Finally, our results indicate that the tonic defect in alertness in right-hemisphere patients contributes to their spatial neglect, because phasically increasing alertness can overcome their spatial deficit in visual awareness. The data show that a nonspatial intervention (that is, the presentation of warning tones, regardless of their location) can have a beneficial spatial effect in right-hemisphere-damaged patients, ameliorating their disabling bias towards the right. This indicates that there may be new approaches to rehabilitation. Most existing treatments of spatial neglect have limited success. They typically seek to direct the patient’s attention to the impaired left side, either by presenting lateralized visual cues at the left (which has the disadvantage that such cues are often themselves neglected), or by encouraging the patient to concentrate voluntarily on the affected side (this treatment typically shows only limited transfer to unsupervised situations). Our results indicate that any manipulation that phasically increases alertness, such as warning sounds, may effectively treat not just the tonic deficit of alertness in right-hemisphere patients, but also their spatial neglect.

Methods

Visual stimuli were presented on an active matrix LCD computer monitor (70 Hz refresh rate), at a viewing distance of ~40 cm. All stimuli appeared white against a uniformly black background. At the start of each trial, a fixation cross (0.9° × 0.9°) appeared at the centre of the display. Subjects were repeatedly requested to maintain their gaze on the central cross, and this was monitored in each trial by an observer. Less than 10% of trials were excluded because of fixation failures. Visual targets consisted of two horizontal bars, each subtending 0.4° in height and 3.1° in width. The bars appeared at symmetrical locations in the left and right visual fields, at the same height as the fixation cross (except for patient S.G.; Fig. 2), with their outer edges 9.8° from fixation.

The sequence of events for a warned trial with a left temporal lead is shown in Fig. 1. In all trials, the fixation cross appeared alone for 600 ms. In 25% of trials, a 300-ms tone burst (randomly either 400 Hz (89 dB) or 1000 Hz (84 dB)) was then presented centrally from two loudspeakers (Macintosh Multimedia Model SK-A10) hidden directly behind the monitor (to the right of the monitor in the follow-up experiment). The use of such warning tones is a standard way to alertness in right-hemisphere patients, but also their spatial neglect.

Increased number of synaptic GABA<sub>A</sub> receptors underlies potentiation at hippocampal inhibitory synapses

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Changes in synaptic efficacy are essential for neuronal development, learning and memory formation and for pathological states of neuronal excitability, including temporal-lobes epilepsy. At synapses, where there is a high probability of opening of postsynaptic receptors, all of which are occupied by the

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released transmitter, the most effective means of augmenting postsynaptic responses is to increase the number of receptors. Here we combine quantal analysis of evoked inhibitory postsynaptic currents with quantitative immunogold localization of synaptic GABA<sub>A</sub> receptors in hippocampal granule cells in order to clarify the basis of inhibitory synaptic plasticity induced by an experimental model of temporal-lobe epilepsy (a process known as kindling). We find that the larger amplitude (66% increase) of elementary synaptic currents (quantal size) after kindling results directly from a 75% increase in the number of GABA<sub>A</sub> receptors at inhibitory synapses on somata and axon initial segments. Receptor density was up by 34-40% and the synaptic junctional area was expanded by 31%. Presynaptic boutons were enlarged, which may account for the 39% decrease in the average number of released transmitter packets (quantal content). Our findings establish the postsynaptic insertion of new GABA<sub>A</sub> receptors and the corresponding increase in postsynaptic responses augmenting the efficacy of mammalian inhibitory synapses.

At excitatory and inhibitory synapses of the adult mammalian central nervous system (CNS) the gain of synaptic transmission can undergo lasting and marked alterations. The plasticity of excitatory (glutamatergic) synapses has been widely studied, but no consensus exists regarding the pre- or postsynaptic site of the alteration responsible for the change in synaptic gain. A few studies have addressed the long-lasting plasticity at central inhibitory (GABA<sub>A</sub>) synapses. A change in the size of miniature inhibitory postsynaptic currents (mIPSCs) occurs at GABA<sub>A</sub> synapses on granule cells of the dentate gyrus after kindling-induced epilepsy, and an increased number of synaptic GABA<sub>A</sub> receptors was inferred from non-stationary noise analysis. Here we have used a direct approach to determine the relationship between the size of synaptic responses and the number of receptors by combining physiological recordings with electron microscopy.

For quantal analysis, IPSCs were evoked by local stimulation of inhibitory axons, which very probably terminate perisomatically on granule cells. Analogously to earlier observations in young animals, this type of stimulation in adult rats evoked IPSCs with amplitude distributions having clearly distinguishable multiple peaks (Fig. 1). The peaks were fitted with multiple gaussian distributions, in each case having equal (quantal) spacings between successive peaks. As generally noted (see, for example, ref. 5), larger peaks fit less well, and the quantal variance fails to increase linearly, but according to an autocorrelation analysis performed in four cells (including the two shown in Fig. 1), the likelihood for the chance occurrence of the observed peaks in the distributions was <4 × 10⁻⁵. The quantal analysis was done in accordance with published methods of Edwards et al. (ref. 5 and Methods), and revealed a significant 66% increase in the quantal size (q) of IPSCs in kindled neurons (Table 1). The quantal content (m) obtained by the method of failures (m<sub>f</sub> = ln(N/N<sub>0</sub>)) assuming Poisson release statistics did not correspond to the value calculated directly (m<sub>d</sub>) by dividing the average evoked IPSC (eIPSC) by q.

Considering full occupancy of postsynaptic GABA<sub>A</sub> receptors by the released transmitter, and a large open probability (P<sub>o</sub> = 0.8) at the peak of synaptic currents, our findings indicate that control synapses have on average 26 GABA<sub>A</sub> receptors (=435 pS/(21 pS × 0.8)) considering an average conductance of 21 pS, and this number increases to 43 after kindling. Alternatively, the 66% increase in q may result from (1) an increase in P<sub>o</sub> or (2) an augmented single-channel conductance. (1) An increase in P<sub>o</sub> to its maximal possible value of 1.0 can account for no more than 25% (1.0/0.8 = 1.25) of the augmented q, but our experimental results are consistent with a decrease, if any, in P<sub>o</sub> after kindling (see below). (2) An enhanced contribution of large conductance states to the average conductance of synaptic GABA<sub>A</sub> receptors can also be excluded. Peak-scaled non-stationary fluctuation analysis revealed similar mean conductances of GABA<sub>A</sub> receptors in control (20.6 ± 0.8 pS; n = 5) and kindled (21.3 ± 1.4 pS; n = 4) granule cells.

To examine the effect of kindling on the expression of GABA<sub>A</sub> receptor subunits in the dentate gyrus, light-microscopic immunocytochemistry was performed for the α1, α2, β2/3 and γ2 subunits. An increased immunostaining of granule cells was observed for all subunits tested (n = 5 pairs of animals), which was apparent from the enhanced staining of the granule cell layer and the neuropile of the molecular layer. No immunostained axon terminals could be detected around somata and axon initial segments (AISs) of granule cells, indicating that all of the detectable change in the expression of GABA<sub>A</sub> receptor subunits takes place postsynaptically.

To investigate directly the relation between the increased quantal size and the number of synaptic receptors, we applied postembedding immunogold localization of the β2 and β3 subunits. The immunosignal was targeted against these subunits because: (1) they are the major β subunits expressed by dentate granule cells; (2) they are expressed at perisomatic synapses and (3) every functional GABA<sub>A</sub> receptor probably contains β subunits. Thus, alterations...
in the total number of GABAA receptors at synapses should be faithfully reflected by changes in the number of β2/3 subunits. As our evoked responses are of proximal origin and mIPSCs are likely to originate from perisomatic synapses, here we examined the β2/3 subunit content of somatic and AIS synapses only. To determine the immunoreactive receptor content of the whole synapse, the entire postsynaptic area was reconstructed from serial sections (for example Fig. 2a1–a3; ref. 9). After kindling, the immunoreactive β2/3 subunit content of synapses was increased on both somata (Fig. 2) and AISs (Fig. 3) of granule cells, with a somewhat larger increase at somatic synapses (Fig. 4a; soma, 94%; AIS, 61%). The larger number of immunoparticles was the consequence of a 34% and 40% increase in their density at somatic and AIS synapses, respectively (Fig. 4a), as well as an average 31% increase in the area (Fig. 4a) of synaptic junctions pooled from both compartments. To exclude the possibility that the increase at synapses was restricted to β2/3 subunits, and to establish whether some of the increased expression observed at the light microscopic level for the other subunits was also localized to synapses, we determined the quantitative distribution of α2 subunits at AIS synapses2,5. All synapses on AISs were found to be consistently immunopositive for α2 subunits (control, 6.4 ± 4.2 particle per synapse; range 1–17; n = 14), and kindling (10.1 ± 6.2; range 6–31; n = 15) increased their immunoreactive receptor content by 58%. The remarkable similarity between the increase in the number of α2 (58%) and β2/3 (61%) subunits at AIS synapses suggests that alterations detected with subunit-selective antibodies represent changes in the total number of synaptic GABAA receptors.

We next examined how the observed increase in the number of GABAA receptors at perisomatic synapses is correlated with the increased q. The immunogold data were obtained from a large number of perisomatic synapses (n = 84 from three control rats and n = 98 from three kindled rats) of several postsynaptic neurons. In contrast, a given q was measured at a few synapses made by a single presynaptic axon2. Therefore, we initially sought to compare the distributions of immunoparticles (Fig. 4b) with those of mIPSCs (Fig. 4c) pooled from several postsynaptic cells. A large variability in the immunoreactive GABAA receptor content was observed in both subcellular compartments of control granule cells (soma, mean = 8.9 ± 0.9 particles per synapse, range 0–30, c.v. = 0.75 ± 0.05; AIS, mean = 13.6 ± 3.4 particles per synapse, range 2–35, c.v. = 0.58 ± 0.12), resulting in a skewed distribution. The large variability and the skewed distribution persisted after kindling (soma, c.v. = 0.78 ± 0.12; AIS, c.v. = 0.62 ± 0.05) with an approximately parallel shift in the cumulative probability plot (Fig. 4b). A similar shift was observed in the cumulative distribution of mIPSC conductance (mIPSCG) recorded in kindled granule cells (Fig. 4c). The relative increase in the median receptor number (75%) after kindling was larger than that observed for mIPSCG (41%), possibly due to changes at some non-overlapping synapse populations sampled for mIPSCs and ultrastructural analysis.

In contrast to mIPSCs, there was a good match between the

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**Table 1** Quantal parameters of inhibitory postsynaptic currents originating from the perisomatic region of control and kindled granule cells

<table>
<thead>
<tr>
<th>Current</th>
<th>n</th>
<th>N – Nf</th>
<th>Nf</th>
<th>q (pS)</th>
<th>m2</th>
<th>m3</th>
<th>c.v. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control eIPSCs</td>
<td>8</td>
<td>467 ± 60</td>
<td>142 ± 19</td>
<td>435 ± 23</td>
<td>2.6 ± 0.3</td>
<td>1.5 ± 0.1</td>
<td>26 ± 2</td>
</tr>
<tr>
<td>Kindled eIPSCs</td>
<td>10</td>
<td>487 ± 64</td>
<td>192 ± 25</td>
<td>722 ± 71*</td>
<td>1.6 ± 0.2*</td>
<td>1.3 ± 0.1</td>
<td>30 ± 3</td>
</tr>
<tr>
<td>Control mIPSCs</td>
<td>4</td>
<td>264 ± 66</td>
<td>–</td>
<td>436 ± 17</td>
<td>–</td>
<td>–</td>
<td>34 ± 3</td>
</tr>
<tr>
<td>Kindled mIPSCs</td>
<td>4</td>
<td>426 ± 89</td>
<td>–</td>
<td>615 ± 17*</td>
<td>–</td>
<td>40 ± 2</td>
<td></td>
</tr>
</tbody>
</table>

N – Nf: number of events per cell, where N is the total number of responses and Nf is the number of failures; q, average quantal size; m2, quantal content (direct method); m3: quantal content (method of failures); c.v., coefficient of variation (after subtraction of baseline noise variance). The q of mIPSCs is given as the median of cumulative probability distributions.

* Significant difference from control (P < 0.06, ANOVA). Data are expressed as mean ± s.e.m.
change in $q$ of eIPSCs and the increase in the number of immuno-
labelled receptors. This direct measurement also allowed us to
derive possible alterations in the $P_o$ of the postsynaptic receptors
after kindling. According to our electrophysiological measure-
ments, 26 functional GABA$_A$ receptors should be present at control
synapses where quantal eIPSCs are generated. The weighted mean
(64% somatic and 36% AIS synapses, to reflec
t their natural
proportion on granule cells$^{23}$) of gold particles was 10.2 for
$\beta^{2/3}$ subunits, corresponding to $\sim 2.5$ functional GABA$_A$ receptors per
particle. On the basis of this relationship, the 18.5 particles per
synapse after kindling should correspond to $\sim 47$ functional recep-
tors, which is within 10% of the predicted 43 channels derived from
the measured $q$ when $P_o = 0.8$. Thus, regardless of the initial
estimate of $P_o$ at control synapses, the increase in $q$ can be solely
attributed to the more receptors present without an accompanying
increase in $P_o$. As more receptors are inserted into synaptic mem-
branes after kindling, and as the size of the synapses increases, at
some synapses the receptors may not be fully occupied by GABA. It
has been reported$^7$ that postsynaptic GABA$_A$ receptors have a very
high degree of occupancy at synapses containing less than $\sim 80$
receptors (corresponding to $\sim 33$ particles in our study). Because
99% of the perisomatic synapses in control granule cells contain
$<33$ particles, full receptor occupancy$^7$ probably occurs at most of
these synapses. In contrast, $\sim 10$% of the kindled synapses contain
$>33$ particles, which is consistent with an incomplete occupancy of
the receptors, whereupon the size of IPSCs at such synapses will
depend on presynaptic factors, such as the transmitter concen-
tration in the cleft.

We have found both anatomical and physiological evidence
for presynaptic changes after kindling. With the postsynaptic
insertion of GABA$_A$ receptors and the enlargement of the postsynap-
tic area, the size of the presynaptic boutons increased as well
(sectioned bouton area in control, $0.20 \pm 0.02 \mu m^2$; kindling,
$0.29 \pm 0.01 \mu m^2$; $P > 0.01$). Larger boutons could decrease the
efficacy of action potentials for evoking release, which might explain
the 39% decrease in quantal content observed after kindling.
Alternative possibilities include changes in the density of presynap-
tic GABA$_A$, metabotropic glutamate, kainate receptors, or Ca$^{2+}$
and K$^+$ channels. It is also interesting to note that in spite of a sufficiently
large postsynaptic space available for the insertion of new GABA$_A$
receptors$^{9,24}$, the postsynaptic area had to increase to accommodate
the new receptors. If one accepts that GABA$_A$ receptors form small
clusters in the postsynaptic membrane$^{9,24}$, our findings are consis-
tent with the necessity for a physical separation between such
clusters.

In summary, our findings demonstrate a direct relationship
between synaptic GABA$_A$ receptor number and quantal size at
potentiated GABAergic synapses in the adult brain. The kindling-
induced change in synaptic efficacy also includes an enlargement of
the synaptic area accompanied by increased presynaptic bouton size.
and decreased quantal content in agreement with the ‘synaptic homeostasis’ principle suggested to occur at Drosophila neuromuscular junctions. At some large kindled synapses the receptors might lose full occupancy, whereupon further insertion of GABAA receptors will no longer linearly increase postsynaptic responses. Larger inhibitory events in epilepsy might be puzzling, but such events might better synchronize granule cell firing. Alternatively, larger inhibitory events in epilepsy might be puzzling, but such events might better synchronize granule cell firing. Alternatively, larger inhibitory events in epilepsy might be puzzling, but such events might better synchronize granule cell firing.

Methods

Preparation of animals and tissue. The procedure of daily kindling of Wistar rats through the hippocampal commissures (100 μA, 60 Hz for 1 s) has been described earlier. Control (implanted but never stimulated, n = 5) and kindled animals (with 43–49 seizures, n = 5) were perfused 48 h after the last seizures with a fixative containing 0.05% glutaraldehyde, 4% paraformaldehyde and ~0.2% picric acid in 0.1 M phosphate buffer for 13–30 min. The brains were then removed, 500-μm-thick Vibratome sections from the dorsal hippocampus were cut and were embedded into an acrylic resin at −50°C (Lowrycril HM20, Chemische Werke Lewi GmbH, Germany). Antibodies and controls. The characterization of polyclonal antibodies to the α1 (ref. 28), α2 and γ2 subunits and the monoclonal antibody (code no. bd17) against the β2 and β3 subunits, has been described earlier. Antibodies were used at the following final protein concentrations (in μg ml⁻¹): α1, 1.3; α2, 1 and 15 for pre- and postembedding reactions, respectively; β2/3, 40 and 120 for pre- and postembedding reactions, respectively; γ2, 1. Selective labelling, resembling that obtained with the specific antibody, could not be detected when the primary antibody was either omitted or replaced by 5% normal serum.

Pre-embedding immunocytochemistry. A standard procedure was applied on 70-μm-thick Vibratome sections using biotinylated secondary antibodies and avidin biotinylated horseradish peroxidase complex. Postembedding immunocytochemistry. Serial sections (20–25 per grid; 70 nm thick) were cut from the upper blade of the dorsal dentate gyrus and were picked up on pioloform-coated slot grids. The sections were incubated in blocking solutions for 30 min, followed by an incubation with the primary and secondary antibodies. Quantification of immunoreactivity for GABAA receptor subunits was performed as described earlier. Briefly, the granule cell layer and the hilus of the dentate gyrus were systematically searched until a symmetrical synapse was found between an axon terminal and the soma or an AIS of a granule cell. The synapse was photographed, followed in serial sections completely through the synaptic specialization, which was measured. Immunoparticles were counted within the membrane specialization. The synaptic area was calculated by multiplying its length in each section by the average thickness of sections (~70 nm). Tangentially sectioned synapses were excluded from the analysis of synaptic area and immunoparticle density. The sectioned area of axon terminals was measured with a digitizing table at the level where the longest synaptic specialization was observed. No correction for shrinkage or expansion was made.

Electrophysiology. Horizontal 400-μm-thick brain slices were prepared after the last seizure (average number of seizures = 65). Whole-cell patch clamp recordings were obtained at room temperature (22–23°C) from dentate granule cells visualized by infrared DIC (differential interference contrast) videomicroscopy (Zeiss Axioscope)30. Slices were perfused with an artificial cerebrospinal fluid containing 2 mM kynurenic acid and 1 μM tetrodotoxin (TTX). For minimal stimulation experiments, the extracellular Ca²⁺/Mg²⁺ ratio was changed to 1:2 (1.5 mM CaCl₂ and 3 mM MgCl₂) and TTX was omitted. The intracellular solution contained (in mM) 130 Cs-gluconate, 2 CsCl, 2 MgCl₂, 20 HEPES, 2 ATP and 1% biocytin (pH 7.25 adjusted with CsOH; osmolality 300–310 mosM). Minimal stimulation (20 μs pulse width) was...
through a theta glass pipette (2 mm diameter, pulled to 1 μm tip diameter) filled with 1 M NaCl and placed 15–30 μm away from the somata. Stimulation at 0.2–1 Hz caused no paired-pulse depression or facilitation. Recordings were done at holding potentials of +10 to 5 mV, and the IPSC reversal potential was always determined. Access resistances (compensated 70–80%) were frequently monitored and remained constant (±15%) during the recordings (control, 12.1 ± 0.5 MΩ; kindled, 12.2 ± 0.5 MΩ). Signals were filtered at 2 kHz, digitized at 6 kHz and analysed with CDR or SCAN software (courtesy of J. Dempster). For the quantal analysis, a Simplex-based least-squares criterion was used to fit non-constrained gaussian distributions to the eIPSC amplitude histograms, in which peaks could be clearly detected by eye. No more than six gaussian distributions were fitted, as the number of very large compound events was very small to be described accurately by a normal curve. Simplex algorithm descriptions did not describe adequately the sequences of failures and peaks in the distributions of eIPSCs. In the absence of any morphological data on the number of release sites, we have refrained from applying a compound binomial analysis. At the recorded holding potentials, the peak of the first distribution was always larger than 1.5 times the variance of the baseline noise (σ0). Quantal amplitudes were calculated as described by Edwards et al., and were converted to quantal conductances to eliminate driving force bias. The c.v. was calculated from the mean of the first quantal peak of eIPSC distributions, or the mean of mIPSCs, divided by the square root of the respective σ0 subtracted variances. Non-stationary noise analysis was performed as described.

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8. Nusser, Z., Call-Candy, S. G. & Farrant, M. Differences in synaptic GABA(A) receptor number underlie mIPSC variability at1 and mIPSCs, divided by the square root of the respective σ0 subtracted variances. Non-stationary noise analysis was performed as described.

**Cloning of inv, a gene that controls left/right asymmetry and kidney development**

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Most vertebrate internal organs show a distinctive left/right asymmetry. The inv (inversion of embryonic turning) mutation in mice was created previously by random insertional mutagenesis; it produces both a constant reversal of left/right polarity (situs inversus) and cyst formation in the kidneys. Asymmetric expression patterns of the genes nodal and lefty are reversed in the inv mutant, indicating that inv may act early in left/right determination. Here we identify a new gene located at the inv locus. The encoded protein contains 15 consecutive repeats of an Ank/Swi6 motif at its amino terminus. Expression of the gene is the highest in the kidneys and liver among adult tissues, and is seen in presomite-stage embryos. Analysis of the transgenic genome and the structure of the candidate gene indicate that the candidate gene is the only gene that is disrupted in inv mutants. Transgenic introduction of a minigene encoding the candidate protein restores normal left/right asymmetry and kidney development in the inv mutant, confirming the identity of the candidate gene.

Organ primordia of vertebrates are symmetric at first, and then acquire distinctive left/right asymmetry. In mice, embryonic turning and heart looping are the earliest manifestations of left/right asymmetry. Before morphological asymmetry develops, several genes, including nodal and lefty in mice, are expressed asymmetrically. Asymmetric expression of nodal and lefty is reversed in the inv mutant. The inv mutation produces a constant reversal of left/right asymmetry, contrasting with several experimental and genetic mechanisms that randomize alterations in left/right asymmetry. In addition, the mutation accompanies cyst formation in the kidney and jaundice.

The inv mutation was found in a family of transgenic mice into which the tyrosinase minigene had been introduced, and is thought to be created by insertional mutagenesis. We previously