Anti-Epileptic Activity

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Contents

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General Considerations

Epilepsy is a disease of high prevalence, being well known since thousands of years as "morbus sacer." In spite of intensive investigations, the pathophysiology of epilepsy is still poorly understood. Studies with various animal models have provided ample evidence for heterogeneity in the mechanisms of epileptogenesis. New evidence derives from investigations of kindling, which involves the delivery of brief, initially subliminal, electrical, or chemical stimuli to various areas of the brain. After 10–15 days of once-daily stimulation, the duration and intensity of afterdischarges reach a stable maximum, and a characteristic seizure is produced. Subsequent stimulation then regularly elicits seizures.

Surveys of methods being used to test compounds with anticonvulsant properties have been provided by Toman and Everett (1964), Woodbury (1972), Hout et al. (1973), Swinyard (1973), Koella (1985), Meldrum (1986), Rump and Kowalczyk (1987), Löscher and Schmidt (1988), Fisher (1989), Rogawski and Porter (1990), and Porter and Rogawski (1992).

Epilepsy becomes drug resistant in 20–30 % of patients. Out of the animal models, the amygdala-kindled rat seems to be a suitable approach (Löscher 1997, 1998, 2002a, b). Furthermore, the rat cortical dysplasia model is recommended (Smyth et al. 2002).

Several biochemical hypotheses have been advanced, involving the inhibitory GABAergic system and the system of the excitatory amino acids glutamate and aspartate. Excitatory receptors have been divided into subtypes according to the actions of specific agonists or antagonists. Agents which reduce $GABA_A$ synaptic function provoke convulsions. A convulsive state is induced by the direct blockade of $GABA_A$ receptors (e.g., to the action of bicuculline) or a reduction in the GABA-mediated opening of the chloride ion channel (e.g., by picrotoxin). One major factor in epileptogenesis seems to be a decreased function of $GABA_A$ synapses.

More recently, research has focused on the therapeutic potential of blocking excitatory amino acids, in particular, glutamate. Of the three receptors of glutamate, the NMDA (*N*-methyl-D-aspartate) receptor is considered one of the most interested in epilepsy, and competitive NMDA receptor antagonists are proposed as potential antiepileptic drugs. Excessive excitatory amino acid neurotransmission is thought to be associated with the neuropathologies of epilepsy, stroke, and other neurodegenerative disorders. Antagonism of NMDA receptor function appears to be the mechanism of action of some novel anticonvulsant and neuroprotective agents. Excitatory amino acid receptors have been classified into at least three subtypes by electrophysiological criteria: NMDA, quisqualic acid (QA), and kainic acid (KA) (Cotman and Iversen 1987; Watkins and Olverman 1987).

Fabene and Sbarbati (2004) underlined the value of in vivo MRI in different models of experimental epilepsy.

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In Vitro Methods

[^{3}H]-GABA Receptor Binding

See chapter "► Tests for Anxiolytic Activity".

$GABA_A$ Receptor Binding

See chapter "► Tests for Anxiolytic Activity".

$GABA_B$ Receptor Binding

See chapter "► Tests for Anxiolytic Activity".

The in vitro assays for GABAergic compounds described in the chapter "► Tests for Anxiolytic Activity" (anxiolytics) are similarly used for evaluation of antiepileptic compounds.

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[^{3}H]-GABA Uptake in Rat Cerebral Cortex Synaptosomes

Purpose and Rationale

Roberts (1974) and others have proposed that the inhibitory action of the amino acid γ-aminobutyric acid (GABA) is the fine tuning control for pacemaker neurons. Disruption of this interplay due to inadequacies of the GABA

system results in various disorders, in particular, convulsive seizures (Roberts 1974; Korgsgaard-Larsen 1985). The nonspecific action of GABA-mimetics makes inhibition of the uptake mechanism, which terminates the neurotransmitter action, the ideal choice for increasing GABA's concentration at specific sites (Roberts 1974; Tapia 1975; Meldrum et al. 1982; Brehm et al. 1979). Demonstration of the high-affinity mechanism that best reflects the in vivo condition utilizes GABA-depleted cerebral cortex synaptosomes (Ryan and Roskoski 1977; Iversen and Bloom 1972; Roskoski 1978). Although the physiological role of GABA transport systems is still unclear, uptake inhibitors such as THPO [4,5,6,7-tetrahydroisoxazolo-(4,5-C)pyridine-3-ol], nipecotic acid, cis-4-hydroxynipecotic acid, and guvacine exhibit anticonvulsant effects (Meldrum et al. 1982; Brehm et al. 1979). Furthermore, a number of neuroleptics have been shown to inhibit GABA uptake (Fjalland 1978). In particular, fluspirilene was found to be equivalent to the most potent uptake inhibitors known.

The assay is used as a biochemical screen for potential anticonvulsants or GABA (γ-aminobutyric acid) mimetic compounds that act by inhibiting GABA uptake.

Procedure

Reagents

1. 0.5 M Tris buffer, pH 7.4.
2. Ringer's solution + 10 mM Tris buffer, pH 7.4 containing.
   - Glucose 10.0 mM,
   - NaCl 150.0 mM
   - KCl 1.0 mM
   - $MgSO_4$ 1.2 mM
   - Na_2HPO_4 1.2 mM
3. Depolarizing Ringer's solution, pH 7.4 reagent 2 containing:
   - KCl 56 mM
   - $CaCl_2$1 mM
4. 0.32 M sucrose.
5. [^{3}H]-GABA is diluted to 2.5×10^{-4} M with distilled water. Forty microliters of this solution in 1 ml of reaction mixture will yield a final concentration of 10^{-5} M.
6. Test compounds.

   A 10 mM stock solution is made up in distilled water, ethanol, or DMSO and serially diluted, such that the final concentration in the assay ranges from 10^{-3} to 10^{-8} M. Total and nonspecific controls should use solvent of test compound.

Tissue Preparation

Male Wistar rats are decapitated and the brains rapidly removed. Cerebral cortex is weighed and homogenized in 9 volumes of ice-cold 0.32 M sucrose using a Potter–Elvehjem homogenizer. The homogenate is centrifuged at 1000 g for 10 min. The supernatant (S_1) is decanted and recentrifuged at 1000 g for 10 min. The pellet (P_2) is resuspended in 9 volumes of 0.32 M sucrose and centrifuged at 24,000 g for 10 min. The washed pellet is resuspended in 15 volumes of depolarizing Ringer's solution, incubated at 25 °C for 10 min and centrifuged at 3000 g for 10 min. The resulting pellet is resuspended in 15 volumes of Ringer's solution and is ready for use.

Assay

60 µl Ringer's solution
100 µl vehicle or appropriate drug concentration
800 µl tissue suspension

Microcentrifuge tubes are set up in triplicate. Nonspecific controls are incubated at 0 °C and total at 25 °C for 10 min. 40 µl of [^{3}H]-GABA are added and the tubes are reincubated for 10 min. All tubes are centrifuged at 13,000 g for 1 min. The supernatant is aspirated and 1 ml of solubilizer (Triton X-100 + 50 % EtOH, 1:4, v/v) is added and mixed to dissolve pellets. Tubes are incubated at 90 °C for 3 min, then centrifuged at 13,000 g for 15 min. 40 µl of supernatant is counted in 10 ml Liquiscint scintillation cocktail.

Evaluation

Active uptake is the difference between cpm at 25 °C and 0 °C. The percent inhibition at each drug concentration is the mean of three

determinations. IC_{50} values are derived from log–probit analysis.

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GABA Uptake and Release in Rat Hippocampal Slices

Purpose and Rationale

The GABA transporter, the subsynaptic $GABA_A$ receptor, and the $GABA_B$ autoreceptor are therapeutically the most relevant targets for drug actions influencing GABAergic synaptic transmission. Uptake inhibitors are potential anticonvulsants.

Procedure

For measurement of GABA uptake, rat hippocampal slices are cut with a McIlwain tissue slicer (100-μm-thick prisms) and dispersed in ice-cold Krebs–Ringer solution with HEPES buffer (pH 7.4). Following two washes, slices (15 mg) are incubated at 37 °C for 15 min in the presence or absence of test compound. [^{3}H]-GABA is added, and samples are incubated for an additional 5 min before filtration through Whatman GF/F filters. Samples are then washed twice with 5 ml ice-chilled 0.9 % saline. Distilled water is added, and samples are allowed to sit at least 60 min before measured for radioactivity by liquid scintillation spectroscopy. Blanks are treated in an identical manner but are left on ice throughout the incubation.

For measurement of GABA release, rat hippocampal slices are prepared and dispersed in ice-cold HEPES-buffered (pH 7.2) Krebs–Ringer solution and incubated with 0.05 μM [^{3}H]-GABA for 15 min at 37 °C. Following two washes, the slices are incubated for an additional 15 min and finally resuspended in medium. Tissue (10 mg) is incubated at 37 °C for a 15 min release period in the presence or absence of test compound. At the end of the release period, the medium is separated from tissue by centrifugation at 500 g for approximately 1 min and poured into 0.5 ml of perchloric acid (0.4 N). The tissue is homogenized in 0.13 N perchloric acid. Radioactivity in the samples is measured by using liquid scintillation spectroscopy.

Evaluation

For GABA uptake, IC_{50} values (μM) are determined.

In GABA release experiments, results are expressed as the amount of radioactivity released as a percent of the total radioactivity.

Modifications of the Method

Roskoski (1978) studied the net uptake of GABA by high-affinity synaptosomal transport systems.

Nilsson et al. (1990, 1992) tested GABA uptake in astroglial primary cultures.

The **isolated nerve-bouton preparation** was used to study GABA release (Jang et al. 2001; Kishimoto et al. 2001; Akaike et al. 2002; Akaike and Moorhouse 2003). The technique was developed by Drewe et al. (1988), Vorobjev (1991), Haage et al. (1998), Rhee et al. (1999), and Koyama et al. (1999).

The method is based on the local application of mechanical vibration directly to the chosen site of a brain slice and does not require the enzymatic pretreatment of the tissue. The mechanical vibration is applied via a glass rod (0.5 mm in diameter) mounted on a piezoelectric bimorph crystal at the site of the chosen brain tissue. The dissociated cells are allowed to settle at the bottom of a Petri dish for 20 min. The cell bodies are usually 10–15 pm at their longest axis, rounded or elongated in shape. Some cells had remaining neurites up to 100 pm long. The majority of cells had neurites less than 15 μm long.

In other studies (Koyama et al. 1999; Kishimoto et al. 2001), a custom-built vibrating stylus was placed in the appropriate region for mechanical dissociation. The glass capillary (1.5 mm o.d.) was pulled to a fine tip and fire polished. The tip was placed within the appropriate region by a manipulator. The vibrating stylus was driven by an electronic relay, and the tip was horizontally moved (excursions of 2–3 mm at 0.5–2 Hz) for 2 min.

Neurons with adherent functional synaptic terminals were investigated by tight-seal whole-cell recordings from the postsynaptic cells.

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Glutamate Receptors: [^{3}H]CPP Binding

Purpose and Rationale

The ionotropic glutamate receptors are ligand-gated ion channels that mediate the vast majority of excitatory neurotransmission in the brain. The family comprises three pharmacologically defined classes that were originally named after reasonably selective ligands: *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and kainate (Cotman and Iversen 1987; Watkins and Olverman 1987; Collingridge and Lester 1989; Monaghan et al. 1989; Carlsson and Carlsson 1990; Young and Fagg 1990; Nakanishi 1992; Cunningham et al. 1994; Herrling 1994; Iversen and Kemp 1994; Mayer et al. 1994; Meldrum and Chapman 1994; Monaghan and Buller 1994; Watkins 1994; Bettler and Mulle 1995; Fletcher and Lodge 1995; Becker et al. 1998; Danysz and Parsons 1998; Meldrum 1998; Chittajallu et al. 1999; Dingledine et al. 1999; Hatt 1999; Gallo and Ghiani 2000; Lees 2000; Meldrum 2000). It turned out that NMDA, AMPA, and kainate receptor subunits are encoded by at least six gene families as defined by sequence homology: a single family of AMPA receptors, two for kainate, and three for NMDA (Dingledine et al. 1999; Mayer and Armstrong 2004).

The NMDA subtype is a hetero-oligomer consisting of an NR1 subunit combined with one or more NR2 subunits and a third subunit, NR3 (Loftis and Janowsky 2003). The receptor has two amino acid recognition sites, one for glutamate and one for glycine, both of which must be occupied to promote channel opening. A variety of drugs have been identified which block the channel selectively (Bräuner-Osboren et al. 2000; Kemp and McKernan 2002).

The AMPA subtype is a hetero-oligomer formed from combinations of iGluR1–4. Selective agonists and competitive antagonists acting at the glutamate recognition site have been useful for defining the physiological and pathophysiological roles played by the receptor. AMPA receptor modulators have been discussed as cognitive enhancers (Lynch 2004).

The kainate subtype consists of hetero-oligomers, comprising five subunits (Hollmann and Heinemann 1994; Huettner 2003).

Excessive excitatory amino acid neurotransmission has been associated with the neuropathologies of epilepsy, stroke, and other neurodegenerative disorders (Cotman and Iversen 1987; Watkins and

Olverman 1987; Parsons et al. 1998). Antagonism of NMDA receptor function appears to be the mechanism of action of some anticonvulsant and neuroprotective agents (Löscher 1998; Tauboll and Gjerstad 1998). The binding site for [^{3}H]2-amino-4-phosphonobutyric acid (AP4) may represent a fourth site which is less well characterized (Thomsen 1997). NMDA receptors are believed to be coupled to a cation channel which converts to an open state with NMDA receptor activation (Kemp et al. 1987; Mukhin et al. 1997). The opening and closing of this cation channel are also modulated by glycine, Mg^{2+}, and Zn^{2+}. Dissociative anesthetics, such as phencyclidine (PCP) and ketamine, and novel anticonvulsants, such as MK-801, block the ion channel and are noncompetitive NMDA receptor antagonists. Competitive NMDA receptor antagonists, such as CPP and the phosphono analogues of L-glutamate, AP7, and AP5 (2-amino-5-phosphonopentanoic acid), are inhibitors at the excitatory amino acid binding site (Olverman et al. 1986; Davies et al. 1986; Harris et al. 1986; Murphy et al. 1987; Lehmann et al. 1987).

The following assay is used to assess the affinity of compounds for the excitatory amino acid binding site of the NMDA receptor complex. [^{3}H]CPP 3-[(±)-2-carboxypiperazin-4-yl]-1-phosphonic acid is a structurally rigid analogue of the selective NMDA receptor antagonist 2-AP7 (2-amino-7-phosphonoheptanoic acid).

Procedure

Reagents

1. Buffer A: 0.5 M Tris HCl, pH 7.6
   60.0 g Tris HCl
   13.9 g Tris base
   q.s. to 1 l with distilled water
2. Buffer B: 50 mM Tris HCl, pH 7.6
   Dilute buffer A 1:10 with distilled water
3. L-Glutamic acid, 5×10^{-3} M
   Dissolve 7.36 mg of L-glutamic acid (Sigma G1251) with 10.0 ml distilled water. Aliquots of 20 µl to the assay tube will give a final concentration of 10^4 M.
4. [^{3}H]CPP is obtained from New England Nuclear, specific activity 25–30 Ci/mmol. For IC_{50} determinations, a 200 nM stock solution is made with distilled water. Aliquots of 50 µl are added to each tube to yield a final concentration of 10 mM.
5. Test compounds. A stock solution of mM is made with a suitable solvent and serially diluted, such that the final concentration in the assay ranges from 10^{-5} to 10^{-8} M. Higher or lower concentrations may be used, depending on the potency of the drug.
6. Triton X-100,10 % (v/v) (National Diagnostics, EC-606). A stock solution of Triton X-100, 10 %, can be prepared and stored in the refrigerator. Dilute 1.0 ml of Triton X-100 to 10.0 ml with distilled water. On the day of the assay, the tissue homogenate (1:15 dilution) is preincubated with an aliquot of Triton X-100, 10 %, to give a final concentration of 0.05 % (v/v).

Tissue Preparation

Cortices of male Wistar rats are dissected over ice and homogenized in ice-cold 0.32 M sucrose, 15 volumes of original wet weight of tissue, for 30 s with a Tissumizer setting at 70. The homogenate is centrifuged at 1000 g for 10 min (SS34, 3000 rpm, 4 °C). The supernatant is centrifuged at 20,000 g (SS34, 12,000 rpm, 4 °C) for 20 min. Resuspend the pellet in 15 volumes of ice-cold distilled water (Tissumizer setting 60, 15 s) and spin at 7600 g (SS34, 8000 rpm, 4 °C) for 20 min. Save the supernatant, swirl off the upper buffy layer of the pellet and add to the supernatant. Centrifuge the supernatant at 48,000 g (SS34, 20,000 rpm, 4 °C) for 20 min. Resuspend the pellet with 15 volumes of cold distilled water and centrifuge. Discard the supernatant and store the pellet at −70 °C.

On the day of the assay, resuspend the pellet in 15 volumes ice-cold 50 mM Tris buffer, pH 7.6. Preincubate the homogenate with Triton X-100 in a final concentration 0.05 % (v/v) for 15 min at 37 °C with agitation. Centrifuge the homogenate at 48,000 g (SS34, 20,000 rpm, 4 °C) for 20 min. Wash the pellet an additional three times by resuspension with cold buffer and centrifugation. The final pellet is resuspended in a volume 20 times the original wet weight.

Assay

1. Prepare assay tubes in triplicate.
   380 µl distilled water
   50 µl buffer A, 0.5 M Tris HCI, pH 7.6
   20 µl L-glutamic acid, 10^{-4} M, or distilled water, or appropriate concentration of inhibitor
   50 µl [^{3}H]CPP
   500 µl tissue homogenate
2. Following the addition of the tissue, the tubes are incubated for 20 min at 25 °C with agitation. Place the tubes in an ice bath at the end of the incubation. Terminate the binding by centrifugation (HS4, 7000 rpm, 4 °C) for 15 min. Return the tubes to ice. Aspirate and then discard the supernatant. Carefully rinse the pellet three times with 1 ml ice-cold buffer, avoiding disruption of the pellet. Transfer the pellet to scintillation vials by vortexing the pellet with 2 ml scintillation fluid, rinse the tubes twice with 2 ml, and add an additional 4 ml scintillation fluid.

Evaluation

Specific binding is determined from the difference of binding in the absence of presence of 10^{-4} M L-glutamic acid and is typically 60–70 % of total binding. IC_{50} values for the competing drug are calculated by log–probit analysis of the data.

Modifications of the Assay

Glutamate (Non Selective)

The assay measures the binding of glutamate, which binds non selectively to ionotropic glutamate receptors including the NMDA, AMPA, and kainate subtypes (Foster and Fagg 1987). In addition, glutamate binds to a family of metabotropic glutamate receptors.

Whole brains (except cerebellum) are obtained from male Wistar rats. A membrane fraction is prepared by standard techniques. Ten mg of membrane preparation is incubated with 1.6 nM [^{3}H]L-glutamate for 10 min at 37 °C. Non-specific binding is estimated in the presence of 50 µM L-glutamate. Membranes are filtered and washed three times to separate bound from free ligand, and filters are counted to determine [^{3}H]L-glutamate bound.

Convulsions induced in mice by intravenous injections of 2.0 mmol/kg L-glutamic acid can be inhibited by glutamate antagonists (Piotrovsky et al. 1991).

Glutamate AMPA

The assay measures the binding of [^{3}H]AMPA (*a*-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid), a selective agonist which binds to the AMPA receptor subtype of glutamate-gated ion channels (Honore et al. 1982; Olsen et al. 1987; Fletcher and Lodge 1995).

Membranes are prepared from male rat brain cortices by standard techniques. Fifteen mg of membrane preparation is incubated with 5 nM [^{3}H]AMPA for 90 min at 4 °C. Nonspecific binding is estimated in the presence of 1 mM L-glutamate. Membranes are filtered and washed three times and the filters are counted to determine [^{3}H]AMPA bound.

Mutel et al. (1998) recommended [^{3}H]Ro 48–8587 as specific for the AMPA receptor.

Fleck et al. (1996) described AMPA receptor heterogeneity in rat hippocampal neurons. AMPA receptor antagonists were described by Kohara et al. (1998), Wahl et al. (1998), Kodama et al. (1999), and Nielsen et al. (1999) and reviewed by Chimirri et al. (1999).

Glutamate Kainate

The assay measures the binding of [^{3}H]kainate, a selective agonist that binds to the kainate subtype of the ionotropic glutamate receptors in rat brain (London and Coyle 1979; Clarke et al. 1997).

Whole brains (except cerebellum) are obtained from male Wistar rats. Fifteen mg of a membrane fraction prepared by standard techniques is incubated with 5.0 nM [^{3}H]kainate for 1 h at 4 °C. Nonspecific binding is estimated in the presence of 1 mM L-glutamate. Membranes are filtered and washed three times to separate free from bound ligand, and filters are counted to determine [^{3}H]kainate bound.

Toms et al. (1997) and Zhou et al. (1997) recommended [^{3}H]-(2S,4R)-4-methylglutamate as kainate receptor selective ligand.

Irreversible inhibition of high-affinity [^{3}H]kainate binding by a photoactivatable analogue was reported by Willis et al. (1997).

Worms et al. (1981) described the behavioral effects of systemically administered kainic acid.

Hu et al. (1998) described neuronal stress and seizure-induced injury in C57/BL mice after systemic kainate administration.

Glutamate NMDA Agonist Site

The assay measures the binding of CGS 19755, a selective antagonist, to the agonist site of the NMDA receptor (Lehmann et al. 1988; Murphy et al. 1988; Jones et al. 1989).

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NMDA Receptor Complex: [^{3}H]TCP Binding

Purpose and Rationale

The purpose of this assay is to determine the binding affinity of potential noncompetitive NMDA antagonists at the phencyclidine (PCP) binding site which is believed to be within or near the NMDA-regulated ion channel. TCP, 1-[1-(2-thienyl)cyclohexyl]-piperidine, is a thienyl derivative of PCP.

Excessive activity of excitatory amino acid neurotransmitters has been associated with the neuropathologies of epilepsy, stroke, and other neurodegenerative disorders (Cotman and Iversen 1987; Watkins and Olverman 1987). Antagonism of NMDA receptor function appears to be the mechanism of action of some novel anticonvulsant and neuroprotective agents. Excitatory amino acid receptors have been classified into at least three subtypes by electrophysiological criteria: NMDA, quisqualic acid (QA), and kainic acid (KA) (Cotman and Iversen 1987; Watkins and Olverman 1987). The binding site for [^{3}H]2-amino-4-phosphonobutyric acid (AP4) may represent a fourth site which is less well characterized. NMDA receptors are believed to be coupled to a cation channel which converts to an open state following activation (Kemp et al. 1987). The opening and closing of this cation channel are also modulated by glycine, Mg^{2+}, Zn^{2+}, and polyamines (Loo et al. 1986; Snell et al. 1987, 1988; Reynolds et al. 1988; Thomson 1989;

Sacaan and Johnson 1989; Thedinga et al. 1989; Williams et al. 1989). Dissociative anesthetics, such as phencyclidine (PCP) and ketamine, and the neuroprotective agent MK-801 block the ion channel and are noncompetitive NMDA receptor antagonists. Competitive NMDA receptor antagonists, such as 3-[(±)-2-carboxypiperazin-4-yl]-1-phosphonic acid (CPP), and the phosphono analogues of L-glutamate, 2-amino-7-phosphonoheptanoic acid (2-AP7), and 2-amino-5-phosphonopentanoic acid (2-AP5) are inhibitors at the excitatory amino acid recognition site.

Molecular cloning and functional expression of rat and mouse NMDA receptors (Moriyoshi et al. 1991; Meguro et al. 1992), a family of AMPA-selective glutamate receptors (Keinänen et al. 1990), and the metabotropic glutamate receptors mGluR1–mGluR6 (Schoepp et al. 1990; Masu et al. 1991; Abe et al. 1992; Bashir et al. 1993; Nakajima et al. 1993; Tanabe et al. 1993) have been reported.

Procedure I

Reagents

1. Buffer A: 0.1 M HEPES, pH 7.5
   Weigh 23.83 g HEPES.
   Add approximately 900 ml distilled water.
   Adjust pH to 7.5 with 10 N NaOH.
   q.s. to 1 l with distilled water.
2. Buffer B: 10 mM HEPES, pH 7.5
   Dilute buffer A 1:10 with distilled water and adjust pH to 7.5.
3. L-glutamic acid, 5×10^{-3} M
   Dissolve 7.36 mg with 10.0 ml distilled water.
   Aliquots of 20 µl to the assay tube will give a final concentration of 10^{-4} M.
4. Glycine, 5×10^{-4} M
   Dissolve 3.75 mg with 10.0 ml distilled water.
   Dilute 1:10 with distilled water.
   Aliquots of 20 µl to the assay tube will give a final concentration of 10^{-5} M.
5. Phencyclidine HCl (PCP) is used for nonspecific binding.
   Dissolve 0.7 mg in 0.5 ml distilled water.
   Aliquots of 20 µl to the assay tube will give a final concentration of 10^{-4} M.
6. [^{3}H]TCP is obtained from New England Nuclear, specific activity 42–60 Ci/mmol. For IC_{50} determinations, a 50 nM stock solution is made with distilled water. Aliquots of 50 µl are added to each tube to yield a final concentration of 2.5 nM.
7. Test compounds. A stock solution of 5 mM is made up with a suitable solvent and serially diluted, such that the final concentration in the assay ranges from 10^{-5} to 10^{-8} M. Higher or lower concentrations may be used, depending on the potency of the drug.

Tissue Preparation

Cerebral cortex of male Wistar rats, 7–10 weeks of age, is dissected over ice and homogenized in ice-cold 0.32 M sucrose, 30 volumes of original tissue weight, for 60 s with a Tissumizer setting at 70. The homogenate is centrifuged at 1000 g for 10 min (SS34, 3000 rpm, 4 °C). The supernatant is centrifuged at 20,000 g for 20 min (SS34, 12,000 rpm, 4 °C). The pellet is resuspended with cold distilled water, to 50 volumes of original tissue weight, using the Tissumizer, 60 s at setting of 70. The homogenate is incubated at 37 °C for 30 min, transferred to centrifuge tubes, and centrifuged at 36,000 g for 20 min (SS34, 16,500 rpm, 4 °C). The pellet is again resuspended in 50 volumes distilled water, incubated and centrifuged. All resuspensions with the Tissumizer are for 60 s at a setting of 70. The resulting pellet is resuspended in 30 volumes of ice-cold 10 mM HEPES buffer, pH 7.5, centrifuged, and washed once again (resuspension and centrifugation) with buffer. Following resuspension in 30 volumes of buffer, the homogenate is frozen in the centrifuge tube and stored at −70 °C until the day of the assay.

On the day of the assay, the homogenate is thawed and centrifuged at 36,000 g for 20 min (SS34, 16,500 rpm, 4 °C). The pellet is washed three times by resuspension with ice-cold 10 mM HEPES buffer, pH 7.5, centrifuged, and finally resuspended in 30 volumes of buffer. Aliquots of 500 µl are used for each assay tube, final volume 1000 µl, and correspond to approximately 0.2 mg protein.

Assay

1. Prepare assay tubes in triplicate. For each test compound, inhibition of [^{3}H]TCP binding is measured both in the absence (basal) and presence (stimulated) of 100 μM L-glutamic acid and 10 mM glycine.

Basal	Stimulated	
380 μl	340 μl	Distilled water
50 μl	50 μl	Buffer A, 0.1 M HEPES, pH 7.5
20 μl	20 μl	PCP (reagent A5) or distilled water, or appropriate concentration of inhibitor
0 μl	20 μl	L-glutamic acid (reagent A3)
0 μl	20 μl	Glycine (reagent A4)
50 μ	50 μl	[^{3}H]TCP (reagent A6)
500 μl	500 μ	Tissue homogenate

2. Following the addition of the tissue, the tubes are incubated for 120 min at 25 °C with agitation. The assay is terminated by separating the bound from nonbound radioligand by rapid filtration with reduced pressure over Whatman GF/B filters, presoaked in 0.05 % polyethyleneimine, using the Brandel cell harvesters. The filters are rinsed once with buffer before filtering the tubes and rinsed two times after filtration. The filters are counted with 10 ml Liquiscint.

Evaluation

Specific binding is determined from the difference of binding in the absence or presence of 10^{-4} M PCP. Specific binding is typically 50 % of total binding in basal conditions and 90 % of total binding when stimulated by L-glutamic acid and glycine. L-glutamic acid and glycine typically increase specific binding to 300 % and 200 % of basal binding, respectively. The combination of L-glutamic acid and glycine typically produce a greater than additive effect, increasing specific binding to 700 % of basal binding. IC_{50} values for the competing drug are calculated by log–probit analysis of the data.

Protocol Modification for Crude Membrane Homogenates

This modified procedure for the preparation of membrane homogenates does not use extensive lysing and washing of the tissue to remove endogenous L-glutamate, glycine, and other endogenous compounds which enhance [^{3}H]TCP binding. This procedure may be used for rapid screening of compounds for inhibition of [^{3}H]TCP binding site without specifically defining an interaction at the ion channel or modulatory sites of the NMDA receptor complex.

Procedure II

Reagents

1. Buffers A and B are prepared as described above.
2. Phencyclidine HCl is used for nonspecific binding and is prepared as described above.
3. [^{3}H]TCP is prepared as described above.
4. Test compounds are prepared as described above.

Tissue Preparation

Cortical tissue is dissected and homogenized in 30 volumes of 0.32 M sucrose, and a crude P_2 pellet is prepared as described above. The pellet is resuspended in 30 volumes of 10 mM HEPES, pH 7.5, centrifuged at 36,000 g (SS34, 16,500 rpm, 4 °C) for 20 min, and again resuspended in 100 volumes of buffer. This homogenate is used directly in the assay in aliquots of 500 μl.

Assay

1. Prepare assay tubes in triplicate.

Volume	Solution
380 μl	Distilled water
50 μl	Buffer A, 0.1 M HEPES, pH 7.5
20 μl	PCP (reagent IA5) or distilled water, appropriate concentration of inhibitor
50 μl	[^{3}H]TCP (reagent IA6)
500 μl	Tissue homogenate

2. Following the addition of the tissue, the tubes are incubated for 120 min at 25 °C with agitation. The assay is terminated by rapid filtration as described above. The filters are rinsed and counted for bound radioactivity as above.

Evaluation

Specific binding is determined from the difference of binding in the presence or absence of 10^{-4} M PCP. Specific binding is typically 90 % of total

binding. IC_{50} values for the competing drug are calculated by log–probit analysis.

Modifications of the Method

Instead of [^{3}H]TCP, radiolabeled [^{3}H]MK-801 has been used as ligand (Wong et al. 1988; Javitt and Zukin 1989; Williams et al. 1989).

Sills et al. (1991) described [^{3}H]CGP 39653 as a *N*-methyl-D-aspartate antagonist radioligand with low nanomolar affinity in rat brain.

Nowak et al. (1995) reported that swim stress increases the potency of glycine to displace 5,7-[^{3}H]dichlorokynurenic acid from the strychnine-insensitive glycine recognition site of the *N*-methyl-D-aspartate receptor complex.

NMDA receptor cloning studies have shown that NMDA receptors contain at least one of seven different NMDAR1 subunits (NR1A–NR1G) (Sugihara et al. 1992) and at least one of four NMDAR2 subunits (NR2A–NR2D) (Kutsuwada et al. 1992; Ishii et al. 1993). While the NR1 subunits are generated by alternative splicing of a single gene, the NR2 subunits are products of four highly homologous genes. Thus, there are thousands of potential subunit combinations yielding complexes of four or five subunits.

Grimwood et al. (1996) reported generation and expression of stable cell lines expressing recombinant human NMDA receptor subtypes, two cell lines expressing NR1A/NR2A receptors, and one cell line expressing NR1A/NR2B receptors.

NR2B selective NMDA antagonists were described by Fischer et al. (1997), Kew et al. (1998), Reyes et al. (1998), and Chenard and Menniti (1999).

For discovery of novel NMDA receptor antagonists, Bednar et al. (2004) developed a high-throughput functional assay based on fluorescence detection of intracellular calcium concentrations. Mouse fibroblasts L(tk-) cells expressing human NR1A/NR2B NMDA receptors were plated in 96-well plates and loaded with fluorescence calcium indicator fluo-3 AM. NR2B antagonists were added after stimulation of NMDA receptors with 10 μM glutamate and 10 μM glycine. Changes in fluorescence after addition of the antagonists were fitted with a single exponential equation providing kobs.

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Metabotropic Glutamate Receptors

Purpose and Rationale

In addition to ionotropic (AMPA, kainate, and NMDA) receptors, glutamate interacts with a second family of receptors, metabotropic or mGlu receptors (Tanabe et al. 1992, 1993; Schoepp

and Conn 1993; Hollmann and Heinemann 1994; Nakanishi and Masu 1994; Okamoto et al. 1994; Watkins and Collingridge 1994; Knöpfel et al. 1995, 1996; Pin and Duvoisin 1995; Conn and Pin 1997; Alexander et al. 2001; Skerry and Genever 2001; DeBlasi et al. 2001; Pin and Acher 2002; Conn 2003). Three groups of native receptors are distinguishable on the basis of similarities of agonist pharmacology, primary sequence, and G protein-effector coupling: Group I ($mGlu_1$ and $mGlu_5$ and splice variants) are coupled via $G_{q/11}$ to phosphoinositide hydrolysis. Group II ($mGlu_2$ and $mGlu_3$) are negatively coupled via Gi/Go to adenylyl cyclase and inhibit the formation of cAMP following exposure of cells to forskolin or activation of an intrinsic G protein-coupled receptor (e.g., adenosine A_2 receptor). Group III receptors ($mGlu_4$, $mGlu_6$, $mGlu_7$, and $mGlu_8$) also inhibit forskolin-stimulated adenylyl cyclase.

Various agonists and antagonists for metabotropic glutamate receptors were described (Ishida et al. 1990, 1994; Porter et al. 1992; Jane et al. 1994; Watkins and Collingridge 1994; Knöpfel et al. 1995; Annoura et al. 1996; Bedingfield et al. 1996; Thomsen et al. 1996; Acher et al. 1997; Doherty et al. 1997; Brauner-Osborne et al. 1998; Kingston et al. 1998; Monn et al. 1999; Jane and Doherty 2000). Schoepp et al. (1999) reviewed pharmacological agents acting at subtypes of metabotropic glutamate receptors. Gasparini et al. (2002) described allosteric modulators of group I metabotropic glutamate receptors as novel subtype-selective ligands and their therapeutic perspectives.

Several radioligands for metabotropic glutamate receptors were described:

- For subtype mGluR4a receptor by Eriksen and Thomsen (1995),
- For group II mGlu receptors by Cartmell et al. (1998), by Ornstein et al. (1998), and by Schaffhauser et al. (1998).

Riedel and Reymann (1996) discussed the role of metabotropic glutamate receptors in hippocampal long-term potentiation and long-term depression and their importance for learning and memory. Furthermore, possible roles in the treatment of neurodegenerative disorders (Nicoletti et al. 1996; Bruno et al. 1998) and of Parkinson's disease (Konieczny et al. 1998) were discussed. Anticonvulsive properties (Atwell et al. 1998; Thomsen and Dalby 1998; Gasparini et al. 1999) as well as anxiolytic properties (Helton et al. 1998) of metabotropic glutamate receptor ligands were reported. Christoffersen et al. (1999) found a positive effect on short-term memory and a negative effect on long-term memory of the class I metabotropic glutamate receptor antagonist, AIDA, in rats.

Procedure

Cultured cells are prepared from the cerebral cortex of 17-day-old embryos of Wistar rats. Prior to the experiments, the culture is maintained for 8–12 days with minimum essential medium (MEM) containing 5 % fetal calf serum and 5 % horse serum.

For **cyclic AMP assays**, the cultured cellsx are preincubated with HEPES-buffered Krebs–Ringer solution containing 5.5 mM glucose (HKR) for 1–1.5 h, then exposed to various agonists for 15 min in the absence or presence of 10 μM forskolin. The content of cyclic AMP is measured using a radioimmunoassay kit after homogenization with 0.1 M HCl.

For **phosphoinositide turnover assays**, the cultured cells are prelabeled with myo-1,2-[^{3}H] inositol in MEM for 8–10 h. The cells are washed twice with HKR containing 10 mM LiCl and then exposed to various agonists in HKR containing 10 mM LiCl for 30 min. The reaction is terminated with 2 % trichloroacetic acid, and the homogenized samples are analyzed for inositol constituents by anion exchange chromatography (Berridge et al. 1982). The extracts are applied to columns containing 1 ml of Dowex 1 in the formate form. The phosphate esters are then eluted by the stepwise addition of solutions containing increasing concentrations of formate. Glycerophosphoinositol and inositol 1:2-cyclic phosphate are eluted with 5 mM sodium tetraborate plus 150 mM sodium formate. The penultimate solution contains 0.1 M formic acid plus 0.3 M ammonium formate, followed by 0.1 M formic acid plus 0.75 M ammonium

formate, each of which removes more polar inositol phosphates. The 1 ml fractions eluted from the columns are counted for radioactivity after addition of 10 ml of Biofluor.

The percentage of radioactivity of inositol phosphates to the total applied to the column is calculated.

Evaluation

Dose–response curves for inhibition of forskolin-stimulated cAMP formation and for percentage of phosphoinositide hydrolysis are established for each test compound.

Modifications of the Method

Thomsen et al. (1993, 1994) used baby hamster kidney (BHK) cells stably expressing $mGluR_{1\alpha}$, $mGluR_2$, or $mGluR_4$ for measurements of phosphoinositol hydrolysis or cAMP formation.

Varney and Suto (2000) recommended functional high throughput screening assay for the discovery of subtype-selective metabotropic glutamate receptor ligands.

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Excitatory Amino Acid Transporters

Purpose and Rationale

Glutamate is not only the predominant excitatory neurotransmitter in the brain but also a potent neurotoxin. Following release of glutamate from presynaptic vesicles into the synapse and activation of a variety of ionotropic and metabotropic glutamate receptors, glutamate is removed from the synapse. This is achieved through active uptake of glutamate by transporters located presynaptically but also postsynaptically, or glutamate can diffuse out of the synapse and be taken up by transporters located on the cell surface of glial cells. The excitatory amino acid transporters form a gene family out of which at least five subtypes were identified (Robinson et al. 1993; Seal and Amara 1999). A role for glutamate transporters has been postulated for acute conditions

such as stroke, CNS ischemia, and seizure, as well as in chronic neurodegenerative diseases, such as Alzheimer's disease and amyotrophic lateral sclerosis. Glutamate transport is coupled to sodium, potassium, and pH gradients across the cell membrane creating an electrogenic process. This allows transport to be measured using electrophysiological techniques (Vandenberg et al. 1997).

Procedure

Complementary DNAs encoding the human glutamate transporters, EAAT1 and EAAT2, are subcloned into pOTV for expression in *X. laevis oocytes* (Arriza et al. 1994; Vandenberg et al. 1995). The plasmids are linearized with BamHI, and cRNA is transcribed from each of the cDNA constructs with T7 RNA polymerase and capped with 5',7-methyl guanosine using the mMESSAGE mMACHINE (Ambion, Austin, TX). cRNA (50 ng) encoding either EAAT1 or EAAT2 is injected into defolliculated Stage 5 *X. laevis oocytes*. Two to 7 days later, transport is measured by two-electrode voltage-clamp recording using a GeneClamp 500 amplifier (Axon Instruments, Foster City, CA) and a MacLab 2e recorder (ADInstruments, Sydney, Australia) and controlled using a pCLAMP 6.01 interfaced to a Digidata 1200 (Axon Instruments). Oocytes are voltage-clamped at – 60 mV and continuously superfused with ND96 buffer (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.5). For transport measurement, this buffer is changed to one containing the indicated concentration of substrate and/or blocker. The voltage dependence of block of glutamate transport is measured by clamping the membrane potential at −30 mV and then applying a series of 100 ms voltage pulses from −100 to 0 mV and measuring the steady-state current at each membrane potential. This protocol is applied both before and during the application of the compound in question and then the baseline current at each membrane potential is subtracted from the current in the presence of the compounds to get a measure for the transport-specific current at the various membrane potentials.

Evaluation

Current (I) as a function of substrate concentration ([S]) is fitted by least squares to

$$I = I_{\max}[\mathrm{S}]/(K_{\mathrm{m}} + [\mathrm{S}])$$

where $I_{\max}$ is the maximal current and K_{m} is the Michaelis transport constant. The $I_{\max}$ values for the various substrates are expressed relative to the current generated by a maximal dose of L-glutamate in the same cell. $I_{\max}$ and K_{m} values are expressed as mean ± standard error and are determined by fitting data from individual oocytes. The potent competitive blockers are characterized by Schild analysis (Arunlakshana and Schild 1959) and the K_{b} estimated from the regression plot. The less potent blockers are assumed to be competitive, and K_{i} values calculated from IC_{50} values using the equation

$$K_{\mathrm{i}} = IC_{50}/(1 + [\mathrm{glutamate}]/K\mathrm{m})$$

where K_{i} is the inhibition constant, IC_{50} is the concentration giving half maximum inhibition, K_{m} is the transport constant, and [glutamate] is 30 μM. The fraction of the membrane electric field sensed by transport blockers when bound to the transporters is estimated using the Woodhull equation (Woodhull 1973),

$$K_{\mathrm{i}} = K_{\mathrm{i}}^{0}\exp(-\zeta\delta FE/RT)$$

where K_{i} is the inhibition constant, K_{i}^{0} is the inhibition constant at 0 mV, ζ is the charge on the blocker, δ is the fraction of the membrane field, F is Faraday's constant, E is the membrane potential, R is the gas constant, and T is temperature in K.

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[^{35}S]TBPS Binding in Rat Cortical Homogenates and Sections

Purpose and Rationale

To screen potential anticonvulsant agents which interact at the convulsant binding site of the benzodiazepine/GABA/chloride ionophore complex by measuring the inhibition of binding of [^{35}S]TBPS to rat cortical membranes.

TBPS, t-butylbicyclophosphorothionate, is a potent convulsant which blocks GABAergic neurotransmission by interacting with the convulsant (or picrotoxin) site of the GABA/benzodiazepine/chloride ionophore receptor complex (Casida et al. 1985; Gee et al. 1986; Olsen et al. 1986; Squires et al. 1983; Supavilai and Karabath 1984). Picrotoxin, pentylenetetrazol, and the so-called cage convulsants are believed to change the state of the chloride channel to a closed conformation and thereby block GABA-induced increases in chloride permeability. Anticonvulsants, such as the barbiturates and the pyrazolopyridines, cartazolate, etazolate, and tracazolate, appear to interact at depressant sites allosterically coupled to the convulsant sites and facilitate the effects of GABA on chloride permeability, by converting the ionophore to the open conformation. Benzodiazepines interact at a separate recognition site to modulate the actions of GABA. Convulsant compounds and some anticonvulsants can inhibit [^{35}S]TBPS binding. These two classes can be differentiated by their effects on dissociation kinetics (Macksay and Ticku 1985; Trifiletti et al. 1984, 1985). [^{35}S]TBPS dissociates slowly, half-life approximately 70 min, in a monophasic manner in the presence of convulsant compounds; anticonvulsants produce a biphasic dissociation, with rapid and slow-phase components. It has been postulated that the rapid and slow phases of [^{35}S]TBPS dissociation may correspond to the open and closed conformation of the chloride ionophore.

Procedure

Reagents

1. Buffer A: 0.05 M Tris with 2 M KCl, pH 7.4
   6.61 g Tris HCl
   0.97 g Tris base
   149.1 g KCl
   q.s. to 1 l with distilled water
2. Buffer B
   A 1:10 dilution of buffer A in distilled water (5 mM Tris, 200 mM KCl, pH 7.4)
3. [^{35}S]TBPS is obtained from New England Nuclear with a high initial specific activity, 90–110 Ci/mmol. For an inhibition assay with a 2 nM final concentration of TBPS, a specific activity of 20–25 Ci/mmol will provide sufficient counts due to a high counting efficiency (87 %) for ^{35}S. The specific activity of [^{35}S]TBPS can be reduced with the addition of 3–5 volumes (accurate measurement with a Hamilton syringe) of an equimolar ethanolic solution of non-radiolabeled TBPS (7.9×10^{-6} M). The new specific activity (Ci/mmol)

is calculated by dividing the number of curies by the number of mmols TBPS. Since [^{35}S] TBPS has a relatively short half-life, 87.1 days, the specific activity is calculated for each assay, based on the exponential rate of decay:

A_0 = initial specific activity

A = specific activity at time t

t = days from date of initial calibration of specific activity

$t_{1/2}$ = half-life of [^{35S}] in days (87.1)

For IC_{50} determinations, a 40 nM stock solution is made with distilled water and 25 µl is added to each tube to yield a final concentration of 2 nM in the assay.

4. Unlabeled TBPS is available from New England Nuclear. A stock dilution of 7.923×10^{-6} M is prepared in ethanol.
5. Picrotoxin is obtained from Aldrich Chemical Company. A solution of 5×10^{-4} M is prepared with distilled water, with sonication if necessary. Aliquots of 10 µl are added to assay tubes to give a final concentration of 10^{-5} M.
6. Test compounds. A stock solution of 1 mM is made up with a suitable solvent and serially diluted, such that the final concentration in the assay ranges from 10^{-5} to 10^{-8} M. Higher or lower concentrations may be used, depending on the potency of the drug.

Tissue Preparation

The whole cerebral cortex of male Wistar rats is dissected over ice and homogenized with a Tekmar Tissumizer, 20 s at setting 40, in 20 volumes of 0.32 M sucrose, ice-cold. The homogenate is centrifuged at 1000 g for 10 min (SS34, 3000 rpm, 4 °C). The supernatant is then centrifuged at 40,000 g for 30 min (SS34, 20,000 rpm, 4 °C). The resulting pellet is resuspended in 20 volumes of ice-cold distilled water with two 6-s bursts of the Tissumizer, setting 40. The homogenate is centrifuged at 40,000 g for 30 min. The pellet is washed (resuspended and centrifuged) once with 20 volumes ice-cold buffer (Tris HCl 5 mM, KCl 200 mM, pH 7.4). The resulting pellet is resuspended with 20 volumes buffer and frozen at −70 °C overnight. The following day, the tissue homogenate is thawed in a beaker of warm water, approximately 15 min, and then centrifuged at 40,000 g for 30 min (SS34, 20,000 rpm, 4 °C). The pellet is washed twice with 20 volumes of ice-cold buffer, and then resuspended and frozen at −70 °C for future use. On the day of the assay, the homogenate is thawed and centrifuged at 40,000 g for 30 min. The resulting pellet is washed once with 20 volumes ice-cold buffer and finally resuspended in 30 volumes buffer. Aliquots of 250 µl are used for each assay tube, final volume 500 µl, and correspond to 8.35 mg original wet weight tissue per tube, approximately 0.2 mg protein.

Assay

1. Prepare assay tubes in triplicate:
   190 µl distilled water
   25 µl Tris 0.05 M, KCl 2 M, pH 7.4
   10 µl picrotoxin, 10^{-5} M final concentration or distilled water or inhibitor 25 µl [^{35S}] TBPS, final concentration 2 nM
   250 µl tissue preparation, 1:30 homogenate
2. Following the addition of the tissue, the tubes are incubated at 25 °C for 150 min with agitation. The assay is terminated by rapid filtration over Whatman GF/B filter circles, presoaked in buffer, with 5 × 4 ml rinses of ice-cold buffer. Vacuum filtration is performed with the 45-well filtration units to avoid contamination of the Brandel harvesters with [^{35}S]. The filters are counted with 10 ml Liquiscint.

Evaluation

Specific binding is determined from the difference between binding in the absence or presence of 10 mM picrotoxin and is typically 85–90 % of total binding. The percent inhibition at each drug concentration is the mean of triplicate determinations. IC_{50} values for the competing drug are calculated by log–probit analysis of the data.

Modifications for Dissociation Experiments

1. Prepare assay tubes as follows:
   185 µl distilled water
   25 µl Tris 50 mM, KCl 2 M, pH 7.4
   10 µl test compound or vehicle
2. Add 250 ml tissue homogenate to tube s. Vortex. Preincubate 30 min at 25 °C.

3. Add 25 ml [^{35}S]TBPS. Vortex. Incubate 180 min at 25 °C.
4. Add 5 ml picrotoxin (10^{-3} M) to give a final concentration of 10^{-5} M. Vortex.
5. At various times after the addition of picrotoxin (0–120 min), tubes are filtered and rinsed as described above.

Modification for [^{35}S]TBPS Autoradiography

1. Sections of rat brain, 20 mm thickness, are collected onto gel-chrome alum-subbed slides, freeze-dried for approximately 1 h, and stored at −70 °C until used.
2. After thawing and drying at room temperature, the sections are preincubated for 30 min in buffer B.
3. Preparation of slide mailers for incubation:
   (a) For scintillation counting:
   2.47 ml distilled water
   0.325 ml buffer A
   3.25 ml buffer B 0.13 ml picrotoxin, 10^{-5} M final concentration or distilled water or inhibitor
   0.325 ml [^{35}S]TBPS, final concentration 2 nM
   6.50 ml final volume
   (b) For autoradiography:
   4.56 ml distilled water
   0.60 ml buffer A
   6.00 ml buffer B
   0.24 ml picrotoxin, 10^{-5} M final concentration or distilled water or inhibitor
   0.60 ml [^{35}S]TBPS, final concentration 2 nM
   12.0 ml final volume
4. Sections are incubated in slide mailers at room temperature with [^{35}S]TBPS in the absence or presence of appropriate inhibitors for 90 min.
5. Slides are transferred to vertical slide holders and rinsed in ice-cold solutions as follows: dip in buffer B, two 5 min rinses in buffer A and a dip in distilled water.
6. Slides are dried under a stream of cool air and desiccated overnight at room temperature.
7. Slides are mounted onto boards with appropriate [^{35}S] brain mash standards.
8. In the dark room under safelight illumination (GBX filter), slides are opposed to Kodak X-OMAT AR film and stored in cassettes for 7–10 days.
9. Develop films as described in "X-OMAT AR Film Processing."

	[^{35}S]TBPS binding parameters	
	Slide-mounted sections	Cortical homogenates
Assay conditions		
Tissue	20 p sections, rat freeze-dried, 1 h	Whole cortex, rat 1:30 homogenate prepared with five washes and two freeze-thaw cycles
	30 min	No preincubation
	Preincubation	
Buffer	5 mM Tris, 200 mM KCl, pH 7.4	5 mM Tris, 200 mM KCl, pH 7.4
Incubation time	90 min, 21–22 °C	150 min, 25 °C
Nonspecific	10^{-5} M picrotoxin	10^{-5} M picrotoxin
Tissue linearity	2.5–25 mg tissue per 0.5 ml assay tube	
Equilibrium constants		
KD (nM)	32.8	25.2
Bmax	1615	2020
(fmol/mg prot)		
Binding kinetics		
Association kobs (min^{-1})	0.0496	0.0138
k + 1 (nM^{-min-1})	0.0164	0.0021
Dissociation k–1 (min^{-1})	0.017	0.001
Dissociation constant	1.03	4.73
k + 1/k – 1 (nM) IC_{50}M		
Picrotoxin	2.8×10^{-7}	3.4×10^{-7}
TBPS	8.7×10^{-8}	8.1×10^{-8}
GABA	1.7×10^{-6}	2.1×10^{-6}
Pentobarbital	1.2×10^{-4}	6.0×10^{-4}
Phenobarbital	None at 10^{-3}	None at 10^{-3}
Clonazepam	None at 10^{-6}	None at 10^{-6}

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[^{3}H]glycine Binding in Rat Cerebral Cortex

Purpose and Rationale

The amino acid glycine is a major inhibitory transmitter in the vertebrate system. Glycinergic synapses are particularly abundant in spinal cord and brain stem, but are also found in higher regions, including the hippocampus. The inhibitory actions of glycine are potently blocked by strychnine. Glycine modulates and may activate the excitatory amino acid receptors of the NMDA subtype (Thomson 1989; Laube et al. 2002).

The strychnine-sensitive, postsynaptic glycine receptor is a ligand-gated chloride channel protein that belongs to the nicotinic acetylcholine receptor family. It is a pentameric transmembrane protein composed of α and β subunits (Lynch 2004).

Glycine has been shown in vitro to potentiate the effects of L-glutamate or NMDA on the stimulation of [^{3}H]TCP binding (Snell et al. 1987, 1988; Bonhaus et al. 1989) and [^{3}H]norepinephrine release (Ransom and Deschenes 1988) and in vivo to act as a positive modulator of the glutamate-activated cGMP response in the cerebellum (Danysz et al. 1989; Rao et al. 1990). The activation of NMDA receptors requiring the presence of glycine is necessary for the induction of long-term potentiation (LTP), a type of synaptic plasticity which may be fundamental to learning processes (Oliver et al. 1990). A [^{3}H]glycine binding site in the brain has been identified and characterized as a strychnine-insensitive site associated with the NMDA receptor complex (Kessler et al. 1989; Monahan et al. 1989; Cotman et al. 1987). Autoradiographic studies have shown a similar distribution of [^{3}H]glycine and [^{3}H]TCP (NMDA ion channel radioligand) binding sites (Jansen et al. 1989). Compounds which interact with the glycine site offer a novel mechanism of action for intervention with NMDA receptor function.

Schmieden and Betz (1995) reviewed the pharmacology of the inhibitory glycine receptor, the agonist and antagonist actions of amino acids, and piperidine carboxylic compounds.

Hyperekplexia is a hereditary neurological disorder in humans characterized by an excessive

startle response which can be caused by mutations in the α1 subunit of the heteropentameric inhibitory glycine receptor (Rees et al. 2002). Becker et al. (2002) generated transgenic mice resembling this disease.

The following assay is used to assess the affinity of compounds for the glycine binding site associated with the *N*-methyl-D-aspartate (NMDA) receptor complex using [^{3}H]glycine as the radioligand.

Procedure

Reagents

1. Buffer A: 0.5 M Tris maleate, pH 7.4 59.3 g Tris maleate
   q.s. to 0.5 l
   Adjust pH to 7.4 with 0.5 M Tris base.
2. Buffer B: 50 mM Tris maleate, pH 7.4
   Dilute buffer A 1:10 with distilled water; adjust pH with 50 mM Tris maleate (acid) or 50 mM Tris base.
3. Glycine, 5×10^{-2} M
   Dissolve 3.755 mg of glycine (Sigma G7126) with 1.0 ml distilled water. Aliquots of 20 µl to the assay tube will give a final concentration of 10^{-3} M.
4. [^{3}H]Glycine is obtained from New England Nuclear, specific activity 45–50 Ci mmol. For IC_{50} determinations, a 200 nM stock solution is made with distilled water. Aliquots of 50 µl are added to yield a final assay concentration of 10 nM.
5. Test compounds. A stock solution of 5 mM is prepared with a suitable solvent and serially diluted, such that the final concentrations in the assay ranges from 10^{-4} to 10^{-7} M. Higher or lower concentrations may be used, depending on the potency of the compound.
6. Triton X-100,10 % (v/v) (National Diagnostics, EC606). A stock solution of Triton X-100, 10 %, can be prepared and stored in the refrigerator. Dilute 1.0 ml of Triton X-100 to 10.0 ml with distilled water. On the day of the assay, the tissue homogenate (1:15 dilution) is preincubated with an aliquot of the 10 % solution to give a final concentration of 0.04 % (v/v).

Tissue Preparation

Cortices of male Wistar rats are dissected over ice and homogenized in ice-cold 0.32 M sucrose, 15 volumes of original wet weight of tissue, for 30 s with a Tissumizer setting at 70. Three cortices are pooled for one preparation. The homogenate is centrifuged at 1000 g for 10 min (SS34, 3000 rpm, 4 °C). The supernatant is centrifuged at 20,000 g (SS34, 12,000 rpm, 4 °C) for 20 min. Resuspend the pellet in 15 volumes of ice-cold distilled water (Tissumizer setting 70, 15 s) and spin at 7600 g (SS34, 8000 rpm 4 °C) for 20 min. The pellet is resuspended with 15 volumes of cold distilled water and centrifuged. Discard the supernatant and store the pellet at −70 °C.

On the day of the assay, the pellet is resuspended in 15 volumes ice-cold 50 mM Tris maleate, pH 7.4. Preincubate the homogenate with Triton X-100 in a final concentration of 0.04 % (v/v) for 30 min at 37 °C with agitation. Centrifuge the suspension at 48,000 g (SS34, 20,000 rpm, 4 °C) for 20 min. Wash the pellet an additional three times by resuspension with cold buffer and centrifugation. The final pellet is resuspended in a volume 25 times the original wet weight.

Assay

1. Prepare assay tubes in quadruplicate.
   380 µl distilled water
   50 µl buffer A, 0.5 M Tris maleate, pH 7.4
   20 µl glycine, 10^{-3} M final concentration, or distilled water or appropriate concentration of inhibitor
   50 µl [^{3}H] glycine, final concentration 10 nM
   500 µl tissue homogenate.
   1000 µl final volume
2. Following the addition of the tissue, the tubes are incubated for 20 min in an ice bath at 0–4 °C. The binding is terminated by centrifugation (HS4, 7000 rpm, 4 °C) for 20 min. Aspirate and discard the supernatant. Carefully rinse the pellet twice with 1 ml ice-cold buffer, avoiding disruption of the pellet. Transfer the pellet to scintillation vials by vortexing the pellet with 2 ml scintillation fluid, rinse the tubes twice with 2 ml, and add an additional 4 ml scintillation fluid.

Evaluation

Specific binding is determined from the difference of binding in the absence or in the presence of 10^{-4} M glycine and is typically 60–70 % of total binding. IC_{50} values for the competing compound are calculated by log–probit analysis of the data.

Modifications of the Method

Baron et al. (1996), Hofner and Wanner (1997), Chazot et al. (1998) described [^{3}H]MDL 105,519 as a high-affinity ligand for the NMDA associated glycine recognition site.

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[^{3}H]Strychnine-Sensitive Glycine Receptor

Purpose and Rationale

The strychnine-sensitive glycine receptor is a member of the family of ligand-gated ion channel receptors. Within this family, the glycine receptor is most closely related to the GABA receptor. Like the $GABA_A$ receptor, the glycine receptor has an inhibitory role, mediating an increase in chloride conductance. However, in contrast to the $GABA_A$ receptor, the glycine receptor is located mainly in the spinal cord and lower brainstem, where glycine appears to be the major inhibitory neurotransmitter. Purification and molecular cloning has shown that the glycine receptor is an oligomeric transmembrane protein complex composed of three α and two β subunits. The inhibitory actions of glycine are potently blocked by strychnine. In addition to strychnine, the steroid derivative RU5135 (Simmonds and Turner 1985), phenylbenzene-α-phosphono-a-amino acid (Saitoh et al. 1996), and 5,7-dichloro-4hydroxyquinoline-3-carboxylic acid (Schmieden et al. 1996) antagonize glycine responses in cultured neurons or cells expressing recombinant glycine receptors.

A glycine receptor agonist may be a potential antispastic agent.

Procedure

Male Wistar rats weighing about 200 g are sacrificed. About 220 mg of frozen pons and medulla are homogenized in 2 × 10 ml ice-cold 50 mM potassium phosphate buffer, pH 7.1, by an Ultra-Turrax homogenizer. The homogenate is centrifuged for 10 min at 30,000 g at 0–4 °C in a refrigerated centrifuge. The pellet is rehomogenized in another 2 × 10 ml portion of

the same buffer and recentrifuged as before. This washing procedure is repeated a total of four times. The final pellet is resuspended in 200 vol/g original tissue in ice-cold 50 mM potassium phosphate buffer, pH 7.1, with or without 1000 mM NaCl, and used directly for binding assays.

Binding assays consist of 1 ml tissue homogenate, 50 µl test solution (water or 5 % v/v ethanol/water is used for serial dilutions), 50 µl water, 5 % ethanol/water or glycine solution (40 mM final concentration), and 25 µl [^{3}H]strychnine working solution, final concentration 2 nM. The samples are mixed well and incubated for 20 min in an ice bath. Free and bound radioactivity are separated by filtration through Whatman GF/C glass fiber filters followed by washing with 2 × 10 ml ice-cold 50 mM potassium phosphate buffer, pH 7.1. Tritium on the filters is monitored by conventional scintillation counting in 3 ml Hydroluma. Nonspecific binding is binding in the presence of 40 mM glycine and is always subtracted from total binding to give specific binding.

Evaluation

K_i values are calculated as

$$K_i = (IC_{50}/1 + [K_D]/[L])$$

whereby IC_{50} are the concentrations that inhibit by 50 % the specific binding of [^{3}H]strychnine determined in two independent experiments using at least three concentrations of the agent in duplicate assays, [L] is the concentration of the radioligand, and K_D is the affinity constant in the absence or the presence of 1000 mM NaCl.

NaCl shift used for differentiating glycine agonists from glycine antagonists is the ratio K_i 1000 mM NaCl versus K_i 0 mM NaCl.

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Electrical Recordings from Hippocampal Slices In Vitro

Purpose and Rationale

The transverse hippocampal slice has been described as a well-defined cortical structure maintained in vitro (Skrede and Westgard 1971). The hippocampus slice has the advantage that each slice may contain all hippocampal structures: The chain of neurons goes from the perforant path to granule cells of the dentate gyrus, through mossy fibers to CA3 pyramidal cells and then through Schaffer collaterals to CA1 cells with their axons leaving the hippocampus through the alveus. The pyramidal cells lie close together and can be easily seen and penetrated with fine microelectrodes.

Procedure

Male guinea pigs weighing 300–400 g are anesthetized with ether, the brains removed, and the hippocampi dissected. Transverse slices of the hippocampus (300–400 pm thick) are cut in parallel to the alvear fibers. After preparation, the slices are submerged in 28 °C warm saline which is equilibrated with 95 % O_2 and 5 % CO_2. After a preincubation period of 2 h, slices are transferred in a Perspex chamber (1.5 × 4 cm) and attached to the bottom consisting of optically plain glass. The chamber is mounted on an inverted microscope allowing detailed inspection of the excised tissue. The slices are superfused by an approximately 3-mm-thick layer of 32 °C warm saline. Intracellular recordings are achieved by means of micropipettes with tip diameters of less than 0.5 pm which are filled with 3 mol/l potassium chloride. Under microscopic control, the tips of the micropipettes are placed within the stratum pyramidale and moved by means of a step motor-driven hydraulic microdrive. For intracellular injections of drugs, e.g., pentylenetetrazol, via the recording microelectrode, a passive bridge is used. Alternatively, drugs are added to the incubation bath.

Evaluation

The resting membrane potential and paroxysmal depolarizations are recorded before and after application of drugs.

Critical Assessment of the Method

The hippocampal slice has been one of the most useful models for the study of basic mechanisms underlying the epilepsies. The model has also been recommended for screening of putative anticonvulsant drugs.

Modifications of the Method

Harrison and Simmonds (1985) performed quantitative studies on some antagonists of *N*-methyl-D-aspartate in slices of rat cerebral cortex consisting of cerebral cortex and corpus callosum.

Tissue culture models of epileptiform activity were described by Crain (1972).

Oh and Dichter (1994) studied the effect of a GABA uptake inhibitor on spontaneous postsynaptic currents in cultured rat hippocampal neurons by the whole-cell patch-clamp method.

Blanton et al. (1989) described whole-cell recordings from neurons in slices of reptilian and mammalian cerebral cortex. Synaptic currents and membrane properties could be studied in voltage and current clamps in cells maintained within their endogenous synaptic currents.

Gähwiler (1988) and Stoppini et al. (1991) described methods for organotypic cultures of nervous tissue. Hippocampal slices from 2 to 23-day old rats were maintained in culture at the interface between air and the culture medium. They were placed on a sterile, transparent, and porous membrane and kept in Petri dishes in an incubator. This yielded thin slices which remained

one to four layers thick and were characterized by a well-preserved organotypic organization. Excitatory and inhibitory synaptic potentials could be analyzed using extra- or intracellular recording techniques. After a few days in culture, long-term potentiation of synaptic responses could reproducibly be induced.

Using this method, Liu et al. (1995) studied dopaminergic regulation of transcription factor expression in organotypic cultures of developing striatum of newborn rats.

Stuart et al. (1993) reported the implementation of infrared differential interference contrast video microscopy to an upright compound microscope and a procedure for making patch pipette recordings from visually identified neuronal somata and dendrites in brain slices.

Bernard and Wheal (1995) described an ex vivo model of chronic epilepsy using slices of rat hippocampus previously lesioned by stereotactic injections of kainic acid. Extracellular population spikes were recorded from the stratum pyramidale of CA1 after stimulation by bipolar twisted wire electrodes placed in the stratum radiatum of CA1 area proximally to stratum pyramidale near the recording electrode.

Using hippocampal slices prepared from brain tissue of patients undergoing neurosurgery for epilepsy, Schlicker et al. (1996) showed that the serotoninergic neurons of the human hippocampus are endowed with presynaptic inhibitory autoreceptors.

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Electrical Recordings from Isolated Nerve Cells

Purpose and Rationale

The use of the cell-attached patch-clamp configuration to record action potential currents has shown to have utility in the testing for drug actions on ion channels in excitable cell membranes (Kay and Wong 1986; McLarnon and Curry 1990; McLarnon 1991).

Procedure

Preparation of Cultured Cells

The cultured cells are obtained from the hippocampus or the hypothalamus of rat brain. The isolation of the hippocampal CA1 neurons is performed according to the procedure of Banker and Cowan (1977). The dissociated hypothalamic neurons are prepared according to Jirikowski et al. (1981). The hippocampal and hypothalamic neurons that are selected for electrophysiological recording are bipolar in shape with the long axis dimension between 10μm and 15 μm. The neurons are studied over a period of 5–10 days after isolation.

Electrophysiology

The cell-attached patch-clamp configuration is used to record spontaneous action potentials in the cultured neurons. The bath solution contains 140 mM NaCl, 5 mM KCl, 0.5 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.3. The composition of the patch pipette solution is the same as the bath solution. The drugs used in the experiments are added to the bath solution.

The patch pipettes (Corning 7052 glass) fabricated using a specific patch pipette puller (PP-83; Narishige, Tokyo) are fire-polished and filled immediately prior to use. The resistance of the pipettes is in the range 4–8 MΩ and the tip diameters are between 1 and 2 pm. An axopatch amplifier (Axon Instruments, Foster City, CA), with low-pass filter set at 5 kHz, is used to record the capacitative currents. After recording, at a sampling frequency of 5 kHz, the data are stored on hard disk or video tape for subsequent analysis. All data are obtained at room temperature (21–24 °C).

Evaluation

The capacitative component of current recorded by the patch pipettes is proportional to the rate of change of membrane potential and can be expressed as $I_C = CdV/dt$, where C is the specific membrane capacitance. Assuming a value of C of 1 μF/cm^2 and a tip diameter of the patch pipette of 2 μm, the membrane area isolated by the patch pipette is about 3×10^{-8} cm^2. Using a value of d V/d t of 100 mV/ms gives an approximate expected magnitude of I_C near 3 pA. When a class III antiarrhythmic drug that blocks a delayed rectifier K^+ channel is added to the bath, the portion of I_C corresponding to the after-hyperpolarization component of the action potential is completely abolished. The Na^+ spike is not altered by the drug. The cell-attached recordings of I_C can also be used to determine effects on the Na^+ spike when tetrodotoxin is included in the bath solution. Thus, the spontaneous action potential can be used for evaluation of drug effects on both K^+ and Na^+ channels in excitable membrane.

Modifications of the Method

Chen et al. (1990) measured current responses mediated by $GABA_A$ receptors in pyramidal cells acutely dissociated from the hippocampus of mature guinea pigs according to the procedure of Kay and Wong (1986) using whole-cell voltage-clamp recordings.

Caulfield and Brown (1992) studied inhibition of calcium current in NG108–15 neuroblastoma cells by cannabinoid receptor agonists using whole-cell voltage-clamp recordings.

Gola et al. (1992, 1993) performed voltage recordings on non-dissociated sympathetic neurons from rabbit coeliac ganglia using the whole-cell configuration of the patch-clamp technique (Neher and Sakmann 1976; Sakmann and Neher 1983).

Stolc (1994) used the voltage-clamp technique in internally dialyzed single neurons isolated from young rat sensory ganglia to study the effects of pyridoindole stobadine on inward sodium and calcium currents and on slow non-inactivating components of potassium outward current.

McGivern et al. (1995) examined the actions of a neuroprotective agent on voltage dependent Na^+ currents in the neuroblastoma cell line, NIE-115, using the whole cell variant of the patch-clamp technique.

Smith (1995) reviewed the use of patch and voltage-clamp procedures to study neurotransmitter transduction mechanisms.

Using whole-cell and perforated-patch recordings, Delmas et al. (1998) examined the part played by endogenous G protein $\beta\gamma$ subunits in neurotransmitter-mediated inhibition of N-type Ca^{2+} channel current in dissociated rat superior cervical sympathetic neurons.

Gonzales et al. (1985) registered membrane potentials with intracellular electrodes in cultured olfactory chemoreceptor cells.

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Isolated Neonatal Rat Spinal Cord

Purpose and Rationale

The spinal cord of the neonatal rat is a useful in vitro preparation, originally proposed by Otsuka and Konishi (1974). In this preparation, ventral root potentials of ten seconds of duration can be recorded after supramaximal electrical stimulation of the lumbar dorsal root. Variously implicated in the generation of these slow ventral root potentials are tachykinins, such as substance P and neurokinin B (Yanagisawa et al. 1982; Akagi et al. 1985; Otsuka and Yanagisawa 1988; Guo et al. 1998) and agonists at the glutamate receptor sites (Evans et al. 1982; Ohno and Warnick 1988, 1990; Shinozaki et al. 1989; Ishida et al. 1990, 1991, 1993; Woodley and Kendig 1991; Bleakman et al. 1992; King et al. 1992; Thompson et al. 1992; Zeman and Lodge 1992; Pook et al. 1993; Jane et al. 1994; Boxall et al. 1996). These long-lasting reflexes are thought to reflect a nociceptive reflex for several reasons: the threshold of activation corresponds to that of C fiber primary afferents (Akagi et al. 1985); they can be depressed by opioids (Yanagisawa et al. 1985; Nussbaumer et al. 1989; Faber et al. 1997) and α_2-adrenoceptor agonists (Kendig et al. 1991); and a similar response can be evoked by peripheral noxious stimulation (Yanagisawa et al. 1995).

Procedure

Preparation of Spinal Cord

Male Wistar rats aged 6–9 days are used. Under ether anesthesia, the spinal column is quickly removed from the animal and placed in a Petri dish, filled with oxygenated physiological

solution. A laminectomy is performed on the dorsal surface of the spinal column at room temperature. The spinal cord of the mid-thoracic to mid-sacral level is then carefully removed from the column and hemisected in the longitudinal plane under a dissecting microscope. After removal of the dura mater, the hemisected cord is completely submerged in the recording chamber (total volume: approximately 0.5 ml), which is perfused with physiological solution (124 mM NaCl, 5 mM KCl, 1.3 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 15 mM $NaHCO_3$, 11 mM glucose) at a flow rate of 1.5–2.5 ml/min. The perfusion medium is continuously bubbled with a gas mixture of 95 % O_2 and 5 % CO_2, and the temperature is kept at 25 ± 0.5 °C. The cut ends of the corresponding dorsal and ventral roots in an $L_{3–5}$ segment are fixed to a pair of suction electrodes for stimulating and recording. The preparation is stabilized in the recording chamber for at least 90 min to allow recovery from the dissection and the sealing of the roots to suction electrodes.

Recording of Monosynaptic Reflexes

Test stimulations, composed of square wave pulses of 0.05–0.2 ms duration and 5–30 V, are applied to the dorsal root every 10 s. The discharges of the corresponding ventral root are recorded with a suction electrode, amplified and monitored on an oscilloscope and stored on an analogue data recorder or computer disks for later analysis. The mean values for the waveform of the monosynaptic reflex (amplitude, area, and latency) are obtained from 6 to 18 successive responses in each experiment before and during application of drugs.

Recording of Single Motoneuron Activity

Test pulses (0.01–0.1 ms duration and 5–15 V) are applied to the dorsal or ventral root every 2 s. The activity of single motoneurons is recorded extracellularly using glass microelectrodes (electrical resistance approximately 10–30 MΩ) filled with 3 M sodium chloride or 2 M sodium acetate. The microelectrode is inserted into the ventral part of the cord through the hemisected surface while monitoring the field potential. The motoneurons in the ventral horn are identified by the short and consistent latency of antidromic spikes (1.66 ± 0.46 ms, $n = 5$), following the stimulation of the ventral root. The motoneurons also produce transsynaptic spikes with orthodromic stimulation of the dorsal root, of which the latency is 10.26 ± 1.05 ms upon supramaximal stimulation. The spike generation of motoneurons is displayed on an oscilloscope and stored on magnetic tapes. The spontaneous firing of the motoneuron is also monitored on an oscilloscope and recorded through a window discriminator and spike counter. The mean number and latency of spikes and latency of the dorsal root-elicited spikes are obtained from 20 to 40 successive responses in each experiment. Comparisons are made before and 3–5 min after application of drugs.

Evaluation

All data are expressed as the mean ± SEM. Statistical significance of the data is determined by repeated measures analysis of variance (ANOVA) and, when appropriate, Student's *t*-test. A *P* value of less than 0.05 is considered statistically significant.

Modifications of the Method

Smith and Feldman (1987) and Wong et al. (1996) described an in vitro neonatal rat brainstem/spinal cord preparation. The brainstem and cervical spinal cord were isolated from 0 to 4 days old ether-anesthetized Sprague–Dawley rats. The en bloc neuraxis was pinned down with ventral surface upward in a recording chamber and superfused continuously with artificial cerebrospinal fluid. Respiratory activity was recorded with suction electrodes from the C_4 ventral root.

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Cell Culture of Neurons

Purpose and Rationale

Cell culture of neurons, especially of hippocampal neurons, has become a widely used tool in pharmacological studies (Banker and Cowan 1977; Skaper et al. 1990, 1993, 2001; Araujo and Cotman 1993; Brewer 1997, 1999; Brewer et al. 1998; Li et al. 1998; Mitoma et al. 1998; Semkowa et al. 1998, 1999; Chaudieu and Privat 1999; May et al. 1999; Hampson et al. 2000; Novitskaya et al. 2000; Pickard et al. 2000; Vergun et al. 2001).

The basic information on methodology of cell culture of rat hippocampal neurons was given by Banker and Cowan (1977). One modification used by Skaper et al. (1990, 2001) studying the role of mast cells on potentiation by histamine of synaptically mediated excitotoxicity in cultured hippocampal neurons is described below.

Procedure

Preparation of Hippocampus

Timed pregnancies are obtained in female Sprague–Dawley rats by daily checking vaginal washings for sperm, the day on which sperm is found being regarded as day 0. At the appropriate stage of gestation, the pregnant rats are anesthetized and the uterus removed to a sterile dish. The remainder of the cell preparations is performed in a sterile hood.

The brains are removed from the fetuses with a pair of fine scissors, and the cerebral hemispheres separated from the brain stem. When the hemisphere of an 18–19-day-old fetus is viewed in a dissecting microscope, the hippocampus can be clearly seen on its medial surface. The hippocampal fissure, usually marked by a conspicuous group of blood vessels, indicates the approximate junction between the hippocampus and the adjoining subicular and entorhinal cortex. The developing fimbria is seen as a white translucent band along the free margin of the hippocampus. Before separating the hippocampus from the hemisphere, the meninges and adherent chorioid plexus are carefully pulled off with fine forceps. At this stage, the full depth of the hippocampal fissure can be seen. Then with iridectomy scissors, the hippocampus is separated from the adjoining cortex by a cut parallel to the hippocampal fissure and by transverse cuts at its rostral and caudal ends.

Cell Culture

Hippocampi isolated from embryonic rats (gestational age 17.5 days) are incubated with 0.08 % trypsin and dissociated in neurobasal medium containing 10 % heat-inactivated calf serum. Cells are pelleted by centrifugation (200 g, 5 min) and resuspended in neurobasal medium containing B27 (Life Technologies, Inc.) supplements (with antioxidants), 25 μM glutamate, 1 mM sodium pyruvate, 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. The cell suspension is plated onto poly-D-lysine (10 μg/ml) coated 48-well culture plates at a density of 4.5×10^4 cells per cm^2. Cultures are maintained at 37 °C in a humidified atmosphere

of 5 % CO_2–95 % air. After 5 days, one-half of the medium is replaced with an equal volume of maintenance medium (plating medium but containing B27 supplements without antioxidants and lacking glutamate). Additional medium exchanges (0.5 volume) are performed every 3–4 days thereafter. Cells are used between 14 and 16 days in culture. During this period, neurons develop extensive neuritic networks and form functional synapses.

Mast cells are collected from the peritoneal lavage of male Sprague–Dawley rats and isolated over a bovine serum albumin gradient to >90 % purity, as judged by toluidine blue and safranin staining.

Neurotoxicity Assays

Cultures are washed once with Locke's solution (pH 7.0–7.4) with or without 1 mM $MgCl_2$. Drug treatments are carried out for 15–30 min (25 °C) in a final volume of 0.5 ml. In the case of mast cell neuron co-cultures, transwell inserts (3-μm pore size, 9 mm diameter) are seeded with 5×10^4 mast cells in RPMI-1640 medium and placed in 24-well plates overnight. Inserts with mast cells are then placed into wells with hippocampal cells. Mast cell activation is achieved using an antigenic stimulus (0.3 μg /ml anti-DNP IgE/0.1 μg /ml DNP albumin). The mast cell-containing inserts are removed at the end of the Mg^{2+} – free incubation. After this time, all cell monolayers are washed with complete Locke's solution and returned to their original culture medium for 24 h. Cytotoxicity is evident during 24 h after the insult. Viable neurons have phase-bright somata of round-to-oval shape, with smooth, intact neurites. Neurons are considered nonviable when they exhibit neurite fragmentation and somatic swelling and vacuolation. Cell survival is quantified 24 h after the insult by a colorimetric reaction with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT).

Evaluation

Data are analyzed by one-way ANOVA with Student-Newman-Keuls post hoc test for differences between groups.

Modifications of the Method

Brewer (1997) reported the isolation and culture of adult rat hippocampal neurons. Using different proteases and special separation techniques, about 90,000 viable neurons could be isolated from each hypothalamus at any age rat from birth to 24–36 months. Neurons were cultured for more than 3 weeks.

Flavin and Ho (1999) found that propentofylline protects hippocampal neurons in culture from death triggered by macrophage or microglia secretory products.

To study neurite outgrowth in cultured hippocampal cells from Wistar rat embryos, 5000-well cells were seeded in 8-well LabTec tissue culture slides with a grown surface of permanox plastic and grown in neurobasal medium supplemented with B27 (Life Technologies, Inc.), 20 mM HEPES, 0.4 % bovine serum albumin, penicillin (100 IU/ml), and streptomycin (100 pg/ml) (Novitskaya et al. 2000). For image analysis, cells were fixed in 4 % paraformaldehyde and stained for 20 min with Coomassie Blue R250. Cover slides were observed in an inverted microscope using phase contrast optics. To measure neurite outgrowth from hippocampal neurons, an unbiased counting frame containing a grid with a number of test lines was superimposed on the images of cells. The number of intersections of cellular processes with the test lines was counted and related to the number of cell bodies, thereby allowing quantification of neurite length per cell.

Cell culture experiments were also performed with **neuronal cells from other areas of the brain** besides the hippocampus.

Brain tissue samples of rat embryos containing either septum plus preoptic area or retrochiasmatic hypothalamus were dissociated and cultured for 14 and 21 days by Jirikowski et al. (1981). By means of immunofluorescence, LHRH, α-MSH, vasopressin, and neurophysin-containing hormones could be identified.

Sinor et al. (2000) studied NMDA and glutamate-evoked excitotoxicity at distinct cellular locations in rat cortical neurons in vitro.

Canals et al. (2001) examined neurotrophic and neurotoxic effects of nitric oxide on

neuronal-enriched fetal midbrain cultures from embryonic Sprague–Dawley rats.

López et al. (2001) investigated the release of amino acid neurotransmitters in cultured cortical neurons obtained from gestation day 19 rats by nicotine stimulation.

Ehret et al. (2001) studied the modulation of electrically evoked acetylcholine release in cultured septal neurons from embryonic Wistar rats.

Tang et al. (2001) found a lack of replicative senescence in cultured rat oligodendrocyte precursor cells.

Yamagishi et al. (2001) used cultured rat cerebellar granule neurons as a model system for studying neuronal apoptosis.

Noh and Koh (2000) prepared mixed **mouse** cortical cultures containing both neurons and astrocytes and pure astrocyte cultures, from fetal (15 days of gestation) and neonatal (1–3 postnatal days) mice.

Saluja et al. (2001) found that PPAR δ agonists stimulate oligodendrocyte differentiation in glial cell culture of mouse cerebra.

Uchida et al. (2000) succeeded to directly isolate clonogenic **human** central nervous system stem cells from fresh human brain tissue, using antibodies to cell surface markers and fluorescence-activated cell sorting.

For further studies with brain cell cultures.

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In Vivo Methods

Electroshock in Mice

Purpose and Rationale

The electroshock assay in mice is used primarily as an indication for compounds which are effective in grand mal epilepsy. Tonic hind limb extensions are evoked by electric stimuli which are suppressed by antiepileptics but also by other centrally active drugs.

Procedure

Groups of 6–10 male NMRI mice (18–30 g) are used. The test is started 30 min after i.p. injection or 60 min after oral treatment with the test compound or the vehicle. An apparatus with corneal or ear electrodes (Woodbury and Davenport 1952) is used to deliver the stimuli. The intensity of stimulus is dependent on the apparatus, e.g., 12 mA, 50 Hz for 0.2 s have been used. Under these

conditions, all vehicle-treated mice show the characteristic extensor tonus.

Evaluation

The animals are observed closely for 2 min. Disappearance of the hind leg extensor tonic convulsion is used as positive criterion. Percent of inhibition of seizures relative to controls is calculated. Using various doses, ED_{50} values and 95 % confidence interval are calculated by probit analysis.

ED_{50} values after oral administration are:

- Diazepam 3.0 mg/kg
- Diphenylhydantoin 20.0 mg/kg

Critical Assessment of the Method

The electroshock test in mice has been proven to be a useful tool to detect compounds with anticonvulsant activity.

Modifications of the Method

Cashin and Jackson (1962) described a simple apparatus for assessing anticonvulsant drugs by the electroshock seizure test in mice.

Kitano et al. (1996) developed the increasing-current electroshock seizure test, a new method for assessment of anti- and proconvulsant activities of drugs in mice. A single train of pulses (square wave, 5 ms, 20 Hz) of linearly increasing intensity from 5 mA to 30 mA was applied via ear electrodes. The current at which tonic hind limb extension occurred was recorded as the seizure threshold. The method allows the determination of seizure threshold current for individual animals.

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Pentylenetetrazol Test in Mice and Rats

See chapter "► Tests for Anxiolytic Activity".

Strychnine-Induced Convulsions in Mice

See chapter "► Tests for Anxiolytic Activity."

Picrotoxin-Induced Convulsions in Mice

See chapter "► Tests for Anxiolytic Activity".

Isoniazid-Induced Convulsions in Mice

See chapter "► Tests for Anxiolytic Activity".

These tests, already described for evaluation of the anticonvulsive activity of anxiolytics, can be used and show activity for antiepileptics.

Many other agents induce seizures in animals and have been used to test the anticonvulsant activity of drugs (Stone 1972), e.g., glutarimides (Hahn and Oberdorf 1960), pilocarpine (Tursky et al. 1987), methionine sulfoximine (Toussi et al. 1987), *N*-methyl-D-aspartic acid (Leander et al. (1988), γ-hydroxybutyrate (Snead 1988).

Shouse et al. (1989) described mechanisms of seizure suppression during rapid eye movement (REM) sleep in cats. Spike–wave paroxysms in the EEG accompanied by bilateral myoclonus of the head and the neck were induced by i.m. injection of 300,000–400,000 IU/kg sodium penicillin G.

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Bicuculline Test in Rats

Purpose and Rationale

Seizures can be induced by the $GAGA_A$ antagonist bicuculline and are antagonized by known antiepileptics.

Procedure

Female Sprague–Dawley rats are injected i.v. with 1 mg/kg bicuculline. At this dose, a tonic convulsion appears in all treated rats within 30 s after injection. Test compounds are administered orally 1 or 2 h before bicuculline injection. Dose–response curves can be obtained.

Evaluation

Percentage of protected animals is evaluated. ED_{50} values and 95 % confidence limits are calculated by probit analysis.

Critical Assessment of the Method

Like the electroshock test, the bicuculline test is considered to be relatively specific for antiepileptic activity.

Modifications of the Method

Czuczwar et al. (1985) studied the antagonism of *N*-methyl-D,L-aspartic acid-induced convulsions by antiepileptic drugs and other agents.

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4-Aminopyridine-Induced Seizures in Mice

Purpose and Rationale

The K^+ channel antagonist 4-aminopyridine is a powerful convulsant in animals and in man. The drug readily penetrates the blood–brain barrier and is believed to induce seizure activity by enhancing spontaneous and evoked neurotransmitter release. Although both excitatory and inhibitory synaptic transmission are facilitated by 4-aminopyridine, the epileptiform activity induced by the drug is predominantly mediated by non-NMDA-type excitatory amino acid receptors. In mice, parenterally administered 4-aminopyridine induces clonic–tonic convulsions and lethality.

Procedure

Male NIH Swiss mice weighing 25–30 g are allowed to acclimatize with free access to food and water for a 24 h period before testing. Test drugs are administered in various doses intraperitoneally 15 min prior to s.c. injection of 4-aminopyridine at a dose of 13.3 mg/kg which was found to be the LD_{97} in this strain of mice. Controls treated with 4-aminopyridine only exhibit characteristic behavioral signs, such as hyperreactivity, trembling, intermitted forelimb/hind limb clonus followed by hind limb extension, tonic seizures, opisthotonus, and death. The mean latency to death at the LD_{97} is about 10 min. Groups of eight mice are used for each dose.

Evaluation

The percentage of protected animals at each dose is used to calculate ED_{50} values. Phenytoin-like anticonvulsants such as carbamazepine and broad-spectrum anticonvulsants such as phenobarbital and valproate are effective whereas GABA enhancers such as diazepam, several NMDA antagonists, and $C\varsigma^{2+}$ channel antagonists such as nimodipine are not.

Critical Assessment of the Method

The profile of drugs effective in this seizure model is distinct from other chemoconvulsant models and more similar to those that prevent tonic hind limb extension in the maximal electroshock seizure test. The test is useful to differentiate the mode of action of anticonvulsant drugs.

Modifications of the Method

Morales-Villagran et al. (1996) described protection against seizures induced by intracerebral or intra-cerebroventricular administration of 4-aminopyridine by NMDA receptor antagonists.

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3-Nitropropionic Acid-Induced Seizures in Mice

Purpose and Rationale

3-Nitropropionic acid is a naturally occurring toxin demonstrated to impair energy metabolism via irreversible inhibition of a mitochondrial complex II component, succinate dehydrogenase (Alston et al. 1977; Ludolph et al. 1991). 3-Nitropropionic acid evokes seizures in mice after i.p. injection of 100–200 mg/kg (Urbańska et al. 1998, 1999). Urbańska et al. (1998) and Zuchora et al. (2005) evaluated anticonvulsants for their protective effect against 3-nitropropionic acid-induced seizures.

Procedure

Male albino Swiss mice weighing 20–25 g were injected i.p. with 210 mg/kg 3-nitropropionic acid, which is equal to the ED_{97} dose (i.e., the dose required to evoke seizures in 97 % of the animals). Groups of eight mice received in addition various doses of the anticonvulsant drugs. Percentage of animals with seizures and latency until occurrence of seizures were determined. Mortality rate was determined 2 h after injection of 3-nitropropionic acid.

Evaluation

ED_{50} and LD_{50} values together with their confidence limits were estimated by computerized fitting of the data by linear regression analysis according to Litchfield and Wilcoxon. Statistical comparisons of latency data were performed by means of one-way analysis of variance (ANOVA) followed by adjustment of P value by the Bonferroni method.

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Epilepsy Induced by Focal Lesions

Purpose and Rationale

Intrahippocampal injections of noxious agents or certain cerebral lesions can induce seizures in animals. Cavalheiro et al. (1982) studied the long-term effects of intrahippocampal kainic acid injections in rats.

Procedure

Adult male Wistar rats are anesthetized with a chloral hydrate/Nembutal mixture and placed in a stereotactic apparatus. For injections, a 0.3 mm cannula is inserted through a burr hole in the calvarium. The coordinates for hippocampal injections are based on a stereotactic atlas, e.g., Albe-Fessard et al. (1971). Kainic acid is dissolved in artificial serum and infused in various doses (0.1–3.0 μg) in a volume of 0.2 μl over a period of 3 min. For recording, bipolar twisted electrodes (100 μm) are positioned stereotaxically and fixed on the skull with dental acrylic cement. Depth recording sites include the dorsal hippocampus and amygdala ipsilateral to the injected side. Surface electrodes are guided from jeweler's screws over the occipital cortex. An additional screw in the frontal sinus serves as indifferent electrode for grounding. Signals are recorded by an EEG polygraph.

Evaluation

EEG recordings and observations of convulsive seizures are performed during the acute phase and during the chronic phase (up to 2 months) with and without drug treatment.

Modifications of the Method

Several agents have been used as convulsants after topical administration, e.g., application of alumina cream (Kopeloff et al. 1942, 1955; Ward 1972; Feria-Velasco et al. 1980), implantation of cobalt powder (Dow et al. 1962; Fischer et al. 1967), injection of a colloidal gel of tungstic acid (Blum and Liban 1960; Black et al. 1967), topical application of penicillin (Matsumoto and Marsan 1964), subpial injection of saturated $FeCl_3$ solution (Reid et al. 1979; Lange et al. 1980), intracerebral injections of zinc sulfate (Pei et al. 1983), intracerebral injection of antibodies to brain gangliosides (Karpiak et al. 1976, 1981), microinjections of cholinergic agonists (Ferguson and Jasper 1971; Turski et al. 1983), topical application of atropine (Daniels and Spehlman 1973), injection of tetanus toxin into the hippocampus (Mellanby et al. 1984; Hawkins and Mellanby 1987), injection of strychnine in the visual or somatosensory cortex (Atsev and Yosiphov 1969), and electrophoretic application of bicuculline from a fluid-filled microelectrode (Campell and Holmes 1984).

Bernhard and Bohm (1955) and Bernhard et al. (1956) evaluated the anticonvulsive effect of local anesthetics in cats and monkeys. The head was fixed in light Nembutal anesthesia, the parietal areas exposed and covered with paraffin oil. Stimulating electrodes were placed at the surface of the parietal region. The cortex was stimulated with repetitive square wave shocks (duration 1–3 ms) with a frequency of 25 per s for 5 s. In order to avoid muscular movements, D-tubocurarine was given. Cortical after discharge was registered before and after injection of local anesthetics.

Cortical epileptic lesions were produced by local freezing (Stalmaster and Hanna 1972; Hanna and Stalmaster 1973; Loiseau et al. 1987).

Repetitive electrical stimulation of discrete regions of the central nervous system has been used as a convenient method for reproduction of the ictal phenomena of epilepsy (Marsan 1972; Racine 1972).

Remler and Marcussen (1986) and Remler et al. (1986) studied the pharmacological response of systemically derived focal epileptic lesions. A defined area of left hemisphere of rats was radiated by α-particles from a cyclotron destroying the blood–brain barrier. After a period of 150 days following irradiation, bicuculline was injected intraperitoneally resulting in focal lesions with EEG spikes and convulsions. Anticonvulsant drugs decreased these effects.

Walton and Treiman (1989) and Walton et al. (1994) described a model of cobalt-lesioned rats in which status epilepticus was induced by injection of homocysteine thiolactone.

Anderer et al. (1993) pointed out that restriction to a limited set of EEG-target variables may lead to misinterpretation of pharmaco-EEG results.

Krupp and Löscher (1998) developed a cortical ramp-stimulation model allowing repeated determinations of seizure threshold at short time intervals in individual rats without inducing postictal threshold increases.

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Kindled Rat Seizure Model

Purpose and Rationale

Kindling, first described by Goddard et al. (1969), results from repetitive subconvulsive electrical stimulation of certain areas of the brain. Initially, local afterdischarge is associated with mild behavioral signs; however, with continued stimulation, electrical activity presumably spreads, and generalized convulsions occur. Although the pathogenesis of kindled seizures is not fully understood, it serves as a useful tool for investigating the efficacy of experimental anticonvulsant agents.

Procedure

Adult female Sprague–Dawley rats (270–400 g) are used. The rats are implanted with an electrode in the right amygdala according to the coordinates of Pellegrino et al. (1979): frontal, 7.0; lateral, −4.7; and horizontal, 2.5. At least 1 week has to elapse before electrical stimulation of the brain is started. Afterdischarge threshold is determined for each rat. Duration and amplitude, behavioral seizure duration, and seizure stage are recorded with increased stimuli afterdischarges. Seizure severity is classified into five stages (Racine 1972). Rats are considered to be kindled on the first stimulation causing a stage 5 seizure which is followed by at least two consecutive stage 5 seizures.

The animals are tested on the day before and after treatment with the test compound (i.p. or orally). Amygdala stimulation is applied at various time intervals.

Evaluation

The occurrence and the degree of seizures are compared between control results and those after administration of the test compound.

Critical Assessment of the Method

The kindled seizure model offers an approach to study anticonvulsive drugs on the basis of a pathophysiological model. This method may give more relevant results than the simpler methods using maximal electroshock or chemically induced convulsions.

Modifications of the Method

Generalized convulsive seizures have been induced by daily amygdaloid stimulation in **baboons** (Wada and Osawa 1976) and in **rhesus monkeys** (Wada et al. 1978).

The kindling effect can be produced by intermittent administration of small doses of pentylenetetrazol (Mason and Cooper 1972).

Dürmüller et al. (1994) tested a competitive (NBQX) and a noncompetitive (GYKI 52446) AMPA antagonist and a competitive NMDA antagonist (D-CPPene) against the development of kindling and against fully kindled seizures in amygdala-kindled rats.

Croucher et al. (1996) described a chemical kindling procedure in rats by daily focal microinjection of NMDA into the right basolateral amygdala and the inhibition of seizures by an NMDA receptor antagonist.

Suzuki et al. (1996) studied the anticonvulsant action of metabotropic glutamate receptor agonists in kindled amygdala of rats.

Löscher et al. (1993), Ebert et al. (1997), and Ebert and Löscher (1999) studied the effect of phenytoin on the spread of seizures in the amygdala kindling model in rats. Sprague–Dawley rats implanted with a stimulation and recording electrode in the basolateral amygdala showed an increase in current intensity necessary for eliciting afterdischarges of about 200 % after administration of phenytoin, while seizure severity at threshold was increased compared to controls. Phenytoin-resistant kindled rats are considered as a model of drug-resistant epilepsy.

Löscher (1998) discussed the pharmacology of glutamate receptor antagonists in the kindling model of epilepsy.

The kindling procedure can also be used to evaluate antidepressant drugs (Babington 1975).

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Posthypoxic Myoclonus in Rats

Purpose and Rationale

The syndrome of posthypoxic myoclonus in man was described by Lance and Adams (1963). Lance (1968), Fahn (1986), Truong et al. (1994), and Jaw et al. (1994, 1995, 1996) reported on a model in rats resembling this human disorder.

Procedure

Male Sprague–Dawley rats which fasted 12–24 h prior to surgery are anesthetized with 100 mg/kg ketamine i.p., supplemented by 0.4 mg/kg atropine. The animal is placed on a circulating water pad and kept at a constant body temperature by a heating lamp. The rat is intubated and ventilated with 30 % O_2 in N_2O. The femoral artery and vein are cannulated for monitoring blood pressure and delivery of drugs, respectively. Electrocardiogram and blood pressure are recorded with a polygraph. The rat is then paralyzed with 2 mg/kg succinlycholine i.v., and ventilator settings are

adjusted to a rate of 60 strokes/min and a volume of 7.5 ml/kg, which yields blood gases of >150 mmHg pO_2, 35–40 mmHg pCO_2, and a pH of 7.35–7.40. N_2O is replaced with N_2 and an equilibrium period of 5 min is allowed.

Cardiac arrest is accomplished with a transthoracic intracardiac injection of KCl and cessation of the respiration. Resuscitation is begun 10 min after the arrest by turning on the ventilator (100 % O_2), manual thoracic compressions, and i.v. injections of 20 µg /kg epinephrine hydrochloride and sodium bicarbonate (4 mEq/kg). The rat is then weaned from the ventilator over 2–4 h and extubated.

Auditory-induced myoclonus: Rats are presented with a series of 45 clicks from a metronome (1 Hz, 95 dB, 40 ms), and the response to each click is scored as follows: 0 = no response, 1 = ear twitch, 2 = ear and head jerk, 3 = ear, head, and shoulder jerk, 4 = whole body jerk, 6 = whole body jerk of such severity that it causes a jump. The total myoclonus score of each rat is determined by summing up the scores yielded over 45 clicks.

Since rats ranging from 3 to 14 days post cardiac arrest show similar susceptibility to audiogenic stimulation, animals within this period are used for pharmacological tests. Myoclonus scores are assessed 30 min before and 60 min after intraperitoneal drug application.

Evaluation

Changes in myoclonus scores are analyzed by paired two-tailed Student's *t*-test.

Critical Assessment of the Test

Some anticonvulsant drugs, such as clonazepam and valproic acid, were reported to be active in this test; however, phenytoin is not. Posthypoxic myoclonus may present a special pathological condition.

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Rat Kainate Model of Epilepsy

Purpose and Rationale

Temporal lobe epilepsy is characterized by complex partial seizures that involve and apparently originate in the mesial temporal structures of the limbic system. These complex partial seizures can evolve into secondarily generalized, tonic–clonic seizures. Patients become resistant to the treatment with the usual antiepileptic drugs. The kainate-treated rat is one of several models used to study temporal lobe epilepsy. Examination of the hippocampus and dentate gyrus from kainate-treated rats has revealed a similar pattern of neurodegeneration in the hippocampus and the presence of mossy fiber sprouting in the inner molecular level of the dentate gyrus. Several authors used this model to find drugs for treatment-resistant epilepsy (Bolanos et al. 1998; Hellier et al. 1998; Longo and Mello 1998; Maj et al. 1998; Bouilleret et al. 1999; Pitkänen et al. 1999; Cilio et al. 2001; Madsen et al. 2001; Ebert et al. 2002; Tamagami et al. 2004). Maj et al. (1998) tested the activity of several drugs

against kainate-induced status epilepticus and hippocampal lesions in the rat.

Procedure

Male Wistar rates weighing 225–250 g are anesthetized with sodium pentobarbital (50 mg/kg i.p.). They are implanted extradurally with electrodes over the frontal and parietal cortex and with a reference electrode on the cerebellum. Caution is taken not to break the inner table of the diploe. All the electrodes are connected to plugs and held to the skull with dental acrylic cement. At least 7 days after surgery, rats are treated with either saline or test drugs intraperitoneally. Then, 15 min later, the rats receive a single i.p. dose of kainic acid (10 mg/kg). EEG recordings and behavioral observations are performed up to 240 min after kainic acid administration. Status epilepticus is defined as a sustained ictal EEG pattern lasting 20 min or longer without any interruption longer than 1 min.

Seven days later, the rats are sacrificed, the brains removed and immersed for 48 h in 10 % formalin. Coronal sections (4 μm) are stained with hematoxylineosin. Hippocampal injury is assessed by counting the number of histologically normal CA4 pyramidal neurons.

Evaluation

The percentage of animals protected from status epilepticus is analyzed using Fisher's exact test. For calculation of the latency to status epilepticus (min) and duration of status epilepticus (min), all animals are included regardless of whether they showed status epilepticus or not. The data are evaluated by analysis of variance (ANOVA) followed by Dunnett's test. Neuronal counts are analyzed using the Mann–Whitney nonparametric test.

Modifications of the Method

Cilio et al. (2001) used immature rats to test the anticonvulsant action and long-term effects of gabapentin.

Hellier et al. (1998) used repeated low-dose systemic treatment in order to reduce the mortality associated with single injections with kainate.

Since intracerebroventricular administration of kainic acid decreases hippocampal neuronal number and increases dopamine receptor binding in the nucleus accumbens, kainic lesions have been discussed as an animal model of schizophrenia (Bardgett et al. 1995; Csernansky et al. 1998).

Humphrey et al. (2001) described methods for inducing neuronal loss in preweanling rats using an intracerebroventricular infusion of kainic acid.

Hu et al. (1998) investigated neuronal stress and injury in C57/BL **mice** after systemic kainic acid administration.

Bouilleret et al. (1999) tested recurrent seizures and hippocampal sclerosis following intrahippocampal kainate injection in adult **mice**.

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Pilocarpine Model of Epilepsy

Purpose and Rationale

Several post-status models are described in which epilepsy develops after a chemically induced status epilepticus, such as the kainate, the pilocarpine, and the lithium–pilocarpine model (Löscher 2002). Several modifications of the pilocarpine and the lithium–pilocarpine model are reported in the literature (Cavalheiro et al. 1991; Leite and Cavalheiro 1995; André et al. 2001; Biagini et al. 2001; Klitgaard et al. 2002; Leite et al. 2002; Wallace et al. 2003; Arida et al. 2004; Leroy et al. 2004; Lyon et al. 2004; Rigoulot et al. 2004; Setkowicz et al. 2004). When rats are pretreated with lithium chloride, status epilepticus can be produced with a substantially lower dose of pilocarpine, and rats display the same clinical and EEG features of status epilepticus as with pilocarpine alone (Honchar et al. 1983). André et al. (2001) and Rigoulot et al. (2004) tested antiepileptic drugs in the lithium–pilocarpine model of epilepsy.

Procedure

Male Wistar rats weighing 225–250 g were anesthetized for electrode implantation by an i.p. injection of 2.5 mg/kg diazepam and 1 mg/kg ketamine hydrochloride. Two single-contact recording electrodes were placed on the skull, one on each side of the parietal cortex, and one bipolar deep-recording electrode was placed in the right hippocampus (Vergnes et al. 1982).

One week after surgery, rats received 3 mEq/kg lithium chloride i.p. On the following day, 1 mg/kg methylscopolamine bromide was administered s.c. to limit the peripheral effects of the convulsant. Status epilepticus was induced by injecting pilocarpine (25 mg/kg s.c.) 30 min after methylscopolamine. Various doses of test drug (i.p.) or 2.5 mg/kg diazepam (i.m.) were injected at 1 h after the onset of status epilepticus. The onset of status epilepticus corresponds to the moment at which rats experience successive seizures without recovery. Continuous spiking of the EEG occurs 30–60 min after pilocarpine

administration. The bilateral EEG cortical activity and the unilateral EEG hippocampal activity were recorded during the whole duration of status epilepticus, and concurrent behavioral changes were noted.

Quantification of neuronal damage was performed 14 days after status epilepticus. Brains of rats sacrificed in pentobarbital anesthesia were removed, and coronal sections containing the hippocampus from the anterior to the posterior level were prepared. Quantification of cell density was performed with a microscopic grid. The numbers of cells obtained in 12 counted fields were averaged.

Evaluation

Statistical analysis of neuronal damage and epilepsy between the different groups was performed by means of analysis of variance followed by a post hoc Dunnett's test for multiple comparisons.

Modifications of the Method

Hort et al. (1999) studied the relation between spontaneous recurrent seizures and the derangement of cognitive function in pilocarpine-induced status epilepticus,

Tang et al. (2004) recorded EEG in freely moving mice after pilocarpine-induced status epilepticus. A transmitter (TSE Systems, Bad Homburg, Germany) was fixed on the electrode socket by plug connection with wires attached to the skull by two screws 3 days before pilocarpine induction. The EEG signals were telemetrically received via an HF receiver which passed the signals to the computer.

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Self-Sustained Status Epilepticus

Purpose and Rationale

Status epilepticus causes neuronal damage that is associated with cognitive impairment. Self-sustained status epilepticus (SSSE) can be induced in rats by electrical stimulation of the perforant pathway (Halonen et al. 1996, 1999, 2001; Pitkänen et al. 1996; De Vasconcelos et al. 1999; Mazarati et al. 1999, 2004). This model is used to find antiepileptic drugs for patients with therapy-resistant epilepsy. Pitkänen et al. (1996), Halonen et al. (1996, 1999, 2001), and Mazarati et al. (2004) studied the effect of drugs on status epilepticus in rats.

Procedure

Under ketamine (60 mg/kg) and xylazine (15 mg/kg) anesthesia, male Wistar rats weighing 260–280 g were implanted with a bipolar stimulation electrode into the angular bundle of the perforant path (0.5 mm anterior and 4.5 mm left to lambda) and a bipolar recording electrode into the ipsilateral dentate gyrus (3 mm posterior and 2.5 mm left to bregma). The depth of the electrode was 3.5–4 mm from the brain surface and was optimized by finding the maximal population spike evoked from the dentate gyrus by stimuli applied to the perforant path.

For induction of self-sustained status epilepticus, perforant path stimulation was delivered using a Grass stimulator model 8800, for 30 min with the following parameters: 10-s, 20-Hz trains for 1 ms, 30-V pulses delivered every minute, together with continuous 2 Hz stimulation using the same parameters.

Test drugs were injected i.v. into the tail vein either 20 min before perforant path stimulation, or 10 or 40 min after the end of perforant path stimulation. Control animals were treated with saline.

Electrographic activity was acquired and analyzed off-line using Harmonie software (Stellate Systems, Montreal), configured for automatic detection and saving spikes and seizures. Analysis of EEG was performed by a “blinded” unbiased investigator. All seizure EEGs were reviewed manually.

Evaluation

The following indices were used to quantify seizure activity: duration of self-sustained status epilepticus (= time between the end of perforant path stimulation and the end of the last electrographic seizure), cumulative seizure time (the sum of the duration of all individual seizures), number of seizure episodes, average duration of individual seizures (cumulative seizure time

divided by number of seizures), and number of spikes per hour. Statistical analysis was carried out with one-way ANOVA followed by Newman–Keuls post hoc test, or, if the normality test failed, ANOVA on ranks followed by Mann–Whitney post hoc test.

Modifications of the Method

Brown et al. (1953) and Barton et al. (2001) characterized the 6 Hz psychomotor seizure model of partial epilepsy in rats.

Nissinen et al. (2000) described a model of chronic temporal lobe epilepsy induced by electrical stimulation of the lateral nucleus of the amygdala in rats.

Walton et al. (1996) induced status epilepticus in rats with actively epileptogenic cortical cobalt lesions by administration of homocysteine thiolactone.

Laurén et al. (2003) described selective changes in gamma-aminobutyric acid type A receptor subunits in the hippocampus in spontaneously seizing rats with chronic temporal lobe epilepsy.

Brandt et al. (2003) studied epileptogenesis and neuropathology after different types of status epilepticus induced by prolonged electrical stimulation of the basolateral amygdala in rats.

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Rat Model of Cortical Dysplasia

Purpose and Rationale

Epilepsy becomes drug resistant in 20–30 % of patients. Cortical dysplasia is implicated as a major contributing factor of many types of epileptic disorders that are resistant to pharmacological intervention (Becker 1991; Aicardi 1994). Several animal models of cortical dysplasia with specific clinical pathologies have been described (Amano et al. 1996; Jacobs 1996; Jacobs et al. 1999; Lee et al. 1997; Hirotsune et al. 1998; Chevassus au Louis et al. 1999; Zhu and Roper 2000; Wenzel et al. 2001; Benardete and Kriegstein 2002; Morimoto et al. 2004; Jacobs and Prince 2005).

Baraban and Schwartzkroin (1995, 1996), Baraban et al. (2000), and Smyth et al. (2002) exposed rats in utero to methylazoxymethanol (MAM).

Procedure

Dysplastic and control rats were generated by injecting pregnant Sprague–Dawley rats on day 15 of gestation with 25 mg/kg i.p. MAM or vehicle (10 % DMSO in 0.3 ml 0.9 % saline).

For in vitro studies, recordings were performed using acute hippocampal slices from adult vehicle or MAM-treated rats. Hippocampi were not dissected out, and all slices included entorhinal cortex and other overlying cortical structures. After cutting, slices remained submerged in a holding chamber containing oxygenated recording medium (NaCF) consisting of (in mM): 124 NaCl, 3 KCl, 1.25NaH_2PO_4, 2$MgSO_4$, 26 $NaHCO_3$, 2 $CaCl_2$, and 10 dextrose. A slice was then transferred to a gas interface recording chamber and perfused with oxygenated NaCF at a flow rate of 2.5 ml/min at 33.5 °C. Borosilicate glass electrodes were pulled, filled with 2 M NaCl (2–8 MΩ) and placed in the CA1 region of stratum pyramidale and/or within neuronal heterotopias under visual microscopic control. A monopolar-stimulating electrode was placed in stratum radiatum. Voltage was recorded with a Neurodata IR-283 amplifier and monitored on a PC running pCLAMP software. Spontaneous field activity and evoked population spikes were stored on hard disk for later blinded analysis. Interictal epileptiform burst activity was initiated with perfusion of NaCF containing 4-aminopyridine (100 μM), a potassium channel blocker known to cause seizures in humans, and spontaneous epileptiform activity in hippocampal slice preparations. The 4-aminopyridine in vitro seizure model is based on blockade of A-type potassium channels leading to the appearance of giant excitatory postsynaptic potentials generated by the prolonged firing of pyramidal neurons in CA3 burst-generating regions of the hippocampus. Burst frequency was determined by counting the number of interictal epileptiform events per second during a 3-min epoch before and after 60 min of antiepileptic drug co-perfusion and was expressed as Hz. Burst amplitude (1.5–6 mV) was determined by measuring the average peak-to-peak interval for ten consecutive representative bursts during the same epoch. Evoked synaptic responses were analyzed by averaging the number of population spikes obtained on ten consecutive sweeps recorded after stratum radiatum stimulation (0.3–3-mA pulses 100 μs pulse width). A downward voltage deflection $\geq$0.5 mV superimposed on the population excitatory postsynaptic potential (EPSP) was defined as a "population spike"; the number of population spikes was compared for each slice during perfusion with normal ACSF (baseline), ACSF plus 4-aminopyridine, and ACSF plus 4-aminopyridine and antiepileptic drug. For each slice experiment, the population spike was monitored every 15 min.

For in vivo studies, control and MAM-exposed rats were administered with 15 mg/kg kainic acid, a concentration that reliably produces acute seizure activity. Behavioral activity was scored on a six-stage scale (Germano and Sperber 1997). Animals were treated with 400 mg/kg i.p. valproate 30 min before kainate injection. Latencies to the first sign of hyperexcitability and to the first tonic–clonic seizure were recorded.

Evaluation

Dates were plotted graphically as "survival" curves, and differences in mean latencies were ranked and analyzed using a nonparametric Kruskal–Wallis one-way ANOVA.

Critical Assessment of the Test

Since the MAM-exposed rats exhibit a dramatically reduced sensitivity to commonly prescribed antiepileptic drugs, this model is considered to be relevant for drug-resistant epilepsy.

Modifications of the Method

Leré et al. (2002) described a model of "epileptic tolerance" for investigating neuroprotection, epileptic susceptibility, and gene expression-related plastic changes. Expression of status epilepticus was triggered by infusion of the excitotoxic agent kainate in the right hippocampus of adult rats. An appropriate dose of kainate was used to cause brain damage to the homolateral, but not contralateral, hippocampus. At various times following the preconditioning insult, kainate was then readministered into the lateral ventricle, and neuroprotection was observed in the contralateral side between 1 and 15 days later.

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Genetic Animal Models of Epilepsy

Purpose and Rationale

Several animal species exhibit epilepsy with spontaneous recurrent seizures such as dogs, rats, and mice (Löscher 1984). Serikawa and Yamada (1986) described spontaneous epileptic rats which are double mutants and exhibit both tonic and absence-like seizures.

Procedure

Spontaneous epileptic rats are obtained by mating the tremor heterozygous rat (*tm*/+) with the zitter homozygous rat (*zi*/*zi*) found in a Sprague–Dawley colony. The behavior of the spontaneous epileptic rats is recorded weekly for 2 h on videotapes. The frequency of tonic convulsions and wild jumping occurring in the absence of external stimuli are recorded. Under anesthesia, silver ball-tipped and monopolar stainless steel electrodes are chronically implanted in the left frontal cortex and hippocampus. An indifferent electrode is placed on the frontal cranium. The frequency of absence-like seizures and tonic convulsions, as well as the duration of each seizure, are measured on the EEG. A mild tactile stimulus is given on the back of the animal every 2.5 min to induce consistent tonic convulsions. Compounds are given i.p. or orally.

Evaluation

The number of seizures and the duration of each seizure are obtained, and the total duration of the seizures (number × duration) is calculated every 5 min before and after injection of the drug. Percent changes between values before and after drug administration are calculated.

Critical Assessment of the Test

Studies in spontaneous epileptic rats and other genetic models are of value for an in-depth investigation of a potential antiepileptic drug.

Modifications of the Method

The **tremor rat (*tm*/*tm*)** was described as a model of petit mal epilepsy (Serikawa and Yamada 1986; Serikawa et al. 1987). Seki et al. (2002) attempted to determine whether gene transfer of aspartoacyclase inhibited absence-like seizures in tremor rats using recombinant adenovirus. Noda et al. (1998) and Iida et al. (1998) described the **NER rat strain** (Noda epileptic rat) as a genetic model in epilepsy research, which was developed by inbreeding rats with spontaneous tonic–clonic seizures in a stock of Crj:Wistar.

The **genetic epileptic WAG/Rij rat** has been recommended as a useful model for general absence epilepsy in humans (Van Luijtelaar and Coenen 1986; Coenen et al. 1992; Budziszewska et al. 1999; Van Luijtelaar et al. 2003; Sarkisova et al. 2003; Bouwman and van Rijn 2004). Danober et al. (1995, 1998), Deransart et al. (2000), Lakaye et al. (2002), and Nehling and Boehrer (2003) studied the **GAERS rat**, the genetic absence epilepsy rat from Strasbourg, which shows generalized nonconvulsive absence seizures characterized by the occurrence of synchronous and bilateral spike and wave discharges.

Amano et al. (1996) developed an **epileptic rat mutant with spontaneous limbic-like seizures** by successive mating and selection from an inherited cataract rat.

Racine et al. (1999) reported selective breeding of **kindling-prone** and kindling-resistant **rats**. The selection of these strains was based on their rates of amygdala kindling. From a parent population of Long–Evans hooded and Wistar rats, the males and females that showed the fastest and slowest amygdala kindling rates were selected and bred.

Sarkisian et al. (1999) described seizures in the **flathead (FH) rat** as a genetic model in early postnatal development.

Tsubota et al. (2003) identified the **Wakayama epileptic rat (WER)** in a colony of Wistar rats, a mutant exhibiting both tonic–clonic seizures and absence-like seizures

Several other genetic animal models have been described (Löscher and Frey 1984; Löscher and Meldrum 1984) showing epilepsy with spontaneous recurrent seizures, such as:

Dogs (Cunningham 1971; Edmonds et al. 1979)
Rats with petit mal epilepsy (Vergnes et al. 1982); rats with two mutations, zitter, and tremor (Serikawa and Yamada 1986; Xie et al. 1990); and rats with absence-like states and spontaneous tonic convulsions (Sasa et al. 1988)
Tottering mice (Green and Sidman 1962; Noebels 1979; Noebels and Sidman 1979; Fletcher et al. 1966; Tehrani et al. 1997)
Leaner mutant mice with severe ataxia and atrophic cerebellum (Herrup and Wilczynsnki 1982; Heckroth and Abbott 1994),
The quaking mouse (Sidman et al. 1966; Chermat et al. 1981) having deficiencies in myelinization in the nervous system (Hogan 1977; Li et al. 1993; Bartoszewicz et al. 1995) and alterations in the dopaminergic (Nikulina et al. 1995) and α_2-adrenergic (Mitrovic et al. 1992) brain system
The stargazer mutant mouse which shows generalized non-convulsive spike–wave seizures with behavioral arrest that resembles the clinical phenotype of general absence epilepsy (Noeberls et al. 1990; Di Pasquale et al. 1997) with a disrupted Cacng2 gene (Letts et al. 2005)
The lethargic (lh/lh) mouse as a model of absence seizures (Hosford et al. 1999)

There are models of epilepsy with reflex seizures, such as:

Baboons with photomyoclonic seizures (Killam et al. 1966, 1967; Stark et al. 1970; Naquet and Meldrum 1972; Killam and Killam 1984; Smith et al. 1991; Chapman et al. 1995)
Photosensitive fowls (Crawford 1969, 1970)
The Fayoumi strain of chickens (Fepi) (Batini et al. 2004)
Audiogenic seizure-susceptible mice (Collins 1972; Seyfried 1979; Chapman et al. 1984; Stenger et al. 1991)
Mechanically stimulated mice (Imaizumi et al. 1959; Oguro et al. 1991)
The EL mouse which is a strain highly susceptible to convulsive seizures after repeated sensory stimulation (Seyfried et al. 1986; King and LaMotte 1989; Green and Seyfried 1991; Wang et al. 1997; Suzuki 2004)
Audiogenic seizure-susceptible rats (Wistar audiogenic rats WAR) (Consroe et al. 1979; Reigel et al. 1986; Smith et al. 1991; Patel et al. 1990; Scarlatelli-Lima et al. 2003; Galvis-Alonzo et al. 2004; Magalhães et al. 2004)

The genetically epilepsy-prone rat GEPR responding to acoustic stimulation has been described by Ko et al. (1982), Dailey and Jobe (1985), Dailey et al. (1989), Faingold (1988), Faingold and Naritoku (1992), Faingold et al. (1994), Jobe et al. (1992, 1995), and Laird (1989). The inferior colliculus is strongly implicated as a critical initiation site within the neuronal network for audiogenic seizures. Two strains were characterized: **GEPR-3** exhibiting moderate or clonic convulsions and **GEPR-9** exhibiting more severe tonic extensor convulsions (Dailey et al. 1996; Kurtz et al. 2001; Moraes et al. 2005).

Gerbils with reflex seizures were described by Thiessen et al. (1968), Loskota et al. (1974), Majkowski and Kaplan (1983), Lee and Lomax (1984), Bartoszyk and Hamer (1987), and Lee et al. (1987).

Löscher et al. (1989) discussed **the sz mutant hamster** as a genetic model of epilepsy or of paroxysmal dystonia.

Quesney (1984) reported **generalized photosensitive epilepsy in cats** after long-term intramuscular administration of low-dose penicillin.

Famula et al. (1997) and Oberbauer et al. (2003) described the epidemiology of epilepsy in **tervurens (Belgian shepherd dogs)** and Srenk et al. (1994) in **golden retrievers**.

Seizure susceptibility was described in *Drosophila* (Kuebler and Tanouye 2000; Kuebler et al. 2001; Zhang et al. 2002).

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Transgenic Animals as Models of Epilepsy

Purpose and Rationale

The availability of transgenic animals has stimulated research on pathogenesis of epilepsy. Several surveys on this topic are available (Allen and Walsh 1999; Meldrum et al. 1999; Noebels 1999; Prasad et al. 1999; Toth and Tecott 1999; Schauwecker 2002; Weinshenker and Szot 2002; Upton and Stratton 2003; Giorgi et al. 2004; Yang and Frankel 2004). Several studies contribute to the understanding of pathology of epilepsy (Butler et al. 1995; Zeng et al. 1997; Campbell et al. 2000; Liang et al. 2000; Musumeci et al. 2000; Viswanath et al. 2000; Kearney et al. 2001; Knuesel et al. 2002; Potschka et al. 2002; Shimizu et al. 2002; Ludwig et al. 2003; Ferri et al. 2004; Diano et al. 2005; Peters et al. 2005).

Lüthi et al. (1997) found that mutant mice overexpressing protease nexin-1 (PN-1) in brain under the control of the Thy-1 promoter (Thy 1/PN-1) or lacking PN-1 (PN-1 −/−) develop epileptic activity in vivo. An endogenous serine protease inhibitor modulated epileptic activity and hippocampal long-term potentiation.

Kunieda et al. (2002) recommended mice with systemic overexpression of the alpha 1B-adrenergic receptor as an animal model of epilepsy.

Some of the studies gave hints for further development of antiepileptic drugs, such as the neuropeptide galanin (Kokaia et al. 2001; Mazarati et al. 2004) or the neuropeptide Y (Shannon and Yang 2004). Several studies were

devoted on the role of brain-derived neurotrophic factor (BDNF) (Lahteinen et al. 2002, 2003, 2004).

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Excitatory Amino Acid Transporters

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Electroshock in Mice

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