Investigation of the mechanisms of action of the novel anticonvulsant topiramate: electrophysiological studies on rat olfactory cortical neurones in vitro and some in vivo rodent models of epilepsy.

A thesis submitted in part fulfilment of the University of London for the award of Doctor of Philosophy in Medicine (Pharmacology)

by

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This thesis describes research conducted in The School of Pharmacy, University of London and The Department of Experimental and Clinical Medicine “G. Salvatore”, Faculty of Medicine and Surgery, University “Magna Graecia” of Catanzaro, Catanzaro, Italy between 5-June-2000 and 16-December-2003 under the supervision of Dr. A. Constanti and Prof. G. De Sarro. I certify that the research described is original and that any parts of the work that have been conducted by collaboration are clearly indicated. I also certify that I have written all the text herein and have clearly indicated by suitable citation, any part of this thesis that has already appeared in publication.

Signed.............................................. Date..............................................

[Signature]

18/07/2005
Abstract

Topiramate (TPM; Topamax®) is a novel anticonvulsant that was originally developed as a possible inhibitor of gluconeogenesis, but was found to be a very effective anticonvulsant drug in many animal models. To date, the proposed mechanisms of action of TPM include: inhibition of neuronal Na⁺ channels, enhancement of GABAₐ-receptor mediated effects; inhibition of AMPA/kainate (glutamate) receptors, inhibition of high voltage-activated Ca²⁺ channels, and inhibition of carbonic anhydrase activity.

In the present study, the effects of TPM were investigated in rat olfactory (piriform) cortex neurones in vitro, using an intracellular current/voltage clamp recording technique. Bath-application of TPM induced a slow, dose-dependent and reversible membrane hyperpolarization, accompanied by a decrease in membrane input resistance and inhibition of repetitive action potential firing. Under voltage clamp at -70 mV holding potential (Vₜ), the TPM response manifested as a slow outward membrane current, developing over 10 min and slowly reversing after washout; the TPM current was partially (~50%) blocked by Ba²⁺ (a general blocker of K⁺ conductances), suggesting it was largely carried by K⁺ ions, but unaffected by Cd²⁺ (200 μM) or bicuculline (10 μM) indicating that a Ca²⁺-dependent conductance or GABAₐ receptors were not involved, respectively. Current/voltage (I/V) plots (Vₜ =-70 mV) constructed in the presence and absence of TPM failed to intersect at very negative potentials, suggesting that the TPM current may comprise of a mixture of ionic conductances, or a contribution from some electrogenic pump mechanism. Topiramate (20 μM) also enhanced and prolonged the slow post-stimulus (Ca²⁺-dependent) afterhyperpolarization (sAHP), that follows a long burst of action potentials (and the underlying slow outward tail current (sIₐHₐP) recorded under voltage clamp). We believe this effect was due to a selective enhancement/prolongation of an underlying L-type Ca²⁺ current that was blocked by nifedipine (20 μM); the modulatory effect of TPM on the sAHP was unlikely to involve an interaction at PKA-dependent phosphorylation sites, since it was unaffected by pre-incubation with forskolin (20 μM), a direct activator of adenylate cyclase (and ultimately PKA). Interestingly, the CA inhibitors acetazolamide (ACTZ, 20 μM) and benzolamide (BZ, 50 μM) both mimicked the membrane effects of TPM, in generating a slow hyperpolarization (slow outward current under voltage clamp) and sAHP enhancement/prolongation. ACTZ and BZ also occluded the effects of TPM in generating the outward current response but were
additive in producing the sAHP modulatory effect, suggesting different underlying response mechanisms. In bicarbonate/CO₂-free, HEPES-buffered bathing medium, all the membrane effects of TPM and ACTZ were reproducible, therefore not dependent on CA inhibition. We propose that other molecules possessing the sulphonamide moiety in their structure might have the same action as TPM on piriform cortical neurones.

In a second series of in vivo experiments, we determined the efficacy of TPM in some animal models of epilepsy and also tested whether L-type Ca²⁺ channel modulators could modify its potency in vivo (as predicted from the in vitro data). The results obtained showed that TPM possesses a wide spectrum of anticonvulsant activity against both convulsive and non-convulsive seizures. In particular, TPM protected against seizures induced by sound stimuli in DBA/2 mice, intracerebroventricular injection of AMPA, subcutaneous administration of 4-aminopyridine or pentylenetetrazole administered intraperitoneally. The co-administration of nifedipine with TPM significantly decreased its anticonvulsant activity only in DBA/2 mice, whereas it did not affect TPM’s anticonvulsant properties in the other animal models. Topiramate was also very effective in two genetic animal models of absence epilepsy (lethargic “lh/lh” mice and WAG/Rij rats). However, rather surprisingly, when TPM was co-administered with nifedipine in these models, contrasting results were observed; in WAG/Rij rats, nifedipine antagonized TPM’s anti-absence activity whereas in the lh/lh mouse model a synergism was found (possibly due to presence of an abnormal L-Ca²⁺ channel β₄ subunit affecting the normal dihydropyridine-TPM-L-channel interaction). Therefore, an involvement of dihydropyridine-sensitive L-type Ca²⁺ channels in the anti-absence effects of TPM might also be considered.

In conclusion, this work has identified two new novel mechanisms of action for TPM, which are not currently shared by any other marketed anticonvulsant drug and could indicate novel targets for the development of new antiepileptic compounds in the future. Furthermore, the interaction of TPM with L-type Ca²⁺ channel antagonists analyzed in in vivo animal experimental models of epilepsy support the proposal that a modulation of neuronal L-type Ca²⁺ channel activity plays an important role in its antiepileptic activity. Finally, our results also predict that nifedipine or other L-type Ca²⁺ channel antagonists could affect TPM’s anticonvulsant efficacy in human epilepsy, therefore their combination or concomitant administration in therapy should be avoided or at least carefully monitored.
“In searching out the truth be ready for the unexpected, for it is difficult to find and puzzling when you find it”.

Heraclitus

“La natura è un tempio ove pilastri viventi emettono talvolta parole confuse. L’uomo la attraversa tra foreste di simboli che lo osservano con sguardi familiari”.

[“Nature is a temple in which living pillars sometimes emit confused words; man crosses it through forests of symbols that observe him with familiar glances].

Charles Baudelaire
To Antonietta Mazza
Acknowledgements

After writing so many pages, I must admit that this page has been the most difficult to write. It is very complicated to find the right words to describe how thankful I am to all these persons. Dr. Andy Constanti has been the craftsman of this project. He received me in his small office more than four years ago, when I was only a young man, who could only poorly understand a few things about English life and also pharmacology! I do not know what he originally saw in me, but this thesis is the proof that he believed in me as a scientist. Thanks Andy for being such a good life teacher and above all thanks for being such a good friend. A special thanks also goes to Prof. Giovambattista De Sarro, who allowed me to access his deep knowledge of epilepsy and epilepsy research. I know he believes I can make a good scientist; thanks for the chance, and I will do my best. Oh! Thanks also for all the good wine. I am disappointed that I cannot create new words to thank my parents Francesca and Antonio for modelling me from nothing to what I am today. For their silent guide that left me to make my choices on my own, for teaching me that big results require big efforts, but the same strength has to be used in daily life. I know you will always be there. Lidia, you know how much I love you; thanks for bearing my terrifying moods. Please, love me forever. The most important finding that I have discovered in London is that drinking and thinking are highly connected. No matter what you drink (beer, wine, tea, coffee), it activates some unknown circuitries that make everything easier in life. For this discovery I must thank Ben and Daniele who made my life in London the most intensive and meaningful experience of my life. My return to Italy has not been as easy and predictable. I have been very lucky to meet Dr. Guido, who I will always continue to thank for his unconditional help in everything I have done and I am doing. Mate, keep on cooking as only you know best. This thesis was also finished thanks to the help given by my favourite veterinary Nicola, who represents the best example of ‘Calabrese’ I have ever met. Also, Rita, Carmen, Salvatore and Luca for making my life in Catanzaro not as bad as it might have been. Let’s go on guys! Dear brother, what can I say?; thanks, I will not disappoint you.

Many more dear friends did not find space on this page, but I know and they know what my feelings are. Let’s go on!
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<td>$\beta$-parachlorophenyl-$\gamma$-aminobutyric acid</td>
</tr>
<tr>
<td>[(±)-CPP</td>
<td>$3\text{-}[(\pm)-2\text{-}\text{carboxy}piperazine\text{-}4\text{-}y] \text{propyl}\text{-}1\text{-}\text{phosphonic acid}</td>
</tr>
<tr>
<td>4-AP</td>
<td>4-aminopyridine</td>
</tr>
<tr>
<td>5-HT</td>
<td>5-Hydr oxytryptamine</td>
</tr>
<tr>
<td>ACTZ</td>
<td>Acetazolamide</td>
</tr>
<tr>
<td>ADNFLE</td>
<td>Autosomal Dominant Nocturnal Frontal Lobe Epilepsy</td>
</tr>
<tr>
<td>AE</td>
<td>Absence Epilepsy</td>
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<tr>
<td>AED</td>
<td>Anti-epileptic drug</td>
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<tr>
<td>AMPA</td>
<td>$\alpha$-amino-$3$-hydroxy-$5$-methyl-isoxazole</td>
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<tr>
<td>AMPA</td>
<td>$\alpha$-amino-$3$-hydroxy-$5$-methylisoxazole-$4$-propionic acid</td>
</tr>
<tr>
<td>APC</td>
<td>Anterior piriform cortex</td>
</tr>
<tr>
<td>AS</td>
<td>Absence seizures</td>
</tr>
<tr>
<td>AT</td>
<td>Area tempestas</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BAPTA</td>
<td>$1\text{-}2\text{-}\text{bis}(O\text{-}\text{aminophenoxy})\text{ethane}-N,N,N',N''\text{-}\text{tetracetic acid}$</td>
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<td>BAPTA-AM</td>
<td>$1\text{-}2\text{-}\text{bis}(O\text{-}\text{aminophenoxy})\text{ethane}-N,N,N',N''\text{-}\text{tetracetic acid}$ acetyl methyl ester</td>
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<td>$S\text{-}1\text{-}4\text{-}\text{dihydro}-2\text{-}6\text{-}\text{dimethyl}-5\text{-}\text{nitro}-4\text{-}[2\text{-}(\text{trifluoromethyl})\text{phenyl}]\text{-}3\text{-}\text{pyridinecarboxylic acid methyl ester}$</td>
</tr>
<tr>
<td>BFNC</td>
<td>Benign familial neonatal convulsions</td>
</tr>
<tr>
<td>BZ</td>
<td>Benzolamide</td>
</tr>
<tr>
<td>BZDs</td>
<td>Benzodiazepines</td>
</tr>
<tr>
<td>CA</td>
<td>Carbonic anhydrase</td>
</tr>
<tr>
<td>$\text{Ca}^{2+}$</td>
<td>Calcium ion</td>
</tr>
<tr>
<td>CAN</td>
<td>$\text{Ca}^{2+}$-activated nonselective cation conductance</td>
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<td>CFM-2</td>
<td>1-$(4\text{'-Aminophenyl})\text{-}3\text{-}5\text{-dihydro}\text{-}7\text{-}8\text{-dimethoxy}\text{-}4\text{H}\text{-}2\text{,3-}$ benzodiaze-pin-4-one</td>
</tr>
<tr>
<td>CI$^-$</td>
<td>Chloride ion</td>
</tr>
<tr>
<td>CNQX</td>
<td>6-cyano-7-nitroquinoline-2,3-dione</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>DAP</td>
<td>Depolarizing after potential</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>ED$_{50}$</td>
<td>Effective Dose 50%</td>
</tr>
<tr>
<td>EEGs</td>
<td>Electroencephalographs</td>
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<tr>
<td>EGTA</td>
<td>Ethylene glycol-bis-(beta-aminomethyl ether)-$N,N'$-tetraacetic acid</td>
</tr>
<tr>
<td>EPSP</td>
<td>Excitatory postsynaptic potential</td>
</tr>
<tr>
<td>ESM</td>
<td>Ethosuximide</td>
</tr>
<tr>
<td>FDA</td>
<td>US Food and Drug Administration</td>
</tr>
<tr>
<td>FLB</td>
<td>Felbamate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>GABA</td>
<td>γ-Aminobutyric acid</td>
</tr>
<tr>
<td>GABA-T</td>
<td>GABA transaminase</td>
</tr>
<tr>
<td>GAD</td>
<td>Glutamic acid decarboxylase</td>
</tr>
<tr>
<td>GAD67</td>
<td>Glutamate decarboxylase 67</td>
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<tr>
<td>GAERS</td>
<td>Genetically Absence Epileptic Rats from Strasbourg</td>
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<tr>
<td>GAT-1</td>
<td>Gamma-aminobutyric acid transporter-1</td>
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<tr>
<td>GBP</td>
<td>Gabapentin</td>
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<td>GEFS&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Generalized epilepsy with febrile seizures plus</td>
</tr>
<tr>
<td>GHB</td>
<td>γ-hydroxybutyric</td>
</tr>
<tr>
<td>GIRK</td>
<td>G-protein coupled inward-rectifying K&lt;sup&gt;+&lt;/sup&gt; conductance</td>
</tr>
<tr>
<td>GYKI 52466</td>
<td>1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine hydrochloride</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)</td>
</tr>
<tr>
<td>HVA</td>
<td>High voltage-activated</td>
</tr>
<tr>
<td>I</td>
<td>Current</td>
</tr>
<tr>
<td>i.c.v.</td>
<td>Intracerebroventricular</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
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<td>I/V</td>
<td>Current/voltage plots</td>
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<td>Ca&lt;sup&gt;2+&lt;/sup&gt;-activated K&lt;sup&gt;+&lt;/sup&gt; conductance</td>
</tr>
<tr>
<td>ILAE</td>
<td>International League Against Epilepsy</td>
</tr>
<tr>
<td>IPSP</td>
<td>Inhibitory postsynaptic potential</td>
</tr>
<tr>
<td>K&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Potassium ion</td>
</tr>
<tr>
<td>LEV</td>
<td>Levetiracetam</td>
</tr>
<tr>
<td>lh/lh</td>
<td>lethargic mice</td>
</tr>
<tr>
<td>LOT</td>
<td>Lateral olfactory tract</td>
</tr>
<tr>
<td>LGT</td>
<td>Lamotrigine</td>
</tr>
<tr>
<td>MES</td>
<td>Maximal electroshock seizure</td>
</tr>
<tr>
<td>mGluR</td>
<td>Metabotropic glutamate receptor</td>
</tr>
<tr>
<td>MHD</td>
<td>10,11-dihydro-10-oxo-CBZ</td>
</tr>
<tr>
<td>MK-801</td>
<td>(+)-methyl-10,11-dihydro-5H-dibenzo(a,d)-cyclohepten-5,10-imine maleate</td>
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<td>MPC</td>
<td>Median piriform cortex</td>
</tr>
<tr>
<td>Na&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Sodium ion</td>
</tr>
<tr>
<td>NBQX</td>
<td>1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[f] quinoxaline-7-sulfonamide</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NMDAR</td>
<td>N-methyl-D-aspartate receptor</td>
</tr>
<tr>
<td>OXC</td>
<td>Oxcarbazepine</td>
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<tr>
<td>OXO-M</td>
<td>Oxotremorine-M</td>
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<tr>
<td>PC</td>
<td>Piriform cortex</td>
</tr>
<tr>
<td>PEMA</td>
<td>Phenylmethylmalonamide</td>
</tr>
<tr>
<td>PHB</td>
<td>Phenobarbital</td>
</tr>
<tr>
<td>pH&lt;sub&gt;i&lt;/sub&gt;</td>
<td>Intracellular concentration of H&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>pH&lt;sub&gt;e&lt;/sub&gt;</td>
<td>Extracellular concentration of H&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>PHT</td>
<td>Phenytoin</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein Kinase A</td>
</tr>
<tr>
<td>PPC</td>
<td>Posterior piriform cortex</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>PRM</td>
<td>Primidone</td>
</tr>
<tr>
<td>PTZ</td>
<td>Pentylenetetrazole; Leptazol</td>
</tr>
<tr>
<td>REM</td>
<td>Rapid eyes movement</td>
</tr>
<tr>
<td>RTN</td>
<td>Reticular thalamic nucleus</td>
</tr>
<tr>
<td>s.c.</td>
<td>Subcutaneously</td>
</tr>
<tr>
<td>s.e.</td>
<td>Standard Error</td>
</tr>
<tr>
<td>sAHP</td>
<td>Slow post-stimulus (Ca(^{2+})-dependent) afterhyperpolarization</td>
</tr>
<tr>
<td>SER</td>
<td>Spontaneously epileptic rats</td>
</tr>
<tr>
<td>SGB-017; ADCI</td>
<td>(±)-5-aminocarbonyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine</td>
</tr>
<tr>
<td>sI(_{AHP})</td>
<td>Slow post-stimulus outward tail current</td>
</tr>
<tr>
<td>SWDs</td>
<td>Spike-wave discharges</td>
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<tr>
<td>TCS</td>
<td>Tonic-clonic seizures</td>
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<td>TEA</td>
<td>Tetraethylammonium</td>
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<td>TGB</td>
<td>Tiagabine</td>
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<td>TPM</td>
<td>Topiramate; Topamax®</td>
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<td>trans-ACPD</td>
<td>Trans-aminocyclopentane-1,3- dicarboxylic acid</td>
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<td>Vigabatrin</td>
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<td>V(_{h})</td>
<td>Holding potential</td>
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<td>Valproate</td>
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<tr>
<td>WAG/Rij rats</td>
<td>Wistar Albino Glaxo rats from Rijswijk</td>
</tr>
<tr>
<td>ZNS</td>
<td>Zonisamide</td>
</tr>
</tbody>
</table>
Chapter I:

General Introduction
I.1 Epilepsy: History and definitions

Epilepsy, from the Greek *epilepsia* (a taking hold of, or ‘seizing’) is a chronic neurological disorder characterized by a spontaneous tendency for recurrent seizures. In general terms, a seizure is defined as the outward manifestation of an abnormal and excessive synchronized electrical discharge of a set of cerebral neurones. The seizures are characteristically sudden and transient in nature, and the clinical manifestations can include a wide variety of motor, psychic and sensory phenomena, which may occur with or without an alteration in consciousness or awareness of the patient (Crill, 1980; Hopkins, 1995; Shorvon, 2000). The symptoms observed depend on the part of the brain involved in the epileptic neuronal discharge. Whereas all patients with epilepsy have recurrent seizures, many more patients have only a single seizure during life (usually early childhood) and are therefore not considered to have epilepsy (Shorvon, 2000). It is important to note that seizure episodes are, in fact, quite common, and according to Sander & Shorvon (1996), between 2% and 5% of the general population will have at least one non-febrile seizure during their lifetimes. Epilepsy is considered the most common serious neurological condition. In studies of most Western populations, it is estimated that the annual incidence of epilepsy is about 80 per 100,000 per year. The lifetime risk (cumulative incidence) of a seizure is about 5%. Single seizures, even if not amounting to epilepsy, often require investigation, and some authorities even recommend antiepileptic treatment (Shorvon, 2000).

The earliest written descriptions of epilepsy may be found on a Babylonian tablet kept in the British Museum, which belongs to a Babylonian textbook of medicine, dated at around 2000BC. Another ancient and quite detailed description of epilepsy is given in the Indian Ayurvedic literature of Charaka Samhita (dated to around 400BC); here, epilepsy is described as “*apasmara*”, meaning “loss of consciousness”. In contrast to the Ayurvedic system of medicine of Charaka Samhita, however, the Babylonian point of view emphasized the “supernatural” property of epilepsy, whereby each type of seizure was associated with the name of a spirit or god. Therefore, treatment was largely considered to be a spiritual matter; (summarized from WHO website- “Epilepsy: historical overview” - http://www.who.int/mediacentre/factsheets/fs168/en/; see also Temkin, 1971). The Babylonian belief was maintained until the 5th century BC when Hippocrates, in his famous treatise “*on the sacred disease*”, suggested that epilepsy was not “sacred”, but a real disorder of the brain. He went on to suggest physical treatments
for the condition, and stated that if the disease became chronic, then it would be incurable. Although Hippocrates provided this less spiritualized vision of the disease, the perception that epilepsy was a chronic disorder of cerebral function started only after the 18th and 19th Centuries AD. Thus, for 2000 years, people with epilepsy were regarded with much suspicion, and treatment was mainly related to supernatural or spiritual matters. In Europe, St Valentine has been the patron saint of people with epilepsy since medieval times and in this period the drinking of human blood and skull cauterization were practiced to cleanse the body of evil spirits and reduce the likelihood of transmission (Temkin, 1971; Hopkins, 1985). Despite the enormous social stigma and alienation assigned to people with epilepsy throughout this time, some well-known individuals did succeed in life and became famous; such people include Julius Caesar, Czar Peter the Great of Russia, Pope Pius IX, the writer Fyodor Dostoyevsky and the poet Lord Byron.

The process of distinguishing epilepsy from madness began in the 19th Century and was linked with the development of neurology as a new and independent discipline, distinct from psychiatry; the concept of epilepsy as a distinct brain disorder then became more widely accepted, especially in Europe and the United States of America (USA). Bromide, introduced in 1857 as the world’s first effective anti-epileptic drug, became widely used in Europe and the USA during the second half of the last century (Hopkins, 1985). In the latter half of the 19th Century, views about epilepsy were radically changed by the famous English physician Dr. John Hughlings-Jackson, the so-called “father of English neurology” who suggested that the word epilepsy should be redefined in neurophysiological rather than clinical terms. In 1873, Jackson proposed that seizures were the result of sudden, excessive, brief electro-chemical discharges of grey matter within the brain. He also suggested that the character of the seizures depended on the location/function of the site of the discharges; (summarized from Medical Dictionary MedicineNet.com, at http://www.medterms.com/script/main/art.asp?articlekey=26727).

This was essentially the first neuronal theory of epilepsy, which formed the foundation of our modern understanding of epilepsy (Hopkins, 1985). In the same period, Drs. David Ferrier in London (based on Jackson’s concepts), Gustav Theodor Fritsch and Eduard Hitzig in Germany discovered the electrical excitability of the brain in animals and man, specifying the areas involved in the five senses. Jackson was the first to localize epileptogenic lesions correlating focal motor seizures with post-mortem
pathological examinations. In 1886, Sir Victor Horsley performed the first surgery for epilepsy; he resected a traumatic cortical scar in a patient with focal motor seizures (Rall & Schleifer, 1992).

The Austrian psychiatrist Hans Berger first recorded electroencephalographs (EEGs) from humans. The EEG revealed for the first time the presence of electrical discharges in the brain. Its important application from the 1930s onwards was in the field of epilepsy, where it revealed different patterns of brainwave discharges associated with different seizure types. The EEG also helped to locate the site of seizure discharges and expanded the possible range of neurosurgical treatments (Trimble & Reynolds, 1986). The first official classification of epileptic seizures was proposed in 1964 by the Commission on Classification and Terminology of the International League Against Epilepsy (ILAE); this was revised in 1981 and again in 1989 (Shorvon, 2000; see Section 1.2). During the first 50 years of the 20th century, the only two drugs available for the treatment of epilepsy have been phenobarbital (first used in 1912) and phenytoin (first used in 1938). Since the 1960s, there has been a rapid process of drug discovery in the antiepileptic field, which reached its peak during the 1990s, when many new drugs were introduced on the market. The development of new drugs was helped in part by a much better understanding of the electrical behaviour of the brain, and especially the nature and function of the principal excitatory and inhibitory neurotransmitters. In addition, in developed countries in recent years, several highly effective "new-generation" drugs have also come onto the market, now enabling seizures to be controlled in ~70% to 80% of newly diagnosed children and adults (Annegers, 1994).

During the last 10-20 years, much greater attention has been paid to the quality of life of epilepsy sufferers, i.e. psychological and social issues, for epileptics; however, progress has been slow and patient care services can still be rather poor. It is also unfortunate, that most of the drug advances made in developed countries are of relevance, but because of their relatively high cost, are not available for ~80% of people with epilepsy who live in underdeveloped or developing countries. For most of these people, supernatural associations, social stigma and discrimination are still abundant. Even in developed countries, epilepsy is still shrouded in secrecy, and people prefer not to reveal or discuss their condition with family, friends or employers. Of the 50 million people around the world that suffer from epilepsy, some 35 million have no access to appropriate treatment. This is either because services are non-existent or because
epilepsy is still not regarded as a medical problem or even as a treatable brain disorder (summarized from WHO website “Epilepsy: historical overview” http://www.who.int/mediacentre/factsheets/fs168/en/).
1.2 Classification of epileptic seizures and syndromes

Epilepsy is not a single disorder, but rather a group of disorders, for which the aetiology is different and relates to each specific disorder. Differences exist between patients who experience a single seizure and those with a tendency for recurrent seizures. Single seizures have various causes, including electrolyte disturbances, drugs, and toxins. An increased extracellular $K^+$ concentration, leading to regenerative neuronal hyperexcitability can underscore seizures that accompany metabolic aberrations. In hyponatremia, the extracellular space shrinks, leading to an increased concentration of extracellular $K^+$ and increased non-synaptic or ephaptic coupling; this rise in $K^+$ facilitates neuronal firing. Similarly, because membrane excitability varies with the extracellular concentration of $Ca^{2+}$, hypocalcaemia can contribute to the synchronization and spread of abnormal electrical activity in the brain. Other metabolic disturbances such as hypomagnesaemia, hyperglycaemia, hypoxia, and ischemia can also induce seizure activity (Calabresi et al., 2003; Somjen, 2002; Stecker et al., 2002; Hopkins, 1995).

Efforts to classify epileptic seizures date back to the earliest medical literature. In 1964, the Commission on Classification and Terminology of the International League Against Epilepsy (ILAE) proposed the first official classification of epileptic seizures, which was revised in 1981 and 1989 (Table 1). Based on this classification, seizures are presently divided into focal (those in which the first clinical and/or electrographical manifestations indicate initial activation of a limited population of neurones in part of one hemisphere), and generalized, (those in which the initial manifestations suggest initial activation of neurones throughout both hemispheres). Focal seizures are further subdivided into simple and complex, based on level of consciousness. Seizures in which consciousness is preserved are referred to as simple partial seizures. Those in which consciousness is impaired are classified as complex partial seizures. Secondarily generalized seizures are generalized seizures preceded by focal clinical and/or electrographical manifestations that occur in patients with focal epilepsy. The revised classification uses seizure semiology and EEG features to establish whether seizures are focal or generalized. For example, a seizure characterized by staring and loss of awareness is classified as absence when generalized spike-wave complexes are noted on the EEG, and as complex partial when associated with focal epileptiform discharges.
(Commission on classification and terminology of the international league against epilepsy, 1981; 1989).

Table 1. Classification of seizures

| I. Partial seizures (72%*) | A. Simple (consciousness not impaired) | A1. With motor manifestations  
A2. With sensory manifestations  
A3. With automatic manifestations  
A4. With psychic manifestations  
B. Complex  
B1. With simple partial features (as above A1–4) at onset followed by impairment of consciousness  
B2. With impairment of consciousness at onset (both B1 and B2 may be followed by automatism)  
C. Secondarily generalised  
C1. Simple partial seizure evolving to generalised seizure  
C2. Complex partial seizure evolving to generalised seizure  
C3. Simple partial seizure evolving to complex partial seizure evolving to generalised seizure  
C3. Simple partial seizure evolving to complex partial seizure evolving to generalised seizure  
II. Generalised seizures (24%*)  
A. Absence seizures (may be typical or atypical)  
B. Myoclonic seizures  
C. Clonic seizures  
D. Tonic seizures  
E. Tonic – clonic seizures  
F. Atonic seizures  
III. Unclassified seizures (4%*)  

Taken from: Services for Patients with Epilepsy; Report of a CSAG Committee chaired by Professor Alison Kitson; Prepared by: Professor Simon Shorvon and others (2000).  
*Frequencies from Shorvon (2000).  

Certain epileptic disorders are characterized by specific clusters of signs and symptoms that are considered epileptic syndromes. Since most of these syndromes have numerous aetiologies, few have been defined as specific diseases. Proposed in 1989, the current classification of the epilepsies subdivides epilepsies and epileptic syndromes into three
categories based on clinical history, electroencephalographic manifestations, and aetiology (Shorvon, 2000). Localization-related epilepsies and syndromes are typified by seizures that originate from a localized cortical region. The generalized epilepsies and epilepsy syndromes are characterized by seizures with initial activation of neurones within both cerebral hemispheres.

The syndromes are further divided into idiopathic and symptomatic types. Idiopathic refers to syndromes that arise spontaneously without a known cause, and usually having a genetic basis. Most affected patients are of normal intelligence and have normal results on neurological examinations. Symptomatic denotes epilepsies with an identified cause such as mesial temporal sclerosis. The term cryptogenic describes syndromes that are presumed to be symptomatic but have no known aetiology and that occur in patients with or without abnormalities on neurological examination. Epilepsies that are characterized by both partial and generalized seizures and focal and generalized epileptiform discharges, but without a clear predominance of one over the other, are classified as undetermined. The term lesional epilepsy refers to focal epilepsy in which a lesion is identified by neuroimaging studies that is the probable cause of seizures. Lesions include mesial temporal sclerosis, congenital malformations, neoplasms, vascular malformations, and ischemic insults. Recent findings suggest, however, that some symptomatic epilepsies have a significant genetic component (Berkovic & Scheffer, 2001).
1.3 Mechanisms underlying epileptic seizure generation

The pathophysiology of epilepsy involves alterations of normal physiological processes. An epileptic seizure is produced by synchronous and sustained firing of a population of neurones in the brain. The behavioural manifestations of a seizure reflect the function of the cortical neurones involved in the generation and spread of abnormal electrical activity. Epileptogenicity refers to the excitability and synchronization of neuronal networks that produce epileptiform activity in the brain. Both excitatory and inhibitory influences may be altered, creating a predisposition to excessive synchrony within neuronal populations (MacDonald & Meldrum, 1995; Rall & Schleifer, 1992). Theoretically, seizures and then epilepsies might arise due to increased excitation and/or decreased inhibition of a network of neurones in the brain; in other words, a seizure is due to a paroxysmal electrical disturbance (imbalance) of brain signalling.

Electrical signals in neurones take two forms: the action potential, which propagates down the axon of the neurone from the soma to the axon terminal, translocating information within a neurone, and transmission of information between neurones, which is accomplished primarily through chemical synapses by means of neurotransmitters and electrical synapses through gap junctions (Serra et al., 1999; Moroni, 1999). A complex series of events underlie these electrical signals. Central to the understanding of these events is that the neuronal membrane is semi-permeable to different ions carrying electrical current. The neuronal membrane permeability exhibits rapid changes that can dramatically alter the voltage across it. At the resting membrane potential (determined largely by the transmembrane K\(^+\) concentration gradient), sodium ions (Na\(^+\)), which are concentrated in the extracellular space, flow into the cell, and intracellular potassium ions (K\(^+\)) flow out. A Na\(^+\)-K\(^+\) pump, utilizing adenosine triphosphate (ATP), eventually replaces the displaced ions. Net influx of positively charged ions (Na\(^+\) and calcium ions (Ca\(^{2+}\))) decreases the membrane potential (depolarization), whereas chloride ion (Cl\(^-\)) influx and K\(^+\) efflux hyperpolarize the membrane. When a cell membrane is depolarized to spike firing threshold, Na\(^+\) channels open, allowing a rapid net flow of the ions intracellularly, producing an action potential. Subsequently, following a Na\(^+\) channel inactivation phase, net K\(^+\) efflux from the cell leads to repolarization of the membrane (Hammond, 1996). The propagation of action potentials along axons transmits information throughout the nervous system. When the presynaptic axon terminal is stimulated by an action potential, there is an influx of Ca\(^{2+}\)
triggering the release of neurotransmitters that bind to postsynaptic membrane receptors. This process produces excitatory and inhibitory postsynaptic potentials (EPSPs and IPSPs) whose summation and synchronization comprise the electrical activity recorded from the surface electroencephalogram (EEG). L-glutamate and L-aspartate are the primary excitatory neurotransmitters in the central nervous system (CNS); γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the brain (Gundersen et al., 2004; Serra et al., 1999; Moroni, 1999; Fonnum, 1984; Roberts et al., 1984; Krogsgaard-Larsen & Arnt, 1979).

Epileptic seizures are triggered by abnormalities in the brain that cause a group of nerve cells in the cerebral cortex to become activated simultaneously, emitting sudden and excessive bursts of electrical energy that lead to seizures. Based on fundamental concepts of neuronal behaviour, two mechanisms seem to be involved in this sudden change from normal pattern of neuronal activity to abnormal electrical discharges: a change in synchronization and intensity of synaptic input (neurotransmission) and/or an alteration of voltage-dependent or passive membrane ionic currents of individual neurones (ion channels).

Neurotransmitters mediate the communication between the neurones in the brain. Some researchers believe that people with epilepsy have an abnormally high level of excitatory neurotransmitters that increase neuronal activity. Others believe epilepsy results from abnormally low levels of inhibitory neurotransmitters that decrease neuronal activity in the brain (Hopkins, 1995). More than 100 neurotransmitters or neuromodulators have been shown to play a role in neuronal processes. Some specific neurotransmitters that relate to epilepsy are GABA, norepinephrine, endogenous opioid peptides, and the excitatory amino acid neurotransmitters, L-glutamate and L-aspartate; although, the most widely studied have been γ-aminobutyric acid (GABA) and glutamate, which act at more than half the neuronal synapses in the brain (Johnston, 1995; Hopkins, 1995).

Ion channels are membrane-spanning proteins forming selective pores for Na⁺, K⁺, Cl⁻, or Ca²⁺ ions. During action potentials, a precise control of ion channel gating is mediated by membrane voltage, and during synaptic transmission by the binding of specific neurotransmitters. With regard to these basic principles, two distinct and structurally conserved classes of ion channels emerged during evolution, the voltage-gated and the ligand-gated channels (Hille, 1992). Ion channels not only provide the
basis for the regulation of excitability in the central nervous system, but also in other excitable tissues such as skeletal and heart muscle. Consequently, mutations in genes encoding ion channels are found in a variety of inherited diseases associated with hyper- or hypoexcitability of the affected tissue, the so-called “channelopathies”. Over the last 10-15 years, the combination of electrophysiological and genetic studies has revealed an increasing number of inherited diseases associated with mutations in ion channel-encoding genes. The first of these so-called ion channel disorders or “channelopathies” were found in skeletal muscle, the myotonias and periodic paralyses, caused by mutations in voltage-gated Na\(^+\), Cl\(^-\), or Ca\(^{2+}\) channels. Subsequently, several disorders of the CNS, the episodic ataxias, familial hemiplegic migraine, spinocerebellar ataxia type-6, startle disease, and several epileptic syndromes, were identified as belonging to the growing family of channelopathies (Ptacek & Fu, 2001; Cannon, 2000; Lehmann-Horn & Jurkat-Rott, 1999, 2000).

An increasing number of epileptic syndromes belong to this group of rare disorders e.g. autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) is caused by mutations in a neuronal nicotinic acetylcholine receptor (affected genes: CHRNA4, CHRNB2); benign familial neonatal convulsions (BFNC) by mutations in K\(^+\) channels that constitute the M-current (KCQ2, KCNQ3) (Biervert & Steinlein, 1999; Miraglia del Giudice et al., 2000; Hirose et al., 2000); generalized epilepsy with febrile seizures plus (GEFS\(^+\)) by mutations in subunits of the voltage-gated Na\(^+\) channel and/or the GABA\(_A\) receptor (SCNB1, SCN1A, GABRG2), and episodic ataxia type-1, which is associated with epilepsy in a few patients, caused by mutations within another voltage-gated potassium channel (KCNA1) (Lerche et al., 2001; Table 2).

Scientists and clinicians increasingly recognize the importance of genetic factors in the origin of epilepsy. Genetics are most relevant to generalized seizures, including absence, generalized tonic-clonic, and myoclonic seizures, but more recently, also some partial epilepsies have been related to genetic causes (Berkovic & Scheffer, 2001; Ottman, 2001). Defects in genes do not lead directly to epilepsy, but they can alter the brain excitability predisposing to seizures. Development of epilepsy can require multiple gene abnormalities, or a gene abnormality in concert with an environmental trigger. Hundreds of gene defects eventually will be related to epilepsy. Only a few of these are now recognized, and even less have been related to ion channel abnormalities (Table 2), but this is one of the fastest growing areas of medicine (Lukasiuk et al., 2003;

The characterization of the products of the channel genes, and of the channel mutations that cause brain diseases has only recently begun. In the brain, a great variety of ion channels are expressed, and the roles played by specific channels are, for the most part, poorly understood. Yet, studies of the first few brain channel disorders indicate that subtle changes in the electrophysiological properties of a single channel type can have important effects on neuronal behaviour. An important challenge now is to analyze more comprehensively the contributions made by a large number of the ion channel genes at the cellular and neuronal network level. Animal genetic models for this work are widely recognized as critical for the understanding of all these processes (Cooper & Jan, 1999). It is noteworthy that studies of inbred lines of mice with epilepsy, cerebellar deficits, and other neurological abnormalities, have resulted in the cloning of channel alleles (Letts et al., 1998; Doyle et al., 1997). Besides these spontaneous mutations, targeted mutation of several different channel genes have also been shown to cause neurological phenotypes, including epilepsy (Felix, 2002; Noebels, 1996).

The discovery of genetic defects and, in particular, the electrophysiological characterization of mutant ion channels in hereditary forms of epilepsy elucidates pathophysiological concepts of hyperexcitability in the CNS. This knowledge enables new therapeutic strategies by antagonizing the epilepsy-causing mechanisms using the defective proteins as pharmacological targets. In the case of BFNC, a completely novel approach in the treatment of epilepsies emerged from identifying retigabine as an activator of M-currents conducted by KCNQ2 and KCNQ3 K⁺ channels. Retigabine shifts the voltage dependence of steady-state activation of these channels by ~ 20 mV in the negative direction so that they are active at the resting membrane potential; this stabilizes the cell membrane via hyperpolarization towards the K⁺ equilibrium potential (Wickenden et al., 2000).
**Table 2.** List of currently known channelopathies causing epilepsy in man and mice (adapted from *Channelopathies*, by F. Lehmann-Horn & K. Jurkat-Rott, 2000).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Locus</th>
<th>Channel protein</th>
<th>Disease</th>
<th>Inheritance</th>
<th>Function change</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sodium channel</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SCN1A</td>
<td>2q24</td>
<td>neuronal α-subunit</td>
<td>Generalized epilepsy with febrile seizures plus (GEFS’2)</td>
<td>Dominant</td>
<td>Gain</td>
</tr>
<tr>
<td>SCN1B</td>
<td>19q1.3</td>
<td>β1 subunit</td>
<td>Generalized epilepsy with febrile seizures plus (GEFS’1)</td>
<td>Dominant</td>
<td>Gain</td>
</tr>
<tr>
<td>SCN8A</td>
<td>12q13</td>
<td>neuronal α-subunit</td>
<td>Mice: <em>MED, jolting</em></td>
<td>Recessive</td>
<td>Loss</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><strong>Calcium channel</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CACNA1A</td>
<td>19p13.1</td>
<td>neuronal P/Q-type α1 subunit</td>
<td>Episodic ataxia 2, absence epilepsy?</td>
<td>Dominant</td>
<td>Loss</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mice: <em>tottering, leaner, rolling, ducky</em></td>
<td>Recessive</td>
<td>Loss</td>
</tr>
<tr>
<td>CACNB4</td>
<td>2q22-23</td>
<td>neuronal β4 subunit</td>
<td>Generalized epilepsy, episodic ataxia 3</td>
<td>Dominant</td>
<td>Gain</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mice: <em>lethargic</em></td>
<td>Recessive</td>
<td>Loss</td>
</tr>
<tr>
<td>CACNG2</td>
<td></td>
<td>neuronal γ2 subunit, stargazer</td>
<td>Mice: <em>stargazer, waggler</em></td>
<td>Recessive</td>
<td>Loss</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><strong>Potassium channel</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KCNA1</td>
<td>12p13</td>
<td>neuronal α1 subunit, A-type, Kv1.1</td>
<td>Episodic ataxia 1</td>
<td>Dominant</td>
<td>Loss</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Drosophila: <em>Shaker</em></td>
<td>Knock out mice: epilepsy</td>
<td>Loss</td>
</tr>
<tr>
<td>KCNQ2</td>
<td>20q13.3</td>
<td>neuronal α subunit, KCNQ2</td>
<td>Benign familial neonatal convulsions 1 (BFNC1)</td>
<td>Dominant</td>
<td>Loss</td>
</tr>
<tr>
<td>KCNQ3</td>
<td>8q24.22-24.3</td>
<td>neuronal α subunit, KCNQ3</td>
<td>Benign familial neonatal convulsions 2 (BFNC2)</td>
<td>Dominant</td>
<td>Loss</td>
</tr>
<tr>
<td>KCNJ6</td>
<td>21q22.1</td>
<td>α subunit, GIRK2, Kir3.2</td>
<td>Mice: <em>weaver</em></td>
<td>Recessive</td>
<td>Gain</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><strong>Nicotinic acetylcholine receptor</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHRNA4</td>
<td>20q13.3</td>
<td>Neuronal α4 subunit</td>
<td>nocturnal frontal lobe epilepsy, ADNFLE</td>
<td>dominant</td>
<td>Loss</td>
</tr>
</tbody>
</table>
I.4 Anticonvulsant drugs: mechanisms of action

The modern approach to treat seizures started in 1850, when bromides were introduced in clinical practice; the use of bromides was based on the belief that epilepsy was caused by an excessive sex drive. In 1910, phenobarbital, which was used for sleep induction, was found to have antiseizure activity and became the drug of choice for many years. A number of medications similar to phenobarbital were then developed, including primidone (Cereghino & Penry, 1995; Swinyard, 1980). In 1937, Tracy Putnam & Houston Merrit introduced the first animal model for screening multiple compounds for antiepileptic activity; they discovered that seizures could be induced in cats by interrupted direct current delivered to the brain for 10 sec through mouth-occipital electrodes (Swinyard, 1980; Putnam & Merrit, 1937). In 1938, phenytoin was introduced as an effective drug for the treatment of epilepsy, and since then it has become a major first-line antiepileptic drug (AED) in the treatment of partial and secondarily generalized seizures (Wilder, 1995; Ramsay et al., 1992; Wilder et al., 1983; Merrit & Putnam, 1938).

Ethosuximide has been used since 1955 as a first-choice drug for the treatment of absence seizures without generalized tonic-clonic seizures (Sherwin, 1995; Browne et al., 1975). Sodium valproate was licensed in Europe in 1960, and now is widely available throughout the world. It became the drug of choice in primary generalized epilepsies and later in the 1990s was approved for treatment of partial seizures (Johannessen & Johannessen, 2003; Bourgeois, 1995; Braathen et al., 1988; Adams et al., 1978; Mattson et al., 1978). In 1965, carbamazepine was approved, initially for the treatment of trigeminal neuralgia; later, in 1974, it was approved for partial seizures (Loiseau & Duché, 1995; Dodson, 1987). These anticonvulsants were the mainstays of seizure treatment until the 1990s, when many newer AEDs with good efficacy, better tolerability and fewer toxic effects were developed. The “new generation” AEDs have been approved in the United Kingdom mainly as add-on therapy only, with the exception of lamotrigine and topiramate which are now approved for conversion to monotherapy (LaRoche & Helmers, 2004; Ängehagen et al., 2003a; Nguyen & Spencer 2003; Asconape, 2002; Dichter & Brodie, 1996; Mattson, 1995; Porter, 1995; Willmore & Dulac, 1995).
1.4.1 Principles of Antiepileptic Drug Treatment

The development of a seizure involves many structures and processes, including neurones, ion channels, receptors, and inhibitory and excitatory synapses; the AEDs modify these processes to favour synaptic inhibition over excitation in order to stop or prevent seizures. A good neurologist should be aware of the mechanism of action and pharmacokinetics of AEDs; this is very important in clinical practice so that AEDs can be used effectively, especially in multidrug regimens.

The selection of an AED is based on efficacy against specific seizure types (c.f. Section 1.3; Table 1) and the potential for producing adverse effects. The goal of treatment is to control seizures using a single agent maintained at serum concentrations that do not produce adverse effects. If seizures persist, the dosage is usually gradually increased until seizure control is achieved or intolerable side effects appear. If polytherapy is required, drugs are generally chosen with different mechanisms of action and side effect profiles to maximize anticonvulsant benefit and minimize toxicity. Interactions among the various AEDs must also be kept in mind in the selection of drug combinations (Table 3; AEDs interactions).

The AEDs can be grouped according to their main mechanism of action, although many of them have multiple sites of action and others have currently unknown (or unclear) mechanisms of action. The main groups include Na\(^+\) channel blockers, Ca\(^{2+}\) channel blockers, \(\gamma\)-aminobutyric acid (GABA) system enhancers, glutamate receptor blockers, carbonic anhydrase (CA) inhibitors and drugs with unknown target effects (Macdonald & Meldrum, 1995). The principal mechanisms of action of the main clinically used AEDs are summarized below.
1.4.2. Drugs used in therapy: established drugs and “newer generation” antiepileptic treatments

Established drugs

Phenytoin

Since its introduction in 1938, phenytoin (PHT) has been a major first-line AED in the treatment of partial and secondarily generalized seizures. PHT is probably the most widely studied anticonvulsant in clinical practice (Wilder, 1995; Ramsay et al., 1992; Wilder et al., 1983). It blocks movements of Na\(^+\) ions through neuronal Na\(^+\) channels during propagation of the action potential, and therefore blocks and prevents posttetanic potentiation, limits development of maximal seizure activity, and reduces the spread of seizures (DeLorenzo, 1995; Tomaselli et al., 1989; MacDonald & McLean, 1982; Woodbury, 1980). It also demonstrates an inhibitory effect on Ca\(^{2+}\) channels and the sequestration of Ca\(^{2+}\) ions in nerve terminals, thereby inhibiting voltage-dependent neurotransmission at the level of the synapse (DeLorenzo, 1980; Selzer, 1978; Yaari et al., 1977). Furthermore, PHT also possess an effect (activation) on the Na\(^+\)/K\(^+\) -ATPase, calmodulin and other secondary messenger systems (cyclic nucleotide); the mechanisms involved and their contribution to its antiepileptic activity are, however, unclear (Vargas et al., 1994; DeLorenzo, 1980, 1986; Woodbury, 1980).

Antiepileptic effect and clinical use: PHT is one of the most commonly used first-line or adjunctive treatments for partial and generalized seizures, Lennox-Gastaut syndrome (a rare symptomatic generalized epilepsy with the following criteria: 1. multiple seizure types; 2. variable degrees of cognitive impairment; 3. abnormal EEG with a slow spike-wave pattern, and other associated EEG changes), status epilepticus, and childhood epileptic syndromes. It is not indicated for myoclonus and absence seizures. This drug is highly effective and economical for the patient; however, tolerability of the drug is a big limit to its utilization (Wilder, 1995; Ramsay et al., 1992; Wilder et al., 1983).

Fosphenytoin

Fosphenytoin sodium injection is a pro-drug intended for parenteral administration. Its active metabolite is PHT. It is safer and better tolerated than PHT and can be infused 3 times faster than intravenous PHT (Sirven & Waterhouse, 2003).
Antiepileptic effect and clinical use: The antiepileptic effect of fosphenytoin is attributable to PHT, but it is clearly better tolerated than the parent compound. Fosphenytoin is indicated for treatment of status epilepticus and for short-term parenteral administration when other routes are not available or inappropriate (Sirven & Waterhouse, 2003).

Carbamazepine

Carbamazepine (CBZ) has been used for epilepsy since 1965, and is a major first-line AED for partial seizures and generalized tonic-clonic seizures (Loiseau & Duché, 1995; Dodson, 1987). It is a tricyclic compound and initially was used primarily for the treatment of trigeminal neuralgia, but its value in the treatment of epilepsy was then discovered quite by chance. CBZ acts mainly by preventing rapid, repetitive, sustained neuronal firing through voltage- and use-dependent blockade of neuronal Na\(^+\) channels and enhancing Na\(^+\) channel inactivation (Dichter, 1993). It also possesses an antagonist action on monoaminergic, cholinergic and NMDA receptors (Cunha et al., 2002; Hough et al., 1996; Miller & Richter, 1985; Consolo et al., 1976).

Antiepileptic effect and clinical use: CBZ is one of the most widely used AEDs in the world. It is highly effective for partial onset seizures, including cryptogenic and symptomatic partial seizures. It has also demonstrated good efficacy in the treatment of generalized tonic-clonic seizures but is not effective (and may even be deleterious), in patients with absence and myoclonic seizures (Loiseau & Duché, 1995; Liporace et al., 1994). The major disadvantages of this drug are transient adverse dose-related effects when initiating therapy (such as nausea, headache, dizziness, tiredness, diplopia) and occasional toxicity (such as skin rash, fever, vasculitis, systemic lupus erythematosus, idiosyncratic haematological toxicity) (Holmes, 1995).

Oxcarbazepine

Oxcarbazepine (OXC) is a recently developed analogue of CBZ, marketed in UK in 2000 and accepted for registration as a first-line AED in several countries (Schwabe, 1994). OXC was developed in an attempt to hold on to the benefits of action of CBZ while avoiding its auto-induction and drug interaction properties; CBZ is a strong inducer of its own liver metabolism and therefore, of other drugs metabolized by the
same enzyme (CYP 3A4). OXC does not produce the epoxide metabolite, which is largely responsible for the adverse effects reported with CBZ. OXC is almost immediately and completely metabolized by reduction to the active metabolite, 10,11-dihydro-10-oxo-CBZ (MHD). The antiepileptic effects of OXC and MHD are comparable in both animals and humans (Dam & Østergaard, 1995). Like CBZ, OXC and its metabolite block neuronal Na⁺ channels during sustained rapid repetitive firing; it also decreases high voltage-activated Ca²⁺ currents in a dose-dependent manner (Stefani et al., 1997).

**Antiepileptic effects and clinical use:** OXC is approved for monotherapy or adjunctive therapy in partial and secondarily generalized seizures. Four randomized, double-blind trials of this agent as monotherapy demonstrated effectiveness superior to that of placebo in patients with refractory epilepsy and in candidates for epilepsy surgery. It is better tolerated and has fewer drug interactions than CBZ. Substitution for CBZ can be made abruptly with an OXC-to-CBZ ratio of 3:2 (Dam & Østergaard, 1995).

**Valproate**

Valproate (VPA) is the drug of choice for primary generalized epilepsies, and is also approved for the treatment of partial seizures. It was discovered by accident; first synthesized in 1882, its antiepileptic properties were recognized when it was used as a solvent for the experimental screening of new antiepileptic compounds. It was licensed in UK in the 1973. It has been used in different chemically-related forms, including Divalproex (sodium, magnesium or calcium salt), or Valpromide. These forms do not differ significantly in their antiepileptic action (Bourgeois, 1995; Braathen et al., 1988; Adams et al., 1978; Mattson et al., 1978).

The precise mechanism of action of VPA is uncertain. It enhances GABA function by inhibiting the GABA metabolic enzyme GABA transaminase (GABA-T) but this effect is observed only at high concentrations (Kerwin & Taberner, 1981; Taberner et al., 1980; Fowler et al., 1975). It may also increase the synthesis of GABA by stimulating glutamic acid decarboxylase (GAD). It also produces selective inhibition of voltage-gated Na⁺ currents during sustained, rapid, repetitive neuronal firing and inhibition of T-type Ca²⁺ channels in neurones (Johannessen & Johannessen, 2003; Fariello et al., 1995; McLean & Macdonald, 1986; Löscher, 1980; Taberner et al., 1980).
**Antiepileptic effect and clinical use:** VPA is a potent AED, effective against a wide range of seizure types. It is the drug of choice in idiopathic generalized epilepsy. Open and comparative studies have shown excellent control rates in patients with newly diagnosed typical absence seizures. It is the drug of choice for juvenile myoclonic epilepsy and can be used in other types of myoclonus. In addition, it is a first-line drug in photosensitive epilepsy and Lennox-Gastaut syndrome. It is a second choice in the treatment of infantile spasms. In focal epilepsy, VPA has been shown to be as effective as other first-line agents (Johannessen & Johannessen, 2003; Bourgeois, 1995; Ramsey et al., 1992; Braathen et al., 1988; Bourgeois et al., 1987; Callaghan et al., 1982; Sato et al., 1982). Sedation, emotional upset, depression and psychosis are common side effects; another potential adverse effect is the association with polycystic ovaries and obesity (Dreifuss, 1995; Dreifuss & Langer, 1988).

**Ethosuximide**

Ethosuximide (ESM) has been used extensively for the treatment of absence seizures. Despite its frequent use, the sites and mechanisms of action of ESM are still poorly defined. The main mechanism of action suggested for this drug is the ability to reduce low-threshold (T-type) Ca\(^{2+}\) currents in thalamic neurones, which seem necessary for the generation of the characteristic firing rhythm of absence seizures. Furthermore, ESM inhibits Na\(^+\)/K\(^+\)-ATPase activity but its effects on neurotransmission remain controversial (Macdonald & Kelly, 1995).

**Antiepileptic effect and clinical use:** ESM is the drug of choice for the treatment of typical and atypical absence seizures (Posner et al., 2003). Nausea, gastrointestinal discomfort and anorexia have been reported to be the most common dose-related side-effects. It has also been reported that ESM could exacerbate various types of seizure, but current data are controversial (Shorvon, 2000; Dreifuss, 1995; Browne et al., 1975).

**Phenobarbital**

Phenobarbital (PHB) is the most commonly prescribed AED of the 20th century. It is a very potent anticonvulsant with a broad spectrum of action. Currently, its use is limited because of its adverse effects such as sedation and cognitive slowing (Painter & Gaus, 1995). It is a free acid, relatively insoluble in water. The sodium salt is soluble in water...
but unstable in solution. It has a direct action on GABA<sub>A</sub> receptors by binding to the barbiturate-modulatory binding site; this results in a prolongation of the duration of Cl<sup>-</sup> channel opening, thereby enhancing GABA<sub>A</sub> receptor-mediated synaptic inhibition. It also reduces neuronal Na<sup>+</sup> and K<sup>+</sup> conductances and Ca<sup>2+</sup> influx and depresses glutamate-evoked neuronal excitability (Prichard & Ransom, 1995).

**Antiepileptic effect and clinical use**: PHB is effective in a wide variety of seizures and is currently the cheapest AED. Although PHB effectiveness is not questioned, it is a second-line drug because of its adverse effects.

**Primidone**

This drug is metabolized to PHB and phenylmethylmalonamide (PEMA). The main action is through the derived PHB. The real clinical effect of primidone or PEMA is unknown and controversial (Smith & DeToledo, 1995).

**Antiepileptic effect and clinical use**: Primidone has the same indications as PHB.

**Benzodiazepines**

The benzodiazepines (BZDs) most commonly used for treatment of epilepsy are lorazepam, diazepam, clonazepam, and clobazam. BZDs act at a specific set of recognition sites on the GABA<sub>A</sub> receptor-Cl<sup>-</sup> ion complex. They act as allosteric modulators of the GABA<sub>A</sub> agonist site increasing the affinity of GABA for its own receptor. BZDs appear to increase the opening frequency of the receptor-ion channel complex without changing the channel conductance (Macdonald & Kelly, 1995). Diazepam and lorazepam are used mainly for emergency treatment of *status epilepticus* seizures because of their quick onset of action, availability of intravenous forms, and strong anticonvulsant effects. Their use for long-term treatment is however, limited because of the development of tolerance. Clonazepam and clobazam are more widely used as adjunctive therapy and for myoclonic seizures; they possess somewhat less side effects than other BZDs, however, sedation and tolerance substantially reduce their usefulness (Booth *et al.*, 1998; Shorvon, 1998 Macdonald & Kelly, 1995).
Acetazolamide

Acetazolamide (ACTZ), an unsubstituted sulphonamide and a potent inhibitor of carbonic anhydrase (CA), was synthesized in 1950 and first used to treat epilepsy in 1952. In most patients, the development of tolerance limits its long-term usefulness as an antiepileptic drug. The only well documented biochemical effect of ACTZ is inhibition of CA. It has been suggested that the mechanism of its anticonvulsant action is through inhibition of this enzyme in the brain (for a review see Woodbury, 1980), however, this still remains controversial (Resor et al., 1995) and its precise mode of anticonvulsant action needs to be further investigated.

**Antiepileptic effect and clinical use:** ACTZ is a beneficial adjunctive agent in the pharmacotherapy of epilepsy and should be considered in refractory epilepsy. Although it may be useful in partial, myoclonic, absence, and primary generalized tonic-clonic seizures uncontrolled by other marketed agents, ACTZ has been inadequately studied by current standards and its clinical use has been limited (Reiss & Oles, 1996; Oles et al., 1989).

**Newer Antiepileptic Treatments**

In the past decade, a number of “newer generation” antiepileptic drugs (AEDs) have been introduced therapeutically, including vigabatrin, lamotrigine, felbamate, gabapentin, tiagabine, levetiracetam, topiramate and zonisamide, which have proved highly effective against various clinical seizure types and offer more favourable pharmacokinetics, and less troublesome side-effects or adverse drug interactions than with other AEDs (Asconape, 2002). Out of all the currently available drugs, topiramate (TPM) is one of the most potent, and shows the most unusually wide spectrum of activity. We have therefore focussed on the effects and clinical uses of this “newer generation” drug in some detail in Section 1.4.3 below.

**Vigabatrin**

Vigabatrin (VGB) has been released for clinical use in 1989 in UK; it is the result of a rational drug design aimed at enhancing brain GABA levels subsequent to an irreversible inhibition of the GABA metabolizing enzyme GABA-transaminase.
(GABA-T), thereby increasing the available pools of presynaptic GABA for release at central synapses. VGB acts as a substrate for this enzyme, the reaction producing an intermediate which binds covalently to the active site of GABA-T. As this inhibition is irreversible, new enzyme molecules must be synthesized to overcome the action of VGB. The inhibition of GABA-T is rapid (within minutes) and long lasting (several days), recovery depending on the synthesis of new enzyme. *In vivo* studies in human and animal subjects have shown that VGB significantly increases extracellular GABA concentrations in the brain. VGB has no other known action (Ånehagen *et al.*, 2003a; Jung & Palfreyman, 1995).

**Antiepileptic effect and clinical use:** VGB has been studied exhaustively in 9 double-blind controlled trials. These trials reported that 40-50% patients with refractory partial seizures had a reduction in seizure frequency of more than 50%, and as many as 10% of patients became seizure free. It is less effective against primarily generalized tonic-clonic seizures and also may worsen myoclonic seizures or generalized absence seizures. The most common side effects are fatigue and sedation. A major serious side effect reported for this drug is visual field defects; it can also induce depression or psychotic disturbances in some patients (Shorvon, 2000; Krauss *et al.*, 1998; Fisher & Kerrigan, 1995). Like TGB, VGB has been reported to cause absence status. Patients with myoclonus or Lennox-Gastaut syndrome do not respond well to VGB. VGB is very effective in the treatment of infantile spasms (West’s syndrome); therefore, it is the drug of choice for this indication in many countries (LaRoche & Helmers, 2004; Aicardi *et al.*, 1996).

**Lamotrigine**

Lamotrigine (LTG) is a phenyltriazine derivative that is chemically unrelated to any of the other AEDs, yet has pharmacological effects similar to those of phenytoin and carbamazepine. It was originally developed as an antifolate agent based on a theory that the mechanism of some AEDs is related to their antifolate property. It was approved in UK in 1995. The proposed mechanism of action of LTG is a reduction of glutamate release subsequent to blockade of voltage-sensitive Na⁺ channels. It has no reported activity at AMPA or NMDA glutamate receptors but inhibits N- & P-type Ca²⁺ channels (Stefani *et al.*, 1997). LTG has a broad spectrum of anticonvulsant effects in
experimental animal models of epilepsy (Messenheimer, 1995; Messenheimer et al., 1994; Lang et al., 1993; Leach et al., 1986).

**Antiepileptic effect and clinical use:** LTG's significant effect on seizures as compared to placebo was demonstrated in 9 out of 10 placebo-controlled trials in which LTG was administered as add-on therapy. It is effective in partial onset and secondarily generalized tonic-clonic seizures, primary generalized seizures (i.e., absence seizures and primary generalized tonic-clonic seizures), atypical absence seizures, tonic/ataxic seizures, and Lennox-Gastaut syndrome. It is sometimes effective for myoclonic seizures, but can cause worsening of myoclonic seizures in some patients with juvenile myoclonic epilepsy or myoclonic epilepsy of infancy. It currently is approved in UK for monotherapy and adjunctive treatment of partial seizures and primary and secondarily generalized tonic-clonic seizures and seizures associated with Lennox-Gastaut syndrome (LaRoche & Helmers, 2004; Goa et al., 1993; Brodie, 1992).

**Felbamate**

Felbamate is a potent anticonvulsant, very effective against multiple seizure types. Unfortunately, after the occurrence of aplastic anaemia and hepatic failure, approval for general use was withdrawn. It is now available only for a very limited use, principally by neurologists in patients for whom potential benefit outweighs the risk. It blocks NMDA receptors and voltage-gated Ca\(^{2+}\) channels, modulates Na\(^+\) channel conductance and possess a barbiturate-like modulatory effect on GABA\(_A\) receptors (Pellock, 1999).

In addition to its activity against seizures, felbamate has been shown to have a neuroprotective effect on models of hypoxic-ischemic injuries (Wallis et al., 1992).

**Antiepileptic effect and clinical use:** Because of its potentially fatal toxic effects, use of felbamate is restricted to patients with severe partial epilepsy or Lennox-Gastaut syndrome who do not respond to other medications. This limited usage is because of the small but definitive risk of aplastic anaemia and hepatic failure (LaRoche & Helmers, 2004; Kaufman et al., 1997).
Gabapentin

Gabapentin (GBP) was approved in UK in 1993 as add-on therapy. It was developed to have a structure similar to that of GABA; however, experimental evidence showed that GBP has, in fact, little or no action on the GABA receptor. It enhances the activity of the enzyme GAD, but only weakly. It binds with the α2δ4 subunit of voltage sensitive Ca\(^{2+}\) channels in the neocortex, hippocampus, and spinal cord; this mechanism of action may be important for its efficacy in pain (Maneuf et al., 2003), although its relevance for antiepileptic activity is unclear.

At the present time, GBP is known to increase the intracellular concentration of GABA, but the exact mechanism by which it does this is unknown (Shorvon, 2000; Taylor, 1995). In vivo MR spectroscopy studies have shown that GBP increases brain levels of GABA and its metabolites homocarnosine and pyrrolidinone. It also may reduce monoamines and affect serotonin release (Maneuf et al., 2003). GBP is a competitive inhibitor of the enzyme branched chain amino acid transferase, which metabolizes the branch chain amino acids (L-leucine, L-isoleucine, and L-valine) to glutamate. Through this mechanism, GBP may reduce brain glutamate levels (Maneuf et al., 2003).

**Antiepileptic effect and clinical use:** Several open and double-blind trials have been conducted with GBP providing unequivocal proof of efficacy as an AED in partial epilepsy. GBP is useful in the treatment of partial and secondarily generalized tonic-clonic seizures but is ineffective in myoclonus and in most generalized seizure disorders. The drug appears to have only a modest efficacy, particularly at low doses (LaRoche & Helmers, 2004; Chadwick et al., 1998; Chadwick, 1994).

Tiagabine

Tiagabine (TGB) is a derivative of the GABA uptake inhibitor nipecotic acid. It acts by inhibition of the GABA transporter-1 (GAT-1). This inhibitory effect is reversible. TGB is lipid soluble and thus is able to cross the blood-brain barrier. It was introduced into clinical practice in 1998. Measurements in human and experimental models have confirmed that extracellular GABA concentrations increase after administration of TGB. Studies have shown little or no effect at other receptor systems (Ångehagen et al., 2003a; Adkins & Noble, 1998).
Antiepileptic effect and clinical use: It is available for use as second-line add-on therapy in patients with partial or secondarily generalized seizures refractory to treatment (LaRoche & Helmers, 2004).

Levetiracetam

Levetiracetam (LEV) is a piracetam (S-enantiomer pyrrolidone) derivative. It was developed in the 1980s to enhance cognitive functions and for anxiolysis. It is a unique AED in that it is ineffective in classic seizure models that screen potential compounds for antiseizure efficacy such as maximal electroshock and PTZ-treatment in rats and mice. During preclinical evaluations, it was found to be effective in several models of seizures, including tonic and clonic audiogenic seizures in mice, tonic seizures in the maximum electroshock-seizure test in mice, and tonic seizures induced in rodents by chemoconvulsants. Interestingly, LEV inhibits the development of pentyleneetetrazole-induced amygdala kindling in mice, a situation in which other drugs such as PHT and CBZ are inactive. Levetiracetam does not modulate Na$^+$ and T-type Ca$^{2+}$ channels (Zona et al., 2001) and does not induce any conventional modulation of the GABAergic system or ionotropic excitatory glutamate receptors. Levetiracetam has however, been shown to potently antagonize negative modulators of both GABA and glycine receptors. Other potential mechanisms underlying its antiepileptic action involve inhibition of high voltage-activated Ca$^{2+}$ currents (Pisani et al., 2004; Lukyanetz et al., 2002; Niespodziany et al., 2001) and a moderate suppression of the delayed rectifier K$^+$ current. Also, levetiracetam’s antiepileptic effect may relate to an interaction with a brain-specific binding site (Fuks et al., 2003; Leppik, 2002), the nature of which was recently revealed to be the synaptic vesicle protein SV2A, and implying that LEV somehow interferes with vesicle exocytosis (Lynch et al., 2004), although it is well known that LEV does not affect normal synaptic transmission (Birnstiel et al., 1997).

Antiepileptic effect and clinical use: Results from placebo-controlled clinical trials and community-based practice have demonstrated that LEV has a broad spectrum of activity in suppressing seizures as add-on treatment and monotherapy and that it is safe and well-tolerated. These results suggest that LEV might have a significant effect in generalized and partial epilepsies (LaRoche & Helmers, 2004).
Zonisamide

Zonisamide (ZNS) was synthesized as a benzisoxazole in 1974. It is chemically unrelated to any of the other AEDs. It is a small-ringed structure related to sulphonamide antibiotics, with a pH-dependent solubility in water. Although ZNS was approved by the US Food and Drug Administration (FDA) in March 2000 for the indication of partial seizures in patients older than 12 years as adjunctive therapy to other AEDs, it has been approved and studied in Japan for more than 10 years; it is not marketed in the UK. The major mechanism of action proposed for ZNS is reduction of neuronal repetitive firing by blocking Na\(^+\) channels and preventing excitatory neurotransmitter release; however, the fact that the ZNS molecule (like topiramate: see below) possesses an essential sulphonamide moiety could suggest a common mode of action for these two AEDs. ZNS also blocks T-type Ca\(^{2+}\) channels in the inactive state, reducing the depolarizing shift due to their activation (Masuda et al., 1998; Kito et al., 1996; Suzuki et al., 1992) to prevent influx of Ca\(^{2+}\), and exhibits neuroprotective effects through free radical scavenging (Leppik, 2002; Oommen & Mathews, 1999; Mori et al., 1998).

**Antiepileptic effect and clinical use:** ZNS has been used for adjunctive therapy for patients with partial seizures who are 12 years or older. It is preferred clinically because of the ease of patient tolerance, degree of seizure reduction, long half-life, and lack of drug interactions with other AEDs (Leppik, 2002; Oommen & Mathews, 1999; Masuda et al., 1998).
Table 3. Known interactions between AEDs (adapted from "Trattamento delle epilessie", Shorvon, 2000).

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<th>Drug</th>
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* The effect on serum concentrations of concomitant AEDs (along top) by the addition of the AEDs in the first column. Possible interactions of newer anticonvulsant drugs may not yet be available. ↑= increase; ↓= decrease; NE= no known effects; †= increase carbamazepine (CBZ) 10, 11-epoxide concentration; Var= variable.
I.4.3 Topiramate: antiepileptic effects, clinical uses and suggested mechanisms of action

**Antiepileptic effect and clinical use:** Topiramate (TPM; 2,3:4,5-bis-O-(1-methylethylidene)-β-D-fructopyranose sulphamate; Topamax®) was originally developed as a possible inhibitor of gluconeogenesis, and is currently used as an additive treatment in adult patients with refractory partial and secondarily generalized seizures (Bauer & Schwalen, 2000); it is also used as adjunctive therapy in children, adolescents and young adults with partial-onset seizures or Lennox-Gastaut syndrome (Coppola et al., 2001, 2002). In recent monotherapy studies, a low dose (100 mg/day) of TPM was effective in treating newly diagnosed epilepsy (Privitera et al., 2003). In animal studies, TPM showed antiseizure activity in the maximal electroshock seizure (MES) test in both rats and mice, with a potency similar to that of PHT, CBZ, PHB and ACTZ, but not against chemically-induced (PTZ) seizures in rats (Wauquier & Zhou, 1996; Shank et al., 1994; Kimishima et al., 1992; Edmonds et al., 1992; Gardocki et al., 1986). TPM (25-200 mg/kg, i.p) effectively and dose-dependently inhibited amygdala-kindled seizures in rats, suggesting it has anti-epileptogenic properties (Reissmüller et al., 2000); the dose-related inhibition was observed with all seizure parameters: behavioural seizures, forelimb clonus, and amygdala and cortical afterdischarges (Wauquier & Zhou, 1996). Furthermore, TPM showed prophylactic properties, significantly increasing the number of stimulations required to obtain the first stage 5 seizure in pretreated rats (Amano et al., 1998). Reissmüller et al. (2000) also demonstrated the efficacy of TPM in PHT-resistant kindled rats (a good model of drug-resistant epilepsy) which is in accordance with recent clinical experience in patients with partial epilepsy (Markind, 1998). In addition, TPM was also able to block both absence-like and tonic-clonic seizures, in spontaneously epileptic rats (SER; zi/zi, tm/tm; a double mutant derived form the zitter and the tremor rat characterized by the presence of both absence and tonic-clonic seizures), whereas CBZ and PHT only blocked tonic seizures but not absence-like seizures (blocked by ESM) (Nakamura et al., 1994).

TPM exhibits an unusually broad therapeutic spectrum, most likely reflecting its multiple sites of action; thus, of the four main mechanisms by which AEDs are thought to exert their antiseizure effects: (1) inhibition of voltage-dependent Na⁺ channels; (2) enhancement of GABA-mediated inhibitory neurotransmission; (3) blockade or
inhibition of excitatory neurotransmitter activity and (4) inhibition of voltage- and receptor-gated Ca^{2+} channels (Macdonald & Meldrum, 1995), TPM seems to be effective via all four routes (White et al., 2000; Sombati et al., 1995; Brown et al., 1993). In cultured hippocampal cells, TPM reduced the duration and frequency of spontaneous action potentials associated with sustained repetitive firing (DeLorenzo et al., 2000), presumably by state-dependent inhibition of neuronal Na^{+} channels (Taverna et al., 1999; Zona et al., 1997). TPM also increased neuronal GABA-induced Cl^{-} influx, acting via a GABA receptor subtype that was not modulated by benzodiazepines (Gordey et al., 2000; White et al., 2000; Shank et al., 2000). In fact, the anticonvulsant effects of TPM on cortical neurones were not reversed by flumazenil, a selective antagonist at the GABA-benzodiazepine receptor complex (White et al., 2000). In addition, recent data on recombinant GABA_A receptors expressed in Xenopus oocytes showed that TPM potentiated and increased the fading rate (decay) of GABA-evoked Cl^{-} currents in oocytes expressing the α6β3γ2S GABA_A receptor (Gordey et al., 2000). Concerning glutamate-mediated excitatory neurotransmission, TPM inhibited the excitatory responses of hippocampal neurones elicited by selective activation of the AMPA/kainate receptor subtype (Ångehagen et al., 2003b, 2004; Gibbs et al., 2000; Skradski & White, 2000). Recent studies have also clearly demonstrated that TPM acts specifically on ionotropic GluR5 and GluR6 AMPA/kainate receptors as a negative modulator. In particular, TPM seems to affect the intracellular phosphorylation sites on the receptor/ion channel complexes in the dephosphorylated state (Ångehagen et al., 2004; Rogawski et al., 2003; Smith et al., 2000; Shank et al., 2000). Finally, TPM has been shown to possess a negative modulatory effect on neuronal L-type high voltage-activated Ca^{2+} channels (White & Privitera, 2000; Zhang et al., 2000). The relative contribution of each mechanism of action to its antiepileptic activity is not known and can probably differ on the type of epilepsy considered.

In addition to being a powerful antiepileptic, TPM is also effective in treating other central disorders (e.g. bipolar disorder, Suppes, 2002; neuralgia, Zwartau-Hind et al., 2000; migraine, Pascual, 1999; bulimia, McElroy et al., 2003, and post-traumatic stress disorder, Berlant & van Kammen, 2002) suggesting that other ion channel, neurotransmitter or biochemical mechanisms may also be targeted by this novel drug.
**Suggested mechanisms of action**

In pyramidal neurones and brain slices displaying spontaneous epileptiform discharges, TPM dose-dependently reduces the duration and frequency of action potentials elicited by repetitive firing (Avoli & Lopantsev, 1997; Coulter *et al.*, 1995), in accordance with its Na^+ and/or Ca^{2+} channel inhibitory effects. Following studies demonstrated that TPM reduces the excitability and suppresses evoked repetitive firing of subicular neurones *in vitro* in a dose-dependent manner. In addition, these neurones exhibited a steady membrane hyperpolarization, and a decrease in input resistance (Kawasaki *et al.*, 1998). Wu *et al.* (1998) also demonstrated a frequency-dependent inhibition of neuronal activity in rat hippocampal slices; in this model, TPM significantly decreased the slope of fEPSPs (field excitatory postsynaptic potentials) and the number of population spikes induced by low frequency stimuli, but did not affect the IPSPs (inhibitory postsynaptic potentials). Furthermore, TPM reduced the number of action potentials elicited by prolonged current pulses; the suggested mechanism was a dose-dependent inhibition of Na^+ currents. These actions on voltage-dependent Na^+ channels has been confirmed by whole cell patch clamp experiments in cerebellar granule cells (Zona *et al.*, 1997), neurones from the enthorinal cortex (Avoli & Lopantsev, 1997), neocortex (Tavema *et al.*, 1999) and cultured spinal neurones (McLean *et al.*, 2000). In this model, TPM produced a voltage-dependent limitation of spontaneous repetitive firing, although, this effect, even at high concentrations, was slower and smaller than that of PHT or LTG, suggesting that this mechanism might not be of primary importance for its antiepileptic action.

Another potential mechanism of action is the enhancement of GABA activity at the level of some receptor subtypes. TPM increased the opening probability of GABA-Cl^- channels in mouse cortical neurones and this action was not blocked by flumazenil, suggesting a non-benzodiazepine action; moreover, it reversibly enhanced GABA-Cl^- currents in cerebellar granule cells and cortical neurones upon GABA application (White *et al.*, 1994). Gordey *et al.* (2000) demonstrated that TPM possessed differential sensitivity in modulating recombinant GABA_A receptors expressed in *Xenopus* oocytes; thus, TPM potentiated and prolonged the fading (decay) phase of GABA-evoked currents when some subunit combinations were expressed, whereas it inhibited or unaffected some other receptor subtypes. In murine cortical neurones, TPM modulated GABA-evoked whole-cell currents with an enhancing effect, being dependent on the
presence of GABA. TPM increased the frequency of channel activation similarly to BZDs, but this effect was not blocked by flumazenil, suggesting a different mechanism (White et al., 2000). More recently, Herrero et al. (2002) observed that TPM in hippocampal slices had an inhibitory effect on GABA_A-mediated depolarizing responses (GDSPs) (in the presence of glutamate ionotropic receptor antagonists), and moreover activated a novel K^+ conductance with consequent hyperpolarization; it was suggested that both effects might be mediated by carbonic anhydrase (CA) inhibition by TPM and the resultant changes in intracellular and extracellular pH. Structurally TPM resembles ACTZ and similarly it inhibits CA. Initial observations however, suggested that this mechanism was not important for its antiepileptic properties, since TPM was 10-100 times less potent than ACTZ at inhibiting this enzyme (Dodgson et al., 2000). More recent observations demonstrated that TPM's CA inhibitory action is indeed comparable to that of ACTZ and that TPM possesses a selective effect, with nanomolar affinity, for the CA II isozyme of the six expressed in the brain, and this action could therefore be relevant for its antiepileptic effect (Casini et al., 2003). Interestingly, Leniger et al. (2004a) have now demonstrated that the anticonvulsant action of TPM on hippocampal CA3 neurones is accompanied by a fall in pH_i, which was attributed to a combined modulatory effect on CA and Na^+-independent Cl^−/HCO_3^− exchange; a similar pH_i lowering effect is also apparently produced by LEV on these cells, via inhibition of Na^+-dependent Cl^−/HCO_3^− exchange (Leniger et al., 2004b), suggesting that pH regulation may be an important factor controlling convulsant activity in central neurones.

It is also been demonstrated that TPM blocks excitatory kainate-mediated responses in cultured hippocampal pyramidal neurones, by probably acting on some AMPA receptor subtypes, but not on NMDA receptors (Gibbs et al., 2000; Coulter et al., 1993, 1995). In addition, TPM blocked AMPA/kainate receptors in cultured cerebellar granule neurones (Skradski & White, 2000). More recently, it has been demonstrated that TPM selectively inhibits GluR5 kainate receptor-mediated currents in rat basolateral amygdala neurones (Gryder & Rogawski, 2003). In spontaneous epileptic rats, TPM reduced by about 45% the high expressed levels of glutamate and aspartate, which is coherent with a similar reduction of tonic extensions (Kanda et al., 1996). TPM (10-100 μM) also reduced dose-dependently, the excitability of CA3 hippocampal neurones in spontaneously epileptic rats by inhibiting glutamate release, but without affecting Na^+
or Ca$^{2+}$ conductances; this inhibition of excitatory neurotransmission could contribute to its antiepileptic activity (Hanaya et al., 1998). Finally, in dentate granule cells, TPM has been shown to selectively inhibit L-type Ca$^{2+}$ channels (Zhang et al., 2000), in particular, TPM at 10 μM significantly decreased the peak and area beneath L-type Ca$^{2+}$ currents whereas this effect was not evident at 50 μM. TPM did not affect the voltage sensitivity of the gating properties of these channels; furthermore, in this cell type, TPM increased the amplitude of non-L-type high voltage-activated Ca$^{2+}$ currents (Zhang et al., 2000).
Summary

Epilepsy is a chronic neurological disorder characterized by a spontaneous tendency towards recurrent seizures (Cereghino & Penry, 1995). The first description of epilepsy goes back to 2000 BC and can be found in the British Museum on a Babylonian tablet. The modern concepts of the classification, treatment and aetiology of epilepsy only started at the end of 19th century with the development of neurology (Trimble & Reynolds, 1986). Today, epilepsy is considered a group of disorders rather than a single disorder and epilepsy classification is based on seizure characteristics and EEG features (Shorvon, 2000). The pathophysiology of epilepsy has been studied extensively but our understanding of underlying mechanisms still appears to be insufficient. The outcome is that epilepsy involves alterations of normal physiological processes that lead to the synchronous activation of a population of neurones producing epileptiform activity. The aetiology of many types of epilepsy has been identified but still many syndromes remain to be clarified. The general belief is that epilepsy can be caused by genetic alterations of the systems regulating neuronal communication (ion channels and neurotransmission) and, in fact, all AEDs act via one or more mechanisms involving these systems. In general, the four main mechanisms that best describe the action of AEDs are:

- Inhibition of voltage-dependent Na⁺ channels;
- Enhancement of GABA_A-mediated inhibitory neurotransmission;
- Inhibition of excitatory (glutamate) neurotransmitter activity;
- Inhibition of voltage- and receptor- dependent Ca²⁺ channels.

All currently available AEDs act via one or more of these mechanisms; e.g. drugs acting on Na⁺ channels (CBZ, OXC, PHT, LTG, ZNS, TPM), GABA inhibitory neurotransmission (PHB, PRI, benzodiazepines, VLP, tiagabine, vigabatrin, TPM), Ca²⁺ channels (ESM, ZNS, GBP, TPM) and glutamate neurotransmission (felbamate, TPM). Many drugs are available for the treatment of epilepsy but still ~ 30% of epileptic patients are refractory to any therapy. Therefore, the need to discover new antiepileptic compounds and in particular, possible new therapeutic targets for such agents remains high.
I.5. Aims and objectives of the present work

In the present work, we have used *in vitro* slices of rat olfactory (piriform) cortex (PC) as a convenient brain slice model (Constanti *et al.*, 1993) to study the direct effects of the novel AED TPM on neuronal membrane properties and post-stimulus afterpotentials recorded under current-clamp or voltage-clamp conditions, and to examine whether these effects were indeed related to CA inhibition. The olfactory cortical brain area was of particular interest, in view of its known susceptibility to limbic epileptogenesis (Löscher & Ebert, 1996). During the early part of the study, the main aim was to learn the techniques for obtaining and maintaining good quality stable intracellular recordings from cells in PC brain slices. These experiments provided an opportunity to observe and understand the behaviour of the neurones impaled and the effects and mechanisms of the responses elicited by the drugs used on the preparation. The techniques for eliciting the post-stimulus slow afterhyperpolarization (sAHP) that follows a burst of action potentials, (s/AHP tail current under voltage clamp), and also standard current clamp current-voltage relationships (CCIVs) were learnt and their significance noted. Use of the Axon Instruments software, CLAMPFIT, for the analysis of collected data, was also learnt. Once the experimental techniques had been established, our preliminary current clamp experiments showed that TPM exerted two main (one of them novel) effects in these cortical cells:

1) induction of a slow membrane hyperpolarization, accompanied by an inhibition of repetitive action potential firing, and

2) *enhancement* and *prolongation* of the sAHP.

In the present study, we wished to examine the mechanism underlying these novel effects of TPM in more detail, using both current-clamp and single microelectrode voltage clamp protocols.

Following considerations on the observed *in vitro* effects of TPM on PC neurones allowed us to speculate on one of the likely mechanisms underlying sAHP enhancement by TPM, namely, the *enhancement* of L-type calcium current. This effect would be unique within the class of AEDs, and particularly of interest, since it has always been believed that the enhancement of neuronal calcium currents is *proconvulsant* rather than anticonvulsant. In fact, one of the numerous animal models of epilepsy is the induction of seizures by intracerebroventricular administration of the L-type calcium channel
opener, S(-)-1,4-dihydro-2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl)phenyl]-3-pyridinecarboxylic acid methyl ester (Bay K 8644) (Wielosz et al., 1997; Palmer et al., 1993; De Sarro et al., 1992a). Furthermore, it has been widely demonstrated that calcium channel blockers possess anticonvulsant properties in different animal models of epilepsy (Kriz et al., 2003; De Sarro et al., 1986, 1988, 1990) and when administered concomitantly with AEDs, they are able to reduce the ED50 values for the induction of seizures (Gasior et al., 1996; De Sarro et al., 1992b). An important question raised by our in vitro observations therefore, was whether calcium channel blockers used in therapy such as nifedipine and verapamil could affect the anticonvulsant efficacy of TPM. It was thus decided to analyze the effects of the concomitant administration of L-type calcium channel modulators on the anticonvulsant efficacy of TPM in different animal models of epilepsy in vivo. These experiments were carried out at the Department of Experimental and Clinical Medicine “G. Salvatore”, School of Medicine, Via T. Campanella, 115, Catanzaro, Italy under the supervision of Prof. Giovambattista De Sarro (Chair of Pharmacology).

After the acquisition of the techniques to perform pharmacological studies in vivo, and familiarity with different chemical and genetic models of epilepsy, I aimed to test and analyse the anticonvulsant efficacy of TPM, and to observe if the concomitant administration of L-type calcium channel modulators changed TPM’s anticonvulsant properties in comparison with some other anticonvulsant agents e.g. glutamate AMPA/kainate receptor antagonists (NBQX and CFM-2) and the carbonic anhydrase inhibitor ACTZ. Preliminary abstracts of this work have been presented to the British Pharmacological Society (Russo & Constanti 2002; Russo et al., 2003) and more full accounts recently published in the British Journal of Pharmacology (Russo & Constanti, 2004) and Neuropharmacology (Russo et al., 2004).
Chapter II:

Experimental Section
II.1  *In vitro* results

II.1.1. Introduction

Electrophysiological recordings from excitable tissue were originally developed around 1940 by J.Z. Young (Brazier, 1988) but the definitive intracellular techniques, including voltage-clamp recordings, were first successfully used on squid axons in 1952 by Hodgkin and Huxley (1952a, b, c), who described for the first time the ionic basis of the action potential (reviewed by Fisher, 1989). Since then, the technique has been further developed and refined, and new recording techniques, such as microelectrode recording (Ling & Gerard, 1949) and patch clamp (Neher & Sakmann, 1976), have been introduced into research. These recordings allow the researcher to directly measure membrane potentials or currents in excitable cells, thus the membrane properties of a cell, and the effects of a drug on these properties can be readily investigated. The ability to study all these parameters has also brought to us a big advance in the understanding of the mechanisms underlying neuronal communication and thus the basic mechanisms by which the brain is working. Over the years, different brain slice preparations have been developed for the study of different brain areas, and many of these have been particularly useful for the study of the mechanism of action of antiepileptic drugs (e.g. hippocampus, entorhinal cortex, neocortex *etc.*); in the present study we have used *in vitro* brain slices of rat olfactory (piriform) cortex (a convenient brain slice model; Constanti *et al.*, 1993; see Section II.1.1.4) to study the direct effects of TPM on neuronal membrane properties using the methods of current- and voltage clamp. We were particularly interested in this brain area, in view of its known susceptibility to limbic epileptogenesis (Lösch & Ebert, 1996); a brief description of the anatomical and physiological characteristics of the piriform cortex (PC) is described in the following sections.

II.1.1.1. Structure and physiology of the mammalian piriform cortex

The mammalian PC is a phylogenetically old part of the brain, that is located bilaterally towards the rostral end of each side of the cerebral cortex (Fig. 1) (Lösch & Ebert, 1996). The lateral olfactory tract (LOT), arising from mitral cell bodies in the olfactory bulb, sends afferent fibres to join the PC and differentiates widely across the cortical surface; in fact, the PC is the largest area of cortex to receive direct sensory input from
the LOT without intermediate thalamic contact (Saar et al., 1999). The PC also receives important afferent inputs from other brain areas (Fig. 3), such as cholinergic innervation from the nucleus of the diagonal band (Eckenstein et al., 1988; Saper, 1985), dopaminergic inputs from the substantia nigra (Fallon et al., 1983), serotonergic inputs from the raphe nuclei (Chabaud et al., 1999), catecholaminergic inputs from the locus coeruleus (Datiche & Cattarelli, 1996) and also projections from the amygdala (Majak et al., 2004). Structurally, the PC is composed of three principal layers (LI-III), with the underlying endopiriform cortex commonly being termed as “layer IV” (Tseng & Haberly, 1989a, b). The laminar structure can clearly be visualised under a stereomicroscope with under illumination (Haberly & Behan, 1983; Haberly, 1983; Haberly & Feig, 1983), making the slice preparation particularly convenient for performing electrophysiological recordings from individual cell layers and for electrically stimulating specific synaptic inputs in those layers (Hori et al., 1988). As with other areas of the cortex, the principal neurones found in the PC are pyramidal cells, located mainly in layer II (superficial) and in layer III ("deep") (Haberly, 1983; Haberly & Feig, 1983). Additionally, there are at least nine other non-pyramidal cell types that have so far been identified (primarily in layer III), including aspiny inhibitory interneurones (Protopapas & Bower, 2000).

The PC is believed to be responsible for assimilating odour information into learning and memory (Wilson & Bower, 1989), and it has been widely used for research into learning and associative memory functions (Dade et al., 2002; Hasselmo et al., 1992). Such functions apparently rely on the presence of intrinsic oscillatory networks (Chabaud et al., 1999), which can predispose some brain areas to epileptogenesis (Draguhn et al., 2000; Löscher & Ebert, 1996; Liljenstrom & Hasselmo, 1995).

II.1.1.2. Properties of the principal cell types found in the piriform cortex

The principal neurone types of the PC cortex can be identified by differences in their morphology and responses to electrical and/or pharmacological stimulation (Ekstrand et al., 2001a; Protopapas & Bower, 2000; Libri et al., 1994; Hoffman & Haberly, 1991; Tseng & Haberly, 1989a, b). Superficial pyramidal neurones are densely packed in LII and have short apical dendrites and moderately branched basal dendritic trees (Haberly & Behan, 1983; Haberly, 1983; Haberly & Feig, 1983). They characteristically respond
to positive electrotonic stimuli with spike pre-potentials early in the evoked spike train, possibly due to an apical "bifurcation trigger zone" (Libri et al., 1994). Because of their relatively low input resistance and poor long-term recording stability, such neurones were excluded from the present analysis. By comparison, “deep” pyramidal neurones located in LIII (from which the majority of recordings in this study were made) have long, thick apical dendrites and a highly branched basal dendritic tree (Libri et al., 1994). The cells exhibit a prominent slow after-hyperpolarization (sAHP; revealed as an underlying slow outward tail current, \( s/AHP \) under voltage clamp) following a long depolarizing stimulus. Under certain conditions, these neurones have also been shown to display epileptiform excitatory postsynaptic potentials (EPSPs) (Tseng & Haberly, 1989c, d; Hoffman & Haberly, 1991). The other major neurone types in LII-III are morphologically varied non-pyramidal cells with no apical dendrite and a diffuse basal dendritic tree (Protopapas & Bower, 2000; Libri et al., 1994); these cells are most likely inhibitory interneurones (Protopapas & Bower, 2000). Interestingly, such neurones respond to depolarizing and hyperpolarizing electrotonic stimuli in a similar manner to deep pyramidal neurones and are therefore indistinguishable electrophysiologically (Libri et al., 1994). Recordings made from LII-III in the present study therefore undoubtedly included recordings from (predominantly) “deep” pyramidal neurones and also interneurones; however, for the purpose of the present analysis, all such data were pooled.

**II.1.1.3. Synaptic connections of the piriform cortex**

The synaptic circuitry of the piriform cortex is characterized by a highly organized structure due to the presence of many different fibre and neurone types distributed within some specific layers (Fig. 2) (Haberly & Bower, 1984; Haberly & Presto, 1986; Rodriguez & Haberly, 1989). During intracellular recordings from a pyramidal neurone of the piriform cortex, an appropriate focal electrical stimulation typically evokes an EPSP/IPSP response characterized by a number of particular features:

1. a fast excitatory component of the EPSP, due to glutamate release acting on its own ionotropic receptors (NMDA and AMPA/kainate receptors),
2. a fast component of the IPSP, due to GABA release acting on GABA\(_A\) receptors (ionotropic) which open Cl\(^-\) channels,
3. a slow IPSP due to activation by GABA of GABA\textsubscript{B} receptors (metabotropic) which selectively open G-protein coupled inward-rectifying K\textsuperscript{+} channels (Malcangio \textit{et al.}, 1995; Libi \textit{et al.}, 1996).

The synaptic connections within the various layers may be summarized as follows:

- LOT afferent terminals in L1a innervate apical dendrites of pyramidal cells and also inhibitory interneurones in L1b, providing feed-forward inhibition (Fig. 2).

- Layer Ib carries cortico-cortical \textit{association fibres} arising from the basal dendrites of (mainly) superficial cells and \textit{intrinsic fibres} arising from the basal dendrites of superficial and deep pyramidal neurones in LII-III that pass rostro-caudally to modulate pyramidal cell responses (Fig. 2) (important for associative memory; Hasselmo & Bower, 1993). Lib also contains \textasciitilde50\% of PC inhibitory interneurones, activated by LOT or association fibre stimulation (Lösch et al., 1998).

- Layer II contains superficial pyramidal neurones and \textasciitilde5\% of the total inhibitory interneurone population.

- Layer III contains “deep” pyramidal neurones and also \textasciitilde15\% of the total interneurones (Haberly & Bower, 1984; Haberly & Price, 1978).

The following sections give a more detailed overview of these connections.

\textit{Afferents from the Lateral olfactory tract (LOT)}

The LOT fibres, originating in the olfactory bulbs, are the main excitatory afferent input that synapse with the neurones of the piriform cortex (Fig. 2; Haberly & Price, 1977). The PC is, in fact, the largest brain area receiving direct inputs from the olfactory bulbs (Johnson \textit{et al.}, 2000). LOT fibres are mainly localized within layer Ia and they typically follow a rostro-caudal direction running parallel to the pial surface (Fig. 2). However, LOT axons frequently descend perpendicularly into layers Ib and II, (Haberly & Behan, 1983), where they make connections with the apical dendrites of superficial and deep pyramidal cells (Fig. 2), and interestingly, it has been shown that each superficial pyramidal cell typically only synapses with a single LOT axon, whilst each deep pyramidal neurone may form di- or polysynaptic connections with LOT afferents (Biedenbach & Stevens, 1969). Therefore, deep pyramidal cells receive a greater
amount of excitatory inputs than superficial cells (Ekstrand et al., 2001b; Hoffman & Haberly, 1991).

**Association fibres (cortico-cortical connections)**

Excitatory synaptic fibres also arise from the basal dendrites of superficial cells and some deep pyramidal cells. These fibres originate within layers II and III and bypassing layer I b reach layer I a, where they leave the piriform cortex to reach other brain regions such as the pre-frontal cortex, amygdala, enthorinal and perirhinal cortices (Fig. 3; Luskin & Price, 1983). It has been shown that these basal dendrites, although branching rarely in the piriform cortex, are highly branched upon other areas and can, traverse an entire cerebral hemisphere, arborizing in a number of brain areas indicating the ability of this area of the brain to communicate with other brain areas in order to process many functions.

**Intrinsic fibres**

Another excitatory synaptic system found in the PC is constituted by the so-called intrinsic fibres. These also originate from the basal dendrites of superficial and deep pyramidal cells as the association fibre system, but they remain within the piriform cortex to form connections with other superficial or deep pyramidal cells (Haberly & Price, 1978; Luskin & Price, 1983; Price, 1973), most probably their role is to distribute excitatory stimuli arriving from LOT afferents. These connections are divided into two types depending on the direction followed by these fibres: a feed-forward (rostro-caudal propagation) system and a feed-back (caudo-rostral propagation) system within the piriform cortex (Haberly & Price, 1978). Of the two identified intrinsic subsystems, the rostro-caudal, feed-forward system has been shown to comprise approximately twice the number of fibres as comprises the caudo-rostral system (Datiche & Cattarelli, 1996b).

**Inhibitory synaptic connections within the piriform cortex**

All the GABA-ergic inhibitory interneurones present in all PC layers are responsible for the inhibitory synaptic connections and therefore, regulation of the activity of this brain area. Both LOT afferents and intrinsic fibres synapse with the non-pyramidal interneurones present within all three layers of the piriform cortex (Scholfield, 1978a; Scholfield, 1978b). These interneurones make synaptic connections with superficial and
deep pyramidal cells and therefore, they participate to feed-forward and feed-back inhibitory pathways. A study has suggested that the association fibres may play a role in the modulation of feed-back inhibitory processes in the piriform cortex (Barkai et al., 1994), whereby association fibres arising from pyramidal neurone stimulate interneurones that, in turn inhibit the pyramidal neurones from which the axons arose. Many electrophysiological (Satou et al., 1982, 1983a, b) and immunocytochemical (Haberly et al., 1987; Loscher et al., 1998) studies have tried to characterize these cell types and they demonstrated that the majority of GABA-ergic neurones are located within layer I (~50%) with ~5% and ~15% found in layers II and III respectively. Kapur et al. (1997) showed that GABA_A-mediated inhibition was dendritically generated and that the circuits responsible for this inhibition were different between the apical dendritic and somatic regions. This was more recently confirmed by a study that identified distinct subpopulations of interneurones within the piriform cortex (Ekstrand et al., 2001a) similarly to what has been described for rat auditory cortex (Aramakis et al., 1997), cat visual cortex (Tamas et al., 1997), rat neocortex (Thomson et al., 1996) and hippocampus (Traub et al., 1987; Vu & Krasne, 1992). Therefore, the inhibitory circuit of a single interneurone depends on the excitatory input initially stimulating the inhibitory interneurone. This creates a complex feed-back and feed-forward inhibitory synaptic circuit within the piriform cortex (Fig. 4), with inhibitory effects upon pyramidal cells being exerted by inhibitory interneurones as a consequence of either excitatory stimulation of the pyramidal cell (feed-back inhibition) or as a result of afferent fibre stimulation of the interneurone, exerting an inhibitory effect upon the pyramidal neurone prior to the arrival of the afferent excitatory stimulus at the pyramidal neurone (feed-forward inhibition).

II.1.1.4. Epileptogenic properties of the piriform cortex

It is now well recognised that the PC is particularly prone to limbic epileptiform events, suggesting that the circuits of the PC area might act as a critical pathway for limbic seizure discharges to affect motor systems. This susceptibility to develop epileptic seizure behaviour has been shown both in vivo and in vitro in response to various pharmacological agents or electrical stimulation (Ekstrand et al., 2001b; Doherty et al., 2000; Ebert et al., 2000; Postlethwaite et al., 1998; de Curtis et al., 1996; Hoffman & Haberly, 1989). Furthermore, the most frequent types of seizure induced in the PC are
complex partial seizures, the most common seizure type encountered in human epilepsy (Lösch & Ebert, 1996). Interestingly, despite the fact that the “deep” layers of the PC are most likely responsible for the initiation of epileptiform activity (Lösch & Ebert, 1996), the neurones usually investigated in this brain area are superficial pyramidal neurones that exhibit very few pro-convulsive responses or characteristics (Hasselmo & Bower, 1990, 1991, 1992; Tang & Hasselmo, 1994). In the last decade, a specific role has been found in the study of experimental kindling development in vivo. Lösch & Ebert (1996), in their review on “the role of the piriform cortex in kindling”, underlined some specific points that make this area particularly prone to limbic seizures: 1) The PC contains the most susceptible neural circuits of all forebrain regions for electrical (or chemical) induction of limbic seizures. 2) During electrical stimulation of other limbic brain regions, prolonged afterdischarges can be observed in the ipsilateral PC, indicating that this region is early activated during the kindling process. 3) The interictal discharge originates in the PC independently from the kindled focus. 4) Bilateral lesioning of the PC blocks seizure generalization following kindling of the hippocampus or olfactory bulb. It may be noted that the PC is not, in fact, a homogeneous structure, which complicates studies on its role in limbic epileptogenesis. Recent reports have attempted to characterize the role of smaller areas inside the PC recruited during the development of kindling (Rigas & Castro-Alamancos, 2004; Doherty et al., 2000; Schwabe et al., 2000; Ebert et al., 2000). It has been demonstrated that neurones in the PC display epileptiform bursting following stimulation by many drugs acting on either inhibitory and/or excitatory systems. For example, proconvulsant drugs in this brain area are: the group I mGluR agonist trans-ACPD (Moldrich et al., 2003; Cartmell & Schoepp, 2000; Constanti & Libri, 1992), penicillin (Yamauchi et al., 1989), picrotoxin, 4-aminopyridine (4-AP), OXO-M (Postlethwaite et al., 1998) and tetraethylammonium (TEA) (Velisek et al., 1995), all of which have been shown to induce persistent epileptiform seizure activity.

In conclusion, both clinical and experimental investigations suggest that, like other limbic structures, the PC displays a low threshold for chemical or electrical seizure induction, and also a unique highly directional association fibre system (Haberly & Price, 1978) which efficiently links to a large number of other brain areas (Litaudon & Cattarelli, 1994), therefore facilitating seizure spread. These characteristics make this area a unique locus from which seizure activity can originate and may, to a certain
extent, account for the overall susceptibility of the temporal lobe to epileptiform bursting (Löschler & Ebert, 1996). Also, taking in account all the documentation present in literature, it is clear that models of epilepsy which involve or use the PC directly can be considered useful in the study of the seizure generation and propagation and, furthermore, as analytical tools for the study of potential or established anticonvulsant drugs.

II.1.1.5. The piriform cortex slice preparation: historical development

The PC slice preparation was initially developed in 1966 and then characterized extensively in the subsequent years. Initially, brain slices were prepared manually by making tangential cuts (incorporating the lateral olfactory tract: LOT) from the surface of guinea pig brains with a bow-cutter and guide, and were used for the development of extracellular recording techniques that, in turn, gave the first detailed information about the electrophysiological properties of this particular area of the brain (Harvey et al., 1974; Richards & Sercombe, 1968; Yamamoto & McIlwain, 1966). Further characterisation was then achieved after the use of intracellular microelectrodes, which defined the main characteristics of the cells of this area and some of their synaptic responses. In vitro intracellular recordings were first made from slices of guinea pig PC by Scholfield (1978a, b, c) following initial in vivo intracellular experiments carried out on cat (Biedenbach & Stevens, 1969) and opossum (Haberly, 1973) PC. The results of the in vitro intracellular investigations obtained by Scholfield provided the first description of the electrical membrane properties and synaptic responses of the “deep” piriform cortical neurones. Thus, large EPSPs responses were obtained after stimulation of LOT afferent fibres, and the presence of strong feedforward and feedback inhibitory synaptic pathways was evidenced by the recording of different IPSPs responses following local stimulation of interneurones. Detailed knowledge of the structure of the PC and various intrinsic synaptic connections along with accumulated electrophysiological data have also allowed the construction of a computer model of this brain area (Wilson & Bower, 1989) which has helped in the understanding of learning and memory processes (Barkai et al., 1994; Haberly & Bower, 1989); however it is notable that deep pyramidal neurones (found in PC layer III), were not included into this simulation, which makes its general usefulness rather limited, particularly for predicting any induced seizure activity in this area (Postlethwaite et al., 1998; Hoffman
& Haberly, 1991). *In vitro* studies have also used slices cut perpendicular to the pial surface (Bower & Haberly, 1986; Constanti & Sim, 1987b), which allows visual access to the pyramidal cell layers and an easy placement of stimulating electrodes within the slice (Hori *et al.*, 1988). It is clear that in slice studies it is important to preserve the fibre system in order to maintain most of the physiological properties of the studied area. In the present study, the transverse slice preparation (~450 μm thick; Libri *et al.*, 1994) was chosen for experiments carried out to define the effect of TPM upon membrane properties; it is considered unlikely that any substantial differences in TPM responses would have been obtained if tangential slices were used instead. In addition, the choice of transverse slices permitted us to prepare a larger number of slices (~6-10) suitable for recordings per animal.
II.1.2. Methods

II.1.2.1 Preparation and storage of brain slices

All the in vitro experiments were carried out using transversely-cut slices of PC prepared from adult Wistar rats (150-200g; either sex), as previously described (Constanti et al., 1993; Constanti & Sim, 1987a). The animals were first decapitated with sharp scissors under deep halothane anaesthesia (May & Baker, Dagenham, U.K.) in accordance with the Home Office, Animals (Scientific Procedures) Act 1986. The brain was carefully exposed by removal of the skull and meninges with forceps, and then removed from the skull with a spatula onto a piece of filter paper moistened with Kreb's solution and placed over a cooled inverted Petri dish. The brain was then bisected longitudinally with a razor blade to separate the two hemispheres. One hemisphere was placed in oxygenated ice cold Kreb's (for later slicing) and from the other, a rectangular block of tissue was cut containing the PC. The block of tissue was then mounted on the teflon stage of a Campden Vibroslice-752M tissue cutter (Campden Instruments, U.K.) and affixed using cyanoacrylate glue (Loctite Ltd, Cheshire, U.K.). The stage and tissue were transferred to the tissue cutter bath containing ice cold (4°C) oxygenated Kreb's solution where ~450 μm thick slices were cut, along the axis of the lateral olfactory tract and perpendicular to the pial surface of the brain. Cut slices were quickly removed into a storage chamber (comprising of a plastic tea strainer partially immersed in a beaker of oxygenated Krebs solution) and maintained at 32°C for at least 30 min before transferring to a Perspex recording bath. Here, they were held in place (completely submerged) between two nylon nets and superfused continuously at ~10 ml/min with pre-warmed oxygenated Krebs solution at 29-30°C. The composition of the normal Krebs solution was (in mM): 118 NaCl, 3 KCl, 1.5 CaCl₂, 25 NaHCO₃, 1 MgCl₂·6H₂O, and 11 D-glucose (bubbled with 95% O₂-5% CO₂, pH 7.4). Bicarbonate-free HEPES-buffered Krebs solution contained (mM): 133 NaCl, 3 KCl, 1.5 CaCl₂, 1 MgCl₂·6H₂O, 10 HEPES and 11 D-glucose (bubbled with 100% O₂, pH 7.4 adjusted with 1N NaOH).

II.1.2.2 Electrophysiological recording

Intracellular current-clamp or voltage clamp recordings were made from the periamygdaloid area of the slices within PC cell layers II-III using glass microelectrodes...
filled with 4M potassium acetate (tip resistances 40-70 MΩ) coupled to an Axoclamp 2A sample-and-hold preamplifier (2-3 kHz switching frequency, 30% duty cycle; Axon Instruments, CA, USA). Intracellular recording electrodes were prepared from GC100F-15 glass capillary tubes (1.0 mm outer diameter, 0.58 mm inner diameter, with inner filament; Clark Electromedical Instruments, Pangbourne, Reading, UK) using a Narashige PN-30 electrode puller (Narashige, Japan). Cells were impaled in “bridge” mode by advancing the microelectrode slowly through the slice and passing brief positive current steps through the electrode via the “positive clear” switch of the preamplifier, or by applying a brief tip oscillation via electrode capacitance overcompensation. After impalement, cells usually settled to a stable membrane potential of between -80 to -85 mV within 5-10 minutes. In order to minimise outward K⁺ conductances, some experiments under voltage clamp where carried out using electrodes filled with 3M caesium acetate (tip resistances 30-50 MΩ) and in the presence of 1 μM tetrodotoxin (TTX) to block generation of fast Na⁺ spikes. Membrane input resistance and firing behaviour were assessed by injecting positive or negative current pulses of varying amplitude (−0.25-3 nA; 160 ms) and the resulting electrotonic potentials or evoked action potentials recorded respectively. First spike amplitudes were routinely measured (from baseline level) under “bridge” recording mode to avoid sampling limitations of the discontinuous sample-and-hold preamplifier. In current clamp experiments, the membrane potential was usually maintained at -70 mV (near firing threshold) by applying steady depolarizing current, while in voltage clamp recordings, the holding membrane potential was set at -70 mV. Current-voltage (I-V) curves were constructed by taking the steady voltage level recorded at the soma following voltage step commands (in TTX). Membrane input resistance was measured using Ohm’s law (R=V/I) applied to current clamp recordings with membrane potential maintained at -70 mV and following the injection of a constant negative current pulse of 0.5nA (160ms); the corresponding peak electrotonic voltage deflection was measured at the end of the current pulse. Measurement of the slow post-stimulus outward tail current (IₙAHP) underlying the sAHP was carried out using a combined “switched” current clamp/voltage clamp approach (“hybrid clamp”; Constanti et al., 1993; Constanti & Sim, 1987a; Lancaster & Adams, 1986) in which a fixed depolarizing stimulus (+2 nA, 1.6 s) was first applied to the cell under current clamp (membrane potential held at -70 mV) to produce a spike train, followed immediately by a manual switch to voltage clamp at -70 mV to reveal the IₙAHP tail. Measurement of the peak sAHP amplitude
relative to the baseline level was made following the end of the positive current pulse, and sAHP duration was estimated as the time necessary for the membrane potential to return at the control value (-70mV), from the end of the current pulse. Under hybrid clamp or voltage clamp conditions, the peak was measured at the beginning of the current tail and the duration was calculated from the end of the positive pulse to the end of the decay phase of the tail. Acceptable recordings typically remained stable for periods of 1-5 h. Sampled membrane current and voltage signals were monitored on a Tektronix 5111A storage oscilloscope (Beaverton, Oregon, USA) and a Gould 2400 ink-jet chart recorder (rise time < 8ms for a square wave input) and also fed directly to a computer (Viglen Ltd. UK) via a Digidata 1200 analogue-to digital interface (Axon Instruments, CA, USA) using pCLAMP 6.03 software (Axon Instruments) for hard disk recording and off-line analysis. Voltage clamp currents following step commands were not corrected for leakage or capacitative currents. All measurements were made before, during, and after bath-superfusion (bath-exchange time ~30 s), of pharmacological agents so that each neurone served as its own control. Unless otherwise stated, each experimental sequence described below was repeated at least three times on different cells. The results presented below are based on recordings obtained from ~150 cortical neurones in ~100 piriform cortex slices.

II.1.2.3 Statistical analysis

Data are expressed as means ± s.e. mean, and when appropriate, statistical significance of the differences between data group means was evaluated using a one-tailed Student’s paired t-test, unless data were presented as percentage change versus control in which case statistical differences were assessed using a nonparametric Wilcoxon signed-rank test.
II.1.4. Results

II.1.4.1 Characterization of the slow hyperpolarizing effect of TPM

Topiramate was successfully tested on 37 olfactory cortical neurones recorded under current clamp conditions; their mean resting potential and input resistance (calculated from ≤ 20 mV hyperpolarizing electrotonic potentials) was -82.2 ± 0.3 mV and 41.1 ± 1.6 MΩ, respectively. When we initially tested the effect of TPM on membrane properties, recordings were performed from cells in their original state (i.e. maintaining their original resting membrane potential); under these conditions, TPM induced a hyperpolarization which usually stabilized the membrane potential at around -87 mV and was accompanied by a noticeable reduction of input resistance (n = 7; data not shown). To better characterize and normalize TPM’s effects, all subsequent recordings were performed on neurones maintained at -70 mV by steady positive current injection.

In 31 out of 37 cells studied (~84%), bath-application of TPM (20 μM, 20-50 min) produced a slow hyperpolarization (mean amplitude = 9 ± 2 mV, significantly different from baseline level; P<0.001) that attained a plateau ~ 10-15 min after onset of application, and was accompanied by a significant decrease in input resistance (mean = 26 ± 7 %; P<0.001); this change was reflected by a reduction in the amplitude of hyperpolarizing electrotonic potentials evoked by injecting negative current pulses into the cell (Fig. 5 A, B). After washing out TPM, both the membrane potential and input resistance recovered slowly to their control levels over 15-30 minutes; this slow hyperpolarizing response was reproducible in all cells if adequate washout time was allowed, with no significant variations in the hyperpolarizing response obtained in the same cell. A similar slow hyperpolarization induced by 100 μM TPM was also reported by Herrero et al. (2002) in hippocampal CA1 neurones, and attributed to an increase in K⁺ conductance (see below). Hyperpolarizing responses to TPM appeared to be dose-dependent within the range of 10-50 μM TPM; however, when a the highest (50 μM) dose of TPM was used, the membrane actions seen were more prominent and longer lasting, with incomplete recovery, despite 1 hr of washout. A standard 20 μM dose of TPM was therefore used in all subsequent experiments in order to facilitate reproducibility and multiple TPM comparisons in any given cell.

When cell firing properties were tested by injecting a brief depolarizing current pulse (160 ms, +0.75 nA) (after correcting for TPM hyperpolarization by steady positive
II.1.3. Drugs

Topiramate (supplied by Johnson & Johnson Pharmaceutical Research & Development, L.L.C., Raritan, NJ, USA) was prepared as a 10mM stock solution in distilled water and was stored for up to 7 days at 4°C without apparent loss of activity (TPM is stable in water solution up to 15 days; R.P. Shank, Johnson & Johnson Pharmaceutical Research and Development, L.L.C., PA, USA; personal communication). Acetazolamide (ACTZ), β-parachlorophenyl-γ-aminobutyric acid ((-)baclofen), (−)-bicuculline methiodide, forskolin, nifedipine, ouabain and tetrodotoxin (TTX), were all purchased from Sigma-Aldrich Co. Ltd., Poole, UK. All these drugs, with the exception of nifedipine, were routinely prepared as stock solutions in distilled water and stored at 4°C; drugs were subsequently diluted in Krebs solution immediately before use. Benzolamide was a gift from Prof. E.R. Swenson, School of Medicine, University of Washington, Seattle, WA USA. All other reagents were obtained from BDH (Analar grade). Nifedipine and benzolamide were dissolved in DMSO and ethanol respectively, and then diluted in Krebs just before use; the final bath concentrations of DMSO (up to 0.5%) or ethanol (0.1%) had no deleterious effects on membrane properties. Due to their light sensitivity, nifedipine, acetazolamide and ouabain were weighed and dissolved in semi-darkness and then stored in containers wrapped in silver foil to exclude light. Exposure to ambient light during application of these drugs to cells was also minimised.
current injection), TPM induced a significant reduction in the number of evoked action potentials (mean number of control spikes = 7.2 ± 1.7, decreased to 2.2 ± 0.9 spikes in TPM; \( P < 0.05, n = 22; \sim 70\% \) reduction), indicating a decreased cell excitability, along with a small (\sim 18\%) reduction in the peak amplitude of the first spike overshoot in the train (mean = 19.8 ± 3.5 mV control vs. = 16.2 ± 2.5 mV in TPM; \( P < 0.05, n = 22 \)) (c.f. Wu et al., 1998); the threshold level for triggering action potentials (typically between \sim -60 to -65 mV) was not notably altered. However, it has to be considered that with the technique used in this study only big variations of the threshold level could be measured. On washout of TPM (40 minutes), these effects were fully reversed (Fig. 5 C). Interestingly, in 6 neurones tested, the membrane potential, input resistance and number of spikes fired were not apparently affected by TPM, (see also Leniger et al., 2004a) suggesting that the action of the drug may be variable depending on neurone-type (i.e. interneurone or deep pyramidal cell; Libri et al., 1994) or possibly, cell metabolic status (see below). In voltage clamp at a holding potential of -70 mV, the slow TPM hyperpolarization was manifested as a slow outward current shift, peaking after \sim 10 minutes of application, and slowly reversing on washout (\sim 30 min) (Fig. 6 A); this current was insensitive to 1 \mu M TTX, therefore was not due to synaptic release endogenous neurotransmitters (\( n = 17 \)). In 32 out of 34 cells, the mean outward TPM current recorded was 0.36 ± 0.26 nA; (2 cells tested in voltage clamp, at a holding potential of -70 mV, failed to respond to TPM - see above). When TPM applications were repeated at different steady holding potentials (between -70 and -110 mV; \( n = 6 \)), the slow outward current decreased in amplitude with increasing hyperpolarization (as would be expected if it were mainly \( K^+ \)-mediated; Herrero et al., 2002) but failed to show a clear reversal to an inward current, even at -110 mV (Fig. 6 A, B). Accordingly, current-voltage (\( I-V \)) curves constructed (in TTX) using 1.5s voltage jumps in the presence and absence of TPM, did not intersect at negative potentials (Fig. 7A), suggesting either a mixture of ionic conductances and/or some electrogenic pump mechanism might be involved in mediating the TPM effect in these cells. In contrast, when the action of TPM was compared with that of the GABA\(_B\) receptor agonist baclofen (20 \mu M), which selectively activates a G-protein coupled inward-rectifying \( K^+ \) conductance (GIRK) in these neurones (Malcangio et al., 1995) (\( n = 4 \) cells) (Fig. 6C), baclofen produced outward currents of similar amplitude (though faster in onset/offset) to those of TPM, that were not occluded when superimposed at the peak of the TPM response, suggesting independent ionic mechanisms; moreover, the (\( I-V \)) curves in the
presence and absence of baclofen showed a clear crossover at negative potentials (Fig. 7 B), giving a mean baclofen reversal potential of \(-90 \pm 5\) mV \((n = 4)\) (close to the calculated \(E_K\) of \(-95\) mV in these cells, taking internal \(K^+\) concentration as \(130\) mM; Harvey et al., 1974).

In the study of Herrero et al. (2002), 100 \(\mu\)M TPM induced a membrane hyperpolarization in hippocampal CA1 neurones that was abolished by 1 mM \(Ba^{2+}\), a known blocker of both outward, and inward-rectifying \(K^+\) conductances (Eaton & Brodwick, 1980). In our experiments, 1 mM \(Ba^{2+}\) only partially blocked the slow outward TPM current (mean = \(47.4 \pm 11.3\) % reduction, \(n = 4\); \(P<0.001\)), suggesting that a \(Ba^{2+}\)-resistant component was present (Fig. 8 A); a similar result was also obtained in \(Cs^+\)-loading experiments (see below). The nature of this other ionic conductance however, remains unclear. In further tests, we found that the TPM current was also unaffected by 200 \(\mu\)M \(Cd^{2+}\) \((n = 9)\), indicating that it was independent of external \(Ca^{2+}\) entry (i.e. it was not a \(Ca^{2+}\)-activated \(K^+\) conductance: \(I_{K(Ca)}\)) (Fig. 8 B), although we cannot exclude the possible induced release of \(Ca^{2+}\) from intracellular stores. In addition, the current was unaffected by 10 \(\mu\)M bicuculline methiodide (in TTX, to prevent spontaneous epileptiform firing activity; \(n = 8\)) (Fig. 8 C), thereby ruling out indirect involvement of GABA\(_A\) receptors, as proposed by Kawasaki et al. (1998) in rat subicular neurones. Finally, blocking \(Na^+\)-\(K^+\)-ATPase activity with 10 \(\mu\)M ouabain (10 min; \(n=4\)) also had no effect on the TPM current, indicating that a \(Na^+\)-\(K^+\) electrogenic pump mechanism was not involved (c.f. Gustafsson & Wigström, 1983). Ouabain also had little or no effect on the sAHP evoked by a burst of action potentials (c.f. Schwindt et al., 1988), or on the ability of TPM to enhance/prolong it (see below).

II.1.4.2 TPM enhances and prolongs the sAHP

In the second main block of experiments, we wished to further characterize the novel enhancing effect of TPM on the sAHP. Figure 9 A shows a representative example of this phenomenon. In control, a long (1.5s) depolarizing current stimulus produced a burst of action potentials followed by a sAHP, due to activation of a \(Ca^{2+}\)-activated \(K^+\) conductance (Constanti & Sim, 1987a) (Fig. 9 A). In TPM (20 \(\mu\)M, 20 min, \(n = 33\)), (after correcting for membrane potential change) there was a significant increase in the amplitude (~20%) (control mean = \(10.5 \pm 3.4\) mV vs \(12.5 \pm 3.8\) mV in TPM; \(P<0.001\))
and particularly, the duration (~63%) (3.39 ± 0.48 s control vs 5.52 ± 1.29 s in TPM; 
P<0.001; paired t-tests) of the sAHP, which reversed slowly after washout (~60 min).
There was also a dramatic increase in spike accommodation during the stimulus pulse.
The corresponding slow outward tail currents underlying the sAHP (sI\textsubscript{ahp}; Sah & Faber, 2002) were revealed using a “hybrid” voltage clamp protocol (Constanti et al., 1993; 
Constanti & Sim, 1987a; Lancaster & Adams, 1986), in which a manual switch to 
voltage clamp at -70 mV was made immediately after a 1.5s depolarizing stimulus (Fig. 
9 B). In TPM, the sI\textsubscript{ahp} tail was significantly enhanced in amplitude (~37 %) (269 ± 
137 pA control vs 369 ± 142 pA in TPM; P<0.001) and prolonged in duration (~ 84 %) 
(3.30 ± 1.04 s vs 6.07 ± 1.16 s in TPM; P<0.001) (n = 14). In this series, 2 out of 16 
n neurones tested (~12%) failed to show an obvious change in sI\textsubscript{ahp} magnitude or time 
course in TPM, although interestingly, these cells still generated a slow outward current 
shift under voltage clamp; this suggests that the underlying response mechanisms 
involved in the two TPM effects are independent of each other.

In an attempt to understand the mechanism underlying the sI\textsubscript{ahp} enhancement by TPM, 
we voltage clamped cells at -70 mV in 1 μM TTX (to prevent fast Na\textsuperscript{+} spike activation)
and applied a large positive voltage step from -70 to -20 mV (1.5s) to evoke outward 
current relaxations. A typical experiment is shown in Fig. 10 A (superimposed records). 
In 20 K acetate-recorded cells, a slow outward relaxation (1.77 ± 0.7 nA peak 
amplitude) and slow outward tail current (sI\textsubscript{ahp}) (115 ± 23 pA peak, 3.37 ± 0.64 s 
duration) were recorded in control solution, largely due to activation of a slow Ca\textsuperscript{2+}- 
activated K\textsuperscript{+} conductance (Constanti & Sim, 1987a). In TPM (20 μM; 20 min; n = 17), 
the amplitude of this outward relaxation was clearly increased (2.23 ± 0.9 nA), along 
with an enhancement (153 ± 34 pA) and dramatic prolongation (7.83 ± 1.32 s) of the slow 
sI\textsubscript{ahp} after-tail current (means significantly different from control; P<0.001; paired 
t-tests); in the corresponding I-V relation (Fig. 10 B and cf. Fig. 7 A), the action of TPM 
appeared as a greater degree of outward current rectification (relative to control) over 
the potential range of -60 to -20 mV. On further adding Cd\textsuperscript{2+} (200 μM; n = 15) both 
enhanced components were eliminated, confirming the Ca\textsuperscript{2+}-dependence of the extra 
TPM-induced current (Fig. 10 A, lower blue record) and the strong outward 
rectification of the I-V plot was abolished (Fig. 10 B). We noted that in 3/20 cells, the 
peak of the outward current relaxation in TPM was smaller than that seen in control, 
even though the sI\textsubscript{ahp} tail current was still enhanced; this could reflect an interplay
between inward and outward current components developing during the voltage jump, that were being differentially affected by TPM (Fig. 11; see Discussion).

II. 1.4.3 Experiments using Cs\(^+\)-filled microelectrodes

In the next set of experiments, we aimed to assess the contribution of K\(^+\) conductances to the extra TPM-induced outward currents; we therefore recorded with 3M Cs acetate-filled microelectrodes, in order to minimise the outward current induced by positive voltage jumps (Galvan et al., 1985), and to hopefully dissect the underlying mechanism of the sAHP enhancement by TPM. The experiments were carried out in the presence of 1 \(\mu\)M TTX to block Na\(^+\) conductances. In 25 Cs\(^+\)-loaded cells (after ~30 min loading), evoked action potentials showed a characteristically prolonged plateau phase due to activation of high voltage-activated (HVA) Ca\(^{2+}\) conductances (Galvan et al., 1985), followed by a prominent slow depolarizing after potential (DAP) (Fig. 12 A). Under voltage clamp, the DAP was manifested as a slow inward tail current following the offset of the depolarizing command (Constanti et al., 1985), most likely mediated by a Ca\(^{2+}\)-activated nonselective (CAN) cation conductance (Kang et al., 1998). Despite Cs\(^+\) loading, TPM still produced a significant enhancement of the residual outward current relaxation (mean amplitude = 1.84 ± 0.48 nA vs 1.56 ± 0.42 nA control; \(P<0.001\)), but now instead of an outward sI\(\text{AHP}\) tail, an enhanced slow inward after-tail current was revealed (124 ± 47 pA amplitude, 7.52 ± 1.15 s duration vs 114 ± 52 pA amplitude, 3.18 ± 0.32 s duration: control) (means significantly different from control; \(P<0.001\); paired \(t\)-tests), most likely composed of a mixture of decaying (enhanced) HVA Ca\(^{2+}\) current and CAN current. Accordingly, both the TPM-induced outward relaxation component and the slow inward tail current were eliminated by now adding Cd\(^{2+}\) (200 \(\mu\)M; \(n = 11\)) indicating they were dependent on external Ca\(^{2+}\) entry (Fig. 12 B).

One distinct possibility, was that this enhanced HVA Ca\(^{2+}\) current component of the inward after-tail current revealed after Cs\(^+\)-loading, was mediated via L-type Ca\(^{2+}\) channels. Indeed, in the presence of nifedipine, a selective L-type Ca\(^{2+}\) channel blocker (20 \(\mu\)M; \(n = 5\)) (Bean, 1989), the TPM-induced tail was blocked, suggesting that Ca\(^{2+}\) influx through L-channels was necessary for its generation (Fig. 13 B; see also Discussion). Nifedipine also abolished the “plateau” region that was characteristically present in the TPM \(I-V\) plot between ~-55 and -35 mV in Cs\(^+\)-loaded cells, and reduced
the TPM-induced extra outward rectification at more positive potentials, causing an unusual crossover of the I-V plots at around -35 mV (Fig. 13 B) (mean crossover potential = -30.6 ± 0.9 mV; n = 5); this suggested a possible interplay between enhanced inward and residual outward rectifying current components in Cs⁺-loaded cells in TPM (see Discussion).

It may be noted that in 17/25 Cs⁺-loaded cells, TPM (20 μM) still evoked a slow outward shift in holding current, although this was significantly smaller (mean = 0.17 ± 0.05 nA; P<0.05) than that recorded in K acetate recorded-cells (0.36 ± 0.26 nA, n = 32); this degree of reduction (~53 %) is comparable to the one obtained with extracellular Ba²⁺ (~47 %) suggesting that these cations could both be blocking the same fraction of TPM outward current. Interestingly, in 3 neurones tested, Cs⁺-loading abolished outward current responses induced by 20 μM baclofen under voltage clamp, despite an incomplete blockade of the outward current produced by TPM in the same neurone (mean = 0.18 ± 0.07 nA); this suggested an effective equilibration with Cs⁺ was occurring under our conditions. In 8/25 Cs⁺-loaded cells, no TPM outward current was generated; (compare with 2/34 occasions where TPM failed to produce slow outward current in normally-recorded cells under voltage clamp). This provides further evidence that the slow TPM-induced current was predominantly carried by K⁺.

II.1.4.4 Is PKA-phosphorylation involved in the modulatory effect of TPM on the sAHP?

According to the “unifying” hypothesis suggested by Shank et al., (2000), TPM prefers to interact with various target ion channels/drug receptors in their de-phosphorylated state; we therefore wished to investigate whether PKA-mediated phosphorylation would affect the modulatory action of TPM on the sAHP. Such a pre-phosphorylation of L-type Ca²⁺ channels and/or the slow Ca²⁺-activated K⁺ channels should occlude the action of TPM. In the representative experiment of Fig. 14, a control sAHP and corresponding outward sI_AHP tail current under “hybrid” voltage clamp were initially recorded in control solution (Fig. 14 A, B). After addition of forskolin (20 μM; 15 min), a direct activator of adenylate cyclase and therefore PKA (Simonds, 1999), the sAHP and sI_AHP were abolished and replaced by a small slow afterdepolarization (sADP) (mean = 4.8 ± 0.96 mV) and small I_AD P tail (95 ± 34 pA; n = 4), most likely due to a rise in
intracellular cAMP and direct inhibitory action on the Ca\(^{2+}\)-activated K\(^+\) current as previously found in hippocampal neurones (Knöpfel et al., 1990; Nicoll, 1988). Forskolin also significantly reduced spike accommodation, as was seen from the greater number of spikes fired during the depolarizing stimulus (means: 38 ± 9 spikes, control; 60 ± 4 spikes in forskolin; \(P<0.05, n = 4\)) (Fig. 14 A). On further addition of TPM, this prior effect of forskolin did not prevent the reappearance of the characteristically prolonged sAHP and corresponding \(sI_{AHP}\) tail (mean = 580 ± 140 pA amplitude, 9.72 ± 4.34 s duration), suggesting that PKA-dependent phosphorylation mechanisms were not occluding the action of TPM on L-channels, or possibly also on the slow Ca\(^{2+}\)-activated K\(^+\) channels (most likely non-SK-BK channels) themselves (Sah & Faber, 2002). Spike firing was reduced in forskolin + TPM (mean = 44 ± 10 spikes), but mean values were not significantly different from forskolin alone (\(P>0.5, n = 4\)). Similar end results were obtained in 4 other neurones, where the converse experiment was carried out (Fig. 15): i.e. TPM was applied first to induce sAHP enhancement, followed by forskolin added in combination. A further prolongation of the sAHP and \(sI_{AHP}\) tail (570 ± 121 pA amplitude, 9.27 ± 3.94 s duration) then appeared, most probably due to forskolin’s own recognised action in enhancing the activity of the underlying L-channels (Dolphin, 1999; Anwyl, 1991), which was supplementing (or possibly synergising with) that of TPM’s. Interestingly, forskolin did not significantly affect spike accommodation in the presence of TPM (mean number of spikes = 28 ± 4 in TPM vs 25 ± 3 in TPM + forskolin, \(P>0.5\); however, both means were significantly different from control: 46 ± 5, \(P<0.05; n = 4\)). Either way, it is clear that TPM can apparently override the inhibitory effect of forskolin on the sAHP and spike accommodation caused by intracellular PKA-dependent phosphorylation.

11.4.5 Carbonic anhydrase (CA) inhibitors mimic the effects of TPM

TPM is known to have a CA inhibitory action, although its potency in this respect has been controversial. In earlier studies, the \(K_i\) value for TPM vs the physiologically important isozyme CA II was quoted to be in the micromolar range, and at least an order of magnitude less than that of the standard membrane-permeant CA inhibitor acetazolamide (ACTZ) (Dodgson et al., 2000). More recently, Casini et al., (2003) reported that TPM was actually quite a potent CA II inhibitor, with a nanomolar affinity similar to that of ACTZ. Acetazolamide is well known for its antiepileptic
properties (Millichap et al., 1955), and is still used today as adjunctive therapy for managing partial refractory seizures (Brodie, 2001) and absences (Panayiotopoulos, 2001); its precise mechanism of anticonvulsant action however, has not been fully established, although it is generally thought to involve CA inhibition, thereby promoting changes in intracellular and extracellular pH and then modulating neuronal excitability indirectly (Resor et al., 1995; see also Discussion). We wished to test whether the observed membrane effects of TPM on olfactory neurones could be partly (or wholly) explained by such changes in pHo (and/or pHj) caused as a consequence of CA inhibition, and to what extent these effects could be mimicked by ACTZ (or other CA inhibitors). We found that ACTZ (20 μM, 10 min; n = 8) elicited a slow, reversible membrane hyperpolarization (mean = 9 ± 2 mV, significantly different from rest level; P<0.001) and a decrease in input resistance (mean = 24 ± 8 %; P<0.001) similar to those elicited by 20 μM TPM (cf. also Herrero et al., 2002). Under voltage-clamp, ACTZ evoked a slow outward current shift (mean = 0.33 ± 0.17 nA; n = 8) with similar amplitude and onset to the TPM-evoked current (cf. Fig. 6 A) but with slower offset on washout. A comparison of the membrane effects of TPM and ACTZ in a single neurone at -70 mV holding potential is shown in Fig. 16. TPM (20 μM) and ACTZ (20 μM) (10 min applications) produced similar amplitude slow outward currents (Fig. 16 A), and when TPM was superimposed at the peak of the ACTZ current, the effect of TPM was occluded, in accordance with the current clamp data of Herrero et al., (2002) on CA1 cells. The converse also applied; in Fig. 16 B, an initial application of ACTZ (20μM, 10 min) produced an outward current response, followed by a TPM response. In TPM, the effect of ACTZ was also clearly occluded; this confirms that both TPM and ACTZ were sharing a common mechanism of action in this respect, although whether this involved CA inhibition was still unclear.

The possible role of CA inhibition in explaining the enhancing effects of TPM on the sAHP was addressed in a second series of tests as shown in Fig. 16 C, D. Here, a control spike burst, sAHP (Fig. 16 C) and corresponding sI_{AHP} tail current under hybrid voltage clamp (Fig. 16 D) were initially recorded in control. Like TPM, 20 μM ACTZ also significantly enhanced (~25 %) (281 ± 122 pA control vs 351 ± 117 pA in ACTZ; P<0.001) and prolonged the duration (~77 %) (3.56 ± 0.83 s vs 6.30 ± 0.9 s in ACTZ P<0.001) (n = 8) of the sI_{AHP} (cf. Fig. 7); however, in contrast with the occlusive slow outward current responses, on co-application with ACTZ, TPM (20 μM) still produced
a further clear enhancement and prolongation of the sAHP and $s_{AHP}$ tail current (351 ± 117 pA amplitude, 6.30 ± 0.9 s duration in ACTZ vs 523 ± 124 pA, 8.24 ± 1.7 s in ACTZ + TPM; t-tests: $P<0.001$), suggesting an additive or possible synergistic effect of these two agents in evoking this response, which could only really be explained if their effects were independent of any CA inhibitory activity.

An important question that remained was whether the abilities of TPM and ACTZ to evoke a slow outward current and enhance/prolong the sAHP (along with their presumed actions on CA, and membrane consequences thereof) were mediated intracellularly or extracellularly (or both). To address this point, we tested the effects of the hydrophilic CA inhibitor benzolamide (CL 11366; BZ) which is generally considered to be poorly membrane permeant (Leniger et al., 2002; Maren, 1977). Surprisingly, BZ mimicked all of the observed effects of TPM (and ACTZ), although with lower potency. Thus, BZ (50 μM, 10 min) induced a slow membrane hyperpolarization (mean = 7 ± 2 mV; $n = 7$) and a decrease in input resistance (mean = 18 ± 8 %; $n = 7$) comparable to the responses produced by TPM or ACTZ (Fig. 17 A). Under voltage clamp, BZ evoked a slow outward current shift (mean = 0.24 ± 0.03 nA; $n = 7$) with similar amplitude and onset/offset to the TPM or ACTZ-evoked currents; also, when TPM (20 μM, $n = 3$) or ACTZ (20 μM, $n = 3$) were applied in the presence of BZ, their usual outward current-inducing effects were occluded (Fig. 17 A). Benzolamide also had a small enhancing/prolonging effect on the sAHP and underlying $s_{AHP}$ tail current (~16 % enhancement) (control mean = 278 ± 129 pA vs 324 ± 131 pA in BZ; $P<0.001$), (~72 % prolongation) (3.59 ± 0.89 s control vs 6.16 ± 0.86 s in BZ; $P<0.001$; paired t-tests; $n = 7$), and moreover, a further co-application of TPM produced its typical additive effect on the outward current tail (324 ± 131 pA amplitude, 6.16 ± 0.86 s duration in BZ vs 562 ± 117 pA, 7.93 ± 1.4 s in BZ + TPM; t-tests: $P<0.001$) (Fig. 17 B, C). These important and novel results with BZ strongly suggest that all of our observed TPM (and ACTZ)-induced effects could be mediated extracellularly via an interaction with the outer part of the neuronal cell membrane; however, we were unsure whether this mechanism still involve CA inhibition and extracellular/intracellular pH changes.
K⁺ conductance mechanism directly (Church, 1992). Despite this suppression, application of 20 μM TPM in HEPES medium still produced a significant enhancement (~83 %) (390 ± 54 pA vs HEPES control; $P<0.002$) and increase in duration (~78 %) (3.42 ± 0.63 s vs 1.91 ± 0.47 s HEPES control; $P<0.002$) of the $sI_{AHP}$, suggesting that this second novel membrane effect of TPM was also not mediated by CA inhibition.
II.1.4.6 Experiments conducted in a HEPES-buffered (bicarbonate-free) bathing medium

To further test whether the common TPM and ACTZ effects were CA-mediated and therefore due to a change in pHo and/or pHj, we carried out experiments (n = 10 cells) in which normal bicarbonate-buffered Krebs bathing medium (bubbled with O2/CO2), was substituted by a solution containing HEPES (at the same pHo) instead of NaHCO3 and bubbled with pure O2 (Church, 1992). Under these conditions, the activity of CA would be blocked, due to absence of its natural substrates. Neurones were first impaled in the normal Krebs medium and control slow outward current responses to TPM or ACTZ recorded under voltage clamp (depending on the drug to be subsequently applied); a switch was then made to HCO3-/CO2-free HEPES-buffered solution and a second drug response obtained after 30 min incubation. On first applying HEPES medium, there was an initial depolarization (mean = 2.4 ± 0.9 mV) within 10 min accompanied by a stable decrease in the input resistance (mean = 8.1 ± 2.3 %), followed by a small hyperpolarization (mean = 3.2 ± 0.6 mV) within the next 10-15 min, which was maintained after 30 min (Fig. 18A) (cf. Church, 1992). In HEPES medium under voltage-clamp at -70 mV, TPM (n = 6) or ACTZ (n = 4) still elicited slow outward currents of comparable time course and amplitude to those measured in control Krebs solution (Fig. 18 B, C) (mean TPM current = 0.38 ± 0.04 nA control vs. 0.40 ± 0.04 nA in HEPES; mean ACTZ current = 0.35 ± 0.03 nA control vs. 0.34 ± 0.03 nA in HEPES). Both means in HEPES were not significantly different from control values; t-tests, P>0.5). This confirms that the common outward currents generated by the two drugs (and most likely also by BZ) are not mediated by CA inhibition, but they probably share a common site of action on the outside of the cell membrane. However, we cannot exclude the possibility that under normal conditions, the CA-inhibitory properties of these compounds (and consequent changes in neuronal pHj/pHo) might also somehow contribute to their observed anticonvulsant activity (see Leniger et al., 2004a). Finally, we also examined whether switching to a HEPES-buffered medium would affect the ability of TPM to modulate the sI\textsubscript{AHP}. In 6 neurones studied under hybrid clamp, the sI\textsubscript{AHP} tail was significantly reduced in amplitude after 30 min exposure to HEPES solution (~48 %) (413 ± 48 pA control vs 213 ± 42 pA in HEPES; P<0.005). A similar effect was reported on the sAHP in hippocampal neurones and is likely to be due to an acidic shift in cytoplasmic pH, affecting the Ca\textsuperscript{2+}-activated...
II.1.5. Discussion

Our study has found that TPM, when applied at therapeutically effective concentrations (10-50 μM) onto rat olfactory cortical neurones, produces two main effects: 1) a slow, dose-dependent and reversible membrane hyperpolarization, accompanied by a decrease in membrane resistance and inhibition of repetitive action potential firing and 2) an enhancement and prolongation of a post-stimulus slow afterhyperpolarization (sAHP), that follows a burst of action potentials. The latter phenomenon has not previously been reported for TPM on other neurones (Herrero et al., 2002; DeLorenzo et al., 2000; Jahromi et al., 2000; Hanaya et al., 1998; Kawasaki et al., 1998) so may well be specific to cortical cells while the former, which has been already reported for other neurone types (Kawasaki et al., 1998; Herrero et al., 2002), presented different properties in our model. Interestingly, the mechanisms responsible for the slow hyperpolarization and sAHP enhancement/prolongation effect appear to be distinct, since in a minority of cells where the sAHP was not enhanced by TPM, the slow hyperpolarization response was still generated; this hypothesis is also supported by the fact that Cd²⁺ was able to completely block the sAHP-enhancement effect, but it did not affect the TPM-induced hyperpolarization.

II.1.5.1 TPM-induces a membrane hyperpolarization and slow outward (clamp) current

Under voltage clamp, the TPM-induced hyperpolarization was manifested as a slowly-developing outward current and increase in membrane conductance, most likely carried largely (but not exclusively) by K⁺ ions, that reversed slowly on drug washout. Similar hyperpolarizing effects of TPM were recorded by Kawasaki et al. (1998) in subicular neurones and also by Herrero et al. (2002) in hippocampal CA1 neurones, using higher concentrations of TPM (200 μM and 100 μM, respectively) than those used here. In the former case, the TPM hyperpolarization was abolished by bicuculline, therefore was attributed to an indirect activation of GABA_A receptors (White et al., 2000), while in the latter, the hyperpolarizing response reversed near the K⁺ equilibrium potential (E_K) and was abolished by the general K⁺ channel blocker Ba²⁺ (Eaton & Brodwick, 1980) suggesting an exclusive increase in a K⁺ conductance. In our experiments, the slow TPM outward current was unaffected by bicuculline, therefore was not GABA_A-mediated; also, the TPM current, although reduced (suggesting it was mainly carried by
K⁺) was incompletely blocked by 1 mM Ba²⁺, and (unlike the baclofen-induced current) did not reverse at negative membrane potentials, indicating that other membrane mechanisms or ionic conductances (yet to be identified) may be involved in its action on these neurones. Alternatively, the apparent Ba²⁺-insensitivity of the postulated K⁺ current activated by TPM could be a feature of the K⁺ channel itself rather than an indication that another conductance mechanism is involved. Indeed, some neuronal K⁺ channels show only partial sensitivity (Krapivinsky et al., 1998) or complete insensitivity (Wagner & Dekin, 1993) to blockade by high concentrations of this cation.

Neuronal hyperpolarizations that do not reverse at E_K, can be produced by a complex combination of ionic conductance changes coupled to possible changes in hyperpolarizing electrogenic pump activity (see Trotier & Doving, 1996; estimated reversal potential of Na⁺ pump current = -143 mV); such hyperpolarizations may or may not be associated with a small increase in membrane conductance (Marinelli et al., 2000; Parker et al., 1996; Mimura et al., 1994; Thompson & Prince, 1986). In our case, involvement of the Na⁺-K⁺-ATPase pump was unlikely, since ouabain was ineffective against the TPM current (c.f. Gustafsson & Wigström, 1983). Other electrogenic pump mechanisms stimulated by TPM with reversal levels outside the normal physiologic range could conceivably generate an outward current e.g. a K⁺-dependent Na⁺-Ca²⁺ exchanger (NCKX2) operating in reverse mode (Dong et al., 2001), however such a process would lead to a rise in cytosolic Ca²⁺, therefore might be neurotoxic. More recently, Leniger et al. (2004a) proposed that TPM activates a Na⁺-independent Cl⁻/HCO₃⁻ exchanger in rat hippocampal CA3 neurones; however, since they did not describe any effect of TPM on the resting membrane potential, it seems unlikely that this transporter (if present) is involved in TPM’s hyperpolarizing action in cortical cells. Another possibility is that the anomalous behaviour of the TPM current was due to activation of a novel K⁺ conductance that is de-activated at very negative potentials; the result illustrated in Figs. 6 A, B would certainly support such a hypothesis. Alternatively, lack of reversal could be due to a simultaneous increase in K⁺ conductance and a decrease in a persistent cationic conductance (TTX-resistant) with a more positive reversal level, or may reflect the fact that it is generated at a remote site(s) from the somatic recording electrode, with a contribution of electrotonic dendritic coupling as appears to happen in locus coeruleus neurones following opioid peptide application (Ishimatsu & Williams, 1996; Travagli et al., 1995; Alreja & Aghajanian,
1993). In the latter case, a limit in voltage clamp recording could explain the lack of
reversal but it would still indicate that this peculiar K\(^+\) channel is not fully blocked by
the general K\(^+\) channel blocker Ba\(^{2+}\). Further experiments would be required to confirm
such complex mechanisms in our cells.

The fact that Cs\(^+\)-loading only partially reduced the TPM slow outward current could be
interpreted in several ways; one explanation is that Cs\(^+\) blocked the proposed K\(^+\)
component of the TPM current, leaving the Ba\(^{2+}\)-resistant (cationic) component, a
hypothesis supported by the similarity in the degree of inhibition of the TPM-induced
slow outward current. Alternatively, a component of the TPM current may have flowed
through Cs\(^+\)-permeable K\(^+\) channels (c.f. Wigmore & Lacey, 2000). Unfortunately, our
present data do not allow us to distinguish between these two possibilities.

Hyperpolarization and reduction in neuronal input resistance by activating a K\(^+\)
conductance is a rather novel mechanism of anticonvulsant action that is not shared by
other conventional AEDs; such a mechanism would reduce neuronal excitability and
therefore inhibit seizure generation and spread in a highly effective manner. Future,
development of new drugs that activate “background” K\(^+\) channels regulating neuronal
cell excitability and resting potential could therefore be therapeutically useful as novel
anticonvulsants (Wickenden, 2002). Presently, only retigabine, a direct opener of
KCNQ K\(^+\) channels, that modulates the activity of the M-type K\(^+\) current (\(I_M\)), is known
to exert its anticonvulsant action via this manner (Main et al., 2000). Similar activation
of M-channels by TPM in our experiments would certainly explain the Ba\(^{2+}\)-sensitive
component of the slow outward current response, and thus cannot presently be
excluded. However, the possibility that the TPM current flows through Ca\(^{2+}\)-activated
K\(^+\) channels (cf. Tricarico et al., 2000) is unlikely, since the response was unaffected by
the general Ca\(^{2+}\) channel blocker Cd\(^{2+}\). The exact mechanism of the TPM-induced
hyperpolarizing response in cortical neurones therefore, still remains to be determined
(see also below).

II.1.5.2 TPM enhances and prolongs the sAHP by modulating an L-type Ca\(^{2+}\) current

The novel enhancement and dramatic prolongation of the sAHP by TPM was found to
be dose-dependent, reversible, and (unlike the slow hyperpolarizing response) reliant on
Ca\(^{2+}\) entry, since it was blocked by Cd\(^{2+}\). Two possibilities could account for this
phenomenon: an increase in Ca\(^{2+}\) entry and/or a direct enhancement in activity of the underlying Ca\(^{2+}\)-activated K\(^{+}\) channels themselves. Intracellular loading with the Ca\(^{2+}\) chelators EGTA or BAPTA to eliminate the Ca\(^{2+}\)-dependent K\(^{+}\) conductance, and therefore confirm the role of Ca\(^{2+}\) in generating the extra TPM-induced outward current relaxation under voltage clamp (Fig. 10) was not attempted in the present study since previous data obtained in our laboratory (Constanti et al., 1993) and those of others (Jahromi et al., 1999; Velumian & Carlen 1999; Zhang et al., 1995; Schwindt et al., 1992) has shown that “sharp” or patch-clamp intracellular recording with EGTA or BAPTA-filled electrodes in cortical or hippocampal neurones can lead to a paradoxical potentiation and prolongation (rather that elimination) of the sAHP; this unusual effect appears to be due to a prolongation of the intracellular Ca\(^{2+}\) signal following stimulus-induced Ca\(^{2+}\) entry into the cell, and relies on an interplay between the intracellular Ca\(^{2+}\) rise, normal Ca\(^{2+}\) extrusion/sequestration and the Ca\(^{2+}\) buffering capacity/binding kinetics of these mobile buffers. Alternative application of the cell membrane-permeable buffer analogue BAPTA-AM also failed to reliably influence the sAHP in our neurones, as previously reported (Constanti et al., 1993). Clearly, such properties of these chelators would complicate rather than clarify any intrinsic effects of TPM on sAHP generation. Likewise, in view of the findings of Lancaster & Batchelor (2000) who found that intracellular loading with BAPTA in whole-cell patch recordings, could itself induce a slow outward K\(^{+}\) current in rat hippocampal CA1 neurones by directly activating the same Ca\(^{2+}\)-activated K\(^{+}\) channels that underlie the sAHP, we also consider it unlikely that such chelator loading experiments would help in resolving the issue of possible Ca\(^{2+}\) mobilization by TPM in generating the slow (hyperpolarizing) outward current shift (see Results above).

Although Cs\(^{+}\) loading alone failed to completely suppress outward current relaxations evoked by positive voltage jumps (indicating the presence of Cs\(^{+}\)-permeable K\(^{+}\) channels; c.f. Wigmore & Lacey, 2000), the experiments did reveal an underlying long post-stimulus inward tail current, that was enhanced and prolonged by TPM. Since this induced tail current was blocked by the specific L-channel antagonist nifedipine (Bean, 1989) it seems likely that an L-type Ca\(^{2+}\) current (activated during the depolarizing stimulus) was the main source of Ca\(^{2+}\) entry and therefore the principal target of action of TPM in our neurones. The observed enhancement of the sAHP by TPM might then be largely, if not entirely explained by the enhancement of an underlying L-type Ca\(^{2+}\)
current, although a direct enhancing effect on the Ca\(^{2+}\)-activated K\(^+\) channels cannot be entirely excluded. An analogous effect has been reported in neostriatal neurones following application of the L-channel agonist Bay K 8644 (Hernandez-Lopez et al., 1996). This result contrasts with the recent findings of Zhang et al., (2000) who observed a block of L-currents by TPM (at 10 \(\mu\)M, but not at 50 \(\mu\)M) in rat dentate granule cells. Their data strongly suggest there may be a two-fold effect of TPM on L-currents, with depression being apparent at low doses, and perhaps a combination of depression and enhancement at higher doses. Interestingly, they also found that non-L-type currents in these cells were transiently increased by TPM at high concentrations (50 \(\mu\)M). In our experiments, only enhancement of the sAHP was ever observed by 20 or 50 \(\mu\)M TPM (see above) and the slow inward current tail revealed after Cs\(^+\)-loading was consistently enhanced by 20 \(\mu\)M TPM.

Regarding the phosphorylation hypothesis of Shank et al. (2000), we found that enhancement and prolongation of the sAHP, and underlying nifedipine-sensitive slow inward tail current by TPM did not appear to involve an interaction of this drug at PKA-dependent phosphorylation sites, since it still occurred in the presence of the direct adenylate cyclase activator forskolin (Simonds, 1999) which itself depressed the sAHP; however, an interaction of TPM at other potential phosphorylation sites on the L-channel (and possibly also the Ca\(^{2+}\)-activated K\(^+\) channel itself) cannot be discounted. The fact that TPM could apparently override the inhibitory effect of a rise in intracellular cAMP produced by forskolin on the sAHP was very interesting, and could well be relevant for its antiepileptic action, since it could overcome the effect of many neurotransmitters (e.g. noradrenaline, 5HT, histamine) that would normally increase neuronal cell excitability by raising intracellular cAMP via a PKA-dependent pathway (Pedarzani & Storm, 1993). To our knowledge, TPM itself is not an effective inhibitor of PKA at therapeutic levels (1-100 \(\mu\)M; R.P. Shank, Johnson & Johnson Pharmaceutical Research and Development, L.L.C., PA, USA; personal communication) therefore, its sAHP-enhancing effect (and apparent reversal of forskolin action) was unlikely to involve a change in basal phosphorylation-dephosphorylation turnover (Pedarzani et al., 1998) and must be taking place via a PKA-independent mechanism. If phosphorylation by forskolin enhances underlying L-currents (Dolphin, 1999; Anwyl, 1991) and also inhibits the sAHP directly, why does the latter effect predominate when forskolin alone is applied (Fig. 14)? Perhaps it is a
question of balance between two opposing physiological effects (*i.e.* functional antagonism). Normally, the sAHP inhibitory action of forskolin may be the stronger effect (with some "spare" capacity). In TPM, Ca\(^{2+}\) entry via \(L\)-channels may be so enhanced that it overrides the inhibitory effect, so only sAHP enhancement is seen.

### II.1.5.3 Are the effects of TPM mediated by carbonic anhydrase (CA) inhibition?

Several reports have appeared showing that CA inhibition (presumably of both intracellular and interstitial isoforms of the enzyme (*c.f.* Tong *et al.*, 2000; Pasternack *et al.*, 1993) by the "classical" CA inhibitor ACTZ can induce external neuronal acidification (Meierkord *et al.*, 2000; Vorstrup *et al.*, 1989), most likely due to CO\(_2\) accumulation and interference with HCO\(_3^-\) availability, therefore decreasing the efficiency of the CO\(_2/\)HCO\(_3^-\) buffering system to handle extracellular metabolic acid shifts (due to H\(^+\) extrusion and/or lactic acid generation). Extracellular acidification is also likely to be mirrored by a comparable reduction in \(\text{pH}_i\) via an unknown mechanism (Church *et al.*, 1998). If TPM and ACTZ were producing a similar extracellular/intracellular acidification via CA inhibition, then could this explain their observed generation of a slow hyperpolarization and sAHP enhancement in our neurones? Changes in \(\text{pH}_o/\text{pH}_i\) are known to affect intrinsic neuronal membrane properties, including outward and inward-rectifying K\(^+\) currents (Zhu *et al.*, 1999; Tombaugh & Somjen, 1996), Ca\(^{2+}\) currents (Tombaugh & Somjen, 1997), and other Ca\(^{2+}\)-dependent conductances (Church *et al.*, 1998; Church, 1999). It is now clear that lowering \(\text{pH}_o\) is anticonvulsant, causing neuronal depolarization and a decrease in input resistance while *depressing* burst firing, sAHP amplitude and HVA Ca\(^{2+}\) currents; the opposite applies for an increase in \(\text{pH}_o\) (Church, 1992, 1999; Church & McLennan, 1989).

Herrero *et al.* (2002) showed that ACTZ elicited a slow hyperpolarizing effect similar to that of TPM on hippocampal cells, and it occluded the TPM-evoked hyperpolarization. We obtained similar results with ACTZ and TPM under voltage clamp in our cells, and further showed that benzolamide (BZ) was also capable of evoking an outward current shift and occlusion of the effect of TPM. Herrero *et al.* (2002) proposed that both TPM and ACTZ activate a common K\(^+\) conductance by virtue of their effects on interstitial (and intracellular?) CA activity. A fast inward rectifier (Kir2.3) channel or background
“leak” TASK-like K⁺ channel (Lesage, 2003) were suggested as likely candidates, both channels being sensitive to changes in pH₀ at near physiological levels; both channels however, are blocked rather than opened by external acidification (Bayliss et al., 2001; Zhu et al., 1999) thus, an alternative channel type(s) must be involved. Our experiments confirmed that similar slow outward currents could be produced by TPM and two other CA inhibitors possessing the sulphonamide moiety, therefore they were all presumably acting via a common mechanism that could possibly have involved alterations in intracellular and/or extracellular pH consequent to CA inhibition (see below). However, since the outward currents induced by TPM or ACTZ were unaffected in a bicarbonate/CO₂-free HEPES-buffered bathing medium, the mechanism of generation could not have involved CA inhibition, as implied by the data of Herrero et al. (2002). Since TPM, ACTZ and BZ all produced similar outward currents, it is possible that all these agents were acting extracellularly to produce these responses, via a common external binding site, unrelated to surface CA activity (Tong et al., 2000); the identity of this binding site however, is currently unknown. It is well known that sulphonamides can interact with many different types of cellular target site to produce a variety of pharmacological effects (Casini et al., 2002). Alternatively, TPM and ACTZ (both membrane permeable) and BZ (originally presumed membrane impermeable) may be interacting with a common intracellular binding sites/receptor to induce outward currents via a CA-independent mechanism. In support of this, Supuran & Scozzafava (2004) have recently shown that BZ is in fact, as permeable as ACTZ through biological membranes.

According to Leniger et al. (2002), ACTZ can also produce an acidic shift in intracellular neuronal pH (although see Munsch & Pape, 1999). By inference from the work of Church (1992, 1999), this would be expected to depress HVA Ca²⁺ conductances, and therefore any Ca²⁺-mediated potentials, which is opposite to the observed enhancing effects on L-type Ca²⁺ tail current and sAHP produced by TPM and ACTZ in our cells. Thus, if these agents were both producing a similar fall in pHᵢ/pH₀ via CA inhibition, then this could not explain their common action in enhancing the sAHP. Moreover, the latter response to TPM and ACTZ was still observed in a HEPES-buffered medium (despite depression of the control sAHP; Church, 1992), further ruling out the involvement of CA. It is worth noting that ACTZ and BZ occluded the effects of TPM on the outward current response but were additive in producing the
enhancement/prolongation effect on the sAHP and $s_{AHP}$ tail current; this could be taken as evidence for different underlying response mechanisms with different pharmacological sensitivities to these compounds. Although Leniger et al., (2002) found no change in neuronal pH, following BZ application (thus supporting the original idea that it is membrane impermeant), as with the outward current response, we are unable to conclude whether the sAHP modulatory effect of TPM and the CA inhibitors is indeed mediated extracellularly or intracellularly (or both).

II.1.5.4 Conclusions and general implications for anticonvulsive therapy

In conclusion, our present study suggests that TPM and possibly other structurally related sulphonamide compounds (e.g. zonisamide; Fig. 19) may share a common anticonvulsant mechanism of action by inducing neuronal outward currents and enhancing the activity of cortical L-type $\text{Ca}^{2+}$ channels, therefore indirectly enhancing the sAHP. Whether other L-type channels e.g. on cardiac, or smooth muscle are also affected by TPM remains to be tested; however, the different subunit properties of L-channels at these different sites could explain its apparently selective effects towards neuronal L-currents. Cardiac, smooth muscle and brain L-channels are known to contain alpha 1C or alpha 1D-type pore-forming subunits (Bell et al., 2001), but variation in splice forms and their association with different beta subunits on neurones could enable them to show distinct functional and pharmacological properties (Catterall, 1998). TPM was tested within the range 1-50 μM (most reversible effects were observed at 20 μM), which is within the therapeutically effective range of free serum levels of TPM observed clinically (~7-100 μM; Wolf et al., 2000). On the basis of our data, we propose, that the sAHP enhancement effect may predominate at low TPM plasma levels, together with the slow hyperpolarization/ conductance increase effect, to give the main anticonvulsant action; whether these effects override or act in combination with other proposed effects of TPM on $\text{Na}^+$ channels, GABA$_A$ receptors and AMPA/kainate glutamate receptors (Ångehagen et al., 2003b, 2004) may be difficult to assess. Furthermore, these two new mechanisms described might open a new field of investigation in the discovery of novel anticonvulsant drugs. Whether these actions of TPM do play a role upon its anticonvulsant activity remains an open question. It is possible to speculate that both mechanisms might stabilize neurones and therefore this might prevent the generation and the spread of seizures throughout the brain.
Interestingly, the relevance of the observed \textit{in vitro} effects of TPM for modulating human motor cortical excitability was recently addressed by Reis \textit{et al.} (2002), who suggested that ion channel blocking mechanisms may not be so important. Despite the ongoing uncertainties over the contributory mechanisms of action of TPM, the importance of L-Ca$^{2+}$ channels in epileptiform bursting is already well established (Straub \textit{et al.}, 2000), therefore a focus on how TPM modulates cortical L-channel activity, and also how dihydropyridine L-channel blockers, already known to possess anticonvulsant activity (De Sarro \textit{et al.}, 1992a, b) might possibly interfere with the anticonvulsive actions of TPM \textit{in vivo} should prove highly informative. The experiments described in the next Section (Chapter II: \textit{in vivo} Results), carried out in the laboratory of Prof. G. De Sarro at the University of Catanzaro, Italy, were specifically designed to address this latter point.
Figure 1: Location of the piriform cortex

The location of the piriform cortex (shaded area) of the rat, as seen from the underside of the brain (modified from Olds, 1979).
Overview of the anatomical structure of the piriform cortex. Left: a segment of piriform cortex shows the LOT entering along the surface and a pyramidal cell with its apical dendrite extending up through layer I. Right: the expanded diagram shows how afferent fibres from the LOT synapse on pyramidal cell distal dendrites in the superficial layer, layer Ia, whereas excitatory intrinsic fibers arising from other pyramidal cells within the cortex terminate on proximal dendrites in layer Ib. Pyramidal cell bodies are tightly packed in layer II (Adapted from Johnson et al., 2000).
Axon from a single pyramidal cell in layer II of rat piriform cortex. Note that axon branches extend over nearly the entire length of the cerebral hemisphere and are widely distributed within the piriform cortex and other olfactory and non-olfactory areas. Spatial distribution of axon branches in surface view. The inset at top right shows the illustrated portion of the rat brain (dashed rectangle) and orientation (45° upward rotation); the hatched area is piriform cortex, and the shaded area is lateral olfactory tract. APC, Anterior piriform cortex; PPC, Posterior piriform cortex; Ant, Anterior; ctx, cortex; nuc, nucleus; olfac, olfactory (Adapted from Johnson et al., 2000).
Simplified diagram showing the principal excitatory and inhibitory synaptic connections affecting both superficial and deep pyramidal neurones of the mammalian piriform cortex (PC). Compartmentalisation of the representative (superficial) pyramidal cell displays the location of the respective parts of the cell within the laminar structure of the PC, and the locations of the synaptic connections it receives. Legend: Red triangles are excitatory synapses, blue triangles are inhibitory synapses. la – layer la, supIb – superficial layer Ib; deepIb – deep layer Ib; II – layer II; III – layer III; FF – feed-forward inhibition; FB – feed-back inhibition. Arrowed lines indicate direction of synaptic transmission. Adapted from http://www.bbb.caltech.edu/GENESIS/illtuts/piriform.html (Wilson & Bower, 1989).
Figure 5: Effects of TPM on the membrane properties of an olfactory cortical neurone.

(A) Slow membrane hyperpolarization accompanied by a decrease in membrane input resistance induced by bath-application of TPM (20 μM; 40 min) from a holding membrane potential of -70 mV (maintained by steady current injection). Negative current pulses (-0.5 nA) were injected every 2s throughout. (B) Superimposed electrotonic potentials evoked by injecting brief (160 ms; -0.25 nA) hyperpolarizing current pulses. TPM induced a clear and reversible decrease in input resistance. (C) Electrotonic response to injection of a depolarizing current pulse (160 ms; +0.75 nA). TPM decreased the number of action potentials elicited during the pulse, indicating a reduction in cell excitability; (corrected for change in membrane potential produced by TPM, by positive current injection).
(A) TPM-induced slow outward currents recorded in a single neurone voltage clamped at various holding potentials from -70 to -110 mV. In each case, TPM (20 μM) was applied for 10 min, followed by a 30 min washout period; note progressive reduction in current amplitude at more negative potentials, but lack of reversal to a slow inward current, even at -110 mV. (B) Corresponding plot of slow current amplitude against holding potential (points represent means ± s.e. mean, n = 6; currents were measured from peak to baseline level). (C) Different neurone clamped at -70 mV; baclofen (20 μM; 3 min) applied initially in control Krebs solution, produced a steady outward current with a relatively rapid rate of onset/offset. Addition of TPM (20 μM; 40 min) induced a similar amplitude outward current, but did not occlude the response to baclofen when applied in combination (note faster chart speed during baclofen responses).
Figure 7: Current/Voltage (I/V) relationship for TPM and Baclofen

Steady-state current-voltage (I-V) relationships obtained under voltage clamp (in TTX) showing the peak amplitude of membrane currents evoked by 1.5 s depolarizing or hyperpolarizing voltage steps from a holding potential of -70 mV (ordinates) vs membrane potential (abscissae). Black symbols: currents recorded in control solution; Red symbols: currents measured during drug application (10 min). (A) I-V plot in the presence and absence of 20 μM TPM shows characteristic upward shift with increase in slope (increased conductance) compared with control, at potentials between -80 and -20 mV, but no crossover at negative potentials. (B) (different cell) I-V plot in the presence and absence of 20 μM baclofen also shows increase in slope, but intersects the control curve at ~-85 mV (estimated $E_{rev}$ for the baclofen-induced current) as might be expected from principal activation of a K$^+$-selective (GIRK) conductance mechanism.
Pharmacology of TPM-induced slow outward current recorded under voltage clamp; (A-C, different neurones clamped at -70 mV). (A) Application of TPM (20 μM; 10 min) in the presence of 1 mM Ba\(^{2+}\) (20 min), produced only a partial block of the TPM current (peak amplitude reduced by ~48%). In contrast, 200 μM Cd\(^{2+}\) (B) or 10 μM bicuculline (C) (10 min pre-applications) did not affect the TPM responses (note different chart speeds in each experiment).
Figure 9: TPM enhances and prolongs the post-stimulus sAHP and \( s_{AHP} \) tail current.

(A) Response to injection of a long (1.5s; +2.0 nA) depolarizing current stimulus, which in control solution evokes a burst of action potentials, followed by a slow afterhyperpolarization (sAHP). In the presence of TPM (20 \( \mu \)M), there was an enhancement and dramatic prolongation of the sAHP, accompanied by a noticeable increase in spike accommodation during the pulse, that reversed on washout (60 min).

(B) Corresponding outward tail current (\( s_{AHP} \)) evoked using a “hybrid” voltage clamp protocol from a holding potential of -70 mV. TPM enhanced and prolonged the time course of the \( s_{AHP} \).
(A) Effect of TPM (red) on clamp currents evoked by a 1.5s voltage command from a holding potential of $-70$ mV to $-20$ mV (in 1μM TTX). Superimposed records show outward current developed during positive command and ensuing slow outward tail current ($sl_{AHP}$) in control Krebs solution, then after 10 min in 20 μM TPM. Note potentiation of outward relaxation and after-tail current. Addition of Cd$^{2+}$ (200 μM) blocked both the extra outward current and $sl_{AHP}$ tail components induced by TPM (blue). A steady outward current of 0.15 nA developed in TPM (not shown), along with a 27 % increase in leak conductance measured around $-70$ mV. (B) Steady-state $I-V$ relation obtained in the same cell shows the clear increase in outward rectification between $-60$ to $-20$ mV in the presence of TPM, that was blocked by Cd$^{2+}$. 

Figure 10: Effects of TPM on clamp currents and relative $I/V$ curves
Variability in action of TPM (red) observed in some olfactory neurones. Cell was voltage clamped at -70 mV and stepped to -20 mV for 1.5s to evoke a characteristic “creeping” outward current relaxation and slow after-tail current ($s_{AHP}$). In this cell, TPM (20 µM, 15 min) reduced the outward relaxation amplitude during the depolarizing stimulus (c.f. Fig. 9) although the $s_{AHP}$ tail was still clearly prolonged.
Figure 12: Effects of TPM recorded in a Cs\(^+\)-loaded neurone (in 1 \(\mu\)M TTX).

(A) Depolarizing electrotonic potential and action potential, (evoked by a +0.3 nA, 160 ms current pulse) recorded 30 min after impalement of a cell with a 3M Cs acetate-filled microelectrode; note the typically prolonged duration and slow rate of onset of the TTX-resistant (Ca\(^{2+}\)) spike, signifying partial blockade of outward (repolarizing) K\(^+\) conductances. The cell membrane potential was set at \(-70\) mV by steady current injection. (B) (Same neurone under voltage clamp). Superimposed clamp currents recorded from \(-70\) mV holding potential following a 1.5s voltage step to \(-20\) mV show enhancement of residual outward relaxation in TPM (20 \(\mu\)M, 15 min; red), although an enhanced inward after-tail current was now revealed. Both outward relaxation and induced inward tail were abolished by Cd\(^{2+}\) (200 \(\mu\)M, 10 min; blue); (note, no steady outward current shift was produced by TPM in this Cs\(^+\)-loaded cell).
(A) Superimposed clamp currents (in TTX) in response to a +50 mV (1.5s) voltage jump recorded in a Cs⁺-loaded neurone held at -70 mV. In the presence of 20 µM TPM, an enhanced outward current and inward after-tail current are induced, both of which are reduced by 20 µM nifedipine (10 min); (note TPM generated a negligible (0.05 nA) steady outward current shift in this cell). (B) Corresponding steady-state I-V relation showing reduction by nifedipine, of extra outward rectification induced by TPM; note blocking effect is only evident between -55 and -25 mV, and the curious crossover of I-V plots at around -35 mV, suggesting an interplay between enhanced inward and outward-rectifying current components.
PKA-mediated phosphorylation does not occlude the action of TPM on the sAHP.

(A) Neurone maintained at –70 mV membrane potential. A prolonged depolarizing stimulus (1.5s, +2.0 nA) induces a post-stimulus sAHP in control solution, which is abolished (converted to a sADP with superimposed repetitive firing) in the presence of the direct adenylate cyclase activator forskolin (20 μM) applied alone. On further adding TPM (20 μM, 10min), a prolonged sAHP tail re-appeared. (B) Corresponding sAHP tail currents recorded under “hybrid” voltage clamp at –70 mV. Traces show currents in control solution, after blockade by forskolin (middle) and after adding TPM; note re-appearance of sAHP tail, with prolonged decay time.
Forskolin further enhances effect of TPM on the sAHP. (A) Shows initial control sAHP recorded in a neurone maintained at −70 mV membrane potential, then 20 min after applying 20 μM TPM alone, showing typical enhancement and prolongation effect. On co-applying forskolin (20 μM), there was a further prolongation of the sAHP. (B) Corresponding sI_{AHP} tail currents recorded under “hybrid” voltage clamp at −70 mV; note usual enhancement of sI_{AHP} in TPM, but additional prolongation on adding forskolin. Set current stimulus parameters were + 2.0 nA, 1.5s throughout.
Figure 16: Acetazolamide (ACTZ; 20 μM) mimics and occludes the neuronal effects of TPM.

(A, B). Comparison of slow outward clamp currents (recorded at -70 mV) produced by TPM and ACTZ respectively in different neurones (10 min applications). (A) Control initial response to TPM (20 μM), followed by 30 min recovery. ACTZ (20 μM; 25 min) applied to the same cell evoked a similar response and occluded the effect of TPM co-applied at the peak of the ACTZ current. (B) Initial response to ACTZ was followed by a similar response to TPM, which occluded the effect of ACTZ co-applied at the peak.

(C) sAHP recorded in control Krebs solution, then after 10 min in ACTZ; note TPM-like enhancement and prolongation of sAHP, along with increased spike accommodation during depolarizing pulse (c.f. Fig. 9). TPM (20 μM) was then applied to the same cell (in ACTZ), producing a further comparable enhancement and prolongation of the sAHP (membrane potential corrected to -70 mV throughout by injecting steady positive current). (D) Corresponding tail currents recorded under "hybrid" voltage clamp at -70 mV, show clear enhancement of the sI_{AHP} tail in ACTZ alone or ACTZ plus TPM respectively. Current stimulus was + 2.0 nA, 1.5s throughout. An outward baseline shift of 0.30 nA induced by ACTZ was omitted for clarity; TPM did not induce any further outward current in the presence of ACTZ.
Figure 17: The carbonic anhydrase inhibitor benzolamide (BZ; 50 μM) mimics the neuronal effects of TPM.

(A) Slow membrane hyperpolarization accompanied by a decrease in membrane input resistance induced by bath-application of BZ (50 μM) from a holding membrane potential of -70 mV (maintained by steady current injection). Negative current pulses (-0.5 nA) were injected every 2s throughout. After correcting for the change in membrane potential induced by BZ (by injecting positive current), on further adding TPM (20 pM; 10 min), the usual hyperpolarization response was clearly occluded by the presence of BZ. (B) (same neurone) sAHP recorded in control Krebs solution, then after 10 min in BZ; note TPM-like enhancement and prolongation of the sAHP, along with increased spike accommodation during depolarizing pulse (c.f. Fig. 9). TPM (20 μM) was then applied to the same cell (in BZ), producing a further comparable enhancement and prolongation of the sAHP (membrane potential corrected to -70 mV throughout). (C) Corresponding tail currents recorded under “hybrid” voltage clamp at -70 mV, show clear enhancement of the sI_{AHP} tail in the presence of BZ alone or BZ plus TPM respectively. Set current stimulus was + 2.0 nA, 1.5s throughout.
Figure 18: TPM and ACTZ effects on outward current generation are unaffected by recording in HEPES-buffered bathing medium.

(A) Slow-speed chart record showing the effect of switching from normal bicarbonate-buffered Krebs solution, to bicarbonate/CO₂-free HEPES-buffered solution on membrane potential and input resistance of a neurone maintained at -70 mV membrane potential by positive current injection. Downward deflections represent hyperpolarizing electrotonic potentials evoked by regular current pulses (-0.5 nA) injected every 1.5s throughout; note the small early depolarization, transient decrease in resistance, and subsequent sustained hyperpolarization in HEPES medium. (B & C) Slow outward currents induced by TPM (20 µM, 10 min; B) or ACTZ (20 µM, 10 min; C) under voltage clamp at -70 mV (followed by slow recoveries on washout) recorded initially in normal Krebs solution (left) then after 30 min exposure to HEPES medium (right). Note both responses are essentially unaltered in the HEPES-buffered solution, therefore not dependent on carbonic anhydrase inhibition for their generation.
Chemical structure of TPM (A) and some related molecules (Acetazolamide, B; Zonisamide, C; Benzolamide, D) possessing the sulphonamide moiety (red bar). A, B and C are known anticonvulsant drugs and the sulphonamide group present in all of them might be able to interact with a common target(s) in the CNS to produce anticonvulsant properties.
Chapter II, Section 2:

*In vivo Results*
II.2 In vivo Results

II.2.1 Introduction

Animal models for seizures and epilepsies have played a fundamental role in our understanding of the physiological and behavioural changes associated with human epilepsies. Since the early half of the 20th century, animal models have lead to the discovery of antiepileptic treatment strategies, and some of those antiepileptic drugs are still commonly prescribed today (Noebels et al., 1997). Animal models range in diversity from Drosophila bang-sensitive mutants (Lee & Wu, 2002) to non-human primates (Kupferberg, 2001) and have proven invaluable for investigating basic mechanisms underlying epileptogenicity (Lee & Wu, 2002; Kupferberg, 2001). Whether it is a specific mutated ion channel or neurotransmitter receptor, fundamental disruptions in neuronal function are consistent across the different animal models (Engel, 1992). In vivo animal models have been categorized into models of seizures and those of epilepsy (for review see: Sarkisian, 2001; Prasad et al., 1999; Seyfried et al., 1999; Löscher, 1997; Engel, 1992). Since human epilepsy is defined by the appearance of multiple spontaneous recurrent seizures, induction of acute seizure activity alone without chronic epileptiform behaviour is considered a model for seizures and not epilepsy (Engel, 1992).

In the present in vivo studies, both models of generalized convulsive and non-convulsive epilepsy have been used. A better classification would distinguish between genetic (WAG/Rij rats, DBA/2 and lethargic mice) and chemical (AMPA-, PTZ-, 4-AP- induced seizure) models of epilepsy or seizures. Moreover, it is possible to classify the chemically-induced and audiogenic-induced seizures as models of generalized tonic-clonic seizures, whereas WAG/Rij rats and lethargic mice belong to the "new family" of genetic models of generalized absence epilepsy. In the following section, we were particularly interested in investigating the possible interactions between TPM and the dihydropyridine antagonist, nifedipine, or the dihydropyridine agonist S(-)-1,4-dihydro-2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl)phenyl]-3-pyridinecarboxylic acid methyl ester (Bay K 8644) at L-type Ca²⁺ channels in the abovementioned models of experimental epilepsy, as suggested by the in vitro intracellular studies carried out on olfactory cortical brain slices. We also compared the effects of TPM administration with those of the AMPA/kainate receptor antagonists NBQX and CFM-2; in addition,
since TPM possesses carbonic anhydrase (CA) inhibitory activity (Casini et al., 2002, 2003; Shank et al., 2000), we compared its effects with that of the classical CA inhibitor, acetazolamide (ACTZ), itself a recognised anticonvulsant agent (Brodie, 2001; Millchap et al., 1955).

II.2.2 Animal models of epilepsy

The following set of experiments was performed in Italy in the animal house facility of the University of Catanzaro under the supervision of Prof. Giovambattista De Sarro. Procedures involving animals and their care were conducted in conformity with the international and national law and policies (European Communities Council Directive of 24th November 1986, 86/609EEC).

Two generalized tonic-clonic animal models of epilepsy were used: DBA/2 mice of either sex (8-12 g, 22-26 days old for audiogenic seizures or 20-28 g, 48-56 days old for AMPA-induced seizures), a strain genetically susceptible to sound-induced seizures, and ICR- CD1 mice (20-30 g, 42-48 days old) for chemically-induced generalized seizures, both purchased from Harlan Italy (Correzzana, Milan, Italy). As genetic animal models of absence epilepsy, we used male WAG/Rij rats (24-36 weeks old; Harlan Italy, Correzzana, Milan, Italy) and male lethargic (lh/lh) mice (8-16 weeks old), inbred in the vivarium facilities of the University of Catanzaro from progenitors originally obtained from Prof. B.S. Meldrum (University of London, UK).

II.2.2.1 Models for generalized tonic-clonic seizures

DBA/2 mice

Sound-induced seizures

The DBA (Dilute Brown Agouti coat colour) strain of the house mouse, Mus Musculus, has been known since 1947 to be susceptible to sound induced seizures (Vicari, 1951). There have been comprehensive reviews on the historical and genetic aspects of audiogenic strains of rodents, including the DBA/2 mice (Chapman et al., 1984; Seyfried, 1979; Collins, 1972; Vicari, 1951). The characteristics of the seizures and the particular stimuli required have been reviewed (Collins, 1972; Bevan, 1955; Vicari, 1951), as well as apparent biochemical, endocrine or developmental abnormalities
Finally, the pharmacology of audiogenic seizures has been the subject of several reviews (De Sarro et al., 2002a, c, 2000a, 1992b, 1988; Chapman et al., 1984; Jobe, 1981; Kellog, 1976; Collins & Horlington, 1969). The following section provides a summary of the main characteristics of this particular strain of mice.

Nearly 100% of the DBA/2 strain of mice undergo an age-dependent, often fatal, sequence of convulsions (a wild running phase followed by clonic convulsions and a tonic extension, ending in respiratory arrest or full recovery) when initially exposed to a loud mixed-frequency sound (12-16 kHz; 90-120 dB) such as a door bell. Different age ranges for maximum susceptibility to sound-induced seizures has been reported: 20-39 days (Suter et al., 1958; Vicari, 1951), 21-28 days (Schlesinger et al., 1965), or 16-26 days (Schreiber, 1981). A number of physiological and biochemical differences between DBA/2 mice and resistant strains have also been described (traditionally, parameters in the DBA/2 strain have been compared with those of the C57 inbred strain of mice, which are resistant to sound-induced seizures at all ages; Vicari, 1951). Parameters that might differ significantly between these two strains have in some cases been shown not to be directly correlated to seizure susceptibility when several strains of resistant and susceptible mice have been compared (Chapman et al., 1984; Seyfried, 1979; Tunnicliff et al., 1973; Collins, 1972; Coleman, 1960). There is no single abnormal parameter reported for the DBA/2 strain that unequivocally correlates to the seizure susceptibility when several susceptible and resistant strains are compared and when the age-factor (of seizure susceptibility) is taken into account (Chapman et al., 1984).

DBA/2 mice are hyperactive, and easily startled, which could relate to an elevated serum level of thyroid hormone (vs C57; Seyfried et al., 1979). The imbalance in thyroid function could also be responsible for a retarded postnatal cerebellar development in DBA/2 mice and could be related to reported changes in brain energy metabolism in this strain (Chapman et al., 1984; Schreiber, 1981; Racagni et al., 1979; Seyfried et al., 1978; Chapman et al., 1977; Rubin & Ferrendelli, 1977). Microsomal Na^+/K^+ ATPase activity in DBA/2 mice was reported to be depressed (vs C57) with a resulting decrease in intracellular K^+ concentration at the peak age of seizure susceptibility (Hertz et al., 1974); however, subsequent studies (Reichert, 1975) have failed to confirm this result (Jensen et al., 1983; Lonsdale, 1982; Worms & Lloyd,
1981; Reichert, 1975; Hertz et al., 1974; Coleman & Schlesinger, 1965; Suter et al., 1958). The relevance of some neurotransmitter systems (GABA, excitatory amino acids, catecholamines and serotonin) in the brain of DBA/2 mice has been the object of many studies. There is no difference in the cerebral levels of GABA between DBA/2 mice and control mice within the ages 8-43 days (Sykes & Horton, 1982), nor are there any abnormalities in the brain levels of the GABA synthesizing enzyme, glutamic acid decarboxylase (GAD) (Tunnicliff et al., 1973; Sykes & Horton, 1982). However, K^+-stimulated release of GABA from brain slices of DBA/2 mice at 30 days of age is significantly lower than that of C57 mice (Hertz et al., 1974). Furthermore, the number of GABA receptor binding sites is reduced in the DBA/2 strain, while the affinities of these sites are higher than in control mice of comparable age (Horton et al., 1982; Ticku, 1979). Benzodiazepine binding to the GABA_A receptor complex also seems to be affected in DBA/2 mice. Horton and co-workers (1982) reported a decreased density of ^3H-flunitrazepam binding sites, but an increased GABA stimulation of benzodiazepine binding in 28 to 29 day old DBA/2 mice, while Robertson (1980) reported an increase in ^3H-flunitrazepam binding sites at 22 days of age.

Abnormalities have been found in the aspartergic and glutamatergic transmitter systems in DBA/2 mice (Chapman et al., 1996; Wieraszko & Seyfried, 1993; Croucher et al., 1982). It has been shown that excitatory amino acid antagonists of the ionotropic (NMDA and AMPA/kainate) glutamate receptors prevent sound-induced seizures (Ferreri et al., 2004; De Sarro et al., 2003; Croucher et al., 1982) and they are also affected by agents acting on metabotropic glutamate receptors (Moldrich et al., 2003; Thomsen et al., 1994). Abnormalities in the catecholamine and serotonin systems in DBA/2 mice may exist (for a review see Chapman et al., 1984). Some of the reported results are however conflicting, which is partly due to the use of different strains of mice for control. Overall, there appear to be decreased levels of noradrenaline (Schlesinger et al., 1965, 1968) and serotonin (Kellogg, 1971; Schlesinger et al., 1965, 1968) in the brains of DBA/2 mice (vs C57) at the seizure susceptible age, but not at other ages (Schreiber, 1981; Kellogg, 1976; McGeer et al., 1969). Kellogg (1976) has shown that particularly in the hindbrain, the noradrenaline level is depressed, and there is an increased turnover of catecholamines in the brains of 14-day-old DBA/2 mice, whereas at other ages the turnover rate is not significantly increased relative to that in control mice. The serotonin turnover in the hindbrain of DBA/2 mice is increased at the
seizure susceptible age (21 days), but not in older (>42 days) animals (Kellogg, 1976). Abnormal activities of enzymes involved in catecholamine and serotonin synthesis and metabolism have also been reported (Tunnicliff et al., 1973).

As a result of all these abnormalities, the seizure susceptibility of DBA/2 mice is greatly affected by pharmacological manipulations of the brain levels of catecholamines and serotonin. All the commonly used anticonvulsant drugs are effective in suppressing the sound-induced seizures in this strain of mice; the validity of this model for predicting the anticonvulsant properties of new molecules has been widely demonstrated and accepted (Gareri et al., 2004; Ferreri et al., 2004; De Sarro et al., 2003, 1998; Löscher & Meldrum, 1984; Chapman et al., 1984).

In conclusion, the DBA/2 strain of mice represents a good model of generalized epilepsy even if it has no direct counterpart in clinical epilepsy (Fisher, 1989). Nevertheless, the true utility of the model lies in its ability to permit a quick and reliable analysis of possible anticonvulsant action of new AED molecules and possible interactions between different drugs.

**AMPA-induced seizures**

Ionotropic glutamate receptors are ligand-gated ion channels that mediate the vast majority of excitatory neurotransmission in the brain. There are three pharmacologically defined classes of receptors, originally named after reasonably selective agonists- NMDA, AMPA and kainate (Dingledine et al., 1999). AMPA and kainate receptors are different from NMDA receptors since they are usually only permeable to Na\(^+\), and are not voltage-sensitive. It is very difficult to differentiate between AMPA and kainate receptors in terms of physiology and biophysics. The original classification of AMPA and kainate subtypes received support from work with recombinant receptors, which provided evidence that these agonists were selective for two different glutamate receptor subclasses. However, some members of each class of receptor can be activated by both agonists and blocked by common competitive blockers such as NBQX (Dingledine et al., 1999).

AMPA administered intracerebroventricularly (i.c.v.) induces seizures in rodents (De Sarro et al., 2004a, b, 2003; Quartarone et al., 2004; Gitto et al., 2004; Ferreri et al., 2004; Zappala et al., 2000). Depending on the dose administered, animals display
seizures of different intensity; in particular, experimental animals go through a series of behavioural changes related to characteristic limbic seizures (rearing, jerking, falling down and forelimb clonus) and subsequent tonic extension and death; similar generalized seizures are also induced by NMDA and kainate administration (De Sarro et al., 2004a, b; Sarkisian, 2001; Prasad et al., 1999; Seyfried et al., 1999; Engel, 1992; Fisher, 1989). The AMPA-induced seizure model has no correspondence in human epilepsy, but it is useful in identifying anticonvulsant drugs acting on the glutamatergic system being, as aforementioned, most likely involved in the aetiology of human epilepsy (see Section 1.3). Topiramate and felbamate are the only two marketed AEDs that possess a direct action on glutamate ionotropic receptors (Kaminski et al., 2004; Poulsen et al., 2004; Ängehagen et al., 2004; Pugliese et al., 1996; Longo et al., 1995; De Sarro et al. 1994) and talampanel, a selective AMPA/kainate antagonist, is currently under phase II clinical trial (Langan et al., 2003; Bialer et al., 2002; Chappel et al., 2002; Solyom & Tarnawa, 2002).

**ICR-CD1 mice**

*Pentylenetetrazole-induced seizures*

Pentylenetetrazole (PTZ; Leptazol) can be defined as the prototype agent in the class of systemic convulsants (Fisher, 1989). PTZ is a tetrazol derivate with consistent convulsive actions in mice, rats, cats and primates, when parentally administered. PTZ initially induces myoclonic jerks, which then become sustained, and usually lead to a generalized tonic-clonic seizure (Stone, 1970). The subcutaneous PTZ test, together with the maximal electroshock (MES) test, has been used extensively for identifying new anticonvulsant drugs (Sarkisian, 2001; White et al., 1995; Fisher, 1989; Swinyard, 1969). For example, it is generally agreed that substances which obtund only the tonic extension of maximal seizures (e.g. phenytoin) may be clinically useful in generalized tonic-clonic seizures, whereas substances which only elevate minimal seizure threshold (e.g. ethosuximide) may be useful in generalized absence seizures (White et al., 1995).

PTZ was synthesized in 1924 and was later shown by Hildebrandt (1926) to have convulsant action. Although it soon replaced other chemical convulsants, its use for evaluating potential AEDs was not considered reliable (Merrit & Brenner, 1947; Putnam & Merrit, 1937). Nevertheless, Richards & Everett, (1944) reported that
trimethadione, a potent analgesic agent (Spielman, 1944), prevented PTZ-induced seizures in rats, and moreover, these seizures were also prevented by phenobarbital but not phenytoin (Everett & Richards, 1944). Goodman et al. confirmed these findings (Goodman & Manuel, 1945; Goodman & Toman, 1945; Goodman et al., 1945). The early history of the research done on this drug was reviewed by Hildebrandt (1937). The drug has been widely studied in experimental animals. Its effects are exerted primarily on the CNS, but other tissues are also affected. PTZ is a useful experimental model for screening anticonvulsant drugs, particularly those that are effective in absence-type seizures in man (see White et al., 1995; Woodbury, 1972). A summary of its use as an experimental model is described by Stone (1972). In experimental animals, threshold convulsive doses of PTZ produce activation of the EEG and motor activity characterized by forelimb and jaw clonus, a response that resembles that produced by electric stimulation of the brain with threshold currents. Slightly higher doses produce generalized asynchronous clonic movements, which are superseded by a tonic convolution similar to that produced by supramaximal electric stimulation of the brain.

In early experiments on dissociated mouse spinal cord neurones, Macdonald & Barker (1977) showed that PTZ antagonized iontophoretic GABA responses, without affecting other neurone properties and responses. Similar results were obtained by Pellmar & Wilson (1977) on Aplysia neurones, suggesting that PTZ was selectively blocking the Cl⁻ channels opened by GABA. However, PTZ can also affect membrane properties directly, thereby altering excitability and stability (Williamson & Crill, 1976a, b; David et al., 1974; Faugier-Grimaud, 1974; Faugier & Willows, 1973; Klee et al., 1973; Speckman & Caspers, 1973). Most of the experiments demonstrating these effects have been conducted in isolated neuronal systems where synaptic influences have been eliminated, or in non-neuronal systems. In such isolated cells, PTZ (10 to 70 mM) was shown to cause slow-wave potential oscillations and a burst pattern of firing (Williamson & Crill, 1976a, b; David et al., 1974; Faugier-Grimaud, 1974; Klee et al., 1973; Speckman & Caspers, 1973). These data demonstrate a direct action of PTZ on neuronal membranes to increase firing ability and spontaneous discharges. Therefore, the inhibition of GABA-mediated Cl⁻ channels is probably the most important mechanism underlying PTZ's convulsant action but very likely, other mechanisms cooperate to produce its final effects (for a review see: Woodbury, 1980).
4-Aminopyridine-induced seizures

The K⁺ channel blocker 4-aminopyridine (4-AP) is a potent convulsant drug that causes epileptiform activity in in vitro preparations and induces convulsions in both animals and man. It has been used as a therapeutic agent in the treatment of many neurological disorders such as Alzheimer's disease, multiple sclerosis and Lambert-Eaton myasthenic syndrome, spinal cord injury and some cases of botulism (Hayes et al., 1994; Bever et al., 1994; McEvoy et al., 1989). However, slight overdose of this compound has been associated with serious side effects, including convulsions (Bever et al., 1994). The pharmacological mechanism involved in the convulsive effect of 4-AP has been addressed in few reports (Boda & Szente, 1996; Yamaguchi & Rogawski, 1992). The inhibitory action of 4-AP on K⁺ channels impairs the repolarization of the action potential, enhances synaptic efficacy and thus may cause hyperexcitability (Muller et al., 1999). Epileptiform activity can readily be induced by 4-AP in vitro (Luhmann et al., 2000; Postlethwaite et al., 1998; Perreault & Avoli, 1991) and in vivo (Morales-Villagran et al., 1996; Fragoso-Veloz et al., 1990). 4-AP administered into the rat lateral cerebral ventricle stimulates the release of glutamate that is involved in the induction and development of seizure activity (Morales-Villagran & Tapia, 1996). Microdialysis infusion of 4-AP in rat motor cerebral cortex induces the release of catecholamines either through a direct effect on nerve endings or as a consequence of seizures (Morales-Villagran et al., 1999). The potent convulsant effect of 4-AP has been described in rodents (Morales-Villagran et al., 1996) and is a valuable chemical model of seizures (Bruckner & Heinemann, 2000). As mentioned above, 4-AP's effects could be explained by its blocking effect on voltage-dependent K⁺ channels (Muller et al., 1999) and/or ability to directly release glutamate and other excitatory amino acids from nerve terminals (Pena & Tapia, 2000; Morales-Villagran & Tapia, 1996). However, the role of glutamate in 4-AP-induced seizures is uncertain. It has been reported that the glutamate receptor antagonists (+)-methyl-10,11-dihydro-5H-dibenzo(a,d)-cyclohepten-5,10-imine maleate (MK-801), 3-[(±)-2-carboxypiperazine-4-yl] propyl-1-phosphonic acid [(±)-CPP], and 1,2,3,4-tetrahydro-6-nitro-2,3-dioxobenzo[f] quinoxaline-7-sulfonamide (NBQX), were unable to block seizures induced by 4-AP (Yamaguchi et al., 1993; Yamaguchi & Rogawski, 1992; Rogawski et al., 1991). On the other hand, 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine hydrochloride (GYKI 52466), and (±)-5-aminocarbonyl-10,11-dihydro-
5H-dibenzo[a,d]cyclohepten-5,10-imine (SGB-017; ADCI), two other glutamate receptor antagonists were protective against seizures and lethality induced by 4-AP (Yamaguchi et al., 1993; Rogawski et al., 1991). Bruckner and Heinemann (2000) have proposed that the epileptiform activity induced by 4-AP in combined entorhinal cortex-hippocampal slices may provide an in vitro model for the development of new drugs against difficult-to-treat focal epilepsy.

The i.p. or i.c.v. administration of 4-AP in mice produced a typical sequence of behavioural activation. The animals initially exhibited hyperactivity, vocalization, salivation, blinking/eye closing, chewing and rearing, followed by straub tail and trembling, then, clonic jerks occurred, followed by explosive running and continuous clonic convulsion. After clonic convulsions, the majority of animals showed generalized tonic seizures and death (Tutka et al., 2002).

II.2.2.2 Models for absence epilepsy

Human absence epilepsy (AE) accounts for 3 to 4% of all seizure disorders (Delgado-Escueta et al., 1983). In general, the diagnosis is easily confirmed by EEG, which is characterized by generalized 3.0-Hz spike-wave discharges (SWDs) superimposed on normal background activity. Bursts of generalized 3.0- to 4.0-Hz SWDs slowing to 2.5- to 3.0-Hz are typically observed during seizures (Fig. 20). Typical absence seizures (AS) occur several to hundreds times per day and can be accompanied by tonic-clonic seizures (TCS). A family history of epilepsy is present in approximately 30% of patients suggesting a genetic inheritance (Berkovic, 2001).

Animal models of generalized absence seizures and in vitro electrophysiological techniques have tried to shed light on the pathogenesis of AS (Snead, 1995). Four leading theories endeavour to explain the origin of the generalized SWDs associated with AS (Coenen & Van Luijctelaar, 2003). Penfield & Jasper (1954) put forward the “centrencephalic” theory, proposing that SWDs originate from a subcortical pacemaker in the upper brainstem and midline thalamus with projections to both sides of the cortex. Buzsáki et al. (1988) proposed the “thalamic clock” theory, in which the reticular thalamic nucleus contains the pacemaker cells for the SWDs; in these two theories, the cortex has only a passive function. On the other hand, the “cortical” theory of Bancaud (1972) and Niedermeyer (1972) implies that the generalized SWDs
originate from a focus in the cortex. In this theory, subcortical structures are not essential for the generation of SWDs but only participate passively. The "corticoreticular" theory postulated by Gloor (1968) is a bridge between the thalamic and cortical theories, implying that a hyperexcitable cortex responds abnormally to initially normal, thalamocortical volleys, thereby initiating, in such a way, abnormal thalamocortical oscillations. In all four views, a prerequisite for the occurrence of SWDs is a highly synchronized activity of thalamocortical or corticothalamic connections. The widespread, bilaterally synchronous SWDs are reflections of highly synchronized oscillations in thalamo-cortical-thalamic networks (Coenen & Van Luijtelaar, 2003). More recently, Meeren and co-workers (2002) discovered that in the WAG/Rij rat model of absence epilepsy, there is a consistent cortical "focus" within the peri-oral region of the somatosensory cortex. The SWDs recorded at other cortical sites consistently lagged behind this focal site, with time delays that increased with electrode distance (corresponding to a mean propagation velocity of 1.4 m/sec). This leads to the hypothesis that epilepsies also considered as generalized might, instead, be secondarily generalized. However, this could be true for this particular animal model but more studies are needed to confirm this hypothesis.

During recent decades, it has become increasingly obvious that genetic factors play a main role in the idiopathic epilepsies, including absence epilepsy. Evidence for a genetic predisposition in this type of epilepsy arose from twin studies, where it was found that both pairs of monozygotic twins suffered more frequently from this disorder than pairs of dizygotic twins (Lennox & Jolly, 1954). Given this genetic predisposition, it seems that genetic animal models more closely resemble absence epilepsy in humans than models in which absences are experimentally induced, such as the feline penicillin, PTZ, and γ-hydroxybutyric models (for reviews see: Hu et al., 2000; Avoli, 1995; Snead, 1992, 1995; Fisher, 1989). The presence of spontaneously occurring SWDs in the EEG and the concomitant presence of clinical signs of epilepsy are the primary demands for a valid absence epilepsy model. Various genetic mouse models of AE have been described such as lh/lh mice and tottering mice, but these mutants generally show other neurological abnormalities, besides the presence of SWDs (Burgess & Noebels, 1999). Interestingly, all these mouse models of AE have mutations on voltage-dependent Ca\(^{2+}\) channel subunits (Felix, 2002). It later emerged that some strains of rats also show spontaneous signs of SWDs. Validation studies were mainly
performed in GAERS (Genetically Absence Epileptic Rats from Strasbourg) and in WAG/Rij rats (Wistar Albino Glaxo rats from Rijswijk) (Danober et al., 1998; Coenen et al., 1992; Marescaux et al., 1992; Van Luijten & Coenen, 1989). The characteristics of the two absence models used in the present experiments are described in the following sections.

**Lethargic (lh/lh) mice**

Recent studies, on mutant mice affected by AE, have identified some genes strictly correlated with the development of this kind of epilepsy, indicating that Ca^{2+} channels probably play a crucial role in the expression of this inherited phenotype (Burgess & Noebels, 1999). Recent progresses in molecular genetics and cellular electrophysiology have demonstrated that AE is particularly connected to abnormalities in P/Q type Ca^{2+} channels. Thus, mutations of the α_{1} subunit of the P/Q channel have been identified in different mutant mice such as: tottering, leaner, rolling Nagoya, and rocker, whereas, the lethargic (lh/lh) mouse possesses a mutation on the β_{4} ancillary subunit (Figure 21). The lh/lh mutant is an example of a channelopathy of a non-pore forming subunit of the Ca^{2+} channel complex that has the ability to modulate more than one channel subtype (Qian & Noebels, 2000). Mutations in the human α_{1A} gene, however, do not appear to be the locus of common idiopathic generalized epilepsy (Sander et al., 1998).

The lh/lh mouse model represents a good animal model of AE; it shares behavioural, electrographic and pharmacological characteristics very similar to those present in the human absence disorder (Hosford & Wang, 1997; Hosford et al., 1992; Löscher & Schmidt, 1988). Lh/lh mice exhibit severe neurological defects including ataxia, episodic dyskinesia and 5-8 Hz SWDs on the cortical EEG together with concurrent behavioural episodes such as vibrissae spasms and increase in breath frequency. The lh mutation deletes the α_{1} subunit interaction domain of the β_{4} subunit (Burgess et al., 1997); specifically, it results in aberrant pre-mRNA and a translational frameshift, which ultimately encodes a severely truncated β_{4} protein missing 60% of the C-terminal relative to wild type, which includes the essential α_{1}-β interaction domain (Felix, 2002). This interaction is important for the regulation of the Ca^{2+} current flowing through voltage-sensitive channels.
As a direct consequence of this mutation, β subunits lose their ability to modulate the function of the pore forming α1 subunit (De Waard et al., 1994). It has been documented that neither full-length nor truncated β4 proteins are expressed as a result of the lh mutation (McEnery et al., 1998). The use of specific antibodies has demonstrated that the β4 subunit is expressed in 30% of N-type calcium channels (Vance et al., 1998; Scott et al., 1996) as well as P/Q type calcium channels (Liu et al., 1996). Felix (2002) has suggested that the hyperexcitability/susceptibility of lh/lh mice could be due to a wrong expression of an immature population of voltage-dependent Ca\(^{2+}\) channels during neuronal development. The β4 subunit interacts with the transmembrane α1 subunit, playing a key role in the assembly and localization of the channel and furthermore, it possesses a unique modulatory site not shared with other β subunits (De Waard et al., 1994), therefore, this alteration can affect both P/Q and N-type calcium channels. The latter are mainly localized at presynaptic terminals where they control neurotransmitters release (Catterall, 1998). Consequently, this mutation is likely to induce alterations in synaptic transmission in the lh/lh mouse brain. Such studies have shown that compensatory mechanisms ensure the entry of Ca\(^{2+}\) at presynaptic sites for exocytotic release; the β4 mutation does not therefore alter presynaptic activity, presynaptic Ca\(^{2+}\) flow or modulation of Ca\(^{2+}\) channels by G proteins (Burgess et al., 1999). Therefore, the lack of β4 subunit does not seem to compromise the function of P/Q and N-type channels in hippocampal synapses, implying the existence of other presynaptic currents mediated by other available subunits. However, the β4 subunit mutation does seem to increase the intrinsic membrane excitability of thalamic neurones, by potentiating low voltage-activated Ca\(^{2+}\) channel activity (Zhang et al., 2002). This alteration increases the probability of an abnormal thalamo-cortical synchronization and therefore, the genesis of absence like activity in lh/lh mutant mice.

The mechanisms underlying the generation of SWDs in the thalamocortical network of lh/lh mice have been extensively studied. From all these studies, it now seems that the numbers of GABA\(_B\) receptors are increased in the neuronal thalamocortical population in lh/lh mutants compared with normal littermates, and these receptors appear to play a pathophysiological role in the expression of absence seizures (Hosford et al., 1999). Some other abnormalities have also been described, but their role in the generation of thalamocortical oscillations has yet to be demonstrated; e.g. Lh/lh mice over express glutamate decarboxylase 67 (GAD67) in the nucleus reticularis thalami (Lin et al.,
and show a reduction in excitatory but not inhibitory synaptic transmission in the ventrobasal thalamic nucleus (Caddick et al., 1999).

Behavioural and pharmacological studies have now widely validated this genetic model (Hosford & Wang, 1997; Hosford et al., 1995b). It has shown a high predictive value in demonstrating the anti-absence clinical efficacies of ethosuximide (Hosford et al., 1992) and valproic acid (Hosford et al., 1995a). Other studies have shown the efficacy of lamotrigine, the possible pro-absence effects of vigabatrin and tiagabine and a lack of effect of gabapentin (Hosford et al., 1997). In addition, in some cases, GABA_A activation in the reticular thalamic nucleus (RTN) suppresses absence seizures in lh/lh mice (Hosford et al., 1999) and in other models; for example, clonazepam, which activates GABA_A receptors, has anti-absence properties. Furthermore, group II metabotropic glutamate receptor agonists (Moldrich et al., 2001), metabotropic glutamate group I antagonists (Chapman et al., 2000), GABA_B (Aizawa et al., 1997; Hosford et al., 1995a, b) and NMDA antagonists (Aizawa et al., 1997) possess anti-absence effects in this animal model.

**WAG/Rij rats**

The Wistar Albino Glaxo rats from Rijswijk (WAG/Rij rats) are widely recognized and used as a valid animal model for AE (Coenen & Van Luijtelaar, 2003). They were first discovered to display SWDs together with mild clinical manifestations by Coenen & Van Luijtelaar (1987). The SWDs appear in the cortical EEG of these animals at the age of 2-3 months and increase with age (Coenen & Van Luijtelaar, 1987). At 6 months of age, both male and female rats show about 16-20 discharges per hour, with an average duration of about 5s. The WAG/Rij strain is a subline of the WAG strain, which was created from Wistar stock by A.L. Bacharach at the Glaxo laboratories in London in 1924. Characteristics in the behaviour of WAG/Rij rats have been studied extensively and are similar to those of Wistar rats with which they are usually compared (Coenen & Van Luijtelaar, 2003). Spike-wave activity in the cortical EEG of adult WAG/Rij rats is bilaterally symmetrical and generalized over the entire cortex (Meeren et al., 2002; Midzianovskaia et al., 2001; Van Luijtelaar & Coenen, 1989). Recently, it has been revealed on the EEG, that a possible focus for the generation of SWDs exists in the perioral area of the somatosensory cortex (Meeren et al., 2002),
however the entire cortex and various thalamic nuclei are primary involved in the
generation and maintenance of the rhythmic oscillations underlying SWDs (Coenen &
Van Luijtelaar, 2003). The SWDs consisted of bursts lasting for 1-30s and a spike-
wave frequency of 7-10 Hz (Drinkenburg et al., 1993); this value is considerably
different from humans, where the frequency is generally 3 Hz. Minimal sex differences
have been reported but they are not significant (Coenen & Van Luijtelaar, 1987).
Seizures in WAG/Rij rats can be accompanied by facial myoclonic jerks, twitching of
the vibrissae, accelerated breathing, head tilting, and eye twitching. The state of
vigilance such as wakefulness, slow-wave sleep, and REM sleep highly influence the
occurrence of SWDs. In more than 80% of cases, SWDs appear during passive
wakefulness or during light slow-wave sleep (Drinkenburg et al., 1991). SWDs follow
a circadian pattern, with the maximum number between the 4th and 5th hour of the dark
period, which mainly consists of light slow-wave sleep and drowsiness (Van Luijtelaar
& Coenen, 1988). Human data also contain indications for a circadian rhythm, with a
maximum likelihood for SWDs at early morning awakenings and at rest periods (Burr
et al., 1991).

Screening of the pharmacological profile of the spike-wave activity of the absence
model has been done with classical AEDs and some of the “novel” drugs recently
marketed. Ethosuximide, valproate and trimethadione were selected as specific anti-
absence drugs, and phenytoin and carbamazepine were used as general anticonvulsant
drugs. As expected, only the anti-absence drugs caused a decrease in the number and
duration of SWDs, whereas the anticonvulsive drugs induced a substantial increase
in their number and duration (Peeters et al., 1988). This result closely corresponds to the
pharmacological profile for humans. Van Rijn et al. (1994) investigated the effects of
lamotrigine on SWDs; in side effect-free doses, it did not inhibit absence seizures. Only
at high doses, a reduction was noticeable but side effects were predominant. This is in
contrast with clinical results, but in accordance with the effects of LTG in the GAERS
model of AE; a possible explanation for this result has not yet been found. Other new
anticonvulsant drugs such as tiagabine and vigabatrin both showed an increase in
epileptic activity (Coenen et al., 1995; Marescaux et al., 1992).

Different biochemical studies have been performed on WAG/Rij rats, to understand the
mechanism underlying the generation of SWDs in this strain. Studies regarded the two
main neurotransmitter systems in the brain (GABA and glutamate) and also the
possible role of dopamine, while some others regarded ion channel functionality. Administration of the GABA_A agonist muscimol enhanced the number of SWDs, while the GABA_A antagonist bicuculline, reduced their number (Peeters et al., 1989). Tiagabine, a GABA reuptake inhibitor, enhanced both the number and duration of SWDs in a dose-related manner (Coenen et al., 1995). These experiments support the view that non-convulsive epilepsy is associated with a hyperfunction of the GABA system, thus underlining the fundamental biochemical difference between convulsive and non-convulsive epilepsy. Interestingly, the administration of muscimol into the specific relay nuclei of the thalamus increased SWDs, whereas injection in the lateral part of the thalamus decreased spike-wave activity (Liu et al., 1991). It should be noted that different type of benzodiazepine receptors are expressed in these areas (Wisden et al., 1992). Whereas SWDs are aggravated by activation of the GABAergic system, they can be inhibited by blocking its counterpart, the glutamatergic system. Thus, the NMDA glutamate receptor antagonist, MK-801 decreased the number of SWDs in a dose-dependent manner (Filakovsky et al., 1999). All agonists at ionotropic glutamate receptors (NMDA, AMPA/kainate), so far, enhance absence epileptic activity, whereas all antagonists decrease the number of SWDs (Peeters et al., 1990, 1994). Dopaminergic neurotransmission has been shown to participate in the control of AE, because dopaminergic agonists reduce, and antagonists enhance, the number of SWDs in both GAERS and WAG/Rij rats (Deransart et al., 2000; Van Luijtelaar et al., 1996; Inoue et al., 1994; Warter et al., 1988). The mechanism by which nigrostriatal dopaminergic activity has been thought to prevent the occurrence of SWDs has been described by Buzsáki et al., (1990). Normally, a sustained level of firing of dopaminergic neurones in the substantia nigra provides a steady inhibitory dorsal striatal output, thereby inhibiting the GABAergic projections from the substantia nigra and globus pallidum to the thalamus (Buzsáki et al., 1990).

Several lines of research suggest that Ca^{2+} and K^{+} ion channel deficits might be involved in increased seizure susceptibility in WAG/Rij rats. Pharmacological studies have confirmed a role for the Ca^{2+}-dependent K^{+} channels in SWDs. The K^{+} blocker apamin enhanced the mean duration of SWDs without causing convulsions. In earlier studies, it was found that a K^{+} channel opener (BRL 34915) decreased the number of SWDs in WAG/Rij rats, while the mean duration was not altered (Gandolfo et al., 1989). It seems certain that Ca^{2+} channels play a role in the pathogenesis of spike-wave
activity, but only pharmacological studies have been performed so far (Van Luijtelaar et al., 1995, 2000). Immunocytochemical data suggest that the number of P/Q Ca\(^{2+}\) channels is enhanced in the rostral pole of the RTN compared to three control groups, the role of these channels await confirmation in \textit{in vivo} experiments (van de Bovenkamp-Janssen et al., 2004).
II.2.3 Methods

II.2.3.1 Experimental protocols in DBA/2 and ICR-CD1 mice

Audiogenic seizures in DBA/2 mice

Experiments were carried out on DBA/2 mice, weighing 8-12 g (22-26 days old). Animals were housed in groups of 8-10 in colony cages at 21-23 °C and a relative humidity of 57 ± 2%, under a 12-h light/dark cycle (lights on at 7:00 am), with free access to food pellets and water available ad libitum. Experimental groups, consisting of 10 animals were assigned according to a randomised schedule, and each mouse was used only once. Control animals were always tested on the same day with respective experimental groups. DBA/2 mice were exposed to auditory stimulation 30 min following intraperitoneal (i.p.) administration of each drug plus vehicle, plus the L-type calcium channel antagonist nifedipine, or the L-type calcium channel agonist Bay K 8644 (De Sarro et al., 1988, 1992a). TPM was also administered concomitantly with the L-type calcium channel antagonist verapamil to evaluate the possible interaction with the L-channel at a different site from the dihydropyridine binding site. Each mouse was placed under a hemispheric perspex dome (diameter 58 cm) and 1 min was allowed for habituation and assessment of locomotor activity. Auditory stimulation (12-16 kHz, 109 dB) was applied for 1 min. The seizure response was assessed using the following scale: 0 = no response, 1 = wild running, 2 = clonus, 3 = tonus, 4 = respiratory arrest, as previously reported (De Sarro et al., 1988). The maximum response was recorded for each animal. ED\textsubscript{50} values for each drug or their combination was calculated using a computer program of the Litchfield and Wilcoxon method (1949). Rectal temperature was recorded immediately prior to auditory testing using an Elektrolaboratoriet thermometer type T.E.3. Behavioural changes were observed during the period between drug administration and auditory testing.

Intracerebroventricular administration of AMPA

Intracerebroventricular (i.c.v.) injection of the glutamate receptor agonist, AMPA, induces seizures in DBA/2 mice (20-28 g, 48-56 days old). For i.c.v. injections, mice were gently hand restrained, anesthetized with halothane and injections were made in the left or right lateral ventricle (coordinates 1mm posterior and 1mm lateral to the bregma; depth 2.4 mm) using a 5 μl Hamilton microsyringe fitted with a nylon cuff on
the needle; injections of drugs by this procedure led to a uniform distribution throughout the ventricular system within 10 min (De Sarro et al., 1988).

Mice treated with AMPA showed the characteristic limbic seizures (rearing, jerking, falling down and forelimb clonus) and 9 out of 10 animals died following tonic extension. Test animals (groups of 10 mice per dose) were pretreated i.p. (30 min before) with TPM (5-40 mg/kg), ACTZ (10-80 mg/kg) or AMPA/kainate receptor antagonists alone or in combination with the L-type calcium channels antagonist nifedipine (2.5 mg/kg) and later by i.c.v. injection of AMPA (9.7 nmol/mouse to induce clonus and 11.7 nmol/mouse for tonus dissolved in 5μl, previously determined CD97 values) (De Sarro et al., 1994).

**Seizures induced by administration of 4-aminopyridine**

The K⁺ channel blocker 4-aminopyridine (4-AP) was administered subcutaneously to ICR-CD1 (20-30g, 42-48 days-old) mice (groups of 10 mice per dose) at a dose of 13.3 mg/kg (previously determined CD97 value) 15 or 30 min after i.p. administration of each drug. Animals showing tonic extension or death were scored as non-protected according to Yamaguchi and Rogawski (1992).

**Pentylenetetrazole-induced seizures in ICR-CD1 mice**

Male ICR-CD1 mice (20-30g, 42-48 days old) were pretreated with vehicle or drugs (groups of 10 mice per dose) 30 min before the i.p. administration of pentylenetetrazole (PTZ) 60 mg/kg. A threshold convulsion was considered as an episode of clonic spasms lasting for at least 5s. Absence of this threshold convulsion over 30 min indicated that the animal was protected from the convulsant-induced seizures (Swinyard & Woodhead, 1982).
II.2.3.2 Experimental protocols in WAG/Rij rats and lethargic mice

Electrode implantation

All animals were chronically implanted with five electrodes under chloral hydrate anaesthesia (Carlo Erba, Milano, Italy) using a Kopf stereotaxic instrument and, when necessary, a guide cannula for i.c.v. administration was also implanted. Small holes were drilled in the skull for epidural implantation of electrodes on the cortex. Stainless steel screw electrodes (200μm diameter; California Fine Wire, Grover Beach, CA) with only the tip non-insulated were implanted on the dura mater over the cortex: two in the frontal region (A = 2.0, L = ±2.5 for WAG/Rij rats; A = 1.5, L = ±2.0 for lh/lh mice) and two in the parietooccipital region (A = -6.0, L = ±2.5 for WAG/Rij rats; A = -4.0, L = ±2.0 for lh/lh mice). The ground electrode was placed over the cerebellum. The electrodes’ leads were soldered to a miniature connector, which was head mounted with cranioplastic cement and mounting screws. All animals were allowed at least 1 week of recovery and handled twice a day. The animals were attached to a multichannel amplifier (Astro-Med, West Warwick, USA) by a flexible recording cable and an electric swivel, fixed above the cages, permitting free movements for the animals. The body heat of the animals was maintained at 37°C using a heating pad placed underneath the animal during the surgery. All stereotactic coordinates are relative to bregma with skull surface flat, according to Paxinos & Watson (1986) for coordinates in rats and Paxinos & Franklin (2001) for mice. All animals were allowed at least 1 week of recovery and handled twice a day after surgery.

Experimental protocol in lethargic (lh/lh) mice

At least 1 week after surgery, each mouse underwent five daily electroencephalogram (EEG) recordings; such recordings where used as controls. Experiments to determine the effects of drugs were organized in 5-h recording sessions. During the experiments, mice received i.c.v. either vehicle (0.01 M sodium phosphate buffer-DMSO) or drug (nifedipine or Bay K 8644, 20 and 40μg/μl) per cannula at 60 min after each baseline recording or intraperitoneally (i.p.) either TPM (10, 20 or 40 mg/kg) or vehicle (DMSO + saline, 1:9) (at least 6 mice per dose were used). For i.c.v. administration of drugs, animals were gently hand-restrained and drug infusions were made using an injector cannula connected by a polyethylene tube to a 5 μl Hamilton syringe. Drugs were
infused in a volume of 1 μl at a rate of 0.2 μl/min, the cannulae kept in situ for one further min. Animals were used only once, and at the end of the experiments, injection sites were verified by both macroscopic and histological examination. During each recording, the behavioural changes after drug treatment in comparison to vehicle were noted. The identification of absence seizures was based on the duration(s) and the number of electroencephalogram spike-wave discharges (SWDs) or poly-spikes, as previously described by Hosford et al. (1992) (i.e. amplitude not less than 60μV and frequency range of 5-6 Hz; seizures must have a duration no shorter than 0.6s). The quantification of absence seizures was based on the duration(s) and the number of EEG SWDs, as previously described by De Sarro et al. (1996). Briefly, the duration and number of SWDs for each rat were summarized in 30 min intervals (epochs) for 1 h before and 4 h after drug or vehicle treatment, and scored by visual inspection of the EEG recordings. In addition, the number of SWDs was also counted and calculated for each 30 min post-injection interval by analysis of the recorded EEG.

Experimental procedure in WAG/Rij rats.

Separate groups of rats (at least n = 6 for each group) were used to determine the effects of i.c.v. injection of nifedipine and Bay K 8644 on electrocortical activity of TPM in WAG/Rij rats. Every recording session lasted 6 h: 1 h baseline without injection, and 5 hours after the injection of vehicle or TPM combined with vehicle, nifedipine or Bay K 8644 in different doses every hour, according to the rules of cumulative dose design. The doses of TPM used were 10, 20, 40 and 80 mg/kg, and the doses of nifedipine or Bay K 8644 were 40, 60 or 80μg/2μl. For i.c.v. administration of drugs, animals were gently hand-restrained and drug infusions were made using an injector cannula connected by a polyethylene tube to a 5 μl Hamilton syringe. Drugs were infused in a volume of 2 μl at a rate of 0.2 μl/min, the cannulae kept in situ for one further min. Animals were used only once, and at the end of the experiments, injection sites were verified by both macroscopic and histological examination. The quantification of absence seizures was based on the duration(s) of EEG SWDs or poly-spikes, as previously described by De Sarro et al. (1992c, 1996). Briefly, the number and duration of SWDs for each rat were summarized in 30 min intervals (epochs) for 1 h before and 5 h after drug or vehicle treatment, and scored by visual inspection of the EEG.
recordings. In addition, the number of SWDs was also counted and calculated for each 30 min post-injection interval by analysis of the recorded EEG.

2.5. Effects on behaviour and motor movements

Animals were trained (just before systemic administration of the drugs tested), to do coordinate motor movements continuously for 5 min on a rotarod 6 cm in diameter for rats and 4 cm in diameter for mice 4.5 rpm (U. Basile, Comerio, Varese, Italy). Impairment of coordinated motor movements was defined as the inability of the animals to remain on the rotarod for a test period of 5 min, according to Dunham and Miya (1957). The locomotor performance was usually assessed at 60 min after drug administration. Behavioural changes and their onset and duration were recorded after drug injection until the time of the rotarod test. In particular, two independent observers followed gross behavioural changes consisting of locomotor activity, ataxia, squatting posture and possible piloerection.
II.2.4. Drugs

Topiramate (TPM) was a gift from Dr. R.P. Shank (Johnson & Johnson Pharmaceutical Research & Development LCC, U.S.A.). 2,3-Dihydroxy-6-nitro-7-sulfamoyl-benzo(F)-quinoxaline (NBQX) was kindly supplied by Novo Nordisk (Malov, Denmark). α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) was purchased from Tocris (Buckhurst Hill, U.K.) and acetazolamide (ACTZ), 4-aminopyridine, verapamil and pentyleneetrazole from Sigma (St. Louis, MO, U.S.A.). Nifedipine and Bay K 8644 were gifts from Research Labs. Bayer (Milan, Italy). 1-(4’-Aminophenyl)-3,5-dihydro-7,8-dimethoxy-4H-2,3-benzodiaze-pin-4-one (CFM-2, MW = 311.3) was kindly supplied by Prof. A. Chimirri (University of Messina). For systemic injections, all compounds were given i.p. as a freshly prepared solution. All drugs administered i.c.v. were dissolved in sodium phosphate buffer 67 mM or dimethyl sulfoxide (DMSO), and microinjected in a volume of 1 μl per mouse. NBQX was dissolved in a minimum quantity of 1 N NaOH; the final volume was made up with sodium phosphate buffer. When necessary, pH was adjusted to 7.3-7.4 by adding 0.2 N HCl. Due to their light sensitivity, some compounds were weighed and dissolved in semi-darkness, and stored in containers wrapped in silver foil to exclude light. Exposure to ambient light during the experiments with these drugs was also minimised.
II.2.5. Results

Anticonvulsant activity of TPM in DBA/2 mice and against seizures induced by chemoconvulsants in ICR-CD1 mice.

Anticonvulsant activity in DBA/2 mice

Audiogenic stimulation in DBA/2 mice regularly induced seizures; administration of TPM (2.5-50 mg/kg i.p.) produced a dose-dependent protection against the clonic and tonic phase of the audiogenic seizure response 30 min after i.p. administration (Fig. 22) with an ED$_{50}$ value of 6.12 and 12.12 mg/kg for tonus and clonus, respectively. Compared with other anticonvulsants, TPM was less effective than the AMPA/kainate receptor antagonists but more effective than ACTZ in preventing seizures in this model; the order of efficacy (based on the calculated ED$_{50}$ values for protecting from tonic and clonic seizures) was CFM-2 > NBQX > TPM > ACTZ (Table 4). Interestingly, administration of a low dose of the L-type calcium channel antagonist nifedipine (2.5 mg/kg, i.p.), which itself did not significantly affect the occurrence or severity of audiogenic seizures (De Sarro et al., 1988, 1992a) in combination with TPM, shifted its dose response curves for protecting from tonic and clonic seizures to the right (Fig. 22) and significantly increased the ED$_{50}$ values by 1.9 and 1.7 fold respectively (Table 4). In contrast, nifedipine, concomitantly administered with ACTZ, or the AMPA/kainate receptor antagonists NBQX or CFM-2, did not significantly affect their anticonvulsant effectiveness (Table 4). Rather surprisingly, when a low dose of the L-type Ca$^{2+}$ channel agonist Bay K 8644 (11µg/µl, i.c.v.) (which alone did not significantly influence the incidence and severity of audiogenic seizures) was administered 15 min after the injection of each anticonvulsant drug, we observed a similar significant rightward shift of the dose-response curves for TPM (Fig. 22) and also for ACTZ (Table 4). After Bay K 8644 (11µg/µl, i.c.v.) the ED$_{50}$ values of TPM and ACTZ, for protecting against tonic and clonic seizures, were increased by 2 and 2.75 fold and 1.77 and 1.8 fold, respectively (Table 4). By comparison, Bay K 8644 (11µg/µl, i.c.v.) increased the ED$_{50}$ values for CFM-2 for protecting against tonic and clonic seizures by only 1.32 and 1.27 fold and for NBQX 1.26 and 1.08 fold, respectively (not significantly different from vehicle control). Finally, the concomitant administration of a low dose of the phenylalkylamine L-type Ca$^{2+}$ channel antagonist verapamil (2.5 mg/kg, i.p.) together with TPM, also shifted its dose-response curves to the right and significantly increased
its ED$_{50}$ values by 1.6 and 1.4 fold for tonus and clonus, respectively, but was somewhat less potent than nifedipine in this respect.

**Anticonvulsant effects of TPM, ACTZ, CFM-2 and NBQX against AMPA-induced seizures in DBA/2 mice**

All the compounds we tested (except for ACTZ) protected against seizures induced by i.c.v. administration of AMPA in DBA/2 mice. NBQX was the most effective compound against tonus induced by AMPA, whereas TPM appeared more potent than CFM-2. ACTZ was ineffective up to the dose of 80 mg/kg (Table 5). Unlike audiogenic-induced seizures, nifedipine was unable to significantly affect the anticonvulsant properties of TPM, CFM-2 or NBQX against AMPA-induced seizures.

**Effects of anticonvulsants against 4-aminopyridine–induced seizures in ICR-CD1 mice**

TPM, ACTZ and CFM-2 protected against seizures induced by 4-aminopyridine (4-AP, 13.3 mg/kg) administered subcutaneously, whereas NBQX was inactive. The observed order of efficacy (based on ED$_{50}$’s for prevention of seizures) was: CFM-2 > TPM > ACTZ (Table 6). Nifedipine (2.5 mg/kg, i.p.) administered alone was unable to antagonize 4-AP-induced seizures, and when the same dose was administered in combination with the other anticonvulsant drugs there were no significant changes in the ED$_{50}$ values, despite a slight rightward shift of the TPM and ACTZ dose-response curves. The ED$_{50}$ values for the concomitant administration of TPM and nifedipine (2.5 mg/kg, i.p.) or ACTZ and nifedipine (2.5 mg/kg, i.p.) were 1.14 and 1.26 fold higher than control (TPM or ACTZ alone) respectively. Verapamil (2.5 mg/kg, i.p.) alone was also unable to significantly antagonize 4-AP-induced seizures or to significantly shift the dose-response curves for TPM, ACTZ or CFM-2 (Table 6).

**Effects of anticonvulsants against pentylenetetrazole–induced seizures in ICR-CD1 mice**

All compounds tested protected against seizures induced by pentylenetetrazole (PTZ, 60 mg/kg) administered i.p. The dose of PTZ used induced seizures in 80% of ICR-CD1
mice, most likely through partial impairment of GABAergic neurotransmission (De Sarro et al., 1999a, b). TPM was more effective than NBQX or ACTZ but less active than CFM-2 in protecting against PTZ-induced seizures (Table 6). When each anticonvulsant compound was administered concomitantly with a low dose of nifedipine or verapamil (2.5 mg/kg, i.p.) there were once again slight increases of the $ED_{50}$ values for all compounds tested (relative to controls), but these were not significant (Table 6).

**Anti-absence activity of TPM in lethargic (lh/lh) mice.**

*Effects of TPM on the number and mean duration of SWDs in lh/lh mice.*

TPM (20 and 40 mg/kg), induced a dose-dependent reduction in the incidence of SWDs, in lh/lh mice, this action being more evident during the 30-90 min post-injection period, even if a clear difference was noticeable during the 30-210 min period ($n = 8$ mice per dose; Fig. 23). The peak effect was observed during the 60-90 min epoch; all data reported below refer to this peak. Multiple range tests ($P < 0.05$ was considered significant) revealed that the mean number of SWD episodes during the 30 min epoch in the vehicle treated group ($19.57 \pm 2.14$) was greater than that in the TPM group ($11.33 \pm 1.53$, at 20 mg/kg and $4.43 \pm 1.06$, at 40 mg/kg) (Fig. 23 A) indicating an anti-absence effect of TPM. A lower dose of TPM (10 mg/kg) was unable to induce a significant reduction in SWD episodes in comparison with a group of mice receiving only vehicle ($n = 8$ mice) (Fig. 23 A). When measuring the mean total duration of SWDs, a multiple range test showed that in the TPM (20 and 40 mg/kg) group it was significantly smaller ($200 \pm 22.9$ s and $167 \pm 13.4$ s, respectively) than in both vehicle control ($315 \pm 15$ s) and TPM (10 mg/kg) groups ($280 \pm 14$ s) (Fig. 23 B).

*Effects of L-type Ca$^{2+}$ channel modulators on TPM-induced changes in the number and mean duration of SWDs in lh/lh mice.*

Nifedipine or Bay K 8644, 20 and 40μg/μl injected i.c.v alone, had no significant effect on the number of SWD episodes ($n = 8$ mice per dose, data not shown); multiple range tests revealed that the number of SWDs were similar to those of the vehicle treated group in each case. However, when nifedipine (20 μg/μl, i.c.v.) was administered
together with TPM (20 or 40 mg/kg) it surprisingly induced a significantly greater reduction in the mean number of SWDs (9.33 ± 1.53, at 20 mg/kg and 3.25 ± 0.87, at 40 mg/kg) compared with a group of mice receiving TPM plus vehicle alone (11.33 ± 1.53, 20 mg/kg or 4.43 ± 1.06, 40 mg/kg; \( P<0.05 \) for both groups; Fig. 24 A, B). This effect was more evident between 30 to 90 min after nifedipine-injection. Furthermore, a multiple range test showed that the mean total duration of SWDs was significantly smaller (170 ± 22.9 s, 20 mg/kg and 151 ± 11.2 s, 40 mg/kg) in the TPM plus nifedipine group than in either vehicle control (315 ± 15) or TPM plus vehicle groups (200 ± 22.9 s, 20 mg/kg and 167 ± 13.4 s, 40 mg/kg; \( P < 0.05 \) for both groups; Fig. 24 C,D). Thus, nifedipine appeared to enhance rather than inhibit the anti-absence effects of TPM in this model. In contrast, Bay K 8644 (20 \( \mu \)g/\( \mu \)l, i.c.v.) administered 30 min after TPM (20 or 40 mg/kg) injection, significantly increased the number and duration of SWDs (Fig. 24). In particular, during the 60-90 min recording epoch, there was a ~37% and a ~50% increase in the number of SWD episodes for 20 and 40 mg/kg TPM (11.33 ± 1.53, at 20 mg/kg and 4.43 ± 1.06, at 40 mg/kg control vs 17.21 ± 3.68, 20 mg/kg and 14.33 ± 3.55, 40 mg/kg TPM + Bay K 8644; \( P < 0.05 \) for both groups) and the duration was increased by ~13% and ~19% for 20 and 40 mg/kg TPM respectively (200 ± 22.9 s, at 20 mg/kg and 167 ± 13.4 s, at 40 mg/kg control vs 226 ± 24.2, 20 mg/kg and 198 ± 23.2, 40 mg/kg TPM + Bay K 8644; \( P < 0.05 \) for both groups).

Anti-absence activity of TPM in WAG/Rij rats.

Effects of TPM in WAG/Rij rats

Figure 25 illustrates a clear dose-dependent decrease in the number and total duration of SWDs in WAG/Rij rats after i.p administration of TPM; thus at 10, 20, 40 or 80 mg/kg TPM, the EEG activity showed a significantly smaller mean number of SWD episodes than control baseline and vehicle (*\( P < 0.05 \) or **\( P < 0.01 \), \( n = 8 \) animals for each dose, Fig. 25 A). We also observed a significant effect (*\( P < 0.05 \) or **\( P < 0.01 \), Fig. 25B) on the mean total duration of SWD episodes in these animals. The two highest concentrations of TPM caused the shortening of the total duration of SWDs by ~67% and ~86 %, respectively; this action was dose-dependent and the effects differed significantly from the control rats receiving only vehicle (Fig. 25). The effects of TPM became more evident during the 30-210 min time period, with the peak activity
occurring within the 60-90 epoch; all data reported refer to this peak. The mean number of SWDs shown by the WAG/Rij rats over a period of 30 min was 19.21 ± 4.07 with a total mean duration of 17.2 ± 1.3 min (n = 8); TPM 10, 20, 40 or 80 mg/kg significantly reduced the number of SWDs by 34.5, 54, 67.5 and 86.5 % (P < 0.01 for each group relative to control) respectively, whereas the duration was decreased significantly only at the three highest doses, by 65.7, 76.2 and 83.7 % respectively (P < 0.01 for each group). The time-course of the effects of TPM 20, 40 and 80mg/kg upon SWDs of WAG/Rij rats is shown in Fig. 26.

Effects of L-type calcium channel modulators alone and after concomitant treatment with nifedipine or Bay K 8644 and TPM in WAG/Rij rats

Administration of nifedipine (40µg/2µl; i.c.v.) alone had no effect on the number and total duration of SWDs in WAG/Rij rats, whereas, in contrast with the lethargic (lh/lh) mouse absence model, the number of episodes of SWDs following treatment with nifedipine 60 and 80µg/2µl i.c.v. showed clear differences from control recordings (Fig. 27). Thus, the mean number of SWDs, was significantly higher after nifedipine 60 or 80µg/2µl i.c.v. administration (18.42 ± 1.08 vehicle control; 25.5 ± 0.83 at 60µg; 27.83 ± 1.55 at 80µg; P < 0.01 at both dose levels) than in the pre-drug administration control or vehicle control (Fig. 27 A), and this effect appeared dose-related. A similar effect was observed for the total duration of SWDs, which increased after giving the two highest doses of nifedipine in comparison to vehicle control (n = 8 for each dose) (Fig. 27 B). Nifedipine was thus pro-epileptic in this WAG/Rij rat absence model. In contrast, when Bay K 8644 (40, 60 or 80µg/2µl i.c.v.) administered alone was tested, we observed a significant reduction in the mean number of SWDs at all doses (10.66 ± 0.7 at 40µg; 7 ± 1.6 at 60µg.; 5.83 ± 0.9 at 80µg; P<0.05 for all groups; n = 8) in comparison to control animals treated with vehicle only (18.83 ± 0.69, n = 8; Fig. 28 A), and this action was also dose-related. A similar effect was observed for the total duration of SWDs (n = 8 for each dose) (Fig. 28 B). The anticonvulsant effects of Bay K 8644 (40, 60 or 80µg/2µl i.c.v.) were always antagonised by a subsequent administration of the same dose of nifedipine and vice versa (data not shown), demonstrating that their effects in the model are due to their interaction with L-type Ca²⁺ channels.
We next tested the effects of the combined administration of TPM (40 mg/kg) with the lowest dose of nifedipine (40 μg/2 μl), which per se did not have any significant pro-absence effect; interestingly, in contrast with the results obtained in lh/lh mice, we found an antagonism of the anti-absence action of TPM. In fact, a significant increment in the mean number of SWDs (19 ± 3.6, \(P < 0.01, n = 8\)) was noticed in comparison with TPM 40 mg/kg administered alone (6.24 ± 1.8, Fig. 29 A). We, then tested the effect of a further administration of Bay K 8644 (40 μg/2 μl) on TPM (40 mg/kg)-treated rats, and found an enhancement of the anti-absence effects of TPM underlined by a further significant (\(P < 0.05\)) reduction in the mean number (3.75 ± 0.87 vs 6.24 ± 1.8 TPM only control; \(n = 8\)) and the duration of SWDs (Fig. 29 A, B).

*Effects of the drugs tested on behaviour and locomotor activity*

All drugs tested (at the doses administered for evaluating antiepileptic effects), did not significantly affect motor coordination in the rotarod test. However, the highest doses of TPM (50 mg/kg) and ACTZ (80 mg/kg) in mice induced a small reduction in the ability to remain on the rotarod for 5 min, but this was not significantly different from mice receiving the vehicle; no difference between the response of DBA/2, lh/lh and ICR-CD1 mice was noticed. All other drugs tested and experimental protocols (drug combinations) did not affect the test. In WAG/Rij rats, the dose of 80 mg/kg of TPM reduced non-significantly, the ability to remain on the rotarod, while all other doses did not modify it. Also, nifedipine and Bay K 8644 or their combination with TPM at all doses did not alter locomotor activity in this strain of rats. Observation of behavioural manifestations indicated that no clear differences between control animals and drug-treated animals where present.
II.2.6. Discussion

The present in vivo data show that TPM possesses potent anticonvulsant properties in various experimental models of genetically-determined or chemically-induced epilepsy, thus confirming its broad spectrum of anticonvulsant activity as previously reported (Rigoulot et al., 2003; De Sarro et al., 2000a, 2002b); in addition, we have demonstrated the anti-absence effects of TPM in two animal models of genetically-predetermined absence epilepsy, the lethargic (lh/lh) mouse and WAG/Rij rat models. The antiseizure properties of TPM were also compared with those of two AMPA/kainate receptor antagonists, CFM-2 and NBQX, and the carbonic anhydrase inhibitor, ACTZ. The wide range of activity shown by TPM in experimental models of generalized tonic-clonic and absence-like seizures is understandable in view of the multiple mechanisms of action possessed by this drug; in fact, it has been suggested that TPM acts as an anticonvulsant by inhibiting fast Na⁺ currents, like carbamazepine or phenytoin (DeLorenzo et al., 2000; Taverna et al., 1999; Zona et al., 1997), by enhancing GABAₐ neurotransmission differently from barbiturates or benzodiazepines (Gordey et al., 2000; White et al., 2000; Shank et al., 2000), by inhibiting excitatory neurotransmission similarly to felbamate but acting on non-NMDA receptors (Ängehagen et al., 2003a, b; Skradski & White, 2000; Gibbs et al., 2000) and by inhibiting carbonic anhydrase, like ACTZ (Stringer, 2000).

The reported effect of TPM on high voltage-activated (HVA) Ca²⁺ conductances however, remains controversial, since Zhang et al. (2000) initially proposed that TPM (at 10μM, but not at 50μM) blocked L-type Ca²⁺ currents in rat dentate granule cells in vitro, while our in vitro data on olfactory cortical brain slice neurones suggested that TPM (20μM) enhances L-currents (blocked by nifedipine); this difference in action could possibly reflect a different composition of the L-channels subunits in the two brain areas. In view of our in vitro findings, we evaluated the effect of the concomitant administration of the L-type calcium channel antagonists, nifedipine and verapamil or the L-type calcium channel agonist, Bay K 8644, on the anticonvulsant activity of TPM observed in various in vivo epilepsy models; our experiments provided some interesting and unexpected results. Firstly, in support of our in vitro data, nifedipine (or verapamil) were clearly able to antagonize the antiepileptic effect of TPM in some models (DBA/2 mouse and WAG/Rij rat) whereas in some others, they remained ineffective (AMPA, 4-AP or PTZ-induced seizures). Surprisingly, in the two absence epilepsy models we
tested, we found opposite effects of nifedipine; in the WAG/Rij rat the dihydropyridine was able to antagonize the anti-absence effect of TPM, while in the lethargic lh/lh mouse, nifedipine and TPM acted synergistically.

L-type Ca\(^{2+}\) channel blockers are known to act as anticonvulsant agents in many animal models of generalized tonic-clonic epilepsy (De Sarro et al., 1986, 1988, 1990), and this has been explained by considering the electrophysiological consequences of neuronal L-type transmembrane Ca\(^{2+}\) entry, subsequent Ca\(^{2+}\) dynamics and Ca\(^{2+}\)-dependent processes and their contribution to epileptogenesis (Pal et al., 2001; Zapater et al., 1998). It is also been reported that the concomitant administration of L-channel blockers with other anticonvulsant drugs leads to a general increment of anticonvulsant potency in many animal models (Gasior et al., 1996; De Sarro et al., 1992b). Furthermore, several of the animal models of absence epilepsy used in research have been associated with mutations in genes encoding specific Ca\(^{2+}\) channel proteins (channelopathies) (Felix, 2002; Pietrobon, 2002). Based on this background, our results show that TPM was very potent against sound-induced seizures in DBA/2 mice with ED\(_{50}\) values of 6.12 and 12.12 mg/kg for tonus and clonus, respectively. When TPM was administered together with a low dose of nifedipine, which alone had no anticonvulsant activity, the latter caused an apparent shift to the right of the TPM dose response curve, and significantly increased its ED\(_{50}\) for protecting from tonic and clonic seizures by 1.9 and 1.7 fold respectively. This antagonism of the anticonvulsant properties of TPM was specific, since it was not evident when nifedipine was administered concomitantly with the glutamate receptor antagonists CFM-2 or NBQX or the carbonic anhydrase inhibitor ACTZ suggesting an interaction at the level of the L-type calcium channel as we predicted. The fact that a low dose of the non-dihydropyridine L-channel blocker verapamil administered with TPM also produced the same effect (TPM ED\(_{50}\) values were increased 1.6 and 1.4 fold for tonus and clonus, respectively) suggests that TPM may interact with the L-channel at a different site from the dihydropyridine binding site. Interestingly, a low dose of the dihydropyridine L-channel agonist Bay K 8644 also shifted the dose response curve for TPM to the right; however, the latter result was not specific for TPM, since all anticonvulsant compounds tested were antagonized.

In order to better evaluate the importance of this postulated L-type Ca\(^{2+}\) channel modulation by TPM in a model in which the generation of seizures was due to an
enhanced glutamatergic neurotransmission, we tested the efficacy of TPM against AMPA-induced seizures in DBA/2 mice. In this model, TPM appeared to be a less potent anticonvulsant than the competitive AMPA/kainate receptor antagonist NBQX, which is in accordance with its reported ability to preferentially inhibit kainate-induced currents (Skradski & White, 2000, Gibbs et al., 2000), but more effective than CFM-2 which is an allosteric AMPA antagonist (De Sarro et al., 1999c). ACTZ (20-80 mg/kg) however, was completely ineffective within the range of doses tested, showing that the possible role of carbonic anhydrase inhibition by TPM in this model is not relevant. The concomitant administration of nifedipine with all drugs did not have any effect on their anticonvulsant properties, suggesting that of all the mechanisms of action possessed by TPM, the modulation of the L-type Ca\(^{2+}\) currents is not crucial in preventing the generation of tonic-clonic seizures by AMPA. TPM was also able to exert a certain protection against seizures induced by PTZ and the K\(^+\) channel blocker 4-AP. It has already been reported that TPM and ACTZ are ineffective against seizures induced by the CD\(_{97}\) dose of PTZ (~85 mg/kg); we used, instead, a lower PTZ dose (60 mg/kg) which is able to produce a partial impairment of GABAergic neurotransmission and to induce seizures in 80% of ICR-CD1 mice (De Sarro et al., 1999a, b). Against this dose of PTZ, TPM was slightly more effective than ACTZ, but less potent than CFM-2, whereas NBQX was ineffective at the doses tested. Regarding, the ability of all compounds tested to block seizures induced by the administration of the CD\(_{97}\) dose of 4-AP, we had a similar result. In particular, NBQX and ACTZ were completely ineffective or weakly effective within the range of doses tested, whereas CFM-2 was more potent than TPM. In both models, when the usual low dose of nifedipine was administered concomitantly with the anticonvulsants, there were no significant changes in the ED\(_{50}\) values for any drug, even though a slight rightward shift of the dose-response curve for TPM was noticeable.

To determine the efficacy of TPM against generalized absence-like seizures we used two genetic animal models, lh/lh mice and WAG/Rij rats, widely recognized to predict the efficacy of drugs in treating absence epilepsy (Coenen & Van Luijtelaar, 2003; Renier & Coenen, 2000; Hosford et al., 1992). No previous data exist on the anti-absence effects of TPM in WAG/Rij rats, whereas Hosford & Wang (1997) reported that TPM was weakly effective in the lh/lh mice absence model. Rigoulot et al. (2003) recently demonstrated that TPM possesses anti-absence properties in the genetic
absence epilepsy rat from Strasbourg (GAERS), another model of absence epilepsy. The lethargic (lhlh) mutant mouse and the WAG/Rij rat express spontaneous absence seizures that share behavioural, electrographic, and pharmacological features similar to those of generalized absence seizures among humans (Renier & Coenen, 2000; Hosford et al., 1999; Van Luijtelaar & Coenen, 1986). The mutation in the Ca\(^{2+}\) channel subunit gene that underlies the development of this characteristics in lhlh mice causes a faulty expression of the \(\beta_4\) subunit (which normally binds to \(\alpha_1\) subunits), resulting in an altered functionality of low and high voltage-activated Ca\(^{2+}\) channels (Zhang et al., 2002; Hosford et al., 1999; Lin et al., 1999). In this model, TPM induced a significant reduction of the number and the mean duration of SWD episodes at the two highest doses tested, in a dose-related manner. This result contrasts with that of Hosford & Wang (1997) where a lack of effect of TPM was found; however, we believe that the different doses we used and our different analytical evaluation (number and duration of SWD episodes every 30 min of recording) permitted us to better evaluate the absence phenomena, and to detect an effect of TPM. The i.c.v. administration of low doses of nifedipine or Bay K 8644 produced no significant changes in the incidence and duration of SWDs, whereas on concomitant administration of TPM with either of the two L-type Ca\(^{2+}\) channel modulators we found that nifedipine enhanced the anti-absence effect of TPM whereas Bay K 8644 antagonized its effectiveness.

The mechanisms that underlie the absence characteristics in WAG/Rij rats are not yet completely understood; all the individuals of this strain spontaneously show a high number of generalized SWDs which are the EEG manifestation of absence seizures. These SWDs, which have a typical spike frequency of 7–11 Hz, are the reflection of highly synchronized and highly rhythmic oscillations in the cortico-thalamocortical network (Inoue et al., 1993). It has recently been shown that LY 300164, an AMPA/kainate receptor antagonist, was able to slightly reduce the number of SWDs at 30-60 min after injection in WAG/Rij rats (Kaminski et al., 2001) and Van Luijtelaar et al. (2000) showed that L-type Ca\(^{2+}\) channel modulators alone were able to influence the incidence of SWDs; in particular, nimodipine, administered i.p. was able to increase the number of SWDs (pro-absence effect) and Bay K 8644, directly injected into the cerebral ventricle was able to decrease the number of SWDs (anti-absence effect). We found that in this genetic animal model of absence epilepsy, TPM was also able to significantly reduce the number and the mean duration of SWDs at all doses tested. The
ability of TPM to selectively antagonise AMPA/kainate currents and to enhance L-channel Ca\(^{2+}\) conductances could eventually explain its activity in this model, in keeping with findings of Van Luijtelaar et al. (2000). When the effects of the i.c.v. administration of either nifedipine or Bay K 8644 at various doses were tested, we confirmed the results obtained Van Luijtelaar et al. (2000), but we noticed that the lowest dose of nifedipine studied (40\(\mu\)g, i.c.v.) had no effect. When this dose of nifedipine was administered concomitantly with a dose of 40 mg/kg of TPM, we observed opposite effects to those obtained in \(lh/lh\) mice; thus, as in the generalized seizure models, nifedipine was apparently able to antagonize the anti-absence effect of TPM also in this model, whereas Bay K 8644 appeared to enhance its anti-absence properties. Therefore, an involvement of dihydropyridine-sensitive L-type Ca\(^{2+}\) channels in the anti-absence effects of TPM might also be considered.

In conclusion, the present results support the very effective broad spectrum of antiepileptic activity of TPM, its multiple mechanisms of action permitting this drug to block seizures in all models used in this present work and confirming the ability of TPM to be effective in both models of tonic-clonic and absence-like epilepsy. We showed for the first time, that in accordance with the predictions of our initial \textit{in vitro} brain slice data, nifedipine was indeed able to modify the anticonvulsant properties possessed by TPM. This action possessed by the dihydropyridine seemed to be modelsensitive, which could be explained if, depending on the model, some of the anticonvulsant mechanisms of action possessed by TPM were more predominant than others. For example, it is well known that antiepileptic drugs such as carbamazepine and phenytoin, that induce an activity-dependent attenuation of voltage-dependent Na\(^{+}\) channels, prevent 4-AP-induced seizures but they exacerbate SWDs in both models of absence epilepsy (Meldrum & Chapman, 1999). The results for the concomitant administration of TPM and nifedipine in the two absence models are apparently in contrast. This could be explained by the different sensitivity of WAG/Rij rats and \(lh/lh\) mice to nifedipine as aforementioned and demonstrated. It may also be considered that \(lh/lh\) mice have a truncated Ca\(^{2+}\) channel \(\beta_4\) subunit (Lin et al., 1999), which probably affects the normal dihydropyridine-TPM-L-channel interaction. Finally, in the light of our data, we would predict that nifedipine or other L-channel blockers could affect TPM’s antiepileptic efficacy in human epilepsy, and therefore their combination or
concomitant administration in therapy should be avoided or at least carefully monitored where appropriate.
### Table 4: Calculated ED$_{50}$’s for audiogenic seizures in DBA/2 mice

Effects of TPM, ACTZ, CFM-2 and NBQX in combination with vehicle, nifedipine, verapamil or Bay K 8644 against audiogenic seizures induced in DBA/2 mice.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose range (mg/kg)</th>
<th>Tonic extension</th>
<th>Clonic seizures</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPM + vehicle</td>
<td>2.5-50</td>
<td>6.12 (3.48-10.74)</td>
<td>12.12 (6.94-21.15)</td>
</tr>
<tr>
<td>TPM + nifedipine</td>
<td>2.5-50</td>
<td>11.93 (6.81-20.88)**</td>
<td>20.34 (12.78-32.36)**</td>
</tr>
<tr>
<td>TPM + Bay K 8644</td>
<td>2.5-50</td>
<td>16.86 (11.95-23.79)**</td>
<td>24.13 (17.41-33.44)**</td>
</tr>
<tr>
<td>TPM + verapamil</td>
<td>2.5-50</td>
<td>10.23 (5.60-18.69) *</td>
<td>17.54 (11.77-26.14) *</td>
</tr>
<tr>
<td>ACTZ + vehicle</td>
<td>5-80</td>
<td>25.5 (14.8-44.0)</td>
<td>36.4 (20.8-63.8)</td>
</tr>
<tr>
<td>ACTZ + nifedipine</td>
<td>5-80</td>
<td>22.1 (13.6-35.9)</td>
<td>31.6 (19.4-51.5)</td>
</tr>
<tr>
<td>ACTZ + Bay K 8644</td>
<td>5-80</td>
<td>44.6 (25.9-76.8)**</td>
<td>65.5 (37.4-114.7)**</td>
</tr>
<tr>
<td>CFM-2 + vehicle</td>
<td>1-10</td>
<td>3.73 (2.37-5.87)</td>
<td>4.44 (2.66-7.41)</td>
</tr>
<tr>
<td>CFM-2 + nifedipine</td>
<td>1-10</td>
<td>3.84 (2.19-6.73)</td>
<td>4.78 (2.46-9.29)</td>
</tr>
<tr>
<td>CFM-2 + Bay K 8644</td>
<td>1-10</td>
<td>4.92 (3.36-7.2)</td>
<td>5.63 (3.42-9.27)</td>
</tr>
<tr>
<td>NBQX + vehicle</td>
<td>2-20</td>
<td>4.07 (2.1-7.89)</td>
<td>6.26 (3.73-10.51)</td>
</tr>
<tr>
<td>NBQX + nifedipine</td>
<td>2-20</td>
<td>4.49 (2.72-7.41)</td>
<td>6.41 (3.56-11.54)</td>
</tr>
<tr>
<td>NBQX + Bay K 8644</td>
<td>2-20</td>
<td>5.15 (3.41-7.78)</td>
<td>6.73 (4.87-9.30)</td>
</tr>
</tbody>
</table>

All data reported above are expressed as mg/kg and were calculated with a computer program according to the method of Litchfield & Wilcoxon (1949). Values in parentheses are 95% confidence limits. * $P<0.05$, **$P<0.01$, statistically significant differences from vehicle-drug control group.
Table 5: Calculated ED$_{50}$'s for AMPA-induced seizures in DBA/2 mice

Effects of TPM, ACTZ, CFM-2 and NBQX in combination with vehicle or nifedipine against AMPA-induced seizures in DBA/2 mice.

<table>
<thead>
<tr>
<th>Treatment (time)</th>
<th>Dose range (mg/kg)</th>
<th>Tonic extension</th>
<th>Clonic seizures</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPM + AMPA</td>
<td>5-40</td>
<td>6.8 (4.9-9.44)</td>
<td>8.21 (6.17-10.92)</td>
</tr>
<tr>
<td>TPM + nifedipine + AMPA</td>
<td>5-40</td>
<td>8.1 (5.88-11.16)</td>
<td>9.85 (7.40-13.11)</td>
</tr>
<tr>
<td>ACTZ + AMPA</td>
<td>10-80</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>ACTZ + nifedipine + AMPA</td>
<td>10-80</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>CFM-2 + AMPA</td>
<td>1-20</td>
<td>7.4 (4.89-11.34)</td>
<td>9.48 (6.87-13.08)</td>
</tr>
<tr>
<td>CFM-2 + nifedipine + AMPA</td>
<td>1-20</td>
<td>7.05 (5.15-9.65)</td>
<td>8.62 (6.18-12.02)</td>
</tr>
<tr>
<td>NBQX + AMPA</td>
<td>1-10</td>
<td>2.53 (1.6-4.0)</td>
<td>5.64 (3.69-8.62)</td>
</tr>
<tr>
<td>NBQX + nifedipine + AMPA</td>
<td>1-10</td>
<td>2.97 (2.01-4.39)</td>
<td>2.62 (2.19-3.13)</td>
</tr>
</tbody>
</table>

All data reported above are expressed as mg/kg and were calculated with a computer program according to the method of Litchfield & Wilcoxon (1949). Values in parentheses are 95% confidence limits.
Table 6: Calculated ED$_{50}$'s for 4-AP- and PTZ-induced seizures in mice

Effects of TPM, ACTZ, CFM-2 and NBQX in combination with vehicle, nifedipine or verapamil against 4-aminopyridine or pentylenetetrazole-induced seizures in ICR-CD1 mice.

<table>
<thead>
<tr>
<th>Treatment (time)</th>
<th>Dose range</th>
<th>4-aminopyridine</th>
<th>pentylentetrazole</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPM</td>
<td>5-40</td>
<td>17.29 (11-27.17)</td>
<td>22.44 (16.17-31.14)</td>
</tr>
<tr>
<td>TPM + verapamil</td>
<td>5-40</td>
<td>16.82 (10.84-26.1)</td>
<td>21.51 (16.76-27.61)</td>
</tr>
<tr>
<td>ACTZ</td>
<td>5-120</td>
<td>95.48 (53.84-169.31)</td>
<td>26.4 (17.56-39.71)</td>
</tr>
<tr>
<td>ACTZ + nifedipine</td>
<td>5-120</td>
<td>&gt;120</td>
<td>31.41 (20.9-47.21)</td>
</tr>
<tr>
<td>ACTZ + verapamil</td>
<td>5-120</td>
<td>&gt;120</td>
<td>29.83 (19.84-44.85)</td>
</tr>
<tr>
<td>CFM-2</td>
<td>1-20</td>
<td>8.44 (6.13-11.61)</td>
<td>7.04 (3.64-13.64)</td>
</tr>
<tr>
<td>CFM-2 + nifedipine</td>
<td>1-20</td>
<td>9.12 (7.04-11.74)</td>
<td>8.65 (6.69-11.18)</td>
</tr>
<tr>
<td>CFM-2 + verapamil</td>
<td>1-20</td>
<td>10.43 (7.56-14.39)</td>
<td>8.2 (5.48-12.27)</td>
</tr>
<tr>
<td>NBQX</td>
<td>5-40</td>
<td>NA</td>
<td>28.88 (24.04-34.74)</td>
</tr>
<tr>
<td>NBQX + nifedipine</td>
<td>5-80</td>
<td>NA</td>
<td>30.6 (12.44-74.99)</td>
</tr>
<tr>
<td>NBQX + verapamil</td>
<td>5-80</td>
<td>NA</td>
<td>29.3 (24.39-35.2)</td>
</tr>
</tbody>
</table>

All data reported above are expressed as mg/kg and were calculated with a computer program according to the method of Litchfield & Wilcoxon (1949). Values in parentheses are 95% confidence limits.
3.0-Hz spike-wave complexes during a seizure in a child with absence epilepsy. Image kindly supplied by Dr. A. Siniscalchi, Department of Neurology, “Annunziata” Hospital, Cosenza, Italy.
Figure 21: Calcium channel structure, pharmacology and distribution

<table>
<thead>
<tr>
<th>Subunit</th>
<th>Voltage (V&lt;sub&gt;net&lt;/sub&gt;)</th>
<th>Name</th>
<th>Isoform</th>
<th>Type</th>
<th>Pharmacology</th>
<th>Tissue distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Membrane spanning and pore forming α&lt;sub&gt;1&lt;/sub&gt;</strong></td>
<td>High (HVA)</td>
<td>Cav1</td>
<td>.1 α&lt;sub&gt;1S&lt;/sub&gt;</td>
<td>L</td>
<td>Dihydropyridine</td>
<td>Skeletal muscle</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>.2 α&lt;sub&gt;1C&lt;/sub&gt;</td>
<td></td>
<td></td>
<td>Heart &amp; smooth muscle, CNS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>.3 α&lt;sub&gt;1D&lt;/sub&gt;</td>
<td></td>
<td></td>
<td>CNS, smooth &amp; heart muscle, endocrine glands</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>.4 α&lt;sub&gt;1F&lt;/sub&gt;</td>
<td></td>
<td></td>
<td>Retina</td>
</tr>
<tr>
<td>Cav2</td>
<td></td>
<td>.1 α&lt;sub&gt;2A&lt;/sub&gt;</td>
<td>P/Q</td>
<td></td>
<td>ω-Agatoxin IVA</td>
<td>CNS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>.2 α&lt;sub&gt;2B&lt;/sub&gt;</td>
<td>N</td>
<td></td>
<td>ω-Conotoxin GVIA</td>
<td>CNS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>.3 α&lt;sub&gt;2E&lt;/sub&gt;</td>
<td>R?</td>
<td></td>
<td>SNX-482</td>
<td>CNS</td>
</tr>
<tr>
<td><strong>Low (LVA)</strong></td>
<td></td>
<td>Cav3</td>
<td>.1 α&lt;sub&gt;3G&lt;/sub&gt;</td>
<td>T</td>
<td>Mibefradil (not very selective)</td>
<td>CNS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>.2 α&lt;sub&gt;3H&lt;/sub&gt;</td>
<td></td>
<td>Ni&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Heart and elsewhere</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>.3 α&lt;sub&gt;3I&lt;/sub&gt;</td>
<td></td>
<td></td>
<td>CNS</td>
</tr>
<tr>
<td>Auxiliary extracellular (α&lt;sub&gt;2&lt;/sub&gt;) &amp; membrane spanning (δ)</td>
<td></td>
<td></td>
<td>(α&lt;sub&gt;2δ&lt;/sub&gt;)&lt;sub&gt;1.3&lt;/sub&gt;</td>
<td></td>
<td>Gabapentin? - with α&lt;sub&gt;2δ&lt;/sub&gt;-2</td>
<td>CNS</td>
</tr>
<tr>
<td>Auxiliary intracellular</td>
<td></td>
<td></td>
<td>β&lt;sub&gt;1-4&lt;/sub&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Auxiliary membrane spanning</td>
<td></td>
<td></td>
<td>γ&lt;sub&gt;1-8&lt;/sub&gt;</td>
<td></td>
<td>γ&lt;sub&gt;1&lt;/sub&gt;-with Cav1.1 in muscle, γ&lt;sub&gt;2&lt;/sub&gt; in brain</td>
<td></td>
</tr>
</tbody>
</table>
Dose-response curves for the anticonvulsant effect of TPM (2.5-50 mg/kg) + vehicle (●), TPM + nifedipine 2.5 mg/kg (■) and TPM + Bay K 8644 11 μg/μl (▲) in DBA/2 mice. Abscissae show the drug doses, ordinates show in (A) % of clonic seizures, and in (B) % of tonic seizures induced by audiogenic stimuli. Note the rightward shift of the TPM dose-response curves by concomitant administration of nifedipine or Bay K 8644.

Dose-response curves where constructed by directly plotting the data, and fitting curves by eye. Therefore, the ED₅₀’s estimated directly from the graph appear slightly different from the ones calculated by computer program with the method of Litchfield and Wilcoxon (1949).
Figure 23: Effects of TPM against absence seizures in lethargic (lh/lh) mice

Anti-absence effects of TPM (10, 20 and 40 mg/kg; n=8 for each dose) monitored by EEG recordings of lethargic (lh/lh) mice: (A) Time- and dose-dependent reduction of the number of epileptic spike wave discharges (nSWDs) by TPM; (B) Effects of increasing doses of TPM on the cumulative duration of SWDs (dSWDs). Values are expressed as mean ± S.E.M. and represent each 30-min period of recording.
Effects of a combined administration of a fixed dose (40 μg/μl) of nifedipine or Bay K 8644 together with TPM (20 or 40 mg/kg) on SWDs in lethargic lh/lh mice: (A) and (C) show the variations in TPM’s (20 mg/kg) anti-absence effect, when co-administered with nifedipine or Bay K 8644, on the number (A) and the total duration (C) of SWDs. (B) and (D) show the modification of anti-absence activity by nifedipine or Bay K 8644, concomitantly administered with a higher dose of TPM (40 mg/kg). Values are expressed as mean ± S.E.M. and represent each 30-min period of recording (n = 8 for each dose).
Dose-dependent anti-absence action of TPM (10, 20, 40, 80 mg/kg administered i.p.; \( n = 8 \) for each dose) on the number (A) and the cumulative duration (B) of SWDs recorded in WAG/Rij rats. Values are expressed as mean ± S.E.M. and represent the 60-90 min period of recording, at the peak of TPM's activity. Significant differences between drug-treated groups and vehicle-treated animals are denoted by * \( P<0.05 \) or ** \( P<0.01 \).
Time- and dose-dependent effects of various doses of TPM (10, 20, 40 and 80 mg/kg; \( n = 8 \) for each dose) on the number (A) and total duration (B) of SWDs in WAG/Rij rats. Values are expressed as mean ± S.E.M. and represent each 30-min period of recording.
Figure 27: Effects of nifedipine against absence seizures in WAG/Rij rats

A

Time- and dose-dependent activity of nifedipine (40, 60, 80 µg/2µl i.c.v.; $n = 8$ for each dose) administered alone on the number (A) and the total duration (B) of SWDs in WAG/Rij rats. Values are expressed as mean ± S.E.M. and represent the 60-90 min period of recording, at the peak of TPM's activity.
Figure 28: Effects of Bay K 8644 against absence seizures in WAG/Rij rats

Time- and dose-dependent activity of Bay K 8644 (40, 60, 80 μg/2μl; n = 8 for each dose) administered i.c.v. on the number (A) and the total duration (B) of SWDs in WAG/Rij rats. Values are expressed as mean ± S.E.M. and represent the 60-90 min period of recording, at the peak of TPM's activity.
Effects of a combined administration of a fixed dose (40 μg/μl) of nifedipine or Bay K 8644 with TPM (40 mg/kg) on EEG recordings of WAG/Rij rats. (A) shows changes in the anti-absence effect of TPM (40 mg/kg), when co-administered with nifedipine or Bay K 8644, on the number of SWDs; (B) shows the ability of nifedipine or Bay K 8644, concomitantly administered with TPM (40 mg/kg), to modify its anti-absence activity on the total duration of SWDs. Values are expressed as mean ± S.E.M. and represent each 30-min period of recording (n = 8 for each dose).
Chapter III:

General Discussion and Conclusions
III. General Discussion and Conclusions

The intricate functions of the brain and their pharmacology are at same time the most studied but the least well understood. The detailed study of neurological diseases has only developed relatively recently, compared with the time these diseases have been in existence. Epilepsy (or the various epilepsies) for example, is truly an ancient disorder, which has probably existed since the dawn of civilization, and most likely evolved along with the development of mankind over the centuries, to become what we regard today as the “family” of epilepsy syndromes. More than a century of studies and efforts has not been enough to classify and fully understand the mechanisms underlying the aetiology of this disease. Nevertheless, many results have been achieved, new pharmacological treatments are now available and some of the underlying mechanisms have been clarified, but still a lot of work needs to be done. The last century has seen the introduction of the most effective drugs for the treatment of epilepsies, whereas in the last 20 years, with the increasing development of new technologies, new light has been shed on the genetics of the disorder and consequently on some (often surprising, and seemingly unrelated) mechanisms involved in this disease. Furthermore, many new techniques and means have now been introduced to help scientists and physicians in their work. The practical result of all these efforts, is the actual use in clinical practice of many effective AEDs and therefore, the improvement in well-being of millions of epilepsy sufferers all around the world. Most of the current drugs available were introduced into the market before their exact mechanism of action was known (e.g. phenobarbitone, diazepam, valproate, carbamazepine, levetiracetam). Certainly, the most important characteristic of an AED is its efficacy. Only few drugs have been the result of a rational drug design (e.g. tiagabine, vigabatrin) whereas most of the others were discovered quite by chance (e.g. valproate, carbamazepine, topiramate). From numerous studies on epilepsy and AEDs, some important fundamental mechanisms of action have been proposed, but many more are probably waiting to be discovered. From what it is known and generally accepted, the four main mechanisms that best suit AEDs are:

- Inhibition of voltage-dependent Na\(^+\) channels;
- Enhancement of GABA\(_A\)-mediated inhibitory neurotransmission;
- Inhibition of excitatory (glutamate) neurotransmitter activity;
Inhibition of voltage- and receptor- dependent Ca\(^{2+}\) channels.

All four points somehow are connected to the general hypothesis that seizure generation in epilepsy is most probably due to a paroxysmal electrical disturbance (imbalance) of brain signalling. Most of the mechanisms of action possessed by AEDs are probably known but the need to further study and define their action is of crucial importance. The discovery of new effects of established AEDs could help in the understanding of the aetiology of epilepsy and the design of new drugs. In this regard, TPM represents one of the most intriguing drugs available today. TPM is widely used in epilepsy therapy and it has also found use in some other neurological diseases (McElroy et al., 2003; Privitera et al., 2003; Suppes, 2002; Berlant & van Kammen, 2002; Zvartau-Hind et al., 2000; Bauer & Schwalen, 2000; Pascual, 1999). Its anticonvulsant properties were discovered quite by chance, during routine testing of a novel series of compounds developed as possible gluconeogenesis inhibitors (Bauer & Schwalen, 2000); it has been demonstrated to possess anticonvulsant actions in many different animal models of epilepsy (see Section I.4.3).

At the beginning of the present project, we set out to compare the possible effects of TPM and other AEDs on the OXO-M-induced bursting phenomenon previously observed in deep pyramidal neurones of immature PC slices by Postlethwaite et al. (1998). In addition, in light of the report by Palmieri et al. (2000) showing that TPM depressed depolarizing plateau potentials induced by carbachol in rat subicular cells (Palmieri et al., 2000), we were particularly interested to test the effects of TPM against the post-stimulus sADP induced by OXO-M in deep PC neurones (Constanti et al., 1993), since this could represent a novel new target for the mechanism of action of this antiepileptic compound. Although our preliminary experiments showed that TPM was indeed an effective inhibitor of OXO-M-induced bursting in immature PC slices and significantly reduced the amplitude of the sADP (E. Russo, B.J. Whalley & A. Constanti, unpublished data), it soon became apparent that TPM had additional unexpected membrane effects that deserved further attention; our study therefore focused on these and other possible novel effects, in an attempt to understand their origin and possible significance towards TPM's anticonvulsant efficacy.

As previously mentioned, the PC is an epilepsy-prone area involved in the generation and the maintenance of limbic seizures (Löschler & Ebert, 1996; see Section II.1.1.3).
When TPM (20 μM) was first tested on rat PC brain slices, (Section II.1) it was noted that it induced two distinct main actions:

1) a slow, dose-dependent and reversible membrane hyperpolarization, accompanied by a decrease in membrane input resistance and inhibition of repetitive action potential firing, and

2) an enhancement and prolongation of the post-stimulus slow afterhyperpolarization (sAHP), that follows a relatively long burst of action potentials.

Both of these effects were considered capable of contributing to TPM’s anticonvulsant activity. Up till then, the known and accepted mechanisms of action for TPM were:

- State-dependent inhibition of neuronal Na⁺ channels (DeLorenzo et al., 2000; Taverna et al., 1999; Zona et al., 1997);
- Increased neuronal GABA-induced Cl⁻ influx (Gordey et al., 2000; White et al., 2000; Shank et al., 2000);
- Inhibition of AMPA/kainate receptors (Ängehagen et al., 2003b; Skradski & White, 2000; Gibbs et al., 2000);
- Inhibition of voltage-activated Ca²⁺ channels (White & Privitera, 2000; Zhang et al., 2000);
- Inhibition of carbonic anhydrase (CA) activity (Casini et al., 2003; Dodgson et al., 2000).

The only report at the time previously describing a hyperpolarizing effect of TPM was by Kawasaki et al. (1998) on rat subicular neurones, but they claimed that this effect was reversed by bath-application of bicuculline, suggesting that the phenomenon was GABA_A-mediated; this type of hyperpolarization has not been described in any other preparation. The standard dose of 20 μM TPM used in our electrophysiological studies is in accordance with the therapeutic range (100-200 mg/day; Wolf et al., 2000) and when a similar dose is administered to rodents no sedation or other behavioural alterations are reported (Shank et al., 2000). This suggests that the effects of TPM on membrane properties, taken together with all its other effects at this dose, do not impair normal brain functions.
The first observed hyperpolarizing action of TPM, (if it were confined to cortical cells), might stabilize these neurones, making them less susceptible to depolarizing stimuli, and blocking the generation and cortical spread of seizures. Unfortunately, the exact mechanism underlying the membrane hyperpolarization has not been completely explained. It was clear that the outward current generated by bath-application of TPM under voltage clamp ($I_{TPM}$) was largely carried out by K$^+$ ions. During the course of our study, this hypothesis was also independently advanced by Herrero et al. 2002 following experiments with TPM action on hippocampal CA1 neurones (see Section II.1). However, our end results differ considerably from those of Kawasaki et al. (1998) and Herrero et al. 2002 in several respects. Thus, our observed TPM-induced hyperpolarization in PC neurones was definitely not GABA$_A$-mediated, not induced by pH changes due to CA inhibition, nor related to Na$^+$/K$^+$ pump activity, or Ca$^{2+}$- or Na$^+$ dependent (via TTX-sensitive channels). The outward current generated by TPM was not completely blocked by Ba$^{2+}$ or Cs$^+$, which are known to be K$^+$ channel blockers and furthermore, in contrast with Herrero et al. (2002), $I_{TPM}$ did not reverse at more negative potentials than the estimated $E_K$ (~95 mV) in these cells (see Sections II.1.3.1 and II.1.4.1). This clearly showed that the TPM-induced outward current is mainly carried by K$^+$ ions but some other electrogenic mechanism(s) might also be involved. However, it is not clear if these other mechanisms are activated at all potentials or only at negative potentials when trying to reverse the outward current. It is also possible that TPM may activate a K$^+$ channel that is inactivated at negative potentials and therefore, $I_{TPM}$ is not easily reversible; in this regard, further specific studies are necessary.

The second observed effect of TPM has not to our knowledge, been reported in any other cell type to date (Leniger et al., 2004a; Herrero et al., 2002; DeLorenzo et al., 2000; Jahromi et al., 2000; Hanaya et al., 1998; Kawasaki et al., 1998). Our results clearly showed that TPM enhanced and prolonged the sAHP elicited after a relatively long burst of action potentials and that this indeed, was due to an increased influx of Ca$^{2+}$ ions through L-type Ca$^{2+}$ channels (although a possible direct enhancing action on the Ca$^{2+}$-activated K$^+$ conductance itself cannot be excluded). This mechanism might also be important for TPM's anticonvulsant properties; it could block both the generation and propagation of seizures by prolonging the refractory state of the neurones in the epileptic foci and/or retard the generation of a second discharge and the
spread to brain areas secondary involved. From the results, it may be underlined that the effect of TPM on both L-type Ca\(^{2+}\) current and the sAHP were mainly *modulatory*, in that the peak of both currents was not as much enhanced as it was prolonged in time; therefore, this suggests that a modulation of the currents is more easily achieved than a pure increase in conductance.

In this project, attention was also focused on a possible role of CA inhibition by TPM and consequent changes in pH\(\text{in}/\text{pHe}\). It was demonstrated that all the membrane effects of TPM could be mimicked by ACTZ (and also by benzolamide), and that moreover, they could be reproduced in a bicarbonate/CO\(_2\)-free HEPES-buffered medium, suggesting they were not dependent on CA modulation. These findings suggest that the common sulphonamide moiety present in these molecules could represent a highly active chemical grouping capable of binding to many natural substrates and modifying cellular function (for most recent review see Winum et al., 2004). It might not be a coincidence that four established AEDs (TPM, ACTZ, sulthiame, and zonisamide) possess this moiety in their structure. Specific structure-activity studies and a detailed comparison of their mechanisms of action might therefore be useful. Interestingly, in a recent study by Lenniger et al. (2004a) on hippocampal CA3 neurones, TPM was found to decrease pH\(\text{i}\) in CO\(_2/\text{HCO}_3^-\)-buffered solution, *without* affecting the membrane potential or input resistance (in contrast to the present data), whereas in the absence of extracellular Na\(^+\), pH\(\text{i}\) was increased (suggesting influx of HCO\(_3^-\)). It was suggested that TPM lowered neuronal pH\(\text{i}\) by a dual effect on Na\(^+\)-independent Cl\(^-/\text{HCO}_3^-\)- exchange and CA, and that these actions were contributing to the anticonvulsive property of TPM in these cells; however, whether Cl\(^-/\text{HCO}_3^-\)- exchange is important for generating the observed membrane hyperpolarizing effects of TPM on PC neurones, remains uncertain. Clearly, TPM is capable of producing multiple effects on central neurones, which suggests that the mechanisms involved in inhibiting seizure generation by this drug may be different in different cell types, being dependent on a combination of several factors.

At present, TPM is the only known AED acting to modulate a Ca\(^{2+}\)-activated K\(^+\) current (albeit indirectly) via the *enhancement* of an underlying L-type Ca\(^{2+}\) conductance. If the latter effect generally predominated, it would clearly be *pro*-convulsant; however, it is normally counterbalanced by the resultant induction of the sAHP. This is in contrast with the widely held hypothesis that anticonvulsant drugs should be *blockers* of Ca\(^{2+}\)
channels. From the results of the electrophysiology section, two important questions were raised:

- Would nifedipine, or any other L-type calcium channel blocker, be able to effectively antagonize the anticonvulsant properties of TPM in vivo?

- What is the role of the sAHP enhancement/prolongation in TPM’s anticonvulsant activity? Accordingly, it was decided to perform some in vivo experiments in an attempt to find an answer to these questions.

TPM is known to be an effective antiepileptic in many animal models (see Section I.4.3). In the present study, the efficacy of TPM was tested against some established generalized seizure models, namely: sound-induced seizures in DBA/2 mice, AMPA-induced seizures, 4-AP-induced seizures and PTZ-induced seizures, as well as two absence seizure models: lh/lh mice and WAG/Rij rats. In each case, we looked for a possible interaction of TPM with L-type Ca^{2+} channel blockers and compared the results with those obtained for some other anticonvulsants: two AMPA receptor antagonists and the CA inhibitor ACTZ. The choice of the animal models was made with regard to the proposed mechanisms of action of TPM. The epilepsy models (DBA/2 mice, lh/lh mice and WAG/Rij rats) were regarded as a better representation of human epilepsies and furthermore, lh/lh mice and WAG/Rij rats are two genetic animal models of absence epilepsy that have been helpful in demonstrating that TPM might be useful in the treatment of absence epilepsy in humans (it has already been demonstrated that TPM is effective in GAERS; Rigolout et al., 2003). It is known that absence seizures in lh/lh mice are most probably due to a mutation of the β4 subunit of voltage-activated Ca^{2+} channels (see Section II.2.1) and that Ca^{2+} channel modulators show different activity in WAG/Rij rats (see Section II.2.1) therefore, TPM’s proposed action on L-type calcium channels might have been important. The seizure models (AMPA-, 4-AP-, PTZ- induced seizures), instead, were considered more useful in discriminating between seizures induced by a dysfunction of excitatory or inhibitory transmission and then trying to speculate about the role played by the TPM-induced enhancement/prolongation of the sAHP. The results obtained confirmed that TPM possesses a wide spectrum of anticonvulsant action, since it was very effective in all the models tested.
To answer our questions, a low dose of nifedipine and/or verapamil (non-effective doses were always used) was co-administered with TPM; in some cases also a low dose of the L-type Ca\(^{2+}\) channel opener, Bay K 8644 was also used. The results obtained indicate that depending on the model, an interaction between L-channel ligands and TPM does indeed exist in vivo. Thus, nifedipine was able to antagonize TPM’s effects in DBA/2 mice and WAG/Rij rats, whereas it was ineffective in AMPA-, 4-AP- and PTZ-induced seizures and surprisingly it acted synergistically with TPM in the lh/lh mouse model of absence epilepsy (see Sections II.2.3 and II.2.4). These results show that, depending on the animal model, the L-current enhancement/prolongation of the sAHP might play a more dominant role in governing the anticonvulsant properties of TPM. In particular, this action seemed to be involved in those animal models where neuronal network circuitries are responsible for the generation of seizures (DBA/2 and lh/lh mice, WAG/Rij rats), whereas it did not seem to be so important in the acute seizure. This apparent selectivity can be explained if TPM’s variability of action does indeed depend on neurone type. From all the studies on this drug, it is clear that depending on the area studied, and therefore on the neurone type, TPM can exert different actions. It is then possible to speculate that TPM’s action to prolong the sAHP might be important in those models where seizures spread from a trigger zone (pacemaker) to secondary involved areas or more generally, in those models where only few brain areas are involved in the generation and maintenance of seizures.

In conclusion, the results of this study have been useful for two main reasons: firstly, the in vitro experimental section identified two new main mechanisms that might underlie the anticonvulsant action of TPM; these effects are not only new for TPM but also for any other anticonvulsant drug. In fact, apart from ACTZ (and possibly also zonisamide), none of the anticonvulsant drugs available is known to possess modulatory effects on the sAHP, either directly or via the enhancement/prolongation of underlying calcium currents. Furthermore, at present, only retigabine is known to directly activate a “background” K\(^+\) current (Main et al., 2000) as we have demonstrated for both TPM and ACTZ, although the mechanism by which this is achieved (opening of Ba\(^{2+}\)-sensitive KCNQ/M-type channels) is clearly different for this drug. Our results might therefore indicate two new target sites for future development of AEDs. Secondly, in the in vivo experimental section, where we tried to elucidate whether the co-
administration of L-type Ca^{2+} channel blockers could modify the efficacy of TPM, we were also able to speculate on the possible role of the sAHP enhancement/prolongation effect in TPM’s anticonvulsive properties. From the results obtained, it seems that more careful attention needs to be given to the possible interaction between TPM and L-type Ca^{2+} channel blockers when these are co-administered in humans, since this could lead to a reduced anticonvulsant efficacy of TPM. This may also depend on the type of epilepsy involved, as clearly indicated by our results. The same consideration can also be drawn for the possible role played by sAHP modulation on TPM’s activity. As a matter of fact, since this action might only be important or play a role in some seizure models (e.g. where nifedipine was able to modify TPM’s activity) it may also be relevant only in some human epilepsies. Further studies will be required to clarify this point.
III.1. Future Work

This electrophysiological study on rat olfactory (piriform) cortical neurones *in vitro* (Russo & Constanti, 2004) suggested that topiramate (TPM) can exert a unique modulatory effect on the slow post-burst afterhyperpolarization (sAHP) response by *enhancing* the amplitude of the underlying L-type Ca\(^{2+}\) currents; however, a direct confirmation of this proposal by measuring L-type currents directly in cortical neurones has not yet been obtained. Good evidence for such a primary interaction of TPM at the L-channel locus was also obtained from *in vivo* studies, in which the specific L-channel antagonist nifedipine was found to interfere with the anticonvulsant effects of TPM in certain animal models of epilepsy (Russo *et al.*, 2004). Interestingly, we have also shown that this modulatory effect of TPM on the sAHP can be mimicked by some other agents possessing an active sulphonamide grouping as part of their molecular structure *e.g.* the carbonic anhydrase (CA) inhibitors acetazolamide and benzolamide; however, our experiments suggest that this action is not mediated by CA itself, but involves an interaction of the common sulphonamide moiety with an unknown modulatory site on the L-channel protein.

Future experiments would be aimed at obtaining some direct evidence for our proposed interaction of TPM (and other CA inhibitors, or molecules containing the active sulphonamide grouping, *e.g.* COX-2 inhibitors: Supuran *et al.*, 2004) at L-type Ca\(^{2+}\) channels, by directly measuring HVA Ca\(^{2+}\) currents from intact or acutely dissociated pyramidal neurones of rat piriform cortex (*c.f.* Magistretti *et al.*, 1999; Castelli *et al.*, 2003) or from oocytes expressing L-type Ca\(^{2+}\) channels using the whole cell patch-clamp technique (*c.f.* Xu & Lipscombe, 2001). In particular, we would probe the underlying mechanistic processes by looking at drug effects on the amplitude and kinetics of L-currents evoked under voltage clamp, the drug concentration-dependence of these effects, and any possible mutual interaction between the drugs when co-applied. Such novel studies should hopefully help to clarify the nature of the binding site(s) implicated in the TPM (or CA)-dependent modification of L-type Ca\(^{2+}\) channel gating in mammalian cortical neurones.

Another point that remained open from this study was the ionic nature of the slow hyperpolarizing current induced by TPM. From the results obtained, it was clear that the outward current generated by bath-application of TPM was largely if not entirely carried by K\(^+\) ions, but with very peculiar characteristics *e.g.* lack of reversal at negative
potentials, and incomplete block by general K⁺ channel blockers (Ba²⁺, Cs⁺). Therefore, a new series of experiments using specific antagonists of more novel types of K⁺ channels could also be performed. For example, TPM might activate one of the channels belonging to the new family of neuronal background K⁺ channels (TASK/TREK family; Lesage, 2003). Recently, much attention has been focused on the role of these channels, revealing that they are all widely expressed in the central nervous system, and are a major determinant of the neuronal resting membrane potential and input resistance, therefore, influencing cell excitability. It is also known that this channel family is activated by drugs such as volatile anaesthetics (Gruss et al., 2004a) and the neuroprotective agent riluzole (Duprat et al., 2000). It may be noted that riluzole also possesses anticonvulsant properties (De Sarro et al., 2000b; Yoshida et al., 2001). On the other hand, local anaesthetics inhibit TASK/TREK channels, with a preference for TASK channels (Kindler et al., 1999), and systemic administration of these drugs can induce convulsions both in humans and rodents (Barat et al., 1996, 1997; Moran et al., 2004; Dernedde et al., 2004; DeToledo, 2000). Zinc also exerts a preferential block of TASK channels (Gruss et al., 2004b). It is then possible to speculate that agents that can selectively block TASK/TREK channels might also interfere with the outward current induced by TPM in cortical neurones; conversely, volatile anaesthetics and riluzole should occlude TPM’s hyperpolarizing effects.

The results obtained in vivo indicate a different interaction between TPM and L-channel antagonists depending on the animal model considered. Therefore, future experiments on some other animal epilepsy models such as different types of kindling or focal kainic acid administration might better elucidate the role of L-type Ca²⁺ channels in TPM’s anticonvulsant action and characterize the pharmacological interaction between TPM and the class of Ca²⁺ channel antagonists. Moreover, further in vitro studies of TPM action on piriform cortical (PC) brain slices prepared from the two genetic absence animal models (lethargic lh/lh mice and WAG/Rij rats), might be able to explain the difference in the results obtained in vivo (nifedipine antagonized TPM’s anti-absence activity in WAG/Rij rats, but a synergism was found in the lh/lh mice), possibly due to presence of abnormal L-Ca²⁺ channel subunits affecting the dihydropyridine-TPM-L-channel interaction. Thus, an altered Ca²⁺ channel assembly in the lethargic lh/lh mice, consequent to the mutation of the β4 subunit and β1-3 subunit reshuffling (Burgess et al., 1999) could significantly influence L-channel
characteristics. Also, it has recently been reported that there are variations in the expression of some neuronal Ca\(^{2+}\) channel subunits in WAG/Rij rats during development, and these might be related to the occurrence of absence seizures in this animal strain (van de Bovenkamp-Janssen et al., 2004). It would therefore be of interest to test the effects of TPM on the sAHP in PC slices prepared during different stages of development in WAG/Rij rats, compared with normal animals.
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ABSTRACTS

