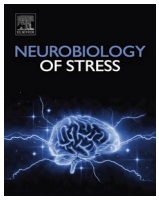
Neurobiology of Stress 36 (2025) 100722

Contents lists available at ScienceDirect

Neurobiology of Stress

journal homepage: www.elsevier.com/locate/ynstr



Dynamic changes of serotonin transporter expression in the prefrontal cortex evoked by aggressive social interactions

Huba Szebik [a,b], Christina Miskolczi [a,b], Bíborka Bruzsik [a], Gyula Balla [a], Soma Szabó [a], László Biró [a,c,1,*], Éva Mikics [a,**,1]

[a] Translational Behavioral Neuroscience Research Group, HUN-REN Institute of Experimental Medicine, Budapest, Hungary
[b] Semmelweis University, Doctoral School, Budapest, Hungary
[c] Thalamus Research Group, HUN-REN Institute of Experimental Medicine, Budapest, Hungary

ARTICLE INFO

Handling Editor: Rita Valentino

Keywords:
Aggression
Serotonin
Prefrontal cortex
Post-weaning social isolation
Serotonin transporter
Super-resolution
Early life stress

ABSTRACT

Aggression is a complex behavior influenced by developmental experiences, internal state, and social context, yet its neurobiological underpinnings remain insufficiently understood. The serotonergic system, particularly the serotonin transporter (SERT), plays a crucial role in aggression regulation. Here, we investigated region-specific, dynamic changes in SERT expression following aggressive interactions and in mice subjected to early-life social adversity. We found that aggressive encounters (resident-intruder test) triggered a significant, rapid increase in SERT immunoreactivity within 90 min, accompanied by neuronal activation in aggression-related brain regions, including the medial prefrontal cortex (mPFC), lateral septum (LS), medial amygdala (MeA), ventromedial hypothalamus (VMHvl), lateral habenula (LH), and dorsal raphe (DR), but not in the paraventricular thalamus (PVT). Notably, this SERT upregulation occurred across the aggression circuitry but was accompanied by a significant increase in 5-HT levels only in the mPFC, a key region in top-down regulation of social and aggressive behavior. This SERT upregulation was not observed following exposure to a non-social challenge, suggesting that it may be more specifically associated with social contexts. Using super-resolution microscopy, we identified an increased density of SERT localization points within serotonergic mPFC axons after an aggressive encounter. Social isolation during adolescence, a model of early social neglect, impaired this rapid SERT response, particularly in the ventral and medial orbitofrontal regions, and altered the relationship between SERT levels and aggression-related behaviors. These findings demonstrate that SERT expression undergoes rapid, experience-dependent plasticity in response to social aggression, and that early-life adversity disrupts this adaptive mechanism, providing new insights into the serotonergic regulation of aggression and its potential relevance for stress-related social dysfunctions.

1. Introduction

Violence places a major and growing health and economic burden on society (Cardia, 2002). Despite this, aggression remains relatively understudied compared to other affective and emotional behaviors (Anderson, 2012; Flanigan and Russo, 2019), and there is a lack of treatments specifically targeting pathological aggression, with existing treatment methods showing modest efficacy (Barlow et al., 2000; van Schalkwyk et al., 2018). Aggression is a highly heterogeneous behavior influenced by developmental experiences, internal state, and social contextual cues, providing additional challenges in the treatment of aggression-related psychiatric symptoms (Volavka and Citrome, 2011). As such, elucidating the neurobiological mechanisms regulating aggression under normal versus pathological conditions remains a crucial issue.

Childhood exposure to abuse or social neglect increases the likelihood of developing psychiatric disorders in adulthood that commonly include abnormal forms of social behavior, such as increased aggressive tendencies (Chapple et al., 2005; Gilbert et al., 2009; Glaser, 2000; Neigh et al., 2009; Stoltenborgh et al., 2011; Susman, 2006; Teicher

* Corresponding author. Thalamus Research Group, HUN-REN Institute of Experimental Medicine, Budapest, Hungary.
** Corresponding author.
E-mail addresses: biro.laszlo@koki.hu (L. Biró), mikics.eva@koki.hun-ren.hu (É. Mikics).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.ynstr.2025.100722
Received 20 December 2024; Received in revised form 17 March 2025; Accepted 25 March 2025
Available online 26 March 2025

et al., 2003, 2016). These behavioral changes are driven by neuronal alterations that also impact brain regions involved in the regulation of aggression (Lee and Hoaken, 2007; Teicher et al., 2003, 2016; Tóth et al., 2008). During early life, several critical periods can be distinguished in the development of the nervous system, during which certain brain areas or neuronal networks show increased plasticity, enabling environmental effects to induce long-term changes in their structure and function (Hensch, 2004).

Of interest, the serotonergic system is strongly implicated in both the development of aggression-related neural networks (Suri et al., 2015) and the acute regulation of aggression (Coccaro et al., 2015), although contradictory results point to a complex and multifaceted role that is not entirely understood yet. Even so, the serotonergic system remains a promising candidate for understanding the link between early-life adversities and pathological aggression in adulthood.

First, the serotonergic system is fundamentally involved in developmental processes (Suri et al., 2015). According to clinical studies, gene polymorphisms that influence the developmental levels of serotonin (5-hydroxytryptamine, 5HT) in the nervous system, such as those involving the serotonin metabolizing enzyme monoamine oxidase A (MAO-A) or the serotonin transporter (SERT), which terminates synaptic transmission of 5HT and controls 5HT volume transmission via its reuptake (Hallikainen et al., 1999; Zhou et al., 1998), may predispose to differences in temperamental traits and emotion regulation, including aggression and impulsivity (Meyer-Lindenberg et al., 2006). Particularly, ontogenic excess of 5HT arising from decreased MAO-A and/or decreased SERT activity has been linked to threat bias, amygdala hyperreactivity, diminished recruitment of prefrontal regulatory control, and trait social hypersensitivity (Buckholtz and Meyer-Lindenberg, 2008; Hariri et al., 2002; Jabbi et al., 2007; Kalin et al., 2008; Thomason et al., 2010; Volman et al., 2013). Gene polymorphisms resulting in lower SERT activity have also been linked to increased sensitivity to the environment (Homberg et al., 2016). Inferring from this, greater emotionality and environmental sensitivity associated with excess developmental 5HT could increase individual vulnerability to early-life social adversities and exacerbate maltreatment-induced abnormal social behavior, including violent tendencies and increased reactive aggression (Buckholtz and Meyer-Lindenberg, 2008; Volman et al., 2013).

Serotonin signaling and its disruption can impact distinct behavioral domains depending on developmental timing (Homberg et al., 2010; Suri et al., 2015). Notably, preclinical studies have outlined adolescence as a serotonin-sensitive period involving aggression and impulsivity (Yu et al., 2014). Adolescence contains a shift in functional connectivity between the prefrontal cortex and the amygdala, reflecting the gradual maturation of top-down prefrontal control over emotional responses (Gee et al., 2013). This shift can be disrupted by adverse early-life events (VanTieghem and Tottenham, 2018). Considering the traits associated with MAO-A and SERT gene polymorphisms, 5HT signaling, particularly in adolescence, could play a key role in establishing prefrontal cognitive control over impulsive responses, including reactive aggression. In line with this, studies report that the use of selective serotonin reuptake inhibitors (SSRI) during childhood and adolescence, but not adulthood, has been associated with an increase in aggression and suicidality (Sharma et al., 2016). Conversely, SSRI treatment in adulthood effectively reduces impulsive aggression (Coccaro et al., 2009; George et al., 2011; Silva et al., 2010), and studies further outline a general inhibitory effect of 5HT on impulsive aggression in adulthood (Audero et al., 2013; Carrillo et al., 2009), suggesting distinct roles of the serotonergic system during development versus adulthood (Audero et al., 2013). Still, contradictory results reflect a more complex role of the serotonergic system in the regulation of aggression, which remains inconclusive (Carrillo et al., 2009).

Neurons that synthesize serotonin are localized in the brainstem in anatomically distinct cell groups (B1-B9), from where they send complex axonal projections that innervate the forebrain (B5-B9) or descend to the spinal cord (B1-B3) (Soiza-Reilly and Gaspar, 2020). Forebrain-projecting axons mainly originate from the dorsal and median raphe nuclei in a complementary way: the median raphe (B5/B8) innervates structures along the medial axis, whereas the dorsal raphe (B4/B6/B9) sends projections to more lateral structures (e.g. the amygdala) and the cortex, including prefrontal areas (Lechin et al., 2006; Muzerelle et al., 2016; Soiza-Reilly and Gaspar, 2020). SERT is distributed along the whole length of serotonergic axons (Zhou et al., 1998), where it controls volume transmission or synaptic levels of 5HT by its reuptake into the axon or the presynaptic terminal, respectively (Hallikainen et al., 1999; Zhou et al., 1998). Further supporting serotonin's involvement in the control of aggression and long-term developmental influence on aggression-related traits, neuroanatomical studies report robust expression of the SERT protein (Linley et al., 2013, 2017; Zhou et al., 1998) in brain regions involved in aggression control, including the medial prefrontal cortex (mPFC) (Biro et al., 2017, 2018; Takahashi et al., 2014), lateral septum (LS) (Koolhaas et al., 1998; Wong et al., 2016), ventrolateral part of the ventromedial hypothalamus (VMHvl) (Hrabovszky et al., 2005; Kruk et al., 1983; Lin et al., 2011), medial amygdala (MeA) (Hong et al., 2014; Newman, 1999; Toth et al., 2012), lateral habenula (LH) (Golden et al., 2016; Takahashi et al., 2022), dorsal raphe (DR) (Takahashi et al., 2010, 2022) and paraventricular thalamus (PVT) (Ahern et al., 2016; Wall et al., 2012). Out of these regions, dysfunctions of the PFC are robustly associated with abnormal aggression in both clinical and preclinical studies (Biro et al., 2017; Grafman et al., 1996; Raine et al., 2000; Toth et al., 2012). Its ventral subregion, the orbitofrontal cortex (OFC) is also strongly implicated in the control of impulsive aggression (Beyer et al., 2015; Coccaro et al., 2007; Nordman and Li, 2020). Moreover, the PFC displays a maturational peak in adolescence that overlaps with the serotonin-sensitive period related to aggression and impulsivity. PFC serotonergic signaling is intricate and can modulate prefrontal activity in diverse ways (Andrade, 2011). 5HT signaling in the PFC during adolescence could be key in establishing normal aggressive behavior, and adverse environmental effects could induce abnormal aggression through changes in the prefrontal serotonergic system (Márquez et al., 2013).

Serotonin transporters are crucial in regulating serotonin levels (West et al., 2019). Examining the role of SERT in aggression offers several advantages in elucidating the role of the serotonergic system in social dysfunctions and stress-related affective disorders. SERT is a presynaptic membrane protein that regulates serotonin transmission via its reuptake of released 5HT, thereby modulating synaptic serotonin concentration (West et al., 2019). Unlike MAO-A, which broadly influences monoamine levels, SERT specifically controls 5HT levels. Until now, investigations of SERT have predominantly focused on genotype-associated traits or SERT binding under baseline conditions (Gressier et al., 2013; Hariri et al., 2002; Spies et al., 2015). To our knowledge, the investigation of acute changes in SERT protein-level expression in response to aggressive social contexts and other immediate challenges remains entirely unexplored. Since the serotonergic system is also involved in mediating fast, adaptive responses to stressful challenges in a context-dependent manner (Hanson and Hurley, 2014; Varga et al., 2020), we hypothesize that SERT is capable of dynamic changes to follow challenge-dependent serotonergic demands. To test this, we subjected mice to either an aggression-related challenge, i.e., the resident-intruder test, or a non-social challenge, i.e. the open field test. Mice were perfused 90 min following the challenge, after which SERT and 5HT levels were investigated in the aggression circuitry using immunohistochemistry, with an extended focus on the PFC.

As SERT is involved in both the neuronal control of aggressive behavior and is impacted by early-life adversity (Márquez et al., 2013), alterations in SERT expression and/or dynamics may contribute to the long-term behavioral consequences of childhood maltreatment. To investigate this, mice were subjected to post-weaning social isolation, a model of early-life social neglect, in which animals are isolated from conspecifics after weaning until adulthood, thus preventing

socialization during the adolescent critical period, a crucial window in the development of social behavior and prefrontal control of impulsive responses (Mikics et al., 2018; Tóth et al., 2008; Toth et al., 2011, 2012; Tulogdi et al., 2014; Wongwitdecha and Marsden, 1996). In adulthood, mice were perfused under baseline conditions or 90 min after the resident-intruder test. SERT and 5HT levels were investigated in the PFC using immunohistochemistry.

Our study investigated the effects of an acute aggressive interaction on 5HT and SERT levels in the prefrontal cortex and key regions of the aggression circuitry, and how the serotonergic system in the prefrontal cortex is impacted by early-life social isolation. We found that SERT and 5HT levels, as well as prefrontal neuronal activity (based on c-Fos counts), undergo acute increases following aggressive interaction, demonstrating a rapid alteration in SERT protein expression within brain regions implicated in aggression control. However, such a significant SERT increase is absent after a non-social emotional challenge. In animals exposed to post-weaning social isolation, aggressive interactions did not trigger such a significant increase in prefrontal SERT expression, indicating that early-life adversity disrupts the dynamic changes in SERT expression during socially challenging situations in adulthood.

Considering the availability and widespread use of medications targeting the serotonergic system in aggression-related psychiatric disorders, our results carry crucial implications and aid in the understanding and refinement of pharmacological treatments for impulsive, abnormal aggression.

2. Materials and methods

2.1. Animals

All experimental subjects were male Crl:CD1 mice (Charles-River Laboratories, RRID: MGI:7384646) obtained from the breeding facility of the Institute of Experimental Medicine (Budapest, Hungary). Mice were maintained at a temperature of 22 ± 1 °C and at a relative humidity of 60 ± 10 %, in a light-dark cycle of 12:12 h with lights off at 7AM. Food and water were available ad libitum. Following weaning on postnatal day 21, animals were co-housed in groups of four in standard Plexiglas cages (36,5 x 20,7 × 14 cm), behavioral experiments started at PD65. Intruders in the resident-intruder tests were group-housed male CD1 mice. For the early-life social isolation experiment, animals were randomly assigned to social or isolated rearing from weaning, animals in the latter group were housed individually for six weeks until the start of the behavioral testing period (Biro et al., 2023). All experiments were carried out in accordance with the Directive of the European Parliament and the Council from September 22, 2010 (2010/63/EU) and were reviewed and approved by the Animals Welfare Committee of the Institute of Experimental Medicine.

2.2. Experimental design

Experiment 1 consisted of a resident-intruder test carried out in adulthood (for the results of behavioral parameters, see Table 1). This was followed by immunohistochemical staining for SERT, 5HT, and c-Fos in areas of the rodent aggression circuitry (n = 6 and n = 12 for control and aggressive interaction, respectively, Fig. 1). SERT immunoreactivity was also investigated with STORM imaging (, n = 3 and n = 3 for control and aggressive interaction, Fig. 3).

Experiment 2 investigated the time dependency of SERT upregulation following aggressive encounters. SERT expression was assessed immediately after the resident-intruder test and 24 h post-test, with a control group included for comparison. A total of 26 mice were used for this experiment (Supplementary Fig. 4).

Experiment 3 aimed to investigate SERT expression at the mRNA level using quantitative PCR. Brain punches were collected from the mPFC of control animals (n = 6) and mice subjected to the resident-intruder test (n = 6). Quantitative PCR analysis was performed to measure mRNA levels of SERT and c-Fos, providing insight into the transcriptional regulation of these markers following aggressive encounters (Fig. 3).

Table 1
The behavior of CD1 mice in the resident–intruder test.

	Frequency of behavior (mean ± SEM)	Duration of behavior (% time, mean ± SEM)
Offense	19.58 ± 1.48	8.30 ± 2.37
Defense	17.92 ± 2.44	8.31 ± 4.65
Submission	3.75 ± 3.34	1.60 ± 0.66
Vigilance	2.42 ± 15.38	0.55 ± 0.28
Sniffing	66.75 ± 0.79	65.08 ± 5.66
Exploration	50.00 ± 2.44	12.61 ± 0.82
Grooming	5.58 ± 1.08	2.34 ± 0.59
Other	8.42 ± 7.37	1.21 ± 0.33
Total behavioral transitions	174.42 ± 15.38	

	Frequency of behavior (mean ± SEM)
Total bites	8.33 ± 4.07
Hard bites	4.50 ± 2.91
Soft bites	3.83 ± 1.59
Attacks targeting vulnerable body parts	1.58 ± 1.15

	Latency of behavior (sec; mean ± SEM)
Latency to attack (s)	504.00 ± 33.72

In Experiment 4, animals were subjected to an open field test for 10 min, as a non-social novelty stressor (n = 8 and n = 10 for control and non-social novelty stressed, respectively, Fig. 4).

In Experiment 5, the effects of post-weaning social isolation were tested on SERT immunoreactivity (social, control: n = 5, social, aggressive interaction: n = 10, isolated, control: n = 6, isolated, aggressive interaction: n = 16, Fig. 5).

2.3. Behavioral tests

2.3.1. Resident-intruder (RI) test

3 days before the test, all animals were single-housed to facilitate the emergence of territorial behavior. Tests were carried out in the early hours of the dark period in dim red lighting. Control animals were not subjected to the test and were kept in their home cages. For animals in the aggressive interaction (AI) group, a smaller, unfamiliar male intruder animal was placed in the cage of the observed, resident animal, and behavior was recorded and analyzed by an experimenter blinded to the experimental conditions (Biro et al., 2023). We examined the resident's latency to the first biting attack, the number of attacks, their intensity (soft versus hard bites), and whether the attacks targeted vulnerable body parts of the intruder, such as the head, throat, or belly (Tóth et al., 2008; Toth et al., 2011, 2012). Hard bites were defined as attacks delivered with a strong body movement from the resident, eliciting a significant startle response in the intruder (e.g., a large jump or defensive behavior), whereas soft bites involve smaller movements of the resident and induce mild responses in the intruder (Biro et al., 2023; Tóth et al., 2008). Additionally, we assessed the duration and frequency of multiple behavioral parameters using the Solomon Coder event-recording software (RRID:SCR_016041). The main behavioral categories were exploration (rearing and exploratory activity that is not directed towards the opponent), sniffing (non-aggressive sniffing of any part of the opponent's body), grooming (self-grooming movements), offensive behavior (chasing, attack bouts, tail-rattling, aggressive grooming, mounting, punching, and kicking), defensive behavior (fleeing, defensive upright posture), submission (remaining still while being sniffed or aggressively groomed, usually crouched with closed eyes), and vigilance (persistent, tense observation of the intruder from a distance, with the body oriented toward the intruder) (Biro et al., 2023; Duque-Wilckens et al., 2018; Newman et al., 2019).

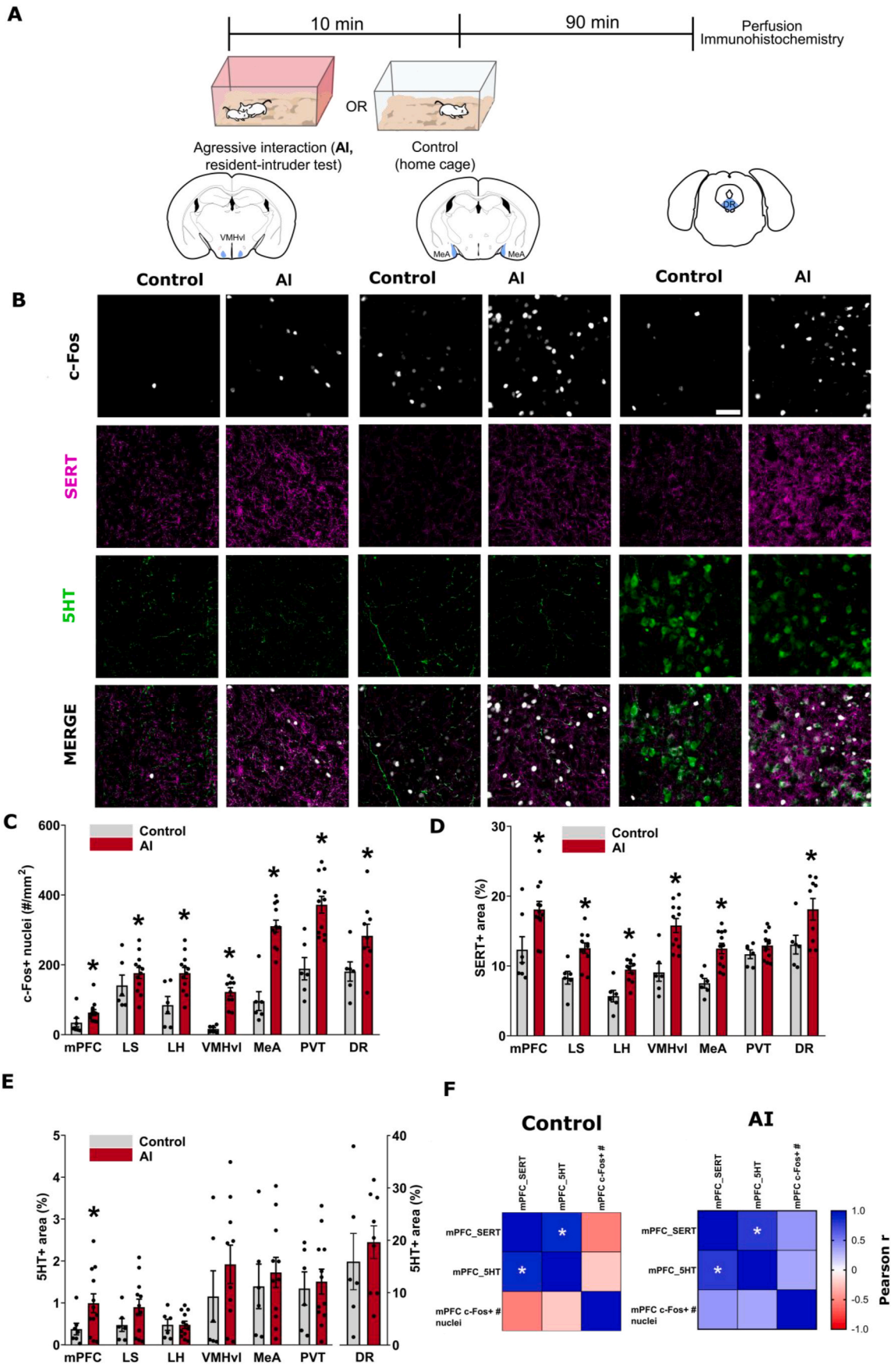


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Fig. 1. Region-specific changes in SERT, 5HT, and c-Fos expression after aggressive interaction.
A, Experimental design. Mice were exposed to an intruder, evoking territorial aggressive behavior (AI), or were kept under baseline conditions (Control). Mice were perfused 90 min after intruder exposure. B, Representative fluorescent microscopy images from the DR, MeA, and VMHvl showing immunolabeling for c-Fos (white), 5HT (green), and SERT (magenta). Scale bar, 50 μm. C-E, Bar graphs showing increased c-Fos counts (C), increased SERT expression (D), and unchanged 5HT levels in the examined brain regions except for the mPFC (E). F, Pearson correlation matrices for control and AI mice showing relationships between SERT-IR, 5HT-IR, and c-Fos expression in the mPFC. Each square indicates the Pearson correlation coefficient, with colors representing correlation strength and labels indicating p-values. All data are presented as mean ± SEM. *p < 0.05. 5HT, 5-hydroxytryptamine; AI, aggressive interaction; LS, lateral septum; LH, lateral habenula; MeA, medial amygdala; PVT, paraventricular nucleus of the thalamus; VMHvl, ventrolateral part of the ventromedial hypothalamus; DR, dorsal raphe.

2.3.2. Open field test

In experiment 2, subjects were exposed to the open field test (OF) as a non-social novel environmental challenge. The open field was a square black Plexiglas box measuring 40 x 36 × 15 cm, subjects were placed into the middle of the box and were allowed to roam the apparatus for 10 min under dim red light illumination (Biro et al., 2023). The apparatus was cleaned with water and dried thoroughly between tests. Behavior was video-recorded, locomotor activity, entries to the center and time spent in the center (defined as the central 20 × 18 cm zone of the apparatus) were analyzed using Ethovision XT 15 software (Noldus, RRID:SCR_000441). Control animals were not subjected to the test and remained in their home cages.

2.4. Immunohistochemistry and imaging

2.4.1. Fixation and tissue processing

Animals were transcardially perfused 90 min after the start of the RI or OF tests, whereas control animals were perfused under baseline conditions. Animals were deeply anesthetized using ketamine-xylazine (16.6 and 0.6 mg/ml, respectively), and transcardially perfused with ice-cold 0.1M PBS followed by 4 % paraformaldehyde in 0.1-M PBS solution, pH 7.4. Brains were removed and postfixed for 3 h, cryoprotected in 30 % sucrose in PBS for 48 h 30 μm slices were cut on a sliding microtome, and sections were stored in a cryoprotectant fluid (50 % sodium phosphate buffer, 30 % ethylene glycol, 20 % glycerol) at −20 °C until immunohistochemical staining.

2.4.2. Immunostaining

Staining was carried out on free-floating slices taken 180 μm apart from each other encompassing the prefrontal cortex, as well as the medial amygdala, the lateral septum, the paraventricular nucleus of the thalamus, the ventrolateral subregion of the ventromedial hypothalamic nucleus, the lateral habenula and the dorsal raphe. Sections were washed in Tris-buffered saline, blocking of nonspecific binding sites was achieved using 10 % normal donkey serum (NDS) (Sigma, USA) with 0.1 % Triton-X-100 (Sigma, USA) for 1h, slices were then incubated in a solution containing goat anti-5HT (Immunostar, Cat. no. 20079, 1:8000, RRID: AB_572262), rabbit anti-SERT (Immunostar, Cat. no. 24330, 1:2500, RRID:AB_572209) or mouse anti-SERT (Synaptic Systems, Cat. no 340011, 1:3000, RRID:AB_2622241) and rat anti-c-Fos (Synaptic Systems, cat. no. 226017, 1:3000, RRID: AB_2864765) antibodies with 5 % NDS and 0.1 % Triton-X-100 in TBS (Thermo Fisher Scientific, USA) for 24 h. After removing the residual antibodies, Alexa 488-conjugated donkey anti-goat (Jackson ImmunoResearch, Cat. no. 705-545-147, 1:500, RRID:AB_2336933), Cy3-conjugated donkey anti-rabbit (Jackson ImmunoResearch, Cat. no. 711-165-152, 1:500, RRID:AB_2307443), Cy3-conjugated donkey anti-mouse (Jackson ImmunoResearch, Cat. no. 715-165-151, 1:500, RRID:AB_2315777), and 647-conjugated donkey anti-rat antibodies were added (Jackson ImmunoResearch, Cat. no 711-605-152, 1:500, RRID:AB_2492288). Sections were washed, mounted and coverslipped using Mowiol 4–88 (Sigma, USA).

To validate the specificity of our rabbit antibody, we utilized an additional mouse anti-SERT antibody raised against amino acids 1–77. This approach ensures that any potential issues related to nonspecific binding are minimized. Co-staining with these antibodies resulted in highly consistent labeling patterns, and the observed data demonstrated strong correlation (Supplementary Fig. 1). The specificity of the rabbit SERT primary and secondary antibodies has been well-established in previous studies (Linley et al., 2013; Vertes et al., 2010). Sections processed without the primary or secondary antibodies showed no immunoreactivity.

For the light microscopy analysis shown in Fig. 5, SERT was labeled using a NiDAB staining method. Briefly, slices (taken 90 μm apart) were incubated in 0,3 % Triton-X100 and 0,3 % hydrogen-peroxide, followed by 0.5 % BSA (Sigma, USA) with 0,25 % T-X-100 in PBS. Rabbit anti-SERT (Immunostar, Cat. No. 24330, 1:5000, RRIR:AB_572209) was used as the primary antibody with 0,1 % BSA and 0,25 % Triton-X-100 for 48h. Next, slices were incubated with a biotinylated anti-rabbit secondary antibody (Jackson ImmunoResearch, Cat. no. 706-066-148, 1:200, RRID:AB_2340452) and Avidin-Biotin Complex (1:400, Vector Laboratories) for 2h and 1h, respectively. The staining was developed using 0,003 % hydrogen-peroxide, 0,2 mg/ml diaminobenzidine tetrahydrochloride and 0,1 % Ni-Al-ammonium-sulfate in TRIS buffer. After mounting, slices were dehydrated using xylene and covered with DPX (Sigma, USA).

2.4.3. Confocal imaging

Images were captured using a Nikon NI-C2 fluorescent confocal microscope (Plan Apo VC 20x, NA 0.75) using 488, 561 and 642 nm lasers (CVI Melles Griot) sequentially. Microscope acquisition settings (laser power, pinhole size, resolution etc.) were kept identical during imaging. Multiple optical planes were imaged 1 μm apart.

2.4.4. Whole slide fluorescent imaging

For the areas shown in Fig. 1, images were taken using a Pannoramic MIDI II slide scanner (Plan Apochromat 20 × , NA 0.8) using sequential LED illumination at 325 nm/px resolution as described perviously (Bősz et al., 2025; Bruzsik et al., 2021; Jász et al., 2025).

2.4.5. Light microscopic imaging

Prefrontal subregions (ACC, IL, PrL, VO and MO) of NiDAB-stained slices were captured using a Olympus DP70 microscope (10x), SERT staining was quantified similar to fluorescent labeling.

2.4.6. Image analysis

Images were analyzed using Fiji (Schindelin et al., 2012). For c-Fos labeling, images were thresholded after background subtraction, and the resulting particles were counted using custom scripts. For SERT and 5HT labeling, the Trainable Weka Segmentation plugin (Arganda-Carreras et al., 2017) was used to train a classifier, which segmented the image to signal (corresponding to fibers positive for the staining) and background pixels based on several preselected image features. The ratio of signal pixels was used as a metric of fiber staining density. A separate classifier was trained for each staining, since fiber intensity levels varied due to both the variability in the applied immunohistochemical staining and the different variance of fiber density across different experiments.

2.4.7. STORM labeling and imaging

For STORM imaging, slices were washed and incubated in TBS containing rabbit anti-SERT primary antibody (1:1000) with 1 % BSA and 0,25 % Triton-X-100 for 24 h. After washing, Alexa 647-conjugated donkey anti-rabbit secondary antibody (Jackson ImmunoResearch, Cat. no 711-605-152, 1:500, RRID:AB_2492288) was added with 0,2 % Triton-X-100 for 2 h. Slices were transferred to the imaging medium (5

m/v% glucose, 0.1M mercaptoethylamine, 1 mg/ml glucose oxidase and 2.5 μl/ml catalase) immediately prior to imaging. Images were taken using a Nikon N-STORM microscope (CFI Apo TIRF 100× objective NA: 1.49, Andor iXon Ultra 897 EMCCD camera), using 405 nm laser as a low-intensity activator with a 300 mW 647 nm laser as reporter (VFL-P-300-647, MPB Communications, Montreal, Kanada). A cylindrical lens was used for 3D imaging. 5000 images were captured at 30 ms intervals, peaks were identified using NIKON NIS-Elements. Images were captured from layers 1 and 2/3 of the prelimbic cortex. Coordinates of localization puncta were exported and analyzed using VividSTORM (Barna et al., 2016), where the number of localization puncta were counted in uniform sized ROIs placed along SERT + fibers.

2.5. Quantitative PCR

Medial prefrontal cortex (mPFC) brain punches were collected from control animals and those subjected to AI, 60 min after the AI test, as described previously (Mikics et al., 2018; Szente et al., 2024). Brains were harvested under RNAase-free conditions, placed on dry ice, and then dissected into 1-mm thick coronal slices using a stainless steel brain mold. Tissue blocks containing the mPFC were identified using distinct landmarks such as the forceps minor and piriform cortex and mPFC was isolated using a sterile razor blade. Total RNA was isolated from the collected brain punches using the RNeasy Lipid Tissue Mini Kit (Qiagen, Germany), following the manufacturer's protocol. The concentration and quality of the total RNA were measured using Qubit RNA BR and Q/I kits (Thermo Fisher Scientific, USA) to ensure suitability for downstream applications. Complementary DNA (cDNA) was synthesized using the Multiscribe Reverse Transcription Kit (Thermo Fisher Scientific, USA), utilizing 250 ng of RNA per reaction. Quantitative PCR (qPCR) was performed using the VIAA7 PCR system (Thermo Fisher Scientific, USA). Reactions were carried out in duplicate using TaqMan assays for *Fos* (Mm00487425_m1, Thermo Fisher Scientific, USA) and *Slc6a4* (Mm00439391_m1, Thermo Fisher Scientific, USA). TaqMan Gene Expression Master Mix (4369514, Thermo Fisher Scientific, USA) was employed for all reactions. Expression levels were quantified using the ΔΔCt method (Livak and Schmittgen, 2001), normalizing to mean levels of *Gapdh* (Mm99999915_g1, Thermo Fisher Scientific, USA) and *Actb* (Mm02619580_g1, Thermo Fisher Scientific, USA). Relative quantities of mRNA were calculated by normalizing to the mean levels of *Actb* and *Gapdh*.

2.6. Machine learning clustering and analysis

We utilized a RandomForest classifier to model the relationship between mPFC molecular markers (c-Fos, SERT, and 5HT levels) and group affiliation (control vs. aggressive interaction). Prior to analysis, these data were standardized. The dataset was divided into training (70 %) and testing (30 %) subsets, stratified to preserve original group proportions. Feature importances were calculated using RandomForest models with 100 estimators. A bootstrapping procedure was implemented, consisting of 30 iterations of independent subsampling and model training. The average model performance (accuracy) and mean absolute error were calculated across the 30 bootstrap iterations using the testing data. Average feature importances were also determined across the 30 iterations. A confusion matrix was calculated by summing the 30 individual confusion matrices from each bootstrap iteration. All analyses were conducted using Python (https://www.python.org/) and Sklearn (https://scikit-learn.org).

2.7. Statistical analysis

Data are expressed as the mean ± standard error of the mean (SEM). Outliers were identified as excluding 2x standard deviation. Statistical differences between groups were shown using Welch's *t*-test or Mann-Whitney U in cases where requirements for t-tests were not fulfilled. For data shown in Fig. 5, a two-way ANOVA was used, followed by Tukey's post hoc analyses. Differences between groups were analyzed using Prism (GraphPad Prism Software Inc. San Diego, California, USA, RRID:SCR_002798), the R software (RRID:SCR_001905), or Python. The significance level was set at $p < 0.05$ for all analyses.

3. Results

3.1. Region-specific serotonergic alterations in 5HT and SERT expression across aggression-related brain regions following acute aggressive interaction

We hypothesized that SERT undergoes dynamic changes to meet challenge-dependent serotonergic demands. To assess the impact of acute aggressive interaction (AI) on the serotonergic system, we labeled 5HT, SERT, and c-Fos, an activity marker, to examine the link between serotonergic function and neuronal activity in aggression-related brain regions (Fig. 1A). During the resident-intruder test, mice displayed species-typical aggressive behavior, with detailed behavioral data provided in Table 1, consistent with previous findings in CD1 mice (Biro et al., 2023). AI induced a significant increase in c-Fos expressing cells within 90min in resident mice across aggression-related regions, including the mPFC ($t = -5.8066$, $p < 0.0001$), LS ($t = -3.6234$, $p = 0.0055$), PVT ($t = -4.5798$, $p = 0.0009$), MeA ($t = -6.7024$, $p < 0.0001$), VMHvl ($t = -8.2521$, $p < 0.0001$), LH ($t = -3.126$, $p = 0.0117$), and DR ($t = -2.3747$, $p = 0.0337$) (Fig. 1B and C). SERT expression was also elevated in all analyzed regions except for the PVT ($t_{PVT} = -1.4889$, $p = 0.1608$; $t_{LS} = -3.6227$, $p = 0.0038$; $t_{MeA} = -4.9143$, $p = 0.0002$; $t_{VMHvl} = -4.159$, $p = 0.0016$; $t_{LH} = -3.9408$, $p = 0.0038$; $t_{DR} = -2.4734$, $p = 0.0281$, Fig. 1B and D). In contrast, 5HT fiber density remained unchanged across these subcortical regions ($t_{LS} = -1.7211$, $p = 0.1051$; $t_{PVT} = -0.3346$, $p = 0.7444$; $t_{MeA} = -0.5166$, $p = 0.6171$; $t_{VMHvl} = -1.0024$, $p = 0.3389$; $t_{LH} = 0.0010$, $p = 0.9992$; $t_{DR} = -0.5665$, $p = 0.5665$, Fig. 1B and E), but showed a significant increase in the mPFC ($t = -2.984$, $p = 0.0096$, Fig. 1B and E), alongside elevated SERT levels ($t = -4.3244$, $p = 0.0007$, Fig. 1B and D). Although no significant correlations were found between agonistic behaviors and serotonergic markers or cellular activity across the analyzed brain regions (Supplementary Table 1), the dynamic elevation of both SERT and 5HT expression in the mPFC suggests a possible involvement of serotonergic changes in the mPFC during aggression. Given these region-specific alterations, we next explored how 5HT, SERT, and c-Fos expression interrelated across aggression-related brain regions through correlation analyses (exploratory analysis without correction for multiple comparisons) (Supplementary Figs. 1 and 2). Control conditions showed a well-structured correlation pattern for 5HT, which was notably disrupted by AI, weakening prefrontal-subcortical connectivity (Supplementary Figs. 1A and 1B). Similar shifts were observed for SERT, with AI reducing its coordinated expression across subcortical hubs and decoupling it from prefrontal regulation (Supplementary Figs. 1C and 1D). AI also altered c-Fos relationships, indicating a transition from top-down control to subcortical-driven aggression (Supplementary Figs. 1E and 1F). Additionally, DR-PVT serotonergic coupling was disrupted (Supplementary Figs. 1G and 1H), and broader serotonergic-neuronal activation patterns shifted. Notably, the mPFC retained a strong positive correlation between 5HT and SERT across conditions, suggesting stable serotonergic regulation despite aggression-induced network changes (Fig. 1F). Such a relationship was not observed in other regions, where serotonergic correlations weakened or shifted following AI (Supplementary Fig. 2).

Next, we employed a RandomForest classifier trained on standardized mPFC c-Fos, SERT, and 5HT expression levels to explore the predictive value of these molecular changes. This model accurately distinguished between control and AI groups, with c-Fos expression emerging as the most important predictor, surpassing SERT and 5HT in relative importance (Supplementary Fig. 3A). Bootstrapping analysis (n

= 30) revealed an average predictive performance of 91.6 % with a mean absolute error of 0.0833 based on the average accuracy of the predictions on the testing data (Supplementary Fig. 3B). A summed confusion matrix demonstrated the overall pattern of correct and incorrect classifications across all bootstrap iterations (Supplementary Fig. 3C). These findings suggest that a combination of mPFC c-Fos, SERT, and 5HT expression serve as reliable indicators of AI-related phenotypic differences.

3.2. mPFC subregion-specific alterations in c-Fos, 5HT, and SERT following social challenge

We next investigated whether mPFC-related alterations were specific to particular subregions. The mPFC-specific changes were evident across its major subregions, including the anterior cingulate (ACC), prelimbic (PrL), and infralimbic (IL) cortices. Socially challenged mice exhibited a significant increase in the numbers of c-Fos expressing cells in the ACC and PrL ($t_{ACC} = -5.8066$, $p_{ACC} < 0.0001$; $t_{PrL} = -3.5174$, $p_{PrL} = 0.0047$, Fig. 2A and B), whereas the IL did not show a significant change (tIL = −1.0696, pIL = 0.3238; Fig. 2A and B). A slightly distinct pattern was observed for 5HT levels, which were significantly elevated in all three mPFC subregions ($t_{ACC} = -2.984$, $p_{ACC} = 0.0096$; $t_{PrL} = -2.4163$, $p_{PrL} = 0.0327$; $t_{IL} = -3.1806$, $p_{IL} = 0.0074$, Fig. 2A and B). Interestingly, SERT area% was also increased in the mPFC, with the most pronounced effect in the IL ($t_{IL} = -6.6528$, $p_{IL} < 0.0001$) followed by the ACC ($t_{ACC} = -4.3244$, $p_{ACC} = 0.0007$) and PrL ($t_{PrL} = -2.705$, $p_{PrL} = 0.0218$; Fig. 2A and B).

3.3. Axonal distribution and rapid regulation of SERT in the mPFC

Serotonin is synthesized exclusively in brainstem raphe nuclei and transported to target regions via long-range axonal projections (Beaudet and Descarries, 1981; Soiza-Reilly and Gaspar, 2020). In the neocortex, including the mPFC, this supply depends entirely on these projections, as intrinsic 5HT or SERT-expressing cells are absent (Kosofsky and Molliver, 1987; Voisin et al., 2016). Once there, serotonergic axons regulate cortical signaling through the release of 5HT and its reuptake via SERT (Fig. 3A) (Belmer et al., 2019; Hallikainen et al., 1999; Zhou et al., 1998). Supporting this, line intensity profile analysis revealed a concomitant increase in 5HT and SERT fluorescence (Fig. 3B and C), indicating a spatially coordinated distribution within serotonergic axons. This was further confirmed by confocal microscopy imaging and Mander's colocalization analysis, which quantified 5HT and SERT co-expression in the mPFC, showing that 85.24 % of 5HT was co-expressed with SERT (n = 10 mice, Fig. 3D).

Since this increase in SERT density is substantially faster than previously discussed expression changes of this molecule, we sought to further verify our results obtained by immunohistochemistry, therefore we analyzed the correlation of SERT expression using two different anti-SERT antibodies. Co-staining with these antibodies resulted in highly consistent labeling patterns (Fig. 3E) and we found a highly positive correlation between the SERT-IR area% in the two immunolabelings ($r = 0.9464$, $p < 0.0001$, Fig. 3F).

Next, we employed stochastic optical reconstruction microscopy (STORM) to achieve nanoscale resolution of SERT distribution, allowing us to examine subcellular changes not detectable with conventional imaging techniques. This approach provided critical insights into whether the observed SERT upregulation in the PrL involved alterations at the level of individual axon terminals (Fig. 3G). We observed increased SERT localization point densities in the PrL (Fig. 3H and I, U = 7976, $p < 0.001$). Together, these results indicate a rapid, challenge-induced increase in SERT expression in the prefrontal cortex.

In a subsequent experiment, we also investigated the time dependency of SERT upregulation and found that the increase was restricted to a specific post-challenge time window, with no similar effect observed at other examined time points (Supplementary Fig. 4).

Next, to investigate serotonin transporter (SERT) expression at the mRNA level, we quantified the levels of *Slc6a4* mRNA, which encodes the SERT protein. Brain punches from the medial prefrontal cortex (mPFC) were collected from both control animals and those exposed to AI. Our analysis revealed that *Fos* mRNA levels (encoding the c-Fos protein) were significantly elevated in AI-exposed mice compared to controls ($p < 0.01$, Fig. 3J), indicating increased neuronal activity. In contrast, *Slc6a4* expression did not show significant upregulation following AI exposure (Fig. 3J). Notably, *Slc6a4* mRNA levels were near the detection limits of our quantitative PCR (qPCR) method. These findings suggest that while *Fos* transcriptional activity is robustly increased after aggressive encounters, the observed upregulation of SERT protein is unlikely to be driven by corresponding local transcriptional changes at the mRNA level. This is consistent with previous studies indicating that SERT protein expression can be regulated post-translationally (Blakely et al., 1998; Cooper et al., 2019; Qian et al., 1997).

3.4. SERT expression is not increased after a non-social challenge

To evaluate whether increased SERT density is specifically associated with aggressive interactions or reflects a general emotional response, we conducted an open field test, presenting a novel, non-social environmental challenge to mice. Control mice were handled similarly to the prior experiment (Fig. 4A). This test was selected because it reliably induces a heightened arousal response when mice are removed from the home cage and forced to confront novelty (Handa et al., 1994; Misslin and Cigrang, 1986). While the open field test induced a significant increase in 5HT + immunolabelled area in the prelimbic cortex ($t = 2.502$, $p = 0.0280$, Fig. 4B and C), SERT expression remains unchanged ($t = 1.231$, $p = 0.2438$, Fig. 4B and C). Thus, increased SERT expression detected after aggression exposure (as seen in Figs. 1–3) seems specific to a social challenge.

3.5. Post-weaning social isolation leads to deficient SERT density changes following aggressive interaction

To test how environmental effects known to lead to serotonergic dysfunction and increased aggressive behavior impact rapid SERT expression changes, we used the post-weaning social isolation model, an established paradigm to investigate the effects of early life maltreatment on rodents (Fig. 5A). Isolation rearing causes disrupted behavioral phenotypes, especially abnormal aggressive behavior (Biro et al., 2023), altered 5HT receptor expression patterns (Bibancos et al., 2007), and disorganized prefrontal activity patterns both within the mPFC and OFC (Biro et al., 2017, 2023; Toth et al., 2012). To investigate the combined effects of early-life social isolation and an acute aggressive interaction, we performed anti-SERT immunostaining on control animals and animals participating in aggressive interaction both in socially-reared and isolated animals (Fig. 5A).

Isolated mice demonstrated heightened aggressive behavior compared to socially-reared mice (Table 2), in line with previous findings. They exhibited shorter attack latencies and delivered more bites, which were frequently directed toward vulnerable areas of their opponents, such as the head, throat, and belly. Additionally, isolated mice displayed an increase in violent hard bites, alongside higher frequencies of offensive and defensive behaviors, while showing reduced submissive behaviors compared to socially-reared counterparts (for detailed behavioral data and statistics, see Table 2). We found that fighting increased SERT area% in all prefrontal subregions (two-way ANOVA main effect of aggressive interaction, $F_{ACC}(1,33) = 7.82$, $p = 0.0085$; $F_{PrL}(1,33) = 9.36$, $p = 0.0044$; $F_{IL}(1,33) = 4.87$, $p = 0.0342$; $F_{MO}(1,33) = 6.69$, $p = 0.0140$, $F_{VO}(1,33) = 5.99$, $p = 0.0201$, Fig. 5B and C). Social isolation did not have a significant effect ($p > 0.05$ in all subregions for the main effect). Nevertheless, a significant interaction was detected between rearing conditions and participation in aggressive interaction

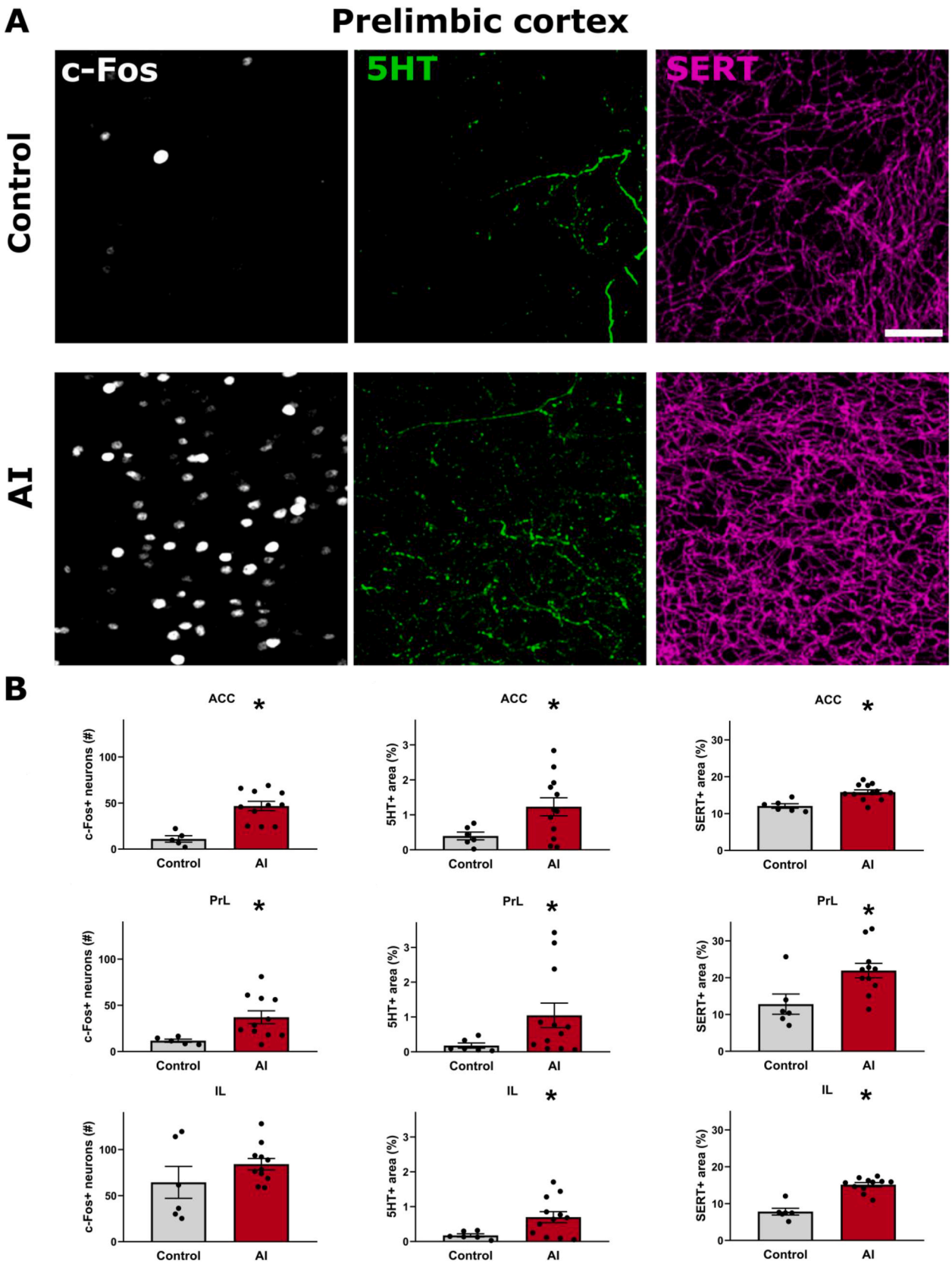

Fig. 2. Social challenge leads to increased SERT and 5HT levels and neuronal activation in the mPFC.
A, Representative single-plane confocal images from the prelimbic cortex showing immunolabeling for c-Fos (white), 5HT (green), and SERT (magenta) in mice exposed to an intruder, evoking territorial aggressive behavior (AI), or maintained under baseline conditions in the homecage. Scale bar: 50 μm. B, Bar graphs showing area fraction of SERT and 5HT immunolabeling as well as c-Fos + nuclei in the prefrontal subregions. All data are represented as mean ± SEM. *$p < 0.05$. 5HT, 5- hydroxytryptamine; ACC, anterior cingulate cortex; PrL, prelimbic cortex; IL, infralimbic cortex.

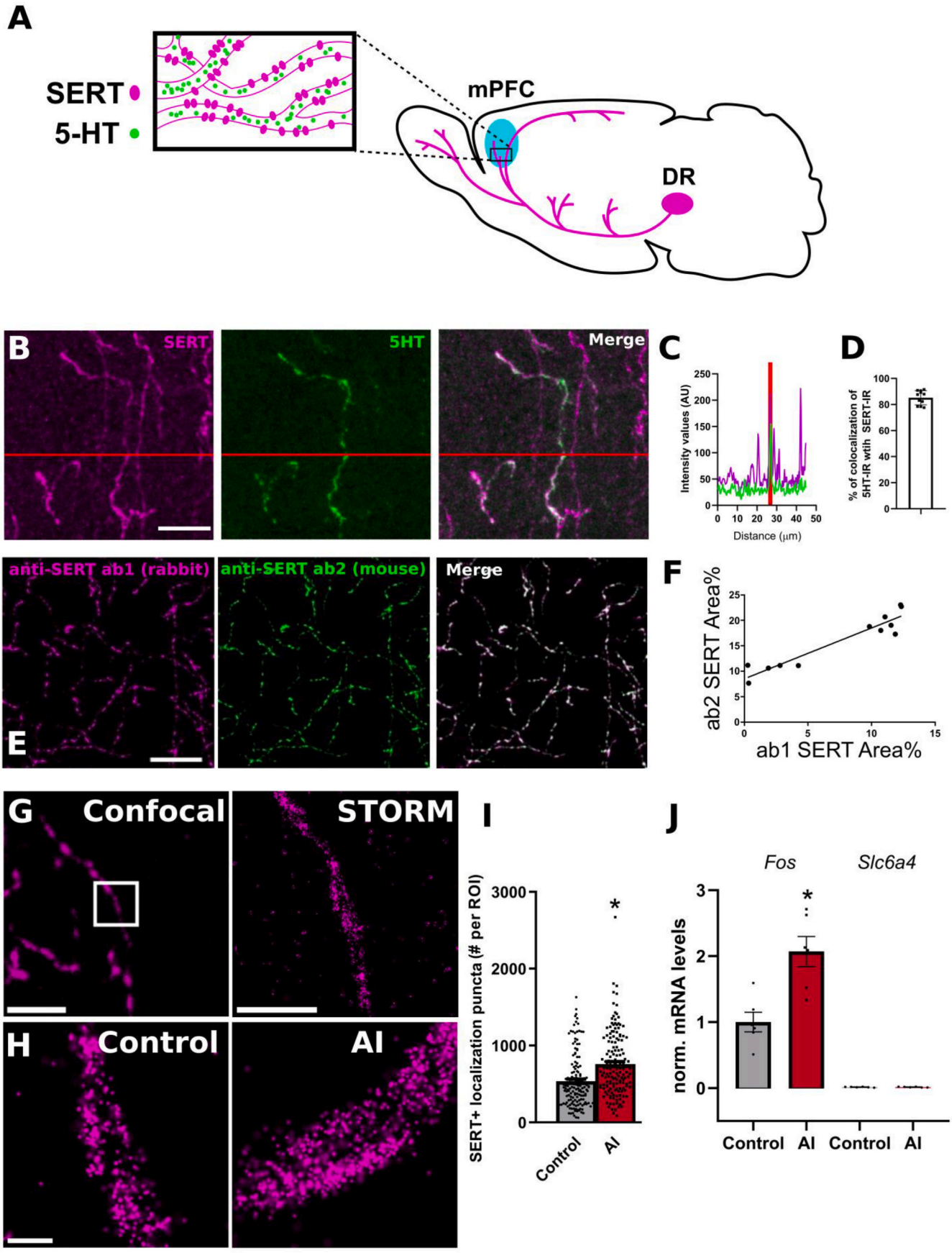

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Fig. 3. Serotonergic axonal organization, SERT-5HT colocalization, and molecular changes in the mPFC following aggressive interaction
A, Schematic representation of serotonergic axons innervating the medial prefrontal cortex (mPFC). Serotonin (5HT) is synthesized in brainstem raphe nuclei and transported to the mPFC via long-range axonal projections. Serotonin release and reuptake are mediated by SERT, which is localized within these axonal structures. B-C, Confocal microscopy images showing SERT immunoreactivity (magenta) and 5HT labeling (green) in serotonergic axons of the mPFC (B). Scale bar, 20 μm. Merged images indicate substantial colocalization of SERT and 5HT (B), supported by line intensity profile analysis (right panel) demonstrating overlapping fluorescence peaks (C). D, Quantification of SERT and 5HT colocalization using Mander's coefficient analysis indicates that 85.24 % of 5HT signal overlaps with SERT immunoreactivity (n = 10 mice), suggesting that 5HT distribution is tightly coupled to SERT + serotonergic axons. E-F, Colocalization analysis of SERT expression with two distinct anti-SERT antibodies (rabbit and mouse). Immunolabeling shows similar axonal patterns with a strong correlation between SERT signals from the two antibodies ($r = 0.9464$, $p < 0.0001$, F). G, Representative images showing resolution differences of conventional confocal (top left) and STORM superresolution imaging for SERT immunolabeling. Scale bar, left: 2 μm, right: 1 μm. H, STORM images from control and AI mice from the mPFC. Scale bar, 200 nm. I, Bar graph representing an increased number of SERT + localization points following aggressive interaction in the PFC ($U = 7976$, $p < 0.001$). J, Quantitative PCR analysis of *Fos* and *Slc6a4* mRNA levels from mPFC tissue punches in control and AI mice. *Fos* mRNA levels were significantly elevated in AI mice ($p < 0.01$), while *Slc6a4* expression remained unchanged. All data are represented as mean ± SEM. *$p < 0.05$. Ab, antibody; STORM, stochastic optical reconstruction microscopy; ROI, region of interest.

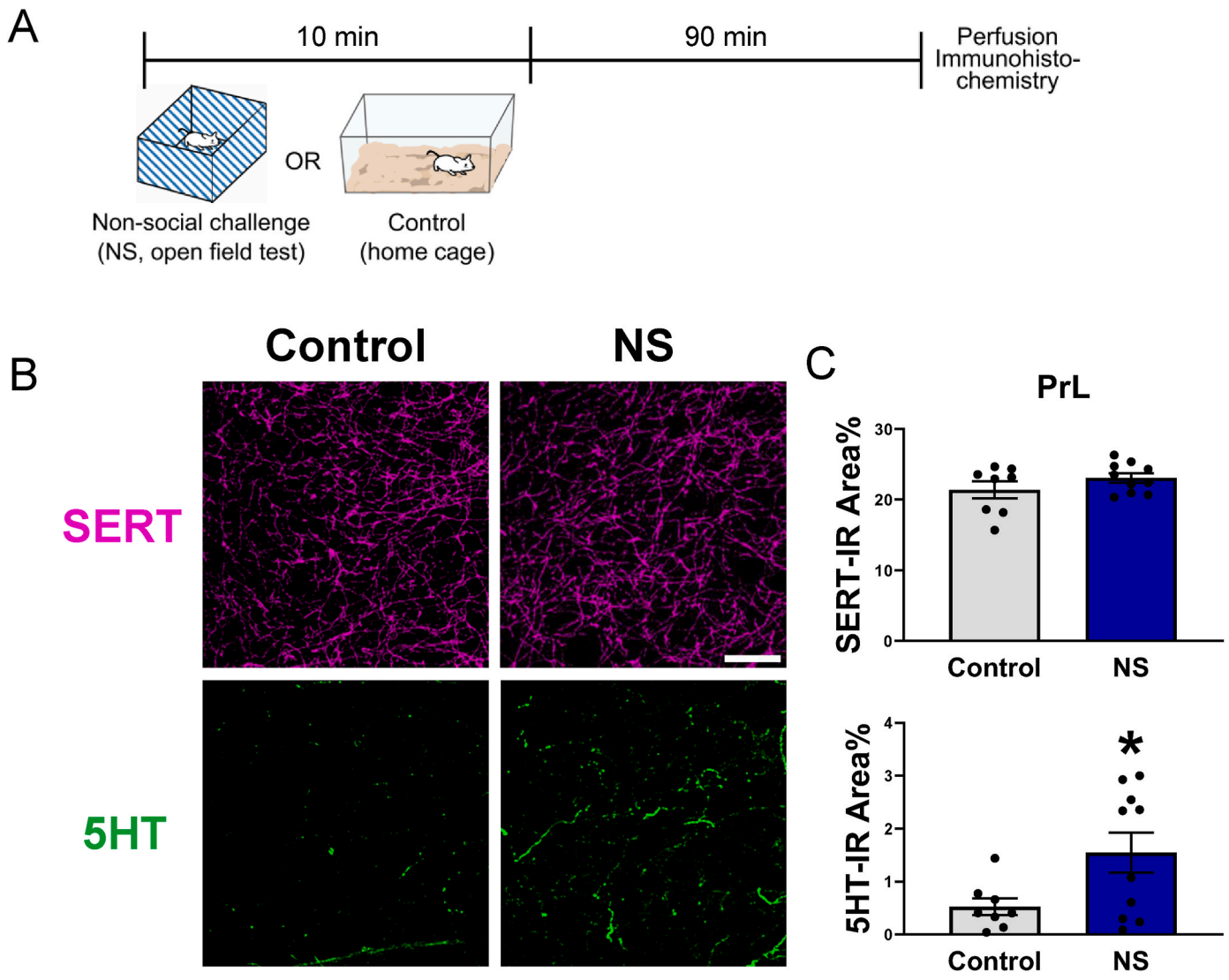

Fig. 4. SERT expression is not increased after a non-social challenge.
A, Experimental design. Mice were exposed to an open-field assay as a non-social novelty stress (NS) or were kept under baseline conditions (Control). Mice were perfused 90 min after open-field exposure. B, Representative single plane confocal images showing immunolabeling for 5HT (green) and SERT (magenta) labeling. Scale bar, 50 μm. C, Bar graphs showing area fraction of SERT and 5HT immunlabelling in the prefrontal subregions. All data are represented as mean ± SEM. *$p < 0.05$. PrL, prelimbic cortex; NS, non-social challenge.

in the VO and ACC (VO: $F_{interaction(1,33)} = 6.91$, $p = 0.013$, ACC: $F_{interaction(1,33)} = 5.51$, $p = 0.025$, Fig. 5C). Tukey's post hoc test analysis found a significant difference between the socially-reared control group and the socially-reared animals participating in aggressive interaction ($p_{VO} = 0.0099$, $p_{ACC} = 0.0088$), however, no significant difference was found between isolated groups. Together, while increased SERT expression in prefrontal subregions was confirmed in socially-reared animals engaging in aggressive interactions, this increase was absent in the VO and ACC of animals reared in isolation, despite their participation in aggressive interactions.

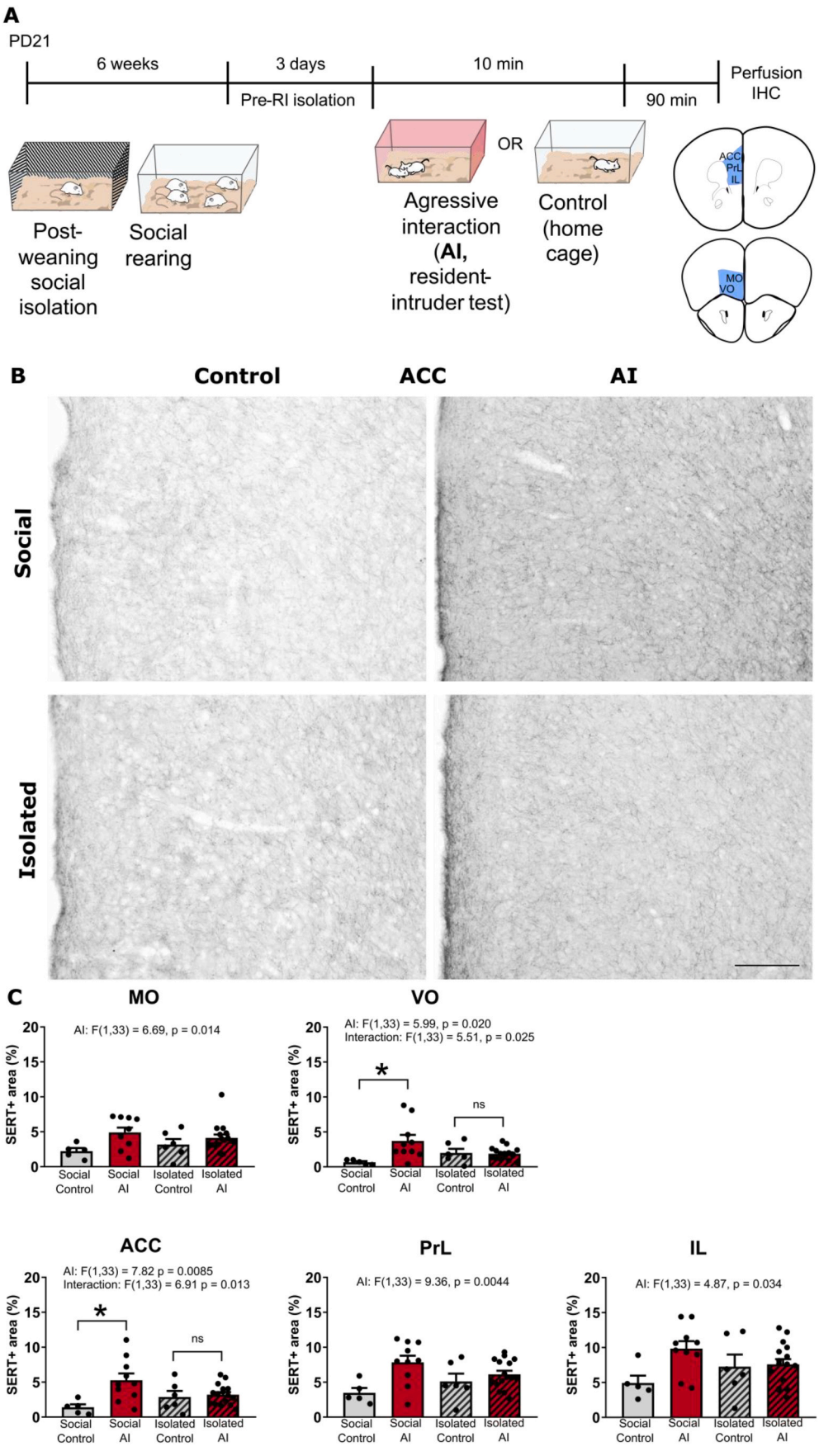

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Fig. 5. Post-weaning social isolation leads to deficient SERT density changes following aggressive interaction.
A, Experimental design. Mice were exposed to post-weaning social isolation (**isolated**) or were group-housed (**social**, 4 mice/cage). After 6 weeks, aggressive behavior was evoked using intruder exposure (**AI**), and control animals were kept under baseline conditions. Animals were perfused 90 min later. B, Representative light microscopy images showing SERT staining in the ACC. Scale bar, 200 μm. C, Bar graphs showing SERT expression changes in the prefrontal subregions. Aggressive interaction increased SERT levels in all subregions, however, in the VO and ACC, a significant effect of aggressive interaction could only be detected in socially reared animals. All data are represented as mean ± SEM. *p < 0.05 Tukey's post hoc test compared to social control. VO, ventral orbitofrontal cortex; MO, medial orbitofrontal cortex; ACC: anterior cingulate cortex; PrL, prelimbic cortex; IL, infralimbic cortex.

Table 2
Behavioral outcomes of social and isolated CD1 mice during the resident-intruder test.

	Frequency of behavior (mean ± SEM)				
	Social	Isolated	t	df	p
Offense	14.10 ± 3.78	40.29 ± 5.02	3.87	22	*0.0008*
Defense	15.80 ± 3.73	32.36 ± 5.09	2.43	22	*0.0238*
Submission	20.20 ± 6.19	2.36 ± 1.61	3.23	22	*0.0038*
Vigilance	3.60 ± 3.17	38.79 ± 6.41	4.36	22	*0.0003*
Sniffing	61.60 ± 7.26	25.79 ± 5.26	4.10	22	*0.0005*
Exploration	66.70 ± 5.15	69.29 ± 4.79	0.36	22	0.7213
Grooming	7.40 ± 2.03	16.79 ± 4.60	1.64	22	0.1159
Other	3.30 ± 1.56	4.79 ± 1.84	0.58	22	0.5665
Total behavioral transitions	211.50 ± 12.83	310.64 ± 22.23	3.47	22	*0.0022*
Total bites	8.40 ± 2.68	32.14 ± 5.30	3.54	22	*0.0018*
Hard bites	4.90 ± 1.56	18.64 ± 3.37	3.26	22	*0.0036*
Soft bites	3.50 ± 1.48	13.50 ± 2.55	3.04	22	*0.0059*
Attacks targeting vulnerable body parts	2.00 ± 1.21	13.21 ± 3.11	2.92	22	*0.0079*
	Latency of behavior (sec, mean ± SEM)				
	Social	Isolated	t	df	p
1st biting attack	295.70 ± 74.38	90.29 ± 34.23	2.76	22	*0.0114*
	Duration of behavior (%time, mean ± SEM)				
	Social	Isolated	t	df	p
Offense	6.74 ± 1.88	19.21 ± 2.53	3.67	22	*0.0013*
Defense	4.32 ± 1.42	9.23 ± 2.31	1.64	22	0.1157
Submission	8.99 ± 2.76	0.77 ± 0.57	3.41	22	*0.0025*
Vigilance	1.51 ± 1.44	14.85 ± 2.74	3.83	22	*0.0009*
Sniffing	44.45 ± 7.26	17.09 ± 5.08	3.54	22	*0.0018*
Exploration	25.39 ± 4.55	31.79 ± 3.52	1.13	22	0.2713
Grooming	2.96 ± 1.05	4.70 ± 1.04	1.15	22	0.2642
Other	1.72 ± 0.74	1.51 ± 0.51	0.24	22	0.8160

A detailed behavioral analysis further confirmed that social isolation significantly altered the relationship between SERT expression and aggressive behaviors (Table 3). In socially-housed mice, correlations between SERT levels and aggression measures were generally weak. However, in isolated mice, MO SERT showed strong positive correlations with bite frequency, offense frequency, and agonistic behavior frequency (all $p < 0.01$, Table 3). Additionally, shorter attack latencies in isolated mice were associated with higher SERT expression in the VO and PrL ($p < 0.05$, Table 3). Interestingly, sniffing behavior was negatively correlated with VO SERT in isolated mice ($p = 0.0154$ Table 3), suggesting a potential shift in the serotonergic modulation of social interactions. These findings indicate that early social isolation amplifies aggressive behaviors and alters their serotonergic regulation, particularly in medial and ventral prefrontal areas.

4. Discussion

Evidence from *in vivo* neuroimaging investigations (da Cunha-Bang and Knudsen, 2021) has established a link between diminished synaptic serotonin levels and heightened impulsive aggression. Interventions that target serotonin modulation affect brain regions associated with impulsive aggression, encompassing the PFC, amygdala, striatum, thalamus, and brainstem (da Cunha-Bang and Knudsen, 2021). Clinical studies have extensively focused on SERT protein availability in psychiatric disorders and genotypic variants, but most measurements were conducted under baseline conditions (Spies et al., 2015). Similarly, preclinical studies have also neglected the investigation of *in vivo* SERT expression in response to acute challenges (Ferrés-Coy et al., 2013; Murphy and Lesch, 2008). Since previous *in vivo* experiments have demonstrated acute increases in 5HT to various stimuli (Takahashi et al., 2015; Unger et al., 2020), and *in vitro* studies show fast regulation of the SERT protein (Steiner et al., 2008), we hypothesized that SERT is capable of rapid, dynamic changes in response to acute challenges. Consequently, we explored SERT expression at pivotal nodes within the aggression circuitry (Lischinsky and Lin, 2020; Nelson and Trainor, 2007; Takahashi and Miczek, 2014). Following a social challenge, i.e. aggressive interaction, we observed escalated neuronal activity, replicating previous findings (Biro et al., 2023; Toth et al., 2012), and heightened SERT expression across examined aggression circuitry brain regions, including the prefrontal cortex, lateral septum, ventrolateral portion of the ventromedial hypothalamus, medial amygdala, lateral habenula, and dorsal raphe.

The mechanism underlying this increased SERT immunoreactivity appears unrelated to protein structural changes, as confirmed by consistent results using multiple antibodies targeting different epitopes. The increase in SERT density is substantially faster than previously discussed expression changes of this molecule *in vivo*. In contrast to our research findings, studies utilizing human neuroimaging have not consistently reported widespread alterations in serotonin transporter (SERT) activity concerning the regulation of aggression. Rather, these investigations have disclosed variations in SERT levels across distinct brain regions. For example, in healthy males exhibiting high levels of impulsive aggression, elevated SERT levels have been detected in the brainstem and midbrain (Rylands et al., 2012). Conversely, an intriguing inverse relationship between anterior cingulate SERT levels and trait aggression was observed among patients diagnosed with intermittent explosive disorder (van de Giessen et al., 2014). These observations underscore the intricate and multifaceted associations between SERT activity and aggressive behavior. It is noteworthy that our research has unveiled rapid alterations in SERT expression, which correspond to the dynamic state of SERT activity. In contrast, human PET data typically reflect a more stable, trait-like profile of SERT functioning.

Analysis of 5HT immunoreactivity revealed that only the mPFC showed changes in the density of 5HT fibers, underscoring the significance of acute prefrontal 5HT release in aggressive behavior control. This aligns with previous microdialysis data emphasizing the role of 5HT signaling in the PFC concerning aggressive behavior (Takahashi et al., 2015). Since the 5HT IR measured in our study was revealed to be intra-axonal, the lack of increase seen in subcortical brain regions may indicate differences in temporal 5HT-signaling dynamics in aggression. We also found a positive correlation between 5HT fiber immunoreactivity and SERT immunoreactivity in the mPFC that remained strong even across the challenge-induced state, whereas in subcortical regions serotonergic correlations weakened or shifted following AI. Exploratory correlation analyses also suggested that AI weakened prefrontal-subcortical connectivity, indicating a transition from top-down to subcortical-driven aggression (Biro et al., 2017, 2018; Potegal, 2012).

The well-documented importance of mPFC in the control of aggression (Gallucci et al., 2020; Giancola, 1995; Raine et al., 2000; Takahashi

Table 3
Correlations between SERT expression and aggressive behaviors in socially housed and isolated CD1 mice.

	Social			Isolated		
	n	r	p	n	r	p
Brain region	**Frequency of bites**					
MO SERT	10	−0.1046	0.7736	14	0.7481	*0.0021*
VO SERT	10	0.0062	0.9865	14	0.2860	0.3215
PrL SERT	10	0.5785	0.0798	14	0.1034	0.7250
IL SERT	10	0.0923	0.7998	14	−0.0088	0.9762
ACC SERT	10	0.1969	0.5855	14	0.2310	0.4268
Brain region	**Frequency of hard bites**					
MO SERT	10	−0.0808	0.8245	14	0.7041	*0.0049*
VO SERT	10	0.0559	0.8781	14	0.4510	0.1055
PrL SERT	10	0.5033	0.1381	14	0.1826	0.5320
IL SERT	10	0.1429	0.6937	14	0.0286	0.9227
ACC SERT	10	0.1802	0.6184	14	0.2530	0.3828
Brain region	**Latency to attack (s)**					
MO SERT	10	0.0675	0.8530	14	−0.3407	0.2333
VO SERT	10	−0.1779	0.6229	14	−0.7846	*0.0009*
PrL SERT	10	−0.2761	0.4400	14	−0.6044	*0.0221*
IL SERT	10	−0.0675	0.8530	14	−0.5341	*0.0492*
ACC SERT	10	−0.0675	0.8530	14	−0.2484	0.3919
Brain region	**Offense frequency**					
MO SERT	10	−0.1707	0.6372	14	0.5391	*0.0467*
VO SERT	10	−0.0549	0.8803	14	0.2442	0.4001
PrL SERT	10	−0.0732	0.8408	14	0.2574	0.3743
IL SERT	10	−0.1768	0.6250	14	0.1738	0.5523
ACC SERT	10	−0.1524	0.6742	14	0.3498	0.2201
Brain region	**Agonistic behavior frequency**					
MO SERT	10	−0.2364	0.5109	14	0.7363	*0.0027*
VO SERT	10	−0.0909	0.8028	14	0.1385	0.6369
PrL SERT	10	0.3939	0.2600	14	0.0505	0.8637
IL SERT	10	−0.0909	0.8028	14	−0.0681	0.8170
ACC SERT	10	0.0545	0.8810	14	0.2703	0.3499
Brain region	**Sniffing frequency**					
MO SERT	10	0.2970	0.4047	14	−0.3344	0.2425
VO SERT	10	0.1758	0.6272	14	−0.6315	*0.0154*
PrL SERT	10	0.0788	0.8287	14	−0.4334	0.1216
IL SERT	10	0.3212	0.3655	14	−0.2794	0.3333
ACC SERT	10	0.2364	0.5109	14	−0.2288	0.4314

et al., 2014) and its distinct pattern of 5HT fiber and SERT upregulation following the aggression-related social challenge prompted us to conduct a more detailed examination focusing on mPFC subregion-specific changes. The number of c-Fos expressing cells increased in the ACC and PrL, but not the IL. Interestingly, these changes were distinct from those affecting 5HT fiber density and SERT area%, which were increased in all three subregions. These differences could arise from distinct temporal dynamics - whereas c-Fos expression indicates neuronal activation during the aggressive encounter, increased SERT and intra-axonal 5HT could either reflect a challenge-induced sustained increase (i.e. the social challenge necessitated a high increase in 5HT levels, which prolonged the reuptake, so at 90 min 5HT has been reuptaken into the serotonergic axons), or multiple fluctuations in serotonin levels following the challenge that may be involved in the post-processing of the experience itself. Supporting the latter, we observed no elevation in SERT immunoreactivity 10 min after the aggressive interaction, and studies investigating 5HT levels in response to social stimuli show a rapid return to baseline following stimulus presentation under normal conditions (Takahashi et al., 2015; Unger et al., 2020). Considering the 5HT system's relationship with neuroplasticity-related factors (Maynard et al., 2016; Mössner et al., 2000) it is plausible that regional fluctuations of serotonergic neurotransmission could induce signaling pathways associated with learning and long-term plasticity, tuning network activity based on previous experiences.

A previous study has shown that *Slc6a4* mRNA (encoding the SERT protein) levels in the prefrontal cortex are susceptible to chronic stress during adolescence and that *Slc6a4* mRNA shows stress-sensitive downregulation 3 h after aggressive interaction (Márquez et al., 2013). Parallel to our findings, these results indicate that prefrontal SERT expression is likely influenced by acute changes, in addition to the well-documented chronic alterations. STORM microscopy similarly revealed an increased SERT localization point density following AI, meaning that immunoreactivity changes detected by fluorescent microscopy are not attributed to altered SERT cellular trafficking. SERT protein is mainly, but not exclusively expressed by serotonergic fibers originating predominantly from the dorsal raphe nucleus (Miner et al., 2000; Soiza-Reilly and Gaspar, 2020). As both axonic transport mechanisms and lateral membrane diffusion were described to have slower temporal dynamics, they cannot account for the fast (<90 min) expression changes in the SERT protein. Thus, the SERT protein expression changes seen are likely the consequence of either local axonic translation in serotonergic fibers, or increased SERT protein trafficking to membrane compartments of raphe serotonergic axons that innervate the prefrontal cortex. Our qPCR results revealed no changes in mPFC *Slc6a4* mRNA expression following AI, this suggests that local axonic translation is unlikely to have occurred. However, the low expression levels near the qPCR detection range may have limited our ability to detect such changes. SERT turnover, as measured using an irreversible inhibitor, has a half-life of 3.4 days (Vicentic et al., 1999); however, *in vitro* studies have demonstrated that SERT can undergo significantly faster, phosphorylation-dependent internalization (Qian et al., 1997). Additionally, the presence of a recruitable pool of endosomal SERT has been proposed, which can be rapidly reintegrated into the cell

membrane in response to external stimuli through SERT dephosphorylation (Blakely et al., 1998). This explanation assumes that antibody-based labeling is more likely to detect SERT molecules on the cell membrane, potentially masking the intracellular SERT pool.

Following a non-social, novelty-related environmental challenge, we found no increase in SERT expression, even though 5HT intra-axonal levels were increased. 5HT levels in the rodent PFC were shown to be increased by a wide range of stimuli, including novelty exposure (Bickerdike et al., 1993), cued fear conditioning, and the introduction of a social partner (Unger et al., 2020). Previous experiments have shown increased 5HT levels mainly coincidentally with stimulus introduction (at the temporal level of seconds or minutes), whereas a 5HT increase in our experiments (both in Figs. 1 and 3) was shown 90 min after stimulus introduction. These differences might be caused by the different detection properties of microdialysis and genetically encoded fluorescent 5HT sensors (mainly measuring 5HT release in the extracellular space) versus the immunohistochemical 5HT detection used in our study (where the measured 5HT content is likely intra-axonal, i.e., inside the axons that raphe serotonergic cells project to the PFC). Since 5HT release is increased both during aggressive interaction and novelty exposure, but not afterwards, increased 5HT staining in our results 90 min after stimulus presentation is likely an indication of increased 5HT reuptake mainly by SERT (West et al., 2019).

Interestingly, SERT expression changes were not present after novelty exposure, even though 5HT staining was increased, whereas following a social challenge SERT expression changes were present in other areas of the aggression circuitry where 5HT increase was not detected. The selective increase in SERT expression following aggressive encounters, but not after exposure to non-social stressors, suggests distinct serotonergic plasticity depending on the stressor type. Social interactions, such as aggressive encounters, engage social threat detection, dominance assessment, and defensive responses, involving key regions like the PFC, medial amygdala, and hypothalamic aggression nodes (Lischinsky and Lin, 2020; Nelson and Trainor, 2007; Toth et al., 2012). SERT upregulation after aggressive interaction—but not after novelty exposure—likely reflects the need to restore baseline serotonergic tone post-social challenge. The absence of SERT increase after a non-social novelty, despite elevated 5HT fiber density, indicates a distinct mechanism from those involved in social stress contexts. This specificity may result from differential circuit activation and/or distinct temporal dynamics, as aggressive encounters necessitate continuous social information processing, fast adaptive responses, and complex processing of the experience for future encounters. Consequently, SERT upregulation appears essential for recalibrating serotonin dynamics following social challenges.

Finally, since both the serotonergic system, as well as SERT itself are highly susceptible to adverse early-life experiences, we were curious to see how this aggressive interaction-induced SERT expression change is affected by post-weaning social isolation (PWSI). Preclinical studies have outlined adolescence as a serotonin-sensitive period involving aggression and impulsivity (Yu et al., 2014). The PFC displays a maturational peak in adolescence that overlaps with the serotonin-sensitive period related to aggression and impulsivity (Petanjek et al., 2011). Additionally, dysfunctions of the PFC are robustly associated with abnormal aggression in both clinical and preclinical studies (Biro et al., 2017; Grafman et al., 1996; Raine et al., 2000; Toth et al., 2012). As such, 5HT signaling in the PFC during adolescence could be key in establishing normal aggressive behavior, and adverse environmental effects could induce abnormal aggression through changes in the prefrontal serotonergic system (Márquez et al., 2013). In line with this, we have shown that PWSI, a model for early-life adversity impacting the adolescent period (Tóth et al., 2008), blunted SERT reactivity in specific prefrontal subregions, indicating impaired serotonergic system adaptability to acute social challenges. Whereas exposure to an aggression-inducing context evoked an increase in SERT expression in all examined prefrontal subregions, in the VO and ACC this increase was exclusive to socially-reared animals, and was absent in the isolated group. This suggests that social isolation may have caused a decrease in the reactivity of the serotonergic system in these areas, since as we have shown, SERT expression is increased by an acute social challenge. Social isolation under resting (control) conditions did not affect SERT fiber density. Social isolation significantly altered the relationship between SERT expression and aggressive behaviors. In socially housed mice, correlations between SERT levels and aggression were weak, but isolated mice showed strong positive correlations between MO SERT and bite frequency, offense frequency, and agonistic behavior. Higher VO and PrL SERT levels were linked to shorter attack latencies, while sniffing behavior negatively correlated with VO SERT. These results suggest that social isolation amplifies aggression and alters serotonergic regulation, especially in medial and ventral prefrontal areas. This shift may reflect maladaptive serotonergic modulation, with the MO and VO OFC becoming more strongly associated with aggressive behaviors rather than social exploration. Early-life adversity can disrupt prefrontal serotonergic circuits, potentially driving these changes (Caramaschi et al., 2008; Homberg et al., 2016). The OFC's role in aggression modulation is supported by findings that 5-HT1B receptor activation reduces aggression (De Almeida et al., 2006; Veiga et al., 2007), although effects can vary by context (Faccidomo et al., 2008). The dorsal raphe-OFC pathway was also shown to be directly involved in regulating attack bouts in mice (Nordman and Li, 2020). Thus, altered SERT dynamics in the OFC following isolation may underlie the observed behavioral changes.

Previous findings suggest that social isolation following maternal separation caused an increase in 5HT-IR fiber density in some subregions of the PFC (Braun et al., 2000), however, the early postnatal period represents a distinct developmental period compared to the post-weaning phase, likely affecting the serotonergic system differently. Similarly, peripubertal stress induced an increase in SERT mRNA expression in the PFC (Márquez et al., 2013), this effect showed a different reactivity to a social stimulus. Since axonal development of raphe serotonergic neurons was shown to reach an adult-like anatomical stage at around P21 (Lauder, 1990; Maddaloni et al., 2017), experience-dependent effects after this developmental stage are probably unable to alter anatomical properties (e.g., fiber density) of serotonergic projections. Our results, in conjunction with those of (Márquez et al., 2013), suggest that adverse social experiences during the juvenile period lead to alterations in the molecular properties and reactivity of the serotonergic system in response to acute challenges, which in turn deepens our understanding of the complex effects of SSRI treatments, highlighting the need for further investigation.

Our results reveal a novel aspect of SERT in behavioral reactivity and highlight the importance of investigating acute dynamic changes in SERT expression. Since pharmacological treatments for aggression-related psychiatric disorders often target the serotonergic system, our study might carry valuable implications and offer new perspectives in the refinement of treatments for impulsive, abnormal aggression.

5. Limitations

In this study, certain limitations warrant consideration. Firstly, we exclusively utilized male mice to investigate the impact of aggressive behavior on the serotonergic system. This choice was made because female mice generally do not exhibit aggressive behavior towards conspecifics (Kuchiiwa and Kuchiiwa, 2016). Secondly, we employed a range of anti-SERT antibodies, each targeting distinct epitopes of the SERT molecule. Nonetheless, it remains uncertain to what degree these antibodies are sensitive to structural alterations in SERT or their capability to discern modifications in the subcellular localization of SERT. Further investigations are required to elucidate these aspects fully. In addition, the article discussed the potential presence of intracellular SERT pools that may not be detected by antibody-based labeling. Investigating the subcellular localization of SERT in more detail,

perhaps through advanced imaging techniques, could clarify this aspect.

CRediT authorship contribution statement

Huba Szebik: Writing – review & editing, Writing – original draft, Visualization, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Christina Miskolczi:** Writing – review & editing, Investigation, Conceptualization. **Bíborka Bruzsik:** Writing – review & editing, Investigation. **Gyula Balla:** Writing – review & editing, Investigation. **Soma Szabó:** Writing – review & editing, Investigation. **László Biró:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Éva Mikics:** Writing – review & editing, Supervision, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Funding

This study was supported by National Research, Development and Innovation Office (NKFIH) Grant# K125390 (to EM) and project no. RRF-2.3.1-21-2022-00011, titled National Laboratory of Translational Neuroscience implemented with the support provided by the Recovery and Resilience Facility of the European Union within the framework of Programme Széchenyi Plan Plus (to EM).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We thank all the core facilities of our institute for their supportive help: the Behavioral Studies Unit for help with behavioral testing (Dr. Kornél Demeter), the Light Microscopy Center for help with microscopy (Dr. László Barna), the Medical Gene Technology Unit for help with mouse breeding. The authors are deeply grateful to Anna Jász and Emília Bősz for their helpful comments on the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ynstr.2025.100722.

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