Analysis of insulin receptor function in the central nervous system by conditional inactivation of its gene in mice

Inaugural-Dissertation
zur
Erlangung des Doktorgrades
der Mathematisch-Naturwissenschaftlichen Fakultät
der Universität zu Köln

Vorgelegt von

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2002

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Tag der mündlichen Prüfung	06-06-2002

Dedicated to my beloved mother Iekha Devi Gautam

and late father Balram U. Gautam

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1. Introduction

1.1 Insulin

Using pancreatic ß-cells from dogs, insulin was isolated in 1922 (Banting and Best, 1922), and Abel crystallized it. (Abel, 1926; Abel *et al.*, 1927). In 1953 the primary sequence of insulin was elucidated (Sanger and Thompson, 1953), and in 1966 the crystal structure of the biologically active form of insulin was elucidated (Dodson *et al* 1966; Harding *et al.*, 1966). In 1966, H. Zahn in Germany (Zahn, 1966) P.G. Katsoyannis in the USA (Katsoyannis *et al.*, 1966) and Niu Ching-I in Wang's laboratory in Shanghai (Niu *et al.*, 1966) independently synthesized insulin by chemical means. In 1967 Steiner discovered that insulin is synthesized as a prohormone – proinsulin –, which is processed to insulin by site-specific protease-dependent cleavage (Steiner *et al.*, 1967).

In humans the insulin gene is located on the short arm of chromosome 11. Expression of the insulin gene yields a precursor protein called preproinsulin, of 104 to 109 amino acids, depending on the animal species, including a 24 amino acid signal peptide. After removal of the signal peptide in the endoplasmic reticulum proinsulin is formed. The next step involves generation of inter- and intra-chain disulphide bridges, facilitated by the removal of the bridging C-peptide. Then thereby separated A- and B-chains of mature insulin are then linked by inter-chain disulphide bridges (Gursky *et al.*, 1992). The excised C-peptide is secreted together with mature insulin into bloodstream. No receptors for the C-peptide have been found, but currently the C-Peptide is supposed to have some, yet unknown, biological activity (Steiner *et al.*, 1997).

1.2 The insulin receptor (IR)

IRs are present on all tissues in mammals, including the classic insulin responsive tissues muscle, fat, and liver and non-classic tissues such as brain, endothelial cells or gonadal cells. The insulin receptor is a prototype of a receptor tyrosine kinase (RTK), which transmits a hormonal signal. The IR is a hetero-tetrameric protein consisting of two a- and two ß-subunits linked by disulphide bonds (Fig. 1.1). The a-chain has a molecular weight of 135 kDa and is exclusively located extra cellular, whereas the ß-chain (95 kDa) contains extra cellular,

transmembrane and cytosolic domains (Van Obberghen *et al.*, 1981). The transmembrane and intracellular portions of the ß-subunit contain the insulin-regulated tyrosine-specific protein kinase that is critical for insulin activity (Kasuga *et al.*, 1983; Chou *et al.*, 1987; Ebina *et al.*, 1987). The extra cellular domain of the a-subunit contains the ligands binding site. The a- and ß-chains of the IR are products of a single gene and are derived from a common polypeptide precursor by proteolysis.

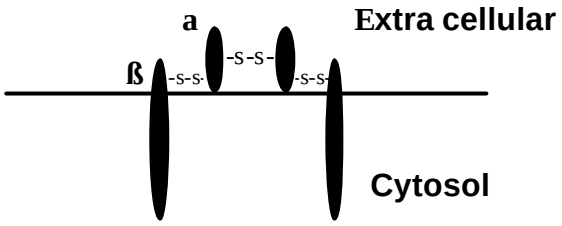

Figure 1.1 Schematic diagram of insulin receptor: A heterodimer consisting of a- and ß-chains linked by disulphide bridges.

The gene for the human insulin receptor is located on chromosome 19 and spans a region of more than 130 kbp comprising 22 exons (Seino *et al.*, 1989, 1990). Exon 1 encodes the signal peptide, exon 15 the transmembrane domain. Unlike other housekeeping genes, the human insulin receptor gene does not contain TATA or CAAT boxes but several binding sites for the transcription factor Sp1.

The complete amino acid sequences, structural details and frame work for the functional domains was determined by the cloning of cDNA of the human insulin receptor precursor (Ebina *et al.*, 1985; Ullrich *et al.* 1985). The mouse and rat insulin receptor were also cloned and exhibit a highly conserved sequence to the human insulin receptor gene (Flores-Riveors *et al.*, 1989; Goldstein *et al.*, 1990). The insulin receptor cDNA encodes an open reading frame of 1370 or 1382 amino acids depending on inclusion of the alternatively spliced exon 11 encoding 12 amino acids near to the C-terminus of the a-subunit. A tetra basic sequence Arg-Lys-Arg-Arg marks the cleavage site for generation of the a-subunit of 732 (720) amino acids and ß-subunit of 620 amino acids, with a predicted molecular weight of 84 kDa and 70 kDa, respectively (Ebina *et al.*, 1985).

There are some receptors sharing similarities with the insulin receptor (IR). Homologues of the mammalian IR are the DAF-2 receptor in *Caenorhabditis elegans* (Kimura *et al.*, 1997)

and the IR-like receptor in *Drosophila* (Fernandez *et al.*, 1995). The DAF-2 receptor shares 35% of its amino acid sequence with the human IR and 34% with the insulin like growth factor receptor-I (IGF-IR). The tyrosine kinase domain of the DAF-2 receptor is 70% similar and 50% identical to the tyrosine kinase domain of the human IR. The most closely related receptors to the insulin receptor are the receptor for insulin-like growth-factor-I (IGF-IR) (Ullrich *et al.*, 1986) and insulin receptor related receptor (IRR) (Shier *et al.*, 1989), both sharing 50 to 60% of the whole IR amino acid sequence, and being more than 80% identical in the kinase domain.

A hybrid form of a receptor containing one a- and one ß-subunit of the insulin receptor and another a- and ß-subunit of IGF-IR receptor, ß- a- a*- ß* has been identified (Soos *et al.*, 1989 and 1990). These hybrids bind IGF-I with high affinity causing IGF-I-stimulated autophosphorylation of their ß-subunits. But the affinity for insulin appears to be relatively low (Moxham *et al.*, 1989).

1.3 Molecular mechanisms of insulin signalling

The IR has diverse signaling pathways that generate the pleiotropic action of the hormone insulin. Binding of insulin to the a-subunit of the IR induces a conformational change of the receptor and activates the tyrosine kinase activity of the ß-subunits leading to autophosphorylation of the intracellular part of the ß-subunit (Kahn *et al.*, 1978). Autophosphorylation occurs in an ordered cascade on seven tyrosine residues (in three groups of phosphotyrosils) located in the carboxyl-terminal tail and in a region close to the membrane of the cytosolic part of the ß-subunit (White *et al.*, 1989; Wilden *et al.*, 1990; Murakami *et al.*, 1991), resulting in activation of the receptor kinase (Feener *et al.*, 1993; Hubbard *et al.*, 1997). Tyrosine-phosphorylated ß-subunits recruit and subsequently phosphorylate tyrosine residues of several intracellular insulin receptor substrates. The insulin receptor substrate (IRS) proteins are a family of structurally and functionally related proteins, comprising the members IRS-1 to 4 and the growth-factor-receptor-binding protein 2-associated binder-1 (Gab-1). IRS-1 was the first identified and cloned substrate of the insulin receptor kinase (Sun *et al.*, 1991), followed by IRS-2 (Sun *et al.*, 1995), which is widely distributed in different tissues, IRS-3, expressed in adipose tissue, fibroblasts and liver cells (Lavan *et al.*, 1997a; Smith-Hall *et al.*, 1997), IRS-4, expressed in embryonic kidney (Lavan

et al., 1997b), and Gab1 (growth factor receptor binding protein 2-associated binder-1), distributed in several mammalian tissues (Holgado-Madruga *et al*., 1996). Other proteins phosphorylated upon IR-activation are $p62^{dok}$ (Yamanashi *et al*., 1997), the SH2- containing protein SHC (Pronk, *et al*., 1993), and the focal adhesion kinase, FAK (Leventhal *et al*., 1997).

All IRS-proteins exhibit a conserved structure with an N-terminal pleckstrin homology (PH) domain, which appears to be responsible for membrane targeting of the IRS-proteins (Musacchio *et al*., 1993). Moreover, the IRS-proteins exhibit a phosphotyrosine-binding (PTB)-domain (Kavanaugh *et al*., 1994), which interacts with tyrosine residue 960 of the IR when phosphorylated. The rest of the protein displays multiple tyrosine residues, which upon phosphorylation serve as a docking platform for Rous sarcoma virus (Src)-homology 2 (SH2) domain containing signaling proteins, such as the growth factor receptor binding protein (Grb)-2 and the regulatory subunit of the phosphatidylinositol (PI) 3-kinase.

The a-collagen protein SHC exists in three isoforms of 46kDa, 52kDa and 66kDa and contains SH2 and PTB domains (Kovanica *et al*., 1993). SHC and IRS proteins compete for the same binding site in the juxtamembrane domain of the insulin receptor (Kaburagi *et al*., 1995; Isakoff *et al*., 1996).

Following phosphorylation, IRS proteins can bind to several SH2-containing cellular signaling proteins, such as phosphatidylinositol 3-kinase (PI 3-kinase), Grb2, Nck, CT10 virus regulator of kinase (Crk) (for detail see review of White, 1997) and SHP-2 (small heterodimerization partner-2), among which the most important and best studied is PI 3-kinase. This enzyme is a heterodimer consisting of a catalytic subunit called p110ß and one out of several different regulatory subunits: p85a, existing in alternatively spliced variants, p50 or p55 ?, either of them recruiting p110ß with high affinity through binding to SH2 domains. Activation of the catalytically active subunit results in phosphorylation of PtdIns bisphosphate (PtdIns-3,4-P2 and PtdIns-4,5 P2) and related lipids at the D-3 position, subsequently phosphorylated by PIP 5-kinase and PIP 3-kinase, respectively (Whiteman *et al*., 1988; Skolnik *et al*., 1991) to generate PtdIns trisphosphate (PtdIns-3,4,5-P3).

PtdIns-3,4-P2 and PtdIns-3,4,5-P3 can bind to the PH domain of protein kinase B (PKB), also known as Akt; (Burgering *et al*., 1995; Datta *et al*., 1996) followed by the translocation of

PKB to the plasma membrane and its co-localization with phosphoinositide-dependent protein kinase 1 (PDK 1) (Alessi *et al.*, 1998; Walker *et al.*, 1998; Chan *et al.*, 1999). The subsequent conformational change enables PDK 1 to phosphorylate PKB on threonine 308 and serine 473, thereby activating the enzyme. PKB itself is a serine/threonine kinase, of which three isoforms have been identified as PKBa, PKBß and PKB? (Walker *et al.*, 1998).

One target for PKB is the glycogen synthase kinase 3 (GSK-3), which undergoes phosphorylation and inhibition. Thus, the PDK/PKB phosphorylation cascade activates glycogen synthesis (Cross *et al.*, 1995).

A further downstream target of PKB is the serine/threonine kinase p70 $^{S6\ kinase}$ (P70S6K). PKB phosphorylates the phosphoinositide-kinase related kinase mammalian target of rapamycin (mTOR), which activates P70S6K, thus leading to phosphorylation of the ribosomal protein S6 and the activation of protein synthesis (Scott *et al.*, 1998).

The activation of the PI 3-Kinase, and the downstream phosphorylation of P70S6K, PKB, GSK-3 and protein kinase C (PKC) results in phosphorylation and activation of the small GTPase Rab and the insulin-stimulated translocation of the GLUT4-glucose transporter from the cytosol of muscle and adipose cells to the plasma membrane, followed by an increased glucose uptake (for review see Taha *et al*, 1999).

Another major physiological role of insulin is the regulation of gene transcription through the MAP kinase cascade. Following insulin stimulation, IRS-proteins, Gab-1 and SHC bind to the SH2 domains of several small adaptor proteins such as GRB-2 (growth-factor receptor binding-2) protein. These proteins will then interact with the GDP/GTP exchange factor son of sevenless (SOS) leading to the activation of the small G-protein RAS (Sklonik *et al.*, 1993). Activated RAS binds to and thereby activates the RAF-kinase. RAF is serine/threonine kinase (Pronk *et al.*, 1994). Activated RAF in turn phosphorylates and activates mitogen-extracellular signal-responsive kinase (MEK), also known as MAPKK by phosphorylating two serine residues (Dent *et al.*, 1990). MEK activates mitogen-activated protein kinase (MAPK) by phosphorylating tyrosine and threonine residues (Crews *et al.*, 1992). Activation of MAPK leads to phosphorylation of the p90 ribosomal S6 kinase (p90 rsk), phospholipase A2 (PLA2) and several transcription factors including the ternary complex factor p62^{TCF} resulting in the activation of gene transcription (Fingar *et al.*, 1994; Gille *et al.*, 1992).

1.4 Role of Insulin-signalling in apoptosis

PKB plays a central role in insulin-stimulated inhibition of programmed cell death (apoptosis). One step in the promotion of cell survival is the phosphorylation of three members of the family of forkhead transcription factors such as FKHR, FKHR L1 and AFX. Phosphorylation of FKHR L1 enables binding of 14-3-3 proteins and retention of FKHR L1 in the cytoplasm, thereby inhibiting the transcription of apoptosis promoting genes such as Fas ligand (Biggs *et al*., 1999; Brunet *et al*., 1999; Rena *et al*., 1999). The role of PKB and the forkhead transcription factors was first established in *C. elegans*, which follows the insulin-like signaling pathway and suppresses the action of the DAF-16 gene, the homologue of the human FKHR which mediates insulin-like metabolic effects and longevity signals in *C. elegans* (Ogg *et al*, 1997). Taken together, insulin through phosphorylation-dependent inactivation of FKHR inhibits Fas-L-transcription and activation of the extrinsic apoptotic pathway (Brunet *et al*., 1999).

PKB also regulates the interaction between Bcl-2 /Bcl-X_L and BAD by phosphorylation to BAD-proteins. BAD and Bcl-X_L are members of the Bcl-2 family and BAD was first identified because of its binding ability to Bcl-2 and Bcl-X_L (Yang *et al*., 1995). Bcl-2 and Bcl-X_L are anti-apoptotic proteins present on the outer membrane of the mitochondria bound to a fourth family member BAX. BAD binds with high affinity to Bcl-X_L, thus breaking up the binding between BAX and Bcl-X_L and reversing its effect as cell death repressor. The following release of cytochrome-*C* from mitochondria into the cytoplasm leads to the activation of apoptosis. PKB specifically phosphorylates BAD on serine 136, enabling the binding of 14-3-3 proteins, thus inhibiting the interaction with Bcl-2/Bcl-X_L and the promotion of apoptosis (Datta *et al*., 1997; Yang *et al*., 1997).

Mitochondria play a critical role in the control of cell death, providing a major intracellular apoptotic signal, cytochrome-*C*. Mitochondria undergo a permeability change that disrupts electron transport causing breakdown of membrane potential. As a result, mitochondrial proteins are shed including cytochrome-*C*, which combines with the APAF (apoptosis-activating factor) and pro-caspase 9, forming a proteolytically active "apoptosome" (Zou *et al*., 1997; Cain *et al*., 1999). Caspases are cysteine melloproteases that cleave proteins after an aspartic acid residue.

1.5 Mutations in the insulin receptor (IR) and other insulin signaling genes

Analysis of naturally occurring mutations and targeted ablation of the gene for the insulin receptor and its signaling proteins in the mouse have provided crucial information about their role in physiology.

Through genetic engineering in mice, a number of the genes playing crucial roles in insulin signaling and action, including insulin, insulin receptor (IR), downstream signalling molecules such as insulin receptor substrate-1 (IRS-1), IRS-2, glucose transporters (GLUT4, GLUT2) and important metabolic enzymes such as glucokinase have been altered to better understand the functional role of these genes *in vivo*. Genes encoding insulin-like growth factors (IGF-I and IGF-II) and their type I receptor (IGF-IR) have also been disrupted. The transgenic and knockout animal model provided novel insight about the pathophysiology of diabetes. A brief summary of these mutations is presented below.

A transgenic mouse over expressing the human IR under the control of promoter/enhancer of rat myosin light-chain exhibited no change in the basal level of blood glucose and insulin in the fasting state as compared to their wild-type controls. The blood glucose and plasma insulin levels after intraperitoneal glucose injection were 25% lower, and blood glucose lowering effect after intraperitoneal injection of insulin was more pronounced in transgenic mice compared to wild-type controls. Thus, moderate increase of IR in muscle causes an increase in insulin-sensitivity (Benecke *et al*., 1993).

The IR gene has been inactivated by conventional gene-targeting introducing stop codons in exon 4 (Acilli *et al*., 1996) and by deleting a part of the IR gene around exon 2 (Joshi *et al*., 1996). The heterozygous mutants (IR+/-) do not exhibit any major metabolic abnormalities and show normal glucose tolerance following an intraperitoneal glucose tolerance test. Moreover, insulin levels were not significantly different compared to their controls. At birth the homozygous null mutant (IR-/-) mice are indistinguishable from their littermates. But they exhibit several major metabolic disorders immediately after suckling. The pups developed severe diabetes mellitus with ketoacidosis, increased levels of triacylglycerol and non-esterified-fatty-acid levels. IR-deficient pups developed marked postnatal growth retardation and skeletal muscle dystrophy. The white and brown adipose tissues were decreased in

amount, and IR-deficient pups died between 3 to 7 days after birth (Acilli *et al.*, 1996; Joshi *et al.*, 1996; Cinti *et al.*, 1998) as a consequence of diabetic ketoacidosis.

The phenotype of transgenic and knockout mice of insulin and insulin-signaling proteins are summarized in the table below (Table 1.1).

Inactivated genes	Viable	Diabetes mellitus	Keto acidosis	Metabolic disorders	Striking phenotypes	Reference
Insulin	No, death within 2 days	Yes	Yes	Hepatic steatosis	Intra-uterine growth retardation, hyperplastic islets	Duvillie *et al.*, 1997.
IR	No, death between 3-7 days	Yes	Yes	Hyperglycaemia, Hyperinsulinaemia	Postnatal growth retardation and skeletal-muscle hypotrophy	Accali *et al.*, 1996; Joshi *et al*, 1996
IRS-1	Yes	No	No	Mild peripheral insulin resistance, hyperglycaemia	Intra-uterine and postnatal growth retardation	Araki *et al.*, 1994; Tamemoto *et al.*, 1994; Abe *et al.*, 1998
IRS-2	Yes	Yes	No	Hyperglycaemia, Hyperinsulinaemia	Intra-uterine and postnatal growth retardation	Withers *et al.*, 1998
IRS-3	Yes	N.D.	No	Normal GTT	Normal growth	Liu *et al.*, 1999
IRS-4	Yes	N.D.	N.D.	Hyperglycaemia	Normal growth	Liu *et al.*, 1999
IGF-I	No/Yes (based on genetic back ground)	No	No	-	Hyperplastic Islets, Dwarfism, Underdeveloped reproductive organs, Infertility, slower long-bone ossification	Liu *et al.*, 1993; Powell-Braxton *et al.*, 1993
IGF-IR	No (death at birth)	No	No		Abnormal development of skin, CNS and bone, muscle hyperplasia, Dwarfism,	Liu *et al.*, 1993
p85(PI3-K)	Yes	Yes	No	Hypoglycaemia	Immunological defects	Terauchi *et al.*, 1999

Inactivated genes	Viable	Diabetes mellitus	Keto acidosis	Metabolic disorders	Striking phenotypes	Reference
Glut 4	Yes (5-7 months)	No	No	Hyperinsulinaemia	Growth retardation, reduced fat deposition	Katz *et al*., 1995
Glut 2	No (death within 2-3 weeks)	Mild	No	Mild hyperglycaemia, mild hypoinsulinaemia, high free fatty acids	Postnatal growth retardation	Guillam *et al*., 1997
GK	No (death within 1 week)	Yes	Yes	High cholesterol and triacylglycerol level, depleted hepatic glycogen		Grupe *et al*., 1995; Terauchi *et al*., 1995
$IR^{+/-}IRS\text{-}1^{+/-}$	Yes	Yes		Hyperinsulinaemia,	Hyperplasia of ß cells	Bruning *et al*., 1997
$IRS\text{-}1^{+/-}GK^{+/-}$	Yes	Yes		Glucose intolerance, fasting huperinsulinaemia	Growth retardation	Terauchi *et al*., 1997

Table 1.1: Summary of the phenotypes of transgenic and conventional knockout animals of insulin and insulin signaling proteins. N.D. indicates not determined.

1.5.1 The Cre-LoxP system

The role of insulin action in individual tissue has been analyzed by the use of a recent developed technique, the cre-Lox-P system (Gu *et al*., 1994) to create tissue specific IR ablation. This conditional inactivation of a gene uses the site-specific bacteriophage P1 Cre-recombinase (Tsien *et al*., 1996). Cre recognises Lox-P-sites and excises DNA, which is flanked by Lox-P-sites (Sauer *et al*., 1987; Sauer *et al*., 1988). In transgenic experiments Lox-P-sites can be integrated in a target gene without interfering with the function of the gene. In a second experiment mice are created, which express the Cre-recombinase in a tissue-specific fashion. If these mouse lines are crossed, the target gene will be inactivated *in vivo*, wherever Cre is expressed.

Through this technology, muscle specific IR knockout (MIRKO) mice have been created by mating mice carrying the IR gene with exon 4 flanked by Lox-P-sites ("floxed") with transgenic mice expressing the *Cre* recombinase under control of the muscle creatinine kinase (MCK)-promoter/enhancer (Bruning *et al*., 1998). The MIRKO mice showed >95% reduction in IR-expression specifically in the skeletal muscle. MIRKO animals exhibited euglycemia up to the age of 20 months; plasma insulin concentration was indistinguishable between knockout and control animals and insulin and glucose tolerance tests showed no evidence for insulin resistance in these mice. The ability to maintain normoglycemia appears to be the result of shunting substrates from muscle to fat. MIRKO mice therefore exhibited a 40% increase in fat depots and exhibited hypertriglyceridemia and high level of serum free fatty acids (Bruning *et al*., 1998). In summary, selective insulin resistance in skeletal muscle appears to be dispensable for glucose homeostasis but can result in obesity and hypertriglyceridemia, which are hallmarks of the metabolic syndrome X (Reaven, 1987).

Tissue-specific knockout of the IR gene in the pancreatic ß cell (ßIRKO) has also been achieved by breeding the IR "floxed" mice with transgenic mice expressing the *Cre* recombinase under control of the rat insulin promoter (Kuhn *et al*., 1995; Kulkarni *et al*., 1999). The ßIRKO females show a dramatic decrease (85%) in insulin secretory response upon glucose stimulation compared to controls. However, the secretion of insulin was gradually increased in ßIRKO animals after 30 minutes upon glucose stimulation and was not significantly different from controls. Upon arginine stimulation, ßIRKO as well as control mice exhibited a 5 to 6 fold increase in insulin secretion, indicating that functional IR-expression is necessary for glucose-stimulated insulin secretion by the pancreatic ß-cell. As a consequence of impaired glucose-stimulated insulin secretion, ßIRKO mice showed impaired glucose tolerance. Therefore, defects of insulin signaling at the level of the ß cell may contribute an alteration in insulin secretion in type 2 diabetes (Kulkarni *et al*., 1999).

Moreover, liver-specific IR knockout (LIRKO) mice have been generated (Michael *et al.*, 2000) by breeding IR "floxed" mice (Bruning *et al.*, 1998) with transgenic mice expressing the *Cre* recombinase from the rat albumin promoter (Postic *et al*., 1999; Postic *et al.*, 2000). Two months old LIRKO male mice displayed markedly elevated levels of blood glucose in the fed state and 20 fold elevated plasma insulin levels. A 40-50% reduction of triglycerides and free fatty acids suggested an impairment of insulin action in the liver to promote triglyceride synthesis. LIRKO mice exhibited impaired glucose tolerance (hyperglycaemia

throughout the test period) and resistance to the blood glucose-lowering effect of insulin. By contrast, at the age of 4 months hyperglycaemia was normalised, but insulin resistance and glucose intolerance were unchanged. The initial body weight (until 6 weeks) of the LIRKO mice was less and the size of the liver was about 50% smaller compared to controls. The above results suggest that isolated liver insulin resistance causes severe defects in glucose and lipid homeostasis and may promote ß cell hyperplasia and hyperinsulinaemia observed in type 2 diabetes (Michael *et al*., 2000).

Similarly, brown adipose tissue (BAT) specific insulin receptor knockout (BATIRKO) mice were generated by breeding IR "floxed" mice with transgenic mice expressing the *Cre* recombinase under control of uncoupling protein (UCP)-1- promoter (Guerra *et al*., 2001). The IR expression was reduced to 95% in BATIRKO mice. The reduction of BAT mass in BATIRKO mice was age dependent, i.e. 50% reduction of BAT was observed in 3 month old mice and 75% in 6 to 12 months old animals compared to controls. BATIRKO mice developed age-dependent hyperglycemia with reduced insulin-stimulated insulin secretion. These results suggest that insulin plays an important role in development and maintenance of BAT that is BAT homeostasis and the regulation of insulin secretion and glucose homeostasis (Guerra *et al*., 2001).

1.6 CNS-regulated food intake and obesity in relation to insulin and leptin

Insulin and leptin receptors expressed on neurons present in the brain appear to be responsible for the regulation of energy homeostasis (Baskin *et al*., 1998 and 1999a; 1999b). It has been demonstrated, that intracerebroventricular (i.c.v.) administration of either peptide acutely causes hypophagia (Woods *et al*., 1979; Weigle *et al*., 1995). Deficiency in leptin as present in the genetically obese ob/ob-mouse causes hyperphagia (Cusin *et al*., 1995). Mechanistically, in the hypothalamic region of the brain, insulin and leptin play a major role to regulate anabolic effectors, neuropeptide Y (NPY) and agouti-related protein (AGRP) and catabolic effectors such as proopiomelanocortin (POMC) and the cocaine- and amphetamine-regulated transcript (CART). The NPY and AGRP are colocalized in neurons of the arcuate nucleus of the hypothalamus (Hahn *et al*., 1998; Broberger *et al*., 1998). Similarly, POMC and CART are colocalised in neurons of the arcuate nucleus (Elias *et al*., 1998). Following intracerebroventricular (i.c.v.) microinjection of leptin, the NPY/AGRP neurons are inhibited,

resulting in a reduced food intake and body weight in rats (Satoh *et al*., 1997), and they are activated when the leptin levels are low in the fed state (Schwart *et al*., 1996; Stephens *et al*., 1995). Other experiments have demonstrated that insulin also inhibited NPY expressing neurons (Sipols *et al*., 1995; Williams *et al*., 1989) and stimulated POMC expression, a precursor of a-melanocortin stimulating hormone (a-MSH). a-MSH binds to and activates the melanocortin receptor 4 (MC4) leading to the inhibition of food intake. The important role of the MC4 receptor in the regulation of energy homeostasis could be demonstrated by the creation of MC4 receptor knockout mice, which exhibit severe hyperphagia and obesity (Huszar *et al*., 1997). AGRP, which is co-expressed with NPY in the arcuate nucleus of the hypothalamus causes hyperphagia when administered i.c.v. The role of insulin and leptin in regulation of food intake and obesity is summarised in Fig. 1.2.

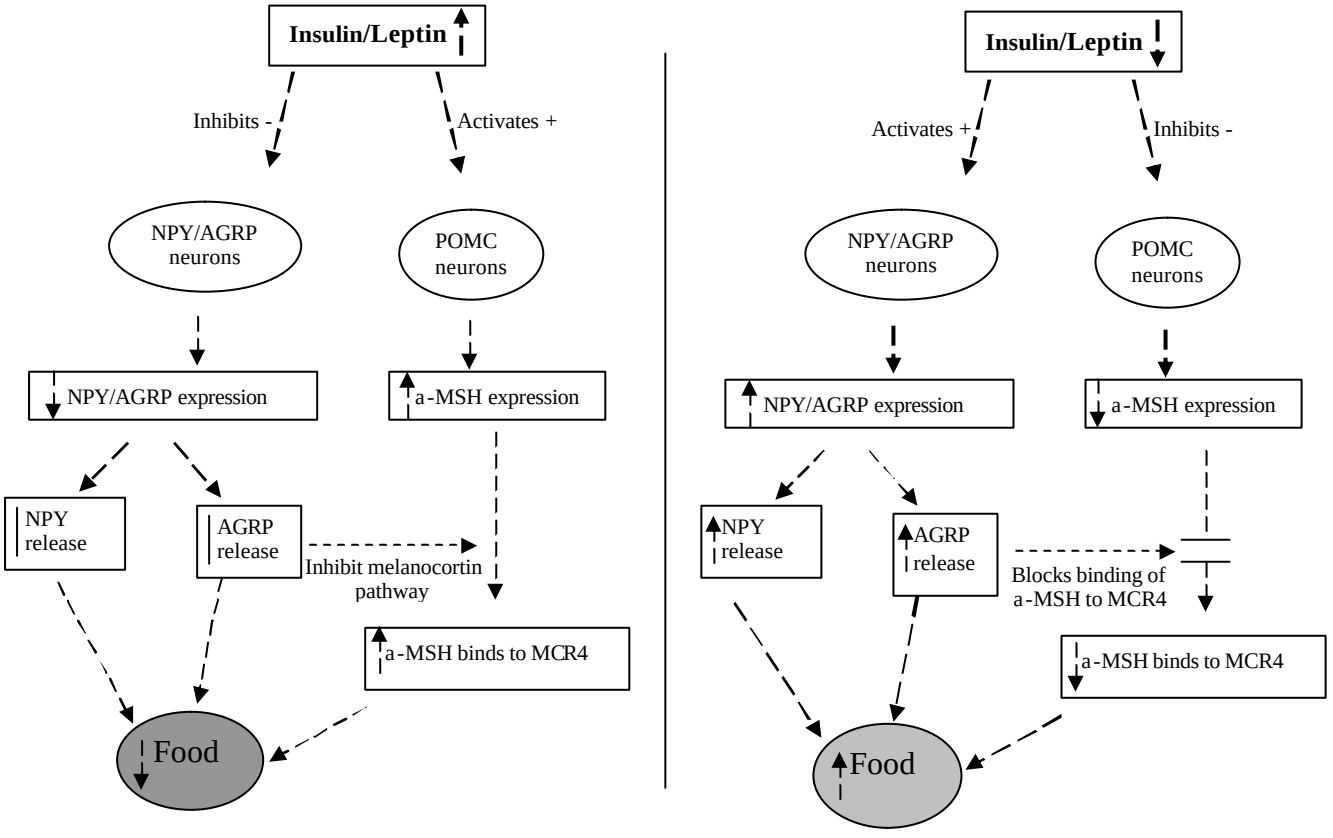

Figure 1.2: Schematic diagram of the role of insulin and leptin in control of food intake and obesity. This figure is taken from Schwartz *et al.,* 2000.Nature, **404**

1.7 Role of insulin and insulin receptor in the central nervous system

Havrankova *et al*. first reported the localization of insulin in the CNS in 1978(a) by immunohistochemical staining of the rat brain with anti-insulin antibodies, which was further broadened by Dorn *et al*. (1981) using fluorescence staining, demonstrating the presence of insulin in neurons of the hypothalamus, thalamus, amygdale and hippocampus of the murine brain. But the concentration of insulin within the different brain regions varies greatly from 10 to 100 fold higher than in plasma (Havrankova *et al*., 1978b; Baskin *et al*., 1983). These findings could indicate the synthesis of insulin within the CNS (Schechter *et al*., 1988; 1990; 1996). It has been hypothesized that adult mammalian brain normally synthesizes an amount of insulin comparable to the peripheral source (Schwartz *et al*., 1992). Moreover, the pancreas-derived insulin enters into the CNS across the blood-brain-barrier (BBB) through an active transport mechanism (Jialal *et al*., 1984; King *et al*., 1985).

In 1978 (b), Havrankova *et al*. demonstrated for the first time the localization of insulin receptor (IR) in the CNS, classically considered to be an insulin-insensitive tissue. The localization of insulin receptors in the CNS was further assessed by various techniques, including *in vitro* binding studies (Hill *et al*., 1986), *in vivo* and *in vitro* autoradiography and computerized densitometry (Van Houten *et al*., 1979; Van Houten *et al*., 1983; Baskin *et al*., 1986; Hill *et al*., 1986; Werther *et al*., 1987) and immunocytochemistry (Unger *et al*., 1989). According to these studies insulin receptors are widely distributed in the brain with highest concentrations in the olfactory bulb, hypothalamus, cerebral cortex and hippocampus. Moreover, insulin receptor mRNA was detected in the olfactory bulb, cerebral cortex, hypothalamus, cerebellum and brain stem by *in situ* hybridisation with radiolabelled oligonucleotides probes (Marks *et al*., 1990). Insulin receptors are not only present in the CNS, but it has been reported that they are also present in the peripheral nervous system such as autonomic ganglia, sciatic nerve and dorsal root ganglia and colocalised with Schwann cells and integrins. It has been hypothesised that the IR in peripheral nerves is responsible for maintenance and repair of myelinated fibres (Waldbiling *et al*., 1987; Sugimoto *et al*., 2000).

Another major role of insulin signaling through its receptors in the brain appears to be a neurotrophic function. The expression of IRs in the brain is developmentally regulated. It has been reported that insulin and its receptor levels change during different stages of CNS development. In general, insulin binding increases during the prenatal period, peaks during

early postnatal life and then rapidly declines and remains at a lower level throughout life (Kappy *et al*., 1984; Bassas *et al*., 1989). Experiments on primary fetal rat brain cell cultures suggested that insulin might play a role in the control of metabolism, growth and development of the CNS. It was shown that insulin could stimulate the activity of ornithine decarboxylase, an enzyme involved in the biosynthesis of polyamines thought to regulate cell growth and metabolism (Yang *et al*., 1981). The role of insulin for the development of the CNS is further supported by the ability of insulin to stimulate neurite outgrowth (Vanhems *et al*., 1990) and protein synthesis by increasing phosphorylation of ribosomal S6 protein in the cultured fetal neurons (Heidenreich *et al*., 1989).

Moreover, it has also been reported that dysregulation of IR-expression could be linked to neurodegenerative disorders such as Alzheimer's (AD) and Parkinson's disease (PD). Like mentioned earlier, insulin concentration and IR density are reduced with aging. It has been reported that insulin concentrations and IR density are dramatically reduced in the brains of Alzheimer's patients compared to their middle-aged controls, whereas the IGF-IR remain unchanged. AD patients also exhibited lower cerebrospinal fluid (CSF) insulin and higher plasma insulin concentrations (Craft *et al*., 1998), indicating an impaired brain insulin metabolism. Concurrently, administration of insulin to AD patients resulted in the improvement in memory and performance (Craft *et al*., 1999). Zhao *et al*. demonstrated in 1999 a significant upregulation of IR mRNA in the CA1 and dentate gyrus area of the hippocampus after a spatial maze learning task. This upregulation of IR mRNA was followed by an increase in IR-protein in the hippocampus further suggesting a role for IR in the regulation of synaptic activities, such as neurotransmission and/or synaptic plasticity during memory formation.

In cultured human neurons, Hong *et al*. (1997) showed that glycogen synthase kinase-3 (GSK-3) can phosphorylate the neuronal protein tau. Hyperphosphorylated tau is the major component of paired helical filaments in neurofibrillary lesions associated with Alzheimer's disease. Hyperphosphorylation reduces the affinity of tau for microtubules and is thought to be a critical event in the pathogenesis of this disease. Insulin and the related hormone IGF-I have been shown to reduce the phosphorylation of tau protein by inhibiting activity of GSK-3.

Intracerebroventricular injection of streptozotocin (STZ) into adult rats is considered as an animal model for chronic neuronal dysfunction that is characterized by a decrease in both the

neuronal metabolism of glucose and the formation of energy, as STZ can inhibit the insulin receptor kinase activity. STZ injections caused long-term and progressive deficit in learning, memory and cognitive behaviour, also indicating a role for the insulin receptor in this context (Lannert *et al*., 1998).

It has been also reported that older diabetic patients (average age over 64 years) perform worse in learning task than age-matched nondiabetic patients (Reaven *et al*., 1990; Elias *et al*., 1997), whereas no evidence of learning and memory impairment was detected in middle-aged (mean age 51 years) type 2 diabetic patients (Ryan *et al*., 2000). This effect could be due to the age-related functional changes in the hippocampus and related structures increasing the vulnerability of the memory processing system to impaired insulin action.

1.8 Aim of the study

As mentioned earlier, the insulin receptor (IR) is distributed throughout the whole body including the classical insulin target tissues liver, fat, and muscle, as well as organs classically considered to be insulin insensitive like brain, kidney etc. IR is activated upon insulin binding and mediates insulin's diverse biological effects such as glucose homeostasis, gene transcription, mitogenesis and inhibition of gluconeogenesis. The role of IR in muscle, fat and liver has been studied in detail; this thesis will focus on the functions of the IR in the central nervous system.

The role of this receptor, which is abundantly expressed in variable densities in the different areas of the CNS such as olfactory bulb, cerebral cortex, hypothalamus, cerebellum and choroids plexus, is still unclear. However, some evidence suggests a role for IR and the corresponding hormone insulin in the CNS: IR in choroids plexus may be essential for regulating insulin concentrations in the cerebrospinal fluid (CSF) by affecting transport of insulin from peripheral system and removing cerebrospinal fluid insulin (Schwartz *et al*., 1992); IR in hypothalamic region is responsible for regulation of food intake and body weight and IR in hippocampus, piryform cortex and amygdale is responsible for learning and memory (Zhao *et al*., 1999). The role of IR in the CNS is mainly focused on food intake and body adiposity, and some studies suggest that IR in cells of the CNS is important for neuronal plasticity and neuronal survival *in vitro,* and that impaired insulin signaling and altered energy

metabolism in CNS may lead to neuronal degenerative disease like Alzheimer's disease and Parkinson's disease.

To study these pathophysiological process *in vivo*, we performed a targeted disruption of the IR gene expression specifically in a tissue specific manner, restricted to the CNS. For this purpose mice carrying Lox-P sites in the flanking regions of exon 4 of the IR gene (Bruning *et al*., 1998) were crossed with mice expressing the *Cre* recombinase under control of the neuron specific nestin promoter (Dahlstrand, 1995). The main objectives of these studies are:

1. To characterize the morphological consequences of selective IR gene disruption in the brain,
2. To study the effect of neuron specific IR gene disruption on glucose homeostasis, body weight regulation, food consumption and cognitive function,
3. To study the role of IR in the neuronal insulin signaling system and
4. To study the function of neuronal IR regarding neuronal cell death.

2. Materials and Methods

2.1 Materials

2.1.1 Chemicals

All analytical grade chemicals were purchased from Sigma-Aldrich, Merck, Serva, Bio-Rad, Pharmacia-Biotech, USB, Gibco BRL, and Applichem. Radiochemicals were obtained from ICN.

Molecular weight markers for proteins (Broad Range) were purchased from Bio-Rad and DNA molecular weight standards from New England Biolabs. Antibodies were purchased from Santa-Cruz Biotechnology. Insulin and Leptin ELISA kits were purchased from Crystal Chem Inc., USA.

2.1.2 Buffers

PBS

136 mM	NaCl
2,6 mM	KCl
10 mM	Na_2HPO_4
1.5 mM	KH_2PO_4, pH 7.4

PBS-T

PBS + 0.1% (v/v) Tween 20

TAE (10x)

2 M	Tris
5.7% (v/v)	Glacial acetic acid
50 mM	EDTA

TBE (10x)

0.89 M	Tris-HCl, pH 8.0
0.89 M	Boric acid
20 mM	EDTA

TBS

1.37 M	NaCl
250 mM	Tris-HCl, pH 7.4

TBS-T

TBS+0.1%(v/v) Tween 20

Protein extraction buffer

50 mM	Hepes, pH 7.4
1% (v/v)	Triton X-100
50 mM	NaCl
100 mM	NaF
10 mM	EDTA
10 mM	Na_3VO_4
0.1%(w/v)	SDS

The buffer was supplemented with a cocktail of protease inhibitors (Leupeptin 10 ng/ml, Aprotinin 10 µg/ml, Benzamidine and PMSF 2mM)

Lysis buffer for tail digestion

100 mM	Tris HCl, pH 8.5
200 mM	NaCl
5 mM	EDTA
0.2% (w/v)	SDS

6x DNA loading buffer

0.25%	Bromophenolblue
0.25%	XylenecyanolFF
40%(w/v)	Sucrose in water

5x SDS protein loading buffer

312,5 mM	Tris-HCl, pH 6.8
500 mM	Dithiothreitol
10 mM	EDTA
10% (w/v)	SDS
0.05% (w/v)	Bromophenolblue
50% (v/v)	Glycerol

Protein transfer buffer

10x Cathode Buffer -1

25 mM	Tris-HCl
40 mM	6,Aminohexamineacid
pH to 9.4	

10x Anode Buffer-1

25 mM	Tris-HCl, pH 10.4

10x Anode Buffer-2

300 mM	Tris-HCl, pH 10.4

2.1.3 Cell culture media and buffers

HHGN

1x	Hanks balanced salt solution (HBSS)
2.5 mM	Hepes, pH 7.3-7.5
2.5 M	Glucose
1 M	$NaHCO_3$

TDn

250 µl	DNAse I (4,000 U/ml)
5 ml	HHGN
50 mg	Trypsin

DnB

500 µl	DNAse I (4,000 U/ml)
10 ml	Basal medium Eagle (BME)

CbC medium (Cerebellar Culture Medium)

100 ml	Basal medium Eagle (BME)
10 ml	Calf serum
1 ml	0.2 M Glutamine
1 ml	Penicillin and streptomycin (10,000 U)
0.8 ml	3M KCL

CbC Starving Medium

100 ml	Basal medium Eagle (BME)
1 ml	0.2M Glutamine
1ml	Penicillin and streptomycin
0.1% (w/v)	Bovine serum albumin (BSA)

2.1.4 Stain for tissue sections

Xyline Eosin stain

1 gm (w/v)	Hamatoxilline (in 1 lit. H_2O)
0.2 gm (w/v)	$NaIO_3$
50 gm (w/v)	$KaI(SO_4)$
50 gm (w/v)	Chloral hydrate
1 gm (w/v)	Acetic acid
0.1 gm (w/v)	Eosin

2.1.5 Animal strains

C57Bl/6J females sham operated, ovarectomised or ovarectomised and implanted with estradiol-releasing pellets were purchased from May and Bakers, Denmark. Insulin Receptor (IR) floxed mice were received from Joslin Diabetes Centre, Boston/USA and have been described previously (Bruning *et al.,* 1998). Mice expressing the Cre-recombinase under control of the rat Nestin promoter were obtained from Rüdiger Klein (EMBL, Heidelberg) and have been previously described (Dahlstrand, 1995).

2.1.6 Synthetic Oligonucleotides

All oligonucleotides HPSF grade, were purchased from MWG Biotech. The following oligonucleotides were used for genotyping of mice by Polymerase Chain Reaction (PCR).

Primer	Sequence (5'-3')	Used for
IR5'	-GATGTGCACCCCATGTCTG-	PCR, to check the presence of IR floxed and wild-type allele
IR3'	-CTGAATAGCTGAGACCACAG-	PCR, to check the presence of IR floxed and wild-type allele
Intron 3'	-ACGCCTACACATCACATGC-	PCR, to check the absence of Exon IV of insulin receptor
Cre5'	-ATGTCCAATTTACTGACCG-	PCR, to check the presence of Nestin-Cre
Cre3'	-CGCCGCATAACCAGTGAAAC-	PCR, to check the presence of Nestin-Cre

2.1.7 Primary and secondary antibodies

Antibody	Type/label	Dilution	Source
IR-ß	Rabbit polyclonal	1:300 (WB)	Joslin Diabetes Center, Boston/USA
IGF-1R ß	Rabbit polyclonal	1:200	Santa-Cruz
IRS-1	Rabbit polyclonal	1:500 (WB)	Joslin Diabetes Center, Boston/USA
IRS-2	Rabbit polyclonal	1:500 (WB)	Joslin Diabetes Center, Boston/USA
p85	Rabbit polyclonal	1:500 (WB)	Joslin Diabetes Center, Boston/USA
IR-ß	Rabbit polyclonal	1:200 (WB)	Santa-Cruz
p-Tyr (PY 99)	Mouse monoclonal	1:100 (WB)	Santa-Cruz
pAkt	Mouse polyclonal	1:500	Santa-Cruz
Goat anti mouse IgG	Peroxide conjugate	1:1000 (WB)	Sigma
Goat anti rabbit IgG	Peroxide conjugate	1:1000 (WB	Sigma

2.2 Methods

2.2.1 Breeding

Female Insulin Receptor (IR) floxed heterozygous (IR flox/+) mice were bred with male mice carrying a transgene expressing the Cre-recombinase under the control of the rat nestin promoter and enhancer to obtain IR floxed heterozygous nestin Cre + pups. These mice were again crossed with IR flox/+ mice to obtain IR flox/flox Cre+ mice and the appropriate control animals.

2.2.2 Backcross breeding

To generate NIRKO mice on a pure C57Bl/6J-background, heterozygous IR flox/+; Nestin Cre+ male mice were bred with C57Bl/6J females (Jackson Laboratory) and resulting pups were genotyped as described below. Resulting IR flox/+; Nestin Cre+ male mice were further bred with C57Bl/6J females. This proceeding was performed for 6 generations to obtain mice, which are more than 98% on a C57Bl/6J-background.

2.2.3 Isolation of genomic DNA from mouse tail

Tail samples (~0.75 cm long) were digested overnight at 55 ^{0}C with vigorous shaking in 600 µl tail lysis buffer containing 60 µg proteinase K. Samples were centrifuged at maximum speed in a bench top centrifuge for 10 min. DNA was precipitated from the clear supernatants by adding an equal volume of isopropanol. Pellets were washed once with 70% ethanol and after a brief air drying step dissolved in 150 µl 10 mM Tris HCl, pH 8.0.

2.2.4 Polymerase Chain Reaction (PCR) for genotyping

A 50 µl reaction mixture usually contained 5 µl of 10x reaction buffer with $MgCl_2$ (AGS Biochemicals), 0.2 mM of each of the dNTPs, 50 pmoles of each primer, 200 – 400 ng of genomic DNA template and 2 U Taq DNA polymerase (AGS Biochemicals).

PCR conditions used to check for the IR floxed allele and the presence of the Nestin-Cre-transgene are described below. All reaction mixtures were submitted to an initial denaturation step at 94 ^{0}C for 2 min.

PCR to detect:	Denaturation	Annealing	Elongation	Cycles
IR floxed and wild-type	92 ^{0}C for 30 sec	61 ^{0}C for 90 sec	72 ^{0}C for 150 sec	30
IR deleted allele	92 ^{0}C for 30 sec	61 ^{0}C for 90 sec	72 ^{0}C for 150 sec	30
Presence of Nestin-Cre	92^{0}C for 30 sec	58 ^{0}C for 60 sec	72 ^{0}C for 120 sec	40

2.2.5 Methods for protein analysis

2.2.5.1 Protein extraction from mouse tissues

Mouse brain, muscle, liver, fat, heart and kidney were isolated, snap frozen in liquid nitrogen and stored at –80 ^{0}C. For isolation of protein, 1 ml protein extraction buffer was added for each 100 mg of tissue. The samples were then homogenised using a rotor-stator homogeniser (Ultra-Turrax T25, Janke & Kunkle). The homogenates were then centrifuged at 30,000 rpm in rotor T175 for 45 min. at 4 ^{0}C (Beckmann). Clear supernatants were collected and protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad).

2.2.5.2 Immunopricipitation

Equal amounts of protein (10 mg) from each tissue were incubated with 10 µl polyclonal anti-IR-antiserum (Joslin Diabetes Center), followed by overnight rotation at 4 ^{0}C. Then 70 µl Protein-A sepharose slurry (50% (v/v) in PBS) were added and allowed to rotate for 1 hour at 4 ^{0}C. Three washings were performed with protein extraction buffer.

2.2.5.3 SDS-polyacrylamide gel electrophoresis (PAGE) and Western Blot analysis

SDS-PAGE was performed as published (Laemmli, 1970). The washed immunopricipitated samples were mixed with 35 µl 2x gel loading buffer and boiled for 5 min. prior to loading.

Equal amounts of protein were resolved on 8% polyacrylamide gels and transferred onto Hybond-C Super^{TM} membranes in a semi-dry blotting chamber (BioRad) according to the manufacturer's instructions. The membrane was then blocked with 4% BSA (w/v) in TBS overnight at 4 ^{0}C, followed by incubation with primary antibodies diluted in blocking buffer for 2 hours at room temperature. After washing 4 times for 15 min. with TBS-T buffer, the bound primary antibodies were detected using 50 µCi of radiolabelled ^{125}I-Protein-A (Pharmacia). After washing 4 times for 15 min. with TBS-T buffer, bound Protein-A was visualised by exposing to an X-ray film.

Alternatively, for non-radioactive detection, the protein transferred onto the membrane was blocked with 1x blocking reagent (Roche) overnight at 4 ^{0}C, followed by incubation with the primary antibodies diluted in 0.5x blocking reagent for two hours at room temperature. After washing 4 times for 15 min. with PBS-T buffer, the bound primary antibodies were detected using the appropriate peroxidase conjugated secondary antibodies. Bound antibodies were visualised by enhanced chemiluminescence according to the manufacturer's instructions (ECL^{TM}, Pharmacia-Biotech).

2.2.6 General performance tests

2.2.6.1 Feeding behavioural study

Feeding studies were conducted for two weeks with equal numbers of mice in each group, knockout and controls, between 4 to 6 months of age. Equal animals with same sex were housed in each cage. Animals were provided with a defined amount of regular chow diet (Altromin). The amount of chow consumed was assessed by weighing the supplied food each morning between 9 to 10 am. Statistical analysis was performed using an unpaired Student's T-test.

2.2.6.2 High fat diet

One group of animals were provided with high fat diet *ad libitum*, containing 60% soybean oil (Altromin) from 3 weeks (after weaning) to 8 months.

2.2.6.3 Growth curve study

To determine the growth rates of the experimental groups of mice, animals were weighed on a weekly basis from 4 weeks up to 40 weeks of age. Body weight of each mouse was recorded and weight gain was compared among the groups of the animals. Statistical analysis was performed using an unpaired Student's T-test.

2.2.6.4 Breeding performance

The breeding performance of the knockout mice for both males and females were compared with their wild-type (WT) littermates. To study breeding performance, animals aged 12 to 16 weeks were selected and breeding was initiated with different possible combinations, such as WT male and WT female mice; WT male with neuronal insulin receptor knockout (NIRKO) females; NIRKO male with WT females. Females were examined for the occurrence of a vaginal mucous plug each morning between 8:00 and 10:00 am. Pregnancies and number of offspring were recorded. Statistical analysis was performed using an unpaired Student's T-test.

2.2.7 Physiological tests

2.2.7.1 Glucose tolerance test

Glucose tolerance tests were performed with 4 to 6 months old mice. Animals were starved for 14 to 15 hours prior to each experiment but allowed free access to water. Animals received intraperitoneal injections of glucose, 2 mg D-glucose/gm body weight (Delta-Pharma GmBH). The blood glucose levels were then measured in blood taken from the tail tip by cutting 1 to 2 mm of tail, before and 15 min., 30 min., 60 min., and 120 min. after intraperitoneal administration of glucose. The glucose concentration was determined using an automatic blood glucose reader, Glucometer Elite Sensor ® (Bayer).

2.2.7.2 Insulin tolerance test

Insulin tolerance tests were performed with 4 to 6 months old mice. Animals were kept fasting for 14 to 15 hours overnight but allowed free access to water. Animals were injected intraperitoneally with human regular insulin (Novo Nordisk Pharma, GmbH) 0.75 IU/kg body weight. The blood glucose level was measured in blood taken from the tail tip by cutting 1 to 2 mm of tail, before and 15 min, 30 min, and 60 min after insulin administration. The glucose concentration was determined using an automatic glucose reader, Glucometer Elite Sensor ® (Bayer).

2.2.7.3 Blood glucose concentration

Blood glucose levels of animals were measured in random fed state and fasting blood glucose level was determined after overnight fasting. The glucose concentration was determined using an automatic glucose reader, Glucometer Elite Sensor ® (Bayer).

2.2.7.4 Serum triglycerides and cholesterol

For this study, 4 to 6 months old animals were selected and kept fasting overnight in fresh cages. The next morning blood was collected from the tail tip in a prechilled eppendorf tube. Immediately after bleeding serum was separated by centrifugation at 8,000 rpm on bench top centrifuge for 10 min. and stored at –80 °C. To determine the triglyceride and cholesterol concentrations, 10 µl of serum were diluted with 90 µl of sterile water and concentrations were determined on a Beckmann Analyzer in a routine procedure in the central analysis laboratory of the Universitätskliniken Köln.

2.2.7.5 Free fatty acids

Free fatty acid concentration was determined in 4 to 6 months old mice. Mice were kept fasting overnight but allowed free access to water. Tail vein blood was collected and serum was separated. Serum samples were then taken to analyze the plasma free fatty acids. The

protocol was used as provided by manufacturer (NEFA, Japan). The absorbance was then measured in a spectrophotometer (Beckman) at 550 nm wavelengths.

2.2.7.6 Analysis of body composition

To determine the triglyceride and protein concentration, 6 to 8 months old neuronal insulin receptor knockout (NIRKO) and wild-type (Wt) mice were selected. Animals were weighed and killed with CO_2. Bodies were digested in 3M KOH dissolved in 70% ethanol. The final volume was brought to 100 ml by adding 3M KOH in 70% ethanol and the bottle was covered tightly and stored for two days at room temperature for complete digestion. 10 µl whole digested body were mixed with 50 µl sterilized water and both triglyceride and protein content were determined on Beckmann Analyzer in a routine procedure in the central analysis laboratory of the Universitatskliniken Koln.

2.2.8 Radioimmunoassay for different hormones

Animals were bled in random fed state from the tail vein and serum were separated immediately by centrifugation at 8,000 rpm in bench top centrifuge for 10 min. at 4 ^{0}C. Serum samples were sent to Prof. A.F.Parlow's laboratory, National Hormone and Peptide Program, Harbor-UCLA Medical Centre, California, USA for the measurement of different anterior pituitary hormones such as Growth Hormone (GH), Luteinizing Hormone (LH), Thyroid Stimulating Hormone (TSH), Prolactin (Prl), Adrenocorticotropic Hormone (ACTH). For determination of ACTH-concentrations, each animal was housed in a single cage to avoid any kind of outside stress and blood was collected from the jugular vein immediately after cervical dislocation.

To assess plasma LH-concentrations under basal and stimulated conditions, blood was taken after injection of Lupron (a GnRH agonist, 200 µg in 100 µl saline/animal) or saline. Tail vein blood was collected before injection and 1 hour after injection of Lupron and serum was separated immediately as mentioned earlier. Similarly, to determine Thyroid Releasing Hormone (TRH) intraperitoneal injection of Antepan®, a TRH agonist, (Henning Berlin GmbH) was injected. One group of animals was injected with 0.250 µg Antepan/animal and control animals received saline. Tail vein blood was collected before Antepan injection and

30 min after injection. Serum samples were then sent to Prof. Parlow's laboratory for determination of hormones by radioimmunoassay.

2.2.9 Insulin and leptin ELISA

Blood was collected from tail vein and serum was separated immediately by centrifugation at 8,000 rpm on bench top centrifuge at 4 ^{0}C. Determination of plasma insulin and plasma leptin concentrations was performed by ELISA according to the protocol provided by the manufacturer (Crystal Chem. Inc, USA).

2.2.10 Spermiogram

Male animals were killed with CO_2 and the epidydimi were isolated and chopped into small pieces in Dulbecco's modified eagle's medium (DMEM, Sigma) to release the spermatozoa into the medium during 15 min. incubation in a cell culture incubator at 37 ^{0}C. After pipetting up and down and further incubation for 45 min. at 37 ^{0}C, the solution was centrifuged for 5 min. at 1000 rpm on bench top centrifuge. The supernatant was discarded and the pellet was dissolved in 2 ml trypan blue. Spermatozoa were counted in a Neubauer chamber.

2.2.11 Estrus detection

After weaning the pups at day 21, monitoring of vaginal opening in NIRKO and their control mice were conducted every day until one and half months of the age. After vaginal opening, vaginal swab was taken every alternate day for one month with the help of sterile swab ("q" tips, Fichier Scientific, USA), and vaginal mucous was smeared on polylysine-L coated glass slides (Menzel-Glaser, Germany). The smeared mucous was air dried and then stained with 0.4% methyline blue for 5 minutes followed by washing with water. The vaginal epithelial cells were then observed under the microscope to detect the state of oestrus (cycling) of the experimental animals.

2.2.12 Behavioural tests

To study the behavioural tests a computer programme was used from Ethovision ®, version 1.91 prepared by Noldus information technology, Wageningen, The Netherland.

2.2.12.1 Learning and memory

2.2.12.1.1 Moris water maze

The cognitive abilities of mice were tested in the Moris water maze (Morris, 1984) tub. A 66 cm diameter and 22 cm deep black circular water tub with a removable 12 cm high escape platform with black top was used for this experiment. The tub was filled with water at room temperature to a level of 14 cm so that the escape platform submerged 2 cm under water. A video camera was mounted in the centre above the water tub, which provided an overview of the pool on a TV monitor that is connected with a computer. The pool was divided into four quadrants, east, west, north and south. Each quadrant was further subdivided by a pattern of lines into a 4X4 matrix of squares. The escape platform was kept in the north quadrant.

Animals were allowed to stay 15 sec. on the escape platform at the beginning of the first experiment. Each animal was allowed to swim for 90 sec., and swimming speed as well as the latency period to find the hidden escape platform were scored in the computer each time the mouse crossed a line dividing the squares with its whole body. According to the computer program, start points in different quadrants were chosen. This procedure was followed for five consecutive days. On the last day of the experiment, the hidden escape platform was removed from the tub and animals were allowed to swim for 90 sec., and their swimming speed and time spent in the different quadrants were monitored and recorded in the computer.

2.2.12.2 Anxiety tests

2.2.12.2.1 Open field test

For this study, a Perspex box of 60 cm^2 and 14 cm height was used. The floor of the box was subdivided into 10 cm^2. A video camera, mounted in the centre above the open field box, provided an overview of the field on a TV monitor. Each animal was allowed to move in this open field box for 300 sec. and their movement towards the walls, centre and corners were recorded in the computer. This test was performed for five consecutive days.

2.2.12.2.2 Light and dark exploration test

A box divided into a bright and a dark compartment was used to perform this test. Between the two compartments a small hole was made so the mice can explore both light and dark sides of the box. A video camera, mounted in the centre above the box, provided an overview of the box on a TV monitor. Each mouse was put into the dark side of the box and then allowed to move for 300 sec. Time spent in the bright and dark side of the box was recorded in the computer. This test was also performed for five consecutive days.

2.2.12.2.3 Elevated plus maze test

A 5 cm wide ring with a diameter of 50 cm and elevated 50 cm high from the ground were taken for this study. The ring was divided into two open and two closed arms. A video camera, mounted in the centre above the elevated maze, provided an overview of the maze on a TV monitor. Each individual mouse was allowed to move on this elevated maze for a period of 300 sec and time spent in the open arms and closed arms were recorded in the computer.

2.2.12.3 Olfactory test

A simple olfactory test was conducted. To study the sense of smell of the animals a small piece of cheese (1 cm^3) was hidden under the bedding of a fresh cage. 300 sec. were provided

to each animal to locate the hidden cheese and the time to locate the cheese was recorded by a stopwatch.

2.2.13 Organ isolation for insulin signalling studies

Animals were anaesthetized with thiopental sodium (Trapanal, BYK Gulden) and an incision was made to open the abdominal cavity. 5 IU human insulin (Novo Nordisk Pharma, GmbH) were injected carefully and slowly into the inferior vena cava. Organs were removed at different time points as follows: liver (after 1 min.), perigonadal fat (2 min.), muscle (3 min.), and brain (10 min.).

2.2.14 Xyline eosin tissue staining

Ovaries and testis were isolated from mice and kept on 10% formaldehyde followed by embedded in paraffin. Paraffin embedded tissues was cut at a thickness of 5 µm using a microtome (Reichert-Jung) and were transferred onto glass slide. Then the tissue sections were dipped into xyline (10 min for 2 times), isopropanol (2 min.) and one min. each in gradient EtOH beginning from 96%, 75% and 50%, respectively followed by 1min. dip in distilled water. After alcohol treatment the tissue section were incubated 4-5 min in hamatotoxilline dye followed by washing on warm running tap water to remove the excess dyes from the tissue. Then the tissue was stained with eosin dye for about 1 min and washed with water to remove the excess dye. The stained tissues were then allowed to dry on room temperature and embedded with glycerine-gelatine.

2.2.15 Hypogonadal animal models

Three groups of 8 weeks old female C57Bl/6J animals were selected for this study. In the first group, females were sham operated, in the second group females were ovarectomised and in the third group, females were ovarectomised with subcutaneous implantation of a 17ß–estradiol tablet with a 90–day release (Innovative Research of America) to maintain blood level of estrogen to 100 pg/ml.

2.2.16 Primary culture of cerebellar granular cells

Five day old pups were killed by decapitation to remove cerebella. The skin was peeled off the back of the head, the skull was removed and the cerebellum was pinched off and kept on HHGN. Meningeges, blood vessels and other attached tissues were removed from the cerebella under a dissection microscope. The processed cerebella were then washed 3 times with HHGN, treated with TDn for 15 min. at room temperature, and washed 3 times with HHGN. To get single cells, cerebella were homogenised in DnB followed by centrifugation at 1000 rpm on bench top centrifuge for 10 min. at 4 ^{0}C The supernatant was removed and cells were redissolved in CbC medium. For protein isolation, 2-5 x 10^6 cells per well were plated on 6-well plates, for apoptosis studies, 10^5 cells per well were plated in 96-well plates coated with poly–L-lysine coating (Becton and Dickinson, USA). Plates were then incubated for 24 hour in a cell culture incubator at 37 °C and 5% CO_2. To further purify the cultured cells and eliminate proliferating cells of other tissues, the cultured cells were treated with 10µM cytosine arabinoside (AraC), which only affects dividing cells but not neuronal cells. After another incubation at 37 ^{0}C for 72 hours, the CbC medium was removed and cells were washed twice with Basal medium eagle (BME) at room temperature. Then the cells were supplemented with low KCl starving medium (5mM KCl), which induces apoptosis of neuronal cells by depolarization of the plasma membrane. For apoptosis studies, cells were treated with different doses of insulin and IGF-I (0.1, 1, 10, 100 nM and 1 µM) followed by an incubation for 36 hours at 37 °C. Fixation of cells were done with 4% paraformaldehyde followed by washing with PBS and apoptotic cells were stained with Hoechst® stain. The portion of apoptotic cells was assessed using a fluorescence microscope.

For protein isolation, cells were stimulated with 10 nM insulin or IGF-I for 10 min., then the medium was removed, dishes were washed with ice cold PBS and cells were scrapped into 70 µl of protein lysis buffer containing a cocktail of protease inhibitors. Protein concentrations were determined by Bradford assay (Bradford, 1976).

3. Results

3.1 Generation of homozygous neuronal insulin receptor knockout (NIRKO) mice

Heterozygous IR(Lox/+) or homozygous IR(Lox/Lox) animals were mated with mice carrying a Cre allele (Cre +/-) under control of the rat nestin promoter and enhancer to generate mice double-heterozygous for the IR and Cre alleles (IR Lox/+ Cre +/-). These animals were then bred with heterozygous IR(Lox/+) mice to obtain IR (Lox/Lox):Nestin Cre(+/-) offspring, that is, mice with neuron-specific insulin receptor knockout (NIRKO).

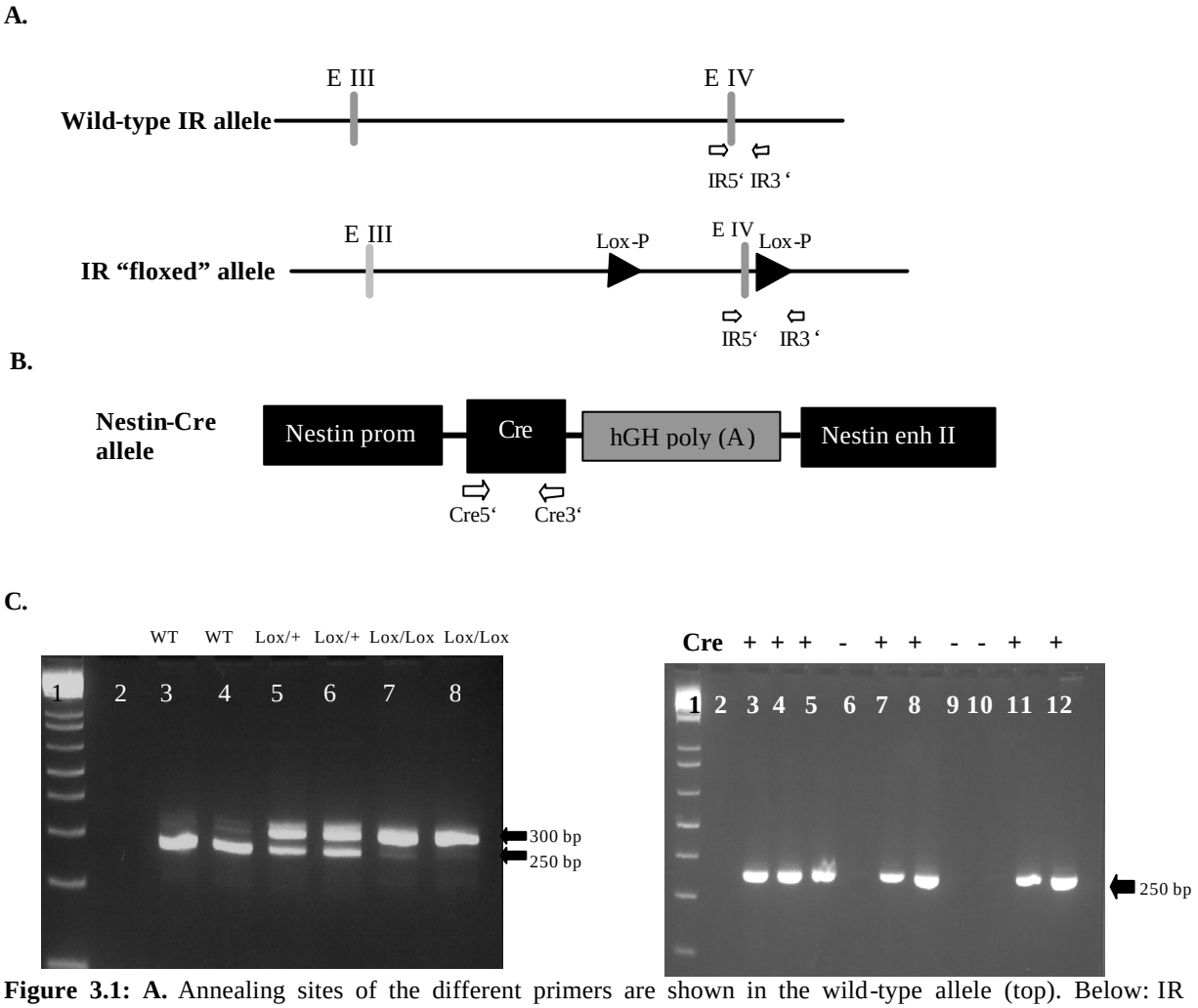

Figure 3.1: A. Annealing sites of the different primers are shown in the wild-type allele (top). Below: IR “floxed” with two Lox-P sites (closed triangles) 5’ and 3’ of exon 3 and 4 (grey box). **B.** Annealing sites of primers in the Cre transgene allele. Cre was expressed under the control of the promoter and enhancer present in the second intron of the rat central nervous system-specific nestin gene. Transcription is terminated by the polyadenylation sequence of the human growth hormone gene (hGHpoly (A)). **C.** PCR product was resolved on a 3% agarose gel. The presence of WT, heterozygous (Lox/+) and homozygous (Lox/Lox) IR alleles was detected by PCR-amplification of products of 250 bp (WT) and 300 bp (floxed) using the PCR primer pair IR3’ and IR5’ (Fig. A) (Fig. C, left panel). The presence and absence of the Cre transgene was assessed by presence or absence of a 250 bp PCR product using PCR primer pair Cre3’ and Cre5’ (Fig. B) (Fig. C, right panel). Lane 1 and 2 (Fig.C, left and right panel) indicates DNA molecular weight markers and negative controls with no DNA templates, respectively.

To facilitate genotyping of large numbers of offspring, genomic DNA isolated from tail biopsies was analysed by a PCR strategy. All primer sequences are listed in 2.1.6 and annealing sites are outlined in Figure 3.1 A and 3.1.B (see page 37).

3.2. Efficiency of recombination

To determine the efficiency of IR gene inactivation in the central nervous system of NIRKO mice, we determined IR-expression on the protein level. Protein was extracted from whole brains of NIRKO and control mice (IR +/+ with Cre – and IR flox/flox with Cre -), age between 6 to 8 months. Protein extracts were subjected to immunoprecipitation with antisera specific for the IR-ß subunit, IRS-1 and IRS-2, respectively. After immunoprecipitation, proteins were resolved on 8% polyacrylamide gels under reducing conditions, and after electrotransfer membranes were probed with the respective antiserum. As shown in Figure 3.2 A, protein extracted from the brains of NIRKO mice exhibited a largely reduced amount of immunodetectable IR-protein. By contrast, expression of IR protein in the brains of wild type and homozygous IR-floxed mice was unaltered. The expression of related insulin signaling proteins, such as IRS-1 and IRS-2 was unaltered in the brain of NIRKO mice. Densitometric analysis of these blots revealed a reduced IR-expression in brain extracts of NIRKO mice by more than 95% compared to controls (Fig.3.2 B).

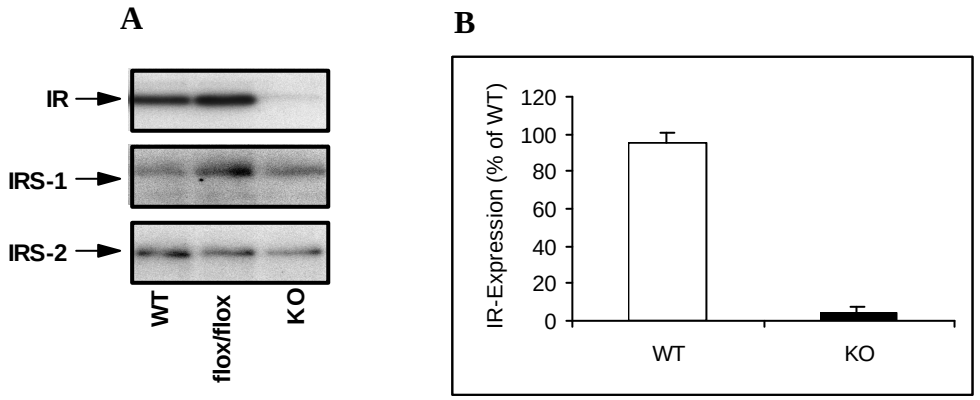

Figure 3.2: A Immunoblot analysis of insulin receptor expression in tissue extracts (10 mg) prepared from wild-type (WT), IR Lox/Lox and NIRKO (KO) mice. Extracts were subjected to immunoprecipitation and proteins were resolved on 8% SDS-polyacrylamide gels under reducing conditions and blotted onto nitrocellulose membranes. The blots were probed with polyclonal antisera specific for the IR-ß subunit (1:300) (upper panel), IRS-1 (1:500) (middle panel) and IRS-2 (1:500) (lower panel) followed by incubation with ^{125}I-labelled Protein A to detect IR expression. **B** Quantification of IR immunoreactivity by densitometric scanning of IR immunoblots as shown in **A**. Data represent the mean ± SEM of *n* = 8 of each genotype and expression is shown relative to that of control mice.

To evaluate the specificity of IR-inactivation in NIRKO mice, we also determined the expression of the IR ß-subunit in different non-neuronal organs such as liver, muscle, heart, kidney, spleen and testis of NIRKO and control mice. This analysis revealed an unaltered IR expression in these organs (Fig. 3.3).

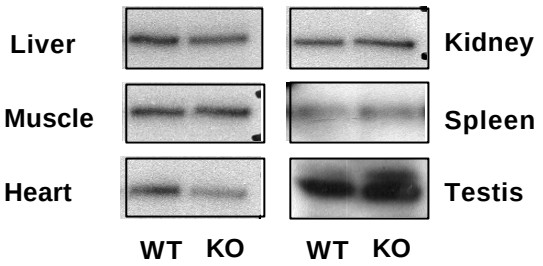

Figure 3.3: Immunoprecipitation and immunoblot analysis of insulin receptor expression from tissue extracts (10 mg) prepared from NIRKO (KO) and control (WT) mice. Extracts were subjected to immunoprecipitation and proteins were resolved on 8% SDS-polyacrylamide gels under reducing conditions and blotted onto nitrocellulose membranes. The blots were probed with polyclonal antisera specific for the IR-ß subunit (1:300) followed by incubation with ^{125}I-labelled Protein A to detect IR expression.

These data show an inactivation of the IR-gene in NIRKO mice with high efficiency and specificity in the central nervous system.

3.3 Reduced IR expression in cultured primary neurons

Since the extracts from whole brain used in the experiments described before (3.2) represent heterogeneous cell populations such as neurons, glia cells and cells from vasculature, we decided to determine whether the faint residual signal for IR-expression detectable in whole brain extracts resulted from unrecombined IR-alleles in neurons or from non-neuronal tissues. Therefore, we analysed the recombination of the floxed IR-allele in neurons cultured from NIRKO and control mice.

To evaluate the efficiency of recombination of the IR Lox allele, DNA was prepared from primary neuronal cultured cells of floxed/deleted IR mice and NIRKO mice. Figure 3.4 shows the position of the different primers used for PCR analysis. 0.6ng of DNA prepared from IR floxed/deleted and KO neuronal culture cells from 5 day old pups were used for PCR using the primers Intron3' and IR5' as forward primer and IR3' as backward primer. In neurons

from IR floxed/deleted mice two bands of equal intensity of 300 bp and 250 bp were observed (Figure 3.4, lane 3 and 5). By contrast, a single band of 250 bp representing the deleted allele was detected in the neuronal DNA obtained from NIRKO mice (figure 3.4, lane 4). These data indicate a complete deletion of exon 4 in the neurons of NIRKO mice.

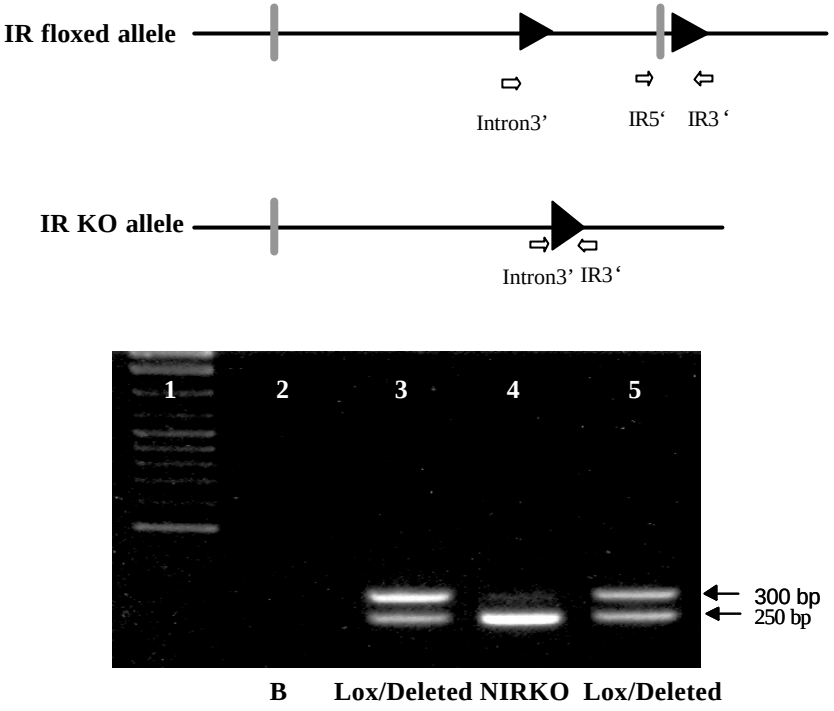

Figure 3.4: Assessment of Cre-mediated recombination of the IR floxed allele in NIRKO mice. Schematic representation of the IR floxed allele (upper panel) showing the position of different primer annealing sites for PCR analysis. The knockout allele (middle panel) is shown indicating the resulting PCR-product of the primers Intron3' and IR3' in case of deletion of exon 4. The lower panel shows a representative PCR analysis of DNA extracted from cultured neurons resolved on a 1.5% agarose gel. DNA derived from IR floxed/deleted mice generated a 300 bp (primers IR5' and IR3') and a 250 bp (primers Intron3' and IR3') PCR product (lane 3 and 5). A single 250 bp (primers Intron3' and IR3') (lane 4) PCR band representing the deleted allele is observed only in the NIRKO mice with PCR primers Intron3' and IR3'. PCR primers Intron3' and IR3' are unable to amplify the IR floxed allele as both primers anneal too far apart. Lane 1 and 2 indicate the DNA molecular weight markers and control with no DNA templates, respectively.

To further confirm the completeness of IR-inactivation in neurons from NIRKO mice, we performed Western Blot analysis on protein extracts from cultured neurons of different experimental mice. As shown in figure 3.5, IR expression in primary cultured neurons derived from NIRKO mice is completely abolished compared to cultured neurons from control littermates.

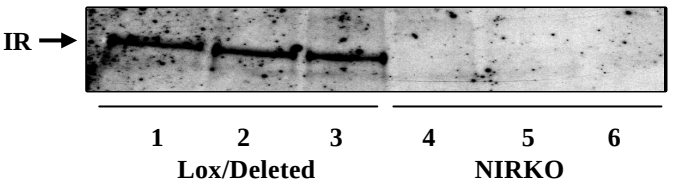

Figure 3.5: Immunoblot analysis of IR expression in the primary cultured neurons. Cerebellar granular cells were prepared from 5 day old pups and cells were grown in poly-L-lysine coated plates. Protein extracts prepared from Lox/Deleted mice (control) and NIRKO mice. Primary cerebellar granular cells were grown for 5 days before protein extraction. Total protein extracts (50 µg/lane) were resolved on a 8% SDS-polyacrylamide gel under reducing conditions and were transferred onto a nitrocellulose membrane. The resulting blot was probed with IR-ß specific antiserum (1:200) anti rabbit IgG (1:1000) as secondary antibodies. Bound antibodies were detected using the ECL system.

3.4. Influence of insulin receptor expression on body weight and food intake

3.4.1 Growth curve

Since previous studies have implicated neuronal insulin receptors in the regulation of food intake and energy homeostasis, we monitored the regulation of body weight in NIRKO and control (IR Lox/Lox, Cre -; IR Lox/+, Cre -, and IR +/+, Cre – or +) mice. At least 16 mice of each genotype and gender were weighed on a weekly basis from 6 to 21 weeks of age. The body weight of male NIRKO mice was indistinguishable from their control littermates during the first 6 months of life under normal chow diet (Fig. 3.6 A). By contrast, female NIRKO mice exhibited a consistent 10 to 15% increase of body weight ($p < 0.05$) as compared to their control littermates under normal chow diet.

To test whether this phenotype of an increased body weight in female NIRKO animals is more pronounced under a high caloric diet, we exposed a second group of mice to a diet enriched in fat (60% soybean oil). Indeed, the mildly obese phenotype in female NIRKO mice was more pronounced when the mice were fed with this diet. Under high-fat diet, by as little as 14 weeks of age male NIRKO mice showed more than 10% elevation of body weight ($p < 0.05$) and female NIRKO mice a 20% increase in body weight ($p < 0.05$) compared to their control littermates (Fig. 3.6 B).

In summary, these data indicate that neuron-specific disruption of the insulin receptor gene in mice results in a diet sensitive increase in body weight.

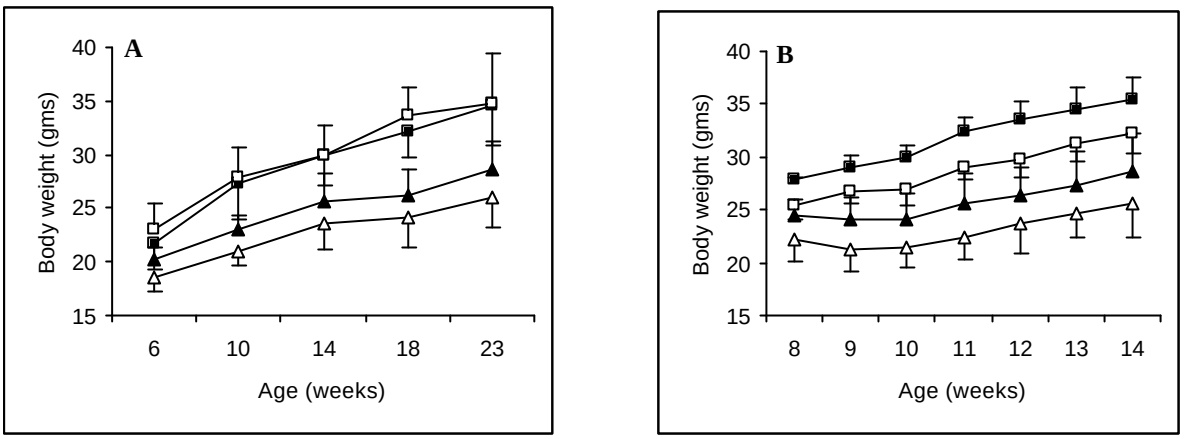

Figure 3.6: Body weight of animals was determined under normal chow (**A**) and under high fat (60%) chow (**B**), NIRKO male (closed square), control male (open square); NIRKO female (closed triangle) and control female (open triangle)). The SEM at each point was below 10% of the indicated value. Body weights of female NIRKO mice were significantly different from their control littermates at every age with $p<0.05$ in an unpaired Student's t-test under normal chow (**A**). Body weights of NIRKO males do not show any statistically significant difference during the same period compared to their controls under normal chow diet. By contrast, under high fat (60%) diet, the body weight of both NIRKO males and females were significantly different ($p<0.05$) as compared to their controls (**B**). Data represent the mean of at least 16 animals in **A** and at least 8 animals in **B** of each gender and genotype. Control indicates IR Lox/Lox, Cre -; IR Lox/+, Cre -, and IR +/+, Cre – or +.

3.4.2 Increased amount of white adipose tissue in NIRKO mice

To determine whether the increase of body weight in the experiments described above resulted from an increased obesity, i.e. increased fat mass, we next determined the weight of white adipose tissue in NIRKO and control (IR Lox/Lox, Cre -; IR Lox/+, Cre -, and IR +/+, Cre – or +) mice. At least eight animals of each genotype and gender between 8 to 12 months of age were selected to determine the parametrial fat depots in female mice and epididymal fat depots in male mice. Both male and female NIRKO mice showed increased adipose tissue mass with an about 2-fold ($p <0.005$) increase in perigonadal white adipose tissue in NIRKO females and a 1.5-fold ($p < 0.05$) increase in male NIRKO mice compared to their controls under normal chow diet.

These data indicate that the increased body weight in female NIRKO mice indeed results from increased obesity and that despite exhibiting a normal body weight as compared to controls, also male NIRKO mice exhibited mildly increased white adipose tissue mass (Fig. 3.7).

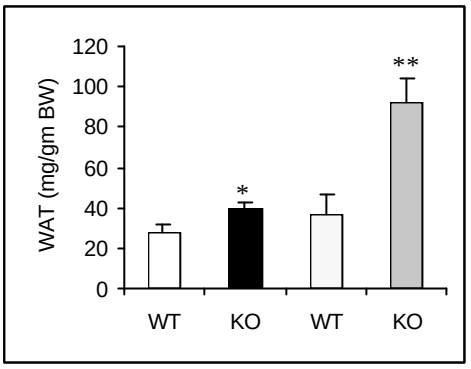

Fig. 3.7: White adipose tissue mass was determined in control indicated as "WT" male (open bar), NIRKO indicated as "KO" male (closed bar), control indicated as "WT" female (dotted bar) and NIRKO indicated as "KO" female (crossed bar) animals under normal chow diet. "WT" indicates control animals (IR Lox/Lox, Cre -; IR Lox/+, Cre -, and IR +/+, Cre – or +). Data represent the mean ± SEM of at least eight animals of each genotype and gender between 8 to 12 months of age (* = $p < 0.05$; ** = $p < 0.005$).

3.4.3 Increased food intake of female NIRKO mice

Since obesity can result from either increased food intake or reduced energy expenditure, we next determined the amount of food consumed by NIRKO mice as compared to their control (IR Lox/Lox, Cre -; IR Lox/+, Cre -, and IR +/+, Cre – or +) littermates. Food intake was assessed in 4 to 6 months old mice under normal chow diet. Food intake and body weight of 14 animals of each genotype and gender was determined daily for a period of one week. The amount of food consumed by the male NIRKO mice did not differ significantly from that of controls (82 mg/gm body weight/day compared to 87 mg/gm body weight/day), whereas female NIRKO mice exhibited an about 20% increase in food intake as compared to their control littermates (121mg/gm body weight/day compared to 100mg/gm body weight/day; $p < 0.01$).

These data indicate, that deficiency in neuronal insulin receptors results in chronically increased food intake, leading to an obese phenotype (fig. 3.8).

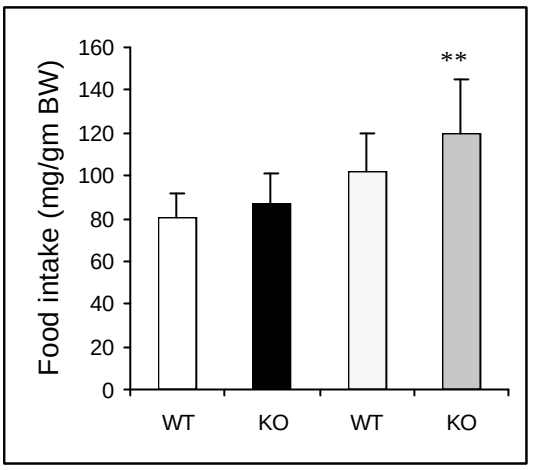

Figure 3.8: Food intake was assessed for a week in control indicated as "WT" male (open bar), NIRKO indicated as "KO" male (closed bar), control indicated as "WT" female (dotted bar) and NIRKO indicated as "KO" female (crossed bar) under regular chow diet. "WT" represents control animals (IR Lox/Lox, Cre -; IR Lox/+, Cre -, and IR +/+, Cre – or +). Data represent the mean ± SEM of at least 14 mice of 4 to 6 months old of each genotype and gender (** = $p<0.01$).

3.5 Physiological study of NIRKO mice

Since neuronal insulin receptor knockout mice exhibited mild obesity compared to their control littermates, we determined the different physiological values like plasma leptin, blood glucose, plasma insulin, plasma cholesterol concentrations, glucose tolerance and leptin sensitivity on those animals.

3.5.1 Plasma leptin

Leptin has been identified as an important regulator of food intake and energy homeostasis (Ingvartsen *et al*., 2001). It is secreted from adipocytes and acts on leptin receptors located in the hypothalamus leading to an inhibition of food intake and increased energy expenditure. To test whether the mild obesity in NIRKO mice results from dysregulated leptin concentrations, we determined plasma leptin concentrations in NIRKO and control (IR Lox/Lox, Cre -; IR Lox/+, Cre -, and IR +/+, Cre – or +) mice. Plasma leptin concentrations were determined by enzyme-linked immunoabsorbent assay (ELISA) in blood samples obtained from 6 to 8 months old mice on regular and high fat chow diet. Data represent the mean of at least 8 to 10 animals from each genotype and gender. Plasma leptin concentrations were elevated 1.5-fold in NIRKO male mice ($p < 0.05$) and 2.5-fold in female NIRKO mice ($p < 0.01$) compared to

their controls under normal chow diet. Similarly, plasma leptin concentrations of these animals under high fat chow were 1.6-fold elevated in NIRKO male and 1.7-fold elevated in NIRKO females as compared to their control littermates (Fig. 3.9).

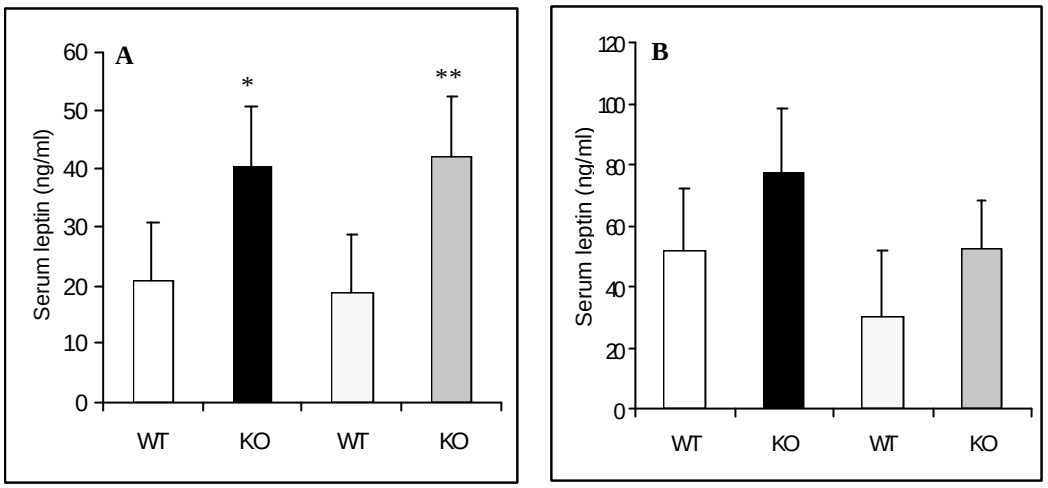

Figure 3.9: Plasma leptin concentrations were determined by ELISA in control indicated as "WT" male (open bar), NIRKO indicated as "KO" male (closed bar), control indicated as "WT" female (dotted bar) and NIRKO indicated as "KO" female (crossed bar) animals either under normal chow diet (**A**) or under high fat chow (60%) (**B**). Control animals indicates IR Lox/Lox, Cre -; IR Lox/+, Cre -, and IR +/+, Cre – or +. Results represent the mean ± SEM of at least 10 animals under regular chow and at least 8 animals under high fat diet of each genotype and gender (* = $p < 0.05$; ** = $p < 0.005$).

3.5.2 Fasting blood glucose measurement

Since massive obesity has been demonstrated to result in systemic insulin resistance (Mittelman *et al.*, 2002), we investigated glucose metabolism in NIRKO and control (IR Lox/Lox, Cre -; IR Lox/+, Cre -, and IR +/+, Cre – or +) mice. Therefore, we first analysed blood glucose concentrations in 6 to 8 months old mice after an overnight fasting period. These experiments revealed that despite the presence of mild obesity in the NIRKO mice, blood glucose concentrations in these mice were unaltered (Fig. 3.10).

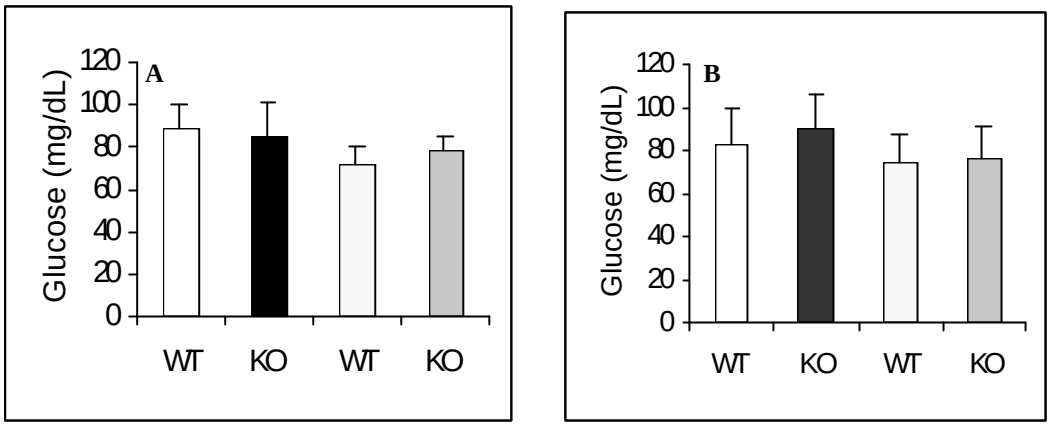

Figure 3.10: Blood glucose concentrations after an overnight fasting period, control indicated as "WT" male (open bar), NIRKO indicated as "KO" male (closed bar), control indicated as "WT" female (dotted bar) and NIRKO indicated as "KO" female (crossed bar) mice. (**A**) under normal chow diet and (**B**) under high fat (60%) chow. Blood glucose concentration was measured in tail tip blood with the help of an automatic blood glucose reader, Glucometer elite sensors. Control animals were IR Lox/Lox, Cre -; IR Lox/+, Cre -, and IR +/+, Cre – or +. Data represent the mean ± SEM of at least 8 animals of each genotype and gender.

3.5.3 Plasma insulin

Plasma insulin concentrations were determined by ELISA in blood samples obtained from 6 to 8 months old mice on normal chow diet (Fig. 3.11 A) and high fat-diet (Fig. 3.11 B). Plasma insulin levels were elevated 1.5-fold in male NIRKO mice and about 2-fold in female NIRKO mice ($p < 0.005$) compared to their controls (IR Lox/Lox, Cre -; IR Lox/+, Cre -, and IR +/+, Cre – or +) under normal chow diet. Whereas in animals under high fat-diet the elevation of plasma insulin levels were opposite to the normal chow diet, NIRKO males showed about 2-fold higher ($p < 0.005$) circulating insulin levels and female NIRKO mice showed about 1.5-fold higher plasma insulin levels compared to their control animals.

This result indicates that NIRKO mice were insulin resistant under normal chow as well high fat diet.

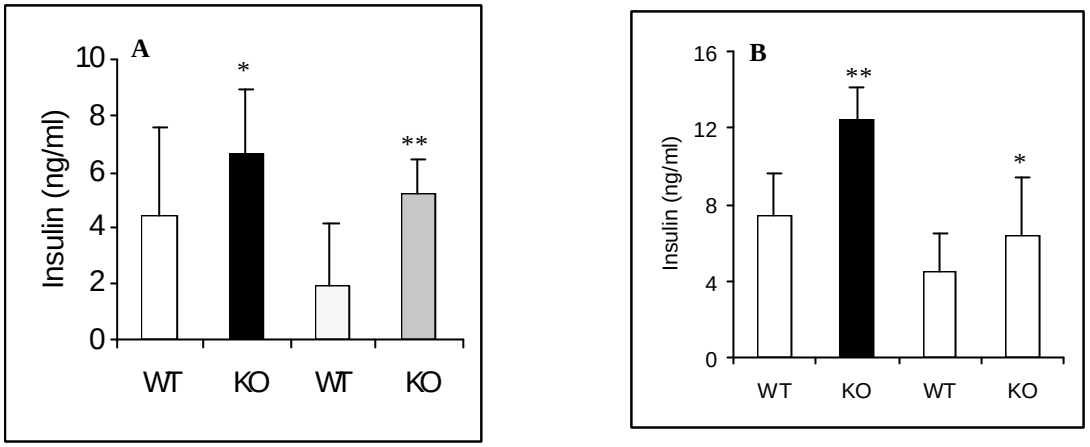

Figure 3.11: Plasma insulin concentration was determined by ELISA in control indicated as "WT" male (open bar), NIRKO indicated as "KO" male (closed bar), control indicated as "WT" female (dotted bar) and NIRKO indicated as "KO" female (crossed bar). (A), animals under normal chow diet and (B), animals under high fat (60%) chow. Control animals indicates IR Lox/Lox, Cre -; IR Lox/+, Cre -, and IR +/+, Cre – or +. Data represent the mean ± SEM of at least 10 animals of each genotype and gender under regular diet and high fat diet, respectively (** = $p<0.005$)

3.5.4 Plasma triglyceride concentrations

Since NIRKO animals exhibited massive obesity, we demonstrated its effect on plasma triglycerides. Plasma triglyceride concentrations were determined in blood samples obtained from 6 to 8 months old mice under normal chow food (Fig 3.12). Both male and female NIRKO mice showed 30% increase in circulating triglycerides compared to their control (IR Lox/Lox, Cre -; IR Lox/+, Cre -, and IR +/+, Cre – or +) littermates, indicating that NIRKO mice show a hypertriglyceridemic phenotype.

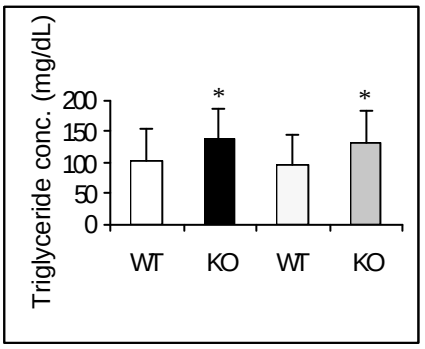

Figure 3.12: Plasma triglyceride concentrations: control indicated as "WT" male (open bar), NIRKO indicated as "KO male" (closed bar), control indicated as "WT" female (dotted bar) and NIRKO indicated as "KO" female (crossed bar), under normal chow diet. Plasma triglyceride was measured in a Beckmann Analyzer. Control animals were IR Lox/Lox, Cre -; IR Lox/+, Cre -, and IR +/+, Cre – or +. Data represent the mean ± SEM of at least 10 animals of each genotype and gender of 6 to 8 months old mice (* = $p<0.05$) under normal chow diet.

3.5.5 Plasma cholesterol concentrations

Plasma cholesterol concentrations were determined in blood samples obtained from 6 to 8 months old mice under normal chow diet. The circulating plasma cholesterol concentrations in control (IR Lox/Lox, Cre -; IR Lox/+, Cre -, and IR +/+, Cre – or +) and NIRKO animals were indistinguishable (Fig. 3.13), indicating that the obesity in NIRKO mice was only associated with hypertriglyceridemia.

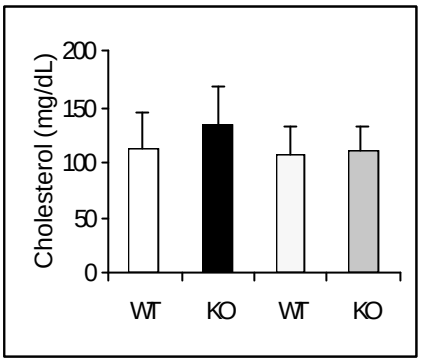

Figure 3.13: Plasma cholesterol concentration: control indicated as "WT" male (open bar), NIRKO indicated as "KO" male (closed bar), control indicated as "WT" female (dotted bar) and NIRKO indicated as "KO" female (crossed bar) under regular chow diet. Plasma cholesterol concentration was determined in a Beckmann Analyser. Control animals were IR Lox/Lox, Cre -; IR Lox/+, Cre -, and IR +/+, Cre – or +. Data represent the mean ± SEM of at least 11 animals of each genotype and gender under normal chow diet ($p > 0.15$).

3.5.6 Glucose tolerance test

To test whether the obese phenotype resulting from isolated insulin resistance in the central nervous system has any impact on whole body glucose metabolism, we performed intraperitoneal glucose tolerance tests in NIRKO and control (IR Lox/Lox, Cre -; IR Lox/+, Cre -, and IR +/+, Cre – or +) mice, both under regular and high fat diet. Fasting blood glucose concentrations were indistinguishable between NIRKO and control mice as was the glucose clearance rate after intraperitoneal glucose challenge (Fig. 3.14 A). However, animals under high fat diet exhibited an impaired glucose tolerance as compared to mice on regular chow diet (Fig. 3.14 B), indicating that high fat-induced obesity alters systemic insulin sensitivity. On the other hand, mild aggravation of obesity by isolated insulin resistance in the central nervous system had no further effect on systemic glucose metabolism.

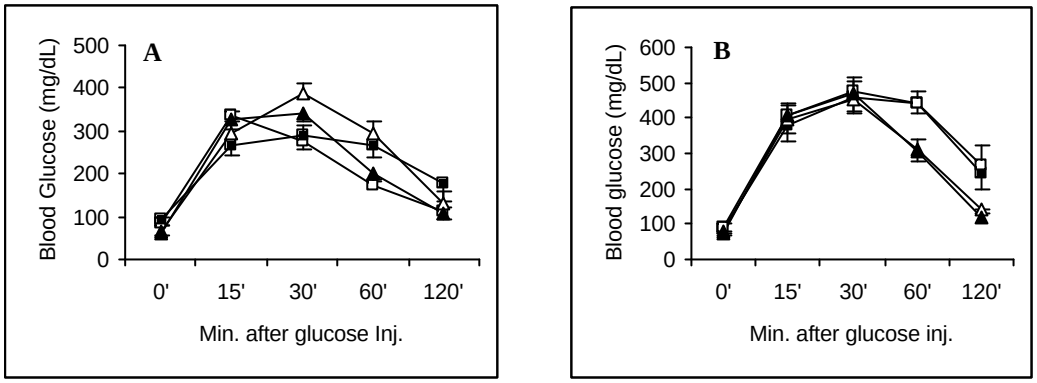

Figure 3.14 Glucose tolerance test of animals under regular chow (A) and under high fat (60%) chow (B). NIRKO male (closed square), control male (open square); NIRKO female (closed triangle) and control female (open triangle). Control animals were IR Lox/Lox, Cre -; IR Lox/+, Cre -, and IR +/+, Cre – or +. Blood glucose was determined with an automatic glucose reader (Glucometer elite sensor, Bayer) before intraperitoneal injection (IP) of glucose, indicating as "0" min. and after IP injection indicating as 15, 30, 60 and 120 min. Data represent the mean ± SEM of at least 8 animals of each genotype and gender under each diet.

3.5.7 Leptin sensitivity study

As described in the introduction, leptin secreted from the adipose tissue acts as an anorectic peptide through its receptors in the hypothalamus. Since obesity in the NIRKO mice occurred despite elevated plasma leptin concentrations, we next investigated whether isolated insulin resistance in the central nervous system results in leptin-resistance, offering a mechanism for the development of obesity in these mice. To study leptin-sensitivity of NIRKO and control (IR Lox/Lox, Cre -; IR Lox/+, Cre -, and IR +/+, Cre – or +) mice, we injected daily either mouse recombinant leptin (10 µg/g body weight) or PBS for one week. Body weight of animals was measured daily in the morning before leptin or PBS injection. While mice receiving a daily injection of PBS showed no difference or even a slight increase in body weight, both control and NIRKO mice receiving leptin injections significantly lost weight over the period of one week (fig. 3.15). These data indicate that NIRKO mice develop obesity and increased plasma leptin concentrations despite unaltered sensitivity to leptin's anorectic effect *in vivo.*

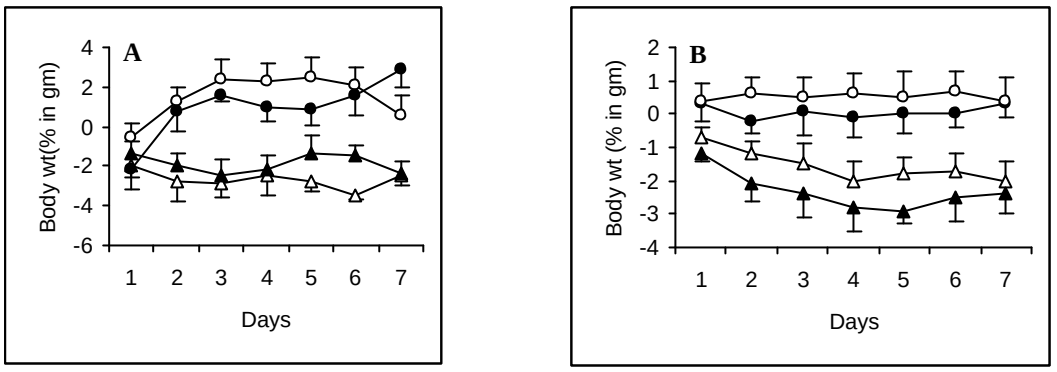

Figure 3.15: Leptin sensitivity study: (**A**), female and (**B**) male mice; control mice received PBS (closed circle), NIRKO mice received PBS (open circle), control mice received recombinant mouse leptin, 10 µg/gm BWT (closed triangle) and NIRKO mice received intraperitoneal recombinant mouse leptin, 10 µg/gm BWT (open triangle) by daily intraperitoneal injection over the period of one week. Shown is the change of body weight in % as compared to the body weight before the respective treatment. Control animals were IR Lox/Lox, Cre -; IR Lox/+, Cre -, and IR +/+, Cre – or +. Data represent the mean ± SEM of at least 8 animals of each genotype and gender.

3.6 Fertility studies

3.6.1 Breeding Study

During the course of these studies we tried to expand the mouse colony more rapidly by mating NIRKO mice with IR flox/flox mice. Since the breeding efficiency from these crossings appeared to be impaired, we decided to study the reproductive performance of NIRKO mice in greater detail. Therefore, breeding were initiated between mice of the different genotypes, i.e. breedings among control mice (C57Bl/6J male (WtM) x C57Bl/6J female (WtF)); breedings between control male and NIRKO females (C57Bl/6J male (WtM) x neuronal insulin knockout-female (KO-F)); breedings between NIRKO (neuronal insulin receptor knockout) male (KO-M) and control (C57Bl/6J) female (WtF)). At least 10 breedings of each combination were monitored for offspring over a period of two months (Fig. 3.16). 76% of the matings established between control mice (WtM x WtF) produced offspring, whereas breeding of male NIRKO mice with control females (KO-M x WtF) produced offspring in only 42% of the cases ($p < 0.05$). Similarly, breeding of control male with NIRKO females (WtM x KO-F) produced offspring in only 40% of the breedings ($p < 0.05$).

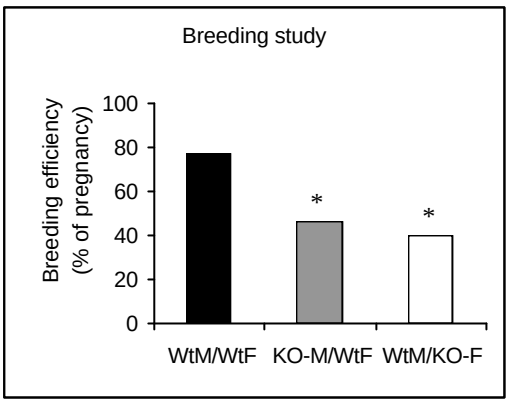

Figure 3.16: Breeding efficiency is indicated for control male with control female (C57Bl/6J male (WtM) X C57Bl/6J female (WtF), (black bar)), NIRKO-male (KO-M) with control female (C57Bl/6J female (WtF), (grey bar)) and control male (C57Bl/6J male (WtM) with NIRKO female (KO-F), (white bar)). The initial values for 10 breedings were set arbitrarily as 100% Data represent the mean of at least 10 breedings in each combination in each group (*, $p < 0.05$).

3.6.2 Reduced spermatogenesis in male NIRKO mice

To further analyse the cause of reduced fertility in male NIRKO mice, we determined the epididymal sperm content in NIRKO and control mice. Therefore, epididymi of control (IR Lox/Lox, Cre -; IR Lox/+, Cre -, and IR +/+, Cre – or +) and NIRKO mice were removed, and spermatozoa were allowed to diffuse into culture medium as described in materials and methods. After centrifugation, total epididymal sperm content was determined as shown in Fig. 3.17. This analysis revealed a 60% reduced epididymal sperm content in NIRKO males compared to their age matched controls ($p < 0.05$).

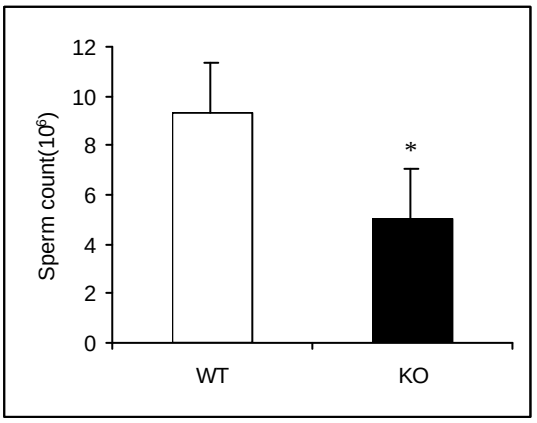

Figure 3.17: Spermiogram of control male indicated as "WT" mice (open bar) and NIRKO male indicated as "KO" mice (closed bar). Total epididymal spermatozoa are counted under a microscope. Control animals were IR Lox/Lox, Cre -; IR Lox/+, Cre -, and IR +/+, Cre – or +. Data represent the mean ± SEM of at least 10 animals of each group (* = $p < 0.05$).

3.6.3 Signs of reduced gonadotropic stimulation in NIRKO mice

To further investigate the reduced fertility in NIRKO mice, we assessed the morphology of testes and ovaries in NIRKO and control (IR Lox/Lox, Cre -; IR Lox/+, Cre -, and IR +/+, Cre – or +) mice. Therefore, the respective organs were removed and fixed in 10% formaldehyde, sectioned to 5 µm and stained with haematoxylin eosin stain as mentioned in materials and methods. This result shows a reduced number of Leydig cells and reduced interstitial stroma in male NIRKO whereas female NIRKO mice exhibited a reduced number of antral follicles (Fig. 3.18).

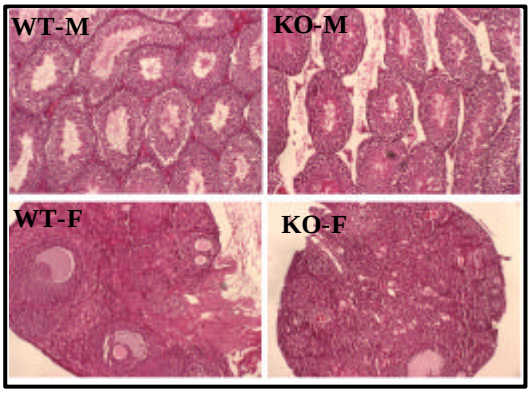

Fig. 3 18: Histological sections of testis and ovary of control and NIRKO mice. Testis section of control male indicated as "WT-M" (upper left panel), testis section of NIRKO male indicated as "KO-M" (upper right panel), ovary section of control female indicated as "WT-F" (lower left panel) and ovary section of NIRKO female indicated as "KO-F" (lower right panel). Testes and ovaries were removed from control and NIRKO mice and fixed in 10% formalin. 5 µm Paraffin-embedded sections were stained with haematoxylin and eosin. The scale bar indicates about 100 mm.

3.6.4 Slightly delayed puberty in NIRKO female mice

The reduced fertility of NIRKO female mice was further investigated by determinig the onset of puberty in these mice by monitoring the ocurrence of vaginal opening in NIRKO and control females (IR Lox/Lox, Cre -; IR Lox/+, Cre -, and IR +/+, Cre – or +). NIRKO females exhibited a slightly delayed onset of puberty compared to their littermates and this delay was statistical significant (Fig. 3.19).

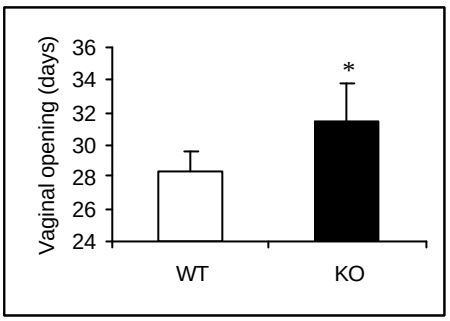

Figure 3.19: Vaginal opening of NIRKO and control females. Control indicated as "WT" female (open bar) and NIRKO indicated as "KO" female (closed bar). Opening of the vagina was monitored after weaning at age 21 to 45 days. Control animals were IR Lox/Lox, Cre -; IR Lox/+, Cre -, and IR +/+, Cre – or +. Data represent the mean ± SEM of at least 8 animals of each genotype (* = $p < 0.05$).

3.6.5 Cycling in NIRKO females

Luteinizing Hormone (LH) has an effect on gonads by inducing ovulation in females (Patton *et al*., 1989). Therefore, we decided to monitor the cycling in NIRKO females and control (IR Lox/Lox, Cre -; IR Lox/+, Cre -, and IR +/+, Cre – or +) littermates. For this study vaginal smears were taken on every alternate day over the period of two months. The smeared vaginal epithelial cells were stained with 0.4% methylene blue and viewed under a microscope. Epithelial cells under the influence of the estrogen exhibit a cuboidal shape. These results revealed a prolonged estrous phase in cycling NIRKO females, and a higher proportion of mice with persistent estrous phase (33% in NIRKO vs. 20% in control mice) (Fig. 3.20).

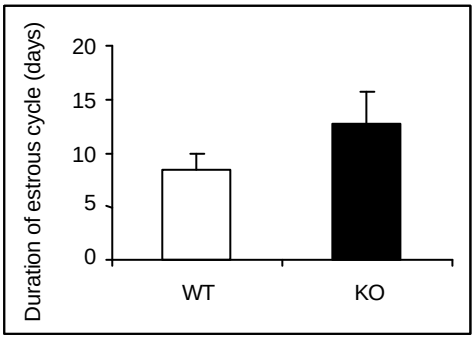

Figure 3.20: Estrous cycle duration in control and NIRKO females. Control indicated as "WT" female (open bar) and NIRKO indicated as "KO" (closed bar). Data represent the mean ± SEM of at least 6 animals of each genotype.

3.7 Hypothalamic control of anterior pituitary function in NIRKO mice

Since reduced fertility in NIRKO mice was associated with less sperm count in males, morphological abnormality of gonads in both males and females, and delayed onset of puberty in females, we investigated the hypothalamic-pituitary-gonadal axis by determining the concentrations of luteinizing hormone, growth hormone, adrenocorticotropic hormone, prolactin, and thyroid stimulating hormone.

3.7.1 Plasma luteinizing hormone (LH) concentration

Since both the histology of reproductive organs and the slightly delayed onset of puberty in NIRKO mice suggested dysregulation of gonadotropin secretion in the presence of isolated insulin resistance in the central nervous system, we decided to directly assess circulating plasma concentrations of luteinizing hormone (LH) in NIRKO and control (IR Lox/Lox, Cre-; IR Lox/+, Cre -; and IR +/+, Cre – or +) mice. LH is responsible for the stimulation of ovarian follicle growth and ovulation in females whereas in males it stimulates the testes to produce spermatozoa and male sex hormones (Patton *et* al., 1989). Plasma LH concentrations were determined by radioimmunoassay in serum samples obtained from 6 to 7 months old mice. This assay showed a 60% reduction of basal LH-concentrations in NIRKO males as compared to control male mice ($p < 0.05$) and a 90% reduction in NIRKO females ($p < 0.01$) compared to their controls (Fig. 3.21).

To further characterise the defect in gonadotropin secretion, we next determined plasma LH concentrations one hour after intraperitoneal injection of lupron (200 µg/animal), a gonadotropin releasing hormone (GnRH) agonist. In this assay male NIRKO mice exhibited an increase in circulating LH concentration comparable to that of their control littermates. Female NIRKO mice exhibited even higher concentrations of circulating LH after lupron stimulation compared to their controls (Fig 3.21 B) ($p < 0.01$).

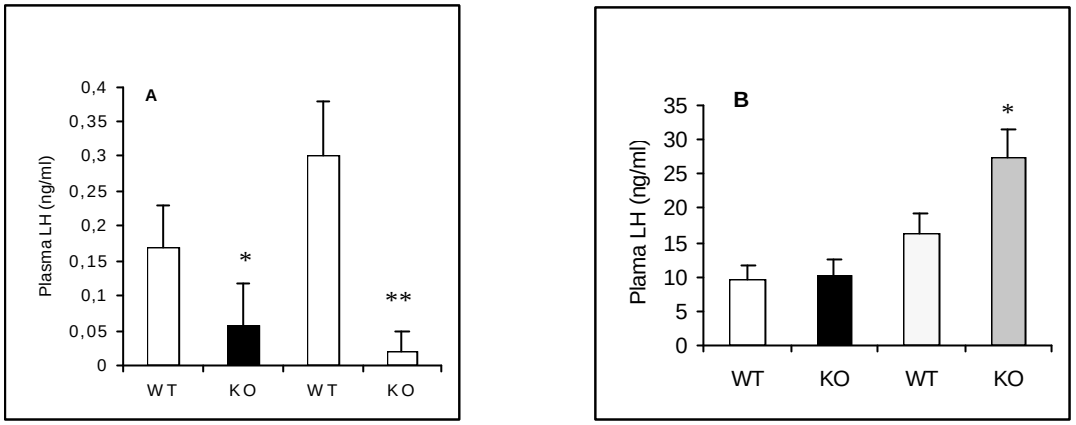

Figure 3.21: Plasma LH concentration (**A**), basal and (**B**), one hour after intraperitoneal injection of lupron (a GnRH agonist). Control indicated as "WT" male (open bar), NIRKO indicated as "KO" male (closed bar), control indicated as "WT" female (dotted bar) and NIRKO indicated as "KO" female (crossed bar). Plasma LH concentration was determined by radioimmunoassay. Control animals were IR Lox/Lox, Cre -; IR Lox/+, Cre -, and IR +/+, Cre – or +. Data represent the mean ± SEM from 6 to 7 months old mice of at least 8 animals of each genotype and gender (* = $p < 0.05$; ** = $p < 0.005$).

These data indicate that reduced concentrations of gonadotropins in NIRKO mice result from hypothalamic dysregulation of GnRH secretion rather than from reduced pituitary LH production. Indeed further immunohistochemical analysis of anterior pituitary sections revealed unaltered LH expression in the pituitary of NIRKO mice as compared to control animals (data not shown).

3.7.2 Plasma adrenocorticotropic hormone (ACTH) concentrations

To test whether NIRKO mice exhibit an isolated defect in hypothalamic gonadotropin regulation or combined defects in the regulation of other anterior pituitary axes, we determined plasma ACTH concentrations in NIRKO and control mice. As ACTH concentrations are strongly influenced by stress (Leal *et al*., 1999; Bulygina *et al*., 2002; Dal-Zotto *et al*., 2002), each animal was housed individually for seven days. Animals were rapidly killed by cervical dislocation and blood was collected from the jugular vein. Plasma ACTH concentration was determined in serum samples of 6 to 7 months old NIRKO and control (IR Lox/Lox, Cre -; IR Lox/+, Cre -; and IR +/+, Cre – or +) mice by radioimmunoassay. This analysis revealed indistinguishable basal plasma ACTH concentrations in both male and female NIRKO animals compared to their controls (Fig. 3.22).

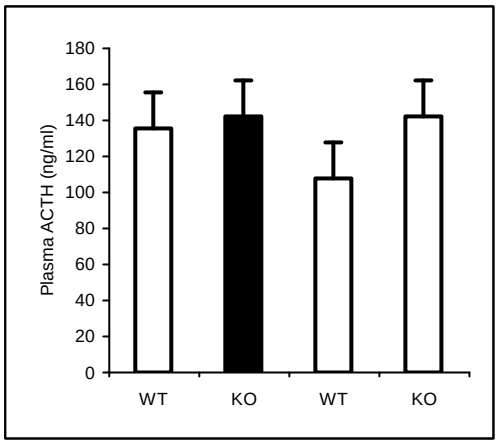

Figure 3.22: Adrenocorticotropic hormone (ACTH) concentrations in NIRKO and control mice: Control indicated as "WT", male (open bar), NIRKO indicated as "KO" male (closed bar), control indicated as "WT" female (dotted bar) and NIRKO indicated as "KO" female (crossed bar). Plasma ACTH concentration was determined by radioimmunoassay. Control animals were IR Lox/Lox, Cre -; IR Lox/+, Cre -, and IR +/+, Cre – or +. Data represent the mean ± SEM of at least 8 animals of each genotype and gender of 6 to 7 months old animals.

To further characterise the endocrine response to stress in NIRKO and control (IR Lox/Lox, Cre -; IR Lox/+, Cre -, and IR +/+, Cre – or +) mice, blood glucose concentrations under restrain stress in NIRKO and control mice were determined. Therefore animals were immobilised and blood glucose concentrations were determined immediately before and 15, 30 and 60 minutes after intraperitoneal glucose injection under restrain condition. This analysis revealed that NIRKO mice exhibited a significantly reduced stress-induced increase in blood glucose concentrations as compared to their controls (Fig. 3.23), indicating that the endocrine response to stress was impaired despite unaltered basal plasma ACTH-concentrations.

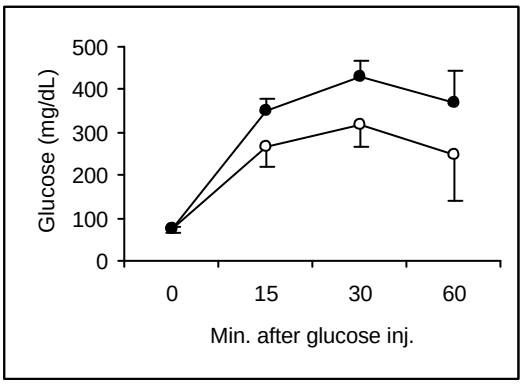

Figure3.23: Stress induced glucose tolerance test in NIRKO and control (IR Lox/Lox, Cre -; IR Lox/+, Cre -, and IR +/+, Cre – or +) mice. NIRKO (open circle) and control (closed circle). Data represent the mean ± SEM of at least 15 animals in each group and are 8 to 9 months old. Blood glucose levels were determined with an automatic blood glucose reader (Glucometer elite sensor, Bayer) in blood obtained from the tail tip before intraperitoneal (IP) injection of glucose (2 mg/g body weight) indicated as 0 min and 15, 30, and 60 min after IP injection.

3.7.3 Unaltered plasma prolactin concentrations in NIRKO mice

To further assess anterior pituitary function in the absence of neuronal insulin signaling, we determined plasma prolactin (prl)-concentrations in NIRKO and control (IR Lox/Lox, Cre -; IR Lox/+, Cre -, and IR +/+, Cre – or +) mice. This analysis revealed that as previously described female mice exhibited significantly higher plasma prl-concentrations as compared to male mice, but there was no difference detectable between NIRKO and control mice, indicating that the dopaminergic regulation of prl-secretion was unaltered in the absence of neuronal insulin receptor signaling (Fig. 3.24).

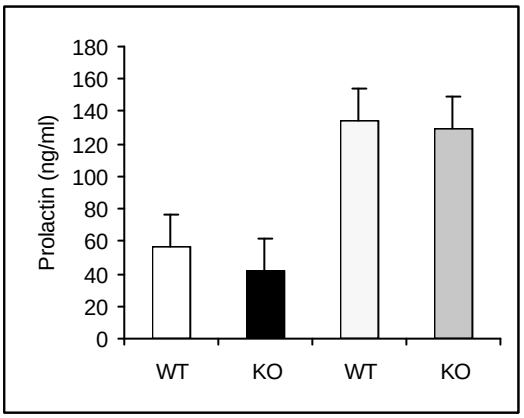

Figure 3.24: Plasma prolactin concentrations in control indicated as "WT" male (open bar), NIRKO indicated as "KO" male (closed bar), control indicated as "WT" female (dotted bar) and NIRKO indicated as "KO" female (crossed bar). Plasma prolactin concentration was estimated by radioimmunoassay. Control animals were IR Lox/Lox, Cre -; IR Lox/+, Cre -, and IR +/+, Cre – or +. Data represent the mean ± SEM of at least 15 animals of each genotype and gender at the age of 6 to 7 months.

Similar to the normal regulation of prolactin secretion, basal growth hormone concentrations were unaltered in NIRKO mice when measured by radioimmunoassay (data not shown).

3.7.4 Unaltered thyroid regulation in NIRKO mice

Since thyroid hormone represents an important factor in the regulation of energy homeostasis (Ingram, *et al*., 1986; Silva, 2001) and given both the obese and hypogonadal phenotype of NIRKO mice, we investigated the hypothalamic/pituitary regulation of thyroid function in NIRKO and control (IR Lox/Lox, Cre -; IR Lox/+, Cre -, and IR +/+, Cre – or +) mice. Therefore, plasma concentrations of thyroid stimulating hormone (TSH) were determined by radioimmunoassay before and 30 minutes after intraperitoneal injection of TSH releasing

hormone (TRH) (0.250 µg/animal) as shown in Fig. 3.25. Both basal TSH concentrations and those after TRH-stimulation were indistinguishable between control and NIRKO mice (Fig. 3.25), indicating an intact regulation of thyroid function in NIRKO animals.

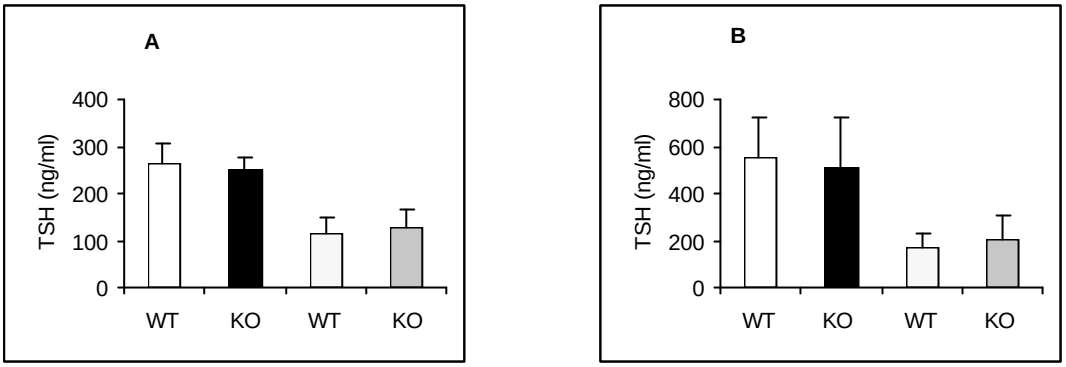

Figure 3.25: Thyroid stimulating hormone (TSH) concentration: (**A**), basal TSH levels and (**B**), TSH levels 30 minutes after TRH injection. Control indicated as "WT" male (open bar), NIRKO indicated as "KO"male (closed bar), control indicated as "WT" female (dotted bar) and NIRKO indicated as "KO" female (crossed bar) mice. Plasma TSH concentration was determined by radioimmunoassay. Control animals were IR Lox/Lox, Cre -; IR Lox/+, Cre -, and IR +/+, Cre – or +. Data represent the mean ± SEM of at least 15 animals of each genotype and gender.

3. 8 Analysis of the interaction of hypogonadism, obesity and insulin action

Since NIRKO mice exhibited both a hypogonadal and mildly obese phenotype our next experiments aimed at the identification of the interaction of these phenotypes. We therefore established a mouse model of defined hypogonadism by using ovarectomised female C57Bl/6J mice at the age of 8 weeks (May and Baker, Denmark). We decided to compare these mice to two different control groups, one being only sham-operated and another being ovarectomised but implanted with pellets releasing estradiol for 90 days to achieve serum estradiol concentrations of 100 pg/ml in the high physiological range.

3.8.1 Ovarectomy results in increased body weight

When the body weight of the three different groups of mice was monitored, there was no significant difference between the animals of the different experimental group at the age of 9 weeks, i.e. one week after surgery. By contrast, at the age of 11 weeks, ovarectomised mice started to show significantly increased body weight as compared to both control groups,. and this phenotype further aggravated with increasing age. Therefore, ovarectomised mice exhibited a 10% increase in body weight at the age of 17 weeks (Fig. 3.26). In conclusion,

isolated hypogonadism results in an increased body weight comparable to that seen in female NIRKO mice.

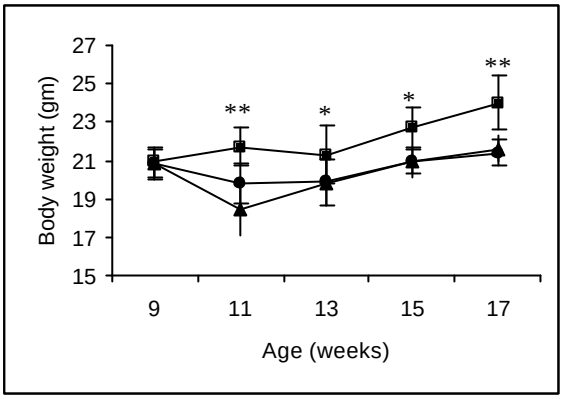

Figure3.26: Body weight of ovarectomised females (closed square), ovarectomised females implanted with estradiol-releasing pellets (closed triangle) and sham-operated females (closed circle). Surgery was performed at the age of 8 weeks. Data represent the mean ± SEM of at least 12 animals in each group (* = $p < 0.05$; ** = $p < 0.005$).

3.8.2 Ovarectomy causes obesity

To determine whether the increase in body weight seen in the ovarectomised females was indeed the consequence of increased obesity, we determined the weight of white adipose tissue depots in the different groups of animals. Therefore, mice were sacrificed at the age of 12 weeks and parametrial fat was removed and weighed (Fig. 3.27). This analysis revealed an approximately 2-fold increased white adipose tissue mass in the ovarectomised mice compared to sham-operated controls. Estradiol-replaced ovarectomised mice exhibited a reduced fat mass compared to the controls.

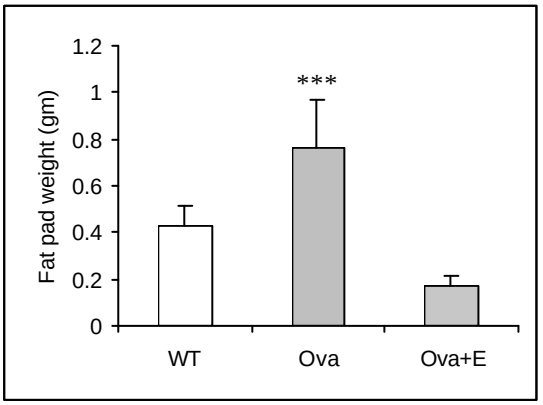

Figure 3.27: Parametrial adipose tissue mass in sham-operated indicated as "WT" (open bar), ovarectomised ("Ova", hatched bar) and ovarectomised females implanted with estradiol-releasing pellets ("Ova+E", crossed bar). Data represent the mean ± SEM of at least 12 animals in each group (*** = $p < 0.005$).

These data indicate that the increased body weight in the ovarectomised mice was indeed due to increased obesity.

3.8.3 Elevated plasma leptin concentrations in ovarectomised mice

To further confirm that the observed obesity is a consequence of hypogonadism, we determined the circulating plasma leptin concentrations in the different groups of mice by ELISA as described in materials and methods. Consistent with the increase in white adipose tissue mass, ovarectomised animals exhibited a 3-fold increase in circulating plasma leptin concentrations (** = $p<0.005$) compared to both control groups (Fig. 3.28).

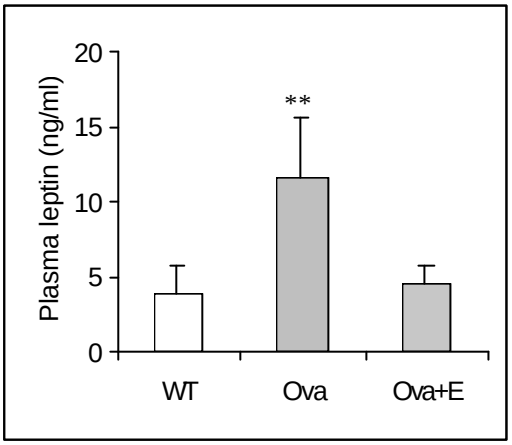

Figure 3.28: Plasma leptin concentrations in sham operated ("WT", open bar), ovarectomised ("Ova", hatched bar) and ovarectomised, estradiol-replaced mice ("Ova+E" crossed bar) was determined by ELISA. Data represent the mean ± SEM of at least 12 animals in each group (** = $p<0.005$).

3.8.4 Hypogonadism causes increased food intake

As obesity can result from either increased food intake or reduced energy expenditure, we determined the amount of food consumed by ovarectomised animals and controls over a period of one week. In these experiments ovarectomised mice exhibited a 10% higher food intake compared to sham-operated or ovarectomised mice under estradiol replacement (Fig. 3.29). In conclusion, hypogonadism in female mice resulted in mild obesity, at least in part due to an increased food intake.

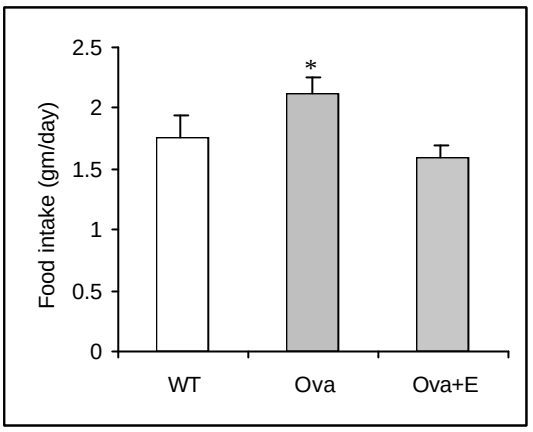

Figure 3.29: Food intake in sham-operated ("WT", open bar), ovarectomised ("Ova", hatched bar) and ovarectomised, estradiol-replaced mice ("Ova+E", crossed bar). Data represent the mean ± SEM of at least 10 animals in each group (* = $p < 0.05$).

3.8.5 Ovarectomised mice remain euglycemic

To determine, whether the obese phenotype of ovarectomised female mice has an effect on glucose metabolism, we first determined the blood glucose concentrations in the different groups of mice with an automated glucose reader in a small drop of tail tip blood. The ovarectomised animals exhibited ~10% higher blood glucose levels than their controls (sham-operated and ovarectomised mice under estradiol-replacement). But this difference did not reach statistical significance (Fig. 3.30).

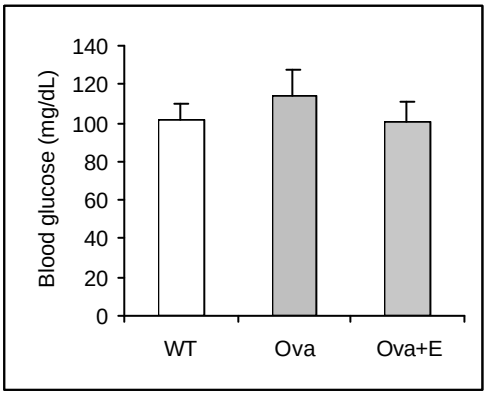

Figure 3.30: Blood glucose levels in sham-operated ("WT", open bar), ovarectomised ("Ova", hatched bar) and ovarectomised, estradiol-replaced mice ("Ova+E", crossed bar) mice were determined by automatic blood glucose reader (Glucometer elite, Bayer) in a drop of tail tip blood. Data represent the mean ± SEM of at least 10 animals of each group.

3.8.6 Unaltered plasma insulin concentrations in ovarectomised mice

To determine whether the slight elevation of blood glucose concentrations in the fed state in ovarectomised mice (Fig. 3.30) resulted from systemic insulin resistance, we also determined plasma insulin concentrations in the different groups of animals by ELISA as described in materials and methods. Again, as for the blood glucose concentrations, there was a trend towards higher plasma insulin concentrations in ovarectomised females compared to both control groups, but this difference did not reach statistical significance (Fig. 3.31).

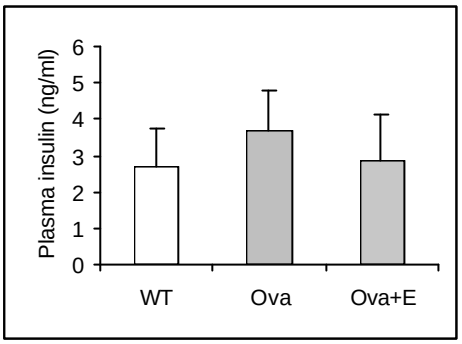

Figure 3.31: Plasma insulin levels in sham-operated ("WT", open bar), ovarectomised ("Ova", hatched bar) and ovarectomised mice under estradiol replacement ("Ova+E", crossed bar). Plasma insulin concentration was determined in serum samples by ELISA. Data represent the mean ± SEM of at least 8 animals in each group.

3.8.7 Unaltered insulin tolerance in ovarectomised females

To further analyse whether estradiol-deficiency may lead to insulin resistance, we investigated the blood glucose lowering effect of intraperitoneally administered insulin (0.75 IU/Kg body weight), following an overnight fasting period. Blood glucose levels were determined with an automatic blood glucose reader (Glucometer elite sensor, Bayer) in tail tip blood before insulin injection (0 min.) and 15, 30 and 60 min after insulin injection. These experiments revealed a similar drop of blood glucose concentrations in response to exogenously administered insulin in all three groups of experimental animals, indicating the absence of significant insulin resistance as a consequence of hypogonadism (Fig. 3.32).

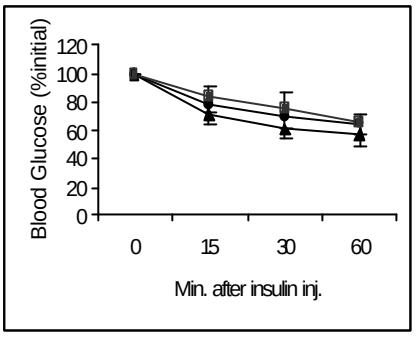

Figure 3.32: Insulin tolerance test in ovarectomised animals: ovarectomised females (closed triangle) and sham-operated females (closed circle), estradiol-replaced females (closed square). Animals were injected intraperitoneally insulin (0.75 IU/Kg body weight), and blood glucose level was determined before injection indicating as 0 min, and 15, 30 and 60 min after insulin injection. The initial values at 0 min (blood glucose level 75 to 84 mg/dL) are set arbitrarily as 100%. Data represent the mean ± SEM of at least 10 animals in each group.

3.8.8 Ovarectomised mice exhibit mildly impaired glucose tolerance

Since dysregulation of glucose homeostasis can result both from impaired insulin action, i.e. insulin resistance, and impaired insulin secretion (Cretti *et al*., 2001; Meyer *et al*., 2002;), we tested the ability of ovarectomised animals to metabolise an intraperitoneal challenge of glucose. Animals were injected with glucose (2 mg/g body weight) intraperitoneally, and blood glucose was measured before injection (0 min) and 15, 30, 60, and 120 min after injection. Blood glucose was assayed with an automatic glucose reader in blood obtained from the tail tip. This analysis revealed that ovarectomised mice exhibited a statistically significant slower decrease in blood glucose concentrations at 30 and 60 minutes time point after intraperitoneal application of glucose (Fig. 3.33) compared to both control groups, indicating that ovarectomy resulted not only in mild obesity but also in impaired glucose metabolism, most likely due to impaired insulin secretion.

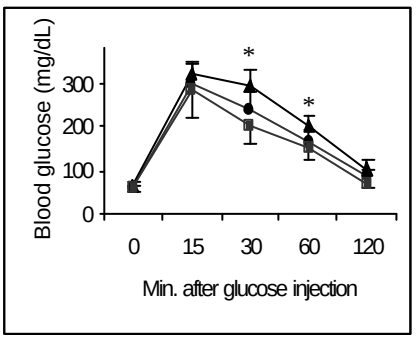

Figure 3.33: Glucose tolerance test in ovarectomised females (closed triangle), sham-operated females (closed circle) and ovarectomised, estradiol-replaced females (closed square). Blood glucose was determined before (0 min.) and after (15, 30, 60 and 120 min) intraperitoneal injection of glucose with an automatic glucose reader (Glucometer elite sensor, Bayer) in blood from tail tips. Data represent the mean ± SEM of at least 15 animals in each group (* = $p < 0.05$).

3.8.9 Analysis of insulin secretion

To evaluate the effect of ovarectomy on the pancreatic ß-cell function, insulin release was measured in response to intraperitoneal application of glucose. To determine the insulin release upon glucose stimulation, animals received an intraperitoneal injection of glucose, (3 mg/g body weight) following an overnight fasting period. Then blood was collected from the tail vein after 2, 5, 15 and 30 minutes. Plasma insulin concentrations were determined by ELISA. While in sham-operated mice a 1-fold and in ovarectomised estradiol-replaced females a 1.5-fold increase in insulin secretion was observed at 2 minutes after glucose injection, there was no significant increase of insulin release detectable in ovarectomised mice at this time point. By contrast, plasma insulin concentrations 30 minutes after glucose injection were indistinguishable between the different groups of mice, indicating that the slow, second phase of glucose-stimulated insulin secretion was intact in the ovarectomised animals (Fig. 3.34).

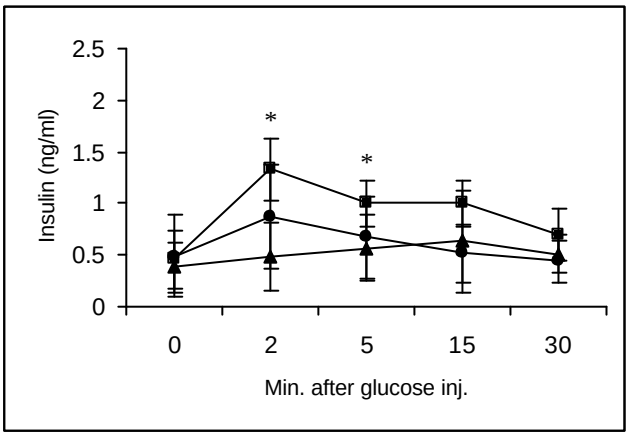

Figure 3.34: Glucose-stimulated insulin secretion in ovarectomised females (closed triangle), sham-operated females (closed circle) and ovarectomised with estradiol-replaced females (closed square). Serum insulin concentrations were determined by ELISA. A statistically significant difference was observed for ovarectomised females compared to ovarectomised estradiol-replaced females at the 2 and 5 minute time intervals. Data represent the mean ± SEM of at least 10 animals in each group (* = $p < 0.05$).

In summary, these data indicate that ovarectomy resulted in a mildly obese phenotype, at least in part due to hyperphagia, a phenotype comparable to that seen in NIRKO-female mice. By contrast, hypogonadism did not result in significant insulin resistance but rather in impaired glucose-stimulated insulin secretion.

3.9 Behavioural analysis of NIRKO mice

3.9.1 Morris water maze test (Morris, 1984)

Since insulin signalling in the central nervous system has been implicated in the regulation of learning and memory (Zhao *et al*., 1999), NIRKO mice represent an excellent model to study the role of IR in this process *in vivo.* Therefore, we analysed the learning capabilities of NIRKO mice in a Morris Water Maze test. Female and male controls (IR Lox/Lox, Cre -; IR Lox/+, Cre -, and IR +/+, Cre – or +) and NIRKO mice aged 4 to 6 months were selected for this study.

Each animal was put into a water filled tub and allowed to swim for 90 seconds to locate and escape to a submerged, hidden platform. The test was performed on 5 consecutive days and both the time spent and distance swom to the platform were recorded. This analysis revealed that escape latencies did not differ for control and NIRKO mice (Fig. 3.35 A).

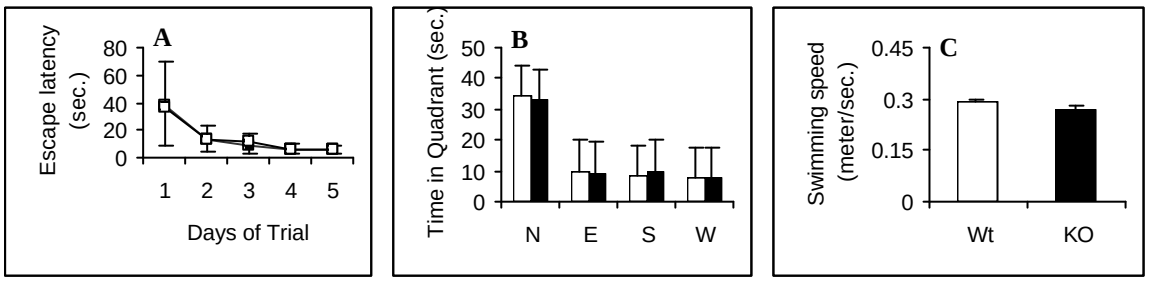

Figure 3.35 A: Escape latency of mice in the Morris water maze test: Control (closed square) and NIRKO (open square). **B:** Time spent in different pool quadrants during the probe trial in the Morris water maze test: Control (open bar) and NIRKO (closed bar); N=north quadrant; E=east quadrant; S=south quadrant and W=west quadrant. **C:** Swimming speed of NIRKO and control mice in the Morris Water Maze test: Control ("WT", open bar) and NIRKO ("KO", closed bar). Controls were IR Lox/Lox, Cre -; IR Lox/+, Cre -, and IR +/+, Cre – or +. Data represent the mean ± SEM of at least 12 animals of each group.

During the 5 day training period, the hidden platform was kept to the north quadrant. After 5 days, a probe trial was performed, in which the platform was removed from the pool. Again, mice were allowed to swim for 90 sec and the time spent in different quadrants of the pool was recorded. Both NIRKO mice and controls performed similarly in the probe trial, spending significantly more time in the north quadrant as compared to the others (Fig. 3.35 B).

These data indicated, that despite severe insulin resistance in the central nervous system, NIRKO mice did not exhibit any severe impairment in learning in this test paradigm.

Moreover, the Morris Water Maze test serves to evaluate the motoric capabilities of NIRKO and control mice. Therefore, the swimming speed was calculated from the time spent and the distance swom to escape to the platform. This analysis revealed that swimming capabilities were not significantly different in NIRKO and control animals (Fig. 3.35 C).

In the course of these different behavioural experiments it became obvious, that the animals within each group exhibited a large variation in terms of their performance. Since neuropsychological performance in mice strongly depends on the genetic background strain, and the NIRKO mouse colony was first kept on a mixed C57Bl/6J/129sv background, we decided to backcross these mice on a C57Bl/6J background, in order to better be able to detect differences in learning and memory capabilities, potentially masked by the intergroup variation. Therefore, NIRKO mice were backcrossed for 6 generations on a C57Bl/6J background, resulting in >95% pure genetic background.

The Morris Water Maze test was then repeated on these animals. This analysis revealed, that by backcrossing, the large intra-group variation was virtually abolished, but that also under these conditions NIRKO and control mice performed similarly (Fig.3.35 D).

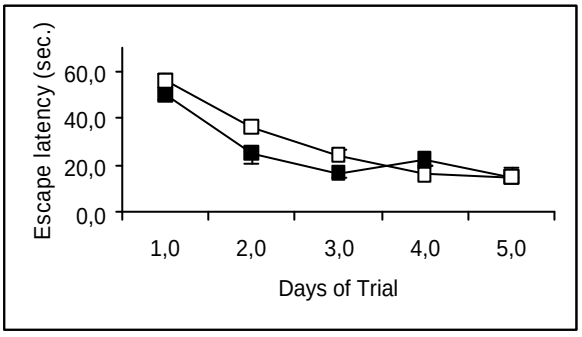

Fig. 3.35 D: Morris Water Maze test with NIRKO and control mice after backcrossing to the C57Bl/6J background. Control mice (closed square) and NIRKO mice (open squares). During 5 consecutive days of trial, the escape latency of NIRKO was reduced and is comparable with their control littermates. Controls were IR Lox/Lox, Cre -; IR Lox/+, Cre -, and IR +/+, Cre – or +. Data represent the mean ± SEM of at least 8 animals of each group.

3.9.2 Anxiety tests in NIRKO and control mice

The implication of anxiety tests was to investigate the role of IR signalling in the central nervous system in the regulation of mechanisms underlying anxiety such as generalised anxiety, panic, phobia and posttraumatic disorders.

3.9.2.1 Open field test (Corman *et al*., 1967; Barclay *et al*., 1982)

Anxiety, phobia and motoric activity of mice can be determined in the open field test. 4 to 6 months old animals (male and female) were selected for this study. They were allowed to move in a Perspex box for 300 seconds, and their movements to the centre, to the corners and along the wall were recorded. Indicating the shelter provided by corners and walls, both NIRKO and control mice spent the majority of the test period in these regions when exposed to this new environment, showing an indistinguishable performance between the two groups of mice (Fig. 3.36).

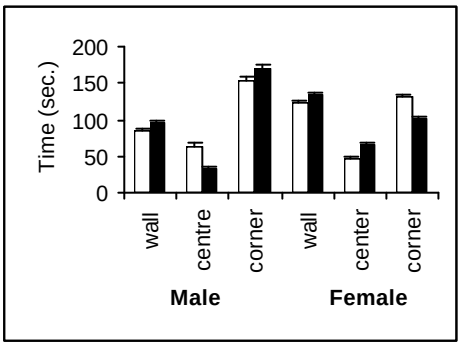

Figure 3.36: Open field test: Control (open bar) and NIRKO mice (closed bar). Open field test was performed according to the standard experimental procedures. In this test each animal was allowed to perform 5 consecutive days and their movements to the wall, centre and corner were recorded in a computer. Controls were IR Lox/Lox, Cre -; IR Lox/+, Cre -, and IR +/+, Cre – or +. Data represent the mean ± SEM of at least 24 animals of each group.

3.9.2.2 Elevated plus maze test (Pellow *et al*., 1985 and 1986)

Another paradigm aimed to assess anxiety-related behaviour is the elevated plus maze test. 4 to 6 months old animals were selected for this study.

In this test, mice can freely choose to walk on a wall-sheltered arm and an unsheltered arm, while the maze is elevated 50 cm above the ground. Mice were allowed to spend 300 sec on this maze and their time spent in the open arm and closed arm was recorded. Both NIRKO

and control (IR Lox/Lox, Cre -; IR Lox/+, Cre -, and IR +/+, Cre – or +) mice spent significantly more time in the closed arm and there was no statistically significant difference observed among these groups (Fig. 3.37).

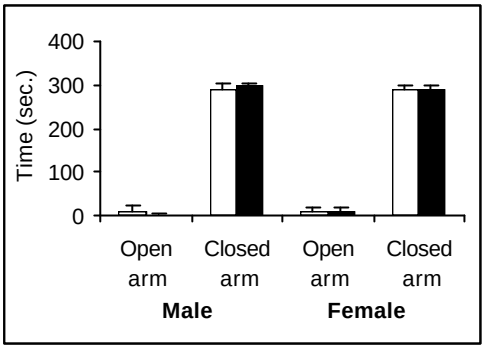

Figure 3.37: Elevated plus maze test: Control (open bars) and NIRKO (closed bars). The elevated plus maze test was performed according to the standard experimental procedures. In this test animals were allowed to perform 5 consecutive days and their movement to the open arm and closed arm was recorded in computer. Control indicates IR Lox/Lox, Cre -; IR Lox/+, Cre -, and IR +/+, Cre – or +. Data represent the mean ± SEM of at least 24 animals of each group.

Consistent with the results obtained in the open field test (Fig. 3.36), the elevated plus maze test did not provide any evidence for altered anxiety-related behaviour in the absence of neuronal insulin receptor signalling.

3.9.2.3 Light and dark exploration test (Ambrogi *et al*., 1984)

To further test behavioural performance, the light and dark exploration test was performed. For this test a box containing a light and a dark compartment was used, the two compartments connected by a small tunnel. The mice were placed in the dark compartment of the test box and both the time of first crossing into the light compartment and the total time spent in each compartment were recorded, each mouse being allowed to explore the test area for 300 seconds. Again consistent with the performance in the open field and elevated plus maze test, there was no significantly different performance detectable between NIRKO and control (IR Lox/Lox, Cre -; IR Lox/+, Cre -, and IR +/+, Cre – or +) mice (Fig. 3.38)

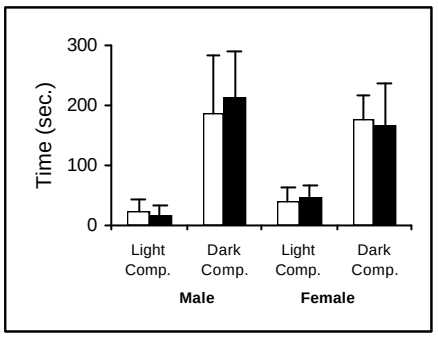

Figure 3.38: Light and dark exploration test: Control (open bars) and NIRKO (closed bars) mice Control animals indicates IR Lox/Lox, Cre -; IR Lox/+, Cre -, and IR +/+, Cre – or +. In this test animal was allowed to perform 5 consecutive days and their movement to the light and dark side of the box was recorded. Controls were IR Lox/Lox, Cre -; IR Lox/+, Cre -, and IR +/+, Cre – or +. Data represent the mean ± SEM of at least 24 animals of each group.

In summary, NIRKO mice did not exhibit any abnormalities in these different anxiety tests. Therefore, neuronal IR signalling appears not to be involved in the formation of behavioural paradigms related to exploration and anxiety.

3.9.3 Olfactory test

Lack of sense of smell will interfere with behaviour of the mice with respect to many behavioural structures such as feeding, social interaction, aggressive encounters, sexual behaviours, and parental behaviours. We therefore tested the impact of lacking neuronal IR-signalling on the ability of mice to locate a hidden cheese (smell stimulus) over a 300 second test period. The time required to locate the cheese (olfactory stimulus) was recorded. Both NIRKO and control (IR Lox/Lox, Cre -; IR Lox/+, Cre -, and IR +/+, Cre – or +) mice exhibited indistinguishable latency periods in locating the olfactory stimulus, indicating that neuronal IR-signalling is dispensable for normal olfactory function (Fig. 3.39).

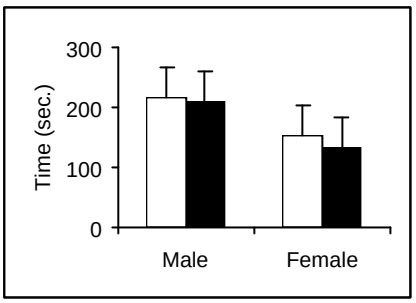

Figure 3.39: Olfactory test: Control (open bars) and NIRKO (closed bars). Mice are allowed to locate hidden cheese inside the cage. In this test animal was allowed to perform once and the time needed to locate the hidden cheese was recorded. Control indicates IR Lox/Lox, Cre -; IR Lox/+, Cre -, and IR +/+, Cre – or +. Data represent the mean ± SEM of at least 12 animals of each group.

3.10 Role of IR expression in the regulation of neuronal development and survival

3.10.1 Brain weight

Since *in vitro* studies have implicated a role for IR signalling in neuronal differentiation and survival (Yamada *et al.*, 1997; Nakamura *et al.*, 2001), we analysed the weight of brains from NIRKO and control (IR Lox/Lox, Cre -; IR Lox/+, Cre -, and IR +/+, Cre – or +) mice. In addition, possible morphological alterations were assessed by histological analysis of brains sections. The first analysis revealed that brain weights of NIRKO and control mice did not exhibit any statistically significant difference (Fig. 3.40), indicating that neuronal IR signalling has no profound impact on brain development in 6 to 8 months old male and female mice.

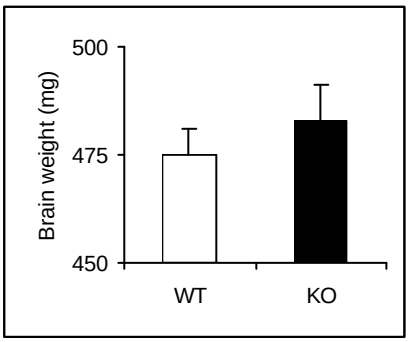

Figure 3.40: Brain weight of control ("WT", open bar) and NIRKO ("KO", closed bar) mice. Data represent the mean SEM ± of at least 20 animals of each genotype. Control animals were IR Lox/Lox, Cre -; IR Lox/+, Cre -, and IR +/+, Cre – or +.

Moreover, histological examination of sections prepared from NIRKO and control brains did not reveal any gross structural alterations due to impaired IR signalling (data not shown). These results indicate that neuronal IRs appear not to be a major factor in normal brain development.

3.10.2 Apoptosis in cultured neurons from NIRKO and control mice

In order to investigate the role of neuronal IR signalling in the regulation of apoptosis, we analysed the ability of insulin and IGF-I to prevent neuronal apoptosis in cultured cerebellar granular cells of NIRKO and control mice. 10^5 cerebellar neuronal cells were plated in 96 wells cell culture plate coated with poly-L-lysine. After 5 days of culture the cerebellar neuronal cells were potassium-depleted for 24 hours to induce apoptosis, with or without addition of insulin or IGF-I. The percentage of apoptotic cells was assessed by Hoechst Dye staining of condensed nuclei. Fig. 3.41 shows a representative example for one of these experiments. Potassium withdrawal leading to membrane depolarisation resulted in profound induction of apoptosis (fig. 3.41; left upper and lower panel), which could be prevented by incubation with increasing doses of IGF-I in case of control mice (fig. 3.41; lower right panel) and also with insulin (fig. 3.41, lower middle panel), while insulin failed to inhibit apoptosis in the neurons cultured from NIRKO mice (fig 3.41; upper middle panel).

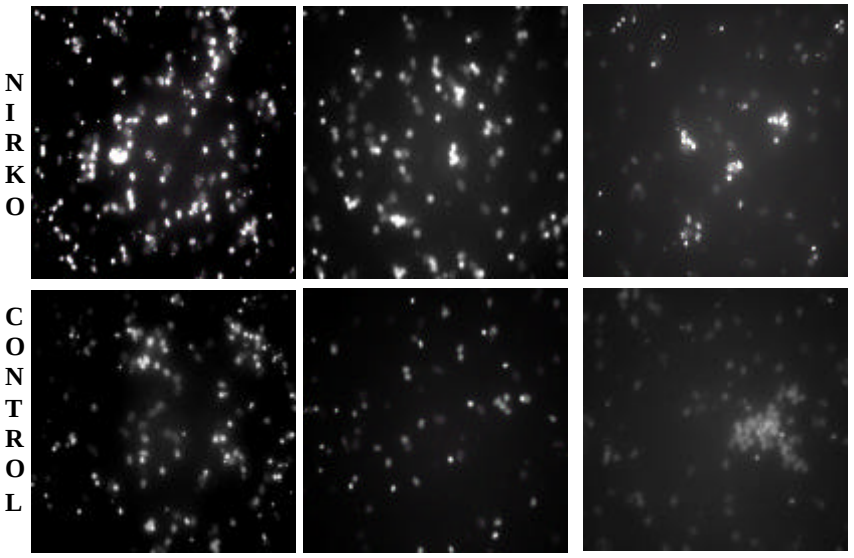

Figure 3.41: Primarily cultured cerebellar granular cells of 5-day old NIRKO (upper panel) and control (lower panel) mice were subjected to low KCl-containing (5mM) medium to induce apoptosis (left lane); cells were incubated (24 hours) with 100nM insulin (middle panel) and with 100nM IGF-I (right panel) and condensed nuclei were stained with Hoechst Dye.

To analyse more detailed the ability of insulin and IGF-I to inhibit apoptosis in cultured neurons, we performed similar experiments with increasing doses of insulin and IGF-I. While in control neurons insulin was able to inhibit apoptosis in a dose-dependent fashion from 77% of apoptotic cells to 50% at a concentration of 10 nM and 35% at 100 nM, insulin was not able to inhibit apoptosis in neurons cultured from NIRKO mice (Fig. 3.42 A). Performing the same type of experiments with increasing doses of IGF-I revealed two major findings. First, in cells obtained from control mice, IGF-I was clearly more potent to inhibit apoptosis than insulin. The percentage of apoptotic cells was reduced to 15% at 100 nM IGF-I (Fig. 3.42 B). Surprisingly, although also neurons cultured from NIRKO mice exhibited a strong response to the anti-apoptotic effect of IGF-I compared to cells from control mice, there was a shift in the dose response to the right (Fig 3.42 B).

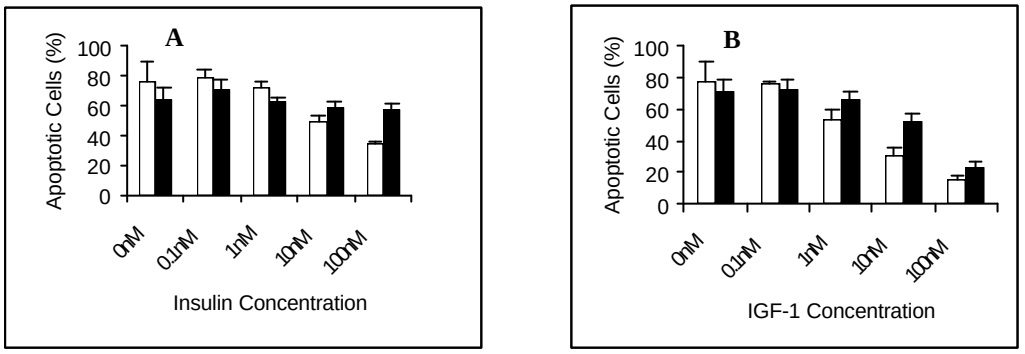

Figure 3.42: Anti-apoptotic effect of insulin and IGF-I on primary cerebellar granular cells. Control (open bars) and NIRKO mice (closed bars). Cerebellar granular neuronals cells were prepared from 5 day old pups from NIRKO and control (IR Lox/Lox, Cre -) mice individually. 10^5 cells per well were plated on 96 well plates coated with poly-L-lysine, and incubated for 5 days in 37 ^{0}C incubator with 5% CO_2. To induce apoptosis, on day 6 cells were grown for 24 hours in KCl-depleted (5mM) medium. To save them from apoptosis, cells were stimulated with 0nM, 0.1nM, 1.0nM, 10nM and 100nM insulin (**A**) or IGF-I (**B**). Cells were fixed in 4% paraformaldehyde and stained with Hoechst dye. Cells from each well were then counted under a fluorescent microscope. Percentages of live and dead cells were assessed from the total cell count. Control indicates neuronal cells from IR Lox/Lox, Cre – pups. Data represent the mean ± SEM of al least 8 animals of each genotype.

To further investigate the molecular mechanisms involved in the anti-apoptotic effect of insulin and IGF-I, we determined the activation of the serine-kinase Akt. In overexpression studies Akt has been demonstrated to be able to inhibit apoptosis, thus representing an important downstream target of the PI 3-kinase pathway following insulin and IGF-I-stimulation (Kuemmerle *et al.*, 1998; Wymann *et al.*, 1998; Imai *et al.*, 1999). Akt itself is activated through serine/threonine-phosphorylation by phospholipid-dependent kinases (PDK) (Bertrand *et al.*, 1999). Phosphorylation of Akt was determined upon insulin and IGF-I

stimulation in neurons cultured from NIRKO and control (IR Lox/Lox with Cre -) mice. For this study cerebellar granular cells were prepared from 5 day old pups from NIRKO and control mice. 3 x 10^6 cells per well were grown for five days in 6-well-plates coated with poly-L-lysine. On day six, cells were stimulated for 10 min with 10 nM insulin or IGF-I and harvested as described above. Proteins were resolved on an 8% polyacrylamide gel under reducing conditions and transferred onto a nitrocellulose membrane. The membrane was the probed with a pAkt specific antibody. This analysis revealed that despite insulin fails to inhibit apoptosis in primary neuronal cells of NIRKO mice, it still can induce Akt phosphorylation although to a lesser extent than IGF-I both in neurons from control and NIRKO mice (Fig. 3.44).

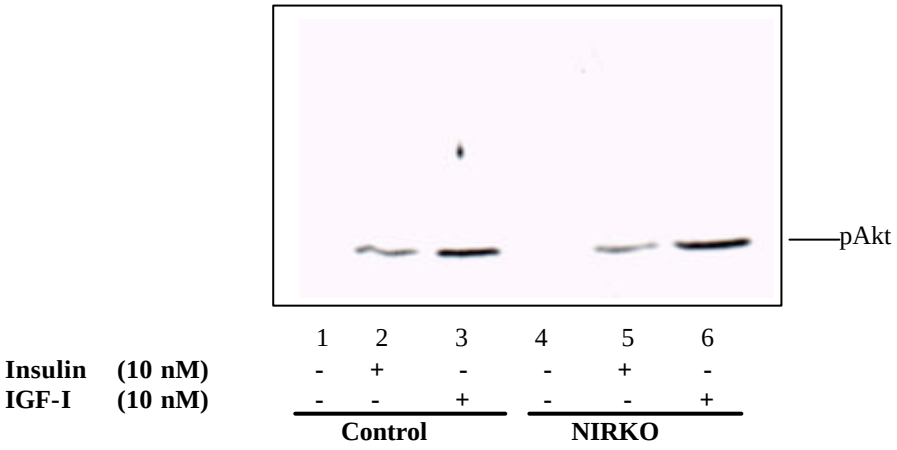

Figure 3.44: Immunoblot analysis of Akt phosphorylation in primary neuronal cell culture following stimulation with insulin or IGF-I (10nM) for 10 min. Cerebellar granular cells from 5-day-old control (IR Lox/Lox Cre -) (lane 1,2,and 3) and NIRKO (lane 4,5 and 6) mice were processed and cultured as described previously. After an overnight starving period in serum-free medium, cells were either left untreated (lane 1 and 4) or stimulated with insulin (lane 2 and 5) or IGF-I (lane 3 and 6). Total protein extracts (50 µg/lane) were resolved on an 8% SDS-polyacrylamide gel under reducing conditions and were transferred onto a nitrocellulose membrane. The resulting blot was probed with pAkt specific antiserum (1:200). Anti rabbit IgG (1:1000) was used as secondary antibodie. Bound antibodies were detected using the ECL system. Data represent the mean ± SEM of al least 6 animals of each genotype.

These data indicate that Akt phosphorylation activated by insulin stimulation even in the absence of IR expression is not sufficient to mediate the anti-apoptotic effect of insulin. Neurons cultured from NIRKO mice therefore represent a useful model to further investigate the exact molecular mechanism of IR mediated inhibition of apoptosis.

3.10.3 Unaltered IGF-I receptor expression in cultured neurons from NIRKO mice

To further investigate whether the deletion of the insulin receptor in the brain of NIRKO mouse leads to alterations in the expression of the closely related IGF-I receptor, we determined the expression of this receptor in neurons cultured from NIRKO and control mice by Western Blot analysis as described in detail in material and methods. As shown in Fig. 3.43 deletion of IR in NIRKO mice did not result in any changes, as for example compensatory upregulation of IGF-I receptor expression.

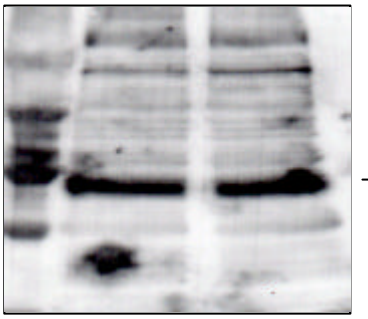

Figure 3.43: Immunoblot analysis of IGF-I Receptor expression in cultured neurons from control (IR Lox/Lox Cre -) and NIRKO pups. Cerebellar granular cells were prepared from 5 day old pups and cells were grown in polylysine-L coated plates for 5 days before protein extraction. Total protein extracts (50 µg/lane) were resolved on an 8% SDS-polyacrilamide gel under reducing conditions and were transferred onto a nitrocellulose membrane. The resulting blot was probed with IGF-1R-ß specific antiserum (1:200). Anti rabbit IgG (1:1000) was used as secondary antibody. Bound antibodies were detected unsing the ECL system. Data represent the mean ± SEM of al least 6 animals of each genotype.

Therefore, cultured neurons from NIRKO mice represent a useful tool to analyse insulin and IGF-I-mediated signalling in the absence of insulin receptors and without the occurrence of compensatory changes in IGF-I-receptor expression.

4. Discussion

Insulin receptors has been reported to be widely expressed in the different areas of the CNS with variable densities such as olfactory bulb, cerebral cortex, hypothalamus, hippocampus, cerebellum and choroids plexus (Baskin *et al*., 1988; LeRoith *et al*., 1988; Adamo *et al*., 1989; Marks *et al*., 1991; Unger *et al*., 1991; Zhao *et al*., 1999). Although subject to research for more than 20 years, the functional role of IR expressed in those areas remained largely unclear.

The first important issue regarding insulin action in the CNS is the question how insulin can cross the blood brain barrier to act on neuronal insulin receptors. There have been several reports demonstrating an active transport mechanism for insulin across the blood brain barrier, which can be inhibited by glucocorticoids (Baura *et al.*, 1993; Baura *et al.*, 1996), on the other hand there has been evidence for insulin production directly in the central nervous system (Schechter *et al.*, 2001). Regardless of the mechanism it appears possible that insulin signaling can occur in the central nervous system under physiological conditions. Since it has been stated that neuronal insulin receptors might participate in the transport of insulin across the blood brain barrier, future experiments using the generated mouse lines with insulin receptor ablation in the CNS (NIRKO mice) will provide an excellent tool to analyse the importance of IR action in this mechanism, by determining insulin concentrations in the cerebral spinal fluid of NIRKO and control mice.

The research on the role of IR expressed in the CNS has been directed by different hypotheses. First, insulin is the key metabolic hormone regulating postprandial glucose disposition and its concentrations rise quickly after ingestion of a carbohydrate-containing meal (Porksen, 2002). Therefore, insulin represents a prime candidate as a negative feedback regulator for food intake, by acting on hypothalamic centers regulating feeding behaviour. Along this line the expression pattern of insulin receptors in the CNS with high levels of expression in the hypothalamus has further prompted this hypothesis (Marks *et al.*, 1990). The hypothesis of insulin acting as a negative feedback mechanism on the regulation of food intake was then further emphasized when Schwartz *et al*. (1992) could demonstrate that intracerebroventricular injection of insulin can acutely inhibit food intake.

Although glucose uptake in the central nervous system has been classically viewed as being insulin-independent, over the last years components of the insulin sensitive glucose transport

signaling system, such as the Glut4-transporter have been identified in the CNS (Alquier *et al*., 2001).

Given the importance for insulin in the regulation of glucose uptake in adipose tissue and skeletal muscle, this has revived research investigating a possible role for neuronal insulin receptors in the regulation of brain glucose metabolism. Therefore, studies have been performed on the expression of insulin signaling proteins in states with impaired brain glucose metabolism, i.e. in brains from patients suffering from neurodegenerative diseases such as Alzheimer's and Parkinson's disease. Indeed these studies revealed reduced expression of insulin receptors under these conditions, leaving the question open, whether this plays any causal role in the development of these diseases (Craft *et al*., 1998). Moreover, further studies in rats could demonstrate the activation of the insulin-signaling cascade upon learning (Zhoa *et al*., 1999). Furthermore, mainly studies on cultured neurons have shown that insulin and IGF-I can promote neuronal differentiation and survival *in vitro* (Aizeman *et al*., 1987; Svrzic *et al*., 1990; Fernyhough *et al*., 1993).

Taken together, there has been emerging evidence that insulin, acting through the insulin receptor in the central nervous system might play an important role in the regulation of essential functions like food intake, obesity, and learning and memory in mammals (Zhoa *et al*., 1999; Schwartz, 2000), but definite proof for its role in the physiological context has been missing. Therefore, the generation and characterisation of mice lacking functional IR expression in the CNS provided an excellent model to test these hypotheses.

4.1 Generation of NIRKO mice

Generation of mice with disruption of the insulin receptor in the central nervous system was achieved by the use of Cre-LoxP-mediated recombination in mice (Gu *et al*., 1994). Mice carrying a "floxed" allele of the IR gene, which had been previously demonstrated to lack functional IR expression upon Cre-mediated deletion (Brüning *et al*., 1998) were mated to mice expressing Cre-recombinase under control of the rat nestin promoter and enhancer (Dahlstrand, 1995). Nestin is a 38 kDa intermediate filament protein specifically expressed in neuroepithelial stem cells (Lendahl *et al*., 1990). Immunoblot analysis using a polyclonal antiserum against the IR-ß subunit showed a > 95% reduction of IR expression in whole brain

extracts obtained from NIRKO animals compared to their controls. Since the CNS represents a complex organ harbouring a variety of different cell types, the question remained, whether the residual expression of IR in the whole brain extracts did arise from cell types different from neuroepithelial stem cells, or represented inefficient deletion in neuroepithelial stem cells. Further experiments therefore focused on the establishment of an immunohistochemistry protocol to detect IR expression *in situ* in brain sections prepared from NIRKO and control mice. Unfortunately, these experiments did not allow a conclusive answer to this question. Although a variety of different anti-IR antibodies have been tested for this purpose, no specific signal for IR expressed in the central nervous system could be achieved. These findings are consistent with the notion, that insulin receptors are expressed at relatively low abundance in the CNS, and that previous studies from other groups yielded conflicting data describing the sites of IR expression in the CNS (Baskin *et al*., 1988; LeRoith *et al*., 1988; Adamo *et al*., 1989; Marks *et al*., 1991; Unger *et al*., 1991; Zhao *et al*., 1999). Moreover, previous studies lacked the negative control as exemplified by NIRKO mice and might therefore have also reported unspecific staining.

To circumvent this problem and to further define the efficiency of IR deletion in neurons of NIRKO mice, we analysed the expression of IR in cultured neurons, i.e. cerebellar granular cells from NIRKO and control mice. The principle of the culture protocol is based on the fact that postnatal neurons can still be cultured, but are postmitotic and can therefore be separated from non-neuronal cells by treatment with the cell cycle-dependent cytostatic agent cytosine arabinoside (AraC), which only affects dividing cells (Oorschot and Jones, 1986). Following this protocol it was possible to obtain a highly enriched neuronal culture from these animals allowing us to detect complete recombination of the floxed IR allele and abolished IR expression as assessed by PCR and Western blot analysis on cultured neuronal cells obtained from NIRKO mice. These findings further support that the inactivation of the IR gene in NIRKO mice appeared to be rather complete in neuroepithelial stem cell derived populations. Along this line it is worth noting that the same line of Cre-transgenic mice has been used to achieve efficient inactivation of the glucocorticoid receptor gene in the CNS of mice (Tronche *et al*., 1999).

In conclusion, the data obtained in our experiments show that inactivation of the IR gene in the CNS of NIRKO mice occurred with high efficiency in neuroepithelial stem cell derived cell populations.

4.2 Obesity in NIRKO mice

The results reported in this study provide for the first time direct evidence for a role of insulin receptors in the regulation of energy homeostasis in a physiological context. Three lines of evidence confirmed the mild obese phenotype of NIRKO mice:

The increased body weight, consistent with an increase in directly assessed white adipose tissue mass, and corresponding to elevated plasma leptin concentrations - a hormone previously shown to correlate well with the fat content of an organism (Shimizu *et al*., 1997). Therefore, we conclude that chronic insulin resistance in the central nervous system indeed can predispose for development of obesity. Nevertheless, the relevance of insulin in the regulation of body weight has to be put into context with other hormones regulating energy homeostasis. It had been demonstrated that after intracerebroventricular injection both leptin and insulin inhibited food intake by regulating the same neuronal populations in the arcuate nucleus of the hypothalamus (Schwartz *et al.*, 1992). Comparing the phenotype of genetically leptin-resistant animals, the ob/ob-mouse, which exhibits a morbidly obese phenotype (Coleman *et al.*, 1973), the obesity detectable in NIRKO mice is relatively mild.

These data indicate that leptin in comparison to insulin could be a much more important factor in the regulation of energy homeostasis. Alternatively, this discrepancy might be explained by the existence of alternative mediators for insulin action in the CNS, i.e. IGF-I receptors. This is further supported by a recent study of Baudry *et al* (2001), in the absence of IR in the muscle cells lead to metabolic effects through the IGF-I receptor with an efficiency that is comparable to that of IR. Interestingly, IGF-IR expression assessed by westernblot analysis on cultured neuronal cells obtained from NIRKO and control mice exhibited equal expression, so any potential compensatory effect mediated through IGF-I receptors does not seem to occur via an upregulation of receptor expression. Therefore, ongoing studies aim to analyse the potential compensatory role for IGF-IR in NIRKO mice by the creation of animals with CNS specific deletion of both the IR and IGF-IR.

Energy homeostasis results from the balance of energy intake and energy expenditure. In the present study only one side of this equilibrium has been analysed. Namely, we could detect an increased food intake, at least in female NIRKO mice as a result of CNS-specific insulin resistance. Hence, it will be important to also determine the energy expenditure in NIRKO

mice by indirect calorimetrie. Indeed, there has been evidence for insulin to regulate sympathetic activity in the CNS, a fact, which certainly needs further proof in NIRKO mice (Ruggeri *et al.*, 2001).

Regarding the increased food intake of NIRKO mice, further studies have provided some evidence for the signaling events involved in insulin-stimulated regulation of feeding behaviour of these animals. In the arcuate nucleus of the hypothalamus, both insulin and leptin regulate expression of orexigenic (NPY and AGRP), and anorexigenic (POMC and CART) peptides in hyperthalamic neurons. It has been observed that following the intracerebroventricular injection of leptin, NPY/AGRP-expressing neurons are inhibited (Satoh *et al*., 1997) and similarly, insulin also inhibited expression of NPY (Sipols *et al*., 1995). Further studies from our laboratory have revealed, that in NIRKO mice under steady state conditions, NPY expression is elevated ~2-fold in NIRKO mice as compared to control animals (J. Gillette, Klinik und Poliklinik für Innere Medizin, personal communication). Although intracerebroventricular injection studies had suggested a negative regulatory function of insulin on the expression of POMC, expression of this peptide-precursor was found unaltered in the NIRKO mice (J. Gillette, Klinik und Poliklinik für Innere Medizin, personal communication). Since also leptin regulates POMC expression and leptin levels are elevated in the NIRKO mice, and we have demonstrated that NIRKO mice are still leptin-sensitive *in vivo*, it appears likely that leptin can compensate for the lack of IR signaling in this pathway or alternatively, that leptin under physiological conditions is the major regulator of this pathway.

Regardless of the molecular mechanisms of cerebral insulin resistance leading to obesity, our data support a unifying hypothesis that insulin resistance also in the central nervous system might be responsible for the close association of obesity and diabetes mellitus type 2 (De Fronzo *et al.*, 1991).

4.3 Hypothalamic hypogonadism in NIRKO mice

Beside the mild obesity, NIRKO mice exhibited reduced fertility due to hypothalamic hypogonadism. This finding can be linked to the evolutionary conservation of insulin signaling. Orthologues of the IR have been identified in *C.elegans*, and *Drosophila* (Fig. 4.1).

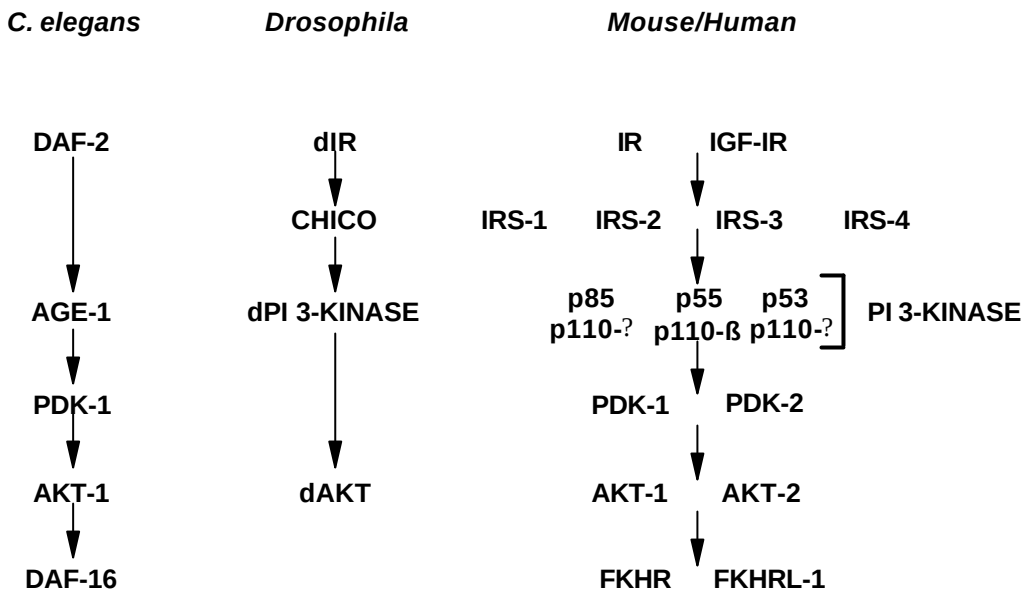

Fig. 4.1: Insulin receptor orthologues in *C.elegans* and *Drosophila melanogaster* and related signaling pathways (Ogg *et al*., 1997; Tissenbaum *et al*, 1998; Taha *et al*, 1999).

Interestingly, an inactivating mutation of the *C.elegans* orthologue DAF-2 results in transition of the organism to the Dauer-stage, representing reduced fertility (Kenyon *et al.,* 1993; Kimura *et al.*, 1997; Thissenbaum *et al.*, 1998). This phenotype is associated with increased lipid storage and longevity (Kimura *et al.,* 1997). Similarly, in *Drosophila* at least in female flies, both single mutations of the insulin receptor orthologue and the insulin receptor substrate orthologue CHICO result in reduced fertility, increased lipid storage and longevity (Clancy *et al.,* 2001; Tatar *et al.,* 2001). While ubiquitous reexpression of DAF-2 in DAF-2-mutant *C.elegans* restores these defects, transgenic rescue of DAF-2 selectively in skeletal muscle fails to do so (Wolkow *et al.,* 2000). Interestingly, selective expression of DAF-1 in the central nervous system is also able to rescue the DAF-1-mutant phenotype in *C.elegans* (Wolkow *et al.,* 2000).

In conclusion, the phenotype observed in NIRKO mice points to an evolutionary conserved signaling pathway regulating fertility and full homeostasis and possibly longevity. Therefore, ongoing studies aim to determine life span of NIRKO mice, to test this tempting hypothesis.

Another striking similarity has been described in mice with conventional targeted disruption of the IRS-2 gene. Matings between $IRS2^{-/-}$ males and females did not result in any pregnancy

and IRS2$^{-/-}$ males exhibited reduced fertility (Burks *et al*., 2000). Like in IRS2$^{-/-}$ females, the histological section of NIRKO females also revealed a reduced number of antral follicles and corpora lutea, indicating that NIRKO mice had insufficient gonadotropin input for the proper maintenance of ovarian follicle maturation. Likewise, in male NIRKO and IRS-2-deficient mice, insufficient gonadotropin results in impaired Leyding cell function as well as spermatogenesis (Burks *et al*., 2000).

Reduced gonadotropin secretion can result from defects on different levels of gonadotropin release. The neurosecretory neurons present in the arcuate nucleus of hypothalamus produce the peptide hormone gonadotropin-releasing hormone (GnRH). GnRH is transported to the anterior pituitary through a capillary network and acts on the gonadotropin-secreting cells to stimulate the production and release of two glycoproteins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Fig. 4.2). These gonadotropins are secreted into the blood stream to be transported to the gonads (Patton *et al*., 1989).

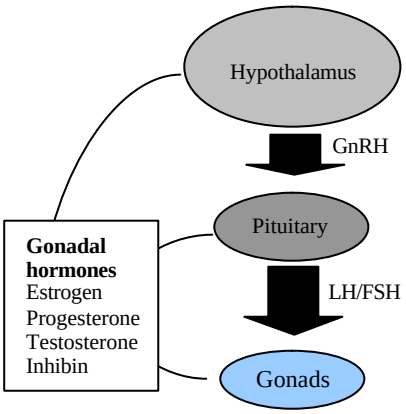

Fig. 4.2: Schematic survey of gonadotropin regulation

In the female, gonadotropin stimulates the growth of ovarian follicles, induces ovulation, and initiate the formation of the corpus luteum. The gonadotropins also stimulate the production of estrogens, progestins, and inhibin. Similarly, in case of the male, LH and FSH stimulate the testis to produce spermatozoa and male sex hormones, including testosterone and inhibin. The sex hormones play a role for the maturation and transport of sperm. Those sex hormones prime the reproductive tract both in female and male, and act as feedback signals to the brain and pituitary axis (Patton *et al*., 1989).

Our further experiments revealed that LH-expression in the anterior pituitary of NIRKO mice as it is in the pituitary of IRS-2-deficient mice was unaltered. Moreover, although basal levels of LH were low both in male and female NIRKO mice, exogenous GnRH was able to stimulate LH secretion in NIRKO mice, again indicating that the pituitary content of LH was unaltered, locating the defect to the hypothalamus. Therefore, we postulate that insulin receptors, likely through recruitment of IRS-2 regulate GnRH-secretion. To date we cannot determine exactly which ligand is responsible for the activation of IR, but it has been demonstrated in cultured hypothalamic neurons, that insulin like growth factor II, a high affinity ligand for the IR, can stimulate GnRH-release (Soldani *et al.,* 1994, Olson *et al.,* 1995). Moreover, proper gonadotropin release requires an exact pattern of pulsatile GnRH-release (Vaquez-Martinez *et al.,* 2001). Therefore, it might be possible that IR mediated signals regulate pulsatility of GnRH-secretion. Unfortunately, this hypothesis is difficult to test directly, since the relatively high sample volume required for LH-detection does not allow serial determinations in the mouse. Interestingly, it could be demonstrated, that GnRH pulsatility is associated with pulsatility in body temperature, and it might therefore be feasible to monitor the oscillation of body temperature in NIRKO and control mice, to obtain an indirect assessment of gonadotropin regulation (H. Jarry, Division of Clinical Endocrinology, Department of Obstetrics and Gynecology, University of Göttingen, Germany; personal communication).

Since hypothalamic hypogonadism occurred in NIRKO mice and hypothalamic dysregulation often result in combined defects of other anterior pituitary axes, we also tested the regulation of ACTH, prolactin, growth hormone and TSH in the NIRKO mice and found them unaltered in these animals. These data suggest, that insulin receptors mediate specifically signals in GnRH-expressing neurons of the hypothalamus.

4.4 Obesity and impaired glucose homeostasis as a consequence of hypogonadism

Since NIRKO mice exhibited a combination of both obesity and hypothalamic hypogonadism, further experiments aimed to identify the link between these two phenotypical changes. Therefore, we investigated the impact of defined hypogonadism in ovarectomised female mice on the regulation of energy homeostasis and glucose metabolism. These experiments revealed that ovarectomised animals exhibited an increase in body weight, white adipose tissue mass and circulating plasma leptin concentrations. Moreover, food intake was

significantly increased. In terms of glucose metabolism, ovarectomy resulted in impaired glucose tolerance and impairment of glucose stimulated insulin secretion. These phenotypical alterations were corrected by estradiol substitution in one group of ovarectomised mice, indicating the specificity of the observed phenotype was due to a lack of estradiol.

These findings are consistent with previous experiments analysing mice with targeted disruption of the aromatase gene, the enzyme required for estradiol-biosynthesis. Both male and female aromatase-deficient mice develop a phenotype of mild obesity and mild impairment of blood glucose metabolism (Jones *et al*., 2000). This phenotype resembles the metabolic profile of mice lacking the estrogen receptor (ER)-a, which also exhibit increased obesity (Ohlsson *et al*., 2000), indicating that estradiol's metabolic effects are mediated through this receptor-isoform. Although Jones *et al*. (2000) describe an impairment of glucose tolerance in aromatase-deficient mice, they do not provide further evidence, whether this results from impaired insulin action, i.e. insulin resistance, or impaired glucose-stimulated insulin secretion. There have been conflicting data with respect to estradiol's effect on insulin action and secretion. Whereas earlier studies concluded that transdermal estrogen therapy in postmenopausal women is associated with a slight, but significant improvement of insulin action and lipid metabolism, recent studies have indicated no alterations in insulin sensitivity after estrogen replacement, while a very recent study even indicates a deterioration of insulin sensitivity following estrogen replacement of postmenopausal women, even when women are of comparable total and abdominal adiposity (Lindheim *et al*., 1994; Duncan *et al*., 1999; Ryan *et al*., 2002). On the other hand, few studies have investigated the effect of hormone replacement of postmenopausal women on insulin secretion, paradoxically showing an impairment of insulin secretion after hormone replacement (Godsland *et al*., 1993).

There is a clear lack of studies investigating the regulation of insulin action by estradiol on a molecular level. Our *in vivo* data indicate that estradiol-deficiency does not result in insulin resistance. Along this line analysis of insulin action in our different experimental groups of mice revealed unaltered expression of IRs and unaltered insulin-stimulated IR-tyrosine phosphorylation, and activation of the PI 3-kinase effector Akt in liver and skeletal muscle in ovarectomised mice (data not shown). Given our results, the impairment of glucose homeostasis in response to ovarectomy appears to be a consequence of alterations in glucose-stimulated insulin secretion. The analysis of the molecular mechanisms for this phenomenon clearly requires further studies.

With respect to the phenotype of NIRKO mice, ovarectomised mice exhibit striking similarities in terms of the development of obesity. Therefore, it cannot be ruled out that the obese phenotype of NIRKO mice – at least in part – results from hypogonadism. On the other hand, hypogonadism in NIRKO mice is only partial, as indicated by the presence of estrous phases in the analysis of vaginal cytology of female NIRKO mice. Therefore, it appears rather unlikely that the metabolic phenotype of NIRKO mice strictly results from hypothalamic hypogonadism. Further studies using subregion-specific disruption of IR-signaling in the central nervous system will therefore try to dissect the role of hypothalamic hypogonadism and the metabolic alterations seen in NIRKO mice.

4.5 Unaltered behavioural performance in NIRKO mice

Another challenging hypothesis for the initiation of our experiments was that IR could be responsible for learning and memory through the regulation of cerebral glucose metabolism (Mayer *et al*., 1990). Moreover, the relatively high density of IR present in hippocampus and parts of the cerebral cortex, brain regions responsible for learning and memory formation, had given rise to speculations in terms of IR regulating this processes (Wickelgren, 1998; Zhao *et al*., 1999). It had also been demonstrated that IR downregulation is associated with neurodegenerative disorders like Alzheimer's disease (AD) (Frolich *et al*., 1998) and Parkinson's disease (PD) (Takahasi *et al*., 1996) and that administration of insulin in AD patients resulted in memory improvement (Craft *et al*., 1995; 1999). Animal studies suggested that learning and memory could be impaired following blockade of neuronal IR function via intracerebroventricular injection of streptozotocin (Biessels *et al*., 1996; Lannert *et al*., 1998). Moreover, recent studies could demonstrate that during hyperinsulinemic, euglycemic clamp conditions memory improved in healthy volunteers (Kern *et al*., 2001).

Therefore, careful analysis of neuropsychological performance of NIRKO mice provides an excellent model to address directly the role of IR mediated signals in the regulation of these processes. Our data revealed unaltered performance of NIRKO mice in the Morris water maze task for learning and memory, as well as different paradigms of anxiety tests. Since the initial tests were performed with mice on a mixed genetic background, and recent work has pointed out extreme variations in neuropsychological performance between different mouse strains

(Owen *et al.,* 1997), we generated NIRKO mice on a >95% pure C57Bl/6J-background by backcrossing them for six generations. But also on this background, there was no significant difference detectable between NIRKO and control mice in the Morris water maze task, although the intra-group variation decreased significantly in the backcross population. These data clearly indicate, that although numerous lines of evidence had suggested a causal role for insulin resistance in the development of memory deficits, CNS-specific disruption of the IR gene in mice has no effect on learning. These findings support the importance of defined transgenic animal models to test hypotheses generated from indirect studies.

5. Summary

To study the role of the insulin receptors expressed in the central nervous system (CNS), we have generated and analysed mice with CNS restricted inactivation of the insulin receptor gene using Cre/loxP-mediated recombination *in vivo*. We could demonstrate that:

1. By crossing mice carrying a loxP-flanked exon 4 of the IR gene with mice expressing Cre-recombinase under control of the rat nestin promoter efficient inactivation of the IR-gene could be achieved in the CNS,
2. NIRKO mice develop a mildly obese phenotype, which is pronounced in female mice and exaggerated under exposure to high caloric diet,
3. Obesity in female NIRKO mice results from hyperphagia, and
4. NIRKO mice exhibit a hypothalamic hypogonadism.
5. Ovarectomy in female mice results in a similar phenotype than that of female NIRKO mice, indicating that part of the metabolic phenotype may result from hypogonadism,
6. NIRKO mice exhibit unaltered neuropsychological performance.

Taken together, these results reveal a novel role for insulin receptors in the central nervous system in the regulation of hypothalamic control of reproduction and energy homeostasis. On the other hand these experiments could rule out a causative role for isolated neuronal insulin resistance in the CNS as a cause for diseases associated with memory deficiencies, such as Alzheimer's disease. Moreover, the NIRKO mice created during the course of these experiments provide an excellent model to further characterise the function of IR-mediated signals in the regulation of brain glucose metabolism *in vivo*.

6. Zusammenfassung

Um die Rolle des Insulinrezeptors (IR) im zentralen Nervensystem (ZNS) zu untersuchen wurden im Rahmen der vorliegenden Arbeit Mäuse geschaffen und analysiert, in denen der Insulinrezeptor mit Hilfe der Cre/loxP-vermittelten Rekombination gezielt im zentralen Nervensystem inaktiviert wurde. Es konnte gezeigt werden, daß:

1. durch Kreuzung von Mäusen, die ein loxP-flankiertes Exon 4 des IR-Gens tragen, mit Mäusen, die die Cre-Rekombinase unter der Kontrolle des Nestin-Promotors der Ratte exprimieren, eine effiziente Inaktivierung des IR-Gens im ZNS erreicht werden konnte,
2. NIRKO-Mäuse einen leicht adipösen Phänotyp entwickeln, der in Weibchen besonders ausgeprägt ist und durch Gabe einer hochkalorischen Diät verstärkt wird,
3. die Adipositas in weiblichen NIRKO-Mäusen durch verstärkte Futteraufnahme zustande kommt,
4. NIRKO-Mäuse einen hypothalamischen Hypogonadismus aufweisen.
5. Ovarektomie in weiblichen Mäusen in einem den NIRKO-Weibchen ähnlichem Phänotyp resultiert, was darauf hindeutet, daß ein Teil des metabolischen Phänotyps vom Hypogonadismus herrührt,
6. NIRKO-Mäuse unveränderte neuropsychologische Leistungen zeigen.

Zusammengenommen bedeuten diese Ergebnisse eine neue Rolle von zentralnervösen Insulinrezeptoren bei der Regulation der hypothalamischen Kontrolle von Reproduktion und Energiehomöostase. Darüberhinaus zeigen unsere Ergebnisse, dass einer isolierten neuronalen Insulinresistenz keine kausale Bedeutung in der Entstehung neurodegenerativer Erkrankungen zukommt.

Außerdem stellen die NIRKO-Mäuse, die im Verlauf dieser Arbeiten geschaffen wurden, ein exzellentes Modell dar, um die Funktion der IR-vermittelten Signaltransduktion bei der Regulation des Glukosestoffwechsels im Gehirn *in vivo* weite

7. Abstract

Analysis of insulin receptor function in the central nervous system by conditional inactivation of its gene in mice

Insulin receptors (IRs) and insulin signaling proteins are widely distributed throughout the central nervous system (CNS). To study the physiological role of insulin signaling in the brain, we created mice with a neuron-specific disruption of the IR gene (NIRKO mice). Inactivation of the IR had no impact on brain development or neuronal survival. However, female NIRKO mice showed increased food intake, and both male and female mice developed diet-sensitive obesity with increases in body fat and plasma leptin levels, mild insulin resistance, elevated plasma insulin levels, and hypertriglyceridemia. NIRKO mice also exhibited impaired spermatogenesis and ovarian follicle maturation because of hypothalamic dysregulation of luteinizing hormone. In vitro studies of apoptosis in pure neuronal cultures derived from NIRKO and control animals revealed a role of IR in insulin and IGF-I mediated prevention of apoptosis. Behavioural studies showed that disruption of IR in the CNS has no effect on learning and memory, and anxiety in our animal model.

Thus, IR signaling in the CNS plays an important role in regulation of energy disposal, fuel metabolism, and reproduction.

Analyse der Funktion des zentralnervösen Insulinrezeptors durch konditionelle Inaktivierung seines Gens in der Maus

Insulinrezeptoren (IR) und Insulin-Signalproteine sind im zentralen Nervensystem weit verbreitet. Um die physiologische Rolle der Insulin-vermittelten Signaltransduktion im Gehirn zu untersuchen, wurde unter Nutzung Cre-loxP-vermittelter Rekombination eine transgene Maus etabliert, deren IR-Gen Neuron-spezifisch ausgeschaltet wurde (NIRKO Maus). Die Inaktivierung des IR-Gens hatte keinen Einfluß auf die Entwicklung des Gehirns und das neuronale Überleben. Dagegen zeigten weibliche NIRKO Mäuse eine erhöhte Futteraufnahme, und sowohl männliche als auch weibliche Mäuse entwickelten eine ernährungsabhängige Adipositas mit erhöhtem Anteil an Körperfett und erhöhtem Leptin-Plasmaspiegel, sowie eine milde Insulinresistenz, erhöhte Insulin-Plasmaspiegel und eine Hypertriglyzeridämie. Außerdem zeigten NIRKO Mäuse eine gestörte Spermatogenese und Follikelreifung aufgrund einer hypothalamischen Dysregulation des Luteinisierenden Hormons. In vitro Apoptosestudien an kultivierten Neuronen aus NIRKO und Kontrolltieren zeigten, dass der Insulinrezeptor eine Rolle in der Insulin- und IGF-I-vermittelten Verhinderung von Apoptose spielt. Verhaltensstudien ergaben keinen Hinweis auf einen Einfluß des Insulinrezeptors auf Lernen, Gedächtnis und Angstverhalten in unserem Tiermodell.

Die vorliegenden Ergebnisse zeigen, dass Insulinrezeptor-vermittelte Signaltransduktion im ZNS eine wichtige Rolle bei der Regulation des Energiehaushalts und der Reproduktion spielt.

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Bruning, J.C., Gautam, D., Burks, D.J., Gillette, J., Schubert, M., Orban, P.C., Klein, R., Krone, W., Muller-Wieland, D., and Kahn, C.R. (2000). Role of brain insulin receptor in control of body weight and reproduction. Science, 289, 2122-2125.

Acknowledgement

I thank Prof. Dr. William Krone who gave me the opportunity to work in his department. It is my immense privilege to work with Dr. Jens C. Brüning whose insight and extraordinary supervision has led this project towards its goal. Without his support and supervision, it is next to impossible to bring this work in this stage. My deepest gratitude goes to him for all kind of supports he has provided during my stay in Germany.

I thank to PD. Dr. Roswitha Nischt for her valuable discussion and I am indebted to her for being a ex-reviewer of my thesis. Her valuable suggestion and keen interest in my work is highly appreciated. Because of some unavoidable circumstances Dr. Nischt was unable to act as reviewer in my thesis committee, and I also would like to thank her for organising Prof. Dr. Dagmar Mörsdorf in her place. I would like to express my sincere thanks to Prof. Dr. Dagmar Mörsdorf from MPI for accepting as a reviewer at the last moment to save me from this crisis. I would like to extend my thanks to Prof. Dr.Thomas Langer for providing his valuable time to go through my thesis and his kind acceptance to be a reviewer. I am indebted to Prof. Dr. Dietmar Schomburg, who has readily accepted to act as a co-examiner.

I would like to extend my thanks to Dr. Staphanie Baudler for her scientific help, discussion and tireless effort. I appericiate her friendly nature for making pleasant working environment in the lab. I thank to Jeniffer Gillette for her help during my study periods. My sincere thanks goes to Philipp Lingohr, Frank Michaelsen, Jens Alber, Mathias Röhl, Julia Baugartl and Andre Kleinridders for their accompany and help during my stay.

I particularly acknowledge the generous helps of Dr. Monzur Murshed, Dr. Markus Schubert, Dr. Marius Vantler, Olaf Holtkotter, Tina Grimm. I would like to thank Dr. Shiva C. Ghimire for his encouragement.

I would like to thank Prof. Dr. Durga Datta Dhakal, Dean, Institute of Agriculture and Animal Science for granting me study leave. My sincere thanks goes to Dr. I.P. Dhakal, Asst. Campus chief, Veterinary Science for his help. I also would like to extend my thanks to Co-Directors (Dr. H.A. Williams and Dr. P. Mainali) of EEC livestock project, Kathmandu and Director General (Dr. N.P.S. Karki) Dept of livestock services for their generous help.

Finally, I thank and express my heartfelt gratitude to my late father and his continuous encouragement for my study. I thank to my mother for her continuous help and support throughout my study periods. I also would like to express my sincere thanks to my parents in law Mr. Gauri Nath Rimal and Mrs. Sushama Rimal, and my brothers and sisters for their support and encouragement. And many many thanks to my wife Suman and my daughter Preetha for their incredible support, encouragement and taking full responsibility of home in Nepal and allowing me to concentrate on my work.

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Abbreviations

AGE-1	advanced-glycation end-product-1
AFX.	Forkhead associated protein family
BAD	Bcl associated protein D
BAX	Bcl-2 associated X protein
Bcl-2	Break point cluster region-2
Bcl-X_L	Bcl-2 associated X_L protein
BME	Basal medium eagle
CbC	Cerebellar culture
DAF-2	deauer formation - 2
dL	decciliter
DMEM	Dulbecco's modified eagle medium
EDTA	Etylene diamino tetra-acetic acid
FKHR	Forkhead related family proteins
FKHR-L	Forkhead associated protein L
FCS	fetal calf serum
GLUT	Glucose transporter
g	gram
HBSS	Hank's Balanced Salt solution
IU	international unit
kg	kilogram
lit	liter
M	Molar
mM	millimolar
MAP	mitogen-activated protein
MAPK	MAP kinase
MAPKK	MAP kinasse kinase
MAPKKK	MAP kinase kinase kinase
mg	milligram
min	minute
ml	milliliter
Nck	small adoptor protein with SH2 and SH3 binding domain
ng	nanogram

nM	nanomolar
O.D.	optical density
PBS	phosphate-buffered saline
PBS-T	PBS-tween
PCR	polymerase chain reaction
pg	picogram
PIP 5-kinase	phosphatidyl inositol phosphate kinase
pmol	picomolar
PTB	Phosphotyrosine binding
Rab	Ras like protein
RAF	serine/threonine protein kinase
RAS	Rat sarcoma
RTK	receptor tyrosine kinase
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacralamide gel electrophoresis
sec	Second
SH2	Src-homology 2 (domain)
SHC	SH2 containing protein
Src	raus sarcoma virus oncogene
TBE	Tris buffer EDTA
TBS	Tris buffer saline
TBS-T	TBS-Tween
Tris	Tris (hydroxy methyl) amino methan
v/v	volume/volume
v/w	volume/weight
WB	western blotting
µg	microgram
µl	micro liter
µm	micromolar

Role of Brain Insulin Receptor in Control of Body Weight and Reproduction

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Insulin receptors (IRs) and insulin signaling proteins are widely distributed throughout the central nervous system (CNS). To study the physiological role of insulin signaling in the brain, we created mice with a neuron-specific disruption of the IR gene (NIRKO mice). Inactivation of the IR had no impact on brain development or neuronal survival. However, female NIRKO mice showed increased food intake, and both male and female mice developed diet-sensitive obesity with increases in body fat and plasma leptin levels, mild insulin resistance, elevated plasma insulin levels, and hypertriglyceridemia. NIRKO mice also exhibited impaired spermatogenesis and ovarian follicle maturation because of hypothalamic dysregulation of luteinizing hormone. Thus, IR signaling in the CNS plays an important role in regulation of energy disposal, fuel metabolism, and reproduction.

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Insulin receptors (IR) are expressed in most tissues of the body, including classic insulin-sensitive tissues (liver, muscle, and fat), as well as "insulin-insensitive" tissue, such as red blood cells and the neuronal tissue of the CNS. In the CNS, the IR displays distinct patterns of expression in the olfactory bulb, the hypothalamus, and the pituitary (1-3), although its function in these regions remains largely unknown. Previous experiments have suggested a role for insulin signaling in the regulation of food intake (4, 5) and neuronal growth and differentiation (6, 7). Moreover, insulin has been shown to regulate neurotransmitter release and synaptic plasticity (8, 9), and dysregulation of insulin signaling in the CNS has been linked to the pathogenesis of neurodegenerative disorders such as Alzheimer's and Parkinson's disease (10, 11).

We have used the Cre-loxP system to generate mice with CNS-specific disruption of the IR gene (12-14). Mice carrying a "floxed" allele of the IR gene (IR-lox mice) were crossed with mice expressing the Cre-recombinase under control of the rat nestin promoter and enhancer. Nestin is an intermediate filament protein that is expressed in neuroepithelial stem cells (15, 16). The resultant brain-specific IR knockout (NIRKO) mice showed a >95% reduction in the level of brain IR protein (Fig. 1, A and B). In contrast, the abundance of other insulin signaling proteins, such as insulin receptor substrates-1 and -2 (IRS-1 and IRS-2), was unaltered in brain extracts of NIRKO mice (Fig. 1A). Inactivation of the IR gene was specific to the brain, as no change in IR expression was detectable in skeletal muscle, heart, liver, kidney, spleen, and gonads (Fig. 1C).

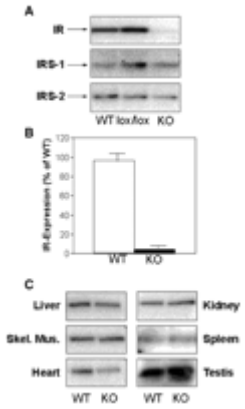

Fig. 1. Insulin receptor expression is specifically disrupted in the brain of NIRKO mice. (**A**) Brain extracts prepared from wild-type (WT), IRlox/lox, and NIRKO (KO) mice were subjected to immunoprecipitation followed by Western blot analyses with antisera specific for the IR-β subunit, IRS-1, and IRS-2 (30). (**B**) IR immunoreactivity was quantified by densitometric scanning of blots similar to that in (A). Data represent the mean ± SEM of $n = 8$ of each genotype and are expressed relative to the control mice. (**C**) Western blot analyses of IR-β subunit content in tissues from control and NIRKO (KO) mice. [View Larger Version of this Image (35K GIF file)]

Because insulin stimulates growth of neurons in culture (16, 17), we investigated the impact of IR deletion on brain development and morphology. Brain weights in NIRKO mice were not significantly different from those in control mice (475 mg compared with 483 mg, $P = 0.18$, $n = 10$ each genotype), and histological analysis revealed no apparent differences in brain development or morphology (18). Immunohistochemical analyses of brain sections with antisera against glial fibrillar acidic protein (GFAP), a marker of glial cell activation, also showed no differences between NIRKO and control mice (18), suggesting that IR expression is not required for neuronal survival in vivo.

Although the body weights of male NIRKO mice were indistinguishable from those of their control littermates during the first 6 months of life on a normal chow diet, female NIRKO mice exhibited a consistent 10 to 15% increase of body weight in comparison with controls (Fig. 2A). In addition, on this diet, both male and female NIRKO mice demonstrated increased adipose tissue mass with an ~twofold increase in perigonadal white adipose tissue (WAT) in NIRKO females and a 1.5-fold increase in NIRKO males (Fig. 2B). Paralleling the increase in adipose mass, plasma leptin concentrations were elevated 2.5-fold in female NIRKO mice ($P < 0.01$) and 1.5-fold in male NIRKO mice ($P < 0.05$) (Fig. 2C). The increased body weight of NIRKO females also correlated with an ~20% increase in food intake as compared with female controls [121 mg per gram of body weight (BW) per day compared with 100 mg per gram of BW per day; $P < 0.01$] (Fig. 2D). In contrast, food intake of male NIRKO mice on the normal chow diet did not differ significantly from that of controls (82 mg per gram of BW per day compared with 87 mg per gram of BW per day; $P = 0.15$, $n = 14$ each genotype). This mild obesity was enhanced when the mice were challenged with a high-fat (60%) diet. Under these conditions, by as little as 14 weeks of age, male NIRKO mice exhibited a 10% elevation of body weight ($P < 0.05$) and female NIRKO mice a 20% increase in body weight ($P < 0.05$) as compared with control mice on the same diet (Fig. 2E).

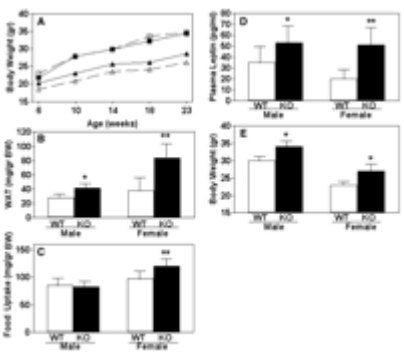

Fig. 2. Absence of IR expression causes obesity. (**A**) Body weights of NIRKO and control mice were determined at the indicated ages. Data represent the mean of at least 16 mice of each gender and genotype. ❑, Wild-type male; ■, NIRKO male; △, wild-type female; ▲, NIRKO female. The SEM at each point was below 10% of the indicated value. Body weights of female NIRKO mice were significantly different from female controls at every age with $P < 0.05$ in an unpaired Student's t test. (**B**) White adipose tissue (WAT)

mass (parametrial fat depots in female mice and epididymal fat depots in male mice) was determined in mice at 8 to 12 months of age. Data represent the mean ± SEM of at least eight animals of each genotype and gender (*, $P < 0.05$; **, $P < 0.005$). (**C**) Plasma leptin concentrations were determined by enzyme-linked immunosorbent assay (ELISA) on blood samples obtained from 6- to 8-month-old mice on a regular chow diet. Data represent the mean ± SEM of at least 10 animals of each genotype and gender (*, $P < 0.05$; **, $P < 0.005$). (**D**) Food intake and body weight of 4- to 6-month-old mice were determined daily over 1 week. Data represent the mean ± SEM of at least eight mice of each genotype and gender (**, $P < 0.01$). (**E**) Body weight of male and female control and NIRKO mice is given at the age of 14 weeks. In these experiments, control and NIRKO mice were put on a high-fat (60%) diet between 5 and 9 weeks of age. Data represent the mean ± SEM of at least six animals of each genotype and gender (*, $P < 0.05$). [View Larger Version of this Image (26K GIF file)]

The obesity in NIRKO mice was associated with insulin resistance and hypertriglyceridemia. At 4 to 6 months of age, the NIRKO mice showed normal fasting blood glucose levels (Fig. 3A), but the circulating plasma insulin levels were elevated by 1.5-fold in males and ~twofold in females (Fig. 3B). Consistent with their obesity phenotype, female NIRKO mice showed a significantly blunted response 15 min after insulin injection and a trend toward elevated blood glucose 30 to 60 min later, whereas after pharmacologic doses of insulin, male NIRKO mice performed similarly to controls (Fig. 3C). Intraperitoneal glucose tolerance tests were normal in both male and female NIRKO mice. Finally, both male and female NIRKO mice showed a 30% increase in circulating triglycerides (Fig. 3D) but had normal plasma cholesterol concentrations (105 mg/dl compared with 109 mg/dl; $P = 0.16$, $n = 9$ each genotype). Thus, brain-specific disruption of the IR gene results in hyperphagia in female mice and causes obesity, hyperleptinemia, insulin resistance, and hypertriglyceridemia in both male and female mice.

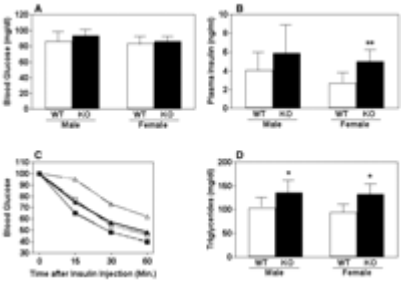

Fig. 3. Obesity in NIRKO mice causes mild insulin resistance and dyslipidemia. (**A**) Blood glucose concentrations were determined on control and NIRKO mice after an overnight fast with a Glucometer Elite. Data represent the mean ± SEM of at least 10 animals of each genotype and gender. (**B**) Plasma insulin concentrations were determined by ELISA on blood samples obtained from 6- to 8-month-old mice on a normal chow diet. Data represent the mean ± SEM of at least 10 animals of each genotype and gender (**, $P < 0.005$). (**C**) Intraperitoneal insulin tolerance tests were performed with 0.75 U of insulin per kg of body weight (30). Data represent the mean ± SEM of at least eight animals of each genotype and gender. ■, wild-type male; □, NIRKO male; ▲, wild-type female; △, NIRKO female. (**D**) Plasma triglyceride concentrations were determined on blood drawn from 6- to 8-month-old mice. Data represent the mean ± SEM of at least 10 animals of each genotype and gender (*, $P < 0.05$). [View Larger Version of this Image (22K GIF file)]

Another phenotype of NIRKO mice manifested itself in breeding experiments. Although 76% of the matings established between control mice yielded offspring, breedings of male NIRKO mice with control females produced offspring in only 46% of the cases ($P < 0.05$). Rates were similarly reduced to 42% when NIRKO females ($P < 0.05$) were bred to male controls. The reduction in male fertility was due to impaired spermatogenesis; epididymal sperm content was reduced by 30% ($P < 0.05$) in NIRKO mice as compared with age-matched controls (Fig.

4A). Histological examination of testis sections revealed that, although spermatogenesis was proceeding normally in many seminiferous tubules of NIRKO males, ~20% of tubules lacked a lumen and presented few, if any, maturing spermatogenic cells. Moreover, there was a reduction of the Leydig cell population, and the interstitial stroma did not support organization of seminiferous tubules within the NIRKO testis (Fig. 4B). The seminal vesicles, prostate, and epididymis did not appear morphologically altered in NIRKO males (20). Histological examination of ovaries from female NIRKO mice also revealed abnormalities. NIRKO ovaries contained reduced numbers of antral follicles (wild type: 2.8 ± 0.46, $n = 8$) and corpora lutea (wild type: 4.0 ± 0.31, $n = 5$, compared with NIRKO: 1.12 ± 0.36, $n = 8$) (Fig. 4B). These observations suggest that NIRKO mice had insufficient gonadotropin input for proper maintenance of ovarian follicle maturation, Leydig cell function, or spermatogenesis.

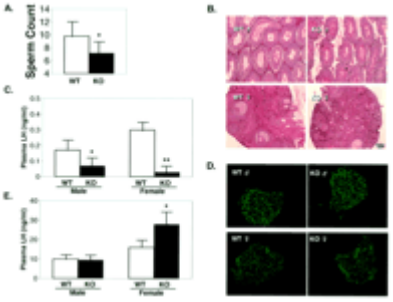

Fig. 4. Absence of brain IR expression results in hypothalamic hypogonadism. (**A**) Epididymi of control and NIRKO mice were removed, and spermatozoa were allowed to diffuse into culture medium. After centrifugation, total epididymal sperm content was determined. Data represent the mean ± SEM of at least 10 animals of each group (*, $P < 0.05$). (**B**) Testes and ovaries were removed from control and NIRKO mice and fixed in 10% formalin. Paraffin-embedded sections were stained with hematoxylin and eosin. The scale bar indicates about 100 μm. (**C**) Plasma LH concentrations were determined by radioimmunoassay on serum samples from 6- to 7-month-old mice. Data represent the mean ± SEM of at least eight animals of each genotype and gender (*, $P < 0.05$; **, $P < 0.01$). (**D**) Pituitaries were dissected from paraformaldehyde-perfused mice. One-micrometer sections were prepared from wild-type and NIRKO mice and stained with polyclonal antibodies to LH. (**E**) Plasma LH concentrations were determined by radioimmunoassay on serum samples obtained 1 hour after intraperitoneal injection of lupron. Data represent the mean ± SEM of at least six animals of each genotype and gender (*, $P < 0.05$). [View Larger Version of this Image (49K GIF file)]

To assess the role of the hypothalamic-pituitary axis in the gonadal insufficiency, we measured plasma levels of luteinizing hormone (LH) in the NIRKO mice. This assay revealed a 60% reduction of circulating LH concentrations in males ($P < 0.05$) and a 90% reduction in females ($P < 0.01$) (Fig. 4C). This decrease occurred with no alteration in pituitary morphology, as determined by methylene blue staining (20), or pituitary LH content, as estimated by immunohistochemical analysis with antisera to LH (Fig. 4D). To test whether the pituitaries of the NIRKO mice respond to LH releasing hormone (LHRH), we injected the mice intraperitoneally with lupron, a GnRH receptor agonist. Male NIRKO mice actually exhibited a normal increase in circulating LH concentrations, whereas female NIRKO mice displayed a twofold enhancement of response compared with controls (Fig. 4E). These data indicate that neuronal expression of the IR is essential for normal regulation of the hypothalamic-pituitary-gonadal axis through its effects on LH secretion.

In summary, this study documents that IR in the CNS plays an important functional role in the regulation of energy homeostasis and reproductive endocrinology. This provides a mechanism for the previous observations that intraventricular injection of insulin inhibits food intake (21, 22) and the evidence that insulin may play a role in regulation of body weight at a central level (4, 23). Thus, insulin acting in the CNS through its receptor appears to provide a negative feedback loop for postprandial inhibition of food uptake. Obesity in NIRKO mice occurs

despite elevated circulating plasma leptin concentrations, suggesting that the CNS insulin resistance is also associated with some degree of CNS resistance to leptin action, creating an interesting link between insulin and leptin action in the regulation of body weight. The current data also suggest a mechanism by which insulin resistance in the CNS can modify the metabolic syndrome by leading to hyperphagia, obesity with hyperleptinemia, and hypertriglyceridemia, thereby further aggravating peripheral insulin resistance. Taken together with our previous studies indicating a role for the insulin receptor in β cells for normal glucose sensing (24), this study demonstrates that genetically determined insulin resistance in classical insulin target tissues, and nonclassical target tissues such as the brain and beta cell, may act synergistically in the induction of obesity, insulin resistance, glucose intolerance, and dyslipidemia, leading to the complex metabolic syndrome associated with type 2 diabetes (14, 24, 25).

Our results also reveal an important link between brain insulin signaling and reproduction. There are at least two possible mechanisms by which insulin might regulate the reproductive axis at a central level. First, although leptin concentrations are only mildly elevated, the elevated plasma leptin concentrations may modify LHRH secretion in NIRKO mice. This seems unlikely, however, because the phenotype of the NIRKO mice differs from leptin-overexpressing mice, which exhibit reduced LH secretion in response to exogenous LHRH (26). Alternatively, the IR expressed on GnRH-producing neurons or at some even higher center may mediate GnRH synthesis or secretion. Indeed, in cultured hypothalamic neurons, IGF-2, a high-affinity ligand for IR, induces GnRH release (27). In a number of severe insulin resistance states, such as the Type A syndrome and lipoatrophic diabetes, hypothalamic-pituitary-gonadal function is perturbed with alterations in menstrual function and even polycystic ovarian disease (28, 29). Thus, the NIRKO mice will provide an important tool for studying insulin action in the CNS and will likely add unexpected aspects to our understanding of genetically determined insulin resistance, obesity, and reproductive function.

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31. We thank A. Parlow for the gift of antisera to LH and M. D. Michael for discussions. Supported by grants from the Deutsche Forschungsgemeinschaft (DFG 1492-2 to J.C.B.), the Volkswagenstiftung (to J.C.B., D.M.-W., and W.K.), NIH (DK31036 to C.R.K. and DK55326-01A2 to D.J.B.) and Joslin Diabetes Center Diabetes Endocrinology Research Center grants (to C.R.K. and D.J.B.).

15 May 2000; accepted 3 August 2000

Volume 289, Number 5487, Issue of 22 Sep 2000, pp. 2122-2125.