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Impairment of VGLUT2 but not VGLUT1 signaling reduces neuropathy-induced hypersensitivity

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ABSTRACT

Glutamate is the major excitatory neurotransmitter in the central nervous system with an important role in nociceptive processing. Storage of glutamate into vesicles is controlled by vesicular glutamate transporters (VGLUT). Null mutants for VGLUT1 and VGLUT2 were poorly viable, thus, pain-related behavior was presently compared between heterozygote VGLUT1 and VGLUT2 mice and their respective wild-type littermates using a test battery that included a variety of assays for thermal and mechanical acute nociception, and inflammatory and neuropathic pain syndromes. Behavioral analysis of VGLUT1 mutant mice did not show important behavioral changes in the pain conditions tested. Reduction of VGLUT2 also resulted in unaltered acute nociceptive and inflammatory-induced pain behavior. Interestingly, VGLUT2 heterozygote mice showed an attenuation or absence of some typical neuropathic pain features (e.g., absence of mechanical and cold allodynia after spared nerve injury). Chronic constriction injury in VGLUT2 heterozygote mice showed also reduced levels of cold allodynia, but had no impact on mechanical thresholds. Together, these data suggest that VGLUT2, but not VGLUT1, plays a role in neuropathy-induced allodynia and hypersensitivity, and might be a therapeutic target to prevent and/or treat neuropathic pain.

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1. Introduction

L-Glutamate (Glu), the major excitatory neurotransmitter in the mammalian brain and spinal cord, plays a pivotal role in both acute and chronic pain (Karim et al., 2001; Minami et al., 2001; Sung et al., 2003; Binns et al., 2005; Liaw et al., 2005; Tao et al., 2005; Moechars et al., 2006). Unfortunately, the neuroanatomical and functional complexity of the glutamatergic system has complicated the development of therapeutic strategies that target specific elements of the system without affecting others (Fundytus, 2001; Neugebauer, 2002). Storage of glutamate in synaptic vesicles, which serve as the neuronal pool for Glu release, is controlled by vesicular Glu transporters (VGLUT). Three distinct VGLUT isoforms have been identified with VGLUT1 and VGLUT2 accounting for most of the presumed excitatory glutamatergic terminals in the central nervous system, whereas VGLUT 3 is diffusely distributed in the brain, defining a discrete subpopulation of nonglutamatergic neurons (Aihara et al., 2000; Bellocchio et al., 2000; Bai et al., 2001; Fremeau et al., 2001; Herzog et al., 2001; Takamori et al., 2001;

Schafer et al., 2002; Gras et al., 2002; Boulland et al., 2004). Although expression of VGLUT1 and 2 overlap in some regions, one transcript always clearly predominates (Fremeau et al., 2001; Herzog et al., 2001; Sakata-Haga et al., 2001; Kaneko and Fujiyama, 2002). The transporter isoforms also display different temporal expression profile as a developmental switch from VGLUT2 to VGLUT1 occurs in some brain regions. In hippocampus, cortex and cerebellum, VGLUT2 is transiently expressed during early development, whereas in adulthood, VGLUT1 takes over and VGLUT2 is only expressed at very low levels in those regions (Fremeau, Jr. et al., 2004; Wojcik et al., 2004). In adults, VGLUT1 is mainly expressed in cerebral cortex, cerebellum and hippocampus, whereas VGLUT2 is most abundant in diencephalon (thalamus, hypothalamus), brainstem and spinal cord. Their differential spatial and complementary expression profiles suggest that individual VGLUTs are associated with distinct functional classes of synapses.

In the present study, we examined the putatively differential function of VGLUT1 and VGLUT2 in pain signaling using genetically altered mice, and the possibility to target these transporters for pain therapy without the adverse effects that have confounded many other manipulations of the Glu system. VGLUT1^{-/-} mice die about 3 weeks after birth, while VGLUT2^{-/-} mice die at or immediately after birth, which is in accordance with the respective developmental expression patterns of VGLUT1 and 2 (Fremeau,

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Jr. et al., 2004; Wojcik et al., 2004; Moechars et al., 2006). Conversely, heterozygous mice survive and were used in the present study for behavioral characterization. A comprehensive and multifaceted test battery was used to examine various clinically relevant pain responses in mice (Leo et al., 2008). The results of VGLUT2 mice were re-inserted (Moechars et al., 2006), and extended to include a second neuropathic pain model (chronic constriction injury). Additionally, the writhing test was added as a visceral pain model. This extended test battery (assays for acute nociception, motor coordination, inflammatory and neuropathic pain) was also applied to VGLUT1 mice to examine the somatosensory system and compare the functions of these isoforms in pain behavior.

2. Material and methods

2.1. Mice

VGLUT1 and VGLUT2 knockout mice were developed in collaboration with Lexicon Genetics (Woodlands, TX, USA) as described previously (Moechars et al., 2006). Clones were isolated by screening of the 129SvEvBrd derived lambda pKOS genomic library (Wattler et al., 1999). For VGLUT1, a 7.4 kb genomic clone spanning the first two coding exons and for VGLUT2, a 9.8 kb genomic clone spanning the first three coding exons was used to generate the targeting vector. An IRESLacZ/MC1neo selection cassette was inserted as a SfiI fragment to replace a 72 bp VGLUT1 genomic fragment starting with the ATG startcodon in exon 1 after yeast-mediated homologous recombination. To replace the 287 bp VGLUT2 genomic fragment spanning exon 2, an IRESBgeo/PGKpuro selection cassette was inserted as a SfiI fragment. The NotI-linearized vector was electroporated into 129Sv/Evbrd (LEX1) embryonic stem (ES) cells and neomycine-resistant (VGLUT1) or puromycine-resistant (VGLUT2) ES cell clones were isolated and analyzed for homologous recombination by Southern blot analysis. Targeted ES cell clones were injected into C57BL/6(albino) blastocysts, and the resulting chimeras were mated to C57BL/6(albino) females to generate animals heterozygous for the mutation. These were subsequently crossed to generate all three genotypes. Heterozygous animals and their wild-type littermates with a mixed 129Sv/EvBrd x C57BL6/J genetic background were used in all experiments. Mice were housed with a 12 h dark-light cycle (light on 6:00 AM) with free access to food and water, except during some of the behavioral testing. All experiments were carried out in accordance with the European Communities Council Directives (86/609/EEC) and were approved by the local ethical committee.

2.2. Hot plate test

Mice were placed on a metal surface maintained at $50.0 \pm 0.3^\circ\text{C}$, $52.0 \pm 0.3^\circ\text{C}$, $54.0 \pm 0.3^\circ\text{C}$ and $56.0 \pm 0.3^\circ\text{C}$. The temperature of the plate was monitored continuously. Locomotion of the mouse on the plate was constrained by a colorless acrylic cylinder ($\varnothing 20$ cm). To remove traces of urine and feces, the plate was wiped with a cloth. Mice were placed on the hot plate, one at a time and the response latency to the heat stimulus was measured. To prevent tissue damage, a cut-off time of 30 s was used. Mice remained on the plate until they performed one of the behaviors regarded as indicative of nociception: hind paw lick, hind paw shake/flutter and hind paw lift, or until the cut-off time had elapsed. Only hind paw responses were used, since fore paw licking and lifting can be components of normal grooming behavior. Mice were tested successively at four different temperatures in increasing order (50°C , 52°C , 54°C , 56°C) with 2 trials for each temperature (≥ 15 min in between), and a 30-min interval between the different temperatures. The mean value of the response latencies of

the two test trials per temperature was taken for statistical analysis.

2.3. Tail withdrawal test

Mice were slightly restrained in a cloth 'pocket' into which they entered voluntarily. The distal half of the tail was then dipped into a bath of warm water, which was thermostatically controlled. Latency to respond to the heat stimulus with vigorous flexion of the tail was measured using a manual stopwatch. Animals were removed immediately after responding and the tail was wiped off with a cloth. A cut-off time of 25 s was adopted to avoid tissue damage. Mice were tested successively at four increasing temperatures (47°C , 49°C , 51°C , 53°C) with 2 trials for each temperature (≥ 15 min in between), and a 30-min interval between the different temperatures. The mean value of the response latencies of the two test trials per temperature was taken for statistical analysis.

2.4. Rotarod

Motor coordination was tested on an accelerating rotarod (Med Associates Inc., St. Albans, VT, USA). The rod is 3 cm in diameter with a ribbed surface to provide suitable grip, and is suspended 12.5 cm above the timer trip plates. It is divided into five test zones such that up to five mice may be tested at the same time. The heads of the animals were directed against the direction of rotation such that the animals had to progress forward to remain on the rod. Mice were first trained on the morning prior to the testing afternoon. Training sessions consisted of 2 parts: the first part, a 3-min trial at gradually increasing speed (2–20 rpm), and the second part, a 1-min trial at constant speed (16 rpm). During these training sessions, mice were replaced on the rod every time they fell off. During the two test trials (intertrial interval, 1 h), mice had to balance on the rod while it accelerated from 4 to 40 rpm in 5 min (300 s). Latency to fall-off the rod was automatically recorded for each mouse. The mean value of the fall-off latencies of the two test trials was taken for statistical analysis.

2.5. von Frey test

To assess mechanical sensitivity, mice were placed individual in wire-mesh floor cages (10×10 cm) to allow insertion of the mechanical probe from below against the plantar surface of the hind paw. The animals were allowed to habituate to the testing chambers for at least 1 h before the first measurement. By using a mechanical force transducer (SENSELab 4 CHAMP, Somedic Sales, Hörby, Sweden) with a metal probe (SENSELab Forcetransducer P/N 739 S/N 110, Somedic Sales, Hörby, Sweden), the mean force required to evoke a withdrawal response was calculated for each animal from three measurements on both hind paws. The individual measurements were taken successively beginning with the right hind paw.

2.6. Writhing test

In this assay of chemical nociception, a noxious substance (acetic acid) is injected into the peritoneal cavity, wherein it activates nociceptors directly and/or produces inflammation of visceral (subdiaphragmatic) and subcutaneous (muscle wall) tissues. This will produce the characteristic 'writhing response': lengthwise stretches of the torso with a concomitant concave arching of the back and extensions of the hind limbs. A 1% acetic acid solution (0.25% Trypan Blue, distilled water) was administered intraperitoneally (ip) in a volume of 0.1 ml/10 g body weight. Prior to injection, mice were habituated for at least 1 h to individual rubber-floored Plexiglas observation cages with a mirror to facilitate observation.

Mice were also habituated to the ip injection by injecting them daily with saline during 3 days before the actual testing day.

The number of abdominal constrictions or writhes was noted from minute 5 to minute 20 after acetic acid injection. One writh was considered to have occurred with the adoption of the typical posture and to have terminated upon resumption of a normal posture. After testing, the site of injection was examined for the presence of blue coloration in the peritoneum. Incorrectly injected animals were excluded.

2.7. Carrageenan test

Injection of λ carrageenan, a polysaccharide obtained from seaweed extract, causes inflammation and hypersensitivity with a peak effect at 3–5 h post-injection. Peripheral inflammation was produced in the mice by a 25 μ l subcutaneous (sc) injection of a 2% λ carrageenan solution (dissolved in saline) into the subplantar side of the left hind paw using a syringe with a 30-gauge needle. Control animals received an injection of saline (25 μ l). Mice were tested for thermal sensitivity on the heat test of Hargreaves, where the IITC Model Plantar Analgesia Meter (IITC Inc. Life Science, Woodland Hills, CA, USA) was used for paw stimulation. The radiant heat source consisted of a high-intensity projector bulb located 50 mm below the glass floor. The test head emits focused, radiant heat, and is placed under a tempered glass surface. The light beam is focused to the top of the glass and creates an intense heat spot (4 $\times$ 6 mm). Before the actual measurement starts, a guide light assists in directing the beam to the precise spot. An adjustable angled mirror is mounted on top of the test head to help the operator viewing the hind paw of the mouse. Prior to testing, mice were acclimatized for at least 30 min in clear plastic testing chambers ($\varnothing$ 14 cm) on a glass floor. Thermal pain thresholds of the hind paws were determined by applying radiant heat to the plantar surface of the hind paws from underneath (Hargreaves et al., 1988). The radiant heat produced a continuous rise in temperature of the paw until the pain threshold was reached, and the animal withdrew its paw. A cut-off time of 20 s was adopted. Prior to injection, baseline latencies were measured twice with an interval of 15 min. Post-injection latencies were measured after 3, 4, 5, 6, 24 and 48 h.

2.8. Formalin test

To assess the paw flinch behavior following injection of formalin, an automated nociception analyzer (University of California, San Diego, CA), specially designed for mice, was used (Rosland et al., 1990). A soft metal band ("C" shaped) was placed on the hind paw with a drop of cyanoacrylate glue. Banded mice were habituated for 2 h in plastic cylindrical testing chambers ($\varnothing$ 8 cm). Afterwards, mice were injected with 25 μ l formalin solution (1%) in the subplantar surface of the banded paw, and placed again into a testing chamber that was mounted on top of a transmitter-receiver coil assembly. During the 1-h registration period, movements of the metal band (in the electric field generated by the transmitter coil) were detected by a loop antenna located under the chamber, and flinch activity was algorithmically calculated. The software sampled flinch counts/minute, thus allowing calculation of total number of flinches during selected intervals (Phase I: 0–9 min; Phase II: 10–60 min; Phase IIa: 10–40 min; Phase IIb: 41–60 min) by accumulating each animal's flinches during that time period.

2.9. Neuropathic pain models

To be able to evaluate pain-related behavior after nerve injury, two different neuropathic pain models were used: spared nerve injury and chronic constriction injury. Surgical procedures were per-

formed under gas anesthesia with a mixture of isoflurane (5% for induction and 3% for maintenance), oxygen and N₂O. After skin and muscle incision of the left hind leg, the sciatic nerve and its branches (sural, common peroneal and tibial nerve) were exposed. The muscle and skin layer were closed separately by using 6–0 Vicryl® suture (Ethicon Inc., Somerville, NJ) and 6–0 Mersilk™ suture (Ethicon Inc., Somerville, NJ), respectively. Sham mice were made by following the same surgical procedure without touching any nerve.

2.9.1. Surgery: spared (tibial) nerve injury (SNI) as a model of nerve injury

After exposing the sciatic nerve and his branches, 2 of the three branches (common peroneal and sural nerve) were tightly ligated with 9–0 virgin silk suture (Ethicon Inc., Somerville, NJ). The ligated branches were then transected distal to the ligation and a 2–4 mm section of the nerve was removed to prevent rejoining. Caution was taken to minimize any contact with the spared nerve (tibial nerve) (Shields et al., 2003).

2.9.2. Surgery: chronic constriction injury (CCI) as a model of nerve injury

The procedure originally proposed by Bennett and Xie was adapted to the mouse (Bennett and Xie, 1988). After exposing the sciatic nerve, 3 ligatures constricting the nerve diameter by approximately 1/3 to 1/2 with chromic catgut (6–0, Ethicon Inc., Somerville, NJ) were placed around the nerve, proximal to the trifurcation. A sham operation was performed by exposing, but not ligating, the sciatic nerve.

2.9.3. Behavioral tests

Prior to surgery, baseline thresholds were recorded in the automated von Frey system, acetone test and balance box. Responses to these mechanical and cold stimuli as well as weight bearing were evaluated 2, 6, 9, 14 and 21 days after injury.

The automated von Frey test (see Section 2.5) was used to evaluate the changed mechanical thresholds after surgery. The acetone test was used to determine the reactivity to a cold, chemical and possibly mechanical stimulus. The mice were caged and acclimatized similar to the von Frey test. From below the grid, 50 μ l of acetone was sprayed onto the plantar surface of the left hind paw with an Eppendorf® multistep pipette. The time (in s) that the mouse spent licking, flinching and lifting its paw during 1 min following acetone application was recorded. The hind paw weight distribution (HPWD) was used as an index of discomfort. The mouse is placed in the balance box (BB SENSELab Platform S/N 011, Somedic, Hörby, Sweden), and positioned with the two hind limbs on the metal plates. The force exerted by each limb (in grams) is averaged over a period of 5 s, thus assessing the tendency of the mouse to shift its weight from one side to the other. For each mouse, three measurements were averaged, which were done successively by repositioning the mouse in the box (straight, looking forward, resting on its four feet). Changes in HPWD between left (operated) and right (contralateral) side were used as an index of neuropathic pain in the spared nerve injury model.

2.10. Statistical analysis

Data are presented as means $\pm$ SEM. Differences between mean values were determined using *t*-test or 2-way repeated measures analysis of variance (RM-ANOVA) procedures with Tukey tests for post hoc comparison. When appropriate, Mann Whitney Rank Sum test was used to compare two groups. All statistical tests were performed at the α level of significance of 0.05.

3. Results

3.1. Baseline thermal nociception

Two different assays were performed to assess acute thermal sensitivity: tail withdrawal (TWR; Fig. 1A) and hot plate (HP; Fig. 1B). Both tests were performed at multiple stimulus intensities ranging from 47 °C to 53 °C for the TWR test and ranging from 50 °C to 56 °C for the HP test. A clear and stable temperature effect was seen, i.e., at higher temperatures, response latency reduced (TWR_{VGLUT1}: $F_{3,76} = 297.05$, $p < 0.001$; HP_{VGLUT1}: $F_{3,76} = 177.84$, $p < 0.001$; TWR_{VGLUT2}: $F_{3,92} = 55.27$, $p < 0.001$; HP_{VGLUT2}: $F_{3,92} = 246.97$, $p < 0.001$).

VGLUT1^{+/+} mice withdrew their tail later compared to their VGLUT1^{+/-} littermates ($F_{1,78} = 11.84$, $p = 0.003$). However, the differences reached only the significance level at 49 °C ($p < 0.001$) and 53 °C ($p < 0.05$). Within the VGLUT2 group, no changed TWR behavior between genotypes was observed ($F_{1,94} = 0.002$, $p = \text{NS}$).

Across VGLUT1 and VGLUT2 groups, there seemed to be a tendency of VGLUT1 mice being less sensitive compared to VGLUT2 mice. Although no overall genotype effect was shown ($F_{3,172} = 1.67$, $p = \text{NS}$), the genotype $\times$ temperature effect was quite obvious ($F_{9,166} = 4.66$, $p < 0.001$), indicating that the lowest temperature (47 °C) differentiated between the two groups.

In the hot plate test, the response latencies of both VGLUT1^{+/+} and VGLUT2^{+/+} mice were not significantly different than those of their respective wild-type littermates at either stimulus intensity (VGLUT1: $F_{1,78} = 2.56$, $p = \text{NS}$; VGLUT2: $F_{1,94} = 2.16$, $p = \text{NS}$).

3.2. Baseline mechanical nociception

The mechanical thresholds in the automated von Frey test of wild-type and heterozygote mice within groups ($n = 12$) were comparable and did not show any differences (left side: VGLUT1^{+/+} = 6.24 ± 0.24 g; VGLUT1^{+/-} = 6.68 ± 0.36 g; VGLUT2^{+/+} = 8.08 ± 0.29 g; VGLUT2^{+/-} = 8.19 ± 0.44 g; right side: VGLUT1^{+/+} = 6.35 ± 0.39 g; VGLUT1^{+/-} = 6.94 ± 0.35 g; VGLUT2^{+/+} = 8.57 ± 0.22 g; VGLUT2^{+/-} = 8.81 ± 0.48 g. Additionally, no sidedness effect (left paw versus right paw) was observed.

3.3. Motor coordination

To assess motor coordination, mice ($n = 10$ – 12) were tested on an accelerating rotarod after being trained. No significant differences were seen within groups: VGLUT1^{+/+} mice stayed on the rod for 114 ± 19 s, VGLUT1^{+/-} mice for 98 ± 16 s, VGLUT2^{+/+} mice for 128 ± 17 s and VGLUT2^{+/-} mice for 129 ± 8 s.

3.4. Inflammatory pain conditions

In the carrageenan test, the response latency to thermal stimulation (Hargreaves' test) of naïve mice (i.e., pre-injection scores) was evaluated first. No significant genotype effect was found neither in VGLUT1 nor in VGLUT2 mice. The mean pre-injection baseline response latencies were 10.83 ± 1.02 s (VGLUT1^{+/+}), 10.47 ± 1.22 s (VGLUT1^{+/-}), 11.80 ± 1.09 s (VGLUT2^{+/+}) and 11.72 ± 1.14 s (VGLUT2^{+/-}), respectively.

To evaluate the effect of carrageenan-induced inflammation on the thermal sensitivity of the mice (CAR_{HT}), results were expressed as percent of baseline. Each animal was normalized to the average pre-injection response latency value of its group. The thermal sensitivity of the mice was followed between 3 and 48 h after carrageenan or NaCl challenge (Fig. 2). Subplantar injection of carrageenan-induced in all mice a significant decrease of paw withdrawal latency, whereas an injection of NaCl did not

(VGLUT1^{+/+} : $F_{1,82} = 14.46$, $p = 0.003$; VGLUT1^{+/-} : $F_{1,82} = 70.85$, $p < 0.001$; VGLUT2^{+/+} : $F_{1,82} = 43.96$, $p < 0.001$; VGLUT2^{+/-} : $F_{1,82} = 13.21$, $p = 0.005$). However, in both groups there was no significant difference between genotypes. VGLUT1^{+/+} and VGLUT2^{+/+} mice showed a comparable behavioral pattern to their respective wild-

Fig. 1. Differences in thermal nociception between VGLUT1^{+/+} and VGLUT2^{+/+} mice and their wild-type littermates, respectively. (A) In the tail withdrawal test, VGLUT1^{+/+} mice withdrew their tail later than VGLUT1^{+/-} mice, although the significance level was only reached at 49 °C and 53 °C. VGLUT2^{+/+} mice behaved similarly to their wild-type littermates. (B) In the hot plate test, there was no effect of genotype in VGLUT1 nor in VGLUT2 mice. Bars represent mean response latencies ($\pm$ SEM, $n = 10$ – 12) following thermal stimulation. Symbol * indicates significant differences between heterozygote mice and wild-type littermates, respectively (* $p < 0.05$; ** $p < 0.001$).

Fig. 2. Differences in pain response after carrageenan-induced inflammation between VGLUT1^{+/-} and VGLUT2^{+/-} mice and their wild-type littermates, respectively. VGLUT1^{+/-}, VGLUT2^{+/-} and wild-type mice developed profound thermal hyperalgesia after carrageenan-injection, as assessed by the Hargreaves' test. Data are presented as means ($\pm$ SEM, $n = 6$). Symbol * indicate significant differences between carrageenan- and NaCl-injected mice in wild-types ($p < 0.05$, ** $p < 0.001$). Symbol \$ indicates significant differences between carrageenan- and NaCl-injected mice in heterozygotes ($^{\$}p < 0.05$, $^{\$ \$}p < 0.001$).

type littermates after being challenged with carrageenan. Across groups (VGLUT1 vs. VGLUT2), some differentiation in development of the carrageenan-induced thermal hypersensitivity was apparent. After 3 h, VGLUT1 mice (VGLUT1^{+/-} and ^{+/-}) showed a mild decrease in response latency, whereas VGLUT2 mice (VGLUT2^{+/-} and ^{+/-}) already reached at that time their maximal level of thermal hyperalgesia.

A second model to examine the possible contribution of VGLUT1 and VGLUT2 to inflammatory pain is the formalin test (Fig. 3). After subplantar injection of formalin, flinch behavior of the mice was evaluated. The flinching of the formalin-injected paw was typically concentrated in two distinct phases. At first, a very active response with peak flinch frequency of 60 flinches/min is seen, which decreases to 20–35 flinches/min around minute 10. Further, the flinch behavior becomes more active again until it extinguishes after one hour (flinch rate of ± 10 flinches/min). These data points were organized and divided into different phases:

Phase I is the summation of the number of flinches from minute 0–9, Phase II from minute 10–60, Phase IIa from minute 10–40 and Phase IIb from minute 41–60. When comparing VGLUT^{+/-} and VGLUT^{+/-} mice in each group, respectively, no change in behavior was observed in the different phases. However, reduced flinching response was observed in VGLUT2 mice compared to VGLUT1 mice in Phase II, more particularly in Phase IIa (genotype effect: $F_{3,190} = 2.34$, $p = \text{NS}$; genotype $\times$ phase effect: $F_{9,186} = 2.48$, $p < 0.05$).

In the visceral pain model ($n = 9–15$), VGLUT1^{+/-} mice behaved similarly as their wild-type littermates within the 15-min observation period after 1% acetic acid ip injection (10.7 ± 2.9 and 12.9 ± 2.7 writhes, respectively; $t = -0.52$, $p = \text{NS}$). VGLUT2^{+/-} mice produced 6.7 ± 1.9 writhes, whereas their wild-type littermates produced 10.8 ± 2.2 writhes ($t = -1.66$, $p = \text{NS}$).

3.5. Neuropathic pain conditions

To gain insight into the role of VGLUTs in neuropathy-related behaviors, two different neuropathic pain models were evaluated: the spared (tibial) nerve injury model (SNI; Fig. 4) and the chronic constriction injury model (CCI; Fig. 5). Three different tests were performed to measure the degree of neuropathy-related sensory dysfunction: mechanical allodynia via the von Frey test, cold allodynia via the acetone test and spontaneous discomfort (weight bearing), as represented by the hind paw weight distribution, in the balance box test.

In the SNI model (Shields et al., 2003), no differences in behavior were observed for VGLUT1, including the von Frey, the acetone, and the balance box test. A significant decrease in mechanical threshold was observed in both VGLUT1^{+/-} ($F_{1,70} = 40.49$, $p < 0.001$) and VGLUT1^{+/-} ($F_{1,70} = 50.76$, $p < 0.001$) SNI-operated mice from day 2 until day 21 post surgery compared to their sham-operated littermates (Fig. 4A, left panel). In the acetone test, VGLUT1^{+/-} mice displayed a mild but significant cold allodynia like behavior after injury ($F_{1,70} = 17.06$, $p = 0.002$), whereas VGLUT1^{+/-} mice did not ($F_{1,70} = 2.75$, $p = \text{NS}$; (Fig. 4)B, left panel). However, post hoc analysis revealed that VGLUT1^{+/-} SNI mice behaved only significantly different from VGLUT1^{+/-}

Fig. 3. Differences in pain response after formalin-induced inflammation between VGLUT1^{+/-} and VGLUT2^{+/-} mice and their wild-type littermates, respectively. After formalin challenge, VGLUT1^{+/-} and VGLUT2^{+/-} mice displayed a similar flinching behavior compared to their wild-type littermates. Data are presented as means ($\pm$ SEM, $n = 11–14$).

sham mice at two different time points: day 2 and day 21 post injury. When observing the weight bearing of the operated animals, again a significant condition effect was seen (VGLUT1^{+/+}: $F_{1,70} = 20.06$, $p < 0.001$; VGLUT1^{+/-}: $F_{1,70} = 61.94$, $p < 0.001$;

(Fig. 4)C, left panel): SNI-operated animals exhibited a clear shift of the weight to the right uninjured side, with a peak effect 2 days post surgery. Sham-operated animals did not show any significant change over time.

Fig. 4. Differences in neuropathic pain behavior in VGLUT1^{+/+} and VGLUT2^{+/+} mice and their wild-type littermates, respectively, after spared (tibial) nerve injury. (A) SNI induced a robust mechanical allodynia in VGLUT1^{+/+}, VGLUT1^{+/-} and VGLUT2^{+/+} mice at all time points post SNI. In contrast, VGLUT2^{+/-} mice did not show altered von Frey thresholds after injury. (B) After SNI, a genotype effect was present in VGLUT2 mice: VGLUT2^{+/+} mice showed significant increased lifting responses following acetone exposure; VGLUT2^{+/-} mice failed to develop a clear cold allodynia-like response after injury. (C) No significant genotype effects were found in the weight distribution after SNI. All genotypes showed a clear weight shift to the right non-injured side, with a peak effect on day 2 post injury. Data are presented as means ($\pm$ SEM, $n = 6-12$). Symbol * indicates significant differences between SNI- and sham-operated mice in wild-types (* $p < 0.05$, ** $p < 0.001$). Symbol \$ indicates significant differences between SNI- and sham-operated mice in heterozygotes (\$ $p < 0.05$, \$\$ $p < 0.001$). Symbol # indicates significant differences between VGLUT2^{+/+} SNI and VGLUT2^{+/-} SNI mice (# $p < 0.05$, ## $p < 0.001$).

VGLUT2 mice display differences between genotypes in SNI-induced sensitivity towards mechanical (VF) and cold (acetone) stimuli, but not in hind paw weight distribution. The SNI did in-

duce mechanical allodynia in VGLUT2^{+/+} mice, but not in VGLUT2^{+/-}. The mechanical thresholds of VGLUT2^{+/-} mice decreased on average 15% after surgery, similar to sham-operated

Fig. 5. Differences in neuropathic pain behavior in VGLUT1^{+/-} and VGLUT2^{+/-} mice and their wild-type littermates, respectively, after chronic constriction injury. (A) CCI induced a robust mechanical allodynia in all genotypes, whereas sham operation did not. (B) The response time to the acetone spray after CCI was significantly increased in VGLUT1^{+/-}, VGLUT1^{+/+}, VGLUT2^{+/-} and VGLUT2^{+/+} mice compared to sham-operated mice. However, VGLUT2^{+/-} mice developed cold allodynia to a lesser extent than VGLUT2^{+/+} mice after injury. (C) No significant genotype effects were found in the weight distribution after CCI. All genotypes showed a clear weight shift to the right non-injured side. Data are presented as means ($\pm$ SEM, $n = 6-15$). Symbol * indicates significant differences between CCI- and sham-operated mice in wild-types ($p < 0.05$, $**p < 0.001$). Symbol \$ indicates significant differences between CCI- and sham-operated mice in heterozygotes ($*p < 0.05$, $ssp < 0.001$). Symbol # indicates significant differences between VGLUT2^{+/-} CCI and VGLUT2^{+/+} CCI mice ($*p < 0.05$).

mice ($F_{1,142} = 0.669$, $p = \text{NS}$; (Fig. 4)A, right panel). In contrast, VGLUT2^{+/-} mice that underwent the spared nerve injury showed robust mechanical allodynia, as represented by the $\pm 50\%$ decrease in thresholds ($F_{1,142} = 40.66$, $p < 0.001$). Also in the response to the acetone spray, VGLUT2^{+/-} mice ($F_{1,142} = 3.38$, $p = \text{NS}$) failed in contrast to their wild-type littermates ($F_{1,142} = 24.85$, $p < 0.001$) to develop a clear and robust cold allodynia-like response (Fig. 4B, right panel). Although significant differences between VGLUT2^{+/-} and their wild-type littermates were apparent in these mechanical and thermal (cold) responses related to neuropathic pain syndromes, no differences between genotypes were seen in the weight distribution. Both genotypes showed a clear weight shift to the right non-injured side, with a peak effect on day 2 post injury.

Additionally, the extent of mechanical and cold allodynia as well as the weight bearing produced by chronic constriction injury (CCI), a second neuropathic pain model, was quantified (Fig. 5). In contrast to SNI, no important differences in the pain-related behavior across genotypes (VGLUT1^{+/+} vs. VGLUT1^{+/-} and VGLUT2^{+/+} vs. VGLUT2^{+/-}) were found, with the exception of the response to the acetone spray in VGLUT2 mice. Both CCI-operated genotypes (VGLUT2^{+/+} and VGLUT2^{+/-}) showed an increased sensitivity to acetone after injury (Fig. 5B, right panel). However, VGLUT2^{+/-} mice developed the cold allodynia to a lesser extent compared to their wild-type littermates (genotype effect: $F_{1,178} = 12.45$, $p < 0.001$), representing reduced signs of neuropathy-induced cold sensitivity. The von Frey (Fig. 5A, right panel) and balance box test (Fig. 5C, right panel), in contrast, could not distinguish between VGLUT2 genotypes.

In VGLUT1 mice, the CCI model demonstrated no differences in pain behavior as assessed by the von Frey, acetone and balance box test (Fig. 5, left panels). These tests clearly distinguished between sham-operated and CCI VGLUT1 animals, but without any differentiation across genotypes.

4. Discussion

Changes in peripheral and central glutamatergic signaling have been reported to affect acute and chronic pain (Fundytus, 2001; Neugebauer, 2002; Bleakman et al., 2006). For example, group I mGluR antagonists and group II mGluR agonists reduce transmission and processing of nociceptive signals (Bhave et al., 2001; Karim et al., 2001). Also, different AMPA and NMDA receptor antagonists display analgesic activity for inflammatory and/or neuropathic pain. However, these and other glutamatergic drugs may be less suitable for clinical development due to their possible sedative, cognitive, ataxic and psychotomimetic side effects (Chiechio et al., 2004). It has been demonstrated that both the expression (initial upregulation followed by downregulation), and uptake activity of Glu transporters contribute to neuropathic pain (Sung et al., 2003). Several authors have therefore suggested that regulation of regional glutamate uptake via Glu transporters could still be an interesting strategy to attenuate glutamate neurotoxicity as well as the pathogenesis of neurological disorders (Minami et al., 2001; Binns et al., 2005; Liaw et al., 2005; Tao et al., 2005). To date, VGLUTs have not been studied for their possible efficacy in different pain conditions. Many pain states are associated with increased release of Glu, and it has been reported that neuropathic-like behaviors were attenuated by reducing Glu release via different pathways (Chen et al., 2002; Coderre et al., 2007). Therefore, the present study evaluated the effect of reduced glutamate release by targeting these transporters.

Here, we compared VGLUT1 and VGLUT2 heterozygote mice with their respective wild-type littermates in a comprehensive test battery to examine various clinically relevant pain responses in

mice (Leo et al., 2008). Homozygote mice appeared to be poorly viable, which further illustrates the developmental importance of these transporters (Wojcik et al., 2004; Moechars et al., 2006). Consequently, pain responses were examined in heterozygote mice using a standard test battery including assays to determine thermal and mechanical acute nociception, as well as pain responses after inflammation or neuropathy. Notably, we demonstrated that mice with VGLUT2 deficiency displayed markedly reduced neuropathic pain symptoms, in contrast to mice impaired in VGLUT1 signaling. However, reduction in VGLUT1 or VGLUT2 did not seem to induce any changes in acute nociceptive processing. Although there was an effect of genotype in the TWR test for VGLUT1 mice, the changed behavior was not consistent across the different temperatures. Furthermore, VF thresholds, HP response latencies and paw withdrawal response latencies in Hargreaves' test (pre-injection scores CAR_{HT}) were unchanged. Therefore, we conclude that adequate glutamatergic signaling is maintained in VGLUT1^{+/-} and VGLUT2^{+/-} mice to ensure acute nociception. The involvement of supra-spinal sensory glutamate signaling was determined with HP responses, while the response in the TWR test is considered to be a spinal reflex. Conditions of inflammation and tissue injury produced by irritating chemicals were also not affected by reduced VGLUT signaling. The formalin test is an experimental model to evaluate an animal's flinching response to moderate, continuous pain generated by tissue damage. Several studies indicated prominent involvement of spinal NMDA receptors in the second phase of the formalin test (Skilling et al., 1988; Dickenson and Sullivan, 1990; Yamamoto and Yaksh, 1992; Coderre and Melzack, 1992a; Coderre and Melzack, 1992b), which is consistent with the involvement of spinal glutamate signaling in this pain response. Accordingly, one might expect changes in formalin-induced flinching behavior especially in VGLUT2 heterozygous mice as VGLUT2 is the predominant isoform in spinal cord. However, it seems that the reduction of spinal glutamate release in VGLUT2 heterozygous mice is not sufficient to influence the response to formalin challenge, as no differences were observed between VGLUT2 heterozygous mice and their wild-type littermates. The remaining protein (50%) might be sufficient to maintain normal glutamate processing. Additionally, VGLUT1 is, although to a much lesser extent, also expressed in spinal cord and might as such also contribute to glutamate release after formalin injection (Li et al., 2003a,b; Landry et al., 2004; Alvarez et al., 2004).

In neuropathic pain conditions, some of the typical features are attenuated or absent in VGLUT2^{+/-} mice, but not VGLUT1^{+/-} mice. Specifically, following SNI, VGLUT2^{+/-} mice displayed no signs whatsoever of mechanical and cold allodynia as already indicated in our previous paper (Moechars et al., 2006). In the present paper, we additionally examined CCI-induced neuropathic pain behavior. Following CCI, VGLUT2^{+/-} mice do develop cold allodynia, but to a lesser extent than their wild-type littermates. No important changes in pain-related behavior were found in response to a reduction in VGLUT1 signaling. The observed analgesic effect in neuropathic VGLUT2^{+/-} mice, but not in VGLUT1^{+/-} mice can probably be explained by their differential expression profile. The regions with most prominent VGLUT2 expression (brainstem, thalamus and spinal cord) are those which are most likely to control changes in neuropathic pain. The most probable involvement of the thalamus in the VGLUT2^{+/-} associated behavior was already stated previously (Moechars et al., 2006) and could explain the observed neuropathic pain defect. The divergent behavior of VGLUT2^{+/-} mice in the SNI and CCI neuropathic pain model is unusual, but it has been reported that the development of neuropathic behavior in the CCI model depends on idiosyncratic immune-mediated response to the chronic suture, in contrast to SNI, which is an axotomy model of neuropathic pain (Maves et al., 1993; Campbell and Meyer, 2006).

There is disagreement in the literature about the importance of the number of vesicular transporters as a determinant of vesicle filling. We share the opinion that the quantal size of glutamate release (i.e., the number of neurotransmitter molecules exocytotically released by a single synaptic vesicle) depends on VGLUT expression level (Schuske and Jorgensen, 2004; Wojcik et al., 2004), whereas some authors have stated that a single vesicular glutamate transporter is sufficient to fill a synaptic vesicle (Freneau, Jr. et al., 2004; Daniels et al., 2006). The former statement is, however, consistent with the present results that show functional consequences of decreased (but not absent) VGLUT expression in heterozygous mice. Our previous study indeed reported 50% reduction in VGLUT protein in these mice (Moechars et al., 2006). We demonstrate that VGLUT levels (and concomitant changes in glutamatergic signaling) appeared to be sufficiently reduced to induce behavioral changes in neuropathic pain conditions, but not in acute nociceptive and inflammatory pain signaling.

However, despite the obvious differentiation in some assays of the test battery across VGLUT genotypes in the present study, the issue of hitchhiking donor gene confound must be addressed (Gerlai, 1996). We and others (Mogil and Wilson, 1997; Leo et al., 2008) already reported that background strains (129 and C57Bl/6) of genetically altered mice can display different patterns of nociceptive performance, which highlight the importance of guarded interpretation of KO studies. Our previous work (Leo et al., 2008) is designed to serve as a reference dataset for future studies with transgenic mice and is as such an interesting tool to check for possible confounds in the first instance. The most important differences between VGLUT genotypes are seen in the neuropathic pain behaviors: VGLUT2^{+/−} SNI mice did not develop mechanical or cold allodynia and VGLUT2^{+/−} CCI mice showed reduced sensitivity to the cold acetone stimulus compared to their wild-type littermates. Our genetic background study revealed a lower degree of neuropathy-like behavior in C57Bl/6J mice as represented by significantly lower mechanical allodynia and lower sensitivity to acetone after SNI compared to 129S6/SvEv mice. Confounding background effects would be expected to result in KO mice that responded like the 129 background strain and WT mice that responded like the C57Bl/6J background strain. As this is clearly not the case, rather the opposite, the phenotypes under investigation do not appear to be subject to this confound.

In conclusion, the distinct expression patterns of VGLUT1 and VGLUT2 are reflected by the presently observed differences in behavioral outcome of nociceptive tests between VGLUT1 and VGLUT2 mutant mice, which also appear to relate to the functional properties of specific regions of the nervous system. By and large, our findings suggest VGLUT2 to be an important modulator of neuropathic pain syndromes that could be an attractive target in prevention and/or treatment of this dramatic clinical condition.

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