular Ca^{2+} handling.

> *(4) The rationale for the use of forskolin across almost all traces is unclear. It is motivated by a desire to 'study the AVP dependence of both alpha and beta cells at the same time'. As best as I can determine, the design choice to conduct all studies under sustained forskolin stimulation is related to the permissive actions of AVP on hormone secretion in response to cAMPgenerating stimuli. The permissive actions by AVP that are cited are on hormone secretion, which in many cell types requires activation of both calcium and cAMP signaling. Whether the activation of V1br and subsequent calcium response is permitted by cAMP is unclear. I believe the argument the authors are making here is that the activation of beta cell calcium by AVP is permitted by forskolin. i.e., the cAMP stimulated by it in beta cells. However, the design does not account for the elevation of cAMP in alpha cells and subsequent release of glucagon, particularly upon co-stimulation with AVP, which permits glucagon release by activating a calcium response in alpha cells. This glucagon could then activate beta cells. If resolving the mechanism of action is the goal, often less is more. The activation of Gaq-mediated calcium is not cAMP dependent (although the downstream hormone secretion clearly often is). As was shown,*

> *AVP does not activate calcium in beta cells in the absence of cAMP. The experiments in Figures 1, 2, and 4 should have been completed in the absence of cAMP first.*

We agree with the reviewer that the use of forskolin needs to be explained more clearly, and we have revised the manuscript accordingly. Our rationale was based on the established permissive role of cAMP in AVP-dependent endocrine responses, but we acknowledge that this does not necessarily imply that the upstream V1bR/Gq-mediated Ca^{2+} response itself is cAMP-dependent. The reviewer is also correct that forskolin elevates cAMP broadly and therefore may affect both alpha and beta cells, including the possibility that AVP-enhanced alpha cell activation and glucagon release secondarily influence beta cell activity.

In fact, our initial experiments were performed without forskolin and revealed an important difficulty: stimulatory glucose alone can increase cAMP levels to a variable extent, as also supported by our previous work on epinephrine signaling, thereby shifting the apparent peak efficacy of AVP stimulation. Thus, forskolin was originally used to reduce this variability and create a more defined cAMP-permissive background in which alpha and beta cell responses could be compared in the same slice. However, we agree that this design works against isolation of beta cell-autonomous AVP effects.

Within a scope of another study we have done an independent series of more focused experiments using GLP-1 receptor stimulation, which preferentially increases cAMP signaling in beta cells compared with the broad cAMP elevation produced by forskolin. We have clarified that the modulation of the AVP-dependent pathway by GLP-1 and related ligands at largely supports beta cell-autonomous AVP effects. It is part of ongoing work and will be reported independently, because a full mechanistic dissection of cAMP–AVP interactions goes far beyond the scope of the present study.

> *(5) It is unexpected that epinephrine in Figure 2 does not activate the alpha cell calcium? A recent paper from the same group (Sluga et al) shows robust calcium activation in alpha cells in a similar prep by 1 nM epinephrine, which is similar to the dose used here.*

We thank the reviewer for pointing this out, but we would like to clarify that epinephrine did significantly activate alpha-cell Ca^{2+} activity in our experiments, as shown in Fig. 3F. This result is consistent with our previous study by Sluga et al., where low nanomolar epinephrine robustly activated alpha cell Ca^{2+} signals in the pancreatic slice preparation. The main point of the present comparison was therefore not that epinephrine is inactive in alpha cells, but that AVP produces a substantially stronger and reproducible alpha cell Ca^{2+} response under comparable experimental conditions. This is also consistent with the data of van der Meulen et al., supporting the view that AVP/V1bR signaling is a particularly potent activator of alpha cell activity. We have revised the text to make this comparison clearer and to avoid the impression that epinephrine failed to activate alpha cells in our preparation.

> *(6) Figure 8 suggests a pharmacological activation of beta cell V1bR in the low pM range. How do the authors reconcile this comparison with the apparent absence of an effect of AVP stimulation at low pM to low nM doses in beta cells (Figure 4A)? I note that there are changes over time with sustained beta cell stimulation with 8 mM glucose, but these changes are relatively subtle, gradual, and quite likely represent the progression of calcium behaviors that would have occurred under sustained glucose, irrespective of these very low AVP concentrations. I will note that the Kd of the V1bR for AVP is around 1 nM, with tracer displacement starting around 100 pM according to the data in figure 5B, which is hard to reconcile with changes in beta cell calcium by AVP doses that start 10-100-fold lower than this dose at 1 and 10 pM (Figure 8).*

We agree that the interpretation of low-pM AVP effects requires caution, particularly when compared with reported V1bR binding affinities. The apparent discrepancy between Fig. 4A and Fig. 8 most likely reflects differences in experimental design, stimulation context, and

readout sensitivity. In Fig. 4A, we assessed acute AVP effects under conditions in which beta cell Ca^{2+} responses are relatively threshold-dependent and where low AVP concentrations produced little or no activation. In contrast, Fig. 8 analyzes prolonged beta cell population dynamics during sustained stimulation with 8 mM glucose, a physiological stimulatory context in which even weak modulatory inputs may become detectable at the level of collective Ca^{2+} activity.

Importantly, the strongest and statistically significant effect was observed at 100 pM AVP, while lower pM concentrations showed only a trend. We therefore do not interpret the low-pM range as evidence for robust direct pharmacological activation of beta cell V1bR. Rather, these data suggest that AVP may exert permissive or modulatory effects within an already active beta cell network, where glucose-dependent excitability, receptor-effector coupling, and Ca^{2+} amplification mechanisms can enhance the apparent efficacy of weak inputs. This interpretation is consistent with the known permissive role of AVP in endocrine responses, where AVP may not act as a primary activator alone but can increase the efficacy of other physiological stimuli.

We have also clarified that sustained 8 mM glucose alone does not account for these effects, since Suppl. Fig. 1 shows no comparable time-dependent progression of Ca^{2+} behavior under sustained glucose stimulation alone. Thus, we now present the low-concentration AVP effects as subtle, contextdependent modulation within the physiological stimulatory range, rather than as evidence for direct beta cell activation at concentrations below the expected receptor affinity range.

> ***Reviewer #3 (Public review):***
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> *Summary:*
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> *This work aims to better understand the role of arginine vasopressin (AVP) in the control of islet hormone secretion. This builds on previous literature in this area reporting on the actions of AVP to stimulate islet hormones. The gap in literature being addressed by these studies is primarily focused on the glucose-dependency of AVP on both insulin and glucagon secretion. A secondary objective is to explore the role of individual receptors with the use of newly generated peptides and existing tools. The methods include the use of Ca2+ imaging in pancreas slices from mice, with additional outcomes including insulin secretion in some areas. The conclusions presented are that AVP acts through V1b receptors in both alpha- and beta-cells, that this activity occurs in the high cAMP environment, and is glucose dependent.*
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> *Strengths:*
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> *The area of research is emerging with plenty of room for new contributions. The concept of AVP stimulating islet hormone secretion is important and deserving of further insight. The use of pancreas tissue to image primary cells makes the experiments physiologically relevant. The advancement of novel tools in this area should be helpful to other groups investigating the actions of AVP.*

We would like to thank the reviewer for recognizing the potential of our emerging area of research.

> *Weaknesses:*
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> *The conclusions are only modestly supported by the data and lack experimental depth and rigor. The rationale for only conducting studies at high cAMP conditions is not entirely clear and limits the conclusions that can be made. The use of Ca2+ is helpful, but it is a surrogate for hormone secretion. Additional measurements of hormone secretion are needed to enhance the robustness of these conclusions. Consideration of paracrine*

> *effects between alpha- and beta-cells is only superficially made and is likely essential in the context of the experimental design. For instance, there is clear literature that alpha-cells secrete several factors that work in paracrine interactions on beta-cells and autocrine actions back on alpha-cells. Conducting these studies in a high cAMP context only completely overlooks these interactions, skewing the interpretations made by the investigators. Finally, the clarity of the experiments and results could be significantly enhanced.*

We thank the reviewer for this balanced assessment and for emphasizing several issues that are central to the interpretation of our study. We agree and now explicitly state in the Limitations, that Ca^{2+} oscillations are a surrogate readout for hormone secretion and that currently used stimulation protocols are not optimized to directly quantify the relationship between Ca^{2+} dynamics and secretory output. To address this limitation, we have now expanded the functional part of the study by adding complete insulin and glucagon secretion measurements during AVP concentration ramps. These new data provide a stronger functional framework for interpreting the Ca^{2+} imaging results, while also clarifying that Ca^{2+} activity and secretion cannot be assumed to correlate linearly under all stimulation protocols.

We have also revised the rationale for the high-cAMP experimental condition. The original aim was to reduce variability arising from glucose-dependent endogenous cAMP signaling and to study alpha and beta cell responses in a common permissive background. However, we agree that broad forskolin stimulation complicates the interpretation of cell-autonomous versus paracrine mechanisms. Independent experiments within a scope of another study demonstrate that using GLP-1 co-stimulation, which provides a more beta-cell-oriented cAMP-permissive condition and supports the interpretation that AVP can modulate beta-cell collective activity in a manner that is not solely secondary to alpha-cell activation.

We fully agree that paracrine interactions within the islet are physiologically important and must be considered, particularly in intact pancreatic slices. Nevertheless, the rapid onset of the AVP effects observed in beta cell Ca^{2+} activity argues against a mechanism mediated predominantly by slower indirect paracrine loops. High AVP concentrations, as shown in Fig. 5, significantly shorten the intervals between Ca^{2+} events in both alpha and beta cells, but that the activity of the two cell populations remains largely noncoordinated. This temporal dissociation does not exclude paracrine modulation altogether, but it argues against a simple alpha-cell-driven explanation for the beta-cell response.

We have revised the manuscript to state these points more clearly and to moderate conclusions where the data support modulation rather than definitive cell-autonomous receptor action. We believe that the added secretion experiments and alpha/beta event-timing analysis substantially strengthen the physiological interpretation of the study, and we thank the reviewer for raising these issues.

> ***Recommendations for the authors:***
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> ***Reviewer #2 (Recommendations for the authors):***
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> *(1) The paragraph discussing the benefits of slice physiology over islets is not reflective of how most - if not all of your colleagues who do islet experiments conduct these. Many labs have reported for years high-quality GSIS experiments, synchronous calcium responses, and a plethora of studies detailing the mechanism of hormone and neurotransmitter actions using islet models, and have done so well. Slice physiology is a unique and helpful model that can have advantages over other models. This particular reviewer uses both models in their lab and each has benefits and - inevitably - drawbacks. Many of the possible drawbacks cited for islet studies apply equally to slices, including the possibility of altered gene expression, lack of innervation, and circulation.*

> *Added drawbacks are the exposure to higher levels of pancreatic enzymes from the slice, which require co-culture with enzyme inhibitors.*

We agree with the reviewer and have revised these limitations accordingly. Our intention was not to imply that isolated islet preparations are generally inferior, since they have provided a highly productive and rigorous experimental platform for GSIS, synchronized Ca^{2+} dynamics, and mechanistic studies of hormonal and neurotransmitter regulation with standardized protocols with all their positive and negative sides. We modified the presentation of pancreatic slices as a complementary model with specific advantages, particularly preservation of local tissue architecture, while also acknowledging their limitations. The revised text therefore avoids a comparative hierarchy between slices and isolated islets and instead emphasizes that both models have distinct strengths and drawbacks depending on the experimental question.

> *(2) If you want to demonstrate direct actions on beta cells, deconstructing the islet would be a better way to go. Less complicated, not more. Dissociated beta cells, instead of slices, were used just to prove or disprove the hypothesis of direct beta cell effects of AVP.*

We agree that dissociated beta cells can be a useful reductionist model to test whether AVP is capable of acting directly on individual beta cells. However, this approach would also remove the collective beta-cell activity that is central to the physiological question addressed in the present study. Since our data indicate that AVP effects emerge within the intact islet as rapid and extensive changes in coordinated Ca^{2+} dynamics, dissociation would not necessarily provide a more informative model for understanding these responses. The fast onset and magnitude of the beta cell response argue against a predominantly indirect non-autonomous mechanism, and this interpretation is further supported by the GLP-1 co-stimulation experiments, which are more consistent with beta cell-autonomous modulation. We have therefore clarified in the revised manuscript that dissociated-cell experiments would be valuable for a narrowly defined receptor-cell autonomy question, but would not resolve the collective islet dynamics that are the focus of this work.

> *(3) If you want to sustain the claim of beta cell expression of V1br, you would have to demonstrate this far more directly by staining (if appropriate antibodies exist), by beta cell-specific deletion of V1br, or by highly selective, well-validated pharmacology. This should include a demonstration of Gaqdependence in isolated beta cells.*

We have added RNAscope in situ hybridization data to the revised manuscript to assess V1b receptor mRNA expression within the pancreas and islet. These new data show a broader expression pattern of V1b receptor transcripts within the islet than originally assumed, suggesting that AVP signaling may not be restricted to a single endocrine cell population. At the same time, the RNAscope analysis confirms previous reports of higher AVP receptor expression in glucagon-positive alpha cells. We have added these results to Figure 1 and revised the corresponding Results and Discussion sections to clarify that the observed functional responses may reflect both direct effects on beta cells and indirect intra-islet effects mediated through alpha-cell signaling.

> *Minor*
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> *(1) O'Carroll et al. should be cited in the context of islet permissive actions of AVP/cAMP. PMID: 18434353, although that paper offers no evidence that the AVP-dependent potentiation of insulin release is mediated directly by beta cells. It does confirm dependence on PKC.*

We agree and have added O'Carroll et al. in the revised manuscript in the context of AVP/cAMP-dependent permissive actions on islet hormone secretion. We therefore use it as support for the broader concept of AVP-dependent amplification of secretion in a permissive signaling context, rather than as direct evidence for beta-cell-autonomous V1bR signaling.

> *(2) Figure 2 E-H, glucose concentration mislabeled.*

We thank the reviewer for pointing this out. The glucose concentration label has been clarified: panels E–H show pooled data from separate experiments performed at 8 mM glucose, whereas panel D shows a representative experiment performed at 9 mM glucose.

> *(3) The insulin secretion in 4E is difficult to interpret without a low-glucose control. If this is hard to do in a slice preparation, a separate static islet secretion experiment would help here. The possibility that the inhibition of insulin secretion traces back to the activation of delta cells by AVP could be considered - I struggle to come up with a plausible mechanistic explanation why AVP (which activates calcium in alpha and in beta cells in the presence of 8 mM G plus forskolin according to your data) would inhibit insulin secretion.*

We agree that the original insulin secretion experiment was difficult to interpret without a clearer low-glucose reference condition. To address this, we have added new insulin release experiments in which glucose was lowered to a non-stimulatory range between AVP concentrations, followed by sequential stimulation with 8 mM glucose and 500 nM forskolin in the same slices. These new data provide a broader dynamic range for assessing insulin secretion and allow a more direct comparison between AVP-dependent Ca^{2+} modulation and secretory output.

We also agree that AVP-dependent inhibition of insulin secretion requires careful interpretation. One possible explanation is not simply activation of delta cells, but a failure of the beta cell collective to maintain coordinated activity at very high AVP concentrations. In the Ca^{2+} imaging data, high AVP concentrations increase activity in many beta cells, but numerous cells within the islet fail to keep pace with the collective oscillatory rhythm, leading to fragmented and less synchronized population activity. Thus, despite increased frequency of Ca^{2+} oscillations in the islet, the integrated beta cell output may become less efficient for insulin secretion. We have added this interpretation to the revised manuscript and now discuss delta cell activation as a possible contributing mechanism, but not as the primary explanation supported by our current data.

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