The KCNQ Channel Opener Retigabine Inhibits the Activity of Mesencephalic Dopaminergic Systems of the Rat

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ABSTRACT

Homo- and heteromeric complexes of KCNQ channel subunits are the molecular correlate of the M-current, a neuron-specific voltage-dependent K+ current with a well established role in control of neural excitability. We investigated the effect of KCNQ channel modulators on the activity of dopaminergic neurons in vitro and in vivo in the rat ventral mesencephalon. The firing of dopaminergic neurons recorded in mesencephalic slices was robustly inhibited in a concentration-dependent manner by the KCNQ channel opener N-(2-amino-4-(4-fluorobenzylamino)-phenyl) carbamic acid ethyl ester (retigabine). The effect of retigabine persisted in the presence of tetrodotoxin (TTX), ZD7288, 4-((2-amino-4-(4-fluorobenzylamino)phenyl) carbamic acid ethyl ester (retigabine), uptake inhibitors, and the KCNQ channel blocker 4-pyridinylmethyl-9(10H)-anthracenone (XE991), indicating a direct effect on KCNQ channels. Likewise, in vivo single unit recordings from dopaminergic neurons revealed a prominent reduction in spike activity after systemic administration of retigabine. Furthermore, retigabine inhibited dopamine synthesis and c-Fos expression in the striatum under basal conditions. Retigabine completely blocked the excitatory effect of dopamine D2 auto-receptor antagonists. Again, the in vitro and in vivo effects of retigabine were completely reversed by preadministration of XE991. Dual immunocytochemistry revealed that KCNQ4 is the major KCNQ channel subunit expressed in all dopaminergic neurons in the mesolimbic and nigrostriatal pathways. Collectively, these observations indicate that retigabine negatively modulates dopaminergic neurotransmission, likely originating from stimulation of mesencephalic KCNQ4 channels.

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KCNQ (also termed Kv7) channels are voltage-dependent potassium channels composed of homo- and heteromeric complexes of five different KCNQ subunits (KCNQ1–5, Kv7.1–Kv7.5). Unlike KCNQ1, all other KCNQ subunits (KCNQ2–5) are expressed in the CNS (Jentsch, 2000). Opening of KCNQ channels leads to neuronal hyperpolarization, thereby stabilizing the membrane potential and decreasing excitability. This makes them particularly interesting as targets in CNS diseases linked to hypereexcitability, including epilepsy, anxiety, pain, and migraine (Blackburn-Munro et al., 2005). The attractiveness of neuronal KCNQ channels in the treatment of such disease states is strongly supported by the identification of mutations within the human KCNQ genes. Thus, mutations in the KCNQ2 and KCNQ3 genes are associated with benign familial neonatal convulsions (Biervert et al., 1998), and certain mutations in the KCNQ4 gene result in progressive hearing loss (Kubicsh et al., 1999).

Several attempts have been made to find pharmacological KCNQ modulators. N-(2-Amino-4-(4-fluorobenzylamino)-9(10H)-anthracenone (XE991), ZD7288, 4-((2-amino-4-(4-fluorobenzylamino)phenyl) carbamic acid ethyl ester (retigabine), 4-pyridinylmethyl-9(10H)-anthracenone (XE991), 2-(3-carboxethoxy-2-propenyl)-3-amino-6-paramethoxy-phenyl-pyridazinium bromide; XE991, 4-pyrindinylmethyl-9(10H)-thiazolo-[5,4-d]-azepindehydrochloride.

ABBREVIATIONS: KCNQ, Kv7 channel; MAO, monoamine-oxidase; CNS, central nervous system; DA, dopamine; PBS, phosphate-buffered saline; TX, Triton X-100; BSA, bovine serum albumin; TH, tyrosine hydroxylase; ACSF, artificial cerebrospinal fluid; SN, substantia nigra; VTA, ventral tegmental area; SNc, substantia nigra pars compacta; SK, small-conductance calcium-activated K+; Ih, hyperpolarization-activated; TTX, tetrodotoxin; ZD7288, 4-((N-ethyl-N-phenylamino)-1,2-dimethyl-6-(methylamino) pyridinium chloride; SR95531, 2-(3-carboxethoxy-2-propenyl)-3-amino-6-paramethoxy-phenyl-pyridazinium bromide; XE991, 4-pyrindinylmethyl-9(10H)-anthracenone; DMSO, dimethyl sulfoxide; ACCshell, shell subpart of the nucleus accumbens; ACCcore, core subpart of the nucleus accumbens; STRdl, dorsolateral part of the rostral striatum; ANOVA, analysis of variance; P-TH, phospho-specific Ser40-tyrosine hydroxylase; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; SNr, substantia nigra pars reticulata; mAHPr, medium afterhyperpolarization phase; Hal, haloperidol; ret/Ret, retigabine; BHT, thiazoloazepin, 2-amino-6-allyl-5,6,7,8-4-thiazolo-[5,4-d]-azepindehydrochloride.
phenyl) carboxylic acid ethyl ester (retigabine) was the first KCNQ opener to be reported and is now in development as an antiepileptic compound (Blackburn-Munro et al., 2005). In vitro, retigabine increases open probability of KCNQ channels by shifting the voltage dependence to more negative voltages (Tatulian et al., 2001). This underlies the hyperpolarization and reduced firing frequency produced by the drug in various neuronal populations, including hippocampal and sympathetic neurons (Passmore et al., 2003; Yue and Yaari, 2004). In these cell types, retigabine is thought to exert its inhibitory action by binding to KCNQ2/3 channels, because this KCNQ channel heteromer is expressed in significant quantities compared with other KCNQ channel subunit combinations (Cooper et al., 2001; Passmore et al., 2003).

However, KCNQ channels also modulate the excitability of other cell types in the CNS. Thus, we have recently shown that retigabine profoundly inhibits haloperidol-induced c-Fos expression in the striatum (Mikkelsen, 2004), but we have not determined the site of action within the basal ganglia. In addition, flupirtine, a structural analog of retigabine, is reported to inhibit haloperidol-induced catalepsy (Schmidt et al., 1997). Haloperidol-induced striatal c-Fos activation and catalepsy are hallmarks of acute blockade of dopamine (DA) D$_2$ autoreceptors (Robertson and Fibiger, 1992), suggesting that retigabine interferes with DA neurotransmission. We therefore aimed at localizing the target area and the mechanism of action for retigabine's pronounced inhibitory effect on striatal excitability.

Using a range of immunohistochemical techniques, in vivo and in vitro electrophysiological recordings as well as in vivo microdialysis, we demonstrate that KCNQ channel stimulation profoundly inhibits neuronal excitability in mesencephalic neurons, which resulted in reduced DA activity in the striatum. This effect likely originates from stimulation of KCNQ4 channels located on DA neurons in the substantia nigra pars compacta and ventral tegmental area.

### Materials and Methods

#### Animals

Unless stated specifically, adult male Wistar rats weighing 250–300 g were used. All experiments were conducted in accordance with guidelines of the National Institute of Health (National Institutes of Health Publication 85-23, 1985) and the Animal Experimentation Inspectorate, Ministry of Justice (Copenhagen, Denmark).

#### Immunocytochemical Staining of KCNQ Subunits in the Ventral Tegmentum

For immunocytochemistry, the rats were anesthetized with mebumal (50 mg/ml; 3 ml/kg). Then, they were perfused with 0.1 M phosphate-buffered saline (PBS; pH 7.4), fixed in 4% paraformaldehyde-PBS for 10 min, and finally immersed in sucrose-PBS for 48 h before sectioning. Forty-micrometer serial frontal sections were cut in series of six through the mesencephalon. Before the immunocytochemical steps, the sections were rinsed for 10 min in 0.01 M PBS, for 10 min in 1% H$_2$O$_2$-PBS to block endogenous peroxidase activity, and for 30 min in 0.01 M PBS containing the midbrain was placed in a vibratome filled with ice-cold (4°C) artificial cerebrospinal fluid (ACSF) of the following composition: 130 mM NaCl, 3.5 mM KCl, 1.25 mM NaH$_2$PO$_4$, 1.25 mM MgSO$_4$, 2 mM CaCl$_2$, 10 mM glucose, and 24 mM NaHCO$_3$, saturated with 95% O$_2$ and 5% CO$_2$, pHi 7.4. A block of tissue containing the midbrain was placed in a vibratome filled with ice-cold ACSF and cut in 400-μm-thick horizontal slices. The mesencephalic slice was placed on a nylon mesh in a recording chamber (volume of 500 μl). The tissue was held in position with an electron microscopy grid weighed down by short pieces of platinum wire. The slice was completely immersed in a continuously flowing (~2 ml/min), heated solution (34.5 ± 0.5°C) of ACSF. The substantia nigra (SN) was identified by transillumination as a semilunar area rostral to the medial terminal nucleus of the accessory optic tract (Paxinos and Watson, 1986). Additional experiments on the ventral tegmental area (VTA) were performed in the lateral part.

Extracellular single-cell recordings were made using glass microelectrodes filled with ACSF (resistance 5–10 MΩ). Action potentials were amplified and displayed on a Fluke digital oscilloscope (model PM 3370B; Fluke Corp., Everett, WA). They were selected with a window discriminator and counted with a digital counter. They were also fed to an analog digital interface (CED1401) connected to a computer. Data were collected and analyzed with the use of the Spike2 version 4 software (Cambridge Electronic Design, Cambridge, UK).

DA neurons in the substantia nigra pars compacta (SNC) recorded in vitro are characterized by long-duration, often triphasic action potentials (~2.5 ms) and a regular firing rate of 0.5 to 4 spikes/s. They are inhibited by nanomolar concentrations of the DA D$_2$ receptor agonists BHT-920, apomorphine, or quinpirole. In all experiments, a 5-min control period was taken to assess the stability of the cells. Drugs were then superfused using three-way taps so that the flow rate remained constant. Each concentration was superfused.
until equilibrium was obtained (duration usually 10–15 min). For each concentration, the percentage of inhibition relative to the mean control period firing rate was calculated, and the concentration producing 50% inhibition (IC50) was determined for each cell. When antagonists/channel blockers were tested, they were first superfused alone for 10 min before testing their ability to block the effect of agonists/channel openers.

**Intracellular Recordings in Rat Midbrain Slices.** The method for the rat midbrain slice preparation is similar to that described above for extracellular electrophysiological recordings. Intracellular recordings were made using glass microelectrodes filled with 2 M KCl (resistance 70–150 MΩ). All recordings were made in the bridge balance mode, using an NI SEClamp amplifier (NPI Electronic GmbH, Tamm, Germany). The accuracy of the bridge was checked throughout the experiments by examining the voltage deflection induced by a small (±50-pA) current injection. The potential of the extracellular medium was measured at the end of each experiment, and its absolute value was within 5 mV of that set to zero at the start. Membrane potentials and injected currents were recorded on a Gould TA240 chart recorder (Gould Instrument Systems Inc., Valley View, OH) and on a Fluke CombiScope oscilloscope (Fluke Corp.). The FlukeView software was used for off-line analysis. The characteristics of DA neurons recorded intracellularly have been described previously (Seutin et al., 1997) and include a slow (0.5–4-Hz) regular pacemaker-like firing, a significant SK channel-mediated afterhyperpolarization, and a robust Ih channel-mediated current.

All experiments were performed in the presence of 0.5 μM tetrodotoxin (TTX; Sigma-Aldrich) to minimize indirect effects. In addition, in a few experiments, the Ih blocker ZD7288 (30 μM; Sigma-Aldrich) and the SK channel blocker apamin (300 nM; Sigma-Aldrich) were used. Finally, the GABAA antagonist SR95531 (10 μM; Sigma-Aldrich) and the SK channel blocker apamin (300 nM; Sigma-Aldrich) were also used in some experiments to exclude the possibility of an action of retigabine on these receptors (van Rijn and Willems-van, 2003).

The KCNQ channel blocker XE991 was synthesized at the Department of Medicinal Chemistry (NeuroSearch A/S, Ballerup, Denmark), dissolved in DMSO (final concentration of DMSO 0.1%), and applied by superfusion using three-way taps. Complete exchange of the bath solution occurred within 2 to 3 min.

**In Vivo Single-Unit Electrophysiology.** Rats (n = 8) were anesthetized with chloral hydrate (400 mg/kg i.p.) and mounted in a Kopf stereotaxic frame with the incisor bar set 3.75 mm below the interaural line. They were fitted with a catheter in the femoral vein to administer drugs (1.0 ml/kg) and supplementary anesthesia (80 mg/kg i.v.). Body temperature was monitored by a rectal thermometer and maintained at 36.5–37.0°C with a thermostatically controlled heating pad. An incision was made in the scalp, the skull was exposed, and a burr whole was drilled overlying the SN. The dura mater was retracted, and a glass microelectrode (filled with 2 M NaCl) was used to perform extracellular single-unit recordings from DA neurons in the SNc. Electrode penetrations were made through the SNc in the dorsoventral direction from 6.0 to 8.0 mm below the dura, 3.0 to 3.3 mm anterior to the interaural line, and 2.2 to 2.4 mm lateral to the midline, respectively, according to Paxinos and Watson (1986). As reported previously, the DA neurons 1) exhibited a characteristic triphasic waveform with a long action potential of 2.5 to 3.5 ms; (2) had low spontaneous firing rates of 0.5 to 10 Hz, and an irregular firing pattern; and (3) were inhibited by apomorphine (0.04 mg/kg i.v.; Sigma-Aldrich) (Grace and Bunney, 1980).

Baseline activity of DA cells was recorded for at least 3 min before intravenous drug administration. Haloperidol (0.08 mg/kg i.v.; Janssen-Cilag, Beesse, Belgium) was administered following recovery of the spike frequency after apomorphine treatment. Upon haloperidol administration, DA cell firing was allowed to reach a stable plateau before administering retigabine (2.5 mg/kg i.v.) or vehicle (10% Tween 80 in 0.9% NaCl, i.v.). Firing rates were collected online via an interface (1401Plus, Cambridge Electronic Design) using the Spike2 software (Cambridge Electronic Design). The total number of spikes was calculated every 10 s. In all experiments, only one cell per animal was monitored unilaterally for its response to drug administration. Recordings were averaged over a total time interval of 3 min and compared with the baseline activity defined as the mean spike level before the first drug injection. The statistical significance of the effect of haloperidol and retigabine, respectively, was determined by comparing the activity of the cell measured as the averaged spike frequency within a time period of 3 min before drug administration and within 2 to 10 min after drug injection. The effect of drug exposure was analyzed using a one-way ANOVA followed by Tukey’s post hoc test.

**Immunohistochemical Analysis of c-Fos Expression.** Rats were injected i.p. with a single dose of 10 mg/kg retigabine (dissolved in 10% Tween 80 in 0.9% NaCl; n = 7) or vehicle (n = 8). To examine the inhibitory role of retigabine, combinations of two drug substances or vehicle (n = 6/group) were given to each rat. Within a 15-min interval, each animal was injected with 0.1 to 10 mg/kg retigabine (dissolved in 10% Tween 80 in 0.9% NaCl) or vehicle followed by i.p. administration of either saline vehicle, 1.0 mg/kg haloperidol (dissolved in 0.9% NaCl), or 0.5 mg/kg raclopride, dissolved in 0.9% NaCl; Sigma-Aldrich). XE991 (3.0 mg/kg; dissolved in 10% Tween 80 in 0.9% NaCl) or vehicle (n = 6) was administered 5 min before retigabine.

The animals were returned to their home cages, and 60 min after the injection of retigabine or corresponding vehicle they were deeply anesthetized with 50 mg/ml (3.0 ml/kg) mebumal (SAD, Copenhagen, Denmark) and perfused transcardially with PBS followed by 4% paraformaldehyde in 0.1 M phosphate buffer for 10 min. For c-Fos immunocytochemistry, the forebrains were immersed in fixative overnight and subsequently submerged in 30% sucrose in PBS at 4°C for 3 days. Forty-micrometer serial coronal sections were cut through the forebrain on a freezing microtome, and representative sections were processed immunocytochemically according to the avidin-biotin immunocytochemical protocol reported previously (Mikkelsen et al., 1998). The primary polyclonal antiserum (1:4000), characterized previously (Mikkelsen et al., 1998), was generated in rabbit against the N-terminal peptide similar to amino acids 2 to 17 of the rat c-Fos protein.

The number of c-Fos-positive cells was counted by means of light microscopy (20× magnification) using a counting grid (500 × 500 μm) placed over the shell (ACShell) and core (ACCcore) of nucleus accumbens as well as the dorsolateral part of the rostral striatum (STRdl). The number of c-Fos-positive cells was averaged from two adjacent sections of each animal, and statistical analysis was performed on group means ± S.E.M. using an unpaired t test or one-way ANOVA with Tukey’s post hoc test where appropriate.

**Protein Analysis and Immunoblotting.** To determine the influence on an effector of DA synthesis an assay detecting the Ser40 phosphorylation level of tyrosine hydroxylase was applied (Håkanson et al., 2004). In an initial experiment, rats (n = 6/group) were injected with 1.0 mg/kg haloperidol and sacrificed at various times after the injection (15, 30, or 60 min). Because the maximal level of Ser40 phosphorylation of TH was observed 30 min post-treatment, this time was used to determine any inhibitory effect of retigabine. Retigabine (10 mg/kg i.p.) was administered 15 min before haloperidol (1.0 mg/kg i.p.). The rats were sacrificed 30 min after haloperidol treatment, and the left and right dorsolateral striatae were rapidly excised, pooled, and snap-frozen in liquid nitrogen, and kept at −80°C until further processing. For Western blotting analysis, total protein was extracted using the Total Protein extraction kit (Chemicon International, Temecula, CA), according to the manufacturer’s instructions. The supernatant was collected and the protein concentration was determined using a modified Lowry method (DC Protein Assay kit; Bio-Rad, Hercules, CA). Protein extracts (20 μg/sample) and a prestained weight marker (Invitrogen) were denatured in sample loading buffer (Invitrogen) under reducing conditions, separated by 10% SDS-polyacrylamide gel electrophoresis, and electro-
transferred in transfer buffer (80 mM Tris-HCl, 39 mM glycine, and 20% methanol) to a nitrocellulose membrane (0.2-μm pore, Protran; Whatman Schleicher and Schuell, Dassel, Germany). The membrane was rinsed with Tris-buffered saline solution with Tween 20 (10 mM Tris, 150 mM NaCl, and 0.1% Tween 20, pH 7.6) and treated with blocking solution (5% nonfat dry milk in Tris-buffered saline solution with Tween 20) for 2 h at room temperature to prevent nonspecific antibody binding. Equal loading and transfer of proteins were initially confirmed by staining the membrane with Ponceau-S solution (Fluka, Buchs, Switzerland). The membrane was incubated overnight at 4°C with a rabbit phospho-specific Ser40-tyrosine hydroxylase antibody (P-TH; 1:3000; Chemicon International) or TH (1:4000) (Chemicon International), respectively. Following incubation with anti-rabbit horseradish peroxidase-labeled secondary antibody (1:30,000; GE Healthcare, Piscataway, NJ) at room temperature for 2 h, the immunoreactive protein was visualized by an enhanced chemiluminescence system (Pierce Chemical, Rockford, IL), and serial exposures were made on autoradiographic films (GE Healthcare). For control of loading and transfer efficacy, the membranes were stripped for 5 min at 37°C using a commercial stripping reagent (Restore Western Blot Stripping buffer; Pierce Chemical) and re-probed with a rabbit polyclonal actin antibody (1:30,000; Sigma-Aldrich). Densitometric analyses of blots were performed using Image-Pro 5.1 (Media Cybernetics, Inc., Silver Spring, MD), and data were expressed as P-TH or TH, respectively, relative to the corresponding actin level.

Microdialysis of Striatal Dopamine and Dopamine Metabolites in the Striatum. Each rat was anesthetized with isoflurane (1.5% isoflurane in a mixture of 20% O<sub>2</sub> and NO<sub>2</sub>) and placed in a stereotaxic frame. The anesthesia was maintained, and the rectal temperature was continuously measured during the experiment. The CMA/12 microdialysis probe (CMA Microdialysis, Solna, Sweden) of 4 mm in length was placed unilaterally into striatum. The probe was placed at the following coordinates: anterior-posterior, + 1.0 mm; lateral, 3.0 mm; and ventral, −6.0 mm (from the dura) (Paxinos and Watson, 1986). The probe was perfused at a flow rate of 2 μl/min with Ringer solution (147 mM NaCl, 4 mM KCl, and 2.3 mM CaCl<sub>2</sub>, pH 6.5) immediately after the implantation. The first three samples were discarded, and the subsequent three samples were collected to determine the baseline of extracellular DA and the DA metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA). Thereafter, retigabine (10 mg/kg i.p.; n = 6) or vehicle (10% Tween 80 in 0.9% NaCl; n = 10) was administered, and the following samples (20 min each) were collected for a total of 180 min. The brain dialysates collected from the probe were used for separate analysis of DA, DOPAC, and HVA. Only rats with a verified correct placement of the probe in the striatum were included in the study.

Concentrations of DA, DOPAC, and HVA were determined by reversed-phase high-performance liquid chromatography with electrochemical detection as described previously (Weikop et al., 2004). The mean area under the curve levels of DA, DOPAC, and HVA, respectively, were calculated from the three last microdialysate fractions collected before retigabine administration, normalized to 100%, and thus considered being the basal level. All subsequent DA, DOPAC, and HVA sample values are expressed as mean levels ± S.E.M. relative to the basal levels. Statistical analysis was carried out using a two-way ANOVA (treatment × time) with Bonferroni’s post hoc test.

Results

Localization of KCNQ Subunits in the Ventral Tegmentum

We examined the anatomical, cellular, and subcellular localization of KCNQ subunits in the ventral mesencephalon using well characterized antisera. Immunoreactivity for the KCNQ channel subunits were observed to be differentially distributed in the SN as well as in other parts of the brain, as reported recently (Kharkovets et al., 2000; Cooper et al., 2001; Yus-Najera et al., 2003). Only KCNQ2 and KCNQ4 immunoreactivity was detected in the SN (Fig. 1, A–D).

Fig. 1. Immunohistochemical localization of KCNQ subunits in the rat mesencephalon. Coronal sections of the ventral tegmentum immunopositive for KCNQ2 (A), KCNQ3 (B), KCNQ4 (C), and KCNQ5 (D), respectively. Moderate level of KCNQ2 immunoreactivity is found in both the SNr and SNc of substantia nigra. Confocal microscopy illustrates simultaneous detection of KCNQ4 (E) and tyrosine hydroxylase (F) immunoreactivity in the rat SNc. There is a complete overlap between TH and KCNQ4 immunoreactivity in SNc DA neurons (G). The cellular distribution of KCNQ4 immunoreactivity demonstrates an accumulation in the cellular membrane. Scale bar, 500 μm (A–D); 10 μm (E–G).
Fig. 2. Retigabine inhibits firing of DA neurons in rat brain slice preparations of the SNc. A, significant reduction of neuronal activity is observed at retigabine concentrations of 2 μM, and spike activity was completely blocked at concentrations of 10 μM retigabine. ***, p < 0.001 (compared with DMSO). B, the capacity of retigabine to inhibit spike activity of SNc was effectively diminished in the presence of the KCNQ channel blocker XE991 (1 μM). Thus, only concentrations of at least 300 μM significantly reversed the effect of XE991. Note that XE991 alone induced a significant increase (22%) of spike frequency. ##, p < 0.01; ###, p < 0.001 (compared with control); ***, p < 0.001 (compared with XE991 + DMSO). C, XE991 at very low concentrations significantly antagonizes the effect of retigabine. By analogy with receptor pharmacology, plotting the log(1 - r) ratio (r being the ratio of the IC50 values of retigabine in the presence and in the absence of XE991) reveals a straight line with an estimated Kd value (the concentration of XE991 that doubles the EC50 of retigabine) of 66 nM. D, retigabine displays a similar inhibitory effect in VTA slice preparations (top trace) and XE991 completely blocks this effect (bottom trace). Traces are from the same VTA DA cell (bottom trace was recorded 15 min after top trace). Control experiments (data not shown) have demonstrated that repeated applications of a given concentration of retigabine yield reproductive inhibition of VTA firing in control conditions.
Bright-field microscopy revealed the presence of KCNQ2 immunoreactive neurons in both the SNc and SN pars reticulata (SNr) compartments; however, it was evident that more KCNQ2-immunopositive neurons were present in the SNr than in the SNc (Fig. 1A). Relatively few KCNQ2-expressing neurons were found in the VTA (Fig. 1A). KCNQ4-positive neurons were widely distributed in the SNc and the adjacent VTA with an extensive number of KCNQ4-positive neurons in both regions (Fig. 1C). A few KCNQ4-positive neurons were present in the SNr (Fig. 1C).

KCNQ4 immunoreactivity seemed to overlap with DA cell-rich areas, i.e., in the SNc, the VTA and the adjoining A8 cell group in the retrorubral area, but not in other parts of the ventral mesencephalon. Confocal immunofluorescence microscopy of sections costained for KCNQ4 and TH revealed that these two antigens completely overlapped in the SNc (Fig. 1, E–G) and VTA (data not shown). As indicated in Fig. 1F, the KCNQ4-labeled profile was observed not only in relation to plasma membranes but also was intracellular and in primary processes, whereas TH immunoreactivity was more homogenously distributed in the cytoplasm.

**Effect of Retigabine and XE991 on Midbrain Dopamine Cell Firing in Vitro**

**Extracellular Recordings.** Retigabine exhibited a potent and robust inhibitory action on neuronal firing of DA neurons in the SNc (Fig. 2A). Thus, 1 μM retigabine significantly inhibited basal spike frequency and a concentration of 10 μM completely blocked neuronal firing (Fig. 2A). The IC50 of retigabine was 2.1 ± 0.4 μM (n = 10).

To determine the target specificity of retigabine, 1 μM XE991 was added to the slice preparation under incrementing concentrations of retigabine. Application of retigabine in a concentration (10 μM) that completely blocks spike activity when applied alone had no activity when coapplied with XE991 (Fig. 2B). Only very high concentrations of retigabine could overcome the blockade by XE991 (Fig. 2B). The IC50 of retigabine in the presence of 1 μM XE991 was 192 ± 40 μM (n = 6). Thus, 1 μM XE991 shifted the IC50 value of retigabine 91-fold. XE991 inhibited retigabine at even lower concentrations, since at 100 nM, it still shifted the IC50 value of retigabine significantly (6.2 ± 1.2 μM in control conditions versus 2.1 ± 0.4 μM; n = 6; p = 0.0072). Further quantitative analysis of the antagonism of retigabine by XE991, revealed that the “Kb” value (in analogy with receptor pharmacology: the concentration that doubles the IC50 of retigabine) of XE991 was 66 nM (Fig. 2C). Interestingly, XE991 produced a modest, but significant, increase (22%) of spike frequency (p = 0.007, t test; Fig. 2B).

In the VTA, retigabine also displayed an inhibitory effect on the basal spike activity. The IC50 of retigabine was 1.7 ± 0.4 μM (n = 5; Fig. 2D) and was not different from the IC50 of retigabine in SNc slice preparations (p = 0.53). The inhibitory effect of retigabine in the VTA was completely blocked by 1 μM XE991 (n = 2) (Fig. 2D). D1 receptor antagonists (haloperidol and raclopride at 1 μM) had no significant influence on the basal DA neuron firing rate in rat midbrain slices, and they did not change the effect of retigabine when coapplied (data not shown).

**Intracellular Recordings.** For intracellular recordings, the effect of retigabine was assessed in five SNc DA neurons. In two neurons, the experiments were performed in the presence of TTX. In three other neurons, the GABA_A antagonist SR95531, the I_h blocker ZD7288, and the SK channel blocker apamin were also added to the control superfusion medium. Since no difference was observed between the two conditions, the results were pooled. The initial membrane potential was set at −50 mV by small DC current injections (+20 to −70 pA). Retigabine (10 μM) induced a hyperpolarization of 9.4 ± 2.0 mV (Fig. 3A) (n = 5). When the membrane potential was brought back to its initial value by current injection, a 54% drop in input resistance was observed (226 ± 46–102 ± 13 MΩ). As shown in Fig. 3A, this effect was maintained during the superfusion of the drug and was only slowly reversible after washout. These effects of retigabine were completely blocked by the simultaneous application of 10 μM XE991 (n = 3; Fig. 3B). XE991 had very little effect on the membrane potential when it was applied alone (data not shown). At −80 mV, retigabine did not induce a hyperpolarization.

A final set of experiments was performed with XE991. At 10 μM, the KCNQ channel blocker did not modify the action potential amplitude (59.2 ± 2 mV in control condition versus 59.1 ± 2 mV with XE991) or duration (0.97 ± 0.1 ms in control condition versus 1.0 ± 0.1 ms with XE991). In addition, 10 μM XE991 did not induce significant changes in the membrane potential (Fig. 4A) and had no influence on the medium afterhyperpolarization (mAHP) phase displayed by DA neurons (Fig. 4B), which is mediated by SK3 channels.
However, 10 μM XE991 induced an increase in the number of spikes produced by depolarizing pulses (≥800 ms) in three of six SNc DA neurons (Fig. 4C).

**Effect of Retigabine on SNc Dopamine Cell Firing in Vivo**

Single unit recordings were used to determine the effect of retigabine on SNc DA neuron firing in vivo. The neurons used for further characterization were selected if they displayed efficient firing inhibition by administration of the D2 receptor agonist apomorphine and excitation following treatment with haloperidol due to acute D2 receptor blockade (Fig. 5A). Retigabine (2.5 mg/kg; n = 8) almost instantly reduced the firing rate in SNc DA neurons from 160 ± 19 spikes/10 s (after haloperidol treatment) to 28 ± 13 spikes/10 s (after retigabine treatment), thus significantly reducing the enhanced spike activity induced by haloperidol (p < 0.0001). It is noteworthy that retigabine reduced the spike activity to a level below baseline (basal 84 ± 6 spikes/10 s versus retigabine, 28 ± 13 spikes/10 s; p = 0.0022), even in the presence of haloperidol (Fig. 5, A and B). The prominent inhibitory effect of retigabine on DA cell firing was sustained throughout the experiment, thus lasting more than 10 min (Fig. 5, A and B).

**Retigabine Inhibits c-Fos Activation in the Striatum**

Functional analysis of retigabine on striatal activity was performed using the immediate-early transcription factor c-Fos as a marker of postsynaptic neuronal excitation. The basal level of c-Fos expression (cells/millimeter²) varied in different parts of the striatum, with the highest level in the ACCshell (199 ± 35, Fig. 6E) and lower in the ACCcore (70 ± 26; Fig. 6I) and STRdl (50 ± 13; Fig. 6A). Administration of retigabine alone produced a significant reduction in basal striatal c-Fos levels. Compared with basal levels in vehicle-treated rats, 10 mg/kg retigabine reduced c-Fos levels to almost undetectable levels in the STRdl (6 ± 4, p = 0.0094; Fig. 6F) and ACCcore (26 ± 10; p = 0.0211; Fig. 6J), whereas basal c-Fos expression in the ACCshell (83 ± 19; p = 0.0153; Fig. 6B) was partially blocked by retigabine.

As expected, haloperidol induced an intense c-Fos immunoreactivity in all parts of the striatum examined (Figs. 6 and 7), i.e., the dorsolateral region (15-fold increase), ACCshell (2-fold increase), and ACCcore (7-fold increase). Pretreatment with retigabine inhibited haloperidol-induced c-Fos dose dependently, being evident in all aspects of the striatum examined (Figs. 6 and 7), because the inhibition was prominent in both the dorsal striatum (Fig. 6, A–D) as well as in ACCshell (Fig. 6, E–H) and ACCcore (Fig. 6, I–L), respectively. The lowest dose of retigabine in all segments of
Fig. 5. Retigabine blocks DA neuronal activity in vivo. A, representative trace of a single unit recording from a SNc DA neuron, showing the characteristic short-lasting inhibitory profile of apomorphine (apo; 0.04 mg/kg i.v.) on DA cell firing. Haloperidol (Hal; 0.08 mg/kg i.v.) significantly increases cell firing, which is completely blocked by retigabine (ret; 2.5 mg/kg i.v.). B, quantitative analysis of the effect of haloperidol and retigabine compared with baseline spike activity. #, p < 0.05; ##, p < 0.01 (compared with vehicle); and ###, p < 0.001 (compared with haloperidol).

Fig. 6. Retigabine strongly inhibits haloperidol-induced c-Fos in all parts of the striatum. Photomicrographs illustrating distribution of c-Fos expression in the dorsolateral striatum (A–D), the shell (E–H), and core (I–L) of nucleus accumbens, respectively. The photomicrographs represent vehicle (A, E, and I), retigabine (B, F, and J), haloperidol (C, G, and K), and retigabine-haloperidol treatment (D, H, and L), respectively. Scale bar, 200 μm.
the striatum showing a statistically significant effect was 1.0 mg/kg.

In the ventral striatum, the haloperidol-activated neurons were organized in the characteristic patches (Fig. 6G). It is noteworthy that retigabine eliminated the accumbal patchy organization of the c-Fos-immunopositive neurons (Fig. 6, H and L).

The inhibition of haloperidol-induced c-Fos levels in the striatum was counteracted by XE991 preadministration. In all striatal areas examined, the inhibitory effect of retigabine was reversed completely to a level indistinguishable from animals treated with haloperidol alone (Fig. 8, A–C). When administered alone, XE991 had no effect on c-Fos expression in any striatal subregion examined (Fig. 8).

The selective D2 receptor antagonist raclopride produced a prominent induction of c-Fos in the striatum, topographically and quantitatively similar to that of haloperidol. The prominent stimulatory effect of raclopride in the ventral striatum was completely blocked by preadministration of retigabine (ACCshell: vehicle, 200 ± 50; raclopride, 410 ± 27; retigabine + raclopride, 187 ± 39; p < 0.001; ACCcore: vehicle, 80 ± 45; raclopride, 347 ± 72; retigabine + raclopride, 70 ± 19; p < 0.001), whereas the inhibitory effect of retigabine was only partial in the STRdl (vehicle, 6 ± 3; raclopride, 802 ± 129; retigabine + raclopride, 255 ± 106; p = 0.0057).

Fig. 7. Quantitative assessment of the dose-related effect of retigabine (0.1–10 mg/kg) on haloperidol-induced (1.0 mg/kg i.p.) c-Fos induction in the dorsolateral striatum (A), nucleus accumbens shell (B), and core (C). A letter not in common signifies a statistical difference (p < 0.05) between treatments.
Retigabine Blocks Induction of TH Phosphorylation in the Striatum

Western blot analysis indicated that acute haloperidol treatment stimulated Ser40 phosphorylation of TH in tissue extracts of the STRdl. TH Ser40 phosphorylation was significantly increased 30 min after haloperidol administration ($p < 0.0012$), whereas TH Ser40 phosphorylation slightly, but nonsignificantly, stimulated at 15 and 60 min after haloperidol administration (Fig. 9A). The short-term effect of haloperidol on TH phosphorylation is in agreement with previous reports (Salvatore et al., 2000; Håkansson et al., 2004). Hence, because of the clear time-dependent response on haloperidol-induced Ser40 phosphorylation, the effect of retigabine was monitored 30 min after haloperidol administration.

As illustrated in Fig. 9B, retigabine had no effect on Ser40 phosphorylation per se, but it completely reversed the stimulatory effect of haloperidol. Subsequent Western blot analysis of total TH levels using a phosphorylation-independent TH antibody indicated that neither haloperidol, retigabine, nor the retigabine-haloperidol combination had any effect on total TH levels compared with control levels (one-way ANOVA, $p = 0.598$) (data not shown).

Retigabine Reduces Extracellular Levels of Dopamine Metabolites in Vivo

In vivo microdialysis was used to measure the effects of retigabine on the level of DA and the corresponding DA metabolites in the striatum (Fig. 10). Administration of retigabine alone did not influence extracellular DA levels in the striatum ($p = 0.6638$; Fig. 10A). By contrast, the levels of the two principal DA metabolites DOPAC (Fig. 10B) and HVA (Fig. 10C) were significantly reduced during retigabine treatment ($p < 0.0001$ for both metabolites). The retigabine-induced inhibitory effect on DOPAC and HVA levels declined gradually. For DOPAC, the reduction was statistically significant at 60 min postinjection, whereas HVA levels were significantly decreased at 100 min, and the response on both DA metabolites continued to decline throughout the experiment.
Discussion

Here, we demonstrate that retigabine profoundly affects neuronal excitability in SNc DA neurons. In vitro, the effect of retigabine was both robust and highly sensitive to the KCNQ channel blocker XE991, indicating that retigabine acted via activation of KCNQ channels. A similar and equipotent effect of retigabine was also observed in the VTA. Our intracellular recordings performed in the presence of TTX and during blockade of GABA_A receptors, SK channels, and Ih channels exclude the possibility that the inhibitory effect of retigabine on the excitability of DA neurons is mediated by an indirect effect in the SNc or by another action of retigabine. The lack of effect at −80 mV is consistent with an action on an M-type current (Delmas and Brown, 2005).

In vivo, the single unit recordings confirmed the in vitro observations by showing that retigabine not only fully blocked haloperidol-induced increase in firing rate but also efficiently reduced the basal firing activity of SNc DA neurons. A XE991-sensitive, typical M-type current has recently been demonstrated in dissociated VTA DA neurons (Koyama and Appel, 2006). Together, those voltage-clamp experiments and our extracellular (both in vitro and in vivo) and intracellular recordings demonstrate the existence of a retigabine-and XE991-sensitive M-current in mesencephalic DA neurons. Our experiments further demonstrate that this current can be potentiated by retigabine under physiological conditions.

XE991 alone had very little effect on the membrane potential of SNc DA neurons, and it did not affect the mAHHP. However, XE991 increased the excitability of a fraction of SNc DA neurons, an effect similar to that reported for VTA DA neurons (Koyama and Appel, 2006). Together, those voltage-clamp experiments and our extracellular (both in vitro and in vivo) and intracellular recordings demonstrate the existence of a retigabine-and XE991-sensitive M-current in mesencephalic DA neurons. Our experiments further demonstrate that this current can be potentiated by retigabine under physiological conditions.

Because KCNQ-immunopositive neurons were found in the SN and VTA, the powerful effect of retigabine is most likely triggered locally within the ventral tegmentum. The SN and VTA neurons displayed subunit-restricted KCNQ-labeling, because only immunoreactivity for KCNQ2 and KCNQ4 was found. In addition, the IC_{50} for tetraethylammonium measured in dissociated VTA DA neurons is reported to lie between the IC_{50} of KCNQ2 and KCNQ4 channels, whereas the IC_{50} of XE991 is closest to that of expressed KCNQ2 channels (Koyama and Appel, 2006). This is interesting, because KCNQ2 and KCNQ4 do not coassemble in vitro to generate functional heteromeric channels (Kubisch et al., 1999). The lack of KCNQ3 immunoreactivity likely rules out that KCNQ2 and KCNQ4 immunoreactivity in the ventral tegmentum represents heteromeric KCNQ channels, and they may therefore operate as homomers. In accordance, homeric KCNQ2 and KCNQ4 channels are capable of generating potassium currents in vitro (Biervert et al., 1998; Kubisch et al., 1999).

KCNQ2 immunoreactivity was present throughout the SN, as also reported in the mouse (Cooper et al., 2001). In contrast, KCNQ4 subunits were observed in the rat SNc, but not in the SNr. The KCNQ4 subunit was predominantly localized to the plasma membrane; however, KCNQ4 immunoreactivity was also observed in intracellular and primary processes, in agreement with Kharkovets et al. (2000). Presumably, these KCNQ4 channel proteins are retained as a consequence of incomplete trafficking to the plasma membrane, or, alternatively, they may be destined for trafficking to axonal, dendritic, or perisynaptic sites, as suggested for KCNQ2 channels (Cooper et al., 2001).

This restricted subregional localization of KCNQ4 subunits is in agreement with a report on murine KCNQ4 distribution (Kharkovets et al., 2000). In the SNc and VTA, KCNQ2 channels were much less expressed than KCNQ4 channels, likely indicating that the KCNQ4 channel is the predominant KCNQ channel responsible for the strong inhibitory effect of retigabine on DA neurotransmission. This is
Fig. 10. Extracellular levels of DA (A), DOPAC (B), and HVA (C) in the rat striatum following systemic administration of retigabine (10 mg/kg i.p.; open circles) or vehicle (10% Tween 80 in saline, closed circles). Retigabine was administered 40 min ($t = 0$) after microdialysis was initiated. Retigabine had no effect on extracellular DA levels in the striatum, but it induced a significant drop in DOPAC ($p < 0.0001$) and HVA ($p < 0.0001$) levels, which progressed throughout the experiment. $*, p < 0.05$; $**, p < 0.01$; and $***$, $p < 0.001$, two-way ANOVA with Bonferroni’s post hoc test. Histograms of cumulated (0- to 180-min) DA, DOPAC, and HVA levels [area under the curve (AUC) ± S.E.M.], respectively, are inserted. $***$, $p < 0.001$, unpaired $t$ test. All data are normalized to the mean concentration sampled in a total of 60 min before vehicle or retigabine injection.
further supported by the immunohistochemical analysis of the principal neuronal phenotype expressing KCNQ4 subunits in the SNc and VTA, revealing that all KCNQ4 channels were present on TH-positive neurons and that KCNQ4 immunoreactivity was extensive, because virtually all mesencephalic DA neurons expressed KCNQ4 channels. We therefore conclude that the mesencephalic homomorphic KCNQ4 channel is the molecular target of retigabine leading to inhibition of DA neuron firing.

The strong inhibitory effect of retigabine on mesencephalic DA activity is intriguing. Since the SNc and the lateral VTA provide essential and extensive DA modulatory inputs to striatal and mesolimbic targets (Swanson, 1982), this suggests that KCNQ channel modulation occurs in both nigrostriatal and limbic pathways. Accordingly, retigabine almost completely eliminated basal excitability in both the dorsolateral and ventral aspects of the striatum, as inferred by the reduced levels of basal c-Fos immunoreactivity, a transcriptional marker commonly used to map changes in synaptic neuronal activity (Morgan and Curran, 1991).

In addition, retigabine reduced basal striatal DOPAC and HVA without affecting DA levels. Since metabolism of DA, by concerted action of monoamine-oxidase and catechol-O-methyltransferase, results in DOPAC and HVA production, changes in DA metabolite levels reflect altered DA synthesis rate and DA neuronal activity (Di Giulio et al., 1978; Nissbrandt et al., 1989), and we can therefore conclude that retigabine reduced DA synthesis in striatal nerve terminals. In accordance, retigabine also reversed haloperidol-induced TH Ser40 phosphorylation, a presynaptic marker of stimulated striatal DA synthesis (Håkanson et al., 2004), indicating that retigabine was also capable of normalizing striatal TH activity in conditions of stimulated DA activity.

Acute blockade of DA D2 receptors effectively depolarizes SNc neurons in vivo (Bunney et al., 1973) and leads to prominent striatal excitation by stimulating DA release (Di Chiara and Imperato, 1988), turnover (Zetterström et al., 1986), and synthesis (Magnusson et al., 1987). Haloperidol caused a pronounced c-Fos induction in the ventral and lateral striatum, interpreted as enhanced neuronal activity caused by acute blockade of somatodendritic and terminal DA D2 receptor function (Robertson and Fibiger, 1992). Retigabine eliminated the strong excitatory response to haloperidol, with an ED50 of approximately 1 mg/kg, and also inhibited the effect of raclopride, emphasizing the inhibitory effect of retigabine on D2 receptor blockade-mediated excitation. The potency of retigabine is higher than found in behavioral studies, where minimum effective doses typically range from 3 to 10 mg/kg (Korsgaard et al., 2005). The negative modulatory effect on haloperidol-induced c-Fos was KCNQ-specific, because XE991 completely reversed this effect. Because virtually all DA neurons in the SNc and VTA express D2 receptors (Khan et al., 1998), this may be of functional relevance to KCNQ channel physiology. The interaction with the D2 receptor-mediated c-Fos response in the striatum is further emphasized by the observation that flupirtine inhibits haloperidol-induced catalepsy, which is a typical extrapyramidal effect of generalized D2 receptor blockade (Schmidt et al., 1997). Furthermore, motor impairment is also observed in rats exposed to acute retigabine administration (Rostock et al., 1996; Korsgaard et al., 2005), which may thus directly relate to the potent inhibitory effect of retigabine on striatal excitability. Although the functional consequences of systemic administration of retigabine likely occur in the SNc and VTA, the striatal effect could potentially also be explained by actions independent on DA input from the SNc and VTA.

Striatal neurons express KCNQ2, KCNQ3, and KCNQ5 but not KCNQ4 channels (Cooper et al., 2001; Shen et al., 2005). In addition, heteromeric KCNQ2/3 channels are suggested to provide inhibitory tonus on striatopallidal/striatonigral neurons (Shen et al., 2005). Striatopallidal neurons express D2 receptors (Gerfen et al., 1990) and respond to excitatory D2 receptor blockade (Robertson and Jian, 1995). Thus, retigabine may also affect neuronal excitability by direct action on striatopallidal neurons. However, because striatal c-Fos induction by pharmacological D2 receptor blockade is critically dependent on mesolimbic DA inputs (Robertson et al., 1992), this likely rules out that intrastriatal KCNQ channels contributed significantly to the inhibitory effect of retigabine. In addition, the cortex and corticostriatal projections express KCNQ channel subunits (Cooper et al., 2001; Yus-Najera et al., 2003), which tentatively suggests that KCNQ channels on corticostriatal projections may play a role in the control of striatal excitability.

In conclusion, the prominent inhibitory effect of KCNQ channel activation in nigrostriatal and mesolimbic pathways suggests that pharmacological enhancement of KCNQ channel function may be a novel approach to improve conditions of DA overactivity in the basal ganglia. Consistent with the localization of KCNQ4 subunits on key sites for control of cerebral DA activity, homomeric KCNQ4 channels may represent a novel target for treating disease states characterized by abnormal DA neurotransmission, e.g., schizophrenia, attention deficit hyperactivity disorder, and drug abuse.

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