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Serotonin transporter deficiency protects mice from mechanical allodynia and heat hyperalgesia in vincristine neuropathy

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ABSTRACT

Painful vincristine (VCR) neuropathy is a frequent and dose-limiting problem in cancer treatment. Here, we investigated how pain behavior is modulated in mice lacking the serotonin transporter (5-HTT−/− mice) after inducing neuropathy by intraperitoneal injections of VCR. We used standard tests for evoked pain, high performance liquid chromatography to measure serotonin (5-HT), and immunohistochemistry of L4/5 dorsal root ganglia (DRG) to assess neuronal injury and inflammation. After injections of VCR, 5-HTT−/− mice did not develop hypersensitivity to heat, in contrast to their wildtype (wt) littermates ($p < 0.05$). Also, 5-HTT−/− mice recovered faster from mechanical hypersensitivity than wt mice ($p < 0.05$). 5-HT levels were lower in the peripheral and central nervous tissue of vehicle or VCR-treated 5-HTT−/− mice compared to wt mice. VCR-treated mice had higher numbers of injured neurons as identified by immunostaining for activating transcription factor 3, and more immunoreactive macrophages in the L4/5 DRG than vehicle-treated mice. There was no difference between genotypes. Thus the 5-HTT−/− genotype did not protect mice from VCR-induced neuronal injury and macrophage infiltration in the DRG. Our results suggest that the reduced peripheral 5-HT levels of 5-HTT−/− mice in VCR neuropathy underlie the lack of heat hyperalgesia. Conversely, attenuation of mechanical allodynia in 5-HTT−/− mice may indicate reduced 5-HT-mediated facilitation in the central nervous system.

Vincristine (VCR) is an anti-cancer drug used to treat many types of cancer, i.e., leukemias, lymphomas and sarcomas. It belongs to the group of Vinca alkaloids; its antineoplastic action is based on the disaggregation of tubulin monomers in mitosis, which inhibits cell division [17]. VCR treatment in rats results in disorganization of the axonal microtubule cytoskeleton, and swelling of unmyelinated axons and large-diameter sensory neurons in the spinal ganglion, suggestive of impaired axonal transport [32,33]. In humans, painful peripheral neuropathy is a frequent and therapy-limiting complication of VCR treatment [12].

There is evidence that the development of pain behavior in mice is modulated by the efficiency of the serotonin transporter (5-HTT) [23,36]. Serotonin (5-HT) plays several roles in pain processing and modulation [6]. In the periphery, 5-HT exerts direct actions on C-fibers [21] after being released from platelets and mast cells [5]. In the rostro-ventral medulla (RVM), 5-HT is released from descending neurons that facilitate spinal nociception [30]. Mice with a 5-HTT deficiency (5-HTT−/− mice) have an overall reduced tissue content of 5-HT [3,15,36]. These mice have attenuated thermal hyperalgesia in the neuropathic pain model of chronic constriction injury (CCI) of the sciatic nerve and in hind paw inflammation induced by complete Freund's adjuvant (CFA) [23,36]. Mechanical hypersensitivity in the mouse CCI model was not attenuated in 5-HTT−/− mice [36], but was reduced by pharmacological depletion of spinal 5-HT in a spinal nerve ligation model in rats [24]. Because of the growing interest in the 5-HTT (SLC6A4) gene in human disorders [22] and the high incidence of chemotherapy-induced neuropathy, we investigated pain behavior in VCR neuropathy in 5-HTT−/− mice. We hypothesized that concomitant with a reduced peripheral 5-HT content, 5-HTT−/− mice might demonstrate attenuated pain behavior compared to wildtype (wt) littermates.

Experiments were performed in 29 adult female mice of C57BL/6J background, 15 wt (5-HTT+/+) and 14 homozygous knockout (5-HTT−/−) littermates. Mean body weight was 35 ± 8 g. The genotype was determined according to Bengel et al. [3]. Mice

Abbreviations: 5-HT, 5-hydroxytryptamine, serotonin; 5-HTT, serotonin transporter; ATF 3, activating transcription factor 3; CNS, central nervous system; DRG, dorsal root ganglia; HPLC, high performance liquid chromatography; ko, knock out; MAC1, mouse macrophages; PNS, peripheral nervous system; sec, seconds; VCR, vincristine; wt, wildtype.

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were housed in groups in a temperature- and humidity-controlled environment with a 12/12 h light/dark cycle and standard rodent nutrition. Experiments were done during the light cycle. All experiments were approved by the Bavarian State authorities and performed in accordance with the European Communities Council Directive of November 1986 (86/609/EEC) for the care and use of laboratory animals. Eight 5-HTT−/− and nine wt mice were treated with VCR i.p. (0.5 mg/kg body weight), and six 5-HTT−/− mice and six wt mice received an equivalent volume of 0.9% saline as placebo treatment. VCR sulfate (Sigma Germany) was dissolved in 0.9% saline to a final concentration of 0.05 mg/ml. VCR was administered for five consecutive days, followed by a two-day pause and subsequent treatment for five more consecutive days. Before the first and sixth injections, the drug dosage was adapted to body weight. The investigator was unaware of the genotype and treatment. Mice were tested on three days to determine baseline thresholds. They were then tested on days 14 and 18 after starting the VCR or saline injections. Both hind paws were tested alternately at an interval of at least 2 min.

Mechanical sensitivity was determined by probing the plantar surface of the hind paw with calibrated von Frey hairs with bending forces from 4 mg to 1.48 g. The 50% mechanical withdrawal threshold (the force of the von Frey hair in grams to which an animal reacts in 50% of the presentations) was determined with hairs applied six times on the basis of the up-and-down method as previously described and modified for mice [28]. The time interval between two trials was at least 1 min on the same paw and at least 30 s on the alternate paw. We refer to a significant decrease in the withdrawal threshold compared to baseline as mechanical allodynia.

The *withdrawal latency to heat stimulation* (*thermal sensitivity*) was tested using a device of Hargreaves et al. [11] purchased from Ugo Basile (Comerio, Italy). The time until the animals reacted by withdrawing from the stimulation by a radiant heat source was determined automatically. Each testing period consisted of at least three presentations for each hind paw. The value of 12 s was assumed if the animal did not react within 12 s, and the stimulation was stopped to prevent tissue damage. A significant decrease in the mean withdrawal latency after injections compared to the control group receiving saline was defined as heat hyperalgesia.

Tissue was collected under deep barbiturate anesthesia on day 19 after the first VCR or saline injection. At first we harvested a 1-cm piece of each sciatic nerve on both sides from the middle of the thigh. The animals were sacrificed by decapitation; the brain was removed and dissected on an ice-cold glass plate. After removal of the hypothalamus, the brain was bisected sagitally to reveal and dissect the hippocampus and thalamus from each hemisphere. Finally the dorsal root ganglia (DRG) L5 were dissected and a section of the spinal cord was cut just proximally to the L4 and distally to the L5 spinal root. Tissue samples for histology were embedded in Tissue Tek® (Sakura Finetek, Zoeterwoude, The Netherlands), frozen in 2-methylbutan cooled in liquid nitrogen, and stored at −80 °C. Tissue for 5-HT analysis was weighed and immediately frozen at −80 °C.

For high performance liquid chromatography (HPLC), samples were thawed, sonicated under argon in ice-cold 150 mM H_3PO_4 and 500 μM diethylene triamine pentaacetic acid and centrifuged at 35,000 × *g* for 20 min at +4 °C. The supernatant was filtered through Milipore Ultrafree-MC filter cups at 9,000 × g, 4 °C, for 1–2 h, and 50 μl of the filtrate was analyzed for 5-HT by reverse-phase HPLC with electrochemical detection as described previously [36].

Cryosections (10 μm) from DRG were mounted on Super Frost Slides from Langenbrinck (Emmerdingen, Germany) and stored at −20 °C. Immunhistochemistry was performed as previously described [26] using primary antibodies to activating transcription factor 3 (ATF 3, 1: 200, rabbit anti-human/rat/mouse, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse macrophages (MAC-1, 1: 50, rat anti-mouse, Camon, Wiesbaden, Germany). Stains were developed with an ABC method (Vector Laboratories, Burlingame, CA, USA) and 0.02% diaminobenzidine as chromogen.

The DRG sections were analyzed using an Axiophot 2 microscope from Zeiss (Oberkochen, Germany), a video camera (DXC 003P) from Sony (Tokyo, Japan), the software SPOT imaging Version 4.5 for Windows XP (SPOT imaging solutions, Burroughs, Sterling Heights, USA) and Image J (Image and Processing Analysing in Java, Wayne R., 1.43r, National Institute of Health, USA, 2010). A counterstain with haemalaun was performed to differentiate anatomical structures. ATF 3 is a sensitive marker of neuronal injury and is activated after diverse noxious chemical stimuli [34]. To determine immunoreactive DRG cells in ATF3 stains, ATF 3 immunoreactive nuclei in DRG neurons were counted and expressed as percent of all neuronal nuclei. To quantify macrophages, the MAC-1 immunoreactive cells were counted in three sections per mouse and expressed as positive cells per DRG section. The total cross-sectional areas of MAC1 and ATF3 sections did not differ between groups (*t*-test: $p > 0.25$).

We used Predictive Analytics Software (PASW Version 18.0; IBM SPSS, New York, 2009) for statistical analysis. Results are presented as mean ± standard error of the mean (SEM). The normal distribution of data was shown by a Kolmogorov–Smirnov test. To compare the behavioral data between groups, test sides and test days, a three-way repeated measures ANOVA was used for parametrical analysis, followed by an LSD post hoc analysis. For nonparametric analysis of the von Frey thresholds, a Mann–Whitney *U* test with a Bonferroni correction for multiple testing was used to compare data between test days. A one-way ANOVA followed by an LSD post hoc analysis was used to compare 5-HT levels between groups. A Student's *t*-test was used to compare ATF3 and MAC-1 immunostaining data between groups. Differences were considered statistically significant if $p < 0.05$.

The withdrawal latencies to thermal and the withdrawal threshold to mechanical stimuli did not differ between the right and left hind paws in all treatment conditions; we therefore pooled the data. Baseline values for withdrawal latencies to thermal stimuli did not differ between wt and 5-HTT−/− mice treated ($n = 8$–10 per genotype). On day 14 after the start of VCR injections wt but not 5-HTT−/− mice revealed heat hypersensitivity with a further reduction in withdrawal latencies on day 18 (LSD post hoc test: baseline vs. day 14, $p = 0.05$, baseline vs. day 18, $p < 0.001$; Fig. 1A).

Baseline withdrawal thresholds to von Frey hairs did not differ between genotypes ($n = 8$–10 per genotype). Withdrawal thresholds decreased significantly in both genotypes on day 14 after the start of VCR injections (Mann–Whitney *U* test: 5-HTT−/− mice and wt mice vs. baseline: $p < 0.05$; Fig. 1B). On day 18 withdrawal thresholds were back to normal in 5-HTT−/− mice (Fig. 1B), whereas wt mice still showed pronounced mechanical hypersensitivity (Mann–Whitney *U* test: VCR-treated wt vs. baseline, $p < 0.05$ vs. baseline, Fig. 1B).

5-HT levels were lower in hippocampus, hypothalamus, thalamus, and L4/5 spinal cord in VCR and saline-treated 5-HTT−/− mice as well as in the sciatic nerve in VCR-treated 5-HTT−/− mice compared to wt mice (LSD post hoc test: $p < 0.05$; Fig. 2A–E). 5-HT levels did not differ between mice with saline and VCR injections within genotypes (Fig. 2A–E).

Quantification of immunohistochemistry revealed a significant increase in ATF 3 positive nuclei in DRG of both wt and 5-HTT−/− mice treated with VCR compared to saline (*t*-test: $p < 0.05$; Fig. 3A). The number of macrophages per DRG section was significantly higher in wt mice treated with VCR than in saline-treated mice (*t*-test: $p < 0.05$). Macrophage numbers did not increase in the 5-HTT−/− mice after VCR (*t*-test: $p = 0.7$; Fig. 3B).

We provide evidence supporting the hypothesis that the 5-HTT−/− genotype protects mice from pain behavior induced by