UNIVERSIDADE DA BEIRA INTERIOR
Ciências da Saúde

The role of transthyretin and thyroid hormones on functional recovery after experimental stroke

Daniela de Fátima Rua Matos Talhada

Tese para a obtenção do grau de Doutor em
Biomedicina
(3º ciclo de estudos)

Orientador: Prof. Doutor Karsten Ruscher
Coorientadora: Prof. Doutora Cecília Reis Alves dos Santos
Coorientadora: Prof. Doutora Isabel Maria Theriaga Mendes Varanda Gonçalves

Covilhã, março de 2020

“To thankful souls
Which devoted their precious life for human health,
We will do our best to make your devotion more useful
And to reduce your sacrifice. '3R' - Replacement, Reduction, Refinement”

Prayer for Precious Souls, memorial stone at Samsung Biomedical Research Institute in Seoul, Korea

This work was supported by FEDER funds through the POCI - COMPETE 2020 - Operational Programme Competitiveness and Internationalization in Axis I - Strengthening research, technological development and innovation (Project No. 007491) cofounded by the European Social Fund and National Funds by FCT - Foundation for Science and Technology (Project UID/Multi/00709/2019) and doctoral fellowship (SFRH/BD/104679/2014). Moreover, this work was also supported "Programa Operacional do Centro, Centro 2020" through the funding of the ICON project (Interdisciplinary Challenges on Neurodegeneration; CENTRO-01-0145-FEDER-. 000013)". Image acquisition for dendritic spine analysis was performed in the Microscopy facility of CICS-UBI, a node of PPBI (Portuguese Platform of BioImaging): POCI-01-0145-FEDER-022122.

Studies presented in this thesis were also supported by the Swedish Brain Fund; the Crafoord Foundation, Sveriges Stroke Riksförbundet; the Hans-Gabriel and Alice Trolle-Wachtmeister Foundation, Sparbanksstiftelsen Färs & Frosta, the Stiftelsen Sven-Olof Jansons livsverk and regional grants from Region Skåne (ALF).

Cofinanciado por:

Acknowledgments

A todos vós que me acompanhastes neste percurso, tendes a minha gratidão.

To you all that supported me in this journey, you have my gratitude.

Karsten Ruscher, I am very grateful for your support as a supervisor and as a such good friend. You gave me this amazing opportunity to search together new therapies for stroke patients. I hope we continue in this demand together in the future.

Tadeusz Wieloch, I was very pleased to have the opportunity to know such an experienced professor. Thank you for endorsing my practical work in the Laboratory for Experimental Brain Research.

Cecília Santos e *Isabel Gonçalves*, muito obrigada pela vossa amizade, orientação e pelas críticas científicas. As duas serão sempre o meu suporte científico na Covilhã.

Elisa Cairrão, estou muito agradecida pela amizade, disponibilidade e empenho na participação deste projeto, que muito valorizou o seu impato para a comunidade científica. Aguardo colaborações futuras!

Ilknur, *Kerstin*, *Miriana* and *Jakob*, thanks for your friendship and support for everything I always needed in the Laboratory for Experimental Brain Research.

Fátima Santos, *Joana Feiteiro*, *Tânia Albuquerque*, *Melissa Mariana* e *Sandra Rocha*, as minhas amigas e colegas do CICS-UBI que estão sempre bem dispostas para bom companheirismo, trabalhar e ajudar. Obrigada pela amizade e pelo vosso apoio constante. Adoro-vos amigas!

Telma Quintela, *Joana Tomás*, *Ana Catarina*, *Ana Raquel Brito* e *Ana Raquel Costa*, é um orgulho ser vossa amiga e trabalhar convosco no Grupo *COMPLEXUS*. *Margarida Almeida*, obrigada também pela tua amizade e por seres a melhor vizinha de gabinete de sempre.

À minha *família*, aos meus pais e irmãos, em especial ao *Tiago Talhada* pela disponibilidade em participar neste projecto, e aos meus *amigos*, muito obrigada pelo vosso apoio constante e incondicional.

A todos muito obrigada!

Resumo

O acidente vascular cerebral (AVC) continua a representar uma das maiores causas de morte e comorbidade a nível mundial, sendo o AVC isquémico o tipo mais comum, que representa 87% dos casos. Em Portugal o AVC é a primeira causa de morte e incapacidade em pessoas idosas. Os sintomas são variáveis de acordo com a região cerebral afetada e com e extensão do AVC, no entanto, a hemiparesia é experienciada por cerca de 75% dos doentes.

A causa mais frequente de um AVC isquémico deve-se à obstrução do fluxo sanguíneo numa artéria cerebral devido à oclusão por um trombo, geralmente um ateroma ou um coágulo, ou por um êmbolo proveniente do coração ou das artérias carótidas. A extensão dos danos depende do tempo em que as células cerebrais ficam privadas de oxigénio e glicose, ou seja, em ambiente de hipóxia. No centro do enfarte, onde o fluxo sanguíneo é inexistente, há uma rápida degeneração do tecido cerebral e morte celular. A morte celular deve-se, principalmente, ao aumento do influxo de cálcio, resultante da excitotoxicidade devido a excesso de glutamato extracelular e ativação excessiva dos canais de glutamato N-metilo-D-aspartato (NMDA) e ácido alfa-amino-3-hidroxi-5-metil-4-isoxazol-propiónico (AMPA). Todavia, na região adjacente ao centro do enfarte ou na penumbra, os danos são menores e potencialmente reversíveis, devido ao fluxo sanguíneo colateral.

As primeiras 4 horas e 30 minutos após o início de um AVC são cruciais para reduzir os efeitos da excitotoxicidade, edema e inflamação aguda. O tratamento com agentes fibrinolíticos ou trombectomia durante esse período após um AVC é eficaz, na medida em que restabelece o fluxo sanguíneo e previne que as células cerebrais sofram danos irreversíveis. Porém, uma minoria de doentes é elegível a este tipo de tratamento na fase aguda, o que limita a sua aplicabilidade. Atualmente, o tratamento de um AVC consiste na recanalização dos vasos obstruídos se possível, e a longo prazo na reabilitação do doente.

Após um AVC, o processo de recuperação funcional ocorre com maiores progressos durante as primeiras quatro semanas e prolonga-se durante meses a anos. Durante este período, o cérebro adota diversos mecanismos de reorganização anatómica e fisiológica espontânea nas áreas subjacentes ao centro do enfarte e na região contralateral, ou seja, neuroplasticidade espontânea. No entanto, a recuperação neurológica espontânea é muito limitada, na medida em que o doente recupera apenas parcialmente algumas funções motoras. Todavia, a recuperação pode ser otimizada através de estímulos terapêuticos. Muitos esforços têm sido feitos no desenvolvimento de terapias adjuvantes que promovam a neuroplasticidade, contudo, nenhuma foi ainda aprovada em ensaios clínicos. De momento, a abordagem clínica a longo prazo em sobreviventes de um AVC continua a ser a reabilitação.

As hormonas da tiroide (TH) e a sua proteína de transporte, transtirretina (TTR), são potenciais alvos terapêuticos para estimular os mecanismos endógenos de reparação neuronal. As TH desempenham funções essenciais durante o desenvolvimento cerebral e na vida adulta. Em particular a forma ativa 3,5,3'-triiodo-L-tironina (T_3) é crucial na regulação de mecanismos de plasticidade neuronal, estimulação da angiogénese e neurogénese, na modulação da função de componentes do citoesqueleto e em processos de transporte intracelular. Estes mecanismos estão também presentes durante o processo de recuperação e estimulam a recuperação da função motora durante as primeiras semanas a meses após um AVC. Independentemente da sua função como proteína de transporte, a TTR tem sido estudada como uma molécula neuroprotetora no cérebro e posta em evidência como alvo promissor durante a fase de recuperação após um AVC, de forma a melhorar as funções neurológicas perdidas.

Os ensaios experimentais apresentados nesta tese tiveram como objetivo investigar o papel das TH e da TTR na reorganização da função neuronal após fototrombose (PT). A administração de T_3 a 50 µg/kg durante as primeiras duas semanas após a indução experimental de AVC, realizada por PT em murganhos, melhorou significativamente a recuperação da função neurológica perdida sem afetar o volume do enfarte. Observámos uma recuperação da função motora, acompanhada por mecanismos de regulação homeostática na periferia do enfarte, em favor da excitabilidade. A nível celular e estrutural demonstramos que a T_3 tem efeitos modulatórios que atuam a diferentes escalas temporais e locais, de forma a assegurar uma eficiente neurotransmissão sináptica.

Mostrámos também que a administração a longo prazo da T_3, após PT, induz alterações estruturais ao aumentar a densidade das espículas dendríticas na periferia do enfarte e na região contralateral. A eficácia da neurotransmissão sináptica parece ter sido aumentada, após administração da T_3, devido ao aumento dos níveis de sinaptotagmina 1 e 2, que são proteínas vesiculares pré-sinápticas envolvidas na libertação de neurotransmissores e ao aumento dos níveis da subunidade 2 dos recetores de glutamato AMPA, na periferia do enfarte. A administração da T_3 também diminui a ação inibitória do neurotransmissor ácido gama-aminobutírico (GABA) na periferia do enfarte devido à redução da atividade dos neurónios que expressam a parvalbumina e à redução dos níveis da descarboxilase do glutamato (GAD) 65/67. Além disso, mostrámos que a T_3 modula, *in vitro*, propriedades intrínsecas da membrana neuronal, com o equilíbrio das correntes dos recetores ionotrópicos ativados pelo glutamato e diminuição dos níveis de sinaptotagmina em neurónios submetidos a privação de oxigénio e glicose. Curiosamente, encontrámos níveis aumentados do recetor TRβ1, que medeia as ações da T_3, no centro do enfarte de amostras *post-mortem* de doentes que sofreram um AVC.

Apesar do seu já demonstrado efeito neuroprotetor, não encontramos expressão do gene nem da proteína TTR no córtex cerebral de murganhos nas duas semanas após PT e, temos dúvidas que a TTR participe em mecanismos de recuperação motora após um AVC.

Em conclusão, os nossos resultados indicam que a T_3 modula a neurotransmissão, excitatória e inibitória, relevante para os processos de plasticidade neuronal após um AVC. A administração da T_3 durante o período crítico para a recuperação regula mecanismos que equilibram o rácio excitação – inibição, em favor da excitação. Nesse contexto, os resultados apresentados parecem muito promissores, no sentido de serem explorados em futuros ensaios clínicos, de forma a desenvolver novas terapias para melhorar a recuperação motora em doentes que sofreram um AVC.

Palavras-chave

acidente vascular cerebral isquémico; espícula dendrítica; fototrombose; hormonas da tiroide; plasticidade neuronal; recuperação de um AVC; transtirretina

Abstract

Stroke remains one of the leading causes of death and disability worldwide. Focal ischemic cortical stroke results in tissue demise in the infarct core and neuronal dysfunction in areas surrounding the core. The loss of neuronal function triggers specific neuroanatomical and neurophysiological changes in both adjacent and remote areas during the first weeks after stroke onset. During this critical time window, there is a profound reorganization of cortical maps that is accompanied with spontaneous neuroplasticity, however, with limited and partially aberrant recovery of motor function. Induced plasticity with external interventions such as rehabilitation, facilitates recovery and promotes improvement of lost neurological function, albeit to a limited extent. Despite much effort has been spent in developing adjuvant therapies to foster spontaneous underlying endogenous mechanisms, none of the treatment attempts reached clinical use. Thus, rehabilitation remains the only evidence-based long-term treatment in stroke survivors.

We hypothesized that by targeting thyroid hormones (TH) and their carrier protein transthyretin (TTR) might be a promising therapeutic strategy to foster endogenous mechanisms of neurorepair. TH are of fundamental importance for brain development and essential factors to warrant brain functions throughout life. In particular, the active form 3,5,3'-triiodo-L-thyronine (T_3) is involved in the regulation of neuronal plasticity, stimulation of angiogenesis and neurogenesis as well as modulation of the dynamics of cytoskeletal elements and intracellular transport processes. These mechanisms overlap with those that have been identified to enhance recovery of lost neurological functions during the first weeks and months after ischemic stroke. Independent of its role as a TH carrier protein, TTR has been studied as a neuroprotective molecule in the brain, which has been emphasized as promising target to enhance lost neurological functions during the recovery phase after stroke.

In the experimental setting, we investigated if TH and TTR are involved in the reorganization of cortical neuronal function after stroke. We found that administration of T_3 50 µg/kg during the first two weeks after photothrombotic stroke in mice significantly enhanced functional recovery of lost neurological function without affecting infarct size. Motor improvement was accompanied by mechanisms of homeostatic regulation in the peri-infarct area in favor for an increased excitability. The mechanisms involved an increased level of the AMPA receptor subunit glutamate receptor 2 and synaptotagmin 1 and 2, which are pre-synaptic vesicles involved in neurotransmitter release. In addition, T_3 increased dendritic spine density of principal neurons in the peri-infarct motor cortex. Moreover, we have shown that T_3 regulates glutamatergic neurotransmission in cortical glutamatergic neurons. In parallel, T_3 suppressed tonic GABAergic signaling in the peri-infarct tissue shown by a reduced number of parvalbumin positive neurons activity and decreased glutamic acid decarboxylase 65/67 levels.

Despite TTR has been demonstrated as neuroprotective after ischemic stroke, we could not find *ttr* or TTR protein expression in the infarct core and peri-infarct area, and it seems unlikely that TTR is involved in mechanisms of tissue reorganization following PT during the recovery phase after stroke.

Our results indicate that T_3 modulates excitatory and inhibitory neurotransmission relevant for plasticity processes in the postischemic brain. T_3 administration during the critical period for brain recovery regulates mechanisms that balance excitation – inhibition in favor of excitation. Further understanding and target those mechanisms might be exploited in future therapies to enhance functional recovery in stroke patients.

Keywords

dendritic spine; ischemic stroke; photothrombosis; neuronal plasticity; stroke recovery; thyroid hormones; transthyretin

Table of contents

List of Figures.. xvii

List of Abbreviations.. xix

List of Scientific publications.. xxi

List of Scientific communications.. xxii

CHAPTER 1

INTRODUCTION.. 3

1. Ischemic stroke.. 3

2. Pathophysiology of ischemic stroke.. 5

2.1. Acute phase after ischemic stroke.. 6

2.2. Recovery phase after ischemic stroke.. 6

3. Therapeutic interventions to enhance motor recovery.. 7

3.1. Acute phase after ischemic stroke.. 7

3.2. Recovery phase after ischemic stroke.. 8

3.2.1. Cortical map reorganization.. 8

3.2.2. Homeostatic plasticity.. 9

3.2.3. Hebbian plasticity.. 12

3.3. Critical period for intervention.. 12

4. Photothrombotic model to study mechanisms of recovery after stroke.. 13

5. Thyroid hormones in the brain.. 15

6. Transthyretin in the brain.. 18

7. References.. 21

CHAPTER 2

GLOBAL AIMS.. 35

CHAPTER 3

Thyroid hormones in the brain and their impact in recovery mechanisms after stroke 39

CHAPTER 4

Triiodothyronine modulates neuronal plasticity mechanisms to enhance functional outcome after stroke.. 59

CHAPTER 5

The role of Transthyretin in the postischemic brain.. 105

CHAPTER 6

CONCLUDING REMARKS AND FUTURE PERSPECTIVES.. 123

List of Figures

Figure 1. Ischemic stroke. Brain ischemia is caused by abrupt interruption of blood supply to a region of the brain. Adapted from strokecenter.org...5

Figure 2. Homeostatic regulation of the excitation – inhibition ratio after ischemic stroke. Pyramidal neurons provide excitatory outputs to other pyramidal neurons (circles in blue a and b) and to inhibitory neurons (circle in red a). Inhibitory neurons in turn feed inhibition back to pyramidal neurons (circle in red b). This activity in surviving cortical networks are affected by loss of synaptic input from neurons in the infarct core. After ischemic stroke, lower activity increases excitatory feedback (circles in blue a' and b') and decreases inhibitory feedback (circle in red b'). Adapted from [54]..10

Figure 3. Induction of photothrombotic stroke. The brain is illuminated through the intact skull of the animal. The photosensitizing dye can be injected intraperitoneally or intravenously. Adapted from [110]...13

List of Abbreviations

AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ATP	adenosine triphosphate
BBB	blood brain barrier
BCSFB	blood cerebrospinal fluid barrier
CNS	central nervous system
CSF	cerebrospinal fluid
CP	choroid plexus
DIO	deiodinase
GABA	gamma-aminobutyric acid
GAD	glutamic acid decarboxylase
GFAP	glial fibrillary acidic protein
HSF1	heat shock transcription factor 1
IV tPA	recombinant tissue plasminogen activator
LAT	L-type amino acid transporters
LTP	long-term potentiation
LRP2	low density lipoprotein-related protein 2
MCT	monocarboxylate transporters
MCAO	middle cerebral artery occlusion
NMDA	*n*-methyl-d-aspartate
OATP	organic anion transporting polypeptides
PT	photothrombotic stroke
PV	parvalbumin
TBG	thyroid binding globulin
TH	thyroid hormones
TR	thyroid hormone receptor
TSH	thyroid stimulating hormone
TTR	transthyretin
T_4	3,5,3',5'-tetraiodo-L-thyronine
T_3	3,5,3'-triiodo-L-thyronine
rT_3	reverse triiodo-L-thyronine
T_2	3,3'-diiodo-L-thyronine

List of Scientific Publications

Papers related to this doctoral thesis

Thyroid hormones in the brain and their impact in recovery mechanisms after stroke
Talhada D, Santos CRA, Gonçalves I, Ruscher K
Frontiers in Neurology (2019), 10:1103

Triiodothyronine modulates neuronal plasticity mechanisms to enhance functional outcome after stroke
Talhada D, Feiteiro J, Costa ARC, Talhada T, Cairrão E, Wieloch T, Englund E, Santos CR, Gonçalves I, Ruscher K
Acta Neuropathologica Communications (2019), 7(1):216

Transthyretin expression in the postischemic brain
Talhada D, Gonçalves I, Santos CR, Ruscher K
PloS One (2019) 14(9):e0221555

List of Scientific Communications

Oral communications related to this doctoral thesis

Effects of thyroid hormone treatment on functional recovery after experimental stroke

Talhada D, Santos C, Ruscher K

I International Congress in Health Sciences Research – Trends in Endocrinology and Neurosciences, 2015. Covilhã, Portugal

Effects of thyroid hormone treatment on functional recovery after experimental stroke

Talhada D, Gonçalves I, Wieloch T, Santos C, Ruscher K

XI Annual Symposium CICS-UBI, 2016. Covilhã, Portugal

Transthyretin expression in the postischemic brain

Talhada D, Gonçalves I, Gomes JC, Saraiva MJ, Santos C, Ruscher K

XII Annual Symposium CICS-UBI, 2017. Covilhã, Portugal

Impact of triiodothyronine in recovery mechanisms after stroke

Talhada D, Feiteiro J, Talhada T, Wieloch T, Santos C, Gonçalves I, Ruscher K

III International Congress in Health Sciences Research – Trends in Aging and Cancer, 2019. Covilhã, Portugal

Poster presentations related to this doctoral thesis

Longterm effects of Thyroid hormones on recovery mechanisms after experimental stroke
Talhada D, Gonçalves I, Wieloch T, Santos C, Ruscher K
9th International Symposium on Neuroprotection Neurorepair, 2016. Leipzig, Germany

Thyroid hormone triiodothyronine modulates mechanisms of brain plasticity after experimental stroke
Talhada D, Gonçalves I, Santos C, Ruscher K
11th FENS Forum of Neuroscience, 2018. Berlin, Germany

Triiodothyronine modulates mechanisms of brain plasticity after experimental stroke
Talhada D, Feiteiro J, Talhada T, Gonçalves I, Santos C, Ruscher K
10th International Symposium on Neuroprotection Neurorepair, 2018. Radebeul, Germany

Triiodothyronine balances glutamatergic neurotransmission - impact for stroke neuroprotection
Talhada D, Feiteiro J, Cairrão E, Wieloch T, Santos C, Gonçalves I, Ruscher K
III International Congress in Health Sciences Research – Trends in Aging and Cancer, 2019. Covilhã, Portugal

CHAPTER 1

INTRODUCTION

INTRODUCTION

1. Ischemic stroke

Stroke is the second leading cause of death above the age of 60 years and is the third leading cause of disability worldwide [1,2]. In Europe, stroke was responsible for 430.000 deaths in 2015, accounting for 8% of all deaths [3]. In Portugal, stroke is the leading cause of death, accounting for 11% of all deaths in 2014, and healthcare costs are estimated to €159.7 million [4]. This represents an enormous burden for national healthcare system, societies and individuals and their relatives, with physical and emotional suffering.

An ischemic stroke occurs when there is an interruption of blood supply to the brain. This leads to death or brain damage due to lack of oxygen and nutrients resulting in sudden neurological deficits that vary depending upon stroke size and location. Stroke victims may experience paralysis or loss of motor function, loss of sensory function and proprioception, impaired speech and/or cognition and loss of vision, that is often accompanied by fatigue and depression [5]. Hemiparesis of the upper extremity is experienced by up to 75% of stroke victims, interfering with quality of life and productivity [5,6].

An ischemic stroke progresses in three time windows, each of them defined by multiple processes and with distinct key therapeutic strategies to overcome the consequences of the injury: the acute phase, the recovery phase and the chronic phase [7]. The acute injury phase is defined by mechanisms of cell death during the initial 48 hours after stroke onset. Thrombolysis and thrombectomy can be very effective to restore blood flow to the brain during the first 4.5 hours after stroke onset, reducing brain damage [8]. However, due to the short treatment window, rapid progression of injury and high risk of hemorrhage, less than 10% of stroke patients are eligible for these treatments [8,9]. Even from stroke patients that receive acute therapies, approximately half of them still have significant long-term disabilities [10,11]. After cell death and accompanied acute inflammation have been subsided, specific inflammatory cascades and mechanisms of spontaneous neuronal recovery arise [12,13]. Adjuvant therapeutic interventions during this phase might be promising and may have the potential to complement acute therapies, due to the possibility to administer them in an extended treatment time window. During this stage, the brain is highly plastic and neuronal connections in the damaged and intact brain areas are susceptible for modulations potentially taking over lost brain functions [7,14]. Adjuvant treatments might also be beneficial for patients once the brain is stable and spontaneous recovery reaches a plateau during the chronic state, which typically occurs during the first months after stroke and continues for the lifetime [7].

Currently, therapeutic strategies to mitigate stroke damage remain suboptimal. The best practice for stroke management is to reduce the initial impact if possible, maximize functional recovery through physiotherapy, the only evidence-based stroke therapy, and prevent secondary stroke complications and further comorbidities [15]. There is still a need to better understand the mechanisms underlying motor recovery and neuronal plasticity, in order to optimize brain’s ability to reorganize in an adaptive way.

2. Pathophysiology of ischemic stroke

The most common type of stroke is an ischemic insult, that accounts for approximately 87% of all strokes (Figure 1). Ischemia is caused by an interruption of blood supply to a region of the brain, due to artery occlusion by an atherosclerotic thrombus formed in a major cerebral artery (about 50%) or an embolus formed in the heart due to atrial fibrillation (about 20%) or carotid arteries [1,16]. As consequence, neurons are exposed to an ischemic environment and due to their dependence on aerobic metabolism, in less than three minutes they become dysfunctional or die [16,17]. In the infarct core, where blood flow drops below a critical level, neurons undergo immediate and irreversible death. Surrounding the infarct core, the penumbra, neuronal damage is slower due to collateral blood supply from adjacent territories and if blood supply is re-established damage might still be reversible. However, if blood flow is not re-established during the first hours after stroke onset, neurons will die in the penumbra [17]. Dependent on blood flow in the tissue in the penumbra, this region will contribute to the final infarct volume or be converted into the peri-infarct area. In the periphery of the infarct or peri-infarct region, partially lesioned surviving neurons remain salvageable.

The events after ischemic stroke evolve in time and are not limited to the lesion itself. The pathophysiological mechanisms following ischemic stroke include changes in blood flow and formation of edema, changes in metabolism, neurotoxicity and inflammation. A delayed phase of injury is characterized by spontaneous neuronal repair and tissue reorganization, that can evolve over several weeks.

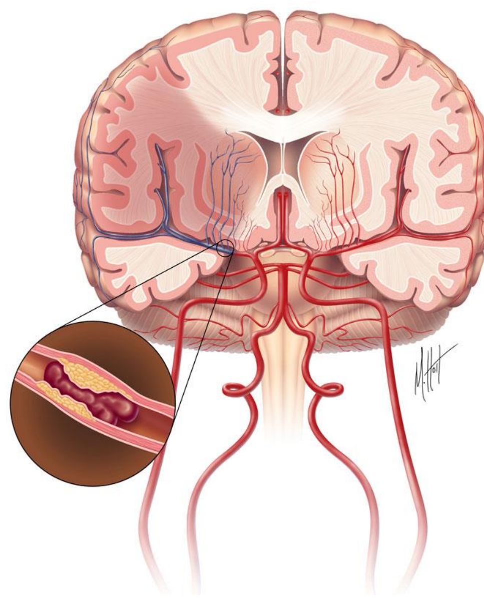


Figure 1. Ischemic stroke. Brain ischemia is caused by abrupt interruption of blood supply to a region of the brain. Adapted from strokecenter.org.

2.1. Acute phase after ischemic stroke

In the acute phase of ischemic stroke, hypoxia triggers a cascade of cellular and molecular events, that results in cell death [16–18]. With the reduction of oxygen and glucose, neurons switch to anaerobic metabolism which is much less effective generating adenosine triphosphate (ATP) than aerobic metabolism. Energy failure leads to malfunction of ionic pumps crucial for ionic gradient maintenance through the neuronal membrane. Dysfunction of the sodium-potassium ATPase contributes to cytoplasmic accumulation of sodium resulting in cytotoxic edema. Dysfunction of the calcium-potassium ATPase leads to intracellular calcium accumulation and as consequence, glutamate neurotransmitter is excessively released and accumulates in the extracellular space [19]. Glutamate is the major excitatory neurotransmitter in the brain and its extracellular accumulation induces overexcitation of neurons post-synaptically that is potentially toxic. Neuronal death following a stroke is mostly due to excitotoxicity elicited by excess of extracellular glutamate [18].

Excitotoxicity overactivates N-methyl-D-aspartate (NMDA) and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) ionotropic glutamate receptors as well as metabotropic glutamate receptors. As consequence there is excess of calcium entry into the cell, which leads to diverse molecular cascades that elicit cell death. Calcium-dependent degradative enzymes are activated resulting in loss of membrane integrity and excessive production of free radicals, nitric oxide and arachidonic acid metabolites. Also, mitochondria are affected by degradative enzymes and release apoptotic factors to the cytoplasm [18,20].

2.2. Recovery phase after ischemic stroke

While in the acute phase of stroke the brain is hyperexcitable due to aberrant glutamate release, the recovery phase is characterized by decrease in glutamate signaling due to cell death and inhibitory inputs. This triggers spontaneous repair-related molecular and cellular changes, although to a limited extent [13]. Spontaneous neuronal repair starts within the first days after acute injury and continues for months in mice [21,22] and up to years in humans [11,23]. Three interrelated mechanisms are suggested to contribute to neuronal repair at this stage. First, with reperfusion following stroke, there is resolution in blood flow, metabolism, edema and subacute inflammation in regions surrounding the infarct tissue. Second, disruption in neuronal activity is restored among surviving neurons by changing neuronal pathways and third, the tissue reorganizes to establish and consolidate new neuronal connections [24–26]. As in the healthy brain this ability to restore and reorganize its connections and structure is defined as neuroplasticity.

3. Therapeutic interventions to enhance motor recovery

Functional improvement is dependent on the individual health status, age, early medical care, the stroke location, the severity of ischemia and the extent of tissue loss. Unfortunately, patients who suffer from stroke unlikely recover function from the damaged tissue that is irreversible lost. However, loss of function after stroke is not only caused by cell death in the infarct core but also by dysfunction in neurons in the penumbra, non-ischemic peri-infarct area and by diaschisis, referent to cell dysfunction in remote uninjured areas but functionally connected to the site of injury [27], occurring due to hypoperfusion and hypometabolism, neurovascular uncoupling and aberrant neurotransmission [28]. The term "improvement after stroke" normally describes a broad of events that include the recovery of the remaining reversible damaged brain areas and the compensatory behavior from new intact brain areas, through motor rehabilitation and neuronal circuitry reorganization, i.e. neuroplasticity [29]. However, the degree of compensatory mechanisms and true recovery contributing for improved neurological function is usually indistinguishable and the term "recovery" has been commonly used to describe both recovery and compensatory mechanisms.

3.1. Acute phase after ischemic stroke

In the early hours after stroke onset, treatment strategies are focused on preserve the penumbra, by resolution of early pathophysiological changes that contribute for motor dysfunction, namely excitotoxicity, edema and inflammation [7]. However, saving the penumbra is very time-limited and cells quickly die without intervention. These processes are activated and occur within minutes after stroke making it very difficult to target them in neuroprotective treatment attempts [21].

The goal is to restore blood flow of the compromised area and optimize collateral blood flow before irreversible damage occurs. According to the current guidelines from the American Heart Association / American Stroke Association, management of stroke in this phase includes recanalization of the occluded vessels [30]. This can be achieved by administration of intravenous fibrinolytics (i.e., recombinant tissue plasminogen activator – IV tPA) within 3 to 4.5 hours after stroke onset. In addition to or in ineligible patients for IV tPA, mechanical thrombectomy is recommended for eligible patients within 6 to 16 hours after stroke onset [30-32]. Recently, the European Stroke Organization also provided new guidelines for mechanical thrombectomy during the first 24 hours after stroke onset [33].

The ischemic penumbra is salvageable during the first hours after stroke onset and the concept that neuroprotection during this interval window preserves the penumbra and extends the time window for recanalization techniques is also subject of several studies [34]. In fact, neuroprotective agents that reduce excessive activation of excitatory receptors and glutamate release enhance neuronal survival in several animal studies, however none has still been successfully translated into clinical trials [35,36]. The most common neuroprotective agents studied so far are NMDA receptor antagonists and, more recently, cell-based therapies are at a very early stage of development [37].

3.2. Recovery phase after ischemic stroke

Beyond of the acute phase of stroke and once the lesion is established, the brain adopts innate mechanisms that enhance brain plasticity [13]. However, brain plasticity is often impaired due to limited self-repair mechanisms and because the activation of brain plasticity inhibitors [13]. Although stroke patients regain some of lost function without any intervention, spontaneous plastic reorganization is suboptimal, and most stroke survivors are left with chronic disability. The only evidence-based plasticity inducing therapy that enhances functional recovery is rehabilitative training [38]. However, rehabilitative training alone in most case of the patients is insufficient to restore lost neurological function.

Current research, therefore, aims at understanding the mechanisms that foster endogenous repair mechanisms that occur spontaneously after stroke [7]. This approach creates an opportunity to identify new targets for adjuvant treatments combined with specific rehabilitative interventions during a wide therapeutic window [39].

3.2.1 Cortical map reorganization

Motor and sensory cortices are organized in somatotopic functional maps, reflecting the brain areas that are responsible to control muscles and to process sensory inputs [40]. These maps are the basis for the learning and expression of movements, representing a type of motor engram, or a memory trace for an action or movement. Brain plasticity is defined as the ability of the adult brain to adapt and learn from experience and to reorganize a structure of motor maps and neuronal connections in response to different stimuli [40]. For instance, behavioral experiences, sensory inputs and learning influence map plasticity by a reorganization of neuronal cortical activity patterns that may drive the growth and maturation of synapses as well as selective survival of synapses [40,41].

Evidence of cortical remapping has been observed in stroke patients by functional Magnetic Resonance Imaging and Transcranial Magnetic Stimulation [42–44] and in animal models of controlled cortical lesions by intracortical microstimulation [45–47], autoradiograms [48] or optogenetics [49]. Cortical remapping is the reflection of neuronal reorganization of connectivity among surviving neurons. Although post-stroke remapping and experience-dependent plasticity may follow different mechanisms, brain plasticity has been considered the basis for spontaneous recovery in stroke rehabilitation [50,51]. Such plastic changes start during the first weeks after focal cortical lesion, involving the peri-infarct tissue, contralateral hemisphere and subcortical and spinal regions [52].

Brain plasticity mechanisms involved in learning, memory, sensory adaptation, development and recovery from brain injury have been a theme of debate among neuroscientists [53]. The actual position is that two opposite mechanisms are implicated in brain plasticity and the brain can adopt both forms of plasticity into a whole. Those are homeostatic plasticity mechanisms that maintain adequate synaptic input through negative feedback processes [54,55] and Hebbian plasticity where individual synaptic connections are strengthened and refined through positive feedback processes [52,56,57]. In analogy to learning and development rules, these mechanisms are also present after ischemic stroke. Homeostatic mechanisms are engaged to restore synaptic input towards a target level and Hebbian mechanisms reinforce appropriate synaptic levels.

3.2.2. Homeostatic plasticity

In the adult brain, homeostatic regulatory mechanisms contribute to stabilize neuronal activity throughout life and maintain appropriate levels of excitation to keep neuronal activity at their firing rate set points [55,58–60]. Synaptic scaling is a form of homeostatic plasticity where neuron’s excitatory synapses regulate synaptic strength in order to maintain a target activity [61–63]. However, tuning of excitatory synaptic strength is not enough to maintain network stability. For this, it is required to balance between excitation and inhibition among neuronal networks aiming at modifying microcircuit function. Upon reduced synaptic activity, excitation between pyramidal neurons is boosted and inhibition is reduced [54,59]. A wide variety of homeostatic mechanisms contribute to the maintenance of overall excitability involving the regulation of neuronal intrinsic excitability and synaptic transmission. These mechanisms include changes in the receptor expression at the post-synaptic level, neurotransmitter release at the pre-synaptic level, ion channel function and synapse number or strength [54,55,60].

After a brain injury such as ischemic stroke, there is a disruption of this homeostatic regulation and as consequence, alteration in the function of neuronal circuits. Tissue adjacent to the infarct undergo structural and functional changes, compromising normal synaptic activity [64].

In response to loss of synaptic inputs, the brain also adopts homeostatic negative feedback mechanisms in order to adjust excitability (Figure 2). The exact molecular mechanisms of homeostatic processes are not completely understood. Several mechanisms have been identified in experimental models and similarly as it happens during development and learning processes, neurons attempt to stabilize the balance between excitatory and inhibitory circuits after stroke [65].

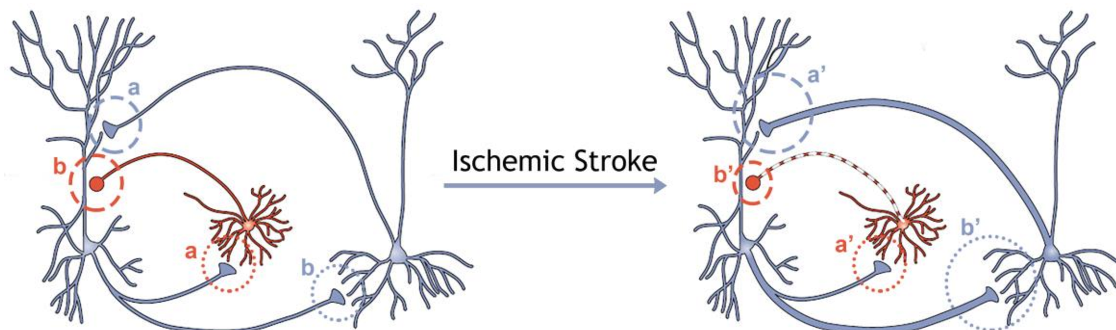


Figure 2. Homeostatic regulation of the excitation – inhibition ratio after ischemic stroke. Pyramidal neurons provide excitatory outputs to other pyramidal neurons (circles in blue a and b) and to inhibitory neurons (circle in red a). Inhibitory neurons in turn feed inhibition back to pyramidal neurons (circle in red b). This activity in surviving cortical networks are affected by loss of synaptic input from neurons in the infarct core. After ischemic stroke, lower activity increases excitatory feedback (circles in blue a' and b') and decreases inhibitory feedback (circle in red b'). Adapted from [54].

Spontaneous increase of neuronal activity after stroke may be determined by an upregulation of pre-synaptic glutamate release and post-synaptic response. Pre-synaptic release of glutamate is dependent on pre-synaptic vesicular proteins, that regulate synaptic vesicle docking, fusion with the membrane and exocytosis. Post-synaptic response to glutamate may be modulated by changing the number or function of ionotropic glutamate receptors, AMPA and NMDA [55,59,66,67], which drive calcium influx and cell excitability. Indeed, stroke-induced glutamate release activates AMPA receptors [68] and NMDA receptors [69], changes that were related with motor recovery. Functional mechanisms involve the modulation of neuron intrinsic membrane properties, with the balance of inward and outward currents of voltage-dependent and ligand-gated channels [59,70].

In addition, homeostatic plasticity triggers structural synaptic plasticity with morphological changes in dendritic spines and the formation of new synapses or the elimination of pre-existing spines [71] and with mechanisms that induce axonal sprouting and increment of dendritic spine density [52,72], which may drive lesion-induced network reorganization [73]. Moreover, there

is an increased expression of genes and proteins important for neuronal growth, synaptogenesis, and proliferation of dendritic spines [25,74,75].

Emerging evidence from experimental studies have pointed out a role of the decrease of tonic gamma-aminobutyric acid (GABA) signaling on the function of neurons and neuronal networks in the recovery phase of stroke [46,76–78]. Tonic GABAergic signaling decrease might be mediated by homeostatic regulation of inhibitory synaptic neurotransmission. Mechanisms may involve a reduction of post-synaptic GABA receptors, such as GABA(A) receptor [46,79–81], reduction of pre-synaptic GABAergic markers such as glutamate decarboxylase (GAD) 65/67 [82] or reduced level or function of GABA transporters [76]. In addition, also growth inhibitory molecules that limit axonal plasticity are reduced [25,83].

Neocortical inhibitory parvalbumin (PV) positive interneurons are important to provide local inhibition on neighboring pyramidal neurons [84]. Modulation of PV activity is also contributing to increase the excitation / inhibition ratio post-stroke. For instance, to block the activity of PV positive neurons induces a decrease in monosynaptic PV inhibition [85]. A decrease in PV immunoreactivity is also related with improved functional recovery after experimental stroke [86].

Insights from animal studies show that several endogenous repair mechanisms occur in the peri-infarct area, that spatially represents a dynamic and important brain region for neurological recovery [87,88]. Moreover, post-stroke hyperexcitability and downregulation of GABA signaling have been demonstrated not only in surviving neurons in the peri-infarct region but also in distant regions functionally connected to the infarct, especially in larger strokes [89–92]. The increased cortical excitability in the peri-infarct area reduces somatotopic modifications and in the contralateral regions reduces lateralized activation, both implicated in stroke recovery [93]. This connectivity between cortical regions enables for the formation of new structural and functional circuits through remapping, to take over the function of the damaged area. This reduces lateralized activation and indicate the ability of compensatory mechanisms to restore function [52].

3.2.3. Hebbian plasticity

Once homeostatic mechanisms are engaged to restore synaptic levels, Hebbian forms of plasticity may be important for strengthening and retain properly wired connections [52,94]. Repeated and persistent hyperexcitability may facilitate action potential dependent activity and strengthen synaptic efficiency.

Experimental and clinical evidence supports a fundamental role for Hebbian mechanisms for brain reorganization, such as rehabilitative training and multisensory stimulation of the brain through non-invasive brain stimulation [7,38,42,95–97]. This reinforces the connection between paretic muscles and brain motor areas. The presence of Hebbian mechanisms such as long-term potentiation (LTP) is observed for seven days in the peri-infarct after focal cortical stroke in rats [98], suggesting a favorable environment for rewiring of synaptic connections. However, there is little direct evidence on Hebbian mechanisms after stroke and whether factors that influence Hebbian processes, such as LTP, may have an impact on stroke recovery.

3.3. Critical period for intervention

Beyond the acute phase after stroke, recovery processes involve molecular, cellular and functional mechanisms of homeostatic and Hebbian plasticity, that can operate at the same time to balance the ratio excitation / inhibition [99]. One challenging strategy to improve stroke recovery is how to optimally modify surviving and new neuronal networks to compensate tissue loss in the infarct core, based in the premise that functional recovery is related with neuronal plasticity. The first four weeks after stroke, when spontaneous lesion-induced reorganization occurs, are particularly sensitive to therapeutic interventions. This is an opportunity window to train the brain to regain functions lost due to tissue damage [51].

The time-dependent sequence of neuronal plasticity mechanisms defines a time window for therapeutic intervention, important to optimize functional recovery. The engagement of additional excitability induced by motor activity (e.g. rehabilitation) or other therapeutic interventions should be carefully investigated. Inappropriate timing between interventions may induce maladaptive (i.e. worsening of function) instead of adaptive (i.e. restoration of function) changes. For instance, early rehabilitation significantly increases improvement of function in rats, but its efficacy declines over time after stroke onset [21]. Clinical studies also indicate that early interventions after stroke are related with better outcomes [100–102]. However if too early, physical activity can be detrimental for stroke recovery, especially if it is provided too intensive [103,104].

4. Photothrombotic model to study mechanisms of recovery after stroke

Different animal models have been used to study cellular and molecular mechanisms underlying ischemic stroke and to develop new neuroprotectors or promising agents for stroke recovery [105]. Experimental stroke is crucial to access stroke pathophysiology and to study drug effects, that are not modeled *in vitro* and cannot be investigated in human stroke, due to high heterogeneity in its causes, symptoms and localization, whereas *in vivo* models are highly reproducible, controllable and standardized [105,106]. Because of heterogeneous etiology of human stroke, different experimental models might be required to address specific pathophysiologic aspects of stroke. The selection of the appropriate animal model is therefore important for the quality of preclinical stroke studies and effective clinical translation.

Photothrombotic stroke (PT) was first used in the rat [107] and later adapted for the mouse brain [108]. This model aims at inducing ischemic damage based on intravascular photo-oxidation of an injected light-sensitive dye (eg, Rose Bengal, erythrosine B) that remains in the vasculature and does not cross the blood brain barrier (BBB). Following illumination of the intact skull in the desired brain region at a specific wavelength, the dye is activated and produces oxygen radicals that damages endothelial cell membranes. Subsequently, there is platelet activation and aggregation, formation of thrombi and local interruption of blood flow (Figure 3). PT induces a well-defined ischemic lesion of the cortex in a reproducible and minimally invasive way, which mimics the most common ischemic damage occurring in humans [108–110].

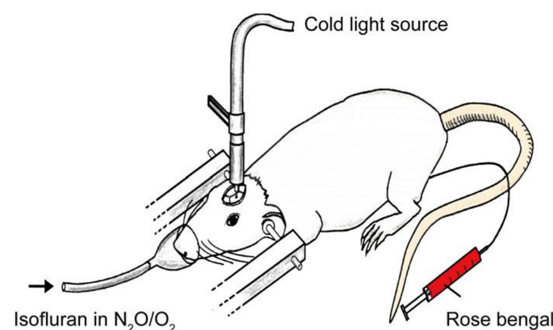


Figure 3. Induction of photothrombotic stroke. The brain is illuminated through the intact skull of the animal. The photosensitizing dye can be injected intraperitoneally or intravenously. Adapted from [110].

As for all experimental models of ischemic stroke, PT has some limitations that need to be considered from the translational point of view. This model is associated with early cytotoxic and vasogenic edema formation (1 to 4 hours after light exposure), due to endothelial damage, and as consequence there is breakdown of the BBB [111] while in acute human stroke vasogenic edema occurs several hours after stroke onset [105,112]. Acute vasogenic edema is therefore also contributing for acute lesion in PT. Due to rapid edema and cell death, there is little collateral blood flow and absence of an ischemic penumbra [113], which limits translational impact of this model of neuroprotective therapies that target the penumbra [112].

Despite limitations associated with the model, ischemic cascades after PT are the same as in human ischemic stroke after thrombus formation and cerebral blood flow occlusion [109]. Therefore, this technique is highly advantageous to study cellular and molecular responses ongoing in the perilesional area and contralateral motor cortices at multiple time points after stroke. Particularly, PT is very suitable for the study of spontaneous functional reorganization in the brain after stroke [49]. It also produces prolonged sensorimotor impairments, allowing long term behavior assessment and recovery associated with the injured area, in particular restoration of brain function due to plasticity [114]. This experimental model allows the identification of molecular and cellular events associated with reorganization of sensory and cortical maps in the peri-infarct and contralateral hemisphere [109], events that also are implicated in functional recovery in human stroke [115].

5. Thyroid hormones and stroke

Thyroid hormones (TH), 3,5,3’,5’-tetraiodo-L-thyronine (T_4) and 3,5,3’-triiodo-L-thyronine (T_3), are important for brain development in mammals, during embryonic and fetal stages, regulating processes of neuronal proliferation, migration and differentiation, neurite outgrowth, synaptic plasticity, dendritic branching and myelination [116–120]. Also, after birth, TH are crucial for normal brain function throughout the entire life. Specifically for the central nervous system (CNS) the active form T_3 is a key regulator for normal metabolism in humans and rodents [118,121,122].

Availability of T_3 to the developing and adult brain is tightly controlled by mechanisms regulating TH secretion, free fraction unbound to thyroxine binding globulins (TBG), transmembrane transporters and the activity of iodothyronine deiodinases (DIO). The pattern of these regulatory processes may vary according to the developmental stage and in the adult brain. T_3 plays an essential role for neurological functions, and minimal disturbances of these mechanisms may have consequences for normal brain development and function [118,123].

In the adult, TH originate in the thyroid gland that secretes approximately 93% as T_4 and 7% as T_3. Once secreted to plasma, TH binding proteins play an important role to maintain TH homeostasis and distribution into tissues, and less than 0.1% of TH circulate free in the blood [124,125]. In humans, 65% of TH bind to TBG, 20% to albumin and about 15% to transthyretin (TTR) [126,127] while in rodents TTR is the main protein carrier in the blood circulation [128,129]. TH also bind to lipoproteins to a less extent [130,131].

In contrary to processes during brain development, a different fraction of T_3 in the adult brain is provided from free T_3 available in the blood circulation and in the cerebrospinal fluid (CSF) [132,133]. Most of TH provided to the brain crosses the blood brain barrier (BBB) and around 20% the blood cerebrospinal fluid barrier (BCSFB) [134,135]. This passage is mediated by transmembrane transporter proteins with overlapping specificity that were identified in endothelial cells of brain microvessels that constitute the BBB and epithelial cells of the choroid plexus (CP) of humans and rodents. These transporters are also important for brain development, and include monocarboxylate transporter (MCT), organic anion transporting polypeptide (OATP), L-type amino acid transporters (LAT) and sodium/taurocholate co-transporting polypeptide (SLC10A1) families [133,136,145,146,137–144]. Among those, MCT8 is of particular importance for T_3 [136,145,146], and in mice deficient for this transporter, T_3 uptake is compromised [147]. In humans, MCT10 also facilitates uptake and efflux of TH, in particular for T_3 [148]. The gene that encodes MCT8 (*Slc16a2*) is also present in membranes of neurons, astrocytes, tanycytes and oligodendrocyte precursor cells and OATP1C1 mRNA has been found in astrocytes [149], mediating intracellular TH transport. In addition, it has been

proposed that either T_4 or T_3 or both are captured via gaps at the endfeet of astrocytes covering brain microvessels [150,151].

In contrast to the rodent, MCT8 deficiency in humans results in low T_3 levels in the brain and high levels in the serum due to TH transport deficiency. Thus, the development of the cerebral cortex is impaired accompanied with severe neurological impairment [152–155]. The lack of alternative TH transporters such as OATP1C1 [144] and MCT10 [156] in the adult human brain to compensate the transport of T_3, may contribute to the neurological deficits observed in humans with MCT8 mutations. The activity of DIO2 and DIO3 in the brain is important to balance neuronal intracellular T_3 levels in the adult brain, according to the developmental stage and brain region [157–159]. DIO enzymes catalyze and remove specific iodine atoms from iodothyronine molecules. In rodents, approximately 50% of T_3 levels localized in the brain relies on local deiodination of T_4 in astrocytes and tanycytes by DIO2 [160]. T_3 produced in glial cells is able to promote T_3 driven transcriptional activity in neurons, demonstrated by *in vitro* experiments in co-cultures of H4 human glioma cells expressing DIO2 and neuroblastoma cells [161]. Homeostasis of T_3 in the CNS is also controlled by DIO3 activity in neurons, that converts T_4 into 3,3',5' reverse triiodo-L-thyronine (rT_3) and inactivates T_3 into 3,3'-diiodo-L-thyronine (T_2) [157,162,163].

Other compensatory mechanisms to maintain sufficient T_3 levels in the rodent brain include the reduction of DIO3 activity and consequently T_3 degradation, the increase of DIO2 activity in astrocytes [133,147,164] and the increase of *Dio2* expression in interneurons in the cerebral cortex [165].

Cellular actions of TH in the adult brain can be mediated by nuclear receptors and transcriptional activity, and also by non-genomic actions [166–168]. Actions of T_3 in the brain are mainly dependent on transcription mediated by T_3 binding to the nuclear receptors and formation of regulatory complexes [166–170]. In the presence of TH, TR are regulated by corepressors and coactivators, proteins, that repress or activate transcription, respectively [151,166–168,171]. TH non-genomic actions that do not require TH binding to nuclear receptors are well described in the literature [172–175]. Actions are immediate and include several interactions of TH with extranuclear receptors located in the cytoplasm, cellular membrane, cytoskeleton and mitochondria, modulating several intracellular pathways.

The complex process of aging is associated with changes in TH metabolism and action in all tissues. During aging, the disruption of circadian rhythm leads to a reduction in thyroid stimulating hormone (TSH) secretion [176–178] and circulating TH levels, in particular T_3, in humans [179,180] and rodents [181]. Nevertheless, TH signaling is well preserved in the aging brain, as demonstrated in mouse models of aging [182]. However, hypothyroidism and decreased TH availability to the brain has been considered a risk factor for the development of neurodegenerative diseases [183,184] and acute ischemic stroke [185,186]. In addition, recent

epidemiological studies have associated low levels of T_3 with poor functional outcome after acute ischemic stroke [187–192]. Interestingly, lower total T_3 levels is not related with poor functional recovery after ischemic stroke in patients below 65 years of age, suggesting that the association between levels of T_3 and stroke recovery may be clinically important in older patients [193]. Nonthyroidal illness syndrome also impairs functional recovery after stroke [194]. Besides, stroke patients with thyroid dysfunction (lower levels of TSH and higher levels of free T_4) are associated with poorer clinical outcome [195]. Together, studies point towards the need for a systemic assessment of thyroid dysfunction and stroke outcome.

Beyond the acute phase after stroke, the brain shows the capacity of spontaneous recovery of lost neurological functions albeit to a limited extent. This process remains slow however, the intrinsic mechanisms are present, and patients may benefit from enhancing those. TH regulate several pathways that are involved in neurorepair, namely regulation of processes of neuronal plasticity, neurogenesis, angiogenesis and glutamate toxicity. Adjuvant therapies that modulate those processes may improve recovery of function after stroke, in particular when applied in combination with physiotherapy in stroke patients.

6. Transthyretin in the brain

Transthyretin (TTR) is a 55 kDa homotetrameric protein, composed of four identical subunits, each containing 127 amino acids [196]. It is well conserved in mammals, and mouse TTR has 80% homology with human TTR [197]. TTR is mainly synthetized by the liver and by the epithelial cells of the CP, which are the sources of TTR in plasma and CSF, respectively [198,199]. In the brain, TTR is the main protein synthesized by the CP, and represents about 25% of total protein in the CSF [198,199]. In the CSF, TTR is the only protein available for TH binding, although TTR has much higher affinity for T_4 than T_3 [200–202]. Percentage of TTR bound to total T_4 in plasma differs between human (10-15%) and rodents (50%) [125,203]. TTR also forms a complex with retinol-binding protein, playing a role in the transport of retinol [204]. TTR has a relatively rapid turnover (half-life time 2 days in blood and 1.5 hours in the CSF) and degradation rate is approximately 650 mg/day [205].

TTR plays an important role as a TH carrier, mainly for T_4, in human and rodent's species [203,206]. In humans, 65% of TH bind to TBG, 20% to albumin and about 15% to TTR, once secreted to plasma [201,205] while in rodents TBG is almost absent, being TTR the main protein carrier [207,208]. TH also bind to lipoproteins to a less extent [131,209]. Hence, free circulating TH in plasma is less than 0.1%, and binding proteins play an important role for TH reservoir, increasing their free fraction in circulation when it is demanded and maintaining an euthyroid status, without changes in free hormone levels [124,125].

Several studies have shown that TH binding proteins are not required for hormone distribution into tissues. Regarding TTR, studies conducted in TTR-null mice showed that TTR only regulates TH levels in the CP and CSF and it is not required for their distribution within the brain parenchyma [8,9]. The administration of EMD 21388, a synthetic flavonoid which displaces TTR from T_4, did not affect T_4 uptake by liver and kidney [210]. In the brain, studies on TTR-null mice demonstrated that in the absence of circulating TTR, the main protein carrier of TH in mice, animals remained in an euthyroid status despite circulating T_4 levels were decreased by 50% [211]. When TTR-null mice receive intravenous injection of T_4, its levels remain normal in brain parenchyma, liver and kidney, with no changes in TH metabolism [212,213]. Also, a morphological study using thaw-mount autoradiography clearly demonstrated that TTR is not required for TH distribution in the brain, since there are no differences in radioactive tracer distribution between wildtype and TTR-null mice brains, after intravenous injection of $[^{125}I]T_3$ and $[^{125}I]T_4$ [206]. Similarly, this occurs with other hormone binding proteins, as humans with increased or decreased TBG levels remain euthyroid [214–216] and kinetic studies in Nagase analbuminemic rats showed that albumin is also not involved in TH distribution into tissues [217].

On the other hand, transmembrane proteins of MCT, LAT and OATP families were identified as intercellular TH transporters [218–220]. The mechanisms of TH transport to the CSF have not been identified but either they are bound to TTR, that is secreted into the CSF; or cross the membrane via MCT8/OATP1C1 proteins, also present in the CP [202]. In either way, even if TTR transports T_4 through the CSF barrier, it is not crucial for TH distribution in the brain. Hence, to date TTR is the only binding protein in the CSF and may provide an exchange compartment of TH to the brain, in particular for T_4.

Together, these studies provide reliable evidences that TH distribution likely relies on free circulating hormones through transmembrane transporters, and there is no substantial role of TTR or other binding proteins to be involved in their distribution into the brain and other tissues. In fact, the presence of TTR in the brain is restricted to the CP and meninges, being totally absent from the hippocampus, cerebellum or cerebral cortex, in wildtype mice [221] shown by *in situ* hybridization and Northern blot analyses [222–224]. Nevertheless, TTR plays an important role as a TH reservoir in blood circulation and CSF and increases hormone bioavailability when demanded and balanced distribution within the tissues [8,10].

Independent of its function as a transporter, attention has been given to TTR as a neuroprotective protein, in particular in Alzheimer´s disease [225,226] and stroke [227–230] through its putative upregulation in the brain. In the context of Alzheimer´s disease, TTR has been suggested as a biological sequester of Aß, since it is able to bind to soluble Aß peptide, preventing the formation of amyloid fibers [231,232]. Some studies in rat and mouse models of Alzheimer´s disease suggest an overexpression and production of TTR in the hippocampus [233-235]. *In vitro* studies also support TTR synthesis in neurons as neuroprotective in Alzheimer´s disease, since primary cultures of mixed cortical and hippocampal neurons from APP23 mice express TTR [236,237], and there is an upregulation of *TTR* in the SH-SY5Y neuroblastoma cell line over-expressing APP_{695} isoform [238]. Activation of neuroprotective pathways is partially mediated by TTR binding to low density lipoprotein-related protein 2 (LRP2). This interaction is also involved in TTR mediated neurite outgrowth since neurons from LRP2 heterozygous/TTR-null mice show reduced neurite outgrowth compared to cells from TTR deficient mice, both treated with TTR [230]. However, the mechanism beyond the assumption of a neuronal regulation of TTR synthesis is unknown and not explored. On the other hand, it has been suggested that the results observed are due to contamination by CP. Indeed, TTR was not found either in the cerebral cortex, hippocampus or cerebellum, verified by TTR immunoreactivity, immunoblotting and TTR mRNA expression in wildtype mice and APP-V717I and Tg2576 mice models of Alzheimer´s disease after a careful laser microdissection [221].

After stroke, TTR deficiency alone has no effect on ischemic damage in mice subjected to permanent middle cerebral artery occlusion (MCAO) [239]. Some immediate effects on lesion size 24 hours after stroke onset were observed in TTR deficient mice heterozygous for heat shock transcription factor 1 (HSF1) [240]. The difference in lesion size and outcome between

TTR deficient mouse strains might be explained by reduced or suppressed levels of HSF1 resulting in a compromised heat shock response during the first hours after stroke onset [241].

In this context, *in situ* TTR may participates in neurorepair mechanisms during the recovery phase after stroke, however, it remains a topic to be investigated.

7. References

1. Benjamin EJ, Virani SS, Callaway CW, Chamberlain AM, Chang AR, Cheng S, et al. Heart disease and stroke statistics - 2018 update: A report from the American Heart Association. Circulation. 2018;137: e67–e492. doi:10.1161/CIR.0000000000000558
2. Feigin VL, Norrving B, Mensah GA. Global Burden of Stroke. Circ Res. 2017;120: 439–448. doi:10.1161/CIRCRESAHA.116.308413
3. OECD/EU. Health at a Glance: Europe 2018: State of Health in the EU Cycle [Internet]. Paris/EU, Brussels: OECD Publishing; 2018. doi:10.1787/health_glance_eur-2018-en
4. OECD, European Observatory on Health Systems and Policies. Portugal: Country Health Profile 2017 [Internet]. Brussels: OECD; 2017 Nov. doi:10.1787/888933593760
5. Hubbard IJ, Carey LM, Budd TW, Levi C, McElduff P, Hudson S, et al. A Randomized Controlled Trial of the Effect of Early Upper-Limb Training on Stroke Recovery and Brain Activation. Neurorehabil Neural Repair. 2015;29: 703–713. doi:10.1177/1545968314562647
6. Langhorne P, Coupar F, Pollock A. Motor recovery after stroke: a systematic review. Lancet Neurol. 2009;8: 741–754. doi:10.1016/S1474-4422(09)70150-4
7. Cramer SC. Treatments to Promote Neural Repair after Stroke. J Stroke. 2018;20: 57–60. doi:10.5853/jos.2017.02796
8. Kim JT, Fonarow GC, Smith EE, Reeves MJ, Navalkele DD, Grotta JC, et al. Treatment With Tissue Plasminogen Activator in the Golden Hour and the Shape of the 4.5-Hour Time-Benefit Curve in the National United States Get With The Guidelines-Stroke Population. Circulation. 2017;135: 128–139. doi:10.1161/CIRCULATIONAHA.116.023336
9. Peisker T, Koznar B, Stetkarova I, Widimsky P. Acute stroke therapy: A review. Trends Cardiovasc Med. 2017;27: 59–66. doi:10.1016/j.tcm.2016.06.009
10. Reed SD, Cramer SC, Blough DK, Meyer K, Jarvik JG. Treatment With Tissue Plasminogen Activator and Inpatient Mortality Rates for Patients With Ischemic Stroke Treated in Community Hospitals. Stroke. 2001;32: 1832–1840. doi:10.1161/01.str.32.8.1832
11. Duncan PW, Min Lai S, Keighley J. Defining post-stroke recovery: Implications for design and interpretation of drug trials. Neuropharmacology. 2000;39: 835–841. doi:10.1016/S0028-3908(00)00003-4
12. Overman JJ, Carmichael ST. Plasticity in the injured brain: More than molecules matter. Neuroscientist. 2014;20: 15–28. doi:10.1177/1073858413491146
13. Cramer SC. Repairing the human brain after stroke: I. Mechanisms of spontaneous recovery. Ann Neurol. 2008;63: 272–287. doi:10.1002/ana.21393
14. Dąbrowski J, Czajka A, Zielińska-Turek J, Jaroszyński J, Furtak-Niczyporuk M, Mela A, et al. Brain Functional Reserve in the Context of Neuroplasticity after Stroke. Neural Plast. 2019; 9708905. doi:10.1155/2019/9708905
15. Gittler M, Davis AM. Guidelines for adult stroke rehabilitation and recovery. JAMA - J Am Med Assoc. 2018;319: 820–821. doi:10.1001/jama.2017.22036
16. Deb P, Sharma S, Hassan KM. Pathophysiologic mechanisms of acute ischemic stroke: An overview with emphasis on therapeutic significance beyond thrombolysis. Pathophysiology. Elsevier Ireland Ltd; 2010;17: 197–218. doi:10.1016/j.pathophys.2009.12.001
17. Moskowitz MA, Lo EH, Iadecola C. The science of stroke: Mechanisms in search of treatments. Neuron. 2010;67: 181–198. doi:10.1016/j.neuron.2010.07.002
18. Hossmann KA. Pathophysiology and therapy of experimental stroke. Cell Mol Neurobiol. 2006;26: 1057–1083. doi:10.1007/s10571-006-9008-1
19. Mongin AA. Disruption of ionic and cell volume homeostasis in cerebral ischemia: The perfect storm. Pathophysiology. 2007;14: 183–193. doi:10.1016/j.pathophys.2007.09.009
20. Arundine M, Tymianski M. Molecular mechanisms of calcium-dependent neurodegeneration in excitotoxicity. Cell Calcium. 2003;34: 325–337. doi:10.1016/S0143-4160(03)00141-6

21. Biernaskie J. Efficacy of Rehabilitative Experience Declines with Time after Focal Ischemic Brain Injury. J Neurosci. 2004;24: 1245–1254. doi:10.1523/JNEUROSCI.3834-03.2004
22. Krakauer JW, Carmichael ST, Corbett D, Wittenberg GF. Getting neurorehabilitation right: What can be learned from animal models? Neurorehabil Neural Repair. 2012;26: 923–931. doi:10.1177/1545968312440745
23. Dromerick AW, Edwardson MA, Edwards DF, Giannetti ML, Barth J, Brady KP, et al. Critical periods after stroke study: translating animal stroke recovery experiments into a clinical trial. Front Hum Neurosci. 2015;9: 231. doi:10.3389/fnhum.2015.00231
24. Wieloch T, Nikolich K. Mechanisms of neural plasticity following brain injury. Curr Opin Neurobiol. 2006;16: 258–264. doi:10.1016/j.conb.2006.05.011
25. Carmichael ST. Cellular and molecular mechanisms of neural repair after stroke: Making waves. Ann Neurol. 2006;59: 735–742. doi:10.1002/ana.20845
26. Nudo RJ. Recovery after brain injury: mechanisms and principles. Front Hum Neurosci. 2013;7: 887. doi:10.3389/fnhum.2013.00887
27. Carrera E, Tononi G. Diaschisis: Past, present, future. Brain. 2014;137: 2408–2422. doi:10.1093/brain/awu101
28. Witte OW, Bidmon HJ, Schiene K, Redecker C, Hagemann G. Functional differentiation of multiple perilesional zones after focal cerebral ischemia. J Cereb Blood Flow Metab. 2000;20: 1149–1165. doi:10.1097/00004647-200008000-00001
29. Kleim JA. Neural plasticity and neurorehabilitation: Teaching the new brain old tricks. J Commun Disord. 2011;44: 521–528. doi:10.1016/j.jcomdis.2011.04.006
30. Powers WJ, Rabinstein AA, Ackerson T, Adeoye OM, Bambakidis NC, Becker K, et al. 2018 Guidelines for the Early Management of Patients With Acute Ischemic Stroke: A Guideline for Healthcare Professionals From the American Heart Association/American Stroke Association. Stroke. 2018;49: e46–e99. doi:10.1161/STR.0000000000000158
31. Nogueira RG, Jadhav AP, Haussen DC, Bonafe A, Budzik RF, Bhuva P, et al. Thrombectomy 6 to 24 hours after stroke with a mismatch between deficit and infarct. N Engl J Med. 2018;378: 11–21. doi:10.1056/NEJMoa1706442
32. Albers GW, Marks MP, Kemp S, Christensen S, Tsai JP, Ortega-Gutierrez S, et al. Thrombectomy for stroke at 6 to 16 hours with selection by perfusion imaging. N Engl J Med. 2018;378: 708–718. doi:10.1056/NEJMoa1713973
33. Turc G, Bhogal P, Fischer U, Khatri P, Lobotesis K, Mazighi M, et al. European Stroke Organisation (ESO)-European Society for Minimally Invasive Neurological Therapy (ESMINT) guidelines on mechanical thrombectomy in acute ischemic stroke. J Neurointerv Surg. 2019;4: 6–12. doi:10.1136/neurintsurg-2018-014568
34. Ramos-Cabrer P, Campos F, Sobrino T, Castillo J. Targeting the ischemic penumbra. Stroke. 2011;42: S7-11. doi:10.1161/STROKEAHA.110.596684
35. O'Collins VE, Macleod MR, Donnan GA, Horky LL, Van Der Worp BH, Howells DW. 1,026 Experimental treatments in acute stroke. Ann Neurol. 2006;59: 467–477. doi:10.1002/ana.20741
36. O'Collins VE, MacLeod MR, Cox SF, Van Raay L, Aleksoska E, Donnan GA, et al. Preclinical drug evaluation for combination therapy in acute stroke using systematic review, meta-analysis, and subsequent experimental testing. J Cereb Blood Flow Metab. 2011;31: 962–975. doi:10.1038/jcbfm.2010.184
37. Moretti A, Ferrari F, Villa RF. Neuroprotection for ischaemic stroke: Current status and challenges. Pharmacol Ther. 2015;146: 23–24. doi:10.1016/j.pharmthera.2014.09.003
38. Hatem SM, Saussez G, della Faille M, Prist V, Zhang X, Dispa D, et al. Rehabilitation of Motor Function after Stroke: A Multiple Systematic Review Focused on Techniques to Stimulate Upper Extremity Recovery. Front Hum Neurosci. 2016;10: 442. doi:10.3389/fnhum.2016.00442
39. Takeuchi N, Izumi SI. Combinations of stroke neurorehabilitation to facilitate motor recovery: Perspectives on Hebbian plasticity and homeostatic metaplasticity. Front Hum Neurosci. 2015;9: 349. doi:10.3389/fnhum.2015.00349
40. Monfils MH, Plautz EJ, Kleim JA. In search of the motor engram: Motor map plasticity as a mechanism for encoding motor experience. Neuroscientist. 2005;11: 471–483. doi:10.1177/1073858405278015

41. Hosp JA, Luft AR. Cortical Plasticity during Motor Learning and Recovery after Ischemic Stroke. Neural Plast. 2011; 871296. doi:10.1155/2011/871296
42. Di Pino G, Pellegrino G, Assenza G, Capone F, Ferreri F, Formica D, et al. Modulation of brain plasticity in stroke: A novel model for neurorehabilitation. Nat Rev Neurol. 2014;10: 597–608. doi:10.1038/nrneurol.2014.162
43. Thickbroom GW, Byrnes ML, Archer SA, Mastaglia FL. Motor outcome after subcortical stroke correlates with the degree of cortical reorganization. Clin Neurophysiol. 2004;115: 2144–2150. doi:10.1016/j.clinph.2004.04.001
44. Grefkes C, Ward NS. Cortical reorganization after stroke: How much and how functional? Neuroscientist. 2014;20: 56–70. doi:10.1177/1073858413491147
45. Nishibe M, Barbay S, Guggenmos D, Nudo RJ. Reorganization of Motor Cortex after Controlled Cortical Impact in Rats and Implications for Functional Recovery. J Neurotrauma. 2010;27: 2221–2232. doi:10.1089/neu.2010.1456
46. Alia C, Spalletti C, Lai S, Panarese A, Micera S, Caleo M. Reducing GABAA-mediated inhibition improves forelimb motor function after focal cortical stroke in mice. Sci Rep. 2016; doi:10.1038/srep37823
47. Ramanathan D, Conner JM, H. Tuszynski M. A form of motor cortical plasticity that correlates with recovery of function after brain injury. Proc Natl Acad Sci. 2006;103: 11370–11375. doi:10.1073/pnas.0601065103
48. Jablonka JA, Burnat K, Witte OW, Kossut M. Remapping of the somatosensory cortex after a photothrombotic stroke: dynamics of the compensatory reorganization. Neuroscience. 2010;165: 90–100. doi:10.1016/j.neuroscience.2009.09.074
49. Harrison TC, Silasi G, Boyd JD, Murphy TH. Displacement of sensory maps and disorganization of motor cortex after targeted stroke in mice. Stroke. 2013;44: 2300–2306. doi:10.1161/STROKEAHA.113.001272
50. Johansson BB. Brain plasticity and stroke rehabilitation: The Willis lecture. Stroke. 2000;31: 223–230. doi:10.1161/01.STR.31.1.223
51. Hara Y. Brain Plasticity and Rehabilitation in Stroke Patients. J Nippon Med Sch. 2015;82: 4–13. doi:10.1272/jnms.82.4
52. Murphy TH, Corbett D. Plasticity during stroke recovery: From synapse to behaviour. Nature Reviews Neuroscience. 2009. doi:10.1038/nrn2735
53. Fox K, Stryker M. Integrating Hebbian and homeostatic plasticity: Introduction. Philos Trans R Soc B Biol Sci. 2017;372: pii: 20160413. doi:10.1098/rstb.2016.0413
54. Turrigiano GG, Nelson SB. Homeostatic plasticity in the developing nervous system. Nat Rev Neurosci. 2004;5: 97–107. doi:10.1038/nrn1327
55. Turrigiano G. Homeostatic synaptic plasticity: Local and global mechanisms for stabilizing neuronal function. Cold Spring Harb Perspect Biol. 2012;4: a005736. doi:10.1101/cshperspect.a005736
56. Attneave F, B. M, Hebb DO. The Organization of Behavior; A Neuropsychological Theory. Wiley, editor. The American Journal of Psychology. New York; 1949. doi:10.2307/1418888
57. Song S, Miller KD, Abbott LF. Competitive Hebbian learning through spike-timing-dependent synaptic plasticity. Nat Neurosci. 2000;3: 919–926. doi:10.1038/78829
58. Davis GW, Bezprozvanny I. Maintaining the Stability of Neural Function: A Homeostatic Hypothesis. Annu Rev Physiol. 2001;63: 847–869. doi:10.1146/annurev.physiol.63.1.847
59. Turrigiano G. Too Many Cooks? Intrinsic and Synaptic Homeostatic Mechanisms in Cortical Circuit Refinement. Annu Rev Neurosci. 2011;34: 89–103. doi:10.1146/annurev-neuro-060909-153238
60. Pozo K, Goda Y. Unraveling mechanisms of homeostatic synaptic plasticity. Neuron. 2010;66: 337–351. doi:10.1016/j.neuron.2010.04.028
61. Marder E, Goaillard J-M. Variability, compensation and homeostasis in neuron and network function. Nat Rev Neurosci. 2006;7: 563–574. doi:10.1038/nrn1949
62. Turrigiano GG. The Self-Tuning Neuron: Synaptic Scaling of Excitatory Synapses. Cell. 2008;135: 422–435. doi:10.1016/j.cell.2008.10.008
63. Lee KFH, Soares C, Béïque JC. Tuning into diversity of homeostatic synaptic plasticity. Neuropharmacology. 2014;78: 31–37. doi:10.1016/j.neuropharm.2013.03.016

64. Zhang S. Rapid Reversible Changes in Dendritic Spine Structure In Vivo Gated by the Degree of Ischemia. J Neurosci. 2005;25: 5333–5338. doi:10.1523/JNEUROSCI.1085-05.2005
65. Dalise S, Ambrosio F, Modo M. Adaptive plasticity and recovery in preclinical models of stroke. Arch Ital Biol. 2014;152: 190–215. doi:10.12871/00039829201442
66. Bredt DS, Nicoll RA. AMPA receptor trafficking at excitatory synapses. Neuron. 2003;40: 361–379. doi:10.1016/S0896-6273(03)00640-8
67. Pérez-Otaño I, Ehlers MD. Homeostatic plasticity and NMDA receptor trafficking. Trends Neurosci. 2005;28: 229–238. doi:10.1016/j.tins.2005.03.004
68. Clarkson AN, Overman JJ, Zhong S, Mueller R, Lynch G, Carmichael ST. AMPA Receptor-Induced Local Brain-Derived Neurotrophic Factor Signaling Mediates Motor Recovery after Stroke. J Neurosci. 2011; doi:10.1523/JNEUROSCI.5780-10.2011
69. Que M, Schiene K, Witte OW, Zilles K. Widespread up-regulation of N-methyl-D-aspartate receptors after focal photothrombotic lesion in rat brain. Neurosci Lett. 1999;273: 77–80. doi:10.1016/S0304-3940(99)00598-4
70. Breton JD, Stuart GJ. Loss of sensory input increases the intrinsic excitability of layer 5 pyramidal neurons in rat barrel cortex. J Physiol. 2009;587: 5107–5119. doi:10.1113/jphysiol.2009.180943
71. Sale A, Berardi N, Maffei L. Environment and brain plasticity: towards an endogenous pharmacotherapy. Physiol Rev. 2014;94: 189–234. doi:10.1152/physrev.00036.2012
72. Dancause N, Barbay S, Frost SB, Plautz EJ, Chen D, Zoubina E V, et al. Extensive cortical rewiring after brain injury. J Neurosci. 2005;25: 10167–10179. doi:10.1523/JNEUROSCI.3256-05.2005
73. Butz M, Steenbuck ID, Van Ooyen A. Homeostatic structural plasticity can account for topology changes following deafferentation and focal stroke. Front Neuroanat. 2014;8: 115. doi:10.3389/fnana.2014.00115
74. Cramer SC, Chopp M. Recovery recapitulates ontogeny. Trends Neurosci. 2000;23: 265–271. doi:10.1016/S0166-2236(00)01562-9
75. Carmichael ST, Archibeque I, Luke L, Nolan T, Momiy J, Li S. Growth-associated gene expression after stroke: Evidence for a growth-promoting region in peri-infarct cortex. Exp Neurol. 2005;193: 291–311. doi:10.1016/j.expneurol.2005.01.004
76. Clarkson AN, Huang BS, MacIsaac SE, Mody I, Carmichael ST. Reducing excessive GABA-mediated tonic inhibition promotes functional recovery after stroke. Nature. 2010;468: 305–309. doi:10.1038/nature09511
77. Jaenisch N, Liebmann L, Guenther M, Hubner CA, Frahm C, Witte OW. Reduced tonic inhibition after stroke promotes motor performance and epileptic seizures. Sci Rep. 2016;6: 26173. doi:10.1038/srep26173
78. Kim YK, Yang EJ, Cho K, Lim JY, Paik NJ. Functional recovery after ischemic stroke is associated with reduced gabaergic inhibition in the cerebral cortex: A GABA PET study. Neurorehabil Neural Repair. 2014;28: 576–583. doi:10.1177/1545968313520411
79. Schiene K, Bruehl C, Zilles K, Qü M, Hagemann G, Kraemer M, et al. Neuronal hyperexcitability and reduction of GABA(A)-receptor expression in the surround of cerebral photothrombosis. J Cereb Blood Flow Metab. 1996;16: 906–914. doi:10.1097/00004647-199609000-00014
80. Schwartz-Bloom RD, Sah R. gamma-Aminobutyric acid(A) neurotransmission and cerebral ischemia. J Neurochem. 2001;77: 353–371. doi:10.1046/j.1471-4159.2001.00274.x
81. Redecker C, Wang W, Fritschy JM, Witte OW. Widespread and long-lasting alterations in GABAA-receptor subtypes after focal cortical infarcts in rats: Mediation by NMDA-dependent processes. J Cereb Blood Flow Metab. 2002;22: 1463–1475. doi:10.1097/01.WCB.0000034149.72481.BD
82. Hartman KN, Pal SK, Burrone J, Murthy VN. Activity-dependent regulation of inhibitory synaptic transmission in hippocampal neurons. Nat Neurosci. 2006;9: 642–649. doi:10.1038/nn1677
83. Mironova YA, Giger RJ. Where no synapses go: Gatekeepers of circuit remodeling and synaptic strength. Trends Neurosci. 2013;36: 363–373. doi:10.1016/j.tins.2013.04.003
84. Rock C, Zurita H, Lebby S, Wilson CJ, Apicella AJ. Cortical Circuits of Callosal GABAergic Neurons. Cereb Cortex. 2018;28: 1154–1167. doi:10.1093/cercor/bhx025
85. Bartley AF, Huang ZJ, Huber KM, Gibson JR. Differential activity-dependent, homeostatic plasticity of two neocortical inhibitory circuits. J Neurophysiol. 2008;100: 1983–1994. doi:10.1152/jn.90635.2008

86. Zeiler SR, Gibson EM, Hoesch RE, Li MY, Worley PF, O'Brien RJ, et al. Medial premotor cortex shows a reduction in inhibitory markers and mediates recovery in a mouse model of focal stroke. Stroke. 2013;44: 483–489. doi:10.1161/STROKEAHA.112.676940
87. Nudo RJ. Mechanisms for recovery of motor function following cortical damage. Curr Opin Neurobiol. 2006;16: 638–644. doi:10.1016/j.conb.2006.10.004
88. Cramer SC, Shah R, Juranek J, Crafton KR, Le V. Activity in the peri-infarct rim in relation to recovery from stroke. Stroke. 2006;37: 111–115. doi:10.1161/01.STR.0000195135.70379.1f
89. Jones TA, Schallert T. Overgrowth and pruning of dendrites in adult rats recovering from neocortical damage. Brain Res. 1992;581: 156–160. doi:10.1016/0006-8993(92)90356-E
90. Ward NS. Neural plasticity and recovery of function. Prog Brain Res. 2005;150: 527–535. doi:10.1016/S0079-6123(05)50036-0
91. Cramer SC, Crafton KR. Somatotopy and movement representation sites following cortical stroke. Exp Brain Res. 2006;168: 25–32. doi:10.1007/s00221-005-0082-2
92. Redecker C, Luhmann HJ, Hagemann G, Fritschy JM, Witte OW. Differential downregulation of GABA(A) receptor subunits in widespread brain regions in the freeze-lesion model of focal cortical malformations. J Neurosci. 2000;20: 5045–5053.
93. Wahl A-S, Schwab ME. Finding an optimal rehabilitation paradigm after stroke: enhancing fiber growth and training of the brain at the right moment. Front Hum Neurosci. 2014;8: 381. doi:10.3389/fnhum.2013.00911
94. Brown CE, Aminoltejari K, Erb H, Winship IR, Murphy TH. In vivo voltage-sensitive dye imaging in adult mice reveals that somatosensory maps lost to stroke are replaced over weeks by new structural and functional circuits with prolonged modes of activation within both the peri-infarct zone and distant sites. J Neurosci. 2009;29: 1719–1734. doi:10.1523/JNEUROSCI.4249-08.2009
95. Alia C, Spalletti C, Lai S, Panarese A, Lamola G, Bertolucci F, et al. Neuroplastic Changes Following Brain Ischemia and their Contribution to Stroke Recovery: Novel Approaches in Neurorehabilitation. Front Cell Neurosci. 2017;11: 1–22. doi:10.3389/fncel.2017.00076
96. Edwardson MA, Lucas TH, Carey JR, Fetz EE. New modalities of brain stimulation for stroke rehabilitation. Exp Brain Res. 2013;224: 335–358. doi:10.1007/s00221-012-3315-1
97. Jones TA, Adkins DL. Motor System Reorganization After Stroke: Stimulating and Training Toward Perfection. Physiology. 2015; doi:10.1152/physiol.00014.2015
98. Hagemann G, Redecker C, Neumann-Haefelin T, Freund HJ, Witte OW. Increased long-term potentiation in the surround of experimentally induced focal cortical infarction. Ann Neurol. 1998;44: 255–258. doi:10.1002/ana.410440217
99. Turrigiano GG, Nelson SB. Hebb and homeostasis in neuronal plasticity. Curr Opin Neurobiol. 2000;10: 358–364. doi:10.1016/S0959-4388(00)00091-X
100. Salter K, Jutai J, Hartley M, Foley N, Bhogal S, Bayona N, et al. Impact of early vs delayed admission to rehabilitation on functional outcomes in persons with stroke. J Rehabil Med. 2006;38: 113–117. doi:10.1080/16501970500314350
101. Horn SD, DeJong G, Smout RJ, Gassaway J, James R, Conroy B. Stroke rehabilitation patients, practice, and outcomes: Is earlier and more aggressive therapy better? Arch Phys Med Rehabil. 2005;86: S101–S114. doi:10.1016/j.apmr.2005.09.016
102. Maulden SA, Gassaway J, Horn SD, Smout RJ, DeJong G. Timing of initiation of rehabilitation after stroke. Arch Phys Med Rehabil. 2005;86: S34–S40. doi:10.1016/j.apmr.2005.08.119
103. Dromerick AW, Lang CE, Birkenmeier RL, Wagner JM, Miller JP, Videen TO, et al. Very early constraint-induced movement during stroke rehabilitation (VECTORS): A single-center RCT. Neurology. 2009;73: 195–201. doi:10.1212/WNL.0b013e3181ab2b27
104. Kozlowski DA, James DC, Schallert T. Use-dependent exaggeration of neuronal injury after unilateral sensorimotor cortex lesions. J Neurosci. 1996;16: 4776–4786.
105. Fluri F, Schuhmann MK, Kleinschnitz C. Animal models of ischemic stroke and their application in clinical research. Drug Des Devel Ther. 2015;9: 3445–3454. doi:10.2147/DDDT.S56071

106. Caleo M. Rehabilitation and plasticity following stroke: Insights from rodent models. Neuroscience. 2015;311: 180–194. doi:10.1016/j.neuroscience.2015.10.029
107. Watson BD, Dietrich WD, Busto R, Wachtel MS, Ginsberg MD. Induction of reproducible brain infarction by photochemically initiated thrombosis. Ann Neurol. 1985;17: 497–504. doi:10.1002/ana.410170513
108. Schroeter M, Jander S, Stoll G. Non-invasive induction of focal cerebral ischemia in mice by photothrombosis of cortical microvessels: Characterization of inflammatory responses. J Neurosci Methods. 2002;117: 43–49. doi:10.1016/S0165-0270(02)00072-9
109. Uzdensky AB. Photothrombotic Stroke as a Model of Ischemic Stroke. Transl Stroke Res. 2018;9: 437–451. doi:10.1007/s12975-017-0593-8
110. Urbach A, Witte OW. Photochemical models of focal brain ischemia. In: Dirnagl U, editor. Rodent Models of Stroke. 2nd ed. New York: Humana Press; 2016. pp. 69–83.
111. Lee VM, Burdett NG, Carpenter TA, Hall LD, Pambakian PS, Patel S, et al. Evolution of photochemically induced focal cerebral ischemia in the rat: Magnetic resonance imaging and histology. Stroke. 1996;27: 2110–2119. doi:10.1161/01.STR.27.11.2110
112. Sommer CJ. Ischemic stroke: experimental models and reality. Acta Neuropathol. 2017;133: 245–261. doi:10.1007/s00401-017-1667-0
113. Liu NW, Ke CC, Zhao Y, Chen YA, Chan KC, Tan DTW, et al. Evolutional Characterization of Photochemically Induced Stroke in Rats: a Multimodality Imaging and Molecular Biological Study. Transl Stroke Res. 2017;8: 244–256. doi:10.1007/s12975-016-0512-4
114. Moon SK, Alaverdashvili M, Cross AR, Whishaw IQ. Both compensation and recovery of skilled reaching following small photothrombotic stroke to motor cortex in the rat. Exp Neurol. 2009;218: 145–153. doi:10.1016/j.expneurol.2009.04.021
115. Schaechter JD, Kraft E, Hilliard TS, Dijkhuizen RM, Benner T, Finklestein SP, et al. Motor Recovery and Cortical Reorganization after Constraint-Induced Movement Therapy in Stroke Patients: A Preliminary Study. Neurorehabil Neural Repair. 2002;16: 326–333. doi:10.1177/154596830201600403
116. Moog NK, Entringer S, Heim C, Wadhwa PD, Kathmann N, Buss C. Influence of maternal thyroid hormones during gestation on fetal brain development. Neuroscience. 2017;342: 68–100. doi:10.1016/j.neuroscience.2015.09.070
117. Gothié JD, Demeneix B, Remaud S. Comparative approaches to understanding thyroid hormone regulation of neurogenesis. Mol Cell Endocrinol. Elsevier Ireland Ltd; 2017;459: 104–115. doi:10.1016/j.mce.2017.05.020
118. Bernal J. Thyroid Hormones in Brain Development and Function. [Updated 2015 Sep 2]. In: Feingold KR, Anawalt B, Boyce A et al, editor. Endotext. South Dartmouth (MA): MDText.com, Inc.; 2000. pp. 1–136. Available: http://www.ncbi.nlm.nih.gov/books/NBK285549/?report=printable
119. Morreale de Escobar G, Obregon MJ, Escobar del Rey F. Role of thyroid hormone during early brain development. Eur J Endocrinol. 2004;151: U25-37. doi:10.1530/EJE.0.151U025
120. Hartley MD, Banerji T, Tagge IJ, Kirkemo LL, Chaudhary P, Calkins E, et al. Myelin repair stimulated by CNS-selective thyroid hormone action. JCI Insight. 2019;4: e126329. doi:10.1172/jci.insight.126329
121. Bárez-López S, Guadaño-Ferraz A. Thyroid Hormone Availability and Action during Brain Development in Rodents. Front Cell Neurosci. 2017;11: 1–7. doi:10.3389/fncel.2017.00240
122. Mullur R, Liu YY, Brent GA. Thyroid Hormone Regulation of Metabolism. Physiol Rev. 2014;94: 355–382. doi:10.1152/physrev.00030.2013
123. Ahmed OM, El-Gareib AW, El-bakry AM, Abd El-Tawab SM, Ahmed RG. Thyroid hormones states and brain development interactions. Int J Dev Neurosci. 2008;26: 147–209. doi:10.1016/j.ijdevneu.2007.09.011
124. Mendel CM, Weisiger RA, Jones AL, Cavalieri RR. Thyroid hormone-binding proteins in plasma facilitate uniform distribution of thyroxine within tissues: A perfused rat liver study. Endocrinology. 1987;120: 1742–1749. doi:10.1210/endo-120-5-1742
125. Palha JA. Transthyretin as a thyroid hormone carrier: Function revisited. Clin Chem Lab Med. 2002;40: 1292–1300. doi:10.1515/CCLM.2002.223
126. Refetoff S. Thyroid hormone serum transport proteins [Updated 2015 Jun 7]. In: Feingold KR, Anawalt B, Boyce A et al, editor. Endotext. South Dartmouth (MA): MDText.com, Inc.; 2015. pp. 1–13.

127. Schreiber G, Richardson SJ. The Evolution of Gene Expression, Structure and Function of Transthyretin. Comp Biochem Physiol Part B Biochem Mol Biol. 1997;116: 137–160. doi:10.1016/S0305-0491(96)00212-X
128. Vranckx R, Rouaze M, Savu L, Nunez EA, Beaumont C, Flink IL. The hepatic biosynthesis of rat thyroxine binding globulin (TBG): Demonstration, ontogenesis, and UP-regulation in experimental hypothyroidism. Biochem Biophys Res Commun. 1990;167: 317–322. doi:10.1016/0006-291X(90)91767-M
129. Vranckx R, Savu L, Maya M, Nunez EA. Characterization of a major development-regulated serum thyroxine-binding globulin in the euthyroid mouse. Biochem J. 1990;271: 373–379. doi:10.1042/bj2710373
130. Benvenga S, Lapa D, Trimarchi F. Re-evaluation of the thyroxine binding to human plasma lipoproteins using three techniques. J Endocrinol Invest. 2001;24: RC16–RC18. doi:10.1007/BF03343863
131. Benvenga S, Cahnmann HJ, Robbins J. Localization of the thyroxine binding sites in apolipoprotein B-100 of human low density lipoproteins. Endocrinology. 1990;127: 2241–2246. doi:10.1210/endo-127-5-2241
132. Morte B, Bernal J. Thyroid hormone action: Astrocyte-neuron communication. Front Endocrinol (Lausanne). 2014;5: 82. doi:10.3389/fendo.2014.00082
133. Bernal J, Guadaño-Ferraz A, Morte B. Thyroid hormone transporters—functions and clinical implications. Nat Rev Endocrinol. 2015;11: 1–12. doi:10.1038/nrendo.2015.66
134. Chanoine JP, Alex S, Fang SL, Stone S, Leonard JL, Korhle J, et al. Role of transthyretin in the transport of thyroxine from the blood to the choroid plexus, the cerebrospinal fluid, and the brain. Endocrinology. 1992;130: 933–938. doi:10.1210/endo.130.2.1733735
135. Richardson SJ, Wijayagunaratne RC, D'Souza DG, Darras VM, Van Herck SLJ. Transport of thyroid hormones via the choroid plexus into the brain: The roles of transthyretin and thyroid hormone transmembrane transporters. Frontiers in Neuroscience. 2015. doi:10.3389/fnins.2015.00066
136. Friesema EC, Ganguly S, Abdalla A, Manning Fox JE, Halestrap AP, Visser TJ. Identification of monocarboxylate transporter 8 as a specific thyroid hormone transporter. J Biol Chem. 2003;278: 40128–40135. doi:10.1074/jbc.M300909200
137. Friesema EC, Jansen J, Milici C, Visser TJ. Thyroid Hormone Transporters. Vitam Horm. 2005;70: 137–167. doi:10.1016/S0083-6729(05)70005-4
138. Visser WE, Friesema EC, Visser TJ. Minireview: thyroid hormone transporters: the knowns and the unknowns. Mol Endocrinol. 2011;25: 1–14. doi:10.1210/me.2010-0095
139. Visser WE, Friesema EC, Jansen J, Visser TJ. Thyroid hormone transport by monocarboxylate transporters. Best Pract Res Clin Endocrinol Metab. 2007;21: 223–236. doi:10.1016/j.beem.2007.03.008
140. Halestrap AP. The monocarboxylate transporter family-Structure and functional characterization. IUBMB Life. 2012;64: 1–9. doi:10.1002/iub.573
141. Halestrap AP, Wilson MC. The monocarboxylate transporter family-Role and regulation. IUBMB Life. 2012;64: 109–119. doi:10.1002/iub.572
142. Hagenbuch B. Cellular entry of thyroid hormones by organic anion transporting polypeptides. Best Pract Res Clin Endocrinol Metab. 2007;21: 209–221. doi:10.1016/j.beem.2007.03.004
143. Kinne A, Schülein R, Krause G. Primary and secondary thyroid hormone transporters. Thyroid Res. 2011;4: S7. doi:10.1186/1756-6614-4-S1-S7
144. Roberts LM, Woodford K, Zhou M, Black DS, Haggerty JE, Tate EH, et al. Expression of the Thyroid Hormone Transporters Monocarboxylate Transporter-8 (*SLC16A2*) and Organic Ion Transporter-14 (*SLCO1C1*) at the Blood-Brain Barrier. Endocrinology. 2008;149: 6251–6261. doi:10.1210/en.2008-0378
145. Van Der Deure WM, Peeters RP, Visser TJ. Molecular aspects of thyroid hormone transporters, including MCT8, MCT10, and OATPs, and the effects of genetic variation in these transporters. J Mol Endocrinol. 2010;44: 1–11. doi:10.1677/JME-09-0042
146. Jansen J, Friesema EC, Milici C, Visser TJ. Thyroid Hormone Transporters in Health and Disease. Thyroid. 2005;15: 757–768. doi:10.1089/thy.2005.15.757
147. Trajkovic M, Visser TJ, Mittag J, Horn S, Lukas J, Darras VM, et al. Abnormal thyroid hormone metabolism in mice lacking the monocarboxylate transporter 8. J Clin Invest. 2007;117: 627–635. doi:10.1172/JCI28253

148. Friesema EC, Jansen J, Jachtenberg J, Visser WE, Kester MHA, Visser TJ. Effective Cellular Uptake and Efflux of Thyroid Hormone by Human Monocarboxylate Transporter 10. Mol Endocrinol. 2008;22: 1357–1369. doi:10.1210/me.2007-0112

149. Zhang Y, Chen K, Sloan SA, Bennett ML, Scholze AR, O'Keeffe S, et al. An RNA-Sequencing Transcriptome and Splicing Database of Glia, Neurons, and Vascular Cells of the Cerebral Cortex. J Neurosci. 2014;34: 11929–11947. doi:10.1523/JNEUROSCI.1860-14.2014

150. Mathiisen TM, Lehre KP, Danbolt NC, Ottersen OP. The perivascular astroglial sheath provides a complete covering of the brain microvessels: An electron microscopic 3D reconstruction. Glia. 2010;58: 1094–1103. doi:10.1002/glia.20990

151. Schroeder AC, Privalsky ML. Thyroid hormones, T3 and T4, in the brain. Front Endocrinol (Lausanne). 2014;5: 40. doi:10.3389/fendo.2014.00040

152. López-Espíndola D, Morales-Bastos C, Grijota-Martínez C, Liao XH, Lev D, Sugo E, et al. Mutations of the thyroid hormone transporter MCT8 cause prenatal brain damage and persistent hypomyelination. J Clin Endocrinol Metab. 2014;99: E2799–E2804. doi:10.1210/jc.2014-2162

153. Dumitrescu AM, Liao XH, Best TB, Brockmann K, Refetoff S. A novel syndrome combining thyroid and neurological abnormalities is associated with mutations in a monocarboxylate transporter gene. Am J Hum Genet. 2004;74: 168–175. doi:10.1086/380999

154. Friesema EC, Grueters A, Biebermann H, Krude H, Von Moers A, Reeser M, et al. Association between mutations in a thyroid hormone transporter and severe X-linked psychomotor retardation. Lancet. 2004;364: 1435–1437. doi:10.1016/S0140-6736(04)17226-7

155. Schwartz CE, May MM, Carpenter NJ, Rogers RC, Martin J, Bialer MG, et al. Allan-Herndon-Dudley Syndrome and the Monocarboxylate Transporter 8 (MCT8) Gene. Am J Hum Genet. 2005;77: 41–53. doi:10.1086/431313

156. Kim DK, Kanai Y, Chairoungdua A, Matsuo H, Cha SH, Endou H. Expression Cloning of a Na+-independent Aromatic Amino Acid Transporter with Structural Similarity to H+/Monocarboxylate Transporters. J Biol Chem. 2001;276: 17221–17228. doi:10.1074/jbc.M009462200

157. Bianco AC, Kim BW. Deiodinases: implications of the local control of thyroid hormone action. J Clin Invest. 2006;116: 2571–2579. doi:10.1172/JCI29812

158. Dentice M, Marsili A, Zavacki A, Larsen PR, Salvatore D. The deiodinases and the control of intracellular thyroid hormone signaling during cellular differentiation. Biochim Biophys Acta - Gen Subj. 2013;1830: 3937–3945. doi:10.1016/j.bbagen.2012.05.007

159. Bianco AC. Minireview: Cracking the metabolic code for thyroid hormone signaling. Endocrinology. 2011;152: 3306–3311. doi:10.1210/en.2011-1104

160. Tu HM, Kim SW, Salvatore D, Bartha T, Legradi G, Larsen PR, et al. Regional distribution of type 2 thyroxine deiodinase messenger ribonucleic acid in rat hypothalamus and pituitary and its regulation by thyroid hormone. Endocrinology. 1997;138: 3359–3368. doi:10.1210/endo.138.8.5318

161. Freitas BC, Gereben B, Castillo M, Kalló I, Zeöld A, Egri P, et al. Paracrine signaling by glial cell-derived triiodothyronine activates neuronal gene expression in the rodent brain and human cells. J Clin Invest. 2010;120: 2206–2217. doi:10.1172/JCI41977

162. Gereben B, Zavacki AM, Ribich S, Kim BW, Huang SA, Simonides WS, et al. Cellular and molecular basis of deiodinase-regulated thyroid hormone signaling. Endocr Rev. 2008;29: 898–938. doi:10.1210/er.2008-0019

163. Tu HM, Legradi G, Bartha T, Salvatore D, Lechan RM, Larsen PR. Regional expression of the type 3 iodothyronine deiodinase messenger ribonucleic acid in the rat central nervous system and its regulation by thyroid hormone. Endocrinology. 1999;140: 784–790. doi:10.1210/endo.140.2.6486

164. Dumitrescu AM, Liao XH, Weiss RE, Millen K, Refetoff S. Tissue-specific thyroid hormone deprivation and excess in monocarboxylate transporter (Mct) 8-deficient mice. Endocrinology. 2006;147: 4036–4043. doi:10.1210/en.2006-0390

165. Guadaño-Ferraz A, Obregón MJ, St Germain DL, Bernal J, Obregon MJ, StGermain DL. The type 2 iodothyronine deiodinase is expressed primarily in glial cells in the neonatal rat brain. Proc Natl Acad Sci U S A. 1997;94: 10391–10396. doi:10.1073/pnas.94.19.10391

166. Cheng SY, Leonard JL, Davis PJ. Molecular aspects of thyroid hormone actions. Endocr Rev. 2010;31: 139–170. doi:10.1210/er.2009-0007
167. Yen PM. Physiological and molecular basis of thyroid hormone action. Physiol Rev. 2001;81: 1097–1142. doi:11427693
168. Sinha R, Yen PM. Cellular Action of Thyroid Hormone [Updated 2018 Jun 20]. In: Feingold KR, Anawalt B, Boyce A et al, editor. Endotext. South Dartmouth (MA): MDText.com, Inc.; 2013. pp. 1–27. doi:10.1089/105072502760143827
169. Bernal J. Action of thyroid hormone in brain. J Endocrinol Invest. 2002;25: 268–288. doi:10.1007/BF03344003
170. Harvey CB, Williams GR. Mechanism of Thyroid Hormone Action. Thyroid. 2002;12: 441–446. doi:10.1089/105072502760143791
171. McKenna NJ, Lanz RB, O'Malley BW. Nuclear receptor coregulators: Cellular and molecular biology. Endocr Rev. 1999;20: 321–344. doi:10.1210/edrv.20.3.0366
172. Osmak-Tizon L, Poussier M, Cottin Y, Rochette L. Non-genomic actions of thyroid hormones: Molecular aspects. Arch Cardiovasc Dis. Elsevier Masson SAS; 2014;107: 207–211. doi:10.1016/j.acvd.2014.02.001
173. Davis PJ, Goglia F, Leonard JL. Nongenomic actions of thyroid hormone. Nat Rev Endocrinol. Nature Publishing Group; 2016;12: 111–121. doi:10.1038/nrendo.2015.205
174. Davis PJ, Leonard JL, Davis FB. Mechanisms of nongenomic actions of thyroid hormone. Front Neuroendocrinol. 2008;29: 211–218. doi:10.1016/j.yfrne.2007.09.003
175. Hiroi Y, Kim HH, Ying H, Furuya F, Huang Z, Simoncini T, et al. Rapid nongenomic actions of thyroid hormone. Proc Natl Acad Sci U S A. 2006;103: 14104–14109. doi:10.1073/pnas.0601600103
176. Campos Costa I, Nogueira Carvalho H, Fernandes L. Aging, circadian rhythms and depressive disorders: a review. Am J Neurodegener Dis. 2013;2: 228–246.
177. Froy O. Circadian Rhythms, Aging, and Life Span in Mammals. Physiology. 2011;26: 225–235. doi:10.1152/physiol.00012.2011
178. Morris CJ, Aeschbach D, Scheer FA. Circadian system, sleep and endocrinology. Mol Cell Endocrinol. 2012;349: 91–104. doi:10.1016/j.mce.2011.09.003
179. Chakraborti S, Chakraborti T, Mandal M, Das S, Batabyal SK. Hypothalamic-pituitary-thyroid axis status of humans during development of ageing process. Clin Chim Acta. 1999;288: 137–145. doi:10.1016/S0009-8981(99)00061-3
180. Bensenor IM, Olmos RD, Lotufo PA. Hypothyroidism in the elderly: Diagnosis and management. Clin Interv Aging. 2012;7: 97–111. doi:10.2147/CIA.S23966
181. Cao L, Jiang W, Wang F, Yang QG, Wang C, Chen YP, et al. The reduced serum free triiodothyronine and increased dorsal hippocampal SNAP-25 and Munc18-1 had existed in middle-aged CD-1 mice with mild spatial cognitive impairment. Brain Res. 2013;1540: 9–20. doi:10.1016/j.brainres.2013.09.034
182. Visser WE, Bombardieri CR, Zevenbergen C, Barnhoorn S, Ottaviani A, Van Der Pluijm I, et al. Tissue-specific suppression of thyroid hormone signaling in various mouse models of aging. PLoS One. 2016;11: 1–25. doi:10.1371/journal.pone.0149941
183. Villanueva I, Alva-Sánchez C, Pacheco-Rosado J. The role of thyroid hormones as inductors of oxidative stress and neurodegeneration. Oxid Med Cell Longev. 2013;2013: 218145. doi:10.1155/2013/218145
184. Fu AL, Zhou CY, Chen X. Thyroid hormone prevents cognitive deficit in a mouse model of Alzheimer's disease. Neuropharmacology. 2010;58: 722–729. doi:10.1016/j.neuropharm.2009.12.020
185. Remmel KS, Wanahita A, Moore K, Gruenthal M. Acute ischemic stroke and hypothyroidism. J Ky Med Assoc. 2006;104: 191–193.
186. Qureshi AI, Suri FK, Nasar A, Kirmani JF, Divani AA, Giles WH. Free thyroxine index and risk of stroke: results from the National Health and Nutrition Examination Survey Follow-up Study. Med Sci Monit. 2006;12: CR501-506.
187. Suda S, Muraga K, Kanamaru T, Okubo S, Abe A, Aoki J, et al. Low free triiodothyronine predicts poor functional outcome after acute ischemic stroke. J Neurol Sci. 2016;368: 89–93. doi:10.1016/j.jns.2016.06.063
188. O'Keefe LM, Conway SE, Czap A, Malchoff CD, Benashski S, Fortunato G, et al. Thyroid hormones and functional outcomes after ischemic stroke. Thyroid Res. 2015;8: 9. doi:10.1186/s13044-015-0021-7

189. Dhital R, Poudel DR, Tachamo N, Gyawali B, Basnet S, Shrestha P, et al. Ischemic Stroke and Impact of Thyroid Profile at Presentation: A Systematic Review and Meta-analysis of Observational Studies. J Stroke Cerebrovasc Dis. 2017;26: 2926–2934. doi:10.1016/j.jstrokecerebrovasdis.2017.07.015
190. Liu J, Wang D, Xiong Y, Yuan R, Tao W, Liu M. Low free triiodothyronine levels are related to symptomatic intracranial hemorrhage and poor functional outcomes after intravenous thrombolysis in acute ischemic stroke patients. Neurol Res. 2016;38: 429–433. doi:10.1080/01616412.2016.1178480
191. Jiang X, Xing H, Wu J, Du R, Liu H, Chen J, et al. Prognostic value of thyroid hormones in acute ischemic stroke – a meta analysis. Sci Rep. London, London; 2017;7: 16256. doi:10.1038/s41598-017-16564-2
192. Lamba N, Liu C, Zaidi H, Broekman MLD, Simjian T, Shi C, et al. A prognostic role for Low tri-iodothyronine syndrome in acute stroke patients: A systematic review and meta-analysis. Clin Neurol Neurosurg. 2018;169: 55–63. doi:10.1016/j.clineuro.2018.03.025
193. Li LQ, Xu XY, Li WY, Hu XY, Lv W. The prognostic value of total T3 after acute cerebral infarction is age-dependent: a retrospective study on 768 patients. BMC Neurol. 2019;19: 54. doi:10.1186/s12883-019-1264-z
194. Hama S, Kitaoka T, Shigenobu M, Watanabe A, Imura I, Seno H, et al. Malnutrition and nonthyroidal illness syndrome after stroke. Metabolism. 2005;54: 699–704. doi:10.1016/j.metabol.2004.11.016
195. Chaker L, Baumgartner C, Den Elzen WPJ, Collet TH, Ikram MA, Blum MR, et al. Thyroid function within the reference range and the risk of stroke: An individual participant data analysis. J Clin Endocrinol Metab. 2016;101: 4270–4282. doi:10.1210/jc.2016-2255
196. Wakasugi S, Maeda S, Shimada K. Structure and expression of the mouse prealbumin gene. J Biochem. 1986;100: 49–58. Available: https://www.ncbi.nlm.nih.gov/pubmed/?term=wakasugi+maeda+shimada+1986
197. Wakasugi S, Maeda S, Shimada K, Nakashima H, Migita S. Structural comparisons between mouse and human prealbumin. J Biochem. 1985;98: 1707–14. Available: http://www.ncbi.nlm.nih.gov/pubmed/3005251
198. Aleshire SL, Bradley CA, Richardson LD, Parl FF. Localization of human prealbumin in choroid plexus epithelium. J Histochem Cytochem. 1983;31: 608–612. doi:10.1177/31.5.6341455
199. Felding P, Fex G. Cellular origin of prealbumin in the rat. BBA - Gen Subj. 1982;716: 446–449. doi:10.1016/0304-4165(82)90040-X
200. Southwell BR, Duan W, Alcorn D, Brack C, Richardson SJ, Kohrle J, et al. Thyroxine transport to the brain: role of protein synthesis by the choroid plexus. Endocrinology. 1993;133: 2116–2126. doi:10.1210/endo.133.5.8404661
201. Schreiber G, Aldred AR, Jaworowski A, Nilsson C, Achen MG, Segal MB. Thyroxine transport from blood to brain via transthyretin synthesis in choroid plexus. Am J Physiol. 1990;258: R338-345. Available: http://www.ncbi.nlm.nih.gov/pubmed/2309926
202. Richardson SJ, Wijayagunaratne RC, D'Souza DG, Darras VM, Van Herck SLJ. Transport of thyroid hormones via the choroid plexus into the brain: The roles of transthyretin and thyroid hormone transmembrane transporters. Front Neurosci. 2015;9: 1–8. doi:10.3389/fnins.2015.00066
203. Kassem NA, Deane R, Segal MB, Preston JE. Role of transthyretin in thyroxine transfer from cerebrospinal fluid to brain and choroid plexus. Am J Physiol Regul Integr Comp Physiol. 2006;291: R1310-1315. doi:10.1152/ajpregu.00789.2005
204. Peterson PA. Characteristics of a vitamin A-transporting protein complex occurring in human serum. J Biol Chem. 1971;246: 34–43.
205. Refetoff S. Thyroid hormone serum transport proteins. In: De Groot LJ, Chrousos G, Dungan K, Feingold KR, Grossman A, Hershman JM, et al., editors. Endotext. South Dartmouth (MA): MDText.com, Inc.; 2015. pp. 1–13. doi:Available from: https://www.ncbi.nlm.nih.gov/books/NBK285566/
206. Palha JA, Nissanov J, Fernandes R, Sousa JC, Bertrand L, Dratman MB, et al. Thyroid hormone distribution in the mouse brain: The role of transthyretin. Neuroscience. 2002;113: 837–847. doi:10.1016/S0306-4522(02)00228-2
207. Vranckx R, Rouaze M, Savu L, Nunez EA, Beaumont C, Flink IL. The hepatic biosynthesis of rat thyroxine binding globulin (TBG): Demonstration, ontogenesis, and UP-regulation in experimental hypothyroidism. Biochem Biophys Res Commun. 1990;167: 317–322. doi:10.1016/0006-291X(90)91767-M

208. Vranckx R, Savu L, Maya M, Nunez EA. Characterization of a major development-regulated serum thyroxine-binding globulin in the euthyroid mouse. Biochem J. 1990;271: 373–379. Available: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1149564&tool=pmcentrez&rendertype=abstract

209. Benvenga S, Lapa D, Trimarchi F. Re-evaluation of the thyroxine binding to human plasma lipoproteins using three techniques. J Endocrinol Invest. 2001;24: RC16--RC18. doi:10.1007/BF03343863

210. Mendel CM, Cavalieri RR, Kohrle J. Thyroxine (T4) transport and distribution in rats treated with EMD 21388, a synthetic flavonoid that displaces T4 from transthyretin. Endocrinology. 1992;130: 1525–1532. doi:10.1210/endo.130.3.1537302

211. Palha JA, Episkopou V, Maeda S, Shimada K, Gottesman ME, Saraiva MJ. Thyroid hormone metabolism in a transthyretin-null mouse strain. J Biol Chem. 1994;269: 33135–33139. Available: http://www.ncbi.nlm.nih.gov/pubmed/7806543

212. Palha JA, Hays MT, Morreale De Escobar G, Episkopou V, Gottesman ME, Saraiva MJ. Transthyretin is not essential for thyroxine to reach the brain and other tissues in transthyretin-null mice. Am J Physiol. 1997;272: E485-493. doi:10.1677/joe.1.06406

213. Palha JA, Fernandes R, De Escobar GM, Episkopou V, Gottesman M, Saraiva MJ. Transthyretin regulates thyroid hormone levels in the choroid plexus, but not in the brain parenchyma: Study in a transthyretin-null mouse model. Endocrinology. 2000;141: 3267–3272. doi:10.1210/en.141.9.3267

214. Refetoff S. Inherited thyroxine-binding globulin abnormalities in man. Endocr Rev. United States; 1989;10: 275–293. doi:10.1210/edrv-10-3-275

215. Bartalena L. Studies on thyroxine-binding globulin. J Endocrinol Invest. Italy; 1993;16: 353–371. doi:10.1007/BF03348858

216. Sarne DH, Refetoff S. Normal cellular uptake of thyroxine from serum of patients with familial dysalbuminemic hyperthyroxinemia or elevated thyroxine-binding globulin. J Clin Endocrinol Metab. United States; 1988;67: 1166–1170. doi:10.1210/jcem-67-6-1166

217. Mendel CM, Cavalieri RR, Gavin LA, Pettersson T, Inoue M. Thyroxine transport and distribution in Nagase analbuminemic rats. J Clin Invest. United States; 1989;83: 143–148. doi:10.1172/JCI113851

218. Friesema ECH, Ganguly S, Abdalla A, Manning Fox JE, Halestrap AP, Visser TJ. Identification of monocarboxylate transporter 8 as a specific thyroid hormone transporter. J Biol Chem. 2003;278: 40128–40135. doi:10.1074/jbc.M300909200

219. Friesema ECH, Jansen J, Milici C, Visser TJ. Thyroid Hormone Transporters. Vitam Horm. 2005;70: 137–167. doi:10.1016/S0083-6729(05)70005-4

220. Visser WE, Friesema ECH, Visser TJ. Minireview: thyroid hormone transporters: the knowns and the unknowns. Mol Endocrinol. 2011;25: 1–14. doi:10.1210/me.2010-0095

221. Sousa JC, Cardoso I, Marques F, Saraiva MJ, Palha JA. Transthyretin and Alzheimer's disease: Where in the brain? Neurobiol Aging. 2007;28: 713–718. doi:10.1016/j.neurobiolaging.2006.03.015

222. Blay P, Nilsson C, Owman C, Aldred A, Schreiber G. Transthyretin expression in the rat brain: effect of thyroid functional state and role in thyroxine transport. Brain Res. 1993;632: 114–120. doi:10.1016/0006-8993(93)91145-I

223. Herbert J, Wilcox JN, Pham KT, Fremeau RTJ, Zeviani M, Dwork A, et al. Transthyretin: a choroid plexus-specific transport protein in human brain. The 1986 S. Weir Mitchell award. Neurology. 1986;36: 900–911. doi:10.1212/WNL.36.7.900

224. Tang YP, Haslam SZ, Conrad SE, Sisk CL. Estrogen increases brain expression of the mRNA encoding transthyretin, an amyloid beta scavenger protein. J Alzheimers Dis. 2004;6: 413–420. Available: http://www.ncbi.nlm.nih.gov/pubmed/15345812

225. González-Marrero I, Giménez-Llort L, Johanson CE, Carmona-Calero EM, Castañeyra-Ruiz L, Brito-Armas JM, et al. Choroid plexus dysfunction impairs beta-amyloid clearance in a triple transgenic mouse model of Alzheimer's disease. Front Cell Neurosci. 2015;9: 1–17. doi:10.3389/fncel.2015.00017

226. Buxbaum JN, Ye Z, Reixach N, Friske L, Levy C, Das P, et al. Transthyretin protects Alzheimer's mice from the behavioral and biochemical effects of Abeta toxicity. Proc Natl Acad Sci U S A. 2008;105: 2681–2686. doi:10.1073/pnas.0712197105
227. Genovese T, Impellizzeri D, Ahmad A, Cornelius C, Campolo M, Cuzzocrea S, et al. Post-ischaemic thyroid hormone treatment in a rat model of acute stroke. Brain Res. 2013;1513: 92–102. doi:10.1016/j.brainres.2013.03.001
228. Hung PL, Huang CC, Huang HM, Tu DG, Chang YC. Thyroxin treatment protects against white matter injury in the immature brain via brain-derived neurotrophic factor. Stroke. 2013;44: 2275–2283. doi:10.1161/STROKEAHA.113.001552
229. Suzuyama K, Shiraishi T, Oishi T, Ueda S, Okamoto H, Furuta M, et al. Combined proteomic approach with SELDI-TOF-MS and peptide mass fingerprinting identified the rapid increase of monomeric transthyretin in rat cerebrospinal fluid after transient focal cerebral ischemia. Mol Brain Res. 2004;129: 44–53. doi:10.1016/j.molbrainres.2004.06.021
230. Gomes JR, Nogueira RS, Vieira M, Santos SD, Ferraz-Nogueira JP, Relvas JB, et al. Transthyretin provides trophic support via megalin by promoting neurite outgrowth and neuroprotection in cerebral ischemia. Cell Death Differ. England; 2016;23: 1749–1764. doi:10.1038/cdd.2016.64
231. Golabek A, Marques M, Lalowski M, Wisniewski T. Amyloid beta binding proteins in vitro and in normal human cerebrospinal fluid. Neurosci Lett. 1995;191: 79–82. doi:10.1016/0304-3940(95)11565-7
232. Schwarzman AL, Gregori L, Vitek MP, Lyubski S, Strittmatter WJ, Enghilde JJ, et al. Transthyretin sequesters amyloid beta protein and prevents amyloid formation. Proc Natl Acad Sci U S A. 1994;91: 8368–8372. doi:10.1073/pnas.91.18.8368
233. Puskas LG, Kitajka K, Nyakas C, Barcelo-Coblijn G, Farkas T. Short-term administration of omega 3 fatty acids from fish oil results in increased transthyretin transcription in old rat hippocampus. Proc Natl Acad Sci U S A. United States; 2003;100: 1580–1585. doi:10.1073/pnas.0337683100
234. Stein TD, Johnson JA. Lack of neurodegeneration in transgenic mice overexpressing mutant amyloid precursor protein is associated with increased levels of transthyretin and the activation of cell survival pathways. J Neurosci. 2002;22: 7380–7388. doi:22/17/7380 [pii]
235. Watanabe CM, Wolffram S, Ader P, Rimbach G, Packer L, Maguire JJ, et al. The in vivo neuromodulatory effects of the herbal medicine ginkgo biloba. Proc Natl Acad Sci U S A. 2001;98: 6577–6580. doi:10.1073/pnas.111126298
236. Wang X, Cattaneo F, Ryno L, Hulleman J, Reixach N, Buxbaum JN. The systemic amyloid precursor transthyretin (TTR) behaves as a neuronal stress protein regulated by HSF1 in SH-SY5Y human neuroblastoma cells and APP23 Alzheimer's disease model mice. J Neurosci. 2014;34: 7253–7265. doi:10.1523/JNEUROSCI.4936-13.2014
237. Li X, Masliah E, Reixach N, Buxbaum JN. Neuronal production of transthyretin in human and murine Alzheimer's disease: is it protective? J Neurosci. 2011;31: 12483–12490. doi:10.1523/JNEUROSCI.2417-11.2011
238. Kerridge C, Belyaev ND, Nalivaeva NN, Turner AJ. The Aß-clearance protein transthyretin, like neprilysin, is epigenetically regulated by the amyloid precursor protein intracellular domain. J Neurochem. 2014;130: 419–431. doi:10.1111/jnc.12680
239. Costa R, Gonçalves A, Saraiva MJ, Cardoso I. Transthyretin binding to A-Beta peptide - Impact on A-Beta fibrillogenesis and toxicity. FEBS Lett. 2008;582: 936–942. doi:10.1016/j.febslet.2008.02.034
240. Santos SD, Lambertsen KL, Clausen BH, Akinc A, Alvarez R, Finsen B, et al. CSF transthyretin neuroprotection in a mouse model of brain ischemia. J Neurochem. 2010;115: 1434–1444. doi:10.1111/j.1471-4159.2010.07047.x
241. Sharp FR, Zhan X, Liu DZ. Heat shock proteins in the brain: role of Hsp70, Hsp27, and HO-1 (Hsp32) and their therapeutic potential. Transl Stroke Res. United States; 2013;4: 685–692. doi:10.1007/s12975-013-0271-4

CHAPTER 2

GLOBAL AIMS

GLOBAL AIMS

The aim of this thesis was to study the contribution of TH and TTR in post-stroke mechanisms underlying stroke recovery.

In Chapter 3 we provide a review summarizing important actions of TH in the brain that may have an impact for stroke recovery.

In the experimental studies included in this thesis, mice were subjected to unilateral PT after intraperitoneal administration of Rose Bengal. The same surgical procedure was performed in Sham operated animals, with injection of a saline solution.

The aim of the first study in Chapter 4 was to investigate the involvement of TH (T_4 and T_3) in the modulation of excitatory and inhibitory neurotransmission relevant for neuronal reorganization and plasticity during a critical period of recovery phase after experimental stroke. To fulfill this aim we assessed (i) recovery of motor function by the rotating pole test; (ii) infarct volumes; (iii) TH receptor (TR) alpha 1 (TRα1) and beta 1 (TRβ1) levels and expression in neuronal and glial cell populations in the peri-infarct and contralateral region; (iv) dendritic spine classification and density in the peri-infarct and homotypic contralateral areas; (v) levels of synaptic proteins relevant for neurotransmitter release and (vi) tonic GABAergic signaling and the activity cortical PV positive interneurons in the peri-infarct area. In addition, we performed *in vitro* studies in cultured glutamatergic neurons in the presence and absence of T_3 to assess (vii) glutamate evoked currents by electrophysiology; (viii) TRα1 and TRβ1 expression and (ix) levels of synaptic proteins after oxygen and glucose (OGD) deprivation. *Post-mortem* human brains from controls and stroke patients were also analyzed in this study to assess (x) levels of TRα1 and TRβ1 in the infarct core, peri-infarct and non-stroke brain regions.

The aim of the second study in Chapter 5 was to investigate the expression of TTR at different time-points after PT, i.e. 24 hours, 48 hours 7 days and 14 days. To fulfill this aim we assessed (i) TTR immunoreactivity in neuronal and microglial population in the peri-infarct and contralateral region; (ii) TTR levels in the postischemic brain and (iii) relative *ttr* expression at different time-points after experimental stroke.

CHAPTER 3

THYROID HORMONES AND STROKE

Thyroid hormones in the brain and their impact in recovery mechanisms after stroke

Short description: This review summarizes essential knowledge on TH regulated mechanisms in the developing and adult brain i.e. maturation of neurons, cell genesis and neuronal plasticity. These mechanisms are also highly relevant to mechanisms of functional recovery after stroke. Unfortunately, and despite the relevance and the importance of TH in the healthy brain and in pathological conditions, the current state of knowledge on this topic (published preclinical studies and clinical trials) so far is scarce. Therefore, in our opinion this overview on functions of TH in the healthy brain and after stroke is of relevance and provides new aspects for future research on recovery mechanisms after stroke.

frontiers in Neurology

REVIEW
published: 18 October 2019
doi: 10.3389/fneur.2019.01103

Thyroid Hormones in the Brain and Their Impact in Recovery Mechanisms After Stroke

Daniela Talhada[1,2,3], *Cecília Reis Alves Santos*[2], *Isabel Gonçalves*[2] *and Karsten Ruscher*[1,3*]

[1] Laboratory for Experimental Brain Research, Division of Neurosurgery, Department of Clinical Sciences, Lund University, Lund, Sweden, [2] CICS-UBI-Health Sciences Research Centre, Faculdade de Ciências da Saúde, Universidade da Beira Interior, Covilha, Portugal, [3] LUBIN Lab-Lunds Laboratorium för Neurokirurgisk Hjärnskadeforskning, Division of Neurosurgery, Department of Clinical Sciences, Lund University, Lund, Sweden

Thyroid hormones are of fundamental importance for brain development and essential factors to warrant brain functions throughout life. Their actions are mediated by binding to specific intracellular and membranous receptors regulating genomic and non-genomic mechanisms in neurons and populations of glial cells, respectively. Among others, mechanisms include the regulation of neuronal plasticity processes, stimulation of angiogenesis and neurogenesis as well modulating the dynamics of cytoskeletal elements and intracellular transport processes. These mechanisms overlap with those that have been identified to enhance recovery of lost neurological functions during the first weeks and months after ischemic stroke. Stimulation of thyroid hormone signaling in the postischemic brain might be a promising therapeutic strategy to foster endogenous mechanisms of repair. Several studies have pointed to a significant association between thyroid hormones and outcome after stroke. With this review, we will provide an overview on functions of thyroid hormones in the healthy brain and summarize their mechanisms of action in the developing and adult brain. Also, we compile the major thyroid-modulated molecular pathways in the pathophysiology of ischemic stroke that can enhance recovery, highlighting thyroid hormones as a potential target for therapeutic intervention.

Keywords: brain, recovery, stroke, thyroid hormones, 3,5,3′,5′-tetraiodo-L-thyronine (T_4), 3,5,3′-triiodo-L-thyronine (T_3)

OPEN ACCESS

Edited by:
Stephan Schilling,
Fraunhofer Society (FHG), Germany

Reviewed by:
Steffen Rossner,
Leipzig University, Germany
Tobias Bobinger,
University Hospital Erlangen, Germany

***Correspondence:**
Karsten Ruscher
karsten.ruscher@med.lu.se

Specialty section:
This article was submitted to Stroke, a section of the journal Frontiers in Neurology

Received: *29 May 2019*
Accepted: *02 October 2019*
Published: *18 October 2019*

Citation:
Talhada D, Santos CRA, Gonçalves I and Ruscher K (2019) Thyroid Hormones in the Brain and Their Impact in Recovery Mechanisms After Stroke. Front. Neurol. 10:1103. doi: 10.3389/fneur.2019.01103

INTRODUCTION

Thyroid hormones (TH), 3,5,3′,5′-tetraiodo-L-thyronine (T_4) and 3,5,3′-triiodo-L-thyronine (T_3), are important for brain development in mammals, during embryonic and fetal stages, regulating processes of neuronal proliferation, migration and differentiation, neurite outgrowth, synaptic plasticity, dendritic branching, and myelination (1–5). Also, after birth, TH are crucial for normal brain function throughout the entire life. Specifically for the central nervous system (CNS) the active form T_3 is a key regulator for normal metabolism in humans and rodents (1, 6, 7).

Availability of T_3 to the developing and adult brain is tightly controlled by mechanisms regulating TH secretion, free fraction unbound to thyroxine binding globulins (TBG), transmembrane transporters and the activity of iodothyronine deiodinases (DIO). The pattern of these regulatory processes may vary according to the developmental stage and in the adult brain. T_3 plays an essential role for neurological functions, and minimal disturbances of these mechanisms may have consequences for normal brain development and function (1, 8).

Dependence of the CNS on T_3 at all stages of development prompted us to review the actions of TH and the relevance of these mechanisms for processes of recovery after ischemic stroke. We will begin to provide an overview on TH signaling in the brain during development and throughout adult life. Thereafter, we will focus on molecules involved in TH signaling after stroke. TH actions at specific time points after the insult, that are dependent of carrier proteins, transmembrane transporters, DIO activity, thyroid hormone receptors (TR) and co-factors, may provide information on underlying molecular and cellular mechanisms that enhance functional recovery of lost neurological functions. Moreover, we will discuss which mechanisms of action of TH in the brain may contribute to enhance functional outcome in stroke patients.

THYROID HORMONE TRANSPORT AND AVAILABILITY TO THE HUMAN AND RODENT BRAIN

In the adult, TH originate in the thyroid gland that secretes ∼93% as T_4 and 7% as T_3. Once secreted to plasma, TH binding proteins play an important role to maintain TH homeostasis and distribution into tissues, and $<0.1\%$ of TH circulate free in the blood (9, 10). In humans, 65% of TH bind to TBG, 20% to albumin and about 15% to transthyretin (TTR) (11, 12) while in rodents TTR is the main protein carrier in the blood circulation (13, 14). TH also bind to lipoproteins to a less extent (15, 16).

In contrary to processes during brain development, a different fraction of T_3 in the adult brain is provided from free T_3 available in the blood circulation and in the cerebrospinal fluid (CSF) (17, 18). Most of TH provided to the brain crosses the blood brain barrier (BBB) and around 20% the blood cerebrospinal fluid barrier (BCSFB) (19, 20). This passage is mediated by transmembrane transporter proteins with overlapping specificity that were identified in endothelial cells of brain microvessels that constitute the BBB and epithelial cells of the choroid plexus (CP) of humans and rodents. These transporters are also important for brain development, and include monocarboxylate transporter (MCT), organic anion transporting polypeptide (OATP), large neutral aminoacid transporter (LAT) and sodium/taurocholate co-transporting polypeptide (SLC10A1) families (17, 21–31). Among those, MCT8 is of particular importance for T_3 (21, 26, 29), and in mice deficient for this transporter, T_3 uptake is compromised (32). In humans, MCT10 also facilitates uptake and efflux of TH, in particular for T_3 (33). The gene that encodes MCT8 (*Slc16a2*) is also present in membranes of neurons, astrocytes, tanycytes and oligodendrocyte precursor cells and OATP1C1 mRNA has been found in astrocytes (34), mediating intracellular TH transport. In addition, it has been proposed that either T_4 or T_3 or both are captured via gaps at the endfeet of astrocytes covering brain microvessels (35, 36).

In contrast to the rodent, MCT8 deficiency in humans results in low T_3 levels in the brain and high levels in the serum due to TH transport deficiency. Thus, the development of the cerebral cortex is impaired accompanied with severe neurological impairment (37–40). The lack of alternative TH transporters such as OATP1C1 (28) and MCT10 (41) in the adult human brain to compensate the transport of T_3, may contribute to the neurological deficits observed in humans with MCT8 mutations. The activity of DIO2 and DIO3 in the brain is important to balance neuronal intracellular T_3 levels in the adult brain, according to the developmental stage and brain region (42–44). DIO enzymes catalyze and remove specific iodine atoms from iodothyronine molecules. In rodents, ∼50% of T_3 levels localized in the brain relies on local deiodination of T_4 in astrocytes and tanycytes by DIO2 (45). T_3 produced in glial cells is able to promote T_3 driven transcriptional activity in neurons, demonstrated by *in vitro* experiments in co-cultures of H4 human glioma cells expressing DIO2 and neuroblastoma cells (46). Homeostasis of T_3 in the CNS is also controlled by DIO3 activity in neurons, that converts T_4 into 3,3′,5′ reverse triiodo-L-thyronine (rT_3) and inactivates T_3 into 3,3′-diiodo-L-thyronine (T_2) (43, 47, 48).

Other compensatory mechanisms to maintain sufficient T_3 levels in the rodent brain include the reduction of DIO3 activity and consequently T_3 degradation, the increase of DIO2 activity in astrocytes (17, 32, 49) and the increase of *Dio2* expression in interneurons in the cerebral cortex (50).

THYROID HORMONES IN BRAIN DEVELOPMENT

It has been shown that mechanisms of brain development might be re-activated in processes of brain reorganization following stroke (51) involving cascades regulated by TH. Therefore, knowledge of TH actions critical and specific for each step of brain development is instrumental to understand their functions following stroke. Epidemiological and clinical studies in humans clearly show that several conditions that compromise maternal TH availability to the fetus impair brain development and are associated with neurological disorders and structural defects, most of them irreversible. A detailed review about TH transport, metabolism and function in the developing brain was recently published (52).

Despite epidemiological and clinical studies have demonstrated the demand of TH during brain development, animal experimental models are of high relevance to identify molecular mechanisms and detailed morphological changes of their biological function during brain development (53). Processes of neurogenesis, proliferation, migration, and maturation show different temporal profiles between humans and rodents, however, basic mechanisms and pathways that regulate brain development are similar (54, 55) allowing the extrapolation of TH deficiency mediated effects in rodents to abnormal TH signaling in humans. Maternal TH are crucial for early cortical neurogenesis, neuronal migration and maturation, during the first trimester of gestation, when fetal brain development occurs (1, 56–58). Both T_4 and T_3 are detected in the human brain embryo even before fetal thyroid gland maturation (59) that occurs at 11–12th week of gestation and starts to secrete TH at week 16 (60).

Also in the rat, the embryo is exposed to maternal TH after embryonic day 11 (E11), before the start of thyroid gland development at E17 (61–64). Experimental hypothyroxinemia induced in rats during this period (before E18) causes abnormal neurogenesis in the cortex and hippocampus, leading to impairment of synaptic plasticity and cognitive deficits (65–67), processes of high relevance in mechanisms of recovery after stroke.

Most of TH dependent processes during brain development are due to the interaction of T_3 with nuclear receptors and regulation of gene expression (68). Increasing levels of protein and mRNA encoding TR alpha and beta (TRα and TRβ) isoforms in the cerebrum and cerebellum start from the 8th to 10th week and increase over gestational time (69, 70) and in rodents there is expression of nuclear TR protein before thyroid gland functioning (71) suggesting transcriptional activity of TH. Several TH dependent genes expressed in the fetal rat brain and neuronal cultures, such as cytoskeletal proteins, are involved in mechanisms of neuronal migration and maturation, branching in neurons and astrocytes (1, 72–74). In both human and rodent species, mutations at the *TRα* and *TRβ* result in several neurological disorders (75–79).

The expression pattern of TR in the brain changes during CNS development. TRα1 is the predominant isoform with mRNA and protein expression in the entire brain, in rodents from E14 (80, 81) and in humans from 8th week of gestation (82, 83) onwards, importantly the expression decreases during brain development (84). TRβ1 is expressed at later stages of brain development and in contrary to TRα, TRβ1 mRNA levels do not decrease over gestational time (80, 84, 85). These studies indicate that gene transcription mediated by nuclear TR has spatiotemporal expression patterns and, therefore, TH actions are distinct in all stages of brain development.

In an *in vitro* model of differentiating mouse embryonic stem cell line (ES-E14TG2a) T_3 treatment (1 nM) enhanced the number of nestin-positive neuronal progenitors, accelerated differentiation and increased survival of pyramidal neurons (86). T_3 mediated differentiation was associated with the regulation of genes involved in corticogenesis namely *nestin*, empty spiracles homeobox 1 *(Emx1)*, T-box brain gene 1 *(Tbr1)*, Calmodulin kinase 4 *(Camk4),* and RC3/Neurogranin *(Nrgn)* (86). Regulation of gene expression during differentiation seems to be inversely correlated with levels of chicken ovalbumin upstream-transcription factor 1 (COUP-TF1) (86), that is crucial for adequate neuronal development (87).

MECHANISMS OF THYROID HORMONES ACTIONS IN THE ADULT BRAIN

Cellular actions of TH in the adult brain can be mediated by nuclear receptors and transcriptional activity, and also by non-genomic actions (85, 88, 89). Here we will elaborate in relevant TH actions described in the literature, and below we will delineate TH actions that might be involved in neurorepair processes.

Genomic Actions of TH

Actions of T_3 in the brain are mainly dependent on transcription mediated by T_3 binding to the nuclear receptors and formation of regulatory complexes (85, 88–91). In the presence of TH, TR are regulated by corepressors (CoR) and coactivators (CoA), proteins, that repress or activate transcription, respectively (36, 85, 88, 89, 92) (**Figure 1**).

In mammals, there are four isoforms of TR (TRα1, TRα2, TRβ1, and TRβ2) encoded by genes alpha (*Thra*) and beta (*Thrb*), which expression and distribution is different to the developmental brain (93). These isoforms are differently distributed in the tissues, regulate the transcription of different genes and exert different biological actions (94).

TRα1 and TRβ1 are the predominant isoforms in the CNS. TRα1 mRNA and protein accounts to 70–80% of TR expression in the brain (36, 95, 96). Thus, genomic actions of T_3 in the brain are mainly, but not exclusively, dependent of TRα1 signaling (97). The analysis of brains from TRα1—green fluorescent protein (GFP) mice revealed that this receptor is expressed in all NeuN positive neurons, especially in the nucleus (83). TRα1 is expressed in both excitatory glutamatergic and inhibitory GABAergic neurons in several brain regions including the striatum, cerebral cortex, hippocampus and dentate gyrus, hypothalamus and cerebellum (83, 98). This isoform of TR is also found in tanycytes lining the third ventricle and oligodendrocytes in the hypothalamus, but not in the striatum, somatosensory cortex or hippocampus (83, 99). Its presence in astrocytes is not completely clear (100), however may be dependent on the activation status. Although lower concentration of the receptor is found in cultured rat astrocytes (101), it is not expressed in glial fibrillary acidic protein (GFAP) positive astrocytes in the naïve rat and mouse brain (83, 99). TRα1 is absent in Purkinje cells in the cerebellum (83). Levels of TRα2, a non T_3 binding isoform, is also detected in the adult brain in a similar pattern as TRα1 (98).

TRβ1 is expressed in the neocortex, and mainly expressed in the pyramidal cell layers of the hippocampus, granule cells of dentate gyrus and paraventricular hypothalamic nucleus (102). It is also expressed in myelin basic protein positive oligodendrocytes (99). In contrary to TRα1, this isoform is highly expressed in Purkinje neurons (94). The TRβ2 isoform is restricted to the anterior pituitary gland and hypothalamus (102–105). As for TRα isoforms, TRβ1 and TRβ2 were not observed in positive GFAP positive astrocytes in the rat brain (99).

Although TR are mainly localized in the cell nucleus and nuclear membrane, TRα1 and TRβ1 isoforms have also been found in the cytoplasm of neurons and astroglia, and this shuttle may increase the rate of T_3 nuclear import (106). It has been suggested that T_4 may also exert genomic actions in the brain through binding to TRα1, that is more susceptible to T_4 than TRβ1 (36), however we did not find experimental studies supporting this hypothesis. So far, a total of 4,108 genes, of which 734 have been identified as being repeatedly regulated by T_3 in the rodents' brain by microarray analysis (72). In this review, we provide an overview on T_3-modulated genes that might be involved in brain repair mechanisms (**Table 1**). Hence, different

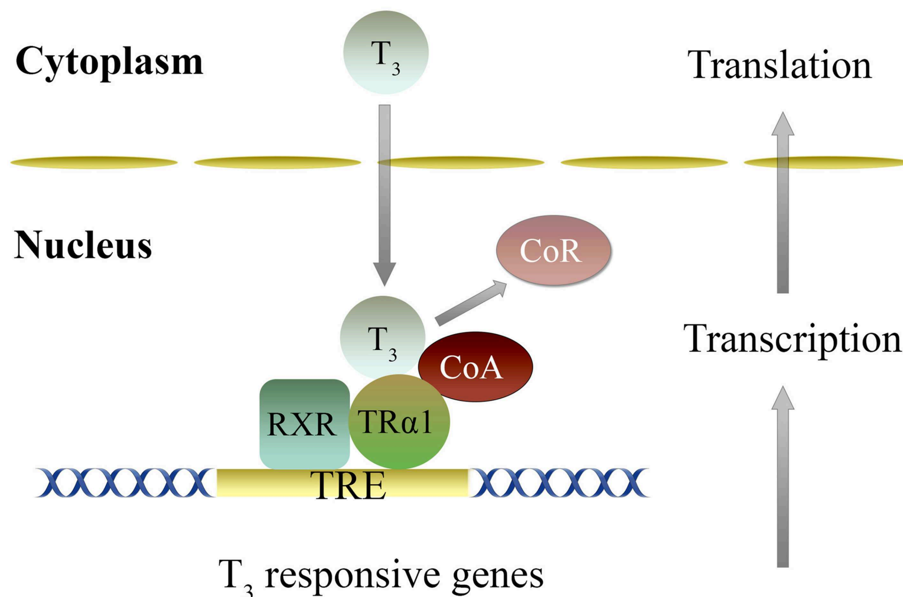


FIGURE 1 | Genomic mechanisms of thyroid hormones action in the brain. Genomic actions of T_3 are dependent on gene transcription mediated by its binding to nuclear TRα and TRβ, and the formation of heterodimer complex with RXR (RXR-TR) that binds to a TRE, located at the regulatory region of T_3 target genes. This activity is regulated by an exchange of CoR for CoA. CoA, Co-activator; CoR, Co-repressor; RXR, Retinoid X receptor; TR, Thyroid hormone receptor; TRE, Thyroid response element; T_3, 3,5,3′-triiodo-L-thyronine.

T_3–dependent transcriptional activities have been observed in different cell types and brain regions.

Non-genomic Actions of TH

TH non-genomic actions that do not require TH binding to nuclear receptors are well-described in the literature (127–130). Actions are immediate and include several interactions of TH with extranuclear receptors, including TRα and TRβ, located in the cytoplasm, cellular membrane, cytoskeleton and mitochondria, modulating several intracellular pathways.

The following points summarize relevant non-genomic actions of TH binding to membranous and cytoplasmic receptors (i–iii), cytoplasmic TH binding proteins affecting ion pumps activity (iv) and the action of TH on the cytoskeleton (v) (**Figure 2**). (i) T_3 complexed to TRβ1 in the cytoplasm interacts with p85α subunit of phosphatidylinositol 3-kinase (PI3K), resulting in phosphorylation and activation of protein kinase (PK) B/Akt signal transduction pathway, rapamycin (mTOR) and phosphorylation of $p70^{S6K}$ (131–136). (ii) T_3 is able to bind to integrin αvβ3 S1 domain in plasma membranes and activates PI3K via Src kinase. T_4 and T_3 interact with integrin αvβ3 S2 domain and activate mitogen-activated protein kinase 1/2 (MAPK 1/2) signaling cascade, through phospholipase C (PLC) and PKC (127, 133, 137–140). Subsequently, it results in an nuclear translocation of TRβ1 (141), estrogen receptor α (142), signal transducing and activator of transcription (STAT) 1α, interferon gamma (IFN-γ) (143) and CoA protein Trip230 (144). In addition, hormone activated MAPK 1/2 phosphorylates TRβ1 at Ser-142, leading to recruitment of CoA proteins (145). (iii) T_4 non-gnomically activates MAPK 1/2 in HeLa and CV-1 cultured cells (146, 147) and phosphorylation of p53 (148) and STAT3 (147). (iv) T_3 modulates Na^+/H^+ exchanger in myoblasts (149); Na-K-ATPase activity in alveolar epithelial cells (150–152), embryonal hepatocytes (153), and synaptosomes (154, 155) through either the PI3K or MAPK pathways (152, 156); the Ca-ATPase activity in erythrocytes (88), the sarcoplasmic reticulum in the heart (157) and in cerebrocortical synaptosomes (158). (v) T_4 and rT_3 stimulate polymerization of actin components of the cytoskeleton neuronal and astrocyte cell cultures, through TH binding to an extranuclear truncated form of TRα1 (TRΔα1) (159–162).

THYROID HORMONES IN THE AGING BRAIN AND ISCHEMIC STROKE

The complex process of aging is associated with changes in TH metabolism and action in all tissues. During aging, the disruption of circadian rhythm leads to a reduction in thyroid stimulating hormone (TSH) secretion (163–165) and circulating TH levels, in particular T_3, in humans (166, 167) and rodents (168). Nevertheless, TH signaling is well-preserved in the aging brain, as demonstrated in mouse models of aging (169).

TABLE 1 | List of genes regulated by thyroid hormones involved in their transport into the brain, mechanisms of tissue repair, and neuronal plasticity following ischemic stroke.

Genes human/rodent	Gene name	Function	Tissue/cultured cells	References
SLC16A2/Slc16a2	MCT8	TH transport	Brain	(107)
DIO2/Dio2	DIO2	TH deiodination		
DIO3/Dio3	DIO3			
Bcl2	Bcl2	Neuronal survival, neurogenesis and neurotrophic factors	Brain cortex	(107–109)
Vegfa	VEGFA			
Sox2	SRY-box2			
Ntf	Neurotrophin			
Nos2	NOS2			
HIF2α	HIF2α		Neuroblastoma cell line	(107)
VEGF	VEGF			
c-JUN	c-Jun			
ENO2	Enolase-2			
Emx1	empty spiracles homeobox 1		mES cell line	(86)
Tbr1	T-box brain gene 1			
Bdnf	Brain derived neurotrophic factor		Hippocampal slices	(110)
Slc12a5	KCC2			
NRGN/Nrgn	Neurogranin	Synaptic plasticity	Hippocampus and forebrain/mES cell line	(86, 111–113)
CAMK4/Camk4	Calmodulin kinase 4		Brain/Neurons/mES cell line	(86, 114–117)
Reln	Reelin		Brain	(118–120)
Srg1	Synaptotagmin-related gene 1		Brain	(121)
Nefh	Neurofilament heavy polypeptide		Neurons	(122)
Nefm	Neurofilament medium polypeptide			(122)
GFAP	GFAP		Astrocytes	(123)
Vim	Vimentin		Mesenchimal cells	(122)
Nes	Nestin		Neurons	(86, 122)
Vegf	VEGF	Angiogenesis	Brain	(124–126)
Angpt2	Angiopoietin-2			

However, hypothyroidism and decreased TH availability to the brain has been considered a risk factor for the development of neurodegenerative diseases (170, 171) and acute ischemic stroke (172, 173). In addition, recent epidemiological studies have associated low levels of T_3 with poor functional outcome after acute ischemic stroke (174–179). Interestingly, lower total T_3 levels is not related with poor functional recovery after ischemic stroke in patients below 65 years of age, suggesting that the association between levels of T_3 and stroke recovery may be clinically important in older patients (180). Non-thyroidal illness syndrome also impairs functional recovery after stroke (181). Besides, stroke patients with thyroid dysfunction (lower levels of TSH and higher levels of free T_4) are associated with poorer clinical outcome (182). Together, studies point toward the need for a systemic assessment of thyroid dysfunction and stroke outcome.

Some reports suggest neuroprotective effects of hypothyroidism prior to brain ischemia in humans (183, 184), as well as experimental studies in rodents (185, 186). In a recent systematic review, stroke patients with subclinical hypothyroidism (higher levels of TSH and normal levels of free T_4 within the reference range) were more prone to suffer a non-fatal stroke and minor adverse events (182). However, we lack mechanistic studies how systemic levels of TH exactly influence processes in the postischemic brain. It is likely that hypothyroid episodes prior to stroke only delayed neuronal death, due to decreased metabolic demand of neurons, decreased glutamate production and delayed oxidative stress (185, 187). There is no evidence from experimental studies that show beneficial effects in hypothyroid animals after stroke. A recent animal study suggested that daily intravenous administrations of rT_3, an inactive form of T_3, prevents ischemic-reperfusion injury in rats subjected to transient MCAO, however authors did not evaluate if rT_3 induced an hypothyroid state (188). Similarly, in rats subjected to permanent middle cerebral artery occlusion (MCAO), TH serum levels are reduced 14 days after injury correlated with increased neurological impairment (189).

On the other hand, hyperthyroidism has been associated with an increased risk for ischemic stroke in humans (190–192). However, the population-based study was performed in patients

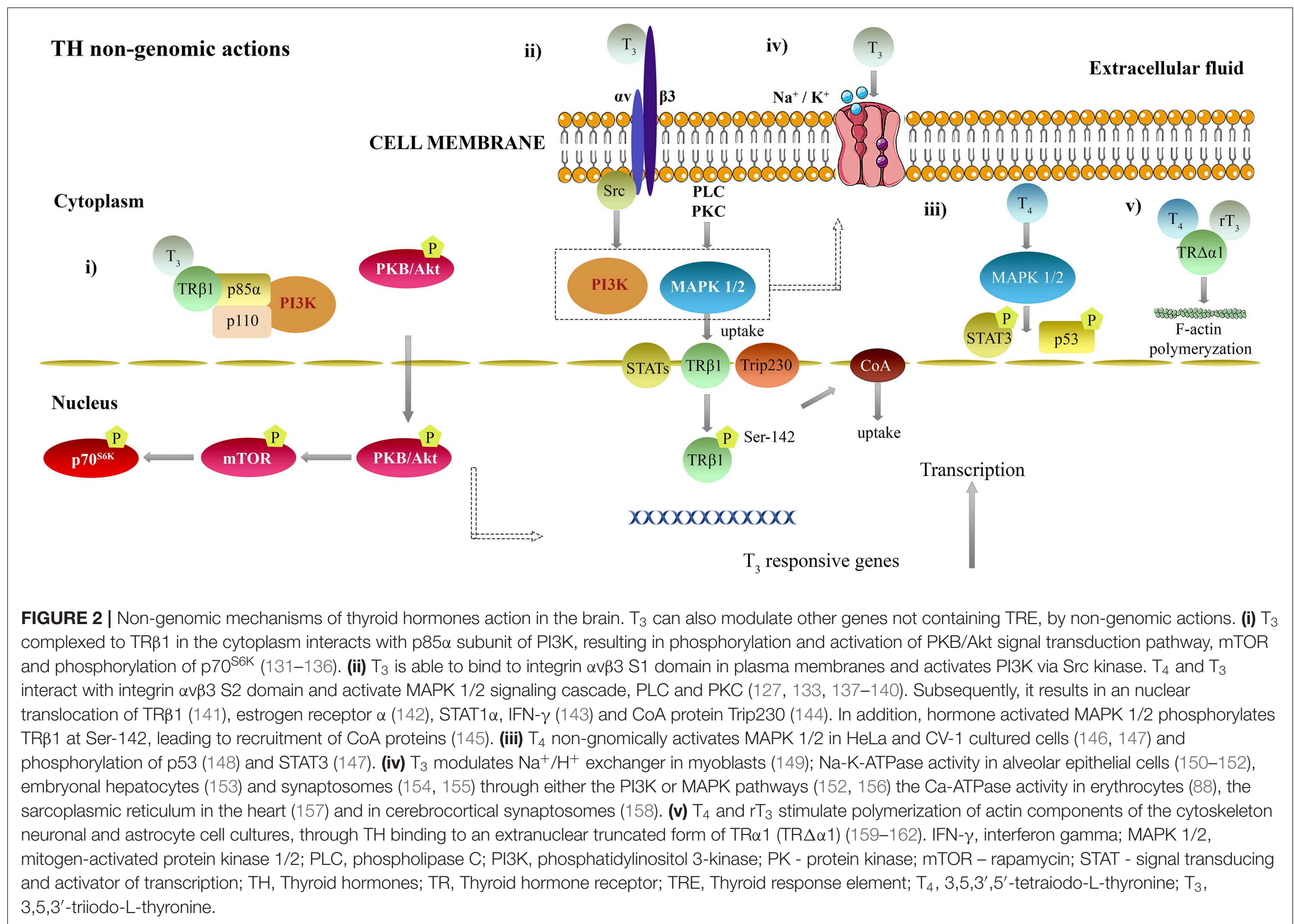


FIGURE 2 | Non-genomic mechanisms of thyroid hormones action in the brain. T_3 can also modulate other genes not containing TRE, by non-genomic actions. **(i)** T_3 complexed to TRβ1 in the cytoplasm interacts with p85α subunit of PI3K, resulting in phosphorylation and activation of PKB/Akt signal transduction pathway, mTOR and phosphorylation of $p70^{S6K}$ (131–136). **(ii)** T_3 is able to bind to integrin αvβ3 S1 domain in plasma membranes and activates PI3K via Src kinase. T_4 and T_3 interact with integrin αvβ3 S2 domain and activate MAPK 1/2 signaling cascade, PLC and PKC (127, 133, 137–140). Subsequently, it results in an nuclear translocation of TRβ1 (141), estrogen receptor α (142), STAT1α, IFN-γ (143) and CoA protein Trip230 (144). In addition, hormone activated MAPK 1/2 phosphorylates TRβ1 at Ser-142, leading to recruitment of CoA proteins (145). **(iii)** T_4 non-gnomically activates MAPK 1/2 in HeLa and CV-1 cultured cells (146, 147) and phosphorylation of p53 (148) and STAT3 (147). **(iv)** T_3 modulates Na^+/H^+ exchanger in myoblasts (149); Na-K-ATPase activity in alveolar epithelial cells (150–152), embryonal hepatocytes (153) and synaptosomes (154, 155) through either the PI3K or MAPK pathways (152, 156) the Ca-ATPase activity in erythrocytes (88), the sarcoplasmic reticulum in the heart (157) and in cerebrocortical synaptosomes (158). **(v)** T_4 and rT_3 stimulate polymerization of actin components of the cytoskeleton neuronal and astrocyte cell cultures, through TH binding to an extranuclear truncated form of TRα1 (TRΔα1) (159–162). IFN-γ, interferon gamma; MAPK 1/2, mitogen-activated protein kinase 1/2; PLC, phospholipase C; PI3K, phosphatidylinositol 3-kinase; PK - protein kinase; mTOR – rapamycin; STAT - signal transducing and activator of transcription; TH, Thyroid hormones; TR, Thyroid hormone receptor; TRE, Thyroid response element; T_4, 3,5,3′,5′-tetraiodo-L-thyronine; T_3, 3,5,3′-triiodo-L-thyronine.

aged 18–44 years. Hence, this study has not been adjusted for other risk factors such as hypertension and atrial fibrillation that may independently contribute for stroke. Larger infarct volumes also have been found in hyperthyroid rats after transient MCAO (193). Hence, hyperthyroid rats (oral administration of TH for 4 weeks) showed profound effects on the cardiovascular system including hypertension and tachyarrhythmia and treatment resulted in a catabolic metabolism (193).

Interestingly, increased mRNA expression of *Dio2* has been found in astrocytes during the first 72 h after transient MCAO (194). Together with modulation of *Thrb* expression, that is reduced in the infarct core and increased in the peri-infarct area, it suggests a local action of T_3 (189). Repeated daily administrations of T_4, before and on days one, two and three after stroke, decrease neuronal damage in the cornu ammonis CA1 pyramidal cells in the hippocampus (195). In an animal model of MCAO, intraperitoneal injection of T_4 (11 µg/kg, 1 h after ischemia and 6 h after reperfusion) reduced cortical and striatal infarct volume 24 h after stroke, with a reduction of GFAP, Iba-1, PKC, and MAPK 1/2 expression (196). Treatment with levothyroxine (25 µg/kg intraperitoneal) 1 h after traumatic brain injury stimulated mRNA expression of genes encoding MCT8, DIO2, and DIO3; genes related with neuronal survival and neurogenesis, namely *Bcl2*, vascular endothelial growth factor A (*Vegfa*), *Sox2*, and neurotrophin (*Ntf*) in the cortex, and of inducible nitrite oxide synthase 2 (*Nos2*) (107).

Moreover, intraperitoneal administration of T_3 at 12 µg/kg 1 h after traumatic brain injury reduced lesion size and inflammation (197). T_3 treatment 25 µg/kg 30 min after transient MCAO also reduced volume of stroke damage in mice through stimulation of fatty acid oxidation by astrocytes (198). A combination therapy of bone marrow stromal cells, daily injections of T_3 200 µg/kg and mild exercise was related to reduce ischemic damage 7 days after transient MCAO in rats (199). Likewise, intraperitoneal administration of thyroxine derivates, 3-iodothyronamine and thyronamine, 50 mg/kg 1 h after MCAO in mice, also reduced infarct volume (200). Neuroprotective action of 3-iodothyronamine administered 2 days before MCAO was associated with hypothermia (200). Although molecular mechanisms have not been evaluated, these studies suggest that non-genomic actions of TH contribute to neuroprotection in the acute phase following stroke. In addition, T_3 treatment prior to brain ischemia (25 µg/kg intravenous) has prevented edema through suppression of aquaporin-4 (AQP4) water channel expression and thereby reducing infarct volume and improving neurological outcome after transient MCAO (109), effects that

were enhanced when T_3 was administrated in nanoparticles at equivalent doses (108). Recently, it has been demonstrated that T_3 modulates AQP4 expression dependent on developmental stage of the CNS. Treatment of mice with T_3 at 1 µg/g increased AQP4 expression in astrocytes in the cerebral cortex until the 60th postnatal day. In contrast, whereas in glioblastoma cell lines stimulation with T_3 (50 nM) treatment was downregulating the expression of AQP4 (201).

Few experimental studies have been performed to identify the mechanisms of TH actions in neuroprotection. Treatment of mouse hippocampal slices lesioned between CA3 and CA1 with T_4 increases levels K-Cl cotransporter (KCC2) mRNA in a brain derived neurotrophic factor (BDNF) dependent manner, that promoted survival and regeneration of damaged neurons in the CA1 region (110). Moreover, it has been demonstrated that treatment with T_3 has a protective effect against glutamate toxicity in cultured astrocytes and neurons (202, 203). This action has been linked to non-genomic actions of TH on Na^+/H^+ exchange activity and glutamate transport (204). Also, T_3 treatment stimulated the expression of *HIF2α*, *VEGF*, *c-JUN,* and Enolase-2 (*ENO2*) in the neuroblastoma in an *in vitro* hypoxia model (107). Although only a few scattered studies have been performed, they indicate an involvement of T_3 in pathways that promote neuronal protection and recovery, through genomic or non-genomic actions.

The expression of TR in the human brain after ischemic stroke have not been studied. Also in experimental models, TR expression has not been investigated, and we found only one experimental study reporting TR expression after permanent MCAO (189). Interestingly, TRα1 expression is increased in microglial cells in the infarct core and in neurons in the peri-infarct area. Astrocytes mildly express nuclear TRα1 and expression of TRβ1 is strongly expressed in the astrocytic scar. If TRα1 and TRβ1 play a crucial role for recovery after brain stroke, in humans and rodents, it remains to be investigated. In fact, TRα1 have been demonstrated to play a crucial role for cardiomyocyte survival after myocardial infarction (205) Therefore, the idea that TRα1 could be a target to promote stroke recovery definitively needs to be further investigated.

MECHANISMS OF THYROID HORMONES THAT MAY ENHANCE MECHANISMS OF RECOVERY AFTER STROKE

Beyond the acute phase after stroke, the brain shows the capacity of spontaneous recovery of lost neurological functions albeit to a limited extent. This process remains slow, however, the intrinsic mechanisms are present and patients may benefit from enhancing those. TH regulate several pathways that are involved in neurorepair, namely regulation of processes of neuronal plasticity, neurogenesis, angiogenesis, and glutamate toxicity. Adjuvant therapies that modulate those processes may improve recovery of function after stroke, in particular when applied in combination with physiotherapy in stroke patients or an enriched environment in rodent models of stroke (206, 207).

Neuronal Plasticity

Neuronal reorganization occurs during the recovery phase of stroke and is initiated by cellular responses to degeneration. Cell death in the infarct core results in synaptic degeneration, instigating regenerative responses among surviving neurons, as the formation of new synaptic connections (208). Neuronal plasticity includes all mechanisms involved in modulation of dendritic and axonal arborization, dendritic spine density and neuronal density that will determine the formation of new synaptic connections and neuronal networks (209, 210).

Neuroplasticity occurs spontaneously during stroke recovery and TH have been identified as a modulator of several genes that may stimulate endogenous neuroplasticity and therefore contributing to facilitate recovery (**Table 1**).

In rodents, T_3 regulates neuron specific RC3/Neurogranin gene (*Nrgn*), that encodes a calmodulin binding protein (112) which binds to calmodulin in the absence of calcium distribution in spines and enhances synaptic plasticity (211). *Nrgn* is highly expressed in dendritic spines in the hippocampus and forebrain and deficiency of *Nrgn* in mice has been reported to induce deficits in spatial learning and anxiety-like tendencies (113). In the human, the homolog gene NRGN is also a direct TH target, during development and in the adult brain (111). TH also regulates *Reelin* (*Reln*) expression during brain development (118). Administration of T_3 increases *Reln* expression in the hippocampus of adult rats (212). Reelin is involved in the migration of multipolar neurons in the developing neocortex (120) and in the adult brain interacts with apoliprotein E receptors and regulates synaptic plasticity and neurogenesis (119).

During brain development, T_3 regulates genes related with the calcium/Calmodulin-activated kinase 4—cAMP responsive element-binding protein 1 (CaMK4/Creb1) signaling pathway, as demonstrated in cultured fetal neurons (213), a mouse embryonic stem cell line (86) and *in vivo* studies (114–117). The CaMK4/Creb1 pathway regulates calcium influx and dendritic growth during development (214), inhibits apoptosis through phosphorylation of Creb and increases anti-apoptotic gene expression. Synaptotagmin-related gene 1 (*Srg1*) is also a TH responsive gene during brain development, that has a putative role as a mediator of synaptic structure and activity (121).

Reorganization of spine cytoskeleton, principally microtubules and actin filaments, can be dynamically modulated and consequently change the pattern of synaptic activity (215). Several studies have shown that TH modulate tubulins (216, 217), microtubule associated proteins (218) and Tau protein (219) in the cytoskeleton during brain development and in the adult brain. TH has also been demonstrated to modulate transcription of genes of intermediate filaments, namely neurofilaments (*Nefh* and *Nefm*), GFAP in mature astrocytes, vimentin in mesenchymal cells and nestin (116, 122). Experiments in cerebral cortex slices suggest that TH activates phosphorylation of cytoskeletal proteins mediated by GABAergic signaling dependent on PKA and PKCaMII activity (220). Studies conducted in cultured glial cells also suggests that TH reorganize the cytoskeleton

through GFAP phosphorylation mediated by RhoA signaling pathway (221).

Actions of TH in the cytoskeleton are particularly important during brain development, to guarantee proper cell migration and to foster neurite outgrowth (160). T_3 also regulates transcription of genes involved in cytoskeleton formation in neurons and astrocytes, during fetal and postnatal brain development (74). Hypothyroidism leads to impaired actin cytoskeleton formation in neurons and astrocytes, affecting neuronal migration and neurite outgrowth. Both rT_3 or T_4 administration can restore polymerization of intracellular filaments F-actin (222, 223) and laminin (223, 224), but this effect was not observed by T_3 administration. Also in neuronal and astrocyte cultures, T_4 and rT_3 stimulate polymerization of the actin cytoskeleton, as already mentioned above (**Figure 2**) (159, 160, 162). As during brain development, basic transcription element-binding protein is upregulated by TH and this protein may play a role in neuronal outgrowth, modulating changes in the cytoskeleton, and cell differentiation (225, 226).

Studies also demonstrate that TH signaling is critical to proper functioning of short term (227) and long term synaptic plasticity (228). Induced hypothyroidism has been related with disruption in synaptic plasticity (229, 230) and long term potentiation in the CA1 neonatal (231, 232) and adult (233) rat hippocampus. Hyperthyroidism has been also related to detrimental effects in dendritic spines. Intraperitoneal injection of T_3 750 µg/kg during 5 consecutive days in adult rats significantly decreased dendritic spine density in CA1 pyramidal cells in the hippocampus (234) and thyroxine induced hyperthyroidism impairs special learning and synaptic plasticity in rats (235).

On the cellular level, TH genomic or non-genomic actions may modulate the activity of ion pumps that are important for normal excitable cell function. Particularly in brain tissues affected by ischemia, directly or indirectly, adapted function of ion pumps is required to avoid intracellular overload of H^+ and Ca^{2+}, preventing cell acidosis and excitotoxicity. T_3 has been shown to decrease the activity of Na, K-ATPase (154, 155) and to stimulate the Na^+/H^+ exchanger (149) and Ca^{2+}/Mg^{2+} ATPase pump activity (158) in cerebrocortical synaptosomes. T_3 increases the transcription of SR Ca^{2+}-ATPase gene (ATP2A2) in the sarcoplasmic reticulum (157). In addition, T_3 has been demonstrated to be benefical in *in vitro* and *in vivo* experimental myocardial ischemia preventing excessive intracellular Ca^{2+} accumulation (236, 237).

Also, T3 contributes to glutamate uptake by astrocytes, protecting neurons from intracellular calcium toxicity and death (203). Neuroprotective effect was attributed to an increased expression of mRNA and protein levels of GLT-1 and GLAST in the astrocytes. It also has been demonstrated that T3 decreases N-methyl-d-aspartate (NMDA)-evoked currents and prevent glutamate-induced neuronal death in hippocampal neurons (202). Together, these actions might be beneficial to prevent cell dysfunction or death of principal neurons during the acute phase after ischemic stroke. Conversely, these mechanisms might be involved to reduce the activity of inhibitory neurons in critical periods of plasticity during the first weeks after stroke. Hence, these actions most likely will dependent on receptor expression profiles in different neuron populations.

The balance between excitation and inhibition is of particular importance for neuronal plasticity processes potentially relevant for recovery. During development, TH increases the level of γ-aminobutyric acid (GABA) in the brain, while the opposite effect is observed in the adult brain. In the developing brain, hypothyroidism impairs the generation of interneurons including reduced proliferation and delayed differentiation of precursor cells in the cerebellum and their migration to the cerebellar cortex (238). These effects could be antagonized by administration of T_3 binding to the TRα1. Likewise, deletion of TRα1 reduced cerebellar GAT-1 expression and Pax-2 precursor cell proliferation (238). TH also affect the release and uptake of GABA from the neuron into the synapse. T_3 stimulates depolarization and release of GABA in synaptosomes from rat cerebral cortex (239). In the adult brain, hypothyroidism is reported to increase glutamic acid decarboxylase (GAD) activity and GABA reuptake, from cerebral cortex homogenates (240, 241) while hyperthyroidism has no effect on GABA uptake (241). In addition, T_3 administration inhibits GABA-induced Cl^- currents, which may affect $GABA_A$ receptors in the cerebral cortex, by non-genomic mechanisms (242).

Adult Neurogenesis

TH signaling is crucial for proper neurogenesis during brain development (1). Several studies have demonstrated that neurogenic events in the adult brain are dependent on TH actions (243–250) and have been reviewed in detail (251, 252). Particularly T_3 is involved in mechanisms of proliferation, survival, differentiation and maturation of neuronal precursors in the adult brain (246, 251). With potential contribution of TH NSPCs from the SVZ may proliferate, migrate and differentiate into neurons, astrocytes or oligodendrocytes in the damaged region and thereby contribute to brain plasticity after ischemic stroke or other brain injury (253, 254). Stem cell therapy and neurogenesis have been explored as a potential therapeutic strategy for neuronal repair after ischemic stroke (255).

Angiogenesis

Therapeutic angiogenesis has been used to enhance brain repair promoting the formation of new blood vessels and restoration of blood flow in the damaged area (256–258). Angiogenic effects of TH have been demonstrated in infarcted tissue of the myocardium (259). Moreover, an increased number of new blood vessels has been found in the brain of hypothyroid rats after administration of 3,5-diiothyroprionic acid (a thyroid hormone analog) or T_4 (260). The proangiogenic effects of TH are apparently mediated by non-genomic actions, through binding to integrin αvβ3 resulting in activation of MAPK 1/2 and STAT3. TH binding to αvβ3 directs transcription of genes that promote angiogenesis, namely fibroblast factors, VEGF and angiopoietin-2 (124–126, 137, 147, 261, 262). To our knowledge, so far no experimental studies have been performed to investigate pro-angiogenic effects of TH after stroke.

TRANSLATION TO CLINICAL STUDIES

Current epidemiological studies in humans and experimental evidence from rodents strongly suggest that TH signaling plays a crucial for stroke recovery. In particular T_3, the active form in the brain, exerts genomic and non-genomic actions that may foster functional outcome after stroke.

Although several epidemiological studies have associated low levels of TH with poor outcome, no clinical trials have been performed to evaluate the recovery promoting effects of TH in stroke patients. At the current stage the first step of translational studies will be to understand exact mechanisms underlying beneficial action of TH after stroke in animal models, in particular T_3. Based on knowledge about mechanisms of action, exact treatment regimens with specific targets can be developed and tested during critical windows of stroke recovery. In this context, the development of cell-specific approaches to target TH signaling in the postischemic brain may result in specific treatments in experimental stroke models, that later, might be translated into clinical studies.

CONCLUSIONS

Several mechanisms in the brain are tightly regulated by TH and T_3 availability to the brain is dependent on factors including (i) maternal TH release before fetus thyroid gland development; (ii) TSH levels and TH release by thyroid gland; (iii) passage of TH through placenta in the fetus; (iv) control of free fraction of TH determined by TH binding proteins; (v) TH transmembrane transport into the cytoplasm; (vi) local activity of iodothyronine deiodinases; (vii) expression and distribution of TR; (viii) and translational activity and non-genomic actions of TH in the brain. Disruption in these mechanisms compromise TH availability and actions in the brain and may result in impairments of neurological functions. There is clinical and preclinical evidence that TH are involved in mechanisms of neuronal plasticity and function of glial cells after ischemic stroke. Further understanding and targeting those might be exploited in future therapies to enhance functional recovery in stroke patients.

AUTHOR CONTRIBUTIONS

DT conceptualized and wrote the review, under supervision of KR. KR, CS, and IG revised the manuscript. All authors approved the final version of the manuscript for submission and publication.

FUNDING

This work was supported by funds from the Health Sciences Research Center (CICS-UBI) through National Funds by FCT—Foundation for Science and Technology (UID/Multi/00709/2019); by a doctoral fellowship (SFRH/BD/104679/2014) from Fundação para a Ciência e Tecnologia; partially supported by Programa Operacional do Centro, Centro 2020 through the funding of the ICON project (Interdisciplinary Challenges on Neurodegeneration; CENTRO-01-0145-FEDER-000013); the Swedish Brain Fund; the Crafoord Foundation, Sveriges Stroke Riksförbundet; the Hans-Gabriel and Alice Wachtmeister Foundation, Sparbanksstiftelsen Färs & Frosta and the Stiftelsen Sven-Olof Jansons livsverk.

REFERENCES

1. Bernal J. Thyroid hormones in brain development and function. In: Feingold KR, Anawalt B, Boyce A, editors. *Endotext*. South Dartmouth, MA: MDText.com, Inc. (2000) 1–136.
2. Gothié JD, Demeneix B, Remaud S. Comparative approaches to understanding thyroid hormone regulation of neurogenesis. *Mol Cell Endocrinol.* (2017) 459:104–15. doi: 10.1016/j.mce.2017.05.020
3. Hartley MD, Banerji T, Tagge IJ, Kirkemo LL, Chaudhary P, Calkins E, et al. Myelin repair stimulated by CNS-selective thyroid hormone action. *JCI Insight.* (2019) 4:e126329. doi: 10.1172/jci.insight.126329
4. Moog NK, Entringer S, Heim C, Wadhwa PD, Kathmann N, Buss C. Influence of maternal thyroid hormones during gestation on fetal brain development. *Neuroscience.* (2017) 342:68–100. doi: 10.1016/j.neuroscience.2015.09.070
5. Morreale de Escobar G, Obregon MJ, Escobar del Rey F. Role of thyroid hormone during early brain development. *Eur J Endocrinol.* (2004) 151:U25–37. doi: 10.1530/EJE.0.151U025
6. Bárez-López S, Guadaño-Ferraz A. Thyroid hormone availability and action during brain development in rodents. *Front Cell Neurosci.* (2017) 11:240. doi: 10.3389/fncel.2017.00240
7. Mullur R, Liu YY, Brent GA. Thyroid hormone regulation of metabolism. *Physiol Rev.* (2014) 94:355–82. doi: 10.1152/physrev.00030.2013
8. Ahmed OM, El-Gareib AW, El-bakry AM, Abd El-Tawab SM, Ahmed RG. Thyroid hormones states and brain development interactions. *Int J Dev Neurosci.* (2008) 26:147–209. doi: 10.1016/j.ijdevneu.2007.09.011
9. Mendel CM, Weisiger RA, Jones AL, Cavalieri RR. Thyroid hormone-binding proteins in plasma facilitate uniform distribution of thyroxine within tissues: a perfused rat liver study. *Endocrinology.* (1987) 120:1742–9. doi: 10.1210/endo-120-5-1742
10. Palha JA. Transthyretin as a thyroid hormone carrier: function revisited. *Clin Chem Lab Med.* (2002) 40:1292–300. doi: 10.1515/CCLM.2002.223
11. Refetoff S. Thyroid hormone serum transport proteins. In: Feingold KR, Anawalt B, Boyce A, editors. *Endotext*. South Dartmouth. MA: MDText.com, Inc. (2015). p. 1–13.
12. Schreiber G, Richardson SJ. The Evolution of Gene Expression, Structure and Function of Transthyretin. *Comp Biochem Physiol Part B Biochem Mol Biol.* (1997) 116:137–60. doi: 10.1016/S0305-0491(96)00212-X
13. Vranckx R, Rouaze M, Savu L, Nunez EA, Beaumont C, Flink IL. The hepatic biosynthesis of rat thyroxine binding globulin (TBG): demonstration, ontogenesis, and UP-regulation in experimental hypothyroidism. *Biochem Biophys Res Commun.* (1990) 167:317–22. doi: 10.1016/0006-291X(90)91767-M
14. Vranckx R, Savu L, Maya M, Nunez EA. Characterization of a major development-regulated serum thyroxine-binding globulin in the euthyroid mouse. *Biochem J.* (1990) 271:373–9. doi: 10.1042/bj2710373
15. Benvenga S, Cahnmann HJ, Robbins J. Localization of the thyroxine binding sites in apolipoprotein B-100 of human low density lipoproteins. *Endocrinology.* (1990) 127:2241–6. doi: 10.1210/endo-127-5-2241
16. Benvenga S, Lapa D, Trimarchi F. Re-evaluation of the thyroxine binding to human plasma lipoproteins using three techniques. *J Endocrinol Invest.* (2001) 24:RC16–8. doi: 10.1007/BF03343863

17. Bernal J, Guadaño-Ferraz A, Morte B. Thyroid hormone transporters—functions and clinical implications. *Nat Rev Endocrinol.* (2015) 11:1–12. doi: 10.1038/nrendo.2015.66
18. Morte B, Bernal J. Thyroid hormone action: astrocyte-neuron communication. *Front Endocrinol.* (2014) 5:82. doi: 10.3389/fendo.2014.00082
19. Chanoine JP, Alex S, Fang SL, Stone S, Leonard JL, Korhle J, et al. Role of transthyretin in the transport of thyroxine from the blood to the choroid plexus, the cerebrospinal fluid, and the brain. *Endocrinology.* (1992) 130:933–8. doi: 10.1210/endo.130.2.1733735
20. Richardson SJ, Wijayagunaratne RC, D'Souza DG, Darras VM, Van Herck SLJ. Transport of thyroid hormones via the choroid plexus into the brain: the roles of transthyretin and thyroid hormone transmembrane transporters. *Front. Neurosci.* (2015) 9:66. doi: 10.3389/fnins.2015.00066
21. Friesema EC, Ganguly S, Abdalla A, Manning Fox JE, Halestrap AP, Visser TJ. Identification of monocarboxylate transporter 8 as a specific thyroid hormone transporter. *J Biol Chem.* (2003) 278:40128–35. doi: 10.1074/jbc.M300909200
22. Friesema EC, Jansen J, Milici C, Visser TJ. Thyroid hormone transporters. *Vitam Horm.* (2005) 70:137–67. doi: 10.1016/S0083-6729(05)70005-4
23. Hagenbuch B. Cellular entry of thyroid hormones by organic anion transporting polypeptides. *Best Pract Res Clin Endocrinol Metab.* (2007) 21:209–21. doi: 10.1016/j.beem.2007.03.004
24. Halestrap AP. The monocarboxylate transporter family-Structure and functional characterization. *IUBMB Life.* (2012) 64:1–9. doi: 10.1002/iub.573
25. Halestrap AP, Wilson MC. The monocarboxylate transporter family-Role and regulation. *IUBMB Life.* (2012) 64:109–19. doi: 10.1002/iub.572
26. Jansen J, Friesema EC, Milici C, Visser TJ. *Thyroid* Hormone Transporters in Health and Disease. *Thyroid.* (2005) 15:757–68. doi: 10.1089/thy.2005.15.757
27. Kinne A, Schülein R, Krause G. Primary and secondary thyroid hormone transporters. *Thyroid Res.* (2011) 4:S7. doi: 10.1186/1756-6614-4-S1-S7
28. Roberts LM, Woodford K, Zhou M, Black DS, Haggerty JE, Tate EH, et al. Expression of the thyroid hormone transporters monocarboxylate transporter-8 (*SLC16A2*) and organic ion transporter-14 (*SLCO1C1*) at the blood-brain barrier. *Endocrinology.* (2008) 149:6251–61. doi: 10.1210/en.2008-0378
29. Van Der Deure WM, Peeters RP, Visser TJ. Molecular aspects of thyroid hormone transporters, including MCT8, MCT10, and OATPs, and the effects of genetic variation in these transporters. *J Mol Endocrinol.* (2010) 44:1–11. doi: 10.1677/JME-09-0042
30. Visser WE, Friesema EC, Jansen J, Visser TJ. Thyroid hormone transport by monocarboxylate transporters. *Best Pract Res Clin Endocrinol Metab.* (2007) 21:223–36. doi: 10.1016/j.beem.2007.03.008
31. Visser WE, Friesema EC, Visser TJ. Minireview: thyroid hormone transporters: the knowns and the unknowns. *Mol Endocrinol.* (2011) 25:1–14. doi: 10.1210/me.2010-0095
32. Trajkovic M, Visser TJ, Mittag J, Horn S, Lukas J, Darras VM, et al. Abnormal thyroid hormone metabolism in mice lacking the monocarboxylate transporter 8. *J Clin Invest.* (2007) 117:627–35. doi: 10.1172/JCI28253
33. Friesema EC, Jansen J, Jachtenberg J, Visser WE, Kester MHA, Visser TJ. Effective cellular uptake and efflux of thyroid hormone by human monocarboxylate transporter 10. *Mol Endocrinol.* (2008) 22:1357–69. doi: 10.1210/me.2007-0112
34. Zhang Y, Chen K, Sloan SA, Bennett ML, Scholze AR, O'Keeffe S, et al. An RNA-sequencing transcriptome and splicing database of glia, neurons, and vascular cells of the cerebral cortex. *J Neurosci.* (2014) 34:11929–47. doi: 10.1523/JNEUROSCI.1860-14.2014
35. Mathiisen TM, Lehre KP, Danbolt NC, Ottersen OP. The perivascular astroglial sheath provides a complete covering of the brain microvessels: an electron microscopic 3D reconstruction. *Glia.* (2010) 58:1094–103. doi: 10.1002/glia.20990
36. Schroeder AC, Privalsky ML. Thyroid hormones, T3 and T4, in the brain. *Front Endocrinol.* (2014) 5:40. doi: 10.3389/fendo.2014.00040
37. Dumitrescu AM, Liao XH, Best TB, Brockmann K, Refetoff S. A novel syndrome combining thyroid and neurological abnormalities is associated with mutations in a monocarboxylate transporter gene. *Am J Hum Genet.* (2004) 74:168–75. doi: 10.1086/380999
38. Friesema EC, Grueters A, Biebermann H, Krude H, Von Moers A, Reeser M, et al. Association between mutations in a thyroid hormone transporter and severe X-linked psychomotor retardation. *Lancet.* (2004) 364:1435–7. doi: 10.1016/S0140-6736(04)17226-7
39. López-Espíndola D, Morales-Bastos C, Grijota-Martínez C, Liao XH, Lev D, Sugo E, et al. Mutations of the thyroid hormone transporter MCT8 cause prenatal brain damage and persistent hypomyelination. *J Clin Endocrinol Metab.* (2014) 99:E2799–E2804. doi: 10.1210/jc.2014-2162
40. Schwartz CE, May MM, Carpenter NJ, Rogers RC, Martin J, Bialer MG, et al. Allan-Herndon-Dudley syndrome and the monocarboxylate transporter 8 (MCT8) gene. *Am J Hum Genet.* (2005) 77:41–53. doi: 10.1086/431313
41. Kim DK, Kanai Y, Chairoungdua A, Matsuo H, Cha SH, Endou H. Expression cloning of a Na+-independent aromatic amino acid transporter with structural similarity to H+/monocarboxylate transporters. *J Biol Chem.* (2001) 276:17221–8. doi: 10.1074/jbc.M009462200
42. Bianco AC. Minireview: cracking the metabolic code for thyroid hormone signaling. *Endocrinology.* (2011) 152:3306–11. doi: 10.1210/en.2011-1104
43. Bianco AC, Kim BW. Deiodinases: implications of the local control of thyroid hormone action. *J Clin Invest.* (2006) 116:2571–9. doi: 10.1172/JCI29812
44. Dentice M, Marsili A, Zavacki A, Larsen PR, Salvatore D. The deiodinases and the control of intracellular thyroid hormone signaling during cellular differentiation. *Biochim Biophys Acta Gen Subj.* (2013) 1830:3937–45. doi: 10.1016/j.bbagen.2012.05.007
45. Tu HM, Kim SW, Salvatore D, Bartha T, Legradi G, Larsen PR, et al. Regional distribution of type 2 thyroxine deiodinase messenger ribonucleic acid in rat hypothalamus and pituitary and its regulation by thyroid hormone. *Endocrinology.* (1997) 138:3359–68. doi: 10.1210/endo.138.8.5318
46. Freitas BC, Gereben B, Castillo M, Kalló I, Zeöld A, Egri P, et al. Paracrine signaling by glial cell-derived triiodothyronine activates neuronal gene expression in the rodent brain and human cells. *J Clin Invest.* (2010) 120:2206–17. doi: 10.1172/JCI41977
47. Gereben B, Zavacki AM, Ribich S, Kim BW, Huang SA, Simonides WS, et al. Cellular and molecular basis of deiodinase-regulated thyroid hormone signaling. *Endocr Rev.* (2008) 29:898–938. doi: 10.1210/er.2008-0019
48. Tu HM, Legradi G, Bartha T, Salvatore D, Lechan RM, Larsen PR. Regional expression of the type 3 iodothyronine deiodinase messenger ribonucleic acid in the rat central nervous system and its regulation by thyroid hormone. *Endocrinology.* (1999) 140:784–90. doi: 10.1210/endo.140.2.6486
49. Dumitrescu AM, Liao XH, Weiss RE, Millen K, Refetoff S. Tissue-specific thyroid hormone deprivation and excess in monocarboxylate transporter (Mct) 8-deficient mice. *Endocrinology.* (2006) 147:4036–43. doi: 10.1210/en.2006-0390
50. Guadaño-Ferraz A, Obregón MJ, St Germain DL, Bernal J, Obregon MJ, StGermain DL. The type 2 iodothyronine deiodinase is expressed primarily in glial cells in the neonatal rat brain. *Proc Natl Acad Sci USA.* (1997) 94:10391–6. doi: 10.1073/pnas.94.19.10391
51. Cramer SC, Chopp M. Recovery recapitulates ontogeny. *Trends Neurosci.* (2000) 23:265–71. doi: 10.1016/S0166-2236(00)01562-9
52. Stepien BK, Huttner WB. Transport, metabolism, and function of thyroid hormones in the developing mammalian brain. *Front Endocrinol.* (2019) 10:209. doi: 10.3389/fendo.2019.00209
53. Anderson GW, Schoonover CM, Jones SA. Control of thyroid hormone action in the developing rat brain. *Thyroid.* (2003) 13:1039–56. doi: 10.1089/105072503770867219
54. Clancy B, Finlay BL, Darlington RB, Anand KJ. Extrapolating brain development from experimental species to humans. *Neurotoxicology.* (2007) 28:931–7. doi: 10.1016/j.neuro.2007.01.014
55. Semple BD, Blomgren K, Gimlin K, Ferriero DM, Noble-Haeusslein LJ. Brain development in rodents and humans: identifying benchmarks of maturation and vulnerability to injury across species. *Prog Neurobiol.* (2013) 106–107:1–16. doi: 10.1016/j.pneurobio.2013.04.001
56. Berbel P, Mestre JL, Santamaría A, Palazón I, Franco A, Graells M, et al. Delayed neurobehavioral development in children born to pregnant women with mild hypothyroxinemia during the first month of gestation: the importance of early iodine supplementation. *Thyroid.* (2009) 19:511–9. doi: 10.1089/thy.2008.0341

57. Carreón-Rodríguez A, Perez-Martínez L. Clinical implications of thyroid hormones effects on nervous system development. *Pediatr Endocrinol Rev.* (2012) 9:644–9.
58. Downing S, Halpern L, Carswell J, Brown RS. Severe maternal hypothyroidism corrected prior to the third trimester is associated with normal cognitive outcome in the offspring. *Thyroid.* (2012) 22:625–30. doi: 10.1089/thy.2011.0257
59. Contempré B, Jauniaux E, Calvo R, Jurkovic D, Campbell S, de Escobar GM. Detection of thyroid hormones in human embryonic cavities during the first trimester of pregnancy. *J Clin Endocrinol Metab.* (1993) 77:1719–22. doi: 10.1210/jcem.77.6.8263162
60. Obregon M, Calvo R, Escobar Del Rey F, Morreale De Escobar G. Ontogenesis of thyroid function and interactions with maternal function. *Endocr Dev.* (2007) 10:86–98. doi: 10.1159/000106821
61. Obregon MJ, Mallol J, Pastor R, Morreale de Escobar G, Escobar Del Rey F. L-thyroxine and 3,5,3'-triiodo-L-thyronine in rat embryos before onset of fetal thyroid function. *Endocrinology.* (1984) 114:305–7. doi: 10.1210/endo-114-1-305
62. Porterfield SP, Hendrich CE. Tissue iodothyronine levels in fetuses of control and hypothyroid rats at 13 and 16 days gestation. *Endocrinology.* (1992) 131:195–200. doi: 10.1210/endo.131.1.1611997
63. Woods RJ, Sinha AK, Ekins RP. Uptake and metabolism of thyroid hormones by the rat foetus in early pregnancy. *Clin Sci.* (1984) 67:359–63.
64. Morreale De Escobar GMD, Pastor R, Obregon MJ, Del Rey FE. Effects of maternal hypothyroidism on the weight and thyroid hormone content of rat embryonic tissues, before and after onset of fetal thyroid function. *Endocrinology.* (1985) 117:1890–900. doi: 10.1210/endo-117-5-1890
65. Ausó E, Lavado-Autric R, Cuevas E, Escobar Del Rey F, Morreale De Escobar G, Berbel P. A moderate and transient deficiency of maternal thyroid function at the beginning of fetal neocorticogenesis alters neuronal migration. *Endocrinology.* (2004) 145:4037–47. doi: 10.1210/en.2004-0274
66. Gilbert ME, Goodman JH, Gomez J, Johnstone AFM, Ramos RL. Adult hippocampal neurogenesis is impaired by transient and moderate developmental thyroid hormone disruption. *Neurotoxicology.* (2017) 59:9–21. doi: 10.1016/j.neuro.2016.12.009
67. Mohan V, Sinha RA, Pathak A, Rastogi L, Kumar P, Pal A, et al. Maternal thyroid hormone deficiency affects the fetal neocorticogenesis by reducing the proliferating pool, rate of neurogenesis and indirect neurogenesis. *Exp Neurol.* (2012) 237:477–88. doi: 10.1016/j.expneurol.2012.07.019
68. Bernal J. Thyroid hormone receptors in brain development and function. *Nat Clin Pract Endocrinol Metab.* (2007) 3:249–59. doi: 10.1038/ncpendmet0424
69. Bernal J, Pekonen F. Ontogenesis of the nuclear 3,5,3'-triiodothyronine receptor in the human fetal brain. *Endocrinology.* (1984) 114:677–9. doi: 10.1210/endo-114-2-677
70. Kilby MD, Gittoes N, McCabe C, Verhaeg J, Franklyn JA. Expression of thyroid receptor isoforms in the human fetal central nervous system and the effects of intrauterine growth restriction. *Clin Endocrinol.* (2000) 53:469–77. doi: 10.1046/j.1365-2265.2000.01074.x
71. Perez-Castillo A, Bernal J, Ferreiro B, Pans T. The early ontogenesis of thyroid hormone receptor in the rat fetus. *Endocrinology.* (1985) 117:2457–61. doi: 10.1210/endo-117-6-2457
72. Chatonnet F, Flamant F, Morte B. A temporary compendium of thyroid hormone target genes in brain. *Biochim Biophys Acta Gene Regul Mech.* (2015) 1849:122–9. doi: 10.1016/j.bbagrm.2014.05.023
73. Horn S, Heuer H. Thyroid hormone action during brain development: more questions than answers. *Mol Cell Endocrinol.* (2010) 315:19–26. doi: 10.1016/j.mce.2009.09.008
74. Sampson D, Pickard MR, Sinha AK, Evans IM, Leonard AJ, Ekins RP. Maternal thyroid status regulates the expression of neuronal and astrocytic cytoskeletal proteins in the fetal brain. *J Endocrinol.* (2000) 167:439–45. doi: 10.1677/joe.0.1670439
75. Bochukova E, Schoenmakers N, Agostini M, Schoenmakers E, Rajanayagam O, Keogh JM, et al. A mutation in the thyroid hormone receptor alpha gene. *N Engl J Med.* (2012) 366:243–9. doi: 10.1056/NEJMoa1110296
76. Forrest D, Golarai G, Connor J, Curran, T. Genetic analysis of thyroid hormone receptors in development and disease. *Recent Prog Horm Res.* (1996) 51:1–22.
77. Jones I, Srinivas M, Ng L, Forrest D. The thyroid hormone receptor beta gene: structure and functions in the brain and sensory systems. *Thyroid.* (2003) 13:1057–68. doi: 10.1089/105072503770867228
78. Refetoff S, Dumitrescu AM. Syndromes of reduced sensitivity to thyroid hormone: genetic defects in hormone receptors, cell transporters and deiodination. *Best Pract Res Clin Endocrinol Metab.* (2007) 21:277–305. doi: 10.1016/j.beem.2007.03.005
79. van Mullem AA, Visser TJ, Peeters RP. Clinical consequences of mutations in thyroid hormone receptor-alpha1. *Eur Thyroid J.* (2014) 3:17–24. doi: 10.1159/000360637
80. Bradley DJ, Towle HC, Young WS. Spatial and temporal expression of alpha- and beta-thyroid hormone receptor mRNAs, including the beta 2-subtype, in the developing mammalian nervous system. *J Neurosci.* (1992) 12:2288–302.
81. Forrest D, Hallböök F, Persson H, Vennström B. Distinct functions for thyroid hormone receptors alpha and beta in brain development indicated by differential expression of receptor genes. *EMBO J.* (1991) 10:269–75.
82. Iskaros J, Pickard M, Evans I, Sinha A, Hardiman P, Ekins R. Thyroid hormone receptor gene expression in first trimester human fetal brain. *J Clin Endocrinol Metab.* (2000) 85:2620–3. doi: 10.1210/jcem.85.7.6766
83. Wallis K, Dudazy S, van Hogerlinden M, Nordström K, Mittag J, Vennström B. The thyroid hormone receptor α1 protein is expressed in embryonic postmitotic neurons and persists in most adult neurons. *Mol Endocrinol.* (2010) 24:1904–16. doi: 10.1210/me.2010-0175
84. Hodin RA, Lazar MA, Chin WW. Differential and tissue-specific regulation of the multiple rat c-erbA messenger RNA species by thyroid hormone. *J Clin Invest.* (1990) 85:101–5. doi: 10.1172/JCI114398
85. Sinha R, Yen PM. Cellular action of thyroid hormone. In: Feingold KR, Anawalt B, Boyce A, editors. *Endotext.* South Dartmouth, MA: MDTextcom, Inc. (2013). p. 1–27. doi: 10.1089/105072502760143827
86. Teng X, Lin YY, Teng W, Brent GA. COUP-TF1 modulates thyroid hormone action in an embryonic stem-cell model of cortical pyramidal neuronal differentiation. *Thyroid.* (2018) 28:667–78. doi: 10.1089/thy.2017.0256
87. Faedo A, Tomassy GS, Ruan Y, Teichmann H, Krauss S, Pleasure SJ, et al. COUP-TFI coordinates cortical patterning, neurogenesis, and laminar fate and modulates MAPK/ERK, AKT, and beta-catenin signaling. *Cereb Cortex.* (2008) 18:2117–31. doi: 10.1093/cercor/bhm238
88. Cheng SY, Leonard JL, Davis PJ. Molecular aspects of thyroid hormone actions. *Endocr Rev.* (2010) 31:139–70. doi: 10.1210/er.2009-0007
89. Yen PM. Physiological and molecular basis of thyroid hormone action. *Physiol Rev.* (2001) 81:1097–142. doi: 10.1152/physrev.2001.81.3.1097
90. Bernal J. Action of thyroid hormone in brain. *J Endocrinol Invest.* (2002) 25:268–88. doi: 10.1007/BF03344003
91. Harvey CB, Williams GR. Mechanism of thyroid hormone action. *Thyroid.* (2002) 12:441–6. doi: 10.1089/105072502760143791
92. McKenna NJ, Lanz RB, O'Malley BW. Nuclear receptor coregulators: cellular and molecular biology. *Endocr Rev.* (1999) 20:321–44. doi: 10.1210/edrv.20.3.0366
93. Brent GA. Mechanisms of thyroid hormone action. *J Clin Invest.* (2012) 122:3035–43. doi: 10.1172/JCI60047
94. Flamant F, Gauthier K. Thyroid hormone receptors: the challenge of elucidating isotype-specific functions and cell-specific response. *Biochim Biophys Acta Gen Subj.* (2013) 1830:3900–7. doi: 10.1016/j.bbagen.2012.06.003
95. Chan IH, Privalsky ML. Isoform-specific transcriptional activity of overlapping target genes that respond to thyroid hormone receptors alpha1 and beta1. *Mol Endocrinol.* (2009) 23:1758–75. doi: 10.1210/me.2009-0025
96. Schwartz HL, Strait KA, Ling NC, Oppenheimer JH. Quantitation of rat tissue thyroid hormone binding receptor isoforms by immunoprecipitation of nuclear triiodothyronine binding capacity. *J Biol Chem.* (1992) 267:11794–9.
97. Gil-Ibañez P, Morte B, Bernal J. Role of thyroid hormone receptor subtypes α and β on gene expression in the cerebral cortex and striatum of postnatal mice. *Endocrinology.* (2013) 154:1940–7. doi: 10.1210/en.2012-2189
98. Constantinou C, Margarity M, Valcana T. Region-specific effects of hypothyroidism on the relative expression of thyroid hormone receptors in adult rat brain. *Mol Cell Biochem.* (2005) 278:93–100. doi: 10.1007/s11010-005-6934-z

99. Carlson DJ, Strait KA, Schwartz HL, Oppenheimer JH. Immunofluorescent localization of thyroid hormone receptor isoforms in glial cells of rat brain. *Endocrinology.* (1994) 135:1831–6. doi: 10.1210/endo.135.5.7525253
100. Mohácsik P, Zeöld A, Bianco AC, Gereben B. Thyroid hormone and the neuroglia: Both source and target. *J Thyroid Res.* (2011) 2011:16. doi: 10.4061/2011/215718
101. Yusta B, Besnard F, Ortiz-Caro J, Pascual A, Aranda A, Sarlieve L. Evidence for the presence of nuclear 3,5,3'- triiodothyronine receptors in secondary cultures of pure rat oligodendrocytes. *Endocrinology.* (1988) 122:2278–84. doi: 10.1210/endo-122-5-2278
102. Bradley DJ, Young WS, Weinberger C. Differential expression of alpha and beta thyroid hormone receptor genes in rat brain and pituitary. *Proc Natl Acad Sci USA.* (1989) 86:7250–4. doi: 10.1073/pnas.86.18.7250
103. Cook CB, Kakucska I, Lechan RM, Koenig RJ. Expression of thyroid hormone receptor beta 2 in rat hypothalamus. *Endocrinology.* (1992) 130:1077–9. doi: 10.1210/endo.130.2.1733708
104. Lechan RM, Qi Y, Berrodin TJ, Davis KD, Schwartz HL, Strait KA, et al. Immunocytochemical delineation of thyroid hormone receptor beta 2-like immunoreactivity in the rat central nervous system. *Endocrinology.* (1993) 132:2461–9. doi: 10.1210/endo.132.6.7684976
105. Williams GR. Cloning and characterization of two novel thyroid hormone receptor beta isoforms. *Mol Cell Biol.* (2000) 20:8329–42. doi: 10.1128/MCB.20.22.8329-8342.2000
106. Anyetei-Anum CS, Roggero VR, Allison LA. Thyroid hormone receptor localization in target tissues. *J Endocrinol.* (2018) 237:R19–34. doi: 10.1530/JOE-17-0708
107. Li J, Donangelo I, Abe K, Scremin O, Ke S, Li F, et al. Thyroid hormone treatment activates protective pathways in both *in vivo* and *in vitro* models of neuronal injury. *Mol Cell Endocrinol.* (2017) 452:120–30. doi: 10.1016/j.mce.2017.05.023
108. Mdzinarishvili A, Sutariya V, Talasila PK, Geldenhuys WJ, Sadana P. Engineering triiodothyronine (T3) nanoparticle for use in ischemic brain stroke. *Drug Deliv Transl Res.* (2013) 3:309–17. doi: 10.1007/s13346-012-0117-8
109. Sadana P, Coughlin L, Burke J, Woods R, Mdzinarishvili A. Anti-edema action of thyroid hormone in MCAO model of ischemic brain stroke: possible association with AQP4 modulation. *J Neurol Sci.* (2015) 354:37–45. doi: 10.1016/j.jns.2015.04.042
110. Shulga A, Blaesse A, Kysenius K, Huttunen HJ, Tanhuanpää K, Saarma M, et al. Thyroxin regulates BDNF expression to promote survival of injured neurons. *Mol Cell Neurosci.* (2009) 42:408–18. doi: 10.1016/j.mcn.2009.09.002
111. De Arrieta CM, Morte B, Coloma A, Bernal J. The human RC3 gene homolog, NRGN contains a thyroid hormone-responsive element located in the first intron. *Endocrinology.* (1999) 140:335–43. doi: 10.1210/en.140.1.335
112. Iniguez MA, De Lecea L, Guadano-Ferraz A, Morte B, Gerendasy D, Sutcliffe JG, et al. Cell-specific effects of thyroid hormone on RC3/neurogranin expression in rat brain. *Endocrinology.* (1996) 137:1032–41. doi: 10.1210/endo.137.3.8603571
113. Miyakawa T, Yared E, Pak JH, Huang FL, Huang KP, Crawley JN. Neurogranin null mutant mice display performance deficits on spatial learning tasks with anxiety related components. *Hippocampus.* (2001) 11:763–75. doi: 10.1002/hipo.1092
114. Krebs J, Honegger P. Calmodulin kinase IV: Expression and function during rat brain development. *Biochim Biophys Acta Mol Cell Res.* (1996) 1313:217–22. doi: 10.1016/0167-4889(96)00092-4
115. Liu YY, Brent GA. Thyroid hormone-dependent gene expression in differentiated embryonic stem cells and embryonal carcinoma cells: identification of novel thyroid hormone target genes by deoxyribonucleic acid microarray analysis. *Endocrinology.* (2005) 146:776–83. doi: 10.1210/en.2004-1177
116. Morte B, Díez D, Ausó E, Belinchón MM, Gil-Ibáñez P, Grijota-Martínez C, et al. Thyroid hormone regulation of gene expression in the developing rat fetal cerebral cortex: prominent role of the Ca2+/calmodulin-dependent protein kinase IV pathway. *Endocrinology.* (2010) 151:810–20. doi: 10.1210/en.2009-0958
117. Navarro D, Alvarado M, Morte B, Berbel D, Sesma J, Pacheco P, et al. Late maternal hypothyroidism alters the expression of CAMK4 in neocortical subplate neurons: a comparison with nurr1 labeling. *Cereb Cortex.* (2014) 24:2694–706. doi: 10.1093/cercor/bht129
118. Alvarez-Dolado M, Ruiz M, Del Río JA, Alcántara S, Burgaya F, Sheldon M, et al. Thyroid hormone regulates reelin and dab1 expression during brain development. *J. Neurosci.* (1999) 19:6979–93.
119. Herz J, Chen Y. Reelin, lipoprotein receptors and synaptic plasticity. *Nat Rev Neurosci.* (2006) 7:850–9. doi: 10.1038/nrn2009
120. Jossin Y, Cooper JA. Reelin, Rap1 and N-cadherin orient the migration of multipolar neurons in the developing neocortex. *Nat Neurosci.* (2011) 14:697–703. doi: 10.1038/nn.2816
121. Thompson CC, Potter GB. Thyroid hormone action in neural development. *Cereb Cortex.* (2000) 10:939–45. doi: 10.1093/cercor/10.10.939
122. Morte B, Ceballos A, Diez D, Grijota-Martínez C, Dumitrescu AM, Di Cosmo C, et al. Thyroid hormone-regulated mouse cerebral cortex genes are differentially dependent on the source of the hormone: a study in monocarboxylate transporter-8- and deiodinase-2-deficient mice. *Endocrinology.* (2010) 151:2381–7. doi: 10.1210/en.2009-0944
123. Morte B, Gil-Ibáñez P, Bernal J. Regulation of gene expression by thyroid hormone in primary astrocytes: factors influencing the genomic response. *Endocrinology.* (2018) 159:2083–92. doi: 10.1210/en.2017-03084
124. Davis FB, Mousa SA, O'Connor L, Mohamed S, Lin HY, Cao HJ, et al. Proangiogenic action of thyroid hormone is fibroblast growth factor-dependent and is initiated at the cell surface. *Circ Res.* (2004) 94:1500–6. doi: 10.1161/01.RES.0000130784.90237.4a
125. Liu X, Zheng N, Shi YN, Yuan J, Li L. Thyroid hormone induced angiogenesis through the integrin αvβ3/protein kinase D/histone deacetylase 5 signaling pathway. *J Mol Endocrinol.* (2014) 52:245–54. doi: 10.1530/JME-13-0252
126. Luidens MK, Mousa SA, Davis FB, Lin HY, Davis PJ. Thyroid hormone and angiogenesis. *Vascul Pharmacol.* (2010) 52:142–5. doi: 10.1016/j.vph.2009.10.007
127. Davis PJ, Leonard JL, Davis FB. Mechanisms of nongenomic actions of thyroid hormone. *Front Neuroendocrinol.* (2008) 29:211–8. doi: 10.1016/j.yfrne.2007.09.003
128. Davis PJ, Goglia F, Leonard JL. Nongenomic actions of thyroid hormone. *Nat Rev Endocrinol.* (2016) 12:111–21. doi: 10.1038/nrendo.2015.205
129. Hiroi Y, Kim HH, Ying H, Furuya F, Huang Z, Simoncini T, et al. Rapid nongenomic actions of thyroid hormone. *Proc Natl Acad Sci USA.* (2006) 103:14104–9. doi: 10.1073/pnas.0601600103
130. Osmak-Tizon L, Poussier M, Cottin Y, Rochette L. Non-genomic actions of thyroid hormones: molecular aspects. *Arch Cardiovasc Dis.* (2014) 107:207–11. doi: 10.1016/j.acvd.2014.02.001
131. Cao X, Kambe F, Moeller LC, Refetoff S, Seo H. Thyroid hormone induces rapid activation of Akt/protein kinase B-mammalian target of rapamycin-p70S6K cascade through phosphatidylinositol 3-kinase in human fibroblasts. *Mol Endocrinol.* (2005) 19:102–12. doi: 10.1210/me.2004-0093
132. Furuya F, Guigon CJ, Zhao L, Lu C, Hanover JA, Cheng S. Nuclear receptor corepressor is a novel regulator of phosphatidylinositol 3-kinase signaling. *Mol Cell Biol.* (2007) 27:6116–26. doi: 10.1128/MCB.00900-07
133. Lin HY, Sun M, Tang HY, Lin C, Luidens MK, Mousa SA, et al. L-Thyroxine vs. 3,5,3'-triiodo-L-thyronine and cell proliferation: activation of mitogen-activated protein kinase and phosphatidylinositol 3-kinase. *Am J Physiol Cell Physiol.* (2009) 296:C980–91. doi: 10.1152/ajpcell.00305.2008
134. Martin NP, Marron De Velasco E, Mizuno F, Scappini EL, Gloss B, Erxleben C, et al. A rapid cytoplasmic mechanism for PI3 kinase regulation by the nuclear thyroid hormone receptor, TRβ, and genetic evidence for its role in the maturation of mouse hippocampal synapses *in vivo*. *Endocrinology.* (2014) 155:3713–24. doi: 10.1210/en.2013-2058
135. Moeller LC, Cao X, Dumitrescu AM, Seo H, Refetoff S. Thyroid hormone mediated changes in gene expression can be initiated by cytosolic action of the thyroid hormone receptor beta through the phosphatidylinositol 3-kinase pathway. *Nucl Recept Signal.* (2006) 4:e020. doi: 10.1621/nrs.04020
136. Storey NM, Gentile S, Ullah H, Russo A, Muessel M, Erxleben C, et al. Rapid signaling at the plasma membrane by a nuclear receptor for thyroid hormone. *Proc Natl Acad Sci USA.* (2006) 103:5197–201. doi: 10.1073/pnas.0600089103
137. Bergh JJ, Lin HY, Lansing L, Mohamed SN, Davis FB, Mousa S, et al. Integrin alphaVbeta3 contains a cell surface receptor site for thyroid hormone that

is linked to activation of mitogen-activated protein kinase and induction of angiogenesis. *Endocrinology.* (2005) 146:2864–71. doi: 10.1210/en.2005-0102
138. Davis PJ, Davis FB, Cody V. Membrane receptors mediating thyroid hormone action. *Trends Endocrinol Metab.* (2005) 16:429–35. doi: 10.1016/j.tem.2005.09.007
139. Davis PJ, Davis FB, Mousa SA, Luidens MK, Lin HY. Membrane receptor for thyroid hormone: physiologic and pharmacologic implications. *Annu Rev Pharmacol Toxicol.* (2011) 51:99–115. doi: 10.1146/annurev-pharmtox-010510-100512
140. Kalyanaraman H, Schwappacher R, Joshua J, Zhuang S, Scott BT, Klos M, et al. Nongenomic thyroid hormone signaling occurs through a plasma membrane-localized receptor. *Sci Signal.* (2014) 7:ra48. doi: 10.1126/scisignal.2004911
141. Cao HJ, Lin HY, Luidens MK, Davis FB, Davis PJ. Cytoplasm-to-nucleus shuttling of thyroid hormone receptor-β1 (TRβ1) is directed from a plasma membrane integrin receptor by thyroid hormone. *Endocr Res.* (2009) 34:31–42. doi: 10.1080/07435800902911810
142. Lin HY, Tang HY, Shih A, Keating T, Cao G, Davis PJ, et al. Thyroid hormone is a MAPK-dependent growth factor for thyroid cancer cells and is anti-apoptotic. *Steroids.* (2007) 72:180–7. doi: 10.1016/j.steroids.2006.11.014
143. Lin HY, Martino LJ, Wilcox BD, Davis FB, Gordinier JK, Davis PJ. Potentiation by thyroid hormone of human IFN-gamma-induced HLA-DR expression. *J Immunol.* (1998) 161:843–9.
144. Chen Y, Chen PL, Chen CF, Sharp ZD, Lee WH. Thyroid hormone, T3-dependent phosphorylation and translocation of Trip230 from the Golgi complex to the nucleus. *Proc Natl Acad Sci USA.* (1999) 96:4443–8. doi: 10.1073/pnas.96.8.4443
145. Davis PJ, Shih A, Lin HY, Martino LJ, Davis FB. Thyroxine promotes association of mitogen-activated protein kinase and nuclear thyroid hormone receptor (TR) and causes serine phosphorylation of TR. *J Biol Chem.* (2000) 275:38032–9. doi: 10.1074/jbc.M002560200
146. Lin HY, Davis FB, Gordinier JK, Martino LJ, Davis PJ. Thyroid hormone induces activation of mitogen-activated protein kinase in cultured cells. *Am J Physiol Physiol.* (1999) 276:C1014–24. doi: 10.1152/ajpcell.1999.276.5.C1014
147. Lin HY, Shih A, Davis FB, Davis PJ. Thyroid hormone promotes the phosphorylation of STAT3 and potentiates the action of epidermal growth factor in cultured cells. *Biochem J.* (1999) 338(Pt 2):427–32. doi: 10.1042/0264-6021:3380427
148. Shih A, Lin HY, Davis FB, Davis PJ. Thyroid hormone promotes serine phosphorylation of p53 by mitogen-activated protein kinase. *Biochemistry.* (2001) 40:2870–8. doi: 10.1021/bi001978b
149. Incerpi S, Luly P, De Vito P, Farias RN. Short-term effects of thyroid hormones on the Na/H antiport in L-6 myoblasts: high molecular specificity for 3,3',5-triiodo-L-thyronine. *Endocrinology.* (1999) 140:683–9. doi: 10.1210/endo.140.2.6535
150. Bhargava M, Lei J, Mariash CN, Ingbar DH. Thyroid hormone rapidly stimulates alveolar Na,K-ATPase by activation of phosphatidylinositol 3-kinase. *Curr Opin Endocrinol Diabetes Obes.* (2007) 14:416–20. doi: 10.1097/MED.0b013e3282f02ae8
151. Lei J, Mariash CN, Ingbar DH. 3,3',5-Triiodo-L-thyronine up-regulation of Na,K-ATPase activity and cell surface expression in alveolar epithelial cells is Src kinase- and phosphoinositide 3-kinase-dependent. *J Biol Chem.* (2004) 279:47589–600. doi: 10.1074/jbc.M405497200
152. Lei J, Mariash CN, Bhargava M, Wattenberg EV, Ingbar DH. T3 increases Na-K-ATPase activity via a MAPK/ERK1/2-dependent pathway in rat adult alveolar epithelial cells. *Am J Physiol Lung Cell Mol Physiol.* (2008) 294:L749–54. doi: 10.1152/ajplung.00335.2007
153. Scapin S, Leoni S, Spagnuolo S, Fiore AM, Incerpi S. Short-term effects of thyroid hormones on Na+-K+-ATPase activity of chick embryo hepatocytes during development: focus on signal transduction. *Am J Physiol Cell Physiol.* (2009) 296:C4–12. doi: 10.1152/ajpcell.90604.2007
154. Sarkar PK, Biswas A, Ray AK, Martin, JV. Mechanisms of L-triiodothyronine-induced inhibition of synaptosomal na(+)-k(+)-ATPase activity in young adult rat brain cerebral cortex. *J Thyroid Res.* (2013) 2013:457953. doi: 10.1155/2013/457953
155. Sarkar PK, Ray AK. Specific binding of L-triiodothyronine modulates Na+-K+-ATPase activity in adult rat cerebrocortical synaptosomes. *Neuroreport.* (1998) 9:1149–52.
156. Davis PJ, Zhou M, Davis FB, Lansing L, Mousa SA, Lin HY. Mini-review: cell surface receptor for thyroid hormone and nongenomic regulation of ion fluxes in excitable cells. *Physiol Behav.* (2010) 99:237–9. doi: 10.1016/j.physbeh.2009.02.015
157. Kahaly GJ, Dillmann WH. Thyroid hormone action in the heart. *Endocr Rev.* (2005) 26:704–28. doi: 10.1210/er.2003-0033
158. Chakrabarti N, Ray, AK. Stimulation of Ca2+/Mg2+-ATPase activity in adult rat cerebrocortical synaptosomes by 3-5-3′-L-triiodothyronine. *Neurosci Res Commun.* (2002) 31:193–201. doi: 10.1002/nrc.10052
159. Farwell AP, Lynch RM, Okulicz WC, Comi AM, Leonard JL. The actin cytoskeleton mediates the hormonally regulated translocation of type II iodothyronine 5'-deiodinase in astrocytes. *J Biol Chem.* (1990) 265:18546–553.
160. Farwell AP, Dubord-tomasetti SA, Pietrzykowski AZ, Stachelek SJ, Leonard JL. Regulation of cerebellar neuronal migration and neurite outgrowth by thyroxine and 3,3′,5′-triiodothyronine. *Dev Brain Res.* (2005) 154:121–35. doi: 10.1016/j.devbrainres.2004.07.016
161. Leonard JL, Farwell AP. *Thyroid.* hormone-regulated actin polymerization in brain. *Thyroid.* (1997) 7:147–51. doi: 10.1089/thy.1997.7.147
162. Siegrist-Kaiser CA, Juge-Aubry C, Tranter MP, Ekenbarger DM, Leonard JL. Thyroxine-dependent modulation of actin polymerization in cultured astrocytes. A novel, extranuclear action of thyroid hormone. *J Biol Chem.* (1990) 265:5296–302.
163. Campos Costa I, Nogueira Carvalho H, Fernandes L. Aging, circadian rhythms and depressive disorders: a review. *Am J Neurodegener Dis.* (2013) 2:228–46.
164. Froy O. Circadian rhythms, aging, and life span in mammals. *Physiology.* (2011) 26:225–35. doi: 10.1152/physiol.00012.2011
165. Morris CJ, Aeschbach D, Scheer FA. Circadian system, sleep and endocrinology. *Mol Cell Endocrinol.* (2012) 349:91–104. doi: 10.1016/j.mce.2011.09.003
166. Bensenor IM, Olmos RD, Lotufo PA. Hypothyroidism in the elderly: diagnosis and management. *Clin Interv Aging.* (2012) 7:97–111. doi: 10.2147/CIA.S23966
167. Chakraborti S, Chakraborti T, Mandal M, Das S, Batabyal SK. Hypothalamic-pituitary-thyroid axis status of humans during development of ageing process. *Clin Chim Acta.* (1999) 288:137–45. doi: 10.1016/S0009-8981(99)00061-3
168. Cao L, Jiang W, Wang F, Yang QG, Wang C, Chen YP, et al. The reduced serum free triiodothyronine and increased dorsal hippocampal SNAP-25 and Munc18-1 had existed in middle-aged CD-1 mice with mild spatial cognitive impairment. *Brain Res.* (2013) 1540:9–20. doi: 10.1016/j.brainres.2013.09.034
169. Visser WE, Bombardieri CR, Zevenbergen C, Barnhoorn S, Ottaviani A, Van Der Pluijm I, et al. Tissue-specific suppression of thyroid hormone signaling in various mouse models of aging. *PLoS ONE.* (2016) 11:1–25. doi: 10.1371/journal.pone.0149941
170. Fu AL, Zhou CY, Chen X. Thyroid hormone prevents cognitive deficit in a mouse model of Alzheimer's disease. *Neuropharmacology.* (2010) 58:722–9. doi: 10.1016/j.neuropharm.2009.12.020
171. Villanueva I, Alva-Sánchez C, Pacheco-Rosado J. The role of thyroid hormones as inductors of oxidative stress and neurodegeneration. *Oxid Med Cell Longev.* (2013) 2013:218145. doi: 10.1155/2013/218145
172. Qureshi AI, Suri FK, Nasar A, Kirmani JF, Divani AA, Giles WH. Free thyroxine index and risk of stroke: results from the National Health and Nutrition Examination Survey Follow-up Study. *Med Sci Monit.* (2006) 12:CR501–6.
173. Remmel KS, Wanahita A, Moore K, Gruenthal M. Acute ischemic stroke and hypothyroidism. *J Ky Med Assoc.* (2006) 104:191–3.
174. Dhital R, Poudel DR, Tachamo N, Gyawali B, Basnet S, Shrestha P, et al. Ischemic stroke and impact of thyroid profile at presentation: a systematic review and meta-analysis of observational studies. *J Stroke Cerebrovasc Dis.* (2017) 26:2926–34. doi: 10.1016/j.jstrokecerebrovasdis.2017.07.015
175. Jiang X, Xing H, Wu J, Du R, Liu H, Chen J, et al. Prognostic value of thyroid hormones in acute ischemic stroke – a meta analysis. *Sci Rep.* (2017) 7:16256. doi: 10.1038/s41598-017-16564-2
176. Lamba N, Liu C, Zaidi H, Broekman MLD, Simjian T, Shi C, et al. A prognostic role for Low tri-iodothyronine syndrome in acute stroke

patients: a systematic review and meta-analysis. *Clin Neurol Neurosurg.* (2018) 169:55–63. doi: 10.1016/j.clineuro.2018.03.025

177. Liu J, Wang D, Xiong Y, Yuan R, Tao W, Liu M. Low free triiodothyronine levels are related to symptomatic intracranial hemorrhage and poor functional outcomes after intravenous thrombolysis in acute ischemic stroke patients. *Neurol Res.* (2016) 38:429–33. doi: 10.1080/01616412.2016.1178480
178. O'Keefe LM, Conway SE, Czap A, Malchoff CD, Benashski S, Fortunato G, et al. Thyroid hormones and functional outcomes after ischemic stroke. *Thyroid Res.* (2015) 8:9. doi: 10.1186/s13044-015-0021-7
179. Suda S, Muraga K, Kanamaru T, Okubo S, Abe A, Aoki J, et al. Low free triiodothyronine predicts poor functional outcome after acute ischemic stroke. *J Neurol Sci.* (2016) 368:89–93. doi: 10.1016/j.jns.2016.06.063
180. Li LQ, Xu XY, Li WY, Hu XY, Lv W. The prognostic value of total T3 after acute cerebral infarction is age-dependent: a retrospective study on 768 patients. *BMC Neurol.* (2019) 19:54. doi: 10.1186/s12883-019-1264-z
181. Hama S, Kitaoka T, Shigenobu M, Watanabe A, Imura I, Seno H, et al. Malnutrition and nonthyroidal illness syndrome after stroke. *Metabolism.* (2005) 54:699–704. doi: 10.1016/j.metabol.2004.11.016
182. Chaker L, Baumgartner C, Den Elzen WPJ, Collet TH, Ikram MA, Blum MR, et al. Thyroid function within the reference range and the risk of stroke: an individual participant data analysis. *J Clin Endocrinol Metab.* (2016) 101:4270–82. doi: 10.1210/jc.2016-2255
183. Akhoundi FH, Ghorbani A, Soltani A, Meysamie A. Favorable functional outcomes in acute ischemic stroke patients with subclinical hypothyroidism. *Neurology.* (2011) 77:349–54. doi: 10.1212/WNL.0b013e3182267ba0
184. Alevizaki M, Synetou M, Xynos K, Alevizaki CC, Vemmos KN. Hypothyroidism as a protective factor in acute stroke patients. *Clin Endocrinol.* (2006) 65:369–72. doi: 10.1111/j.1365-2265.2006.02606.x
185. Rastogi L, Godbole MM, Ray M, Rathore P, Rathore P, Pradhan S, et al. Reduction in oxidative stress and cell death explains hypothyroidism induced neuroprotection subsequent to ischemia/reperfusion insult. *Exp Neurol.* (2006) 200:290–300. doi: 10.1016/j.expneurol.2006.02.013
186. Shuaib A, Ijaz S, Mazagri R, Kalra J, Hemmings S, Senthilsvlan A, et al. Hypothyroidism protects the brain during transient forebrain ischemia in gerbils. *Exp Neurol.* (1994) 127:119–25. doi: 10.1006/exnr.1994.1085
187. Lee CH, Yoo KY, Hwang IK, Choi JH, Park OK, Li H, et al. Hypothyroid state does not protect but delays neuronal death in the hippocampal CA1 region following transient cerebral ischemia: focus on oxidative stress and gliosis. *J Neurosci Res.* (2010) 88:2661–8. doi: 10.1002/jnr.22436
188. Rastogi L, Godbole MM, Sinha RA, Pradhan S. Reverse triiodothyronine (rT3) attenuates ischemia-reperfusion injury. *Biochem Biophys Res Commun.* (2018) 506:597–603. doi: 10.1016/j.bbrc.2018.10.031
189. Lourbopoulos A, Mourouzis I, Karapanayiotides T, Nousiopoulou E, Chatzigeorgiou S, Mavridis T, et al. Changes in thyroid hormone receptors after permanent cerebral ischemia in male rats. *J Mol Neurosci.* (2014) 54:78–91. doi: 10.1007/s12031-014-0253-3
190. Bengtsson D, Brudin L, Wanby P, Carlsson M. Previously unknown thyroid dysfunction in patients with acute ischemic stroke. *Acta Neurol Scand.* (2012) 126:98–102. doi: 10.1111/j.1600-0404.2011.01604.x
191. Sheu JJ, Kang JH, Lin HC, Lin HC. Hyperthyroidism and risk of ischemic stroke in young adults: a 5-year follow-up study. *Stroke.* (2010) 41:961–6. doi: 10.1161/STROKEAHA.109.577742
192. Wollenweber FA, Zietemann V, Gschwendtner A, Opherk C, Dichgans M. Subclinical hyperthyroidism is a risk factor for poor functional outcome after ischemic stroke. *Stroke.* (2013) 44:1446–8. doi: 10.1161/STROKEAHA.113.000833
193. Keshavarz S, Dehghani GA. Cerebral ischemia/reperfusion injury in the hyperthyroid rat. *Iran J Med Sci.* (2017) 42, 48–56.
194. Margaill I, Royer J, Lerouet D, Ramaugé M, Le Goascogne C, Li WW, et al. Induction of type 2 iodothyronine deiodinase in astrocytes after transient focal cerebral ischemia in the rat. *J Cereb Blood Flow Metab.* (2005) 25:468–76. doi: 10.1038/sj.jcbfm.9600041
195. Rami A, Krieglstein J. Thyroxine attenuates hippocampal neuronal damage caused by ischemia in the rat. *Life Sci.* (1992) 50:645–50. doi: 10.1016/0024-3205(92)90251-J
196. Genovese T, Impellizzeri D, Ahmad A, Cornelius C, Campolo M, Cuzzocrea S, et al. Post-ischaemic thyroid hormone treatment in a rat model of acute stroke. *Brain Res.* (2013) 1513:92–102. doi: 10.1016/j.brainres.2013.03.001
197. Crupi R, Paterniti I, Campolo M, Di Paola R, Cuzzocrea S, Esposito E. Exogenous T3 administration provides neuroprotection in a murine model of traumatic brain injury. *Pharmacol Res.* (2013) 70:80–9. doi: 10.1016/j.phrs.2012.12.009
198. Sayre NL, Sifuentes M, Holstein D, Cheng SY, Zhu X, Lechleiter JD. Stimulation of astrocyte fatty acid oxidation by thyroid hormone is protective against ischemic stroke-induced damage. *J Cereb Blood Flow Metab.* (2017) 37:514–27. doi: 10.1177/0271678X16629153
199. Akhoundzadeh K, Vakili A, Sameni HR, Vafaei AA, Rashidy-Pour A, Safari M, et al. Effects of the combined treatment of bone marrow stromal cells with mild exercise and thyroid hormone on brain damage and apoptosis in a mouse focal cerebral ischemia model. *Metab Brain Dis.* (2017) 32:1267–77. doi: 10.1007/s11011-017-0034-0
200. Doyle KP, Suchland KL, Ciesielski TMP, Lessov NS, Grandy DK, Scanlan TS, et al. Novel thyroxine derivatives, thyronamine and 3-iodothyronamine, induce transient hypothermia and marked neuroprotection against stroke injury. *Stroke.* (2007) 38:2569–76. doi: 10.1161/STROKEAHA.106.480277
201. Costa LES, Clementino-Neto J, Mendes CB, Franzon NH, De Oliveira Costa E, Moura-Neto V, et al. Evidence of aquaporin 4 regulation by thyroid hormone during mouse brain development and in cultured human glioblastoma multiforme cells. *Front Neurosci.* (2019) 13:317. doi: 10.3389/fnins.2019.00317
202. Losi G, Garzon G, Puia G. Nongenomic regulation of glutamatergic neurotransmission in hippocampus by thyroid hormones. *Neuroscience.* (2008) 151:155–63. doi: 10.1016/j.neuroscience.2007.09.064
203. Mendes-de-Aguiar CBN, Alchini R, Decker H, Alvarez-Silva M, Tasca CI, Trentin, AG. Thyroid hormone increases astrocytic glutamate uptake and protects astrocytes and neurons against glutamate toxicity. *J Neurosci Res.* (2008) 86:3117–25. doi: 10.1002/jnr.21755
204. Guo ZH, Li F, Wang WZ. The mechanisms of brain ischemic insult and potential protective interventions. *Neurosci Bull.* (2009) 25:139–52. doi: 10.1007/s12264-009-0104-3
205. Pantos C, Mourouzis I. Thyroid hormone receptor α1 as a novel therapeutic target for tissue repair. *Ann Transl Med.* (2018) 6:254. doi: 10.21037/atm.2018.06.12
206. Madinier A, Quattromani MJ, Sjölund C, Ruscher K, Wieloch T. Enriched housing enhances recovery of limb placement ability and reduces aggrecan-containing perineuronal nets in the rat somatosensory cortex after experimental stroke. *PLoS ONE.* (2014) 9:e93121. doi: 10.1371/journal.pone.0093121
207. Quattromani MJ, Cordeau P, Ruscher K, Kriz J, Wieloch T. Enriched housing down-regulates the Toll-like receptor 2 response in the mouse brain after experimental stroke. *Neurobiol Dis.* (2014) 66:66–73. doi: 10.1016/j.nbd.2014.02.010
208. Wieloch T, Nikolich K. Mechanisms of neural plasticity following brain injury. *Curr Opin Neurobiol.* (2006) 16:258–64. doi: 10.1016/j.conb.2006.05.011
209. Pekna M, Pekny M, Nilsson M. Modulation of neural plasticity as a basis for stroke rehabilitation. *Stroke.* (2012) 43:2819–28. doi: 10.1161/STROKEAHA.112.654228
210. Sharma N, Classen J, Cohen LG. Neural plasticity and its contribution to functional recovery. *Handb Clin Neurol.* (2013) 110:3–12. doi: 10.1016/B978-0-444-52901-5.00001-0
211. Zhong L, Gerges NZ. Neurogranin targets calmodulin and lowers the threshold for the induction of long-term potentiation. *PLoS ONE.* (2012) 7:e41275. doi: 10.1371/journal.pone.0041275
212. Sui L, Ren WW, Li BM. Administration of thyroid hormone increases reelin and brain-derived neurotrophic factor expression in rat hippocampus *in vivo*. *Brain Res.* (2010) 1313:9–24. doi: 10.1016/j.brainres.2009.12.010
213. Krebs J, Means RL, Honegger P. Induction of calmodulin kinase IV by the thyroid hormone during the development of rat brain. *J Biol Chem.* (1996) 271:11055–8. doi: 10.1074/jbc.271.19.11055
214. Redmond L, Kashani AH, Ghosh A. Calcium regulation of dendritic growth via CaM kinase IV and CREB-mediated transcription. *Neuron.* (2002) 34:999–1010. doi: 10.1016/S0896-6273(02)00737-7
215. Gordon-Weeks PR, Fournier AE. Neuronal cytoskeleton in synaptic plasticity and regeneration. *J Neurochem.* (2014) 129:206–12. doi: 10.1111/jnc.12502

216. Aniello F, Couchie D, Gripois D, Nunez J. Regulation of five tubulin isotypes by thyroid hormone during brain development. *J Neurochem.* (1991) 57:1781–6. doi: 10.1111/j.1471-4159.1991.tb06381.x
217. Lorenzo PI, Ménard C, Miller FD, Bernal J. Thyroid hormone-dependent regulation of Talpha1 alpha-tubulin during brain development. *Mol Cell Neurosci.* (2002) 19:333–43. doi: 10.1006/mcne.2001.1087
218. Silva JE, Rudas P. Effects of congenital hypothyroidism on microtubule-associated protein-2 expression in the cerebellum of the rat. *Endocrinology.* (1990) 126:1276–82. doi: 10.1210/endo-126-2-1276
219. Aniello F, Couchie D, Bridoux AM, Gripois D, Nunez J. Splicing of juvenile and adult tau mRNA variants is regulated by thyroid hormone. *Proc Natl Acad Sci USA.* (1991) 88:4035–9. doi: 10.1073/pnas.88.9.4035
220. Zamoner A, Funchal C, Heimfarth L, Silva FR, Pessoa-Pureur R. Short-term effects of thyroid hormones on cytoskeletal proteins are mediated by GABAergic mechanisms in slices of cerebral cortex from young rats. *Cell Mol Neurobiol.* (2006) 26:209–24. doi: 10.1007/s10571-006-9027-y
221. Zamoner A, Funchal C, Jacques-Silva MC, Gottfried C, Barreto Silva FR, Pessoa-Pureur R. Thyroid hormones reorganize the cytoskeleton of glial cells through Gfap phosphorylation and Rhoa-dependent mechanisms. *Cell Mol Neurobiol.* (2007) 27:845–65. doi: 10.1007/s10571-006-9084-2
222. Faivre-Sarrailh C, Rabié A. Developmental study of factors controlling microtubule *in vitro* cold-stability in rat cerebrum. *Dev Brain Res.* (1988) 42:199–204. doi: 10.1016/0165-3806(88)90238-6
223. Farwell AP, Dubord-Tomasetti SA, Pietrzykowski AZ, Leonard JL. Dynamic nongenomic actions of thyroid hormone in the developing rat brain. *Endocrinology.* (2006) 147:2567–74. doi: 10.1210/en.2005-1272
224. Farwell AP, Dubord-Tomasetti SA. Thyroid hormone regulates the expression of laminin in the developing rat cerebellum. *Endocrinology.* (1999) 140:4221–7. doi: 10.1210/endo.140.9.7007
225. Cayrou C, Denver RJ, Puymirat J. Suppression of the basic transcription element-binding protein in brain neuronal cultures inhibits thyroid hormone-induced neurite branching. *Endocrinology.* (2002) 143:2242–9. doi: 10.1210/en.143.6.2242
226. Martel J, Cayrou C, Puymirat J. Identification of new thyroid hormone-regulated genes in rat brain neuronal cultures. *Neuroreporte.* (2002) 13:1849–51. doi: 10.1097/00001756-200210280-00003
227. Vara H, Martínez B, Santos A, Colino A. Thyroid hormone regulates neurotransmitter release in neonatal rat hippocampus. *Neuroscience.* (2002) 110:19–28. doi: 10.1016/S0306-4522(01)00541-3
228. Liu W, Dong J, Wang Y, Xi Q, Chen J. Developmental iodine deficiency and hypothyroidism impaired *in vivo* synaptic plasticity and altered PKC activity and GAP-43 expression in rat hippocampus. *Nutr Neurosci.* (2010) 11:50. doi: 10.1179/147683010x12611460764525
229. Dong J, Yin H, Liu W, Wang P, Jiang Y, Chen J. Congenital iodine deficiency and hypothyroidism impair LTP and decrease c-fos and c-jun expression in rat hippocampus. *Neurotoxicology.* (2005) 26:417–26. doi: 10.1016/j.neuro.2005.03.003
230. Sui L, Gilbert ME. Pre- and postnatal propylthiouracil-induced hypothyroidism impairs synaptic transmission and plasticity in area CA1 of the neonatal rat hippocampus. *Endocrinology.* (2003) 144:4195–203. doi: 10.1210/en.2003-0395
231. Gerges NZ, Alkadhi KA. Hypothyroidism impairs late LTP in CA1 region but not in dentate gyrus of the intact rat hippocampus: MAPK involvement. *Hippocampus.* (2004) 14:40–5. doi: 10.1002/hipo.10165
232. Niemi WD, Slivinski K, Audi J, Rej R, Carpenter DO. Propylthiouracil treatment reduces long-term potentiation in area CA1 of neonatal rat hippocampus. *Neurosci Lett.* (1996) 210:127–9. doi: 10.1016/0304-3940(96)12676-8
233. Gilbert ME. Alterations in synaptic transmission and plasticity in area CA1 of adult hippocampus following developmental hypothyroidism. *Dev Brain Res.* (2004) 148:11–8. doi: 10.1016/j.devbrainres.2003.09.018
234. Gould E, Allan MD, McEwen BS. Dendritic spine density of adult hippocampal pyramidal cells is sensitive to thyroid hormone. *Brain Res.* (1990). doi: 10.1016/0006-8993(90)90884-E
235. Taşkin E, Artis AS, Bitiktas S, Dolu N, Liman N, Süer C. Experimentally induced hyperthyroidism disrupts hippocampal long-term potentiation in adult rats. *Neuroendocrinology.* (2011) 94:218–27. doi: 10.1159/000328513
236. Forini F, Kusmic C, Nicolini G, Mariani L, Zucchi R, Matteucci M, et al. Triiodothyronine prevents cardiac ischemia/reperfusion mitochondrial impairment and cell loss by regulating miR30a/p53 axis. *Endocrinology.* (2014) 155:4581–90. doi: 10.1210/en.2014-1106
237. Zinman T, Shneyvays V, Tribulova N, Manoach M, Shainberg A. Acute, nongenomic effect of thyroid hormones in preventing calcium overload in newborn rat cardiocytes. *J Cell Physiol.* (2006) 207:220–31. doi: 10.1002/jcp.20562
238. Manzano J, Cuadrado M, Morte B, Bernal J. Influence of thyroid hormone and thyroid hormone receptors in the generation of cerebellar γ-aminobutyric acid-ergic interneurons from precursor cells. *Endocrinology.* (2007) 148:5746–51. doi: 10.1210/en.2007-0567
239. Hashimoto H, Walker CH, Prange AJJ, Mason GA. The effects of thyroid hormones on potassium-stimulated release of 3H-GABA by synaptosomes of rat cerebral cortex. *Neuropsychopharmacology.* (1991) 5:49–54.
240. Kalaria RN, Prince AK. Effects of thyroid deficiency on the development of cholinergic, GABA, dopaminergic and glutamate neuron markers and DNA concentrations in the rat corpus striatum. *Int J Dev Neurosci.* (1985) 3:655–66. doi: 10.1016/0736-5748(85)90056-5
241. Mason GA, Walker CH, Prange AJ, Bondy SC. GABA uptake is inhibited by thyroid hormones: implications for depression. *Psychoneuroendocrinology.* (1987) 12:53–9. doi: 10.1016/0306-4530(87)90022-9
242. Martin JV, Williams DB, Fitzgerald RM, Im HK, Vonvoigtlander PF. Thyroid hormonal modulation of the binding and activity of the GABA(A) receptor complex of brain. *Neuroscience.* (1996) 73:705–13. doi: 10.1016/0306-4522(96)00052-8
243. Ambrogini P, Cuppini R, Ferri P, Mancini C, Ciaroni S, Voci A, et al. Thyroid hormones affect neurogenesis in the dentate gyrus of adult rat. *Neuroendocrinology.* (2005) 81:244–53. doi: 10.1159/000087648
244. Desouza LA, Ladiwala U, Daniel SM, Agashe S, Vaidya RA, Vaidya VA. Thyroid hormone regulates hippocampal neurogenesis in the adult rat brain. *Mol Cell Neurosci.* (2005) 29:414–26. doi: 10.1016/j.mcn.2005.03.010
245. Fanibunda SE, Desouza LA, Kapoor R, Vaidya RA, Vaidya VA. Thyroid hormone regulation of adult neurogenesis. *Vitam Horm.* (2017) 106:211–51. doi: 10.1016/bs.vh.2017.04.006
246. Kapoor R, Desouza LA, Nanavaty IN, Kernie SG, Vaidya VA. Thyroid hormone accelerates the differentiation of adult hippocampal progenitors. *J Neuroendocrinol.* (2012) 24:1259–71. doi: 10.1111/j.1365-2826.2012.02329.x
247. Lemkine GF, Raj A, Alfama G, Turque N, Hassani Z, Alegria-Prévot O, et al. Adult neural stem cell cycling *in vivo* requires thyroid hormone and its alpha receptor. *FASEB J.* (2005) 19:863–5. doi: 10.1096/fj.04-2916fje
248. López-Juárez A, Remaud S, Hassani Z, Jolivet P, Pierre Simons J, Sontag T, et al. Thyroid hormone signaling acts as a neurogenic switch by repressing Sox2 in the adult neural stem cell niche. *Cell Stem Cell.* (2012) 10:531–43. doi: 10.1016/j.stem.2012.04.008
249. Montero-Pedrazuela A, Venero C, Lavado-Autric R, Fernández-Lamo I, García-Verdugo JM, Bernal J, et al. Modulation of adult hippocampal neurogenesis by thyroid hormones: implications in depressive-like behavior. *Mol Psychiatry.* (2006) 11:361–71. doi: 10.1038/sj.mp.4001802
250. Zhang L, Blomgren K, Kuhn HG, Cooper-Kuhn CM. Effects of postnatal thyroid hormone deficiency on neurogenesis in the juvenile and adult rat. *Neurobiol Dis.* (2009) 34:366–74. doi: 10.1016/j.nbd.2009.02.006
251. Kapoor R, Fanibunda SE, Desouza LA, Guha SK, Vaidya VA. Perspectives on thyroid hormone action in adult neurogenesis. *J Neurochem.* (2015) 133:599–616. doi: 10.1111/jnc.13093
252. Remaud S, Gothié JD, Morvan-Dubois G, Demeneix BA. Thyroid hormone signaling and adult neurogenesis in mammals. *Front Endocrinol.* (2014) 5:62. doi: 10.3389/fendo.2014.00062
253. Christie KJ, Turnley AM. Regulation of endogenous neural stem/progenitor cells for neural repair—factors that promote neurogenesis and gliogenesis in the normal and damaged brain. *Front Cell Neurosci.* (2013) 6:70. doi: 10.3389/fncel.2012.00070
254. Ming GL, Song H. Adult neurogenesis in the mammalian brain: significant answers and significant questions. *Neuron.* (2011) 70:687–702. doi: 10.1016/j.neuron.2011.05.001
255. Lu J, Manaenko A, Hu Q. Targeting adult neurogenesis for poststroke therapy. *Stem Cells Int.* (2017) 2017:5868632. doi: 10.1155/2017/5868632

256. Angels Font M, Arboix A, Krupinski J. Angiogenesis, neurogenesis and neuroplasticity in ischemic stroke. *Curr Cardiol Rev.* (2010) 6:238–44. doi: 10.2174/157340310791658802
257. Arai K, Jin G, Navaratna D, Lo EH. Brain angiogenesis in developmental and pathological processes: neurovascular injury and angiogenic recovery after stroke. *FEBS J.* (2009) 276:4644–52. doi: 10.1111/j.1742-4658.2009.07176.x
258. Arenillas JF, Sobrino T, Castillo J, Dávalos A. The role of angiogenesis in damage and recovery from ischemic stroke. *Curr Treat Options Cardiovasc Med.* (2007) 9:205–12. doi: 10.1007/s11936-007-0014-5
259. Tomanek RJ, Zimmerman MB, Suvarna PR, Morkin E, Pennock GD, Goldman S. A thyroid hormone analog stimulates angiogenesis in the post-infarcted rat heart. *J Mol Cell Cardiol.* (1998) 30:923–32. doi: 10.1006/jmcc.1998.0671
260. Schlenker EH, Hora M, Liu Y, Redetzke RA, Morkin E, Gerdes AM. Effects of thyroidectomy, T4, and DITPA replacement on brain blood vessel density in adult rats. *AJP Regul Integr Comp Physiol.* (2008) 294:R1504–9. doi: 10.1152/ajpregu.00027.2008
261. Davis PJ, Sudha T, Lin HY, Mousa SA. Thyroid hormone, hormone analogs, and angiogenesis. *Compr Physiol.* (2016) 6:353–62. doi: 10.1002/cphy.c150011
262. Mousa SA, O'Connor LJ, Bergh JJ, Davis FB, Scanlan TS, Davis PJ. The proangiogenic action of thyroid hormone analogue GC-1 is initiated at an integrin. *J Cardiovasc Pharmacol.* (2005) 46:356–60. doi: 10.1097/01.fjc.0000175438.94906.a0

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Talhada, Santos, Gonçalves and Ruscher. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

CHAPTER 4

TRIIODOTHYRONINE AND STROKE

Triiodothyronine modulates neuronal plasticity mechanisms to enhance functional outcome after stroke

Short description: This study addresses important findings of 3,5,3'-triiodo-L-thyronine (T_3) in the regulation of homeostatic mechanisms that adjust excitability – inhibition ratio in the postischemic brain during the first two weeks after experimental stroke induced by photothrombosis and in cultured glutamatergic neurons subjected to an *in vitro* model of acute cerebral ischemia. Moreover, we assessed the expression pattern of thyroid hormone receptors protein levels important to mediate T_3 actions, in the human post-stroke brain. Our results show that T_3 modulates several plasticity mechanisms that may operate on different temporal and spatial scales as compensatory mechanisms to assure appropriate synaptic neurotransmission.

Acta Neuropathologica
Communications

RESEARCH **Open Access**

Triiodothyronine modulates neuronal plasticity mechanisms to enhance functional outcome after stroke

Daniela Talhada[1,2], Joana Feiteiro[2], Ana Raquel Costa[2], Tiago Talhada[1], Elisa Cairrão[2], Tadeusz Wieloch[1], Elisabet Englund[3], Cecília Reis Santos[2], Isabel Gonçalves[2] and Karsten Ruscher[1,4*]

Abstract

The development of new therapeutic approaches for stroke patients requires a detailed understanding of the mechanisms that enhance recovery of lost neurological functions. The efficacy to enhance homeostatic mechanisms during the first weeks after stroke will influence functional outcome. Thyroid hormones (TH) are essential regulators of neuronal plasticity, however, their role in recovery related mechanisms of neuronal plasticity after stroke remains unknown. This study addresses important findings of 3,5,3′-triiodo-L-thyronine (T_3) in the regulation of homeostatic mechanisms that adjust excitability – inhibition ratio in the post-ischemic brain. This is valid during the first 2 weeks after experimental stroke induced by photothrombosis (PT) and in cultured neurons subjected to an in vitro model of acute cerebral ischemia. In the human post-stroke brain, we assessed the expression pattern of TH receptors (TR) protein levels, important for mediating T_3 actions. Our results show that T_3 modulates several plasticity mechanisms that may operate on different temporal and spatial scales as compensatory mechanisms to assure appropriate synaptic neurotransmission. We have shown in vivo that long-term administration of T_3 after PT significantly (1) enhances lost sensorimotor function; (2) increases levels of synaptotagmin 1&2 and levels of the post-synaptic GluR2 subunit in AMPA receptors in the peri-infarct area; (3) increases dendritic spine density in the peri-infarct and contralateral region and (4) decreases tonic GABAergic signaling in the peri-infarct area by a reduced number of parvalbumin$^+$ / c-fos$^+$ neurons and glutamic acid decarboxylase 65/67 levels. In addition, we have shown that T_3 modulates in vitro neuron membrane properties with the balance of inward glutamate ligand-gated channels currents and decreases synaptotagmin levels in conditions of deprived oxygen and glucose. Interestingly, we found increased levels of TRβ1 in the infarct core of *post-mortem* human stroke patients, which mediate T_3 actions. Summarizing, our data identify T_3 as a potential key therapeutic agent to enhance recovery of lost neurological functions after ischemic stroke.

Keywords: Ischemia, Photothrombosis, Recovery, Stroke, Thyroid hormones, Thyroid hormone receptors, 3,5,3′-triiodo-L-thyronine (T_3), 3,5,3′,5′-tetraiodo-L-thyronine (T_4)

Introduction

Loss of motor function following ischemic stroke is the most enduring and disabling consequence [4, 26]. Despite the attempt to find neuroprotective treatments that mitigate tissue damage and loss of motor function, their translation into clinical practice has been disappointing. So far, thrombectomy and thrombolysis in the acute phase after stroke are the only effective treatments to restore blood flow and minimize brain damage. However, acute therapies are limited to the first 4.5 h for thrombolysis or up to 24 h for thrombectomy after stroke onset and are accessible to less than 10% of stroke patients [36, 49]. Beyond the acute phase constant and consistent specific rehabilitation programs are instrumental to partially regain brain function, dependent on size and brain regions affected by stroke [37]. Therefore, the options to minimize the damage after ischemic stroke remains

* Correspondence: karsten.ruscher@med.lu.se
[1]Laboratory for Experimental Brain Research, Division of Neurosurgery, Department of Clinical Sciences, Lund University, BMC A13, S-22184 Lund, Sweden
[4]LUBIN Lab - Lunds Laboratorium för Neurokirurgisk Hjärnskadeforskning, Division of Neurosurgery, Department of Clinical Sciences, Lund University, Lund, Sweden
Full list of author information is available at the end of the article

© The Author(s). 2019 **Open Access** This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated.

sub-optimal and there is need for new therapeutic approaches that target restorative processes.

In response to loss of input from the infarct core, surviving neurons adopt self-repair and self-organizing homeostatic mechanisms in order to stabilize the ratio between excitatory and inhibitory circuits and maintain adequate synaptic input [21]. First, blood flow is restored and there is a temporary resolution in neuronal activity and metabolism in regions surrounding and connected to the infarct [33, 82]. Concomitantly, in response to cell death in the infarct core, there is a change in neuronal pathways and reorganization of neuronal connectivity, namely axonal growth, spine remodeling and dendritic arborization [29, 82]. A wide variety of homeostatic mechanisms contribute to the maintenance of overall excitability, involving the regulation of neuronal intrinsic excitability and synaptic transmission. These mechanisms include changes in receptor expression at the post-synaptic level, neurotransmitter release at the pre-synaptic level, ion channel function and synapse number or synaptic strength [52, 76, 77].

Processes of neuronal reorganization and cellular responses to the infarct occur during the first weeks after stroke in mice [7] and up to months and years in humans [23, 24]. During this period the brain is highly plastic, and distinct overlapping events promoting recovery of neurological function can be modulated by external interventions [82]. Therefore, current research is focused to understand mechanisms of post-injury plasticity that occur spontaneously after stroke [50, 82].

Current epidemiological human studies suggest that thyroid hormones (TH) signaling is related to a better outcome after stroke although the mechanisms involved are poorly investigated [71]. Several studies have pointed out that TH contribute to neuroprotection when administered before [46, 61] or during the first hours after stroke or traumatic brain injury [20, 22, 27, 40, 55]. TH also protect cortical neurons against glutamate-induced neuronal damage [42].

However, long-term effects of TH during the recovery phase after stroke remain largely unknown [71]. Here we hypothesized that 3,5,3′,5′-tetraiodo-L-thyronine (T_4) and 3,5,3′-triiodo-L-thyronine (T_3) might be actively involved to enhance post-stroke recovery, since they are essential in several mechanisms for brain development [3, 5] and normal function of the adult brain [47]. Summarizing, TH are involved in mechanisms of neuronal proliferation, migration and differentiation, neurite outgrowth, synaptic plasticity, dendritic branching and myelination during brain development [5, 28]. In the adult brain, several processes of neurorepair are particularly dependent on T_3 action, namely neuronal plasticity and neurogenesis [35, 56].

To study the role of TH in mechanisms of neuronal repair, we analyzed post-ischemic brains of mice subjected to intraperitoneal (i.p.) administration of T_4 and T_3 at 5 or 50 μg/kg starting at day two after photothrombosis (PT) and every second day, in a total of six administrations; we assessed T_3 effects in ionotropic glutamate receptors (iGluRs) in cultured glutamatergic neurons; and we analyzed expression pattern of TH receptors (TR) alpha 1 (TRα1) and beta 1 (TRβ1) in post-ischemic brains of mice and human patients. In the present investigation we demonstrate that T_3 modulates pathways during critical periods of recovery after stroke involved in reorganization of neuronal circuits and synaptic plasticity, functional connectivity and motor recovery. Summarizing, we demonstrate that (1) T_3 enhanced recovery of lost motor function in an experimental model of stroke, (2) T_3 increased levels of synaptotagmin 1&2 and levels of post-synaptic glutamate receptor 2 (GluR2) subunit in alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors in the peri-infarct area, (3) T_3 increased dendritic spine density in the ipsilateral and contralateral regions and (4) T_3 decreased tonic GABAergic signaling in the peri-infarct area by a reduced number of parvalbumin-positive (PV^+) / c-fos^+ neurons and glutamic acid decarboxylase 65/67 (GAD 65/67) protein levels. In cultured neurons (5) T_3 modulates membrane properties with the balance of inward glutamate ligand-gated channels currents and (6) T_3 modulates synaptotagmin levels in an in vitro model of ischemia. In the human post-ischemic brain (7) TRβ1 has a spatial expression pattern, which may drive T_3 transcriptional activity.

Materials and methods

Ethical considerations

Mice were bred and genotyped at the conventional facility of the Biomedical Centre, (BMC, Lund, Sweden). All animal experiments (*Studies I and II*) were carried out in accordance with the international guidelines on experimental animal research, with the approval of the Malmö-Lund Ethical Committee (ethical permit no. M50/2015) and followed the ARRIVE guidelines. All in vitro experiments (*Study III*) were carried out in compliance with directives on animal experimentation (Decreto-Lei 113/2013 and 2010/63/EU) in Portugal and European Union and with approval of the committee of Animal Research at Universidade da Beira Interior (CICS-UBI, Covilhã, Portugal). Human brain tissue used in this study was used with the approval of the Lund Ethical Review Board for research involving humans (Dnr 2011/80).

Thyroid hormones effects after experimental stroke (study I)

For this study, 117 C57BL/6 male mice (20 to 26 g, aged 9 to 10 weeks, purchased from Charles River) were used. Out of 117 animals, 12 were excluded due to problems during surgery and mortality before entering the treatment phase and 105 animals were randomly assigned into the treatment groups (Fig. 1). Treatment was initiated on day two after PT and every other day until the endpoint of the study. Vehicle (Vh, NaCl 0.9%), T_3 (5 or 50 μg/kg) or T_4 (5 or 50 μg/kg) were administered by i.p. injection in a total of six administrations. On days two, seven and 14 after stroke onset or sham surgery, animals were evaluated for motor function.

Photothrombosis

Focal ischemic stroke was induced by PT, as described previously [65, 81]. Ischemic stroke was induced in the right hemisphere through illumination of a squared aperture measuring 4.0 to 2.0 mm (equal to an area of 8.0 mm^2). The light position related to bregma (+ 1.5 mm lateral and + 0.5 mm anterior) affected the mouse primary motor cortex of forelimb-responsive sites, in the left body side [72]. The same procedure was performed in sham operated animals, with saline injection instead of photosensitizing dye.

Behavior analysis

Motor function and exploratory behavior after TH treatment was assessed using a neuroscore consisting of the rotating pole test (RPT) and the open field test, respectively [60, 79]. These assessments were performed in a blinded fashion to the investigator that performed the surgeries and treatments.

The RPT was used to assess postural and locomotor asymmetry that results from an unilateral brain lesion [57]. After stroke or sham surgery, animals were evaluated on day two for randomization into treatment groups. Each trial was video recorded, and videos were used to assess motor dysfunction by using a zero to six scoring system (Table 1). Animals that did not fulfill inclusion criteria were excluded from behavior analysis (see Additional file 1: Supplementary methods).

The open field test was performed 14 days after stroke to assess both spontaneous post-ischemic locomotor activity and post-ischemic exploration behavior [78].

Immunohistochemistry and immunofluorescence

Tissue collection for immunostainings was performed as described before [53, 63]. Primary antibodies used for immunofluorescence were rabbit TRβ1 (Millipore, 1:1000), rabbit TRα1 (Abcam, 1:1000), goat parvalbumin (PV235, Swant, 1:5000), mouse neuronal nuclei (NeuN, Millipore, 1:1000), glial fibrillary acidic protein (GFAP)-Cy3 (Sigma, 1:5000), rat cluster of differentiation (CD) 68 (Abd Serotec, 1:300), and mouse glutathione S-transferase (GST)-pi isoform (BD Transduction Laboratories, 1:1000).

Infarct size measurement

Coronal brain sections from the start until the end of the infarct and spaced one millimeter were collected and stained for NeuN (rabbit NeuN, Millipore, 1:5000). The non-injured portion of the ipsilateral and contralateral hemisphere were encircled and the indirect infarct volume was calculated by integration of areas from serial sections of each brain as described previously [70] using Fiji software [64].

Counting of parvalbumin positive cells

For each animal one coronal section (– 2.0 mm relative to bregma) was stained for PV^+ neurons using a monoclonal

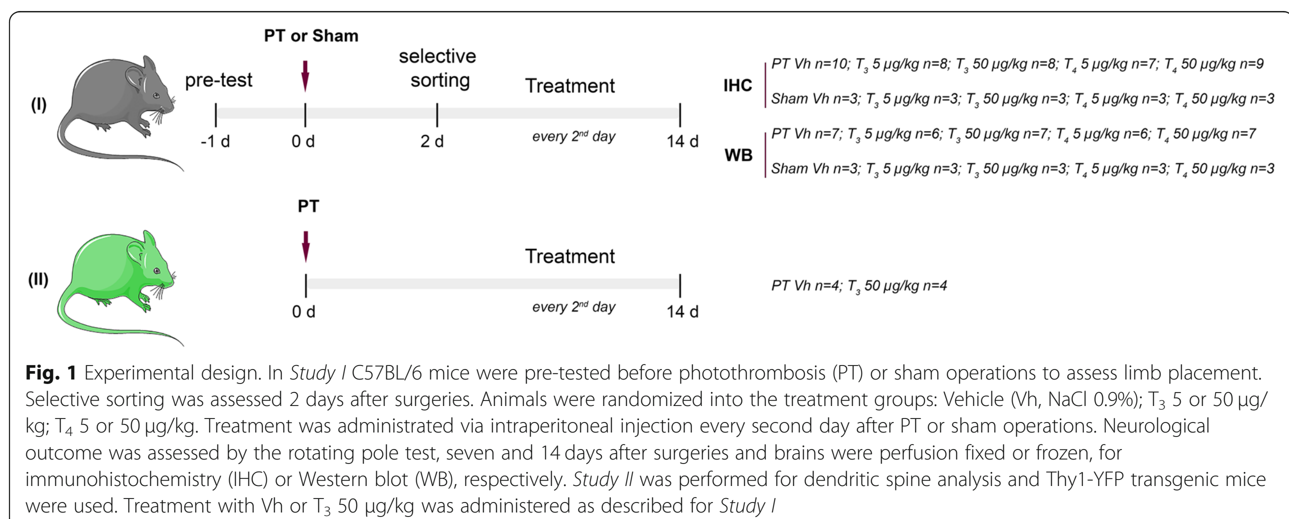


Fig. 1 Experimental design. In *Study I* C57BL/6 mice were pre-tested before photothrombosis (PT) or sham operations to assess limb placement. Selective sorting was assessed 2 days after surgeries. Animals were randomized into the treatment groups: Vehicle (Vh, NaCl 0.9%); T_3 5 or 50 μg/kg; T_4 5 or 50 μg/kg. Treatment was administrated via intraperitoneal injection every second day after PT or sham operations. Neurological outcome was assessed by the rotating pole test, seven and 14 days after surgeries and brains were perfusion fixed or frozen, for immunohistochemistry (IHC) or Western blot (WB), respectively. *Study II* was performed for dendritic spine analysis and Thy1-YFP transgenic mice were used. Treatment with Vh or T_3 50 μg/kg was administered as described for *Study I*

Table 1 Motor function assessed by the rotating pole test before and after photothrombosis at days 2, 7 and 14

Score	Criteria
0	animal falls off immediately upon entry onto the pole
1	animal remains embraced to the pole unable to cross and eventually falls off the pole
2	animal falls off during crossing or if the hindlimbs do not contribute to forward movement
3	animal crosses the pole while continuously slipping with the forelimbs or hindlimbs
4	animal crosses pole with > 3 ft slips
5	animal traverses the pole with 1–3 ft slips
6	animal crosses the pole without any foot slips

goat primary antibody (PV235, Swant, 1:5000), and visualization accessed using a VECTOR NovaRED Peroxidase (HRP) Substrate Kit (Vector Laboratories, CA, USA). Rabbit c-fos (Santa Cruz, 1:500) positive immunoreactivity (c-fos$^+$) was accessed using the avidin–biotin–HRP system.

Immunoblotting

Brains from mice were collected as previously described [63] and the tissue correspondent to the infarct core and peri-infarct was collected. Tissue from human brains were dissected out by a pathologist following autopsy. Primary antibodies used for Western blots were rabbit TRα1 (Abcam, 1:1000), rabbit TRβ1 (Millipore, 1:20000), mouse postsynaptic protein 95 (PSD95; BD Transduction Laboratories, 1:1000), rabbit synaptophysin (Thermoscientific, 1:15000), rabbit GluR1 (Millipore, 1:2000), mouse GluR2 (Millipore, 1:1000), mouse *N*-methyl-D-aspartate receptor 1 (NMDAR1) (BD Transduction Laboratories, 1:1000), rabbit synaptotagmin 1&2 (Abcam, 1:1000) and rabbit GAD 65/67 (Millipore, 1:2000). Membranes were reprobed with anti β-actin HRP conjugated (1:150000, Sigma-Aldrich). Levels were calculated as a percentage of β-actin expression, after densitometric analysis using Fiji software.

Dynamics of dendritic spines after administration with T_3 (study II)

To study the effects of T_3 on dendritic spine dynamics in mouse neocortical neurons after experimental stroke, eight Thy1-yellow fluorescent protein (YFP) transgenic mice (25 to 40 g, aged 1 year, own breeding), that express YFP in neuronal population were used. Mice were randomly assigned in the following treatment groups: PT/Vh, $n = 4$; PT/T_3 50 μg/kg, $n = 4$ (Fig. 1). Treatment was administered as described above for *Study I*. Fourteen days after the surgery, mice were sacrificed, perfusion fixed with paraformaldehyde 4% and brains were collected for further infarct volume assessment and dendritic spine analysis.

Photothrombosis

To induce PT in animals for dendritic spine analysis (*Study II*) the surgical procedure was performed as in *Study I*, and the left hemisphere was illuminated with a cold light source through a round aperture measuring 1.5 mm in diameter (equal to an area of 1.767 mm^2) for 20 min. This approach induced smaller infarct sizes so that dendritic spines could be analyzed in different regions in the peri-infarct area. The same procedure was performed in Sham operated animals, with saline injection instead of photosensitizing dye.

Detection and classification of dendritic spines from fluorescence laser scanning microscopy

Three coronal sections per animal were collected at different levels: + 2.0 mm, + 1.0 mm and 0 mm relatively to bregma, corresponding to the rostral pole, center and caudal pole of the infarct, respectively. For each animal, we analyzed layers II/III correspondent to the apical pyramidal neurons in the ipsilateral motor cortex (Region 1, R1), ipsilateral somatosensory cortex (Region 2, R2), contralateral motor cortex (Region 3, R3) and contralateral somatosensory cortex (Region 4, R4).

Dendritic spine density and shape classification was accurately quantified and characterized using a three-dimensional computational approach as previously described, after image deconvolution [58].

For each region, three to five dendritic branches were randomly selected. Dendrites were manually selected, and spines were automatically detected using NeuronStudio software. Dendritic spines were classified according to the head to neck ratio and head diameter as stubby, mushroom or thin [30, 58], using default parameters from NeuronStudio. Dendritic spine density was calculated with the ratio number of spines / dendrite length.

In vitro modulation of T_3 in glutamatergic neurons (study III)

An in vitro model of cerebral ischemia and electrophysiology studies were performed to study immediate effects of T_3 in homeostatic plastic mechanisms, namely modulation of synaptic proteins crucial for neurotransmission and NMDA and AMPA evoked currents.

Cell cultures

Cultured cortical neurons were used after 7–8 days in vitro (DIV). Primary cortical neuronal cultures were prepared as described before [59]. Cells were obtained from the cerebral cortex from Wistar rats on embryonic day 16–18. Briefly, meninges were removed, and the

cortex dissected and subjected to enzymatic dissociation, using 0.05 / 0.02% w/v in phosphate buffered saline (PBS) trypsin / EDTA (#15400054, Thermofisher) for 15 min at 37 °C. The homogenized was rinsed with Dulbecco's Modified Eagle's medium (#11880036, DMEM, GIBCO) with 10% fetal bovine serum (#10500–064, GIBCO), 100 U penicillin and streptomycin/ml (#15140122, Thermofisher), 2 mM L-glutamine (#G5792, Sigma-Aldrich), dissociated with a Pasteur pipette, centrifuged and redissociated in starter medium (#21103049, Neurobasal medium, GIBCO) supplemented with B27 (#17504044, GIBCO), 100 U penicillin and streptomycin/ml, 2 mM L-glutamine (#G5792, Sigma-Aldrich) and 25 μM glutamate (#49621, Sigma-Aldrich). The cells were plated onto poly-L-lysine (#P4707, Sigma-Aldrich) pre-coated multiwells at 1.5×10^5 cells/cm^2 and grown in starter medium at 37 °C and 5% CO_2. One-half of the medium was replaced with cultivating medium (starter medium without glutamate) from 4 DIV. Cells were used after 7–8 DIV for in vitro assays.

In vitro ischemic model and experimental treatments

After 7 DIV neurobasal medium was collected and stored to be replaced after the experiments. Neuron cultures were washed with PBS, and oxygen and glucose deprivation (OGD) was induced with a deoxygenated aglycemic solution. OGD was generated in a hypoxia incubator chamber (StemCell Technologies), flushed with gas: 5% CO_2, 95% N_2. In control cultures, medium was replaced by basic salt solution (BSS) after washing with PBS and cells were incubated in a normoxic atmosphere containing 5% CO_2. Cultures were in OGD or BSS solutions for 120 min and after replaced by the previous collected medium. After OGD / BSS conditions, cells were incubated with Vh (DMSO in PBS, 0.01%) or T_3 1 μM for 48 h. Subsequently, cells were washed with cold PBS to remove excess of culture medium and cells collected and frozen at − 80 °C until protein extraction.

Immunocytochemistry

For immunocytochemistry, neurons were plated on glass coverslips and fixed after 7 DIV. Antibodies used for immunofluorescence were rabbit TRα1 (Thermoscientific, 1:500) or rabbit TRβ1 (Millipore, 1.500). The next day, neurons were stained with Hoechst-33,342 (4 μg/ml, Life Technologies).

Immunobloting

Protein extraction was performed as previously described [38, 63]. Western blot was performed to evaluate levels of mouse synaptotagmin (BD Transduction Laboratories, 1:2000).

Electrophysiological recording of membrane currents

To study ligand-gated channels AMPA and NMDA, we adopted the voltage-ramp method [85].

Individual currents were recorded after incubation with T_3 1 μM (n = 4) or Vh (n = 3) during the 48 h preceding the experiments. A sequence of voltage ramps at a rate of 0.23 mV/millisecond were applied at a holding potential of − 80 mV. To obtain the agonist induced current-voltage (I-V) relation, ramps I-V curves were constructed applying a 500 milliseconds voltage ramp ranging from − 110 mV to + 20 mV elicited every 8 s. Voltage ramps were applied in the absence and in the presence of AMPA and NMDA agonist glutamate at 50 μM and co-agonist of NMDA channels glycine at 3 μM, to enable subtraction of leak currents. The antagonists of AMPA and NMDA channels, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; Sigma-Aldrich) and dizocilpinehydrogen maleate (MK-801; Sigma-Aldrich), respectively, were used both at 10 μM.

Cell currents were recorded sequentially in the presence of specific K^+- channel blockers tetraethylammonium sodium salt (5 mM) and 4-Aminopyridine (1 mM), that were applied in the perfusion system together with the other drugs. Voltage-gated K^+ channels needed to be blocked, since those channels were contributing to the conductance as well to the reversal potential obtained.

Statistical analysis

Data are expressed as means ± standard error of the mean (SEM) for parametric data or as medians for non-parametric data. P values < 0.05 were considered as statistically significant. Statistical analysis was performed using IBM SPSS statistics 24 software for dendritic spine analysis or GraphPad Prism 6.0 software (GraphPad, San Diego, CA, USA), using one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test when three or more groups were present or two-tailed unpaired Student's t-test when comparing two groups. For non-parametric data, Kruskal Wallis test was employed for more than two groups followed by the Dunn's multiple comparisons test and the Mann-Whitney U-test for comparison of two groups. Graphs were designed using GraphPad Prism 6.0 software.

For additional details about techniques and analysis performed, please refer to the Additional file 1: Supplementary Methods.

Results

Treatment with T_3 improves functional recovery after PT without affecting infarct size

We first assessed if treatment with T_3 or T_4 at 5 or 50 μg/kg enhances motor function in mice subjected to unilateral PT. Motor function was assessed by RPT on day 7 and 14 after

stroke onset. We observed some degree of spontaneous recovery in mice of all groups subjected to PT. T_3-treated mice at 50 μg/kg could traverse the pole with a score higher than three at 10 rpm, to the right and left sides, showing that all animals crossed the pole without falling (Additional file 2: Video S1, Additional file 3: Video S2, Additional file 4: Video S3 and Additional file 5: Video S4). However, a significantly enhanced functional recovery was only observed when the pole rotated at 10 rpm to the left, in animals treated with T_3 at 50 μg/kg, when compared to Vh-treated animals (Fig. 2a). Fourteen days after stroke, 73% (eight out of 11) and 64% (seven out of 11) of mice treated with T_3 50 μg/kg had a score higher or equal to four points, at 3 rpm and 10 rpm to the left, respectively. In contrast, only 9% (one out of 11) of mice subjected to PT and treated with saline had a score of four points and not higher, at 3 and 10 rpm to the left (Additional file 1: Figure S1).

Infarct size influences the severity of neurological deficits and differences of infarct size among treatment groups may influence behavior assessment to evaluate motor recovery over time. Overall the infarct volume did not differ between animals assigned to treatment groups (2.5 ± 0.78 mm^3 Vh, 3.2 ± 0.97 mm^3 T_3 5 μg/kg, 1.6 ± 0.47 mm^3 T_3 50 μg/kg, 3.1 ± 1.5 mm^3 T_4 5 μg/kg, 4.0 ± 1.3 mm^3 T_4 50 μg/kg; mean ± SEM) as shown in Fig. 2b. All treatments had no influence on the behavior of sham-operated mice (data not shown).

The doses used in the present studies have been determined in preliminary studies (data not shown). No adverse effects related to hyperthyroidism were seen following any of the given doses. In addition, no differences were observed in body weight or temperature in animals from all groups throughout the studies (Additional file 1: Table S1). In all experimental groups, plasma levels of T_3 and T_4 were in physiological range at the endpoint of the study (Additional file 1: Figure S2).

We performed the open field test to ascertain that TH administration was not associated with anxiety or depression-like behavior. Treatment with TH did not affect open field scores, indicative that the treatment did not induce anxiety (Additional file 1: Figure S3).

Treatment with T_3 did not affect the expression of TH receptors after PT

To characterize if functional improvement after T_3 administration was mediated by its binding to respective TR, we assessed their expression in the post-ischemic brain. We found that both isoforms, TRα1 and TRβ1, were ubiquitously expressed in the brain. TR were expressed in the cytoplasm of NeuN and PV^+ neurons in the peri-infarct region and in GFAP positive reactive astrocytes in the glial scar surrounding the infarct (Fig. 2c). In contrast, CD68 positive monocytic phagocytes and GST-pi positive oligodendrocytes were not immunoreactive for TR (Additional file 1: Figure S4).

Importantly, treatment with T_3 or T_4 at 5 or 50 μg/kg did not change the levels of TRα1 (Fig. 2d), despite there was a nonsignificant elevation of TRα1 protein levels found in protein extracts obtained from the peri-infarct area (0.67 ± 0.22 Vh, 0.15 ± 0.05 T_3, 1.15 ± 0.12 T_4; arbitrary units, mean ± SEM). Likewise, no changes have been found in TRβ1 levels (1.59 ± 0.51 Vh, 1.61 ± 0.20 T_3, 1.74 ± 0.42 T_4; arbitrary units, mean ± SEM) (Fig. 2e).

Thyroid hormone receptor pattern expression in human stroke patients

Both receptor isoforms were also found in *post-mortem* brain tissues. The levels for both isoforms did not differ between the peri-infarct area from stroke patients and cortex samples from non-stroke patients. However, differences were observed in the infarct core. Here, TRβ1 protein levels increased (0.37 ± 0.02 Ctrl, 0.32 ± 0.03 PI, 0.63 ± 0.07 IC; arbitrary units, mean ± SEM) while levels of TRα1 decreased (0.85 ± 0.13 Ctrl, 0.91 ± 0.16 PI, 0.38 ± 0.15 IC; arbitrary units, mean ± SEM) (Fig. 2f, g).

Treatment with T_3 increases dendritic spine density in principal neurons and modulates synaptic neurotransmission

Using Thy1-YFP transgenic mice, we performed a second study to evaluate if T_3 at 50 μg/kg was involved in modulation of dendritic spine density and morphology as an estimate of structural plasticity in the postischemic brain. The study design including surgeries and treatment with T_3 at 50 μg/kg or Vh were adopted from *Study I*. To determine the possibility of formation of new synaptic connections 14 days after T_3 administration, we evaluated dendritic spine density and morphologic classification in four regions corresponding to the peri-infarct area and remote areas to stroke (Fig. 3a).

Infarct volumes did not differ between the treatments (1.0 ± 0.45 mm^3 Vh, 1.32 ± 0.41 mm^3 T_3; mean ± SEM) and did not affect dendritic spines in regions of interest. Representative dendritic branches from mice treated either with T_3 at 50 μg/kg or Vh are shown in Fig. 3b. Each dendritic spine was classified as mushroom, thin or stubby using the NeuronStudio software (Fig. 3c).

Throughout all three levels covering the anterior, middle and posterior peri-infarct area and homotypic regions of the contralateral hemisphere, the overall number of dendritic spines was increased in T_3-treated animals compared to Vh-treated animals. In particular, a significant increase in mushroom type spines was observed in R1, level 1, thin spines in R3 and R4 from level 1 and R1 and R2 from level 2 and stubby spines in R2 and R4 of levels 2 and 3 ($p < 0.001$, all regions). Together, we found an increment of dendritic spine density

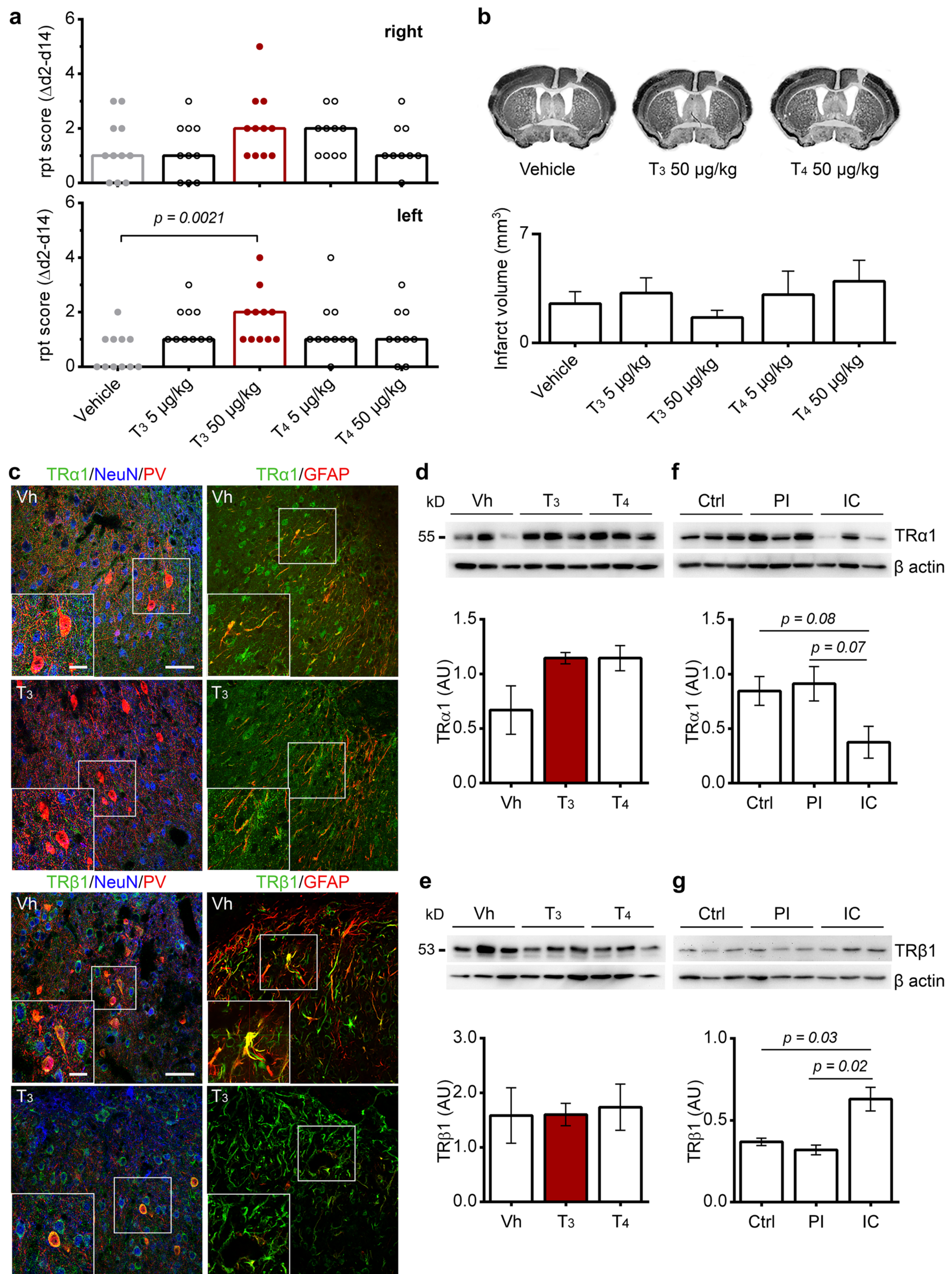


Fig. 2 (See legend on next page.)

(See figure on previous page.)
Fig. 2 Treatment with T_3 50 μg/kg improves functional recovery 14 days after photothrombosis (PT) without affecting infarct size. **a** Difference between the rotating pole test (rpt) scores from day 2 (selective sorting) and 14 (Δd2-d14) at 10 rotations per minute to the right and to the left sides, from mice subjected to PT (right hemisphere). Scores are shown as individual data and group median. Statistical analysis was performed by Kruskal-Wallis test followed by Mann-Whitney test ($p = 0.0021$ in T_3 50 μg/kg versus Vehicle treatment). Vehicle ($n = 11$), T_3 5 μg/kg ($n = 10$), T_3 50 μg/kg ($n = 11$), T_4 5 μg/kg ($n = 10$), T_4 50 μg/kg ($n = 9$). **b** Representative coronal brain sections from stroke mice treated with Vehicle, T_3 50 μg/kg or T_4 50 μg/kg. Staining with NeuN was performed to measure cortical infarcts. Infarct volumes are displayed as means ± SEM. On day 14 after PT in mice treated with Vehicle ($n = 10$), T_3 5 μg/kg ($n = 8$), T_3 50 μg/kg ($n = 8$), T_4 5 μg/kg ($n = 7$) or T_4 50 μg/kg ($n = 7$). Statistical analysis was performed by one-way ANOVA and Bonferroni's multiple comparisons test. **c** Thyroid hormone receptors (TR) α1 and TRβ1 (AF488, green) expression in mouse brain cell populations. Both TR isoforms are expressed in NeuN (Cy5, blue) positive neurons and Parvalbumin (Cy3, red) positive neurons. GFAP (Cy3, red) immunoreactive astrocytes express TRβ1 in the ischemic territory, 14 days after PT. Scale bars 50 μm and 10 μm for insets at higher magnification. **d** Levels of TRα1 and **e** TRβ1 in the infarct core and peri-infarct area were analyzed 14 days after PT and after treatment with Vehicle ($n = 3$), T_3 50 μg/kg ($n = 3$) or T_4 50 μg/kg ($n = 3$). No difference was observed in levels of TRα1 and TRβ1. **f** Levels of TRα1 and **g** TRβ1 in the grey matter of human brain in non-stroke (Ctrl), and stroke cases, including the peri-infarct (PI) and infarct core (IC). For uncropped images of western blots see Additional file 1: Figure S5. Levels of TRβ1 are increased in the IC. Statistical analysis was performed by One-way ANOVA and Bonferroni's multiple comparisons test. Two-tailed unpaired Student's *t* test was employed to determine *p* values. Data are expressed as mean ± SEM

in T_3-treated animals, in all regions and sections analyzed, particularly in the region correspondent of ipsilateral somatosensory cortex (Fig. 3d).

These findings prompted us to investigate if treatment with T_3 at 50 μg/kg modulates pre- and/or postsynaptic proteins, which reflects structural changes in dendritic spines and the number of functional synapses relevant for synaptic neurotransmission in the peri-infarct area. We observed no differences in the level of the presynaptic synaptophysin and the PSD95 (Fig. 4). Likewise, no differences were detected in NMDAR1. Interestingly, we found that glutamate receptor 2 (GluR2), one of the AMPA receptor subunits, was significantly increased in mice subjected to PT and treated with T_3 while levels of GluR1 remained stable (Fig. 4). Accompanied we found increased levels of synaptotagmin 1&2. In sham operated control experiments, treatment with T_3 had no effect on all studied proteins.

Synaptotagmins are downregulated by T_3 in an in vitro model of ischemia and are downregulated in the infarct core of human stroke

The finding that T_3 at 50 μg/kg modulates levels of synaptotagmin 1&2 in vivo prompted us to evaluate its expression in OGD-treated neuronal cultures pre-treated with T_3 1 μM for 48 h. Levels of synaptotagmin were significantly decreased in neuronal cultures in the presence of T_3 (Fig. 5a). This pre-synaptic protein was also expressed in the ischemic territory of stroke patients, being significantly reduced in the infarct core (Fig. 5b).

T_3 inhibits glutamate evoked currents in glutamatergic cortical neurons

To study the relevance of T_3 for neuron function we used the method of voltage ramp to establish information about the I-V relations of calcium permeable NMDA and AMPA post-synaptic receptors in the presence and absence of T_3. For each cell tested, membrane current amplitudes were normalized in order to obtain current density (pA/pF).

Glutamatergic neurons responsiveness to T_3 stimulation was consistent with the positive immunoreactivity for TRα1 and TRβ1 (Fig. 5c). Application of agonist glutamate at 50 μM and NMDA co-agonist glycine at 3 μM elicited an inward component at negative potentials. Glycine together with glutamate potentiated the glutamate induced current, even in the presence of Mg^{2+} in the extracellular bath. We also examined the possibility of glycine to induce currents by itself. Application of glycine at 3 μM did not induce a current in any of the neurons tested (Additional file 1: Figure S8). We also tested if response was mediated by postsynaptic iGluRs NMDA and AMPA, by application of non-competitive antagonists MK-801 and CNQX at 10 μM, respectively. After application, currents were almost reversed (Fig. 5d). Similarly, to the application of the antagonists, currents are also almost reversed after washout with extracellular bath (data not shown).

Compared with cells in control conditions, the presence of T_3 (1 μM) in cell cultures for 48 h before the experiments significantly decreased glutamate / glycine response in the neurons analyzed (Fig. 5e).

T_3 downregulates GABA synthesis and activity of cortical Parvalbumin immunoreactive cells

To determine whether functional recovery mediated by i.p. injection of T_3 at 50 μg/kg modulates GABAergic signaling, we evaluated GAD 65/67 expression in stroke mice treated with T_3 compared with Vh. Longterm administration of T_3 50 μg/kg for 14 days after ischemic stroke significantly reduced GAD 65/67 expression in the ischemic territory. In sham operated animals, administration of T_3 did not alter the expression of GAD 65/67 (Fig. 6a).

To understand the significance of lower GAD 65/67 expression in animals treated with T_3, we assessed the

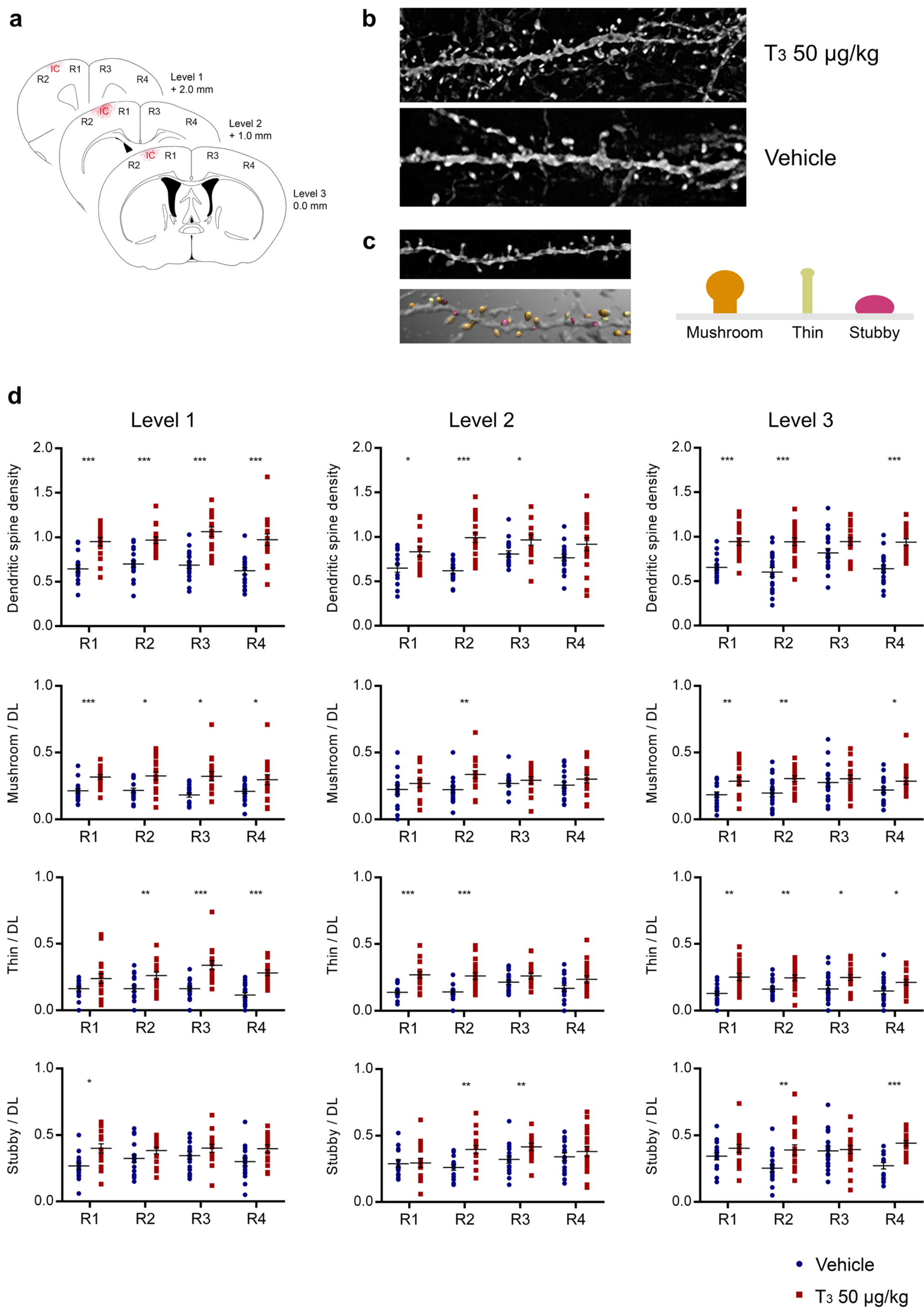


Fig. 3 (See legend on next page.)

(See figure on previous page.)
Fig. 3 Treatment with T_3 50 μg/kg increases dendritic spine density 14 days after photothrombosis (PT). **a** Dendritic spine analysis 14 days after PT at different distances from bregma correspondent to the rostral pole (level 1), center (level 2) and caudal pole (level 3) of the cortical infarct. The regions analyzed correspond to the ipsilateral (R1) and contralateral (R3) motor cortex; and ipsilateral (R2) and contralateral (R4) somatosensory cortex. **b** Representative dendritic segments from animals treated with T_3 50 μg/kg ($n = 4$) and Vehicle (Vh; $n = 4$). **c** Apical dendritic spines from cortex layers II/III were automatically detected by NeuronStudio software and classified as mushroom, thin or stubby. Three to five dendritic segments were analyzed per animal. **d** Dendritic spine density (number of total spines / dendritic length) per region and classification of dendritic spines as mushroom, thin or stubby and their density per region, at each level analyzed. Results are displayed as means ± SEM. Statistical analysis was performed with two-tailed unpaired Student *t*-test, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

activity of cortical PV neurons, a class of interneurons that regulate GABA neurotransmission. PV immunoreactivity was co-localized with the activity-dependent marker c-fos, through NovaRED Peroxidase (PV) and DAB (c-fos) immunohistochemistry (Fig. 6b, c). As shown in Fig. 6d, there is a significant reduction in PV^+ / c-fos^+ ratio between Vh and T_3 50 μg/kg-treated animals in the peri-infarct region (40.46 ± 4.26 Vh; 14.62 ± 3.4 T_3 50 μg/kg; mean ± SEM) and the homotypic region in the contralateral hemisphere (43.61 ± 9.43 Vh, 11.54 ± 4.12 T_3 50 μg/kg; mean ± SEM). In contrast, treatment with T_4 50 μg/kg did not change the activity of PV^+ cells in the same regions (Ipsilateral 33.97 ± 7.59; Contralateral 35.91 ± 2.65; mean ± SEM). Importantly, treatment with TH did not influence the total number of PV immunoreactive cells in the ipsilateral and contralateral hemispheres (Fig. 6d).

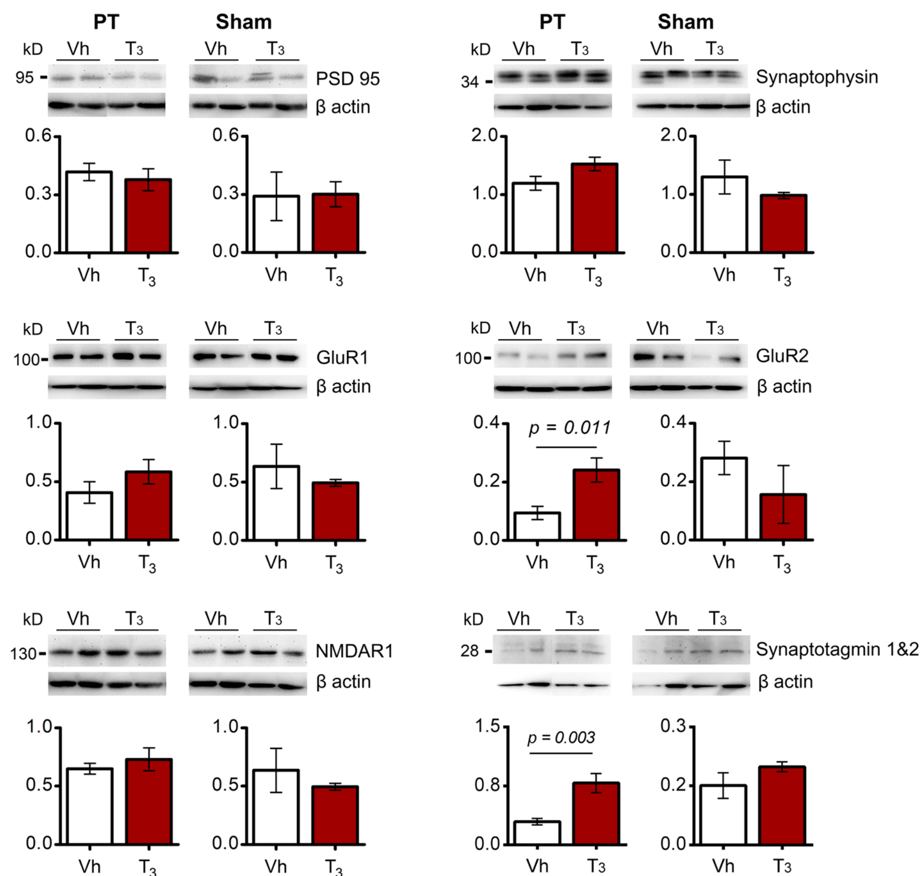


Fig. 4 Levels of synaptic proteins in the infarct core and peri-infarct area 14 days after photothrombosis (PT) or in the homotypic area in sham operated mice have been analyzed after treatment with Vehicle (Vh; $n = 6$ for PT and $n = 3$ for sham) or T_3 50 μg/kg ($n = 6$ for PT and $n = 3$ for sham). There are no significant differences between levels of postsynaptic density protein 95 (PSD95), synaptophysin, glutamate receptor 1 (GluR1) and NMDA receptor 1 in the infarct core and peri-infarct in T_3-treated mice compared with Vh. Levels of AMPA receptor subunit GluR2 and synaptotagmin 1&2 are increased in the infarct core and peri-infarct in T_3-treated mice compared with Vh. Synaptotagmins are vesicle-associated synaptic proteins involved in neurotransmitter release. For uncropped images of western blots see Additional file 1: Figure S6. No differences were observed in sham operated mice. Results are displayed as means ± SEM. Statistical analysis was performed with two-tailed unpaired Student's *t* test

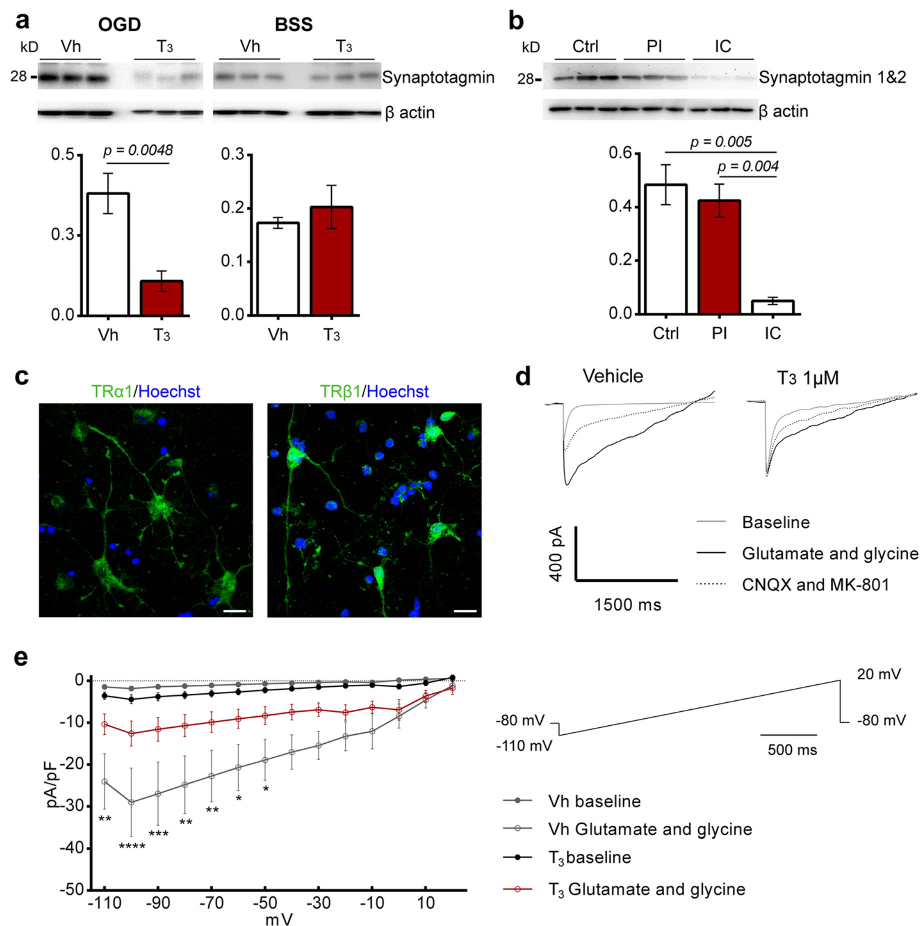


Fig. 5 Treatment with T_3 1 μM for 48 h inhibits iGluRs evoked currents in cultured cortical glutamatergic neurons and downregulates synaptotagmin levels after oxygen and glucose deprivation (OGD). **a** In an in vitro model of acute cerebral ischemia, levels of synaptotagmin are decreased in cells pre-treated with T_3 1 μM for 48 h but no difference was observed in the control conditions (Vh, $n = 5$; T_3 1 μM, $n = 5$). **b** Human brains of stroke and non-stroke control cases have been analyzed for levels of synaptotagmin 1&2. Levels of synaptotagmin are decreased in the infarct core (IC) in comparison with control (Ctrl) and peri-infarct (PI) regions ($n = 3$ for each brain region). For uncropped images of western blots see Additional file 1: Figure S7 **c** Representative images of expression of Thyroid hormone receptors TRα1 and TRβ1 (AF488, green) in cultured cortical glutamatergic neurons. TRα1 was mainly localized in the cytoplasm and TRβ1 was expressed in the cytoplasm and nucleus. Scale bar 20 μm. **d** Representative traces obtained during voltage ramps from − 110 to + 20 mV after application of glutamate 50 μM and glycine 3 μM, held at − 80 mV. After application of AMPA and NMDA antagonists, CNQX and MK-801 respectively, currents were almost fully reverted. **e** I-V relationship of glutamate 50 μM and glycine 3 μM induced current in cortical neurons under voltage clamp condition under the membrane potential of − 80 mV. Each trace is the result of the average of three ramps for each 10 mV (Vh, $n = 3$; T_3 1 μM, $n = 4$). Results are displayed as means ± SEM. Statistical analysis was performed with two-tailed unpaired Student's *t* test to compare glutamate induced currents in cells pre-treated with T_3 1 μM for 48 h, *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Discussion

After an ischemic stroke, there is a disruption of normal neuron function i.e. synaptic activity due to cell death occurring in the infarct core and therefore, disruption in the normal neuronal circuity [87]. As consequence, surviving neurons adjacent to the infarct spontaneously adopt homeostatic mechanisms that contribute to maintain overall excitability, although to a limited extent [18, 21, 48]. The molecular mechanisms of homeostatic processes characterize the recovery phase of ischemic stroke and enhancing those with adjuvant interventions might be a key therapeutic strategy [19]. This may create a wider therapeutic window to optimize and restore lost neurological function.

TH have been recently proposed as a key modulator in stroke [71] and brain injury recovery [41]. The lacking evidence of the underlying mechanisms of TH promoting functional recovery after stroke prompted us to evaluate the role of TH in the post-ischemic brain. Summarizing, our work demonstrates for the first time that T_3 modulates key homeostatic regulatory mechanisms that are crucial to maintain appropriate levels of excitation and mechanisms that stabilize neuronal activity in the post-

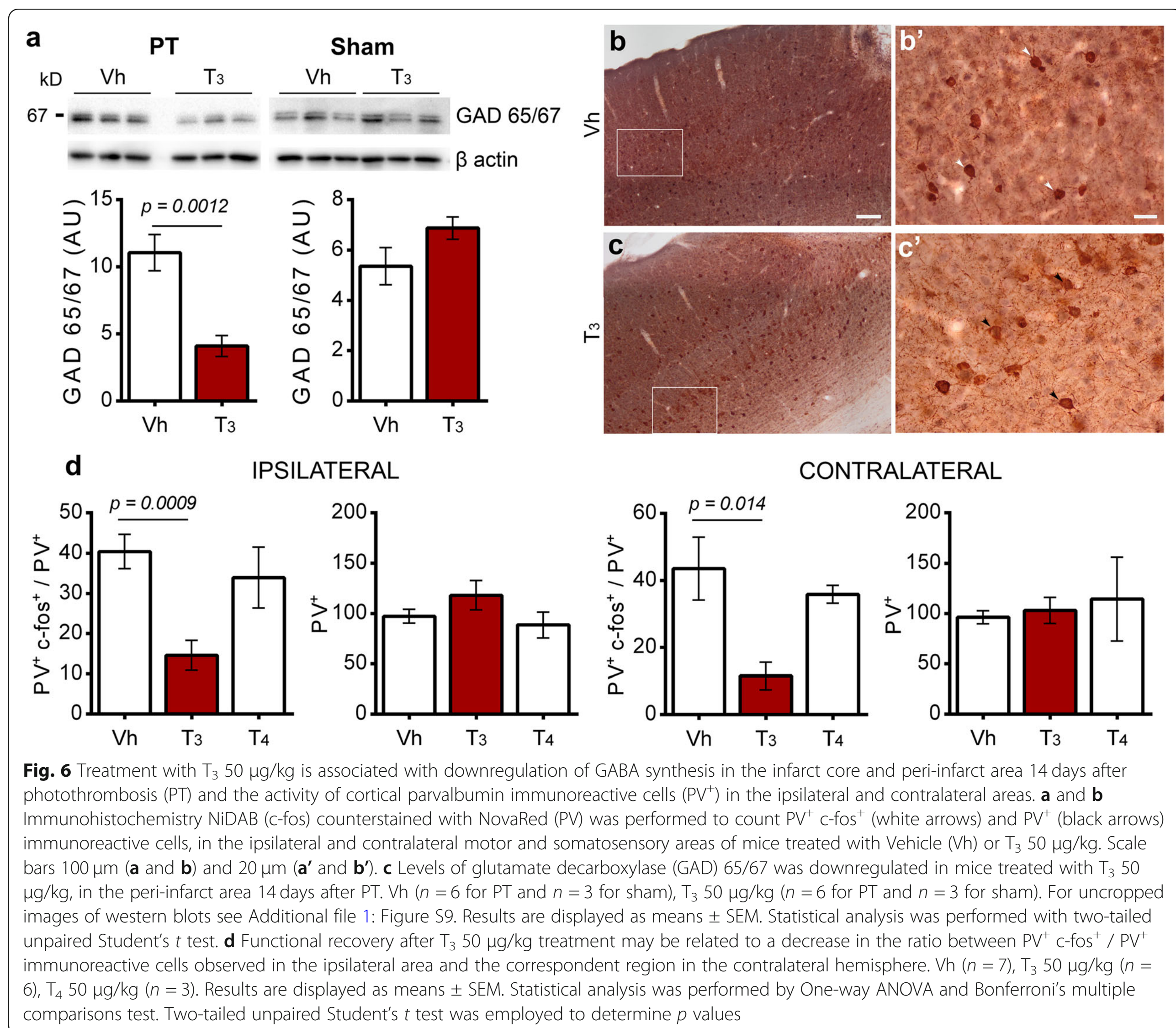


Fig. 6 Treatment with T_3 50 μg/kg is associated with downregulation of GABA synthesis in the infarct core and peri-infarct area 14 days after photothrombosis (PT) and the activity of cortical parvalbumin immunoreactive cells (PV^+) in the ipsilateral and contralateral areas. **a** and **b** Immunohistochemistry NiDAB (c-fos) counterstained with NovaRed (PV) was performed to count PV^+ c-fos^+ (white arrows) and PV^+ (black arrows) immunoreactive cells, in the ipsilateral and contralateral motor and somatosensory areas of mice treated with Vehicle (Vh) or T_3 50 μg/kg. Scale bars 100 μm (**a** and **b**) and 20 μm (**a'** and **b'**). **c** Levels of glutamate decarboxylase (GAD) 65/67 was downregulated in mice treated with T_3 50 μg/kg, in the peri-infarct area 14 days after PT. Vh (n = 6 for PT and n = 3 for sham), T_3 50 μg/kg (n = 6 for PT and n = 3 for sham). For uncropped images of western blots see Additional file 1: Figure S9. Results are displayed as means ± SEM. Statistical analysis was performed with two-tailed unpaired Student's t test. **d** Functional recovery after T_3 50 μg/kg treatment may be related to a decrease in the ratio between PV^+ c-fos^+ / PV^+ immunoreactive cells observed in the ipsilateral area and the correspondent region in the contralateral hemisphere. Vh (n = 7), T_3 50 μg/kg (n = 6), T_4 50 μg/kg (n = 3). Results are displayed as means ± SEM. Statistical analysis was performed by One-way ANOVA and Bonferroni's multiple comparisons test. Two-tailed unpaired Student's t test was employed to determine p values

ischemic brain, contributing to cortical reorganization and to functional recovery.

Given that TH signaling could be related to better outcome, we first assessed behavioral recovery after experimental stroke in mice treated with T_4 or T_3 at 5 or 50 μg/kg. The photothrombotic model adopted for our study induced a well-defined ischemic damage in the primary motor cortex that produced consistent hemiparesis 2 days after stroke [53], allowing behavioral assessment of motor function following ischemia. As expected, all mice spontaneously recovered some of lost motor function over time in analogy to spontaneous recovery in humans [23, 24]. Interestingly, the group treated with T_3 50 μg/kg had significant higher neurological scores 14 days after PT, with no difference in the infarct size compared to control group. However no significant statistical differences were observed in the T_4-treated mice groups. T_4 is the prohormone and it needs to be converted to T_3 before it can exert any biological effect [47]. In the rodent, half of T_3 levels in the brain is provided from its free fraction in blood circulation and cerebrospinal fluid and the other half relies in local deiodination of T_4 in astrocytes and tanycytes, which concentration is regulated by deiodinases activity [44, 74]. Although we did not verify deiodinase expression in the post ischemic brain, the possible scenario is that administration of T_4 is less effective to exert action in the brain, since it still needs to be converted to the active form T_3.

Next, we investigated the key T_3-mechanisms that might contribute for stroke recovery. Taking into account that genomic actions of T_3 in the brain are mainly mediated by binding to TRα1 and TRβ1 [71], we assessed their levels and expression pattern in the post-ischemic mouse brain. TR levels were not altered after

administration of T_4 or T_3 at 50 μg/kg, suggesting that recovery induced by T_3 was mediated by other mechanisms. However, our results do not exclude the possibility that genomic actions in the brain have an impact on stroke recovery also at different temporal and spatial scales, in other animal models or in humans. Indeed, one study reported a reduction of TRβ1 expression in the infarct core compared with unaffected peri-infarct cortex and contralateral hemisphere 14 days after permanent middle cerebral occlusion (MCAO) [43]. We also found that TRβ1 was significantly increased in the infarct core in the human brain, when compared to non-stroke patients. Taken together, we show that cerebral ischemia induces heterogenic changes in human brain TR expression, which may imply an important role for T_3 signaling.

Although TR are mainly nuclear, TRα1 and TRβ1 have been also found in the cytoplasm, which may increase T_3 nuclear import [2]. Interestingly, we observed that TRα1 and TRβ1 was heterogeneously expressed in the cytoplasm of neurons and in reactive astrocytes from the glial scar, in accordance with a previous study performed 14 days after MCAO [43]. However, none of TR isoforms were found in positive GFAP astrocytes in the naïve rodent brain [14]. If TR expression has implications in the formation and function of the glial scar should be the subject for subsequent studies.

Besides genomic actions, other TH-mediated non-genomic mechanisms may contribute for stroke [71] and brain injury [41] recovery. After ischemic stroke, there is an extensive and rapid loss of neurons and degeneration of their axons and dendritic spines in remote areas [87], in both ipsilateral and contralateral cortex [31], leading to a disruption in normal function of neuronal circuits and loss of brain function. In analogy to brain development and learning/plasticity mechanisms, surviving neurons after stroke attempt to stabilize the ratio between excitatory – inhibitory circuits, in order to adjust brain excitability [21]. A wide variety of homeostatic mechanisms might contribute to the maintenance of overall excitability, involving the regulation of neuronal intrinsic excitability and synaptic transmission [52, 76, 77]. Here, we have identified for the first time T_3-modulated mechanisms of homeostatic plasticity that were related to motor recovery after experimental stroke. In particular, we have shown that T_3 modulates plasticity mechanisms that may operate on different temporal and spatial scales as compensatory mechanisms to assure appropriate synaptic neurotransmission.

Dendritic spines are highly dynamic [6, 84] and especially after stroke it occurs an extensive reorganization in dendritic arbors, which includes an increase in spine density and spine turnover [12, 13, 25], particularly in apical cortical pyramidal neurons within the first 2 weeks [11]. In *Study II* we observed overall enhanced cortical reorganization in T_3-treated Thy1-YFP mice reflected in increased spine density in cortical layers II/III, especially in the peri-infarct area, which may contribute for spontaneous recovery. The process of spine formation or spinogenesis includes the formation of thin and long dendritic filopodia that are highly dynamic and establish contact with presynaptic axons. The presence of appropriate signals would result in stabilization of the contact and maturation of filopodia into functional dendritic spines [6]. Interestingly, we found increased density of thin protrusions in T_3-treated animals, especially in the peri-infarct area, although in a temporal scale we could not distinguish newly formed protrusions from the pre-existing ones. We also observed an increased number of mushroom-like spines in the peri-infarct region in all sections analyzed from T_3-treated mice. Although we could not assure that all protrusions are or will be transformed in more stable thin or mushroom-like spines over time, this was a direct finding that T_3 modulated the reorganization of spines in numbers and structure 2 weeks after stroke onset.

Based on these findings, we further evaluated synaptic efficacy. To address this question, we studied levels of pre-synaptic proteins synaptophysin and synaptotagmin, important to regulate endocytosis and exocytosis of synaptic vesicles, respectively [39, 68, 69] and therefore neurotransmitter release. In particular, synaptotagmins are crucial for the docking of synaptic vesicles and fusion with neuron membrane [69]. We demonstrate that in the human ischemic infarct core, levels of synaptotagmin 1&2 were very low due to cell death and loss of synaptic neurotransmission. Nevertheless, their levels in the peri-infarct remained as the same as non-stroke brain tissue, which makes synaptotagmin a molecular target. The increase in synaptotagmin 1&2 levels in the post-ischemic brain of T_3-treated mice supports an increase of neurotransmitter release probability, which in turn may increase synaptic efficacy [9]. In contrast, we observed that synaptotagmin is reduced in OGD T_3-treated cultured glutamatergic neurons, which demonstrated homeostatic regulation by T_3 in order to reduce neurotransmitter release and hyperexcitability in an in vitro model of acute brain ischemia. Synaptotagmin related gene 1 is a TH responsive gene during brain development, regulating synaptic activity and structure [73] and T_4 has been reported to restore synaptotagmin 1 levels to normal in hypothyroid rats [83]. However, how T_3 activates / inhibits synaptic vesicles for synaptotagmin action remains to be elucidated.

Besides neurotransmitter release, efficacy of neurotransmission is dependent on post-synaptic response to

glutamate in neuron terminals, that can be modulated by changing the number or function of iGluRs AMPA and NMDA [10, 51, 75, 76]. Indeed, stroke-induced glutamate release activates AMPA receptors [17] and NMDA receptors [54], changes that are related with excitatory synaptic transmission and motor recovery. Here we show an increase in levels of AMPA receptor subunit GluR2 in the peri-infarct area of mice treated with T_3. The AMPA receptor subunit GluR2 regulates critical aspects of AMPA receptor function, neurotransmission and synaptic plasticity [32, 66] which ultimately contributes to increased excitability in the post-ischemic brain and recovery [67].

We characterized AMPA and NMDA excitatory post-synaptic currents with a voltage-clamp method in cultured glutamatergic neurons pre-treated for 48 h with T_3 1 μM. Interestingly, we found that glutamate evoked currents were significantly lower in neurons previously incubated with T_3. Similarly, in a previous study, T_3 at 10 μM has been implicated in the reduction of miniature excitatory post-synaptic currents frequency and glutamate induced toxicity in hippocampal neurons [42]. Interestingly, we found that T_3 recruits

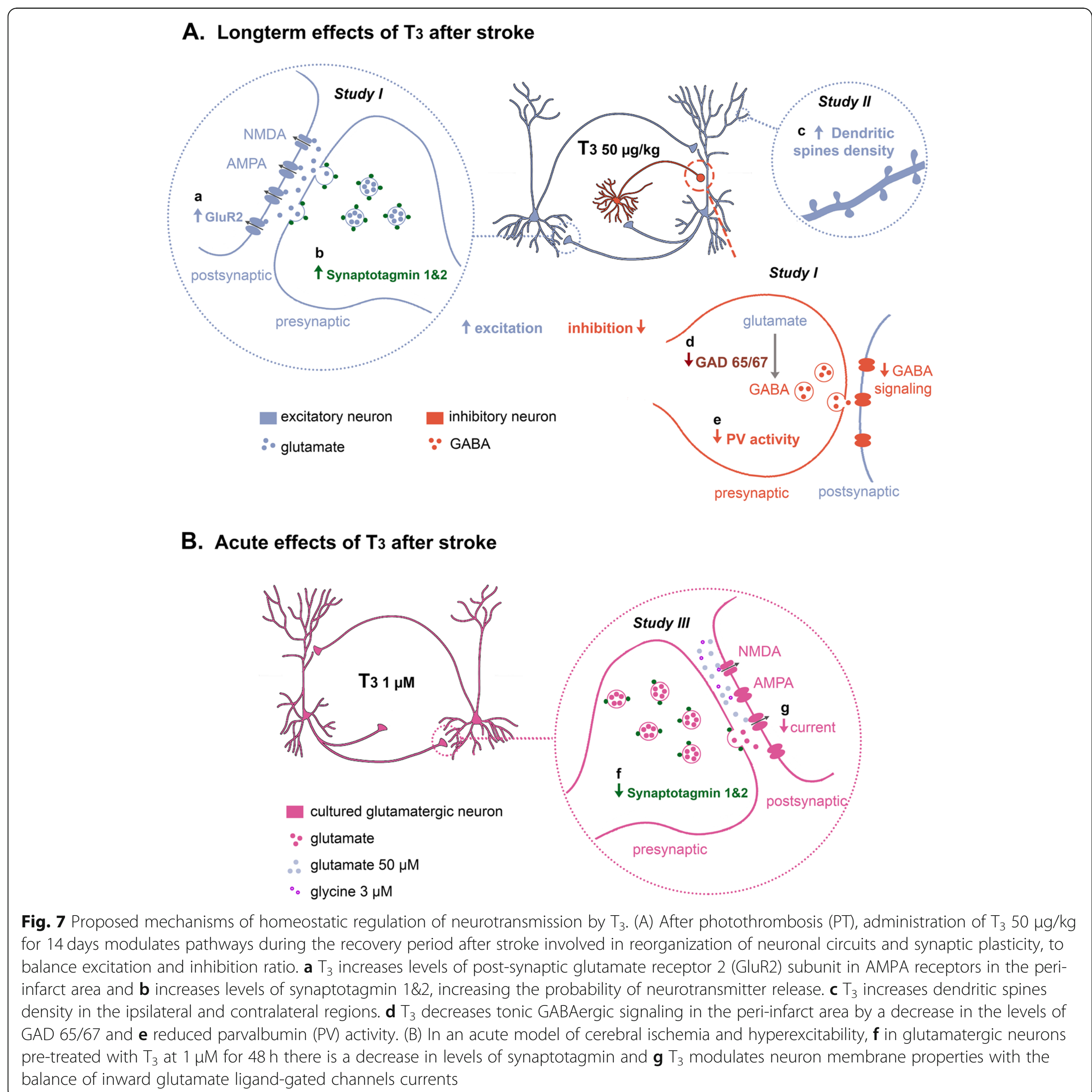


Fig. 7 Proposed mechanisms of homeostatic regulation of neurotransmission by T_3. (A) After photothrombosis (PT), administration of T_3 50 μg/kg for 14 days modulates pathways during the recovery period after stroke involved in reorganization of neuronal circuits and synaptic plasticity, to balance excitation and inhibition ratio. **a** T_3 increases levels of post-synaptic glutamate receptor 2 (GluR2) subunit in AMPA receptors in the peri-infarct area and **b** increases levels of synaptotagmin 1&2, increasing the probability of neurotransmitter release. **c** T_3 increases dendritic spines density in the ipsilateral and contralateral regions. **d** T_3 decreases tonic GABAergic signaling in the peri-infarct area by a decrease in the levels of GAD 65/67 and **e** reduced parvalbumin (PV) activity. (B) In an acute model of cerebral ischemia and hyperexcitability, **f** in glutamatergic neurons pre-treated with T_3 at 1 μM for 48 h there is a decrease in levels of synaptotagmin and **g** T_3 modulates neuron membrane properties with the balance of inward glutamate ligand-gated channels currents

divergent mechanisms to achieve homeostasis in two different systems regarding synaptic network organization, i.e., in vitro and in vivo and dependent on the activation status of neurons and brain tissues, respectively. Important for stroke recovery, T_3 could modulate synaptic neurotransmission to an optimal firing rate.

After an ischemic insult, synaptic glutamate signaling is depressed also due to tonic inhibition of neuronal circuits, which ultimately restricts the process of recovery [8, 15, 80]. Modulation to shift the excitation - inhibition ratio by stimulation of glutamate signaling [15, 17] and reducing GABA inhibition [1, 15, 16] in the motor and somatosensory cortex accelerates motor recovery in mice. GABAergic neurotransmission is mediated by cortical interneurons, a group of cells expressing calcium-binding proteins, including PV. In fact, a correlation between reduction of PV/GABA cells and functional recovery in rodents subjected to stroke has been shown [86]. Also, different therapeutic approaches such as environment enrichment [29], benzodiazepine inverse agonist [1], but also intravenous infusion of human bone marrow mesenchymal stromal cells after transient MCAO [62] decreased cortical PV immunoreactivity or activity and were associated with enhanced recovery of lost neurological function. Treatment with T_3 reduced the activity of PV immunoreactive cells in the peri-infarct area and in the contralateral hemisphere, without affecting the total number of PV^+ cells.

Concomitantly, in the peri-infarct area of animals treated with T_3 50 μg/kg, expression levels of GAD 65/67 was significantly reduced, and directly GABA production. Our results are in accordance with studies describing an increased GAD activity and GABA uptake in neurons in hypothyroid state [34] and the finding that T_3 administration inhibits GABA-induced Cl^- currents [45]. Thus, the decrease in PV cortical activity may facilitate experience dependent plasticity and decrease GABA availability and tonic inhibition, and therefore contribute to restoration of neuronal networks.

Together, our findings reveal important implications of T_3-mediated mechanisms in stroke recovery (Fig. 7). At the cellular and structural level, we demonstrated that T_3 is involved in mechanisms of neuronal plasticity that collectively contributed to functional recovery following experimental stroke. Based on our findings it will be possible to develop specific approaches targeting T_3-mediated mechanisms in the post-ischemic brain. Those may result in specific treatments to be tested in clinical trials.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s40478-019-0866-4.

Additional file 1. Supplementary Methods and Results.

Additional file 2. Rotating pole test mouse 1 selective sorting after photothrombosis.

Additional file 3. Rotating pole test mouse 1 after vehicle treatment at 14 days.

Additional file 4. Rotating pole test mouse 2 selective sorting after photothrombosis.

Additional file 5. Rotating pole test mouse 2 after T_3 treatment (50 μg/kg) at 14 days.

Acknowledgements

This work was supported by funds from the Health Sciences Research Center (CICS-UBI) through National Funds by FCT - Foundation for Science and Technology UID / Multi / 00709/2019 (CRS and IG); by a doctoral fellowship (SFRH/BD/104679/2014) from FCT (DT); partially supported by "Programa Operacional do Centro, Centro 2020" through the funding of the ICON project (Interdisciplinary Challenges on Neurodegeneration; CENTRO-01-0145-FEDER-000013) (ARC, CRS and IG)"; the Swedish Brain Fund; the Crafoord Foundation, Sveriges Stroke Riksförbundet; the Hans-Gabriel and Alice Trolle-Wachtmeister Foundation, The Alborada Research Fund; Sparbanksstiftelsen Färs & Frosta and a regional grant - ALF-Skåne (TW and KR). Image acquisition for dendritic spine analysis was performed in the Microscopy facility of CICS-UBI, a node of PPBI (Portuguese Platform of BioImaging): POCI-01-0145-FEDER-022122.

Authors' contributions

DT and KR designed the research; DT, JF, ARC, TT, EC and KR performed research studies. EE provided human *post-mortem* brain tissues. EC, TW, CRS, IG and KR provided funding and material for all the experiments. EC, CRS, IG and KR supervised the studies. DT wrote the paper with input from all authors. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Author details

[1]Laboratory for Experimental Brain Research, Division of Neurosurgery, Department of Clinical Sciences, Lund University, BMC A13, S-22184 Lund, Sweden. [2]CICS-UBI-Health Sciences Research Centre, Faculdade de Ciências da Saúde, Universidade da Beira Interior, Covilhã, Portugal. [3]Division of Oncology and Pathology, Lund University Hospital, Lund, Sweden. [4]LUBIN Lab - Lunds Laboratorium för Neurokirurgisk Hjärnskadeforskning, Division of Neurosurgery, Department of Clinical Sciences, Lund University, Lund, Sweden.

Received: 29 October 2019 Accepted: 8 December 2019
Published online: 21 December 2019

References

1. Alia C, Spalletti C, Lai S, Panarese A, Micera S, Caleo M (2016) Reducing GABA A-mediated inhibition improves forelimb motor function after focal cortical stroke in mice. Sci Rep 29:37823. https://doi.org/10.1038/srep37823
2. Anyetei-Anum CS, Roggero VR, Allison LA (2018) Thyroid hormone receptor localization in target tissues. J Endocrinol 237:R19–R34. https://doi.org/10.1530/JOE-17-0708
3. Bárez-López S, Guadaño-Ferraz A (2017) Thyroid hormone availability and action during brain development in rodents. Front Cell Neurosci 11:1–7. https://doi.org/10.3389/fncel.2017.00240
4. Benjamin EJ, Blaha MJ, Chiuve SE, Cushman M, Das SR, Deo R et al (2017) Heart disease and stroke Statistics'2017 update: a report from the American Heart Association. Circulation. 135(10):e146–e603. https://doi.org/10.1161/CIR.0000000000000485.
5. Bernal J (2000) Thyroid hormones in brain development and function. [updated 2015 Sep 2]. In: Feingold KR, Anawalt B, Boyce A et al (eds) Endotext. MDText.com, Inc., South Dartmouth, pp 1–136
6. Bhatt DH, Zhang S, Gan W-B (2009) Dendritic spine dynamics. Annu Rev Physiol 71:261–282. https://doi.org/10.1146/annurev.physiol.010908.163140
7. Biernaskie J (2004) Efficacy of rehabilitative experience declines with time after focal ischemic brain injury. J Neurosci 24:1245–1254. https://doi.org/10.1523/JNEUROSCI.3834-03.2004

8. Boddington LJ, Reynolds JNJ (2017) Targeting interhemispheric inhibition with neuromodulation to enhance stroke rehabilitation. Brain Stimul 10: 214–222. https://doi.org/10.1016/j.brs.2017.01.006
9. Branco T, Staras K (2009) The probability of neurotransmitter release: variability and feedback control at single synapses. Nat Rev Neurosci 10: 373–383. https://doi.org/10.1038/nrn2634
10. Bredt DS, Nicoll RA (2003) AMPA receptor trafficking at excitatory synapses. Neuron 40:361–379. https://doi.org/10.1016/S0896-6273(03)00640-8
11. Brown CE, Boyd JD, Murphy TH (2010) Longitudinal in vivo imaging reveals balanced and branch-specific remodeling of mature cortical pyramidal dendritic arbors after stroke. J Cereb Blood Flow Metab 30:783–791. https://doi.org/10.1038/jcbfm.2009.241
12. Brown CE, Li P, Boyd JD, Delaney KR, Murphy TH (2007) Extensive turnover of dendritic spines and vascular remodeling in cortical tissues recovering from stroke. J Neurosci 27:4101–4109. https://doi.org/10.1523/JNEUROSCI.4295-06.2007
13. Brown CE, Wong C, Murphy TH (2008) Rapid morphologic plasticity of peri-infarct dendritic spines after focal ischemic stroke. Stroke 39:1286–1291. https://doi.org/10.1161/STROKEAHA.107.498238
14. Carlson DJ, Strait KA, Schwartz HL, Oppenheimer JH (1994) Immunofluorescent localization of thyroid hormone receptor isoforms in glial cells of rat brain. Endocrinology 135:1831–1836. https://doi.org/10.1210/endo.135.5.7525253
15. Carmichael ST (2012) Brain excitability in stroke: the yin and yang of stroke progression. Arch Neurol 69:161–167. https://doi.org/10.1001/archneurol.2011.1175
16. Clarkson AN, Huang BS, Macisaac SE, Mody I, Carmichael ST (2010) Reducing excessive GABA-mediated tonic inhibition promotes functional recovery after stroke. Nature 468:305–309. https://doi.org/10.1038/nature09511
17. Clarkson AN, Overman JJ, Zhong S, Mueller R, Lynch G, Carmichael ST (2011) AMPA receptor-induced local brain-derived Neurotrophic factor signaling mediates motor recovery after stroke. J Neurosci. https://doi.org/10.1523/JNEUROSCI.5780-10.2011
18. Cramer SC (2008) Repairing the human brain after stroke: I. mechanisms of spontaneous recovery. Ann Neurol 63:272–287. https://doi.org/10.1002/ana.21393
19. Cramer SC (2018) Treatments to promote neural repair after stroke. J Stroke 20:57–60. https://doi.org/10.5853/jos.2017.02796
20. Crupi R, Paterniti I, Campolo M, Di Paola R, Cuzzocrea S, Esposito E (2013) Exogenous T3 administration provides neuroprotection in a murine model of traumatic brain injury. Pharmacol Res 70:80–89. https://doi.org/10.1016/j.phrs.2012.12.009
21. Dalise S, Ambrosio F, Modo M (2014) Adaptive plasticity and recovery in preclinical models of stroke. Arch Ital Biol 152:190–215. https://doi.org/10.12871/00039829201442
22. Doyle KP, Suchland KL, Ciesielski TMP, Lessov NS, Grandy DK, Scanlan TS et al (2007) Novel thyroxine derivatives, thyronamine and 3-iodothyronamine, induce transient hypothermia and marked neuroprotection against stroke injury. Stroke 38:2569–2576. https://doi.org/10.1161/STROKEAHA.106.480277
23. Dromerick AW, Edwardson MA, Edwards DF, Giannetti ML, Barth J, Brady KP et al (2015) Critical periods after stroke study: translating animal stroke recovery experiments into a clinical trial. Front Hum Neurosci 9:231. https://doi.org/10.3389/fnhum.2015.00231
24. Duncan PW, Min Lai S, Keighley J (2000) Defining post-stroke recovery: implications for design and interpretation of drug trials. Neuropharmacology 39:835–841. https://doi.org/10.1016/S0028-3908(00)00003-4
25. Enright LE, Zhang S, Murphy TH (2007) Fine mapping of the spatial relationship between acute ischemia and dendritic structure indicates selective vulnerability of layer V neuron dendritic tufts within single neurons in vivo. J Cereb Blood Flow Metab 27:1185–1200. https://doi.org/10.1038/sj.jcbfm.9600428
26. Feigin VL, Norrving B, Mensah GA (2017) Global burden of stroke. Circ Res 120:439–448. https://doi.org/10.1161/CIRCRESAHA.116.308413
27. Genovese T, Impellizzeri D, Ahmad A, Cornelius C, Campolo M, Cuzzocrea S et al (2013) Post-ischaemic thyroid hormone treatment in a rat model of acute stroke. Brain Res 1513:92–102. https://doi.org/10.1016/j.brainres.2013.03.001
28. Gothié JD, Demeneix B, Remaud S (2017) Comparative approaches to understanding thyroid hormone regulation of neurogenesis. Mol Cell Endocrinol 459:104–115. https://doi.org/10.1016/j.mce.2017.05.020
29. Hakon J, Quattromani MJ, Sjölund C, Tomasevic G, Carey L, Lee JM et al (2018) Multisensory stimulation improves functional recovery and resting-state functional connectivity in the mouse brain after stroke. NeuroImage Clin 17:717–730. https://doi.org/10.1016/j.nicl.2017.11.022
30. Harris KM, Jensen FE, Tsao B (1992) Three-dimensional structure of dendritic spines and synapses in rat hippocampus (CA1) at postnatal day 15 and adult ages: implications for the maturation of synaptic physiology and long-term potentiation. J Neurosci 12:2685–2705
31. Huang SY, Chang CH, Hung HY, Lin YW, Lee EJ (2018) Neuroanatomical and electrophysiological recovery in the contralateral intact cortex following transient focal cerebral ischemia in rats. Neurol Res 40:130–138. https://doi.org/10.1080/01616412.2017.1411454
32. Isaac JTR, Ashby M, McBain CJ (2007) The role of the GluR2 subunit in AMPA receptor function and synaptic plasticity. Neuron 54:859–871. https://doi.org/10.1016/j.neuron.2007.06.001
33. Jones TA, Adkins DL (2015) Motor system reorganization after stroke: stimulating and training toward perfection. Physiology 30:358–370. https://doi.org/10.1152/physiol.00014.2015
34. Kalaria RN, Prince AK (1985) Effects of thyroid deficiency on the development of cholinergic, GABA, dopaminergic and glutamate neuron markers and DNA concentrations in the rat corpus striatum. Int J Dev Neurosci 3:655–666. https://doi.org/10.1016/0736-5748(85)90056-5
35. Kapoor R, Fanibunda SE, Desouza LA, Guha SK, Vaidya VA (2015) Perspectives on thyroid hormone action in adult neurogenesis. J Neurochem 133:599–616. https://doi.org/10.1111/jnc.13093
36. Kim JT, Fonarow GC, Smith EE, Reeves MJ, Navalkele DD, Grotta JC et al (2017) Treatment with tissue plasminogen activator in the Golden hour and the shape of the 4.5-hour time-benefit curve in the National United States get with the Guidelines-Stroke Population. Circulation 135:128–139. https://doi.org/10.1161/CIRCULATIONAHA.116.023336
37. Knecht S, Hesse S, Oster P (2011) Rehabilitation after stroke. Dtsch Arztebl Int 108:600–606. https://doi.org/10.3238/arztebl.2011.0600
38. Kuric E, Ruscher K (2014) Reduction of rat brain CD8+ T-cells by levodopa/benserazide treatment after experimental stroke. Eur J Neurosci 40:2463–2470. https://doi.org/10.1111/ejn.12598
39. Kwon SE, Chapman ER (2011) Synaptophysin regulates the kinetics of synaptic vesicle endocytosis in central neurons. Neuron 70:847–854. https://doi.org/10.1016/j.neuron.2011.04.001
40. Li J, Donangelo I, Abe K, Scremin O, Ke S, Li F et al (2017) Thyroid hormone treatment activates protective pathways in both in vivo and in vitro models of neuronal injury. Mol Cell Endocrinol 452:120–130. https://doi.org/10.1016/j.mce.2017.05.023
41. Liu YY, Brent GA (2018) Thyroid hormone and the brain: mechanisms of action in development and role in protection and promotion of recovery after brain injury. Pharmacol Ther 186:176–185. https://doi.org/10.1016/j.pharmthera.2018.01.007
42. Losi G, Garzon G, Puia G (2008) Nongenomic regulation of glutamatergic neurotransmission in hippocampus by thyroid hormones. Neuroscience 151: 155–163. https://doi.org/10.1016/j.neuroscience.2007.09.064
43. Lourbopoulos A, Mourouzis I, Karapanayiotides T, Nousiopoulou E, Chatzigeorgiou S, Mavridis T et al (2014) Changes in thyroid hormone receptors after permanent cerebral ischemia in male rats. J Mol Neurosci 54: 78–91. https://doi.org/10.1007/s12031-014-0253-3
44. Maia AL, Kim BW, Huang SA, Harney JW, Larsen PR (2005) Type 2 iodothyronine deiodinase is the major source of plasma T3 in euthyroid humans. J Clin Invest 115:2524–2533. https://doi.org/10.1172/JCI25083
45. Martin JV, Williams DB, Fitzgerald RM, Im HK, Vonvoigtlander PF (1996) Thyroid hormonal modulation of the binding and activity of the GABA(a) receptor complex of brain. Neuroscience 73:705–713. https://doi.org/10.1016/0306-4522(96)00052-8
46. Mdzinarishvili A, Sutariya V, Talasila PK, Geldenhuys WJ, Sadana P (2013) Engineering triiodothyronine (T3) nanoparticle for use in ischemic brain stroke. Drug Deliv Transl Res 3:309–317. https://doi.org/10.1007/s13346-012-0117-8
47. Mullur R, Liu YY, Brent GA (2014) Thyroid hormone regulation of metabolism. Physiol Rev 94:355–382. https://doi.org/10.1152/physrev.00030.2013
48. Overman JJ, Carmichael ST (2014) Plasticity in the injured brain: more than molecules matter. Neuroscientist 20:15–28. https://doi.org/10.1177/1073858413491146
49. Peisker T, Koznar B, Stetkarova I, Widimsky P (2017) Acute stroke therapy: a review. Trends Cardiovasc Med 27:59–66. https://doi.org/10.1016/j.tcm.2016.06.009

50. Pekna M, Pekny M, Nilsson M (2012) Modulation of neural plasticity as a basis for stroke rehabilitation. Stroke 43:2819–2828. https://doi.org/10.1161/STROKEAHA.112.654228
51. Pérez-Otaño I, Ehlers MD (2005) Homeostatic plasticity and NMDA receptor trafficking. Trends Neurosci 28:229–238. https://doi.org/10.1016/j.tins.2005.03.004
52. Pozo K, Goda Y (2010) Unraveling mechanisms of homeostatic synaptic plasticity. Neuron 66:337–351. https://doi.org/10.1016/j.neuron.2010.04.028
53. Quattromani MJ, Cordeau P, Ruscher K, Kriz J, Wieloch T (2014) Enriched housing down-regulates the toll-like receptor 2 response in the mouse brain after experimental stroke. Neurobiol Dis 66:66–73. https://doi.org/10.1016/j.nbd.2014.02.010
54. Que M, Schiene K, Witte OW, Zilles K (1999) Widespread up-regulation of N-methyl-D-aspartate receptors after focal photothrombotic lesion in rat brain. Neurosci Lett 273:77–80. https://doi.org/10.1016/S0304-3940(99)00598-4
55. Rami A, Krieglstein J (1992) Thyroxine attenuates hippocampal neuronal damage caused by ischemia in the rat. Life Sci 50:645–650. https://doi.org/10.1016/0024-3205(92)90251-J
56. Remaud S, Gothié JD, Morvan-Dubois G, Demeneix BA (2014) Thyroid hormone signaling and adult neurogenesis in mammals. Front Endocrinol (Lausanne) 5:62. https://doi.org/10.3389/fendo.2014.00062
57. Rickhag M, Deierborg T, Patel S, Ruscher K, Wieloch T (2008) Apolipoprotein D is elevated in oligodendrocytes in the peri-infarct region after experimental stroke: influence of enriched environment. J Cereb Blood Flow Metab 28:551–562. https://doi.org/10.1038/sj.jcbfm.9600552
58. Rodriguez A, Ehlenberger DB, Dickstein DL, Hof PR, Susan L (2008) Automated Three-Dimensional Detection and Shape Classification of Dendritic Spines from Fluorescence Microscopy Images. PLoS One 3:e1997. https://doi.org/10.1371/journal.pone.0001997
59. Ruscher K, Freyer D, Karsch M, Isaev N, Megow D, Sawitzki B et al (2002) Erythropoietin is a paracrine mediator of ischemic tolerance in the brain: evidence from an in vitro model. J Neurosci 22:10291–10301. https://doi.org/10.1523/jneurosci.22-23-10291.2002
60. Ruscher K, Johannesson E, Brugiere E, Erickson A, Rickhag M, Wieloch T (2009) Enriched environment reduces apolipoprotein E (ApoE) in reactive astrocytes and attenuates inflammation of the peri-infarct tissue after experimental stroke. J Cereb Blood Flow Metab 29:1796–1805. https://doi.org/10.1038/jcbfm.2009.96
61. Sadana P, Coughlin L, Burke J, Woods R, Mdzinarishvili A (2015) Anti-edema action of thyroid hormone in MCAO model of ischemic brain stroke: possible association with AQP4 modulation. J Neurol Sci 354:37–45. https://doi.org/10.1016/j.jns.2015.04.042
62. Sammali E, Alia C, Vegliante G, Colombo V, Giordano N, Pischiutta F et al (2017) Intravenous infusion of human bone marrow mesenchymal stromal cells promotes functional recovery and neuroplasticity after ischemic stroke in mice. Sci Rep 7:6962. https://doi.org/10.1038/s41598-017-07274-w
63. Saraiva C, Talhada D, Rai A, Ferreira R, Ferreira L, Bernardino L et al (2018) MicroRNA-124-loaded nanoparticles increase survival and neuronal differentiation of neural stem cells in vitro but do not contribute to stroke outcome in vivo. PLoS One 13:e0193609. https://doi.org/10.1371/journal.pone.0193609
64. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T et al (2012) Fiji: an open-source platform for biological-image analysis. Nat Methods 9:676–682
65. Schroeter M, Jander S, Stoll G (2002) Non-invasive induction of focal cerebral ischemia in mice by photothrombosis of cortical microvessels: characterization of inflammatory responses. J Neurosci Methods 117:43–49. https://doi.org/10.1016/S0165-0270(02)00072-9
66. Seung HJ, Hyeong SC, Ki JK, Qing ZL, Sung KW (2009) Electrophysiological characterization of AMPA and NMDA receptors in rat dorsal striatum. Korean J Physiol Pharmacol 13:209–214. https://doi.org/10.4196/kjpp.2009.13.3.209
67. Soundarapandian MM, Wei HT, Peng PL, Zervos AS, Lu YM (2005) AMPA receptor subunit GluR2 gates injurious signals in ischemic stroke. Mol Neurobiol 32:145–155. https://doi.org/10.1385/MN:32:2:145
68. Südhof TC (2012) Calcium control of neurotransmitter release. Cold Spring Harb Perspect Biol 4:a011353. https://doi.org/10.1101/cshperspect.a011353
69. Südhof TC (2013) A molecular machine for neurotransmitter release: Synaptotagmin and beyond. Nat Med 19:1227–1231. https://doi.org/10.1038/nm.3338
70. Swanson RA, Morton MT, Tsao-Wu G, Savalos RA, Davidson C, Sharp FR (1990) A semiautomated method for measuring brain infarct volume. J Cereb Blood Flow Metab 10:290–293. https://doi.org/10.1038/jcbfm.1990.47
71. Talhada D, Santos CRA, Gonçalves I, Ruscher K (2019) Thyroid hormones in the brain and their impact in recovery mechanisms after stroke. Front Neurol 10:1103. https://doi.org/10.3389/fneur.2019.01103
72. Tennant KA, Adkins DL, Donlan NA, Asay AL, Thomas N, Kleim JA et al (2011) The organization of the forelimb representation of the C57BL/6 mouse motor cortex as defined by intracortical microstimulation and cytoarchitecture. Cereb Cortex 21:865–876. https://doi.org/10.1093/cercor/bhq159
73. Thompson CC, Potter GB (2000) Thyroid hormone action in neural development. Cereb Cortex 10:939–945. https://doi.org/10.1093/cercor/10.10.939
74. Tu HM, Kim SW, Salvatore D, Bartha T, Legradi G, Larsen PR et al (1997) Regional distribution of type 2 thyroxine deiodinase messenger ribonucleic acid in rat hypothalamus and pituitary and its regulation by thyroid hormone. Endocrinology 138:3359–3368. https://doi.org/10.1210/endo.138.8.5318
75. Turrigiano G (2011) Too many cooks? Intrinsic and synaptic homeostatic mechanisms in cortical circuit refinement. Annu Rev Neurosci 34:89–103. https://doi.org/10.1146/annurev-neuro-060909-153238
76. Turrigiano G (2012) Homeostatic synaptic plasticity: local and global mechanisms for stabilizing neuronal function. Cold Spring Harb Perspect Biol 4:a005736. https://doi.org/10.1101/cshperspect.a005736
77. Turrigiano GG, Nelson SB (2004) Homeostatic plasticity in the developing nervous system. Nat Rev Neurosci 5:97–107. https://doi.org/10.1038/nrn1327
78. Walsh RN, Cummins RA (1976) The open-field test: a critical review. Psychol Bull 83:482–504. https://doi.org/10.1037/0033-2909.83.3.482
79. Walter HL, van der Maten G, Antunes AR, Wieloch T, Ruscher K (2015) Treatment with AMD3100 attenuates the microglial response and improves outcome after experimental stroke. J Neuroinflammation 12:24. https://doi.org/10.1186/s12974-014-0232-1
80. Ward NS (2017) Restoring brain function after stroke — bridging the gap between animals and humans. Nat Rev Neurol 13:244–255. https://doi.org/10.1038/nrneurol.2017.34
81. Watson BD, Dietrich WD, Busto R, Wachtel MS, Ginsberg MD (1985) Induction of reproducible brain infarction by photochemically initiated thrombosis. Ann Neurol 17:497–504. https://doi.org/10.1002/ana.410170513
82. Wieloch T, Nikolich K (2006) Mechanisms of neural plasticity following brain injury. Curr Opin Neurobiol 16:258–264. https://doi.org/10.1016/j.conb.2006.05.011
83. Yang HY, Sun CP, Jia XM, Gui L, Zhu DF, Ma WQ (2012) Effect of thyroxine on SNARE complex and synaptotagmin-1 expression in the prefrontal cortex of rats with adult-onset hypothyroidism. J Endocrinol Investig 35:312–316. https://doi.org/10.3275/7767
84. Yasuda R (2017) Biophysics of biochemical signaling in dendritic spines: implications in synaptic plasticity. Biophys J 113:2152–2159. https://doi.org/10.1016/j.bpj.2017.07.029
85. Yelhekar TD, Druzin M, Karlsson U, Blomqvist E, Johansson S (2016) How to properly measure a current-voltage relation?—interpolation vs. ramp methods applied to studies of GABAA receptors. Front cell Neurosci 10:10. https://doi.org/10.3389/fncel.2016.00010
86. Zeiler SR, Gibson EM, Hoesch RE, Li MY, Worley PF, O'Brien RJ et al (2013) Medial premotor cortex shows a reduction in inhibitory markers and mediates recovery in a mouse model of focal stroke. Stroke 44:483–489. https://doi.org/10.1161/STROKEAHA.112.676940
87. Zhang S (2005) Rapid reversible changes in dendritic spine structure in vivo gated by the degree of ischemia. J Neurosci 25:5333–5338. https://doi.org/10.1523/JNEUROSCI.1085-05.2005

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Online Resource 1 Supplemental Material

Triiodothyronine modulates neuronal plasticity mechanisms to enhance functional outcome after stroke

Daniela Talhada[1,2], Joana Feiteiro[2], Ana Raquel Costa[2], Tiago Talhada[1], Elisa Cairrão[2], Tadeusz Wieloch[1], Elisabet Englund[3], Cecília Reis Santos[2], Isabel Gonçalves[2], Karsten Ruscher[1,4*]

[1]Laboratory for Experimental Brain Research, Division of Neurosurgery, Department of Clinical Sciences, Lund University, Sweden.

[2]CICS-UBI-Health Sciences Research Centre, Faculdade de Ciências da Saúde, Universidade da Beira Interior, Covilhã, Portugal.

[3]Division of Oncology and Pathology, Lund University Hospital, Lund, Sweden.

[4]LUBIN Lab - Lunds Laboratorium för Neurokirurgisk Hjärnskadeforskning, Division of Neurosurgery, Department of Clinical Sciences, Lund University, Sweden.

*__Corresponding Author:__ Dr. Karsten Ruscher, Associate Professor, Laboratory for Experimental Brain Research, Wallenberg Neuroscience Center, Lund University, BMC A13, S-22184 Lund, Sweden. Tel +46-46-2220613. Fax +46-46-2220615. email: karsten.ruscher@med.lu.se

This PDF includes:

Supplementary Figures 1-9

Supplementary Table 1

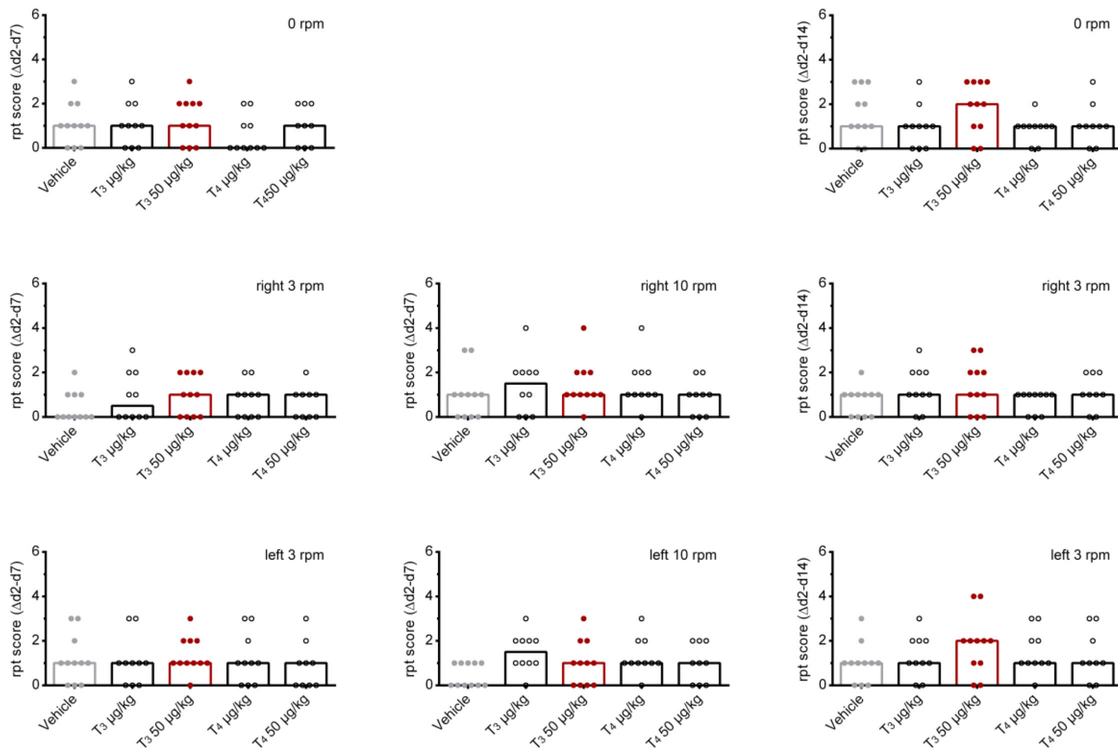
Legends for Supplementary Videos 1-4

Supplementary Methods

Other Supplementary material for this manuscript includes:

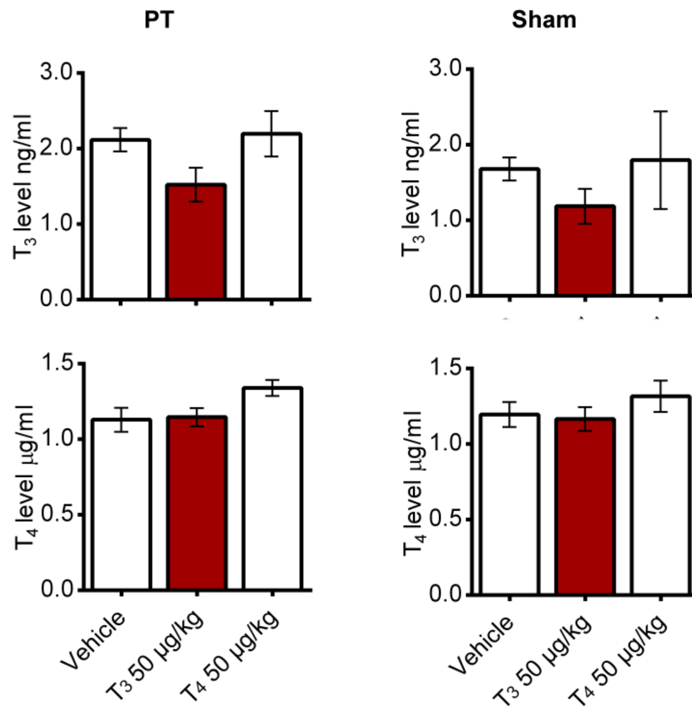
Supplementary Videos 1-4

Supplementary Figures



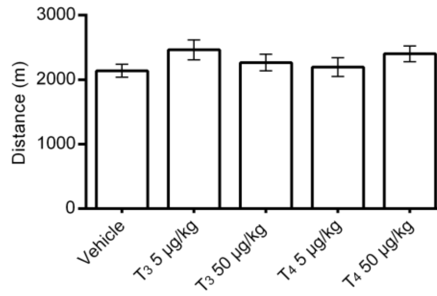
Supplementary Fig. 1

Rotating pole test after photothrombosis (PT). Difference between the rotating pole test (rpt) scores from day two (selective sorting) and seven (Δd2-d7) and from day two and 14 (Δd2-d14) at 0, 3 and 10 rotations per minute (rpm) to the right and to the left sides, from mice subjected to PT (right hemisphere). Scores are shown as individual data and group median. Statistical analysis was performed by Kruskal-Wallis test followed by Mann-Whitney test. Vehicle (n = 11), T_3 5 µg/kg (n = 10), T_3 50 µg/kg (n = 11), T_4 5 µg/kg (n = 10), T_4 50 µg/kg (n = 9)



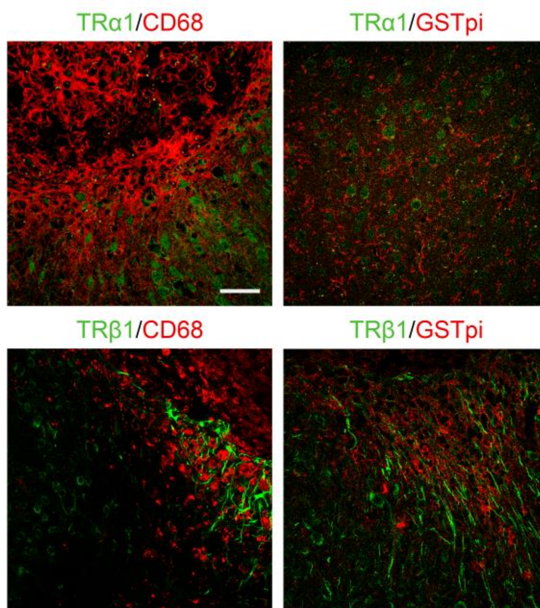
Supplementary Fig. 2

Levels of thyroid hormones in plasma collected from mice 14 days after photothrombosis (PT) or sham surgeries were analyzed after treatment with vehicle, T_3 50 µg/kg or T_4 50 µg/kg (n = 6 for PT and n = 3 for sham, for each condition). Results are displayed as means ± SEM. No statistical differences were seen among experimental groups. Statistical analysis was performed by One-way ANOVA and Bonferroni's multiple comparisons test



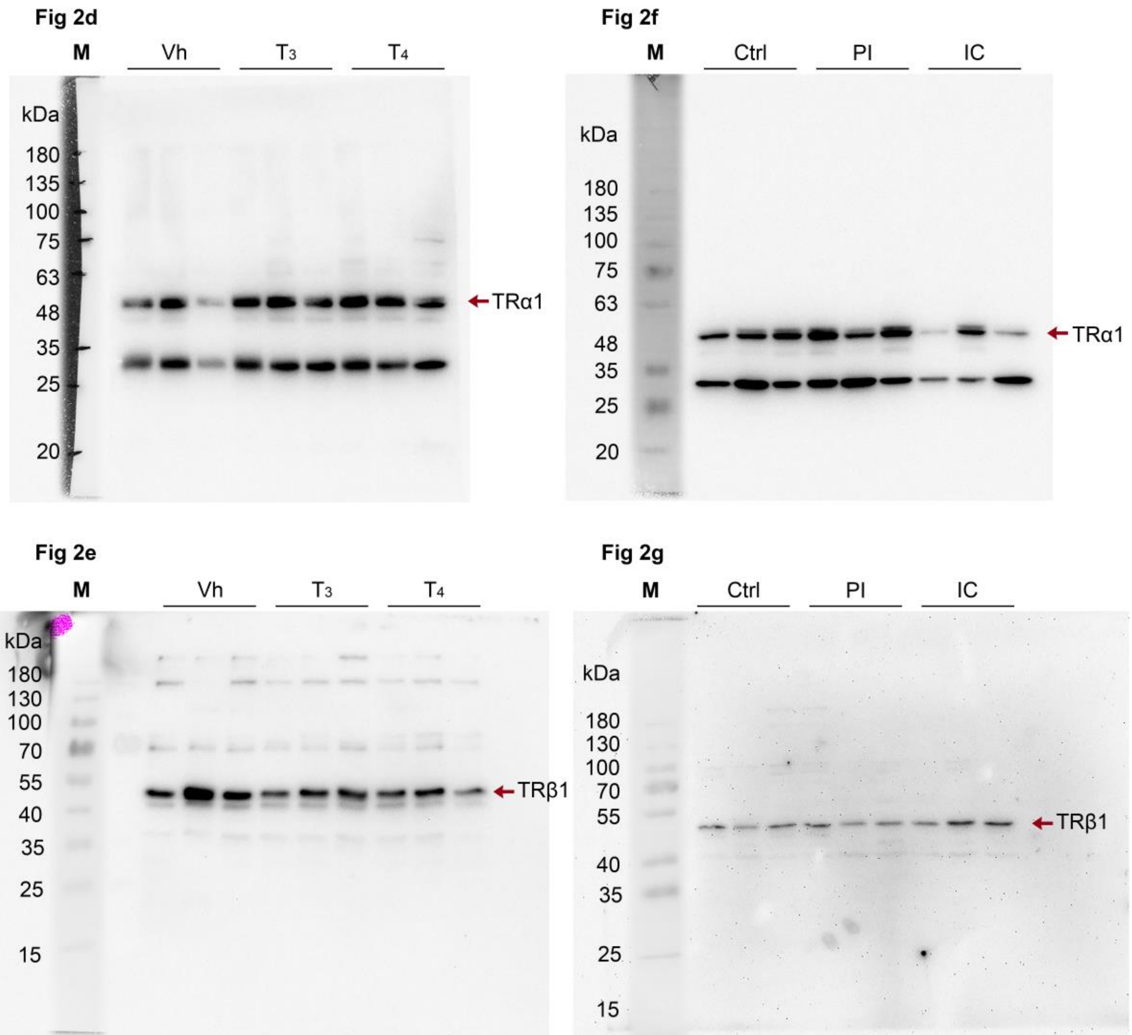
Supplementary Fig. 3

Locomotor activity in the open field test. The total distance traveled by mice did not differ among treatment groups. Statistical analysis was performed by One-way ANOVA and Bonferroni's multiple comparisons test. Data are expressed as mean ± SEM. Vehicle (n = 15), T_3 5 µg/kg (n = 13), T_3 50 µg/kg (n = 13), T_4 5 µg/kg (n = 10), T_4 50 µg/kg (n = 11)



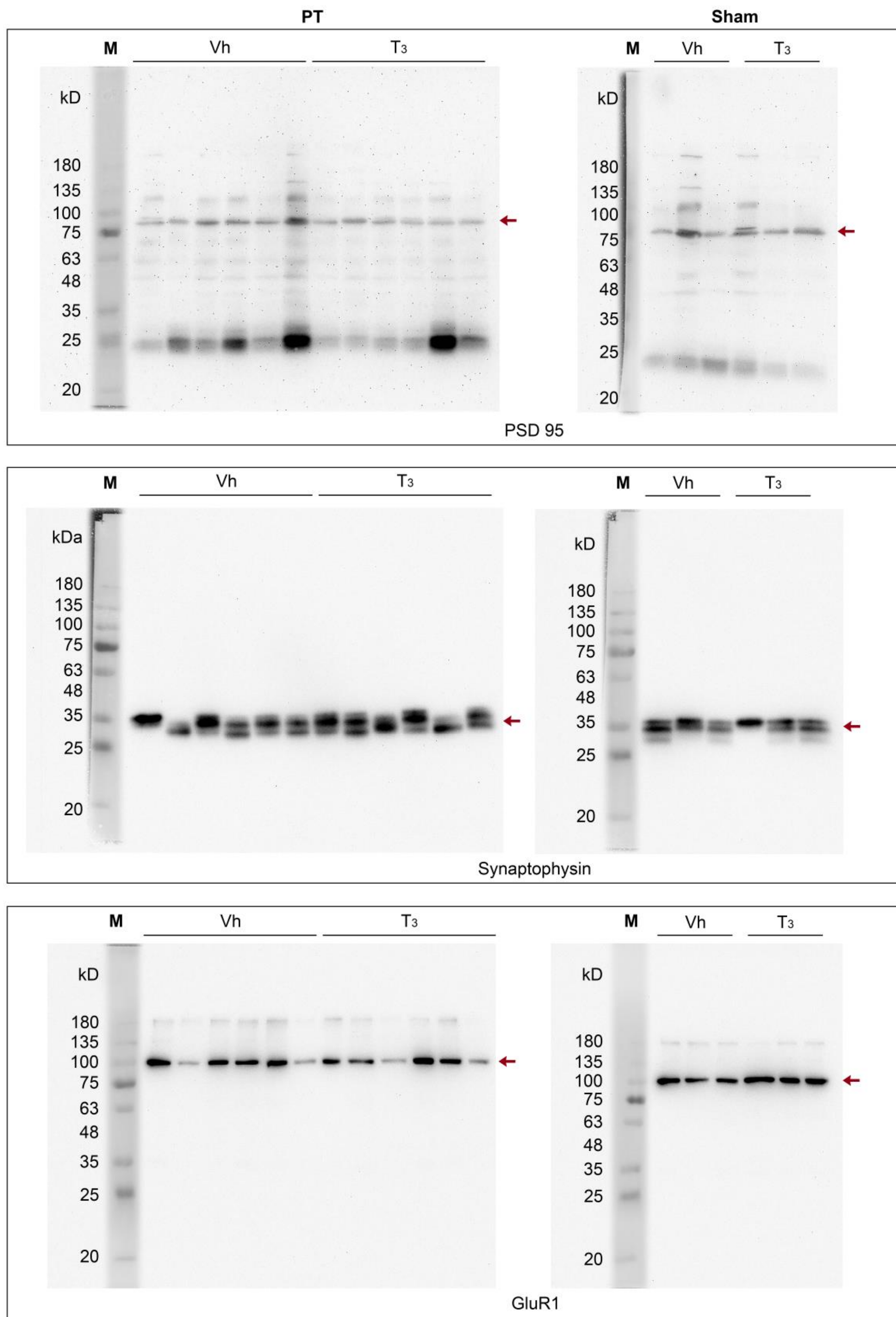
Supplementary Fig. 4

Thyroid hormone receptors (TR) α1 and TRβ1 (AF488, green) expression in mouse brain 14 days after photothrombosis and treated with vehicle (NaCl, 0.9 %). We did not find co-expression in both TR isoforms in CD68 positive microglia (Cy3, red) or GSTpi positive olygodendrocytes (Cy3, red). Scale bar 50 µm



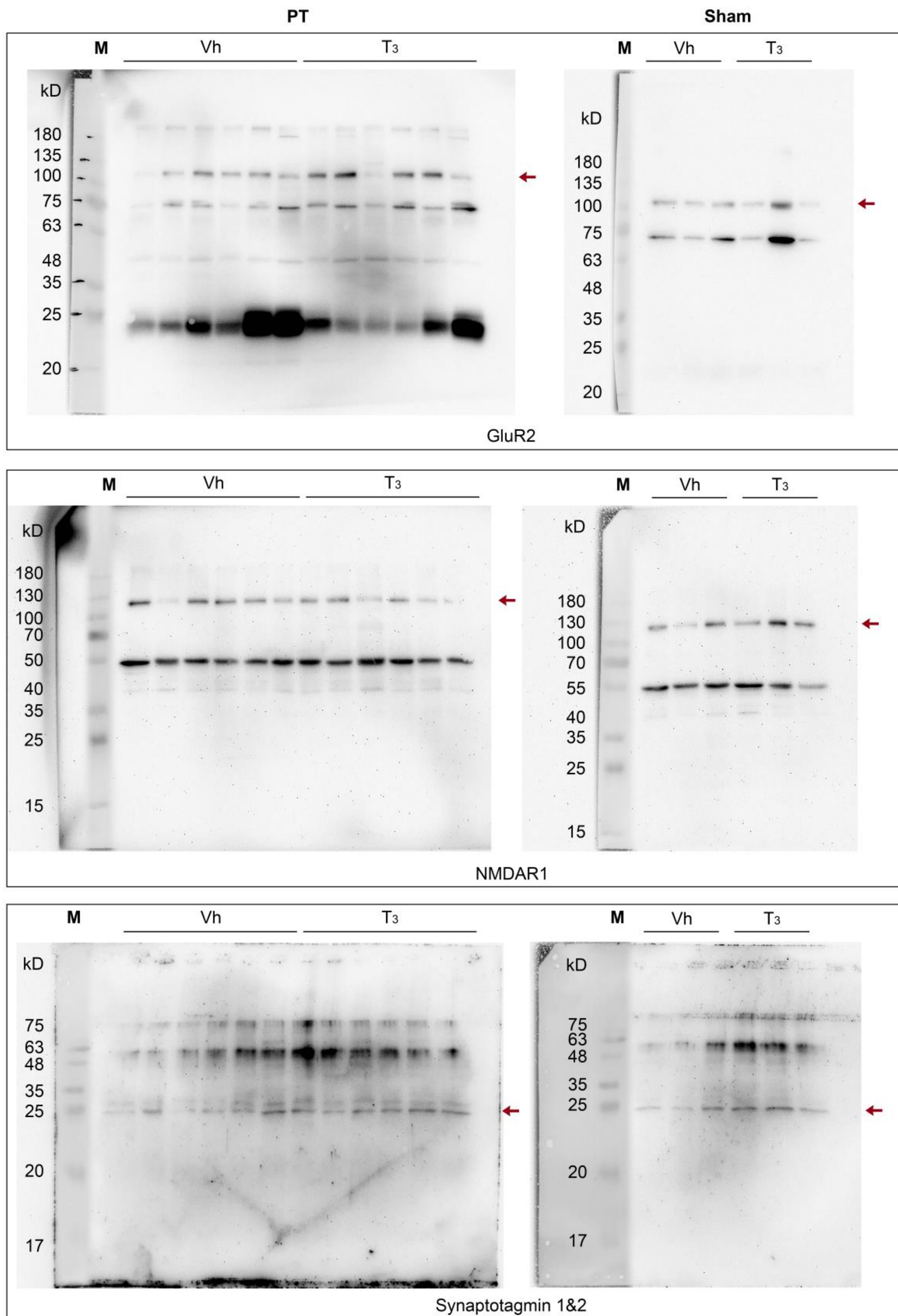
Supplementary Fig. 5

Corresponding uncropped images of western blots shown in Fig. 2 panels d, e, f and g. The protein analyzed is depicted with an arrow



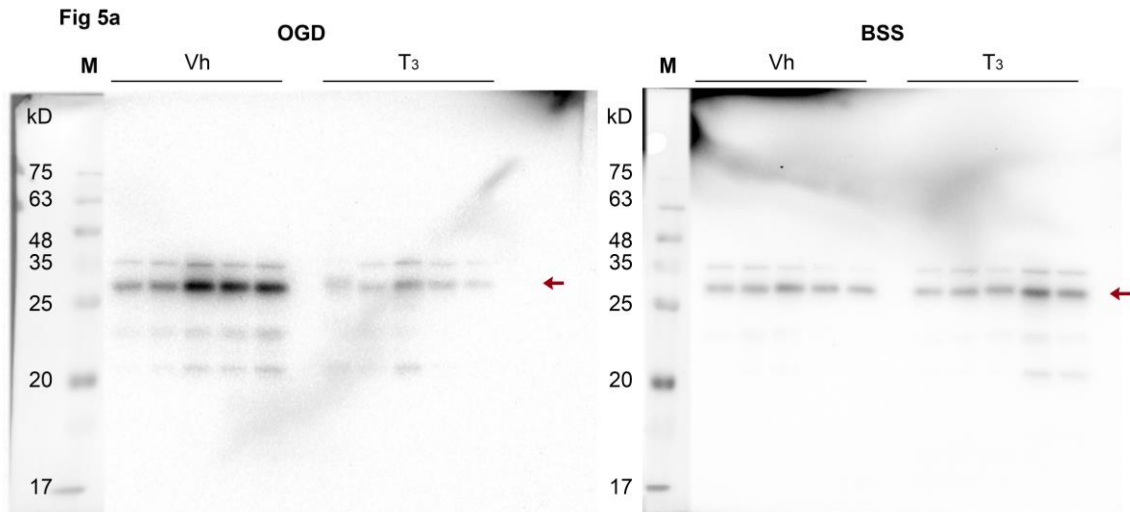
Supplementary Fig. 6 (continue next page)

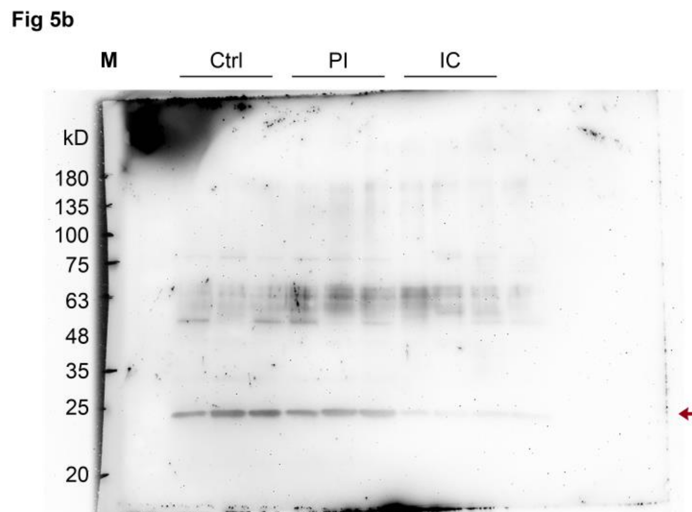
Corresponding uncropped images of western blots shown in Fig. 4. The protein analyzed is depicted with an arrow



Supplementary Fig. 6

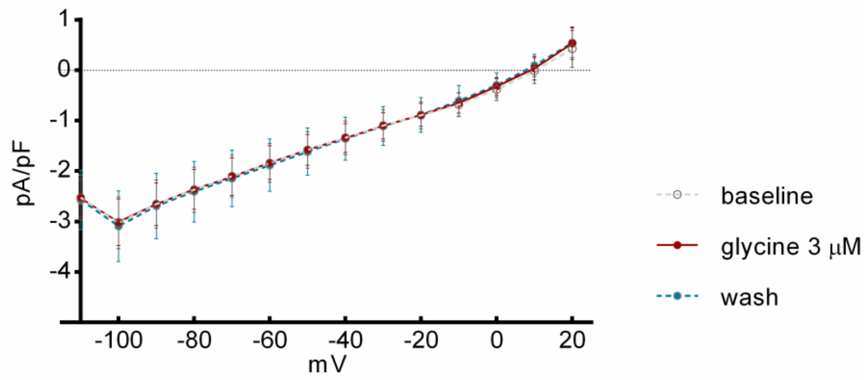
Corresponding uncropped images of western blots shown in Fig. 4. The protein analyzed is depicted with an arrow





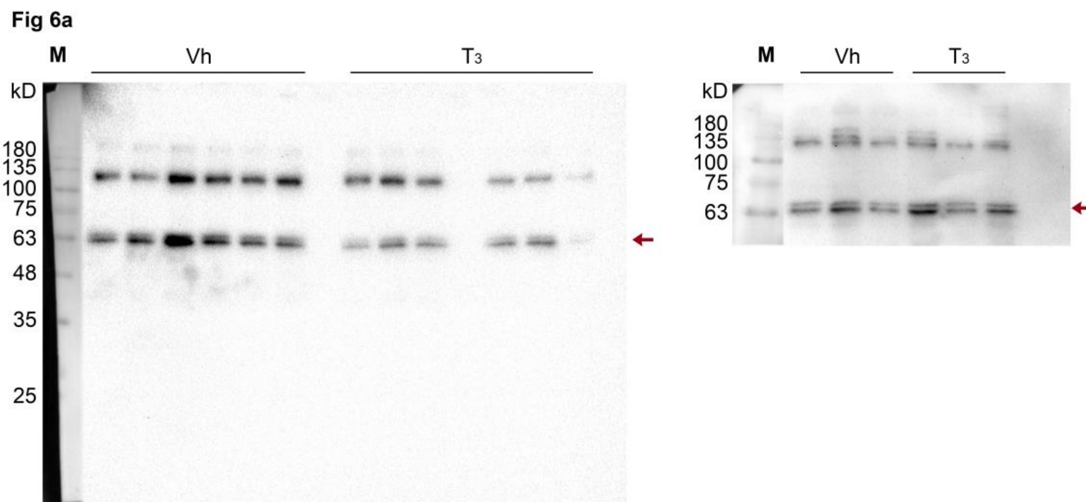
Supplementary Fig. 7

Corresponding uncropped images of western blots for synaptotagmin shown in Fig. 5. The protein analyzed is depicted with an arrow



Supplementary Fig. 8

Glycine did not induce inward currents in cultured glutamatergic neurons (*n* = 3). Representative traces obtained during voltage ramps from -110 to + 20 mV after application of glycine 3 µM, held at -80 mV. Results are displayed as means ± SEM



Supplementary Fig. 9

Corresponding uncropped images of western blots shown in Fig. 6. The protein analyzed GAD 65/67 is depicted with an arrow

Supplementary Video legends

Supplementary Video 1. Selective sorting two days after photothrombosis. The mouse could not transverse the pole at 10 rotations per minute to the left, with a final score of 2.

Supplementary Video 2. The same mouse from Video 1 was treated with vehicle (NaCl 0.9) during 14 days after photothrombosis. The mouse could not transverse the pole at 10 rotations per minute to the left, with a final score of 2.

Supplementary Video 3. Selective sorting two days after photothrombosis. The mouse could not transverse the pole at 10 rotations per minute to the left, with a final score of 2.

Supplementary Video 4. The same mouse from Video 3 was treated with T_3 50 μg/kg during 14 days after photothrombosis. The mouse was able to transverse the pole at 10 rotations per minute to the left, with a final score of 5.

Supplementary Table 1. List of body weights (BW) and temperatures (Temp) from animals included in our experimental studies, before and after days 2, 7 and 14 of photothrombosis (PT) or sham surgeries. Results are displayed as means ± SEM

TREATMENT GROUPS			Before		Day 2		Day 7		Day 14	
			BW g (mean ± SEM)	Temp ºC (mean ± SEM)	BW g (mean ± SEM)	Temp ºC (mean ± SEM)	BW g (mean ± SEM)	Temp ºC (mean ± SEM)	BW g (mean ± SEM)	Temp ºC (mean ± SEM)
***Study I* PT**	Vehicle	(*n* = 17)	23.9 ± 0.4	37.6 ± 0.1	22.5 ± 0.4	37.6 ± 0.2	23.3 ± 0.4	38.1 ± 0.1	23.7 ± 0.4	37.9 ± 0.2
	T_3 5 µg/kg	(*n* = 14)	23.3 ± 0.5	37.5 ± 0.1	21.7 ± 0.4	37.7 ± 0.2	22.1 ± 0.4	38.3 ± 0.2	22.8 ± 0.4	38.0 ± 0.2
	T_3 50 µg/kg	(*n* = 15)	24.0 ± 0.4	37.4 ± 0.2	22.3 ± 0.4	37.4 ± 0.1	23.4 ± 0.3	38.3 ± 0.1	24.7 ± 0.3	37.8 ± 0.1
	T_4 5 µg/kg	(*n* = 13)	23.9 ± 0.5	37.6 ± 0.1	22.4 ± 0.4	37.5 ± 0.1	23.1 ± 0.4	38.0 ± 0.2	23.5 ± 0.4	38.0 ± 0.1
	T_4 50 µg/kg	(*n* = 16)	23.8 ± 0.3	37.7 ± 0.1	22.5 ± 0.3	37.7 ± 0.1	23.2 ± 0.3	38.2 ± 0.1	23.7 ± 0.3	37.8 ± 0.1
***Study I* Sham**	Vehicle	(*n* = 6)	24.1 ± 0.5	37.1 ± 0.3	23.3 ± 0.6	37.5 ± 0.2	23.8 ± 0.6	38.0 ± 0.3	24.3 ± 0.7	38.0 ± 0.4
	T_3 5 µg/kg	(*n* = 6)	24.2 ± 0.9	37.3 ± 0.2	23.1 ± 1.1	37.7 ± 0.1	23.7 ± 1.0	37.7 ± 0.3	24.3 ± 1.1	38.2 ± 0.4
	T_3 50 µg/kg	(*n* = 6)	23.6 ± 0.4	38.0 ± 0.3	23.0 ± 0.6	37.5 ± 0.2	24.0 ± 0.6	38.0 ± 0.1	25.5 ± 0.9	37.9 ± 0.1
	T_4 5 µg/kg	(*n* = 6)	23.4 ± 0.5	37.2 ± 0.3	22.2 ± 0.7	37.3 ± 0.2	22.9 ± 0.6	37.9 ± 0.3	23.3 ± 0.7	37.7 ± 0.3
	T_4 50 µg/kg	(*n* = 6)	23.5 ± 0.3	37.1 ± 0.1	22.5 ± 0.3	37.3 ± 0.2	23.1 ± 0.4	37.9 ± 0.3	23.5 ± 0.5	37.8 ± 0.3
Study II	Vehicle	(*n* = 4)	25.3 ± 0.2	36.9 ± 0.1	23.6 ± 0.3	37.5 ± 0.2	24.6 ± 0.4	38.0 ± 0.2	24.6 ± 0.4	38.0 ± 0.04
	T_3 50 µg/kg	(*n* = 4)	25.7 ± 0.3	37.3 ± 0.2	24.9 ± 0.5	37.7 ± 0.1	25.7 ± 0.6	38.7 ± 0.2	26.2 ± 0.4	38.1 ± 0.3

Supplementary Methods

Mice

Mice were bred and genotyped at conventional facility of Biomedical Centre, (BMC, Lund, Sweden). All animal experiments (*Studies I and II*) were carried in accordance with the international guidelines on experimental animal research and with the approval of the Malmö-Lund Ethical Committee (ethical permit no. M50/2015) and followed the ARRIVE guidelines. Animals were housed in a controlled environment with a 12:12 hour light cycle, room temperature of 22°C and food and water *ad libitum*. In all animal studies, body weight was monitored every day and body temperature was monitored before and on days two, seven and 14 after the surgeries. The studies were performed pre-specified, subjected to randomization and performed in a blinded fashion to the investigator who performed behavior assessments and dendritic spine analysis. All *in vitro* experiments (*Study III*) were carried out in compliance with directives on animal experimentation (Decreto-Lei 113/2013 and 2010/63/EU) in Portugal and European Union and with approval of the comitte of Animal Research at Universidade da Beira Interior (CICS-UBI, Covilhã, Portugal). Human brain tissue used in this study were carried out with the approval of the Lund Ethical Review Board for research involving humans (Dnr 2011/80).

Drugs and drug delivery

Drugs used in this study were T_3 and T_4 (Sigma-Aldrich, Deisenhofen, Germany), glutamic acid (Sigma-Aldrich), glycine (Fisher Scientific), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; Sigma-Aldrich) and Dizocilpinehydrogen maleate (MK-801; Sigma-Aldrich). For intraperitoneal administration in mice (*Study I and Study II*), T_3 and T_4 stocks were dissolved in NaOH and diluted in NaCl 0.9% at the desired concentrations. For *in vitro* experiments, a T_3 stock solution was prepared in dimethyl sulfoxide (DMSO) and further diluted in phosphate buffered saline (PBS). For whole cell electrophysiology studies (*Study III*) stock solutions of drugs were first dissolved in distilled water, except for CNQX and MK-801 that were first diluted in DMSO, and then diluted in artificial cerebral-spinal fluid (aCSF) at the desired final concentrations. In all *in vitro* experiments, final concentration of DMSO did not exceed 0.01%.

Thyroid hormones effects after experimental stroke (Study I)

For this study, 117 C57BL/6 male mice (20 to 26 g, aged nine to ten weeks, purchased from Charles River) were used. Out of 117 animals, 12 were excluded due to problems during surgery and mortality before entering the treatment phase and 105 animals were randomly assigned into the treatment groups (Fig. 1). Treatment was initiated on day two after photothrombosis (PT) and every other day until the endpoint of the study. Vehicle (Vh, NaCl 0.9%), T_3 (5 or 50 µg/kg) or T_4 (5 or 50 µg/kg) were administered by intraperitoneal injection in a total of six administrations. On days two, seven and 14 after stroke onset or sham surgery, animals were evaluated for motor function.

Photothrombosis. Focal ischemic stroke was induced by PT, as described previously [11, 16]. Briefly, animals were anesthetized with isoflurane in N_2O / O_2 (0.7: 0.3, 5% induction and 1.5 - 2 % maintenance) and placed into a stereotactic frame. After local anesthesia (bupivacaine 0.25 mg/ml), a sagittal skin incision was made on the scalp, subcutaneous connective tissue was removed, and the skull bone was dried. Five minutes after intraperitoneal injection (i.p.) of 1% (w/v) solution of the photosensitizing dye Rose Bengal (0.15 ml/mouse; Sigma-Aldrich, Taufkirchen, Germany) in NaCl 0.9%, the right hemisphere was illuminated with a cold light source (Schott KL 1500 LCD, intensity: 3200 K/5D) through an squared aperture measuring 4.0 to 2.0 mm (equal to an illumination area of 8.0 mm^2) for 20 minutes. The light position related to bregma (+1.5 mm lateral and +0.5 mm anterior) affected the mouse primary motor cortex of forelimb-responsive sites, in the left body side [13]. The same procedure was performed in Sham operated animals, with saline injection instead of photosensitizing dye. During anesthesia, the body temperature was monitored by a rectal probe (Linton Instrumentation, Norfolk, UK) connected to a heating pad maintaining body temperature at 36 - 37°C.

Behavior analysis. Motor function and exploratory behavior after thyroid hormones treatment was assessed using a neuroscore consisting of the rotating pole test (RPT) and the open field test, respectively [8, 15]. These assessments were performed in a blinded fashion to the investigator that performed the surgeries and treatments.

Rotating pole test. The RPT was used to assess postural and locomotor asymmetry that results from an unilateral brain lesion [4]. In brief, mice traversed a rotating wooden pole (length 1500 mm, diameter 40 mm, and elevation 700 mm) at zero, three, and ten rotations

per minute (rpm), to the right and left sides. Every animal was trained during three days before surgery and tested the day before PT. After stroke or sham surgery, animals were evaluated on day two for randomization into treatment groups. Each trial was video recorded, and videos were used to assess sensorimotor dysfunction by using a zero to six scoring system (Table 1). Animals that did not perform the behaviour test before the surgery (total score RPT < 20 points) or did not have motor deficits two days after PT (total score RPT > 15 points), were excluded from behaviour analysis. Behavioural analysis was performed in a blinded fashion to the investigator. In total, 42 animals were excluded from behaviour analysis and the following included: PT/Vh, n = 11; PT/T_3 5 μg/kg, n = 10; PT/T_3 50 μg/kg, n = 11; PT/T_4 5 μg/kg, n = 10; PT/T_4 50 μg/kg, n = 9.

Open field. The open field test was performed 14 days after stroke to assess both spontaneous post-ischemic locomotor activity and post-ischemic exploration behaviour [14]. Briefly, mice where placed into a square arena (44.5 cm × 44.5 cm) surrounded by 44.5 cm high sidewalls. The mouse was always placed in the center of the box and locomotion was recorded and the total distance traveled measured for five minutes.

Thyroid hormone levels determination. Animals were anesthetized with pentobarbital and plasma was collected 14 days after experimental stroke. Blood was collected from the heart into heparinized syringes and maintained at 2-8 ºC while handling. Plasma was collected after centrifugation 2000 x g for 10 minutes at 4 ºC and further stored at -80 ºC for further analysis. ELISA kit assay was used to determine TH levels in the plasma of mice. Plasma levels of T_3 and T_4 were determined by a commercial ELISA kits (ThermoFisher Scientific cat #EIAT3C and cat #EIAT4C, respectively) according to manufacturers instructions. In brief, plasma samples were incubated with specific primary antibodies in donkey anti sheep or goat anti mouse coated 96-well plates. After washing in respective buffer, plates were incubated with 3,3′,5,5′-tetramethylbenzidine substrate and absorbance was measured at 450 nm.

Immunohistochemistry and Immunofluorescence. Tissue collection for immunostainings was performed as described before [3, 9]. Fourteen days after PT animals were deeply anesthetized with pentobarbital and perfused fixed with paraformaldehyde (PFA) 4% and brains collected for immunohistochemistry analysis. Brain sagittal sections (thickness 30 μm) were washed three times in PBS and quenched with 3% H_2O_2 for

20 minutes. Brain sections were blocked for one hour at room temperature (RT), with 5% normal donkey serum (Jackson Immunoresearch, UK) in PBS supplemented with 0.25% Triton X-100. Sections were incubated with primary antibodies at 4 °C overnight, diluted in blocking solution.

Subsequently, slices were incubated with appropriate secondary biotinylated antibodies (donkey anti-rabbit / goat, Vector Laboratories, USA; 1:400) during 90 minutes at RT. Visualization was achieved through the Vectorstain ABC Elite kit (Vector Laboratories, CA, USA), 3,3'-diaminobenzidine tetrahydrochloride (DAB, DabSafe, Saveen Werner, Sweden), 8% $NiCl_2$ and 3% H_2O_2. Bright-field pictures were acquired using an Olympus BX60 microscope (Solna, Sweden), under standard conditions.

For immunofluorescence, brain sections were blocked as described above and incubated with primary antibodies at 4 ºC overnight. Primary antibodies used for immunofluorescence were rabbit TRβ1 (Millipore, 1:1000), rabbit TRα1 (Abcam, 1:1000), goat parvalbumin (PV235, Swant, 1:5000), mouse neuronal nuclei (NeuN, Millipore, 1:1000), glial fibrillary acidic protein (GFAP-Cy3; Sigma, 1:5000), rat CD68 (Abd Serotec, 1:300), and mouse GST-pi (BD Transduction Laboratories, 1:1000). The next day sections were incubated with appropriate secondary antibodies (donkey anti-rabbit/mouse/rat, 1:400) conjugated with fluorescent dyes Cy3, Cy5 (Jackson Immunoresearch, UK) or Alexa 488 (molecular Probes, Invitrogen, USA) for 90 minutes at RT. Images were acquired using an LSM510 confocal laser scanning fluorescence microscope (Carl Zeiss).

Infarct size measurement. Coronal brain sections from the start until the end of the infarct and spaced one millimeter were collected and stained for NeuN (rabbit NeuN, Millipore, 1:5000). Brain slices were mounted in Pertex and digitalized (CanoScan 8800F, Canon, Tokyo, Japan). The non-injured portion of the ipsilateral and contralateral hemisphere were encircled and the indirect infarct volume was calculated by integration of areas from serial sections of each brain as described previously [12], using Fiji software [10].

Counting of parvalbumin positive cells. For each animal one coronal section (-2.0 mm relative to bregma) was stained for Parvalbumin-positive (PV^+) neurons using a monoclonal goat primary antibody (PV235, Swant, 1:5000), and visualization accessed using a VECTOR NovaRED Peroxidase (HRP) Substrate Kit (Vector Laboratories, CA, USA). Rabbit c-fos (Santa Cruz, 1:500) positive immunoreactivity (c-fos^+) was accessed using the avidin–biotin–HRP system, as described before.

The following animals were included in this analysis: PT/Vh, n = 7; PT/T_3 50 µg/kg, n = 6; and PT/T_4 50 µg/kg, n = 4. Bright field images were acquired with 4x magnification objective and Fiji software was used to draw regions of interest, using an optical grid to define the distances and draw the regions. PV^+ cells and PV^+/c-fos^+ in the peri-infarct somatosensory cortex (area of 0.8 mm^2) and homotypic area in the contralateral hemisphere were counted. The infarct core was identified by the lack of NeuN immunoreactivity in subsequent sections.

Immunoblotting. Brains from mice were collected as previously described [9]. Fourteen days after PT animals were deeply anesthetized with pentobarbital and brains were immediately frozen (−40°C) in isopentane (Sigma-Aldrich, Taufkirchen, Germany) and further cooled down to −70°C on dry ice for immunoblotting. Fresh frozen brains were placed into a brain matrix and cut (+2.2 mm to -2.2 mm relatively to bregma). For each four millimeters thick section, the tissue correspondent to the infarct core and peri-infarct was collected. The procedure was performed in a refrigerated chamber at -20°C.

Tissue from human brains were dissected out by a pathologist following autopsy. Brain tissues were immediately frozen and stored at -80 ºC, temporarily moved to a refrigerated chamber at -20 ºC to excise small specimens and stored at -80 ºC until protein extraction.

Western blot. Proteins were dissected from brain tissue as described before [2, 9]. Briefly, brain tissue was mechanically homogenized by a Dounce homogenizer in lysis buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM sodium orthovanadate (Na_3VO_4), 1 mM phenylmethanesulfonyl fluoride (PMSF), 1% triton-X 100 and supplemented with protease inhibitor cocktail (Sigma-Aldrich, Deisenhofen, Germany). The homogenates were centrifuged 14.000g at 4°C for 20 minutes, after 20 minutes of incubation on ice. The supernatant was collected and stored at -20°C for further analysis. Whole protein concentrations were determined by the Bradford method using bovine serum albumin (BSA, Sigma-Aldrich) in lysis buffer as standard.

Brain lysates were diluted in sodium dodecyl sulfate (SDS) sample buffer and proteins denatured at 94 °C for five minutes. Five or ten micrograms of protein were separated on a 10% or 15% SDS polyacrylamide gel. After transferring proteins onto polyvinylidene difluoride membranes, these were blocked with 5% non-fat dry milk solution in tris buffered saline with Tween 20 1% (TBS-T). Following blocking, membranes were incubated with primary antibody in 5% BSA solution in TBS-T, at 4 °C overnight. Primary antibodies used for western blot were rabbit TRα1 (Abcam, 1:1000), rabbit TRβ1 (Millipore, 1:20000), mouse

postsynaptic protein 95 (PSD95; BD Transduction Laboratories, 1:1000), rabbit synaptophysin (Thermoscientific, 1:15000), rabbit glutamate receptor 1 (GluR1; Millipore, 1:2000), mouse GluR2 (Millipore, 1:1000), mouse *N*-methyl-D-aspartate receptor 1 (NMDAR1; BD Transduction Laboratories, 1:1000), rabbit synaptotagmin 1&2 (Abcam, 1:1000) and rabbit glutamic acid decarboxylase 65/67 (GAD 65/67; Millipore, 1:2000). After blocking, the membranes were incubated with a rabbit / mouse secondary HRP-conjugated antibody (Sigma-Aldrich, Stockholm, Sweden, 1:15000 / 1:10000, respectively) for one hour at RT. The signals were visualized by using a chemiluminescence kit (Merck Millipore, Germany) and CCD camera (Fujifilm LAS 1000, Fujifilm, Tokyo, Japan). Membranes were reprobed with anti β-actin HRP conjugated (1:150000, Sigma-Aldrich). Levels were calculated as a percentage of β-actin expression, after densitometric analysis using Fiji software.

Dynamics of dendritic spines after administration with T_3 (Study II)

To study the effects of T_3 on dendritic spine dynamics in mouse neocortical neurons after experimental stroke, eight Thy1- yellow fluorescent protein (YFP) transgenic mice (25 to 40 g, aged one year, own breeding), that express YFP in neuronal population were used. Mice were randomly assigned in the following treatment groups: PT/Vh, n = 4; PT/T_3 50 µg/kg, n = 4 (Fig. 1). Treatment was administered as described above for *Study I*. Fourteen days after the surgery, mice were sacrificed, perfusion fixed with PFA 4% and brains were collected for further infarct volume assessment and dendritic spine analysis.

Photothrombosis. To induce PT in animals for dendritic spine analysis (*Study II*) the surgical procedure as described for *Study I*, and the left hemisphere was illuminated with a cold light source (Schott KL 1500 LCD, intensity: 3050 K/4D) through a round aperture measuring 1.5 mm in diameter (equal to an illumination area of 1.767 mm^2) for 20 minutes. This approach induced smaller infarct sizes so that dendritic spines could be analyzed in different regions in the peri-infarct area. The same procedure was performed in Sham operated animals, with saline injection instead of photosensitizing dye.

Detection and classification of dendritic spines from fluorescence Laser Scanning Microscopy. Brain coronal sections (thickness 30 μm) were rinsed in PBS, mounted on super charged slides, and cover-slipped with PVA-DABCO (Sigma-Aldrich). Images were acquired using an AxioObserver LSM 710 confocal (Carl Zeiss) using a Plan-Apochromat 63x/1.4 DIC M27 oil immersion objective (Carl Zeiss, Jena, Germany) and ZEN 2010 imaging software.

Three coronal sections per animal were collected at different levels: +2.0 mm, +1.0 mm and 0 mm relatively to bregma, corresponding to the rostral pole, center and caudal pole of the infarct, respectively. For each animal, we analyzed layers II/III correspondent to the apical pyramidal neurons in the ipsilateral motor cortex (Region 1, R1), ipsilateral somatosensory cortex (Region 2, R2), contralateral motor cortex (Region 3, R3) and contralateral somatosensory cortex (Region 4, R4).

Images were obtained in z-stack planes of 224.8 × 224.8 μm in *xy* and 15 to 16 μm in *z* (*x/y/z*, 0.11 pixel/μm), resolution 2048 x 2048 pixels in *xy* and spaced 0.2 μm in *z*.

Dendritic spine density and shape classification was accurately quantified and characterized using a three dimensional (3D) computational approach as previously described [5]. After median filter application to reduce noise, radius of 1.0 pixel, we performed proper image 3D deconvolution using interactive deconvolve 3D plugin from Fiji software, after theoretical point spread function (PSF) generation using diffraction PSF 3D plugin. Deconvolution restores image contrast that is lost during image recording due to the optical smearing introduced by the PSF of the microscope, and it is an important systematic error correction for dendritic spine analysis by improvement of signal to noise ratio [5].

For each region, three to five dendritic branches were randomly selected. Dendrites were manually selected, and spines were automatically detected using NeuronStudio software. Minimum and maximum height for spines were set to 0.2 μm and 2.0 μm, respectively, and voxel dimensions were adjusted for our images (0.098 μm, 0.098 μm and 0.2 μm for *xyz*, respectively). For spine shape classification, we used the Rayburst algorithm provided by NeuronStudio software [5, 6], which allowed to use all the information from a LSM image stack, and also provided procedures as declumping of merged spines and spine stem reattachment, making spine detection more accurate than manual or 2D method, where spines are masked along *z* axis. Dendritic spines were classified according to head to neck ratio and head diameter as stubby, mushroom or thin [1, 5], using default parameters from NeuronStudio. Some detected dendritic spines from neighbor dendritic branches were manually deleted and not included in statistical analysis. Dendritic spine density was calculated with the ratio number of spines / dendrite length.

In vitro modulation of T_3 in glutamatergic neurons (Study III)

An *in vitro* model of cerebral ischemia and electrophysiology studies were performed to study immediate effects of T_3 in homeostatic plastic mechanisms, namely modulation of synaptic proteins crucial for neurotransmission and ionotropic glutamate receptors alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and *N*-methyl-D-aspartate (NMDA) evoked currents.

Cell cultures. *C*ultured glutamatergic cortical neurons were used after 7 - 8 days *in vitro* (DIV). Primary cortical neuronal cultures were prepared as described before [7]. Cells were obtained from the cerebral cortex from Wistar rats on embryonic day 16 - 18. Briefly, meninges were removed, and the cortex dissected and subjected to enzymatic dissociation, using 0.05 / 0.02% w/v in PBS trypsin / EDTA (#15400054, Thermofisher) for 15 minutes at 37 ºC. The homogenized was rinsed with Dulbecco's Modified Eagle's medium (#11880036, DMEM, GIBCO) with 10% fetal bovine serum (#10500-064, GIBCO), 100 U penicillin and streptomycin/ml (#15140122, Thermofisher), 2 mM L-glutamine (#G5792, Sigma-Aldrich), dissociated with a Pasteur pipette, centrifuged and redissociated in starter medium (#21103049, Neurobasal medium, GIBCO) supplemented with B27 (#17504044, GIBCO), 100 U penicillin and streptomycin/ml, 2 mM L-glutamine (#G5792, Sigma-Aldrich) and 25 µM glutamate (#49621, Sigma-Aldrich). The cells were plated onto poly-L-lysine (#P4707, Sigma-Aldrich) pre-coated multiwells at 1.5×10^5 cells/cm^2 and grown in starter medium at 37 ºC and 5% CO_2. One-half of the medium was replaced with cultivating medium (starter medium without glutamate) from 4 DIV. Cells were used after 7-8 DIV for *in vitro* assays.

In vitro ischemic model and experimental treatments. After 7 DIV, neurobasal medium was collected and stored to be replaced after experiments. Neuron cultures were washed with PBS, and oxygen and glucose deprivation (OGD) was induced with deoxygenated aglycemic solution (in mM): 143.8 Na^+, 5.5 K^+, 1.8 Ca^{2+}, 1.8 Mg^{2+}, 125.3 Cl^-, 26.2 HCO^{3-}, 1.0 PO_4^{3-}, 0.8 SO_4^{2-}, pH 7.4) in an anoxic atmosphere. OGD was generated in a hypoxia incubator chamber (StemCell Technologies), flushed with gas: 5% CO_2, 95% N_2. In control cultures, medium was replaced by basic salt solution (BSS) after washing with PBS (in mM): 143.8 Na^+, 5.5 K^+, 1.8 Ca^{2+}, 1.8 Mg^{2+}, 125.3 Cl^-, 26.2 HCO^{3-}, 1.0 PO_4^{3-}, 0.8 SO_4^{2-}, 20 glucose, pH 7.4), and cells were incubated in a normoxic atmosphere containing 5% CO_2. Cultures were in OGD or BSS solutions for 120 minutes and after replaced by previous collected medium. After OGD / BSS conditions, cells were incubated with Vh (DMSO in

PBS, 0.01%) or T_3 1 μM for 48 hours. Subsequently, cells were washed with cold PBS to remove excess of culture medium and cells collected and frozen at -80 ºC until protein extraction.

Immunocytochemistry. For immunocytochemistry, neurons were plated on glass coverslips and fixed in PFA 4% for 10 min after 7 DIV. Immunofluorescence was performed as described for brain sections, and coverslips were incubated overnight with rabbit TRα1 (Thermoscientific, 1:500) or rabbit TRβ1 (Millipore, 1.500). The next day, neurons were incubated with secondary biotinylated antibody donkey anti-rabbit, 1:400 (Jackson Immunoresearch, UK) for 90 minutes at RT and Streptavidin Alexa 488 (molecular Probes, Invitrogen, USA) for 60 minutes at RT. Next, glass coverslips were stained with Hoechst-33342 (4 μg/ml, Life Technologies) for 5 min at RT and mounted in Dako fluorescent mounting medium. Photomicrographs were obtained using an AxioObserver LSM710 confocal microscope (Carl Zeiss).

Immunobloting. Protein extraction was performed as previously described [2, 9]. Western blots were performed as described above for brain extracts, and mouse synaptotagmin (BD Transduction Laboratories, 1:2000) was incubated overnight at 4 ºC. After blocking, the membranes were incubated with a mouse secondary HRP-conjugated antibody (Sigma-Aldrich, Stockholm, Sweden, 1:10000) for one hour at RT.

Electrophysiological recording of membrane currents. To study ligand-gated channels AMPA and NMDA, we adopted the voltage-ramp method [17]. Currents were amplified with an Axopatch 200B (Axon Instruments, USA) and digitized at a frequency of 10 kHz and filtered at 0.1 kHz using the analog-to-digital converter Digidata 1322A (Axon Instruments, USA) and pClamp software (version 8, Axon Instruments, USA). During recording, cells were maintained at RT (21 – 25 ºC) in aCSF filtered through a 0.45 μm mesh, of the following composition (in mM): 126 NaCl, 3 KCl, 2 $MgSO_4$, 2 $CaCl_2$, 1.25 NaH_2PO_4, 10 glucose and 10 HEPES, 290 mOsmol. The internal solution of the recording electrode was composed of (in mM) 115 K-gluconate, 4 NaCl, 0.3 GTP and 2 ATP-Mg, pH 7.2 with KOH, 270 mOsmol.

Electrodes were pulled on a vertical puller (PC-10, Narishige), from borosilicate glass capillaries (Harvard Apparatus). The initial patch microelectrode had a resistance of 5.6 –

7.4 MΩ, when filled with internal solution. The electrode was sealed against cells at least 1.0 GΩ, and membrane was ruptured by suction pulses, which allows the recording of the intracellular membrane potential. Only cells with Ra (Access resistance) values < 10 MΩ were included.

Solutions were delivered diluted in the bath solution through a custom-made perfusion system, where capillary tubes with 250 µm inner diameter merged into a common outlet. Drugs were applied close to cell at approximately 50 to 100 µm, at a rate of 20 µl/min.

Individual currents were recorded in the presence of T_3 1 µM (n = 4) or Vh (n = 3), that were incubated 48 hours before the experiments. A sequence of voltage ramps at a rate of 0.23 mV/millisecond were applied at a holding potential of -80 mV. To obtain the agonist induced current-voltage (I-V) relation, ramps I-V curves were constructed applying a 500 milliseconds voltage ramp ranging from -110 mV to +20 mV elicited every 8 seconds. Voltage ramps were applied in the absence and in the presence of AMPA and NMDA agonist glutamate at 50 µM and co-agonist of NMDA channels glycine at 3 µM, to enable subtraction of leak currents. CNQX and MK-801 were used both at 10 µM as antagonists of AMPA and NMDA channels, respectively. For stabilization of background currents, a minimum of 80 seconds was recorded before agonists and CNQX / MK-801 application.

Cell currents were recorded sequentially in the presence of specific K^+- channel blockers tetraethylammonium sodium salt (5 mM) and 4-Aminopyridine (1 mM), that were applied in the perfusion system together with the other drugs. Voltage-gated K^+ channels needed to be blocked, since those channels were contributing to the conductance as well to the reversal potential obtained.

References

1. Harris KM, Jensen FE, Tsao B (1992) Three-dimensional structure of dendritic spines and synapses in rat hippocampus (CA1) at postnatal day 15 and adult ages: implications for the maturation of synaptic physiology and long-term potentiation. J Neurosci 12:2685–2705

2. Kuric E, Ruscher K (2014) Reduction of rat brain CD8+ T-cells by levodopa/benserazide treatment after experimental stroke. Eur J Neurosci 40:2463–2470. doi: 10.1111/ejn.12598

3. Quattromani MJ, Cordeau P, Ruscher K, Kriz J, Wieloch T (2014) Enriched housing down-regulates the Toll-like receptor 2 response in the mouse brain after experimental stroke. Neurobiol Dis 66:66–73. doi: 10.1016/j.nbd.2014.02.010

4. Rickhag M, Deierborg T, Patel S, Ruscher K, Wieloch T (2008) Apolipoprotein D is elevated in oligodendrocytes in the peri-infarct region after experimental stroke: Influence of enriched environment. J Cereb Blood Flow Metab 28:551–562. doi: 10.1038/sj.jcbfm.9600552

5. Rodriguez A, Ehlenberger DB, Dickstein DL, Hof PR, Susan L (2008) Automated Three-Dimensional Detection and Shape Classification of Dendritic Spines from Fluorescence Microscopy Images. 3:e1997. doi: 10.1371/journal.pone.0001997

6. Rodriguez A, Ehlenberger DB, Hof PR, Wearne SL (2006) Rayburst Sampling, an Algorithm for Automated Three-dimensional Shape Analysis from Laser Scanning Microscopy Images. Nat Protoc 1:2152–2161. doi: 10.1038/nprot.2006.313

7. Ruscher K, Freyer D, Karsch M, Isaev N, Megow D, Sawitzki B, Priller J, Dirnagl U, Meisel A (2002) Erythropoietin Is a Paracrine Mediator of Ischemic Tolerance in the Brain: Evidence from an In Vitro Model. J Neurosci 22:10291–10301. doi: 10.1523/jneurosci.22-23-10291.2002

8. Ruscher K, Johannesson E, Brugiere E, Erickson A, Rickhag M, Wieloch T (2009) Enriched environment reduces apolipoprotein E (ApoE) in reactive astrocytes and attenuates inflammation of the peri-infarct tissue after experimental stroke. J Cereb Blood Flow Metab 29:1796–1805. doi: 10.1038/jcbfm.2009.96

9. Saraiva C, Talhada D, Rai A, Ferreira R, Ferreira L, Bernardino L, Ruscher K (2018) MicroRNA-124-loaded nanoparticles increase survival and neuronal differentiation of neural stem cells in vitro but do not contribute to stroke outcome in vivo. PLoS One 13:e0193609. doi: 10.1371/journal.pone.0193609

10. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez JY, White DJ, Hartenstein V, Eliceiri K, Tomancak P, Cardona A (2012) Fiji: An open-source platform for biological-image analysis. Nat. Methods 9:676–682

11. Schroeter M, Jander S, Stoll G (2002) Non-invasive induction of focal cerebral ischemia in mice by photothrombosis of cortical microvessels: Characterization of inflammatory responses. J Neurosci Methods 117:43–49. doi: 10.1016/S0165-0270(02)00072-9

12. Swanson RA, Morton MT, Tsao-Wu G, Savalos RA, Davidson C, Sharp FR (1990) A semiautomated method for measuring brain infarct volume. J Cereb Blood Flow Metab 10:290–293. doi: 10.1038/jcbfm.1990.47

13. Tennant KA, Adkins DL, Donlan NA, Asay AL, Thomas N, Kleim JA, Jones TA (2011) The organization of the forelimb representation of the C57BL/6 mouse motor cortex as defined by intracortical microstimulation and cytoarchitecture. Cereb Cortex 21:865–876. doi: 10.1093/cercor/bhq159

14. Walsh RN, Cummins R a (1976) The Open-Field Test: a critical review. Psychol Bull 83:482–504. doi: 10.1037/0033-2909.83.3.482

15. Walter HL, van der Maten G, Antunes AR, Wieloch T, Ruscher K (2015) Treatment with AMD3100 attenuates the microglial response and improves outcome after experimental stroke. J Neuroinflammation 12:24. doi: 10.1186/s12974-014-0232-1

16. Watson BD, Dietrich WD, Busto R, Wachtel MS, Ginsberg MD (1985) Induction of reproducible brain infarction by photochemically initiated thrombosis. Ann Neurol 17:497–504. doi: 10.1002/ana.410170513

17. Yelhekar TD, Druzin M, Karlsson U, Blomqvist E, Johansson S (2016) How to Properly Measure a Current-Voltage Relation?—Interpolation vs. Ramp Methods Applied to Studies of GABAA Receptors. Front Cell Neurosci 10:10. doi: 10.3389/fncel.2016.00010

CHAPTER 5

TRANSTHYRETIN AND STROKE

Transthyretin expression in the postischemic brain

Short description: This original paper evaluates the expression of TTR in the ischemic territory following experimental stroke. Brain tissues were analyzed for *ttr* expression and TTR levels at 24 hours, 48 hours, 7 days and 14 days following PT. Together, our results indicate that TTR is not synthesized in brain resident cells in the ischemic infarct core and adjacent peri-infarct area. Thus, it seems unlikely that *in situ* synthesized TTR is involved in mechanisms of tissue reorganization during the first 14 days following PT.

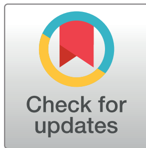
RESEARCH ARTICLE

Transthyretin expression in the postischemic brain

Daniela Talhada[1,2,3], Isabel Gonçalves[2], Cecília Reis Santos[2], Karsten Ruscher[1,3]*

1 Laboratory for Experimental Brain Research, Division of Neurosurgery, Department of Clinical Sciences, Lund University, Lund, Sweden, **2** CICS-UBI-Health Sciences Research Centre, Faculdade de Ciências da Saúde, Universidade da Beira Interior, Covilhã, Portugal, **3** LUBIN Lab—Lunds Laboratorium för Neurokirurgisk Hjärnskadeforskning, Division of Neurosurgery, Department of Clinical Sciences, Lund University, Lund, Sweden

* karsten.ruscher@med.lu.se



OPEN ACCESS

Citation: Talhada D, Gonçalves I, Reis Santos C, Ruscher K (2019) Transthyretin expression in the postischemic brain. PLoS ONE 14(9): e0221555. https://doi.org/10.1371/journal.pone.0221555

Editor: Johannes Boltze, University of Warwick, UNITED KINGDOM

Received: May 31, 2018

Accepted: August 11, 2019

Published: September 3, 2019

Copyright: © 2019 Talhada et al. This is an open access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This study was supported by National Funds by FCT - Foundation for Science and Technology (grant SFRH/BD/104679/2014) (DT), the "Programa Operacional do Centro, Centro 2020" through the funding of the ICON project (Interdisciplinary Challenges on Neurodegeneration; CENTRO-01-0145-FEDER-000013) (IG and CS), Health Sciences Research Center (CICS-UBI) through National Funds by FCT - (UID / Multi / 00709/2019) (IG and CS), the

Abstract

The unknown role of the carrier protein transthyretin (TTR) in mechanisms of functional recovery in the postischemic brain prompted us to study its expression following experimental stroke. Male C57/B6 mice (age 9 to 10 weeks) were subjected to permanent focal ischemia induced by photothrombosis (PT) and brain tissues were analyzed for *ttr* expression and TTR levels at 24 hours, 48 hours, 7 days and 14 days following the insult by RT-PCR, Western blot and immunohistochemistry. Fourteen days after PT, non-specific TTR-like immunoreactive globules were found in the ischemic core and surrounding peri-infarct region by immunohistochemistry that could not be allocated to DAPI positive cells. No TTR immunoreactivity was found when stainings were performed with markers for neurons (Neuronal Nuclei, NeuN), reactive astrocytes (glial fibrillary acidic protein, GFAP) or microglia (cluster of differentiation 68, CD68). In addition, we could not find TTR by immunoblotting in protein extracts obtained from the ischemic territory nor *ttr* expression by RT-PCR at all time points following PT. In all experiments, *ttr* expression in the choroid plexus and TTR in the mouse serum served as positive controls and recombinant legumain peptide as negative control. Together, our results indicate that TTR is not synthesized in brain resident cells in the ischemic infarct core and adjacent peri-infarct area. Thus, it seems unlikely that *in situ* synthesized TTR is involved in mechanisms of tissue reorganization during the first 14 days following PT.

1. Introduction

Transthyretin (TTR) is a 55 kDa homotetrameric protein, composed of four identical subunits, each containing 127 amino acids [1]. TTR is mainly synthesized by the liver and by the epithelial cells of the choroid plexus (CP), which are the sources of TTR in plasma and cerebrospinal fluid (CSF), respectively [2,3]. TTR is one of the most abundant proteins in the CSF (up to 25% of total CSF protein) and, of both humans and rodents, it is the carrier protein of 3,5,3',5'-tetraiodo-L-thyronine (T_4) and 3,5,3'-triodo-L-thyronine (T_3), although TTR has much higher affinity for T_4 than T_3.

Swedish Brain Fund (No. FO2019-0254 and FO2018-0316) (KR), ALF Skåne (KR), Sparbanksstiftelsen Färs & Frosta (KR), the Crafoord Foundation (KR), Sveriges Stroke Riksförbundet (KR), the Hans-Christian and Alice Wachtmeister Foundation (KR) and the Stiftelsen Sven-Olof Jansons livsverk (KR). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

TTR mRNA expression is usually described as being restricted to the CP and meninges, being totally absent from the hippocampus, cerebellum or cerebral cortex, in wildtype mice, shown by *in situ* hybridization and Northern blot analyses as well as RT-PCR of microdissected tissue from different brain regions and RNase protection assay [4–7]. Other studies have demonstrated that neurons express TTR mRNA and have the capacity to synthesize the protein in cortical [8] and in cerebellar neurons [9]. However, how TTR appears in brain areas other than its site of synthesis and secretion, remains to be investigated.

Based on the possibility that specific pathologies may induce the expression of TTR in different brain regions, some *in vitro* and *in vivo* studies have been conducted to assess a potential neuroprotective role of TTR in brain ischemia [10–12] and Alzheimer´s disease. In particular in Alzheimer´s disease, TTR may have the capacity to sequester amyloid beta (Aβ) [13–15]. Moreover, TTR synthesis by neurons from cortex and hippocampus has been interpreted as a natural neuroprotective response in Alzheimer´s disease [16–18]. Hence, further studies need to clarify if TTR is upregulated in the hippocampus and cerebellum [19] or if it cannot been found in the cerebral cortex, hippocampus and cerebellum in models of Alzheimer´s disease [4]. After stroke, TTR immunopositive cells were found in the ischemic territory 24 hours after permanent middle cerebral artery occlusion (MCAO) and migration of TTR from CSF has been suggested [11,12]. On the other hand, it was also shown that in the acute phase of ischemic stroke, TTR production by CP cells is upregulated, but absent in brain tissue [10].

The unknown role of TTR in recovery processes after stroke prompted us to study its expression in the post-ischemic brain. In this study, we assessed the expression of TTR in the ischemic territory at different time points, i.e. 24 hours, 48 hours, 7 days and 14 days after photothrombosis (PT).

2. Material and methods

2.1 Experimental design and animals

All animal experiments were carried out with the approval of the Malmö-Lund Ethical Committee and followed the ARRIVE guidelines (permit no. M50/2015) (S1 Table). Animals were housed in a controlled environment with a 12:12 hour light cycle, room temperature of 22˚C and food and water *ad libitum*. For this experiment, 59 male C57BL/6 mice (20 to 26 g, aged 9 to 10 weeks, purchased from Charles River) were used.

Experimental design is described in Fig 1. For immunohistochemistry analysis, 13 animals were randomly assigned into the following groups: PT (n = 10) and Sham (n = 3). Fourteen days after the surgery, animals were perfusion fixed with 4% paraformaldehyde (PFA) and brains were collected for immunohistochemistry analysis.

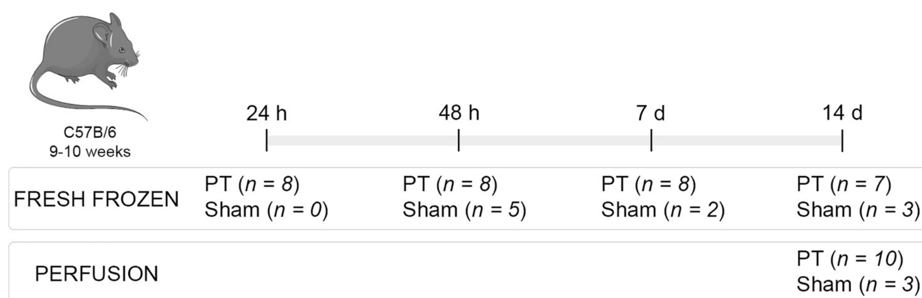


Fig 1. Experimental design. Brains were collected at 24 hours, 48 hours, 7 days and 14 days after brain ischemia or sham surgery to obtain protein extracts for immunoblotting. For immunohistochemistry/immunofluorescence, brains were perfusion-fixed with paraformaldehyde 4%, 14 days after PT. Abbreviation: PT—Photothrombosis.

https://doi.org/10.1371/journal.pone.0221555.g001

To analyze TTR and *ttr* expression in the brain at different time points, 41 animals were used: PT (n = 31) and Sham (n = 10). Animals were sacrificed at 24 hours, 48 hours, 7 days and 14 days after PT or Sham surgery, as detailed in Fig 1. Animals were deeply anesthetized with pentobarbital and blood and CSF were collected. Brains were collected and immediately frozen in isopentane at −40˚C (Sigma-Aldrich, Taufkirchen, Germany) and further cooled down to −70˚C on dry ice. Mouse serum and CP were used as TTR positive controls and recombinant legumain (Abcam) as negative control.

Human serum samples were obtained from healthy volunteers enrolled in another study approved by the regional ethical review board of Lund University, Sweden (permit no. 2013/181).

2.2 Photothrombosis

Photothrombosis (PT) was carried out as described previously [20]. Briefly, animals were anesthetized with isoflurane (induction 4%, and surgery maintenance 1.5–2%) and placed into a stereotactic frame. A sagittal skin incision was made on the scalp, subcutaneous connective tissue was removed, and the skull bone was dried. Five minutes after intraperitoneal injection of the photosensitizer dye Rose Bengal (0.15 mL at 10 mg/mL; Sigma-Aldrich, Taufkirchen, Germany), the right hemisphere was illuminated with a cold light source (Schott KL 1500 LCD, intensity: 3200 K/5D) through an aperture measuring 4.0 vs 2.0 mm (equal to an illumination area of 8.0 mm^2) for 20 minutes. During the surgery, body temperature was measured continuously through a rectal probe and maintained at 36.5 to 37.0˚C by placing the mouse on a heating pad. In total 5 animals died during surgery outside of planned euthanasia or humane endpoints. Before and after PT, temperatures and body weights were monitored throughout the experiments (S2 Table). During surgery, the animals spontaneously breathe through a face mask delivering 1.5% isoflurane in a gas mixture of 30% O_2 and 70% N_2O. Local analgesic bupivacaine (marcain) was subcutaneously injected 3 minutes before scalp incision. During the experiment, animals were monitored every day and did not show any signs of suffering, an there was no need for additional analgesia. Body weights were assessed every day and body temperatures were assessed before and during photothrombosis, 2 days, 7 days and 14 days after surgery.

2.3 Immunohistochemistry

Brain coronal sections (thickness 30 μm) from PFA perfused animals were gently washed three times in phosphate buffered saline (PBS) at room temperature (rt) and quenched with 3% H_2O_2 for 20 minutes. After washing (PBS 3x10 min rt), brain sections were blocked with 5% normal donkey serum (NDS) in PBS supplemented with 0.25% Triton X-100 (PBS-T) for one hour. Sections were incubated with anti-mouse polyclonal TTR primary antibody (rabbit cat# PA5-27220 Thermo Scientific Rockford, USA diluted at 1:1000), in 3% NDS PBS-T at 4˚C overnight. After incubation with primary antibody, slices were washed in PBS-T (3x10 min rt) and incubated with a secondary biotinylated donkey anti-rabbit antibody (1:400 Jackson Immunoresearch, Baltimore, USA), in 3% NDS PBS-T, at room temperature for 90 minutes. Thereafter, sections were washed in PBS-T (3x10 min rt). Visualization was achieved through the Vectorstain ABC Elite kit (Vector Laboratories, CA, USA), washed in PBS in between (3x10 min rt), and using 3,3-diaminobenzidine-tetrahydrochloride (Dabsafe, Saveen Werner AB, Limhamn, Sweden), 8% $NiCl_2$ and 3% H_2O_2. Sections were dehydrated in consecutive higher concentrations of ethanol, followed by xylene and mounted using Pertex (Histolab AB, Gothenburg, Sweden). To access specificity, primary TTR antibody was pre-incubated with TTR blocking peptide (cat# SBP3500378, Sigma-Aldrich, Saint Louis, USA), during 30 minutes at room temperature.

2.4 Immunofluorescence

Brain coronal sections (thickness 30 μm) from perfused animals were gently washed three times in PBS and blocked with 5% NDS in PBS-T for one hour at room temperature. Sections were incubated with rabbit anti-TTR (ThermoScientific, Rockford, USA, diluted at 1:500) together with either a mouse anti neuronal nuclei (NeuN, Merck Millipore; diluted 1:1000), or rat anti CD68 (AbD Serotec; diluted 1:200–1:500), diluted in 2–3% NDS PBS-T at 4˚C overnight. Monoclonal directly Cy3 conjugated anti-glial fibrillary acidic protein (GFAP) was used to stain astrocytes (diluted at 1:5000, Sigma-Aldrich, St Louis, USA) in 3% NDS PBS-T for one hour at room temperature. After incubation with primary TTR antibody, sections were rinsed and incubated with a secondary biotinylated donkey anti rabbit antibody (Jackson Immunoresearch, Baltimore, USA) and secondary donkey anti-mouse and anti-rat antibodies conjugated with fluorescent dye Cy3 (Jackson Immunoresearch, UK), all diluted at 1:400 in 3% NDS PBS-T at room temperature for 90 minutes. Thereafter, sections were incubated with an Alexa 488 Streptavidin conjugate (Jackson Immunoresearch, UK, diluted at 1:400), for one hour at room temperature. Thereafter, sections were incubated with DAPI (final concentration 0.5 μg/ml) for 5 minutes at room temperature. Co-stainings were visualized using a confocal microscopy system (LSM510 Zeiss, Jena, Germany) and the AIM LSM 4.2 software (Zeiss).

2.5 Western blotting

2.5.1 Brain Sample Preparation. Fresh frozen brains were placed into a brain matrix and cut, 2.2 mm to -2.2 mm relatively to bregma. For each four mm thick section, the infarct core (IC) and peri-infarct (PI) area in the ipsilateral (IPS) region were collected. The same brain regions were collected from sham operated animals. All the procedures were performed in a glove chamber at -20˚C.

2.5.2 Preparation of protein extracts from brain. Brain tissue was mechanically homogenized by a Dounce homogenizer in lysis buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM sodium orthovanadate (Na_3VO_4), 1 mM phenylmethanesulfonyl fluoride (PMSF), 1% Triton-X100 and supplemented with protease inhibitor cocktail (Sigma-Aldrich, Deisenhofen, Germany). The homogenates were centrifuged 14000xg at 4˚C for 20 minutes, after 20 minutes of incubation on ice. The supernatant was collected and stored at -20˚C for further analysis. Total protein concentrations were determined with the Bradford assay using bovine serum albumin (BSA) as standard.

2.5.3 Immunoblotting. Samples were diluted in dodecyl sulfate sodium (SDS) sample buffer and proteins denatured at 100˚C for 15 minutes. Proteins were separated on a 15% SDS polyacrylamide gel. After transferring proteins onto polyvinylidene difluoride (PVDF) membranes, blocking was accomplished by incubation of the membranes in a 5% non-fat dry milk solution in Tris-Buffered Saline supplemented with 0.1% Tween (TBS-T). Following blocking, membranes were incubated with polyclonal TTR primary antibodies (rabbit cat# PA5-27220 diluted at 1:3000, ThermoScientific Rockford, USA; chicken cat# SAB3500378 diluted at 1:3000, Sigma-Aldrich) in 5% BSA (cat# A4503, Sigma-Aldrich) in TBS-T at 4˚C overnight. The membranes were incubated with a secondary antibody anti-rabbit horse radish peroxidase (HRP) conjugated (diluted 1:15000, Sigma-Aldrich, Germany) in 5% non-fat dry milk solution for one hour at room temperature, or secondary biotinylated donkey anti-chicken (diluted at 1:80000), followed by anti-biotin HRP conjugated (diluted at 1:3000, Cell Signaling, USA), in 5% non-fat dry milk solution, for one hour at room temperature. To assess specificity, primary TTR antibody was pre-incubated with TTR blocking peptide (cat# SBP3500378, Merck Sigma-Aldrich, Saint Louis, USA), for 60 minutes at room temperature. The signals were visualized

using a chemiluminiscence kit (Merck Millipore, Darmstadt, Germany) and charge-coupled device camera (Fujifilm LAS 1000, Fujifilm, Tokyo, Japan). Membranes were stripped and reprobed with anti β-actin HRP conjugated (diluted at 1:50000, Sigma-Aldrich, Deisenhofen, Germany).

2.6. mRNA quantification by Real-time PCR

Brain tissue samples and CP were homogenized in RNeasy mRNA kit (Qiagen) and total RNA extraction was performed according to the manufacturers protocol. Synthesis of cDNA was carried out using the Scrip cDNA Synthesis Kit (Bio-Rad). cDNA was analyzed using real-time PCR SsoAdvance SYBR Green Supermix from Bio-Rad using appropriate primers (sequences: fw 95 –GGTGCTGGAGAATCCAA; rev 345 –CATCCGCGAATTCATGGA) and run on a Bio-Rad CFX96 real-time quantitative PCR (qPCR) system. Reactions were performed using 0.2 μM of each primer and 1 μL of diluted cDNA (1:20) in a final volume of 10 μL. Quantification cycle threshold (Cq = 39) values per target were manually estimated. Gene expression was normalized to the housekeeping gene GAPDH and calculated using the $2^{-\Delta Ct}$ method. Melt curve analyses were performed to ensure the specificity of qPCR products. All assays included at least two biological replicates with two technical replicates each, positive control and a non-template control.

3. Results

3.1 Transthyretin expression in the ischemic territory 14 days after permanent stroke

We performed immunohistochemistry analysis to evaluate if cells express TTR in the postischemic brain, i.e the infarct core (IC) and peri-infarct (PI) area 7 and 14 days after PT. As shown in Fig 2A and 2B we found some scattered TTR-like immunoreactive globules in the IC and in the PI area, however, their appearance was inconsistent and immunoreactivity (ir) was not found in all animals subjected to PT. Globules showed irregular morphology, mostly in a triangular shape and TTR appeared in granules indicative of dying cells. In addition, ir could not be allocated to DAPI (Fig 2G to 2J). Dilution of primary antibody revealed a slight reduction of this unspecific ir. Importantly, these signals could not be blocked by a specific TTR blocking peptide (Fig 2D and 2E) while signals in cells positive for TTR in the CP were partially blocked (Fig 2F). In Sham operated animals, TTR was only found in the CP.

3.2 Phenotyping of Transthyretin expressing cells in the ischemic territory

To further characterize these cells, we performed immunofluorescence co-stainings with neuronal, astrocytic and microglia markers, in brain sections from animals subjected to PT. While specific TTR ir was found consistently in the CP of all animals, no specific TTR ir was detected in the ischemic territory. In particular, we did not observe TTR ir in NeuN positive neurons and GFAP positive reactive astrocytes in the proximal peri-infarct area. Also, CD68 positive microglia/macrophages mainly accumulating in the IC did not show TTR staining (Fig 3).

3.3 Transthyretin levels in the ischemic territory

To further evaluate the presence of TTR in the ischemic territory at different time points after stroke, protein extracts from the ischemic territory (peri-infarct and infarct core border) were used for Western blotting (Fig 4A). Samples from mice subjected to PT and sham operated did not show a specific 15 kDa TTR band at 24 hours, 48 hours, 7 days and 14 days after PT corresponding to monomeric or tetrameric TTR, respectively. Likewise, no signals were detected in

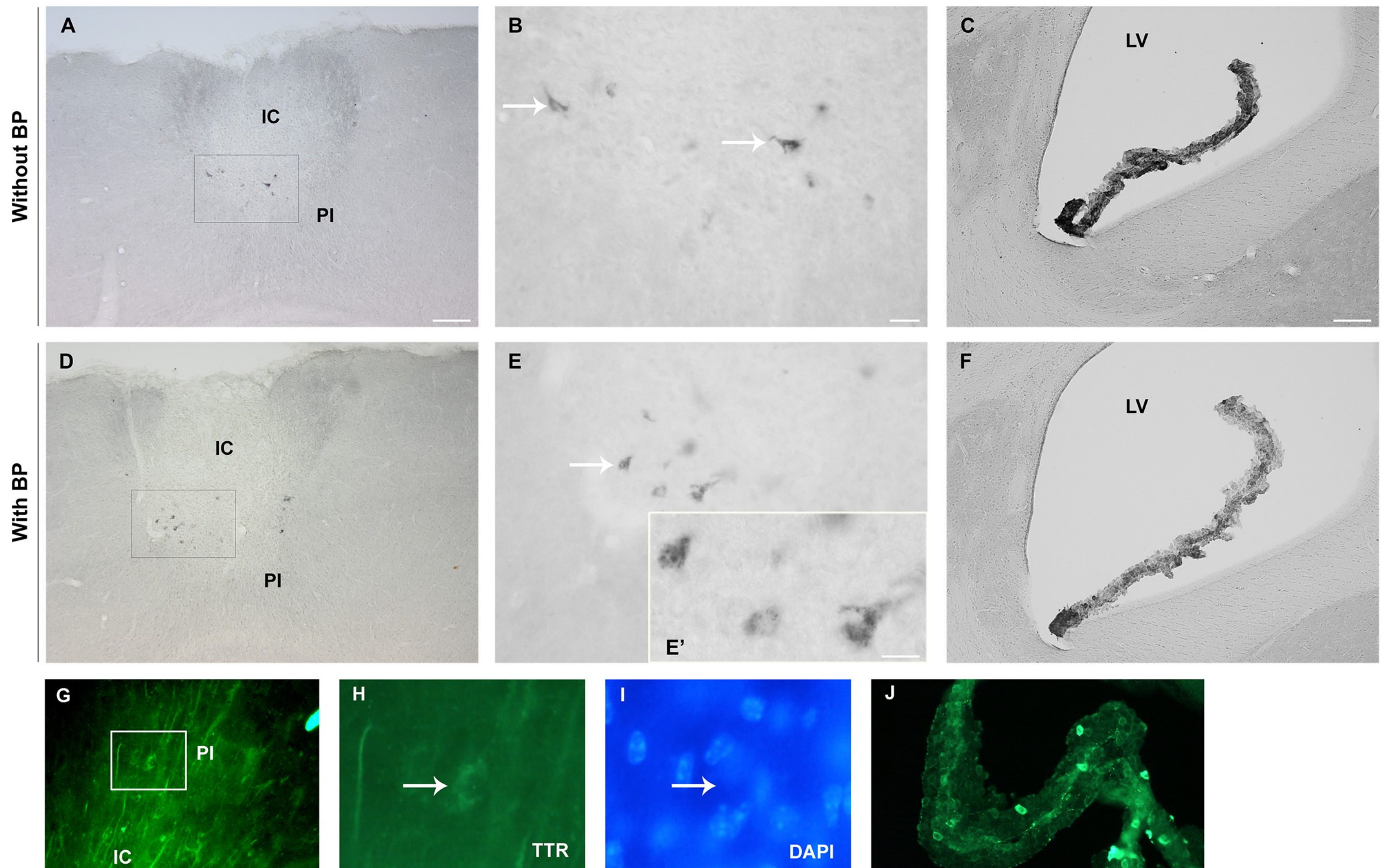


Fig 2. Immunohistochemistry in the postischemic brain. Scattered cells were immunoreactive for TTR in the IC and PI 14 days after ischemic stroke. Low magnification overview of the ischemic territory using 1:1000 anti-TTR rabbit antibody (ThermoScientific cat# PA5-27220, Rockford, USA) **(A)** without and **(D)** with BP. **(B, E and E´)** Higher magnification micrographs of images (A) showing cells immunoreactivity for TTR (white arrows). **(C)** Epithelial cells from CP were positive for TTR and **(F)** partially blocked with BP. **(G)** Staining for TTR (green, AF488) and with higher magnification in (J) together with DAPI (H). **(J)** Epithelial cells from CP from the same section. Scale bars: **A**, **C**, **D** and **F**—100 μm, **B** and **E**—20 μm and **E´** - 10 μm. BP–Blocking peptide; CP—Choroid plexus; IC—Infarct core; PI–Peri-infarct area; TTR–Transthyretin; LV–Lateral ventricle.

https://doi.org/10.1371/journal.pone.0221555.g002

the negative control. In contrast, mouse serum and mouse CSF showed a specific 15 kDa band (Fig 4B to 4D).

Since we essentially did not find TTR in the postischemic brain, we performed additional Western blots using a chicken anti TTR antibody. Again, we observed specific TTR bands in lanes loaded with mouse serum as well as in lanes loaded with human serum supporting the specificity of this TTR antibody. However, in contrast to Western blots using the rabbit primary antibody we obtained an additional band of approximately 60 to 65 kDa. This band could not be blocked as the 15 kDa monomer of TTR, further corroborating that this band does not correspond to the TTR tetramer (Fig 5C). Interestingly, this band is also present in absence of primary antibody suggesting a direct binding of the secondary antibody (Fig 5D). Together, only the 15 and the 55 kDa band was found to be specific for TTR. Both antibodies were recognizing the monomeric form of TTR at 15 kDa in positive controls (human and mouse serum).

To confirm negative results from immunohistochemistry and Western blot experiments, we performed quantitative RT-PCRs. As shown in Fig 6, expression of *ttr* was found in all CP samples (relative expression 1.24 ± 0.48, n = 5). In contrast, we found extremely low expression

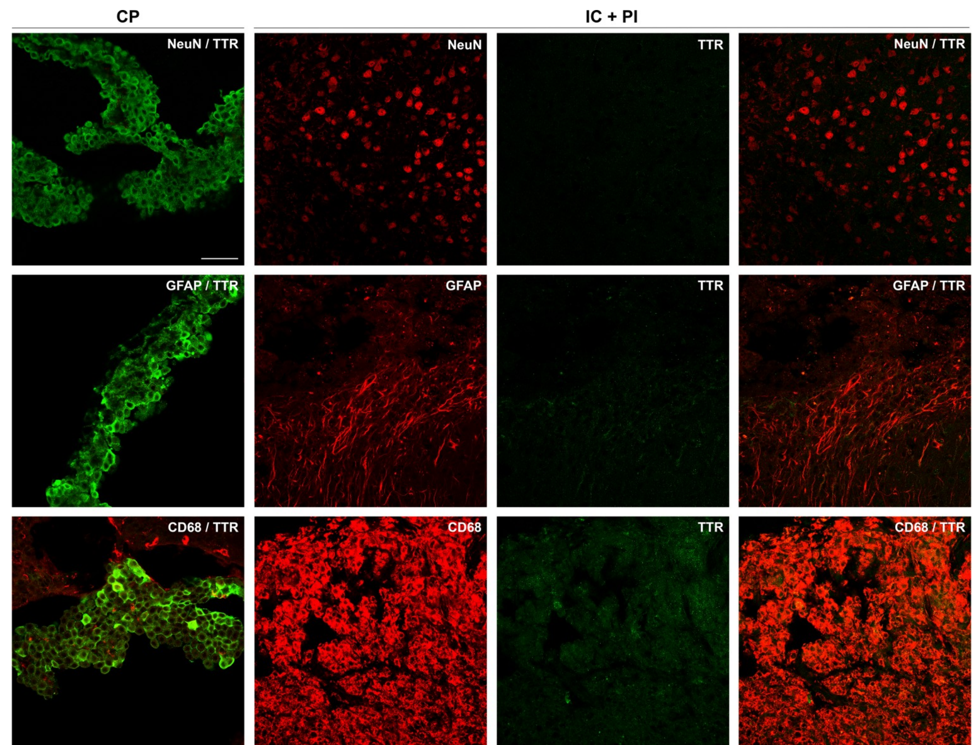


Fig 3. Phenotyping of TTR positive cells. TTR immunoreactivity (AF488, green) was not found in NeuN positive neurons, GFAP positive astrocytes or CD68 positive microglia/macrophages, 14 days after PT. NeuN, GFAP or CD68 are shown in red (Cy3). TTR is expressed in the epithelial cells of CP, used as a positive control. Scale bars 50 μm. Rabbit anti-TTR cat# PA5-27220 ThermoScientific Rockford, USA diluted at 1:500. CP–Choroid plexus; GFAP–Glial fibrillary acidic protein; IC–Infarct core; NeuN–Neuronal nuclei; PI–peri-infarct area; PT–Photothrombosis; TTR–Transthyretin.

https://doi.org/10.1371/journal.pone.0221555.g003

levels in samples obtained from the ischemic territory at all time points (48 h: 0.0008 ± 0.0008, n = 5; 7 d: 7.22E-06 ± 5.94E-06, n = 4; 14 d: 5.29E-05 ± 2.56E-05, n = 3) and respective sham operated mice (48 h: 7.57E-06 ± 4.17E-06, n = 3; 7 d: 7.42E-07 ± 1.64E-07, n = 2; 14 d: 6.68E-06 ± 3.30E-06, n = 2). From these experiments, we conclude that compared to CP samples *ttr* expression is not detectable in brain parenchyma. Moreover, low levels of *ttr* expression did not result in relevant protein levels of TTR in brain parenchyma.

4. Discussion

The present study was conducted to evaluate if transthyretin (TTR), a major transport protein for thyroid hormones and retinol-binding protein in the plasma and CSF, is expressed in the postischemic brain during the first 14 days following permanent focal ischemia induced by photothrombosis (PT). Analysis of the infarcted and peri-infarct brain tissue by immunoblotting, immunohistochemistry and RT-PCR revealed that there is no evidence for the expression of TTR in the brain parenchyma. Nevertheless, some scattered cell bodies positive for TTR were found in the ischemic infarct core and proximal peri-infarcted tissue 14 days after PT. We did not observe TTR positive neurons, any expression of the protein in astroglial cell populations or microglia/macrophages in the peri-infarct area. During the discussion, we will elaborate on possible functions of TTR in the brain and discuss results regarding the expression of TTR after stroke. Moreover, we will discuss our results showing scattered TTR positive cells in the ischemic infarct core.

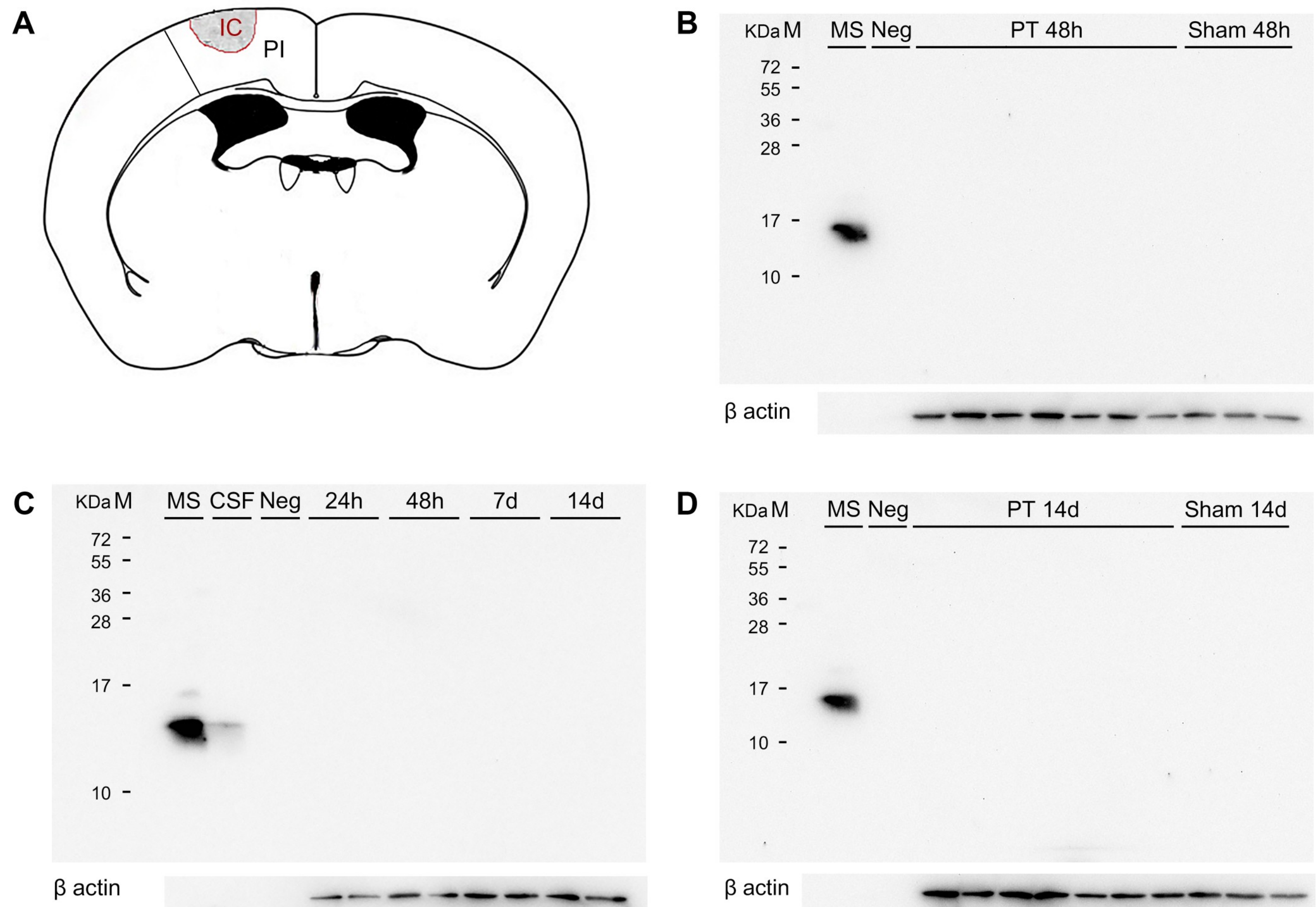


Fig 4. TTR levels at different time points after PT. **(A)** The ischemic territory region was sampled for protein extraction (coronal section of an adult mouse brain adapted from [21]. **(B)** TTR protein is absent in the ischemic territory 48 hours after PT and absent in the brain parenchyma of wild type mice not subjected to ischemic stroke. **(C)** TTR is not expressed in the ischemic territory at different time points after PT: 24 hours, 48 hours, 7 days and 14 days, respectively. **(D)** TTR protein is absent in the ischemic territory 14 days after PT and absent in the brain parenchyma of sham operated mice. Loading of 10 μg of total protein. Anti-TTR rabbit antibody diluted at 1:3000 (ThermoScientific cat# PA5-27220, Rockford, USA). CSF–cerebrospinal fluid; IC—Infarct Core; M–Marker; Neg–negative control (recombinant legumain 2 μg); PI–Peri-infarct area; MS–Positive control (mouse serum); PT–Photothrombosis; TTR–Transthyretin.

https://doi.org/10.1371/journal.pone.0221555.g004

Attention has been given to TTR as a neuroprotective protein, in particular in Alzheimer´s disease [13–15] and stroke [10–12] through its putative upregulation in the brain. In the context of Alzheimer´s disease, TTR has been suggested as a biological sequester of Aβ, since it is able to bind to soluble Aβ peptide, preventing the formation of amyloid fibers [22,23]. Some studies in rat and mouse models of Alzheimer´s disease show an overexpression and production of mRNA transcription of TTR in the hippocampus [19,24,25]. *In vitro* studies also support TTR synthesis in neurons as neuroprotective in Alzheimer´s disease, since primary cultures of mixed cortical and hippocampal neurons from APP23 mice express TTR [17,18], and there is an upregulation of *ttr* in the SH-SY5Y neuroblastoma cell line over-expressing APP_{695} isoform [16]. *TTR* expression has also been found in the frontal cortex of postmortem brain tissues from patients with Alzheimer´s disease [26].

After stroke, TTR immunopositive cells have been detected in the infarct core 24 h after permanent occlusion of the middle cerebral artery with no expression of *ttr* in the postischemic brain [12]. Recently, it has been reported that intranasal and intracerebroventricular

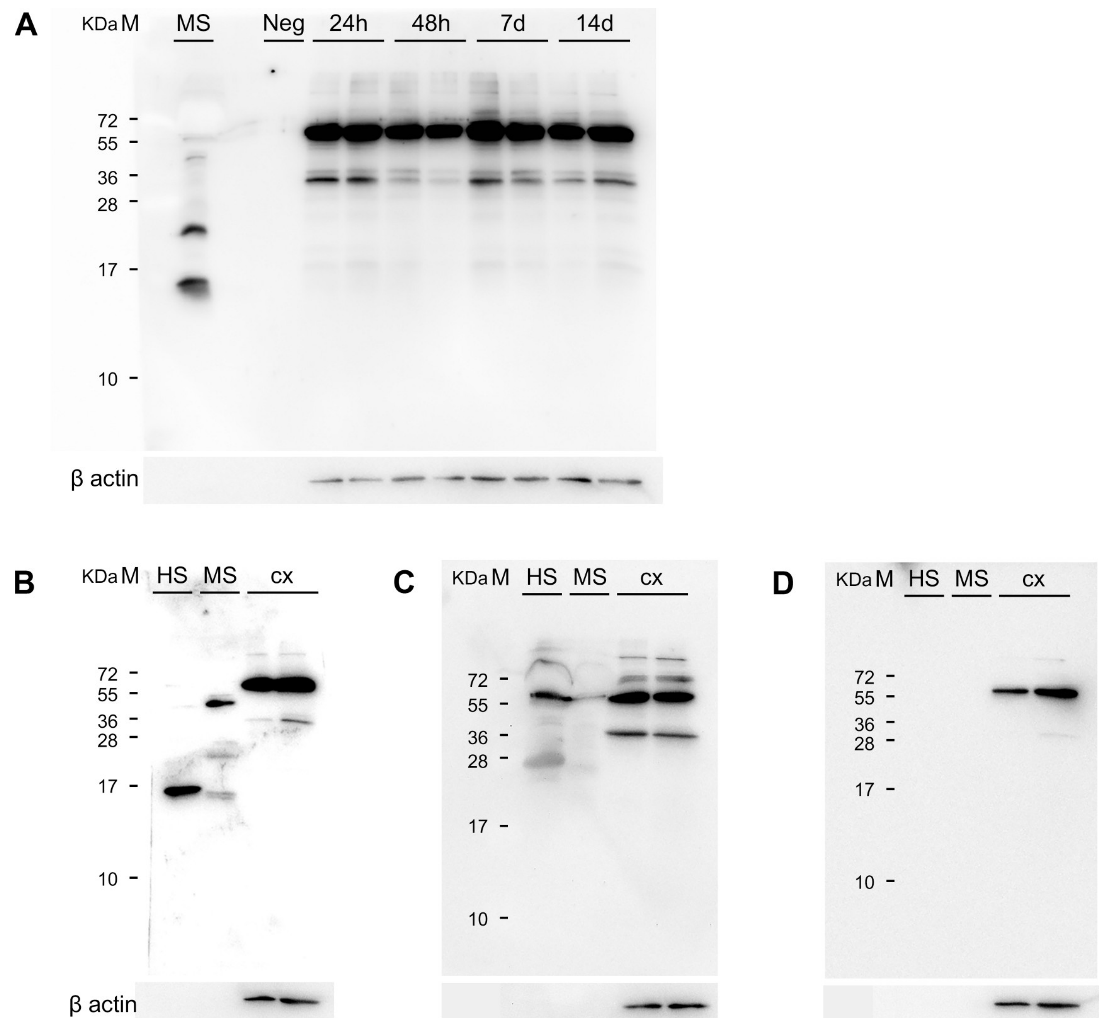


Fig 5. Evaluation of a chicken TTR antibody and TTR levels in brain parenchyma. (A) In contrast to protein extracts obtained from ischemic territory (infarct core–peri-infarct area) at 24 hours, 48 hours, 7 days and 14 days after PT, the monomeric form of TTR is found in samples of mouse serum, used as positive control. **(B)** TTR in human and mouse serum and absence of the protein in the cortical ischemic territory 14 days after PT. Instead, a band of approximately 60 to 65 kDa is found, not corresponding to the TTR protein **(C)** Blocking of specific TTR bands by preincubation with a specific TTR peptide. **(D)** Western blot performed without primary antibody incubation. HS–Human serum; M–Marker; MS–mouse serum; PT–Photothrombosis; TTR–Transthyretin; cx–cortex.

https://doi.org/10.1371/journal.pone.0221555.g005

delivery of an anti-TTR nanobody 169F7 reached several brain regions and spinal cord in naïve mice. From differences in the concentration of nanobodies in different regions of the CNS the authors concluded that TTR is synthesized in these tissues [27]. On the other hand, it has been suggested that the results observed in brain tissues are due to contamination by CP. TTR was not found either in the cerebral cortex, hippocampus or cerebellum, verified by TTR immunoreactivity, immunoblotting and TTR mRNA expression in wildtype mice and APP-V717I and Tg2576 mice models of Alzheimer´s disease after laser microdissection [4]. Likewise, upregulation of TTR has been shown exclusively in the CP in a proteomic study in rats subjected to transient MCAO supporting the idea that TTR expression is restricted to the CP [10]. In the present study, we found TTR synthesized in the CP but not in brain parenchyma. Our study clearly shows that TTR is not expressed in brain resident cells of the ischemic territory of C57/B6 mice, 24 hours, 48 hours, 7 days and 14 days after permanent focal

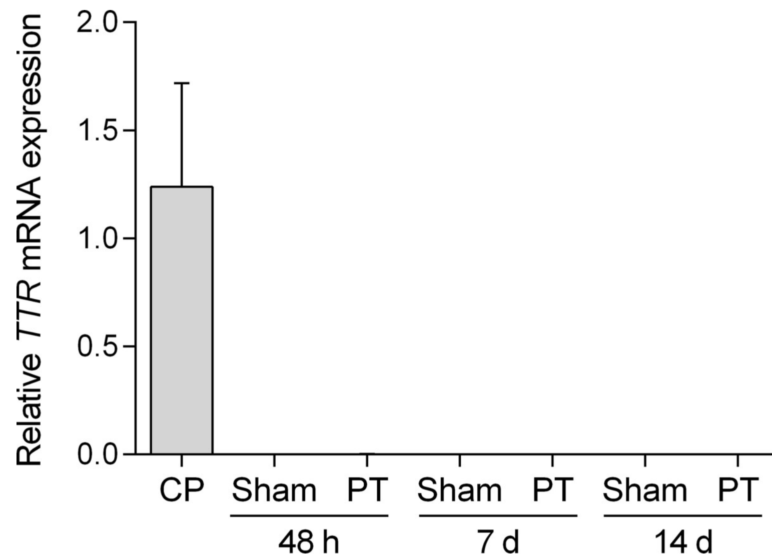


Fig 6. Expression of *ttr* in the postischemic brain. Relative expression *ttr* to GAPDH mRNA was semi-quantified by real time PCR in the choroid plexus (positive control) and brain parenchyma. Compared to the control, *ttr* is not expressed in brain parenchyma after photothrombosis or sham surgeries after 48 hours, 7 days and 14 days, respectively. Data represents means ± SEM of n = 2 to 5 mice. CP–Choroid Plexus; PT–Photothrombosis; TTR–Transthyretin.

https://doi.org/10.1371/journal.pone.0221555.g006

ischemia. Scattered and inconsistently appearing TTR positive cells found in the ischemic territory are considered as cellular debris supported by their random morphology. Positive cells were found randomly throughout the study. Immunoreactivity was not responsive to titration of antibodies neither to a specific TTR blocking peptide. We excluded that immunoreactivity reflects a *de novo* TTR synthesis in neurons since RT-PCR experiments showed non-detectable levels of *ttr*, neither found we evidences for TTR transport from the blood or CSF. Results from our RT-PCR confirm previous results showing that *ttr* is not expressed in brain parenchyma 24 hours after permanent MCAO [12]. If TTR like proteins such as TTR-52, a bridging molecule in the phagocytic process by phagocytes and in the regeneration of axons, are expressed, remains open and might be focus of future studies. In *Caenorhabditis elegans*, a TTR-like protein mediates the recognition of apoptotic cells by crosslinking with Phosphatidyl Serine (Eat me Signal) with a receptor in the phagocyte (macrophages/microglia), being involved in the engulfment of apoptotic cells [28,29].

Nevertheless, and despite of its absence in brain parenchyma, TTR has been shown to promote neuroprotection. Activation of neuroprotective pathways is partially mediated by binding of high concentrated exogenous TTR to low density lipoprotein-related protein 2 (LRP2) in neuronal cultures. This interaction is also involved in TTR mediated neurite outgrowth. Neuronal cultures from LRP2 +/- /TTR deficient mice showed reduced neurite outgrowth compared to cell cultures from LRP2 +/+ /TTR deficient mice, both treated with TTR [11]. In contrast, TTR deficiency alone has no effect on ischemic damage in mice subjected to permanent MCAO [15]. Some immediate effects on lesion size 24 hours after stroke onset were observed in TTR deficient mice heterozygous for heat shock transcription factor 1 (HSF1) [12]. The difference in lesion size and outcome between TTR deficient mouse strains might be explained by reduced or suppressed levels of HSF1 resulting in a compromised heat shock response during the first hours after stroke onset [30]. In addition, TTR is reported as a neurogenic factor in the peripheral nervous system, enhancing neurite outgrowth *in vitro* and nerve regeneration *in vivo* studies [31,32].

5. Conclusions

Independent of its role as a transport protein, TTR has been studied as neuroprotective molecule in brain pathologies such as stroke. Here we found that TTR is not expressed in the infarct core and peri-infarct area during the first 14 days after photothrombosis, being restricted to the choroid plexus. Nevertheless, scattered cells showing TTR-like immunoreactivity were found in the infarct core in some animals, which may correspond to unspecific binding of the antibody to cellular debris, due to their morphology and inconsistent appearance. Although TTR is absent from brain parenchyma after stroke, it is neuroprotective *in vitro* models of ischemia, and we cannot exclude that CP derived TTR may provide similar effects *in vivo*.

Supporting information

S1 Table. The ARRIVE guidelines checklist animal research: Reporting in vivo experiments.
(PDF)

S2 Table. Mean of body weights and temperatures of animals over the time course of the experimental procedures. BW–Body weight; IHC–Immunohistochemistry; PT–Photothrombosis; SD–Standard deviation; Temp–Temperature; WB–Western Blotting.
(DOCX)

Author Contributions

Conceptualization: Daniela Talhada, Cecília Reis Santos, Karsten Ruscher.

Data curation: Daniela Talhada, Karsten Ruscher.

Formal analysis: Daniela Talhada.

Funding acquisition: Isabel Gonçalves, Cecília Reis Santos, Karsten Ruscher.

Investigation: Daniela Talhada, Karsten Ruscher.

Methodology: Daniela Talhada, Karsten Ruscher.

Project administration: Karsten Ruscher.

Resources: Karsten Ruscher.

Supervision: Isabel Gonçalves, Cecília Reis Santos, Karsten Ruscher.

Validation: Daniela Talhada, Isabel Gonçalves, Cecília Reis Santos, Karsten Ruscher.

Writing – original draft: Daniela Talhada, Karsten Ruscher.

Writing – review & editing: Daniela Talhada, Isabel Gonçalves, Cecília Reis Santos, Karsten Ruscher.

References

1. Wakasugi S, Maeda S, Shimada K. Structure and expression of the mouse prealbumin gene. J Biochem. 1986; 100: 49–58. Available: https://www.ncbi.nlm.nih.gov/pubmed/?term=wakasugi+maeda+shimada+1986 https://doi.org/10.1093/oxfordjournals.jbchem.a121705 PMID: 3020014
2. Aleshire SL, Bradley CA, Richardson LD, Parl FF. Localization of human prealbumin in choroid plexus epithelium. J Histochem Cytochem. 1983; 31: 608–612. https://doi.org/10.1177/31.5.6341455 PMID: 6341455
3. Felding P, Fex G. Cellular origin of prealbumin in the rat. BBA—Gen Subj. 1982; 716: 446–449. https://doi.org/10.1016/0304-4165(82)90040-X

4. Sousa JC, Cardoso I, Marques F, Saraiva MJ, Palha JA. Transthyretin and Alzheimer's disease: Where in the brain? Neurobiol Aging. 2007; 28: 713–718. https://doi.org/10.1016/j.neurobiolaging.2006.03.015 PMID: 16698124

5. Tang YP, Haslam SZ, Conrad SE, Sisk CL. Estrogen increases brain expression of the mRNA encoding transthyretin, an amyloid beta scavenger protein. J Alzheimers Dis. 2004; 6: 413–420. Available: http://www.ncbi.nlm.nih.gov/pubmed/15345812 PMID: 15345812

6. Blay P, Nilsson C, Owman C, Aldred A, Schreiber G. Transthyretin expression in the rat brain: effect of thyroid functional state and role in thyroxine transport. Brain Res. 1993; 632: 114–120. https://doi.org/10.1016/0006-8993(93)91145-i PMID: 8149219

7. Herbert J, Wilcox JN, Pham KT, Fremeau RTJ, Zeviani M, Dwork A, et al. Transthyretin: a choroid plexus-specific transport protein in human brain. The 1986 S. Weir Mitchell award. Neurology. 1986; 36: 900–911. https://doi.org/10.1212/wnl.36.7.900 PMID: 3714052

8. Li X, Buxbaum JN. Transthyretin and the brain re-visited: is neuronal synthesis of transthyretin protective in Alzheimer's disease? Mol Neurodegener. 2011; 6: 79. https://doi.org/10.1186/1750-1326-6-79 PMID: 22112803

9. Zhou L, Tang X, Li X, Bai Y, Buxbaum JN, Chen G. Identification of transthyretin as a novel interacting partner for the δ subunit of GABAA receptors. PLoS One. 2019; 14: e0210094. https://doi.org/10.1371/journal.pone.0210094 PMID: 30615651

10. Suzuyama K, Shiraishi T, Oishi T, Ueda S, Okamoto H, Furuta M, et al. Combined proteomic approach with SELDI-TOF-MS and peptide mass fingerprinting identified the rapid increase of monomeric transthyretin in rat cerebrospinal fluid after transient focal cerebral ischemia. Mol Brain Res. 2004; 129: 44–53. https://doi.org/10.1016/j.molbrainres.2004.06.021 PMID: 15469881

11. Gomes JR, Nogueira RS, Vieira M, Santos SD, Ferraz-Nogueira JP, Relvas JB, et al. Transthyretin provides trophic support via megalin by promoting neurite outgrowth and neuroprotection in cerebral ischemia. Cell Death Differ. England; 2016; 23: 1749–1764. https://doi.org/10.1038/cdd.2016.64 PMID: 27518433

12. Santos SD, Lambertsen KL, Clausen BH, Akinc A, Alvarez R, Finsen B, et al. CSF transthyretin neuroprotection in a mouse model of brain ischemia. J Neurochem. 2010; 115: 1434–1444. https://doi.org/10.1111/j.1471-4159.2010.07047.x PMID: 21044072

13. González-Marrero I, Giménez-Llort L, Johanson CE, Carmona-Calero EM, Castañeyra-Ruiz L, Brito-Armas JM, et al. Choroid plexus dysfunction impairs beta-amyloid clearance in a triple transgenic mouse model of Alzheimer's disease. Front Cell Neurosci. 2015; 9: 1–17. https://doi.org/10.3389/fncel.2015.00001

14. Buxbaum JN, Ye Z, Reixach N, Friske L, Levy C, Das P, et al. Transthyretin protects Alzheimer's mice from the behavioral and biochemical effects of Abeta toxicity. Proc Natl Acad Sci U S A. 2008; 105: 2681–2686. https://doi.org/10.1073/pnas.0712197105 PMID: 18272491

15. Costa R, Gonçalves A, Saraiva MJ, Cardoso I. Transthyretin binding to A-Beta peptide—Impact on A-Beta fibrillogenesis and toxicity. FEBS Lett. 2008; 582: 936–942. https://doi.org/10.1016/j.febslet.2008.02.034 PMID: 18295603

16. Kerridge C, Belyaev ND, Nalivaeva NN, Turner AJ. The Aβ-clearance protein transthyretin, like neprilysin, is epigenetically regulated by the amyloid precursor protein intracellular domain. J Neurochem. 2014; 130: 419–431. https://doi.org/10.1111/jnc.12680 PMID: 24528201

17. Wang X, Cattaneo F, Ryno L, Hulleman J, Reixach N, Buxbaum JN. The systemic amyloid precursor transthyretin (TTR) behaves as a neuronal stress protein regulated by HSF1 in SH-SY5Y human neuroblastoma cells and APP23 Alzheimer's disease model mice. J Neurosci. 2014; 34: 7253–7265. https://doi.org/10.1523/JNEUROSCI.4936-13.2014 PMID: 24849358

18. Li X, Masliah E, Reixach N, Buxbaum JN. Neuronal production of transthyretin in human and murine Alzheimer's disease: is it protective? J Neurosci. 2011; 31: 12483–12490. https://doi.org/10.1523/JNEUROSCI.2417-11.2011 PMID: 21880910

19. Stein TD, Johnson JA. Lack of neurodegeneration in transgenic mice overexpressing mutant amyloid precursor protein is associated with increased levels of transthyretin and the activation of cell survival pathways. J Neurosci. 2002; 22: 7380–7388. 22/17/7380 [pii] PMID: 12196559

20. Walter HL, van der Maten G, Antunes AR, Wieloch T, Ruscher K. Treatment with AMD3100 attenuates the microglial response and improves outcome after experimental stroke. J Neuroinflammation. 2015; 12: 1–24. https://doi.org/10.1186/s12974-014-0232-1 PMID: 25881123

21. Paxinos G, Franklin KBJ. The mouse brain in stereotaxic coordinates [Internet]. Academic Press. 2004. https://doi.org/10.1016/S0306-4530(03)00088-X

22. Golabek A, Marques M, Lalowski M, Wisniewski T. Amyloid beta binding proteins in vitro and in normal human cerebrospinal fluid. Neurosci Lett. 1995; 191: 79–82. https://doi.org/10.1016/0304-3940(95)11565-7 PMID: 7659297

23. Schwarzman AL, Gregori L, Vitek MP, Lyubski S, Strittmatter WJ, Enghilde JJ, et al. Transthyretin sequesters amyloid beta protein and prevents amyloid formation. Proc Natl Acad Sci U S A. 1994; 91: 8368–8372. https://doi.org/10.1073/pnas.91.18.8368 PMID: 8078889

24. Puskas LG, Kitajka K, Nyakas C, Barcelo-Coblijn G, Farkas T. Short-term administration of omega 3 fatty acids from fish oil results in increased transthyretin transcription in old rat hippocampus. Proc Natl Acad Sci U S A. United States; 2003; 100: 1580–1585. https://doi.org/10.1073/pnas.0337683100 PMID: 12566565

25. Watanabe CM, Wolffram S, Ader P, Rimbach G, Packer L, Maguire JJ, et al. The in vivo neuromodulatory effects of the herbal medicine ginkgo biloba. Proc Natl Acad Sci U S A. 2001; 98: 6577–6580. https://doi.org/10.1073/pnas.111126298 PMID: 11381109

26. Laiterä T, Kurki MI, Pursiheimo JP, Zetterberg H, Helisalmi S, Rauramaa T, et al. The expression of transthyretin and amyloid-β protein precursor is altered in the brain of idiopathic normal pressure hydrocephalus patients. J Alzheimer's Dis. 2015; https://doi.org/10.3233/JAD-150268 PMID: 26444765

27. Gomes JR, Cabrito I, Soares HR, Costelha S, Teixeira A, Wittelsberger A, et al. Delivery of an anti-transthyretin Nanobody to the brain through intranasal administration reveals transthyretin expression and secretion by motor neurons. J Neurochem. 2018; https://doi.org/10.1111/jnc.14332 PMID: 29527688

28. Wang X, Li W, Zhao D, Liu B, Shi Y, Chen B, et al. Caenorhabditis elegans transthyretin-like protein TTR-52 mediates recognition of apoptotic cells by the CED-1 phagocyte receptor. Nat Cell Biol. 2010; 12: 655–64. https://doi.org/10.1038/ncb2068 PMID: 20526330

29. Neumann B, Coakley S, Giordano-Santini R, Linton C, Lee ES, Nakagawa A, et al. EFF-1-mediated regenerative axonal fusion requires components of the apoptotic pathway. Nature. 2015; 517: 219–222. https://doi.org/10.1038/nature14102 PMID: 25567286

30. Sharp FR, Zhan X, Liu DZ. Heat shock proteins in the brain: role of Hsp70, Hsp27, and HO-1 (Hsp32) and their therapeutic potential. Transl Stroke Res. United States; 2013; 4: 685–692. https://doi.org/10.1007/s12975-013-0271-4 PMID: 24323422

31. Fleming CE, Mar FM, Franquinho F, Saraiva MJ, Sousa MM. Transthyretin internalization by sensory neurons is megalin mediated and necessary for its neuritogenic activity. J Neurosci. 2009; 29: 3220–32. https://doi.org/10.1523/JNEUROSCI.6012-08.2009 PMID: 19279259

32. Fleming CE, Saraiva MJ, Sousa MM. Transthyretin enhances nerve regeneration. J Neurochem. 2007; 103: 831–839. https://doi.org/10.1111/j.1471-4159.2007.04828.x PMID: 17897357

CHAPTER 6

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

In analogy to brain development and experience learning processes, reorganization of cortical networks after experimental stroke is dependent on molecular, cellular and structural self-repair changes that peak in rodents during the first weeks following the injury. The efficacy to enhance those mechanisms will influence functional outcome. This thesis addresses first, the importance of TH in the brain as a key component that contributes to motor recovery, based in epidemiological and preclinical studies. Second, the influence of TH on homeostatic mechanisms that adjust excitability – inhibition ratio in the postischemic brain and cultured glutamatergic neurons that are important for motor recovery. Third the expression pattern of TR in human post-stroke brain and fourth, the role of TH carrier protein TTR in the postischemic brain.

In Chapter 4, our work demonstrates that T_3 modulates homeostatic regulatory mechanisms that maintain appropriate levels of excitation in the postischemic brain, stabilizes neuronal activity and contributes for neuronal reorganization and functional recovery. At the cellular and structural level, we show that T_3 adjusts several plasticity mechanisms that can operate on different temporal and spatial scales to assure appropriate synaptic neurotransmission. We have shown *in vivo* that long-term administration of T_3 after PT significantly (i) enhances lost motor function; (ii) increases levels of synaptotagmin and levels of the post-synaptic GluR2 subunit in AMPA receptors in the peri-infarct area; (iii) increases dendritic spine density in the peri-infarct and contralateral region and (iv) decreases tonic GABAergic signaling in the peri-infarct area by a reduced number of PV^+ / c-fos^+ neurons and GAD 65/67 levels. In addition, we have shown that T_3 modulates *in vitro* neuron membrane properties with the balance of inward glutamate ligand-gated channels currents and decreases synaptotagmin levels in OGD conditions. Interestingly, we found increased levels of TRβ1 in the infarct core of *post-mortem* human stroke patients, which mediate T_3 actions. On the other hand, the TH carrier protein TTR was not expressed in the cortex of the postischemic brain as we show in Chapter 5, and TTR is unlikely involved in recovery mechanisms during the first two weeks following experimental stroke induced by PT.

Summarizing, our data identify T_3 as potential therapeutic agent to enhance recovery of lost neurological functions after ischemic stroke. We have shown that T_3 can engage divergent signaling pathways to adjust brain excitability. However, much work will be needed to address many other remaining questions. What is the origin of the mechanisms involved in homeostatic plasticity engaged by T_3? How does T_3 engage homeostatic synaptic responses in neurons? What are the molecular players implicated in homeostatic plasticity? What is the exact timescale and spatial extent optimal for actions of T_3 implemented in changes of neuronal activity?

Moreover, motor recovery after stroke is a complex, multifactorial and dynamic process, dependent on many other pathophysiological events that were not addressed in this thesis. It remains to be elucidated if TH are implicated in the attenuation of post-stroke inflammation, in glial scar formation, in the modulation of inhibitory proteins of plasticity, in neurogenesis and in angiogenesis.

In conclusion, mechanisms of homeostatic regulation are diverse due to heterogenous nature of stroke. Biological targets for restorative therapies change over time and proper maintenance of neural activity can differ among individual cases. It is of note, that induced plasticity might not have the desired effect when preclinical studies are translated to the human. In this context, unraveling the underlying mechanisms of T_3 actions in the postischemic brain is crucial to define a time window for intervention and the development of cell-specific treatments to target T_3 signaling, that later might be translated into clinical trials.