Focal ischemic reperfusion stroke model in rats and the role of galanin

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The cover illustration depicts the overall structure of the rat brain

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Printed in Sweden by LiU-tryck, Linköping, Sweden, 2011
To my beloved family, Peter, Mattias and Daniel

To the memory of my father Lennart Petersson
Abstract

Stroke is the third most common cause for mortality in industrialised countries and amongst the major causes of long-time morbidity. While the mortality due to myocardial infarction has been dramatically reduced during the last 10-15 years, mortality due to stroke remains almost the same, despite the fact that the two share similar basic pathogenic mechanisms including atherosclerosis, hypertension and diabetes. Treatment modalities of reperfusion therapy for acute ischemic stroke, including the use of tissue plasminogen activator for thrombolysis and endovascular treatments, are effective if applied early after onset of the first symptoms. The more frequent use of reperfusion therapy, especially in the most common type of stroke affecting the middle cerebral artery (MCA), increase the clinical relevance and demand for experimental models of temporary and focal ischemia of the brain. The primary goal of the present work was to develop a model in rats for studying the mechanisms underlying focal and temporary ischemia in brain regions supplied by the MCA.

We have modified the intracranial method of occluding the MCA originally described by Tamura et al. in the early 1980es by introducing a microclip to occlude the artery and induce reperfusion under direct visual control through an operating microscope. The goal was to create a mild ischemia model with low morbidity and mortality, optimizing conditions for the animals postoperatively and allowing long-term (weeks) observation periods of high relevance for human stroke. Morbidity and mortality in experimental stroke models are crucial confounders. Change of anesthesia from intraperitoneally administrated chloral hydrate to isoflurane inhalation anesthesia with endotracheal intubation and controlled ventilation reduced mortality markedly from 25% to ~10%. Improved overall skills in anesthesia and surgical techniques further reduced mortality to <3%.

Hypothermia reduces brain lesions caused by ischemia not only when administered before and during the ischemic episode, but also afterwards. Several studies have shown that galanin concentrations are increased in response to various types of lesions to the nervous system, and galanin may be amongst the factors supporting neuronal survival and functions. We therefore investigated whether or not hypothermia-induced alterations in galanin concentrations could constitute a part of the established neuroprotective effect of hypothermia in our rat stroke model. Hypothermia induced an overall increase in the concentrations of immunoreactive galanin (p < 0.001). The elevated galanin levels were predominantly found in the non-ischemic control hemisphere. The galanin concentrations were lower in the ischemic hemisphere in both the normo- and hypothermic animals compared to the corresponding contralateral intact hemisphere (p = 0.049). The hypothermia and not the ischemic/reperfusion lesions explained the major part of the observed changes in galanin concentrations. Hypothermia-induced elevation in galanin concentration is therefore...
not likely to be amongst the major protective mechanisms of hypothermia. Our results support the notion that hypothermia-induced increase in tissue concentrations of galanin in the brain are the result of changes from optimal homeostatic conditions – the hypothermia-induced stress – rather than the ischemic/reperfusion lesion-induced changes in galanin concentrations.

Whether the lesion-induced increase in galanin concentrations is primarily a signal that a lesion has occurred, a consequence of the lesion or a mechanism for facilitating neuronal survival is an open question. We therefore infused three different concentrations of galanin intracerebroventricularly in a direct attempt to investigate whether or not galanin has neuroprotective properties in a rat model of MCA occlusion. Furthermore, we infused the GalR2/3 agonist Gal(2-11) (AR-M1896) shown to subserve neuroprotective functions. The lesion was 98% larger seven days after a 60 min transient MCA occlusion and continuous administration of the GalR2/3 agonist Gal(2-11). No differences were found after seven days in the groups treated with galanin in three different concentrations (0.24, 2.4 and 24 nmol/day; p = 0.939, 0.715 and 0.977, respectively). There was also no difference in the size of the ischemic lesion measured after three days in the galanin-treated group (2.4 nmol/d) compared to artificial cerebrospinal fluid (p = 0.925).

The expression of the galanin, GalR1, GalR2 and GalR3 receptor genes were investigated in the female rat brain seven days after a 60 min unilateral occlusion/reperfusion of the MCA. Galanin gene expression showed a 2.5-fold increase and GalR1 a 1.5-fold increase in the locus coeruleus of the ischemic hemisphere compared to the control side, and the GalR1 mRNA levels decreased by 35% in the cortex of the ischemic hemisphere. Thus, stroke-induced forebrain lesion upregulates synthesis of galanin and GalR1 in the locus coeruleus, a noradrenergic cell group projecting to many forebrain areas, including cortex and the hippocampal formation, supporting the notion that galanin may play a role in the response of the central nervous system to injury and have trophic effects.
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Abbreviations

CCA   Common carotid artery
CNS   Central nervous system
DRG   Dorsal root ganglion
CSF   Cerebrospinal fluid
ECA   External carotid artery
Gal   Galanin
GALP  Galanin-like peptide
GalR1, -R2, -R3  Galanin receptors 1,2,3
Gi    G-protein (inhibitory)
HiFo  Hippocampal formation
ICA   Internal carotid artery
ICH   Intracerebral haemorrhage
LC    Locus coeruleus
LDCV  Large dense core vesicle
MCA   Middle cerebral artery
MCAo  Middle cerebral artery occlusion
NO    Nitric oxide
RT-PCR Reverse Transcription Polymerase Chain Reaction
SAH   Subarachnoid haemorrhage
SD    Spraque Dawley
SHR   Spontaneously Hypertensive Rats
TTC   2,3,5- Triphenyltetrazolium hydrochloride
Introduction

Stroke is the third leading cause of death in Sweden (Socialstyrelsen 2010) as in other industrialised countries (Goetz et al. 1999; Gorelick et al. 1999; Bradley 2008). It is currently the somatic disease category resulting in the largest number of patient days spent in hospital and a major cause of long-lasting disability in the workplace, since about a fifth of the cases occur before retirement.

According to the World Health Organization (WHO) stroke is “the rapidly developing loss of brain function(s) due to disturbance in the blood supply to the brain” (WHO 1978). The time dimension was later included in the definition “rapidly developed clinical signs of focal or global disturbance of cerebral function, lasting more than 24 hours or until death, with no apparent non-vascular cause” (WHO 1988).

Stroke is caused by ischemia resulting from obstruction to blood flow to the brain by thrombosis or arterial embolism, or by haemorrhage. The brain is amongst the tissues of the body most vulnerable to ischemia due to its high oxygen demand, partly needed for nerve impulse propagation and chemical neurotransmission. The human brain represents only 2% of the total body weight, but uses 15% of the cardiac output, 25% of the total oxygen consumption of the body at rest, 75 L of molecular oxygen and 120g glucose daily (Bradley 2008).

Since brain damage is an important cause of mortality and morbidity in society, more reliable therapeutic options are needed, that can minimize the neuronal damage caused by cerebrovascular diseases and traumatic brain injury.

Stroke

Types of stroke

Stroke consists of two pathological subtypes: ischemic and haemorrhagic (Figure 1) (Bradley 2008). Ischemic stroke constitutes 80% of the cases and is caused either by a local blood clot (thrombus) which blocks blood flow in an artery or by a wandering clot or some other particle (an embolus) which forms away from the brain, usually from the heart or from the bifurcation of the carotid artery and is transported to the brain by the blood. Haemorrhagic stroke consists of intracerebral haemorrhage (ICH) or subarachnoid haemorrhage (SAH). ICH are caused either by a defective artery in the brain parenchyma which bursts, flooding the surrounding tissue with blood or by a weakness in the wall of a medium sized artery (aneurysm) which bursts, sending blood to the subarachnoidal room covering the brain. Several of the patients suffering from haemorrhagic strokes die before reaching a hospital due to increased intracranial pressure which in itself causes brain ischemia due to the external pressure exerted mainly on minor vessels. However, survivors of haemorrhagic stroke usually enjoy a more favourable recovery than the sufferers of ischemic stroke. The reason is that when a blood vessel is blocked from within, a part of the brain dies and is not regenerated. In
haemorrhagic stroke the pressure and the brain dysfunctions it causes are relieved as time passes and many of the lost brain functions are thereby regained.

Figure 1
Types of stroke
Vital neurosurgical procedures e.g. as a result of vascular events, tumours or accidents sometimes necessitate temporary occlusion of the arterial blood flow to parts of the brain risking ischemic brain damage.

Risk factors for stroke
Atherosclerosis is the main risk factor in ischemic stroke, and its risk factors are thus shared with all other disease states caused by atherosclerosis including myocardial infarction (Gorelick et al. 1999). Several risk factors for stroke have been identified including hypertension, atrial fibrillation, myocardial infarction, diabetes mellitus, smoking, elevated blood lipids and asymptomatic carotid artery disease. Bradley (2008) classifies the risk factors according to whether they are modifiable or not (Table I):

Table 1
Nonmodifiable and modifiable risk factors for stroke (Bradley 2008)

<table>
<thead>
<tr>
<th>Nonmodifiable</th>
<th>Modifiable</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>Arterial hypertension</td>
</tr>
<tr>
<td>Gender</td>
<td>Transient ischemic attacks</td>
</tr>
<tr>
<td>Race/ethnicity</td>
<td>Prior stroke</td>
</tr>
<tr>
<td>Family history</td>
<td>Asymptomatic carotid bruit/stenosis</td>
</tr>
<tr>
<td>Genetics</td>
<td>Cardiac disease</td>
</tr>
<tr>
<td></td>
<td>Aortic arch atheromatosis</td>
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<tr>
<td></td>
<td>Diabetes mellitus</td>
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<td>Dyslipidemia</td>
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<tr>
<td></td>
<td>Cigarette smoking</td>
</tr>
<tr>
<td></td>
<td>Alcohol consumption</td>
</tr>
</tbody>
</table>
Introduction

Nonmodifiable | Modifiable
---|---
Increased fibrinogen | Elevate homocystine
Low serum folate | Elevated anticardiolipin antibodies
Oral contraceptive use | Obesity

Successful modification of the risk factors for stroke has substantial impact on the risk of stroke. Lowering diastolic blood pressure by as little as 5 mmHg can reduce stroke risk by 42% (Gorelick et al. 1999). At least 25% of adults suffer from hypertension defined as diastolic blood pressure of more than 90 mmHg or systolic blood pressure of 140 mmHg or more (Bradley 2008). This excellent opportunity for prevention is unfortunately as yet not fully and properly exploited even in countries such as Sweden with health care systems that should have sufficient resources to cope with the problem. Statin treatment of hyperlipidaemia and vascular inflammation is also very effective in preventing stroke, as exemplified by the study of the Scandinavian Simvastatin Survival Study Group which showed a 28% reduction in fatal or nonfatal stroke and transient ischemic attacks (Pedersen et al. 1998).

Stroke symptoms
Due to the multitude and complexity of brain functions and the many locations in the brain affected by stroke, it causes a wide range of symptoms, each corresponding to the affected location.

The most common symptom is sudden weakness or paralysis of the face, arm or leg, most often affecting one side of the body. Other symptoms are confusion, trouble speaking or understanding, difficulty seeing with one or both eyes, difficulty in walking, and loss of balance or coordination (Bradley 2008). A stroke survivor is frequently also prone to emotional instability and sudden moods swings, even after long periods of time.

Treatment of stroke
Most stroke survivors are left with lifelong disability. With the exception of early administered thrombolytic therapy by means of tissue-type plasminogen activator (t-PA) and rare opportunities for endovascular interventions, no clinically proven, practical and causal therapy exists as yet for the management of acute ischemic stroke (Stapf et al. 2002; Benchenane et al. 2004). Since brain damage is an important cause of mortality and morbidity in society, additional reliable therapeutic options are needed. However, the development and use of multiple endovascular modalities of reperfusion therapy for acute ischemic stroke has reported promising results, i.e. intra-arterial thrombolysis and/or stent deployment increase the chance of recanalization (Gupta et al. 2011).
Introduction

**Stroke outcomes**

The severity of the hypoxia-ischemia and the ability of the brain including its collateral circulation determines the extent of the lesions and its neurological consequences as shown in Figure 2.

**Figure 2**

Possible outcomes when the brain is affected by ischemic lesions. The fate of brain tissues is partially determined by the severity of the initial insult. Mild or short ischemic conditions engage compensatory mechanisms in the cells including the activation or inhibition of pre-existing proteins and new gene expression. The possibility of viable tissue is thereby improved. When the ischemia is moderate adaptive homeostasis is again engaged succeeding only partially (e.g. in the penumbra part of the ischemic tissues). Apoptosis occurs in neurons that sustained irreparable damage at the initial insult thereby removing nonfunctional neurons. Severe ischemia occurs in the center of the infarction resulting in necrosis Adapted from (Fisher et al. 2003).

The brain has homeostatic mechanisms able to deal with mild ischemic attacks. Larger ischemic challenges result in a mixture of damaged and surviving cells, whereas severe ischemia overwhelms the homeostatic defences and causes cell death in smaller or larger parts of the brain.

**Types of cerebral ischemia**

Ischemia of the brain occurs in several varieties (Goetz 2003; Bradley 2008)(Figure 3). Global ischemia reduces blood flow to the entire brain. It occurs in cardiac arrest, severe hypotension, or occasionally during surgical procedures that alter blood flow. Focal ischemia affects circumscribed part(s) of the brain e.g.
the internal capsule, caudoputamen or the cortex commonly from occlusion of the middle cerebral artery (MCAo). Focal ischemia occurs in response to transient or permanent MCAo. The degree of brain damage in response to ischemia depends on duration of occlusion, site along the MCAo, and amount of collateral blood flow into the middle cerebral artery (MCA) territory. Characteristic of focal ischemia is an ischemic core, where cell death is most extensive – or complete surrounded by a penumbra zone, of partially damaged but still surviving brain cells with an undecided long-time fate. The ischemic core is surrounded by the penumbra zone where cells suffer from the consequences of hypoxia (Astrup et al. 1981), but where the final fate of the cells is not yet decided (Arvidsson et al. 2002). Extensive ischemia in the brain causes cell death within minutes. Permanent ischemia is caused e.g. by an embolus occluding the MCA for extended periods of time (several hours), sufficiently to cause cell death. Temporary ischemia is e.g. caused when an embolus is dissolved by fibrinolytic therapy, removed using endovascular technique or when a vessel is occluded during neurosurgical procedures.

Figure 3
Types of experimental models of cerebral ischemia. Several types of global and focal ischemia are illustrated.

Experimental stroke models
The increased use of thrombolytic therapy for treating patients suffering from cerebral ischemia and temporary ischemia during neurosurgical procedures increases the clinical relevance and demand for experimental models of temporary and focal ischemia of the brain. Previous experimental paradigms described in the recent literature consist mainly of permanent MCAo. The primary goal of the present work was to develop a model in rats for studying focal and temporary ischemia, since this is a state of considerable clinical importance. Temporary MCAo was chosen, since it is the vessel most commonly affected in human stroke (Goetz et al. 1999).
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Ten years ago when we started developing the present method we experienced rat mortality of 25% when performing temporary clipping of the MCA in the rat. This – in our opinion – high mortality prompted us to abandon the intraperitoneal anesthesia by chloral hydrate and ventilation by tracheotomy in favour of intubation, and isoflurane anesthesia (1% isoflurane in 30%/70% O₂/N₂O) in order to favour better survival rates and recovery after surgery.

We have worked strenuously to develop and perfect a model for temporary MCAo by micro clip applied on the MCA exposed by craniotomy. This paradigm caters for a visually controlled application of a micro clip and visual control that the blood-flow in the MCA is inhibited. The method results in an atraumatic pressure on the artery with minimal risk for post occlusion thrombosis. Our experimental paradigm thus caters for clear-cut but limited damage, allowing the rat to feed and thrive well for days to weeks in order for the long-time effects of the temporary ischemia to be studied. Even if the surgical methods and skills as such are important for the success of this kind of experiments, we have found the anesthetic procedures to be just as important.

Most experimental paradigms of stroke in experimental animals cater for observations done during a short period of only 1-3 days. To study perhaps more relevant observation of the end of outcome of brain ischemia, we set out to design a model of a mild reperfusion ischemic damage to the rat brain compatible with survival for days and weeks.

Many details in the basic mechanisms causing cell death in brain ischemia/stroke are preferably studied in individual cells in culture. However, the brain is the most extensively integrated and communicative organ in the body, and a comprehensive assessment of its integrated functions can therefore only be studied in intact organisms/animals. Human brain tissues are – for natural reasons – not available for this type of studies except in cases, where noninvasive imaging techniques can be used and when microdialysis probes can be inserted in conditions, when that type of monitoring is deemed beneficial for the patient.

It is therefore crucial to have ready access to experimental animal models mimicking human stroke in order to investigate stroke mechanisms and discover new treatment options. Animal models of stroke have been established in several species including mice, rats, cats, dogs, rabbits, monkeys (Sundt et al. 1966; Hudgins et al. 1970; Suzuki et al. 1980; Lyden et al. 1987). Rodents, in particular rats and mice, are the most commonly used species. Small animals are easy to maintain, entail comparatively low costs for storage and feeding, and have proven less controversial from an ethical point of view than higher animals including primates. The anatomy of the arterial and nerve supply to the rat cerebral hemispheres is similar to that of humans, and several aspects of the biochemical and molecular mechanisms of injury are also similar (Yamori et al. 1976). However, it is crucial to realize that the brain of rats and mice have different details in their anatomy and physiology compared to humans, and it should not be expected that all mechanisms and therapeutic opportunities discovered in rodents
Introduction

automatically will also be useful in humans. Rats have very little white matter compared to humans, and their grey matter is not gyrated (Hoyte et al. 2004). It should also be realized that the highest cognitive brain functions in humans are absent from animals and may be affected by drugs proven neuroprotective in animals without cognitive side-effects in the animals.

In contrast to some other rodents (e.g. gerbils), rats and mice have a complete circle of Willis (Dirnagl et al. 1999). Rats have more effective collaterals between large cerebral vessels than humans, and suffer severe ischemia when proximal occlusion models are used (Maeda et al. 2000). In the common Spraque-Dawley (SD) rats, the wild type strain shows most consistent infarct, compared to, for example, the spontaneously hypertensive rats (SHR) which develop large and more variable-sized infarcts (Ginsberg 2003).

Since stroke is a disease of the highly integrated and complex brain, treatment options found valuable in cell cultures may not work when tested in intact animals or in humans for that matter. Treatment modalities found valuable in animal models of stroke may also not work in humans.

Several variables that may affect the experimental outcome need to be controlled in experimental animal models. Male animals are commonly used instead of females to avoid the effects of varying concentrations of female sex hormones during the ovarian cycle. Young and healthy animals are commonly used in experimental studies, in contrast to humans suffering from stroke, who are commonly elderly and hypertensive, suffer from generalized atherosclerosis and/or suffer from diabetes. Even design features which could have been built into stroke studies in experimental animals, including extended observation periods of more than 1-3 days are seldom included in the study design. There is therefore a multitude of reasons, why treatment strategies proven effective in the laboratory have failed when put to the test in the clinic (Howells et al. 2010a; Howells et al. 2010b; van der Worp et al. 2010).

Since the original description of a stroke model in dogs (Hill et al. 1955), a plethora of experimental stroke models in animals have been described and used in a variety of study paradigms (Stefanovich 1983; del Zoppo 1990; Overgaard 1994; Wang-Fischer 2009; Dirnagl 2010).

Ischemic stroke models are basically either global or focal models or models which create permanent ischemia or induce reperfusion after ischemic lesions (Table II). Furthermore, they include opening of the skull or not.
Introduction

Table II

Rodent models of cerebral ischemia Modified from (Ginsberg et al. 1989).

Models of global cerebral ischemia in rats
Two-vessel occlusion model of forebrain ischemia
  Transient bilateral common carotid artery (CCA) occlusions plus hypotension
Four-vessel occlusion model of forebrain ischemia
  Transient bilateral CCA occlusions plus permanent vertebral artery occlusions
Ischemia models involving elevated cerebrospinal fluid pressure
  Bihemispheral forebrain compression-ischemia
  Unihemispheral forebrain ischemia
Miscellaneous global ischemia-producing strategies
  Neck tourniquet
  Decapitation
  Levine preparation of hypoxia-ischemia and its modifications

Models of focal cerebral ischemia in rats
Middle cerebral artery occlusion and its variants (Laing et al. 1993)
  • Direct occlusion of the MCA through craniotomy by electro cauterization or by a clip (Tamura et al. 1981a; Bederson et al. 1986b)
  • Inserting a poly-lysine coated suture thread into the carotid artery in the neck until it occludes the MCA (Koizumi 1986; Longa et al. 1989; Belayev et al. 1996)
  • By introducing an embolus (blood clot or synthetic embolus) to the carotid artery in the neck making it travel to and occlude the MCA (Hill et al. 1955; Kudo et al. 1982; Kaneko et al. 1985; Zhang et al. 1997b).
  • Using light of a specific wavelength to activate a polymer injected into the blood, thus occluding the artery (Futrell et al. 1989; Matsuno et al. 1993; Zhao et al. 2002)
Stroke in the SHR
Miscellaneous models of cerebral embolism and thrombosis
  Blood clot embolization
  Microsphere embolization
  Photochemically initiated thromboembolism
  Arachidonate-induced thrombosis

Models of cerebral ischemia in gerbils
Unilateral CCA occlusion
Bilateral CCA occlusions
Models of focal cerebral ischemia

The most common stroke model, due to its relevance to human stroke, is focal MCAo. It may, as listed above, be induced by several different approaches including temporary or permanent, proximal or distal occlusion of the artery. MCAo is sometimes combined with carotid artery occlusion (ipsi-, contra-, or bilateral; temporary or permanent) in order to increase the extent of the ischemic lesion(s). The MCA can be occluded in several different ways, including direct clipping, intraluminal suture or by an embolus (blood clot). A widely used invasive permanent occlusion technique is cauterization of the MCA through craniectomy (Tamura et al. 1981a). A craniectomy technique allowing reperfusion is occlusion by means of micro clip (Theodorsson et al. 2005b) or ligature which can be released. Even some methods for photothrombic occlusion of the vessel have been claimed to result in reperfusion.

Middle cerebral artery occlusion (MCAo) through craniectomy

MCAo in rats through a craniectomy has been used in experimental models of cerebral ischemia since 1975 (Robinson et al. 1975; Robinson 1979; Bederson et al. 1986b). The technique of directly and permanently (by electro coagulation) occluding the MCA has been optimized and characterized by Tamura et al. (1981a), and has been used for several recent studies of focal cerebral ischemia (Tamura et al. 1981b). Clips and ligatures have also been used to permanently or transiently occlude the MCA (Shigeno et al. 1985; Buchan et al. 1992; van Bruggen et al. 1999; Theodorsson et al. 2005b).

The advantages of the craniectomy techniques are the ability to visually identify the artery to be occluded (Figure 4), and to visually verify that it has been occluded. Furthermore, reperfusion of the artery can also be verified, when the clip is removed. The disadvantage of the method is its technical complexity and steep learning curve and the mortality due to the operation itself.
Introduction

Figure 4
The rat MCA seen through an operation microscope by means of a drill hole in the skull. The use of the optic tract to locate the MCA is apparent.

Thromboembolic stroke model
In 1955, Hill and colleagues pioneered in using injection of homologous blood clots into the carotid artery as an experimental model for cerebral ischemia in dogs (Hill et al. 1955). The thromboembolic stroke models in rats are the most frequently used models for studies of experimental thrombolytic therapies (Kudo et al. 1982; Kaneko et al. 1985). To induce microembolization, Kudo and co-workers used suspension of blood clots (≤ 100 μm) injected into the CCA, while Kaneko et al. used larger blood clots averaging between 100 and 200 μm in diameter. It is, however, difficult to exert detailed/sufficient control of the size of the blood clots. Depending on the size of the clot lesions of different sizes are induced. The smallest blood clots cause microembolization, whereas the big clots run the risk of occluding even the entire targeted arterial circulation.

Zhang and co-workers 1997 inserted a modified PE50 catheter close to the MCA origin through internal carotid artery (ICA), and then occluded the MCA with injection of a single clot (Zhang et al. 1997b). This technique was improved by Busch et al. (1997) by inserting a PE50 catheter to the ICA through the external carotid artery (ECA), injection of a number (12) of clots (350 x 1500 μm), small enough to reach the MCA origin, during which the CCA was temporarily closed.
Photochemical thrombotic stroke model
Watson et al. (1985) introduced the photochemical stroke model in rats. A potent photosensitive dye (Rose-bengal) was injected intravenously into the circulation, and external illumination with green light (530-590 nm) from a filtered xenon arc lamp is performed through the intact tissue to perform the ischemic lesion.

Intraluminal suture stroke model
The most commonly used method for occluding the MCA permanently or transiently is the intraluminal suture technique (so-called filament or suture model) initially developed in rats by Koizumi et al. (1986). The MCA is reached from the ECA by occlusion of the CCA, thereby forcing the suture to find its way into the ICA, thereby enabling the suture to find its way into the ICA and the MCA. The lesion it induces includes the cortex and striatum. This model represents a relatively non-invasive way of achieving MCAo, since the artery is reached intraluminally from the ECA and the skull itself remains intact. The technique was modified by Longa and co-workers (1989) using a 4-0 uncoated nylon thread with a tip blunted by heating near a flame, while the Koizumi group used a silicone-coated 4-0 nylon surgical thread. Belaev et al. (1996) made a modification of the initial model of Longa et al. (1989) by blunting the tip of thread and then coating it with poly-L-lysine thereby making the ischemia and the ischemic lesion more reproducible. The poly-L-lysine coating increases the adhesion of the suture to the vascular wall (Mazia et al. 1975), thereby increasing reproducibility of the method. In addition the incidence of the complication of SAH is reduced.

There are major differences in the outcome of focal cerebral ischemia between rat strains. Studies during the last twenty years, using transient intraluminal filament MCAo method have been SD in 41% of the cases, Wistar in 34% of the cases, and 10% were SHR and 2% Long-Evans rats and, finally, other inbred strains in 2% of the cases including Fischer F-344 rats (Dittmar et al. 2006). Dittmar et al. showed significant differences in the success and complication rates in use of the MCAo filament models of transient cerebral ischemia applied to Fischer-344, SD and Wistar rats. The Fischer-344 rat had low success rates (40%) and frequent (50%) complication of SAH in contrast to the SD, and Wistar rats which showed negligible complication rates. The reason for this was shown by magnetic resonance angiographies (MRA) to be the different anatomical course of the ICA, especially in the petrous segment in Fischer rats compared to the Wistars rat’s carotid arteries (Dittmar et al. 2006).

Staining methods for infarct volume determination
Several methods have been used to determine the extent of the infarction in stroke models. They can be subdivided into morphological and functional methods. Morphological methods show cell structures and the effect of the ischemia on them and include the classical hematoxylin-eosin and silver staining methods. The 2,3,5-Triphenyltetrazolium hydrochloride (TTC) staining method used in the present studies is a classical functional method which shows colour related to the function of the mitochondria in the tissues.
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Triphenyltetrazolium hydrochloride staining
TTC was first synthesized in 1894 (von Pechman et al. 1894) and initially used for testing the viability of seeds (Glenner 1969). Since 1958 TTC is used to detect by staining ischemic lesions in human tissues, i. a. the myocardium (Sandritter et al. 1958). TTC, a water soluble, colourless salt reacting with intact oxidative enzymes, dehydrogenases, of the inner mitochondrial membrane, is reduced and forms a fat soluble compound (formazan) that turns normal tissue deep red and thereby delineates abnormal areas (Glenner 1969; Orten et al. 1975). Staining by TTC has been used to determine the location and extent of the infarcted areas in cerebral tissue after ischemic injury (Altman 1976; Bederson et al. 1986a; Lundy et al. 1986; Goldlust et al. 1996).

Pathophysiological mechanisms in cerebral ischemia
The extraordinary vulnerability of cerebral tissues to ischemic damage reflects: 1) its high metabolic rate and oxygen demand, varying between different cerebral regions (Rosner et al. 1986) and 2) the unique pathological mechanisms that the normal neurotransmission/neurochemical mechanisms in the brain exert in ischemic conditions damaging effects on its own and other cells.

A more or less orderly row of events – the ischemic cascade – has been established as the backbone in the pathogenesis of stroke. Each of the steps in this cascade needs to be studied as potential target for treatment. The currently most favoured excitotoxic mechanism of permanent ischemic damage in the brain is that hypoxia releases excitatory amino acids, in particular glutamate, which influences all cells in the vicinity (Siesjö et al. 1989; Siesjö 1992; Siesjö et al. 1998; Siegel et al. 1999; Ginsberg 2003; Fawcett 2006). Glutamate is an excitatory amino acid normally of crucial importance for, among others, memory and is the neurotransmitter present in highest concentrations in the brain. When present in excessive concentrations, it causes depolarisation of cell membranes and increased intracellular calcium levels which trigger the cell damage. Glutamate in high concentrations is therefore toxic to neurons, and an important part in the mechanisms of excitotoxicity (Siesjö et al. 1989). Glutamate increases the entry of calcium into the cells which induces cell death, e.g. through apoptosis. The primary reason for the particular vulnerability of the brain in cerebral ischemia is the fact that the inter- and intra-cellular signalling mechanisms crucial for normal functions of the brain, become harmful under ischemic conditions; energy failure is accelerated enhancing the final pathways underlying ischemic cell death, including free radical production, activation of catabolic enzymes, membrane failure, apoptosis and inflammation (Calabresi et al. 2000; Centonze et al. 2001).

A fundamental pathophysiological mechanism of cell death in brain ischemia is lack of energy supply to the cells due to lack of oxygen leading within minutes to insufficient cell respiration and to depletion of the cells ATP supplies (Siesjö et al. 1998). ATP is i. a. needed to power the ion pumps of the cell membranes which uphold the membrane potentials required i. a. for neurotransmission and
Introduction for the synthesis of chemical neurotransmitters. Lacking ATP, the cells enter into a state of anoxic depolarization which opens up voltage-sensitive ion channels allowing pathological entry of calcium, sodium and chloride ions into the cells. Passive and excessive entry of water into the cells subsequently results in cytotoxic oedema (Siesjö 1992).

Temporary brain ischemia is characterized by hypoxia during a short period of time followed by a period of hyper perfusion of the tissue with well-oxygenated blood. The reperfusion generates huge amounts of free radicals in the tissue, which are particularly damaging to macromolecules including proteins, nucleic acids and cell membranes. The pathophysiological end result is variable depending on the cell organelle or macromolecules affected.

The increased intracellular calcium levels cause cell death by various mechanisms, including activation of proteases and lipases, formation of free radicals, lipid peroxidation and formation of nitric oxide (NO) and arachidonic acid. The generation of high levels of NO is results in free radicals which damage important biomolecules, including membrane lipids, enzymes and DNA.

The various mechanisms that normally protect the neurons against excitotoxicity are the calcium transport systems/ion pumps, mitochondrial function and radical scavengers. Transport systems are not able to counteract the increase in calcium concentrations, when ATP is missing. The mitochondrial function is disrupted, when mitochondrial stores are overloaded with calcium, and this results in even further reduced ATP synthesis.

When reperfusion occurs and the oxygenation is restored, it can lead to even further damage because of generation of reactive oxygen species. They include e.g. superoxide, hydroxyl radicals and hydrogen peroxide which are generated as side products in mitochondrial ATP synthesis. When oxygenation is restored, reactive oxygen species accumulate and can cause damage to important macromolecules in the cell.

The cerebral circulation in rats
The brain is supplied through four primary arteries; the anterior and posterior circulation, connected through the circle of Willis formed by the anterior cerebral and posterior communicating arteries. From the arch section of aorta the innominate, left common carotid and left subclavian arteries arise. The innominate is divided to the right subclavian and right CCA. The ICA and ECA are derived from respective CCA. The ICA – anterior circulation - branches intracranially into several arteries; the posterior communicating artery, anterior cerebral artery and MCA are the major. The posterior cerebral artery is a branch of the posterior communication artery (Figure 5).

The vertebral arteries – the posterior circulation – arise from the subclavian arteries, enters the skull through foramen magnum, and form the basilar artery which is a component of the circle of Willis (Wang-Fischer 2009).
Introduction

Anterior Cerebral artery (0.28 mm in diameter)
Middle Cerebral artery (0.24 mm in diameter)
Posterior cerebral artery (0.26 mm in diameter)
Internal carotid artery (0.71 mm in diameter)
Basilar artery (0.36 mm in diameter)
Vertebral artery (0.34 mm in diameter)
External carotid artery (0.77 mm in diameter)
Common carotid artery (0.90 mm in diameter)

Figure 5
The cerebrovascular anatomy of the brain of rats. The cerebrovascular hemispheres are supplied through four primary arteries, the right and left internal carotid arteries and the right and left vertebral arteries. The anterior, middle, and posterior cerebral arteries are derived mainly from the internal carotid arteries to form a modified circle of Willis (modified by kind permission from (Longa et al. 1989)).

Hypothermia and brain ischemia
The neuroprotective properties of hypothermia in brain ischemia have long been suggested (O’Keeffe 1977; Chinard 1978). People suffering from global cerebral hypoxia during near-drowning events have been reported to experience remarkable neurological recovery, if hypothermia is present through cold water drowning (Young et al. 1980; Nunney 2008). Hypothermia in humans also improves the neurological outcome in survivors of cardiac arrest which has resulted in global cerebral hypoxia (Bernard et al. 2002; Group 2002). The use of hypothermia is today recommended after cardiac arrest, i. a. by the International Liaison Committee on Resuscitation (ILCOR) (Nolan et al. 2003). Furthermore, whole-body hypothermia has been shown to reduce mortality and to improve the neurodevelopmental outcome in neonates suffering from hypoxic-ischemic encephalopathy (Shankaran et al. 2005).

The effects of hypothermia in acute ischemic stroke in humans have as yet been tested only in a few clinical studies, i. a. by the COOL-AID study group in two clinical trials using surface cooling (Krieger et al. 2001) and by endovascular cooling (De Georgia et al. 2004). However, both studies are too small to allow comprehensive conclusions. There is i. a. an uncertainty about the optimal depth and duration of the hypothermia. Furthermore, cooling below 35°C, which is used in most experimental animal models, requires controlled mechanical ven-
tilation and sedation only available in intensive care units. This limits the enrolling of patients in clinical trials as well as the practical implementation of any therapy shown to be effective. On the other hand, prospective observational studies have reported that elevated body temperatures are associated with poor outcome after stroke (Reith et al. 1996; Castillo et al. 1998).

Figure 6
The role of hypothermia in maintaining the integrity and functional status of neurons affected by cerebral ischemia. Adapted from (Gonzalez-Ibarra et al. 2011).

Hypothermia inhibits the release of excitatory amino acids and calcium ions released by the ischemic insult. This inhibits elevation of intracellular concentrations of calcium ions and the mitochondrial dysfunction/cell death. Moreover, hypothermia decreases DNA lesions in the cells, thereby improving the chance that cells in the penumbra zone survive. Finally, hypothermia also inhibits the activation of astroglia and thus release of protein S-100 B, cytokines and free radicals.

Several studies in experimental animals have been made on the effects of induced hypothermia in focal cerebral ischemia (Ginsberg 1997). van der Worp and co-workers (2007) recently reported a systematic review and meta-analysis of the evidence for efficacy of hypothermia in animal models of ischemic stroke, identifying 101 publications on the effects of hypothermia on infarction size or functional outcome, including data from a total of 3353 animals. Overall, hypothermia reduced infarction size by 44%. The effect was most pronounced when cooling to temperatures even below 31°C, when hypothermia was induced before or at the onset of the ischemic insult, and in transient stroke models compared to permanent ischemic models. A 30% reduction in infarction volume was reported with cooling to 35°C and with initiation of hypothermia between 90 and 180 min after the onset of the ischemic insult. The effects of hypothermia were also more pro-
nounced in hypertensive animals compared to normotensives.

The neuroprotective mechanisms of hypothermia are most likely multiple and act in synergy on a broad spectrum of biochemical pathways (Figures 6 and 7, Table III). The brain metabolism decreases by hypothermia by slowing down the rate of oxygen and glucose utilization, and of ATP breakdown (Erecinska et al. 2003). The brain oxygen consumption is reduced by approximately 5% per every degree of fall in body temperature in the temperature range of 22-37°C (Hagerdal et al. 1975).

Excitatory amino acids including glutamate are toxic to neurons in high extracellular concentrations and are amongst the most important mediators of excitotoxicity and the damage caused by cerebral ischemia (Siesjö et al. 1989). Hypothermia reduces the excitotoxic damage by reducing glutamate release (Nakashima et al. 1996). In addition, hypothermia impairs glutamate-mediated calcium influx (Takata et al. 1997). This reduces damage due to uncontrolled rise in the concentrations of intracellular calcium ion which activate enzymes (i. a. proteases and nucleases) that degrade proteins, nucleic acids and enzymes synthesizing NO.

Hypothermia also inhibits free radical formation (Yenari et al. 2002). The neuroinflammatory response is attenuated (Inamasu et al. 2001), and sustained responses as late as one week after hypothermia have been reported (Wang et al. 2002). Furthermore, hypothermia is reported to limit brain oedema formation and alter necrosis/apoptosis (Eberspacher et al. 2005; Wang et al. 2005).

The effect of an ischemic insult on markers of the biological activity of the neuropeptide galanin is still a matter of debate, since the galanin response has been reported to be increased (Barbelivien et al. 2004; De Michele et al. 2006), decreased (Raghavendra Rao et al. 2002; Theodorsson et al. 2005c), as well as biphasic (Hwang et al. 2004). Since galanin has been claimed neuroprotective in models of cerebral ischemia, a possible component in the neuroprotective effects of hypothermia-induced alterations in galanin concentrations was studied in the current thesis (Theodorsson et al. 2008).

**Table III**

Neuroprotective mechanisms of hypothermia  from i. a. (Sinclair et al. 2010; Gonzalez-Ibarra et al. 2011)

<table>
<thead>
<tr>
<th>Mechanism</th>
<th>Explanation</th>
<th>Time frame</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prevention of apoptosis</td>
<td>Ischemia induces apoptosis and calpain-mediated proteolysis which both are reduced or even prevented by hypothermia.</td>
<td>Hours, days to weeks</td>
</tr>
</tbody>
</table>
### Introduction

<table>
<thead>
<tr>
<th>Mechanism</th>
<th>Explanation</th>
<th>Time frame</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reduced mitochondrial dysfunction and improved energy homeostasis</td>
<td>Ischemia leads to mitochondrial dysfunction and apoptosis. Hypothermia reduces metabolic demands and improves energy homeostasis and mitochondrial functions.</td>
<td>Hours to days</td>
</tr>
<tr>
<td>Reduced free radical production</td>
<td>Mild to moderate hypothermia (30°C to 35°C) reduces the production of free radicals including superoxide peroxynitrate, hydrogen peroxide and hydroxyl radicals.</td>
<td>Hours to days</td>
</tr>
<tr>
<td>Mitigation of reperfusion injury</td>
<td>Reperfusion-related reactions are inhibited by hypothermia, including free radical production.</td>
<td>Hours to days</td>
</tr>
<tr>
<td>Reduced permeability of the blood-brain barrier and the vascular wall and reduced oedema formation</td>
<td>Blood-brain barrier disruptions, vascular permeability and capillary leakage induced by trauma or ischemia are decreased by hypothermia.</td>
<td>Hours to days</td>
</tr>
<tr>
<td>Reduced permeability of cellular membranes</td>
<td>Decreased membrane leakage, resulting in improved cellular homeostasis, mitigation of DNA injury and decrease in intracellular acidosis.</td>
<td>Hours to days</td>
</tr>
<tr>
<td>Improved ion homeostasis</td>
<td>Ischemia induces the excitotoxic cascade including release of calcium and excitatory neurotransmitters including glutamate. This cascade is inhibited by hypothermia.</td>
<td>Minutes to 72 hours</td>
</tr>
<tr>
<td>Reduced metabolism</td>
<td>The requirements of the cells for oxygen and glucose are reduced 5 - 8% by each centigrade decrease in core body temperature.</td>
<td>Hours to days</td>
</tr>
<tr>
<td>Decrease in potentially harmful immune- and pro-inflammatory responses</td>
<td>Hypothermia blocks destructive inflammatory reactions and secretion of pro-inflammatory cytokines in response to ischemic damage of the brain.</td>
<td>Hours to days</td>
</tr>
<tr>
<td>Reduction in cerebral thermopooling</td>
<td>Hyperthermia increases damage to injured brain cells. Hypothermia blocks the increase in brain temperatures in certain injured brain regions of up to 3°C which can be induced by ischemic damage.</td>
<td>Minutes to days</td>
</tr>
<tr>
<td>Anticoagulant effects</td>
<td>Hypothermia exert anticoagulant effects which prevent microthrombus formation, adding to brain ischemia.</td>
<td>Minutes to days</td>
</tr>
</tbody>
</table>
Introduction

<table>
<thead>
<tr>
<th>Mechanism</th>
<th>Explanation</th>
<th>Time frame</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suppression of seizures</td>
<td>Seizures after ischemic injury increase brain injury and hypothermia mitigates them.</td>
<td>Hours to days</td>
</tr>
</tbody>
</table>

**Figure 7**

The effects of therapeutic hypothermia on the oxidative stress and neuronal metabolism induced by cerebral ischemia Adapted from (Gonzalez-Ibarra et al. 2011).

Hypothermia inhibits the increase in thromboxin A2 induced by ischemia and thereby the subsequent platelet aggregation and vessel occlusion. Hypothermia reduces metabolic demands, reducing glucose use, generation of lactate and the subsequent acidosis with its detrimental effects on the mitochondria.
Neuropeptides

Neuropeptides constitute the oldest neurotransmitter system known, found already in the phylogenetically ancient Hydra (Grimmelikhuijzen 1983). Currently more than hundred neuropeptides have been characterized and studied, creating an innovative field of scientific enquiry – neuropeptide research (Klavdieva 1995; Klavdieva 1996a; Klavdieva 1996b; Klavdieva 1996c; Strand 1999; Hökfelt et al. 2000; Hökfelt et al. 2003; Kastin 2006; Burbach 2010).

Neuropeptides are synthesized in, and released from neurons of the central (CNS) and peripheral (PNS) nervous system - hence the name neuropeptides. They are regularly co-expressed with at least one classical neurotransmitter e.g. a monoamine and/or an amino acid, and often with more than one other neuropeptide (Hökfelt et al. 1980). Many neurons in the CNS are able to release a 'cocktail' of chemical messengers, including a fast-acting, excitatory transmitter amino acid such as glutamate together with a monoamine and even one or more neuropeptides. This caters for a more 'efficient' signalling suited for the purpose than the simple on/off signal that would be available, if neurons had one transmitter only.

Neuropeptides are actually a subgroup within the broader group of regulatory peptides which in addition to neuropeptides also include peptides present in and released from widely distributed endocrine cells. The concept of APUD cells was put forward already in the 1960’s by A. G. Pearse. The concept groups together seemingly unrelated endocrine cells having in common 1) high Amine content, 2) substantial Precursor Uptake, 3) and the enzyme amino acid Decarboxylase (Pearse 1969; Pearse 1974). In addition to amines, APUD cells also contain regulatory peptides which exert their effects in three different ways: 1) by being released into the bloodstream (endocrine transmission); 2) via local diffusion to adjacent cells (paracrine transmission) or to the cell which released the peptide (autocrine transmission); 3) through modulation of signal transmission in or outside nerve cell synapses (neurocrine or synaptic/non-synaptic transmission). Regulatory peptides in endocrine cells of the gut participate i. a. in the regulation of gut secretion and motility and in the absorption and utilization of nutrients.

Neuropeptides are 2-100 amino acids in length and have up to 100 times higher molecular weight than the classical neurotransmitters. However, they are smaller than regular proteins, including e.g. common metabolic enzymes and have a less complex three dimensional structure (Hökfelt et al. 2003). They expose more numerous binding sites and thereby convey 'more' chemical information to their receptors than classical transmitters and bind more slowly but more tightly than smaller neurotransmitters. The binding affinity between neuropeptides and their receptors is in the nmol/L or higher range, 1000 times higher than classical transmitters which have binding affinity in the μmol/L range.
Neuropeptide biosynthesis and release

There are several differences between neuropeptides and classical transmitters with regard to their synthesis, storage and release mechanisms (Figure 8).

Figure 8

The synthesis, neuronal transport, release and effect of neuropeptides from (Hökfelt et al. 2003), with permission from the copyright holders. Neuropeptides are mainly synthesized as peptide precursors in the cell body, packaged into dense-core vesicles which also can contain and co-release classic/monoamine transmitters. The vesicle also contains convertases cleaving the bioactive peptide from its precursor. The peptide receptors contain seven transmembrane spanning regions of the G-protein-coupled type, and are present on cell soma, dendrites, axons, and nerve endings. Classic/monoamine transmitters are synthesized in the nerve terminal and released upon lower frequency stimulus than the neuropeptide transmitters. Classic/monoamine transmitters, in contrast to the neuropeptides, have reuptake mechanisms resulting in termination of their action and their re-cycling. Neuropeptides, on the other hand, are inactivated by extracellular proteases and only replaced by axonal transport from the cell body which can take up to days when long axons are involved. Therefore classical/monoamine neurotransmission has very large capacity and is not exhausted, whereas neuropeptides are selectively released and have comparatively low capacity over time.

Neuropeptides are produced by ribosomes in the cell body of their neuron as precursor peptides (prepropeptides), and subsequently packaged into large dense core vesicles (LDCVs, 90-250 nm in diameter) for further processing. They reach the nerve endings by fast calcium ion dependent active, fast transport in the axons and dendrites and are released extrasynaptically (Gainer 1981; Lund-
berg et al. 1986a). In contrast classical neurotransmitters are produced locally and presynaptically by dedicated enzyme mechanisms and stored in small clear synaptic vesicles (40-60 nm in diameter) located in nerve endings close to the release site of the synapse.

The neuropeptides are released by high frequency firing in the synapse, whereas the classical neurotransmitters are released into the synaptic cleft during low frequency activity (Lundberg et al. 1986b; Tallent 2008). The classical transmitters, in contrast to neuropeptides, have dedicated reuptake mechanisms located in the presynaptic cell membrane, wherefrom they are incorporated into synaptic vesicles using vesicular transporter molecules. In contrast, peptides are cleaved and their activity terminated by extracellular peptidases. They are only re-supplied to the site of release through axonal transport. Taken together - the peptides are produced far from the site of release and their transport takes long time. Therefore peptidergic neurotransmission is much more easily exhausted compared to classic monoaminergic transmission.

In some instances neuropeptides are present in high concentrations and ‘functional’ all the time. In other instances neuropeptides are expressed in low or undetectable concentrations, or not at all and then upregulated under certain conditions, for example in response to nerve injury. Neuropeptides may also be expressed early during development, often only prenatally, and then downregulated postnatally.

The biological functions of neuropeptides range from neurotransmitter to growth factor. They are hormones in the endocrine system, and are messenger in the immune system. Much evidence indicates that neuropeptides play a role mainly when the nervous system is challenged by different physiological/pathophysiological processes (Hökfelt et al. 2003) (e. g. by stress, nociception, mood, feeding, injury, drug addiction, learning and memory or diseases).

It is difficult to use neuropeptides as medicines because they decompose rapidly in the gastrointestinal tract and the bloodstream. Instead, research has focused on the identification of their receptors and exploits the knowledge of how these and neuropeptides are structured. The idea is to produce substances called antagonists, which cancels the effects of a neuropeptide by blocking the receptor and which, preferably, are small and pass the blood-brain barrier.

**Galanin**

Galanin was extracted from porcine small intestines based on a novel isolation method (Tatemoto et al. 1978). Galanin consists of 29 amino acids Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-His-Ala-Ile-Asp-Asn-His-Arg-Ser-Phe-His-Asp-Lys-Tyr-Gly-Leu-Ala-NH2, and is C-terminally amidated. Its name stems from the fact that it contains an N-terminal glycine residue and a C-terminal alanine (Tatemoto et al. 1983). The sequence of amino acids in human galanin was determined in 1991. It consists of 30 amino acids, lacks the C-terminal amide but includes the additional amino acid serine (Evans et al. 1991).

It was early noted that galanin does not share any structural features with any oth-
Introduction

Galanin is a member of the family of biological active neuropeptides (Vrontakis et al. 1987) and exerts its biological effects by its N-terminal end, and it was long assumed to constitute a peptide family of its own (Bedecs et al. 1995). Galanin is phylogenetically old and highly conserved among different species, showing over 85% homology between rat, mouse, porcine, bovine and human sequences (Bedecs et al. 1995). In all species (the tuna fish being the exception), the first 15 amino acids from the N-terminal are identical, but amino acids differ at several positions at the C-terminal end of the peptide (Kask et al. 1995). These slight differences in protein structure have far reaching implications on their biological effects. For example, porcine and rat galanin inhibit glucose-induced insulin secretion in rats and dogs but have no effect on insulin secretion in humans. This demonstrates that it is essential to study the effects of galanin, and other regulatory peptides, in their autologous species (Bersani et al. 1991). Galanin is produced from the cleavage of a 123 amino acid long precursor, known as preprogalanin, which is produced from the preprogalanin gene.

![Galanin – containing neuronal pathways in the rat brain. From Kang Zheng 2011 with permission.](image)

The galanin family of neuropeptides consists of four members. After galanin itself, galanin message associated protein (GMAP), a 59 or 60 amino acid peptide formed from the cleavage of preprogalanin, was characterized in 1986 (Rökaeus et al. 1986). The two remaining peptides, galanin-like peptide (GALP) and alarin, were identified relatively recently and are both encoded for by the same gene, the preproGALP gene. GALP and alarin are produced by different post-translational splicing of this gene (Lang et al. 2007).
Introduction

Expression of galanin

Galanin is widely expressed in the central and peripheral as well as in the endocrine nervous system and co-exists with a number of classical neurotransmitters (Melander et al. 1986b) and has strong inhibitory actions on synaptic transmission by reducing the release of these neurotransmitters, for example acetylcholine (Fisone et al. 1987) and noradrenaline (Morilak et al. 2003), and also interacts with other neuromodulators including neuropeptide Y, substance P and vasoactive intestinal polypeptide (Figure 9).

The inhibitory actions of galanin result in a diverse range of physiological/pathophysiological functions, such as reproduction, memory and food intake (Liu et al. 2002; Taylor et al. 2009; Merchenthaler 2010; Crawley 2010; Barson et al. 2010), it also has roles in development and as a trophic factor (Hobson et al. 2010).

Galanin is also thought to play a role in a number of diseases including pain (Xu et al. 2010), Alzheimer’s disease (Counts et al. 2010), epilepsy (Lerner et al. 2010) as well as depression (Kuteeva et al. 2010), and cancer (Rauch et al. 2010).

Galanin coexists with choline acetyltransferase in basal forebrain cell bodies in several species. In the rat, galanin is expressed after colchicine treatment in 50–70% of cholinergic choline acetyltransferasepositive neurons in the medial septal nucleus and diagonal band of Broca area, some of which project to the hippocampus, i.e. a septohippocampal projection (Melander et al. 1985; Senut et al. 1989). However, there are important species differences. In humans, galanin is not co-localized in cholinergic neurons of the nucleus basalis of Meynert (Kordower et al. 1990), the main source of cortical cholinergic innervation in humans. In the rat, the majority of hippocampal galanin-containing cholinergic neurons project to the ventral hippocampal region. It is important to note that a substantial number of galanin nerve terminals within the hippocampal formation (HiFo) are noradrenergic, derived from locus coeruleus (LC) somata (Melander et al. 1986c; Xu et al. 1998a). Galanin is also expressed after colchicine treatment in a population of 5-hydroxytryptamine (5-HT) neurons in the dorsal raphe (Melander et al. 1986b; Xu et al. 1998b).

Galanin binding sites have been detected in the ventral HiFo, septum, and ventral aspect of the amygdala complex and entorhinal and perirhinal areas with relatively low binding in the dorsal cortex and in the striatum (Skofitsch et al. 1986b; Melander et al. 1988). In the HiFo the binding sites are concentrated to the most ventral part with medium dense labelling in CA3, CA1 and CA2 regions, with a high density labelling in the subiculum.

Galanin and pain

Numerous studies have demonstrated that galanin and its receptors are involved in the transmission and modulation of nociceptive information at spinal levels (Zhang et al. 2000; Liu et al. 2002; Hua et al. 2004; Wiesenfeld-Hallin et al. 2005; Xu et al. 2010). In the brain, studies have demonstrated that galanin plays an antinociceptive role in the hypothalamic arcuate nucleus in intact rats, in rats with inflammation and in rats with chronic neuropathic pain (Sun et al. 2003; Gu et al. 2007).
Introduction

Galanin in the central nervous system (CNS)
Galanin is distributed throughout the CNS of several species, including rat (Skoðfitsch and Jacobowitz 1985; Melander et al. 1986a; Skoðfitsch and Jacobowitz 1986a; Ryan et al. 1996), where it co-exists with classical neurotransmitters (Melander et al. 1986b; Merchenthaler et al. 1993; Jacobowitz et al. 2004).

Galanin mRNA is most abundant in hypothalamus and brainstem of rats (Jacobowitz et al. 1990; Jacobowitz et al. 2004), with very high levels in the preoptic, periventricular, and dorsomedial hypothalamic nuclei, bed nucleus of the stria terminalis, medial and lateral amygdala, LC, and nuclei of the solitary tract. Low to medium galanin mRNA levels are observed in olfactory bulb, septal nuclei, thalamus, parabrachial nucleus, and the spinal trigeminal tract nucleus. Shen et al. (2003) have demonstrated galanin mRNA in the proliferative zones of developing and adult brain – the subventricular zone and subgranular zone of hippocampus, and in oligodendrocyte precursor cells in the corpus callosum.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Receptors</th>
<th>Site of action</th>
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<tbody>
<tr>
<td>Galanin</td>
<td>GalR1</td>
<td>Peripheral</td>
</tr>
<tr>
<td></td>
<td>GalR2</td>
<td>Nerve injury/pain</td>
</tr>
<tr>
<td></td>
<td>GalR3</td>
<td>Pancreatic function</td>
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<td>Aging</td>
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<td>Alzheimer Disease</td>
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</table>

Figure 10
Galanin exerts its effects through three G-protein coupled receptors widely distributed both in the central and peripheral nervous system (adapted from (Lundström et al. 2005a)).

Galanin and neuronal injury
Galanin has been shown to be markedly upregulated after injury, both in the central and peripheral nervous system and both mRNA and peptide levels. Examples of such lesion studies include the upregulation of galanin in (1) dorsal root ganglion (DRG) neurons after peripheral axotomy (Hökfelt et al. 1987; Villar et al. 1989); (2) trigeminal ganglion neurons after damage of the vibrissae of rats (White et al. 1994); (3) medial septum-vertical diagonal band neurons after (i) electrocoagulation lesions of the ventral hippocampus or decortication (Cortes et al. 1990), (ii) transection of the septohippocampal pathway (Agoston et al. 1994) or (iii) tetrodotoxin injections into the vertical diagonal band (Agoston et al. 1994); (4) LC neurons after olfactory bulbectomy (Holmes et al. 1996); and
(5) magnocellular hypothalamic neurons after hypophysectomy, a procedure that transects the axons of these neurons (Villar et al. 1994).

As much as a 120-fold increase has been seen in dorsal root ganglia after nerve injury (Hökfelt et al. 1987; Villar et al. 1989). Galanin transcription is regulated in a tissue-specific manner both by enhancer and silencer sequences under the control of several transcription factors (see Vrontakis 2002).

These studies have led a number of investigators to suggest that galanin might play a cell survival or growth promoting role in addition to its classical neuromodulatory effects.

To test this hypothesis, transgenic animals were generated, bearing loss- or gain-of-function mutations in the galanin gene (Bacon et al. 2002; Holmes et al. 2000; Steiner et al. 2001; Blakeman et al. 2001). Phenotypic analysis of galanin knockout animals demonstrated that, surprisingly, the peptide acts as a survival factor to subsets of neurons in the developing peripheral and central nervous system (Holmes, 2000; O'Meara et al. 2000). It has also been demonstrated that this neuronal survival role is also relevant to the adult DRG. Sensory neurons are dependent upon galanin for neurite extension after injury, mediated by activation of the second galanin receptor subtype in a protein kinase C-dependent manner (Mahoney et al. 2003). There are several studies providing evidence that galanin might also act in a similar manner in the CNS, reducing cell death in animal models of brain injury, damage or disease.

**Galanin receptors**

It took more than ten years after the galanin discovery for the first galanin receptor (GalR1) to be cloned, by Habert-Ortoli and collaborators (1994). Then the remaining two (GalR2 and GalR3) were cloned fairly soon after that (Branchek et al. 2000). The distribution of galanin receptors was first studied in the rat brain, originally by ligand binding autoradiography (Skoﬁtsch et al. 1986b; Melander et al. 1988), then all three galanin receptors subtypes by in situ hybridization in particular by Dajan O’Donnell and associates at AstraZeneca, Montreal (see O’Donnell et al. 1999; Burazin et al. 2000; Waters et al. 2000; Mennicken et al. 2002; O’Donnell et al. 2003; Xu et al. 2005) and others. Galanin receptors are expressed in the CNS in peripheral tissues, including in the pancreas as well as on solid tumours (Figure 10). The level of expression of the different receptors varies at each location, and this distribution changes after injury to neurons (Figure 11).
Galanin expression is increased after peripheral nerve injury, in the basal forebrain in Alzheimer’s disease, during neuronal development and after stimulation with estrogen. Inflammation suppresses expression, and seizure activity depletes galanin in the hippocampus (adapted from Lundström et al. 2005b). The biological effect of galanin are mediated by the activation of one or more of the three known, cloned G-protein-coupled galanin receptor subtypes, designated GalR1, GalR2 and GalR3 (Branchek et al. 2000) which are all part of the G-protein-coupled receptor (GPCR) super family. The receptors show high interspecies homology and moderate homology to each other. All three receptors couple to $G_{i/o}$ and inhibit adenylyl cyclase (Habert-Ortoli et al. 1994; Smith et al. 1998) but GalR2 can in addition signal via $G_{q/11}$ to activate phospholipase C and protein kinase C (Wang et al. 1998b; Wittau et al. 2000). Many galanin receptor-specific ligands exist (Mitsukawa et al. 2010). One instrumental tool has been the galanin fragment Gal(2-11) (Liu et al. 2001).

Lu et al. (2005) has demonstrated binding of $^{125}$I-galanin and Gal(2-11) to receptor subtypes on isolated membranes, showing for example high GalR1 expression in the hypothalamic paraventricular nucleus, and predominantly GalR2 in the dorsal raphe, HiFo and the amygdala. Rafael Rodriguez-Puertas’ group used the [35S] GTPγS assay and autoradiography to analyse galanin receptor-coupling to G-proteins (Barreda-Gomez et al. 2005). In most areas agreement with earlier $^{125}$I-galanin binding studies and with GalR1 mRNA distribution was reported, but apparent discrepancies were also found The same group used a similar approach to study the effect of intraventricularly administered galanin
on muscarinic and galaninergic G-protein coupling and found, for example, that this treatment increases the coupling of both galanin and muscarine type of receptor in the medial amygdala nucleus, whereas in other areas only one type of receptor-coupling was modulated (Barreda-Gomez et al. 2005).

The third galanin receptor was first described by Wang et al. (1997). There are only a few studies describing the tissue expression profile of GalR3 in the rat with using a variety of RNA profiling techniques and certain discrepancies have emerged, particularly with respect to its CNS distribution.

By Northern blot, Wang and co-workers detected GalR3 mRNA in heart, spleen and testis but not in brain; isolation of GalR3 from a rat hypothalamic cDNA library, however indicates that it is present in rat CNS at low abundance. Indeed, using the more sensitive RNase protection assay, Smith et al. (1998) detected GalR3 transcripts in discrete regions of the rat CNS with highest levels in the hypothalamus, lower levels in the olfactory bulb, cerebral cortex, medulla oblongata, caudate putamen, cerebellum, and spinal cord, and no significant detection in hippocampus or substantia nigra.

In the peripheral nervous system the highest levels of GalR3 mRNA were found in the rat pituitary gland. More recently, Waters and Krause (2000) described a similar GalR3 distribution profile using both reverse transcription/polymerase chain reaction (RT/PCR) and RNase protection assays. However, these authors observed GalR3 expression in the rat hippocampus, whereas Smith et al. did not. Mennicken et al. (2002) used in situ hybridization (ISH) with a CDNA riboprobe to study the cellular distribution of GalR3 within the rat CNS. They displayed low and discrete labelling throughout the CNS. GalR3 expression is most prominent in the preoptic/hypothalamic area and the subfornical organ, but is also evident in discrete regions of the basal forebrain, pons, medulla and dorsal horn of the spinal cord. The regions in which Mennicken et al. (2002) detected GalR3 mRNA are also known to contain high levels of galanin and galanin binding sites, representing targets for the central action of galanin.

**Galanin agonists**

The introduction of Gal(2-11) which acts as an agonist with 500-fold selectivity for GalR2 (Liu et al. 2001) compared with GalR1, was an important advance in the field, although a latter publication showed that Gal(2-11) also binds and activates GalR3 in a transfected cell line with similar affinity to GalR2 (Lu et al. 2005). Gal(2-11) has since then been employed in several studies, as a non-GalR1 ligand, as no ligand with higher selectivity has been available (Jimenez-Andrade et al. 2006; Alier et al. 2008).

GalR2, along with the other galanin receptor subtypes, could in the future be an important target in several disease states such as epileptic seizure, Alzheimer's disease, mood disorders, anxiety, alcohol intake in addiction, metabolic disease, pain and solid tumors (Mitsukawa et al. 2010). If so, a subtype specific ligand is needed, to downsize unwanted side-effects.
Introduction

The GalR2/3 agonist Gal(2-11) has been evaluated in several studies (Liu et al. 2001; Elliott-Hunt et al. 2004; Hua et al. 2005; Mazarati et al. 2005; Pirondi et al. 2005; Ding et al. 2006; Kuteeva et al. 2008) and its effects have in most cases been considered as mediated via GalR2, when interpreting the result. The exclusion of effects mediated via GalR3 are somewhat doubtful, as the expression pattern of GalR3 in the CNS is disparate in the literature (Smith et al. 1998; Agranoff et al. 1999; Waters et al. 2000; Mennicken et al. 2002). The introduction of a small molecule GalR3 antagonist has provided possibilities to study presence and importance of GalR3 in the CNS (Swanson et al. 2005; Barr et al. 2006).

Galanin receptors have been characterized in expression models. Sebastian Wirz in Bartfai’s group (2005) analysed GalR1 dimerization and internalization using fluorescence resonance energy transfer (FRET) technique and Chinese hamster ovarian cells (CHO) (Wirz et al. 2005). No evidence for dimerization was obtained, but internalization was demonstrated using time lapse fluorescence imaging. Zhi-Qing David Xu from the Karolinska group has presented studies on GalR1 and GalR2 receptors transfected to PC12 cells, demonstrating both constitutive and ligand induced internalization for the GalR2 receptor (Xia et al. 2004; Xia et al. 2005; Xia et al. 2008). The data strongly suggest that GalR2 receptor trafficking utilizes the clathrin-dependent endocytic recycling pathway, which also seems to be the case for the GalR1 receptor, whereas no evidence for constitutive internalization was found for the latter receptor.
Aims of the thesis

The main focus on the present thesis was to develop an experimental stroke model catering for focal and transient ischemia, modern anesthesia and intraoperative monitoring with low mortality.

Using this reperfusion stroke model in rats the effects on the neuropeptide galanin and its receptors were studied in order to

- investigate the effects of hypothermia on the size of the ischemic lesions and on galanin concentrations in brain tissues after transient MCAo in naïve female rats.
- investigate the effects of intracerebroventricular galanin and galanin receptor 2/3 agonist on the ischemic brain lesions after transient MCAo in naïve female rats.
- investigate the effects on galanin and its three receptors gene expression in discrete brain regions after transient MCAo in naïve female rats.
Material and Methods

Animals and surgery

Ethical aspects
The guidelines issued by the National Committee for Animal Research in Sweden and Principles of Laboratory Animal Care (NIH publication No 86-23, revised 1985) were followed. All studies and their experimental protocol were approved by the Local Ethics Committee for Animal Care and Use at Linköping University.

Housing
Female SD rats were used in all studies. Animals from the breeder B&K Universal (Sollentuna, Sweden) were used in paper I-III and from Taconic (Germany) in paper IV. Adult females with a body weight of 216-350g were housed at the Animal Department of Linköping University for at least 1 week before the start of the experiments. The rats were kept two in each cage (Macrolone, MAC3, 974 cm² at the bottom, and 15,5 cm high and containing 300g aspen material (Tapvei, Finland)) at constant room temperature (21 ± 1°C), with free access to water and standard rat chow (Lactamine, Vadstena, Sweden), and with 12 hours light-dark (light on at 8.00 am).

Anesthetic procedures
The animal was kept in its own cage with free access to water and standard rat chow until induction of anesthesia. The rat was placed in an induction chamber (50216 Stoelting, Wood Dale, IL, USA) and anesthesia was induced by 4% isoflurane (Forene; Abbott Scandinavia, Kista, Sweden) in a mixture (30%/70%) of oxygen/nitrous oxide.

Endotracheal intubation
A specially designed sloping board was used to position the anaesthetized rat with the teeth’s on the upper jaw fixed to extend the head in order to identify the epiglottis and to intubate the trachea. The tube, a venflon 14G (Venflon Viggo, Helsingborg, Sweden), without its mandrin, was introduced into the trachea with a specially modified laryngoscope for neonates under direct visualization (Figure 12).
Material and Methods

Figure 12

The technique of endotracheal intubation. A. Anesthesia is induced in the induction chamber. B. The neck is extended with the animal placed on a sloping board. C. Epiglottis and trachea opening is visualized. D. The tube is positioned.

The tube in the trachea was connected to an animal ventilator (Zoovent, CWC 600AP, ULV, Newport, UK), and the anesthesia was continued with 1% isoflurane in a mixture (30%/70%) of oxygen/nitrous oxide. The animals were kept normoventilated and the tidal volume and the frequency were carefully regulated according to the results of repeated blood gas analysis. Normally a peak airway pressure of 10-12 cmH2O and a rate of 80-85 breaths per minute were required to maintain appropriate blood gas tensions.

The depth of anesthesia was determined by sensory and motor responses. The absence of foot withdrawal when pinched, and the absence of eye blink when the eyelid was touched, were used as indices of an adequate anesthesia.
Material and Methods

Preparation of rat for surgery and intraoperative monitoring

Eye gel (Lubrithal 15 g Leo laboratories, Dublin, Ireland) was applied to protect the rat’s eyes during the surgery. Saline (NaCl 9 mg/mL, B. Braun Medical AB, Bromma, Sweden), 6 mL/kg body weight, was administered subcutaneously at the start of operation for substituting loss of fluid. A long-acting analgetic (Rimadyl® 50 mg/mL, Pfizer, Dundee, Scotland) was given at the same time in a dose of 0.1 mL/kg. A surgical head holder was used to position the head during surgery by means of an adjustable nose clamp (Figure 13).

To keep the animal’s body temperature at the desired levels according the study protocols (37.0 ± 0.5°C paper I – IV and 33.0 ± 0.5°C paper II) the rat was placed on a heating pad (Homeothermic Blanket System Harvard, Edenbridge, UK) (temperature controller) to keep the core temperature at intended level, and monitored through a rectal probe.

Catheterizing of the femoral artery

Before insertion the catheter (Micro-Renathane® tubing 36 ft [MRE-25 Braintree Scientific, Inc., MA, USA]) was prepared with a needle (27 G) and flushed with Heparin (100 IU/mL, Lövens Läkemedel, Malmö, Sweden). The left femoral triangle was shaved carefully to avoid abrading the skin and thoroughly cleaned with 70% ethanol. A 1-2 cm long skin incision was made over the femoral vessels, and the fascia was opened to expose the femoral sheath. Through blunt dissection the artery was separated from the nerve and the vein (Figure 14).
Material and Methods

The femoral artery was gently retracted with a pair of sutures (Ethicon Vicryl 4/0, Johnson & Johnson Intl., Sollentuna, Sweden), first "downstream" then "upstream" to the area into which the catheter was introduced. A micro scissor was used to cut a minute hole in the vessel. The catheter was inserted 1-1½cm in the artery, and the catheter was secured to the artery with ligatures. To inhibit coagulation the vessel was flushed with heparin (4 IU/mL) (Figure 15).

Data registration
All data from the monitors of rectal temperature, arterial blood pressure and pulse were transferred through an interface module (UIM 100, BIOPAC Systems, Inc., Goleta, CA, USA) and an acquisition unit (MP 100, BIOPAC Systems, Inc.) to the computer (IBM PC, Dublin, Irland). The data were processed by AcqKnowledge® software (BIOPAC Systems, Inc.), displaying parameters graphically on-line. All events during the operation were documented in real time in a surgical protocol included in the AcqKnowledge® computer for that specific operation.

Occlusion of the middle cerebral artery
The rat was carefully shaved on the left side of the head, between the eye and the ear, to avoid abrading the skin and thoroughly cleaned with 70% ethanol. Care was taken to avoid absicising or shortening the rat’s whiskers when shaving the operation area, since this would have major impact on the behavior of the animal. The left MCA was exposed through a subtemporal approach, using an operating microscope (Figure 16). Transection of the facial nerve was avoided during exposure of the temporalis muscle, which was divided posteriorly and retracted in the anterior– inferior direction. The zygoma was partially removed (4–5 mm). A 3 mm craniectomy was drilled up 3 mm anterior and 1 mm lateral to the foramen ovale, where the mandibular nerve exits. To prevent heating of the underlying cortex physiological saline was flushed at the drill site. By a fine micro hook the dura was opened by a cruciate incision. The MCA was visualized as it runs forwards initially, than turning laterally running over the olfactory tract.
Figure 16  A general overview from the operating room.

Figure 17  The MCA and the microclip positioned just before clipping the artery as visualized through the operating microscope.
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The arachnoid membrane was opened on either side of the artery by a fine needle. The MCA was occluded with a microclip between the rhino cortical branch and the lenticulostriate artery (Figure 17). After 1h occlusion of the MCA the microclip was carefully removed. The craniectomy was covered by a small piece of surgical soft tissue (Surgicel, Johnson & Johnson, Intl.) and the wound was closed using re-absorbable suture (4-0 Vicryl, Ethicon, Inc., Johnson & Johnson, Intl.).

Application of hypothermia

By the application of icepacks around the animal it was cooled at an average rate of 0.25°C/min beginning after intubation. The temperature of 33°C was therefore achieved after an average of 15 min. The rats were maintained at the desired level of core/rectum temperature at 33.0 ± 0.5°C for the duration of the experiment by means of a thermostatic heating pad. In case the body temperature of a rat deviated more than 0.5°C from the set point for more than 5 min, that rat was excluded from the study. The hypothermic animals were rewarmed to 37°C with a heating lamp during a 1-hour session after termination of the MCAo operation and before being returned to a clean cage with free access to water and standard rat chow (paper II).

Figure 18

Stererotactic application of the catheter for intracerebroventricular administration

Intracerebroventricular administration of galanin and galanin receptor 2/3 agonist

An Alzet® brain infusion kit including a 28 G stainless steel cannula and a height adjustment spacer (Alzet® Infusion Kit II, 3-5 mm, Durect Corporations, Cupertino, CA, USA) was used together with an Alzet® osmotic pump, (volume 100 μL, releasing rate 0.5 μL/h, lasting 1 week) (Alzet® Osmotic Pump 1007D, Durect Corporations) (Figure 18 and 19). The cannula was operated into the rats left ventricle using the stereotactic coordinates: Bregma (B) -1.3 mm anterior/posterior, B -3.8 mm dorsal/ventral and +1.8 mm lateral to midline. The
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height adjustment spacer was 0.5 mm. The cannula was secured with three stainless steel screws and glue (Dental® plus, Heraeus Kutzer, Dormagen, Germany) to secure lasting position. The Alzet® osmotic pump was inserted in the rat neck and connected to the brain infusion cannula. The osmotic pump was filled with rat galanin (SC 936, Neosystem, Strasbourg, France) dissolved in the isotonic Ringer-Acetate (Braun) to a concentration of 8.4 μg/100μL, 84 μg/100μL or 840 μg/100μL releasing a dose of 0.77 μg/day (0.24 nmol/day), 7.68 μg/day (2.4 nmol/day) or 76.8 μg/day (24 nmol/day). The GalR2/3 agonist Gal(2-11), (AR-M1896, Tocris Cookson Ltd., Bristol, UK), was dissolved as above to a concentration of 84 μg/100μL, releasing a dose of 7.68 μg/day (2.4 nmol/day) and processed as mentioned above (paper III).

Figure 19
The Alzet® brain infusion kit with a stainless cannula using one height adjustment spacer was operated into the left ventricle by stereotactical technique for intracerebroventricular administration of galanin, galanin R2/3 agonist or artificial CSF. The osmotic pump was inserted subcutaneously in the rat neck and connected to the brain infusion cannula by a thin tube.

Termination of the surgery
The catheter in the femoral artery used for measuring the pulse and blood pressure was carefully removed and permanently ligated (Ethicon Vicryl 4/0, Johnson & Johnson Intl.), and the skin was sutured. The flow of isoflurane and nitrous oxide to the respirator was stopped, and the rat was allowed to breathe oxygen until spontaneous breathing was regained. The rat was subsequently transferred to a clean cage with free access to water and standard rat chow. The next day, a new dose of analgesic Rimadyl® (50 mg/mL, Pfizer) 0.1 mL/kg was administered. Members of the staff of the Animal Department made daily post-operative controls of the rats.
Material and Methods

Brain biopsies

Brain slices and biopsies
The rats were sacrificed after anesthesia by pure carbon dioxide using a guillotine (Small animal decapitator, AH 55-0012, Harvard Apparatus, Edenbridge, UK) and the brain was carefully dissected out from the skull, avoiding damage from sharp pieces of bone and the meninges and cooled in +4°C saline (paper I – IV).

After a period (2-4 min) of cooling in a solution of 0.9% NaCl the brain was transferred to a rat brain matrix (RBM-4000, ASI Instrument, Inc., Warren, MI, USA). The brain was cut into 2 mm thick coronary slices, by razor blades, using bregma as position zero (Paxinos 1995) (Figure 20). In paper II five slices were cut at bregma and 2, 4, 6 and 8 mm posterior to bregma. In paper I, III and IV two more slices were added, 2 and 4 mm anterior to bregma. Furthermore, in paper IV two more slices were added, 10 and 12 mm posterior to bregma.

Figure 20
Slicing the rat brain using a brain matrix and razor blades

Punch biopsies
Punch biopsies were taken for measurement of the concentrations of galanin (paper II) and galanin/galanin receptor gene expression (paper IV). The biopsies were taken with a biopsy needle (Punch tip, 2 mm, ASI Instrument Inc.) and were 2 mm in diameter and 2 mm thick. The location of punch biopsies (paper II) are shown in Figure 21, and were taken from both the lesioned and the contra lateral control side; at bregma – frontal cortex, bregma minus 2 mm – parietal cortex and inferior striatum, bregma minus 4 mm – parietal cortex and medial thalamus, bregma minus 6 mm – medial thalamus, bregma minus 8 mm – dorsal and ventral hippocampus. For galanin gene expression punch biopsies were taken from from LC (bregma minus 12 mm), cortex (bregma minus 2 mm) and dorsal hippocampus (bregma minus 8 mm) (paper IV). The biopsies were rapidly collected and snap-frozen on dry ice and stored at -70°C prior to analysis.
Material and Methods

Figure 21
Location of punch biopsies in the brain slices from both the lesioned and the contra-lateral control side for measurement of galanin or galanin receptor gene expression.

Measuring the size of the ischemic brain lesions
Each brain slice was carefully transferred to a Petri dish. The 2 mm thick coronary slices were freed from dura mater and soaked for 10 minutes in a solution of 2% TTC in 0.1 mol/L PBS (pH 7.4) in a Petri dish, maintained at 37°C in a heater. Gentle stirring of the slices was used to ensure even exposure of the surfaces to staining. Excess TTC was then drained, and the slices were scanned (ScanJet IIc, Hewlett-Packard, Cupertino, CA, USA) into a computer file (Microsoft Windows) for image analysis.

The size of the brain lesion was measured using the image analysis software SigmaScan Pro version 5 (Systat Software Inc., Richmond, CA, USA). The image was divided into its red, green and blue colour spectra. The red spectrum was used to measure the total area of the slice and the green spectrum for measuring the ischemic lesion area. To sharpen the boundary between the slice itself and its surroundings the intensity of the red spectra was maximized before the measurement. In the green spectra the outer boundaries of the ischemic area were marked in the "overlay draw mode" to demark the area from normal structures in the brain. To mark the ischemic area a threshold of 40% in the
green spectrum was used in the function "fill mode". The lesioned area was measured as percentage of the total area of each slice (Bederson et al. 1986a; Bederson et al. 1986b; Goldlust et al. 1996) (Figure 22) (papers I-IV).

Figure 22
Measuring the size of the ischemic brain lesions. The original scanned image (A). The red spectrum of the original image (B) is used to estimate the size of the total area (C). The green spectrum of the original image (D) is used to estimate the size of the ischemic brain lesion (E).
Material and Methods

**Galanin measurements**

**Extraction of brain punch biopsies**
The punch biopsies from the brain slices were extracted in 2 mL of boiling 1 mol/L acetic acid (MERCK, Darmstadt, Germany) and boiled for 10 min. The tissues were homogenized with a polytron (CAT X520D, Scientific Industries, New York, NY, USA) and centrifuged at 1500 g in 4°C for 10 min. The supernatant was collected, and a second extraction was immediately performed of the supernatants with 10 mL of distilled water per gram tissue to increase the recovery. The supernatants from each sample were combined, lyophilized and stored at -70°C prior to analysis (paper II).

**Measuring galanin concentrations in brain tissue extracts**
Galanin was analysed using antiserum RatGal4 raised against conjugated synthetic rat galanin(1-29). The antiserum does not cross react with neurokinin A, neuropeptide K, substance P, neurokinin B, neuropeptide Y, gastrin, pancreatic polypeptide, glucagon or neurotensin. HPLC-puriﬁed 125I rat galanin was used as radioligand and rat galanin as standard. The detection limit of the assay was 8 pmol/L. Intra- and interassay coefﬁcients of variation were 6% and 10%, respectively (Theodorsson et al. 2000) (paper II).

**Real-time reverse transcriptase-polymerase chain reaction in brain punch biopsies**
Gene expression was analysed by RT-PCR in punch biopsies from the brain slices from LC (bregma minus 12 mm), cortex (bregma minus 2 mm) and the dorsal hippocampus (bregma minus 8 mm). The biopsies were rapidly collected using a micro-dissection protocol and snap-frozen on dry ice and stored at -70°C. Total RNA was extracted using the RNeasy Micro kit (Qiagen, Sollentuna, Sweden), according to the manufacturer’s instructions, including deoxyribonuclease treatment with ribonuclease-free deoxyribonuclease set (RNase-Free DNase set, Qiagen). The concentrations and purity of the RNA were measured with NanoDrop ND-1000 Spectrophotometer (Saveen & Werner AB, Limhamn, Sweden). RNA samples were kept at -70°C prior to analysis.

A sample of 80 ng of RNA from LC, dorsal HiFo and cortex, were reversely transcribed to single-stranded cDNA by random hexamer priming using High Capacity cDNA RT kit (Applied Biosystem, Stockholm, Sweden), in a volume of 20 μL, using the following program: 25°C for 10 min, 37°C for 60 min, 85°C for 5 sec, 4°C ∞. The samples were diluted 1:10 and kept at -70°C prior to analysis.

For RT-PCR, 7 μL of cDNA was added to TaqMan® Fast Universal PCR Master Mix (2X), RNase-free water and TaqMan® Gene Expression Assay primer (Applied Biosystems) to achieve a ﬁnal reaction volume of 20 μL. The PCR protocol consisted of: initiation at 1 cycle at 95°C for 20 sec, followed by ampliﬁcation for 40 cycles at 95°C for 1 sec and 60°C for 20 sec.

The following TaqMan® Gene Expression assays were used: Galanin: Rn
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Ywahz Rn 00755072_m1 and Sdha Rn 00590475_m1 (Applied Biosystems) were used as endogenous reference genes for study of the effects of lesions (Gubern et al. 2009) and of the estrous cycle (Hvid, Ekström et al. 2010).

The reactions were carried out in 96-well plates covered with optical adhesive film (Applied Biosystems). Each sample was analyzed in duplicate and average values were calculated. No-template reactions with water instead of cDNA were included as negative controls on all plates.

Gene expression was calculated using the ΔΔCt method (Ct = threshold cycle). Each gene was normalized with the corresponding average of Sdha and Ywahz expression in the same animal and expressed as the fold-difference in relation to the control group (paper IV).

Statistical methods

Paper II

The mean and standard error of the mean were used as measures of central tendency and variation respectively throughout the study. Multivariate analysis of variance (ANOVA) was used for significance testing (SYSTAT version 11, Systat Software Inc.) using galanin concentration and infarction size, respectively, as dependent variable and body temperature, observation periods and ischemia as independent factors. We refrained from making detailed multiple comparisons e.g. between regions and groups, since they were not an inherent part of the original hypothesis. p < 0.05 were considered significant.

Paper III

The mean and standard error of the mean were used as measures of central tendency and variation, respectively, throughout the study. Multivariate analysis of variance (ANOVA) and Tukey’s post hoc test were used for significance testing (SYSTAT version 11, Systat Software Inc.). p < 0.05 were considered significant.

Paper IV

Data were analyzed using two-tailed t test for independent samples, only of the groups deemed of interest when the study was designed. A p < 0.05 was considered statistically significant. Data are reported as mean± SEM.
Results and discussion

Stroke model – methodological aspects (paper I)

Ten years ago when I became a member of the research group there was a need for an experimental stroke model to study the physiological and pathophysiological role(s) of estrogen-responsive neuropeptides, e.g. galanin, in ischemic brain lesions. Our primary goal was to mimic the clinical situation of an appropriately treated ischemic stroke in humans with an initial ischemic episode followed by reperfusion, as seen after successful thrombolytic therapy. Therefore a focal, MCAo, and transient – allowing reperfusion – stroke model was chosen. The MCAo could be done in a direct or in an indirect way; i.e. by craniectomy or not and by different occlusions methods. Our choice was MCAo by a microclip under direct visualization, instead of photochemically induced thrombus formation (Wester et al. 1995), the intraluminal filament method (Kozumi 1986; Longa et al. 1989) or embolic cerebral ischemia (Zhang et al. 1997a; Zhang et al. 1997b).

The reason for choosing the direct clip method was from the outset the wish to visually verify the occlusion and the availability of microsurgical skills in the group. However, as we soon discovered, this was also combined with our underestimation of the technical obstacles this method would entail in rats.

The technique of focal MCAo through craniectomy was first described by Robinson and colleagues in 1975 using suture ligation (Robinson et al. 1975). A couple of years later came a detailed description of a stroke model by Tamura and co-workers (1981a) which became the basis of our model. Through a sub-temporal approach ad modum Tamura the MCA was visualized and occluded, but with an important modification of the Tamura method; we used temporary microclip occlusion catering for reperfusion, instead of permanent electrocoagulation. Our method was not combined with occlusion of vertebral- or carotid arteries, and systemic hypotension and hypoxia were avoided. Thus, the apparent risk of damage to other organs including the heart and the kidneys was reduced. The goal was to have a mild ischemia model with low morbidity and mortality, optimizing conditions for the animals thriving well postoperatively and allowing long-term (weeks) observation periods.

Different commercially available microclips were tested in the beginning, i.e. Sundt AVM micro clip No. 1 (Codman, Johnson & Johnson). However, there were serious draw-backs with these clips, including the fact that they could only be used once, they were very expensive and – in our hands – hard to use. Taken together, this prompted us, together with the Department of Medical Engineering, University Hospital, Linköping, Sweden to specially modify and design a reusable microclip (Modified microvascular clamp size 8 mm (98397A, Rehsstock Instruments GmbH, Durbheim, Germany)) (Figure 23).

As described by Tamura and colleagues, the MCA was occluded between the
Results and discussion

lenticulostriate artery and the arterial branch of the rhinal cortex, causing an ischemic damage to cortex and striatum. Since it is of uttermost importance that the microclip maintains its mechanical stability and strength, it has been regularly checked for its biomechanical properties at the Department of Medical Engineering.

Using a stroke model with craniectomy and microclip occlusion of the MCA involves several challenges and risks for complications specific for this model. A subtemporal approach may cause bleedings from the structures passed to reach the MCA, temperature elevation of the cortex when drilling a hole in the skull, brain contusion, changes in intracranial pressure, damage to and bleeding from the MCA (0.24 mm in diameter), leakage of cerebrospinal fluid and infections. After acquiring the necessary technical skills, bleeding is the most common complication. We have not observed any complications of infection post operatively, nor cerebrospinal fluid leakage.

An advantage of a direct microclipping method is that occlusion of the artery is visually verified, compared to the intraluminal filament method, where occlusion of other cerebral vessel orifices and insufficient MCAo are complications described (Schmid-Elsaesser et al. 1998; Furuya et al. 2004), as well as SAH and endothelial lesions causing thrombotic complication. A mortality rate up to 92.7% within 48h after MCAo have been reported when a silicone rubber cylinder is attached to the filament (Nagasawa et al. 1989). Spontaneous hyperthermia up to 40°C early after MCAo with the filament method has been observed (Li et al. 1999; Gerriets et al. 2003). Increased body temperature is associated with damage to the hypothalamus and is likely the result of reduced blood flow to this brain region due to incorrect placed filament.

The visually controlled temporary MCAo between the rhino-cortical branch and the lenticulostrate artery results in small ischemic brain lesions and short-term (hours) neurological deficits allowing the animals to thrive well for at least 2 weeks. The size of the ischemic lesions are small – less than 10% of the total slice area and variably engaging cortex and/or striatum, predominantly the lateral part. The lesions are largest at bregma and 2 mm anterior and pos-
terior to bregma. There is a difference in collateral circulation between species, and in the rat – the animal used in the current stroke model – the variability in MCA and its branches including collateral anastomoses is well known (Oliff et al. 1995a; Oliff et al. 1995b; Oliff et al. 1997). These circumstances are likely to play an important role in the observed variation in the size of the ischemic lesions observed in our studies.

**Anesthesia model – methodological aspects (paper I)**

When we started developing the present method most studies in the field used intraperitoneal anesthesia (Bederson et al. 1986b; Goldlust et al. 1996; Simpkins et al. 1997; Dubal et al. 1998; Wise 2000; Wise et al. 2000; Ardehali et al. 2003) and spontaneous respiration. This reason, in combination with the fact that no other anesthetic techniques were available at our Local Animal Department at that time, led us to an initial use of intraperitoneal anesthesia by chloral hydrate. However, we used oxygen ventilation by tracheotomy. With this method we experienced difficulties in maintaining control of anesthesia during the surgical procedure, both concerning the time from induction to sufficient anesthetic depth and the period of time when an additional dose of the anesthetic agent was needed. Furthermore, the rats – in our experience - recovered slowly from anesthesia, and often developed respiratory depression and hypotension after the surgery and extubation. Contributing factors to this could be inadvertent injections into the gut and urinary bladder (Zausinger et al. 2002). Thus, using intraperitoneal drug administration increased the risk of over as well as under dosage. Considerable difficulties in controlling the anesthesia and a mortality of about 25% in the 40 initially operated rats prompted us to abandon intraperitoneal anesthesia by chloral hydrate and ventilation by tracheotomy in favour of endotracheal intubation and isoflurane anesthesia (1% isoflurane in 30%/70% O₂/N₂O).

The techniques for endotracheal intubation was described in the literature (Jaffe et al. 1973; Pena et al. 1980). However, the crucial equipment needed was not commercially available. Therefore we designed, and the Department of Medical Engineering produced the modified laryngoscope (initially designed for use in neonates) and the necessary sloping board extending the rat neck, facilitating the insertion of the tube into trachea (Figure 24).

The use of face-mask and spontaneously ventilated rats was not chosen, since this method critically affects the outcome after cerebral ischemia through intraoperative physiological parameters like blood pressure (Patel et al. 1991; Chileuitt et al. 1996), arterial blood content of O₂ and CO₂ (Browning et al. 1997; Smrcka et al. 1998) and pH in blood (Siesjö et al. 1996). Zausinger and co-workers (2002) reported a significantly increased ischemic damage and a higher mortality rate in spontaneously breathing SD male rats (using inhalation of halothane administered with a face mask, as well as intraperitoneal chloral hydrate anesthesia) compared to mechanically ventilated rats with halothane.
Results and discussion

anesthesia in a transient filament stroke model. In addition to the apparent risk of hypercapnia, acidosis and low blood pressure influencing the final outcome after an ischemic insult, there are other drawbacks of the face mask method; e.g. inadequate fitting masks and interference of the mask with the proper access to the operating area. Furthermore, there is an apparent risk of waste of anesthetic gases and hazardous pollution of the operating area, if an operation mask is used.

Modern available inhalational agents at the time of our start up were isoflurane, halothane and sevoflurane, all reported to exert a degree of neuroprotection similar to that of barbiturates in the setting of focal cerebral ischemia (Zausinger et al. 2002). The underlying mechanisms of neuroprotection may be related to the excitotoxic glutamate response (Martin et al. 1995), prostanoid production (Moore et al. 1994) and NO synthesis (Tobin et al. 1994). Our choice was isoflurane and we use it in our experimental stroke model throughout all studies.

To avoid stress prior anesthesia the rats were kept in their own cages with free access to water and standard chow until transferred to the induction chamber for the anesthesia and subsequent anesthesia induction. Other groups report pre operative fasting (Yang et al. 1994) or only access to water (Zausinger et al. 2002). An important reason for preanesthesia fasting is to reduce the risk of vomiting and aspiration of vomit. Since rats do not vomit there is no risk for these complications (Wang-Fischer 2009). This is also easily verified in practice.

Figure 24
Specially designed sloping intubation board (A) and the modified neonate laryngoscope (B). The size of the instruments is detailed (mm) in the figure. The lump of metal behind the sloping board itself gives the instrumentation the necessary weight to stand still during the intubation procedure.
General methodological aspects (paper I)

Intra- and postoperative mortality
When we started developing the present stroke model, we experienced an overall rat mortality of 25% when performing MCAo. Morbidity and mortality in studies employing experimental stroke models are crucial confounding factors, which unfortunately are rarely comprehensively reported. This – in our opinion – high mortality of 25% prompted us to abandon this anesthesia method and to develop a method of endotracheal intubation and isoflurane anesthesia. These anesthetic techniques reduced the intraoperative and first 24 hours post-operative mortality from 25% to 10.6%. Improved overall skills in the anesthesia and operation methods further reduced the mortality to 2.7%.

Blood glucose
The level of blood glucose is an important factor to be monitored and maintained in the normal range in experimental animal stroke models, since hyperglycemia is reported to impair outcome of stroke both in humans and in animal studies (Kagansky et al. 2001; Johan Groeneveld et al. 2002). When we designed our stroke model we measured blood glucose in about 30-40 rats using the Hemocue® apparatus, and all samples were found within normal range (Hemocue, Angelholm, Sweden). A reasonable explanation to this was that the rats were allowed free access to rat chow until anesthesia was induced. Unfortunately the current blood gas analyzer (AVLOPTI 1 Medical Nordic AB, Stockholm, Sweden) we are using does not measure blood glucose and is the main reason, why this parameter is not reported in the studies.

Animal sex
The majority of experimental animal stroke studies are performed in male rats, avoiding the impact of the cyclic variation in ovarial hormone concentrations in females known to influence the ischemic brain damage (Ström et al. 2010). When submitting manuscripts we have, surprisingly enough and at repeated occasions, got the question why our studies were done on female rats. Spectrum, concentrations and cyclicity of steroid hormones are evidently widely different in female and male rats. However, all our previous experience in the field relates to female rats (Hilke et al. 2005; Theodorsson et al. 2005a; Theodorsson et al. 2005c; Ström et al. 2008a; Ström et al. 2008b; Ström et al. 2009; Ström et al. 2010), which prompted us to use female rats. It is evident that a direct comparison of on our own data with studies made on male rats cannot be made.

Effect of animal strain and vendor
The rat is the species most frequently used in experimental animal stroke models, even though mice and gerbils are studied as well. It is known that genetic factors influence the ischemic brain damage in animal stroke models. For example, using SHR and stroke-prone SHRs; e.g. genetically hypertensive strains, usually results in larger ischemic brain lesions and lower infarct size variabi-
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Sensitivity than reported in normotensive strains (Brint et al. 1988; Duverger et al. 1988). Furthermore, intra-strain differences in cerebral infarction after MCAo depending on the animals’ origin/vendor is reported (Oliff et al. 1995a). We have used the same strain (SD females) in all studies and from the same vendor, except for paper IV where the animals were delivered from another breeder.

Age of animals
Young adult healthy animals are routinely used in animal stroke models (Gladstone et al. 2002), including ours, despite the importance of aging in cerebro-vascular disease in humans. Davis et al. (1995) report an age-dependent increase in the cerebral ischemic damage in a focal stroke model in male Wistar rats, indicating the relevance and need for further studies in old rats.

The staining method for measuring the size of the ischemic lesion
The TTC method of measuring the brain lesion (Bederson et al. 1986a; Goldlust et al. 1996) is carefully validated for use up to 24 hours after the occlusion, delineating both the ischemic core and its surrounding penumbra (Astrup et al. 1981), and partially validated after 3 days (Lin et al. 1993; Vergouwen et al. 2000). The affected area is continuous and well delineated after few hours, when the lesions are extensive, and becomes more irregular in shape as the days pass and the surviving cells in the penumbra are revitalized. These irregular shapes preclude the use of volume-morphometric methods, and therefore area measurements were used. Brain oedema, of cytotoxic origin, developing the first days after the ischemic insult, may artificially and markedly increase the infarct volume (Brint et al. 1988). Swanson and co-workers (1990) have proposed a method compensating for the oedema when measuring the infarct volume, but this approach was not used in the current method.
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Effects of hypothermia on the ischemic brain lesion and on tissue concentrations of galanin in the stroke model (paper II)

Hypothermia is known to reduce brain lesions caused by ischemia (Churchill-Davidson 1954; Holzer et al. 2010), not only when administered before and during the ischemic episode, but also afterwards (Rosomoff et al. 1954). The neuroprotective role of moderate hypothermia has been well established in humans (Hemmen et al. 2009; Yenari et al. 2010), and in experimental animals (Kawai et al. 2000; Yanamoto et al. 2001; Miyazawa et al. 2003), and clinical use of hypothermia dates back to the 1960s (Westin 2006).

Several studies have shown that galanin concentrations are increased in response to various types of lesions to the central and peripheral nervous systems (Hökfelt et al. 1987; Villar et al. 1989; Cortes et al. 1990) and, as mainly shown by Wynick and colleagues (2001), galanin may be amongst the factors contributing to maintenance of neuronal survival and functions (Xu et al. 1996; Holmes et al. 2000; O’Meara et al. 2000; Elliott-Hunt et al. 2004). We therefore considered it of interest to investigate whether or not hypothermia-induced alterations in galanin concentrations could constitute a part of the established neuroprotective effect of hypothermia in a focal and transient rat stroke model (Theodorsson et al. 2008).

Female rats were allocated to hypothermia (33°C) and normothermia (37°C) treatment during a 60 min microclip MCAo. The ischemic lesions were visualized using the TTC method and quantified after observation periods of 2 and 7 days, respectively. Concentrations of galanin were measured using a specific competitive radioimmunoassy in extracts from punch biopsies from various relevant brain regions. After 2 days at 33°C the ischemic lesions were generally larger than in the 37°C group, but after 7 days a significant reduction (more than 50%) was observed in the ischemic brain of the 33°C group lesions (p = 0.011).

This results corroborate those of Maier et al (2001) who showed that intraluminal transient (1.5 h) MCAo during intra-ischemic hypothermia (33°C) in rats resulted in a decreased size of the brain lesions monitored after 3 days (-37% [initiated at the onset of ischemia], -48% [initiated with 90 min delay from the onset of the ischemia] and -56% [initiated with 180 min delay from the onset of ischemia]).

Most studies reporting a hypothermia-induced decrease in ischemic brain lesions used observation periods of 1-3 days (Xue et al. 1992; Karibe et al. 1994; Maier et al. 1998; Zausinger et al. 2000) in contrast to the 7 days used in our study (Theodorsson et al. 2008). The reason for the prolonged observation period used in our study was our interest in investigating whether galanin was a reactant acting for short period of time, e.g. 2 days, or whether galanin also was
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A long time reactant/neuroprotective/neurotrophic factor likely to act over extended periods of time, e.g., 7 days.

Hypothermia induced an overall increase in the concentrations of immunoreactive galanin (p < 0.001). The elevated galanin levels were predominantly found in the non-ischemic control hemisphere, in the HiFo, thalamus, and the posterior part of parietal cortex. The galanin concentrations were lower in the ischemic hemisphere in both the normo- and hypothermic animals compared to the corresponding contralateral intact hemisphere (p = 0.049). The factor of time, 2 respectively 7 days, did not show any significant difference regarding the galanin concentrations (p = 0.844). Multivariate analyses of variance revealed significant effect of ischemia on the size of the ischemic brain lesions (p = 0.001) but no overall effect of temperature, when data from both 2 and 7 days observation periods were analysed together. Thus, the hypothermia and not the ischemic/reperfusion lesions, may explain the major part of the observed changes in galanin concentrations. Hypothermia-induced elevation in galanin concentration is therefore not likely to be amongst the major protective mechanisms of hypothermia.

The ischemic lesion created in our focal and transient stroke model leads to decreased galanin concentrations in the lesioned hemisphere, indicating that different mechanisms of tissue damage induce opposite responses in the tissue concentrations of galanin. Our data support the notion that hypothermia-induced increase in tissue concentrations of galanin in the brain are the result of changes from optimal homeostatic conditions – the hypothermia-induced stress – rather than the ischemic/reperfusion lesion-induced changes in galanin concentrations.

A study in gerbils by transient (5 min) global ischemia, showed increased galanin mRNA expression and galanin in the CA1-region of hippocampus 12 h after ischemia, but after 4 days the galanin mRNA and glanin peptide levels were lower than in the controls (Hwang et al. 2004). However, focal transient (intraluminal, 1 h) MCAo in SHR resulted in upregulation of galanin mRNA in the ipsilateral vs. contralateral cortex after 24 h, but not after 6 h (Raghavendra Rao et al. 2002). In a permanent MCAo model in rats, a delayed upregulation of galanin in the peri-infarction area was reported 3 days after the ischemic episode. This upregulation was barely detectable after 24 h and was not seen after 1 and 4 h (De Michele et al. 2006). Unfortunately, De Michele and co-workers do not report the changes in galanin levels in the ischemic hemisphere compared to the unlesioned contralateral control hemisphere (De Michele et al. 2006). Thus, different stroke models (global vs. focal, permanent vs. transient) and methods used to measure galanin responses (immunohistochemical analysis, in situ hybridization, quantitative radioimmunoassay methods) apparently result in different conclusions regarding the responses of the brain galanin systems to injury.
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Effects of intracerebroventricular galanin and galanin receptor 2/3 agonist on the ischemic brain lesion (paper III)

Several studies have shown upregulation of the expression of galanin mRNA and increased concentrations of galanin itself in neurons of the central and peripheral nervous systems exposed to various types of lesions. An early example was the dramatic increase in the expression of galanin mRNA in the DRG neurons after peripheral axotomy (Hökfelt et al. 1987).

Several studies have corroborated this finding in several species and locations of the nervous system (Villar et al. 1989; White et al. 1994). Whether this lesion-induced increase in galanin concentrations is primarily a signal that a lesion has occurred or a consequence of the lesion or – more interestingly – a mechanism for limiting tissue damage or facilitating neuronal survival and repair is still an open question. The upregulation of galanin in response to injury has logically prompted several investigators to hypothesize that galanin may play a neuroprotective role (Branchek et al. 2000; Holmes et al. 2000; O’Meara et al. 2000; Elliott-Hunt et al. 2004), an effect primarily mediated by activation of the GalR2 receptor (Mahoney et al. 2003; Pirondi et al. 2005; Elliott-Hunt et al. 2007).

Several studies have addressed the question of whether galanin is neuroprotective, however indirectly. We therefore considered it of interest to infuse various concentrations of galanin intracerebroventricularly in a direct attempt to address the question of whether galanin has neuroprotective properties in a rat model of MCAo. We infused three different concentrations of galanin into the cerebral ventricles expecting to reduce the brain lesions in response to mild ischemia – reperfusion lesions in the female rat brain. Furthermore we infused, in one concentration, the GalR2/3 agonist Gal(2-11) (AR-M1896) shown to preferably bind to galanin receptor subtypes subserving neuroprotective functions; that is to elucidate whether or not the possible neuroprotective properties of galanin are dependent on effects on a specific receptor subtype (Liu et al. 2001), as visualized in the current experimental paradigm.

The biological effects of galanin are generally inhibitory, in particular when acting through the GalR1- and -3 subtypes. Since neuronal lesions are caused by excessive release of excitatory amino acids, a possible mechanism which galanin may subserve in neuronal lesions could be to decrease release of glutamate at noxious concentrations. The general inhibitory properties of galanin may however also hamper the upregulation of molecules needed for neuronal repair and metabolic activity in the neurons crucial for survival. Both of these perspectives may actually be too simplistic, and what really matters may be the known direct neurotrophic effects exerted by galanin, superseding possible inhibitory effects on cell growth.
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Biological effects often, but not always, show linear dose-response relations, but non-linear dose/response relation also occurs, including U-shaped curves. We therefore considered it of interest to study the dose/response relationship of the effect of intracerebroventriculatly infused galanin on ischemic brain lesions over a wide range of concentrations.

In the present stroke model using female rats the lesion was 98% larger seven days after 60 min transient MCAo and continuous intracerebroventricular administration of the GalR2/3 agonist Gal(2-11), as compared to controls. No differences were found after seven days in the groups treated with galanin in three different concentrations (0.24, 2.4 and 24 nmol/day; p = 0.939, 0.715 and 0.977, respectively). There was also no difference in the size of the ischemic lesions measured after three days in the galanin-treated group (2.4 nmol/d) compared to artificial CSF (p = 0.925).

In view of the many studies cited above showing neuroprotective effects of galanin, mostly exerted via GalR2, we expected to find that the GalR2/3 agonist Gal(2-11) (and actually galanin – for that matter) would decrease the size of the ischemic lesion. However, the results of the current study were contrary to our initial hypothesis. Thus further studies are needed to clarify underlying mechanisms.
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The effects of ischemic injury on galanin and galanin receptor gene expression in discrete brain regions (paper IV)

Injury to neurons results in upregulation of galanin in some central and peripheral systems (Hökfelt et al. 1987; Villar et al. 1989; Cortes et al. 1990), and it has been suggested that this neuropeptide may play a protective and trophic role (Holmes et al. 2000; Kerr et al. 2000; O’Meara et al. 2000; Zigmond 2001; Pirondi et al. 2005; Hobson et al. 2008). The effects of galanin are mediated by the activation of three G-protein-coupled galanin receptor subtypes, GalR1, GalR2 or GalR3 (Habert-Ortoli et al. 1994; Wang et al. 1998a; O’Donnell et al. 1999; Branchek et al. 2000; Waters et al. 2000). In addition, the galanin fragment Gal(2–11) (AR-M1896) (Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-NH2) acts as an agonist with 500-fold affinity for GalR2 versus GalR1 (Liu et al. 2001). However, Gal(2–11) can also bind and activate GalR3 as shown in transfected cell lines, with a similar affinity as to GalR2 (Lu et al. 2005).

The objective of the present study was to investigate whether temporary and focal ischemic lesions (Theodorsson et al. 2005b) to the brain influence gene expression of galanin, GalR1, GalR2 and GalR3 in various brain regions. Quantitative real-time PCR was employed to study gene expression in punch-biopsies from the LC, cortex and dorsal HiFo, including sham-operated female rats as controls.

We selected three brain regions, the parietal cortex which is partly lesioned by the occlusion (extending from the main lesion in the striatum), the HiFo which is apparently unaffected, and the LC which provides noradrenergic/galaninergic innervations of both these regions, probably partly representing collaterals (Room et al. 1981). This means that one and the same neuron can send afferents to both parietal cortex and HiFo.

A 259% increase in the galanin transcript levels and a 45% increase in GalR1 mRNA levels were found in the LC in the ischemic hemisphere compared to the intact control side seven days after a 60-min-microclip MCAo in female rats. In contrast, expression of GalR1 mRNA decreased by 35% in the parietal cortex. However, in this area the Ct values were close to - or at detection limit (average Ct 35) and caution should be made when interpreting the significance of these results. These low values of galanin and galanin receptor mRNA in this brain region is in accordance with previous studies using in situ hybridization. Thus, under normal circumstances transcripts for galanin (Jacobowitz et al. 1990) and galanin receptors (O’Donnell et al. 2003) could not be detected within cortex or in the HiFo. However, after treatment with cholchicine, a toxic natural product inhibiting microtubule polymerization and therefore resulting in an accumulation of axonally transported neurotransmitters (Dahlström 1968; Kreutzberg 1969), galanin- positive cells can be seen in cortex and hippocampus, as well as in striatum (Skofitsch et al. 1985; Melander et al. 1986c), and
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in glia (Xu et al. 1992; Calza et al. 1998; Shen et al. 2003). In addition, when measuring the galanin peptide by means of radioimmunoassay, low levels of galanin were found in cortical areas (Skofitsch et al. 1986a). In the LC, on the other hand, galanin levels are around 4- and 3-fold higher compared to parietal cortex and HiFo, respectively. Immunohistochemical studies suggest that under normal circumstances galanin in dorsal cortical and hippocampal areas is mainly in afferent from the brain stem, especially in NA fibers originating in the LC (Melander et al. 1986c; Gabriel et al. 1995; Xu et al. 1998a).

Several earlier studies in male rats involving different stroke models and time periods have reported on expression of the galanin system after MCAo. Gender, species, dissection protocols and/or time schedules however, make direct comparison with the present results difficult (Bond et al. 2002; Raghavendra Rao et al. 2002; Hwang et al. 2004; Lee et al. 2005; De Michele et al. 2006). Two array studies monitored transcripts, that is similar to our own study. Thus, Raghavendra Rao et al. (2002) performed the occlusion for 60 min followed by reperfusion for 6 or 24 h and carefully dissected cortical "MCA territory" in SHR. They found a 12.5-fold increase in galanin mRNA at 24 h but no change at 6h. They also observed a 3.2-fold increase in GalR1 at 6h and a 2.6-fold increase at 24 h. Bond et al. (2002) used normotensive SD rats and occluded the MCA for 90 min followed by reperfusion for 24 h. They found that galanin mRNA was increased in the ipsilateral versus the contra-lateral "hemisphere" ("no local dissection was performed in order to minimize any variance due to anatomy or sampling"). However, the results were dependent on reference gene. When using cyclophilin as reference gene, the galanin transcript increase was 133% and significant, whereas with actin there was a 54%, not significant ("borderline") increase. Even if actin was stable across treatment groups, also this gene had shortcomings. Thus, arrays of MCAo brains appear difficult.

Is there a relation between the expression of the GalR2 demonstrated in the present study and our earlier findings of increased ischemic brain lesions caused by the continuous infusion of a galanin agonist, Gal(2-11), for three or seven days (Holm et al. 2011)? This agonist mainly acts through GalR2, which in many other studies has been shown to exert trophic effects (see above). Interestingly, in the present study there was a trend towards lower GalR2 mRNA levels in the HiFo. This may indicate that stimulation of the GalR2 increases ischemic lesions. Decreased expression of the receptor might thus contribute to the amelioration of ischemic lesions.

Taken together our data support the notion that galanin may play a role in the response of the CNS to injury, but its relation to mechanisms underlying galanin’s trophic effects remains to be elucidated.
Conclusions

- Modern anesthesia methods using endotracheal intubation and controlled ventilation is a crucial determinant of morbidity and mortality in the present experimental animal stroke in rats. Since morbidity and mortality are important confounders in this kind of experimental paradigms, methods for administrating anesthesia are crucial.
- Mild per-operative hypothermia during transient MCAo decreased the size of the ischemic lesions and increased the concentrations of immunoreactive galanin in the non-ischemic control hemisphere. The galanin concentrations in the ischemic hemisphere, in both normo- and hypothermic animals, were lower compared to the contralateral control hemisphere.
- Continuous administration of galanin or galanin receptor 2/3 agonist intracerebroventricularly increases the ischemic lesions seven days after transient MCAo.
- Transient MCAo upregulates galanin and GalR1 gene expression in the locus coeruleus.
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