Triac treatment prevents neurodevelopmental and locomotor impairments in Mct8/Oatp1c1 double knockout mice

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Abstract

Background

Patients with inactive thyroid hormone (TH) transporter MCT8 display intellectual disability due to an insufficient TH transport and action in the CNS. As a therapeutic strategy, application of Triac (3, 5, 3'-triiodothyroacetic acid) and Ditpa (3, 5 -diiodo-thyropropionic acid) have been proposed as both thyromimetic compounds are not dependent on MCT8 for cellular entry. Here, we tested and directly compared the thyromimetic actions of Triac versus Ditpa in Mct8/Oatp1c1 double knockout mice (Dko), a suitable mouse model for human MCT8 deficiency.

Methods:

Newborn Dko mice were daily injected during the first three postnatal weeks with either Triac (50 ng/g or 400 ng/g) or Ditpa (400 ng/g or 4000 ng/g) and compared with Wt and Dko mice receiving saline injections. A second cohort of Dko mice was daily injected with Triac (400 ng/g) only between postnatal week 3 and 6. Thyromimetic effects in the CNS and peripheral tissues were monitored at different postnatal time points by immunofluorescence stainings for neural marker proteins, in situ hybridization and quantitative real time PCR. Locomotor performance was assessed in rotarod and hanging wire test. Acute brain slices of Triac treated Dko mice and their respective controls were used for electrophysiological recordings.

Results:

Only Dko mice injected with Triac (400 ng/g) during the first three postnatal weeks showed normalized myelination, differentiation of cortical GABAergic interneurons as well as locomotor performance. Electrophysiological recordings revealed an increased frequencies of cortical spontaneous miniature inhibitory postsynaptic currents in Dko mice and a normalization of this parameter in Triac treated Dko mice. In comparison, treatment of Dko mice with Ditpa at 4000 ng/g during the first three postnatal weeks resulted in normal

myelination and cerebellar development but was less effective in restoring neuronal parameters and locomotor function. Finally, Triac was more potent than Ditpa in suppressing *Trh* and *Tshb* expression, respectively, and exerts stronger thyromimetic effects in liver and kidneys.

Conclusions:

In newborn Dko deficient mice, Triac is highly effective and more efficient than Ditpa in promoting CNS maturation and function. Yet, Triac treatment needs to be initiated directly after birth to achieve the most beneficial effects.

53 **Introduction**

54 Patients carrying inactivating mutations in the thyroid hormone (TH)-specific
55 monocarboxylate transporter 8 (MCT8) show global developmental delays and a complex
56 cluster of severe intellectual and motor disabilities (Allan-Herndon-Dudley syndrome (AHDS))
57 (1-4). Additionally, patients exhibit characteristic changes in the TH serum profile with highly
58 elevated T3 in the presence of normal/low T4 and normal/elevated TSH. According to the
59 prevailing hypothesis, the neurological impairments of AHDS patients are caused by an
60 insufficient TH transport into the brain while peripheral tissues rather sense the elevated
61 serum T3 concentrations and are therefore in a hyperthyroid state (4-6). That the CNS of
62 MCT8 patients is indeed in a TH deficient state could be substantiated by histopathological
63 findings that include hypomyelination, decreased cerebral expression of parvalbumin (PV), a
64 Calcium binding protein present in a distinct subset of inhibitory neurons, and a delayed
65 cerebellar development (6, 7). Tissue-specific alterations in TH content are also a
66 characteristic feature of Mct8 ko mice that fully replicate the abnormal serum TH profile and
67 show increased TH concentrations in peripheral tissues. Mct8 ko mice also exhibit a
68 diminished passage of the T3 into the CNS but are still capable of transporting T4 into the
69 CNS (8-10). This latter observation can be explained by the presence of the T4-specific
70 transporter Oatp1c1 in murine blood-brain barrier cells (11, 12). Indeed, only Mct8/Oatp1c1
71 double knock-out mice (Dko) show a strongly diminished TH content in the CNS, similar
72 morphological alterations and disturbed neural differentiation as seen in patients, as well as
73 profound locomotor impairments (13). In light of these similarities, Dko mice represent a
74 suitable mouse model for AHDS and are, therefore, a valuable tool for testing therapeutic
75 interventions.

76 One of the most promising therapeutic approaches is the application of TH analogs
77 such as 3, 5 -diiodo-thyropropionic acid (Ditpa) and 3, 5, 3'-triiodothyroacetic acid (Triac) that
78 activate TH receptors and that do not depend on MCT8 for cellular entry (14-17). Studies in
79 Mct8 deficient mice and AHDS patients have already revealed that both compounds are

80 capable of lowering endogenous TH production and normalizing symptoms of a peripheral
81 thyrotoxicosis (such as hypermetabolism, muscle wasting, and increased heart rate) (18-21).
82 It is, however, still a matter of debate to which extent and during which time period both
83 compounds can exert beneficial effects on brain maturation and function.

84 Here, we provide the first direct comparison of Triac versus Ditpa action on brain
85 parameters in Dko mice. Our results clearly indicate that Triac is more effective than Ditpa in
86 promoting a normal brain development and maturation. Moreover, our studies revealed that
87 the most beneficial effects are obtained if Triac treatment is initiated directly after birth while a
88 delayed treatment onset significantly compromises the efficacy of the TH analog.
89

90 **Materials and Methods**

91 *Animals*

92 Generation and genotyping of mice lacking concomitantly Mct8 (Slc16a2^{tm1Dgen}; MGI:
93 3710233) and the organic anion transporting protein Oatp1c1 (Slco1c1$^{tm1.1Arte}$; MGI: 5308446)
94 were reported elsewhere (8, 11, 13). Heterozygous breeding pairs on C57Bl/6N background
95 were set up to obtain Mct8/Oatp1c1 double knockout (Dko) offspring and wildtype (Wt)
96 animals that served as controls (13). All animals were supplied with regular chow and water
97 *ad libitum* and housed in IVC cages at a constant temperature (22°C) and light cycle (12 h
98 light, 12 h dark). Since the Mct8 encoding gene Slc16a2 is encoded on the X-chromosome,
99 only male mice were used in this study.

100 Animals were daily s.c. injected with TH analogs for indicated periods: Triac (3,5,3´-L-
101 triioothyroacetic acid; Sigma-Aldrich) was applied at a concentration of 50 ng/g bw (T(50)) or
102 400 ng/g bw (T(400)) while Ditpa (3,5-diiodo-thyropropionic acid; Sigma-Aldrich) was given at
103 a dose of 400 ng/g bw (D(400)) or 4000 ng/g bw (D(4000)). Control mice received saline
104 injections.

105 For immuno-histochemical analysis, animals were intracardially perfused with 4% PFA
106 in PBS and brains were post-fixed over-night. Coronal forebrain and sagittal cerebellar
107 sections (50 µm) were produced with a vibratome (Thermo Scientific). Animals intended for *in*
108 *situ* hybridization studies (ISH), quantitative PCR analysis (qPCR), and TH measurements
109 were killed by CO2 inhalation. For ISH, tissues were snap-frozen in 2-methylbutane on dry-
110 ice and stored at -80°C until further processing. Coronal forebrain cryo-sections (20 µm) and
111 pituitary sections (14 µm) were produced with a cryostat (Leica), mounted on superfrost plus
112 slides and stored at -80°C. For qPCR analyses, tissue was frozen on dry ice. Serum was
113 obtained from whole blood samples collected by cardiac puncture using microvette tubes
114 (Sarstedt) and stored at -20°C. Serum TH concentrations were determined as described
115 elsewhere (22, 23).

116

117 *Immunohistochemistry*

118 Staining and analysis was carried out as detailed elsewhere (13). In brief, mid-sagittal
119 cerebellar vibratome sections were blocked and permeabilized with 10% normal goat serum
120 in PBS containing 0.2% Triton X-100 and immunostained with a mouse anti-Calbindin D28k
121 antibody (Sigma-Aldrich, 1:1000) followed by incubation with Alexa Fluor 555-labeled goat
122 anti-mouse secondary antibody (Thermo Fisher, 1:1000).

123 Coronal forebrain sections were blocked and permeabilized as above, stained with rat
124 anti-MBP (Millipore, 1:300), mouse anti-Parvalbumin (PV, Millipore, 1:1000) or mouse anti-
125 GAD67 (Millipore, 1:200) and incubated with Alexa Fluor 555-labeled secondary antibody
126 produced in goat (Thermo Fisher, all 1:1000). To visualize myelin, sections were incubated
127 with FluoroMyelin Green Stain according to the manufacturer´s instructions (Molecular Probes,
128 1:300 dye dilution). Sections between Bregma 1.045 and -1.555 were employed for these
129 analyses.

130 Pictures were taken with an Olympus AX70 microscope or Zeiss ApoTome2. For
131 quantification of the Purkinje cell (PC) outgrowth, thickness of the molecular layer (ML) that
132 reflects the dimension of the PC dendritic tree was determined at three different positions in
133 lobules III, IV and V using ImageJ. PV positive neurons were counted in all layers of the
134 somatosensory and retrosplenial cortex and normalized to the size of the analyzed area. For
135 quantifying MBP, GAD67 and FluoroMyelin staining intensities, the respective integrated
136 fluorescence signal intensities per area were measured using ImageJ software. Wt average
137 values were set as 1.0. Blinding was achieved by attributing random numbers to the pictures.
138 For each analysis, four brain sections per animal from 3-5 mice per experimental group were
139 employed.

140

141 *ISH histochemistry*

142 CDNA fragments complementary to mouse *Trh* (NM_009426.2, nt 1251-1876), mouse
143 *Tshb* (NM_009432.2, nt 190-445), and mouse *Hr* (NM_021877.2, nt 902-1598) were used as
144 templates for in vitro transcription using [^{35}S]-UTP (Hartmann Analytik) as a substrate.

145 Radioactively labeled cRNA probes were purified and diluted in hybridization buffer (50%
146 formamide, 10% dextrane sulfate, 0.6 M NaCl, 10 mM Tris-HCl pH 7.5, 1x Denhardt's solution,
147 100 µg/ml sonicated salmon sperm DNA, 1 mM EDTA and 0.5 mg/ml t-RNA) to a final
148 concentration of 1 x 10^4 cpm/µl. In order to prevent overexposure of the ISH signal, the
149 radioactively labeled cRNA probes for *Trh* and *Tsh*b were further diluted with the respective
150 unlabeled cRNA probes (5 ng/µl in hybridization buffer) at a ratio of 1:10 (for *Trh*) and 1:3 (for
151 *Tshb*).

152 ISH was performed according to the hybridization procedures described in detail
153 previously (13). For detection of radioactive ISH signals dehydrated sections were dipped in
154 Kodak NTB nuclear emulsion and stored at 4°C for 3 days (*Trh* and *Tshb*) and 8 days (*Hr*).
155 Autoradiograms were developed and analyzed under darkfield illumination with an Olympus
156 microscope. For quantification of ISH signals, 4-6 tissue sections of 4-5 animals per
157 experimental group were analyzed using ImageJ to determine the integrated signal intensities
158 as described previously (13). Experiments carried out using the respective sense cRNA
159 probes did not produce any specific ISH signals.

160

161 *qPCR*

162 Total tissue RNA was isolated using the NucleoSpin RNA II Kit (Macherey-Nagel).
163 cDNA synthesis was performed by reverse transcription using the Transcriptor High Fidelity
164 cDNA-Synthesis Kit (Roche) according to the manufacture's protocol. For each replicate, 5 ng
165 of cDNA were employed. To exclude the presence of genomic DNA, one sample without
166 reverse transcriptase was included as well. qPCR was performed using the iQ SYBR Green
167 SupermixTM (Bio-Rad) and a Bio-Rad CFX384 detection system. Following primers were
168 used:
169 Cyclophillin D: 5'-GCAAGGATGGCA-AGGATTGA-3' and 5'- AGCAATTCTGCCTGGATAGC-
170 3'; Dio1: 5'-CGTGACTCCTGAAGATGATG-3' and 5'-CCAATGCCTATGGTTCCTAC-3'. The
171 primer pairs were designed for an annealing temperature of 55°C. Transcript levels of Dio1

172 were normalized to expression levels of Cyclophillin D as a house-keeping gene. Four
173 samples per experimental group were subjected to analysis.
174
175 *Electrophysiology*
176 For electrophysiological recordings, 350-μm-thick brain slices were prepared from 3
177 weeks old mice and equilibrated in aCSF (in mM): 120 NaCl, 3 KCl, 1.3 MgSO4, 1.25
178 NaH2PO4, 2.5 CaCl2, 10 D-glucose, 25.0 NaHCO3, gassed with 95% O2 / 5% CO2, pH 7.3
179 at room temperature for at least 1 hour as described previously (24).
180 Patch clamp recordings were performed on coronal slices that were placed in a
181 submerged recording chamber mounted on an upright microscope (BX51WI, Olympus). Slices
182 were continuously superfused with gassed aCSF (2–3 ml/min, 32 °C, pH 7.3). Recordings of
183 mIPSCs were performed using a CsCl-based intracellular solution (in mM): 122 CsCl, 8 NaCl,
184 0.2 MgCl2, 10 HEPES, 2 EGTA, 2 Mg-ATP, 0.5 Na-GTP, 10 QX-314 [*N*-(2,6-
185 dimethylphenylcarbamoylmethyl) triethylammonium bromide], pH adjusted to 7.3 with CsOH.
186 dl-APV (30 μM), CNQX (10 μM) and tetrodotoxin (0.5 μM) were added to the perfusate.
187 mIPSCs were recorded at a holding potential of −70 mV for at least 5 min in aCSF. Data
188 analysis was performed off-line with the detection threshold levels set to 5 pA. The following
189 parameters were determined: frequency, peak amplitude, rise time, time constant of decay
190 (τdecay), half-width, and electrical charge transfer.
191
192 *Behavioral studies*
193 Motor coordination was evaluated using an Accelerating Rotarod (TSE Systems).
194 Briefly, animals were familiarized to system by allowing them to run once with a velocity of 5
195 rpm. On the following day, animals were exposed to acceleration of rod rotation from 5-50 rpm
196 within the maximum testing period of 300 seconds. The riding time of each animal was
197 recorded twice daily and averaged for five consecutive days. Ten to fourteen animals were
198 included in each experimental group.

Muscle strength was assessed in the hanging wire test. Animals were placed on top of a wire cage lid. After shaking gently and turning the cage upside down, the animal's capability to cling to the wire was monitored. Time was taken until the mouse fell down or else the trial was terminated after 60 seconds. Each mouse was tested twice daily for three consecutive days and values were averaged. Twelve to sixteen mice were used per experimental group.

Statistics

All data are presented as mean ± SD. Comparison between groups was performed by two-way ANOVA for experiments including two doses of each compound while one-way ANOVA analysis was applied for experiments using single doses followed by pairwise Tukey's *post hoc* test, if not otherwise indicated. Differences were considered statistically significant if $p < 0.05$ and labelled *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; a – statistically significant difference to Wt animals; b – statistically significant difference to saline-treated Dko animals.

Study approval

All animal procedures were in accordance with the European Union (EU) directive 2010/63/EU and approved by the Animal Welfare Committees of the Thüringer Landesamt für Lebensmittelsicherheit und Verbraucherschutz (03-011/11; TLLV; Bad Langensalza, Germany) and the Landesamt für Natur-, Umwelt- und Verbraucherschutz Nordrhein-Westfalen (84-02-04.2015.A331; LANUV; Recklinghausen, Germany).

224 **Results**

225 *Comparison of Triac versus Ditpa treatment during early postnatal development*

226 In a pilot study, we demonstrated that treatment of Dko mice between postnatal day 1
227 (P1) and P11 with Triac at a concentration of 50 ng/g bw (T(50)) or 400 ng/g bw (T(400))
228 resulted in a dose-dependent improvement of Purkinje cell (PC) dendritogenesis and
229 myelination in the cerebral cortex at P12 (16). To directly compare the thyromimetic effects of
230 Triac and Ditpa, we expanded this study and injected newborn Dko animals during the same
231 postnatal period with Ditpa at either 400 ng/g bw (D(400)) or 4000 ng/g bw (D(4000).

232 Visualizing cerebellar PC dendritogenesis by Calbindin immunofluorescence staining
233 confirmed a strongly reduced dendritic outgrowth in Dko mice and, consequently, a much
234 smaller molecular layer that reached only 60% of the thickness found in Wt animals at P12
235 (Fig. 1A, B). Application of high doses of Triac or Ditpa to Dko mice fully restored PC outgrowth
236 while the low dose applications were less effective. Analysis of MBP immunoreactivity in the
237 cerebral cortex revealed similar findings. Only in Dko mice treated with Triac or Ditpa at a high
238 dose normal MBP immunoreactivity could be achieved (Fig. 1A, C). As another indicator for
239 thyromimetic action in the CNS, we analyzed the number of GABAergic interneurons
240 expressing the calcium-binding protein parvalbumin (PV) in the primary somatosensory cortex
241 as well as the retrosplenial cortex. In agreement with previous data (16), Dko mice exhibited
242 a highly reduced number of PV+ cells in both regions (Fig. 1A, D, E). High dose Triac treatment
243 restored 80% of the PV+ interneuron population in both areas wheras high dose Ditpa
244 treatment improved PV+ cell numbers only to 50% of the respective Wt values.

245 Application of TH analogs will affect the activity of the hypothalamus-pituitary-thyroid
246 (HPT) axis by downregulating *Trh* and *Tshb* expression. To directly compare Triac versus
247 Ditpa effects, we injected Dko mice between P1 and P20 with the same low and high doses
248 paradigm as above and sacrificed the animals at P21. Radioactive ISH studies on brain and
249 pituitary sections confirmed elevated *Trh* expression in hypothalamic paraventricular nucleus
250 (PVN) neurons and increased *Tshb* expression in the anterior pituitary in Dko mice (Fig. 2A,

251 B). Only high dose Triac treatment reduced *Trh* expression whereas both Triac concentrations
252 caused a profound downregulation of pituitary *Tshb* mRNA expression. In response to Ditpa
253 treatment, Dko mice only showed a mild reduction in Trh transcript expression independent of
254 the injected dose and a moderate decrease in *Tshb* specific expression signals upon high
255 dose Ditpa application. These data indicate that Triac exerts stronger thyromimetic action than
256 Ditpa on the HPT axis.

257 We further assessed the expression of TH-sensitive markers in different organs to
258 evaluate the respective tissue- specific thyroidal state. In the CNS, we analyzed mRNA
259 expression of Hairless (*Hr*), a gene well-established to be positively regulated by TH, by ISH
260 (Fig. 2C). *Hr*-specific signal intensities were strongly reduced in the cerebral cortex of Dko
261 mice and increased dose-dependently following Triac treatment whereas Ditpa had only little
262 stimulating effect on *Hr* mRNA expression in the CNS. In liver and kidney, increased *Dio1*
263 expression (Fig. 2D) confirmed a hyperthyroid state of these tissues in Dko mice (13). Low
264 dose Triac treatment significantly reduced *Dio1* transcript levels in both organs whereas Dko
265 animals treated with the high dose of Triac showed similarly elevated *Dio1* levels as found in
266 Dko mice. Low dose Ditpa treatment caused slightly reduced renal and hepatic *Dio1*
267 expression while application of high dose of Ditpa resulted in normal hepatic *Dio1* levels and
268 slightly elevated renal *Dio1* expression. Altogether, these results suggest that Triac exerts a
269 much stronger thyromimetic effect than Ditpa in all analyzed organs.

270

271 *Locomotor behavior of Triac versus Ditpa treated Dko animals*

272 We next addressed the question whether a postnatal treatment with TH analogs had
273 any beneficial effect on locomotion. To this end, Dko mice were injected with either a high
274 dose of Triac (400 ng/g bw) or Ditpa (4000 ng/g bw) for the first three postnatal weeks.
275 Thereafter, TH analog treatment was terminated, and adult animals at the age of 6-7 weeks
276 were subjected to locomotor tests.

277 In accordance with previous data (13), Dko mice spent significantly shorter time on the
278 accelerating rotarod compared to control mice and did not display any visible improvement in
279 their performance during the 5 days-training period (Fig. 3A). In contrast, Triac-treated Dko
280 mice stayed on the rotating wheel as long as Wt mice and significantly improved their skills
281 during the training period. The performance of Ditpa-treated Dko mice was initially not
282 significantly different from that of Wt mice. However, these animals failed to further improve
283 their riding time during the 5 days testing period pointing to motor learning deficits.
284 Subsequently, animals were subjected to a hanging wire test for muscle strength assessment
285 (Fig. 3B). Wt animals could easily cling on the metal wire for 60 s whereas Dko animals fell off
286 after 20 s. Both Triac and Ditpa treatment significantly increased the hanging time of Dko mice.
287

288 *Long-term effects of Triac versus Ditpa on the HPT axis and brain morphology*

289 Following locomotor assessment, animals were sacrificed at the age of 10 weeks, and
290 the activity of the HPT axis was evaluated by analyzing *Trh* and *Tshb* transcript levels by ISH
291 and determining serum TH values. At this time point, animals have not been injected with TH
292 analogs for 7 weeks, and, therefore, a reconstitution of the abnormal HPT axis parameters in
293 all Dko animals could be envisioned. Hypothalamic *Trh* mRNA expression as assessed by
294 ISH (Suppl. Fig. 1A) was equally high and serum T4 levels were equally low (Suppl. Fig. 1B)
295 in all Dko mice independent of their treatment during the first three postnatal weeks. Serum
296 T3 concentrations were elevated in Ditpa- and Triac-treated Dko mice, but to a significantly
297 lesser extent than in Dko animals (Suppl. Fig. 1B). Likewise, TH-analog treated Dko animals
298 showed lower *Tshb* expression than Dko mice (Suppl. Fig. 1A). These findings suggest that
299 early postnatal treatment with TH analogs induces long-lasting changes in the set point of the
300 HPT axis in Dko animals.

301 In addition, we investigated the long-term effects of early postnatal TH-analog
302 treatment on brain morphology. To examine CNS myelination, coronal vibratome sections
303 were subjected to FluoroMyelin staining and fluorescence intensities were quantified in the
304 corpus callosum area (Fig. 4A). Compared to control animals, Dko mice showed strongly

305 reduced myelin content. Triac and Ditpa-treated Dko mice exhibited similar FluoroMyelin
306 staining intensities as Wt animals suggesting a normalization of myelin formation. In order to
307 assess the maturation state of PV-expressing GABAergic neurons in retrosplenial and
308 somatosensory cortex, PV+ cells were visualized by immunofluorescence staining. In contrast
309 to Dko mice, Triac treated Dko animals showed similar numbers of PV+ cells in both areas as
310 found in Wt mice (Fig. 4B). Interestingly, Ditpa treatment only slightly increased PV+ cell
311 numbers in the somatosensory cortex, but did not exert any beneficial effect in the retrosplenial
312 cortex. As PV is present only in a subset of GABAergic neurons, we additionally investigated
313 expression of glutamate decarboxylase GAD67 as a key marker for all GABA producing
314 interneurons. Quantification of GAD67 immunoreactivity in the somatosensory and
315 retrosplenial cortex showed significantly reduced values in saline-treated Dko mice (Fig. 4C).
316 Importantly, application of Triac, but not Ditpa restored normal GAD67 expression in Dko mice.
317 Altogether, our data demonstrate that a transient treatment of Dko mice with TH analogs
318 during early postnatal stages induces long lasting morphological changes in the CNS with
319 Triac being more effective than Ditpa in normalizing brain parameters of Dko animals.
320
321 *Electrophysiological studies*
322 Low GAD67 expression may result in diminished local GABA production and inhibition
323 in the cerebral cortex that in turn will greatly influence cortical network activity. Therefore, we
324 assessed GABAergic synaptic transmission in the somatosensory cortex in slices obtained
325 from Wt, untreated Dko and Dko mice treated with high dose Triac for the first three postnatal
326 weeks. Patch-clamp recordings were performed and mIPSC (spontaneous miniature inhibitory
327 postsynaptic current) kinetics were analyzed. Representative traces recorded from pyramidal
328 neurons in cortical layers II/III are shown in Fig. 5A. No differences in passive membrane
329 properties (capacity: Wt 43.7±4.3 pF, Dko 36.8±1.8 pF; Dko + T(400) 35.8±2.0 pF and input
330 resistance: Wt 67.0±4.6 MΩ; Dko 69.3±6.4 MΩ; Dko + T(400) 65.2±6.8 MΩ) were observed.
331 In contrast, mean frequencies of mIPSCs recorded from pyramidal neurons of the
332 somatosensory cortex were significantly increased in Dko slices compared to Wt slices (Fig.

333 5B) suggesting that Mct8/Oatp1c1 inactivation has a major effect on GABAergic transmission
334 in the cerebral cortex. Other recorded mIPSC kinetics parameters such as amplitudes, rise
335 time, half-width, time constant of decay, and transported electric charges did not differ
336 between the genotypes (Fig. 5C). Interestingly, Triac-treated Dko mice showed fully
337 normalized mIPSC frequencies. This observation underscores the prominent and beneficial
338 effects of Triac treatment on the development and function of the inhibitory system in Dko
339 mice.
340
341 *Determining the critical time window of Triac action*
342 Obviously, high dose Triac application restored myelination and improved maturation
343 and function of the GABAergic system when applied between P1 and P21. In order to
344 determine the critical time window of Triac action, we repeated our studies with a second
345 cohort of Dko mice that only received Triac treatment between postnatal days P22-P42 (Fig.
346 6). Only Dko mice that received Triac during the first three postnatal weeks showed a full
347 normalization of PV+ cell numbers in retrosplenial (Fig. 6B) and somatosensory cortex (Fig.
348 6C) as well as normal myelination in the corpus callosum assessed by FluoroMyelin (Fig. 6D).
349 In contrast, in Dko mice that received Triac only between postnatal week 3 and 6, myelination
350 was not improved while a partial recovery of PV+ immunoreactivity could still be detected.
351 Altogether, our data indicate that the most pronounced beneficial effects of Triac on different
352 brain parameters of Dko mice can only be achieved if postnatal treatment is initiated as early
353 as possible.
354
355

356 **Discussion**

357 AHDS represents a devastating neurodevelopmental disease for which no therapy is
358 currently registered. Early treatment strategies such as the application of T4 in combination
359 with PTU in order to block T4 to T3 conversion aimed to normalize serum TH parameters and
360 to ameliorate the symptoms of peripheral thyrotoxicosis (25). Such a treatment regiment,
361 however, will not improve neurological symptoms given that a diminished TH transport across
362 brain barriers and thus a reduced TH action inside the CNS represent the major pathogenic
363 mechanisms. This uptake blockage may be circumvented by applying thyromimetic
364 substances that bypass MCT8 and are able to activate TH receptors in neural target cells. In
365 a compassionate study, Ditpa was given for 26-40 months to four affected children at the age
366 of 8-25 months and was able to both normalize high serum T3 concentrations and reduce
367 symptoms of hypermetabolism (18). Improvements in neurological functions, however, have
368 not been reported. Likewise, in a first international clinical trial with forty-six AHDS patients
369 (median age 7.1 years) which were treated with Triac for 12 months, a rapid reduction of the
370 high serum T3 levels as well as beneficial and sustainable effects regarding clinical sings of a
371 peripheral thyrotoxicosis could be observed (21). This Triac trial was not designed to detect
372 any impact of the treatment on neurodevelopmental outcomes in human MCT8 deficiency
373 although a trend towards neurodevelopmental improvement was noted in a subset of patients
374 in an exploratory analysis. Thus, it is still an open question whether Triac (and Ditpa) treatment
375 has any long-lasting beneficial effects on CNS development. A second clinical phase 2 study,
376 Triac trial II (NCT02396459), has recently been initiated to specifically assess Triac effects in
377 patients younger than 30 months of age at the start of the treatment whereas a similar clinical
378 trial for Ditpa is in preparation (NCT04143295).

379 Although both TH analogs have already been tested in different model systems (15,
380 16, 19, 26-30), previous studies did not allow to draw any conclusions as to which of the two
381 compounds exerts stronger beneficial effects on CNS development due to a variety of
382 confounding factors (i.e. usage of different Mct8 mutants, treatment regimens, read-out

383 parameters and drug concentrations). The major aim of our study was therefore to evaluate
384 and directly compare the thyromimetic potential of Ditpa and Triac in Mct8/Oatp1c1 double
385 knock-out (Dko) mice as a preclinical AHDS model. Due to a profound CNS specific TH
386 deprivation, these Dko mice exhibit distinct brain histomorphological abnormalities
387 (hypomyelination, compromised cerebral and cerebellar neuronal differentiation) that were
388 also seen in AHDS patients (6, 7, 13). Moreover, by detecting a rise in cortical mIPSC
389 frequencies in Dko mice (Fig. 5), we provide another relevant read-out parameter that
390 underscores the impact of M/O deficiency on inhibitory neuronal network activity.

391 Here, we provide a first systematical testing of different concentrations of Triac and
392 Ditpa in parallel. Two doses from previous concentration-finding pilot studies were employed
393 for Triac (16). For Ditpa, we used 10-fold higher concentrations compared to Triac as such an
394 order-of-magnitude-increase was needed to achieve similar thyromimetic effects in cerebellar
395 Purkinje cell outgrowth *in vitro* (Kersseboom; not published). Likewise, 10-times higher
396 concentration of Ditpa compared to Triac were needed to induce myelin marker p0 mRNA
397 expression in mct8 mutant zebrafish to a similar extent (28). Moreover, we focused on brain
398 parameters as read-outs that are well-established TH targets and that have been reported to
399 be affected in AHDS patients as well as in our AHDS mouse model of Mct8/Oatp1c1 double
400 deficiency (7, 13).

401 As major findings of our study, we could demonstrate for the first time robust and long-
402 lasting beneficial effects of Triac on various brain parameters in Dko mice only if a high-dose
403 Triac treatment was initiated directly after birth. This regimen was sufficient to fully restore
404 cerebellar development, myelination as well as maturation of cortical GABAergic neurons in
405 Dko mice as evidenced by immunofluorescence analysis (Fig. 1). Cortical mIPSC frequencies
406 recorded in acute brain slices and elevated in Dko animals, showed normal values in high-
407 dose Triac treated Dko animals (Fig. 5). Possibly, these electrophysiological findings reflect
408 the altered expression pattern of PV and other Calcium binding proteins in Dko mice as a
409 reduced neuronal Ca^{2+} buffering capacity will cause an elevated release probability and thus
410 a rise in mIPSC frequency. Finally, high dose Triac treatment of Dko mice restored locomotor

411 performance as assessed by rotarod and hanging wire test (Fig. 3). In comparison, high dose
412 Ditpa treatment during the first three postnatal weeks also stimulated myelination and
413 cerebellar development but was significantly less effective in mending cortical GABAergic
414 neuron maturation and normalizing locomotor function, suggesting that Triac has a stronger
415 potential for the treatment of MCT8 patients.

416 Several aspects, however, have to be considered before translating these preclinical
417 data into clinical practice. The high concentration of Triac needed for effective normalization
418 of brain parameters in our mouse model resulted in a fully downregulated HPT axis with *Tshb*
419 transcript below the detection limit (Fig. 2A,B). Although we were not able to determine serum
420 Triac versus TH concentrations in Triac treated animals due to antibody cross-reactivity issues
421 (31) we speculate that endogenous serum TH concentrations are highly reduced in Triac
422 treated Dko mice. Triac is most likely the only TH receptor active substance in these animals
423 with such a high thyromimetic activity (at the highest dose) that it still causes a peripheral
424 thyrotoxic state as indicated by the elevated hepatic and renal *Dio1* expression (Fig. 2D). This
425 scenario is certainly in contrast to the situation of the AHDS patients in the first clinical Triac
426 trial (21). In fact, one major aim of this clinical study was to achieve a reduction in the highly
427 elevated serum T3 levels in order to ameliorate peripheral thyrotoxicosis while T4 serum levels
428 should still be detectable. To that end, an average dose of 38.3 µg/kg/day was given, which
429 is in the same range as the low Triac dose (T(50)) used in this study. However, treating Dko
430 mice with such a low Triac dose had only little beneficial effects on brain parameters such as
431 myelination or interneuron maturation (Fig. 1). It is therefore tempting to speculate that with
432 respect to the neurological outcome, MCT8 patients might benefit more from a treatment with
433 a higher dose of Triac even if under these circumstances signs of e.g. hepatic thyrotoxicosis
434 are still present.

435 Another intriguing observation is the optimal time window of Triac application. In our
436 study, only the early onset Triac treatment regiments exerted strong beneficial effects on brain
437 parameters in Dko while late onset Triac application starting at postnatal day 22 and lasting
438 for 3 weeks did not improve myelination and only insufficiently restored cortical PV+ cell

439 numbers (Fig. 6). Why the CNS of Dko mice showed very little response to the late-onset Triac
440 treatment is still elusive. As one hypothesis, the not-yet identified transport systems by which
441 Triac enters the CNS and neural target cells might be down-regulated in the mature mouse
442 CNS (26). Additionally, the critical time window during which neural differentiation processes
443 are still sensitive to TH might be closed. The latter scenario would underscore the utmost
444 importance of an early postnatal diagnosis of AHDS followed by an immediate treatment
445 initiation.

446 As an alternative to TH analog treatment, gene therapy approaches exploiting AAV
447 vector constructs have been considered (32-34). These interventions aim to express a
448 functional MCT8 transporter in brain endothelial cells and eventually restore TH action in other
449 neural cell types. Intravenous injection of endothelial cell specific AAV-BR1-Mct8 constructs
450 in newborn Dko mice resulted in improved cerebellar development, myelination and
451 GABAergic marker expression (34). Yet, these beneficial effects were less profound compared
452 to the alterations seen here upon high dose Triac treatment in Dko mice. Treatment of juvenile
453 Dko mice with AAV-BR1-Mct8 at P30 induced expression of well-established T3-target genes,
454 yet failed to improve myelination and only slightly induced GABAergic marker expression,
455 similar to our observations with late onset Triac treatment. Likewise, in Dko mice treated at
456 P30 with AAV9-MCT8 constructs, serum TH parameters remained abnormal indicating that a
457 peripheral hyperthyroidism is preserved, while TH-target genes in the CNS showed only a
458 partial response (33).

459 By comparing different treatment strategies and similar read-out parameters in the
460 same Dko mouse model, Triac appears as the most promising treatment approach for AHDS
461 to date. Yet, Triac presumably needs to be given at a high dose and the treatment should be
462 initiated as early as possible to achieve the best outcome for the patients.

463

464

465

466 **Author contributions:**

467 JC, SM, WEV and HH devised the study. JC, ES, SMS, DD and SM conducted the in vivo
468 work and all histo-morphological analyses. LL performed and analysed the
469 electrophysiological experiments. AB determined serum TH levels. JC, LL, CAH, AB, SM and
470 HH interpreted the results. JC, SM, WEV and HH wrote the manuscript.

471

472 **Author Disclosure Statement:**

473 The authors have nothing to disclose.

474

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480

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584

Fig. 1

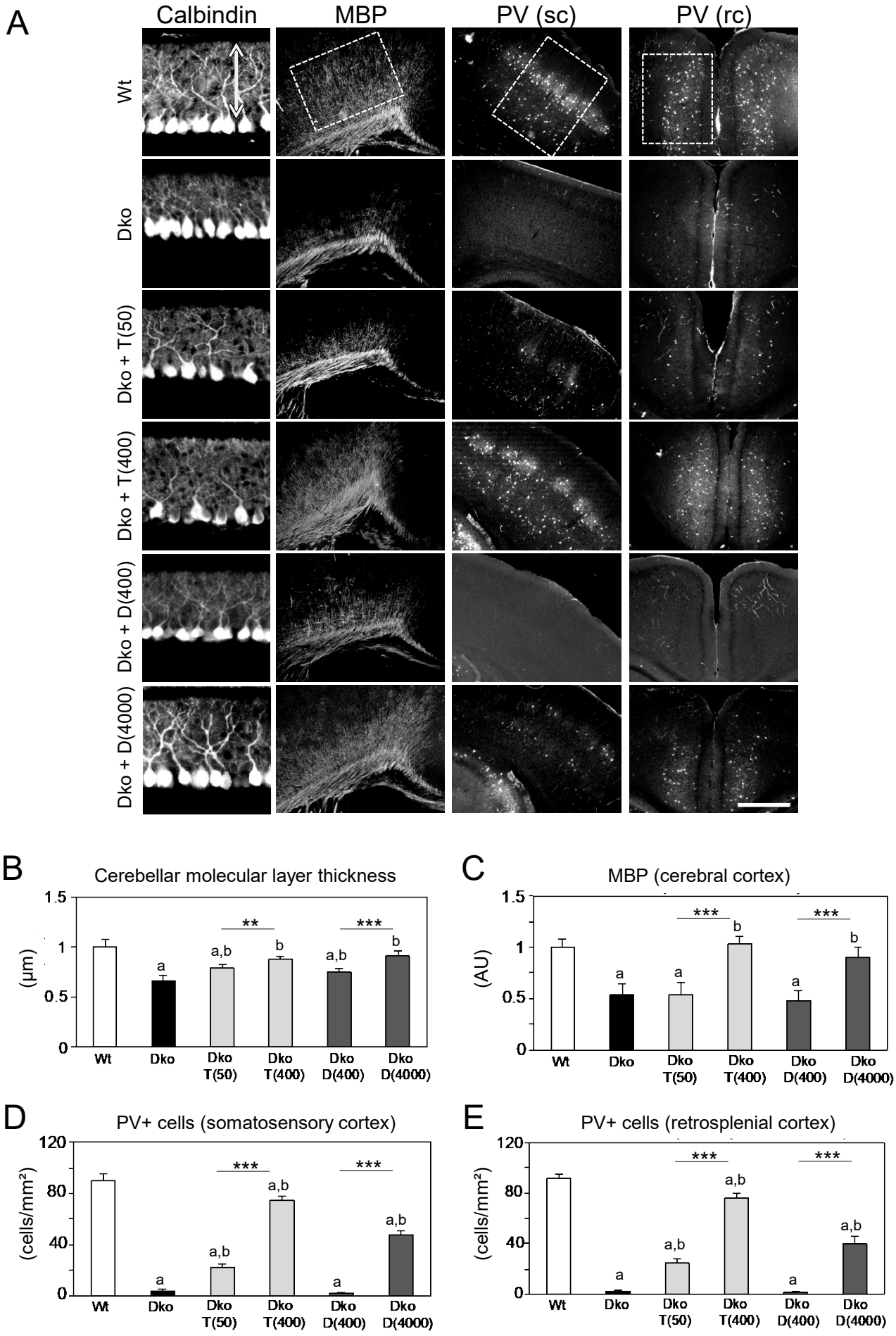

A
Calbindin
MBP
PV (sc)
PV (rc)
Wt
Dko
Dko + T(50)
Dko + T(400)
Dko + D(400)
Dko + D(4000)
B
Cerebellar molecular layer thickness
(μm)
1.5
1
0.5
0
**
***
a
a,b
b
Wt
Dko
Dko T(50)
Dko T(400)
Dko D(400)
Dko D(4000)
C
MBP (cerebral cortex)
(AU)
***
D
PV+ cells (somatosensory cortex)
(cells/mm²)
120
80
40
E
PV+ cells (retrosplenial cortex)

Fig. 1: TH analog treatment initiated after birth improves brain development and maturation.

Newborn mice received a daily injection of saline (control) or different doses (in ng/g bw) of TH analogs Triac or Ditpa between postnatal days 0 and 11 as indicated and brain morphology was analyzed at P12. A) Cerebellar Purkinje cell development was monitored by Calbindin immuno-staining, myelination in the cerebral cortex was assessed by MBP immuno-reactivity and PV+ interneurons were visualized in the somatosensory cortex (sc) and retrosplenial cortex (rc). B) Dimension of the Purkinje cell dendritic tree (arrow in A) was measured as a readout for the thickness of the molecular layer. Thickness was reduced in Dko animals and restored by Triac and Ditpa treatment in a dose-dependent manner. C) Integrated density of MBP-specific signals was determined in the inner layers of the cerebral cortex (box in A delineating the measured area). MBP levels were reduced in Dko animals and normalized only upon high dose Triac or Ditpa administration. Number of PV+ interneurons was enumerated in the somatosensory (D) and retrosplenial (E) cortex (boxes in A indicate areas of interest). PV+ cells were almost absent in M/O Dko mice and increased dose-dependently following Triac or high dose Ditpa application. n=3-5. Scale bars 50 µm (Calbindin), 500 µm (MBP), 250 µm (PV).

Fig. 2

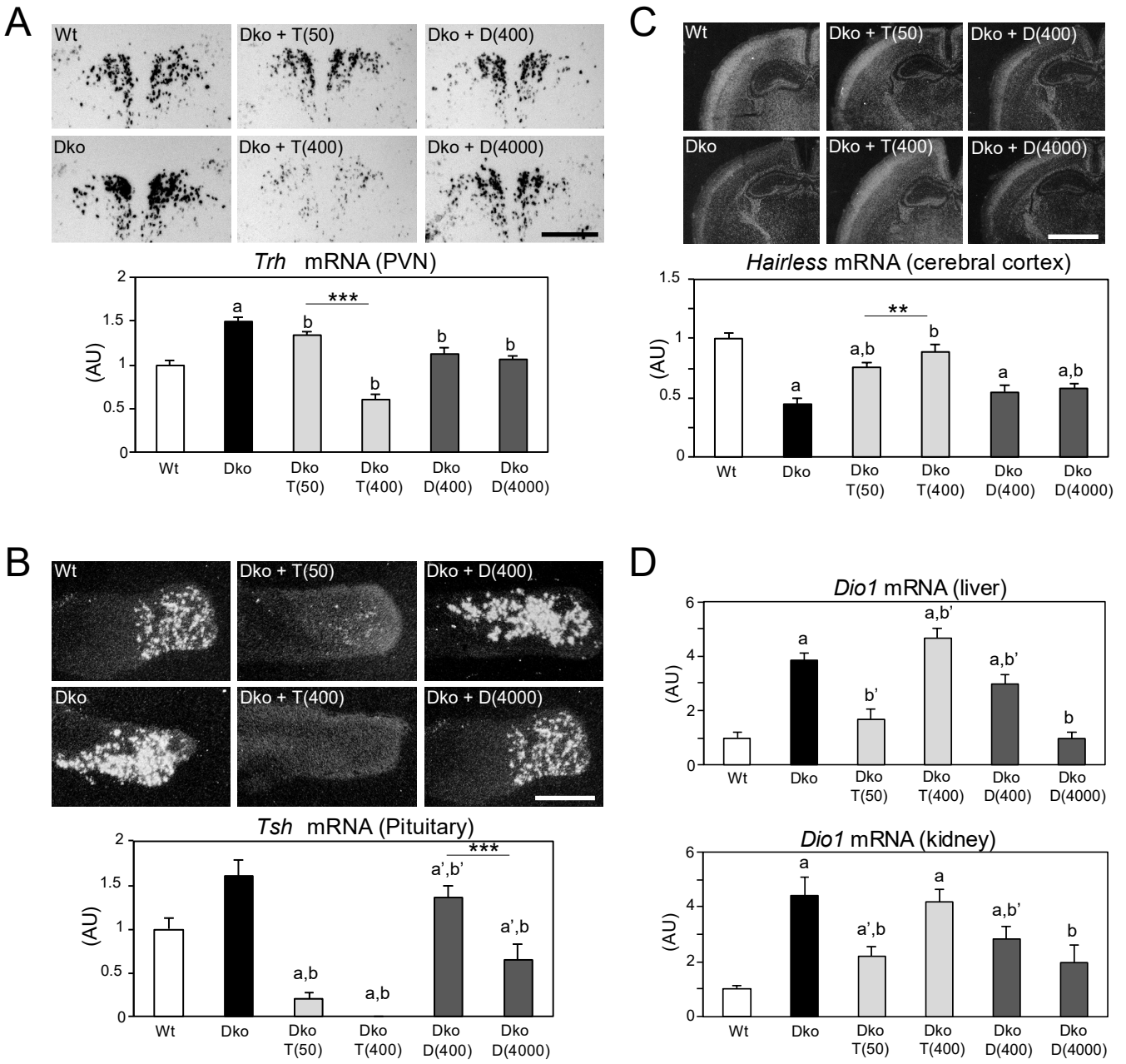

A
Wt
Dko + T(50)
Dko + D(400)
Dko
Dko + T(400)
Dko + D(4000)
Trh mRNA (PVN)
(AU)
Wt
Dko
Dko T(50)
Dko T(400)
Dko D(400)
Dko D(4000)
C
Hairless mRNA (cerebral cortex)
B
Tsh mRNA (Pituitary)
D
Dio1 mRNA (liver)
Dio1 mRNA (kidney)

Fig. 2: Triac executes stronger thyromimetic actions on the HPT axis than Ditpa.

Dko mice were injected daily with either a saline solution or different concentrations of Triac and Ditpa between P0 and P20. At P21, activity of the HPT axis was monitored. A) Saline-treated DKo mice exhibited strongly increased Trh-specific ISH signals in PVN neurons, which were reduced in intensity following Triac application in a dose dependent manner. Ditpa had only little effect on Trh expression. Bright-field images are depicted while dark-field illuminations were employed for quantification. B) Dark-field autoradiograms illustrate alterations in Tsh beta transcripts in the pituitary with increased levels in Dko mice that were almost completely suppressed by Triac application, but only moderately so by Ditpa treatment. C) Hairless-specific hybridization signals were used to examine thyromimetic effects of TH analogs in the brain. Centrally severely hypothyroid Dko mice present with strongly decreased Hairless expression that was restored by Ditpa and, even more efficiently, by Triac application. D) Hepatic and renal Dio1 expression were studied by qPCR. Mice receiving the low dose of Triac showed a reduction in Dio1 expression in both organs while high dose Triac maintained elevated Dio1 transcript levels as seen in saline-treated Dko mice. Ditpa decreased Dio1 expression dose dependently. n=4-10. Scale bars 200 µm (TRH), 500 µm (TSH), 1 mm (Hairless).

Fig. 3

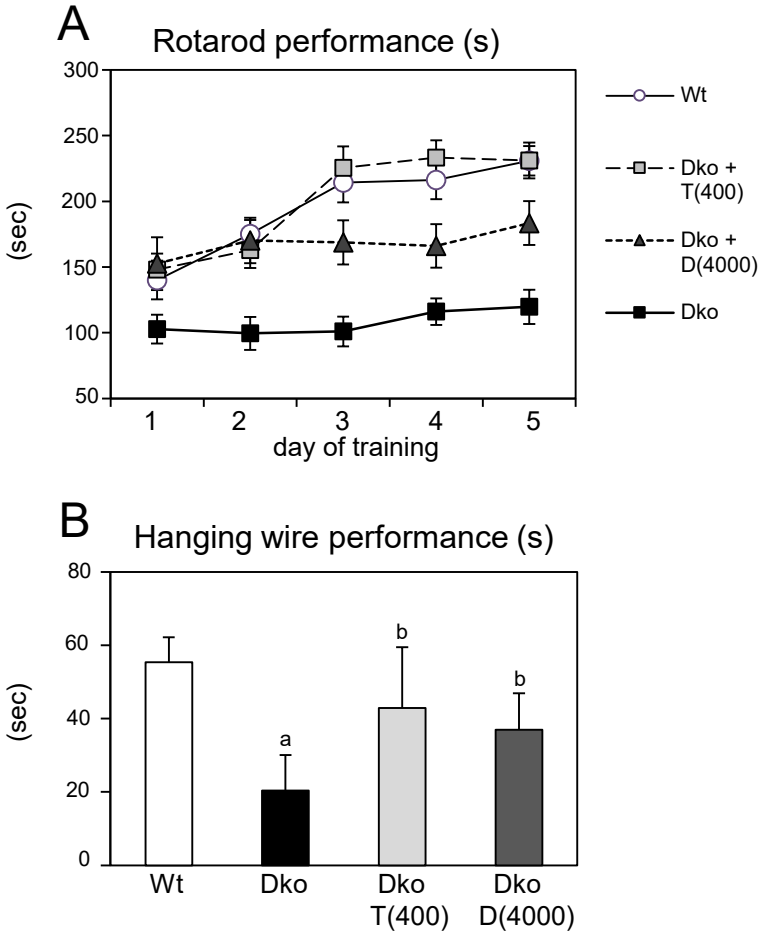

A
Rotarod performance (s)
300
250
200
150
100
50
(sec)
1
2
3
4
5
day of training
Wt
Dko +
T(400)
Dko +
D(4000)
Dko
B
Hanging wire performance (s)
80
60
40
20
0
(sec)
a
b
b
Wt
Dko
Dko
T(400)
Dko
D(4000)

Fig. 3: Transient early TH analog treatment improves locomotion an adulthood.

Dko mice received daily injections with saline, 400 ng/g bw Triac (Dko+T(400)) or 4000 ng/g bw Ditpa (Dko+D(4000)) between P0 and P20. Thereafter, treatment was ceased and locomotor behavior was addressed at 6-7 weeks. A) In contrast to Dko mice receiving saline and showing severe locomotor deficiencies, Ditpa application moderately and Triac treatment fully normalized performance on an accelerating rotarod. B) Neuromuscular abnormalities were further examined by a hanging wire test. Dko mice clung to the wire for only a short time period, which was significantly improved in TH analog-treated experimental groups. n=10-14.

Fig. 4

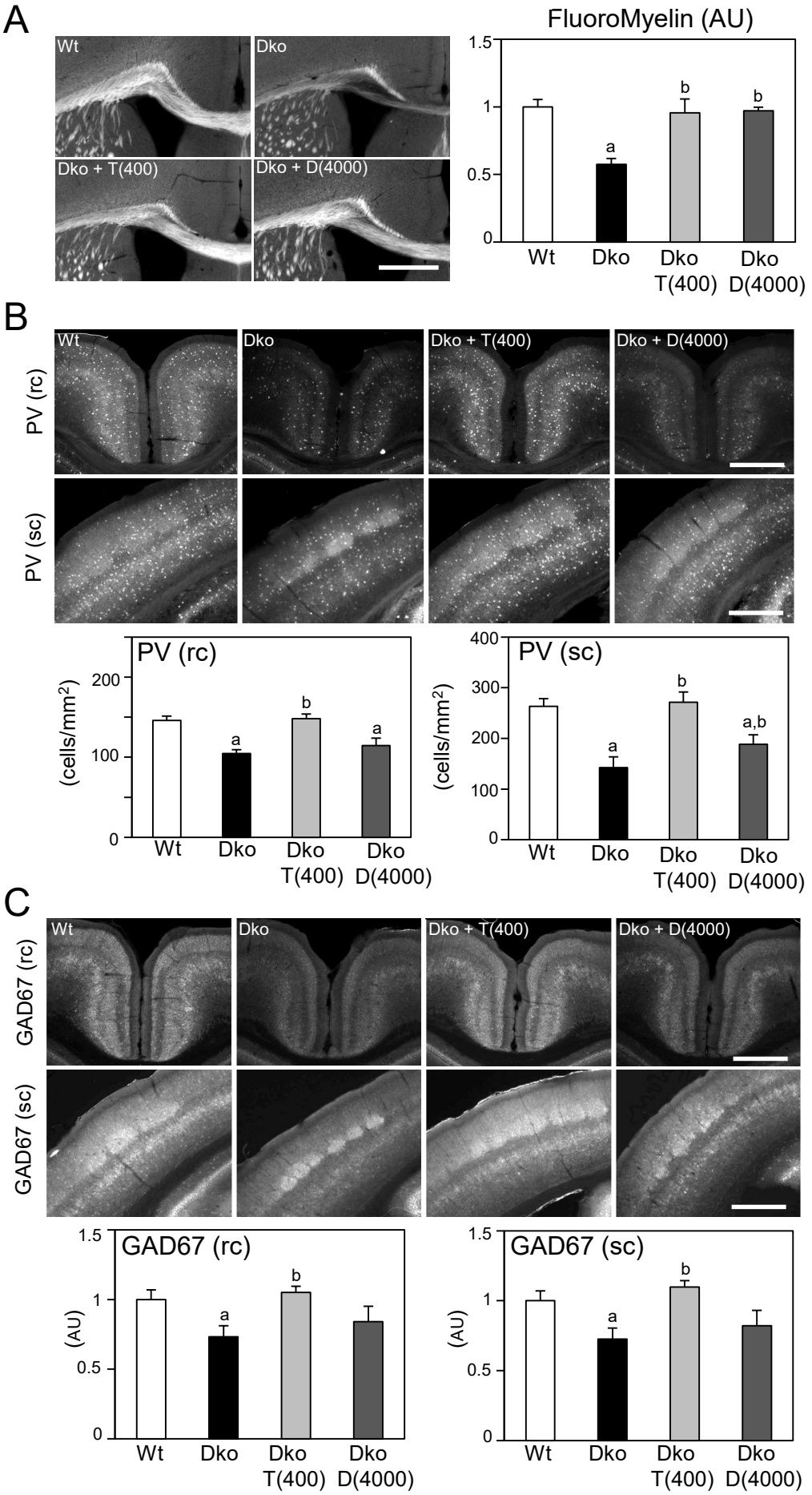

A
Wt
Dko
Dko + T(400)
Dko + D(4000)
FluoroMyelin (AU)
1.5
1
0.5
0
a
b
b
Wt
Dko
Dko T(400)
Dko D(4000)
B
PV (rc)
PV (sc)
Wt
Dko
Dko + T(400)
Dko + D(4000)
PV (rc)
(cells/mm2)
200
100
0
a
b
a
Wt
Dko
Dko T(400)
Dko D(4000)
PV (sc)
(cells/mm2)
400
300
200
100
0
a
b
a,b
Wt
Dko
Dko T(400)
Dko D(4000)
C
GAD67 (rc)
GAD67 (sc)
Wt
Dko
Dko + T(400)
Dko + D(4000)
GAD67 (rc)
(AU)
1.5
1
0.5
0
a
b
Wt
Dko
Dko T(400)
Dko D(4000)
GAD67 (sc)
(AU)
1.5
1
0.5
0
a
b
Wt
Dko
Dko T(400)
Dko D(4000)

Fig. 4: Long-term morphological alterations are induced by early postnatal TH analog application.

Following a transient treatment with TH analogs Triac and Ditpa (concentrations in ng/g bw as indicated) between P0 and P20, brain parameters were examined at 10 weeks of age. A) FluoroMyelin staining was used to evaluate myelination in the corpus callosum. Hypomyelination phenotype of Dko mice could be rescued by TH analog treatment. B) PV+ interneurons were visualized in the retrosplenial cortex (rc) and somatosensory cortex (sc). Cell numbers were enumerated showing strong reductions in saline-injected Dko animals. Triac application fully restored PV+ numbers in both areas, while Ditpa only mildly improved PV+ numbers in the sc. C) GAD67 immuno-reactivity was analyzed in the same cortical regions demonstrating reduced integrated densities in saline-injected Dko mice that was unaffected by Ditpa, but fully restored upon Triac application early in life. n=3-4. Scale bars 500 µm (FluoroMyelin), 250 µm (PV, GAD67).

Fig. 5

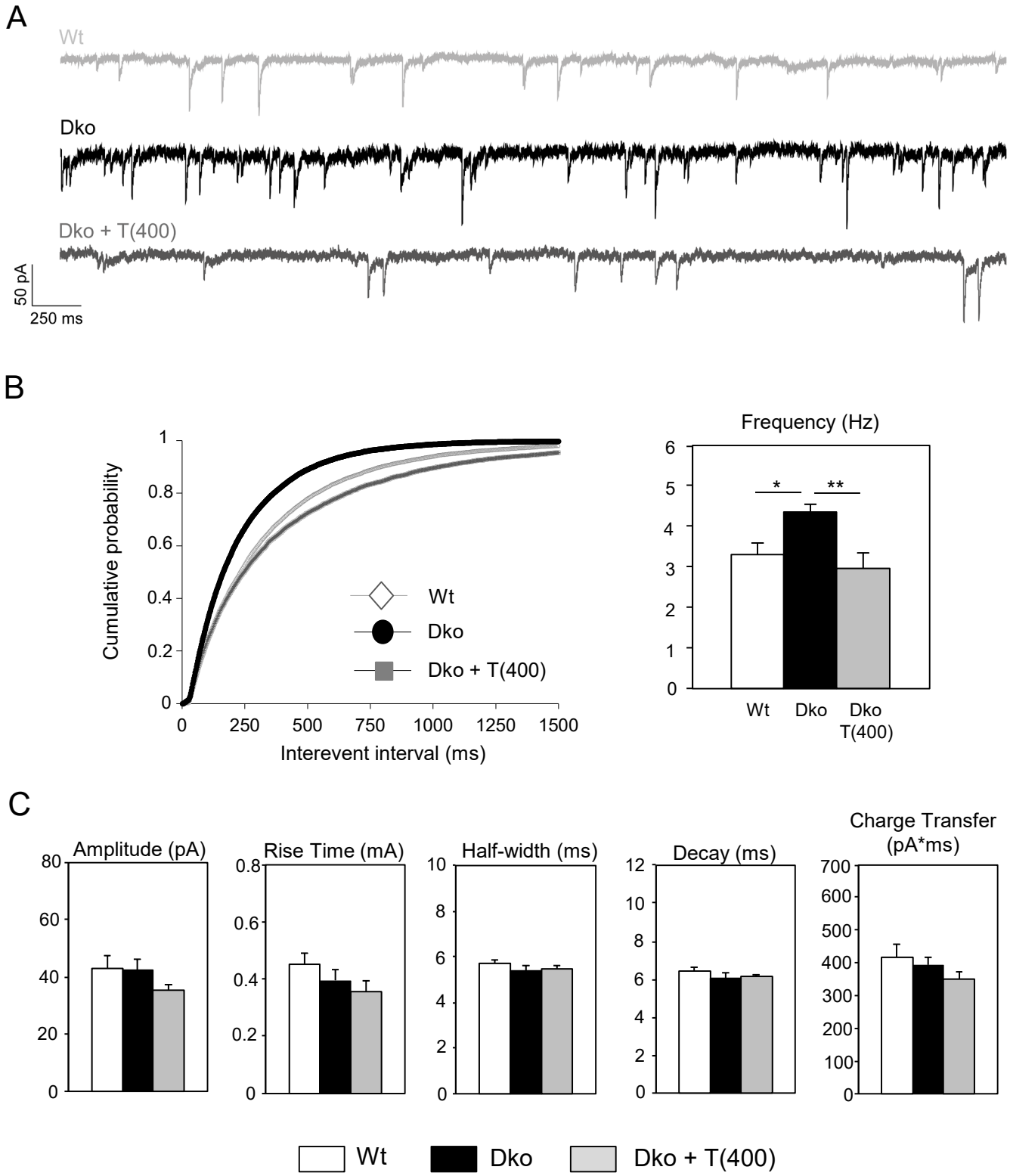

A
Wt
Dko
Dko + T(400)
50 pA
250 ms
B
Cumulative probability
Interevent interval (ms)
Wt
Dko
Dko + T(400)
Frequency (Hz)
*
**
Wt
Dko
Dko T(400)
C
Amplitude (pA)
Rise Time (mA)
Half-width (ms)
Decay (ms)
Charge Transfer (pA*ms)
Wt
Dko
Dko + T(400)

Fig. 5: Deletion of Mct8/Oatp1c1 modulates GABAergic transmission in the cortex

Electrophysiological parameters were recorded on coronal forebrain sections using Patch-clamp technique. A) Representative traces of mIPSCs of Wt, Dko and Dko + T(400) recorded from pyramidal neurons of the somatosensory cortex. B) Cumulative plot and bar chart of mIPSC frequencies demonstrating a significant increase in Dko animals that can be rescued by application of Triac for 3 weeks. C) Amplitude, rise time, half-width, time constant of decay, and transported electric charges are not modified in neurons from Dko animals. n=17-18.

Fig. 6

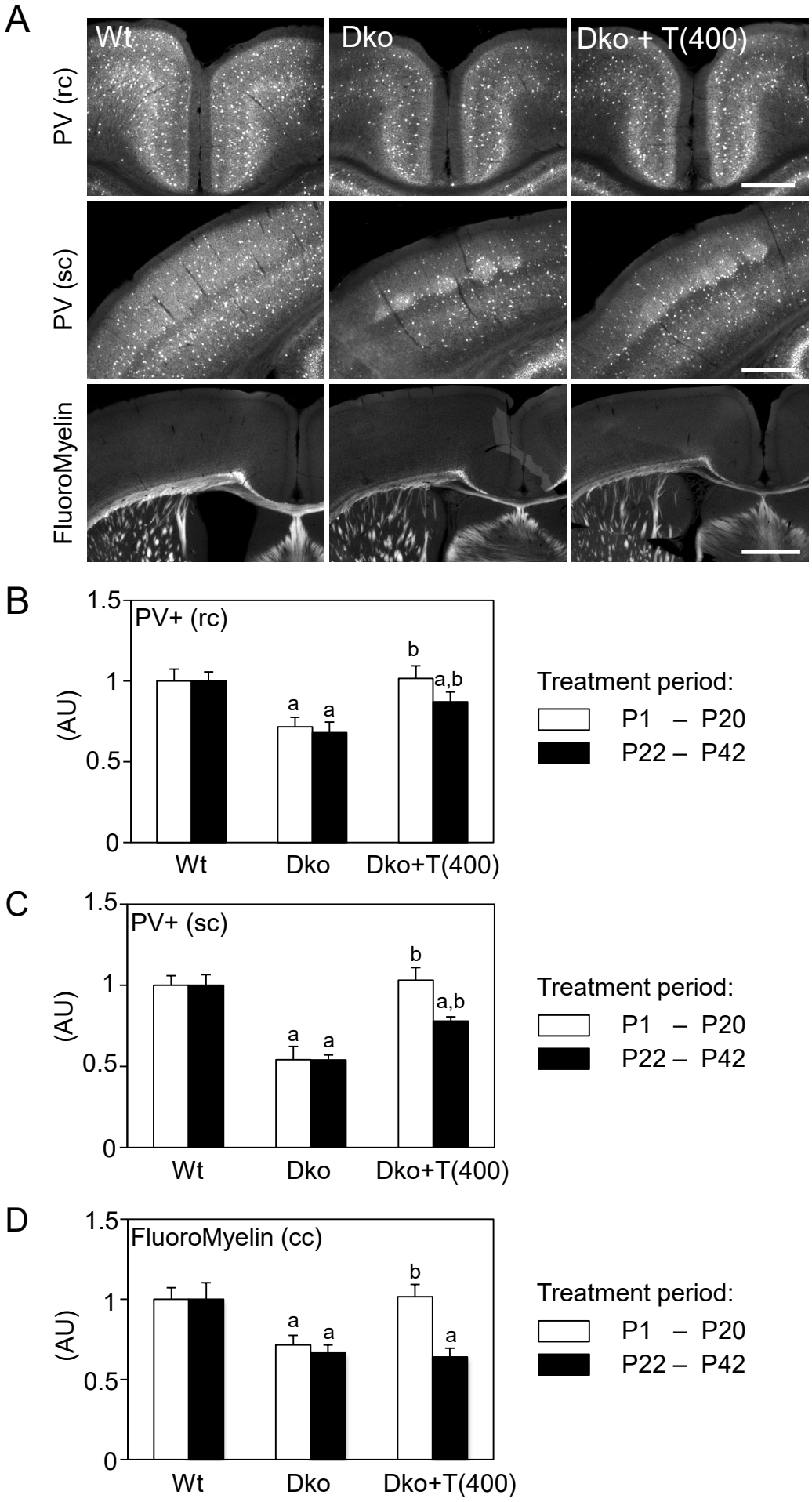
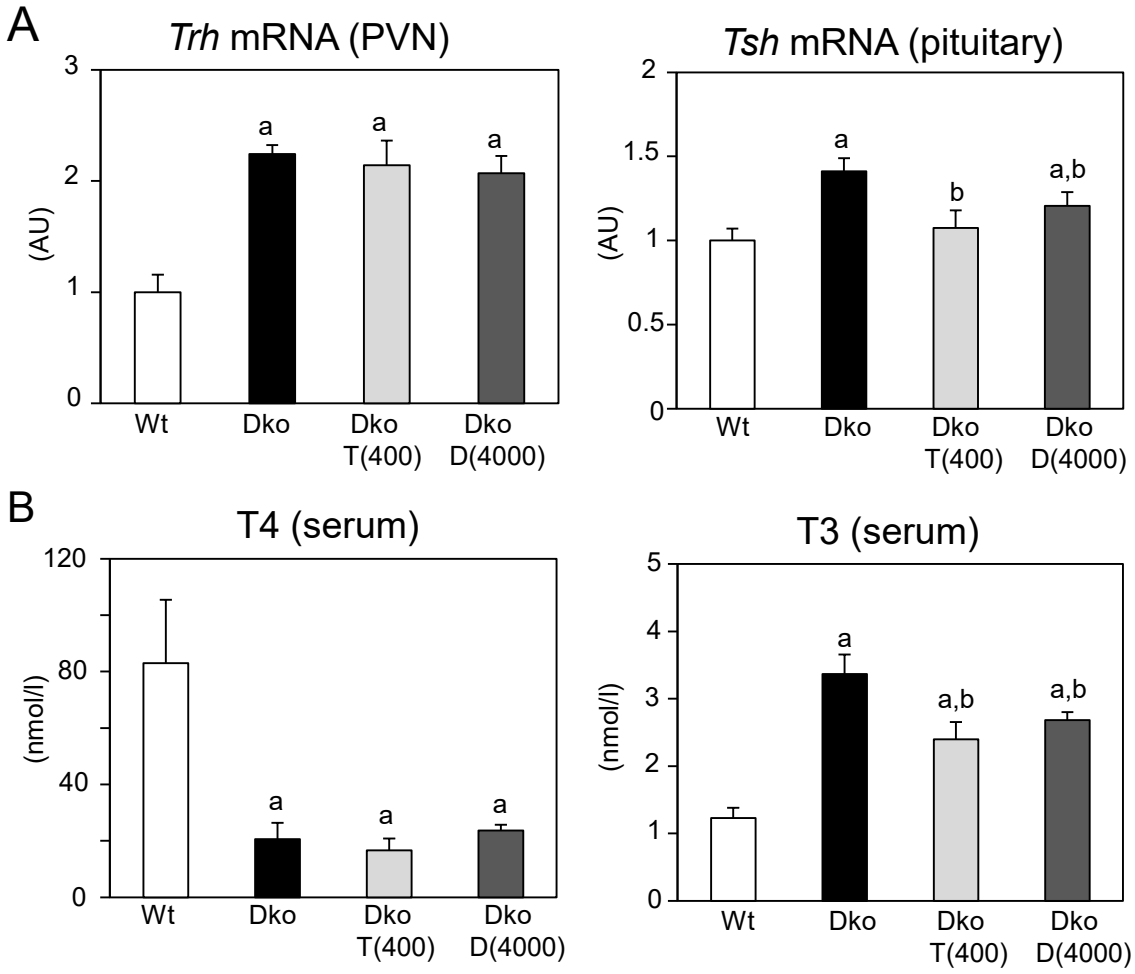

A
Wt
Dko
Dko + T(400)
PV (rc)
PV (sc)
FluoroMyelin
B
PV+ (rc)
(AU)
1.5
1
0.5
0
a
a
b
a,b
Wt
Dko
Dko+T(400)
Treatment period:
P1 – P20
P22 – P42
C
PV+ (sc)
(AU)
1.5
1
0.5
0
a
a
b
a,b
Wt
Dko
Dko+T(400)
Treatment period:
P1 – P20
P22 – P42
D
FluoroMyelin (cc)
(AU)
1.5
1
0.5
0
a
a
b
a
Wt
Dko
Dko+T(400)
Treatment period:
P1 – P20
P22 – P42

Fig. 6: Delayed onset of Triac application compromises efficacy of the treatment.

Dko mice were injected with saline or 400 ng/g bw Triac either between P0 and P20 or P22-P42 and analyzed at P43. A) PV+ interneurons were visualized in the retrosplenial cortex (rc) or somatosensory cortex (sc) and myelination was assessed in the corpus callosum by FluoroMyelin staining. Pictures of animals treated with Triac between P22-P42 are shown. B) PV+ cell numbers were quantified in the rc (B) and sc (C) and were normalized to respective Wt numbers. Low numbers of PV+ neurons in saline-injected Dko mice were fully restored following early onset Triac treatment while upon late onset only a partial recovery was observed. D) FluoroMyelin integrated density was measured in the corpus callosum (cc). Hypomyelination phenotype of Dko mice could only be rescued by Triac application between P0-P20. Late onset of the treatment did not have any effects. n=3-4. Scale bars 250 µm (PV), 500 µm (FluoroMyelin).

Suppl. Fig. 1


A
Trh mRNA (PVN)
(AU)
3
2
1
0
a
a
a
Wt
Dko
Dko T(400)
Dko D(4000)
Tsh mRNA (pituitary)
(AU)
2
1.5
1
0.5
0
a
b
a,b
Wt
Dko
Dko T(400)
Dko D(4000)
B
T4 (serum)
(nmol/l)
120
80
40
0
a
a
a
Wt
Dko
Dko T(400)
Dko D(4000)
T3 (serum)
(nmol/l)
5
4
3
2
1
0
a
a,b
a,b
Wt
Dko
Dko T(400)
Dko D(4000)

Suppl. Fig. 1: Early postnatal TH analog application permanently modulates the set point of the HPT axis.

Activity of the HPT axis was monitored at 10 weeks of age in animals receiving TH analogs Triac or Ditpa daily between P0 and P20 only. A) Radioactive ISH was performed and Trh mRNA expression in the PVN and levels of Tsh beta transcripts in the pituitary were quantified. Following cessation of TH analog treatment, Trh levels returned to abnormally high values as seen in saline-injected Dko mice. In the pituitary, Tsh expression was still decreased in animals that received an early, transient treatment with TH analogs. B) TH serum levels were determined. T4 was equally low in all Dko animals independent of the treatment, whereas T3 concentrations significantly reduced in Triac and Ditpa-treated Dko mice in comparison to saline-injected genotype controls. n=3-5.