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1 **Vesicular Acetylcholine Transporter (VACHT) overexpression induces major**
2 **modifications of striatal cholinergic interneuron morphology and function**

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22 **Running title: VACHT over-expression in striatal cholinergic interneuron**

23
24 **Keywords** : ChAT–ChR2 mice, VACHT overexpression, cholinergic interneurons, cholinergic
25 varicosities, electron microscopy, STED

26
27 **Abbreviations** :

28 ACh: Acetylcholine; AChE: Acetylcholinesterase; CathD: Cathepsin D; ChAT : Choline
29 Acetyltransferase; CHC: Clathrin heavy chain; CHT: High-affinity choline transporter; ER:
30 Endoplasmic Reticulum; CIN: Striatal cholinergic interneurons; GM130: Golgi matrix protein
31 of 130kDa; IP : immunoparticle; PBS: Phosphate buffer saline; *pc*: Pearson's coefficient; PDI:
32 protein disulphide isomerase; TGN38: Trans-Golgi network specific integral membrane
33 protein; VACHT: vesicular acetylcholine transporter.

1 **Title : Vesicular Acetylcholine Transporter (VACHT) overexpression induces major**
2 **modifications of striatal cholinergic interneuron morphology and function**

3
4
5 **Abstract**

6
7 Striatal cholinergic interneurons (CIN) are pivotal for the regulation of the striatal network.
8 Acetylcholine (ACh) released by CIN is centrally involved in reward behavior as well as
9 locomotor or cognitive functions. Recently, BAC transgenic mice expressing
10 channelrhodopsin-2 (ChR2) protein under the control of the choline acetyltransferase (ChAT)
11 promoter (ChAT-ChR2) and displaying almost 50 extra copies of the VACHT gene were
12 used to dissect cholinergic circuit connectivity and function using optogenetic approaches.
13 These mice display overexpression of the vesicular acetylcholine transporter (VACHT) and
14 increased cholinergic tone. Consequently, ChAT-ChR2 mice are a valuable model to
15 investigate hypercholinergic phenotypes. Previous experiments established that ChAT-ChR2
16 mice display an increased sensitivity to amphetamine induced-locomotor activity and
17 stereotypies. In the present report, we analyzed the impact of VACHT overexpression in the
18 striatum of ChAT-ChR2 mice. ChAT-ChR2 mice displayed increased locomotor sensitization
19 in response to low dose of cocaine. In addition, we observed a dramatic remodeling of the
20 morphology of CIN in ChAT-ChR2 transgenic mice. VACHT immunolabeling was markedly
21 enhanced in the soma and terminal of CIN from ChAT-ChR2 mice as previously shown
22 (Crittenden *et al.* 2014). Interestingly, the number of cholinergic varicosities was markedly
23 reduced (-87%) whereas their size was significantly increased (+177%). Moreover, VACHT
24 overexpression dramatically modified its trafficking along the somatodendritic and axonal
25 arbor. These findings demonstrate that ChAT-ChR2 mice present major alterations of CIN
26 neuronal morphology and increased behavioral sensitization to cocaine, supporting the
27 notion that the increased levels of VACHT observed in these mice make them fundamentally
28 different from wild-type mice.

1 Introduction

2

3 Acetylcholine (ACh) is a major neuromodulator of the striatal network. Dysfunctions of striatal
4 cholinergic activity contribute to the pathophysiology of neurodegenerative diseases like
5 Parkinson's disease or to the development of repetitive and compulsive behaviors, as well as
6 to addiction (Di Chiara *et al.* 1994, Sakae *et al.* 2015, Zhao & Keen 2008, Ztaou *et al.* 2016).
7 Although CIN represent less than 2% of striatal neurons, they display dense axonal arbors
8 and release locally high levels of ACh (Lim *et al.* 2014). In CIN, ACh is transported into
9 synaptic vesicles by the vesicular acetylcholine transporter (VACHT, *Slc18a3*). Therefore,
10 VACHT is a pivotal actor of ACh-mediated neurotransmission (Prado *et al.* 2013). CIN also
11 express the atypical vesicular glutamate transporter 3 (VGLUT3) and are able to release
12 glutamate in addition to ACh (Gras *et al.* 2008, Sakae *et al.* 2015). Optogenetics has
13 contributed to our improved understanding of specific neurochemical networks (Deisseroth
14 2015, Kreitzer & Berke 2011). It is still common for the study of cholinergic circuits the use of
15 mouse lines expressing ChR2 under the ChAT promoter (Ren *et al.* 2011, Steidl *et al.* 2016,
16 Wang *et al.* 2014, Zhao *et al.* 2011, Zhao & Keen 2008).

17 The cholinergic gene *locus* includes, between the first and second exons of the ChAT gene,
18 an intronless open reading frame for VACHT (Cervini *et al.* 1995, Eiden 1998, Erickson *et al.*
19 1994, Roghani *et al.* 1994). Therefore, the BAC used to generate many reporter mouse lines
20 (GFP, CRE and ChR2) carries also a copy of the VACHT gene (Crittenden *et al.* 2014,
21 Kolisnyk *et al.* 2013, Ting & Feng 2014). Therefore, the ChAT-BAC mouse and rat lines
22 overexpress VACHT in the striatum and other brain regions (Nagy and Aubert. 2013,
23 Kolisnyk *et al.* 2013, Crittenden *et al.* 2014). Consequently, in these transgenic animals, ACh
24 levels are increased in synaptic vesicles and there is significant increase in ACh release in
25 the hippocampus and in their neuromuscular junction (Kolisnyk *et al.* 2013, Sugita *et al.*
26 2016). ChAT-ChR2 mice display marked improvement in motor endurance and cognitive
27 deficits, including attention deficits and deficits in working and spatial memory (Kolisnyk *et al.*
28 2013). These mice also display an increase of amphetamine-induced stereotypies
29 (Crittenden *et al.* 2014). Taken together, these results show that increased VACHT
30 expression disrupts critical steps in information processing and exacerbates drug-induced
31 behaviors.

32 Changes in cholinergic activity have the potential to cause long-term changes in neuronal
33 function by disturbing gene expression pathways and critical signalling (Kolisnyk *et al.* 2013).
34 For example, ChAT-ChR2 mice have increased levels of hnRNPA2/B1, an essential splicing
35 regulator (Kolisnyk *et al.* 2016). However, although these abnormalities in gene expression in
36 targeted tissues and behavioral functions are now documented, whether VACHT
37 overexpression can have long-term consequences for cholinergic neuron function is

1 unknown. Elevated levels of VACHT could affect its own intraneuronal trafficking as well as
2 that of other proteins.

3 Vesicular transporters undergo complex regulation of their trafficking, which has been well
4 studied in the case of VACHT and vesicular monoamine transporters (VMAT1-2) (Barbosa *et al.*
5 *et al.* 2002, Fei *et al.* 2008, Ferreira *et al.* 2005, Krantz *et al.* 2000, Pothos *et al.* 2000, Santos
6 *et al.* 2001, Santos *et al.* 2009, Tan *et al.* 1998, Yao & Hersh 2007). For example, VACHT
7 follows the clathrin dependent endocytotic pathways before its targeting to varicosities (Tan
8 *et al.* 1998, Krantz *et al.* 2000, Pothos *et al.* 2000, Santos *et al.* 2001, Barbosa *et al.* 2002,
9 Ferreira *et al.* 2005, Fei *et al.* 2008, Santos *et al.* 2009).

10 In support to previous findings with amphetamine (Crittenden *et al.* 2014), we found that
11 ChAT-ChR2 mice display increased behavioral sensitization in response to low dose of
12 cocaine administration. Furthermore, VACHT overexpression dramatically remodelled CIN
13 morphology and reduced the number and increased the size of cholinergic varicosities.
14 Finally, we show that these mice presented altered VACHT trafficking in CIN. Our findings
15 show functional changes and long-term modification of CIN morphology presumably due to
16 VACHT overexpression. Furthermore, our results stress the need for careful use of ChAT-
17 ChR2 mice and other ChAT-BAC transgenic rodents for functional and imaging experiments.

18

1 **Methods**

2

3 **Animals**

4 Experiments followed the Canadian Council of Animal Care (CCAC) guidelines for care and
5 use of animals. The animal protocol was approved by the University of Western Ontario
6 (protocol # 2008-127). ChAT-ChR2 mice [B6.Cg-Tg(Chat-COP4*H134R/EYFP)6Gfng/J; The
7 Jackson Laboratory] were described previously (Ting & Feng 2014, Zhao *et al.* 2011). Mice
8 were maintained as hemizygous on C57BL/6J background. Only male mice were used in the
9 study and wildtype (WT) littermates were used as controls.

10 For behavioral experiments, animals were housed in groups of two to four per cage in a
11 temperature-controlled room (22-23°C) with a 12:12 light–dark cycle (lights ON at 7am).
12 Food and water were provided ad libitum. Mice older than 2 months were used in all
13 experiments and the age range in individual behavioral experiments was as follows:
14 behavioral sensitization experiments without habituation 2-3 months; behavioral sensitization
15 experiments in which mice were habituated to locomotor boxes 2-5 months (cocaine 10
16 mg/kg) and 3-7 months of age (cocaine 20 mg/kg). Mice were randomized into treatment
17 groups using a randomization table and the experimenter was blind to the genotypes
18 [ARRIVE guidelines (Kilkenny *et al.* 2010)].

19

20 **Behavioral experiments**

21 *Determination of sample size in behavioral experiments*

22 The initial data from the first behavioral sensitization experiment (protocol without
23 habituation, see below) were used to determine the expected difference between genotypes
24 (approximately 20%) and the approximate standard deviation associated with the
25 measurements. Based on these values we calculated the number of animals required for the
26 statistical power of 0.8 as minimum of 10 mice per each group. The sample size and
27 statistical power was calculated for repeated measures ANOVA test that was later used for
28 statistical analysis. We used G*Power software (Faul *et al.* 2009) to calculate sample sizes
29 and statistical power.

30

31 *Behavioral sensitization*

32 Two complementary protocols with or without habituation to the locomotor boxes were used
33 to assess behavioral sensitization to cocaine. In experiments in which mice were not
34 habituated, the animals were repeatedly injected intraperitoneally with cocaine (i.p., 10 mg/kg
35 in 0.01 ml/g) in six successive days (day 1-6). After each injection, mice were immediately
36 placed into the open field apparatus and locomotor activity was measured for 1 hour. An
37 automated activity monitor (AccuScan Instruments Inc.; Columbus, OH; 20 cm x 20 cm

1 platform with 30 cm high walls) was used to measure locomotor activity. Distance travelled
2 (converted from beam breaks to cm) was recorded at 5-min blocks and the first 20 minutes
3 after injection were used for analysis (Guzman *et al.* 2011).

4 In our second experimental design, mice were first habituated to the open field apparatus
5 and to i.p. injection by receiving saline injection repeatedly for 3 successive days (day 1-3).
6 Mice were habituated to the apparatus 60 minutes before the injection then removed from
7 the apparatus for injection and immediately placed back after injection. Locomotion was
8 recorded 60 minutes before and after injection but only the first 30 minutes after injection
9 were used for analysis. From day 4, mice were separated into 2 groups: group 1 (saline)
10 continued to receive saline injections while group 2 (cocaine) received cocaine i.p. injections
11 (10 or 20 mg/kg in 0.005 ml/g) in five successive days (day 4-8). After the injection on day 8,
12 mice were left undisturbed in their cages for the subsequent 4 days. All mice (group 1 and 2)
13 received a last cocaine injection on day 13. Locomotion was recorded and analysed as
14 described above in the same apparatus.

15 In all 3 experiments together, one mouse was excluded because it died during experiment
16 and two were excluded (from experiment 1 and 2, cocaine treated and one saline treated
17 respectively) as outliers, based on the formula: group average \pm 2x standard deviation.

18

19 **Tissue preparation for immunohistochemistry**

20 For all anatomical studies we used 2-5 months old male mice. Stimulated-emission-depletion
21 (STED) and electron microscopy (EM) experiments were performed on WT and ChAT-ChR2
22 mice. Briefly, animals were deeply anaesthetised and perfused transcardially with
23 paraformaldehyde (2%). For EM experiments, 0.2% glutaraldehyde was added in the
24 perfusion solution. Brains were dissected, fixed over-night in 2% paraformaldehyde and
25 stored in PBS until use. Sections (70 μ m-thick) from forebrain including striatum were cut on
26 a vibrating microtome (Leica, VT1000S).

27

28 **Antisera**

29 VACHT was detected with an anti-VACHT polyclonal antiserum raised in guinea pig (Gras *et*
30 *al.* 2008). VGLUT3 was detected with an anti-VGLUT3 polyclonal antiserum raised in rabbit
31 (Synaptic Systems Cat# 135 203 Lot# RRID:AB_887886). To identify subcellular
32 compartments associated with VACHT, the following antisera were used: anti-Clathrin heavy
33 chain (CHC ; mouse ; BD Biosciences Cat# 610499 Lot# RRID:AB_397865) ; anti- trans-
34 Golgi network specific integral membrane protein (TGN38, mouse; Thermo Fisher Scientific
35 Cat# MA3-063 Lot# RRID:AB_325484); anti-Golgi matrix protein of 130kDa (GM130; mouse;
36 BD Biosciences Cat# 610822 Lot# RRID:AB_398141); anti-Rab5 (mouse; BD Biosciences
37 Cat# 610281 Lot# RRID:AB_397676); anti-Rab9 (mouse; Thermo Fisher Scientific Cat#

1 MA3-067 Lot# RRID:AB_2175599); anti-protein disulphide isomerase (PDI; mouse; Thermo
2 Fisher Scientific Cat# MA3-019 Lot# RRID:AB_2163120); anti-cathepsin D (CathD (G-19);
3 mouse; Santa Cruz Biotechnology Cat# sc-6494 Lot# RRID:AB_2087097). The following
4 markers of the cholinergic system were used: anti-choline acetyltransferase (ChAT; goat;
5 Millipore Cat# AB144P Lot# RRID:AB_2079751), anti-acetylcholinesterase (AChE, a gift from
6 Palmer Taylor, UCSanDiego, USA), anti-high-affinity choline transporter (CHT; mouse;
7 Synaptic Systems Cat# 216 011 Lot# RRID:AB_2301977).

8 The specificity of VACHT labelling was assessed by the disappearance of the staining in
9 VACHT null mice (data not shown, Guzman et al. 2011).

10

11 **Immunofluorescent detection of VACHT at confocal microscopic level**

12 VACHT was detected alone or in association with VGLUT3 or with various markers of
13 subcellular compartments in perikarya and axonal varicosities of CIN of the mouse striatum.
14 Sections were incubated with antisera against: VACHT (1:5000) and VGLUT3 (1:1000), CHC
15 (1:500), TGN38 (1:500), GM130 (1:200), Rab5 (1:100), Rab9 (1:500), PDI (1:100), AChE
16 (1:1000), CHT (1:300), CathD (1:1000), ChAT (1:300). Sections were then incubated in a
17 mixture of secondary antibodies: goat anti-guinea pig coupled to Alexa 594 for VACHT
18 detection (1:1000, Molecular Probes Cat# A-11076 also A11076 Lot# RRID:AB_141930) and
19 goat anti-mouse (for CHC, TGN38, GM130, Rab5, Rab9, PDI, CHT; 1:1000, Thermo Fisher
20 Scientific Cat# A10524 Lot# RRID:AB_2534033) or goat anti-rabbit (for AChE and VGLUT3;
21 1:1000, Thermo Fisher Scientific Cat# A10523 Lot# RRID:AB_2534032); all coupled to CY5.
22 For the simultaneous detection of VACHT and ChAT or CathD, sections were first incubated
23 with a donkey anti-goat coupled to Alexa 633 (ChAT or CathD; 1:1000, Molecular Probes
24 Cat# A-21082 also A21082 Lot# RRID:AB_141493), washed 3 times in PBS, incubated in
25 4% normal goat serum, then in goat anti-guinea pig coupled to Alexa594 (VACHT), washed
26 in PBS, and mounted in Prolong gold (ThermoFisher Scientific). Sections were observed
27 using an inverted TCS SP5 confocal microscope (Leica Microsystems) equipped with a 63X
28 HCX Plan APO CS NA=1.4 oil immersion objective. The pixel size was set to 60 nm and two
29 diode lasers at 561nm and 633nm were used. VACHT labeling was first identified by
30 epifluorescence under the confocal microscope using a filter specific for the red fluorochrome
31 (filter N2.1, Bandpas 515-560 nm dichroic: 580, Longpass 590 nm). Images were acquired in
32 the confocal mode. The confocal microscope used an Acousto-Optical Beam Splitter (AOBS)
33 system without any filter of excitation. Two laser diodes were used: DPSS: 561 nm, 10mW
34 and HeNe : 633 nm, 10mW to excite Alexa594 and 633 fluorochromes, respectively.
35 The cross-talk and bleed through between channels were minimized by two means.
36 First, we have chosen the spectra band of emission of Alexa594 in order to avoid to collect
37 emission due to the excitation of Alexa633 or CY5 by the 561nm laser. The spectra band to

1 detect emission of Alexa594 was set to 570-615nm for Alexa594-Alexa633 colocalization
2 experiments. The spectra band to detect emission of Alexa594 was set to 570-620nm for
3 Alexa594-CY5 colocalization experiments. The spectra band to detect emission of Alexa633
4 and CY5 was set to 660-711nm.

5 Second, we have checked that no signal was detected in the Alexa633/CY5 or Alexa594
6 channels when Alexa594 or Alexa633/CY5 were excited in single labeled sections.

7 Images were treated using ImageJ and Adobe Photoshop software.

8

9 **Immunofluorescent detection of VACHT at STED microscopic level**

10 Sections were successively incubated in anti-VACHT guinea pig polyclonal antiserum
11 (1:5000) overnight then with goat anti-guinea pig IgG coupled to Alexa 594 (1:100;
12 ThermoFisher Scientific) and mounted in ProLong Gold (Thermo Fisher Scientific).

13 Sections were observed using a SP8 gated-STED 315 microscope (Leica Microsystems)
14 equipped with a 775nm depletion laser. Alexa 594 was excited at 594nm. All acquisitions
15 were performed using the same excitation laser power (2%). Sequential scanning of
16 individual channels has been used in all colocalizations analyses. Images were submitted to
17 deconvolution (Huygens software, Scientific Volume Imaging) that allows the recovery of
18 objects that are degraded by blurring and noise. Finally, images were analyzed using ImageJ
19 (National Institutes of Health) and Adobe Photoshop.

20

21 **Quantification of the density, surface and intensity of VACHT immunopositive-spots**

22 Striatal sections labeled for VACHT immunoreactivity were observed using the 63x objective
23 and acquisitions were performed under the confocal microscope using the same excitation
24 laser intensity and parameters for WT and ChAT-ChR2 mice sections. Labeling was then
25 analyzed using the ImageJ software (National Institutes of Health). Because of the VACHT
26 overstaining in ChAT-ChR2 mice, overlapping immunopositive puncta could not be resolved
27 leading to lower quantal counts. To correct this bias, we analyzed our images using a
28 method that allows separation of touching fluorescent objects. This method, called the
29 watershed separation, is performed with the ImageJ software. Briefly, after thresholding, the
30 objects (here varicosities) are separated from the background (Fig. S1b, g). Then,
31 varicosities are separated one from another, with the watershed function (Fig. S1d,i) and
32 counted with the analyze particles function (Fig. S1e,j). The density of VACHT
33 immunopositive spots and of the surface of cholinergic varicosities were quantified. For that,
34 seventy five fields (surface: $655\mu\text{m}^2$) in striatum per animal in 6 mice per group were
35 acquired under the confocal microscope.

36 The relative optical density corresponding to the relative intensity of spots was automatically
37 calculated by the ImageJ software.

1

2 **Quantification of Colocalization.**

3 In perikarya, the quantification of colocalization of VAcHT with organelle or cholinergic
4 markers was analyzed with the “Just Another Colocalization Program” (JACoP) plug-in
5 (ImageJ, National Institutes of Health), and statistical data are reported from the Costes’s
6 randomization-based colocalization module (Bolte & Cordelieres 2006). Costes’s
7 randomization method for measurement of colocalization was used to confirm, with >95%
8 certainty, that the colocalization observed between the VAcHT and organelle or cholinergic
9 markers immunofluorescent signal was not caused by chance coincidence (Costes *et al.*
10 2004). A Pearson’s coefficient (ρ) was calculated. Costes’ randomization was applied on 5
11 neurons from 4 mice of each genotype using at least 150 iterations per image. Analysis have
12 been restricted to the somatic area. Just individual images were analyzed.

13 In varicosities, the quantification of colocalization of VAcHT with organelle markers was
14 performed from the labeling on image observed with the 63x objective (surface of the field:
15 $655\mu\text{m}^2$). Labeling was then analyzed using the ImageJ software (National Institutes of
16 Health). Briefly, after thresholding, puncta labeling (i.e. varicosities) were automatically
17 cropped and touching varicosities were separated by the watershed function (see above).
18 Their number was automatically calculated. Every image was carefully analyzed and the
19 number of puncta displaying labeling for both VAcHT and another protein was marked using
20 the cell counter plugin of ImageJ (National Institutes of Health). Results were expressed as
21 the percentage of VAcHT immunoreactive spots, displaying also labeling for a marker of
22 organelles. Three images (surface of the field: $655\mu\text{m}^2$) from 4 mice of each genotype were
23 quantified. A mean of 2018 varicosities per WT mouse and 558 varicosities ChAT-ChR2
24 animal were analyzed.

25

26 **Electron microscopy**

27 Electron microscopic experiments were performed on WT and ChAT-ChR2 mice brain tissue
28 as previously described (Bernard *et al.* 1998). Briefly, animals were deeply anesthetized and
29 perfused transcardially with a mixture of 2% paraformaldehyde in 0.1 M phosphate buffer
30 (pH7.4) and 0.2% glutaraldehyde. Brains were dissected, fixed overnight in 2%
31 paraformaldehyde and stored in PBS until use. Sections ($50\ \mu\text{m}$) from midbrain including
32 striatum were cut on a vibrating microtome (Leica, VT1000S). Sections were successively
33 incubated in anti-VAcHT guinea pig polyclonal antiserum (1:5000), then with goat anti-guinea
34 pig IgG coupled to biotin (Vector laboratories) and in streptavidin coupled to gold particles
35 (1.4 nm in diameter; Nanoprobes; 1:100 in a buffer containing PBS, bovine serum albumin
36 and gelatin). The signal of the gold immunoparticles was increased using a silver
37 enhancement kit (HQ silver; Nanoprobes) for 2 min at RT in the dark. Finally, after treatment

1 of sections with 1% osmium, dehydration and embedding in resin, ultrathin sections were cut,
2 stained with lead citrate and examined with a transmission electron microscope (EM 912
3 OMEGA, Zeiss) equipped with a LaB6 filament at 80kV. Images were captured with digital
4 camera (SS-CCD, 2kx2k, Veleta). Alternatively, a Field Emission scanning electron
5 microscope GeminiSEM 500 (Zeiss) operating at 20kV with a 20 μ m aperture was also used.
6 Transmitted electrons are collected in bright field mode with the STEM detector located
7 beneath the sample. Finally, the surface of axonal varicosities labeled for VACHT and the
8 surface of their mitochondria were measured using ImageJ.

9 10 **Quantification of the surface of varicosities and mitochondria and of VACHT-** 11 **immunoparticles for VACHT at EM level**

12 The quantification of the surface of varicosities and mitochondria was performed from EM
13 images with the ImageJ software. As an index of the relative number of VACHT molecules
14 per varicosity at EM level, we have quantified the surface occupied by immunoparticles (IPs)
15 per surface of varicosity. The number of IPs is proportional to the quantity of molecules
16 detected (as previously validated (Bernard *et al.* 2003, Bernard *et al.* 1999)). Briefly, after
17 thresholding at the same level for both genotypes of labeled varicosities on EM images,
18 black dots corresponding to IP were automatically cropped and their surface was calculated.
19 These values were divided by the surface of varicosities (excluding the surface occupied by
20 mitochondria).

21 22 **Statistical analyses**

23 Statistical analysis of behavioral experiments was done with Prism 7 software (Graph Pad).
24 Student's *t* test was used to compare total distance travelled after the first cocaine injection.
25 Two-way analysis of variance (ANOVA) was used to evaluate the effect of cocaine dose (10
26 or 20mg/kg) on the total distance travelled on day 8 of behavioral sensitization in the control
27 and mutant mice. Repeated measures two-way ANOVA was used to evaluate the effect of
28 genotype on the distance travelled throughout the behavioral sensitization experiments.
29 Bonferroni's test was used for post hoc comparisons. P values < 0.05 were considered as
30 statistically significant.

31 Statistical analyses for anatomical investigations were performed with Prism 4 software
32 compare (Graph Pad). The density of VACHT immunoreactive spots, the surface of
33 varicosities and mitochondria, the surface of IP for VACHT and the *pc* calculated in
34 colocalizations analyses in WT and ChAT-ChR2 were compared using a Mann-Whitney U
35 test. The percentage of VACHT immunopositive varicosities expressing markers of
36 organelles were compared using the Fisher's exact test. All data are shown as the means \pm
37 SEM; * *p* < 0.01; **: *p*<0.005; ***: *p*<0.0001.

1 Results

2 3 ChAT-ChR2 mice are more sensitive to cocaine

4
5 ChAT-ChR2 mice have more pronounced repetitive behavior after administration of
6 amphetamine (Crittenden et al. 2014). To test whether ChAT-ChR2 mice have abnormal
7 responses to other psychostimulants, ChAT-ChR2 mice and WT littermates (n=8 for each
8 genotype) were injected with cocaine (i.p., 10 mg/kg) daily during 6 successive days.
9 Locomotor sensitization was measured (Fig. 1a). On the day 1, we saw no significant
10 difference in locomotor activity between groups (mean±SEM of total distance traveled in 20
11 minutes after first injection for controls and mutants, respectively: 571±132 and 756±133 cm;
12 $t_{(14)}=0.989$, $p=0.34$, two-tailed t test) (Fig. 1a). However, after repeated administrations of
13 cocaine on days 2-6, ChAT-ChR2 mice showed increased activity compared to wildtype
14 littermates (repeated measures ANOVA main effect of genotype $F(1, 14)=18.07$; $p=0.0008$)
15 (Fig. 1a). Moreover, post-hoc analysis with Bonferroni's multiple comparisons test showed
16 that the ChAT-ChR2 mice moved significantly more than controls already on the day 2
17 (mean±SEM of total distance traveled for controls and mutants, respectively: 1109±169 and
18 2185±222, $p=0.0052$).

19 To further test abnormal behavioral sensitization in ChAT-ChR2 mice, we used a new cohort
20 of mice. We first habituated them extensively to locomotor boxes, handling and injections.
21 This protocol was used to separate the specific effect of drug on behaviour sensitization from
22 the effect of novelty. As can be seen, WT mice and ChAT-ChR2 mice receiving only saline
23 injection showed no locomotor sensitisation (Fig. 1b). In contrast, ChAT-ChR2 mice
24 presented increased behavioural sensitization after cocaine injection (repeated measures
25 ANOVA main effect of genotype, $F_{(1, 20)}=7.875$; $p=0.0109$) (Fig. 1b). The *post-hoc* analysis
26 (Bonferroni's multiple comparisons test) revealed that the ChAT-ChR2 mice receiving
27 cocaine were significantly different from wildtype mice receiving cocaine after the third
28 injection of cocaine on day 6 (mean±SEM of total distance traveled for controls and mutants,
29 respectively: 576±157 and 1416±255, $p=0.0378$). There also appeared to be a trend towards
30 hyperactivity in ChAT-ChR2 mice during the habituation period and after the first injection of
31 cocaine that was not statistically significant (difference in locomotion between genotypes on
32 day 4 $t_{(180)}=1.896$, adjusted $p=0.536$, Bonferroni's *post-hoc* test).

33 We also tested whether the increased behavioral sensitization of ChAT-ChR2 mice is dose-
34 dependent or if the mice show increased sensitization to both low and high doses of cocaine.
35 For this, a new cohort of WT mice and ChAT-ChR2 mice were injected with 20 mg/kg (i.p.) of
36 cocaine. As expected, the sensitization elicited in both WT and ChAT-ChR2 mice by 20
37 mg/kg of cocaine was higher than the sensitization elicited by 10 mg/kg (mean±SEM of

1 distance travelled on the day 8 after 10 mg/kg in wildtypes and mutants, respectively:
2 762±202 and 1719±307; and after 20 mg/kg in wildtypes and mutants, respectively:
3 4205±471 and 3858±465; two-way ANOVA main effect of cocaine dose, $F(1, 39)=54.3$,
4 $p<0.0001$). However, the locomotion of ChAT-ChR2 and control mice after cocaine
5 administration was almost identical throughout the experiment (Fig. 1c). High doses of
6 stimulants such as the one used here can also induce stereotypies in mice which would
7 result in decreased locomotion. Because we did not visually evaluate stereotypies in our
8 experiment we cannot exclude the possibility that while the locomotion appeared to be the
9 same, the ChAT-ChR2 mice were actually displaying more stereotypies and thus higher
10 sensitivity to cocaine. Therefore, based on our data, we can only conclude that ChAT-ChR2
11 mice are more sensitive to low doses of cocaine.

12

13 **VACHT expression is dramatically modified in striatum of ChAT-ChR2 mice.**

14 Given the predominant role of the striatum in the response to drugs of abuse, we
15 investigated the expression of VACHT and morphology of CIN. Using confocal microscope,
16 VACHT is observed in the soma of scattered large-sized neurons in the striatum of WT mice
17 (Fig. 2a, a'). In addition, VACHT-immunolabelling is also present abundantly as punctiform
18 labeling corresponding to staining in axonal varicosities (Fig. 2a, a'; arrows). A similar
19 distribution is observed in the dorsal striatum as well as in the nucleus accumbens (data not
20 shown). As shown in Fig. 2b, b', VACHT labelling is dramatically modified in ChAT-ChR2
21 mice. The intensity of VACHT immunolabeling is strongly increased in both soma and
22 varicosities, as expected, due to overexpression. In perikarya of ChAT-ChR2 mice, VACHT
23 staining was detected in large puncta in the cytoplasm and close to the plasma membrane.
24 Furthermore, the number of VACHT-immunopositive varicosities seemed dramatically
25 reduced compared to WT animals (Fig. 2b; -87 %; WT: 0.957 ± 0.038 immunoreactive
26 spots/ μm^2 ; ChATChR2 : 0.125 ± 0.016 immunoreactive spots/ μm^2 ; Mann-Whitney U
27 test: $p<0.01$; $n=6$ mice per group). It is possible that individual puncta visible in the control
28 mice are overlapping in the ChAT-ChR2 mice, leading to lower quantal counts only because they
29 cannot be resolved. We have used an additional marker, VGLUT3 staining, to confirm that
30 the decrease of our quantification concerning VACHT varicosities is real. Indeed VGLUT3
31 and VACHT are co-expressed in CIN varicosities (see Gras et al. 2002 for example).
32 Interestingly, in contrast to VACHT, VGLUT3 immunolabeling intensity is unchanged in CIN
33 from ChAT-ChR2 mice relatively to WT mice (Fig. 2 c-d'; Relative optical density in
34 varicosities: WT: 26.16 ± 1.14 ; ChATChR2 : 26.43 ± 1.59 ; Mann-Whitney U test : NS, $n=6$ for
35 each genotype). Importantly, the decreases of the density of VACHT and VGLUT3
36 varicosities in ChAT-ChR2 mice are similar (-87% and -74%, respectively). These data

1 support the notion that the number of cholinergic varicosities is reduced in mice over-
2 expressing VACht, likely impacting both VACht and VGLUT3 neurotransmission.
3 In addition, the surface of cholinergic VACht immunopositive varicosities is significantly
4 higher in mutant mice compared to WT animals (Fig. 2b; +177%; WT: $0.060 \pm 0.006 \mu\text{m}^2$;
5 ChATChR2 : $0.165 \pm 0.010 \mu\text{m}^2$; Mann–Whitney U test: $p < 0.01$).
6 In order to increase the resolution and better resolve how additional VACht-positive material
7 is distributed in cholinergic axonal varicosities of ChAT-ChR2 mice, we used super-resolution
8 STED microscopy (Fig. 2e-l). At low magnification, we detected small spots of VACht
9 immunolabeling in varicosities of WT mice (Fig. 2e). In contrast, in ChAT-ChR2 mice,
10 varicosities are much larger and VACht labeling is observed at the periphery of the
11 varicosities (Fig. 2f, arrows). At higher magnification, STED experiments show groups of
12 labeled spots in varicosities in WT mice, (Fig. 2g,h,i). In contrast, in the enlarged boutons
13 from CIN of ChAT-ChR2 mice, immuno-positive spots seemed clustered at the periphery
14 (Fig. 2j,k,l). This was confirmed by EM experiments (Fig. 3h,i).
15 In summary, our data show that ChAT-ChR2 mice have a lower number of larger cholinergic
16 striatal varicosities when compared to controls and that VACht is located mostly at the
17 periphery of these large boutons in ChAT-ChR2.

18

19 **Ultrastructural localization of VACht in ChAT-ChR2 mice**

20 We performed pre-embedding immunogold detection, in order to determine with a higher
21 resolution VACht localization and its distribution within CIN. EM observations demonstrated
22 that in WT mice, VACht is exclusively associated with membranous organelles in the
23 perikarya and varicosities (Fig. 3 a, c, e and f). In perikarya, some IPs for VACht are
24 associated with the endoplasmic reticulum (ER) and Golgi apparatus (Go) (Fig. 3a arrows,
25 c). In ChAT-ChR2 mice, a higher density of IP is identified in perikarya in association with ER
26 and Go (Fig. 3b (arrows), d). VACht is also detected at the surface of large-sized vesicles in
27 the perikarya (Fig. 3, b', b''). EM observations confirmed the substantial increased size of
28 axonal varicosities in mutant mice (Fig. 3j; +177%, Mann–Whitney U test: $p < 0.01$; $n = 6$ mice
29 in each group). In WT mice, VACht IP are evenly distributed inside the axonal varicosities
30 and are associated with synaptic vesicles (Fig. 3 e, f; arrow-heads). In ChAT-ChR2 mice, the
31 density of VACht IP was dramatically increased and often located underneath the plasma
32 membrane (Fig. 3h,i). Because of the size of the IP (20nm in diameter), it was difficult to
33 determine whether VACht is associated with plasma membrane, or with sub-membranous
34 organelles that may be hidden by the high density of IP. Within enlarged cholinergic
35 varicosities, the clustering of VACht IP prevented quantitative analysis of synaptic vesicles
36 (Fig. 3h,i). We found a significant increase of the surface occupied by IP in cholinergic
37 varicosities of ChAT-ChR2 mice compared to WT mice (+108%, WT: 0.1128 ± 0.0185 ; ChAT-

1 ChR2: 0.2344 ± 0.0164 ; $n=6$, Mann-Whitney U test $p < 0.01$), suggesting an increase of VACHT
2 molecules in mutants.
3 Of the 41 varicosities observed in ChAT-ChR2 mice, 17 (41%) of them contained one large
4 round-shaped compartment (Fig. 3g). Of 10 perikarya observed, all of them contained a
5 mean of 12 large round-shaped compartments (Fig. 3b,b',b').
6 Surprisingly, the number of mitochondria per varicosity appeared increased in ChAT-ChR2
7 mice relatively to WT mice. Indeed, only 16% out of 120 VACHT immunopositive varicosities
8 in WT mice contain one mitochondrion. In contrast, 65% of 125 varicosities in ChAT-ChR2
9 mice contain at least one mitochondrion and some of them showed two mitochondria.
10 Moreover, the quantification demonstrated that the surface of mitochondria in ChAT-ChR2
11 mice is increased compared to WT animals (Fig. 3k; +93%; Mann-Whitney U test: $p < 0.01$).
12 In summary, the overexpression of VACHT in ChAT-ChR2 mice profoundly alters the shape
13 and organization of cholinergic striatal varicosities.

14

15 **VACHT overexpression induces redistribution of VACHT in subcellular compartments**

16 The trafficking of VACHT has been previously investigated (Santos et al. 2001; Barbosa et al.
17 2002, Kim & Hersh 2004, Ferreira et al. 2005). We then investigated whether over-
18 expression of VACHT would impact its synthesis in the ER, maturation in Golgi apparatus,
19 endocytosis in clathrin coated pits in perikarya and varicosities of ChAT-ChR2 mice.

20 Colocalization of VACHT and organelle or cholinergic markers in perikarya were analyzed
21 (Bolte & Cordelieres 2006, Costes et al. 2004) (see methods). Colocalization observed
22 between the VACHT and organelle or cholinergic markers immunofluorescent signal was not
23 caused by chance coincidence in all WT and ChAT-ChR2 and for all combination of markers.
24 In WT mice, PDI, GM130 or TGN38, specific markers of the endoplasmic reticulum, cis- or
25 trans-Golgi apparatus, respectively, were detected in small proportion in subcellular
26 compartments expressing VACHT (Fig. 4a,c,e,g,i,k; Fig. S2; Fig. S3; $pc = 0.32 \pm 0.01$,
27 0.34 ± 0.04 or 0.24 ± 0.03 , respectively). The colocalization of VACHT with PDI, GM130 and
28 TGN38 (Fig. 4b,c,f,g,j,k, arrow-heads; $pc = 0.66 \pm 0.05$, 0.53 ± 0.01 or 0.70 ± 0.05 , respectively)
29 was significantly higher in perikarya of ChAT-ChR2 mice compared to WT mice (Mann-
30 Whitney U test: $p < 0.0001$). In varicosities of WT mice, PDI, GM130 or TGN38 were
31 detected in a very weak proportion of VACHT immunopositives varicosities (Fig. 4a',d,e',h,i'
32 and l; $3.45\% \pm 1.45$, $0.26\% \pm 0.08$ or $1.30\% \pm 0.42$, respectively). In contrast, in ChAT-ChR2
33 mice, PDI, GM130 or TGN38 were present in almost all VACHT immunopositives varicosities
34 (Fig. 4b',d',f',h,j' and l; $94.91\% \pm 1.17$, $98.48\% \pm 0.52$ or $95.78\% \pm 0.35$, respectively). The
35 Fisher's test demonstrated a highly significant increase in the proportion of varicosities
36 expressing VACHT and PDI, GM130 and TGN38 (Fig. 4d,h,l; $p < 0.0001$).

1 In soma or varicosities of WT animals, clathrin heavy chain (CHC), Rab5 or Rab9, markers of
2 clathrin-coated pits or early or late endosomes, respectively, were rarely detected in VACHT
3 immunopositive puncta (Fig. 5a,a',e,e',i and i'; perikarya : $pc = 0.27 \pm 0.03$, 0.26 ± 0.01 and
4 0.29 ± 0.03 , respectively; varicosities : $2.96\% \pm 0.47$, $3.67\% \pm 0.11$ or $2.49\% \pm 0.30$, respectively).
5 In contrast, in perikarya and varicosities from the striatum of ChAT-ChR2 mice, CHC, Rab5
6 and Rab9 were often detected in subcellular compartments immunopositive for VACHT (Fig.
7 5b,b',c,d,f,f',g,h,j,j',o and p; Fig. S3; Fig. S5; arrow-heads; perikarya: $pc = 0.56 \pm 0.08$,
8 0.67 ± 0.03 and 0.71 ± 0.05 , respectively; varicosities : $94.52\% \pm 1.75$, $94.22\% \pm 1.97$ and
9 $94.47\% \pm 1.03$, respectively). The colocalization of VACHT with CHC, Rab5 and Rab9 was
10 significantly higher in perikarya of ChAT-ChR2 mice compared to WT mice (Fig. 5c,g and k;
11 Mann–Whitney U test: $p < 0.0001$). The Fisher's test demonstrated a highly significant
12 increase in the proportion of varicosities expressing VACHT and CHC, Rab5 and Rab9 (Fig.
13 5d,h,l; $p < 0.0001$).

14 Finally, in WT mice, CathD, a marker of lysosomal vesicles, was detected in VACHT
15 expressing compartments in the soma or varicosities of CIN (Fig. 5m,m' and o; perikarya :
16 $pc = 0.46 \pm 0.04$; varicosities : $0.47\% \pm 0.14$), whereas this was occasionally the case in the
17 striatum of ChAT-ChR2 mice (Fig. 5n and n'). In perikarya and varicosities from the striatum
18 of ChAT-ChR2 mice, CathD was often detected in subcellular compartments immunopositive
19 for VACHT (Fig. 5n,n',o, and p; arrow-heads; perikarya: $pc = 0.76 \pm 0.02$; varicosities :
20 $98.54\% \pm 0.60$). The colocalization of VACHT with CathD is significantly higher in perikarya of
21 ChAT-ChR2 mice compared to those from WT (Fig. 5o; Mann–Whitney U test: $p < 0.0001$).
22 The Fisher's test demonstrated a significant increase in the proportion of varicosities
23 expressing VACHT and CathD (Fig. 5p; $p < 0.0001$). These data demonstrate that in ChAT-
24 ChR2 mice, overexpressed VACHT is largely redistributed in CIN and overflows to the
25 endoplasmic reticulum, cis- and trans-Golgi apparatus, clathrin-coated pits, in early and late
26 endosomes and lysosomes.

27

28 **VACHT overexpression does not modify expression of cholinergic markers**

29 We next inspected whether VACHT overexpression impacted the expression of other
30 cholinergic markers, such as ChAT, AChE and CHT in CINs of ChAT-ChR2 mice.

31 The intensity of the immunolabeling of ChAT, AChE, and CHT were quantified in WT and in
32 ChAT-ChR2 mice at the level of varicosities and perikarya. We observed no modification of
33 the intensity of the various immunolabeling (Relative optical density in varicosities: ChAT :
34 WT: 16.73 ± 0.63 ; ChATChR2 : 14.84 ± 0.27 ; AChE : WT: 27.71 ± 2.16 ; ChATChR2 :
35 27.45 ± 2.045 ; CHT : WT: 32.20 ± 3.07 ; ChATChR2 : 31.99 ± 2.79 ; For all three markers : Mann-
36 Whitney U test : NS, $n=4$ for each genotype).

1 In perikarya of WT mice ChAT immunoreactivity was homogeneously detected in the
2 cytoplasm of cell bodies (Fig. 6a). Labeling was detected in the cytoplasm which sometimes
3 colocalized with VACHT (Fig. 6a,c; $pc=0.49\pm0.06$). AChE immunoreactivity was detected
4 mostly at the plasma membrane of perikarya (as previously shown, (Dobbertin *et al.* 2009)
5 (Fig. 6d; arrows). A faint staining was detected in the cytoplasm that sometimes colocalized
6 with VACHT ($pc=0.42\pm0.01$). As previously established (Ferguson *et al.* 2003, Ribeiro *et al.*
7 2003), CHT immunoreactivity was detected mostly in the cytoplasm, in vesicles-like
8 structures in perikarya (Fig. 6g), sometimes associated with VACHT staining ($pc=0.60\pm0.04$).
9 The same pattern was generally observed in ChAT-ChR2 mice, however, ChAT, AChE and
10 CHT showed partial colocalization with VACHT (Fig. 6b,c,e,f,h,i; arrow-heads; $pc=0.80\pm0.05$,
11 0.69 ± 0.03 and 0.88 ± 0.02). The colocalization of VACHT with ChAT, AChE and CHT is
12 significantly increased in perikarya of ChAT-ChR2 mice compared to those from WT mice
13 (Fig. 6c,f and i; Mann–Whitney U test: $p<0.0001$).

14

15 **Discussion**

16

17 The gene encoding for VACHT is embedded within the first intron of the ChAT gene (Cervini
18 *et al.* 1995, Eiden 1998, Erickson *et al.* 1994, Roghani *et al.* 1994). The ChAT-ChR2 mouse
19 line incorporated almost 50 extra copies of the VACHT gene (Kolisnyk *et al.* 2013). Hence,
20 this mouse line is a convenient model to study the functional and anatomical consequences
21 of hyper-cholinergy. This is particularly relevant in the striatum, the brain area with the
22 highest content in ACh. The increase in VACHT expression augments ACh storage and
23 release from synaptic vesicles in the neuromuscular junction and in the brain (Kolisnyk *et al.*
24 2013, Sugita *et al.* 2016). Interestingly, mutations in VACHT in humans (O’Grady *et al.*, 2016;
25 Aran *et al.*, 2017) phenocopy mice with decreased levels of VACHT suggesting a high degree
26 of functional conservation (Aran *et al.* 2017, Lima Rde *et al.* 2010, Martyn *et al.* 2012,
27 O’Grady *et al.* 2016, Prado *et al.* 2006, Roy *et al.* 2013). We show in the present paper that
28 overexpression of VACHT, consistently with changed cholinergic function, increases
29 behavioral sensitization in response to cocaine administration. Moreover, ChAT-ChR2 BAC
30 mice display profound alterations in cholinergic neuropil within the striatum, likely due to
31 VACHT spilling over to several different membranous compartments involved with the
32 synthesis and trafficking of membrane proteins.

33 **VACHT overexpression induces increased behavioral sensitization in response to** 34 **cocaine administration.**

35 VACHT overexpression disrupts a number of cognitive functions, presumably due to
36 excessive increase in cholinergic tone (Kolisnyk *et al.* 2013). In addition, ChAT-ChR2

1 response to amphetamine is also altered as the mice are more prone to amphetamine-
2 induced stereotypies and show increased behavioral sensitization induced by low doses of
3 amphetamine (Crittenden et al. 2014). The present study supports and further extends these
4 observations, by showing that these mice also show increased behavioral sensitization
5 induced by low doses of cocaine.

6 The mechanism by which the putative increased cholinergic tone affects the responses to
7 stimulants is not fully understood, but it is likely to be mediated by increased VACHT levels in
8 ChAT-ChR2 BAC mice. Although several brain regions contribute to behavioral sensitization
9 to cocaine (e.g. medial prefrontal cortex, ventral tegmental area or pedunculopontine and
10 laterodorsal tegmental nucleus, both containing cholinergic neurons), the nucleus
11 accumbens (ventral striatum) is usually seen as a key structure controlling increased motor
12 response to repeated administration of stimulant drugs (Steketee and Kalivas, 2011).
13 However, the relationship between striatal ACh and sensitivity to cocaine is complex and
14 cocaine-induced effects can be both decreased and increased as a result of cholinergic
15 manipulations (Gonzales & Smith 2015, Williams & Adinoff 2008). It should not be surprising
16 then that while ablation of striatal CINs leads to higher sensitivity to cocaine (Hikida *et al.*
17 2003, Kitabatake *et al.* 2003), the ChAT-ChR2 mice with putative increase in cholinergic tone
18 display a similar effect. Some of the conflictual observations can probably be explained by
19 the fact that CIN release glutamate in addition to ACh (El Mestikawy *et al.* 2011, Gras *et al.*
20 2002). Therefore, glutamate released by CINs could account for some effects previously
21 attributed to ACh in CIN ablation experiments (Guzman et al. 2011, Sakae et al. 2015).
22 Deleting VGLUT3, which alters the balance between glutamate and ACh, increases
23 sensitivity to cocaine psychostimulant effects (Gras et al. 2002, Sakae et al. 2015). Increased
24 VACHT expression may change the balance between ACh and glutamate released by CIN
25 similar to removal of VGLUT3. Alternatively, increased VACHT expression may have long-
26 term effects in gene expression as we previously observed (Kolisnyk et al. 2016). This may
27 also contribute to the profound morphological changes we detected in these cholinergic
28 interneurons as discussed below.

29 **VACHT overexpression induces deep remodeling of striatal cholinergic interneurons** 30 **morphology**

31 VACHT overexpression deeply remodels the morphology of CIN. Changes induced by
32 VACHT overexpression are particularly spectacular at the level of axonal varicosities as
33 shown by the increased size of axonal varicosities. The molecular and cellular pathways
34 underlying the enlargement of axonal varicosities is not yet determined. However, it can be
35 hypothesized that these modifications could result from a putative cholinergic overactivity

1 induced by VACHT overexpression. Cholinergic overactivity was described in the
2 hippocampus and neuromuscular junction of mice over-expressing VACHT (Kolisnyk et al.
3 2013, Sugita et al. 2016). However, this is not yet formally established in the striatum of
4 ChAT-ChR2 mice.

5 Our morphological observations lead to a central question: can putative cholinergic
6 overactivity be responsible for these changes of axonal morphology? Interestingly, another
7 mutant mouse line, which lacks the proline-rich membrane anchor (PRIMA) for AChE has \approx
8 90% loss of AChE activity (Dobbertin et al. 2009). Genetic ablation or pharmacological
9 inhibition of AChE is a classic method to increase ACh transmission (Mount *et al.* 1994, Ray
10 *et al.* 2009). Interestingly CIN varicosities were not altered in PRIMA-KO (Dobbertin et al.
11 2009). However, it should be noted there might be important differences between these
12 mouse lines regarding cholinergic tone. PRIMA-KO mice have substantial receptor
13 desensitization, likely due to constant increased levels of ACh in the synaptic cleft. ChAT-
14 ChR2 mice in contrast release more ACh, but the neurotransmitter can be quickly degraded
15 in synapses, as AChE activity and expression is not altered (present findings and (Sugita et
16 al. 2016)). ChAT-ChR2 mice are unlikely to present similar levels of receptor dysfunction as
17 PRIMA-KO, although we did detect substantial change in M2 autoreceptors that are
18 consistent with downregulation or desensitization. Alternatively, it cannot be excluded that
19 compensatory mechanisms prevent generalized cholinergic overactivity and that these
20 effects are independent from cholinergic tone. Therefore, the formal confirmation (or
21 information) of the existence of an overactive cholinergic tone will be necessary to fully
22 interpret the present results.

23 The putative increased striatal cholinergic tone could activate ACh nicotinic and muscarinic
24 receptors. The M2 and M4 receptors are good candidates to mediate this effect since they
25 are both known to be expressed by these neurons and to be involved in modulation of ACh
26 release (Bernard *et al.* 2006, Bernard *et al.* 1992, Zhang *et al.* 2002). The increased size of
27 varicosities could also result from a trophic role of muscarinic receptors as previously
28 reported (Di Liberto *et al.* 2017, Mount et al. 1994).

29 In addition to changes in the shape of neuronal varicosities in VACHT overexpressing mice,
30 we found that varicosities display more mitochondria with increased size compared to WT
31 animals. The distribution of mitochondria in neurons depends on the energy needed to
32 accomplish specialized functions. Areas with high demands for ATP, such as axonal
33 terminals, contain more mitochondria than other cellular domains (Brodin *et al.* 1999, Li *et al.*
34 2004, Nguyen *et al.* 1997). Moreover, the dynamics of mitochondria, including mitochondrial
35 fission and fusion cycles, adapt the shape of mitochondria to the metabolic conditions of the

1 cell (Westermann 2012). Here, the presence of additional mitochondria supports the
2 possibility that larger and less numerous CIN varicosities are more active.

3 It should be kept in mind that these large cholinergic varicosities could also be extrinsic to the
4 striatum and originate from the brainstem (Dautan *et al.* 2016), as our experiments would be
5 unable to differentiate varicosities from interneurons from those of projecting cholinergic
6 neurons.

7 8 **VACHT overexpression induces redistribution of VACHT in exo- and endocytotic** 9 **pathways**

10 To understand the trafficking of an overexpressed transporter in CIN and to identify how a
11 putative cholinergic over-activity could modify the distribution of VACHT in CINs we identified
12 which organelles expressed VACHT in CHAT-ChR2 mice. Our observations of VACHT
13 accumulation in various exo- and endocytotic pathways suggest that, in ChAT-ChR2 mice,
14 the excessive amounts of VACHT likely saturate each step normally used for its trafficking.
15 Furthermore, our study suggests the pathways taken by the transporter between the cell
16 body and varicosities in cholinergic neurons in vivo (see Fig. 7).

17 VACHT overexpression in the exocytotic pathway may result from activation of VACHT
18 synthesis in the ER and maturation in the Golgi apparatus (as previously shown, (Kolisnyk *et*
19 *al.* 2013)). Alternatively, we cannot exclude that the synthesis and maturation are normal.
20 The excess of VACHT may thus be trapped in the ER and Golgi apparatus. This could be
21 due to overwhelming saturation in the trafficking machinery required to direct proteins to
22 post-synthesis compartments, including the plasma membrane, as previously reported for
23 the muscarinic M2 receptor in a model of striatal cholinergic overactivity (Bernard *et al.* 2003,
24 Bernard *et al.* 2006).

25 Our observation on VACHT distribution on the endocytic pathway (Figs 5 and 7) is in line with
26 previous data describing endocytosis motifs in the cytoplasmic tail of VACHT that potentially
27 interact with clathrin-associated protein adaptor protein 1 and 2 (Barbosa *et al.* 2002, Kim &
28 Hersh 2004, Santos *et al.* 2001). According to our data, during its trafficking towards
29 varicosities, VACHT could be directed first to the plasma membrane in cell bodies.
30 Interestingly, VACHT contains a molecular motif involved in membrane addressing and in
31 targeting to synaptic varicosities (Ferreira *et al.* 2005, Santos *et al.* 2001). In WT animals,
32 VACHT is never co-detected with AChE, a membrane bound protein (Fig. 6d). Therefore, the
33 presence of VACHT near the plasma membrane of perikarya in over-expressing mice may
34 represent a very transient state in WT mice. At varicosities, EM experiments show that
35 VACHT is often detected close to or at the plasma membrane. Due to the tiny shape of
36 varicosities, it is hard to determine whether VACHT is associated with the membrane of with
37 sub-membrane vesicles. The functional signification of the putative association of VACHT

1 with the plasma membrane is still unclear. This observation could reflect the increased
2 activity of these enlarged terminals and in particular a jammed endocytic pathway.

3 The highest number and size of mitochondria shown in varicosities from VACHT
4 overexpressing mice in our study suggests an overall activation of synaptic vesicle cycling
5 and endocytosis. Indeed, the local demand in energy (and thus the number of mitochondria
6 per terminals) likely correlates with the number of synaptic vesicles that undergo endocytosis
7 (Marland *et al.* 2016).

8 The post-endocytic fate of VACHT may take different forms. VACHT may be degraded in
9 lysosomes as suggested by co-localization of VACHT and cathepsin D. Alternatively, VACHT
10 produced in perikarya may be transported along the axon towards varicosities and synaptic
11 vesicle membrane (Fig. 7). This phenomenon called transcytosis has been reported for Trk
12 receptors (Ascaño *et al.* 2009) but never for a vesicular transporter of neurotransmitter.
13 Additional experiments are needed to test both hypotheses. Experiments using a pulse-
14 chase approach may be necessary to confirm the proposed model. Time course to follow
15 VACHT targeting will be needed to confirm the suggested model.

17 **VACHT overexpression does not modify other markers of the cholinergic transmission**

18 Our results show no modification of the overall expression of cholinergic markers. In the
19 striatum, the major part of ChAT, AChE and CHT labeling is present in varicosities.
20 Therefore, the absence of overall modification of cholinergic marker probably reflects that
21 these markers are unchanged in varicosities. In addition, there is an increased colocalization
22 of VACHT and cholinergic markers. This suggests a redistribution of these markers in the
23 same subcellular compartments. Alternatively, it cannot be excluded that there is an increase
24 of expression of these markers in the perikarya.

25 VACHT overexpression does not change drastically the localization of other pivotal markers
26 of cholinergic transmission such as ChAT, AChE and CHT. Therefore, all morphological
27 changes observed in these mice seems to be related to the excess of VACHT in CIN.

29 **Conclusion**

30 The present study describes morphological abnormalities of CIN and intraneuronal VACHT
31 redistribution in VACHT overexpressing mice. These data allow us to propose a putative
32 model of VACHT trafficking in CIN *in vivo* (Fig. 7). After synthesis and maturation, VACHT is
33 targeted to the plasma membrane of the perikaryon. Then, VACHT undergoes endocytosis
34 and is either sent to lysosomes to be degraded, or sent through the axon up to axonal
35 varicosities. Taken together, our model is compatible with the trafficking process previously
36 proposed in cultured cells overexpressing the transporter (Barbosa *et al.* 2002, Ferreira *et al.*
37 2005, Santos *et al.* 2001).

1 It is tempting to use ChAT-ChR2 mice to decipher the role of CIN in the regulation of striatal
2 functions. However, it should be kept in mind that these mice display abnormal cholinergic
3 neurotransmission and major morphological alterations that make them fundamental different
4 from wild-type mice. Our work suggests the need for caution when using rodents in which
5 ChAT- BAC transgenesis is used to regulate expression of proteins in cholinergic neurons.
6

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2

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16

17 **Conflict of interest statement**

18

19 Marco Prado is a handling editor for the Journal of Neurochemistry. The other authors have
20 no conflict of interest.

21

22

23

1 **Figure Legends**

2

3 **Figure 1**

4 **ChAT-ChR2 mice are more sensitive to low dose of cocaine in behavioral sensitization**
5 **paradigm.** Sensitivity to different doses of cocaine was tested in ChAT-ChR2 mice using
6 behavioral sensitization paradigm. a : mice were injected with cocaine i.p. (10 mg/kg) for six
7 successive days and distance travelled in the open field during the first 20 minutes after
8 injection was analysed (n = 8 for both genotype). b and c : Mice were habituated to the open
9 field and saline i.p. injections for three days. From the day 4, they received i.p. cocaine
10 injection (10 or 20 mg/kg as indicated) and the distance travelled during first 30 minutes was
11 recorded. After 5 days of withdrawal, locomotor response to cocaine was tested again on the
12 day 13. Note that ranges of Y axes in a, b and c are set differently for better resolution,
13 because the performance of WT mice differs depending on cocaine dose. Number of mice
14 used are as follows: in b, n=4 (WT saline), n=10 (WT cocaine), n=4 (ChAT-ChR2 saline) and
15 n=12 (ChAT-ChR2 cocaine); in c, n=5 (WT saline), n=10 (WT cocaine), n=5 (ChAT-ChR2
16 saline) and n=11 (ChAT-ChR2 cocaine). Significant differences between genotypes are
17 indicated as **, $p < 0.05$ and ***, $p < 0.001$.

18

19 **Figure 2**

20 **Immunohistochemical localization of VACHT and VGLUT3 in the striatum of WT and**
21 **ChAT-ChR2 mice.** Images were taken with a confocal (a-d') or a STED microscope (e-l). In
22 the striatum of a WT (a, a') or a ChAT-ChR2 (b, b') mouse, VACHT is detected in the
23 cytoplasm of a neuron (asterisk) and in axonal varicosities (arrows). In the striatum of a WT
24 (c, c') or a ChAT-ChR2 (d, d') mouse, VGLUT3 is also detected in the cytoplasm of a neuron
25 (asterisk) and in axonal varicosities (arrows). Note that the intensity of VACHT labeling is
26 much increased in ChAT-ChR2 mice. In contrast, the intensity of VGLUT3 labeling is similar
27 in WT and in ChAT-ChR2 mice. Using STED microscopy, at low magnification, VACHT is
28 detected as spots of labeling (arrows in e) in a WT mouse. In a ChAT-ChR2 mouse, spots of
29 labeling appear much larger (arrows in f). At higher magnification, VACHT immunostaining is
30 observed as round-shaped spots within axonal varicosities in WT and ChAT-ChR2 mice. In
31 WT mice, VACHT-positive spots entirely filled the varicosities (g-i). In ChAT-ChR2 mice
32 VACHT labelling was located at the periphery of varicosities (j-l). Scale bars: a, b, c, d :
33 10µm; a', b', c', d', e, f : 5µm; e-j: 200nm.

34

35 **Figure 3**

36 **Immunogold localization of VACHT in striatum of WT and ChAT-ChR2 mice at the**
37 **electron microscopic level.** a-d : Distribution of VACHT immunoparticles (IP) in the

1 perikaryon of WT and ChAT-CHR2 mice. In a WT animal, VACHT IP were detected mostly in
2 the cytoplasm in association with endoplasmic reticulum (arrows) or Golgi apparatus (Go; a
3 and c). In a perikaryon of ChAT-ChR2 mice, VACHT IP are abundantly detected in the
4 cytoplasm in association with endoplasmic reticulum (b ; arrows), with large round-shaped
5 compartments (inserts in b' and b'') and with Golgi apparatus (d ; Go). In WT mice, synaptic
6 vesicles filled the varicosities (e, f). IP for VACHT were evenly distributed in the varicosity and
7 some of them were seen in association with synaptic vesicles (arrow-heads). In varicosities
8 from ChAT-ChR2 mice, synaptic vesicles were rarely identified (g-i, arrow-heads). VACHT IP
9 were often located at or close to the plasma membrane. Sometimes, IP are associated with
10 large round-shaped compartments (arrow in g). The surface of varicosities was larger in the
11 striatum of ChAT-ChR2 mice (g-j) than in WT mice (e-f,j). The surface of mitochondria was
12 also increased in varicosities of ChAT-ChR2 mice compared to those of WT animals (g-i,k).
13 Quantification on EM images demonstrated that the size of varicosities and of the
14 mitochondria in varicosities were significantly higher in ChAT-ChR2 mice compared to WT
15 mice (surface of varicosities: +177%; surface of mitochondria : +93%; Mann Whitney U test :
16 * : $p < 0.01$; $n = 6$ mice ; 20 varicosities/mouse ; 3 or 13 mitochondria/mouse in WT or ChAT-
17 ChR2 animals). Scale bars : a-d: 500nm; b', b'': 100nm; e-l : 200nm.

18

19 **Figure 4**

20 **Immunohistochemical localization of VACHT in neuronal compartments involved in**
21 **synthesis and maturation in the striatum of WT mice and ChAT-ChR2 mice.** In WT
22 animals (a,a',e,e',i,i'), VACHT colocalized rarely or never with PDI in the endoplasmic
23 reticulum (a, arrow-head), TGN38 and GM130 in the Golgi apparatus of perikarya (e,i) or
24 varicosities (insets, a',e',i'). In ChAT-ChR2 mice (b,f,j), VACHT was abundantly detected in
25 the cytoplasm of perikarya. VACHT often colocalized with PDI, GM130 and TGN38 (arrows-
26 heads. In axonal varicosities (insets, b',f',j') , VACHT colocalizes with all three markers
27 (arrows-heads). The analysis of the colocalization of VACHT and PDI, GM130 and TGN38 in
28 perikarya was performed using the Jacop pluggin of ImageJ and statistical data are reported
29 from the Costes 's randomization-based colocalization module (see methods). For perikarya
30 (c,g,k), data were expressed as a Pearson's coefficient (pc) and pc were compared using the
31 Mann–Whitney U test. Our analysis has shown that the colocalization observed between the
32 VACHT and immunofluorescent signal for organelle markers was not caused by chance
33 coincidence in all WT and ChAT-ChR2 and for all combination of marker. Moreover, the
34 colocalization of VACHT with PDI, GM130 and TGN38 (b,c,f,g,j,k, arrow-heads) is
35 significantly higher in perikarya of ChAT-ChR2 mice compared to those from WT (Mann–
36 Whitney U test: ***: $p < 0.0001$). The Fisher's test demonstrated a highly significant increase
37 in the proportion of varicosities expressing VACHT and PDI, GM130 and TGN38 (Fig. d,h,i;

1 *** : $p < 0.0001$). Scale bars: a-f: 5 μ m; a'-f': 1 μ m.

3 **Figure 5**

4 **Immunohistochemical localization of VACht in neuronal compartments involved in** 5 **endocytosis and degradation in the striatum of WT and ChAT-ChR2 mice.**

6 In WT animals (a,a',e,e',i,i'), VACht did not colocalize with CHC in clathrin-coated pits, Rab5
7 in early endosomes and Rab9 in late endosomes in perikarya (a,e,i) and varicosities (insets;
8 a',e',i'). In ChAT-ChR2 mice (b,d,f), VACht often colocalizes with CHC, Rab5 and Rab9 in
9 perikarya (b,f,j) and in axonal varicosities (arrows-heads, b',f',j'). In WT animals (m, m'),
10 VACht did not colocalize with CathD in perikarya and varicosities (inset). In ChAT-ChR2
11 mice (n,n'), VACht sometimes colocalized with CathD in perikarya (arrows-heads) and
12 varicosities (inset, arrows-heads). The analysis of the colocalization of VACht and CHC,
13 Rab5, Rab9 and CathD in perikarya was performed using the Jacop plugin of ImageJ and
14 statistical data are reported from the Costes 's randomization-based colocalization module
15 (see methods). For perikarya (c,g,k and o), data were expressed as a Pearson's coefficient
16 (pc) and pc were compared using the Mann–Whitney U test. Our analysis has shown that the
17 colocalization observed between the VACht and immunofluorescent signal for organelle
18 markers was not caused by chance coincidence in all WT and ChAT-ChR2 and for all
19 combination of marker. Moreover, the colocalization of VACht with CHC, Rab5, Rab9 and
20 CathD (c,g,k and o) is significantly higher in perikarya of ChAT-ChR2 mice compared to
21 those from WT (Mann–Whitney U test: ***: $p < 0.0001$). The Fisher's test demonstrated a
22 highly significant increase in the proportion of varicosities expressing VACht and CHC,
23 Rab5, Rab9 and CathD (Fig. d,h,l and p; *** : $p < 0.0001$). Scale bars: a-h : 5 μ m; a'-h': 1 μ m.

25 **Figure 6**

26 **Immunohistochemical detection of markers of the cholinergic system in CIN of WT** 27 **and ChAT-ChR2 mice at confocal microscopic level.**

28 ChAT, AChE, CHT were detected in the cytoplasm of perikarya of WT and ChAT-ChR2 mice
29 (a-l). AChE was present at the plasma membrane (arrows, d and e). The analysis of the
30 colocalization of VACht and ChAT, AChE and CHT in perikarya was performed using the
31 Jacop plugin of ImageJ and statistical data are reported from the Costes 's randomization-
32 based colocalization module (see methods). For perikarya (c,f,i and l), data were expressed
33 as a Pearson's coefficient (pc) and pc were compared using the Mann–Whitney U test. Our
34 analysis has shown that the colocalization observed between the VACht and
35 immunofluorescent signal for cholinergic markers was not caused by chance coincidence in
36 all WT and ChAT-ChR2 and for all combination of marker. Moreover, the colocalization of
37 VACht with ChAT, AChE and CHT (b,c,e,f,h and i; arrow-heads) is significantly higher in

1 perikarya of ChAT-ChR2 mice compared to those from WT (Mann–Whitney U test: ***:
2 $p < 0.0001$). Scale bars: 1 μm .

3

4 **Figure 7**

5 **Schema proposed for VACht trafficking in CIN in WT and ChAT-ChR2 mice.**

6 This schema indicates progression of VACht among the intracellular compartments
7 suggested by these data. In WT mice, VACht is mostly located at the level of axonal
8 varicosities. VACht follows synthesis and maturation and endocytotic pathways likely in a
9 very transient way, before being transported to axonal varicosities. In ChAT-ChR2 mice,
10 VACht is abundantly present in the maturation and endocytotic compartments (endoplasmic
11 reticulum and Golgi apparatus), then addressed to the plasma membrane from where it is
12 internalized by clathrin-mediated endocytosis. Then VACht would be either sent to
13 lysosomes to be degraded, or transported up to axonal varicosities through a transcytotic
14 mechanism.

15

16

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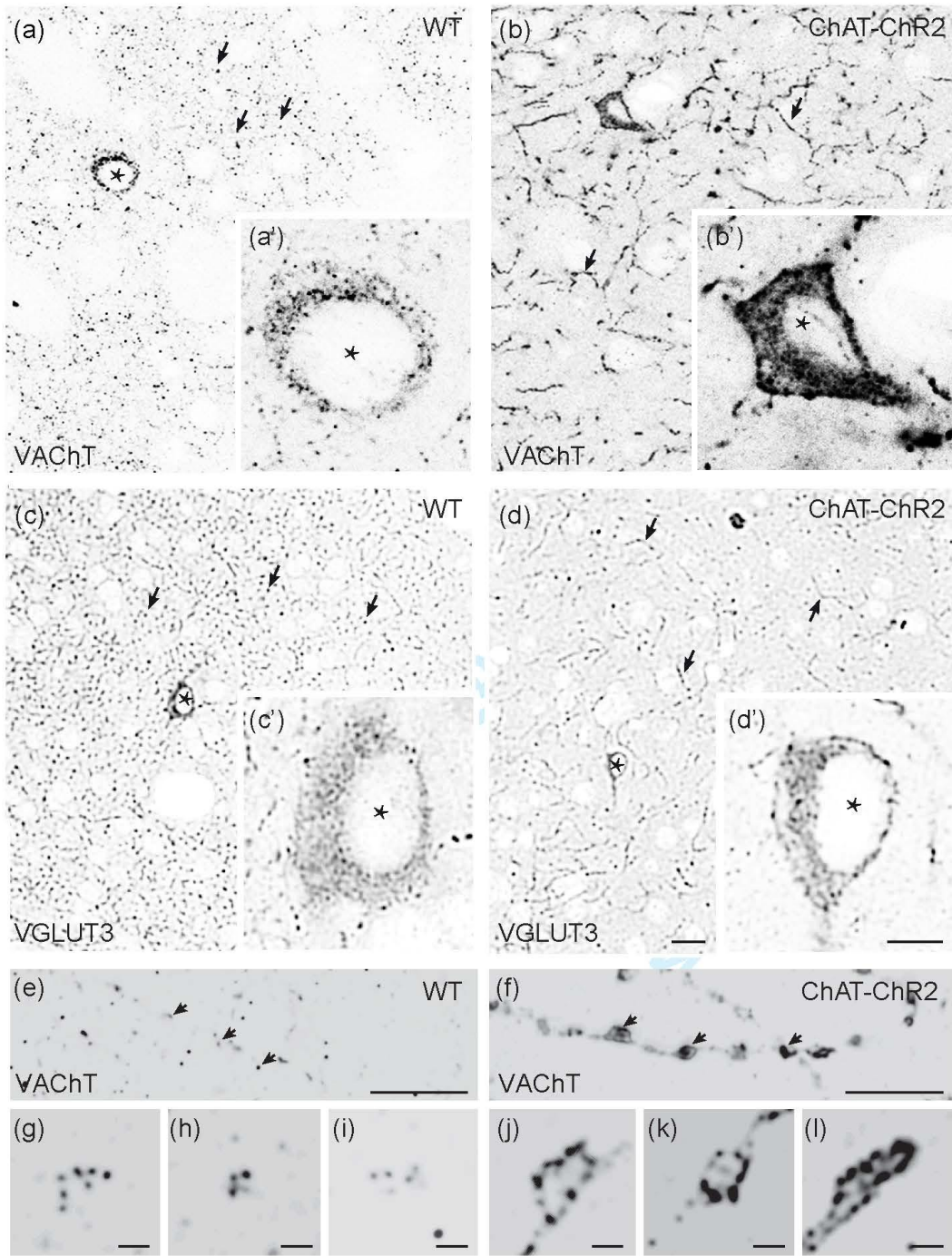


Figure 2 Janickova et al.

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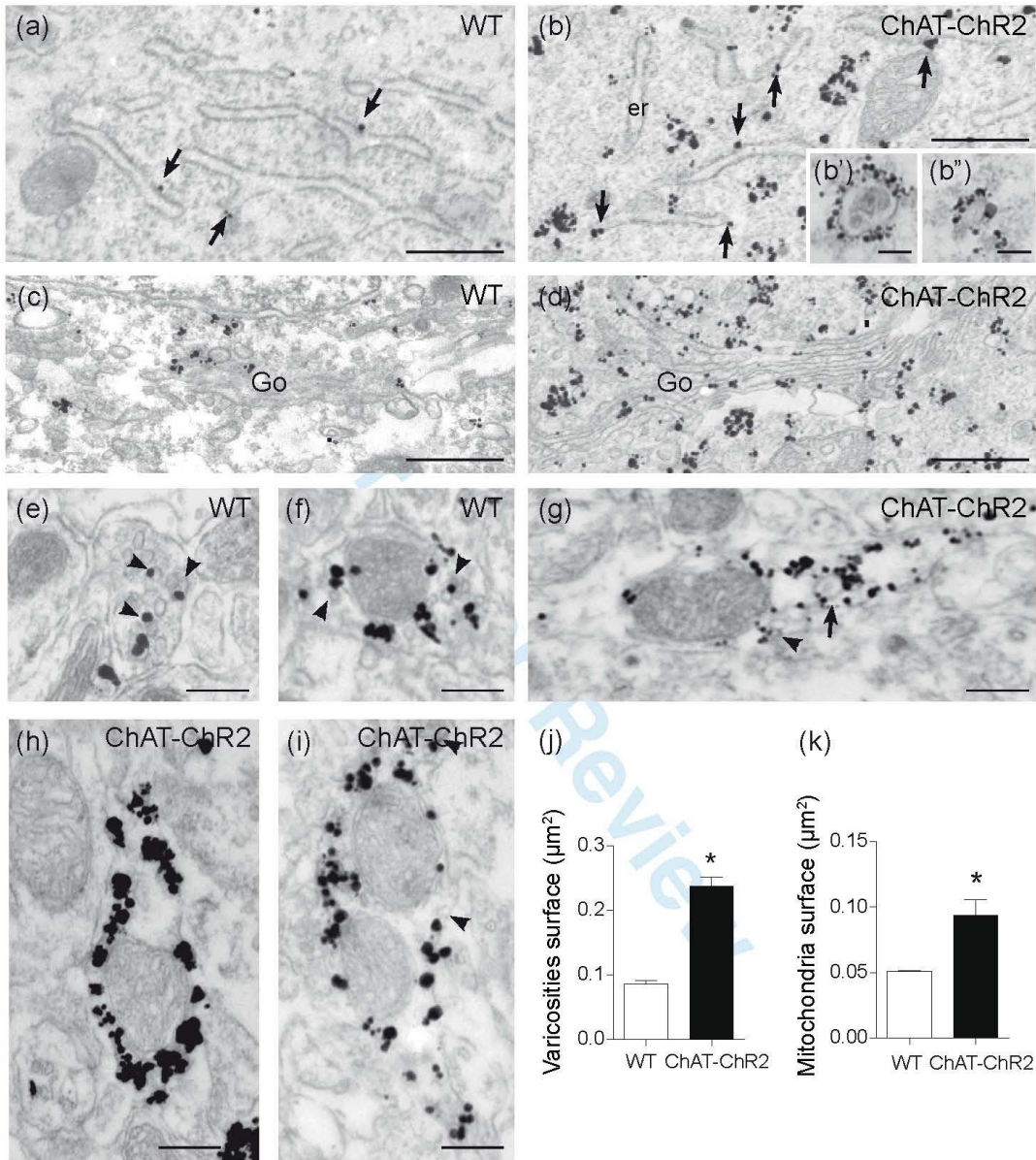
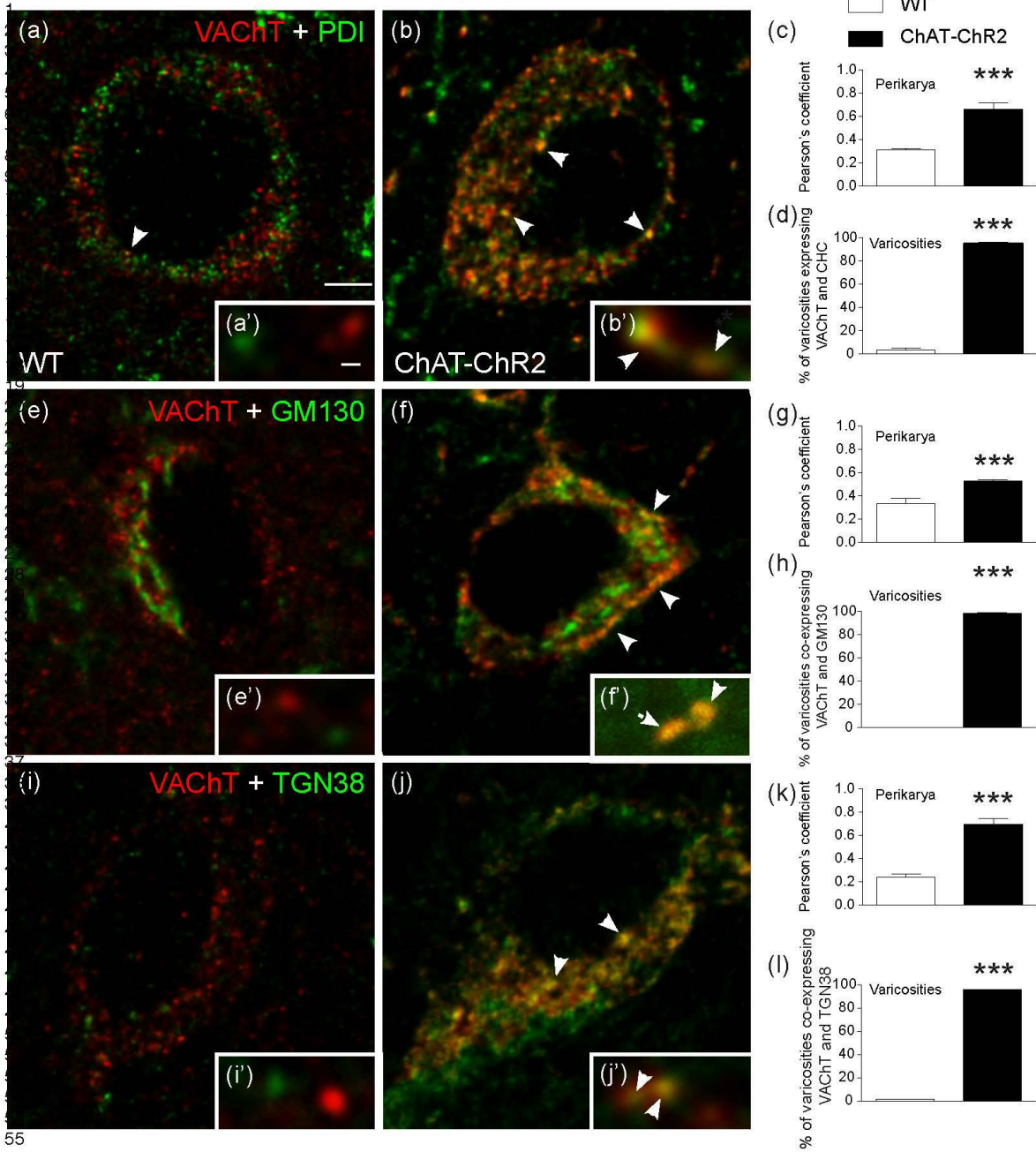


Figure 3 Janickova et al.



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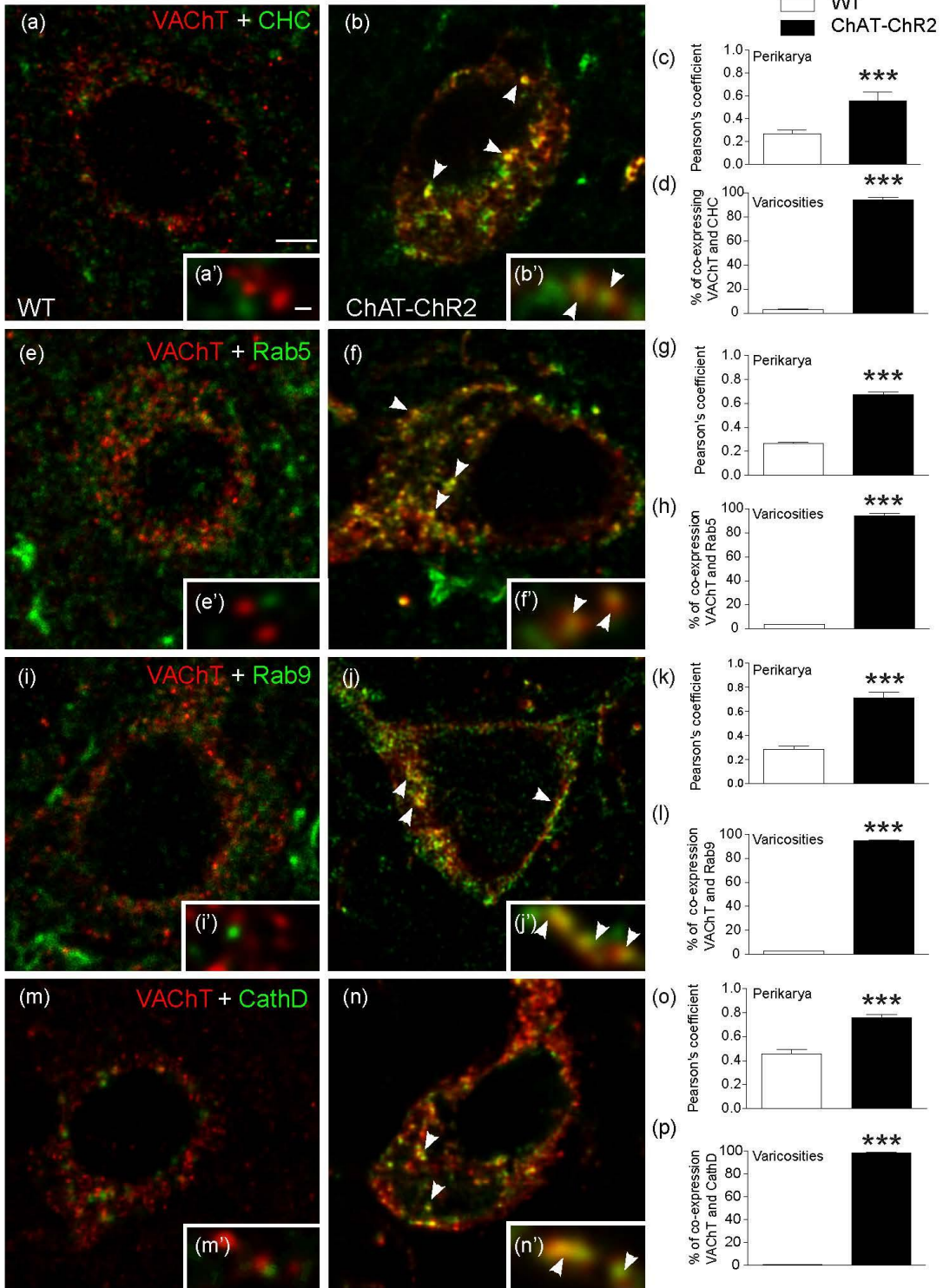


Figure 5 Janickova et al.

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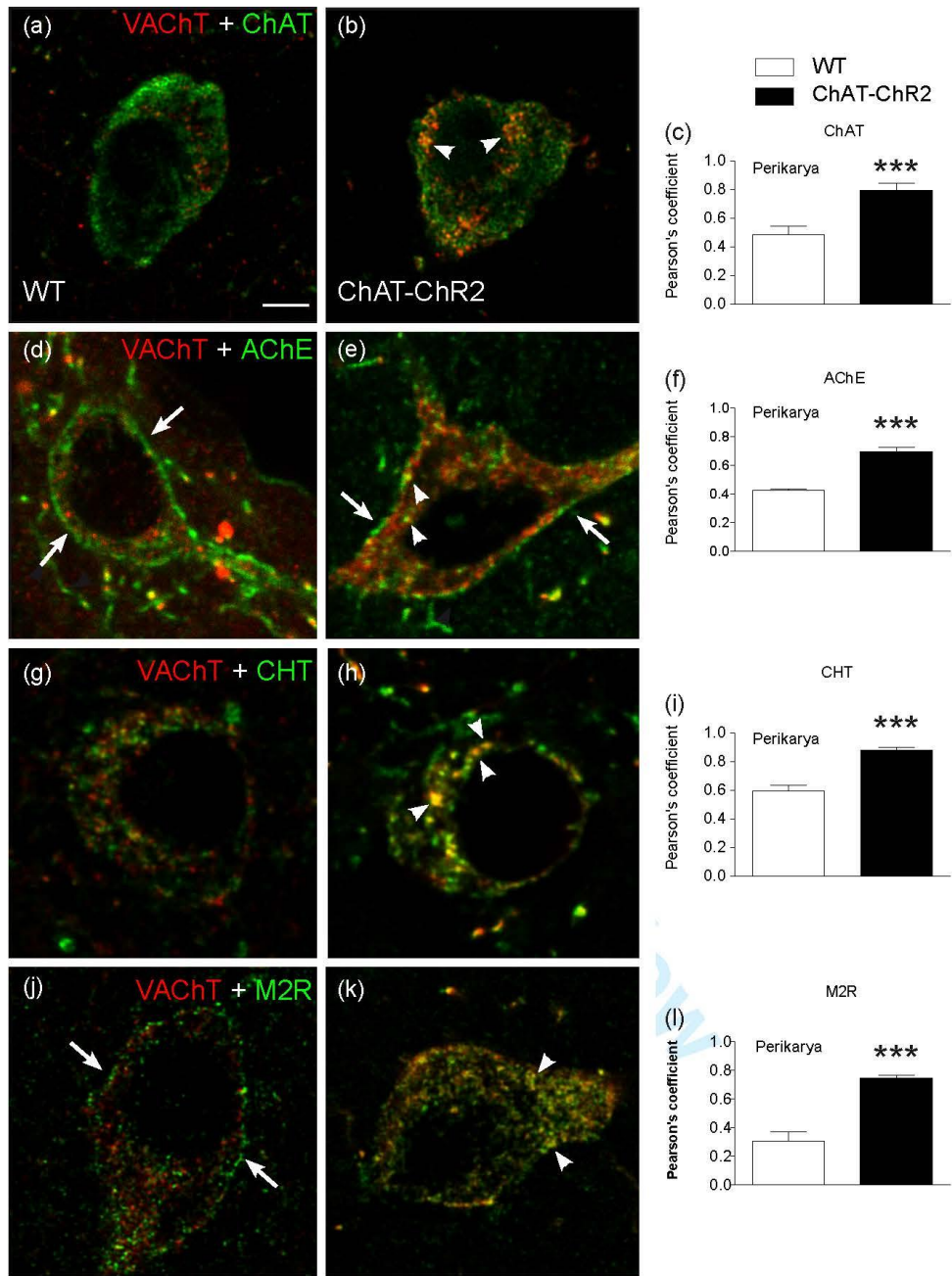


Figure 6 Janickova et al.

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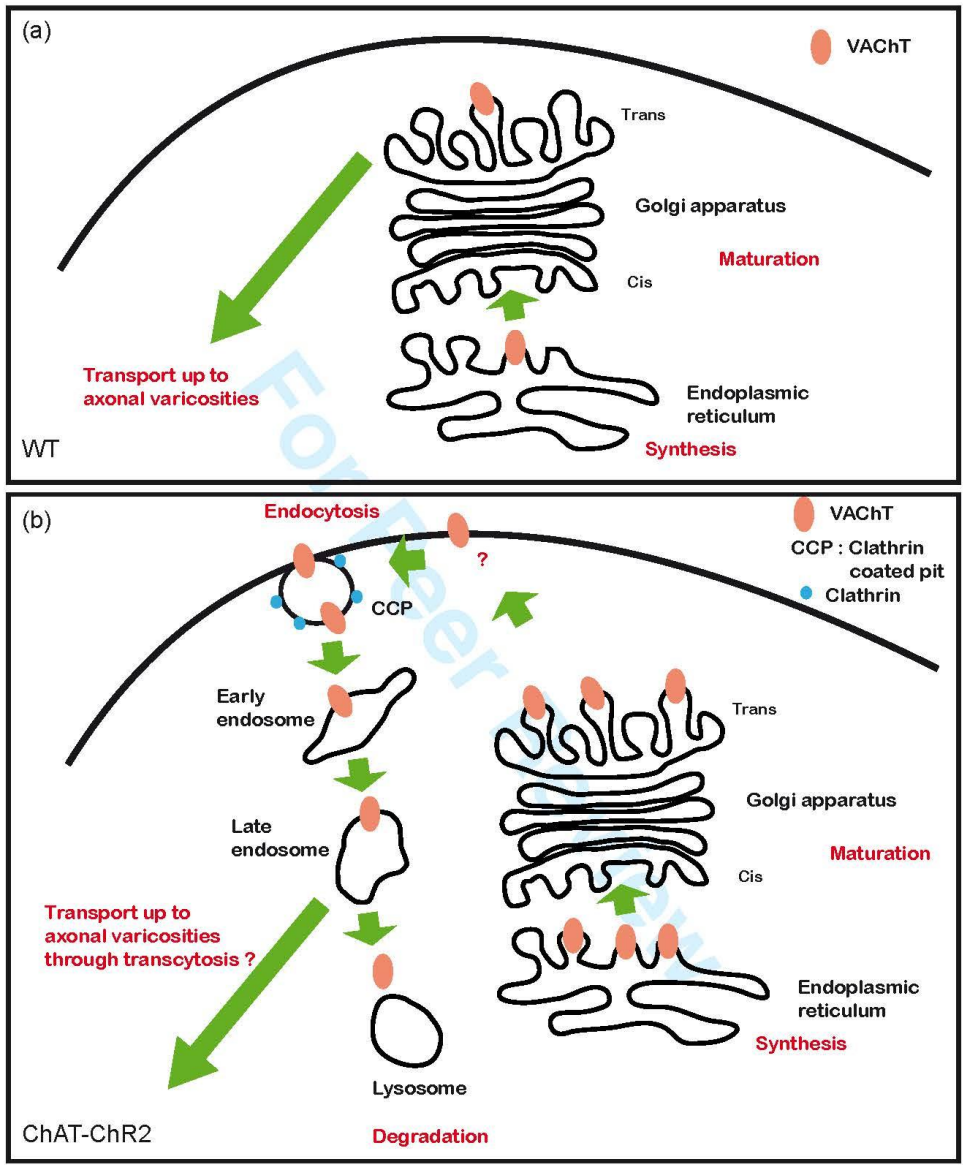


Figure 7 Janickova et al.

Supporting Information

Title : Vesicular Acetylcholine Transporter (VACHT) overexpression induces major modifications of striatal cholinergic interneuron morphology and function

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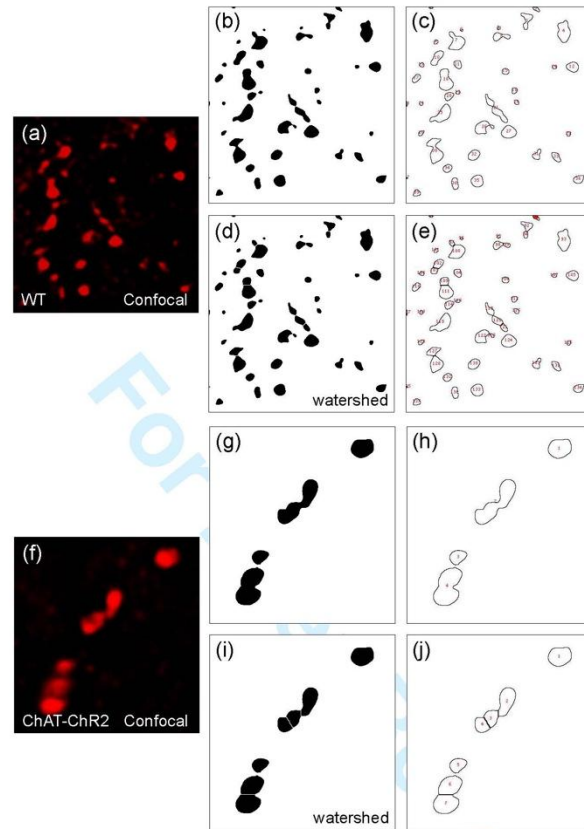
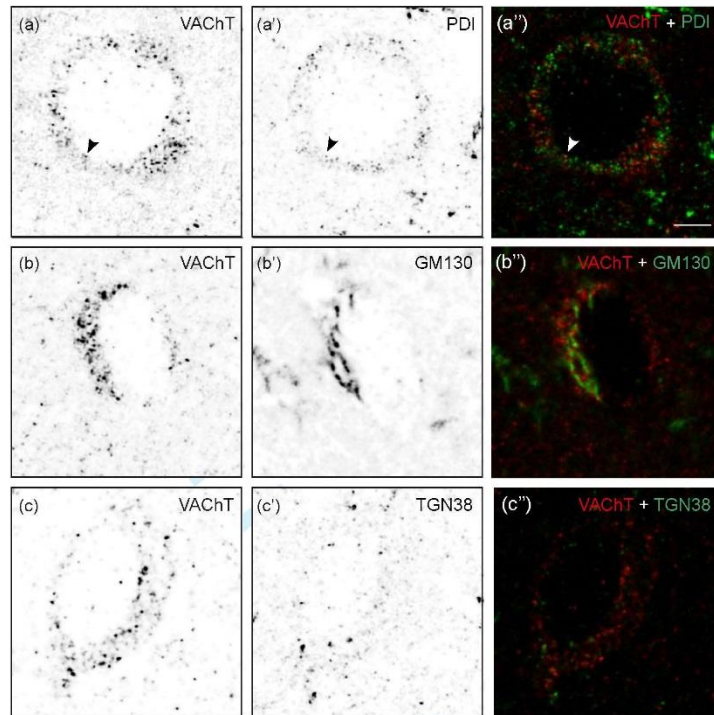


Figure S1

Method of counting of the density of VAcHT immunopositive varicosities using the watershed segmentation.

Striatal sections labeled for VAcHT immunoreactivity were observed using the x63 objective and acquisitions were performed under the confocal microscope. Labeling was then analyzed using the ImageJ software. Because there is overstaining for VAcHT in the BAC mice, it could be that the individual puncta visible in the control mice are overlapping in the BAC mice leading to lower quantal counts only because they cannot be resolved. To correct this bias, we have analyzed our pictures using a method that allow to separate touching fluorescent objects. This method, called the watershed separation, is performed with the ImageJ software. Briefly, after thresholding, the varicosities are separated from the background (b,g). Then, the varicosities are separated one from another, with the watershed function (1d,i) and counted with the analyze particles function (1e,j). The density of spots corresponds to the number of spots divided by the surface of the fields ($655\mu\text{m}^2$).

**Figure S2****Colocalization of VACHT in neuronal compartments involved in synthesis and maturation in CIN perikarya of the striatum of WT mice.**

We show here the individual channels first before the overlay is presented in order to get an unbiased impression of potential co-labeling. The first channel shows VACHT labeling (a-c) and the second one presents PDI (a'), GM130 (b') and TGN38 (c') staining. Panels a'', b'' and c'' correspond to the overlay. Almost no detectable colocalization of VACHT with PDI, GM130 and TGN38 was seen with PDI, GM130 and TGN38 (arrows-heads). Scale bars: a-c'' : 5 μ m.

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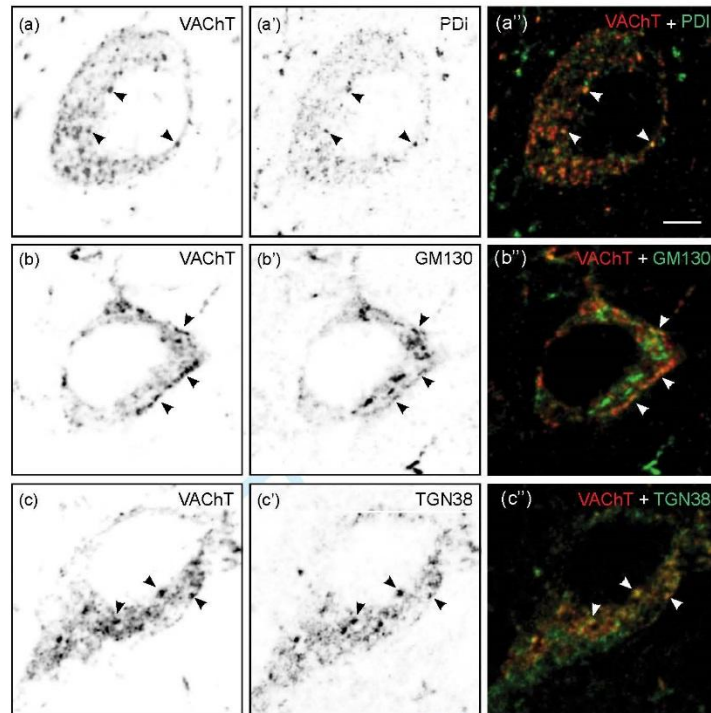
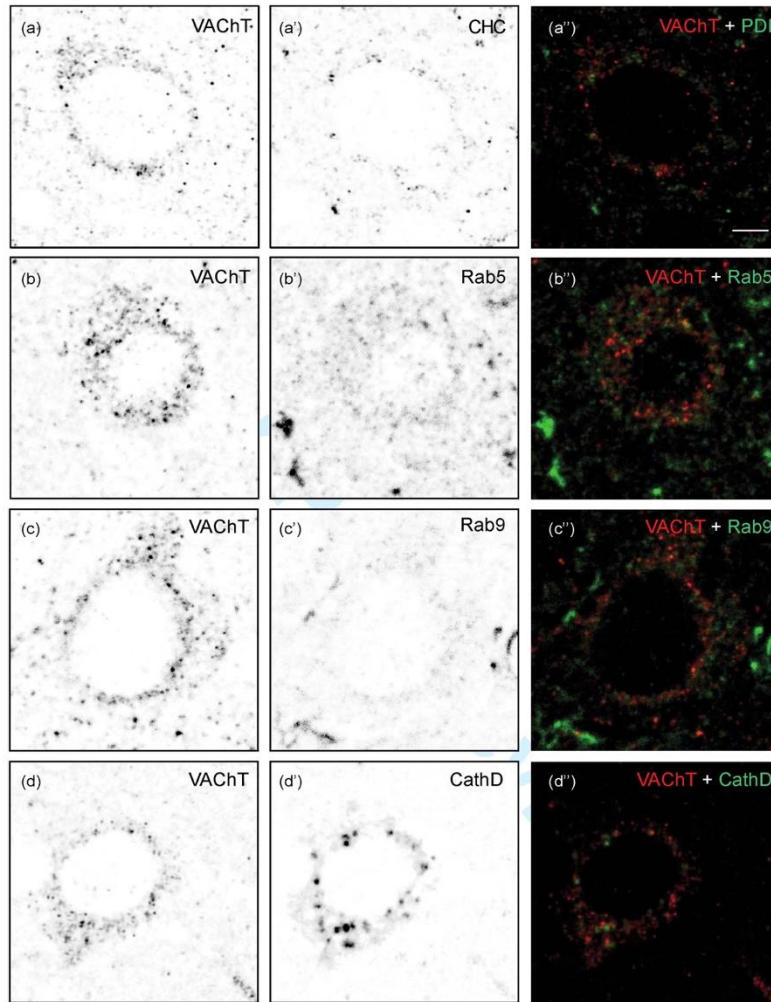


Figure S3

Colocalization of VACHT in neuronal compartments involved in synthesis and maturation in CIN perikarya of the striatum of ChAT-ChR2 mice.

We show here the individual channels first before the overlay is presented in order to get an unbiased impression of potential co-labeling. The first channel shows VACHT labeling (a-c) and the second one presents PDI (a'), GM130 (b') and TGN38 (c') staining. Pannels a'', b'' and c'' correspond to the overlay. VACHT often colocalized with PDI, GM130 and TGN38 (arrows-heads). Scale bars: a-c'' : 5µm.

**Figure S4****Colocalization of VACHT in neuronal compartments involved in endocytosis and degradation in CIN perikarya of the striatum of WT mice.**

We show here the individual channels first before the overlay is presented in order to get an unbiased impression of potential co-labeling. The first channel shows VACHT labeling (a-d) and the second one presents CHC (a'), Rab5 (b'), Rab9 (c') and CathD (d') staining. No detectable colocalization of VACHT with PDI, GM130 and TGN38 was seen (arrows-heads). Pannels a'', b'', c'' and d'' correspond to the overlay. Scale bars: a-d'' : 5 μ m.

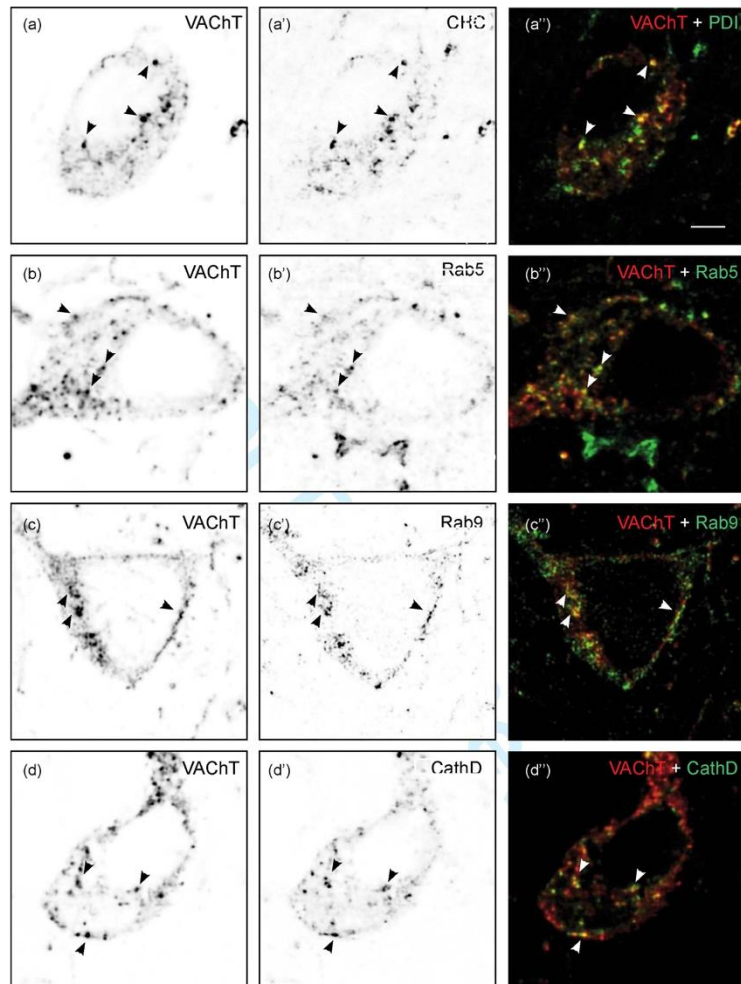


Figure S5

Colocalization of VACHT in neuronal compartments involved in endocytosis and degradation in CIN perikarya of the striatum of ChAT-ChR2 mice.

We show here the individual channels first before the overlay is presented in order to get an unbiased impression of potential co-labeling. The first channel shows VACHT labeling (a-d) and the second one presents CHC (a'), Rab5 (b'), Rab9 (c') and CathD (d') staining. VACHT often colocalized with PDI, GM130 and TGN38 (arrows-heads). Pannels a'', b'', c'' and d'' correspond to the overlay. Scale bars: a-d'' : 5µm.