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Physiological effects of SSRI administration: Negative feedback control of serotonin production and release

by

Gerard Honig

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Neuroscience

in the

GRADUATE DIVISION

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by
Gerard Honig

Dedication and acknowledgements

I would like to dedicate this work to three of the great teachers in my life: my father, Jacques Honig; my sensei, Nobuyoshi Higashi; and the UCSF community.

The following individuals made significant contributions to the work described: Minke Jongsma; Marieke van der Hart; Melodi Bowman; Thomas Cremers; Angela Liou; Miles Berger; Elaine Carlson; Michael German and Larry Tecott.

Abstract

Serotonin (5-HT) is a neuromodulator with important roles in the regulation of physiological processes, particularly those regulating affect in humans. Drugs which selectively potentiate serotonergic neurotransmission by inhibiting the reuptake of serotonin (SSRIs) are widely used for the treatment of psychiatric disorders. It is thought that the therapeutic efficacy of SSRIs is limited by homeostatic mechanisms which counteract the ability of the drug to potentiate serotonergic neurotransmission. Here, I describe my work on two such mechanisms. First, I describe a role for 5-HT 2C receptors in counteracting the physiological and behavioral effect of SSRIs. In serotonin 2C receptor null mutant mice, extracellular 5-HT was unaltered at baseline. Upon administration of the SSRI fluoxetine, extracellular 5-HT rapidly increased in both wild-type and null mutant mice; this effect was potentiated in mutant mice. Furthermore, mutant mice had a greater response to fluoxetine in a behavioral assay for antidepressant effect. Pharmacological blockade of 5-HT 2C receptors also potentiated the effects of fluoxetine and another SSRI, citalopram, in wild-type mice and rats. In a second line of investigation, we investigated the effects of chronic citalopram treatment on 5-HT synthesis, which is an important factor in the clinical effects of SSRIs. Administration of citalopram for 2 days, 14 days or 28 days suppressed 5-HT synthesis. 5-HT content was not significantly reduced by citalopram at any time point. However, chronic citalopram did cause a significant reduction in forebrain 5-HT content when monoamine synthesis was completely inhibited by acute administration of an amino acid decarboxylase

inhibitor. 5-HIAA content was reduced in citalopram-treated brains at all time points. These results demonstrate a sustained suppression of serotonin synthesis by chronic citalopram administration and suggest that serotonergic neurons are particularly dependent on *de novo* synthesis of 5-HT when the reuptake of 5-HT is inhibited. In technical appendices I describe novel tools for the study of serotonin biology. Taken together, these studies improve our understanding of the physiological actions of SSRIs and suggest strategies for improving their efficacy.

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Introduction

Serotonin: An ancient signaling molecule which regulates physiology and affect

The indoleamine 5-hydroxytryptamine (serotonin, or 5-HT) is an evolutionarily ancient signaling molecule which regulates an extraordinary array of behavioral and physiological processes(Jacobs and Azmitia 1992; Whitaker-Azmitia 1999; Richerson 2004; Tecott 2007). 5-HT was first identified in the enterochromaffin cells of the gut and, later, in blood, where it acts as a vasoconstrictor(Whitaker-Azmitia 1999). The discovery of 5-HT in the mammalian brain provided the first evidence that 5-HT could act as a neurotransmitter(Twarog and Page 1953). In fact, 5-HT is a neurotransmitter and neuromodulator in the nervous systems of all known phyla(Tecott 2007). Although 5-HT synthesized and released only by a tiny proportion of the total neurons in the nervous system, it participates in neural circuits regulating activity, metabolism, respiration, aggression, development, affect, cognition and many other functions(Jacobs and Azmitia 1992; Tecott 2007).

In the mammalian brain, 5-HT is synthesized in and released by a small set of specialized neurons in the raphe nuclei of the brainstem(Twarog and Page 1953; Jacobs and Azmitia 1992). These serotonergic neurons are defined by the expression of two key genes. The first, *tph2*, encodes an enzyme which catalyzes tryptophan hydroxylation, the rate-limiting step in the synthesis of serotonin from the essential acid tryptophan(Dahlstrom and Fuxe 1964; Lovenberg, Jequier et al. 1967; Jacobs and Azmitia 1992). The second, *slc6a4*, encodes the high-affinity plasma membrane serotonin transporter (SERT) which

recovers serotonin from the extracellular space and regulates intracellular and extracellular levels of 5-HT(Jacobs and Azmitia 1992). Other key molecular players in 5-HT signaling include transporters, degrading enzymes and receptors. VMAT2, the vesicular monoamine transporter, packages the 5-HT that is synthesized in the cytoplasm into synaptic vesicles for eventual release(Pothos, Larsen et al. 2000). Two monoamine oxidase enzymes (isoforms A and B) reside in the mitochondrial membrane of serotonergic and non-serotonergic cells and catalyze the degradation of 5-HT into physiologically inactive 5-hydroxyindoleacetic acid (5-HIAA). Finally, an extraordinary variety of ionotropic and metabotropic receptors transduce extracellular 5-HT into myriad cellular effects in almost every class of neuron (including the serotonergic neuron itself, which expresses multiple autoreceptors). The serotonergic neurons of the raphe nuclei produce extraordinarily ramified projections, innervating the entire brain and spinal cord(Jacobs and Azmitia 1992). 5-HT is released by these neurons at release sites which resemble classical synapses or non-synaptic paracrine release sites on both axonal and somatodendritic portions of the cell(Chazal and Ralston 1987).

Serotonin and psychopharmacology

Serotonin has played an important role in the development of the disciplines of neuroscience, pharmacology and biological psychiatry. 5-HT was one of the first compounds shown to act as a neurotransmitter(Twarog and Page 1953). This discovery was followed by a series of seminal observations in clinical pharmacology which revolutionized the treatment of psychiatric and neurological

conditions. Reserpine, which depletes synaptic vesicles of monoamines (including 5-HT), was used for the treatment of hypertension and produced severe depression in some patients(Achor, Hanson et al. 1955). Lysergic acid diethylamine, a potent hallucinogen, was found to act through 5-HT receptors(Woolley and Shaw 1954; Green 2008). The final and crucial set of observations concerned the mode of action of effective psychiatric drugs: the vast majority of drugs used to treat psychiatric disorders were found to bind 5-HT receptors, transporters and/or degrading enzymes(Kramer 1997). Antidepressant drugs, in particular, profoundly affect multiple features of serotonergic neurotransmission(Castren 2005; Green 2008). Monoamine oxidase inhibitors (MAOIs) inhibit the intracellular degradation of 5-HT into 5-HIAA, resulting in increased brain content of 5-HT and increased 5-HT release(Youdim, Edmondson et al. 2006). Tricyclic antidepressants inhibit the reuptake of serotonin from the extracellular space by SERT(Axelrod and Inscoe 1963; Castren 2005; Berton and Nestler 2006). Based on the hypothesized importance of SERT inhibition in the therapeutic action of tricyclic antidepressant drugs, selective serotonin reuptake inhibitors (SSRIs) were developed(Kramer 1997). When a molecule of 5-HT is transported back into the neuron by SERT, it can no longer diffuse and bind to its cognate extracellular receptors. Inhibition of this transport thus alters the spatiotemporal dynamics of serotonin signaling such that activity in the serotonergic neuron causes greater increases in extracellular serotonin than would normally occur.

Selective serotonin reuptake inhibitors: Physiological effects and limitations

Depression is a devastating illness which is one of the major causes of disability in the world, affecting over 120 million people(Michaud, Murray et al. 2001; 2008). SSRIs are widely prescribed as the first line of treatment for depression and many other psychiatric disorders due to the broad efficacy and safety of these drugs(Kramer 1997; Katzung 2004; Parker 2009). Although the pharmacological targets of SSRIs have been well characterized for decades (fluoxetine being an early example of rational drug design(Kramer 1997)), major scientific and clinical issues regarding their use remain unresolved. Salient clinical issues include a possible latency of several weeks for therapeutic effects to occur(Hervas, Vilaro et al. 2001; Taylor, Freemantle et al. 2006). This phenomenon has inspired the hypothesis that antidepressants exert their behavioral effects by gradually inducing plasticity, neurotrophin release and neurogenesis(Berton and Nestler 2006). The mechanisms downstream of SERT blockade which are responsible for the therapeutic effects of SSRIs remain elusive. However, given the high affinity of SSRIs for SERT(2008), it is thought that these downstream events, whatever they may be, must be initiated and sustained by effects on serotonergic neurotransmission(Delgado, Price et al. 1994). Although many SSRIs such as fluoxetine and paroxetine may have significant pharmacological interactions with targets other than SERT(Heurteaux, Lucas et al. 2006; Pinna, Costa et al. 2006; O'Leary, Bechtholt et al. 2007), citalopram (CIT) and escitalopram are extremely selective for SERT(2008).

The efficacy of antidepressant drugs is another clinical issue which has recently received considerable attention(Kirsch, Deacon et al. 2008; Thase 2008). Although SSRIs are preferred over older antidepressant formulations because of the lowered

incidence of undesirable effects, SSRIs are comparable to tricyclic antidepressants in terms of alleviating the symptoms of depression(Berton and Nestler 2006). Accurate quantification of antidepressant response is difficult (Parker 2009); however, clinical evidence suggests that a significant proportion of patients will not respond to the SSRI initially prescribed or even to any SSRI, and other patients will attain only a partial remission of symptoms(Kirsch, Deacon et al. 2008). Our lack of fundamental understanding of how SSRIs affect multiple aspects of brain function currently hampers efforts to optimize SSRIs and develop novel antidepressants.

SSRIs affect many aspects of serotonergic neurotransmission in addition to (or secondarily to) reuptake inhibition. These effects may play a major role in the therapeutic response(Blier, Chaput et al. 1988; Hervas, Vilaro et al. 2001). One effect which has been widely studied is the suppression of activity in serotonergic neurons, which is thought to be mediated by an acute increase in extracellular 5-HT followed by activation of inhibitory 5-HT 1A somatodendritic autoreceptors(Chaput, de Montigny et al. 1986). This suppression of activity, in turn, limits the ability of the SSRI to produce further elevations in extracellular release(Adell, Celada et al. 2002). The potency of this effect dissipates over the course of chronic treatment(Chaput, de Montigny et al. 1986). This has been suggested to be one mechanism for the therapeutic delay(Hervas, Vilaro et al. 2001; Artigas, Adell et al. 2006) although attempts to accelerate the clinical effects of SSRIs using 5-HT 1A antagonists have generate mixed results(Artigas, Celada et al. 2001), perhaps due to the limitations of the 5-HT 1A antagonist which was used for these studies(Cremers, Wiersma et al. 2001).

Multiple additional mechanisms exist which can limit the increase of

extracellular 5-HT via a process of negative feedback. Receptors located on serotonergic terminals can respond to local signals to suppress 5-HT release. For example, inhibitory 5-HT 1B autoreceptors perform this function(Adell, Celada et al. 2002). Activity in serotonergic neurons could also be suppressed via local GABAergic interneurons in the raphe expressing excitatory 5-HT receptors and responding to somatodendritically released 5-HT(Liu, Jolas et al. 2000; Adell, Celada et al. 2002). Alternatively, 5-HT released by serotonergic terminals could feed back and inhibit its release by exciting GABAergic interneurons which innervate those same terminals(Liu, Jolas et al. 2000). Nonspecific 5-HT2 receptor agonists have been found to enhance GABAergic neurotransmission in both the prefrontal cortex, the CA1 region of the hippocampus and the dorsal raphe(Shen and Andrade 1998; Abi-Saab, Bubser et al. 1999; Liu, Jolas et al. 2000).

Negative feedback control of 5-HT synthesis is another plausible mechanism which could limit the physiological effects of SSRIs. Experimental evidence indicates that the rate of serotonin synthesis is a critical factor in SSRI response. *In vitro* data suggest that the rate of neurotransmitter synthesis is an important determinant of signaling efficacy for monoaminergic neurons(Pothos, Larsen et al. 2000). Serotonin synthesis rate is regulated *in vivo* by many factors, including the availability of tryptophan(Fernstrom and Wurtman 1971; Carlsson and Lindqvist 1978); acute depletion of plasma tryptophan causes immediate reductions in brain 5-HT synthesis and content(Olivier, Jans et al. 2008). Direct pharmacological inhibition of serotonin synthesis(Shopsin, Gershon et al. 1975) and tryptophan depletion(Delgado, Charney et al. 1990; Delgado, Price et al. 1991;

Benkelfat, Ellenbogen et al. 1994; Delgado, Price et al. 1994; Leyton, Young et al. 1997; Moore, Gillin et al. 1998; Smith, Morris et al. 1999; Leyton, Ghadirian et al. 2000; Booij, Van der Does et al. 2002) can induce an immediate relapse of depression symptoms in SSRI-treated patients with remitted depression, an effect which is not readily observed in subjects with no history of SSRI administration (Delgado, Charney et al. 1990; Delgado, Price et al. 1991; Benkelfat, Ellenbogen et al. 1994; Delgado, Price et al. 1994; Leyton, Young et al. 1997; Moore, Gillin et al. 1998; Smith, Morris et al. 1999; Leyton, Ghadirian et al. 2000; Booij, Van der Does et al. 2002). Tryptophan, itself a relatively ineffective antidepressant agent(d'Elia, Hanson et al. 1978), has been reported to potentiate the therapeutic action of 5-HT reuptake inhibitors(Walinder, Skott et al. 1975; Walinder, Skott et al. 1976; Møller 1990), although this result was not replicated in one study(Walinder 1981).

The central importance of 5-HT synthesis in antidepressant response immediately raises the question of how 5-HT synthesis is affected by SSRI treatment. There is substantial evidence that acute administration of citalopram and other SSRIs suppresses serotonin synthesis throughout the brain(Carlsson and Lindqvist 1978; Muck-Seler, Jevric-Causevic et al. 1996; Moret and Briley 1997; Barton CL 1999; Stenfors, Yu et al. 2001; Yamane, Okazawa et al. 2001; Cervo, Canetta et al. 2005). Using the decarboxylase inhibition assay (described in Results), Carlsson found that acute injection of CIT and other antidepressants causes a dose-dependant 20-60% suppression of 5-HT synthesis(Carlsson and Lindqvist 1978). Consistent results in many brain regions of mouse and rat have been reported by multiple investigators(Moret and Briley 1997; Barton CL 1999; Stenfors, Yu et al. 2001; Cervo, Canetta et al. 2005); the mechanism for

this effect is unknown(Stenfors, Yu et al. 2001). Diksic and colleagues, using an index based on brain trapping of the radiotracer α-[^{14}C]-methyl-tryptophan, reported increased 5-HT synthesis throughout the brain following acute injection of fluoxetine(Tsuiki, Yamamoto et al. 1995; Muck-Seler, Jevric-Causevic et al. 1996) and decreased 5-HT synthesis following acute administration of paroxetine(Yamane, Okazawa et al. 2001). However, there is disagreement in the literature as to whether or not α-[^{14}C]-methyl-tryptophan trapping is a measure of 5-HT synthesis(Shoaf, Carson et al. 1998; Benkelfat, Young et al. 1999; Shoaf, Carson et al. 2000).

SSRI administration immediately triggers multiple physiological responses in the brain, for example the suppression of activity in serotonergic neurons via activation of inhibitory autoreceptors(Chaput, de Montigny et al. 1986; de Montigny, Chaput et al. 1990). Many of these effects dissipate upon chronic treatment, and in fact this adaptation may be required for the beneficial effects of antidepressants to emerge following the therapeutic delay period(Artigas, Celada et al. 2001; Hervas, Vilaro et al. 2001). As a first step in understanding how 5-HT synthesis rate might be involved in SSRI efficacy, it is critical to determine the effect of SSRIs, administered chronically and continuously as in patients, on 5-HT synthesis rate. Published studies addressing this issue have reported contradictory results, perhaps due to methodological issues. Moret et al(Moret and Briley 1992) and Stenfors et al(Stenfors and Ross 2002) reported increased 5-HT synthesis rate following chronic treatment with fluoxetine, whereas Esteban et al(Esteban, Lladó et al. 1999) report no effect of chronic citalopram. For these studies, SSRI administration was withheld following the chronic treatment and prior to determination of 5-HT synthesis rate in order to allow the SSRI to 'wash out'. SSRI washout has been demonstrated to

induce rapid changes in serotonergic physiology which may be opposite to the effect of continuously administered drug(Anthony, Sexton et al. 2000; Jongsma 2006). Notably, Trouvin et al report reduced brain 5-HT and 5-HIAA content following chronic fluoxetine administration; however, this effect reverses rapidly with washout(Trouvin, Gardier et al. 1993). Yamane et al report reduced brain trapping of the radiotracer α-[^{14}C]-methyl-tryptophan following chronic treatment with paroxetine without washout, which is consistent with reduced 5-HT synthesis(Yamane, Okazawa et al. 2001).

Under normal circumstances, serotonergic neurons have two potential sources of 5-HT: synthesis from tryptophan and reuptake from the extracellular space. When reuptake is inhibited, as with SSRI administration, brain 5-HT content should be more dependant on 5-HT synthesis. Experimental evidence suggests that this is in fact the case(Walinder, Skott et al. 1976; Buus Lassen 1978; Núria 1996; Kim, Tolliver et al. 2005; Jongsma 2006; Lowe, Yeo et al. 2006; Fox, Jensen et al. 2007; Fox, Jensen et al.; Olivier, Jans et al. 2008). Therefore, an effect of SSRI administration on 5-HT synthesis could lead to pronounced changes in brain 5-HT and 5-HIAA content which could limit, amplify or otherwise affect SSRI function. A number of studies have demonstrated that chronic SSRI treatment causes a sustained decrease in 5-HIAA in the rodent brain(Hwang, Magnussen et al. 1980; Caccia, Fracasso et al. 1992; Trouvin, Chanut et al. 1992; Caccia, Anelli et al. 1993; Trouvin, Gardier et al. 1993; Dygalo, Shishkina et al. 2006). The effect of SSRI treatment on total brain 5-HT is less clearly defined. Surprisingly, although SSRI administration leads to increased extracellular 5-HT, the total amount of 5-HT in the brain (intracellular and extracellular) may actually be reduced with chronic SSRI treatment. Trouvin et al reported 20-50% decreases in total 5-HT tissue

content in several brain regions following a 21-day treatment with fluoxetine(Trouvin, Chanut et al. 1992; Trouvin, Gardier et al. 1993). Dygalo et al(Dygalo, Shishkina et al. 2006) and Caccia et al(Caccia, Fracasso et al. 1992; Caccia, Anelli et al. 1993) reported comparable results, although this effect was not observed in all brain regions examined. Marsteller also observed reduced tissue 5-HT following chronic treatment with CIT(Marsteller, Barbarich-Marsteller et al. 2007). This effect was, however, not observed in another study using chronic fluoxetine administration(Hwang, Magnussen et al. 1980) and another series of studies using acute administration of multiple SSRIs has suggested administration of these drugs actually leads to increased brain 5-HT content by inhibiting liver degradation of tryptophan(Badawy and Evans 1981; Badawy and Evans 1982; Badawy and Morgan 1991; Badawy 1996; Bano, Morgan et al. 1999).

In this work I describe two novel mechanisms which may limit the physiological and behavioral effects of SSRIs. First, I identify a role for 5-HT 2C receptors in negative feedback control of 5-HT release following SSRI administration. Second, I address the question of how administration of citalopram affects forebrain 5-HT synthesis rate and forebrain 5-HT and 5-HIAA content over the course of chronic treatment. In two appendices I describes novel tools for the study of serotonin biology in genetically modified mice.

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Chapter 1:

Inactivation of 5-HT 2C Receptors Potentiates Consequences of Serotonin Reuptake Blockade

Abstract

The enhancement of central serotonin system function underlies the therapeutic effects of selective serotonin reuptake inhibitors (SSRIs), which have become the most commonly used class of antidepressant agents. However, many individuals experience depressive episodes that are resistant to SSRI treatment. Homeostatic mechanisms that limit the extent to which SSRIs enhance serotonergic neurotransmission have been implicated in this phenomenon. Here I report a novel strategy for enhancing the efficacy of SSRIs. Using *in vivo* microdialysis methods in 5-HT 2C receptor null mutant mice, I found that although this mutation did not affect baseline extracellular serotonin levels or tail suspension test (TST) behavior, it enhanced fluoxetine's effects on serotonin levels and immobility in the TST. In rats, the nonselective 5-HT2 receptor antagonist ketanserin was observed to produce a robust augmentation of citalopram-, fluoxetine- and sertraline-induced elevations of hippocampal extracellular serotonin levels. Similar effects were also observed in prefrontal cortex. The potentiation of SSRI-induced increases in hippocampal serotonin levels was reproduced by the 5-HT 2C receptor-selective antagonists SB 242084 and RS 102221, but not by the 5-HT2A receptor-selective antagonist MDL 100,907. Although 5-HT 2C receptor antagonists augmented the actions of SSRIs, they had no effect on extracellular serotonin levels or tail suspension

responses when administered alone. These findings reveal an unanticipated pharmacological action of 5-HT 2C receptors that warrants consideration in the development of novel strategies for the treatment of depression.

Introduction

Major depressive disorder is among the most common and debilitating psychiatric conditions, with a lifetime prevalence of 20% for females and 10% for males (Judd, 1995). The high incidence and chronic recurrent course of unipolar depression has led the World Health Organization to predict that it will become the second-ranking global cause of illness-related disability by 2020 (Murray and Lopez, 1997). Enhancement of central serotonin system function has been associated with the actions of a wide variety of antidepressant treatments, as indicated by the widespread use of selective serotonin reuptake inhibitors (SSRIs). The SSRIs, which are generally considered to be the first line antidepressants of choice (Fava, 2000), block the plasma membrane serotonin reuptake transporter (SERT) and thus augment the availability of synaptic serotonin.

However, this augmentation is blunted by SSRI-induced increases in serotonin levels in the vicinity of brainstem raphe nuclei serotonergic neurons. In response to elevated serotonin levels, somatodendritic 5-HT1A autoreceptors are activated, reducing serotonergic neuronal firing rates and forebrain serotonin release (Artigas et al., 1996). The characteristic delay in the onset of antidepressant effects of SSRIs has been attributed to a gradual decrease in this

inhibition, resulting from partial 5-HT 1A autoreceptor desensitization (Hervas et al., 2001). These findings led to the proposal that the coadministration of SSRIs with 5-HT 1A receptor antagonists could result in accelerated and possibly enhanced antidepressant responses (Artigas, 1993). This hypothesis was examined in clinical studies using the nonselective 5-HT 1A receptor antagonist pindolol. There was some indication that the onset of antidepressant responses was accelerated by the combined pharmacotherapy, although results were mixed (Artigas et al., 2001; Brousse et al., 2003). Because pindolol has partial 5-HT1A receptor agonist activity, along with very high affinity at adrenoceptors, the receptor mechanisms through which pindolol influences antidepressant action have been called into question (Artigas et al., 2001; Cremers et al., 2001; Brousse et al., 2003). Thus, clinical tests of this antidepressant augmentation strategy await the availability of safe, potent and selective 5-HT1A receptor antagonist
compounds.

The 5-HT 2C receptor is another 5-HT receptor subtype that warrants consideration in the development of novel treatment strategies for depression. Although a wide variety of antidepressants have high-affinity antagonist activity at 5-HT 2C receptors (Jenck et al., 1994; Palvimaki et al., 1996), the contributions of this activity to their therapeutic effects are poorly understood. Indirect activation of 5-HT 2C receptors by SSRI treatment may contribute to the transient anorectic and anxiogenic effects of these compounds (Halford and Blundell, 2000; Bagdy et al., 2001). However, the contribution of these receptors

to the neurochemical and antidepressant behavioral effects of SSRIs remains unclear. Recent findings indicating that RNA editing of these receptors is regulated by SSRI treatment and altered in the prefrontal cortex of suicide victims highlights the importance of clarifying the contributions of these receptors to antidepressant action (Niswender et al., 2001; Gurevich et al., 2002a; Gurevich et al., 2002b).

Here I report a novel interaction through which pharmacological blockade or a null mutation in 5-HT 2C receptors potentiates both the neurochemical and the antidepressant-like behavioral actions of SSRIs. These results suggest a potential treatment strategy that may counter SSRI induced negative feedback of serotonergic neurotransmission and augment the antidepressant actions of these drugs.

Results

Effect of *htr2c* gene inactivation on neurochemical to fluoxetine. To examine the contributions of 5-HT 2C receptors to the actions of SSRIs I used a mouse molecular genetic approach. Two *in vivo* microdialysis studies were performed to examine the effect of fluoxetine (20 mg/kg, i.p.) on extracellular serotonin levels in the PFC of wild-type and 5-HT 2C receptor null mutant mice. In an initial experiment, fluoxetine or vehicle was administered 40 minutes prior to TST (Fig. 5). No phenotypic effects on basal extracellular serotonin levels were observed (5.8±0.7 and 5.6±0.6 fmol/sample (mean ± sem) in the PFC of mutant and wild type mice, respectively. TST caused a rapid and short-lived increase in 5-HT efflux with no effect of genotype in mice injected with vehicle (Fig. 6). By

contrast, the mutation significantly augmented the effect of fluoxetine followed by TST on extracellular serotonin levels in the PFC ($p<0.05$) (Fig. 7). In order to determine whether the effect of fluoxetine alone (without TST) would be potenitated by the null mutation, I repeated this experiment omitting TST and found that the mutation did indeed retain its potentiating effect ($p<0.05$) (Fig. 4).

Potentiation of SSRI-induced serotonin release by ketanserin. The effect of coadministration of the nonselective 5-HT2 receptor antagonist ketanserin on SSRI-induced serotonin release was examined in rats using *in vivo* microdialysis. Microdialysis probes were placed in the ventral hippocampal formation, where baseline extracellular serotonin levels were 4.81 + 0.38 fmol/sample (n = 95). Whereas the SSRI citalopram (3 mg/kg s.c.) produced 400-500% increases in serotonin levels relative to baseline ($p<0.0001$), ketanserin administration (40 µg/kg s.c.) alone produced no effect (Fig. 1a). However, the effect of citalopram on extracellular serotonin was substantially augmented, in a dose-dependent manner, by co-administration with ketanserin. Whereas 0.4 µg/kg ketanserin was devoid of any effect, 4 and 40 µg/kg augmented the effect of citalopram, with the 40 µg/kg dose producing 1000% increases from baseline values ($F(1,106)=15.82$ $p<0.005$).

I then determined the extent to which the ability of ketanserin to potentiate citalopram-induced serotonin release generalizes to additional SSRIs. Whereas fluoxetine alone (4 mg/kg) increased serotonin levels to approximately 350% of the baseline value ($p<0.0005$), ketanserin co-administration (40 µg/kg s.c.) augmented this effect to produce a 600% increase relative to baseline values

(F(1,108)=6.44, p<0.05) (Fig. 1b). Sertraline (9 mg/kg, s.c.) also increased serotonin levels (p<0.0005) and ketanserin (40 μg/kg, s.c.) potentiated this effect (F(1,87)=4.19, p<0.0005) (Fig. 1c). To determine whether the ability of ketanserin to potentiate citalopram-induced serotonin release generalizes to other forebrain regions implicated in the regulation of affect, the experiment was repeated with microdialysis probes located in the prefrontal cortex (PFC). In this region, baseline extracellular serotonin levels were 3.79 ± 0.37 fmol/sample (mean ± sem, n = 19). Citalopram administration (3 mg/kg s.c.) increased extracellular serotonin levels in the PFC (p<0.0001) and ketanserin potentiated this effect (F(1,163)=6.27, p<0.05) (Fig. 1d).

Identification of receptor subtype mediating potentiation of SSRI-induced serotonin release. Ketanserin interacts with high affinity at 5-HT2 receptors and at several non-5-HT receptor subtypes (Roth et al., 2000). To determine the extent to which these receptors contribute to the ability of ketanserin to potentiate SSRI-induced serotonin release, antagonists of these receptors were coadministered with citalopram. The α1-adrenoceptor antagonist prazosin (0.4 mg/kg s.c.) significantly decreased 5-HT levels when administered alone (p<0.05), but did not alter the effect of citalopram (Fig. 2a). The histamine H1-receptor antagonist mepyramine (0.3 mg/kg s.c.) did not alter 5-HT levels when administered alone, nor did it modify the effect of citalopram (Fig. 2b). Similarly, the selective 5-HT2A receptor antagonist MDL 100,907 (0.4 mg/kg s.c.) had no effect on 5-HT levels when administered alone or in combination with citalopram (Fig. 2c). In contrast, two selective 5-HT 2C receptor antagonists, SB 242084

and RS 102221, potentiated citalopram-induced serotonin release. Although these agents did not produce significant alterations of extracellular serotonin levels when given alone (0.4 and 0.6 mg/kg s.c., respectively), they substantially augmented the serotonergic response to citalopram, as indicated by 900% increases of extracellular serotonin levels from baseline values (SB 242084: $F(1,117) = 9.19$, $p< 0.05$; RS 102221: $F(1,120) = 12.14$, $p<0.005$; Figs. 3a, 3b.)

Effect of *htr2c* gene inactivation and 5-HT 2C receptor antagonist treatment on tail suspension responses. Saline-treated mutant and wild type mice displayed similar immobility times in the TST. Fluoxetine treatment (20 mg/kg i.p., 40 min prior to testing) significantly enhanced immobility, revealed by 2-way ANOVA as a significant overall effect of drug treatment ($F(1,27) = 139$, $p<0.001$). This effect of fluoxetine was potentiated in 5-HT 2C receptor mutant mice, as indicated by a significant genotype X drug interaction ($F(3,27) = 8.7$, $p<0.01$; Fig. 5a). A similar effect was observed in wild type mice treated with the 5-HT 2C receptor antagonist SB206553. Although neither citalopram nor SB206553 produced significant effects on immobility, coadministration of these compounds (SB206553 5 mg/kg, s.c., 30 min prior to testing; citalopram (16 mg/kg s.c.) produced a significant anti-immobility response ($F(3,28) = 4.35$, $p<0.02$) (Fig. 5b).

Discussion

These findings indicate that serotonin 5-HT 2C receptors have substantial influence on both neurochemical and antidepressant-like behavioral responses to SSRIs. The observed inability of ketanserin administered alone to alter central serotonin levels was consistent with previous studies indicating that 5-HT2

receptor agonists and antagonists produce at most small effects on serotonin levels when administered alone (Gobert and Millan, 1999; Gobert et al., 2000). We were therefore surprised to find that ketanserin produced a robust augmentation of citalopram-induced elevations of serotonin levels. This effect of ketanserin was also observed with the SSRIs fluoxetine and sertraline, indicating a general ability of ketanserin to potentiate the neurochemical effects of serotonin reuptake blockade.

Determination of the mechanism through which ketanserin potentiates SSRI action required consideration of the potential contributions of the multiple receptor subtypes for which the ketanserin has substantial antagonist affinity, such as the 5-HT2A, 5-HT 2C, histamine H1, and α1-adrenergic receptors (Leysen et al., 1992; Roth et al., 2000). When corresponding subtype-selective antagonists were coadministered with citalopram, only the 5-HT 2C receptor antagonists SB 242084 and RS 102221 reproduced the effects of ketanserin. However, neither antagonist altered extracellular serotonin levels when administered alone. The potentiation of SSRI-induced serotonin release by 5-HT 2C receptor inactivation was observed in both the hippocampal formation and prefrontal cortex, two regions that have been strongly implicated in the pathophysiology of depressive disorders and in responses to antidepressant drugs (Stockmeier, 1997; Davidson et al., 2002). The potential antidepressant efficacy of drug treatments has been associated with reductions of immobility time in a tail suspension assay (Steru et al., 1985; Porsolt, 2000). Because the enhancement of brain serotonin function is thought to be central to the

antidepressant effects of SSRIs, we hypothesized that the antidepressant-like behavioral effects of SSRIs would be potentiated by 5-HT 2C receptor inactivation. Accordingly, pharmacological blockade of 5-HT 2C receptors produced a small potentiation of citalopram's effect in the TST, yet produced no observable effects in this test in the absence of SSRIs. Additional studies are required to determine the extent to which behavioral responses to the pharmacological blockade of 5-HT 2C receptors generalizes to additional SSRIs.

A striking degree of concordance exists between the results obtained using pharmacological or genetic inactivation of 5-HT 2C receptors: 1) both the 5-HT 2C receptor mutation and the 5-HT2C receptor antagonists enhance fluoxetine-induced extracellular serotonin levels in prefrontal cortex, 2) neither the mutation nor the antagonists themselves alter extracellular serotonin levels. Taken together, these complementary pharmacological and genetic studies provide strong evidence for the potentiating effects of 5-HT 2C receptor inactivation on SSRI action.

Prior investigations of the influence of 5-HT 2C receptors in assays of “behavioral despair” have yielded inconsistent results. In one previous study, several 5-HT2 receptor agonist and antagonist compounds failed to influence immobility in the mouse forced swim test (Redrobe and Bourin, 1997). By contrast, in a study utilizing the rat forced swim test, several 5-HT 2C receptor agonists, but not mCPP, reduced immobility in the forced swim test; these effects were blocked by 5-HT 2C receptor antagonists (Cryan and Lucki, 2000). Differences between these findings and those in the present study may relate to

differences in the assays (forced swim vs. TST), drug doses, and the species (rats vs. mice) used.

The observed influence of 5-HT 2C receptors on extracellular serotonin levels during SSRI treatment, but not under baseline conditions was not anticipated. The possibility that 5-HT2 receptor antagonism could influence forebrain serotonin levels had been suggested by a prior study with the nonspecific 5-HT 2C receptor antagonist mianserin (Kreiss and Lucki, 1995). However, the high affinity of mianserin for 5-HT 2C receptors, adrenoceptors and histamine receptors precludes attribution of this effect to its activity at 5-HT 2C receptors (NIMH Psychoactive Drug Screening Program Database: http://pdsp.cwru.edu/pdspf.asp). To my knowledge, 5-HT 2C receptors have not been demonstrated on the presynaptic elements of serotonergic neurons and have not been considered to have a classic autoreceptor function. The ability of both pharmacological and genetic inactivation of 5-HT 2C receptors to alter serotonin levels during SSRI treatment, but not under baseline conditions, suggests that 5-HT 2C receptors may contribute to a negative feedback mechanism recruited under conditions of elevated serotonergic tone. Such a role for these receptors in the homeostatic regulation of serotonin levels could account for prior observations that 5-HT 2C receptor inactivation enhances sensitivity to the motoric effects of the serotonin-releasing agents dexfenfluramine and MDMA (Vickers et al., 1999; Bankson and Cunningham, 2002).

Several possible mechanisms could underlie the augmentation of SSRI-

induced serotonin release by 5-HT 2C receptor inactivation. Although an autoreceptor function has not been demonstrated for 5-HT 2C receptors, it is notable that these receptors are expressed in the vicinity of mesencephalic raphe serotonergic neurons that project widely to the forebrain (Clemett et al., 2000). Neurochemical and electrophysiological studies indicate that these neurons are subject to GABAergic inhibitory regulation (Gervasoni et al., 2000; Tao and Auerbach, 2003). Notably, nonspecific 5-HT receptor agonists suppress serotonin neuronal firing rates (Boothman et al., 2003), and bath application of serotonin to mesencephalic slices increases the frequency of inhibitory postsynaptic potentials in these serotonergic neurons (Liu et al., 2000). These effects are attenuated by 5-HT2A receptor antagonists, and to a much lesser extent, by 5-HT 2C receptor antagonists (Liu et al., 2000; Martin-Ruiz et al., 2001; Boothman et al., 2003). It is therefore possible that functional blockade of 5-HT 2C receptors in the DRN could reduce the magnitude of serotonin-mediated excitation of raphe GABA neurons. Alternatively, it is possible that serotonin release could be influenced by 5-HT 2C receptors expressed within interneurons of the PFC and hippocampus (Wright et al., 1995). Nonspecific 5-HT2 receptor agonists have been found to enhance GABAergic neurotransmission in both the prefrontal cortex and CA1 region of the hippocampus (Shen and Andrade, 1998; Abi-Saab et al., 1999). The relative contributions of the 5-HT2A and 5-HT 2C receptor subtypes to these effects remain to be determined.

Altogether, these results define a novel action of 5-HT 2C receptors in the modulation of serotonin levels within brain regions implicated in the regulation of

affect. These results further suggest that co-administration of 5-HT 2C receptor antagonists with SSRIs warrants consideration as a novel approach for overcoming homeostatic mechanisms that attenuate the therapeutic efficacy of SSRIs.

Materials and Methods

Animals. All animals were group-housed at 22°C on a 12-hr light/dark cycle, with standard food and water *ad libitum.* Adult male albino rats of a Wistar-derived strain (285-320 g; Harlan, Zeist, Netherlands) were used. Effects of 5-HT 2C receptor antagonism in the mouse tail suspension test were determined using C57BL/6 mice (M & B A/S, Denmark). To examine the effects of genetic inactivation of 5-HT 2C receptors, male 8-12 week old mice hemizygous for a null mutation of the X-linked *htr2c* gene (backcrossed to a C57BL/6J background for at least 24 generations) and wild-type littermates were used (Tecott et al., 1995). The line has been maintained through matings of heterozygous females with C57BL/6J males (Jackson Laboratory, Bar Harbor, ME). Experiments were conducted in accordance with the Declaration of Helsinki and the NIH Guide for the Care and Use of Laboratory Animals.

Drugs. For rat studies, citalopram hydrobromide, fluoxetine hydrochloride, irindalone, sertraline hydrochloride, olanzapine, RS 102221 and SB 242084 were obtained from Lundbeck A/S (Copenhagen, Denmark). Mepyramine, prazosin, and ketanserin were obtained from RBI (Natick, USA). MDL 100907 was synthesized at the University of Groningen. These drugs were dissolved in saline and injected subcutaneously in a volume of 1 ml/kg. Antagonists were

administered immediately (within 15 sec) prior to either SSRI or vehicle treatment. For mutant mouse studies, fluoxetine hydrochloride was obtained from Tocris (Ellisville, MO). Fluoxetine was dissolved in a sterile saline solution for i.p. injections. Control injections consisted of vehicle only.

Surgical implantation of microdialysis probes. For rats, microdialysis was performed using I-shaped probes, composed of polyacrylonitrile/sodium methyl sulphonate copolymer dialysis fiber (i.d. 220 µm, o.d. 310 µm, AN 69, Hospal, Italy). The exposed length of the membranes was 4 mm for both ventral hippocampus and prefrontal cortex. Prior to surgery, rats were anaesthetized using ketamine (50 mg/kg ip) and xylazine (8 mg/kg ip), after premedication with midazolam (5mg/kg s.c.). Lidocaine-HCl (10 % (m/v)) was used for local anaesthesia. Rats were placed in a stereotaxic frame (Kopf, USA), and probes were implanted into the ventral hippocampus (L +4.8 mm, IA: +3.7 mm, V: -8.0 mm) and prefrontal cortex (L –0.9 mm, AP: +3.5 mm, V: -6.0 mm). After insertion, probes were secured with dental cement. For mice, concentric microdialysis probes were constructed using 23 gauge stainless steel and silica capillary tubing. The dialysis membrane (i.d. 240 µm, o.d. 290 µm, AN 69HF, Hospal, Italy) consisted of polyacrilonitril/sodium methalyl sulphonate copolymer with an average pore size of 29 A. Dialysis probes had 2 mm of exposed membrane. Prior to surgery, mice were anesthetized using ketamine (100 mg/kg ip) and xylazine (10 mg/kg ip) and then placed in a stereotaxic frame. Microdialysis probes were implanted into the right prefrontal cortex (PFC) at the following coordinates relative to bregma (AP +2 mm; L –0.5 mm; DV –4 mm; (Franklin and

Paxinos, 1997)).

In vivo microdialysis. All animals used in microdialysis studies were allowed to recover for at least 24 hr following surgery. For rat studies, probes were perfused with artificial cerebrospinal fluid containing (in mM): NaCl 147, KCl 3.0, CaCl2 1.2, and MgCl2 1.2, at a flow-rate of 1.5 µl/min (Harvard Apparatus pump, South Natick, Ma., USA). Microdialysis samples were collected at 15 min intervals in HPLC vials containing 7.5 µl 0.02 M acetic acid for serotonin analysis (animals were injected at t=0, data were not corrected for lag time). For mouse studies, probes were perfused with artificial cerebrospinal fluid containing (in mM): NaCl 145, KCl 2.7, MgCl2 1, CaCl2 1.2, Na2HPO4 2, pH 7.40, at a flow rate of 1 µl/min. Microdialysis samples were collected at 20 min intervals. Fluoxetine was administered immediately before sample 6.

Determination of dialysate serotonin levels. Serotonin concentrations were determined using HPLC coupled with electrochemical detection. For rat studies, 20 µl microdialysate fractions were injected via an autoinjector (CMA/200 refrigerated microsampler, CMA, Sweden) onto a 100 x 2.0 mm C18 Hypersil 3 µm column (Bester, Amstelveen, the Netherlands) and separated with a mobile phase consisting of 5 g/L di-ammoniumsulfate, 500 mg/L Na2-EDTA, 50 mg/L heptane sulphonic acid, 4% methanol v/v and 30 µl/L of triethylamine, pH 4.75 at a flow rate of 0.4 ml/min (Shimadzu LC-10 AD). 5-HT was detected amperometrically at a glassy carbon electrode at 500 mV vs Ag/AgCl (Antec Leyden, Leiden, Netherlands). The detection limit was 0.5 fmol 5-HT per 20 µl sample (signal to noise ratio 3). For mouse studies, the mobile phase consisted

of a mixture of acetonitrile, phosphate buffer and an ion-pairing agent (ESA, Chelmsford MA). The mobile phase was delivered by a pump (model 582, Solvent Delivery Module; ESA) at a flow rate of 0.25 µl/min through a MD-150 narrowbore column (ESA). 5-HT was detected using a coulometric detector (Coulochem II; ESA) coupled to a single channel enhanced amperometric cell (Model 5041; ESA). The potential applied to the electrode was +175 mV; under these conditions, the limit of detection for 5-HT was approximately 2-3 fmol. Data were analyzed using a basic integrator (Model 3395; Hewlett-Packard). For microdialysis data, basal 5-HT levels were defined as the average level of the first five samples. Differences between treatments were analyzed using a two-way ANOVA with repeated measurements, followed by Students Newman Keuls post-hoc analysis. In separate analyses for evaluating the effects of treatments relative to baseline, one-way ANOVA for repeated measurements on ranks were used, followed by Dunnet's test. Level of significance was set at $p \leq 0.05$.

Tail suspension test (TST). The mouse tail suspension test was designed as a screening assay for antidepressant compounds, and is sensitive to the major classes of antidepressants (Steru et al., 1985; Porsolt, 2000). Animals were suspended by the tail using adhesive tape from either a 1.2-cm diameter metal bar (5-HT 2C receptor mutant studies) or a 1 cm diameter hook (5-HT 2C receptor antagonist studies) elevated 30-35 cm. When suspended, rodents either make apparent escape attempt movements or adopt a characteristic immobile posture. The total immobility time was measured

(manually for 5-HT 2C receptor mutant studies, and by automated strain gauge for 5-HT 2C receptor antagonist studies (Med Associates, St. Albans, VT)) during the 6 min test period. The effects of treatments relative to baseline were analyzed using a one-way ANOVA, followed by Dunnet's test. Interactions between antagonist treatment and SSRI treatment, or between genotype and SSRI treatment, were analyzed by a two-way ANOVA.

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Figure 1

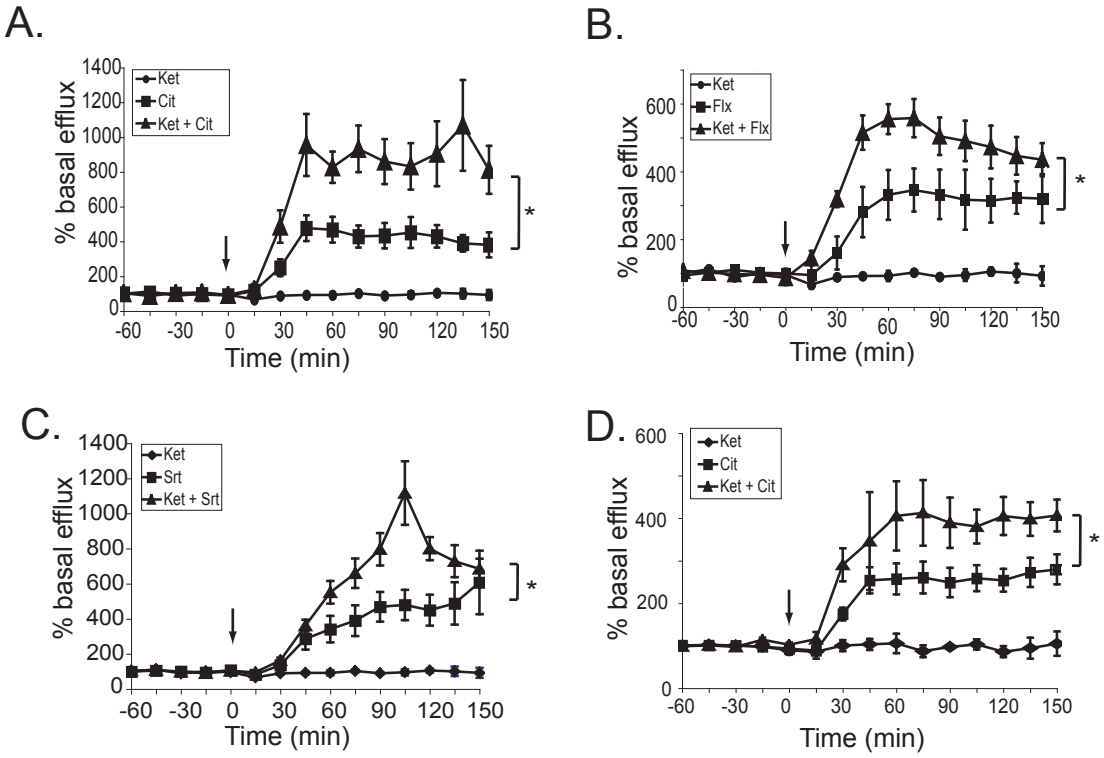

A.
B.
C.
D.
% basal efflux
Time (min)
Ket
Cit
Ket + Cit
Flx
Ket + Flx
Srt
Ket + Srt
*

Figure 1. Augmentation of SSRI-induced serotonin release in hippocampus (a-c) and prefrontal cortex (d) of freely moving rats by coadministration of the 5-HT2 receptor antagonist ketanserin. All drugs were administered at time 0.

A. Citalopram administration (3 mg/kg s.c.) significantly increased extracellular serotonin levels in the ventral hippocampus ($p<0.0001$, $n = 7$). Ketanserin (40 µg/kg s.c.) did not alter hippocampal serotonin levels when administered alone ($n = 4$). However, when co-administered with citalopram, it augmented the serotonin-releasing effects of the SSRI ($F(1,106)=15.82$, $^{**}p<0.005$, $n = 4$).

B. Fluoxetine administration (4 mg/kg s.c.) significantly increased hippocampal extracellular serotonin levels ($p<0.0005$, $n = 5$). Ketanserin (40 µg/kg s.c.) augmented the serotonin-releasing effects of fluoxetine ($F(1,108)=6.44$, $^{*}p<0.05$, $n = 5$).

C. Sertraline administration (9 mg/kg s.c.) significantly increased hippocampal serotonin extracellular levels ($p<0.0005$, $n = 4$). Ketanserin (40 µg/kg s.c.) augmented the serotonin-releasing effects of sertraline ($F(1,87)=4.19$, $^{***}p<0.0005$, $n = 4$).

D. In the PFC, citalopram administration (3 mg/kg s.c.) significantly increased extracellular serotonin levels ($p<0.0001$, $n = 10$). As observed in the hippocampus, ketanserin (40 µg/kg s.c.) alone produced no effect ($n = 7$), but it significantly augmented the effect of citalopram (3 mg/kg s.c.) ($F(1,163)=6.27$, $^{*}p<0.05$, $n = 4$).

Figure 2

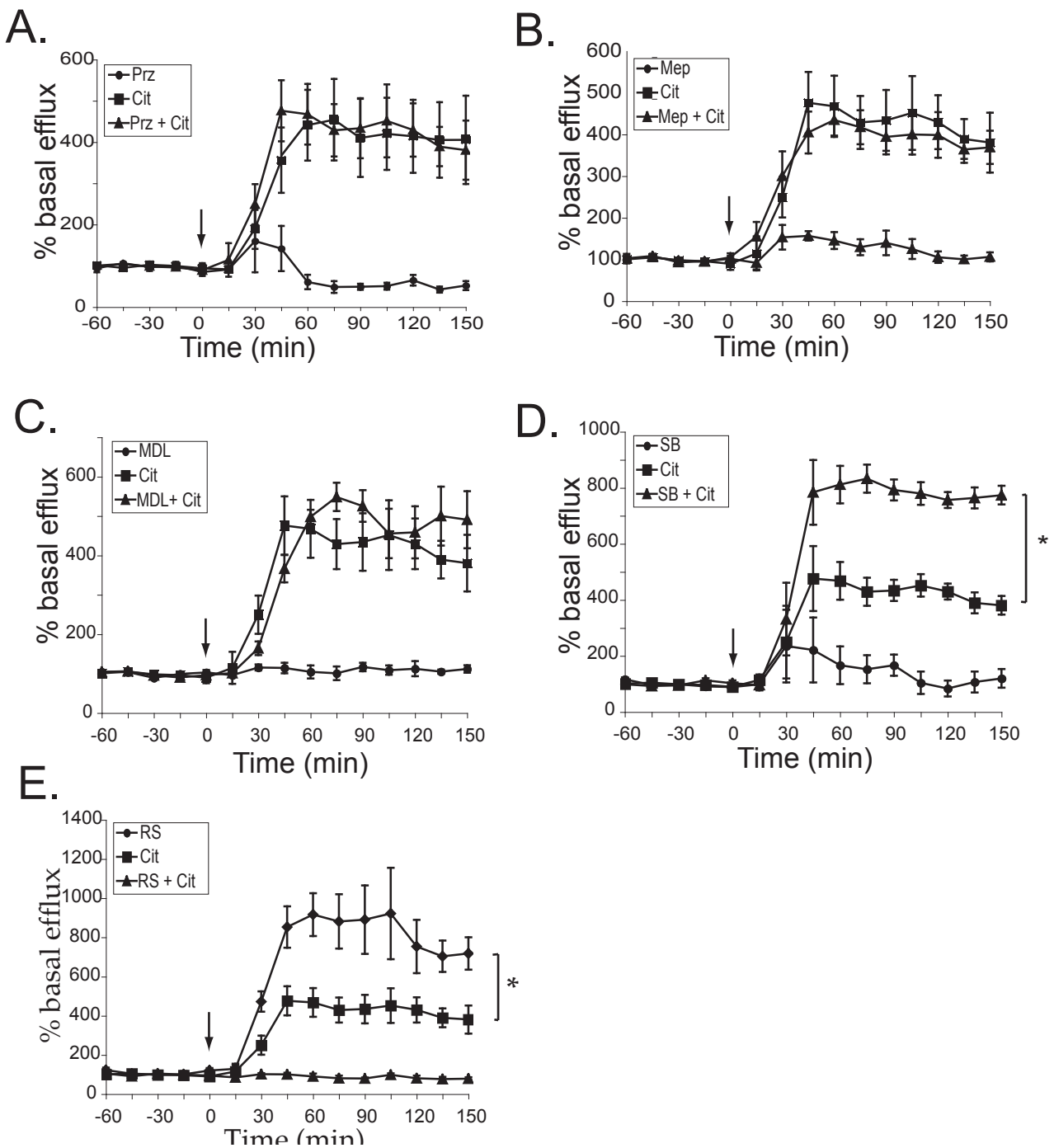

A.
Prz
Cit
Prz + Cit
% basal efflux
Time (min)
B.
Mep
Cit
Mep + Cit
% basal efflux
Time (min)
C.
MDL
Cit
MDL+ Cit
% basal efflux
Time (min)
D.
SB
Cit
SB + Cit
*
% basal efflux
Time (min)
E.
RS
Cit
RS + Cit
*
% basal efflux
Time (min)

Figure 2. SSRI-induced hippocampal serotonin release in freely moving rats is not potentiated by co-administration of an α1-adrenoceptor antagonist, a histamine receptor 1 antagonist or a 5-HT2A receptor antagonist. All drugs were administered at time 0.

A. Administration of the α1-adrenoceptor antagonist prazosin (0.4/kg s.c.) alone significantly reduced 5-HT extracellular levels ($p<0.05$, n = 4). However, prazosin did not alter serotonin release induced by citalopram (3 mg/kg s.c.; coadministration n = 8, citalopram alone n = 4).

B. Administration of the H1 receptor antagonist mepyramine (0.3 mg/kg s.c.) did not alter extracellular serotonin levels when administered alone (n = 5) or in combination with citalopram (10 μmol/kg s.c.; coadministration n = 7, citalopram alone n = 7).

C. Administration of the selective 5-HT2A receptor antagonist MDL 100,907 (0.4 mg/kg s.c.) did not alter extracellular serotonin levels when administered alone (n = 5) or in combination with citalopram (3 mg/kg s.c.; coadministration n = 5, citalopram alone n = 7).

Figure 3

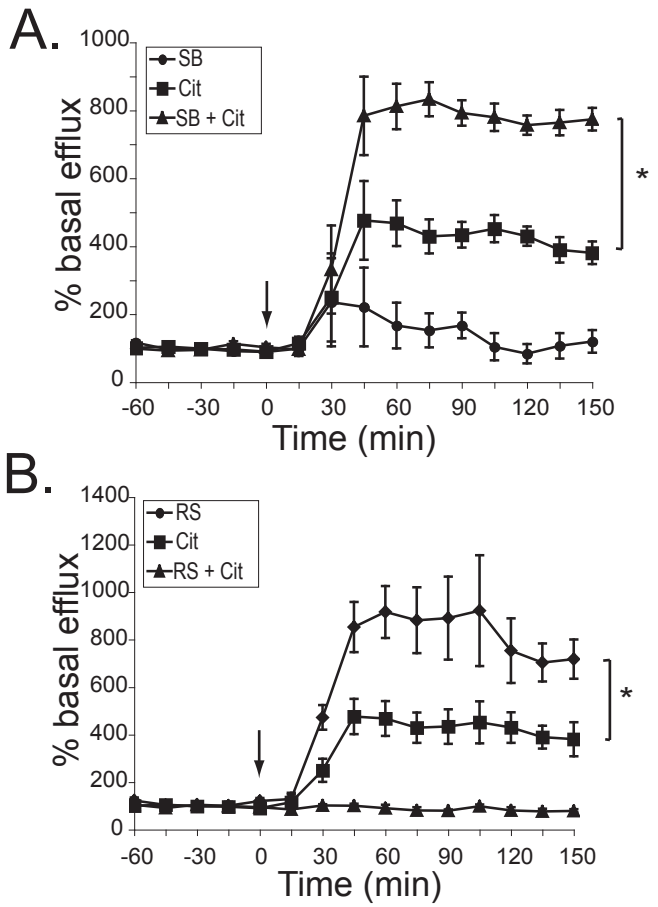

A.
SB
Cit
SB + Cit
% basal efflux
1000
800
600
400
200
0
-60
-30
0
30
60
90
120
150
Time (min)
*
B.
RS
Cit
RS + Cit
% basal efflux
1400
1200
1000
800
600
400
200
0
-60
-30
0
30
60
90
120
150
Time (min)
*

Figure 3. Co-administration of 5-HT 2C receptor antagonists potentiated SSRI-induced hippocampal serotonin release in freely moving rats. All drugs were administered at time 0.

A. Whereas the selective 5-HT 2C receptor antagonist SB 242084 (0.4 mg/kg s.c.) did not alter serotonin levels when administered alone (n = 5), it significantly augmented the serotonergic neurochemical response to 3 mg/kg s.c. citalopram ($F(1,117) = 9.19$, $^{*}p < 0.05$, n = 5; citalopram alone n = 7).

B. Another 5-HT 2C receptor antagonist, RS 102221 (0.6 mg/kg s.c.) did not alter serotonin levels when administered alone (n = 5), but significantly augmented the serotonergic neurochemical response to 3 mg/kg s.c. citalopram ($F(1,120) = 12.14$, $^{**}p < 0.005$, n = 5; citalopram alone n = 7)**.**

Figure 4

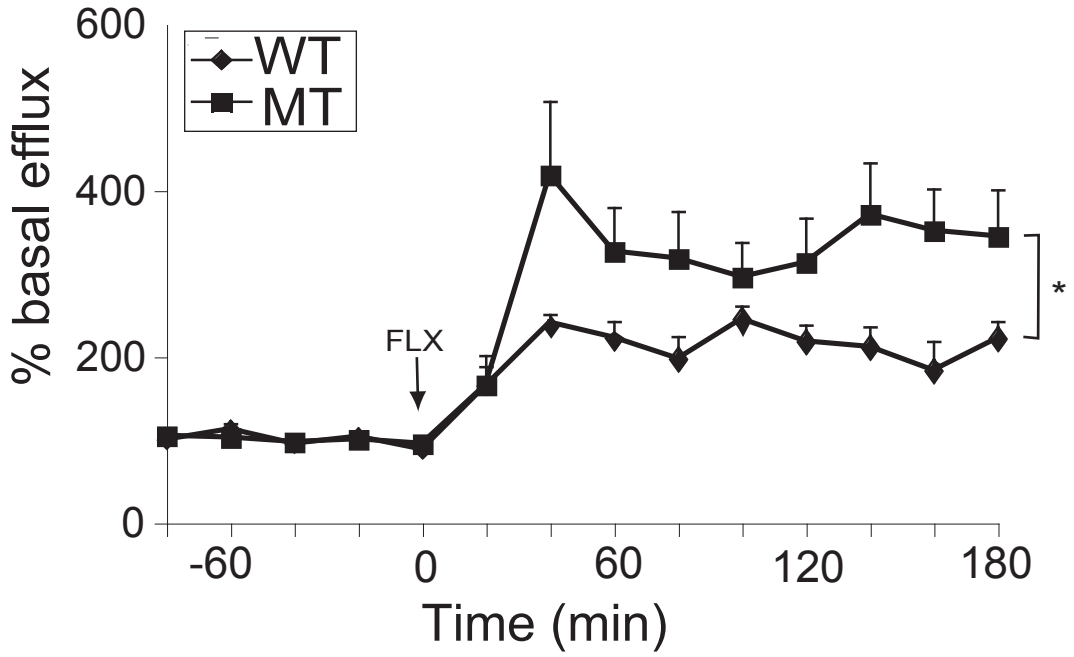


Figure 4. Neurochemical effects of fluoxetine are potentiated in 5-HT 2C receptor null mutant mice. Fluoxetine was administered at time 0. Basal extracellular serotonin levels were (mean ± sem) 5.8 + 0.7 fmol/sample in the PFC of 5-HT 2C receptor mutant mice and 5.6 + 0.6 fmol/sample in the PFC of wild type animals and did not significantly differ between the two groups. Fluoxetine-induced (18 mg/kg i.p.) increases in serotonin levels were potentiated in mutant mice ($F(1,18) = 6.018$, $^*p<0.05$, n = 9 WT and 11 MT).

Figure 5

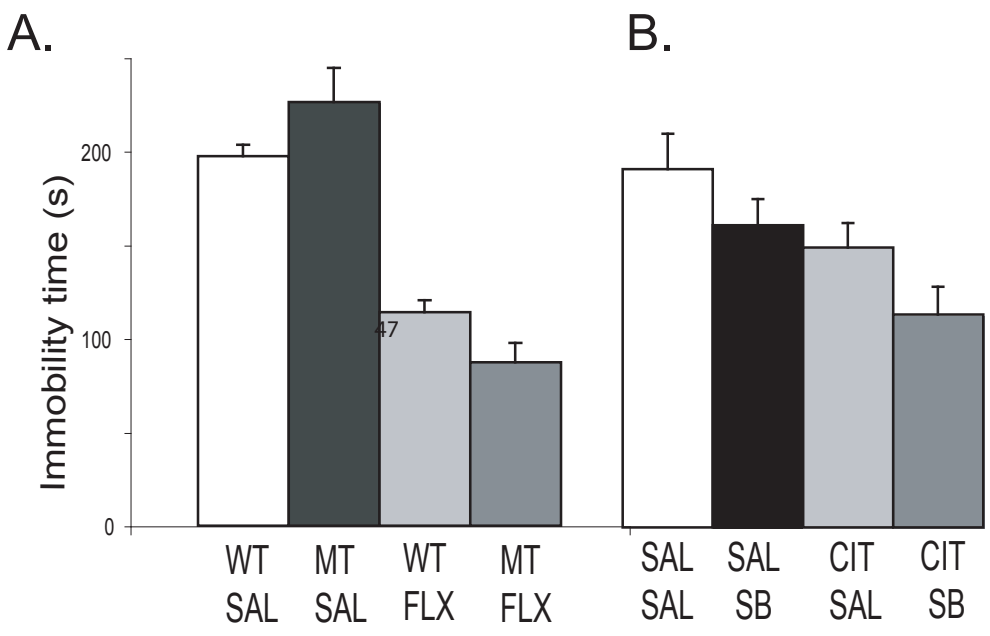
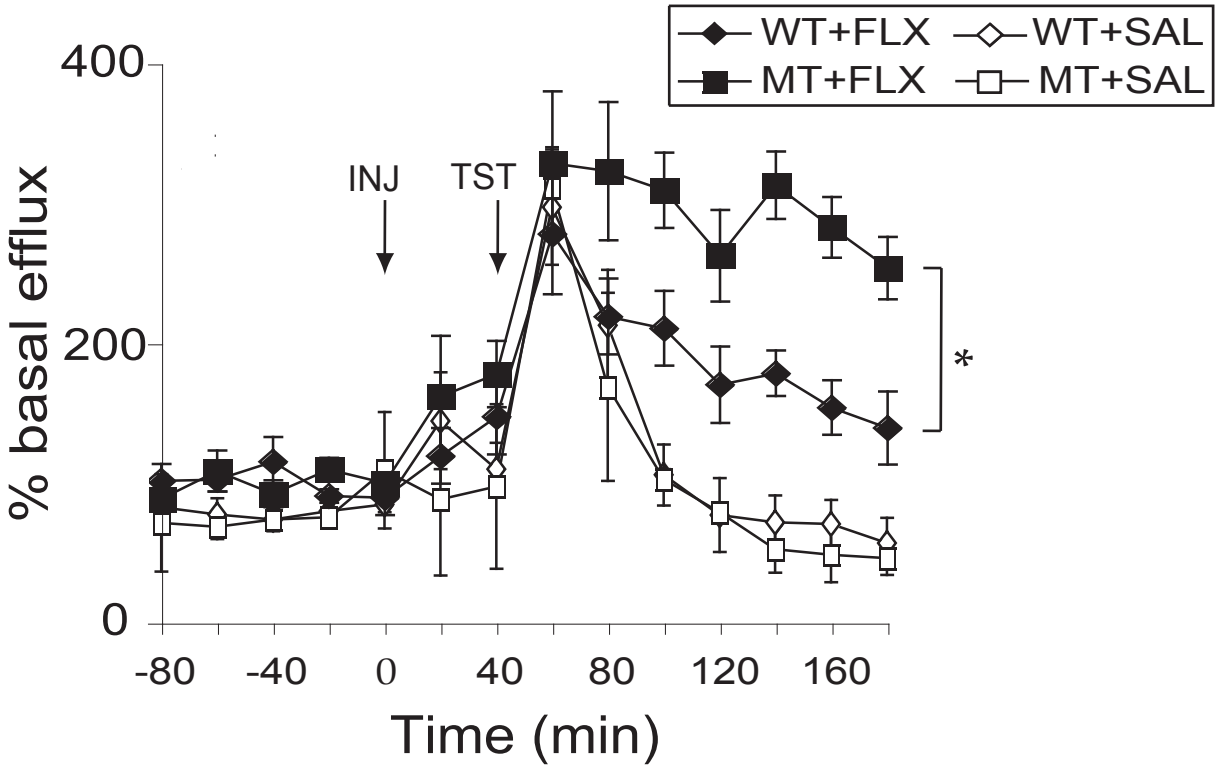

A.
B.
Immobility time (s)
200
100
0
47
WT SAL
MT SAL
WT FLX
MT FLX
SAL SAL
SAL SB
CIT SAL
CIT SB

Figure 5. Potentiation of behavioral effects of citalopram and fluoxetine by 5-HT2C receptor inactivation.

A. Immobility times of 5-HT 2C receptor mutant and wild type mice during tailsuspension assay. No significant effect of genotype on immobility time was observed in saline-treated animals. However, fluoxetine administration (20 mg/kg i.p.) elicited a significantly greater effect on immobility time in 5-HT 2C receptor mutant mice than in wild type controls ($F(1,27) = 8.7$, **$p<0.01$; n = 6-10 mice per group).

B. Coadministration of a 5-HT 2C receptor antagonist potentiates the effect of fluoxetine on immobility time in the tail suspension test. Administration of the 5-HT 2C receptor antagonist SB 206553 (5 mg/kg s.c.) produced no significant alteration in behavioral responses to tail-suspension. However, when co-administered with citalopram (16 mg/kg s.c.), SB 206553 significantly potentiated the anti-immobility effects of the SSRI ($F(3,28) = 4.35$, *$p<0.01$, n = 8 per group).

Figure 6


WT+FLX
WT+SAL
MT+FLX
MT+SAL
400
200
0
% basal efflux
INJ
TST
*
-80
-40
0
40
80
120
160
Time (min)

49

Figure 6. Neurochemical effects of fluoxetine followed by TST are potentiated in 5-HT 2C receptor null mutant mice. Fluoxetine (16 mg/kg i.p.) or vehicle was administered following the collection of 5 basal samples. 40 minutes later, TST was performed. Basal extracellular serotonin levels did not significantly differ between any groups. TST caused a robust, short-lived increase in 5-HT efflux in saline-treated mice with no effect of genotype. In fluoxetine-treated mice, 5-HT efflux increased to a greater extent in mutant mice (*$p<0.05$).

Chapter 2:

Chronic citalopram administration causes a sustained suppression of serotonin synthesis in the mouse forebrain

Abstract

Serotonin is a neurotransmitter with important roles in the regulation of physiological processes, particularly those regulating affect in humans. Drugs that selectively potentiate serotonergic neurotransmission by inhibiting the reuptake of serotonin (SSRIs) are widely used for the treatment of psychiatric disorders. Although the regulation of serotonin synthesis may be an important factor in the effects of SSRIs, the effect of chronic SSRI administration on 5-HT synthesis is not well understood. Here, I describe the effect of chronic administration of the SSRI citalopram (CIT) on 5-HT synthesis and content in the mouse forebrain. Administration of citalopram for 2 days, 14 days or 28 days via osmotic minipump suppressed 5-HT synthesis. 5-HT content was not significantly reduced by CIT administration at any time point, although a pooled analysis suggested that 5-HT was modestly reduced in CIT-treated mice. When mice were challenged with an acute injection of a decarboxylase inhibitor (which dramatically reduces monoamine synthesis), CIT treatment caused a depletion of brain 5-HT. 5-HIAA content was reduced in CIT-treated brains at all time points. Taken together, these results demonstrate a sustained suppression of serotonin synthesis by chronic citalopram administration and indicate that the 5-HT content of the brain is highly dependant on *de novo* synthesis of 5-HT under conditions of 5-HT reuptake blockade.

Introduction

Depression is a devastating illness and one of the major causes of disability in the world, affecting over 120 million people (Michaud, Murray et al. 2001; WHO, 2008). Selective serotonin reuptake inhibitors (SSRIs) are widely prescribed as a first line of treatment for depression and many other psychiatric disorders (Kramer 1997; Katzung 2004; Parker 2009). The primary pharmacological activity of SSRIs is the inhibition the serotonin transporter (SERT), which is responsible for the reuptake of serotonin (5-HT) from the extracellular space back into the specialized neurons that release it (Castren 2005; Berton and Nestler 2006). Inhibition of this transport alters the spatiotemporal dynamics of serotonin signaling such that activity in the serotonergic neuron causes greater increases in extracellular serotonin than would normally occur.

Although the pharmacological targets of SSRIs have been well characterized for decades, major scientific and clinical issues regarding their use remain unresolved. Accurate quantification of antidepressant response is difficult (Kirsch, Deacon et al. 2008; Thase 2008; Parker 2009); however, clinical evidence suggests that while SSRIs are generally effective for the treatment of many psychiatric disorders, a significant proportion of patients will not respond to the SSRI initially prescribed or even to any SSRI, and other patients will attain only a partial remission of symptoms (Kirsch, Deacon et al. 2008).

Another salient clinical issue is the latency of several weeks for therapeutic effects to occur (Hervas, Vilaro et al. 2001; Taylor, Freemantle et al. 2006). This phenomenon has inspired the hypothesis that antidepressants exert their behavioral effects by gradually inducing neuronal plasticity, neurotrophin release and neurogenesis (Berton and Nestler 2006). However, the mechanisms which are responsible for the therapeutic

effects of SSRIs (downstream of SERT blockade) remain elusive. Given the high affinity of SSRIs for SERT (PDSP, 2008), it is generally believed that these downstream events, whatever they may be, are initiated and sustained by effects on serotonergic neurotransmission (Delgado, Price et al. 1994). (Although many SSRIs such as fluoxetine and paroxetine have significant pharmacological interactions with targets other than SERT(Heurteaux, Lucas et al. 2006; Pinna, Costa et al. 2006; O'Leary, Bechtholt et al. 2007), citalopram (CIT) is extremely selective for SERT (PDSP, 2008).) In general, the lack of fundamental understanding of how SSRIs affect multiple aspects of brain function currently hampers efforts to develop novel antidepressant strategies.

SSRIs affect many aspects of serotonergic neurotransmission in addition to (or secondarily to) reuptake inhibition. These effects may play a major role in the therapeutic response (Blier, Chaput et al. 1988; Hervas, Vilaro et al. 2001). 5-HT synthesis is a likely determinant of SSRI response. In vitro, the rate of neurotransmitter synthesis is an important factor in monoaminergic neurophysiology (Pothos, Larsen et al. 2000). In vivo, 5-HT synthesis rate is regulated by many factors, including the availability of the dietary precursor tryptophan (Fernstrom and Wurtman 1971; Carlsson and Lindqvist 1978; Olivier, Jans et al. 2008). Pharmacological inhibition of 5-HT synthesis can induce an rapid relapse of depression symptoms in SSRI-treated patients with remitted depression, an effect which is not readily observed in subjects with no history of SSRI administration (Delgado, Charney et al. 1990; Delgado, Price et al. 1991; Benkelfat, Ellenbogen et al. 1994; Delgado, Price et al. 1994; Leyton, Young et al. 1997; Moore, Gillin et al. 1998; Smith, Morris et al. 1999; Leyton, Ghadirian et al. 2000; Booij, Van der Does et al. 2002) (also see (Shopsin, Gershon et al. 1975)). Tryptophan, itself considered to be ineffective

as an antidepressant (d'Elia, Hanson et al. 1978), has been reported to potentiate the therapeutic action of a 5-HT reuptake inhibitor (Walinder, Skott et al. 1975; Walinder, Skott et al. 1976; Møller 1990) (but see (Walinder 1981)). A putative genetic deficiency in 5-HT synthesis in mice (Zhang, Beaulieu et al. 2004; Osipova, Kulikov et al. 2008; Tenner, Qadri et al. 2008) may be associated with blunted responses to citalopram (Cervo, Canetta et al. 2005; Calcagno, Canetta et al. 2007).

These findings raise the question of how chronic SSRI administration affects 5-HT synthesis. SSRI administration immediately triggers multiple physiological responses in the brain, for example the suppression of activity in serotonergic neurons (Chaput, de Montigny et al. 1986; de Montigny, Chaput et al. 1990). Many of these effects dissipate upon chronic treatment; in fact, this adaptation may be required for the beneficial effects of antidepressants to emerge (Artigas, Celada et al. 2001; Hervas, Vilaro et al. 2001). There is substantial evidence that acute administration of citalopram and other SSRIs suppresses serotonin synthesis throughout the brain (Carlsson and Lindqvist 1978; Muck-Seler, Jevric-Causevic et al. 1996; Moret and Briley 1997; Barton CL 1999; Stenfors, Yu et al. 2001; Yamane, Okazawa et al. 2001; Cervo, Canetta et al. 2005). However, is not clear how chronic, continuous administraton of SSRIs affect 5-HT synthesis rate. Prior studies addressing this issue have reported contradictory results, perhaps due to methodological issues (Moret and Briley 1992; Esteban, Lladó et al. 1999; Yamane, Okazawa et al. 2001; Stenfors and Ross 2002). Although SSRI treatment produces robust increases in extracellular 5-HT, there is evidence that SSRI administration can actually deplete brain 5-HT and 5-HIAA stores (Hwang, Magnussen et al. 1980; Caccia, Bizzi et al. 1992; Trouvin, Chanut et al. 1992; Caccia, Anelli et al. 1993;

Trouvin, Gardier et al. 1993; Dygalo, Shishkina et al. 2006; Marsteller, Barbarich-Marsteller et al. 2007), as would be predicted if 5-HT synthesis was suppressed and serotonergic neurons were unable to effectively recapture released 5-HT. (For details of these studies, see Discussion.)

In this study, I addressed the question of how chronic reuptake blockade affects forebrain 5-HT synthesis rate and forebrain 5-HT and 5-HIAA content over the course of chronic treatment in mice. Furthermore, in order to address whether CIT administration renders 5-HT stores more sensitive to manipulations of 5-HT synthesis, I examined forebrain 5-HT and 5-HIAA content in CIT- and vehicle-treated mice following acute inhibition of amino acid decarboxylase.

Results

Male adult C57Bl6/J mice were treated with CIT or vehicle using osmotic minipumps to deliver a 10% CIT solution or saline vehicle solution at a constant low rate of infusion (0.25 uL/hour) for 2, 14 or 28 days. The dose and route of administration were chosen to approximate typical clinical pharmacokinetics, whereby CIT rapidly reaches a steady-state plasma concentration with relatively little variation throughout the day (Kragh-Sorensen, Overo et al. 1981; Rasmussen and Brosen 2000; Brøsen and Naranjo 2001). I used high performance liquid chromatography coupled with mass spectroscopy (HPLC-MS) to quantify CIT concentrations in plasma collected at the time of sacrifice (without drug washout). Following 2 or 4 weeks of CIT administration, plasma levels were approximately 400 nM, which is a typical therapeutic plasma concentration (Fig. 1A) (Kragh-Sorensen, Overo et al. 1981; Fredricson Overo 1982; Rasmussen and Brosen 2000; Brøsen and Naranjo 2001).

5-HT synthesis rate was assessed using the decarboxylase inhibition method. 3-hydroxybenzylhydrazine dihydrochloride (NSD-1015) was injected 30 minutes prior to sacrifice (100 mg/kg, intraperitoneal). Under normal circumstances, the amino acid decarboxylase enzyme (AADC) catalyzes the decarboxylation of 5-hydroxytryptophan (5-HTP), the product of tryptophan hydroxylation, into 5-HT. NSD-1015 inhibits AADC, leading to accumulation of 5-hydroxytryptophan (5-HTP), which is ordinarily present only in minute concentrations due to its rapid decarboxylation. 5-HTP can be precisely identified and quantified by HPLC coupled to electrochemical detection (HPLC-ED) (Carlsson and Lindqvist 1970; Carlsson, Bedard et al. 1972; Carlsson and Lindqvist 1972; Carlsson and Lindqvist 1973; Carlsson and Lindqvist 1978). Since tryptophan hydroxylation is the rate-limiting reaction in 5-HT synthesis, the amount of 5-HTP which accumulates over a specific period of time is considered an index of the rate of 5-HT synthesis (Carlsson and Lindqvist 1970; Carlsson, Bedard et al. 1972; Carlsson and Lindqvist 1972; Costa 1972; Carlsson and Lindqvist 1973; Carlsson and Lindqvist 1978; Carlsson and Lindqvist 1978; Moret and Briley 1997).

For each time point (surgery, dissection and analysis), CIT- and vehicle-treated mice and samples were handled in parallel by a blind experimenter. I observed a significant effect of time on 5-HTP accumulation in vehicle-treated mice ($p=0.0002$, ANOVA) (Fig. 1B). Comparable effects of time were observed for 5-HT, 5-HIAA and 5-HT/5-HIAA ratio in all vehicle-treated groups.

Citalopram treatment resulted in a sustained suppression of 5-HT synthesis rate in the forebrain (Fig. 1B) ($p<0.0001$, ANOVA with Bonferroni correction). 5-HTP accumulation was lower in CIT-treated mice than in control mice at all time points ($p<0.01$

for all comparisons, 2-tailed t-test with Welch's correction). The effect of CIT was greatest after 2 days of treatment (36% decrease, $p<0.0001$, t-test) and was comparable at 2 weeks (24% decrease, $p=0.003$, t-test) and 4 weeks (20% decrease, $p=0.008$, t-test). These results indicate that 5-HT synthesis remains significantly inhibited following a prolonged treatment with a highly selective SSRI at a clinically relevant dose.

It is not clear whether physiologically relevant reductions in 5-HT synthesis rate significantly affect brain content and release of 5-HT. Reuptake inhibition could interact synergistically with decreased synthesis to deplete brain 5-HT (Kim, Tolliver et al. 2005). Using HPLC-ED, I examined brain 5-HT content in forebrain in mice treated for 2, 14 and 28 days with CIT or vehicle (Fig. 2A). For this experiment, no NSD-1015 was administered prior to sacrifice. A significant overall effect of treatment on 5-HT was observed ($p=0.04$, ANOVA); however, criteria for Bonferroni correction were not met for any individual comparison, as reflected by a lack of significant difference between CIT- and vehicle-treated groups at any specific time point ($p>0.07$, t-test). These data suggest that mice treated with CIT at a clinically relevant dose have a modest reduction in forebrain 5-HT content.

Multiple mechanisms may exist to buffer brain 5-HT content and stabilize neurotransmission in the face of reduced 5-HT synthesis. One potential mechanism for buffering brain 5-HT content might be reduced intracellular degradation of 5-HT into 5-HIAA. To test whether SSRI administration affects 5-HT degradation, I quantified brain 5-HIAA content using HPLC-ED (Fig. 3A & B). Brain 5-HIAA content was reduced in CIT-treated mice at all time points, both with NSD-1015 administration ($p<0.0001$, ANOVA for treatment effect) (Fig. 3B) and without ($p<0.0001$, ANOVA) (Fig. 3A). The difference was

comparable after 2 days (without NSD-1015, 36% decrease, $p<0.0001$, t-test) and 2 weeks of treatment (without NSD-1015, 39% decrease, $p=0.007$) and was of lower magnitude after 4 weeks (22% decrease, $p=0.049$, t-test). The ratio of 5-HT to 5-HIAA has been considered to be an index of 5-HT turnover. I observed a significant effect of CIT administration on this ratio ($p<0.0001$, ANOVA), with a significant reduction following 2 days ($p<0.0001$, t-test) and 2 weeks ($p=0.044$, t-test) but not 4 weeks ($p=0.056$, t-test) of treatment.

To test the hypothesis that 5-HT reuptake inhibition would deplete brain 5-HT in a condition when 5-HT synthesis is seriously compromised, I examined forebrain 5-HT content in CIT- and vehicle-treated mice following acute injection of NSD-1015 (100 mg/kg intraperitoneal 30 minutes before sacrifice). This NSD-1015 treatment is thought to cause a near-complete inhibition of monoamine synthesis in mice(Carlsson and Lindqvist 1973). In this condition, chronic CIT administration caused a significant reduction in brain 5-HT content ($p<0.0001$, ANOVA) (Fig. 2B). This effect was not observed at 2 days ($p=0.12$) and was comparable at 2 weeks (18% decrease; $p<0.0001$, t-test) and 4 weeks of treatment (20% decrease; $p=0.004$, t-test).

Discussion

SSRI treatment is a critically important component of mental health care. Nevertheless, SSRIs have serious limitations. A complete understanding of the effects of SSRI administration on brain function, and particularly on serotonergic neurotransmission, is an important goal in biological psychiatry. In this study, I demonstrate that chronic treatment with a widely prescribed and highly selective SERT inhibitor(Katzung 2004; 2008) at a clinically relevant dose causes a significant suppression of 5-HT

synthesis in the mouse brain (Fig. 1B). This effect is most pronounced early in treatment but persists chronically and is likely to occur throughout the brain, as I detected this difference using a homogenate of the entire right forebrain.

Numerous investigators have reported that acute treatment SSRIs produces a suppression of 5-HT synthesis (Carlsson and Lindqvist 1978; Moret and Briley 1997; Barton CL 1999; Stenfors, Yu et al. 2001; Cervo, Canetta et al. 2005). Although several previous studies have addressed the issue of how chronic SSRI administration affects 5-HT synthesis, methodological considerations limit the interpretation of these results. Moret et al (1992) and Stenfors et al (2002) reported increased 5-HT synthesis rate with chronic treatment with citalopram and fluoxetine, respectively, whereas Esteban et al (1999) report no effect of chronic fluoxetine. For these studies, SSRIs were administered by repeated injection and administration was withheld following the chronic treatment and prior to determination of 5-HT synthesis rate in order to allow the SSRI to 'wash out'. It is notable, however, that SSRI washout can induce rapid changes in serotonergic physiology which are opposite to the effect of the same drug continuously administered (Anthony, Sexton et al. 2000; Jongsma 2006). Notably, Trouvin et al report reduced brain 5-HT and 5-HIAA content following chronic fluoxetine administration; however, this effect reverses rapidly with washout (1993). Therefore, I did not include a washout period in our study design.

In previous studies of chronic SSRIs and 5-HT synthesis, SSRIs were administered by repeated injection (Moret and Briley 1992; Esteban, Lladó et al. 1999; Stenfors and Ross 2002). This treatment regimen can lead to large daily fluctuations in plasma citalopram in rodents (Cremers, de Boer et al. 2000) which complicate the

interpretation of resulting effects. I used osmotic minipumps to deliver CIT at a constant rate, resulting in plasma CIT levels in the typical clinical range (Fig. 1A) (Kragh-Sorensen, Overo et al. 1981; Fredricson Overo 1982; Rasmussen and Brosen 2000; Brøsen and Naranjo 2001). Yamane et al used osmotic minipumps to administer chronic paroxetine without washout and reported reduced brain trapping of the radiotracer α-[^{14}C]-methyl-tryptophan, which is consistent with reduced 5-HT synthesis (Yamane, Okazawa et al. 2001). However, there is disagreement in the literature as to whether or not α-[^{14}C]-methyl-tryptophan trapping is a reliable measure of 5-HT synthesis (Shoaf, Carson et al. 1998; Benkelfat, Young et al. 1999; Shoaf, Carson et al. 2000). The decarboxylase inhibition assay used in the present study has the advantage that 5-HTP can be unambiguously identified and quantified by HPLC-ED; however, as a caveat, 5-HT synthesis is assessed while monoamine systems are significantly disrupted by inhibition of synthesis of multiple active neurotransmitters (Muck-Seler and Diksic 1995).

I observed a significant effect of time on 5-HT synthesis and other neurochemical measures in vehicle-treated mice. Although the design of our study does not allow us to interpret the specific causes for this effect, one may speculate that different levels of peri-operative stress may have affected serotonergic function, as 5-HT synthesis was particularly high in saline-treated mice 2 days after minipump implantation (Fig. 1B). SSRI administration affects neurochemical responses to stress (Kawahara, Kawahara et al. 2007) and thus stress and CIT administration may interact to affect these results.

How might these data regarding 5-HT synthesis inhibition be explained in terms of monoamine neurophysiology? SSRI administration causes an immediate increase in extracellular 5-HT (Bosker, Folgering et al. 2009) but not in intracellular 5-HT (which

constitutes the vast majority of brain 5-HT) (Fig. 2A). Carlsson and colleagues proposed that high extracellular 5-HT feeds back on serotonergic neurons to inhibit 5-HT synthesis (1976). The mechanism whereby this might occur remains unknown. Activation of the known 5-HT autoreceptors does not appear to be required for acute suppression of 5-HT synthesis by citalopram (Stenfors, Yu et al. 2001). Furthermore, the functional activation of autoreceptors is thought to dissipate over the course of SSRI treatment (Blier, Chaput et al. 1988; Hervas, Vilaro et al. 2001) whereas the effect on synthesis persists. Citalopram might also affect 5-HT synthesis by restricting absorption of tryptophan into the brain. This appears unlikely as many antidepressants have been reported to increase brain tryptophan by inhibiting metabolism of tryptophan in the liver (Badawy and Evans 1981; Badawy and Evans 1982; Badawy and Morgan 1991; Bano, Morgan et al. 1999). Studies on tryptophan hydroxylase enzyme function in CIT-treated mice could elucidate the mechanisms for the effect I observed.

What might be the functional consequences of chronic suppression of 5-HT synthesis? Neurotransmitter synthesis and its regulation are fundamental features of neurophysiology; however, the functional significance of physiologically relevant alterations in 5-HT synthesis (Azmitia and McEwen 1969; Boadle-Biber 1978) is not well understood. Prolonged suppression of 5-HT synthesis by SSRI treatment might deplete 5-HT brain stores, which could limit or otherwise affect SSRI response. I did not observe overt changes in total brain 5-HT content at any time point using our treatment regimen; however, when data from all time points were analyzed together using ANOVA, I detected a significant effect of treatment on brain 5-HT content, suggesting that there is a modest decrease in CIT-treated mice. Trouvin et al reported 20-50% decreases in total

5-HT tissue content in several brain regions following a 21-day treatment with fluoxetine(Trouvin, Chanut et al. 1992; Trouvin, Gardier et al. 1993). Dygalo et al (2006) and Caccia et al (Caccia, Fracasso et al. 1992; Caccia, Anelli et al. 1993) reported comparable results, although this effect was not observed in all brain regions examined. Marsteller also observed reduced tissue 5-HT following chronic treatment with CIT (Marsteller, Barbarich-Marsteller et al. 2007). This effect was, however, not observed in two studies of chronic fluoxetine administration (Hwang, Magnussen et al. 1980; Holt and Baker 1996). Taken together, these results suggest that SSRI treatment produces a modest, dose-dependant reduction in brain 5-HT content, with unknown functional consequences.

Multiple mechanisms may exist to buffer neurotransmitter stores and stabilize neurotransmission in the face of reduced synthesis. For example, glutamatergic synaptic transmission is remarkably resistant to manipulation of glutamate synthesis (Kam and Nicoll 2007). Under conditions of reuptake blockade, intracellular 5-HT might be buffered by a suppression of 5-HT degradation. In accord with this hypothesis, I observed a reduction of brain 5-HIAA content with CIT treatment, as has been observed previously (Hwang, Magnussen et al. 1980; Caccia, Fracasso et al. 1992; Trouvin, Chanut et al. 1992; Caccia, Anelli et al. 1993; Trouvin, Gardier et al. 1993; Kabuto, Yokoi et al. 1994; Holt and Baker 1996; Dygalo, Shishkina et al. 2006) (but see Kabuto, Yokoi et al. 1994; Kabuto, Yokoi et al. 1994; Marsteller, Barbarich-Marsteller et al. 2007). 5-HT degradation may be suppressed due to SSRI-induced inhibition of monoamine oxidases, as has been reported (Kabuto, Yokoi et al. 1994; Holt and Baker 1996)

Although I did not observe overt changes in 5-HT stores despite suppressed synthesis of 5-HT with CIT administration, I hypothesized that there might be conditions under which SSRI administration would deplete brain 5-HT. In fact, when I challenged mice by injecting NSD-1015 immediately prior to sacrifice, prolonged CIT treatment led to a depletion of brain 5-HT. In accord with this finding, SERT null mutant mice and rats have much more pronounced depletion of brain 5-HT content than wild-type animals in response to tryptophan depletion (Kim, Tolliver et al. 2005; Olivier, Jans et al. 2008); SERT inactivation also leads to exaggerated neurochemical and behavioral responses to drugs which enhance 5-HT synthesis (Buus Lassen 1978; Fox, Jensen et al. 2008); and tryptophan depletion has marked effects on extracellular 5-HT in SSRI-treated rats but not in control rats (Núria 1996; Jongsma 2006). Two potential mechanisms could explain these results. SSRI treatment and NSD-1015 administration could have independent inhibitory effects on 5-HT synthesis; co-administration could then lower synthesis below a critical threshold and lead synergistically to 5-HT depletion. However, this interpretation is not supported by data on the mouse SERT null mutation, which causes increased 5-HT synthesis at baseline which nevertheless accelerates NSD-1015-induced depletion of 5-HT stores (Kim, Tolliver et al. 2005). A more plausible hypothesis is suggested by the fact that serotonergic neurons have only two sources of 5-HT: synthesis from tryptophan and reuptake from the extracellular space. When reuptake is inhibited, as with SSRI administration, brain 5-HT content should be more dependant on 5-HT synthesis. It is not clear why this depletion effect would be observed with 12 or 28 days but not 2 days of treatment. At 2 days of CIT treatment as compared to 14 and 28 days, serotonergic neurons might have a greater reservoir of intracellular 5-HT, or a heightened tendency to

retain intracellular 5-HT due to the reduction in neuronal activity which dissipates upon chronic treatment.

These results suggest that SSRI administration might cause a form of "serotonergic vulnerability"(Jans, Riedel et al. 2007) whereby serotonergic neurotransmission becomes more sensitive to environmental or genetic factors which would inhibit 5-HT synthesis, such as an unbalanced diet (Agazzi, De Ponti et al. 2003; Lakhan and Vieira 2008). In fact, tryptophan depletion has pronounced effects on patients taking SSRIs (Delgado, Charney et al. 1990; Delgado, Price et al. 1991; Benkelfat, Ellenbogen et al. 1994; Delgado, Price et al. 1994; Leyton, Young et al. 1997; Moore, Gillin et al. 1998; Smith, Morris et al. 1999; Leyton, Ghadirian et al. 2000; Booij, Van der Does et al. 2002).

Taken together, these data suggest that pharmacological augmentation of 5-HT synthesis, for example with tryptophan loading, may be beneficial for the treatment of depression if administered in conjunction with a 5-HT reuptake inhibitor. In fact, this indication is supported by a limited number of clinical trials (Walinder, Skott et al. 1975; Walinder, Skott et al. 1976). These data are, however, not definitive (d'Elia, Hanson et al. 1978; Walinder 1981) and have not been followed up by a large double-blind clinical trial, perhaps for financial reasons as tryptophan is in the public domain and thus cannot be patented. Behavioral and physiological relevance of the suppression of 5-HT synthesis by CIT could be assessed in the mouse model by augmenting 5-HT synthesis in CIT-treated mice with tryptophan loading.

In conclusion, I demonstrate that chronic administration of an SSRI causes a sustained suppression of 5-HT synthesis rate in the mouse forebrain. This work

emphasizes the importance of understanding neurotransmitter synthesis in elucidating how SSRIs and serotonergic physiology operate to regulate behavior.

Materials and Methods

Animals & Surgery. All procedures involving mice were approved by the UCSF Institutional Animal Care and Use Committee. 6-week-old male C57BL/6J mice were obtained from Jackson Laboratories and housed under standard conditions (7 AM to 7 PM light; PicoLab 5053 diet ad lib; individually aerated cages; Specific Pathogen Free facility; 3 mice per cage). 2-3 weeks following shipment, mice underwent surgery between 1 and 4 PM. Mice were anesthetized with 2-4% isoflurane and osmotic minipumps (Alzet) were implanted as per manufacturer's instructions. Minipumps (Model 1002 for 2-day and 2-week treatment groups; Model 2004 for 4-week treatment groups) were filled with sterile 0.9% NaCl vehicle (Hospira) or 10% citalopram hydrobromide (kindly provided by Lundbeck A/S) dissolved in vehicle. Citalopram solution was briefly warmed to 37◦C to facilitate dissolution and was sterile filtered.

Sample collection and preparation. All sample preparation and analysis was performed blind to treatment group. Following the appropriate treatment period, mice were sacrificed by decapitation under brief isoflurane anesthesia between 1 and 4 PM. For one set of CIT- and SAL-treated mice, dissections were performed without additional treatment. For another set, mice were injected with saline 1 hour prior to sacrifice and with NSD-1015 (3-hydroxybenzylhydrazine dihydrochloride, Sigma, 54880). Trunk blood was collected in K2EDTA tubes (365974, BD) and spun at 1000 g for 10 minutes. Plasma supernatant was stored at -80◦C. Brains were rapidly dissected by removing the pineal gland,

olfactory bulb and cerebellum and sectioning coronally immediately caudal to the hypothalamus. Forebrains were hemisected saggitally. Brain samples were frozen immediately over powdered dry ice and stored at -80◦C. Right forebrain samples were then homogenized using a glass mortar and pestle in 600 µL cold 0.1 M perchloric acid (Sigma) and spun at 16000 g for 15 minutes at 4◦C. Supernatant was stored at -80◦C and analyzed without further dilution.

Analysis of plasma citalopram. Plasma citalopram was analyzed by HPLC-MS as follows. 3 HPLC pumps (Shimadzu LC10ADVp, Kyoto, Japan) were used, two for the mobile phase and one for postcolumn addition, working at a flow rate of 0.5 ml/min and 0.1 ml/min, respectively. Separation was performed on a reversed phase 150x4.6 mm Zorbax Eclipse XDB-C8 column (Agilent Technologies) with a Zorbax Eclipse XDB C8 guard column (Agilent). The mobile phase consisted of 50% water and 50% acetonitrile supplemented with 0.1% formic acid. 30 uL samples were injected using an autosampler (Shimadzu SIL-10, Kyoto, Japan). Citalopram was detected using an API4000 mass spectrometer consisting of a turbospray interface (Applied Biosystems). The acquisitions were performed in positive ionization mode with ion spray voltage set at 5.5 kV and probe temperature of 400°C. The instrument was operated in multi-reaction-monitoring (MRM) mode for detection of citalopram (precursor 325, product ion 262) and Lu-10-202 (precursor 341, product ion 278). Quantification was performed by the external standard method using the Analyst 1.4.2 data system (Applied Biosystems). Citalopram peak heights were normalized to peak heights of Lu-10-202 internal standard.

Analysis of brain 5-HT, 5-HIAA and 5-HTP. 5-HT, 5-HIAA and 5-HTP were analyzed by HPLC-ED as follows. Mobile phase consisted of 50 mM sodium acetate, 0.51 mM EDTA, 0.9 mM 1-Octanesulfonic acid sodium salt, and 14% methanol (pH 4.4) and was delivered at a flow rate of 1 mL/min using a Shimadzu Prominence pump. Analytes were separated using a reversed phase SupelcoSil LC-18-DB 58993 15cmX4.6mmX3um column (Supelco) heated to 30◦C. 50 µL samples were cooled to 12◦C in a refrigerated tray and injected using a Gilson 231 autosampler. Fresh standards (Sigma) were prepared in formic acid and run at beginning of run. Samples were interspersed with quality control brain sample replicates to monitor sensitivity, chromatography and sample degradation. Dihydroxybenzylamine hydrobromide (Sigma, 858781) was added to each sample and used as an internal standard. Analytes were detected using a two-electrode electrochemical cell (model 5011, ESA) with the reference electrode set at 50 mV and a working electrode set at 250 mV. A Coulochem II detector (ESA) was used to control the cell. Peak height was measured using EZChrom Elite software (Scientific Software).

Protein assays. A BCA microplate assay (Pierce 23225) was used for the determination of protein content in perchloric acid homogenates. Homogenates were allowed to equilibrate at room temperature. 12 µL of each sample was added to 200 µL BCA working reagent, prepared as per the manufacturer's instructions, in quadruplicate in a flat-bottomed 96-well plate (Nunc). BSA standards were prepared in 0.1 M perchloric acid. Plates were sealed with

adhesive plate covers (ABI) and well vortexed immediately, then incubated at 37◦C for 40 min. Plate covers were removed and absorbance at 562 nm was read on a SpectroMax 190 plate reader (Molecular Devices).

Statistical analysis. Data was exported from into a custom Microsoft Access database. For all peaks, peak height was normalized to the height of the internal standard peak and converted to molarity using the appropriate standard. For single factor comparisons of two groups, two-tailed Student's t-test with Welch's correction was applied. For multiple-factor comparison, ANOVA with Bonferroni post-test was applied.

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Figure 1

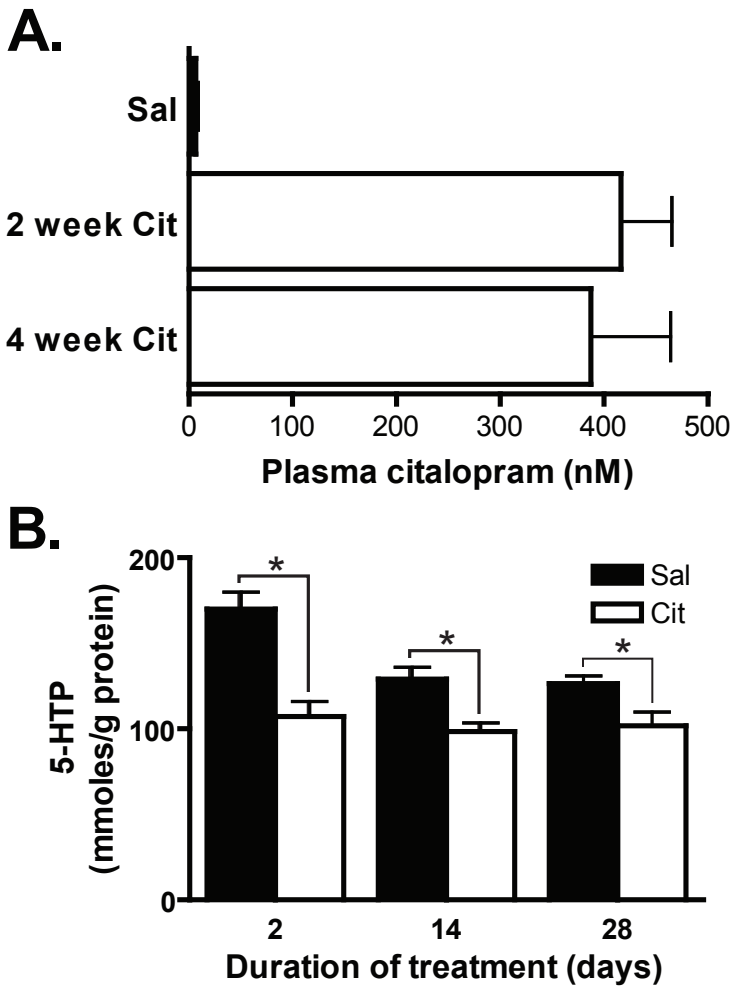

A.
Sal
2 week Cit
4 week Cit
0
100
200
300
400
500
Plasma citalopram (nM)
B.
200
100
0
5-HTP
(mmoles/g protein)
*
*
*
Sal
Cit
2
14
28
Duration of treatment (days)

Figure 1: Chronic citalopram treatment: Plasma drug concentrations and inhibitory effect on 5-HT synthesis.

A. Plasma levels of CIT were assessed by HPLC-MS in C57Bl6/J adult male mice following 2 or 4 weeks of citalopram or saline administration by osmotic minipump. As no significant amount of citalopram was found in mice implanted with vehicle-filled minipumps for 2 or 4 weeks, these groups were combined.

B. 5-HTP accumulation, an index of 5-HT synthesis, was quantified in the forebrains of mice following chronic CIT or saline administration by osmotic minipump (10% solution, 0.25 µL/hour) with acute injection of NSD-1015 (100 mg/kg I.P.) 30 minutes before sacrifice. 5-HTP accumulation was significantly suppressed at all 3 time points ($p<0.0001$, ANOVA with Bonferroni correction). *Significant effect by two-tailed t-test ($p<0.01$).

Figure 2

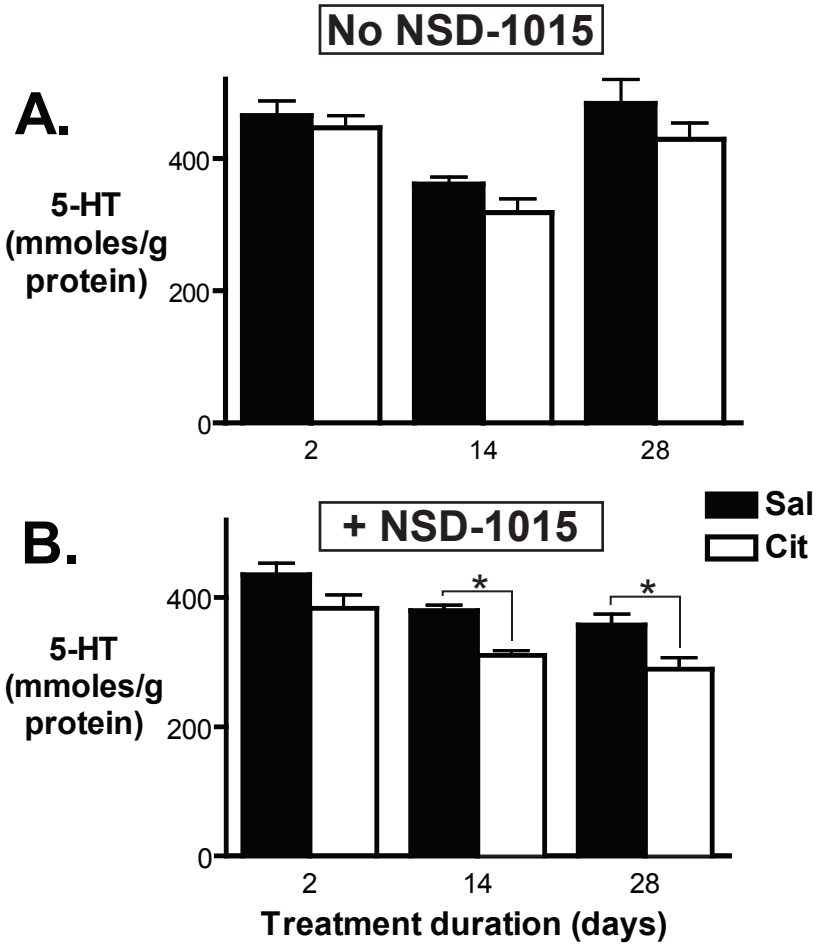

No NSD-1015
A.
5-HT (mmoles/g protein)
400
200
0
2
14
28
+ NSD-1015
B.
Sal
Cit
*
*
Treatment duration (days)

Figure 2: Effect of chronic citalopram treatment on forebrain 5-HT content

A. 5-HT forebrain content in mice treated chronically with CIT or vehicle. 5-HT content was not significantly affected by CIT treatment at any time point ($p>0.07$, t-test).

B. 5-HT forebrain content in mice treated chronically with CIT or vehicle and acutely with NSD-1015 (100 mg/kg IP) 30 minutes before sacrifice. Forebrain 5-HT content was reduced in mice treated with CIT for 2 weeks ($p<0.0001$, t-test) and 4 weeks ($p=0.004$) but not 2 days ($p=0.12$). *Significant effect by two-tailed t-test ($p<0.005$).

Figure 3

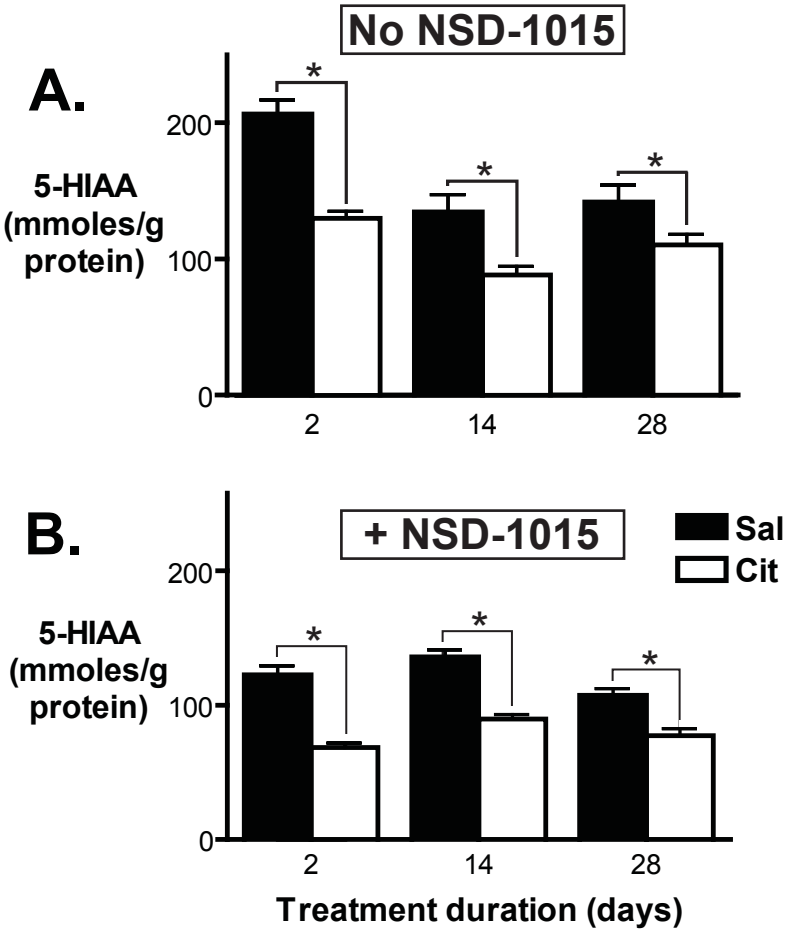

A.
No NSD-1015
5-HIAA (mmoles/g protein)
200
100
0
2
14
28
*
B.
+ NSD-1015
Sal
Cit
5-HIAA (mmoles/g protein)
200
100
0
2
14
28
Treatment duration (days)

Figure 3: Reduced forebrain 5-HIAA and 5-HIAA/5-HT ratio in mice treated with CIT.

5-HIAA forebrain content was significantly lower in mice treated chronically with CIT both with acute NSD-1015 injection ($p<0.0001$, ANOVA) (B) and without NSD-1015 ($p<0.0001$) (A). 5-HIAA/5-HT ratio was also reduced in CIT-treated mice following 2 days ($p<0.0001$, t-test) and 2 ($p=0.044$) weeks but not 4 weeks ($p=0.056$) of treatment (C). *Significant effect by two-tailed t-test ($p<0.05$).

Conclusion

Pharmacological inhibition of neurotransmitter reuptake is an ancient and widespread practice. The coca leaf has been consumed in South America for thousands of years(Hastorf 1987) to treat altitude sickness(Knuepfer 2003) and as a sacrament(Martin 1970). Its primary psychoactive compound, cocaine, has its highest pharmacological affinity as SERT blocker(Ritz, Cone et al. 1990; 2008). Today, more selective 5-HT reuptake inhibitors are widely by millions of adults and children for the treatment of psychiatric disorders(Kramer 1997; Katzung 2004).

SSRIs are now the first-line treatment for depression, a devastating illness which is a leading cause of disability worldwide, affecting approximately 120 million people(2008). Unfortunately, fewer than 25% of those affected by this disorder have access to effective treatments(2008). The availability of SSRIs in the developed world has been one critical factor in improving treatment of depression and other psychiatric disorders, due to the efficacy and safety of these medications. However, SSRIs are sub-optimal. Although many patients may respond to one SSRI and not another, it is not possible to predict which medication would be best for a given patient. Furthermore, many cases of depression are refractory to pharmacological treatment. In many other patients, SSRIs produce a relatively modest alleviation of symptoms(Kirsch, Deacon et al. 2008). Although SSRIs have advantages over older antidepressant drug formulations, the efficacy of SSRIs is comparable to that of monoamine oxidase inhibitors and tricyclic antidepressants.

SSRIs have remarkably complex effects on many features of neurophysiology and behavior. The first cause of these effects is thought to be potentiation of serotonergic neurotransmission. Therefore, a complete understanding of how SSRIs affect the physiology of serotonergic neurotransmission would be an invaluable asset for biological psychiatry. Such knowledge would also provide basic insight into the fascinating physiology of the serotonergic system, an evolutionarily ancient component of the nervous system. In this work I describe two novel physiological effects of SSRI adminstration, both of which have the potential to influence therapeutic response to SSRIs and both of which suggest pharmacological strategies for augmenting the therapeutic effects of SSRIs. These results suggest new avenues for research on how antidepressant drugs affect neurophysiology; how their effects may be related to therapeutic efficacy; and how this efficacy might be improved.

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Appendix A:

A general approach for high-throughput cost-effective mouse genotyping using melting curve analysis

Addressing key unresolved questions in biology requires the development of increasingly complex genetic manipulations. Accurate and rapid genotyping of mutant organisms, particularly mice, is a critically important task which consumes significant labor, resources and time in many laboratories. In order to improve the efficiency of mouse genotyping in our laboratory, we have assembled and adapted a set of techniques commonly used for human genetics studies humans (Ririe, Rasmussen et al. 1997; Germer and Higuchi 1999; Akey, Sosnoski et al. 2001; Lipsky, Mazzanti et al. 2001; Hladnik, Braida et al. 2002; Papp, Pinsonneault et al. 2003; Wang, Chuang et al. 2005).into a comprehensive approach for mouse genotyping. Ultra-low-volume, small-product multiplex PCR reactions are performed using an optical cycler in the presence of SYBR dye, which fluoresces in the presence of double-stranded DNA. At the end of the amplification reaction, PCR products corresponding to specific alleles are detected by progressively heating the reaction and plotting the derivative of SYBR fluorescence; annealing and melting of a specific product generates a peak at a specific melting temperature. The PCR reaction well is never opened and no gels are required. Melting curve peaks can be manipulated for specific purposes using simple, inexpensive primer modification, such that this approach can readily address almost any genotyping application.

Our system offers the following key advantages:

- *Throughput*. Using our protocol, one person can set up hundreds or thousands of genotyping reactions in an afternoon with relatively little manual labor, and with a single 96-well optical cycler can determine 96 genotypes in one 2 hour run. With an increasing number of reactions and higher capacity (384 well) instrumentation, the time savings increase dramatically.
- *Lower cost*. The high sensitivity of this method allows for very small volume reactions. The reduced need for human labor and the elimination of agarose gels also represent major savings. No expensive custom reagents are required. We have reduced our expenditures for genotyping materials alone from $0.72 per genotyping reaction to $0.25 per reaction.
- *Universal and robust PCR conditions*. Short PCR products ensure robust amplification with a simple, universal thermal cycling protocol enabling concurrent execution of multiple assays.
- *General applicability and simplicity*. Simple design criteria allow assays to be developed for almost any genotyping need
- *Reduction of post-PCR contamination*. PCR reaction wells are never opened post-PCR, dramatically reducing nefarious post-PCR contamination.
- *Reduction of environmental waste*. The micro-scaled reactions and elimination of agarose gels reduce the generation of waste and notably eliminate the use of carcinogenic ethidium bromide.

- *Opportunity for automation*. All DNA preparations and reactions are performed in strip tubes or multiwell plates, allowing the use of multichannel pipettes or robotic systems. Uniform reaction conditions simplify automation of plate setup.
- *Animal welfare*. Low-volume reactions require little template DNA, which can be obtained from very small, minimally invasive biopsies.
- *Rapid setup*. No custom reagents are required other than standard oligonucleotide primers. An optical cycler is the only specialized equipment required.

Our protocol presents a simple step-by-step process to apply this combination of proven methods for any common mouse genotyping application and includes a number of validated assays for commonly used mouse transgenes (Table 2) (Soriano 1999; Novak, Guo et al. 2000; Gu, Dubauskaite et al. 2002; Holland, Hale et al. 2002; Tumbar, Guasch et al. 2004; Yu, Power et al. 2004).

Procedure

Template DNA preparation

To minimize labor and reagent consumption, small-volume crude DNA preparations can be prepared for use as PCR templates. Small biopsies (e.g. ear punches) are collected in 0.2 mL strip tubes. To tubes is added: 50 uL of DirectPCR Lysis Reagent (402-E, Viagen Biotech) diluted 1:2 in PCR-grade water; and 2 µL (0.05 U) proteinase K solution (03115887001, Roche). The tubes are rotated overnight at 55◦C, heated to 80◦C for 30 minutes and stored at -20 or 4 ◦C. 0.15 µL is used as template for one PCR reaction.

Locus sequencing

The first step in designing a genotyping assay is to choose a specific polymorphic locus. For transgenic mice, constructs usually consist of a cDNA cassette, for example encoding a reporter or recombinase, ligated to regulatory DNA elements designed to direct regulated expression of the cDNA. The resulting transgene is introduced into the genome at a random insertion site. Since this site is usually unmapped, it is difficult (and usually not advantageous) to distinguish hemizygotic transgenic mice from homozygous transgenic mice. Therefore, an appropriate multiplex assay should consist of a transgene-specific primer set and a PCR control primer set which amplifies at an unrelated genomic locus and allows failed reactions to be distinguished from a wild-type genotype result.

For genotyping mice generated using a randomly inserted transgene, one may choose PCR primers which amplify a region contained within a cDNA cassette. Such assays ('generic') can be used to genotype mice from any transgenic line for which the transgene contained that cassette. We describe in Table 1 several such assays for *cre*, *ER-Cre*, *GFP* and *tTA*. While generic assays may be convenient in some instances (for example when a mouse line has recently been imported), assays which are as allele-specific as possible are generally preferable. To design such assays, one should choose to amplify over a point of junction between two cassettes within the transgene (for example, between the 3' end of a cell type-specific promoter and the 5' end of a cDNA).

In the case of targeted mutations, it is desirable to distinguish homozygous wild-type mice, heterozygous mice and homozygous wild-type mice. Therefore, one should design a multiplex assay which allows for simultaneous detection of two distinct PCR products, one for each allele. This may be accomplished using a 3-primer design, in which one primer is common to both the transgenic and wild-type-product; another primer primes only at the wild-type allele, generating one specific product with a characteristic Tm; and a third primer primes only the transgenic allele. For this design, the point of junction between a transgene cassette and the endogenous genomic DNA is therefore an ideal locus for assay design. Alternatively, one may employ a 4-primer design, where one primer set amplifies only the wild-type allele (for example, within a region which is ablated in the mutant allele) and another primer set amplifies only the transgenic allele (for example, within an inserted cassette).

Given the small size of the eventual PCR products, accurate sequence information is critical. Published papers often provide only general information on transgene sequence. Fortunately, genomic sequence is now readily available from public databases (e.g., http://www.genome.ucsc.edu) and recent technological advances have facilitated rapid, accurate and inexpensive sequencing. For high-throughput sequencing without gel separation of PCR product, the following protocol may be employed. Several sets of PCR primers are designed flanking a region of interest. The predicted resulting PCR product should be 500-1000 base pairs (bp) in size. A standard 10 µL PCR reaction is performed using transgenic template DNA in a strip tube or plate compatible with

Applied Biosystems optical instruments (e.g., ABI 4316567). To 5 µL of the resulting PCR product is added 2 µL of ExoSAP-IT (78250, USB), a cocktail of exonuclease I and shrimp alkaline phosphatase, and 0.7 µL of SYBR Green I Nucleic Acid Stain (S-7563, Invitrogen) diluted 1:1000 in DMSO. The reaction is incubated at 37○ C for 15 min to allow the enzymes to digest primers and nucleotides which interfere with sequencing, then at 80○ C for 15 min to heat-inactivate the enzymes. The resulting reaction is run on an ABI 7300 or 7900 optical cycler using an Absolute Quantification program with the following modified dissociation stage: 95°C for 1 min; 60°C for 2min; 95°C for 15sec. SYBR I should be added as a detector with no passive reference. If the PCR reaction generated a single product, the melting curve (a.k.a. dissociation curve, the derivative of the normalized SYBR I fluorescence) should exhibit a single major peak. In this case the reaction can then be sequenced without further purification. If multiple peaks are visible in the melting curve, the desired PCR product should be purified by gel electrophoresis or an alternative PCR design should be used.

Assay design: Primer sets

Once accurate sequence information has been obtained, several primer sets should be designed which amplify the region of interest. Primers may be designed using Primer 3 or a similar software program, using the following criteria: product size, 50-150 bp (preferably 50-100 bp, although products larger than 150 bp may work well); primer size, 18-27 bp (18 bp optimal); primer Tm, 58-62◦ C (60◦ C optimal); primer GC content, 30-80%; checked against rodent

mispriming/repeat library. PCR conditions should be as follows: PCRs are performed in ABI-compatible optical strip tubes or plates. Master mix (5 µL per reaction) is prepared as follows: SYBR GreenER 2X PCR mix, 2.5 µL per reaction; 200 nM of each primer; up to 5 µL with PCR-grade water. Master mix is aliquoted and 0.15 µL genomic template DNA is added to each well. Cycling is performed on an ABI 7300 or 7900 instrument (or similar optical cycler) with SYBR detector and ROX passive reference; thermal parameters are as follows: 95◦C for 10 min; 95◦C for 15 s followed by 60◦C for 1 min (40 cycles) (data is collected for amplification plot during 60◦C step); 95◦C for 15 s; 60◦C for 15 s; followed by a 2% ramp rate up to 95◦C (data is collected for dissociation curve during this step). PCR reactions can be set up rapidly through the use of plate layout spreadsheets and single- and multi-channel repeater pipettes or pipetting robots.

For transgenic mouse lines, a primer set which specifically amplifies the transgene is required. For targeted mutations, two primer sets are required, one for the mutant allele and one for the wild-type allele. (If a 3-primer design is used, one primer will be common to both primer sets.) Figure 1A depicts an amplification plot for a primer set which amplifies a short fragment spanning the junction point between *cre* coding region and a regulatory region for the *pdx1* gene. The amplification plot should exhibit the expected exponential and robust amplification, with a threshold cycle value between 17 and 25. In the final cycles of the reaction, fluorescence should reach a plateau, indicating that reagents (such as nucleotides) have become limiting; this ensures that different primer

sets will generate approximately equal amounts of product. The corresponding melting curve should contain only one significant peak; if multiple peaks are present, this may indicate that primer-dimer products are forming and that an alternative primer set should be chosen. As oligonucleotide primers are relatively inexpensive, it is advisable to order several sets of primers for each allele, as certain primer sets will not amplify effectively and as availability of multiple primer sets may simplify the design of multiplex assays.

In some cases, a given primer set will generate robust amplification but will not result in a sharp peak in the melting curve. Figure 3B depicts such a situation. This primer set is specific for targeted mutations at the *rosa26* locus generated using a standard targeting vector(Soriano 1999). Peak sharpness in the melting curve is a function of the dynamics of annealing and melting of a specific PCR product. We have found that addition of GC 'clamp' tail sequences to the 5' end of primers can improve the sharpness of peaks, probably by increasing the bonding energy between the two single-stranded complementary DNA molecules. In the case of the *rosa26* mutant allele assay, addition of 10 GC repeats to the 5’ end of the forward primer resulted in a sharper, more well-defined peak. GC tails will also increase the Tm of the product, as more energy is required to melt it; this shift is highly useful for the design of multiplex assays (see below).

A number of useful validated primer sets are presented in Table 1.

Multiplex assay development

In order for primer sets to be effectively combined in a multiplex assay, several conditions should be met. First, the Tms of the products should be sufficiently distinct (over 2◦C difference). Second, the PCRs resulting from each primer set should be of comparable efficiency; this may be estimated by the Ct value (the Ct values for uniplex assays with each primer set should not differ by more than 4 cycles).

For randomly inserted transgenes, a multiplex assay should consist of a transgene-specific primer set and a PCR control primer set. Once a transgene-specific primer set has been designed, choice of a PCR control primer set is relatively straightforward because any genomic locus can be chosen for amplification. In Table 1, we list a number of validated PCR control assays, with a range of Tms and C values. For the assay presented in Figure 1, the transgene-specific primer set generated a product with a Tm of 79.5◦C (Fig. 1B). Therefore, a PCR control primer set which generates a product with a Tm of 82◦C was chosen (*gdf*) (Fig. 1D). A multiplex reaction performed on transgenic DNA yields a dissociation curve with two distinct peaks (Fig 1F). This multiplex assay readily discriminates between a wild-type DNA sample (Fig. 2B) and a transgenic sample (Fig. 2D).

For targeted mutations, design of multiplex assays is more involved. Since two specific alleles must be genotyped, situations may arise where the wild-type and transgenic sequences chosen for amplification generate amplicons with overlapping Tms. In such cases it is advantageous to shift the Tm of one product with the addition of GC tails. For example, in an attempt to design an

assay for targeted mutations at the *rosa26* locus, a 3-primer design was employed. Two primer sets were generated, with a shared forward primer; one set was specific for the transgenic allele (Fig. 1B) and one for the wild-type allele (Fig. 1A), both with a product Tm of 81.6◦C. Addition of 10 GC repeats to the forward primer shifts the Tm of the wild-type product to 83.8◦C. Addition of 10 GC repeats to the forward primer and 20 GC repeats to the reverse primer shifts product Tm to 86.6◦C. The addition of the 10 GC tail to the forward primer had the additional benefit of sharpening the transgenic product peak; the Tm of this product was shifted to 83.3◦C, which still allowed a multiplex assay to be designed using the reverse transgene-specific primer with 20 GC repeats. This multiplex assay correctly discriminated homozygous wild-type samples (Fig. 4B), heterozygote samples (Fig. 4D) and homozygous mutant samples (Fig. 4F).

In some cases, it may be necessary to adjust the concentrations of primers in a multiplex reaction, for example to compensate for large copy number for randomly inserted transgenes or for particularly efficient primer sets, to avoid one PCR product from predominating and consuming the majority of the reagents in the PCR reaction. This may also be advisable when one primer set amplifies particularly efficiently, for example for the *gdf* PCR control primers. For such primers, primers at 30 nM concentration are sufficient to amplify a product which can be detected on a melting curve.

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Table 1: Validated primer sets

Primer set name	Target	Target locus	Ref.	Primer 1 name	Primer 1 sequence	Primer 2 name	Primer 2 sequence	Product Tm
Cre	Any transgenic line containing cre	cre (internal)		CRE_F	acatttgggccagctaaacat	CRE_R	cggcatcaacgttttctttt	79
EGFP_1	Any transgenic line containing gfp	gfp (internal)		EGFP_5_F	ACCACATGAAGCAGCACGAC	EGFP_5_R	CGTCGTCCTTGAAGAAGATGG	81
CreER-TM	Any transgenic line containing CreER	CreER (internal)		Cre_4_F	ggatagtgaaacaggggcaat	ERTM_1_R	TCTCCTGAAGCACCCATTTC	81
tTA_1	Any transgenic line containing tTA	tTA (internal)		tTA_1_F	AAAAATAAGCGGGCTTTGCT	tTA_1_R	CCCCTTCTAAAGGGCAAAAG	77.5
tTA_2	Any transgenic line containing rtTA	tTA (internal)		tTA_2_F	ACAGCGCATTAGAGCTGCTT	tTA_2_R	GGCGAGTTTACGGGTTGTTA	76
Gdf	PCR control	gdf		Gdf_F	AGCAGCAGGCAGGGCTTT	Gdf_R	GTCTGGACACGGGAGCACTT	85
K17	PCR control	k17		K17_F	ggcgagagcagagtgtggat	K17_R	aagtcggcaggcacaggag	79
TEAP	PCR control	teap		TEAPrt-s	aaatggtctccgtcgccag	TEAPrt-as	cccagccactgtgcttcat	78
Zeg	Any transgenic line generated using the pCAGG construct (Z/EG, Z/AP, Z/ED)		Novak et al, 2000	Zeg6670_1_F	tcgatgcaggataacttcgt	Zeg6767_F	ggtaccgtcgactgcagaat	77.5
H2B-GFP	Fusion between histone 2B subunit and GFP	H2Bgfp	Tumbar et al, 2004	H2B_1_F	cgtgtccgagggtactaagg	5'EGFP_4_R	AACAGCTCCTCGCCCTTG	83
PdxA	tTA targeted to the pdx1 locus	pdx1	Holland et al. 2002	pdx_5'gen_1_F	CTCTGGTTCCCCAGGAGAG	Xen-bglob_2_R	CTGCCAAAGTTGAGCGTTTA	80.5
PdxF	tTA targeted to the pdx1 locus	pdx1	Holland et al. 2002	pdx_5'gen_1_F	CTCTGGTTCCCCAGGAGAG	5-pdxTTA_3_R	TGCAAAAAGAACAAGCAAGC	79
PdxE	tTA targeted to the pdx1 locus	pdx1	Holland et al. 2002	pdx_5'gen_1_F	CTCTGGTTCCCCAGGAGAG	5-pdxTTA_5_R	CGTTTATTCTGAGCTTCTGCAA	79
PdxB	Wild-type pdx1	pdx1	Holland et al. 2002	pdx_ex2_F	GAGCTGGCAGTGATGTTGAA	pdx_ex2_R	CACTTCATGCGACGGTTT	78
KirA	kir2.1-lacZ fusion transgene	kir2.1	Yu et al, 2004	TKITLZ_F	atcctgcggatcgagttaat	Kir2.1_R	gatgctgtagcggttggttc	79
RosaWT_B	Wild-type rosa26 locus	rosa26	Soriano et al, 1999	RosaWT_2_F	GAGAATCCCTTCCCCCTCTT	RosaWT_2_R	TAAGCCTGCCCAGAAGACTC	80
RosaWT_C	Wild-type rosa26 locus	rosa26	Soriano et al, 1999	RosaWT_2_F	GAGAATCCCTTCCCCCTCTT	RosaWt_R	CACACCAGGTTAGCCTTTAAGC	80
RosaWT_A	Wild-type rosa26 locus	rosa26	Soriano et al, 1999	Rosa_F	GTGATCTGCAACTCCAGTCTTTC	RosaWt_R	CACACCAGGTTAGCCTTTAAGC	79
RosaWT_A_30GC	Wild-type rosa26 locus	rosa26	Soriano et al, 1999	10GC_Rosa_F	GCGCGCGCGCGTGATCTGCAACTCCAGTCTTTC	20GC_RosaWt_R	GCGCGCGCGCGCGCGCGCGCCACACCAGGTTAGCCTTTAAGC	86.6
RosaTG_A	Targeted rosa26 locus	rosa26	Soriano et al, 1999	Rosa_F	GTGATCTGCAACTCCAGTCTTTC	RosaLacZ_R	GACAGGATAAGTATGACATCATCAAGG	80.5
RosaTG_A_10GC	Targeted rosa26 locus	rosa26	Soriano et al, 1999	10GC_Rosa_F	GCGCGCGCGCGTGATCTGCAACTCCAGTCTTTC	RosaLacZ_R	GACAGGATAAGTATGACATCATCAAGG	83.3
Pdx1-Cre_A	cre fused to 5' regulatory region of pdx1 gene	pdx1-cre	Gu et al, 2002	pdx_pr_1_F	TAAGGCCTGGCTTGTAGCTC	Cre_3_R	accggtaatgcaggcaaat	80

Table 2: Validated multiplex assays

Assay name	Target locus	Primer set 1	Concentration (nM)	Primer set 2	Concentration (nM)
Z/EG	Any transgenic line generated using the pCAGG construct (Z/EG, Z/AP, Z/ED)	ZegB	400	Gdf	50
H2B-GFP	Fusion between histone 2B subunit and GFP	H2B-GfpA	200	K1	200
Kir	kir2.1-lacZ fusion transgene	KirA	200	Gdf	50
Cre	Any transgenic line containing cre	Cre	200	K17	200
CreER-TM	Any transgenic line containing CreER	CreER_A	200	K1	200
CreER-TM_B	Any transgenic line containing CreER	CreER_A	200	k17	200
PdxCre	cre fused to 5' regulatory region of pdx1 gene	PdxCre_A	200	Gdf	30
Rosa-tTA	tTA	tTA_B	200	RosaWT_C	200
Rosa26	Targeted rosa26 locus	RosaWT_A_30GC	200	RosaTG_A_10GC	200

Figure 1: Allele-specific PCR with discrimination of products by Tm

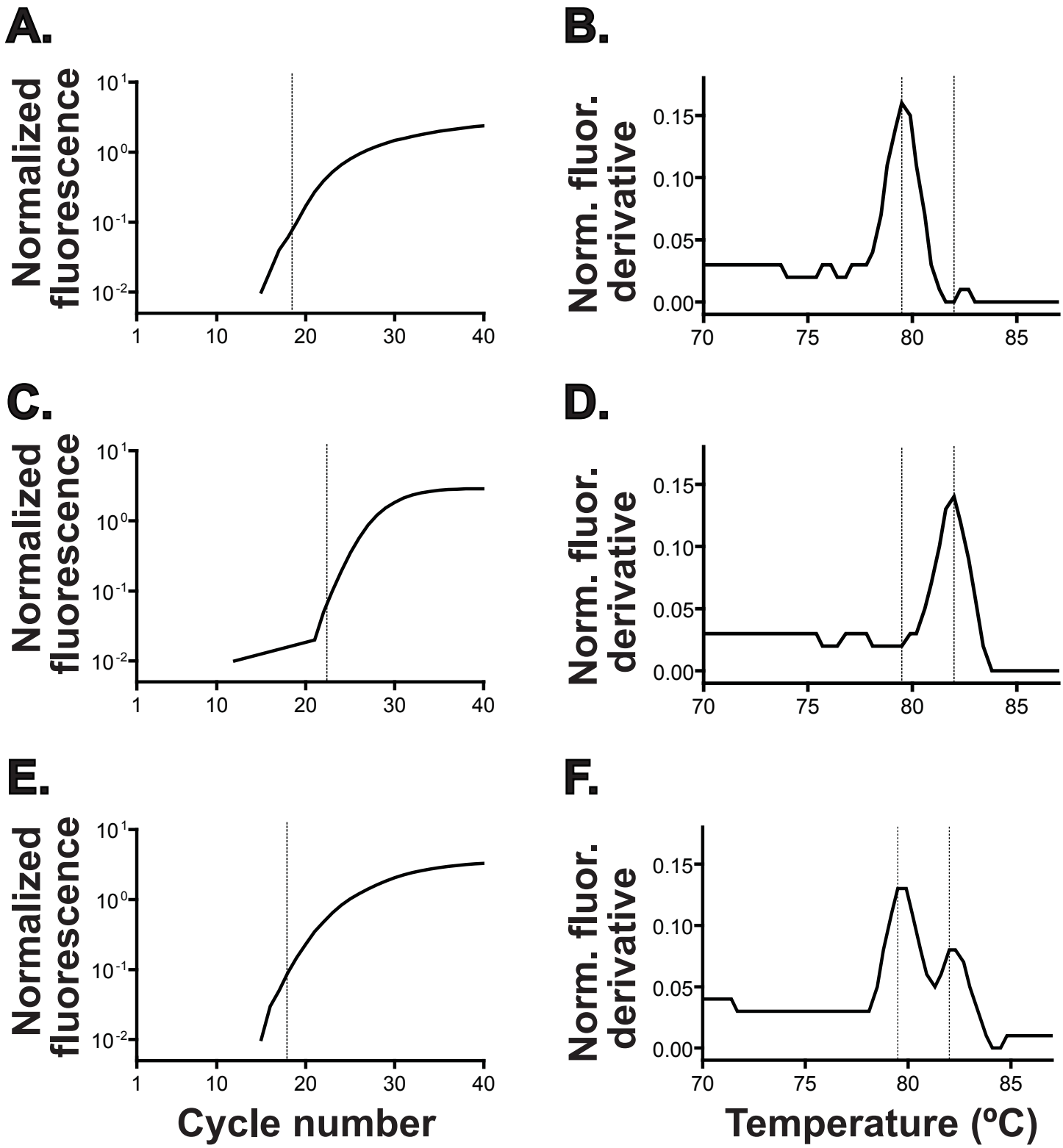


Figure 1: Allele-specific PCR with discrimination of products by Tm

Amplification plots (A, C, E) and melting curves (B, D, F). Normalized fluorescence is the ratio of SYBR signal to passive reference (ROX) signal minus baseline ratio. Genomic DNA template was from a *PdxCre*$^{Cre/0}$ hemizygote mouse for all reactions.

A, B: PCR with primers (PdxCre_F and PdxCre_R) specific for the *pdx-cre* transgene. Note product melting at 79.5◦C (B).

C, D: PCR with primers (Gdf_F and Gdf_R) specific for a genomic control locus. Note product melting at 82◦C (D).

E, F: Multiplex PCR with both primer sets (PdxCre_F, PdxCre_R, Gdf_F and Gdf_R). Note two peaks in melting curve: at 79.5◦C (*pdx-cre* product) and 82◦C (*gdf* product) (F).

Figure 2: Genotyping random-insertion transgenic mice: Multiplex PCR with identification of transgene-specific and a PCR control products by Tm

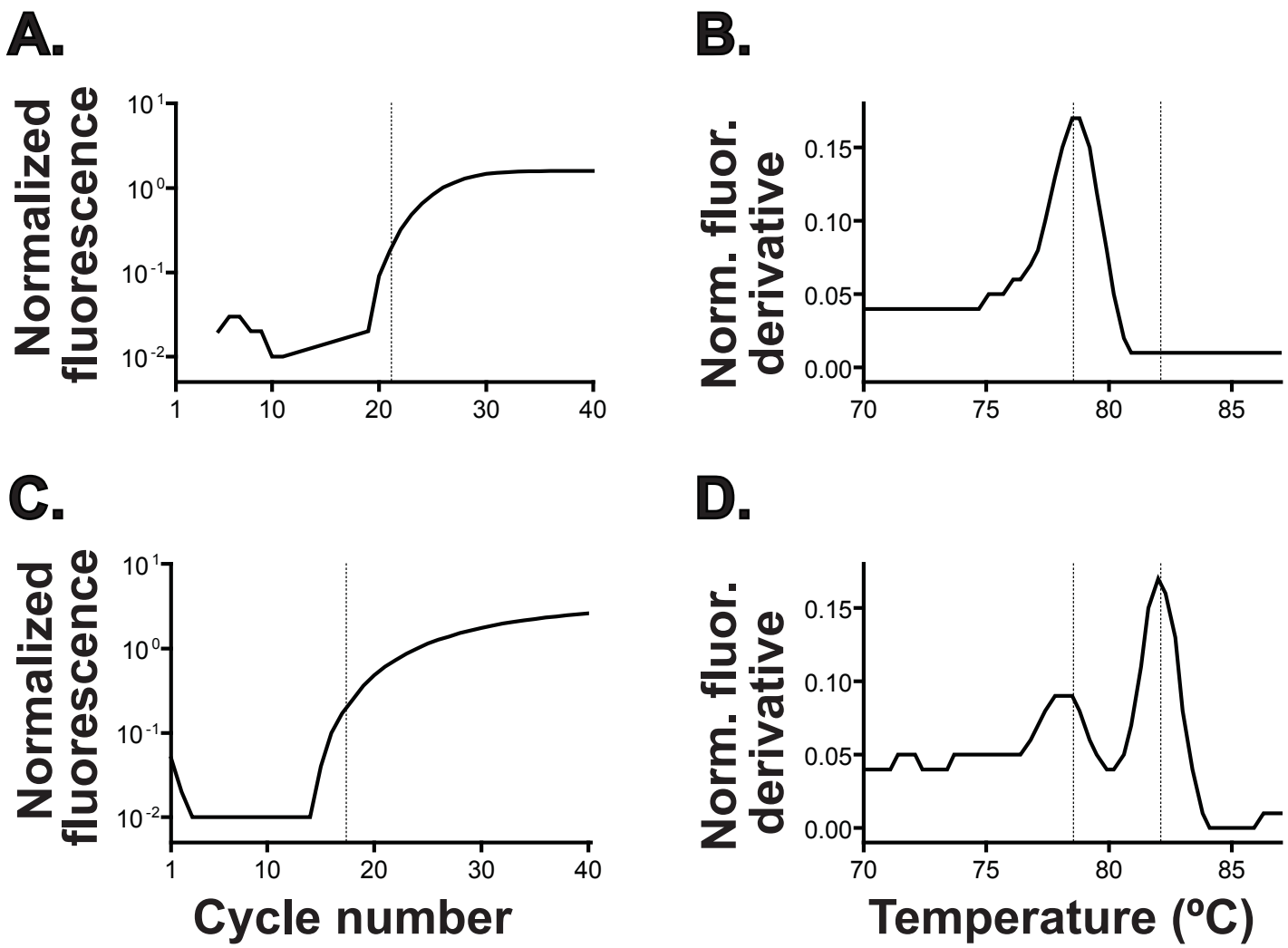


Figure 2: Genotyping random-insertion transgenic mice: Multiplex PCR with identification of transgene-specific and genomic control products by Tm

Amplification plots (A, C) and melting curves (B, D) for multiplex PCR reactions containing primers specific for the *cre* recombinase transgene (Cre_F, Cre_R) and for the *k17* genomic control locus (K17_F and K17_R).

A, B: Identification of a wild-type mouse. Note single peak in melting curve at 78.4◦C (B).

C, D: Identification of a *PdxCre*$^{Cre/0}$ hemizygote mouse. Note two peaks in melting curve: at 78.4◦C (*k17* control product) and 82.4◦C (*cre* product) (D).

Figure 3: Genotyping mice with targeted mutations: Shifting product Tm and sharpening melting peaks with GC-tails

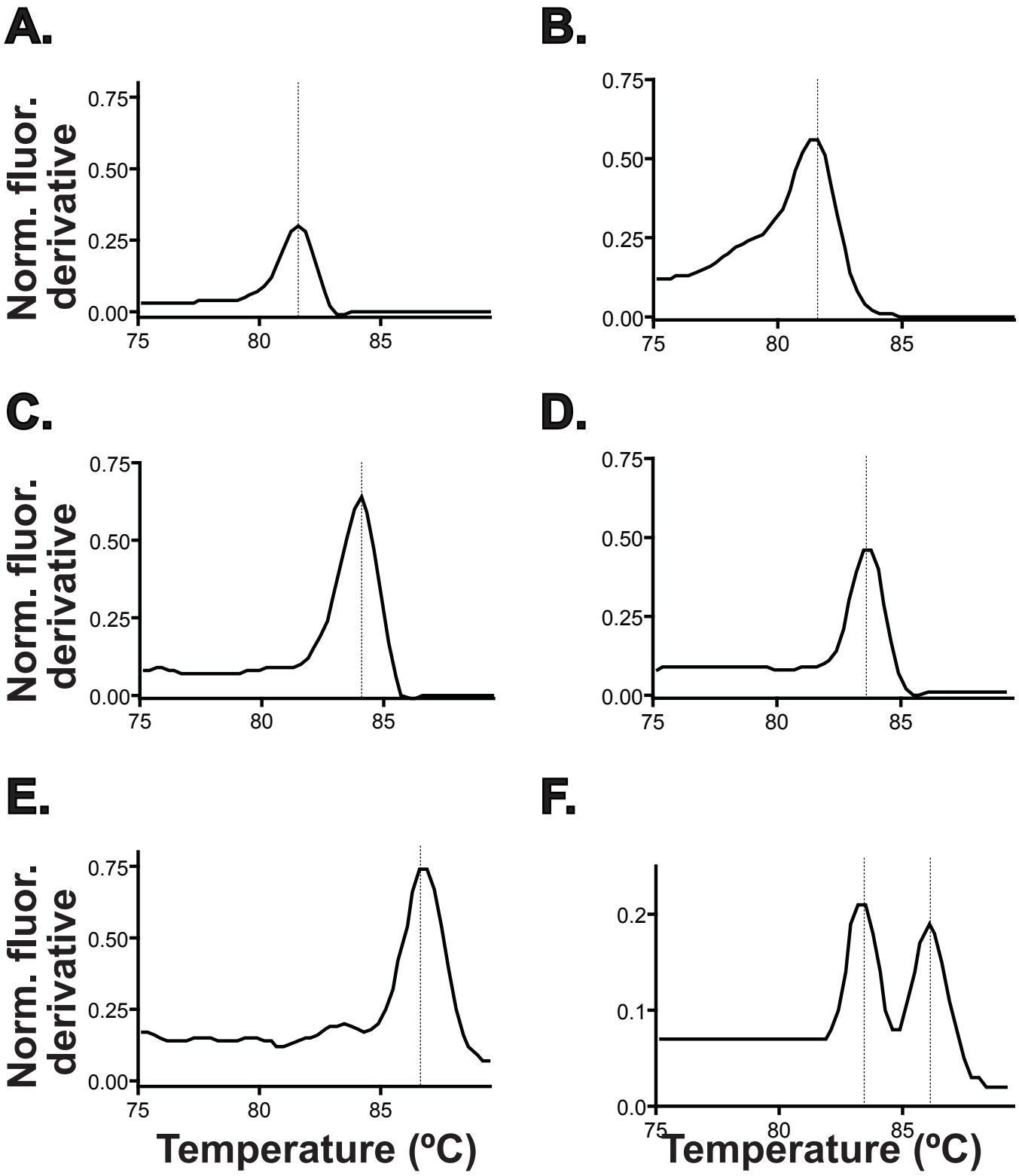


Figure 3: Genotyping mice with targeted mutations: Shifting product Tm and sharpening melting peaks with GC-tails

Melting curves are shown. For reactions shown in A, C, E and F, genomic template DNA from a *rosa26*$^{Ptx/0}$ heterozygote mouse was used. For reactions shown in B and D, template DNA from a *rosa26*$^{Ptx/Ptx}$ homozygote mouse was used.

A, B: In order to identify homozygote, heterozygote and wild-type mice, a multiplex PCR assay is required in which two distinguishable products are generated, one for mutant alleles and one for the wild-type allele. As an initial step in designing an assay for the rosa26 locus, a 3-primer design was used. Primers Rosa_F and RosaWT_R amplify the wild-type allele (A). Primers Rosa_F and RosaTG_R amplify mutant alleles generated using a standard targeting vector (Soriano 1999) (B). Note 2 issues: Products have the same Tm (81.6◦C), rendering discrimination impossible; and transgenic product (B) has broad melting peak.

C, E: Shifting Tm of the wild-type product through the addition of GC repeat sequences to the 5' end of primers. C: Addition of 10 GC repeats to forward primer shifts product Tm to 83.8◦C (10GC_Rosa_F and RosaWT_R primers). E: Addition of 10 GC repeats to forward primer and 20 GC repeats to reverse primer shifts product Tm to 86.6◦C (10GC_Rosa_F and 20GC_RosaWT_R primers). Note increased amplitude of melting peak.

D: Improving melting peak detection. Addition of 10 GC repeats to 5' end of forward primer sharpens melting peak and shifts Tm to 83.3◦C.

E: 3-primer multiplex assay using 10GC_Rosa_F, 20GC_RosaWT_R and RosaTG_R primers. Note 2 distinct peaks at 83.3◦C (mutant product) and 86.6◦C (wild-type product).

Figure 4: Genotyping mice with targeted mutations: Multiplex PCR with simultaneous identification of mutant and wild-type alleles by product Tm

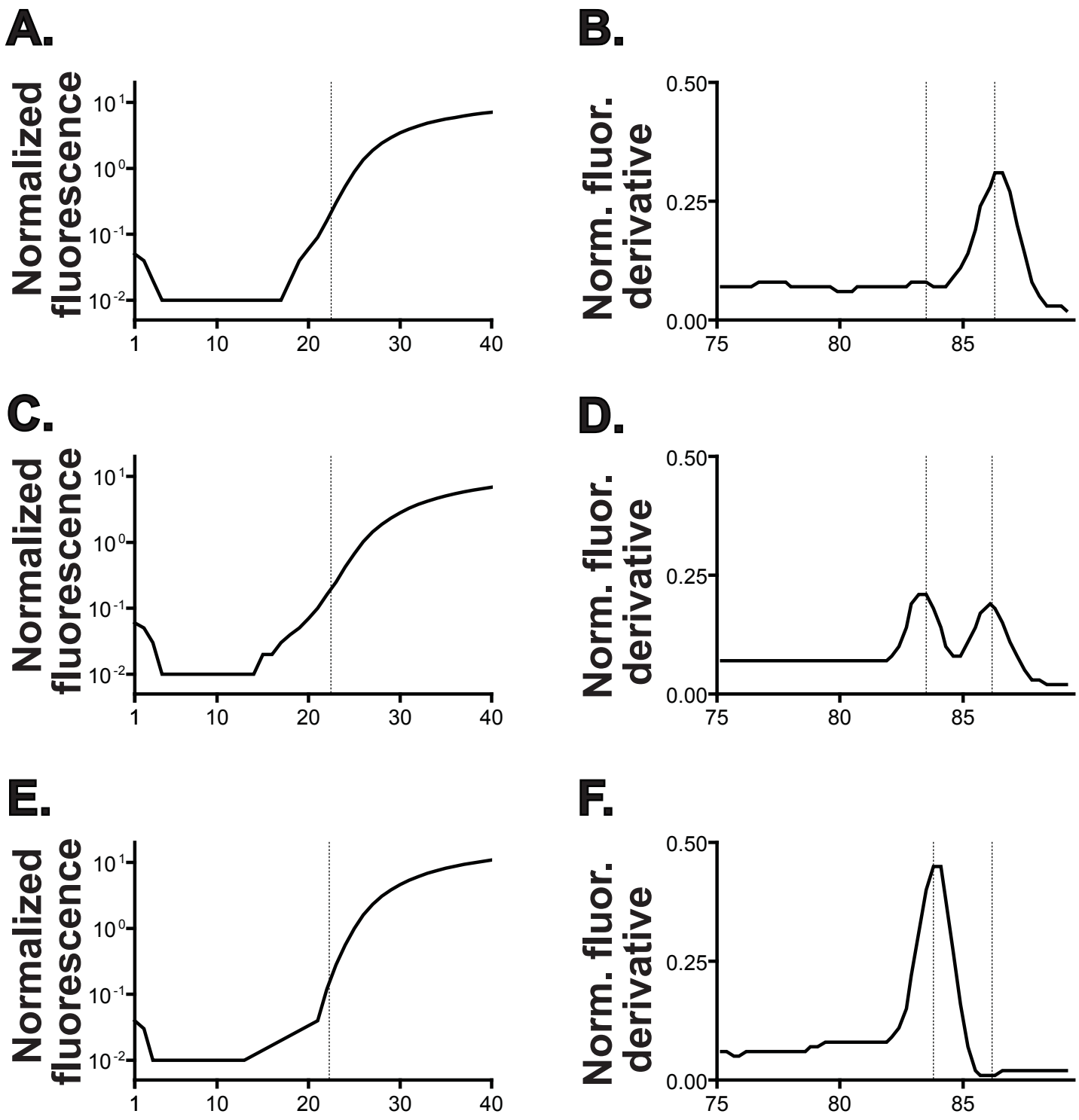


Figure 4: Genotyping mice with targeted mutations: Multiplex PCR with simultaneous identification of mutant and wild-type alleles by product Tm

Amplification plots (A, C, E) and melting curves (B, D, F) for 3-primer multiplex assay for targeted mutations at the *rosa26* locus.

A, B: Identification of a wild-type mouse. Note single peak in melting curve at 86.6◦C (wild-type product) (B).

C, D: Identification of a *rosa26*$^{Ptx/0}$ mouse. Note two peaks in melting curve: at 83.3◦C (mutant product) and 86.6◦C (wild-type product) (D).

E, F: Identification of a *rosa26*$^{Ptx/Ptx}$ mouse. Note single peak in melting curve at 83.3◦C (mutant product) (F).

Appendix B:

A *pdx1-cre* transgenic mouse line with recombination in rostral but not caudal serotonergic neurons

Mammalian serotonergic neurons are anatomically and functionally diverse (Jacobs and Azmitia 1992). The importance of these neurons in the regulation of physiology and affect has long been recognized. Transgenic methods of manipulating gene expression through the expression of recombinases in specific cellular populations and/or at specific developmental time points are a valuable asset in understanding the development, physiology and function of specialized neuronal populations (Branda and Dymecki 2004). This technology has only recently been applied to the study of serotonergic neurons (Jensen, Farago et al. 2008). Several mouse lines are reported to exhibit Cre-mediated recombination in all serotonergic neurons (Scott, Wylie et al. 2005; Gong, Doughty et al. 2007; Jensen, Farago et al. 2008). A Cre transgenic mouse line in which recombination is restricted to rostral serotonergic neurons which innervate the forebrain would be useful in elucidating the function of these neurons.

Here, I describe neuronal recombination in a widely used mouse line commonly known as *pdx1-cre* (formal designation, Tg(Ipf1-cre)89.1Dam) (Gu, Dubauskaite et al. 2002). This mouse line has been employed in at least 30 published studies, as it exhibits robust recombination in the developing endocrine pancreas (Gu, Dubauskaite et al. 2002; Aguirre, Bardeesy et al. 2003; Lammert, Gu et al. 2003; Murtaugh, Stanger et al. 2003; Lee and Hennighausen 2005; Murtaugh, Law et al. 2005; Stanger, Datar et al. 2005; Stanger, Stiles et al. 2005;

Bardeesy, Aguirre et al. 2006; Bardeesy, Cheng et al. 2006; Cano, Sekine et al. 2006; Fujikura, Hosoda et al. 2006; Miralles, Hebrard et al. 2006; Pasca di Magliano, Sekine et al. 2006; Ueki, Okada et al. 2006; Zhang, Ackermann et al. 2006; Zhang, Feng et al. 2006; Collombat, Hecksher-Sorensen et al. 2007; Fujikura, Hosoda et al. 2007; Ivashchenko, Duan et al. 2007; Kojima, Vickers et al. 2007; Lynn, Skewes-Cox et al. 2007; Seymour, Freude et al. 2007; Siveke, Einwachter et al. 2007; Wang, Zhang et al. 2007; Gupta, Jetton et al. 2008; Gupta, Jetton et al. 2008; Heiser, Cano et al. 2008; Hezel, Gurumurthy et al. 2008; Lynda Elghazi 2008; Nekrep, Wang et al. 2008). Using two Cre reporter lines, Z/EG and rosa26R (Soriano 1999; Novak, Guo et al. 2000), I found that that this line also exhibits recombination in the developing inner ear; in rostral serotonergic neurons; in the hypothalamus; and in non-serotonergic neurons of the caudal hindbrain. Using these lines, expression of GFP (Z/EG line) or LacZ (rosa26R line) provided a specific marker of Cre-mediated recombination as no expression was observed in single-transgenic, Cre-negative control tissues (Figs. 1C; 2A; 4A; 4C; 4E; 4G). Mice were generated on a mixed Cd1 / C57Bl6/J background. Embryonic stage was estimated by plug date.

Recombination was first detected at e10.5 in the pancreas (Fig. 1A), as reported (Gu, Dubauskaite et al. 2002), and in the inner ear formation (patchy expression in the developing anterior and posterior semicircular canal region with enriched expression in two anterior and posterior medial domains) (Fig. 1B). The earliest recombination in the brain was observed at e11.5, in serotonergic and non-serotonergic neurons in rhombomeres 1 (Figs. 2B, 2C) and 2 (Fig. 2E); non-

serotonergic neurons in caudal rhombomeres (Fig. 2F); and in the diencephalon (Figs. 3A, 3B). Little reporter expression was observed in rhombomere 4 (Fig. 2E)In the hindbrain, serotonin and reporter immunoreactivity was always observed in the same sections; however, the degree of overlap diminished with increasingly caudal locations. At e16.5, reporter expression in the rostral hindbrain was restricted to the rostral raphe nuclei, particularly the dorsal raphe nucleus (Fig. 4B) and caudal linear raphe nucleus (Fig. 4D). In the caudal hindbrain, reporter expression was observed in the non-serotonergic inferior olive nucleus (Fig. 4F) and in the hypothalamus (Fig. 4H).

In adult tissues, confocal microscopy was employed for analysis of hindbrain sections in order to determine the extent of colocalization of GFP with 5-HT. In the dorsal raphe nucleus, which is generated in the most rostral portion of the hindbrain (Jensen, Farago et al. 2008), a majority of serotonergic neurons expressed GFP and all GFP-positive cells were serotonergic (Figs. 5B, 5B', 5B''). In the median raphe nucleus, which is generated more caudally (Jensen, Farago et al. 2008), there was partial overlap between GFP and 5-HT expression (Figs. C, C', C''). In the caudal hindbrain, 5-HT and GFP expression were completely non-overlapping, although occurring in the same sections; GFP expression was restricted to the inferior olive nucleus (Figs. D, D', D'', E). In the hypothalamus, recombination occurred in the arcuate nucleus, dorsomedial nucleus and lateral hypothalamus (Fig. 5F).

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Figure 1: Recombination in pancreatic primordium and developing inner ear at e10.5

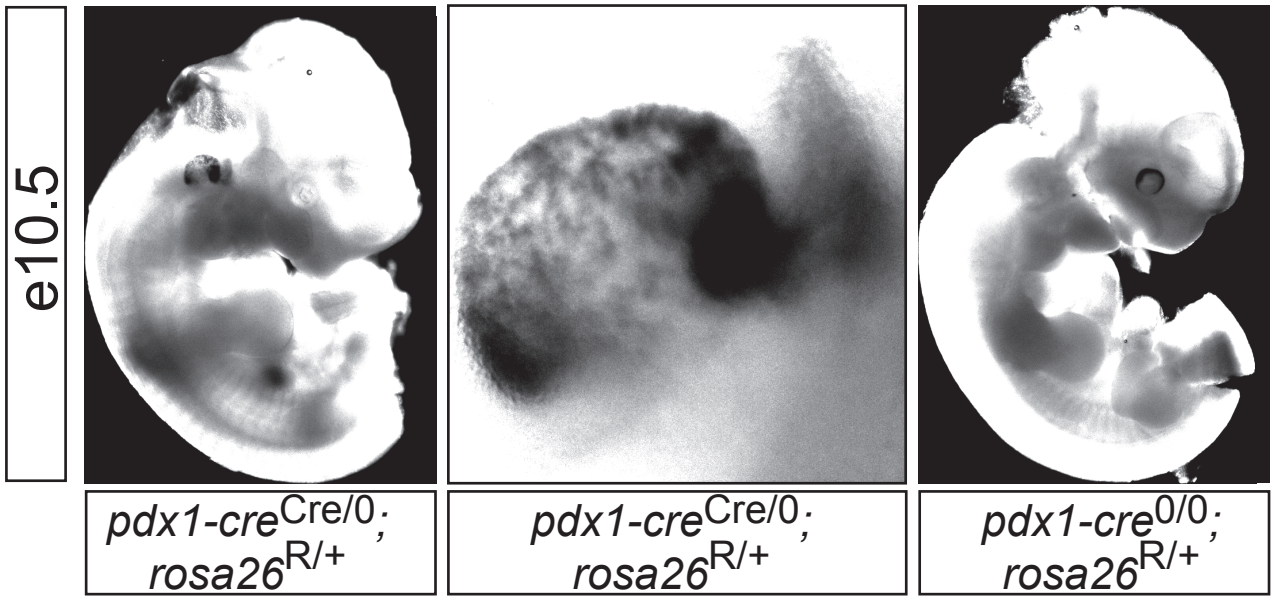


Figure 2: Recombination in rostral serotonergic neurons and caudal hindbrain non-serotonergic neurons

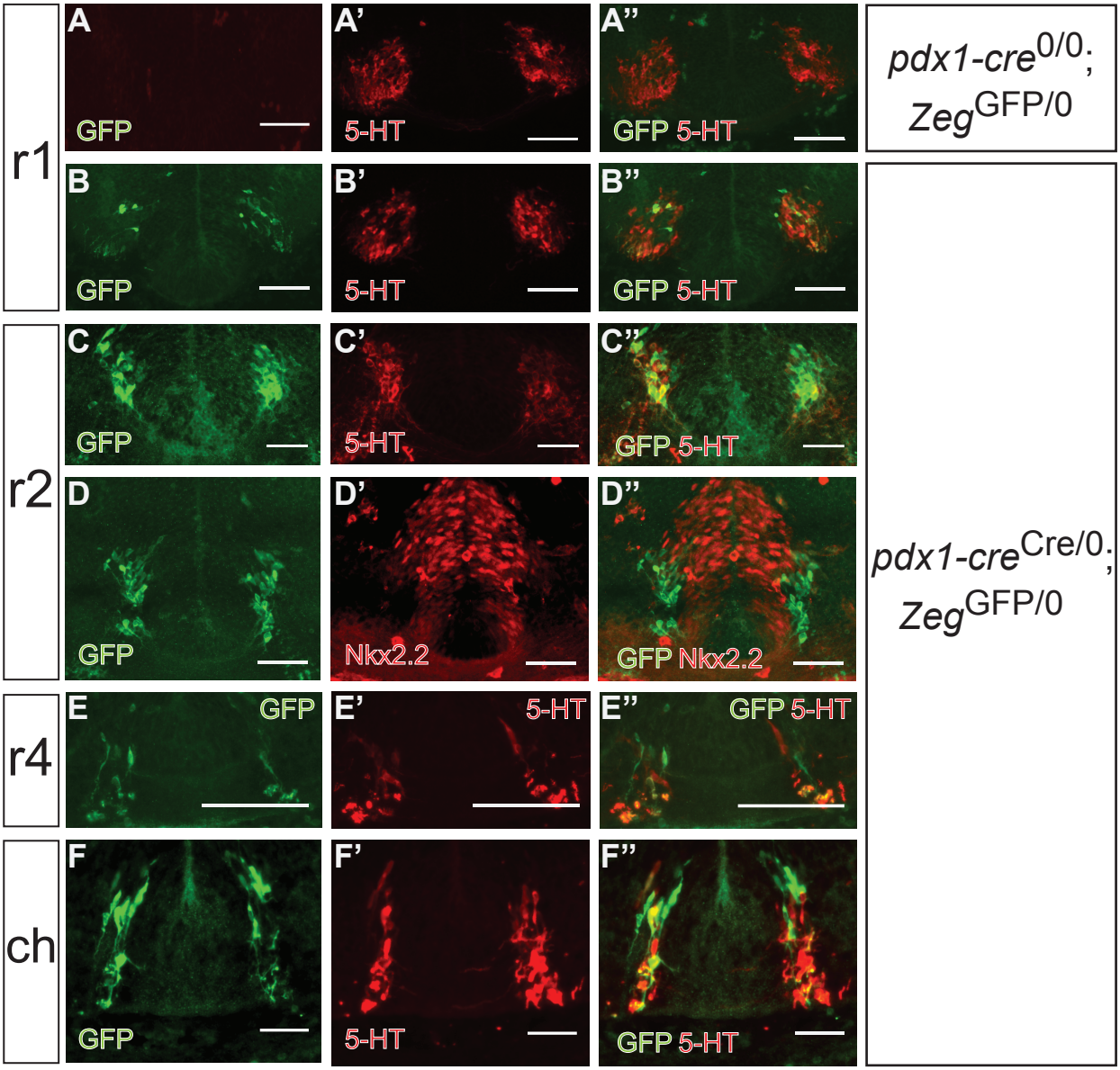


Figure 3: Recombination in the diencephalon at e11.5

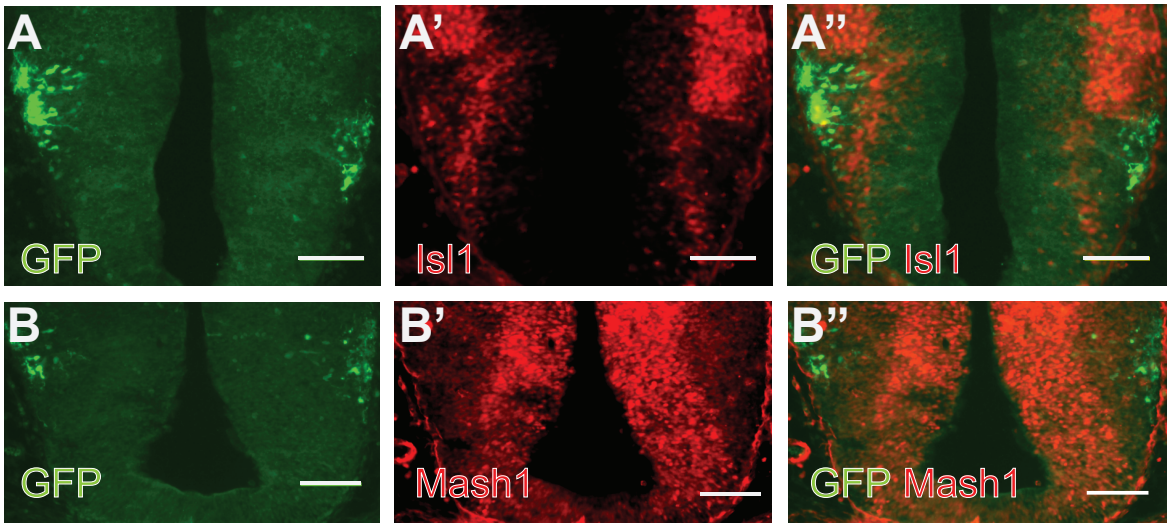


Figure 4: Recombination in the rostral raphe nuclei, inferior olive and hypothalamus at e16.5

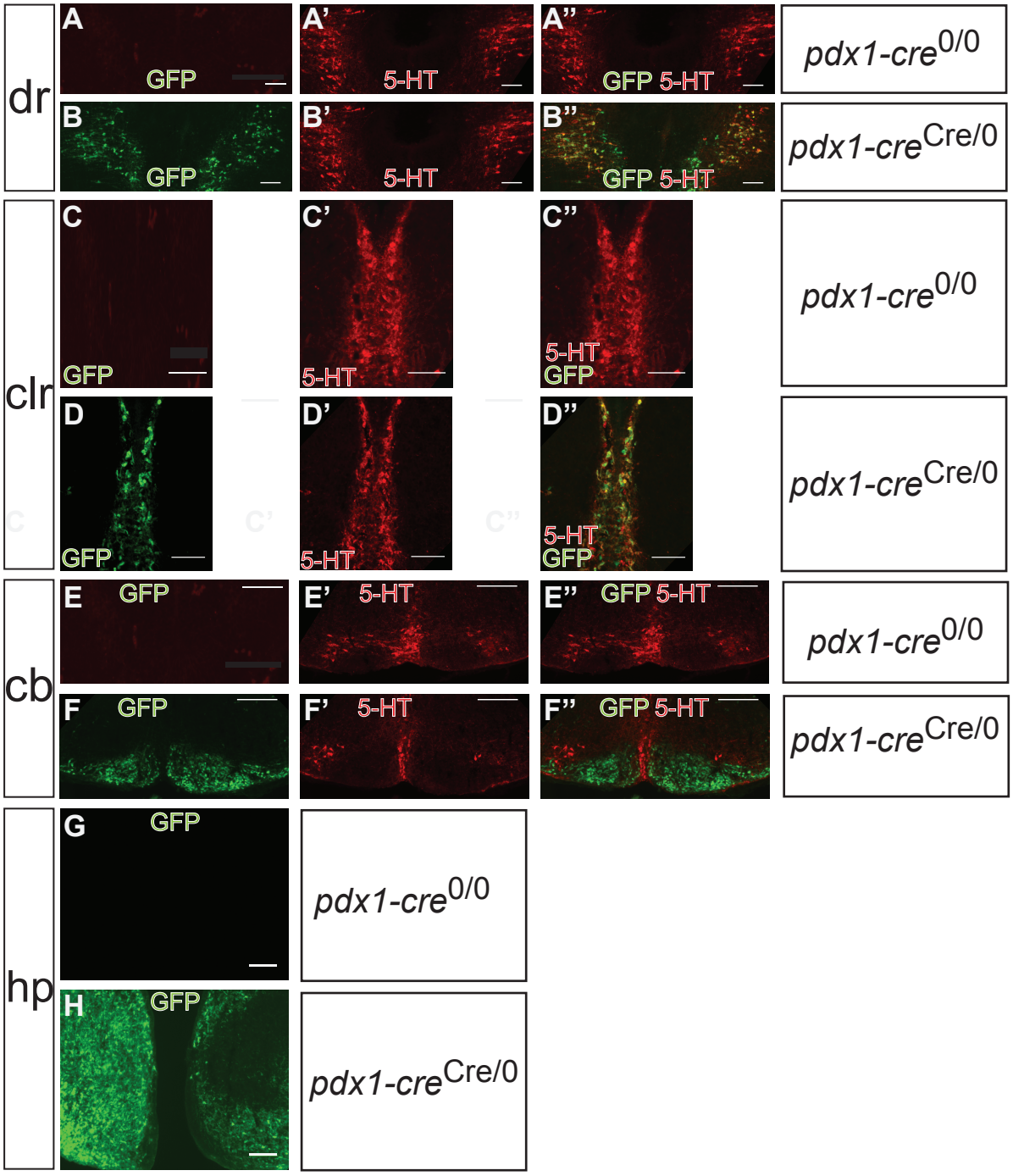
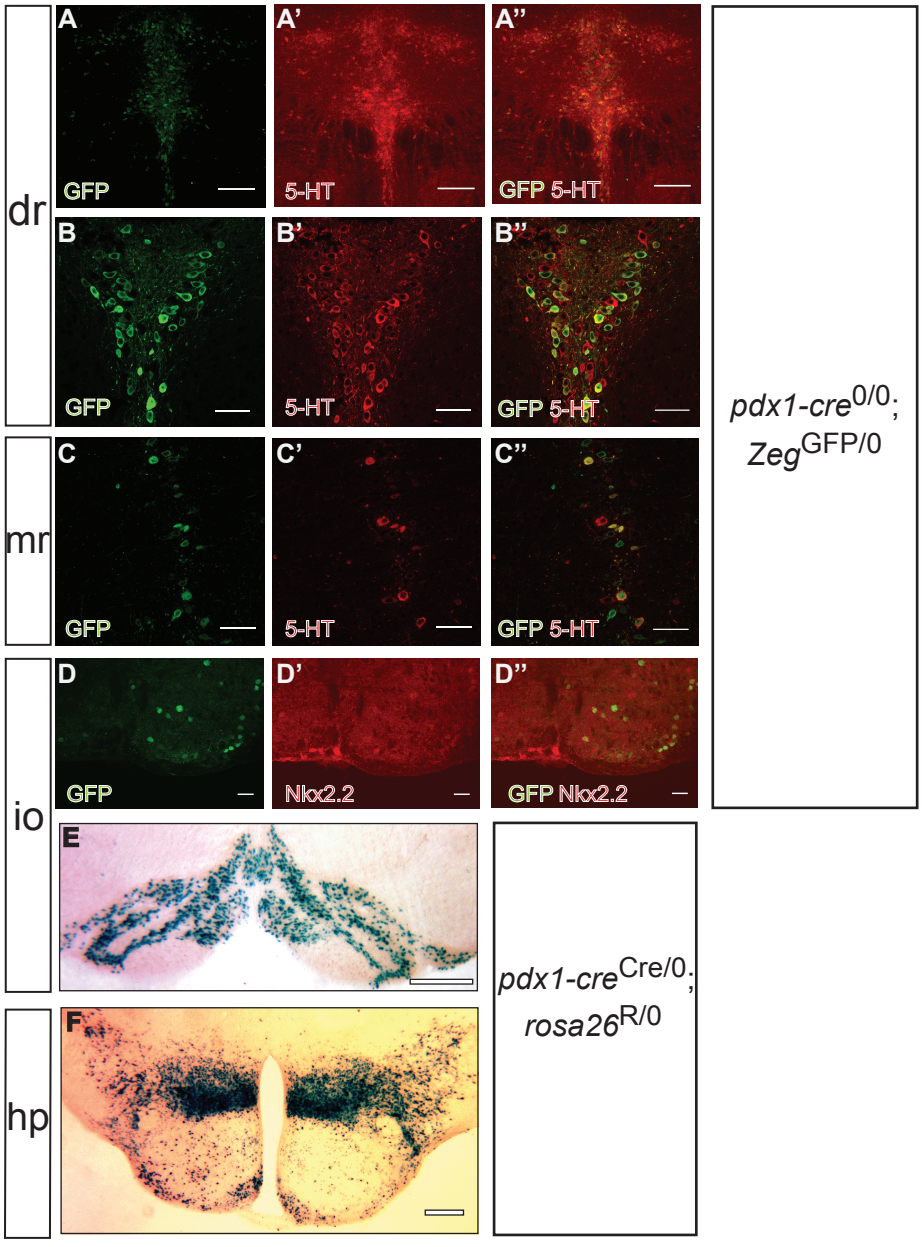


Figure 5: Recombination in adult rostral serotonergic neurons, inferior olive neurons and arcuate and dorsomedial nuclei of the hypothalamus



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