Chronic SSRI stimulation of astrocytic 5-HT$_{2B}$ receptors change multiple gene expressions/editings and metabolism of glutamate, glucose and glycogen: a potential paradigm shift

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INTRODUCTION: SERT, THE 5-HT$_{2B}$ RECEPTOR AND SSRI

**SSRI TARGETS**

It is firmly believed that the mechanism of action of SSRI's in major depression is to inhibit the serotonin transporter, SERT, and increase extracellular concentration of serotonin. However, this undisputed observation does not prove that SERT inhibition is the mechanism, let alone the only mechanism, by which SSRI's exert their therapeutic effects. It has recently been demonstrated that 5-HT$_{2B}$ receptor stimulation is needed for the antidepressant effect of fluoxetine in vivo. The ability of all five currently used SSRIs to stimulate the 5-HT$_{2B}$ receptor equipotentially in cultured astrocytes has been known for several years, and increasing evidence has shown the importance of astrocytes and astrocyte-neuronal interactions for neuroplasticity and complex brain activity. This paper reviews acute and chronic effects of 5-HT$_{2B}$ receptor stimulation in cultured astrocytes and in astrocytes freshly isolated from brains of mice treated with fluoxetine for 14 days together with effects of anti-depressant therapy on turnover of glutamate and GABA and metabolism of glucose and glycogen. It is suggested that these events are causally related to the mechanism of action of SSRI's and of interest for development of newer antidepressant drugs.

**Keywords:** calcium homeostasis, fluoxetine signaling, 5-HT$_2$ receptors, glucose metabolism, glutamate/GABA, glycogen, major depression, PLA$_2$

**SSRI TARGETS**

It is generally thought that the molecular mechanism of SSRI's is the long-known blockage of serotonin (5-HT) reuptake by its transporter SERT (Fuller and Wong, 1977; Wong and Bymaster, 1995). Binding of SSRI's to this transporter (Langer et al., 1986; Launay et al., 2006; Diaz et al., 2012) leads to elevated extracellular 5-HT levels, assumed to cause their antidepressant effects. SSRIs do exert a direct inhibitory effect on the SERT protein on the neuronal presynaptic plasma membrane (Zhou et al., 2009). Recently the molecular basis for high-affinity recognition of fluoxetine in the SERT molecule was unraveled (Andersen et al., 2014). However, these findings do not prove that SERT inhibition is the mechanism, let alone the only mechanism, by which SSRI's exert their therapeutic effects in major depression.

Recently Diaz et al. (2012) demonstrated in intact brain a raphe nuclei 5-HT$_{2B}$ receptor with relatively high affinity for SSRIs, which was indispensable for the antidepressant effect of fluoxetine. However, SSRI effects exerted via the 5-HT$_{2B}$ receptor had previously been described not only in cultured neurons (Launay et al., 2006) but also in cultured astrocytes (Kong et al., 2002), where they have been thoroughly studied (Li et al., 2008a, 2009; Zhang et al., 2010). The resultant induction of signaling pathways in glia and neurons has been further characterized in astrocytic and neuronal fractions from mice treated for 2 weeks with fluoxetine. They may play a key role in the antidepressant mechanism of SSRIs. Given the dominant role ascribed to neurons in the mechanisms of SSRIs and other psychiatric drugs it may appear surprising that studies found the major chronic impact of SSRIs in the fluoxetine-treated mice to be on astrocytes. However they are consistent with the growing evidence for a major role of these cells in major depression and their therapy studied in patients (Abdallah et al., 2014a,b), postmortem brain (Rajkowska and Stockmeier, 2013) or in models of this disease (Gosselin et al., 2009; Banasr et al., 2010).

Astrocytes account for $\sim$25% of brain cortical volume and are responsible for at least a corresponding fraction of oxidative energy metabolism, mainly required for maintaining extracellular glutamate, GABA, and K$^+$ homeostasis, and synthesis of glutamate and GABA via the glutamine-glutamate (GABA) cycle (reviewed in Hertz, 2011). This cycle is well established in the brain in vivo, where it represents the quantitatively most important interaction between neurons and astrocytes. It will be described in more detail below. Astrocytes synthesize
all transmitter glutamate and GABA and accumulate most after neuronal release. According to recent research (Duarte and Gruetter, 2013), astrocytes account for an even larger fraction of oxidative brain metabolism, when their role in subsequent metabolism of released GABA before its partial return via astrocytes to neurons also is taken into account. These major roles of astrocytes are likely to be relevant for antidepressant effects on glutamate homeostasis, excitatory and inhibitory signaling, and glucose metabolism. Involvement of the glutamine-glutamate (GABA) cycle remains to be studied after SSRI administration, but it has been investigated in patients suffering from major depression (Abdallah et al., 2014a). Moreover, studies of the rapidly acting anti-depressant drugs ketamine or riluzole (which have no known effect on 5-HT<sub>2B</sub> receptors) have shown that increases in flux in this cycle parallel recovery from experimental and clinical depression (Chowdhury et al., 2008, 2012; Brennan et al., 2010).

Besides discussing the 5-HT<sub>2B</sub> receptor as an SSRI target, this review will deal with 5-HT<sub>2B</sub> receptors' cellular locations; the signaling pathways activated; short term effect on cell signaling; and long-term-effects in cultured astrocytes and in fluoxetine-treated animals. Consequences of long term (14 days) SSRI treatment on gene up-regulation and editing in primary cultures of astrocytes and in neurons and astrocytes freshly isolated from the brains of mice treated with fluoxetine and/or in whole brains from such animals are described in detail. Some of these effects are exerted on genes mediating glutamate/glutamine transport and interconversion and on glutamate and GABA receptor genes. Other effects are exercised on genes which are not directly related to glutamate signaling, but are important for the well-established correlation between recovery from major depression and increase in glucose metabolism in brain (Buchsbaum et al., 1997; Mayberg et al., 2000; Kennedy et al., 2001). Finally, the reviewed studies pinpoint acute and chronic effects on pathways for glycogen turnover. This is important because glycogenolysis is known to impact glutamate formation, learning and longer term neuroplasticity (Gibbs et al., 2007, 2008; Duran et al., 2013). Together, these changes may be the link between the molecular and cellular changes due to 5-HT<sub>2B</sub> receptor binding and the longer-term impact on depressive symptoms. Selective activation of this receptor or intermediates of its downstream pathways may accordingly constitute potential targets for pharmaceutical development. Such development would be important, since (i) a considerable fraction of patients suffering from major depression do not respond adequately to current antidepressant therapy; (ii) the response is slow except for a few recently tested drugs (O’Leary et al., 2014); and (iii) even the relatively safe SSRIs can have severe side effects if used in pregnant women (Ellfolk and Malm, 2010) or after acute coronary occlusion (Rieckmann et al., 2013).

**THE CRITICAL IMPORTANCE OF THE 5-HT<sub>2B</sub> RECEPTOR FOR SSRI EFFECTS**

The 5-HT<sub>2B</sub> receptor was identified in 1987 (Cohen and Fludzinski, 1987) and was thus unknown when SSRIs were introduced and believed to lack relevant receptor effects. Like other 5-HT<sub>2</sub> receptors, the 5-HT<sub>2B</sub> receptor is G<sub>q/11</sub> protein-coupled and stimulates phospholipase C (PLC) to generate diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP<sub>3</sub>) by hydrolysis of phosphatidyl-inositol 4,5-bisphosphate (PIP<sub>2</sub>). This triggers IP<sub>3</sub> receptor-mediated increase of free cytosolic calcium concentration ([Ca<sup>2+</sup>]<sub>i</sub>) (Deecher et al., 1993; Roth et al., 1998; Porter et al., 1999) and additional second messenger effects. During chronic exposure to fluoxetine these second messenger effects may be responsible for the many reported changes in gene expression, as well as alterations in metabolism and the glutamine-glutamate (GABA) cycle that will be described later.

The 5-HT<sub>2B</sub> receptor is expressed in mouse, rat, and human brain (Kursar et al., 1994; Baez et al., 1995; Bonhaus et al., 1995; Choi and Maroteaux, 1996). In astrocytes obtained from mouse brain (Zhang et al., 2010) its mRNA expression is ~2 times higher than in neurons (Li et al., 2012, see Figure 1). It is also a major 5-HT<sub>2</sub> receptor in astrocyte cultures (Kong et al., 2002). Neuronal expression has been reported in Purkinje cells (Choi and Maroteaux, 1996) and raphe nuclei (Diaz et al., 2012). Diaz et al. (2012) showed in an in vivo rat model that 5-HT<sub>2B</sub> receptors are needed for long-term behavioral effects of fluoxetine. These effects were abolished in 5-HT<sub>2B</sub><sup>−/−</sup> mice or after pharmacologic inactivation of 5-HT<sub>2B</sub> receptors, whereas stimulation by a selective 5-HT<sub>2B</sub> receptor agonist induced similar responses as fluoxetine in behavioral assays. Fluoxetine-mediated neurogenesis (Manev et al., 2003) was eliminated in 5-HT<sub>2B</sub> knock-out animals, and acute fluoxetine administration to 5-HT<sub>2B</sub><sup>−/−</sup> mice induced a much smaller increase in hippocampal 5-HT levels than in wild type mice.

**ACUTE EFFECTS OF SSRIs ON 5-HT<sub>2B</sub> RECEPTORS AND THEIR TARGETS**

**ACUTE EFFECTS ON 5-HT<sub>2B</sub> RECEPTORS ON NEURONS**

5-HT<sub>2B</sub> receptor effects on SERT or inhibition of 5-HT release has been shown in very young cultures of serotonergic neurons and in neuronal-enriched cultures from the raphe nuclei (Azmitia et al., 1990; Launay et al., 2006). However, these nuclei in addition contain glia cells (Azmitia and Whitaker-Azmitia, 1991), which may also be present in the cultures. Unfortunately our own cultured neurons tolerate fluoxetine poorly. Moreover, they are also young and the immature nervous system is different from the mature brain (e.g., Hertz, 2013). However, future studies to establish potential direct neuronal responses to fluoxetine would be of high importance.

**GLIAL 5-HT<sub>2B</sub> BINDING IS CRITICAL FOR IN VIVO EFFECTS OF SSRIs**

Support for the concept that direct binding to 5-HT<sub>2B</sub> receptors is important for SSRI's mechanisms of action is that all presently used SSRIs have virtually identical affinity for the 5-HT<sub>2B</sub> Receptor in cultured astrocytes (Zhang et al., 2010). This contrasts the huge differences in their potency as inhibitors of 5-HT uptake (Wong and Bymaster, 1995; Popik, 1999). Also inconsistent with SSRIs owing their therapeutic effects solely to an action on SERT are findings that substantial SERT occupancy (about 80%) occurs at sub-therapeutic SSRI doses, and that increasing doses to clinically
Studies on post receptor signaling in these glia cells have shown effects on signaling targets such as growth factors and glycogen synthesis. Both of these are known to influence neuroplasticity, which is believed to be key factor in the efficacy of SSRIs as antidepressants (Eom and Jope, 2009; Freitas et al., 2013). Acute administration of fluoxetine increases [Ca\(^{2+}\)]\(_i\) and stimulates glycogenolysis (a [Ca\(^{2+}\)]\(_i\)-dependent process) in cultured astrocytes (Zhang et al., 1993; Chen et al., 1995). These effects are not limited to cultured cells, since both fluoxetine and paroxetine support learning in day-old chickens. This effect is inhibited by an antagonist of the 5-HT\(_{2B}\) receptor and by a glycogenolytic inhibitor (Gibbs and Hertz, 2014). Further evidence that the effects were specific to 5-HT\(_{2B}\) binding as opposed to SERT was that the same effects were obtained with almost identical doses of SSRIs (Zhang et al., 2010) that affect 5-HT uptake with widely different potency (Wong and Bymaster, 1995).

The signaling pathway activated by fluoxetine in cultured astrocytes (Li et al., 2008a) is complex (Figure 2). Abolishment of 5-HT\(_{2B}\) receptor activity by its siRNA or administration of the 5-HT\(_{2B}\) receptor inhibitor SB204741 prevented any response, further validating the concept that the 5-HT\(_{2B}\) receptor is directly stimulated. Phosphorylation of extracellular kinases 1 and 2 (ERK\(_{1/2}\)) was established as an end point without examining further downstream effects, except for a rapid increase in the expression of the immediate early genes cfas and fosB. The increase in [Ca\(^{2+}\)]\(_i\) (Chen et al., 1995) leads to activation of metalloproteinases (MMPs) and shedding of growth factor(s), perhaps mainly heparin-binding epidermal growth factor (HB-EGF). This EGF receptor agonist is known to be present in adult brain and to be required for synaptic plasticity (Oyagi et al., 2011).

Growth factor release may link glial 5-HT\(_{2B}\) binding to the longer-term cellular and behavioral changes induced by SSRI stimulation of receptor tyrosine kinases of the epidermal growth factor (EGF) receptor (EGFR). Such an effect of G protein-coupled receptors represents a transactivation process, a common mechanism in astrocytes (Daub et al., 1997; Peavy et al., 2001; Peng, 2004; Du et al., 2009). mRNA expression of the EGRF is approximately 4 times higher in freshly isolated mouse brain astrocytes than in corresponding neurons (Peng et al., 2014). EGFR phosphorylation leads to activation of ERK\(_{1/2}\) and of PI3K, with the latter causing AKT phosphorylation (Hertz et al., 2012; Peng et al., 2014). Moreover, released growth factor also acts on neurons (Li et al., 2008b) and may be at least one of the stimuli for the well known fluoxetine-stimulated neurogenesis (Manev et al., 2003) and effects on synaptic activity (Oyagi et al., 2011). Within 1 h the phosphorylation of ERK\(_{1/2}\) induces gene expression of cfos and fosB in astrocytes (Li et al., 2008a), shown in Figure 3 to be abolished by inhibitors of the pathway indicated in Figure 2. Fluoxetine also rapidly induces ERK\(_{1/2}\)-dependent enhancement of gene expression of glial-derived nerve factor (GDNF) in the cultured astrocytes used by Mercier et al. (2004). These cultures differ in several respects from ours, e.g., by being prepared from rats instead of mice and without exposing the cells to the differentiating agent dibutylryl...
FIGURE 2 | Schematic illustration of pathways leading to stimulation of extracellular-regulated kinase (ERK) and AKT phosphorylation by fluoxetine in astrocytes. Fluoxetine binds to 5-HT$_{2B}$ receptors. The activation of the receptors induces protein kinase C (PKC) activity and increase of intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]$^i$) by Ca$^{2+}$ release from intracellular stores. The latter activates Zn-dependent metalloproteinases (MMPs) and leads to shedding of growth factor(s). The released epidermal growth factor receptor (EGFR) ligand stimulates phosphorylation of the EGFR. The downstream target of EGFR (ERK) ligand stimulates phosphorylation of the EGFR. The downstream target of EGFR, ERK (shown in blue) is phosphorylated via the Ras/Raf/MEK pathway, and AKT is phosphorylated via PI3K pathway. PIK3 is also known to catalyze the formation of PIP$_3$ from PIP$_2$. During fluoxetine administration, phosphorylation of ERK and AKT was prevented after siRNA administration against the 5-HT$_{2B}$ receptor or after administration of inhibitors (shown in yellow) of this receptor (SB204741), of PKC (GF 109293X), of intracellular Ca$^{2+}$ homeostasis (BAPTA/AM, an intracellular Ca$^{2+}$ chelator), of Zn-dependent MMPs (GM6001), of the receptor-tyrosine kinase of the EGFR (AG1478), of ERK phosphorylation (U0126, a mitogen-activated kinase (MEK) inhibitor) or of the AKT pathway (LY294002, a PI3K inhibitor). This inhibition is an indication of participation of all the inhibited factors in the normal signaling pathway. (Hertz et al., 2012).

cyclic AMP. It is re-assuring that several aspects of fluoxetine effects are the same. However, some differences were also found, which is not unexpected when cultured cells are used.

A further important step where glial 5-HT$_{2B}$ receptor binding may influence the longer-term cellular and behavioral effects of SSRIs is the impact on glycogen synthesis. Glycogen turnover, i.e., interspersed glycogen synthesis and glycogenolysis, is indispensable during learning (Gibbs and Hutchinson, 2012; Hertz et al., 2013a). The acute memory-enhancing, glycogenolysis-dependent effect of both fluoxetine and paroxetine has been mentioned (Gibbs and Hertz, 2014). Knock-out of brain glycogen synthase abolishes learning of new motor and cognitive skills (Duran et al., 2013). It is likely that fluoxetine also affects glycogen synthesis, since activation of glycogen synthase by GSK-3 decreases its activity (Embi et al., 1980; De Sarno et al., 2002).

Consistent with these findings in cultured astrocytes Plenge (1976) found an acute increase in brain glycogen after administration of Li$^+$ (“lithium”), a known inhibitor of GSK3. As shown in Figure 4, this reflected increased glycogen synthesis (Plenge, 1982). Effects of fluoxetine (and of electroconvulsive therapy) by inhibitory phosphorylation of GSK-3 have also been summarized by Gould et al. (2006), and the role of GSK-3 in synaptic plasticity, including memory, discussed by Bradley et al. (2012).

The Jope group found in whole brain that 5-HT$_{2B}$ stimulation or acute fluoxetine administration decreases the levels of phosphorylated GSK3 (Li et al., 2004; Polter et al., 2012). This response was blunted or absent in young mice (Beurel et al., 2012), consistent with astrocytic localization since astrocytes are mainly generated postnatally (Schousboe, 1971; Ge et al., 2012). Other papers from this group showed that deficiency in inhibitory phosphorylation of GSK-3 increases sensitivity to mood disturbances (Polter et al., 2010) and that GSK-3 is required for the antidepressant effect of ketamine (Beurel et al., 2011).
COMPOUNDS RELEASE "GLIOTRANSMITTERS" AND OTHER NEUROACTIVE
ASTROCYTES RECEIVE TRANSMITTER SIGNALS FROM NEURONS AND
BRAIN NEURONS REQUIRED FOR NORMAL FUNCTION IN ADULT
FURTHER INTERACTIONS BETWEEN ASTROCYTES AND
NEURONS REQUIRED FOR NORMAL FUNCTION IN ADULT
BRAIN
ASTROCYTES RECEIVE TRANSMITTER SIGNALS FROM NEURONS AND
RELEASE "GLIOTRANSMITTERS" AND OTHER NEUROACTIVE
COMPOUNDS
The response of astrocytes to the SSRI 5-HT<sub>2B</sub> agonists is only one example of how astrocyte neurotransmitter receptors may modulate synaptic strength and plasticity. An increasing number of studies have demonstrated that astrocytes express receptors for most neurotransmitters and release neuroactive substances that modulate neuronal activity (Ben Achour and Pascual, 2010). Besides the already mentioned growth factor release in response to stimulation by 5-HT<sub>2B</sub> receptors (and many other neurotransmitter receptors) they release compounds like adenosine triphosphate (ATP) and glutamate as gliotransmitters. The amount of glutamate released is much smaller than that released from glutamatergic neurons, but it is of special importance because it acts on glutamate receptors which are not located postsynaptically. In this manner astrocytes contribute to regulation of long-term potentiation (LTP), long-term depression (LTD) and neuroplasticity (Ben Achour and Pascual, 2010).

ASTROCYTES SYNTHESIZE ALL TRANSMITTER GLUTAMATE AND GABA
AND ACCUMULATE MOST AFTER NEURONAL RELEASE
Neurons cannot carry out glutamate synthesis from glucose because they lack an enzyme (pyruvate carboxylase), which is critical for glutamate synthesis in vivo (Shank et al., 1985) and in culture (Yu et al., 1983). The majority of glutamate released as a neurotransmitter is taken up by glial cells. Subsequently glutamate is transferred to neurons in the glutamine-glutamate (GABA) cycle via astrocytic glutamine formation, release of glutamate, and its uptake in neurons, where it is deamidated to glutamate (Hertz and Zielke, 2004; Schousboe et al., 2013). In glutamatergic neurons glutamate is used as a transmitter, and in GABAergic neurons it is converted to GABA (Figure 5).

In gray matter (Lebon et al., 2002) of the awake human brain the rate of this process equals about 75% of the rate of total glucose consumption, and in the deeply anesthetized, isoelectric brain it is abolished (Sibson et al., 1998; Hyder and Rothman, 2012; Duarte and Gruetter, 2013). The correlation between cycle flux and brain glucose utilization is linear (Sibson et al., 1998; Hyder and Rothman, 2012). Key processes in this massive astrocyte-neuronal interaction are strictly regulated and/or complex. This applies to astrocytic release of glutamine (Nissen-Meyer and Chaudhry, 2013) and neuronal formation of glutamate from glutamine (Palaiologos et al., 1989), which requires concomitant stimulation of glycogenesis (Chowdhury et al., 2014; Verkhratsky et al., 2014). This requirement explains part of the linear correlation between glucose utilization and the glutamine-glutamate (GABA) cycle. The cycle also brings previously released transmitter amino acids back to neurons after an initial uptake in astrocytes (Figure 5). This is associated with a small amount of energy expenditure (for glutamate uptake and glutamine formation). Return to astrocytes of previously released transmitter and its transport back to neurons represents the major part of the flux in the glutamine-glutamate (GABA) cycle. Only 15–25% of the flux serves to transfer newly synthesized glutamate from astrocytes to neurons (an anaplerotic process) and return glutamate to astrocytes for oxidative degradation (a cataplerotic process). The anaplerotic de novo synthesis requires glucose utilization in astrocytes (one glucose molecule for each molecule of glutamate). Cycle flux is also significantly correlated with glutamate content in human brain (Abdallah et al., 2014a). Since the glutamate content in astrocytes is very low (Lebon et al., 2002), this mainly represents neuronal glutamate.

The GABA component of the glutamine-glutamate (GABA) cycle accounts for ~20% of cycle flux from neurons to astrocytes (Patel et al., 2005; Duarte and Gruetter, 2013). GABA synthesis and metabolism to glutamine shows similarities and differences from production and degradation of glutamate. In contrast to direct astrocytic uptake of most neuronally released glutamate, astrocytically accumulated GABA must first be converted to glutamate in the TCA cycle as described below. This requires condensation of oxaloacetate (OAA) with acetyl Coenzyme A (ac-CoA; Figure 5) but no pyruvate carboxylation (Patel et al., 2005; Duarte and Gruetter, 2013; Lanz et al., 2013). The generated glutamate might be directly converted to GABA. Cultured GABAergic neurons show also a substantial uptake and reutilization of released GABA (Schousboe et al., 2013). Both of these mechanisms may make GABA synthesis less dependent upon the classical glutamate-glutamine (GABA) cycle. However a recent in vivo study in which neuronal reuptake was inhibited showed minimal impact on the fraction of GABA synthesis from glutamine suggesting that direct reuptake may be significantly lower at least in the cerebral cortex (Patel et al., 2015). Further studies will be needed to fully understand the regional and
activity dependance of direct neuronal reuptake vs. astrocyte uptake.

The conversion of GABA to glutamate is a complex process, in which GABA initially enters the astrocytic mitochondria, possibly in exchange with glutamate allowing exit of newly synthesized glutamate to the cytosol. It is then transaminated to succinic semialdehyde and oxidized to succinate, which enters the TCA cycle and via oxaloacetate (OAA) and citrate is converted to α-ketoglutarate, from which glutamate is generated by the same transamination that catalyzes the conversion of GABA to succinic semialdehyde. The participation of TCA cycle activity means that complete oxidation also could have occurred after exit of malate (Figure 5). However, Duarte and Gruetter (2013) found no increase in pyruvate carboxylation from previously reported values, indicating no major increase in anaplerosis/cataplerosis, compared to cycling related to glutamatergic signaling.

In the long term, rates of anaplerosis and cataplerosis must be identical (Lebon et al., 2002; Sonnewald, 2014), but this is not necessarily so in the short term, since brain glutamate content can transiently increase. This happens during specific phases of learning (Gibbs et al., 2007), although children with reading difficulties (Pugh et al., 2014) or with ADHD (Carrey et al., 2007) have an increased brain glutamate content. The latter findings might be related to hyperexcitability. In cases of epileptic seizure and even physiological visual stimulation there is an acute increase in brain glutamate (Peca et al., 2010; Mangia et al., 2012). The larger involvement of astrocytic metabolism during anaplerosis/cataplerosis provides increased possibilities for astrocytic regulation of the cycle. Nevertheless even simple astrocytic involvement in return of previously released transmitter provides a possibility for astrocytic regulation, because glutamine can traverse the astrocytic syncytium (Cruz et al., 2007). It cannot even be excluded that incoming glutamate from neuronal release might be re-directed to GABA-ergic neurons and vice-versa.

**K⁺ HOMEOSTASIS**

There is increasing evidence that increased extracellular K⁺ resulting from neuronal excitation is initially accumulated in astrocytes by the astrocytic Na⁺, K⁺-ATPase, which in contrast to the neuronal enzyme is stimulated by above-normal extracellular K⁺ concentrations (reviewed by Hertz et al., 2014b). It is subsequently released via Kir4.1 channels, probably after transport through the astrocytic syncytium, distributing the amount released over a larger area and thus preventing excessive local extracellular K⁺ increase. This allows secondary uptake by the neuronal Na⁺, K⁺-ATPase, preventing neuronal K⁺ depletion. This process is strikingly reminiscent of how astrocytes handle released neuronal glutamate. However in neither case is it known which advantages the energetically costly double uptake provides for the brain.
Table 1 | Some genes affected by chronic fluoxetine treatment in different brain preparations.

| Gene   | FACS, cerebral astrocytes* | Cultured astrocytes | FACS or otherwise identified cerebral neurons** | Brain (different regions) | Raphe |
|--------|-----------------------------|---------------------|-----------------------------------------------|---------------------------|-------|
| ADAR1  | Unaltered                   | Unaltered           | Unaltered                                     |                            |       |
| ADAR2  | Up                          | Up                  | Unaltered                                     |                           |       |
| GluK1  | Absent                      | Absent              | Absent**                                       |                            |       |
| GluK2  | Up                          | Up                  | Unaltered                                     | Up                         |       |
| GluK2 editing | Up                  | Up                  | Unaltered                                     | Up                         |       |
| GluK3  | Unaltered                   | Unaltered           | Unaltered                                     | Unaltered                  |       |
| GluK4  | Unaltered                   | Unaltered           | Unaltered                                     | Up                         |       |
| GluA1  | Li et al. (2012)            | Li et al. (2011a)   | Li et al. (2012)                               | Up Barbon et al. (2011)    |       |
| GluA2  | Absent                      | Up in dendrites     | Up                                             | Ampuero et al. (2010),    |       |
| GluA3  | Up                          | Up                  | Up                                             | Valou et al. (2010),      |       |
| GluA4  | Up                          | Up                  | Up                                             | Barbon et al. (2011)      |       |
| mGlu5  | Unaltered                   | Unaltered           | Unaltered                                     | Up                         |       |
| mGlu7  | Hertz et al. (2014a)        | Hertz et al. (2014a)|                                               |                            |       |
| cPLA2a | Up                          | Up                  | Unaltered                                     | Ampuero et al. (2010),    |       |
| sPLA2  | Unaltered                   | Unaltered           | Unaltered                                     | O’Connor et al. (2013)    |       |
| iPLA2  | Unaltered                   | Unaltered           | Unaltered                                     | Rao et al. (2006)         |       |

(Continued)
Table 1 | Continued

| Gene            | FACS, cerebral astrocytes* | Cultured astrocytes | FACS or otherwise identified cerebral neurons** | Brain (different regions) | Raphe                      |
|-----------------|-----------------------------|---------------------|-----------------------------------------------|---------------------------|---------------------------|
| Ca_v1.2         | Up                          | Up                  | Unaltered                                     |                            |                           |
|                 | Du et al. (2014)            | Du et al. (2014)    | Du et al. (2014)                              |                           |                           |
| Ca_v1.3         | Unaltered                   | Du et al. (2014)    | Unaltered                                     |                           |                           |
| 5-HT_2A receptor| Unaltered                   | Li et al. (2012)    | Unaltered                                     |                           |                           |
| 5-HT_2B receptor| Up                          | Li et al. (2012)    | Up                                            |                           |                           |
| 5-HT_2B editing | Up                          | Li et al. (2012)    | Up                                            |                           |                           |
| 5-HT_2C receptor| Unaltered                   | Li et al. (2012)    | Unaltered                                     |                           |                           |
| 5-HT_2C editing | Unaltered                   | Li et al. (2012)    | Up                                            |                           |                           |
| 5-HT_1A receptor| Unaltered                   | Peng et al. (2014)  | Unaltered                                     |                            |                           |
| 5-HT_1B receptor| Absent                      | Kong et al. (2002)  | Absent                                        |                            |                           |
| SERT            | Absent                      | Li et al. (2012)    | Absent                                        |                            |                           |
| EGF receptor    | Unaltered                   | Peng et al. (2014)  | Unaltered                                     |                            |                           |
| Nucleoside transporter | Up                             | Li et al. (2013)    | Unaltered                                     |                            |                           |
| ENT2            |                              |                     |                                               |                            |                           |

* The cells isolated by FACS were freshly obtained (see text) from mice treated for 2 weeks with fluoxetine hydrochloride, 10 mg/kg per day, i.p. The cultured cells were treated with fluoxetine concentrations between 1 and 10 µM for 14 days. For brain and raphe the treatment varied between 7 and 14 days (e., 14 days for sPLA_2, but 7 days for GluK2). *FACS unless otherwise indicated. **Present at low density in cultured hippocampal neurons (Li et al., 2011a).
CHRONIC EFFECTS OF FLUOXETINE
OBJECTIVES AND METHODOLOGIES
The several weeks delay between SSRI administration and improvement in depressive symptoms show that chronic effects are the most clinically relevant (Nierenberg et al., 2000). The long life span of astrocyte cultures allows chronic studies of both functional properties and gene expression and editing. In the studies described below all effects on gene expression were confirmed in isolated neuronal and astrocytic cell fractions (Lovatt et al., 2007) prepared from chronically fluoxetine-treated animals as described in the legend of Figure 1. Our own observations using these methodologies will be supplemented with information from studies by other authors, which provide no information about cellular location(s). While the absence of SERT in cultured astrocytes excludes that the gene effects could be secondary to SERT inhibition, those shown in neurons in intact animals could be SERT-related.

The effects of SSRIs on gene expression and editing in both neurons and astrocytes from fluoxetine-treated animals (10 mg fluoxetine hydrochloride/kg per day for 14 days) are summarized in Table 1. The expression and editing changes in both neurons and astrocytes are present in whole brain as also shown in the Table. Many of the genes studied are relevant for major depression. This includes a key role for glutamate in major depression and its treatment (Barbon et al., 2006; Sanacora et al., 2007, 2012; Chowdhury et al., 2008, 2012; Banasr et al., 2010; Hertz et al., 2012; Li et al., 2012; Sanacora and Banasr, 2013; Niciu et al., 2014). Furthermore some oppositely-directed gene changes have been shown in an animal model of depression, indicating their therapeutic relevance (Li et al., 2012; Peng et al., 2014).

EFFECTS ON GLUTAMATE, GABA AND ENERGY METABOLISM
Patients suffering from major depression show evidence of increased glutamatergic activity (Mitani et al., 2006; Hashimoto et al., 2007; Kanner, 2014) and decreased GABAergic activity (Bajbouj et al., 2006; Kanner, 2014) as well as of cortical GABA levels (Sanacora et al., 2004). Successful therapy of major depression lowers cortical glutamate in brain (Abdallah et al., 2014a) and raises GABA (Sanacora et al., 2002; Bhagwagar et al., 2004).

Glutamine synthetase is down-regulated in brains from depressed patients (Rajkowska and Stockmeier, 2013). This is consistent with the increased amino acid neurotransmitter (glutamate and GABA) cycling between astrocytes and neurons after antidepressant treatment with ketamine or riluzole (Chowdhury et al., 2008, 2012). Moreover, inhibition of glutamine synthetase or of the transporter mediating glutamine uptake into neurons causes depression-like symptoms in animal models (Lee et al., 2013).

In a recent 13C MRS study no significant difference in cycling flux, determined from glutamate and glutamine 13C labeling from [1-13C]glucose, (0.19 ± 0.05 (SD) vs. 0.18 ± 0.04 (SD) µmol/g per min) was found between healthy and depressed individuals (Abdallah et al., 2014a). The rate of GABA synthesis was also unaltered. However, inhibition of glutamine synthase or glutamate uptake does not necessarily decrease glutamate flux to glutamine. The flux can still be maintained if glutamate release is not inhibited but at the cost of higher extracellular and glial glutamate levels, as has been seen when glial glutamate uptake or glutamine synthase is inhibited in animal models (Rothstein et al., 1996; Eid et al., 2012). The elevated glutamate would be expected to alter synaptic function. Furthermore it could lead to a change in the ratio of released glutamate that is oxidized in astrocytes vs. glutamate that is directly converted to glutamine.

In patients suffering from depression, brain glucose metabolism is reduced (Little et al., 1996, 2005; Videbech, 2000; Rasgon et al., 2008; Abdallah et al., 2014a) in parallel with the severity of the illness (Kimbrell et al., 2002). Normalization occurs following SSRI treatment (Buchsbaum et al., 1997; Mayberg et al., 2000; Kennedy et al., 2001). Anti-depressant doses of ketamine increase oxidative metabolism in neuronal and glial cells in the brain in vivo (Chowdhury et al., 2012). Consistent with this therapeutic effect Abdallah et al. (2014a) found a large (one quarter) decrease in the rate of glucose oxidation by glutamatergic neurons in depressed patients.

On account of the linear relationship between neuronal activity and neuronal oxidative demand beyond isoelectricity, the metabolic decrease in glutamatergic neurons suggests a drastic reduction of neuronal activity. An increased cycling after treatment with riluzole or ketamine might remedy this deficiency regardless whether or not decreased glutamate cycling is a key component of the pathophysiology of major depression. Increased energy metabolism in glutamatergic neurons is required not only for release of neurotransmitter but probably even more for effects exerted via their glutamate receptors.

In conclusion, in spite of convincing evidence for alterations in glutamate-glutamate (GABA) fluxes and associated energetics in animal models of major depression and its treatment there are still unanswered questions in human depression. This is perhaps partly because the cycle is of key importance for both glutamatergic and GABAergic signaling, and partly because there are many different glutamate receptors. Effects of chronic fluoxetine treatment of mice on glutamate and GABA receptors in whole brain, astrocytes and or neurons might provide further clues.

GLUTAMATE AND GABA RECEPTOR AND TRANSPORTER GENES
Fluoxetine effects on up-regulation and/or editing of genes of several glutamate receptors are shown in Table 1. Receptor up-regulation might potentially correct the abnormal ratio between energetics and the glutamine-glutamate (GABA) cycle observed by Abdallah et al. (2014a), whereas the effects of editing may vary between receptors and editing sites. Editing requires ADARs, a family of adenosine deaminases, which catalyze deamination of adenosine to inosine in mRNAs. This changes the amino acids in the translated protein sequence, since inosine is read as guanosine (Bass, 2002). ADAR 2 is upregulated in astrocytes but not in neurons in mice treated with fluoxetine (Table 1). GluK2 is up-regulated and edited in astrocytes at all its 3 editing sites (Li et al., 2011a, 2012), and GluK4 is upregulated in neurons (Li et al., 2012). The GluK2 editing may be the reason why a normal increase in [Ca2+]i in cultured astrocytes in response to 100 µM glutamate is abolished by fluoxetine treatment (Li et al., 2011a).
The human GluK2c splice variant in brain is mainly expressed in non-neuronal cells (Barbon et al., 2008). Mice with GluK2 receptor knock-out, exhibit less anxious or more risk-taking type behavior and less manifestation of despair (Shaltiel et al., 2008).

Obsessive-compulsive disorder (OCD) is genetically linked to abnormalities in the GluK2 gene, Grik2 (Delorme et al., 2004; Sampaio et al., 2011). The neuronal up-regulation of GluK4 by fluoxetine may appear paradoxical since genetic ablation of this receptor subunit causes anxiolytic and antidepressant-like behavior in mice (Catches et al., 2012). However, there is a risk of elevated suicidality during initiation of antidepressant therapy (Fava and Rosenbaum, 1991; Kraus et al., 2010; Trivedi et al., 2011; Singh et al., 2013), which might be related to this up-regulation.

The GluA2 gene is expressed in both neurons and astrocytes (Cahoy et al., 2008), and it is up-regulated in whole brain by fluoxetine treatment (Ampuero et al., 2010; Vialou et al., 2010; Barbon et al., 2011). Up-regulation in neuronal dendrites leads to structural plasticity (Rubio et al., 2013). Mice susceptible to chronic social defeat show a significant decrease in GluA2 levels, while resilient mice showed increased GluA2 levels (Vialou et al., 2010). GluA1 (Barbon et al., 2006; Ampuero et al., 2010) and GluA4 (Barbon et al., 2011) are also upregulated in brains of fluoxetine-treated animals, but GluA3 is less affected (Barbon et al., 2006). In contrast to the lack of fluoxetine effect on mGlu5 in both astrocytes and neurons (Table 1) and of an unaltered mGluR2 and mGluR3 expression in whole brain in major depression (Muguruza et al., 2014), mGluR7 is up-regulated in whole brain after fluoxetine treatment (Ampuero et al., 2010; O’Connor et al., 2013). Very little information is available about possible fluoxetine effect on NMDA receptors. The GABA-synthesizing enzyme glutamic acid decarboxylase (GAD) is upregulated in hippocampus but not in prefrontal cortex by 2 weeks of fluoxetine treatment (Guirado et al., 2012). A hippocampal up-regulation of the GABAβ receptor has also been reported (Sands et al., 2004). A decrease in cerebrospinal fluid of patients with depression of the neurosteroid 3α-hydroxy 5α-pregn-20-one (allopregnanolone), which enhances GABA action at GABA_A receptors, is corrected by fluoxetine treatment (Pinna et al., 2009). The likely reason for this is that fluoxetine, sertraline, and paroxetine acutely cause a large decrease of the K_m for conversion of 5α-dihydropregosterone to allopregnanolone by human 3α-HSD type III (Griffin and Mellon, 1999). In turn, 5α-dihydropregosterone is formed from progesterone (and some other substrates) by the rate-limiting enzyme 5α-reductase. In cerebellum this enzyme is mainly expressed in astrocytes and oligodendrocytes (Kiyokage et al., 2014). However, in frontal cortex, where allopregnanolone is down-regulated in depressed patients, it was reported to be synthesized in glutamatergic neurons (Agis-Balboa et al., 2014). A decrease of corticolibic allopregnanolone content induced in mice by social isolation as well as the induced behavioral changes are sterospecifically normalized by fluoxetine by a mechanism independent from 5-HT reuptake inhibition (Pinna et al., 2009). Thus, fluoxetine facilitates GABA_A receptor neurotransmission and effectively ameliorates depression by stimulating brain steroidogenesis in a SERT-independent manner.

### PHOSPHOLIPASE GENES

A polymorphism in the calcium-dependent phospholipase 2 (cPLA_2) gene is connected with increased risk for major depression (Pae et al., 2004). This enzyme is strongly expressed in astrocytes (Lautens et al., 1998; Balboa and Balsinde, 2002; Sun et al., 2004). In cultured astrocytes cPLA_2 causes transactivation of the EGF receptor and ERK_1,2 phosphorylation (Xia and Zhu, 2011). Its activation releases the unsaturated fatty acid arachidonic acid from membrane-bound phospholipids (Felder et al., 1990; Qu et al., 2003; Rapoport, 2008). In agreement with an up-regulation reported in whole brain (Rapoport, 2008), Li et al. (2009) showed a slow and selective up-regulation of mRNA and protein expression of cPLA_2, the major cPLA_2 isofrom, in cultured mouse astrocytes during chronic incubation with fluoxetine (Table 1). The up-regulation was abrogated by the 5-HT_2b antagonist SB 204741, and by inhibitors of the fluoxetine signaling pathway shown in Figure 2. Up-regulation, specifically of cPLA_2, was confirmed in freshly dissociated mouse astrocytes after 2 weeks fluoxetine treatment, whereas no effect was found in neurons (Li et al., 2012).

sPLA_2 is upregulated by fluoxetine in neurons and whole brain, but not in astrocytes (Table 1). Some subtypes of sPLA_2 also stimulate arachidonic acid release (Murakami et al., 1998). Exogenously applied sPLA_2 causes an increase in neurotransmitter release from cultured hippocampal neurons (Wei et al., 2003).

One of the many effects of arachidonic acid is to stimulate glucose metabolism in cultured astrocytes (Yu et al., 1993). So does treatment with 10 μM fluoxetine for 24 h (Allaman et al., 2011), which might have sufficed to induce an increase in cPLA_2, whereas acute exposure to fluoxetine has no corresponding effect (L. Peng and L. Hertz, unpublished experiments). Stimulation of glucose metabolism by arachidonic acid in vivo (Sublette et al., 2009) may be important in the treatment of depressive illness, as shown in Figure 6. Arachidonic acid in addition stimulates glycogenolysis (Sorg et al., 1995; Hertz et al., 2015) and seems to enhance signaling via glutamatergic receptors of the AMPA and mGluR subtypes (Schaeffer and Gattaz, 2008).

Brain glycogenolysis is activated by a multitude of transmitters (Xu et al., 2014; Hertz et al., 2015). Increased glycogenolysis has been found in paroxetine-treated mice (Webhofer et al., 2013) and in astrocyte cultures treated with 10 μM fluoxetine for more than 1 week, whereas shorter treatment led to a decrease (Kong et al., 2002). Astrocytic glutamate production depends on glycogenolysis (Gibbs et al., 2007). However, glycogenolysis has additional wide-reaching effects on neuroplastic changes, which are likely to be associated with chronic actions of SSRIs. Thus, Duran et al. (2013) found an absence of appropriate postsynaptic LTP responses to high-frequency stimulation in alert, behaving mice with a specific knock-out of brain glycogen synthase.

The acute effect of fluoxetine on GSK-3 via PI3K/AKT and the demonstration that the GSK-3 inhibitor lithium stimulates glycogen synthesis (Plenge, 1982) suggest that potential chronic effects of fluoxetine on glycogen formation might be very important for its mechanism of action. This conclusion is consistent with the suggestion by Li and Jope (2010) that GSK-3 is a central modulator of mood regulation. Recent studies by Gu and
Peng showed a tripling of astrocytic glycogen content after 14 days of treatment with 1 µM fluoxetine, but a caveat is that with this concentration of fluoxetine the transient decrease in glycogenolysis described above (Kong et al., 2002) may not have subsided after 2 weeks. However, the findings by Plenge (1982) of acute effects of lithium strongly suggest that the stimulation of the PI3K/AKT pathway by chronic treatment with 10 µM fluoxetine does stimulate glycogen synthesis, and treatment of cultured astrocytes with 10 µM fluoxetine for 14 days increased fluoxetine-induced AKT phosphorylation (Hertz et al., 2012). In contrast, chronic lithium treatment abolishes the increased incorporation of glucose into glycogen seen after acute exposure to lithium (Plenge, 1982). The importance of SSRI-induced effects on glycogen is consistent with its crucial involvement in astrocytic metabolism and plasma arachidonic acid levels.

**5-HT RECEPTORS GENES AND SERT**

Fluoxetine interactions with 5-HT receptors are age-dependent (Sarkar et al., 2014) and only effects on adult individuals will be discussed. In contrast to the 5-HT2B receptor’s up-regulation by 14 days of *in vivo* treatment with fluoxetine, the astrocytic 5-HT2A and 5-HT2C receptors are unaltered (Table 1). After 2 weeks fluoxetine treatment all editing sites in the 5-HT2B receptor become edited in astrocytes (Li et al., 2012). In cultured astrocytes up-regulation of the 5-HT2B receptor occurs slowly (Hertz et al., 2014a). Editing of the receptor is faster and abolishes the 5-HT-induced increase in IP3 turnover. This together with a similar up-regulation and editing of 5-HT2C receptors in neurons (Table 1) are consistent with an early suggestion that chronic SSRI treatment reduces 5-HT2C/5-HT2B receptor responsivity in rats (Lightowler et al., 1994).

In whole brain fluoxetine-induced editing of the HT2C receptor has repeatedly been described (Niswender et al., 2001; Gurevich et al., 2002; Englander et al., 2005). It requires PKC and ADAR activity (Schmauss et al., 2010). The fluoxetine-induced changes in editing are opposite to those seen in suicide victims (Gurevich et al., 2002). These findings might suggest that a reduced agonist activity after editing (see above) neutralizes a reported anxiogenic effect of the un-edited 5-HT2C receptor (Kemetti et al., 1997; Vicente and Zangrossi, 2014). It is consistent with this conclusion that Vicente and Zangrossi (2014) found acute injection of the 5-HT2C receptor agonist MK-212 into the amygdala to have anxiogenic effect, and that chronic treatment with fluoxetine abolished the anxiety. However, there is also strong evidence that acute administration of *specific* agonists of 5-HT2C receptor are therapeutically effective in depression, obsessive compulsive disorder and anxiety (Jenck et al., 1998; Martin et al., 1998; Lysen, 1999; Cryan and Lucki, 2000; Rosenzweig-Lipson et al., 2007).

The expression of the inhibitory 5-HT1A autoreceptor, suggested to contribute to the slow manifestation of the...
therapeutic effect of SSRIs (Blier and De Montigny, 1983), was unaltered after fluoxetine treatment in both neurons and astrocytes (Peng et al., 2014) and whole brain (Le Poul et al., 2000; Johnson et al., 2009). This does not mean that its activity could not have decreased, since a fluoxetine-induced internalization of the receptor was shown by Descarries and Riad (2012) and a reduced 5-HT$_{1A}$ receptor expression was found in the raphe nuclei (Le Poul et al., 2000).

The cell culture finding that SERT is absent in astrocytes (Kong et al., 2002) was confirmed in freshly isolated astrocytes from the cerebral hemispheres, excluding raphe nuclei (Peng et al., 2014). This applies to neurons, although a minor presence cannot be excluded (Table 1). Expression of the EGF receptor, involved in 5-HT$_{2B}$ receptor signaling (Figure 2), is not altered by chronic fluoxetine treatment in either astrocytes or neurons (Peng et al., 2014). However mRNA of the equilibrative nucleoside transporter ENT2 is increased in neurons and even more in astrocytes after fluoxetine treatment (Table 1), probably a reflection of the fluoxetine-induced increase in cell generation (Manev et al., 2003).

The 5-HT$_{2B}$ receptor is downregulated in mice becoming anhedonic after chronic stress (Li et al., 2012). No similar downregulation was found of the 5-HT$_{2C}$ receptor, but anhedonia is only one component of depressive symptomatology, and effects on the 5-HT$_{2C}$ receptor may mainly influence anxiety.

**CONCLUSIONS**

The importance of the 5-HT$_{2B}$ receptor in the mechanism of action of SSRIs has been demonstrated both in vivo (Diaz et al., 2012) and in cultured astrocytes (Li et al., 2008a). This paper elucidates its pathway, emphasizes astrocytic-neuronal interactions in brain function and shows identical effects of fluoxetine on astrocytic gene expression in our cultured astrocytes, expressing no SERT, and in drug treated animals. The latter study also found neuronal effects. The signaling pathway, studies by other authors and the observed gene effects point towards the importance in SSRIs's mechanism of action of glucose and glycogen metabolism, glutamate and GABA turnover and signaling, cPLA$_2$ and sPLA$_2$ activities, arachidonic acid, cellular Ca$^{2+}$ regulation, and alterations in 5-HT$_2$ receptor expression and editing. Many of the observed effects might be useful targets for drug development.

**CONTRIBUTION STATEMENT**

Professors Hertz and Rothman wrote and revised the manuscript. Professor Rothman is also a member of many of the studies referred to. Professor Peng approved and corrected the initially submitted manuscript, had designed the many experiments from her own group (partly with some input from Professor Hertz) and supported all research from her group. Dr. Li provided essential help to Professor Hertz by preparing the reference list and and quite complex Table 1. She also performed the majority of the experiments in Professor Peng’s group related to this review.

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5-HT 5-HT receptor as SSRIs target

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