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Neural and Behavioural Effects of the *Ginkgo biloba* Leaf Extract Egb 761

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by

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Abstract

Numerous studies have documented the cognitive-enhancing effects of standardized *Ginkgo biloba* leaf extracts (*Gingko*) both in humans and in rodents. In addition to its antioxidant and platelet-activating factor inhibiting properties, *Ginkgo* has been shown to protect cellular membrane fluidity, to enhance cerebral blood flow and microcirculation, and to modulate cholinergic system functioning, properties that have been suggested to underlie its cognitive-enhancing effects. However, the possible neurogenic-enhancing effects of *Ginkgo* on adult hippocampal neurogenesis as a possible mechanism underlying its nootropic effects have not been evaluated. The experiments discussed in the present thesis assessed such a possibility.

Chapter 1 reviews studies that have evaluated the nootropic effects of *Ginkgo* in humans and in rodents. The anti-stress properties of *Ginkgo* as well as several molecular mechanisms proposed to account for its beneficial effects on central nervous system functioning are also discussed. Experiments presented in Chapter 2 used c-Fos immunoreactivity to characterize functional activity in selected brain regions following acute oral (150 mg/kg) and intraperitoneal (2.5-25 mg/kg, i.p.) administration of *Ginkgo*. Both routes of administration increased c-Fos immunoreactivity in the insular cortex and amygdala. However, some additional regions including the nucleus accumbens and dentate gyrus showed increased c-Fos immunoreactivity only in response to parenteral administration.

Experiments presented in Chapter 3 evaluated the possible neurogenic properties of *Ginkgo*. The anxiolytic effects of the extract using the elevated plus maze, light/dark emergence and social interaction tests were also assessed. Acute administration of

Ginkgo (2.5-25 mg/kg, i.p.) had neither stimulating nor depressive effects on cell proliferation in the adult dentate gyrus. Survival of newborn neurons in the dentate gyrus of adult male rats was also not affected by chronic *Ginkgo* administration. Furthermore, the results showed that chronic administration of *Ginkgo* treatment had neither anxiolytic nor anxiogenic properties; however acute administration of a high and moderate dose of *Ginkgo* induced anxiety in some measures.

Experiments described in Chapter 4 investigated whether suppressed neurogenesis induced by corticosterone (CORT) treatment could be restored by *Ginkgo* co-treatment. The results revealed that survival of newborn cells was inhibited in the dorsal dentate gyrus of rats chronically treated with CORT; however, *Ginkgo* co-treatment did not buffer this effect.

Finally, experiments described in Chapter 5 evaluated the cognitive-enhancing effects of *Ginkgo* on young adult male rats using a food-reinforced two-component double Y-maze task. Results showed that *Ginkgo*-treated rats reached the training criteria faster, and made fewer errors. Rats treated with *Ginkgo* during the learning phase made fewer working memory errors. However, neither acute nor chronic post-training treatment with *Ginkgo* enhanced spatial working memory.

Taken together, the results of the present dissertation showed that *Ginkgo* increased c-Fos immunoreactivity in several brain structures involved in learning and memory. Moreover, *Ginkgo* enhanced acquisition and performance of a double-Y maze task, but did not offer any continued benefits in an already-learned working memory task. Interestingly, a dose of *Ginkgo* (13.75 mg/kg) that was found to improve cognitive function failed to promote either cell proliferation or cell survival in the adult dentate

gyrus. It also failed to restore the suppressive effects of CORT on dentate gyrus cell survival. It is concluded that *Ginkgo* pre-treatment subtly enhances learning, but this effect is likely not related to a modulatory effect on adult hippocampal neurogenesis.

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Table of Contents

List of Figures

Abbreviations

2-HβC:	2-hydroxypropyl-β-cyclodextrin
5-HT	5-hydroxytryptamine
ANOVA:	Analysis of variance
β-CCE:	ethyl β-carboline-3-carboxylate
BNA:	Basolateral nucleus of amygdala
BNST:	Bed nucleus of the stria terminalis
BrdU:	5-bromo-2'-deoxyuridine
C:	Centre box
CA1:	Cornu ammonis area 1
CA3:	Cornu ammonis area 3
CEA:	Central nucleus of amygdala
CORT:	Corticosterone
CPu:	Caudate-putamen
DAB:	3,3'-Diaminobenzidine-tetrahydrochloride hydrate
DCX:	Doublecortin
EGb 761:	Extract of *Ginkgo biloba*
Fos IR:	c-Fos-immunoreactivity
G1:	Goal box 1
G2:	Goal box 2
GABA:	Gamma-aminobutyric acid
$GABA_A$:	Gamma-aminobutyric acid receptor A
Ginkgo:	*Ginkgo biloba* leaf extract
i.p.:	Intraperitoneally
LSV:	Lateral septal nucleus, ventral part
lux:	The international system of Unit of illuminance
MNA:	Medial nucleus of amygdala
nAcc:	Nucleus accumbense
NeuN:	Neuron-specific nuclear protein
PAF:	Platelet-activating factor
PB:	Phosphate buffer
PBG:	Phosphate buffered goat serum
PSA-NCAM:	Polysiallylated form of the neural cell adhesion molecule
PV:	Paraventricular nucleus
ROS:	Reactive oxygen species
s.c.:	Subcutaneously
S1:	Start box 1
S2:	Start box 2
SEM:	Standard error of the mean
SGZ:	Subgranular zone
SSRI:	Selective serotonin reuptake inhibitors
SVZ:	Subventricular zone
Veh:	Vehicle

Chapter 1: Introduction

Ginkgo biloba (*Ginkgo*) is an ancient tree native to eastern Asia. It has survived over millions of years and as such is referred to as a 'living fossil' (Bombardelli, Cristoni, & Morazzoni, 2000; Jacobs & Browner, 2000; Major, 1967). *Ginkgo* is a dioecious tree, meaning that the male and female blossoms are formed separately on two individual trees. It grows to a height of 30 to 40 m and to a diameter of 1 to 4 m and can live for hundreds of years (Del Tredici, 2000; Melzheimer & Lichius, 2000). *Ginkgo* trees are highly resistant to pests, bacteria, fungi, viruses, and air pollution, and are considered ideal ornamental trees in large cities (Laurain, 2000; Major, 1967). It is noteworthy that *Ginkgo* trees were the only trees reported to sprout one year after their destruction by the Hiroshima atomic bomb (Jacobs & Browner, 2000; Laurain, 2000)

The unusual fan-shaped *Ginkgo* leaves are dark green in colour during the summer, and turn bright yellow in autumn (Laurain, 2000). *Ginkgo* fruits resemble small apricots and contain an almond-shaped nut. *Ginkgo* leaves and seeds have long been used in traditional Chinese medicine to treat various kidney and bladder disorders, as well as lung-related illnesses such as asthma and bronchitis (Del Tredici, 2000). However, pharmaceutical use of the extract of the *Ginkgo biloba* leaf originated in Germany in 1965. The first commercially available and standardized *Ginkgo biloba* leaf extract, EGb 761, was registered in 1974 in France (Del Tredici, 2000).

The Ginkgo biloba leaf extract EGb 761

Ginkgo extract EGb 761 is a water-acetone extract of dried green leaves of *Ginkgo biloba.* It is prepared with a defined 27-step extraction procedure that yields 1 pound of the extract from 50 pounds of dried leaves. During the extraction process, EGb 761 is standardized to contain 24% flavonoid glycosides (including quercetin,

kaempferol, isorhamnetin and myricetin), 6% terpenoids (containing 3.1 % ginkgolides A, B, C, and J and 2.9% bilobalide), and less than 9% proanthocyanidins and organic acids (Chan, Xia, & Fu, 2007; DeFeudis & Drieu, 2000; Maclennan, Darlington, & Smith, 2002; Ponto & Schultz, 2003; Zimmermann, Colciaghi, Cattabeni, & Di Luca, 2002). Each EGb 761 constituent has a unique pharmacology; however, its overall therapeutic properties are believed to be the result of agonistic, antagonistic and synergistic effects of these active components (DeFeudis & Drieu, 2000; Nathan, 2000; Yoshikawa, Naito, & Kondo, 1999).

Dr. Willmar Schwabe Pharmaceuticals (Karlsruhe, Germany) and Beaufor-Ipsen Pharma (Paris, France) are two well-recognized manufacturers of EGb 761. Today, EGb 761 marketed under the trade names of *Rökan*, *Tanakan*, *Tebonin* and *Ginkgold*, is one of the best-selling herbal medicines in Europe and the United States (Chan et al., 2007). In fact, retail sales of *Ginkgo biloba* products in the United States alone reached $150 million in 1998, ranking first among 13 most purchased herbal medicines in the mainstream market (Blumenthal, 1999). In addition, the annual worldwide sales of *Ginkgo biloba* products have been estimated at half a billion US dollars (Warrier & Corzine, 2000).

Ginkgo biloba extracts are commonly prescribed in Germany and in France for the treatment of "cerebral insufficiency", age-related disorders, cardiovascular disease, vertigo (dizziness), tinnitus (ringing in ears) of vascular origin, and intermittent claudication characterized by cramping and pain in the lower limbs during walking or running due to insufficient oxygen. However, *Ginkgo* is also purchased as an over-the-counter supplement as a nootropic, or cognitive enhancer (Darlington, Smith, &

Maclennan, 2000; Gold, Cahill, & Wenk, 2002; Yoshikawa et al., 1999). The present summary is centered on the effects of a *Ginkgo biloba* leaf extract on cognition, stress and anxiety. The possible mechanisms of actions of the extract are then discussed.

Cognitive-enhancing effects of Ginkgo biloba leaf extracts

Standardized *Ginkgo* extracts, such as EGb 761, appear to enhance cognitive functioning both in humans and in rodents (Kennedy, Haskell, Mauri, & Scholey, 2007; Kennedy, Jackson, Haskell, & Scholey, 2007; Kennedy, Scholey, & Wesnes, 2000, 2002; Le Bars et al., 1997; Mix & Crews, 2000, 2002; Stackman et al., 2003; Stoll, Scheuer, Pohl, & Muller, 1996; Stough, Clarke, Lloyd, & Nathan, 2001; Y. Wang, Wang, Wu, & Cai, 2006; E. Winter, 1991; J. C. Winter, 1998; Wirth, Stemmelin, Will, Christen, & Di Scala, 2000). However there are also contradictory results, particularly in the human clinical data, regarding its putative cognitive-enhancing efficacy.

For example, a randomized double blind placebo-controlled study (Le Bars et al., 1997) reported significant improvements in cognitive performance and social functioning of demented patients who were treated with EGb 761 (120 mg/day for 6 to 12 months). However, another study (van Dongen, van Rossum, Kessels, Sielhorst, & Knipschild, 2000) reported no significant improvements in either cognitive or social functioning of a similar population, but after only 6 months of treatment with EGb 761 (160 and 240 mg/day). A meta-analysis of four randomized, placebo-controlled double blind studies with the total sample size of 212 in each *Ginkgo* and placebo groups reported a modest effect on objective measurements of cognitive abilities on elderly patients with dementia. The effect was evident after three to six months of treatment with 120 and 240 mg daily dose (Oken, Storzbach, & Kaye, 1998). Nevertheless, a more recent meta-analysis, which

included 35 randomized, double blind studies with a total of 4247 participants, concluded that the evidence for beneficial effects of *Ginkgo* on cognitive abilities of patients with dementia or cognitive impairment was 'inconsistent and unconvincing' (Birks & Grimley Evans, 2007). In fact, results of a recent longitudinal double-blind, placebo-controlled study following participants for a median of 6.1 years, have shown that EGb 761 (120 mg, twice daily) failed to prevent cognitive decline in aged (75 years and older) volunteers with mild cognitive impairments (DeKosky et al., 2008).

The influence of the extract on the cognitive abilities of healthy older adults has also been investigated. For example, Mix and Crews (2000; 2002) using a double-blind, placebo-controlled design, reported that EGb 761 (180 mg/day for 6 weeks) enhanced certain aspects of cognitive performance in healthy elderly volunteers. In contrast, Solomon et al. (2002) reported lack of effectiveness of a similar *Ginkgo* extract (*Ginkoba*, 120 mg/day for 6 weeks) in a similar population (i.e. healthy elderly).

In regards to young healthy individuals, acute administration of a *Ginkgo* extract (GK-501, 120 mg, 240 mg and 360 mg) has been reported to dose-dependently (240 and 360 mg) improve performance on the 'speed of attention', measured by combining the reaction times of three different attentional tasks. Albeit, the lowest dose (120 mg) tested in that study negatively affected 'speed of attention', but it enhanced 'quality of memory' at 1 and 4 h following the treatment (Kennedy et al., 2000). In a different study the same group of researchers investigated the effects of acute administration of similar doses of *Ginkgo* extract on performance of more demanding cognitive tasks in young healthy volunteers and reported a significant dose-dependent enhancement in speed of responding during one of the tasks, namely the serial three subtraction test (Kennedy et al., 2002).

Recently, acute administration of a lower dose of *Ginkgo* extract (120 mg) complexed with soy-phospholipid (phosphatidylserine), which enhances bioavailability of active components of the extract, has been reported to significantly enhance performance and accuracy of memory tasks as well as speed of memory task performance measured 1 to 6 h following administration in young healthy adults. Such observed effects were not evident after administration of the extract alone (120 mg) or complexed with phosphatidylcholine (Kennedy, Haskell et al., 2007). Moreover, chronic administration of EGb 761 (120 mg/day for 30 days), in young healthy volunteers, has been shown to facilitate speed of information processing, executive processing, and working memory (Stough et al., 2001). Nonetheless, results of a recent meta-analysis of 15 randomized clinical trials examining the cognitive enhancing effects of the extract in young healthy individuals were less than compelling (Canter & Ernst, 2007). Seven of the studies included in the meta-analysis were acute studies with sample sizes ranging from 8 to 70, leading to a total sample size of 202. Doses of the extract administered ranged from 120 to 600 mg. Eight other studies included in the above meta-analysis were longer-term studies with sample sizes ranging from 8 to 104 making a total sample size of 349. Doses of the extract used ranged from 40 to 300 mg per day and the treatment duration ranged from 2 to 91 days. The meta-analysis results showed no favourable effects of the extracts on any aspects of cognitive performance in young healthy adults after either acute or chronic administrations (Canter & Ernst, 2007). Following the aforementioned meta-analysis study Kennedy et al. (2007) reanalyzed data from three placebo-controlled, double blind studies, which were identical in their methodologies, with a total sample size of 78. The results revealed that the120 mg dose of the extract acutely administered to

young healthy volunteers significantly improved accuracy of memory task performance, but significantly slowed 'speed of attention' measured by combining the reaction times of three different attentional tasks. These effects were not due to a 'trade off' between speed and accuracy as the authors found no direct effect on the speed of memory task performance and accuracy of attention task performance. Taken together, data from clinical studies in regards to the cognitive enhancing effects of *Ginkgo* are inconclusive.

The effects of *Ginkgo* on cognitive functioning of young and aged rodents has also been investigated (Stackman et al., 2003; Stoll et al., 1996; Y. Wang et al., 2006; E. Winter, 1991; J. C. Winter, 1998; Wirth et al., 2000). For example, chronic pre-treatment with EGb 761 has been shown to enhance acquisition and performance of an appetitive operant conditioning task. After 4 or 8 weeks of EGb 761 (100 mg/kg) treatment, young healthy mice (9 or 13 weeks old) were trained in a two-lever operant response sequence for food reward for 30 days. The number of correct responses as well as the speed of acquisition was enhanced in EGb 761-treated groups. EGb 761 treatment was maintained until the retention test 10 weeks later, and an improvement of retention of the learned response was evident in EGb 761-treated mice (E. Winter, 1991).

Chronic daily pre-session treatment of *Ginkgo* (50 mg/kg) has also been shown to facilitate learning an eight-arm radial maze in rats. The radial maze task was designed to measure both working memory, measured by re-entries into the already visited arms, and reference memory, measured by entries into the arms that have never been baited. Interestingly, the life span of the *Ginkgo*-treated rats was significantly increased (J. C. Winter, 1998). Moreover, in a follow up experiment it was shown that chronic pre-session *Ginkgo* (100 and 200 mg/kg) treatment enhanced learning in a delayed non-

matching to position task, in which a 30-min delay was imposed between the two sessions, in 20-months-old rats (J. C. Winter, 1998).

In another study, young (3 months old), middle-aged (12 months old) and aged (22 to 24 months old) mice were treated with EGb 761 (100 mg/kg for 3 weeks) prior to learning a passive avoidance task. Short-term memory measured by avoidance latency 60 s after shock was significantly improved in aged mice, but no effect was found in long-term memory measured by avoidance latency 24 h after the shock. No improvement was evident in the younger groups (Stoll et al., 1996). However, the facilitative effects of acute injection of *Ginkgo* (60 and 120 mg/kg, i.p.) on an olfactory short-term recognition test in both young and aged rats have been demonstrated (Wirth et al., 2000).

Moreover, spatial memory deficits in aged rats (Y. Wang et al., 2006) and in transgenic mice model of Alzheimer's disease (Tg 2576) were prevented by chronic *Ginkgo* treatment (Stackman et al., 2003)-- effects that have been shown and attributed to enhancement of hippocampal long-term potentiation by *Ginkgo* (Y. Wang et al., 2006). After chronic treatment with EGb 761 (30 and 60 mg/kg, 30 days), aged rats were anesthetized and underwent electrophysiological recordings. Field excitatory postsynaptic potentials were recorded from the CA1 region of the hippocampus in response to ipsilateral high-frequency stimulation of the Schaffer collateral/commissural pathway, and a significant enhancement in long-term potentiation induced by high frequency stimulation was observed in *Ginkgo*-treated rats. The authors concluded that *Ginkgo* might ameliorate spatial learning and memory deficits associated with aging by increasing hippocampal long-term potentiation (Y. Wang et al., 2006). In support of this, an earlier study found that acute exposure to EGb 761 to brain slices taken from aged

mice significantly increased neuronal excitability by decreasing the population spike threshold, and increased the early phase of long-term potentiation. In addition, the population spike threshold and long-term potentiation in slices of aged mice fed chronically with EGb 761 (300 mg/kg for 30 days) was significantly increased (Williams, Watanabe, Schultz, Rimbach, & Krucker, 2004).

However, one study reported lack of improvements in both acquisition or retention of memory in the water maze task in aged mice chronically treated with EGb 761 (100 mg/kg) for 28 and 70 days. In this latter study, there was a significant difference in baseline anxiety levels measured using the elevated plus maze prior to water maze training. Thus, in a subsequent experiment, to reduce pre-existing differences in anxiety levels, a matched-pair design was applied and EGb 761 was found to significantly reduce stress induced by cold water exposure. The authors suggested that *Ginkgo* might act as an "anti-stress buffer" rather than a cognitive enhancer (Ward et al., 2002). Indeed, anxiolytic properties of EGb 761 have been recognized (e.g., see DeFeudis & Drieu, 2004) and are discussed in the following section.

It is noteworthy that most animal studies that have evaluated the putative cognitive enhancing effects of *Ginkgo* treatment have focused on its effects on the acquisition of information (i.e., learning), rather than the post-training effects in a working memory task. To the author's knowledge, only one study has examined the post-training effects of *Ginkgo* treatment in young adult rats. This study reported no significant effects in a Morris water maze task, or in radial arm maze tasks designed to assess short-term working and long-term reference memory (Shif et al., 2006).

Anti-stress and anxiolytic effects of Ginkgo biloba leaf extracts

Ginkgo extracts possess "stress-alleviating" and "vigilance-enhancing" properties (DeFeudis & Drieu, 2004). For example, chronic treatment with a standardized *Ginkgo* extract (LI 1370, 120 mg per day for 4, 6 and 10 months) has been found to improve mood in healthy geriatric volunteers who reported being better able to cope with the stressful demands of daily living (Trick, Boyle, & Hindmarch, 2004). A recent double-blind, placebo-controlled study also reported that EGb 761 (480 and 240 mg/kg for 4 weeks) alleviated symptoms of anxiety in a dose-dependent manner in younger patients who were either diagnosed with generalized anxiety disorder or adjustment disorder with anxious mood. For example, a four-week exposure to EGb 761 led to considerably reduced reports of somatic symptoms by patients. In addition, significant improvements were seen in the clinician's rating scores using Hamilton Rating Scale for Anxiety test, a standard measure of the severity of anxiety (Woelk, Arnoldt, Kieser, & Hoerr, 2007). A single dose of EGb 761 (120 mg) was also found to significantly reduce stress-induced rises in systolic and diastolic blood pressure, without influencing the heart rate, in young healthy volunteers in a double blind placebo-controlled study. The stress used in this study involved a combination of static exercise (handgrip) and mental load (memory test). In addition, male participants showed an increase in salivary cortisol levels measured in the afternoon that was effectively reduced by single EGb 761 treatment (Jezova, Duncko, Lassanova, Kriska, & Moncek, 2002).

The anti-stress effects of *Ginkgo* in rodents using various behavioural models of stress have also been documented. For example, rats pre-treated chronically with EGb 761 (50 and 100 mg/kg for 5 days) prior to inescapable shocks in the learned helplessness

paradigm showed fewer escape failures in subsequent tests (Porsolt, Martin, Lenegre, Fromage, & Drieu, 1990). Mice pre-treated chronically with the extract (50 and 100 mg/kg for 5 days) also showed significantly reduced stress in the emotional hypophagia test evident by increased novel food consumption in a novel environment (Porsolt et al., 1990). Chronic EGb 761 (100 mg/kg for 15 days) treatment was also found to prevent development of stress-induced polydipsia resulting from stress-induced handling and intubation feeding under light ether anesthesia (Rodriguez de Turco, Droy-Lefaix, & Bazan, 1993). Additionally, chronic *Ginkgo* pre-treatment considerably decreased stress induced by cold water exposure (Ward et al., 2002), stress-induced learning deficits in a discrimination task (Rapin, Lamproglou, Drieu, & DeFeudis, 1994) and stress-induced memory deficits in a passive avoidance task (Walesiuk, Trofimiuk, & Braszko, 2005). Moreover, mice treated with *Ginkgo* (125 mg/kg for one week) spent significantly more time on the open arms of the elevated plus maze compared to vehicle-treated mice, suggesting an anxiolytic-like effect of the extract (Kuribara, Weintraub, Yoshihama, & Maruyama, 2003). Additionally, zingicomb, a preparation of *Ginkgo* and ginger extract, has been reported to have anxiolytic properties in the elevated plus maze (Hasenöhrl et al., 1996).

Chermat and colleagues (1997) investigated the interaction of *Ginkgo*, diazepam (an anxiolytic agent and full agonist at the $GABA_A$/benzodiazepine/Cl^- channel complex) and ethyl β-carboline-3-carboxylate (β-CCE; an anxiogenic agent and partial inverse agonist at the $GABA_A$ receptor complex) using the social interaction test of anxiety. Interactions of pairs of unfamiliar rats from similar treatment groups were assessed in a novel and brightly illuminated environment for 10 minutes. Both acute injection (8 and

16 mg/kg, 30 min prior to testing) and chronic oral pre-treatment (48 and 96 mg/kg for 8 days) of EGb 761 reduced social interaction. Acute diazepam administration however increased social interaction, an effect that was potentiated by concurrent EGb 761 administration. Conversely, acute β-CCE administration reduced social interaction, but concurrent *Ginkgo* treatment significantly reversed that effect to a level similar to that of vehicle-treated rats (Chermat et al., 1997). The apparent anxiogenic activity of *Ginkgo* administered on its own in this latter study, evident by reduced social contact, is surprising; however, the authors (Chermat et al., 1997) suggested that *Ginkgo* possibly increased arousal or vigilance of the animals. It has also been suggested that the existing state of the animal may govern the effects of EGb 761 such that EGb 761 potentiated the anxiolytic effects of diazepam, but neutralized the anxiogenic effects of β-CCE (DeFeudis & Drieu, 2004)

More recently, it was reported that *Ginkgo* produced antidepressant effects measured by a significant decrease in immobility duration in the forced swimming test in rats chronically pre-treated with *Ginkgo* extract (Ginkgolon-24, 10 and 50 mg/kg for 14 days). An acute intraperitoneal *Ginkgo* injection (50 and 100 mg/kg) was also found to decrease immobility duration in the tail suspension test in mice (Sakakibara et al., 2006). However, chronic or acute EGb 761 treatment has been ineffective in some other behavioural models of stress and anxiety including shock-suppressed licking (Vogal conflict test) in rats, and shock-suppressed exploration and spontaneous exploration in mice, leading to the suggestion that the underlying mechanisms of anxiolytic or anti-stress properties of *Ginkgo* may differ from that of classical antidepressants such as

impiramine, or anxiolytics such as diazepam, which have been shown to be effective in the above tests (Porsolt et al., 1990).

Mechanisms of actions of Ginkgo biloba leaf extracts

Various pharmacological effects of *Ginkgo* may be the result of agonistic, antagonistic and synergistic effects of its active constituents (DeFeudis & Drieu, 2000; Nathan, 2000; Yoshikawa et al., 1999). Here, several molecular mechanisms proposed to account for the beneficial effects of *Ginkgo* and its active components including its neuroprotective, nootropic and anxiolytic effects are summarized.

Antioxidant and free radical scavenging

Ginkgo has been suggested to serve as a useful therapy in the treatment and prevention of disorders and conditions that are associated with oxidative stress such as ischemia and neurodegenerative diseases, as well as the aging process (Drieu & DeFeudis, 2000). Reactive oxygen species (ROS) such as nitric oxide (NO•), superoxide (O2•-) and hydroxyl radicals (•OH) are potent free radicals. They are by-products of normal cellular functions such as mitochondrial respiration, fatty acid metabolism, or microphage function. Under normal biological conditions, enzymatic antioxidants such as superoxide dismutase, catalase, and glutathione peroxidase, and non-enzymatic antioxidants such as vitamin C and E, scavenge or prevent the formation of ROS. However, under some pathological conditions as well as aging, there may be an imbalance between the production of ROS and antioxidant defence mechanisms. This state of imbalance is called oxidative stress (Beckman & Ames, 1998). The abnormal production of ROS can damage lipids in cell membranes by peroxidation, can cause

protein fragmentation by oxidating amino acid chains, and can lead to breakdown of DNA by reacting with thymine in nuclear and mitochondrial DNA (Farber, Kyle, & Coleman, 1990).

Using cell-free biochemical techniques, superoxide scavenging effects and superoxide dismutase activity of *Ginkgo* have been demonstrated (Pincemail et al., 1989). Using flow cytometric analysis it has also been shown that pre-treatment of cortical neurons with *Ginkgo* protected them from oxidative stress or apoptosis induced by hydrogen peroxide (Ni, Zhao, Hou, & Xin, 1996; Oyama, Chikahisa, Ueha, Kanemaru, & Noda, 1996). Pre-treatment of macrophage cells (Kobuchi, Droy-Lefaix, Christen, & Packer, 1997), endothelial cells (Cheung, Siow, Chen, & O, 1999) and hippocampal neurons (Bastianetto, Zheng, & Quirion, 2000) with *Ginkgo* has also been shown to protect them from nitric oxide-induced toxicity. Chronic EGb 761 treatment (100 mg/kg for 14 days) in rats has also been found to significantly reduce lipid peroxidation in the hippocampus, while increasing the activity of the antioxidant enzymes superoxide dismutase and catalase in the hippocampus, substantia nigra and striatum (Bridi, Crossetti, Steffen, & Henriques, 2001). These studies suggest that the neuroprotective effects of *Ginkgo* may, in part, be the result of its antioxidant and free radical scavenging properties.

Inhibition of platelet-activating factor

Platelet-activating factor (PAF) is a name commonly used for a large number of structurally related, biologically potent, phospholipids that are produced by various cell types (Stafforini, McIntyre, Zimmerman, & Prescott, 2003) including neurons (Yue, Lysko, & Feuerstein, 1990). They mediate diverse physiological effects by interacting

with specific PAF surface receptors that are expressed on a wide range of cell types. However, up-regulation of PAF, which may be the result of either excessive synthesis or impaired degradation of PAF, is associated with many pathological conditions that are usually accompanied by inflammation and thrombosis (Stafforini et al., 2003). Neuropathology of the brain following hypoxia and ischemia has been shown to be related to the up-regulation of PAF (for a review, see Yue & Feuerstein, 1994). For example, physiological levels of PAF have been shown to increase significantly in the hippocampus, cortex, and thalamus in response to ischemia at early stages of reperfusion leading to neuronal death and damage (Nishida & Markey, 1996).

Neuroprotective effects of *Ginkgo* have been attributed to its PAF antagonistic activity. For example, the *Ginkgo* constituents ginkgolides A and B are potent PAF receptor antagonists, and it has been demonstrated that acute pre- and post-treatment (25 mg/kg, once pre and once post ischemia) with ginkgolides A and B significantly decreased ischemia-induced neuronal death in the occipital and parietal cerebral cortex, as well as in CA1 and CA3 hippocampal subfields in rats (Prehn & Krieglstein, 1993). Pre-treatment with EGb 761 (25, 50 and 100 mg/kg for 7 days) and bilobalide (3 and 6 mg/kg for 7 days) has also been shown to protect CA1 neurons against neuronal damage induced by ischemia in rats (Chandrasekaran et al., 2002). EGb 761 also reduces infarct volume after permanent and transient focal cerebral ischemia. Additionally, rats treated with EGb 761 showed significant improvement in neurobehavioural testing 3 to 7 days following transient focal cerebral artery occlusion (Lee et al., 2002). Bilobalide administered one hour before or immediately after ischemia has also been found to significantly and dose-dependently decrease the infarct volume in mouse brain

(Krieglstein et al., 1995). In addition, EGb 761, bilobalide, ginkgolide A and B have been found to dose-dependently protect primary neuronal cultures against glutamate-induced excitotoxicity (Chandrasekaran et al., 2002; Prehn & Krieglstein, 1993). *In vitro* studies have also shown that EGb 761 suppressed the formation of amyloid beta fibril, which is one of the characteristics of Alzheimer's disease. Furthermore, EGb 761 inhibited activation of the apoptotic enzyme caspase-3 (Luo et al., 2002). Neurotoxicity induced by beta-amyloid was also found to be significantly and dose-dependently reduced in hippocampal cultured neurons incubated with EGb 761 or the flavonoid fraction of the extract, but not with the terpenoid fraction (Bastianetto, Ramassamy et al., 2000), suggesting that neuroprotective effects of the extract may also be attributed to antioxidant and free radical scavenging activities of its flavonoid fraction.

Down-regulation of glucocorticoids

It has been suggested that EGb 761 facilitates behavioural adaptation under stressful conditions by reducing glucocorticoid synthesis (Amri, Ogwuegbu, Boujrad, Drieu, & Papadopoulos, 1996). Repeated treatment with EGb 761 (50 and 100 mg/kg for 8 days) and ginkgolide A and B (2 mg/kg for 8 days), but not with bilobalide (4 mg/kg for 8 days), has been demonstrated to dose-dependently down-regulate glucocorticoid synthesis by reducing levels of messenger RNA protein and drug binding of adrenocortical mitochondrial peripheral-type benzodiazepine receptors in rats (Amri et al., 1996). Accordingly, *Ginkgo* pre-treatment has been reported to normalize elevated levels of circulating corticosterone induced by restraint stress in rats (Rai, Bhatia, Sen, & Palit, 2003; Shah, Sharma, & Vohora, 2003). Repeated treatment with EGb 761 (50 and 100 mg/kg for 20 days) was also found to significantly and dose-dependently reduce the

auditory stress-induced elevation of circulating levels of epinephrine, norepinephrine and corticosterone in both young and aged rats (Rapin et al., 1994). It has been suggested that the ability of *Ginkgo* to down-regulate glucocorticoid synthesis may contribute to its anti-stress activity, which in turn increases vigilance and awareness leading to improved cognition (Drieu & DeFeudis, 2000).

Improving blood flow and membrane fluidity

It has also been proposed that the cognitive enhancing effects of *Ginkgo* may be related to positive effects on cerebral blood flow and microcirculation (Drieu & DeFeudis, 2000). Under normal conditions *Ginkgo* treatment is associated with vasodilatation and improved blood flow in the whole body, including the brain (Ahlemeyer & Krieglstein, 2003; Iliff & Auer, 1983). Intravenous injection of *Ginkgo* (0.3 mg/kg/min for 30 min) in cats has been shown to significantly enhance dilation of pial arteries, which led to an increase in cerebral blood flow and microcirculation (Iliff & Auer, 1983). Acute intravenous *Ginkgo* (130 mg/kg) injection has also been shown to enhance cerebral blood flow and microcirculation in rats by 50 to 100% in 39 brain structures analyzed including the dentate gyrus of the hippocampus (Krieglstein, Beck, & Seibert, 1986). It appears that the enhancement effects of *Ginkgo* on cerebral blood flow and microcirculation is related to its terpenoid fraction (Ahlemeyer & Krieglstein, 2003).

Under pathological conditions *Ginkgo* treatment is also associated with enhancement of blood flow. For example immediately after ischemia, cerebral blood flow increases and then decreases to 40 to 60% of pre-ischemia for several hours. Treating rats with EGb 761 5 and 45 min following global cerebral ischemia1 increased cerebral blood flow to relatively normal levels (Krieglstein et al., 1995). Treatment with EGb 761 (100

and 200 mg/kg) 2 h post transient middle cerebral artery occlusion has also been shown to significantly increase local cortical blood flow 24 h after such reperfusion injury (Lee et al., 2002).

Ginkgo treatment has also been shown to enhance *in vitro* proliferation of endothelial progenitor cells in a dose-dependent manner. Migration, adhesion and *in vitro* vasculogenesis capacity of endothelial cells was also improved by *Ginkgo* treatment (Chen et al., 2004). Endothelial progenitor cells are derived from bone marrow and contribute to endothelial cells in newly formed neovascularization (Murayama et al., 2002).

In addition, *Ginkgo* appears to enhance cell membrane fluidity, which is reduced by aging or by agents such as excessive ascorbic acid or ferrous ions. In one study synaptosomes obtained by homogenization followed by centrifugation of brain cortices of young mice were incubated with or without ascorbic acid and varying doses of EGb 761 (2-16 μg/ml) for 60 min. The reduction of membrane fluidity induced by ascorbic acid was significantly reduced by EGb 761 treatment in a dose-dependent manner (Ramassamy, Girbe, Christen, & Costentin, 1993). Furthermore, *in vivo* treatment of aged mice (22-24 months old) with EGb 761 (100 mg/kg) for 21 days was found to significantly protect neuronal membrane fluidity, which is considerably reduced by ageing (Stoll et al., 1996).

Neurotransmitter and receptor modulation

The effects of *Ginkgo* on neurotransmitters and receptors have also been investigated. Aging is associated with reduced density of serotonin 5-hydroxytryptamine (5-HT) type 1A receptors in the cerebral cortex of rats, and chronic EGb 761 treatment

was found to reverse this effect by 33% (Huguet, Drieu, & Piriou, 1994). Synaptosomes, prepared from the cerebral cortex of mice, incubated with EGb 761 and CP 202 (EGb 761 devoid of terpenoid fraction) but not with BN 52063 (EGb 761 devoid of flavonoid fraction) displayed increased uptake of 5-HT. However, concurrent treatment of synaptosomes with clomipramine, a 5-HT uptake antagonist, diminished this effect (Ramassamy, Christen, Clostre, & Costentin, 1992). Chronic *Ginkgo* (5 mg/kg for 21 days) treatment has also been shown to reverse the age-related decrease in the number of α_2 -adrenoceptors in the hippocampus (Huguet & Tarrade, 1992). Moreover, oral administration of a single dose of *Ginkgo* (50, 100 and 200 mg/kg) attenuated working memory deficits in rats induced by the α_2 -adrenoceptors antagonist yohimbine (Zhang & Cai, 2005). Chronic *Ginkgo* treatment has also been shown to reduce whole brain levels of dopamine, norepinephrine and serotonin induced by restraint stress (Shah et al., 2003).

Of particular interest is the modulatory effect of *Ginkgo* on the cholinergic system (Chopin & Briley, 1992; Das et al., 2002; Kristofikova, Benesova, & Tejkalova, 1992; Taylor, 1986; Yamamoto, Adachi, Fujii, & Kamei, 2007). For example, long-term *Ginkgo* (100 mg/kg for 28 days) treatment has been shown to increase hippocampal cholinergic muscarinic receptor numbers (Taylor, 1986). Chronic *Ginkgo* (50 mg/kg for 30 days) treatment has also been shown to enhance the release of acetylcholine by increasing sodium-dependent high-affinity choline uptake into hippocampal synaptosomes (Kristofikova et al., 1992). *In vitro* dose-dependent inhibitory effects of *Ginkgo* on acetylcholinesterase activity have also been reported (Das et al., 2002), and it has been shown that *Ginkgo* reversed amnesia induced by the cholinergic muscarinic receptor antagonist scopolamine in passive avoidance (Chopin & Briley, 1992; Das et al.,

2002), and radial arm maze tasks (Yamamoto et al., 2007). In addition, doses of *Ginkgo* (30 and 60 mg/kg) that reversed scopolamine-induced amnesia were found to inhibit acetylcholinesterase activity *ex vivo,* but these inhibitory effects on acetylcholinesterase activity were not evident *ex vivo* when a lower dose of the extract (15 mg/kg) was administered. Furthermore, this lower dose failed to reverse scopolamine-induced amnesia. Thus, it has been suggested that the ability of *Ginkgo* to enhance activity of the cholinergic system may contribute to its cognitive enhancing properties (Das et al., 2002). Of particular relevance to the present dissertation are recent findings suggesting a role for the cholinergic system in regulating adult hippocampal neurogenesis (Cooper-Kuhn, Winkler, & Kuhn, 2004; Kaneko, Okano, & Sawamoto, 2006; Kotani, Yamauchi, Teramoto, & Ogura, 2006, 2008). For example, adult hippocampal neurogenesis has been found to be suppressed in rats by immunotoxic lesioning of neurons of the cholinergic basal forebrain that project to the dentate gyrus (Cooper-Kuhn et al., 2004).

It is also noteworthy that the cognitive enhancing effects of *Ginkgo* are often associated with long-term use (Muller & Chatterjee, 2003). This corresponds well with the hypothesis that *Ginkgo* enhances adult hippocampal neurogenesis, since newborn neurons require at least a few weeks to become fully functional and to integrate into the existing neuronal circuitry (van Praag et al., 2002). As one of the aims of the present dissertation was to investigate such a hypothesis, the following section provides an overview of adult hippocampal neurogenesis.

Adult hippocampal neurogenesis

Adult neurogenesis is the process of birth and growth of functionally integrated neurons in the postnatal brain (H. Song et al., 2005). This phenomenon has only been

recently confirmed in the adult mammalian brain. Lack of methodological advances to distinguish between multiplying glial cells from mitotic neurons led Santiago Roman y Cajal, the founder of modern neuroscience, to conclude that neurogenesis was only limited to prenatal brains (Gross, 2000). In the late 1950s, [H^3]-thymidine autoradiography, which allowed labeling replicating DNA, was developed. By taking advantage of this new technique, Altman and Das (1965) challenged the dogma that "new neurons are not added to the adult mammalian brain" (Gross, 2000) and provided evidence for neurogenesis in specific regions of adult rat brain including the dentate gyrus of the hippocampus (Altman & Das, 1965), olfactory bulb (Altman, 1969) and neocortex (Altman & Das, 1966).

Towards the early 1990s, a new method was developed for labeling dividing cells using bromodeoxyuridine (BrdU), which is incorporated into replicating DNA and can be detected by immunohistochemistry. Before the end of the twentieth century, using this technique in combination with cell-specific markers for immunohistochemical identification of newly generated cells and stereological quantification, the presence of two neurogenic regions in the central nervous system of all mammals examined including humans was confirmed (Eriksson et al., 1998). Neurogenesis in the adult mammalian brain is believed to be limited to the subventricular zone (SVZ) of the lateral ventricle, and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus. Adult neurogenesis outside these two regions has been suggested, but at present remains controversial (Gould, 2007; Rakic, 2002).

Adult neuronal stem cells reside in the neurogenic niche of the SGZ of the dentate gyrus (Seri, Garcia-Verdugo, McEwen, & Alvarez-Buylla, 2001) and give rise to

transient amplifying progenitor cells (Kempermann, Jessberger, Steiner, & Kronenberg, 2004; Ming & Song, 2005). The niche has a special microenvironment that not only maintains neuronal stem and progenitor cells, but also promotes their proliferation and fate determination. Resident astrocytes and vasculatures are two integrative components of the neurogenic niche. Clusters of proliferating cells in the granule cell layers have been shown to reside in close proximity to fine capillaries. It appears that astrocytes and blood factors released from vasculature may play important roles in proliferation and fate specification of neuronal stem cells (Palmer, Willhoite, & Gage, 2000; H. J. Song, Stevens, & Gage, 2002).

Transient amplifying progenitors have limited potential for self-renewal. Some will die, while others divide symmetrically and rapidly give rise to a pool of progenitors that in turn differentiate into immature neurons expressing doublecortin (DCX) and the polysiallylated form of the neural cell adhesion molecule (PSA-NCAM). The immature neurons migrate locally and move into the granule cell layer (Ming & Song, 2005; van Praag et al., 2002). As they migrate, immature neurons undergo unique morphological (Zhao, Teng, Summers, Ming, & Gage, 2006) and physiological (Ge et al., 2006; Schmidt-Hieber, Jonas, & Bischofberger, 2004; S. Wang, Scott, & Wojtowicz, 2000) changes.

Neurite outgrowth initiates soon after newborn neurons exit the progenitor cell cycle. In a few days, dendritic and axonal growth starts. Newly generated axons interact initially with hilar mossy cells and interneurons located in the hilus (Zhao et al., 2006). At this stage, immature neurons have a higher intracellular concentration of chloride ions, and unlike mature neurons depolarize in response to GABA (Ge et al., 2006).

Approximately 8 to10 days later, axons of immature neurons reach CA3 pyramidal neurons and begin synaptic interactions with them. At about 16 days after birth, dendritic spines of immature neurons begin to grow (Zhao et al., 2006). Interestingly, the immature neurons have a much lower threshold for long-term potentiation compared to the existing older granule neurons (Schmidt-Hieber et al., 2004). The excitatory action of GABA on newborn neurons changes to inhibitory at approximately 2 to 4 weeks after birth. Glutamatergic synapses develop once the dendritic branches of newborn neurons cross the molecular cell layer of the dentate gyrus (Ambrogini et al., 2004). The density and complexity of spines, however, continue to increase for several months (Zhao et al., 2006). The inhibitory GABAergic inputs probably precede excitatory glutamatergic inputs to allow integration of newborn neurons into the existing circuit without the risk of excitation-induced neurotoxicity (Lledo, Alonso, & Grubb, 2006).

It has been estimated that about 9000 new BrdU-labeled cells are generated in the adult rat dentate gyrus every day (Cameron & McKay, 2001). However, half of the newborn neurons die within the first 3 weeks after birth. If they survive this critical period, they can survive for at least 11 more months in the dentate gyrus of the rodents (Kempermann, Gast, Kronenberg, Yamaguchi, & Gage, 2003) and for at least 2 more years in the human dentate gyrus (Eriksson et al., 1998). At 4 weeks of age, newborn neurons start expressing neuron-specific nuclear protein (NeuN), a marker of mature neurons (Ming & Song, 2005). Newborn granular neurons integrate functionally into the existing neuronal circuit in the dentate gyrus, receiving inputs from the perforant path and sending the outputs to CA3 pyramidal cells (van Praag et al., 2002). At 4 months of age, they morphologically (Zhao, Deng, & Gage, 2008) resemble existing mature neurons in

the granular cell layer, suggesting that newly generated neurons might be involved in hippocampal activity. In fact, recent studies that have relied on activity-dependent immediate-early gene expression have provided the most convincing support for the involvement of newborn dentate gyrus neurons in cognitive processes and their relevance to hippocampal activities (Kee, Teixeira, Wang, & Frankland, 2007; Ramirez-Amaya, Marrone, Gage, Worley, & Barnes, 2006).

Adult hippocampal neurogenesis and cognition

Several studies suggest that the number of newborn neurons in the adult dentate gyrus correlates with learning abilities (e.g., see Kempermann, Kuhn, & Gage, 1997b; van Praag, Christie, Sejnowski, & Gage, 1999). Factors that have been shown to affect adult hippocampal neurogenesis have also been found to influence learning and memory. For example, survival of newborn granule neurons has been shown to increase considerably in adult mice that lived in an enriched environment (Kempermann, Kuhn, & Gage, 1997a). Cell proliferation in the dentate gyrus of adult mice is also significantly enhanced by voluntary wheel running (van Praag, Kempermann, & Gage, 1999). Both enriched environment (Kempermann et al., 1997a) and voluntary exercise (van Praag, Christie et al., 1999) have been shown to enhance spatial navigation in the Morris water maze, a hippocampal-dependent task. On the other hand, stress (Tanapat, Hastings, Rydel, Galea, & Gould, 2001), high levels of glucocorticoids (Cameron & Gould, 1994), and aging (McDonald & Wojtowicz, 2005)--all associated with a reduction of adult hippocampal neurogenesis--are also associated with impairments in hippocampal-dependent learning tasks (Bodnoff et al., 1995; Conrad, Galea, Kuroda, & McEwen, 1996; Drapeau et al., 2003). However, learning ability in the hidden platform version of

the water maze was reported not to be predictable by the number of newborn cells in the dentate gyrus of aged male Long-Evans (Bizon & Gallagher, 2003; Bizon, Lee, & Gallagher, 2004) and aged female Fischer 344 rats (Merrill, Karim, Darraq, Chiba, & Tuszynski, 2003). That is, although aging significantly reduced the rate of neurogenesis, there was no correlation between the number of newborn cells and spatial learning performance.

As described earlier in this chapter, *Ginkgo* treatment has been shown to improve cognitive abilities in rodents. For example, chronic *Ginkgo* pre-treatment has been shown to enhance acquisition and performance of a two-lever response sequence task as well as retention of a learned response (E. Winter, 1991), improve learning in an eight-arm radial maze task, enhance learning in a delayed non-matching to position task (J. C. Winter, 1998) and promote learning in a passive avoidance task (Stoll et al., 1996). Moreover, chronic *Ginkgo* treatment has been shown to prevent spatial memory deficits in aged rats (Y. Wang et al., 2006) and in a transgenic mouse model (Tg 2576) of Alzheimer's disease (Stackman et al., 2003). The exact mechanism by which *Ginkgo* enhances cognitive abilities is not fully understood, but if the number of newborn neurons in the adult dentate gyrus correlates positively with learning abilities, then *Ginkgo* treatment might also lead to an enhancement of adult hippocampal neurogenesis.

Related to this point, it is noteworthy that pharmacological manipulation of the cholinergic system--which no doubt plays an important role in cognitive function--has also been found to modify adult hippocampal neurogenesis. For example, it has been shown that chronic (4-week) exposure to the selective acetylcholinesterase inhibitor treatment donepezil significantly increased neurogenesis, while scopolamine

administration significantly decreased this effect. The effects were specific to survival of newborn neurons in the dentate gyrus as neither donepezil nor scopolamine treatment influenced cell proliferation in the dentate gyrus (Kaneko et al., 2006; Kotani et al., 2006, 2008). Thus, it is possible that *Ginkgo* may promote adult hippocampal neurogenesis by modulating cholinergic system activity, which in turn improves cognitive function. In fact, *in utero* exposure to *Ginkgo* has been shown to enhance neuronal survival and growth of the developing hippocampus. During gestational days 12 to 16, pregnant dams were injected with EGb 761 (100 or 300 mg/kg). The hippocampi of the fetuses were dissected and cultured at embryonic day 17. After 8 days, neuronal cells obtained from the EGb-treated group were more viable and had more branches compared to controls. Using a gene microarray assay it was also demonstrated that prenatal exposure to EGb 761 (25, 50 or 100 mg/kg for 5 days) modified expression of a number of specific genes that are mainly involved in the development, maturation and repair of neuronal cells (Li et al., 2003).

Furthermore, transcription of a gene that encodes for transthyretin has been found to increase more than three-fold in the hippocampus of adult mice fed a diet supplemented with EGb 761 for 4 weeks (Watanabe et al., 2001). Transthyretin protein is of particular interest given its involvement in the transport of the thyroid hormone thyroxine to cerebrospinal fluid and serum (Kuchler-Bopp, Dietrich, Zaepfel, & Delaunoy, 2000). Thyroid hormones have also been shown to play roles in the regulation of adult hippocampal neurogenesis (Ambrogini et al., 2005). Taken together, these studies suggest that *Ginkgo* may have a proliferative- and survival-promoting effect on hippocampal neurons. However, whether or not *Ginkgo* enhances survival as well as

proliferation of newborn granular cells--which are continuously generated in the dentate gyrus of adult brain--remains a matter of investigation.

The purpose of the present dissertation

The purpose of the present dissertation was twofold. Firstly, it aimed to identify neural sites involved in the cognitive enhancing effects of *Ginkgo,* and explore its possible neurogenic-enhancing properties on adult hippocampal neurogenesis. Secondly, the present dissertation aimed to examine separately the putative cognitive enhancing effects of *Ginkgo* on learning and on memory of a working memory task using a double Y-maze.

Using c-Fos immunohistochemistry, the experiments described in Chapter 2 characterized the neural effects of *Ginkgo* by quantifying functional activity in several brain regions known to be involved in learning and memory in rats. Furthermore, as described above, several lines of evidence strongly suggest that *Ginkgo* may enhance adult hippocampal neurogenesis. First, *Ginkgo* positively modulates activity of the cholinergic system (Chopin & Briley, 1992; Das et al., 2002; Kristofikova et al., 1992; Taylor, 1986; Yamamoto et al., 2007), which is involved in the regulation of adult hippocampal neurogenesis (Cooper-Kuhn et al., 2004; Kaneko et al., 2006; Kotani et al., 2006, 2008). Second, the ability of *Ginkgo* to enhance microcirculation (Krieglstein et al., 1986) and proliferation of endothelial progenitor cells (Chen et al., 2004) may suggest its neurogenic potential. Newborn cells in the granule cell layers reside in close proximity to fine capillaries and in fact, 37% of all dividing cells have been shown to be immunoreacive for endothelial markers (Palmer et al., 2000). Third, *in utero* exposure to *Ginkgo* enhances neuronal survival and growth in the developing hippocampus and

modifies expression of specific genes that are involved in development, maturation and repair of neuronal cells (Li et al., 2003). Fourth, long-term rather than short-term *Ginkgo* treatment is often associated with improved learning. This corresponds to the hypothesis that *Ginkgo* enhances neurogenesis, as newborn neurons require several weeks before they integrate into the neuronal circuitry and become fully functional (van Praag et al., 2002). Thus the experiments presented in Chapter 3 evaluated the possible neurogenic properties of *Ginkgo*. Because *Ginkgo* treatment has been shown to reduce basal and stress-induced anxiety, the experiments described in Chapter 3 were supplemented with behavioural tests of anxiety. A battery of behavioural measures including the light/dark emergence, elevated plus maze and social interaction tests were employed to assess the acute and chronic effects of *Ginkgo* on anxiety.

As previously reviewed, *Ginkgo* treatment counteracts the effects of glucocorticoids by reducing levels of messenger RNA protein and drug binding of adrenocortical mitochondrial peripheral-type benzodiazepine receptors (Amri et al., 1996). For example, it normalizes elevated levels of circulating corticosterone induced by stress (Rai et al., 2003; Rapin et al., 1994; Shah et al., 2003). In addition, it has recently been shown that chronic *Ginkgo* treatment enhances cell proliferation in young and aged transgenic mice believed to be a model of Alzheimer's disease, as well as in aged wild type control mice (Tchantchou, Xu, Wu, Christen, & Luo, 2007). These studies raise the possibility that *Ginkgo* may have different effects in the normal healthy nervous system, compared to in dysfunctional nervous systems where the constitutive level of neurogenesis has been down-regulated. To examine this possibility, experiments in

Chapter 4 investigated whether corticosterone-induced suppression of hippocampal neurogenesis could be restored by *Ginkgo* co-administration.

Several well-designed studies evaluating the cognitive-enhancing abilities of *Ginkgo* on rodents were summarized above. In most of those studies, *Ginkgo* treatment normally began weeks or months prior to the training sessions. Thus, it is not clear whether *Ginkgo* is effective in improving acquisition or memory of a task without prior chronic treatment. It is not also clear whether ongoing *Ginkgo* treatment offers any continued beneficial effects in an already-learned working memory task. Thus, the first experiment described in Chapter 5 sought to evaluate the effectiveness of *Ginkgo* on acquisition and performance of a double Y-maze task in young adult rats. Moreover, the effect of *Ginkgo* on memory, per se, is not clear since in most previous studies *Ginkgo* treatment was initiated prior to and/or during the acquisition phase. Thus, the second experiment in Chapter 5 aimed to examine the acute and chronic effects of *Ginkgo* directly on working memory function by administrating *Ginkgo* in the interval between acquisition and testing.

Foreword to Chapter 2

The following chapter is based on a manuscript submitted to the journal *Phytomedicine* in April 2008. The manuscript was accepted for publication in August 2008 and is now published (Apr 2009, vol 16, pg 361-368). Dr. Paul E. Mallet and his students Clinton A. Moore and Melissa T. Collie are co-authors. Brains were collected from animals treated at the University of New England, Australia, and the immunohistochemical processing was conducted by Melissa Collie and Clinton Moore. The author of the present dissertation conducted the microscopic quantification of labelled nuclei, and conducted preliminary data analyses. The first draft of the manuscript was written by Dr. Mallet.

Chapter 2: Regional distribution of *Ginkgo biloba*-induced c-Fos immunoreactivity

Abstract

A growing literature supports the notion that *Gingko biloba* has cognitive enhancing and anxiolytic properties; however, its effects on neuronal populations have yet to be characterized. The present study used c-Fos immunoreactivity (Fos-IR) to characterize neuronal activity in selected brain regions following administration of a standardized *Ginkgo biloba* extract. Because *Ginkgo* is typically consumed orally, Experiment 1 sought to identify patterns of neural activity induced by oral administration. To ensure that the alterations in functional neural activity observed in Experiment 1 were not simply due to novel gustatory experience, Experiment 2 characterized patterns of Fos-IR following intraperitoneal administration of *Ginkgo*. Rats were habituated to handling and experimental conditions. In Experiment 1, rats self-administered 150 mg/kg *Ginkgo* or vehicle alone (strawberry jam) orally. In Experiment 2, rats were injected with *Ginkgo* (2.5 or 25 mg/kg, i.p.) or vehicle (0.3% gum Arabic). Animals were anaesthetized and perfused transcardially. Brains were sectioned; immunostained using a c-Fos antibody, then the number of labeled cells was quantified microscopically in selected brain regions. In both experiments *Ginkgo* increased Fos-IR in numerous brain regions including the insular cortex and amygdala. Intraperitoneal administration induced Fos-IR in some additional regions including the nucleus accumbens and dentate gyrus. Results provide important preliminary data serving to identify several candidate neural sites involved in the cognitive enhancing and anxiolytic effects of *Ginkgo biloba.*

EGb 761 is a water-acetone extract of dried *Ginkgo biloba* leaves, standardized to contain 24% flavonoid glycosides, 6% terpenoids, and less than 9% proanthocyanidins and organic acids (DeFeudis & Drieu, 2000; Maclennan et al., 2002). Extracts of *Gingko biloba,* such as EGb 761, are commonly used to increase blood circulation peripherally and centrally, and to protect the lipid portion of cellular membranes against damage induced by free radicals (reviewed by Ahlemeyer & Krieglstein, 2003). A growing literature supports the notion that *Gingko biloba* has cognitive enhancing properties. For example, studies using rats have shown that *Gingko biloba* facilitates olfactory recognition (Wirth et al., 2000), enhances performance in a radial maze (J. C. Winter, 1998), and facilitates learning in a water maze (Y. Wang et al., 2006). In humans, extracts of *Gingko biloba* have been found to improve cognitive function in young healthy individuals (Stough et al., 2001), in the elderly (Winther, Randlov, Rein, & Mehlsen, 1998), and in patients with Alzheimer's disease (Maurer, Ihl, Dierks, & Frolich, 1997; Oken et al., 1998). Studies also generally support the notion that extracts of *Ginkgo biloba* reduce basal (Kuribara et al., 2003) or stress-induced anxiety (Ward et al., 2002), and attenuate the cognitive impairments induced by exposure to stress (Walesiuk & Braszko, 2009; Walesiuk et al., 2005; Walesiuk, Trofimiuk, & Braszko, 2006).

The neural mechanisms responsible for the cognitive and behavioural effects of *Ginkgo biloba* extracts are poorly understood; however, several candidate modes of action have so far been identified. Administration of *Ginkgo biloba* dose-dependently inhibits acetylcholinesterase *in vitro* (Das et al., 2002), and increases cortical blood flow *in vivo* (Krieglstein et al., 1986). In aged rats, oral administration of an extract of *Ginkgo biloba* increases the density of hippocampal muscarinic receptors (Taylor, 1986). Other

neurotransmitter systems may also be affected by exposure to *Ginkgo biloba* including adrenergic, dopaminergic, serotonergic, glutamatergic and GABAergic systems (reviewed by Ahlemeyer & Krieglstein, 2003). Hippocampal electrophysiological alterations have also been noted. For example, EGb 761 was found to reduce the population spike threshold and increase the early phase of long-term potentiation in aged mouse hippocampal slices (Williams et al., 2004). Furthermore, administration of EGb 761 to aged rats increased the magnitude of inducible long-term potentiation in the CA1 region (Y. Wang et al., 2006).

The aim of this study was to further characterize the neural effects of an extract of *Ginkgo biloba* by using c-Fos immunoreactivity (Fos-IR) in rats to quantify functional activity in several brain regions known to be involved in learning and memory. Expression of the immediate early gene *c-fos* is largely dependent on synaptic stimulation (reviewed by Kaczmarek & Chaudhuri, 1997). The immunohistochemical localization of c-Fos can therefore serve as a useful neurobiological tool to map functional activity. Because *Ginkgo biloba* is typically consumed orally, the first experiment sought to identify patterns of neural activity induced by orally self-administered *Ginkgo*. To control for the influence and novel gustatory experience on *c-fos* expression following oral administration, a second experiment served to characterize patterns of Fos-IR following parenteral *Ginkgo* administration.

Methods

Animals were treated in accordance with the National Health and Medical Research Council's "Australian code of practice for the care and use of animals for scientific purposes" (7th edition, 2004) and the "Principles of laboratory animal care"

(National Research Council, 1996). Approval for this research was obtained from the University of New England Animal Ethics Committee.

Subjects. Forty male albino Wistar rats (16 for Experiment 1 and 24 for Experiment 2, mean weight 320 g), bred at the University of New England, were group-housed in a temperature-controlled room (21 °C) maintained on a 12:12 h reversed light: dark cycle (lights on at 1900 hours). Experiments were conducted during the dark cycle. Animals had free access to lab chow (Barastoc, Ridley AgriProducts, Australia) and tap water throughout the study. Rats were handled for 7 days prior to the commencement of the experiment.

Ginkgo 761 Preparation and Administration. An injectable solution was prepared by mixing the *Ginkgo biloba* extract EGb 761 (*Ginkgoselect*, Indena S.p.A, Milan, Italy) in 0.3% Gum Arabic (Sigma-Aldrich, Castle Hill, NSW). The solution was stored at 4 °C until needed. *Ginkgoselect* is composed of EGb 761--standardized to contain 24% ginkgoflavonglucosides, 6% ginkgolides and bilobalide, and less than 5 ppm ginkgolic acids--complexed with soy phospholipids at a ratio of 1:2 (w/w) to increase bioavailability. Injections were administered intraperitoneally in a volume of 2 ml/kg body weight at doses of 2.5 and 25 mg/kg body weight. Doses are expressed to reflect the EGb 761 content of the phospholipid complex. The i.p. administration of a similar dose of Ginkgoselect was found to enhance acquisition of a double Y-maze task (Satvat & Mallet, 2009).

A *Ginkgo biloba* suspension was also prepared for oral consumption using commercial water-soluble EGb 761 tablets (AIM *Ginkgo* 3000™, AIM International,

Somerton Victoria, Australia). Tablets were crushed into a fine powder and mixed with strawberry jam (Savings brand, Coles Myer Ltd., Tooronga, Victoria, Australia) such that each g of jam contained 30 mg EGb 761. Rats were fed 5 g jam per kg body weight per dose, yielding an EGb 761 dose of 150 mg/kg.

Procedure.

EXPERIMENT 1: ORAL ADMINISTRATION

To reduce flavor neophobia, a glass dish containing 30 g strawberry jam without *Ginkgo* was placed in each group home cage containing six rats on each of 2 consecutive days.

The next phase of the experiment, lasting 21 days, served to reduce *c-fos* expression in response to novelty, handling, and experimental procedures. Each day animals were weighed and placed individually in 40 × 30 × 25 cm seclusion cages for 3 h, in a dimly lit (two 40 W incandescent red lamps) quiet room with soft pink noise (noise generator model K2135, Altronics, Perth, WA, Australia). Seclusion cages contained a dish with 5 g/kg strawberry jam, which later served as the vehicle for *Ginkgo*. Placement in the seclusion cages was staggered to habituate the animals to the experimenter entering the room at 10 min intervals, which was required for the precise timing of the perfusions later in the experiment.

On the next day the experiment was conducted as usual, except that the strawberry jam was replaced with the *Ginkgo* jam mixture for half the rats (n=8). At the end of the 3-h seclusion session, rats were deeply anesthetized with sodium pentobarbital (120 mg/kg, i.p.), and then perfused transcardially first with 100 ml phosphate-buffered

saline (0.9%, pH 7.2), then with 150 ml phosphate-buffered paraformaldehyde (4%, pH 7.2). To ensure group differences in *c-fos* expression were not related to circadian rhythms, odd-numbered rats in the sequence were assigned to the vehicle group, and even-numbered rats in the sequence were assigned to the *Ginkgo* group.

EXPERIMENT 2: INTRAPERITONEAL ADMINISTRATION

On each of 4 consecutive days, animals were weighed, injected with 0.3% gum Arabic (i.p.), then placed individually in 40 × 30 × 25 cm seclusion cages in a dimly-lit quiet room (details above) for 2 h. On the following day, the experiment was conducted as usual, except that rats were injected with the gum Arabic vehicle (n=8), 2.5 mg/kg *Ginkgo* (n=8), or 25 mg/kg *Ginkgo* (n=8) prior to the 2-h seclusion session. Rats were then anesthetized and perfused as described above. To ensure that group differences in Fos-IR were not related to circadian rhythms, the order of treatments was counterbalanced such that one rat from each group was perfused every 45 minutes (i.e., one perfusion was performed every 15 min).

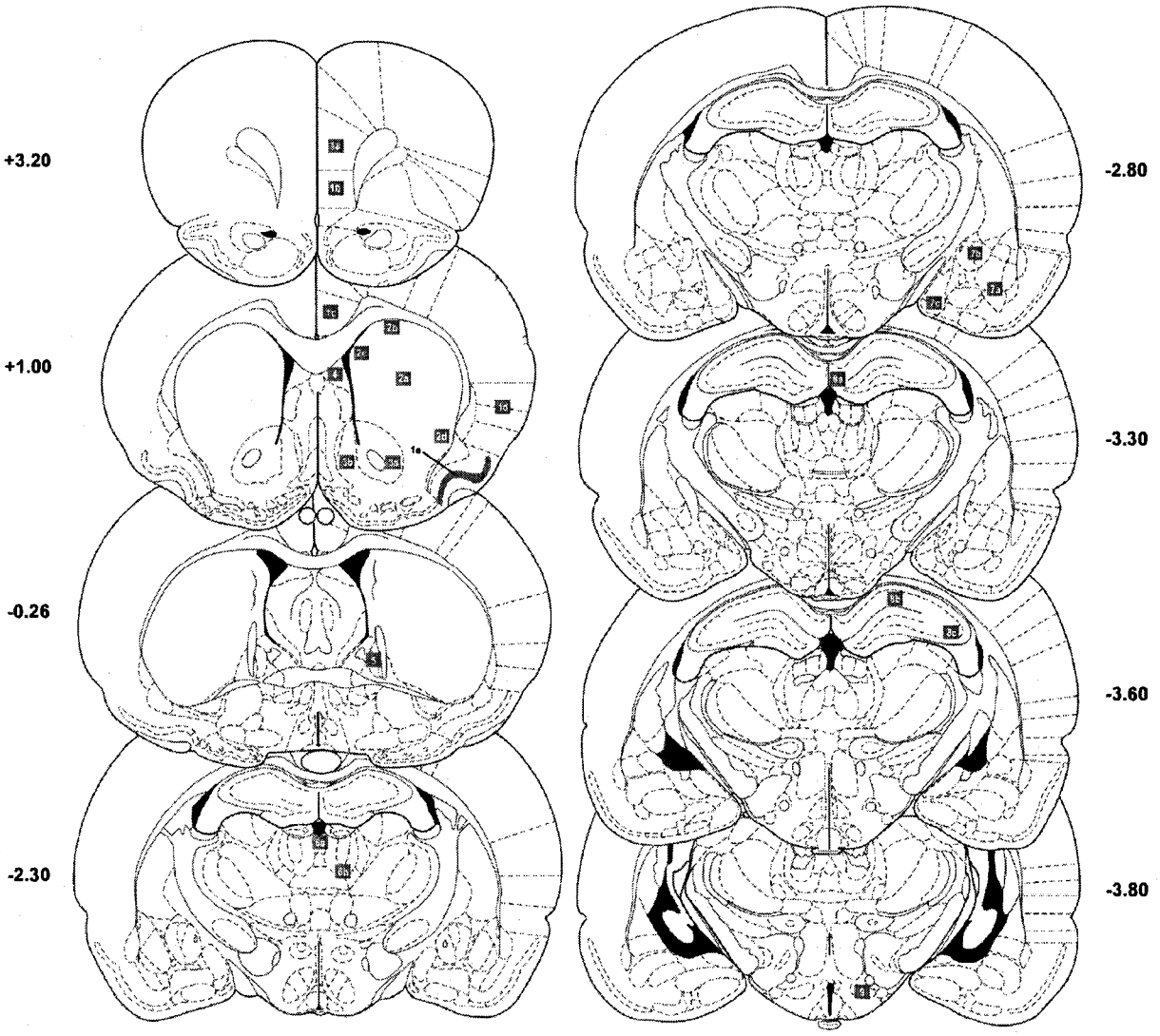
Immunochemistry. Immunohistochemical staining was performed in a manner similar to that described earlier (Singh, McGregor, & Mallet, 2005, 2006). Following perfusions, brains were extracted and post-fixed in 4% phosphate-buffered paraformaldehyde (pH 7.2) for 48 h at 4 °C, then placed in 15% w/v phosphate-buffered sucrose (pH 7.2) for 24 h at 4 °C, and then finally transferred into 30% w/v phosphate-buffered sucrose for 48-72 h at 4 °C. Brains were sectioned coronally at 40 µm using a cryostat (Leica CM1850), and the sections were collected in phosphate buffer (PB, pH 7.2).

Free-floating sections were washed in 0.9% hydrogen peroxide for 30 min to inhibit endogenous peroxidase, and then were bathed for 30 min in 3% phosphate-buffered goat serum. Sections then were incubated for 72 h at 4 °C in the primary c-Fos antibody (s52, Santa Cruz Biotechnology, Santa Cruz, CA; rabbit polyclonal, specific for the amino acid terminus of c-Fos p62, non cross-reactive with FosB, Fra-1 or Fra-2) diluted 1:2000 in phosphate buffered goat serum (PBG, 0.1% bovine serum albumin, 0.2% Triton X-100, 2% normal goat serum in PB). Sections were washed for 30 min in PB, then were placed in biotinylated anti-rabbit IgG (Vector Laboratories, Burlingame, CA) diluted 1:500 in PBG for 60 min at room temperature. Next, the tissue was washed in PB for 30 min, and subsequently incubated for 60 min in extrAvidin-horseradish peroxidase (Sigma-Aldrich, Castle Hill, NSW) diluted 1:1000 in PBG. After three additional 30-min washes in PB, horseradish peroxidase activity was visualized using the nickel diaminobenzidine and glucose oxidase reaction with nickel enhancement, as described by Shu et al. (1988). The reaction was terminated 10 min later by washing in PB. Sections were mounted onto gelatine-coated slides, dehydrated in ethanol, cleared with dipentene (Histolene, Fronine Laboratory Supplies Pty Ltd, Riverstone, NSW, Australia), and coverslipped.

Microscopy. Each microscope slide was coded to ensure "blind" counting. A total of 20 brain regions were examined using a Leica DMR upright light microscope at approximately 200× magnification. The atlas of Paxinos and Watson (1997) was used to identify the brain regions shown in Figure 1. A 10 x 10 square graticule was positioned over a standardized region of each structure and the number of labeled nuclei within the graticule, which covered a 500 × 500 μm area, was counted manually. Only round and

Figure 1. Schematic diagrams of coronal sections of the rat brain (adapted from Paxinos & Watson, 1997). The number of c-Fos immunoreactive nuclei was quantified within the areas numbered and shaded in gray (shown to scale). Labels correspond to the brain regions listed in Table 1.

Figure 1



oval nuclei that were completely black were counted. Structures examined included the caudate-putamen (CPu): central, dorsal, dorsomedial, and ventrolateral regions; cortex: cingulate, insular and piriform regions; nucleus accumbens (nAcc): core and shell regions; lateral septal nucleus, ventral part (LSV); bed nucleus of the stria terminalis, lateral division, dorsal region (BNST); thalamus: paraventricular nucleus (PV) and dorsomedial; amygdala: basolateral nucleus (BNA), central nucleus (CEA), and medial nucleus (MNA); hippocampal formation: dentate gyrus, CA1 and CA3 regions; and the premammillary nucleus.

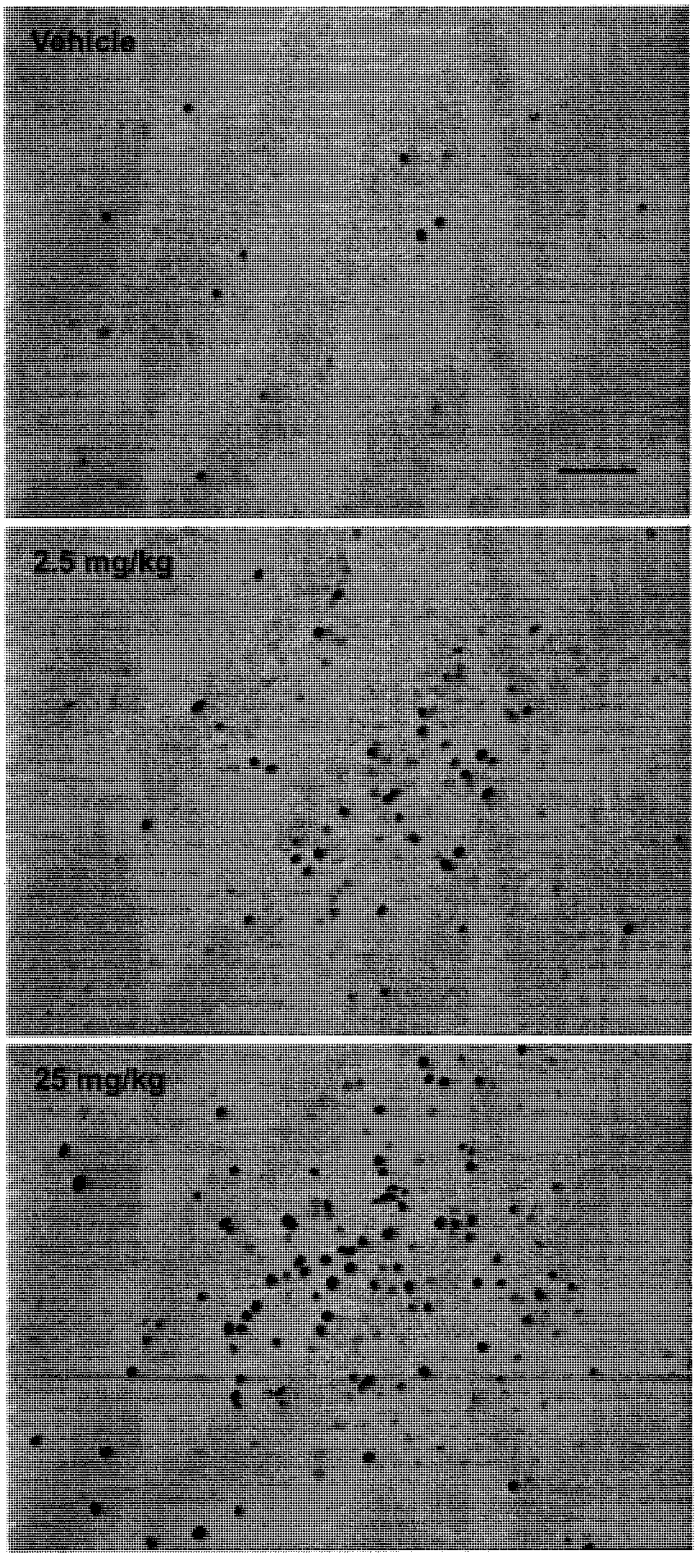
Statistical Analysis. In Experiment 1 (oral administration), the number of c-Fos immunoreactive cells was compared between groups using unpaired *t*-tests. In Experiment 2 (i.p. administration), the number of labeled cells was compared between groups using one-way analyses of variance. (ANOVA). When a significant ANOVA was found, groups were further compared using post hoc Tukey tests. Because the homogeneity of variance and normality assumptions were frequently violated, independent groups randomization tests were also conducted using the program NPFact (May, Hunter, & Gabriel, 1993) to confirm the outcome of the parametric tests.

Results

The numbers of c-Fos immunoreactive cells for each brain region examined are shown in Table 1. In addition, representative photomicrographs of c-Fos immunoreactive nuclei are presented in Figure 2. In all cases but one, the randomization tests yielded the same outcome as the parametric tests, so for ease of interpretation only the outcome of the randomization tests are shown. In one case the ANOVA was marginally significant,

Figure 2. Photomicrographs showing c-Fos immunoreactive cells in the central nucleus of the amygdala from representative animals treated with vehicle, 2.5 mg/kg or 25 mg/kg *Ginkgo* from Experiment 2. Scale bar is 100 μm.

Figure 2



but the randomization test was not. Because the ANOVA homogeneity of variance assumption was violated, the non-significant outcome of the randomization test was adopted.

Oral administration of *Ginkgo* (Experiment 1) increased Fos-IR moderately, but significantly, in the insular cortex (t_{13}=2.67, P<0.05) and amygdala (basolateral and central nuclei; t_{14}=5.56, P<0.001 and t_{14}=5.56, P<0.05, respectively). The i.p. administration of *Ginkgo* (Experiment 2) also significantly increased Fos-IR in several brain regions including the prelimbic ($F_{2,21}$=8.18, P<0.01) and insular cortices ($F_{2,21}$=4.38, P<0.05), nAcc shell ($F_{2,21}$=4.01, P<0.05), LSV ($F_{2,21}$=4.71, P<0.05), BNST ($F_{2,21}$=5.29, P<0.05), PV ($F_{2,21}$=7.62, P<0.01), CEA ($F_{2,21}$=7.09, P<0.01), and dentate gyrus ($F_{2,21}$=2.62, P<0.05). Outcomes of the post hoc pairwise comparisons are presented in Table 1

Discussion

Results revealed that both oral and parenteral administration of *Ginkgo* increased *c-fos* expression in selected limbic and related structures, suggesting that *Ginkgo* increases neural activity in a region-specific manner. *Ginkgo* significantly increased Fos-IR in the insular cortex and CEA using both routes of administration. However, for some brain regions the pattern of neural activation differed depending on the route of administration. For example, Fos-IR was significantly increased in the PV, nAcc shell, and dentate gyrus following i.p. administration, but not following oral administration of *Ginkgo*. Conversely, Fos-IR was increased significantly in the BNA by oral, but not i.p. administration of *Ginkgo*. This should not be taken as evidence that different routes of administration produce unique neural (and consequently behavioural) effects. Indeed a

Table 1.

Region	Bregma	Experiment 1: Oral		Experiment 2: Intraperitoneal		
		Vehicle	150 mg/kg	Vehicle	2.5 mg/kg	25 mg/kg
1. Cortex						
a. Prelimbic	+3.20	43.2 ± 16.0	71.4 ± 17.0	35.0 ± 9.0	32.5 ± 7.0	75.0 ± 8.9 **, ††
b. Infralimbic	+3.20	52.8 ± 18.0	86.3 ± 25.1	42.5 ± 13.0	40.0 ± 9.5	52.5 ± 6.6
c. Cingulate	+1.00	26.9 ± 6.2	31.5 ± 6.6	31.0 ± 10.7	25.0 ± 5.2	36.5 ± 3.4
d. Insular	+1.00	29.7 ± 4.4	103.0 ± 25.3 *	15.0 ± 6.9	15.5 ± 3.0	38.5 ± 8.2 *, †
e. Piriform	+1.00	27.4 ± 4.6	33.5 ± 4.1	28.1 ± 3.6	29.9 ± 8.3	39.0 ± 7.1
2. Caudate-putamen						
a. Central region	+1.00	1.1 ± 0.7	7.0 ± 4.4	0.5 ± 0.5	4.0 ± 3.5	9.5 ± 3.6
b. Dorsal region	+1.00	2.3 ± 1.5	2.5 ± 1.1	0.0 ± 0.0	0.0 ± 0.0	2.5 ± 1.3
c. Dorsomedial region	+1.00	10.3 ± 3.1	13.5 ± 1.7	4.5 ± 2.1	3.0 ± 1.6	4.0 ± 1.1
d. Ventrolateral region	+1.00	6.3 ± 4.4	11.5 ± 3.6	1.5 ± 1.1	2.5 ± 2.0	12.0 ± 5.4
3. Nucleus accumbens						
a. Core region	+1.00	8.0 ± 3.6	26.0 ± 6.9	3.0 ± 2.5	2.0 ± 1.1	3.5 ± 1.2
b. Shell region	+1.00	19.4 ± 2.7	25.5 ± 6.1	8.5 ± 2.6	12.5 ± 4.0	29.0 ± 8.1 *
4. Lateral septal nucleus, ventral part	+1.00	46.9 ± 19.5	53.0 ± 11.5	20.0 ± 7.6	25.5 ± 8.0	58.5 ± 12.4 *
5. BNST lateral division, dorsal	–0.26	33.7 ± 10.0	53.3 ± 12.6	5.5 ± 1.3	24.5 ± 6.0 *	26.5 ± 6.2 *
6. Thalamus						
a. Paraventricular nucleus	–2.30	121.7 ± 19.0	167.5 ± 16.9	69.5 ± 14.2	98.5 ± 17.6	163.5 ± 20.1 **, †
b. Dorsomedial	–2.30	14.9 ± 7.8	23.5 ± 5.6	2.0 ± 0.8	3.0 ± 2.5	1.0 ± 0.7
7. Amygdala						
a. Basolateral nucleus	–2.30	15.5 ± 4.0	47.5 ± 4.2 **	8.0 ± 3.1	7.0 ± 2.2	10.0 ± 2.4
b. Central nucleus	–2.30	44.0 ± 14.8	100.0 ± 15.4 *	17.5 ± 4.6	28.0 ± 9.6	72.0 ± 15.5 **, †
c. Medial nucleus	–2.30	23.0 ± 5.2	36.0 ± 7.4	7.5 ± 3.2	19.0 ± 5.5	22.0 ± 6.4
8. Hippocampal formation						
a. Dentate gyrus	–3.30	21.5 ± 5.5	36.5 ± 5.6	4.5 ± 2.3	15.0 ± 5.1	31.0 ± 9.2 **
b. CA1	–3.60	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.5 ± 0.5
c. CA3	–3.60	6.5 ± 2.0	9.1 ± 2.1	0.0 ± 0.0	0.5 ± 0.5	0.0 ± 0.0
9. Pre-mammillary nucleus, ventral	–3.80	7.0 ± 3.8	16.0 ± 4.6	10.0 ± 2.9	10.5 ± 4.4	27.0 ± 7.2

direct comparison of these two routes of administration is not possible given differing pharmacodynamics and the necessary procedural differences required to vary the route of administration (e.g., dose, injection, exposure to jam). Rather, Experiment 1 served to characterize neural activity induced by oral administration given that this is the most commonly employed route of administration, while Experiment 2 served to demonstrate that many of the observed changes in Fos-IR found in Experiment 1 cannot be simply explained by novel gustatory experience (i.e., a noticeable change in jam flavour by the addition of *Ginkgo*).

Growing evidence suggests a critical role for the CEA and insular cortex in learning and memory. For example, the insular cortex has been shown to be involved in incentive memory (Balleine & Dickinson, 2000), the consolidation of object memory (Bermudez-Rattoni, Okuda, Roozendaal, & McGaugh, 2005), the acquisition and consolidation of inhibitory avoidance (Mello e Souza et al., 2001; Miranda & McGaugh, 2004), and the consolidation of conditioned taste aversion (Miranda & McGaugh, 2004). The CEA plays a critical role in the acquisition, consolidation, and expression of Pavlovian fear conditioning (Wilensky, Schafe, Kristensen, & LeDoux, 2006). Thus, the finding that acute administration of *Ginkgo* increased neural activity in the insular cortex, CEA, and dentate gyrus indicates that limbic structures may have potentially important roles in the long-term neurocognitive effects of *Ginkgo*.

The significant increase in Fos-IR found in the dentate gyrus is particularly interesting given the important role of the hippocampal formation in learning and memory (Squire, Stark, & Clark, 2004). It is not, however, clear why the increase in Fos-IR was specific to the i.p. route of administration, but this may have been related to the

different time courses and doses of *Ginkgo* used in the two experiments. That is, the time between administration of *Ginkgo* and perfusion was 1 h longer in the experiment using oral administration to allow for the slower rate of absorption using this route of administration. It is worth noting that although the regional activity in the dentate gyrus was not significantly increased by oral administration of *Ginkgo*, a non-significant trend (p=0.088) was observed. Furthermore, different doses were used in each experiment. In Experiment 1, the dose used was 150 mg/kg because pilot studies revealed that this dose approached the maximum concentration in the jam vehicle tolerated by rats. That is, higher concentrations were not well consumed, presumably because of an undesirable taste. In Experiment 2, doses were 2.5 and 25 mg/kg. The higher dose approached the maximum quantity of *Ginkgo* that could be suspended in vehicle using an injection volume of 2 ml/kg. Further studies comparing plasma and brain concentrations of *Ginkgo* constituents following both routes of administration and time courses would be useful to clarify this point.

The increase in Fos-IR in the PV and prelimbic cortex are noteworthy given the role of midline intralaminar nuclei in attention. Thus, PV efferents project to the amygdala, nucleus accumbens, and ventral aspects of the medial prefrontal cortex (reviewed by Huang, Ghosh, & van den Pol, 2006), all of which are limbic regions involved in motivation and attention. Fos-IR in the PV and prelimbic cortex was significantly increased by i.p. *Ginkgo*, but the increase following oral administration fell short of statistical significance in both cases. The diminished drug effect following oral administration despite a longer habituation period may be dose, route, or time course related, but is more likely due to the high baseline level of Fos-IR observed in the vehicle

group (oral consumption of jam), which may have been a consequence of consuming a highly palatable food. Support for this notion comes from the finding that PV *c-fos* expression is increased by anticipatory feeding (Nakahara, Fukui, & Murakami, 2004). It is therefore possible that at least some of the cognitive enhancing effects of *Ginkgo* may be attributed to enhanced cortical arousal and attention.

It is not possible to determine conclusively from the present results alone whether the increase in Fos-IR found in the BNA following oral consumption of *Ginkgo* was the result of a central pharmacological effect of *Ginkgo*, or was simply caused by the subjective experience of the novel taste of *Ginkgo*. However, the finding that Fos-IR was not increased significantly in this brain region by parenteral administration of *Ginkgo* suggests that taste novelty cannot be discounted. In support of this notion, it has been shown that amygdalar *c-fos* expression is increased by exposure to a novel saccharin solution in mice (Montag-Sallaz, Welzl, Kuhl, Montag, & Schachner, 1999) and rats (Koh, Wilkins, & Bernstein, 2003).

The nature of the neuroplastic changes responsible for the cognitive enhancing effects of chronic administration of *Ginkgo* have yet to be characterized. Nonetheless, results from the present study provide novel evidence that acute administration of *Ginkgo* induces neural activity in a region-specific manner. Given that the beneficial cognitive effects of *Ginkgo* are typically more pronounced following chronic dosing (reviewed by Muller & Chatterjee, 2003), it remains the goal of future research to determine whether chronic alterations in regional activity by *Ginkgo* induces longer-term neural plasticity responsible for cognitive improvements.

Chapter 3: Effects of *Ginkgo biloba* leaf extract on proliferation and survival of newborn cells in the dentate gyrus of the hippocampus in young adult male rats

Abstract

The present study aimed to explore the possible neurogenic-enhancing effects of a *Ginkgo biloba* leaf extract (*Ginkgo*) on adult hippocampal neurogenesis as a means to explain its cognitive-enhancing properties. The acute and chronic effects of *Ginkgo* on anxiety were also assessed using a battery of behavioural measures including the light/dark emergence, elevated plus maze and social interaction tests. In Experiment 1, to evaluate the acute effects of *Ginkgo* on anxiety, rats (n=12) were injected with *Ginkgo* (0, 2.5, 13.75, or 25 mg/kg), 30 min prior to behavioural testing. Two weeks later, to evaluate the acute effects of *Ginkgo* on hippocampal cell proliferation, half of the rats (n=6) in each group received a single *Ginkgo* (0, 2.5, 13.75, or 25 mg/kg) injection, followed by a single 5-bromo-2'-deoxyuridine (BrdU, 75 mg/kg) injection 30 min later. Animals were perfused 24 hours later. Administration of 13.75 and 25 mg/kg doses appeared to induce anxiety in the light/dark emergence test as indicated by increased duration of time spent in the hide box and longer exit latency. Locomotor activity was not significantly influenced by *Ginkgo*. Furthermore, cell proliferation in both the dorsal and ventral dentate gyrus was unaffected by acute *Ginkgo* injection. In Experiment 2, to evaluate the effects of *Ginkgo* on cell survival, rats (n=10) received 3 days of BrdU (50 mg/kg twice daily) injection. Twenty-four hours later, rats received daily injections of *Ginkgo* (0, 2.5, 13.75, or 25 mg/kg) for 21 days. On day 22, anxiety was assessed and rats were perfused the next day. *Ginkgo* had no significant effects in all behavioural tests. In addition, chronic *Ginkgo* treatment did not influence cell survival in either the dorsal or the ventral dentate gyrus. In sum, results revealed that both acute and chronic administration of varying doses of *Ginkgo* had no effect on hippocampal cell proliferation

or survival. *Ginkgo* may exert its nootropic effects in young rats via mechanisms other than enhancement of adult hippocampal neurogenesis.

The *Ginkgo* extract EGb 761 contains 24% flavonoid glycosides, 6% terpenoids, and less than 9% proanthocyanidins and organic acids (Chan et al., 2007; DeFeudis & Drieu, 2000; Maclennan et al., 2002; Ponto & Schultz, 2003; Zimmermann et al., 2002). Each of these active components has its own unique pharmacology, making it difficult to identify a specific mechanism that may contribute to its beneficial effects on cognitive function. In fact, the favorable effects of the extract on cognition are believed to be the result of agonistic, antagonistic and synergistic effects of its various constituents (DeFeudis & Drieu, 2000).

The flavonoid and proanthocyanidins components possess antioxidant and free radical scavenging properties (DeFeudis & Drieu, 2000), and the ginkgolide components antagonize the effects of platelet activating factor and improve cerebral metabolism (Braquet & Hosford, 1991). The bilobalide fraction of *Ginkgo* has neuroprotective properties and has been shown to protect neuronal damage induced by ischemia (Krieglstein et al., 1995). In addition, *Ginkgo* has been shown to protect cellular membrane fluidity (Stoll et al., 1996), and to enhance cerebral blood flow and microcirculation (Krieglstein et al., 1986), properties that have been suggested to underlie its cognitive-enhancing effects (Drieu & DeFeudis, 2000).

The involvement of *Ginkgo* on promoting adult hippocampal neurogenesis, as an additional mechanism of action responsible for its pro-mnemonic effects, has not been evaluated. Given that the number of newborn dentate gyrus neurons has been shown to correlate with learning abilities (e.g., see Kempermann et al., 1997b; van Praag, Christie et al., 1999), and that newborn neurons are involved in cognitive functioning (Kee et al., 2007; Ramirez-Amaya et al., 2006), it was hypothesized that the beneficial effects of

Ginkgo on learning may, at least to some extent, be attributed to its neurogenic potential; that is, *Ginkgo* may enhance learning by positively affecting proliferation and/or survival of newborn neurons in the dentate gyrus of the adult hippocampus. Several lines of evidence support this hypothesis. First, *Ginkgo* positively modulates activity of the cholinergic system (Chopin & Briley, 1992; Das et al., 2002; Kristofikova et al., 1992; Taylor, 1986; Yamamoto et al., 2007), which is involved in the regulation of adult hippocampal neurogenesis (Cooper-Kuhn et al., 2004; Kaneko et al., 2006; Kotani et al., 2006, 2008). Second, the ability of *Ginkgo* to enhance microcirculation (Krieglstein et al., 1986) and proliferation of endothelial progenitor cells (Chen et al., 2004) may suggest its neurogenic potential. Newborn cells in the granule cell layers are in close proximity to fine capillaries and in fact, 37% of all dividing cells have been shown to be immunoreacive for endothelial markers (Palmer et al., 2000). Third, *in utero* exposure to *Ginkgo* enhances neuronal survival and growth in the developing hippocampus and modifies expression of specific genes that are involved in development, maturation and repair of neuronal cells (Li et al., 2003). Fourth, results from the previous chapter also provided evidence that administration of *Ginkgo* induced neural activity in the dentate gyrus. Fifth, long-term rather than short-term *Ginkgo* treatment is often associated with improved learning. This corresponds to the hypothesis that *Ginkgo* enhances neurogenesis, as newborn neurons require several weeks before they integrate into the neuronal circuitry and become fully functional (van Praag et al., 2002). Sixth, transcription of a gene that encodes for transthyretin has been found to increase more than three-fold in the hippocampus of adult mice fed a *Ginkgo* supplemented diet for 4 weeks (Watanabe et al., 2001). Transthyretin is involved in the transport of the thyroid

hormone thyroxine to cerebrospinal fluid and serum (Kuchler-Bopp et al., 2000). Thyroid hormones have also been shown to play roles in the regulation of adult hippocampal neurogenesis (Ambrogini et al., 2005). Thus, Experiment 1 examined the acute effects of *Ginkgo* on cell proliferation in the dentate gyrus of adult rats, and Experiment 2 investigated whether chronic *Ginkgo* administration influences adult hippocampal cell survival.

Antidepressants such as selective serotonin reuptake inhibitors (SSRI) have potent neurogenic properties and have been shown to enhance both proliferation (Malberg, Eisch, Nestler, & Duman, 2000) and survival (Nakagawa et al., 2002) of the dentate gyrus newborn neurons. Thus, the influence of fluoxetine, a SSRI antidepressant, on hippocampal cell survival was also examined as a positive control to verify the immunohistochemical and cell quantification methods employed in the present study.

There is evidence that *Ginkgo* treatment reduces basal and stress-induced anxiety (for a review, see Chapter 1). For example, chronic *Ginkgo* pre-treatment has been shown to significantly decrease stress induced by cold water exposure (Ward et al., 2002), stress-induced learning deficits in a discrimination task (Rapin et al., 1994) and stress-induced memory deficits in a passive avoidance task (Walesiuk et al., 2005). Mice treated with *Ginkgo* (125 mg/kg for one week) have also been shown to spend significantly more time on the open arms of the elevated plus maze compared to vehicle-treated mice, suggesting an anxiolytic-like effect of the extract (Kuribara et al., 2003). Thus, Experiments 1 and 2 were supplemented with behavioural tests of anxiety. A battery of behavioural measures including the light/dark emergence, elevated plus maze and social

interaction tests were employed to assess the acute and chronic effects of *Ginkgo* on anxiety.

Experiment 1: Acute effects of Ginkgo on hippocampal cell proliferation

Method

Subject. Forty-eight male Sprague-Dawley rats (Charles River, Canada), weighing between 210 and 250 g upon arrival to the facility were used. Rats were pair-housed in standard plastic shoebox cages (45 × 25 × 20 cm) maintained at 21-22°C in a colony room on a 12 hour reversed light-dark cycle (lights off at 0700 h). Testing was conducted exclusively during the dark cycle. Experimental procedures for this and the next experiment followed Canadian Council on Animal Care guidelines, and were approved by the Wilfrid Laurier University Animal Care Committee.

Drugs. Ginkgoselect (Indena S.p.A., Milan, Italy) was dissolved in 10% w/v 2-hydroxypropyl-β-cyclodextrin (2-HβC; OnBio Inc.). Ginkgoselect is composed of EGb 761 complexed with soy phospholipids at a ratio of 1:2 (w/w) to increase bioavailability. Doses are expressed to reflect the EGb 761 content of the phospholipid complex. Half of the rats in each group (n=6) also received a single injection of 5-bromo-2'-deoxyuridine (BrdU, Sigma-Aldrich) administered at a dose of 75 mg/kg. BrdU was dissolved in 15% w/v 2-HβC at 37 °C. The drugs were administered intraperitoneally (i.p.) at a volume of 2 ml/kg of body weight.

Apparatus.

LIGHT/DARK EMERGENCE

The light/dark emergence apparatus consists of two chambers, one of which is relatively smaller and dimly illuminated, while the other one is larger with brighter illumination. The test involves a conflict between the desire to explore a novel environment and the natural desire to avoid an open and typically brighter space (Lister, 1990). Anxiolytic drugs have been found to increase exploration in the light compartment (i.e. increase the number of light/dark transitions or hide box exits), at doses that do not influence locomotor activity. Conversely, anxiogenic drugs have been found to reduce light/dark transitions or reduce exploratory behaviours in the light compartment (Crawley & Goodwin, 1980; Hascoet & Bourin, 1998; Lister, 1990). In a different version of the test the light and dark compartments are of the same size (Belzung, Misslin, Vogel, Dodd, & Chapouthier, 1987). The other version of the test, employed in the present study, consists of a small black-coloured hide box, which is placed inside a large arena maintained under red light or low level illumination (McGregor et al., 2003).

The light/dark emergence apparatus used in the present experiments consisted of a white square open field (120 × 120), and a black wooden hide box (40 × 24 × 15 cm) that was placed inside the open field. The walls of the open field were 45 cm in height, and were made out of white melamine. The floor was constructed of black ABS plastic. The hide box was placed 2 cm away from the back wall of the open field in a location equidistant from the left and right corners of the arena. The open field was illuminated with red light (5 lux at the floor level). The distance traveled was tracked and measured by the ANY-maze video tracking system (version 4.3; Stoelting Co.).

On test days, rats were placed inside the hide box and their behaviours were recorded for 5 min. The percent duration of time spent in the hide box (time in the hide box ÷ total duration of the test × 100), exit latency (s) and distance traveled (m) were all measured. The apparatus was cleaned between test sessions with a detergent (Coverage 256, Steris Corp) diluted 1:1000 in water, and then thoroughly dried.

ELEVATED PLUS MAZE

The elevated plus-maze apparatus consisted of two open and two enclosed arms, arranged such that the two open and enclosed arms were opposite to each other. The plus maze was elevated 50 cm above the floor to take advantage of the reluctance of animals to explore the elevated open arms. The test has been shown to be sensitive to the effects of both anxiolytic and anxiogenic drugs (Lister, 1990).

The apparatus used in the present experiments consisted of two open arms (10 × 50 cm) and two enclosed arms (10 × 50 cm) with walls 40 cm in height. A central square (10 × 10 cm) connected the four arms. The walls of the maze were made out of black plastic (UHMW polyethylene) and the floors were made out of black ABS plastic. The testing room was illuminated with a red light (5 lux at the maze floor). The amount of time spent in each arm as well as the number of entries into each arm and locomotor activity were measured using the ANY-maze video tracking system (version 4.3; Stoelting Co.). On the test day, rats were placed in the centre of the maze, facing one of the open arms, and were tested for 5 min. The apparatus was cleaned between each test session with a detergent (Coverage 256, Steris Corp) diluted 1:1000 in warm water and then thoroughly dried.

SOCIAL INTERACTION

The social interaction test measures levels of anxiety that rats display when confronted by an unfamiliar conspecific (File, 1980). The model has been validated for demonstration of both anxiogenic and anxiolytic effects of different types of drugs (File, 1980, 1985; File & Seth, 2003). Whereas anxiolytic drugs increase social interaction, anxiogenic drugs reduce social interaction without significantly influencing locomotor activity (Guy & Gardner, 1985). Two testing conditions, including lighting level and familiarity of the rats to the test environment, have been shown to influence social interactions. Unfamiliarity with the testing arena and high levels of illumination both decrease social interaction, whereas familiarity to the test environment and low levels of illumination both increase social interaction (File, 1980). In the present experiment, social interaction was measured in a familiar arena under low light illumination.

The apparatus was a rectangular enclosure (40 × 60 × 25 cm), placed on a table 90 cm above the floor. The top and three side walls were made out of aluminum, and the front wall was constructed of clear acrylic. The floor was made out of black ABS plastic. The testing room was illuminated with dim white light (65 lux at the enclosure floor). A Panasonic colour CCTV camera, mounted on a tripod next to the social interaction box, was connected to a monitor as well as a video recorder in an adjacent room. The video recorded the interactions of the animal. Each interaction was coded with a random number so that the experimenter could subsequently "blindly" score social interaction behaviours. The ODLog software package (Macropod Software, ODLog for Mac OS X version 2.3; www.macropodsoftware.com) was used to manually score behaviours of interest.

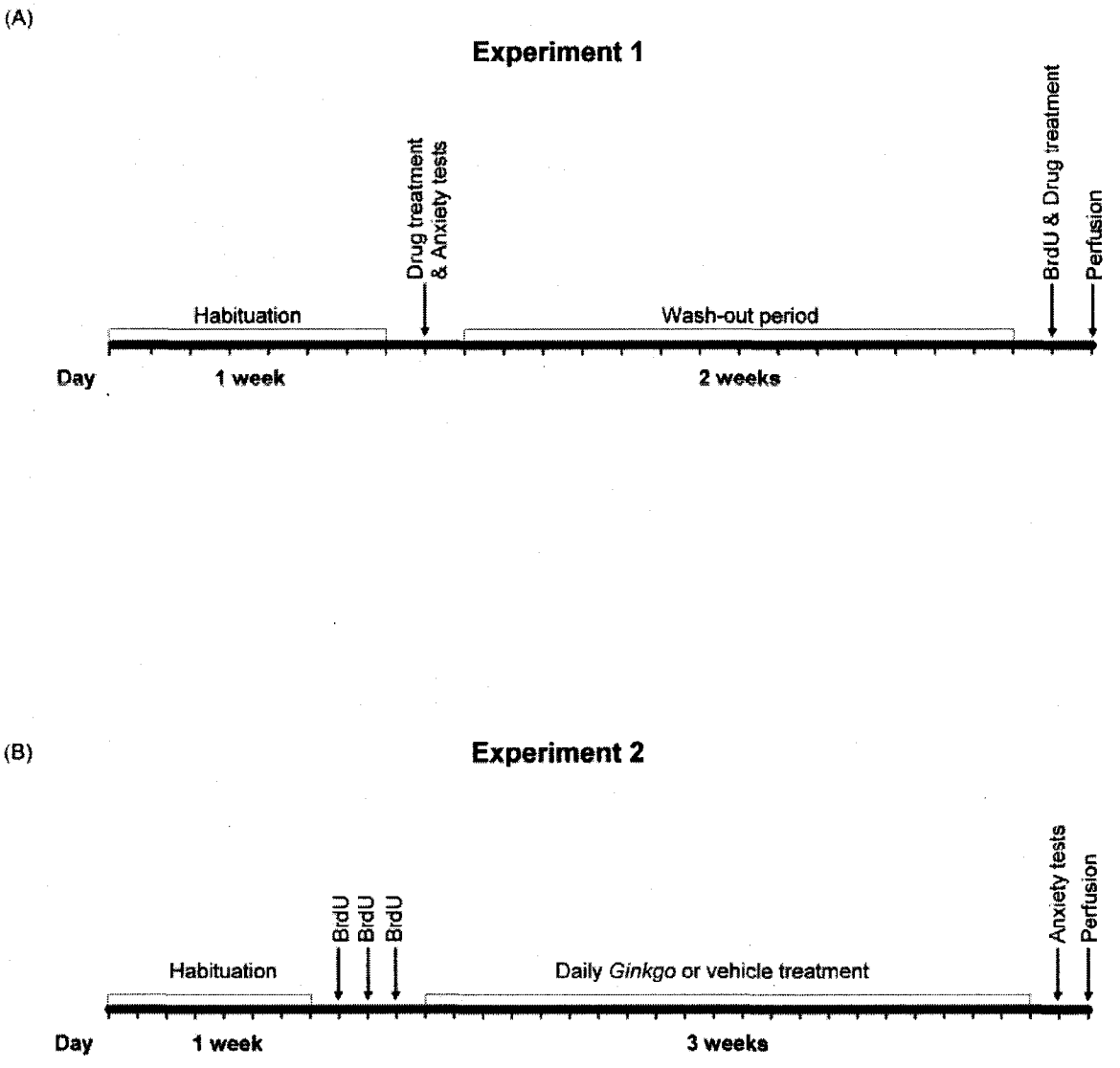
Rats were habituated to the social interaction apparatus one day before testing. During the habituation, each rat was placed in the social interaction enclosure for two non-consecutive 5 min periods. On the test day, immediately after plus maze or light/dark emergence tests, rats were placed in the social interaction apparatus for a 10 min period. Each pair of rats was approximately the same weight, and was in the same drug condition. Duration of behaviours including sniffing, following, grooming, crawling under and over the other rat were scored and considered as active social interactions (File, 1980). Duration of rearing, defined as when the rat lifted itself upright on its hind limbs, was also scored and used as an index of locomotor activity in the social interaction apparatus. The apparatus was cleaned with a detergent (Coverage 256, Steris Corp) diluted 1:1000 in warm water and thoroughly dried between test sessions.

Procedure. An overview of the design of Experiment 1 is shown in Figure 3 (A). Rats were handled and acclimatized to the colony for one week. On the test day, pairs of unfamiliar rats were injected with vehicle or *Ginkgo* (2.5, 13.75, or 25 mg/kg). Thirty min following the injection pairs of unfamiliar rats from each group were tested so that one of the rats was tested in the light/dark emergence apparatus while the other one was tested in the elevated plus maze.

Rats were placed in the plus maze facing the open arm and were placed inside the hide box of the light/dark emergence apparatus. The animals' activities in both the elevated plus maze and light/dark emergence apparatuses were tracked and recorded using the ANY-maze video tracking system for 5 min. The rats were then placed back into their home cages for 5 min, during which time both apparatuses were cleaned then thoroughly dried. The rats were brought back to the testing room and were placed into the

Figure 3. Timeline of Experiment 1 (A) and Experiment 2 (B) in Chapter 3.

Figure 3



untested apparatuses for 5 min. Immediately afterwards, both rats were placed in the social interaction enclosure, where they met for the first time for a 10 min period. This procedure allowed for unfamiliar rats to visit in the social interaction box after the same amount of time had elapsed after the injection.

After a two week wash-out period, during which time the rats were weighed daily, the acute effects of *Ginkgo* on cell proliferation was investigated in half of the rats in each drug treatment group (n=6). The rats received a single injection of either vehicle or *Ginkgo* (2.5, 13.75, or 25 mg/kg), followed 30 min later by an injection of BrdU (75 mg/kg). To detect the effect of acute *Ginkgo* injection on cell proliferation, rats were perfused 24 hours after BrdU injection.

Perfusion. Rats were anaesthetized with a lethal dose of sodium pentobarbital (120 mg/kg, i.p.). Once non-reflexive, rats were transcardially perfused with 150 ml 0.1 M phosphate buffer saline (PBS, pH 7.2) followed by 150 ml 4% paraformaldehyde (Mallinckrodt Baker, Inc) in 0.1 M PBS (pH= 7.2). Brains were extracted and fixed for 48 hours in 4% paraformaldehyde in PBS solution at 4 °C. Brains were cryoprotected first in 15% w/v phosphate-buffered sucrose at 4 °C for 24 h, and then in 30% w/v phosphate-buffered sucrose at 4°C until they sank.

Sectioning. Brains were sectioned coronally at 40 μm using a cryostat (Model CM 3050 S; Leica Microsystems Nussloch GmbH) set at a temperature of -16 °C. Brain sections were collected in five PBS-filled vials so that each vial contained each fifth section (i.e. each vial contained sections 200 μm apart). To protect tissue antigenicity,

brain sections were transferred into polyglycerine anti-freeze solution and stored at -20 °C until immunohistochemical processing.

Immunohistochemistry. Only one fifth of the brains were removed from the freezer for immunohistochemistry. Prior to BrdU processing, all sections were washed three times in PBS and were stained with 0.5% cresyl violet (Sigma-Aldrich). To denature DNA, free-floating sections were incubated with 2N HCl at 37°C for 30 min, followed by two 20-min washes in borate buffer (pH 8.5), and a 20-min wash in PBS. Sections were blocked in 3% normal goat serum in PBS for 30 min, and were incubated overnight at 4° C in primary antibody raised against BrdU (mouse monoclonal IgG, Santa Cruz Biotechnology, sc-20045) at 1:300 dilution in phosphate buffered goat serum (PBG) containing bovine serum albumin (CALLBIOCHEM), normal goat serum (PAA Laboratories Inc.), Triton-X 100 (ICN Biomedicals Inc) and PBS. The next day after one PBS wash, endogenous tissue peroxidase activity was blocked by washing the sections in 0.9% hydrogen peroxide for 30 min. This was followed by three 20-min washes in PBS. Sections were then incubated at room temperature for 60 min in biotinylated secondary antibody (goat anti-mouse IgG-B, Santa Cruz Biotechnology, sc-2039) at 1:500 dilution in PBG. After a 20-min PBS wash, sections were incubated in ExtrAvidin-Peroxidasè (Sigma-Aldrich) at 1:1000 dilution in PBG at room temperature. After three 20-min washes in PBS, sections were incubated in 0.05% 3,3'-diaminobenzidine-tetrahydrochloride hydrate (DAB, Sigma-Aldrich) 0.015% H_2O_2 in PBS for 10 min to stain BrdU-positive cells. Sections were washed twice in PBS and were stored at 4°C until mounted on gelatin-coated microscope slides. For unbiased counting, the slides were coded. The mounted slides were submerged in 75%, 90% and 100% ethanol baths

for 5 min each and cleared with Harleco (clarification Neo-Clear, EMD^{TM}) for 20 min. The slides were then cover slipped using Permount Mounting Medium (Fisher Scientific) solution and allowed to dry for 3 days.

Data quantification and analysis. The number of BrdU-labeled cells was quantified with a Leica DMR upright light microscope at approximately 400 × magnification on a series of every fifth section at 200 µm intervals. All the circular encapsulated BrdU-labeled cells along the granular cell layers of the dentate gyrus were counted separately in the dorsal (bregma -2.56 to -4.52 mm, Figure 4) and ventral (bregma -5.80 to -6.72 mm, Figure 5) hippocampus (Paxinos & Watson, 1997). BrdU-labeled cells that had blood cell phenotypes were not counted. Images of the dentate gyrus were captured using a Leica DC camera and Leica Image Manager 50 (version 1.10) program under approximately 50× and 12.5× magnification for the dorsal and ventral dentate gyrus, respectively. The outline of the dentate gyrus was traced manually on each photomicrograph using an Intuos3 (PTZ-930, WACOM Co. Ltd.) graphic tablet system. The area of each tracing was then calculated using ImageJ (Wayne Rasband, National Institutes of Health, version 1.37). The area of dorsal and ventral dentate gyrus were summed separately and multiplied by the tissue thickness (40 µm) and by 5 (to account for 4/5 of the sections that were not processed) to estimate the volume of the dorsal and ventral dentate gyrus (Gundersen & Jensen, 1987). To estimate the total number of BrdU-labeled cells in the dorsal and ventral dentate gyrus, the BrdU-labeled cell counts were multiplied by 5 to account for the samples that were not processed and by 8 (40 µm tissue thickness/5 µm depth of microscope focus) to account for the microscope depth of focus. BrdU-labeled cell densities in the dorsal and ventral dentate

Figure 4. Schematic illustration of coronal sections of the rat brain used for quantifying BrdU-labeled cells in the dorsal dentate gyrus. The dentate gyrus is coloured in red; bregma -2.56 to -4.52 mm (Paxinos & Watson, 1997).

Figure 4

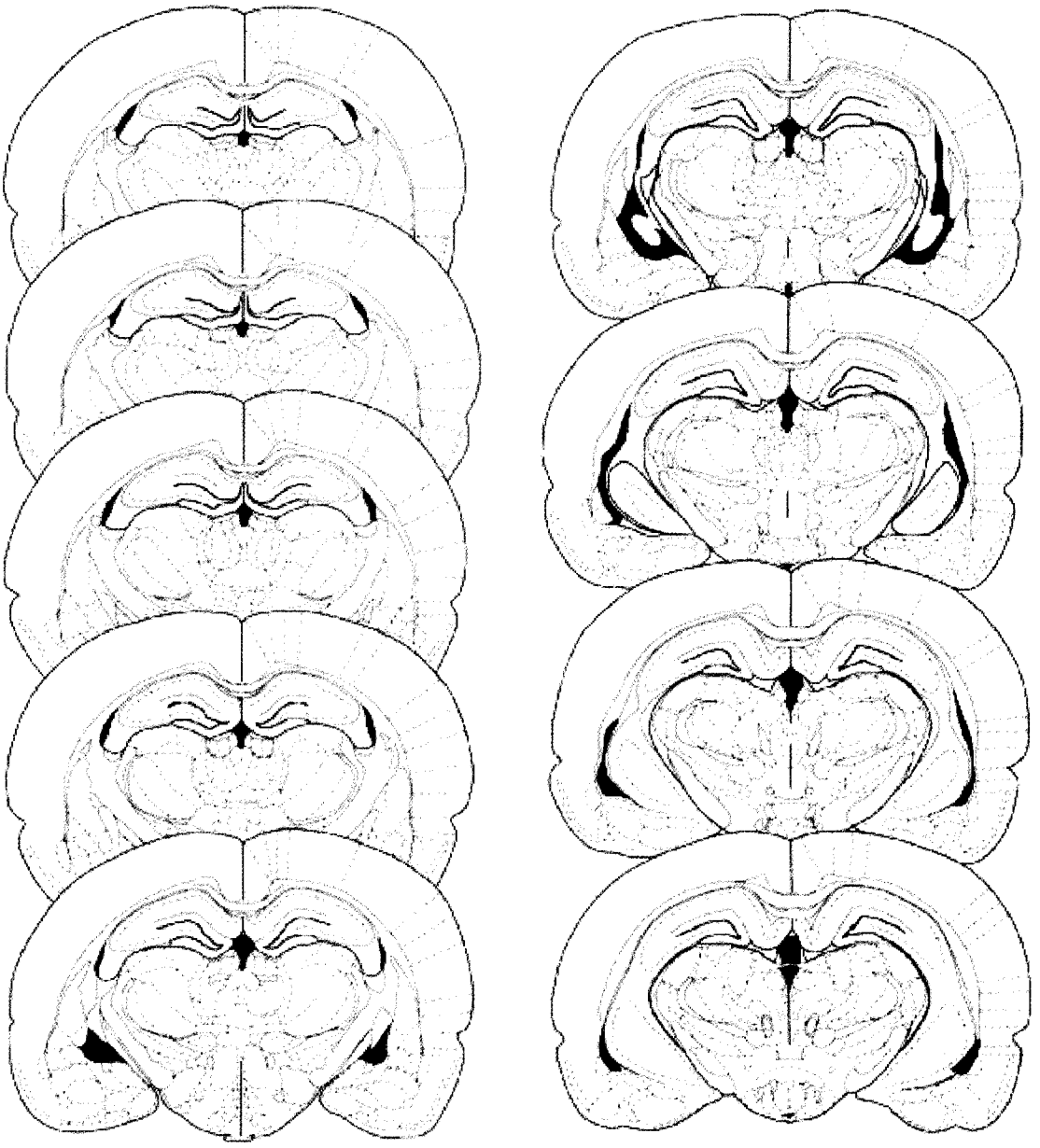
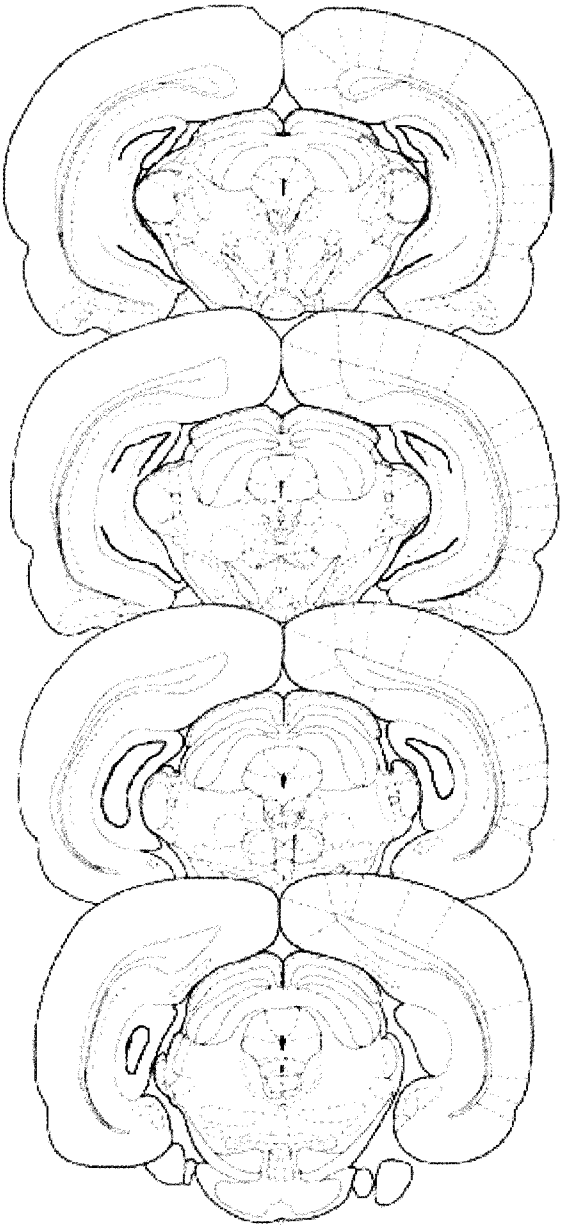


Figure 5. Schematic illustration of coronal sections of the rat brain used for quantifying BrdU-labeled cells in the ventral dentate gyrus. The dentate gyrus is coloured in red; bregma -5.80 to -6.72 mm (Paxinos & Watson, 1997).

Figure 5



gyrus were calculated by dividing the estimated total number of BrdU-labeled cells in the dorsal and ventral dentate gyrus by their estimated volumes. There were a few missing sections. The number of BrdU-labeled cells and the area of the missing sections were estimated by taking the average of the number of labeled cells and area from the sections prior to and after the sections.

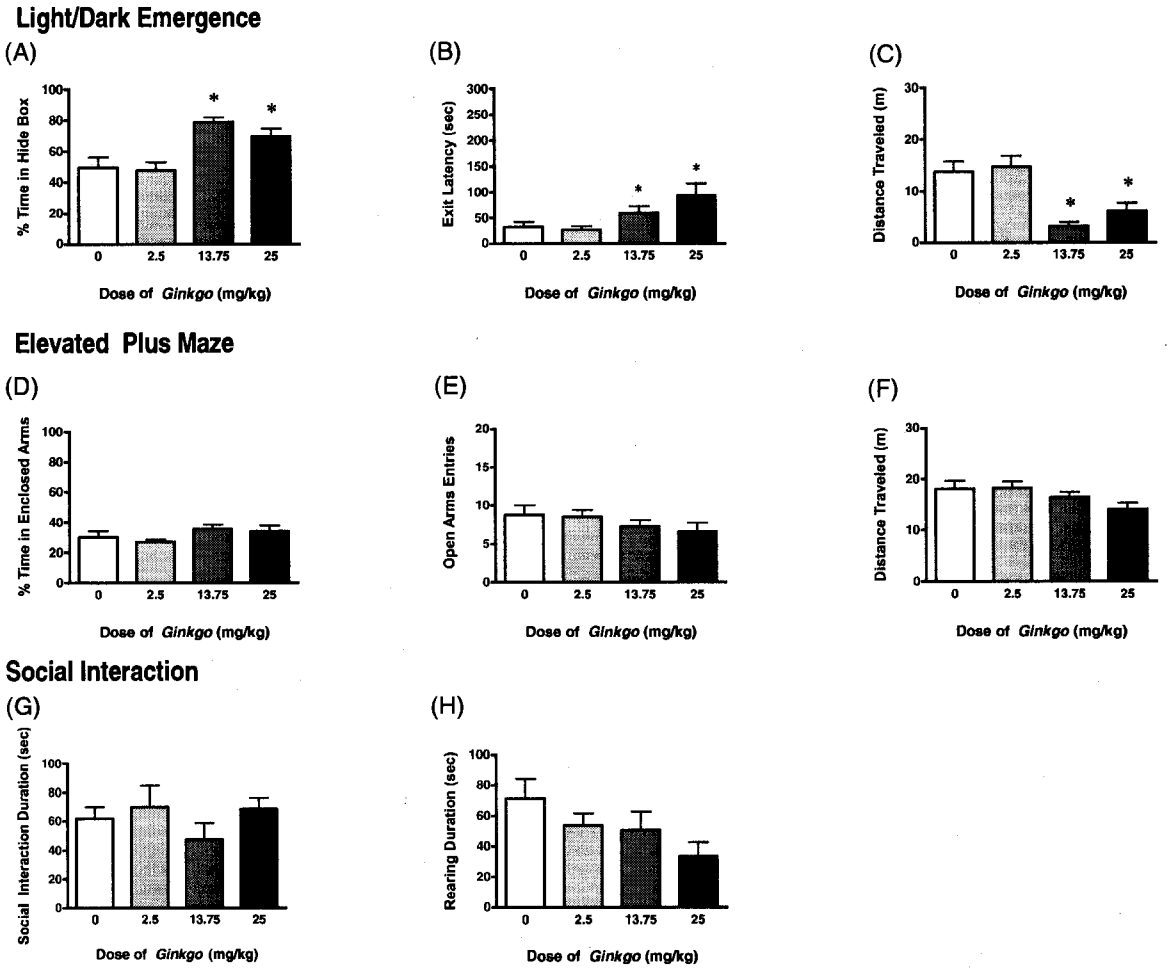
Data analysis. Data from the anxiety tests were analyzed separately using one-way analysis of variance (ANOVA). The number of BrdU-labeled cells, dentate gyrus volume and BrdU-labeled cell density were analyzed separately for the dorsal and ventral hippocampus using one-way ANOVAs. Significant effects were followed by Tukey post-hoc tests.

Results

Light/dark emergence test. The mean (+SEM) percentage of time spent in the hide box, mean (+SEM) exit latency, and mean (+SEM) distance traveled in the light/dark emergence apparatus are shown in Figure 6 (A, B, and C, respectively). The data obtained for percentage duration of time spent in the hide box, exit latency, and distance traveled were analyzed separately by one-way ANOVAs. The ANOVA found an overall significant effect of treatment on percentage duration of time spent in the hide box, $F(3, 44)=8.43$, $p<0.001$, exit latency $F(3,43)=4.17$, $p<0.05$, and distance traveled, $F(3, 44)=11.52$, $p<0.001$. Post-hoc analysis showed that the 13.75 and 25 mg/kg *Ginkgo*-treated groups spent significantly more time in the hide box (Figure 6, A), had significantly longer exit latencies (Figure 6, B) and traveled significantly shorter distances (Figure 6, C) compared to both vehicle- and 2.5 mg/kg *Ginkgo*-treated animals

Figure 6. Mean (+SEM) percentage of time spent in the hide box (A), mean (+SEM) exit latency (B) and mean (+SEM) distance traveled (C) in the light/dark emergence apparatus. Mean (+SEM) percentage of time spent in the enclosed arms (D), mean (+SEM) number of open arms entries (E) and mean (+SEM) distance traveled (F) in the elevated plus maze. Mean (+SEM) duration of time unfamiliar rats spent interacting with each other (G), and mean (+SEM) duration of time rearing (H) in the social interaction test in Experiment 1. Rats received a single injection of 0, 2.5, 13.75 or 25 mg/kg *Ginkgo* 30 min prior to testing. *$p<0.05$, significantly different from vehicle and 2.5 mg/kg *Ginkgo*.

Figure 6


Light/Dark Emergence
(A)
% Time in Hide Box
Dose of Ginkgo (mg/kg)
(B)
Exit Latency (sec)
Dose of Ginkgo (mg/kg)
(C)
Distance Traveled (m)
Dose of Ginkgo (mg/kg)
Elevated Plus Maze
(D)
% Time in Enclosed Arms
Dose of Ginkgo (mg/kg)
(E)
Open Arms Entries
Dose of Ginkgo (mg/kg)
(F)
Distance Traveled (m)
Dose of Ginkgo (mg/kg)
Social Interaction
(G)
Social Interaction Duration (sec)
Dose of Ginkgo (mg/kg)
(H)
Rearing Duration (sec)
Dose of Ginkgo (mg/kg)

($p<0.05$). Because half the rats were first tested in the elevated plus maze, and the other half in the emergence test, two-way ANOVAs (order x treatment) were performed to also investigate the effect of test order on percentage duration of time spent in the hide box, exit latency and distance traveled. No significant effects of order were observed (data not shown).

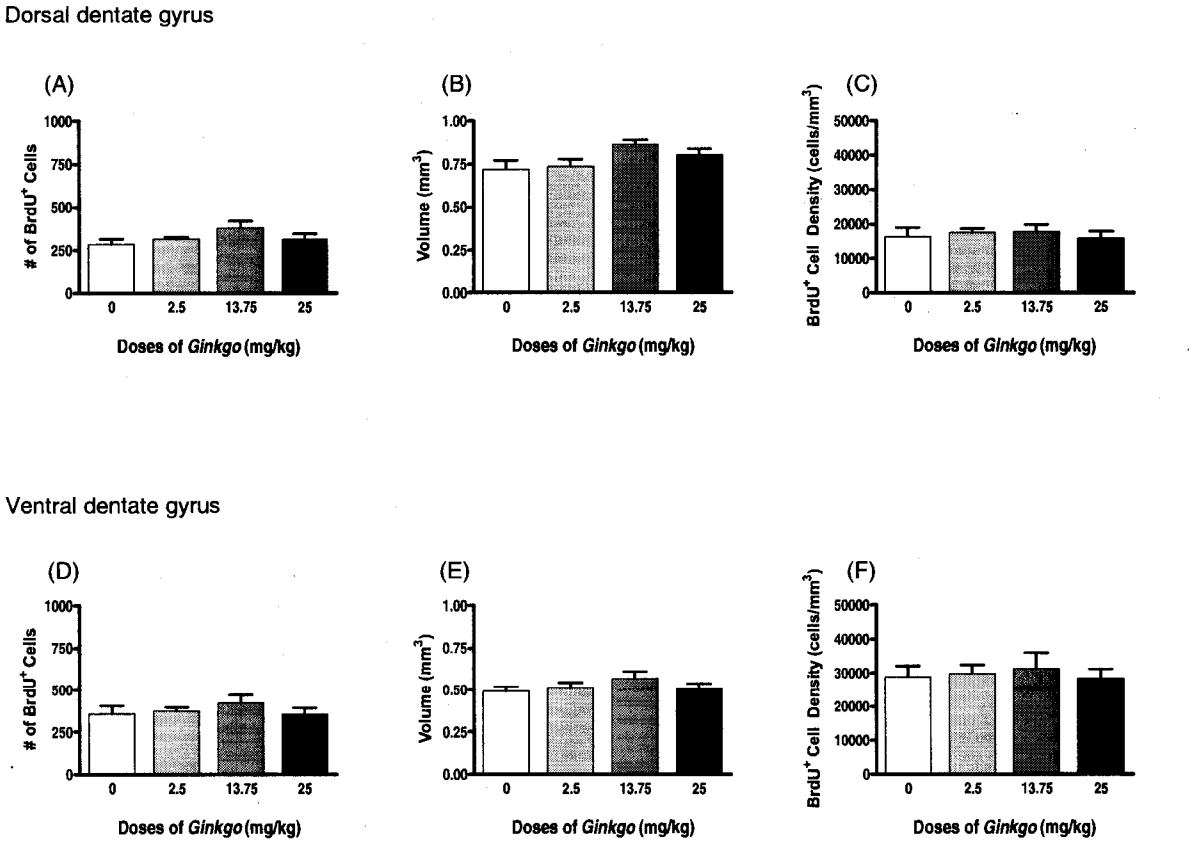
Elevated plus maze test. The percentage of time spent in the enclosed arm was calculated according to the formula: time spent in the enclosed arms ÷ total duration of the test × 100. Separate one-way ANOVAs for the percentage duration of time spent in the enclosed arms (Figure 6, D), number of entries to the open arms (Figure 6, E), and distance traveled (Figure 6, D) revealed no overall significant effect of treatment. Furthermore, two-way ANOVAs revealed no significant order effect (data not shown).

Social interaction test. Figure 6 (G) shows the total duration of active social interaction calculated by adding the duration of sniffing, following, and grooming of the social partner (File, 1980). Duration of rearing, defined as the rat lifting itself upright on its hind paws, was used as an index of locomotor activity and is shown in Figure 6 (H). The two separate one-way ANOVAs for the total duration of social interaction and rearing duration found no significant treatment effects, although as evident in Figure 6 (H) there was a dose-dependent trend toward suppression of rearing.

Acute effects of Ginkgo on hippocampal cell proliferation. None of the doses of *Ginkgo* tested acutely had any effect on the number of BrdU-labeled cells, volume or cell density in the dorsal or ventral dentate gyrus as shown in Figure 7. The number of BrdU-

Figure 7. Mean (+SEM) number of BrdU-labeled cells (A), mean (+SEM) volume of the dentate gyrus (B) and mean (+SEM) BrdU-labeled cell density (C) in the dorsal region of the dentate gyrus. Mean (+SEM) number of BrdU-labeled cells (D), mean (+SEM) volume of the dentate gyrus (E) and mean (+SEM) BrdU-labeled cell density (F) in the ventral region. Rats were injected with a single dose of BrdU (75 mg/kg) followed by a single injection of 0, 2.5, 13.75 or 25 mg/kg *Ginkgo* 30 min later. Rats were perfused 24 h after the BrdU injection.

Figure 7



labeled cells, volume and BrdU-labeled cell density were analyzed separately for the dorsal and ventral hippocampus using one-way ANOVAs. None of the ANOVAs revealed significant treatment effects, suggesting that acute *Ginkgo* treatment had no effect on hippocampal cell proliferation either in the dorsal or in the ventral dentate gyrus. Representative photomicrographs of BrdU-labeled cells in the dentate gyrus of four groups of rats are shown in Figure 8.

Experiment 2: Chronic effects of Ginkgo and fluoxetine on hippocampal cell survival

This experiment was designed to investigate whether chronic *Ginkgo* administration influences hippocampal cell survival and anxiety using light/dark emergence, elevated plus maze and social interaction tests. Fluoxetine has been shown to enhance both proliferation and survival of newborn neurons (Malberg et al., 2000; Nakagawa et al., 2002); thus, an additional 10 rats were used as a positive control group for the immunohistochemistry procedures.

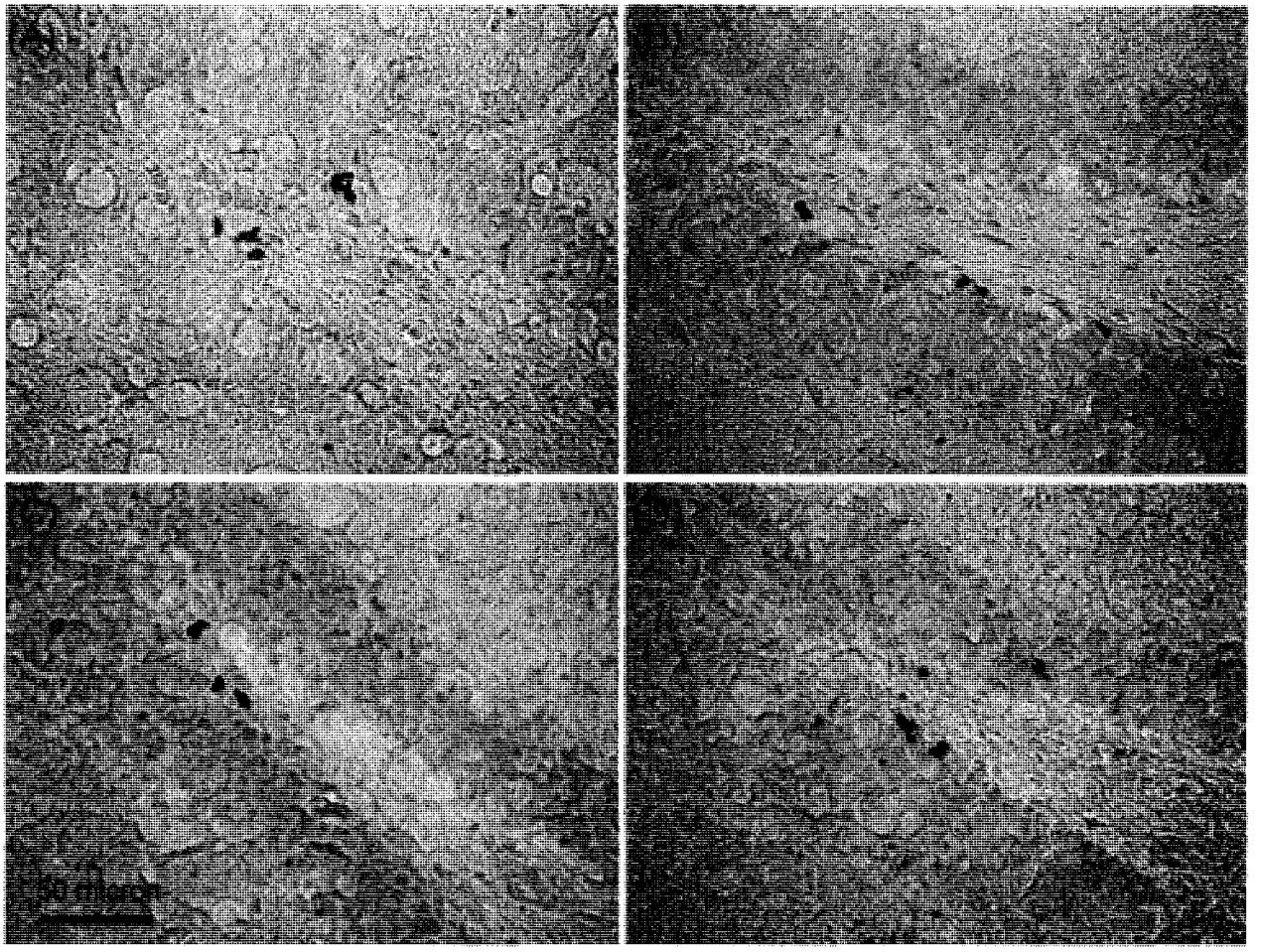
Method

Subject. Forty male Sprague-Dawley rats (Charles River, Canada), weighing between 230 and 270 g upon arrival to the colony were used. As in the first experiment, rats were pair-housed in standard plastic shoebox cages (45 × 25 × 20 cm) maintained at 21-22°C in a colony room on a 12 hour reversed light-dark cycle (lights off at 0700 h). Testing was conducted exclusively during the dark cycle.

Drugs. Ginkgoselect (Indena S.p.A., Milan, Italy) was dissolved in 10% w/v 2-HβC. BrdU was dissolved in 10% w/v 2-HβC in a 37 ° C water bath. The drugs were

Figure 8. Photomicrographs (at approximately 400× magnification) of BrdU-labeled cells in the dentate gyrus of 0 (A), 2.5 (B), 13.75 (C) and 25 (D) mg/kg *Ginkgo*-treated rats in Experiment 1.

Figure 8



administered i.p. in a volume of 2 ml/kg of body weight. Fluoxetine hydrochloride (Spectrum Chemical, MFG. CORP. New Brunswick) was dissolved in 10% w/v 2-HβC and was administered i.p. at a dose of 5 mg/kg in a volume of 2 ml/kg body weight daily for 21 days.

Procedures. An overview of the design of Experiment 2 is shown in Figure 3 (B). After a one week handling period, all animals were injected with 50 mg/kg BrdU twice daily at 1100 and 1500 h for three consecutive days. This BrdU injection protocol served to label a relatively large number of dividing cells. To examine the effects of chronic *Ginkgo* administration on anxiety and cell survival, rats were injected with either a daily dose of vehicle or *Ginkgo* (2.5, 13.75, or 25 mg/kg) for the next 21 days. The fluoxetine positive control group received 5 mg/kg fluoxetine daily for 21 days.

On the last day of drug injections, all rats were habituated to the social interaction apparatus. They were individually placed in the enclosure for 5 min, twice non-consecutively. On day 22, the chronic effects of *Ginkgo* on anxiety were assessed using light/dark emergence, elevated plus maze, and social interaction tests of anxiety as described in the first experiment. Rats did not receive drug treatments on this day. On day 23, the rats were perfused transcardially 24 hours after the anxiety tests. Although the fluoxetine group was used as a positive control to verify the immunohistochemical procedures employed, animals in that group underwent similar behavioural testing. The behavioural data were not analyzed for the fluoxetine-treated group. All procedures for the perfusion, brain sectioning, immunohistochemistry, quantification of BrdU-labeled cells and data analysis were similar to those described in the first experiment. The

number of BrdU-labeled cells, dentate gyrus volume and BrdU-labeled cell density were compared separately for the dorsal and ventral hippocampus for fluoxetine- and vehicle-treated rats using independent samples *t*-tests.

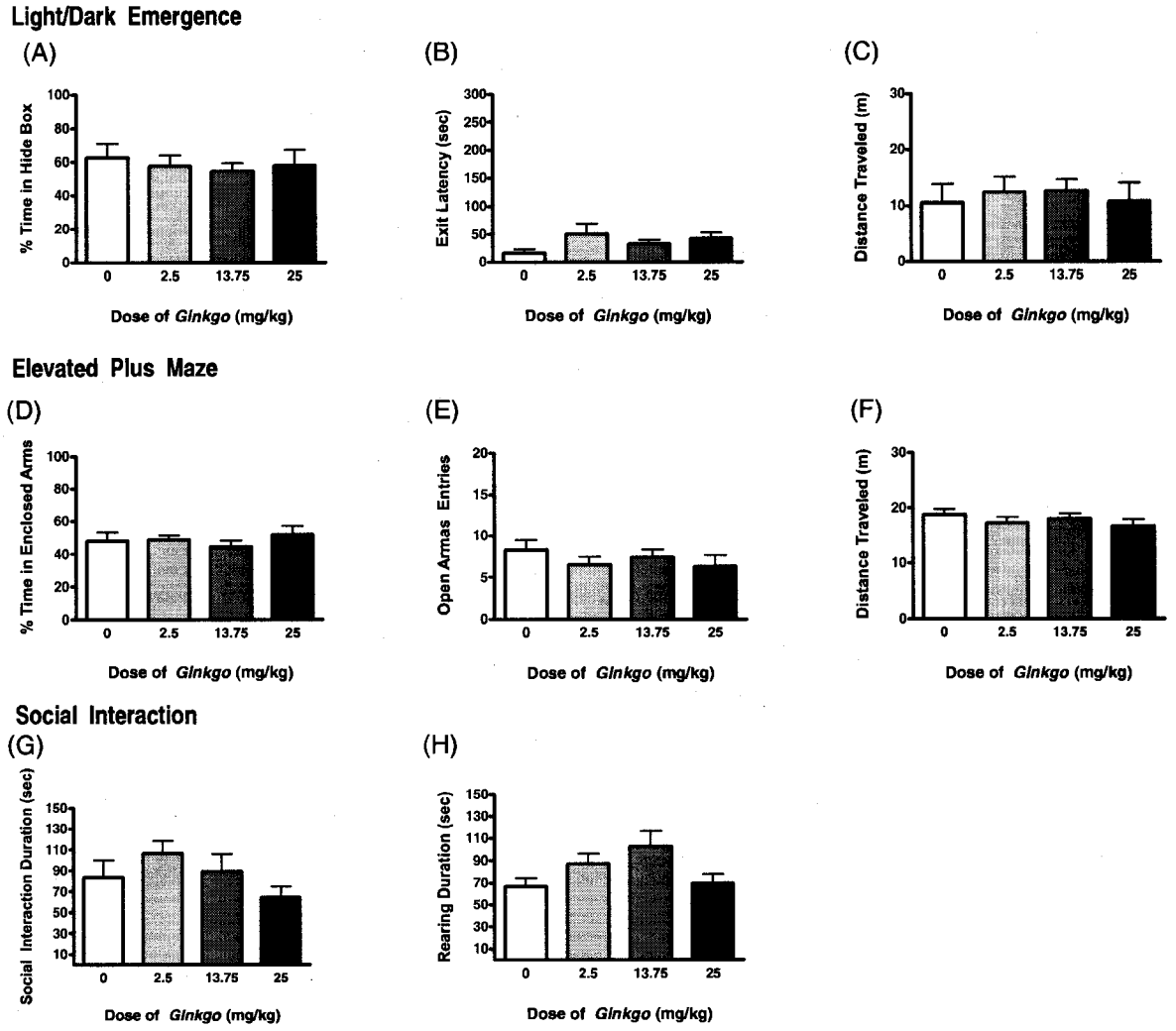
Results

Light/dark emergence test. Chronic administration of *Ginkgo* for 21 days appeared to be neither anxiogenic nor anxiolytic as all the groups spent similar amounts of time in the hide box and had similar exit latencies as shown in Figure 9 (A and B, respectively). Hence, separate one-way ANOVAs performed for percentage duration of time spent in the hide box and exit latency revealed no significant effect of treatment. In addition, the one-way ANOVA for distance traveled also revealed no treatment effect (Figure 9, C). Two-way ANOVAs (order x treatment) were also performed to investigate whether the order of the test influenced exit latency, percentage duration of time spent in the hide box, and distance traveled. The results revealed a significant order effect for distance traveled, $F(1, 32)=6.51$, $p=0.016$ and for the percentage duration of time spent in the hide box, $F(1, 32)=7.22$, $p=0.011$ (see Appendix A for additional details).

Elevated plus maze. Figure 9 (D and E) shows that measures of anxiety in the elevated plus maze, namely duration of time in the enclosed arms and open arms entries, were not affected by chronic treatment with any dose of *Ginkgo*. No overall significant effects of treatment were revealed by one-way ANOVAs for the percentage duration of time spent in the enclosed arms, number of entries to the open arms, or distance traveled (Figure 9 D-F). To investigate the order effect on measures of anxiety in the plus maze,

Figure 9. Mean (+SEM) percentage of time spent in the hide box (A), mean (+SEM) exit latency (B) and mean (+SEM) distance traveled (C) in the light/dark emergence test. Mean (+SEM) percentage of time spent in the enclosed arms (D), mean (+SEM) number of open arms entries (E) and mean (+SEM) distance traveled (F) in the elevated plus maze. Mean (+SEM) duration of time unfamiliar rats spent interacting with each other (G), and mean (+SEM) duration of time rearing (H) in the social interaction test in Experiment 2. Rats were injected with 0, 2.5, 13.75, and 25 mg/kg *Ginkgo* for 21 days prior to testing.

Figure 9


Light/Dark Emergence
(A)
% Time in Hide Box
100
80
60
40
20
0
0
2.5
13.75
25
Dose of Ginkgo (mg/kg)
(B)
Exit Latency (sec)
300
250
200
150
100
50
0
0
2.5
13.75
25
Dose of Ginkgo (mg/kg)
(C)
Distance Traveled (m)
30
20
10
0
0
2.5
13.75
25
Dose of Ginkgo (mg/kg)
Elevated Plus Maze
(D)
% Time in Enclosed Arms
100
80
60
40
20
0
0
2.5
13.75
25
Dose of Ginkgo (mg/kg)
(E)
Open Armas Entries
20
15
10
5
0
0
2.5
13.75
25
Dose of Ginkgo (mg/kg)
(F)
Distance Traveled (m)
30
20
10
0
0
2.5
13.75
25
Dose of Ginkgo (mg/kg)
Social Interaction
(G)
Social Interaction Duration (sec)
150
130
110
90
70
50
30
10
0
2.5
13.75
25
Dose of Ginkgo (mg/kg)
(H)
Rearing Duration (sec)
150
130
110
90
70
50
30
10
0
2.5
13.75
25
Dose of Ginkgo (mg/kg)

two-way ANOVAs (order x treatment) were also performed, but no significant effect was found (data now shown).

Social interaction test. Figure 9 (G) shows the duration of active social interaction in the four *Ginkgo*-treated groups. It seems that there was a slight increase in the active social interaction in the rats treated chronically with 2.5 mg/kg *Ginkgo* compared to the other groups, but the one-way ANOVA conducted for total duration of social interaction found no significant treatment effect. The one-way ANOVA for rearing duration, however, was significant, $F(3, 36)=2.90$, $p=0.048$, but post-hoc multiple comparison failed to find any significant differences between treatments (Figure 9, H).

Chronic effects of Ginkgo on hippocampal cell survival. As can be seen in Figure 10, the number of BrdU-labeled cells, volume and BrdU-labeled cell density were similar across groups for both the dorsal and the ventral dentate gyrus. Consequently, none of the ANOVAs performed separately for the number of BrdU-labeled cells, volume and cell density in the dorsal and ventral dentate gyrus found any significant treatment effects. See Figure 11 for representative photomicrographs showing BrdU-labeled cells in the dentate gyrus of rats receiving various doses of *Ginkgo*.

Chronic effects of fluoxetine on hippocampal cell survival. Figure 12 (A-C) shows the number of BrdU-labeled cells, volume and BrdU-labeled cell density of fluoxetine- and vehicle-treated rats. There was no difference between the vehicle- and fluoxetine-treated rats in the number of BrdU-labeled cells and for BrdU-labeled cell density. Figure 13 shows photomicrographs of BrdU-labeled cells in the dorsal dentate gyrus of vehicle-

Figure 10. Mean (+SEM) number of BrdU-labeled cells (A), mean (+SEM) volume of the dentate gyrus (B) and mean (+SEM) BrdU-labeled cell density (C) in the dorsal region used for quantifying BrdU-labeled cells. Mean (+SEM) number of BrdU-labeled cells (D), mean (+SEM) volume of the dentate gyrus (E) and mean (+SEM) BrdU-labeled cell density (F) in the ventral region. Rats received 50 mg/kg BrdU injections twice daily for 3 days followed by 21 days of 0, 2.5, 13.75, or 25 mg/kg *Ginkgo*. Rats were perfused 48 hours after the last drug injection.

Figure 10

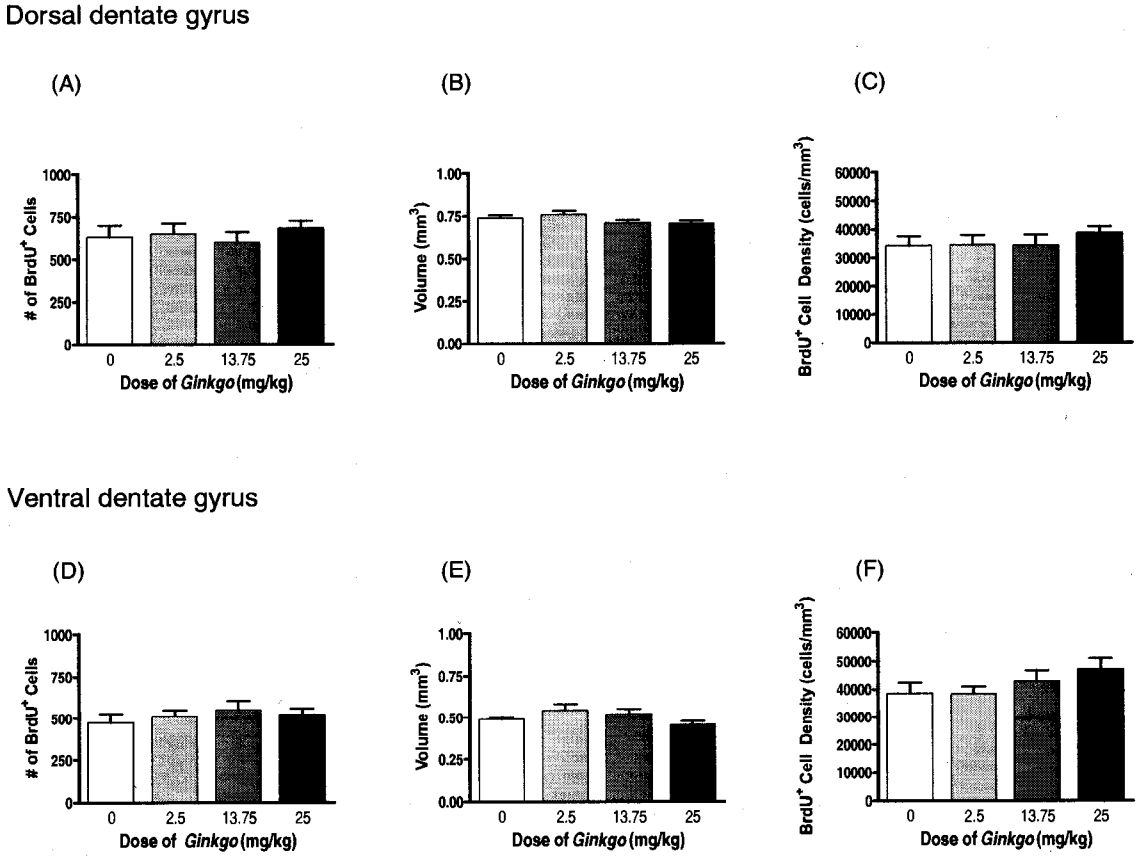


Figure 11. Photomicrographs (at approximately 400× magnification) of BrdU-labeled cells in the dentate gyrus of 0 (A), 2.5 (B), 13.75 (C) and 25 (D) mg/kg *Ginkgo*-treated rats in Experiment 2.

Figure 11

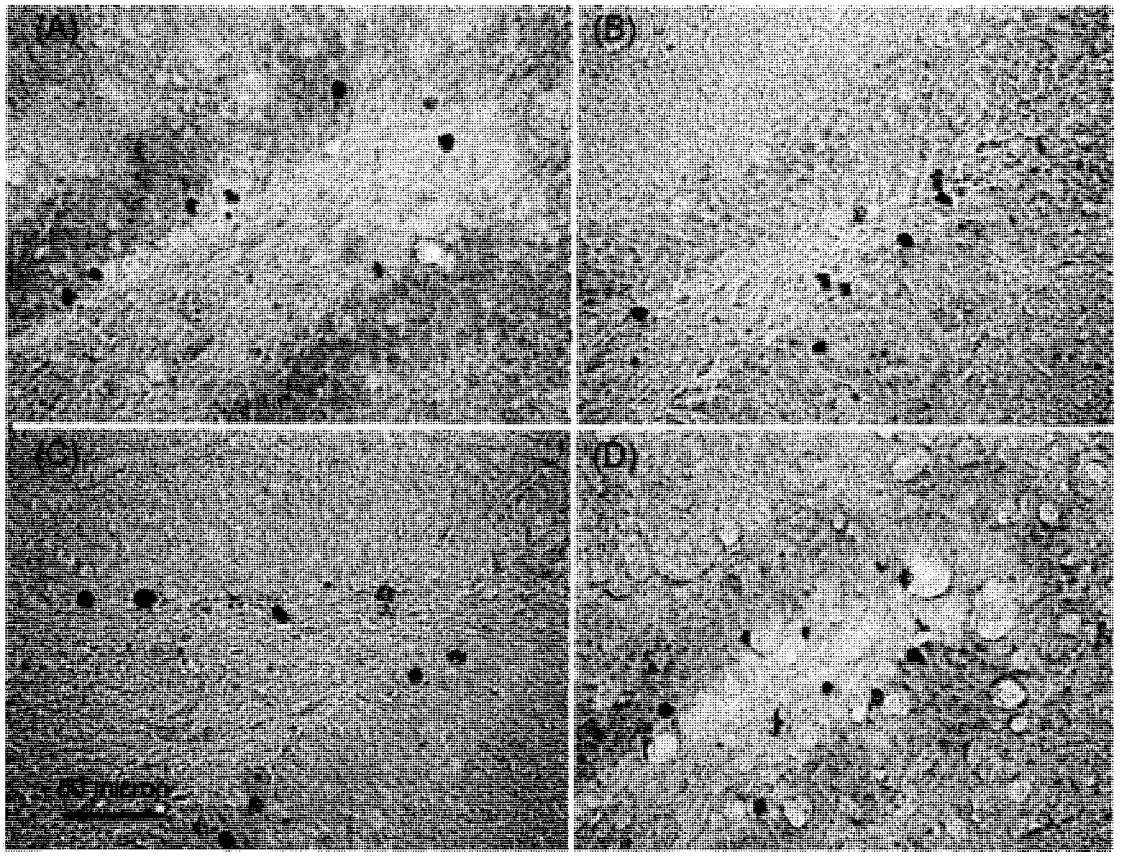


Figure 12. Mean (+SEM) number of BrdU-labeled cells (A), mean (+SEM) volume of the dentate gyrus (B) and mean (+SEM) BrdU-labeled cell density (C) in the dorsal and ventral dentate gyrus in rats that received 50 mg/kg BrdU injections twice daily for 3 days followed by 21 days of vehicle or fluoxetine (5 mg/kg). *p<0.01, significantly different from vehicle.

Figure 12

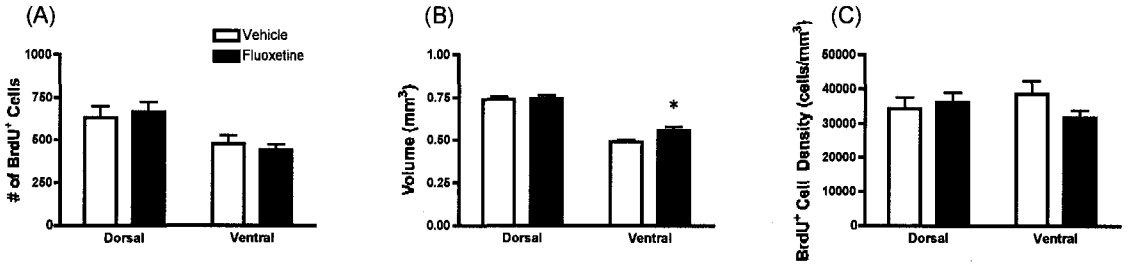
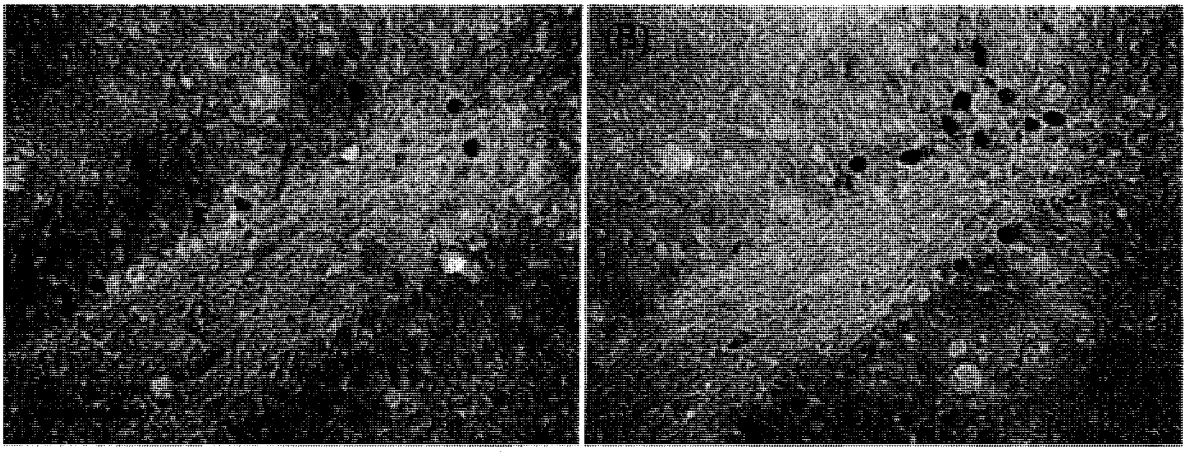


Figure 13. Photomicrographs (at approximately 400× magnification) of BrdU-labeled cells in the dentate gyrus of vehicle- (A) and 5 mg/kg fluoxetine- (D) treated rats.

Figure 13



and fluoxetine-treated rats. Two separate independent samples *t*-tests for the number of BrdU-labeled cells and cell density were not significant. However, as shown in Figure 12 (B), the volume of the ventral dentate gyrus appeared larger in fluoxetine-treated rats compared to that of the vehicle-treated rats. Accordingly, an independent samples *t*-test comparing these groups was significant, $t(18) = 2.91$, $p<0.01$.

Discussion

The results of the present study revealed that *Ginkgo* had no influence on adult hippocampal neurogenesis. Firstly, cell proliferation was not affected by acute injection of *Ginkgo* either in the dorsal (Figure 7, A & C) or in the ventral dentate gyrus (Figure 7, D & F). Secondly, the survival of newborn cells in either the dorsal (Figure 10, A & C) or the ventral (Figure 10, D & F) dentate gyrus was not affected by chronic administration of *Ginkgo*. It has been suggested that adult neurogenesis in the dorsal dentate gyrus has a distinct role from that of the ventral dentate gyrus (Sahay & Hen, 2007). There is differential neuronal connectivity along the dorsal-ventral axis of the hippocampus. The dorsal and ventral hippocampus receive inputs from different layers of the entorhinal cortex and project their outputs to different structures. It appears, specifically from lesion studies, that the dorsal hippocampus is involved in learning behaviours and the ventral hippocampus is involved in response to stress and anxiety (for review, see Bannerman et al., 2004). For this reason, in the present study, the number of $BrdU^+$ cells was counted separately in the dorsal and ventral hippocampus in order to examine whether or not *Ginkgo* and fluoxetine differentially influence dorsal and ventral hippocampal neurogenesis.

Unexpectedly, in the present study, 21 days of daily fluoxetine during the post-BrdU survival period failed to reveal any changes in cell survival in either the dorsal or ventral dentate gyrus (Figure 12, A & C). However, the volume of the ventral dentate gyrus was found to be larger in the fluoxetine treated rats (Figure 12, B). It has been reported that 3 (J. W. Wang, David, Monckton, Battaglia, & Hen, 2008) and 4 (Nakagawa et al., 2002) weeks of fluoxetine treatment significantly increased the number of BrdU-labeled cells in the dentate gyrus. However, 2 weeks of fluoxetine treatment followed by 2 weeks of no treatment had no effect on the number of BrdU-labeled cells (Malberg et al., 2000). In parallel with present results, a recent study also reported no enhancement of cell survival or cell proliferation in either the dorsal or ventral dentate gyrus of rats treated chronically for 25 days with 5 mg/kg fluoxetine (Cowen, Takase, Fornal, & Jacobs, 2008).

While the present study was in progress, another group reported that chronic *Ginkgo* treatment enhanced cell proliferation in young and aged transgenic mice (TgAPP/PS1) believed to be a model of Alzheimer's disease (Tchantchou et al., 2007). In that study age-matched wild type mice were also used as controls. Consistent with our results, *Ginkgo* treatment had no effect on cell proliferation in young healthy mice (6 months). This study did not investigate the effects of *Ginkgo* on cell survival. However, a critical finding of the study was that *Ginkgo* treatment significantly enhanced neuronal proliferation in aged (22 months) wild type mice. Results of the present study, together with the study by Tchantchou et al. (2007), raise the possibility that *Ginkgo* may have different effects in the normal healthy nervous system, compared to in dysfunctional nervous systems where the constitutive level of neurogenesis has been down-regulated.

Thus future studies should characterize the neurogenic potential of *Ginkgo* on hippocampal cells under abnormal conditions.

The present results also revealed an apparent anxiogenic effect induced by the high (25 mg/kg) and moderate (13.75 mg/kg) doses of *Ginkgo,* when administered acutely and only in the light/dark emergence test as indicated by increased duration of time spent in the hide box and longer exit latency (Figure 6, A & B) . Rats treated acutely with the high and moderate doses of *Ginkgo* also traveled significantly shorter distances in the light/dark emergence box compared to the other groups (Figure 6, C). This effect was not likely the result of a general suppressive effect of *Ginkgo* on locomotor activities because other measures of locomotion, namely distance traveled in the plus maze (Figure 6, F) and rearing duration in the social interaction test (Figure 6, H) were unaffected by the same doses of *Ginkgo.* Nevertheless, suppression of motor activity by a single oral dose of *Ginkgo biloba* extract (500 and 1000 mg/kg but not 250 mg/kg) have been reported (Kuribara et al., 2003).

When *Ginkgo* was administered chronically for 21 days, it induced neither anxiogenic nor anxiolytic effects in the light/dark emergence test (Figure 9, A & B) at all doses tested. In addition, acute and chronic administration of all doses of *Ginkgo* tested failed to alter the duration of time spent in the enclosed arms, number of entries into the open arms of the elevated plus maze (Figure 9, D & E), and duration of active social interactions (Figure 9, G).

It seems surprising that administration of the acute high and moderate doses of *Ginkgo* induced anxiety only in the light/dark emergence test, and not in the other two anxiety tests. This suggests that the nature of the anxiety evoked by the light/dark

emergence, elevated plus maze, and social interaction tests are dissimilar. Both the light/dark emergence and the elevated plus maze are based on exploratory behaviours, and anxiety is generated by stimuli that are ethologically relevant to the rats. That is, the open space in the emergence apparatus, and the height and openness of the open arms of the plus maze generate behavioural and physiological changes (File, 1992). In contrast, unfamiliarity of a conspecific in the social interaction test generates behavioural changes that are used as measures of anxiety-like behaviour (File, 1980). Chronic *Ginkgo* treatment did not alter measures of anxiety in any of these ethologically based tests of anxiety. Thus, results of the present study do not support previous reports suggesting that *Ginkgo* can reduce anxiety (Hasenöhrl et al., 1996; Kuribara et al., 2003; Porsolt et al., 1990; Rodriguez de Turco et al., 1993; Walesiuk et al., 2005; Ward et al., 2002). Reasons for these inconsistencies are unclear, but may stem from different routes of administration, dosages or the specific behavioural tests of anxiety used in this study.

The results of the social interaction test in the present study differed from that of Chermat and colleagues (1997) who found a reduction of social interaction in pairs of unfamiliar rats treated either acutely or chronically with *Ginkgo*. This discrepancy may be due to the use of different doses, route of administration, or testing environment. In the present study, social interaction was tested in a familiar arena under low light illumination. Unfamiliarity to the testing environment as well as bright lighting, conditions used by Chermat et al. (1997), have been shown to decrease social interaction (File, 1980).

In sum, the results showed that chronic parenteral administration of *Ginkgo* treatment had neither anxiolytic nor anxiogenic effects in young adult male rats. Acute

parenteral administration of high and moderate doses of *Ginkgo,* however, exerted anxiogenic effects in some measures of anxiety. In addition, the results of the present study showed that cell proliferation and survival in the dentate gyrus of hippocampus were not affected by *Ginkgo* treatment in young male rats. *Ginkgo* may therefore exert its cognitive-enhancing effects in young healthy rats via mechanisms other than the enhancement of hippocampal neurogenesis.

Chapter 4: Effects of *Ginkgo biloba* leaf extract on corticosterone-induced suppression of hippocampal neurogenesis in young adult male rats

Abstract

The *Ginkgo biloba* leaf extract *(Ginkgo)* has been shown to decrease circulating levels of corticosterone (CORT) and to normalize stress-induced plasma CORT elevation. The involvement of CORT in stress-induced suppression of adult hippocampal neurogenesis has been recognized. Stress and elevated levels of CORT suppress cell proliferation, survival, and differentiation of newborn cells in the dentate gyrus of the hippocampus. The present study investigated whether CORT-induced suppression of adult hippocampal neurogenesis could be restored by *Ginkgo* co-treatment. After one week of habituation, 32 young adult male Sprague-Dawley rats were injected i.p. with 50 mg/kg BrdU twice daily for three consecutive days. Starting the next day, rats were injected i.p. with vehicle or 25 mg/kg *Ginkgo* daily for 22 days. Half of the rats in the vehicle and *Ginkgo* groups were also injected immediately with 35 mg/kg CORT subcutaneously, while the other half in each group received the vehicle used for CORT. On days 20 and 21, one hour after their daily drug treatments, rats were subjected to the light/dark emergence test followed immediately by the elevated plus maze. On day 22, one hour after their last daily drug injections, rats were sacrificed. Adverse physiological effects of chronic CORT treatment included body weight loss as well as atrophy of the thymus and adrenal glands. These effects were not reversed by co-treatment with *Ginkgo*. Results also showed that CORT treatment significantly suppressed newborn cell density in the dorsal dentate gyrus. *Ginkgo* did not attenuate the suppressive effect of CORT on cell survival. In addition, *Ginkgo* by itself had no effect on survival of newborn cells in either the dorsal or ventral dentate gyrus. In summary, the results of the present study suggest that chronic CORT treatment suppressed survival of newborn cells in the dorsal dentate

gyrus, caused body weight loss, thymus and adrenal gland atrophy, but *Ginkgo* co-treatment did not buffer these effects.

Standardized *Ginkgo biloba* leaf extracts (*Ginkgo*) have been shown to improve cognitive functioning both in humans and in rodents (Kennedy et al., 2000; Le Bars et al., 1997; Mix & Crews, 2000, 2002; Satvat & Mallet, 2009; Stackman et al., 2003; Stoll et al., 1996; Stough et al., 2001; Y. Wang et al., 2006; E. Winter, 1991; J. C. Winter, 1998; Wirth et al., 2000). Various biological activities of *Ginkgo* have been suggested to underlie its beneficial effects on cognition. *Ginkgo* improves cerebral metabolism (Braquet & Hosford, 1991), enhances cerebral blood flow and microcirculation (Krieglstein et al., 1986), protects cellular membrane fluidity (Stoll et al., 1996), and modulates activity of the cholinergic system (Chopin & Briley, 1992; Das et al., 2002; Kristofikova et al., 1992; Taylor, 1986; Yamamoto et al., 2007). Since several lines of evidence strongly suggested that *Ginkgo* might enhance adult hippocampal neurogenesis the experiments in the preceding chapter explored its possible neurogenic-enhancing properties as another means to explain its nootropic effects. However, results showed that *Ginkgo* treatment affected neither cell proliferation nor survival of newborn cells in the dentate gyrus of the hippocampus in young adult male rats.

Nonetheless, a recent report showed enhancing effects of *Ginkgo* on adult hippocampal cell proliferation in both young and old mice using a transgenic model of Alzheimer's disease (TgAPP/PS1) (Tchantchou et al., 2007). That study did not investigate the influence of *Ginkgo* on cell survival, but notably, *Ginkgo* treatment was shown to significantly enhance neuronal proliferation in aged (22 months)-matched wild type mice as it did in both aged and young transgenic mice. However, *Ginkgo* treatment had no effect on hippocampal cell proliferation in younger mice (6 months). Those findings, together with results presented earlier raise the possibility that *Ginkgo* may

exert its effect on hippocampal neurogenesis only in a brain compromised by age, stress or Alzheimer's disease. Thus, the aim of the present study was to investigate the effects of *Ginkgo* on hippocampal neurogenesis under conditions in which the baseline rate of neurogenesis is suppressed.

Adult hippocampal neurogenesis has been shown to be sensitive to circulating levels of glucocorticoids (Cameron & Gould, 1994; Gould, Cameron, Daniels, Woolley, & McEwen, 1992). Glucocorticoids are released from the adrenal cortex in response to diurnal cues as well as in response to various stressors. The primary adrenal glucocorticoids are cortisol and corticosterone (CORT) in humans and in rodents, respectively (Hibberd, Yau, & Seckl, 2000). Proliferation of progenitor cells is believed to be regulated, at least in part, by circulating glucocorticoids. For instance, elevated levels of CORT have been shown to reduce cell proliferation (Cameron & Gould, 1994). In addition, survival of newly born cells in the dentate gyrus (Wong & Herbert, 2004), and their differentiation into mature neurons (Wong & Herbert, 2006) have also been shown to be sensitive to circulating CORT levels. A CORT-free environment by adrenalectomy, on the other hand, has been shown to significantly increase proliferation and survival of newborn cells in the dentate gyrus (Cameron & Gould, 1994; Wong & Herbert, 2004). Consistent with these findings, other experimental manipulations, which alter levels of adrenal glucocorticoids, have been shown to modulate adult hippocampal neurogenesis (Tanapat et al., 2001). For example, elevated levels of CORT by aging decrease the rate of hippocampal neurogenesis (Kuhn, Dickinson-Anson, & Gage, 1996; McDonald & Wojtowicz, 2005), but this effect can be reversed by adrenalectomy (Cameron & McKay, 1999). In addition, stress such as predator odour in rats (Tanapat et

al., 2001), or psychosocial stress in tree shrews (Gould, McEwen, Tanapat, Galea, & Fuchs, 1997) and marmoset monkeys (Gould, Tanapat, McEwen, Flugge, & Fuchs, 1998), causes significant suppression of cell proliferation in the dentate gyrus. Chronic restraint stress has also been shown to inhibit survival of newborn cells of the dentate gyrus (Pham, Nacher, Hof, & McEwen, 2003). Thus, the increase in the baseline CORT levels brought about by aging or stress is detrimental for adult hippocampal neurogenesis. Therefore, treatments that normalize the elevated CORT may reverse CORT-induced suppression of cell proliferation in the dentate gyrus.

Repeated treatment with *Ginkgo* has been shown to decrease circulating levels of CORT by reducing levels of messenger RNA protein and drug binding of adrenocortical mitochondrial peripheral-type benzodiazepine receptors in rats (Amri et al., 1996). *Ginkgo* pre-treatment has also been found to normalize elevated levels of circulating CORT induced by restraint stress in rats (Rai et al., 2003; Shah et al., 2003). Thus, the ability of *Ginkgo* to enhance hippocampal neurogenesis in aged (Tchantchou et al., 2007), but not young rodents (Tchantchou et al., 2007), might have been, in part, due to the normalizing effects of *Ginkgo* on aged-induced CORT elevations. Accordingly, the present study examined whether the reduced number of newborn cells in the dentate gyrus of young adult rats treated with CORT could be restored to normal levels by *Ginkgo* co-treatment.

At the molecular level, 21 days of repeated daily CORT injections (40 mg/kg, i.p.) cause suppression of hippocampal neurogenesis in rats (Wong & Herbert, 2004, 2006) and at the behavioural level, a similar regimen of CORT injections produces emotional changes that correspond to symptoms of clinical depression but not anxiety

(Gregus, Wintink, Davis, & Kalynchuk, 2005). For example, chronic CORT treatment (40 mg/kg i.p. for 21 days) has been shown to increase depressive like behaviour measured by longer immobility time and shorter swimming time in the forced swim tests (Gregus et al., 2005; Johnson, Fournier, & Kalynchuk, 2006). However, a similar CORT regimen and also implantation of a 100 mg CORT pellet for one week have been found to be ineffective in altering anxiety measured by the open field, elevated plus maze, and social interaction tests (Fernandes, McKittrick, File, & McEwen, 1997; Gregus et al., 2005). However, CORT has been shown to induce anxiety in the light/dark emergence test. Female mice treated chronically with CORT (13 mg/kg for 17 or 18 days) significantly decreased exploratory behaviours in the light compartment of the light/dark emergence apparatus (Ardayfio & Kim, 2006). Because different animal tests of anxiety are sensitive to different 'states of anxiety' (File, 1992), the present study compared the chronic effects of high dose CORT treatment in two different tests of anxiety: the light/dark emergence and the elevated plus maze.

It has been shown that the nature of anxiety measured in the second trial of the elevated plus maze is distinct from that measured in the first trial (Fernandes & File, 1996), thus rats were tested in each of the anxiety tests twice over two consecutive days. In the previous chapter the acute and chronic effects of *Ginkgo* on anxiety were examined using the elevated plus maze, light/dark emergence, and social interaction tests only once. While an acute high dose of *Ginkgo* treatment appeared to be anxiogenic in some measures of anxiety (only in the light/dark emergence test), the chronic treatment was neither anxiogenic nor anxiolytic. Animals had 21 days of drug exposure before the test day and were tested while drug-free. In the present study, rats were tested twice in the

light/dark emergence and the elevated plus maze and unlike the previous study rats were tested under the influence of the drug treatment on both days.

The purpose of the present investigation was two-fold. First, it investigated whether CORT-induced suppression of cell survival can be attenuated by *Ginkgo* co-treatment. Second, it investigated any possible interactions that may exist between *Ginkgo* and CORT in anxiety-like behaviour by utilizing two behavioural tests of anxiety namely, the elevated plus maze and the emergence test.

Method

Subjects. Thirty-two male Sprague-Dawley rats (Charles River, Canada), weighing between 230 and 250 g upon arrival to the colony were used. Rats were pair-housed in standard plastic shoebox cages (45 × 25 × 20 cm) maintained at 21-22°C in a colony room on a 12 hour reversed light-dark cycle (lights off at 0700 h). Testing was conducted exclusively during the dark cycle. Experimental procedures followed Canadian Council on Animal Care guidelines, and were approved by the Wilfrid Laurier University Animal Care Committee.

Drugs. Ginkgoselect (*Ginkgo*, Indena S.p.A., Milan, Italy), composed of EGb 761 complexed with soy phospholipids at a ratio of 1:2 (w/w) to increase bioavailability, was dissolved in 10% w/v 2-hydroxypropyl-β-cyclodextrin (2-HβC; OnBio Inc.). The dose (25 mg/kg) is expressed to reflect the EGb 761 content of the phospholipid complex. 5-bromo-2'-deoxyuridine (BrdU, Sigma-Aldrich), dissolved in 10% w/v 2-HβC in a 37 ° C water bath, was administered at a dose of 50 mg/kg. Both *Ginkgo* and BrdU were administered intraperitoneally (i.p.) in a volume of 2 ml/kg of body weight.

Corticosterone (Sigma-Aldrich), dissolved in 40% w/v 2-HβC, was administered subcutaneously (s.c.) at a dose of 35 mg/kg in a volume of 1 ml/kg body weight.

Apparatus.

LIGHT/DARK EMERGENCE

The light/dark emergence apparatus was similar to the one described in the previous chapter. Briefly, it consisted of a white square open field (120 × 120), and a black wooden hide box (40 × 24 × 15 cm) placed inside the open field. The open field was illuminated with red light (5 lux at floor level). The activities of the rats were tracked and measured by the ANY-maze video tracking system (version 4.3; Stoelting Co.). On test days, rats were placed inside the hide box and their behaviours were recorded for 5 min. The percentage of time spent in the hide box (time in the hide box ÷ total duration of the test × 100), exit latency, and distance traveled were measured. The apparatus was cleaned between test sessions with a detergent (Coverage 256, Steris Corp) diluted 1:1000 in water, and then thoroughly dried.

ELEVATED PLUS MAZE

The elevated plus-maze apparatus was similar to the one used and described in the preceding chapter. Briefly, it consisted of two open arms (10 × 50 cm) and two opposite closed arms (10 × 50 cm) with walls 40 cm in height. A central square (10 × 10 cm) connected the four arms. The maze was 50 cm elevated from the floor. The testing room was illuminated with a red light (5 lux at the maze floor). The percentage of time spent in the enclosed arms (time in the enclosed arms ÷ total duration of the test × 100) as well as the number of entries into each arm and locomotor activity were determined using ANY-

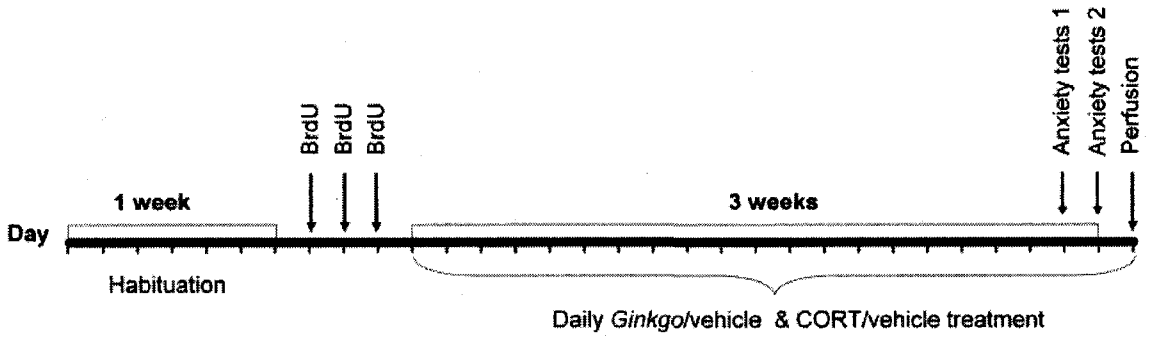
maze (version 4.3; Stoelting Co.). On test days, rats were placed in the centre part of the maze, facing one of the open arms and were tested for 5 min. The apparatus was cleaned between test sessions and thoroughly dried.

Procedure. An overview of the design of the study is shown in Figure 14. After one week of habituation, rats were injected with 50 mg/kg BrdU twice daily at 1100 and 1500 h for three consecutive days. The following day, chronic administration of *Ginkgo* and CORT began. Rats first received an i.p injection of either *Ginkgo* (25 mg/kg) or its vehicle and immediately afterwards received an s.c. injection of either CORT (35 mg/kg) or its vehicle. Thus, four groups of rats (n=8) were formed: vehicle-vehicle (Veh-Veh), vehicle-CORT (Veh-CORT), *Ginkgo*-vehicle (*Ginkgo*-Veh), and *Ginkgo*-CORT (*Ginkgo*-CORT). Chronic CORT treatment is typically accompanied by weight loss (e.g., see Kalynchuk, Gregus, Boudreau, & Perrot-Sinal, 2004) and for ethical reasons, it was decided that rats whose individual body weights reached less than 80% of that of the control group (Veh-Veh) would be removed from the study.

On both days 20 and 21, one hour after drug treatment, rats were subjected to the light/dark emergence test followed immediately by the elevated plus maze. On day 22, one hour after the last daily drug injections, rats were anaesthetized with an overdose of sodium pentobarbital. Once non-reflexive, rats were perfused transcardially with 150 ml 0.1 M phosphate buffer saline (PBS, pH =7.2) followed by 150 ml 4% paraformaldehyde (Mallinckrodt Baker, Inc) in 0.1 M PBS (pH= 7.2). Just prior to the perfusion blood was collected directly from the right atrium in EDTA coated tubes, and kept on ice until centrifuged at 4000 rpm for 15 minutes at 4° C. Plasma was collected and stored at -20° C to be assayed for CORT content. Brains, thymus and adrenal glands were removed

Figure 14. Timeline of the Experiment in Chapter 4.

Figure 14



immediately after the perfusion and were kept in 4% paraformaldehyde solution in PBS. One day after the perfusion, any fat or extra tissue attached to the thymus and adrenal glands was removed and the wet weight was measured immediately.

All the procedures for the perfusion, brain sectioning, immunohistochemistry and BrdU-labeled cell quantification were similar to those described in the previous chapter.

Measuring plasma CORT level. CORT levels were determined using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Assaypro, St. Charles, MO), which employs a quantitative competitive sandwich enzyme immunoassay technique. Plasma samples were thawed and diluted with a buffer provided with the kit at 1:50 for the samples from Veh-Veh and *Ginkgo*-Veh groups and at 1:500 for the samples collected from Veh-CORT and *Ginkgo*-CORT groups. All the samples were assayed in duplicate in one kit along with triplicate of CORT standards ranging from 0 to 100 ng/ml. The absorbance (optical density at a wavelength of 450 nm) was read using a microplate reader (MRX ® Microplate Absorbance Reader and Revelation™ application program, DYNEX Technologies, Inc.). The standard curve was plotted as optical density against log concentrations of CORT using Prism (GraphPad Software, version 5.0a). Plasma CORT concentrations of the samples were interpolated from the standard curve using a non-linear regression analysis perfumed in Prism and were corrected by the dilution factors.

Data analysis. Body weights were analyzed using a three-way (*Ginkgo* × CORT × day) analysis of variance with repeated measures on 'day'. When a three-way interaction was found data were further analyzed by two-way (*Ginkgo*/Veh or CORT/Veh by day)

ANOVAs with repeated measures on 'day'. Significant day by CORT or day by *Ginkgo* interactions were further analyzed by comparing the groups at each level of day using independent sample *t*-tests.

Plasma CORT level presented as ng/ml was analyzed by a two-way ANOVA (*Ginkgo* × CORT). Adrenal glands and thymus weights were expressed as mg per 100 g body weight on the last day of the study and were analyzed using two-way ANOVAs (*Ginkgo* × CORT). Significant interactions were followed by independent sample *t*-tests to compare the groups at each level of *Ginkgo* or CORT.

For the light dark emergence tests, the percentage of time spent in the hide box, exit latency, and distance traveled were analyzed using three-way ANOVAs (CORT × *Ginkgo* × trial) with repeated measures on 'trial'. For the elevated plus maze test, the percentage of time spent in the enclosed arms, number of entries to the open arms, and distance traveled were also analyzed by separate three-way ANOVAs (CORT × *Ginkgo* × trial) with repeated measures on 'trial'.

The number of BrdU-labeled cells, dentate gyrus volume and BrdU-labeled cell density in the dorsal and ventral dentate gyrus were analyzed separately using two-way ANOVAs (CORT × *Ginkgo*).

Results

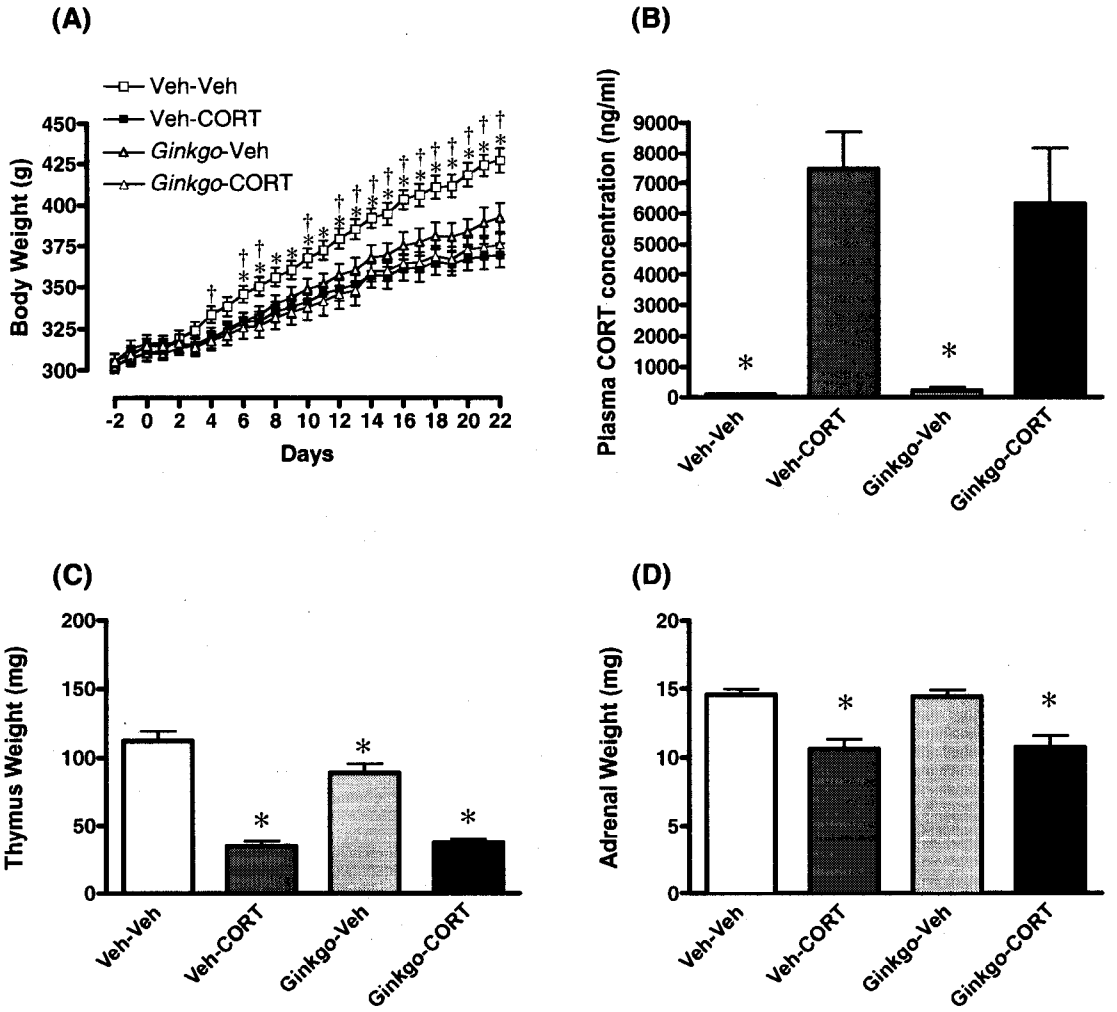
Body weights. One rat in the *Ginkgo*-CORT group was excluded from the study because its body weight reached less than 80% of that of the control group (Veh-Veh) 14 days into the CORT treatments. A two-way ANOVA comparing body weights of the four groups of rats over the three BrdU treatment days before initiation of the CORT or

vehicle treatments found no significant difference across groups. All rats were similarly gaining weights over the three days as shown in Figure 15 (A). This was supported by a significant main effect of day, $F(2, 56)=89.61$, $p<0.001$. There was no significant group by day interaction.

The three-way ANOVA comparing body weights over the 22 days of drug exposure revealed significant main effects of CORT, $F(1,28)=9.16$ $p<0.05$ and day, $F(21, 588)=538.04$, $p<0.001$. The main effect of *Ginkgo* was not significant. All the rats gained weight over 22 days. However, the two CORT-treated groups (Veh-CORT and *Ginkgo*-CORT) gained significantly less weight (Figure 15, A). The ANOVA also revealed significant interactions of day by CORT, $F(21, 588)=25.12$, $p<0.001$, day by *Ginkgo* $F(21, 588)=2.63$, $p<0.001$, and day by CORT by *Ginkgo* $(21, 588)=7.35$, $p<0.001$. To better understand the nature of the significant interactions separate two-way ANOVAs (at CORT and at *Ginkgo* levels) with repeated measure on 'day' factor were performed. For the two groups treated with CORT (i.e., comparing the Veh-CORT and *Ginkgo*-CORT groups over 22 days), the two-way ANOVA revealed only a significant effect of day, $F(21, 294)=140.97$, $p<0.001$. For the two no-CORT groups (i.e., comparing Veh-Veh and *Ginkgo*-Veh groups over 22 days), the two way ANOVA showed a main effect of *Ginkgo*, $F(1,14)=6.01$ $p<0.05$, a significant effect of day, $F(21, 294)=487.80$, $p<0.001$ and a significant interactions of day by *Ginkgo,* $F(21, 294)=10.65$, $p<0.001$. To further characterize the significant day by *Ginkgo* interaction, the body weights of the two groups of rats (Veh-Veh and *Ginkgo*-Veh) were compared at each level of day by independent sample *t*-tests. The *t*-tests revealed that the significant group differences were evident at days 4, 6, 7 and 10 until the last day. These results, as evident in Figure

Figure 15. (A) Mean (±SEM) body weight of four groups of rats over 22 days of drug treatments. *p<0.05, Veh-Veh significantly different from Veh-CORT, †p<0.05 Veh-Veh significantly different from *Ginkgo*-Veh. (B) Mean (+SEM) plasma CORT concentration (ng/ml) of four groups of rats. *p<0.001, significantly different from Veh-CORT and *Ginkgo*-CORT. (C) Mean (+SEM) thymus weight (mg) per 100 g body weight, and (D) mean (+SEM) adrenal gland weight (mg) per 100 g body weight in four groups of rats. *p<0.05, significantly different from Veh-Veh.

Figure 15



15 (A), indicate that *Ginkgo* on its own (*Ginkgo*-Veh) suppressed body weight over the 22 days of the study.

For the two groups treated with *Ginkgo* (i.e., comparing *Ginkgo*-Veh and *Ginkgo*-CORT), the two-way ANOVA showed only a main effect of day, $F(21, 294)=152.90$, $p<0.001$. for the two no-*Ginkgo* groups (i.e., comparing Veh-Veh and Veh-CORT), the two-way ANOVA revealed a main effect of CORT, $F(1,14)=11.31$ $p<0.01$, a significant effect of day, $F(21, 294)=677.84$, $p<0.001$ and a significant interaction of day by CORT, $F(21, 294)=65.39$, $p<0.001$. Consequently, the significant day by CORT interaction was followed by independent sample *t*-tests to compare the body weight of the two groups of rats (Veh-Veh and Veh-CORT) at each level of day. The *t*-tests revealed that the significant group differences began on day 6 and continued to the last day. These results, as evident in Figure 15 (A), indicate that CORT significantly decreased body weight in Veh-treated rats; however, a similar suppression was evident in *Ginkgo*-treated rats regardless of CORT treatment.

Plasma CORT concentrations. Plasma CORT levels were expressed as ng/ml and were analyzed by a two-way (*Ginkgo* by CORT) ANOVA, which only found a significant main effect of CORT, $F (1, 27)=41.31$, $p<0.001$. As shown in Figure 15 (B), plasma CORT levels in both CORT-treated groups (Veh-CORT and *Ginkgo*-CORT) were significantly higher than the other two groups (Veh-Veh and *Ginkgo*-Veh). The non-significant main effect of *Ginkgo* and the non-significant interaction indicated that *Ginkgo* either by itself or in combination with CORT injection had no effects on plasma CORT levels.

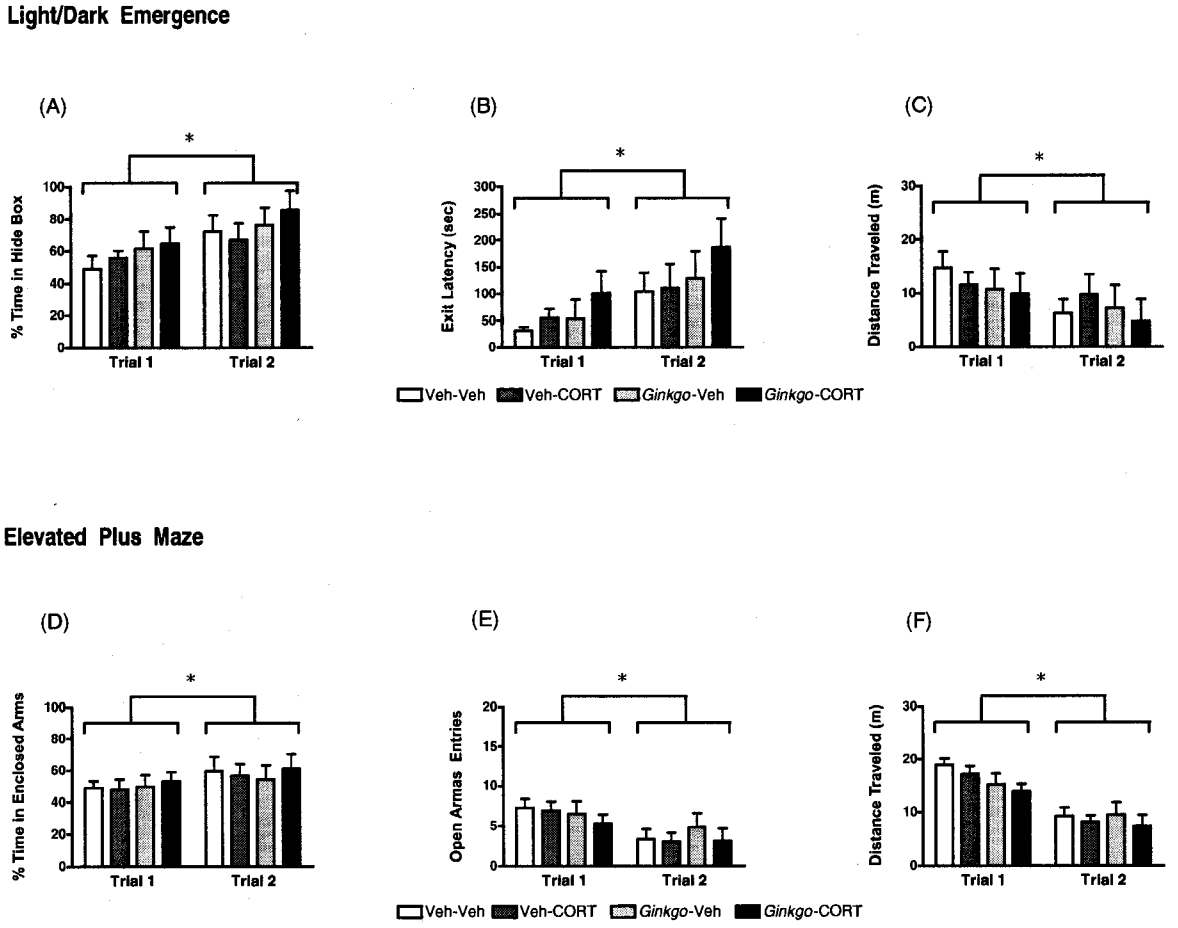
Thymus and adrenal gland weights. Figure 15 (C) shows the mean (+ SEM) thymus gland weights in four groups of rats. The thymus weight was calculated as mg per 100 g body weight and was analyzed by a two-way (*Ginkgo* by CORT) ANOVA. The ANOVA revealed a significant effect of CORT, $F(1, 27)=151.87$, $p<0.001$, and a significant CORT by *Ginkgo* interaction, $F(1, 27)=5.78$, $p<0.05$. The main effect of *Ginkgo* was not significant. To characterize the significant interaction effect, two independent sample *t*-tests were performed to compare thymus weights between the Veh-Veh and *Ginkgo*-Veh groups and between the Veh-CORT and *Ginkgo*-CORT groups. Thymus weight was significantly reduced in the *Ginkgo*-Veh group relative to the Veh-Veh group, $t(14)=2.46$, $p<0.05$.

The adrenal gland weights were also expressed as mg per 100 g body weight and were analyzed by a two-way (*Ginkgo* by CORT) ANOVA which revealed only a significant main effect of CORT, $F (1, 27)=42.46$, $p<0.001$. This indicated adrenal atrophy in both CORT-treated groups (Veh-CORT and *Ginkgo*-CORT) as shown in Figure 15 (D).

Light/dark emergence test. For the percentage of time spent in the hide box the three-way ANOVA found only a significant effect of trial, $F(1, 27)=19.29$, $p<0.001$. Neither CORT nor *Ginkgo* nor their interaction were significant (Figure 16, A). The ANOVA for exit latency also revealed a significant effect of trial, $F(1, 27)=10.50$, $p<0.01$ (Figure 16, B). The effect of trial, $F(1, 27)=16.02$, $p<0.001$ was also found to be significant for the ANOVA comparing distance traveled across groups (Figure 16, C). These results indicate that regardless of the treatment, rats were more anxious in the second trial compared to the first one. All rats spent more time in the hide box, took

Figure 16. Mean (+SEM) percentage of time spent in the hide box in Trials 1 and 2 (A), mean (+SEM) exit latency in Trials 1 and 2 (B) and mean (+SEM) distance traveled in the Trial 1 and 2 (C) in the light/dark emergence test. Mean (+SEM) percentage of time spent in the enclosed arms in Trials 1 and 2 (D), mean (+SEM) number of open arms entries in Trials 1 and 2 (E) and mean (+SEM) distance traveled in Trials 1 and 2 (F) in the elevated plus maze. Rats received chronic injections of vehicle and vehicle (Veh-Veh), 25 mg/kg Ginkgo and vehicle (Ginkgo-Veh), vehicle and 35 mg/kg corticosterone (Veh-CORT) or 25 mg/kg Ginkgo and 35 mg/kg corticosterone (Ginkgo-CORT) and were subjected to the anxiety tests on two days (Trial 1 and Trial 2). *$p<0.05$, Trial 1 significantly different from Trial 2. †$p<0.05$, significantly different from Veh-Veh and Veh-CORT groups.

Figure 16


Light/Dark Emergence
(A)
(B)
(C)
% Time in Hide Box
Exit Latency (sec)
Distance Traveled (m)
Trial 1
Trial 2
Veh-Veh
Veh-CORT
Ginkgo-Veh
Ginkgo-CORT
Elevated Plus Maze
(D)
(E)
(F)
% Time in Enclosed Arms
Open Armas Entries
Distance Traveled (m)

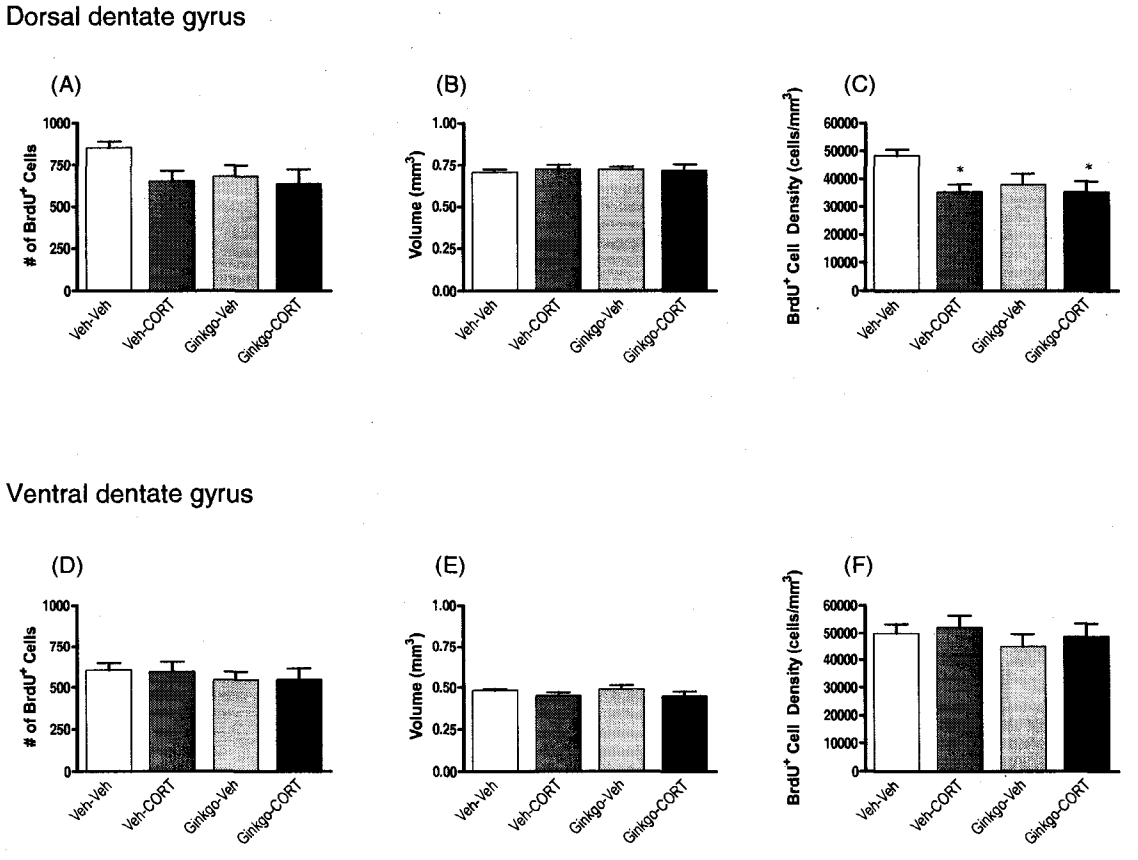
longer to exit the hide box and traveled a shorter distance in the second trial compared to the first trial.

Elevated plus maze test. For the percentage of time spent in the enclosed arms, the three-way ANOVA found only a significant effect of trial, $F(1, 27)=7.69$, $p<0.05$. Neither CORT nor *Ginkgo* nor their interaction was significant (Figure 16, D). The ANOVA for the open arms entries also revealed a significant trial effect, $F(1, 27)=33.99$, $p<0.001$ (Figure 16, E). The ANOVA for distance traveled revealed a significant effect of trial, $F(1, 27)=155.70$, $p<0.001$, and a significant trial by *Ginkgo* interaction $F(1, 27)=6.69$, $p<0.05$. Thus, each trial was analyzed separately by two-way ANOVAs, which for distance traveled found a significant effect of *Ginkgo* $F(1, 27)=4.65$, $p<0.05$ in the first trial only. These results also indicate that the rats were more anxious in the second trial compared to the first trial and that there was no group differences either in trial 1 or in trial 2. All four groups of rats traveled less distance in the second elevated plus maze trial compared to the first one; however, *Ginkgo*-treated rats (*Ginkgo*-Veh and *Ginkgo*-CORT) traveled less distance compared to the other groups (Veh-Veh and Veh-CORT) only in the first trial (Figure 16, F).

Hippocampal neurogenesis. Data from one rat in the *Ginkgo*-CORT group was lost due to technical difficulties during the perfusion and as mentioned previously another rat from the same group was excluded from the study due to weight loss (final n=6 for the *Ginkgo*-CORT group). Figure 17 shows, the number of BrdU-labeled cells, volume and BrdU-labeled cell density for both the dorsal and the ventral dentate gyrus in the four groups of rats. The number of BrdU-labeled cells, dentate gyrus volume and BrdU-

Figure 17. Mean (+SEM) number of BrdU-labeled cells (A), mean (+SEM) volume of dentate gyrus (B) and mean (+SEM) BrdU-labeled cell density (C) in the dorsal region used for quantifying BrdU-labeled cells. Mean (+SEM) number of BrdU-labeled cells (D), mean (+SEM) volume of the dentate gyrus (E) and mean (+SEM) BrdU-labeled cell density (F) in the ventral region. Rats received 50 mg/kg BrdU injections twice daily for 3 days followed by 22 days of vehicle and vehicle (Veh-Veh), 25 mg/kg *Ginkgo* and vehicle (*Ginkgo*-Veh), vehicle and 35 mg/kg corticosterone (Veh-CORT) or 25 mg/kg *Ginkgo* and 35 mg/kg corticosterone (*Ginkgo*-CORT) injections. Rats were perfused one hour after the last drug injection.

Figure 17



labeled cell density were analyzed separately for the dorsal and ventral hippocampus using two-way ANOVAs. There was only a significant CORT effect, $F(1, 26)=4.72$, $p<0.05$, for cell density in the dorsal dentate gyrus, indicating that both CORT-treated groups had significantly less BrdU-labeled cell density compared to the Veh-Veh and *Ginkgo*-Veh groups. These results show that *Ginkgo* did not reverse the suppression of hippocampal neurogenesis induced by CORT treatment. See Figure 18 for representative photomicrographs showing BrdU-labeled cells in the dentate gyrus of the four groups of rats.

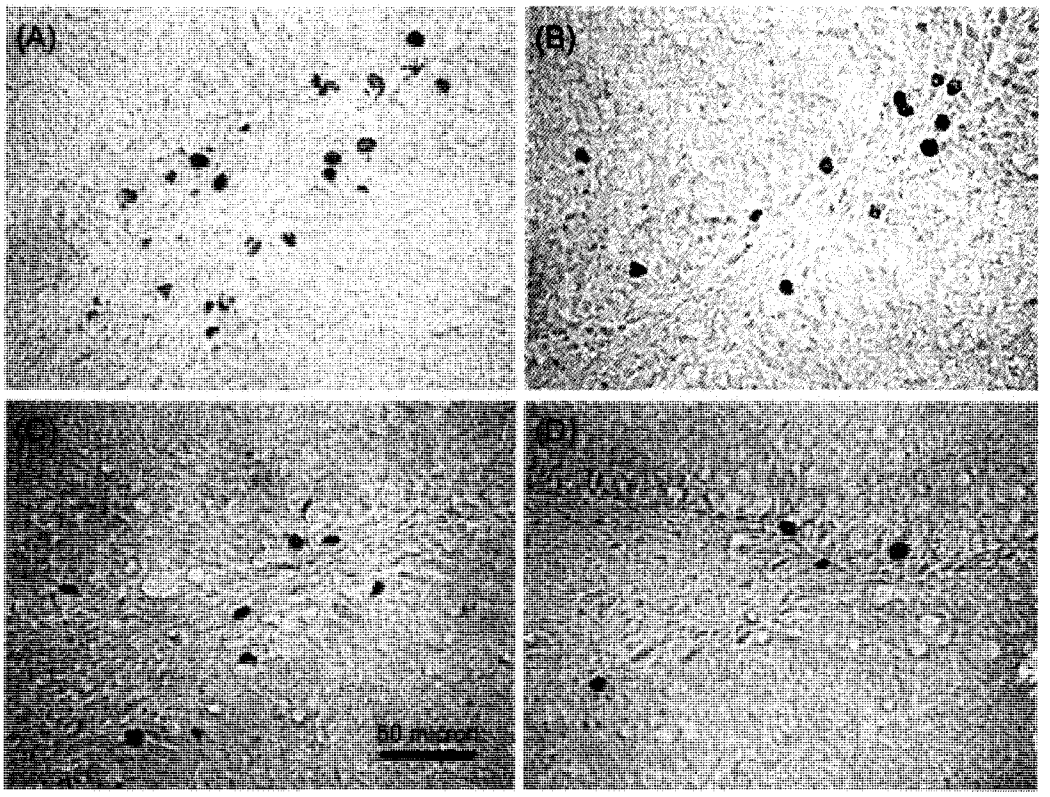
Discussion

The aim of the present study was to determine whether CORT-induced suppression of adult hippocampal neurogenesis could be restored by *Ginkgo* co-treatment. Results showed that survival of newborn cells was inhibited in the dorsal dentate gyrus of rats chronically treated with CORT; however, *Ginkgo* co-treatment failed to attenuate this effect. The ability of chronic CORT administration to induce anxiety and whether *Ginkgo* co-treatment could influence any observed anxiogenic properties of repeated CORT injection were also examined. Measures of anxiety were not significantly affected by repeated daily CORT treatment; however, regardless of the treatments, all rats appeared to be more anxious in the second trial of testing compared to the first trial.

Chronic CORT treatment clearly increased plasma CORT concentrations and induced adverse physiological effects. The baseline level of CORT in young Sprague_Dawley rats is approximately 50 ng/ml (Rai et al., 2003). The mean (± SEM) concentration of CORT was 90.03 (±15.39) ng/ml and 221.64 (±92.13) ng/ml for the

Figure 18. Photomicrographs (at approximately 400× magnification) of BrdU-labeled cells in the dentate gyrus of rats treated with Veh-Veh (A), *Ginkgo*-Veh (B), Veh-CORT (C) and *Ginkgo*-CORT (D).

Figure 18



Veh-Veh and *Ginkgo*-Veh groups, respectively. Stress induced by two daily injections in the Veh-Veh group may explain the increased level of circulating CORT in these animals. Repeated treatment with *Ginkgo* (50 and 100 mg/kg for 8 administered orally) has been reported to decrease circulating levels of CORT (Amri et al., 1996), however the dose of *Ginkgo* used in the present study administered i.p. failed to reduce circulating CORT levels in the *Ginkgo*-Veh group. With chronic CORT treatment the plasma CORT levels reached 7468.11 (±1227.53) ng/ml in the Veh-CORT group, and *Ginkgo* co-administration failed to significantly reduce plasma CORT levels and was found to be 6320.75 (±1854.20) ng/ml in the *Ginkgo*-CORT group. Plasma CORT levels have been shown to increase dramatically to 450 ng/ml after a single exposure to restraint stress (150 min) and to approximately 300 ng/ml after chronic (150 min a day for 7 consecutive days) exposure to restraint stress (Rai et al., 2003). The plasma CORT concentration in the present study was much higher than the level of CORT reported in stressed animals and may explain the discrepant results.

Consistent with previous studies (e.g., see Fernandes et al., 1997; Gregus et al., 2005), rats chronically treated with CORT exhibited significant reductions in thymus, adrenal and body weights (Figure 15). *Ginkgo* co-treatment did not restore the effects of CORT on body weights or thymus and adrenal gland atrophy. In fact, the body weights of animals treated with *Ginkgo* (*Ginkgo*-Veh) fell significantly lower than those of the control rats (Veh-Veh) on days 4, 6, 7 and 10 to the end of treatment. Surprisingly, the thymus weight of *Ginkgo*-treated rats (*Ginkgo* -Veh) was also found to be significantly lower than that of the control (Veh-Veh) group (Figure 15, B). This is in contrast with a report showing rejuvenating effects of *Ginkgo* on degenerated thymus glands in aged female mice (Tian, Tian, Zhang, & Dai, 2003). The reason for this discrepancy is not clear; however in this study, aged female mice (22-months-old) were fed a relatively low

dose (1.5 mg) of *Ginkgo* extract for 60 days. It is therefore possible that gender differences, age at exposure, length of treatment, route of administration, drastic differences in dose, or some combination of these factors, may have accounted for the discrepant results. Although adrenal weight was not affected by chronic *Ginkgo* administration in the present study, the fact that there was weight loss as well as thymus atrophy suggests that i.p. administration of this dose of the extract may have induced some kind of malaise. Note that this dose of *Ginkgo* also failed to influence baseline plasma CORT level in the control rats (Veh-Veh), although repeated treatment with *Ginkgo* (50 and 100 mg/kg for 8) has been reported to decrease circulating levels of CORT (Amri et al., 1996) and to normalize elevated levels of circulating CORT induced by restraint stress (Rai et al., 2003; Shah et al., 2003).

The present study evaluated the anxiogenic effects of chronic CORT administration using a battery of anxiety tests, including light/dark emergence and the elevated plus maze. The relationship between CORT and *Ginkgo* co-treatment in measures of anxiety was also evaluated. Despite the significant elevation of plasma CORT levels in the CORT-treated rats, there were no significant effects on measures of anxiety. In the light/dark emergence test the four groups of rats spent similar amounts of time in the hide box (Figure 16, A), took similar amounts of time to exit the hide box (Figure 16, B) and traveled similar distances in the open field of the light/dark emergence apparatus (Figure 16, C). In the elevated plus maze test, regardless of the treatment, rats spent similar amounts of time in the enclosed arms (Figure 16, D) and entered into the open arms of the elevated plus maze similar number of times (Figure 16, E). However, the two groups of rats treated with *Ginkgo* (*Ginkgo*-Veh and *Ginkgo*-CORT) traveled less

distance compared to the other two groups that did not receive *Ginkgo*. This effect was only evident in the first trial (Figure 16, F).

Previous studies have shown that elevated CORT levels due to chronic CORT injection (Gregus et al., 2005; Kalynchuk et al., 2004) or CORT pellet implantation (Fernandes et al., 1997) do not change measures of anxiety in the elevated plus maze, social interaction, open field and resistance-to-capture tests. In fact, it has been suggested that chronic CORT administration produces a double-dissociation between elevated corticosteroid concentration and anxiety (Fernandes et al., 1997). It seems that emotional changes brought about by chronic daily CORT treatment, both in male and in female rats, correspond to symptoms of clinical depression rather than anxiety (Gregus et al., 2005; Kalynchuk et al., 2004). For example, chronic CORT injection (40 mg/kg for 21 days) has been shown to increase depression-like behaviours in the forced swim test (Kalynchuk et al., 2004). The present study did not evaluate the chronic effects of CORT and *Ginkgo* co-treatment on animal models of depression, but the lack of significant effects in the two animal tests of anxiety employed agrees with the aforementioned studies. Nonetheless, one study has reported that measures of anxiety in the light/dark emergence test were influenced by chronic CORT treatment (13 mg/kg for 17 or 18 days). Exit latency significantly increased in mice chronically treated with CORT (Ardayfio & Kim, 2006). This is in contrast with the light/dark emergence data obtained in the present study and may be due to the lower dose of CORT employed.

Consistent with the results presented in the previous chapter, chronic *Ginkgo* treatment on its own was not found to influence anxiety. In our previous study, rats received their last daily injection of *Ginkgo* 1 day before behavioural tests. In the present

study, rats were under the influence of *Ginkgo* treatment during both tests and both trials and yet no effect was evident in the anxiety measures. Our results showing that chronic *Ginkgo* treatment had no anxiolytic effects contrast with another study which reported anxiolytic-like effects of *Ginkgo* (125 mg/kg) in the elevated plus maze after one week of oral administration in mice (Kuribara et al., 2003).

The anxiety data also revealed that regardless of treatment, rats were more anxious in the second trial compared to the first trial (Figure 16). All rats spent more time in the hide box, had longer exit latencies, and traveled shorter distances in the second light/dark emergence trial compared to the first. Similarly, all rats spent more time in the enclosed arms, entered less into the open arms, and traveled shorter distances in the second elevated plus maze trial compared to the first. These results are consistent with other studies that have shown repeated exposure to the elevated plus maze reduces open arm exploration in the following trials even when rats are treated with diazepam (e.g., see Treit, Menard, & Royan, 1993).

Finally, the present study investigated whether *Ginkgo* could attenuate CORT-induced suppression of adult hippocampal neurogenesis. Survival of newborn cells was inhibited in the dorsal dentate gyrus of rats chronically treated with CORT (35 mg/kg for 21 days). This was evident by the decreased density of newborn cells in the dorsal dentate gyrus. This finding is consistent with previous reports that suggest the rate of neurogenesis is regulated by levels of circulating CORT (Cameron & Gould, 1994). Previous studies have shown that CORT treatment suppresses proliferation (Cameron & Gould, 1994; Gould et al., 1992) as well as survival (Wong & Herbert, 2004) of newborn cells in the dentate gyrus of the hippocampus. Differentiation of newborn cells into

mature neurons has also been shown to be inhibited by repeated CORT treatment (Wong & Herbert, 2006). However, previous studies did not differentiate between the influence of CORT on neurogenesis in the dorsal and ventral regions of the dentate gyrus. A novel aspect of the present study was that chronic CORT treatment suppressed survival of newborn cells exclusively in the dorsal, but not ventral dentate gyrus (Figure 17). In the preceding chapter the influence of fluoxetine on hippocampal cell survival were also included as a positive control to verify the immunohistochemical and cell quantification methods employed. However, fluoxetine in our study failed to increase survival of newborn cells in the dentate gyrus. In the present study, the fact that CORT treatment was found to reduce cell survival confirmed the immunohistochemical methods used in both studies.

Consistent with the results presented in the previous chapter, chronic *Ginkgo* treatment on its own had no effect on the survival of newborn cells in the dentate gyrus, confirming that the beneficial effects of *Ginkgo* on cognitive processing do not appear to be mediated by the enhancement of hippocampal neurogenesis. However, in contrast to the expected results, *Ginkgo* co-treatment did not attenuate CORT-induced suppression of newborn cell survival (Figure 17). Since repeated treatment with *Ginkgo* has been found to decrease circulating levels of CORT (Amri et al., 1996) and to normalize the elevated levels of circulating CORT induced by restraint stress (Rai et al., 2003; Shah et al., 2003), it was hypothesized that the reduced number of newborn cells in the dentate gyrus of young adult rats treated with CORT could be restored to normal levels by *Ginkgo* co-treatment. Unexpected findings may, in part, be related to the timing of the injections. That is, rats were treated with both *Ginkgo* and CORT at about the same time,

but it is possible that the two drugs had different bioavailability. Pharmacokinetic information on the dose of *Ginkgo* (25 mg/kg administered i.p.) and CORT (35 mg/kg administered s.c.) used in the present study is not available. However, the half-lives of ginkgolide A, B, and bilobalide (active constituents of *Ginkgo*), after 100 mg/kg oral administration have been shown to be 1.8, 2.0 and 3.0 h, respectively (Biber & Koch, 1999). It is also possible that the dose of *Ginkgo* used in the present study was not high enough to counteract the effects of such a high dose of CORT. The present study only investigated the influence of chronic CORT and *Ginkgo* treatment on cell survival. Future studies might aim to examine whether chronic *Ginkgo* pre-treatment can protect CORT-induced suppression of cell proliferation and survival.

To summarize, results of the present study showed that chronic CORT treatment had no significant effect on measures of anxiety in adult male rats. It significantly reduced body weight and induced thymus and adrenal gland atrophy. Moreover, it suppressed survival of newborn cells in the dorsal dentate gyrus. None of these adverse effects were restored by *Ginkgo* co-treatment.

Foreword to Chapter 5

The following chapter is based on a manuscript submitted to the journal *Psychopharmacology* in April 2008. The manuscript was accepted for publication in July 2008 and is now published (Jan 2009, vol 202, pg 173-185). Dr. Paul E. Mallet is a co-author. The author of the present dissertation collected and analyzed all data, and wrote the first manuscript draft.

Chapter 5: Chronic administration of a *Ginkgo biloba* leaf extract facilitates acquisition but not performance of a working memory task

Abstract

Ginkgo biloba leaf extracts have been shown to improve learning and memory when administered chronically prior to the learning phase. However, the influence of *Ginkgo* on learning without prior chronic treatment and on memory per se (i.e., post-training administration) is less clear. Thus, Experiment 1 investigated the influence of *Ginkgo* on acquisition, and Experiment 2 examined the acute and chronic effects of *Ginkgo* on memory in young adult rats using a food-reinforced two-component double Y-maze task. In Experiment 1, seventeen rats were treated daily with a standardized *Ginkgo biloba* extract (13.75 mg/kg, i.p.) or vehicle 30 min prior to daily maze training for 14 days. *Ginkgo*-treated rats reached the training criteria slightly faster, and made fewer errors. In Experiment 2, twelve rats received 24 training trials daily, then received *Ginkgo* (0, 0.25, 2.5, 13.75, or 25 mg/kg, i.p.) 30 min prior to each test session. Post-training *Ginkgo* administration did not enhance memory. Subsequently, the same rats received daily injections of either *Ginkgo* (13.75 mg/kg, i.p.) or its vehicle. Memory was tested after 10 and 20 days of drug treatment, once under the influence of the drug, and once in a drug-free state. In all cases *Ginkgo* produced no significant effects. Taken together, results demonstrate that repeated daily pre-session *Ginkgo* injection subtly facilitates acquisition of a spatial working memory task, but neither acute nor chronic post-training exposure enhances spatial working memory. We conclude that ongoing *Ginkgo* administration does not offer any continued beneficial effects in an already-learned working memory task.

Ginkgo biloba is an evolutionarily ancient tree native to China (Major, 1967). Its fruits and seeds have been used in traditional Chinese medicine to treat a wide variety of health disorders for thousands of years (Maclennan et al., 2002); however, the standardized *Ginkgo biloba* leaf extract EGb 761 was developed for pharmaceutical use only recently (Maclennan et al., 2002). Today, EGb 761, marketed under multiple trade names including Rökan, Tanakan, Tebonin and Ginkgold, is one of the best-selling herbal medicines in Europe and the United States (Chan et al., 2007). In fact, retail sales of *Ginkgo biloba* products in the United States reached $150 million in 1998, making it the most commonly purchased herbal supplement in the mainstream market (Blumenthal, 1999). *Ginkgo biloba* extracts are commonly prescribed for the treatment of intermittent claudication, "cerebral insufficiency", vertigo, and tinnitus of vascular origin. However, it is also purchased as an over-the-counter supplement for use by healthy individuals as a nootropic, or cognitive enhancer (Darlington et al., 2000; Gold et al., 2002; Yoshikawa et al., 1999).

EGb 761 is a water-acetone extract of dried green leaves of *Ginkgo biloba*, standardized to contain 24% flavonoid glycosides (including quercetin, kaempferol, isorhamnetin), 6% terpenoids (containing 3.1 % ginkgolides A, B, C, and J and 2.9% bilobalide), and less than 9% proanthocyanidins and organic acids (DeFeudis & Drieu, 2000; Maclennan et al., 2002). The beneficial effects of EGb 761 are believed to be the result of agonistic, antagonistic and synergistic effects of its active ingredients (DeFeudis & Drieu, 2000). The pharmacological actions of each active constituent have been extensively studied. For example, flavonoid and proanthocyanidins components contribute to its antioxidant and free radical scavenging effects (DeFeudis & Drieu,

2000). Ginkgolide components are known to antagonize effects of platelet activating factor, to improve cerebral metabolism, and to protect neural tissues against hypoxic damage (Braquet & Hosford, 1991). Additionally, EGb 761 protects cellular membrane fluidity (Stoll et al., 1996), reverses neurotransmitter receptor density loss induced by aging (Taylor, 1986), and decreases circulating corticosteroid levels by reducing levels of messenger RNA protein and drug binding of adrenocortical mitochondrial peripheral-type benzodiazepine receptors (Amri et al., 1996). These beneficial properties of the extract may underlie its apparent therapeutic effects on a wide range of disorders (DeFeudis & Drieu, 2000).

Nonetheless, there are conflicting results in clinical studies regarding the putative beneficial effects of *Ginkgo biloba* extracts on cognitive function. For example, while Le Bars et al. (1997) reported that EGb 761 improved cognitive decline associated with Alzheimer's disease or multi-infarct dementia, van Dongen et al. (2000) found contradictory results. Mix and Crews (2000; , 2002) reported that EGb 761 enhanced certain aspects of cognitive performance of healthy older adults, but Solomon et al. (2002) found the *Ginkgo biloba* extract Ginkoba to be ineffective in improving cognitive functioning in a similar population. In young healthy individuals administration of the *Ginkgo biloba* leaf extract (GK 501) improves attention (Kennedy et al., 2000), while chronic administration of EGb 761 facilitates speed of processing and working memory (Stough et al., 2001). However, in a meta-analysis of 15 randomized clinical trials, Canter and Ernst (2007) reported no favourable effects of *Ginkgo biloba* extracts on any aspect of cognitive performance of young healthy adults after both acute and chronic administration.

While the clinical data are somewhat inconclusive, a few well-designed animal studies support the notion that *Ginkgo biloba* extracts enhance cognitive functioning in both young and old animals (Stackman et al., 2003; Stoll et al., 1996; Y. Wang et al., 2006; E. Winter, 1991; J. C. Winter, 1998; Wirth et al., 2000). In one study, acquisition and retention of a learned response was enhanced in young healthy mice treated for 4 or 8 weeks with 100 mg/kg *Ginkgo* prior to learning a 2-lever response sequence for food (E. Winter, 1991). In another study, aged mice (22 to 24 months old) treated with *Ginkgo* daily for 3 weeks prior to learning a passive avoidance task showed significant improvements in short-term but not long-term memory measured by avoidance latency 60 s and 24 h after shock, respectively. No improvement was evident in young (3 months old) and middle-aged (12 months old) groups (Stoll et al., 1996), perhaps due to a ceiling effect in the younger mice. Chronic daily pre-session treatment of *Ginkgo* has been shown to facilitate learning an eight-arm radial maze in young rats and a delayed non-matching to position task in aged rats. Interestingly, the life span of the *Ginkgo*-treated rats was significantly increased (J. C. Winter, 1998).

Spatial memory deficits have been also shown to be prevented by chronic *Ginkgo* treatment in both aged rats (Y. Wang et al., 2006) and in a transgenic mouse model of Alzheimer's disease (Stackman et al., 2003), effects that were attributed to enhancement of hippocampal long-term potentiation by *Ginkgo* (Y. Wang et al., 2006). However, one study reported no effect of chronic treatment (28 and 70 days) on either acquisition or retention of memory in a water maze task in aged mice (Ward et al., 2002). In this latter experiment, there was a significant difference in baseline anxiety levels measured using an elevated plus maze prior to water maze training. In a subsequent experiment, to reduce

pre-existing differences in anxiety level, a matched-pair design was applied and the results indicated that *Ginkgo* significantly reduced stress induced by cold water exposure. The authors suggested that *Ginkgo* may act as an anti-stress buffer rather than a cognitive enhancer (Ward et al., 2002). Indeed, it has been demonstrated, for example, that stress-induced learning deficits in a discrimination task (Rapin et al., 1994) and stress-induced memory deficits in a passive avoidance task (Walesiuk et al., 2005) can be diminished by repeated pre-treatment of *Ginkgo*.

Most studies that have investigated the effects of *Ginkgo* on cognitive functioning have utilized the Morris water maze, or tasks that involve inhibitory avoidance learning or appetitive operant conditioning. Because it is difficult to determine the relative contribution of non-mnemonic processes in these tasks, the present study employed a double Y-maze task comprising separate spatial discrimination and delayed alternation components. The task is unique in that it includes control procedures that guard against misinterpretation of possible non-mnemonic effects such as altered motor function, motivation, or sensory-perceptual abilities (Mallet & Beninger, 1993; Mason, Mallet, Jhamandas, Boegman, & Beninger, 1999; Smith, Beninger, Mallet, Jhamandas, & Boegman, 1994). The delayed alternation task, but not the spatial discrimination task, requires the use of working memory. A selective impairment in the delayed alternation task can therefore be confidently attributed to a deficit of working memory.

In previous studies, *Ginkgo biloba* extract treatment typically began weeks or months prior to the first training session. Thus, it is not clear whether *Ginkgo* is effective in improving acquisition and memory of a task without prior chronic treatment. Accordingly, the first experiment in the present study was undertaken to elucidate the

effects of *Ginkgo* on training and memory of a double Y-maze task without prior *Ginkgo* treatment. Furthermore, the possible acute effects of *Ginkgo* on memory have rarely been investigated. In fact, only a single study has reported the facilitative effects of acute injection of *Ginkgo* (60 and 120 mg/kg, i.p.) on an olfactory short-term recognition test in both young and aged rats (Wirth et al., 2000). More studies are clearly needed to clarify the acute effects of *Ginkgo* on memory. Therefore, the second experiment explored the acute post-training effects of *Ginkgo* on memory of a learned double Y-maze task; that is, by administrating the extract after completion of training. After the acute test, rats were injected daily with either *Ginkgo* or vehicle and the chronic post-treatment effects on spatial working memory in a learned double Y-maze task were examined.

Experiment 1: Effects of Ginkgo on maze task acquisition

Method

Subject. Eighteen male Sprague-Dawley rats (Charles River, Canada), weighing between 300 and 340 g at the start of the experiment were used. Rats were pair-housed in standard plastic shoebox cages (45 × 25 × 20 cm) maintained at 21-22°C in a colony room on a 12 hour reversed light-dark cycle (lights off at 0700 h). Testing was conducted exclusively during the dark cycle. Experimental procedures followed Canadian Council on Animal Care guidelines, and were approved by the Wilfrid Laurier University Animal Care Committee.

Rats were acclimatized to the colony and handling procedures, then were given 3 g of Kellogg's Froot Loops breakfast cereal in the home cage once daily for 2 days. Beginning the following day, food access was limited to 2 h per day. The quantity of food

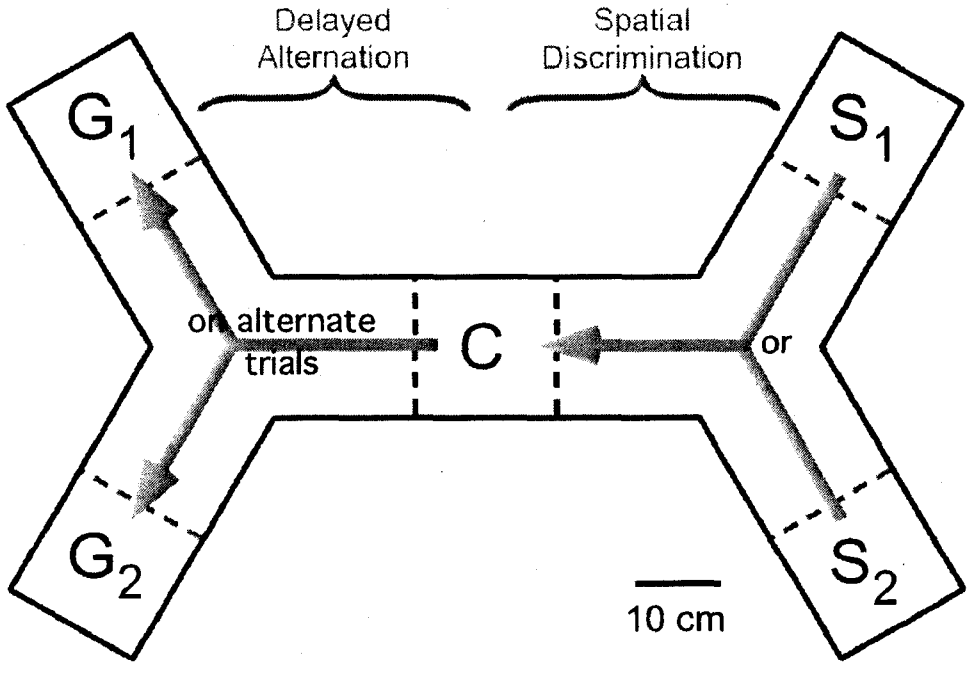
was adjusted to maintain body weights at 90% of individual baseline free-feeding weights, adjusted for growth. Rats were fed in individual cages after maze training or testing, then were placed back into their home cages for the remainder of the day. Water was freely available in the home and feeding cages.

Drug. Ginkgoselect (Indena S.p.A., Milan, Italy) was dissolved in 10% w/v 2-hydroxypropyl-β-cyclodextrin (2-HβC; OnBio Inc., Richmond Hill, Ontario, Canada). Ginkgoselect is composed of EGb 761 complexed with soy phospholipids at a ratio of 1:2 (w/w) to increase bioavailability. Ginkgoselect and vehicle control injections (10% w/v HβC) were injected intraperitoneally (i.p.) in a volume of 2 ml/kg. The i.p. route of administration was used to reduce stress associated with more traditional gavage procedures, and to better control doses and time course compared to more traditional drinking water exposure procedures. Doses are expressed to reflect the EGb 761 content of the phospholipid complex.

Apparatus. The double Y-maze (see Figure 19) was similar to that used previously (Mallet & Beninger, 1993; O'Shea & Mallet, 2005; Smith et al., 1994), but with minor modifications. The walls of the maze were constructed of melamine, and the floor was made of black ABS plastic sheeting. The walls of the first 'Y' were white, while those of the second 'Y' were dark purple. Passageways measured 16.5 cm wide and 25 cm high. There were four arms, each 35 cm long, that extended at 120° angles from a 45-cm long central stem. Removable guillotine doors were used to control access to certain parts of the maze. Opaque food cups (2 cm high, 7 cm diameter) were placed at the ends of the arms and in the central stem. Small pieces of Froot Loops cereal

Figure 19. Schematic representation of a top view of the double Y-maze. Dashed lines represent the locations of removable guillotine doors that restricted access to different parts of the maze. Food cups were located in start boxes (S_1 and S_2), the centre box (C), and goal boxes (G_1 and G_2). Arrows show possible correct paths. In the spatial discrimination, rats were placed quasi-randomly into either start box S_1 or S_2. Proceeding to box C was correct, while entering the other start box constituted an incorrect choice. In the delayed alternation, the correct response was to alternate between G_1 and G_2 on successive trials.

Figure 19



(approximately one-sixth of a full piece) were placed in the food cups and were used to reward correct responses. To mask possible food odor cues, cereal dust was scattered throughout the maze. The entire maze was placed on a 76-cm high table in a testing room illuminated with moderate white lighting (360 lux at the maze floor). Constant visual cues such as posters, light fixtures, and the researcher were visible from within the maze. Between individual tests, the walls and floor of the maze were cleaned with a detergent (Coverage 256, Steris Corp, Mentor, OH, USA) diluted 1:1000 in water, and then thoroughly dried.

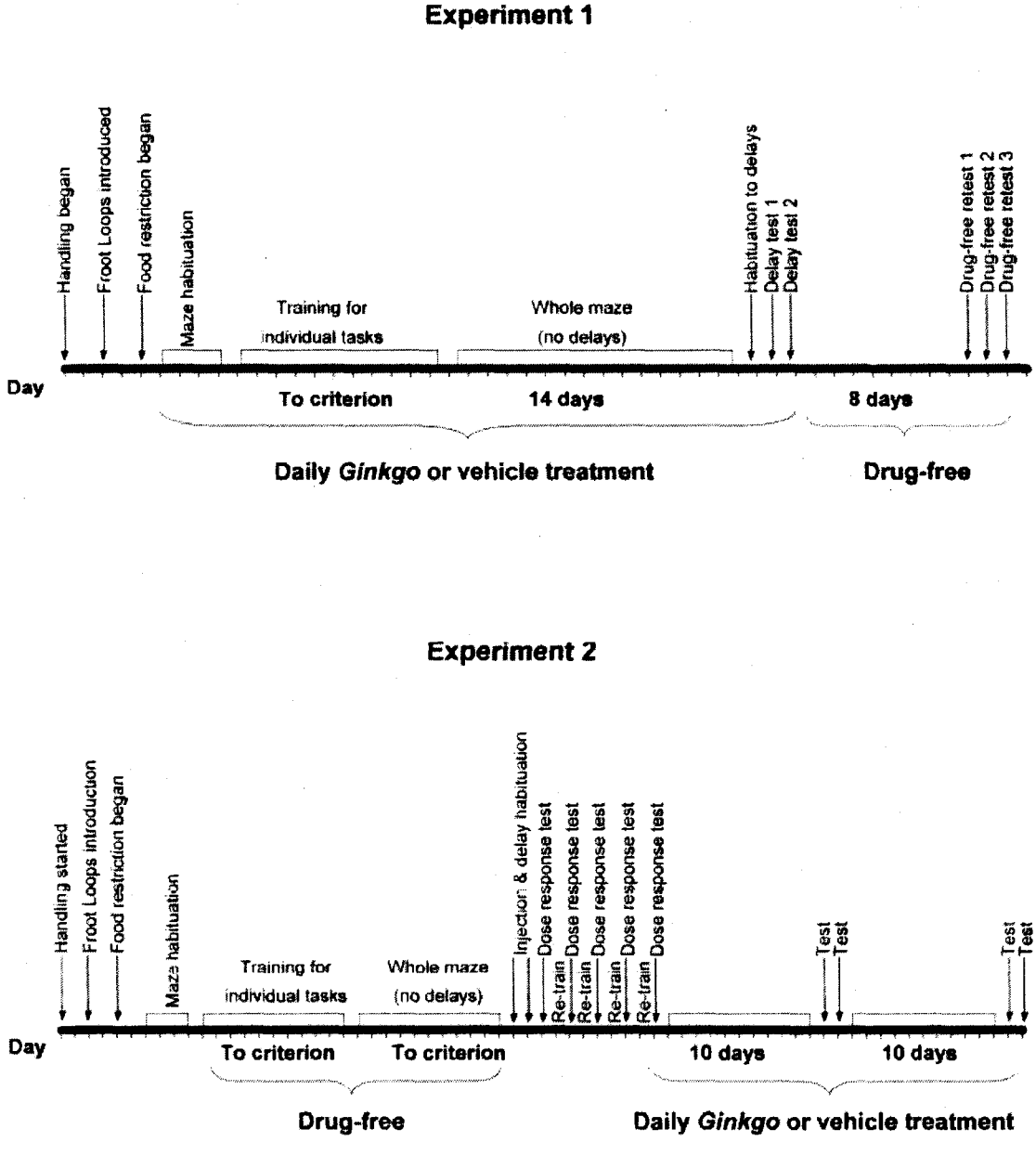
Procedure. An overview of the timeline of the phases of Experiment 1 is shown in Figure 20 (Top).

HABITUATION

Two days after initiation of food restriction, rats were habituated to the maze over four consecutive days. Daily i.p. injections of either 10% w/v 2-HβC (vehicle) or 13.75 mg/kg *Ginkgo* began on the first day of habituation. Each day, rats received 15 min of free access to all maze areas 30 min after drug injection, and were permitted to collect cereal pieces from all food cups. Beginning on the second day of habituation, rats were gradually introduced to the movement of the guillotine doors. One rat from the *Ginkgo* group still displayed overt signs of fear (defecating, urinating and freezing or jumping out of the maze) on the last day of habituation and was therefore excluded from the study.

Figure 20. Timeline of the various phases of Experiment 1 (top) and Experiment 2 (bottom) in chapter 5.

Figure 20



TRAINING

Following habituation, rats were given one 24-trial training session per day, 30 min after injection of either vehicle or *Ginkgo*. Half the rats in each group were randomly chosen to be initially trained in the first 'Y' (i.e., the spatial discrimination task), while the other half were initially trained in the second 'Y' (i.e., the delayed alternation task). Training in each task continued until animals reached an acquisition criterion of at least 21 out of 24 trials correct on 2 consecutive days.

Spatial discrimination training involved placing a rat into one of the randomly chosen start boxes (S1 or S2, see Figure 19), with the guillotine door lowered. The guillotine door at the distal end of box 'C' was also lowered to prevent entry into the second 'Y' maze. A small piece of cereal reward was placed in the food cup located in 'C'. The start box guillotine door was raised and the rat was allowed to find the reward in box 'C'. If the animal first entered the other start box, the guillotine door was lowered behind it, an error was scored and the animal was immediately placed back into the start box to start the next trial. Entering 'C' without first entering the other start box was scored as a correct choice. Once the rat was in 'C' the guillotine door behind it was lowered, and the animal was permitted to consume the food. The animal was then immediately placed into a randomly chosen start box to begin the next trial.

For delayed alternation training, each trial began by placing an animal in 'C' (see Figure 19) with the guillotine doors lowered in front of and behind the animal. Box 'C' therefore served as a start box. The task was to choose alternate arms on consecutive trials. That is, if the reward was placed in G1 on the first trial, it was placed in G2 on the second, and so on. On the first trial of each session, the initial goal box (G1 or G2) was

chosen randomly and was baited with a piece of cereal. On this first un-scored trial only, a guillotine door blocked the incorrect goal box such that an error was not possible. On subsequent trials, the start box door was raised and the rat was permitted to collect food by choosing the alternate arm on each trial. After a correct choice, the guillotine door was lowered behind the rat. After consuming its reward, the rat was removed immediately from the goal box and was placed back into 'C' to begin the next trial. Incorrect choices were not reinforced, and the guillotine door was lowered to prevent further choices. The animal was immediately placed back into 'C' box to start the next trial.

Once rats had reached the acquisition criterion in the first task, training in the second task was undertaken. When the rats reached the training criteria for both individual tasks, they were tested on the whole maze task. That is, each trial consisted of a spatial discrimination, followed by a delayed alternation. The initial start box was chosen randomly on each trial, with the proviso that an equal number of each start box was chosen in each session, and the same box was not used consecutively more than three times. Each rat was placed into one of the randomly chosen start boxes. At this stage, the distal door of the 'C' box was closed. If the rat entered the other start box, a door was placed behind it, and a spatial discrimination task error was noted. The rat was placed back into the start box to initiate the trial again. When the rat entered the correct 'C' box, a door was placed behind it to prevent the animal's retreat back in to the first Y. After consuming the reward in box 'C', the distal door of box 'C' was opened and the rat was presented with the alternation task. The rat had to choose alternate arms on successive trials. Rats were tested on the full maze task for 14 days.

DELAY TESTING

Delay testing was identical to full maze testing, except that the working memory load needed to solve the delayed alternation task was increased by the addition of inter-trial delays. Delays were imposed by retaining the rat in the start box for 0, 15, or 60 s before opening the door at the start of each trial. The order of delays was quasi-random, with an equal number of each delay (i.e., eight) used within each session. Approximately 5 s was required between trials to remove the rat from the goal box, place it back in the start box, bait the appropriate food cups, and lower the guillotine doors. Thus, the inter-trial delays should be understood as 0, 15, or 60 s plus the few seconds required to prepare the maze for the next trial. Rats were habituated to the delay intervals for one day before being tested for two consecutive days. Rats were injected with vehicle or *Ginkgo*, 30 min prior to testing.

DRUG-FREE RE-TEST

To determine whether *Ginkgo* had any lasting residual effects, injections were discontinued after the last delay test, and 8 days later rats were tested in the maze without any treatments or delays for 3 consecutive days.

Data analysis. The number of trials required to reach the training criterion and the total number of errors committed during the individual task training phases were analyzed separately using two-way (group × task) ANOVAs with repeated measures on the 'task' factor.

For the whole-maze (no delay) test phase, the number of correct choices was converted to percentage correct scores for both the spatial discrimination and delayed

alternation tasks for each of the 14 test sessions. Two-way ANOVAs (group × day) with repeated measures on the 'day' factor were performed separately for the spatial discrimination and delayed alternation tasks. Significant day main effects were further analyzed using Bonferroni-corrected pairwise comparisons to compare the first day with the remaining 13 days. Planned comparisons using independent sample *t*-tests were also used to compare groups at each day.

Raw scores for the number of correct responses in the two delay tests were converted to percentage correct responses. For each test, the spatial discrimination and delayed alternation tasks were analyzed separately using two-way ANOVAs (group × delay) with repeated measures on the 'delay' factor. Significant interactions were followed by tests of the simple main effects to compare groups at each level of delay. In addition, the mean time (s) required to complete both delay tests was compared across groups using independent sample *t*-tests.

Raw scores for the number of correct choices in drug-free retention tests were also converted to percentage correct choices. The correct choices for the spatial discrimination and delayed alternation components were analyzed separately using two-way (group × day) ANOVAs with repeated measures on the 'day' factor. Independent sample *t*-tests were used to compare the total number of errors made by the two groups over the three retention tests.

Whenever the sphericity assumption was violated, determined by a significant Mauchly's test, the Huynh-Feldt epsilon correction was applied. In all cases the corrected F-tests agreed with the uncorrected tests; therefore for ease of interpretation of the results only non-adjusted degrees of freedom are reported. For the independent sample *t*-tests,

Levene's test of homogeneity of variance was considered. When the homogeneity of variance assumption was violated, adjusted t statistics were used.

Results

Training in individual tasks. Regardless of treatment, animals required fewer trials to acquire the spatial discrimination task relative to the delayed alternation task (see Figure 21, A), shown by a significant main effect of task, $F(1,15)=26.33$, $p<0.001$. Furthermore, *Ginkgo*-treated animals required fewer trials to reach the combined acquisition criteria, shown by a significant main effect of group, $F(1,15)=5.45$, $p<0.05$. The group by task interaction was not significant.

Regardless of treatment, animals also committed significantly more errors in the delayed alternation relative to the spatial discrimination, as shown by a significant main effect of task, $F(1,15)=26.02$, $p<0.001$. However, *Ginkgo* had no significant effect on the total number of errors committed during training (see Figure 21, B), as shown by a non-significant main effect of group, and a non-significant group by task interaction.

Whole maze testing. Performance of the spatial discrimination task during the 14 days of whole-maze testing was highly accurate, but was similar across groups (see Figure 22, A). A two-way ANOVA revealed no significant main effects of group or day, or group by day interaction.

Baseline response accuracy in the delayed alternation task began lower (see Figure 22, B), then improved steadily across days in both groups; however, *Ginkgo*-treated animals attained a high level of accuracy considerably earlier than the vehicle-treated animals. Two-way ANOVA revealed significant main effects of group,

Figure 21. Mean (+SEM) number of trials required to reach the training criteria (A) and mean (+SEM) number of errors committed (B) in the spatial discrimination (SD) and delayed alternation (DA) tasks in Experiment 1. Rats were injected with a standardized *Ginkgo biloba* extract (13.75 mg/kg, i.p.) or vehicle 30 min prior to all training sessions. *p<0.05, significant main effect of group; †p<0.001, significant main effect of task.

Figure 21

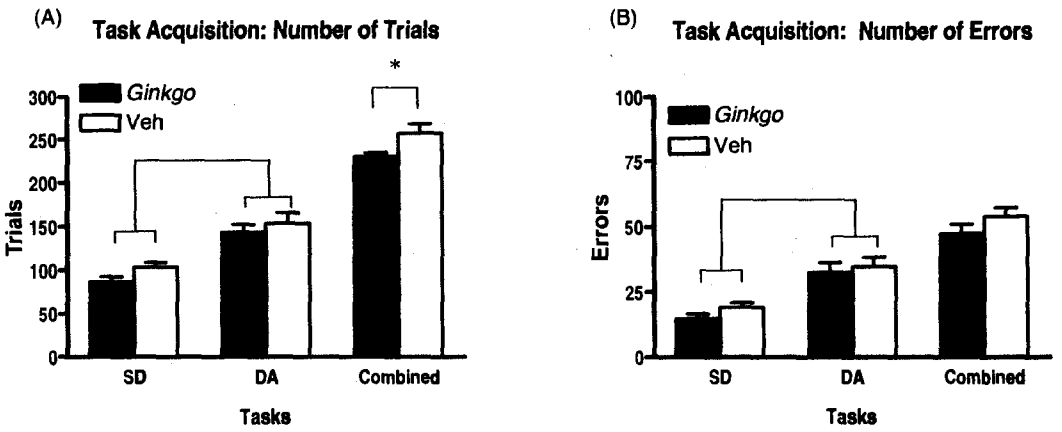
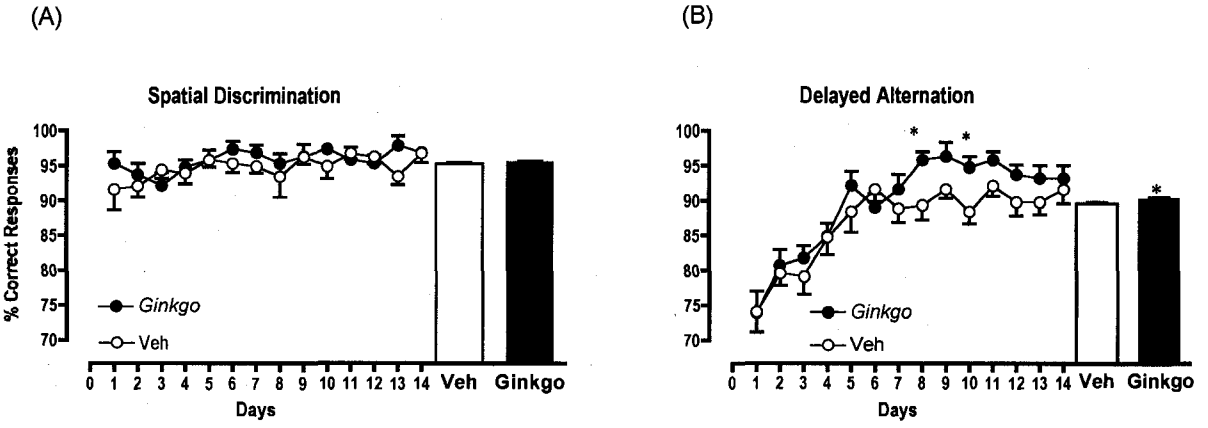


Figure 22. Mean (±SEM) percentage of correct responses in the spatial discrimination (A) and delayed alternation (B) tasks over 14 days of whole-maze testing in Experiment 1. Bar graphs on the right represent the mean (+SEM) percentage of correct responses over 14 days. Rats were injected with a standardized *Ginkgo biloba* extract (13.75 mg/kg, i.p.) or its vehicle 30 min prior to each test. *p<0.05, significantly different from vehicle.

Figure 22



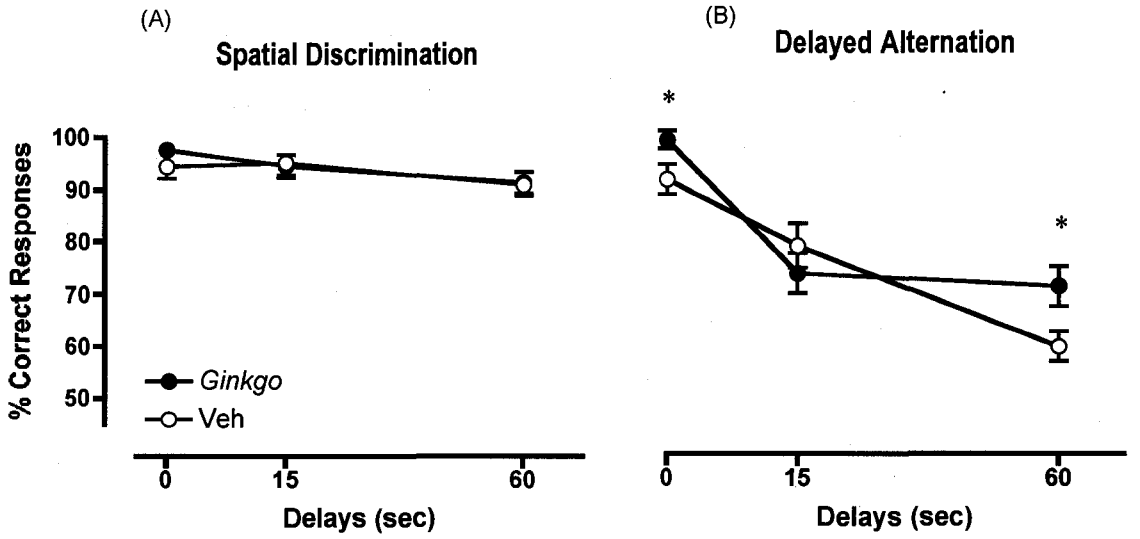
$F(1,15)=6.20$, $p<0.05$, and day, $F(13,195)=19.53$, $p<0.001$, but the group by day interaction was not significant. To further characterize the significant day main effect in the delayed alternation, scores from the first day were compared with those from all other days. Response accuracy was found to be higher at days 5 through 14 relative to day 1, $p<0.05$. Furthermore, the planned pairwise comparisons of groups at each level of day revealed that the *Ginkgo*-treated group made significantly fewer errors on training days 8 and 10 ($p<0.05$). A similar trend was observed on days 9 and 11, but the group difference was not significant ($p=0.07$).

The time (s) required to finish the whole maze test over 14 days of the experiment was analyzed by a two-way ANOVAs (group × day) with repeated measures on the 'day' factor and found only a significant main effect of day $F(13,195)=15.32$, $p<0.001$. The main effect of group and day by group interaction were not significant. Regardless of treatment, rats required significantly less time to finish the whole maze test over days. The mean (± SEM) time required to complete the test session on the first day was 388.88 (±17.46) and 381.33 (±14.50) s for the *Ginkgo* and vehicle groups, respectively. The mean (± SEM) time required to complete the test session on the last day significantly reduced to 290 (±14.69) and 267.11 (±8.68) s for the *Ginkgo* and vehicle groups, respectively.

Delay testing. Regardless of treatment, response accuracy in the spatial discrimination task decreased slightly, but significantly, as a function of delay (see Figure 23, A), shown by a significant main effect of delay, $F(2,30)=3.72$, $p<0.05$. The main effect of group and the group by delay interaction were not significant. Post-hoc paired sample *t*-tests comparing each delay interval to the other two revealed that the 60-s delay

Figure 23. Mean (±SEM) percentage of correct responses in the spatial discrimination (A) and delayed alternation (B) tasks across three delay intervals (0, 15 and 60 s) in Experiment 1. *p<0.05, significantly different from vehicle.

Figure 23



differed significantly from the 0 and 15 s delays ($p<0.05$). None of the other comparisons was significant.

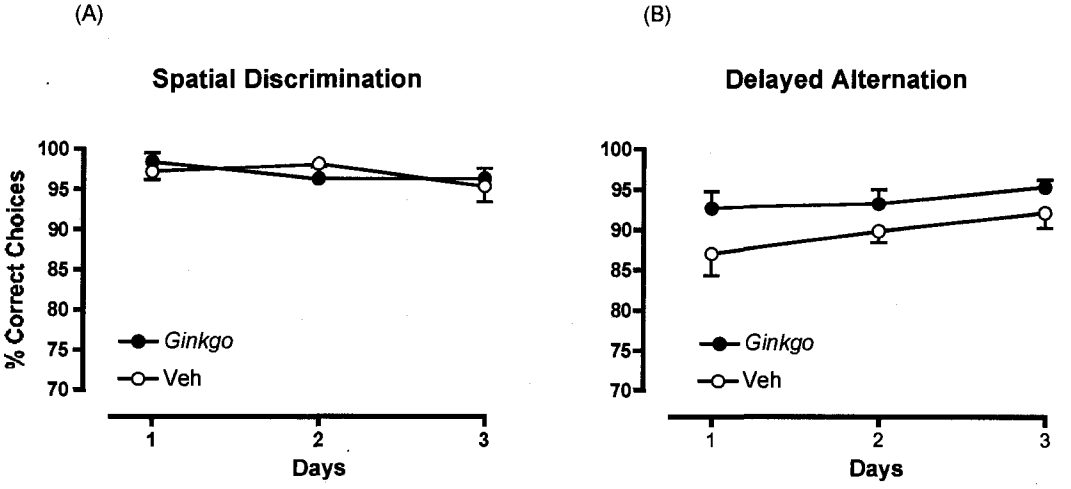
As expected, a pronounced delay-dependent decline in response accuracy was observed for both groups in the delayed alternation (see Figure 23, B). Furthermore, response accuracy was better in *Ginkgo*-treated animals at the shortest and longest delay intervals. ANOVA revealed a significant main effect of delay, $F(2,30)=52.49$, $p<0.001$, and a significant group by delay interaction, $F(2,30)=4.38$, $p<0.05$. The main effect of group was non-significant. Post hoc paired sample *t*-tests revealed that the 0-s delay differed from both the 15- and 60-s delays ($p<0.05$), and the 15-s delay differed significantly from the 60-s delay ($p<0.05$). To further characterize the significant interaction, pairwise group comparisons conducted at each level of delay revealed that response accuracy was significantly better in the *Ginkgo*-treated animals at the 0- and 60-s delays ($p<0.05$).

The mean (± SEM) time required to complete each delay test sessions was 916.2 ± 28.1 and 873.5 ± 10.1 s for the vehicle and *Ginkgo* groups, respectively. An independent samples *t*-test comparing these values was not significant. Taken together, results demonstrated that *Ginkgo* improved performance of the delayed alternation task at both the 0- and 60-s delay intervals.

Drug-free retest. In the drug-free retest, spatial discrimination performance remained highly accurate across groups over three days of testing (see Figure 24, A). ANOVA revealed no significant effects of group, day, or group by day interaction. Response accuracy in the delayed alternation task improved slightly, but not significantly

Figure 24. Mean (±SEM) percentage of correct responses in the spatial discrimination (A) and delayed alternation (B) tasks during the drug-free re-test in Experiment 1. Rats were re-tested for 3 consecutive days 8 days after the last administration of a standardized *Ginkgo biloba* extract or its vehicle.

Figure 24



$F(2,30)=3.26$, $p=0.052$, over the three days of testing (see Figure 24, B). In addition, response accuracy was slightly higher in the *Ginkgo*-treated group relative to the vehicle control group, but the difference did not reach statistical significance. The group by day interaction was not also significant.

The mean (± SEM) amount of time required to complete the drug-free retest sessions ranged from 259.9 (± 6.9) to 307.0 (± 17.0) s. A two-way ANOVAs (group × day) with repeated measures on the 'day' factor revealed that the main effects of day and group, and the day by group interaction were not significant.

Experiment 2: Post-training effects of Ginkgo

Results from Experiment 1 revealed that *Ginkgo* not only enhanced acquisition of the double Y-maze task, but also improved the post-training performance of the delayed alternation task. However, it was not clear whether the improvement in response accuracy was due to improved working memory per se, or was simply the result of enhanced task acquisition by *Ginkgo* (i.e., higher baseline performance). Thus, Experiment 2 examined the acute post-training effects of several doses of *Ginkgo* in experimentally naïve rats with no prior exposure to *Ginkgo*. At the completion of testing, animals also received sub-chronic exposure to *Ginkgo* or vehicle, then memory was re-assessed both under the influence of *Ginkgo* and in a drug-free state.

Method

Subject. Twelve male Sprague-Dawley rats (Charles River, Canada), weighing 270-300 g at the start of the experiment were used. Animals were housed and maintained as described in Experiment 1.

Drugs. EGb 761 (*Ginkgo*) and vehicle solutions were prepared as described in Experiment 1.

Procedures. An overview of the design of Experiment 2 is shown in Figure 20 (Bottom). Habituation and maze training was similar to that described in Experiment 1, but rather than being tested for 14 days, rats were trained to an acquisition criterion of two or fewer errors over 2 successive days prior to drug testing. Rats were then habituated to the injection procedure (1 ml/kg saline, i.p.) and delay intervals for 2 days prior to drug tests as described in Experiment 1.

PART 1: ACUTE EFFECTS OF *GINKGO*

Thirty min prior to test sessions, rats were injected with vehicle or one dose of *Ginkgo* (0.25, 2.5, 13.75, or 25 mg/kg) using a within subjects design. Rats received one experimental treatment every 2 days, with the order of treatments being counterbalanced across sessions. To ensure washout between treatments, rats received an identical drug-free test on the days between treatments.

PART 2: CHRONIC EFFECTS OF *GINKGO*

One day after the last acute test, animals were assigned randomly to two groups of six animals. One group received sub-chronic exposure to *Ginkgo* (13.75 mg/kg, i.p.) once daily for 10 days, and the other half received comparable vehicle injections. Rats were not exposed to the maze during this period. Animals were then re-tested using delays for 2 consecutive days as described in Experiment 1. Rats continued to receive *Ginkgo* or vehicle during the two delay tests. However, to determine whether any possible improvements in performance were dependent on continued exposure to *Ginkgo*, half the

animals received *Ginkgo* or vehicle 30 min prior to the first test session (pre-test exposure), while the other half received *Ginkgo* or vehicle 30 min after the first test session (post-test exposure). On the second day of testing, the timing of injections was reversed so that the rats previously receiving pre-test exposure were injected post-test, and vise versa. Daily sub-chronic treatments continued for 10 additional days, then animals were again tested on the maze for 2 consecutive days as described above.

Data analysis. Raw scores were converted to percentage correct responses. Data for the acute administration experiment (part 1) were analyzed separately for the spatial discrimination and delayed alternation tasks using two-way (treatment × delay) repeated measures ANOVAs. Where significant interactions were found, tests of the simple main effects were used to compare treatments at each level of delay. The possible influence of *Ginkgo* on the time needed to complete the maze was also analyzed using a one-way repeated measure ANOVA.

For the chronic administration experiment (part 2), three-way (group × delay × test) ANOVAs with repeated measures on the 'delay' and 'test' (10 and 20 days) factors were performed separately for the spatial discrimination and delayed alternation results. When a significant group by delay interaction was found, independent sample *t*-tests were used to compare vehicle- and *Ginkgo*-treated groups at each level of delay. Significant main effects of delay were followed by Bonferroni-adjusted paired *t*-tests to compare each delay interval to all others. Finally, the time required to complete the maze was analyzed using a two-way (group × test) ANOVA with repeated measures on the 'test' factor. Independent *t*-tests were then used to compare groups separately at pre- and post-session tests.

When the assumption of homogeneity of variance was violated, adjusted t statistics were reported. Whenever the Mauchly's test of sphericity was found to be significant, the Huynh-Feldt epsilon correction was used. For ease of interpretation, only the non-adjusted degrees of freedom are reported.

Results

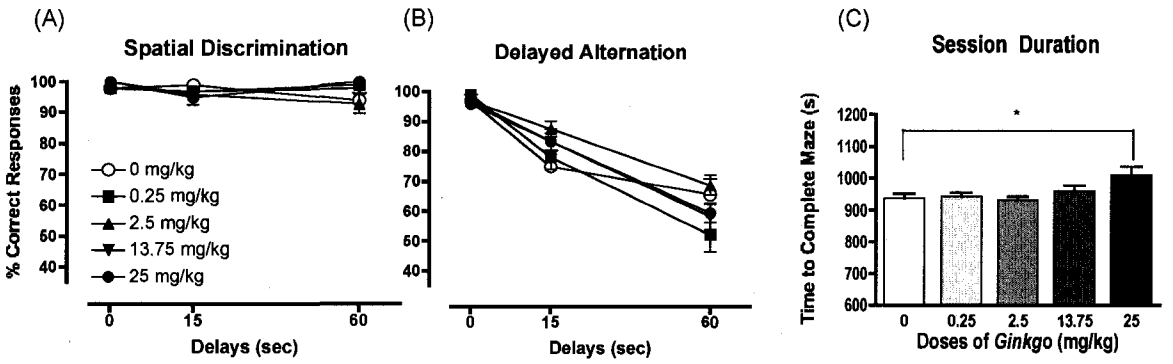
Part 1: Acute effects of Ginkgo. Response accuracy was high across all three delay intervals in the spatial discrimination task following administration of vehicle or *Ginkgo* (see Figure 25, A). The ANOVA revealed no significant main effects of treatment or delay. The treatment by delay interaction was marginally significant, $F(8,88)=2.19$, $p=0.04$; however, tests of the simple main effects revealed no significant differences between treatments at any delay intervals, indicating that response accuracy was not influenced by any dose of *Ginkgo* at any single delay interval in the spatial discrimination task.

As expected, response accuracy decreased in a delay-dependent manner in the delayed alternation task (see Figure 25, B), shown by a significant main effect of delay, $F(2,22)=134.49$, $p<0.001$. Pairwise comparisons revealed that response accuracy was significantly higher at 0-s delay relative to both the 15- and 60-s delays ($p<0.05$) and significantly higher at the 15-s delay relative to the 60-s delay ($p<0.05$). *Ginkgo* failed to influence response accuracy in the delayed alternation task, shown by a non-significant main effect of treatment, and a non-significant treatment by delay interaction.

The mean (+SEM) amount of time needed to complete the task was increased significantly by *Ginkgo* (see Figure 25, C). A one-way repeated measures ANOVA revealed a significant effect of treatment, $F(4,44)=7.61$, $p<0.001$. Pairwise comparisons

Figure 25. Mean (±SEM) percentage of correct responses in the spatial discrimination (A) and delayed alternation (B) tasks across 3 delay intervals 30 min following administration of 0, 0.25, 2.5, 13.75 or 25 mg/kg *Ginkgo* in Experiment 2. Data are from animals receiving a standardized *Ginkgo biloba* extract for the first time (in a counterbalanced order) after acquiring both maze tasks in a drug-free state. (C) Mean (+SEM) amount of time (s) required to complete the test sessions for each of the five treatments.

Figure 25



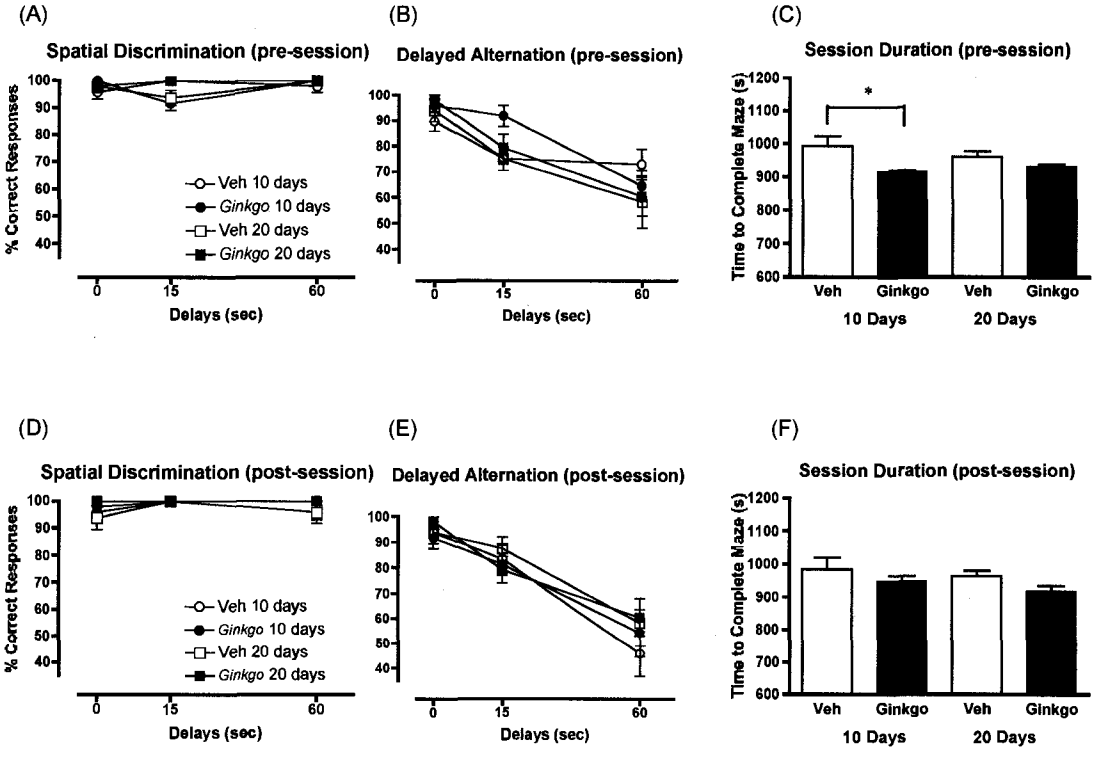
showed that animals receiving the 25 mg/kg dose required significantly longer to complete the maze relative to vehicle controls, $p<0.05$; none of the other doses significantly influenced maze completion times.

Taken together, results indicate that acute post-training administration of *Ginkgo* does not enhance response accuracy in the double Y-maze. Administration of a high dose of *Ginkgo*, however, may influence locomotor activity, as shown by increased time required to complete the maze.

Part 2: Chronic effects of Ginkgo. Pre-session test. Figure 26 (A and B) shows the mean (+SEM) percentage of correct responses over three delay intervals following 10 and 20 days of *Ginkgo* exposure when animals were also given *Ginkgo* or vehicle just prior to maze testing. For the spatial discrimination task, the three-way ANOVA revealed no significant main effects of group or test, but the delay effect was significant, $F(2,20)=4.35$, $p=0.027$. Both the delay by test and delay by group interactions were non-significant, but the test by group and the three-way (group × delay × test) interaction were significant, $F(1,10)=5.71$, $p=0.038$ and $F(2,20)=6.19$, $p<0.01$, respectively. Separate group by delay ANOVAs with repeated measures on 'delay' were performed for each test (10 and 20 days). At 10 days of treatment, the ANOVA revealed no significant effects of group or delay, but the group by delay interaction was significant, $F(2,20)=6.74$, $p< 0.01$. Independent samples *t*-tests comparing groups at each level of delay revealed that *Ginkgo*-treated rats made fewer correct responses than vehicle-treated animals at the 15-s delay, $t(5)=3.16$, $p<0.05$. None of the other comparisons was significant. At 20 days of treatment, the two-way ANOVA revealed no significant main effects or interaction.

Figure 26. Mean (±SEM) percentage of correct responses in the pre-session tests for the spatial discrimination (A) and delayed alternation (B) tasks, respectively. (C) Mean (+SEM) amount of time (s) required to complete the pre-session tests after 10 or 20 days of daily treatment with a standardized extract of *Ginkgo biloba* (13.75 mg/kg, i.p.). Mean (±SEM) percentage of correct responses in the post-session tests for the spatial discrimination (D) and delayed alternation (E) tasks, respectively. (F) Mean (+SEM) amount of time (s) required to complete the post-session tests after 10 or 20 days of drug treatment.

Figure 26



For the delayed alternation task, the three-way ANOVA found only a significant main effect of delay $F(2,20)=25.17$, $p<0.001$ (see Figure 26, B). Pairwise comparisons revealed that the 0-s delay differed significantly from the 15- and 60-s delays ($p<0.05$) and that the 15-s delay differed significantly from the 60-s delay interval ($p<0.05$).

For both tests there was a tendency for *Ginkgo*-treated animals to complete the maze in less time relative to vehicle-treated animals (see Figure 26, C). A two-way ANOVA with repeated measures on 'test' revealed a significant main effect of group, $F(1,10)=5.57$, $p<0.05$, and a significant group by test interaction, $F(1,10)=7.35$, $p<0.05$. Pairwise group comparisons conducted separately at each test found a significant difference between groups at the first test only (10 days treatment), $t(10)=2.66$, $p<0.05$.

To summarize, results showed that the spatial discrimination was selectively impaired at the 15-s delay interval following 10 days of *Ginkgo* exposure when animals were also given *Ginkgo* just prior to testing. Although this finding is surprising, the fact that the *Ginkgo*-treated rats also required significantly less time to finish the task suggests that the observed impairment may have been related to impaired attention. Regardless of the treatment, response accuracy declined as a function of delay in the delayed alternation task as expected, but prior exposure to *Ginkgo* did not significantly influence response accuracy.

Post-session test. Figure 26 (D) shows the mean (+SEM) percentage of correct responses over the three delay intervals after 10 and 20 days of drug treatment for the spatial discrimination task for animals receiving *Ginkgo* or vehicle just after each maze test. The three-way ANOVA for the spatial discrimination task found only a significant

main effect of delay, $F(2, 20) = 4.29$, $p = 0.028$. Pairwise comparisons revealed that none of the delays differed significantly from any other delays ($p>0.05$).

In the delayed alternation, response accuracy declined as the delay interval was increased regardless of treatment (see Figure 26, E) after both 10 and 20 days of drug treatment. The three-way ANOVA for the delayed alternation task revealed only a significant main effect of delay, $F(2, 20) = 43.49$, $p <0.001$. Pairwise comparisons revealed that response accuracy was significantly higher at the 0-s delay relative to both the 15- and 60-s delays ($p<0.05$), and was significantly higher at the 15-s delay relative to 60 s ($p<0.05$).

The two-way ANOVA for test 2 (20 days treatment) found only a significant effect of delay $F(2, 20) = 19.56$, $p < 0.001$. Post hoc paired sample *t*-tests found that the 60-s delay was significantly different from both the 0- and 15-s delays ($p<0.05$) and the 5-s delay differed significantly from the 0-s delay ($p<0.05$). The main effect of group and group by delay interaction were found to be non-significant. When comparing Figures 26 (B) and 26 (E) at the 60-sec delay interval, there appear to be group differences, such that pre-session vehicle-treated rats performed better than post-session vehicle- and *Ginkgo*-treated rats. Thus data were also analyzed using three-way (group × delay × pre-post) ANOVAs with repeated measures on the 'delay' and 'pre-post' (pre-session and post-session) factors once after 10 days and once after 20 days of drug treatment, but no group effect was found (see Appendix B for details).

The mean (+SEM) amount of time required to complete the tasks for both post-session tests are shown in Figure 26 (F). Two-way ANOVA with repeated measure on the 'test' factor found no significant effects of group, test or group by test interaction.

Discussion

The present study demonstrates that repeated daily pre-session parenteral administration of a *Ginkgo biloba* leaf extract subtly, but significantly, facilitates acquisition and performance of a working memory task in young adult rats, as shown by improved response accuracy in a delayed alternation task. Results also revealed that the cognitive-enhancing effects of *Ginkgo* are specific to the learning phase, since neither acute nor chronic post-training *Ginkgo* administration improved memory. In most previous studies, *Ginkgo* treatment was shown to improve learning when treatment began weeks or months before initiation of training sessions (e.g., see Stoll et al., 1996; Y. Wang et al., 2006; Ward et al., 2002; E. Winter, 1991). The present study, however, shows that chronic pre-training *Ginkgo* treatment is not required to reveal improved learning in young adult animals.

Experiment 1 examined the effects of *Ginkgo* on learning using a double Y-maze comprising two distinctive tasks: a spatial discrimination and a delayed alternation. Rats treated with *Ginkgo* shortly before each training session learned the individual tasks significantly faster than vehicle-treated animals (see Figure 21). Animals then received 14 additional days of whole-maze testing during which each trial consisted of both a spatial discrimination and delayed alternation task. Here, *Ginkgo*-treated rats made significantly fewer errors compared to vehicle-treated animals in the delayed alternation task, especially during the latter half of testing (see Figure 22). No significant group

differences were observed for the spatial discrimination task, suggesting that the improvement of learning produced by *Ginkgo* was due to a facilitation of working memory. That is, both double Y-maze tasks involved a food-motivated two-choice discrimination in a Y-maze, but only the delayed alternation task required working memory.

The working memory load was further increased by introducing delays between successive trials. Results showed that response accuracy in the delayed alternation task declined in a delay-dependent manner (see Figure 23), confirming that in trained animals the delayed alternation, but not the spatial discrimination task requires working memory as reported previously (Mallet & Beninger, 1993; Mason et al., 1999; Smith et al., 1994). A comparison of groups revealed that response accuracy was higher in animals treated with *Ginkgo*, but unexpectedly the improvement was not limited to the longer delay intervals; rather, it was restricted to the 0- and 60-s delays (see Figure 23, B). It is not clear why response accuracy was not improved at the 15-s delay. *Ginkgo* treatment was then withdrawn, and animals were re-tested 8 days later. Although response accuracy remained slightly higher in rats previously treated with *Ginkgo* (see Figure 24), the difference was not statistically significant. It therefore appears that the beneficial effects of *Ginkgo* on task acquisition subside following a period of abstinence.

Experiment 2 evaluated the acute post-training effects on memory by administering *Ginkgo* just prior to each test session in animals that had already been well trained in the double Y-maze. The finding that response accuracy declined in a delay-dependent manner regardless of treatment in the delayed alternation task again confirmed that the task depends on intact working memory. However, acute post-training treatment

across a wide range of doses of *Ginkgo* failed to enhance response accuracy in either the spatial discrimination or delayed alternation tasks (see Figure 25). These findings are at odds with those of Wirth et al. (2000) who reported facilitative effects of acute *Ginkgo* administration (60 and 120 mg/kg, i.p.) in young and aged rats in a spontaneous olfactory recognition task. This discrepancy may be due to differences in behavioural tasks, drug dosages, or age and strain of the animals used. It is noteworthy that the highest doses tested here (25 mg/kg) significantly increased the time required to complete the maze, suggesting some non-mnomonic effects may occur following high dose parenteral *Ginkgo* administration. Suppression of motor activity by a single oral dose of *Ginkgo biloba* extract (500 and 1000 mg/kg but not 250 mg/kg) have also been reported elsewhere (Kuribara et al., 2003).

Experiment 2 further examined the short-term (10 days) and longer-term (20 days) chronic post-training effects of *Ginkgo* on memory both while the rats were under the influence of the drug, and while they were in a drug-free state. Results revealed that *Ginkgo* did not improve response accuracy of either the spatial discrimination task or the delayed alternation task (see Figure 26). In fact, surprisingly, response accuracy for the spatial discrimination task was reduced at the 15-s delay at 10 days of exposure when *Ginkgo* was also administered pre-test. However, the pre-session *Ginkgo*-treated rats required significantly less time to complete the maze (see Figure 26, C) suggesting that the spatial discrimination task impairment may have been caused by reduced attention. The finding that chronic post-training *Ginkgo* failed to enhance performance of spatial discrimination and delayed alternation tasks agrees with a recent report showing that repeated post-training administration of an extract of *Ginkgo biloba* in young adult rats

had no significant effects in a Morris water maze task, or in radial arm maze tasks designed to assess short-term working and long-term reference memory (Shif et al., 2006).

Taken together, results from the present study revealed that acquisition of a spatial working memory task was amenable to the cognitive-enhancing effects of *Ginkgo.* It is worth stressing, however, that the facilitative effects of *Ginkgo* on maze acquisition were subtle. Previous studies of the cognitive effects of *Ginkgo* have typically employed an oral route of administration (e.g., see Stackman et al., 2003; Stoll et al., 1996; Y. Wang et al., 2006; E. Winter, 1991; J. C. Winter, 1998; , but see Wirth et al., 2000); the present study therefore provides evidence that parenteral administration of an extract of *Ginkgo biloba* is equally effective in improving learning, provided that the treatment is administered before training sessions. Performance of a working memory task, however, was largely unaffected by both acute and chronic post-training *Ginkgo* administration. Results therefore support the notion that ongoing *Ginkgo* administration does not appear to offer any continued beneficial effects, at least in an already-learned working memory task.

Chapter 6: General discussion

The present dissertation aimed to identify neural sites involved in the cognitive enhancing effects of the *Ginkgo biloba* leaf extract, EGb 761, and to explore its possible neurogenic-enhancing properties on adult hippocampal neurogenesis. Additionally, this dissertation aimed to assess anxiolytic-or anxiogenic-like properties as well as cognitive-enhancing effects of this widely used herbal supplement.

Experiments described in Chapter 2 used c-Fos immunoreactivity (Fos-IR) to map regional activity in selected limbic and related structures in response to oral or parenteral *Ginkgo* administration. Immediate early genes and their protein products such as c-Fos have been extensively utilized in mapping neuronal activity (for a review, see Kaczmarek & Chaudhuri, 1997). After acute oral or intraperitoneal (i.p.) administration of *Ginkgo*, c-Fos expression was increased in selected structures in a region-specific manner. Increased Fos-IR in the insular cortex and central nucleus of amygdala was observed after both oral and i.p. *Ginkgo* administration. However, while a single i.p. treatment of *Ginkgo* significantly increased Fos-IR in the paraventricular nucleus, nucleus accumbens shell, and dentate gyrus, a single oral treatment of the extract did not produce similar effects. On the other hand, oral but not i.p. administration of the extract significantly increased Fos-IR in the basolateral nucleus of the amygdala. As previously noted, these differential observations using two different routes of administration do not necessarily imply that each route produces unique neural (and consequently behavioural) effects. However, it is possible that different doses of the extract, as well as different time courses (the time between administration and perfusion), may have induced Fos-IR specific to each route of administration. Due to the different pharmacodynamics of the two routes of administration employed, it is difficult to compare the results. However, the i.p. route of

administration was employed to demonstrate that any observed changes in Fos-IR found with oral administration of the extract were not due to novel gustatory experience. In future studies plasma and brain concentrations of *Ginkgo* constituents following the two routes of administration should be quantified to facilitate the comparison of results.

Increased neural activity in the insular cortex, central nucleus of the amygdala, and dentate gyrus by acute *Ginkgo* treatment indicates that these limbic structures may possibly play roles in the long-term cognitive enhancing properties of the extract. The central nucleus of the amygdala and the insular cortex are believed to be involved in learning and memory. For example, the insular cortex has been shown to play a role in incentive memory (Balleine & Dickinson, 2000), the consolidation of object memory (Bermudez-Rattoni et al., 2005), the acquisition and consolidation of inhibitory avoidance (Mello e Souza et al., 2001; Miranda & McGaugh, 2004), and the consolidation of conditioned taste aversion (Miranda & McGaugh, 2004). The central nucleus of the amygdala is involved in the acquisition, consolidation, and expression of Pavlovian fear conditioning (Wilensky et al., 2006).

It was particularly interesting to find a significant increase in Fos-IR in the dentate gyrus after i.p. administration; however, it is noteworthy that a non-significant trend (p=0.088) in Fos-IR was also observed in the dentate gyrus after oral administration of *Ginkgo*. This hippocampal sub-region plays a pivotal role in learning and memory and mediates mnemonic processing of spatial information (for a review, see Kesner, 2007). In addition, the dentate gyrus is one of only two undisputed neurogenic regions of the adult mammalian brain (Ming & Song, 2005). Recent studies have taken advantage of the fact that synaptic activation induces expression of immediate early genes and have provided

convincing support for the involvement of newborn dentate gyrus neurons in cognitive processes and their relevance to hippocampal activity. Incredibly, newborn neurons are two to three times more likely to be activated during water maze learning or spatial exploration task than older neighboring neurons (Kee et al., 2007; Ramirez-Amaya et al., 2006). The c-Fos immunohistochemical study presented in Chapter 2 did not investigate the expression of immediate early gene products in newly born dentate gyrus neurons, nor after animals performed a hippocampal-dependent task. Rather, it assessed cell responsiveness in selected limbic structures including the dentate gyrus. Given that newborn dentate gyrus neurons are more responsive to hippocampal-dependent tasks, it might be possible that they are also more responsive (in terms of immediate early genes and their encoding protein product expression) to nootropic agents. To the best of the author's knowledge this possibility has not been investigated.

The increased Fos-IR found in the dentate gyrus, together with other lines of evidence suggesting the possible neurogenic-enhancing potential of *Ginkgo* formed the basis for the experiments described in Chapter 3, which investigated the effects of varying doses of *Ginkgo* on cell proliferation and cell survival. Neural stem cells reside in the neurogenic niche of the SGZ of the dentate gyrus and can unlimitedly self-renew through symmetric cell division, and can give rise through asymmetric cell division to neurons, astrocytes or oligodandrocytes (Gage, 2000). Adult neural stem cells appear to be a subset of astrocytes, expressing GFAP and nestin. (Seri, Garcia-Verdugo, McEwen, & Alvarez-Buylla, 2001). The niche has a special microenvironment that not only maintains the stem cells but also regulates their proliferation and fate determination. For example, the stem cells are intimately associated with resident astrocytes and

vasculatures. Astrocytes and blood factors released from vasculatures have been shown to play important roles in proliferation and fate specification of neural stem cells (Palmer et al 2000). The proliferating neural stem cells give rise to transient amplifying progenitor cells, which in turn differentiate into immature neurons (Kempermann et al., 2004; Ming & Song, 2005). Transient amplifying progenitors have limited potential for self-renewal. Some will die while some symmetrically divide and rapidly give rise to a pool of progenitors that in turn differentiate into immature neurons, which migrate locally and move into the granule cell layer (Ming & Song, 2005; van Praag et al., 2002). After newborn cells exit the progenitor cell cycle, neurite and axonal growth start. New generated axons initially interact with hilar mossy cells and interneurons located in the hilus (Zhao, Teng, Summers, Ming, & Gage, 2006). At this stage, immature neurons depolarize in response to GABA as they have a higher intracellular concentration of chloride ions (Ge et al., 2006). Around 8 to10 days later, the axons of immature neurons reach CA3 pyramidal neurons. At around 16 days after birth, dendritic spines of immature neurons begin to grow (Zhao, Teng, Summers, Ming, & Gage, 2006). The excitatory action of GABA on newborn neurons changes to inhibitory action at 2 to 4 weeks after birth. Glutamatergic synapses develop, once the dendritic branches of newborn neurons cross the molecular cell layer of the dentate gyrus (Ambrogini et al., 2004). The density and complexity of spines however continue to increase for the next several months (Zhao, Teng, Summers, Ming, & Gage, 2006). It has been estimated that about 9000 new cells are generated in the dentate gyrus of an adult rat every day (Cameron & McKay, 2001). However, half of the newborn neurons die within the first 3 weeks after birth. If they survive this critical time window, they can survival for at least

11 more months after birth in the dentate gyrus of rodents (Kempermann, Gast, Kronenberg, Yamaguchi, & Gage, 2003) and for at least 2 more years in the human dentate gyrus (Eriksson et al., 1998). At 4 weeks of age, newborn neurons start expressing NeuN, a marker of mature neurons (Ming & Song, 2005). Finally, newborn granular neurons functionally integrate into existing neuronal circuits in the dentate gyrus, receiving inputs from the perforant path, and sending outputs to CA3 pyramidal cells (van Praag et al., 2002).

Many factors have been shown to affect adult hippocampal neurogenesis. For example, survival of newborn granule neurons has been shown to increase considerably in adult mice that lived in an enriched environment (Kempermann, Kuhn, & Gage, 1997). Cell proliferation in the dentate gyrus of adult mice is also significantly enhanced by voluntary wheel running (van Praag, Kempermann, & Gage, 1999). Both enriched environment (Kempermann, Kuhn, & Gage, 1997) and voluntary exercise (van Praag, Christie, Sejnowski, & Gage, 1999) have been shown to positively influence learning and memory. Thus, we hypothesized that *Ginkgo* might also enhance learning and memory through enhancemnet of cell proliferation and/or cell survival in the dentate gyrus. Other lines of evidence described in Chapter 3 supported the hypothesis that *Ginkgo* might have pro-neurogenic potential. In two different sets of experiments this possibility was investigated by separately investigating the effects of the extract on cell proliferation after a single administration and cell survival after prolonged treatment. Results, however, indicated that neither cell proliferation nor cell survival was affected by *Ginkgo* treatment in neither the dorsal nor ventral dentate gyrus in young adult male rats.

It has been shown that *Ginkgo* has a positive effect on blood flow and microcirculation in the dentate gyrus and other brain regions (Krieglstein et al., 1986). The microenvironment of the neurogenic niche of the dentate gyrus has been shown to play a key role in neurogenesis. Newborn cells in the granule cell layers are situated in close proximity to fine blood vessels (Palmer et al., 2000). The negative results suggest that enhancement of dentate gyrus blood supply by *Ginkgo* is not sufficient to enhance neurogenesis and other factors might be required to promote this process. Consistent with the present findings, a recent study examining the influence of (-)-epicatechin--a plant-derived flavanol--on adult hippocampal neurogenesis also failed to find any enhancing effects on cell genesis; despite the fact that (-)-epicatechin significantly improved memory function. However, results revealed that (-)-epicatechin selectively enhanced angiogenesis (generation of new blood capillaries from pre-existing blood vessels) in the dentate gyrus, suggesting an alternative neural mechanism for the enhancement of memory observed (van Praag et al., 2007). *Ginkgo* is similar to (-)-epicatechin in that it is rich in flavanol. In fact, *Ginkgo* contains 24% flavonoid glycosides. However, the experiments presented in Chapter 3 did not investigate the effects of *Ginkgo* on hippocampal angiogenesis. Nonetheless, *Ginkgo* treatment has been shown to increase proliferation of endothelial progenitor cells (Chen et al., 2004). Whether or not *Ginkgo* treatment enhances hippocampal angiogenesis remains to be investigated in the future studies.

As previously described, positive modulatory effects of *Ginkgo* on the cholinergic system have been demonstrated. For example, *Ginkgo* treatment has been shown to increase hippocampal cholinergic muscarinic receptor numbers (Taylor, 1986), enhance

the release of acetylcholine (Kristofikova et al., 1992), and inhibit acetylcholinesterase activity (Chopin & Briley, 1992; Das et al., 2002; Yamamoto et al., 2007). Since the cholinergic system is involved in adult hippocampal neurogenesis (Cooper-Kuhn et al., 2004; Kaneko et al., 2006; Kotani et al., 2006, 2008), it was expected that *Ginkgo* may indirectly affect adult hippocampal neurogenesis through its influence on the cholinergic system. However, the results did not support this hypothesis.

Taken together, the results presented in Chapter 3 suggested that the nootropic effects of *Ginkgo* are unlikely the result of enhanced adult hippocampal neurogenesis, at least in young adult rats. Other forms of plasticity might explain the nootropic effects of the extract. Indeed, a recent study investigated whether the cognitive enhancing effect of the extract was related to enhanced LTP. Electrophysiological recordings of field excitatory postsynaptic potentials were performed from the CA1 in response to ipsilateral stimulation of the Schaffer collateral/commissural pathway in aged rats chronically treated with *Ginkgo* (60 mg/kg for 30 days) and found significant enhancement of *in vivo* LTP in aged but not young rats. Moreover, results of a recent study showing enhancement of adult hippocampal cell proliferation by *Ginkgo* treatment in a transgenic mouse model of Alzheimer's Disease (Tchantchou et al., 2007) raise the possibility that *Ginkgo* may have different effects in the dysfunctional brain where the constitutive level of neurogenesis is down-regulated compared to normal healthy neural systems. Thus, the experiments described in Chapter 4 addressed this possibility by investigating whether *Ginkgo* co-treatment could counteract the suppressive effects of corticosterone (CORT) treatment on cell survival (Wong & Herbert, 2004). Three weeks of CORT (35 mg/kg) treatment clearly increased plasma CORT concentrations, and consistent with previous

studies (e.g., see Fernandes et al., 1997; Gregus et al., 2005) induced adverse physiological effects including significant reductions in thymus, adrenal gland and body weights. The survival of newborn cells was also significantly suppressed in the dentate gyrus of rats chronically treated with CORT. However, none of the CORT-induced effects were significantly attenuated by *Ginkgo* co-treatment. In fact, the body and thymus weights of animals treated only with *Ginkgo* fell significantly below those of the control rats. This suggests that i.p. administration of the dose of *Ginkgo* chosen may have induced some kind of malaise. Support for this possibility comes from the finding that acute administration of the high dose of *Ginkgo* increased measures of anxiety in the light-dark emergence test in Chapter 3. One way to investigate whether a high dose of *Ginkgo* induces malaise is to use the conditioned taste aversion paradigm. Conditioned taste aversion is a form of classical conditioning by which an animal associates a novel taste such as a sucrose solution with a sickness-inducing agent such as lithium chloride. This robust association prevents subsequent consumption of the novel taste that was paired with the sickness-inducing agent. However, it has been shown that rewarding drugs such as cocaine also induce conditioned taste aversion. Thus, researchers use taste reactivity test to measure orofacial reactions elicited by a flavoured solution infused directly into the animal's mouth. Two types of reactions that are typically elicited by the animals are ingestive and disgust reactions. Disgust reactions are evident by infusing a flavoured solution that was previously paired with a sickness-inducding agent (e.g. lithium choloride) and ingestive reactions are observed by infusing a flavoured solution previously paired with an agent (e.g. cocaine) that changed the "physiological state" of the animal (for a review, see Parker, 2003). To the author's knowledge *Ginkgo* does not

induce any rewarding or hedonic state changes; however, the results presented in Chapter 3 showed that acute high dose of *Ginkgo* increased some measures of anxiety evident by increased duration of time spent in the hide box and exit latency. It remains the goal of future research to test *Ginkgo* in a taste reactivity test.

Additionally, consistent with the results presented in Chapter 3, chronic *Ginkgo* treatment on its own had no effect on the survival of newborn cells in the dentate gyrus, confirming that the beneficial effects of *Ginkgo* on cognitive processing do not appear to be mediated by enhanced hippocampal cell genesis. Furthermore, as expected, chronic CORT treatment suppressed survival of newborn cells, but contrary to the expected results, *Ginkgo* co-treatment did not attenuate this effect.

Proliferation of progenitor cells is believed to be regulated, at least in part, by circulating CORT, as elevated levels of CORT suppresses proliferation (Gould et al., 1992; Hellsten et al., 2002), and adrenalectomy stimulates this process (Cameron & Gould, 1994; Gould et al., 1992). Additionally, circulating CORT levels also play a role in the survival of newborn cells since chronic CORT treatment both before and after cell division has been shown to suppress survival of newborn neurons (Wong & Herbert, 2004). CORT acts on two receptors: glucocorticoid receptors and mineralocorticoid receptors. Both receptor genes have been found to be expressed at high levels by granular cells in the dentate gyrus (Van Eekelen, Jiang, De Kloet, & Bohn, 1988). However, mineralocorticoid receptors have a 10-fold higher affinity to CORT relative to glucocorticoid receptors, which are only fully occupied when CORT levels are elevated (Joels, 2007). Glucocorticoid receptors are expressed by only 50% of early progenitors and presumably are more sensitive to the suppressive effects of CORT on cell

proliferation than mineralocorticoid receptors, which are expressed only by mature granule cells (Garcia, Steiner, Kronenberg, Bick-Sander, & Kempermann, 2004). The role these two receptors play in survival of newborn neurons is presently not clear, but the fact that both receptors are expressed in mature neurons suggests that both may be involved in regulating survival of newborn cells.

One novel aspect of the results worth further discussion was the finding that chronic CORT treatment selectively suppressed cell survival in the dorsal dentate gyrus. While the dorsal hippocampus appears to be involved in spatial learning and memory tasks, the ventral hippocampus processes motivational and emotional information (for a review, see Bannerman et al., 2004). Accordingly, it has also been suggested that adult neurogenesis in the dorsal dentate gyrus has a distinct role from that of the ventral dentate gyrus (Sahay & Hen, 2007). Previous studies have not differentiated between the influence of CORT on neurogenesis in the dorsal and ventral regions of the dentate gyrus (Cameron & Gould, 1994; Wong & Herbert, 2004, 2006). Additionally, to the best of the author's knowledge, the distribution of glucocorticoid and mineralocorticoid receptors along the septo-temporal axis of the dentate gyrus has not been investigated. Possible differential distribution of glucocorticoid and mineralocorticoid receptors along the dorsal and ventral dentate gyrus, assuming that cell survival is regulated by these receptors, may explain the selective suppression of cell survival in the dorsal dentate gyrus.

Moreover, high levels of stress and high concentrations of exogenous CORT have been shown to impair spatial memory (de Quervain, Roozendaal, & McGaugh, 1998; Diamond, Fleshner, Ingersoll, & Rose, 1996). Given the preferential role for the dorsal

hippocampus in spatial learning and memory tasks (Bannerman et al., 2004), it may be assumed that CORT might induce spatial memory deficits in part by selectively impairing the survival of newborn cells in the dorsal dentate gyrus. Unfortunately, Chapter 4 did not investigate learning and memory in a spatial task. Nonetheless, there is evidence that chronic CORT treatment also induces 'depressive-like' behaviour (Gregus et al., 2005; Kalynchuk et al., 2004), which presumably should be associated with suppression of neurogenesis in the ventral dentate gyrus (Sahay & Hen, 2007). Future studies that aim to identify the roles of CORT and its receptors in adult hippocampal neurogenesis along the septo-temporal axis of the dentate gyrus are required.

One limitation of the experiments described above was the lack of double labeling. That is, the identification of newborn neurons is often confirmed using double-labeling techniques to detect BrdU cells that express neuronal markers such as neuron-specific nuclear protein (NeuN). Glial cells, on the other hand, can be identified by detecting BrdU in cells that co-express glial cell markers such as glial fibrillary acidic protein (GFAP) (Ming & Song, 2005). Because *Ginkgo* failed to significantly alter the number of BrdU labeled cells, a decision was made not to carry out immunohistochemical procedures for double labeling because it is very unlikely that *Ginkgo* selectively increased the number of neurons while at the same time reducing the number of glial cells (or vise versa) such that the total number of BrdU labeled cells in the *Ginkgo*-treated rats became similar to that of the vehicle-treated rats. In support of this argument, more than 95% of newborn cells differentiated into neurons, but less than 1% differentiated into glial cells in the Tchantchou et al (Tchantchou et al., 2007) study

showing enhancement of adult hippocampal cell proliferation by chronic *Ginkgo* treatment in a transgenic mouse model of Alzheimer's Disease.

In Chapter 3, in order to validate the immunohistochemical protocol employed, a group of rats were treated with fluoxetine and used as positive controls in the second experiment in which the survival of newborn cells were studied. Fluoxetine has been reported to enhance cell survival in the dentate gyrus (Nakagawa et al., 2002; J. W. Wang et al., 2008). The lack of fluoxetine effect on the number of BrdU labeled cells in that experiment raised concerns regarding the immunohistochemical methods employed. However, using the same immunohistochemical protocol, results presented in Chapter 4 found suppression of cell survival in the dentate gyrus by chronic CORT treatment (Wong & Herbert, 2004). Another recent study also failed to replicate the enhancement of cell survival as well as cell proliferation in either the dorsal or ventral dentate gyrus by chronic fluoxetine treatment (5 mg/kg for 25 days) in adolescent, adult and aged rats (Cowen et al., 2008).

Since *Ginkgo* treatment has been shown to reduce basal and stress-induced anxiety, the experiments described in Chapter 3 also assessed measures of anxiety after single or multiple treatments with *Ginkgo* using a battery of behavioural tests including the light/dark emergence, elevated plus maze, and social interaction tests. A single high (25 mg/kg) or moderate (13.75 mg/kg) dose of *Ginkgo* appeared to induce anxiety-like behaviour in the light/dark emergence test as indicated by increased exit latency, and increased duration of time spent in the hide box. This effect was not evident after multiple treatments with *Ginkgo*. Additionally, acute and chronic administration of all doses of *Ginkgo* tested failed to alter the duration of time spent in the enclosed arms,

number of entries into the open arms of the elevated plus maze, and duration of active social interactions. Chronic *Ginkgo* treatment did not alter measures of anxiety in any of these tests of anxiety, which contrasts with previous studies showing anxiolytic-like effects of the extract (Hasenöhrl et al., 1996; Kuribara et al., 2003; Porsolt et al., 1990; Rodriguez de Turco et al., 1993; Ward et al., 2002). Reasons for these inconsistencies may be due to different routes of administration, dose, or the specific behavioural measures of anxiety employed. However, consistent with the present results, one other study reported that chronic *Ginkgo* (100 mg/kg for 21 days) treatment failed to change the duration of time spent in the open arms, or the number of entries into the open arms of the elevated plus maze (Walesiuk et al., 2005). In this latter study, rats had 21 days of drug exposure in a manner similar to that used in Chapter 3, but the test was conducted 24 h after the last drug treatment such that rats were tested while drug-free. The results of the acute study presented in Chapter 3, however, showed that a single high or moderate dose of *Ginkgo* administered prior to the light/dark emergence test increased duration of time spent in the hide box and exit latency. Thus, it was decided to continue the drug treatment on the test day and examine anxiety-like behaviour induced by chronic *Ginkgo* treatment. In addition, it has been shown that the nature of anxiety measured in the second trial of the elevated plus maze is distinct from that measured in the first trial (Fernandes & File, 1996; File, Zangrossi, Viana, & Graeff, 1993). Thus, in Chapter 4 rats were tested twice in the light/dark emergence and the elevated plus maze over two consecutive days, and unlike the study presented in Chapter 3 rats were under the influence of the drug treatment on both days of testing. Additionally, the anxiogenic- or

anxiolytic-like effect of chronic CORT treatment alone or in combination with *Ginkgo* was assessed.

Consistent with the results presented in Chapter 3, chronic *Ginkgo* treatment was not found to influence anxiety even when the rats were pre-treated with *Ginkgo* just prior to the testing. Thus, it appears that the seemingly anxiogenic effect of a single high dose of *Ginkgo* diminishes with repeated treatment. It is likely that rats receiving multiple *Ginkgo* treatments habituated to the active constituents of the extract that might have induced anxiogenic-like effects in the light/dark emergence test following a single treatment. Moreover, despite the significant elevation of plasma CORT levels in the CORT-treated rats, there were no significant effects on any of the measures of anxiety. Interestingly, the two groups of rats treated with *Ginkgo* (*Ginkgo*-Veh and *Ginkgo*-CORT) traveled shorter distances in the elevated plus maze compared to the other two groups that did not receive *Ginkgo*. This effect was only evident in the first trial and may suggest a simple suppression of locomotor activity.

The fact that CORT did not induce any anxiogenic-like effects is in agreement with previous studies that have shown elevated CORT levels due to chronic CORT injection (Gregus et al., 2005; Kalynchuk et al., 2004) or CORT pellet implantation (Fernandes et al., 1997) did not change measures of anxiety in the elevated plus maze, social interaction, open field, or resistance-to-capture tests. In fact, it has been suggested that chronic CORT administration produces a double-dissociation between elevated corticosteroid concentration and anxiety (Fernandes et al., 1997). It seems that emotional changes brought about by chronic daily CORT treatment, both in male and female rats, correspond to symptoms of clinical depression rather than anxiety (Gregus et al., 2005;

Kalynchuk et al., 2004). For example, chronic CORT injection (40 mg/kg for 21 days) has been shown to increase depression-like behaviours in the forced swim test (Kalynchuk et al., 2004). The present study did not evaluate the chronic effects of CORT, *Ginkgo* and their co-treatment on animal models of depression, but the lack of significant effects in the two animal tests of anxiety employed agrees with the aforementioned studies. One study, however, reported a significant increase in exit latency in female mice chronically treated with CORT (Ardayfio & Kim, 2006). This anxiolytic-like effect of CORT contrasts with results presented here, but might be related to differences in dose, species and/or gender employed. Indeed, it has been shown that low doses of CORT can produce anxiolytic-like effects (File, Vellucci, & Wendlandt, 1979).

Consistent with other studies (e.g., see Treit et al., 1993), the data showed that regardless of treatment, rats appeared to be more anxious in the second trial compared to the first trial. All rats spent more time in the hide box, had longer exit latencies, and traveled shorter distances in the second light/dark emergence trial compared to the first. Similarly, all rats spent more time in the enclosed arms, entered less frequently into the open arms, and traveled shorter distances in the second elevated plus maze trial compared to the first. It has been argued that in the first trial of the elevated plus maze, rats acquire a fear of heights and thus in the second trial the response is considered a phobic response (File et al., 1993). It is noteworthy that in Experiment 2 in Chapter 3, regardless of the drug treatments, rats that were first tested in the elevated plus maze and then in the emergence test appeared more anxious measured by increased percentage of time in the hide box and by decreased distance traveled. This may suggest that experiencing the elevated plus maze first increased baseline levels of anxiety and thus potentiated

measures of anxiety in the emergence test. It has also been suggested that aversion to the open arms is learned during the first trial of the elevated plus maze, and thus during the second trial animals tend to avoid the open arms (Bertoglio & Carobrez, 2000). An alternate interpretation is that rats learn that the enclosed arms are safer than the open arms, and thus spend significantly more time in the enclosed arms during the second trial (Roy, Chapillon, Jeljeli, Caston, & Belzung, in press). These interpretations may also be applied to the second trial of the light/dark emergence test. That is, rats may have learned to avoid the open space in the second trial of the light/dark emergence test or conversely found the dark compartment safer, and thus spent significantly more time in the dark compartment during the second trial. In any case there were no treatment effects in either the first or second trials, and the results suggested that chronic treatment with *Ginkgo*, CORT or their combination induce neither anxiolytic- nor anxiogenic-like effects.

Finally, the effects of *Ginkgo* on acquisition and performance of a working memory task were examined in two experiments described in Chapter 5. The double Y-maze, which consists of two separate spatial discrimination and delayed alternation components, was employed. The double Y-maze includes control procedures that guard against misinterpretation of possible non-mnemonic effects such as altered motor function, motivation, or sensory-perceptual abilities (Mallet & Beninger, 1993; Mason et al., 1999; Smith et al., 1994). Experiment 1 examined the effects of pre-session treatment of *Ginkgo* on learning of the double Y-maze task. Acquisition of the individual tasks was found to be enhanced in rats pre-treated with *Ginkgo*. Animals then received 14 additional days of whole-maze testing during which each trial consisted of both a spatial discrimination and delayed alternation task. *Ginkgo*-treated rats made significantly fewer

errors compared to vehicle-treated animals in the delayed alternation task. However, no significant group differences were observed in the spatial discrimination task, suggesting that the improvement of learning produced by *Ginkgo* was due to a facilitation of working memory. When the working memory load was further increased by introducing delays between successive trials, response accuracy declined in a delay-dependent manner in the delayed alternation task. This confirmed that in trained animals the delayed alternation, but not the spatial discrimination task, requires working memory (Mallet & Beninger, 1993; Mason et al., 1999; Smith et al., 1994). When *Ginkgo* treatment was withdrawn and animals were re-tested 8 days later, response accuracy was no longer higher in rats previously treated with *Ginkgo*, suggesting that *Ginkgo* does not have an ongoing beneficial effect.

The effect of *Ginkgo* on memory, per se, by administrating *Ginkgo* in the interval between acquisition and testing is not clear. Thus, the acute post-training effects of various doses of *Ginkgo* on memory by administering *Ginkgo* just prior to each test session in a different group of animals that had already been well trained in the double Y-maze was also evaluated in Chapter 5. Although response accuracy declined in a delay-dependent manner confirming that the task depends on intact working memory, there was no treatment effect. Furthermore, the highest dose tested (25 mg/kg) significantly increased the time required to complete the maze, suggesting some non-mnemonic effects may occur following administration of that dose. This same dose administered acutely was found to induce anxiety-like behaviour in the light dark/emergence test in Chapter 3. Furthermore, in Chapter 4, this same dose administered chronically was found to reduce thymus and body weight, suggesting adverse physiological effects.

Short-term (10 days) and longer-term (20 days) chronic post-training effects of *Ginkgo* on memory were also assessed in Chapter 5. *Ginkgo* did not improve response accuracy of either the spatial discrimination task or the delayed alternation task. Consistent with these results, another study also showed that repeated post-training administration of an extract of *Ginkgo biloba* in young adult rats had no significant effects in a Morris water maze, or in radial arm maze tasks designed to assess working and reference memory (Shif et al., 2006). Thus, the cognitive-enhancing effects of *Ginkgo* were specific to the learning phase, since neither acute nor chronic post-training *Ginkgo* administration improved memory.

Ginkgo has been shown to protect cellular membrane fluidity, to enhance cerebral blood flow and microcirculation, and to modulate cholinergic system functioning, properties that have been suggested to underlie its cognitive-enhancing effects (Maclennan et al., 2002). It has also been suggested that the cognitive-enhancing ability of *Ginkgo* is due to its "anti-stress" properties. That is, *Ginkgo* may reduce stress associated with behavioural learning tasks and results in better performance (Ward et al., 2002). However, some of the results presented in this dissertation do not support this hypothesis. Neither acute nor chronic *Ginkgo* treatment reduced measures of anxiety in the elevated plus maze, social interaction or light/dark emergence tests. In fact, measures of anxiety in the light/dark emergence test were increased after a single injection of high or medium doses of *Ginkgo* extract.

With the assumption that adult hippocampal neurogenesis is positively correlated with cognitive ability, the present dissertation tested the hypothesis that the cognitive-enhancing ability of *Ginkgo* may be related to its neurogenic-promoting effects. A dose

of *Ginkgo* (13.75 mg/kg) that was found to improve acquisition of a working memory task failed to influence cell proliferation or cell survival in the dentate gyrus of young adult rats. Several studies suggest that the number of newborn neurons in the adult dentate gyrus correlates with learning abilities (e.g., see Kempermann et al., 1997b; van Praag, Christie et al., 1999); however, there is also evidence that this is not always the case. For example, learning ability in the hidden platform version of the water maze has been found to be a poor predictor of the number of newborn cells in the dentate gyrus of aged rats (Bizon & Gallagher, 2003; Bizon et al., 2004; Merrill et al., 2003). As mentioned earlier, (-)-epicatechin has also been shown to improve memory function despite its lack of effect on adult hippocampal neurogenesis (van Praag et al., 2007).

Taken together, the experiments described in the present dissertation identified limbic structures that are targeted by active constituents of the *Ginkgo biloba* leaf extract EGb 761. Prominent c-Fos immunoreactivity was observed in the insular cortex, central nucleus of the amygdala, and dentate gyrus following a single administration of the extract, indicating that these limbic structures may possibly play roles in the long-term cognitive enhancing properties of the extract. However, *Ginkgo* treatment had no effect on cell proliferation or survival in the dentate gyrus of young adult healthy rats. *Ginkgo* also failed to restore the detrimental effects of CORT-induced suppression of cell survival in the dentate gyrus. Furthermore, repeated treatment with *Ginkgo* failed to produce any anxiolytic- or anxiogenic-like effects, while single high and moderate doses of the extract were found to increase some measures of anxiety. Lastly, while neither acute nor chronic post-training *Ginkgo* treatment improved memory of an already-learned

working memory task, repeated daily pre-session *Ginkgo* treatment facilitated acquisition and performance of such a task in a double Y-maze.

It is concluded that the cognitive-enhancing effects of *Ginkgo* is limited to the enhancement of learning, rather than memory per se, and this effect is likely not related to a modulatory effect on adult hippocampal neurogenesis, at least in healthy young adult individuals.

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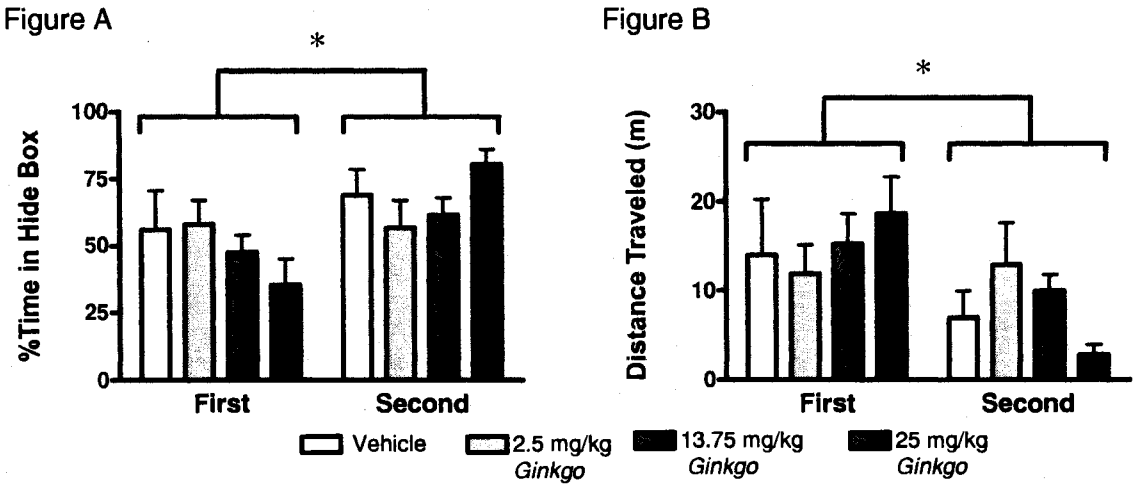
Appendix A



Mean (+SEM) percentage of time spent in the hide box (Figure A) and mean (+SEM) distance traveled (Figure B) in the light/dark emergence apparatus for rats that were tested in the emergence test either first or second (i.e., before or after being tested in the elevated plus maze). *$p<0.05$, second test significantly different from first.

Half of the rats in each group were first tested in the elevated plus maze, while the other half began with the emergence test. The rats then were tested in the apparatus that was not previously tested. For this reason, two-way ANOVAs (order x treatment) were also performed to investigate both order and treatment effects in measures of anxiety in both the elevated plus maze and emergence tests. There were only significant effects of order for distance traveled in the emergence test, $F(1, 32)=6.51$, $p=0.016$ and in the percentage duration of time spent in the hide box, $F(1, 32)=7.22$, $p=0.011$. These results suggest that regardless of the treatment, rats appeared more anxious when they were tested in the emergence box after being tested in the elevated plus maze. All rats spent more time in the hide box and traveled a shorter distance when they were tested in the plus maze before emergence test. This suggests that regardless of drug treatment, experiencing the elevated plus maze first increased baseline level of anxiety and thus potentiated measures of anxiety in the emergence test.

Appendix B

Figure A

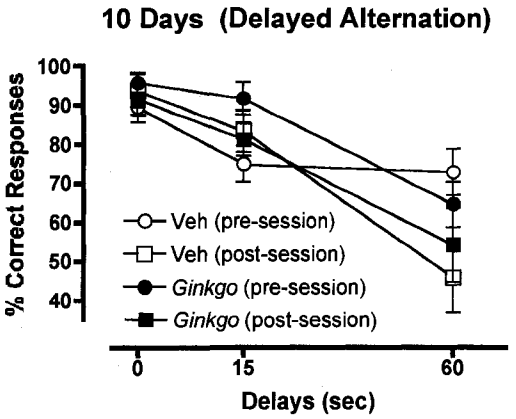
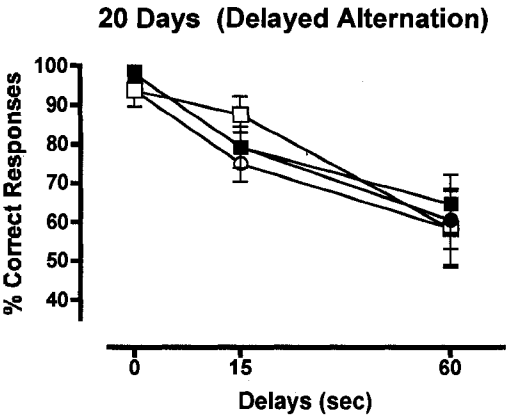


Figure B



Mean (±SEM) percentage of correct responses after 10 (Figure A) and 20 (Figure B) days of drug treatment in the pre- and post session tests for the delayed alternation task.

The three-way ANOVA (group × delay × pre-post) for the first 10 days found the main effect of group to be non-significant. There were significant effects of pre-post, $F(2, 20) = 4.39$, $p < 0.05$, delay $F(2, 20) = 33.07$, $p < 0.001$, and the pre-post by delay interaction, $F(1, 10) = 5.69$, $p < 0.05$. Pairwise comparisons for delay revealed that response accuracy was significantly higher at the 0-s delay relative to both the 15- and 60-s delays ($p<0.05$), and was significantly higher at the 15-s delay relative to 60 s ($p<0.05$). Pairwise comparisons for pre-post found that response accuracy was significantly higher at pre-test relative to post-test at 60-sec delay interval ($p<0.05$).

The three-way ANOVA (group × delay × pre-post) for 20 days found only a significant effect of delay, $F(2, 20) = 51.24$, $p < 0.001$. Pairwise comparisons revealed that response accuracy was significantly higher at the 0-s delay relative to both the 15- and 60-s delays ($p<0.05$), and was significantly higher at the 15-s delay relative to 60-s ($p<0.05$). The main effect of group and the group by delay interaction were not significant.