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Title: Deletion of the serotonin transporter perturbs BDNF signaling in the central amygdala following long-access cocaine self-administration

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Abstract: Background: Human neuroimaging studies indicate that the amygdala plays a key role in cocaine addiction. One key plasticity factor that modulates effects of cocaine on the brain is Brain-Derived Neurotrophic Factor (BDNF). A wealth of evidence shows that cocaine exposure alters BDNF signaling in corticolimbic structures, but, surprisingly, such evidence is very limited for the amygdala. Additionally, while BDNF is strongly regulated by serotonin levels and inherited serotonin transporter down-regulation is associated with increased vulnerability to cocaine addiction, the effects of serotonin transporter genotype on BDNF signaling in the amygdala under naïve and cocaine exposure conditions are unknown. Methods: We measured BDNF signaling in the central amygdala of wild-type and serotonin transporter knockout rats 24 hours into withdrawal from long-access cocaine self-administration. Results: In wild-type rats mature BDNF (mBDNF) protein levels were decreased, whereas the phosphorylation of its receptor TrkB as well as of its intracellular signaling molecules Akt and ERK1 were increased. mBDNF protein expression and its signaling in cocaine-naïve serotonin transporter knockout rats resembled that of wild-type rats with a history of long-access cocaine self-administration. Interestingly, cocaine-exposed serotonin transporter knockout rats showed increased BDNF levels, with no signs of phospho-TrkB receptor coupling to phospho-Akt and phospho-ERK1. Conclusions: Long-access cocaine self-administration dysregulates BDNF signaling in the central amygdala. Vulnerability to cocaine addiction is associated with dysregulation of this signaling.

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Contributors

Michel Verheij and Danielle Mendes performed the cocaine self-administration study. Lucia Caffino and Francesca Mottarlini performed the molecular analyses. Lucia Caffino, Michel Verheij and Francesca Mottarlini did the statistical analyses. Lucia Caffino, Francesca Mottarlini and Danielle Mendes managed the literature searches. Lucia Caffino, Michel Verheij, Fabio Fumagalli and Judith Homberg designed the study, wrote the protocol and interpreted the data. Lucia Caffino, Michel Verheij, Judith Homberg and Fabio Fumagalli wrote the manuscript. All authors contributed to and have approved the final manuscript.

Conflict of interest

There are no conflicts of interest

Highlights:

- Cocaine self-administration decreases BDNF levels in the amygdala of wild-type rats
- This decrease in BDNF levels upregulates TrkB-Akt-ERK1 signaling
- Cocaine-induced changes are copied in naïve serotonin transporter knockout rats
- Cocaine decouples BDNF and TrkB-AKt-ERK1 signaling in the knockout rats

Deletion of the serotonin transporter perturbs BDNF signaling in the central amygdala following long-access cocaine self-administration

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Running title: SERT, BDNF and amygdala

Abstract

Background: Human neuroimaging studies indicate that the amygdala plays a key role in cocaine addiction. One key plasticity factor that modulates effects of cocaine on the brain is Brain-Derived Neurotrophic Factor (BDNF). A wealth of evidence shows that cocaine exposure alters BDNF signaling in corticolimbic structures, but, surprisingly, such evidence is very limited for the amygdala. Additionally, while BDNF is strongly regulated by serotonin levels and inherited serotonin transporter down-regulation is associated with increased vulnerability to cocaine addiction, the effects of serotonin transporter genotype on BDNF signaling in the amygdala under naïve and cocaine exposure conditions are unknown. **Methods**: We measured BDNF signaling in the central amygdala of wild-type and serotonin transporter knockout rats 24 hours into withdrawal from long-access cocaine self-administration. **Results**: In wild-type rats mature BDNF (mBDNF) protein levels were decreased, whereas the phosphorylation of its receptor TrkB as well as of its intracellular signaling molecules Akt and ERK1 were increased. mBDNF protein expression and its signaling in cocaine-naïve serotonin transporter knockout rats resembled that of wild-type rats with a history of long-access cocaine self-administration. Interestingly, cocaine-exposed serotonin transporter knockout rats showed increased BDNF levels, with no signs of phospho-TrkB receptor coupling to phospho-Akt and phospho-ERK1. **Conclusions**: Long-access cocaine self-administration dysregulates BDNF signaling in the central amygdala. Vulnerability to cocaine addiction is associated with dysregulation of this signaling.

Key words: serotonin, BDNF, amygdala, cocaine self-administration

1.0 Introduction

It has been suggested that compulsive use of drugs of abuse is, at least in part, mediated by structural and functional changes in the amygdala, an area that signals negative emotional states (Koob & Le Moal 2008b). This is supported by several lines of evidence in humans. For instance, amygdala volume is reduced in cocaine addicts (Makris *et al*. 2004; but see Mei *et al*. 2015), amygdala-striatum connectivity is increased in cocaine addicts in response to losing outcomes (Worhunsky *et al*. 2017) and cocaine users show amygdala hyperresponsiveness during fear conditioning and in response to angry and fearful facial expressions (Crunelle *et al*. 2015; Kaag *et al*. 2016). Preclinical evidence also supports the role of amygdala in cocaine addiction. As such, MRI studies have revealed that, among a few other regions, amygdala exhibits an increased clustering coefficient during the first day of abstinence from cocaine self-administration (Orisini *et al*., 2018). In addition, amygdala volume changes correlate with the inability to refrain from cocaine seeking and taking behaviour (Cannella *et al*., 2018). Taken together, both preclinical and clinical studies suggest that the amygdala undergoes plasticity changes during cocaine use, but the precise nature of such changes remains to be elucidated.

The neurotrophin Brain-Derived Neurotrophic Factor (BDNF) plays a critical role in brain plasticity, in experimental models of addictive disorders (Caffino et al., 2016; Fumagalli et al., 2013; Li and Wolf, 2015; Verheij et al., 2016) and in corresponding human situations (Castrén and Kojima, 2017; Ornell et al., 2018). While studies on BDNF in cocaine addiction merely focussed on the prefrontal cortex (Li et al., 2018; McGinty *et al*., 2010), nucleus accumbens (Caputi et al., 2019) and ventral tegmental area (Wang et al., 2016; Schmidt *et al*., 2012), insight in the role of BDNF in amygdala in relation to cocaine addiction is surprisingly limited. Thus far, it has been reported that a decrease in *Bdnf* mRNA levels in the amygdala, induced through a virally-mediated approach, impaired cocaine-induced conditioned place preference (Heldt *et al*. 2014). Furthermore, during withdrawal from cocaine self-adminstration, time-dependent increase in *Bdnf* levels in the amygdala

were found related to incubation of craving (Grimm *et al*. 2003).

Besides BDNF, serotonin plays a key role in neuronal plasticity and cocaine addiction (for review: Müller & Homberg 2015). It is known that antidepressant serotonin reuptake inhibitors upregulate BDNF levels, positively influencing neuronal plasticity (Dias et al., 2003; Molteni et al., 2006). For instance, human studies have shown that the plasmalemmal serotonin transporter (SERT), which regulates the extracellular levels of serotonin, is associated with cocaine addiction (Ersche et al., 2008; Gerra et al., 2007). In line, we previously found that serotonin transporter knockout ($SERT^{-/-}$) rats show increased cocaine self-administration. This was specifically observed under long-access (LgA) conditions (Karel *et al*. 2018; Verheij *et al*. 2018), a condition modeling compulsive drug intake (Ahmed & Koob 1998; Koob & Volkow 2010). These rats also display an increased motivation to work for cocaine, and a reduced extinction of cocaine seeking behaviour when cocaine is no longer available (Homberg *et al*. 2008; Karel *et al*. 2018). It is interesting to note that increased LgA cocaine self-administration was found to be associated with changes in the expression of corticotropin releasing factor (CRF) in the central amygdala (Verheij *et al*. 2018), a subarea of the amygdala that modulates the negative emotional state observed during withdrawal from cocaine (for review: Koob & Le Moal 2008b a; Koob & Volkow 2010). It has been shown that CRF is required for stress-induced BDNF release (Walsh et al., 2014). We suggest that, similar to an increase of CRF, BDNF up-regulation in the central amygdala may enhance the negative emotional state in SERT KO rats, contributing to enhanced escalation. The involvement of the amygdala in the increased cocaine self-administration in $SERT^{-/-}$ rats is supported by the observation that amygdala volume was reduced in $SERT^{-/-}$ rats after a history of LgA cocaine self-administration (Karel *et al*. 2019). Since there is a bi-directional interaction between serotonin and BDNF (Homberg *et al*. 2014), it is plausible that BDNF signaling in the (central) amygdala contributes to the increased cocaine intake in $SERT^{-/-}$ rats.

The aim of this study was to clarify changes in BDNF signaling in the central amygdala in

association with compulsive cocaine self-administration, and how these changes are affected by SERT knockout. To this end, wild-type ($SERT^{+/+}$) and $SERT^{-/-}$ rats were allowed to self-administer cocaine under LgA conditions. Twenty-four (24) hours into withdrawal from LgA cocaine self-administration, i.e. at the time point the next self-administration session would start, we sacrificed the animals. Additionally, we included a naïve control group. In the post-mortem central amygdala of these rats we evaluated the levels of BDNF, its high affinity receptor TrkB, as well as BDNF-induced major intracellular signaling pathways, i.e. MAP kinase and Akt. Furthermore, given the role of serotonin and BDNF in the regulation of brain remodeling (Brivio et al., 2019; Leal et al., 2014), we measured Arc/Arg3.1 and PSD95 as markers of structural integrity (Caffino et al., 2017; Fumagalli et al., 2006).

2.0. Material and Methods

2.1 Behaviour

2.1.1 Animals.

SERT$^{-/-}$ rats (SLC6A41Hubr) were generated as previously reported (Smits et al., 2006) and outcrossed with commercially available Wistar rats (Harlan, Ter Horst, the Netherlands) for at least ten generations (Homberg et al., 2007). Male SERT$^{-/-}$ and wild-type (SERT$^{+/+}$) rats, weighing 250-300 g at the beginning of the study, were equipped with jugular vein catheters and subjected to cocaine- self-administration according to the procedures described below (SERT$^{+/+}$: n=12, SERT$^{-/-}$: n=14). All rats were housed in groups of 2-3 in Macrolon type III cages (42 x 26 x 15 cm) under a 12 h / 12 h reversed day/night cycle (lights off at 8:00 AM) in a temperature-controlled room (22±2 °C). Food pellets and water were available ad libitum, except during the cocaine- self-administration sessions. All procedures were carried out in agreement with the current National Research Council Guide for the Care and Use of Laboratory Animals and were approved by local Institutional Animal Care and Use Committees. All efforts were made to reduce the number of animals used and their suffering.

2.1.2. Drug.

Cocaine (NIDA, Rockville, MD) was dissolved in saline 0.9%.

2.1.3. Operant testing

Cocaine self-administration was performed in standard operant chambers (28 x 26 x 20 cm, Med Associates Inc., St Albans, VT) placed in a ventilated, light- and sound-attenuating cubicle. Chambers were equipped with a swivel system allowing rats to move freely during cocaine self-administration sessions. Cocaine was delivered by a 15 r.p.m. syringe pump (Razel Scientific Instruments, Georgia, VT). The start of a session was signaled by the presentation of 2 retractable

levers into the self-administration chamber. Pressing the right lever delivered cocaine (volume: 0.1 mL in 4s) whereas pressing the left lever had no programmed consequences. During drug self-administration, a stimulus light above the active lever was illuminated and illumination lasted throughout a time-out period of 40s, during which operant responding was not reinforced. One week after surgery, rats were trained to self-administer cocaine (0.5 mg/kg/infusion) under a fixed ratio 1 (FR1) schedule of reinforcement for 10 days during 1 hour/day. Two days after cocaine self-administration training, rats were allowed to self-administer cocaine during daily 6 h sessions (extended or Long-Access (LgA) group of rats), for a total of 15 days (Ahmed and Koob, 1998). For both training and LgA cocaine self-administration there was no maximum set for the number of infusions. After each infusion there was a time out for 40 seconds to prevent overdosing.

Additional groups of cocaine-naive $SERT^{-/-}$ (n=8) and $SERT^{+/+}$ (n=6) rats also underwent intravenous catheterization, were handled daily, and received daily infusions of heparinized saline, but were not exposed to the self-administration chambers and the discrete cues that predict cocaine availability (Verheij *et al*. 2016, 2018).

2.2. Molecular analyses

2.2.1. Collection of central amygdala tissue.

Twenty-four hours following the last cocaine self-administration session, a randomized part of the rats that had self-administered cocaine (n=6-8) was sacrificed by decapitation. The control animals were also decapitated. After decapitation brains were quickly collected and stored at -80^0C. According to the rat brain atlas of Paxinos and Watson (2007), a 1 mm diameter region containing bilateral central amygdala was punched (coordinates between bregma -1.72 mm and bregma -3.48 mm) from frozen dissected brain sections of 220 µm (Giannotti *et al*. 2016). Central amygdala tissue was stored at −80°C until being processed for molecular analysis (see below). Material of one hemisphere was used for RNA isolation, and material of the other hemisphere for protein analysis.

2.2.2. RNA Preparation and Real-Time Polymerase Chain Reaction

Total RNA of 6-8 randomly selected rats/genotype/treatment was isolated by single step guanidinium isothiocyanate/phenol extraction using PureZol RNA isolation reagent (Bio-Rad Laboratories, Segrate, Milan, Italy) according to the manufacturer's instructions and quantified by spectrophotometric analysis. Following total RNA extraction, the samples were processed for real-time reverse transcription polymerase chain reaction (real time RT-PCR) to assess mRNA levels, as previously described (Caffino *et al*. 2018). Briefly, an aliquot of each sample was treated with DNase to avoid DNA contamination. RNA was analyzed by TaqMan qRT-PCR instrument (CFX384 real time system, Bio-Rad Laboratories) using the iScriptTM one-step RT-PCR kit for probes (Bio-Rad Laboratories). Samples were run in 384 wells formats in triplicate as multiplexed reactions. Thermal cycling was initiated with an incubation at 50°C for 10 min (RNA retrotranscription) and then at 95°C for 5 min (TaqMan polymerase activation). After this initial step, 39 cycles of PCR were performed. Each PCR cycle consisted of heating the samples at 95°C for 10 s to enable the melting process and then for 30 s at 60°C for the annealing and extension reaction. Data were analyzed with the comparative threshold cycle (ΔΔCt) method using either *36B4* or *β-actin* as reference genes. The primer efficiencies were experimentally set up for each couple of primers. Primers and probe for *Bdnf exon IV* and *VI* were purchased from Applied Biosystem (*BDNF exon IV*: ID Rn01484927_m1 and *Bdnf exon VI*: ID Rn01484928_m1). Primers and probe for total *Bdnf, Bdnf exon I* and *36B4* were purchased from Eurofins MWG-Operon. Their sequences are shown below:

- total *Bdnf*: forward primer 5'-AAGTCTGCATTACATTCCTCGA-3', reverse primer 5'-GTTTTCTGAAAGAGGGACAGTTTAT-3', probe 5'-TGTGGTTTGTTGCCGTTGCCAAG-3';

- *Bdnf exon I*: forward primer 5'-GGGAGACGAGATTTTAAGACACTG-3', reverse primer 5'-GTCATCACTCTTCTCACCTGG-3', probe 5'-TTGTGGCTTTGCTGTCCTGGAGA-3';

- *36B4*: forward primer 5'-TTCCCACTGGCTGAAAAGGT-3', reverse primer 5'-CGCAGCCGCAAATGC-3', probe 5'-AAGGCCTTCCTGGCCGATCCATC-3'.

2.2.3. Protein extraction and Western blot analyses

Central amygdala tissues of 6 randomly selected rats/genotype/treatment randomly selected animals were homogenized in a glass-glass potter using a cold buffer containing 0.32 M sucrose, 1 mM Hepes solution, 0.1 mM EGTA, 0.1 mM PMSF, pH = 7.4, in presence of a complete set of protease inhibitors and a phosphatase inhibitor cocktail. Total proteins have been measured in the whole homogenate by the Bio-Rad Protein Assay (Bio-Rad Laboratories). Western blots were run as previously described (Caffino *et al*. 2016). Briefly, fifteen micro-grams of proteins for each sample were run under reducing conditions on the criterion TGX precast gels (Bio-Rad Laboratories, Milan, Italy) under reducing conditions and then electrophoretically transferred onto nitrocellulose membranes (GE Healthcare, Milan, Italy). Blots were blocked 1 h at room temperature with 10% non-fat dry milk in tris buffered saline + 0.1% Tween-20 buffer and then incubated with antibodies against the total proteins of interest.

The conditions of the primary antibodies were the following: anti mBDNF (1:1000, Icosagen, USA); anti phospho-TrkB Tyr816 (1:1000, Cell Signaling Technology, USA), anti phospho-TrkB Tyr706 (1:500, Cell Signaling Technology, USA), anti phospho-Akt Ser473 (1:1000, Cell Signaling Technology, USA), anti phospho-ERK1 Thr202/Tyr204 and phospho-ERK2 Thr185/Tyr187 (1:1000, Cell Signaling Technology, USA), anti-total TrkB (1:500, Cell Signaling Technology, USA), Akt (1:1000, Cell Signaling Technology, USA), ERK2 (1:5000, Cell Signaling Technology), anti PSD95 (1:4000, Cell Signaling Technology, USA), Arc/Arg3.1 (1:500, BD Transduction Laboratories, USA) and anti β-actin (1:10,000, Sigma-Aldrich). Results were standardized to β-actin control protein, which was detected by evaluating the band density at 43 kDa. Immunocomplexes were visualized by chemiluminescence using the Chemidoc MP Imaging

System (Bio-Rad Laboratories). Activation of the proteins investigated were expressed as a ratio between the phosphorylated and the respective total forms and analyzed using the Image Lab software from Bio-Rad Laboratories.

2.3. Statistical analysis

Data were collected in individual animals (independent determinations) and are presented as means ± standard errors. The effects of genotype on cocaine self-administration were analyzed using a two-way analysis of variance (ANOVA) with a correction for repeated measures followed by a Student's t test.

To enable visual comparisons across genotypes with different degrees of expression of BDNF and its signaling pathways, values are presented as percent of the control group, namely the $SERT^{+/+}$-naive group that was not exposed to LgA cocaine self-administration. Molecular changes produced by genotype and cocaine exposure alone as well as by their combination were analyzed using a two-way ANOVA, with genotype and cocaine self-administration as independent variables. When dictated by relevant interaction terms, Bonferroni multiple comparisons test was used to characterize differences among individual groups of rats. When no interaction between genotype and cocaine self-administration was observed, only the main effects were reported. Significance for all tests was assumed at $p < 0.05$.

3.0. Results

3.1. Cocaine intake

During the acquisition of cocaine self-administration, no genotype differences were observed (Fig. 1A). When the rats were allowed to self-administer cocaine under long access conditions, the daily number of cocaine-reinforced lever presses was higher in $SERT^{-/-}$ versus $SERT^{+/+}$ rats (Fig. 1B: genotype x session effect: $F_{(14,336)}=1.797$, p=0.038), with a higher total cocaine intake in $SERT^{-/-}$ (1204 +/- 84 infusions) versus $SERT^{+/+}$ (832 +/- 157 infusions) rats (t-test: p=0.020). The number of inactive lever presses was similar in both genotypes (Fig. 1C).

3.2. Molecular analyses

Molecular analyses were performed on the central amygdala from rats sacrificed 24 hours following the last cocaine self-administration session. We first analyzed *total Bdnf* mRNA levels in $SERT^{-/-}$ and $SERT^{+/+}$ rats, with and without LgA cocaine self-administration. Fig. 2 shows that SERT knockout modulated the neurotrophin expression in both cocaine-naïve and LgA cocaine-exposed rats. Two-way ANOVA of *total Bdnf* revealed a significant effect of treatment ($F_{(1,22)}=9.563$, p=0.0053), genotype ($F_{(1,22)}=6.324$, p=0.0197) and a significant genotype x treatment interaction effect ($F_{(1,22)}=5.822$, p=0.0246). Examining the individual treatment effect, we found that LgA cocaine self-administration increased *Bdnf* mRNA levels only in $SERT^{-/-}$ rats (+43% vs $SERT^{-/-}$-naïve rats, p=0.0063; +39% vs $SERT^{+/+}$- LgA rats, p=0.0164; Fig. 2A).

Given the complexity of the *Bdnf* gene, consisting of different isoforms driven by separate promoters (Aid et al., 2007), and taking into account that such isoforms may respond to different stimuli and be differently located in neurons, we expanded our analysis by investigating *exon I*, *exon IV* and *exon VI Bdnf* mRNA levels. Analysis of *exon I* revealed a significant effect of treatment ($F_{(1,22)}=37.23$, p<0.0001) and a genotype x treatment interaction effect ($F_{(1,22)}=18.00$, p=0.0003). Compared to naïve $SERT^{+/+}$ rats, basal levels of *Bdnf exon I* were reduced in $SERT^{-/-}$

rats (-34% vs SERT$^{+/+}$-naïve rats, p=0.0252). Additionally, similar to the pattern seen for *total Bdnf*, cocaine self-administration was associated with up-regulated *Bdnf exon I* gene expression in SERT$^{-/-}$ rats (+81% vs SERT$^{-/-}$-naïve rats, p<0.0001; Fig. 2B). We then analyzed *exon IV* expression levels, the most abundant activity-dependent transcription factor. We found an effect of genotype ($F_{(1,22)}$=17.56, p=0.0004) and treatment ($F_{(1,22)}$=16.62, p=0.0005), and a genotype x treatment interaction effect ($F_{(1,22)}$=5.211, p=0.0325; Fig 2C). In particular, LgA cocaine self-administration was associated with a marked increase in *Bdnf exon IV* levels only in SERT$^{-/-}$ rats (+75% vs SERT$^{-/-}$-naïve rats, p=0.0015; +77% vs SERT$^{+/+}$- LgA cocaine self-administration rats, p=0.0013). Finally, we examined the expression of *Bdnf exon VI*, located in dendrites (Chiaruttini et al., 2008), a feature that allows its prompt synthesis in case of demand. Two-way ANOVA of *Bdnf exon VI* revealed an effect of treatment ($F_{(1,22)}$=8.644, p=0.0076), no effect of genotype ($F_{(1,22)}$=5.629, p=0.0268) and a genotype x treatment interaction effect ($F_{(1,22)}$=6.997, p=0.0148). Post-hoc testing indicated a marked and significant increase of *Bdnf exon VI* in the central amygdala of cocaine exposed SERT$^{-/-}$ rats compared to SERT$^{-/-}$ naïve rats (+78%, p=0.0056) and SERT$^{+/+}$ rats with a history of LgA cocaine self-administration (+70%, p=0.0142) (Fig. 2D).

We then analyzed protein expression of the mature form of BDNF (mBDNF) in the central amygdala of SERT$^{-/-}$ and wild-type rats, with and without LgA cocaine self-administration (Fig 3A). Two-way ANOVA of mBDNF expression revealed a significant genotype x treatment interaction effect ($F_{(1,22)}$=34.24, p<0.0001). In particular, SERT deletion evoked a significant decrease in mBDNF protein levels in cocaine naïve rats (-26% vs SERT$^{+/+}$-naïve rats, p=0.0205). Interestingly, LgA cocaine self-administration was associated with a decrease in mBDNF levels in SERT$^{+/+}$ rats (-26% vs SERT$^{+/+}$-naïve rats, p=0.0175) but an increase in SERT$^{-/-}$ rats (+41% vs SERT$^{-/-}$-naïve rats, p=0.0004; +41% vs SERT$^{+/+}$-LgA cocaine self-administration rats, p=0.0004) (Fig. 3A).

We next analyzed the expression and phosphorylation of the BDNF receptor TrkB in the central amygdala. Figure 3B and 3C shows activation of TrkB in two distinct sites of phosphorylation, i.e. pTrkB (Tyr816) and pTrkB (Y706). No significant effects were found when examining pTrkBY816/TrkB levels. Conversely, two-way ANOVA of pTrkBY706/TrkB levels revealed an effect of genotype ($F_{(1,22)}$=6.017, p=0.0226) and treatment ($F_{(1,22)}$=9.926, p=0.0046) as well as a significant genotype x treatment interaction effect ($F_{(1,22)}$=5.173, p=0.033). In wild-type rats, LgA cocaine self-administration was associated with an increased activation of pTrkB (Tyr706) (+84% vs SERT$^{-/-}$-naïve rats, p=0.0039). Naïve SERT$^{-/-}$ displayed a significant increase in pTrkB (Tyr706) (+73% vs SERT$^{-/-}$-naïve rats, p=0.0134), but LgA self-administration cocaine had no further effect on TrkB phosphorylation (Tyr706) in the central amygdala of SERT$^{-/-}$ rats (+14% vs SERT$^{-/-}$-naïve rats, p>0.99).

Next, we investigated whether the changes in the BDNF-TrkB system would translate to the recruitment of BDNF intracellular pathways, as previously shown (Giannotti et al., 2014). Two-way ANOVA of Akt activation revealed a significant treatment effect ($F_{(1,22)}$=4.834, p=0.0387), and a genotype x treatment interaction effect ($F_{(1,22)}$=4.532, p=0.0447; Fig. 4A). In wild-type rats with a history of LgA cocaine self-administration, as well as cocaine-naïve SERT$^{-/-}$ rats, pAktS473/Akt levels were upregulated (+33% SERT$^{-/-}$-naïve rats vs SERT$^{+/+}$-naïve, p=0.0358; +32% vs SERT$^{+/+}$-LgA cocaine self-administration vs SERT$^{+/+}$-naïve rats, p=0.0268), whereas there was no further change in pAktS473/Akt levels after LgA cocaine self-administration in SERT$^{-/-}$ rats (0% vs SERT$^{-/-}$-naïve rats, p>0.99).

Two-way ANOVA of pERK1(Thr202/Tyr204)/ERK1 levels revealed an effect of treatment ($F_{(1,22)}$=8.201, p=0.009) and a significant genotype x treatment interaction effect ($F_{(1,22)}$=5.538, p=0.028; Fig. 4B). In wild-type rats, LgA cocaine self-administration was associated with increased pERK1 levels (+47% vs SERT$^{-/-}$-naïve rats, p=0.0056) whereas no changes in pERK1 levels were observed in SERT$^{-/-}$ rats (+2% vs SERT$^{-/-}$-LgA rats, p>0.999). Conversely, investigation of ERK2

expression and its phosphorylation (Thr185/Tyr187) revealed no effect of treatment ($F_{(1,22)}$=0.9566, p=0.3387), genotype ($F_{(1,22)}$=1.634, p=0.2145) and genotype x treatment interaction effect ($F_{(1,22)}$=0.163, p=0.6903; Fig. 4C).

We finally analyzed the expression of two major structural synaptic proteins, PSD95 and Arc/Arg3.1, to evaluate whether LgA cocaine self-administration may result in changes in structural or functional integrity of the post-synaptic density (see also: Fumagalli et al., 2006; Fumagalli et al., 2009; Caffino et al., 2017; Caffino et al., 2018). With respect to PSD95 (Fig. 5A), we found an effect of genotype ($F_{(1,20)}$=12.59, p=0.002) and a significant genotype x treatment interaction effect ($F_{(1,20)}$=19.75, p=0.0002). LgA cocaine self-administration was associated with enhanced PSD95 levels in the central amygdala of wild-type rats (+42% vs $SERT^{+/+}$-naïve rats, p=0.0144), but reduced PSD95 levels in $SERT^{-/-}$ rats (-68% vs $SERT^{+/+}$-LgA cocaine self-administration rats, p=0.0002) (Fig. 5A). Examining Arc/Arg3.1 expression we found only a significant genotype x treatment interaction effect ($F_{(1,21)}$=27.49, p=0.0001). LgA cocaine self-administration was associated with increased Arc/Arg3.1 levels in the central amygdala of wild-type rats (+43% vs $SERT^{+/+}$-naïve rats, p=0.0222). Furthermore, $SERT^{-/-}$ was associated with an up-regulation of Arc/Arg3.1 protein levels in cocaine-naive $SERT^{-/-}$ rats (+47% vs $SERT^{+/+}$-naïve rats, p=0.0111). Finally, LgA cocaine self-administration was associated with diminished Arc/Arg3.1 expression in $SERT^{-/-}$ rats (-57% vs $SERT^{-/-}$-naïve rats, p=0.0028; -53% vs $SERT^{+/+}$-LgA cocaine self-administration rats, p=0.0056; Fig. 5B).

4.0. Discussion

Our findings show that short-term (24 h) withdrawal from Long-Access (LgA) cocaine self-administration decreases mBDNF protein levels in the central amygdala of $SERT^{+/+}$ rats. This goes along with increased TrkB (Y706) receptor phosphorylation (indicative of increased activity) and an increase in ERK1- and Akt-dependent intracellular signaling. Additionally, the state of the BDNF system in cocaine-naïve $SERT^{-/-}$ rats resembles that of the BDNF system in cocaine-exposed $SERT^{+/+}$ rats. Finally, we observed that LgA cocaine self-administration led to an overall dysregulation of BDNF intracellular signaling in the central amygdala of $SERT^{-/-}$ rats (for summary, see Fig. 6).

The decreased mBDNF protein levels in cocaine-exposed $SERT^{+/+}$ rats may reflect a compensatory response to the increased TrkB receptor and Akt- and ERK1-dependent intracellular signaling. Since increased TrkB receptor signaling generally facilitates neuroplasticity (Duman and Voleti, 2012), our findings could be interpreted as adaptive changes to support homeostasis with high levels of cocaine on board.

Like in cocaine exposed $SERT^{+/+}$ rats, we found an overall reduction in the expression of BDNF protein in cocaine-naïve $SERT^{-/-}$ rats. This suggests that increased extracellular serotonin negatively affects the synthesis of BDNF, an effect previously seen for the prefrontal cortex and hippocampus (Calabrese et al., 2013; Molteni et al., 2010). In cocaine-exposed $SERT^{-/-}$ rats, we found that changes in basal *Bdnf* mRNA levels (*total BDNF* as well as *BDNF exons* with the exception of *BDNF exon IV*) were paralleled by changes in basal mBDNF protein expression. This association between gene and protein expression is not necessarily expected as we have previously shown (Fumagalli et al., 2007). The combined *Bdnf* gene and protein changes indicate that the changes caused by SERT knockout are enduring and stable. Although the functional consequences of multiple *Bdnf transcripts* are still obscure, specific exons may undergo different subcellular localization (Aid et al., 2007), which may be functionally relevant. In fact, changes in *Bdnf exon*

I and *IV*, localized at somatic level, may be indicative of altered neuronal activity whereas *Bdnf exon VI* changes may reflect an altered dendritic targeting of this isoform. Following LgA cocaine self-administration *BDNF exons I, IV* and *VI* increased in SERT$^{-/-}$ rats, in line with *total BDNF* mRNA levels, ruling out the possibility of a cocaine-induced effect on a specific neurotrophin pool (i.e. somatic vs. dendritic). Previous data have shown that, following repeated cocaine self-administration, *Bdnf* expression is increased in the rat amygdala after 30 and 90, but not 1, day(s) of withdrawal, an effect related to the incubation of craving (Grimm et al., 2003). Our results suggest that changes in extracellular serotonin induced by SERT deletion may shorten the latency of BDNF elevation, providing a mechanism for the higher cocaine intake in these animals.

TrkB is the high affinity receptor that mediates the action of BDNF by activating its intracellular pathways. Its specific action strictly depends on its phosphorylation sites, whose stimulation addresses the activation of a different BDNF-dependent signaling pathway. Accordingly, we measured two different phosphorylation sites of TrkB, namely pTrkB (Y816), which regulates PLCγ-mediated signaling, and pTrkB (Y706), which regulates both ERK and Akt phosphorylation (Minichiello, 2009). First, we noted that the baseline phosphorylation level is different between the phosphorylation sites, since pTrkB (Y816) is unaltered while pTrkB (Y706) is increased in the central amygdala of cocaine-naïve SERT$^{-/-}$ rats. Accordingly, basal ERK1 and Akt phosphorylation is increased in SERT$^{-/-}$ rats, at variance from ERK2 phosphorylation that is unchanged. Thus, these data unravel a previously unappreciated difference between ERK 1 and ERK2 as ERK2 appears to be uncoupled from its related phosphorylation site of TrkB. The role of each of these two isoforms has not been extensively characterized in the amygdala. While being aware that phosphorylation of a given protein may depend upon the presence and expression level of protein phosphatases, these findings suggest separate regulatory mechanisms for ERK1 and ERK2 in the central amygdala. Second, we observed that LgA cocaine self-administration was associated with changes in the phosphorylation level of the 2 pathways in SERT$^{+/+}$ and SERT$^{-/-}$ rats.

Whereas no changes were observed in ERK2 phosphorylation following cocaine exposure in both genotypes, $SERT^{+/+}$ rats showed a marked increase in ERK1 and Akt phosphorylation. In contrast, the knockout of SERT could not promote a further increase of ERK1 and Akt activation. The cocaine-induced activation of the Akt-mediated pathway in $SERT^{+/+}$ rats may have functional relevance. In fact, it has been demonstrated that BDNF, through Akt (Yoshii and Constantine-Paton, 2007), influences dendritic spine formation and stability (Berry et al., 2018; Kim and Sheng, 2004). The analysis of PSD95, a structural marker of dendritic spines, revealed that the cocaine mediated activation of the BDNF-TrkBY706-Akt pathway in $SERT^{+/+}$ rats may up-regulate PSD95 expression, which is instead reduced in the central amygdala of $SERT^{-/-}$ rats. Notably, we found that the expression of the other BDNF downstream target Arc/Arg3.1, which is engaged in structural synaptic plasticity in the amygdala (You et al., 2014) and regulated by cocaine (Caffino et al., 2017, 2015), fully overlaps with the PSD95 expression profile. Accordingly, we point to Arc/Arg3.1 and PSD95 as signatures of amygdaloid structural dysregulation following LgA cocaine self-administration in $SERT^{-/-}$ rats. These findings suggest that LgA cocaine self-administration alters the physiological structural remodeling as a function of the serotonergic tone likely through, at least partially, the BDNF-TrkB-Akt cascade. As a very big step, the cocaine-induced reduction in PSD95 and Arc/Arg3.1 may contribute to the smaller amygdala volume of cocaine-exposed $SERT^{-/-}$ rats (Karel et al., 2019) and cocaine addicts (Makris et al., 2004). Furthermore, the increase of *Bdnf* mRNA and protein levels observed in $SERT^{-/-}$ rats exposed to LgA cocaine self-administration may reflect an attempt to compensate for the structural alterations shown by the reduced expression of PSD95 and Arc/Arg3.1 in these animals.

While we believe that our manuscript shows several strengths, we are also aware that it suffers from some limitations. First, our study does not show whether cocaine intake leads to changes in BDNF, or whether BDNF lead to changes in the intake of cocaine. Second, mBDNF may have affected the phosphorylation of TrkB sites other than Tyr706 or Tyr816. Third, while the

amygdala is specifically linked to emotional changes in addiction, we did not measure emotion in this study. From our previous work, however, we know that anxiety is increased in SERT$^{-/-}$ rats, relative to wild-type rats, 24 hrs into withdrawal from LgA cocaine self-administration (Verheij et al., 2018). This suggests that the BDNF-Arc/Arg3.1-PSD95 reductions found in SERT$^{-/-}$ rats following LgA cocaine self-administration is related to increased anxiety, as previously demonstrated (Moonat et al., 2011).

In conclusion, we here show that 24 hours withdrawal from long-access cocaine self-administration is associated with decreases in mBDNF protein expression levels and increases in TrkB signalling in the central amygdala of wild-type animals. The knockout of SERT increases BDNF protein expression and perturbs TrkB signaling in the central amygdala following long-access cocaine self-administration. This implies that BDNF and serotonin at least partly mediate compulsive cocaine-self-administration.

Figure legends

Figure 1 Training of cocaine self-administration in SERT$^{+/+}$ (n = 12) and SERT$^{-/-}$ (n = 14) rats preceding the long access phase (panel a). Number of active (panel a) and inactive (panel b) lever presses during 15 session of cocaine self-administration under long access conditions. Data represent the mean ± SEM. See Material and Methods for further details.

$^{*}p < 0.05$, $^{**}p < 0.01$ versus SERT$^{+/+}$ (two-way ANOVA: genotype x session effect)

Figure 2 Interaction between SERT deletion and cocaine self-administration on gene expression of the neurotrophin BDNF and its main isoforms in the central amygdala.

mRNA levels of *total BDNF* (panel a), *BDNF exon I* (panel b), *BDNF exon IV* (panel c) and *BDNF exon VI* (panel d) are expressed as percentages of SERT$^{+/+}$-naive rats. Histograms represent the mean ± SEM of eight to six rats per group.

$^{*}p < 0.05$ versus SERT$^{+/+}$-naive; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ versus SERT$^{-/-}$-naive; $^{\$}p < 0.05$ versus SERT$^{+/+}$-LgA (two-way ANOVA followed by Bonferroni multiple comparisons test)

Figure 3 Interaction between SERT deletion and cocaine self-administration on BDNF protein levels and on its high affinity receptor TrkB in the whole homogenate of the central amygdala.

Panel (a) shows mBDNF protein levels whereas panel (b) and (c) show the ratio between the phosphorylated (Tyr816 and Tyr706, respectively) and the total form of the protein. Below the

graphs, representative immunoblots are shown for mBDNF (14 kDa), pTrkB Tyr816/TrkB (145 kDa) and pTrkB Tyr706/TrkB (145 kDa) proteins in the homogenate of central amygdala. Histograms, expressed as percentages of $SERT^{+/+}$-naive rats, represent the mean ± SEM of eight to six rats per group.

$^{*}p < 0.05$ versus $SERT^{+/+}$-naive; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ versus $SERT^{-/-}$-naive; $^{\$}p < 0.05$ versus $SERT^{+/+}$-LgA (two-way ANOVA followed by Bonferroni multiple comparisons test)

Figure 4 Interaction between SERT deletion and cocaine self-administration on BDNF-induced intracellular signaling pathways in the whole homogenate of the central amygdala.

Panel a, b and c show the phosphorylation of Akt, ERK1 and ERK2, respectively, expressed as the ratio between the phosphorylated and the total form of the protein. Below the graphs, representative immunoblots are shown for pAkt S473/Akt (60 kDa), pERK1Thr202-Tyr204/ERK1 (44 kDa) and pERK2Thr185-Tyr187/ERK2 (42 kDa) proteins in the homogenate of central amygdala. Histograms, expressed as percentages of $SERT^{+/+}$-naive rats, represent the mean ± SEM of eight to six rats per group.

$^{*}p < 0.05$ versus $SERT^{+/+}$-naive (two-way ANOVA followed by Bonferroni multiple comparisons test)

Figure 5 Interaction between SERT deletion and cocaine self-administration on PSD95 (panel a) and Arc/Arg3.1 (panel b) protein expression in the whole homogenate of the central amygdala.

Below the graphs, representative immunoblots are shown for PSD95 (95 kDa) and Arc/Arg3.1 (55 kDa) proteins in the homogenate of central amygdala. Histograms, expressed as percentages of $SERT^{+/+}$-naive rats, represent the mean ± SEM of eight to six rats per group.

$^{*}p < 0.05$, $^{\#\#}p < 0.01$ versus $SERT^{+/+}$-naive; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ versus $SERT^{-/-}$-naive; $^{\$\$}p <$

0.01, $^{\$\$\$}p < 0.001$ versus SERT$^{+/+}$-LgA (two-way ANOVA followed by Bonferroni multiple comparisons test)

Figure 6 Schematic representation of the changes of BDNF and its associated network set in motion by the combination of SERT knockout and long-access cocaine self-administration: comparison with between SERT$^{+/+}$ and SERT$^{-/-}$rats.

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*Revision Notes

Dear Prof.dr. Porrino,

Thank you for handling our manuscript entitled “Deletion of the serotonin transporter perturbs BDNF signaling in the central amygdala following long-access cocaine self-administration”. We thank the reviewers for carefully evaluating our edits. A few comments were remaining after our revision. Below, we set out how we addressed these comments. Our edits in the manuscript are highlighted in green. We believe that our manuscript is now suitable for publication in *Drug and Alcohol Dependence*.

Reviewer #1: The authors did not address my first and fourth concerns, which are minor. The Highlights contain grammatical errors, which at the least should be corrected. They do not synthesize the findings of the manuscript, however.
Reply: Thank you. We have further revised the highlights and removed the grammar errors we could detect.

As a refresher- here are the comments from the prior review that are referred to by the reviewer.

1. The data from the 10 days of 1 h short-access cocaine self-administration should also be shown in Figure 1, in addition to the 15 days of 6 h long-access.
Reply: We have added the 10 days of 1 h short-access cocaine self-administration to the manuscript as figure 1A.

4. The Highlights could be improved to reflect a more synthesized view of the BDNF dysregulations observed in SERT-/- rats.
Reply: We have substantially changed the highlights. We hope they convey now better the core findings of the manuscript, including the dysregulations found in the SERT-/- rats.

Reviewer #2: This revised manuscript has undergone a great improvement in text and figure clarity. Only a couple minor issues were noted in the review of this manuscript:
1. In the abstract, the abbreviation "mBDNF" is not explained before the first appearance in the text.
Reply: Apologies. We have now provided the explanation in the abstract.

2. The link between CRF and BDNF is not well established in the introduction. A more in depth description of the relationship between CRF and BDNF is needed to support a link for the roles of CRF and BDNF in the central amygdala being similar as they are functionally different.
Reply: Fair point. We have now mentioned in the introduction how CRF influences BDNF signaling.

Author's contribution

Michel Verheij and Danielle Mendes performed the cocaine self-administration study. Lucia Caffino and Francesca Mottarlini performed the molecular analyses. Lucia Caffino, Michel Verheij and Francesca Mottarlini did the statistical analyses. Lucia Caffino, Francesca Mottarlini and Danielle Mendes managed the literature searches. Lucia Caffino, Michel Verheij, Fabio Fumagalli and Judith Homberg designed the study, wrote the protocol and interpreted the data. Lucia Caffino, Michel Verheij, Judith Homberg and Fabio Fumagalli wrote the manuscript. All authors contributed to and have approved the final manuscript.

Conflicts of Interest

The authors declare no conflict of interest in relation to the work herein described.

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Nijmegen, July 9th 2019

Dear Editor,

Please find attached an **Original Article** entitled "**Deletion of the serotonin transporter perturbs BDNF signaling in the central amygdala following long-access cocaine self-administration**" by Caffino and colleagues, for consideration for publication in **Drug and Alcohol Dependence**.

There is a great deal of interest in understanding the mechanisms through which the amygdala plays a key role in cocaine addiction. It has been well-established that cocaine exerts its effects, amongst others, through the neurotrophin BDNF. Surprisingly, data on amygdaloid BDNF signaling in the context of cocaine addiction is virtually non-existent. Here, we investigate BDNF signaling in the amygdala of rats exposed to long access cocaine self-administration. We not only measured BDNF levels, but also assessed expression levels of its receptor TrkB and down-stream intracellular signaling molecules. Both phosphorylated and non-phosphorylated levels were determined to determine whether the signaling pathways were functional. We found that, at 24 hours into withdrawal from cocaine exposure, BDNF mRNA and protein levels were decreased, whereas the phosphorylation of its receptor TrkB as well as of its intracellular signaling molecule Akt were increased.

Because BDNF is strongly regulated by serotonin levels and inherited serotonin transporter down-regulation is associated with increased vulnerability to cocaine addiction, we also studied the effect of serotonin transporter knockout on cocaine-mediated amygdaloid BDNF signaling. We found that these knockout rats showed increased cocaine self-administration, in line with our previous findings with this rat model. Additionally, we observed that BDNF signaling in cocaine-naïve serotonin transporter knockout rats resembled that of wild-type rats with a history of long access cocaine self-administration. This provides a mechanistic account for the increased vulnerability to compulsive

cocaine intake in these animals. Interestingly, at 24 hours into withdrawal from cocaine self-administration, this baseline profile in serotonin transporter knockout rats completely changed. We found that BDNF protein levels increased, whereas phosphoTrkB receptor coupling to Akt was decreased. We conclude that long access cocaine self-administration dysregulates BDNF signaling in the amygdala, and that vulnerability to cocaine addiction is associated with dysregulation of this signaling.

Our results represent an original research, which has not been previously published or been submitted for publication elsewhere.

All listed authors concur with the submission and have approved the final manuscript and none of the authors have competing financial interests related to the research described.

A list of potential reviewers for the manuscript and their expertise is included.

I trust that our manuscript could be of interest to **Drug and Alcohol Dependence** and I am looking forward to hearing from you.

On behalf of all authors,

Yours sincerely,

Prof.dr. Judith Homberg

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Figure 1

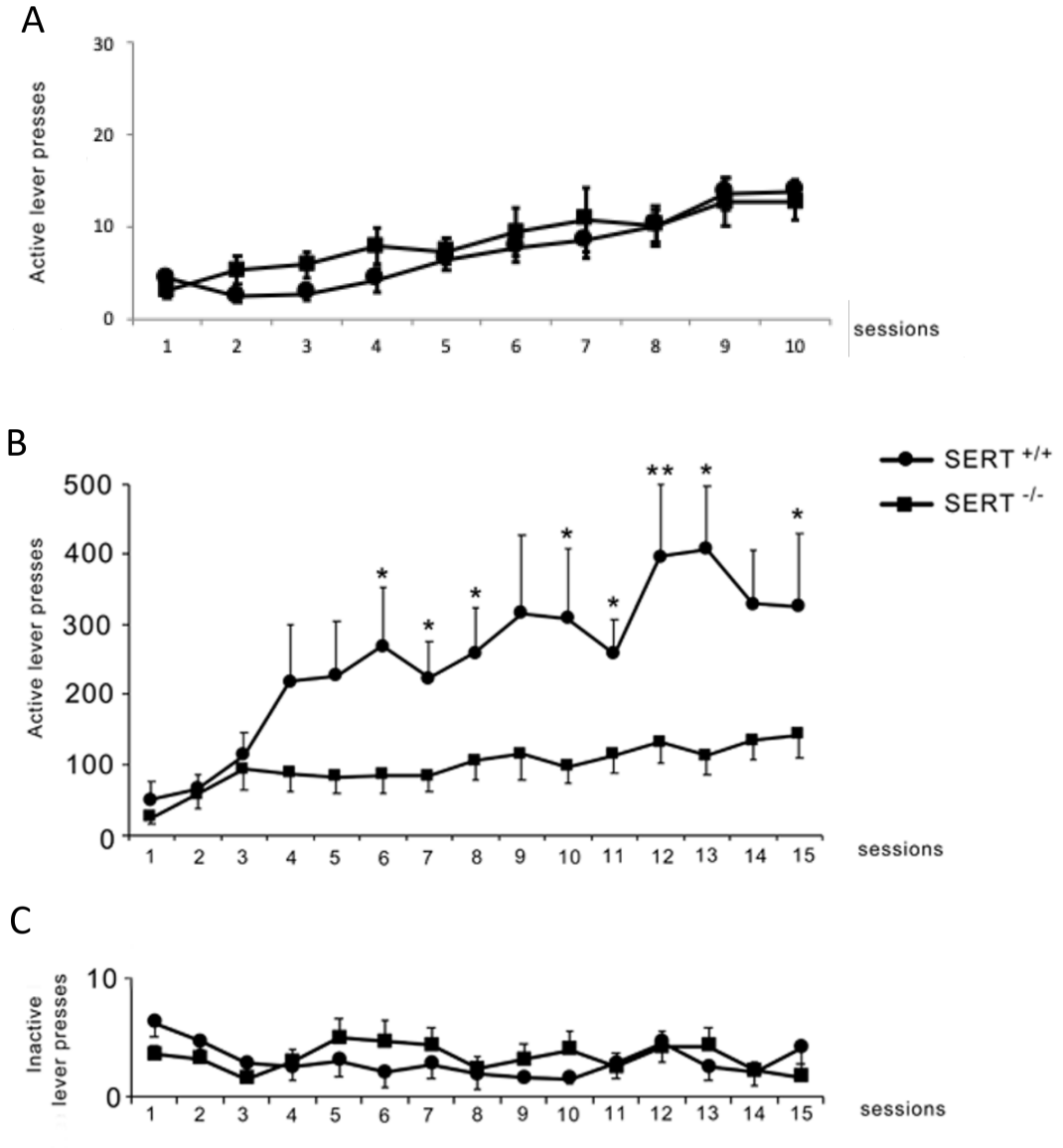
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Figure 2
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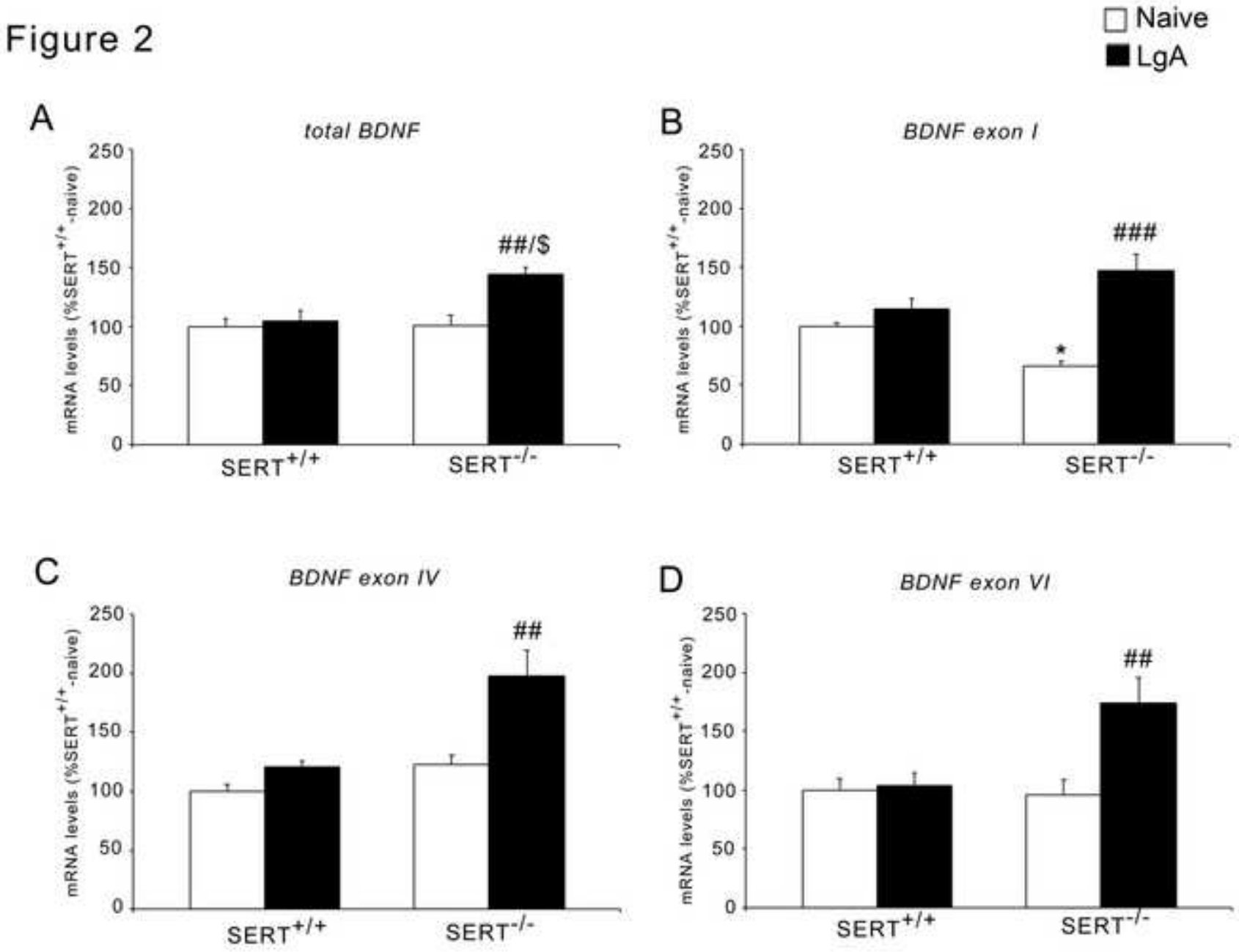

Figure 3

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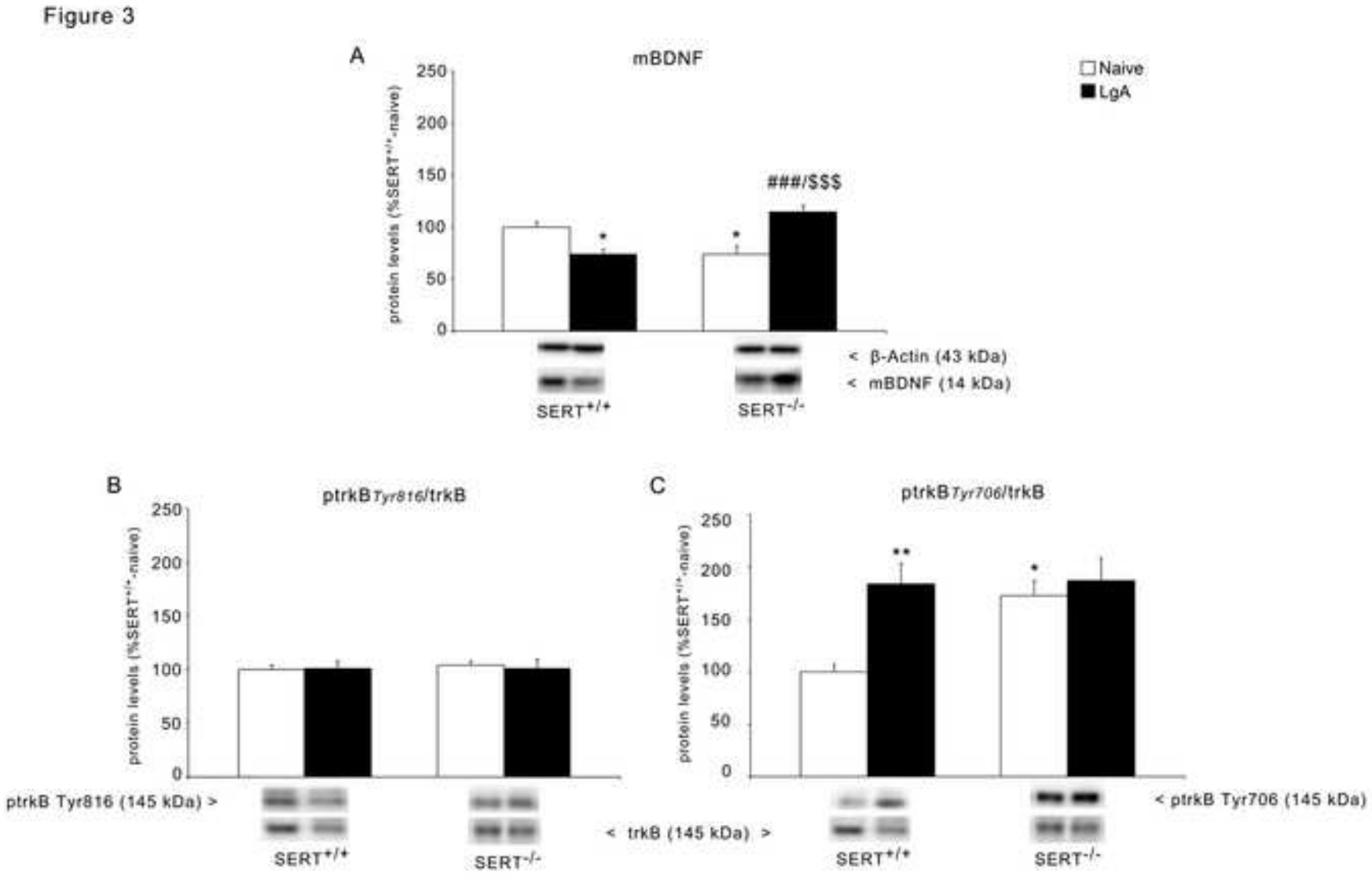

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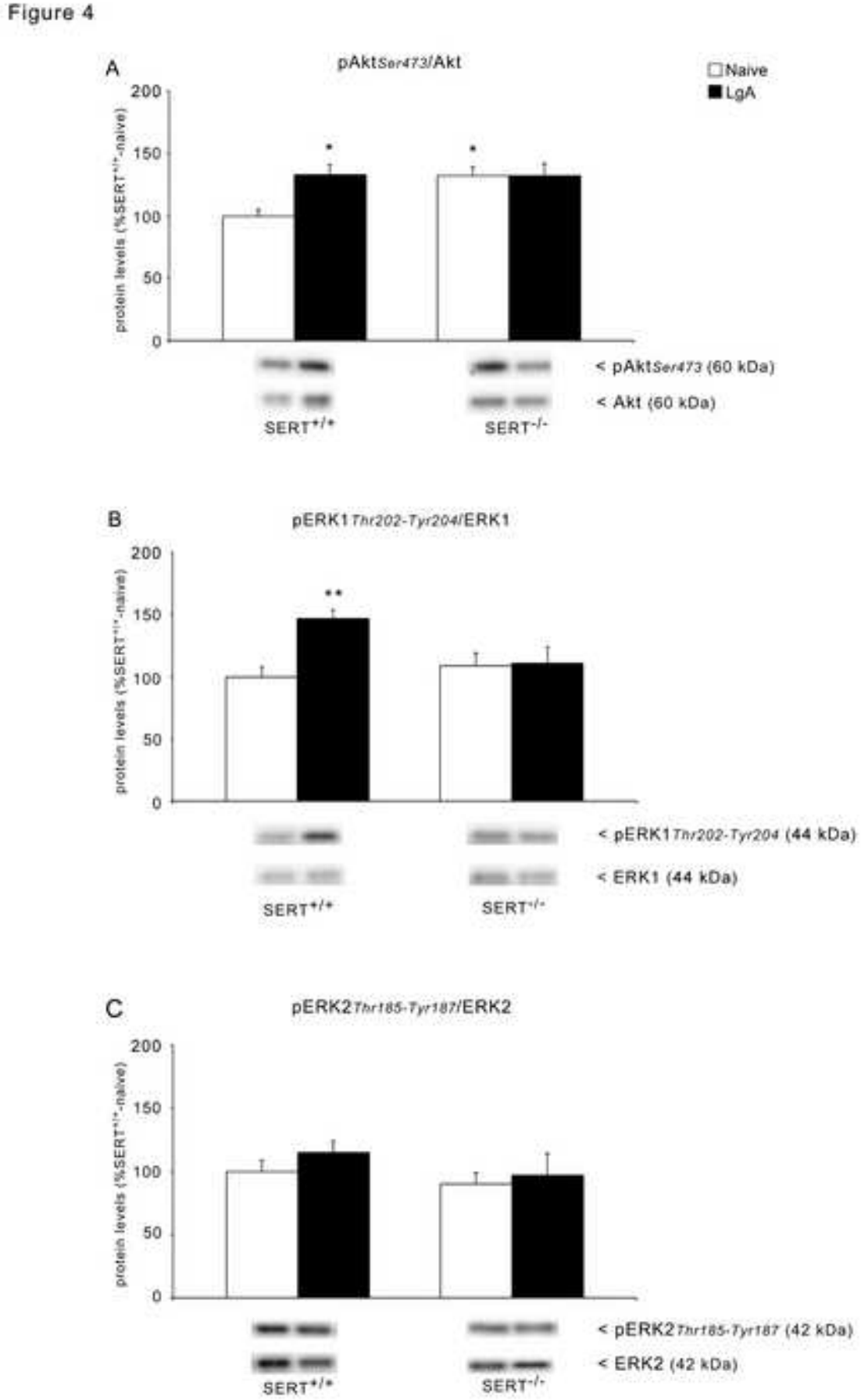

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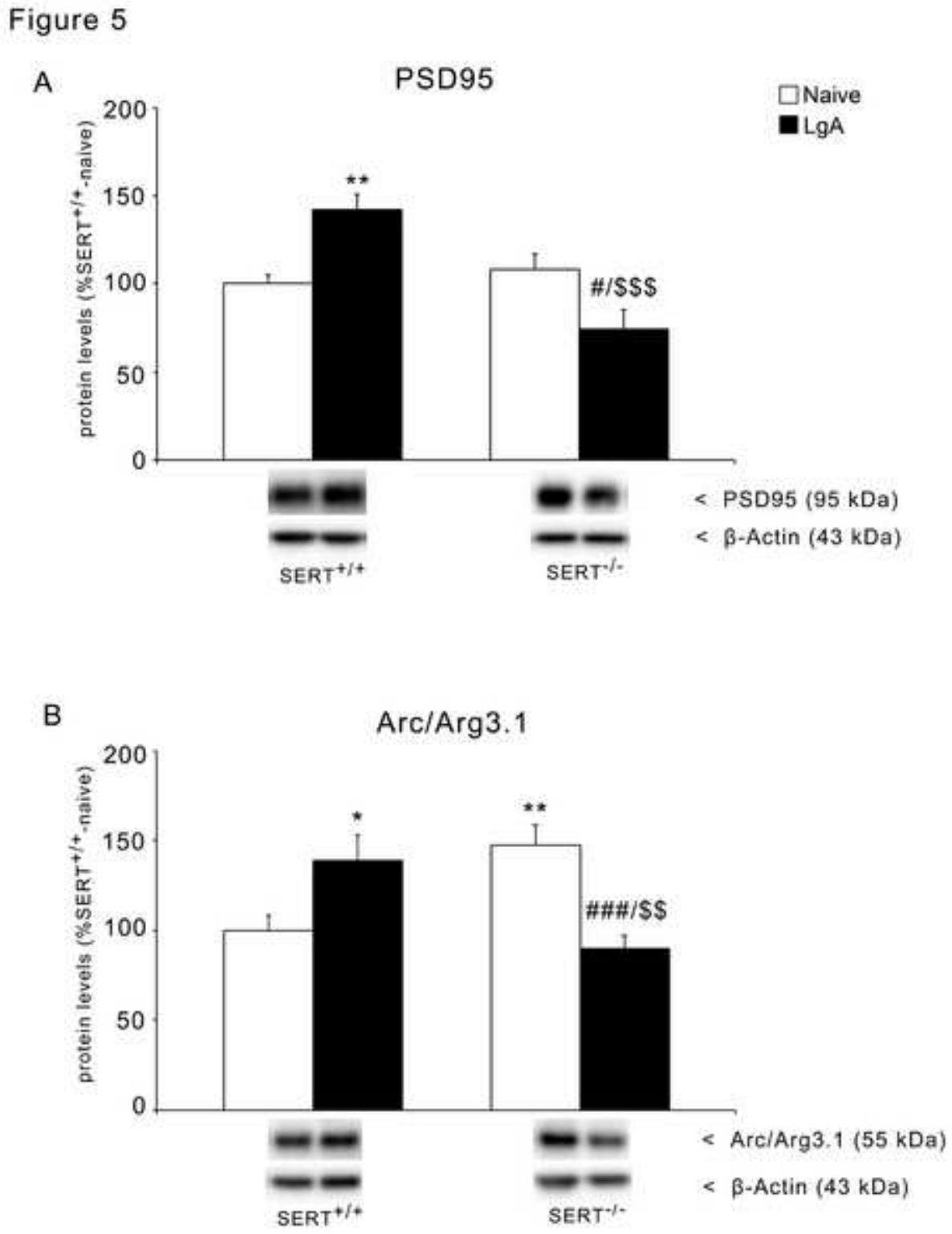

Figure 6
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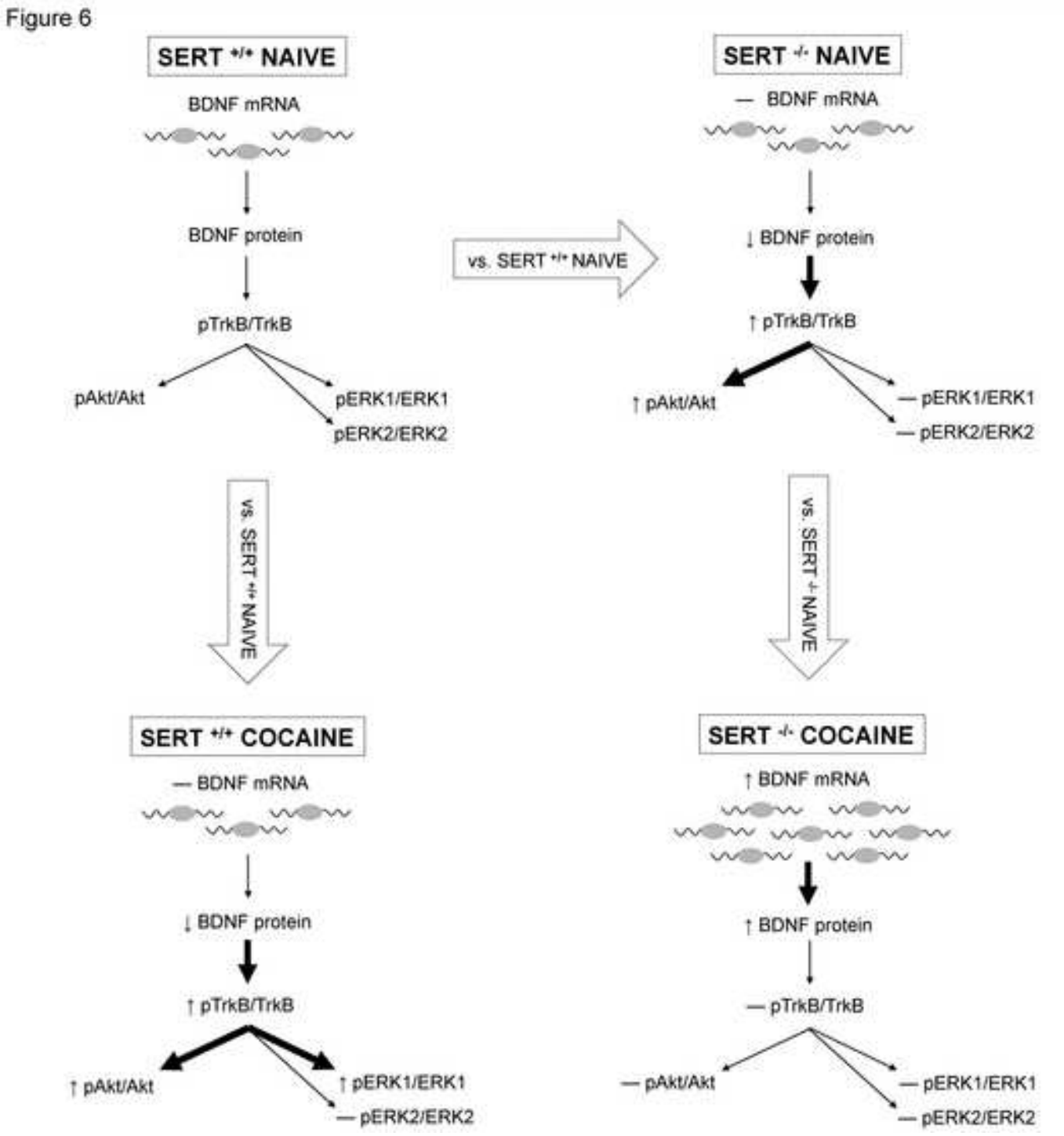

Supplementary Material

Click here to download Supplementary Material: Supplementary figures BLOT.pptx