A role for astroctye-related genes in suicide

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ABSTRACT

Astrocytes are the most abundant cell-type in the nervous system, yet are perhaps the least acknowledged players in brain function. Long considered support cells or 'glue', astrocytes are gradually being recognized as integral not only to the cytoarchitecture and the inflammatory process, but also to cell signaling and synaptic plasticity.

This thesis will describe how a network of astrocyte-related genes may play a role in suicide. Through global gene expression analysis, a network of astrocyte-related genes was identified in brain of suicide completers as down-regulated compared to people who did not die by suicide. In particular, we identified an astrocyte-expressed TrkB gene, whose reduction of expression in suicide brain seems partially accounted for by epigenetic mechanisms. Finally, we investigated an astrocyte-expressed transcription factor, Sox9, that may partially regulate this astrocyte-related gene network.

This work identifies a novel contributor to suicide, namely astrocyte dysfunction, which may impact behavior and mood.

RÉSUMÉ

Les cellules en plus grand nombre dans le système nerveux sont les astrocytes, mais leur rôle dans le fonctionnement du cerveau demeure nébuleux. Longtemps considérées comme des cellules de support permettant à plusieurs types cellulaires d'interagir entre eux, les astrocytes sont désormais mieux reconnues comme faisant partie intégrale de la cytoarchitecture du cerveau et de la plasticité neuronale.

Cette thèse décrira le rôle que jouent les gènes astrocytaires sur le suicide. Par une analyse de l'expression génétique globale, des gènes astrocytaires ont été identifiés dans le cerveau de personnes décédées par suicide qui présentaient un niveau d'expression beaucoup plus faible chez les suicidaires que chez les sujets contrôles décédés de causes naturelles. Plus particulièrement, la variante astrocytaire du gène du récepteur trkB, dont l'expression est significativement réduite dans les cerveaux des suicidaires, semble être partiellement sous contrôle de mécanismes de régulation épigénétiques. Finalement, nous avons examiné l'implication du facteur de transcription astrocytaire sox9 dans la régulation partielle de l'expression de la variante astrocytaire du récepteur trkB et dans la formation des réseaux neuronaux.

Pour conclure, cet ouvrage identifie un nouveau marqueur du suicide, notamment la dysfonction astrocytaire, comme ayant un rôle prédominant dans la régulation de l'humeur et la susceptibilité au suicide.

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CHAPTER 1: INTRODUCTION

1. Historical perspective

1.1 Early views on suicide

The published record of the study of suicide began by statistical accounts of cause of death in different communities. The earliest reports of the kind were published in the early XIXth century by the American Board of Health (BoardofHealth, 1809). From the late 1830's, more detailed statistical data about suicide became available. For instance, a study conducted by health departments in England over a 24-year period included information on age and sex of suicide completers as well as the month and method of suicide (London, 1838). Over the next 100 years, research into suicide and suicidality focused on clinical issues and descriptive statistics. Worthy of mention are Kallman's assessment of suicide in twin pairs and only children (Kallmann et al., 1949) and a series of comments about attempted suicide published in 1952 in both the *American Journal of Psychiatry* and the *British Journal of Psychiatry*. A literature search on Medline for the 1950's through the mid-1960's period leads mainly to reports describing the methods used to commit suicide. A succinct summary of the literature at this point is provided below (Sifneos et al., 1956):

"*The literature on suicide is voluminous. Summaries of some of the findings follow. The role of social sciences in the study of suicide cannot be overemphasized. Dynamic formulations are very important in individual cases. In "successful" suicides, men outnumber women in a ratio of 3 to 1. In attempted suicides, men are outnumbered by women in a ratio of 1 to 3. In age, the peak for men is from 25 to 29 years; and for*

women, from 20 to 24 (William Blake, "Auguries of Innocence."). Northern Europeans outnumbered Southern Europeans. Negroes attempt suicide less frequently than any other race. More Protestants than Catholics try to kill themselves. Marriage seems to be a protective influence; the more people in the family, the fewer the suicides. Attempts increase in May and June in the Northern hemisphere and in January and February in the Southern hemisphere. Suicide is rare in the morning, frequent in the afternoon. With war it decreases, and with peace it increases. Poison (iodine was first of the poisons years ago) is the favorite method, with gas second. Slashing, firearms, hanging, leaping, and drowning follow in that order in men; leaping, slashing, firearms, drowning, and hanging, in women. Physical incapability was the major motivating factor in 8 out of 200 patients. The incidence of a history of broken homes is higher among suicidal individuals than among those with any other type of adult nervous or mental illness."

In the late 1950's, research on suicide took a distinctly more complex approach. Instead of focusing on raw population statistics, investigators began to focus on clinical variables that relate to suicide rather than simply to novel methods of death (Simon and Gilberstadt, 1958; Walton, 1958). In 1965, the idea of studying the brains of suicide completers was first explored. While clinical factors related to suicide began being explored more thoroughly with the purpose of increasing prevention (Friedman, 1962), the concept of suicide as a neurobiological entity emerged. Thus, pioneering biochemical studies were conducted (Bunney and Fawcett, 1965) with urine samples from suicide completers, which lead to the observation that levels of 17-hydroxycorticosteroid, a breakdown product of cortisol, were elevated prior to suicide. The rationale of an

neurobiological disturbance in suicide was further explored by these investigators and their colleagues, with a study measuring levels of serotonin (5-HT), its main metabolite 5-Hydroxyindoleacetic acid (5-HIAA), and noradrenaline (NA) in the hindbrain of suicide and control subjects (Bourne et al., 1968). This study showed reduced levels of 5-HIAA in the brains of suicide completers compared to control subjects. Two subsequent studies of significance assessed levels of 5-HT and other electrolytes in the brain of suicide completers (Shaw et al., 1967; Shaw et al., 1969). During the same period, Pare and collaborators performed a comprehensive comparative analysis of 5-HT, NA and dopamine (DA) levels in three different brain regions of suicide and control subjects (Pare et al., 1969). Stemming from these analyses, but also from the emerging stress hypothesis of mood disorders as well as the actions of certain treatments (Monoamine Oxidase Inhibitors, Tri-cyclic antidepressants), the majority of subsequent studies on neurobiological factors underlying suicide remained focused on the 5-HT and corticosteroid systems (Brooksbank et al., 1971; Brooksbank et al., 1972). From this period onwards suicide emerged as a distinct neurobiological entity. This concept was put forward by three excellent review papers in the late 1990's (Bachus et al., 1997; Gross-Isseroff et al., 1998; Mann, 1998).

1.2. The birth of suicide neurobiology

Suicide is hypothesized to be an independent neurobiological entity, distinct - but not exclusive from - psychopathology. The main rationale behind this hypothesis is that not all people who share risk factors for suicide kill themselves. For example, why do some people who suffer from major depression commit suicide while others never consider it?

People who commit suicide, then, may represent distinct entities within particular clinical groups. For example, despite the fact that the motivations underlying suicide are heterogeneous across subjects, people with major depression who kill themselves may be biologically different from people with major depression who do not.

While psychiatric studies are often hampered by the presence of unclear phenotypes (Mannuzza et al., 1995; Biederman et al., 2003), the same cannot be said for studies that investigate the neurobiology of suicide, as suicide completion is unambiguous. In this perspective, addressing the neurobiology of depression is of interest to any discussion pertaining to suicide, as it is the major psychopathology associated with suicide. In this review, we have made an effort to separate studies that specifically addressed the neurobiology of suicide from those addressing major depression. Below, we review the evidence supporting (or not) the notion that suicide is accompanied by dysregulations in some of the major neurotransmitter and neuromodulator systems in the brain. Attention will be mainly given to the central systems for which there is substantial evidence, namely the monoaminergic innervations and the fast excitatory (glutamate) and inhibitory (GABA) systems. Each system will first be discussed in the light of neurochemical and ligand binding studies (sections 1-5). We will then review the recent progress made in understanding if there is a genetic component to suicide, and whether the putative neurotransmitter dysfunctions are associated with altered genetic or epigenetic states/processes (section 6).

2. Serotonin

The 5-HT system has been the most widely investigated neuromodulatory system in studies of suicide attempters and completers. The idea of a dysfunction in 5-HT transmission leading to depressed mood and possibly suicide emerged from the studies discussed above, and is supported by the clinical benefits of mood stabilizing selective 5-HT reuptake inhibitors (SSRIs). To date however, no clinical trial has investigated the benefits of SSRIs to prevent suicide risk (Mann et al., 2005). In fact, there is some suggestion that medications such as SSRIs, which increase extracellular 5-HT, may increase suicide risk in youth (Bridge et al., 2007).

The dense 5-HT innervations pervading all brain regions originate exclusively from projection neurons located in the brainstem's raphe nuclei. Tryptophan hydroxylase (TPH), the enzyme of synthesis of 5-HT, has been widely used as a marker of 5-HT activity. Quantitative immunocytochemical and immunoautoradiographic studies in the dorsal raphe nucleus (DRN), which projects mainly to the cerebral cortex and hippocampus, have shown significantly higher numbers and densities of TPH-immunoreactive (TPH-IR) neurons in MDD subjects (Underwood, et al 1999), depressed suicides (Boldrini, et al 2005) and alcohol-dependent depressed suicides (Bonkale, et al 2004) compared to controls. In accordance with these studies, Bach-Mizrachi and colleagues (2006) reported a 33% increase in expression (mRNA) of TPH2 in the raphe of suicide victims compared to controls. Given the multiple lines of evidence indicating an overall reduction in 5-HT transmission in both cortical and subcortical regions of MDD and suicide subjects (see below), increased TPH expression could constitute a compensatory mechanism to alleviate central 5-HT transmission, and/or a response to

increased stress, which has also been associated with alterations of TPH protein and mRNA levels (Azmitia, et al 1993; Chamas, et al 2004). Alternatively, a predisposing neurodevelopmental phenomenon may exist which increases the number of 5-HT neurons in the DRN, as suggested by the observation of significantly higher numbers of TPH-IR neurons documented across the lifespan, even at a young age, in suicide subjects (Underwood, et al 1999). A possible mechanisms accounting for such an increase would be an alteration in the programmed cell death normally occurring during brainstem development.

John Mann's group examined [^{3}H]-paroxetine binding to the 5-HT transporter (SERT) in the DRN of suicides and matched controls, and reported fewer SERT-expressing neurons accompanied by a greater expression of SERT per neuron (Arango et al., 2001). This study also reported a decrease in 5-HT1A binding, thus suggesting a loss of receptors in the raphe, in contrast to earlier data from an independent group (Stockmeier et al., 1998). With regards to 5-HT innervations in terminal fields, Mann's team reported a decrease of [^{3}H]-paroxetine binding in prefrontal cortex (Mann et al., 1996) and ventro-lateral prefrontal cortex (Arango et al., 1995) of suicide subjects. Athough these data were not consistently reproduced by others (e.g. Bligh-Glover et al., 2000; (Lawrence et al., 1990)Hrdina *et al.*, 1993) SERT-immunocytochemistry has supported the notion of a decrease in densities of 5-HT innervations in prefrontal cortical areas (Austin et al., 2002).

A few 5-HT receptor binding studies have compared terminal fields between suicides and controls. Cheetham and collaborators reported no difference in 5-HT2 binding sites (Cheetham et al., 1988) in cortex and amygdala, nor in 5-HT1 and 5-HT1B binding (Cheetham et al., 1990) in frontal and temporal cortex of suicides versus matched controls. They did, however, report a reduction in the number and affinity of 5-HT1 binding sites in the hippocampus and amygdala, respectively (Cheetham et al., 1990). A subsequent study found a significant increase in [^{3}H]-ketanserin (5-HT2 receptors) binding in both PFC and amygdala (67% and 96%, respectively) (Hrdina et al., 1993). However, using a very large sample of 73 suicides and 70 controls, no difference between groups was observed in [^{3}H]-ketanserin binding in any of six brain regions assessed (Lowther et al., 1994). In a similar sample, these investigators found no difference in [^{3}H]8-OH-DPAT (5-HT1A) binding either (Lowther et al., 1997). Thus, although there are some regional inconsistencies that may have to do with medication or other confounding factors, most binding data on 5-HT receptors in the suicide brain have tended to be negative.

3. Catecholamines

3.1 Dopamine

The major dopaminergic innervations in the brain take their origin in the ventral tegmental area and substantia nigra in the mesencephalon. To date, very little evidence has implicated dopaminergic transmission in suicide. Measurements of dopamine concentrations in cortical and subcortical regions in suicide versus matched control subjects did not reveal any significant difference between groups. However, it has been

hypothesized that mesolimbic dopaminergic transmission is reduced in depression and suicide which could account for the dopaminergic-based antidepressant pharmacotherapies. In support for this, radioligand binding data have shown that there is a significant reduction in dopamine transport coupled with an increase in D2/D3 receptors in the amygdala of major depressed subjects. It is thus conceivable that regional changes in dopaminergic transmission may be involved in mood disorders (e.g. by contributing to anhedonia) and lead to suicide.

3.2 Adrenaline and Noradrenaline

Adrenaline (epinephrine) is synthesized from tyrosine and phenylalanine in both the adrenal gland and the brain, and is considered both a hormone and a neurotransmitter. Adrenaline is an activator molecule well known to induce several physiological effects (e.g. increased heart rate) and general cognitive enhancement (e.g. increased awareness and attention). Although adrenergic transmission has been hypothesized to play a role in mood and possibly suicide (Lipinski et al., 1987), no study has examined in suicide the morphological features of the small adrenergic nuclei located in the brainstem. Instead, most of the attention has been devoted to central noradrenergic transmission, including the adrenaline- and noradrenaline-binding adrenergic receptors; a system also known to interact with the stress response through a feed-forward mechanism.

Noradrenaline-synthesizing neurons are located in the locus coeruleus (LC) and lateral tegmental area of the brainstem and project to all brain regions (Foote, et al 1983; Moore and Bloom, 1979), of which the LC is the best studied.. The LC has been reported to present neurochemical alterations in MDD and suicide subjects (Klimek, et

al 1997; Merali, et al 2006), but there is no compelling evidence in the literature suggesting that LC cells display morphological signs of altered plasticity in these conditions. Thus, most studies have failed to find differences between MDD suicides and control subjects in the number of neuromelanin containing (NA-producing) cells in the LC (Baumann, et al 1999a, 1999b; Syed, et al 2005; Zhu, et al 1999; Ordway, 1999). One report has indicated significant reductions in the total number and average density of pigmented LC neurons (left hemisphere only) in suicide completers (Arango, et al 1996), and another, the inverse trend (Ordway, et al 1994). In general, no differences have been reported in the numbers of LC TH-immunoreactive neurons (TH-IR) between depressed suicides and control subjects (Biegon and Fieldust, 1992; Baumann, et al 1999; Syed, et al 2005). Interestingly, a secondary analysis by Baumann and colleagues (1999) revealed that the patients with mood disorders not committing suicide had significantly fewer TH-IR neurons. Arguably, this observation suggests that the LC undergoes similar increases in local neurotransmitter synthesis than those in the DRN (see 2.0) in subjects with mood disorders which are prone to suicide. In line with this argument, increased levels of TH protein have been observed in the LC of suicide subjects (Ordway, et al 1994; Zhu, et al 1999; but see Biegon and Fieldust, 1992).

In the CNS, the metabotropic adrenergic receptors are subdivided into β - and α-subtypes (Hein, 2006). The β2-adrenergic receptors have received the most attention with regards to suicide, and this has stemmed mainly from studies conducted by Garcia-Sevilla's team. These investigators have consistently reported increases in β2-adrenergic receptor densities in the hypothalamus (Meana et al., 1992) and frontal cortex of

depressed suicides compared to matched controls, with no changes in binding affinity (Meana and Garcia-Sevilla, 1987). These data were supported by subsequent findings obtained with a similar sample set, where increased β2-adrenergic receptor binding (Callado et al., 1998) and mRNA levels (Garcia-Sevilla et al., 1999) were described in frontal cortex of depressed suicides compared to controls. Employing the agonist [^{3}H]UK-14304, another β2-adrenergic agonist, these authors further reported a significant increase in [^{35}S]GTPγs binding in hippocampal and frontal cortical tissues of suicides and depressed suicides compared to controls (Meana et al., 1992). More recent studies by this group have provided further evidence the β2-adrenergic receptor expression is altered in the brains of depressed suicides (Gonzalez-Maeso et al., 2002; Escriba et al., 2004). However, a number of negative studies by independent groups suggest that this may not be the case (Arango et al., 1993; De Paermentier et al., 1997; Gross-Isseroff et al., 2000). Other studies suggest that β2-adrenergic receptors may be involved in non-frontal cortical regions (Ordway et al., 1994; De Paermentier et al., 1997).

4. Glutamate and GABA

Glutamate and GABA are respectively the main excitatory and inhibitory neurotransmitters in the mature brain. As a rule of thumb, glutamate is mainly synthesized by projection neurons, whereas GABA is the fast-acting transmitter used mainly by interneurons. Glutamine is a core requirement for the synthesis of both glutamate and GABA. In nerve terminals, the enzyme glutaminase converts glutamine to glutamate, and the latter can be used for the synthesis of GABA via the enzymatic actions

of GAD. In glial cells, glutamine synthetase is the enzyme required to synthesize glutamine from the re-uptake of either glutamate or GABA (Weiler et al., 1979).

In recent years, morphological evidence has been brought forward suggesting an alteration in the organization and morphology of glutamatergic neurons in key brain regions associated with mood disorders and suicide, and particularly the dorsolateral prefrontal cortex (Brodmann area 9; BA9). Despite the fact that most of these studies have focused on MDD, it is important not to rule out that the majority of the samples under study were from subjects that had committed suicide. Rajkowska and collaborators (1999) first reported variations in neuronal densities in layers II, III and VI of MDD subjects compared to controls; the former presenting significant reductions and increases in the densities of the largest and smallest neurons, respectively. Although these findings were not replicated by some subsequent studies (Cotter, et al 2002; Miguel-Hidalgo et al., 2005), others provided evidence for reductions in supra-granular pyramidal neurons in MDD subjects (Law and Harrisson, 2003).

Recently, it was reported that a highly significant increase in density of GABA neurons (GAD 65/67-immunoreactive (-IR)) occurs in the hippocampus and in several neocortical areas in MDD suicide subjects versus controls (Bielau et al., 2007), suggesting GABA dysfunction as a widespread phenomenon in the brain. However, these results need to be further investigated as studies using different methodologies have indicated a somewhat opposite trend. Recently, Rajkowska, et al (2007) focused on subpopulations of cortical GABA interneurons based on the co-expression of GABAergic markers with calcium

binding proteins and found significant reductions in both neuron somal size and density for BA9 calbindin (CB)-IR neurons in MDD (majority of suicides) versus control subjects. In contrast, these authors found no differences in the density and somal size of parvalbumin-immunoreactive (PV-IR) interneurons in the same sample (Rajkowska, et al 2007).

There are three major classes of glutamate receptors: AMPA, NMDA, and metabotropic glutamate receptors. AMPA receptors are composed of 4 subunits (GluR1-GluR4; (Wisden and Seeburg, 1993), whereas NMDA receptors are composed of one NR1 receptor and one of NR2A, NR2B, NR2C, NR2D subunits (Wenthold et al., 2003). The class of metabotropic receptors is divided into three families and comprises mGluR1-mGluR8 (Conn and Pin, 1997). One study of NMDA receptors with the ligand MK-801 showed no difference between suicide and control subjects (Holemans et al., 1993). Functional activation of NMDA receptors requires co-binding of glycine and glutamate at distinct sites. To test the hypothesis that glycine displaceable sites may be altered in suicide brains, Nowak and colleagues assessed the proportion of high affinity glycine displaceable [3H]CGP-39653 binding sites in the brain, and found that binding was reduced from 45 ± 5% in controls to 27 ± 6% in suicide victims (Nowak et al., 1995). A later study also suggested that the density of AMPA receptors may be increased in the caudate nucleus of suicide subjects (Noga et al., 1997). Taken together, these binding studies suggest that both NMDA and AMPA receptor functions are altered in the suicide brain.

Despite a slight difference observed in the hypothalamus, regional GABA levels in the brain do not seem to differ between suicide and control brains (Korpi et al., 1988). Similarly, assessing binding to GABA transporter-1 (GAT-1) with the ligand [^{3}H]-tiagabine in frontal cortex and anterior cingulate cortex of suicide and control subjects led to no significant difference between groups (Sundman-Eriksson and Allard, 2002). GABAA and GABAB receptors, the two GABA receptor subtypes (Macdonald and Olsen, 1994), have received significant attention from suicide researchers, and studies have largely come up negative. Manchon and collaboratos conducted one of the first GABA receptor binding studies in suicide brains (Manchon et al., 1987). These authors reported an increase in the number of type I benzodiazepine binding sites in the hippocampus of suicides versus controls. This increase was accompanied by a slightly greater binding affinity (Manchon et al., 1987). Using a larger sample, the same group later reproduced these results (Rochet et al., 1992). However, an independent study using the same radioligand with hippocampal and amygdalar tissues found no difference between subject groups (Stocks et al., 1990). Similarly, investigating GABAA receptor binding with [^{3}H]-flunitrazepam at three anatomically defined levels of the locus coreleus revealed no significant difference between depressed suicides and controls (Zhu et al., 2006). A subsequent study of benzodiazepine receptors in BA10 using [^{3}H]-RO15-1788 realized in schizophrenic suicides, non- schizophrenic suicides and controls found a small increase in benzodiazepine receptors in the suicide group, although this seemed mostly due to neuroleptic treatment (Pandey et al., 1997). Similarly, Cheetham and colleagues (1988b) reported an incease in GABAA binding in the frontal (but not temporal) cortex of suicides versus controls. Two studies investigating metabotropic GABAB binding

sites in the brain found no difference between suicide and control subjects (Cross et al., 1988; Arranz et al., 1992).

5. Other systems

5.1 Opioids

Opioids have long been known to have an effect on mood, but not since the discovery of endogenous opioids and opioid receptors (Cox et al., 1975; Hughes et al., 1975; Goldstein, 1976), and their effects (Belluzzi and Stein, 1977) have these molecules been examined as potential etiological factors in mood disorders (Ball, 1987). The first study to examine the relationship between opioid receptors and suicide was performed with quantitative [^{3}H]-DAGO autoradiography to assess μ-opioid receptors in the brain (Gross-Isseroff et al., 1990). This group reported that younger suicide completers had a higher density of μ-opioid receptors in frontal and temporal cortex than matched controls. These results were later independently replicated with the same technique on a comparable sample (15 subjects per group) (Gabilondo et al., 1995). In this study, a 40% increase in μ-opioid receptors was observed in frontal cortex and thalamus. Interestingly, a recent investigation employing the same approach found no difference in μ-opioid density or affinity in prefrontal cortex or pre-post central gyrus of suicide completers compared to matched controls (Zalsman et al., 2005). This may be attributable to smaller sample size (n = 9/group). To this day, no study has addressed the distribution and affinity of other opioid receptor subtypes in the brain of suicide subjects.

5.2 Acetylcholine

Acetylcholine (ACh) innervations in the brain originate from the basal forebrain, the pedunculopontine tegmentum, and from a population of giant interneurons in the striatum. ACh has been implicated by several investigators in cognitive states and functions, such as attention and memory (Sarter et al., 2003). There are two main types of acetylcholine receptors: nicotinic (nAChRs) and muscarinc receptors (mAChRs) (Birdsall and Hulme, 1976; McCarthy et al., 1986; Berg et al., 1989). Cholinergic dysfunction was fist considered in psychiatric disorders when sleep disturbances in mood disorders were related to myasthenia gravis, an autoimmune disorder where antibodies inappropriately attack nAChRs and generate weakened states (Gillin et al., 1979; Sitaram et al., 1982). To our knowledge, no study has ever examined the morphology of ACh neurons and their axonal projections in suicide, and none have reported in this context on changes in nAChR binding in the brain. In contrast to Meyerson and colleagues (1982) who reported that [^{3}H]-quinuclidinyl benzilate binding to mAChRs increased by 47% in the cerebral cortex of suicide completers compared to homicide victims (Meyerson et al., 1982), two subsequent studies using the same approach reported no difference in mAChR binding affinity nor density between groups in the frontal cortex (Stanley, 1984) as well as in the pons and hypothalamus (Kaufmann et al., 1984). An investigation using [^{35}S]-GTP□S binding to G-proteins largely supported the view that mAChRs are generally unaltered in suicide brains (Gonzalez-Maeso et al., 2002). More recently, however, using [^{3}H]-pirenzepine, a ligand specific to M1 and M4 receptors, an effect of suicide on binding was reported (Zavitsanou et al., 2004) in one patient population (SCZ) but not in others (bipolar and major depressive disorders). It remains to be seen if this result will be replicated in other samples.

6. Cell signalling

Extra and intra-cellular signaling refers to the interaction and resulting effector cascade between proteins inside or outside the cell. The processes can be seen as continuous in the sense that nuclear effects generate gene products which can be exported from the cell to stimulate receptors on other cells' membranes which can then activate an intracellular signal cascade which stimulates nuclear effectors. In suicide research, the study of signaling molecules is largely restricted to either measuring the quantity of an RNA or protein molecule post-mortem known from basic neuroscience studies to be involved in cell signaling or, in some cases, from the attempt to measure activity of a protein extracted from suicide brain (Shelton *et al.*, 2009).

Neurotrophic factors are extracellular signaling molecules and have emerged as candidate molecules in the neurobiology of suicide. Neurotrophins are well known for their roles in neuronal and plasticity, and their altered expression could underlie, at least in part, changes in plasticity observed in the brains of suicides. Neurotrophic receptors, meanwhile, are mostly found on the cell membrane and, after binding the ligand, can stimulate intracellular effects. While the major neurotrophic factors include NGF, NT3/4, FGF, and BDNF, only BDNF, FGF, or their respective receptors have been associated with suicide or major depression by multiple investigators.

FGF is an important molecule for cell and organ development throughout the body, including the brain (Vaccarino et al., 1999; Turner et al., 2006). Two different studies have implicated the fibroblast growth factor (FGF) system in depression and suicide.

Using brains collected as part of the Pritzker consortium, Evans and colleagues used frontal cortical tissue from 14 controls, 6 bipolar, and 13 MDD subjects, of which 6 were female (Evans *et al.*, 2004). The most robust findings from their work was that two FGF receptors (FGFR2 and FGFR3) are downregulated in both DLPFC and anterior cingulated cortex specifically in MDD subjects. The FGF system is of interest to studies and depression and has received some support from other groups. For example, microarray data derived from BA10 from Stanley subjects (15 bipolar, 15 depressed, 15 SCZ, and 15 controls donated by Stanley Foundation) yielded alterations in the FGFR1, NCAM1, and CAMK2A (Tochigi et al., 2008a). The FGFR3 gene was also shown to be reduced in frontal cortex in suicide brain in the Kim et al. microarray expression study (Kim et al., 2007a). Using our own microarray data, we confirmed that FGFR3 and FGFR2 are down regulated in multiple brain regions of suicide completers (unpublished observations). In support of these human gene expression studies are rodent studies where chronic defeat stress has been shown to decrease multiple elements of the FGF system and increase in response to continuous anti-depressant treatment (Mallei et al., 2002; Bachis et al., 2008; Turner et al., 2008b; Turner et al., 2008a).

TrkB is a trans-membrane receptor capable of high affinity binding to BDNF, a growth factor that acts to stimulate cell signaling cascades to affect cell growth and proliferation in the CNS. The TrkB gene has three main splice products, where full length TrkB and TrkB.T2 expression are mostly restricted to neurons and TrkB.T1 expression is restricted to astrocytes. A number of brain post-mortem studies as well as serum BDNF level studies have been performed both in suicide completers and subjects with major

depression. Much of this work, that demonstrates a decrease in the BDNF/TrkB system, has been supported by the animal literature.

Anti-depressants increase the expression of BDNF in human. Early reports in rodent suggested that treatment with antidepressants or electroconvulsive therapy led to an increase in BDNF expression (Nibuya *et al.*, 1995), and it was these studies that encouraged studies in human psychopathology to move forward. While the conceptual model, that individuals with depression have chronic under expression of TrkB/BDNF, is difficult to test, testing the affect of antidepressants on TrkB/BDNF expression in human brain is more feasible. These studies followed a general procedure where post mortem brain sections were obtained from a particular brain region, usually hippocampus or frontal cortex, and assessed for gene expression level (Chen *et al.*, 2001). These studies showed increased expression of BDNF after pharmacological treatment. Other studies addressing the effects of antidepressants on BDNF collected serum from treated patients, largely with similar results to the post mortem studies (Matrisciano *et al.*, 2009). The idea that antidepressants can up-regulate BDNF expression seems clear and a meta-analysis of this effect has been conducted (Sen *et al.*, 2008); the role of anti-depressants on TrkB expression is less clear, however (Linden *et al.*, 2000).

If antidepressants up-regulate BDNF, might a chronic decrease in BDNF/TRKB may be an underlying cause of major depression? To assess this idea, a number of studies have been performed in both depressed people and suicide completers. Some studies have suggested that both BDNF and full length TRKB are down regulated in different brain

regions of suicide completers, most of who were diagnosed with major depression (Dwivedi *et al.*, 2003; Pandey *et al.*, 2008). A number of serum studies suggest, controlling for medication effects, that BDNF is down-regulated in subjects with major depression (Brunoni *et al.*, 2008).

Microarray expression studies have not observed differences in BDNF in either depressed patients or suicide completers, but have detected decreased expression of TrkB. As mentioned, there are three main variants of TrkB and most microarray platforms are able to detect all three variants. To date, three different microarray studies have implicated TrkB decrease in depressed mood (Aston et al., 2005; Nakatani et al., 2006; Ernst et al., 2009), yet only one of these studies, performed by our group, disclosed which variant was analyzed. Interestingly, in our own study it was the T1 variant that was downregulated in suicide brain with no alteration in either other TRKB variants. The T1 variant is specific to astroglial cells and plays a role in calcium signaling (Rose *et al.*, 2003), suggesting a role for astrocytes and TRKB in depression and suicide. As astrocytes are the neural stem cell of the adult CNS (Doetsch *et al.*, 1999), and it is these cell that are implicated in the neurogenesis hypothesis of depression (Duman *et al.*, 1997; Kempermann and Kronenberg, 2003), a decrease expression in TrkB.T1 could have an affect on astroglial growth and signalling.

Intacellular signalling cascades often involve second messenger systems where an external signal is transduced in the cell by a series of enzymatic reaction that can significantly amplify a signal. This can come in the form, for example, of the addition of

phosphate groups or from protein conformational changes. Other signalling cascades involve the influx of ions in the cell, such as calcium, which can generate numerous effects in cell. Molecules in intracellular signalling cascades have received much less attention and support than extracellular signalling cascades so we refer the reader to reviews that summarize the relation of different signals to mood disorders and suicide which have largely focused on the cAMP second messenger system (Sulser, 2002; Dwivedi and Pandey, 2008)

7. Stress

7.1 HPA Axis

The hypothalamic-pituitary-adrenal (HPA) axis is the major biological infrastructure of the human stress system with interconnections between the structures by the hormones CRH, ACTH, and cortisol. It is the over- or under-activity of these hormones that is often tested in suicide and depression studies and how their long term effects can alter brain structures. In particular, the stress-related theory of depression postulates that long-term chronic activation of the HPA axis leads to detrimental effects on hippocampal neurons (Duman *et al.*, 1997; McKinnon *et al.*, 2009).

The dexamethasone suppression test is used to assess HPA axis functioning. Dexamethasone is a glucocorticoid receptor (GR) antagonist and acts to suppress the release of ACTH from the hypothalamus; ACTH can no longer stimulate the release of cortisol from the adrenal gland. Cortisol can be measured in saliva and it is these salivary

cortisol measurements that have been assessed in different studies of suicidality and depression.

There is a lack of consensus in whether cortisol response is increased or decreased after dexamethasone challenge in suicide attempters compared to controls. This has been reviewed (Westrin, 2000; Mann and Currier, 2007), so we describe only a few recent studies which suggest that this divergence continues. A recent large study examined the levels of cortisol after dexamethasone challenge in depressed patients that recently attempted suicide and found lower levels of cortisol in depressed, suicidal patients compared to depressed, non-suicidal patients (Pfennig *et al.*, 2005). Jokinen and Nordstrom (2008) recently assessed cortisol response to dexamethasone in suicide attempters and found hyperactive cortisol response (or non-supressors) in attempters (Jokinen and Nordstrom, 2008). Lindqvist et al, examined suicide HPA activity in suicide attempters and found hypoactive cortisol response (Lindqvist *et al.*, 2008). While almost negative studies are published in this area, one of the initial studies in this field reported no differences in cortisol levels in suicide attempters (Roy, 1992).

Hypoactivity of cortisol in suicide attempters after dexamethasone has been interpreted as a 'burnt-out' HPA axis; that is, that chronic stress has over-exerted the system and cortisol can no longer be released properly. Alternatively, hyperactivity of cortisol after dexamethasone challenge has been interpreted as an HPA axis that releases too much cortisol leading to detrimental brain effects and feelings of chronic stress. How can these divergent interpretations be interpreted? Most likely differences in sampling procedure,

analysis methodology, and time of the suicide attempt in relation to the dexamethasone challenge. There are also suggestions that CRH stimulation after dexamethasone challenge and more complex analysis methods may be important in interpreting data (Sher et al., 2006; Fountoulakis et al., 2008).

7.2 Polyamines

Polyamines are ubiquitously expressed, highly regulated compounds found in all organisms and contain two or more amine (NH2) groups (Tabor and Tabor, 1984). While their function in cells in not entirely clear, they are known to bind DNA through an intermediary and play a role in cell system response (Moinard *et al.*, 2005). Inhibition of the most widely studied polyamines, which include spermidine, putrescine, and spermine, leads to cell death (Gilad and Gilad, 2003). A number of these compounds or the rate-limiting enzymes in the metabolic pathways, have been related to psychiatric illness, particularly schizophrenia, anxiety, and major depression (Gilad et al., 1995; Fiori and Turecki, 2008).

Both human and animal studies have associated polyamine dysfunction with psychopathology. In animal models of depression, levels of putrescine, spermine and spermidine have been shown to be down-regulated (Genedani *et al.*, 2001) and antidepressant effects of different medication have been shown to alter the polyamine system, particularly the interaction of agmantine or putrescine on NMDA receptors (Aricioglu and Altunbas, 2003; Li et al., 2003; Zomkowski et al., 2006; Zeidan et al., 2007). Stress has also been shown to have an affect on the polyamine system in rodents,

including stress-increased putrescine levels in rodent frontal cortex (Gilad and Gilad, 2002; Sohn et al., 2002; Lee et al., 2006), as well as non-human primate models of depression (Karssen *et al.*, 2007). In human, most studies involve only assessments of the rate-limiting enzymes in polyamine metabolic pathways; these include spermidine/spermine N^1-acetyltransferase (SSAT), ornithine decarboxylase (ODC), and polyamine oxidase (PAO). Dahel et al. found an increase in PAO in serum of depressed patients which returned to more normal levels after electro-convulsive therapy (Dahel *et al.*, 2001). Our own group has demonstrated in multiple brain regions of suicide completers that SSAT expression is down-regulated in frontal cortex of suicide completers with or without major depression (Sequeira *et al.*, 2006). Other reports have also emerged indicating a possible role of (SSAT) in suicide and major depression (Sequeira et al., 2006; Guipponi et al., 2008; Klempan et al., 2009). Some studies in human have found no differences in polyamine metabolic enzymes (Gilad *et al.*, 1995).

8. Astrocytes and oligodendrocytes

Glial cells include primarily astrocytes, oligodendrocytes, and microglia and their role and importance in the central nervous system continues to expand (Barres, 2008). Microglial progenitor cells are derived from mesodermal origins, unlike oligodendrocytes and astrocytes which are derived from ectodermal origins (Chan *et al.*, 2007). Microglia are mononuclear phagocytes in the CNS, and one of their key roles is to clear cell debris and to support cell survival (Gonzalez-Scarano and Baltuch, 1999). They are the housekeepers of the resting nervous system, continuously surveying the brain with small mobile processes (Nimmerjahn *et al.*, 2005). Oligodendrocytes are the myelin generating

cells of the CNS and myleination of axonal membranes allows for action potential functioning in neurons (Peters, 1960; McTigue and Tripathi, 2008). More recent work also expands the role of oligodendrocyes to receptor clustering on the neuronal membrane and neuron survival (Wilkins *et al.*, 2003). Astrocytes are a heterogeneous group of cells with many functions (Wang and Bordey, 2008) and this category of cells is likely the most diverse of glial cells. Astrocytes release and take-up neurotransmitters, propagate calcium waves, play a role in synapse development and maintenance, and are involved in the immune response (Kettenman, 2005), amongst many other roles not least of which is acting as the neural progenitors cells in the adult brain (Doetsch, 2003; Bonfanti and Peretto, 2007). Glial cells, particularly astrocytes and oligodendrocytes have been implicated in major depression and suicide.

Astrocytes were first implicated in depression and suicide through neuro-anatomical and hypothesis-driven gene expression studies. Using post-mortem brain sections, initial anatomical studies reported less glial cells in brain of people with depression (Ongur et al., 1998; Rajkowska et al., 1999; Cotter et al., 2001a; Bowley et al., 2002; Rajkowska, 2003). Due to the type of staining techniques used it was impossible to distinguish which type of glial cell that was reduced, but still independent reports suggested that the frequency of non-neuronal cells in the brain was lasss in depressed subjects than in controls. These findings suggest a general decrease in astrocytes, oligodendrocytes, and microglia in brains from depressed subjects but the lack of cell-type specificity as well as the difficulty in accurately counting cells in post mortem brain (Gundersen *et al.*, 1988) left a requirement for more evidence to demonstrate global glial reduction in depression.

Investigating expression levels of astrocyte-specific genes is a different way to assess a reduction in astrocytes. The glial fibrillary acidic protein (GFAP), an astrocyte-specific marker, has been used in studies using frontal or anterior cingulated tissue from depressed subjects. These studies have suggested that astrocyte levels are normal or increased in the depressed brain (Miguel-Hidalgo et al., 2000; Davis et al., 2002; Webster et al., 2005).

Astrocyte dysfunction, without reduction in cell number, may be a factor in major depression and suicide. Microarray studies have implicated a number of genes that are specific to astrocytes and a number of these genes have been replicated by independent groups. Astrocyte expressed genes that are detected across multiple microarray studies using frontal cortical tissue from depressed subjects or suicide completers include FGFR2/3, SLC1A3, SLC1A2, and GLUL. We note the distinct glutamate involvement of some of these astrocyte-specific genes (Evans et al., 2004; Choudary et al., 2005; Kim et al., 2007a; Ernst et al., 2009). Given the role of glutamate in mood disorders and the role of astrocytes in glutamate metabolism, astrocytes may be mediators of glutamate dysfunction detected in major depression and suicide. Astrocytes have a number of roles in the brain and slight alterations in functioning could affect mood. Two roles of distinct curiosity are astrocyte glutamate release at the synapse and the long-range propagation of calcium waves throughout the brain.

Oligodendrocytes and myelin-related disturbances have been noted in multiple psychiatric disorders, particularly schizophrenia, but also in major depression. A number

of histochemical studies of oligodendroglia in depression, (Regenold *et al.*, 2007) used a detection technique that stains all cells, requiring experimenters to make challenging cell-type decisions based on cell morphology. These studies found a consistent decrease in oligodendrocytes in brain areas of depressed subjects; however, the interpretation is difficult due to the staining technique used (Uranova *et al.*, 2004; Vostrikov *et al.*, 2007). One study done in amygdala that suggests reduction in cell number is due to oligodendrocytes used immunochemical stains for astrocytes and microglia and a general stain fro all cells. By detecting an overall cell number deficit in depressed subjects, with non-significant differences in cell number of astrocytes or microglia this data points to the idea that cell number reduction is due to oligodendrocytes (Hamidi *et al.*, 2004). Other studies using specific chemical stains for myelin observed decreased myelin in the depressed brain (Regenold *et al.*, 2007)

White matter (myelin) scarring, as detected by hyper-intensities using magnetic resonance imaging is suggested to be increased in people with depression (Dupont *et al.*, 1995). While this appears to be due to cerebrovascular disease risk (Lenze *et al.*, 1999), there does appear to be an increase in white matter scarring in people with late-life depression (Herrmann *et al.*, 2008)

A number of gene expression studies have implicated oligodendroglial genes in depression and suicide. One exploratory gene expression study found a large number of oligo-specific genes to be down-regulated in post mortem brain of depressed subjects (Aston *et al.*, 2005). Research from our group also suggests down-regulation of multiple

oligodendroglial genes, particularly the RNA binding protein QKI (unpublished data). In a mouse study of depression using unpredictable chronic stress, a gene set enriched with oligodendroglial genes was observed to be significantly down-regulated. Because of the wide variety of confounding factors that can affect gene expression studies done using human brain such as post mortem interval or pH (Vawter *et al.*, 2006), an animal study such as this supports oligodendrocyte involvement in chronic stress/depression and suggests that oligodendroglial reduction is not due to post mortem artefacts.

9. Evaluating the genetic contribution to suicidal behaviors

The search for genes whose variation may contribute to suicidal behaviors is now in its third decade. Spurred by evidence indicating that suicide risk may have a genetic component, association-based studies have investigated over thirty candidate genes. As has been the case with related psychiatric phenotypes, attention has centered on monoaminergic genes, primarily those regulating the availability of 5-HT. The most studied are serotonin transporter (SLC6A4) and TPH genes, each the focus of over thirty studies. In the case of SLC6A4, only two variants have been investigated: the triallelic promoter polymorphism (LPR) and a multiallelic, intronic, VNTR. In contrast to the weak association evidence for the VNTR variant, support for the role of LPR, namely its low-activity S allele, is more substantial. The S allele and genotypes have been found to confer odds ratios between 1.7-4.8 and 2.3-6.5 respectively for a number of phenotypes, especially for violent, impulsive, and repeated suicide attempts (Bondy et al., 2006). Two meta-analyses confirmed its involvement in predisposition to suicide attempts and violent suicides (Anguelova et al., 2003; Lin and Tsai, 2004). Unlike the SLC6A4 gene research which focused on only two variants, the selection of polymorphisms in TPH genes was more comprehensive encompassing multiple introns, single nucleotide polymorphisms (SNPs) and microsatellites. This is especially true for TPH2, a more recent candidate, which has so far been studied only in relation to completed suicides and with only one positive finding in a German sample (Zill et al., 2004). As for the TPH1 gene, studies have focused on the variation in intron 7, especially on two closely linked SNPs: A218C and A779C. The evidence implicating these polymorphisms is controversial. While an earlier meta-analysis found no association (Lalovic and Turecki,

2002), a more recent one indicated that A218C confers a risk of 1.62 for suicidal behaviors (Bellivier et al., 2004).

The exploration of candidate genes has also been extended to include 5-HT receptors and catabolizing enzymes. 5HTR2A receptor's C102T variant, for example, was found to be associated with both suicidal ideation and attempts, with CC genotypes increasing the risk 3.1 and 5.5 times respectively (Du et al., 2000; Arias et al., 2001). A meta-analysis, however, did not support the contribution of the 5HTR2A gene to suicidality (Anguelova et al., 2003). 5HTR1B receptor's G861C SNP was the only promising variant among the three examined to date (New et al., 2001). G allele of the C1019G polymorphism was, similarly, the only allele in the 5HTR1A receptor with relevance to suicidality (Lemonde et al., 2003). No association was found with variants in serotonin receptors 5HTR1D, 5HTR1E, 5HTR1F, 5HTR2C, 5HTR5A, 5HTR1A, or 5HTR6.

The focus of such studies has also expanded to include COMT and MAOA genes, two important components of catabolism in several neurotransmitter systems. MAOA's VNTR and COMT's Val/Met polymorphisms showed significant and in some cases gender-specific association with violent suicide attempts (Courtet et al., 2005);(Nolan et al., 2000). Other systems, involving dopaminergic, noradrenergic, glutamatergic, GABAergic, and neurotrophic genes, have been studied to a lesser extent and with less encouraging results. Researchers have also targeted individual genes rather than systems involved in detoxification (MNSOD), transport (ABCG, SCN8A), and signaling (CCK), with no evidence of association with examined suicidal phenotypes.

Thirty years and close to 200 studies later, the goal of identifying putative susceptibility genes remains elusive. As with other psychiatric phenotypes, association-based searches for genes with small-to-moderate effects in complex diseases are plagued by insufficient power, population stratification, and phenotype heterogeneity (Craddock et al., 2006). Additionally, the effects of some variants may only be obvious when considered in the context of other genes and environments (Caspi et al., 2003; De Luca et al., 2006). Along with methodological improvements, future research in the field should benefit from better characterization of final and intermediate phenotypes, quantification of both main and interactive genetic effects within and across systems, and consideration of environmental and epigenetic factors.

9.1 Microarray expression studies

Microarray expression studies offer the possibility to assess transcription levels of most known genes. In this way, it is possible to extract RNA from a brain region of interest from a psychiatric sample set and monitor the transcriptome (the complete set of transcripts generated from the genome). Microarray technology allows for non-hypothesis driven work, but requires a major new focus in bioinformatics and statistics to understand the large quantities of data produced. A number of microarray studies have been produced in recent years for both the study of suicide and other psychopathogies. Sibille and colleagues produced the first comprehensive microarray study in the depressed suicide brain (Sibille et al., 2004). In this study, 19 depressed suicides were matched to controls and RNA extracted from two frontal cortical regions was processed

by microarray. Brain samples were from the Sylvio Conte Center, at the New York State Psychiatric Institute. After stringent microarray statistical processing, the authors found no significant differences between groups. A follow-up analysis examining only genes of the 5-HT system also yielded no results of significance. This study is of particular interest because it represents the first global analysis of gene transcription in the suicide brain; however, the statistical analysis was likely overly-stringent, accounting for the lack of significant results. With the refinement of mathematical techniques to process microarray data, different groups have since performed microarray analysis using brains from suicide completers and depressed subjects and found more promising results.

Two different studies have implicated the fibroblast growth factor (FGF) system in depression and suicide. FGF is an important molecule for cell and organ development throughout the body, including the brain (Vaccarino et al., 1999; Turner et al., 2006). Using brains collected as part of the Pritzker consortium, Evans and colleagues used frontal cortical tissue from 14 controls, 6 bipolar, and 13 MDD subjects, of which 6 were female (Evans et al., 2004). The most robust findings from their work was that two FGF receptors (FGFR2 and FGFR3) are downregulated in both DLPFC and anterior cingulated cortex specifically in MDD subjects. The FGF system is of interest to studies and depression and has received some support from independent groups. For example, microarray data derived from BA10 from Stanley subjects (15 bipolar, 15 depressed, 15 SCZ, and 15 controls donated by Stanley Foundation) yielded alterations in the FGFR1, NCAM1, and CAMK2A (Tochigi et al., 2008a). Of note, FGFR1 was not shown to be dysregulated in the Evans et al. paper (2004).

GABA- and glutamate-associated dysregulations have been the most consistent finding in microarray studies in psychiatry. Choudary and collaborators used tissue from BA9 and anterior cingulate cortex from 7 controls, 9 MDD subjects and 6 BPD to generate microarray data (Choudary et al., 2005) – these subjects appear to be identical to those subjects used in the paper by Evans et al. (2004). In subjects with MDD, this group showed that SLC1A2, SLIC1A3, and glutamine synthase (GS) were all downregulated in the sample of MDD compared to controls in both regions, while $GABA_A\alpha5$ was upregulated. The findings of SLC1A2, SLIC1A3, and GS down-regulation are of interest as they are all glia specific genes, suggesting support for a glial hypothesis of mood abnormalities (Coyle and Schwarcz, 2000). With regards to $GABA_A\alpha5$, Kim and colleagues also showed an upregulation of $GABA_A\alpha5$ in a large microarray sample (>50 microarray chips for both controls and suicdes) in frontal cortex of suicde completers with mixed phenotype and controls subjects (Kim et al., 2007a). Our own group has conducted a series of studies using microarray data generated from suicide completers and controls using brain tissues from the Quebec Suicide Brian Bank (Douglas Institute). Our data has implicated GABA and glutamate dysfunction (Klempan et al., 2007; Sequeira et al., 2007), and the same gene transcripts identified by Choudary et al (2005) as down-regulated are found in the samples we analyzed.

For both glutamate and GABA associations with psychopathology, the lack of specificity to a particular mental illness is of some concern. For example, is glutamate/GABA dysfunction consistently present in schizophrenia, MDD, and bipolar disorder? Given the

differing phenotypes, this would be surprising, although glutamate/GABA systems may be involved in different CNS systems or perhaps at the level of different receptor subunit stoichiometry for different diseases. In this perspective, subtle differences in the glutamate/GABA system, beyond analysis of up-down regulation, will need to be investigated.

Some reports have emerged indicating a possible role of spermidine/spermine N^1-acetyltransferase (SSAT) (Sequeira et al., 2006), and the neurogenesis-associated gene 14-3-3 epsilon (Yanagi et al., 2005). The observation of notable oligodendroglial misexpression in the brains of individuals with major depression (Aston et al., 2005) also appears to be evident in the context of completed suicide (Klempan et al., Submitted). This suggests that fundamental processes related to a specific cell lineage are perturbed in the suicidal brain, similar to the accumulating evidence for myelination defects in schizophrenia.

While there is a level of consistency between microarray studies that does not exist in other areas of psychiatric genetics, no gene has been consistently demonstrated across platforms, laboratories, and subjects. This might be due to the confounding factors that afflict post-mortem brain studies in psychiatry: tissue heterogeneity, phenotypic variation across subjects, and processing procedures. While this is a perpetual issue of studies of this nature, what is less clear is what the role of reduced expression of a given gene signifies, or what the implication is in the brain of altered expression. Does a 1.3 fold change in gene product affect the way the brain works? Even if these questions are

answered in the affirmative, the potential mechanism for these expression differences remains elusive. Despite these yet unanswered questions, the use of microarrays has supported previous evidence for dysregulation of some of the neurotransmitter systems discussed above (e.g. glutamate and GABA) but not of others (e.g. 5-HT). This approach has also led to the identifiation of other systems possibly involved in suicide, namely polyamines. Interestingly, neurotrophic factors have also emerged as candidate molecules in the neurobiology of suicide. Neurotrophins are well known for their roles in neuronal and plasticity, and their altered expression could underlie, at least in part, changes in plasticity observed in the brains of suicides.

9.2 Whole genome association studies

The years 2005-2009 will undoubtedly be remembered as the years of whole genome association (WGA) studies of a wide range of diseases. The basic premise of this technique is to screen at least 500K SNPs using chip-based technology and very large sample sizes. Isolation of DNA from thousands of people with disease X and matched control subjects, followed by SNP-chip DNA hybridization and bioinformatics analysis, has led to many significant findings for multiple diseases including diabetes, restless leg syndrome and others. This chip based technology leaves researchers with a small number of SNPs that are more highly represented in a disease group compared to a control group. These SNPs can be suggestive of linkage to other genetic problems (similar to initial linkage studies, an identified SNP may be linked to a disease allele) or may themselves be associated with an illness.

Psychiatric studies may also benefit from WGA studies. One major challenge of this will be ensuring all people in the disease group have clear phenotypes. For example, instead of using only a diagnosis of bipolar disorder based on DSMIV criteria, bipolar subjects may also have to be lithium responsive. This type of approach might decrease the phenotypic heterogeneity and provide more meaningful results. To date, one WGA study has been performed in bipolar disorder (Sklar et al., 2008). This study used 1461 BP subjects and found a small number of SNPs that seemed to associate with the disorder. This was not validated when the group used two independent samples of bipolar disorder subjects.

9.3 Epigenetics

After the identification and crystallization of 5-methyl-cytosine (Johnson, 1925), more reports emerged as to the presence of a methyl group on cytosine residues in DNA in the late 1940's and 1950's in vertebrate cells (Wyatt, 1951). What remained unclear about DNA methylation, however, was what exactly its function was. Work throughout the 1960's determined not the function of DNA methylation, but more of the biochemical properties. For example, it was discovered that the process occurs after DNA synthesis (Scarano et al., 1965) and that cytosine residues in a CpG sequence can be methylated (Grippo et al., 1968).

The function of methylation was suspected to be the alteration of gene expression patterns (Doerfler, 1981; Bird, 1984). That is, that genes that were heavily methylated were associated with less transcription and less methylated genes had more gene

transcription. One of the first direct attempts to test this hypothesis arrived via experiments using the gamma globin gene. In this study, a fully methylated globin gene was transferred into mouse oocytes. Examination of RNA from transformed cells revealed that methylation of the 5' region upstream of the gene led to significant decreases in gene transcription (Busslinger et al., 1983). Since then, more evidence has accumulated to support the idea that DNA methylation has a role in gene expression. In particular the role of DNA methyalion in X Chromosome inactivation (Gartler and Riggs, 1983) and Fragile X syndrome (Bell et al., 1991). Methylation aberrations are also frequently associated with tumor in multiple cancer types. Indeed, one reason for metastasis may be the aberrant gene expression of genes whose promoters lack methylation (Esteller, 2005).

Methylation of DNA and its effects on gene transcription may have a role in psychiatric disorders. The first suggestion that a methylation-related mechanism was involved in psychiatric illness was probably by Osmond and Smythies (1952), when they observed that some hallucinogens (e.g. mescaline) are methylated versions of catecholamines – major neurotransmitters in the brain. They then proposed that psychosis is due to abnormal methylation (in this case of an amine and not of DNA) of catechols in the brain. They termed this the "transmethylation hypothesis" (Osmond and Smythies, 1952).

The epigenome (the pattern of methylation throughout the genome) is programmed during development, but recent data suggests that it could be responsive to environmental cues during life. Signaling pathways activated by neuronal and environmental stimuli

could target DNA methylation to specific loci, resulting in inter-individual epigenetic alterations that would cause changes in gene expression. It is these inter-individual differences in methylation that may mediate the interaction between the genome and the environment that may predispose people to the development or persistence of a mood disorder. Recent work has implicated altered methylation patterns of a number of different genes in a psychiatric context. In an animal model of early life adversity, Michael Meaney's group has suggested that mice raised in environments with poor maternal care have increased methylation at a CpG nucleotide that forms a transcription factor binding site in the glucocorticoid receptor gene (Weaver et al., 2004). These data suggest that early life events can alter methylation status and affect gene transcription of an important gene in stress reactivity. A number of different genes have also been investigated in humans in relation to psychiatric illness. The first gene to be implicated in the pathology of schizophrenia related to DNA methylation was Reelin. In an initial study of Reelin in schizophrenia, increased methylation at the Reelin promoter in the occipital lobe of schizophrenia patient seemed to associate with Reelin gene expression in frontal cortex (Grayson et al., 2005). The validity of this study has recently been questioned, however (Tochigi et al., 2008b). Epigenetic analysis in schizophrenia studies have also been performed for Sox10 and COMT (Iwamoto et al., 2005; Abdolmaleky et al., 2006), and rRNA (McGowan et al., 2008) and $GABA_A$ (Poulter et al., 2008) in the brains of suicide completers. The current flux of papers relating DNA methylation to gene expression in relation to psychopathology will need to be evaluated in future years to see if any data can be independently replicated.

As with other analysis methods (genetic polymorphisms, quantitative-PCR), methylation techniques can also be performed in a high-throughput fashion. A recent paper assessing methylation effects in psychosis highlights how this approach may work for an analysis of DNA extracted from brain tissue (Mill et al., 2008). After immuno-precipation of fragmented, methylated DNA, DNA is labelled and hybridized to a chip with un-methylated DNA from the same subject. Currently, commercially available chips have probes for CpG islands or promoter regions present throughout the genome. This allows for screening of methylation of thousands of different regions and the potential identification of methylation differences between a psychiatric group and a control group.

Other epigenetic studies involve the analysis of the methylation, acetylation, or phosphorylation state of histone molecules, though most studies have centered on the methylation of histone residues. Similar to methylation of DNA, methylation of histones is a possible mechanism for decreased gene expression (Weisbrod, 1982; Richards and Elgin, 2002; Shilatifard, 2006). The first analysis of methylation state of a histone residue was done comparing schizophrenia subjects to control subject using chromatin extracted from frontal cortex (Akbarian et al., 2005). While these authors examined phosphorylation, acetylation and methylation at different histone residues they found a significant association of increased methylation in a subgroup of schizophrenia subjects at arginine 17 on histone 3 related to decreased expression of four metabolic genes. Some other work has been done on enzymes that add methyl groups to histone complexes; however, these results are preliminary and need replication (Huang et al., 2007; Sharma et al., 2008). Anti-depressant drug action may also affect histone

methylation. Nestler and colleagues showed that promoter IV of the BDNF gene is associated with increased methylation at lysine 27 at histone 3 and chronic defeat stress in rats.

Epigenetic analyses in psychopathology offer an explanation for the difficulty geneticist have experienced trying to link genetic polymorphisms to disease. Epigenetic effects alter gene expression without altering the genome and there is some evidence that environmental factors can influence methylation status. More work is required in this area to clarify the role of epigenetic modification to DNA or histones and the relation ship to psychopathology.

10. Rationale and objectives

It is clear from the literature review that most areas studied in molecular suicide research show contradictory findings. Some groups suggest a change in gene X, while other groups fail to replicate the findings. More concerning is the potential number of negative results studies in these same areas that may never have been published (Ioannidis, 2005).

Suicide is a complex phenotype that requires a more complex and thorough analysis than what has been previously used. For my work, I want to move beyond candidate gene approaches, association studies, and binding studies. Yet, is there a way to address the heterogeneity of the suicide phenotype in a non-biased way?

The rationale for this thesis is to assess extreme variation in global gene expression on a subject-specific basis in brains from suicide completers and control subjects.

Objectives:

1. To design a valid analysis method to explore gene expression data by individual instead of by group.
2. To use this method to determine if any expression differences exist in the brains of suicide completers as compared to control subjects.
3. To understand the underlying causes of any expression differences observed.

CHAPTER 2: PREFACE

Through 2003-2005, brain sections from suicide completers and controls were dissected and sent to a company for gene expression profiling. These data form the McGill Group for Suicide Studies Gene Expression database, and it is this information that forms the foundation of the following study. An initial exploration of the data had already been done in frontal cortex (Sequeira et al., 2006) using traditonal microarray analysis techniques and dividing suicide completers by diagnostic criteria. For my purposes, I wanted to analyze the data somewhat differently. First, to increase power, I studied only the suicide phenotype so that I did not need to sub-divide the suicide completers by psychiatric diagnosis. Second, I wanted to screen the data using our newly developed technique that assessed microarray data for extreme expression signatures (Appendix 1).

The following study will describe how this idea led to our investigation of decreased expression levels of a TrkB variant in the brains of suicide completers. I will refer the reader to Appendix 1 where I lay out the foundation for the outlier-detection approach in a broad way, describe the technique mathematically, and use the technique in a real data set to demonstrate how it works.

This study also investigated the TrkB promoter methylation state. Prior to performing the experiments described in Study 1, we performed a number of technical experiments to ensure that DNA could be analyzed for methylation state from post-mortem human brain. For these studies, please see Appendix 2.

Alternative splicing, methylation state, and expression profile of TrkB in frontal cortex of suicide completers

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Abstract

Context: While most of the effort to understand the neurobiology of depressive states and suicide has focused on neuronal processes, recent studies suggest that astroglial dysfunction may play an important role. TrkB.T1 is expressed in astrocytes and BDNF/TrkB signaling has been linked to mood disorders.

Objective: We tested the hypothesis that TrkB.T1 expression is downregulated in suicide completers and that this downregulation is mediated by an epigenetic process.

Design: Post-mortem case control study

Main outcome measures: Thirty-nine French-Canadian males were screened using the HG-U133 plus 2 microarray chip. Nine frontal cortical regions and the cerebellum were assessed using a microarray screening approach for extreme expression differences across subjects and a conventional screening approach. Results were validated by quantitative polymerase chain reaction and Western Blot. Animal experiments were performed to control for drug and alcohol effects. Genetic and epigenetic studies were carried out by direct sequencing and bisulfite mapping.

Results: We found that 35% of suicide completers demonstrated significant decreases in different probe sets specific to TrkB.T1 in BA 8/9. These findings were generalizable to other frontal regions, but not to the cerebellum. The decrease in TrkB was specific to the T1 splice variant. Our results were not accounted for by substance comorbidity or by reduction in astrocyte number. We found no effect of genetic variation in a 2500 base pair promoter region or at relevant splice junctions; however, we detected an effect of methylation state at particular CpG dinucleotides on TrkB.T1 expression.

Conclusion: A reduction of TrkB.T1 in frontal cortex of a subpopulation of suicide completers is associated with the methylation state of the promoter region.

Introduction

Suicide is most often associated with depressed mood and hopelessness and is a leading cause of death in many regions of the world(Murray and Lopez, 1997; Mann, 1998; Boelle and Flahault, 1999; Okasha, 1999; Phillips et al., 2002; Anderson et al., 2006). While much of the effort to understand the biology of these mood states has centered on neuronal processes, some recent studies have suggested that astroglial dysfunction may play an important role(Coyle and Schwarcz, 2000; Cotter et al., 2001a; Czeh et al., 2006; Hasler et al., 2007). Astrocytes, the most prevalent cell type in the central nervous system, are involved in synaptic communication(Araque et al., 1999), express serotonergic, GABAergic, glutamatergic, dopaminergic, as well as many other receptor types(Porter and McCarthy, 1997), and are capable of rapid, long range signaling throughout the brain(Scemes and Giaume, 2006). The role of astrocytes in brain homeostasis, metabolism, and injury has been extensively documented(Kettenman, 2005). More recently it has become clear that astrocytes play an important role in many brain functions(Wilkin et al., 1990), and may be involved in mood and other neurological disorders(Seifert et al., 2006).

TrkB.T1 is the only TrkB isoform expressed in astrocytes under normal conditions and BDNF/TrkB signaling has been linked to major depression and suicide(Rudge et al., 1994; Rose et al., 2003; Duman and Monteggia, 2006; Kim et al., 2007b). TrkB.T1, a truncated form of the TrkB receptor incapable of catalytic activity(Frisen et al., 1993; Condorelli et al., 1994), is known to mediate BDNF-induced calcium signaling through astrocyte networks(Rose et al., 2003). Importantly, TrkB.T1 is upregulated in cultured

astrocytes in response to antidepressant treatment(Mercier et al., 2004), and a number of microarray studies have linked decreased TrkB gene expression in human cortex to mood disorders(Aston et al., 2005; Nakatani et al., 2006), although the specific TrkB variant identified in these studies was not presented.

In this study, we screened microarray data for extreme expression values and found severely decreased expression of TrkB.T1 in 35% of suicide cases. When we analyzed microarray data following more conventional methodology, we still found TrkB.T1 to be significantly down-regulated in the suicide group. Our results were present in the frontal cortex and were specific to the T1 variant as we could detect no difference in expression level in the cerebellum or in other TrkB isoforms. We attempted to explain the mechanism of this finding by first analyzing the TrkB promoter region for genetic variation, and secondly, for methylation patterns. We found a significant association between methylation at two specific CpG dinucleotides in the promoter region and decreased TrkB.T1 expression in the frontal cortex of suicide completers.

Methods

Brain tissue used in this study was obtained from the Quebec Suicide Brain Bank, and selected for limited post-mortem intervals (not exceeding 48 hours (Tomita et al., 2004; Vawter et al., 2006)). Subjects were French-Canadian in origin, considered members of a homogeneous population with a well-identified founder effect(Labuda et al., 1996). All individuals were male, and groups were matched for age, post-mortem interval (PMI), and pH. All subjects died suddenly and could not have undergone any resuscitation procedures or other type of medical intervention, thus did not have prolonged agonal states. Twenty-eight suicide completers and eleven controls were recruited for this study. Brodmann areas 4, 6, 10, 11, 44, 45, 46, 47, and the region encompassing BA 8 and 9 (BA 8/9) from the left hemisphere (gray matter only), and cerebellum were carefully dissected. Sectioning was performed at 4^{o}C and snap frozen in isopentene at -80^{o}C; we note that different numbers of sections were taken per Brodmann area, therefore sections used for microarray analysis were adjacent but different to those used for biological replication by semi-quantitative PCR, Western blot, and epigenetic analysis.

For identification and dissection of neuroanatomical regions, we relied on experienced histopathologists using reference neuroanatomical maps(Haines, 2000; Nolte, 2002). Briefly, we used gyri and sulci to landmark specific frontal cortical areas and removed gray matter tissue blocks. In all cases, 1cm^3 human tissue blocks were paraffin-embedded, cryostat-sectioned, slide-mounted, and examined for any signs of disease by

two independent pathologists in at least 3 different brain regions. All sections were cryostat-cut at 15 □m. Brain tissue was assessed for any obvious signs of pathology.

We used strict inclusion criteria to ensure a sample as homogenous as possible. Inclusion criteria for suicide completers included: 1) subject must be Caucasian and of French Canadian origin. This was assessed systematically by verifying that both sets of grandparents of the subject were born in the province of Quebec. 2) Male sex, 3) Post mortem interval under 48 hours (Tomita et al., 2004; Vawter et al., 2006). Exclusion criterion for suicide completers was the presence of schizophrenia and other psychotic disorders at any point in the life of the subject. Inclusion criteria for control subjects were the same as suicide completers for criterion 1, 2, and 3. Further inclusion criteria were: Sudden death without a prolonged agonal state. Medical records, family reports, and interviews with the best possible informant were screened to ensure that the subject was not in a prolonged agonal state. Most control subjects died in accidents or by cardiac arrest. Case reports for each subject were prepared by way of psychological autopsy carried out by trained clinicians and medical doctors with the best possible informants, as reported elsewhere (Dumais et al., 2005). Psychological autopsies were completed with medical charts and a consensus diagnosis was reached by a panel of clinicians. Relevant clinical information for all subjects can be found in Table 1.

We propose a different approach to screen microarray data, one that accounts for the likely heterogeneous nature of psychiatric diseases. The method, termed extreme values analysis (EVA), functions to screen microarray data individual-by-individual in search

for any extreme values that may signify some possible abnormality. Given the lack of replication in psychiatric genetic research(Ebmeier et al., 2006), it seems plausible that an investigative approach focusing on subgroup rather than on mean group effects is reasonable. In psychiatric research an approach that subdivides the experimental sample has been applied (Akbarian et al., 2005) and indeed, several promising findings in schizophrenia (Millar et al., 2005), autism (Sebat et al., 2007), and mental retardation(Friedman et al., 2006) have all been identified through genome wide screening on a subject-by-subject basis. The rationale for this approach is the fact that etiologic heterogeneity is likely to occur in psychiatric conditions, including suicide(Weissman, 2002). Identification of extreme values or 'outlier detection' has been used successfully with microarray data, and the approach put forth here (EVA) is a slight modification on previously applied methods (Lyons-Weiler et al., 2004; Tomlins et al., 2005; Tibshirani and Hastie, 2007). Specifically, EVA uses the standard deviation and fold-change from the mean whereas the other methods use percentile rankings. Briefly, all methods normalize microarray data and examine individual subject scores outside of a particular range, compared across experimental and control groups. The EVA protocol is fully described in the supplemental section.

Methods for microarray analysis, Quantitative PCR (QPCR) western blot, DNA sequencing and methylation mapping of the TrkB promoter, and luciferase experiments for promoter function can all be found in the supplemental section (all primers can be found in table S1). Full protocols for animal behavior experiments to control for drug and alcohol effects can also be found in the supplemental section.

Microarray analyses were carried out using AVADIS (Strand Life Sciences, Carlsbad, USA). All other statistical comparisons were done using SPSS v15.0 (SPSS, Chicago, USA). All data are reported as mean $\pm$ standard error of the mean (SEM).

Results

Figure 1 demonstrates a flow chart outlining the experimental design of this study.

BA 8/9: Extreme Values Analysis (EVA)

We initially focused on the dorsolateral prefrontal cortex, Brodmann area (BA) 8/9, because this brain region is thought to play an important role in the neurobiology of suicide(Drevets et al., 1997; Cotter et al., 2002; Dwivedi et al., 2003). Data was log-transformed and the standard deviation (SD) for each probe set was ranked according to SD value and the top 1000 probe sets were kept. Following this first step, we proceeded to screen all remaining probe sets to find at least one suicide subject that deviated three-fold from the control mean. 287 probe sets had at least one suicide subject that had an intensity value that was three-fold different from the control mean. To account for variation in the control means, we removed any probe sets where the intensity value from the suicide subject that was three fold from the control mean was not also outside of 1.5 SD's from the control mean; 99 probe sets had at least one suicide subject that had, as defined here, an extreme expression value (Supplemental table 2).

Two of these 99 probe sets were specific to the TrkB gene (T1 variant: 221796_at and 221795_at; Figure 2A). We chose to further explore TrkB because: A) there were two different TrkB probe sets that were included in the final filtered list, suggestive of internal replication, B) there was a large number of subjects with extreme expression values for both TrkB probe sets, all of whom were the same for both TrkB probe sets and C) TrkB has been related to suicidal behavior before(Dwivedi et al., 2003). The results

observed for TrkB using the extreme value analysis were also significant when standard microarray analysis methods were used (Supplemental figure 1). We note that an analysis of BDNF expression level between groups did not reveal any statistically significant findings (supplemental table S3).

Analysis of TrkB probe sets in Affymetrix chips

There are 6 probe sets specific to TrkB on the HG-U133 chips. Using the Ensembl genome browser program (www.ensembl.org) and a recent paper describing the genomic organization of TrkB(Stoilov et al., 2002), we found that 214680_at, 221795_at, and 221796_at, two of which are the probe sets we observed as differentially expressed following the EVA, are specific to exon 16, while 236095_at was specific to exon 19 and 207152_at and 229463_at are specific to exon 24. Each of these exons is specific to a different splice variant of TrkB. Exon 16 is particular only to TrkB.T1, while exon 19 and exon 24 are specific to TrkB.T2 and full length TrkB (TrkB.FL), respectively (Stoilov et al., 2002).

We re-performed our microarray analysis with subjects that had previously undergone analysis using the HG-U133 A,B chip set to further assess the reliability of our findings (Supplemental material). This analysis supported our initial findings.

Strong correlation within TrkB.T1 probe sets across subjects

Given that 3 different probe sets are designed to interrogate TrkB.T1, we asked how well the 3 probe sets correlated with each other across all subjects. The Pearson correlation

values between the TrkB.T1 probe sets were excellent, suggesting a high degree of consistency as that expected for probe sets measuring the same gene transcript: 221796_at and 221795_at, r =0.901; p<0.001; 221795 and 214680, r = 0.914, p<0.001; 214680_at and 221796_at, r = 0.867 p<0.001. We note that the 214680_at probe set was narrowly filtered out at one of the early stages of the EVA.

Semi-quantitative PCR and Western analysis of TrkB.T1 low expressors and matched controls.

The 10 suicide completers who were identified in the EVA screen as being low expressors of TrkB.T1 were matched to 10 controls for age, PMI, and pH (Table 2). We performed both semi-quantitative PCR (Figure 2B) and western blot (Figure 2C) analysis to validate and further investigate at the protein level the results from the microarray analysis.

To confirm the validity of the TrkB.T1 microarray results, we designed primers to probe set binding sites specific to exon 16 of TrkB for semi-quantitative PCR analysis. We used mRNA extracted from independent BA 8/9 brain samples, which were from tissue adjacent to the initial section used for the microarray experiment. There was a significant difference in the levels of mRNA between the TrkB.T1 low expressors and matched controls (Figure 2B; t_{18}=3.47, p=0.003). To ensure the reliability of this finding, we redesigned primers that bound to both exon 15 and exon 16, guaranteeing non-contamination of genomic DNA (t_{18}=4.88, p=0.001). We then investigated whether

TrkB.T1 protein was similarly reduced using western blots and found a significant reduction in the protein levels of TrkB.T1 (Figure 2C; t_{18}=4.98, p=0.001).

There was excellent predictive value between QPCR, Western, and microarray experiments. (QPCR:Western (n=20), r = 0.79; QPCR: Microarray (N=19), r = 0.64; Western, Microarray (N=19), r = 0.44).

Analysis across eight other frontal cortical regions and the cerebellum

We screened eight other frontal cortical regions to determine A) If the TrkB.T1 low expressors from the BA 8/9 analysis also show reduced expression in neighboring regions with regard to the TrkB.T1 probe sets, and B) Whether low expression of TrkB.T1 was specific to the frontal cortex. For this second analysis, we performed microarray analysis using RNA extracted from the cerebellum.

There were 10 TrkB.T1 low expressor suicides. To understand whether these TrkB.T1 low expressors from BA 8/9 were also TrkB.T1 low expressors in other brain regions, we compared all of these subjects to controls across eight frontal cortical regions and the cerebellum (Figure 3A). We note that there was some variability in the number of subjects across regions because of sample and microarray quality control parameters. N values for subjects per group in each region are indicated in Figure 3. We observed a highly significant difference in the expression level of TrkB.T1 across all frontal cortical regions in these subjects, but not in the cerebellum. All TrkB.T1 probe sets were

significant across regions in the frontal cortex and not the cerebellum, while neither TrkB.FL nor TrkB.T2 were ever significant in any region (Figure 3A-3D).

To validate the microarray results in frontal areas other than BA 8/9, we extracted new tissue from three frontal cortical regions (BA 11, BA 44, and BA 45) for each subject from the TrkB.T1 low expressors and matched controls (N=20 subjects) and conducted semi-quantitative PCR experiments. This tissue was independent from the tissue used in the microarray experiments. The p-values from t-tests between suicide and control subjects in each region were less than 0.01. In all frontal regions, the TrkB.T1 low expressors showed reduced expression of TrkB.T1 mRNA compared to control subjects.

Control experiments: PMI, pH, age, and toxicology

Effects of pH, PMI, and age for all subjects across all experiments are shown in Table 2. To further understand the role of these variables on TrkB.T1 expression we performed correlation analyses using semi-quantitative PCR TrkB.T1 data from the TrkB.T1 low expressors (N=10) and the matched controls (N=10) from BA 8/9. Our main question was: Does PMI, pH, or age predict levels of TrkB.T1, irrespective of group membership? We found no significant correlations between any of these variables and the level of expression of TrkB.T1 (pH: r =-.23, p=0.33; PMI: r=-0.07, p=0.78; Age: r = 0.23, p=0.32). There was also no significant correlation between PMI and protein level (r=-0.18, p=0.45). All analyses in this study were re-computed using age, pH, and PMI as covariates (supplemental table S4).

Very few subjects had positive toxicology for psychotropics (Table 1). While certain psychotropic medications are known to alter the expression of BDNF in the brain of laboratory animals(Hashimoto et al., 2002; Yasuda et al., 2007), their effect on levels of TrkB is less clear(Linden et al., 2000). There were three subjects in this study who had positive toxicological evidence of psychotropic medications, none of whom were TrkB.T1 low expressors.

We next assessed the effects of lifetime history of alcohol abuse/dependence and history of cocaine use on the expression level of TrkB.T1 in BA 8/9. There were three control subjects and 3 TrkB.T1 low expressors with a history of alcohol abuse/dependence and 2 TrkB.T1 low expressors with a history of cocaine abuse (Table 1). We grouped semi-quantitative PCR data from the 6 subjects with a history of alcohol abuse/dependence and compared them to all other subjects (no history of alcohol). A history of alcohol abuse/dependence did not significantly affect the expression level of TrkB.T1 ($t_{(18)}$ = 0.68, p= 0.50). A history of cocaine abuse also did not affect levels of trkB.T1 ($t_{(18)}$ = 0.83, p = 0.42). To experimentally rule out the possibility that our results are a consequence of comorbidity with alcohol or cocaine use, we modeled both cocaine and chronic and acute alcohol abuse in rats to further clarify their role on TrkB.T1 expression (supplemental material)

Astrocyte control experiments

TrkB.T1 is the TrkB variant expressed in astrocytes in the adult brain(Rose et al., 2003; Ohira et al., 2005b) To determine if the effect we observed was a result of a reduction in

the number of astrocytes(Rajkowska, 2000; Si et al., 2004), we analyzed the mean of glial fibrillary acid protein (GFAP; probe set 203540_at) between groups using microarray data and QPCR. GFAP is the most commonly used molecular marker of astrocytes in the brain; we could not detect a difference in the expression level of GFAP between sudden death controls and suicide completers with low levels of expression of TrkB.T1. For all 3 probe sets specific to TrkB.T1, the ratio of TrkB.T1/GFAP was always at least two-fold lower in the low-TrkB.T1 suicide subgroup compared to the control group. Finally, we performed QPCR experiments to confirm that there was no significant difference in the levels of GFAP between groups ($t_{(18)=}1.1$, p=0.27).

Promoter sequencing

We next asked whether any genetic variation in particular regions of this gene was associated with a reduction in expression of TrkB.T1. Obvious sites of genetic variation that could affect expression level were the promoter region and the splice junctions surrounding exon 16 (Figure 4A)(Barettino et al., 1999; Stoilov et al., 2002). We defined the promoter region similar to that proposed in mouse(Martens et al., 2007b). We then confirmed that this region has promoter activity in humans (supplemental material). We found no association between any promoter variants and TrkB.T1 expression level (supplemental material)

Assessment of methylation state of the TrkB promoter in BA 8/9 and cerebellum

We next asked whether epigenetic differences might account for the expression differences observed in the TrkB gene. We reasoned that this could be a possible

mechanism, despite there being two other unchanged transcripts potentially from the same promoter region, given that TrkB.T1 is the only TrkB isoform expressed in astrocytes under normal conditions. In order to control for assay variability and other stochastic effects leading to erroneous conclusions, we randomized important variables in every experimental step (sodium bisulfite treatment, cloning, and sequencing were done blind to group status and in pairs).

Technical issues involving the conversion of cytosine to uracil using bisulphite treatment has been the subject of some debate(Grayson et al., 2005; Tochigi et al., 2007). Amplification bias of bisulfite-converted products is also a potential confound of methylation mapping(Warnecke et al., 1997). Control experiments related to these technical issues can be found in the supplemental section.

We cloned a 440 bp sequence from the 10 TrkB.T1 low expressors and 10 matched controls used throughout this study in BA 8/9 (all raw data in supplemental figures S4 and S5). We found a significant difference ($t_{(18)}$ =2.60, p=0.018) in mean methylation level per clone when we compared suicide completers (1.72 methylated sites/clone + 0.1) and control subjects (1.17 methylated sites/clone + 0.18). Most methylation was reserved to two CpG dinucleotides, site 2 and site 5 (Figures 4, 5, S4, S5). We therefore generated methylation frequencies for each subject at both Site 2 and Site 5 (e.g. number of methylated cytosines at Site 2/total clones). At Site 2, a t-test analysis revealed a marginally non-significant p-value of 0.074 ($t_{(18)}$ =1.90). At Site 5, the t-test p-value was 0.028 ($t_{(18)}$=2.40). To determine if there was an association between methylation and

expression level, we $\log_2$ transformed microarray data and performed correlations between expression level of TrkB.T1 (221795_at) and methylation frequency across all subjects (Figure 6). The Pearson correlation was significant for both Site 2 ($r= -0.563$, $p=0.010$) and Site 5 ($r= -0.615$, $p=0.004$). We also investigated the Pearson value when only suicide or control subjects were examined (supplemental figure 6).

To demonstrate the specificity of this finding we also analyzed methylation patterns in CpG's 2 and 5 from the TrkB promoter using DNA extracted from the cerebellum (Figure 7). We found a paucity of methylation in DNA extracted from the cerebellum from all subjects.

Six of ten subjects with low expression of TrkB.T1 had MDD, which could suggest that the relationship observed between TrkB.T1 expression level and methylation state is due to the presence of MDD. To fully rule out this possibility, we selected 5 MDD subjects from the Suicide group who did not previously undergo epigenetic analysis. We isolated DNA from BA 8/9 of the frontal cortex of these subjects, bisulfite treated the DNA, and cloned the PCR product (10 clones/subject). Of these five subjects, 1 subject had two clones methylated at CpG site 5 and 1 other subject had 1 clone methylated at site 5. Therefore, there was very little methylation at sites 2 and 5 from these suicide subjects with MDD and high expression of TrkB.T1.

Comment

This study has demonstrated, in a subset of suicide completers, that TrkB.T1 is significantly down-regulated in frontal cortex compared to control subjects and that this down-regulation is associated with methylation at specific CpG dinucleotides proximal to the coding region.

TrkB.T1 is the only TrkB isoform expressed in astrocytes and binds BDNF, an important neurotrophic factor in the brain(Rudge et al., 1994). The initial reports that characterized TrkB.T1 investigated its *in situ* hybridization pattern and reported that TrkB.T1 was expressed in non-overlapping patterns with the full length TrkB isoform(Klein et al., 1990; Beck et al., 1993), where the full length TrkB isoform was shown to be expressed in neurons and the TrkB.T1 isoform mostly in glia and to a lesser extent in ependymal and choroid plexus cells. Importantly, the expression of TrkB.T2 was shown to overlap with TrkB.FL and be restricted to neuronal cells, a pattern also confirmed by independent studies(Wetmore and Olson, 1995). To our knowledge, there are no published reports suggesting that TrkB.T1 is expressed in either microglia or oligodendrocyte, which suggests that the glial cells where TrkB.T1 is expressed are astrocytes, an idea confirmed by many groups(Condorelli et al., 1994; Rudge et al., 1994; Ohira et al., 2007). A more recent report using more refined anatomical techniques confirms that TrkB.T1 expression is essentially localized to glial cells(Silhol et al., 2005). We note that after brain lesions (chronic brain injury), reactive astrocytes can express TrkB.FL(McKeon et al., 1997) and that there is one report of TrkB.T1 expression in neurons in one layer of monkey cortex(Ohira et al., 2005a).

We found that TrkB.T1 expression was associated with more methylation at two particular sites in the TrkB promoter. While we made every effort to control for confounders such as PMI, pH, age, toxicology, and presence of major depression, we cannot fully rule out the potential effect of other factors such as anti-depressant medications stoped in the past and not detected in the toxicological exam, other stress factors, or as-of-yet unknown enviornmental factors.

We observed an association between methylation state and decreased expression of TrkB.T1, yet two other variants that may be transcribed from the same promoter region were unaffected. First, it could be that the promoter region examined in this study is specific to the T1 variant of TrkB. One study of the TrkB promoter region in mouse identified two different promoters, although both seemed to promote transcription equally for all transcripts(Barettino et al., 1999). Second, it could be that this methylation pattern occurs only in astrocytes, where only TrkB.T1 is expressed(Rudge et al., 1994; Silhol et al., 2005). This would suggest that the cellular machinery targeting methylation to this region is astrocyte-specific. Methylation events that occur in a cell-type specific way are known in assorted tissue types(Boquest et al., 2007; Yamasaki-Ishizaki et al., 2007). As astrocytes express exclusively TrkB.T1, the methylation of this promoter region would affect only this isoform of TrkB. Third, it could be that the methylation pattern is present in most cells of the affected brain, but that the regulation of the promoter region in astrocytes is different than that of neurons or other cells in the brain.

Sub-stratifying subjects from psychiatric post-mortem samples based on low expression level of one marker could be seen as an invalid experimental design. In this study, we used an *a priori* method to detect extreme values in suicide subjects, controlling for variation in control subjects. This non-biased mathematical algorithm is similar to other methods used to detect extreme values(Lyons-Weiler et al., 2004; Tomlins et al., 2005) in microarray data. We note also, that even when mean group effects were analyzed for TrkB.T1 expression level, there was a significant difference when suicide subjects were compared to control subjects. Individual markers may be important for some diseases and irrelevant for others. For instance, in the case of Alzheimer's and ALS, a single marker is enough to define a subset of affected subjects(Poirier, 1999; Valdmanis et al., 2008). Given that psychiatric disorders are widely believed to be etiologically heterogeneous, a fact often credited for frequent lack of replication in biological psychiatric studies, it seems that at least attempting this approach is valid. In this regard, promising findings based on subgroup or individual effects were recently published about autism(Sebat et al., 2007) and schizophrenia(Walsh et al., 2008).

Suicide is a heterogeneous phenomenon that is almost always associated to psychopathology, particularly mood disorders, substance disorders and schizophrenia(Arsenault-Lapierre et al., 2004) Many of these factors are comorbid (Kim et al., 2003). Studies investigating the neurobiology of suicide suggest that individuals who die by suicide have a certain individual predisposition that is independent from that associated with psychopathology(Brent et al., 1996; Turecki, 2005). The heterogeneity

associated with suicide leads to difficulties in experimental design, particularly if group mean effects are assessed. We have proposed an analysis method that attempts to study the complicated phenotype more on an individual basis, where individual data points are assessed.

Dwivedi et al.(Dwivedi et al., 2003) have shown in a similar study that the TrkB.FL isoform is decreased in BA 9 of suicide completers. There are a number of possible reasons for the discrepant findings between the current study and the Dwivedi et al. study. Using semi-QPCR analysis for TrkB and BDNF, Dwivedi et al. reported a strong down-regulation in both TrkB and BDNF in suicide completers and identified corresponding changes at the protein level, although it is unclear which truncated form of TrkB their antibody deteceted(Gestwa et al., 1999). The forward primer used by Dwivedi et al. for TrkB semi-QPCR analysis, according to the published structure of the TrkB gene(Stoilov et al., 2002), is specific to exon 10 of TrkB and their reverse primer is specific to exon 12. Both of these exons are present in all TrkB isoforms.

TrkB.T1 has no cytoplasmic catalytic activity, and it may therefore act by sequestering BDNF, thus competing for this ligand with neuronal, full-length TrkB and decreasing TrkB 2nd messenger activation. However, recent data suggests that TrkB.T1 plays a role beyond passive competition with the full length TrkB receptor. This truncated form of the TrkB receptor has been convincingly shown to mediate neurotrophin-evoked calcium signaling in astrocytes(Rose et al., 2003). While glutamate and ATP are both known to induce calcium transients in astrocytes(Haydon, 2001), BDNF is the most potent

endogenous agonist for this process, required only at nanomolar concentrations(Rose et al., 2003). Thus it is feasible that reduction in TrkB.T1 in the frontal cortex may affect calcium signaling in astrocytes.

Figures

1. **Flow chart of study stages.**

2. **Reduced expression of TrkB.T1 in BA 8/9 of suicide completers.** A) HG-U133 plus 2 microarray results from the Extreme Values Analysis (EVA). Each probe set is represented by 2 groups: Normal control subjects (Control; N=9) and the subgroup of suicide subjects (Suicide_ext) who passed EVA filtering (N=10). TrkB.FL probe set order is: 207152_at and 229463_at.TrkB.T1 probe set order as listed on graph are: 214680_at, 221795_at, 221796_at. B) Semi-QPCR analysis of the 10 suicide subjects who passed EVA filtering (TrkB.T1 low expressors) and 10 matched controls. C) Western analysis of the 10 suicide subjects who passed EVA filtering (TrkB.T1 low expressors) and 10 matched controls. D) Example of QPCR and Western gels used to generate data. C=control subject; S=Suicide TrkB.T1 low expressor.

3. **Reduced expression of TrkB.T1 in 8 frontal cortical regions but not in cerebellum.** A) Global expression of TrkB.T1 across frontal cortex and cerebellum (probe set 221795_at). N for the TrkB.T1 low expressors (T1) and control subjects across all the regions was (Cerebellum: T1=9,C=11; BA 4: T1=7, C=8; BA 6: T1=5,C=7; BA 10: T1=4, C=6; BA 11: T1=6, C=7; BA 44=T1=9, C=11; BA 45: T1=8, C=11; BA 46: T1= 6, C=7; BA 47: T1=8, C=9). TrkB.T1 probe set order as listed on graph are: 214680_at, 221795_at, 221796_at. B) All TrkB probe sets from BA 11 from all TrkB.T1 low expressors (Suicide_ext) and controls that passed microarray quality control. Note that probe sets 229643_at failed quality control in BA 11. C) Signal intensity of all TrkB probe sets in BA 44. D) Signal intensity of all TrkB probe sets in BA 45.

4. **Structure of the TrkB gene and promoter region**. A) Exon/intron view of TrkB gene. Vertical lines represent exons, while horizontal line represents intronic regions. Break in intronic region is a 250 Kb space. Down arrows represent exons whose presence in a transcript defines major TrkB isoforms (all have stop codons). Exon 16 (arrow 1), exon 19 (arrow 2), and exon 24 (arrow 3) are specific to TrkB.T1, TrkB.T2, and TrkB.FL, respectively. B) A portion of the TrkB promoter. Numbers in superscript represent CpG nucleotides referenced in the text. Underlined segments are the primer binding sites for primers specific for bisulfite treated DNA from BA 8/9. Numbers in subscript indicate the nucleotide position number (accession number AF410902).

5. **Methylation mapping of the TrkB promoter region**. A) Example of a sequencing result from bisulphite treated DNA (Site 2). B) Example of a sequencing result from bisulfite treated DNA (Site 5). C) Representative data from one suicide subject with low expression of TrkB.T1. Each circle represents a CpG position in DNA. Dark circles represent those CpG sites that are methylated. Selected CpG sites are numbered and each line represents one clone. Clones from frontal cortex (BA 8/9) are shown. D) Representative methylation results from one Control subject.

6. **Correlation analysis of TrkB.T1 expression and methylation frequencty at CpG 2 or CpG 5 of TrkB.T1 promoter**

7. **Analysis of methylation state of CpG sites 2 and 5 in DNA extracted from cerebellum.** Ten subjects are represented in the suicide and control groups and each subject comprises 10 clones (rows), Each subject consists of 2 colums which represent the methylation state of sites 2 and 5 from TrkB promoter. Filled-in circles represent methylation whereas white circles demonstrate lack of methylation.

Tables

1. **Relevant clinical information (N=39).** C=control subject; S_ext=Suicide subject extreme expressor for TrkB.T1; S=suicide subject. Toxicology refers to substances detected in a toxicological screen at time-of-death.. MDD: Major depressive disorder; BPD: Bipolar disorder; OCD: Obsessive Compulsive disorder.
2. **Age, pH, and PMI summary for all comparisons.** MA = microarray experiment

Group	Cause of death	DSM-IV Diagnosis	Toxicology	PMI	pH	Age
C	CAR ACCIDENT	Alcohol dependence	Alcohol	15	6.83	51
C	MOTOR VEHICLE ACCIDENT			29.5	6.67	31
C	CARDIAC ARREST			20.5	6.55	27
C	MYOCARDIAL INFARCTION			19.5	6.42	46
C	MOTOR VEHICLE ACCIDENT			27	6.32	28
C	MOTOR VEHICLE ACCIDENT			24	6.0	41
C	CARDIAC ARREST			24	6.42	21
C	CARDIAC ARREST	Alcohol dependence		24	6.67	31
C	CARDIAC ARREST	Alcohol abuse		12	6.49	47
C	CARDIAC ARREST			31	6.34	58
C	CAR ACCIDENT			24	6.75	55
S_ext	ASPHYXIATION	OCD, Alcohol dependence		21	6.59	21
S_ext	SUICIDE BY HANGING			18	6.68	33
S_ext	SUICIDE BY HANGING	MDD	Cocaine	34	6.0	26
S_ext	SUICIDE BY HANGING	MDD, alcohol dependence		31	6.61	35
S_ext	SUICIDE BY HANGING	MDD, alcohol dependence		29	6.3	53
S_ext	HANGING	MDD		29.5	6.17	19
S_ext	SELF INFLICTED GUN SHOT			36	6.66	36
S_ext	HANGING	MDD, Alcohol abuse		32	6.57	49
S_ext	HANGING			27	6.77	29
S_ext	OVERDOSE	BPD I, alcohol	Cocaine	30	6.68	24

		dependence				
S	SUICIDE BY HANGING			32.5	6.27	31
S	SUICIDE BY HANGING	alcohol dependence, cocaine dependence	Alcohol	23	6.0	38
S	SUICIDE BY HANGING			25	6.54	36
S	SELF-INFLICTED GUNSHOT WOUND	MDD, pathological gambling		20.5	6.57	45
S	SUICIDE BY HANGING	MDD		20	6.71	24
S	SUICIDE BY HANGING	MDD, alcohol dependence		11.5	6.35	22
S	SUICIDE BY HANGING	MDD, alcohol dependence	Alcohol	20	6.84	28
S	SUICIDE BY HANGING	MDD, panic w/o agarophobia		22	6.96	40
S	SUICIDE BY CARBON MONOXIDE POISONING	MDD		30	6.6	53
S	SELF INFLICTED GUN SHOT	Alcohol dependence		21	6.12	51
S	HANGING	MDD		25.5	6.6	39
S	OVERDOSE	MDD	Fluoxetine	19	6.0	39
S	CARBON MONOXIDE	MDD		27	7.28	18
S	HANGING	MDD		24	6.64	22
S	HANGING			36	6.73	25
S	HANGING		Valproic acid	46	6.75	72
S	HANGINIG	BPD I		16	6.48	28
S	HANGING	BPD I	Lithium	30	6.71	51

Table 1

Design	**Group$_{(N)}$**	**Age (years)**	**pH**	**PMI (hours)**
	$C_{(11)}$	39.6 $\pm$ 3.8	6.50 $\pm$ 0.08	22.7 $\pm$ 1.7
	$S_{(28)}$	39.5 $\pm$ 2.4	6.57 $\pm$ 0.06	26.3 $\pm$ 1.4
Total MA		$t_{(37)} = 0.96$, p=0.35	$t_{(37)} = 0.79$, p=0.43	$t_{(37)} = 1.43$, p=0.16
(N=39)	Range	18 – 58	6.0 – 6.96	12 – 45
BA 8/9 MA	$C_{(9)}$	43.1 $\pm$ 3.7	6.50 $\pm$ 0.09	22.9 $\pm$ 2.1
(N=31)	$S_{(22)}$	35.3 $\pm$ 2.9	6.54 $\pm$ 0.06	26.4 $\pm$ 1.6
		$t_{(29)} = 1.55$, p=0.13	$t_{(29)} = 0.42$, p=0.68	$t_{(29)} = 1.43$, p=0.16

	Range	19 – 58	6.0 – 6.96	12 – 45
QPCR, Epigenetics (N=20)	$C_{(10)}$	39.5 ± 4.2	6.54 ± 0.06	22.7 ± 1.9
	$S_{(10)}$	32.5 ± 3.6	6.59 ± 0.06	28.8 ± 1.8
		$t_{(18)} = 1.33$, p=0.22	$t_{(18)} = 0.52$, p=0.61	$t_{(18)} = 1.81$, p=0.13
	Range	21 – 58	6.17 – 6.75	12 – 34

Table 2

Figures

Figure 1

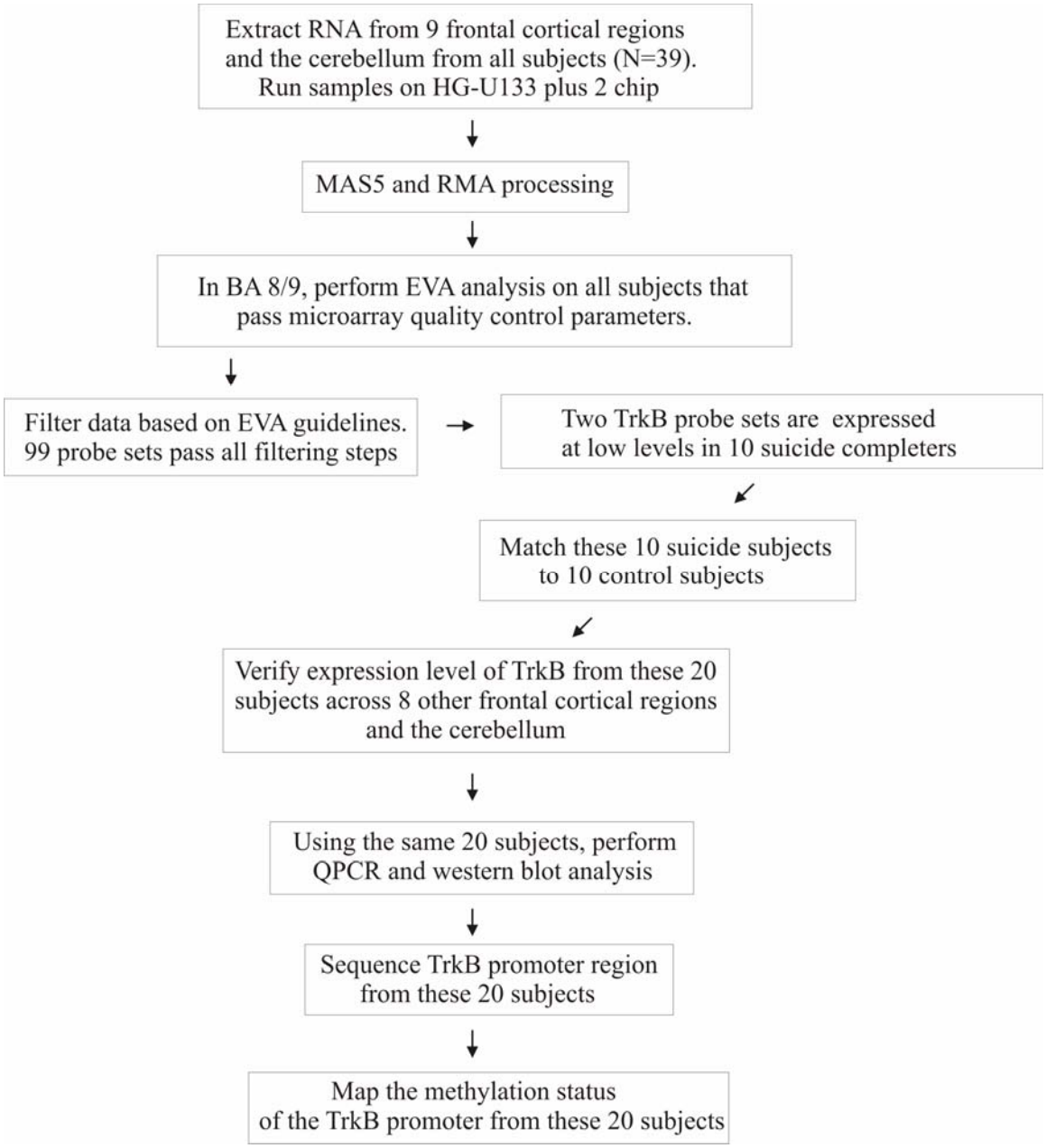


Figure 2

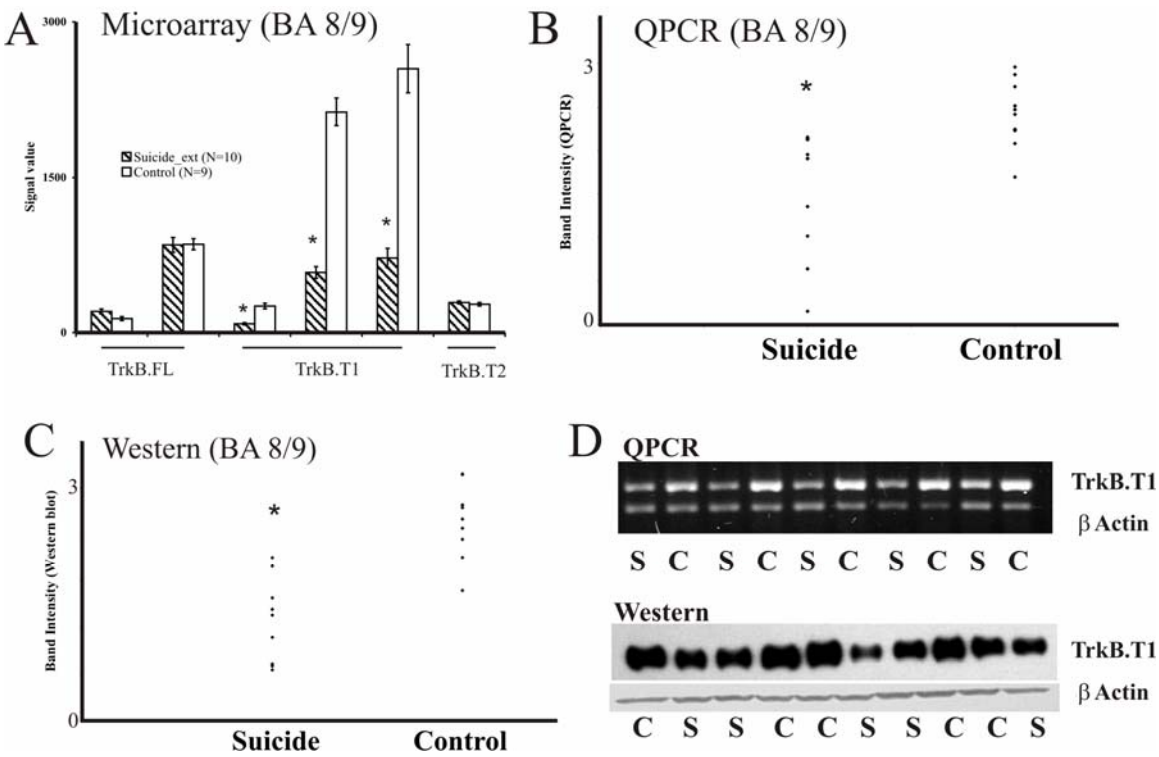

A
Microarray (BA 8/9)
Signal value
3000
1500
0
Suicide_ext (N=10)
Control (N=9)
TrkB.FL
TrkB.T1
TrkB.T2
B
QPCR (BA 8/9)
Band Intensity (QPCR)
3
0
Suicide
Control
C
Western (BA 8/9)
Band Intensity (Western blot)
3
0
Suicide
Control
D
QPCR
TrkB.T1
β Actin
S C S C S C S C S C
Western
TrkB.T1
β Actin
C S S C C S S C C S

Figure 3

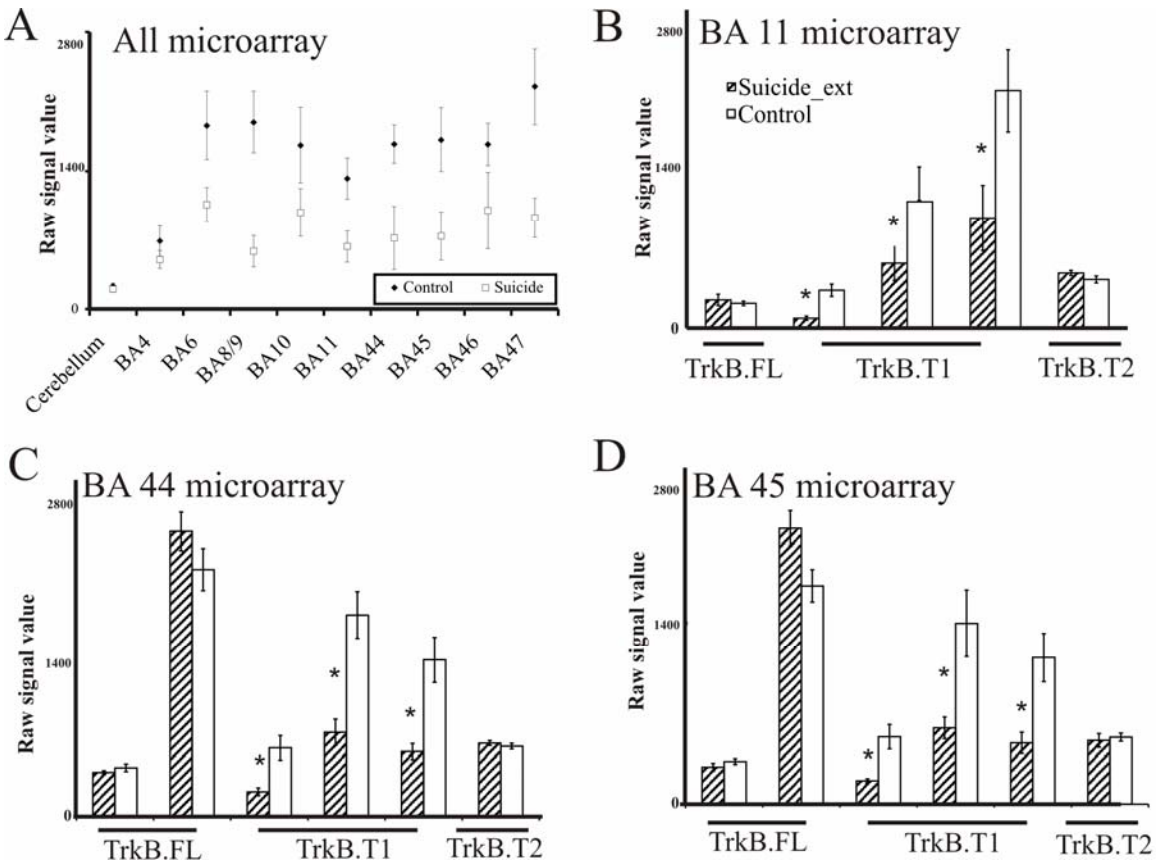
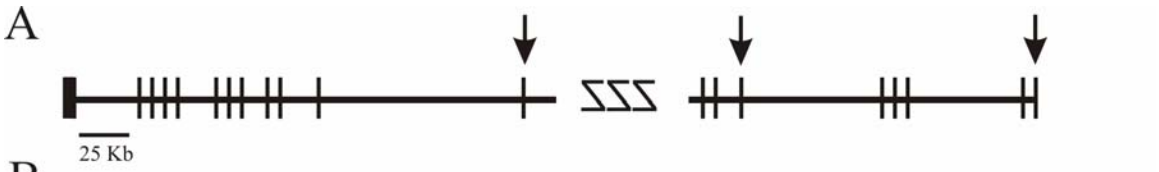

A
All microarray
Raw signal value
2800
1400
0
Control
Suicide
Cerebellum
BA4
BA6
BA8/9
BA10
BA11
BA44
BA45
BA46
BA47
B
BA 11 microarray
Suicide_ext
Control
Raw signal value
TrkB.FL
TrkB.T1
TrkB.T2
C
BA 44 microarray
Raw signal value
TrkB.FL
TrkB.T1
TrkB.T2
D
BA 45 microarray
Raw signal value
TrkB.FL
TrkB.T1
TrkB.T2

Figure 4



B

(3458)GAGAGTGGGCACACTGGTGGCTCCAG[1]CGG[2]CGTCAGTGCCAT[3]CG[4]CG
GGGCAAGTTGATTCCTGGGC[5]CGGAGCTGGGCACTCATCCATCCACAGTC
TC[6]CGGGCTGGGGT[7]CGGGGTGGGGATGA[8]CG[9]CGAGCAGAGAGGGAGAG
TGCCCCAATTAGTGGTGTTGGGGGTCCTA[10]CGCTCAGTCTTA[11]CG[12]CGTG
TCTGTTTGTCCTCAGCCT[13]CGAGGTGCATAC[14]CGGACCCCCATT[15]CGCAT
CTAACAAGGAATCTG[16]CGCCCCAGAGAGTCC[17]CGGGAG[18]CGC[19]CGC[20]CG
GT[21]CGGTGCC[22]CGG[23]CG[24]CGC[25]CGGGCCATGCAG[26]CGA[27]CGGC[28]CGC[29]C
G[30]CGGAGCTC[31]CGAGCAG[32]CGGTAG[33]CGCCCCCCTGTAAAG[34]CGGTT[35]C
GCTATGC[36]CGGGGCCACTGTGAACCCTGC[37]CGCCTGC[38]CGGAACACTCT
T[39]CGCTC[40]CGGACCAGCTCAGCCTCTGATA AGCTG (3898)

Figure 5

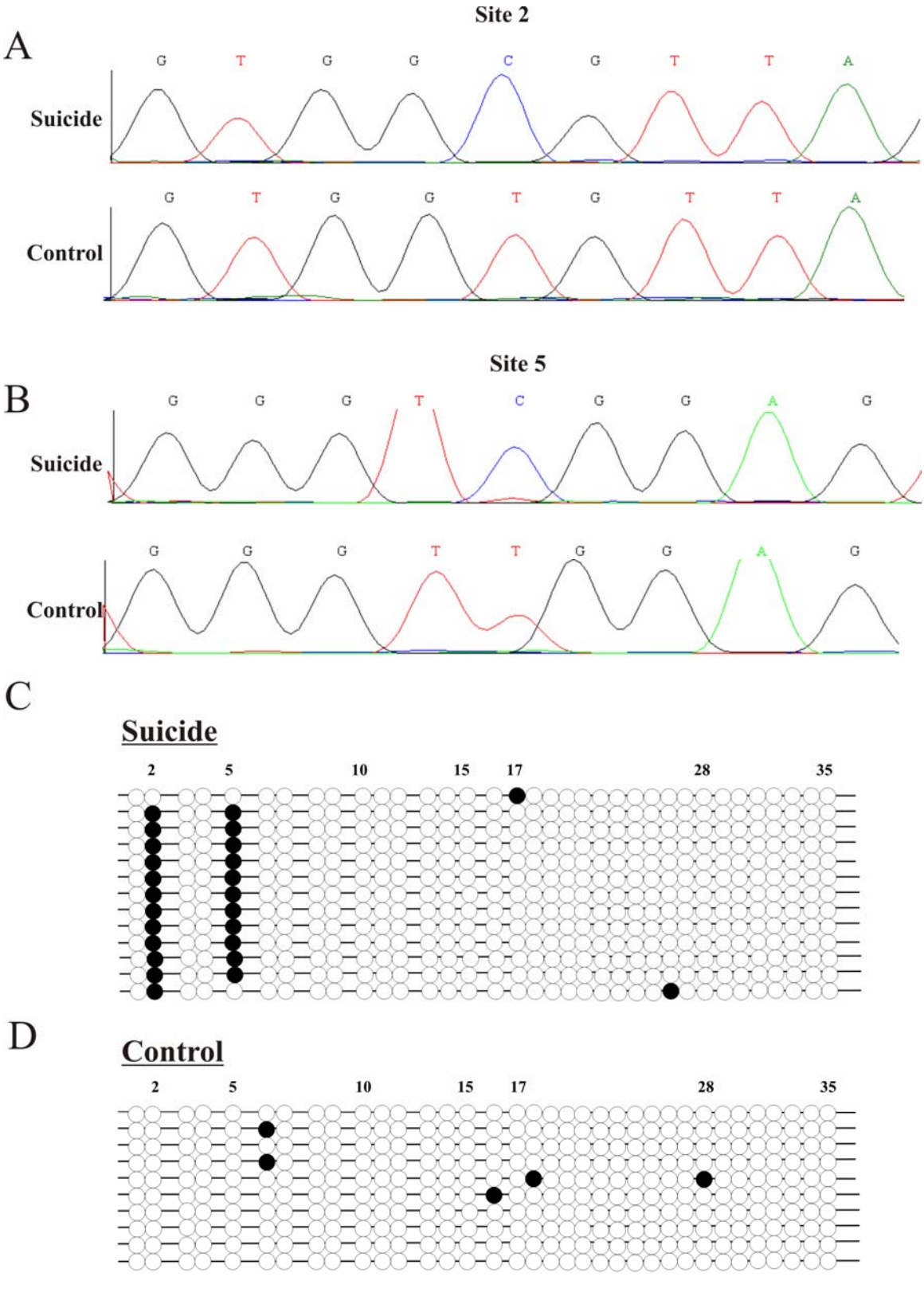

A
Site 2
Suicide
G T G G C G T T A
Control
G T G G T G T T A
B
Site 5
Suicide
G G G T C G G A G
Control
G G G T T G G A G
C
Suicide
2 5 10 15 17 28 35
D
Control
2 5 10 15 17 28 35

Figure 6

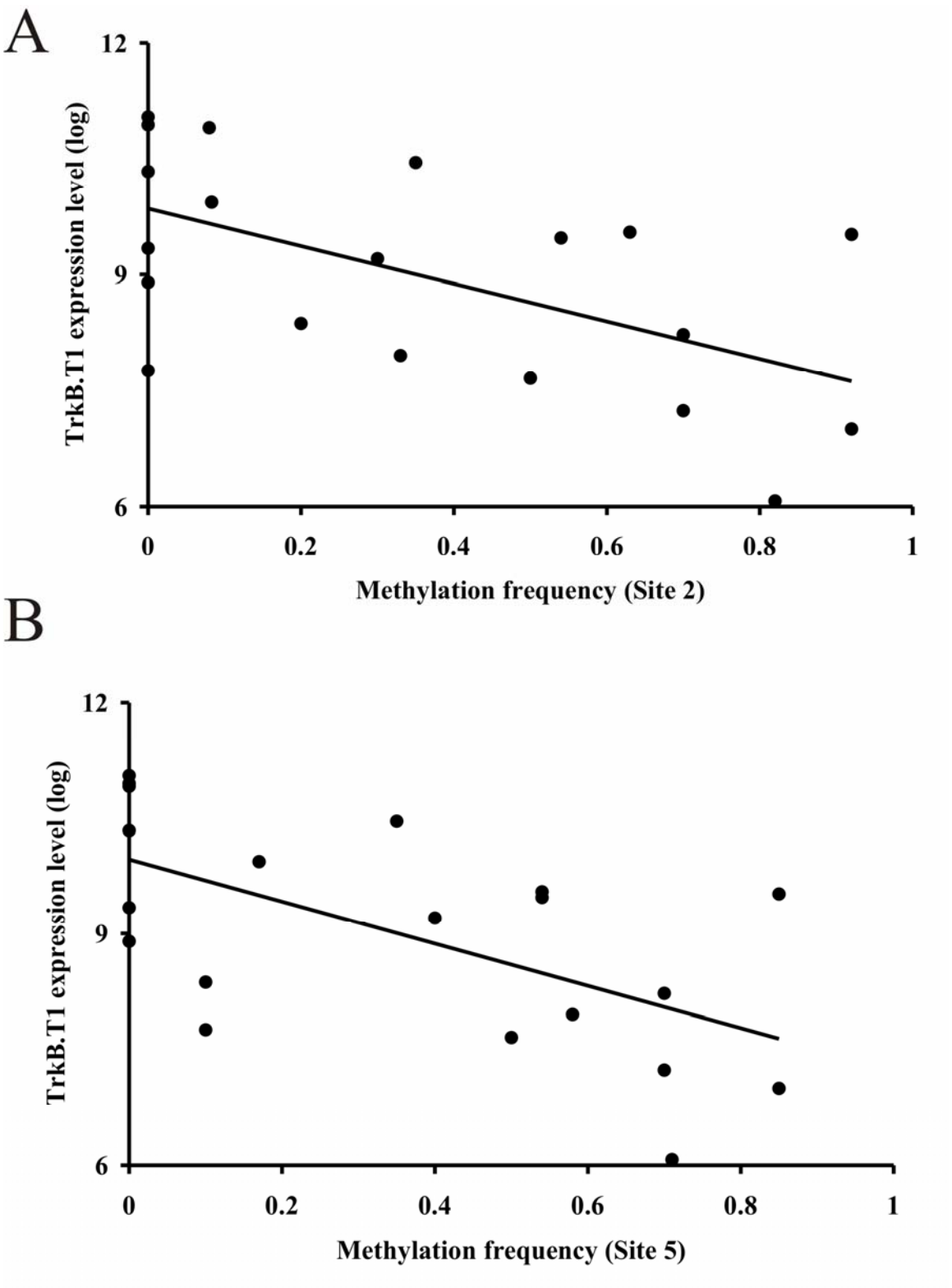
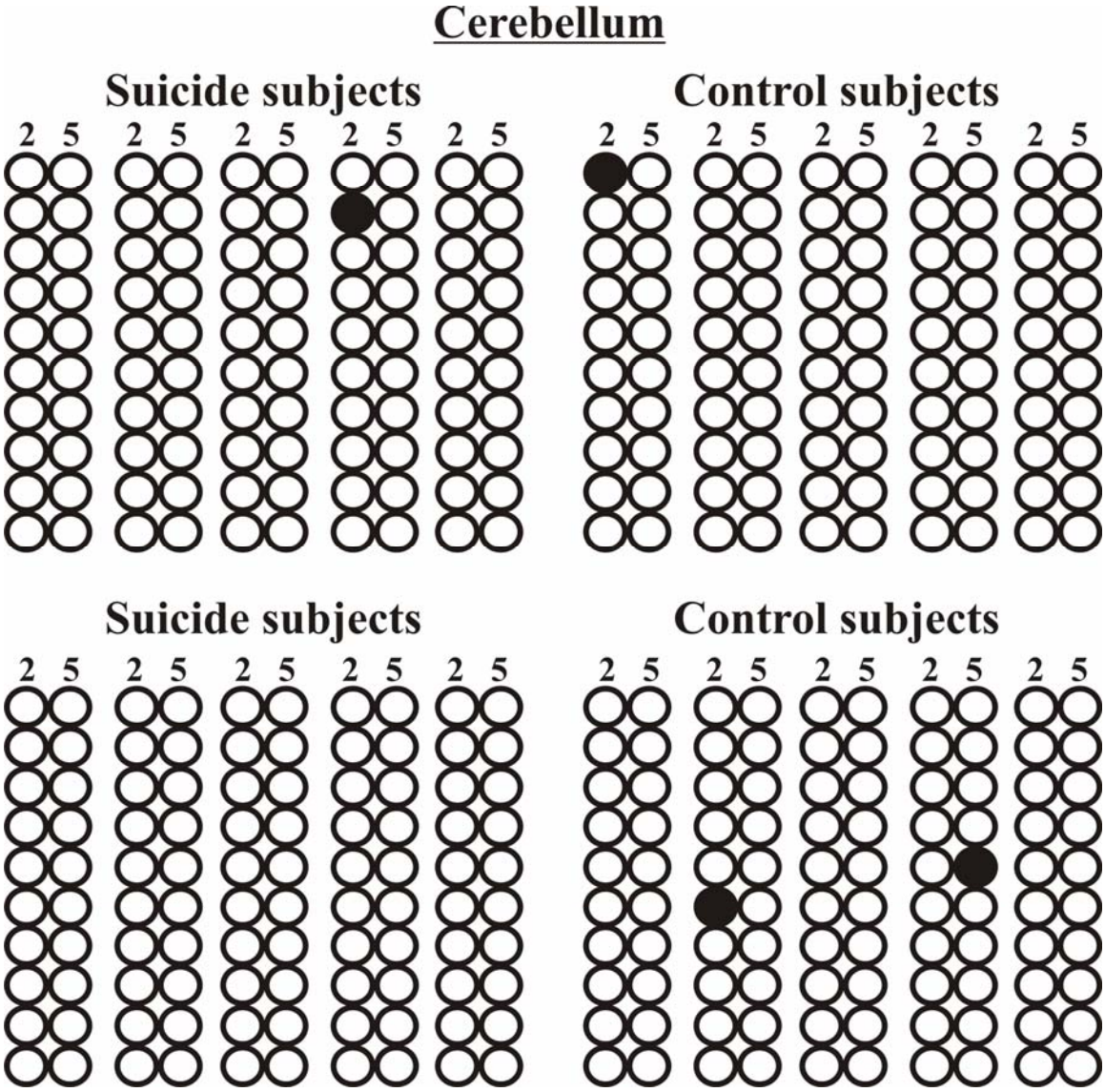

A
12
9
6
TrkB.T1 expression level (log)
0
0.2
0.4
0.6
0.8
1
Methylation frequency (Site 2)
B
12
9
6
TrkB.T1 expression level (log)
0
0.2
0.4
0.6
0.8
1
Methylation frequency (Site 5)

Figure 7


Cerebellum
Suicide subjects
Control subjects
2 5 2 5 2 5 2 5 2 5
Suicide subjects
Control subjects
2 5 2 5 2 5 2 5 2 5

SUPPLEMENTAL MATERIAL

Methods

Microarray analysis

Microarray analysis was performed on 39 subjects using the Affymetrix Human Genome (HG)-U133 plus 2 chip. RNA samples used had a minimum A260/A280 ratio of > 1.9. The samples were further checked for evidence of degradation and integrity. Samples had a minimum 28S/18S ratio of > 1.6 (2100-Bioanalyzer, Agilent Technologies).

Expression data was analyzed using Genesis 2.0 (GeneLogic Inc, Gaithersburg, MD) and AVADIS (Strand Genomics, Redwood City, Calif). Several RNA integrity measures were used in the screen to detect samples with poor RNA quality before final analysis. Microarray quality control parameters used included the following: noise (RawQ), consistent scale factors, and consistent β-actin and GAPDH 5'/3' signal ratios (>0.3 and 0.5, for all probes respectively). For this study, we define 'consistent' as any subject within two standard deviations of the mean. We performed our analyses using both the Robust Multiarray Average algorithm(Irizarry et al., 2003) (RMA) and Microarray Suite 5.0 (MAS5.0) to ensure that any results observed were not a factor of microarray normalization procedure.

In total, 39 subjects underwent microarray analysis in 10 brain regions. However, there was some variability in number of subjects analyzed per region, as not all samples passed microarray quality control in all regions. In BA 8/9, the brain region used for initial

screening, 31 subjects (9 controls and 22 suicide completers) passed microarray quality control.

Extreme Values Analysis

EVA was carried out according to the following steps in BA 8/9. All data was $\log_2$ transformed and a standard deviation (SD) was generated for the signal intensity of each probe set. The 1000 probe sets with the highest SD values were selected. The purpose of this step was to select for probe sets that have large variations among individual data points. Probe sets with large SD's likely have individual data points that vary widely from the mean (i.e. have extreme values).

The next steps of the EVA process involve trying to identify probe sets where a particular signal intensity value from a suicide subject diverges significantly from the mean of control subjects. The mean expression level and standard deviation of all probe sets generated for all control subjects was generated and this was used as a standard with which to compare individual expression levels from suicide subjects. Probe sets were selected that had at least 1 suicide subject 3-fold different from the control group's mean expression value and outside of the expression range corresponding to the control group's mean expression value $\pm$ 1.5 SDs.

Animal experiments

Adult male Sprague-Dawley rats (Charles River, St. Constant, Québec) weighing between 300-350g at the beginning of the experiment were used in these studies. Rats

were housed (in pairs for cocaine studies, individually for alcohol studies) in clear Plexiglas cages (46 cm × 18 cm × 30 cm) on a 12-hr reverse-light cycle (lights on at 12:00 pm) with food and water available *ad libitum*, unless otherwise stated. Rats were permitted to acclimatize to the colony one week prior to testing. All procedures were conducted in accordance with guidelines established by the Canadian Council on Animal Care.

Cocaine administration

Rats were randomly assigned to either vehicle (n = 4; 1 ml/kg saline ip) or cocaine (n = 4; 10 mg/kg ip) conditions and received daily injections of the designated treatment for 5 consecutive days. Rats were sacrificed 20 min following the final treatment. The frontal cortex was quickly dissected, flash frozen in isopentene and stored at -80°C until further analysis. This experiment was repeated in 10 new rats (n = 5; 1 ml/kg saline ip) or cocaine (n = 5; 10 mg/kg ip), and TrkB.T1 RNA levels were assessed in frontal cortex (Rat primers: TrkB.T1: F,5′-CAACCTAACGACTAACAGAGCC-3′ , R, 5′-TTGGTTCAAGTCCACACTCC-3′; □-Actin: F, 5'-TGTCACCAACTGGGACGATA-3', R, 5'- GGGGTGTTGAAGGTCTCAAA-3').

Acute Ethanol (EtOH) Administration

Rats were randomly assigned to either vehicle (n = 5; 1 ml/kg ip) or EtOH (n = 5; 2.5g/kg EtOH ip; 15% v/v EtOH in 0.9% saline) treatment conditions. Doses were based upon previous studies (Schulz et al., 1980; Seizinger et al., 1983). Each rat received a single injection of the designated treatment and was sacrificed 3 hr later by live decapitation.

The frontal cortex was quickly dissected, flash frozen in isopentene and stored at -80°C until further analysis.

Chronic Ethanol Administration

Food and water consumption were monitored for 3 days prior to treatment to ensure no differences in baseline consumption existed between the treatment groups. Rats were then randomly assigned to one of 3 treatment conditions: water control (n = 5), sucrose control (n =5; 10% sucrose solution) or EtOH (n = 5; 15% EtOH in a 10% sucrose solution). Sucrose was added to the EtOH solution so that the rats would consume the ethanol without previous training(Czachowski et al., 1999). Once rats in the EtOH group readily drank the 10% sucrose solution (1 day) they were gradually habituated to the 15% EtOH solution(Samson et al., 1999). EtOH rats received 5% EtOH in 10 % sucrose for 1 day, followed by 10% EtOH in 10% sucrose for 2 days. The solution was then changed to the 15% EtOH in 10% sucrose. Chronic EtOH treatment persisted for 28 days once the rats had access to the 15% EtOH solution. During this time, rats received only the designated treatment as their sole source of liquid. Food and liquid consumption was monitored daily at the beginning of the dark-cycle and body weight was measured weekly. Twenty-nine days after the 15% EtOH treatment began, rats were sacrificed prior to lights off. Brains were removed and frontal cortex was quickly dissected, flash frozen in isopentene, and stored at -80°C until subsequent analysis.

Semi-Quantitative polymerase chain reaction (semi-QPCR)

DNaseI was used in all QPCR experiments in this study. All primers can be found in a supplementary table. RNA was extracted from adjacent samples to those taken for the microarray analysis. Quality of RNA was assessed using an Agilent Bioanalyzer and no RNA was used that had an RNA Integrity number (RIN) value of less than 5. For semi-QPCR procedure, we used the QuantiTect probe PCR kit (Qiagen) and followed the instructions from the manufacturer. Linear range analysis was performed prior to semi-QPCR experiments with cycles from 20-35. Finally, we sequenced the PCR product to ensure that the single band observed was measuring the appropriate product. All samples were run and processed in randomized order. All samples were PCR amplified and run on a gel with both the internal control (□-Actin) and TrkB.T1 PCR products processed together. Optical density readings for bands were determined using Gene Tools software, a computer-assisted densitometry system.

Western blotting

Twenty-five ug of brain homogenate and sample buffer (0.25M Tris-HCl, 20% glycerol, 4% SDS, 0.005% bromophenol blue, and 5% □-mercaptoethanol) were boiled at 95^{o}C for 5 minutes. Next, samples were loaded on a Novex sure-lock electrophoresis system (Invitrogen), using 4-20% precast gels (Invitrogen). All gels were loaded pseudo-randomly. After samples were run and the gel transfer was complete, membranes were blocked with a 5% milk solution (Carnation fat free milk, in Tris-buffered saline; TBS) shaking for 1 hour. Membranes were submerged in a 5% albumin solution (Albumin in TBS with 0.2% Tween) with primary antibody (1:1000, rabbit anti-TrkBT1, sc119; Santa Cruz.) and left shaking overnight at 4^{o}C. Sixteen hours later, membranes were

thoroughly washed and left shaking for 1 hour with secondary antibody (1:5000, donkey-HRP anti rabbit, Santa Cruz). Membranes were then exposed using an ECL kit (Amersham) and apposed to film. In order to verify the accuracy of loading, membranes were stripped and re-probed with an antibody to □-actin (Santa Cruz). Both TrkB.T1 and □-actin antibodies used in this study gave only a single band on the blot. Optical density readings for bands were determined using Gene Tools software, a computer-assisted densitometry system.

DNA sequencing

We sequenced the entire putative promoter region of the TrkB gene and the splice junctions at exon 16(Stoilov et al., 2002). We note that genomic organization (intron/exon boundaries) as defined by Stoilov et al.(Stoilov et al., 2002) includes the putative promoter region from mouse in exons 4 and 5(Barettino et al., 1999; Wagner et al., 2005; Martens et al., 2007b). To account for this apparent discrepancy, we use the nomenclature of Stoilov et al. for exon numbering, but the promoter region suggested by the work of Barettino et al.(Barettino et al., 1999), Wagner et al. (Wagner et al., 2005) and Martens et al.(Martens et al., 2007b). The promoter region conservatively begins 2500 bp upstream from the sequence of primer 1 forward to 100 bps passed the ATG translation start site of the TrkB gene. All sequencing was done in duplicate, using both forward and reverse primers. All primers can be found in the supplementary table.

Analysis of methylation state

DNA was extracted from BA 8/9 from 20 subjects (10 suicide completers and 10 matched control subjects). DNA was treated with sodium bisulphite (Qiagen EpiTec Bisulfite Kit) to convert all non-methylated cytosine bases to uracil(Frommer et al., 1992; Clark et al., 1994; Flanagan et al., 2006). DNA was then amplified by PCR; we used Methprimer(Li and Dahiya, 2002) to design primers specific to a 440 bp region of the putative TrkB promoter. The amplified product was extracted from the gel, ligated into a pDrive vector, and transformed into competent *E.coli* cells (Qiagen PCR Cloning*Plus* Kit). Incorporation of the correct DNA fragment was verified by restriction enzyme digestion using EcoRI. All sequencing was done at the Genome Quebec Innovation Centre. We note that only the 35 5' CpG sites in this PCR fragment could be assessed due to technical reasons.

Luciferase experiments

We cloned a 2,182 bp PCR product into the pDrive vector. To do this, we used primers from TrkB sequencing experiments. After endonuclease excision, the product was subcloned into the pGL3 vector (Promega), verified for orientation, and sequenced. Plasmid propagation was done by transforming competent *E.coli* cells (Qiagen PCR Cloning*Plus* Kit). Bacterial cells were grown overnight on an agar media at 37 °C and positive colonies were selected. Plasmids were extracted from cells using Qiagen plasmid MIDI kits. Plasmids were then co-transfected into COS7 cells (ATCC) with a pRL plasmid (Promega - internal control). Transfections were done using lipofectamine 2000 and left overnight in DMEM media (Gibco). Luciferase experiments were performed using a dual injection Luminometer (Berthold) and the Dual-Luciferase Reporter Assay system (Promega). Plasmid-transfected cells were lysed with passive

lysis buffer and incubated with Luciferase Assay Reagent II. Firefly luciferase (pGL3 vector) activity was then measured. Immediately following activity reading, Stop-and-Glo reagent was injected, and *Renilla* luciferase (internal control) activity was measured. Stop-and-Glo reagent quenches the luciferase signal and provides the substrate (coelenterazine) for the Renilla signal to be generated. Experiments were run in triplicate and were compared to the pGL3_basic plasmid in all experiments.

Results

BA 8/9: Conventional analysis of microarray data

In BA 8/9 (N= 9 controls subjects and 22 suicide completers), we compared the fold-change difference between all suicide completers and all control subjects. Using RMA data, we removed all probe sets that did not vary by greater than $\pm$1.5 fold between suicide and control groups; 282 probe sets remained after this analysis. Probe set values were $\log_{(2)}$ transformed and t-tests were performed for all probe sets across all suicide and control subjects. Only probe sets below the 0.05 level were kept after this filtering step. In total, 179 probe sets showed fold-change differences of $\pm$1.5 and had p-values less than 0.05. From these 179 probe sets, two TrkB probe sets also identified using the EVA analysis were present (Figure S1): probe sets 221795_at (FC=-1.63; p=0.017) and 221796 (FC=-1.70; p=0.009). Notably, the 214680_at TrkB probe set identified from the EVA screen that was filtered out in this statistical analysis had very similar fold-change and p-values to the other two TrkB probe sets (214680_at: FC=-1.69; p=0.051; Figure S1A).

We also demonstrate the individual expression values for each subject from microarray data in BA 8/9 (Figure S1B).

Investigation of BDNF probe sets in BA 8/9

We found no evidence of alterations in BDNF, despite differences that we found in TrkB.T1 expression between suicide completers and control subjects. There are 7 probe sets specific to BDNF on the HG-U133 plus 2 arrays. First, we compared all subjects

that passed quality control in BA 8/9 (N=9 control subjects and 22 suicide completers) for expression levels of BDNF and then we compared the 10 suicide completers with low expression of TrkB.T1 to 9 control subject (supplemental table 3)

Cross-platform technical validation: Re-processing of identical RNA samples using the HG-U133 A,B platform

For the probe set reliability measure (n=20), microarrays were performed for each subject using both the HG-U133A,B chip and the HG-U133 plus 2 chip (www.affymetrix.com) using RNA extracted from Brodmann 8/9. Data was processed using Microarray Suite 5 (MAS 5.0) with Genesis software (GeneLogic, Gaithersburg, Md). Micorarray data from HU-133A,B and HU-133 plus 2 was first screened to remove any probe sets that were not similar to both chips using Microsoft Access. 44 592 probe sets are common to both HG-U133 A,B and HG-U133 plus 2 chips.

To confirm the reliability of the chips themselves we compared the 20 subjects in BA 8/9 on both HG-U133 A,B and HG-U133 plus 2. We plotted all common probe sets from both chips for all subjects and generated a correlation coefficient. The Pearson correlation co-efficient was a statistically significant 0.46 between chips and within subjects. To assess the reliability of our TrkB.T1 findings, we correlated those subjects present on both the HG-U133 plus 2 chip and the HG-U133 A,B chip across the 3 TrkB.T1 probe sets. The Pearson correlations between the chips for the TrkB.T1 probe sets were: 221796 (0.92, $p<0.001$); 221795_at (0.66, $p<0.001$); 214680_at (0.59, $p<0.001$).

Rat control experiments: Modeling cocaine and alcohol abuse

To fully rule out possible effects of alcohol and cocaine on the regulation of the TrkB.T1 protein, we performed three experiments in rats. We chose three experimental conditions that best model toxicological and informant reports, as well as frequency of reports in our sample: binge cocaine, binge drinking, and chronic alcohol abuse. An independent samples t-test revealed no difference in the level of TrkB.T1 protein in rats injected with cocaine compared to rats injected with saline (t_6=0.52, p=0.62). The 5-day cocaine administration protocol was repeated in 10 new rats (5 administered cocaine and 5 administered saline) to ensure the reliability of this finding, and RNA was extracted from frontal cortex. There was no significant difference in the level of TrkB.T1 RNA between cocaine or saline treated rats (t_8=1.35, p=0.90). A similar result was found in the acute alcohol experiments: We could detect no difference in TrkB.T1 protein in rats injected with alcohol compared to rats injected with saline (t_8=0.44, p=0.67). ANOVA one way analysis revealed no group differences in the chronic alcohol group ($F_{2,13}$=0.58, p=0.57) when the level of TrkB.T1 protein was compared across rats given water, sucrose, or an alcohol diet (supplemental figure S2).

Promoter sequencing

We defined the promoter region similar to that proposed in mouse(Wagner et al., 2005). To confirm that this region is a promoter region in human, we cloned a ~2.1Kb product and performed luciferase assays. We found a 4.15-fold increase in promoter activity

when the TrkB fragment was cloned upstream of the Luciferase gene compared to a control plasmid.

We sequenced a 2500 bp region downstream of the start site of TrkB that included the previously cloned fragment from the Luciferase experiments. These experiments were done using the 10 TrkB.T1 low expressors identified from the microarray analysis and 10 matched controls. We observed rs1187322 and rs1187323 as well as one novel SNP 23 base pairs from the start site. None of these variants was associated with a reduced expression of TrkB.T1. One control subject and one suicide subject had a variant at each of these three positions.

Technical experiments for methylation mapping

To ensure that our bisulfite conversion procedure fully converted all non-methylated cytosines to uracil, we treated a pGL3 plasmid (Promega) after it had been propagated in *E.Coli* cells with SSSI methyltransferase, an enzyme that methylates all cytosine nucleotides in a CpG dinucleotide.

DNA samples were treated with sodium bisulfite following the manufacturer's protocol (Qiagen EpiTec Bisulfite Kit) and purified. Primers specific for bisulfited-treated pGL3-CH3 were designed using Methyl Primer Express (Forward: 5' AAGATGTTTTTTGTGATTGGT 3'; Reverse: 5' TTCCTATTTTACTCACCCAAA 3'). We sequenced >10 clones in this fashion and found that all cytosines residing in the dinucleotide CpG sequence remained methylated in all clones, while all cytosines found

in other sequence contexts were detected as thymidine bases, indicating lack of methylation.

After bisulfite treatment not all PCR products are similar, although all are the same length. This is due not only to complementary DNA strands no longer being equivalent, but also to the fact that the PCR reaction amplifies DNA from different cells, with potentially different DNA methylation patterns. If DNA from a given cell has a different methylation pattern than another cell, the PCR products will not be equivalent after bisulfite treatment. PCR primers specific to bisulfite-treated DNA may have an amplification bias for DNA with less initial methylation. DNA with a higher cytosine content (and thus with more heavily methylated DNA prior to bisulfite treatment) has a higher melting temperature and has a higher probability of forming secondary structures than DNA with a lower G:C content.

To test whether the region amplified in this study gives way to amplification bias we performed several control experiments. Using the plasmid containing the promoter region used in this study for the luciferase experiments, we propagated the plasmid in *E.coli* and subjected the plasmid solution to bisulfite treatment. This provided a TrkB promoter region that was initially fully unmethylated (*E.coli* lack methyltransferases). To generate a plasmid that was initially fully methylated, we again propagated the TrkB promoter plasmid in *E.coli*. This time, before bisulfite treatment we treated the plasmid solution with SSSI methyltransferase – thereby converting all cytosine residues in CG

dinucleotides to 5'-methylcytosine. After confirming that this step functioned, via a restriction enzyme digest test, we bisulfite treated this plasmid solution.

Two plasmid solutions post-bisulfite treatment were set to be tested for amplification bias: One solution which was initially fully unmethylated and the other solution which was initially fully methylated. First, we quantified the relative concentration of plasmid in each of the two solutions. After ensuring equal concentrations, we made a series of solutions that differed in the ratio of plasmid. The starting percentages of methylated DNA was as follows: 20, 25, 33, 50, 66, 75. Using the primers designed for methylation mapping the TrkB promoter in this study, we PCR amplified each of these solutions. After amplification, we used the MluI restriction enzyme to digest the product of the amplification. This enzyme cuts the PCR product derived from methylated DNA once, and leaves the PCR product derived from unmethylated DNA intact. Digital images were captured from a gel and quantified (Figure S3). In this way, we were able to determine whether a PCR bias existed for either the fully methylated plasmid or the fully un-methylate4d plasmid. We note that this experiment is conservative given that we never detected DNA from actual human brain that was fully methylated. Indeed, this region of DNA is rarely methylated in frontal cortex of human brain, except for specific sites. Using the equation: $b=[y(100-x)]/[x(100-y)]$, where b is reflects the value of bias to give the line of best fit to the graph in figure S3 (Werenecke et al. 1997). For these primers we found a (b) bias value 0f 1.06 – a 1.06 fold preference for methylated DNA over un-methylated DNA, suggesting that both methylated and unmethylated DNA are amplified with equal frequency.

Supplemental Tables

Primer	Forward	Reverse
TrkB promoter 1	AACGAGACTCCAACCCATTG	GCATCCAGCTACGAAGAAGC
TrkB promoter 2	AGGCACTGCGGTGTATTTTC	TAACCTGACGGGATGTAGCC
TrkB promoter 3	GGCTTCTTCGTAGCTGGATG	GCGTGTGTGTGTCTGTGTGT
TrkB promoter 4	CAGCCTCTACCGCGATTGTA	GCTACTGCTGGCGAAGTGAC
TrkB promoter 5	GAGCGTGTGTGTGTTTTTGG	TAAAGGGGAATGCGGAGACT
TrkB promoter 6	GGTGAGCAGCGCAGATAGT	GCTGAGGACAAACAGACACG
TrkB promoter 7	CAGGCTCGAAGAGAGAGTGG	TGCGGCTCTCTTAACTCCTC
TrkB promoter 8	CCCCCTGTAAAGCGGTTC	AGATGACCCTGTCCTGCATC
TrkB_exon16_5'	TTCCCCCAACTTCTGTTCTG	TCAGTGTGCACTTATACCTGTT
TrkB_exon16_3'	GCTGACTTGACTCCAAGGAA	CAAAGGGCTTTGCCATAGAA
TrkB_QPCR15_16	TGGGATTTTGCCTTTTGGTA	GGATAAGCCAACAGCAGTCC
TrkB_QPCR16	AGCTGTGGATTTCTGCATCC	CTGGCTCTGAAGTCCTCCTG
GFAP_QPCR	GCTTCCTGGAACAGCAAAAC	GGCTTCATCTGCTTCCTGTC
TrkB_CH3	GAGAGTGGGTATATTGGTGGTTTTA	CCAACTTATCAAAAACTAAACTAAT

Supplemental table 1. Primers used in this study

Probe_set	**Gene Name**	**Gene Symbol**
200799_at	(Heat shock 70kDa protein 1A, Heat shock 70kDa protein 1B)	(HSPA1A, HSPA1B
200800_s_at	(Heat shock 70kDa protein 1A, Heat shock 70kDa protein 1B)	(HSPA1A, HSPA1B
214359_s_at	(Heat shock protein 90kDa alpha (cytosolic), class B member 1)	(HSP90AB1, HSP90AB2P)
217356_s_at	(Phosphoglycerate kinase 1, Similar to Phosphoglycerate kinase 1)	(LOC644774, PGK1
201034_at	Adducin 3 (gamma)	ADD3
201753_s_at	Adducin 3 (gamma)	ADD3
221008_s_at	Alanine-glyoxylate aminotransferase 2-like 1	AGXT2L1
217757_at	Alpha-2-macroglobulin	A2M
202834_at	Angiotensinogen (serpin peptidase inhibitor, clade A, member 8)	AGT
226228_at	Aquaporin 4	AQP4
213738_s_at	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1	ATP5A1
203296_s_at	ATPase, Na+/K+ transporting, alpha 2 (+) polypeptide	ATP1A2
214432_at	ATPase, Na+/K+ transporting, alpha 3 polypeptide	ATP1A3
227556_at	ATPase, Na+/K+ transporting, beta 1 polypeptide	ATP1B1
204311_at	ATPase, Na+/K+ transporting, beta 2 polypeptide	ATP1B2
217911_s_at	BCL2-associated athanogene 3	BAG3
213812_s_at	Calcium/calmodulin-dependent protein kinase kinase 2, beta	CAMKK2
222484_s_at	Chemokine (C-X-C motif) ligand 14	CXCL14
218597_s_at	Chromosome 10 open reading frame 70	ZCD1
205328_at	Claudin 10	CLDN10

208669_s_at	CREBBP/EP300 inhibitor 1	CRI1
201360_at	Cystatin C (amyloid angiopathy and cerebral hemorrhage)	CST3
200664_s_at	DnaJ (Hsp40) homolog, subfamily B, member 1	DNAJB1
231930_at	ELMO/CED-12 domain containing 1	ELMOD1
201313_at	Enolase 2 (gamma, neuronal)	ENO2
207547_s_at	Family with sequence similarity 107, member A	FAM107A
209074_s_at	Family with sequence similarity 107, member A	FAM107A
204379_s_at	Fibroblast growth factor receptor 3 (achondroplasia, thanatophoric dwarfism)	FGFR3
201667_at	Gap junction protein, alpha 1, 43kDa (connexin 43)	GJA1
231771_at	Gap junction protein, beta 6 (connexin 30)	GJB6
200947_s_at	Glutamate dehydrogenase 1	GLUD1
215001_s_at	Glutamate-ammonia ligase (glutamine synthetase)	GLUL
208813_at	Glutamic-oxaloacetic transaminase 1, soluble (aspartate aminotransferase 1)	GOT1
203178_at	Glycine amidinotransferase (L-arginine:glycine amidinotransferase)	GATM
209170_s_at	Glycoprotein M6B	GPM6B
201841_s_at	Heat shock 27kDa protein 1	HSPB1
200806_s_at	Heat shock 60kDa protein 1 (chaperonin)	(HSPD1)
210338_s_at	Heat shock 70kDa protein 8	HSPA8
201185_at	HtrA serine peptidase 1	HTRA1
222920_s_at	KIAA0748	KIAA0748
212877_at	Kinesin 2	KNS2
207761_s_at	LETM1 domain containing 1	(LETMD1, METTL
200624_s_at	Matrin 3	MATR3
213395_at	Megalencephalic leukoencephalopathy with subcortical cysts 1	MLC1
213629_x_at	Metallothionein 1F (functional)	MT1F
212859_x_at	Metallothionein 1M	(MT1E, MT1M)
224918_x_at	Microsomal glutathione S-transferase 1	MGST1
207323_s_at	Myelin basic protein	MBP
201976_s_at	Myosin X	MYO10
201669_s_at	Myristoylated alanine-rich protein kinase C substrate	MARCKS
214279_s_at	NDRG family member 2	NDRG2
33767_at	Neurofilament, heavy polypeptide 200kDa	NEFH
204412_s_at	Neurofilament, heavy polypeptide 200kDa	NEFH
221805_at	Neurofilament, light polypeptide 68kDa	NEFL
221796_at	Neurotrophic tyrosine kinase, receptor, type 2	NTRK2
221795_at	Neurotrophic tyrosine kinase, receptor, type 2	NTRK2
218224_at	Paraneoplastic antigen MA1	PNMA1
201876_at	Paraoxonase 2	PON2
200845_s_at	Peroxiredoxin 6	PRDX6
212230_at	Phosphatidic acid phosphatase type 2B	PPAP2B
212226_s_at	Phosphatidic acid phosphatase type 2B	PPAP2B
200738_s_at	Phosphoglycerate kinase 1	PGK1
223062_s_at	Phosphoserine aminotransferase 1	PSAT1
209466_x_at	Pleiotrophin (heparin binding growth factor 8	PTN

209465_x_at	Pleiotrophin (heparin binding growth factor 8	PTN
211737_x_at	Pleiotrophin (heparin binding growth factor 8	PTN
204679_at	Potassium channel, subfamily K, member 1	KCNK1
228581_at	Potassium inwardly-rectifying channel, subfamily J, member 10	KCNJ10
215707_s_at	Prion protein (p27-30)	PRNP
205825_at	Proprotein convertase subtilisin/kexin type 1	PCSK1
200603_at	Protein kinase, cAMP-dependent, regulatory, type I, alpha	PRKAR1A
228222_at	Protein phosphatase 1, catalytic subunit, beta isoform	PPP1CB
204284_at	Protein phosphatase 1, regulatory (inhibitor) subunit 3C	PPP1R3C
212636_at	Quaking homolog, KH domain RNA binding (mouse)	QKI
217764_s_at	RAB31, member RAS oncogene family	RAB31
217762_s_at	RAB31, member RAS oncogene family	RAB31
202388_at	Regulator of G-protein signalling 2, 24kDa	RGS2
225202_at	Rho-related BTB domain containing 3	RHOBTB3
208671_at	Serine incorporator 1	SERINC1
225491_at	Solute carrier family 1 (glial high affinity glutamate transporter), member 2	SLC1A2
208389_s_at	Solute carrier family 1 (glial high affinity glutamate transporter), member 2	SLC1A2
202800_at	Solute carrier family 1 (glial high affinity glutamate transporter), member 3	SLC1A3
203908_at	Solute carrier family 4, sodium bicarbonate cotransporter, member 4	SLC4A4
207974_s_at	S-phase kinase-associated protein 1A (p19A)	SKP1A
228038_at	SRY (sex determining region Y)-box 2	SOX2
202930_s_at	Succinate-CoA ligase, ADP-forming, beta subunit	SUCLA2
229039_at	Synapsin II	SYN2
217853_at	Tensin 3	TNS3
208850_s_at	Thy-1 cell surface antigen	THY1
213710_s_at	Transcribed locus	
203381_s_at	Translocase of outer mitochondrial membrane 40 homolog (yeast)	(APOE, TOMM40)
203382_s_at	Translocase of outer mitochondrial membrane 40 homolog (yeast)	(APOE, TOMM40)
214023_x_at	Tubulin, beta 2B	(TUBB2A, TUBB2I
212664_at	Tubulin, beta 4	TUBB4
200693_at	Tyrosine 3-monooxygenase/, theta polypeptide	YWHAQ
213326_at	Vesicle-associated membrane protein 1 (synaptobrevin 1)	VAMP1
201426_s_at	Vimentin	VIM
204712_at	WNT inhibitory factor 1	WIF1

Supplemental table 2. Probe sets that passed EVA criteria in BA 8/9

Probe set	Microarray (N=31)	Microarray (N=19)
206382_s_at	0.865686	0.350286

239367_at	0.841139	0.977369
1565265_at	0.327641	0.59776
1567359_at	0.409887	0.438575
1567361_at	0.317153	0.604333
1567575_at	0.84225	0.759438
1567576_at	0.92885	0.833732

Supplemental table 3. No change in expression level of BDNF across 7 different probe sets on HG-133 plus 2 in BA 8/9. Numbers shown are p-values generated by comparing control subjects to suicide completers. The microarray column with 31 subjects represents 22 suicide completers and 9 controls and the microarray column with 19 subjects represents 9 controls and the 10 suicide completers with low expression levels of TrkB.T1.

Covariate	Microarray (N=31)	Microarray (N=19)	QPCR (N=20)	Western (N=20)
pH	0.009	0.003	0.004	0.001
Age	0.017	0.008	0.008	0.001
PMI	0.015	0.001	0.005	0.001

Supplemental table 4. Statistical analysis using PMI, pH and age as covariates when comparing control subjects to suicide completers for different analyses. Adjusted p-values in BA 8/9 comparing suicide completers to control subjects for all analyses. Performed using SPSS (general linear model; univariate) using group as the fixed factor, effect (QPCR, Western, Microarray) as the dependent variable and the variables in the column marked, 'covariates' as covariates.

Probe set	***TrkB isoform***
207152_at	Full length
229463_at	Full length
214680_at	T1
221795_at	T1
221796_at	T1
236095_at	T2

Supplemental table 5. TrkB probe sets from Affymetrix gene chips. Indicated is the isoform specificity of each probe set.

Supplemental Figures

Supplemental figure 1. **Results of TrkB probe sets when screening data using a conventional microarray approach.** A) Using a conventional microarray screening approach, the total suicide group (N=22) has significantly reduced expression in two TrkB.T1 probe sets compared to control subjects (N=9) in BA 8/9. B) Example of individual expression distribution for TrkB.T1 in BA 8/9 using probe set 221795_at (N=31). Suicide = Suicide subjects who failed EVA criteria for extreme expression value for TrkB.T1; Suicide_ext= Suicide subjects who pass EVA criteria for extreme expression value for TrkB.T1.

Supplemental figure 2. **Assessment of the effects of binge cocaine, binge alcohol and chronic alcohol treatment on the TrkB.T1 protein in frontal cortex of rat.** A) No significant differences in the ratio of TrkB.T1 protein/actin protein were detected in any group. B) Example of western blots from some animals from the chronic alcohol experiments.

Supplemental figure 3. **No amplification bias in methylation assessment experiments.** Gel demonstrating different percentages of methylated DNA compared to unmethylated DNA. The slow migrating (top) band represents the uncut PCR product. DNA that gave rise to this band was initially unmethylated. The two fast moving (bottom) bands represent DNA that was initially fully methylated. Lane 1 is closest to the 100 bp ladder. Percentage of methylated DNA in each of lanes 1-6 were: 75, 66, 50, 33, 25, 20. Graph shows the relationship between percentage of methylated DNA in the PCR reaction and the percentage of methylated DNA that was amplified.

Supplemental figure 4. **All raw data from methylation mapping experiments in 10 control subjects**

Supplemental figure 5. **All raw data from methylation mapping experiments in 10 suicide completers with low TrkB.T1 expression level**

Supplemental figure 6. **Correlation analysis of TrkB.T1 expression and methylation state by group.** A) Methylation frequency at site 5 compared to expression level of TrkB.T1 expression level for control subjects. B) Methylation frequency at site 2 compared to expression level of TrkB.T1 for control subjects. C) Methylation frequency at site 5 compared to expression level of TrkB.T1 for suicide completers. D) Methylation frequency at site 2 compared to expression level of TrkB.T1 for suicide completers.

Supplemental figures

Figure S1

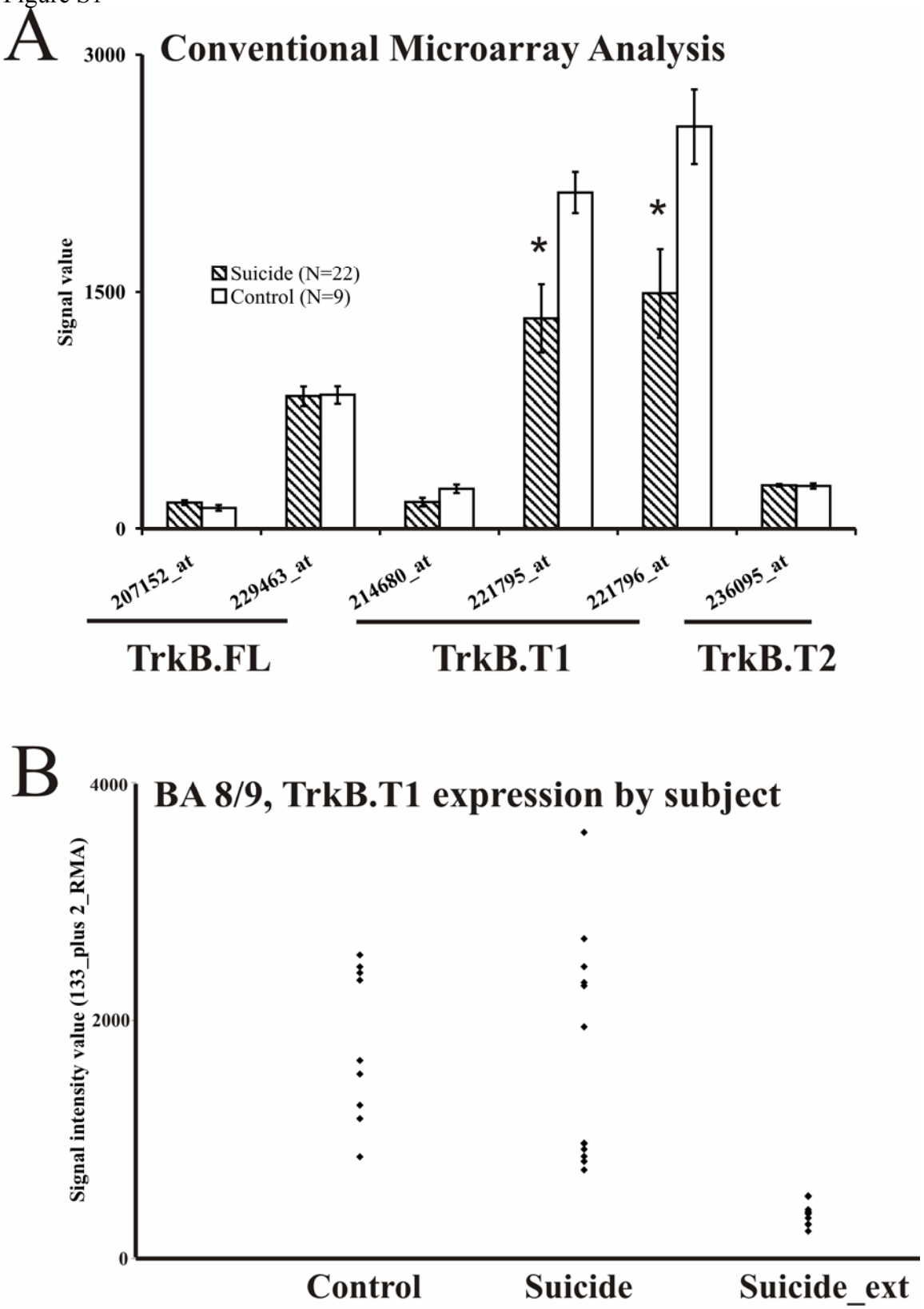


Figure S2

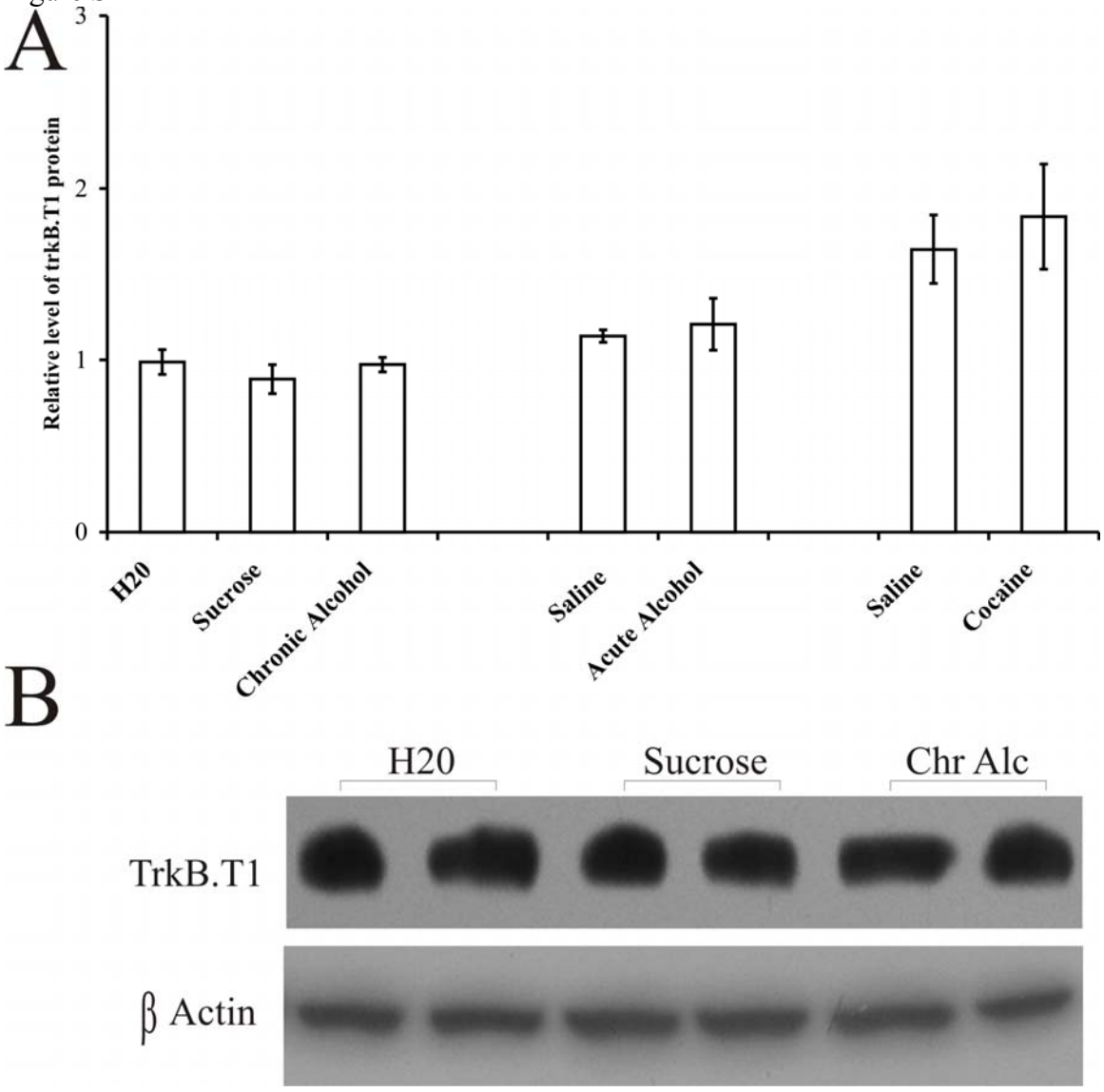

A
Relative level of trkB.T1 protein
3
2
1
0
H20
Sucrose
Chronic Alcohol
Saline
Acute Alcohol
Saline
Cocaine
B
H20
Sucrose
Chr Alc
TrkB.T1
β Actin

Figure S3

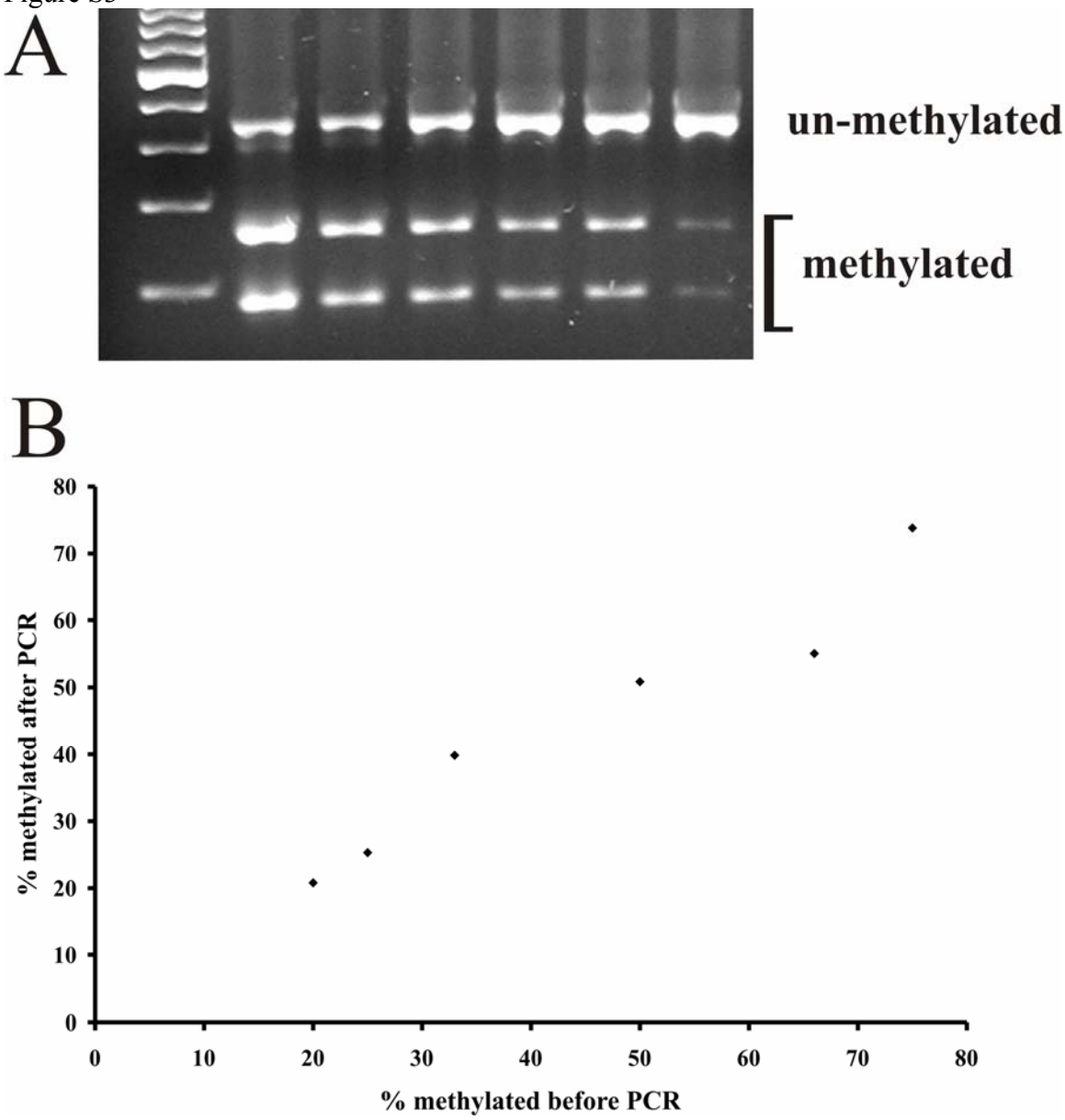

A
un-methylated
methylated
B
% methylated after PCR
% methylated before PCR
0
10
20
30
40
50
60
70
80

Figure S4

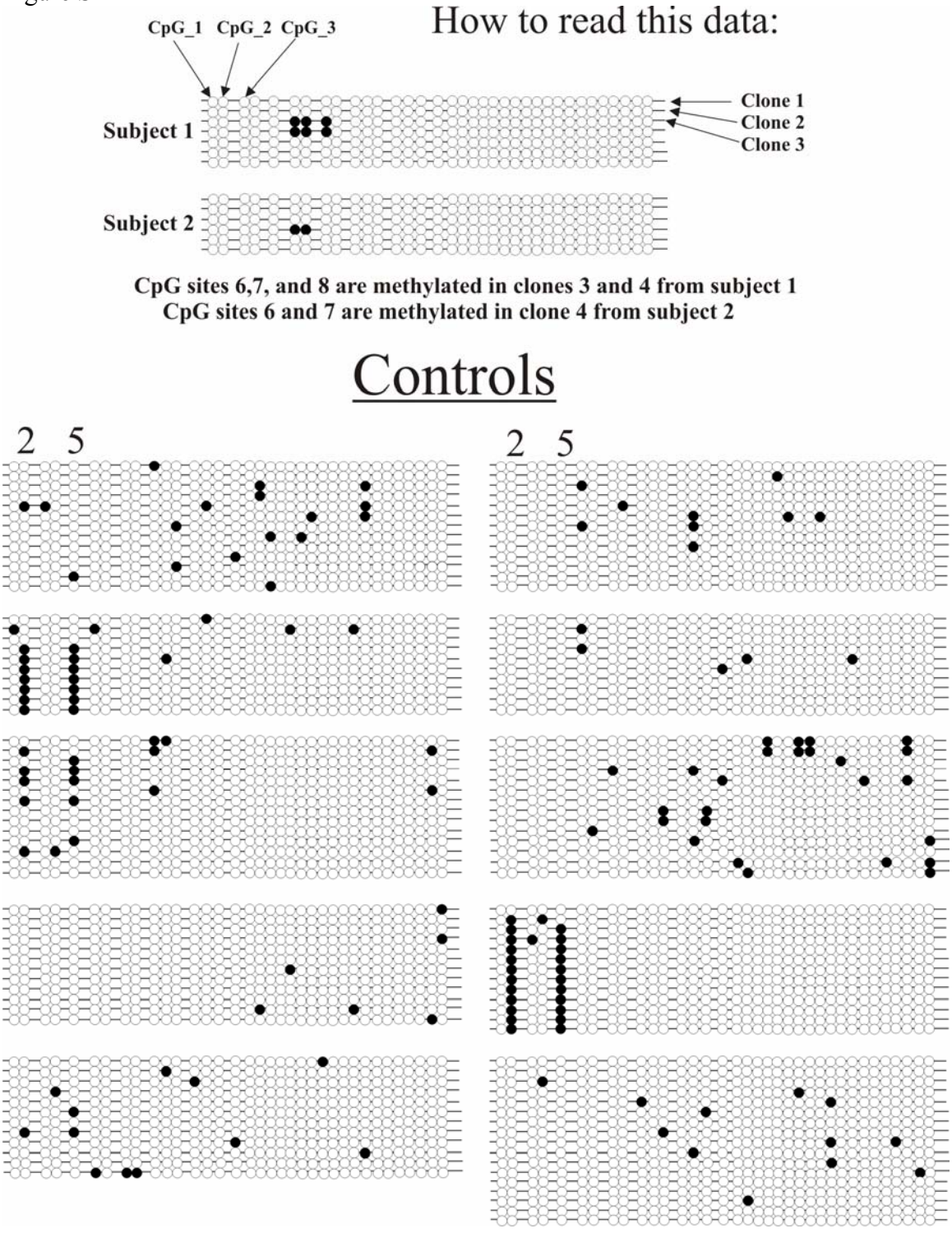

How to read this data:
CpG_1 CpG_2 CpG_3
Clone 1
Clone 2
Clone 3
Subject 1
Subject 2
CpG sites 6,7, and 8 are methylated in clones 3 and 4 from subject 1
CpG sites 6 and 7 are methylated in clone 4 from subject 2
Controls
2 5
2 5

Figure S5

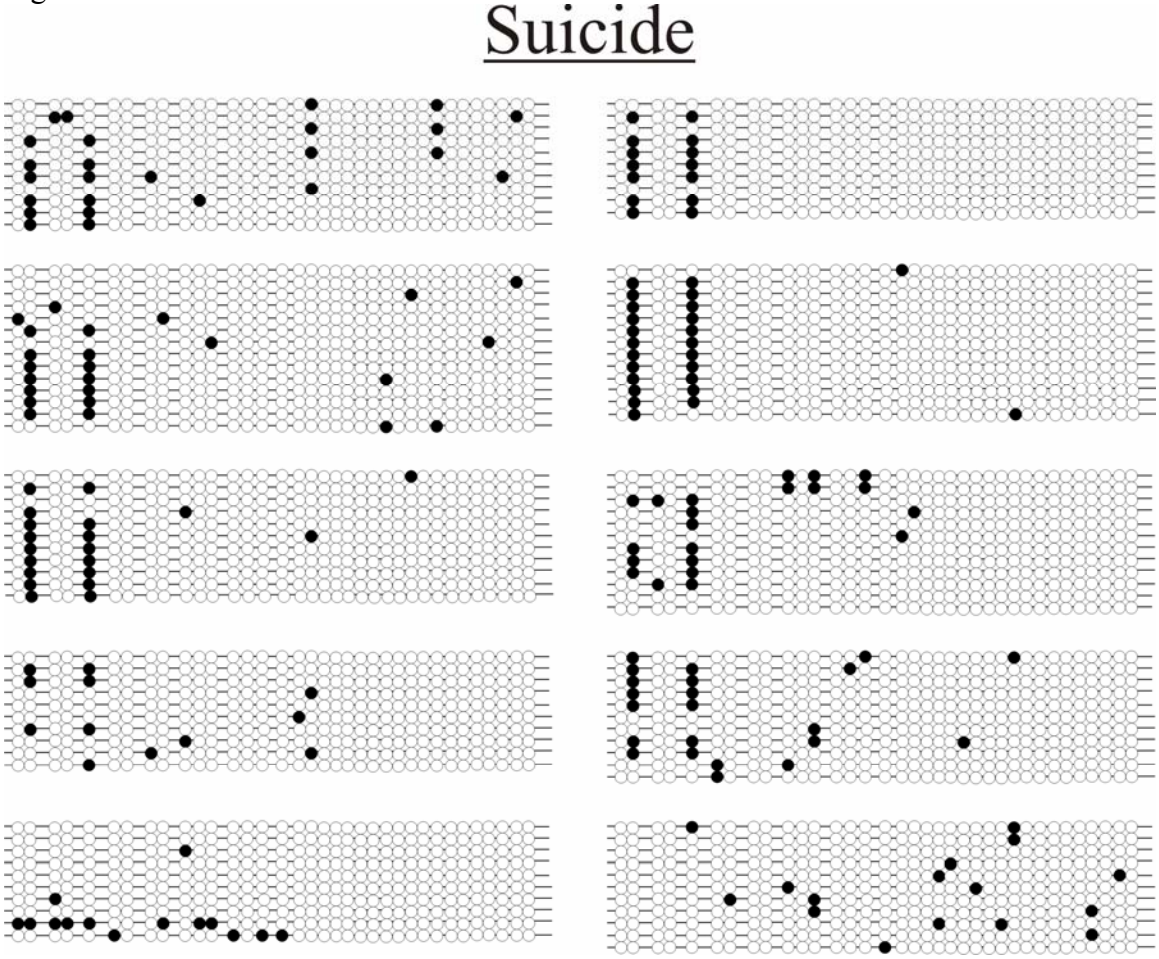
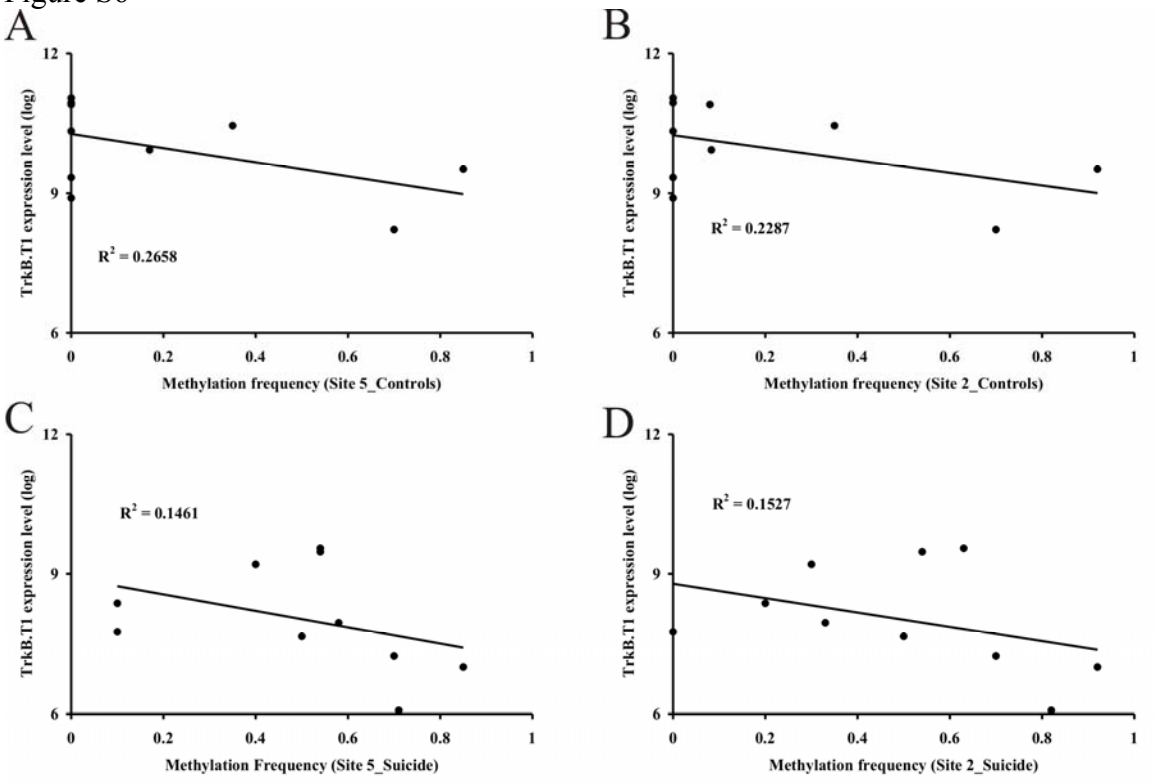


Figure S6


A
TrkB.T1 expression level (log)
12
9
6
0
0.2
0.4
0.6
0.8
1
R² = 0.2658
Methylation frequency (Site 5_Controls)
B
TrkB.T1 expression level (log)
R² = 0.2287
Methylation frequency (Site 2_Controls)
C
TrkB.T1 expression level (log)
R² = 0.1461
Methylation Frequency (Site 5_Suicide)
D
TrkB.T1 expression level (log)
R² = 0.1527
Methylation frequency (Site 2_Suicide)

CHAPTER 3: PREFACE

While our findings concerning reduced expression of TrkB.T1 and the relationship with promoter methylation state were interesting, they did not fully account for the severe decrease in TrkB.T1 expression observed. For the following study, we investigated what other molecular mechanisms might explain the decrease in TrkB.T1. We opted to test the hypothesis that another epigenetic mechanism may be at play. More specifically, our question was:

Does increased methylation at histone (H) 3, lysine 27 at the TrkB genomic region associate with decreased expression of TrkB.T1?

The following study will describe how our assessment of DNA extracted from BA10 and cerebellum in suicide completers and controls led to a better understanding of the role of H3 lysine 27 and TrkB.T1 expression.

Of interest also, is that we serendipitously uncovered a deletion in the TrkB gene while performing this study. For this project, I would direct the reader to Appendix 3

Histone methylation and decreased expression of TrkB.T1 in orbital frontal cortex of suicide completers

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Decreased expression of full length TrkB and TrkB.T1 have been implicated in suicide and major depression and this may be due to an epigenetic mechanism (Dwivedi et al., 2006, Castren and Rantamaki, 2008, Ernst et al., 2009).

Methylation of certain histone residues is thought to decrease transcription at DNA close to the histone complex. Residues studied to date include lysine 4, 9, 27, and 36, all of which are present on the histone H3 protein. Of particular interest to the current study is H3 lysine 27. This residue has been shown to have increased methylation at the P3 and P4 BDNF promoters in rats who underwent chronic defeat stress (an animal model of depression) compared to a control group (Tsankova et al., 2006). As the molecular mechanism of H3 lysine 27 methylation related to decreased transcription is known (Cao et al., 2002), and histone methylation analysis in post-mortem brain is experimentally feasible (Huang et al., 2006), we opted to test whether H3 lysine 27 methylation at a *TRKB* promoter locus was increased in orbital frontal cortex of suicide completers as compared to control subjects.

Ten control subjects and 20 suicide completers were included in this study. Subjects were recruited at the Montreal morgue and underwent full psychological autopsy procedures, as previously described (Dumais et al., 2005). All subjects were Caucasians of French-Canadian descent. No subjects were used with a history of psychotic symptoms, including bipolar disorder and schizophrenia. One subject of 30 was female. There was no significant differences across groups in pH (t_{28}=0.87, p=0.40; Mean Control = 6.49 + 0.08, Mean Suicide = 6.58 $\pm$ 0.06), age (t_{28}=0.83, p=0.41; Mean Control = 38.7 years $\pm$

4.3 years, Mean Suicide = 35.2 years $\pm$ 2.6 years), or PMI (t_{28}=0.50, p=0.62; Mean Control = 24.9 hours $\pm$ 1.9 hours, Mean Suicide = 26.3 hours $\pm$ 1.8 hours). Nor were any correlations between histone methylation status and clinical variables significant. Sixteen of 20 suicides and eight of 10 controls in this study are identical to those used in the our previous study (Ernst et al., 2009).

For histone immunoprecipitation, we followed procedures laid out expertly here (Huang et al., 2006). DNA was extracted from BA10 and cerebellum from all subjects. We first tested that the IP reaction had worked by using positive (GRIN2A) and negative (HBB) control primers described by Huang et al. (2006). Each fraction of isolated DNA (input and bound) underwent quantitative PCR analysis for each subject.

RNA extraction was performed using Qiagen RNeasy kits, and cDNA conversion was performed using random hexamer primers (Invitrogen) according to the manufacturer's instructions (Roche Molecular Biochemicals). RNA Integrity Numbers (RINs) ranged from 6.0-8.0.

A mixture of 10 µL, containing DNA 5µL LightCycler® 480 SYBR Green I Master and 0.1 µM of the *TRKB* sense 5'-CCCTAGCACACATGAACACG-3' and *TRKB* antisense primers 5'-ATGTAGCCATTCCCAGATCG-3' (112 bp product), were loaded into LightCycler capillaries (Roche Molecular Biochemicals). In separate experiments we evaluated the *GLUL* promoter. Sense: 5'-GTGCCTTTAGCCACCACAAT-3', antisense 5'- TGGGATGTTTCAGACTGGTG-3'. Quality control for primer specificity included

ensuring the presence of a single melting peak followed by pictorial analysis of standard curve and specificity. Reactions were repeated in triplicate. To determine the relative concentrations of *TRKB* promoter immunoprecipatated, a standard curve of 10-fold serial dilutions of a mixture of each of the sample DNA was used to plot the relative Ct value on the y-axis and the amount of DNA used on the x-axis. To calculate the fold-change, the relative amount of the bound fraction was divided by the relative amount of input for each subject. Primers directed against TrkB.T1 mRNA were: Forward: 5'-GGATAAGCCAACAGCAGTCC-3'

Reverse: 5'-GGATAAGCCAACAGCAGTCC-3'

We have previously shown that TrkB.T1 is down-regulated in the frontal cortex of suicide completers in BA 10 (Ernst et al., 2009). There, we found a down-regulation in two different TrkB probe sets that are specific to the TrkB.T1 isoform. We performed RT-PCR in all samples in the current study to determine if the decrease of TrkB.T1 could be validated (t_{28}=2.26, p=0.035).

Methylation analysis of H3 lysine 27 was performed in both orbital frontal cortex (BA10) and cerebellum. After immunoprecipitation using antibodies directed against methylated lysine 27 (H3), we performed DNA RT-PCR on a region of the TrkB promoter on both input and bound fractions from both cerebellum and BA 10, in triplicate for each subject and brain region. In cerebellum, we found no significant difference in methylation at this residue (t_{28}=0.646, p=0.53) between suicides and controls. In BA 10, we found a

significant difference in methylation state at Lysine 27 (H3) between suicides and controls (t_{28}=2.60, p=0.015). Methylation was increased at Lysine 27 in suicide brains.

We next asked whether any segment of DNA, known to contain an astrocyte expressed gene such as TrkB.T1, would be associated with increased methylation at lysine 27 H3 in BA 10. To this end, we performed RT-PCR in BA10 for the glutamine synthetase (GLUL) promoter. We found no significant association between histone methylation and the suicide phenotype in BA 10 (t_{28}=0.67, p=0.51).

We next asked whether expression level of TrkB.T1 inversely correlated with the level of H3 lysine 27 methylation in BA 10. We found consistent trends of significant Pearson values of -0.34, -0.14, -0.16 for TrkB.T1 probe sets 214680_at, 221795_at, and 221796, respectively. In subjects with low levels of TrkB.T1 (0.5 SD below expression level in controls; Pearson =0.61, p=0.046, N=11 suicide completers), however, we found a statistically significant correlation between increased H3 lysine 27 methylation and TrkB.T1 expression level.

This finding suggests that H3 lysine 27 may need to be more heavily methylated before any effect on transcription is observed.

Reference:

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CHAPTER 4: PREFACE

The outlier detection strategy detected the TrkB.T1 gene, but there were a number of other genes as well that showed extreme expression differences in our Gene Expression database. The following study will describe the follow-up with this data-set and how we detected a number of genes that were expressed almost exclusively in astrocytes. For this study, we began our investigation with the Connexin 30 (aka Cx30, GJB6) and Connexin 43 (aka Cx43, GJA1) genes, as it was these probe sets with the most extreme expression levels in the most subjects. For this study, our main questions were:

1) Can the extreme expression levels observed for Cx30 and Cx43 be validated in the laboratory?
2) What other genes correlate with the expression level of Cx30 and Cx43?
3) Can an independent microarray set replicate our findings?
4) What is the molecular mechanisms underlying the decrease in expression levels of the astrocyte-related genes?

An astrocyte-related gene network for suicide

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Abstract
Suicide is a major public health concern, yet little is known of the etiology. Here, we identify six astrocyte related genes as severely down-regulated in brains of suicide completers from two independent samples, representing close to 150 subjects. We use both western blot and RT-PCR to show that the astrocyte related genes Cx30, Cx43, FGFR3, Sox9, SLC1A3, and GLUL are severely down-regulated. These findings are not a result of pH, post-mortem state, or reduced astrocyte number. Finally, we identify Sox9 as a potential transcription factor regulating this network. These results suggest that astrocyte dysfunction may play a role in suicide.

Introduction

Astrocytes are traditionally considered the support cells of the central nervous system, where they are known to serve a number of functions. Some of these functions include sequestering charged molecules to maintain electrical balance in the brain (Somjen, 2002), responding to brain insults (Skaper, 2007), initiating long-range signaling to affect vascular tone (Mulligan and MacVicar, 2004), and playing a role in the formation and maintenance of the blood-brain barrier (Bradbury, 1985), to name a few. Recently, astrocytes have also been identified as neural stem cells in both the developing (Laywell et al., 2000) and adult brain (Doetsch et al., 1999) and have been suggested to be a major factor in neurological disorders (Seifert et al., 2006). Clearly, there are many important roles for astrocytes beyond acting as passive supporters of neurons.

Astrocytes have been implicated in psychiatric disorders. In schizophrenia, astrocyte numbers are reported to be reduced (Cotter et al., 2001b; Steffek et al., 2008), although chronic treatement with antipsychotic treatment results in approximately a 20% reduction in astrocytes (Konopaske et al., 2008), so it is unclear whether the relationship is due to medications. There is evidence to suggest that astroyctic dysfunction in schizophrenia is not merely a reduction in astrocyte number or a result of antipsychotic treatment. For example, Matute et al (2005) report increased expression of an astrocyte-specific glutamate transporter in prefrontal cortex of schizophrenic subjects (Matute et al., 2005). The idea of glial cell dysfunction without a reduction in astrocyte number in schizophrenia is supported by numerous studies (Uranova et al., 1996; Niizato et al., 2001; Rothermundt et al., 2004). In major depression, contradictory studies exist as to

whether there are more or less astrocytes in different brain regions of depressed subjects (Cotter et al., 2001a; Damadzic et al., 2001; Fatemi et al., 2004; Rajkowska and Miguel-Hidalgo, 2007). Astrocytic dysfunction may directly be involved in major depression, however. For example, glia-derived factors stimulate synaptogenesis (Mauch et al., 2001; Nagler et al., 2001; McNally et al., 2008). Another intriguing example of astrocyte dysfunction without loss of cell number is the affect of riluzole in treating depression (Zarate et al., 2004), a drug thought to up-regulate the (SLC1A3/A2) glial glutamate receptors (McNally et al., 2008).

This study began with no pre-conceived notions of which genes may be dysregulated in suicide. Using a microarray screen for individual differences in extreme expression values (Ernst et al., 2008) combined with a conventional microarray analysis (Allison et al., 2006), we identified SLC1A3, FGFR3, Cx43, Cx30, Sox9 and GLUL as severely down-regulated in brains from suicide completers from both the Quebec Suicide Brain Bank and the Stanley Foundation. These genes are all astrocyte-related and suggest a common regulatory pathway as well as astrocyte dysfunction in suicide.

Methods

Subjects

All subjects were recruited from the Montreal morgue. Upon case assessment, next-of-kin were contacted and asked to participate in the study. Suicide cases and controls passed this initial assessment if there were no reports of agony at time-of-death and if the post-mortem delay was under two days. Families that answered in the affirmative to participate in the study consented to brain removal and the release of medical records of the deceased, as well as to participate in structured clinical interviews to determine likely psychiatric diagnoses. All subjects underwent toxicological analysis, where over 50 different agents are assessed (e.g. antidepressant, alcohol, cocaine). All procedures in this study were approved by the ethical review board of McGill University

After psychological autopsies and assessment of medical records, subjects were enrolled in the study if 1) they were of French-Canadian origin (both grandparents born in Quebec) and 2) no psychotic diagnoses were detected (including Bipolar Disorder), and 3) were Caucasian. The final two criteria were to reduce the heterogeneity of the sample.

Neuroanatomy

Brains were analyzed and dissected at the Douglas Hospital Research Institute. Brain were dissected into Brodmann regions based on histological maps (Haines, 2000; Nolte, 2002) and gyri/sulci landmarks. Three different brain regions were also sectioned and Nissl stained for any signs of pathology. After dissection, brain sections were flash frozen in isopentene and stored at -80C. DNA and RNA was isolated with appropriate extraction kits (Qiagen). All concentrations were done using NanoDrop.

Microarray analysis

Microarray analysis was performed on 30 subjects using the Affymetrix Human Genome (HG)-U133 plus 2 chip. RNA samples used had a minimum A260/A280 ratio of > 1.9. The samples were further checked for evidence of degradation and integrity. Samples had a minimum 28S/18S ratio of > 1.6 (2100-Bioanalyzer, Agilent Technologies). Expression data was analyzed using Genesis 2.0 (GeneLogic Inc, Gaithersburg, MD) and AVADIS (Strand Genomics, Redwood City, Calif). Several RNA integrity measures were used in the screen to detect samples with poor RNA quality before final analysis. Microarray quality control parameters used included the following: noise (RawQ), consistent scale factors, and consistent β-actin and GAPDH 5'/3' signal ratios (>0.3 and 0.5, for all probes respectively). We performed our analysis using the Robust Multiarray Average algorithm (Irizarry et al., 2003).

Stanley brain microarray analysis

The brain tissues were meticulously collected in a standardized manner via pathologists in the offices of the Medical Examiner in several states with the families' permission under the aegis of the Stanley Foundation Brain Collection (Array Collection plus Consortium Collection). The selection of specimens, clinical information, diagnoses of patients, and processing of tissues were conducted by Stanley Foundation Consortium as described previously Gene expression profiling utilized post-mortem prefrontal cortex

(Brodmann's Area 46/10) mRNA and Affymetrix Human Genome U133 Set A (HGU133A) using standardized techniques as described.

The robust multi-array averages (RMA)-normalized microarray data from four independent studies were downloaded from the SMRIDB. Microarray data from the same platform, Affymetrix Human Genome U133 Set A (HGU133A), were used to avoid platform-to-platform variation. The platform contains 22,215 probe sets.
For the bipolar disorder cohort, the total dataset consisted of 49 suicide completers' gene chips and 58 non-suicide gene chips, while for schizophrenia cohort, the total dataset consisted of 22 suicide completer gene chips and 89 non-suicide chips. Among 45 bipolar samples, there were 22 suicide cases, and 23 non-suicide cases. Among 45 schizophrenia patients, there were 10 suicide cases, and 35 non-suicide cases. Two to three microarray chip datasets were generated from the each patient's sample. These repeated microarray data from each patient were treated as technical replicates.

Microarray data was analyzed by a statistical method described previously with slight modifications. Briefly the following steps were followed within each diagnostic group. First, the differentially expressed genes between suicide completers vs. non-suicide groups were filtered by average fold change ($FC \geq |1.3|$) using the BRB-array tool[30] without covariates. Second, the influence of continuous demographic variables (such as age, post-mortem interval (PMI) and brain pH) with the nominal variable suicide was tested using ANOVA. Then, categorical variables such as sex, smoking, alcohol and drug abuse were tested using chi square tests of association (Statview software SAS, Cary,

NC). In addition, correlation analyses of the demographic factors with expression levels of the differentially expressed probe sets from step 1 were performed. Continuous variables were analyzed by Spearman's rank correlation and categorical variables were tested by ANOVA. P-values were adjusted by False Discovery Rate (FDR) in both tests. Third, significant confounding factors were tested as possible covariates for ANCOVA model inclusion.

Westerns

Twenty-five □g of brain homogenate and sample buffer (0.25M Tris-HCl, 20% glycerol, 4% SDS, 0.005% bromophenol blue, and 5% □-mercaptoethanol) were boiled at 95^{o}C for 5 minutes. Next, samples were loaded on a Novex sure-lock electrophoresis system (Invitrogen), using 4-20% precast gels (Invitrogen). All gels were loaded pseudo-randomly. After samples were run and the gel transfer was complete, membranes were blocked with a 5% milk solution (Carnation fat free milk, in Tris-buffered saline; TBS) shaking for 1 hour. Membranes were submerged in a 5% albumin solution (Albumin in TBS with 0.2% Tween) with primary antibody and left shaking overnight at 4^{o}C. Sixteen hours later, membranes were thoroughly washed and left shaking for 1 hour with secondary antibody. Membranes were then exposed using an ECL kit (Amersham) and apposed to film. In order to verify the accuracy of loading, membranes were stripped and re-probed with an antibody to □-actin (Santa Cruz). Optical density readings for bands were determined using Gene Tools software, a computer-assisted densitometry system.

QPCR

RNA was extracted from adjacent samples to those taken for the microarray analysis. Quality of RNA was assessed using an Agilent Bioanalyzer and no RNA was used that had an RNA Integrity number (RIN) value of less than 5. For semi-QPCR procedure, we

used the QuantiTect probe PCR kit (Qiagen) and followed the instructions from the manufacturer. Linear range analysis was performed prior to semi-QPCR experiments with cycles from 20-35. Finally, we sequenced the PCR product to ensure that the single band observed was measuring the appropriate product. All samples were PCR amplified and run on a gel with both the internal control (□-Actin) and gene-of interest PCR products processed together. Optical density readings for bands were determined using Gene Tools software, a computer-assisted densitometry system.

Animal experiments

Cocaine administration

Rats were randomly assigned to either vehicle (*n* = 5; 1 ml/kg saline ip) or cocaine (*n* = 5; 10 mg/kg ip) conditions and received daily injections of the designated treatment for 5 consecutive days. Rats were sacrificed 20 min following the final treatment. The frontal cortex was quickly dissected, flash frozen in isopentene and stored at -80°C until further analysis. This experiment was repeated in 10 new rats (*n* = 5; 1 ml/kg saline ip) or cocaine (*n* = 5; 10 mg/kg ip), and RNA levels were assessed in frontal cortex

Acute Ethanol (EtOH) Administration

Rats were randomly assigned to either vehicle (*n* = 5; 1 ml/kg ip) or EtOH (*n* = 5; 2.5g/kg EtOH ip; 15% v/v EtOH in 0.9% saline) treatment conditions. Doses were based upon previous studies (Schulz et al., 1980; Seizinger et al., 1983). Each rat received a single injection of the designated treatment and was sacrificed 3 hr later by live decapitation. The frontal cortex was quickly dissected, flash frozen in isopentene and stored at -80°C until further analysis.

Chronic Ethanol Administration

Food and water consumption were monitored for 3 days prior to treatment to ensure no differences in baseline consumption existed between the treatment groups. Rats were then randomly assigned to one of 3 treatment conditions: water control (n = 5), sucrose control (n =5; 10% sucrose solution) or EtOH (n = 5; 15% EtOH in a 10% sucrose solution). Sucrose was added to the EtOH solution so that the rats would consume the ethanol without previous training (Czachowski et al., 1999). Once rats in the EtOH group readily drank the 10% sucrose solution (1 day) they were gradually habituated to the 15% EtOH solution(Samson et al., 1999). EtOH rats received 5% EtOH in 10 % sucrose for 1 day, followed by 10% EtOH in 10% sucrose for 2 days. The solution was then changed to the 15% EtOH in 10% sucrose. Chronic EtOH treatment persisted for 28 days once the rats had access to the 15% EtOH solution. During this time, rats received only the designated treatment as their sole source of liquid. Food and liquid consumption was monitored daily at the beginning of the dark-cycle and body weight was measured weekly. Twenty-nine days after the 15% EtOH treatment began, rats were sacrificed prior to lights off. Brains were removed and frontal cortex was quickly dissected, flash frozen in isopentene, and stored at -80°C until subsequent analysis

Sox9 knock-down experiments

In rat astrocyte cell lines we used shRNAi (Invitrogen) directly from the manufacturer to transiently block Sox9. In human testicular cell line, we used RNAi (Invitrogen) for transfection. All transfection were performed with Lipofectamine2000 over a 24 hour period, unless otherwise noted.

Results

We isolated RNA from 12 cortical regions (BA 4, 6, 8/9, 11, 44, 45, 46, 47, 20, 21, 24, 38) from the brains of controls (n=12) and suicide completers (n=24). Each control was matched to two suicide completers fro age, PMI, and pH. To begin our analysis, we performed a standard microarray analysis in BA 8/9 (Allison et al., 2006). We selected for further processing those probe sets that differed by greater than $\pm$ 1.5 ($p<0.05$) between suicide and control groups. We complemented this approach with EVA (Ernst et al., 2008), an approach that attempts to account for heterogeneity in psychiatric data research. Next, we selected all probe sets that were common across both the standard analysis and EVA (Table 1). We chose to investigate the Cx30 and Cx43 genes because of the magnitude of the effect in the EVA analysis in 4 subjects (i.e absence of Cx30) and the fact that these two genes interact in brain, and their potential role in mood disorders, based on mice Knock-out studies (Dere et al., 2003; Frisch et al., 2003).

We analyzed Cx30 across all 12 cortical regions and found almost identical patterns expression to BA 8/9 throughout, (Figure 1A). We note that using standard microarray analysis, GJB6 is significantly different from controls in 10/12 regions and that the same subjects had extremely decreased expression of Cx30 across all cortical regions. The correlation (r-sq) between Cx30 and Cx43 is >0.80 in all regions.

While we observed a significant reduction in both Cx30 and Cx43 when comparing suicide to control groups, we opted to continue our analyses in only a subgroup of suicide subjects; these subjects were selected based on EVA criteria and in attempt to study a

more homogeneous population (i.e. suicide completers with profound reduction in Cx30/43).

We matched 4 subjects with extreme expression values of Cx30 and Cx43 and matched them to 2 controls each (n=8 controls). We performed both QPCR and western blot experiments and observed a profound reduction in Cx RNA and protein in brain of suicide subjects (Figure 1B, C, D, E), as would be expected from microarray results. For QPCR experiments, we isolated new RNA from six frontal regions.

This finding was not explained by age, PMI, or pH values as control subjects and suicide completers did not differ in any of these variable in the total microarray group (n=36; table 2), or when the 4 extreme Cx30 expressors were matched to 8 control subjects (n=12, table 3).

From psychological autopsies and chart information, some subjects had cocaine (N=3) and alcohol (N=6) in their system at time-of-death. To understand if alcohol or cocaine exposure decreases expression of Cx30 and Cx43, we treated rats with either cocaine or alcohol (chronic and acute), extracted RNA from the frontal cortex, and performed QPCR (Figure 2A).

Cx30 is an astrocyte-specific protein. To determine if the level of Cx30 is only a function of the number of astrocytes we interrogated GFAP, the most commonly used

marker of astrocytes, in the same four suicide subjects and eight matched controls (Figure 2B).

Correlational analyses of Cx30 with all other expressed genes across cortical regions

To better understand the reason for the profound decrease in Cx30/Cx43 proteins in the brains of some suicide completers, we correlated all genes in the microarray experiment to Cx30 across all brain regions and all subjects. The purpose of this experiment was to determine if any genes correlated well with Cx30, which may be a sign of mutual regulation, or relationship.

In 12 cortical brain regions, we correlated expression level for all probe sets across all subjects to Cx30 expression level. Next, we calculated a mean r-squared value for all probe sets across all brain regions, and ranked them. We calculated the number of 'hits' which refers to the number of times a given probe set in a given region met criteria (>0.75) The probe sets with the highest rank (Table 4) may co-regulate with Cx30. Presented in table 4 are all probe sets that had r-squared values >0.75. Of note is the number of genes that are preferentially expressed in astrocytes.

External validation with Stanley brains

A publicly available microarray dataset (Kim et al., 2007a), also investigating the suicide phenotype, was also independently analyzed in BA46/BA10. This analysis was performed blind to the results of this study. Brains from this study were from the Stanley brain bank where varying numbers of subjects were used in different diagnoses: Bipolar

disorder (N = 45; 22 suicide completers; 23 non-suicide), Schizophrenia (N = 45; 10 suicide completers ; 35 non-suicide). 243 genes passed false discovery rate criteria and were expressed differentially from between suicides and non-suicides were compared. We cross checked all probe sets from our correlational analysis with the Stanley replication results (Table 4). To ensure the validity of the Stanley analysis, we selected Sox9 and Cx43 for RT-PCR analysis (Figure 3)

Over-representation of astrocyte-related genes in Stanley and QSSB involved in cell growth and glutamate maintenance

From the correlational analysis and the replication in Stanley brains, we noted a series of astrocyte related genes. This is not a function in gene ontology propgrammes, such as DAVID. Known astrocyte-related genes include Sox9 (Pompolo and Harley, 2001), GLUL (Hertz and Zielke, 2004), FGFR3 (Pringle et al., 2003), SLC1A3 (Lee et al., 2007) and TrkB.T1 (Rudge et al., 1994; Rose et al., 2003), Cx30 (Nagy et al., 1999), and Cx43 (Bennett et al., 2003).

First, we validated the expression levels of Sox9, GLUL, FGFR3, and SLC1A3 in the same study design as for Cx30/43 (4 suicide completers vs 8 matched controls – Figure 4). We note that TrkB.T1 was validated and thoroughly explored in a different study (Ernst et al., 2009).

We again performed QPCR with rat frontal cortex RNA from acute-cocaine and acute and chronic-alcohol affected rats. We found no associations between the expression level

of these genes and presence of alcohol and cocaine; however, there was a significant increase in Cx43 on cocaine treated rats ($t=2.43$, $p=0.04$).

From our correlational analysis and the independent replication of the suicide phenotype, we chose to investigate the gene with the strongest effect, the transcription factor Sox9 (the number 1 ranked gene from the Stanley analysis, with two different probe sets), which could also be a regulator of all of the astrocyte-expressed genes. In brain, Sox9 is expressed solely in astrocytes, although its function is unknown. Mutations in Sox9 cause campomelic dysplasia (Wagner et al., 1994). Campomelic dysplasia is thought to be an autosomal dominant disorder, occurring sporadically in effected individuals. We note the overlapping phenotype of Sox9 and Cx30 mutations, namely hearing loss and skeletal abnormalities (Kibar et al., 1996; Olney et al., 1999).

Interactions of Sox9

The Sox9 consensus site is 10 bps long and Sox9 is thought to bind as a monomer. AACAAT is the general SOX consensus site, while the addition of AG and GG to the ends define the specificity for Sox9 (AGAACAATGG) (Mertin et al., 1999). We searched DNA in Cx43/30, GLUL, SLC1A3, FGFR3, TrkB for any Sox9 consensus sites. All of these genes had potential Sox9 binding sites.

RNAi

We investigated whether transiently reducing Sox9 expression could affect the expression of any of: Cx30, Cx43, SLC1A3, TrkB.T1, GLUL or FGFR3. To perform this

experiment we used a testicular human cell line (wrong tissue, right species), known to express Sox9, and a rat astrocyte cell line (wrong species, right tissue).

Human testicular cell line

We transfected a human cell line with an RNAi molecule specific to SOX9 mRNA and a plasmid that generated a non-specific RNAi molecule (negative control). To begin, we extracted RNA from cells 24 hours after transfection and measured relative levels of the genes of interest in knock-down and negative control transfected wells. We found that neither TrkB.T1 nor Cx30 was expressed in this cell line, as anticipated. We performed transfection experiments in triplicate across 4 different concentration ranges for the shRNA plasmid (90 pmol, 120 pmol, 140 pmol, or 160 pmol). First, we found that our transfection protocol was able to knock down Sox9 by 40-50%, with a decrease in Sox9 expression as the RNAi concentration increased (Figure 5A) We found a significant decrease in Cx43 (four concentrations done in triplicate, N=4, $p=0.04$ and SLC1A3 ($p=0.003$) but not in GLUL ($p=0.57$) or FGFR3 ($p=0.20$) (Figure 5). In the case of Cx43 we observed what appeared to be a dose specific decrease in Cx43 expression as Sox9 RNAi concentrations increased. This relationship was not observed in the case of SLC1A3.

Rat astrocyte cell line

We next assessed what the effects of knocking down Sox9 were on the expression of the validated genes from human testicle (Cx43 and SLC1A3) as well as the genes that are not expressed in human testicle (Cx30). Using two different plasmids that express RNAi

molecules targeting different regions of Sox9 mRNA as well as a plasmid expressing an RNAi molecule not specific to Sox9 (negative control), we extracted RNA and protein from astrocyte cells 24 hours after transfection and performed semiQ PCR. First we found a decrease in Sox9 of > 40% for both plasmids. We found a decrease in Cx43 of >16% and a large decrease in Cx30 of close to 100%. We found a decrease in SLC1A3 of >10% for both Sox9 shRNA plasmids.

There was a severe decrease in Cx30 with a decrease of Sox9 of only 40%. Firstly, we note that these variations in expression level are very similar to that observed in suicide brain (i.e. profound reduction in Cx30 with a significant decrease in Sox9). While it is not necessarily expected that a decrease in Sox9 will lead to a corresponding decrease in Cx30, so we opted to perform further tests. We again transfected Sox9 shRNA but extracted RNA at 4, 8, and 24 hours. We found that, even within four hours, Cx30 expression was reduced by 70%, by eight hours 95%, and absent at 24 hours. Finally, we asked whether transfection of shRNA sox9 could affect the protein level of Cx30, based on our observations from the RNA experiments. First we note that, in this rat astrocyte cell line, Cx30 is detected at 36 KDa including in control lanes as compared to Cx30 from human brain. This may be due addition of glutathione groups in rat Cx30 at the CYLLLKVC motif in this particular astrocyte cell culture. We observed a large reduction in Cx30 protein when shRNA sox9 was transfected into this cell line.

Discussion

Pathway connections

Sox9 interacts with the genes in the pathway identified. Sox9 has a known role in chondrogenesis (bone formation), where it was first shown to be a transcriptional regulator (Lefebvre et al., 1997). In the case of FGFR3, FGF18 has been shown to signal through the FGF receptor 3 to stimulate chondrogenesis (Davidson et al., 2005). FGFR2, a similar gene to FGFR3 has also been reported to be a downstream regulator of Sox9 (Ross and Capel, 2005) and FGF9 mutations cause sex –reversal, a similar phenotype to Sox9 mutations (Colvin et al., 2001) FGF5 and FGF9 are also know to stimulate upregulation of Cx43 (Reuss et al., 2000) We note also overlapping phenotypes in Clouston syndrome (non-functional Cx30) and Campomelic dysplasia (non-functional Sox9) in the region where both are expressed (ear): severe hearing loss. Cx30 and Cx43 are known to be the major mediators of calcium transients in the CNS. A recent study reported that stimulation of astrocytes with BDNF stimulates these calcium waves through the TrkB.T1 receptor (Rose et al., 2003).

We note that a number of these genes have also been reported to be activated by, or interact with, glucocorticoids in bone including GLUL and SLC1A3 (Chandrasekhar et al., 1999; Kalariti et al., 2007), FGFR3 (Walsh et al., 2000), and brain for TrkB (Schaaf et al., 1997; Numakawa et al., 2009). We note also that glucocorticoids are potent stimulators of calcium astrocyte signaling, a process mediated by Cx30 and Cx43 (Simard et al., 1999). In these cases, stress (glucocorticoids) up-regulates this gene

network. Given that most of this work has been done in bone, it will be interesting to see whether increased stress levels increase the expression of these genes.

Hypoxia and down regulation of astrocyte-related genes

While we made every effort to control for post-mortem factors, method of death cannot be controlled for given the nature of the study. As most suicide completers died by hanging we investigated the literature to determine what the effect of hypoxia is on the astrocyte related genes. First, we note that a number of studies have assessed Cx43 under hypoxic conditions in multiple different tissues and found an upregulation of the mRNA and protein. Under hypoxic conditions, Cx43 is up-regulated, for example after occlusion of the middle cerebral artery in mice (Lin et al., 2008). More importantly, Cx43 is also up-regulated in the area surrounding a neural infarct in human (Nakase et al., 2006). Indeed, Cx43 seems to have a neuroprotective affect and Cx43 upregulation in response to hypoxia in kidney has been recently reviewed, with multiple examples cited (Lin et al., 2008). Cx43 gap junction channels are thought to be blocked and down-regulated in response to inflammation however, for example in Alzheimer's disease (Kielian, 2008). In our study, brains were screened AD. As subjects were free of inflammatory diseases and hypoxic conditions are thought to increase expression of Cx43, we suggest that hanging-induced hypoxia likely does not account for the severe decrease in Cx43 observed in suicide brains. A specific study was done to test the effect of hypoxia on SLC1A3; SLC1A3 is not affected by hypoxic conditions (Cimarosti et al., 2005). Glutamine synthetase, similar to Cx43, is known to be increased in cultured astrocytes in response to hypoxia (Kobayashi and Millhorn, 2001). This report also

confirms that hypoxic conditions have no effect on SLC1A3. In vivo experiments assessing the effects of hypoxia on GLUL suggest no long term decrease in expression (Krajnc et al., 1996). Similarly, FGFR3 expression levels do not decrease under hypoxic conditions (Khnykin et al., 2006). While no work has been done on Cx30 and TrkB.T1 in hypoxia to our knowledge, TrkB. FL is known to be up-regulated in response to hypoxia (Meng et al., 2005; Martens et al., 2007a). While we acknowledge that hypoxia in experimentally induced in rat or cell culture does not fully replicate the cellular processes that occur after death by hanging, it does give some indication that lack of oxygen does not seem to affect the astrocyte gene netwok investigated here.

Astrocyte connexins in mood disorders

Cx30 and Cx43 are both strongly expressed in astrocytes in brain. They function to form a channel between astrocyte cells that allows the flow of material though astrocytes. We note that, in animal KO models, both Cx30 and Cx43 have been implicated in Depression-like symptoms (Dere et al., 2003; Frisch et al., 2003). Further, Lithium treatment (Li+ is used to treat bipolar disorder) is known to increase Cx43 through a WNT-mediated pathway (van der Heyden et al., 1998; Ai et al., 2000). Finally, a role of astrocytes in the brain is to create calcium waves, a process mediated by Connexin 43 and Connexin 30. This calcium wave velocity and Cx43 expression is increased in brain sections exposed to serotonin (Blomstrand et al., 1999a; Blomstrand et al., 1999b). Finally, we note that the application of pure serotonin to non-CNS connexin channels increases their open probability (Moore and Burt, 1995).

Clinical applications

Our study generates a number of potential clinical applications. We hypothesize that drugs targeting any of the genes identified in this study could have an impact on depressive symptoms in humans. There is good evidence that this may already be the case. We note that the SLC1A3 up-regulator, riluzole, has demonstrated initial efficacy in treating depressive symptoms (Zarate et al., 2004). Mefloquine, an antimalarial drug, is known to induce psychotic symptoms and is contraindicated people with mood disorders (Stuiver et al., 1989; Wooltorton, 2002). Intriguingly, this drug acts to block connexin channels, at low concentrations Cx36 and Cx50 but also Cx43 and other connexins at higher concentrations (Cruikshank et al., 2004). While this drug has the opposite of a therapeutic effect, it suggests that drug treatment to connexins can have an effect on mood. Finally, a main function of Cx30/Cx43 binding is to generate calcium transients in astrocytes. The full reason for the function of these waves is unknown, yet a decreased expression of Cx30/Cx43 could have a negative effect on calcium wave propagation. Targeting systems that increase calcium transient in astrocytes , like TrkB.T1, could be a potential drug target.

Figures

Figure 1. **Identification of 4 suicide subjects with severely reduced levels of Cx30 across 12 brain regions.** A) Raw microarray values from control subjects (n=12), suicide subjects who were not extreme expressors of Cx30 (S_normal, n=20) and suicide subjects who were extremely low expressors of Cx30 (S_extexp, n=4). All values are specific to the Cx30 probeset (231771_at). BA = Brodmann Area. B) Semi-quantitative PCR values from 4 suicide subjects and 8 matched control subjects in 3 brain regions. The 4 suicide subjects are the first 4 bands on the left. C) T- and p-values from semi-quantitative PCR in 6 cortical regions (n=12) for Cx30 and Cx43 expression. The pictures in (B) were used to generate the values in (C). D) Protein analysis of Cx30 and Cx43 in 4 suicide subjects and 8 matched control in BA 11. The 4 suicide subjects are the 4 lightest bands, surrounded by 4 control subjects on either side. Arrow head indicates Cx30 or Cx43. .

Figure 2. **Reduced expression of Cx30 and Cx43 is not due to cocaine, alcohol, or astrocyte number.** A) Representative figures from alcohol and cocaine experiments from rat demonstrating no difference in Cx43 or Cx30 in rat frontal cortex. B) GFAP expression in human frontal brain region is unchanged in subjects with low expression of Cx30 and Cx43

Figure 3. Validation of Sox9 and Cx43 in RNA from frontal cortex of brains from the Stanley collection

Figure 4. Down regulation of genes that correlated with Cx30 expression in the brain of a subset of suicide completers. A) Sox9 pictures and values from QPCR experiments in 6 cortical regions (N=4 suicide subjects and 8 matched control subjects). B) QPCR of GLUL, FGFR3, and SLC1A3 in subjects with low levels of Cx30 and Cx43.

Figure 5. **Transient knock-down using Sox9 RNAi reduces the expression of astrocyte-related genes in human testicle cell lines at different concentrations.** A) Dose dependent knock-down of Sox9 B) Dose dependent reduction in Cx43 C) Reduction of SLC1A3. No effect of Sox9 knock-down on FGFR3 (D) or GLUL (E). Abbreviations: NegC = Negative control RNAi

Figure 6. **Transient knock-down of Sox9 reduces the expression of Cx30, Cx43, and SLC1A3 in rat astrocyte cell lines.** A) Sox9, Cx30, Cx43, and SLC1A3 are reduced after transient transfection with two different plasmids (p1 and p2) containing shRNA targeting different regions of the Sox9 gene. NC is negative control shRNA. Profound reductions in Cx30 after 4, 8, and 24 hour transfection with the p1 shRNA sox9 plasmid. Western blot showing control human brain tissue (HB - no knock-down), and rat astrocytes protein extract showing untransfected control (UT), negative control (NC), and Sox9 shRNA (S9).

Tables

gene symbol	probe set	Gene name
AQP4	226228_at	Aquaporin 4
BAG3	217911_s_at	BCL2-associated athanogene 3
CAMKK2	213812_s_at	Calcium/calmodulin-dependent protein kinase kinase 2, beta
CLDN10	205328_at	Claudin 10
DNAJB1	200664_s_at	DnaJ (Hsp40) homolog, subfamily B, member 1
GJA1	201667_at	Gap junction protein, alpha 1, 43kDa (connexin 43)
GJB6	231771_at	Gap junction protein, beta 6 (connexin 30)
HSPB1	201841_s_at	Heat shock 27kDa protein 1
(HSPA1A, HSPA1B)	200806_s_at	Heat shock 60kDa protein 1 (chaperonin)
(HSPA1A, HSPA1B)	200799_at	Heat shock 70kDa protein 1A, Heat shock 70kDa protein 1B)
(HSPA1A, HSPA1B)	200800_s_at	Heat shock 70kDa protein 1A, Heat shock 70kDa protein 1B)
(HSPA1A, HSPA1B)	214359_s_at	Heat shock protein 90kDa alpha (cytosolic), B member 1,
(LETMD1,	207761_s_at	LETM1 domain containing 1
MGST1	224918_x_at	Microsomal glutathione S-transferase 1
NTRK2	221795_at	Neurotrophic tyrosine kinase, receptor, type 2
NTRK2	221796_at	Neurotrophic tyrosine kinase, receptor, type 2
PPAP2B	212226_s_at	Phosphatidic acid phosphatase type 2B
PPAP2B	212230_at	Phosphatidic acid phosphatase type 2B
PPP1R3C	204284_at	Protein phosphatase 1, regulatory (inhibitor) subunit 3C
QKI	212636_at	Quaking homolog, KH domain RNA binding (mouse)
SLC1A2	208389_s_at	(glial high affinity glutamate transporter), member 2
SLC1A2	225491_at	(glial high affinity glutamate transporter), member 2
SLC1A3	202800_at	(glial high affinity glutamate transporter), member 3
SLC4A4	203908_at	sodium bicarbonate cotransporter, member 4
SYN2	229039_at	Synapsin II
WIF1	204712_at	WNT inhibitory factor 1

Table 1. Probe sets that meet both conventional microarray analysis and EVA criteria in BA 8/9.

	Mean ± SEM		T	p
	Control (N=12)	Suicide (N=24)		
PMI (hours)	23.4 ± 1.7	26.0 ± 1.3	1.23	0.22
Age (years)	38.8 ± 3.6	34.9 ± 2.1	1.10	0.31
pH	6.48 ± 0.06	6.55 ± 0.05	0.83	0.41

Table 2. PMI, Age, and pH comparisons from all subjects in microarray experiment (n=36 people)

	Mean ± SEM		T	p
	Control	**Suicide**		
PMI (hours)	**(N=8)**	**(N=4)**	**0.84**	**0.42**
	23.5 ± 1.5	**26.0 ± 3.0**		
	Control	**Suicide**		
Age (years)	**34.4 ± 3.8**	**29.0 ± 4.0**	**0.88**	**0.40**
	Control	**Suicide**		
pH	**6.54 ± 0.1**	**6.66 ± 0.04**	**0.84**	**0.42**

Table 3. PMI, age, and pH from four suicide completers with low expression of Cx30 and eight matched controls

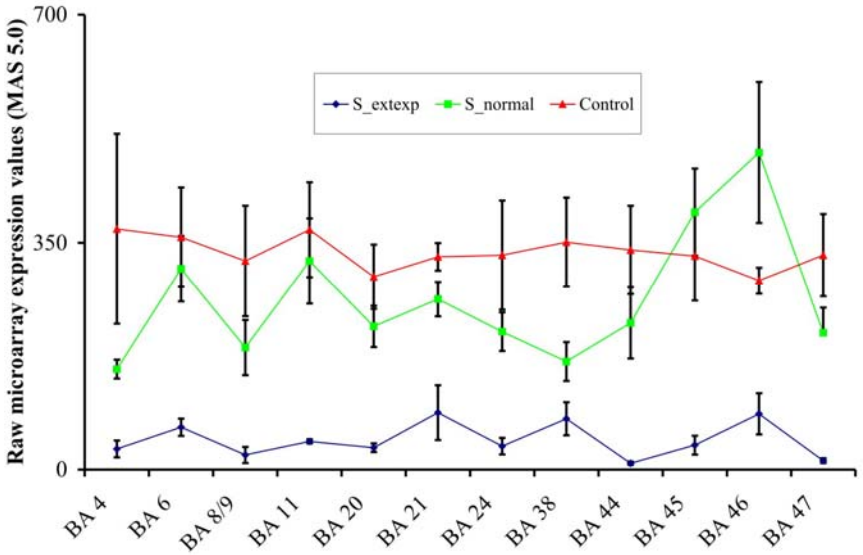
gene name	Hits	MEAN r^2	# of probesets	Stanley FDR p-value
gap junction protein, alpha 1, 43kDa (connexin 43)	7	0.89	1	0.0325
phosphatidic acid phosphatase type 2B	15	0.88	2	0.0348
neurotrophic tyrosine kinase, receptor, type 2 (T1)	18	0.86	3	0.0325 0.0364
solute carrier family 1 (glial high affinity glutamate transporter), member 3	7	0.86	1	0.0364
solute carrier family 4, sodium bicarbonate cotransporter, member 4	14	0.85	2	NA
SRY (sex determining region Y)-box 9 (campomelic dysplasia,	13	0.82	2	0.032 0.032

autosomal sex-reversal)				
aquaporin 4	13	0.81	2	0.0364
solute carrier organic anion transporter family, member 1C1	8	0.81	1	NA
solute carrier family 1 (glial high affinity glutamate transporter), member 2	12	0.8	2	NA
DKFZP586A0522 protein	10	0.8	1	NA
fibroblast growth factor receptor 3 (achondroplasia, thanatophoric dwarfism)	10	0.78	2	0.0483
low density lipoprotein receptor-related protein 4	10	0.77	1	NA
myosin X	8	0.77	1	0.0364
cystatin C (amyloid angiopathy and cerebral hemorrhage)	6	0.77	1	0.0371
glutamate-ammonia ligase (glutamine synthase)	18	0.76	3	0.0418
KIAA0644 gene product	10	0.75	1	0.0421

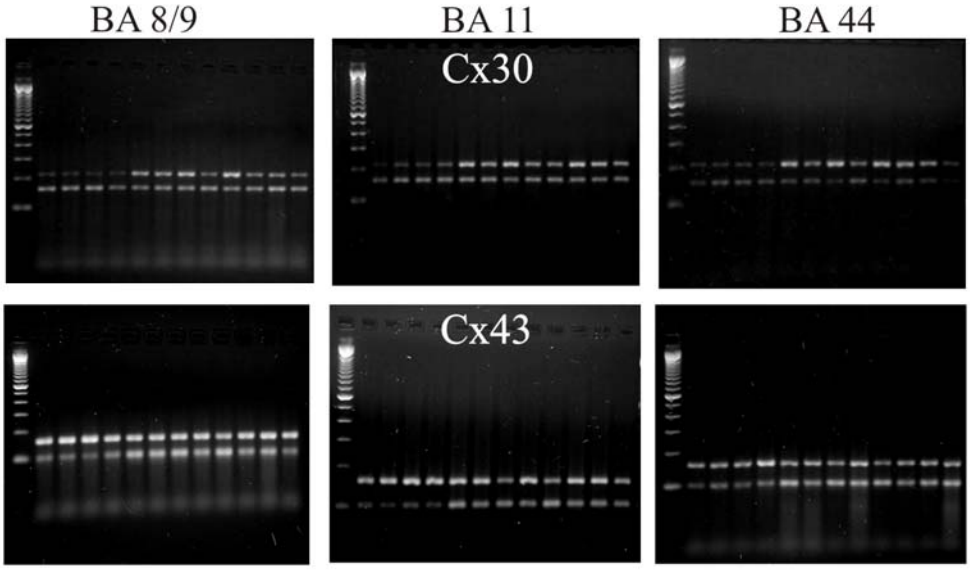
Table 4. **Correlational analysis using microarray data.** All genes were correlated to Cx30 across all brain regions (n=12) and all subjects (n=36), irrespective of mode-of-death. Hits refers to the number of times a probe set corresponding to the same gene .was present. Shown here are the genes with the highest correlation to Cx30. Probe sets that were also screened an independent screen from the Stanley brain bank are noted by the presence of a p-value in the final column.

Figures
Figure1

A



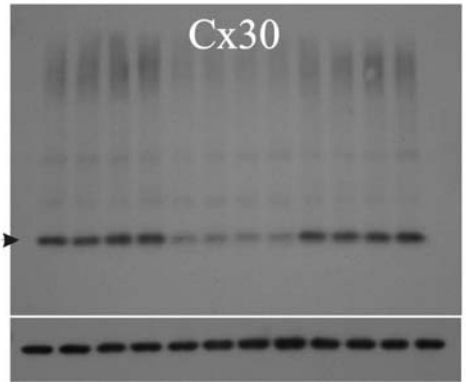
B



C

QPCR	BA4	BA8/9	BA11	BA44	BA45	BA47
Cx30 (T,p)	3.23, 0.009	2.5, 0.031	2.9, 0.016	2.96, 0.014	4.4, <0.001	2.72, 0.021
CX43 (T,p)	4.6, <0.001	2.45, 0.035	5.72, <0.001	3.90, 0.003	5.12, <0.001	3.33, 0.008

D



E

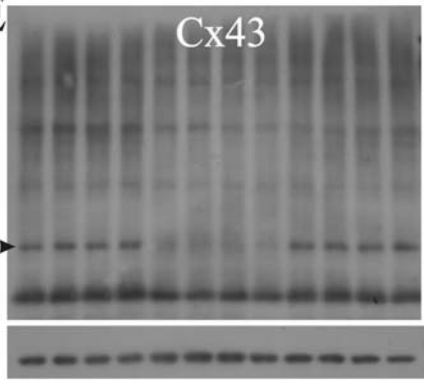
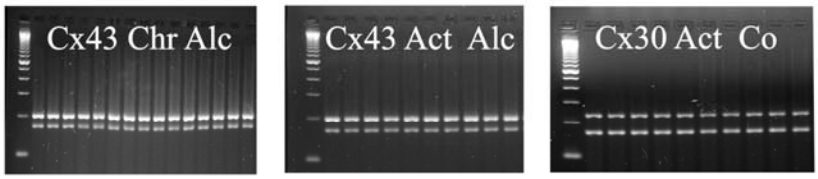


Figure 2

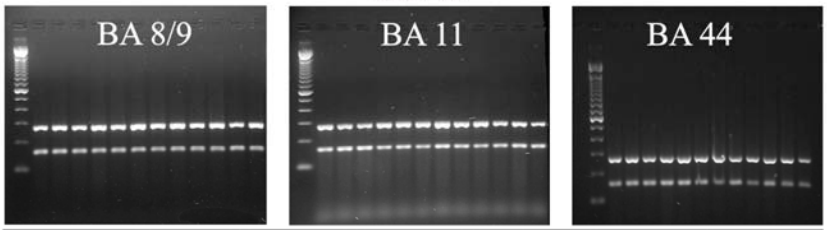
A



Gene	Direction of change	Cocaine	Acute Alcohol	Chronic Alcohol
Cx30 (T/F, p)	increase	1.04, 0.320	1.58, 0.19	0.324, 0.73
Cx43 (T/F,p)	increase	2.47, 0.053	1.44, 0.19	0.432, 0.66

B

GFAP



QPCR	BA4	BA8/9	BA11	BA44	BA45	BA47
GFAP (T,p)	1.08, 0.30	1.17, 0.27	0.95, 0.37	0.30, 0.77	0.52, 0.61	1.46, 0.19

Figure 3

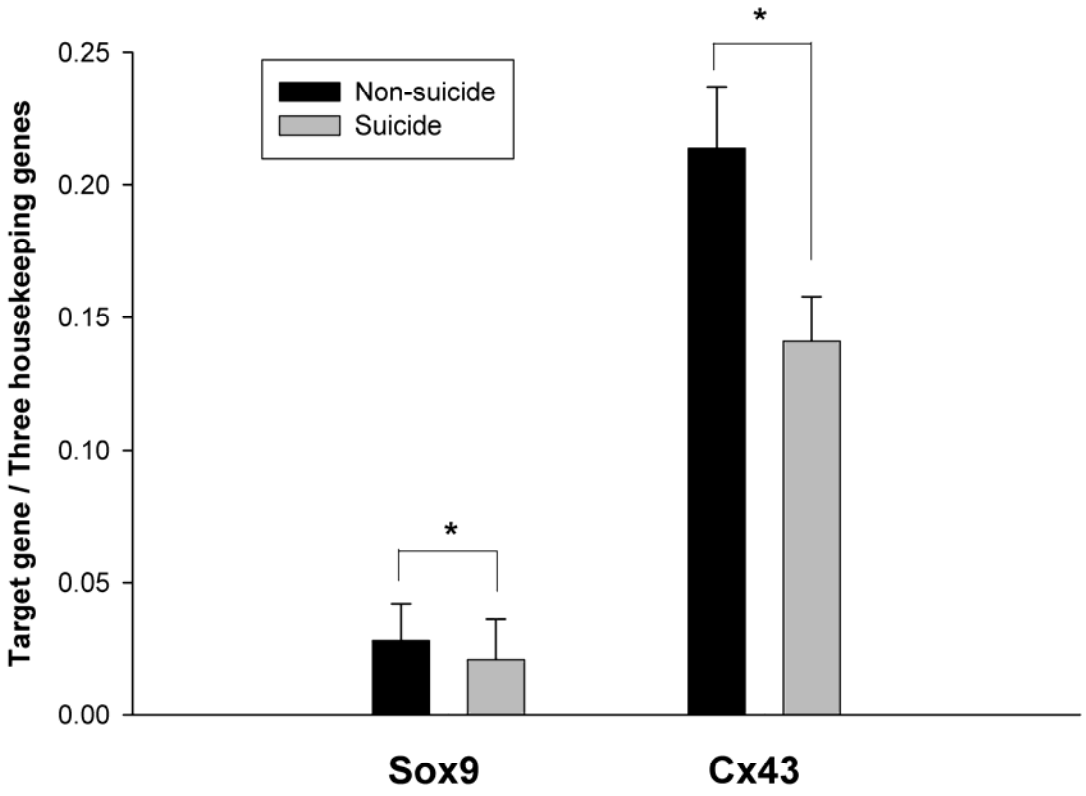
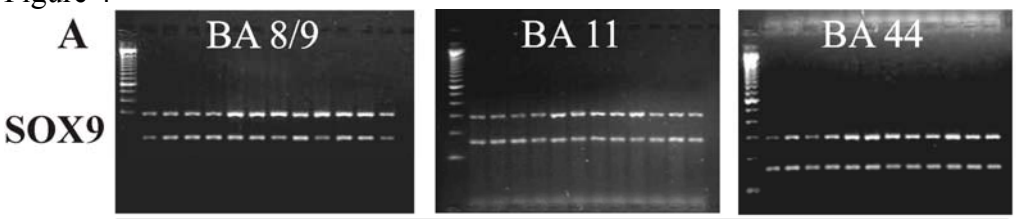
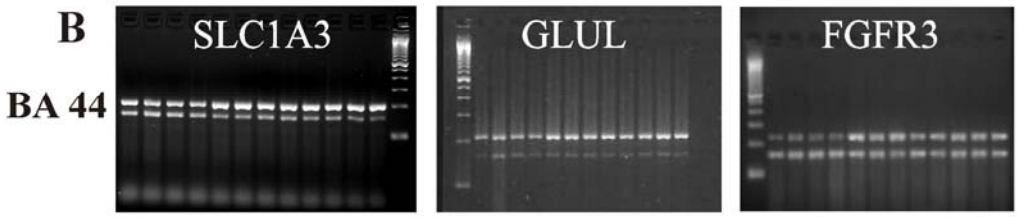

Target gene / Three housekeeping genes
0.25
0.20
0.15
0.10
0.05
0.00
Non-suicide
Suicide
*
*
Sox9
Cx43

Figure 4



QPCR	BA4	BA8/9	BA11	BA44	BA45	BA47
SOX9 (T,p)	3.21, 0.009	4.98, 0.001	3.85, 0.003	2.32, 0.043	5.66, <0.001	3.31, 0.008



QPCR	BA8/9	BA11	BA44	BA47
SLC1A3 (T,p)	4.23, 0.002	2.25, 0.048	3.10, 0.011	4.28, 0.002
GLUL (T,p)	0.97,0.36	2.37, 0.045	3.93, 0.003	1.49, 0.18
FGFR3 (T,p)	3.93, 0.003	2.46, 0.034	5.00, 0.001	4.75, 0.001

Figure 5

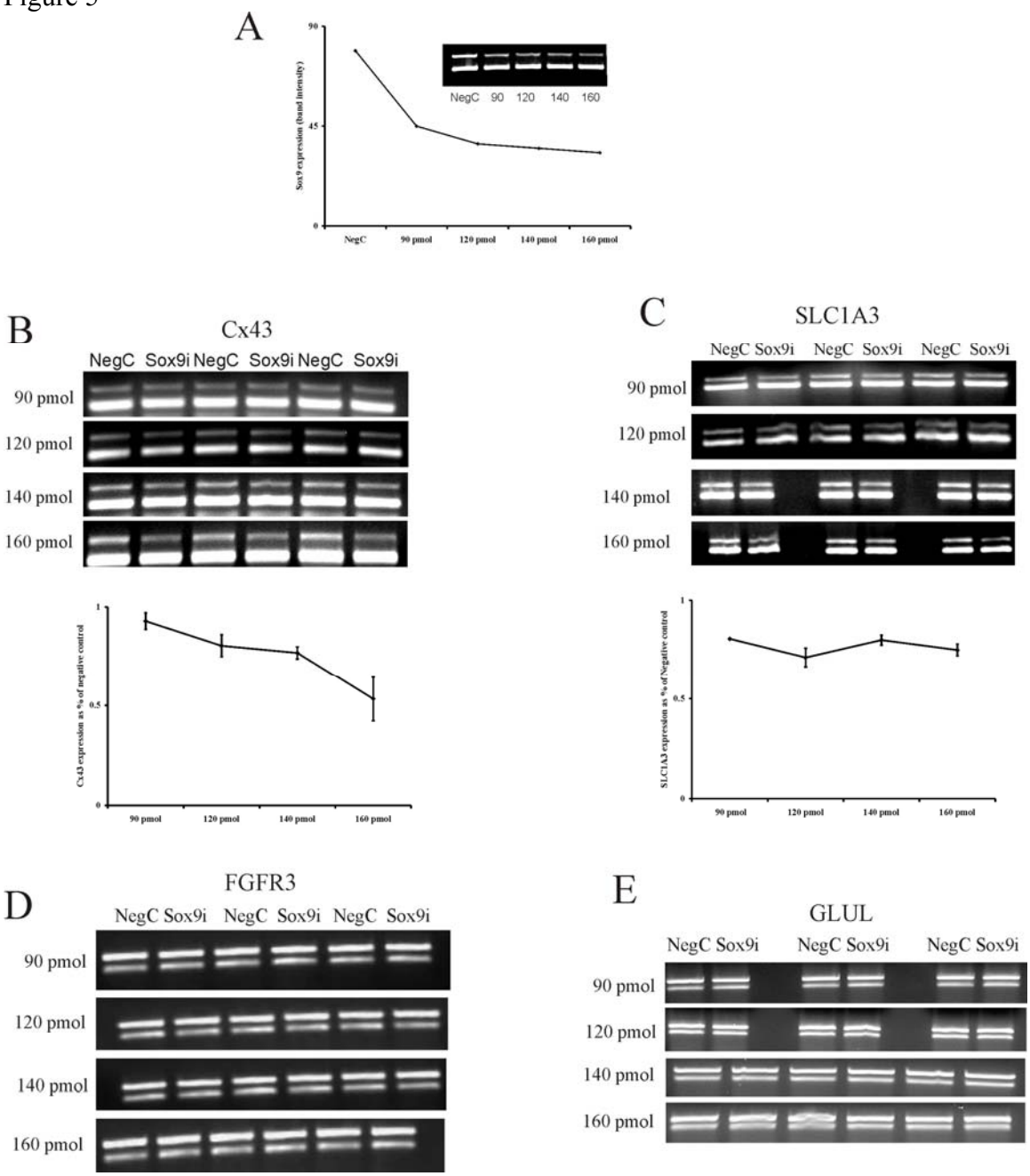
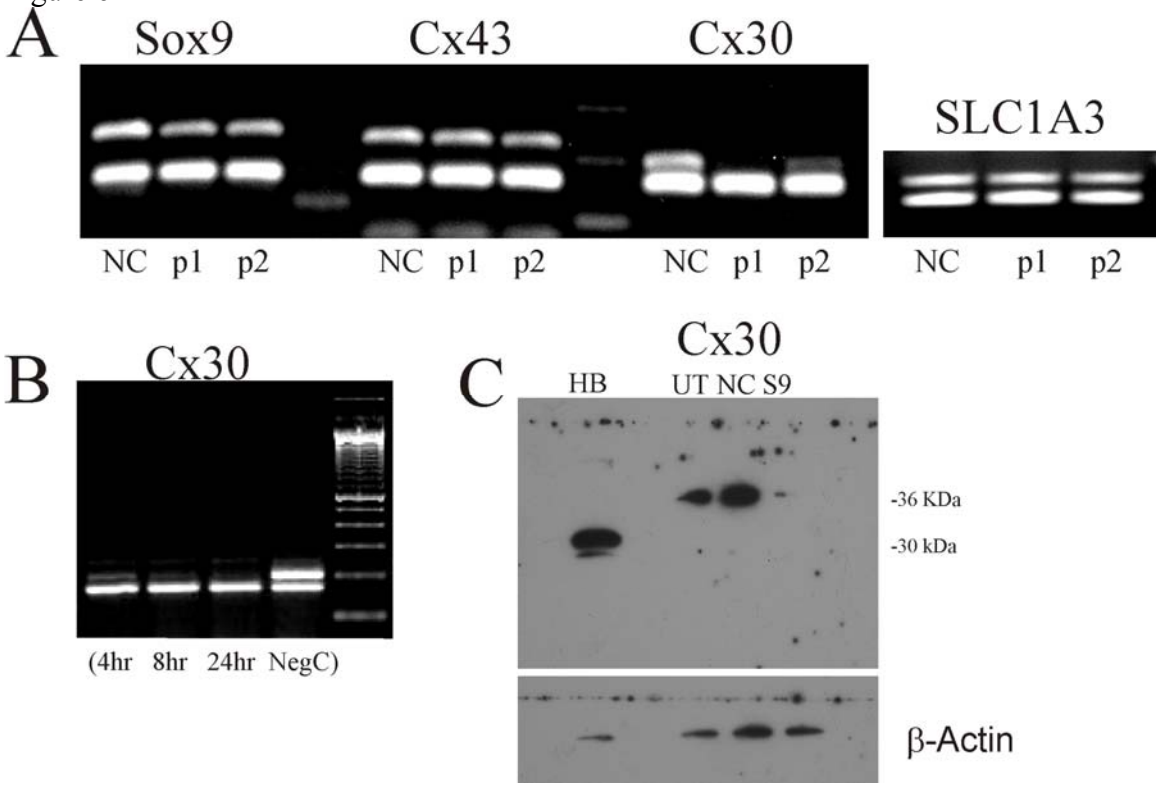

A
NegC 90 120 140 160
Sox9 expression (band intensity)
NegC
90 pmol
120 pmol
140 pmol
160 pmol
B
Cx43
NegC Sox9i NegC Sox9i NegC Sox9i
90 pmol
120 pmol
140 pmol
160 pmol
Cx43 expression as % of negative control
C
SLC1A3
NegC Sox9i NegC Sox9i NegC Sox9i
90 pmol
120 pmol
140 pmol
160 pmol
SLC1A3 expression as % of Negative control
D
FGFR3
NegC Sox9i NegC Sox9i NegC Sox9i
90 pmol
120 pmol
140 pmol
160 pmol
E
GLUL
NegC Sox9i NegC Sox9i NegC Sox9i
90 pmol
120 pmol
140 pmol
160 pmol

Figure 6


A
Sox9
Cx43
Cx30
SLC1A3
NC p1 p2
NC p1 p2
NC p1 p2
NC p1 p2
B
Cx30
(4hr 8hr 24hr NegC)
C
Cx30
HB
UT NC S9
-36 KDa
-30 kDa
β-Actin

CHAPTER 5: CONCLUSIONS

5.1 Research and clinical implications

At the core of this thesis, is the possibility of astrocyte dysfunction in the suicide brain. In a non-biased way, we analyzed gene expression data and the most robust finding was that brains from suicide completers had lower expression levels of astrocyte related genes, such as the glial glutamate transporter (SLC1A3) and TrkB.T1. We attempted to explain this finding at the molecular level by trying to understand how these genes are regulated, and what may affect their regulation (such as methylation patterns). We also attempted to understand how the transcription factor Sox9 could drive some of the genes in this network.

As referenced throughout, astrocytes have been suggested to be linked to psychiatric disorders such as depression and schizophrenia. Most of these studies, though, looked only at cell morphology or total cell numbers using stereologic techniques. While my work supports the idea of astrocyte dysfunction in mood disorders, it does not support the idea that the reason for astrocyte dysfunction is the reduction in astrocyte number. Rather, my work suggests that a particular gene network in astrocytes that is involved in cell signalling and glutamate uptake is dysregulated. I note that cell signalling and glutamate turnover have also been linked to mood disorders, as outlined in my literature review.

There are potential clinical applications for this work. First, this study supports the use of the SLC1A3 up-regulator, riluzole, in treating major depression. My work suggests that SLC1A3 is down-regulated in the brains of suicide completers, therefore any drug that up-regulates this gene could be an effective therapy in depression. Second, the assessment of the effect of calcium transients in astrocytes has never been assessed in the clinic. My work has generated a testable hypothesis to understand better the role of long range calcium signaling in astrocytes and its potential effect on mood. This idea is supported by two converging findings from my work: 1) that the TrkB.T1 is down-regulated in the brains of suicide completers and stimulation of TrkB.T1 is known generate these astrocyte calcium waves and 2) that major mediators of calcium flow through astrocytes, the Cx30 and Cx43 genes, are down-regulated in the brains of suicide completers. My work suggests that astrocyte calcium wave signaling would be hamperered in the brains of suicide completers. Finally, my work supports the idea that glutamate dysfunction is a factor in major depression. I have shown data where an important glutamate synthesis enzyme and the glutmate transporter were downregulated.

5.2 Future Directions

Studies of the post-translational modification of proteins deviate conceptually from current research lines in that it is no longer necessarily tied to ‘amount ‘of a given gene product, but to differences in form, structure, and stoichiometry that may influence function. After translation in the cytoplasm, most proteins undergo a series of different modifications. After protein synthesis, proteins are targeted to the Golgi network where they are packaged into vessicles and delivered to other sub-cellullar locations such as the

cell membrane or the cytoskeleton. Within the Golgi network, proteins may undergo different modifications including ubiquitination (Bonifacino and Traub, 2003), phosphorylation (Mostov et al., 1995), sulfation (Monigatti et al., 2006), glycosylation (Helenius and Aebi, 2004) and fatty acylation (Resh, 1999; el-Husseini Ael and Bredt, 2002). All of these modifications, and there are many types within each category, can affect proteins function, and this alteration in protein function could affect mood and behaviour.

Studies in depression and suicide have largely focused on environmental (e.g. childhood abuse) and genetic factors (e.g. SNPs in 5-HTT). While the technology to study brain circuitries is in its infancy, the actual connections of cells and their micro-environment remain largely unstudied in these disorders. Studying genetics alone, on the assumption that it is a genetic change that leads to a change in brain circuitry, may miss out on important differences in brains from psychiatric patients and control subjects. For example, even in identical genetic environments, axon path-finding during development never occurs identically across individuals. While axons may terminate in the same general area based on chemo-attractant and repellent cues, the intense cellular environment-dependent competition that ensues during synaptogenesis (and even throughout life) makes it impossible that two individuals share the same brain circuitries. In this way, diversity can be generated across individuals, without any genetic differences. As imaging technologies progress towards increased sensitivity, the ability to understand how neurons, astrocytes, and other brain cells connect with each other in living or even post-mortem human brains will be of tremendous interest.

MicroRNAs are small, single stranded 21 base RNA strands that bind to mRNA and lead to the degradation of mRNA. MicroRNA's are encoded in the genome with approximately 400 known so far (Kosik, 2006). After transcription and subsequent transport from the nucleus, microRNAs bind to any cytoplasmic mRNA molecule with complementary bases. As double stranded RNA in the cytoplasm is destroyed any mRNA with fully or partially complimentary bases to a microRNA molecule is degraded. This leads to less mRNA from a given gene, and therefore, less protein product. MicroRNA's could conceivably bind to multiple different mRNAs, causing major changes in cell function.

MicroRNAs have been related to psychiatric disease. The binding site of hsa-mir-189 in SLITRK1, a gene important for neuronal pathfinding, was recently shown to be changed in patients with Tourette's syndrome (Abelson et al., 2005). In this case, a G to A base substitution led to enhanced mir-189 binding, leading to increased SLITRK1 repression. Two recent studies used microRNA profiling in brain samples to compare different psychiatric sample sets and controls. In the first study, microRNA differences were found in frontal cortex of schizophrenia when compared to microRNA expression in frontal cortex of controls (Perkins et al., 2007). Specifically, this study profiled the expression of 254 different microRNA's using chip-based technology in 15 schizophrenic patients and 21 normal controls. This study found that 16 different microRNAs were differentially regulated between groups. A study of similar design examined microRNA expression in cerebellum of people with Autism spectrum disorders compared to controls (Abu-Elneel et al., 2008). While this study also used a chip based approach to investigate

450 different microRNA's (n = 13 autistic and 13 controls), the analysis was different in that group mean effects were not assessed, but rather autistic subjects were individually compared against the mean level of a given microRNA in the control group. This type of analysis yielded two gene targets of interest, *Neurexin* and *SHANK3,* two genes previously linked to autism spectrum disorders.

Data from these studies suggest that (a) chip based technology directed at investigating microRNA levels in brain is experimentally sound; (b) this type of approach can generate meaningful results that survive laboratory follow-up experiments; (c) different types of analyses can be used to evaluate microRNA data to generate biologically relevant data, and (d) microRNA may be an important factor in the pathophysiology of mental disorders.

Other areas of future study include transcriptomics (Garlow, 2002), the variation in different RNAs from the same gene, of which a good example is RNA editing. RNA editing is the alterations of particular nucleotides in an RNA molecule that alters the protein code. One example of this, and one studied in psychiatry (Schmauss, 2003; Dracheva et al., 2008), is the 5-HT2C receptor mRNA which has five adenosine sites that can be edited to inosine which can lead to alterations in the triplet codon sequence, affecting the protein sequence. Other genes of interest to mood researchers have also shown RNA editing (Paschen et al., 1994), yet have not been explored in psychiatric studies.

Appendix 1

Application of microarray outlier detection methodology to psychiatric research

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[2] Centre de recherche Université Laval Robert-Giffard and Department of social and preventive medicine, Université Laval, Canada.

Published in: *BMC Psychiatry*. 2008 Apr 23;8:29.

Abstract

Background Most microarray data processing methods negate extreme expression values or alter them so that they do not lie outside the mean level of variation of the system. While microarrays generate a substantial amount of false positive and spurious results, some of the extreme expression values may be valid and could represent true biological findings.

Methods We propose a simple method to screen brain microarray data to detect individual differences across a psychiatric sample set. We demonstrate in two different samples how this method can be applied.

Results This method targets high-throughput technology to psychiatric research on a subject-specific basis.

Conclusions Assessing microarray data for both mean group effects and individual effects can lead to more robust findings in psychiatric genetics.

Background

Currently used psychiatric nosology is based on a compilation of clinical symptoms into categories based primarily on symptom clustering and course. Diagnostic systems such as the current version of the DSM, allow for certain flexibility in the definition of diagnostic categories, with no assumption that each category of mental disorder is a completely discrete entity. As such, individuals diagnosed under a certain diagnostic class are not clinically homogeneous, there are no clear boundaries between classes, and different classes are not mutually exclusive. It is, therefore, unrealistic to expect that all subjects diagnosed with a given disorder will share a common psychopathological process, which would be associated with a common underlying biological process.

Most research efforts in psychiatry are directed towards the identification of group effects, negating the fact that significant etiological heterogeneity may exist. This limitation is particularly true for microarray research in psychiatry, where gene expression from different brain areas has been assessed comparing all affected subjects to non-affected subjects. A possible solution would be to carry out studies aiming at the identification of biologically meaningful effects focusing on single individuals or subgroups. This approach would mimic fruitful efforts in the identification of genetic factors underlying heterogeneous conditions such as, among others, spinocerebellar ataxia (Schols et al., 2004) and Alzheimer's disease (Bertram and Tanzi, 2004).

Microarray data from psychiatric subjects can be investigated for individual or subgroup effects that may be of genuine biological significance. Specifically, we hypothesize that

specific subgroups can be identified through microarray data screening for extreme expression values. Three previous studies have described how microarray data can be investigated on a subject-specific basis when analyzing data from cancer studies (Lyons-Weiler et al., 2004; Tomlins et al., 2005; Tibshirani and Hastie, 2007). We suggest here that microarray experiments using brain-gene expression levels from psychiatric experiments (e.g. schizophrenia group vs non- schizophrenia group) can utilize microarray data not only for group mean effects (i.e. standard microarray analysis) but also, whenever possible, should evaluate expression levels by individual subjects.

Most microarray projects in psychiatry involve examining more than one neural region (Roth et al., 2006; Sequeira et al., 2006; Karssen et al., 2007; Kim et al., 2007a). Specifically, researchers studying gene expression in brain tend to analyze more than one brain region on more than one array. This leaves researchers with gene expression data from multiple brain regions for each subject. This offers the possibility of using data from different arrays as confirmations of findings which may appear to be outliers. Any outlier on one chip that is also an outlier on a different chip may represent a valid finding.

Human brain has been categorized in two main ways: either by gross anatomical structure, the preference of imaging specialists, or by Brodmann region, the preference of neuro-anatomists. Irrespective of how the human brain is categorized anatomically, what is less obvious is whether gene expression varies between neighboring regions. Two recent, replicating studies suggest that brain gene expression of samples from the same individual, while non-identical, are biologically-related (Roth et al., 2006; Ernst et al.,

2007). This sharing of similar expression patterns across samples allows for the exclusion of extreme values in the microarray data due to noise. This provides a potential to validate microarray data, particularly for variables that are extreme values, across chips. Those extreme values present across chips for the same probe set and the same individual may represent a true biological effect.

We have designed a method that can assess extreme values and utilizes expression data across chips from the same individual. The method will allow for the detection of any subjects that have probe set values that differ drastically from a mean and outside of a certain threshold (e.g. Standard deviations from a mean). This method, termed Extreme Values Analysis (EVA), takes into account the complex and heterogeneous nature of psychiatric diseases. We illustrate this approach in two different situations. First, in a publicly available sample where extreme values were simulated, and second, in a sample of subjects who died by suicide and sudden death controls. EVA functions to screen microarray data individual-by-individual in search for any extreme values that may signify some abnormality.

Methods

We used a publicly available data set to first evaluate EVA. The data set comprises 9 subjects screened over 20 regions of the CNS and can be found here (Roth et al., 2006). In this dataset, one of the authors (AB) inputted simulated extreme values for two subjects across all CNS regions for one randomly selected probe set each. The expression values were multiplied by $4^{(1+0.25Z)}$ for one of the probe sets and by $0.25^{(1+0.25Z)}$ for the other, where Z is a standard normal random variable which was generated independently for each CNS region. Another of the authors (CE), blinded to the experimental manipulation, applied the method to detect the inputted value(s). The rationale for this experiment is to determine if EVA can detect an artificially generated extreme value in one probe set from > 11 million different data points (10 subjects X 20 regions X~55,000 probe sets).

We assessed EVA in a second sample that comprised a group of suicide completers and sudden death controls. Information on the subjects, clinical variables, and microarray data quality of the suicide and sudden death controls can be found in Sequeira et al.,(Sequeira et al., 2006).

EVA can be applied under a control:experimental design (suicide and sudden death controls example) or in a one sample design (CNS screening example). We describe the control:experimental setting, although the description applies also to the one sample

design. In the one sample design, all individual values are compared to the group to which they belong.

The mean and standard deviation (SD) of $\log_2$-transformed expression level is computed in the experimental group for all probe sets in every region. In our example, this was done in 2 cortical brain regions from suicide subjects. Log transformation stabilizes the variance, allowing comparison of SD across probe sets. After this step, the probe sets with the highest SD values were selected for further analysis. We used only those probe sets in the top 5% of SD values. We reasoned that these probe sets likely have individual values that are extreme, which accounts for a high SD value.

To buffer against detecting mathematical artifacts, EVA selects only those probe sets with high SD values in all regions. In our example, we selected probe sets that were common across both cortical regions. Next, we assess whether the same subject is responsible for the high SD value across brain regions. We set as criterion for an extreme expression a value of ± 3 fold greater than the mean expression level of the specific probe set among the control group (in our example, the sudden death controls). This approach operates on the assumption that neighboring brain regions are not discrete units and that gene expression should not vary widely from one cortical region to another. Even if brain region-specific expression is more common, it is not expected that a subject that is an outlier in one region is necessarily an outlier in a neighboring region. In other words, extreme values that are detected across multiple brain regions are more likely to represent real biological phenomena. We note that this method is conservative.

Individual expression values also have to be outside of 1.5 SD's of the control group, after having met the above criteria. While we selected 1.5 SD's from the mean of the opposite group, this number can be changed depending on the false discovery level acceptable to the experimenter. Manipulating the SD threshold establishes the false discovery rate (FDR) of the experiment.

The statistical significance of each identified outlier can be assessed by computing the p-value of the subject's expression values for a probe set in the multiple brain regions compared to the multivariate distribution of the expression values in the control group. The null distribution of the $\log_2$-transformed probe set-specific expression is estimated by fitting a normal mixed model where the subject effect is random. Letting, X_{ij} be the probe set-specific expression of the i^{th} subject in the j^{th} brain region, and $Y_{ij} = \log_2(X_{ij})$, this model has the form:

$$Y_{ij} = \mu_j + a_i + e_{ij},\ a_i \sim N(0,\sigma^2),\ e_{ij} \sim N(0,\sigma^2)$$

where μ_j is the region-specific mean expression, a_i is the subject random effect and e_{ij} is the residual. We fit such a model by restricted maximum likelihood (REML) using the maanova package (Kerr et al., 2000) for the R statistical software (Offord et al., 1989). The subject random effect captures the expected correlation between expression in different brain regions of the same subject. The p-value for the observed deviation of the

$\log_2$-transformed expression level of the i^{th} subject from the mean of the group of reference $y_{ij} - \hat{\mu}_j$, j = 1,…, J (or observed fold change on the original scale) is given by

$$P\left(|Y_1 - \hat{\mu}_1| > |y_{i1} - \hat{\mu}_1|, \ldots, |Y_J - \hat{\mu}_J| > |y_{iJ} - \hat{\mu}_J|\right)$$

which we compute using a multivariate t-distribution with the covariance matrix estimated under the normal mixed model.

Results

EVA in partially simulated data

We tested EVA in a sample data set that included 20 different CNS regions (Roth et al., 2006). This dataset was selected because A) we could test how the method works with the RMA algorithm and B) we could demonstrate the method in a one-sample case.

We began by computing the standard deviation (SD) for three of the 20 CNS regions described in this data set. The probe sets in the top 5% of SD values was selected for each of three regions and those probe sets that were common to all regions were selected. Five hundred forty-five probe sets were common to all three regions. Next, we screened for any individual values that lay outside of $\pm$1.5 SD's and was three-fold different from the mean. There were 14 genes that were found to be 3-fold greater than the mean and outside of +1.5SD's and 245 values that were three fold below the mean and outside of -1.5SDs. Each of these values was then cross-referenced across all 20 CNS regions. Two probe sets were found that met all criteria (1 above the mean for one subject and one below the mean for another subject). These were the probe sets that had been artificially altered (Figure 1).

EVA in real microarray data

To demonstrate this technique, we used a sample that included 6 control subjects and 8 suicide completers with microarray data from BA 8/9 and BA 11. We first screened all expression values for MAS 5.0 present/absent call leaving 14,896 probe sets in BA 8/9 and 14,412 probe sets in BA 11. We next calculated a standard deviation for all probe

sets from suicide subjects. This was done using log_2-transformed expression values. We then selected the probe sets with the highest SD values (top 5%) from both BA 8/9 and BA11.

Any probe sets that was identical to both BA 8/9 and BA 11 after SD filtering was selected. There were 180 probe sets that were common to both regions. Next, to account for the variability of expression in control values, we searched the data for any suicide data point greater than 3-fold from the control mean and outside of 1.5 SD's. We reasoned that an extreme value across all regions for the same subject(s) could represent a biologically relevant event.

Beginning in BA 8/9, we filtered the 180 probe sets for those probe sets from suicide completers outside of 3-fold from the control mean. There were 20 probe sets where X_{ij} (a particular expression value from a particular subject in a given brain region) was not outside of 3-fold from the control mean. From the 160 remaining probe sets, 108 probe sets were also outside of 1.5 SD's in BA 8/9. Probe sets from BA 11 were then filtered for these probe sets.

Table 1 lists the probe sets across the eight suicide completers and the individual p-values associated with each subject. From 108 probe sets that passed all EVA criteria in BA 8/9, 69 passed all EVA criteria in BA 11. Included in this list of probe sets are a number of genes that have been linked to suicide before including the FGF family (Evans et al., 2004), NTRK2 (Dwivedi et al., 2003), and members of the ubiquitin family (Ryan et al.,

2006). Of note, from the table, is that for a number of probe sets there is more than one subject who has an extreme expression value reaching a significance level.

Discussion

The extreme values analysis, or EVA, is a method to detect individual or subsets of outliers for a given probe set in microarray experiments. The rationale for this type of experiment is that psychopathology is not necessarily group specific but more likely sub-group or subject specific. The method outlined here uses log-transformed data to determine which probe sets have the highest variance and screens out those probe sets with little variation. This step is intended to select those probe sets with values that deviate widely from the mean. Next the method compares individual data points to a control mean, and searches for any 3-fold changes. Selected values also have to be outside of 1.5 SD's from the mean. These values were considered extreme expression values. These extreme expression values were next verified in one other cortical region to determine if they were extreme expression values in other cortical regions as well. We reasoned that the use of other cortical regions functioned as replicate experiments and enforced the finding.

We also evaluated this method in a one sample case after inputting artificial values for one probe set across all CNS regions in RMA data. EVA was able to detect the inputted value; the only difference between the control:experimental case and one sample case is the mean value used: In the one sample case the mean used includes the extreme value while in the control:experimental case it does not.

The use of multiple cortical regions as within-subject replicates is a way to detect true extreme expression values in individual subjects. Operating under the assumption the

gene expression in one cortical region is generally similar in neighboring cortical regions, we propose that different chips for the same subject can be used as replicate experiments, if probe set outliers on an individual specific basis are being investigated. If an observed outlier is a real biological event, it is very probable that the same subject on the same probe set will also be an outlier in a neighboring region. Consider, for example, the family with a deletion in the MAOA gene (Brunner et al., 1993). Had this family undergone post-mortem microarray analysis as a part of a larger sample of subjects, EVA would have detected the MAOA decrease in expression whereas microarray analysis using mean group effects would not have. Using multiple brain regions as replicates does undermine the idea that gene expression is different across different brain regions, which it is (Ernst et al., 2007) (Lein et al., 2005); however, it means that if an effect is detected, it is likely real and robust.

Comparison to PPST method

The PPST method (Lyons-Weiler et al., 2004) counts the number of subjects in both control and experimental group outside of the 95th percentile of the opposite group. The FDR is therefore controlled by altering the percentile threshold. EVA uses the SD from the opposite group and counts the number of subjects that are outside a given SD value ($\pm$1.5 SDs in this study). Selecting more stringent SD values allows for direct manipulation of the FDR. In this study a liberal cutoff was chosen (outside of 1.5SD's). The FDR among the detected outliers could be estimated from the p-value of the subject's expression values using standard methods such as that of Reiner et al. (Reiner et al., 2003)

Comparison to COPA method

Cancer outlier profile analysis (COPA) is another outlier detection that has proved fruitful in the past(Tomlins et al., 2005). This technique normalizes all probe sets (one sample design) and calculates the 75^{th}, 90^{th}, and 95^{th} percentiles for each probe set and rank-orders them by percentile score. A prioritized list of probe set with some subjects that have extreme expression values is then investigated. Tibshirani and Hastie [3] introduce the outlier-sum statistic in their paper to improve on the COPA method. Their method differs from COPA by the standardization procedure of each probe set expression level using the median and median absolute deviation.

There are some caveats to be aware of before proceeding with this approach to screen microarray data. Firstly, the method is very conservative and likely has a high beta error rate. It is very likely that there were a number of true positives that were not detected because of the rigidity of the design. Some parameters may need to be adjusted to allow more probe sets to pass filtering (e.g. top 10% of SD values instead of the top 5% being used). Second, this method has the disadvantage of requiring a number of replicates per individual, a component that could be cost-prohibitive. Third, the method can only be used to study genes whose expression levels are similar across brain regions. Finally, we note that all probe-level microarray algorithms dampen extreme values at the scanner. This method is conservative and could only be used to investigate extreme values after initial processing.

Our view for this technique is as another analysis technique to further explore microarray data, in conjunction with more mainstream techniques (Allison et al., 2006). This method, termed Extreme Values Analysis, can detect extreme differences in gene expression on a subject-by-subject basis from microarray data across different chips. The method uses high-throughput technology in a non-biased way to understand psychiatric disease for each subject investigated.

Table 1. **HG-U133 plus 2 probe sets that met all EVA criteria**. Numbers represent p-values generated for each probe set across each subject (S). Subjects who met EVA criteria have p-values underlined. Note that p-values are generated from the number of SD's from the mean, therefore some subjects with very small p-values may be outside of a given number of SD's but <3-fold different than the mean.

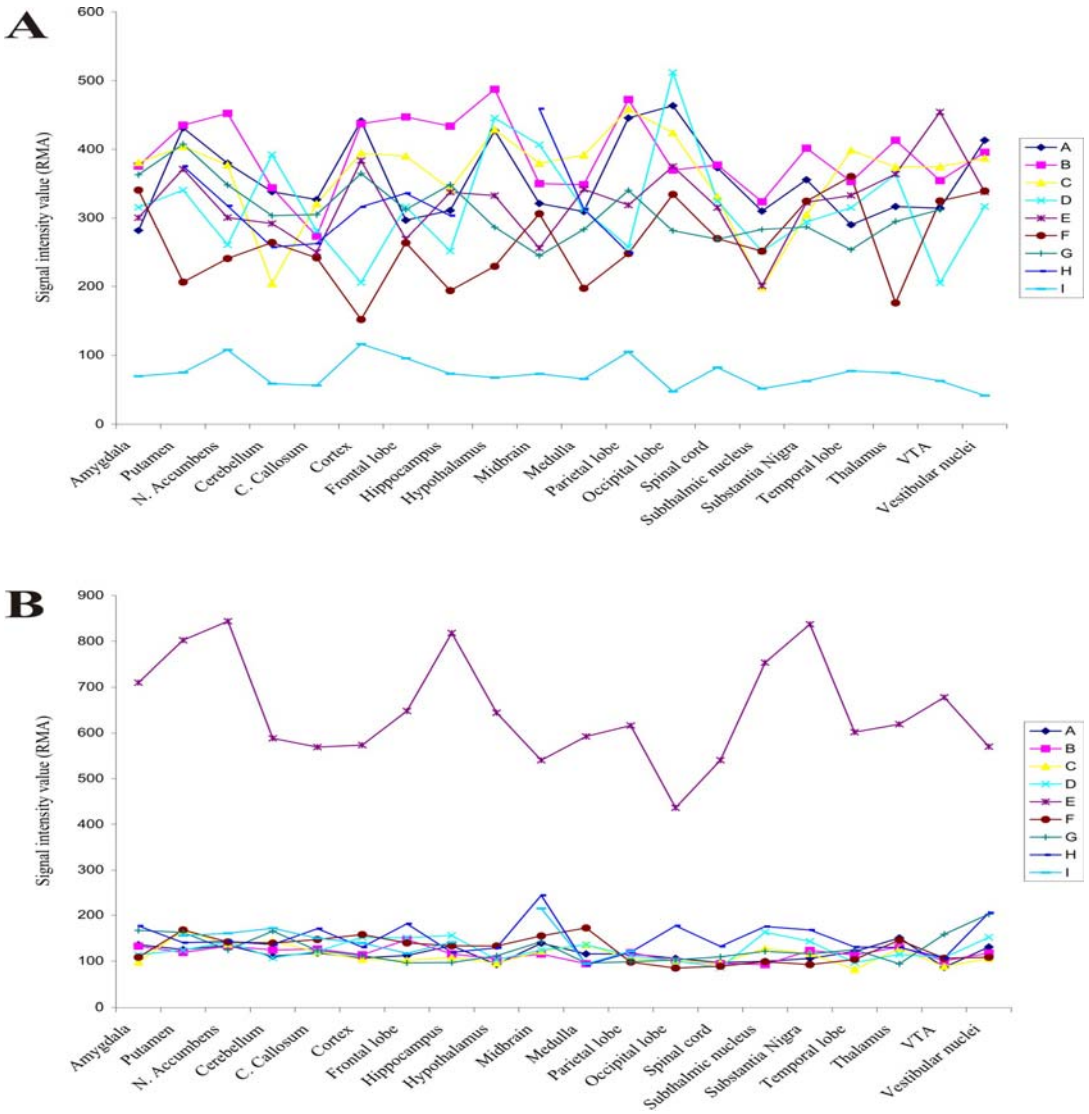
Probe set	S1	S2	S3	S4	S5	S6	S7	S8
233814_at	0.00011	0.262901	0.060973	0.119425	0.340339	0.065993	0.291037	0.044823
225440_at	0.003777	0.041856	0.549775	0.617546	0.000754	0.044895	0.52789	0.000355
203638_s_at	0.008775	0.076607	0.099598	0.154338	0.000159	0.089298	0.01575	0.357839
214680_at	2.89E-05	0.010459	0.007155	0.001488	0.000348	0.007818	0.000768	0.148031
37170_at	0.001734	0.002016	0.095708	0.010663	0.000198	0.000259	0.003458	0.140702
227556_at	0.00074	0.397533	0.000597	0.010661	0.291994	7.80E-07	0.100161	0.010813
229917_at	0.00895	0.170125	0.421083	0.044559	0.401754	0.000248	0.055834	0.003505
227330_x_at	0.018916	0.412934	0.378829	0.053889	0.002547	0.576196	0.037467	0.46594
231804_at	0.411319	0.075227	0.566597	0.115634	0.485137	0.489949	0.285547	0.012676
200904_at	0.066736	0.080689	0.236431	0.504115	0.009175	0.255437	0.466512	0.105319
230141_at	0.041214	0.157873	0.408831	0.098357	0.013597	0.067947	0.333108	0.004167
222020_s_at	6.92E-05	0.071294	0.239087	0.018151	0.271669	0.043433	0.044077	0.428814
213812_s_at	0.004793	0.002408	0.151259	0.121657	0.154226	0.030286	0.022571	0.02784
201505_at	0.316616	0.570798	0.160905	0.604017	0.000337	0.193244	0.157064	0.00128
240467_at	0.103838	0.007113	0.076142	0.001703	0.050236	0.332126	0.148029	0.199109
229861_at	0.005266	0.047216	0.055746	0.000202	0.028745	0.028233	0.019457	0.00037
225872_at	0.001483	0.528714	0.628325	0.427906	0.000266	0.396505	0.019428	0.194352
241758_at	0.62524	0.62922	0.43515	0.45219	0.00547	0.28296	0.22366	0.0017

	6	5	3	4	3	2	2	18
214449_s_at	0.400041	0.272717	0.307379	0.020469	0.000229	0.040009	0.340049	0.000813
200648_s_at	0.011284	0.109397	0.371707	0.027636	0.013746	0.075645	0.025063	0.264293
221795_at	0.000617	0.006302	0.070084	0.008107	0.005545	0.039509	0.018253	0.284639
204379_s_at	0.046084	0.083475	0.391619	0.237209	0.025689	0.079905	0.073448	0.22916
215172_at	0.109908	0.139031	0.199485	0.152187	0.155703	0.203207	0.144057	0.001802
203324_s_at	0.005691	0.092966	0.150765	0.000126	0.000217	0.056637	0.205541	0.183977
202800__at	0.077088	0.491247	0.411825	0.162812	0.013449	0.102756	0.028244	0.452363
236223_s_at	0.030208	0.196886	0.209618	0.000686	0.258376	0.130886	0.373756	0.268783
209023_s_at	5.10E-05	0.000708	0.030401	0.001934	0.251739	0.002706	2.10E-05	0.054205
213593_s_at	0.005575	0.001263	0.108932	0.005416	0.007774	0.209469	0.083709	0.036244
222249_at	0.101364	0.131587	0.040535	0.007024	0.009912	0.096597	0.000144	0.012586
220460_at	0.018979	0.539318	0.195645	0.120094	0.009469	0.067115	0.013541	0.346847
201656_at	0.000481	0.130316	0.064596	0.010041	0.000455	0.018925	0.145398	0.117989
235775_at	0.000373	0.079169	0.040214	0.112246	0.000676	0.222721	0.208163	0.049906
204516_at	9.40E-05	0.051213	0.096458	0.027735	0.003274	0.277745	0.208683	0.17238
201843_s_at	0.047846	0.096131	0.228376	0.016614	0.010238	0.104263	0.005888	0.155651
204712_at	0.024623	0.417385	0.556647	0.127233	0.010306	0.182687	0.121824	0.39341
224736_at	0.002159	0.022778	0.118888	0.114733	0.126571	0.333625	0.187404	0.021966
214203_s_at	0.018997	0.038021	0.062828	0.074293	0.001333	0.017613	0.003911	0.047244
200914_x_at	0.010589	0.005141	0.075284	0.058115	0.042784	0.274358	0.103149	0.103501
222404_x_at	0.003613	0.040237	0.059019	0.220239	0.151453	0.188272	0.358947	0.103866
229553_at	0.008515	0.022552	0.027346	0.070236	0.144188	0.145378	0.331622	0.223119
203249_at	0.139980	0.138380	0.229280	0.100470	0.004920	0.094850	0.075730	0.002400

	1	9	3	7	1	4		53
203041_s_at		0.11118	0.22779	0.40269	0.01421	0.31023		0.1744
	0.01742	2	2	4	4	8	0.14055	57
209292_at	0.06817	0.34599	0.50725		0.00661	0.09967	0.00243	0.2758
	5	2	3	0.01934	1	3	8	59
226084_at		0.00527	0.05975	0.09679	0.07670	0.64222	0.10113	0.1531
	0.00373	2	4	2	2	7	7	24
204976_s_at		0.08366	0.10033	0.15299		0.15924	0.44999	0.5837
	0.00165	7	6	2	0.00468	4	2	33
212368_at	0.01328	0.01686		0.08618		0.24617	0.12679	0.2932
	3	4	0.07334	3	0.13413	1	9	17
211962_s_at			0.17837	0.02632		0.01755	0.01185	0.2787
	0.00228	0.17037	6	4	0.0011	7	4	36
226228_at	0.09014	0.42342	0.57241	0.12590	0.01524	0.10057	0.07767	0.6332
	7	5	5	2	2	4	7	29
213954_at	0.00275	0.00191	0.02284	0.01345	8.47E-	0.01891	0.07572	0.0775
	3	8	1	4	05	1	5	12
213922_at	0.00471	0.00340	0.10955	0.02476		0.41703	0.04262	0.1999
	9	8	5	6	0.07629	4	1	03
221517_s_at	0.00116		0.04829	0.02265	0.00327	0.15088	0.08003	0.0110
	7	0.00413	3	4	1	2	9	72
227099_s_at	0.04754	0.09224	0.06982	0.33391	0.00042		0.20093	0.0091
	9	1	1	7	5	0.00361	3	89
201737_s_at	0.00015	0.01297	0.21939	0.11093	0.09283	0.27389	0.07892	0.0352
	9	2	3	6	6	1	8	64
214279_s_at	0.00859	0.36344	0.36589		0.03621	0.01418	0.05912	0.0665
	4	1	8	0.01142	8	1	7	75
205709_s_at	0.00705	0.02199	0.11040	0.35952		0.21843	0.30623	0.2063
	9	8	2	9	0.08021	8	5	74
225810_at	0.03905	0.25639	0.15914	0.45217	0.00152	0.01067	0.01758	0.1305
	4	6	2	3	5	6	1	46
226435_at	0.15099	0.24859	0.43252	0.00458	0.03236		0.07201	0.2160
	6	3	5	9	8	0.12243	6	67
226364_at	0.31052	0.02606	0.01170		0.02688	0.17898	0.04868	0.0052
	4	2	4	0.02649	9	3	3	22
240482_at	0.31833	0.47526	0.19551	0.21471	0.15166	0.39087	0.00357	0.0013
	5	3	2	8	3	2	8	16
204881_s_at		0.06896	0.07529	0.11720	0.02075	0.03967	0.31076	0.0843
	0.00017	9	4	2	9	3	9	19
203841_x_at	0.00020	0.00582	0.08929	0.00684	0.05279	0.11416	0.03791	0.0528
	1	3	7	3	1	9	1	24
212677_s_at	0.02141	0.00319	0.03398	0.02231		0.12809	0.09066	0.0602
	2	3	8	2	0.03778	7	5	85
201502_s_at	0.00137	0.00637	0.16733	0.04153	0.03542	0.07065	0.26315	0.6141
	2	9	5	1	3	7	8	72
240299_at	0.01765	0.27652	0.19769	0.00820	0.0622	0.21590	0.08793	0.0257

	8	9	9	6		9	7	87
212423_at	0.02407	0.01584	0.14017	0.14094		0.02046		0.3998
	3	7	3	8	0.19045	9	0.19758	76
228811_at	0.29058	0.34914	0.14006	0.37157		0.00217	0.06632	0.0022
	4	3	8	8	0.50686	6	4	91
224737_x_at	0.00036	0.28885	0.11245	0.00264	0.09718		0.21953	0.0216
	7	1	7	1	1	0.24341	5	62
201019_s_at	0.00344		0.27613	0.18183	0.06450	0.68040	0.17936	0.0995
	6	0.00455	2	2	1	6	1	58
229281_at	0.09071	0.33316		0.24131	0.00073	0.01918	0.02505	0.3141
	3	5	0.42729	6	2	7	4	21

Figure 1. **Microarray expression values demonstrating two subjects who passed all EVA criteria in a one sample case.** A) Extreme low expressor (light blue trace) compared to other subjects in the same sample for one probe set identified across multiple CNS regions. Each subject is represented by a letter (A, B, C…). B) Extreme high expressor (purple trace) compared to other subjects in the same sample for one probe set across multiple CNS regions.



Appendix 2

The effects of pH on DNA methylation state: *in-vitro* and post-mortem brain studies

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Abstract

Assessment of methylation state of DNA extracted from brain is becoming one of the most investigated issues in the study of epigenetics and psychopathology. pH effects in brain are known to affect gene transcription, though pH effects on DNA methylation state are unknown. We demonstrate *in vitro* using an artificially methylated plasmid that DNA methylation state remains stable, even under extreme pH conditions**.** Next, using two different genomic regions from human DNA, we assess methylation state from both cortical and sub-cortical brain regions using subjects with varying pH levels. No correlation was found between DNA methylation state and pH. These results suggest that DNA methylation state is stable in post-mortem brain.

Introduction

A wealth of epigenetic studies in brain is currently under way (Abdolmaleky et al., 2004; Mill and Petronis, 2007), and methylation is one epigenetic mechanism that affects gene transcription which could mediate the interaction of genes and environment (Weaver et al., 2004). Methylation refers to the process of the addition of a methyl group to DNA and a number of proteins are known that bind methylated DNA or that add methyl groups to DNA. These protein/DNA interactions can have important repercussions on gene expression (Amir et al., 1999).

Of particular interest to psychopathological research is the methylation status of DNA isolated from post-mortem brain, but a better understanding of the potential effect of confounding factors, such as pH, is needed before associations between methylation state and certain illnesses are made. pH effects in post-mortem brain are a major caveat of gene expression studies (Vawter et al., 2006), though the effect of pH on methylation state of DNA extracted from post-mortem brain is unknown. It is possible that DNA exposed to more acidic conditions even in the absence of any biological function, could affect DNA methylation state.

This study addresses the effects of pH in post-mortem brain on DNA methylation state. Using both *in vitro* and post-mortem brain experiments, we find that DNA methylation state is stable in post-mortem brain.

Materials and Methods

In vitro analysis of pH effects on DNA methylation

We treated the pGL3 plasmid (Promega) with SSSI methyltransferase, an enzyme that methylates all cytosine nucleotides in a CpG dinucleotide. To ensure that this step was effective, we took two sub-samples of the methylated plasmid (pGL3-CH3) solution and exposed them to two restriction enzyme digestions: HPAII and MSPI. Both of these enzymes recognize the same site (CCGG), but HPAII is blocked from cutting DNA when the internal C is methylated. The pGL3 plasmid is 4,800 basepairs long and has 25 CCGG sites. MSPI is insensitive to the methylation status of the internal C. The *in vitro* methylated pGL3 was purified by standard phenol-chloroform extraction procedures.

Following ethanol precipitation the plasmid DNA was re-suspended and incubated for 48 h at 22 C^{o} in pure water and a series of solutions that differed in pH (3.8, 6.1, 6.62, 7.2, 10, 12). pH solutions were made using HPLC-grade water with NaOH and HCl. Following incubation, the DNA samples were treated with sodium bisulfite following the manufacturer's protocol (Qiagen EpiTec Bisulfite Kit).

Bisulfite treatment converts all cytosine residues to uracil, but methylated cytosines remain intact (Clark et al., 1994). This treatment creates a sequence difference between methylated and unmethylated cytosines which enables mapping of the methylation pattern at single base resolution. Primers specific for pGL3-CH3 were designed using Methyl Primer Express (Figure 1B; Forward: 5' AAGATGTTTTTTTGTGATTGGT 3';

Reverse: 5' TTCCTATTTTTACTCACCCAAA 3'). Using these primers in a PCR reaction, a product of 278 basepairs was generated.

pH measurements from human brain

We followed the protocol used by Vawter et al. (2006) to take pH measurements. Eighty to 120 mg of cerebellar tissue was homogenized in Chromosolv water (ultrapure water normally used for high performance liqid chromotagraphy - Sigma-Aldrich) at a 10:1 water to tissue ratio. All tissue was taken from previously frozen brains. Tissue was homogenized with a TissueTearor (Biospec Productions Inc), on ice, until no brain fragments were visible. After re-equilibration to room temperature, solutions were measured with a Corning pH meter.

Subjects

All subjects in this study were recruited at the Montreal Morgue as part of on-going recruitment of subjects for the Douglas Hospital Brain Bank. All subjects were male and did not die in an extreme agonal state, according to medical charts and/or informant reports. After death and permission from next-of-kin, brains were extracted, sectioned based on Brodmann region at 4^{o}C and snap frozen in isopentene at -80^{o}C. Brains were then stored at -80^{o}C. DNA was extracted from the dorsolateral prefrontal cortex (BA 9) and hippocampus from each subject and bisulfite treated.

Post-mortem analysis of pH effects on DNA methylation state

Two different primer pairs were used to assess methylation status in CpG rich promoter regions: Ribosomal RNA gene regulatory region (U13369): Forward: 5′-GTT TTT GGG TTG ATT AGA-3′; Reverse: 5′-AAA ACC CAA CCT CTC C-3’. DNA used was from hippocampus. NTRK2 promoter, (NM_000346): Forward: 5’-GAGAGTGGGTATATTGGTGGTTTTA-3’; Reverse, 5’-CCAACTTATCAAAAACTAAACTAATCC – 3’. DNA used was from BA 9. The amplified products were extracted from the gel, ligated into a pDrive vector, and transformed into competent *E.coli* cells (Qiagen PCR Cloning*Plus* Kit). Incorporation of the correct DNA fragment was verified by restriction enzyme digestion. All sequencing was done at the Genome Quebec Innovation Centre. At least 8 clones were used for each subject and for each primer pair.

Results

To test the effects of pH *in vitro* we first needed a fully methylated DNA sequence with which to perform the experiment. We selected the pGL3 plasmid due to plasmid availability and presence of a number of CpG dinucleotides inside of an easily amplifiable region. We first exposed the plasmid to SSSI methyltransferase to methylate all CpG dinulceotides. To test the effectiveness of this step, we exposed the experimentally methylated plasmid (pGL3-CH3) to two restriction enzymes: The first (HPAII), an enzyme incapable of cleaving methylated CpG dinucleotides and the second (MSPI), an enzyme fully capable of cleaving methylated CpG dinucleotides. Both enzymes recognize CCGG site for cleavage. Figure 1A demonstrates the resulting gel from the pGL3-CH3 plasmid being treated with each of the two enzymes.

We next used the pGL3-CH3 plasmid to assess the effects of pH on DNA methylation patterns *in vitro*. We made a wide range of pH solutions (3.8, 6.1, 6.62, 7.2, 10, 12) and incubated pGL3-CH3 for 48 hours with differing pH solutions and pure water. After incubation, we extracted pGL3-CH3 from the solutions, bisulfite treated the extract, and amplified a small region of DNA within the plasmid. The amplified product was then cloned into a pDrive vector (at least 8 clones per solution) and sequenced. We found that the sequences from plasmids incubated in varying pH solutions were indistinguishable from those incubated in pure water (Figure 1B,1C). All cytosines residing in the dinucleotide CpG sequence remained methylated under all conditions while all cytosines found in other sequence contexts were detected as thymidine bases, indicating lack of methylation.

We next tested the effects of pH on DNA methylation status in post-mortem brain. All subjects used in this study underwent full psychological autopsy procedures at the McGill Group for Suicide Studies (Dumais et al., 2005). We used two different primer pairs and two different brain regions for this study.

We first analysed ribosomal RNA (rRNA), a gene known to have a heavily methylated promoter region (Ghoshal et al., 2004). After sequencing clones from 10 individuals using DNA extracted from hippocampus, we found no significant correlation between pH and methylation state of the rRNA gene (Figure 1D; PCR product size of 250 bps with 27 CpG dinucleotides). We next analyzed the promoter of a gene that has been investigated as a candidate in psychiatric disorders (NTRK2- (Dwivedi et al., 2003), in 20 subjects (10 of whom were the same as for the rRNA analysis) in frontal cortex. No correlation was found between pH and methylation state (Figure 1E; PCR product size of 440 bps with 35 potential CpG dinucleotides).

Discussion

This study has demonstrated that DNA methylation state is a stable phenomenon, at least in regards to acidity and alkalinity in post-mortem brain and *in vitro*. We first demonstrated this under extreme pH conditions (i.e. pH conditions outside of physiological range for human brain) *in vitro* and then under physiological pH conditions in post-mortem brain tissue.

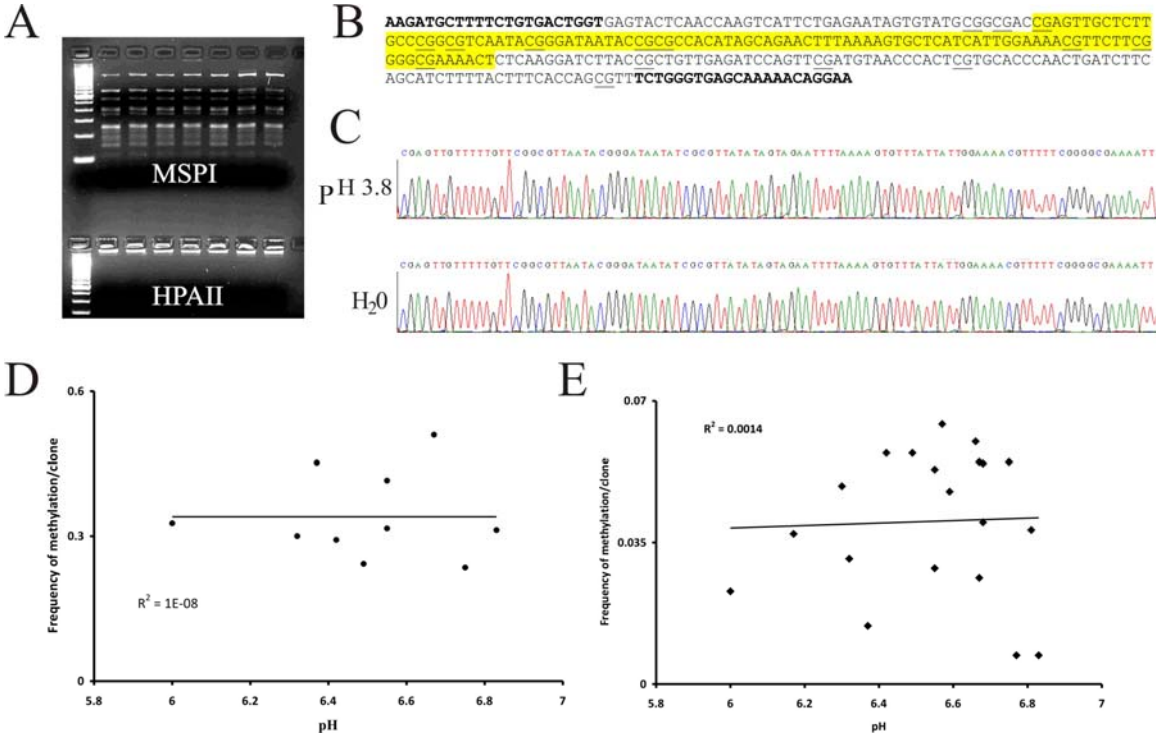
This study did not investigate *in vivo* effects of pH on DNA methylation. The purpose of this study was to understand whether pH in post-mortem tissue is a relevant confounding factor in experiments where DNA is extracted from brain to be used for methylation analyses. If DNA methylation is an active process, as has been suggested (Kangaspeska et al., 2008; Metivier et al., 2008), than pH changes may affect DNA methylation *in vivo*.

One time point of interest that could not be addressed by this study is the point from death to brain preservation in cold storage, i.e. methylation state changes during the post mortem interval. Immediately after death, some brain cells are still alive and lactic acidosis may occur (Ravid et al., 1992; Alafuzoff and Winblad, 1993). This potential change in pH as brain cells die is accompanied by a host of other physiological conditions (e.g. hypoxia, apoptosis, necrosis) each of which is a variable that could alter DNA methylation state. Studying only pH effects on DNA methylation status during brain death, even in a controlled laboratory setting using animals, is technically very challenging.

What this study can conclude, however, is that once pH is set (once the brain is in cold storage) there is no correlation across subjects between pH and methylation state of DNA. This could be directly assessed using DNA incubated in solutions that differed in pH. This allowed the examination of pH effects on DNA methylation state without the interference of any other biological factors. We caution that our post-mortem correlation results apply only to the range of pH reported in this study (6.0 – 7.0).

This study suggests that pH does not affect methylation state, either in post-mortem brain or under experimentally induced extreme pH conditions *in vitro*. These findings should be of use to studies examining methylation state of DNA extracted from any human tissue where pH could be a factor.

Figure 1. pH does not affect methylation status of DNA *in vitro* or in post-mortem tissue. A) Successful methylation of the pGL3 plasmid. B) Tested sequence from pGL3 plasmid for the described experiment. pGL3 primers specific for bisulfite-treated plasmid are emboldened. CpG sites are underlined. Yellow highlight demonstrates area where sequence traces in 1C are taken. C) Sequences from pure water and pH 3.8. Note that all CpG sites are methylated and C sites not in a CpG dinucleotide are detected as Thymidine. D) pH versus frequency of methylation, rRNA, and E) NTRK2.



Concordance of methylation patterning across brain and blood in an rRNA gene promoter

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Abstract

DNA methylation patterns are thought to be highly variable and region specific, yet no analysis of this has been done in human brain tissue using a genomic region known to be heavily methylated. We assessed the methylation pattern of an rRNA gene promoter in blood, frontal cortex, and cerebellum. We find that methylation patterns are highly variable across clones from the same individual, but that site and region methylation frequencies are remarkably similar across subjects and regions.

Introduction

Methylation analysis of a promoter region can be performed to test the hypothesis that epigenetic processes are involved in programming of gene expression in an experimental group compared to a control group. Hypermethylated promoters are usually silenced, thus methylation differences inversely correlate in many instances with gene expression and might provide information as to the mechanisms responsible for these changes in expression. This line of experimentation to explain why gene product is reduced is confounded though, by the suggestion that methylation patterns between individuals have high levels of variability irrespective of disease state or treatment conditions. While this high level of variability has been shown in sperm (Flanagan et al., 2006), no experiment has yet been performed in brain and a comparison region using a promoter region known to be methylated (i.e. positive control).

To perform such a study, we focused on the rRNA gene promoter, which is known to be heavily methylated (Ghoshal et al., 2004). In human cells there are 400 copies of rRNA genes per nucleus. A fraction of this population is unmethylated and expressed while the majority of the alleles are methylated and silenced (Brown and Szyf, 2007). We have previously shown a significant difference in the state of methylation of rRNA genes between suicide completers who were exposed to adversity in early childhood and controls (McGowan et al., 2008). This difference was seen in the hippocampus but not in the cerebellum. However, the question remains as to the normal distribution of methylation patterns across individuals. The extent of normal variation in methylation patterns of rRNA genes between different tissue types is also a question of significance.

To what extent do methylation patterns in the same promoter region from the same individual vary between blood and different brain regions?

Methods

We isolated DNA from blood, cerebellum, and frontal cortex from three disease-free subjects and assessed the methylation pattern of rRNA genes. The goal of this analysis was to understand whether methylation patterns of rRNA genes exhibit either brain-region specificity or subject specificity.

Subjects were recruited at the Montreal morgue and brain and blood samples were extracted after permission from the next-of-kin. All subjects were screened for psychopathological indicators using the psychological autopsy method with the best informant (Dumais et al., 2005), and all subjects with a history of psychiatric illness were excluded from this study (McGowan et al., 2008). DNA was extracted and bisulfite converted (Clark et al., 1994) and purified using standardized kits from Qiagen. DNA sequencing was performed using an automatic DNA sequencer at the Genome Quebec facilities. For each subject, we analyzed at least 10 clones per region.

Results

The raw data from this study is shown in Figure 1. Methylated CpG sites are represented by black circles and non-methylated CpG regions are represented by white circles. We analyzed a region of the rRNA promoter region that had 24 CpG sites. A general inspection of the data suggested that high variability existed between clones and within subjects but that little variability existed across regions and subjects.

We first assessed whether there was a difference in the state of methylation across frontal cortex, blood or cerebellum. Table 1 demonstrates that there is very little region-specific variation across the 24 sites analyzed although there are site-specific differences in methylation that persists in all regions. That is, if a CpG site is methylated in 30% of clones in frontal cortex, than the same CpG site is methylated approximately 30% of the time in cerebellum and in blood. This suggests that methylation patterns of rRNA genes promoters are not specific to a given region, but rather are consistent across both blood and brain.

Next, we assessed whether there were any subject-specific effects (Table 2). We found that the methylation effects were remarkably similar across subjects. For example, we note that the total methylation for this region is 37%, 38%, and 39% across subjects. Individual CpG sites showed very little variability as well. For example, at CpG site 18, the methylation frequency was 48%, 49%, and 50%. This suggests that methylation patterning in the rRNA gene promoter in different individuals is very similar.

The methylation patterning of the rRNA gene promoter is highly variable across clones in the same subject (Table 3, Figure 1). While a general level of methylation seems to exist (e.g. 40% of CpG's methylated in the rRNA gene promoter), the specific pattern of methylation at specific CpG sites is highly variable. For example, a number of clones in the same subject have no methylation on any CpG site, while some clones from the same region and subject are methylated at every site (Figure 1).

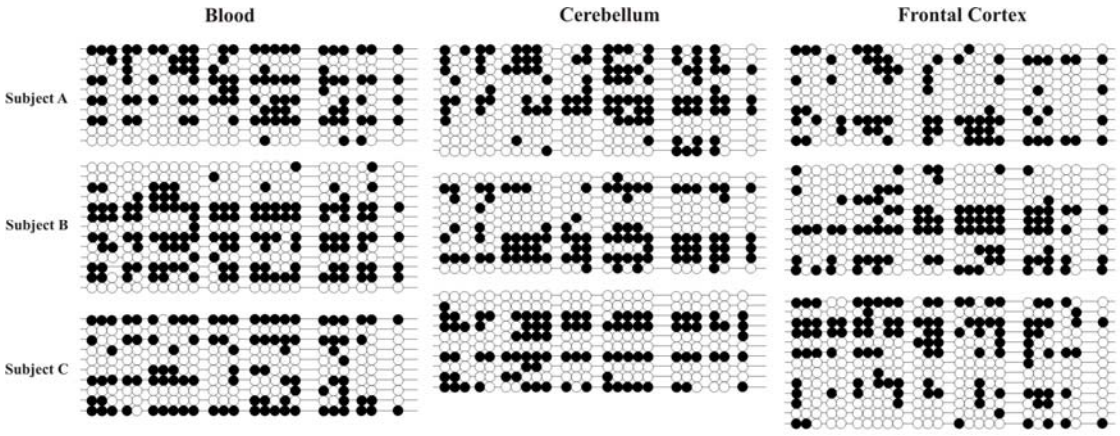
Discussion

These results suggest that the methylation pattern of the rRNA gene promoter in human blood and brain is stable across subjects and regions, but that a high level of variation exists between clones in the same individual. This finding is in contrast to what was described in germ cells where high levels of variability were found across clones and across individuals (Flanagan et al., 2006).

We have previously shown that rRNA genes promoter methylation state varies between suicide completers and controls. The current report shows low variation in the methylation state of rRNA gene promoters across individuals and tissues. The lack of variation in the population suggests that a relatively small number of individuals need to be studied to obtain significant results. In contrast however, this report highlights the need for large numbers of clones when analyses are performed in a single tissue-type due to the high level of variability across clones in the same individual. The fact that although the methylation state of CpG sites in single alleles seem to be stochastic, the overall methylation levels in a tissue or brain region is consistent across individuals suggests that the overall methylation pattern is defined by robust developmental processes. These processes overide possible genetic differences between individuals.

Figure

Figure 1. Raw methylation data for all subjects across all regions



Tables

Site	Total methylation	Blood	Cerebellum	Frontal cortex
CpG_1	45	14	15	16
CpG_2	33	14	11	8
CpG_3	19	9	4	6
CpG_4	36	14	14	8
CpG_5	24	11	8	5
CpG_6	32	13	8	11
CpG_7	33	10	12	11
CpG_8	42	12	15	15
CpG_9	37	11	13	13
CpG_10	37	15	11	11
CpG_11	24	11	8	5
CpG_12	42	14	10	18
CpG_13	45	16	15	14
CpG_14	36	14	13	9
CpG_15	44	18	16	10
CpG_16	40	12	14	14
CpG_17	39	15	13	11
CpG_18	48	16	15	17
CpG_19	44	14	14	16
CpG_20	39	16	12	11
CpG_21	53	21	15	17
CpG_22	39	13	16	10
CpG_23	28	12	10	6
CpG_24	33	12	12	9
Total		**327**	**294**	**271**

Table 1. Total methylation by region, irrespective of subject

Clone #	Subject A	Subject B	Subject C
CpG_1	0.50	0.37	0.51
CpG_2	0.30	0.34	0.36
CpG_3	0.13	0.20	0.24
CpG_4	0.43	0.37	0.30
CpG_5	0.27	0.31	0.15
CpG_6	0.23	0.40	0.33
CpG_7	0.30	0.40	0.30
CpG_8	0.40	0.46	0.42
CpG_9	0.47	0.31	0.36
CpG_10	0.33	0.37	0.42
CpG_11	0.13	0.31	0.27
CpG_12	0.47	0.43	0.39
CpG_13	0.47	0.46	0.45
CpG_14	0.40	0.34	0.36
CpG_15	0.53	0.49	0.33
CpG_16	0.43	0.40	0.39
CpG_17	0.43	0.37	0.39
CpG_18	0.50	0.49	0.48
CpG_19	0.47	0.43	0.45
CpG_20	0.37	0.37	0.45
CpG_21	0.60	0.51	0.52
CpG_22	0.47	0.43	0.30
CpG_23	0.27	0.37	0.21
CpG_24	0.33	0.34	0.33
Total	0.39	0.39	0.37

Table 2. Total methylation by subject, irrespective of region

	Subject A			Subject B			Subject C		
Site	Blood	cerebellum	frontal	Blood	cerebellum	frontal	Blood	cerebellum	frontal
CpG_1	0.4	0.55	0.5	0.46	0.27	0.36	0.4	0.6	0.54
CpG_2	0.4	0.18	0.3	0.54	0.36	0.09	0.3	0.5	0.31
CpG_3	0.2	0.09	0.1	0.31	0.09	0.18	0.3	0.2	0.23
CpG_4	0.6	0.45	0.2	0.38	0.45	0.27	0.3	0.4	0.23
CpG_5	0.3	0.27	0.2	0.46	0.27	0.18	0.2	0.2	0.08
CpG_6	0.3	0.18	0.2	0.46	0.36	0.36	0.4	0.2	0.38
CpG_7	0.1	0.36	0.4	0.54	0.36	0.27	0.2	0.4	0.31
CpG_8	0.2	0.45	0.5	0.46	0.36	0.55	0.4	0.6	0.31
CpG_9	0.5	0.36	0.5	0.31	0.27	0.36	0.2	0.6	0.31
CpG_10	0.6	0.27	0.1	0.46	0.27	0.36	0.3	0.5	0.46
CpG_11	0.3	0.09	0	0.38	0.27	0.27	0.3	0.4	0.15
CpG_12	0.5	0.27	0.6	0.46	0.36	0.45	0.3	0.3	0.54
CpG_13	0.5	0.55	0.3	0.46	0.45	0.45	0.5	0.4	0.46
CpG_14	0.4	0.55	0.2	0.38	0.27	0.36	0.5	0.4	0.23
CpG_15	0.7	0.55	0.4	0.54	0.55	0.36	0.5	0.4	0.15
CpG_16	0.5	0.45	0.3	0.31	0.45	0.45	0.3	0.4	0.46
CpG_17	0.5	0.36	0.4	0.23	0.45	0.45	0.7	0.4	0.15
CpG_18	0.4	0.64	0.4	0.54	0.36	0.55	0.5	0.4	0.54
CpG_19	0.5	0.55	0.3	0.38	0.36	0.55	0.4	0.4	0.54
CpG_20	0.4	0.36	0.3	0.46	0.36	0.27	0.6	0.4	0.38
CpG_21	0.7	0.73	0.4	0.54	0.36	0.64	0.8	0.3	0.46
CpG_22	0.5	0.73	0.2	0.46	0.45	0.36	0.33	0.3	0.31
CpG_23	0.3	0.27	0.2	0.54	0.45	0.09	0.22	0.2	0.23
CpG_24	0.4	0.27	0.3	0.31	0.36	0.36	0.44	0.5	0.15

Table 3. Methylation by subject and region

Appendix 3

A deletion in TRKB and development of human anxiety disorders

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Abstract

We identified a novel 11-base deletion in the promoter of TrkB that impairs transcription. In a 22-year longitudinal cohort sample representative of the Quebec general population, we demonstrate that this deletion is significantly associated with anxiety traits during childhood and the development of anxiety disorders in adulthood. The identification of this deletion provides additional support for the role of TrkB in anxiety-related traits.

Introduction

TrkB is a fundamental neurotrophic factor receptor in the brain that plays a critical role in synaptogenesis, neurodevelopment, and cell signaling. Functional studies of TrkB in mice suggest that the gene may play a key role in anxious traits. In mouse transgenic studies, over-expression of TrkB reduces anxiety (Saarelainen et al., 2003), while deletion of TrkB in forebrain induces impulsive reactions to novel stimuli and inappropriate coping responses when facing stressful paradigms (Zorner et al., 2003). Defective neuronal release of BDNF, a high affinity ligand for TrkB, leads to increased anxiety-like traits in mice (Berton et al., 2006; Chen et al., 2006). Conceptually, TrkB has been linked to psychiatric illness through the neurotrophin hypothesis of stress-related mood disorders (Duman et al., 1997; Duman and Monteggia, 2006).

Results

While screening the TrkB gene in 39 subjects from the French-Canadian population, we found two subjects with an 11 base pair deletion (Figure 1A,B). While the structure of TrkB is complex (Stoilov et al., 2002; Martens et al., 2007b), this deletion is located in a region thought to be the promoter (Martens et al., 2007b). To investigate this novel deletion, we designed a series of assays (supplemental material). First, the wildtype TrkB sequence contains a single *AluI* digestion site, allowing for identification of deletion carriers by means of a restriction enzyme assay (Figure 1C). Second, given the size of the deletion, we could detect deletion carriers by running the PCR product of DNA amplified through primers flanking the deletion site in agarose gels; in individuals with the deletion, we observed two bands (Figure 1D). Finally, we sequenced both mutant and wild-type bands (Figure 1E), and cloned the deletion (Figure 1F,H).

We cloned a ~2.1 Kb fragment that included the deletion region as well as downstream sequences (supplemental material). Consistent with a previous report (Martens et al., 2007b), this region showed clear promoter activity (Figure 1G) both in COS7 cells and HEK293 cells, where constructs with the deletion showed less luciferase activity than wildtype constructs (COS7: $t=4.33$, $p=0.012$; 1.41 fold decrease; HEK293: $t=4.1$, $p=0.002$; 1.37 fold decrease).

Given the evidence suggesting that decreased TrkB activity is associated with anxiety-related behaviors in animal, we hypothesized that individuals carrying this deletion would

be more prone to present increased measures of anxiety traits. To test this hypothesis, we investigated 640 participants from a 22-year longitudinal study with a representative sample of kindergarten children from the province of Quebec (Zoccolillo et al., 1999). Included in the present study were subjects that had complete childhood and adult data on behavioural traits through all assessment waves, complete adult psychiatric information, and provided a DNA sample. In total, we found 20 subjects (3.1%) with the deletion, suggesting that this is not a common genetic variant.

Using a clustering technique for longitudinal data (Nagin, 1999), we identified 5 trajectories of anxiety with different longitudinal profiles based on annual parent ratings of anxiety traits from age 6 to 12 (Figure 2A). Individuals following the high (N = 146; N_{DEL} = 6; 4.1%) and moderately high (N = 321; N_{DEL} = 13; 4.0 %) anxiety trajectories were significantly more likely to carry a deletion (Fisher's p=0.01) when compared to individuals following a decreasing low (N = 26; N_{DEL} = 0), low (N = 118; N_{DEL} =1, 0.6 %) or very low (N = 29; N_{DEL} = 0) trajectories. Similar results were observed for teacher-rated scores of anxiety from ages 6 to 12 (supplemental material).

At age 21-23 years, individuals were re-assessed for anxiety traits using a personality trait questionnaire (Livesley and Jackson, 1986; Livesley and Jackson, in press). Analyses showed that the deletion was associated with higher anxiousness scores (β =.09, t (1) = 2.27, p < .05), even in subjects who were diagnosed with GAD (Figure 2B). This association was robust, and in fact strengthened, when covariates were considered (β =.09, t (1) = 2.21), p < .05). In addition, at this age period, all subjects were assessed for

the presence of psychiatric disorders. As shown in Figure 2C, individuals carrying the deletion were significantly more likely to be diagnosed with generalized anxiety disorder (GAD) ($\chi^2 = 4.58$, df = 1; $p < .05$) and panic disorder (PD) ($\chi^2 = 5.33$, df = 1; $p < .05$), even after adjusting for significant covariates (gender and early family adversity). Having the deletion increased the odds of GAD by about three times (OR = 2.86; 95% CI 1.1-7.5) and of PD by about 3.5 times (OR = 3.69; 95% CI 1.2-11.2) and explained 3% of the variance in both PD and GAD.

We also examined whether anxiety trajectory membership on the basis of teacher or parent ratings were differentially related to adult anxiety measures in order to establish predictive validity of the trajectories after controlling for gender and family adversity (Supplemental material).

Disucussion

The functional deletion in TrkB identified here provides additional support for the role of TrkB in anxiety-related traits. This deletion has never been documented and is relatively infrequent in the general French-Canadian population. At the 5'-end immediately preceding the deleted region are the following five bases: 5'-CTGGGC-3'; we note that the same five bases (deleted bases are: 5'-CGGAGCTGGGC-3') are present at the 3'-end of the deleted region. It is possible that this deletion arose due to strand slippage, effectively excluding the deleted region from the DNA replication process.

Promising association studies in schizophrenia and mood disorders have not often been replicated in independent analyses. The strength of the current study is the multifaceted longitudinal study approach; therefore, potential replication studies need similar developmental designs.

Materials and methods

Subjects

Comprehensive descriptions of the community cohort used in this study are available elsewhere (Tremblay et al., 1994; Brezo et al., 2006). Briefly, 640 (364 female participants, 57%) members of a cohort followed since 1986 were studied in this report; they are currently aged 26-28. These individuals were randomly selected from French speaking public schools in Quebec, Canada when they were in kindergarten. Only subjects whose parents were born in Quebec and whose mother tongue was French were included in this study(Tremblay and Schaal, 1996).

Behavioral and Psychiatric Assessments

Social Behaviour Questionnaire(Masse and Tremblay, 1997) (SBQ) :

Cohort members were assessed yearly during childhood from age 6 to 12 using the Social Behavior Questionnaire(Masse and Tremblay, 1997) which uses teacher and parent reports to score several childhood traits. Anxiousness was tested with six items from the Social Behavior Questionnaire: 1) Fearful or afraid of things or new situations; 2) is worried, worries about many things; 3) cries easily; 4) has a tendency to work alone; 5) looks sad, unhappy, tearful; 6) easily distracted. These items were used to identify developmental behavioral scores of anxiety (Cronbach α: .72 to .77). The SBQ was scored independently by the classroom teacher and parent.

Diagnostic Assessment of Personality Pathology – Brief Questionnaire(Livesley et al., 1998) (DAPP-BQ): Anxiety traits in adulthood (age 21-23) were obtained by means of

the DAPP-BQ, which is a self-administered questionnaire containing 290 questions measuring personality traits. To obtain anxiety scores, we used the anxiousness subscale, which is composed of 16 items (Cronbach α: .92).

Diagnostic Interview Schedule(Robbins LN, 1995) (DIS)

Diagnoses and symptom counts of anxiety disorders in adulthood (age 21-23) were obtained using DSM-IIIR criteria on information collected by means of interviews using the Diagnostic Interview Schedule. This is a fully structured diagnostic interview specially designed for epidemiological studies.

Laboratory procedures

DNA was extracted using the blood DNA extraction kit (Qiagen), following the manufacturer's protocol. Polymerase chain reaction (PCR) was performed using an ABI 2720 thermocylcer and platinum Taq polymerase (ABI). All PCR reagents were purchased from Qiagen. We had two different forward primers to assess the deleted fragment, both of which were amplified using the same reverse primer. For product amplification studies, we used PCR primers forward 5'- GGTGAGCAGCGCAGATAGT and reverse 5' – GCTGAGGACAAACAGACACG. This PCR product is 495 bases. For molecular cloning and the restriction enzyme digestion, we used the forward 5' - CAGGCTCGAAGAGAGAGTGG, with the same reverse primer as for the amplification experiment. *AluI* digestion was performed according to the manufacturer's protocol (New England Biolabs). Briefly, the PCR product was amplified and incubated with *AluI* overnight at 37 C. *AluI* cuts DNA at AGCT, a site that occurs once in the 223 basepair

PCR product. The 11 bp deletion occurs at the *AluI* site, protecting the fragment from endonuclease activity. In wildtype subjects, use of this enzyme with these primers results in 83 and 140 base products. All DNA sequencing was done at the Genome Quebec Innovation Centre. For detection of the deletion, all subjects were screened by DNA sequencing. RNAse was used at relevant points in all DNA experiments.

We cloned a 2,182 bp product (wildtype) and a 2,171 bp product (mutant) into the pDrive vector. To do this, we used primers forward 5'- AGGCACTGCGGTGTATTTTC and reverse 5' – TGCGGCTCTCTTAACTCCTC. After endonuclease excision, the product was subcloned into the pGL3 vector (Promega), verified for orientation, and sequenced. Plasmid propagation was done by transforming competent *E.coli* cells (Qiagen PCR Cloning*Plus* Kit). Bacterial cells were grown overnight on an agar media at 37 C and positive colonies were selected. Plasmids were extracted from cells using Qiagen plasmid MINI kits. Plasmids were co-transfected into HEK293 or COS7 (ATCC) cells with a pRL plasmid (internal control). Transfections were done using lipofectamine 2000 and left overnight in DMEM media (Gibco). All transfection experiments were compared to a baseline level of luciferase activity using the pGL3 basic plasmid. Luciferase experiments were performed using a Berthold dual injection Luminometer and the Dual-Luciferase Reporter Assay system (Promega). Plasmid-transfected cells were lysed with passive lysis buffer and incubated with Luciferase Assay Reagent II. Firefly luciferase activity was then measured. Immediately following activity reading, Stop-and-Glo reagent was injected, and *Renilla* luciferase activity was measured. Experiments done in HEK293 cells were performed in triplicate each time for each transfected plasmid

across different days. Statistics were generated by pooling all experiments. Experiments done in COS7 cells were done independently from those done in HEK293 cells and were done in triplicate.

Figures

Figure 1. **Assays designed to test for a deletion in TrkB**. A) Structure of the TrkB gene; exons are represented by individual lines. ChAB4 is a 35K repeat in the gene, spanning approximately 250 Kb. B) A promoter region, including the deletion (highlighted) and primer binding sites (underlined). C) Restriction digestion (*AluI*) of the deleted fragment. Subjects with the deletion (MUT) are all heterozygous for the mutation, and therefore half of the DNA product is protected from *AluI* digestion. Note the presence of the full length band in MUT lanes, which are absent in all wildtype (WT) lanes. D) Gel electrophoresis showing MUT and WT subjects. Note the presence of an extra band, 11 bps less than the full length band, in MUT lanes. E) Sequencing from mutant (MUT) and wildtype (WT) bands. Note the doubling of peaks after the deletion (arrow) in the MUT lanes. F) Gel electrophoresis of cloned fragment, lower molecular weight bands are present in MUT lanes. G) Luciferase assay to assess functionality of the TrkB deletion. H) Sequencing of the cloned bands in MUT and WT subjects from (E).

Figure 2. Relationship between TrkB deletion, anxiety traits and anxiety disorders A) Longitudinal trajectory profiles of parent ratings of anxiety while cohort members were children aged 6 to 12 and their relationship to the TrkB promoter deletion. B) Distribution of anxiety traits using the DAPP and C) Proportion of GAD and PD diagnoses in cohort members during early adulthood (age 21-23) according to their TrkB deletion status.

Figures

Figure 1

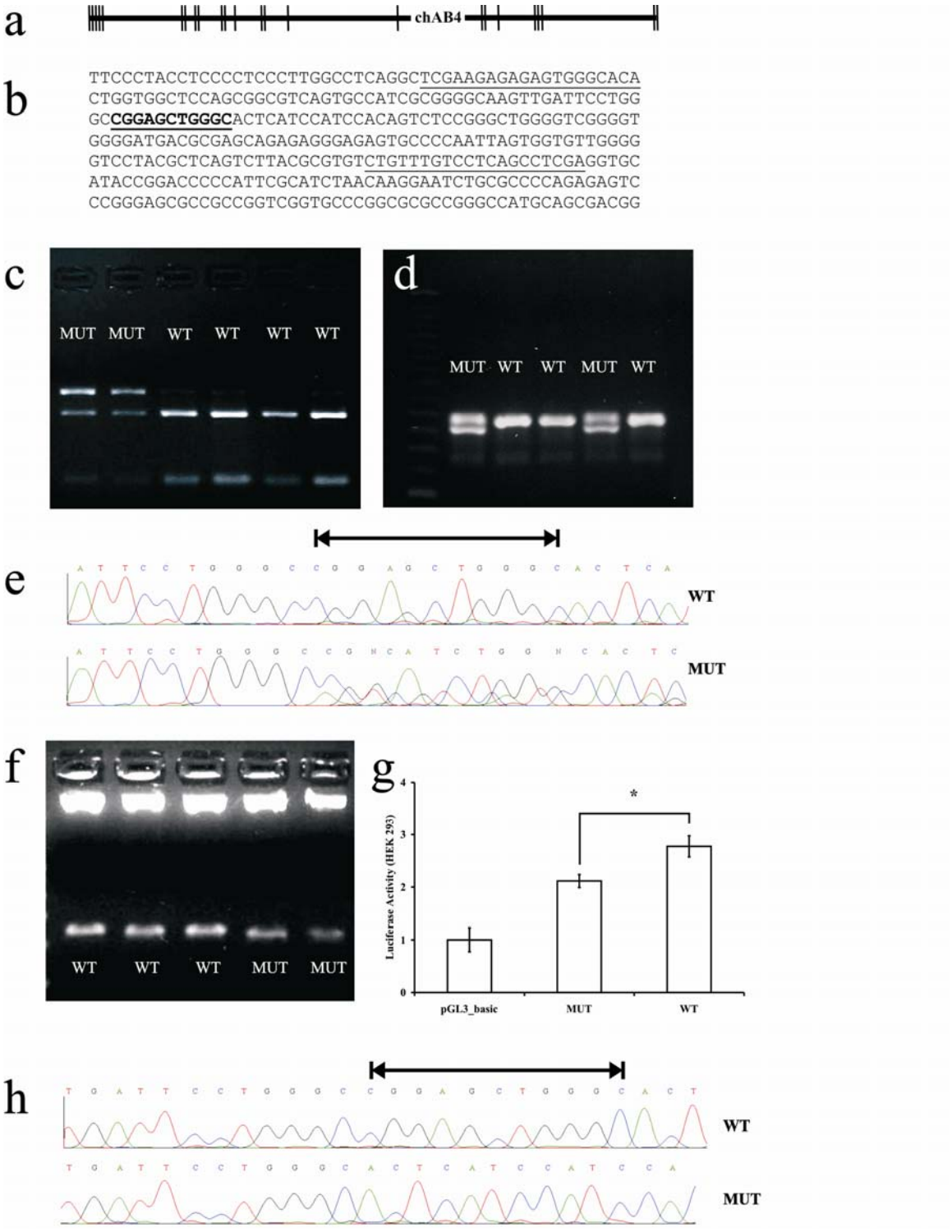
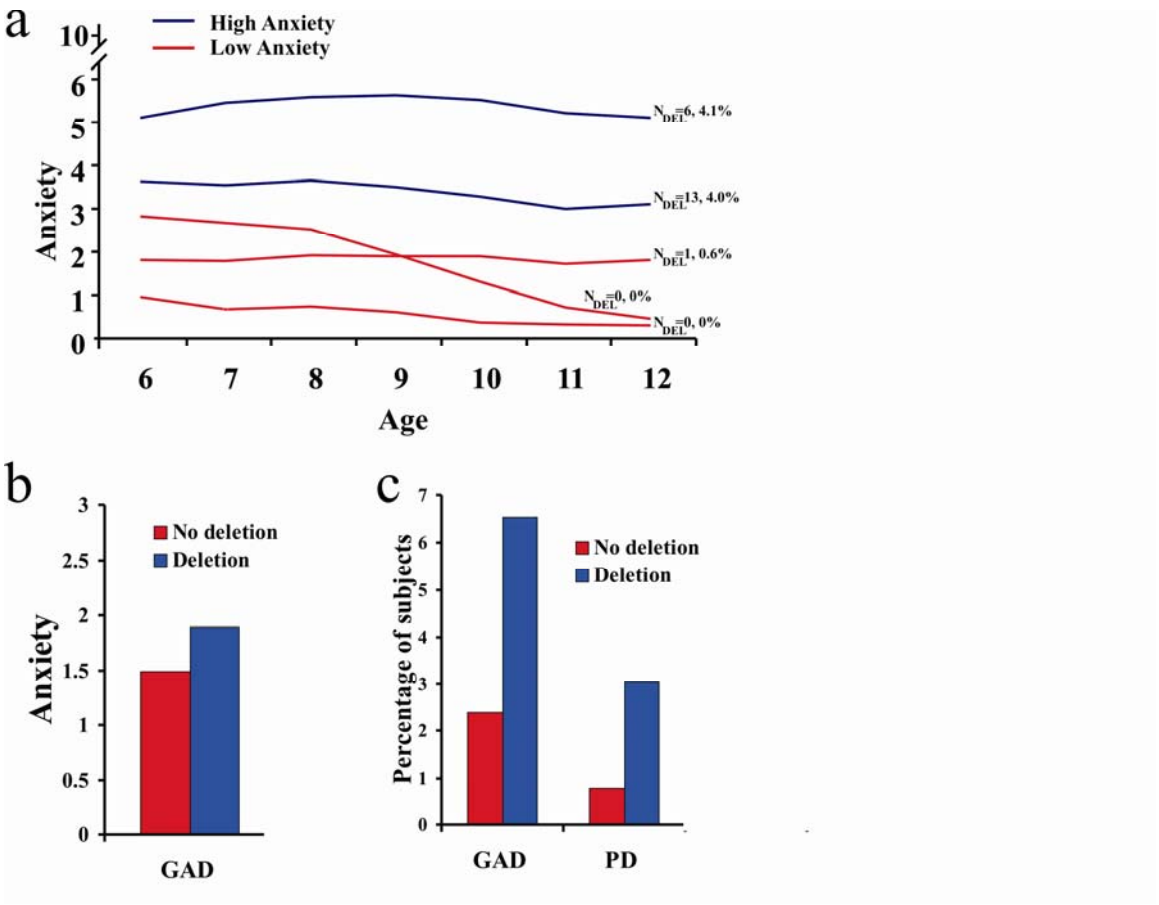


Figure 2


a
High Anxiety
Low Anxiety
Anxiety
Age
N_{DEL}=6, 4.1%
N_{DEL}=13, 4.0%
N_{DEL}=1, 0.6%
N_{DEL}=0, 0%
N_{DEL}=0, 0%
b
No deletion
Deletion
Anxiety
GAD
c
No deletion
Deletion
Percentage of subjects
GAD
PD

Supplemental material

Childhood trajectories of anxiety

Using a semi-parametric clustering technique for longitudinal data(Nagin, 1999; Nagin and Tremblay, 1999), we identified trajectories with distinct longitudinal profiles of teacher and parent ratings of annually assessed anxiety scores between ages 6 and 12 years on the SBQ (Figure 2A and Fig S1). For both teacher and parent ratings of anxiety, gender composition was balanced across the trajectory groups.

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To test the hypothesis that the deletion is predictive of high levels of anxiety using the teacher-rated anxiety scores, we calculated a series of Fisher's exact tests. The results revealed that a significant ($p < .05$) higher percentage of members ($N_{DEL} = 7$; 5.7%) of the teacher-rated extreme trajectory group carried a deletion compared to only one percent of the members of the teacher-rated low trajectory group ($N_{DEL} = 2$; 1.2%), whereas the remaining trajectory groups were similar to the low group in this regard ($N_{DEL} = 8$; 3.3% and $N_{DEL} = 3$; 2.6%, for the decreasing and increasing trajectory group, respectively). Hence, for both teacher and parent respondents, children with consistently high anxiety trajectories were more likely to carry a deletion compared to children whose anxiety levels were comparably lower between ages 6 and 12 years.

Adult anxiety traits

At age 21 years, ordinary least squares regression analyses showed that the deletion was associated with higher DAPP-BQ anxiousness scores (β =.09, F (1, 640) = 5.16, $p < .05$) and it explained 1% of the variance. The inclusion of gender and early family adversity did not affect the strength of the association between deletion and DAPP-BQ anxiousness scores, but increased the amount of the variance explained by the deletion to 3% (β =.09, F (3, 640) = 7.52), $p < .01$).

Adult anxiety disorders

We hypothesized that individuals carrying the deletion would be at higher risk for anxiety disorders in adulthood. At age 21-23 years, all participants were assessed for the presence of psychiatric disorder symptoms by means of the Diagnostic Interview Schedule for Adults (DIS) using DSM-III-R criteria. Individuals carrying the deletion were significantly more likely to have symptoms of generalized anxiety disorder (GAD) (χ^2 = 4.58, df = 1; $p < .05$) and panic disorder (PD) (χ^2 = 5.33, df = 1; $p < .05$), as indicated in multivariate negative binomial regression models that adjusted for significant covariates (gender and early family adversity). Having the deletion increased the odds of GAD about three times (OR = 2.86; 95% CI 1.1-7.5) and of PD symptoms about three and a half times (OR = 3.69; 95% CI 1.2-11.2). Being male significantly decreased the number of GAD (OR = 0.53; 95% CI 0.4-0.7; $p < .01$), but not PD. Early life adversity did not contribute to a statistically significant degree to either phenotype.

Moderation

We also tested the hypothesis that the deletion represents a common diathesis that underlies each adult anxiety measure. For this purpose, we included interaction terms in the previous regression models. When including interaction terms in the regression models, the results showed that the deletion neither moderated the link between PD and DIS GAD ($\chi^2 = 0.21$, df = 1; $p = .64$) nor between PD and DAPP-BQ anxiousness (t (1, 640) = 0.62, $p = .53$).

We also tested the common diathesis hypothesis in a linear manner. Specifically, we tested whether the deletion explained part of the common variances of the adult anxiety measures (Mplus 3.011 software). Prior to controlling for the deletion but accounting for gender and family adversity, the partial correlations were $r = .43$, $z = 6.11$ and $r = .23$, $z = 5.03$, for PD_DIS GAD and PD_DAPP-BQ anxiousness, respectively. After controlling for the deletion from these partial correlations (i.e., PD, DIS GAD, and DAPP-BQ anxiousness were regressed on the deletion), the partial correlations were $r = .40$, $z = 6.17$ and $r = .21$, $z = 4.77$ for PD_DIS GAD and PD_DAPP-BQ anxiousness, respectively. Although only a small percentage of participants carried the deletion, the deletion explained 3% of the variance in both DIS PD and DIS GAD and 1% of the variance in DAPP-BQ anxiousness.

Appendix 4

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Appendix 5

Ethics Approval forms

Le 30 octobre 2008

Dr Gustavo Turecki
Centre de recherche de l'Institut Douglas
Pavillon FBC

Object : **Protocole 04/15** *Predisposition to Suicide : A Brain Expression Study*
Renouvellement annuel

Dr Turecki,

Nous désirons vous remercier pour le rapport annuel que vous avez soumis pour approbation pour le protocole cité en rubrique. J'ai examiné votre rapport et l'ai jugé satisfaisant. À titre de président, j'accorde donc une approbation accélérée puisque le rapport est complet et qu'il rencontre les exigences du CÉR.

Cette étude est à ré-approuvée pour une période d'une année.

Merci de votre collaboration.

pour :
Serge Gauthier, M.D., F.R.C.P(c)
Président
Comité d'éthique de la recherche
de l'Institut Douglas

/lb

Comité d'éthique de la recherche

Lors d'une réunion du Comité d'éthique de la recherche de l'Hôpital Douglas tenue le *11 novembre 2008*

Un comité composé de:

Dr Serge Gauthier, Président & Ethicien	Neurologue
Dr Kenneth Bruce	Psychologue
Dr Anne Crocker	Chercheure
Me Johane de Champlain	Avocate
M. Denis DeChazal	Représentant la communauté
Mme Moira Edwards	Représentant la communauté
Mme Iris Jaitovitch Groisman, Éthicienne interim.	Chercheure
Mme Sylvie Mador	Archiviste
Dr Valentin Mbekou	Psychologue
Mme Françoise Thomas	Représentant la communauté
Dr Jacques Tremblay	Omnipraticien

A confirmé l'approbation du rapport annuel soumis pour le protocole intitulé:

Predisposition to Suicide : A Brain Expression Study

Tel que proposé par: Dr Gustavo Turecki

Ce protocole est approuvé pour une période d'une année

Serge Gauthier, M.D., F.R.C.P(c), Président
Comité d'éthique de la recherche de l'Hôpital Douglas
/lb

Date: 11/11/08
 # CÉR: 04/15

Appendix 6

Copyright waiver forms

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From: Carl Philip Ernst [mailto:carlphilip.ernst@mail.mcgill.ca]
Sent: Tuesday, January 13, 2009 2:50 PM
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Hi,

I would like to re-produce an article I published in Arch Gen Psy in my PhD thesis at McGill University.

my name and address:
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Article (I want to reproduce the entire article):

Arch Gen Psychiatry. 2009;66(1):22-32.

Alternative Splicing, Methylation State, and Expression Profile of Tropomyosin-Related Kinase B in the Frontal Cortex of Suicide Completers

Carl Ernst, MSc; Vesselina Deleva, MSc; Xiaoming Deng, MD; Adolfo Sequeira, PhD; Amanda Pomarenski, BSc; Tim Klempan, PhD; Neil Ernst, MSc; Remi Quirion, PhD; Alain Gratton, PhD; Moshe Szyf, PhD; Gustavo Turecki, MD, PhD

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