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Food restriction and polyunsaturated fatty acids enriched diet attenuate learning and memory impairments following global ischemia in rats.

A Doctoral Dissertation

By

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Cette thèse est dédiée à mes parents, Michel et Suzanne Roberge

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Abréviations

AA:	arachidonic acid
ADN:	acide désoxyribonucléique
AGL:	average grey level
AIF:	apoptosis inducing factor
AL:	ad libitum feeding
AMPA:	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPA-GluR1:	AMPA receptor 1
ANCOVA:	analysis of covariance
ANOVA:	analysis of variance
Apaf-1	apoptotic protease activating factor 1
ARN:	acide ribonucléique
ATP:	adénosine triphosphate
AVC:	accident vasculaire cérébral
BDNF:	brain-derived nerve factor
Ca^{2+}:	calcium intracellulaire
CA1:	field CA1 of ammon's horn
CA2:	field CA2 of ammon's horn
CA3:	field CA3 of ammon's horn
CBF:	cerebral blood flow
CO:	corn oil
CORT	corticostérone
CRH1:	corticotrophin-releasing hormone receptor 1
DHA:	docosahexaenoic acid
DMTS:	delayed matching-to-sample
DNMTS:	delayed non-matching-to-sample
EPA:	eicosapentaenoic acid
EPM:	elevated plus maze
FADD:	fas associated death domain
FO+CO:	fish and corn oils supplemented diet
FO:	fish oil
FR:	food restriction
GPR-40:	G-protein coupled receptor 40
GRP-75:	glucose-regulated protein, 75-KD
GRP-78:	glucose-regulated protein, 78-KD
HPC:	hippocampal lesion
HSP-70:	heat shock protein, 70-KD
IL-1ß:	interleukine-1ß
ISCH:	ischemic surgery
LTP:	long-term potentiation
MPTP:	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NGF:	nerve growth factor
NMDA:	N-methyl-D-aspartic acid
NMDA-R1:	NMDA receptor 1
NO:	oxyde nitrique

n-3:	omega-3 fatty acids
n-6:	omega-6 fatty acids
OF4:	open field 4 days post-ischemia
OF30:	open field 30 days post-ischemia
PBS:	phosphate buffer solution
PFC:	prefrontal cortex lesion
PTPC:	permeability transition pore complex
PUFA:	polyunsaturated fatty acids
RAM:	radial arm maze
SD:	standard diet
SEM:	standard error of the mean
SHAM:	sham surgery
SNL:	signal to noise ratio
TNF-1 :	récepteur 1 du facteur de nécrose des tumeurs
TNFα:	facteur nécrotique des tumeurs de type alpha
vGAT:	vesicular gamma(γ)-aminobutyric (GABA) transporter
vGluT1:	vesicular glutamate transporter 1
2DG:	2-deoxyglucose
2-VO:	two-vessel occlusion
2-VO + hypo	occlusion à deux vaisseaux combiné à de l'hypotension
3NP:	acide 3-nitropropionique
4-VO:	four-vessel occlusion

Résumé

L'objectif principal de cette thèse était de caractériser l'impact d'une consommation alimentaire quotidienne réduite en calorie ou enrichie de gras polyinsaturés (PUFA) sur les répercussions neuronales et fonctionnelles d'une ischémie cérébrale globale. Différents groupes de rats furent assignés aléatoirement aux conditions expérimentales, déterminées par la diète consommée et la procédure chirurgicale administrée. Les régimes alimentaires des deux premières études de cette thèse incluaient l'ingestion alimentaire ad libitum ou une restriction alimentaire de 40% comparée à l'ingestion des rats des groupes ad libitum. Dans une troisième étude, nous avons évalué l'impact de la consommation d'une diète enrichie de gras polyinsaturés de type oméga 3 et oméga 6, via l'ajout de 11.5% d'huile de poisson et de 3.5% d'huile de maïs à la diète contrôle. Ces différentes diètes furent toutes initiées chez des rats âgés de 4 semaines et maintenues pendant une période de 18 semaines (pré et post-chirurgie). Les procédures chirurgicales, administrées durant la 13^{ième} semaine d'ingestion alimentaire, incluaient soit une chirurgie vasculaire impliquant 8 ou 12 minutes d'ischémie cérébrale globale ou une opération contrôle (chirurgie sham). Suite à ces procédures, l'administration d'une série de paradigmes comportementaux débuta pour l'ensemble des animaux au 4^{ième} ou 5^{ième} jour suivant la chirurgie, et se terminant 7, 31 ou 70 jours suivant l'ischémie, dépendamment de l'expérimentation. Les tests comportementaux utilisés incluaient l'aire ouverte, le labyrinthe en croix et le labyrinthe radial à huit bras. À l'instar des recherches suggérant des effets neuroprotecteurs de la restriction alimentaire et de l'ingestion de suppléments d'acides gras polyinsaturés, nos résultats démontrent que la survie neuronale dans le CA1 de l'hippocampe est comparable entre les rats ischémiés nourris des diètes

expérimentales et ceux nourris à volonté. Cette absence de différence s'observe 7, 31 et 70 jours suivant la procédure chirurgicale. Également, nos résultats comportementaux révèlent une réduction significative, voire la prévention, des déficits d'apprentissage et de mémoire spatiale induits par l'ischémie globale chez les rats restreints et nourris d'une diète enrichie de PUFA. Les performances mnésiques de ces animaux sont comparables à celle des rats contrôles et significativement meilleure que celle des rats ischémiés nourris d'une diète régulière. Fait plus impressionnant, les animaux ischémiés restreints démontrent également la capacité de compléter une série de tâches différées complexes appariées ou non à l'échantillon (DMTS/DNMTS) dans le labyrinthe radial, contrairement aux rats ischémiés nourris à volonté. Nos résultats suggèrent que la restriction alimentaire et l'ingestion de suppléments d'acides gras polyinsaturés induisent des changements plastiques et/ou l'activation de mécanismes endogènes compensatoires capables de protéger les animaux des déficits d'apprentissage et de mémoire spatiale généralement produits par l'ischémie globale.

Introduction Générale

Ces dernières années, plusieurs recherches épidémiologiques ont été entreprises dans le but d'étudier l'évolution des régimes alimentaires à travers le monde et leur impact sur la santé humaine. Ces études ont permis de déceler d'importantes différences dans certains marqueurs de santé selon l'environnement géographique et culturel, mais également de réaliser l'importance de la nature des produits alimentaires consommés par ces différentes populations. Ainsi, Harper et Jacobson [180] répertorient un taux élevé de maladies coronariennes chez la population danoise qui consomme une diète riche en gras (environ 42% de la quantité totale de calories ingérées). À l'opposé, malgré une consommation équivalente de gras dans leur alimentation, les Eskimos du Groenland sont moins susceptibles de mourir des suites des maladies cardiovasculaires [266,180]. De façon similaire, certaines populations méditerranéennes d'Europe démontrent de faible taux de mortalité de maladies cardiovasculaires de type coronarienne [251,293,294].

Plusieurs auteurs ont tenté d'extraire les éléments nutritionnels bénéfiques de la diète méditerranéenne. Ce régime alimentaire se caractérise principalement par une consommation élevée en huile d'olive, en fruits et légumes et en grains et céréales (incluant le pain), augmentant ainsi le ratio d'acides gras monoinsaturés [389,445]. L'alimentation de ces populations se distingue également par une consommation régulière modérée d'alcool (principalement le vin rouge), de produits de la mer et laitiers ainsi que par une faible consommation de viandes et produits dérivés [445]. La diète méditerranéenne demeure donc une diète élevée en gras, dont la particularité provient du fait que près de 50% de ces gras sont monoinsaturés (dérivés de la consommation d'huile d'olive). Les bienfaits de ce régime sont ainsi associés à un apport élevé en minéraux, en

antioxydants et en acides gras monoinsaturés et polyinsaturés, associés à une meilleure régulation du système vasculaire [137,389,445]. En analysant davantage ces différentes populations, les chercheurs ont constaté l'importance de la contribution distincte de différents types de matières grasses et de la composition du régime alimentaire d'une population sur l'apparition de problèmes physiques.

À l'instar des autres mammifères, le régime alimentaire de l'humain est varié et complexifié par de multiples combinaisons alimentaires. Dans ce contexte, l'utilisation de modèles animaux permet de mieux cerner la contribution de composantes alimentaires spécifiques. Au cours de la dernière décennie, la recherche animale a permis de démontrer les effets bénéfiques de la restriction calorique, de la consommation d'aliments riches en antioxydants et/ou d'acides gras polyinsaturés sur des variables physiologiques ou cognitives associées au vieillissement, au diabète, et aux maladies auto-immunes, neurodégénératives et cardiovasculaires [29,44,57,58,64,75,82, 99,107,108,111,117, 192, 276,281,288,302,306,314,337,366,426,432,477,489,494]. Toutefois, peu d'études se sont penchées sur les effets possibles de ces diètes sur les maladies vasculaires cérébrales.

L'objectif principal de cette thèse de doctorat visait à caractériser les répercussions possibles aux niveaux neuronal et fonctionnel de l'adoption de comportements alimentaires distincts lors d'une ischémie cérébrale globale chez le rat. Ce type d'accident vasculaire cérébral entraîne la perte des fonctions cérébrales dûe à l'interruption temporaire complète de l'apport sanguin au cerveau, un phénomène similaire à celui produit lors d'un arrêt cardiaque chez l'humain. Les modifications alimentaires testées chez le rat visent à déterminer si elles peuvent induire des effets physiologiques et/ou neuronaux capables d'altérer le recouvrement fonctionnel suivant

une ischémie cérébrale globale et d'agir comme facteurs de prévention efficace.

1. Ischémie cérébrale

1.1. Maladies vasculaires cérébrales

Les maladies vasculaires cérébrales se classent au quatrième rang des causes de décès au Canada où entre 40 000 et 50 000 nouveaux cas sont rapportés annuellement totalisant des coûts en santé de 2.7 milliards de dollars. Le risque de souffrir d'un accident vasculaire cérébral (AVC) double tous les dix ans après l'âge de 55 ans. À l'heure actuelle, 16 000 canadiens meurent annuellement des suites d'un ACV et quelques 300 000 en conservent des séquelles. Les femmes représentent 60% des nouveaux cas annuels et ont une plus grande probabilité de décès puisque les AVC se produisent à un âge plus avancé comparativement aux hommes [138].

L'AVC consiste en « la perte soudaine de fonction(s) cérébrale(s) attribuable à la rupture de vaisseaux sanguins (AVC hémorragique) ou à l'interruption de l'apport sanguin (AVC ischémique) au cerveau » [138]. Les conséquences d'un AVC dépendent de « l'étendue des dommages encourus » et peuvent affecter différentes fonctions intellectuelles, motrices, sensorielles et émotionnelles. « 20% des AVC sont de nature hémorragique, se produisant lors d'un saignement incontrôlé au cerveau » compromettant la survie neuronale des zones cérébrales irriguées par le vaisseau sanguin affecté. L'AVC hémorragique est généralement causé par des problèmes structuraux de vaisseaux sanguins pathologiques et l'origine du saignement peut être soit sous-arachnoïdienne ou intracérébrale. La majorité des AVC (80%) sont de nature ischémique et surviennent lors d'une interruption de l'apport sanguin au cerveau. La cessation du flot sanguin peut être d'origine thrombotique, causée par

un caillot s'étant formé dans une artère allant au cerveau, ou embolique, lorsqu'une artère cérébrale est obstruée par un caillot sanguin formé ailleurs dans l'organisme et transporté au cerveau par le sang. L'AVC ischémique peut être focal ou global. L'ischémie focale se caractérise par l'arrêt temporaire du flot sanguin dans une région particulière, l'artère cérébrale moyenne étant la plus fréquemment affectée chez l'humain. L'ischémie globale, perturbe quant à elle l'intégrité du flot sanguin afférant au cerveau via les artères vertébrales et carotides internes. Chez l'humain, cette situation survient principalement lors d'un arrêt cardiaque, et sa durée est généralement courte.

1.2. Ischémie et modèles animaux

Les modèles animaux demeurent fondamentaux dans la compréhension des mécanismes physiologiques impliqués lors d'une ischémie cérébrale et l'étude de différents traitements expérimentaux. Trois modèles sont largement utilisés par les chercheurs pour induire une ischémie cérébrale globale. Le premier est l'occlusion à deux vaisseaux (2-VO) combinée à l'hypotension (2-VO + hypo) chez le rat. Ce modèle consiste en l'occlusion bilatérale temporaire des artères carotides conjointement avec l'induction d'hypotension artérielle causée par l'absence du flot sanguin [39]. L'occlusion à deux vaisseaux mène à des dommages focaux, localisés dans une ou plusieurs régions cérébrales. Le deuxième modèle est l'occlusion à deux vaisseaux chez les gerbilles (2-VO). Jusqu'à présent, on croyait à un polygone de Willis incomplet chez ces animaux, rendant l'occlusion à deux vaisseaux similaire à l'occlusion de quatre vaisseaux chez les rats. Ce principe a récemment été remis en question de même que l'efficacité de ce modèle chez différentes souches de gerbilles [252]. Finalement, le troisième modèle est l'occlusion à quatre vaisseaux (4-VO) caractérisée

par l'occlusion permanente des artères vertébrales et temporaire des artères carotides, pour une durée variant de 6 à 30 minutes [39]. Dépendamment de la durée de l'occlusion, ce modèle produit des dommages cérébraux sélectifs ou diffus et plus importants que ceux produits par l'occlusion à deux vaisseaux. Ce modèle demeure le plus fréquemment utilisé pour étudier les conséquences neuronales, physiologiques et fonctionnelles secondaires à une ischémie globale chez le rat.

Tableau 1 : Modèles animaux d'ischémie cérébrale.

Ischémie Globale	Ischémie Focale
- Décapitation - Occlusion de l'aorte/veine cave - Arrêt cardiaque - Hémorragie ou hypotension - Ischémie hypoxique - Hypertension intracrânienne combinée à l'occlusion des artères carotides - Occlusion à deux vaisseaux combinée avec une hypotension artérielle - Occlusion des quatre vaisseaux	- Occlusion de deux vaisseaux (rat) - Infarctus spontané au cerveau (chez certaines espèces animales) - Occlusion de l'artère cérébrale moyenne sans craniotomie - Occlusion de l'artère cérébrale moyenne avec craniotomie - Dommage direct au tissu cérébral (photothrombose cérébrocorticale)

1.3. Mécanismes pathophysiologiques de l'ischémie globale

L'interruption du flot sanguin lors d'une ischémie cérébrale réduit considérablement l'apport d'oxygène et de glucose, compromettant la production d'adénosine triphosphate (ATP) et les fonctions cellulaires globales. Cette réduction soudaine des processus cellulaires stimule la glycolyse, un mécanisme anaérobique de régénération de l'ATP qui se déroule en l'absence d'oxygène. L'augmentation des concentrations de phosphate inorganique, d'acide

lactique et d'hydrogène qui en résulte favorise l'acidose cellulaire. Une cascade physiopathologique s'ensuit, occasionnant une mort neuronale différée dans les régions cérébrales les plus vulnérables à l'événement ischémique. Cette série de réactions biochimiques s'identifie via trois phases : la phase d'excitotoxicité (initiée durant les premières heures suivant l'ischémie), la phase d'inflammation (s'installant durant les premiers jours suivant l'ischémie) et la phase d'apoptose (qui débute quelques jours suivant l'ischémie et peut durer quelques semaines) [39].

1.3.1. Phase d'excitoxicité

Durant la phase d'excitotoxicité, la réduction du flot sanguin entraîne un dysfonctionnement des transporteurs membranaires ioniques dépendants de l'ATP menant à un déséquilibre électrolytique et à une dépolarisation massive des neurones. Cette dépolarisation persistante conduit à l'accumulation de calcium (Ca^{2+}) intracellulaire, provoquant la libération de neurotransmetteurs, incluant le glutamate [63]. L'activation subséquente combinée des récepteurs AMPA et NMDA favorise la libération intracellulaire de Ca^{2+} et l'activation d'une gamme d'enzymes incluant les protéases, les phospholipases, les endonucléases et les enzymes de synthèse de l'oxyde nitrique [1]. Cette prolifération enzymatique compromet l'intégrité des composantes cellulaires et/ou entraîne une surproduction de radicaux libres en diminuant du même élan l'activité des phagocytes endogènes chargés de leur élimination [14,15]. Ultimement, le nombre élevé de radicaux libres devient toxique pour le neurone et enclenche des processus conduisant à la mort neuronale.

1.3.2. Processus de nécrose cellulaire

Les processus excitotoxiques initiés dans les premières 24 heures suivant une ischémie cérébrale globale conduisent à une dégénérescence neuronale par nécrose [136,384,395]. Ce type de mort cellulaire non programmée se produit lorsque l'homéostasie cellulaire est compromise par un manque énergétique, découlant de dommages affectant le fonctionnement des mitochondries de la cellule [292]. Durant la phase d'excitotoxicité, l'activation enzymatique des phospholipases stimulée par l'influx massif de Ca^{2+} intracellulaire favorise l'apparition de renflements membranaires ("blebbing"). Ces derniers sont dûs à l'infiltration massive de fluide dans le neurone menant à la rupture de la membrane cellulaire. Cette dégradation progressive de la membrane cellulaire est associée à l'infiltration progressive d'ions et de métabolites toxiques dans la cellule

Parallèlement, on observe une dilatation du réticulum endoplasmique rugueux et la désintégration des polyribosomes et de l'appareil de Golgi, une condensation de la chromatine en amas compact aux contours irréguliers et un renflement des mitochondries [292,304,305,448]. L'ensemble de ces changements conduit à la fragmentation de la chromatine nucléaire et la lyse du neurone, libérant les constituants cellulaires dans le milieu extracellulaire [292,304,305]. La libération de métabolites toxiques et de glutamate dans le milieu extracellulaire affecte les neurones adjacents, accélérant la lyse de ces derniers. De ce fait, la nécrose s'accompagne d'une réaction inflammatoire importante du tissu cellulaire, facilitant son identification. Elle modifie irréversiblement l'homéostasie cellulaire calcique et dégrade progressivement la chromatine nucléaire s'accompagnant d'une fragmentation aléatoire de l'ADN.

1.3.3. Rôle de l'inflammation dans l'événement ischémique

La phase d'inflammation constitue une réaction physiologique naturelle apparaissant dès les premières heures suivant une ischémie cérébrale. Elle se caractérise par « la migration de composants immunologiques tels les leucocytes polynucléaires (LP) dans les tissus endommagés et l'activation des cellules gliales (microglies et astrocytes) » [39]. En réponse à l'inflammation, la sécrétion d'interleukines (dont l'IL-1ß) et du facteur nécrotique des tumeurs de type alpha (TNFα) caractérise la phase aigüe de la réponse de l'organisme. Les LP stimulent la production d'oxyde nitrique (NO) impliqué dans l'élimination des bactéries pathogènes et exerçant des effets anti- ou pro-apoptotiques sur la mort cellulaire. En présence d'un taux élevé d'oxygène superoxydé (superoxide O_2), comme l'on remarque suivant l'ischémie, la formation d'ions peroxonitriques est responsable de la modification du potentiel de membrane mitochondrial et d'autres phénomènes favorisant l'apoptose cellulaire et la mort de cellules immunitaires (c.f., monocytes et lymphocytes T) impliquées dans la phagocytose. Ainsi, la multiplication d'IL-1ß, de TNFα et de NO suivant l'ischémie constitue un événement toxique majeur au niveau neuronal [407].

1.3.4. Processus d'apoptose cellulaire

Outre l'accentuation de la mort cellulaire par nécrose, la lyse des mitochondries libère des toxines et autres vecteurs cellulaires initiant le processus d'apoptose cellulaire. Ce type de mort cellulaire dite programmée est initiée de façon tardive (3 à 4 jours suivant un événement ischémique) et peut s'établir sur une période pouvant durer jusqu'à trois semaines suivant l'accident vasculaire [345]. En raison de l'activation de multiples

gènes qui caractérise ce processus et sa présence en période normale de développement cellulaire chez tout organisme vivant, ce phénomène est parfois défini comme un « suicide cellulaire programmé ». Contrairement à la nécrose, l'apoptose déclenche l'autodestruction des neurones en réponse à des signaux physiologiques spécifiques et n'implique pas d'inflammation et /ou de lyse de la membrane cellulaire [230]. Parmi les caractéristiques cellulaires qui lui sont propres, on note la condensation du cytoplasme, l'augmentation de la densité de chromatine et son agglomération en amas compact à la périphérie du noyau et la fragmentation de l'ADN, conduisant à la formation des fragments nucléaires et cytoplasmiques [301,448]. Ultimement, malgré une membrane plasmique intacte, la cellule se compose de corps apoptotiques, organelles cellulaires et/ou matériel nucléaire fragmenté et cesse de fonctionner [448].

Le processus d'apoptose est régularisé via deux voies cellulaires principales, que l'on nomme intrinsèque et extrinsèque, qui sont intimement liées à l'activation de protéines cystéiniques appelées caspases [112,127,230]. Parmi ces dernières, on retrouve les caspases initiatrices (caspases-2,-8,-9, et -10) et effectrices (caspases-3,-6, et -7) [278]. Ces protéines sont responsables des changements morphologiques et biochimiques observés lors du processus d'apoptose cellulaire.

La voie intrinsèque de la régulation apoptotique est gouvernée par des gènes et protéines appartenant à la famille des Bcl-2 [43,112]. L'activation de cette voie apoptotique découle principalement de la lyse des mitochondries et de la libération de molécules pro-apoptotiques dans le cytosol via « l'ouverture du PTPC (Permeability Transition Pore Complex) un complexe multiprotéique de la membrane interne mitochondriale ». Subséquemment, la protéine intermembranaire AIF (Apoptosis

Inducing Factor), le cytochrome c et les caspases (-2,-3 et -9), sont libérées dans le cytosol, ce qui initie la phase de dégradation. La famille des Bcl-2 contient des protéines anti-apoptotiques (Bcl-2, Ced-9, Bcl-X_L, Mcl-l, A1, Bcl-W, Bfl-1, Brag-1) et pro-apoptotiques (Bax, Bad, Bak, Bik, Bcl-X_S, Bid, Hrk) qui bloquent ou facilitent la libération de ces protéines intermembranaires [11,43,171,181]. Libéré par les mitochondries, le cytochrome c participe à la respiration cellulaire et régularise le transport ionique intracellulaire. Lorsqu'il est libéré dans le cytosol suite à l'ischémie, il forme, en liaison avec l'Apaf-1 (apoptotic protease activating factor 1) et le déoxyadénosine triphosphate, un complexe multiprotéique qui active les enzymes de type caspase-9 [278]. Ces derniers stimulent à leur tour l'activation de caspase-3, principale responsable de la fragmentation de l'ADN et de la mort par apoptose.

La voie extrinsèque implique, quant à elle, l'activation du récepteur Fas ou du récepteur 1 du facteur de nécrose des tumeurs (TNF-1) [112,278]. La signalisation intracellulaire par le récepteur Fas enrôle la molécule FADD (Fas Associated Death Domain) et l'enzyme caspase-8, entraînant le clivage de la caspase-8 qui active subséquemment la caspase-3 via 2 voies de transmission [5,335,410]. La première voie consiste en l'activation directe de la caspase-3 par la caspase-8. La deuxième voie se caractérise par l'activation indirecte de la caspase-3 par la caspase-9 agissant via la libération du cytochrome c. Tout comme dans la voie intrinsèque, la caspase-3 module ultimement la fragmentation de l'ADN et de la mort cellulaire apoptotique.

L'ensemble de ces phénomènes illustre la complexité de la cascade physiologique initiée suivant l'ischémie globale et engendrant la mort neuronale par nécrose ou apoptose. La nécrose découle de processus dégénératifs associés à un manque d'énergie des cellules

atteintes lors d'accidents vasculaires, alors que l'apoptose induit via une activation génique l'autodestruction des neurones. Ce type de mort neuronale est retardé par rapport à la nécrose et se produit généralement quelques jours à quelques semaines suivant l'ischémie cérébrale.

1.4. Régions cérébrales vulnérables et mort neuronale différée suite à une ischémie globale

L'ischémie cérébrale globale engendre des dommages sélectifs affectant particulièrement les neurones pyramidaux de la région du CA1 de l'hippocampe. Selon l'intensité du choc ischémique, les cellules Purkinje du cervelet, les neurones du striatum et des couches corticales 3, 5 et 6 peuvent être affectées [39]. L'étendue des dommages neuronaux dépend principalement de la durée de l'épisode ischémique globale; plus la durée de l'épisode ischémique est prolongée, plus les dommages neuronaux sont élargis et sévères. Ainsi, une occlusion inférieure ou égale à 10 minutes produit généralement des dommages restreints au CA1 de l'hippocampe. À l'opposé, une occlusion de 20 minutes affecte significativement les régions hippocampiques du CA1, CA2, CA3, l'hippocampe paramédian, le gyrus dentelé, alors que les neurones du striatum et de certaines couches corticales (3, 5, et 6) ne sont que légèrement endommagés [39]. Ultimement, lors d'une occlusion durant plus de 30 minutes, des dommages sévères sont induits dans l'ensemble des couches pyramidales de l'hippocampe affectant également diverses structures extra-hippocampiques, incluant le striatum, le thalamus, la substance noire et le cervelet [54,347,392,459]. La mort des neurones pyramidaux du CA1 ne s'observe que plusieurs heures suivant la réperfusion (débutant environ 96 h suivant une ischémie globale de 10 min), un phénomène appelé « mort neuronale différée ». Ce constat suggère l'existence d'une

fenêtre thérapeutique de quelques jours.

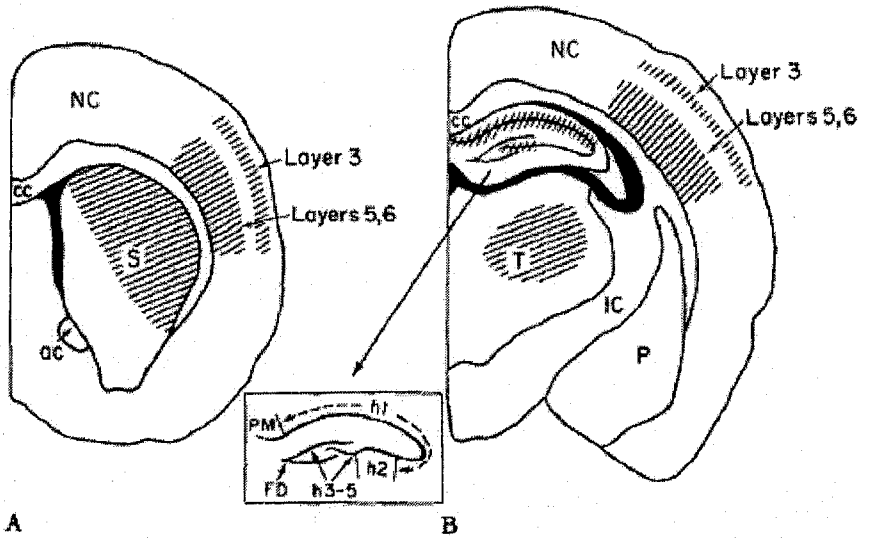

Figure 1: Hémisections coronales du cerveau d'un rat montrant les dommages neuronaux engendrés par l'ischémie globale [392]. (A) Section coronale au niveau du striatum (noyau caudé); (B) Section coronale de l'hipocampe antérieur. NC = néocortex; S = Striatum; P = Putamen; T = Thalamus; PM = hippocampe paramédian; FD = fascia dentata; h1, h2, h3-4 = zones de l'hippocampe; IC = capsule interne; cc = corps calleux; ac = commissure antérieure. Les aires hachurées représentent les régions ayant encourues des dommages neuronaux suite à l'ischémie globale. Les zones noires représentent les ventricules cérébraux.

2. Déficits comportementaux produits par l'ischémie globale

À ce jour, plusieurs études ont évalué la relation entre la densité cellulaire hippocampique et la récupération fonctionnelle suite à un événement ischémique. L'étendue des dommages cérébraux, plus particulièrement une dégénérescence neuronale modérée à sévère dans la portion dorsale de l'hippocampe, est associée à la présence de déficits mnésiques et comportementaux chez l'humain et l'animal [39,53,190,241,459]. Chez l'humain, un infarctus du myocarde et/ou une occlusion de l'artère coronaire, deux pathologies équivalentes à l'occlusion à quatre vaisseaux chez les animaux, engendrent

d'importants troubles de reconnaissance et d'apprentissage affectant la mémoire antérograde [347,374]. Chez l'animal, une ischémie globale est associée à une hyperactivité locomotrice temporaire et à des déficits mnésiques de reconnaissance et de mémoire spatiale et d'apprentissage [39].

2.1. Activité locomotrice et exploratoire

Maintes études rapportent une hyperactivité transitoire des animaux ischémiés placés dans une aire ouverte [18,78,156,225,250,379,382]. Cette hyperactivité est typiquement observable dans les 24-72 heures suivant l'ischémie globale et prédominante lors des premières 24 heures suivant l'ischémie. Elle se résorbe par la suite progressivement pour généralement disparaître 5 à 7 jours suivant la réperfusion [39,40]. Certains chercheurs associent l'hyperactivité locomotrice à la mort neuronale dans le CA1 et l'attribuent à des déficits mnésiques qui retardent la familiarisation des animaux ischémiés à un nouvel environnement [10,225,250]. Différentes études ont démontré l'efficacité de certains traitements pharmacologiques (tels la fluoxetine, le tacrolimus, le nizeferrone, la clonidine, le lithium, et le dextromethorphane) et du préconditionnement ischémique cérébral à diminuer les dommages hippocampiques et l'hyperactivité locomotrice chez les animaux ischémiés [40,124,225,235,480]. De façon similaire, Colbourne et collègues [89] observent que l'hypothermie est efficace pour réduire les dommages ischémiques des cellules pyramidales CA1 et les déficits comportementaux dans l'aire ouverte et le labyrinthe en T. Étant donné une relation étroite entre dommages neuronaux et hyperactivité locomotrice suivant l'ischémie cérébrale globale, plusieurs chercheurs en sont venus à la conclusion que ces deux événements étaient intimement reliés.

D'autres chercheurs ont par ailleurs suggéré un lien de ce comportement exploratoire avec l'activité neuronale accrue des cellules pyramidales du CA1 présente 24 h suivant l'occlusion à deux vaisseaux chez la gerbille [435] et 48-72 h suivant une ischémie focale chez le rat [81]. Cette excitabilité neuronale coïnciderait davantage au niveau temporel avec l'hyperactivité locomotrice observée 24 heures suivant un événement ischémique et précéderait la dégénérescence neuronale. Ainsi, l'hyperactivité est saillante 24 heures suivant l'ischémie globale alors que la mort neuronale par nécrose est différée et établie environ 96 h suivant l'ischémie. Par ailleurs, certaines études n'établissent pas de corrélation entre le niveau d'hyperactivité et le nombre de neurones pyramidaux dans le CA1 [375]. À l'inverse, d'autres études montrent que certains traitements neuroprotecteurs connus, tels le préconditionnement ischémique et l'administration de l'inhibiteur calcique flunarizine, n'affectent pas l'hyperactivité [96,378,382]. De plus, Plamondon et Khan [379] observent que l'antagoniste CRH1 inhibe l'hyperactivité produite par une ischémie globale (4-VO) chez le rat sans toutefois affecter la mort neuronale dans l'hippocampe et les déficits de mémoire spatiale et d'apprentissage dans le labyrinthe à huit bras. De façon similaire, Puurunen et collègues [393] notent qu'un environnement enrichi en stimulations prévient l'expression de l'hyperactivité locomotrice produite par l'ischémie globale chez le rat sans avoir d'impact sur la dégénération neuronale. Finalement, Nelson et al. [341] ne rapportent aucune différence d'exploration horizontale et verticale dans l'aire ouverte entre les rats ischémiés et contrôles, malgré une dégénérescence neuronale importante chez les rats ischémiés.

À l'heure actuelle, il n'existe donc pas de consensus confirmant ou infirmant le fait que l'hyperactivité locomotrice produite par une ischémie globale soit dépendante des

neurones de la région du CA1 de l'hippocampe. Malgré les divergences présentées, peu d'études ont analysé les phénomènes pouvant y être sous-jacents. Une étude récente de notre laboratoire a démontré l'impact considérable et différencié des conditions d'éclairage (brillant versus tamisé; 450 versus 40 lux) sur les comportements des animaux ischémiés et contrôles dans l'aire ouverte, un phénomène susceptible d'expliquer les divergences et l'absence de consensus observés dans la littérature [322].

2.2. Anxiété et ischémie cérébrale

De nombreuses recherches se sont intéressées aux répercussions mnésiques et locomotrices de l'ischémie cérébrale et peu aux changements émotionnels qui découlent de l'accident vasculaire. Pourtant, les données cliniques montrent que suivant un accident vasculaire cardiaque, les survivants souffrent non seulement de déficits mnésiques et attentionnels, mais également de perturbations émotionnelles, incluant la dépression et l'anxiété [109]. Ainsi, il semble probable que l'ischémie globale influence des processus neurochimiques et/ou cellulaires affectant les réponses émotionnelles des animaux. L'hyperactivité locomotrice peut en soi représenter une de ces manifestations comportementales chez un animal placé dans un environnement inconnu et possiblement anxiogène. Dans un contexte naturel, un tel événement peut représenter une menace pour un animal, associée à des processus de vigilance et de mobilisation énergétique accrus et une activation du système nerveux sympathique et de l'axe hypothalamo-hypophysaire-surrénalien combinée à l'inhibition temporaire de certains comportements incluant le besoin de se nourrir [32,350,388]. En soi, l'hyperactivité exploratoire observée dans un

environnement nouveau chez les rats ischémiés va à l'encontre de la tendance naturelle des mammifères de n'explorer que graduellement un environnement anxiogène non familier.

À cet égard, il est à souligner que l'activité dans l'aire ouverte est utilisée pour déterminer le niveau d'anxiété chez les animaux [32,100,361]. Une augmentation des comportements exploratoires en général et/ou de ceux effectués dans le centre de l'aire ouverte représentent des comportements associés à une atténuation du niveau d'anxiété [380,388,421]. Malgré une utilisation fréquente dans l'évaluation des effets anxiolytiques de substances pharmacologiques, la mesure de l'activité dans le centre de l'aire ouverte a été peu utilisée auprès d'animaux ischémiés comparativement à celle du labyrinthe surélevé en forme de croix (elevated plus maze) [179]. Il est toutefois informatif de combiner les mesures de ces deux tests afin de quantifier les comportements associés à l'anxiété des animaux et leur volonté à explorer des environnements non familiers [32].

À l'instar des résultats obtenus dans l'aire ouverte, on retrouve une variabilité d'observations de l'activité exploratoire post-ischémique dans le labyrinthe en croix. Plusieurs recherches démontrent que les rongeurs ayant subi une ischémie globale ou focale effectuent plus d'entrées et passent significativement plus de temps dans les bras ouverts du labyrinthe en croix [341,379,471,480]. À l'opposé, Nakashima et al. [339] indiquent que deux jours suivant l'occlusion bilatérale des artères carotides, les souris ischémiées passent moins de temps dans les bras ouverts du labyrinthe en croix. Dhooper et collègues [109] observent également une réduction du nombre d'entrées dans les bras ouverts du labyrinthe deux jours suivant une ischémie globale de 7 minutes chez le rat, démontrant ainsi un niveau d'anxiété plus élevé que chez les rats contrôles. Ce comportement se stabilise partiellement chez ces animaux, sans toutefois devenir équivalent à celui des animaux contrôles.

Finalement, d'autres études ne rapportent pas de différence significative entre les animaux ischémiés et contrôles au niveau du temps passé et du nombre d'entrées dans les bras ouverts du labyrinthe en croix [26,107]. Tout comme le test de l'aire ouverte, des différences méthodologiques et/ou environnementales lors du test (c.f., familiarité des lieux d'évaluation; intensité de l'éclairage) sont susceptibles d'influencer les résultats, rendant plus difficile l'identification d'un profil typique chez les animaux.

2.3. Mémoire et apprentissage

Les déficits d'apprentissage et de mémoire spatiale sont souvent associés aux dommages sélectifs des neurones pyramidaux de la région CA1 de l'hippocampe suite à une ischémie globale. Plusieurs paradigmes expérimentaux sont utilisés dans la recherche pour évaluer ces déficits chez les animaux, les plus fréquemment utilisés étant (1) l'apprentissage à l'aide de repères visuo-spatiaux de l'emplacement de nourriture dans un labyrinthe radial à plusieurs bras ou d'une plateforme immergée dans un labyrinthe aquatique comme le 'Morris water Maze', et (2) les tâches impliquant la mémoire de travail, comme les tâches différées appariées ou non avec l'échantillon initialement présenté («delayed-matching ou non-matching-to-sample-tasks»; DMTS et DNMTS, respectivement).

2.3.1. Les labyrinthes en forme de T et de Y

Les labyrinthes en forme de T et de Y mesurent la préférence exploratoire naturelle des animaux d'alterner de bras en bras, leur habileté à récolter une récompense dans un bras prédéterminé lors d'essais successifs, et leur habileté à associer une réponse exploratoire à l'obtention d'une récompense ou d'une conséquence aversive (paradigmes

DNMTS et/ou DMTS). La mesure la plus fréquente demeure l'alternance spontanée des animaux, mesurant leurs comportements de navigation naturels sans privation de nourriture ou de procédures aversives.

De nombreuses recherches démontrent que les animaux ischémiés ont un taux d'alternance spontanée nettement inférieur à celui des animaux contrôles. Par exemple, Kofler et collègues [245] notent une réduction importante du taux d'alternance des souris et ce, jusqu'à sept jours suivant une ischémie globale. De façon similaire, Ishibashi et al. [205] et Sarti et al. [412] observent des déficits d'alternance chez des gerbilles et des rats ischémiés 30, 60 et 90 jours suivant l'occlusion bilatérale des artères carotides. Ce phénomène est également observé suite à une ischémie globale de 10 minutes chez le rat [326]. Certains traitements dont l'administration d'aminoguanidine, un inhibiteur de l'enzyme de synthèse de l'oxyde nitrique, 30 minutes précédant l'occlusion sanguine et à toutes les 24 heures pendant quatre jours suivant l'ischémie parviennent à atténuer significativement ce déficit. Cette diminution des comportements d'alternance spontanée chez des gerbilles ischémiées est présente malgré un nombre total d'entrées supérieur dans les bras du labyrinthe [175]. Parmi les observations discordantes, Wahl et al. [461] n'observent toutefois aucun effet d'une occlusion focale de l'artère cérébrale moyenne sur les taux d'alternance spontanée des animaux.

2.3.2. Le labyrinthe radial à huit bras

Le labyrinthe radial représente un des tests comportementaux les plus utilisés pour quantifier la mémoire spatiale chez l'animal. Il est sensible aux lésions hippocampiques, incluant celles produites par l'ischémie cérébrale, et les résultats

obtenus sont facilement reproduits. Dans cette tâche, la mémoire spatiale est évaluée par le nombre d'erreurs de mémoire de référence et/ou de mémoire de travail durant la tâche. Une erreur de mémoire de référence est commise lorsque l'animal entre dans un bras non ciblé par la procédure choisie et qui ne contient pas de récompense. Une erreur de mémoire de travail est commise lorsque l'animal pénètre à nouveau dans un bras qu'il a préalablement visité. Le temps requis par l'animal pour effectuer la tâche, le temps requis avant de faire une première erreur de mémoire de travail, le nombre de réponses exactes et le nombre de récompenses ingérées lors de chaque session expérimentale représentent des mesures additionnelles.

De nombreuses études ont démontré des déficits d'apprentissage et de mémoire spatiale dans le labyrinthe à huit bras chez les animaux ischémiés [39,183,346,352]. Ces animaux se distinguent par des déficits importants de mémoire de travail et de référence [146,158, 207, 380]. Kiyota et al. [241] notent que les erreurs de mémoire de travail semblent davantage dépendantes de la durée de l'ischémie globale. Ainsi, des animaux ayant reçu 20 minutes d'ischémie commettent davantage d'erreurs de ce type que des animaux n'ayant reçu qu'une ischémie de 5 minutes, malgré un nombre d'erreurs de mémoire de référence comparable. De plus, Schwartz et collègues [415] observent que la mémoire de travail est également affectée avec l'âge; les animaux âgés (> 2 ans) commettent davantage d'erreurs de mémoire de travail que les animaux juvéniles (3 mois).

Finalement, des déficits permanents de mémoire de travail sont observés chez des animaux ischémiés, alors que des déficits de mémoire de référence sont absents ou réversibles [472]. À ce sujet, Davis et al. [104] rapportent que suite à 70 essais d'entraînement dans le labyrinthe précédant l'induction de 30 minutes d'ischémie globale, la

mémoire de travail des rats est altérée suivant l'occlusion alors que la mémoire de référence est intacte. Cette différence est observée malgré des dommages neuronaux importants dans l'hippocampe. Dans un même ordre d'idée, Volpe et al. [456] notent qu'une période d'entraînement de 36 essais pré-ischémie n'a pas d'impact sur les déficits de mémoire de travail suivant l'ischémie. Toutefois, cet entraînement préalable atténue considérablement les déficits de mémoire de référence des animaux ischémiés. Fait intéressant, une période d'entraînement pré-ischémique prolongée incluant 80 essais parvient à décroître significativement le nombre d'erreurs de mémoire de travail et de référence suivant l'ischémie et ce, malgré des dommages importants à la région du CA1 de l'hippocampe.

L'ensemble de ces résultats indique la présence d'importants déficits de mémoire spatiale suivant l'ischémie globale, qui peuvent être atténués par la maîtrise de certaines habiletés avant l'ischémie. Ces déficits sont davantage observés lors de l'évaluation de la mémoire de travail dans le labyrinthe radial et sont influencés par la durée de l'ischémie et l'âge de l'animal. Finalement, la présence de déficits de mémoire de référence est plus aléatoire et ces déficits sont plus facilement réversibles, ce qui suggère que les mécanismes physiologiques et/ou structures cérébrales sous-jacents à la mémoire de référence diffèrent de ceux associés à la mémoire de travail.

2.3.3. Le labyrinthe aquatique de Morris

Le deuxième type de tests de mémoire spatiale le plus utilisé est le labyrinthe aquatique de Morris. De nombreux groupes rapportent des déficits d'apprentissage spatial dans ce test chez les animaux ischémiés dans les tâches simples d'acquisition et de rétention [39,40,41,42,167,270,341,346,354]. En général, le temps nécessaire pour compléter la tâche

ainsi que la distance parcourue à chaque essai sont significativement plus élevés chez ces animaux comparativement aux animaux contrôles avec ou sans indices visuels supplémentaires [183]. En plus des déficits de la mémoire de référence, les animaux ischémiés présentent des déficits de mémoire de travail dans les tâches d'apprentissage complexes, c'est-à-dire lorsque la position de la plateforme change aléatoirement à chaque essai ou bloc d'essais. L'expérimentation de Nunn et al. [345] comparant la performance de rats ischémiés (4-VO de 15 minutes) à celle de rats ayant uniquement une lésion du CA1 ou de rats contrôles dans plusieurs variations du labyrinthe aquatique de Morris, supportent ces observations. Toutefois, les rats ayant une lésion exclusive du CA1 et les rats ischémiés malgré des dommages hippocampiques similaires présentent un profil comportemental distinct. Ainsi, bien que les rats ayant une lésion du CA1 soient capables d'accomplir les tâches d'acquisition et de rétention, les rats ischémiés démontrent des déficits permanents sévères dans l'apprentissage et la complétion de l'ensemble de ces tâches mnésiques.

D'autres études sont plus nuancées et exposent des déficits d'apprentissage temporaires suite à une ischémie globale. Par exemple, bien que l'acquisition et la rétention de la tâche demeure déficiente lors d'une ischémie globale de 5 ou 10 minutes [53], les rats ayant reçu une ischémie de 5 minutes démontrent des capacités d'apprentissage moins affectées. De même, suivant plusieurs essais, ces derniers atteignent des niveaux de performance équivalents à ceux des rats contrôles, en termes de vitesse de nage et de distance parcourue pour atteindre la plateforme. Ces résultats suggèrent une gradation des déficits en fonction de la durée de l'ischémie et des dommages neuronaux encourus. Toutefois, Hagan et Beaughard [177] n'observent que des déficits d'apprentissage transitoires dans l'acquisition et la rétention de la tâche du labyrinthe aquatique de Morris chez des rats ayant subi une

ischémie globale de 15 minutes, malgré des déficits d'alternation dans le labyrinthe en T et d'importants dommages aux neurones pyramidaux du CA1.

Enfin, d'autres recherches suggèrent que malgré des déficits sévères de mémoire de travail et de référence dans le labyrinthe à huit bras, l'acquisition et la rétention de ces animaux demeurent comparables à celles de rats contrôles dans le labyrinthe aquatique de Morris [241]. Green et collègues [166] rapportent des résultats similaires lors de tâches d'acquisition et de rétention avec une plateforme visible ou submergée. Toutefois, l'utilisation d'une tâche d'apprentissage plus complexe impliquant un changement régulier de la position de la plateforme est associée à une meilleure discrimination de la performance des rats ischémiés et contrôles [17,166]. Ainsi, tel qu'observé dans les labyrinthes en T, en Y et radial, les résultats dans le labyrinthe aquatique de Morris varient en fonction de la durée de l'ischémie, des dommages cellulaires encourus et de la complexité du paradigme expérimental sélectionné.

2.3.4. Tâches différées appariées ou non à l'échantillon (DMTS/DNMTS)

Dans l'ensemble, très peu de recherches utilisent des tâches complexes, telles que les tâches DMTS/DNMTS, pour caractériser les déficits comportementaux induits par l'ischémie cérébrale chez des rongeurs et primates. Toutefois, ces tests permettent une évaluation approfondie de la mémoire nécessitant une capacité de rétention temporelle et visuo-spatiale plus complexe de l'information et une flexibilité cognitive des animaux associée à l'accomplissement de tâches différées distinctes. Dans une tâche différée non appariée à l'échantillon (DNMTS), Zola-Morgan et collègues [496] démontrent que les performances de singes cynomolgus ayant subi une ischémie

cérébrale ou une lésion spécifique du CA1 sont significativement inférieures à celles des singes contrôles lorsqu'ils doivent apprendre à déplacer le bon objet suivant un délai de 15, 60, 600 ou 2400 secondes. Woods et al. [477] observent des déficits comparables chez des rats ischémiés utilisant des délais de 4, 15, 30 et 60 secondes, mettant en évidence les déficits d'acquisition de la tâche lors de tests de rétention différée. De manière similaire, Mumby et al. [334] rapportent des déficits sévères dans une tâche différée de reconnaissance d'objets chez des rats ischémiés. À l'opposé, certaines études ne rapportent aucun déficit mnésique chez des animaux ischémiés dans ce type de tâche [341,413]. Ces différents résultats mettent en evidence les difficultés à établir les habilités cognitives résiduelles des animaux ischémiés avec une tâche DNMTS incluant la reconnaissance d'objets comme principale composante.

Une proportion importante des études animales utilise le labyrinthe en T pour évaluer la mémoire de travail des animaux ischémiés lors de tâches d'alternance différée. Les animaux ischémiés démontrent des déficits d'apprentissage dans ces tâches [358], qui sont plus sévères lorsque la reconnaissance implique un même objet et emplacement que lors de la présentation initiale (DMTS) et une position aléatoire des récompenses et/ou délais pour chaque essai [9,461]. Malgré qu'un pré-entraînement à la tâche n'atténue pas les déficits post-ischémiques de mémoire de travail chez des gerbilles lors d'appariements différés distincts (DNMTS), il améliore significativement leur performance mnésique lors de tâche différée appariée à l'échantillon (DMTS). La performance des gerbilles ischémiées dans cette tâche devient ainsi équivalente à celle des gerbilles contrôles [19].

Parmi les méthodes ayant un impact sur la performance mnésique post-ischémique, Farrell et al. [134] observent que des gerbilles ayant reçu un préconditionnement ischémique

(via une courte ischémie induite quelques jours avant l'ischémie prolongée) et/ou ayant été logées dans un environnement stimulant démontrent la capacité d'apprendre une tâche DNMTS dans un labyrinthe en T 60 jours suivant l'ischémie globale. À l'opposé, les gerbilles ischémiées non traitées sont incapables d'apprendre cette tâche. De plus, seules les gerbilles des groupes contrôles ou ischémiés ayant reçu un préconditionnement ischémique sont en mesure d'apprendre la tâche DMTS. Dans une étude antérieure, Colbourne et Corbett [90] observent également des déficits d'apprentissage dans la tâche DMTS chez des gerbilles ayant reçu 5 minutes d'ischémie globale. D'autres chercheurs observent une diminution significative des réponses exactes lorsqu'un délai de plus de 10 secondes est introduit entre les phases d'acquisition et de rétention dans les premiers jours suivant l'ischémie. Ce déficit se résorbe graduellement chez ces animaux qui rejoignent éventuellement la performance des gerbilles contrôles [201]. Finalement, l'hypothermie durant et dans les heures suivant l'ischémie globale diminue considérablement la mort neuronale dans le CA1 et améliore significativement la performance des gerbilles dans les tâches DNMTS et DMTS.

Il est surprenant de constater que malgré la popularité du labyrinthe radial dans l'étude des processus mnésiques, l'intégration de tâches DMTS/DNMTS dans ce test est rarement utilisée afin d'évaluer plus spécifiquement l'étendue des déficits de mémoire spatiale suivant l'ischémie. Il est raisonnable de penser que l'observation de déficits cognitifs significatifs en utilisant des tâches simples dans ce test et le fait que les tâches plus complexes nécessitent généralement l'atteinte d'un critère d'apprentissage (ce qui peut s'avérer laborieux pour les chercheurs au niveau du temps et de l'argent investis dans la recherche) expliquent en partie cette situation.

3. Ischémie et traitements neuroprotecteurs

Au cours des dernières décennies, plusieurs travaux scientifiques ont évalué l'impact de divers traitements pharmacologiques sur les dommages neuronaux et déficits fonctionnels subis lors d'une ischémie cérébrale. D'autres études ont analysé l'impact de facteurs associés à la durée de l'épisode ischémique ou à sa répétition, de même qu'exploré des mécanismes physiologiques de défense endogènes activés lors du préconditionnement ischémique cérébral et favorisant l'adaptation au stress ischémique et la survie neuronale.

La méthode thérapeutique la plus efficace à ce jour pour contrer la mort neuronale et accroître la préservation fonctionnelle demeure l'abaissement significatif de la température cérébrale, qui réduit les processus de dégénérescence cellulaire. Ces derniers sont associés à une diminution du métabolisme cérébral, de l'accumulation de calcium intracellulaire et du relargage de neurotransmetteurs excitateurs de même qu'une préservation de l'intégrité de la barrière hémato-encéphalique souvent compromise lors d'accidents vasculaires. Plusieurs études ont démontré que l'induction post-ischémique brève (3 à 6 heures) ou prolongée (12 à 24 heures) de période d'hypothermie chez les rongeurs réduit significativement les dommages neuronaux engendrés et la dégénérescence dans le CA1 et ce, jusqu'à 6 mois suivant l'ischémie [90,91,257,447].

Plusieurs chercheurs ont également évalué l'impact de divers facteurs environnementaux sur la dégénérescence neuronale et la récupération fonctionnelle suivant une ischémie cérébrale. Ces études démontrent en autre les effets bénéfiques de l'exercice sur la résistance des muscles cardiaques, la réperfusion sanguine ou la survie neuronale lors d'événements ischémiques subséquents [48,49,218,269]. De même, l'exercice restaure les effets neuroprotecteurs du préconditionnement ischémique chez des rats âgés de 24 mois

suite à une ischémie globale de 20 minutes suivie de 40 minutes de réperfusion cardiaque [2]. Finalement, certains auteurs montrent que l'activité physique chez les rongeurs réduit la mort neuronale par apoptose, augmente la restructuration cellulaire suite à des atteintes neuronales, et accroît la neurogénèse dans les zones sous-ventriculaire et sous-granulaires des ventricules latéraux et du gyrus dentelé de l'hippocampe, respectivement [50,218, 229, 381, 395,449,451,452].

De façon intéressante, plusieurs recherches ont démontré des bénéfices neuronaux et fonctionnels à l'exposition à un environnement riche en stimulations, proposant des contacts animaliers, des aires d'habitat plus spacieuses et des activités physiques variées (roues, tubes, jouets, etc) renouvelées régulièrement. Plusieurs études ont évalué des animaux vivant en groupe partageant de tels environnements. Ces dernières rapportent des avantages significatifs de ce mode de vie par rapport aux rongeurs vivant seul dans une cage de dimension régulière, notamment en ce qui a trait aux effets de ce type d'environnement sur les maladies neurodégénératives et cardio- ou cérébro-vasculaires. Parmi de multiples effets répertoriés, ces recherches démontrent que l'exposition à ce type d'environnement 'enrichi' favorise une plus grande plasticité neuronale et influence le fonctionnement cellulaire, en partie via l'accroissement des arborisations et épines dendritiques et/ou de la densité synaptique et une synthèse protéinique accrue [36,50,103,214,215,249,393,490]. Un habitat proposant de multiples stimulations sensorielles favorise également la présence de neurogénèse dans l'hippocampe [186,228,247,248,348]. Malgré cette neurogénèse, différentes études rapportent que ce type de stimulations environnementales ne confère pas de protection contre les effets dévastateurs de la cascade ischémique sur les cellules vulnérables de l'hippocampe [102,134,160,216,373]. Ainsi, l'étendue des dommages

cérébraux encourus ne diffère pas entre les animaux ischémiques ayant vécus dans un environnement enrichi ou régulier [299]. Belayev et collègues [31] suggèrent qu'un habitat riche en stimuation retarde plutôt que prévient la mort neuronale à long terme. Malgré cette observation, les rongeurs ischémiés logés dans de telles conditions présentent des améliorations significatives de leur performance dans des tâches d'apprentissage et de mémoire spatiale telles que le labyrinthe aquatique de Morris et le labyrinthe radial [36,51,52,55,102,214,216,299,351,428]. En bref, la présence de stimulations sensorielles, physiques et sociales entraîne des changements cérébraux importants chez les animaux ischémiés qui facilitent la récupération fonctionnelle post-ischémie et ce, malgré la dégénérescence maintenue des neurones hippocampiques du CA1.

4. Ischémie et régimes alimentaires

Un nombre croissant de recherches scientifiques étudient les effets bénéfiques de certains nutriments et /ou catégories de produits alimentaires. La motivation initiale de ces études découle principalement des possibilités démontrées par divers aliments ou suppléments nutritionnels à réduire la production de radicaux libres associée au vieillissement [75,220,221,255,424,484]. Ces derniers endommagent la structure membranaire plasmique et les acides nucléiques (ADN, ARN), compromettant la synthèse des protéines et le fonctionnement cellulaire [219,425,442]. Le déclin des capacités du cerveau âgé à combattre les effets du stress oxydatif et de l'inflammation accroît sa vulnérabilité au développement de maladies neurodégénératives.

Les manipulations alimentaires expérimentales sont diverses et incluent l'ajout de divers phytonutriments et vitamines (C et E, principalement). Les phytonutriments les plus

connus comprennent les composés polyphénoliques et les terpènes. Malgré que les phytonutriments ne représentent pas des aliments essentiels au développement humain, ils possèdent des propriétés anti-oxydantes, anti-inflammatoires, anti-allergènes et réduisent le développement de divers types de cancers [75,113,196,307, 455, 470]. Tout comme les phytonutriments, les sources de vitamine C ou E ont également démontré des effets antioxydants bénéfiques, un accroissement des capacités immunitaires et sont associés à une réduction de l'apparition de certains cancers et des maladies coronariennes associées au vieillissement [62,163,170,319,355,357,363].

Au cours de la dernière décennie, d'autres types de modifications alimentaires ont démontré des effets bénéfiques. En particulier, deux stratégies alimentaires se sont avérées des pistes expérimentales prometteuses dans l'amélioration d'une variété de conditions, incluant le vieillissement, le cancer, l'inflammation, les maladies neurodégénératives, et les maladies cardio- et cérébro-vasculaires. La première de ces stratégies suggère des bénéfices d'une alimentation réduite en termes de consommation énergétique quotidienne, via une diminution substantielle du nombre de calories et/ou de la ration alimentaire quotidienne fournie à l'animal. La deuxième stratégie consiste à intégrer à son régime alimentaire une consommation accrue d'aliments ou de suppléments alimentaires riches en gras polyinsaturés.

4.1. Restriction alimentaire

La restriction alimentaire employée en recherche animale consiste à réduire le nombre de calories ingérées par une diminution de la ration de nourriture quotidienne fournie à l'animal. Deux protocoles expérimentaux principaux sont utilisés dans ce type

d'expérimentations. Certaines études utilisent une procédure basée sur des rations de nourriture illimitées uniquement disponibles un jour sur deux. D'autres expérimentations optent pour un protocole utilisant une ingestion alimentaire quotidienne, mais restreinte de la diète régulière. Typiquement, la ration alimentaire quotidienne fournie aux animaux dans ces études varie entre 40-60% de la ration de nourriture journalière des animaux du groupe contrôle étant nourris ad libitum. La restriction alimentaire est typiquement administrée pour des périodes variant de 15 à 120 jours dans des protocoles de recherche sur les maladies neurodégénératives et cardiovasculaires. Elle peut toutefois atteindre une période de deux ans lors d'étude de l'impact de ce type de diète sur le vieillissement. Plusieurs recherches étudiant divers paramètres chez les animaux rapportent que la restriction alimentaire produit de nombreux changements physiques, incluant une réduction de la température corporelle, du poids, du pourcentage de gras/cholestérol, et une meilleure régulation de la pression artérielle, du rythme cardiaque et de l'insuline et du glucose sanguin [303].

4.1.1. Restriction alimentaire et vieillissement

Plusieurs études démontrent une augmentation de la durée de vie chez de nombreuses espèces animales dont les rongeurs et les primates exposés pour des périodes variables à une consommation alimentaire restreinte [38,123,172,195,227, 295,296,403, 417,426, 464,465]. Parmi les effets bénéfiques, la restriction alimentaire préserve les fonctions protectrices du système immunitaire, et prévient ou retarde le déclin des lymphocytes de type T du système immunitaire associé à l'âge. En freinant la production de cytokines pro-inflammatoires par l'organisme, la restriction alimentaire réduit

l'incidence de cancers et de maladies cardiovasculaires chez ces animaux [135]. De plus, la diminution significative du gras viscéral et l'accroissement de la sensibilité à l'insuline observé chez les animaux restreints exerce un impact positif sur l'incidence des maladies dégénératives telles que le diabète [132,151]. Ainsi, Cefalu et al. [76] rapportent qu'une restriction alimentaire de 30% pour une période d'un an est associée à une réduction significative du poids corporel et de l'accumulation de gras intra-abdominal chez un groupe de primates. Ces animaux présentent également une meilleure régulation du cholestérol sanguin, affichant des concentrations plus élevées de « bon » cholestérol et une amélioration significative de la sensibilité à l'insuline des tissus périphériques. Finalement, Holt et collègues [192] observent une diminution de la mort des cellules épithéliales et intestinales (petit intestin et colon) chez les rats âgés nourris d'une restriction alimentaire. Ces observations suggèrent une meilleure préservation cellulaire et fonctionnelle de ces organes.

En lien avec ces observations, la restriction alimentaire est également associée à des changements cérébraux, incluant une amélioration de certains processus synaptiques, du fonctionnement des mitochondries et des récepteurs dopaminergiques (D2) dans le néostriatum [173,271,330,402]. De plus, certaines recherches montrent que la restriction alimentaire réduit les dommages à l'ADN liés au vieillissement [189,425,434]. Celle-ci protègerait les neurones en préservant les enzymes de réparation de l'ADN et en favorisant l'expression de protéines antiapoptotiques impliquées dans la mort neuronale. Finalement, la restriction alimentaire augmente la résistance cellulaire via une régulation de l'expression de certains gènes. Ces derniers incluent une variété de protéines

chaperonnes, de facteurs de croissance, de neurotrophines et de cytokines qui stimulent la neurogénèse et la plasticité synaptique [210,303,311,418].

Finalement, diverses études montrent également un impact positif de la restriction alimentaire sur le fonctionnement cognitif et comportemental des animaux lors du vieillissement [86,202,376,377]. Par exemple, Idrobo et al. [199] notent un apprentissage accéléré et un nombre d'erreurs significativement réduit dans le labyrinthe à huit bras chez des souris soumises à une restriction alimentaire pour une période de 12 mois. Goodrick [162] observe que des rats restreints (âgés de 30 mois) démontrent une performance supérieure aux rats nourris à volonté dans un labyrinthe complexe (14-unit maze). Leur performance est en fait comparable à celle des rats adultes âgés de 6 mois. De manière similaire, Stewart et al. [432] montrent qu'une restriction alimentaire de 24 mois prévient les déficits de mémoire associés à l'âge dans un labyrinthe aquatique, en plus d'améliorer l'apprentissage des rats adultes âgés de 8 et 16 mois. D'autres études rapportent que la restriction alimentaire accroît la coordination motrice, l'activité locomotrice, le score d'alternance spontanée et l'apprentissage dans plusieurs paradigmes comportementaux chez des animaux âgés [118-122,202,287,313,376,377,487]. En contrepartie, d'autres études ne démontrent aucun effet significatif de la restriction alimentaire sur la performance mnésique de rats âgés dans des tâches de navigation spatiale [29,45,288].

4.1.2. Restriction alimentaire et maladies neurodégénératives, cérébro- et cardiovasculaires

Plus récemment, des recherches ont appuyé les effets de la restriction alimentaire sur la résistance aux effets de maladies neurodégénératives chroniques ou de traumatismes crâniens [178,290,302-305,367,463]. Par exemple, Zhu et collègues [493] notent qu'une restriction alimentaire d'une durée de 3 mois, basée sur un régime d'ingestion par alternance journalière, réduit de façon appréciable le stress oxydatif dans un modèle animal de la maladie d'Alzheimer caractérisé par une mutation de la presenilin-1. Utilisant ce modèle animal, ces chercheurs observent une résistance accrue des neurones du CA1 et du CA3 de l'hippocampe aux dommages induits par l'administration d'acide kaïnique chez les animaux mutants maintenus en restriction alimentaire. Ces résultats suggèrent que la restriction alimentaire exerce un impact sur la vulnérabilité neuronale associée à cette mutation génétique, supportant le rôle neuroprotecteur d'une telle diète. En évaluant l'impact de l'alternance journalière chez des souris, Lee et al. [264] rapportent également une résistance accrue des neurones de l'hippocampe et du cortex cérébral aux pathologies associées à l'âge, principalement la maladie d'Alzheimer et la dégénérescence associée à des conditions aigües telles que l'ischémie. Pour leur part, d'autres chercheurs notent qu'une période de 2 à 4 mois d'alternance journalière chez des rats est suffisante pour accroître la résistance des neurones de l'hippocampe et du striatum aux dommages excitotoxiques causés par l'administration intra-hippocampique d'acide kaïnique et systémique d'acide 3-nitropropionique (3NP) [59]. Ces effets cellulaires sont associés à une amélioration des habilités motrices et mnésiques dans un exerciseur (e.g., le rotary rod apparatus) et le labyrinthe aquatique de Morris, respectivement. Enfin, une période de trois mois d'alternance journalière chez des rats

atténue significativement la vulnérabilité des neurones dopaminergiques de la substance noire aux effets du 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), une toxine connue pour activer une cascade neurodégénérative similaire à celle impliquée dans la maladie de Parkinson [117]. De concert avec la diminution des dommages neuronaux, les déficits moteurs induits par le MPTP sont significativement réduits chez ces animaux. Des résultats analogues sont obtenus chez des singes rhésus, suggérant que ces effets bénéfiques pourraient éventuellement s'appliquer aux humains [298].

Les bénéfices de la restriction alimentaire sur les fonctions cardiaques et la récupération suivant un accident cardiovasculaire de type ischémique sont également documentés [57,82]. Pionniers dans ce domaine de recherche, Crandall et collègues [99] rapportent que la restriction alimentaire et l'exercice combinés représentent des facteurs influençant de façon significative la sévérité d'une attaque ischémique du myocarde. Ces chercheurs notent également une atténuation des cardiomyopathies reliée à une augmentation de la tolérance des cellules cardiaques aux attaques ischémiques, chez des rats soumis à une restriction alimentaire de 30 à 40% sur une période de 10 mois. Dans un même ordre d'idée, Broderick et al. [58] rapportent qu'une restriction alimentaire administrée sur une période de huit mois (réduction de 45%), seule ou combinée avec de l'exercice, favorise le recouvrement des propriétés mécaniques de contraction du cœur. Cette restriction augmente la capacité du coeur à maintenir un débit sanguin constant suivant une ischémie et d'optimiser son fonctionnement. D'autres chercheurs observent que la restriction alimentaire administrée depuis la naissance chez des rats adultes (10 mois) et âgés (24 mois) favorise la préservation de la protection cellulaire et fonctionnelle octroyée par le préconditionnement ischémique cardiaque contre les effets d'un accident vasculaire cardiaque [3,276].

Au niveau cérébral, Lynch et collègues [281] ont étudié les effets de la restriction alimentaire sur la densité vasculaire et le flot sanguin cérébral chez une population vieillissante de rongeurs. Leurs résultats indiquent une résistance accrue de la microvasculature du cerveau (artérioles) et une amélioration de l'irrigation sanguine de l'hippocampe, associée à une diminution significative de la mort neuronale dans cette structure chez les animaux soumis à la restriction alimentaire. À ce jour, il n'existe qu'une étude ayant évalué les effets de la restriction alimentaire sur l'ischémie cérébrale. Les travaux de Yu et Mattson [488] montrent une réduction significative des dommages causés par l'ischémie focale chez des rats nourris par alternance journalière pendant une période de trois mois. La réduction du volume de la zone cortico-striatale affectée est associée à une diminution importante des déficits neurologiques chez ces animaux.

La restriction alimentaire semble donc influencer divers processus cellulaires susceptibles d'influencer les mécanismes physiologiques de survie et/ou de mort cellulaires. Les effets bénéfiques de cette procédure au niveau cellulaire se traduisent par un meilleur recouvrement fonctionnel et une atténuation significative des déficits moteurs et mnésiques secondaires aux traumatismes crâniens et maladies neurodégénératives et cardio- ou cérébrovasculaires.

4.2. Les acides gras polyinsaturés

Les acides gras polyinsaturés jouent un rôle nutritionnel important et exercent des effets bénéfiques multiples sur la santé humaine [266]. Ils se divisent en deux grandes catégories établies à partir de leur structure moléculaire: les gras oméga 3 (n-3) et oméga 6 (n-6) [180]. Ces acides gras ne sont pas synthétisés par le corps humain et doivent être

obtenus via une consommation d'aliments riches de ce type de gras. Au-delà de la quantité totale d'acides gras polyinsaturés consommée, c'est le ratio d'oméga 6 et 3 ((n-6)/(n-3)) qui importe, le ratio optimal étant inférieur ou égal à 4 [266,422]. Les acides gras de type oméga 6 se retrouvent dans une variété d'aliments comprenant des huiles de tournesol, de maïs, de sésame, de fève de soya et d'arachides et sont plus facilement intégrés à l'alimentation quotidienne, tandis que les gras de type oméga 3 se retrouvent principalement dans les huiles de poissons, de lin et de chanvre. Généralement, tout produit alimentaire se compose d'une quantité variable d'acides gras polyinsaturés incorporés dans la quantité lipidique totale. La nature et la quantité des produits alimentaires ingérés déterminent la consommation de ces acides gras.

Plusieurs études ont démontré les effets bénéfiques d'une consommation prévalente d'acides gras polyinsaturés par rapport à celle de gras saturés, notamment quant à l'impact de tels choix sur l'incidence des maladies cardiovasculaires, le diabète et le développement de maladies auto-immunes [44,64,107,108,111,143,206,312,337,338,476,481]. Ces études proposent une corrélation négative entre la quantité d'oméga 3 consommée et l'occurrence des maladies coronariennes, et suggèrent qu'une diète élevée en acides gras de type oméga 3 aide à prévenir le développement de maladies cardiovasculaires (voir Connor [93]).

Des recherches récentes ont cherché à élucider certains des mécanismes physiologiques via lesquels les gras de type oméga 3 induisent ces effets vasculaires protecteurs [4,93,152,180,182,251,258,266,327,422]. Les propriétés anti-arythmiques constituent une des actions associées à la diminution des maladies coronariennes [93,180,266,327,371]. Ainsi, différents mécanismes ou actions physiologiques impliqués dans l'arythmie (c.f., fonctionnement des canaux ioniques de la membrane cellulaire,

fibrillation ventriculaire, fréquence des battements cardiaques) sont influencés par la consommation d'oméga 3, un phénomène limitant les dommages de nature ischémique [266]. À cet effet, Mori et Beilin [327] suggèrent que l'ingestion de ce type de gras altère la composition phospholipidique des membranes cellulaires, ce qui affecte le transport cellulaire et/ou l'activité enzymatique des cellules. Ces changements altèrent conséquemment certaines fonctions cardiaques, prévenant le développement de problèmes arythmique cardiaque. Finalement, suite à l'ingestion de gras oméga 3, Pepe et McLennan [371] observent une résistance accrue des cellules cardiaques au stress de nature ischémique, réduisant l'accumulation intracellulaire de calcium impliquée dans les perturbations cellulaires et l'excitotoxicité.

Les acides gras de type oméga 3 possèdent également des propriétés antiathérogènes, qui favorisent la réduction de l'accumulation de plaques lipidiques (c.f., triglycérides) sur la paroi interne de grosses ou moyennes artères. Ces effets atténuent le risque de thromboses en réduisant l'agrégation plaquettaire donnant naissance au thrombus (le caillot occluant le vaisseau sanguin), en diminuant la viscosité du plasma sanguin et en régularisant les facteurs de coagulation. Finalement, la consommation d'acides gras de type oméga 3 favorise un meilleur fonctionnement vasculaire des cellules endothéliales, améliore les interactions avec les récepteurs membranaires, accentue les propriétés anti-inflammatoires de l'organisme et réduit la pression sanguine et les dommages induits aux plaques terminales des fibres nerveuses myélinisées des muscles striés [4,93,180,182,266,327,422].

Plus récemment, les bénéfices d'une consommation accrue d'acides gras polyinsaturés ont été démontrés au niveau cérébrovasculaire [206,338,369,481]. Ainsi, certaines études suggèrent qu'une consommation à court terme de supplément d'huile de

poisson atténue les dommages cérébraux subséquents à une occlusion de l'artère cérébrale moyenne chez le rat [86,139,398]. Bas et al. [28] ont également observé une réduction significative de l'oxydation cellulaire et de la mort cellulaire par apoptose dans la formation hippocampique de rats ischémiés suite à la consommation d'un supplément alimentaire d'huile de poisson pendant deux semaines. De façon similaire, les travaux de de Wilde et collègues [107] démontrent des effets significatifs de la consommation de ce type de supplément d'acides gras polyinsaturés sur le recouvrement de paramètres microvasculaires suite à une ischémie focale, associé à une préservation de l'intégrité de la barrière hémato-cérébrale.

À ce jour, peu d'études ont analysé l'impact d'une consommation accrue d'acides gras polyinsaturés auprès des mécanismes cellulaires impliqués lors d'une ischémie globale, produisant de la dégénérescence neuronale et des déficits d'apprentissage et de mémoire spatiale. Blondeau et al. [44] notent qu'un traitement d'acide linoléique administré pendant trois jours précédant une ischémie à quatre vaisseaux de 6 minutes augmente considérablement l'expression des protéines de choc thermique (heat shock protein; HSP70) et inhibe la mort neuronale secondaire à l'ischémie. Farkas et collègues [133] observent, quant à eux, une augmentation de la densité des récepteurs muscariniques M1 dans la région hippocampique et une préservation des habiletés d'apprentissage et processus mnésiques chez des animaux ischémiés ayant reçu un supplément en acides gras polyinsaturés pendant une période de 3 mois. Finalement, Okada et al. [353] montrent qu'une administration chronique d'acide docosahexaenoic (DHA 1, 10, 100 ou 200 mg/kg pendant 21 jours atténue également la mort des neurones hippocampiques et améliore significativement les déficits mnésiques spatiaux dans la tâche du labyrinthe radial à 8 bras suivant une ischémie globale.

Ces résultats suggèrent qu'un supplément d'acides gras polyinsaturés altère les processus cellulaires et déficits fonctionnels typiquement observés suite à une ischémie globale. La portée de ces résultats demeure toutefois limitée puisque ces études n'ont évalué que les effets associés à la consommation à court terme d'acides gras polyinsaturés ($\leq$ 6 semaines), ainsi qu'une récupération cellulaire, moléculaire et fonctionnelle immédiate suite à l'ischémie. Les effets à long terme d'un supplément d'acides gras polyinsaturés sur la récupération neuronale et fonctionnelle suite à une ischémie demeurent méconnus.

En résumé, les propriétés anti-arythmiques, antiathérogènes, anticoagulantes et la préservation cellulaire induite suite à la consommation d'acides gras polyinsaturés représentent des événements prometteurs dans la prévention et le traitement de maladies cardiovasculaires. Les études récentes suggèrent que les acides gras polyinsaturés exercent également des effets positifs sur les mécanismes cérébraux et la récupération fonctionnelle suite à un événement ischémique.

5. Thesis research objectives

The overall objective of the current thesis is to further characterize the contribution of two dietary regimens on hippocampal neurodegeneration and functional recovery following global cerebral ischemia. This current thesis is presented as a series of three articles, each of which addresses specific research objectives.

Manuscript 1: Food restriction attenuates ischemia-induced spatial learning and memory deficits despite extensive CA1 ischemic injury. Food restriction is amongst the most efficient and reproducible interventions for reducing the incidence of various age-associated and neurodegenerative diseases and increasing life span in a variety of species, including mammals. Short and long-term food restriction also significantly attenuates age-associated spatial memory impairments as well as functional deficits secondary to chronic neurodegenerative conditions or acute brain injuries. To our knowledge, only one study has assessed the impact of food restriction on cerebral ischemia. This first experiment determines 1) whether 40% food restriction for a period of three months prevents and/or attenuates CA1 damage following a 12 min global ischemia in rats, and 2) how short-term food restriction affects ischemia-induced behavioural and memory outcomes in the radial arm maze.

Manuscript 2: Food restriction induces long-lasting recovery of spatial memory deficits following global ischemia in delayed matching and non-matching-to-sample radial arm maze tasks. In this second study, our research work aimed to determine long-term impact of food restriction on histological and functional outcomes following global ischemia in rats. We selected delayed matching or non-matching to sample (DMTS/

DNMTS) tasks, which provide more complex behavioural paradigms to assess memory and cognitive flexibility in the radial arm maze. This second experiment is the first to characterize 1) the effects of food restriction on hippocampal damage 70 days following ischemia, 2) the cognitive abilities of ad libitum fed (AL) and food-restricted (FR) animals using complex delayed non-matching- and matching-to-sample tasks in the radial arm maze and 3) expression of vesicular transporters for GABA and glutamate in 11 subfields of the hippocampal formation 70 days post ischemia.

Manuscript 3: Dietary PUFA supplements reduce memory deficits but not CA1 ischemic injury in rats. Numerous studies have suggested that polyunsaturated fatty acids (PUFA) exert beneficial effects on various pathological states including cardiovascular and autoimmune diseases. To date, investigation of the contribution of PUFA supplemented diets on neuronal damage and memory deficits following global ischemia remains limited. The purpose of the present study was to examine the impact of nutritional supplementation with essential omega 3 and 6 fatty acids for twelve weeks prior to ischemia and in the 6 post ischemic weeks on CA1 neuronal death and on locomotor behaviour in the open field, anxiety in the elevated plus-maze and spatial memory in the radial maze following global ischemia.

Manuscript 1

Food restriction attenuates ischemia-induced spatial learning and memory deficits despite extensive CA1 ischemic injury.

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Author Contribution

Marie-Claude Roberge: The first author conducted all experiments on animals that were euthanized at 31 days post-ischemia, under the supervision of Dr. Hélène Plamondon.

Judith Hotte-Bernard: The second author conducted experiments on animals that were euthanized at 7 days post-ischemia, under the co-supervision of Marie-Claude Roberge and Dr. Hélène Plamondon.

Claude Messier: This author provided help with the writing and editing of the manuscript.

Hélène Plamondon: This author supervised the research work of this paper, edited the manuscript, and provided financial support.

Abstract

The purpose of the present study was to examine whether short-term food restriction (40% less food over a three month period) can attenuate ischemia-induced CA1 neuronal degeneration, and whether this attenuation translated into improved recovery of functional impairments following global ischemia. There was a significant loss of pyramidal CA1 neurons in ischemic compared to sham-operated rats but no difference between the ad lib and food-restricted ischemic animals. Although the diet did not influence neuronal damage in ischemic animals, the performance of food-restricted ischemic rats in spatial task such as the radial arm maze was significantly better than that of ad lib fed ischemic rats. Food-restricted ischemic rats made equivalent numbers of working memory errors as sham-operated animals and took the same time to complete a standard 8-arm radial arm maze task. They also displayed higher activity level in the open field compared to ad libitum fed ischemic rats, and spent considerably more time in the open arms of the elevated plus maze compared to the other groups, suggesting decreased anxiety in these ischemic rats. The relative sparing of spatial memory performance in food-restricted ischemic animals suggest that food restriction facilitate functional recovery.

Introduction

Numerous studies have reported increased life expectancy, improvement of cognitive functions and reduction of cardiovascular and neurodegenerative diseases risk factors associated with food restriction (FR; reduction in energy intake with maintenance of nutrition) [117,123,276,281,302,306,403,426,493]. Broderick et al. [57,58] showed enhanced contractile properties of the heart and better blood flow regulation and resistance to cerebral ischemia in rats maintained on an 8-month food restriction diet (45% reduction) compared to ad libitum fed animals. Similarly, 30 to 40% FR over a 10-month period significantly reduced myocardial infarction in rodents [99]. Chandrasekar et al. [80] reported decreased inflammation and oxidative damage to heart cells following occlusion of the left anterior descending coronary artery in FR rats. Studies also indicate preservation of cardiac ischemic preconditioning cellular and functional protection in middle aged (10 months) and aged (24 months) rats maintained on life-long food restriction diets [3,276].

Recently, various studies have demonstrated the impact of food restriction on acute brain injuries and chronic neurodegenerative conditions [178,290,304]. Bruce-Keller et al. [59] observed that 2 to 4 months of alternate day fasting was sufficient to enhance the resistance of hippocampal and striatal neurons to kainate-induced excitotoxicity and prevent toxin-induced mitochondrial damage in the dorsal hippocampus. Cellular recovery in food-restricted rats translated into improved motor abilities and memory performance in the water maze. Consistent with such findings, 3-month alternate day fasting significantly reduced 1-methyl-4phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced damage of dopaminergic neurons and motor

impairments [117]. Similar results were obtained with MPTP-treated rhesus monkeys suggesting that these observations could extend to humans [298].

To our knowledge, only one study has assessed the impact of food restriction on cerebral ischemia. In that study, Yu and Mattson [488] observed a significant reduction of focal ischemic damage in animals exposed to a 3-month alternate-day fasting schedule. The reduction of cortico-striatal infarct volume was associated with significant inhibition of behavioural deficits in food-restricted rats. The present study examines whether 40% food restriction for a period of three months prevents CA1 damage following a 12 min global ischemia in rats, and determines how short-term food restriction affect ischemia-induced behavioural and memory outcomes.

Materials and Methods

Animals and dietary protocols

One hundred and eight male Wistar rats, weighing between 100-125 g at the time of arrival were obtained from Charles River Laboratories (Rochefort, Quebec, Canada). The animals were individually housed and maintained on a 12 h light/dark cycle (lights on at 7:00 AM), with free access to water and standard (Purina) rat chow. The room temperature was maintained at 21-23 °C with 60% relative humidity. One week following their arrival, animals were separated into two dietary groups. Rats in the ad lib group (AL; n = 55) continued to have free access to a standard (Purina) rat chow, while rats in the restricted group (FR; n = 53) received 60% of the average amount of Purina rat chow consumed by the ad lib group. A pilot study indicated that adult rats consumed on average 30-32 grams of standard Purina rat chow per day. Based on these observations,

ad libitum fed rats received a daily ration of 30-32 grams standard Purina rat chow while food-restricted rats received 60% of that amount (i.e., 18-19.2 grams of standard Purina rat chow per day). This means that food-restricted animals received the same nutrients in the same relative proportions but, overall, received less of all nutrients. Although it is possible that the restricted diet may have led to specific nutrient deficiencies, the often replicated observation of increased lifespan and general better health of food-restricted animals suggest that there is little deleterious impact of food restriction. Food intake was measured daily between 8:00-9:00AM. All animals had free access to water throughout the experiment. Rats were handled daily and weighted every two to three days.

The diets were provided for a 3 month period prior to sham or ischemic surgeries (See figure 2 for the experimental protocol). Rats of the two diet groups were randomly assigned to one of the following experimental conditions. Four groups of ad libitum (AL) fed animals and four groups of 40% food-restricted (FR) rats received either a sham surgery (SHAM) or a 12 min global ischemia (ISCH), and were euthanized 7 or 30 days following reperfusion (SHAM-AL-7: n = 9; SHAM-AL-30: n = 10; ISCH-AL-7: n = 11; ISCH-AL-30: n = 10; SHAM-FR-7: n = 9; SHAM-FR-30: n = 10; ISCH-FR-7: n = 13; ISCH-FR-30: n = 11). Of the initial 108 rats, twenty-four ischemic animals died during or after surgery (4 ISCH-AL-7, 2 ISCH-FR-7, 11 ISCH-AL-30 and 7 ISCH-FR-30). One ISCH-FR-30 rat displayed atypical behaviour and was excluded from all analyses. All experiments conformed to the NIH guide for the Care and Use of laboratory animals (NIH publications N°80-23, revised 1996) and procedures were in accordance with the guidelines set by the Canadian Council of Animal Care and approved by the University of Ottawa Animal Care Committee. Efforts were made to minimize the number of

animals used and their suffering.

Surgical Procedure

Forebrain ischemia was performed using the four-vessel occlusion model as previously described by Pulsinelli and Brierley [391]. Briefly, rats were deeply anesthetized by inhalation of 2 to 4% halothane (weight dependent) vaporized by oxygen (1.5 to 2 L/min). Vertebral arteries were irreversibly occluded by electrocoagulation, and a small-diameter silk thread looped around the carotid arteries to facilitate subsequent occlusion. Twenty-four hours later, the common carotid arteries were clamped with microaneurysm clamps for 12 minutes in awake and spontaneously ventilating animals. Sham-operated animals underwent anesthesia and received the same dorsal and ventral surgical incisions as the two ischemic groups, with the exception of electrocoagulation of the vertebral arteries. Twenty-four hours later, the carotid arteries were exposed, but not clamped. Body temperature was kept at 37°C ± 0.5 throughout the surgery using a feedback-regulated heating blanket connected to a rectal thermometer (Homeothermic Blanket Control Unit, Harvard Instruments, Natick, MA). Subjects' body temperature was further supported with a heating pad in the hours following surgery and reperfusion.

Behavioural tests

Open field

The arena was made of gray Plexiglas (LWH: 75 X 75 X 30 cm) with a clear Plexiglas floor. A painted grid divided the Plexiglas floor into 36 identical squares each measuring 15 X 15 cm. The arena was located on a table 90 cm above the floor, and was

dimly illuminated. Animals were brought to the test room at least 30 minutes prior to test administration. Black curtains surrounded the arena and behaviour was monitored using an overhead video camera. Four days following reperfusion, sham and ischemic rats were placed in the open field and behaviour were monitored for 15 min by the experimenter using a PC computer and data logging software. Animals were re-tested at 30 days post-ischemia to assess long-term activity levels. Rats were monitored for frequency of line-crossing and rearing behaviour, and time (sec) spent grooming or being inactive. Frequency of behaviour was assessed in the peripheral zone (defined as the 20 squares directly adjacent to the walls) and central zone (all remaining squares).

Elevated plus maze

The elevated plus maze (EPM) consisted of two opposing open arms (50 X 10 cm with a 5 mm clear Plexiglas lip), an open 10 X 10 cm area in the center and two opposing closed arms (50 X 10 cm with 40 cm high walls). The maze was 60 cm above the floor. Animals were tested 5 days following reperfusion and brought to the test room 30 min prior to test administration. Black curtains surrounded the maze and behaviour was monitored using an overhead video camera. Each rat was placed in the open field (75 X 75 cm with 30 cm high walls) for a 5-min habituation period. Immediately thereafter, the rat was placed in the center of the elevated plus maze facing one of the open arms of the maze. The time spent and the number of entries in the open or closed arms, risk assessment and crossing behaviour were recorded during a 5 min test interval. A close or open-arm entry was defined as placing all four paws in an arm. Risk assessment was operationally defined as a stretch-attend response, whereas the rat stretched its body

forward and either sniffed or visually scanned the open arm. In stretch-attend behaviour, head entry into the open arm was required while paws and body remained in the center area or closed arm. Crossing behaviour was recorded when the animal crossed the center zone going from one arm to its opposite arm.

Radial arm maze

Twenty-four hours after the completion of the elevated plus maze, the animals began training in the radial arm maze. The maze consisted of eight arms (60 X 12 cm with a 5 cm lip around each arm) extending radially from a central octagonal area (32 cm in diameter with a 30 cm high clear Plexiglas wall). Plexiglas sliding doors were moved up to allow entry into each arm. The floor of the arms and central area was covered with black rubber lining. The apparatus was elevated 50 cm above the floor and surrounded by extra-maze cues placed on the walls, such as posters or calendars. The experimenter was sitting behind a panel where he could observe and record behaviour unobtrusively as well as manipulate the overhead strings to open and close the maze doors. At the end of each arm, a food cup (1 cm deep into the floor) contained a piece of Fruit Loop. Animals were brought to the room 30 minutes prior to the testing. The weights of all animals fed ad libitum were gradually reduced (over a 4-day period) and maintained at approximately 85% of their free-feeding rate during the testing period to ensure adequate motivation. The rats fed a reduced diet were not further deprived.

During training, rats were habituated to the radial arm maze during four 10-min daily sessions on successive days. The baits (small pieces of Fruit Loop cereal) were initially available throughout the maze to encourage exploration, and gradually moved

towards the end of each arm to finally be restricted to the food cups. Following this shaping period, each animal was individually placed in the center of the maze, with doors to all arms closed. Upon opening the doors, the rat was allowed to enter any of the eight arms. When the rat had consumed the reward at the end of one arm and returned to the center of the maze, all doors were closed again to confine the rat to the center zone for a 10 s delay. Doors were then reopened, and the procedure repeated. The test continued until all rewards were consumed or 15 min had elapsed. An arm entry was recorded when all four paws of the rat were within an arm. The orientation of the animal's head when initially placed in the central area varied from trial to trial to minimize development of a response pattern based on position. The floor and center walls of the maze were cleaned after each trial to reduce olfactory cues. The number of working memory errors (re-entering an arm that was previously visited) and the time taken to consume all rewards were recorded. Animals were tested once daily for a 16-day period.

Analysis of neuronal density on thionin-stained sections

Twenty-four hours upon completion of the behavioural testing, rats were deeply anesthetized using sodium pentobarbital and perfused using 4% paraformaldehyde. Brains were removed and stored at -84°C. Serial coronal sections (14 μm) of the hippocampal region were obtained using a cryostat (Leica Instruments) and stained with thionin. Neuronal density of the hippocampal CA1 subfield was determined by the method of Kirino et al. [239]. Analysis of neuronal density was performed on coronal sections located between 3.14 and 4.16 mm posterior to bregma [370]. The total linear length of the CA1 (as defined by Paxinos and Watson [370]) was measured using a

digitizer. The number of intact neurons in the stratum pyramidale of the CA1 subfield was counted using LEICA DAS microscope attached to a SONY digital camera and computer-assisted cell counting performed using the Norton Eclipse software (v 6.0). Neurons with shrunken cell bodies and surrounding empty spaces were excluded. The neuronal density of the CA1 sector, i.e. the number of intact pyramidal cells per mm linear length of the CA1 stratum pyramidale observed in each 14 μm section, was quantified. A mean value for each hippocampal CA1 substructure was obtained from 6 bilateral measurements in each animal. Neuronal density for a given animal represents the average of the right and left measures.

Statistical Analyses

Body weight, open field, and radial arm maze data were analyzed using mixed ANOVA designs with two levels of the independent factors *surgery* and *diet,* and repeated time factors. The Hundt-Felt correction for violations to the assumption of sphericity was applied when appropriate: when used, adjusted degrees of freedom are reported. Significant interactions were further tested using simple main effect tests with a Bonferroni adjustment of the critical alpha level. Data obtained from the elevated plus maze and neuronal density were analyzed using two factors (*surgery and diet*) ANOVAs. Again, simple main effect tests were used to analyze significant interactions. All data were analyzed using the SPSS (V.14) software package. Results are presented as means ± S.E.M.

Results

Body weight

Figure 3 presents the mean body weight of ad libitum fed and food-restricted animals throughout the experiment. Main effects of time ($F(2.44,83.01) = 1914.42$, $p < .001$), and diet ($F(1,34) = 185.36$, $p < .001$), and an interaction of time x diet ($F(2.44,83.01) = 74.91$, $p < .001$) were found. In general, body weight increased over time. The body weight of restricted rats increased at a lower rate than that of ad libitum fed animals. In our study, all animals consumed the entire ration of food provided daily and food intake measured daily. Casual observations indicated that food-restricted rats appeared to more readily eat once awoke following vessel occlusion, and were generally more active than ad libitum fed rats. Compared to ad lib animals, food-restricted animals gained more weight following surgery most likely because food restriction was postponed for five days after surgery. During these five days, all animals had unlimited access to food and water, and were given palatable food (Cheerios, Fruit Loop, and graham powder) to hasten their recovery to pre-surgical weights. The observations that food-restricted animals appeared more active, reestablished pre-surgical body weight more rapidly than ad lib fed animals, and their preserved performance in the standard radial arm maze suggest that these animals were healthy and that they received sufficient nutrients for the required daily needs.

Open field

Figures 4a and 4b present the combined frequencies of line crossing and rearing (general activity) in the open field every 5-min over the 15-min monitoring period

performed 4 days following reperfusion. Main effects of time ($F(1.9,138.42) = 107.13$, $p < .001$), and diet ($F(1,73) = 4.95$, $p = .029$), and an interaction of surgery x diet ($F(1,73) = 4.14$, $p = .046$) were observed. General activity decreased over time for all groups. Food-restricted ischemic rats were more active than ad libitum fed ischemic rats across the testing period ($p = 0.003$). Additionally, food-restricted animals displayed a higher level of activity throughout the test and made significantly more behaviour than ad libitum fed animals in the central area of the open field (see figure 4a; $F(1,73) = 5.88$, $p = .018$). No other significant difference was found.

Figures 5a and 5b present the general activity in the open field every 5-min over the 15-min period for the subset of animals tested 4 (T1) and 30 (T2) days following reperfusion. There were main effects of time ($F(2,72) = 124,34$, $p < .001$), days after reperfusion ($F(1,36) = 8,71$, $p = .006$), diet ($F(1,36) = 8,54$, $p = .006$), and an interaction of surgery x diet ($F(1,36) = 4,43$, $p = .042$). All rat groups showed decreased activity as time elapsed within an open field session. However, activity level was higher in all groups when tested 30 days following surgery compared to the initial testing 4 days post surgery. Simple effect tests revealed that food-restricted ischemic rats were significantly more active than all other groups across the testing period on day 4 ($p = 0.01$). But on day 30, the only difference that remained significant was that food-restricted ischemic rats were significantly more active than ad libitum ischemic rats ($p = 0.01$). On day 4, ad libitum sham-operated rats displayed higher activity level than ischemic rats ($p = 0.01$) fed the same diet, a phenomenon no longer present at day 30.

Figures 5c and 5d present the general activity in the central zone of the open field every 5-min over the 15-min period for the subset of animals tested 4 (T1) and 30 (T2)

days following reperfusion. Ischemic rats fed an ad libitum diet were significantly less active than food-restricted ischemic and sham-operated rats in the central area of the open field on day 4 ($p = 0.016$). Both ischemic groups (ISCH-AL and ISCH-FR) showed increased activity in the central area in the initial two 5-min blocks on day 30 ($p = 0.04$). Five rats (1 ISCH-AL-7, 1 ISCH-FR-7, 1 ISCH-FR-30, 1 SHAM-AL-7 and 1 SHAM-FR-7) were excluded from these analyses as they remained immobile during most of the testing period. No motor dysfunction appears to account for this, because these animals completed all the other behavioural tests. One additional rat (ISCH-AL-7) was excluded from statistical analysis as it showed significant increase in global activity at both time intervals, a profile that was discordant with the general decreased activity levels.

Elevated plus maze

Figure 6 presents the performance of all animals in the EPM. There was a main effect of diet ($F(1,78) = 5.12$, $p = .026$) for the time spent in the open arms of the EPM. Simple effect tests showed that food-restricted ischemic rats spent more time than ad lib ischemic rats in the open arms of the maze ($p = 0.003$). We also found a main effect of surgery for risk assessment behaviour ($F(1,77) = 4,37$, $p = 0.04$). Both ischemic groups made fewer risk assessments behaviour than sham groups ($p = 0.04$). No other significant difference was found. One animal (ISCH-AL-30) was excluded from this analysis because it fell on the floor during the task, and remained immobile throughout the remainder of the test.

Radial arm maze

Figure 7 shows the performance of all animals in the radial arm maze and the impact of diet on working memory errors. There were main effects of time ($F(5.06,172.02) = 35.09$, $p < .001$), surgery ($F(1,34) = 5.63$, $p = .023$), diet ($F(1,34) = 7.19$, $p = .011$), and a significant surgery x diet interaction ($F(1,34) = 5.33$, $p = .027$). All animals made significantly less working memory errors with time. Simple effect tests showed that ischemic rats fed an ad libitum diet made considerably more working memory errors than food-restricted ischemic and sham-operated rats across time. The food-restricted ischemic rats' performance was equivalent to that of sham-operated animals.

Figure 8 presents the time required by each group to complete the task. There were main effects of time ($F(3.06,104.07) = 39.64$, $p < .001$), surgery ($F(1,34) = 8.55$, $p = .006$), diet ($F(1,34) = 21.81$, $p < .001$) and interactions of time x diet ($F(3.06,104.07) = 2.93$, $p = .036$), and surgery x diet ($F(1,34) = 6.81$, $p = .013$). As testing progressed, all groups took significantly less time to complete the task. Food-restricted animals took generally less time to complete the task than ad lib fed animals. Simple effect tests revealed that ad lib fed ischemic rats took more time to complete the task than all other animals. Food-restricted ischemic rats were as quick as sham-operated rats to complete this task. One animal (ISCH-FR-30) was eliminated from analyses because he remained immobile during most trials, suggesting increased anxiogenic behaviour.

Histopathological changes at the hippocampus

Table 2 shows the effects of global ischemia and food restriction on hippocampal CA1 neuronal density 7 and 31 days following reperfusion. There was a main effect of surgery ($F(1,74) = 185.04$, $p < .001$). Sham-operated animals had significantly more neurons within the CA1 subfield of the hippocampus relative to ischemic rats. We found no significant effect of food restriction on neuronal density.

Covariances

CA1 neuronal density in both ischemic groups was somewhat variable. In order to determine the possible impact of this factor on functional recovery, we re-analyzed the behavioural data using neuronal density as a covariate every time a significant correlation between a particular behavioural measure and the neuronal density was observed. There was no significant correlation of neuronal counts and open field and elevated plus maze data (open field: behavioural activity (global/centre): r = -0.018/-0.09; EPM: open entries: r = 0.06, time spent in the open arms: r = 0.064, risk assessment behaviour: r = 0.14; and crossing behaviour: r = 0.06). However, working memory errors in the radial arm maze were correlated with CA1 neuronal density (working memory errors: r = -0.49*, p = 002). There was no significant correlation between the neuronal density and the time to complete the task (r = -0.26). Mixed ANCOVA analysis for working memory errors indicated a main effects of time ($F(5.29,174.45) = 4.72$, $p < .001$) and diet ($F(1,33) = 7.51$, $p = .01$) as well as a surgery x diet interaction ($F(1,33) = 4.42$, $p = .043$).

Neuronal density for a given animal represents the average of the six right and left measures. Variability within CA1 included animals with asymmetry between the left and

right hemisphere (i.e., either left or right side had more CA1 intact neurons). In order to determine if a higher unilateral number of cells had an impact on the spatial learning and memory deficits, we performed a second correlation between the number of working memory errors and the neuronal density (only using the cellular count of the side with the highest CA1 intact neurons). There was only a similar significant negative correlation between these two variables (working memory errors: $r = -0.46^*$, $p = 004$. Therefore, CA1 mean neuronal counts (or the highest of bilateral counts) did not account for the behaviour observed in the radial arm maze. Thus, using neuronal counts as a correlate did not change the conclusions drawn from the analysis of the working memory errors, namely, that all animals made significantly less working memory errors with time, food-restricted ischemic rats made less working memory errors than ad lib ischemic rats across time, and had a better performance in the radial arm maze irrespective of CA1 neuronal counts.

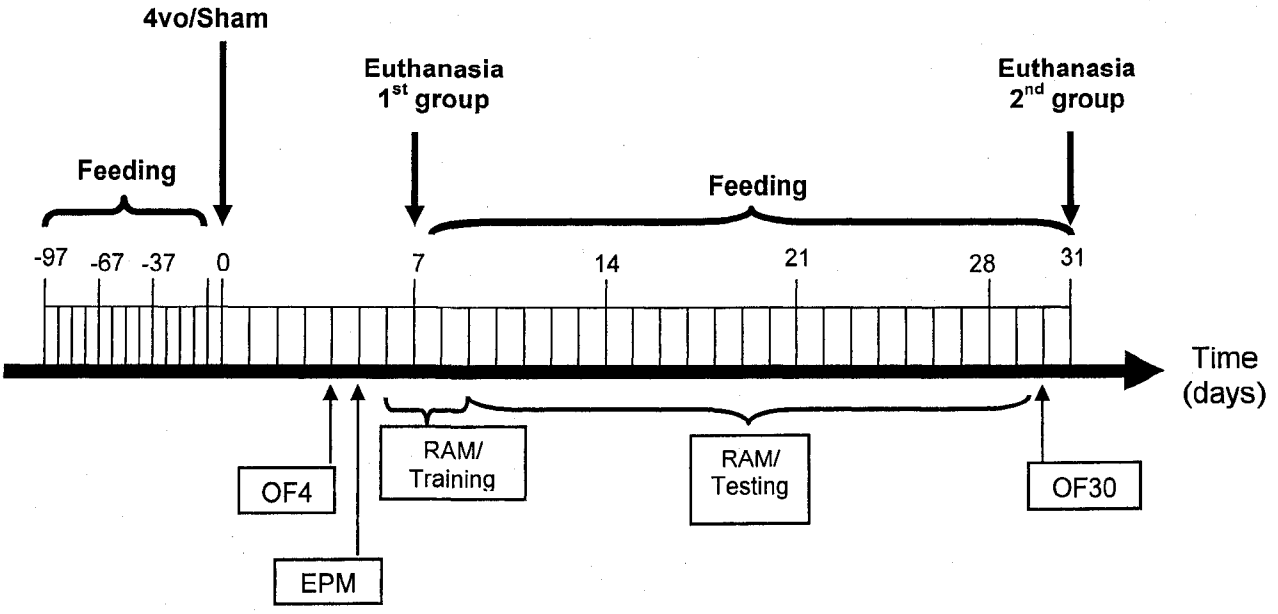

Figure 2: Experimental protocol. Feeding = Presurgical period of ad libitum or food restriction; 4-VO = 4 vessel occlusion; OF4 = Open field 4 days post-ischemia; EPM = Elevated plus maze 5 days post-ischemia; RAM/Training = Training on the radial arm maze from days 6 to 9 post-ischemia; RAM/Testing = Testing on the radial arm maze from days 10 to 29; OF30 = Open field 30 days post-ischemia. Day 0 refers to the day of surgery. 5 days following surgery, rats returned to their respective pre-surgical feeding period (without treats). From days 6 to 9, the weight of animals fed ad libitum that were tested in the radial arm maze were gradually reduced and maintained at approximately 85% of their free-feeding rate during the testing period to ensure adequate motivation while food-restricted animals were not further deprived.

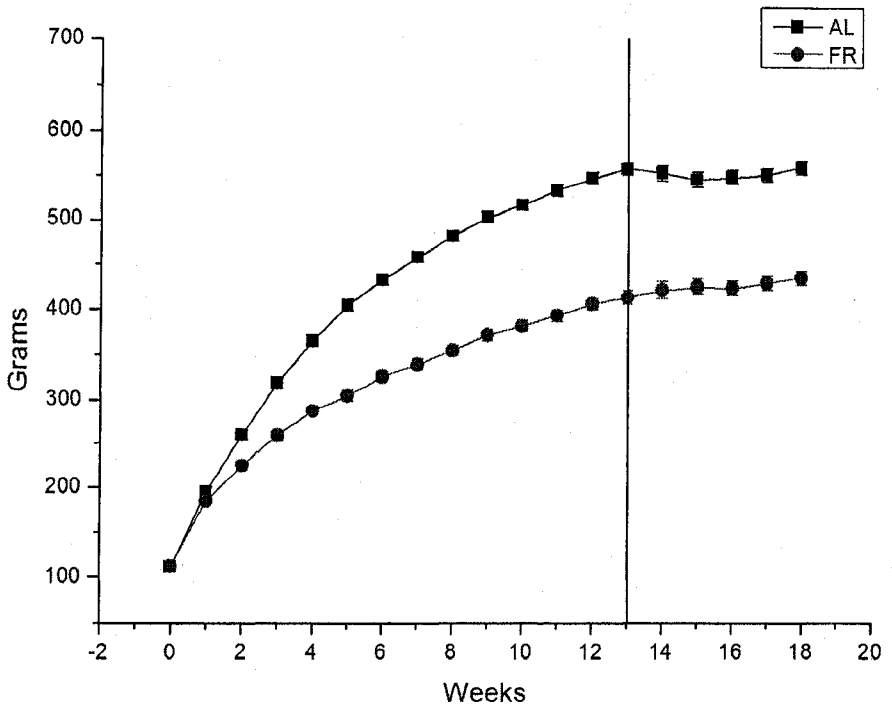

Figure 3: Mean body weight of ad libitum fed and food-restricted rats over an 18-week period. Sham or global ischemia surgery is indicated by the vertical line. Rats fed a restricted diet weighted significantly less than ad lib rats throughout the experiment.

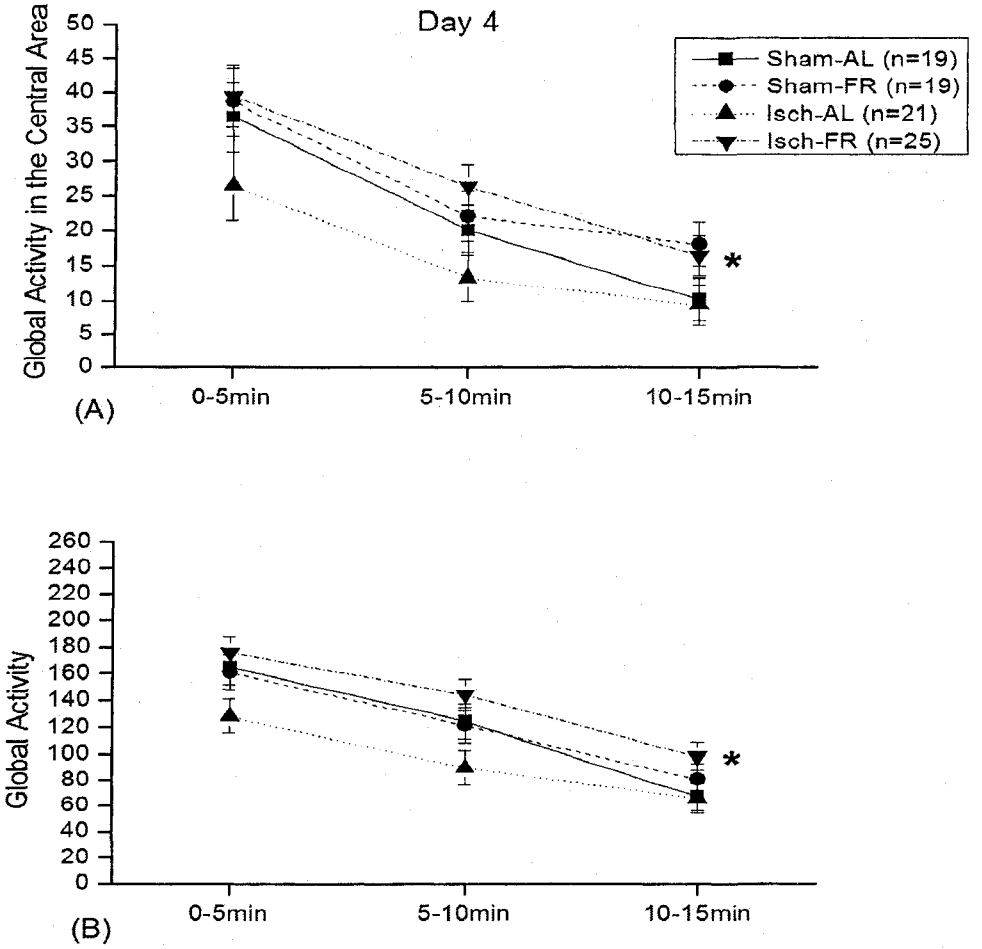

Figure 4: Effect of ischemia and food restriction on locomotion (number of squares crossed) and rearing behaviour in the open field 4 days following reperfusion. **4a**: presents activity levels in the central zone of the open field. (*) indicates significantly higher activity levels food-restricted compared to ad libitum fed rats, for both ischemic and sham-operated animals ($p = .018$). **4b**: presents global activity levels monitored in the entire open field arena. (*) indicates significantly higher activity levels in food-restricted compared to ad lib fed ischemic rats ($p = .003$).

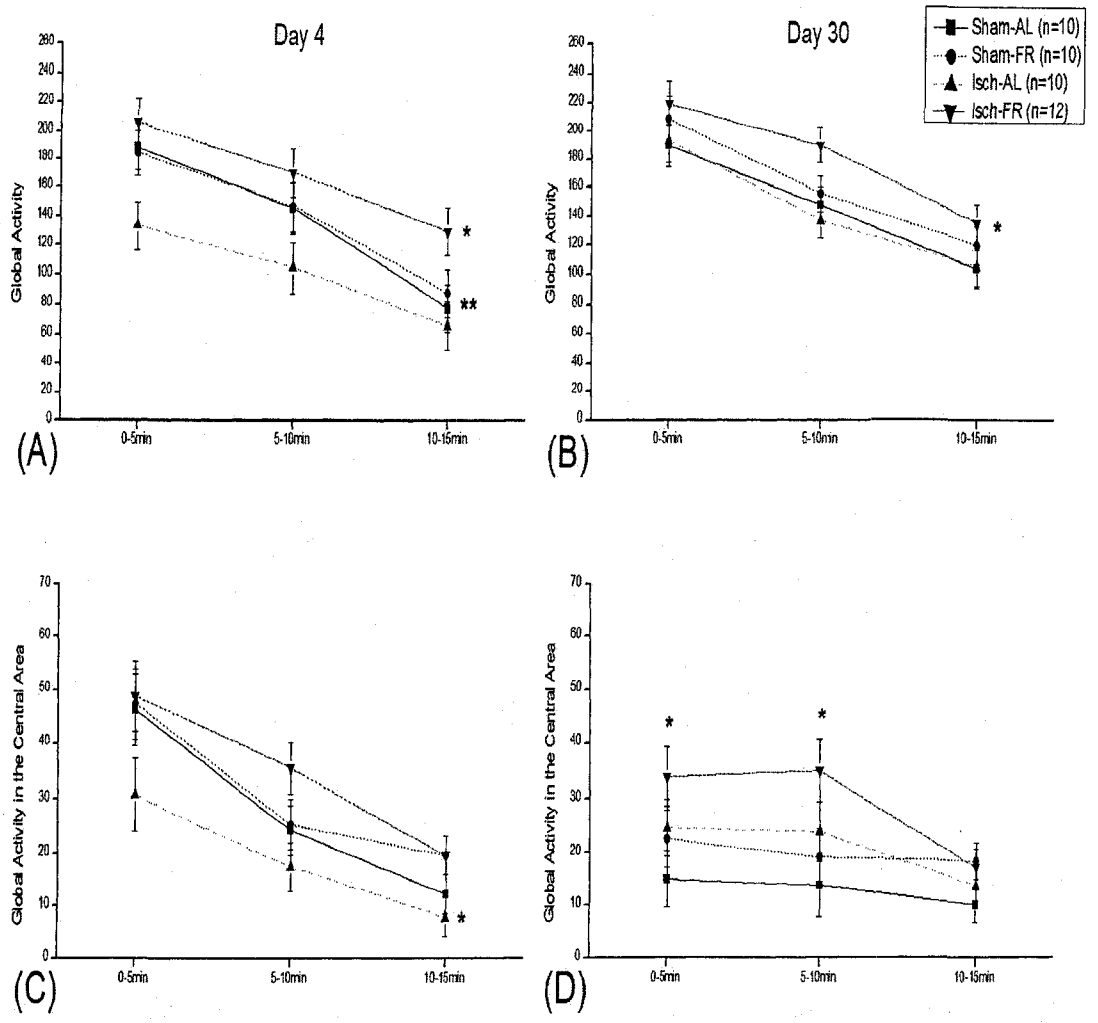

Figure 5: Open field activity (number of crossed squares and rearing frequencies) in animals tested 4 and 30 following reperfusion. The overall activity level of all experimental groups was higher at day 30 than at day 4. **5a, 5b**: Global activity levels at day 4 and day 30, respectively. Day 4 - (*) indicates higher activity levels across all time periods in food-restricted ischemic rats compared to all other groups ($p = .01$). (**) indicates elevated activity levels in ad libitum fed sham as compared to ischemic rats ($p = .01$). Day 30 - (*) indicates higher activity levels across testing in food-restricted compared to ad libitum ischemic rats ($p = .01$). **5c, 5d**: Global activity levels in the center zone open field at day 4 and day 30, respectively. Day 4 - (*) ad libitum ischemic rats showed less center field activity compared to food-restricted animals ($p = .016$). Day 30 - (*) indicates that both ischemic groups executed an increased numbers of behaviour in the central area in the initial two 5-min blocks on day 30 ($p = .04$).

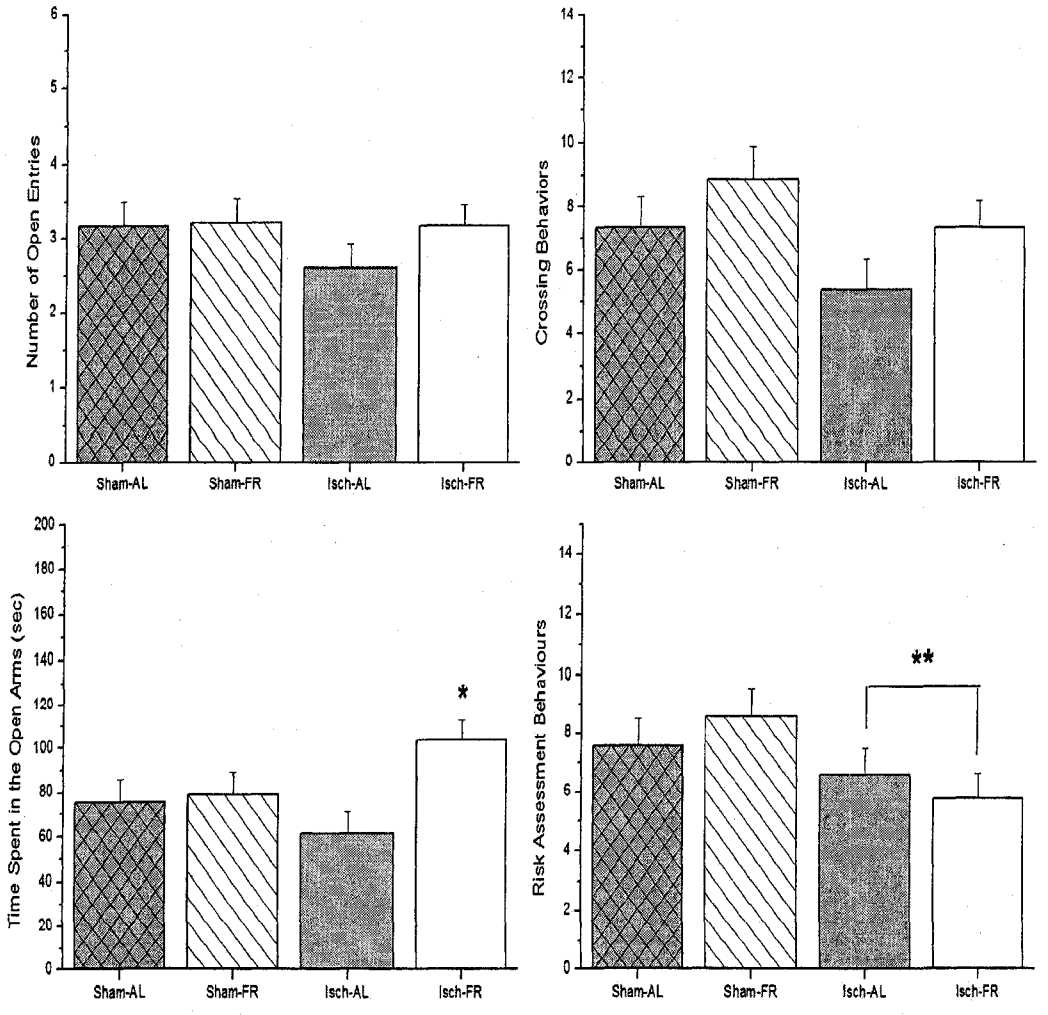

Figure 6: Frequencies of behaviour and time spent in the open arms of the elevated plus maze for ad libitum and food-restricted sham and ischemic rats. (*) Ischemic rats fed a food-restricted diet spent significantly more time in the open arms of the maze as compared to ischemic rats fed a standard diet ($p = .003$). (**) indicates that both ischemic groups made fewer risk assessment behaviour than sham-operated rats ($p = .004$).

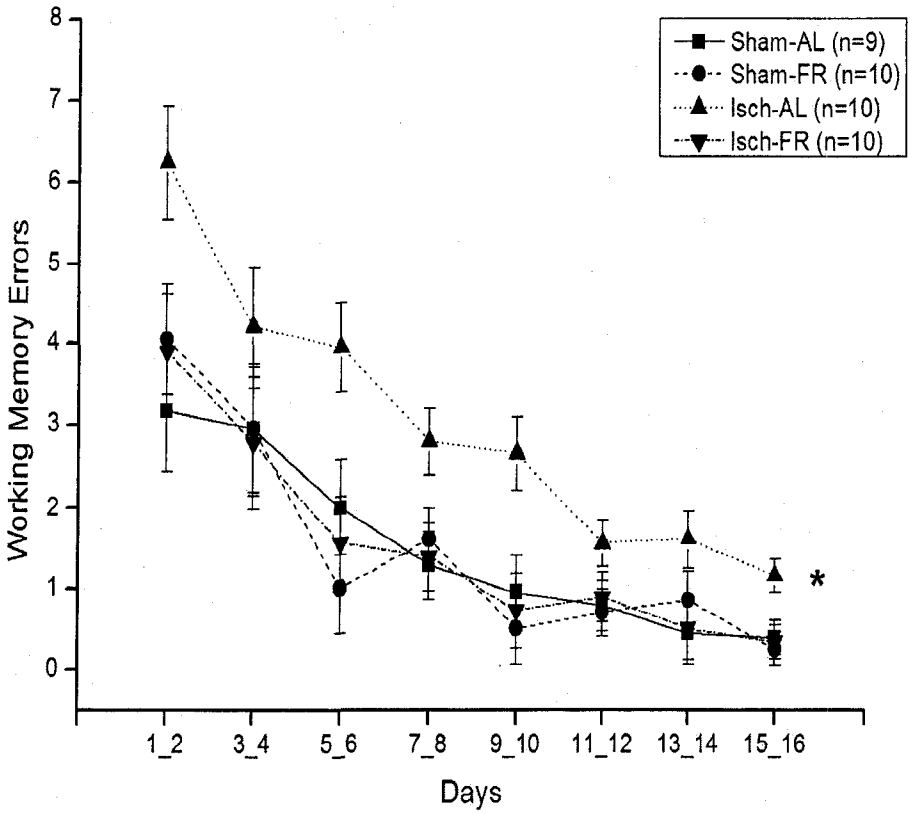

Figure 7: Effect of ischemia and food restriction on spatial memory performance in the radial arm maze. (*) shows a significant increase in number of working memory errors in ad libitum fed ischemic rats compared to food-restricted ischemic, and sham-operated rats over the entire testing period ($p = .017$). Food-restricted ischemic rats showed comparable performance as sham-operated animals.

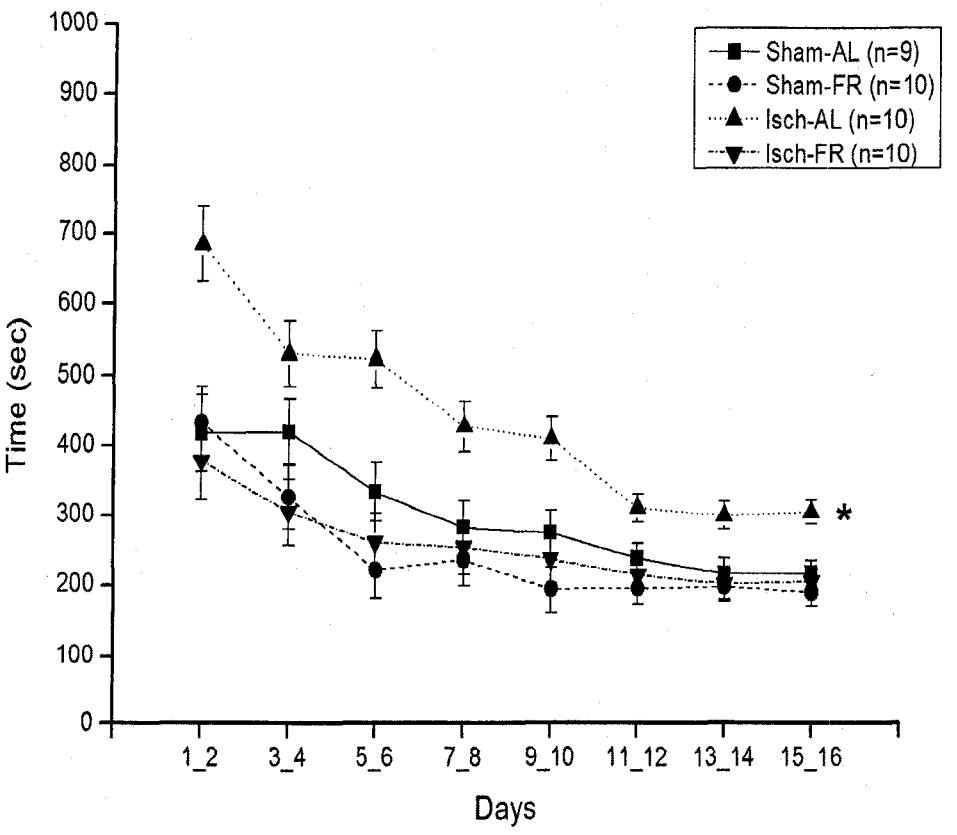

Figure 8: Effect of ischemia and food restriction on time required to perform the radial arm maze task. (*) indicates significantly increased time required by ad libitum ischemic rats to perform the radial arm maze task compared to food-restricted ischemic and sham-operated rats ($p = .013$).

Table 2: Density of hippocampal CA1 neurons (cells/1mm, CA1 tissue) in AL and FR sham and ischemic animals, 7 and 30 days following reperfusion.

	SHAM-AL	SHAM-FR	ISCH-AL	ISCH-FR
At 7 days	267.7±17.01	266.93±16.14	104.21±15.39*	118.86±15.39*
At 30 days	269.09±16.14	264.51±18.05	110.65±16.14*	115.518±14.16*

(*) indicates that neuronal density of both ischemic groups was significantly reduced as compared to sham-operated rats ($p < .001$).

Discussion

To our knowledge, the current study is the first to examine the effects of food restriction on ischemia-induced behavioural deficits and CA1 neuronal degeneration, using a variety of behavioural paradigms. Among the most interesting findings is the sparing of spatial memory in the radial maze and the speed at which ischemic rats maintained on a 40% food-restricted diet performed the task compared to ad libitum fed ischemic animals, despite comparable CA1 neuronal damage in both groups. This suggests the possibility of adaptive changes and/or compensatory mechanisms initiated prior to or subsequent to ischemic lesion, enabling food-restricted rats to functionally recover from ischemia in this spatial task. In addition to effects on memory, our findings demonstrated different behavioural patterns of food-restricted and ad libitum fed ischemic rats in the open field and elevated plus maze (EPM). These results raise a number of questions that need to be addressed.

The first question is why food-restricted ischemic rats show comparable behavioural performance as sham-operated controls even though hippocampal CA1 neurons were equally injured in both ischemic groups? A number of hypotheses could explain these results. The first one is that pyramidal CA1 neurons are not necessary for the expression of the behaviour measured in our experiment. The obvious corollary is that other brain regions were protected from ischemic damage by food restriction and the sparing of these regions forms the basis for the behavioural sparing of the food-restricted rats. This hypothesis has indirectly been tested by previous studies described below that examined the association between behaviour and CA1 neuronal density.

Relationship between ischemia-induced CA1 neuronal damage and behaviour has

been examined for ischemia-induced hyperactivity and is typically observed within 24-72 h following reperfusion when rodents are exposed to a novel environment [18,78]. Some studies have associated hyperactivity with the amount of CA1 neuronal loss and suggested that increased activity among ischemic animals results from memory impairments, which delay familiarization to the novel environment in animals with hippocampal damage [225]. Other studies have reported an absence of correlation between the extent of CA1 neuronal protection and increased locomotor activity [96,375], suggesting that the relationship between hyperactivity and CA1 damage is not always observed. Thus, even within the ischemia literature, there is some variability in the relationship between behavioural deficits and hippocampal damage. The alternative explanation that the restricted diet reduced ischemia-induced behavioural changes is also suggested by the demonstration that enriched housing environments eliminated ischemia-induced hyperactivity, while having little impact on hippocampal cell death [393]. In the present experiment, food-restricted ischemic animals showed increased open field activity as compared to ad lib ischemic rats 4 days following ischemia, despite comparable CA1 neuronal damage. When re-tested at day 30, all groups had enhanced open field exploration suggesting that all groups reacted in a similar fashion to novelty.

Open field activity is widely used in the assessment of exploratory anxiety. Increased time spent exploring an open arena and/or its center zone is associated with decreased anxiety [380,388,421]. It is common to combine measures of open field behaviour with open-arm activity in the elevated plus maze (EPM) in the assessment, as both assess the willingness of rodents to explore anxiogenic environments [32,100,361]. Contrary to previous observations [342,380] ad libitum ischemic animals did not show

significant increase in the time spent in the open arm of the EPM, as compared to sham-operated animals. Rather, food-restricted ischemic animals in our experiment which were more active in the open field, also displayed increased open arm activity in the EPM, suggesting reduced anxiety in these animals. The reasons for divergent observations in the current and past studies are unknown. These could be attributable to methodological differences: rats in the present experiment were handled daily and tested in a room located outside of the vivarium while previous studies tested animals in their housing chambers in the vivarium, potentially affecting basal stress and/or anxiety levels. Food restriction per se did not appear to modulate anxiety since food-restricted and ad libitum fed sham rats spent comparable time in the open arms of the maze.

Numerous studies have examined the relationship between hippocampal density and performance on a variety of memory tasks (i.e., split stem t-maze, radial maze, delayed-no-match-to-sample (DNMTS), water maze, and learning set tasks). Moderate to severe loss of CA1 neurons typically produces spatial memory and learning impairments [39,53,190,241,459]. However, there are discordant findings showing that CA1 cell loss is not always correlated with impairments in various cognitive tasks [280]. For example, different studies reported no correlation between acquisition latency in the Morris water maze and CA1 cell loss following forebrain ischemia [343,347,354]. Moreover, spatial memory impairments were observed following transient occlusion of the common carotid arteries (2-VO) in rats despite the absence of neuronal necrosis in the CA1 sector of the hippocampus [213]. These findings suggest that these tasks do not solely depend on hippocampal CA1 pyramidal neurons and that ischemia-induced functional impairment in tasks involving delays, object recognition or spatial memory may involve a number of

cortical and subcortical structures [20,190].

Our findings indicate a reversal of ischemia-induced spatial memory in rats maintained on a 40% food-restricted diet. The performance of food-restricted ischemic rats was almost identical as that of sham-operated animal: they made reduced working memory errors and were as fast as sham rats to complete the task. However, food restriction did not alter the behaviour of sham-operated rats. In the present study, food restriction also failed to reduce CA1 neuronal death demonstrating an interesting dissociation between CA1 neuron survival and functional sparing of behaviour thought to depend on that same region. Although asymmetry between the left and right CA1 hippocampus was observed in some ischemic animals (i.e., ischemia-induced damage was more pronounced on one side only), these lateralized differences did not account for distinct behavioural responses. Our findings indicate that neuronal density was not the sole factor that accounted for the effect of surgery and/or diet on the rats' performance in the radial arm maze.

The present results are at odds with the significant neuronal protection produced by food restriction in other lesion models [59,488,493]. A number of factors may account for this discrepancy. First, these experiments measured neuronal death within short reperfusion intervals (12, 24 and 30h, respectively) using either excitotoxic or focal ischemic models. The use of these short intervals could have underestimated the impact of global ischemia which typically leads to delayed cell death in rats and humans [238,374,390,392]. Second, there is no indication that "protected" neurons observed a few hours post-surgery in previous studies are functional and will survive at longer reperfusion intervals as used in the present study. Finally, neuronal protection observed

shortly following insult may not correlate with behavioural outcomes observed at remote intervals when function could be recuperated through plastic remodeling of the remaining neural networks. However, the functional alterations observed at 4-6 days post-ischemia in the present experiment makes it unlikely that functional recuperation could induce significant neuronal remodeling in such a short delay.

Another question raised by the present results pertains to the absence of beneficial effects of food restriction in sham-operated rats. Previous studies have shown beneficial effects of food restriction on age-related cognitive impairments [29,202,376,377]. Thus, a 24-month food restriction deferred age-associated impairments in a water maze task, and increased the learning ability of younger rats restricted for either 8 or 16 months [432]. Similarly, Idrobo and colleagues [199] reported enhanced performance of C57BL/6 female mice in the 8-arm radial maze following a 12-month food restriction. Other studies have shown increased motor coordination, locomotor activity, and spontaneous alternation following food restriction [202,313]. One obvious difference between these studies and the present experiment is the relatively shorter period of food restriction (3 months) in our experiment. Thus, the benefits of food restriction may be observable after longer exposure to restricted diets in normal or sham-surgery controls but still lead to improvements following global ischemia.

Current hypotheses to explain increased cellular survival in injured tissue include decreased free radical production and increased cellular resistance in response to stress [75,306,315]. Food restriction has been shown to significantly attenuate mitochondrial free radical induction and oxidative damage to mitochondrial-DNA in rat liver [277,394], heart [164,165], skeletal muscles [114,254,489], and brain [140,173,411]. In addition,

changes in the expression of several genes, particularly those encoding chaperone proteins and neurotrophic factors have been demonstrated [387]. Food restriction significantly enhanced expression of heat-shock proteins in heart tissues and brain [92,416,488] and the expression of brain-derived neurotrophic factor [115,116,174,298], the latter being associated with enhanced antioxidative enzymes and Bcl-2 expression [303] and neurogenesis in the dentate gyrus [263,265]. The presence of similar physiological effects in our study remains to be established. Comparable performance of food-restricted ischemic rats and sham-operated controls in the radial maze and the fact that restricted ischemic animals performed significantly better and faster than ad lib ischemic animals in a spatial task despite similar CA1 neuronal loss suggest an impact of food restriction on mechanisms that play a role in neuronal plasticity and/or promote compensatory mechanisms or adaptive changes within brain structures.

Manuscript 2

Food restriction induces long-lasting recovery of spatial memory deficits following global ischemia in delayed matching and non-matching-to-sample radial arm maze tasks.

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Author Contribution

Marie-Claude Roberge: Unless otherwise indicated all experiments were performed by the first author under the supervision of Dr. Hélène Plamondon.

Claude Messier: This author co-supervised the first author on some immunohistochemistry experiments (i.e., Caspase-3 and Double Cortin), and co-edited this manuscript with Dr. Staines and Dr. Plamondon.

William A. Staines: This author trained and supervised the immunohistochemical work presented in this study (i.e., vGluT1 and vGAT). This author also co-edited the manuscript with Dr. Messier and Dr. Plamondon.

Hélène Plamondon: This author supervised the research work of this paper, edited the manuscript, and provided financial support.

Abstract

Food restriction has been shown to be beneficial for a number of brain processes. In the current study, we characterized the impact of food restriction on hippocampal damage 70 days following ischemia. We assessed memory and cognitive flexibility of ad libitum fed (AL) and food-restricted (FR) animals using complex delayed non-matching- and matching-to-sample tasks in the radial arm maze. Our findings demonstrate that food restriction led to significant improvement of ischemia-induced memory impairments. FR ischemic animals rapidly reached comparable performance as both AL and FR sham animals in delayed-non-matching (win-shift) and matching (win-stay) radial arm maze tasks. They also made considerably less microchoices in the retention trials than AL ischemic animals. In contrast, AL ischemic rats showed persistent spatial memory impairments in the same paradigms. Assessment of basal and stress-induced corticosterone (CORT) secretion revealed no significant differences in baseline levels in AL and FR rats prior or following global ischemia. However, FR animals showed a more pronounced attenuation of CORT secretion 45 min following restraint. Both FR and AL ischemic rats had comparable CA1 and CA3 cell loss at 70 days following reperfusion, although a trend toward increased CA3 cell survival was observed in FR ischemic rats. The functional sparing in the FR ischemic animals in the face of equivalent hippocampal cell loss suggests that food restriction somehow enhanced the efficacy of remaining hippocampal or extrahippocampal neurons following ischemia. In the current study, this phenomenon was not associated with diet- and or ischemia-related alterations of vGluT1 expression in various hippocampal regions although lower vGAT immunostaining was

present in the CA1stratum oriens and the CA3 stratum radiatum in FR sham and ischemic rats.

Introduction

The ability of the brain to recover functionally and adapt physiologically to damage reveals both adaptation to disease and more generally its ability to change following new events. Treatments and conditions that facilitate these adaptive processes are of interest for the alleviation of functional deficits following brain injury. Food restriction (FR) is amongst the most efficient and reproducible intervention for reducing the incidence of various age-associated and neurodegenerative diseases and increasing life span in a variety of species, including mammals [198,306,426]. Short and long-term food restriction also significantly attenuates age-associated spatial memory impairments [29,199,202,313,376,432] as well as functional deficits secondary to chronic neurodegenerative conditions or acute brain injuries [59,117,178,298,493]. Yu and Mattson [488] also reported reduced brain damage and neurological deficits following focal ischemia in rats that were subjected to a 3-month alternate-day fasting schedule. To date, no studies have determined long-term histological and functional outcomes of food restriction following global ischemia in rats, a model developed to mimic the neuronal damage observed following the global forebrain ischemia produced by heart failure [237,392].

This neuronal damage, which typically includes the death of CA1 hippocampal neurons, has been shown to produce working and spatial memory impairments in rodents [90,177,241]. These deficits model the severe anterograde amnesia observed in humans

with moderate to extensive loss of hippocampal CA1 pyramidal cells following global cerebral ischemia [374,495]. In rat models of ischemia, the radial arm maze and the Morris water maze are typically used to assess spatial memory in rats. Although less commonly used, delayed matching or non-matching to sample (DMTS/ DNMTS) procedures can provide a richer picture of memory deficits and cognitive flexibility in spatial or non-spatial tasks. An early study by Ordy et al. [358] reported persistent memory impairment in a DNMTS T-maze task 2 and 4 months following global ischemia (4-VO for 30 min) in rats. The usefulness of DMTS/ DNMTS procedures was also demonstrated in a study that showed that exposure of gerbils to an enriched environment prior to an ischemic insult preserved the ability to learn a win-shift rule (DNMTS) but not a win-stay rule (DMTS) in a T-maze, 60 days post-occlusion [134]. Together, these findings demonstrate that the combined use of DNMTS and DMTS spatial tasks could provide a more precise evaluation of neuroprotective treatments following ischemia.

The current study is the first to examine the long-term effects of food restriction on global ischemia-related neuronal damage and cognitive impairments using DNMTS and DMTS radial arm maze tasks. Quite unexpectedly, we found that food-restricted ischemic rats, despite considerable CA1 and CA3 cell loss, had comparable performance to sham-operated rats in both the DNMTS and DMTS radial arm maze tasks while ad lib fed ischemic rats, with equivalent lesions, were significantly impaired in both delayed spatial tasks. The current study also determined corticosterone secretion in ad lib and food-restricted animals at baseline and following restraint stress, before and following sham or ischemic surgery. Post mortem tissue analysis assessed vGluT1 and vGAT immunohistochemical expression within various hippocampal regions in ad libitum fed

and food-restricted sham and ischemic animals possibly related to cognitive impairments and/or functional recovery.

Materials and Methods

Subjects and dietary protocols

43 male Wistar rats, weighing between 100-125g were obtained from Charles River Laboratories (Rochefort, Quebec, Canada). Upon arrival, animals were individually housed and maintained on a 12 h light/dark cycle (lights on at 7:00 AM), with free access to water and standard rat chow (Purina 5012). Room temperature was maintained at 21-23°C with 60% relative humidity. One week following their arrival, animals were assigned to two dietary groups. Rats in the ad lib group (AL; n = 21) continued to have free access to a standard (Purina) rat chow, while rats in the food-restricted group (FR; n = 22) received 60% of the average amount of Purina rat chow consumed by the ad lib group. A pilot study indicated that adult rats consumed on average 30-32 grams of standard Purina rat chow per day. Based on these observations, ad libitum fed rats received a daily ration of 30-32 grams standard Purina rat chow while food-restricted rats received 60% of that amount (i.e., 18-19.2 grams of standard Purina rat chow per day). This meant that food-restricted animals received the same nutrients in the same relative proportions but, overall, received less of all nutrients. Although it is possible that the restricted diet may have led to specific nutrient deficiencies, the often replicated observation of increased lifespan and general better health of food-restricted animals suggest that there is little deleterious impact of food restriction. Food intake was measured daily between 8:00-9:00AM. All animals had free access to water throughout

the experiment. Rats were handled daily and weighed every two to three days.

The diets were provided for a 3-month period prior to sham or ischemic surgeries and up until euthanasia (i.e., 70 days post-surgery). The four experimental groups included: ad lib food with either sham surgery (SHAM-AL; n=10) or ischemia (ISCH-AL; n=10), and restricted food with either sham surgery (SHAM-FR; n=10) or ischemia (ISCH-FR; n=11). Two ischemic animals (1 ISCH-AL and 1 ISCH-FR) died following surgery and were not included. All experiments conformed to the NIH guide for the Care and Use of laboratory animals (NIH publications N°80-23, revised 1996) and procedures were in accordance with the guidelines set by the Canadian Council of Animal Care and approved by the University of Ottawa Animal Care Committee. Efforts were made to minimize the number of animals used and their suffering.

Forebrain ischemia

Forebrain ischemia was performed using the four-vessel occlusion model as previously described by Pulsinelli and Brierley [391]. Briefly, rats were deeply anesthetized by inhalation of 2 to 3.5% halothane vaporized by oxygen (1.5 to 2L/min). Vertebral arteries were irreversibly occluded by electrocoagulation, and a small-diameter silk thread looped around the carotid arteries to facilitate subsequent occlusion. Twenty-four hours later, common carotid arteries were occluded with microaneurysm clamps for 12 minutes in spontaneously ventilating animals. Sham-operated animals underwent anesthesia and received the same dorsal and ventral surgical incisions as the two ischemic groups, with the exception of electrocoagulation of the vertebral arteries. Twenty-four hours later, carotid arteries were exposed, but not clamped. Core temperature was kept at

37°C ± 0.5 throughout the surgery using a feedback-regulated heating blanket connected to a rectal thermometer (Homeothermic Blanket Control Unit, Harvard Instruments, Natick, MA). Rats' body temperature was further supported with a heating pad in the hours following surgery and reperfusion.

Behavioural Tests

Functional recovery in the different behavioural tests was sequentially assessed over seventy days following reperfusion (see Figure 9).

Delayed-non-matching- and matching- to-sample (DNMTS; DMTS) radial arm maze: Apparatus

The radial maze consisted of eight arms (60 X 12 cm with a 5 cm lip around each arm) extending radially from a central octagonal area (32 cm in diameter with a 30 cm high clear Plexiglas wall). Plexiglas sliding doors allowed entry into each arm. The floor of the arms and central area were covered with black rubber lining. The apparatus was elevated 50 cm above the floor and surrounded by extra-maze cues such as posters or calendars along the sidewalls. The experimenter sat behind a panel where he could observe and record behaviour unobtrusively and manipulate the overhead strings to open and close the maze doors. At the end of each arm, a food cup (1 cm deep into the floor) contained a reward (a piece of Fruit Loop).

Training procedures

From day 6 to day 12 post-ischemia, rats began training in the radial maze. Daily food ration of ad lib animals was gradually reduced (over a 4-day period) and rats maintained at approximately 85% of their free-feeding rate during the testing period to ensure adequate motivation. The food-restricted rats were not further deprived. Rats were familiarized with the radial arm maze during four daily sessions each lasting 10 min on successive days. Rewards were initially available throughout the maze to encourage exploration, but were gradually restricted to the food cups. Each animal was individually placed in the center of the maze with the doors to all arms closed. Upon opening the doors, the rat was permitted to enter any of the eight arms. When the rat had consumed the reward at the end of one arm and returned to the center of the maze, all doors were closed again to confine the rat to the center zone of the maze for a 10 s delay. The doors were then reopened, and the procedure repeated. The test continued until all baits were consumed or until 10 min had elapsed. An arm entry was counted when the rat had its four paws within an arm. The orientation of the animal's head when placed into the central area was randomly permuted from trial to trial to minimize the development of a response pattern based on position. The floor of the maze's eight arms and central area was cleaned after each trial to reduce olfactory cues.

DNMTS tasks: win-shift

The DNMTS task was initiated 13 days post-ischemia, and consisted in daily acquisition and retention trials separated by a 15-min delay. In the acquisition trials, four arms were blocked with transparent Plexiglas doors. Rats were free to navigate to the

four available arms, which were baited with a small piece of Fruit Loop. After consuming all food rewards (or when 10 min had elapsed), rats were removed from the maze and returned to their home cage for 15 min. After this delay, rats were placed back in the clean maze with a different body orientation for the retention trial. At this stage, all eight arms were available for entry, but only those previously blocked, contained a reward. At the completion of each day's retention trial, rats were given their daily food ration. The arms blocked in the acquisition trials were individually and randomly assigned to rats from a list of 30 possible patterns, and arm sequences were counterbalanced across groups. One sequence was assigned to each animal and maintained for the 15-day testing period. Following this period, rats were tested for an additional three days in a DNMTS task using the same arm sequence but a different inter-trial delay on each day (5, 30 or 240 min). Also, to determine if the rats had learned rules involved in the DNMTS task rather than simply memorized the arm sequences, we tested rats for an additional six days using the same methodology but novel arm sequences. Measures recorded in the acquisition trial (pre-delay) included total number of working memory errors, latencies to complete the task and microchoices (in which a rat visually scanned cues corresponding to an individual arm, but only his head and/or front paws extended into the corresponding arm). In the retention trials, the error score was further divided into retroactive errors (entry into a non-rewarded arm), and proactive errors (re-entry into an arm within the test trial).

DMTS task: win-stay

Following the DNMTS tasks, animals underwent a DMTS task for an additional 8 days using a new arm sequence and a 15-min inter-trial delay. In this paradigm, animals were rewarded for selecting previously baited arms in the retention trials. Measures recorded in the acquisition and retention trials of the DMTS were identical as the DNMTS.

Blood Collection

Two month following initiation of the feeding conditions (pre-surgical measures) and 68-69 days following reperfusion (post-surgical measures), tail blood samples were obtained for each rat by lancing the tail close to its tip and collecting a few drops of blood on a Schleicher and Schuell filter paper. Immediately following collection of a baseline sample, the rat was slid into a rodent restraint cone for 15 min to evaluate the impact of an acute stressor on the corticosterone response profile. However the rats were not otherwise restrained during the blood collection procedure. Blood collection was repeated 30 and 60 min following baseline (i.e., 15 and 45 min following restraint stress). Following the last sample collection, animals were returned to their home-cage. Filter papers holding the blood samples were dried overnight in the dark in a semi-closed box and stored at -84 °C for subsequent analysis. Blood sampling took place between 12:00 and 6:00 pm. The time of test for animals in each of the groups was randomized to control for possible circadian rhythm effects on CORT levels. Blood collection was performed during the same time window as that selected to perform behavioural testing.

Corticosterone assay

Blood samples were eluted from the filter paper by taking a 3.2 mm punch and placing it in a glass tube and adding 100 μl of Dulbecco's phosphate-buffered saline (containing 0.1% gelatin) to each tube. These were shaken at room temperature for 1h at 50 rpm, refrigerated overnight, and shaken for an additional hour the following morning. Plasma corticosterone levels were determined using a commercially available radioimmunoassay kit (ICN Biomedicals, Costa Mesa, CA). The intra-assay variability was < 10% and testing all blood samples in a single radioimmunoassay reduced inter-assay variability. Total corticosterone concentrations were determined as outlined by Wortham and Stallings [478].

Analysis of neuronal density on thionin-stained sections

Seventy days following reperfusion, rats were deeply anesthetized using sodium pentobarbital and perfused using a 4% paraformaldehyde solution. Their brain was removed and stored at -84°C. Serial coronal sections (14 μm) of the hippocampal regions were subsequently obtained using a cryostat and stained with thionin. Neuronal density of the hippocampal CA1 and CA3 subfields was determined using the method of Kirino et al. [239] and performed on coronal sections located between 3.14 and 4.16 mm posterior to bregma [370]. The total linear length of the CA1 and CA3 sectors (as defined by Paxinos and Watson [370] was measured by means of a digitizer. The number of intact neurons in the stratum pyramidale within CA1 and CA3 subfields was counted using a Leica DAS microscope attached to a Sony digital camera and computer-assisted cell counting was performed using Norton Eclipse (v 6.0). Neurons that had shrunken cell

bodies with surrounding empty spaces were excluded. The neuronal density of the CA1 and CA3 sectors, *i.e.* the number of intact pyramidal cells per 1 mm linear length of stratum pyramidale was quantified. A mean value for each hippocampal substructures was obtained from 6 bilateral measurements per animal in each of the experimental groups. The neuronal density for a given animal represents the average of both the right and left hippocampal measures.

Immunohistochemistry

Slide mounted tissue sections were incubated with primary antibodies diluted in PBS containing 0.3% Triton-X 100. Vesicular GABA transporters (vGAT) were visualized using a rabbit anti-rat vGAT 11 (1:500; Alpha Diagnostic; Unites States), and vesicular glutamate transporters were visualized using a guinea pig anti-mouse vGluT1 (1:500, Chemicon; Unites States). Secondary antibodies used were donkey anti-rabbit Alexa Fluor 488 (1:400; Invitrogen; Canada), and goat anti-guinea pig Alexa Fluor 488 (1:400; Invitrogen; Canada). Antibody solution mixing was achieved using low-amplitude vibration applied by attaching a small fish tank air pump to the top of the slide incubation chamber which sat on a mouse pad [208]. After incubation for 3 h at room temperature with the primary antibodies, slides were rinsed in PBS for 10 min and then incubated with the secondary antibodies for 30 min at 37 ◦C without vibration. Sections were examined using a Zeiss Axiophot II microscope equipped with standard filter sets for FITC (490/525 nm) and Texas-Red (590/620 nm). Image data were acquired using a Retiga Qimage CCD camera and the Northern Eclipse imaging software (EMPIX; Toronto, Canada). Image analysis was performed on some images to extract gray level

information (Image J). The signal to noise ratio (SNR) was calculated by dividing the average gray level of the specific immunofluorescence minus the average gray level of the background immunofluorescence by the average gray level of the background immunofluorescence time 100. SNR were calculated for vGluT1 and vGAT. Immunofluorescence intensity was determined in 11 subfields within the hippocampal formation, including the CA1 and CA3 pyramidal layers, the associated stratum oriens and radiatum, the stratum lucidum, the lacunosum molecular and molecular layers of the dentate gyrus as well as the granular cell layer and hilus of the dentate gyrus (see Figure 10). For each of these hippocampal substructures, the average grey level (AGL) was obtained from 2 to 4 bilateral measurements in each animal, caring for comparable selection of anterior-posterior coordinates between animals. Measures for a given animal represent the average of both the right and left hippocampal measures. SNR and standard error of the mean were calculated.

Statistical analyses

All statistical analyses were conducted using SPSS (V.14) software package. Body weight and radial arm maze data were analysed using mixed ANOVA designs with two independent factors *surgery and diet* and various levels of the repeated factor *time*. Plasma corticosterone levels data were analyzed using a mixed ANOVA design with two independent factors *surgery and diet* and two repeated factors *time and minutes.* The Huynh-Feldt correction for violations to the assumption of sphericity was applied when appropriate. The degrees of freedom have been adjusted when the correction was used. Significant interactions were further analysed using simple effect tests with Bonferroni

modification of critical alpha level. Data obtained from neuronal densities and signals to noise ratio of immunofluorescence were analysed using two factors (*surgery and diet*) ANOVAs. Simple effect tests were used to further analyse significant interactions. In the radial arm maze, data are expressed as total errors (± SEM) for blocks of either 3 days (15 day DNMTS task) or 2 days (DNMTS - *novel sequence* and DMTS). Data from the different inter-trials delays in the DNMTS tasks were analysed separately. Values are expressed as mean ± SEM for all tests. A difference was considered significant when $p \leq 0.05$.

Results

Body weight

Figure 11 presents the mean body weight of each rat group throughout the experiment. There were main effects of time ($F(4.85,145.48) = 1601.38$, $p < .001$), diet ($F(1,30) = 88.77$, $p < .001$), and an interaction of time x diet ($F(4.85,145.48) = 32.01$, $p < .001$). Body weight increased over time, although at a lower rate in food-restricted as compared to ad lib fed rats. Simple effect tests showed that from week 3 to the end of the experiment, food-restricted animals weighted considerably less than ad lib fed animals ($p < .001$). Casual observations indicated that food-restricted rats appeared to recover faster from global ischemia, eating more rapidly and being more active than ad lib fed rats upon awakening from vessel occlusion. They also gained more weight following surgery compared to ad lib fed animals, most likely because the caloric restriction was only reinstated five days following surgery and that all rats were offered palatable food (Cheerios, Fruit Loop, and graham powder) to hasten recovery of pre-surgical weights.

Behavioural tests

Delayed spatial radial arm maze

Two animals (1 ISCH-AL and 1 SHAM-FR) were unable to complete the radial maze tasks due to immobility and were excluded from statistical analyses. The number of working memory errors observed during acquisition trials for the different DNMTS and DMTS conditions are seen in *Figures 12a, 12c, 13a* and *14a.* Analyses revealed main effects of surgery on the number of working memory errors in the acquisition trials for the initial DNMTS task ($F(1,35) = 17.28$, $p < .001$), DNMTS tasks using inter-trial delays of 5, 30, and 240 min ($F(1,35) = 5.98$, $p = .002$), the DNMTS task using a novel sequence ($F(1,35) = 4.95$, $p = .033$) and the DMTS task (win-stay) ($F(1,35) = 12.24$, $p = .001$). Ischemic rats made more working memory errors in the acquisition trials as compared to sham-operated rats independent of diet regimen. Our data also revealed that animals, once trained with the initial DNMTS task, significantly improved their performance in subsequent acquisition trials reducing between-group differences. These findings suggest that all groups did ultimately acquire the set rule but ischemia slowed this acquisition.

Total numbers of errors in retention trials of the DNMTS and DMTS tasks are highlighted in Figure 12b, 12d, 13b and 14b. In the initial DNMTS task, we found main effects of time ($F(3.85,134.64) = 39.07$, $p < .001$), surgery ($F(1,35) = 37.93$, $p < .001$), diet ($F(1,35) = 17.17$, $p < .001$), and a significant surgery x diet interaction ($F(1,35) = 15.89$, $p < .001$). Simple effect tests revealed that these effects were attributable to increased proactive and retroactive errors in ad lib ischemic rats as compared to food-restricted ischemic rats and sham animals over the entire 15-day testing period ($p < .001$). Analysis of the number of errors with inter-trials delays of 5, 30, or 240 min revealed

main effects of time ($F(2,70) = 6.28$, $p = .003$), surgery ($F(1,35) = 19.58$, $p < .001$), and diet ($F(1,35) = 9.26$, $p = .004$), and significant time x surgery ($F(2,70) = 3.85$, $p = .025$) and surgery x diet ($F(1,35) = 14.45$, $p = .001$) interactions. Simple effect tests indicated that ad lib ischemic rats made more errors at all delays ($p \leq 0.02$). Analysis of the number of errors on the 2nd DNMTS revealed main effects of time ($F(2,70) = 17.49$, $p < .001$), surgery ($F(1,35) = 41.82$, $p < .001$), diet ($F(1,35) = 13.76$, $p = .001$) and a significant surgery x diet interaction ($F(1,35) = 15.36$, $p < .001$). When rats were tested using a novel arm sequence (figure 13b), sham-operated and food-restricted ischemic rats made more errors on the first two days of testing, but the number of errors rapidly decreased over the remaining four days, while ad lib ischemic rats showed no significant improvement over time. Figure 14b presents the number of errors in the DMTS (win-stay) task. Statistical analyses indicated main effects of time ($F(3,105) = 30.62$, $p < .001$), surgery ($F(1,35) = 41.82$, $p < .001$), diet ($F(1,35) = 53,62$, $p < .001$), and significant surgery x diet ($F(1,35) = 29.57$, $p < .001$), and time x surgery x diet ($F(3,105) = 2.84$, $p = .042$) interactions. Sham-operated and food-restricted ischemic rats rapidly mastered this task while ad lib ischemic rats took longer to learn the rule.

Retroactive and proactive errors

The total number of errors in the retention trials was further divided into retroactive and proactive errors. Statistical analyses revealed nearly identical patterns of results as for the total number of errors, and are not further analysed to avoid redundancy. Hence, ischemic rats fed an ad lib diet made significantly more retroactive and proactive errors than all other groups on all DNMTS and DMTS tasks.

Latencies to complete the delayed radial arm task

Table 3 presents the latencies to complete the acquisition trials for all DNMTS and DMTS tasks and the latencies of the retention trials of the 1st DNMTS tasks. There were main effects of time ($F(3.9,136,43) = 61.39$, $p < .001$) and diet ($F(1,35) = 6.55$, $p = .015$), and a surgery x diet interaction ($F(1,35) = 6.83$, $p = .013$) for the latencies to complete the acquisition trials. A gradual reduction in time taken to complete the task was observed for all animals. Simple effect tests indicated that ad lib fed ischemic rats took considerably more time to complete the task than food-restricted ischemic ($p = .001$) and sham ($p = 0.004$) rats. No significant difference was found in the time to complete the task for the different inter-trial delay. In the second DNMTS, food-restricted rats readily adapted to the change in sequence and the number of errors and latency to complete the task rapidly diminished (main effect of diet ($F(1,35) = 4.65$, $p = .038$). As expected, all animal groups progressively took less time to complete the DMTS task ($F(2.63,92.16) = 6.2$, $p = .001$). A similar trend was observed for the retention trials where main effects of time for all DNMTS and DMTS tasks (1-15 days: $F(2.77,96.82) = 81.06$, $p < .001$; 1-6 days: $F(2,70) = 9.88$, $p < .001$; 1-8 days: $F(2.86,100.12) = 6.62$, $p < .001$) indicating that during each test phase, there was a progressive reduction in time required to complete. Latencies of the retention trials of the variable delays, 2nd DNMTS and DMTS are not presented as no significant difference was observed between groups.

Microchoices in the radial arm maze

In general, no significant differences were found between the animal groups in the frequencies of microchoices performed during acquisition trials in any of the radial maze

paradigms. All rat groups made significantly less microchoices as time went by (data not shown). However, data revealed group differences in the number of microchoices performed in the retention trials (see Figure 15). Statistical analyses revealed main effects of time ($F(1.62,56.79) = 8.95$, $p = .001$), and diet ($F(1,35) = 7.52$, $p = .01$) in the initial DNMTS, attributable to reduced number of microchoices in food-restricted compared to ad lib fed animals although all animals showed a progressive decrease in the number of microchoices with repeated exposure. During the variable inter-trial delay period, we observed a main effect of diet ($F(1,35) = 7.16$, $p = .011$), and a surgery x diet interaction ($F(1,35) = 5.23$, $p = .028$). Food-restricted rats made fewer microchoices in each of the delay intervals (Figure 15b). Figures 15c and 15d present the number of microchoices made in the second DNMTS and the DMTS tasks, respectively. There was a significant surgery x diet interaction ($F(1,35) = 8.04$, $p = .008$) for the DNMTS task and a main effect of diet ($F(1,35) = 4.74$, $p = .036$) and a surgery x diet interaction ($F(1,35) = 4.14$, $p = .049$) for the DMTS task. For all these testing conditions, simple effect tests indicated that there was an increased number of microchoices made by ad lib fed ischemic rats.

Plasma Corticosterone levels

Figure 16 shows the effects of feeding conditions and 12 min global ischemia on plasma corticosterone levels one month prior and 68-69 days post surgery. One animal (SHAM-FR) was excluded from statistical analyses because its plasma corticosterone levels significantly differed across time from values of its reference group. A mixed ANOVA design with two repeated factors (time=pre/post; minutes= baseline/

30min/60min) revealed main effects of minutes ($F(1.87,67.4) = 264.15$, $p < .001$), and diet ($F(1,36) = 5.79$, $p = .021$), and a significant interaction of time x minutes ($F(2,72) = 3.27$, $p = .044$). Corticosterone levels showed time-dependent increases, reaching peaked plasma concentrations 15 min following restraint stress. Overall, food-restricted rats displayed relatively lower corticosterone levels across time (pre and post-surgery), and effect attributable to a more rapid decrease in CORT secretion in food-restricted rats 45 min following stress ($p=0.017$).

Histopathological findings in the hippocampus

Table 4 and Figure 17 show the effects of food restriction and 12-min global ischemia on hippocampal CA1 and CA3 neuronal densities. There were main effects of surgery for CA1 ($F(1,37) = 169.12$, $p <.001$) and CA3 ($F(1,37) = 45.75$, $p <.001$). Sham-operated animals had significantly more neurons within the CA1 and CA3 subfields of the hippocampus relative to ischemic rats irrespective of feeding regimen. Our data suggested the existence of a trend toward a slight increase in CA3 cellular survival in food-restricted ischemic rats; however, this observation was not supported by statistical analyses.

Correlation of functional impairments with the extent of the lesion

Some variability was observed within CA1 cell loss for both ischemic groups. Therefore, to determine the possible impact of this neuronal variability on functional recovery, all behavioural data were re-analysed using neuronal density as a covariate when there was a significant correlation between a particular behavioural measure and

the neuronal density. The total number of errors for the DNMTS [i.e., day 1-15, 5, 30 and 240 min inter-trial delays, and second 6 day test] and DMTS tasks were significantly correlated with CA1 neuronal density ($r = -0.63$, $p < .001$; $r = -0.45$, $p = 0.004$; $r = -0.51$, $p = .001$; $r = -0.37$, $p = .021$; $r = -0.61$, $p < .001$; and $r = -0.51$, $p = .001$, respectively).

A mixed ANCOVA design indicated main effects of time ($F(3.94,133.91) = 5.04$, $p = .001$) and diet ($F(1,34) = 16.22$, $p < .001$) and a surgery x diet interaction ($F(1,34) = 16.37$, $p < .001$) for the first 15 days of testing (1st DNMTS task). A main effect of diet ($F(1,34) = 8.32$, $p = .007$) and a surgery x diet interaction ($F(1,34) = 14.51$, $p = .001$) were found for the delay intervals. There were a main effect of diet ($F(1,34) = 12.62$, $p = .001$) and significant time x diet ($F(2,68) = 3.26$, $p = .045$) and surgery x diet interactions ($F(1,34) = 15.02$, $p < .001$) for the 2nd DNMTS task. Main effects of time ($F(3,102) = 4.5$ $p = .005$), diet ($F(1,34) = 51.38$, $p < .001$) and surgery x diet ($F(1,34) = 29.34$, $p < .001$), and time x surgery x diet ($F(3,102) = 2.78$, $p = .045$) interactions were observed for the DMTS task. Post-hoc tests indicated that, as found previously, ad lib ischemic rats made significantly more errors than food-restricted ischemic rats and sham animals over the first DNMTS task, interval delays, second DNMTS, and DMTS tasks. There was no significant correlation between CA1 neuronal density and latencies to complete any of the tasks. Finally, a negative correlation was found between CA1 cells and the number of microchoices in the retention trials of the DMTS task ($r = -0.33$, $p = .041$). Nonetheless, the covariance analysis did not reveal significant difference between groups.

These results suggest that the impact of diet on the behaviour in the delayed radial arm maze tasks is not solely dependent on the extent of neuronal death within the CA1

subfield of the hippocampus. The statistical differences observed after CA1 neuronal density was used as covariate suggest that factors other than neuronal counts determined the performance of ischemic rats with and without the restricted diet.

Immunohistochemistry

vGluT1: Figure 18 shows representative photomicrographs of vesicular glutamate transporter (vGluT1) immunostaining observed within various regions of the hippocampal formation. Independent of diet, sham and ischemic animals showed pronounced vGluT1 expression in all hippocampal regions, except the pyramidal and dentate gyrus granular cell layers. The distribution profile reported is consistent with previous studies [12,223,233]. Signal to noise ratio analyses revealed main effects of surgery for the stratum oriens at the Ca1 level ($F(1,21) = 25.54$, $p < .001$), the CA1 ($F(1,21) = 17.47$, $p < .001$) and CA3 ($F(1,21) = 4.79$, $p = .004$) pyramidal layer, the stratum radiatum at the CA1 level ($F(1,21) = 8.32$, $p = .009$), the hilus ($F(1,21) = 4.84$, $p = .039$), and the lacunosum molecular ($F(1,21) = 6.76$, $p = .017$) and molecular ($F(1,21) = 4.91$, $p = .038$) layers of the dentate gyrus. Sham-operated animals generally had a significantly higher signal to noise ratio than ischemic animals in most hippocampal regions, for the exception of the CA3 pyramidal layer, and the molecular layer and hilus of the dentate gyrus (see Table 5).

vGAT: Figure 19 shows representative photomicrographs of vesicular Gamma(γ)-aminobutyric acid transporter (vGAT) immunostaining observed within various regions of the hippocampal formation. In sham animals, we observed pronounced expression of vGAT immunofluorescence along the granular, molecular and lacunosum molecular

layers of the dentate gyrus, and along the CA1 and CA3 pyramidal cell layers. We also observed moderate expression of vGAT throughout the stratum oriens and radiatum, but not in the granular and pyramidal cells nor in the stratum lucidum. Again, this distribution pattern seemed consistent with previous literature [12,83,429,454]. The distribution pattern of ischemic animals was similar to that of sham-operated animals, for the exception of the CA1 pyramidal cell layer. Signal to noise ratio analyses revealed main effects of surgery for the CA1 pyramidal cell layer ($F(1,19) = 13.74$, $p = .001$), the stratum radiatum at the CA1 level ($F(1,19) = 7.84$, $p = .011$), and the hilus of the dentate gyrus ($F(1,19) = 6.07$, $p = .023$). Main effects of diet were observed for the stratum oriens at the CA1 level ($F(1,19) = 5.71$, $p = .027$) and the stratum radiatum at the CA3 level ($F(1,19) = 5.04$, $p = .037$). Sham-operated animals generally had a significantly higher signal to noise ratio than ischemic animals in the CA1 pyramidal cell layer and the hilus of the dentate gyrus. Ischemic animals' signal to noise ratio were superior of that of sham-operated animals in the stratum radiatum at the CA1 level. Ad lib fed animals had a significantly higher signal to noise ratio than food-restricted animals in the stratum oriens at the CA1 level and the stratum radiatum at the CA3 level (see Table 6).

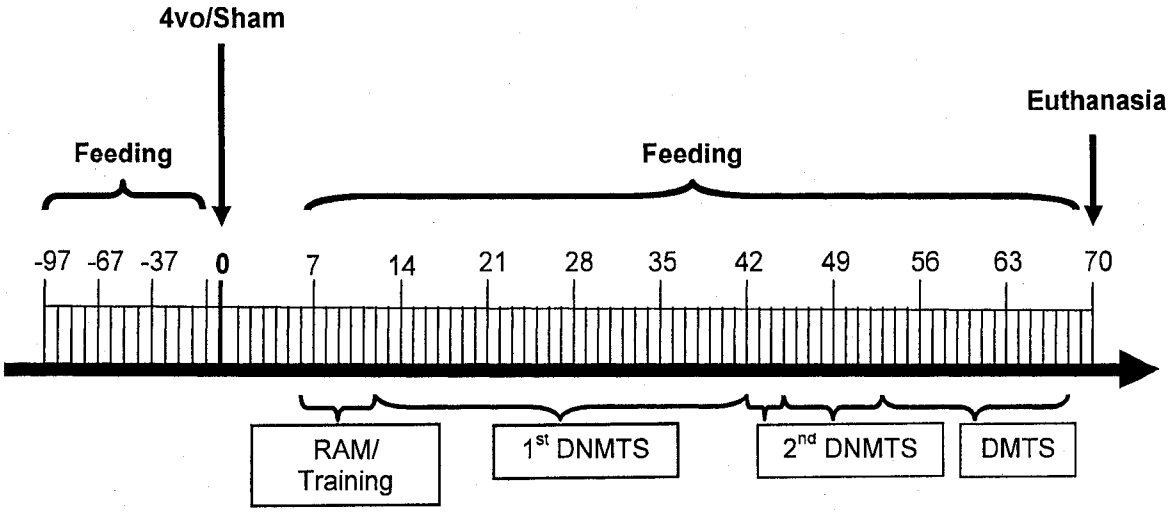
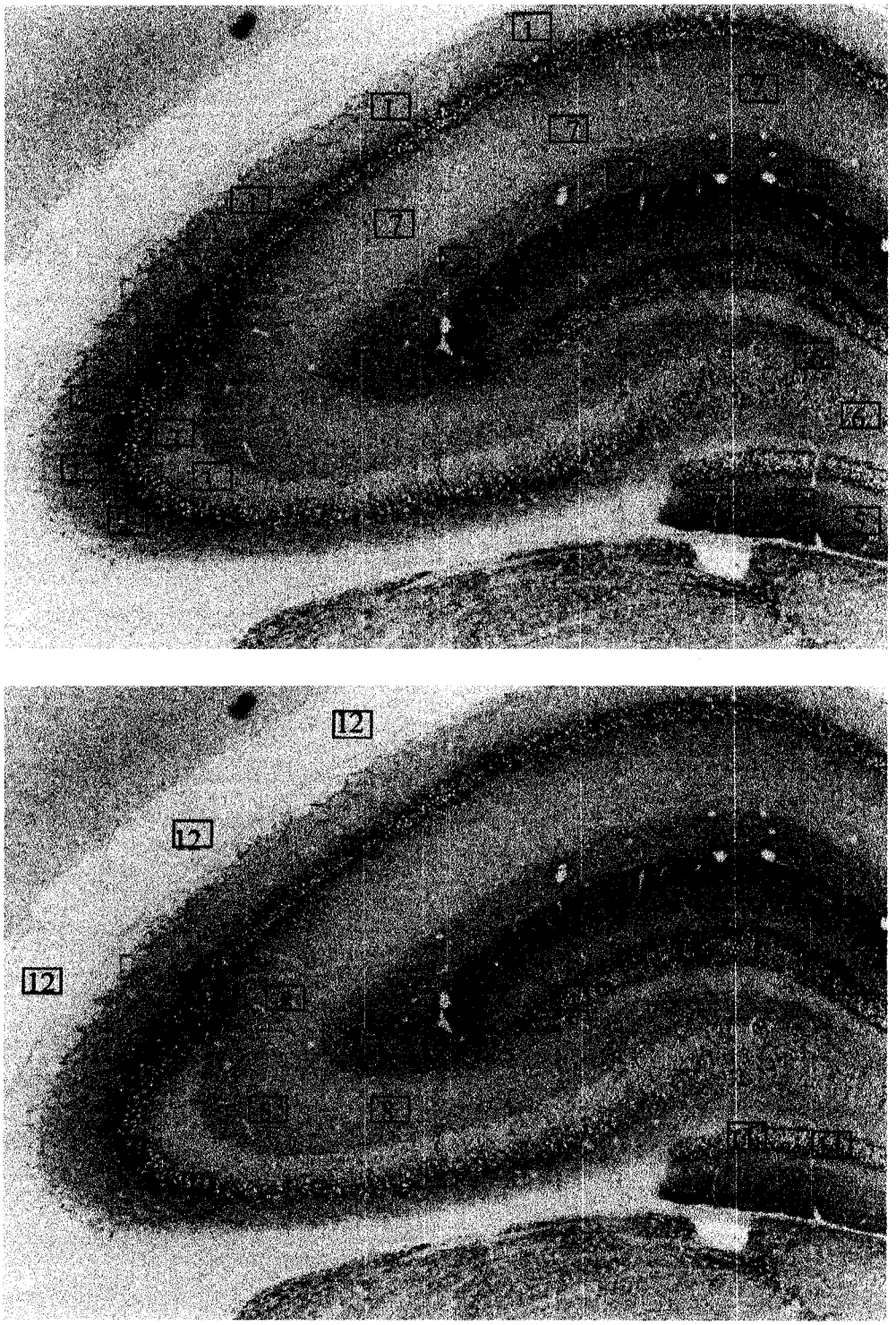


Figure 9: Experimental protocol. Feeding : Presurgical period of differential feeding: ad libitum or food restriction. 4-VO : 4 vessel occlusion. After surgery, all animals were given 5 days of ad lib access to food and treats to hasten recovery. After the recovery period, food-restricted animals were subjected to their previous restricted diet for the remaining testing period. From days 6 to 9, the weight of animals fed ad libitum were gradually reduced and maintained at approximately 85% of their free-feeding rate during the remaining testing period to ensure adequate motivation while food-restricted animals were not further deprived. RAM/Training: Training on the radial arm maze from days 6 to 12 post-ischemia; 1st DNMTS task from days 12 to 42; DNMTS – variable delays (5, 30, 240 min) from days 43 to 45; 2nd DNMTS task from days 46 to 53; DMTS task from days 54 to 68. Day 0 refers to the day of surgery.

Figure 10: Localizations of the 11 hippocampal substructures sampled for vGluT1 and vGAT immunofluorescence intensity. Average Grey Level (AGL) for each area was obtained from 2 to 4 bilateral measurements per animal in each of the experimental groups. AGL for a given animal represents the average of both the right and left hippocampal measures. SNR mean and standard error of the mean were calculated. (1) stratum oriens at the CA1 level; (2) stratum oriens at the CA3 level; (3) stratum lucidum; (4) stratum lacunosum molecular; (5) stratum molecular of the dentate gyrus; (6) hilus of the dentate gyrus; (7) stratum radiatum at the CA1 level; (8) stratum radiatum at the CA3 level; (9) CA1 pyramidal cell layer; (10) CA3 pyramidal cell layer; (11) granular cell layer of the dentate gyrus; (12) corpus callosum (used as background).

Figure 10.



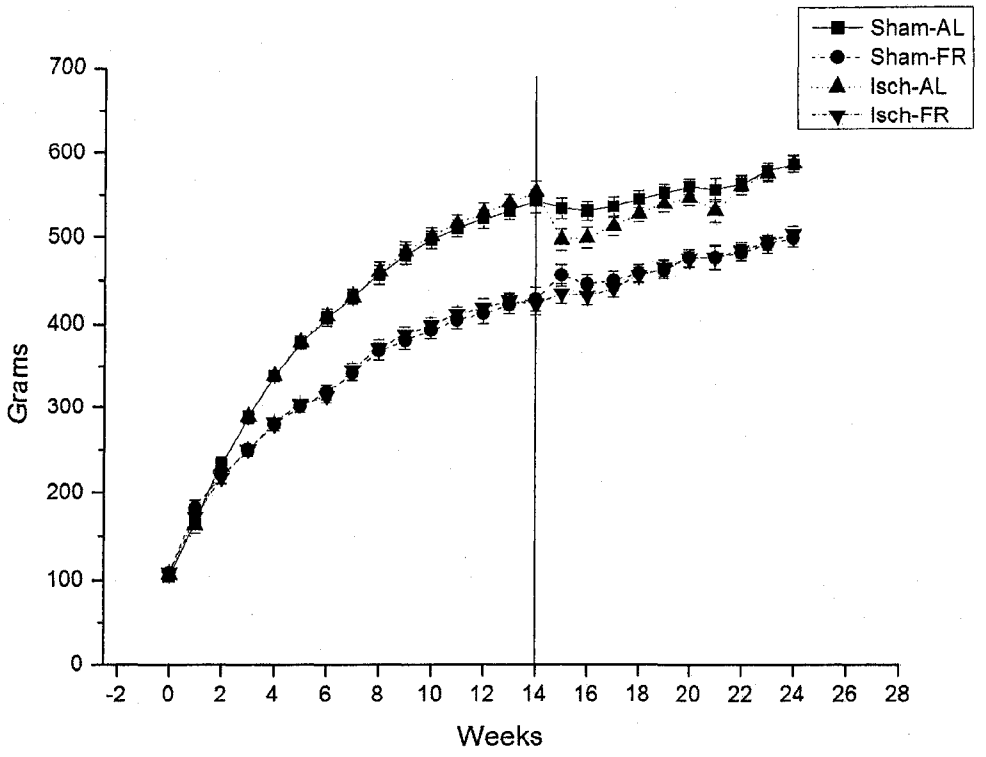


*Figure 11: Body weight of rats either fed ad lib or 60% of the ad lib food weight for three months prior to the induction of global ischemia (indicated by the vertical lin*e). Rats fed a restricted diet weighted less than ad lib rats throughout the experiment.

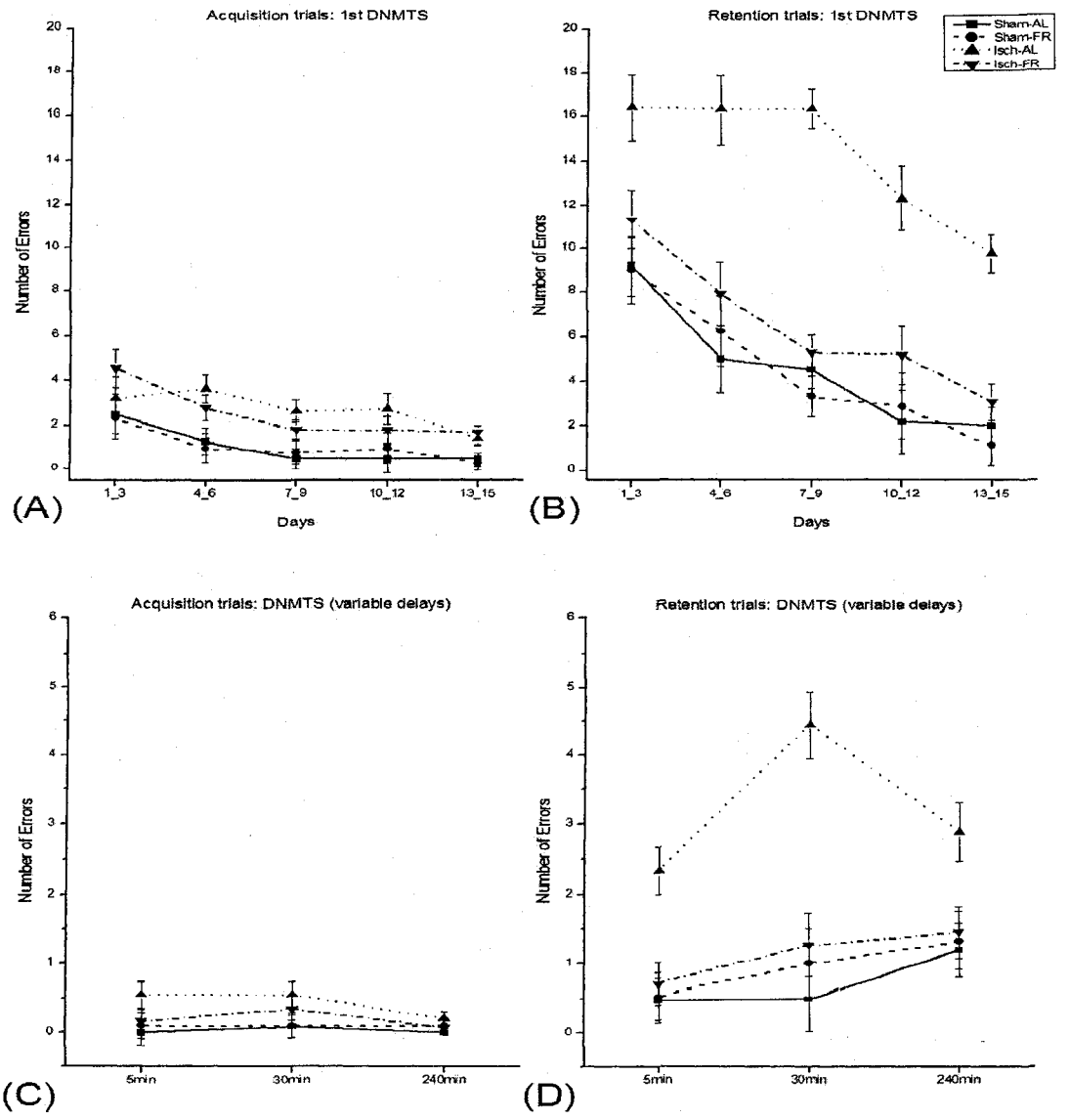


Figure 12: Effect of ischemia and food restriction on the total number of errors in the first DNMTS (15 days) and DNMTS (variable delays) tasks in the radial arm maze. **12a and 12c:** present the number of errors made by all groups during the acquisition trials of the first DNMTS and DNMTS (variable delays) respectively. Both ischemic groups made considerably more errors than sham-operated groups. **12b:** presents the number of errors made during the retention trials of the first DNMTS task. Ad lib fed ischemic rats made significantly more errors than food-restricted ischemic and sham-operated rats ($p < .001$). The performance of food-restricted ischemic rats did not significantly differ from that of sham-operated animals. **12d:** shows the number of errors made during the retention trials of the DNMTS (variable delays). Again, ad lib fed ischemic rats made more errors at all delays, particularly at the 30-min delay than all other groups ($p \leq 0.02$).

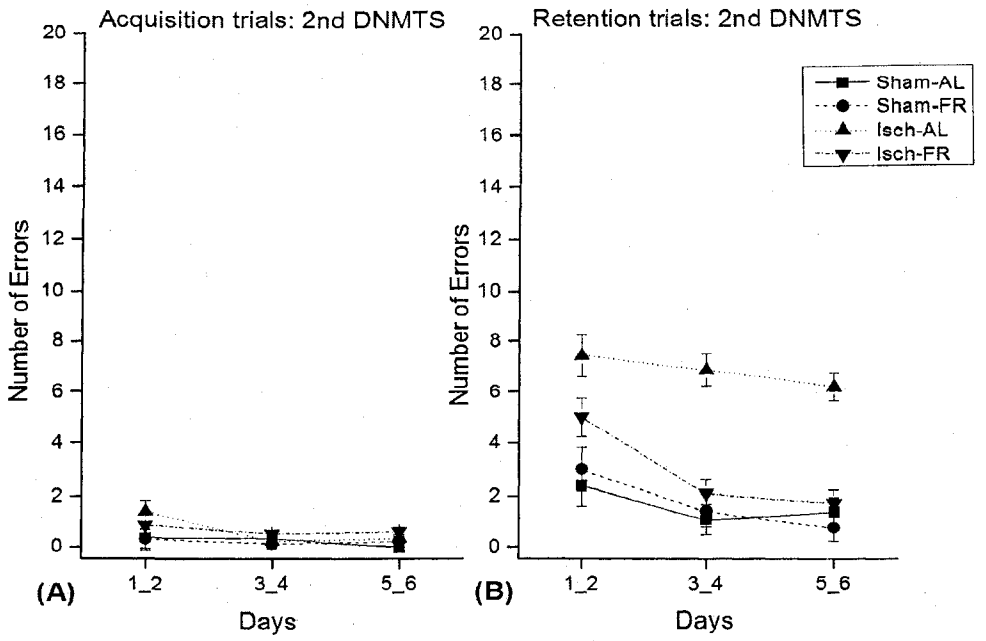


Figure 13: Effect of ischemia and food restriction on the total number of errors made during the 2nd DNMTS task in the radial arm maze. **13a:** presents the number of errors made by all groups during the acquisition trials the 2nd DNMTS. Both ischemic groups made considerably more errors than sham-operated groups. **13b:** presents the number of errors made during the retention trials the 2nd DNMTS. Sham-operated animals and food-restricted ischemic rats made more errors on the first two days of testing, but the number of errors rapidly decreased over the remaining four days while ad lib ischemic rats failed to show any significant memory improvement.

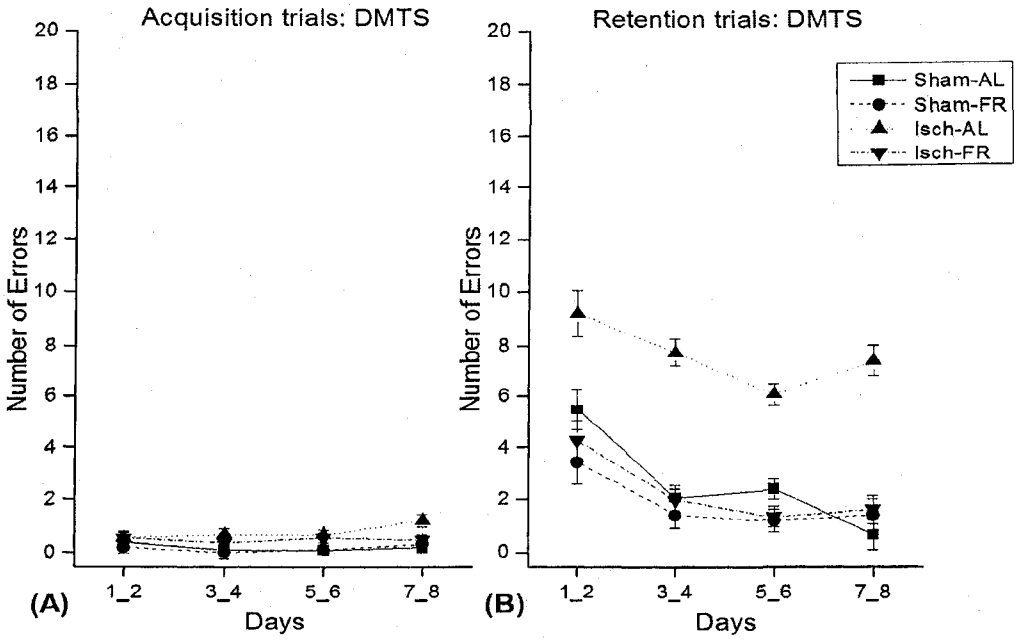


Figure 14: Effect of ischemia and food restriction on the total number of errors made during the DMTS task in the radial arm maze. **14a:** presents the number of errors made by all groups during the acquisition trials the DMTS. Both ischemic groups made considerably more errors than sham-operated groups. **14b:** presents the number of errors made during the retention trials the DMTS. Again, sham-operated and food-restricted ischemic rats mastered this task after two days while ad lib ischemic rats did not readily improve.

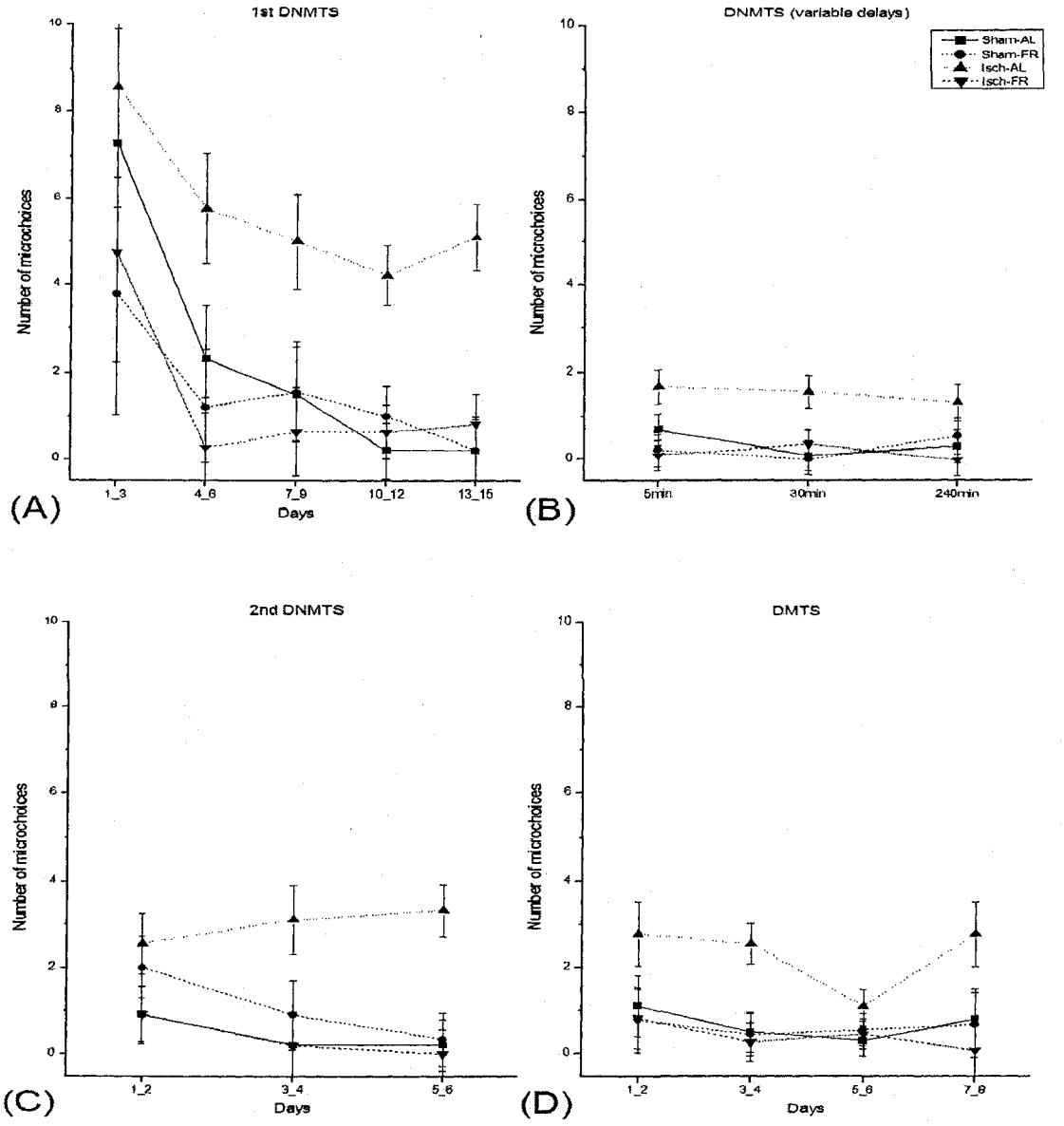


Figure 15: Number of microchoices made by all groups during the retention trials of the DNMTS and DMTS tasks in the radial arm maze. **15a:** presents the microchoices made during the first DNMTS task. **15b:** shows the microchoices made during the DNMTS (variable delays). **15c:** presents the microchoices made during the 2nd DNMTS tasks. **15d:** shows the microchoices made during the DMTS task. No significant difference was found between groups. Across all retention trials ad lib fed ischemic rats made more microchoices than sham-operated and food-restricted ischemic rats.

Table 3: Latencies to complete the acquisition trials of the DNMTS and DMTS tasks and the retention trials of the initial DNMTS radial arm maze task.

Acquisition trials	Sham-AL	Sham-FR	Isch-AL	Isch-FR
1st DNMTS (15 days)				
1-3	442.30±41.82	459.04±42.78	517.70±34.37*	390.15±33.20
4-6	301.97±25.99	296.52±50.93	466.85±41.59*	284.49±39.49
7-9	250.90±31.78	219.22±25.02	370.04±49.63*	231.79±20.10
10-12	194.97±24.23	231.63±29.32	327.56±54.58*	176.49±13.02
13-15	188.50±25.11	179.30±15.39	277.15±49.60*	187.33±23.26
DNMTS (delay intervals)				
5 min	205.90±37.42	165.00±17.28	158.79±38.66	155.82±20.49
30 min	257.20±26.94	173.11±22.02	183.44±34.78	161.27±29.06
240 min	257.50±36.48	208.44±27.14	187.79±32.16	144.46±13.25
2nd DNMTS (6 days)				
1-2	278.45±45.61	193.00±12.97#	196.39±26.53	195.68±18.86#
3-4	290.10±41.11	174.17±21.94#	172.61±33.59	152.27±9.49#
5-6	260.80±42.56	171.50±13.30#	211.06±39.48	172.91±21.06#
DMTS (8 days)				
1-2	239.90±24.68	184.94±13.75	218.06±39.37	192.82±10.68
3-4	241.15±33.42	167.61±11.66	196.89±51.58	163.86±15.35
5-6	201.10±26.10	160.44±19.53	200.17±52.09	144.46±12.87
7-8	186.45±20.18	143.56±13.62	209.28±51.61	143.55±7.64

Retention trials	Sham-AL	Sham-FR	Isch-AL	Isch-FR
1st DNMTS (15 days)				
1-3	450.97±43.54	383.78±46.47	439.00±47.75	425.18±38.86
4-6	327.17±43.37	275.63±53.36	347.63±56.92	234.72±45.20
7-9	264.57±40.97	234.78±36.83	230.07±42.21	192.88±44.65
10-12	215.80±32.20	171.70±23.26	196.30±50.24	180.91±27.41
13-15	188.60±31.07	160.04±14.96	167.22±49.94	178.79±41.92

(*) indicates that ischemic rats fed an ad lib diet took considerably more time to complete the first DNMTS task than sham-operated rats ($p = .004$) and food-restricted ischemic rats ($p = .001$). (#) indicates that food-restricted rats took considerably less time than ad lib fed rats to complete the acquisition trials of the 2nd DNMTS task ($p = .038$).

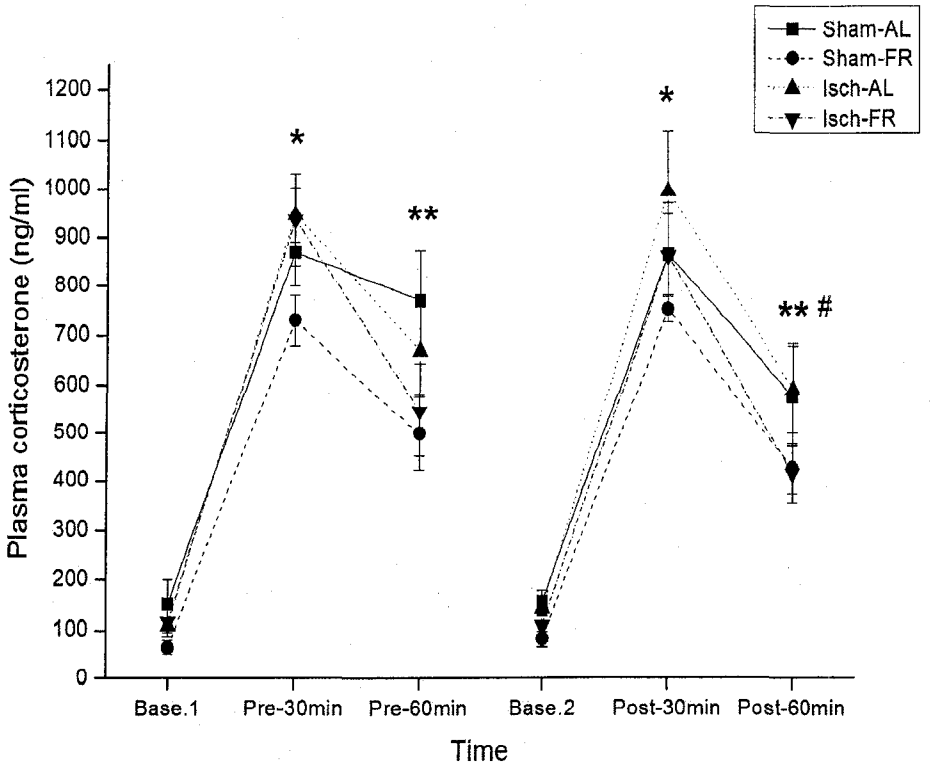


Figure 16: Effects of the ad lib and restricted diet on baseline CORT concentrations and the impact of a 15 min restraint stress on blood concentrations 30 and 60 min following stress induction. Blood collection was performed one month prior (Pre – left panel), and 68 days following sham surgery or 12-min global ischemia (Post – right panel). (*,**) indicates significant differences from respective baseline levels for all animal groups ($p \leq .001$). (#) reveals a significant difference in CORT levels between food-restricted and ad libitum fed rats 45 min following restraint stress ($p=0.017$).

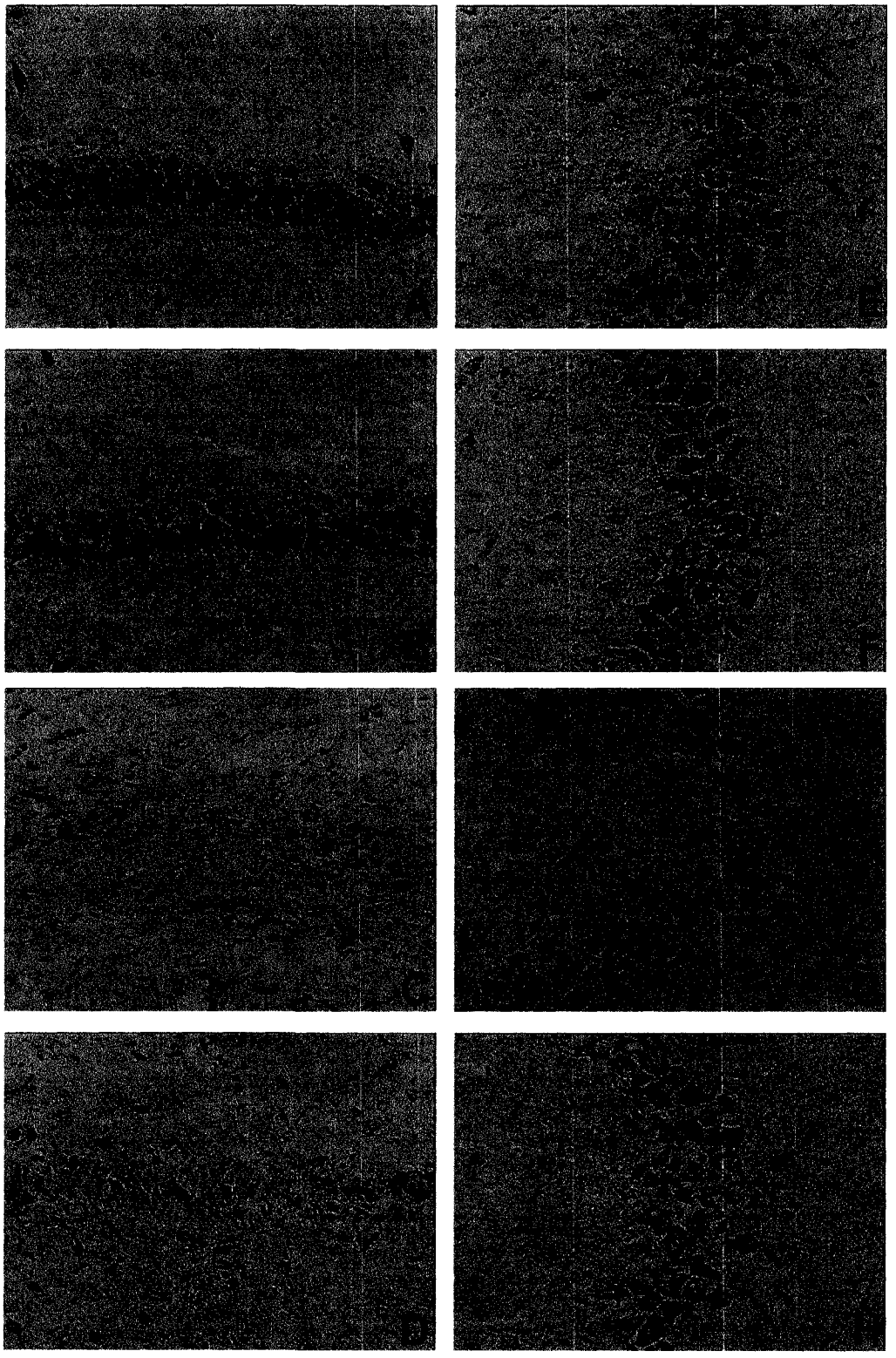
Table 4: Density of hippocampal CA1 and CA3 neurons (cells/1mm, CA1 and CA3 tissue) in AL and FR sham and ischemic animals, 70days following reperfusion.

	SHAM-AL	SHAM-FR	ISCH-AL	ISCH-FR
CA1	271.01±3.43	280.39±5.13	63.3±17.67*	83.11± 23.41*
CA3	237.73±6.38	237.15±8.21	135.71±18.72*	168.05±13.26*

(*) indicates that neuronal density of both ischemic groups was significantly reduced as compared to sham-operated rats ($p < .001$).

Figure 17: Representative photomicrographs of neuronal density in the hippocampal CA1 (A, B, C, and D; left column) and CA3 (E, F, G, and H; right column) pyramidal cell layers in ad libitum and food-restricted sham-operated and ischemic rats 70 days following surgery: (A and E) ad lib fed sham-operated rats; (B and F) food-restricted sham-operated rats; (C and G) ad lib fed ischemic rats; (D and H) food-restricted ischemic rats.

Figure 17.



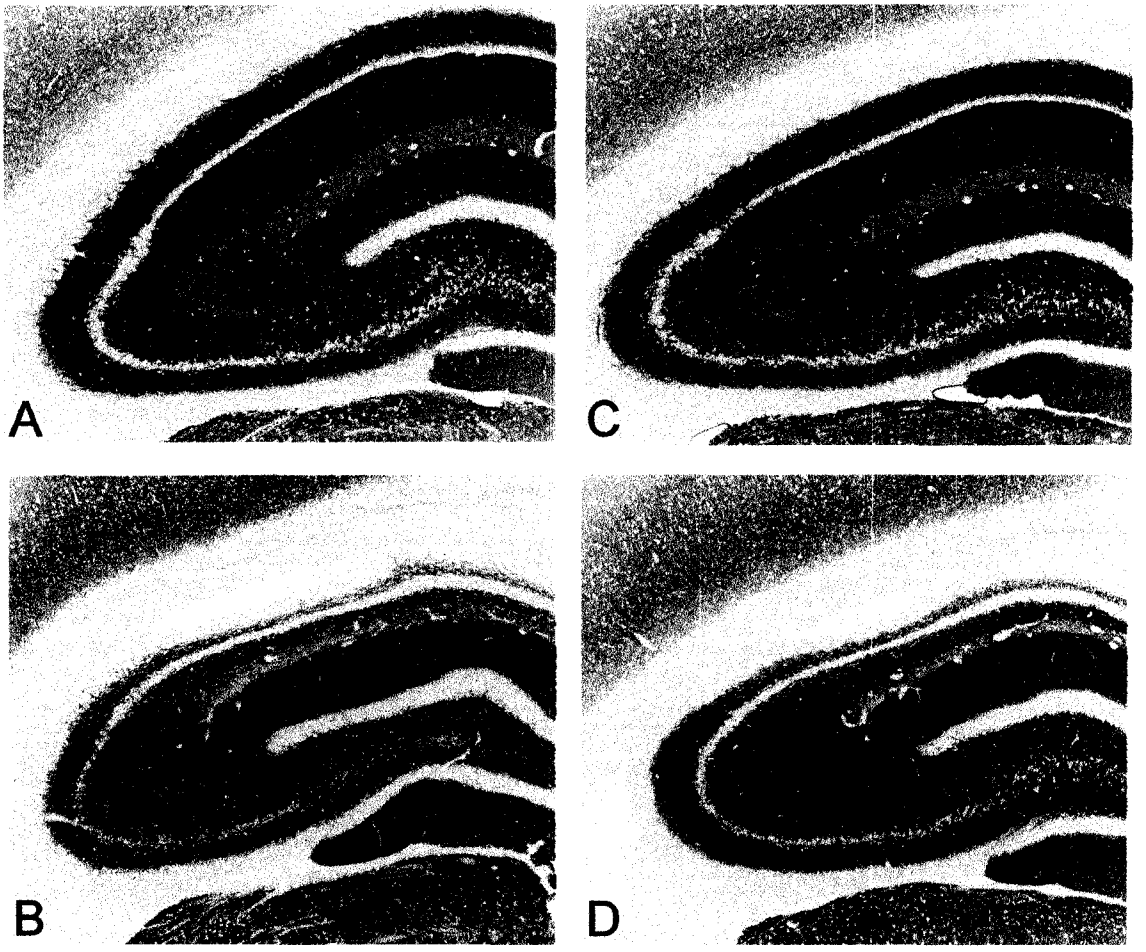


Figure 18: Representative photomicrographs of vGluT1 immunofluorescence in ad libitum (A and B; left column) and food-restricted (C and D; right column) sham-operated (A and C) and ischemic (B and D) rats 70 days following surgery.

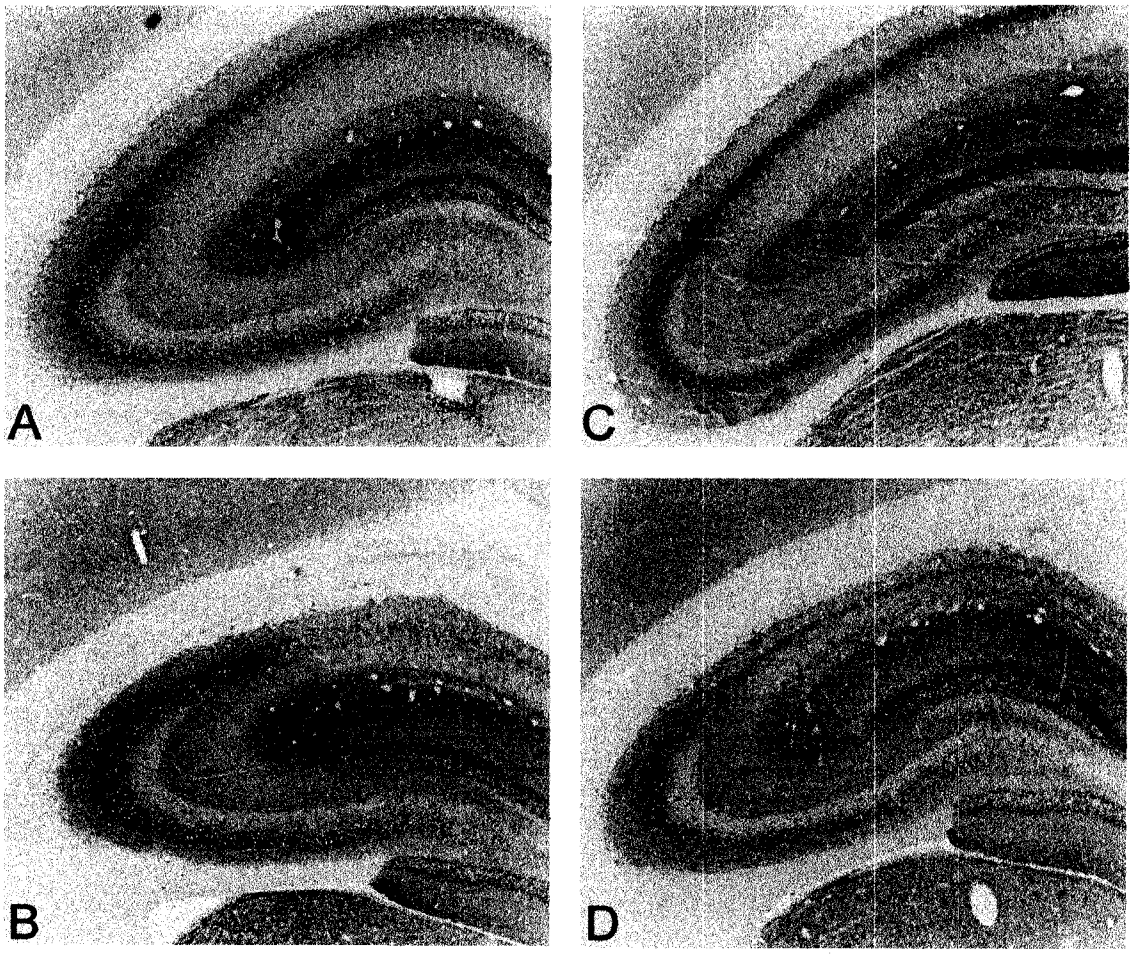


Figure 19: Representative photomicrographs of vGAT immunofluorescence in ad libitum (A and B; left column) and food-restricted (C and D; right column) sham-operated (A and C) and ischemic (B and D) rats 70 days following surgery.

Table 5: Signal to noise ratio of vGluT1 immunofluorescence in 14 μm tissue sections.

Areas	**Sham-AL**	**Sham-FR**	**Isch-AL**	**Isch-FR**
1. CA1 stratum oriens	382.51±12.9	359.22±25.8	192.88±43.7*	188.27±39.3*
2. CA3 stratum oriens	367.89±18.3	361.81±13.6	306.65±37.6	354.02±37.0
3. Stratum lucidum	336.90±10.5	339.88±4.9	348.85±18.1	349.44±19.6
4. DG lacunosum molecular layer	208.87±12.4	201.38±10.7	169.87±13.8*	161.15±18.9*
5. DG molecular layer	286.58±21.9	309.97±11.2	348.63±29.9#	356.72±23.4#
6. DG hilus	246.68±21.4	258.82±19.0	311.93±31.2#	312.63±27.8#
7. CA1 stratum radiatum	363.37±15.2	352.26±25.8	262.09±41.4*	269.59±30.2*
8. CA3 stratum radiatum	281.07±7.6	294.68±11.6	273.53±24.4	312.46±28.6
9. CA1 pyramidal cell layer	97.30±7.8	94.08±7.8	58.93±12.2*	46.47±10.1*
10. CA3 pyramidal cell layer	72.77±8.0	77.93±6.0	95.76±9.1#	106.38±16.7#
11. DG granular cell layer	13.96±7.0	15.60±4.2	25.50±6.3	20.79±4.1
12. Corpus Callosum	39.15±1.6	40.14±0.8	40.23±1.5	37.43±1.2

Signal to noise ratio is defined by the relationship (specific IF – background IF) / (background IF) x 100.

(*) indicates that SNR of both ischemic groups was significantly reduced as compared to sham-operated rats, in the stratum oriens at the CA1 level ($p < .001$), the CA1 pyramidal cell layer ($p < .001$), the stratum radiatum at the CA1 level ($p = .009$), and the lacunosum molecular layer of the dentate gyrus ($p = .017$).
(#) indicates that SNR of both ischemic groups was significantly superior of that of sham-operated rats, in the CA3 pyramidal cell layer ($p = .004$), and the molecular layer ($p = .038$), and hilus of the dentate gyrus ($p = .039$).

Table 6: Signal to noise ratio of vGAT immunofluorescence in 14 μm tissue sections.

Areas	Sham-AL	Sham-FR	Isch-AL	Isch-FR
1. CA1 stratum oriens	34.10±1.7	29.79±2.8#	43.01±4.1	32.98±2.2#
2. CA3 stratum oriens	40.89±2.9	40.20±3.7	59.94±8.6	40.24±9.6
3. Stratum lucidum	32.64±3.1	32.42±2.2	33.40±5.5	24.08±2.9
4. DG lacunosum molecular layer	57.23±5.2	56.09±2.1	55.91±8.4	43.78±3.0
5. DG molecular layer	63.83±6.2	69.20±5.8	66.54±7.1	52.05±4.3
6. DG hilus	88.38±3.5	90.83±10.1	88.40±5.3*	73.81±7.0*
7. CA1 stratum radiatum	32.15±4.1	29.65±2.6	48.38±4.5**	40.24±4.9**
8. CA3 stratum radiatum	42.90±1.8	41.51±3.3#	52.09±3.6	39.62±2.6#
9. CA1 pyramidal cell layer	86.72±2.2	84.14±4.6	70.85±9.6*	56.95±2.5*
10. CA3 pyramidal cell layer	95.61±6.1	90.41±6.5	96.34±15.2	74.18±7.2
11. DG granular cell layer	39.80±1.4	37.40±5.1	28.86±4.0	27.37±4.3
12. Corpus Callosum	72.89±2.3	70.64±2.7	72.05±2.2	73.83±1.5

(*) indicates that SNR of both ischemic groups was significantly reduced as compared to sham-operated rats in the CA1 pyramidal cell layer ($p < .001$), and the molecular layer of the dentate gyrus ($p = .023$).
(**) indicates that SNR of both ischemic groups was superior of that of sham-operated rats in the stratum radiatum at the CA1 level ($p = .011$). (#) indicates that SNR of food-restricted animals was significantly lower of that of ad lib fed animals, in the stratum oriens at the CA1 level ($p = .027$) and the stratum radiatum at the CA3 level ($p = .037$).

Discussion

The purpose of this study was to assess the impact of 3-month food restriction on ischemia-induced learning and memory deficits using spatial delayed non-matching- and matching-to-sample radial arm maze tasks. These tasks are believed to be more complex than the standard radial arm maze task based on the cognitive abilities that they required. The standard radial arm maze task is a measure of allocentric spatial memory and working memory. DNMTS/DMTS tasks are also measures of allocentric spatial memory and working memory but also include reference memory and winshift/winstay procedures. Consequently, more brain structures are involved in the latter tasks. The main finding of this experiment was the preserved spatial memory observed in food-restricted ischemic rats in all delayed tasks. In contrast, ad lib fed ischemic animals made significantly more errors in both tasks. The findings are intriguing because ad lib and food-restricted ischemic animals had comparable CA1 and CA3 cellular damage.

These findings raise a number of questions. The first one is whether food restriction, as such, produces changes in the brain that allow more efficient learning and memory for example by recruiting other brain areas to perform spatial tasks. These changes could be conceivably induced by food scarcity and be an adaptive response to increase foraging abilities. In the present experiment, however, the sham-operated food-restricted animals did not perform better in the spatial tasks even though there was a slight advantage to food-restricted sham animals immediately following the switch from DNMTS to DMTS. This lack of effect in sham animals is at odds with previous demonstrations of the beneficial effects of food restriction on memory performance [29,199,202,313,376,432]. A possible explanation is the presence of a ceiling effect in the

tasks for the sham animals. However, that explanation does not account for lack of difference in the retention tests at the beginning of the series of tests, where improvement was possible. Thus, at least within the limits of the present experiment, the effect of food-deprivation in the ischemic group does not appear to be linked to a general improvement in learning and memory abilities.

The second question is whether food restriction is conducive to improved neuronal plasticity following injury and thus leads to improved learning abilities in the face of equal neuronal damage through a more efficient reorganization of the remaining neuronal pathways. Because animals were euthanized 70 days post-ischemia, there was limited opportunity to test this hypothesis. We conducted preliminary experiments on other groups of rats that underwent similar food restriction euthanized at various delays (i.e., 7, 31, and 70 days) after ischemia. We did not find, at any of the delays, any difference in the number of apoptotic cells using caspase-3 immunolabeling or any increase in the number of doublecortin-positive cells (newly-formed neurons) in the hippocampus of food-restricted ischemic animals compared to ad lib ischemic ones. Casual examination of these brains did not reveal obvious differences in caspase-3 labeling in other brain regions involved in spatial memory. Although these observations are from other animals, they suggest that food restriction does not produce its beneficial effect on learning and memory through a large reduction of neuronal damage or an increase of adult neurogenesis.

Alternatively, the trend towards increased survival of CA3 neurons in the food-restricted rats could be entertained as a possible basis for functional sparing in these animals. However, CA3 neurons are part of a pathway projecting to the CA1 and CA2

layers before projecting back the entorhinal cortex, perirhinal cortex and neocortex [30,106,243]. Although a small increase in cell survival in the CA3 region could promote reduction of ischemia-induced memory impairments in food-restricted animals, the extensive cell loss that we observed in the CA1 in these animals makes it unlikely that the surviving CA3 neurons played an extensive role in the functional sparing observed in food-restricted rats.

A final thought on the possibility that food restriction could exert some impact post-ischemia. Although possible, the fact that "ad lib" animals were essentially food-deprived starting 6 days post-ischemia until the end of the behavioural testing and that "food-deprived" rats were actually not deprived during the immediate post-operative period seems to rule out this possibility. Indeed, since the "ad lib" animals did have important neuronal losses and limited success in the behavioural tasks, it is unlikely that food restriction produced its effects in the immediate post-operative period (when all animals had ad lib access to food) or during the lengthy behavioural testing period (when all animals were food-deprived). Moreover, the fact that the food restriction was begun well before the ischemia suggests that it must either provide protection from memory deficits or facilitate recovery from a deficit: the results of Figure 12 indicate a protection rather than a facilitation of recovery. Taken together, these observations suggest that the pre-ischemic food deprivation is the crucial step that led to better functional sparing in the food-restricted animals. Obviously, this hypothesis will need to be further addressed before a final conclusion is reached.

A number of physiological hypotheses could have been entertained had the design of the present experiments allowed us to make observations at other time point than the

end of the behavioural observations. Beyond the doublecortin and caspase-3 observations described above, we chose to examine GABA and glutamate vesicular transporters because of the central role of these neurotransmitters in excitation and inhibition. Overall, the distribution patterns of vGluT1 and vGAT expression in sham-operated and ischemic animals in this experiment were consistent with previous reports [12,83,223,233,429, 454].Our findings revealed higher vGluT1 immunofluorescence in most hippocampal substructures in sham-operated animals. Moderate to intense vGAT immunofluorescence was found along the granular, molecular, and lacunosum molecular layers of the dentate gyrus, and along the CA1 and CA3 pyramidal cell layers, and the stratum oriens and radiatum in these animals. In contrast, ischemic rats showed significantly higher vGluT1 expression in the CA3 pyramidal cell layer, and the molecular layer and hilus of the dentate gyrus. vGAT distribution in the hippocampal formation was similar in ischemic and sham-operated animals, with the exception of reduced vGAT expression in the CA1 pyramidal cell layer in ischemic animals. These alterations were independent of the consumed diet.

In the current study, comparable vGluT1 expression was observed in food-restricted and ad lib fed rats in various hippocampal substructures. This study is the first to observe changes in the vesicular transporters in food-restricted animals rendering direct comparisons impossible. To date, studies assessing glutamatergic transmission in food-restricted animals have generated mixed finding. Thus, while studies have reported significant inhibition of aged-related decline of NMDA-R1 and AMPA-GluR1 receptors expression and long term potentiation deficits in food-restricted rodents [60,128,153,154, 176,218,443], Newton et al. [344] and Shi et al. [419] demonstrated lower level of

NMDA-NR1 and AMPA-GluR1 subunits in young food-restricted animals as compared to ad lib fed animals, levels that remained stable throughout lifespan. Of interest, Guo et al. [173] demonstrated that HSP-70 and GRP-78 protein levels are more elevated in food-restricted rats' synaptosomes as compared to ad lib fed rats. As a result, their synaptosomes are more resistant to oxidative and metabolic damage given a better glucose preservation, glutamate transportation, and mitochondrial functions. Furthermore, alternate day feeding has been shown to delay age-related dendritic spines losses or configuration changes within pyramidal cells [330]. These findings suggest plastic changes at the synaptic levels following food restriction that may promote better neurotransmission despite discrete alterations in glutamatergic transporter density.

In addition, food-restricted rats showed lower vGAT expression in the stratum oriens and radiatum at the CA1 and CA3 levels, respectively. This is interesting because the inputs to the CA3 pyramidal cell layer from the dentate gyrus via the perforant path and the mossy fibers travel through the CA3 stratum radiatum. Moreover, the outputs of the CA1 pyramidal cell layer to the subiculum and entorhinal cortex travel through the stratum oriens at the CA1 level. A decrease of inhibition in these two hippocampal substructures as suggested by lowered vGAT expression could lead to enhanced excitatory signals, allowing better information transfer to the remaining neurons in the CA1 pyramidal cell layer, subiculum, and entorhinal cortex. Although the functional relevance of these observations will have to be specifically assessed, they could conceivably have led to better consolidation of spatial information in food-restricted ischemic rats despite significant CA1 and CA3 neuronal loss.

One month prior to induction of surgery and 68 days following reperfusion, we collected three blood samples (baseline, 30 and 60 min following restraint onset) in order to evaluate the effect of the surgery and the diet on basal corticosterone levels and variations in the different conditions upon acute stress exposure. Studies have demonstrated that corticosterone (CORT) levels increase considerably with age in ad libitum fed rodents, and that chronic elevations of CORT have deleterious effects on the animals' brain and behaviour including hippocampal atrophy, cognitive impairments, decreased long-term potentiation, and reduced neurogenesis [366]. In recent years, food restriction has been shown to considerably increase the endogenous peak of plasma and serum CORT levels in the diurnal period preceding feeding [13,77,342,406]. Under food restriction regimens, circadian synchronization of food-restricted and ad libitum fed animals is usually not concordant because ad lib animals feed throughout the dark photoperiod while food-restricted rats typically consume food in fewer episodes usually during the light period. In animals fed during the light period, food restriction produces a peak of CORT in the morning whereas CORT levels are usually low during that period in ad libitum fed animals [259]. In the current study, the observations of comparable baseline CORT levels in ad lib and food-restricted rats pre and post surgery and increased CORT inhibition in food-restricted rats only following stress make it unlikely that CORT levels could explain the behavioural differences observed between food-restricted and ad lib animals.

Studies that have assessed blood glucose levels in ad libitum and following daily food restriction have shown reduction in blood glucose as compared to ad lib rats, with concentrations between 120-130 mg/dL in food-restricted compared to 140-160 mg/dL

for ab libitum ischemic rats. Interestingly, although plasma glucose are lower, food-restricted rats use glucose fuel at the same rate per unit of metabolic mass per day as rats fed ad libitum [297]. At present, the effects of hypoglycemia are far less established and Auer demonstrated hypoglycemic brain damage after glucose levels have fallen below 1mM (18mg/dL) [16] or between 1-3 mM [492]. In contrast, blood levels in the range of 6-7 mM (108 – 126 mg/dL) had no impact on neuronal necrosis [492]. Thus, it appears that drastic variations in glucose are unlikely to have played a determinant effect on cell survival. Nonetheless, given that the FR regimen lasted for 3 months prior to the ischemic insult, it is possible that other physiological processes were affected. Such differences (e.g., 1°C lower temperature, slightly improved CBF) might lessen cell death in structures (e.g., hilus, subiculum, posterior CA1 and CA3) that were not assessed in the current study.

The observation of opposite behavioural profiles in the radial arm maze despite comparable neuronal degeneration in the CA1 and CA3 hippocampal subfields of ad lib fed and food-restricted ischemic rats raises the question whether hippocampal pyramidal neurons and their associated neuronal circuits are necessary for the acquisition and retention of DNMTS or DMTS. Two aspects of our tasks are relevant for hippocampal function: the first one is the delay in receiving the reinforcement and the second is the spatial navigation required to successfully perform the tasks. Although performance on DNMTS and DMTS tasks based on object recognition was initially claimed to be hippocampal dependent [88,397,469], a number of studies showed that hippocampal lesions do not always impair DNMTS or DMTS performance. For example, ischemic monkeys with mild to moderate CA1 cell loss had no memory impairment in a similar

DNMTS task [413]. Also, ischemic rats with CA1 damage were not impaired in a delayed-non-matching-to-position task in a Skinner box; however, they were unable to use allocentric spatial information in the Morris Water Maze [341]. Contrary to ischemic rats with partial hippocampal lesions which showed impairments in DNMTS and DMTS object recognition tasks, rats with complete hippocampal ablation were found to be unimpaired or only mildly impaired in these tasks [332,334]. Furthermore, ischemia followed by hippocampal ablation failed to impair object recognition performance in rats, again suggesting the contribution of extrahippocampal structures in delayed recognition task. Finally, Duva and colleagues [125] observed that partial NMDA-induced lesions of the dorsal hippocampus, which mimic the hippocampal damage following a 2-VO occlusion, failed to alter performance in a similar DNMTS task in rats with or without pre-operative training. However, these animals showed important acquisition deficits on a spatial reference memory task in the water maze. Taken together, these results suggest that delayed aspects in non-spatial recognition tasks are not solely dependent on hippocampal function, but involve extrahippocampal regions in rodents and primates. Alternatively, these extrahippocampal regions could become involved only after the hippocampus is damaged. Bachevalier and Mishkin [21] suggested that partial hippocampal damage may result in a functional disorganization of the remaining hippocampal circuits interfering with afferent connections to extrahippocampal regions. Some evidence suggests that cortical and subcortical structures including the perirhinal, subicular and parasubicular regions and the medial prefrontal cortex, may play an important role in tasks involving delays, object recognition or spatial memory [20,190,440].

More recently, Lee and Kesner [260] demonstrated that HPC or PFC lesioned-rats initially showed deficits using a short delay (10s) in a DNMTS task in the radial arm maze; however, both groups improved to sham level over time. In contrast, rats with combined lesions of HPC and PFC showed lasting impairments. Interestingly, a 5-min inter-trial delay was shown to selectively affect hippocampal-lesioned rats whose performance worsened while PFC-lesioned rats learned the win-shift rule with such delay. In a similar experiment, learning deficits were found in rats with lesions of either CA3 subfield of the hippocampus or the dentate gyrus in DNMTS radial maze using 10-sec and 5-min delays while impairments in CA1 lesioned animals were observed only with the longer 5-min inter-trial delay [261]. These results demonstrate that hippocampal lesions lead to deficits in spatial tasks with a relatively long delay ($\geq$ 5 min) such as DNMTS and DMTS in the radial arm maze but there are indications that with shorter delays, animals can overcome the effect of hippocampal lesions with extended training. In the present study, it is difficult to ascertain that deficits observed in the DNMTS radial maze task in ad lib fed ischemic animals are delay-dependent. To make such statement, it would have been useful to vary delay in the initial testing period to appreciate change in acquisition of the task with distinct delays. Our findings show that a 15-min inter-trial delay led to significant memory impairments in ischemic ad lib rats that persisted during the 15-day testing period. However, once DNMTS training had taken place, additional testing using variable delays had no impact on rats' performance. Thus, performance of ischemic ad lib animals using a 30-min delay was equivalent to that seen on the 15th testing day in the initial DNMTS task, while performance using 5- and 240-min delays appeared slightly improved. In contrast, memory improvement gradually observed in

food-restricted ischemic rats in the first DNMTS task suggests that their performance was not delay dependent. This is further supported by the fact that they reached criterion in about the same time in the 5, 30, or 240-min inter-trial delay conditions as they did in the initial DNMTS task. Food-restricted ischemic rats also had identical performance as that of sham-operated rats fed either diet. Thus, our results indicated that once the task was learned, increasing delays did not impair retention.

In our experiment, ad lib fed ischemic rats were significantly impaired in the DMTS task as they persevered in visiting alternate arms in retention trials normally baited in DNMTS task. Surprisingly, they appeared to predominantly use a DMTS strategy when they initially had to perform a DNMTS task and a DNMTS strategy in the DMTS task. As they made progress using DNMTS strategies, they appeared unable to rapidly shift from one strategy to another suggesting a lack of cognitive flexibility. In addition, although no significant differences were found between the animal groups in the frequencies of microchoices performed during acquisition trials, ad lib fed ischemic rats made consistently more microchoices in the retention trials on both DMNTS and DMTS tasks than food-restricted ischemic rats and sham-operated rats fed either diet. In order to complete the behavioural tasks, they made more macrochoices than other groups, which included correct responses and more particularly proactive and retroactive errors. Taken together, these results demonstrate spatial memory impairments and lack of cognitive flexibility in ad lib fed ischemic rats. In contrast, food-restricted animals made fewer microchoices and demonstrated more adaptive cognitive responses in both DNMTS and DMTS tasks within few days.

A final consideration is that repeated testing over 70 days can be taken as a form

of cognitive rehabilitation and that food restriction may have facilitated this process and led to improved neuronal plasticity and learning abilities. This hypothesis remains to be tested but could be significant.

In summary, our study is the first to demonstrate that 40% food restriction confer protection against cognitive impairments produced by global ischemia. Food-restricted ischemic rats were able to learn complex memory tasks and demonstrated superior adaptive behaviour and cognitive flexibility despite comparable CA1 and CA3 neuronal loss as ad lib fed ischemic rats. These results suggest an impact of the diet regimen on endogenous mechanisms that play a role in neuronal plasticity and/or promote compensatory mechanisms or adaptive changes within brain structures.

Manuscript 3

Dietary PUFA supplements reduce memory deficits but not CA1 ischemic injury in rats.

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Author Contribution

Marie-Claude Roberge: Unless otherwise indicated all experiments were performed by the first author under the supervision of Dr. Hélène Plamondon.

Hélène Plamondon: This author supervised the research work of this paper, edited the manuscript, and provided financial support.

Abstract

The purpose of the present study was to examine the impact of nutritional supplementation with essential omega 3 and 6 fatty acids on CA1 neuronal death and recovery of functional impairments following global ischemia. Groups of Wistar male rats were randomly assigned to four experimental conditions determined by the consumed diet and surgical condition. Rats either received a standard diet (SD; Purina 5012) or a 15% PUFA supplemented diet (FO+CO) prepared by adding 11.5% (w/w) fish oil from menhaden fish and 3.5% corn oil to standard rat chow. Diet conditions were initiated in 30-day old rats and maintained for an 18-week period (pre and post surgery). Sham or 8-min global ischemic surgeries occurred during the 13th feeding week and behavioural testing took place following reperfusion for an additional 4 weeks, after which all rats were euthanized. Our findings revealed significant loss of pyramidal CA1 neurons 31 days post-ischemia in ischemic as compared to sham-operated rats but no difference between ischemic animals fed the SD or PUFA supplemented diet. In the radial arm maze, SD ischemic rats took longer time to complete the task and made significantly more working memory errors than PUFA ischemic and sham-operated animals. Independent of the diet, ischemic animals appeared less anxious in the elevated plus maze, spending considerably more time in the open arms as compared to sham-operated rats. Taken together, these results suggest that a PUFA supplemented diet exerts beneficial effect on ischemia-induced spatial memory deficits despite absence of protective effects on CA1 hippocampal neurons.

Introduction

Numerous studies have suggested that polyunsaturated fatty acids (PUFA) exert beneficial effects on various pathological states including cardiovascular and autoimmune diseases [4,64,111,337,369]. At the cellular level, PUFA have been shown to promote structural integrity of neuronal membranes, enhance hippocampal dendritic spine density and confer protection against ischemia-induced apoptotic cell death [28,408]. Unable to synthesize these molecules, mammals consume these essential fatty acids as part of their diet. High levels of omega-3 PUFA, including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are derived from the tissues of oily fish.

Recently, different studies have suggested that short-term consumption of fish oil supplemented diet significantly attenuated brain damage following middle cerebral artery occlusion in rats [86,139,398]. Similarly, when administered for 14 days prior to bilateral common carotid occlusion, fish oil (0.4g/kg/day) significantly attenuated ischemia-induced cellular oxidation and apoptosis in rats' hippocampal formation [28]. Concomitant to improvement of cerebral microvascular functions, consumption of PUFA supplemented diets significantly reduced learning impairments in the Morris water maze in ischemic animals [107]. At present, investigation of the contribution of PUFA supplemented diets on neuronal damage and memory deficits following global ischemia remains limited. Administration of alpha-linolenic acid for three days prior to 6-min ischemia in rats led to significant increase of heat shock proteins (HSP70) expression associated with reduced ischemia-induced CA1 neuronal injury [44]. Moreover, DHA administration for 21 days significantly attenuated ischemia-induced hippocampal injury and spatial memory deficits in the radial maze [353]. These findings suggest that PUFA

supplementation can alter ischemia-induced cellular processes and/or functional deficits. However, all studies have assessed short-term PUFA exposure (≤ 6 weeks) and cellular molecular and recovery at early reperfusion intervals, leaving the long-term effects of prolonged PUFA intake on ischemia-induced neuronal and functional outcomes undetermined.

Thus, the aim of the current study was two fold: to examine the impact of a three month PUFA supplemented diet on hippocampal cellular damage following 8 min global ischemia, and determine the effects of prolonged PUFA supplement intake on locomotion, spatial memory and anxiety in control and ischemic animals.

Materials and methods

Animals and dietary protocols

Male Wistar rats (N=39), weighing between 100-125 g at arrival into the animal care facility, were obtained from Charles River Laboratories (Rochefort, Quebec, Canada). The animals were individually housed and maintained on a 12 h light/dark cycle (lights on at 7:00 AM), with free access to water and standard rat chow. The room temperature was maintained at 21-23 °C with 60% relative humidity. Rats were fed a standard laboratory chow for the first week while adapting to the new environment. Following this period, rats were randomly assigned to one of two dietary conditions for an 18-week period, the surgical procedure occurring on week 13 following initiation of the diet (see Figure 20 for the experimental protocol). Dietary regimens continued until euthanasia (i.e., 31 days following reperfusion). One group of animals continued to have free access to

a standard rat chow (SD: Purina # 5012), consisting of 22.5% protein, 4.02% fat, 4.6% fiber, and 52.9% carbohydrates. The second group received a 15% PUFA supplemented diet (FO+CO), prepared by adding 11.5% (w/w) fish oil from menhaden fish (Sigma-Aldrich, Canada) and 3.5% corn oil (commercial oil, No Name brand) to the standard diet, which increased n3 and n6 fatty acid content, respectively. The 4.02% fatty acids content of the regular chow included Linoleic acid (1.81%), Linolenic acid (18:3n-3; 0.12%), Arachidonic acid (20:4n-6; ≤0.01%), Omega-3 (0.31%), monounsaturated (1.03%) and saturated (0.74%) fatty acids. In the supplemented diet, the fish oil contained approximately 25% n-3 (octadecatetraenoic, eicosapentaenoic acid - EPA - and docosahexaenoic - DHA) fatty acids. The fatty acid composition of fish oil was approximately as follows: 0.1% 12:0, 10.8% 14:0, 23.2% 16:0, 4.2% 18:0, 0.4% 20:0, 0.1% 22:0, 11.4% 16:1, 10.6% 18:1n-9, 1.3% 20:1, 1.8% 18:2n-6, 1.7% 18:3n-3, 0.9% 16:2, 13.5% 20:5n-3, 1.7% 22:1, 9.9% 22:6n-3 (percent of total fatty acids) [320]. The corn oil contains at least 98% of lipids, including more than 60% of linoleic acid and less than 1.5% of linolenic acid [349]. Table 7 shows the approximate polyunsaturated fatty acids content of the experimental diets (g/100g).

To control PUFA intake by individual rats, the oil supplement was added daily to individual portion of standard diet. A pilot study done in our lab indicated that adult rats consumed on average 30 grams of standard rat chow per day. Based on these observations, the amount of oil supplemented to the diet was calculated individually using the formula: (FO: food weight (g) × 0.115; CO: food weight (g) × 0.035). The standard chow contained 4.02 g of fat per 100 g, providing 1.2 g fat

per 30 g individual portion. Individual portion of the FO+CO diet contained 3.45 g of menhaden oil and 1.05 g of corn oil added to 30 g of standard chow (already containing 1.2 g of fat). The resulting product weighted 34.5 g and contained 16.5% fat, of which 12.5% came from PUFA supplementation. Therefore, the FO+CO supplemented diet provided additional lipid energy of about 110 kcal/100 g of diet compared to the standard chow. To minimize oxidation, fresh food pellets were prepared daily. All food and oil were stored in the dark at 4°C. Food intake was recorded every morning between 8:00-9:00 AM. All animals had free access to food and water throughout the experiment. Rats were handled daily and weighted every two or three days, immediately after food was replenished.

Nineteen rats were fed the standard rat chow while twenty rats received the FO+CO supplemented diet. They were randomly assigned to one of the four experimental groups: rats fed a standard diet receiving sham surgery (SHAM-SD; n=9) or 8-min global ischemia (ISCH-SD; n=9), and PUFA supplemented groups receiving sham surgery (SHAM-FO+CO; n=9) or 8-min global ischemia (ISCH-FO+CO; n=10). These groups do not include the 3 animals that died following ischemic surgery (2 ISCH-SD, and 1 ISCH-FO+CO). All experiments are conform to NIH guide for the Care and Use of laboratory animals (NIH publications N°80-23, revised 1996) and procedures were carried out in accordance with the guidelines set by the Canadian Council of Animal Care and were approved by the University of Ottawa Animal Care Committee. All efforts were made to minimize the number of animals used and their suffering.

Surgical Procedures

Forebrain ischemia was performed using the four-vessel occlusion model as previously described by Pulsinelli and Brierley [391]. Briefly, rats were deeply anesthetized by inhalation of 2% halothane vaporized by oxygen (1.5 to 2 L/min). Vertebral arteries were irreversibly occluded by electrocoagulation, and a small-diameter silk thread looped around the carotid arteries to facilitate subsequent occlusion. Twenty-four hours later, the common carotid arteries were clamped with microaneurysm clamps for 8 minutes in awake and spontaneously ventilating animals. Sham-operated animals underwent anesthesia and received the same dorsal and ventral surgical incisions as the two ischemic groups, with the exception of electrocoagulation of the vertebral arteries. Twenty-four hours later, the carotid arteries were exposed, but not clamped. Body temperature was kept at 37°C ± 0.5 throughout the surgery using a feedback-regulated heating blanket connected to a rectal thermometer (Homeothermic Blanket Control Unit, Harvard Instruments, Natick, MA). Subjects' body temperature was further supported with a heating pad in the hours following surgery and reperfusion.

Behavioural testing

Open field

The arena was made of gray Plexiglas (LWH: 75 X 75 X 30 cm) with a clear Plexiglas floor. A painted grid divided the Plexiglas floor into 36 identical squares each measuring 15 X 15 cm. The arena was located on a table 90 cm above the floor, and was dimly illuminated. Black curtains surrounded the arena and behaviour was monitored using an overhead video camera. Animals were brought to the test room 30 min prior to

test administration. Four and thirty days following reperfusion, sham and ischemic rats were placed in the open field and behaviour were monitored for 15 min by the experimenter using a PC computer and data logging software. Rats were monitored for frequency of line-crossing and rearing behaviour, and time (sec) spent grooming or being inactive. Frequency of behaviour was assessed in the peripheral zone (defined as the 20 squares directly adjacent to the walls) and central zone (all remaining squares).

Elevated plus maze

The elevated plus maze (EPM) consisted of two opposing open arms (50 X 10 cm with a 5 mm clear Plexiglas lip), an open 10 X 10 cm area in the center and two opposing closed arms (50 X 10 cm with 40 cm high walls). The maze was 60 cm above the floor. Animals were tested 5 days following reperfusion and brought to the test room 30 min prior to test administration. Black curtains surrounded the maze and behaviour was monitored using an overhead video camera. Each rat was placed in the open field (75 X 75 cm with 30 cm high walls) for a 5-min habituation period. Immediately thereafter, the rat was placed in the center of the elevated plus maze facing one of the open arms of the maze. The time spent and the number of entries in the open or closed arms, as well as risk assessment and crossing behaviour were recorded. Crossing behaviour refers to the situation where the animal cross the center zone going from one arm to its opposite arm and were recorded during a 5 min test interval. A close or open-arm entry was defined as placing all four paws in an arm. Risk assessment was operationally defined as a stretch-attend response, whereas the rat stretched its body forward and either sniffed or visually scanned the open arm. In stretch-attend behaviour, head entry into the open arm was

required while paws and body remained in the center area or closed arm.

8-arm radial maze

The radial arm maze consisted of eight arms (60 X 12 cm with a 5 cm lip around each arm) extending radially from a central octagonal area (32 cm in diameter with a 30 cm high clear Plexiglas wall). Plexiglas sliding doors were moved up to allow entry into each arm. The floor of the arms and central area was covered with black rubber lining. The apparatus was elevated 50 cm above the floor and surrounded by extra-maze cues placed on the walls, such as posters or calendars. The experimenter was sitting behind a panel where he could observe and record behaviour unobtrusively as well as manipulate the overhead strings to open and close the maze doors. At the end of each arm, a food cup (1 cm deep into the floor) contained a piece of Fruit Loop. Twenty-four hours after the completion of the elevated plus maze (6 days following reperfusion), the animals began training in the radial maze. Again, animals were brought to the room 30 minutes prior to the testing. The weight of all animals were gradually reduced (over a 4-day period) and maintained at approximately 85% of their free-feeding rate during the testing period to ensure adequate motivation.

During training, rats were habituated to the radial arm maze during four 10-min daily sessions on successive days. The baits (small pieces of Fruit Loop cereal) were initially available throughout the maze to encourage exploration, and gradually moved towards the end of each arm to finally be restricted to the food cups. Following this shaping period, each animal was individually placed in the center of the maze, with doors to all arms closed. Upon opening the doors, the rat was allowed to enter any of the baited

arms. When the rat had consumed the bait at the end of one arm, it returned to the center of the maze and all doors were closed again, confining the rat to the center of the maze for a 10 sec delay. The doors were then reopened, and the procedure repeated. The trial continued until the eight baits were consumed or 15 min had elapsed. An arm entry was recorded when all four paws of the rat were within an arm. The orientation of the animal's head when initially placed in the central area varied from trial to trial to minimize development of a response pattern based on position. The number of working memory errors (re-entering an arm that was previously visited) and the time taken to consume all rewards were recorded. Animals were tested once daily (5 days per week) for a 16-day period. The floor and center walls of the maze were extensively cleaned after each trial, and identical lining used for each of the radial maze arm, minimizing the possible use of olfactory or tactile cues by rats. Although visual acuity is hard to directly measure in rats, daily manipulation of the animals showed no difference in home cage guided behaviour, or when tested in the radial arm maze.

Assessment of CA1 neuronal density on thionin-stained sections

Twenty-four hours upon completion of the behavioural testing, rats were deeply anesthetized using sodium pentobarbital and perfused using 4% paraformaldehyde. Brains were removed and stored at -84°C. Serial coronal sections (14 μm) of the hippocampal region were obtained using a cryostat (Leica Instruments) and stained with thionin. Neuronal density of the hippocampal CA1 subfield was determined by the method of Kirino et al. [239]. Analysis of neuronal density was performed on coronal sections located between 3.14 and 4.16 mm posterior to bregma [370]. The total linear

length of the CA1 (as defined by Paxinos and Watson [370]) was measured using a digitizer. The number of intact neurons in the stratum pyramidale of the CA1 subfield was counted using LEICA DAS microscope attached to a SONY digital camera and computer-assisted cell counting performed using the Norton Eclipse software (v 6.0). Neurons with shrunken cell bodies and surrounding empty spaces were excluded. The neuronal density of the CA1 sector, i.e. the number of intact pyramidal cells per mm linear length of the CA1 stratum pyramidale observed in each 14 μm section, was quantified. A mean value for each hippocampal CA1 substructure was obtained from 6 bilateral measurements in each animal. Neuronal density for a given animal represents the average of the right and left measures.

Statistical Analyses

Body weight, open field, and 8-arm radial arm maze data were analyzed using mixed ANOVA designs with two levels of the independent factors *surgery* and *diet,* and repeated time factors. The Hundt-Felt correction for violations to the assumption of sphericity was applied when appropriate: when used, adjusted degrees of freedom are reported. Significant interactions were further tested using simple main effect tests with a Bonferroni adjustment of the critical alpha level. Data obtained from the elevated plus maze and neuronal density were analyzed using two factors (*surgery and diet*) ANOVAs. Again, simple main effect tests were used to analyze significant interactions. All data were analyzed using the SPSS (V.14) software package.

Results

Body weight

Figure 21 presents the body weight of each group throughout the experiment. There was a main effect of time ($F(2.23,66.95) = 2712.34$, $p < .001$), surgery ($F(1,30) = 9.49$, $p = .004$), and interactions of time x surgery ($F(2.23,66.95) = 6.17$, $p = .003$) and time x diet ($F(2.23,66.95) = 9.49$, $p < .001$). In general, body weight increased over time. Simple effect tests revealed that ischemic rats lost more weight than sham-operated controls following surgery. Interestingly, ischemic rats fed the PUFA supplemented diet gained weight more rapidly than ischemic rats fed the standard diet on weeks 17 and 18 while being slightly deprived (maintained at approximately 85% of their free-feeding rate) to perform the radial arm maze.

Open field

Figure 22 presents the activity level in the open field every 5 min over the 15-min period 4 and 30 days following reperfusion. There were main effects of day ($F(1,31) = 33.06$, $p < .001$), time intervals ($F(2,62) = 106.16$, $p < .001$), surgery ($F(1,31) = 8.34$, $p = .007$), and interactions of days x time ($F(2,62) = 3.26$, $p = .045$) and days x time x surgery x diet ($F(2,62) = 3.22$, $p = .047$). Behavioural activity decreased with time within each testing period. Independent of groups, rats appeared more active 30 days following reperfusion. Ischemic animals fed either diet were considerably less active than sham-operated animals fed either diet across time. Simple effect tests revealed that sham rats fed a PUFA supplemented diet displayed higher activity level than ad lib sham rats in the initial 5 min interval of

day 4 ($p = 0.015$). Figure 23 presents the activity level in the central area of the open field 4 and 30 days following reperfusion. There was only a main effect of time within each test session ($F(2,62) = 38.74$, $p < .001$). Animals in all groups showed less activity in the central area of the open field as time elapsed.

Elevated plus maze

Figure 24 presents the behaviour of the animals in the EPM. There were main effects of surgery for the time spent in the open arms of the EPM ($F(1,30) = 5.34$, $p = 0.028$) and for crossing behaviour ($F(1,30) = 18.2$, $p < 0.001$). Ischemic rats fed either diet appeared less anxious than sham-operated animals as measured by a progressively longer time spent in the open arms within each test. However, sham rats made more crossing behaviour, indicating increased locomotion. Two rats were eliminated from analysis as one rat fell on the floor (ISCH-FO+CO) and one (ISCH-SD) remained immobile throughout the testing period.

8-arm radial arm maze

Figure 25 presents the performance in the radial arm maze and the impact of diet on working memory errors. There were main effects of time ($F(5.77,167.24) = 11.98$, $p < .001$), surgery ($F(1,29) = 7.8$, $p = .009$), and a significant surgery x diet interaction ($F(1,29) = 6.59$, $p = .016$). All animals made progressively less working memory errors across test sessions. Simple effect tests showed that, in general, ischemic rats fed a standard diet made considerably more working memory errors across time, as compared to ischemic rats fed the fish and corn oils supplemented

diet ($p = .03$), and both sham groups ($p < .001$). Table 8 shows the time taken by each group to complete the task. Statistical analyses revealed main effects of time ($F(3.75,108.81) = 29.5$, $p < .001$), and surgery ($F(1,29) = 6.8$, $p = .014$), and an interaction of surgery x diet ($F(1,29) = 5.94$, $p = .021$). As testing progressed, all animals took significantly less time to complete the task. Simple effect tests revealed that ischemic rats fed a standard diet generally took more time to complete the test than sham rats fed the same diet ($p = .001$). Three animals (1 ISCH-FO+CO, 2 SHAM-FO+CO) that did not eat the rewards following many trials were excluded from statistical analysis.

Histopathological changes at the hippocampus

Table 9 presents hippocampal CA1 neuronal density of animals that underwent an 8-min global ischemia or sham operation and were fed either a standard diet or fish oil supplemented diet. There was only a main effect of surgery ($F(1,32) = 156.23$, $p < .001$), sham-operated rats showing higher CA1 neuronal density than ischemic rats, irrespective of diet.

Covariances

Variability was observed within CA1 neuronal density of both ischemic groups. Therefore, to determine the impact of this neuronal variability on functional recovery, all behavioural analyses were re-analyzed using neuronal density as a covariate when there was a significant correlation between a particular behavioural measure and the neuronal density. Working memory errors and time to complete the

task in the radial maze were negatively correlated with the CA1 neuronal density (working memory errors: $r = -0.59$, $p < .001$; time to complete the task: $r = -0.43$, $p = 0.014$). The ANCOVA analysis revealed a main effect of diet on working memory errors ($F(1,28) = 4.26$, $p = .048$), and a significant surgery x diet interaction on the time spent to complete the radial maze task ($F(1,28) = 5.84$, $p = .022$). PUFA supplemented rats made less working memory errors than SD fed rats. Simple effect tests of the time taken to complete the radial maze task did not reveal any significant difference between groups. These results suggest that the impact of surgery or diet on the rats' performance in the 8-arm radial maze is not completely determined by the extent of CA1 neuronal death.

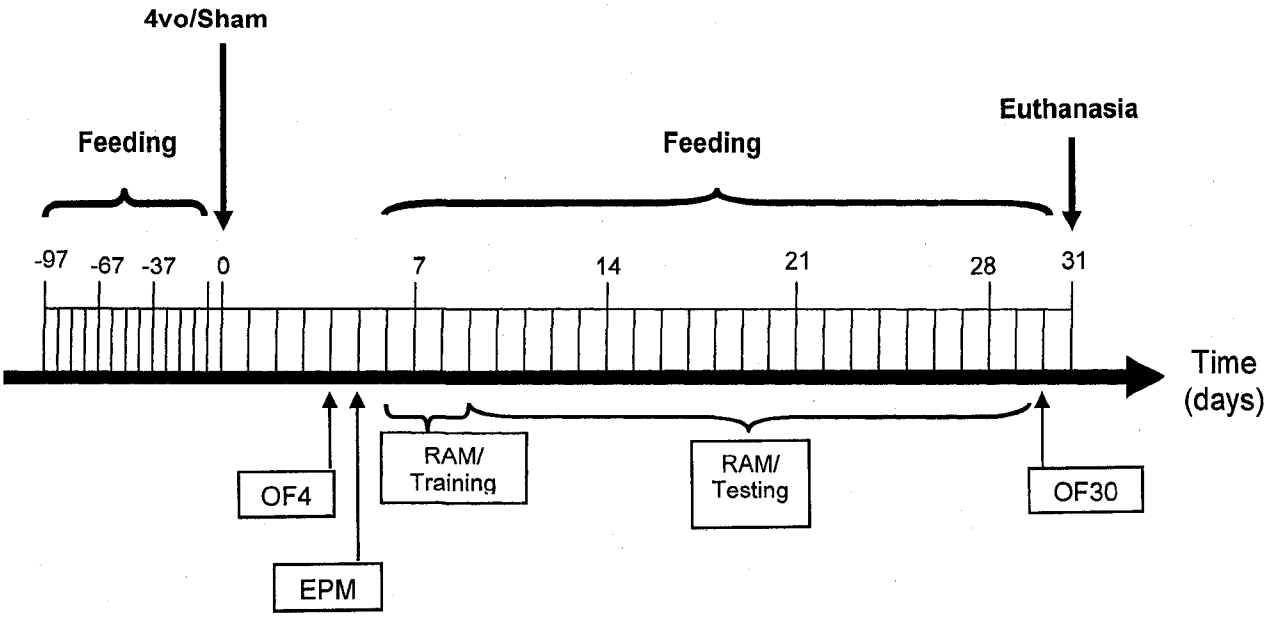


Figure 20: Experimental protocol. Feeding = Presurgical period of a standard or a fish + corn oils supplemented diets. 4-VO = 4 vessel occlusion. Day 0 refers to the day of surgery. After surgery, all animals were given 5 days of ad lib access to food and treats to hasten recovery. OF4 = Open field 4 days post-ischemia. EPM = Elevated plus maze 5 days post-ischemia. From days 6 to 9, the weight of all animals were gradually reduced and maintained at approximately 85% of their free-feeding rate during the remaining testing period to ensure adequate motivation. RAM/Training = Training on the radial arm maze from days 6 to 9 post-ischemia. RAM/Testing = Testing on the radial arm maze from days 10 to 29; OF30 = Open field 30 days post-ischemia.

Table 7: Approximate polyunsaturated fatty acids content of the experimental diets (g/100g).

Fatty acid	Experimental diets	
	Control (SD)	FO+CO
18:2n-6 (LA)	1.81	1.81(SD); 0.2(FO); 2.1[†] (CO)
20:4n-6 (AA)	≤ 0.01	≤ 0.01
Total n-6	1.81	4.1
18:3n3 (ALA)	0.12	0.12(SD); 0.2(FO); 0.05[†] (CO)
20:5n-3 (EPA)	0.31[*]	0.31(SD); 1.55(FO)
22:6n-3 (DHA)		1.14(FO)
Total n-3	0.43	3.37
n-6/n-3 ratio	4.21	0.89

[†] calculated from typical fatty acids composition of corn oil [15].
[*] reported total % omega-3 fatty acids from fish meal (EPA & DHA) in Purina 5012 diet.

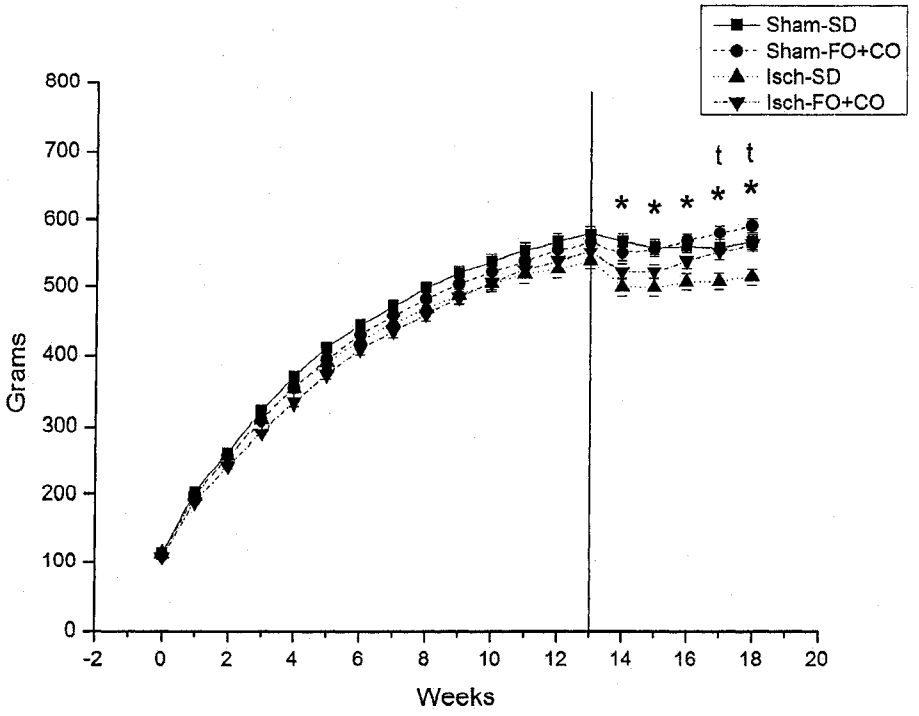


Figure 21: Mean body weight of rats fed either a standard or a PUFA supplemented diet over an 18-week period. Sham or global ischemia surgery is indicated by the vertical line. (*) indicates that ischemic rats weighted significantly less than sham-operated rats post-surgery ($p \leq .025$). (t) indicates that FO+CO ischemic rats gained weight more rapidly than SD ischemic rats on week 17 and 18 while being deprived from food for the radial arm maze task ($p \leq .05$).

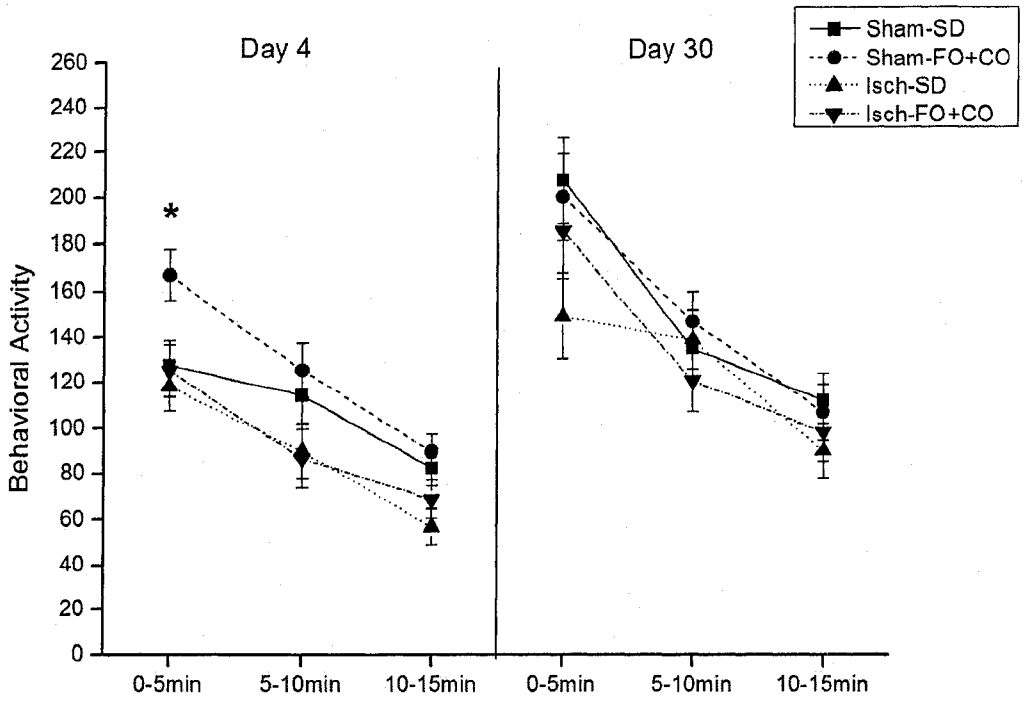


Figure 22: Open field activity (number of crossed squares and rearing frequencies) in animals tested 4 and 30 following reperfusion. The overall activity level of all experimental groups was higher at day 30 than at day 4. (*) indicates higher activity levels in FO+CO sham-operated rats as compared to SD sham-operated rats ($p = 0.015$).

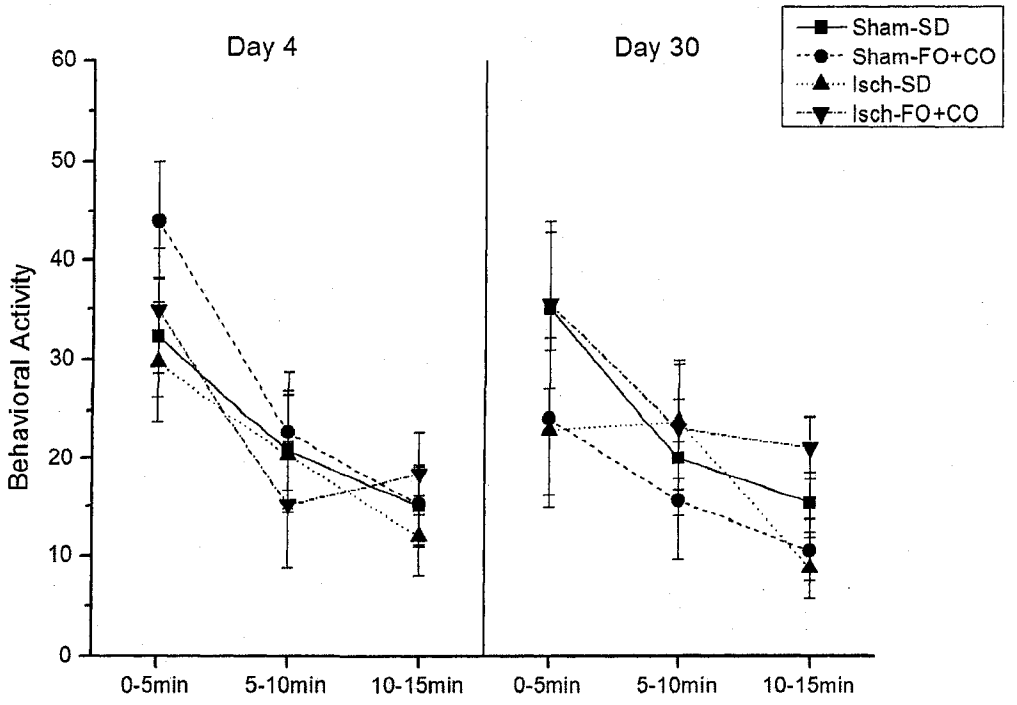


Figure 23: Open field activity (number of crossed squares and rearing frequencies in the central area of the open field) in animals tested 4 and 30 following reperfusion. For the exception of a main effect of time within each test, no significant difference was found between groups.

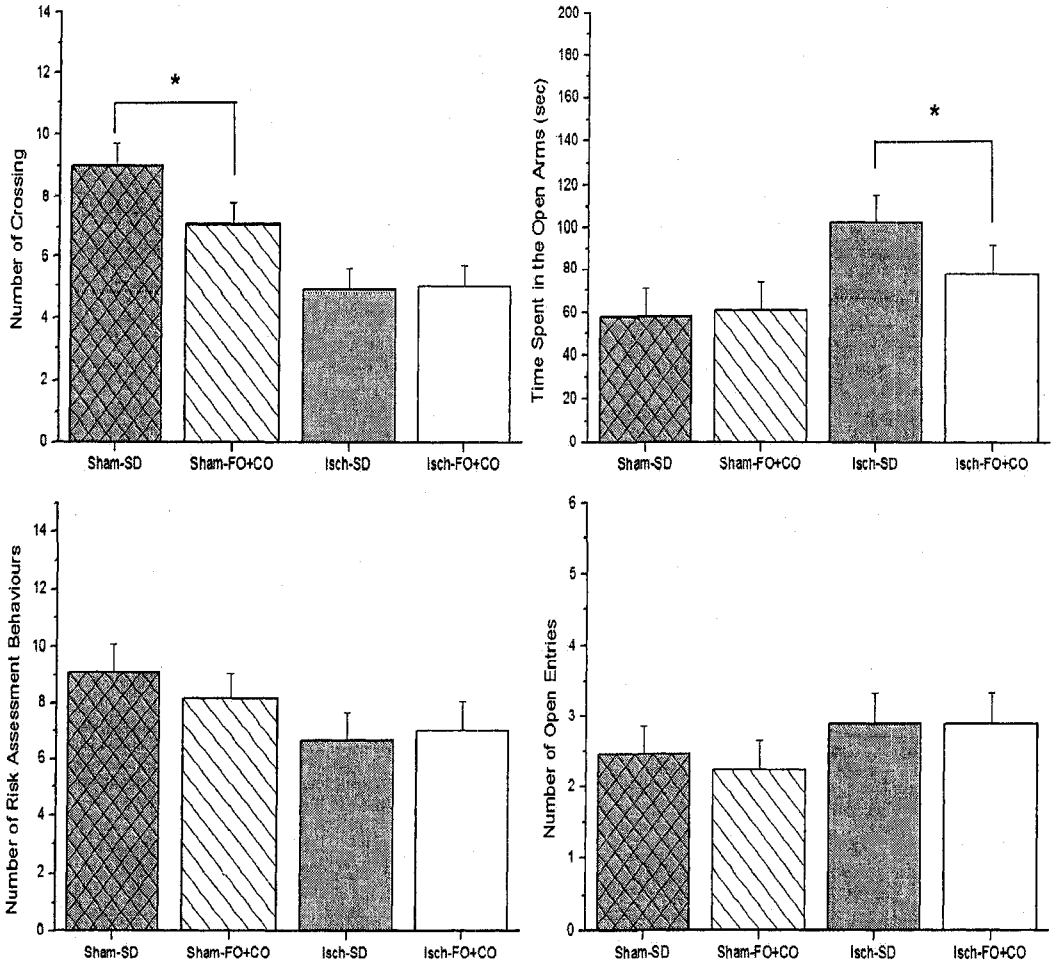


Figure 24: Frequencies of behaviour and time spent in the open arms of the elevated plus maze. Ischemic rats fed either diet spent significantly more time than sham-operated rats in the open arms ($p = .028$). Sham-operated rats made considerably more crossing behaviour than ischemic rats ($p < .001$).

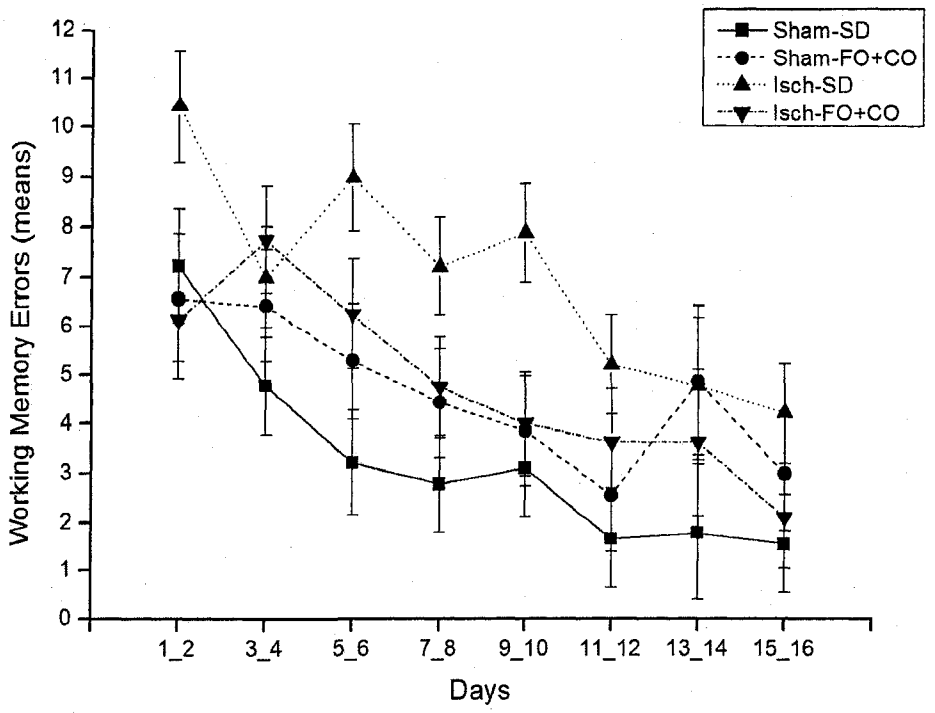


Figure 25: Effect of ischemia and PUFA supplemented diet on spatial memory performance in the radial arm maze. SD ischemic rats made significantly more working memory errors across time than FO+CO ischemic rats ($p = .003$) and both sham groups ($p < .001$).

Table 8: Latencies to complete the standard radial arm maze task.

Days	Sham-SD	Sham-FO+CO	Isch-SD	Isch FO+CO
1-2	598.44±73.73	628.29±83.60	766.00±73.73*	652.13±78.20
3-4	385.06±74.19	577.57±84.12	633.39±74.19*	620.69±78.69
5-6	328.50±64.81	512.71±73.48	683.83±64.81*	528.56±68.74
7-8	278.78±48.07	432.71±54.51	557.44±48.07*	382.50±50.99
9-10	297.00±51.34	385.64±58.21	592.61±51.34*	399.44±54.45
11-12	235.56±51.28	327.57±58.15	425.39±51.28*	401.50±54.39
13-14	204.61±51.74	397.14±58.67	393.33±51.74*	369.55±54.88
15-16	237.56±47.92	375.71±54.33	399.72±47.92*	347.00±50.82

(*) indicates that SD ischemic rats took significantly more time to complete the task than FO+CO ischemic rats and both sham-operated groups ($p = .001$).

Table 9: Density of hippocampal CA1 neurons (cells/1mm CA1 tissue) in sham and ischemic animals fed either a standard or a PUFA supplemented diet.

Groups	CA1 cell
Sham-SD	279.09±3.32
Sham-FO+CO	275.96±3.96
Isch-SD	99.33±20.69*
Isch-FO+CO	87.84±20.28*

(*) indicates that neuronal density of both ischemic groups was significantly reduced as compared to sham-operated rats ($p < .001$).

Discussion

The current study examined the impact of 3-month consumption of a PUFA enriched diet on CA1 neuronal injury, spatial memory impairments, locomotion, and anxiety following global ischemia in rats. In contrast to previous studies assessing shorter-term PUFA supplementation and reperfusion intervals, this study found no impact of the PUFA supplement on CA1 neuronal damage measured 31 days following ischemia. However, PUFA supplemented ischemic animals were less impaired than ad libitum fed controls in the 8-arm radial maze. Finally, all ischemic animals regardless of diet showed decreased anxiety in the elevated plus maze.

PUFA-enriched diets and ischemic damage

The first question raised by these results concerns the lack of protective effects of our PUFA supplemented diet on CA1 neuronal damage. Our findings contrast with the significant neuronal protection observed after n-3 polyunsaturated fatty acid supplementation in other lesion models [69,353,398]. Among the factors that may contribute to such differences is the post-ischemic interval, because different treatments have been shown to gradually lose their beneficial effects after prolonged delay following reperfusion (for a review, see Corbett and Nurse [97]). Thus, while previous experiments have assessed neuronal injury following short intervals (5-8 days), the 31-day post-ischemic interval used in the current study may in part explain vanished neuronal protection. It is also possible that the insult produced by an 8-min ischemia may have been too severe for our diet supplementation to counteract cell death in highly vulnerable hippocampal neurons. In contrast to most previous studies that have administered fish oil

or DHA by gavage or intraperitoneal injections [184,224], we chose a natural feeding procedure so that our behavioural measures were not contaminated by the stress associated with the feeding regimen. However, as a consequence, exact estimation of daily ingested PUFAs was not possible and may have resulted in more variable PUFAs intake. On the other hand, animals usually ate their entire food ration and daily ingestion was comparable between rats. Thus, group differences are unlikely attributable to differences in the amount of PUFA ingested, although smaller quantities ingested by all groups compared to force fed animals may have led to an absence of neuronal protection.

Possible mechanisms

Among physiological effects, increased DHA in brain tissue following omega-3 PUFA supplements has been associated with enhanced free radical scavenging and inhibition of prostaglandin synthesis and lipid peroxidation [28,69,73,194]. Additionally, by increasing the expression of heat shock proteins (HSP70), PUFA enriched diets administered prior ischemia likely improve neuronal stress resistance [44]. Fish oil supplements have been shown to significantly diminish post-ischemic brain infarction volume and edema, a phenomenon attributed to changes in the fatty acids composition and antioxidant enzymatic activity in the brain [37,86,398]. It is suggested that these changes exert an impact on the oxidative levels and apoptotic changes in the rat hippocampus.

In the current study, the fact that phospholipid fatty acid brain content was not measured prevents establishment of a relationship between penetration of PUFA into brain tissue and neuronal or functional observations. To date, the majority of studies

showing increased DHA concentrations in brain tissues following omega-3 PUFA supplements have used animals that were depleted in total omega-3 fatty acids throughout one or two generations, so that the omega-3 supplementation at weaning restored DHA in the growing brain [7,73,329,409]. Our animals had not been deprived of omega-3 fatty acids before ingesting the FO+CO supplemented diet. Therefore, it is possible that the additional gain produced by the FO+CO supplementation over the feeding period was not substantial in comparison with the DHA content in brains from standard rats. Of interest, Force et al. [139] demonstrated no effects of a 6-week mixed supplement of 20% menhaden oil and 3% corn oil in fat free chow on cardiac ischemic injury despite significant myocardial increase in phospholipids PUFA content in n-3 supplemented animals.

In contrast to many studies using PUFA supplementation, our experimental diet included mixed supplementation of n-3 and n-6 fatty acids in the regular diet. Addition of 3.5% corn oil to 11.5% fish oil aimed to enhance final n-3 fatty acid consumption while achieving optimal n-6/n-3 fatty acid ratio in the supplemented diet [422]. An increased ratio of n-6 to n-3 fatty acids in platelet phospholipids has been associated with higher incidence of death from cardiovascular diseases and neurodegenerative or inflammatory disorders in humans [65,94,286]. Although intrinsic PUFA effects on cell injury and behaviour was in part controlled using PUFA supplemented sham animals, inclusion of sham and ischemic groups receiving a 'neutral' oil (e.g., rich in monounsaturated fatty acid such as olive oil) or alternate proportion of the fish and corn oil supplements could have help further define possible interaction of n-3 and n-6 PUFA supplements and the impact of extra daily caloric intake on pre and post-ischemic observations. Thus,

increased concentrations of arachidonic acid (AA) from corn oil supplements may have enhanced synthesis of pro-inflammatory cytokines in PUFA supplemented ischemic rats [65,427], possibly counteracting inhibition of ischemia-induced inflammation by n-3 fatty acids and reducing PUFA effects on neuronal injury. Supplemented alone, 6 or 15 week of corn oil showed no preventive effects on myocardial ischemic injury [139,369].

PUFA-enriched diets and behaviour

We now turn to the behavioural measurements in the present study which showed a reduced impact of ischemia on memory processes in PUFA-supplemented rats but little effects of this diet on open field behaviour and anxiety.

Open field activity is widely used to assess ischemia-induced hyperactivity which typically occurs within 24-72h after ischemia, and is often negatively correlated with the remaining number of CA1 hippocampal neurons. Among other hypotheses, habituation deficits associated to memory impairment has been proposed to underlay this phenomenon [10,18,78,156,225]. Diet-induced effects on this behaviour are variable. Wainwright et al. [462] reported no effect of variable PUFA ratios on open field activity level in naïve rats. Similarly, Naliwaiko et al. [340] showed that all rats presented comparable behaviour in the open field and elevated plus maze tests despite that chronic fish oil supplementation was associated with longer immobility period in the forced swimming test. In contrast, Carrié and colleagues [74] demonstrated that only young rats (but not adult or older ones) supplemented with fish oil showed increased open field exploratory activity.

Previous studies from different laboratories, including our own, have reported

ischemia-induced hyperactivity [78,156,225,250,379]. In the current study, ischemic animals of both feeding groups showed reduced activity level as compared to sham-operated rats, 4 and 30 days post-occlusion, a phenomenon positively correlated with CA1 neuronal counts. This finding is difficult to explain. Obvious differences between this study and other performed with ischemic animals are the older age of tested animals in the current study. In addition, the 90 day pre-ischemic feeding regimen involved daily manipulation of all animals, requiring regular removal of the animal from their home cage. In that sense, the extended behavioural testing in the present experiment may be viewed as a treatment that could influence behaviour in general. As for the impact of the diet per se, our observations are consistent with findings of Wainwright et al. [462] and Naliwaiko et al. [340] showing no impact of fish oil supplemented diet on global activity levels.

The elevated plus maze (EPM) assesses the willingness of rodents to explore anxiogenic environments [32,100,361]. There appears to be no clear consensus in the literature regarding the behavioural pattern of these animals in the EPM. Studies assessing the anxiety levels of rodents fed a PUFA supplemented diet have for most used n-3 fatty acids deficient animals as controls. Some studies showed that time spent and number of entries in the open arms is higher in n-3 fatty acid deficient mice as compared to mice with adequate ratio of n-3 fatty acids, suggesting reduced anxiety in deficient mice [141,339]. In contrast, other groups showed higher anxiety levels in n-3 deficient animals as compared to animals fed a diet containing adequate levels of linoleic and α-linoleic fatty acids. This phenomenon was significantly attenuated when adequate levels of fatty acids were provided to deficient animals [33,438]. Finally, some studies found no

difference in the EPM between n-3 deficient and adequately fed rats [272,328].

Increased number of entries and/or time spent in the open arms of the elevated plus maze has been reported in ischemic animals, suggesting reduced anxiety level following ischemia [341,379]. To date, one study assessed anxiety in ischemic rats either fed a control diet or one of two PUFA enriched diets [107]. de Wilde and colleagues reported no diet related differences in the elevated plus maze; animals of all groups spending equal time in the open arms and visiting them at the same frequency [107]. Concordant with previous findings, we observed increased exploration of the open arms in ischemic as compared to sham-operated rats, independent of the fed diets. In addition, similar to de Wilde et al. [107], fish oil supplementation had no impact on anxiety-related behaviour in this study as no difference was observed between FO+CO and SD sham animals. These findings contrast with studies performed using n-3 deficient animals which showed reduced anxiety of these animals upon short-term PUFA supplementation. In the current study, the 15% PUFA supplement was provided for a three month period prior to ischemia in rats that had no prevalent deficiency, hence distinct brain DHA baseline concentrations in the groups.

To date, numerous studies have shown spatial learning and memory deficits in the radial arm maze in ischemic animals [39,183,241,346,352], these deficits being caracterized by increased number of working and reference memory errors [146,158,207,380]. In our study, ad lib fed ischemic rats took longer time to complete the radial arm maze task and made significantly more working memory errors than PUFA supplemented ischemic and all sham-operated animals while PUFA supplemented diet had no impact on the performance of sham-operated rats.

Behaviour and CA1 neuronal loss

Moderate to severe neuronal death within CA1 subfield of the hippocampus has commonly been associated with spatial learning and memory impairments [39,53,190,241]. In our study, PUFA supplementation improved ischemia-induced working memory deficits in the radial arm maze, despite comparable CA1 neuronal injury in the SD and FO+CO ischemic groups. Although intriguing, these findings are consistent with various reports demonstrating no impact of CA1 cell injury on cognitive performance in the radial arm and Morris water mazes [280,341,343,347]. In the current study, data analysis using CA1 neuronal injury as a covariate demonstrated that a significant proportion of the variance remains unexplained by CA1 neuronal density, suggesting that other factors influenced the performance of ischemic rats.

Proposed mechanisms for PUFA functional effects

The precise mechanisms by which PUFA supplementation exerts functional effects remain poorly defined but a number of hypotheses have been proposed. PUFA supplementation has been associated with increased membrane fluidity and excitability, modification of the production and activity of neurotransmitters and receptors (including dopamine in the mesolimbic and mesocortical pathways and acetylcholine in the hippocampus), as well as specific activation of membrane-bound G-coupled receptors [193,483]. In addition, several studies have demonstrated that PUFA facilitates long-term potentiation (LTP) induction in hippocampal slices and mammal's brain [144,145], and inhibitates LTP deficits in aged rodents [281,282,308-310]. Although the majority of studies have focused on neuronal and functional effects of DHA, beneficial effect of

PUFA supplementation on spatial memory could also be attributable to increased eicosapentaenoic acid (EPA) in PUFA supplemented rats. Indeed, Song et al. [427] recently reported inhibition of IL-1-induced spatial memory impairments in the Morris water maze in rats supplemented with 0.5% EPA for 7 weeks prior to IL treatment, an effect that was not observed in rats receiving AA (1%) or γ -linolenic acid (0.5%) diet supplements. These functional effects were associated with decreased plasma corticosterone levels and increase in post mortem dopamine levels in the frontal cortex of EPA-treated rats. Noteworthy, significant decrease in IL-induced inflammation was observed in both EPA and γ–linolenic acid supplemented animals, suggesting that this phenomenon may not significantly contribute to EPA-induced memory effects.

Plasticity and neurogenesis

Recent studies have demonstrated behavioural effects and enhanced learning and memory functions associated with neurogenesis in the subgranular zone of the dentate gyrus and the CA1 following global ischemia [34,451,452]. In recent years, PUFA supplementation have been shown to play a significant role in neurogenesis within the hippocampus and cortex, by enhancing neurite outgrowth and promoting differentiation of neural progenitor cells into neurons [66,68,226]. Enhanced dendritic spine density within CA1 hippocampal neurons following ingestion of PUFA enriched diets also suggest that PUFA promote synaptogenesis [67,408]. Dyall and colleagues [126] also demonstrated prevention of the age-associated decline in GluR2 and NR2B subunits in the prefrontal cortex, striatum and hippocampus of aged rats (24-25 months) fed omega-3 enriched diet. In addition to these effects, PUFA have been shown to bind to the G-

protein coupled receptor 40 (GPR40) in peripheral tissue [56]. Recently, Ma et al. [285] identified this receptor throughout the central nervous system of primates, suggesting that long chain PUFA may act as extracellular signaling molecules at the membrane surface to regulate neuronal function. Interestingly, GPR40 protein is significantly upregulated in the dentate gyrus post ischemia, with maximal expression detected in newborn hippocampal neurons 15 days following ischemia [284]. This observation raises the possibility that PUFA-GPR40 interaction in newborn neurons may play a role in enhanced memory function of PUFA supplemented ischemic rats.

In conclusion, our findings show that a 15% PUFA supplemented diet had no effect on global activity level in the open field, anxiety-related behaviour in the elevated plus maze and ischemia-induced CA1 neuronal degeneration. Nonetheless, PUFA supplementation exerted an impact on ischemia-induced working memory deficits in the radial arm maze. Whether such improvement could be associated with reorganization of discrete neuronal networks or functional remodeling in PUFA supplemented ischemic rats requires further investigation.

Discussion Générale

L'objectif principal de cette thèse visait à caractériser l'impact d'une consommation alimentaire quotidienne réduite en calorie et/ou enrichie de gras polyinsaturés [huiles de poisson (oméga 3) et de maïs (oméga-6)] sur les répercussions neuronales et fonctionnelles d'une ischémie cérébrale globale. Nos résultats démontrent l'absence d'effets protecteurs de ces diètes sur la dégénérescence des cellules pyramidales de l'hippocampe dans le modèle d'ischémie cérébrale sélectionné par nos études. Toutefois, malgré les dommages observés dans le CA1 et/ou CA3, les animaux ischémiés nourris de ces diètes pour une période de 12 semaines pré-ischémie ont démontré une atténuation significative des déficits d'apprentissage et de mémoire spatiale induits suivant l'ischémie cérébrale globale. Les prochains paragraphes résument brièvement les résultats des travaux scientifiques de cette thèse et discutent les implications de ces observations quant aux mécanismes physiologiques sous-jacents au recouvrement fonctionnel, à la résistance cellulaire et à la plasticité neuronale.

1. Restriction alimentaire et ischémie cérébrale globale.

1.1. La restriction alimentaire atténue les déficits d'apprentissage et de mémoire spatiale induits par l'ischémie globale.

Nos résultats suggèrent une réduction significative, voire la prévention, des déficits d'apprentissage et de mémoire spatiale chez les animaux dont l'ingestion alimentaire a été restreinte pour une période de trois mois précédant un accident vasculaire cérébral. La performance post-ischémique comportementale de ces animaux est comparable à celle des rats contrôles et significativement meilleure (en terme du

nombre d'erreurs de mémoire de travail et de la rapidité dans l'exécution de la tâche) que celle des rats ischémiés nourris d'une diète régulière. Par contre, la restriction alimentaire n'a pas d'impact sur la performance des rats contrôles sham.

Dans le but de mieux définir l'amélioration fonctionnelle des rats ischémiés restreints, la deuxième étude de cette thèse a intégré à l'évaluation fonctionnelle des animaux, une série de tests mnésiques plus complexes dans le labyrinthe radial, soit des tâches différées similaires ou non à l'échantillon initial. Ce type de tâches permet d'évaluer, au-delà de la mémoire de travail, la flexibilité cognitive des animaux puisque le succès du deuxième essai dépend de l'apprentissage de la règle établie. Malgré des tests comportementaux plus difficiles, la courbe d'apprentissage des rats ischémiés restreints s'est avérée comparable à celle des rats shams, démontrant la capacité de ces animaux à planifier une action en liaison avec les contraintes expérimentales établies. À l'opposé, les rats ischémiés nourris à volonté ne sont pas parvenus à maîtriser ces tâches complexes, continuant malgré plusieurs essais à commettre significativement plus d'erreurs rétroactives (mémoire de référence) et proactives (mémoire de travail) que l'ensemble des autres groupes expérimentaux. De même, ils ne présentaient que peu d'amélioration (tel que démontré par l'absence de courbe d'apprentissage chez ce groupe) à travers les 70 jours d'évaluation dans le labyrinthe. Curieusement, ces rats ischémiés semblaient utiliser la stratégie applicable au paradigme DMTS lorsqu'ils devaient compléter une tâche DNMTS et une stratégie DNMTS lorsqu'ils devaient compléter une tâche DMTS.

1.2. La restriction alimentaire ne protège pas les neurones hippocampiques du CA1.

Différentes hypothèses ont été proposées pour expliquer les effets neuroprotecteurs de la restriction alimentaire lors du vieillissement et/ou de maladies neurodégénératives, cardio- ou cérébrovasculaires. Une d'entre elles soutient que la restriction alimentaire affecte directement les processus associés à la dégénérescence cellulaire par l'atténuation de la production de radicaux libres et des dommages secondaires à l'ADN, aux protéines et aux transporteurs membranaires ioniques dépendants de l'ATP [301-303,425]. L'explication physiologique sous-jacente à ce phénomène réside dans l'habituation graduelle des animaux restreints à une diminution de l'apport de glucose au cerveau et à une baisse des ressources énergétiques cellulaires disponibles. Il en résulte chez ces animaux la capacité d'assurer le fonctionnement neuronal et cognitif à partir de ressources énergétiques limitées. De ce fait, les perturbations anaérobiques induites par l'ischémie cérébrale n'entraîne pas une dépolarisation aussi massive des neurones à la base de l'initiation de la cascade physiopathologique et de la production de radicaux libres. En se faisant, il se produit une réduction des dommages secondaires et une meilleure survie neuronale [118,426].

Tel que mentionné, nos résultats ne nous ont pas permis d'observer de réductions significatives des dommages neuronaux par la restriction alimentaire dans les neurones de la couche pyramidale du CA1 de l'hippocampe 7, 31 et 70 jours suivant une ischémie cérébrale globale de 12 minutes. Toutefois, le comptage cellulaire ne permet pas de distinguer la mort cellulaire par nécrose de celle par apoptose, la dernière étant associée à une cascade cellulaire distincte et modulée par l'activation de gènes liés à l'initiation

d'une cascade enzymatique délétère. Il n'est donc pas exclus que la mort neuronale par apoptose dans l'hippocampe diffère entre les groupes. Lors d'une étude pilote, nous n'avons observé aucune différence significative dans l'expression du marqueur pro-apoptotique caspase-3 dans les couches cellulaires pyramidales ou le gyrus dentelé de l'hippocampe 7, 31 ou 70 jours suivant l'ischémie globale. Ces résultats effectués sur de petits groupes d'animaux demeurent toutefois préliminaires.

À ce jour, aucune étude ne s'est intéressée aux effets de la restriction alimentaire sur les principaux neurotransmetteurs impliqués dans l'ischémie globale et/ou dans l'apprentissage et la mémoire (le glutamate et le GABA étant deux importants neuromodulateurs de ces effets). Néanmoins, des études récentes ont démontré que la restriction alimentaire prévient la réduction de l'expression des récepteurs NMDA-R1 et AMPA-GluR1 chez les rongeurs et les déficits de potentialisation à long terme associés au vieillissement [60,128,153,154,176,218,443]. Fait intéressant, on note une réduction de l'expression de ces neuromodulateurs en période juvénile chez les animaux restreints comparativement à ceux nourris à volonté, ce qui suggère que les observations subséquentes sont probablement associées à une plus grande stabilité de ces vecteurs physiologiques avec l'âge [6,344,419]. Une meilleure préservation du glucose, du transport glutaminergique et des fonctions mitochondriales est également associée à la résistance accrue des synaptosomes aux dommages oxydatif et métabolique chez ces animaux. De manière congruente, Guo et collègues [173] observent des niveaux supérieurs de protéines HSP-70 et GRP-78 dans les synaptosomes de rats nourris par alternance journalière pour une période de trois mois, contrairement à ceux de rats nourris à volonté.

Un des objectifs de nos travaux a donc tenté de vérifier la possibilité que des altérations biochimiques impliquant les neurotransmetteurs GABA et glutamate influencent la performance mnésique des animaux restreints. Pour ce faire, nous avons réalisé une analyse post-mortem sur l'ensemble des animaux sham et ischémiés (nourris ad libitum et restreints) dont les cerveaux ont été prélevés suivant la complétion de l'évaluation comportementale, 70 jours suivant l'ischémie. Via l'utilisation de techniques immunohistochimiques sur coupes, nous avons démontré une expression intensifiée des transporteurs vésiculaires du glutamate (vGluT1) dans la plupart des régions hippocampiques des rats contrôles. Chez les rats ischémiés, le patron d'expression est distinct et l'expression des vGluT1 s'avère plus intense comparativement aux rats contrôles dans la couche pyramidale du CA3, la couche moléculaire et le hilus du gyrus dentelé.

Nos résultats révèlent également une expression distincte des transporteurs vésiculaires du GABA (vGAT). Bien que la distribution des vGAT chez les animaux ischémiés soit similaire à celle des rats contrôles dans plusieurs régions de l'hippocampe, il existe une réduction importante des vGAT le long des cellules pyramidales du CA1. De manière générale, la distribution des transporteurs vésiculaires de glutamate et de GABA observée chez les animaux contrôles et ischémiés nourris ad libitum est comparable avec celle précédemment documentée [12,83,223,233,429,454].

Il est à présent impossible de comparer les observations immunohistochimiques de ces marqueurs avec celles d'autres chercheurs, nos travaux étant les premiers à quantifier l'expression des transporteurs vésiculaires du glutamate et du GABA chez des animaux ischémiés restreints. Dans une perspective fonctionnelle, l'expression réduite de

vGAT dans les couches radiatum à la hauteur du CA3 et oriens à la hauteur du CA1 chez les animaux restreints comparativement aux animaux nourris à volonté est toutefois intéressante. Ainsi, les afférences vers les cellules pyramidales du CA3 provenant du gyrus dentelé via la voie perforante et les fibres moussues voyagent à travers la couche radiatum à la hauteur du CA3. De même, les efférences des cellules pyramidales du CA1 aux cortex subiculaire et entorhinal circulent par la couche oriens à la hauteur du CA1. Une réduction des circuits inhibiteurs dans ces deux régions, même si elle est minimale, pourrait améliorer la transmission synaptique chez les animaux ischémiés restreints. Ce phénomène pourrait favoriser un transfert plus efficace de l'information spatiale aux cellules pyramidales du CA1 et subséquemment aux cortex subiculaire et enthorinal. Des analyses complémentaires sont nécessaires afin de déterminer l'importance de ces changements synaptiques. Cependant, cette hypothèse pourrait en partie expliquer l'apprentissage de tâches de mémoire spatiale complexes et la flexibilité cognitive supérieure des rats ischémiés restreints malgré une perte neuronale comparable à celle des rats ischémiés nourris à volonté.

1.3. La restriction alimentaire favorise un accroissement de la résistance cellulaire, la plasticité cellulaire et la neurogénèse.

En complément aux études démontrant une réduction de la mort cellulaire, d'autres recherches suggèrent que la restriction alimentaire augmente la résistance cellulaire via une régulation de l'expression de certains gènes [8,129,185,262,306,488]. La dernière décennie a été particulièrement riche en découvertes à ce chapitre. Plusieurs travaux scientifiques ont démontré une meilleure utilisation des ressources cellulaires

suite à une période de restriction alimentaire rendant l'organisme plus efficace à combattre différents stress endogènes [302]. Dans ces conditions physiologiques plus contraignantes, les neurones d'animaux restreints démontrent une activation accrue de certains gènes, dont ceux d'une variété de protéines chaperonnes, de facteurs de croissance et de cytokines associés aux processus de neurogénèse et de plasticité synaptique [210,311,418].

1.3.1. Restriction alimentaire et protéines chaperonnes

Le rôle des protéines chaperonnes dans la préservation fonctionnelle des différentes systèmes cellulaires de l'organisme est connu. Ces protéines, synthétisées lorsque l'organisme est soumis à des agressions de type physique, chimique ou métabolique, permettent à la cellule de se protéger contre un stress ou tout facteur pouvant induire la mort cellulaire. Elles permettent également de maintenir intacte ou de restaurer la structure de certaines autres protéines, en bloquant les processus de dégradation enzymatique, favorisant leur réparation suite au stress, via l'inhibition ou la stimulation de l'expression de certains gènes et hormones. Bien que les mécanismes neuroprotecteurs de ces protéines demeurent méconnus, leur expression est corrélée à la régulation homéostasique du calcium intracellulaire, le recouvrement des fonctions mitochondriales ainsi qu'une réduction du stress oxydatif [232,274,279,488].

À ce jour, de nombreuses recherches ont démontré que la restriction alimentaire stimule la production de diverses protéines impliquées dans la réponse au stress, telles les protéines régulatrices de la production énergétique mitochondriale (GRP-75) et glucogénique (GRP-78), les protéines chaperonnes (dont les 'heat shock proteins' - HSP-

70 étant la plus étudiée) et certains gènes anti-apoptotiques (dont le Bcl-2) [262]. Plus particulièrement, la restriction alimentaire augmente l'expression de HSP-70 et de GRP-78 dans le foie, les intestins et les régions cérébrales corticales, striatales et hippocampiques de rongeurs [8,129,185,416,488]. Par exemple, Duan et Mattson [117] ont démontré qu'un pré-traitement au 2-Deoxyglucose (2DG; un analogue non métabolisable du glucose) mime les effets de la restriction alimentaire et augmente considérablement l'expression post-mortem de HSP-70 dans le striatum de souris. Cet accroissement de l'expression de HSP-70 est corrélé à la résistance accrue des neurones dopaminergiques de la substance noire aux effets du 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Lee et collègues [262] notent qu'un pré-traitement au 2DG augmente également les niveaux de HSP-70 et GRP-78 dans les neurones hippocampiques observés suite à l'injection d'acide kainique dans l'hippocampe dorsal et ce, sans affecter les niveaux de Bcl-2 et GRP-75. Ces résultats suggèrent qu'une atténuation du glucose disponible par un traitement au 2DG, qui imite les effets bénéfiques de la restriction alimentaire et conduit à l'augmentation de la résistance neuronale aux dommages oxydatif et métabolique. Finalement, Yu et Mattson [488] observent une augmentation accrue de l'expression d'HSP-70 dans le striatum de rats nourris par alternance journalière ayant reçu une ischémie focale. Cet accroissement d'HSP-70 est associé à une réduction significative des dommages cortico-striataux et des déficits neurologiques secondaires à l'ischémie. De façon similaire à d'autres stress endogènes comme le préconditionnement ischémique, la restriction alimentaire active des mécanismes de défense endogènes qui favorise une meilleure adaptation de l'organisme à un stress physiologique comme celui d'une ischémie cérébrale [27,279,300-303]. Nos

travaux n'ont pas examiné l'expression de protéines chaperonnes au niveau cortical et hippocampique chez les animaux restreints, ces derniers ayant dépassés les intervalles optimaux pour ce genre d'analyses habituellement effectuées dans de brefs délais suivant l'occlusion des vaisseaux, et précédant la mort neuronale.

Malgré une survie neuronale statistiquement comparable, les rats ischémiés restreints possèdent un peu plus de neurones dans le CA3 de l'hippocampe. Une activité métabolique et/ou neurochimique de ces neurones pourrait possiblement contribuer à la performance cognitive supérieure dans des tâches simples et complexes de mémoire spatiale. Néanmoins, les facteurs possiblement associés à une meilleure résistance des cellules du CA3 chez ces animaux ne sont pas connus.

Les études de cette thèse ont particulièrement ciblé les effets fonctionnels à long terme de la restriction alimentaire suite à une ischémie cérébrale globale chez le rat. Suite à nos observations, il apparaît maintenant nécessaire d'identifier les effets de ce protocole alimentaire sur différentes variables physiologiques susceptibles de jouer un rôle dans la préservation fonctionnelle observée. Dans cette optique, la quantification de changements métaboliques, neurochimiques et cellulaires au niveau cérébral doit être établie à des intervalles post-ischémiques en étroite liaison avec les changements fonctionnels observés. À cet égard, l'expression des protéines chaperonnes dans les régions cérébrales, représente une étape importante en vue d'établir les mécanismes endogènes protecteurs de la restriction alimentaire et identifier les groupes cellulaires possiblement impliqués. Toutefois, étant donné l'expression maximale de ces protéines à de courts délais suivant l'induction d'un stress physique, métabolique ou chimiques, l'intensité et/ou les changements de l'expression de ce marqueur ne permettent d'établir seuls des liens

fonctionnels directs (impliquant une causalité) avec les processus cognitifs et/ou la réadaptation fonctionnelle observés à de plus long intervalles suivant l'établissement des dommages cérébraux.

1.3.2. Restriction alimentaire et facteurs neurotrophiques

Les neurotrophines, regroupant le facteur de croissance neuronale (nerve growth factor - NGF), le facteur neurotrophique des cellules cérébrales (Brain-derived nerve factor - BDNF) et les neurotrophines 3 et 4, représentent des éléments essentiels à la prolifération, la différenciation et la survie des neurones au cours du développement. Par leurs actions sur les processus de signalisation cellulaire, ces protéines régularisent l'activation enzymatique intracellulaire des caspases. En se faisant, elles empêchent les neurones cibles d'initier leur autodestruction lors de blessures entraînant des dommages oxydatifs et métaboliques [84,131,253,399,446]. Certaines recherches suggèrent l'activation par ces facteurs neurotrophiques de composants neutralisant le stress oxydatif (enzymes antioxydantes et Bcl-2) et stabilisant les niveaux de calcium intracellulaire [302,304,305].

Plusieurs recherches révèlent que certains stress cellulaires (e.g., ischémie cérébrale, traumatisme crânien, épilepsie et hypoglycémie), de même que la réadaptation physique et cognitive (e.g., exercice physique, environnement enrichi, et apprentissage de tâches mnésiques) augmentent la production de neurotrophines et le développement subséquent de nouveaux neurones dans des régions spécifiques du cerveau [22,157,160, 168,169,187,188,217,234,242,273,274,275,323,331,368,372, 405, 439,453,486,495]. D'autres études rapportent que la restriction alimentaire accroît l'expression de plusieurs

neurotrophines dans le cortex cérébral, la formation hippocampique et le striatum chez des rongeurs [115]. Plus précisément, Duan et al. [116] et Lee et al. [263,265] ont démontré une élévation du BDNF associée à une résistance accrue des neurones pyramidaux de l'hippocampe chez des animaux nourris par alternance journalière comparativement à des animaux nourris à volonté.

Suite à la restriction alimentaire, l'expression du BDNF a été principalement observée dans le cortex cérébral, l'hippocampe et certaines régions sous-corticales antérieures dont le septum. En plus de favoriser la survie neuronale via une restructuration cellulaire, le BDNF favorise la neurogénèse dans les zones para-ventriculaires et sous-granulaires des ventricules latéraux et du gyrus dentelé de l'hippocampe suivant une atteinte neuronale [50,218,229, 381,395,449,451,452]. Ce facteur de croissance participe au développement d'axones et de dendrites et à l'établissement de synapses permettant la différenciation des nouveaux neurones. Cette plasticité synaptique accrue facilite subséquemment l'apprentissage et la mémoire [236].

Certaines études rapportent une augmentation de la neurogénèse dans l'hippocampe chez des rongeurs nourris d'une restriction alimentaire. Ainsi, Lee et collègues [263-265] révèlent que des animaux nourris par alternance journalière, pour des périodes variant de 14 à 90 jours, possèdent un nombre plus élevé de nouvelles cellules dans la couche granulaire du gyrus dentelé comparativement à des animaux nourris à volonté. Cette neurogénèse est observée en présence de concentrations supérieures de BDNF et de neurotrophine 3 dans le CA1, le CA3 et le gyrus dentelé de ces animaux. Plus récemment, Lee et al [265] ont rapporté une augmentation significative de la neurogénèse chez des souris saines suite à une restriction alimentaire, mais aucun effet neurogénique chez les animaux restreints ayant subi

une lésion hippocampique suite à une injection d'acide kainique. Pour leur part, Bondolfi et al. [46] n'observent aucun effet significatif de la restriction alimentaire sur la prolifération, la survie et la différenciation des nouveaux neurones hippocampiques chez des souris C57BL/6 adultes, comparativement aux observations réalisées chez des souris nourries à volonté. Ils notent toutefois une augmentation des cellules gliales dans le hilus du gyrus dentelé chez les animaux restreints.

De même, la restriction alimentaire est associée à des changements plastiques et une meilleure préservation de la configuration des épines dendritiques des neurones pyramidaux lors du vieillissement chez des rongeurs [330]. Ces dernières reçoivent et intègrent l'information afférente des terminaisons axonales et jouent un rôle important dans l'apprentissage et la mémoire. Par contre, d'autres études démontrent que cette restriction a peu d'impact sur les processus dégénératifs des synapses inhibitrices du cortex sensorimoteur et ou sur le nombre de synapses par neurone dans le gyrus dentelé de rats âgés [344,420].

1.4. Hypothèses sous-jacentes des effets fonctionnels de la restriction alimentaire

Dans l'ensemble, les résultats de cette thèse suggèrent que la restriction alimentaire favorise des changements neuroplastiques et/ou la présence de mécanismes compensatoires permettant de réduire significativement les déficits d'apprentissage et de mémoire spatiale induits par l'ischémie cérébrale globale. Diverses avenues méritent d'être évaluées afin de mieux comprendre les mécanismes cellulaires et ré-organisationnels associés à la restriction alimentaire.

Une *première hypothèse* propose que la restriction alimentaire facilite le recrutement de régions extra-hippocampiques lors de la complétion de tâches mnésiques spatiales, permettant de compenser l'importante dégénérescence des neurones pyramidaux du CA1 [21]. De nombreuses structures extra-hippocampiques corticales et sous-corticales, dont les cortex périrhinal, entorhinal, et parahippocampique, sont impliquées dans la consolidation à long terme des apprentissages explicites [430]. Il est possible que le rôle des structures de ce circuit neuronal soit modifié lorsque l'hippocampe est endommagé. De plus, une lésion partielle de l'hippocampe peut engendrer une désorganisation fonctionnelle des circuits hippocampiques, interférant avec les connections afférentes aux régions extra-hippocampiques. Une telle désorganisation pourrait de manière subséquente empêcher la consolidation en mémoire à long terme [21]. Certains chercheurs proposent que cette désorganisation soit atténuée par la restriction alimentaire. Parallèlement, une *deuxième hypothèse* stipule que la restriction alimentaire favorise une plus grande plasticité neuronale liée à une meilleure réorganisation de circuits neuronaux encore fonctionnels. Un réseautage accru entre les régions du lobe temporal médian, intégrant l'efficacité de la transmission électrique et neurochimique à la synaptogénèse, pourrait contribuer à la réduction des déficits mnésiques ischémiques [173]. En préambule à une discussion de ces deux possibilités, il s'avère important de présenter brièvement l'anatomie et les circuits neuronaux principaux du lobe temporal médian.

1.4.1. Organisation cytoarchitectonique du lobe temporal médian

Le lobe temporal médian, comprenant la formation hippocampique et différentes structures extra-hippocampiques, exerce un rôle important dans la consolidation à long terme des mémoires explicites, incluant la récupération intentionnelle de l'information et des expériences passées et la formation des mémoires spatiales [430]. Chez les mammifères, la formation hippocampique se compose du gyrus dentelé, du cortex subiculaire et de l'hippocampe. Les structures extra-hippocampiques incluent des régions corticales adjacentes à l'hippocampe, soit les cortex pré-, para-, et postsubiculaires, entorhinal, périrhinal, postrhinal, et parahippocampique. La boucle néocortex - hippocampe – néocortex se caractérise par une organisation hiérarchique de réseaux associatifs participant à l'intégration d'informations multimodales à l'intérieur du système de mémoire du lobe temporal médian.

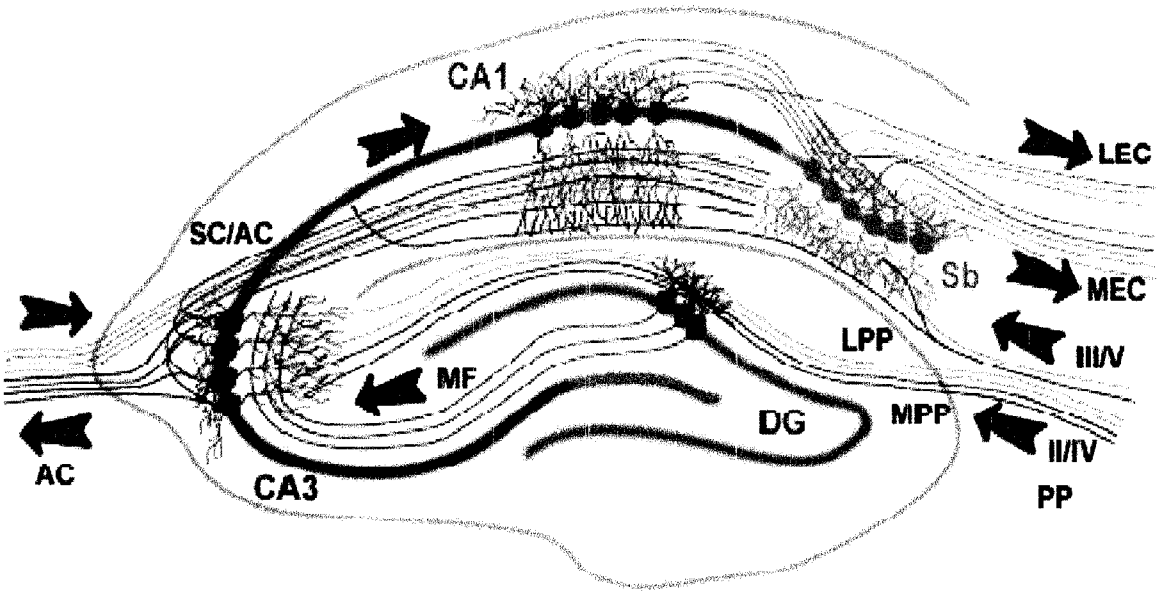
La principale voie de communication entre le néocortex et l'hippocampe est la voie perforante (« perforant pathway » - voir figure 26) [30,244,321,474]. Les deux tiers de l'information en provenance du néocortex sont transmis au cortex entorhinal par les cortex périrhinal et parahippocampique via des connexions réciproques [203,204,256, 321,436,437]. Le cortex entorhinal reçoit également des signaux afférents des bulbes olfactifs, des régions corticales infralimbiques, orbitofrontales, et cingulaires, du cerveau antérieur basal, de l'amygdale, du noyau raphé, du locus coeruleus, de l'aire tegmentale ventrale et du thalamus [35,159,203,204]. Il est à noter que ces diverses connexions projettent vers des couches cellulaires distinctes [321]. Ainsi, l'information sensorielle afférente en provenance du néocortex converge de manière directe ou via des régions adjacentes vers les couches I, II et III du cortex entorhinal.

Chez les rongeurs et les primates, la voie perforante se divise en deux faisceaux partant de la couche II ou III du cortex entorhinal [473]. Essentiellement, les neurones de la couche II projettent leurs axones vers les cellules granulaires du gyrus dentelé, ces dernières projetant vers les cellules pyramidales du CA3, via les fibres moussues (Mossy fibers). Les cellules pyramidales du CA3 communiquent à leur tour avec les cellules pyramidales du CA1 en empruntant le faisceau collatéral de Schaffer. Il existe également une voie commissurale associative via laquelle les cellules pyramidales des deux hémisphères partagent l'information reçue. Quant aux neurones entorhinaux de la couche III du cortex, ils projettent exclusivement vers la couche pyramidale du CA1 et le cortex subiculaire. Ce sont ces neurones (du CA1 et du cortex subiculaire) qui ferment cette boucle hippocampique en retournant l'information transformée à chacun des niveaux vers la couche V du cortex entorhinal [30,106,243,244,321,479]. Les neurones des couches V et VI envoient alors l'information vers le néocortex, de manière à l'entreposer dans la mémoire à long terme [161]. De plus, des projections entre les neurones de la couche V et ceux des couches superficielles I-III permettent d'intégrer l'information en provenance de l'hippocampe à l'information afférente provenant du cortex entorhinal [450,479,482]. L'information transférée aux couches superficielles se trouve ainsi à entrer de nouveau dans la boucle hippocampique. Ce mécanisme serait impliqué dans l'entreposage de l'information dans des réseaux neuronaux et serait dépendant de bonnes connections inter-neuronales entre les couches profondes et superficielles du cortex entorhinal [450].

La voie mamillothalamique représente un autre lien important via lequel les cellules pyramidales du cortex subiculaire projettent vers le fornix. Ce dernier projette ensuite aux noyaux médian et latéraux des corps mamillaires et au noyau thalamique

antérieur. Les efférences du noyau thalamique antérieur empruntent alors la capsule interne pour rejoindre le cortex cingulaire, le gyrus parahippocampique, le cortex entorhinal pour retourner à la formation hippocampique via la voie perforante. Cet ensemble de relais neuronaux limbiques représente le « circuit de Papez », connu pour son implication dans le contrôle des émotions et la consolidation mnésique.

Figure 26: Organisation neuronale dans l'hippocampe [497].



The Hippocampal Network: The hippocampus forms a principally uni-directional network, with input from the Entorhinal Cortex (EC) that forms connections with the Dentate Gyrus (DG) and CA3 pyramidal neurons via the Perforant Path (PP - split into lateral and medial). CA3 neurons also receive input from the DG via the mossy fibres (MF). They send axons to CA1 pyramidal cells via the Schaffer Collateral Pathway (SC), as well as to CA1 cells in the contralateral hippocampus via the Associational Commissural pathway (AC). CA1 neurons also receive input directly from the Perforant Path and send axons to the Subiculum (Sb). These neuron in turn send the main hippocampal output back to the EC, forming a loop.

1.4.2. Formation hippocampique, apprentissage et mémoire spatiale

De nombreuses études ont examiné la relation entre la densité neuronale dans l'hippocampe (principalement dans la couche pyramidale du CA1) et la performance comportementale dans une variété de paradigmes expérimentaux. Une perte modérée à sévère des neurones pyramidaux du CA1 engendre typiquement des déficits d'apprentissage et de mémoire spatiale importants dans des tâches simples (labyrinthe aquatique, radial à huit bras, en forme de Y ou de T) et complexes (tâches différées appariées ou non à l'échantillon impliquant une composante spatiale) [23,39,53,190,241, 400,457,459,477]. À l'opposé, d'autres recherches montrent que la mort neuronale dans le CA1 suivant une ischémie globale n'est pas toujours associée à des déficits mnésiques dans des tâches spatiales comme le labyrinthe aquatique de Morris [343,347,354]. De plus, la mémoire spatiale de rats suite à une occlusion temporaire des artères carotides (2VO) est significativement affectée malgré l'absence de dégénérescence des cellules pyramidales du CA1 de l'hippocampe [213,280]. À l'inverse, d'autres études rapportent des niveaux modérés à sévères de mort neuronale dans le CA1 en l'absence de déficits mnésiques [17,413]. Ces résultats suggèrent que la performance des animaux ischémiés dans ces tâches ne dépend pas essentiellement de l'intégrité fonctionnelle des neurones pyramidaux du CA1, présentant certaines divergences avec des liens d'interdépendance classiquement établis entre mémoire spatiale et fonctions hippocampiques.

1.4.3. Structures extra-hippocampiques, apprentissage et mémoire spatiale

De nombreuses études supportent un rôle important de plusieurs régions extra-hippocampiques dans la consolidation de l'information spatiale, incluant les cortex péri et

postrhinal [274,336,360], para-hippocampique [87,212,356,396], et pré-, para-, et post-subiculaire [148,150,231,475]. Les projections afférentes et efférentes du cortex entorhinal jouent un rôle significatif dans l'encodage, la consolidation et le rappel d'information spatiale [130,142,147-149,197,365,431,444]. Parron et collègues [364] ajoutent que les liaisons corticales et sous-corticales vers le cortex entorhinal participent au traitement de l'information sensorielle multimodale et des repères visuels simples, permettant une navigation adaptée dans l'espace. Opérationnellement, les déficits de navigation spatiale se traduisent par un nombre supérieur d'erreurs de mémoire de travail, une latence significativement plus élevée pour accomplir la tâche, et une augmentation de la distance parcourue dans les labyrinthes radial ou aquatique de Morris.

Certains chercheurs ne rapportent aucun déficit d'apprentissage et de mémoire spatiale suite à des lésions du cortex enthorinal [24,25,47,61,211,359,386]. Steffenach et collègues [431] suggèrent que ces situations sont attribuables à des lésions incomplètes qui n'affectent que partiellement les circuits hippocampiques impliqués dans la mémoire spatiale. Ces auteurs montrent qu'une lésion à l'intérieur de la bande dorso-latérale du cortex entorhinal interrompt complètement et de manière consistante le rappel de l'information spatiale encodée précédemment par des rats dans le labyrinthe aquatique de Morris. À l'opposé, la mémoire spatiale semble être préservée en présence de lésions dans la bande ventro-médiale du cortex entorhinal, qui communique prioritairement avec l'hippocampe ventral et joue un rôle plus important dans les comportements d'exploration et d'anxiété. Ces résultats suggèrent que les différences fonctionnelles entre l'hippocampe ventral et dorsal sont tributaires de liens neuronaux spécifiques avec le

cortex entorhinal, affectant de manière distincte la navigation spatiale et la consolidation d'information en mémoire à long terme.

1.4.4. Implications de régions situées à l'extérieur du lobe temporal médian dans l'apprentissage et la mémoire spatiale

Un nombre croissant de recherches se sont intéressées à l'implication de plusieurs régions situées à l'extérieur du lobe temporal médian dans la l'apprentissage, la mémoire spatiale et la mémoire de travail. Tout d'abord, le cortex pariétal et ses connections avec le cortex rétrosplénial et l'hippocampe joue un rôle significatif dans le mémoire spatiale égocentrique (i.e., la perception de l'espace basée sur la position de l'animal) et allocentrique (i.e., la relation spatiale entre les objects et l'environnement spatial) [501]. Le cortex pariétal intègre l'information en provenance des cortex moteur, prémoteur, visuel, auditif, et somatosensoriel, ce qui permet à l'animal de former une carte spatiale de l'environnement et de discriminer les environnements vis-à-vis de sa position dans l'espace et des indices visuels disponibles [87,507-509,515,516].

Le cortex rétrosplénial, quant à lui, intègre les indices visuels disponibles avec l'information générée par les mouvements de l'animal en utilisant des indices internes comme la rétroaction proprioceptive et l'activation vestibulaire durant le mouvement [95,240]. De par ses connections avec l'hippocampe, le cortex rétrosplénial utilise la mémoire spatiale à long terme pour corriger les erreurs qui surviennent lors de l'intégration de l'information, ce qui améliore la navigation spatiale de l'animal [502-504,513]. Des lésions au cortex rétrosplénial et/ou à ses connexions engendrent des

déficits de mémoire de référence et de travail dans les labyrinthes aquatique et radial chez des rongeurs [517].

D'autres recherches ont mis en évidence un rôle considérable du thalamus dans la cognition, notamment dans l'attention, la mémoire, et les comportements dirigés vers un but [333]. Le noyau thalamique antérieur est impliqué dans le traitement de l'information visuelle et est nécessaire pour la mémoire spatiale allocentrique. Le noyau thalamique intralaminaire joue également un rôle dans la mémoire spatiale et les fonctions motrices intentionelles alors que le noyau thalamique médian est associé à l'attention et à la mémoire de travail qui sont aussi gérées par le cortex préfrontal. Ainsi, des lésions aux noyaux thalamiques médian, antérieur, et intralaminaire entraînent des problèmes d'apprentissage et/ou de mémoire de travail dans des tâches spatiales allocentriques [498, 500,511,512,514].

Le cortex préfrontal est également une région importante dans l'accomplissement de tâches de mémoire spatiale allocentrique. Le cortex préfrontal médian et dorsolatéral s'active lors de tâches de mémoire de travail et de tâches différées (DNMTS/DMTS) [499,505,506,510]. Le cortex prélimbique joue aussi un rôle dans la mémoire de travail, son rôle étant de récupérer l'information spatiale et d'organiser la séquence motrice subséquente. Le cortex prélimbique s'active davantage lors de tâches différées. Finalement, les noyaux gris centraux sont également nécessaires à l'accomplissement de tâches spatiales [518]. Le globus pallidus permet une connexion entre le system limbique et le thalamus médian ainsi qu'entre le stiatum, le cortex préfrontal et le thalamus. Le noyau caudé intègre l'information limbique et corticale et produit de manière subséquente les comportements dirigés vers un but, particulièrement dans le labyrinthe

radial. Finalement, le striatum est essentiel pour l'apprentissage de séquences motrices et de comportements automatiques.

1.4.5. Ischémie, régions extrahippocampiques, apprentissage et mémoire spatiale

Au cours des dernières décennies, la présence de lésions dans des régions extra-hipocampiques et/ou à l'extérieur du lobe temporal médian suite à un épisode ischémique a été observée chez plusieurs espèces animales [20]. Par exemple, des évaluations post-mortem de patients décédés des suites d'un arrêt cardiaque et/ou des résultats d'études d'imagerie entreprises auprès de patients ayant survécus un arrêt cardiorespiratoire révèlent des dommages localisés dans plusieurs régions corticales et sous-corticales. Ces dernières incluent entre autres les cortex frontal, pariétal et temporal antérieur ainsi que le subiculum, l'insula, le striatum, l'amygdale et le thalamus et le cervelet [101,374,458]. De même, Zola-Morgan et collègues [496] ont démontré en plus des dommages significatifs aux neurones hippocampiques des lésions au gyrus dentelé, cortex subiculaire, putamen ventral, cervelet, corps mamillaires et thalamus chez des singes ayant subi une ischémie globale de 15 minutes. Plusieurs auteurs observent également des lésions extra-hippocampiques chez des rongeurs ischémiés, localisées dans le striatum, le thalamus, le néocortex, le cervelet, et certains noyaux du tronc cérébral [104,105,459,460]. Il faut donc considérer la possibilité que l'attrition légère à modérée observée dans ces sites neuronaux puisse contribuer aux déficits mnésiques suite à l'ischémie cérébrale. Plus précisément, les cortex rhinal, subiculaire, para-hippocampique et cingulaire contribuent à la reconnaissance mnésique et à la mémoire spatiale [289,316-318,364,466-468].

À ce jour, aucune étude n'a déterminé l'activation de ces régions extra-hippocampiques chez des animaux restreints sains ou ayant subi une lésion cérébrale. L'hypothèse du recrutement de ces structures au niveau fonctionnel demeure donc théorique malgré son intérêt en liens avec les résultats présentés dans cette thèse. Il est possible que la restriction alimentaire favorise le recrutement de régions extra-hippocampique lors de la complétion de tâches mnésiques spatiales permettant de compenser la perte des neurones pyramidaux du CA1. Des recherches supplémentaires s'avèrent nécessaire afin de valider une telle hypothèse.

1.4.6. Restriction alimentaire et réorganisation fonctionnelle de circuits neuronaux.

Une *deuxième* hypothèse stipule que la restriction alimentaire favorise la plasticité neuronale stimulant la réorganisation de circuits neuronaux fonctionnels. À ce jour, certains effets de la restriction alimentaire affectant la transmission dopaminergique habituellement réduite avec l'âge ont été documentés. Ainsi, la restriction alimentaire est associée à une augmentation de la libération de dopamine dans le striatum de rats âgés et une prolongation de sa présence dans la fente synaptique [110,404,491]. Les travaux de Carr et collègues [72] suggèrent également que la restriction alimentaire modifie les récepteurs post-synaptiques D1 et D2 et facilite la signalisation de la dopamine. De même, une sensibilité accrue aux agonistes des récepteurs dopaminergiques D1 et D2, tels que le SKF-82958, est observée chez des animaux restreints, induisant une activité locomotrice supérieure et une expression accrue du gène précoce c-fos dans le striatum [70-72]. Par ailleurs, Holmer et al. [191] montrent que lorsqu'administrée suite à une

lésion de la voie nigrostriatale chez des souris, la restriction alimentaire réduit le niveau de glutamate extracellulaire libéré par une injection de 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Cette réduction de glutamate s'observe en présence d'une perte importante de signaux inhibiteurs dopaminergiques dans le striatum. Parallèlement, ces auteurs démontrent que la restriction alimentaire accroît significativement l'activité neuromodulatrice transmise par la voie thalamo-cortico-striatale en augmentant le niveau de glutamate dans le striatum comparativement aux autres groupes. Dans un même ordre d'idée, Masswood et al. [298] rapportent qu'une restriction alimentaire de 30%, administrée pendant une période de 6 mois, augmente la résistance des neurones dopaminergiques dans le striatum et réduit les déficits moteurs induits par le MPTP chez des singes rhésus. Les résultats de Morgan et al. [325] vont toutefois à l'encontre de ces observations et notent l'incapacité de la restriction alimentaire à protéger les neurones dopaminergiques de la substance noire de souris âgées d'un an des dommages cellulaires induits par une dose toxique (mais plus faible) de MPTP.

D'autres études démontrent des changements dans les concentrations cérébrales de sérotonine et de dopamine chez les animaux restreints, qui sont associés à des changements émotionnels incluant des comportements anxiogènes et dépressifs [362,484]. Par exemple, l'étude de Jahng et al. [209] démontre qu'une période de restriction alimentaire de cinq semaines réduit la synthèse de sérotonine dans l'hippocampe et l'hypothalamus de rongeurs, ce qui augmente significativement les niveaux de corticostérone chez ces derniers. La restriction alimentaire est également associée à des changements neurochimiques affectant l'ingestion alimentaire et la régularisation de l'humeur de façon prolongée et ultérieure à la cessation de cette

restriction [79]. Notamment, ces auteurs démontrent qu'une restriction alimentaire cyclique altère la relation entre les neurotransmetteurs sérotonine et dopamine dans le noyau caudé. De plus, lorsqu'elle est combinée avec un accès intermittent à la nourriture, elle possède un effet anorexigène, réduit les niveaux de sérotonine et de dopamine dans le cortex préfrontal médian et est associée avec certains comportements dépressifs chez les rates de cette étude.

1.5. Une conclusion sur les effets de la restriction alimentaire sur l'ischémie globale

De manière générale, les résultats de cette thèse ouvrent des pistes intéressantes dans la compréhension des effets fonctionnels de la restriction alimentaire et de la participation du GABA et du glutamate à ces observations. Nous avons démontré des performances cognitives impressionnantes des animaux ischémiés restreints dans des tests complexes peu souvent utilisés de par la durée exigée de l'évaluation comportementale. Ces résultats aident à préciser et valider des observations antérieures démontrant des effets via l'utilisation des tests comportementaux plus simples. L'absence d'effets protecteurs de la restriction alimentaire contre les dommages ischémiques massifs ou de changements dans l'expression du marqueur pro-apoptotique caspase-3 suggèrent que d'autres facteurs jouent un rôle déterminant dans les effets mnésiques observés. L'étude de la réorganisation de circuits fonctionnels et/ou des différences de la neurotransmission chimique chez les animaux restreints à des intervalles multiples suivant l'ischémie permettra de déterminer les fluctuations endogènes et le rôle possible

de ces neurotransmetteurs dans la survie neuronale et/ou d'établier un lien plus direct avec les observations fonctionnelles établies.

2. Diète enrichie de gras polyinsaturés et ischémie globale

Un second objectif de cette thèse visait à évaluer les effets de changements alimentaires plus directement applicables aux habitudes de consommation humaine, soit d'une diète enrichie de gras polyinsaturés (PUFA; 11.5% d'huile de poisson combinée à 3.5% d'huile de maïs). Tel que précédemment décris, les recherches rapportent de plus en plus d'effets bénéfiques de la consommation de gras polyinsaturés sur plusieurs variables physiologiques. Une consommation privilégiée de ces gras est associée à une réduction de l'incidence d'accidents vasculaires cérébraux et des séquelles physiologiques et comportementales subséquentes.

Comme avec la restriction alimentaire, la diète enrichie de PUFA n'a pas induits d'effets préventifs sur la dégénérescence des neurones du CA1 de l'hippocampe 31 jours suivant l'ischémie. Cependant, la consommation de cette diète pour une période de trois mois est associée à une atténuation des déficits d'apprentissage et de mémoire spatiale chez les animaux ischémiés. Les mécanismes physiologiques et/ou neuronaux induits par la consommation d'une diète enrichie de PUFA demeurent méconnus. Toutefois, les effets récemment démontrés de ces diètes sur la neurogénèse et la plasticité cellulaire sont des avenues intéressantes à explorer dans le cadre des effets fonctionnels observés dans notre étude (voir manuscrit 3). Ces travaux documentent entre autre l'augmentation de la résistance cellulaire via l'expression de protéines chaperonnes (HSP-70), la diminution du volume de l'infarctus et l'œdème post-ischémique, l'inhibition de la

synthèse de prostaglandines, une phagocytose accrue, une plus grande fluidité et excitabilité de la membrane, la modification de la production et de l'activité des neurotransmetteurs et récepteurs, l'activation de récepteurs spécifiques couplés aux protéines G (GRP40), la réduction des déficits de potentiation à long terme associés à l'âge, et la promotion de la neurogénèse et la synaptogénèse.

À partir de nos résultats, il est difficile de déterminer par quels moyens notre diète enrichie de PUFA a préservé les fonctions mnésiques des rats ischémiés. Contrairement aux recherches précédentes employant des méthodes de supplémentation des gras par injection ou gavage, nous avons opté pour une intégration des suppléments de PUFA à la ration de nourriture quotidienne des animaux. Cette méthode plus naturelle visait à réduire le niveau de stress des animaux puisque l'apport principal de notre expérimentation consistait en l'analyse du comportement. En employant cette méthode, l'estimation de la quantité exacte de PUFA s'est avérée plus ardue dû à la possibilité d'ingestion variable. Ce facteur ne semble toutefois pas avoir joué un rôle déterminant, les animaux de l'ensemble des groupes consommant en entier la ration quotidienne fournie.

Un autre facteur contrastant avec la plupart des études employant des suppléments de gras polyinsaturés est une intégration combinée d'acides gras de type oméga 3 et oméga 6 à la diète régulière. Cette combinaison avait pour objectif d'augmenter la consommation finale de gras oméga 3 tout en maintenant un ratio optimal de gras n-6/n-3. Un ratio supérieur de gras oméga 3 est généralement associé à un taux inférieur de mortalité du aux maladies cardiovasculaires, neurodégénératives et/ou inflammatoires [65,94,286]. Toutefois, une limite importante de notre étude est le fait que nous n'ayons

pas quantifié via une analyse chromatographique de la diète les concentrations exactes d'acides gras polyinsaturés [133,222]. En conséquence, nous ne pouvons qu'estimer le ratio final de n-6/n-3 PUFA de notre diète (voir manuscrit 3, Tableau 7)

Afin de clarifier les effets neuronaux de notre diète, la détermination de la composition de gras polyinsaturés dans les membranes phospholipides cérébrales de chaque groupe expérimental permettrait d'établir la perméabilité de ces acides gras à travers la barrière hémato-encéphalique et de mieux définir la relation entre la biodistribution de ces gras polyinsaturés et nos observations neuronales et comportementales. De manière à mieux caractériser l'impact de chaque PUFA, il serait également intéressant d'inclure des groupes recevant soit un seul type de gras polyinsaturés, ou d'étudier parallèlement les effets de l'intégration de différents ratios de PUFA à la diète.

Notre étude des effets comportementaux de l'ingestion de PUFA répartie sur 30 jours post-ischémie, incluant l'aire ouverte (open field), le labyrinthe en croix (elevated plus maze) et le labyrinthe radial (8-arm radial arm maze), demeure une force considérable de notre étude. Nos résultats soutiennent encore une fois la dissociation des effets comportementaux des dommages cellulaires au CA1 de l'hippocampe. La consommation de cette diète riche en gras polyinsaturés possède des effets préventifs sur les déficits mnésiques dans le labyrinthe radial..Ces effets sont toutefois moindres que ceux induits par la restriction alimentaire.

Considérant les applications possibles chez l'humain, il serait intéressant de déterminer les effets de la diète enrichie de PUFA lorsqu'elle est initiée à différents stades dans la vie des animaux et pour des durées d'ingestions variées, de manière à

éclaircir la relation entre la durée de l'ingestion de ces gras et l'observation d'effets physiologiques et/ou comportementaux.

Une autre avenue attrayante serait de caractériser les effets de différents PUFA en utilisant une série de tâches cognitives complexes similaires à celles employées dans cette thèse avec la restriction alimentaire, combinant l'apprentissage, la mémoire spatiale et la flexibilité cognitive.

3. Apport des travaux de recherche et avenues d'investigations futures

Les travaux de cette thèse de doctorat sont les premiers à évaluer l'impact de la restriction alimentaire sur la survie neuronale suite à une ischémie globale et à déterminer les effets de cette procédure sur les déficits mnésiques et comportementaux y étant associés. Cette série d'études visaie l'inclusion de tests comportementaux proposant des défis mnésiques, en intégrant l'apprentissage de différentes tâches mnésiques dans le labyrinthe et des changements procéduraux permettant d'évaluer la flexibilité cognitive.

Un second élément distinctif de nos expérimentations consiste en la durée de l'évaluation comportementale. Les recherches évaluant les mécanismes neuroprotecteurs des diètes utilisées dans cette thèse n'incluent que rarement l'analyse du comportement et lorsque qu'elles le font, les tests sont simples, l'intervalle de passation des tests est de courte durée, et restreint aux jours suivant immédiatement l'induction du trauma. Ces divergences sont importantes et nos résultats indiquent que ces facteurs ont une incidence sur la protection neuronale conférée par ces diètes [59,489,494]. Il est certain que le stress ischémique induit dans nos études est lié à l'initiation d'une cascade physiologique associée à la dégénérescence neuronale. Il constitue un stress physiologique intense,

possiblement supérieur à celui de plusieurs études démontrant des effets protecteurs.

De plus, la sélection d'un intervalle de reperfusion plus long dans nos études pourrait expliquer l'incapacité des seuls changements organiques initiés par la restriction alimentaire à protéger les neurones hippocampiques suite à une ischémie. À cet effet, plusieurs recherches ont démontré une dégradation graduelle des effets protecteurs de différents traitements lorsqu'accompagnés de plus longue périodes de reperfusion (pour une revue, consultez Corbett et Nurse [97]). Néanmoins, au-delà des effets observés au niveau neuronal, la restriction alimentaire favorise la récupération cognitive et fonctionnelle des rats ischémiés. Ainsi, nos résultats suggèrent que les effets de la restriction alimentaire sur l'apprentissage et la mémoire spatiale sont importants et possiblement durables. Ces observations proposent de nouvelles fenêtres d'investigations intéressantes, notamment quant aux effets de la restriction alimentaire sur les changements neurochimiques et neuroplastiques suite à l'ischémie cérébrale.

Une limite de nos expérimentations consiste au fait d'une inclusion unique de rats mâles adultes. Peu d'études se sont attardées à caractériser les effets de changements nutritionnels auprès des femelles. Par exemple, Martin et collègues [291] ont démontré une émaciation des femelles suite à une restriction alimentaire de 40% associée à une interruption du cycle reproducteur et l'expression d'un profil endocrinien masculinisé. Elles montrent également une accentuation de leur réponse physiologique au stress et un niveau accru d'activité spontanée. Nonobstant ces changements physiologiques importants, ces rates ont démontré une performance mnésique supérieure et des concentrations accrues du facteur neurotrophique BDNF comparés aux femelles contrôles. Aucune recherche jusqu'à ce jour n'a déterminé les effets de la restriction

alimentaire auprès de femelles ischémiées. Il serait donc intéressant de caractériser ces effets de manière à déterminer s'ils se produisent également et à la même intensité chez ces dernières.

Jusqu'à présent, plusieurs recherches ont démontré que la restriction alimentaire prévenait ou retardait les déficits mnésiques associés au vieillissement chez les rongeurs [29,155,202,246,432]. Il est possible que les résultats puissent être différents advenant l'adoption d'une restriction alimentaire plus tard dans la vie des animaux et/ou que les effets ne peuvent se reproduire auprès d'animaux recevant un choc ischémique à un âge plus avancé. Afin de pleinement valider les effets de cette procédure, il s'avère important d'introduire la restriction alimentaire à différent âge, et d'inclure une période d'ingestion minimale nécessaire à l'obtention d'effets fonctionnels. L'examen fonctionnel devrait également se poursuivre sur de plus longs intervalles de manière à déterminer si les effets de cette diète demeurent stables avec le temps.

Finalement, il s'avère impératif à la lueur de nos observations et de celles de plusieurs travaux récents de mieux cerner dans les études à venir les changements survenant dans les régions extra-hippocampiques. À ce titre, il serait important d'intégrer plusieurs marqueurs neuronaux et synaptiques aux études du comportement de manière à accentuer la compréhension des mécanismes protecteurs de la restriction alimentaire par un pairage physiologie-comportement à l'intérieur des mêmes travaux scientifiques. Puisque que la restriction alimentaire semble préserver la mémoire spatiale et la flexibilité cognitive des animaux ischémiés tel qu'observé dans cette thèse, il serait intéressant de caractériser ces marqueurs avant, pendant et après l'apprentissage de tâches davantage complexes.

En conclusion, l'ensemble des résultats de cette thèse de doctorat a contribué à mieux cerner l'impact de changements alimentaires et à définir l'importance de ces modifications comme facteurs de prévention efficace lors d'un événement ischémique. Plus précisément, la consommation de diètes restreinte en calories et/ou enrichie de gras polyinsaturés pour une période de trois mois précédent l'administration d'une ischémie cérébrale globale ne protège pas les neurones hippocampiques du CA1 7, 31 et 70 jours post-ischémie. Ces deux diètes induisent néanmoins des effets physiologiques qui subséquemment altèrent ou préservent les habiletés mnésiques post-ischémiques dans un labyrinthe radial. Les résultats de cette thèse sont également les premiers à démontrer des habiletés mnésiques et une flexibilité cognitive comparable à celles des rats sham et des changements au sein des transporteurs vésiculaires chez des animaux ischémiés restreints. Malgré que ces résultats soient préliminaires, ils appuient l'hypothèse d'une meilleure plasticité synaptique et possiblement une réorganisation des circuits neuronaux survivants. Il est souhaité que ces derniers ouvrent de nouvelles pistes de recherche qui sauront nous éclairer davantage quant aux mécanismes protecteurs de ces diètes.

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