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Glucocorticoid-induced, rapid serotonin release by serotonergic neurons *in vitro*

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Nicolas Paul contributed to the following:

- Performing experiments for the colocalization of GR/synaptotagmin 1, FFN511 live cell imaging and FM4-64FX staining after treatment with potassium chloride and dexamethasone.
- Analyzing and visualizing data of the colocalization of GR/synaptotagmin 1, GR/5-HT and GR/FM4-64FX, of the live cell imaging, and of the immunofluorescences. The data of the colocalization of GR/synaptotagmin 1 are presented in both, the publication and this dissertation thesis.
- Preparing the manuscript.
- Preparing Figures 1 to 5, Supplements 1 and 2 as well as the graphical abstract.
- Submitting the final manuscript.
- Preparing revisions and rebuttal letters.

Table of Contents

Abbreviations III

Figures IV

1 Introduction 1

2 Background 3

2.1 Stress and the hypothalamus-pituitary-adrenal axis 3

2.2 The serotonergic system 5

2.3 Interactions between stress and the serotonergic system 8

3 Aims of this work 10

3.1 Mechanism of rapid serotonin release after glucocorticoid receptor activation 10

3.2 Interaction between glucocorticoid receptor and synaptic structures 10

3.3 Interaction between activated glucocorticoid receptor and the serotonin transporter 11

4 Materials 12

4.1 Consumables and chemicals 12

4.2 Stock solutions 14

4.3 Cell culture 14

4.4 FFN511 life cell imaging of 1C11-5HT 14

4.5 FM4-64FX staining of 1C11-5HT 15

4.6 Subcellular fractionation and detection of glucocorticoid receptor and synaptic markers 15

4.7 Immunofluorescence: Colocalization of glucocorticoid receptor and synaptotagmin 1 in 1C11-5HT 16

4.8 Immunofluorescence: Colocalization of glucocorticoid receptor and the serotonin transporter in 1C11-5HT 16

5 Methods 17

5.1 Culture and differentiation of 1C11 17

5.2 Confocal laser scanning microscopy 18

5.3 Colocalization analysis in fluorescent microscopy 19
5.4 FFN511 life cell imaging of 1C11-5HT 21
5.5 FM4-64FX staining of 1C11-5HT 22
5.6 Subcellular fractionation and detection of glucocorticoid receptor and synaptic markers 23
5.7 Immunofluorescence: Colocalization of glucocorticoid receptor and synaptotagmin 1 in 1C11-5HT 25
5.8 Immunofluorescence: Colocalization of glucocorticoid receptor and the serotonin transporter in 1C11-5HT 25
5.9 Statistics 26

6 Results 27

6.1 FFN511 life cell imaging of 1C11-5HT 27
6.2 FM4-64FX staining of 1C11-5HT 29
6.3 Subcellular fractionation and detection of glucocorticoid receptor and synaptic markers 33
6.4 Immunofluorescence: Colocalization of glucocorticoid receptor and synaptotagmin 1 in 1C11-5HT 34
6.5 Immunofluorescence: Colocalization of glucocorticoid receptor and the serotonin transporter in 1C11-5HT 36

7 Discussion 39

7.1 Mechanism of rapid serotonin release after glucocorticoid receptor activation 39
7.2 Interaction between glucocorticoid receptor and synaptic structures 43
7.3 Interaction between activated glucocorticoid receptor and the serotonin transporter 46
7.4 Conclusions and areas of further research 48

8 Summary 50

9 Bibliography 52

10 Curriculum vitae 73

11 Acknowledgements 74

Abbreviations

1C11	Cell line of immortalized, murine teratocarcinoma cells
1C11-5HT	Serotonergic phenotype of 1C11
5-HT	5-hydroxytryptamine, serotonin
5-HT1-7	Serotonin receptors 1-7
ACTH	Adrenocorticotropic hormone
cAMP	Cyclic adenosine monophosphate
CLSM	Confocal laser scanning microscopy
CNS	Central nervous system
CRH	Corticotropin-releasing hormone
db cAMP	N^6,2'-O-dibutyryladenosine 3',5'-cyclic monophosphate
ddH_2O	Double-distilled water
Dex	Dexamethasone
DRN	Dorsal raphé nuclei
GR	Glucocorticoid receptor
HPA axis	Hypothalamic-pituitary-adrenal axis
hSERT	Human serotonin transporter
KCl	Potassium chloride
Mif	Mifepristone
PCC	Pearson's correlation coefficient
ROI	Region of interest
RT	Room temperature
SERT	Serotonin transporter
WT	Wild type

Figures

Figure 1: Serotonergic neurons and their projections in the central nervous system. 5

Figure 2: Differentiated 1C11-5HT. 18

Figure 3: Schematic imaging with confocal laser scanning microscopy. 19

Figure 4: Exemplary colocalization analysis. 21

Figure 5: FFN511 life cell imaging with 1C11-5HT before and after administration of potassium chloride or dexamethasone. 28

Figure 6: Signal intensities of 1C11-5HT loaded with FFN511 after administration of potassium chloride or dexamethasone. 29

Figure 7: 1C11-5HT stained with FM4-64FX after administration of dexamethasone or potassium chloride. 31

Figure 8: FM4-64FX uptake of (1) neurites and (2) somas in 1C11-5HT after administration of dexamethasone or potassium chloride. 32

Figure 9: Immunological detection of glucocorticoid receptor and synaptic markers in the subcellular fractions of central nervous systems of wild-type mice and mice carrying the human serotonin transporter. 34

Figure 10: Colocalization of glucocorticoid receptor and synaptotagmin 1 in neurites of 1C11-5HT. 35

Figure 11: Exemplary images of colocalization of the serotonin transporter and glucocorticoid receptor in neurites of 1C11-5HT. 37

Figure 12: Colocalization of membrane-bound serotonin transporter and glucocorticoid receptor in 1C11-5HT after administration of dexamethasone. 38

Figure 13: Schematic illustration of rapid, dexamethasone-induced release of serotonin. 40

Figure 14: Schematic illustration of the proximity between activated glucocorticoid receptor and the serotonin transporter. 47

1 Introduction

Depression is the most common affective disorder. Patients feel sad, empty, lose interest in almost all daily activities and frequently have suicidal thoughts (American Psychiatric Association, 2013). Symptoms cause distress, interfere with the patient's social or occupational functioning, and persist for at least two weeks (American Psychiatric Association, 2013). The overall lifetime prevalence of depression in the United States is 16.6 % (Kessler et al., 2005), and a survey from 2018 reported that 7.2 % of adults aged 18 and above had a depressive episode in the United States in the previous year (Substance Abuse and Mental Health Services Administration, 2019).

The disease's pathogenesis is not thoroughly understood. For decades, there has been the monoamine theory of depression (Meyer et al., 2006). It proposes the importance of low levels of monoamines, such as serotonin (5-HT), dopamine, and norepinephrine (Duman, Heninger, & Nestler, 1997; Schildkraut, 1965; Schildkraut & Kety, 1967). Particular focus of research has been on the role of 5-HT. Studies have reported lowered concentrations of 5-hydroxyindoleacetic acid, the metabolite of 5-HT degradation, in the cerebrospinal fluid of depressive patients (Asberg, Thoren, Traskman, Bertilsson, & Ringberger, 1976; Van Praag & Korf, 1971; Van Praag, Korf, & Puite, 1970), which correlates with low 5-hydroxyindoleacetic acid concentration in the brain (Mignot et al., 1985; Stanley, Traskman-Bendz, & Dorovini-Zis, 1985; Van Praag, 2004). The monoamine theory is supported by today's clinical practice, which evidences that symptoms of depression are lowered by substances that enhance monoamine levels. In Germany, these substances are recommended as part of the first-line treatment for patients with moderate or severe depression (Deutsche Gesellschaft für Psychiatrie und Psychotherapie, 2015).

It is unlikely that disturbances in the monoamine systems of the brain, particularly the 5-HT system, are solely responsible for the outbreak of a depressive episode. Additional factors come into play. Besides environmental and biographic aspects as well as a genetic vulnerability of the individual (Caspi & Moffitt, 2006; Chaouloff, 2000),

a special focus has been on the role of stress, which frequently precedes depression (Christine & Markus, 2006; Kendler, 1995; Lanfumey, Mongeau, Cohen-Salmon, & Hamon, 2008; Shelton, 2004; Van Praag, 2004). Individuals experiencing multiple stressful life events are more likely to become depressed (Brown, Bifulco, & Harris, 1987; Heim & Nemeroff, 2001), and depressive patients show hyperactivity of the hypothalamic-pituitary-adrenal axis (HPA axis) (Heuser, 1998), which mediates the body's stress response. Patients have insensitive feedback loops of the HPA axis, resulting in an overshooting cortisol response (Heuser, 1998; Holsboer, 1995), and an insufficient cortisol suppression after administration of the glucocorticoid receptor (GR) agonist dexamethasone (dex) (Carroll, 1982).

Bidirectional relations have been described between the 5-HT system of the central nervous system (CNS) and the body's stress response (Van Praag, 2004). Understanding the interplay between the two systems can help understand the underlying pathogenesis of psychiatric diseases such as depression. 5-HT triggers activity of the HPA axis (Dinan, 1996; R. W. Fuller, 1995; Lefebvre et al., 1992). In turn, stress elevates the turnover and synthesis of cerebral 5-HT (Christine & Markus, 2006; Davis, Stanford, & Heal, 1995; E. De Kloet et al., 1982; E. De Kloet, Versteeg, & Kovacs, 1983). Additionally, it has been shown that stress and the administration of glucocorticoids acutely enhance extracellular 5-HT levels in the hippocampus of rats and lizards (Barr & Forster, 2011; Rex, Voigt, & Fink, 2005; Summers et al., 2003; Wright, Upton, & Marsden, 1992). 5-HT then inhibits the HPA axis, therefore acting as a negative feedback loop (Nuller & Ostroumova, 1980; Van Praag, 2004). On a cellular level, however, it is unknown how glucocorticoids elevate extracellular 5-HT. To understand the interplay between glucocorticoids and 5-HT neurons, this thesis investigates the following question: *How do glucocorticoids increase extracellular 5-HT on a cellular level?*

Firstly, it is explored whether glucocorticoids trigger vesicular 5-HT release *in vitro*. Secondly, it is analyzed whether there is spatial proximity between the GR and synaptic release sites *in vivo* and *in vitro,* which is a prerequisite for a possible interaction. Thirdly, it is investigated if the GR has a spatial proximity to the serotonin

transporter (SERT), potentially altering 5-HT uptake. The thesis is structured as follows: In the background section, the body's stress response is explained, the brain's 5-HT system is described, and the interdependencies between the two systems are elicited. In the materials and methods section, the monoclonal 1C11 cell line is introduced, and confocal laser scanning microscopy (CLSM), colocalization analysis, as well as the experimental protocols for immunofluorescence, life cell imaging, and subcellular fractionation are described. In the subsequent sections, results of those experiments are shown, the implications of the results are discussed, and conclusions are drawn.

2 Background

2.1 Stress and the hypothalamus-pituitary-adrenal axis

Stress has been described as a *"nonspecific response of the body to any demand"* (Selye, 1976, p. 53). By its very nature, stress is nonspecific in its cause and triggers a nonspecific systemic response. Any change in the environment threatening the body's homeostasis can act as a stressor, which results in a stress response to restore homeostasis (Chrousos, 2009; Chrousos & Gold, 1992; Sapolsky, 2003). Among cardiovascular and behavioral changes, the HPA axis is activated, which consists of three components (Christine & Markus, 2006): (1) The paraventricular nucleus in the hypothalamus releases corticotropin-releasing hormone (CRH) and arginine-vasopressin (Lanfumey et al., 2008), which prompts the (2) anterior pituitary gland to release adrenocorticotropic hormone (ACTH) and other peptides from the precursor pro-opiomelanocortin (Christine & Markus, 2006; Lanfumey et al., 2008). (3) ACTH triggers release of the glucocorticoids cortisol in humans and corticosterone in rodents from the cortex of the adrenal gland (Christine & Markus, 2006). The stress response is terminated by negative feedback of glucocorticoids on the adrenal gland, anterior pituitary gland, hypothalamus, and hippocampus (Lanfumey et al., 2008).

Corticosteroids unfold their effects via the mineralocorticoid receptor (MR or type I) and the GR (type II) (E Ronald De Kloet, Vreugdenhil, Oitzl, & Joels, 1998; Gass, Reichardt, Strekalova, Henn, & Tronche, 2001). The MR binds to mineralocorticoids

such as aldosterone, but has an affinity to corticosterone ten times higher than the GR. As a result, basal corticosterone levels occupy 90 % of MR but only 10 % of GR (Lanfumey et al., 2008; J. M. H. M. Reul & De Kloet, 1986). The MR is responsible for a tonic inhibition of the HPA axis, whereas the GR inhibits the HPA axis during stress and circadian peaks (Bradbury, Akana, & Dallman, 1994; E Ronald De Kloet et al., 1998). Lipophilic corticosteroids transcend the cell membrane and bind to the nuclear MR and GR (Beato & Sánchez-Pacheco, 1996; Tasker, Di, & Malcher-Lopes, 2006). The receptors form complexes with heat shock proteins and immunophilin (Jenkins, Pullen, & Darimont, 2001), which dissociate upon glucocorticoid binding. The ligand-receptor complexes dimerize, translocate to the nucleus, and attach to glucocorticoid response element sequences of the DNA in the promotor regions of myriad genes, thereby positively or negatively influencing transcription (Stockner, Sterk, Kaptein, & Bonvin, 2003; Truss & Beato, 1993). Some of the corticosteroid-mediated effects are initiated too fast to be mediated by alterations of genomic expression. Therefore, it has been suggested that corticosteroids have rapid, non-genomic effects, potentially by interacting with target receptors located in or associated with the cell membrane (E. Ronald De Kloet, Karst, & Joëls, 2008; Groeneweg, Karst, de Kloet, & Joëls, 2012; Tasker et al., 2006).

The HPA axis and the body's stress response are altered in depressed patients (Heuser, 1998). Board, Persky and Hamburg (1956) observed hypercortisolemia, and Sachar et al. (1973) showed enhanced cortisol levels in depression throughout the day. Depressed patients also show a greater cortisol response to stressors (Van Praag, 2004), elevated ACTH plasma levels (Arborelius, Owens, Plotsky, & Nemeroff, 1999; Holsboer, 2000), and enhanced cortisol responsiveness of the adrenal gland to ACTH (Krishnan et al., 1991). The HPA axis becomes irresponsive to negative feedback mechanisms, which partially explains high CRH (Carroll, 1982). If the GR agonist dex is administered, it suppresses ACTH and cortisol release. Depressive patients require higher doses of dex to decrease plasma cortisol levels than healthy individuals (Modell, Yassouridis, Huber, & Holsboer, 1997). Interestingly, treatment with various antidepressants reverse alterations of the HPA axis parallel to the patient's clinical response (Heuser, 1998): GR gene expression is up-regulated (J. M.

Reul, Stec, Söder, & Holsboer, 1993) and the negative feedback of the HPA axis is strengthened, resulting in a decrease in CRH (De Bellis et al., 1999), ACTH and cortisol, and normalization of the dex suppression test (Van Praag, 2004; Zobel, Yassouridis, Frieboes, & Holsboer, 1999).

2.2 The serotonergic system

Neurons containing the monoamine neurotransmitter 5-HT are rare, with one per 1,000,000 neurons in rodents (Jacobs & Azmitia, 1992) and one per 172,000 neurons in humans (Azevedo et al., 2009; Hornung, 2003). Their somas are primarily located in the raphé nuclei, which is a collection of neurons around the midline of the rostrocaudal extension of the brain stem (Taber, Brodal, & Walberg, 1960) (**Figure 1**). The rostral group harbors 85 % of the brain's 5-HT neurons (Hornung, 2003), the caudal group makes up 15 %. The 5-HT system projects to various areas of the CNS (Hornung, 2003; Puig & Gulledge, 2011). Neurotransmission is either done by one-to-one chemical synapses or via diffuse, volume-based transmission at varicosities (Hornung, 2003). It modulates the perception of pain (Mason, 1999), thermoregulation, the circadian rhythm, appetite, behavior and learning (Lucki, 1998).

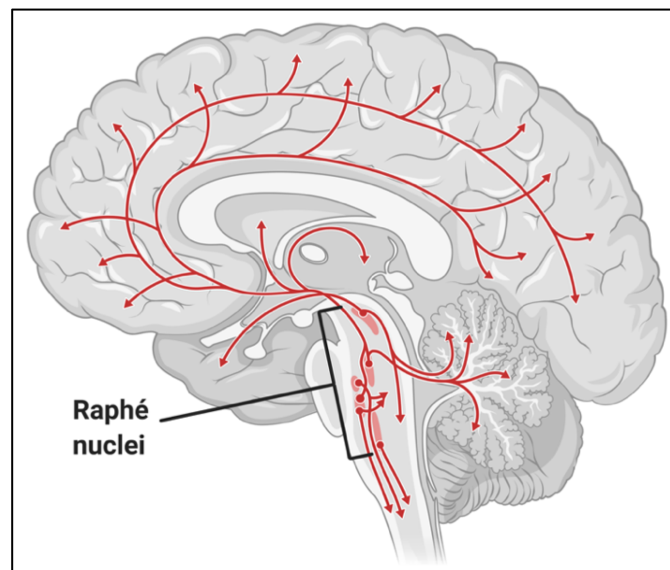

Figure 1: Serotonergic neurons and their projections in the central nervous system. Serotonergic neurons are primarily located in the raphé nuclei, from where they project to various areas of the central nervous system and the spinal cord. Own illustration created with BioRender.com.

5-HT is synthesized from L-tryptophan, which is hydroxylated by the tryptophan hydroxylase (Chen & Miller, 2012). In the second step, the aromatic amino acid decarboxylase catalyzes the synthesis of 5-HT (Walther & Bader, 2003). The vesicular monoamine transporter 2 pumps 5-HT from the cytosol proton-dependently into vesicles (Charnay & Léger, 2010). Mediated by calcium-influx and merge of the vesicles with the cell membrane, 5-HT is released into the synaptic cleft (Charnay & Léger, 2010). 5-HT either degrades or is ATP-dependently taken up into the nerve terminal by the SERT, where it is either metabolized by the monoamine oxidase or packed into vesicles by vesicular monoamine transporter 2 (Charnay & Léger, 2010). 5-HT receptors can be grouped in seven families (5-HT1–5-HT7) with more than 15 receptors (Charnay & Léger, 2010; Raymond et al., 2001). They are metabotropic receptors and act via G-proteins (Barnes & Sharp, 1999; Raymond et al., 2001). Only the 5-HT3 is ionotropic, increasing the membrane's permeability to potassium and sodium (Derkach, Surprenant, & North, 1989). All 5-HT receptors can be found throughout the brain (Barnes & Sharp, 1999), but a particular focus has been on the 5-HT1A, which acts as an autoreceptor and causes inhibitory feedback on 5-HT neurons (Lanfumey et al., 2008; Richer, Hen, & Blier, 2002).

The SERT regulates 5-HT neurotransmission, as it is responsible for the uptake of 5-HT (Kish et al., 2005). The SERT transporter belong to a larger family of neurotransmitter:sodium symporters (Nelson, 1998), has twelve transmembrane segments and multiple phosphorylation sites (K-P Lesch, Wolozin, Estler, Murphy, & Riederer, 1993; K. P. Lesch, Wolozin, Murphy, & Riederer, 1993). Using the transmembrane sodium gradient, one 5-HT molecule is transported with one sodium and one chloride molecule, one potassium molecule is effluxed (Rudnick & Clark, 1993). Amphetamines can reverse the transportation direction, resulting in an efflux of 5-HT (Sitte & Freissmuth, 2010). The SERT is expressed in 5-HT neurons, astrocytes and platelets (Mercado & Kilic, 2010; Pickel & Chan, 1999; Steiner, Carneiro, & Blakely, 2008). In neurons, the SERT is expressed in the area of synapses, on somas, on axons as well as dendrites outside of synaptic contacts (Matthäus et al., 2016; Miner, Schroeter, Blakely, & Sesack, 2000; Qian, Melikian, Rye, Levey, & Blakely, 1995; F. C. Zhou, Tao-Cheng, Segu, Patel, & Wang, 1998), and in intracellular

compartments (Qian et al., 1997; Tao-Cheng & Zhou, 1999; Whitworth, Herndon, & Quick, 2002).

SERT activity is modified by the dynamic variation of its localization, trafficking from the extracellular membrane to intracellular compartments and vice-versa (Steiner et al., 2008). The focal adhesion protein hic-5, the neuronal nitric oxide synthase 1, treatment with selective 5-HT reuptake inhibitors, α-synuclein, γ-synuclein, and secretory carrier membrane protein 2 interact with SERT and foster its internalization (Benmansour et al., 1999; Benmansour, Owens, Cecchi, Morilak, & Frazer, 2002; Carneiro & Blakely, 2006; Chanrion et al., 2007; Horschitz, Hummerich, & Schloss, 2001; Kittler, Lau, & Schloss, 2010; Lau, Horschitz, Bartsch, & Schloss, 2009; Lau, Horschitz, Berger, Bartsch, & Schloss, 2008; Müller, Wiborg, & Haase, 2006; Wersinger & Sidhu, 2009). Syntaxin 1A associates with SERT and determines whether 5-HT is moved electroneutrally coupled with ions or electrogenically (Quick, 2003). Further, SERT surface expression is down-regulated by binding of the active, GTP-binding form of rab4 (Ahmed et al., 2008). The α2 adrenergic receptor also causes a calcium-dependent downregulation of the SERT (Ansah, Ramamoorthy, Montañez, Daws, & Blakely, 2003). Activation of the protein kinase C for less than 5 min decreases SERT activity without affecting its cell surface expression, and activation of protein kinase C for more than 30 min decreases SERT surface expression (Jayanthi, Samuvel, Blakely, & Ramamoorthy, 2005). On the other hand, activation of the adenosine receptor (A3AR) recruits intracellular SERT to the cell membrane by a protein kinase G-linked pathway (Zhu, Hewlett, Feoktistov, Biaggioni, & Blakely, 2004; Zhu et al., 2007). By a different pathway, protein kinase G activates p38 mitogen-activated protein kinase (Zhu et al., 2004), which enhances the 5-HT affinity of the SERT, a process dependent on the activity of the protein phosphatase 2A (PP2A) (Zhu, Carneiro, Dostmann, Hewlett, & Blakely, 2005). Additionally, the integrin subunits αIIb and β3 enhance SERT's 5-HT affinity (Carneiro, Cook, Murphy, & Blakely, 2008).

Alterations of the 5-HT neurotransmitter system have been associated with psychiatric disorders such as panic disorder and schizophrenia (Lucki, 1998). In depression,

changes of the 5-HT neurotransmission concern the availability of tryptophan, 5-HT synthesis, release, metabolism, reuptake and 5-HT receptors (Lucki, 1998). Selective, tryptophan-depleted nutrition can cause a CNS 5-HT deficiency (Delgado, Charney, Price, Landis, & Heninger, 1989; Moja, Cipolla, Castoldi, & Tofanetti, 1989; Young, Smith, Pihl, & Ervin, 1985). This leads to mood lowering in healthy individuals with a family history of depression (Benkelfat, Ellenbogen, Dean, Palmour, & Young, 1994), and can cause a relapse in patients successfully treated for recent depressive episodes, but neither causes depression in healthy individuals nor worsens the clinical condition in depressed individuals (Heninger, Delgado, & Charney, 1996). Some depressed patients showed lower tryptophan availability from the plasma (Maes et al., 1990; Van Praag, 2004), reduced serum tryptophan levels and faster tryptophan clearance after intake of high amounts of tryptophan (Deakin, Pennell, Upadhyaya, & Lofthouse, 1990; Van Praag, 2004), and a lower uptake of 5-hydroxytryptophan across the blood-brain barrier (Ågren & Reibring, 1994; Ågren et al., 1991; Van Praag, 2004). Using SPECT imaging, reduced SERT binding-sites were detected in the brain stem of depressed patients, which increased with the remission of depressive symptoms (Freeman et al., 1993; Malison et al., 1998). Moreover, the SERT gene has a polymorphic region (5-HTTLPR) with two variants, a short form (s) and a long form (l) (Heils et al., 1996; K-P Lesch et al., 1994). The s-allele is associated with lower SERT expression (Heils et al., 1996), anxiety-related personality traits (Klaus-Peter Lesch et al., 1996), and is more likely to be found in depressive patients as well as suicide victims (Bondy, Erfurth, De Jonge, Krüger, & Meyer, 2000; Collier et al., 1996).

2.3 Interactions between stress and the serotonergic system

The HPA axis and the 5-HT neurotransmitter system share a reciprocal relationship. 5-HT fibers innervate the CRH-containing neurons in the paraventricular nucleus (Liposits, Phelix, & Paull, 1987) and can cause CRH release (Calogero et al., 1989). 5-HT directly stimulates ACTH release in the pituitary (Spinedi & Negro-Vilar, 1983) and corticosteroid release in the adrenal gland (R. Fuller, 1992; Klaassen, Riedel, van Praag, Menheere, & Griez, 2002; Lefebvre et al., 1992). The 5-HT innervation of the HPA axis influences the circadian rhythm of serum glucocorticoid levels (Banky,

Halasz, & Nagy, 1986) and modulates GR and MR expression (Budziszewska, Siwanowicz, & Przegaliński, 1995; Seckl & Fink, 1991).

In return, the 5-HT system is influenced by the HPA axis. Inescapable stress triggers 5-HT release from the DRN (José Amat et al., 2005; Maswood, Barter, Watkins, & Maier, 1998; Takase et al., 2004) and escapable stress enhances 5-HT release in the periaqueductal grey (Jose Amat, Matus-Amat, Watkins, & Maier, 1998a, 1998b). CRH-containing fibers directly interact with 5-HT neurons of the DRN (Kirby, Rice, & Valentino, 2000; Ruggiero, Underwood, Rice, Mann, & Arango, 1999), and the DRN also contains a small population of neurons co-expressing CRH and 5-HT (Commons, Connolley, & Valentino, 2003). CRH binds to CRH1 and CRH2 receptors that adversely affect target cells (Valentino, Lucki, & Van Bockstaele, 2010). Initial stress events and low levels of CRH primarily activate CRH1 and inhibit the DRN 5-HT neurons (Kirby, Allen, & Lucki, 1995; Kirby et al., 2000; Price, Kirby, Valentino, & Lucki, 2002; Waselus, Nazzaro, Valentino, & Van Bockstaele, 2009). Repeated stress causes an externalization of CRH2 and an internalization of CRH1 (Waselus et al., 2009), which then has an excitatory effect on DRN neurons (Kirby et al., 2000).

Glucocorticoids alter 5-HT levels in different brain regions. Corticosterone treatment decreases 5-HT levels in the frontal cortex and enhances 5-HT levels in the hippocampus (Luine, Spencer, & McEwen, 1993). In rats, stress was found to increase the extracellular 5-HT in the ventral hippocampus (Barr & Forster, 2011; Rex et al., 2005; Wright et al., 1992), which was suppressed by the GR antagonist mifepristone (mif) (Barr & Forster, 2011). Corticosterone binding to the MR also increases 5-HT turnover in the hippocampus and raphé nuclei (E. De Kloet et al., 1983). Glucocorticoids have direct effects on the 5-HT metabolism as well. They induce tryptophan-degrading enzymes (Badawy, 1977), stimulate the uptake of L-tryptophan in synaptosomes (Neckers & Sze, 1975), and affect the expression of tryptophan hydroxylase 2 (Heydendael & Jacobson, 2009; Malek, Sage, Pévet, & Raison, 2007).

In addition to 5-HT synthesis, glucocorticoids impact the re-uptake of 5-HT with ambiguous evidence. Acute stress and elevated corticosteroid levels decreased SERT

mRNA in raphé nuclei (Vollmayr, Keck, Henn, & Schloss, 2000), but did not alter 5-HT uptake or SERT density (Berton, Durand, Aguerre, Mormede, & Chaouloff, 1999; Telegdy & Vermes, 1975). Chronic exposure to stress or dex reduced SERT mRNA expression (Fumagalli, Jones, Caron, Seidler, & Slotkin, 1996), but in a different study, chronic stress enhanced SERT agonist binding in the raphé nuclei (Paré & Tejani-Butt, 1996). In line with this result, repeated social defeat enhanced SERT mRNA levels (Filipenko, Beilina, Alekseyenko, Dolgov, & Kudryavtseva, 2002). In humane B-lymphoblast cells, 24-hour exposure to dex caused a dose-dependent increase in SERT agonist binding and 5-HT uptake (Glatz, Mössner, Heils, & Lesch, 2003). In serotonergic 1C11 cells (1C11-5HT), low concentrations reduced and high concentrations enhanced SERT expression on the cell surface (Kittler, 2012).

3 Aims of this work

3.1 Mechanism of rapid serotonin release after glucocorticoid receptor activation

Acute administration of glucocorticoids and stress increase extracellular 5-HT levels in the hippocampus, which is mediated by the GR (Barr & Forster, 2011; Rex et al., 2005; Summers et al., 2003; Weber & Bartsch, 2014; Wright et al., 1992). On a cellular level, however, it remains unknown how glucocorticoids elevate extracellular 5-HT. This thesis hypothesizes that GR activation triggers a rapid vesicular 5-HT release. This question was addressed by using life cell imaging of 1C11-5HT incubated with fluorescent false neurotransmitter 511 (FFN511), which accumulates in synaptic vesicles (Lau, Proissl, Ziegler, & Schloss, 2015), and immunofluorescence with FM4-64FX to visualize vesicular 5-HT release (Trueta, Kuffler, & De-Miguel, 2012).

3.2 Interaction between glucocorticoid receptor and synaptic structures

The elevation of extracellular 5-HT levels happens within minutes after the administration of glucocorticoids, which is most likely too fast to be mediated by alterations of transcription and protein synthesis. More likely, this effect is caused by rapid, non-genomic effects of glucocorticoids. The second question addressed in this

work is if there is spatial proximity and therefore potential interaction between the GR and synaptic release sites *in vivo* and *in vitro*. Using subcellular fractionation, proteins of mice brain lysates were separated and GR as well as synaptic markers were immunologically detected in the membrane/vesicular fraction. Employing immunofluorescence of 1C11-5HT, colocalization between GR and synaptotagmin 1, which is pivotal for vesicular release (Pang & Südhof, 2010), was quantified.

3.3 Interaction between activated glucocorticoid receptor and the serotonin transporter

Extracellular 5-HT levels do not only depend on 5-HT release, but also on 5-HT uptake by the SERT. *In vivo*, the acute effects of glucocorticoids and stress on the SERT are ambiguous (Berton et al., 1999; Telegdy & Vermes, 1975; Vollmayr et al., 2000). *In vitro*, glucocorticoids dose-dependently increase SERT cell surface expression one hour after application (Lau, Heimann, Bartsch, Schloss, & Weber, 2013). However, it remains unknown whether the activated GR rapidly and directly interacts with SERT that is already on the cell surface (such as, e.g., hic-5, secretory carrier membrane protein 2, or syntaxin 1A). To answer this question, 1C11-5HT were incubated with the GR agonist dex and stained for the membrane-bound SERT and GR. Then, colocalization of the two proteins was quantified.

4 Materials

4.1 Consumables and chemicals

4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	Carl Roth
Amersham™ Hybond™ ECL Nitrocellulose	GE Healthcare
Ammonium sulfate	Sigma-Aldrich
Calcium chloride	Grüssing GmbH
Cell culture plate, 100x20 mm	Sarstedt
Cell culture plate, 35 mm	ibidi
Cell culture plate, 6 well, 35 mm	Sarstedt
Cell scraper, 16 cm, soft	Sarstedt
Coverslips, 15 mm	ThermoFisher
CryoTube™ tubes – CryoPlus, 1 ml	Sarstedt
Cyclohexanecarboxylic acid (CCA)	Sigma-Aldrich
N^6,2'-O-dibutyryladenosine 3',5'-cyclic monophosphate (db cAMP)	Sigma-Aldrich
D-Glucose	Merck
Double-distilled water (ddH_2O)	Millipore
Fluorescent mounting medium	Agilent Dako
Dex (water soluble)	Sigma-Aldrich
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich
Disodium hydrogen phosphate	Sigma-Aldrich
DMEM Glutamax®	ThermoFisher
Ethanol	Riedel-de-Haen
Fetal bovine serum	ThermoFisher
Gelatine	Sigma-Aldrich
Glycine	Sigma-Aldrich
Syringe, 1 ml	Hamilton
Horse serum	Invitrogen
Hydrochloric acid (HCl)	AnalaR NORMAPUR
Isopropanol	AppliChem
L-glutamine	Invitrogen

Materials

μ-slide, 8 well, 9.4 x 10.7 mm	ibidi
Mif	Sigma-Aldrich
Methanol	J. T. Baker
Mercaptoethanol	Sigma-Aldrich
Milk powder	Carl Roth
Sodium chloride	J. T. Baker
Sodium hydroxide	AppliChem
Microscope slides	Engelbrecht
Non-essential amino acids	Invitrogen
Neubauer counting chamber	Optic Labor
Odyssey® protein molecular weight marker	LICOR
Para-formaldehyde	Riedel-de-Haen
Parafilm “M”	American Can
Penicillin/streptomycin	Invitrogen
Phosphate-buffered saline, cell culture use	ThermoFisher
Pipettes 5 ml, 10 ml, 25 ml, 50 ml	Sarstedt
Pipette tips 1000 µl, 200 µl, 20 µl, 10 µl	Sarstedt
Ponceau S solution	Sigma-Aldrich
KCl	AppliCem
Potassium dihydrogen phosphate	Sigma-Aldrich
ProLong™ Gold antifade mountant	ThermoFisher
Rotiphorese® Gel 30	Carl-Roth
Saponin	Sigma-Aldrich
Sodium dodecyl sulfate (SDS)	Carl-Roth
SDS probe buffer	BioLabs
N,N,N’,N’-Tetramethylethylendiamine (TEMED)	Sigma-Aldrich
Tris(hydroxymethyl)aminomethane (TRIS)	Carl-Roth
Trypsin-ethylenediaminetetraacetic acid (Trypsin-EDTA)	Invitrogen
Tube 0.5 ml, 1.5 ml, 2 ml	Sarstedt
Tube 15 ml, 50 ml	Sarstedt
TWEEN 20®	Sigma-Aldrich
Whatman® 3MM Paper	Sigma-Aldrich

4.2 Stock solutions

Dex, water-soluble: [c] = 50 µM: 100 g powder contains 56 mg dex (M = 392.47 g/mol; remaining volume is 2-Hydroxypropyl-b-CycloDextrin); 17.52 mg powder are dissolved in 500 ml ddH_2O ([c] = 50 μM); stored at -20 ^oC; no exposure to light

Mif: [c] = 1 mM: 4.29 mg mif (M = 429.6 g/mol) in 10 ml ethanol; stored at 4 °C for 1 week

NaOH solution: [c] = 3 M NaOH in ddH_2O

10x PBS: 80 g NaCl; 2 g KCl; 14.4 g Na_2HPO_4; 2.4 g KH_2PO_4; dissolved in 800 ml ddH_2O; pH adjusted with HCl to 7.4; filled with ddH_2O to 1,000 ml; autoclaved and stored at room temperature (RT)

4.3 Cell culture

1C11 cells: Kindly provided by PD Dr. Thorsten Lau

1x PBS: 10x PBS diluted in ddH_2O (1:10); then autoclaved and stored at RT

Growth medium: DMEM Glutamax with 10 % fetal bovine serum; 1 % non-essential amino acids; 1 % penicillin/streptomycin; 1 % L-glutamine

Differentiation medium: Growth medium with 1 mM db cAMP; 0.05 % CCA

Freezing mix: 70 % growth medium; 20 % fetal bovine serum; 10 % dimethyl sulfoxide (DMSO)

4.4 FFN511 life cell imaging of 1C11-5HT

Dye: FFN511, Abcam

Reaction buffer: 130 mM NaCl; 20 mM HEPES; 5 mM $CaCl_2$; 10 mM D-glucose; 60 mM KCl; in ddH_2O; pH adjusted to 7.2-7.4 with 3 M NaOH solution

Loading/washing buffer: 130 mM NaCl; 20 mM HEPES; 5 mM $CaCl_2$; 10 mM D-glucose; in ddH_2O; pH adjusted to 7.2-7.4 with 3 M NaOH solution

Loading/washing buffer with dex: 130 mM NaCl; 20 mM HEPES; 5 mM $CaCl_2$; 10 mM D-glucose; 20 nM dex; in ddH_2O; pH adjusted to 7.2-7.4 with 3 M NaOH solution

Calcium-free buffer with dex: 130 mM NaCl; 20 mM HEPES; 10 mM D-glucose; 20 nM dex; in ddH_2O; pH adjusted to 7.2-7.4 with 3 M NaOH solution

4.5 FM4-64FX staining of 1C11-5HT

Dye: FM4-64FX, ThermoFisher

Washing buffer: 130 mM NaCl; 20 mM HEPES; 5 mM $CaCl_2$; 10 mM D-glucose; in ddH_2O; pH adjusted to 7.2-7.4 with 3 M NaOH solution

Reaction buffer: 130 mM NaCl; 20 mM HEPES; 5 mM $CaCl_2$; 10 mM D-glucose; 60 mM KCl; in ddH_2O; pH adjusted to 7.2-7.4 with 3 M NaOH solution

Dex buffer: 130 mM NaCl; 20 mM HEPES; 5 mM $CaCl_2$; 10 mM D-glucose; 20 nM dex; in ddH_2O; pH adjusted to 7.2-7.4 with 3 M NaOH solution

4.6 Subcellular fractionation and detection of glucocorticoid receptor and synaptic markers

Antibodies:

Rabbit anti-GR polyclonal, Santa Cruz

Mouse anti-synapsin 1 monoclonal, Synaptic Systems

Mouse anti-rab3 monoclonal, Synaptic Systems

IRDye® 680RD donkey anti-rabbit IgG, LICOR

IRDye® 800CW donkey anti-mouse IgG, LICOR

Marker: Odyssey® Protein Molecular Weight Marker, LICOR

9 % running SDS gel: 8.7 ml H_2O; 6 ml Rotiphorese gel; 5 ml of 1.5 M TRIS (pH 8.7); 200 µl 10 % SDS; 80 µl ammonium sulfate; 40 µl TEMED

12 % running SDS gel: 7 ml H_2O; 8 ml Rotiphorese gel; 5 ml 1,5 M TRIS (ph 8,7); 200 µl 10 % SDS; 80 µl ammonium sulfate; 40 µl TEMED

5 % stacking SDS gel: 5.5 ml H_2O; 1.3 ml Rotiphorese gel; 1 ml of 1 M TRIS (pH 6.8); 80 µl 10 % SDS; 80 µl ammonium sulfate; 16 µl TEMED

Running buffer: ddH_2O with 0.386 mol/l glycine; 41.3 mmol/l TRIS; 6.9 mmol/l SDS

Transfer buffer: 80 % ddH20; 20 % methanol with 0.1918 mol/l glycine; 25 mmol/l TRIS; 1.3 mmol/l SDS

TRIS-buffered saline: ddH_2O with 50 mM TRIS (ph 8.0); 120 nM NaCl

TWB: 1000 ml ddH_2O with 50 mM TRIS (pH 8.0); 120 mM NaCl; 5 g TWEEN

4.7 Immunofluorescence: Colocalization of glucocorticoid receptor and synaptotagmin 1 in 1C11-5HT

Antibodies:

Rabbit anti-GR polyclonal, Santa Cruz

Guinea pig anti-synaptotagmin 1 polyclonal, Synaptic Systems

Donkey anti-rabbit 568 nm, Life Technologies

Goat anti-guinea pig 488 nm, Life Technologies

Blocking solution B: 0.2 % gelatin; 0.1 % saponin; in 1x PBS

Antibody solution: 0.2 % gelatin; 0.01 % saponin; in 1x PBS

4.0 % para-formaldehyde: 40 g para-formaldehyde in 500 ml 1x PBS (at 60-65 ^{o}C), titrated with 1 M NaOH until the solution is clear. Filled with 1x PBS to 1000 ml. After cooling down to RT, pH is adjusted to 7.2-7.4 with HCl. Stored at -20 ^{o}C.

4.8 Immunofluorescence: Colocalization of glucocorticoid receptor and the serotonin transporter in 1C11-5HT

Antibodies:

Mouse anti-SERT monoclonal, ATS

Rabbit anti-GR polyclonal, Santa Cruz

Donkey anti-mouse 488 nm, Life Technologies

Donkey anti-rabbit 568 nm, Life Technologies

Dex medium: DMEM Glutamax with 10 % fetal bovine serum; 1 % non-essential amino acids; 1 % penicillin/streptomycin; 1 % L-glutamine; 1 mM db cAMP; 0.05 % CCA; 20 nM dex

Mif and dex medium: Dex medium (above) supplemented with 10 μM mif

Blocking solution A: 0.2 % gelatin; 10 % horse serum; in 1x PBS

Blocking solution B, antibody solution, 4.0 % para-formaldehyde: see above

5 Methods

5.1 Culture and differentiation of 1C11

1C11 cell line: 1C11 is a murine teratocarcinoma cell line, immortalized by the expression of the simian virus 40 oncogenes (Buc-Caron, Launay, Lamblin, & Kellermann, 1990). They have an immature precursor state and differentiate to 1C11-5HT, a neuron-like phenotype that expresses neuronal markers such as synaptophysin (found in the membrane of synaptic vehicles) and N-CAM (a neuronal adhesion molecule) (Buc-Caron et al., 1990). 1C11-5HT constitute a complete 5-HT metabolism (tryptophan hydroxylase, aromatic amino acid decarboxylase, monoamine oxidase A, 5-hydroxyindoleacetic acid, 5-hydroxytryptophan, 5-HT) (Mouillet-Richard et al., 2000), express several 5-HT receptors (5-HT1B, 5-HT1D, 5-HT2A, 5-HT2B) (Kellermann, Loric, Maroteaux, & Launay, 1996), and show 5-HT uptake via SERT similar to 5-HT neurons (Buc-Caron et al., 1990).

Culture: 1C11 were cultivated in 100 mm plates coated with 0.1 % gelatin in 1C11 growth medium at 37 °C and 5 % CO_2. Every second day, 1/6 or 1/8 of 1C11 were sub-cultured onto a new 100 mm plate, depending on density. For this, growth medium was removed, cells were washed three times with 1x PBS and incubated with 2 ml of trypsin for 10 min at 37 °C. Cells were gently removed from the plate's surface using a cell scraper, and either 4 ml (sub-culturing 1/6) or 6 ml (sub-culturing 1/8) of growth medium were added. 1 ml was transferred to a 100 mm plate coated with 0.1 % gelatin and 9 ml of growth medium was added.

Freezing 1C11: Cells were washed, trypsinated and removed from the surface as described. 4 ml growth medium was added, and the plate's content transferred to a 15 ml falcon, which was centrifuged with 1000 rpm for 10 min at 4 °C. The supernatant was removed, the pellet resuspended in 1ml freezing medium, and transferred to a CryoTube™ to be stored at -80 °C.

Differentiation to 1C11-5HT: 1C11 were washed, trypsinated and removed from the surface as previously described. 4 ml of growth medium were added, and the content

of the plate transferred to a 15 ml falcon. After centrifugation with 1000 rpm for 5 min at 4 °C, supernatant was removed, and the pellet resuspended in 4 ml differentiation medium. 1C11 density was determined in a Neubauer counting chamber and 40,000 cells were transferred to a 35 mm plate with three 15-mm coverslips in 3 ml differentiation medium. **Figure 2** shows exemplary images of 1C11-5HT.

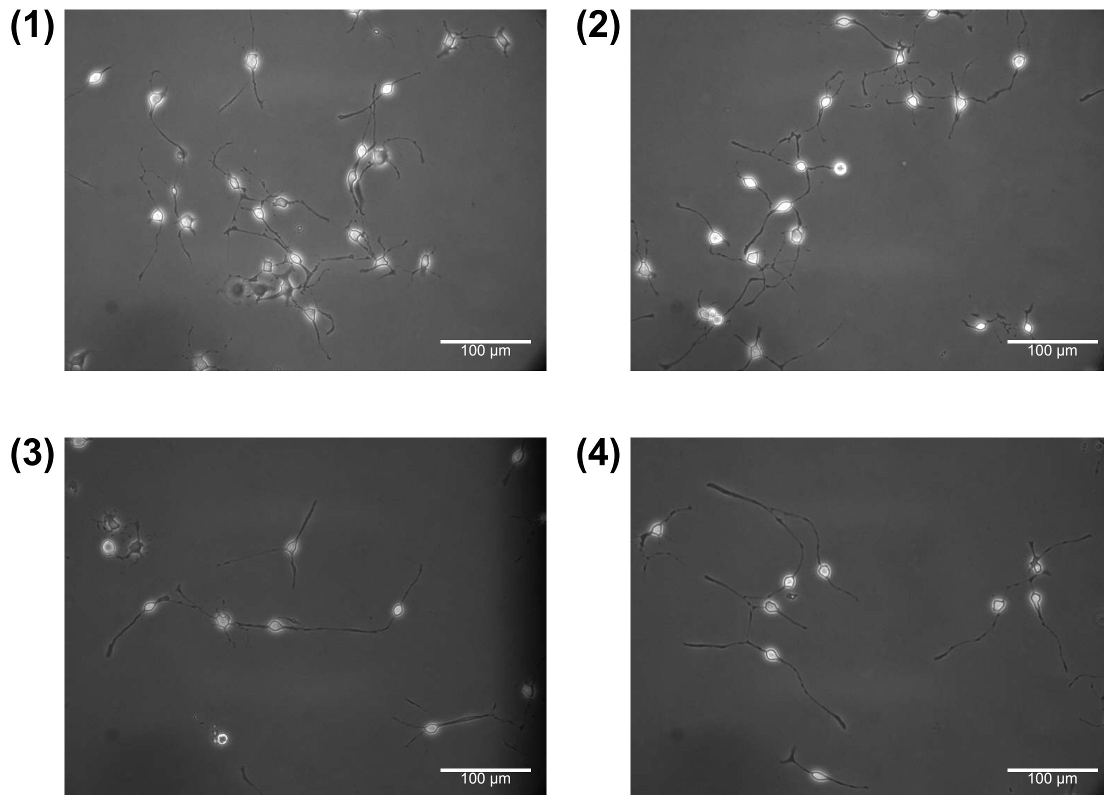

Figure 2: Differentiated 1C11-5HT.
Images were taken on a 6-well plate, 35 mm, with a density of 40,000 1C11-5HT per well, with a Leica DMR IB plan 10x/0.25. In differentiation medium, 1C11 transform from a fibroblast-like shape to a neuron-like shape. Within four days, they develop a complete 5-HT phenotype (Mouillet-Richard et al., 2000). Experiments were performed on day 4 (d4).

5.2 Confocal laser scanning microscopy

Fluorescent images were acquired using a Leica TCS SP5 Confocal imaging system on a DM IRE2 microscope, equipped with an acousto-optical beam splitter, argon ion laser (458 nm – 514 nm), a diode-pumped solid-state laser (561 nm), and a helium neon laser (633 nm). Laser lines were used as recommended by the applied dyes. In CLSM, laser light passes through a pinhole and is reflected by a beam splitter. The pinhole ensures that only a small spot of the specimen is illuminated by the laser. A

lens focuses the laser light on a specific point of the specimen. The emitted fluorescent signal passes the beam splitter, because it has a different wavelength compared to the laser signal (blue vs. red in **Figure 3**). The emitted fluorescent signal passes through another pinhole, which ensures that only the signal from the point of interest reaches the detector, which transforms the light into an electric signal. As a result, signals for one particular point in the specimen are caught. To obtain a 2D image, the laser scans multiple points of the focal plane, and a computer compounds the point signals to an image. Varying the focal plane produces three-dimensional z-stacks.

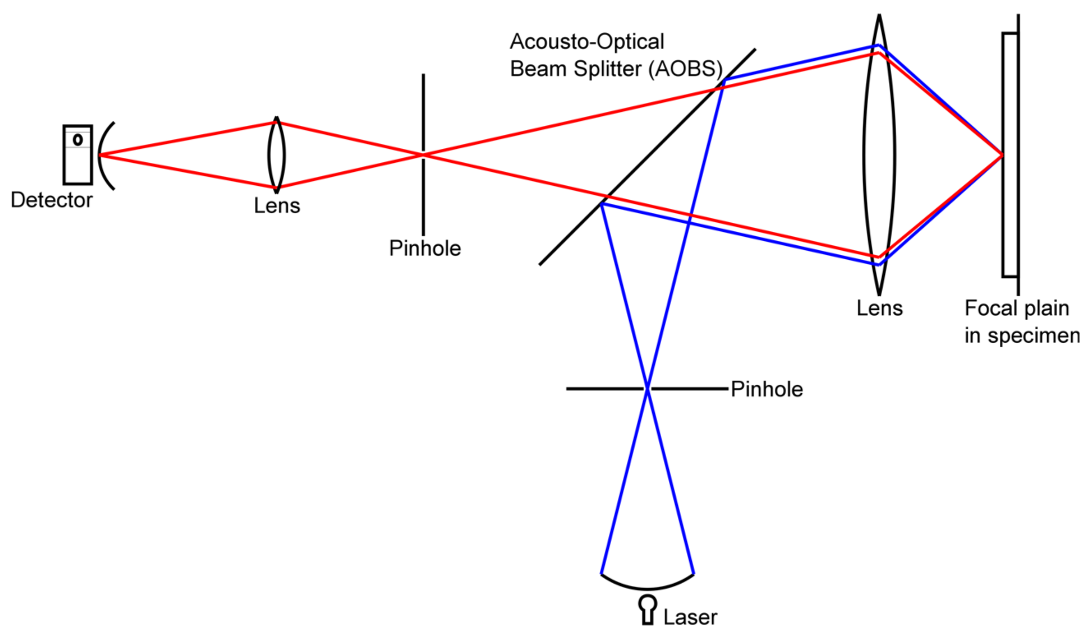

Figure 3: Schematic imaging with confocal laser scanning microscopy.
The laser emits laser light (blue), which passes through a pinhole, is reflected by a beam splitter (AOBS) and focused on the specimen. The emitted signal (red) passes through the beam splitter, because it has a different wavelength than the initial laser signal. After another pinhole, emissions from the specific point of interest are transformed to an electric signal. Scanning many points of the focal plane and varying the focal plane produces z-stacks.

5.3 Colocalization analysis in fluorescent microscopy

Colocalization of fluorescent signals can be broken down to co-occurrence, which is the overlap of the fluorescence of two probes, and correlation, which implies that one fluorescent signal is in a constant proportion to the other (Dunn, Kamocka, & McDonald, 2011). Colocalization of fluorescent signals does not indicate direct

molecular interaction but indicates spatial association between two molecules, which is limited by microscope resolution (Dunn et al., 2011). In this work, colocalization was quantified using Pearson's correlation coefficient (PCC) (Dunn et al., 2011). PCC measures the degree to which signal intensities in two channels are linearly correlated with each other. Therefore, it is a measure of co-occurrence and correlation. The formula to calculate PCC for two channels red (R) and green (G) is:

$$PCC = \frac{\sum_i (R_i - \bar{R}) \times (G_i - \bar{G})}{\sqrt{\sum_i (R_i - \bar{R})^2 \times \sum_i (G_i - \bar{G})^2}}$$

R_i and G_i refer to the red and green intensity values at pixel i, whereas $\bar{R}$ and $\bar{G}$ refer to the red and green channels' mean intensity values over the entire images (Dunn et al., 2011). A PCC of 1 depicts a perfect linear relationship, -1 describes a perfect, inverse linear relationship (Dunn et al., 2011). The square of PCC, called R^2 or coefficient of determination, indicates the proportion of the variability of G explained by the variability of R (Dunn et al., 2011). **Figure 4** exemplifies the determination of PCC in two images.

PCC is not influenced by background noise, because the image's mean intensity value is subtracted from each pixel (Dunn et al., 2011). However, as large areas of background artificially inflate PCC, a region of interest (ROI) should be selected (Dunn et al., 2011). Further, as the staining intensity might differ between cells, simultaneous colocalization analysis for multiple cells in one image can reduce the overall PCC. To avoid this effect, colocalization should be analyzed on the level of individual cells (Dunn et al., 2011). PCC is insufficient to cover complex, non-linear relationships, i.e., if correlation varies between cell compartments. It is also not suitable to measure the mere overlap of signals, because PCC also covers proportionality (Dunn et al., 2011).

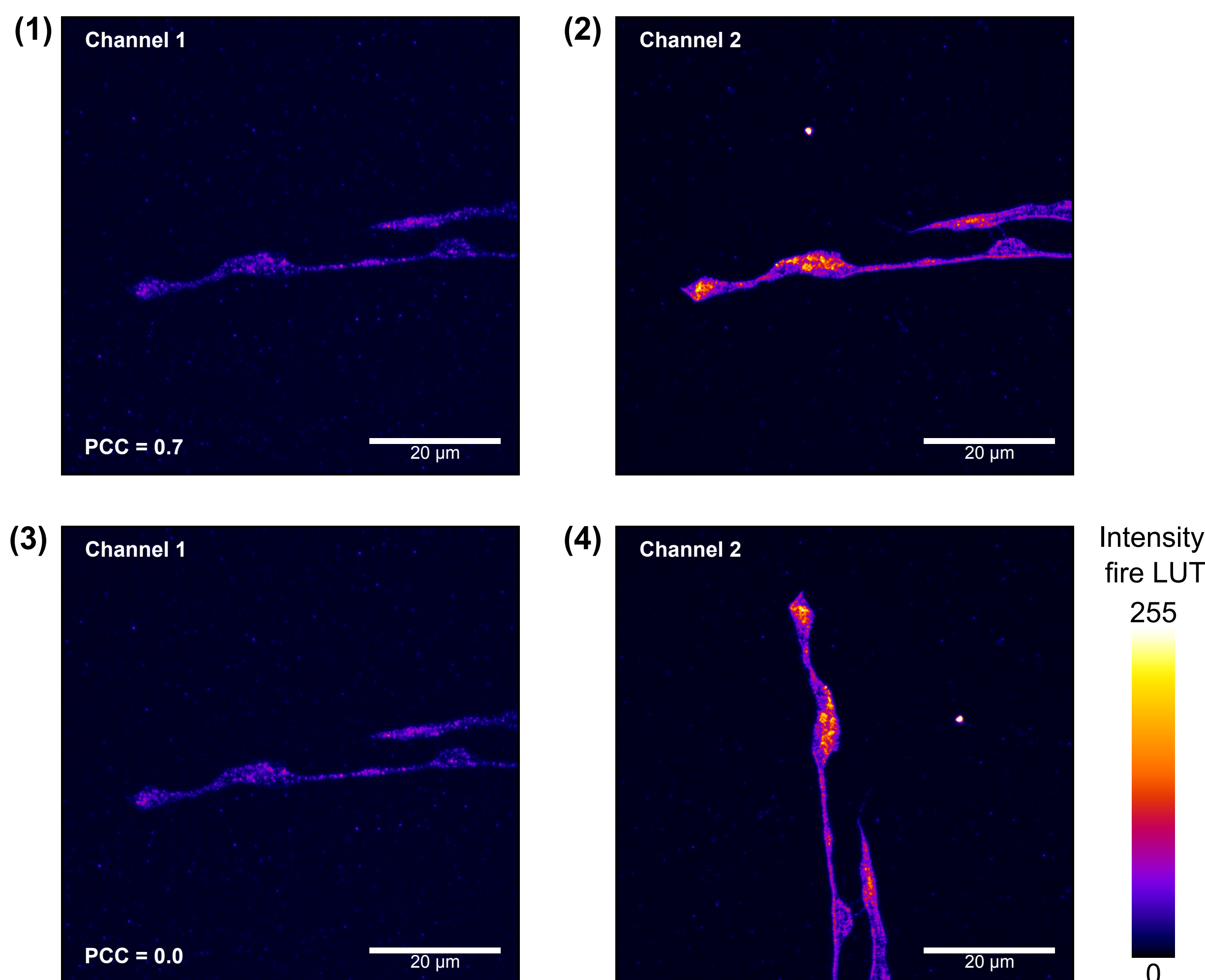

Figure 4: Exemplary colocalization analysis.
Image (4) is a 90° rotation of image (2). After 90° rotation, PCC drops from 0.71 (images (1) and (2)) to 0.0 (images (3) and (4)). This means that there is no linear correlation between signals in image (3) and image (4). PCC = Pearson's correlation coefficient.

5.4 FFN511 life cell imaging of 1C11-5HT

1C11 preparation and CLSM images: 1C11-5HT were differentiated on a μ-slide 8 well plate with 10,000 cells per well. Medium was removed, cells were incubated with loading buffer with 10 μM FFN511 for 10 min. Loading buffer with FFN511 was substituted by washing buffer. Then, control images were taken. Washing buffer was substituted either by reaction buffer, by loading/washing buffer with dex, or by calcium-free buffer with dex. After 2-3 min, images were taken with an argon laser 458 nm (laser output power 15 %-21 %). Laser excitation intensity, photomultiplier range and gain were set to minimize background signal and avoid saturated images. Settings

were kept constant during image acquisitions. Image stacks containing multiple neurons were taken with 0.5 μm sections.

Image analysis: In ImageJ, z-projections were created using the projection type *Sum Slices* and transformed to 8bit. Background correction was done by selecting an area without cells as a ROI and applying the *BG Subtraction from ROI* function. Then, neurons (soma and neurite) were selected as ROI. The mean intensity value of the ROI was measured. *Prism 7* was used to create charts of normalized data and perform statistics.

5.5 FM4-64FX staining of 1C11-5HT

1C11 preparation and CLSM images: 1C11-5HT were differentiated on six 15 mm coverslips in two 35 mm plates (40,000 cells per plate) and washed with 1x PBS. 2 μM FM4-64FX was added to the reaction buffer, washing buffer, and dex buffer. Two 100 μl drops of each buffer containing 2 μM FM4-64FX were placed on a heater with 37 °C. One coverslip was placed on each drop and incubated for 10 min at RT. Coverslips were washed three times with 1x PBS, incubated with 4 % para-formaldehyde, washed three times with 1x PBS and one time with ddH20, and mounted with fluorescence mounting medium. Images were taken with a diode-pumped solid-state laser at 561 nm. Laser excitation intensity, photomultiplier range and gain were set to avoid saturated images and background signal and settings were kept constant during image acquisitions. Somas or dendrites were selected and zoomed in on. Stacks of somas and dendrites were taken separately with 0.5 μm sections.

Image analysis: In ImageJ, z-projections were created using the projection type *Sum Slices* and transformed to 8bit. The length of neurites and the area of somas was measured using ROIs. Globular structures on somas and on neurites were manually counted and divided by the respective length or area. *Prism 7* was used to create charts of normalized data and perform statistics.

5.6 Subcellular fractionation and detection of glucocorticoid receptor and synaptic markers

GR and the synaptic markers synapsin 1 and rab3 were detected in the subcellular fractions of lysed CNS of WT mice and mice carrying the human SERT (hSERT), which were provided by Marc Caron, Duke University, USA (Jacobsen et al., 2014).

Subcellular fractionation: Mice were kept under standard conditions in the core animal facility of the Central Institute of Mental Health, Mannheim. The CNS was extracted from five WT and five hSERT mice by trained and certified personnel, homogenized by ten strokes with an ice-cooled mortar and pestle, and dissolved in PBS. The lysate was centrifuged with 1500 rpm for 3 min, and the supernatant was transferred to a new tube and centrifuged with 18,000 rpm for 20 min. The second supernatant, which contained enriched cytosolic cell components, was transferred to a new tube, resuspended in ice-cooled PBS with 5 % glycerol (1 ml per mouse CNS), and transferred in 0.5 ml aliquots to ice-cooled 1.5 ml tubes, which were blast-frozen in liquid nitrogen (-80 °C) and stored. The pellet of the second centrifugation, which contained enriched cell membrane and vesicles, was processed similarly.

Preparation of probes: The protein concentrations of the probes were quantified according to Markwell (Markwell, Haas, Bieber, & Tolbert, 1978) and for each probe, 40 μg protein was transferred to a 1.5 ml tube and denaturized with SDS probe buffer/mercaptoethanol (9:1). SDS probe buffer/mercaptoethanol equivalent to half of the volume of the protein probe was added. Each probe was filled with ddH_2O to a total volume of 30 μl and incubated at 95 °C for 5 min.

Preparation of SDS gels: Gels were prepared with either a 9 % or a 12 % running SDS gel and a 5 % stacking SDS gel. The running SDS gel was filled between two 1.5 cm glass plates, separated by a spacer, up to 1 cm below the glass rim. The remaining space was filled with isopropanol. After 20 min and polymerization of the running SDS gel, the isopropanol was removed and the remaining space between the glass plates was filled with stacking SDS gel up to the glass rim. Pockets were created with a 1.5

cm plastic comb. After 30 min at RT, the gel was wrapped in wet pulp cloths, placed in a sealed plastic bag, and stored at 4 °C to be used the following day.

Electrophoresis and blotting: Two gels (12 % and 9 % running gel) were fixed in the Mini-PROTEAN 3 electrophoresis cell (Bio-Rad Laboratories). The outer chamber was halfway filled and the inner chamber completely filled with running buffer. The gel pockets were filled with the probes using a Hamilton syringe, one pocket was filled with Odyssey® Protein Molecular Weight Marker. 100 V was applied for 15 min and 120 V for 60 min. Gels were taken out and, after removal of the glass plates, wrapped between nitrocellulose membrane, four layers of Whatman paper and a sponge on each side. The layers were compressed with a tensioner and placed in a vertical blotting chamber, which was placed in the electrophoresis chamber with a cooling element. The chamber was filled with transfer buffer and 160 mA were applied for 105 min. The nitrocellulose membrane was placed in Ponceau S solution to visualize protein bands and washed with TRIS-buffered saline.

Immunologic detection of the proteins: Membranes were incubated with 50 ml TWB with 5 % milk powder for 60 min at RT to block residual binding sites, and then with 20 ml TWB with 2.5 % milk powder and the primary antibodies for 60 min at RT (GR antibody (1:500); synapsin 1 antibody (1:2000) with the membrane of the 9 % gel; rab3 antibody (1:1000) with the membrane of the 12 % gel). The nitrocellulose membranes were washed twice for 4 min with TRIS-buffered saline, twice for 2 min with TWB, and twice for 4 min with TRIS-buffered saline. The nitrocellulose membranes were incubated with 20 ml TWB with 2.5 % milk powder and secondary antibodies (anti-rabbit 680RD; anti-mouse 800CW (both 1:10,000)) for 45' at RT without light exposure. After washing the membranes twice for 4 min with TRIS-buffered saline, twice for 2 min with TWB, and twice for 4 min with TRIS-buffered saline, they were scanned on the Licor Odyssey Scanner at 700 nm and 800 nm.

5.7 Immunofluorescence: Colocalization of glucocorticoid receptor and synaptotagmin 1 in 1C11-5HT

1C11 preparation and CLSM images: 1C11-5HT were differentiated on two 35 mm plates with three 15 mm coverslips each and 40,000 cells per plate. Coverslips were incubated with 1 % para-formaldehyde for 15 min at RT, washed three times with 1x PBS and blocked as well as permeabilized for 10 min at RT with blocking solution B. Coverslips were placed on 100 μl droplets of antibody solution with antibodies against GR (1:200) and synaptotagmin 1 (1:250) for 60 min at RT. Coverslips were washed three times with blocking solution B and placed on 250 μl droplets of antibody solution with donkey anti-rabbit 568 nm and goat anti-guinea pig 488 nm (both 1:1000) for 45 min at RT without light exposure. After washing coverslips three times with 1x PBS and one time with ddH2O, they were mounted with ProLong™ Gold antifade mountant. For probe excitation, an argon laser at 488 nm (20 % output power) and a diode-pumped solid-state laser at 561 nm were used. Using non-stained negative control slides, photomultiplier range, laser intensity and gain were adjusted to minimize unspecific fluorescent signal. Control images of probe slides were taken to adjust settings to avoid saturated signals. Settings were kept constant during image acquisitions. Neurites were selected for imaging, zoomed in on, and z-stacks with 0.5 μm sections were acquired (using non-simultaneous imaging).

Image analysis: In ImageJ, z-projections of channels 1 and 2 were created using the projection type *Max Intensity*, transformed to 8bit, and background signal was corrected. Neurites were selected as a ROI and PCC between channels 1 and 2 was determined using the ImageJ colocalization plugin. Data was visualized with *Prism 7*.

5.8 Immunofluorescence: Colocalization of glucocorticoid receptor and the serotonin transporter in 1C11-5HT

1C11 preparation and CLSM images: 1C11-5HT were differentiated on three 35 mm plates with three 15 mm coverslips each and 40,000 cells per plate. Two coverslips were incubated in mif medium, two coverslips were incubated in mif and dex medium, and two coverslips were kept in differentiation medium at 37 °C and 5 % CO_2 for either

15 min or 30 min. Coverslips were washed three times with 1x PBS, fixed with 1 % para-formaldehyde for 15 min at RT, washed three times with 1x PBS and incubated with blocking solution A for 60 min. Coverslips were placed on 100 *µ*l droplets of blocking solution A with the antibody against extracellular epitope of the SERT (1:200) for 60 min. Coverslips were washed three times with blocking solution A and permeabilized with blocking solution B for 10 min. Coverslips were placed on 100 *µ*l droplets of antibody solution with the GR antibody (1:200) for 60 min. Coverslips were washed three times with blocking solution B, placed on 250 *µ*l droplets of antibody solution with donkey anti-rabbit 568 nm and donkey anti-mouse 488 nm (both 1:1000) for 45' at RT without light exposure. Coverslips were washed three times with 1x PBS, washed one time with ddH2O and mounted using ProLong™ Gold antifade mountant. CLSM images of neurites were taken as described in section **5.7**.

Image analysis: Images were analyzed in ImageJ as described in section **5.7**. Data was visualized in *Prism 7*.

5.9 Statistics

Analyses were performed with cells or neurites as independent variables (n). Experiments were conducted at least three times on different days using different batches of 1C11. Data is expressed as mean ± standard error of the mean. For the colocalization analysis, one-sample, one-tailed t-test was used to determine if PCC was significantly greater than 0 (no correlation), as proposed by McDonald and Dunn (2013). To compare the means of multiple groups, non-parametric Kruskal-Wallis test was used. After the Kruskal-Wallis test, post-hoc analysis using Dunn's Multiple Comparisons test was performed to test whether means of individual groups were significantly different from each other; $p < .05$ was considered significant.

6 Results

This thesis aims at answering the question how glucocorticoids rapidly increase extracellular 5-HT levels. First, results of the 1C11-5HT life cell imaging with FFN511 are described. Second, results of the FM4-64FX staining in 1C11-5HT are presented. After that, findings of the subcellular fractionation of mice CNS, followed by electrophoresis, blotting and detection of GR, rab3, and synapsin 1 are shown. Then, results of the colocalization analysis of GR and synaptotagmin 1 in 1C11-5HT are presented (data also shown in Paul et al. (2021)). At last, results of the colocalization analysis of GR and the membrane-bound SERT in 1C11-5HT are described.

6.1 FFN511 life cell imaging of 1C11-5HT

To analyze if GR activation triggers vesicle release, 1C11-5HT were loaded with FFN511, which accumulates in synaptic vesicles (Sames, Dunn, Karpowicz, & Sulzer, 2013), and fluorescent signal intensities were measured upon administration of dex. **Figure 5** illustrates example images. FFN511 signal intensities decreased after the administration of any of the buffers. Results of the quantification of the intensities of the fluorescent signals are shown in **Figure 6**. After the administration of 60 mM KCl, FFN511 signal intensities decreased to 47.6 ± 3.6 % (Kruskal-Wallis test: $\chi2(5, N = 204) = 78.93$, $p < .001$; post-hoc Dunn's multiple comparison test: $p < .001$). The application of KCl causes a neuronal depolarization with subsequent calcium influx and vesicle release (Sarkar et al., 2012; Trueta, Méndez, & De-Miguel, 2003). Incubation with 20 nM dex and 5 mM $CaCl_2$ caused a similar decrease of FFN511 signal intensity to 61.3 ± 6.3 % (post-hoc Dunn's multiple comparison test: $p < .001$). This indicates that in the presence of calcium, dex causes a release of synaptic vesicles comparable to depolarization with KCl. After the incubation with 20 nM dex in the absence of calcium, FFN511 signals dropped to 59.5 ± 5.2 % (post-hoc Dunn's multiple comparison test: $p < .001$), indicating that extracellular calcium may be dispensable for dex-triggered vesicle release.

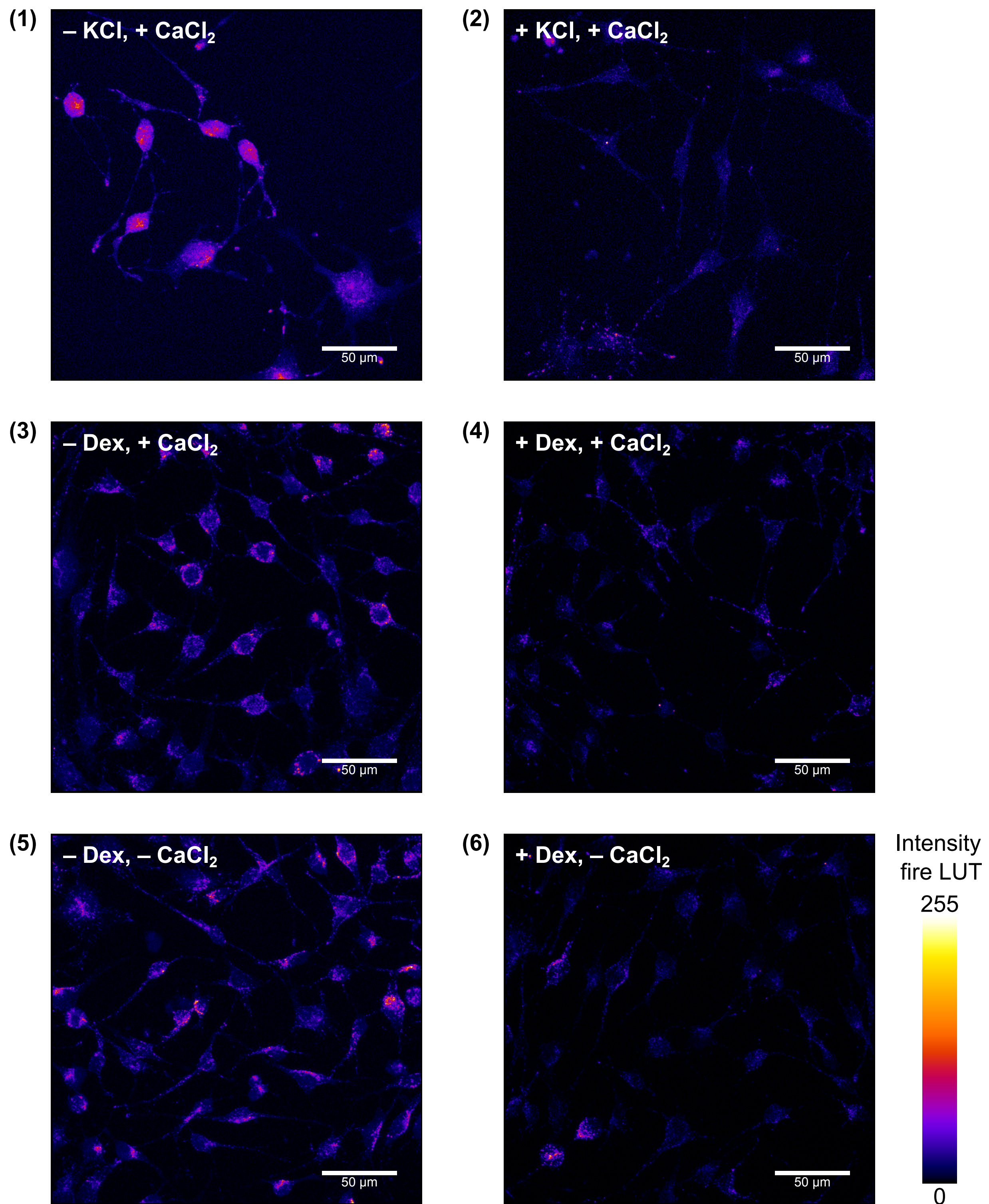

Figure 5: FFN511 life cell imaging with 1C11-5HT before and after administration of potassium chloride or dexamethasone.
(1): pre pulse KCl. **(2):** pulse KCl. **(3):** pre pulse dex with $CaCl_2$. **(4):** pulse dex with $CaCl_2$. **(5):** pre pulse dex without $CaCl_2$. **(6):** pulse dex without $CaCl_2$. Signal intensities of 1C11-5HT loaded with FFN511 dropped after application of KCl or dex in the presence and absence of $CaCl_2$. $CaCl_2$ = calcium chloride; Dex = dexamethasone; KCl = potassium chloride.

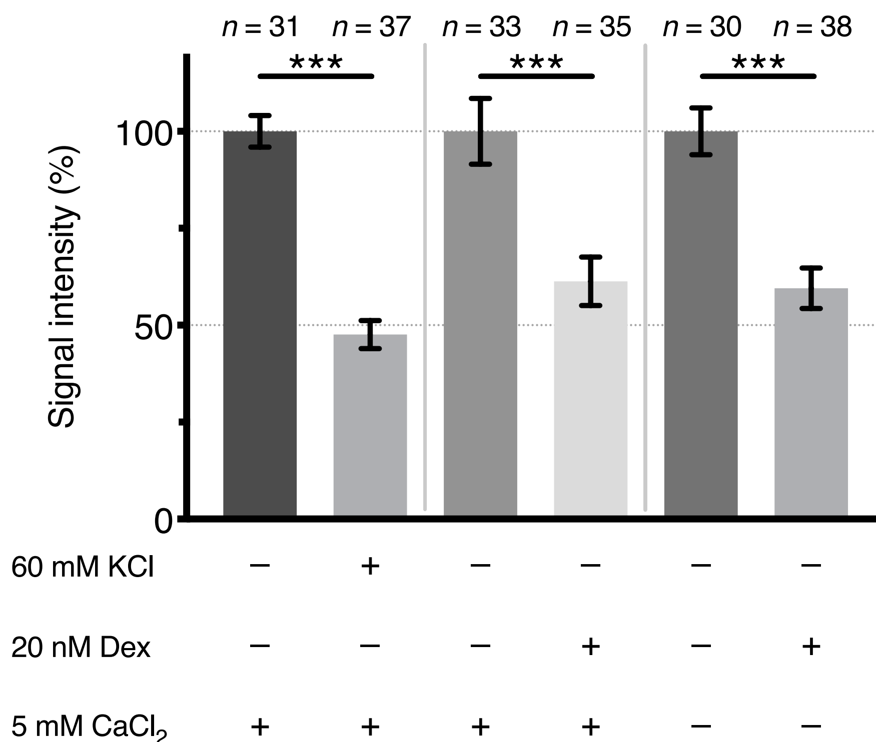

Figure 6: Signal intensities of 1C11-5HT loaded with FFN511 after administration of potassium chloride or dexamethasone.
1C11-5HT loaded with FFN511 were incubated with either KCl or dex in the absence or presence of $CaCl_2$. After administration of KCl, FFN511 signal intensity decreased to 47.6 ± 3.6 %. After administration of dex, FFN511 signal intensity decreased to 61.3 ± 6.3 %. After administration of dex in the absence of $CaCl_2$, FFN511 signal intensity decreased to 59.5 ± 5.2 %. Results indicate a rapid vesicular release after KCl or dex administration, irrespective of the presence of extracellular $CaCl_2$. Bars depict mean ± standard error of the mean; *** ($p < .001$). Groups were compared using Kruskal Wallis test and post-hoc Dunn's multiple comparison test. $CaCl_2$ = calcium chloride; Dex = dexamethasone; KCl = potassium chloride.

6.2 FM4-64FX staining of 1C11-5HT

To analyze whether glucocorticoids trigger rapid vesicle release in 1C11-5HT, cells were loaded with the amphiphilic styryl dye FM4-64FX, which has previously been used to visualize exocytosis and endocytosis of vesicles (W. J. Betz, Mao, & Smith, 1996). 1C11-5HT were incubated with KCl or dex and images were taken for neurites and somas separately. An increase in the number of intracellular globular structures indicates an increase in the number of recycled vesicles, which is an indirect sign of vesicular release; each globular spot represents a recycled vesicle cluster (W. Betz, Bewick, & Ridge, 1992; W. J. Betz & Bewick, 1992; W. J. Betz, Mao, & Bewick, 1992; W. J. Betz et al., 1996; Sarkar et al., 2012; Trueta et al., 2003).

Figure 7 exemplifies images taken after the administration of KCl or dex. After the incubation with 60 mM KCl or 20 nM dex, an increase in globular structures was observed compared to control images. This observation applied to both, somas and neurites. **Figure 8 (1)** depicts results of the quantification of globular structures in neurites. Administration of KCl caused an increase in the number of globular structures in neurites to 286.0 ± 18.4 % (Kruskal-Wallis test: $\chi2(2, N = 568) = 137.8$, $p < .001$; post-hoc Dunn's multiple comparison test: $p < .001$) compared to control. Administration of dex resulted in an increase in the number of globular structures in neurites to 232.4 ± 12.2 % (post-hoc Dunn's multiple comparison test: $p < .001$) compared to control. **Figure 8 (2)** shows the results of the quantification of globular structures in somas. Administration of KCl increased the number of globular structures in somas to 202.0 ± 11.6 % (Kruskal-Wallis test: $\chi2(2, N = 431) = 69$, $p < .001$; post-hoc Dunn's multiple comparison test: $p < .001$) compared to control. After the incubation with dex, the number of globular structures in somas increased to 164.5 ± 7.8 % (post-hoc Dunn's multiple comparison test: $p < .001$) compared to control. In accordance with the results shown in **6.1**, these findings indicate that dex application rapidly triggers release of synaptic vesicles in 1C11-5HT in neurites as well as in somas.

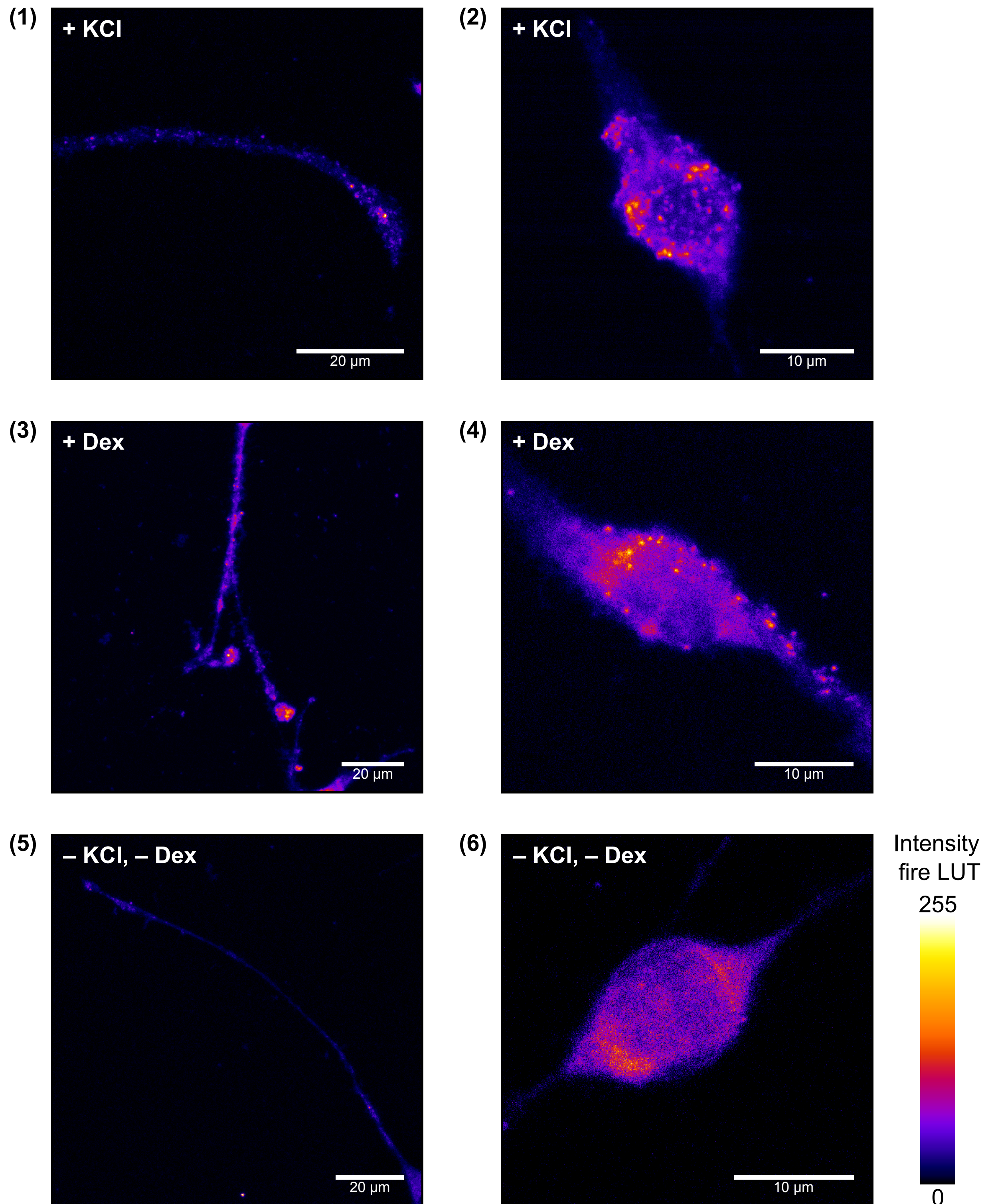

Figure 7: 1C11-5HT stained with FM4-64FX after administration of dexamethasone or potassium chloride.
(1): 60mM KCl, neurites. **(2):** 60mM KCl, soma. **(3):** 20nM dex, neurite. **(4):** 20nM dex, soma. **(5):** control, neurite. **(6):** control, soma. After administration of KCl or dex, FM4-64FX uptake increased compared to the control in both, neurites and somas. This indicates an increased vesicle uptake and release upon administration of KCl or dex. Dex = dexamethasone; KCl = potassium chloride.

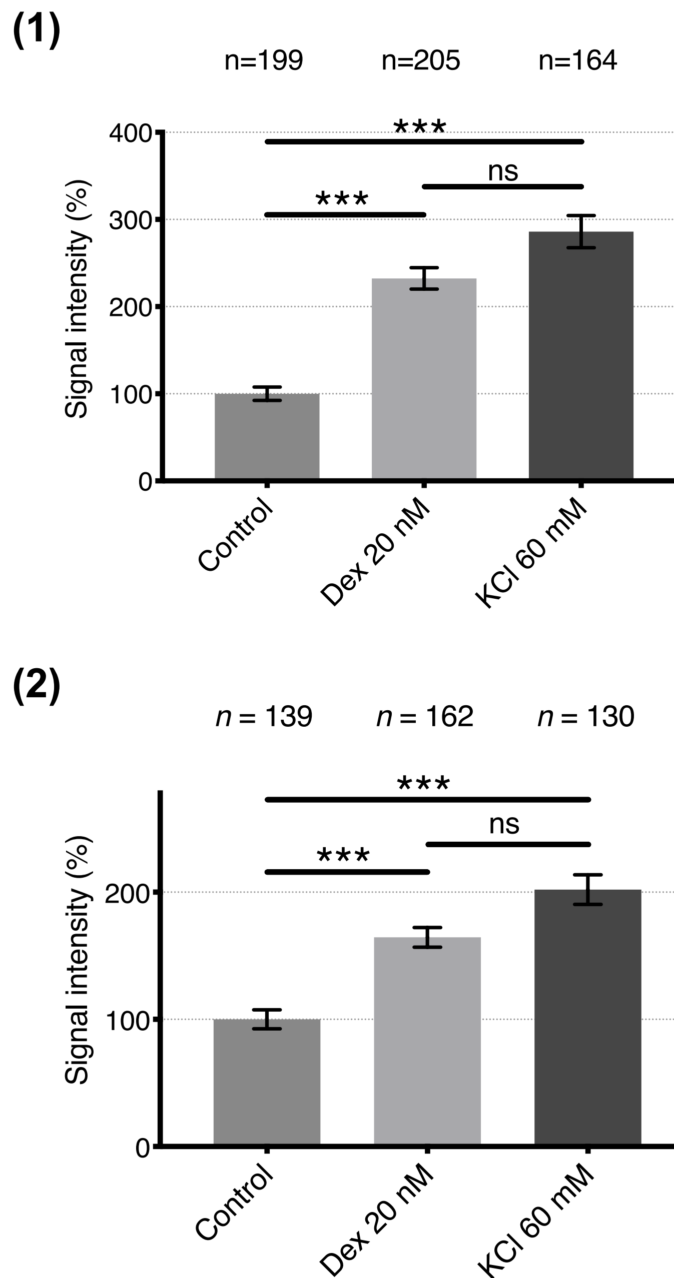

Figure 8: FM4-64FX uptake of (1) neurites and (2) somas in 1C11-5HT after administration of dexamethasone or potassium chloride.
(1): In neurites, treatment with dex caused a rapid increase in FM4-64FX uptake to 232.4 ± 12.2 % compared to control. Treatment with KCl triggered an increase in FM4-64FX uptake to 286.0 ± 18.4 % compared to control. **(2):** In somas, treatment with dex caused a rapid increase in FM4-64FX uptake to 164.5 ± 7.8 % compared to control. Treatment with KCl triggered an increase in FM4-64FX uptake to 202.0 ± 11.6 % compared to control. Results indicate rapid somatodendritic vesicle release and uptake upon dex administration. Bars depict mean ± standard error of the mean; *** ($p < .001$); ns (not significant). Groups were compared using Kruskal Wallis test and post-hoc Dunn's multiple comparison test. $CaCl_2$ = calcium chloride; Dex = dexamethasone; KCl = potassium chloride.

6.3 Subcellular fractionation and detection of glucocorticoid receptor and synaptic markers

To investigate if there is a possible interaction between GR and synaptic release sites to mediate rapid vesicular release, the distribution of GR between the membrane/vesicular compartment and the cytosol was analyzed *in vivo*. After subcellular fractionation of mice CNS, GR and vesicular markers were immunologically detected in the probes. **Figure 9 (1)** shows the results of the detection of GR and rab3 in the subcellular fractionation probes. Rab3 is a G protein localized in synaptic vesicles and pivotal for vesicular neurotransmitter release (Tanaka et al., 2001). We found Rab3 primarily in the membrane/vesicular fraction of CNS cells, and small amounts were also detectable in the cytosolic fractions. GR was primarily present in the cytosol, but we also found that GR was present in the membrane/vesicular fraction of the brain cells, which indicates spatial proximity of GR and the cell membrane as well as vesicles. **Figure 9 (2)** shows the results of the detection of GR and synapsin 1 in the subcellular fractionation probes. Synapsin 1 is a neuron-specific marker of synapses, which is present in small vesicles. We found Synapsin 1 in the membrane/vesicular fraction of CNS cells, and to a smaller extend also in the cytosolic fraction. GR was primarily located in the cytosolic fraction but, again, was also present in the membrane/vesicular fraction. This indicates spatial proximity of GR and the cell membrane as well as vesicles.

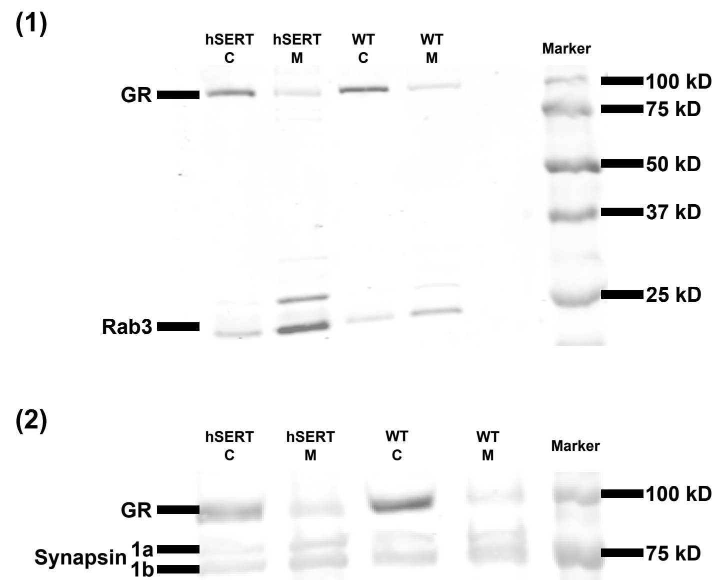

Figure 9: Immunological detection of glucocorticoid receptor and synaptic markers in the subcellular fractions of central nervous systems of wild-type mice and mice carrying the human serotonin transporter.
(1): The vesicular marker protein rab3 was detected in the membrane/vesicular fraction, and to a smaller extent in the cytosolic fraction. **(2):** The synaptic marker synapsin 1 was detectable in the membrane/vesicular fraction, and to a smaller extent in the cytosolic fraction. Synapsin 1a and 1b are two splicing variants. In (1) and (2), the GR was present in the cytosolic fraction, but was also found in the membrane/vesicular fraction of wild-type and humane SERT mice, which indicates a spatial proximity of the GR and membrane or vesicular proteins *in vivo*. Image colors were inverted for visibility, but are unmodified apart from that. GR = glucocorticoid receptor; hSERT C = human SERT mice, cytosolic fraction; hSERT M = human SERT mice, membrane/vesicular fraction; kD = kilodalton; SERT = serotonin transporter; WT C = wild-type mice, cytosolic fraction; WT M = wild-type mice, membrane/vesicular fraction.

6.4 Immunofluorescence: Colocalization of glucocorticoid receptor and synaptotagmin 1 in 1C11-5HT

To investigate if GR can be found in proximity to synaptic release sites, which is a prerequisite for an interaction, colocalization between GR and synaptotagmin 1 was quantified in 1C11-5HT. **Figure 10** shows exemplary colocalization images and the quantification of colocalization using PCC, which was 0.73 ± 0.01 (this data is also shown in Paul et al. (2021)). PCC was significantly different to 0 (comparison to a PCC of 0: $t(66) = 77.95$, $p < .001$). R^2 was 0.53, meaning that 53 % of the variability in the GR fluorescent signal could be explained by the variability in the synaptotagmin 1 signal. Applying the framework proposed by Zinchuk and colleagues (2013) to interpret PCC values, 0.73 is can be considered a strong colocalization, meaning that

signal intensities of the GR staining strongly overlapped and positively correlated with signal intensities of the synaptotagmin 1 staining. This provides evidence for spatial proximity between the GR and vesicular release sites in 1C11-5HT.

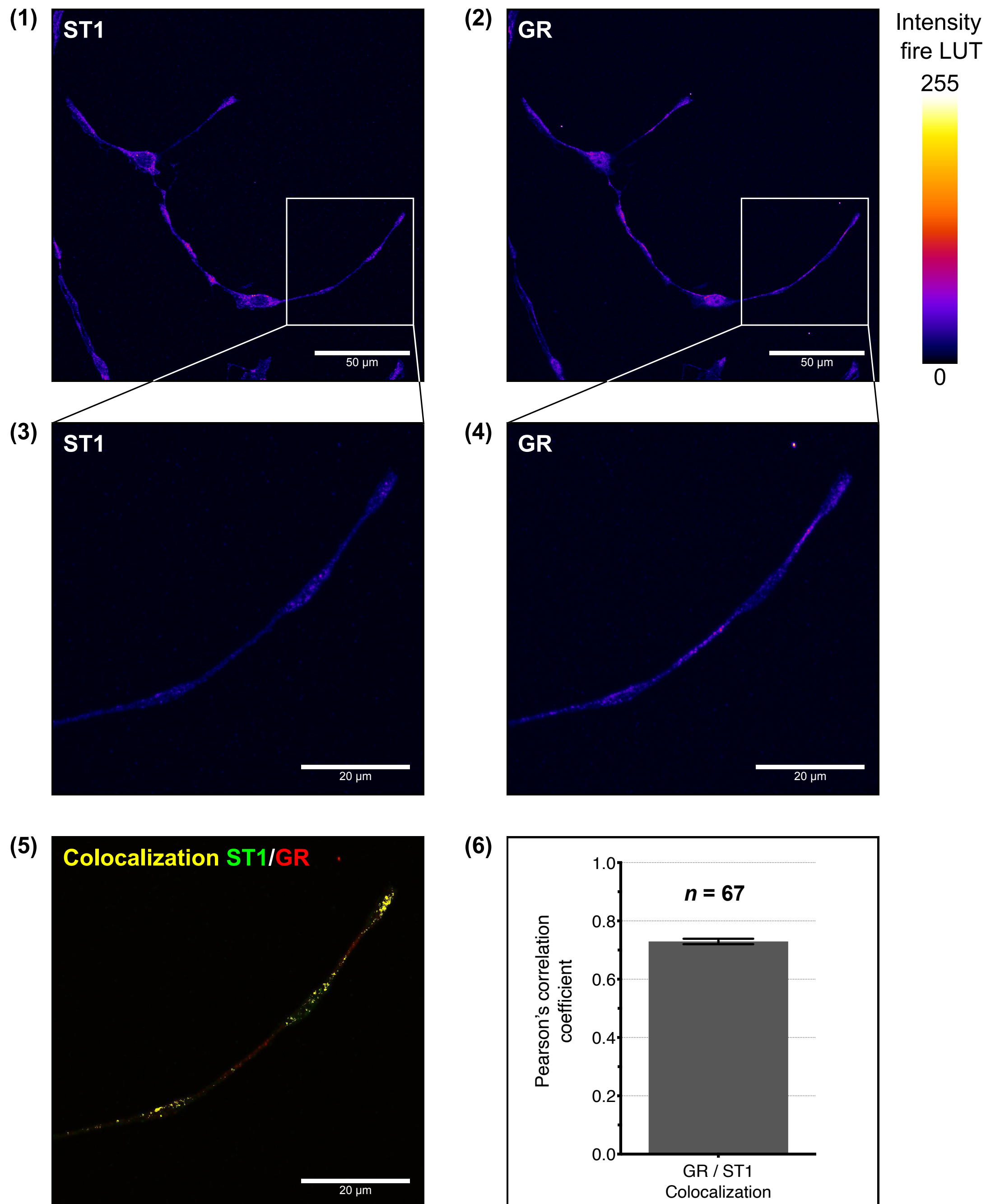

Figure 10: Colocalization of glucocorticoid receptor and synaptotagmin 1 in neurites of 1C11-5HT.
(1),(3): ST1 staining of permeabilized 1C11-5HT. **(2),(4):** GR staining of permeabilized 1C11-5HT. **(5):** Merged images (3) and (4) with colocalized particles highlighted in yellow, ST1 signal in green and GR signal in red. **(6):** Pearson's correlation coefficient of GR and ST1 signals was 0.73 ± 0.01 (Paul et al., 2021). The results indicate strong colocalization and spatial proximity of GR and ST1 signals in 1C11-5HT. Bar depicts mean ± standard error of mean. GR = glucocorticoid receptor; ST1 = synaptotagmin 1.

6.5 Immunofluorescence: Colocalization of glucocorticoid receptor and the serotonin transporter in 1C11-5HT

It was investigated if activated GR has spatial proximity to the membrane-bound SERT, which is a prerequisite for GR to rapidly alter 5-HT uptake. 1C11-5HT were incubated with dex for 15 min or 30 min, stained for GR and the membrane-bound SERT, and colocalization of GR and the membrane-bound SERT was quantified in neurites. **Figure 11** shows exemplary fluorescent images. As shown in **Figure 12 (1)**, colocalization between the membrane-bound SERT and GR increased compared to control when 1C11-5HT were incubated with dex for 15 min (PCC of 0.30 ± 0.02 vs. 0.39 ± 0.01; Kruskal-Wallis test: $\chi 2(2, N = 231) = 31.81$, $p < .001$; post-hoc Dunn's multiple comparison test: $p = .003$). This effect did not occur in the presence of the GR antagonist mif (PCC of 0.24 ± 0.02; post-hoc Dunn's multiple comparison test compared to control: $p = .102$). This indicates a recruitment of GR right after its activation towards the SERT. As shown in **Figure 12 (2)**, colocalization of the membrane-bound SERT and GR decreased compared to control when 1C11-5HT were incubated with dex for 30 min (PCC of 0.62 ± 0.01 vs. 0.53 ± 0.02; Kruskal-Wallis test: $\chi 2(2, N = 190) = 10.89$, $p = .004$; post-hoc Dunn's multiple comparison test: $p = .003$). This effect did not occur in the presence of the GR antagonist mif (PCC of 0.60 ± 0.02; post-hoc Dunn's multiple comparison test compared to control: $p > .999$).

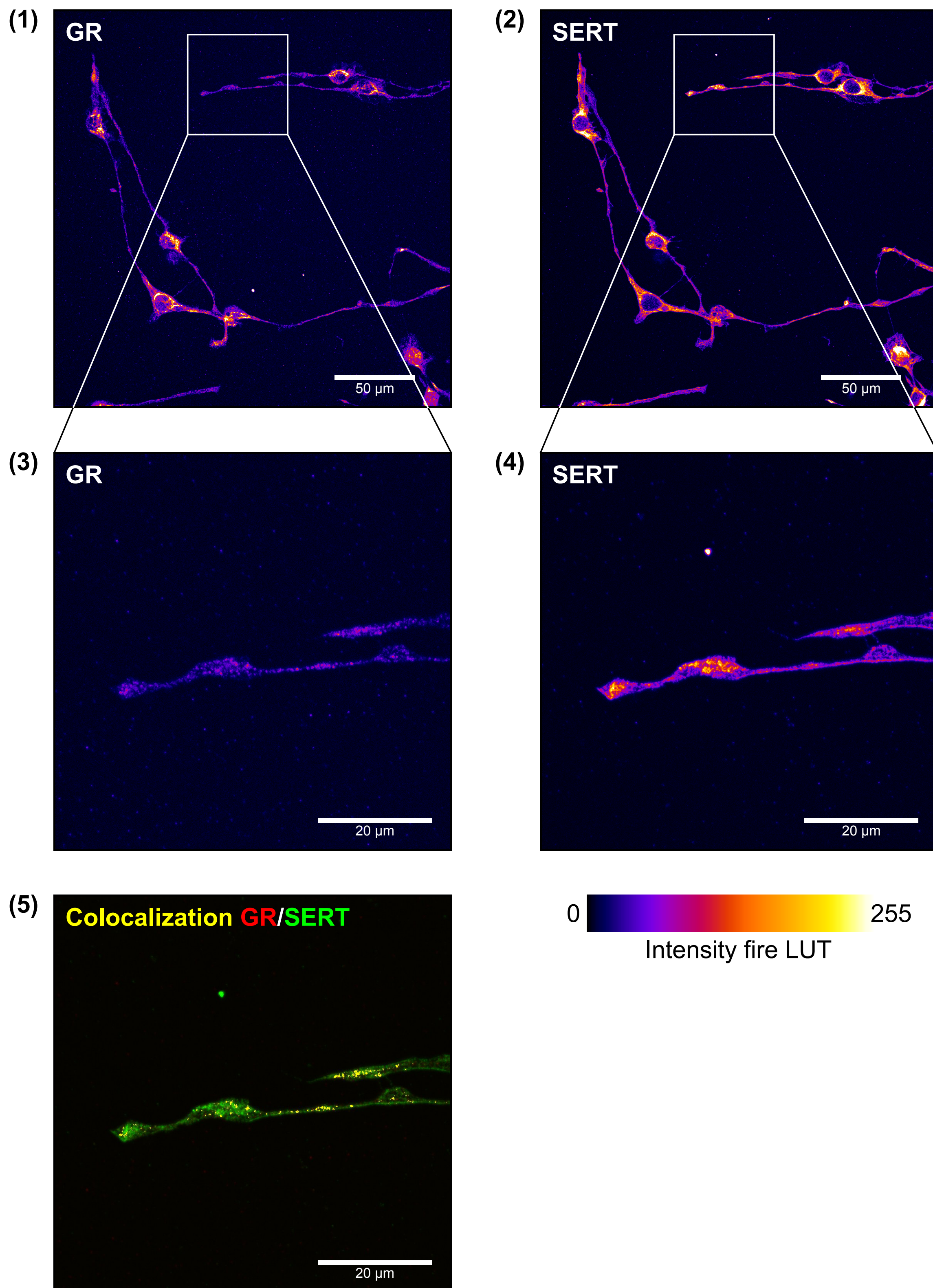

Figure 11: Exemplary images of colocalization of the serotonin transporter and glucocorticoid receptor in neurites of 1C11-5HT.
(1),(3): GR staining of 1C11-5HT. **(2),(4):** Membrane-bound SERT staining of 1C11-5HT. **(5):** Merged images (3) and (4) with colocalized particles highlighted in yellow, GR signal in red and SERT signal in green. Pearson's correlation coefficient for the images (3) and (4) is 0.71. GR = glucocorticoid receptor; SERT = serotonin transporter.

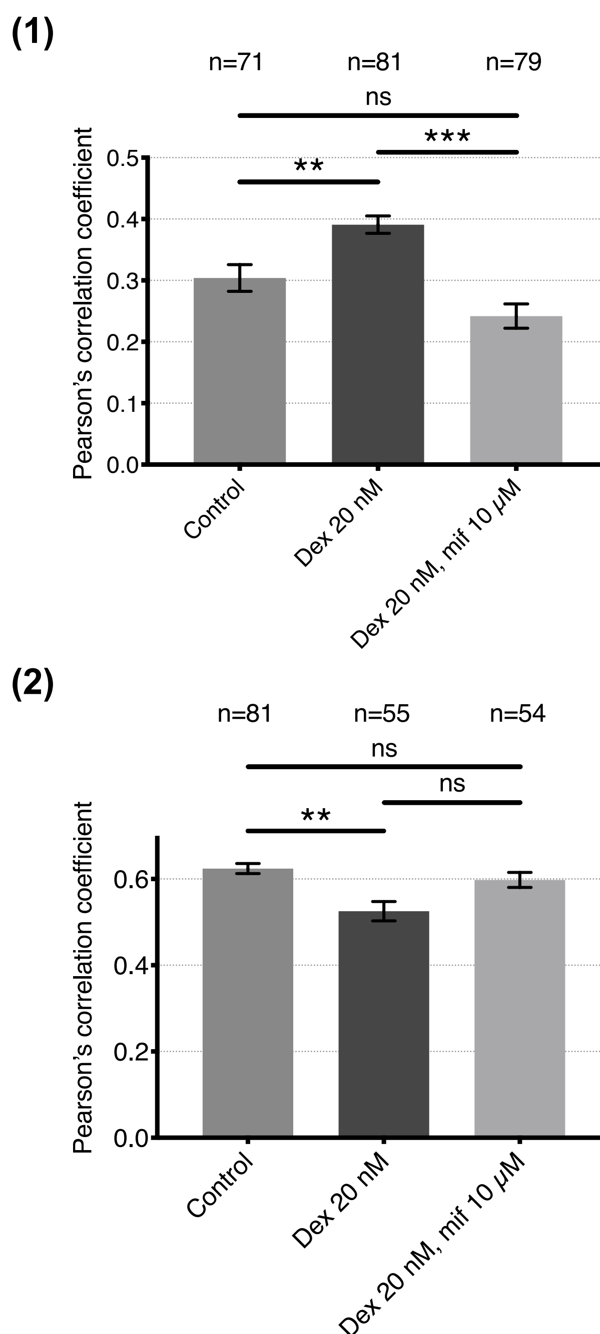

Figure 12: Colocalization of membrane-bound serotonin transporter and glucocorticoid receptor in 1C11-5HT after administration of dexamethasone.
1C11-5HT were treated for either (1) 15 min or (2) 30 min with dex, stained for GR and the membrane-bound serotonin transporter, and colocalization was calculated using Pearson's correlation coefficient. **(1):** Treatment with dex for 15 min enhanced Pearson's correlation coefficient from 0.30 ± 0.02 to 0.39 ± 0.01. This effect was not observable in the presence of mif (Pearson's correlation coefficient of 0.24 ± 0.02). **(2):** Treatment with dex for 30 min reduced Pearson's correlation coefficient from 0.62 ± 0.01 to 0.53 ± 0.02. This effect was not observable in the presence of mif (Pearson's correlation coefficient of 0.60 ± 0.02). Following an initial increase in colocalization, spatial proximity between GR and membrane-bound serotonin transporter decreased 30 min after GR activation. Bars depict mean ± standard error of the mean; ** ($p < .01$); *** ($p < .001$); ns (not significant). Groups were compared using Kruskal Wallis test and post-hoc Dunn's multiple comparison test. Dex = dexamethasone; GR = glucocorticoid receptor; mif = mifepristone.

7 Discussion

7.1 Mechanism of rapid serotonin release after glucocorticoid receptor activation

It has been shown that acute stress and GR activation rapidly enhance extracellular 5-HT levels *in vivo*. However, it remained unknown how GR activation cause 5-HT release on a cellular level. Results of this work indicate that GR activation triggers rapid vesicular 5-HT release from somas and neurites. Further, dex-induced vesicular release was independent of extracellular calcium depletion (summarized in **Figure 13**). FFN511, which is taken into presynaptic vesicles by the vesicular monoamine transporter 2, directly visualizes vesicular release. Using life cell imaging, FFN511 signal intensities were determined after administration of KCl and dex in the presence or absence of calcium. KCl sparked vesicular release by depolarization of the cell membrane (Sarkar et al., 2012; Trueta et al., 2003), and dex caused vesicular release comparable to KCl-induced release. In a second experiment, 1C11-5HT were stained with FM4-64FX, which binds to cellular and vesicular membranes (W. J. Betz et al., 1996), before administration of either KCl or dex. Using fluorescent microscopy, vesicular FM4-64FX uptake was quantified as an indirect measure of vesicular release (W. J. Betz & Bewick, 1992; W. J. Betz et al., 1992; Sarkar et al., 2012; Trueta et al., 2003). Confirming results from the FFN511 live cell imaging, vesicle formation in somas and neurites caused by dex was comparable to vesicle formation observed after KCl administration, indicating vesicular release from somas and neurites.

The work at hand extends the existing *in vivo* literature on extracellular 5-HT levels after stress and glucocorticoid administration. It was shown that acute restraint stress caused a rapid increase in extracellular 5-HT levels in the hippocampus of WT mice (Weber & Bartsch, 2014). Within minutes after stress cessation, 5-HT levels plunged back to baseline. This effect was mediated by the GR, as GR-knockout mice did not show an increase in extracellular 5-HT. In a different study, using *in vivo* microdialysis and HPLC coupled with electrochemical detection, stress significantly enhanced extracellular 5-HT levels in the ventral hippocampus of rats (Wright et al., 1992). Not only stress, but also the direct *in vivo* application of corticosteroids caused a dose-

dependent increase in extracellular 5-HT, as shown for the hippocampus of lizards and rats (Barr & Forster, 2011; Summers et al., 2003). The increase in extracellular 5-HT levels after administration of corticosterone was blocked by the GR antagonist mif, indicating GR-dependency (Barr & Forster, 2011). Our *in vitro* data support the finding that 5-HT release depends on GR activation. Further, our work shows that glucocorticoids, which are the physiological mediator of the stress response, trigger immediate vesicular release, thereby enhancing short-term extracellular 5-HT levels.

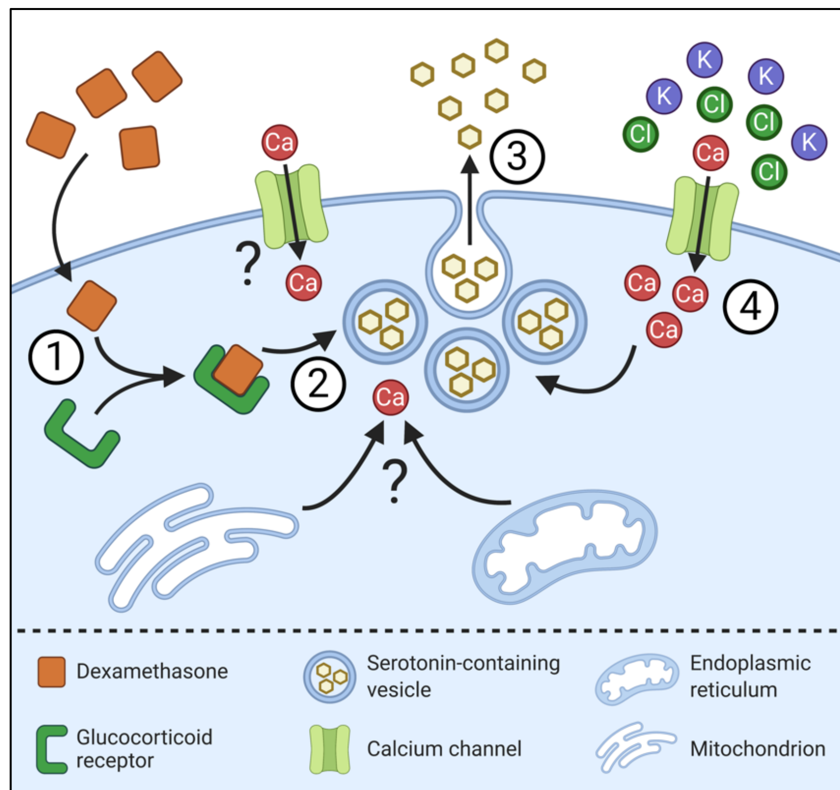

Figure 13: Schematic illustration of rapid, dexamethasone-induced release of serotonin. Dexamethasone activates the glucocorticoid receptor (1), which resides in proximity to vesicular release sites. This rapidly provokes somatodendritic vesicular release (2) of serotonin in the extracellular space (3), similar to depolarization with potassium chloride (4). Vesicular release also takes place in absence of extracellular calcium, indicating that calcium might also be recruited from intracellular storage sites such as the endoplasmic reticulum or mitochondria (question marks). Own illustration created with BioRender.com.

In 1C11-5HT, vesicular 5-HT release took place not only along neurites but also at somas. Somatic vesicular release after depolarization has been shown for 5-HT neurons of the raphé nuclei of rats and for 5-HT perikarya of nodosa ganglia of cats (Fueri, Faudon, Hery, & Hery, 1984; Sarkar et al., 2012). For serotonergic neurons of

the leech, a frequency-dependent somatic 5-HT vesicle release was described (De-Miguel & Trueta, 2005; Trueta et al., 2003; Trueta, Sánchez-Armass, Morales, & De-Miguel, 2004). Additional *in vivo* studies with rats and cats also indicated that 5-HT release did not only take place at axons but also at somas and dendrites (Adell, Carceller, & Artigas, 1993; Becquet, Faudon, & Hery, 1990; Piñeyro & Blier, 1999). Further, electrophysiological recordings of dorsal raphé neurons in mice suggested somatodendritic 5-HT release (Matthäus et al., 2016). For murine embryonic stem cell-derived 5-HT neurons, Lau et al. (2010) showed the existence of 5-HT vesicles over cell bodies and neurites using FM4-64FX and FM1-43 dyes. Depolarization triggered calcium influx visualized by Fluo4 and vesicle-mediated neurotransmitter release. Somatodendritic 5-HT release concurs with the understanding that 5-HT is not primarily released via one-to-one intercellular synaptic communications at the axons of neurons (also called wiring transmission), but via volume transmission to the extracellular space at varicosities alongside the somatodendritic cell membranes (Quentin, Belmer, & Maroteaux, 2018; Vizi, Kiss, & Lendvai, 2004).

Interestingly, our results indicate that extracellular calcium is dispensable for the dex-triggered vesicle release, which is contradictory to previous studies. Kerwin and Pycock (1979) showed that KCl enhances extracellular 5-HT only in the presence of calcium in the medium. Further, 5-HT release observed after electrical stimulation of raphé nuclei in rats was calcium dependent, most likely mediated via N-type voltage-gated calcium channels (Bagdy & Harsing, 1995). Even the spontaneous 5-HT release *in vivo* in cats and rats was diminished in the absence of extracellular calcium (Adell & Artigas, 1998; Hery, Faudon, & Ternaux, 1982). Only one study by Adell et al. (1993) did not find any effect of extracellular calcium depletion on 5-HT release. A recent study from our working group employed 1C11-5HT and showed that vesicular 5-HT release from neurites depended on extracellular calcium influx via L-type voltage-gated calcium channels (Paul et al., 2021). However, Paul et al. (2021) demonstrated extracellular calcium dependency for vesicular 5-HT release from neurites only, whereas the study at hand investigated calcium dependency for entire 5-HT neurons including the soma. Thus, while neurites of 1C11-5HT seem to depend on extracellular

calcium influx for vesicular release, the work at hand indicates that extracellular calcium is dispensable for somatic release in 1C11-5HT.

Generally, the intracellular presence of calcium is required for somatic vesicular neurotransmitter release (Lang & Jahn, 2008; Pang & Südhof, 2010; Südhof, 2004), but it might also stem from other sources than the extracellular medium. Our results suggest that somatic vesicle release upon dex application in 1C11-5HT in calcium-depleted medium may be mediated by recruitment of intracellular calcium storages. This explanation is supported by the work of Trueta et al. (2003; 2004) on somatic vesicular release in 5-HT neurons of the leech. Similar to findings of Paul et al. (2021) for neurites of 1C11-5HT, Trueta et al. (2003; 2004) demonstrated that extracellular calcium depletion and blockage of L-type voltage-gated calcium channels reduced somatic 5-HT secretion. Yet, the recruitment of calcium from intracellular storages by caffeine triggered somatic vesicle release, even in case of external calcium depletion and blocked external calcium influx. Underscoring this finding, depleting intracellular calcium storages with the calcium-ATPase inhibitor thapsigargin reduced caffeine-induced, somatic vesicle release (Trueta et al., 2004). Further, somatic, external calcium influx and internal calcium release were functionally coupled by ryanodine receptors (Trueta et al., 2004). Possible mobilization sites of intracellular calcium for vesicular release in 1C11-5HT are the endoplasmic reticulum, mitochondria, or even synaptic vesicles themselves (A. J. Verkhratsky & Petersen, 1998). Electron microscopy of somas of leech 5-HT neurons showed high proximity between vesicle clusters, cell membrane and smooth endoplasmic reticulum (Trueta et al., 2004), suggesting internal calcium release from the smooth endoplasmic reticulum. Other studies have also proven that calcium release from the smooth endoplasmic reticulum can trigger neurotransmitter release (A. Verkhratsky, 2005). Future investigations should comprise vesicular release experiments with inhibitors of internal and external calcium fluxes to investigate co-dependent regulation of dex-induced 5-HT release.

As a strength of our work, FFN511 and FM4-64FX are well-established agents to visualize neuronal vesicular release. Nevertheless, the experiments bear weaknesses inherent to the nature of the fluorescence microscopy. First of all, images inevitably

carry background noise. Settings of the microscope were optimized to minimize background while having good fluorescent signals; nevertheless, background noise could not be entirely excluded. To tackle this problem, equal background correction was performed to all images during image analysis. Further, 1C11-5HT show minimal autofluorescence, which is visible in fluorescence microscopy images of unstained 1C11-5HT. For the FM4-64FX dye, the mild, homogeneous staining of the cell membrane overlay the staining of vesicle clusters in the fluorescence images. A quencher could have been used to suppress fluorescent signal of the cell membrane and to better visualize vesicle cluster. However, globular structures in the images were very apparent and easily identifiable despite the overlying, slight staining of the cell membrane.

7.2 Interaction between glucocorticoid receptor and synaptic structures

Vesicular 5-HT release upon GR activation happens too rapidly to be mediated by the slow, genomic pathway of glucocorticoid action. In this pathway, the GR binds to glucocorticoids which passage through the cell membrane. GR-bound proteins such as heat shock protein 90 dissociate, and the activated GR is actively translocated in the nucleus (Beato & Sánchez-Pacheco, 1996; J. Zhou & Cidlowski, 2005). GR homodimers bind to glucocorticoid responsive elements in promotors of target genes and alter gene transcription (J. Zhou & Cidlowski, 2005). The rapid effect of the activated GR on vesicular 5-HT release, however, is most likely mediated by fast, non-genomic GR actions. Rapid, non-genomic effects by glucocorticoids on target cells have been described for multiple contexts in the brain and periphery. For instance, it was shown that glucocorticoids quickly inhibit glutamatergic synaptic connections to neurons of the paraventricular nucleus in rats (Di, Malcher-Lopes, Halmos, & Tasker, 2003). A different study found that the *in vivo* application of corticosterone in rats caused a quick increase in AMPA receptor surface diffusion (Groc, Choquet, & Chaouloff, 2008).

To investigate the role of GR in the rapid vesicle release, we determined the spatial proximity of GR and vesicular release sites, which is a prerequisite for a potential

interaction. First, GR and the synaptic markers rab3 and synapsin 1 were immunologically detected in the cytosolic and membrane/vesicular fractions of homogenized murine CNS. In a second experiment, colocalization between synaptotagmin 1 and GR was quantified in 1C11-5HT (data also presented in Paul et al. (2021)). Our results indicate that GR is located in the same cellular compartment as synaptic structures *in vivo*, and that GR has strong spatial proximity to the synaptic marker synaptotagmin 1 *in vitro*. Employing 1C11-5HT, Paul et al. (2021) also show a strong colocalization between GR and 5-HT as well as GR and FM4-64FX, which underscores the results presented in this thesis. The high proximity between the GR and synaptic markers indicates that the GR might have a relatively direct effect on synaptic structures, ultimately causing rapid vesicular 5-HT release. This could both be an immediate interaction, or an intermediate interaction mediated by other proteins.

Three possible ways of rapid, non-genomic actions of glucocorticoids have been proposed. Firstly, there is evidence of the existence of a membrane-bound GR and MR. Studies have shown that the direct, intracellular application of glucocorticoids with a patch pipette did not trigger rapid effects in the target cells, but the use of dex bound to bovine serum albumin, which cannot surpass cell membranes, had a similar effect on target cells as free dex (Di et al., 2003). Further, electron microscopy suggested the presence of the GR and MR in postsynaptic membrane densities (Johnson, Farb, Morrison, McEwen, & LeDoux, 2005; Prager, Brielmaier, Bergstrom, McGuire, & Johnson, 2010). Secondly, rapid, non-genomic actions of glucocorticoids might be mediated by a novel, up to this point unknown membrane-associated receptor for glucocorticoids. This hypothesis is based on the observation that some rapid effects of glucocorticoids on target cells are still observable in the presence of antagonists against the GR and MR. The novel GR is most likely G protein coupled (Groeneweg et al., 2012). To support this hypothesis, a G protein-coupled, membrane-bound GR has been identified in the amphibian *Taricha granulosa,* but has not been found in humans so far (Orchinik, Murray, & Moore, 1991). Thirdly, it has been proposed that the rapid, non-genomic effects of glucocorticoids are mediated by cytosolic GR and MR that associate with the cell membrane (Tasker et al., 2006). This hypothesis was based on the observation that the cytosolic estrogen receptors (not the GR or MR) can

associate with cell membrane caveolae (Razandi, Oh, Pedram, Schnitzer, & Levin, 2002). Two downstream signaling pathways have been identified for rapid GR effects. It was shown that corticosterone activates, via G proteins, the cAMP–protein kinase A pathway as well as the mitogen-activated protein kinase pathway (Malcher-Lopes et al., 2006; Xiao, Feng, & Chen, 2010). Potentially, the dex-activated GR, which might be membrane-bound or membrane-associated with close proximity to synaptic structures, mobilizes secondary signaling molecules and causes a release of surrounding 5-HT vesicles via activation of the cAMP-protein kinase A pathway or mitogen-activated protein kinase pathway.

The GR and the synaptic markers synapsin 1 as well as rab3 were detected in lysates of entire CNS of WT and hSERT mice. As 5-HT neurons are scarce in the CNS *in vivo*, the CNS lysates contained only few 5-HT neurons and primarily other CNS cells (astrocytes, microglia, non-5-HT neurons). Therefore, the detection of GR in membrane/vesicular fractions cannot be attributed to 5-HT neurons but to CNS cells in general. Further, as noted by Dreger (2003), the subcellular fractionation process is the methodological centerpiece of assigning proteins to specific compartments. The protocol in this work enriched cellular proteins in two fractions, the cytosolic fraction and the membrane/vesicular fraction, using differential centrifugation of tissue homogenate. It was noted that resolution of this method can be limited, with contaminating proteins from other organelles in the subcellular fractions (Lee, Tan, & Chung, 2010). Nevertheless, the detection of the synaptic proteins rab3 and synapsin 1 primarily in the membrane/vesicular fractions and barely in the cytosolic fractions indicated a good level of separation. The weak signal in the cytosolic fractions might also partially be due to trafficking of the respective proteins between compartments, as for example the membranes of the Golgi apparatus and endoplasmic reticulum are not enriched in the membrane preparation. The signals of the GR, rab3 and synapsin 1 were clearly identifiable, indicating that potential proteins bound to Golgi apparatus and endoplasmic reticulum membranes do not interfere with our qualitative detection.

In vivo results were confirmed using *in vitro* colocalization of GR and the vesicular marker synaptotagmin 1. The immunofluorescence protocol used in this experiment is

well-established, and potential crosstalk between laser channels was prevented by non-simultaneous imaging. The colocalization marker, PCC, has been used in several previous studies (Dunn et al., 2011). It is robust to background noise and does not only detect colocalization but also proportionality of corresponding signals (Dunn et al., 2011). PCC between the GR and synaptotagmin 1 was 0.73 and significantly greater than 0 (no correlation), which indicated a strong positive colocalization (Zinchuk et al., 2013) of GR and synaptotagmin 1 signals in 1C11-5HT. Taken together, our results indicate a spatial proximity between the GR and synaptic structures.

7.3 Interaction between activated glucocorticoid receptor and the serotonin transporter

The extracellular 5-HT concentration does not only depend on neuronal 5-HT release but also on 5-HT re-uptake; responsible for re-uptake is the SERT. It was shown that multiple proteins interact with the SERT to alter its activity or cell surface expression (Lau & Schloss, 2012). However, it is unclear whether activated GR rapidly interacts with the SERT to influence 5-HT uptake. 1C11-5HT were incubated with the GR agonist dex for either 15 min or 30 min. Subsequently, 1C11-5HT were stained for GR and the membrane-bound SERT, and colocalization was quantified using PCC. As illustrated in **Figure 14**, our results indicate a rapid increase in proximity between GR and membrane-bound SERT upon GR activation.

We found that 15 min after its activation, the spatial proximity of GR to the membrane-bound SERT increased. This effect was suppressed by the simultaneous application of the GR antagonist mif. SERT can be found in the cell membrane as well as in intracellular compartments (Qian et al., 1997; Tao-Cheng & Zhou, 1999; Whitworth et al., 2002). Its activity is modified by trafficking to the cell membrane and vice versa, or by directly affecting activity of the transporter (Lau & Schloss, 2012). Proteins such as SCAMP-2, hic-5, syntaxin 1A, neuronal nitric oxide synthase 1, rab4, as well as integrin subunits αIIb and β3 directly interact with SERT and either modify its activity or cause internalization (Ahmed et al., 2008; Carneiro & Blakely, 2006; Carneiro et al., 2008; Chanrion et al., 2007; Müller et al., 2006; Quick, 2003). Further, it was shown

that the activation of protein kinases such as protein kinase C plays a role in the allosteric modulation of SERT activity (Mnie-Filali et al., 2016). In the work at hand, the GR was rapidly recruited to the SERT after its activation, as indicated by an increase in colocalization. In spatial proximity, the GR might directly or via mediator proteins interact with the SERT and decrease its activity, which would subsequently increase extracellular 5-HT.

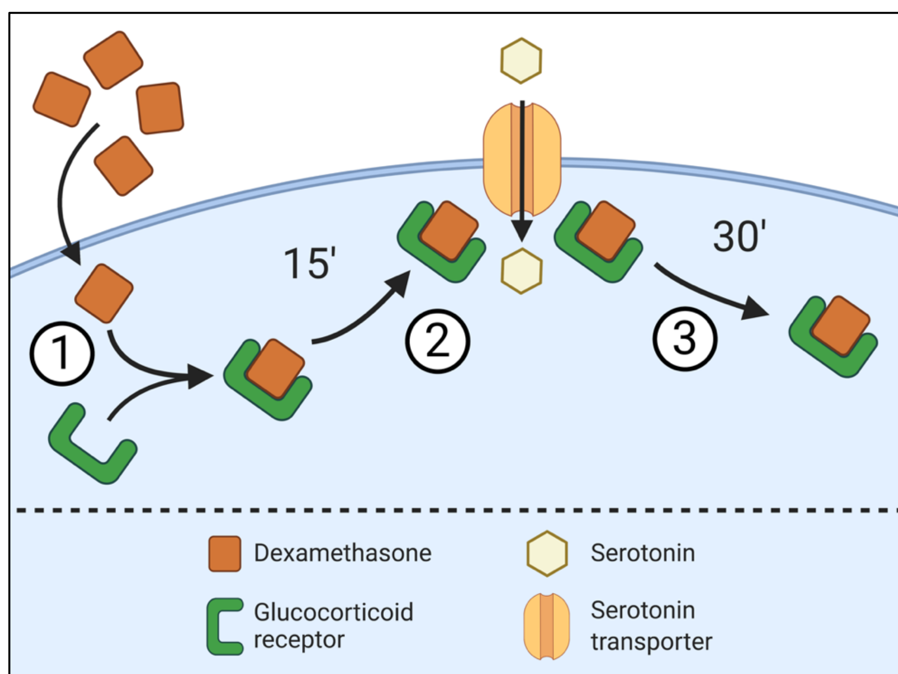

Figure 14: Schematic illustration of the proximity between activated glucocorticoid receptor and the serotonin transporter.
Upon activation with dexamethasone (1), the glucocorticoid receptor is rapidly recruited to the serotonin transporter (2). After 30 min, dropping levels of colocalization indicate that the glucocorticoid receptor dissociates from the serotonin transporter (3). Own illustration created with BioRender.com.

Our results show that 30 min after GR activation, the spatial proximity between GR and the membrane-bound SERT dropped below the initial level. This effect was not observable in the presence of the GR antagonist mif. One possible explanation is that upon activation, the GR is quickly recruited to the membrane-bound SERT, but then recedes again. Another, more convincing explanation is offered by the findings of Lau et al. (2013). Their study with embryonic stem cell-derived serotonergic neurons showed that already one hour after dex application, SERT concentration on the cell

surface increases in the presence of the protein synthesis inhibitor cycloheximide. Although SERT concentrations on the cell surface were not quantified for shorter time frames below one hour after dex application, they concluded that dex recruits SERT rapidly and non-genomically from intracellular compartments to the cell surface. The additional recruitment of SERT molecules to the cell surface could potentially mask the increase in colocalization of activated GR to membrane-bound SERT that we found in our study.

The PCC of the control was 0.30 for 15 min dex treatment and 0.62 for 30 min dex treatment. This difference can be explained by different antibody uptake and staining quality of the GR and membrane-bound SERT. The cellular dye uptake differs across experiments (Dunn et al., 2011), with a lower cellular dye uptake resulting in generally lower PCC values. We account for this variability across cells by comparing the PCC after dex application to its respective control. The advantage of the use of PCC as a measure of colocalization is its robustness (Dunn et al., 2011). PCC is well-established for quantitative colocalization analyses and its use is independent of image background. That is a major advantage to Manders' Colocalization Coefficients, which is very sensitive to background signal and requires image pre-processing prior to application (Dunn et al., 2011). As a limitation, PCC is unable to comprehend non-linear, complex relationships. This is another difference to Manders' Colocalization Coefficients, which solely calculate signal overlap and ignore the nature of the relationship between signal intensities (Dunn et al., 2011). Because it was shown that high background-to-cell ratios artificially inflate PCC values (Dunn et al., 2011), we determined PCC for ROIs around neurons, and to avoid crosstalk between channels, fluorescent images were taken with non-simultaneous imaging.

7.4 Conclusions and areas of further research

This thesis expands the knowledge on how glucocorticoids interact with 5-HT neurons. More specifically, it was investigated how the activation of GR triggers a rapid increase in extracellular 5-HT. Employing murine stem cell-derived 5-HT neurons, it was shown that activated GR rapidly causes vesicular release of 5-HT *in vitro*. Upon GR activation, vesicles are released from both, somas and neurites. In this work,

extracellular calcium was dispensable for dex-induced, vesicular 5-HT release, which indicates that upon dex administration, calcium is recruited from intracellular calcium depots, for example mitochondria or the endoplasmic reticulum (A. J. Verkhratsky & Petersen, 1998). Further, we demonstrate that GR resides in spatial proximity to synaptic release sites, which indicates that GR might cause rapid 5-HT release by interacting with synaptic release sites (Paul et al., 2021). This interaction could either be direct or indirect via the cAMP–protein kinase A or the mitogen-activated protein kinase pathway (Malcher-Lopes et al., 2006; Xiao et al., 2010). Additionally, within 15 min after its activation, the GR is recruited towards the membrane-bound SERT, as shown by enhanced colocalization, which is a prerequisite for interaction. Thus, GR might directly or via mediating molecules interact with the membrane-bound SERT and reduce 5-HT uptake, thereby increasing extracellular 5-HT. Colocalization between GR and membrane-bound SERT dropped again 30 min after GR activation, which could imply a dissociation of GR and the SERT, or could be explained by a GR-induced recruitment of additional SERT molecules from intracellular compartments to the membrane, as suggested by Lau et al. (2013).

Several additional areas of research evolve from this work. Vesicular release upon dex application was shown for murine stem cell-derived 1C11-5HT *in vitro*. 1C11-5HT show most properties of 5-HT neurons, like a 5-HT metabolism, expression of 5-HT receptors, and expression of the SERT (Baudry, Mouillet-Richard, Schneider, Launay, & Kellermann, 2010; Buc-Caron et al., 1990; Launay, Mouillet-Richard, Baudry, Pietri, & Kellermann, 2011; Mouillet-Richard et al., 2000). The 1C11-5HT model of rodent 5-HT neurons offers the chance to conduct experiments on 5-HT neurotransmission on the molecular level *in vitro,* which are difficult to conduct *in vivo* (Henke et al., 2018). Reasons include the overall low number of 5-HT neurons in the CNS and the arborization of their dendrites to various CNS regions (Hornung, 2003). In contrast, the 1C11-5HT model establishes an isolated environment of interacting 5-HT neurons, where influences of surrounding cells can be excluded. However, 1C11-5HT are neither human cells, nor do they resemble the complex environment of the brain *in vivo*, which puts limitations on this work. Hence, future studies should employ human 5-HT neurons and *in vivo* rodent models to validate the results of the work at hand.

Another area of future research is the identification of potential synaptic interaction partners of the GR and signaling pathways that mediate rapid, vesicular 5-HT release. A direct interaction, for example, could be examined in experiments using fluorescence resonance energy transfer or co-immunoprecipitation, and signaling pathways could be identified by the application of inhibitors of the respective pathways, such as the cAMP–protein kinase A pathway as well as the mitogen-activated protein kinase pathway (Malcher-Lopes et al., 2006; Xiao et al., 2010).

8 Summary

Disturbances in the serotonergic neurotransmitter system are known to play a pivotal role in the pathogenesis of many psychiatric diseases, particularly in depression. Further, depressive patients show alterations in the stress response, namely in the hypothalamic-pituitary-adrenal axis. The hypothalamic-pituitary-adrenal axis and the serotonergic neurotransmitter system share close interrelationships; glucocorticoids, as mediators of the body's stress response, affect serotonergic neurons and vice versa. Understanding the interplay between those systems can help understand the pathogenesis of psychiatric diseases such as depression.

Previous research suggested that glucocorticoids cause an immediate increase in extracellular hippocampal serotonin. However, the mechanisms of glucocorticoid receptor-induced serotonin release are unknown. This work examined the molecular process of immediate, glucocorticoid receptor-mediated serotonin release and uptake by employing murine stem cell-derived 5-HT neurons (1C11-5HT). It was investigated if glucocorticoids trigger rapid, vesicular serotonin release *in vitro*, and if there is spatial proximity between the glucocorticoid receptor and vesicular release sites, which is a prerequisite for a possible interaction. Lastly, it was investigated whether the activated glucocorticoid receptor shows spatial proximity to the membrane-bound serotonin transporter, which is a prerequisite to alter serotonin uptake.

Employing life cell imaging with the vesicular dye FFN511 and the styryl dye FM4-64FX, it was shown that dexamethasone causes immediate vesicular serotonin

release and subsequent vesicle recycling and uptake, comparable to the vesicle release and uptake observed after depolarization with potassium chloride. Vesicle release was also observable in the absence of calcium in the extracellular medium and occurred on the level of neurites as well as somas. To further investigate the role of the glucocorticoid receptor in the release process, subcellular fractionation of mice central nervous system and subsequent immunological detection showed that the glucocorticoid receptor has spatial proximity to the vesicular and synaptic markers rab3 and synapsin 1 *in vivo*. Those results were confirmed *in vitro*, as it was shown that the glucocorticoid receptor colocalizes with the vesicular protein synaptotagmin 1. To examine whether the activated glucocorticoid receptor could potentially affect serotonin uptake by interacting with the membrane-bound serotonin transporter, colocalization analysis in 1C11-5HT showed an increased spatial proximity between the membrane-bound serotonin transporter and the activated glucocorticoid receptor 15 min after glucocorticoid receptor activation. 30 min after glucocorticoid receptor activation, colocalization decreased below the initial level. Both effects were not observable under the simultaneous application of the glucocorticoid receptor antagonist mifepristone.

This work extends the knowledge of the interplay between glucocorticoids and serotonergic neurons. Firstly, it was shown that glucocorticoids cause an immediate release of serotonergic vesicles, which was independent from extracellular calcium and took place at neurites and somas. Secondly, the high proximity between the glucocorticoid receptor and vesicular markers is an indication that the glucocorticoid receptor might directly or via signaling pathways interact with vesicular release sites to trigger serotonin release. Thirdly, an increase of short-term colocalization between the membrane-bound serotonin transporter and the activated glucocorticoid receptor could indicate a possible interaction between the glucocorticoid receptor and the serotonin transporter to modify serotonin re-uptake. Extending the knowledge of the interrelationship between glucocorticoids as mediators of the body's stress response and the serotonergic neurotransmitter system might ultimately help understand the underlying mechanisms in the pathogenesis of depression and other psychiatric disorders.

9 Bibliography

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10 Curriculum vitae

Name:	Nicolas Paul
Date of birth:	November 10, 1992
Place of birth:	Hamburg

Secondary education

2003 – 2008	Heilwig-Gymnasium, Hamburg
2008 – 2009	Summit High School, Bend, Oregon, USA
2009 – 2011	Heilwig-Gymnasium, Hamburg
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University education

WS 2011	Commencement of medical studies at Heidelberg University
05.09.2013	1st part of medical state examination (M1); very good (1.0)
WS 2014	Commencement of studies for the Master of Science in Health Economics at Heidelberg University
12.10.2017	2nd part of medical state examination (M2); very good (1.0)
20.11.2018	3rd part of medical state examination (M3); good (2.0)
	Final grade of medical studies: very good (1.33)
21.11.2018	Master of Science in Health Economics; very good (1.3)

Employment

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