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Review of the Neuroanatomic Landscape Implicated in Glucose Sensing and Regulation of Nutrient Signaling: Immunophenotypic Localization of Diabetes Gene *Tcf7l2* in the Developing Murine Brain

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Abstract

Genetic variants in the transcription factor 7-like 2 (*Tcf7l2*) gene have been found to confer a significant risk of type 2 diabetes and attenuated insulin secretion. Based on its genomic wide association *Tcf7l2* is considered the single most important predictor of diabetes to date. Previous studies of *Tcf7l2* mRNA localization in the adult brain suggest a putative role of *Tcf7l2* in the CNS regulation of energy homeostasis. The present study further characterizes the immunophenotypic distribution of peptide expression in the brains of *Tcf7l2* progeny during developmental time periods between E12.5 and P1. *Tcf7l2*^{-/-} is lethal beyond P1. Results show that while negligible TCF7L2 expression is found in the developing brains of *Tcf7l2*^{-/-} mice, TCF7L2 protein is relatively widespread and robustly expressed in the brain by E18.5 and exhibits specific expression within neuronal populations and regions of the brain in *Tcf7l2*^{+/-} and *Tcf7l2*^{+/+} progeny. Strong immunophenotypic labeling was found in the diencephalic structure of the thalamus that suggests a role of *Tcf7l2* in the development and maintenance of thalamic activity. Strongly expressed TCF7L2 was localized in select hypothalamic and preoptic nuclei indicative of *Tcf7l2* function within neurons controlling energy balance. Definitive neuronal staining for TCF7L2 within nuclei of the brain stem and circumventricular organs extends TCF7L2 localization within autonomic neurons and its potential integration with autonomic function. In addition robust TCF7L2 expression was found in the tectal and tegmental structures of the superior and inferior colliculi as well as transient expression in neuroepithelium of the cerebral and hippocampal cortices of E16 and E18.5. Patterns of TCF7L2 peptide localization when compared to the adult protein synthetic chemical/anatomical landscape of glucose sensing exhibit a good correlational fit between its expression and regions, nuclei, and pathways regulating energy homeostasis via integration and response to peripheral endocrine, metabolic and neuronal signaling. TCF was also found co-localized with peptides that regulate energy homeostasis

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Ethical Statement

The protocol and animals used in this study were reviewed and approved by the university IACUC which adheres to the NIH and AAALAC guidelines for the ethical and humane treatment of animals.

including AgRP, POMC and NPY. TCF7L2, some variants of which have been shown to impair GLP-1-induced insulin secretion, was also found co-localize with GLP-1 in adult TCF wild type progeny. Impaired *Tcf7l2*-mediated neural regulation may contribute to the risk and/or underlying pathophysiology of type 2 diabetes that has found high expression in genomic studies of *Tcf7l2* variants.

Keywords

type 2 diabetes mellitus; *Tcf7l2* gene; TCF7L2 peptide; transcription factor 7-like 2; developing brain; glucose sensing; embryonic development; neuroanatomy; immunohistochemistry; neural development

1. Introduction

The *Tcf7l2* (Transcription factor 7-like-2) gene is a transcription factor that plays a key role as a downstream effector in the canonical Wnt signaling pathway (Huelsen and Birchmeier 2001, Korinek et al., 1998). Numerous studies have subsequently confirmed the initial report that a variant of the *Tcf7l2* gene confers an increased risk of type 2 diabetes (Grant et al., 2006; Zeggini and McCarthy 2007). Variants of *Tcf7l2* have been shown specifically to impair GLP-1-induced insulin secretion (Schäfer et al., 2007), insulin secretion and conversion of proinsulin to insulin (Kirchhoff et al., 2008), insulin secretion as well as incretin effects (Lyssenko et al., 2007) and impairment of glucose tolerance (Florez et al., 2006). Aside from epidemiological studies, *Tcf7l2* expression and function has also been studied in animal tissues including intestine (Korinek et al., 1998), pancreatic islets (da Silva Xavier et al., 2009; Shu, et al., 2008) and adipocytes in adipogenesis (Johansson et al., 2006; Ahlzen et al., 2008). Recently studies have localized *Tcf7l2* in the brain including the general online ISH mapping site of the Allen Brain Institute (Lein et al., 2007), as well as more specific studies of the thalamus (Murray et al., 2007), splice isoform variability in cerebellar anlage (Nazwar et al., 2009) and the combined ISH-IHC study of the adult mouse brain (Lee et al., 2009). Aside from pituitary organogenesis (Brinkmeier et al., 2003, 2007), no studies to date however have been carried out that have used the specific *Tcf7l2*-deficient mouse model to compare the developmental dynamics in neuronal expression and spatial localization of the TCF7L2 peptide. The primary goals of this study was to compare and contrast earlier studies that have mapped the topography of *Tcf7l2* mRNA hybridization in the adult brain with the distribution of protein expression in the brains of wild type, null and heterozygote *Tcf7l2* progeny at E12.5, 16, 18.5 and P1 neonates. Secondly, since no attempt has been made to date to explain the neuronal localization of this important gene and gene product that have been hypothesized to play a role in the pathogenesis of type 2 diabetes, the present study includes a review and comparison of the cellular localization of TCF7L2 peptide within key regions and nuclei of the brain. This analysis characterizes the local milieu within which the TCF7L2 peptide is expressed in the context of glucose utilization, glucose sensing and other potential interactions that may implicate this transcription factor in glucose neuromodulation.

2. Materials and Methods

2.1 Animals and Tissue Processing

Breeding pairs of our current *Tcf7l2* mouse colony heterozygous for targeted attenuation of the *Tcf7l2* locus was originally provided by Dr. Hans Clevers, University Medical Center Utrecht, Heidelberglaan. Gel preparations of primers specific for *Tcf7l2* were used to identify the wild type allele while those recognizing hygromycin identified the knockout

allele (Brinkmeier, et al. 2003). The colony was maintained in isolated quarters and received a 12 light/12 hour dark cycle and food and water *ad libitum*. Animal procedures and handling were carried out according to Animal Welfare regulations of the university IACUC and RAR auspices. Pregnant dams were collected at timed intervals post-copulation plugging at 12.5-14.5, 15-16.5 and 17-20. Neonatal pups were collected between the P₀-P₁ interval. Sample measurements were collected on wild type progeny to corroborate gestational age using crown-rump length (CRL) in which E 12.5-13.5, 7.5-8.5mm; E14.5-15.5, 9.0-12.0mm; E15.5-17.5, 13-14mm; E19-20, 18.8-20.4mm and exhibited a good correlative fit. All animals were killed by CO₂ asphyxiation. Tissues were immediately collected and fixed by submersion fixation using Zamboni's fixative. These were fixed for 24 hrs, washed in PBS, dehydrated, cleared and infiltrated with paraffin. Specimens either as whole bodies or heads were placed in liquid paraffin, oriented to yield coronal and/or sagittal planes for subsequent sectioning and the paraffin solidified. Brains were sectioned at 7µm and collected as serial sections which were mounted on SuperFrost Plus slides as multiple sections ranging from four to five sections per slide.

2.2 Immunohistochemistry

Sections collected at known intervals were stained with Nissl (.5% cresyl violet acetate) and served as reference slides for each brain set. Selected intervening sections were stained for either brightfield or fluorescence immunohistochemistry for selected neuronal markers. Sections allocated for brightfield microscopy were deparaffinized in xylene, rehydrated, and washed in PBS (pH 7.2, 3×5min). Antigen retrieval was performed using the microwave technique and citric acid unmasking buffer (Vector Labs) for 30 min at 95°C. Sections were cooled to 4°C and washed in cold PBS (3×5min). Endogenous peroxidase was reduced by incubation of sections in 3% H₂O₂ in cold PBS for 30 min at 4°C. Sections were washed in cold PBS (3×5min), encircled with a PAP pen and non-specific binding blocked by incubation of sections in normal serum at room temperature for 30 min. Excess sera were removed and the primary antibody applied and sections incubated overnight at 4°C. Primary antibodies and their host and dilutions included: TCF-4, Millipore, clone 6H5-3(05-511), mouse monoclonal, 1:800; TCF-4, Millipore, clone EP2033Y(04-1080), rabbit monoclonal, 1:250; TCF7L2, Novus Biologicals, H00006934-M06, mouse monoclonal, 3µg/ml; Neuronal Nuclei, Millipore MAB377, mouse monoclonal, 1:100; Oligodendrocyte transcription factor (Olig 2), Millipore, AD9610, rabbit polyclonal, 1:1000; Anti-phospho-Histone (H3), Millipore, 06-570, rabbit polyclonal, 10mg/ml; Sox 21, Abcam, 24604, rabbit polyclonal, 1:300; Nestin, Abcam, 5468, rabbit polyclonal, 1:1000; Agouti-Related Protein (AgRP), Neuromics, GT15023, goat polyclonal, 5µg/ml; AgRP, Phoenix Pharmaceuticals, H003-57, rabbit polyclonal, 1:200; AgRP, Santa Cruz, sc-18334, goat polyclonal, 1:100; Neuronal Peptide Y (NPY), Millipore, 1583, sheep polyclonal, 1:1000; NPY, Immunostar, AB22940, rabbit polyclonal, 1:5000; Proopiomelanocortin Precursor (POMC), Santa Cruz, sc-20148, rabbit polyclonal, 1:100; POMC, Abcam, ab32893, goat polyclonal, 1:100; Neuron specific β II Tubulin, Abcam, 78078, mouse monoclonal, 1µg/ml; and Doublecortin (phospho 528), Abcam, ab23544, rabbit polyclonal, 1:1000. Initially all antibodies were substituted with buffer or adsorbed with antigen to assess immunological specificity and to serve as controls. Following incubation in the primary antibody, the sections were rinsed in PBS as above and incubated in the appropriate biotinylated secondary antibody (Vector Labs) at room temperature for 30 min. Following secondary incubation, sections were washed in PBS and the ABC complex applied for 30 minutes at room temperature. These were washed for a final time and the biotin/avidin complex developed using several substrates including oxidized DAB-Ni (Vector, SK-4100), NovaRED (Vector, SK-4800) and Blue Alkaline Phosphatase (SK-5300). Sections were counterstained with either a modified Van Gieson or haematoxylin, dehydrated and coverslipped with Permount. Sections were viewed and photographed on an M1 Zeiss

upright microscope. Sections allocated for fluorescence microscopy were treated as above except for the application of secondary antibodies. Depending upon the host of the primary antibody, the secondary antibody was selected from the following: DyLight(DL) 488, donkey, anti-mouse; DL 688, donkey, antirabbit; DL 488, donkey, anti-goat; FITC, donkey anti-sheep; DL Cy 3, donkey, anti-rabbit, DL Cy 3, donkey, anti-mouse, DL Cy 3, goat, anti-rabbit; DL 405, goat, anti-mouse; DL 350, goat, anti-mouse, Cy 5, donkey, anti-rabbit; AlexaFluor(AF) 488, goat, anti-mouse, AF 488, donkey, anti-rabbit; AF 680, donkey, anti-rabbit; AF 680, donkey, anti-mouse; AF 594, rabbit, anti-goat; AF 405, donkey, anti-goat. All secondary antibodies were incubated at room temperature for two hours and at a dilution of 1:200. Following secondary incubation, sections were washed in PBS and mounted with VECTASHIELD®(Vector Labs) media with or without DAPI. Sections were viewed and photographed on an M1 Zeiss upright microscope equipped with fluorescence filters.

3. Results

Although the earliest expression of TCF7L2 can be found in wild type and heterozygote embryos between E12.5, the staining is restricted largely to the thalamus and colliculi. All null progeny including those surviving to P₀-P₁ exhibited negligible labeling(See: Supplemental Figure 1, A-D). *Tcf7l2* heterozygote staining was similar to that found in wild type progeny and included in the study to ascertain the degree of expression of TCF7L2 in the heterozygote state. As shown in Figure 1(A-L) taken from a series of coronal brain sections, the immunohistochemical localization of TCF7L2 is relatively widespread and varies in its expression within neuronal populations and regions of the brain in the wild type and het progeny at E18.5.

3.1 Rhombencephalon

Beginning at the caudal pole, TCF7L2 immunoreactivity is robustly expressed among the densely populated neurons of the central nucleus of the inferior colliculus(Fig 1A, IC) and the less dense cells of its ventricular zone(Fig 1B, V). Strong labeling was also found in the more sparsely populated cells of the superior colliculus(Fig 1C, SC). The colliculi remained strongly labeled throughout subsequent levels(Fig 1A-G) as did the cells of the superior colliculus(Fig 1A-G). Throughout the lower medullary levels cells of the medial longitudinal fasciculus were strongly labeled for TCF7L2(Fig 1A-C, mlf; Fig 2A). In these lower levels, cells of the prepositus hypoglossal nucleus showed moderate to strong TCF7L2 labeling(Fig 1C, Pr, Fig 2B) while scattered and lightly stained cells of the raphe magnus and obscurus nuclei were found as well(Fig 1A-C, RMg; ROb). Throughout middle to higher medullary levels moderately stained cells were also found in the dorsal tegmental nucleus(Fig 1C, DTg; Fig 2C) while those of the lateral tegmental nucleus were lightly stained(Fig 1C, LTg). At these and subsequent levels, the periaqueductal gray region was characterized by densely populated TCF7L2 immunoreactive cells(Fig 1C-G, PAG; Fig 2D). Within the medullary regions moderately to strongly labeled neurons were found that represented a number of nuclei including those of the reticular formation(Fig. 1B-C, RF), medial vestibular nucleus(Fig 1B, MVe), parapyramidal reticular nucleus(Fig 1B, PPy), solitary tract nucleus(Fig 1B, Sol), and dorsal cochlear nucleus(Fig 1B, DC). Moving cranially, higher medullary levels consisted of strongly labeled cells within the dorsal tegmental nucleus(Fig 1C-D, DTg), dorsal raphe nucleus(Fig 1D, DR), the mesencephalic trigeminal nucleus(Fig 1D, Me5), superior vestibular nucleus(Fig 1D, SuVe), the posterior dorsal tegmental nucleus(Fig 1D, PDTg), the lateral reticular nucleus(Fig 1D, LRt), the lateral vestibular nucleus(Fig 1D, LVe) and the gigantocellular reticular nucleus(Fig 1D, Gi). At pontine levels, scattered cells of the medial longitudinal fasciculus exhibited moderate to light immunoreactive staining(Fig 1E, mlf) while at higher pontine levels small nuclei representative of the microcellular tegmental nucleus(Fig 1F; MiTg; Fig 2F), the

medial raphe nucleus(Fig 1F, MnR; Fig 2F) and the reticular formation of pons(Fig 1E-F, RFp; Fig 2G) exhibited moderate to light TCF staining. Cells of the substantia nigra compactus exhibited moderate TCF expression(Fig. 1E, SN; Fig 2H) as well as scattered lightly stained cells of the red nucleus(Fig 1E, R).

3.2 Mesencephalon

At the mesencephalic level which marked the beginning of thalamic and hypothalamic regions, robustly stained cells included those of the medial geniculate nucleus(Fig 1G, MG), anterior pretectal nucleus(Fig 1G, APT) and the posterior thalamic nucleus(Fig 1G, Po) while cells of the ventral posterior medial and ventral posterior lateral thalamic nuclei exhibited a strong to robust staining(Fig 1G, VPM, VPL). Rostrally the initial cells of the ventromedial hypothalamus exhibited moderate to light staining(Fig 1G, VMH; Fig 2I). Cells of the arcuate nucleus stained with a strong to light immunoreactivity(Fig 1G, Arc; Fig 2J). At the caudal-most region of the next ascending level cells of the habenular nuclei(Fig 1H, Hb), lateral posterior thalamic nucleus(Fig 1H, LP), nucleus of the posterior commissure(Fig 1H, PCom), the anterior pretectal nucleus(Fig 1H, APT), the dorsal lateral geniculate nucleus(Fig 1H, DLG), the ventral posterior and ventral posterior medial thalamic nuclei(Fig 1H, VP, VPM) and the intergeniculate leaflet(Fig 1H, IGL) all exhibited strong TCF7L2 labeling. Moving more rostrally the cells of the ventral lateral geniculate nucleus(Fig 1H, VLG), ventral medial thalamic(Fig 1H, VM), subparafascicular thalamic nucleus(Fig 1H, SPF), paraventricular thalamic nucleus(Fig 1H, PV) and the cells of the retrouniens area(Fig 1H, Re) all expressed intense labeling for TCF7L2. In the medial rostral region, the sparse cells of the posterior hypothalamic nucleus exhibited moderate labeling(1H, PH). Laterally the cells of the subgeniculate thalamic nucleus(Fig 1G-H, SubG) and the parasubthalamic nucleus(1H, PSTh) all exhibited moderately strong staining. In the rostral-most region lie the moderately to strongly labeled cells of the ventromedial hypothalamic nucleus(Fig 1H, VMH), as well as those of the arcuate hypothalamic nucleus and the medial tuberal nucleus(Fig 1H, Arc, MT; Fig 2J; Fig 2K). Very few labeled cells were found within the dorsomedial hypothalamic nucleus(1H, DM). At the next level intensely labeled TCF7L2 cells were found in the medial and lateral habenular nuclei(Fig 1J, MHb, LHb), the paraventricular thalamic nucleus(Fig 1I, PV) and more rostrally the lateral dorsal thalamic nucleus(Fig 1I, LD), lateral part of the mediodorsal thalamic nucleus(Fig 1I, MDL), the anterioventral thalamic nucleus(Fig 1I, AV), the central medial thalamic nucleus(Fig 1I, CM), the ventral lateral thalamic nucleus(Fig 1I, VL) and the reticular thalamic nucleus (Fig 1I, Ret). More rostrally, moderate to lightly stained cells were found in the lateral hypothalamic nucleus(Fig 1I, LH), lateral and medial preoptic nuclei(Fig 1I, LPO, MPO) and the substantia innomina(Fig 1H, SI). Moderately to strongly stained cells were also found in the globus pallidus(Fig 1H, GP) and paraventricular hypothalamic nucleus(Fig 1I, Ph). Moving cranially, TCF7L2 labeled cells of the next level included the moderately stained neurons of the medial and lateral habenular nuclei(Fig 1J, MHb, LHb) within the epithalamus. More strongly stained neurons were found in the mediodorsal, lateral dorsal, and ventral lateral thalamic nuclei (Fig 1J, MD, LD, VL) as well as the reuniens thalamic nucleus(Fig 1J, Re). Scattered lightly to moderately stained neurons were found in the area of the globus pallidus(Fig 1J, GP). The cells of the lateral hypothalamic nucleus(Fig 1J, LH; Fig 2K) exhibited moderate to strong staining and were more densely arrayed.

3.3 Prosencephalon

Beginning at the forebrain level the distribution of TCF7L2-labeled cells included the robustly stained neurons of the subfornical organ(Fig 1K, SFO; Fig 2L), those of the lateral preoptic area(Fig 1K, LPO; Fig 3A), the medial preoptic nucleus(Fig 1K, MPO; Fig 3B) and smaller nuclei including the nucleus of horizontal limb of diagonal band(Fig 1K, DBh),

accumbens nucleus(Fig 1K, Acb) and magnocellular preoptic nucleus(Fig 1K, MCPO). At the remaining levels, intensely labeled TCF7L2 cells composed several layers lining the ventricular and subventricular zones of the lateral ganglionic eminence(Fig 1L, LG) which form the septal(Fig 1L, Sn, Fig 3C) and striatal neuroepithelium. Although present in the same zones of the lateral ganglionic eminence forming cortical neuroepithelium(Fig 1L, Cn) and medial ganglionic eminence forming the striatal neuroepithelium(Fig 1L, Mge), the cells of this layer exhibited considerably less intense labeling for TCF7L2. The strongest expression of TCF7L2 in the neuroepithelium was found between E14 and E18.5. A few moderately stained and scattered cells were found in the medial septal nucleus(Fig 1L, MS).

3.4 Parasagittal Planes

Parasagittal sections provided further insight to the distribution and expression of TCF7L2. The robust staining of the dense collection of neurons shown in Fig 3D-E which compose the parafascicular(PF), mediodorsal(MD), subparafascicular(SPF), anteriomedial(AM), ventral medial(VM), ventral posterior medial(VPM), and reticular(Ret) thalamic nuclei as well as the unstained fibers of the fasciculus retroflexus(fr) above and the cells of the zona incerta(ZI) below contributed to the well-defined demarcation of thalamic boundaries from the preteum above and the hypothalamus below. This sagittal section also illustrates labeled TCF7L2 cells of the tectum including moderately labeled cells of the preteum(Fig 3D-E, Ptec), periaqueductal gray(Fig 3D, PAG), dorsomedial periaqueductal gray(Fig 3D, DMPAG) and superior colliculus(Fig 3D, SC) as well as cells of the epithalamus including posterior commissural(pc), precommissural(PrC) and the medial and lateral habenular nuclei(Fig 3E, MHb, LHb) and the ventrolateral periaqueductal gray area(Fig 3D, VLPAG). In Fig 3F, moving subthalamically a clear profile of robust TCF7L2 labeled cells can be seen composing the ventromedial hypothalamic nucleus(VMH) near the pituitary(P) in this parasagittal section near the midline. In the brain stem adjacent to the thalamus the reticular cells of the pons were found to exhibit a strong immunoreactive label for TCF7L2(Fig 3F, RFp). This was found to be characteristic of the reticular cells throughout the brain stem including those reticular cells of the medulla(Fig 3F, RFm) and the gigantocellular reticular nuclei(Figs 3F-G, Gi). The latter extended inferiorly from the dorsogigantocellular reticular nucleus below the prepositus nucleus(Fig 3F, Pr) as lightly to strongly stained cells which became more intense within the anterior gigantocellular reticular nucleus. The typical netlike structure of the cells and fibers that populate the medulla, pons and mesencephalon of the brain stem is shown in Figure 3G. Moderate to intensely labeled cells were found within the nucleus of the solitary tract(Fig 3F, Sol) and the medial vestibular nucleus(3F, MVe) respectively. Sagittal sections also provided in many cases a clearer distribution of TCF7L2 immunoreactive cells within select nuclei. In Fig 4A the sagittal section shows definitively strong immunoreactive cells that populate the ventromedial and periventricular hypothalamic nuclei(Fig 4A, VMH, Pe) while moderate to lightly stained TCF7L2 immunoreactive cells were found in the dorsomedial and posterior hypothalamic nuclei(Fig 4A, DM, PH). Inferior to the ventromedial hypothalamic nuclei, strongly to moderately stained neurons were found in the retrochiasmatic area(Fig 4A, Fig 4B-C, RCh; arrows). Very robustly stained neurons were found widely dispersed throughout the lateral hypothalamic and the lateral preoptic nuclei(Fig 4D, LH, LPO) in sagittal sections that yielded an extensive view of these nuclear groups. The sagittal section shows robust TCF7L2-stained cells throughout the reticular formation including cells of the gigantocellular reticular nucleus of the medulla(Fig 4E, Gi) and the cells of the pedunculopontine, reticular pontine and reticular medullary regions(Fig 4E, PP, RP, RM). Given the midline position of this sagittal section, a thin strip of hypothalamic neuroepithelium was observed and within which was found moderate to intensely stained cells scattered within the border area adjacent to the peduncular fossa(Fig 4E, Hn). Distinct and strongly stained TCF7L2 immunoreactive cells were found in the ventromedial and

arcuate hypothalamic nuclei and preoptic area (Fig 4A-E, VMH, Arc, POA). Sagittal sections afforded a brief but more laterally expanded view of the arcuate nucleus compared to coronal sections. In Fig 5A-C, TCF7L2-immunoreactive cells are compared to NPY-(Fig 5A) and Agouti-related peptide-stained cells (Fig 5B) and POMC neurons in the arcuate nucleus. Not all cells of the arcuate nucleus stained for TCF7L2 and their pattern of staining ranged from light to intensely stained nuclei. In Figure 6, the distribution of strongly stained TCF7L2 cells are found distributed in the neocortical and tectal plates (Fig. 6A, N,T) as well as in the outer cell layers of the cerebellum (Fig 6A, Cb). More specifically the cells of the lateral septal neuroepithelium of the lateral ganglionic eminence exhibited strong TCF7L2 staining in the ventricular and subventricular zones (Fig 6A, LSn). These extended inferiorly to the region of the olfactory bulb where TCF7L2 stained cells exhibited strong labeling within the ventricular and subventricular zones (Fig 6A, OB) and projected outward to include the less dense cells of the granule cell, internal plexiform and mitral cell layers (Fig 6A, Gl, IPl, Mi; 6B). Some moderately stained cells were observed in the glomerular and external plexiform layers as well. Overlying the immunoreactive proliferative zones were the primary and secondary motor cortices (Fig 6A, M1/M2), the intermediate cortical layer (Fig 6A, ICx), and the cortical plate and subplate (Fig 6A, Cxp, Cxs) all of which exhibited negligible to light staining. This same pattern of an over-riding mantle of lightly stained cells composed the layers of the primary somatosensory and visual cortices of the retrosplenial granular cortex (Fig 6A, S1Tr, V1, RSC) and the presubiculum (PrS), subiculum (S), fields of CA1 and CA3 (Fig 6A, CA1, CA3) the dentate gyrus (Fig 6A, DG) and the ventral hippocampal commissure of the hippocampus (Fig 6A, vhc, Hi). The strongly stained TCF7L2 cells of the ventricular and subventricular zones below the dorsomedial periaqueductal gray of the tectum extended around the periphery of the ventricle becoming highly condensed within the inferior colliculus (Fig 6A, IC). TCF7L2 immunoreactive cells of the cerebellum showed intense labeling in the dense laminae formed by the external germinal (Fig 6E, Eg) and molecular layers (Fig 6E, MI). Scattered cells within the Purkinje cell layer were also strongly stained (Fig 6E, Pc). The intense staining pattern outlined clearly the ventricular and subventricular layers as they extended around the periphery of the cerebellum from the region of the superior cerebellar peduncle (Fig 6A, Scp), the tip of the germinal trigone (Fig 6E, GT) to the choroid plexus (Fig 6A, CP). Very few cells of the plexus itself expressed TCF7L2. Although TCF7L2 label was not co-localized within the same section, the general staining pattern within the germinal zones was found identical to Nestin immunohistochemistry (Fig 6F, 6L) indicative of Class IV intermediate filament expression in embryonic neuroepithelium, with Olig 2 marker in the region of the septal and olfactory neuroepithelium and the mitosis marker, anti-phospho-Histone H3 (Fig 6H, 6J). The germinal layers remained conspicuously unlabeled when stained with NEUronal Nuclei which is detectable at developmental time points that correspond with the withdrawal of neurons from the cell cycle and/or with the initiation of terminal differentiation of the neuron (Mullen et al., 1992) as seen in the labeled cells of the intermediate and cortical plates of the neocortex and dentate gyrus (Fig 6I, N, DG, GK). Intensely stained TCF7L2 cells were also found in the area postrema (Fig 6A, AP). Lightly to moderately stained TCF7L2-immunoreactive cells were also found scattered within the dorsal raphe (Fig 6A, DR), the dorsal tegmental nucleus (Fig 6A, DTg) and the posterior dorsal tegmental nucleus (Fig 6A, PdT) near the neuroepithelium bordering the aqueduct and fourth ventricle (Fig 6A, ne).

4. Discussion

From the preceding description of cellular localization and expression, the TCF7L2 protein has a ubiquitous phenotypic distribution throughout the developing murine brain. The protein expression found throughout the early (E12.5), intermediate (E16.0-18.5) and late (P0-P1) stages of brain development in TCF7L2 wild type and het progeny shows a striking correlation with a recent study of mRNA mapping of TCF7L2 in the adult mouse brain

which concluded that *Tcf7l2* may be linked to glucose sensing (see Lee et al., 2009). It should also be noted that the immunophenotypic distribution of TCF7L2 in the developing brain was found to be the same in adult wild type TCF progeny which was subsequently included to provide a comparative basis of study (See: Supplemental Figure 2). In reviewing the literature regional patterns of neural organization do emerge that may restrict TCF7L2 expression and may indicate functional dynamics of the *Tcf7l2* gene especially in the context of glucose sensing in both forebrain and hindbrain regions. Glucose as a regulatory signal not only controls endocrine secretion but activates neurons in the peripheral and central nervous system. The neurons of this system are broad-ranged cellular metabolic sensors that allow them to sense and interpret peripheral signals via transporters, metabolite-sensing kinases, ion channels and receptors (Dzeja and Terzic, 2009). Furthermore, GLP-1, a neuropeptide with diverse systemic effects, has been shown to be operative in the central regulation of feeding (Turton et al., 1996) via modulation of gut motility and inhibition of gastric acid and glucagon secretion as well as the GLP-1 receptor which permits the effect of GLP-1 metabolism in the brain (Alvarez et al., 2005). This becomes relevant in the context of *Tcf7l2* impairment of GLP-1-induced insulin secretion as a potentially important variable in the increased risk for type 2 diabetes. Subsequent examination of co-localization of TCF7L2 with GLP-1 was found in the adult TCF wild type in both hypothalamic as well as medullary regions (see Supplemental Figure 3). The evidence for a correlation between TCF7L2 protein expression and glucose sensing as well as its potential interaction with neural GLP-1 expression is considered in the following discussion.

4.1 Brain Stem Nuclei

A number of principal brain stem nuclei were found to express TCF7L2 protein. However it should be noted that in Lee's systematic mapping of TCF7L2 mRNA of two nuclei showed less labeling than that obtained in the labeled protein expression. For example, the area postrema (AP) exhibited moderate density (Lee et al., 2009) compared to the robust protein expression found in the present study. Furthermore, their staining of the NTS exhibited labeling inconsistency compared to the moderate TCF7L2 label for the protein. Other than the disparity arising from the state of synthetic activity possibly expressed as a factor of metabolism it is difficult to state a definitive reason for the discrepancy. On the other hand the comparative paucity of labeled cells expressing mRNA may arise from the coronal versus parasagittal presentation of the AP and NTS which afford a more extensive longitudinal view of each nuclei within the parasagittal plane as preferred and presented in the present study.

4.2 Reticular formation

TCF7L2 exhibits a striking distribution within the reticular formation that extends throughout the medulla, pons and mesencephalon of the brain stem (see Figs 1A-F, 3F-G, 4E, 6A). Recent studies have begun to reveal the role of the reticular formation (RF) in glucose sensing. Expression of insulin-responsive glucose transporter GLUT4 mRNA has been found in the reticular field of the rat hindbrain (Messari et al., 2002). Furthermore GLUT2 protein expression has been found in the caudal ventral RF and has been suggested to function at least in part in glucose sensing (Arluison et al., 2004). Perhaps more importantly TCF7L2 involvement in the reticular system may be via modulation of GLP-1 which is expressed in the medullary reticular nucleus (Jin et al, 1988). As noted earlier, in a test series of GLP-1 expression in the present study, GLP-1 was found co-localized with TCF7L2 in cells of the medullary reticular formation of adult TCF wild type mice (see Supplemental Data: Figure 2). No expression however was found in developing TCF progeny examined (data not shown).

4.3 Raphe nuclei

In further support of hindbrain involvement, TCF7L2 labeled cells were found in the present study within the raphe nuclei(RN), cells of the nucleus of the solitary tract(NTS) and the medial vestibular nucleus(MVe). Previous studies have not only shown that the nucleus raphe obscurus controls pancreatic hormone secretion(Krowicki and Hornby 1995) but that both the dorsal and median raphe nuclei undergo a decrease in cell number in streptozotocin-induced diabetic Wistar rats(Hassanzadeh et al., 2010). Additional studies have included the RN in a widespread network of glucokinase glucose sensing cells that maintain glucose homeostasis (Matschinsky 2008; Maekawa et al, 2000).

4.4 Solitary tract

Numerous studies of the nucleus of the solitary tract(NTS) which exhibited TCF7L2-immunoreactive cells in the present study have shown that the NTS is operative in the regulation of food intake and energy expenditure(Hayes, et al. 2009). The most relevant studies, given the observation that TCF7L2 as been shown to impair GLP-1-induced insulin secretion, are those that have shown the NTS as a primary source of GLP-1 containing neurons including those that have shown de novo synthesis of GLP-1 in the cells of the NTS similar to that produced in pancreas(Han et al., 186; Tachibana et al, 2005), GLP-1 protein localization in the NTS(Jin et al, 1988) and that cells of the NTS process preproglucagon to glucagons-like peptide 1(GLP-1) in response to the metabolic demands of obesity(Vrang et al, 2007). Furthermore, recent studies linking NTS to metabolic modulation have shown that endogenous NTS GLP-1R activation driven by gastric satiation signals contributes to the control of normal feeding(Hayes et al., 2009).

4.5 Medial Vestibular nucleus(MVe)

In the present work the medial vestibular nucleus(MVe) was found to contain a dense population of TCF7L2 labeled cells(Fig 3F, MVe). In light of recent work showing an association of TCF7L2 and insulin receptor substrate 1(IRS-1) leading to GLUT-4 translocation defect and reduced levels of IRS-1(Wang et al., 2008), TCF7L2 could conceivably participate in the modulation of the insulin-regulated glucose transporter(GLUT4) whose mRNA transcripts have been expressed in cells of the vestibular nucleus(Kobayshi et al., 1996). Other membrane receptors relevant to insulin regulation including K_{ATP} channel-forming Kir6.2 subunit and sulfonylurea receptor SUR1 expressed in the cells of the vestibular nucleus(Karschin et al., 1997) and more specifically cells of the MVe(Miki et al., 2001) may also be associated with TCF7L2. Recent evidence has also demonstrated lactate as a crucial “sensed” variable in the caudal hindbrain monitoring CNS metabolic status indicating the. Specifically it has be shown that the caudal fourth ventricular administration(CV4) of monocarbocylate transporter inhibitor α -cyano-4-hydroxycinnate(4CIN) enhances Fos immunoreativity in cells of the NTS, the adjacent area postrema and the MVe. Furthermore, CV4 L-lactate infusion increases Fos labeling within the MVe subsequent to insulin-induced hypoglycemia. The authors conclude that lactate is a crucial “sensed” monitored metabolic variable in the caudal hindbrain detection of energy imbalance arising from glucoprivation(Patil and Briski 2005). Any attenuation in the fuel triggers neural mechanisms that enhance systemic glucose availability. This may hold significance for TCF7L2 participation via GLP-1 given a recent study that provides evidence for GLP-1 stimulation of ^{13}C lactate labeling from glucose(Gu et al, 2011).

4.6 Circumventricular organs

TCF7L2 immunoreactive cells were found within the circumventricular organs(CVOs) located in the walls of the lateral, third and fourth cerebral ventricles and whose neurons are well facilitated to detect and transduce signals which can be effectively relayed to

hypothalamic and brain stem nuclei. Recent work clearly demonstrates that circulating signals carrying information to the CNS about nutritional status arise from the gastrointestinal system. These signals impact feeding behavior as well as metabolic control as a result of their ability to modulate the neural circuitry component of metabolic homeostasis (Hoyda et al., 2009). Many of these molecules have receptors in the CVO's. In the present study, a dense collection of strongly TCF7L2-labeled cells were found namely within the area postrema (AP), subfornical organ (SF) and the vascular organ of the lamina terminalis (VOLT) all of which represent sensory CVOs that contain neuronal cell bodies. Light to moderately TCF7L2-stained cells were found in the subcommissural organ and choroid plexus while light to moderately stained cells were found in the secretory CVOs that compose the median eminence, neurohypophysis, intermediate lobe of the pituitary and the pineal gland.

4.7 Area postrema

Clearly defined and intensely stained TCF7L2 neurons were found in the area postrema (Fig 6A, AP). This CVO is located on the dorsal surface of the medulla at the obex of the fourth ventricle and adjacent to the NTS to which it extends abundant projections. Numerous studies have shown that fibers projecting from the AP/NTS extend to the pontine parabrachial nucleus where they divide by way of the thalamocortical taste pathway to numerous structures implicated in nutritive homeostasis. More compelling evidence lies in the work that has demonstrated that the AP/NTS is necessary for metabolic control of food intake in rats whereby 2-deoxy-D-glucose (2DG) which normally increases food consumption (Epstein et al., 1975; Ritter 1986; Smith and Epstein 1969) is abolished in animals in which the AP/NTS has been lesioned (Bird et al., 1983; Contreras et al., 1982; Hyde and Miselis 1983; Ritter and Taylor 1990; Schneider and Zhu 1994) as well as in studies that show an increase in neuronal activity in the AP/NTS in both rats (Ritter et al., 1992) and hamsters (Schneider and Zhu 1994) that have been treated with 2DG. The role of the AP itself has been dissected out in several studies. Earlier studies where the AP alone has been removed show complete attenuation in the inhibitory effect of insulin-induced hypoglycemic stress on pulsatile LH release (Cates and O'Byrne 2000). The AP has also been found to express a high density of receptors for amylin (Sexton, et al. 1994) the pancreatic hormone cosecreted with insulin in response to food intake and increased levels of blood glucose (Cooper 1994) and which functions to retard gastric emptying (Young et al., 1996), to reduce centrally mediated food intake (Lutz, et al. 1998) and along with glucose co-activates the AP (Riediger et al., 2002). Furthermore GLP-1 receptors are densely expressed in the AP and recent work has suggested that catecholamine neurons of the AP provide a link between peripheral GLP-1 and the central autonomic control areas mediating neuroendocrine and autonomic actions of peripheral GLP-1 (Yamamoto et al., 2003) and with co-localization as shown in the present study could provide a potential site of TCF7L2 interaction. These and other studies imply that the AP is not simply a conduit for outflow signals to the brain, but is the initial site of integration of the signals as they engage the circuitry of the CNS (Price et al., 2007). The extent to which TCF7L2 may become operative in GLP-1/AP sites could prove significant.

4.8 Subfornical Organ (SFO)

The SFO is situated dorsal to the anterior commissure within the dorsal region of the lamina terminalis and projects rostrally into the third ventricle. In the present study, the SFO was found to contain a dense population of strongly stained TCF7L2 cells (see Fig 2K; Fig 2I). Aside from studies have defined efferent projections from the SFO to the hypothalamic centers regulating food intake (Cottrell and Ferguson 2004), recent work has provided evidence that supports the SFO as a center where ghrelin and amylin may influence discrete subpopulations of neurons to influence the hypothalamic regulation of feeding elevating the

SFO to a primary target for circulating feeding signals. Furthermore, ^{125}I -GLP-1 binding studies have shown particularly high binding densities in the subfornical organ (McKinley et al., 2003) that may provide for TCF7L2 interaction. Many of the SFO linked nuclei have been shown in the present study to exhibit definitive TCF7L2 expression. With the recent and increasing trends supportive of CVOs as targets for circulating signal controlling ingestive behavior (Fry and Ferguson 2007; Fry et al., 2007), a role for TCF7L2 may become evident in the linkage of both forebrain and hindbrain control of food intake via CVO integration.

4.9 Hypothalamus

The hypothalamus has long been implicated in the central control of feeding behavior and nutritive homeostasis and has been found to express *Tcf7l2* mRNA in previous studies (Lee, et al. 2009; Lein et al., 2007). In the present study some of the strongest staining for TCF7L2 expression was found in cells of the ventromedial and lateral hypothalamic nuclei.

4.10 Ventromedial hypothalamic nucleus (VMH)

Bilateral lesions of the VMH have long been known to produce hypertriglyceridemia and hyperinsulinemia with normal food intake (Froehman et al., 1969; Schnatz et al., 1971; Schnatz et al., 1973). Furthermore, the cells of the VMH (see Figs 1G; 1H; 3F; 4A,E: VM) are linked to glucose sensing given their ability to respond to both systemic hypoglycemia (Beverly et al., 2001) and local extracellular changes in glucose levels (Levin et al., 1999; Song et al., 2001; Silver and Erecinska 1998). Furthermore *in vitro* studies show approximately 30% of VMH neurons alter their firing rate to accommodate changes in glucose concentration (Levin et al., 1999; Lee et al., 1999; Silver and Erecinska 1998; Song et al., 2001). The ability of VMH neurons to dynamically respond to glucose lies in their membrane-bound sulfonyleurea receptors which are associated with K_{ATP} channels. In addition to glucose sensing VMH integrates neuronal input from the forebrain with information arising from the brain stem and in turn projects to areas implicated in numerous activities and physiological responses (Swanson 1987). These projections extend to diverse sites many of which exhibited TCF7L2 expression in the present study including the medial and lateral hypothalamic nuclei, zona incerta, portions of the thalamus, bed nucleus of the stria terminalis, central, lateral and medial amygdalar nuclei, periaqueductal gray, telencephalic regions and numerous terminal fields within the brain stem. Recent studies have also shown that VMH projects via direct glutamatergic fibers to adjacent POMC neurons which have been shown in the present study to co-localize with TCF7L2 may exert regulation of among others ingestive behavior as well as counter regulatory adjustments to hypoglycemia (Sternson et al., 2005; Canteras 2002; Choi et al., 2005; King 2006; Borg et al., 1994, 1995, 1997; Tong et al., 2007). Aside from potential modulation of TCF7L2 in regional VMN glucose sensing, VMH-TCF7L2 interaction may involve the regulation of its effects via GLP-1. A recent study using manganese enhanced magnetic resonance imaging (MEMRI) has shown the peripheral administration of GLP-1 induces a significant reduction in signal intensity within the VMH (Parkinson et al., 2009). Still others have found dual labeling *in situ* hybridization histochemistry of GLP-1 receptors and GLUT-2 or glucokinase mRNAs in VME of the human brain (Alvarez et al., 2005).

4.11 Lateral Hypothalamic Nucleus (LH)

Well delineated and strong TCF7L2 localization in the LH provide a potential network within this nucleus that may exert energy regulation. Older studies of the LH have demonstrated that lesions of the LH suppress food intake (Anand and Brobeck, 1951; Baillie and Morrison, 1963). With the discovery that LH nuclei (see Figs 1I; 2K; 4D: LH) contain the peptide transmitters orexins (Sakurai et al., 1998) came further evidence for glucose-sensing properties in these neurons of the hypothalamus. It is now thought that about 95% of LH

orexin neurons *in situ* which stimulate among other behavioral parameters food intake are directly hyperpolarized and inhibited by physiological concentrations of glucose (Burdakov et al., 2005). Thus the existing hypothesis suggests that LH orexin neurons are inhibited by elevated levels of glucose and stimulated by its decrease. In addition to orexin-LH neurons a melanin-concentrating hormone (MCH) is also present within cells populating the LH. While similar to orexin-LH neurons in their extensive projections throughout the brain their physiological roles appear to function in opposition to orexin neurons. In mice where the MCH gene is knocked out (Shimada et al., 1998) or is lacking (Marsh et al., 2002) the metabolic rate increases and there is a reduction in body weight. The potential interaction of TCF7L2-labeled cells of the LH and glucose regulation could also arise within the milieu of GLP-1 integration. The mRNA mapping of GLP-1 receptors have shown GLP-1-R to label cells of the LH (Shughrue et al., 1996) while GLP-1 has been demonstrated to act on the LH to significantly suppress food intake (Schick et al., 2002). Furthermore, GLP-1 has been shown to excite both directly and indirectly the hypocretin/Orexin neurons of the LH (Acuna-Goycolea and van den Pol, 2004).

4.12 Arcuate Nucleus(Arc)

In the present study, light to robustly stained TCF7L2 immunoreactive cells were found within the hypothalamic Arc (see Figs 1G; 1H; 2j; 4E, 5B) which is a well defined center regulating metabolism and appetite. This regulation is based in part on integration of metabolic signals including monoamines, insulin, leptin and ghrelin and glucose sensing. TCF7L2-immunoreactive cells of the arcuate nucleus were found to co-localize with several key peptides that regulate energy homeostasis that have not been shown elsewhere. The Arc nucleus contains both neuropeptide Y (NPY) and proopiomelanocortin (POMC) neurons that function antagonistically to regulate food intake and energy. The NPY neurons (see Fig 5A) which also co-express Agouti-related peptide (ArRP) (see Fig 5B) are held to promote weight increase by their stimulation of appetite and suppression of energy expenditure whereas POMC neurons (Fig 5C) inhibit food intake and activate energy expenditure (Broberger and Hökfelt 2001; Williams et al., 2001; Schwartz et al., 2000; Cone et al., 2001; Billington and Levine 1992). Both in turn project to ample regions within the CNS that regulate appetite and metabolism (Burdakov et al., 2005). The mechanism that modulates the smooth synergism between the counter regulatory impulses of NPY and POMC is derived from the opposing influences exerted by their signals. Thus, the hormone leptin that suppresses appetite excites POMC neurons and inhibits NPY neurons while the hormone ghrelin that stimulates appetite inhibits POMC neurons and activates NPY neurons (Cowley et al. 2001; Cowley et al., 2003; van den Top et al., 2004). In addition, the Arc nucleus contains a population of glucose sensing neurons (Spanswick et al., 2000; Spanswick et al., 1997; Li et al., 2003) that have been shown to serve an integrative role in the regulation of energy balance (Wang et al., 2004). Aside from direct interaction of TCF7L2-labeled arcuate neurons in glucose sensing, its indirect involvement may be reflected in recent studies which have shown that GLP-1 may function to regulate both insulin secretion as well as glucose production via GLP-1 receptors of the Arc (Sandoval et al., 2008). Older studies have shown that destruction of the Arc eliminates the effect of ICV administration of GLP-1 on food intake and appetite (Tang-Christensen et al., 1998).

4.13 Retrochiasmatic Area(RCA)

The RCA lies adjacent to the Arc, caudal to the optic chiasm at the base of the hypothalamus on either side of the midline and is composed of cells that lie within the fibers of the supraoptic decussations. Although not reported in previous studies localizing *Tcf7l2* mRNA hybridization, the cells of this region were found in the present study to exhibit light to moderate TCF7L2 immunoreactive labeling that clearly demarcated their perimeter from adjacent unlabeled cells lying cranially and the fibers of the optic tract and chiasm that

formed their anterior and caudal boundaries respectively (Figs 4A-C, RCh). Although the role of the RCA in energy balance has not been extensively studied, several studies do implicate this region in the regulation of energy homeostasis. From each midline arcuate neurons extend into each area and integrate with many neurons of the RCA that express the POMC gene and produce α -MSH (Cechetto and Saper 1988) and cocaine- and amphetamine-regulated transcript (CART) (Elias et al., 1998). Subsequent to intravenous administration of leptin, a delimited pattern of Fos expression has been found in hypothalamic nuclei that include arcuate/retrochiasmatic neurons (Elias et al., 2000; Elmquist et al., 1999). Furthermore, following retrograde labeling used to identify leptin-activated sympathetic sources, only neurons of the arcuate/retrochiasmatic area were labeled excluding other sources of hypothalamo-spinal projections regardless of spinal level (Elias et al., 1998; Saper 2002). These same cells double-labeled for CART and α -MSH. From these data the leptin activates the CART/POMC neurons of the RCA that innervate sympathetic neurons of the spinal cord which increase thermogenesis and energy expenditure leading to decreased body weight that is observed subsequent to leptin administration. Thus leptin acts to coordinate regulation of CART, POMC and α -MSH in energy balance via neurons of the RCA. TCF7L2 co-localization with POMC neurons implicate potential synergy that may ultimately regulate energy homeostasis. It is of further interest to note that neurons of the RCA exclusively express an unusual gene called "beacon" in proportion to body fat in lean and obese sand rats, *Psammomys obesus*. This gene encodes for a small protein of 73 amino acids and increases food intake and body weight when administered ICV. (Collier et al., 2000). TCF7L2 protein expression here as in the case of beacon could suggest that it too may form part of an integrated network of molecules regulating energy balance. The potential link of TCF7L2 in RCA energy regulatory activities may be its association with GLP-1 interaction. Recent studies have shown the 10.7% of POMC neurons of the RCA also coexpress GLP-1 receptor mRNA (Sandoval et al., 2008).

4.14 Preoptic Area(PreA)

In the present study cells within the anterior region of the hypothalamus collectively known as the preoptic area exhibited moderate to strong immunoreactive labeling for TCF7L2 protein (Figs 11, LPO, MPO; Fig 4, POA). TCF7L2-labeled cells were found throughout the preoptic region and included lateral, medial, median and periventricular areas with a preponderance of labeled cells within the periventricular nucleus. Although cells of the PreA are generally known to participate in thermoregulation and thirst regulation several studies have specifically implicated the preoptic area in glucose sensing. Early work on glucose sensing in the PreA focused on the effects of glycopenia has shown site-specific induction of Fox expression in the preoptic neurons in response to 2-deoxy-D-glucose-induced glucoprivation (Briski and Sylvester 1999) and have provided evidence for the transactivational effects of glucopenia on γ opioid receptor-expressing neurons within the preoptic area (Briski and Sylvester 2001). Furthermore studies have localized glucokinase specifically within the preoptic area (Lynch et al., 2000) while others have demonstrated that administration of selective dopamine D₁ and D₂ receptor agonists produces an increase in glucose utilization in the preoptic nucleus (Huang et al., 1997). Aside from the local sites of glucose interaction, in the larger framework of metabolic regulation, cold activation of the preoptic area and its neuronal circuitry with the DMH induces sympathetic outflow to brown adipose tissue (BAT) and thermogenesis in the same manner that leptin exerts activation of sympathetic nerves controlling BAT (Mounien et al., 2010). Based on ICV GLP-1 injection and the subsequent c-fos labeling, GLP-1 has been associated within the paraventricular nucleus (PVN) of the hypothalamus (Turton et al., 1996). This has recently been confirmed by GLP-2 immunophenotyping indicating the PVN as a site of action for GLP-1 signaling (Tauchi et al., 2008). TCF7L2 co-localization with GLP-1 observed in the present study provide the basis of TCF7L2 participation in the metabolic facets of this nucleus.

4.15 Thalamus

In previous studies of TCF7L2 gene the thalamus exhibited robust and extensive mRNA hybridization in humans(Lein et al., 2007), adult mouse(Lee et al., 2009) and monkey(Murray et al., 2007). In the present study, TCF7L2 was strongly expressed throughout thalamic nuclei without exception(Figs 1H-J; Figs 3D-E). Although no quantitative determination was made, it was clear that TCF7L2-stained cells predominated throughout the thalamic fields. The preponderance of classical studies of thalamus have solidified its characterization as first and foremost a second-order relay of sensory signals between diverse subcortical areas and their associated cerebral cortical areas. Although it is also associated with the maintenance of restive and awake states, nevertheless existing data do suggest thalamic involvement with glucose sensing as well. Earlier work examining hypoglycemic encephalopathy in animal models has demonstrated manifest neuronal necrosis in the reticular thalamic nuclei of rats subsequent to cardiac arrest but not in hypoglycemic brain damage (Auer et al., 1984; Kawai et al., 1992; Paschen et al., 1991). This was subsequently found to be true in humans as well where symmetrical thalamic lesions occurred in patients with post-cardiac ischemic encephalopathy but absent in those with hypoglycemic encephalopathy (Fujioka et al., 1997). The difference may lie with the predominant loss of aspartate into extracellular space, the availability of residual glucose and the oxidation of endogenous non-glucose fuel by the brain in hypoglycemic encephalopathy. Under these conditions ATP is significantly increased(45% of control values) in the thalamus compared to other regions of the brain(Paschen et al., 1991). More recent studies have further shown thalamic activation during induced hypoglycemia(Teves et al., 2004) and the functional role of specific thalamic nuclei in response to hypoglycemia. Arbelaez et al., (2008) have shown by mapping blood flow that the dorsal medial and paraventricular thalamic nuclei which contained densely-stained TCF7L2 cells play an active inhibitory role in the reduction of sympathoadrenal and symptomatic responses to hypoglycemia where previous hypoglycemia has occurred and is a key facet of hypoglycemia-associated autonomic failure(HAAF) in diabetes. The posterior paraventricular thalamic nucleus(PPV) alone has also been shown to contribute to the sympathoadrenal stimulation induced by hypoglycemia and thus may function in a coordinated CNS response to metabolic stressors(Al-Noori et al., 2008). This is reasonable in light of the fact that glucose kinase(GK) has been found in areas of the brain without known glucose sensors including the PPV(Lynch et al., 2000). In the context of energy deprivation a reduction of energy intake also leads to an increased expression of the metabolic-sensor protein SIRT1 in nuclei of the epithalamus, hypothalamus and thalamus. Recent studies have shown that the nicotinamide adenosine dinucleotide-dependent deacetylase, SIRT1 regulates not only key metabolic adaptations to nutrient deprivation in peripheral tissues but is induced in the brain as well by a reduction of energy intake(Cohen et al. 2004; Ramadori et al., 2008). Under conditions of deprivation Ramadori et al.,(2008) have shown SIRT1 mRNA to exhibit moderate to high levels of expression in the anterodorsal, anterior paraventricular thalamic nuclei and low to moderate expression in all other thalamic sites. Furthermore a hypothalamic-specific, fasting-induced SIRT1 is abolished in leptin-deficient, obese mice, an observation that further suggests SIRT1's role in brain-mediated regulation of energy homeostasis. SIRT1 was also found in all other key TCF7L2 labeled neurons of the present study in addition to thalamus and hypothalamus including area postrema and solitary tract of the hindbrain. Aside from the intrinsic role of TCF7L2-labelled cells within the context of thalamic glucose sensing, several studies have demonstrated substantial data on thalamic GLP-1 receptor distribution and activity. While some studies have shown RIA levels of GLP-1-1R to be the highest in the thalamus(Shimizu et al., 1987) others have demonstrated by receptor autoradiography the highest densities of [¹²⁵I]GLP-1 binding sites to include the thalamus(Goke et al., 1995).

4.16 Neuroepithelium

As shown in Fig. 6A, cells exhibiting intense TCF7L2 labeling were found in the neuroepithelium lining the developing cerebral and hippocampal cortices of TCF7L2 mice. As in the case of the thalamus, the potential relevance of TCF7L2 with germinal centers of the neuroepithelium may reside with TCF's role in the canonical Wnt signaling pathway since it has been shown in numerous studies that the Wnt pathway is critical in cell proliferation, morphogenesis of the neural plate, subsequent cell fate decisions, neuronal differentiation and patterning and lineage decision events in the embryonic nervous system (Castelo-Branco et al., 2003; Chenn and Walsh 2002; Gulacsi and Anderson 2008; Gunhaga et al., 2003; Hirabayashi et al., 2004; Ille and Sommer 2005; Lee, et al. 2000; Patapoutian and Reichardt 2000). More specifically, recent work has shown Wnt signaling maintains the mitotic activity of neuroepithelial precursors in developing ventricular zones (Yu et al., 2007). Aside from this potential role of the transactivating complex formed by Tcf transcription factor and β -catenin as a downstream effector of Wnt serving to translate extracellular signals into morphological events, TCF7L2 expression in neuroepithelium may also indicate its role in glucose sensing. Bondy et al., (1992) mapped the hybridization of the facilitative hexose transporters GLUTs 1-4 in rat brains from E11 through adulthood and found that prior to the formation of the blood brain barrier (BBB) GLUT1 mRNA is localized exclusively in the ependymal cell layer of the germinal neuroepithelium lining the ventricles while subsequent to the formation of the BBB it was limited to vascular endothelium. Furthermore, while GLUT2 and GLUT4 mRNA were not detected at any stage, GLUT3 was detected only in differentiated neurons suggestive of a transition from GLUT1 to GLUT3 gene expression by the neuroepithelial cell lineage during terminal neural differentiation. Finally GLUT3 mRNA increased exponentially from E14 and its expression reached their highest levels in neurons found in the present study to have TCF7L2 protein expression namely in neurons of the olfactory, hippocampal formation, neocortex, cerebellar, pontine and brainstem nuclei. The study concluded that the ontogeny and neuroanatomical localization of GLUT3 gene expression correlates with developmental and regional patterns of brain glucose utilization, and that while GLUT1 may function in the transfer of glucose via the BBB it is GLUT3 expression that determines brain glucose utilization (Bondy et al., 1992). Others have found a similar GLUT expression in fetal rat brains (Vannucci 1994). In a recent study Maurer et al., (2006) have found expression of GLUT1 and GLUT3 mRNA and protein in neurospheres from the adult rat subventricular zone which is retained postnatally as a neurogenic niche (Alvarez-Buylla and Lim 2004). Furthermore, GLUT1 and GLUT3 mRNA and GLUT3 protein expression is upregulated by hypoxia, elevated glucose concentrations or a combination of both. An mRNA mapping study of preproglucagon (PPG) mRNA localized PPG mRNA expressing perikarya in the olfactory bulb while GLP-1R mRNA was restricted to the mitral cell layer of the olfactory bulb (Merchenthaler et al., 1999). This restricted expression may indicate a more specialized interplay of glucose monitoring and GLP-1 within this region of the prosencephalon.

4.17 Superior and Inferior Colliculi

As in previous studies that have demonstrated TCF7L2 mRNA hybridization in the superior and inferior colliculi, the present study has confirmed the same pattern for TCF7L2 protein expression. One common element shared between all TCF studies to date is the striking robust collicular staining for mRNA and protein. No attempt however has been made to explain this strong localization of TCF in the tectal collicular nuclei although existing data do support glucose regulation within the colliculi.

4.18 Inferior Colliculus(IC)

The IC has been shown to respond to systemic changes in nutrient dynamics. Although the functional significance of diet-induced changes in brain insulin growth-like factor (IGF)

system expression is not fully understood, Cheng et al., (2003) have shown that IGF mRNA is decreased and IGF receptors increased in the IC among selected brain regions in response to both calorie-restricted standard chow (CRS) or calorie-restricted ketogenic diet (CRK). Furthermore, GLUT3 is increased in the IC of animals fed a CRK diet. This reflects metabolic flexibility in the IC since a CRK diet results in a transition in brain metabolic pathways. As glucose becomes unavailable as fuel or substrate the resulting shift to nonglycolytic pathways utilizes the readily available fatty acids and ketone bodies. The increase in IC GLUT3 may ensue as a consequence of a reduction in systemic glucose levels that occurs in CRK-treated animals since hypoglycemia has been shown to promote GLUT expression (Boado and Pardridge 2002; Cheng et al., 2003; Duelli and Kuschinsky 2001; Lee et al., 2000). Furthermore, a high abundance of the key enzyme of the phosphogluconate pathway, transketolase, has been found in the IC (Becker et al., 2008). In its generation of glyceraldehyde-3-phosphate and fructose, transketolase reconfigures the phosphogluconate pathway with glycolysis. This latter pathway however unlike glycolysis generates NADPH/H⁺ which instead of being used in oxidative phosphorylation is used in synthesizing steroids and lipids. High levels of glycogen phosphorylase, an enzyme responsible for mobilizing glucose from glycogen to preserve energy reserves that serve to maintain primed nutrient levels, have also been found in the IC (Becker et al., 2008). It should also be noted that the inhibition of glycogen phosphorylase has been proposed as a means of treating type 2 diabetes (Somsak et al., 2003). Since in type 2 diabetic patients, hepatic glucose production increases (Moller 2001), inhibiting the glucose from the liver may serve to reduce the levels of excess. Given the prevalence and robust expression of TCF7L2 in cells of the IC, it is reasonable that TCF7L2 levels linked to glycogen phosphorylation could conceivably alter dynamics of glucose processing in the IC. Finally although previously the homeodomain transcription factor IDX/IPF1 encoded by a Hox-like homeodomain gene was thought restricted to pancreas and duodenum, it is now known to be expressed in embryonic brain during active neurogenesis (Perez-Villamil et al., 1999). Furthermore, it has been shown that IDX1/IPF1 and somatostatin are colocalized in several areas of the developing brain including the cortex, neuroepithelium, hypothalamus and inferior colliculus (Schwartz et al., 2000) indicative of its potential role of regulating somatostatin gene expression in the neural cells of these regions and/or regulation of neural cell-specific neuropeptide gene expression including TCF7L2. While it has been shown that IDX1/IPF1 regulates the expression of multiple pancreatic genes including insulin, somatostatin, islet amyloid polypeptide and glucose transporter type 2 (Waeber et al., 1996; Ohlsson et al., 1993; Miller et al., 1994; Carty et al., 1997) which has been confirmed by the finding that mutations in the human IPF-1 gene are associated with a type of autosomal dominant type of diabetes in the young known as MODY4 (Staffers et al., 1997), the implications for neural expression remain unknown.

4.19 Superior Colliculus(SC)

Studies have shown that the cells of the SC as well as those of the IC contain the orexigenic peptide neuropeptide Y (Borostyánkői-Baldauf 2002; Covenas et al., 1990; Mensah-Brown and Garey 2004). Among other targets, NPY is a major neuromodulator that serves as a potent inducer of appetite and feeding behavior (Levine and Morley 1984; Stanley and Leibowitz 1984). Furthermore, studies that have focused on NPY G-protein coupled receptor subtypes, NPY Y₁-Y₆, have shown that the NPY Y₁ receptor plays a dominant role in the regulation of feeding behavior and nutrient signaling (Eva et al., 2006). The ICV administration of NPY Y₁ receptor-selective antagonists have been shown to abolish NPY-induced intake and significantly reduce native daily consumption (Hipskind et al., 1997; Kanatani et al., 1999; Mullins et al., 2001) as in the case of oral administration (Ishihara et al., 2002). Furthermore, suppressed feeding behavior is manifest when NPY Y₁ receptors are blocked by the antisense to NPY Y₁ (Lopez-Valpuesta et al., 1996) or in mice with Y₁

receptor deficiency (Kanatani et al., 2000). Although its role remains to be fully characterized, recent studies have demonstrated that among several regions of the brain NPY Y₁ receptors are localized within the SC (Kishi et al., 2005; Kopp et al., 2002) and which would suggest that the SC is a region that is implicated in orexigenic processing. Given its colocalization with NPY found elsewhere in the present study, TCF7L2 may be equally implicated in the orexigenic activity within the SC.

5. Summary

This study provides detailed mapping of the sites of TCF7L2 immunophenotypic protein expression in the developing brain. It represents an important first step in assessing the function of *Tcf7l2* in the brain which at present remains unknown. The ubiquitous distribution of TCF7L2 expressing cells strongly suggest that *Tcf7l2* may be implicated in a neuromodulatory role in specific areas of the brain. When the distribution patterns of TCF7L2 protein expression studied here developmentally are compared with the adult brain of adult TCF wild types as well as previous work of *Tcf7l2* mRNA expression in the adult brain and superimposed on areas, nuclei and pathways of the brain linked to glucose sensing there is strong reciprocal correlation between the data sets. Detailed review of what is known about glucose sensing in these areas of correlation as well as localization of GLP-1 and its receptors known to have a role in *Tcf7l2* attenuation of insulin secretion provide a context within which *Tcf7l2* could conceivably function in neuromodulation of glucose. Likewise, molecular defects in *Tcf7l2* expression would understandably be linked to underlying dysfunction in the regulation of energy homeostasis that precede Type 2 diabetes mellitus with which *Tcf7l2* variants have been widely and genomically associated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

IC	inferior colliculus
SC	superior colliculus
mlf	medial longitudinal fasciculus
Pr	prepositus nucleus
RMg	raphe magnus nucleus
ROb	raphe obscurus nucleus
DTg	dorsal tegmental nucleus
LTg	lateral tegmental nucleus
PAG	periaqueductal gray

RF	reticular formation
MVe	medial vestibular nucleus
PPy	parapyramidal reticular nucleus
Sol	solitary tract nucleus
DC	dorsal cochlear nucleus
DR	dorsal raphe nucleus
Me5	mesencephalic trigeminal nucleus
SvVe	superior vestibular nucleus
PDTg	posterior dorsal tegmental nucleus
LRt	lateral reticular nucleus
LVe	lateral vestibular nucleus
Gi	gigantocellular reticular nucleus
MiTg	microcellular tegmental nucleus
MnR	median raphe nucleus
RFp	reticular formation of pons
RFm	reticular formation of medulla
SN	substantia nigra
R	red nucleus
MG	medial geniculate nucleus
APT	anterior pretectal nucleus
Po	posterior thalamic nucleus
VPM	ventral posterior medial thalamic nucleus
VPL	ventral posterior lateral thalamic nucleus
VMH	ventromedial hypothalamic nucleus
Arc	arcuate nucleus
LP	lateral posterior thalamic nucleus
PCom	nucleus of the posterior commissure
DLG	dorsal lateral geniculate nucleus
VP	ventral posterior thalamic nucleus
IGL	intergeniculate leaflet
VLG	ventral lateral geniculate nucleus
VM	ventral medial thalamic nucleus
SPF	subparafascicular thalamic nucleus
PV	paraventricular thalamic nucleus
Re	retrochiasmatic area
PH	posterior hypothalamic nucleus

SubG	subgeniculate nucleus
PSTh	parasubthalamic nucleus
MT	medial tuberal nucleus
DM	dorsomedial hypothalamic nucleus
MHb	medial habenular nucleus
LHb	lateral habenular nucleus
MD	mediodorsal thalamic nucleus
LD	lateral dorsal thalamic nucleus
VL	ventral lateral thalamic nucleus
Ret	reticular thalamic nucleus
LPO	lateral preoptic nucleus
MPO	medial preoptic nucleus
POA	preoptic area
SI	substantia innomina
GP	globus pallidus
Re	reunions thalamic nucleus
LH	lateral hypothalamic nucleus
SFO	subfornical organ
DBh	horizontal limb of the diagonal band
Acb	accumbens nucleus
MCPO	magnocellular preoptic nucleus
Sn	septal neuroepithelium
SM	stria medullaris
Cn	cortical neuroepithelium
MS	medial septal nucleus
Pf	parafascicular thalamic nucleus
AM	anteromedial thalamic nucleus
fr	fasciculus retroflexus
ZI	zona incerta
Ptec	pretectum
DMPAG	dorsomedial periaqueductal gray
Pc	posterior commissure
PrC	precommissural nucleus
VLPAG	ventrolateral periaqueductal gray
P	pituitary
Pe	periventricular hypothalamic nucleus

RCh	retrochiasmatic area
PP	pedunculopontine region
RP	reticular pontine region
RM	reticular medullary region
N	neocortical plate
T	tectal plate
Cb	cerebellum
LS	lateral septum
LSn	lateral septal neuroepithelium
OB	olfactory lobe
Gl	granule cell layer
IPI	internal plexiform layer
Mi	mitral cell layer
M1	primary motor cortex
M2	secondary motor cortex
ICx	intermediate cortical layer
Cxp	cortical plate
Cxs	cortical subplate
S1Tr	primary somatosensory cortex
V1	visual cortex
RSC	retrosplenial granular cortex
PrS	presubiculum
S	subiculum
CA1	CA1 fields
CA3	CA3 fields
DG	dentate gyrus
vhc	ventral hippocampal commissure
Hi	hippocampus
Eg	external germinal layer of cerebellum
MI	molecular layer of cerebellum
Pc	Purkinje cells
GT	germinal trigone
CP	choroids plexus
AP	area postrema
PdT	posterior dorsal tegmental nucleus
ne	neuroepithelium

Lr	lateral reticular nucleus
LV	lateral ventricle
III	third ventricle
Aq	aqueduct
VOLT	vascular organ of the lamina terminalis
V	ventricular layer of IC
TH	thalamus
Sep	superior cerebellar peduncle
Aq	cerebral aqueduct
CnA	central nucleus of amygdala
OT	optic tract

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Highlights

- > Genetic variants in the transcription factor 7-like 2(Tcf7l2) gene confer a significant risk of type 2 diabetes.
- > Tcf7l2 has been suggested in the adult to function in glucose sensing in the brain.
- > We mapped the immunophenotypic expression of TCF7L2 protein in the developing brain of Tcf7l2 progeny.
- > TCF7L2 is widely expressed in the developing mouse brain and within regions of the brain involved in energy balance and nutrient regulation.
- > It is concluded that the Tcf7l2 gene may be operative in neuromodulation of glucose within glucose sensing systems of the CNS.

Figure 1.

A-I. Medullary to midbrain coronal sections of an E18.5 *Tcf7l2*^{+/+} mouse showing the widespread distribution of TCF7L2 peptide. A-F show the distribution of the peptide within nuclei of the hindbrain while G-I are midbrain sections illustrating the prevalence of TCF7L2 peptide within principal nuclei of the thalamus and hypothalamus. A full list of terms and their abbreviations is given in the list of abbreviations. Scale line in I = 500 microns and is the same for A-H Figure 1. J-L. The continuing series of coronal sections showing the midbrain to forebrain regions and localization of TCF7L2. K-L illustrate the peptide staining of the circumventricular organs (SFO and VOLT) as well as the stained neuroepithelium of the germinal zones lying adjacent to the walls of the lateral ventricles. Scale line in L = 500 microns and is the same for J-K.

Figure 2.

A-L show labeled cells from the boxed areas of Fig 1A-L at a higher magnification(20 $\times$). These include cells of medial longitudinal fasciculus(A), prepositus hypoglossal nucleus(B), dorsal tegmental nucleus(C), periaqueductal gray(D), microcellular tegmental nucleus(E), medial raphe nucleus(F), reticular formation of pons(G), substantia nigra compactus(H), ventromedial hypothalamic nucleus(I) arcuate nucleus(J), medial tuberal nucleus(K), and subfornical organ(l). Scale line of A,C,D-F, I, K-L = 100 microns; B,G,H,J = 50 microns.

Figure 3.

A-C. Labeled cells of the lateral preoptic nucleus(A), medial preoptic nucleus(B) and septal neuroepithelium(C). 20× D. Parasagittal view of a P1 *Tcf7l2*^{+/-} head showing the distribution of TCF7L2 within the thalamus and midbrain regions. 1× E. Higher magnification of the inset from D showing TCF7L2 labeled cells clearly defining the thalamus and its adjacent areas. The fasciculus retroflexus(fr) separates the thalamic cells from the pretectal(Ptec), habenular(MHb) and commissural cells above(PrC) and the zona incerta(ZI) below. 10× F. Parasagittal view showing the stained hindbrain nuclei including the reticular formation of the pons(RFp), medial vestibular(MVe), prepositus hypoglossal nucleus(Pr), Solitary tract nucleus(Sol), reticular formation medulla(RFm) and the gigantocellular nucleus(Gi) as well as the midbrain thalamus(TH), and ventromedial hypothalamic nucleus(VMH). 5× G. A higher magnification of the inset region F showing in detail the staining of neurons within the gigantocellular reticular nucleus. Scale line of in A-C = 50 microns; D = 1mm; E = 100 microns and F = 200 microns.

Figure 4.

A parasagittal view of labeled cells of the hypothalamic region including the ventromedial (VMH), retrochiasmatic (RC), dorsomedial (DM), posterior (PH) and periventricular (Pe) hypothalamic nuclei. 10× B-C. Magnified views of the inset region of A showing detailed staining of the labeled cells of the retrochiasmatic nucleus using red (B) and blue (C) counterstaining to highlight the staining of these cells lying adjacent to the optic tract (OT) which have not been reported in earlier works. 20× D. Parasagittal section showing the stained cells of the thalamus with relation to the cells of the lateral hypothalamic (LH) and lateral preoptic nuclei (LPO). 5× E. Parasagittal section showing the stained cells of the hindbrain including the gigantocellular (Gi), medullary reticular (RM), pontine reticular (RP) and the pedunculopontine (PP) nuclei and those of the midbrain including the arcuate (Arc) and ventromedial (VMH) hypothalamic nuclei. Other stained cells shown include the periaqueductal gray (PAG), hypothalamic neuroepithelium (Hn) and cells within the preoptic area (POA). Scale line in A = 100 microns; B-C = 50 microns; D-E = 300 microns.

Figure 5.

A-C. Stained cells within the arcuate hypothalamic nucleus. A. NPY-stained cells showing immunoreactive fibers extending beyond the nucleus into the region adjacent to the arcuate nucleus. B. TCF7L2 stained nuclei of arcuate neurons. C. Agouti-related protein immunoreactive cells within the arcuate nucleus.

Figure 6.

A. Parasagittal section of an E18.5 *Tcf7l2*^{+/-} showing the distribution of stained neuroepithelium in the neocortical(N) and hippocampal(Hi) cortices. Immunoreactive neuroepithelial cells were also found in the germinal layers of the cerebellum(Cb) and those lining the cerebral aqueduct(CAq). These also extended into regions of stained cells found in the dorsal raphe (DR, dorsal tegmental(DTg) and posterior dorsal tegmental(PdT) nuclei. 5× B-D. Magnified insets of A including details of stained cells within the ventricular(V), subventricular(SV) and granular zones of the olfactory bulb(B), olfactory lobe(C), the retrosplenial lobe(D) and the external granular(Eg), molecular layer(MI) and Purkinje cells(Pc) of the cerebellum(E). 20× F-I. Serially stained parasagittal sections of an E16 *Tcf7l2*^{+/-} showing sequential staining for nestin(F), oligo 2(G), neuronal nuclei(H) and H3(I) highlighting the similarity in staining patterns for TCF7L2(A). 5× J-L. Magnified insets of G-I, showing detailed staining for H3(J), neuronal nuclei (K) and oligo 2(L). Compared to Fig A, TCF7L2 stained those mitotic figures of neuroepithelium in F and I, oligodendrocytic maturation(G and L) and those cells lacking stained expression in post-mitotic neurons in H and K. Scale line in A = 500 microns; B-E = 100 microns; F-I = .1cm; J-L = 100 microns.