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An interplay between the serotonin transporter (SERT) and 5-HT receptors controls stimulus-secretion coupling in sympathoadrenal chromaffin cells

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Abstract

Adrenal chromaffin cells (ACCs), the neuroendocrine arm of the sympathetic nervous system, secrete catecholamines to mediate the physiological response to stress. Although ACCs do not synthesize 5-HT, they express the serotonin transporter (SERT). Genetic variations in SERT are linked to several CNS disorders but the role(s) of SERT / 5-HT in ACCs has remained unclear. Adrenal glands from wild-type mice contained 5-HT at $\approx$ 750 fold lower abundance than adrenaline, and in SERT^{-/-} mice this was reduced by $\approx$ 80% with no change in catecholamines. Carbon fibre amperometry showed that SERT modulated the ability of 5-HT_{1A} receptors to inhibit exocytosis. 5-HT reduced the number of amperometric spikes (vesicular fusion events) evoked by KCl in SERT^{-/-} cells and wild-type cells treated with escitalopram, a SERT antagonist. The 5-HT_{1A} receptor antagonist WAY100635 blocked the inhibition by 5-HT which was mimicked by the 5-HT_{1A} agonist 8-OH-DPAT but not the 5-HT_{1B} agonist CP93129. There was no effect on voltage-gated Ca²⁺ channels, K⁺ channels, or intracellular [Ca²⁺] handling, showing the 5-HT receptors recruit an atypical inhibitory mechanism. Spike charge and kinetics were not altered by 5-HT receptors but were reduced in SERT^{-/-} cells compared to wild-type cells. Our data reveal a novel role for SERT and suggest that adrenal chromaffin cells might be a previously unrecognized hub for serotonergic control of the sympathetic stress response.

HIGHLIGHTS

- Adrenal chromaffin cells (ACCs) in the sympathetic nervous system express SERT

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Chemical compounds studied in this article:

Serotonin hydrochloride (PubChem CID: 160436); 8-OH-DPAT (PubChem CID: 6917794); CP93129 (PubChem CID: 124007); WAY100635 (PubChem CID: 11957721); Escitalopram (PubChem CID: 146571); Gallein (PubChem CID: 73685).

- ACCs contain small amounts of 5-HT due to uptake by SERT
- SERT modulates the ability of 5-HT_{1A} receptors to inhibit catecholamine release
- 5-HT_{1A} receptors recruit an atypical mechanism independent from Ca²⁺ or K⁺ channels
- ACCs might be an unrecognized hub for control of sympathoadrenal stress by SSRIs

Graphical abstract

Keywords

serotonin transporter; adrenal chromaffin cell; calcium channel; exocytosis; catecholamine; 5-HT receptor; amperometry; calcium imaging; GPCR

1. INTRODUCTION

The serotonin transporter (SERT; SLC6A4) is one of the main routes for clearing extracellular serotonin (5-hydroxytryptamine; 5-HT) in the central nervous system. As such, SERT plays an important role in controlling serotonergic neurotransmission / neuromodulation. Underscoring this, genetic variations that alter the expression or activity of SERT are linked to several neurobehavioural disorders (e.g. depression, anxiety, autism), which are often treated by drugs that target SERT (e.g. selective serotonin reuptake inhibitors - SSRIs) (for reviews see (Daws and Gould, 2011; Muller et al., 2016; Murphy et al., 2008; Ye and Blakely, 2011)). Although much attention has focused on serotonergic signaling in the CNS, the majority of 5-HT is produced in the periphery, primarily in the gut where it has multiple actions (Gershon, 2013). SERT plays diverse physiological roles in the periphery by modulating 5-HT signaling and homeostasis. Of relevance for this paper, SERT is prominently expressed in adrenal chromaffin cells which comprise the neuroendocrine arm of the sympathetic nervous system (Schroeter et al., 1997). However, the role(s) of SERT and 5-HT signaling in the peripheral sympathetic nervous system remain poorly understood.

Sympathoadrenal chromaffin cells synthesize and release a cocktail of catecholamines (primarily adrenaline and noradrenaline) and neuropeptides to mediate the response to physiological and emotional stressors (Livett et al., 1981; Winkler and Westhead, 1980). Preganglionic splanchnic nerves relay the central drive and trigger electrical excitability, Ca²⁺ entry through voltage-gated Ca²⁺ channels, and subsequent exocytosis of secretory vesicles (stimulus-secretion coupling) (Garcia et al., 2006). A variety of G protein coupled receptors (GPCRs) expressed by chromaffin cells detect and respond to neuronal, autocrine/

paracrine, and circulating (endocrine) mediators to modulate stimulus-secretion coupling (Currie, 2010; Garcia et al., 2006; Jewell and Currie, 2013). In mammals, adrenal chromaffin cells lack the rate limiting enzyme for synthesis of 5-HT, tryptophan hydroxylase. However, they do contain a small amount of 5-HT (≈ 750 fold lower abundance than adrenaline) which appears to be the result of SERT-mediated uptake (Kent and Coupland, 1984; Linder et al., 2009; Tjurmina et al., 2002; Verhofstad and Jonsson, 1983). This 5-HT is presumably packaged into secretory vesicles by the vesicular monoamine transporter; otherwise it would be subject to metabolism by monoamine oxidase. Thus, one role of SERT in the adrenal gland could be to endow a serotonergic phenotype onto these catecholaminergic cells. SERT^{-/-} mice display an exaggerated sympathoadrenal response to restraint stress (i.e. a greater increase in plasma adrenaline compared to wild-type controls) (Tjurmina et al., 2002), and treatment with SSRIs enhanced the sympathoadrenal response to hypoglycemia in rodents and humans (Briscoe et al., 2008; Sanders et al., 2008). Taken together, these data suggest that serotonergic signaling helps control the sympathetic stress response, but it is unclear if this reflects local serotonergic signaling within the adrenal gland and/or altered central drive due to loss/block of SERT in the CNS.

In this paper we confirmed that mouse adrenal glands contain 5-HT (≈ 750 fold lower than adrenaline) and this was dramatically reduced in SERT^{-/-} mice with no change in the catecholamine content. We show for the first time that SERT modulated the ability of 5-HT_{1A} receptors to inhibit stimulus-secretion coupling. Receptor activation reduced the number of vesicular fusion events but not the amount or kinetics of transmitter release from each vesicle. The inhibition did not involve voltage-gated Ca²⁺ channels or K⁺ channels, the usual targets for inhibitory GPCR signaling. Spike charge and kinetics were reduced in SERT^{-/-} cells compared to wild-type cells, indicating that SERT has an additional, distinct effect. Overall, our data reveal a novel role for SERT, and suggest that adrenal chromaffin cells might be a previously unrecognized hub for serotonergic control of the sympathetic stress response.

2. METHODS

2.1. Ethical approval / animals

All procedures were subject to prior approval by the Institutional Animal Care and Use Committee of Vanderbilt University Medical Center and complied with appropriate laws and guidelines set forth by the National Institutes of Health guide for the care and use of laboratory animals. Male C57BL/6J mice were group housed on a 12:12 hour light / dark cycle with food and water available *ad libitum*. Mice were euthanized using carbon dioxide inhalation followed by cervical dislocation. SERT knockout mice (SERT^{-/-} mice) have been described previously (Bengel et al., 1998) and were bred onsite at Vanderbilt University Medical Center. Wild-type mice were littermate controls of SERT^{-/-} or were purchased from The Jackson Laboratory (Bar Harbor, ME).

2.2. Determination of tissue monoamine content by HPLC

The right adrenal gland was harvested from euthanized male mice (9 -20 weeks old), carefully trimmed of fat and immediately frozen and maintained at -80°C until use. Tissue monoamines were then extracted and determined by the Vanderbilt Brain Institute Neurochemistry Core at Vanderbilt University. Briefly, the adrenal glands were homogenized using a tissue dismembrator in 150 μl of 0.1M TCA (which contained 10^{-2} M sodium acetate, 10^{-4} M EDTA, 5ng/ml isoproterenol (as internal standard) and 7.5% methanol (pH 3.8)). Samples were spun in a microcentrifuge at 10,000 g for 20 minutes and the supernatant removed for biogenic amine analysis while the pellet was saved for protein analysis. Biogenic amines were quantified using HPLC (Waters 515 pump; Waters Corporation, Milford, MA US) with electrochemical detection (Antec Decade II [oxidation: 0.65] electrochemical detector operated at 33°C ; Antec, Zoeterwoude, The Netherlands). Twenty microliter samples of the supernatant were injected onto a Phenomenex Kinetex C18 HPLC column (100×4.60 mm, 2.6 μ). Biogenic amines were eluted with a mobile phase consisting of 89.5% 0.1M TCA, 10^{-2} M sodium acetate, 10^{-4} M EDTA and 7.5 % methanol (pH 3.8) with a flow rate of 0.6 mL/min. HPLC control and data acquisition are managed by Empower 3 software.

2.3. Isolation of adrenal chromaffin cells

Adrenal glands were harvested from euthanized male mice (7-14 weeks old), placed on ice in Locke's solution (154mM NaCl, 5.6mM KCl, 2.1 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 0.8mM Na_2HPO_4 , 10mM glucose and 10mM HEPES, pH 7.4) and trimmed of fat. The adrenal cortex was dissected from the glands, and the medullae were incubated for 20 minutes at 37°C in NeuroPapain solution (1.5mg/ml; Genlantis, San Diego, CA) supplemented with bovine serum albumin (0.5mg/ml), CaCl_2 (1mM), L-cysteine (0.5mM) and EDTA (0.5mM). This was followed by a 5-minute incubation in ovomucoid trypsin inhibitor (2.5mg/ml; Worthington Biochemical Corporation, Lakewood, NJ) with BSA (0.5mg/ml) and three washes in Locke's solution. The medullae were transferred to growth medium consisting of Dulbecco's modified Eagle medium / F12 with Glutamax (Life Technologies, Grand Island, NY), fetal bovine serum (10%; GE Healthcare Life Sciences, Logan, UT) and penicillin / streptomycin (100 units ml^{-1} / 100 μg ml^{-1} ; Mediatech, Inc., Manassas, VA) and triturated with a series of fire polished glass pipettes with decreasing tip diameters. The resulting chromaffin cell suspension was plated onto glass coverslips thinly coated with growth-factor reduced Matrigel (BD Biosciences, Bedford, MA) in 35mm dishes. Cells were allowed to adhere to the coverslips for 1 hour at 37°C in a humidified, 5% CO_2 atmosphere before addition of 2ml of growth medium to the dish. Culture medium was replaced the following day and cells were recorded from 2-5 days after isolation. In one set of experiments, pertussis toxin (300ng/ml) (EMD Biosciences, La Jolla, CA) was added to the media 2 days after cell isolation and cells were recorded from 18-24 hours later.

2.4. Carbon fibre amperometry recording

Amperometric data were collected using commercially available carbon fibre electrodes (5 μm diameter) (CFE-2; ALA Scientific Instruments, Farmingdale, NY). A potential of +700mV was applied to the electrode and data was recorded using a VA-10X amplifier (NPI

Electronic, GmbH, Tamm, Germany), a 16-bit BNC-2090 analog to digital converter (National Instruments, Austin, TX), and WinEDR software written by Dr. John Dempster (Strathclyde University, Glasgow, Scotland). Amperometric currents were filtered at 2kHz and continuously sampled at 10kHz. The shaft of the CFE-2 carbon fibre electrodes is insulated with parylene and the end of the fibre cut to expose a fresh disk of bare carbon that acts as the working electrode. Each carbon fibre electrode was used multiple times with the fibre carefully cut back between every cell to reveal a fresh oxidation surface and to minimize electrode fouling. Each electrode was carefully inspected under a microscope to ensure a clean perpendicular cut. The electrode was mounted on a Burleigh PCS-5200 micromanipulator (Thorlabs, Newton, NJ) to enable accurate and stable positioning in the recording bath. Chromaffin cells were placed into the recording bath (volume ~ 300 μ L) and continually perfused with extracellular solution from gravity fed reservoirs at a rate of ~3 ml / min for 5-10 minutes prior to and throughout the recording. Following initial testing of the electrode response to the +700 mV step, the electrode was positioned so it just touched the surface of a chromaffin cell. The extracellular recording solution contained (in mM): 145 NaCl, 2 KCl, 1 $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 10 glucose, 10 HEPES, 2 CaCl_2 , pH 7.3 and osmolarity ~ 305 mOsm. To evoke exocytosis, cells were stimulated with 30mM KCl for 60s; the composition of this solution was the same except KCl was increased to 30mM and NaCl reduced to 117mM to maintain osmolarity. The experimental / drug application paradigm for each set of data is explained in the Results section and/or figure legends. All recordings were alternated between control cells and cells with the various treatments for that particular set of experiments (e.g. with/without 5-HT or escitalopram; wild-type cells vs $\text{SERT}^{-/-}$ cells). This was done to randomize the influence of any variability due to cell preparations, recording solutions, electrode characteristics, etc.

In preliminary experiments, batches of electrodes were tested to assess baseline properties and the response to adrenaline. The response to increasing concentrations of adrenaline (100nM, 1 μ M, 10 μ M and 100 μ M) was linear and consistent across fibres (1.25 [0.3] pA / μ M (mean [SD], n = 14)). Repeated application of adrenaline showed that electrode sensitivity did not change over the time-course / conditions of the experimental protocols used in this study (4.5 [2.7] % change (mean [SD])). The electrode properties were also assessed in extracellular solution with no monoamine present. As expected, when the voltage applied to the electrode was stepped from 0 mV to +700 mV there was a large, transient charging (capacitive) current. The decay of this transient reflects electrode capacitance which is proportional to the surface area of bare carbon; with well-insulated disk electrodes (i.e. small area of exposed carbon fibre) the decay was relatively fast (tens to a few hundred milliseconds). The amplitude and noise of the stable current step following the charging transient also increase with electrode surface area. Under our recording conditions the amplitude of the baseline current step was 2.9 [0.7] pA (mean [SD]) and the peak-to-peak noise was very low ($\approx$ 0.5 - 0.7 pA at 2kHz filtering). Deliberate fouling of the electrode surface to reduce sensitivity to adrenaline also reduced the amplitude of the baseline current. Together, the decay of the charging transient along with the amplitude and noise of the baseline current are indicators of electrode quality. If the electrode is broken or the working surface occluded, the voltage step will produce no increment in baseline current and the exponentially decaying charging spike is absent. If the fibre is not well insulated

(large surface area), then decay of the charging transient will be slower, and the amplitude / noise of the baseline current will be increased. These features were carefully monitored before every recording by initially positioning the electrode away from the cell to be recorded, then applying the +700 mV potential and allowing the electrode baseline current to stabilize.

2.5 Analysis of amperometry data

Amperometric spikes were detected and analyzed using the Quanta Analysis macros written by Dr. Eugene Mosharov (Mosharov and Sulzer, 2005) in IgorPro software (Wavemetrics Inc., Oswego, OR). An amplitude threshold of 3pA was used for spike detection. These events were readily resolved due to the low baseline noise (rms noise of the baseline current was 0.16 [0.07] pA (mean [SD]); n = 568 cells). Cells were subjected to two rounds of analysis: first, to assess the number of vesicular fusion events, all spikes 3pA with rise time (25-75%) between 0.5 and 100ms were counted. This included spikes that overlapped by 50%. All detected spikes were confirmed by visual inspection. Cells with < 15 spikes evoked by KCl were considered to be non-responsive and excluded from analysis. Cells with an unusually large number of spikes (> 200 events) were also excluded due to difficulty in resolving individual non-overlapping events in the second round of analysis (see below). The second round of detection included additional criteria to ensure accurate analysis of individual spike parameters (charge, amplitude, half-width, slope). Detection criteria were: amplitude 3pA; only non-overlapping spikes were included (defined as < 10% overlap in the spike detection software); only spikes with a rise time < 10ms were included to minimize detection of spikes that likely occurred distant to the carbon fibre and whose parameters cannot be accurately analyzed. All detected spikes were confirmed by visual inspection. The more stringent detection criteria (i.e. non-overlapping spikes) included $65 \pm 0.7\%$ of the spikes detected in the first round of analysis (n = 568 cells). After detection of spike number and parameters using the IgorPro macros, the data were collated and analyzed using OriginPro7 (OriginLab Corporation, Northampton, MA) and Prism 5 software (GraphPad Software Inc., La Jolla, CA). When comparing the individual amperometric spike parameters, median values were calculated for each cell and these were pooled for statistical comparison. This was done to ensure that each cell has the same statistical weight in the analysis (for further discussion see (Colliver et al., 2001; Mosharov and Sulzer, 2005)).

2.6. Patch clamp electrophysiology

Patch pipette electrodes were pulled from borosilicate glass capillary tubes (World Precision Instruments, Sarasota, FL) using a Flaming/Brown P-97 pipette puller (Sutter Instruments, Novato, CA). Pipettes were coated with dental wax (Electron Microscopy Services, Hatfield, PA) and fire polished using a Narishige MF-830 micro forge (Narishige, Amityville, NY) to a final resistance of ~2M Ω when filled with internal solution. Cells were recorded using the amphotericin perforated whole-cell recording configuration. Electrode tips were filled with amphotericin free patch pipette solution (see below for composition) and backfilled with patch pipette solution containing 0.5mg/ml amphotericin B (EMD Biosciences, La Jolla, CA). Chromaffin cells were voltage-clamped using a HEKA EPC10 amplifier and Patchmaster acquisition software (HEKA Instruments Inc., Holliston, MA, USA). Analog data were filtered at 2.9 kHz and sampled at 50kHz. Typically, series resistance < 15M Ω was

achieved within 15 mins of seal formation. Cells that did not display low series resistance within this time frame were discarded, as were cells with unstable holding current. Series resistance was partially compensated (~50-80%) using the EPC10 / Patchmaster software. Ca^{2+} channel currents (I_{Ca}) were activated by a 15ms step depolarization to 0mV from a holding potential of -80mV, applied every 15s. I_{Ca} were subjected to linear capacitance and leak subtraction using P/-6 protocols with leak pulses applied after test pulses. K^{+} channel currents (I_{K}) were evoked from a holding potential of -70mV by a series of 50 ms step depolarizations ranging from -120 mV to + 80mV. Amplitude was measured in the last 5ms of the step depolarization and normalized to cell capacitance to yield current density. Raw data were analyzed using HEKA FitMaster software, and subsequently collated and subjected to statistical comparison using OriginPro7 software (OriginLab Corporation, Northampton, MA) and Prism 5 (GraphPad Software Inc., La Jolla, CA).

The extracellular recording solution was the same as used for amperometry and contained (in mM): 145 NaCl, 2 KCl, 1 $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 10 glucose, 10 HEPES, 2 CaCl_2 , pH 7.3 and osmolarity ~ 305 mOsm. For recording I_{Ca} , 750nM tetrodotoxin (TTX) was added to block voltage-gated sodium channel currents. The patch pipette solution for recording I_{Ca} contained (in mM): 145 cesium glutamate, 10 HEPES, 10 NaCl, 1 tetraethylammonium chloride, pH 7.3 and osmolarity ~ 309mOsm. The patch pipette solution for recording I_{K} contained (in mM): K-aspartate 110, KCl 25, NaCl 8, MgCl_2 2, HEPES 20, pH 7.3 and osmolarity ~ 305 mOsm. Stock solution of amphotericin B (50 mg/ml) was prepared in dimethyl sulfoxide (DMSO) on the day of use and diluted to a final concentration of 0.5 mg/ml in the appropriate patch pipette solution.

2.7. $[\text{Ca}^{2+}]_{\text{i}}$ measurements

Free cytosolic $[\text{Ca}^{2+}]_{\text{i}}$ was measured in cells loaded with the fluorescent Ca^{2+} indicator Fura-2AM (Molecular Probes, Eugene, OR or Setareh Biotech, LLC, Eugene, OR) using an InCyt IM2 fluorescence imaging system (Intracellular Imaging Inc, Cincinnati, OH). Cells were washed twice with HEPES-buffered Hank's balanced salt solution and incubated with 5 μM Fura-2AM at 37°C for ~45 mins, protected from light. Cells were then washed in Fura free solution for 30-60 minutes at room temperature, protected from light. Coverslips were transferred to a recording chamber mounted on a Nikon TE2000 fluorescence microscope (Nikon Instruments Inc., Melville, NY). The recording bath was continually perfused with extracellular solution or 30mM KCl stimulation solution from gravity fed reservoirs at a flow rate of ~3-4 ml / min. Typically, between 5-15 individual cells were selected in the field of view for analysis. Cells were excited at wavelengths of 340 nm and 380 nm and emission detected at 510 nm with a PixelFly digital camera. The ratio of fluorescence emission at the two excitation wavelengths (340nm / 380nm) was measured every 1s and converted to $[\text{Ca}^{2+}]_{\text{i}}$ using an *in vitro* calibration curve generated by adding 15.8 μM Fura-2 pentapotassium salt to solutions from a calibration kit containing 1mM MgCl_2 and known concentrations of Ca^{2+} (0-1350 nM) (Molecular Probes, Eugene OR). Data were transferred to OriginPro7 or Prism 5 software for analyses and statistical comparison.

2.8. Solutions, drugs and reagents

5-HT (Sigma-Aldrich, St Louis, MO) was prepared fresh daily as a 2mM aqueous stock and diluted into extracellular solution at final working concentrations of 25 nM - 10 μ M. Escitalopram oxalate (20mM), (R)-(+)-8OH-DPAT hydrobromide (50mM) (both Sigma-Aldrich, St Louis, MO), WAY100635 maleate (25mM) (Abcam, Cambridge, MA), CP93129 dihydrochloride (50mM) (R&D systems, Minneapolis, MN) and TTX (1mM) (R&D systems, Minneapolis, MN) were all prepared as aqueous stock solutions and frozen in aliquots. All stock solutions were diluted ($> 1000\times$) to a final working concentration in extracellular solution or KCl stimulation solution on the day of use. Gallein (R&D systems, Minneapolis, MN) was prepared as a 30mM stock solution in DMSO on the day of use and diluted to 10 μ M working concentration (0.03% DMSO). Fura-2AM was prepared as a 1mM stock solution in DMSO and aliquots stored at -20°C for no more than 1-2 weeks. Stocks were diluted into HEPES-buffered Hank's balanced salt solution on the day of use.

2.9. Statistical analysis

Graphing and statistical analyses were performed using OriginPro7 and GraphPad Prism 5 software. The specific tests that were used are noted in the results section and/or figure legends. Data from calcium imaging, patch clamp electrophysiology, and the number of amperometric spikes per cell were normally distributed and reported as mean with standard deviation or standard error as noted in the text. Data for the individual amperometric spike parameters were not normally distributed (determined in Prism 5 software using a Shapiro-Wilk or D'Agostino & Pearson omnibus normality test), so are reported as median with interquartile range. Nonparametric tests were used for statistical comparison of these data, or in some cases the data were first log-transformed to enable comparison using two-way analysis of variance (ANOVA) (see results section for figure 3). When comparing two data sets, Students t-test (paired or independent) or Mann-Whitney test was used. When comparing multiple (>2) groups, ANOVA or Kruskal-Wallis tests were used with appropriate post-hoc tests for multiple pairwise comparisons (as noted for each dataset). For each experimental dataset, "n" denotes the number of cells recorded, and these were from between 2-7 independent cell preparations (mean = 3.9 ± 0.6) for each dataset.

3. RESULTS

3.1. The 5-HT content was dramatically reduced but catecholamines were unaltered in the adrenal glands from SERT^{-/-} mice

Adrenal chromaffin cells robustly express SERT (Schroeter et al., 1997) and one role of SERT likely would be to accumulate 5-HT into these catecholaminergic cells. We used HPLC to analyze the monoamine content of adrenal glands from nine male wild-type and nine male SERT^{-/-} mice (figure 1). The glands from SERT^{-/-} mice were of similar size to wild-type: the total protein content was 350 (SD 97) μg for wild-type and 366 (SD 81) μg for SERT^{-/-} glands (not significant; $P = 0.71$, unpaired t-test). There was a striking reduction in the 5-HT content of the adrenal glands, from 55.25 (SD 8.19) pmoles/mg protein in wild-type mice ($n = 9$) to 10.92 (SD 7.89) pmoles/mg protein in SERT knockouts ($n = 9$) ($P < 0.0001$, unpaired t-test) (figure 1A). In the same glands, there was no significant difference in the amount of adrenaline, noradrenaline, or dopamine between SERT^{-/-} and wild-type

mice (figure 1A). Our data also confirmed that the 5-HT content was much lower than catecholamines (note the difference in Y-axis scale in figure 1A). The molar ratio of 5-HT to adrenaline was 0.13 % (SD 0.03) in wild-type mice, and this was reduced in SERT^{-/-} mice to 0.03 % (SD 0.02) ($P < 0.0001$, unpaired t-test) (figure 1B). The molar ratio of noradrenaline to adrenaline was not changed ($P = 0.84$, unpaired t-test) and there was a modest increase for dopamine ($P = 0.004$, unpaired t-test). To summarize, these data showed that the 5-HT content of the adrenal gland was very small compared to adrenaline (0.13%). In SERT^{-/-} mice, 5-HT was dramatically reduced but there was no significant change in catecholamines (or the overall monoamine content).

3.2. The serotonin transporter modulates stimulus-secretion coupling in adrenal chromaffin cells.

We used carbon fibre amperometry to determine if / how serotonergic signaling regulates stimulus-secretion coupling in cells isolated from wild-type and SERT^{-/-} mice. The carbon fibre electrode was positioned immediately adjacent to a chromaffin cell and secretion evoked by continuously perfusing the cell with 30mM KCl, a stimulus that evokes similar secretion to stress-like firing patterns (Fulop and Smith, 2007) (figure 2A). KCl depolarizes the cell, leading to Ca²⁺ entry through voltage-gated Ca²⁺ channels and triggering of exocytosis. Each upward “spike” on the current trace reflects oxidation of the monoamine transmitter released from a single secretory vesicle (primarily adrenaline and noradrenaline) (figure 2A). First we analyzed the number of spikes (vesicular fusion events) evoked by KCl. Both wild-type and SERT^{-/-} chromaffin cells displayed robust secretory responses: in the absence of 5-HT, KCl evoked 95 ± 10 spikes in wild-type cells ($n = 24$) and 84 ± 8 spikes in SERT^{-/-} cells ($n = 35$) (figure 2B, D). 5-HT (25nM or 1μM) did not significantly change the number of spikes evoked by KCl in wild-type cells (figure 2B) ($F = 0.32$, $P = 0.73$, one-way ANOVA), or the time-course of secretion ($F = 0.69$, $P = 0.41$, two-way ANOVA) (figure 2C). However, in SERT^{-/-} cells 5-HT did significantly reduce the number of spikes evoked by KCl (figure 2D) ($F = 4.43$, $P = 0.015$, one-way ANOVA) and altered the time course of secretion ($F = 8.17$, $P = 0.006$, two-way ANOVA) (figure 2E).

We also analyzed the amount and kinetics of transmitter release from individual fusion events to determine if these were altered by SERT and/or 5-HT (figure 3). Various parameters (charge, amplitude, duration, and slope) were determined from non-overlapping spikes and a median value was calculated for each cell. These median spike parameters are plotted in figure 3. Prior to statistical comparison using two-way ANOVA, the data were log-transformed to normalize the distribution. These data revealed that 5-HT did not significantly influence spike charge, amplitude, duration, or slope in wild-type or SERT^{-/-} cells. However, SERT genotype did influence some spike parameters; spike charge (integral) (figure 3A) is directly proportional to the number of oxidizable transmitter molecules released from a single vesicle (i.e. “quantal size”). In control conditions (no 5-HT present) the median spike charge was $\approx 35\%$ smaller in SERT^{-/-} cells compared to wild-type cells (0.17 [0.11, 0.21] pC; $n = 35$ compared to 0.26 [0.18 - 0.33] pC; $n = 24$) (median [interquartile range]). Statistical analysis confirmed that spike charge was influenced by SERT genotype, but not by 5-HT (for genotype $F = 42.2$, $P < 0.0001$; for 5-HT $F = 0.01$, $P = 0.94$, two-way ANOVA). Pairwise comparison using Bonferroni post-tests also found no

statistically significant effect of 5-HT in either genotype (figure 3A). The peak amplitude of the spikes (which reflects the maximal concentration of catecholamine at the electrode tip) was smaller in SERT^{-/-} cells but was not influenced by 5-HT (for genotype $F = 19.5$, $P < 0.0001$; for 5-HT $F = 0.77$, $P = 0.38$; two-way ANOVA). Pairwise comparison using Bonferroni post-tests found no statistically significant effect ($P > 0.05$) of 5-HT in either genotype (figure 3B). The duration of spikes at half maximal amplitude (“half-width”) was shorter in SERT^{-/-} cells, and statistical comparison revealed it was influenced by genotype and 5-HT (for genotype $F = 16.48$, $P < 0.0001$; for 5-HT $F = 4.55$, $P = 0.04$; two-way ANOVA). However, direct pairwise comparison using Bonferroni post-tests found no statistically significant difference between control and 5-HT treated cells in either genotype (figure 3C). The rising slope of the spike (initial rate of transmitter release) was not significantly influenced by genotype or 5-HT (for genotype $F = 0.28$, $P = 0.60$; for 5-HT $F = 2.81$, $P = 0.10$; two-way ANOVA). Pairwise comparison using Bonferroni post-test found no statistically significant difference between control conditions and 5-HT for either genotype (figure 3D).

To summarize, these data (figures 2 and 3) suggest that SERT has two distinct effects on secretion from adrenal chromaffin cells. In the absence of extracellular 5-HT, loss of SERT *per se* (SERT^{-/-} cells) did not alter the number of amperometric spikes evoked by KCl. However, loss of SERT did reduce the charge, amplitude, and duration of individual spikes. Application of extracellular 5-HT (1 μ M) had no effect on individual spike properties (charge, amplitude, slope, duration) in cells from either genotype, but did significantly reduce the number of spikes released from SERT^{-/-} cells. This latter effect suggests that SERT function in wild-type cells opposes the ability of extracellular 5-HT to inhibit the number of amperometric spikes (vesicular fusion events).

3.3. Acute pharmacological block of SERT unmasks a 5-HT receptor-mediated inhibition of secretion in wild-type chromaffin cells

As described in the results above (section 3.2, figure 2), 1 μ M 5-HT significantly reduced the number of spikes evoked by KCl in SERT^{-/-} cells (figure 2D). It is well established that G protein coupled receptors (GPCRs) can regulate neurotransmitter and hormone release, and in particular receptors that couple to pertussis toxin sensitive ($G_{i/o}$ -type) G proteins inhibit secretion (Betke et al., 2012; Currie, 2010). To test the involvement of this pathway we pretreated SERT^{-/-} cells with pertussis toxin (300ng/ml for ~18-24 hours) and found that this prevented the inhibition of spike number by 1 μ M 5-HT: the number of spikes evoked by KCl in the absence of 5-HT was 64 ± 12 ($n = 10$) and in the presence of 5-HT (1 μ M) was 77 ± 15 ($n = 10$) ($P = 0.49$, unpaired t-test).

This effect of pertussis toxin suggested that a $G_{i/o}$ -coupled receptor mediated the inhibition of secretion by 5-HT in SERT^{-/-} cells. Compensatory changes in 5-HT receptor expression have been reported previously in the CNS of SERT^{-/-} mice (Fabre et al., 2000; Li et al., 2000). Thus, one explanation for why 5-HT inhibited secretion in SERT^{-/-} but not wild-type cells could be upregulation of receptor expression in the SERT^{-/-} mice. Alternatively, SERT function in the wild-type cells could control / prevent the receptor-mediated inhibition. To distinguish these possibilities, we pharmacologically blocked SERT in wild-type cells to

determine if this unmasked a serotonergic inhibition of secretion (figure 4A, B). Acute application of 1 μ M escitalopram, a selective serotonin reuptake inhibitor, had no effect on secretion when applied alone (absence of 5-HT). However, the combined presence of 5-HT (25 nM) and escitalopram significantly reduced the number of spikes evoked by KCl in wild-type cells ($F = 3.42$, $P = 0.02$, one-way ANOVA) (figure 4A). Spike charge was not significantly altered (figure 4A). Thus, acute pharmacological block of SERT in wild-type cells revealed an inhibition of spike number by 5-HT similar to that seen in cells isolated from SERT^{-/-} mice. To summarize, these data are consistent with 5-HT acting via a G_{i/o}-coupled receptor to inhibit the number of amperometric spikes (vesicular fusion events). SERT function in wild-type cells restricts this receptor-mediated inhibition.

3.4. Pharmacological evidence that 5-HT_{1A} receptors inhibit secretion

The 5-HT₁ family of receptors couple to G_{i/o}-type G proteins, and it is well established that somatodendritic 5-HT_{1A} receptors and presynaptic 5-HT_{1B} receptors can inhibit serotonergic transmission in the CNS (Bayliss et al., 1997a; Daws et al., 2000; Hagan et al., 2012; Maejima et al., 2013; McDevitt and Neumaier, 2011; Penington et al., 1993). To test the involvement of 5-HT_{1A} receptors, wild-type cells were recorded in the presence of WAY100635 (25nM), a selective 5-HT_{1A} receptor antagonist. WAY100635 prevented the inhibition of secretion produced by combined 5-HT and escitalopram treatment (figure 4B). In another series of experiments 8-OH-DPAT (300nM), a selective 5-HT_{1A} receptor agonist, inhibited the number of spikes evoked by KCl in wild-type cells, but CP93129 (300 nM), a selective 5-HT_{1B} agonist, had no effect ($F = 6.22$, $P = 0.003$, one-way ANOVA) (figure 5A). 8-OH-DPAT (1 μ M) also significantly inhibited the number of spikes evoked by KCl in SERT^{-/-} cells ($P = 0.024$, unpaired t-test) (figure 5B). Spike charge was not significantly altered in wild-type cells ($P = 0.70$, Kruskal-Wallis test) or SERT^{-/-} cells ($P = 0.26$, Mann-Whitney test) (figure 5C, D). To summarize, we show that activation of a 5-HT receptor (likely 5-HT_{1A}) can inhibit secretion (the number of spikes) from chromaffin cells, and SERT activity modulates the ability of 5-HT to recruit this pathway.

3.5. The inhibition of secretion by 5-HT receptors was not mediated by altered voltage-gated Ca²⁺ or K⁺ channel activity, or changes in intracellular [Ca²⁺]

Modulation of voltage-gated Ca²⁺ channels is a widespread mechanism by which GPCRs inhibit transmitter release. In adrenal chromaffin cells, several GPCRs including P2Y purinergic receptors inhibit Ca²⁺ channels and catecholamine secretion (Albillos et al., 1996; Currie and Fox, 1996; Powell et al., 2000). Therefore, we used amphotericin perforated whole-cell patch clamp recording to determine if 5-HT_{1A} receptors modulated voltage-gated Ca²⁺ channel currents (I_{Ca}). I_{Ca} was evoked every 15s by a brief step depolarization from -80mV to 0mV (the peak of the I-V relationship) and each cell was sequentially exposed to 8-OH-DPAT (300nM) and then the P2Y receptor agonist ATP (100 μ M) which served as a positive control (figure 6A). 8-OH-DPAT had no effect on I_{Ca} in either wild-type or SERT^{-/-} cells, while ATP (the positive control) produced a significant inhibition (~ 32 – 36 %) in both genotypes (figure 6B). We also found no effect of 5-HT (1 μ M) on I_{Ca} in SERT^{-/-} cells, or in wild-type cells either in the absence or presence of escitalopram (1 μ M) (figure 6C). G protein activated inwardly rectifying K⁺ channels (GIRK channels) are another well-known target of G_{i/o}-coupled GPCRs and G $\beta\gamma$ signaling. In serotonergic neurons, 5-HT_{1A} receptors

activate GIRK channels to reduce membrane excitability and action potential firing (Bayliss et al., 1997a; Penington et al., 1993). However, we found no evidence for such an effect in chromaffin cells; 8-OH-DPAT did not change whole cell K^+ channel currents evoked over a wide voltage range (figure 6D, E).

To complement our patch clamp data, we used ratiometric Ca^{2+} imaging of Fura-2 loaded chromaffin cells isolated from wild-type mice (figure 7). The protocol mimicked that used to evoke secretion in the amperometry experiments: cells were exposed to 5-HT (1 - 10 μ M) for 5-minutes before (and during) stimulation with 30mM KCl (figure 7A). 5-HT did not change the baseline (resting) $[Ca^{2+}]$ or the KCl-evoked increase in $[Ca^{2+}]_i$ (figure 7B). We repeated the experiment in the presence of escitalopram (1 μ M). While the combined presence of 5-HT and escitalopram inhibited secretion (figure 4), this drug combination did not significantly alter baseline $[Ca^{2+}]$ or the Ca^{2+} response to 30mM KCl (figure 7C).

As the 5-HT receptor mediated inhibition did not involve the typical $G\beta\gamma$ targets (i.e. Ca^{2+} channels or K^+ channels) we investigated the impact of gallein, a small molecule reported to block $G\beta\gamma$ interaction with downstream effectors (Lehmann et al., 2008). Wild-type chromaffin cells were preincubated for 10 minutes with vehicle (DMSO) or gallein (10 μ M). The cells were then treated for an additional 5 minutes $\pm$ 8-OH-DPAT (300 nM). Consistent with our previous data (figure 5), 8-OH-DPAT (300 nM) significantly inhibited the number of amperometric spikes evoked by KCl in DMSO treated control cells ($P = 0.01$, unpaired t-test) and had no effect on spike charge (figure 8B). This inhibition was abolished in cells treated with gallein ($P = 0.19$, unpaired t-test) (figure 8C), thus implicating $G\beta\gamma$ signaling. To summarize, these data (figures 6-8) suggest that 5-HT_{1A} receptors signal via $G\beta\gamma$ to inhibit secretion (the number of vesicular fusion events), but this inhibition is unusual as it is not mediated via the typical targets, voltage-gated Ca^{2+} channels or K^+ channels.

4. DISCUSSION

4.1. SERT confers a serotonergic phenotype onto adrenal chromaffin cells but does not influence catecholamine content

Adrenal chromaffin cells synthesize and release catecholamines (primarily adrenaline and noradrenaline) and neuropeptides to mediate the physiological response to stress. Even though they lack the rate limiting enzyme for 5-HT synthesis, tryptophan hydroxylase, mammalian adrenal chromaffin cells contain small amounts of 5-HT, (Kent and Coupland, 1984; Linder et al., 2009; Verhofstad and Jonsson, 1983). It has been proposed that this 5-HT is accumulated by uptake via SERT which is highly expressed in the adrenal medulla (specifically in chromaffin cells) but not in the adrenal cortex (Schroeter et al., 1997). We confirmed that 5-HT was present in adrenal glands isolated from wild-type mice at $\approx$ 750 fold lower abundance than adrenaline (figure 1). In glands isolated from SERT^{-/-} mice, 5-HT was reduced by $\approx$ 80% but there was no change in the catecholamine content (figure 1). This shows that loss of SERT had no effect on the synthesis or storage of adrenal catecholamines, consistent with previously published work (Linder et al., 2009; Tjurmina et al., 2002).

4.2. SERT modulates the quantal size of vesicular fusion events

Amperometric spikes from SERT^{-/-} cells had significantly smaller charge, amplitude, and duration than wild-type cells (figure 3). Spike charge is directly proportional to the number of oxidizable transmitter molecules released by a single vesicular fusion event and was $\approx 35\%$ smaller in SERT^{-/-} cells (figure 3A). This was a surprisingly large difference because the catecholamine content in SERT^{-/-} mice was not altered, and although the 5-HT content was reduced by $\approx 80\%$, it comprised only a small fraction of the total monoamines even in wild-type glands (≈ 750 fold lower abundance than adrenaline) (figure 1). Even accounting for the fact that up to four electrons can be generated for each molecule of 5-HT that is oxidized compared to two electrons for each molecule of catecholamine that is oxidized (Bruns and Jahn, 1995), the decrease in spike charge is much larger than expected based on loss of 5-HT alone. The difference in spike charge, duration and amplitude was related to SERT genotype but there was no effect of 5-HT or the 5-HT_{1A} agonist 8-OH-DPAT on these spike parameters (figures 3, 5). Therefore, the change in spike parameters was distinct from the inhibition of spike number by 5-HT receptor activation (see below; section 4.3.). It is possible that loss of SERT (and thus intracellular 5-HT) favors exocytosis of a distinct subset of secretory vesicles, or partial rather than complete emptying of the vesicular monoamine content as reported for different peptide cargos (Weiss et al., 2014). Further investigations will be needed to elucidate the underlying mechanism(s) by which SERT controls quantal size.

4.3. 5-HT_{1A} receptors inhibit the number of amperometric spikes by an atypical mechanism which is modulated by SERT

Activation of 5-HT_{1A} receptors inhibited the number of amperometric spikes but had no effect on spike charge (figures 2, 4, 5). In wild-type cells, SERT function modulated this inhibition by opposing the action of 5-HT (figures 2, 4). The most straightforward interpretation is that SERT-mediated uptake of 5-HT precluded activation of nearby 5-HT receptors. This would require efficient uptake by SERT to reduce the local [5-HT] which might be favored by close co-localization and/or a relatively high abundance of SERT compared to 5-HT receptor expression. Other reciprocal interactions between SERT and 5-HT receptors are known to occur; for example, 5-HT_{1B} receptors can modulate SERT function in the brain (Daws et al., 2000; Hagan et al., 2012). Furthermore, in some cell types it has been reported that intracellular 5-HT can be covalently bound to target proteins (dubbed serotonylation) following uptake by SERT, and that this results in altered signaling (Muma and Mi, 2015; Walther et al., 2011).

The 5-HT_{1A} receptor-mediated inhibition seems to involve G $\beta\gamma$ signaling because it was prevented by treating cells with gallein, a small molecule blocker of some G $\beta\gamma$ -effector interactions (figure 8). Control of transmitter release by GPCRs is widespread in both the central and peripheral nervous systems and in most cases G_{i/o}-coupled GPCRs act through inhibition of I_{Ca} (Boehm and Kubista, 2002; Zamponi and Currie, 2013). Indeed, 5-HT receptors inhibit I_{Ca} in neurons (Bayliss et al., 1997b; Foehring, 1996; Penington and Kelly, 1990) and several GPCRs use this mechanism in adrenal chromaffin cells, including P2Y purinergic, μ -opioid, prostaglandin EP3, and alpha adrenergic receptors (Albillos et al., 1996; Brede et al., 2003; Carabelli et al., 1998; Currie and Fox, 1996; Jewell et al., 2011).

However, we found the 5-HT_{1A} receptor had no effect on I_{Ca} in chromaffin cells (figure 6), and also had no effect on GIRK channels (figure 6) which help control membrane excitability in the CNS (Courtney and Ford, 2016; Luscher et al., 1997; Penington et al., 1993). Using fluorescent Ca²⁺ imaging we confirmed that Ca²⁺ entry was unaltered and intracellular Ca²⁺ handling (clearance, release from intracellular stores, etc.) was not perturbed (figure 7). The most common pathway by which GPCRs inhibit I_{Ca} involves direct binding of Gβγ to the channels (Zamponi and Currie, 2013). This requires close co-localization of the channels and GPCRs, so it is possible that a lack of physical proximity or specific orientation within a macromolecular complex could account for why the 5-HT receptors did not inhibit I_{Ca} (see (Hamid et al., 2014)).

Gβγ can inhibit secretion independently from calcium channel regulation in chromaffin cells (Chen et al., 2005; Yoon et al., 2008), although spike charge was reduced in those previous papers which was not the case with the 5-HT_{1A} receptors. The “downstream” target(s) of Gβγ remains uncertain but might include the SNARE proteins of the exocytotic fusion machinery (Betke et al., 2012). It is unusual that the 5-HT_{1A} receptors act exclusively by the “downstream” mechanism, because other inhibitory GPCRs in chromaffin cells target I_{Ca} . It is worth noting that Gβγ-mediated inhibition of I_{Ca} is voltage-dependent and can be reversed by strong depolarization / repetitive firing, thereby diminishing the inhibitory effect on transmitter release / (Currie and Fox, 2002; Zamponi and Currie, 2013) .. As the inhibition by 5-HT receptors does not target I_{Ca} , it might persist even during strong, sustained depolarization as might occur during stress.

4.4. Possible future directions and physiological / pharmacological relevance

In this paper we identify a functional interplay between SERT and 5-HT receptors that controls stimulus-secretion coupling. This provides the foundation for future investigations and raises the possibility that adrenal chromaffin cells are a previously unrecognized hub for serotonergic control of sympathetic stress responses. One question to consider is the source of 5-HT that might activate the 5-HT_{1A} receptors *in vivo*. Adrenal chromaffin cells themselves contain small amounts of 5-HT due to SERT-mediated uptake, so this 5-HT could act in an autocrine feedback pathway. The pathway might become more prominent when strong stimulation (stress) enhances chromaffin cell exocytosis and the local 5-HT concentration increases to levels that overwhelm SERT-mediated reuptake. Other sources could contribute to changes in the local 5-HT content and chromaffin cells might detect and respond to some or all of these sources of 5-HT depending on the physiological context. Blood contains large amounts of 5-HT, most of which is sequestered into platelets (Watts et al., 2012). There are also resident mast cells in the adrenal gland which synthesize and release 5-HT (Lefebvre et al., 2001). Although innervation of chromaffin cells is primarily by cholinergic preganglionic splanchnic nerve terminals, there is also some sensory and vagal innervation (Parker et al., 1993). So, it is possible that a subset of these neurons might contain 5-HT. Acute block by SSRIs might be expected to enhance inhibition by favoring 5-HT_{1A} receptor activation (regardless of where the 5-HT is coming from). This enhanced inhibition might persist with chronic block of SERT, but the 5-HT stores in adrenal chromaffin cells might be depleted (as seen in the SERT^{-/-} mice) which would ultimately reduce or prevent the autocrine feedback loop.

Under physiological conditions SERT function will not be fully ablated as it is in SERT^{-/-} mice. However, the expression and / or function of SERT can be altered by genetic polymorphisms, promoter variants, and cell signaling pathways (Baganz and Blakely, 2013; Daws and Gould, 2011; Murphy and Lesch, 2008). From a pharmacological perspective, clinically relevant doses of selective serotonin reuptake inhibitors (SSRIs), which are widely prescribed for depression and anxiety disorders, result in ~80 - 90 % occupancy of SERT in the CNS (Meyer et al., 2004; Owens et al., 2008). It will be of interest to determine how these (patho)physiologically relevant perturbations of SERT impact sympathoadrenal function both *in vivo* and at the cellular / mechanistic level. Heterozygous SERT^{+/-} mice are a useful model of the reduced protein expression seen with some promoter variants (Murphy and Lesch, 2008), and development of floxed mice enabling tissue specific excision of SERT (Chen et al., 2015) is another exciting prospect that could help dissect the impact of serotonergic signaling on peripheral sympathetic nervous system function.

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Abbreviations

8-OH-DPAT	7-(dipropylamino)-5,6,7,8-tetrahydronaphthalen-1-ol
DMSO	dimethyl sulfoxide
GIRK channel	G protein activated inwardly rectifying potassium channel
GPCR	G protein coupled receptor
I_{Ca}	voltage-gated Ca ²⁺ channel current
SERT	serotonin transporter
SERT^{-/-}	SERT knockout mice
SSRI	selective serotonin reuptake inhibitor
TTX	tetrodotoxin

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Figure 1. 5-HT was dramatically reduced but catecholamines were unaltered in the adrenal gland of SERT^{-/-} mice

The monoamine content of whole adrenal glands from wild-type (WT; $n = 9$ mice) or SERT^{-/-} mice (KO; $n = 9$) was determined using HPLC. **A**) 5-HT, adrenaline (Adr), noradrenaline (NA), and dopamine content expressed as nmoles / mg total protein (box denotes interquartile range, line denotes median, whiskers denote standard deviation). The amounts of 5-HT and dopamine were much lower than adrenaline / noradrenaline (note change in scale). Glands from SERT^{-/-} mice had dramatically reduced 5-HT but no change in catecholamines (*** $P < 0.0001$; ns = not significantly different: Adr, $P = 0.39$; NA, $P = 0.65$; dopamine, $P = 0.07$; unpaired t-tests). **B**) The molar ratio of each monoamine relative to adrenaline (mean $\pm$ sem). Note the change in scale due to the much lower relative abundance of dopamine and 5-HT (ns , $P = 0.84$; ** $P = 0.004$; *** $P < 0.0001$; unpaired t-tests).

Figure 2. The number of amperometric spikes evoked by KCl was modulated by 5-HT in a SERT-dependent manner

A) Chromaffin cells isolated from wild-type or SERT^{-/-} mice were pretreated for 5 minutes in the absence (ctl) or presence of 5-HT (25nM or 1μM) before secretion was evoked by 30mM KCl (60 s stimulation). Amperometry recordings are shown from a representative wild-type (left) and SERT^{-/-} chromaffin cell (right trace), both in the presence of 1μM 5-HT. Some examples of individual spikes from a wild-type cell are shown on an expanded time scale. **B)** The total number of spikes evoked by KCl in wild-type cells was not altered by 5-HT ($F = 0.32$, $P = 0.73$, one-way ANOVA). **C)** Time-course of secretion (cumulative number of amperometric spikes grouped by 10s bins) from wild-type cells in the absence (ctl) or presence of 5-HT (1μM) (mean $\pm$ sem) (for 5-HT, $F = 0.69$, $P = 0.41$, two-way ANOVA). **D)** The total number of spikes evoked by KCl in SERT^{-/-} cells was significantly reduced by 5-HT ($F = 4.43$, $P = 0.01$, one-way ANOVA; “ns” denotes not significant, ** $P < 0.01$, Bonferroni multiple comparison test compared to control). **E)** Time-course of secretion from SERT^{-/-} cells in the absence (ctl) or presence of 5-HT (1μM) (for 5-HT, $F = 8.17$, $P = 0.006$, two-way ANOVA).

Figure 3. SERT genotype, but not the presence of 5-HT, altered the charge and kinetics of amperometric spikes

Amperometry spike parameters were analyzed from the same cells as figure 2 (control conditions or in the presence of 1 μ M 5-HT). The median spike parameters for each cell were calculated and shown in the box plots (25%, median, 75%; whiskers denote 10% and 90%). Statistical comparison was performed using two-way ANOVA (genotype and 5-HT) with pairwise comparison using Bonferroni post-tests. The number of cells in each group was: wild-type control = 24; wild-type 5-HT = 22; SERT^{-/-} control = 35; SERT^{-/-} 5-HT = 27. **A)** Spike charge was smaller in SERT^{-/-} cells than in wild-type cells, but was not altered by 5-HT (two-way ANOVA: for genotype $F = 42.2$, *** $P < 0.0001$; for 5-HT $F = 0.01$, $P = 0.94$) (“ns” not significant, Bonferroni post-test). **B)** Spike amplitude was smaller in SERT^{-/-} cells than in wild-type cells, but was not influenced by 5-HT (two-way ANOVA: for genotype $F = 19.5$, *** $P < 0.0001$; for 5-HT $F = 0.77$, $P = 0.38$) (“ns” not significant, Bonferroni post-test). **C)** The half-width (spike duration at half maximal amplitude) was shorter in SERT^{-/-} cells than in wild-type cells and 5-HT had a modest influence (two-way ANOVA: for genotype $F = 16.48$, *** $P < 0.0001$; for 5-HT $F = 4.55$, $P = 0.04$). Pairwise comparison using Bonferroni post-tests found no statistically significant difference between control and 5-HT treated cells in either genotype (“ns” not significant, Bonferroni post-test). **D)** Spike slope was not significantly altered by genotype or 5-HT (two-way ANOVA: for genotype $F = 0.28$, $P = 0.60$; for 5-HT $F = 2.81$, $P = 0.10$) (“ns” not significant, Bonferroni post-test).

Figure 4. SERT function opposed a 5-HT receptor-mediated inhibition of secretion in wild-type chromaffin cells

The experimental paradigm is depicted at the top of each panel. **A) Left Panel:** Escitalopram (1 μ M) alone had no effect on the number of amperometric spikes evoked by KCl, but combined with 5-HT (25nM) produced a significant inhibition ($F = 3.42$, $P = 0.02$, one-way ANOVA; “ns” not significant, ** $P < 0.01$, Bonferroni post-tests). **Right Panel:** Spike charge was not significantly altered by drug treatments ($P = 0.22$, Kruskal-Wallis test; “ns” not significant, Dunn’s multiple comparison post-test). **B) The same experiment was repeated in the presence of the 5-HT_{1A} receptor antagonist WAY100635 (25nM). Left Panel:** WAY100635 prevented the inhibition of spike number produced by escitalopram + 5-HT ($F = 1.55$, $P = 0.21$, one-way ANOVA; “ns” not significant, Bonferroni post-test). **Right Panel:** Spike charge was not significantly altered by drug treatments in the presence of WAY100635 ($P = 0.61$, Kruskal-Wallis test; “ns” not significant, Dunn’s multiple comparison post-test).

Figure 5. The 5-HT_{1A} receptor agonist 8-OH-DPAT inhibited the number of evoked amperometric spikes

The upper panel depicts the experimental paradigm. Cells were perfused with NaCl-based extracellular solution in the absence (control) or presence of 8-OH-DPAT (a 5-HT_{1A} receptor agonist) or CP93129 (5-HT_{1B} receptor agonist). Secretion was then evoked by perfusing the cells for 60s with KCl in the continued absence (controls) or presence of the agonists. **A)** The 5-HT_{1A} receptor agonist 8-OH-DPAT (300 nM) significantly reduced the number of amperometry spikes evoked by KCl in wild-type cells, but the 5-HT_{1B} receptor agonist CP93129 (300 nM) had no effect ($F = 6.22$, $P = 0.003$, one-way ANOVA) (“n.s.” not significant; ** $P < 0.01$ for multiple pairwise comparisons to control cells using Dunnett’s post-test). **B)** 8-OH-DPAT significantly reduced the number of KCl-evoked spikes in SERT^{-/-} chromaffin cells, (* $P = 0.024$, unpaired t-test). **C)** Spike charge was not significantly altered by 8-OH-DPAT or CP93129 in wild-type cells ($P = 0.70$, Kruskal-Wallis test; “ns” not significant, Dunn’s multiple comparison post-test). **D)** Spike charge was not significantly altered by 8-OH-DPAT in SERT^{-/-} cells ($P = 0.26$, Mann-Whitney test).

Figure 6. Voltage-gated Ca^{2+} channel or K^{+} channel currents were not modulated by 5-HT or the 5-HT_{1A} receptor agonist 8-OH-DPAT

A) Amphotericin perforated whole-cell patch clamp recording of voltage-gated Ca^{2+} channel currents (I_{Ca}). Peak amplitude of I_{Ca} is plotted against time from a representative wild-type cell. The inset shows I_{Ca} corresponding to the numbered points. 8-OH-DPAT (DPAT) (300 nM) had no effect on I_{Ca} , but ATP (100 μM) (a P2Y purinergic receptor agonist) reversibly inhibited I_{Ca} . **B)** Pooled data (mean $\pm$ sem) showing the fractional change in I_{Ca} amplitude for wild-type cells ($n = 6$) and $\text{SERT}^{-/-}$ cells ($n = 5$). 8-OH-DPAT had no effect while ATP significantly inhibited I_{Ca} in wild-type cells ($F = 30.47$, $P < 0.0001$, repeated measures ANOVA; “ns”, not significant, *** $P < 0.0001$ using Bonferroni post-test) and $\text{SERT}^{-/-}$ cells ($F = 20.47$, $P < 0.0001$, repeated measures ANOVA; “ns”, not significant, *** $P < 0.0001$ using Bonferroni post-test). **C)** Pooled data (mean $\pm$ sem) showing the fractional change in whole-cell I_{Ca} amplitude for wild-type cells (WT) exposed to 1 μM 5-HT ($n = 5$) or 5-HT + escitalopram (1 μM) ($n = 4$) and $\text{SERT}^{-/-}$ cells exposed to 1 μM 5-HT ($n = 8$). None of the drugs produced a significant change in I_{Ca} amplitude (ns, not significant compared to within cell controls, paired t-test). **D)** Representative amphotericin perforated whole-cell patch clamp recording of K^{+} channel currents (I_{K}) from a wild-type chromaffin cell. I_{K} were evoked by steps to +80 mV in the absence (control) and then in the presence of 8-OH-DPAT (300 nM). **E)** Current density plotted against voltage (inset shows stimulus protocol) showing no effect of 8-OH-DPAT (two-way repeated measures ANOVA; for 8-OH-DPAT treatment $F = 0.11$, $P = 0.75$; $n = 6$).

Figure 7. 5-HT did not alter resting or KCl-evoked intracellular $[Ca^{2+}]$

Time-course showing intracellular $[Ca^{2+}]$ monitored in FURA-2 loaded chromaffin cells isolated from wild-type mice (mean $\pm$ sem; $n = 61$ cells). The experimental paradigm mimicked that used to evoke catecholamine secretion in previous amperometry experiments (60s stimulation with 30mM KCl after 5 min exposure to 1 μ M 5-HT). Labels: “a” = baseline before application of 5-HT; “b” = baseline at the end of the 5 min drug exposure; “peak” = maximal response to KCl; “end” = response at the end of KCl stimulation. **B) Left panel:** 5-HT had no effect on baseline $[Ca^{2+}]$. Intracellular $[Ca^{2+}]$ before (“a”) and during (“b”) 5-HT exposure for three experimental groups (see labels “a” and “b” on panel-A): controls (0 μ M 5-HT; $n = 53$; “ns” denotes $P = 0.24$, paired t-test); 1 μ M 5-HT ($n = 61$; “ns” denotes $P = 0.39$, paired t-test); 10 μ M 5-HT ($n = 28$; “ns” denotes $P = 0.06$, paired t-test). **Right panel:** 5-HT did not significantly change $[Ca^{2+}]$ at the “peak” or “end” of the KCl response (“ns” denotes not significant, one-way ANOVA: “Peak” response, $F = 0.56$, $P = 0.58$; “End” response, $F = 2.93$, $P = 0.06$). **C) The same experimental paradigm as in panels A and B except cells were exposed to 1 μ M escitalopram (escital $n = 30$) or a combination of 1 μ M escitalopram + 1 μ M 5-HT (escit + 5-HT, $n = 38$) for 5 minutes prior to KCl. **Left panel:** Baseline $[Ca^{2+}]$ was not altered by escitalopram ($P = 0.62$, paired t-test) or by escitalopram + 5-HT ($P = 0.99$, paired t-test). **Right panel:** Intracellular $[Ca^{2+}]$ at the “peak” and “end” of the KCl response in the presence of escitalopram alone or escitalopram $\pm$ 5-HT (“ns”, no significant difference, paired t-test; peak, $P = 0.06$; end, $P = 0.22$).**

Figure 8. The 5-HT receptor-mediated inhibition of secretion was prevented by gallein, an antagonist of G $\beta\gamma$ signaling

A) Depicts the experimental paradigm. Chromaffin cells from wild-type mice were pretreated with DMSO (vehicle controls) or gallein (10 μ M) and stimulated with 30mM KCl in the absence or presence of 8-OH-DPAT (300 nM). Secretion was detected using carbon fiber amperometry. **B)** In DMSO treated cells, 8-OH-DPAT (DPAT) significantly inhibited the number of amperometric spikes evoked by KCl compared to matched controls (ctl) (* P = 0.01, unpaired t-test). There was no effect on spike charge (“ns” P = 0.57, Mann-Whitney test). **C)** Gallein treatment prevented the inhibition of spike number by 8-OH-DPAT (“ns”, P = 0.19, unpaired t-test). There was no change in spike charge (“ns” P = 0.56, Mann-Whitney test).